PROCEEDINGS OF THE WORKSHOP ON TECHNIQUES IN PARASITOLOGY

ZOOLOGICAL SURVEY OF INDIA, CALCUTTA

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COLLECTION AND PRESERVATION OF PARASITIC PROTOZOA

By

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The parasitic protozoa being unicellular and microscopic, require special effort for collection and preservation for proper scientific study and laboratory diagnosis. Like all other parasitic groups, they are specialised to inhabit different microhabitats *viz.*, lumen of the digestive tracts, blood, and other tissues of the hosts and accordingly different techniques have to be employed for their collection.

The various parasitic protozoa can be broadly divided into : (A) Lumenicolous forms (*i. e.*, those found inside the lumen of the digestive tract) and (B) Tissue inhabiting forms (*i.e.*, those living in the blood and other tissues of the host) for collection and preservation.

Several techniques have been developed for the collection and preservation of parasitic protozoa in recent years. However, those that are found most useful and easy to adopt in the laboratory have been dealt with in this paper.

A. LUMENICOLOUS FORMS

A variety of protozoan parasites may be found in the digestive tract and its associated organs or lumen of the body of the host. The examination of all the inhabiting parasites present in the gut or lumen requires time and patience.

However, for the collection of lumenicolous protozoa one must have at hand a plenty of physiological saline (0.7% NaCl or Ringer's solution for cold-blooded vertebrates ; 0.85%NaCl or Locke's solution for birds and mammals), containers, slides, and cover glasses, dissecting instruments including bone shears, fixatives, and staining set. Accurate records are to be kept including the name of the host, locality, site of infection, preservation or processing technique used alongwith the date and name of the collector.

The host animal should be freshly killed and the parasitic protozoa should be quickly collected, because some of the parasitic protozoa are likely to degenerate soon after the death of the host animal. Fish may be stunned by a blow on the head or the base of the brain may be pierced with a sharp instrument. The brain and spinal cord of frogs and toads should be pithed. Turtles may be decapitated and other vertebrates, if not shot in the field are killed with a drug or anaesthetic in a tight container.

Generally the vertebrates are cut through the body wall along the ventral line and extend the incision backward through the pubic symphsis and around the anus so that the posterior part of the digestive and urinogenital systems can be removed intact later. The incision can be extended anteriorly to a point between the mandibles, through sternum and ribs, if necessary. In turtles, the ventral part of the shell may first be removed by cutting through the bridge on each side and dissecting the plastron from the attached skin and underlying tissues. The viscera is to be exposed fully making transverse incisions of the body wall as per requirement. It is desirable not to cut the large blood vessels until a preliminary examination has been made.

The viscera is to be separated and removed carefully and the various organs are placed in different containers to avoid mixing of the parasites from one to the other. In removing the digestive tract, the mesenteries are to be cut close to the intestine, leaving them *in situ.* Then the various portions of the tract are separated. The small intestine of large animals should be cut into smaller bits, for convenience in handling. The digestive tract is cut open to its entire length. A small quantity of gut content is removed from various levels of the gut length and examined in saline smears under a microscope. The intestine is opened and washed in saline to remove the contents and subsequently transferred to fresh saline. Then, scraping the tissues to the level of muscle layers of the intestine is to be carried out for collection of tissue-protozoa.

(a) METHOD OF CONCENTRATION

Scanty infections may be easily missed if only direct examination of the faecal samples are made. If possible, a method of concentrating cysts is necessary (these methods, however, kill trophozoite stages).

A simple method of concentrating is the suspension of a small portion of the faeces (about 15 ml=one cubic inch) in a 33.1% aqueous solution of Zinc sulphate ($ZnSO_4$ $7H_2O$). The suspension is placed in a glass cylinder measuring about 5 × 2 cm. (2 × 3/4 in.) which is filled upto the brim and then a cover-slip is placed over the mouth of the cylinder touching the fluid. After 20-30 minutes, the coverslip is removed by a straight upward motion and placed (fluid side down) on a slide and examined under microscope. Cysts if present, will be floating on the surface of the zinc sulphate solution and adhere to the cover-slip along with the thin liquid film. If cysts are present, they can subsequently be stained by placing a drop of 1% freshly prepared iodine solution alongside the coverslip and drawing the solution beneath the latter by a piece of filter paper to the opposite edge of the coverslip.

Another method of concentration of cysts of intestinal protozoa is the Formol-Ether technique which requires the use of a centrifuge. Generally 1-2 gm of faeces is emulsified in 10 ml of 10% Formol-Saline and strained through a wire sieve (16 mesh/cm, = 40 mesh/in.) into a small centrifuge tube ; Formol-Saline is added to bring the fluid level to about 2.5 cm. (1 in.), from the top of the tube ; about 3 ml. of Ether is then added, a rubber stopper is tightly fixed to the mouth of the centrifuge tube and then the tube is vigorously shaken for a minute. Then centrifuged for 5 minutes at 2000 rpm. Fatty debris can be discarded along the interface and the supernatant fluid. The remaining bottom deposit is resuspended in a small drop of fluid present in the tube. Cysts may be examined as described above from this suspension.

The oocysts of intestinal coccidia are also concentrated preferably by $ZnSO_4$ soln., centrifugation-floatation method. However, in order to identify the species it is often necessary to keep the oocysts *in vitro* at room temperature for 2-3 days or more for sporulation to be completed, which can not be done after Formol-Ether concentration as this kills the parasites. Zinc sulphate solution is also likely to be harmful. The simplest method to concentrate coccidian oocysts is to suspend the faeces in a saturated aqueous solution of NaCI and centrifuge the suspension at 1,500 rpm (about 400 × g) for 2 minutes. The surface layer of solution, containing the oocysts, is at once pipetted into 3 or 4 times its volume of water, which dilutes the saline sufficiently to allow the oocysts to sink (by gravity or after further centrifugation) ; the sedimented oocysts are finally resuspended in 2% aqueous Potassium dichromate solution and kept in a shallow layer of this solution in a petridish (or specimen tube) at room temperature, until sporulation is completed.

(b) TEMPORARY PREPARATION

i) *Unstained saline preparation* :—A small amount of physiological saline solution is placed on a watch glass and small amount of faeces is mixed thoroughly in it. Care must be taken so that the suspension should not be too thick. A drop of suspension is placed on a slide and covered with a coverslip for microscopic examination.

Ciliates and flagellates can be seen actively moving in saline smears. Trophozoites of amoebae may show various grades of movement or may remain stationary, and in some, with protruded pseudopodia. Oocysts of coccidia (and associated helminth ova) can be recognized by the shape and size. Many other objects may be mistaken as protozoa such as flagellated bacteria, yeast, fungal spores, pollen grains, and ingested pseudoparasites of grain mites or coccidian oocysts animal contaminated through the food swallowed by the animal under examination. The microscopic examination may be carried under high dry objective by ordinary (× 400) or better under a suitable phase-contrast microscope.

ii) *Stained preparation* :—Temporary stained preparation can be made using 1% aqueous Eosin solution and double strength of Lugol's iodine solution (4% Potassium Iodide and 2% Iodine in distilled water). In Eosin preparation, if correctly made (without an excess of Eosin), live protozoan cysts (and helminth ova) will appear as small unstained (white) objects against the coloured background of Eosin solution and the redstained debris. They can thus be readily detected and subjected to further examination at higher magnification, if necessary.

In order to visualize certain details which are not visible in a live specimen, wet smears may be stained with Iodine. The faecal suspension can be mixed with an equal amount of D'Antoni's aqueous Iodine solution or Lugol's solution (1 part) diluted with distilled water (4 parts). Cysts may be studied with Iodine staining, which stains the nuclei of cysts, and their internal structures can also be seen. Glycogen vacuoles if present, appear as light or deep golden-brown in colour.

(c) PERMANENT PREPARATION

The preparation and mounting of permanent slides are carried as follows :—

i) *Killing and fixation* :—Chemical methods are employed for killing and fixing protozoan parasites. A good fixing fluid should penetrate rapidly and stop metabolic processes with a minimum of distortion or degeneration. Most fixatives are protoplasmic poisons and act rapidly when warm ; some of them may also serve as preservatives in which material can be left more or less indefinitely. However, specimens become hard and brittle, if kept for a longer period in some of the fixatives, hence, they must be removed soon after complete fixation. The slides with protozoan smears are then washed and placed in a preservative (usually 70% alcohol).

After fixation in fluids containing Corossive sublimate (Sat. Mercuric Chloride), material must be treated with alcoholic Iodine solution to remove all traces of Mercury.

Specimens fixed in fluids containing coloured ingredients such as Picric or Chromic acid must be washed until the colour is removed. Smears are made either on glass slide and stained in coplin jars ; or they may be made on coverslips and stained in columbia jars (now available in India). The latter method has the advantage because small amounts of reagents are sufficient. A clean preparation is obtained (since there is no possibility of a portion of the smear extending beyond the coverslip). Further, when the smear is made on the coverslip (No. 1) the objective can come much closer to the smear proper and this particularly desirable when examination is being made under an oil immersion objective.

Parasitic Protozoa living in a medium rich in albuminous substances, easily adhere to the cover-glass at the time of smear preparation. Generally the thin smears on coverglasses are always uniform and in some cases it is necessary to mix the faeces with a little physiological saline in order to make it thin enough to spread well. Smears should be, semi-dried, but not completely dried except, at the margin.

The smears are then quickly fixed. The most commonly used fixative for intestinal protozoa is Schaudinn's fluid (Cold saturated (6-7%) Mercuric bichloride soln. (66 ml.) Absolute or 95% alcohol (33 ml.), glacial acetic acid (1 ml.). The first two ingredients can be kept mixed without deterioration, but the glacial acetic acid must be added just before use. Generally, it is fixed at room temperature or warmed to 50°C. The fixative is placed in a petridish or in a container and the smear on a coverslip is gently dropped on it with the smeared surface facing downwards. With some experience, air bubbles can be avoided and make coverslips float on the surface of the fixative. Smear, if made on slides, may be placed on glass-rods inside just covered with fixative inside a petridish with the smear surface downwards. Fixation is carried for 15-20 minutes.

A satisfactory method of fixing a single specimen for type collection may be accomplished by isolating the desired specimen with the help of a microfine dropper on a clean slide from the saline suspensoin using a microscope. With the drying of the fluid the movement of the specimen will be slowed down and as soon as it comes to rest the fixative is immediately poured on the specimen.

The other fixatives commonly used for parasitic protozoa are as follows : a) Bouin's fluid : Sat. Picric acid (aqeuous/alcoholic) (75 ml.), Formaldehyde (25 ml.), Glacial acetic acid (5 ml.) with fixation time of 5-30 min,, and washed in 70 % alcohol. b) Carnoy's fluid : Absolute alcohol (30 ml.) Glacial acetic acid (10 ml.) and the fixation time 5-30 min., and washed in 95% alcohol.

(ii) *Staining :* Best results are obtained with materials stained soon after fixation. Stains may vary greatly in their properties since some are in aqueous, and the others in alcoholic media. Hence the preparations must be processed accordingly. Initially the smears containing intestinal protozoa should be passed through increasing or decreasing grades of alcohol as required. Staining may be either progessive, *i.e.*, by exposure to dilute stain for a longer time until the desired intensity is attained or retrogressive in which material is overstained initially and then gradually destained (or differentiated) up to a desired point, when internal structures of the protozoan specimens are clearly seen. The general tendency is to destain the preparations which requires considerable judgement. Certain stains require mordants (usually an alum or acid), either before, or more often, during the staining process. At a time two or more stains can be used for differential staining of various parts of the protozoa. The smears after fixation are transferred to a staining jar, containing 70% alcohol for at least half-an hour with two changes during the period. The smears are then passed through lower concentration of alcohol in water, keeping for 1-2 minutes in each grade. After 15 minutes, it is once again rinsed in distilled water and stained. Heidenhain's iron haematoxylin is the most commonly used stain as it is dependable and gives a clear nuclear picture. This is employed both for parasitological and histological works. Haematoxylin alone has a very poor staining property, and a mordant must be employed to make it more effective. Starting with the smears in 70% alcohol after passing through alcoholic (70%) iodine soln. for removal of mercury used in Schaudinn's fixative (when Schaudinn's fixative is used), the staining schedule is as follows :- 50% alcohol (1 min.) ; 30% alcohol (5 min.) ; distilled water (5 min.) ; 3% aqueous Iron alum (at least 1 hr.) ; thorough rinsing in distilled water (1 min.) ; 0.5% aqueous haematoxylin (2 hrs.) ; rinsing again distilled water (differentiated or controlled destaining with the help of 1% Iron alum, until the structures assume the proper intensity of colour). This process should be controlled by periodic microscopic examination running tap water (15 min). The smears are washed in dist. water and upgraded in alcohol (30%, 50%, 70%, 90%) ; Abs. alcohol twice ; xylol (5 min.)

Gradual changes in alcohol grades are required in all staining and dehydration procedures to avoid distortion. Haematoxylin-stained smears and sections can be kept in 70% alcohol over night or indefinitely, if necessary.

If desired, the smears can be counter-stained with Eosin-Y However, this has a tendency to obscure finer nuclear details. To counterstain the smears, it is transferred from 70% alcohol to 0.5% solution of Eosin-Y in alcohol, stained for 45 sec. to 3 min. Then the slide is transferred to 95% alcohol for washing the excess dye, and proceeded as usual with the absolute alcohol.

(iii) *Dehydration and clearing* : Water must be thoroughly removed from the preparations that are to be cleared and mounted. Dehydration is usually accomplished by passing the material through ascending grades of alcohol. A number of natural or synthetic oils as well as xylol and other hydrocarbons are used as clearing agents. Clearing is a critical process and it is at this stage the vesicular or delicate structures are most likely to collapse. If non-volatile oils are used, the excess must be removed from specimens before mounting, usually after washing in xylol.

(iv) *Mounting media :* Specimens are mounted in a resinous solution which hardens as the solvent evaporates. The media most commonly used are Canada balsam, D. P. X., Damar and synthetic resins such as Euparal, Napharx, Permount and Clearite. Synthetic resins are preferred especially for thin preparations stained with dyes that generally fade due to acidity. The D. P. X. is preferable to Balsam and has the additional advantage of hardening more rapidly. The consistency of the mounting medium, size and thickness of the coverslip are determined by the type of the medium used. Very thin medium and No. 0 or thin coverslips are absolutely necessary in preparations that are to be observed under oil immersion lens.

The smears must not be allowed to dry in air during processing in any circumstance.

(v) *Labelling :* Slides should be labelled with pertinent information including the scientific name, host, site of infection, date of collection, collector's name, etc.

B. TISSUE INHABITING FORMS

Blood inhabiting Protozoa may be obtained from live hosts by pricking the blood vessel with the help of a sterilised insect pin or sewing needle. The site for obtaining blood varies from host to host and the convenient sites of different vertebrates are tabulated as follows :

Table I : *Puncturing sites in different vertebrate hosts.*

Host	Sites
1. Man	Finger-tips
2. Other mammals	Tail, ear
3. Birds	Brachial vein tarso-metatarsal vein ; clipped toe.
4. Reptiles	Tail ; finger or toe ; mandibular vein.
5. Amphibians	Finger or toe ; or tail
6. Fish	Caudal vein or branchial vessel.

Thick or thin smears of the blood are prepared for detection of blood parasites. Thick smears are preferable to thinner ones for mammalian blood because the former permits examination of relatively larger quantity of blood within a short time. But in case of non-mammalian hosts, thin smears are prepared because of their nucleated erythrocytes. However, for the detection of trypanosomes thick smears of non-mammalian blood are also useful.

(a) PREPARATION OF BLOOD FILM

Two slides are to be cleaned by rinsing in 95% alcohol and subsequently wiped with a clean cloth. Slides are to be handled only by their edges to avoid finger marks, on the surface of the slides. A small drop of blood is placed at the end of a scrupulously clean slide. Another slide is placed at an angle of 30 to 40 degrees vertically on the first, so that the drop of blood may spread along the zone of contact between the slides. Now, the second slide is pushed on the first rapidly, thus drawing the blood into a thin film. The thickness of the film is dependent on the size of the drop, the angle between the slides, and the rapidity with which the smear is drawn. The film is dried rapidly by waving in the air.

Thick film smear is prepared as for thin smear and a medium-sized drop of blood, or several small ones are placed on the slide and spread or mixed evenly with a needle or with the corner of another slide.

Because of density, thick films are dehaemoglobinized by putting the slide in dist. water. In this process however, such cytozoic forms as malarial parasites, are liable to be distorted.

(b) PREPARATION OF ORGAN IMPRINT SMEARS

For the collection of endogenous stages of various protozoan parasites, small pieces of tissue of the suspected organs such as lung, liver, heart, kidney and spleen are dissected out from the host. The cut surfaces are dabbed several times on filter-paper or blotting-paper to remove most of the blood, and then pressed (not smeared) once on to a clean and grease-free slide (several dabs may be made on one slide). After drying in air, such smears are fixed, stained and examined in the way as described for thin blood smears.

Imprint-smears of brain may be made by placing a small piece (pin head size) at one end of a slide. It is then crushed by placing another slide on it and spread out by sliding the two slides perpendicular to one another. Smears of bone marrow are usually made by spreading a small fragment on a slide with the aid of a needle.

Fixation and Staining : Methyl alcohol is used as a fixative Giemsa's stain is used and Romanowsky stains both fix and stain the blood and tissue inhabiting protozoa.

i) *Method for Giemsa's stain* :—1) The air dried smear is fixed in Acetone and moisture-free Methyl alcohol for 5 minutes, and allowed to dry ; 2) Unless ready for use, the stock solution of the stain is diluted at the rate of 1 drop to 1 ml., of 7.0-7.2 pH distilled water or buffer ; 3) The slide is placed with smear side up on a staining rack and covered the same with the diluted stain. It is left for 40-50 min., and if necessary more stain is added ; 4) Neutral distilled water or buffer solution is poured over the preparation for washing. The back of the slide is rubbed clean with a moist cotton wool or cloth ; 5) It is then drained and dried ; 6) Uncovered smears, if kept clean and wrapped in tracing or tissue paper, may keep the stained preparation for a long time ; 7) For mounting a drop of a neutral mounting medium is placed on the dried smear, and covered over with a clean cover slip.

ii) *Method for Wright's/Leishman's stain* :—1) The dried smear is placed on a staining rack and enough undiluted stain is added to cover well for 1-2 min., 2) Neutral distilled or buffered water (pH 7.0-7.2) twice the quantity of stain is added drop by drop or until a metallic scum appears and left in it for 5-10 minutes ; 3) The slide is then kept in horizontal position and flushed rapidly with neutral water ; 4) Water is drained off, back of the slide is cleaned with moist cotton wool or cloth and dried rapidly. If desired, it may be covered and labelled as in the above process.

C. HISTOLOGICAL PREPARATION

Organs suspected to contain protozoan parasites can be fixed (Carnoy's fixative or Bouin's fixative), embedded (in paraffin) and sections are cut, following by the standard histological procedure (Pearse, 1960). The sections may then be stained with Haematoxylin and Eosin (if counterstaining necessary) or with Giemsa's stain.

Method of staining :— 1) For Haematoxylin and Eosin staining the procedure is similar as in permanent preparations for lumenicolous forms. 2) For Giemsa's stain : Small pieces of tissue are fixed for about 3 hours in Carnoy's fluid (Ethanol (6 parts) ; Chloroform (3 parts) ; Acetic acid (1 part) and washed in 90% Ethanol before dehydration, clearing and embedding in the usual way. The actual staining procedure is as follows (*vide* Bray and Garnham, 1962).

1. After dehydration, the slide is placed in a mixture of equal volumes of Giemsa's one volume of methanol with 10 volumes of distilled water at pH 7.2 for an hour.

2. The slides are removed from the stain and washed in tap water.

3. The sections are differentiated by pouring (15% solution of Colophonium resin in Acetone on to the wet slides, rocking the latter to and fro 3-10 seconds. The solution is then drained off. It is necessary to repeat the process 2 or 3 times until the blue-green dye streams out in the differentiating solution.

4. The slides are washed rapidly in a mixture of xylol (7 parts) and Acetone (3 parts), followed by several washings in pure Xylol.

5. Finally it is mounted in a neutral medium (D.P.X. or Euparal).

REFERENCES

BRAY, R. S. and GARNHAM, P. C. C. 1962. The Giemsa-Colophonium method for staining protozoa in tissue sections. *Indian J. Malariol.*, **16** : 153-155.

PEARSE, A. G. E. 1960. *Histochemistry*. - [J. A. Churchil], London, 2nd edition.

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IN VITRO CULTIVATION OF PARASITIC PROTOZOA

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In studying the life-cycle, metabolism, physiology and ecology of parasitic protozoa, it is necessary to isolate and maintain them *in vitro* in the laboratory. Therefore, *in vitro* cultivation of parasitic protozoa forms an integrated part of Protozoology. For our convenience, the techniques of *in vitro* cultivation of parasitic protozoa may be discussed under two major heads : (i) blood or other tissue inhabiting species, which can be subdivided in to extra and intra-cellular forms, and (ii) the enteric forms, found inside the enteron of various hosts.

I. *General precautions to be taken for the in vitro cultivation of parasitic protozoa.*

Some basic and important precautions are discussed here. The glass-ware, instruments etc. to be used, must be clean, dry and sterilised properly. Generally, cleaning of glass-ware is necessary to remove all traces of dirt and oily substances. Hardwater and soap are avoided. The detergent of choice should be easily soluble in water, capable to soften the water, easily removable on washing, leaves no precipitate in hot and boiling water, contains no free alkali and should not irritate hand. Usually the glass-ware are soaked in warm water containing approximately 1-2% detergent, brushed clean and washed thoroughly at least 3 times in warm water. After final rinsing in distilled water, the glass-ware are allowed to dry in air or inside a 'dryer' The dried glass ware and other equipment are then packed in aluminium foil and/ or in good quality brown paper. Flasks, bottles and pipettes should be properly plugged. The packed material can be sterilised in a hot air oven by raising the temperature of the oven to 100° C., and maintaining it for at least one hour at that temperature. Sterilisation can also be made in an autoclave at 15 lbs. pressure for 20-30 minutes. Caps of the screwcap bottles should be kept loose during sterilisation and should be screwed tight after cooling to room temperature.

Solutions are generally sterilised by millipore filtration however, simple salt solutions may be sterilised in an autoclave. Both Seitz and membrane filters (Maxflow) are now available in India. These are best be suited for removing contaminant micro-organisms particularly from the liquids, where exposure to heat is not conducive.

Antibodies are generally used in culture media for suppressing the growth and subsequent removal of unwanted bacterial and fungal contaminants. Sodium Penicillin G 100 units/ml., and Streptomycin 100 microgramme/ml., are effective against a wide range of bacterial contaminants. For the fungal contaminants, fungicide and antibiotic, like Mycostatin, 20 microgramme/ml., may be used. Good quality chemicals e.g. analytical reagent grade inorganic chemical, and grade A, organic chemicals should be used for culture media preparations. A double glass distilled or deionised water is preferably used. Usually concentrated (10X) stock solutions are prepared, sterilised and stored.

Commercially available liquid/dehydrated media may be used for isolation and maintenance of parasitic protozoa. Some are now readily available in India. Enzymes (amylase, trypsin etc.) used in culture media are mixed either in distilled water or in balanced salt solution (BSS) without bicarbonate. The pH of culture media should be checked and properly adjusted by addition of appropriate alkali or acid. Occasionally, CO_2 gas bubbling may be necessary for pH stabilisation of the culture media.

The pH of contaminated culture media readily fluctuates and the use of proper staining methods would disclose the identity of the microorganism contaminants.

II. *Methods of cultivation of blood and other tissue inhabiting forms.*

(*a*) *The haemoflagellates :*

(i) *Monogenetic trypanosomes :—*

Many haemoflagellates show extracellular stages in their life-cycle, but *in vitro* culture for all the stages have yet to be developed. Monogenetic trypanosomes can be grown in simple medium comprising Peptone, Glucose, Sodium chloride and water. Guttman (1963) has devised a defined medium for *Crithidia* and *Blastocrithidia*.

(ii) *Digenetic trypanosomes :—*

Trypanosomes are generally found to be in promastigote and epimastigote stages in the invertebrate vector and amastigote, epimastigote and trypomastigote forms in vertebrate host. Besides this, transitional stages may also be encountered among both the hosts.

Digenetic trypanosomes may be classified as stercorarian or salivarian type according to their site of development and mode of transmission.

In stercorarian type, the development in the invertebrate vector is completed with the formation of metatrypanosome stage (metacyclic infective form) in the posterior section of the gut and is transmitted by contamination.

In salivarian, the development takes place in the anterior gut of the invertebrate vector and finally the metatrypanosome is transmitted during biting (inoculative).

The extracellular forms of digenetic trypanosome are normally cultured in diphasic (undefined) medium. The most classical medium is NNN medium (Nicolle, 1908). Nicolle himself modified Navy and Mac Neal's medium ; Wenyon (1926) again slightly modified the medium, and the ingredients are as follows : Dissolve (by heat) Agar (14 g.) and Sodium chloride (6 g.) in distilled water (900 ml.). Autoclave and dispense into tubes (about 5 ml., each). Add to each tube 20 drops (1 ml.) of fresh rabbit blood in an hygienic way. Allow to set in a slant and then incubate at 37° C. for 2 days to check contamination. Refrigerate before use. Incubate the medium at 25° C. (after inoculation) and transfer to fresh medium every week. Many modifications of this blood-agar medium have been carried by subsequent workers. Sometimes an overlay of saline, Glucose-saline, Locke's fluid etc., are added so as to prevent the medium from absolute drying and enable the growth and multiplication of the flagellates. Some workers autoclave the diphasic medium after the addition of blood to the base, in which case the medium not only becomes perfectly sterile but also remains firm from which erythrocytes will not be liberated. The blood of man, rabbit, guineapig, horse, sheep etc. can be used for the medium.

In salivarian trypanosomes, true cultivation of the form found in vertebrate (the haematozoic form) has not been achieved. Lemma and Schiller (1964) successively subjected three species of *Leishmania* and succeeded to transform *Leishmania* promastigote to amastigote at 34°C. Bhattacharya and Janovy (1975) successfully maintained amastigote stages of *L. donovani* in infected cell culture by the following method. Peritoneal exudate cells were collected from golden hamster stimulated intraperitoneally with 3 ml., of 2% starch suspension in Locke's solution from 24 to 48 hrs., prior to harvest. Ten ml., sterile McCoy's 5A (modified) medium with 30% Faetal bovine serum (GIBCO) were injected into the peritoneal cavity. The abdomen was gently kneaded and the fluid was withdrawn into the same needle and syringe which were not removed. Exudate cells from more than one hamster were pooled in siliconized (250 ml.), screwcap Erlenmeyer flask and counted by haemocytometer. The concentration of macrophage cells was adjusted to approximately 1.25×10[illegible] cells/ml., by the addition of McCoy's medium. Exudates containing less than 90% macrophage cells were discarded. Two ml. of the medium containing macrophage cells were dispensed in siliconized (16×125 mm.), cover slip. Promastigotes in 0.5 ml., Tanabe's medium at a ratio of 2 parasites per macrophage cell were introduced into the leighton tube. Macrophage cell cultures were kept at 35°C. Fresh medium was added to culture every 48 hours. The culture could be maintained for one week.

(b) *Sporozoa*

(i) *Plasmodium*

The malarial parasites have been obviously well studied for their importance. The techniques have been modified for cultivation of (1) the erythrocytic stages (2) the exo-erythrocytic schizonts (3) the sporogonic stages which also develop in mosquitoes.

(1) *The erythrocytic stages* :

The multiplication of the intra-erythrocytic stages of the parasite suspension of host erythrocytes has been demonstrated in human malarial parasite *Plasmodium falciparum* (Bass and Johns 1912). Trager (1958) has further supplemented the red cell medium with folic acid for culturing the *P. falciparum* and obtained a better result. Similar work has been done on *P. lophura* (Trager 1947, Mc Ghee and Trager, 1950, etc.) and *P. knowlesi* (Ball *et al.* 1945, Gieman *et al.* 1946).

(2) *The exo erythrocytic stages* :

This phase develops within the cells of the reticulo-endothelial system in birds, whereas in primates it is restricted to the hepatic cells of the liver. For avian malarial parasite, the culture was prepared with infected chick brain tissue in roller tube with Tyrode's solution and chick embryo extract.

(3) *The mosquito stage* :

Oocysts of *Plasmodium* have been grown in a medium similar to mosquito haemolymph. Ball and Chao (1960) devised a good medium for mosquito tissue culture in which the parasites can be successfully cultured.

(ii) *Toxoplasma :*

T gonodii, present in many vertebrates has been grown successfully in vertebrate cell suspension with any medium that keeps the host cells alive. It has also been cultured in monolayer culture in a roller tube. Chernin (1954) maintained RH strain of *Toxoplasma* for more than 100 days in peritoneal exudate from infected mice. The culture was maintained in a medium of 90% beef amniotic fluid, and 5% inactivated horse serum.

(c) *Parasitic flagellates* (other than trypanosomatidae)

(i) *Giardia*

The common intestinal flagellate *Giardia* can be cultured in a medium made up of yeast like fungus *Candida* and chick fibroblast. The other types of media are : egg medium with Ringer and Locke's solution, serum with Ringer's solution, and agar solid phase with an overlay of serum and egg albumen.

(ii) *Trichomonas*

T vaginalis can be grown in a variety of media in association with mixed bacteria and fungal flora. For axenic culture (and also for termite flagellates) TYM (trypticase, yeast, maltose), TTY (Tryptose, trypticase, yeast extract) have proved successful. Certain trichomonads of cold blooded vertebrates have been grown in defined medium 5F.

(d) *Parasitic ciliates*

The ciliates like *Balantidium coli* have been grown non-axenically in several media e.g., diphasic Tanabe's medium, or saline serum solution. There are some special media for rumen inhabiting ciliates.

Other parasitic protozoa

Organisms like *Entamoeba histolytica* has been grown in many media in association with bacteria. The common media are : Agar, Locke's and serum medium, egg yolk-liver extract medium, liver infusion agar medium, and horse serum base with hen's egg overlay medium etc.

Parasitic *Hartmanella* is commonly grown in laboratory in agar base medium with *Aerobacter aerogens.*

Microsporidia can be cultivated in roller tube in a medium (Morris *et al.*, 1956) containing 50% horse serum, 40% balanced salt solution and 10% beef embryo extract.

Conclusion

Many parasitic protozoa are isolated and maintained *in vitro.* Some are defined media and many others are non-defined. Defined media for *in vitro* cultivation of several parasitic protozoa need to be developed.

REFERENCES

BALL, E. G., ANFINSEN, C. B., GEIMAN, Q. M. MCKEE, R. W. and ORMSBEE, R. A. 1945. *In vitro* growth and multiplication of the malarial parasite, *Plasmodium knowlesi.—Science*, N. Y., 101 : 542-544.

BALL, G. H. and CHAO, J. 1960. *In vitro* development of the mosquito phase of *Plasmodium relictum.—Exp. Parasit.*, N. Y., 9 : 47-55.

BASS, C. C. and JOHNS, F. M. 1912. The cultivation of malarial plasmodia (*Plasmodium vivax* and *Plasmodium falciparum*) *in vitro.*— *J. exp. Med.*, 16 : 567-579.

BHATTACHARYA, A. and JANOVY, J. 1975. *Leishmania donovani* : Autoradiographic evidence for molecular exchanges between parasites and host cells.—*Exp. Parasit.*, N. Y., 37 (3) : 353-360.

CHERNIN, E. and WELLER, T. H. 1954. Serial propagation of *Toxoplasma gondii* in roller tube cultures of mouse and of human tissues.—*Proc. Soc. exp. Biol. and Med.*, 85 : 68-72.

GIEMAN, Q. M., ANFINSEN, C. B., MCKEE, R. W., ORMSBEE, R. A. and BALL, E. G. 1946. Studies on malarial parasites. VII. Methods and techniques for cultivation.—*J. exp. Med.* 84 : 583-606.

GUTTMAN, HELENE N. 1961. Experimental glimpses at the lower Trypanosomatidae.—*Exp. Parasit.*, N. Y., 13 : 129-142.

LEMMA, A. and SCHILLER, E. L. 1964. Extracellular cultivation of the leishmanial bodies of species belonging to the protozoan genus *Leishmania,—Exp. Parasit.*, N. Y., 15 : 503-513.

MCGHEE, R. E. and TRAGER, W. 1950. The cultivation of *Plasmodium lophurae in vitro* in chicken erythrocytic suspensions and the effects of some constituents of the culture medium upon its growth and multiplication.—*J. Parasit.*, 36 : 123-127.

MORRIS, J. A., MCCOWN, J. M. and BLOUNT, R. I. 1956. Ascites and hepatospleenomegaly in mice associated with protozoan-like cytoplasmic structures.—*J. Dis., Infect.* 98 : 306-311.

NICOLLE, C. 1908. Nouvelles acquisitions sur le Kala-azar : cultures ; inoculations an chien, etiologie.—*C.r. hebd. seane. Acad. Sci.*, Paris, 146 : 498-499.

TRAGER, W. 1947. The development of the malarial parasite *Plasmodium lophurae* in red blood cell suspensions *in vitro., J. Parasit.*, 33 : 345-350.

TRAGER, W. 1958. Folic acid and non-dialyzable materials in the nutrition of malarial parasites.—*J. exp. Med.*, 108 : 753-772.

TRAGER, W. 1959. Tse-tsefly tissue culture and the development of trypanosomes to the infective stage.—*Ann. trop. Med. Parasit.*, 53 : 473-491.

WENYON, C. M. 1926. *Protozoology*, Bailliere, Tindall and Cox, London, 2 : ix—779-1563.

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A NEW APPARATUS FOR REARING MIRACIDIA

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MacMullen and Beaver (1945) suggested the use of a 1000 ml., flat bottom flask with a side tube at its neck for the rearing of the miracidia. Stunkard (1946) introduced the use of Erlen-meyer flask. Many workers preferred to the use of the former over the latter. In the hatching experiments for the miracidia of *Paryphostomum* sp. (Family : Echinostomatidae), the present author noted that during the repeated changes of the water, the resulting water bubbles dislocate or disturb the developing eggs in MacMullen and Beaver's flat bottom flask. Therefore, the new apparatus described below (Fig. 1) is designed which gave better results.

I. DESCRIPTION OF THE NEW APPARATUS

The new apparatus, essentially consists of a 20-21 cm., long glass tube with an internal diameter of 2.5 cm. A little below the upper or open end of the tube a side 'J' tube of about 5 cm., in length and 1.25 cm., diameter is attached. The bent portion of the side tube is about 2 cm., in length. The rims of the main and the side tube should be at the same level. The whole apparatus is wrapped with a black paper, except for about 1.5 - 2.0 cm., length of the side arm.

II. METHOD OF REARING

The worms are teased, the eggs separated, washed thoroughly and a large number of them are placed in the main tube. Water, is added and changed daily through the side arm only, so that the eggs in the larger tube are least disturbed. The miracidia when hatched, being positively phototropic, migrate to the side tube, into the uncovered portion and can be readily detected by their movement in the water.

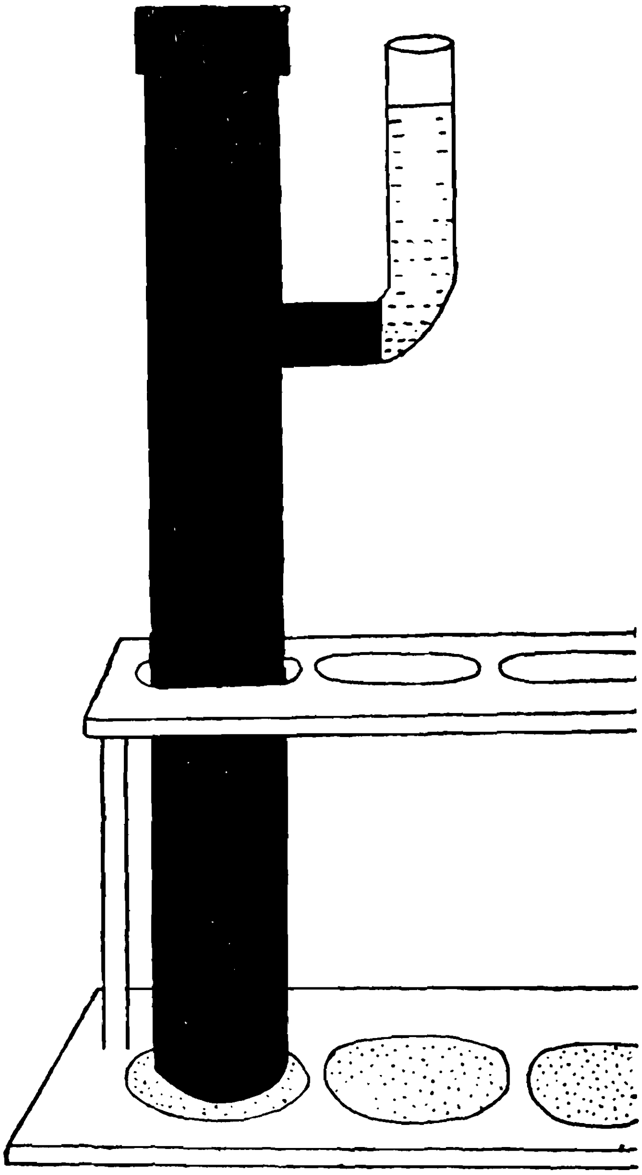

Fig. 1

REFERENCES

MACMULLEN, B. D. and BEAVER, P. C. 1945. Studies on *Schistosoma* dermatitis IX. The life cycles of the three dermatitis producing *Schistosoma* from birds and a discussion of the subfamily Bilharziellinae.—*Amer. J. Hyg.*, 42 : 128-154.

STUNKARD, H. W. 1946. Possible snail hosts of human schistosomes in the United States.—*J. Parasit.*, 32 : 539-552.

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COLLECTION AND STUDY OF LARVAL TREMATODES (PLATYHELMINTHES)

By

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Large number of trematodes have been reported from different parts of the world. These parasites infect both cold and warm blooded animals. India is well known for the abundance of these parasites. Various species of trematodes parasitising fishes, amphibians, reptiles, birds and mammals including man have been reported from this country. Some of these trematodes are highly pathogenic and cause diseases. They infect different organs of the host and the damage caused by the infection depends very much on the intensity of infection and the organs infected. The loss of cattle wealth every year in India due to trematode infection only is very heavy, although the exact loss is not known. Trematode infection proved to be one of the major factors in undermining the health of our domestic animals and cause considerable monetary loss to the live-stock owners. Trematodes not only infect the domestic animals but they are equally responsible, in certain cases, for the death of our wild animals. Data on the type of trematode infection, percentage of infection, mortality etc., in wild animals have not yet been thoroughly worked out. Our knowledge on the incidence of infection, distribution, pathogenicity, treatment, life-history, control etc., of the various species of trematodes is very scanty.

Studies on the life history of trematodes help to understand properly the morphology, taxonomy, pathogenicity and in formulating control measures against them. However, the life histories of only a few trematodes have so far been worked out in India. To complete the life history a trematode invariably needs an intermediate host which is generally a snail. Therefore the distribution and the survival of the trematodes depend upon the availability of suitable intermediate hosts and their distribution.

The miracidia after hatching from the eggs, laid by the trematodes, may swim for some time in the water and finally enter into a suitable intermediate snail host. The miracidia after entering into the snail body, shed their body cilia and develop into sporocysts, which in turn develop into rediae, or may directly give rise to cercariae. Generally the rediae give rise to cercariae and these immature cercariae emerge from the rediae into the snail body. Further development of the cercariae takes place inside the snail host and finally the fully developed cercariae emerge from the snail into the water. Those cercariae swim in water for sometimes and either form the cysts or enter into the final host. The cercariae on encystment, form the infective stage and these cysts develop further only when they are ingested by a suitable host. A cercaria is generally provided with a body and tail and morphologically it resembles its adult. Some of the cercariae may also be provided with a trunk.

METHOD OF COLLECTION

The larval trematodes can be studied when they emerge from the infected snails or the developmental stages can be obtained by dissecting out the snails. The snails should be collected throughout the year from all possible sources, with the help of a hand net. The aquatic snails are also found to adhere on the aquatic plants and on floating or submerged objects like pieces of wood or stick. The adhering snails can be picked up by hand from these sources. The snails thus collected are throughly washed in running tap water, sorted species wise, counted and then kept singly in specimen tubes containing little tap water. Periodically each tube is examined, by holding it against the light, for the discharge of cercariae for a number of days. Snails found to discharge cercariae are transferred into aquaria beakers, glass jars or small enamel basins and are provided with aquatic plants and algae. The water of these containers and the tubes should be changed daily. It is also necessary to expose the tubes daily to the sunlight or against a glowing electric bulb for sometime as it stimulates the positively phototropic cercariae to come out of the infected snails. The snails which on repeated examination found negative of trematode infection should be seperated species-wise and transferred into big glass jars, enamel basins or in big earthen tubs and maintained in the laboratory on boiled algae and on well washed aquatic plants. A regular supply of clean laboratory breed snails can be obtained, for carrying out further studies on the life history, from the eggs laid by these snails.

Detailed records of the locality from which the snails were collected, source of collection, species of snails collected, date of collection, name of the collector, percentage of infected snails, intensity of infection, type of cercariae shed by the infected snails, time of emergence of the cercariae from the infected snails etc., should be maintained properly. It is also desirable to note down the approximate age of the infected snails, organs infected and mortality rate of the infected snails in different age groups. A single species of snail may act as an intermediate host of a single trematode species or a number of species. A single snail as a rule sheds one type of cercariae but occasionally it may discharge two types of cercariae simultaneously.

MODE OF EXAMINATION

Live cercariae should be first studied when sufficient number of cercariae are shed by a snail, they should be transferred by means of a fine dropper into a beaker containing enough clean water. All the activities of the cercariae such as their mode of swimming, movement towards the illuminated side of the container or away from it, the level on which they swim etc., should be carefully noted down.

(a) *Temporary mounts*

For the detailed microscopical studies a few cercariae with a drop of water should be taken on a clean slide by means of a fine dropper, covered with a cover slip pressure and examined under a low power of microscope. The slight cover slip pressure is sufficient to flatten the cercariae properly which facilitate the study of the internal morphology. All the morphological characters such as shape of body, trunk and tail structure, distribution and nature of the body pigment, distribution and structure of the cuticular spines, shape and structure of eye-spots and cystogenous cells, structure and position of the oral and ventral suckers etc., should be carefully studied in the live specimens. The excretory system of a cercaria can only be studied in the live specimens. The excretory, digestive and reproductive system, nature and structure of the different cells and their ducts, arrangement of muscles, number and arrangement of spines can be studied only after staining the cercariae with intra vital stains like neutral red, briliant cresyl blue, janus green, trypan blue, methylene blue, nile blue, gentian violet, etc. The digestive and reproductive systems can also be stained by borax carmine or acetic alum carmine.

Other stages such as sporocysts, rediae and the various stages of development of the cercariae can be obtained by dissecting out the infected snails in the normal saline. These stages can be taken out on a slide with the help of a fine dropper and can be studied alive and after staining with intra vital stains as mentioned above. The measurements of the various body organs of sporocysts, rediae and cercariae should be taken with the help of an eye-piece micrometer.

(b) *Permanent mounts*

The cercariae can be killed by putting them in a drop of water on a slide and holding it over a flame. They can also be killed and fixed on a slide by hot Bouin's fixative or by hot 70% alcohol. The cercariae should be placed on a slide under a cover slip and few drops of hot alcoholic Bouin's solution or 70% alcohol are put on one side of the cover slip and from the other side the fluid is drained out by means of a piece of filter or blotting paper. This will not only kill the cercariae, but also fix them on to the slide. The cover slip should be removed carefully, and if the Bouin's solution is used it should be washed well with alcohol before using any stain. The cercariae are then dehydrated in different grades of alcohol, and then kept in xylol and mounted in a thin film of Canada balsam.

REFERENCES

MUKHERJEE, R. P. 1962. Studies on some amphistomatous trematodes of domesticated animals.—*Agra Univ. J. Res.* (*Sci*). 11(2) : 131-135.

PORTER, A. 1938. The larval trematodes found in certain South African mollusca with special reference to schisto-somiasis (Bilharziasis).— *Publ. Sth. African Inst. Med. Res.* No. 42 : 1-492.

SEWELL, R. B. S. 1922. Cercariae indicae. — *Indian J. Med. Res.* (Suppl.) 10 : 1-376.

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COLLECTION AND PRESERVATION OF TREMATODES AND CESTODES

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The trematodes and cestodes play a very significant role in the day-to-day economy of man and his domestic animals. The trematodes, occuring as active adults in vertebrates, comprise two broad divisions, based on their life histories - the monogenetic and the digenetic. The digenetic trematodes are a more important group, found practically in every part of the body of the final host, and require one or more intermediate hosts for completing their life-cycle. The monogenetic trematodes are ectoparasitic, and their entire life-cycles are completed in a single host. The cestodes, are mainly found as adults in the intestine of various vertebrates and as larvae in practically every body part of the intermediate host.

I. COLLECTION

For collection of the parasitic helminths, the host is carefully dissected and the gut and other prospective visceral parts are transferred to 1% saline, when they are likely to leave the site of infection. In case of very small trematodes, it is necessary to separate the gross contents by decantation. If alive and scanty, they can be discerned by their movements. For this aid of an hand lens or a microscope may be necessary.

II. PRESERVATION

After collection, they are cleaned in 1% saline. The smaller forms should be transferred to a clean slide with a pipette and fixed in any one of the following fixatives : Bouin's fluid, 70% alcohol, 2-5% Formalin, or Alcohol-Formalin-Acetic acid fixative (Commercial Formalin (10 pts), 95% alcohol (50 pts), Glacial Acetic acid (2 pts), Distilled water (40 pts). Then they may be very carefully set under a cover slip. The preliminary fixation of large forms requires a slight variation. Sometimes, narcotisation with rectified spirit etc., may be necessary. Also the larger forms are set between two slides held together by rubber bands or by winding a thread. Very long cestodes are coiled carefully round a suitable base, before being immersed in the fixative. After the specimens are allowed to set in the fixative, they should be finally transferred to 70% alcohol.

The stains generally used are Borax Carmine, or Aceto-Carmine, or Ehrlich's Haematoxylin. For proper examination, permanent mounts in Canada Balsam are to be made.

Sometimes, it may be necessary to examine specimens in live condition.

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ESTIMATION OF HELMINTHIC INFECTIONS

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Helminths are an important group of animal parasites occurring in the adult stage in vertebrate hosts, practically invading every organ system of the host, and in the larval stages in the invertebrate hosts. They are wide spread in all animals above the protozoans in almost every part of the world, though the intensity of the infection may differ from time to time or place to place. Secondly, they produce a wide variety of direct effects. Thus, they play a vital role in undermining the welfare of man and the animals with which he is associated to a smaller or greater extent. The latter is mainly responsible for the development of the helminthological research, specially by the veterinary parasitologists all over the world.

A perusal of helminthological literature from India, indicates that more attention has been paid to the taxonomic studies of these worms. It is needless to state that, while taxonomy forms the backbone of all biological investigations, it alone does not fulfill the objectives of medical, veterinary, and other parasitologists. Very little effort has been made to ascertain the extent of damage caused and losses incurred due to these helminths. Information on the reservoir hosts, exact distribution, bionomics, etc., with regard to most of the species is still wanting. These lacunae are mainly responsible for our failure in combating the helminthic infections.

The parasites, whether helminths or other groups, are always under the influence of two types of environments, viz., the host body providing the first type, while the external environment in which the host itself is living forming the second type. It is the inter action of the influence of this environment and the strategy adopted by the parasite to counter influence that develops the resistance potential of the parasite, host specificity, and host parasite relationships etc. To plan an all out war against the parasitic diseases, therefore, one needs to study several of the bioecological factors including the host-parasite relationship.

From the bio-medical point of view, we have to assess first the parasite intensity or concentration index, and the parasite frequency index. These two factors are outlined below :

I. PARASITE INTENSITY OR CONCENTRATION INDEX

In order to calculate the intensity or concentration index, the number of individual hosts and number of each parasite species encountered have to be recorded. The degree of infection can be grouped into four categories : negative (nil), light (1-9 parasites), medium (10-49 parasites), and heavy (over 50 parasites). The average number of each parasite species in each host in a given ecological niche indicate the parasite intensity or concentration index for that parasite species in that niche. Likewise, the concentration index with respect of each parasite species in different ecological niches have to be calculated. The average of the aggregate index of each parasite species in different ecological niches with reference to an individual or group of host species gives the total intensity of the infection or concentration index of the parasite in a given area, ecosystem.

II. PARASITE FREQUENCY INDEX

The parasite frequency index is calculated by taking the percentage of the number of hosts infected by an individual parasite species against the total number of hosts examined in a particular area under investigation. The number of parasites recovered from a single host is not taken into consideration. This frequency index can also be classified into : rare (0.1-9%), occasional (10-29%), common (30-69%), and abundant (70-100%). The parasite frequency index may be calculated for each ecological niche, or for the entire area under investigation.

A generalized data sheet is given, which can be used while collecting the data.

By combining these two indices, a graphic picture of both the intensity and frequency of the parasitic infection can be obtained in any ecosystem or with reference to a given area for that period of observation.

These indices, if calculated for each host (both vertebrate and invertebrate) will give us the nature and status of infection. If charts for each parasite, host, or area-wise, over long period are prepared, they will not only give us the parasite burden, but also the different physico-chemical, or biological factors influencing the parasite infections, which will in turn aid in formulating an effective control programme against the helminthic diseases.

For additional information, Cinderman (1953), Petrushevski and Petrushevskaya (1960), and Paperna (1964) are recommended.

REFERENCES

CINDERMAN, C. J. 1953. *Fisheries Report for lakes of North Central Massachusetts.* [Div Fish. & Game, Dept. Conservation] Boston, (1950) : 1-28.

PAPERNA, I. 1964. The metazoan parasite fauna of Israel inland water fishes.- *Bamidgeh,* 16 (1/2) : 1-66.

PETRUSHEVSKI, G. K. and PETRUSHEVSKAYA, M. G. 1960. The accuracy of quantitative indices under the study of parasito-fauna of fishes.- *Paras. Sobr. Zool.,* 19 : 333-343.

DATA SHEET

Serial No.
Date.
Locality
Name of host
Number of host examined
Number of host found infected
Location of parasite
Location of parasite recovered
Name of parasite recovered
Number of parasite recovered

$$\text{Frequency index} = \frac{I}{E} \times 100,$$ where I = Total Number of host infected and E = Total Number of host examined.

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DETECTION AND RECOVERY OF EGGS AND INFECTIVE LARVAE OF GASTRO-INTESTINAL NEMATODES FROM FAECAL SAMPLE OF SHEEP AND GOATS

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The definitive diagnosis of the intestinal helminthic diseases has always been either on the identification of the parasites after post-mortem examination or the identification of their eggs in the faeces host. In majority of cases the latter method of diagnosis is not reliable because of similarity in the size and shape of eggs. Morphological characters of third stage larvae, therefore, would be useful in identification of the species and for estimating the intensity of infestation. Differentiation of the eggs and larvae of the numerous strongyle and trichostrongyle worms, which infest sheep and goats is essential for a precise ante-mortem diagnosis. Differentiation of the infective larvae, chiefly based on the length of the tail, is relatively simple, and may be carried out quickly and accurately, without actual measurements, by some experience.

The faecal samples are by far the most important because the eggs of larvae of all gastro-intestinal nematodes leave the body through this medium. No eggs will however, be found if the worms are still immature, or if only males are present, and if the host animals do not harbour the helminthic infections.

Fresh faecal sample is directly collected from the rectum of the animal so that the faeces does not get contaminated. For routine work, the selection of a few simple methods, is desirable than the exposition of all the known methods.

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A. EGG RECOVERY TECHNIQUES

(i) *Direct Smear*

A small quantity of faeces is placed on a glass slide mixed with a drop of water, spread out with the tip of a thick needle and examined directly under a low power objective of a microscope. A few slides should be examined from the same faecal sample to come to any conclusion, as lighter infestations may escape our notice. This method is suitable for a very rapid examination.

(ii) *Concentration Method*

The purpose of this method is to detect lighter infestations. This enables saving of time and also eliminates the disturbing large faecal particles. The low specific gravity of most nematode eggs is an added advantage for separating them from the faeces. About 1 gm of the faecal specimen is thoroughly mixed with 20 ml., of concentrated salt solution in a suitable narrow cylinder which is filled to the brim with the liquid. A clean slide is placed over the top of the cylinder, so that it is in contact with the liquid. Care should be taken that no air bubbles come between the slide and the salt solution. After about 45 minutes the slide is quickly removed with a straight upward motion and examined under a low power objective of a microscope.

(iii) *Centrifugal Floatation*

In this method greater accuracy is obtained. A faecal sample, about 25 gm is well mixed with about 500 ml., of water and strained through a seive of 1 mm., mesh. The mixture is left to settle for 15 minutes and the supernatant fluid poured off leaving the sediment. This washing is repeated several times till the sediment is quite clear. The sediment is stirred and a sample is taken into a centrifuge tube, mixed thoroughly with an equal volume of concentrated solution of sugar or salt, and centrifuged at 1000 r.p.m. for 2 minutes. The nematode eggs collected at the top layer may be examined as stated above.

B. EGG COUNTING TECHNIQUE

For a quantitative estimation of the worm load the number of eggs/gm faeces should be determined. For determining eggs/gm of faeces Chandler's (1925) modification of Stoll's method is quite suitable.

C. CULTURING

(i) *Faecal Culture*

The species identification of gastro-intestinal nematodes can not always be made accurately from the eggs ; therefore, it will be necessary to cultivate the larvae from these eggs. For this purpose, a quantity of faeces is broken up and placed in a glass jar, then closed and kept at a temperature of about 27°C., for a suitable time — usually 7 days. After the incubation, the jar is placed in dull light and larvae of many species will creep on the wall of the jar. Then, they can be removed with a needle, or fine brush for examination in a drop of water on a glass slide.

Another suitable method is described by Roberts and O'sullivan (1950). Equal amount of sterilized sheep faeces is mixed with the above fresh faeces. Instead of sterilized faeces, fine saw dust may also be added to the faeces culture. Due to the granular nature, it mixes well with faeces, and at the same time does not form a thick paste as in the case with the sterilized faeces. It also forms a porous mass and sufficient aeration is available for the developing larvae during incubation period. For collecting infective larvae, the jars are filled upto the brim with tap water at 45° C., and covered by petridishes. The petridish is firmly held in position and the bottle is then carefully inverted upside down. A little water is put in the petridishes so that migrating larvae are not dried. After about 12 hours nearly all the larvae migrate through the culture medium and collect in the petridishes. The larvae in water can be pipetted out in clean specimen tubes.

(ii) *Smear Method*

About 5 gm. faeces is converted into thick paste on a clean glass plate by means of a spatula and spread uniformly over a small square piece of white *khadi* cloth. The slides thus prepared are placed in petridishes covered with lids lined with moist filter paper to prevent drying of cultures. The cultures are maintained at 27° C. for a week or more. On 8th day infective larvae are separated from the culture with a Baerman's apparatus.

D. FIXATION AND EXAMINATION OF THE LARVAE

For studying infective larvae, it is advisible to kill the larvae by heating the glass slides carefully over a small spirit lamp flame until movement ceases and infective larvae lie fully extended. Earlier literature on the subject has been reviewed by Dikmans and Andrews (1933) in their comprehensive article on the comparative morphology of the infective larvae of common nematodes of sheep. Several other workers, *viz.* Threlkeld (1934 and 1946), Andrews (1935). Mayhew (1939), Sprent (1946), Keith (1953), Wetzal and Marholdt (1955) and Tripathi (1968) also have described the morphological characters of the infective larvae of species belonging to the following genera *Haemonchus, Bunostomum, Oesophagostomum, Strongyloides, Trichostrongylus, Ostertagi, Cooperia, Nematodirus* and *Chabertia* etc. infesting sheep and goats.

REFERENCES

ANDREWS, J. S. 1935. A note on the morphology of the anterior ends of the infective larvae of some nematodes parasitic in the alimentary tract of sheep.- *Proc. Helminth. Soc. Wash.* 2 : 89.

CHANDLER, A. C. 1925. The species of *Strongyloides* (Nematoda). *Parasitology,* 17 : 426.

DIKMANS, G. and ANDREWS, J. S. 1933. A comparative morphological study of the infective larvae of the common nematodes parasitic in the alimentary tract of sheep. *Trans. Amer. Micros. Soc.,* 52 (1) : 1-25.

KEITH, R. K. 1953. The differentiation of infective larvae of some common nematode parasites of cattle.- *Austr. J. Zool.,* 1 : 223-235.

MAYHEW, R. L. 1939. Studies on bovine gastro-intestinal parasites I. The mode of infection of the hookworms and nodular worm. - *Cornell Vet.,* 29 : 367-376.

ROBERTS, F. H. S. and O'Sullivan, P. J. 1950. Methods for egg counts and larval cultures for strongyles infesting the gastro-intestinal tract of Cattle. - *Austr. J. agric. Res.,* 1 : 99-102.

SPRENT, J. F. 1946. Some observations on the bionomics of *Bunostomum phlebotomum* a hookworm of Cattle. - *Parasitology,* 37 : 202.

THRELKELD, W. L. 1934. The life history of *Ostertagia circumcincta*. - *Tech. Bull. Va. Agric. Expt. Sta.*, 52 : 24.

THRELKELD, W. L. 1946. Helminth parasites in sheep. - *Vir. Agric. Exp. Sta. Bull.*, 68 : 46.

TRIPATHI, J. C. 1968. Morphological studies of infective larvae of some common gastro-intestinal nematodes of goats. - *Indian J. Helminth.*, 20 (1) : 57-69.

WETZEL, R. and MARHOLDT, D. 1955. Morphology of infective larvae. *Helm. Abstrs.*, 24 (3) : 224 (Abstract).

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COLLECTION AND PRESERVATION OF NEMATODE PARASITES OF ANIMALS

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Nematodes, play a significant role in the economy of man. They are very successfully adapted to a variety of habitats. Widely distributed as free-living forms in different terrestrial and aquatic habitats, they are also found parasitic in several hosts, both plants and animals including man.

Nematodes parasitic in vertebrates show a wide range of adaptability. In these hosts, these worms are usually found in the alimentary canal. However, they may be found in the body cavity, lungs, heart, blood vessels, urinogenital system etc. Very often they have also been found in the connective tissue, serous membrane, oral, nasal and orbital cavities. Their larvae, usually encysted, have been found unrestrictedly distributed in the host body.

I. COLLECTION

The collection of parasitic nematodes begins first, by thoroughly exploring all parts of a suspected host. To avoid the deleterious effects of the post-mortem changes of the host, these parasites, both intestinal and extra-intestinal, are collected soon after the death of the host animal. The extra-intestinal nematodes may be found in nodules or tumours on the wall of the alimentary canal or embedded deep in the tissues. It is desirable to note the exact site of infection in the host body, where the parasite has been primarily located.

The intestinal nematodes are collected by opening the alimentary canal lengthwise with a pair of blunt-pointed scissors and collecting the entire contents. The mucous membrane is then washed in 1% saline. The gut content, washings of the mucous membrane and the mucous membrane itself need to be thoroughly examined for the parasites.

The portions of the infected body parts and washings with nematodes (if collected in water) should not be kept for a long time, because the worms, particularly the delicate ones are likely to be damaged in water. In case of smaller hosts the gut is cut open, thoroughly submerged in saline and kept in a suitable dissecting tray or a petri dish.

In case of nematodes infesting bird caeca, it is advisable to scrap out the mucous membrane in saline and shake the liquid. Nematodes, if present, will settle down at the bottom, if the saline is allowed to stand for some time. The worms are collected after decanting the supernatant.

II. PRESERVATION

Intestinal worms, collected are washed thoroughly to remove extraneous materials adhering to them. Then they are laid straight in a clean petri dish and some steaming 70% alcohol is poured over so as to submerge them completely. For tissue-inhabiting nematodes, cold alcohol is preferably used to prevent damage to the specimens. When the specimens are satisfactorily fixed, usually after a few minutes, they are transferred to 70% glycerine-alcohol mixture for storage in sealed containers.

Temporary mounts are preferable to permanent ones, and hence only these are discussed here. Whenever, the specimen is to be examined, it may be cleared either in Creosote (beechwood) or Lactophenol. Before re-storing them into the 70% glycerine-alcohol, they should be washed for a few minutes in 70% acid alcohol to prevent their darkening. In case of very delicate and small nematodes, glycerine may be substituted as clearing agent, but manipulation of the specimen will be hindered. For special type of examination, such as an end-on view of the head, it is necessary to detach the head with a clean knife and roll in the desired position under a cover glass effectively supported with four small plasticine "feet" at the coners.

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COLLECTION AND PRESERVATION OF ACANTHOCEPHALA

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Acanthocephala were first noticed by Redi (1684 see also 1708) and later by Leeuwenhoek (1695). Koelreuther (1771) named them as 'Acanthecephali', because of the hooks on the head. Acanthocephala is an important group of animal endoparasites, occurring in the adult stage widely, in vertebrates including those of direct economic importance to man. Though, Chandler (1925) reported for the first time a new species from the intestine of a Calcutta cat, but Thapur (1927) was the first to initiate systematic studies on this group in India.

The Acanthocephala are pseudocoelomate worms, usually elongated and cylindrical, ranging from 1.5 mm-65 cm. The smaller species are the parasites of fishes (Neoechinorhynchidea), and the large and specialized are parasitic in mammals (Gigantorhynchidea) (cf. Szidat Rule in Eichler 1949). The shape varies from plump, fusiform, clavate, long and slender to even laterally flattened forms. They may be straight, bowed, or rarely coiled. Where the species is curved, the concave side is referred to as the ventral side, while in others, this side is determined with reference to the nerve ganglion on the proboscis sheath, or the larger hooks on the proboscis, since both are located on the ventral side. The body surface may be smooth, wrinkled, or pseudometameric. The body is divided into a short, narrow *presoma*, and a longer and stout body proper or the trunk. The *presoma* comprises externally a proboscis bearing the hooks, and a neck devoid of hooks ; and internally a proboscis sheath, or receptacle into which the proboscis retracts or invaginates, the liminisici and the nerve ganglion located on the proboscis sheath. The neck is generally short, and rarely very long, and with or without a bulbous swelling. The trunk is either spinose or aspinose. The body wall is composed of a cuticle, epidermis (hypodermis), and the dermis. The inner layer of the hypodermis is provided with lacunar canal and nuclei. The digestive system is absent. The excretory system, when present, consists of protonephridia or a branched mass of flame bulbs attached to a stem. The sexes are separate. The females are larger than the males. The gonopore lies at or near the posterior end. There are a pair of testes, sperm ducts, and a seminal vesicle, also clusters of unicellular gland cells known as the cement glands, or the prostrate glands, and a muscular bursa in the male. In the female there is a single or paired ovaries which break into a large number of ovarian balls, they remain floating for some time in the dorsal ligamental sac, and then liberated in to the body cavity after bursting of the sac. There is a muscular funnel-shaped uterine bell leading into an uterine tube, followed by a uterus which leads to a vagina. The latter opens to the exterior by the genital pore.

The life-history of these worms involves at least one intermediate host (an arthropod), or sometimes even two intermediate hosts, of which the second host is referred to as a transport or carrier host (generally a lower vertebrate). The mature eggs are discharged by the female and are transported to the outside by the host faeces. The eggs when swallowed by the arthropod intermediate host hatch immediately into an *Acanthor*. The *Acanthor* burrows through the intestinal wall, and either encysts on the outer intestinal wall, (as in Cockroach), or remains in the body cavity (as in the case of Ostracods). Several weeks after, it develops into the next stage larva, the *Acanthella*, which is infective stage. If the Second intermediate host (a lower vertebrate) eats the first intermediate host, the *Acanthella* burrows through the intestinal wall and then encysts in the liver, till the final (definitive) host ingests the second intermediate host, when the *Acanthella* develops into an adult. In the case of the cockroach, the final development takes place, when rats eat the cockroach, and here the carrier host is absent.

The group is divided into four orders *viz*., Apororhynchidea (with a single family), Neoechinorhynchidea, Echinorhynchidea, and Gigantorhynchidea (all with several families).

I. METHOD OF COLLECTION

Specimens are preferably collected from the freshly killed host animals (cysts from the outer intestinal wall of the insect, liver of lower vertebrates, and adults from the intestine of the vertebrate host). The adult worms are kept in distilled water for several hours, or left overnight in a refrigerator so that they die in a relaxed condition.

The specimens are then pressed between a slide and a rectangular cover slip, and wound round by a thread, and fixed in AFA solution

(Alcohol 96% (100 ml.) : Formaline 40% (30 ml.) : Acetic acid (5 ml.) and dist. water (200 ml.) from few hrs., to overnight. Other fixatives usually used are the 10% neutral Formaline, Bouin's fluid, and sometimes 70% alchohol. After the specimens are properly fixed, they are transferred into 70% alcohol with a little (5%) Glycerine, and stored indefinitely in this medium.

The eggs are collected from the body cavity of the female or the uterus, etc., or from the host faeces also. They are of different shapes, and outer shell sculpturing ; sometimes the middle shell is prolonged or the embryo is provided with minute spines or larval hooklets, and therefore they also help in the identification of the species.

II. TEMPORARY MOUNTS

The eggs are usually studied in Ringer's solution, or normal saline in the live condition (if necessary, permanent mounts can be prepared). The adults are studied either in the live condition in normal Saline, or in Creosote.

III. PERMANENT PREPARATIONS

The specimens are pricked at different places with entomological pin (No. 16 or 20) and transferred to Grenacher's Borax Carmine solution. The material is allowed to remain in the stain for 7-8 hrs, and a drop of Conc. HCl, is added to the stain, (1 drop of acid for every 5 ml., of stain is required), and then thoroughly mixed by quick shaking of the tube. The specimens are left overnight in the stain again, and then transferred to acid alcohol (10% HCl., in 70% alcohol). The material attains a light pink colour in the acid alcohol. The specimens should not remain in acid alcohol for long time. The stained specimens are dehydrated in alcohol grades *viz.*, 90% (three changes), Abs. alcohol (two changes), and in xylol (once) and left in Clove oil overnight. The material is again transferred to Xylol to remove the traces of the Clove oil, and mounted in the D.P.X. mountant.

Another method, which gives better results is as follows :—

The specimens after staining, and washing in acid alcohol, are processed through 90% Abs. alcohol, 25%, 50%, and 75% Terpineol (each treatment may be given for 6-18 hrs), and pure Terpineol (for about 18-24 hrs. and mounted in the D.P.X. mountant.

References

CHANDLER, A. C. 1925. The helminthic parasites of cats in Calcutta and the relation of cats to human helminthic infections.— *Indian J. med. Res.*, 13 : 213.

EICHLER, Wd. 1949. Some rules in ecto-parasitism.— *Ann. Mag. nat. Hist.*, (12) 1 : 588-598.

KOELREUTHER, J. T. 1771. Descriptio cyprini rutili quem Halawel Russi vocant, historie-anatomica.—*Nov. Comment. Ac. Sc.* Petrogr., 15 : 494.

LEEUWENHOEK, A. 1695. *Arcana naturae.* Frontispiece, 2 p.l. [Delphis] 568 pp., 7 1., 27 pls. port.

REDI, F. 1684. *Osservazioni...intorno agli animali viventi che si trovano negli animali viventi.*, [Firenze] 2 p.l. : 253 pp. 26 pls.

REDI, F. 1708. *De animalculis vivis, quae in corporibus animalium viventi um reperiuntur observationes, Ex etruscis latinas fecit Petrus Costae* [Amstelaedami] (Transl. *cf.* 1684) 11 p.l. : 342 pp. 1 1., 26 pls.

THAPAR, G. S. 1927. On *Acanthogyrus* n. G. from the intestine of the Indian fish *Labeo rohita*, with a note on the classification of the Acanthocephala.— *J. Helminth.*, 5(2) : 109-120.

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THE COLLECTION AND PRESERVATION OF PHTHIRAPTERA (INSECTA)

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The order Phthiraptera includes small, flattened ectoparasitic insects, popularly referred to as the 'biting-, bird-, or chewing-lice' (Mallophaga *s.l.*) and the 'true-, or sucking-lice' (Siphunculata or Anoplura *auct.*). The order is now considered to contain four suborders *viz.*, Amblycerophthirina (=Amblycera), Ischnocerophthirina (=Ischnocera), Rhynchophthirina, and Siphunculophthirina (=Siphunculata) (Clay, 1970; Lakshminarayana, 1970, 1976). The first three suborders (or superfamilies of some) constitute the erstwhile Mallophaga. While the Amblycerophthirina and Ischnocerophthirina are parasitic both on birds and mammals, the Rhyncophthirina and Siphunculophthirina are exclusively parasitic on mammals.

The chewing-lice have usually biting type of mouthparts with dentated mandibles, thoracic segmentation, two segmented tarsi with single or paired claws, and ventrally placed thoracic spiracles. In contrast the sucking-lice have highly modified mouth parts for piercing and sucking, with obliterated or fused thoracic segmentation, single segmented tarsi with unpaired claws, and dorsally placed thoracic spiracles. The Ischnocerophthirina and Amblycerophthirina normally feed on the plumage or pelage, and in addition may ingest blood also, while the Rhynchophthirina feed on sebaceous matter and blood like the Siphunculophthirina.

The lice normally cause considerable annoyance to their hosts by their movement and feeding. The damage caused to the wool by the sheep infesting chewing-lice and fall in egg production and even death in heavily infested poultry birds are well known. The chewing-lice are known to transmit typhus, rickettsias, and some are definitive vectors of bird filaria, dog tape-worm, infectious equine anaemia, and produce dermatitis. Because of the sucking habits, the sucking-lice definitely inject toxins and are known to transmit typhus, trench-fever, relapsing fever and probably trachoma in man and tularaemia, murine typhus, surra, hog-cholera, rat trypansomiasis, canine Leishmaniasis and the dog filariasis.

I. METHODS OF COLLECTION

The Amblycerophthirina are not normally restricted to any part of the host body, but the Ischnocerophthirina have selected ecological niches. The lice of mammals are tightly attached to the hairs, and often it is difficult to extricate them. A list of references on the methods of collection and preservation were given by Kéler (1939) and Eichler and Zotorzycka (1969). Hopkins (1949), and Lakshminarayana (1977) discussed in detail the collection techniques for Phthiraptera and the same procedure more or less universally followed with slight modifications. Phthiraptera can be collected by any of the following methods :

(1) Mechanical

(a) *Hand Picking*

Using a fine watch-repairer's forceps (BB or NN) one can conveniently pick up the lice. Plucking the barbs of the feathers, or hairs, holding a little below the louse will avoid crushing of the specimen in the process. Though laborious, the method can be used for collecting the lice from a live host.

(b) *Brushing Technique*

The host (a mammal or bird) should be placed in a separate polythene bag, and the lice which can be seen actively moving about, should preferably be killed by Chloroform, Ether, or Carbon disulphide, sprinkled over a cotton swab kept inside the bag. Care should be taken so that the fumigant should not come in direct contact with the host body, with the head always kept outside of the fumigating jar or bag (when a live host is to be fumigated), and that each host is fumigated in a separate bag to avoid contamination. After about thirty minutes, the live animal or the skin should be vigorously tapped or rubbed with hand over a clean (preferably white) sheet. The dropped scales, hairs, feathers, and the dust should be exmined for the ectoparasites. Some parasites also leave the host body when fumigated, and therefore, the empty bag may also be examined for the parasites. The specimens can be preserved in 70-95% alcohol.

Dusting the host animal with pyrethrum powder, sodium flouride (Rao *et al.*, 1954) or silicon dioxide and ammonium silicon dioxide and ammonium silicoflouride or 4% ammonium flourosilicate and commercially available as 'DRI-DIE or DRY-DIE' (Biswas, *in litt.*) (this method is particularly useful for collecting the parasites on migratory birds), and spraying with diluted DDT (Mani, *in litt.*) also yield the parasites.

(2) CHEMICAL EXTRACTION (DISSOLVING TECHNIQUE)

This procedure is useful for extracting parasites from a dead host and cannot be used, if the skin of the animal is required for identification or permanent preservation.

Hopkins (1949) described it in detail and it is more useful for mammal infesting forms. Briefly, the procedure is as follows : Pieces of freshly killed or dried host skin should be placed in 5-10% potassium or sodium hydroxide (KOH or NaOH) solution, until the hair becomes soft and can be scrapped off. The scrapped and partly softened hairs were then heated with additional quantity of 5% KOH or NaOH solution over a water bath, till the hairs dissolve completely. The contents of the beaker were then filtered, while hot, through a fine mesh of stainless steel gauge. The solid residue on the gauge was washed well into a petridish and examined for the parasites, which if present, can be preserved in 70-95% alcohol.

Hopkins' method was used by several workers for extracting bird parasites also (Ash, 1960 and Ledger, 1970). Recently, Hilton (1970) slightly modified the technique. The modifications involve essentially the following : the entire host animal can be kept for about 12-24 hrs depending on the size in KOH solution ; heating the scrapped hair or feathers for about 1-2 hrs ; keeping the solution for about 12 hrs till the solid material like the parasites settle down ; discarding the supernatant liquid and centrifuging the solid residue with a little KOH solution for about 5 minutes at about 1200 r.p.m. ; discarding the supernatant solution in the centrifuge tubes and refilling them with $ZnSO_4$ solution (386 gm of Zinc sulphate in 1000 ml. of water) ; centrifuging the solution a second time ; decanting or removing with an aspirator the bodies of the parasites that float over the $ZnSO_4$ solution.

In this process care should be taken to avoid handling the KOH or NaOH solution with bare hands and also inhalation of the hot fumes.

This method of collection is useful for making the whole mounts and for section cutting purposes this method is not suitable (*vide infra*).

II. METHODS OF PRESERVATION

The material collected can be conveniently preserved in a liquid medium or mounted on slides.

(1) Storing in a liquid medium

The material can be preserved in small glass vials containing 70-95% alcohol, plugged with cotton or air tight plastic stoppers (cork stoppers should not be used), which are in turn placed in a large jar containing 95% alcohol. Hopkins (1949) quoted Werneck's technique in which the mouths of the vials were sealed over a blow pipe flame.

(2) Whole mounts

(*a*) *Resin mounts*

The freshly collected material (if not already treated with 10% KOH or NaOH during extraction) should be heated for about 20 minutes, or even less (depending on the size) over a water bath (or the specimens were left overnight) in 10% KOH or NaOH solution. With the help of entomological pins No. 16 the gut should be punctured through the intersegmental membrane. The specimens were then gently pressed with the head of the pin, so that the inner contents drain out. The specimens were washed well through three or four washing with water, and gradually passed through the ascending series of alcohols, *viz.*, 70% to Absolute alcohol. Alcohol-Xylol (50 : 50), Xylol, and finally mounted on glass slides in Canada balsam.

Elbel (1967) quoted Emerson's procedure of mounting. In this process, after treating in KOH or NaOH solution, the specimens were washed well with water (about 20 min.) and then placed in 40% alcohol (15 min.). They were then transferred to a solution of Ziehl Nielson's Carbol-Fuchsin (see below) for about 30 minutes. The material then passed through 70% alcohol (30 min.), 95% alcohol (15 min.) washed in Abs. alcohol, and then placed in Beechwood creosote (1 hr to overnight), and finally mounted in Damar gum or Canada balsam.

The present author found an alternate method most suitable where in, the processing through the ascending series of alcohols and xylol could be avoided by the present author (Lakshminarayana, 1970, 1977). This method was originally used for mounting aphids, and was equally good for Phthiraptera. In this process, after clearing the inner contents in 10% KOH or NaOH solution, the specimens were washed well with water, and then transferred into a cavity block (embryo dish) containing glacial Acetic acid for about 15-30 minutes (two or three washings would be ideal), and then transferred to another cavity block containing Clove oil. The material should be left in Clove oil till it attains sufficient clearance (which normally takes about 10 min.), and then mounted in Canada balsam.

(b) *Gum chloral mounts*

Kéler (1960) used Faure's Gum Chloral mountant (Hopkins, 1949). The essential ingredients of Faure's medium are : Chloral hydrate (50 gm), gum Arabic (30 gm), Glycerine (30 ml), dist. water (50 ml), which are mixed well and filtered through bolting silk. The treated specimens either from water or alcohol were placed in a mixture of equal volumes of Chloral hydrate and Lacto-Phenol and then mounted in the mountant.

Symmons (1952) suggested the use of Swan's mountant. She treated the specimens with Lactic acid for some time over a hot plate and when sufficiently cleared, were transferred into liquid Phenol for 30 min., and mounted in Swan's mountant. Swan's mountant consists of the following : dist. water 20 ml., gum Arabic (15 gm), Chloral hydrate (60 gm), Glucose syrup (10 ml.), Acetic acid (5 ml.).

For Indian conditions, experience shows that Chloral hydrate mountants are not well suited for permanent slides.

(c) *Polyvinyl Alcohol-Lacto-Phenol mountant*

Hopkins (1949) stated that he used Polyvinyl alcohol-Lacto Phenol medium. Boudreaux and Dosse (1963) gave a modified formula following after Heinze for mounting mites. This mountant was found quite suitable for other arthropods also. The mountant can be prepared by the following method : Polyvinyl alcohol (10gm) and dist. water (40-60 ml.) were mixed in a large beaker and then gently heated over water bath, stirring constantly. To this solution Lactic acid (30 ml.) was added, stirred well, and then Glycerine (10ml.) was also added to the mixture. Another mixture containing Chloral hydrate (100gm) in Phenol-water solution (1.5%) (25ml.) was prepared and both the mixtures were added together. The polyvinyl alcohol-Lacto-phenol mixture was then filtered through a filter paper in a suction funnel. The treated specimens can be mounted directly in this medium. Edwards (1961) states that the disadvantages of using Polyvinyl alcohol is that it does not completely clean the matter in the alimentary tract, the slides must be examined periodically to fill in the vacuities that appear under the coverslip, and the medium occasionally hardens with dispersion of small crystal like objects in it.

(3) SERIAL SECTION MOUNTS

Symmons (1952) described section cutting and mounting of the serial sections. In this method, live specimens of Phthiraptera have to be fixed in hot Bouin (60°C.) or Zenker's solution and the lice were dehydrated and cleared for few hours to 1-2 days in Supercedrol. Celloidin mixture (2-6%) is prepared with equal volume of absolute alcohol and ether, and then diluted further in Supercedrol so as to make 1¼ celloidin solution. The dehydrated specimens were then transferred into celloidin solution in which they were left over for a day or two. The specimens were then placed in a saturated mixture of Chloroform and wax at 37-40°C., for about 12 hours. They were then removed to melted wax (M 60°C., and previously heated to smoking point and filtered) in a vacuum embedding bath. Impregnation of wax under vacuum may take about 2 hours. Embedding of the specimens in watch glass, embryo dish, or paper boat, section cutting, and mounting can be carried in the conventional way.

(4) STAINING

Normally, the phthiraptera are heavily sclerotized and therefore, staining of the specimens is not required. However, the following stains would be useful wherever required.

(a) *Ziehl Nielson's Carbol-Fuchsin*

Elbel (1967) quoted Emerson's technique in which this stain is employed. The stain consists of the following : basic Fuchsin (1 gm), acid Carbolic (5 gm), Alcohol (10 ml.), and dist. water (100 ml.).

(*b*) *Acid Fuchsin*

The present author used acid fuchsin (B. D. H.) satisfactorily (Lakshminarayana, 1970, 1977). After processing the specimens through KOH, water, Acetic acid, the specimens were kept for few seconds in acid fuchsin in 70% alcohol. The specimens were then differentiated to the desirable degree in acetic acid and cleared in Clove oil. Mounting was done as given above.

(*c*) *Symmon's modified Heidenhain's Azan stain*

This stain is useful for staining microtome sections. Symmons (1952) suggested the modified method using Heidenhain's Azan stain. In this process, after removing the wax from the serial scctions and the conventional way of passing the sections through the descending grades of alcohols and water, the slides were placed in Azocaramine solution for few minutes at room temperature. The sections were slightly overstained, and if necessary differentiated with aniline water. They were then rinsed in water, and mordanted in 10% Phosphotungstic acid in water, for the Aniline Blue, and fixed for the red dye for about an hour. The slides were then washed and placed in Azan solution containing Aniline Blue and orange G for about 30 mins. The excess stain was removed with 90% alcohol, followed by changes with Absolute alcohol, Absolute alcohol and xylol mixture (50 : 50), cleared in xylol, and mounted in Canada balsam.

(5) SCANNING ELECTRON MICROSCOPE PREPARATIONS

Attempts to utilize scanning microscope for the study of Phthiraptera have been made elsewhere (Clay. 1959, 1960). The material for the study has to be processed through a special technique and is not discussed here, since the scanning microscope is not usually available for many workers. However, those interested may refer to Grimstone (1970).

III. LABELLING

For all parasitic groups the host name is necessary, hence *proper identification of the host is a must.* The following data have to be provided both for wet collections as well as for the slides *viz.*, Tube No., Host, Locality, Date of collection, and Collector's name. The name of the species, its sex along with the name of the person who determined it has to be provided on another label. It is preferable to have a uniform labels for the slides. The left hand label should bear the collection particulars, and the right hand one, the name of the species etc., and the registered number. It is always desirable to mount single specimen on a slide and the labels be pasted on either side of the cover slip, parallel to the anteroposterior axis of the specimen in the centre.

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REFERENCES

ASH, J. S. 1960. A study of the Mallophaga of birds with particular reference to their ecology.— *Ibis*, 102 : 93-110.

BISWAS. B. *Personal communication.*

COUDREAUX, I. B. & DOSSE, G. 1963. The usefulness of new taxonomic characters in females of the genus *Tetranychus* Dufour (Acari : Tetranychidae). *Acarologia*, 5 : 13-33, figs 1-23.

CLAY, T. 1969. A key to the genera of the Menoponidae (Amblycera : Mallophaga : Insecta).—*Bull. Br. Mus.(nat. Hist.) Ent.*, 24 (1) : 1-26, 29 figs, 7 pls.

CLAY, T. 1970. The Amblycera (Phthiraptera : Insecta). — *Bull. Br. Mus. (nat. Hist.) Ent.*, 25(3) : 73-98, 9 figs. 7 pls.

EDWARDS, R. L. 1961. Studies of the Philopteridae (Mallophaga) from birds of the order *Procellariformes*. 1. The genus *Halipeurus* Thompson.—*J. Parasit.*, 47 (1) : 125-157.

EICHLER, WD. & RIBBECK, R. & ZOTORZYCKA, J. 1973. Zeitgenoessiche Mallophagen — Literatur (II). — *Mitt. Zool. Mus. Berl.* 49(2) : 423-461.

ELBEL, R. E. 1967. Amblyceran Mallophaga (Biting lice) found on the Bucerotidae (Hornbills).—*Proc. U.S. nat. Mus.*, 120, No. 3558 : 1-76, 72 figs.

GRIMSTONE, A. W. 1970. *The Electron Microscope in Biology*, / Edw. Arnold/ : 54.

HILTON, D.F.J. 1970. A technique for collecting ectoparasites from small birds and mammals. — *Can. J. Zool.*, 1445-1446.

HOPKINS, G. H. E. 1949. The host-associations of the lice of mammals. —*Proc. zool. Soc. Lond.*, 119(2) : 387-604.

KELER, S. 1939. Ueber eine erprobte Behandlungswethode alter Alkoholexemplare von Mallophagen. — *Z. ParasitKde*, 11 : 42-46.

KELER, S. 1960. Bibliographie der Mallophagen. — *Milt. Zool. Mus. Berl.*, 36 (2) 1 : 146-403.

LAKSHMINARAYANA, K. V 1970. *Systematic studies on Mallophaga on Indian birds and Mammals.*—Ph. D. thesis (Zoology), Agra Univ., Agra, I & II : xvii — 572 (Text) & A-U (Text-figs), 33 (Photomicrographs).

LAKSHMINARAYANA, K. V. 1975. On the collection and preservation of Phthiraptera (Insecta). — *Labdev. J. Sci. & Tech.* (Life Sci.), 13-B : 141-146.

LEDGER, J. A. 1969. Ectoparasite load in a laughing dove with a deformed mandible. — *Ostrich*, 41, : 191-194.

MANI, M. S. *Personal Communication.*

RAO, V. T., RAO, P. G., & RAO, P. V. 1954. *Guide showing methods to Control crop pests and diseases.* —/ Dept. Agric. & Fisheries, Andhra/, Bapatla, Bull. No. 1 : 1-141:

SYMMONS, S., 1952. Comparative anatomy of the mallophagan head.— *Trans. zool. Soc. Lond.*, 27(4) : 349-436, 60 figs.

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A NOTE ON THE *IN VITRO* STUDIES OF THE CHEWING-LICE (PHTHIRAPTERA)

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It is well known that in certain cases where sibling species are involved, the conventional morphological attributes of the adults may not help us in their identification, and one has to look to other criteria like the larval morphology behaviour, feeding habits, etc. While the study of developmental stages may be easy with other groups, it is not so in case of obligatory parasites like the 'chewing-lice' which show high degree of not only the host-specificity, but also niche-specificity. The life-span of individuals outside the host has been found insignificantly small, and is one of the reasons why these parasites rarely infest other hosts and secondarily establish on them. Another reason is their extreme food preference for the plumage or pelage of the natural host. Therefore, the biology and the nymphal taxonomy of very few species only are known and we know nothing of a very many species, especially those inhabiting non-domesticated host groups. Unfortunately, most of our laboratory collections also include only the adults, and rarely we find even a single nymph for examination, or collectors may ignore the juveniles while making the collections. At times, we do come across the nymphal instars and also the eggs or nits. In such cases, we may not be able to correlate them with their adult stages, unless they are also collected along with the nymphs, or when more than one species (say a sympatric one) harbours the host. We have no alternative than to rear them for study in the laboratory and the following will give a general outline of the requirements for the *in vitro* culture of the chewing-lice.

I. TEMPERATURE

Temperature plays an important role in *in vitro* culture, since the lice are very much conditioned to the host feather or hair cover which insulates them from the external atmospheric fluctuations in the tem-perature. The lice occupy different levels of the host body, as for example, under the feather cover, which varies from species to species and also for different nymphal instars and the eggs (Ash, 1960). Ash (*op. cit.*) also states that the actual skin temperature and at different levels of the feather cover differ from each other. Ignoring these facts might have been one of the reasons for the failure of the *in vitro* cultures. The temperature requirement varies from species to species. For example, Agarwal (1967) successfully bred *Falcolipeurus frater* (Giebel) at 33°C. (±0.5°C.), 33°C.-34°C. for *Cuclotogester heterographus* (Nitzsch) (Wilson, 1934), 32°C.-33°C., for *Lipeurus caponis* Linne (Wilson, 1939), 37.5°C., for *Philopterus citrinellae* (Schrank), and *P. turdi* Denny (Ash, 1960), 30°C.-40°C., for *Goniodes colchici* (Denny) (Williams, 1970 a, b), 35°C., for *L. tropicalis* Peters (Arora and Chopra, 1959), and 32°C.-37°C., for *Colpocephalum turbinatum* Denny (Nelson, 1971). Rakshapal (1959) fixed the thermopreferences of *Columbicola columbae* (Linne) lie between 33°C.-36°C., and Bair (1950) calculated it as 42.49°C.-0.34°C., for *Cuclotogaster heterographus* (Nitzsch) (see Wilson, 1934) and that the optimum culture temperature lies in the vicinity of 42°C., while the average skin temperature of the head and neck of the host where the parasites are found as 41.5°C. Both these latter authors have outlined the devices they used for selecting the temperature preferences of the species. We may reasonably assume that the avian infesting forms (not of the water inhabiting hosts) in general require 30°-42°C. While most of the studies have been centred round the Ischnocerophthirina, Nelson (1971) very successfully bred Amblycerophthirina.

II. HUMIDITY

Williams (1970 a,b) rightly pointed that the humidity requirements of the chewing-lice are little known. This author considers the thres-hold of relative humidity (R. H.) is around 60% and that most *in vitro* cultures can be maintained at 80% R. H. It is interesting to investigate the water conservation mechanism or source of water supply in the chewing-lice.

III. FOOD SUPPLY

The Ischnocerophthirina and Amblycerophthirina are mostly feather or hair feeders and the latter sometimes ingest blood also. The Rhynchophthirina like the sucking-lice (Siphunculophthirina) takes blood and sebaceous matter. As already stated, the chewing-lice or niche—, and host-specific. Therefore, these lice do not feed on the feathers from other regions of the host body than of its usual ones, or from other hosts. Ash (1960) however, stated that they may live in one niche, but may feed on feathers of other regions. The voracious feeding and destruction to the wool in the sheep is a well known phenomenon. Ash (1960) reported that feathers of other hosts and other feathers (and not the usual ones on which they normally feed) may either lead to the death of the parasite, or if forcibly fed on the altered food, it fails to breed. Wilson (1934) though stated that *Cuclotogaster heterographus* (Nitzsch) from the hen feed and disgest the feathers of Little green heron, his results indicate that the cycle has not been completed in any of the cases referred to by him. Mukherji and Sen-Sarma (1955) stated that *Haematomyzus elephantis* Piaget does not survive for more than 3 hrs, if seperated from the elephant host and kept in glass vials or transferred on to the guinea-pig or other mammals. Therefore, for a proper maintenance of an *in vitro* culture, one has to provide the correct feed for the parasite, failing which the culture cannot be maintained for long. Nelson (1971) reported that *Colpocephalum turbinatum* Denny predates its own eggs and juvenile stages and this may be one of the limiting factors for a successful maintenance of the *in vitro* culture.

No serious attempts have so far been made on artificial diet feeding, and if we succeed in it, it will be a major break-through for *in vitro* culture of the chewing-lice. Perhaps, Ischnocerophthirina may be induced to feed on artificial diets in the form of flakes.

IV. EQUIPMENT

At present it is possible to rear the chewing-lice at least for few generations with the following set up.

a. An incubator with thermostat control, which can be suitably fixed between 30°C.-40°C. (±5°C.) ; the actual temperature tolerance can be found out by trial and error method or by methods suggested by Rakshpal (*op. cit*), or Bair (*op. cit.*).

b. Water in a wide mouthed beaker or pan for providing required humidity. Williams (*op. cit.*) gave details of another technique for balanced humidity.

c. The lice may be kept in petri-dishes with the appropriate food. The food should be periodically replenished and examined for eggs and nymphs. Nelson (*op. cit.*) considers healthy colonies can be maintained, if the examination schedule is not too close in between each. The soft feathers are usually preferred as the food and wing-feathers for egg-laying purposes be provided. The eggs are laid on the dorsal grooves of the rami and therefore one has to look for them there. Williams (1970a, b) and Nelson (1971) also detailed the methods of arranging the feathers, simulating the bird.

d. When a new culture is raised, it is preferable to leave both the sexes in the petri dishes, (although cases of probable parthenogenesis are known) for successful egg laying.

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REFERENCES

AGARWAL, G. P. 1967. Studies on the bionomics and life-history of *Falcolipeurus frater* (Giebel, 1874) (Mallophaga : Ischnocera). — *The Indian J. Zoot.*, 8(1) : 21-40, 14 figs.

ARORA, G. L. & CHOPRA, N. P. 1957. Some observations on the biology of *Lipeurus tropicalis* Peters — *Res. Bull. Punjab Univ.*, 130 : 485-491.

ASH, J. 1960. A study of the Mallophaga of birds with particular reference to their ecology. — *Ibis.*, 102 : 93-110.

BAIR, T. D. 1950. Experimental determination of the autoselected temperature in chicken louse, *Cuclotogaster heterographus* (Nitzsch). — *Ecology*, 31 : 474-477.

MUKHERJI, D. D. & SEN-SARMA, P. 1955. Anatomy and affinity of the elephant louse (*Haematomyzus elephantis* Piaget, Insecta, Rhynchophthiraptera). — *Parasitology*, 45 (1/2) : 5-30, 40 figs.

NELSON, B. C. 1971. Successful rearing of *Colpocephalum turbinatum* (Phthiraptera). — *Nature New Biology*, 232 (34) : 255.

WILLIAMS, R. T. 1970a,b. *In vitro* studies on the environmental biology of *Goniodes colchici* (Denny) (Mallophaga : Ischnocera) I. The effects of temperature and humidity on the bionomics of *G. colchici*. — *Austral. J. Zool.*, 18 (4) : 379-389. II. The effects of temperature and humidity on water loss. — *Ibid.*, 18 (4) : 391-398.

WILSON, F. H. 1934. The life-history and bionomics of *Lipeurus heterographus* (Nitzsch). — *J. Parasit.*, 20 : 304-311, 2 figs.

WILSON, F. H. 1939. The life-history and bionomics of *Lipeurus caponis* (Linn.). — *Ann. ent. Soc. Amer.*, 32 : 318-321.

RAKSHAPAL, R. 1959. On the behaviour of pigeon louse, *Columbicola columbae* Linn. (Mallophaga). — *Parasitology*, 49 (1/2) : 232-241.

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COLLECTION AND PRESERVATION OF PHLEBOTOMID SANDFLIES
(DIPTERA PHLEBOTOMIDAE)

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The recent havoc caused by the kala-azar in our neighbouring state, Bihar, is well known. The epidemiology of kala-azar, as Adler (1964) puts it, "is replete with problems which call attention of the ecologist, pathologist, entomologist, immunologist and protozoologist" respectively. Because of vector-potential of leishmaniases, phlebotomine sandflies have come to a great prominence during the last fifty years. *Leishmania* species have so far been reported from the mammals and some old world lizards and curiously not yet described from the birds. Sandflies are the only known vectors of the human leishmaniases (Adler & Theoder, 1957). The human leishmaniases comprise two types, *viz.*, the dermal, and visceral. The resevoir hosts are largely rodents and canids. *Leishmania* species are generally correlated clinically or serologically. *Leishmania tropica* (Wright), the causative agent of dermal leishmaniasis (oriental sore), is widely distributed from Africa bordering the Mediterranean sea, Europe, Asia, and Australia. *L. donovani* (Laveran and Mesnil) causes the visceral leishmaniasis or kala-azar in Asia, Africa, Europe and South America, with which *L. infantum* Nicolle, the causative agent of Mediterranean kala-azar, is considered by many as conspecific, and by others as distinct species. *L. brasiliensis* Vianna with at least five recognizable varieties causes espundia, bubos, or American naso-oral leishmaniasis in South and Central America.

The vector of kala-azar in the Eastern India is the sandfly, *Phlebotomus argentipes* Annandale and Brunetti, and in Kashmir probably *P. chinensis* Newstead (Lewis, 1973a). The sandflies are also known as vectors of trypanosomes of some vertebrates (Wallace and Hertig, 1968). A reptilian malarial parasite has been found to develop into sporozoites in two species of *Lutzomyia*, a new world genus of sandfly. The sandfly fever occurs in the southern part of the Palaearctic Region and extends upto the Nile and also India. It is transmitted by *P. papatasi* Scopoli and the virus can possibly pass from the female through the egg to larva, i.e., trans-ovarian route in nature. Yellow fever virus and several other strains have been isolated from the sandflies, but the relative significance of these findings is yet to be assessed. In India, twenty viral strains of *Phlebotomus* fever have been isolated (Goverdhan *et al.*, 1976), of which eleven were identified as Naples, and the other nine as Sicilian types. The epidemiology of the diseases is largely dependent on the ecology of the sandfly vectors. The study of the sandflies and their correlation to different diseases is rather laborious. Adult flies are often hard to locate and their developmental stages usually impossible to find. Therefore, one has to collect the adult flies and maintain a culture in the laboratory. Marsden (1970) rightly remarked that sandflies are the most difficult of the vector arthropods to study in the laboratory, and many of the leishmanial parasites transmitted by them are morphologically indistinguishable.

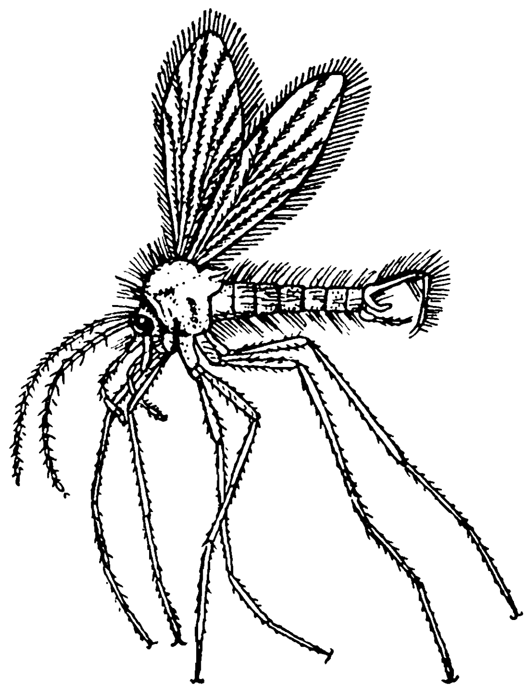

Fig. 1. *Phlebotomus* sp. ♂

Sandflies (called 'Unki' in Bengali) (Figs. 1 & 2) are small (1.5-3.0 mm in length), brown, heavily setose, elusive biting flies belonging to the dipteran family Phlebotomidae. They often fly in short characteristic hops, but many can travel to a considerable distance in clear weather. With a little experience they can be recognized by their nearly erect and rather narrow wings and the short hops. Six genera and over 550 species of Phlebotomidae are recorded from different parts of the world.

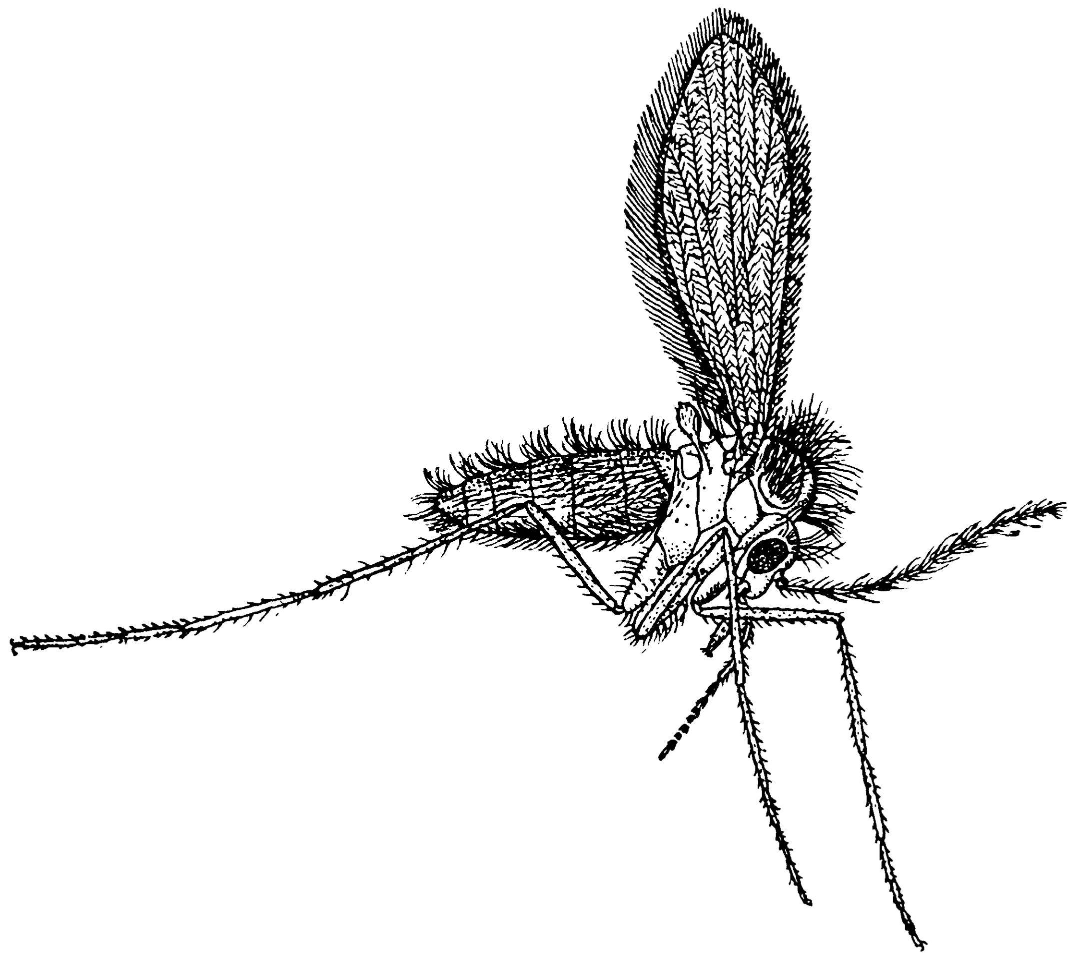

Fig. 2. *Phlebotomus* sp. ♀

According to Lewis (1973a) 32 species belonging to two genera, viz., *Phlebotomus* and *Sergentomyia*, are known from India, to these, a few more recent discoveries are to be added.

Sandflies bite generally at night. Their resting sites during the day include tree holes, between the buttresses of trees, the foliage of the forest undergrowth, animal burrows, termite hills, dwelling houses and cracks. They enter the houses to feed on man and many females remain there for the whole day. The flies are also found in rodent burrows, near the human habitations. Quate (1965) reported their association with termites, while some species of *Sergentomyia* are found by the present authors, associated with the bugs or Hemiptera. Generally, in the temperate regions of the world the sandflies abundantly occur in summer, and diapause during the cold winter. In the tropics on the other hand, some species flourish throughout the year. They generally occupy dark corners, preferably of the ground floor, and rarely on the first floor of the dwelling houses. They are also more common in the rural than urban areas and not generally seen in the crowded parts of the city. *Sergentomyia* species are very common in the houses especially in the latrines or lavatories of the suburban houses, (this may be due to their preference to flth) and also tree holes. *Phlebotomus argentipes* is found in cattle sheds as well as human dwellings, while *P. papatasi* is more in houses. The present authors collected species of both *Phlebotomus* and *Sergentomyia* under endophilic and exophilic conditions in and around Calcutta.

The eggs, larvae, and pupae of the sandflies are found in loose soil, or in the leaf litter in forests. The larvae feed on various plants and animal matter including faeces. Development is slow and the duration varies with the temperature. Emergence of adults takes about 100 days at 18° — 20° C. and 53 days at 28° — 29° C. from the time of ovi-position (Foster *et al.*, 1970). The larvae undergo diapause in areas with prolonged cold winter season. It is important to note that infection rate of *Leishmania* in wild sandflies has been significantly higher during the rainy season (10.6%) than the dry season (4.1%) (Adler, 1964).

I. COLLECTION

Sandflies have always been difficult to collect, because of their small size and delicate nature. The flies can disappear suddenly from the nets and such other common collection equipments. The following methods of collection are useful.

(a) *Killing tube*

A 15 cm. × 2.5 cm. test tube with a little cotton wool dipped in some killing agent placed at the bottom can be used as a collecting tube. The specimens are caught alive by using empty test tubes. A test tube is placed over the fly while it is resting and a piece of paper is introduced between the wall and the mouth of the test tube and the fly is caught. This method is time consuming and can be used only when the flies rest on the surface of the walls. A wide mouthed killing jar can be used for collection in the same manner, when the flies occur in abundance.

(b) *Traps*

Different types of baited and unbaited traps can be used to collect sandflies on wings. Baited traps are not of much use, when the natural host population is dense, but they are useful when the sandflies are in abundance. The design of the traps vary according to the choice of the investigator. The different designs of traps and the variable nature of the rodent hosts may make it difficult to assess the results of the catches. In Sudan it was found that *Phlebotomus langeroni orientalis*, a species which attacks man in large numbers and is the suspected main vector of kala-azar in that area, could be trapped only in insignificant numbers though four different types of forest rodents were used as baits (Turner and Hoogstral, 1965).

1. *Human bait trap*. This is widely used for collection of tsetse flies, but not of much use in sandfly collection, since the latter bites during night. A fine net suspended above a human bait may be lowered at intervals and the flies captured therein can be collected.

2. *Animal bait trap*. These traps are used all over the world with good results. It is also used for assessing the vertical distribution of sandflies. In this, the animal baits are used and the flies attracted to them are collected by various methods.

The trap fabricated by the present authors (Fig. 3) consists of a slotted (15 cm. interval) angle iron frame of 1 m. sq. base and 1.35 m. height. They are interconnected by thin iron bars fixed with nuts and bolts. Two wire mesh shelves are fitted at desired height. In the centre of the wire mesh shelf a cage of $30 \times 30 \times 20$ cm. is placed with the bait inside which is surrounded by four aluminium trays of 65×35 cm. On the lower shelf in the middle is placed a blotting paper and the four trays are placed in the same way as on the shelf above. The trap is protected from rain by a thin tin roof of 1.2×1.2m. The trays are filled with castor oil to a height of 2 mm. and are placed as mentioned above. The sandflies which are attracted to the bait cannot fly for a long distance after the heavy blood meal, and usually settle in the surrounding oil trays, and those trying to escape from the lower side are caught in the lower shelf. Albino mouse, guinea pig, house lizard and chicken were tried as baits in our experiments. More than 50 sandfly specimens could be collected per trap night depending upon the bait and the fly activity.

3. *Glass jar traps* (Figs. 4). An ordinary glass jar of about half or one litre capacity can be converted into a trap to collect sandflies from rodent burrows (Perfilov, 1966). A paper funnel with an opening of 15 mm. is inserted into the jar. The funnel is tied to the neck of the jar after folding its outer margin over the rim of the jar.

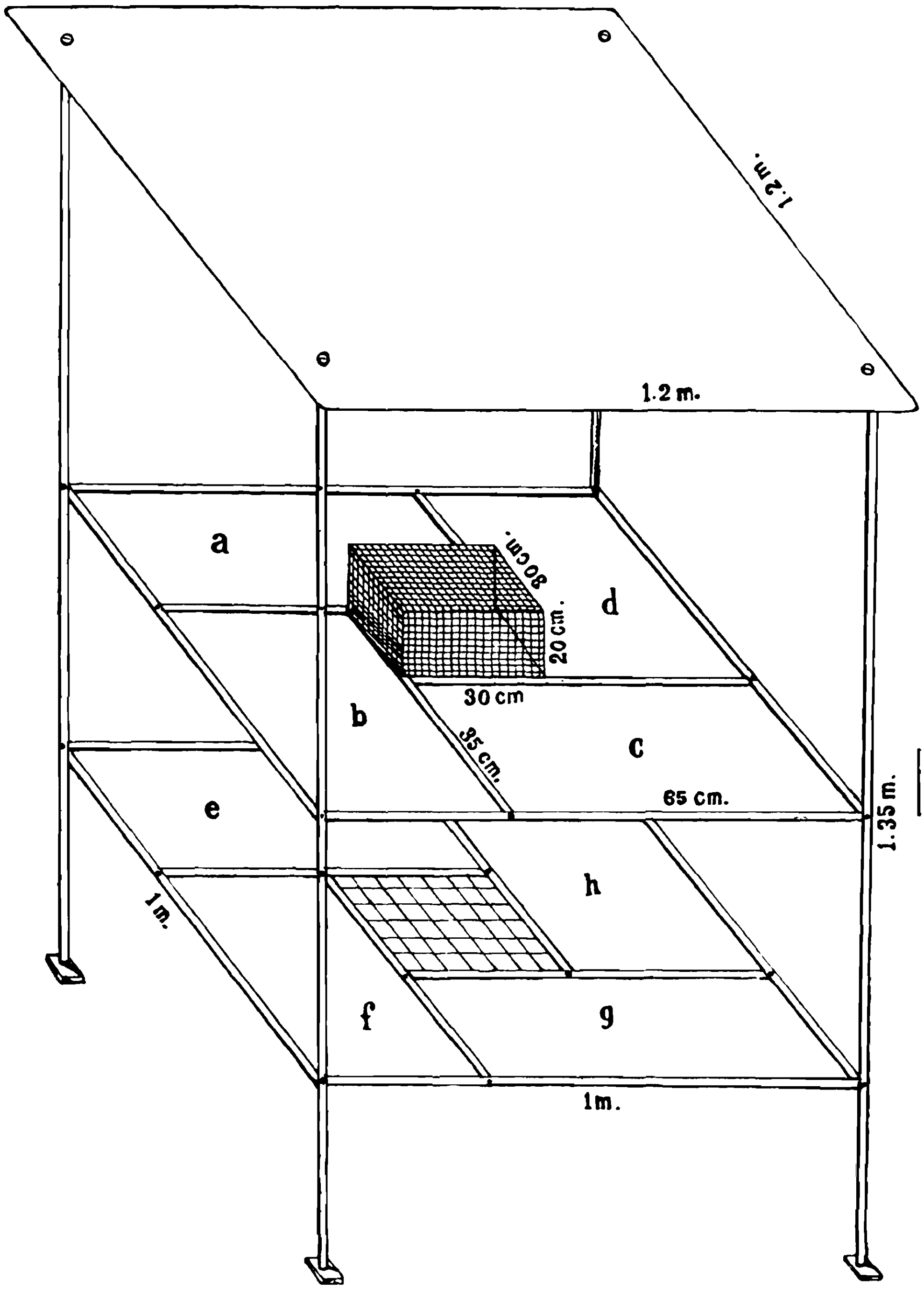

Fig. 3. Animal bait trap (a-h, aluminium trays).

The jars are tightly inserted into the burrows to a third of their length, half an hour to one hour before the sun-set. Spaces between the jar and the entrance are plugged with soil or cotton wool. The jars are collected one to two hours after the sun-set. At the time of the collection the opening of the paper funnel is closed with a cotton plug to prevent escape of the flies. A piece of cotton wool dipped in some killing agent is placed in the jar and the lid of the jar is replaced. If live flies are to be collected, the mouth of the jar is opened into a cage and the flies are set free into it. A maximum of six flies only could be collected by the present authors from a burrow, with this trap.

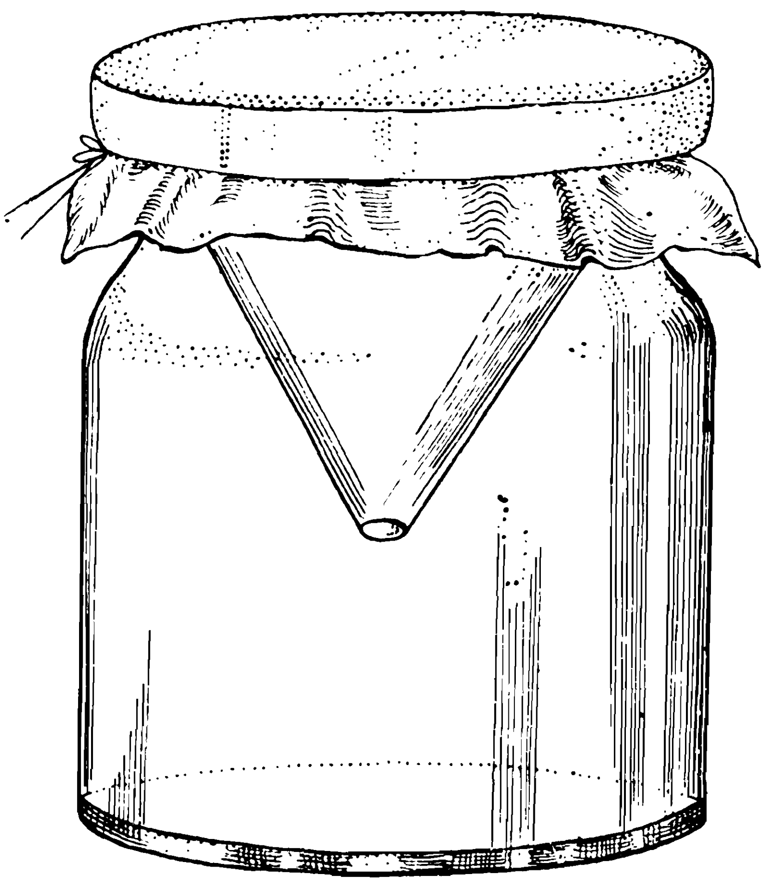

Fig. 4. Glass jar trap.

4. *Sticky traps.* Trapping on a sticky surface is one of the conventional methods of insect collection. Sandfly collection is relatively easy by this method. A piece of paper is smeared with castor oil. The oiled paper traps are an excellent device for detection and collection of sandflies. However, the insects are damaged in this method as setae, scales, antennae, wings, legs and part of abdomen may often stick to the trap. Hence, all the specimens caught on this trap are difficult to identify. Sheets of thick oil paper, clipped to thin bamboo frame, can be used as paper traps. A standard size will facilitate easy counting. Castor oil is applied to either side or one side only of the paper and is hung where there is sufficient fly activity. The standflies are taken off the oiled paper with a brush soaked in alcohol. They should not be transferred immediately to 75% alcohol as the flies stick together in clumps. The specimens are repeatedly washed in 96% alcohol to remove the oil. It has been found that 10% KOH can easily remove the oil from the flies within a few minutes.

Another simple device to collect sandflies from rodent burrows is a tin sheet cone lined with oiled paper (Perfilev, 1968). This device has the same limitations of the oiled paper traps described above.

(c) *Aspirators*

It is easy to collect sandflies with an aspirator even from the uneven surfaces, corners, cracks, small holes, etc. There are different types of aspirators.

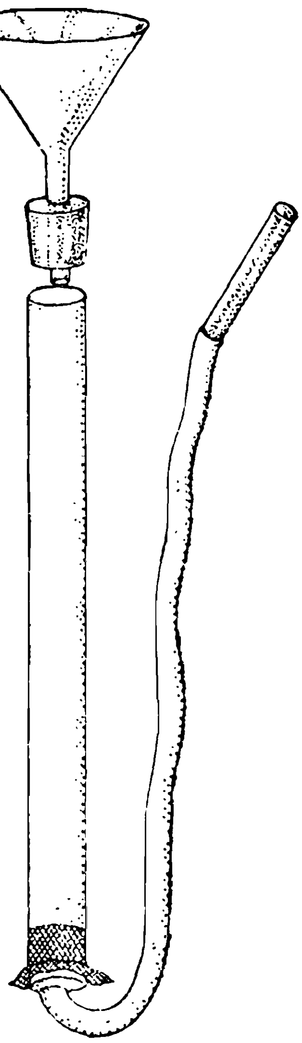

Fig. 5. Aspirator.

A simple aspirator is used in the authors work., (Fig. 5). It consists of a hard transparent plastic tube measuring 60 cm. × 1.5 cm., one end of which is fitted with a rubber tube of 90 cm. × 1.25 cm. This end of the rubber tube is covered with a piece of thin muslin to prevent entry of flies and other dust particles while sucking. The other end of the rubber tube is fitted to a 15 cm. × 1.5 cm. glass tube. This aspirator is being successfully used by the authors in collecting sandflies blindly from their possible hide-outs. The captured flies can easily be transferred to a tube containing a little alcohol, which is of slightly larger diameter than the aspirator tube, by introducing the aspirator tube into it and blowing the flies out. With this aspirator 150 or more flies could be collected per man hour depending upon the fly population.

If the collecting surface is plain and the flies are plentiful, this aspirator can be modified for quick collection by inserting a funnel of 4 cm. diameter fitted to a rubber stopper to the other end of the aspirator tube. The funnel is removed when the flies are to be transferred.

Perfilev's (1968) modified aspirator with a fixed inverted funnel has a disadvantage, since the flexible tube has to be disconnected while collecting the flies from the aspirator, which is inconvenient and time consuming.

II. PRESERVATION

A) *Temporary Preservation*

Flies can be preserved dry or wet (75% alcohol). The flies caught on sticky traps are to be preserved only after removing the oil.

B) *Permanent Preservation*

1) *Clearing*

Sandflies are rarely pinned, but are usually mounted on a slide. They may be macerated in 5-10%. KOH, stained, and mounted in Canada balsam. This method is cumbersome, and there is every possibility of losing the setae and scales, when treated with KOH.

As the internal structures such as the pharyngeal armature, the cibarial teeth and the spermathecae are of great taxonomic value, the flies are to be carefully cleared before mounting. For this different clearing media are, for example Lacto-phenol and Lacto-Chlorophenol, used with effective results.

Aman's Lacto-Phenol solution consists of phenol or carbolic acid (chemically pure crystals) (1 part), lactic acid (1 part), glycerine (2 parts) and distilled water (1 part). It has the advantage of clearing the specimens without shrinkage. Lacto-Phenol is both a clearing and mounting medium which can be used in the field because of its simplicity and rapidity. The specimens are kept in 75% alcohol for a few minutes. Then they are transferred to Lacto-Phenol for a few hours and mounted. Lacto-Phenol does not remove the scales and setae of the sandflies to the same extent as caustic potash.

Lacto-Chlorophenol is better than Lacto-Phenol as a clearing medium. It is composed of Chloral hydrate (crystals) (2 parts), acid carbolic (crystals) (1 part) and pure lactic acid (1 part). This medium, not only clears the specimens rapidly, but also quickly softens the dried ones, for an easy dissection. Specimens cleared in this medium show very distinctly the details of the buccal and pharyngeal armature as well as the spermathecae. The portion to be studied is placed in this fluid for 2 to 3 hours, or longer, if necessary, depending upon the nature and size of the specimens. Specimens of *Sergentomyia* species require more than 3 hours for clearing, while *Phlebotomus* species even a longer time. The females of *Sergentomyia* species are damaged in more than seven hours and the males in nine hours. From the authors' experience, it is seen that if the specimens are to be mounted in Balsam medium, they should be dehydrated in carbol-xylol mixture for about 15 minutes.

2) *Fixing*

The cleared specimens can be fixed on slides with Carnony's fixative 96% alcohol (6 parts), chloroform (3 parts), and glacial Acetic acid (1 part). The specimens are oriented in the fixative and are left for about 3 minutes. Then the slides are washed in 96% alcohol and a drop of celloidin is dropped over the specimens. After about a minute the slides are washed in 96% alcohol, in which they can be left for a longer time without any damage. This process may be of considerable help to keep them in position when mounted in balsam. But it is not necessary, when the specimens are mounted in quick drying mounting media like Berlese's fluid, Hoyer's fluid or Stroyan's fluid.

3) *Staining*

The shape of spermathecae is in important taxonomic character for the identification of females. In *Sergentomyia* species the spermathecae are entire, whereas they are crenulated in *Phlebotomus* species. Spermathecae can be better studied when stained with Pyrogallic acid (Perfilev, 1968). After clearing and washing, the specimens are placed in a freshly prepared mixture of Acetic acid (5 parts) and 20% Pyrogallic acid (1 part). They are kept in this mixture for about 20 hours at 60°C. If the specimens are over stained, they are treated with 10% KOH for 1 to 3 hours to remove the excess stain. After staining, the flies are washed in distilled water. It is not advisable to stain the head of the sandfly as it impairs the visibility of the buccal teeth.

4) *Mounting*

Even though Canada balsam is the ideal mounting medium, it is time consuming and also takes much longer time for drying. So any one of the following media can be used with advantage :

	Hoyer's medium	*Berlese's medium*	*Stroyan's medium*
Gum Arabic	6.25%	7.25%	16.66%
Chloral hydrate	41.66%	76.2%	27.77%
Glycerine	41.66%	—	—
Distilled water	10.43%	9.3%	38.91%
Glucose syrup	—	4.75%	8.33%
Glacial Acetic acid	—	2.5%	8.33%

III. REARING

Sandflies are reared in the laboratory by keeping gravid females in an ovulation tube (Fig. 6) for laying eggs. They lay eggs normally on wet sufaces in humid atmosphere, and so a piece of wet blotting paper is placed at the bottom of the tube. The tube is then kept in a dark box containing a tray of distilled water to maintain humidity. The flies do not oviposit if it is excessively wet. They normally oviposit in darkness at a temperature of 28° to 30°C. After the oviposition, the eggs are transferred to an earthen rearing pot, containing a few grammes of moist animal droppings which serve as a rearing medium. As the rearing pot has to be maintained moderately wet, it is kept either in a large petridish filled with wet sand or for better results in a desiccator containing a little water (Fig. 7). The larval feed is added in smaller quantities once in a fortnight or so. When the larvae pupate, they are transferred to hatching tubes to prevent the escape of the emerged imagos.

IV. SMEAR PREPARATION

The smears for leishmanial parasites are prepared on the fifth to seventh day after blood meal of the fly. For better results, the flies can

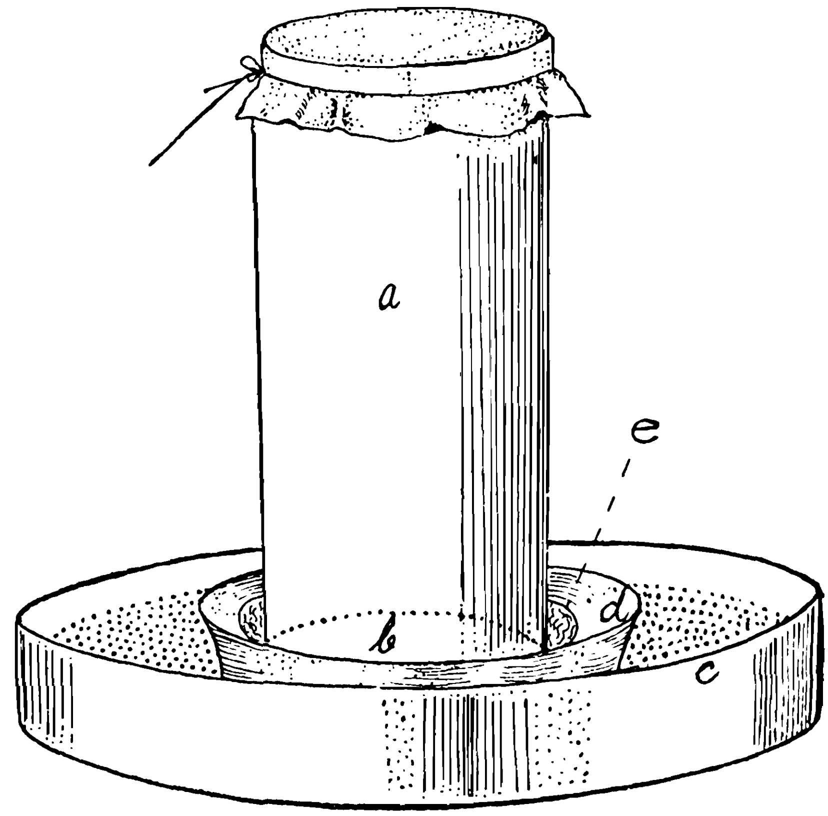

Fig. 6. Ovulation tube (a, tube ; b, blotting paper ; c, petridish with wet sand ; d, earthern pot ; e, wet cottonwool).

be fed with 5% glucose solution on the fourth day. The foregut of the flies are dissected out, spread and fixed on slides in methyl alcohol and stained with Leishman's stain.

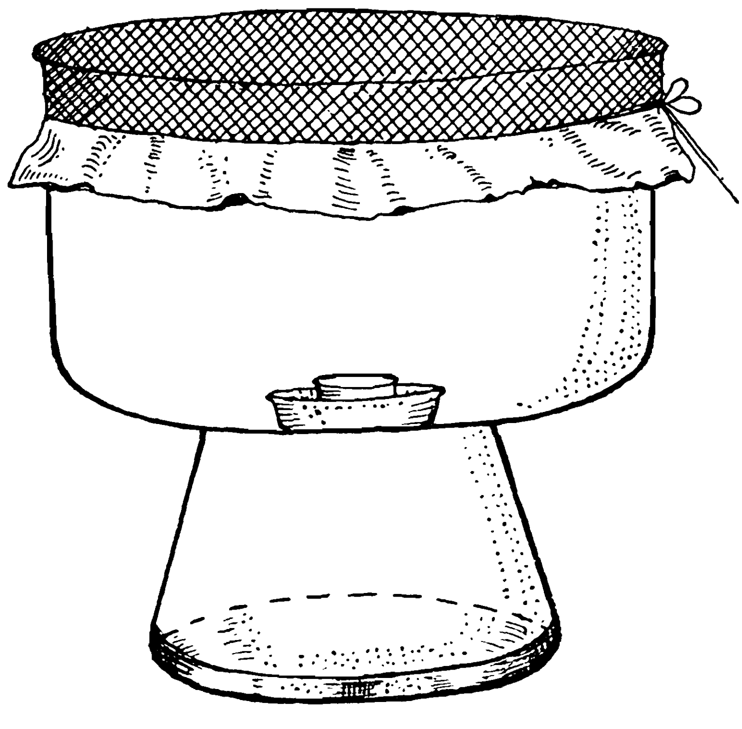

Fig. 7. Rearing pot.

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REFERENCES

ADLER, S. 1964. *Advances in Parasitology*, [Academic Press,] London and New York, **2** : 35-96.

ADLER, S. and THEODOR, 0. 1957. Transmission of disease agents by phlebotominae sandflies. —*A. Rev. Ent.*, **2** : 205-226.

FOSTER, W. A., TESFA-YOHANNES, T. M. and TESFA TECLE, 1970. Studies on leishmaniasis in Ethiopia II. —*Ann. trop. Med. Parasit.*, **64** : 403-409.

GOVERDHAN, M. K., DHANDA, V., MODI, G. B., BHATT, P. N., BHAGWAT, R. B., DAMDWATE, C. N. and PAVRI, K. M. 1976. Isolation of *Phlebotomus* (Sandfly) fever virus from Sandflies and Humans during the same season in Aurangabad District, Maharashtra State. —*Indian J. med. Res.*, **46** : 57-63.

LEWIS, D. J. 1973a. *A catalog of the Diptera of the Oriental Region.* —[The University Press of Hawai], Honolulu, **1** : 245-254.

LEWIS, D. J. 1973b. Phlebotomidae and Psychodidae (Sandflies and Mothflies) in K. G. V. Smith ed. *Insects and other Arthropods of Medical Importance*, Trustees of the British Museum (Natural History), London, 155-187.

PERFILEV, P. P. 1966. *Fauna of U.S.S.R.* [Academy of Sciences of the USSR. Zoological Institute.] New Series No. 93. Volume 3, No. 2 : X+363 pp. (Israel Program for Scientific Translations, Jerusalem, 1968).

QUATE, L. W. 1965. A taxonomic study of Philippine Phlebotomines (Diptera : Psychodidae). —*J. med. Ent.*, **2** (1) : 17-37.

TURNER, E. R. and HOOGSTRAAL, H. 1965. Leishmaniasis in the Sudan Republic, 23. Sandflies attracted to rodent baited traps (Diptera : Psychodidae). —*J. med. Ent.*, **2** : 136-139.

WALLACE, F. G. and HERTIG, M. 1968. Ultrastructural comparison of promastigote flagellates (leptomonads) of wildcaught Panamanian *Phlebotomus*. —*J. Parasitol.*, **54** : 606-612.

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COLLECTION AND PRESERVATION OF BLACKFLIES (DIPTERA SIMULIIDAE)

By

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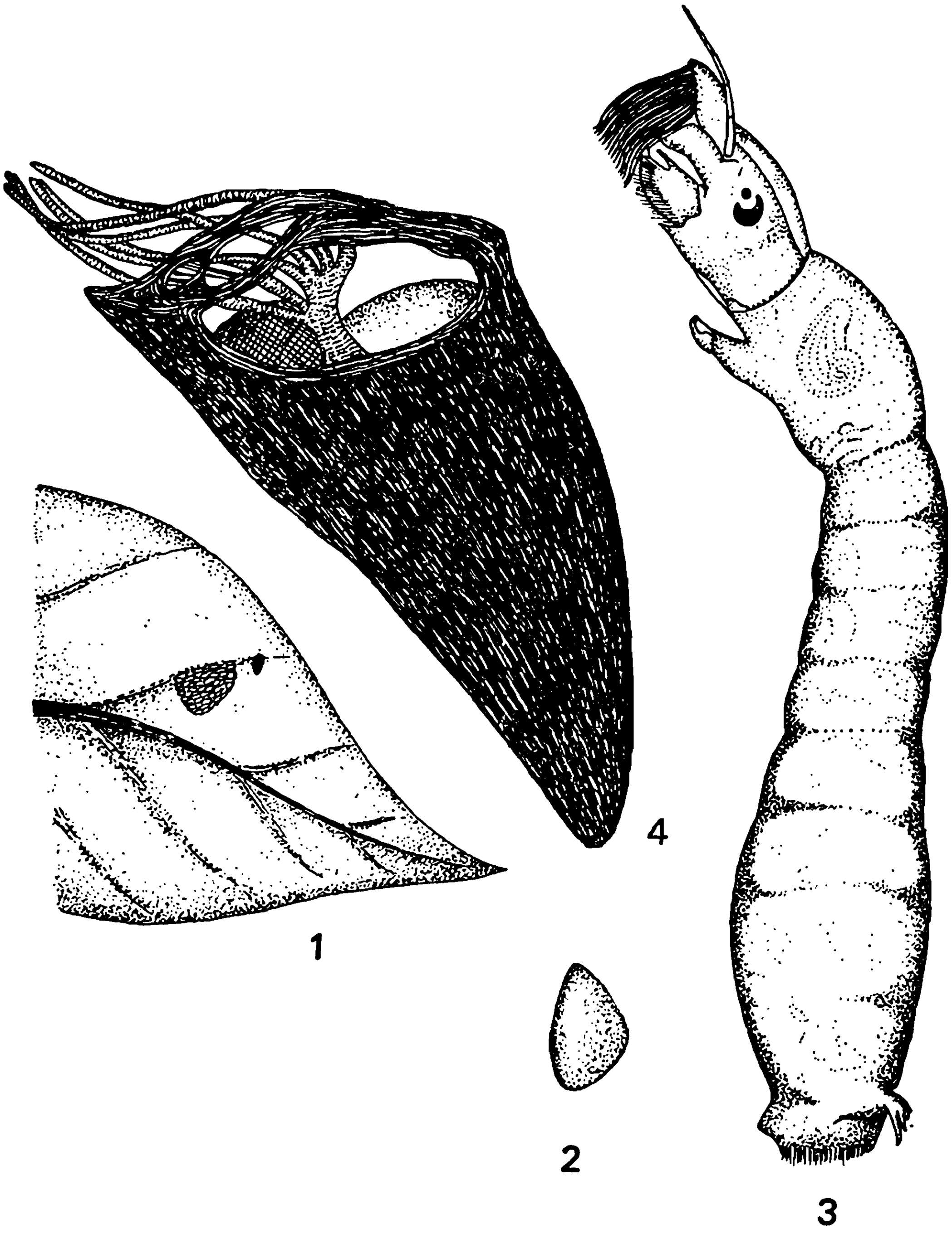
INTRODUCTION

Blackflies or dimdam flies as they are often called in the North-East India are a homogeneous group of dipterans of almost cosmopolitan distribution. These flies are economically important since the females of most species are haematophagous and their blood-feeding habit is sometimes closely associated with the transmission of certain diseases, namely, human and non-human onchocerciasis, avian trypanosomiasis and leucocytozoonosis, and probably rabbit myxomatosis in many parts of the world (Fallis, 1964). In India, there are several records of their biting and resultant inconvenience (Lewis, 1974; and Datta and Dasgupta, 1975), but the direct evidence of disease transmission is yet to be established. This communication deals with the identification of different developmental stages of black flies and the techniques for their collection, rearing and preservation.

OCCURRENCE OF BLACKFLIES

Blackflies occur most abundantly either in hilly terrain with water-courses in form of trickles, ditches, channels and streams, or in the vicinity of large rivers which are their breeding places. Females lay their eggs in masses on the live or dead vegetation (Fig. 1), rocks and other suitable submerged substrata or scattered loosely in the bottom of the flowing watercourses (Datta, Day, Paul and Pal, 1975). The larvae upon hatching remain attached to the same substratum, or more often travel to other suitable substrata. The pupae are usually spread over the substratum, but are sometimes crowded together in extraordinary dense masses (Datta *et al.*, 1975). The adults on emergence are capable of flying soon after reaching the water surface. Most species appear to mate in mid-air, and mainly alight on evergreen tree-tops or bushes.



Figs. (1-4) 1. Eggs of *Simulium* sp. deposited on a leaf. 2. An egg of a *Simulium* sp. 3. A mature larva of *Simulium* sp. 4. A pupa of a *Simulium* sp. within the cocoon

MORPHOLOGICAL CHARACTERISTICS OF BLACKFLIES

The morphological characteristics of different developmental stages in the life-history of blackflies are enumerated below for easy identification.

Egg : An egg (Fig. 2) is approximately 0.3 mm. long, ovoid with a bulge on one side. The surface is smooth, whitish when freshly laid, which later turns dark brown.

Larva : A mature larva (last instar) (Fig. 3) approximately 6.0 mm. long, cylindrical and somewhat swollen posteriorly. The head is usually with a pair of large fan-like structures used for capturing food. The thorax is provided with a pair of dark spots (histoblasts) laterally and a proleg ventrally. The last abdominal segment is provided with a disc encircled by a series of parallel rows of small hooks.

Pupa : Pupae are sedentary, each (Fig. 4) being encased in a slipper- or shoe-shaped cocoon. The thorax bears a pair of slender, branched, filamentous or bulbous respiratory organs. The abdomen is provided with various hooklets for attachment with the cocoon.

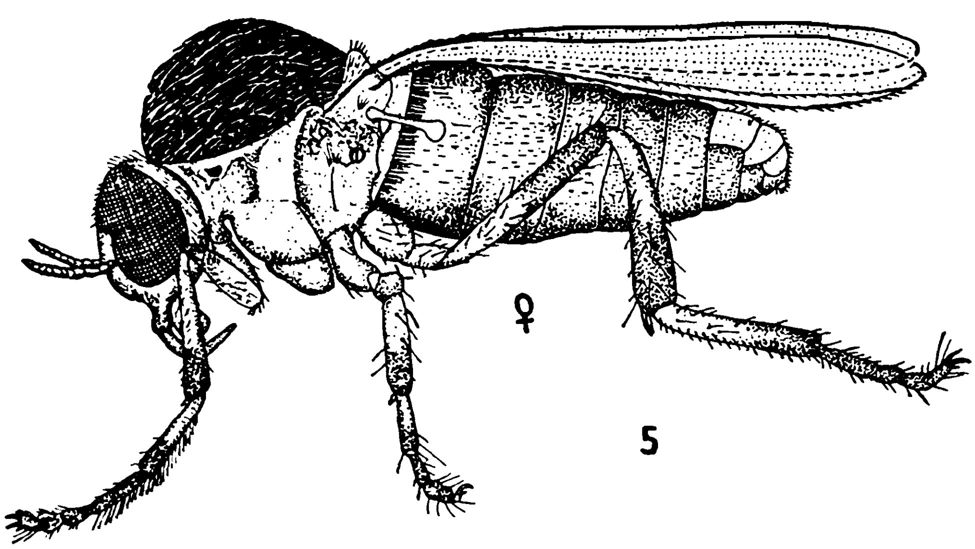


Fig. 5. An adult female of *Simulium* sp.

Imago : Imagines (Figs. 5 & 6) are small, usually dark-coloured flies. The males are holoptic while the females are dichoptic, and without ocelli in both sexes. The antennae are short horn-like and generally 11-segmented. The mouthparts are mainly adapted for cutting and sucking. The thorax is remarkably arched and without transverse sutures. The wing is broad, the three anterior veins are more prominent than the posterior ones ; a forked vein-like crease, the submedian fold is present between M_2 and Cu_1. The legs are short, stout and with elongated first tarsomeres. The first abdominal tergum is modified into a basal scale, usually bearing long setae. The male genitalia are non-rotate type.

COLLECTION OF IMMATURE STAGES

Datta and Dasgupta (1972) have briefly described the techniques of collection of different developmental stages. The eggs laid on the

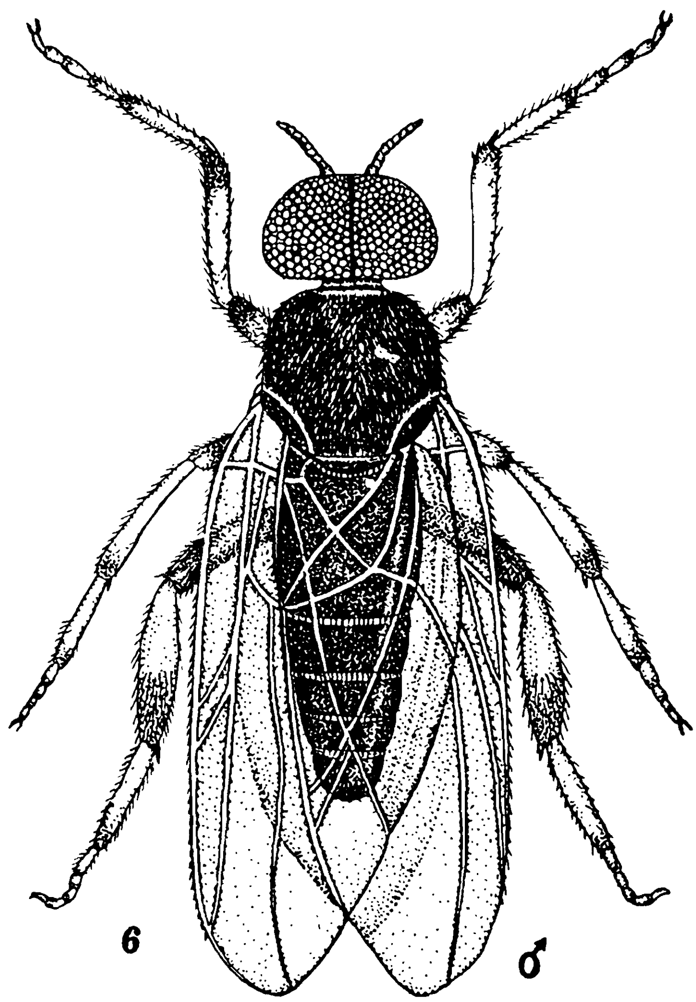


Fig. 6. An adult male of *Simulium* sp.

vegetation, are collected by cutting with scissors portions of the vegetation together with the eggs. Removing egg-masses from rocks or stones is rather difficult, and therefore, can be collected by careful scraping or if desired, along with the substratum. The larvae are collected by lifting the submerged substrata and then, picking up the attached larvae gently with forceps. The larvae attached to the immovable substrata, can also be collected by the same method, but care must be taken to pick them up before they are washed away by the water current. The pupae are collected along with the substrata in the same way as the eggs, but those from immovable substrata are collected carefully by scraping with the help of needles.

REARING OF PUPAE

The rearing of pupae to adults is indispensable for species-determination. The device of Wood and Davies (1966) is used with some modifications (Datta and Dasgupta, 1972). Some small, numbered vials (Fig. 7), each with a cotton bed moistened with water are arranged in a petri dish. The pupae (preferably dark pupae being mature) are separated specieswise (depending upon the number and mode of arrangement of the respiratory filaments of the thorax) under a stereoscopic binocular microscope and are transferred singly on to the cotton bed. Then the vials are loosely plugged with dry cotton. It is mandatory to keep the pupae moist but caution is to be taken to avoid bathing in surplus water. The whole device is then kept in a place at the room temperature, preferably away from direct sun light. The hatched imagines with the pupal exuviae of a given species are taken out of the vials at intervals of 5 or 6 hours, the time required for hardening and darkening of the different parts of the insect body. Thus, a good number of male and female specimens are obtained for studies.

COLLECTION OF ADULTS

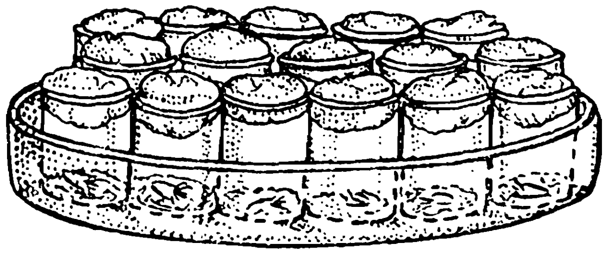
The adult blackflies are mainly collected by the following techniques :

1. Sweeping forest canopy near or far off the breeding sites with a collection net ;
2. Providing human and animal baits ;
3. Setting traps, particularly, the light traps.

As the first two methods are well-known, the last one is only discussed in detail. Several light traps are in use. One used by the present author has been described elsewhere (Datta and Dasgupta, 1972).

However, this trap combines the essential features of the New Jersey light trap, Rothamsted light trap, Robinson light trap, Pennsylvania light trap and Chinsurah light trap. The body (B) of the light trap (Fig. 8) is a four-walled chamber with a sloping roof (R) made up of plain tin sheets and the rear (approximately) (60 cms. × 60 cms.) is completely open. This roofed chamber is placed on an open terrace of a building facing a deep forest area with breeding pockets of blackflies. A light source (L) of 500 wattage electric bulb having a reflector (E) is hung from the roof, holding very near the middle of the open end of the chamber. An iron-wire netting (N) of 4 mesh per sq.cm. is fixed inside the chamber to cover the light source. Approximately 12 cms. beneath the open end of the chamber there is a platform (P) fixed to the wall by two iron brackets (C). A rectangular tray (T) measuring 45 cms. × 63 cms. × 5 cms. contains the trapping liquid, a 5% aqueous

Acetic acid solution at a depth of about 2.3 cms and this is covered with an iron-wire netting (not shown in the diagram) of 4 mesh per sq. cm. The tray is then placed on the platform during operation.



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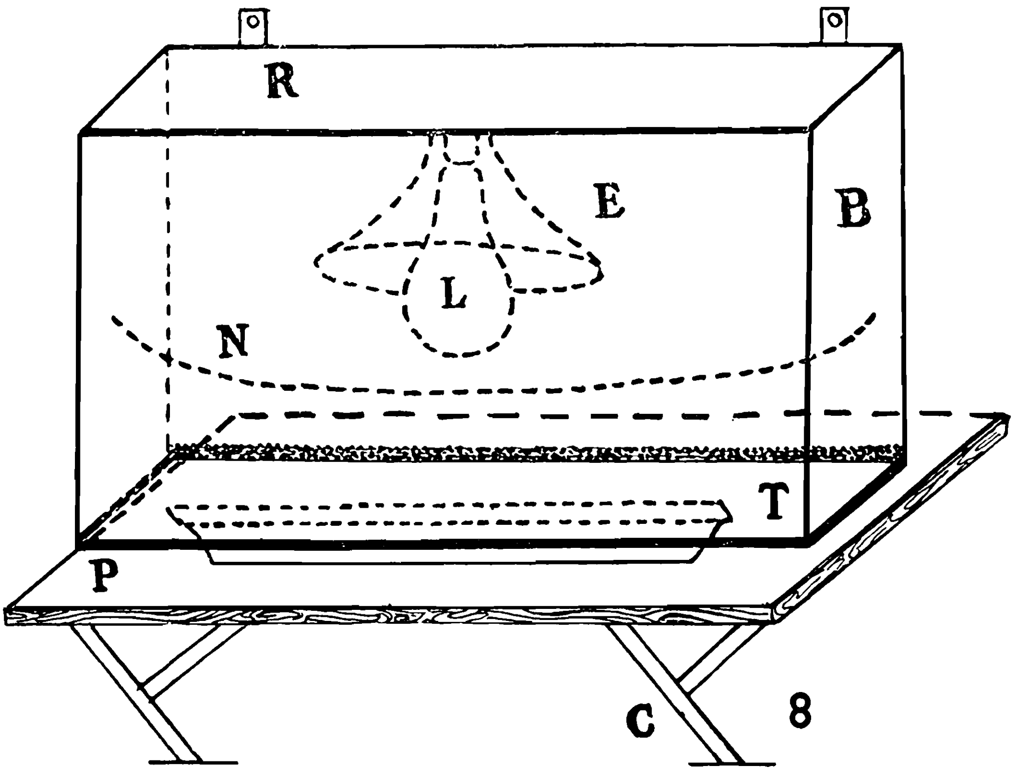

Figs. (7-8) 7. The rearing device. 8. The light-trap device.

After a night-long operation the tray of the trap device is brought to the laboratory. Next, the iron-wire net is removed and fresh water is added to the tray-contents to increase the volume of the existing liquid for removal of the pungent stench of Acetic acid prior to collecting the trapped blackflies from the tray.

PRESERVATION OF BLACKFLIES

The specimens preserved in Ethyl Alcohol are the best for taxonomic purposes for measuring various parts of the body and for making microscopic preparations. The eggs with the substrata are washed and directly preserved in 70% alcohol. The larvae are preserved in 90% alcohol which helps spread their cephalic fans and extend their rectal "gills" It is better to preserve live specimens directly in the field. One or more changes of alcohol will give better results before final preservation. Some pupae are washed and preserved in 70% alcohol, while some are kept for rearing. Some adults are killed in Chloroform or Ether vapour and are pinned, and others are directly preserved in 70% alcohol. Each pupal exuvium is either fixed along with the adult or preserved in 70% alcohol with a reference to the adult emerged out of it. In each case, however, collection data, *viz.*, name of the species, place of collection, date, collector's name and reference number, if any, must be provided with every specimen either collected or reared. Microscopic observations are made with slide - mounted specimens in Phenol balsam (saturated Phenol solution in absolute alcohol plus Canada balsam in equal proportions) after clearing in saturated Phenolic solution in absolute alcohol at 60°C.

Blackflies caught in nature in fed and gravid conditions are mainly used for parasitological investigatoins. The specimens are killed by the methods described earlier and are identified. The head and the thorax-abdomen portions are severed and fixed in Bouin's fluid composing saturated aqueous Picric acid solution (30 parts), 40% Formaldehyde, (10 parts) and Glacial Acetic acid (2 parts) or preferably in Carnoy's fluid composing Ethyl alcohol (6 parts), Chloroform (3 parts) and Glacial Acetic acid (1 part) for about half-an-hour or more depending upon the size of the material. A change of the fixative is necessary for the larger material at regular intervals. The tissue is then removed to 90% alcohol for two changes of an hour each, and is then gradually dehydrated through 95% alcohol and two changes of absolute alcohol for one hour each. The material is finally washed in absolute alcohol and is kept in Cedar wood oil for storage. If longer period of storage is required, the material can also be kept in 70% alcohol avoiding immediate dehydration.

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REFERENCES

DATTA, M. & DASGUPTA, B. 1972. On the collection and rearing of blackflies (Simuliidae : Diptera) of Darjeeling. —*Indian Biol.*, **4** : 54-59.

DATTA, M. & DASGUPTA, B. 1975. Host preferences of blackflies (Diptera : Simuliidae) of Darjeeling, West Bengal.—*Proc. zool. Soc. Calcutta*, 28 : 147-153.

DATTA, M., DEY, R. K., PAUL, A. K. & PAL, T K. 1975. Ecology of blackflies (Diptera : Simuliidae) in Darjeeling area, India.—*Proc. Indian Acad. Sci.* (B), **81** : 7-19.

FALLIS, A. M. 1964. Feeding and related behaviour of female Simuliidae (Diptera).—*Exptl. Parasitol.*, 15 : 439-470.

LEWIS, D. J. 1974. Man-biting Simuliidae (Diptera) of Northern India. —*Israel J. Ent.*, 9 : 23-53.

WOOD, D. M. & DAVIES, D. M. 1966. Some methods of rearing and collecting blackflies (Diptera : Simuliidae).—*Proc. Ent. Soc. Ontario*, 96 : 81-90.

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COLLECTION AND PRESERVATION OF MOSQUITOES

By

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The word mosquito is of spanish derivation meaning a little fly. These tiny creatures have been coming to man and making a nuisance of themselves for nearly a million years. But far from the fact of their complex life-histories and that of their visits for our blood, it is the discovery that these flying visits could be the prelude to sickness and death, leading to epidemics, which in the past even shaped human history, that rivetted man's attention to these tiny creatures.

With over 2500 species belonging to 33 genera, they are predominent in the tropics though there is no land mass where they do not make their presence felt. Some species like *Culex pipens* are found almost all over the world, except in the far north and south, while a few may be restricted to particular habitats. Mosquitoes are found practically everywhere, from the arctic to the equator, in cities and in the rural areas, in forests and even in the deserts.

I. IMMATURE STAGES AND BREEDING SITES

a) *Eggs*

Majority of mosquitoes lay their eggs singly or in clusters attached to floating vegetation, while in four genera eggs are laid in egg-rafts. In cases where the eggs are laid singly they may be laid in the open, fully exposed pools (e.g. *Anopheles maculipennis*), while others among thick vegetation or under over hanging banks (e.g. *A. funestus*). *A. culicifacies* an important malaria transmitter in India lay the eggs in paddy fields. The typical egg raft of *Culex* consists of several hundred eggs laid at a single 'sitting' ; the shape, size and number of eggs in a raft may often help in determining the genera and in some even upto the species. Some rafts are attached by the mother (eg. *Culiseta morsitans*) to the moist substrate some five cms above the surface of water. Some species lay their eggs inside damaged bamboos and reeds.

Species of subgenera *Aedimorphus*, *Neomelanicorion* etc., lay their eggs on or over more or less dry ground which will be inundated by flooding, by melting snow, or by tidal waves.

b) *Larvae*

It is often said that mosquitoes breed in stagnant water, but in fact, almost every type of fresh water is exploited by one species or another. While the gully trap and pit latrines may be suitable for some, others are much particular and will breed only at the edges of clear-running streams and rivers. There are forest-pool, swamp, brackish water, tree hole, plant axil and even empty snail shell breeders while still others may breed in small quantities of water held by the fallen leaves or in hollow reeds. Larvae can be found wriggling in these water pockets.

II. ADULTS

Adults can be found flying around or resting in shady places including huts, thatched houses, cattle sheds, bushes, cracks and crevices, under bricks, tree-holes and bamboo holes or even in the most modern buildings. All mosquitoes, both male and female, feed on sugar in one form or another, but most females require a blood meal if they are to produce viable eggs. Some females like *Culex pipens molestus* can lay the first batch of eggs without a blood meal but the meal is needed for the subsequent batches.

Adult mosquitoes are incredibly efficient and their sense organs have attained a high degree of development not having many such parallels in the animal world. For a mosquito flying through rain, each drop may appear as a gigantic missile several times its own weight, yet the insect can fly safely through them and land for a meal. The circadian activity cycles are very common throughout the animal world and these rhythms are better understood in the mosquitoes. "Endogenous circadian rhythms are maintained by a physiological clock within the organism ; this must first be 'set' by an external stimulus such/a change from light to dark, but/as thereafter the rhythm : maintains without further time cues (Harker, 1958, 1961). The knowledge of this rhythm is of special significance in deciding when and where to look for collection, and also in tackling problems associated with maintaining colonies of mosquitoes in the laboratory.

III. COLLECTION OF ADULTS

Generally the collections are made for two types of studies *viz.*, a) Qualitative studies (when we need to study the presence, distribution, species and type of behaviour of different species in various macro & micro environmental conditions) and b) Quantitative studies (to measure the value and variability of vector relative density and abundance, longevity, infectivity, impact of anti-vector measures on the vector population, impact on transmission etc.).

1. *Collection by Hand*

Mosquitoes resting on different surfaces are collected using a test tube or a sucking tube.

i) *By Test tube :* When collecting the mosquitoes in test tube, the collector should apply the mouth of the tube perpendicularly over the wings of the mosquito so that, when it attempts to fly off, it will fly directly into the tube.

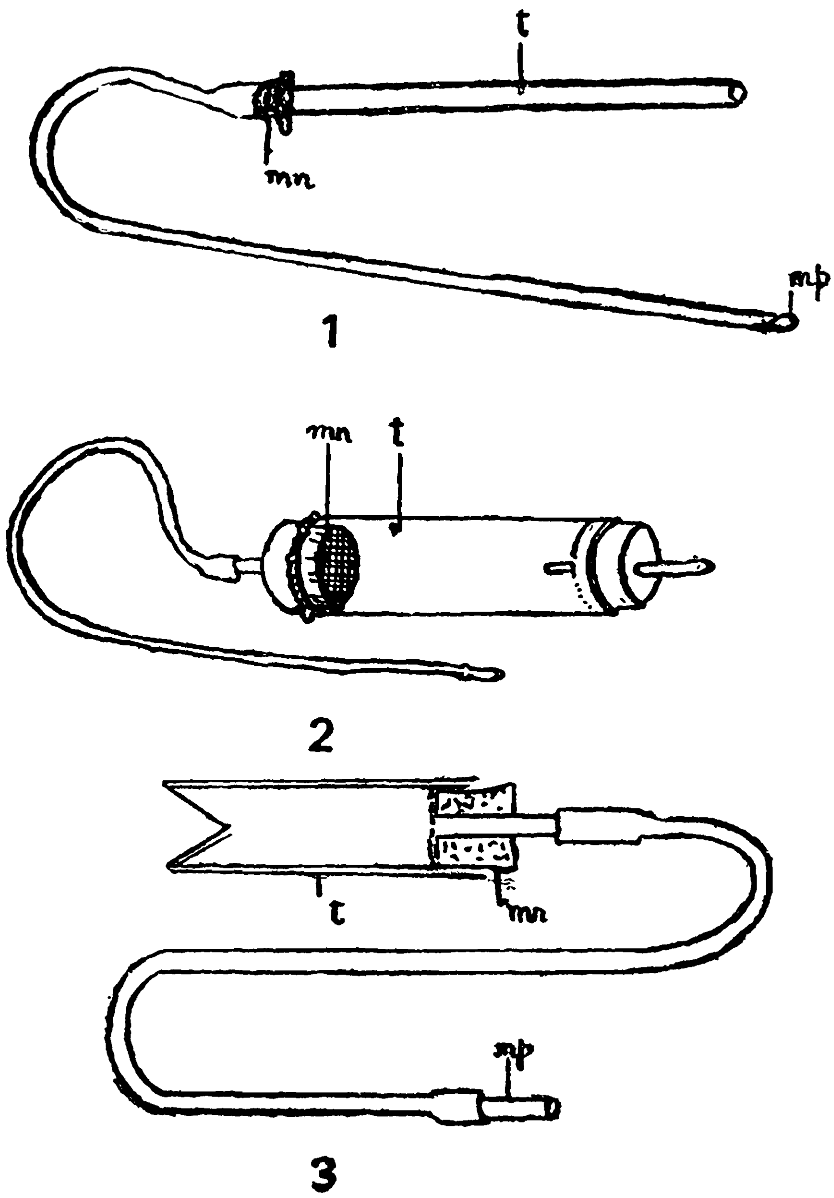
ii) *By Aspirators :* In figs. (1, 2 & 3) three types of collecting aspirators, or sucking tubes 1 represents the common type, composed of a glass or plastic tube 15 mm., in diameter, a rubber or plastic tube (pt) and a mouth piece (mp). 2 & 3 represent the Italian type with details of the component parts and dimensions are shown.

2. *By Insecticidal Sprays*

This method consists of the collection of indoor resting mosquitoes on a white cotton sheet after knock down by spraying with Pyrethrum solution.

3. *By Baiting*

Mosquitoes are collected when they land on the host, man or animal. While using man as a bait, the collector himself, or a member of his team or a local inhabitant can serve as bait. Local animals (donkey, buffalo, cow etc.) can serve as animal bait. Mosquitoes are collected directly off the bait using a sucking tube or test tubes.



Figs. (1-3) Aspirators, t-glass or plastic tubing ; mn-mosquito netting ; mp-mouthpiece.

4. *Trap Collection*

There are several types of traps which can be classified into 3 categories.

A. *Fixed traps :* These are attached to existing structure., viz.,

a) *Exit traps* (window, door, caves, walls and verandah traps)

b) *Entry traps*

a. *Exit traps* are of importance in studies on the effect of insecticides on endophilic species and house frequenting exophilic species. It gives indirect information on the circulation of mosquitoes in different physiological stages from outside to inside and *vice versa.*

b. *Entry traps.* Entry window or door traps are placed indoors with the aperture of entry cone fixed in the space through which mosquitoes enter.

B. *Portable traps enclosing bait :*

The portable traps are not fixed and contain a bait.

a. *Bed net traps :* Mosquitoes are attracted by a human bait sleeping on a bed unprotected or protected by a closer inner net under a big mosquito net.

b. *Animal baited traps :* These are used to detect the presence and relative density of mosquitoes biting animals in a given area before or after spraying.

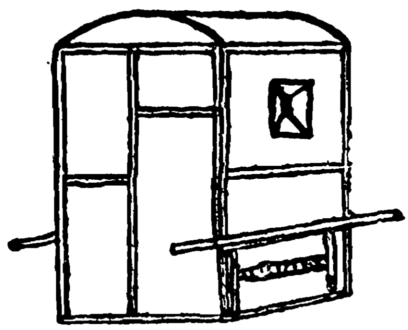
i) *Magoon traps :* These are portable wooden huts made up of panels in which the upper halves of the walls are removed and replaced by gauze netting and an entry slit is provided all the way round (figs. 4 & 5). The parts can be dismantled at will. A calf or another animal is kept as a bait in the trap. Large number of mosquitoes can be caught in a single trap.

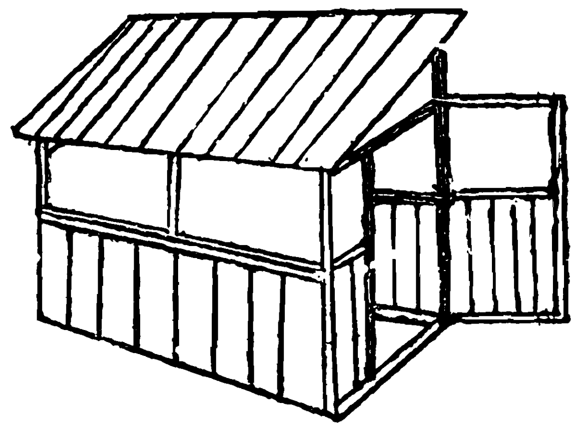
ii) *Steer baited traps* (figs. 6, 7) : It is a more refined trap based on the principle of the magoon trap.

iii) *Trap nets* : These are made of ordinary mosquito netting supported on a metal or wooden skeleton and big enough to enclose a large animal with a man bait, if desired. Precipitin test will reveal the host preference.

C. *Mechanical Devices for Collection of Outdoor Or/And Indoor Mosquitoes*

a. *Fixed traps* using an attractant light alone, light and a small animal or lights and CO_2. The mosquitoes after being attracted by light, are blown into a small reservoir (cage or bottle) by a current of air produced by an electric fan (fig. 8).





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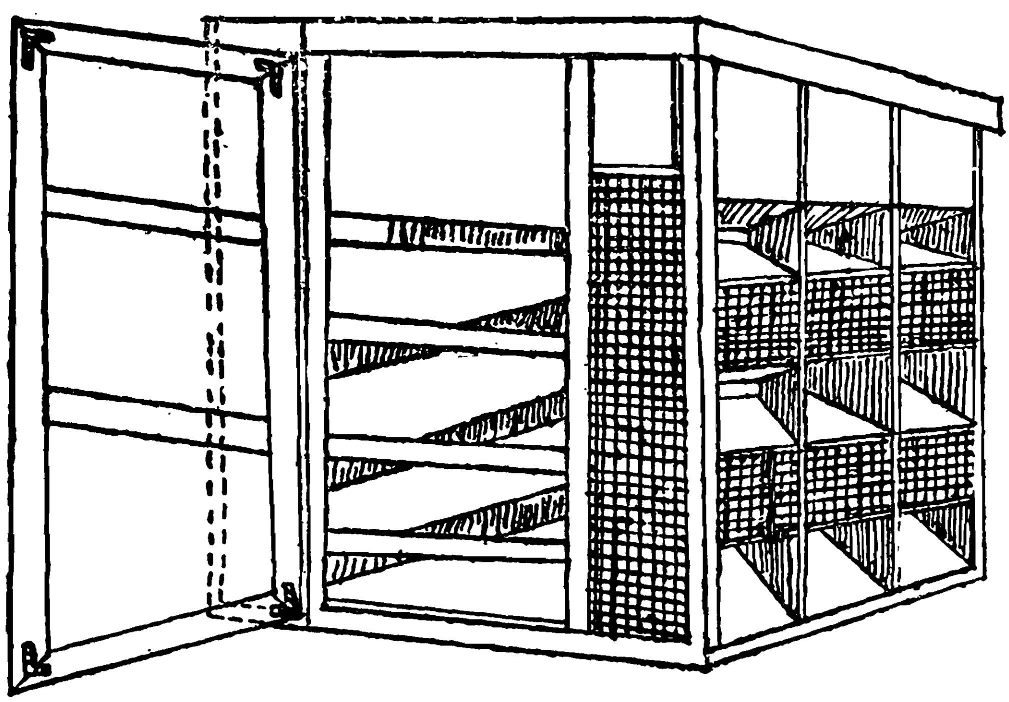

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Figs. (4-6) 4-5. Magoon traps. 6. Steer baited trap.

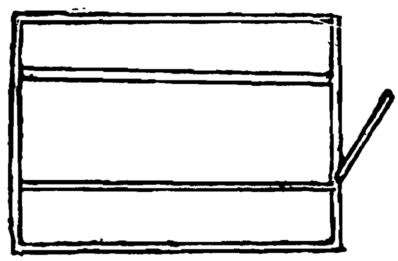
b. *Mobile traps* mounted on a vehicle (car, lorry or bicycle)

D. *Experimental Bait*

These baits are used to evaluate the normal behaviour of house-hold mosquitoes and their reaction to the insecticides.

E. *Catches in out door Shelters*

Mosquitoes resting outdoors are caught by hand or with the aid of an aspirator. One of the following devices can be used.



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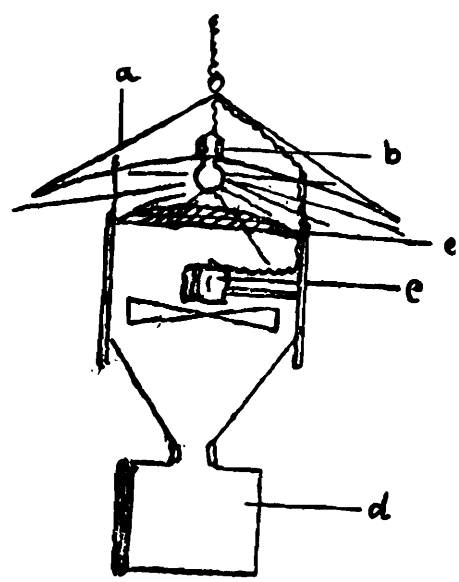

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Fig. (7-8) 7. Steer baited trap. 8. Light trap. a = shield ; b = source of light ; c = electric fan ; d = collecting reservoir ; e = metallic grill to retain large insects.

i) Suction tube and a torch for dark places.

ii) Mechanical aspirator (sweeper) for collecting mosquitoes from vegetation, holes etc.

iii) Artificial resting shelters (pit shelters, barrels, boxes etc.)

iv) Drop net for collecting from grass or low vegetation.

IV. COLLECTION OF EGGS LARVAE

Collection of eggs and larvae may also be required.

a. for studying the breeding habits of different species ; b. to know the geographical distribution of vectors ; c. to establish the active breeding places ; d. to evaluate the dynamics of development of aquatic stages ; e. to evalaute the impact of adulticides on the larval density or to demonstrate the persistence or absence of a species whose adults could not be found ; f. to evaluate the impact of antilarval measures and larval density ; g. to rear adults for taxonomic studies and biological observations.

A. I. *Larval collection* :

Three methods are in common use. a) Dipping ; b) Netting and c) Pipetting.

a. *Dipping* :

i) A white enamel bowl may be used to collect larvae from the edge of the swamps, ditches and streams, rice fields or other large bodies of water.

ii) A rectangular or round frying pan about 25 cm., in diameter, with a long handle to collect from more inaccessible parts of above mentioned breeding places.

iii) A round palette made up of a wire, 25 cm., in diameter, to which a nylon gauze is attached, with a handle which may be attached to a long stick, can be used for collecting larvae and eggs from all types of breeding places.

Dipping method is most frequently used for collection of mosquito larvae. The collecting instruments (enamel bowl, frying pan, ladle) should be immersed in the breeding places at an angle of 45° (fig. 9.). The palette should be used as shown in fig. 10 and immersed with a quick movement. Palette can be dragged and more areas can be covered at a time.

b. *Netting* :

Using this method larvae are collected by sweeping the surface layer of the water with a net. A hand net can be made from a ring of iron wire to which a nylon bag is attached. The upper part of the bag is reinforced to a depth of about 10 cm. A round hole is cut in the bottom of the bag and a transparent plastic cylinder is attached. A cone or bamboo handle is attached to the ring with some modifications. A well net (fig. 11) can be made to collect larvae from wells.

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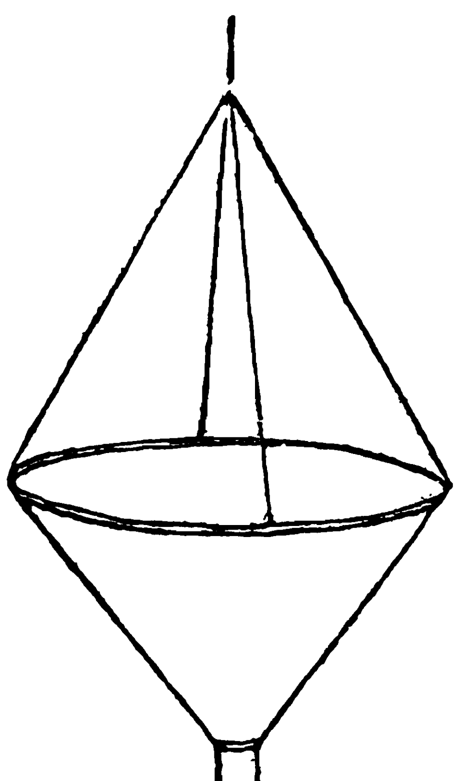

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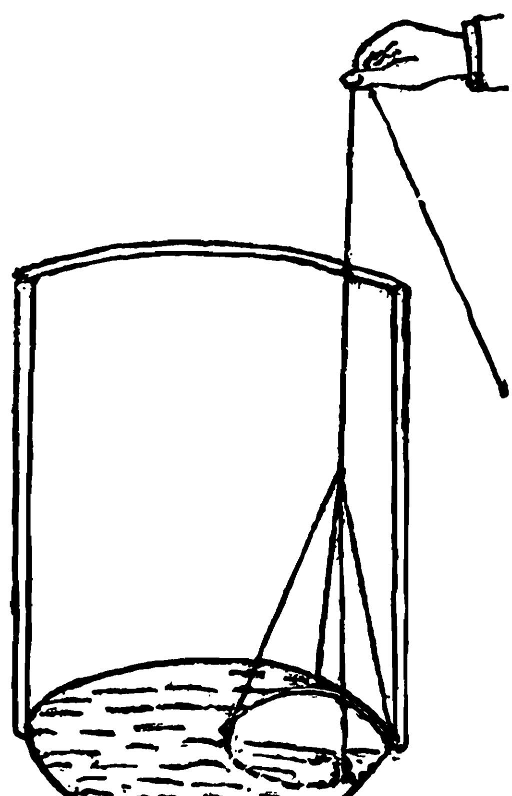

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Figs. (9-11) 9. Dipper made from frying pan. 10. Well net. 11. Use of well-net.

c. *Pipetting* :

Small pipetts (glass tube with a rubber bulb) can be used for collecting larvae from the surface of breeding places.

A II. *Collection of larvae from tree holes and/or axils of leaves* :

A ladle or small net can be used for quantitative studies of large, deep tree holes or the water can be siphoned off rubber tubing depending on the diameter of the tree holes. The holes can be washed 2 or 3 times with extra water. Wide pipettes or a special siphon (fig. 12) can be used to collect larvae in small narrow holes or from axils of leaves. Eggs, larvae and pupae can be collected by this method.

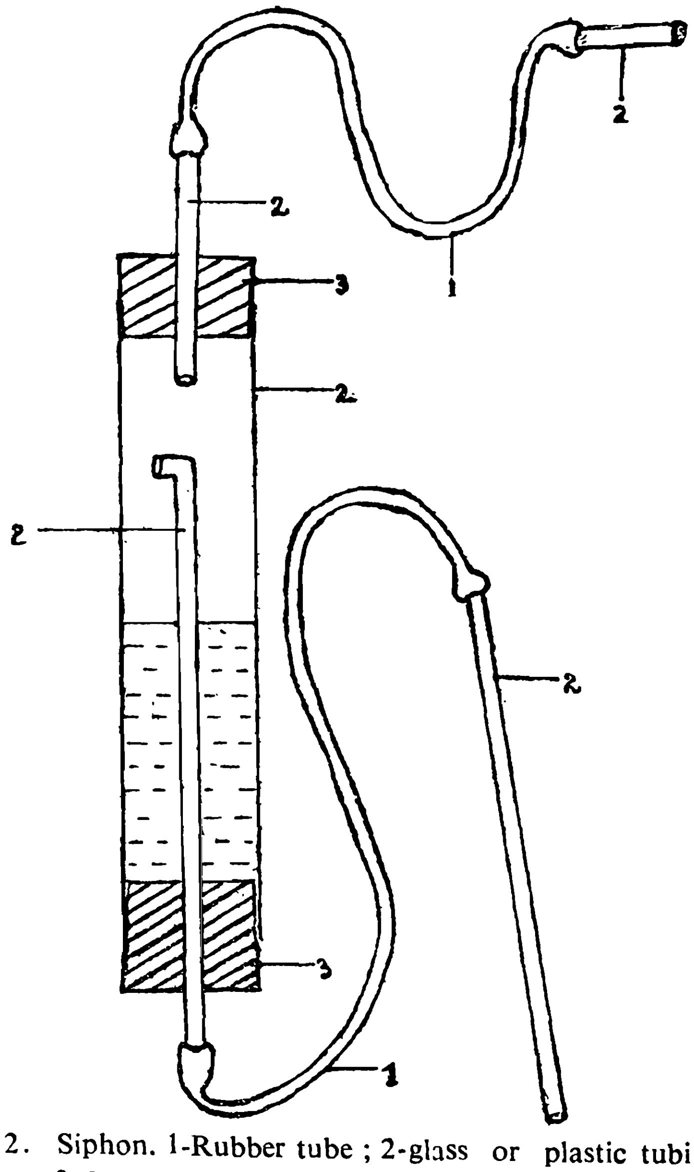

Fig. 12. Siphon. 1-Rubber tube ; 2-glass or plastic tubing. 3-Cork or rubber stopper.

V. BREEDING AND MAINTENANCE OF MOSQUITOES UNDER LABORATORY CONDITIONS

Mosquitoes are bred in the laboratory to provide a constant supply for biological observations, to study the effect of insecticides, to provide materials for training, to identify sibling species and to mass produce sterile hybrid males. These objective can be easily attained in a research institution as well as under field conditions.

Mosquitoes can be reared in an insectary which may consist of a single room or several rooms, in which larvae and adults are reared. The insectary should have a low ceiling and the walls should not exceed 220 cms. The floor should be cemented. The larval room should have large windows or there should be provision for artificial lighting, either together or seperate.

Rearing of larvae :

i) *Collection of eggs* : Batches of eggs are obtained by isolating gravid females in glass or plastic tubes in which a wet support of water is provided.

ii) *Hatching of larvae* : White polythene bowls filled with chlorine free rain water, fresh distilled water or water from breeding places can be used for the eggs to hatch into. The incubation period normally lasts about 2 days at 25°C. Solid food in the form of very fine powder is used for breeding most species. The quantity of dry food and the frequency of application depend on the number of larvae in the breeding pan. Liquid food is recommended for species of *Anopheline* mosquitoes whose larvae feed on the bottom as well as at the surface. The temperature of the breeding place should be around 28°C, but temperatures 30-32°C., will not be harmful for species which breed in nature in places exposed to the sun.

Rearing of adults :

Pupae are collected from the breeding pans and placed in glass jars kept in the breeding cages. The majority of adults will emerge in about 2 days at 25-27°C. To maintain the colony, the females have to be fertilized by natural or artificial mating. Adult mosquitoes need an optimum temperature of about 25-26°C and a relative humidity of 75-85% which can be provided by automatic apparatus. The above were the optimum conditions in our own experience with *Armigeres* sp.

i) *Feeding of adult mosquitoes* : To maintain a colony, the female should be given blood meals at intervals of 2-3 days. Males are fed with 5-10% sugar or glucose solution. In our experiment with *Armigeres* sp., white mice was used to feed on by the mosquitoes. When egg development is not required, feeding with 2% to 10% sugar solution will keep the mosquitoes alive for long periods.

2) MOSQUITOES REARING UNDER FIELD CONDITIONS

It is not difficult to rear mosquitoes under field conditions in the tropics as the appropriate temperature & humidity required exist in any shaded room or can be obtained by modifying the room. Mosquitoes can be reared in three ways in the field.

i) Breeding mosquitoes from larvae, collected from natural breeding places.

ii) From eggs laid in captivity by wild females.

iii) A self perpetuating colony, which is automatically maintained in the case of stenogamous mosquitoes or by artificially mating in case of eurygamous mosquitoes.

VI. METHODS FOR MARKING MOSQUITOES

Mosquitoes are marked to study their dispersion, longevity and calender age and also the average duration of gonotrophic cycle. It can be done by dusting with powders, marking with paints or by labelling with a radio-active isotope.

VII. PRESERVATION AND MOUNTING OF DIFFERENT STAGES

1. *Killing*

Chloroform can be used successfully, as in the case of most other flies, for killing mosquitoes causing least damage.

2. *Mounting*

a) *Mounting of adults on pins or on card points*

Method I. As in the case of most other Dipterans, small stainless steel pins are essential for mounting. Freshly killed specimens are best suited for pinning, though those hardened due to long storage or drying, can still be mounted.

Method II. The following equipments are needed for this method *viz.*, i) Insect pins — No. 3 ; ii) Card points-these are made by using an "insect mounting point punch" and punching out points from a 3″ x 5″ index card ; iii) Clear finger nail polish ; iv) Plastic setter.

The No. 3 insect pin is thrust through the thick end of the triangular point. Finger nail polish is then applied to the tip of the card point using the brush. The mosquito is then glued at the thoracic pleurites.

b) *Mounting of Slides*

i) *Mounting of male genitalia* : After the genitalia is detached from the abdomen it should be boiled in 10% NaOH or KOH and mildly heated for 3 to 4 minutes or kept for a longer duration in cold NaOH or KOH. After washing it in pure water the specimen is transferred and kept in 50% Cellosolve for 10 minutes before transferring it to pure Cellosolve. Later it is mounted in Canada Balsam.

ii) *Mounting of eggs* : The eggs are kept in Euparol for 10 minutes, before mounting in Canada Balsam.

iii) *Mounting of larvae & pupae* : For preparing permanent slides the larvae or pupae should be killed in hot water (60°C). This will avoid shrinkage and distortion of the specimen. It also prevents darkening of the specimen as well, since the heat destroys the enzyme which converts proteins to tyrosine and tyrosine to melanin.

c) *Preservation in liquids*

Preservation of larvae : The common method is to preserve in 10% Ethyl alcohol. But there are a few more methods yielding excellent results like using Mac Gregor's solution with the following composition :

i) Borax dissolved in small quantity of distilled water 5 gms ; ii) Glycerin — 2.5 ml., and iii) Formaldehyde — (39%) — 100 ml., and sufficient water to make 1000 ml. This solution has the advantage of preventing the specimen from hardening and helps in conserving the setae. To make good mounts for study the digestive tract of the larvae should be rid of opaque materials, which can be accomplished by starving the larvae for 12 hrs.

VIII. EXAMINATION FOR INFECTIONS

Mosquito stomachs positive for oocysts are required to be stained and mounted using the following procedure : —

A drop of 2% aqueous solution of Mercurochrome is placed on the stomach after it is dissected out and later removed of saline. The gut is washed twice in Formolglycerine solution. On a glass slide, previously ringed with Ducoenamel paint, the gut is mounted in Formolglycerine, using a cover slip. This procedure is less time consuming and is recommended especially for specimens in which the infection is doubtful.

Besides the above, Precipitin test is used in the laboratory for identifying the blood meal and mixed agglutination test for identifying the blood available even in minute quantity.

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REFERENCES

ANONYMOUS, 1975. *Manual of Practical Entomology in Malaria.* [World Health Organization] Geneva, pts 1 & 2.

GILLETT, J. D. 1971. Mosquitoes. In Richard Carrington (ed). *The World Naturalist.* —[The Chancer press Ltd.] Bungay, Suffolk.

HARKER, J. E. 1958. *Bio. Rev.*, Cambridge, **33** : 1 - 52.

HARKER, J. E. 1961. *Ann. Rev. Ent.*, Pale Alto, **6** : 131 - 146.

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COLLECTION AND PRESERVATION OF ACARINA OF MEDICAL AND VETERINARY IMPORTANCE

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The subclass Acarina includes arthropods popularly known as 'ticks' and 'mites' Though, they are often erroneously associated with insects under "Entomology", the ticks and mites have usually four pairs of legs in the adult. Several parasitic acarina are pathogenic and little is known of others. Some ticks and mites are transmitters of dangerous disease causing protozoa, bacteria, viruses. Some house-dust mites cause asthma and allergic reactions in man. The food grains, flour infested with acarid mites may be toxic to man and domestic animals. When the mites are consumed together with food they cause intestinal and urinary afflictions in animals and in more serious cases even abortion and paralysis as for example, in mares and saws (Speransky, 1971). A few species of soil mites though not parasitic, however, act as intermediate hosts of cestodes parasitic in domestic animals. A good number of parasitic acarina harm directly by blood feeding or by causing injury to the skin, etc., of the host. The common clinical signs (in snakes, birds and mammals) are irritation, restlessness, loss of sleep, pruritis or dermatitis, thickening of skin, weakness, loss of blood, anaemia, sometimes even death. In birds, they also produce inflammation, crusts of leg and face, cough, deformity of beak and legs, lameness, decreased egg production, matting or sometimes loss of feathers, etc. Some parasitic acarina cause haemorrhage, pain, inflammation of the ear, deafness, twisted neck, loss of equilibrium, circling, convulsions, meningitis, foci in lung, chronic pneumonitis, or asthma, baldness, wart-like horny outgrowths of ears, nose, etc., in mammals.

Most of the species of the subclass Acarina are small, less than a millimetre long ; but a few others are relatively larger. Those acari that are parasitic on wild animals, man and his live stock, either directly or indirectly endangering the health belong to orders Metastigmata, Mesostigmata, Astigmata, Cryptostigmata and Prostigmata. At least fifty of the more than 180 acarine families contain parasitic species (Nutting, 1968).

The complete life history of most of the parasitic acarina has not yet been studied. Nearly all of them lay eggs, although a few are larviparous. The eggs are laid either on the host body, where they inhabit or deposit their eggs in soil, crevices, etc. The eggs are laid one to several thousand at a time. The eggs may be smooth, spherical, opaque white, or variously coloured. In some cases the eggs are sticky. Eggs of some species of ticks are protected from drying by thin layer of water proof waxy coating applied by the female prior to deposition. The eggs hatch into six-legged larva, which may or may not feed. The larva moults, into a eight-legged protonymph which is a feeding stage. The protonymph metamorphoses into a deutonymph, usually a feeding stage. The deutonymph may pass through a tritonymphal stage before becoming adult.

Larval and nymphal stages of most metastigmatid mites require feeding on the host. Certain species of ticks pass through as many as eight nymphal stages, but the morphological differences between various nymphal instars have not yet been fully established.

Parasitic Mesostigmata usually pass through egg, larva, protonymph, deutonymph and adult stages in their life cycles. A few species, however, do not lay eggs, but give birth direct to larvae. Larva does not feed, but protonymph feeds, and the deutonymph again may not feed.

All species of Cryptostigmata pass through five developmental stages, *viz.*, egg, larva, protonymph, deutonymph and adult.

The developmental stages of Astigmata in general consist of an egg and four active stages (larva, protonymph tritonymph and adult), but in some species an inactive, non-feeding hypopus stage may appear.

The trombiculid mites have five stages, the larva, protonymph, deutonymph, tritonymph, and adult after hatching from the egg. The larvae are parasitic on birds and mammals, where as the deutonymph and adult are non-parasitic and predacious on other arthropods, or their eggs.

I. COLLECTION

Parasitic acarina occur on feathers, pelage, or hair follicles, skin, beneath epidermal scales of legs, nasal passages, trachea, bronchii, lungs, air sacs, body cavity, or the surface of the liver or other organs, frontal sinus, etc., of snakes, birds, and mammals. Since a wide range of organs are infested by the parasitic acarina, it is necessary to have a thorough examination of the host for their collection.

A number of techniques for the collection of parasitic acarina have been developed from time to time, and each technique is considered as the most suitable to its designer. The choice of the technique depends entirely upon the aim of the investigator. However, some simpler techniques are given below. Parasitic acari can be collected either from the live or dead host, as the case may be.

(A) MECHANICAL EXTRACTION

i) *Flag dragging* :

Adult ticks and the juveniles (not attached to the host) may be collected from vegetation by dragging a meter square flannel cloth, when they adhere to the cloth.

ii) *Hand Picking* :

Ticks from the hosts may be picked up individually by forceps, brush etc. Many ectoparasitic acarina are collected by keeping the host for 2-4 days in a screening cage over a pan of water. Since many of them are not obligatory parasites, they are detached after feeding from the host. Parasites can also be collected by combing carcasses over a polythene sheet or a tray. Feather mites are generally seen along the rachis or on the remiges. These mites may be collected with a fine brush moistened with water or alcohol, from the feathers under a binocular microscope.

iii) *Flushing* :

The nasal cavities are flushed with a stream of water under high pressure for collecting internasal mites. Yunker (1961) described the flushing technique in detail. The tip of a 20-gauge needle is cut off 2 mm, from its base, and the tip is filed smooth. The dead animal is grasped firmly by the throat, in order to close the trachea and oesophagus. The needle is attached to a 5 cc., syringe filled with water, and introduced into one of the nostrils. The water is injected forcibly through one of the nostrils and collected as it comes through the other, and the mouth. The procedure is repeated 2 or 3 times alternately in the two nostrils. Internasal chiggers, if present, will generally be found in the sediment along with mucous strands and occasionally, blood. Epidermoptid, and Speleognathid mites may also be recovered by this method. The latter, being extremely hydrophobic, are nearly always found floating on the surface of the washings.

iv) *Scraping* :

Suspected wounds caused by mites may be scraped gently with a scalpel, until the skin is roseate, and can be examined for mites in a suitable medium like water, or alcohol.

v) *Autopsy* :

Splitting of the bill between nares of the dead host often facilitates the recovery of mites. The nasal cavities of dogs are examined by sawing transverse sections through the head, buccal cavity, frontal sinus. Lungs also may be examined for mites.

vi) *Heat-desiccation* :

Feather mites can also be collected by spreading the feathers on a fine meshed wire gauze (preferably a 2 mm mesh) placed on a funnel, with a over hanging electric bulb to drive the parasites by heat desiccation. A small container is placed below the stem of the funnel. The container is filled with water, (if live parasites are to be collected), or with 70% alcohol. Parasites from detritus have to be sorted out under the dissecting microscope. Nidicolous ectoparasites, soil inhabiting species which act as intermediate hosts, or house dust mites can also be collected by this technique.

(B) CHEMICAL EXTRACTION

i) *Application of detergents* :

Ectoparasitic acari may be collected by shaking vigorously the carcasses of birds, small mammals etc., in available detergents, like soap. The mites then leave the host and can be collected from the decanted washings.

ii) *Application of acaricides* :

Insecticides or acaricides such as compounds containing Sulphur, Pyrethrum, systemic insecticides or commercially available "Dry-die", Chloroform etc., may be applied either directly by spraying or dusting on the host. Care should be taken so that the nose, eyes, and mouth or bill are protected sufficiently, if a live host is involved. When these acaricides or detergents are applied the acarina leave the host body, which may also be tapped on a cloth and the mites can be collected from the cloth or from the polythene bag.

iii) *Dissolution technique* :

Parasitic mites may be recovered from the skin of the dead host by Hopkins dissolution technique. Fresh or dried skin pieces should be placed in 5-10% Potassium or Sodium hydroxide (KOH or NaOH) till the hairs or feathers become soft and can be scraped off. The scraped and partly softened material was then treated with additional quantity of KOH or NaOH solution over a water bath, till it dissolves completely in the solution. The contents of the beaker were then filtered while hot, through a fine mesh of stainless steel gauze. The solid residue on the gauze was washed well into a petridish and examined for parasites. This technique has been modified by several workers. The residue may also be treated with $ZnSO_4$ solution so that the parasites float on the surface and can be easily removed. (cf. paper no. V.1.).

II. PRESERVATION

Preservation of acari can be either in liquid medium or mounted on slides.

i) *Preservation in liquid* :

Ticks and mites are preserved in 70% alcohol. Glycerine (upto 5%) may be added to prevent the evaporation of the alcohol during storage. Dried specimens may be relaxed by warming in 60% Lactic acid.

ii) *Mounting on slides* :

Adult and some immature stages of ticks are examined under the dissecting microscope. In some cases, the immature stages are mounted on glass slides for specific identification. The mounting techniques of ticks and mites are as follows :

a) *Temporary slide preparation* :

The mites have to be made transparent prior to the examination under microscope. The specimens are directly transferred from the preservative to Lactic acid on a slide, oriented, and covered with a coverslip ; placed on a warm plate until the specimen is sufficiently cleared. Warming in Lactic acid reduces the normal opacity of the mites and also cause the legs to extend. Clearing time depends upon the strength of Lactic acid and the degree of sclerotization of the specimen.

b) *Permanent slide preparation* :

Heinze-PVA mounting medium and Hoyer's medium are found quite satisfactory for permanent slide preparation. The former is more satisfactory in hot and humid climates.

Heinze mountant contains the following : polyvinyl alcohol (10 gm). Distilled water (40-60 cc.); 85-92% Lactic acid (35 cc.) ; Glycerine (10 cc.) ; 1.5% Phenol-water solution (25 cc.) ; Chloral hydrate (100 gm). All ingredients must be of very good quality. Water is added to the PVA powder in a large beaker and heated on a water bath, constantly stirring the mixture. After a while Lactic acid is added. Then Glycerine is added and once again stirred. The mixture is allowed to cool to lukewarm temperature and then Chloral hydrate (dissolved previously in the Phenol solution) is added. After a thorough stirring, the mixture is filtered using a filter paper, which may take about a day or so. Glass wool filtering is not quite satisfactory. The Heinze-PVA medium should always be stored in brown bottles. Slide preparations with Heinze-PVA medium need not be warmed and are dried at room temperature. Refractive index of the mountant is 1.515. The slide should be properly labelled.

Hoyer's medium consists of Distilled water (50 cc.) ; Gum arabic (crystals) (30 gm) ; Chloral hydrate (200 gm) ; glycerine (20 cc.). The ingredients are added in the order shown above at room temperature and filtered through a clean gauze to remove the sediments. The edge of coverslip should be sealed with 'gold size', 'cutex' or any other suitable cement.

Both Heinze and Hoyer's media are water soluble and therefore, the mounted specimens can be easily removed and remounted, if necessary by dipping the slide in water.

III. PRECAUTIONS

1. Hosts must be placed in mite-proof containers as soon as collected, so that the mites can not crawl away from one host to another ;

2. Each host must be kept in a separate container so as to avoid the mixing of the parasites of other hosts ;

3. When the hosts are transported to the laboratory, they should not be crowded and so it is necessary to pack them in larger boxes ;

4. Parasites from each host also should be kept in separate tubes for an indirect study of the host specificity ;

5. Tubes with collections should be properly labelled with the name of the host, locality and date of collection, name of the collector etc., the exact location or micro-habitat need to be mentioned on the labels ; and

6. When the specimens are sent to specialists for identification, *they should be in alcohol (and not mounted on slides)*, since the examination technique often differs from individual to individual, or species to species.

IV. REARING OF TICKS AND MITES

Evans *et al.* (1961) discussed the rearing of various ticks and mites in detail, and hence it is not dealt with here.

REFERENCES

EVANS, G. O., SHEALS, J. G. and MACFARLANE, D. 1961. The *Terrestrial Acari of the British Isles. Vol. I. (Introduction and Biology)*.—[British Museum (Natural History)]. London : 219 pp.

NUTTING, W. B. 1968. Host specificity in parasitic acarines.—*Acarologia*, **10** (2) : 165-180.

SPERANSKY, V. V. 1971. Acaridoses in man caused by tirogliphoid mites (Obzor literatury).—*Med. parasitol.*, (Moskova), **49** : 488-491.

YUNKER, C. E. 1961. A sampling technique for intranasal chiggers (Trombiculidae).—*J. Parasit.*, 47 : 720.

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COLLECTION AND PRESPRVATION OF FLEAS (SIPHONAPTERA INSECTA)

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The order Siphonaptera comprises insects popularly known as 'fleas' They are small wingless and are found on many warm-blooded animals (i.e. birds and mammals). The fleas, in addition, to their direct injury to the host by biting, they are also known to transmit *Pasteurella pestis* which causes bubonic plague (epizootic and sylvatic), *Rickettsia typhi* responsible for endemic typhus and *Trypanosoma lewisi* the agent of rat *trypanosomiasis*. They are also reported to be the intermediate hosts of some cat and dog tape worms.

The body of the fleas are laterally compresssed, facilitating movement through the hairs or feathers of the host ; and covered with strongly sclerotised cuticle. Mouth parts of the adult are piercing and sucking type. A blood meal is necessary for egg laying. The antenna consists of 3 segments. The eyes are simple, may be present, absent or vestigial. Legs are adapted for clinging and leaping.

The egg is oval, transparent, white or cream coloured, soft and liable to be damaged during careless handling. Larva is hyper active, whitish, apodous and vermiform with masticatory mouth parts.

Eggs and larvae are found in places where the hosts rest, or roost in the accumulated rubbish, debris and dust.

1. COLLECTION

For collection of developmental stages (egg, larva and pupa) of fleas, floor litter from the nest and sleeping places of the hosts are collected and the specimens are searched with great care by using a soft brush.

Adults may be collected from the body surface of the hosts or from their haunts or living places. It is difficult to collect the fleas because of their swift and leaping habits. However, they may be collected from the live host with forceps, or with a cotton swab fixed to the end of stick moistened with chloroform or alcohol ; where necessary, the hosts are also anaesthetised. Combing sometimes help in collecting the fleas. Domestic animals may be searched for and the wild animals are killed by shooting. Small mammals may be trapped using various types of traps available. Small birds and mammals soon after their death should be placed in polythene bags or jars (separate container is used for each specimen) with great care, so that the fleas do not escape. The material is brought to the laboratory and left for few hours. The fleas begin to leave the host body as the cadaver becomes cold. Cotton soaked in chloroform may be kept inside the glass jar or polythene bag as a result of which the fleas jump off from the host body and die, and can be easily picked up. Miles (1968) devised a carbon dioxide bait trap for collection of ticks and fleas from mammalian burrows. Deoras (1965), George (1972), Greenwood (1973) and Beaucournu (1974) also discussed different methods for collection of fleas.

2. PRESERVATION

(a) *Preservation in liquid* :

Adult fleas are preserved in small vials containing 70% alcohol added with a few drops of glycerine. The addition of the latter prevent the specimens from hardening on prolonged storage. Separate vials are used for fleas collected from each host, suitably labelled with data of collection, locality, name of the host, and collector's name.

Larvae are preferably killed in hot water, before preservation in alcohol.

(b) *Mounting on slides*

Fleas are mounted on slides for morphological studies and identification. They are cleared in 10% cold KOH solution for 24-48 hrs. depending on the degree of sclerotization of cuticle, washed several times in distilled water to remove traces of the alkali solution. Then the specimens are dehydrated in ascending grades of alcohol from 30% to absolute alcohol keeping the specimens in each grade for several minutes, with longer duration in higher grades of alcohol. Then the specimen is cleared in clove oil and mounted in neutral canada balsam.

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REFERENCES

BEAUCOURNU, J. C. 1974. Techniques of collecting Siphonaptera.—*Entomologist*, **30**(2) : 80-86.

DEORAS, P. J. 1965. Studies on bombay rats. A modified technique for flea indices.—*Curr. Sci.*, 34 (18) : 540-542.

GEORGE, R. S. 1972. Notes on collecting fleas.—*Entomol. Gaz.*, **23** (2) : 140-142.

GREENWOOD, M. T. 1973. An introduction to the collection of fleas of small mammals.—*J. Biol. Edc.*, 7 (6) : 5-14.

MILES, V. I. 1968. A carbon dioxide bait trap for collecting ticks and fleas from mammalian burrows. *J. Med. Ent.*, **5** (4) : 491-495.

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TECHNIQUES OF COLLECTION AND PRESERVATION OF PARASITIC CRUSTACEANS

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INTRODUCTION

The Crustacea, embrace forms commonly called prawns, crabs, lobsters, hermit-crabs, wood-lice, barnacles, etc. Majority of these are aquatic, breath by gills, or by the general surface of the body. These creatures play a dominant role in the economy of man. A number of them are directly useful to him as food, while a vast majority, virtually myriads, form the important constituent of the food chain of other valued animals, and thus largely contribute to the maintenance of certain fisheries. They also endanger the health of many animals including man, either directly as parasites or vectors of other parasites.

The crustacea form one of the divisions of a comprehensive group *viz.* Arthropoda among the members of the animal kingdom and broadly classified into seven sub-classes namely, Branchiopoda, Ostracoda, Mystacocarida, Copepoda, Branchiura, Cirripedia and Malacostraca.

True parasitic crustaceans pertain mainly to three groups namely, Copepoda, Cirripedia and the Isopoda. In each of these groups, there are some species with modifications of such a profound nature, that without knowing the life history of the animal concerned, it would be quite impossible to identify it as a crustacean. Less profoundly modified parasites are found among the amphipods and the Branchiura. It will, therefore, be convenient to deal with each of these groups separately.

COLLECTION, PRESERVATION AND EXAMINATION

Copepods : Parasitic copepods are quite common on both elasmobranch and teleost fishes. These parasites inhabit either the buccal cavity, the branchial chamber or surface of the body of the host. Such of those inhabiting the buccal cavity or the gills usually remain in their positions even after the death of the host. But others which occur on the body surface will fall off after the death of the host or they may be rubbed off while handling the fishes. Hence it is very essential to examine the fishes as soon as they are caught.

It is easy to locate and collect large Copepods such as the Caligids, but there are many others which need careful examination of the host. Parasites occurring in the gill chambers, for example, are sometimes confused with the gill filaments. In such cases, it is very essential to cut open the gills and tease the filaments with the help of mounted needles in a watch glass, and the dissected material will reveal the presence of a large number of copepods. There are still other forms such as those pertaining to the group Lernaeopodids fused with the body of the host and extraction of such forms are done by dissection and tracing the parasite upto its head. Since the structure of the head is an important character in the determination of these forms, it is quite necessary to locate the head and collect the material intact with its head.

Certain other copepods, specially the Cyclopoids are not visible to the naked eye as they are mixed with the surrounding mucus of the gills of dead fishes. In such cases, it is necessary to flush the material. Since most of the parasitic copepods are covered with mucus secreted by the host, it is essential that the material is cleared earlier prior to its preservation. Otherwise, the mucus hardens and sticks to the body and the appendages and makes the determination difficult. To obviate this difficulty, the mucus can be removed from the parasite by immersing the material in a dilute solution of Sodium bicarbonate for about 10 to 15 minutes. The best preservative is alcohol and 75% to 90% may serve the purpose, in case alcohol is not immediately available. Polyvenol solution is also used as a preservative.

For identification of the parasitic copepods, the material preserved in alcohol or other preservatives may be used as such. In case the material is opaque due to preservative, it is better to mount it temporarily in Lactic acid, which has the capacity to clear the material and retain the shape and other characters. Such temporary mounts are always preferred for studies compared with permanent mounts after proper staining.

Branchiura : All the members of this small group are parasites. The genus *Argulus* is well known and is remarkable for the development of a large pair of adhesive suckers in place of the first maxillae. Most of them are quite small in size, except in some species which grow over an inch. These creatures attach themselves to the skin of fishes. In addition, they have also been recorded from tadpoles of frogs and newts. All these forms are not permanent parasites, since they may be found quite often swimming freely. However, the genus *Chonopeltis* lost its ability to swim and it dies within a couple of days, in case it is removed from its host.

The damage caused by the branchiurans is probably not very serious, but the small wound it inflicts on the host may become infected with fungus, which may kill the fish in due course.

The branchiurans may be looked for mostly on the body of the host, specially in the gill region and often near the fins. They may be easily collected with the help of forceps and mounted needles. The preservative used is 75% to 90% alcohol.

Cirripedia : Barnacles (both stalked and the sessile forms) attach themselves to other animals and enjoy free transport. Some of the stalked forms develop root-like processes and embed themselves in the skin of the host. True parasites are found only among the Rhizicephala of which *Sacculina* is well known. It is seen from *Sacculina*, that the roots are developed from a small central body developed from a mass of undifferentiated cells, instead of the head as the name Rhizocephala indicates. Further, the roots spread throughout the host body extending even to the tips of the legs. *Sacculina* is quite commonly found attached to the abdomen of crabs. The adult parasite appears as a sac on the undersurface of the crab. Hence collection of the parasite from the host is a difficult task and needs care and patience. It is only by means of dissection and teasing musculature, etc. with the assistance of needles, it may be possible to extricate the entire parasite. After narcotisation in 4% Formalin, and wash, the material may be preserved in 75% to 90% alcohol.

Isopods : Among the Isopods, one sub order *viz.* Epicaridea are exclusively parasites in nature and most of them are observed as parasites in other crustacea. The life histories of these forms are complicated and involve two hosts. The eggs give rise to larval forms which are essentially typical Isopods. These larvae swim freely in the sea until they find a host, *viz.*, a copepod. The Isopods remain for six or seven days and pass through couple of moults. The larva is now termed as *cryptoniscus* and leaves the copepod and seeks out the final host viz. a prawn or crab. Among the Bopyridae (a family of Epicaridea), the cryptoniscus makes its way into the gill chamber of a young decapod. The larva moults and loses the pelopods from its abdomen. It grows through a series of moults. The parasite feeds by sucking the blood. The first *cryptoniscus* which establishes itself in the gill chamber invariably develops into a female and any later arrivals develop as males. Although the bopyrids are external parasites, the draining of blood from the host has serious internal effects. In general these are similar to the effects of *Sacculina* on crabs. The reproductive organs of the host are reduced and parasitised males, become effeminate. The female is asymmetrical and large, while the males are small and less asymetrical.

Most of the other Isopods which are parasitic are confined to hosts other than crustacea. The forms pertaining to Gnathiidae are the most interesting, since they are parasitic during the early stages only. The free living adults are found in small groups in the crevices of rocks or in tunnels in the banks of estuaries.

Most members of the family Cymothoidae are parasitic on fish, but only as adults. In some Cymothoids the mature females are somewhat asymmetrical recalling the asymmetry found in the Bopyridae.

The Isopod parasites are mostly external except for the freshwater form *Ichthyoxenus*, which, occurs either in the gill chamber or near the pectoral fins. Some of them are found to occur in other areas attached to the skin of the host. In the case of *Ichthyoxenus*, the parasites are internal and they are always found in pairs (a male and female, the male being smaller in size). The males are symmetrical compared with the females. All these parasites referred to above can be easily collected with forceps and needles, except in the case of bopyrids, where a little cutting of the membrane covering the gill is necessary. The material thus collected may be killed with 4% Formalin, and after a wash, transferred to 75% to 90% alcohol.

LABELLING AND PACKING

Unless proper interest is taken in preparing the labels for the collection, the material thus collected would lose its purpose. While giving the details of the locality, collector, date of collection and other details, care should be taken to give the name of the host, in case the host is not preserved along with the parasite. The material should be carefully packed before transhipment.

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ON SOME ASPECTS OF PROCESSING OF HAEMATOPHAGOUS ARTHROPODS FOR VIROLOGICAL STUDIES

By

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Blood sucking (haematophagous) arthropods *viz.*, mosquitoes, sandflies, culicoides, tabanids, fleas and lice (Insecta), ticks, and mites (Acarina), in addition to direct injury by their bites, play an important role in the maintenance and spread of certain dreadful viral diseases of animals and man. They also act as vectors of other pathogenic organisms *viz.*, Rickettsia, spirochaetes, bacteria etc.

The viruses, biologically transmitted by arthropods, are known as arboviruses. Several arboviruses pathogenic to man and animal are reported from many parts of the world including India. Mosquitoes are the most important vectors and are actively associated in the transmistion of several viruses *e.g.* Dengue, Japanese encephalitis Chickengunya, West Nile yellow fever, and several other encephalitis causing large scale epidemics in different parts of the World. Ticks rank next to mosquitoes as potential virus transmitters. In India, the well known Kyasanur Forest Disease (KFD) virus, producing several epidemics to both monkey and human populations in Karnataka during the last 20 years, and Ganjam, Kaisodi etc. has been isolated from ticks. The ticks are also the main vectors of Russian Summer-Spring encephalitis and Congo viruses.

Several papers, in this workshop, have reviewed the collection and preservation techniques of important haematophagous ectoparasitic arthropods and their hosts. But they are chiefly aimed at collection and the preservation of postmortem material for taxonomic and other studies. On the other hand, the present paper provides a brief outline for handling of these arthropods for isolation of arboviruses.

I. EVALUATION OF THE PREVALENCE OF A VIRUS

Activity of an arbovirus in a particular area is suspected first, by clinical diagnosis and symptomatology of the disease caused by them.

In the suspected areas, attempts have to be made for the isolation of the potential pathogens from the haematophagous arthropods. Isolation of viruses from the clinical, biopsy, or autopsy materials collected from the patients and determination of the specific antibodies help us in the final confirmation of their involvement.

II. STUDY OF ARTHROPOD FAUNA

It is well known that, areas, endemic or having epidemic of arbovirus must have arthropod vectors. Further, for culminating in epidemic, the vector population must reach an optimum degree of density in a given area. Number of vectors is not alone important, but the density of the specific developmental stage of the arthropod vector, which acts as potential carrier of arboviruses, influencing the transmission of the disease is also equally important. For example, a peak of KFD cases, both in monkey and man, has been found coinciding with the maximum activity of the nymphal stages of *Haemaphysalis spinigera*. It has been found later, that the nymphs of this tick are the most effective potential transmitters of this disease.

III. COLLECTION OF ARTHROPODS FOR ISOLATION OF VIRUSES

Collection of arthropods is essentially the same as described by various authors in this workshop. However, care is taken to collect live uninjured forms, and preferably, a good number of them. These arthropods are to be kept alive for a few days, before preservation, till the blood meal taken previously by them, is apparently digested. If possible, they are identified in live condition and pooled species-wise from different localities. Such groups as sandflies, trombiculid mites etc., where identification is not possible up to specific level in the live condition, some parts such as mouthparts (in case of sandflies) or a representative sample are preserved for subsequent identification in the usual manner.

Pooled specimens are properly registered giving all the details and then despatched to the specialized laboratories for further processing. Specimens may be sent alive or physiologically immobilised at a very low temperature. Small cages with suitable arrangements for humidity control, or large tubes with water at both the end compartments and live specimens in the middle chamber may be used according to the convenience, depending on the group of arthropods involved. They can also be preserved in air tight, screw cap sterlized vials having suitable preservative like 'Buffer-Albumin-Phosphate Solution' Liquid nitrogen refrigerators having temperature at — 200°C., can also be used for storage of arthropods during transportation. In such low temperature the viruses remain dormant and can be revived later. Subsequent long term storage is carried at low temperatures between — 60°C, to — 80°C., in the ultra low temperature refrigerators.

Further processing of the arthropods for isolation and identification of viruses is a specialized job. In India, this is undertaken by the National Institute of Virology, Pune, and School of Tropical Medicine, Calcutta.

IV. COLLECTION OF BLOOD FOR ARBOVIRAL ANTIBODIES

Man and animals including bats, rodents, shrews and birds, known to have an important role in the natural cycle of arboviruses, develop antibodies against the viruses, once they are infected with them.

To determine the sero-epidemiology of the disease, blood samples are collected at periodical intervals from the affected areas. The blood sample is allowed to clot and serum is separated by centrifugation. These samples are asceptically collected, stored in screw cap sterlized vials on dry ice and transported to the specialized laboratories. Long term storage is to be carried at — 20°C. Specific antibodies are determined by several tests, Haemagglutination inhibition, Neutralization tests etc.

It is possible that Zoological Survey of India (ZSI) may have occasion to work in endemic, epidemic, or pevalent areas of some arboviruses, in India. In such cases and in areas hitherto, unknown for such diseases, the Department can contribute significantly by working on some aspects of epidemiology such as population dynamics of vector species etc. It can also play a very significant role by extending their assistance, providing samples of properly collected and stored specimens of arthropods for virological assessment.

ACKNOWLEDGEMENT

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CHROMOSOMAL STUDIES IN DIGENETIC TREMATODES AND SMALL ARTHROPODS THE TECHNIQUES FOR CHROMOSOME PREPARATION FOR CYTOTAXONOMICAL STUDIES

By

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Karyological analysis has come to be recognized as an important tool in understanding the problem of taxonomy and phylogeny. For proper understanding of population dynamics and inter-specific diversity, it is necessary to have a detailed karyological analysis of different species and their populations. In the case of higher vertebrates considerable advances have been made in karyological studies by inducing longitudinal differentiation of chromosome into the banded patterns. This facilitates the proper understanding of various subdivisions in a chromosome and for positive identification of all the chromosomes of a complement. It is also possible through the application of this technique to compare the relative phylogenetic affinities between the near or closely related species and establish criterion for proper identification of these animals at specific and population level.

The basic prerequisite for cytological studies of any group is procurement of live material. This live material has to undergo certain pretreatments before their particular tissue is made worth for cytological investigation. The purpose of this communication is to enumerate certain known methods for preparation of material for chromosomal studies and guideline for preservation of tissue, and making slides in the fields which could be brought to the laboratory for the specialist. The scope of this paper is thus limited to the techniques in helminths and smaller arthropods.

TECHNIQUE FOR DIGENETIC TREMATODES

A technique for the preparation of chromosomes in digenetic trematodes has been devised by Reddy and Subramanyam (1971) which has proved successful.

The parasites isolated from the animal are washed in Ringer's B solution to give a vertebrate environment. For getting good spreading of the chromosomes, the parasites are pretreated for 2 hours at room temperature in 10 ml., Ringer's B solution with 2 ml., of 0.025% Colchicine. The parasites are immobilized on a microscopic slide by a drop wise addition of Ethyl alcohol and Acetic acid (3 : 1), flattened well between the two slides. After fixation for 6 hours the specimens are dissected out for their testes, located one above the other in the posterior end. The testes are either employed directly from the fixative or after storage in 70% alcohol following preservation. After a wash in distilled water for 5 minutes, hydrolysis in 1 N HCl at 60°C. for 8 minutes, mordanting in 4% Ferric Ammonium Sulphate for 30 minutes, wash in water for 10 minutes, the testes are stained in Heidenhain's haematoxylin for 60 minutes, squashed and made permanent.

TECHNIQUE FOR SMALL ARTHROPODS

Because of the hard cuticle covering in these insects the penetration of pretreating solution is very difficult. If the size of the gonads is fairly big, the gonads particularly testes are dissected out, otherwise the abdomen is ruptured on the slide with a few drops of normal saline (·67% NaCl) and the tissues are fixed in aceto-methanol (1 part Acetic acid and 3 parts methanol) after 15 minutes of hypotonic treatment in .56% KCl. In the field the fixed material is stored in 70% Ethyl alcohol. To avoid hardening of the material, a few drops of glycerine are added in the vials containing the fixed tissues. In the laboratory the material is refixed in aceto-methanol (1 : 3) and is stored at 0°C. The fixed material is squashed in 50% Acetic acid on albuminized slides. The slides are kept overnight in the vapours of 50% Acetic acid at 5°C. The slides are then kept at room temperature for 15 minutes and are immersed in Aceto—methanol (1 : 3) for one hour. The coverslips are removed from the slides while still immersed in acetomethanol, and both are air dried. The slides are stained in 5% solution of Giemsa in Phosphate buffer pH 6.8 for 20 minutes. The stained slides are differentiated in phosphate buffer (pH 6.8), air dried and finally mounted in DPX.

With best chromosome preparations one can proceed for C-bands (constitutive heterochromatin bands) with the following technique :

The slides are kept in 0.2 N HCl for an hour. After rinsing the slides in triple distilled water, these are kept in $Ba(OH)_2$ solution at 50°C for 5-15 minutes. Immediately the slides are rinsed twice in triple distilled water and are put in 2xSSC for one hour at 60°C. After a rinse in triple distilled water the slides are passed through different grades of alcohol, *i.e.*, 70%, 90% and absolute alcohol. The slides are stained in 5% solution of Giemsa for one and half an hour. Lastly the slides are rinsed in triple distilled water, air dried and are finally mounted in DPX.

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COLLECTION AND PRESERVATION OF VECTOR MOLLUSCS

By

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The medical, veterinary and economic role of molluscs is the least worked out aspect in Indian Malacology. Molluscs act as the main or one of the intermediate hosts of the digenetic trematodes, cestodes and nematodes. But unfortunately, the role of molluscs as intermediate hosts and their relationship with the parasites was neither clearly understood nor given due importance.

Of the seven classes of the Phylum Mollusca, only two (class Gastropoda and class Pelecypoda) are important because they are the intermediate hosts of many helminth parasites and play distinct roles in public health. The completion of the life history of the parasite and its distribution is entirely dependant on the intermediate molluscan host. A correct identification of these molluscs, their ecology, and distribution are important to note for the containment of the diseases they transmit. These facts closely link malacology with parasitology.

Although many of the snails (Gastropoda) are known to harbour some parasites or other, freshwater forms are by far the most important. Land snails and slugs were recorded as intermediate hosts of a few parasites. Among bivalves (Pelecypoda), while marine forms serve as intermediate hosts of many parasites, freshwater forms were recorded as intermediate hosts in limited cases.

I. METHOD OF COLLECTION AND EQUIPMENT

Freshwater snails are generally found adhering to the aquatic vegetation, floating objects or stones in stagnant waters. They are also common in paddy fields and irrigation canals. All the data pertaining to the snails *viz* : hydrographic information like the type of water body, temparature, nature of bottom, and other ecological details should be collected. A standardized data sheet for snails, which may be filled in the field is appended here. (after Cheng and Malek, 1974).

Periodical surveys covering at least one calendar year need to be conducted and the data collected as indicated in the sheets. These data are then, analysed which would disclose the bionomics, life-cycle etc., of the intermediate molluscan host.

The following articles are useful in the collection of the snails — 1) a hand-,or water-, or a scoop-net, 2) a small piece of cloth, 3) a few cloth or polythene bags, 4) specimen bottles/ tubes (assorted sizes), 5) a thermometor, 6) hip-wading boots, 7) forceps 8) enamel trays, 9) necessary chemicals, 10) field note book and data sheets, 11) labels.

A hand net is made of a fine mosquito net fixed to a round iron-ring and fitted to a wooden handle. A scoop-net is usually a metallic one with 30 cm. × 30 cm. frame of steel bars and wire netting and the scoop is 10 cm., deep with a blade of 8 cm., width is soldered to the frame. A wooden handle is attached at the other end. The hand net or scoop net is dragged over the aquatic vegetation, and when it is filled with aquatic weeds, the contents are then poured out on a spread out cloth piece. The snails can then be picked up with a forceps from the weeds.

While making collections the following precautions are taken : i) Boots should always be used and one should not go bare footed. ii) Hands and arms should be repeatedly cleaned with 70% alcohol, if bare hands are used. But the collector should avoid using bare hands.

II. FIXATION AND PRESERVATION

A. *Histological Study*

Snails are fixed, either in hot Bouin's fluid (sat. aq. Picric acid, 75 ml. Formalin, 25 ml., Glacial Acetic acid 5 ml.) or Alcohol-Formalin-Acetic acid (AFA) (70% Ethyl alcohol 100 ml., Formalin 5 ml., and Glacial Acetic acid 5 ml.)

Time of fixation varies 20 - 24 hours and then the snails are removed and thoroughly washed in running tap water.

B. *For Routine Studies*

Identification of a mollusc is not entirely based on shell characters alone, as it is usually believed, especially of the members of the family

Lymnaeidae. Morphological details like the structure of the radula, shape of the prostrate gland etc., are also required for accurate identification of a species. It is therefore, needless to mention that the soft parts of the animal are to be preserved along with the shell.

(i) *Narcotization*

The aquatic molluscs collected are to be placed in an enamel tray with pond water. Then finely powdered Menthol or Magnesium sulphate or Chloral hydrate should be sprinkled over the water surface, and covered by a suitable lid. The specimens are kept for 24 hours usually.

Nembutal (Sodium pentabarbitone) has been successfully used elsewhere. Specimens are placed in a 0.08% pure Nembutal and allowed to relax. Time required for complete relaxation depends on the species, and hence a close watch has to be kept during narcotization. Urethane has also been successfully used for this purpose.

During narcotization land snails and slugs secrete a large amount of mucus. Therefore, specimens should be thoroughly washed under running tap water after narcotization. Freshwater snails can also be relaxed in boiling water.

(ii) *Asphyxiation*

Land snails and slugs can be killed by asphyxiation. A glass bottle or jar is fully filled with water, and the specimens are dropped into it, then the mouth of the jar is closed by an air tight lid, or screw cap. Deoxygenated water or addition of a few drops of spirit at intervals, may help in rapid killing. The specimen may extend fully and get relaxed in 20 to 24 hrs.

(iii) *Preservation*

a. *Soft Parts*

After narcotization, the snails may be preserved either in 70% alcohol or rectified spirit, but never in Formaline. Slugs, can also be preserved in 4% Formaline.

b. *Dry Shells*

While preserving some shells with the animal intact, it is a good practice to preserve a few dry shells separately. After narcotization, the animal is extracted from the shell with a bent tipped a forceps. Empty shells may be boiled in water for about half an hour or so to remove any soft tissue sticking inside the shell. The shells may then further be cleaned with a smooth brush, and then dried in air. In case of operculate shells, operculum should be retained by pasting it to a cotton plug inserted into the aperture of the shell.

Dry shells may be packed in cotton in small glass vials or tubes or card board boxes depending upon the size of the snail sample. Relevant data *viz* : field No. locality, date of collection, collector's name etc., should be provided with each sample.

C. *Quantitative Studies*

The population density of snails in a given area may be estimated using any one of the following methods.

(i) *Quadrate Method*

In this method a metal ring or square is dropped in the area of study, and all the snails found within that ring or square are collected and counted.

(ii) *Standard Scoop or Dredge Method*

A scoop or dredge of standard size is passed over the required area and number of snails collected in each operation are counted.

(iii) *Counts Per unit time*

It involves counting the number of snails, collected systematically with sieves by one or more trained collectors in a measured or marked area of the biotope for a given length of time.

(iv) *Palm-leaf Trap method*

Palm leaves are placed at regular intervals along the shore or bank of a water body under study. After exposing for a definite period of time, the leaves are carefully removed and the snails sticking to them are counted. This method has been successfully followed in many Middle East countries during surveys of snails of *Biomphalaria* and *Bulinus*. It is interesting to note that where other methods have failed, this method has given better results (Malek and Cheng, 1974).

D. *Laboratory Rearing of Molluscs*

Molluscs can be conveniently cultured in the laboratory, in glass aquariam, or jars, or in enamel trays, and dishes. It will give good results, if the natural conditions of a mollusc are simulated in the laboratory. The paramenters to be noted are vegetation and food temparature and water composition. In this connection the field data will be useful.

Water, preferably, from the same pond may be used in the aquarium. If tap water is to be used it should be first, allowed to stand about a week, so that it gets dechlorinated. Providing vegetation in the aquarium helps in oxygenation and removal of carbondioxide. It not only provides food for the snails, but also the necessary surface for egg-laying.

Land snails or slugs are easy to rear in laboratory. They can be maintained on a herbivorous diet in a terrarium. They are equally fond of fresh and decaying vegetation. An inch of moist and at the bottom with a covering of dead leaves provides a suitable substratum for land forms. Periodical moistening of the substratum ensures necessary humidity for the snails.

SNAIL-COLLECTION DATA SHEET

Date	Time	Locality	Artificial :
Water body :	Types :	Natural river, lake, creek etc. Permanent, Stagnant, clear Seasonal, running, muddy	Man made : reservoir, irrigation canal, fish pond etc.

Nature of bottom : rocky, sandy, clayey, humus, decaying matter.
Pollution :
Aquatic vegetation : dense, light : Type :
Density of snails : Many, few.
Egg masses : Present, absent
Snails collected :

Trematode infection :	Snail	Cercaria	Metacercaria
Nematode infection ;			
Other parasites :			
Sota exposure of water to Sun :			
Colour of water :			
Temperature : Air	Surface	water	Microhabitat
Water analysis :	Dissolved	oxygen	Hardness
Aquatic birds or mammals :			
Contact with and use by human beings :			
Any other information :			

REFERENCE

MALEK, E. A. and CHENG, T. C. 1974. *Medical and Economic Malacology.* — [Academic Press], New York and London : IX+ 398 pp.

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COLLECTION, PRESERVATION, REARING AND MAINTENANCE OF VECTORS LEECHES AND THEIR USE IN LABORATORY EXPERIMENTS

By

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Leeches are well known for their role as intermediate or carrier hosts of some haemo-protozoans and some metazoan parasites. In India, they are also implicated as potential transmitters of cattle diseases. The land leeches are known to transmit the flagellates (*Harpetomonas*) causing gangrenous ulcer in man. Leeches may even act as simple mechanical transmitters of bacterial infections.

Any one interested in the sudy of haemoprotozoans of aquatic vertebrates must have a good leechary. The details of initiating and maintaining a leech colony vary according to the involved species. However, in our country, a trained leech-breeder is not readily available. Unfortunately, the reproductive biology of many species of leeches is yet to be known. A guideline in maintaining the vector leeches in the laboratory is given below.

I. COLLECTION

The aquatic leeches are collected from the ponds, pools, lakes and sometimes from running streams. Generally, they hide under the thick growth of the weeds, stones, plants in fresh water. They are very sensitive and when they get the response of some vertebrates nearby, they come out for their blood meal. They can easily be collected by sweeping with a thick meshed hand net. Beating the weeds sometimes produce good results. They can also be collected by carefully searching the body surface of fishes, amphibians and some reptiles, such as water snakes, turtles and tortoises. It is often difficult to remove them from the host body, and application of a little salt water or salt crystals on the leech, will detach them easily.

Some bivalve molluscs like *Lamellidens*, and some gastropod snails, like *Limnaea*, also harbour leeches viz., *Glossiphonia*, *Helobdella* and *Hemiclepsis*, as endocommensals in the mantle cavity, and they can be easily collected by opening the mantle of these molluscs.

The land leeches of the genus *Haemadipsa* may be picked up´by hand after careful examination of the marshy, and shady places and sometimes, the deep bushes.

II. PRESERVATION

For exact identification, the vector leeches are narcotised and preserved in suitable media.

Leeches are particularly difficult to preserve with all the diagonostic characters intact, and great care should be taken in treating them after capture. Leeches invariably should be anaesthetized, before placing in the preservative.

The leeches should be preserved straight, moderately extended, undistorted and, of course, neither macerated, and over hardened nor dried. To ensure best results the following procedures are recommended and found suitable in our laboratory.

a) *Narcotization*

The leeches are placed in a glass jar containing a small quantity of water and are anaesthetised as follow :—

Carbon-di-oxide (as in soda-water syphons) ; Chloroform vapours ; Chloral hydrate or Chlorotone, Cocaine hypochlorate (1 : 1000 strength), weak Nicotine, Magnesium Sulphate, Low percentage of alcohol, weak acids, in which leeches usually die extended. When they no longer respond to pinching with the forceps or similar such stimulus, and before maceration begins, they are rapidly passed between the fingers to remove the surplus body mucus, straightened out, and laid extended side by side in a flat dish.

b) *Fixation and Preservation*

The fixative fluid, 50% Alcohol or 2% Formaldehyde is gradually added to the dish containing the narcotised and straightened leeches. After fixation the leeches are transferred to 85-90% Alcohol or 4% Formaldehyde for preservation. They should be placed in tubes or vials of suitable size to keep them straight, and to avail crowding and distortion. The tubes or vials are labelled with essential information such as the exact locality of collection, date of collection, name of the host, name of the collector, etc.

III. REARING

The fresh water leeches of tropical and temperate climates usually begin to breed in spring or early summer. Breeding time is indicated by the differentiation of the clitellum or by eggs or seminal fluid visible through the ventral body wall.

Fully engorged and gravid leeches are used for laboratory propagation. Each leech should be kept seperately in finger bowls or glass vials containing aerated freshwater. They are kept at room temperature or a little below the room temperature. A glass plate is placed on the top of each container, because the leeches tend to move away from the open container. The water of the container should be changed at regular intervals. Eggs (30-50) are carried on the ventral surface of the parent leech for about a week or two. Hatching of the eggs is completed within 24 hours. Yolk in the young leeches disappear within the next 2 weeks, and young leeches will be ready for feeding. The young leeches are very small, white with transparent body and showing the tendency to congregate in and around the ventral side of the parent.

Clotted human blood found to serve as an excellent food for *Macrobdella decora* (Moore, 1937). Blood clots placed in the rearing jar were immediately attached to and devoured within a few minutes by the young leeches. Attempts to feed *Helobdella nociva*, *Glossiphonia* sp. and *Hemiclepsis marginata* by this method were only partially successful, therefore, they were fed directly on otherwise uninfected fishes or amphibians.

IV. LABORATORY FEEDING

The newly hatched young leeches are placed on the experimental animals kept in the aquarium or glass jars containing fresh water, at a depth of about 5-10 mm. They are generally placed on the legs, knee joints, belly, back and loose skins of the amphibians and on the carapace, attached to the legs, neck and loose skin between the plastron and carapace of the turtles and tortoises. In case of fishes, the leeches are placed at the base of the dorsal or pectoral fins or by the side of the anal and genital apertures, or directly on the gills by opening the operculum. Occasionally, the animal is allowed to bleed by a pin prick and the feeding of the young is facilitated on the spot.

As soon as the leeches are fully engorged, they detach from the sucking site. The engorged leeches should be kept seperately in finger bowls wlth aerated fresh water, after necessary marking of the bowls.

V. INFECTING THE LEECH WITH PARASITES

A number of hungry leeches (about 25) are selected. They may be marked as Group-I, (of 20 leeches) fed on an infected host (amphibian) as experimental, and Group-II, of (five leeches) fed on an uninfected host (amphibian) as control.

Control leeches are dissected on the 3rd, 4th, 6th, 8th and 10th day after removal from the uninfected host.

Experimental leeches are dissected on the 2nd, 3rd, 4th, 6th, 8th, 10th, 13th, 16th, 22nd, 28th, 34th day and onwards after the infective meal to study the various stages of the life-cycle of parasites, like *Trypanosoma, Haemogregarina* etc.

Before the dissection the leeches are narcotized in aquaeous 4% Chlorobutanol (Pennak, 1953) and washed 3-4 times in Ringer's solution. The proboscis, proboscis sheath, crop, salivary glands and gastric caeca are removed and examined under a microscope. Smears are made from the caecal content and crop puncture material and fixed in buffered Formaline. Citrated saline solution may also be used for dilution of the caecal or crop content to study movement and behaviour of live parasites.

Air dried crop content smears are fixed and stained with any Romanowsky stain e.g. the Leishman or Giemsa stain. Some of the fixed smears may be stained with Iron alum-haematoxylin. Eosin may be used as counter stain.

VI. INFECTION OR INOCULATION OF THE VERTEBRATE HOST

Usually the infected leech is allowed to feed on the uninfected vertebrate host for fresh infection. However, it is desirable to know precisely the stage of infection being transmitted experimentally, therefore, a small amount of infective inoculum is squeezed out of the infective leech, examined, and injected to the experimental host.

The caecal and the crop contents of infected leeches are mixed with citrated saline solution (about 1 : 3) and 0.5 ml., of the same is injected subcutaneously or intraperitoneally. The amount of inoculum may vary according to the sex and age of the experimental host.

VII. HISTOLOGICAL PREPARATION

For the study of the developmental stages of the parasites inside the leech gut and tissues, it is essential to make histological sections of the leeches. Properly narcotized leeches should be well fixed in alcoholic Bouin's fixative containing saturated solution of Picric acid, (75 parts) Formaldehyde, (25 parts), and Glacial Acetic acid, (5 parts), and further processing for section cutting is carried out as per the general histological techniques (Pearse 1960).

REFERENCES

MOORE, J. P. 1937. *Laboratory care of leeches* — In Galtsoff, P. S. *et al.* (ed), Ithaca : 201-204.

PENNAK, R. W, 1953. *Fresh water invertebrates of the United States.* — [The Ronald Press Company], New York.

PEARSE, A. G. E. 1960. *Histochemistry,* — [J. & A. Churchill], London, 2nd Ed.

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BIRDS : THEIR COLLECTION AND PRESERVATION FOR STUDY

By

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I. COLLECTION

Specimens of birds are usually collected with firearms. A 12- or 16-bore and a.410-bore shotgun, and cartridges loaded with Nos. 3 or 4 and 8 or 9 shots will cover most needs. The larger bore gun is used for taking larger specimens and/or from long range, and the smaller one for smaller birds and/or from close range. Shot Nos. 3 or 4 are meant for larger birds, and Nos. 8 or 9 for smaller ones. Various types of traps and nets are also sometimes used for collecting birds.

II. ARTICLES REQUIRED FOR PRESERVING BIRDS

1) A scalpel with about 4 cm. long blade. 2) A pair of dissecting scissors with about 6 cm., long blades one of which is with pointed end the other with rounded end. 3) A pair of dissecting forceps with pointed ends, about 12-13 cm. long. 4) A pair of long forceps with rounded ends, about 25 cm. long. 5) An ordinary pen knife or kitchen knife. 6) A pair of bone-cutters. 7) A pair of cutting pliers. 8) Galvanized iron wire, 11, 16 or 18, 20 or 22 gauges ; or smooth, straight sticks, such as bamboo splinters, of various lengths and thicknesses. 9) A nail or tooth-brush. 10) Sewing needles (ordinary), Nos. 2-8. 11) Cotton sewing thread, Nos. 8, 60 and 90. 12) A hoghair brush (round), No. 8, as used by artists. 13) A 10- or 20 ml., metal-covered glass (or all glass) hypodermic syringe, with stout (No. 1, 2, 8, etc.) needles. 14) A medium-sized carborandum hone. 15) Chloroform. 16) Formalin (38-40% solution of Formaldehyde). 17) Rectified spirit. 18) Common salt. 19) Benzene or Carbon tetrachloride. 20) Heavy Magnesium carbonate (Magnesium carbonate pond.) or Magnesium oxide. 21) Arsenical soap. (Melt 1 kg. of soft soap, such as "Sunlight", cut up into small bits in a little water over a low fire. Add 230 g of white arsenic (=Arsenic trioxide),

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125 g of Borax. Boil for a few minutes, stirring all the time. Removė from fire, add 63 g of camphor, and continue stirring until it cools. The consistency of arsenical soap depends on the amount of water initially used for melting soap. By adjusting it, the arsenical soap may be made thick enough to solidify into cakes). 22) Teased non-absorbent cotton wool. 23) Absorbent cotton wool. This must be of very good quality with long fibres, that can be easily peeled into thin layers. 24) Old newspapers for carrying dead birds and for wrapping dry skins.

III. PRESERVATION

From the moment a specimen is shot it should be treated very carefully. With bits of cotton wool wipe off blood and dirt from plumage Plug the throat and cloaca with cotton wool. Note the colours of soft parts, especially of the iris, which in many species change soon after death. Wrap specimen in a piece of paper or cloth so that plumage is not disturbed. Specimens should be thus carried to the field laboratory.

a) *Preparation of study skins*

Replace throat and cloacal plugs of cotton wool with fresh plugs. Lay specimen on its back and part the feathers down the middle of the breast. With a scalpel make a longitudinal incision through the skin from about the middle of breast to vent, taking care not to cut the abdominal wall (Fig. 1). Separate the skin from flesh with fingers or handle of scalpel, and continue doing so until the knee-joint is bared. Keep exposed flesh dry by frequently dusting with Magnesium carbonate. Sever freed knee-joint one after another. Separate skin round to base of tail and cut through with scissors or scalpel, taking care not to cut bases of tail feathers : a good bit of flesh and bones of tail may be safely kept with skin. Continue forward freeing skin from

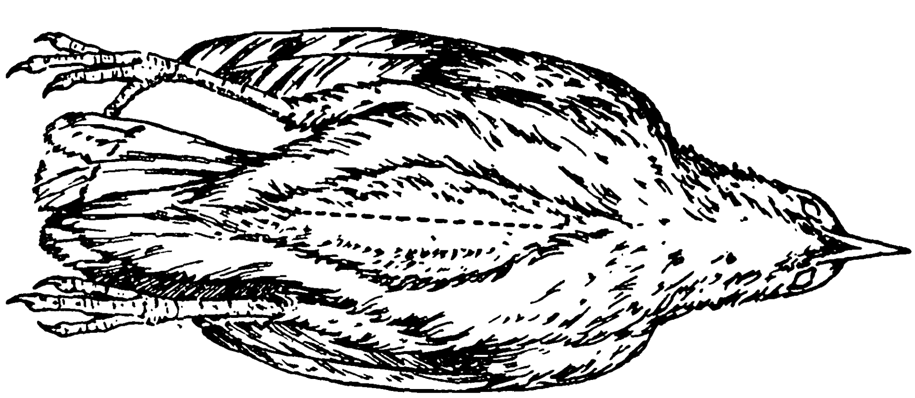

Fig. 1. — Line of initial incision (dotted) on the ventral side of bird.

body, turning it inside out. Work on the wings like legs, and proceed skinning forward till ears are reached. Pull skin out of ear-holes with fingernail or forceps. A little anteriorly over the eyeballs, cut thin transparent membranes which attach eyelids to eyeballs, taking care not to cut the lids. Continue freeing skin as far as base of bill. Remove the eyeballs and cut away floor of mouth-cavity, including tongue. Cut across back of skull and continue the cut on to roof of mouth-cavity, and thus sever neck and body from head. Clean out brain and as much soft tissue from skull as possible.

The skin is now completely inside out. Cut off upper arm bone just short of elbow-joint and remove it along with flesh. Clean off the flesh from leg bones and base of tail, and as much fat and flesh from skin as possible. Apply arsenical soap all over inner surface of skin and on parts of bones where bits of flesh might still adhere, such as roof of mouth-cavity, base of tail, etc. Make up leg muscles with cotton wool wound round leg bones. Fill up eye-sockets with cotton wool. Turn head back to its normal position, but do not pull skin of neck. Turn the whole skin right side out. Lay wing with inner surface exposed. Make a longitudinal incision on the skin of forearm from elbow-joint to wrist. Free skin and remove flesh from wing bones. Apply aresenical soap and replace skin so as to cover bones. Tie the forearm bones of one side with those of the other side by passing a string through the loop formed by these bones at elbow-joint (Fig. 2). The gap between them should be as much as it is in a natural state.

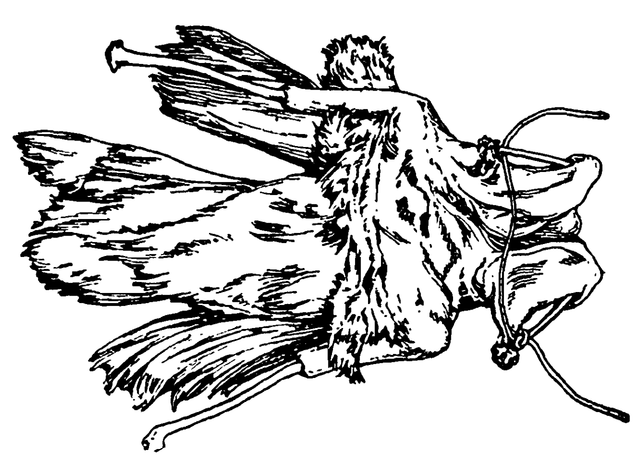

Fig. 2. — Bird skin inside out, showing the method of tying the forearm bones.

The skin of neck of bird with narrow neck or large head (*e.g.*, woodpecker, duck, etc.) cannot be drawn over head. In such a case, skin neck as far forward as can be easily done, then cut off neck. Make an incision through the skin from middle or back of crown to nape (Fig. 3). Turn out skull through this opening, clean, poison, place back and sew up the cut. Remove mud and blood stains, if any from plumage with cotton wool soaked in clean water. Dry wet patches with liberal use of Magnesium carbonate. Shake off powder from plumage.

Fig. 3. — Line of incision (dotted) on the dorsal side of the crown of a bird with large head or narrow neck.

On a piece of wire or stick (pointed at one end) a little longer than body, roll cotton wool tightly (tying from time to time with strong cord) so as to make an artificial body roughly similar in shape and size to those of the body which has been removed from skin. Pass narrow end of artificial body up neck and place whole "body" within skin. Insert pointed end into back of skull so that head and bill lie in the same line as body. Arrange the skin properly, and pack small pieces of cotton wool wherever required to given a good shape to specimen. Cut off projecting part of wire or stick. Expose the leg bones and pass their heads through strings with which body is tied (Fig. 4). Sew base of tail into the cotton body by at least two stitches. Sew up cut on the skin of belly. Apply a very thin solution of arsenical soap on bill, orbital skin, legs, feet, claws, pads, etc. Tie mandibles together with a fine thread so that bill remains closed. Cross legs and extend toes moderately. Tie the string of label at the crossing of legs very firmly. Arrange feathers so that they lie flat, and place wings and tail in position. Wrap specimen in a thin layer of cotton wool or a piece of lint, muslin or mosquito-netting, and lay aside in a shady, airy place for drying. Take special care so that ants and other insects, rats, mice, dogs, cats, etc., have no access to specimen.

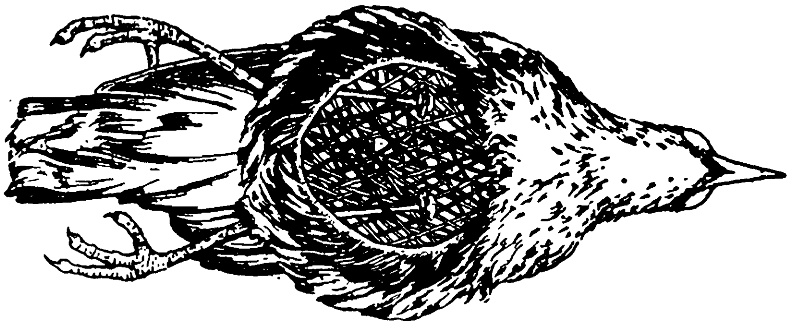

Fig. 4. — Ventral view of a bird skin with the artificial body inside, showing the positions of the heads of the leg bones before sewing up the ventral cut.

b) *Determination of sex*

After the body is removed from the skin, cut open abdominal cavity. Locate the gonads which lie close to the backbone near anterior end of the kidneys. The male gonads are a pair of testes, smooth globular, ovoid of elongated bodies, large during breeding season, but quite small (and inconspicuous in small birds) in non-breeding period. The female gonad consists usually of a single (left) ovary which is an irregular mass of granular substance. During breeding season the granules enlarge into globular ova of various sizes. Make a note of sex of specimen on its label.

c) *Preservation of whole specimens*

Specimens of birds are also preserved whole. For this, 75-80% alcohol (dilute 80 parts of rectified spirit 95-96% strength with 20 parts of clean water) or formalin solution (dilute 1 part of strong formalin 38 - 40% strength in 9-14 parts of water, and add three table-spoonfuls of common salt per litre of solution) may be used as preservative. Inject a liberal dose of preservative into abdominal cavity of specimen (or make an incision on abdominal wall large enough to expose viscera), tie a label, and drop specimen in preservative. If alcohol is used, replace with fresh alcohol after two or three days ; for large and fatty specimen change alcohol two or three times. The volume of preservative should be about 10 times that of specimen.

IV. LABELS

(Fig. 5)

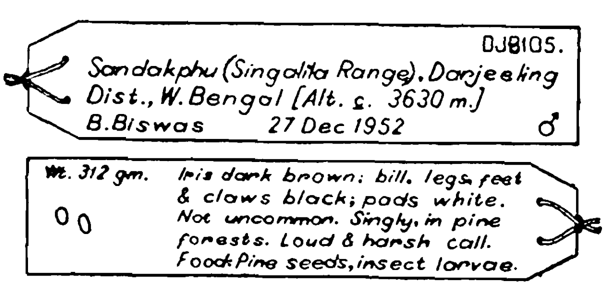

Fig. 5. — Front (upper fig.) and back (lower fig.) of an ideal label for a bird skin.

Every specimen must bear a label made up of good, strong, durable paper, approximately 7 × 2 cm. in dimensions, with a cotton string (No. 8 or so) attached at one end. The following are the minimum data that should be entered on each label :

1. *Locality of collection*, followed by names of district and State.

2. *Date of collection* : the day of the month followed by first three letters of the name of month and year in full. Example : 14 Mar 1962, but *not* 14. 3. 62.

3. *Name of collector*.

4. *Sex* of *specimen* : '♂' for male, '♀' for female and '—' for doubtful cases and for specimens whose gonads could not be examined.

Additional data on size and condition of gonads, colours of soft parts, etc., are also desirable.

All entries on the label should be made with water-proof black Indian ink.

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MAMMALS THEIR COLLECTION AND PRESERVATION FOR STUDY

By

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I. COLLECTION

Specimens of mammals are usually collected with firearms and traps. A 12-bore shotgun with cartridges loaded with LG, SG, SSG and Nos. 1, 4 and 8 or 9 shots ; and a. 410-bore shotgun with cartridges having shot Nos. 4, 6 and 8 or 9, serve most purposes. Besides, cartridges loaded with No. 12 or 'Dust' shots may prove useful in collecting flying bats ; and one or more sporting rifle (.375", .275", 30"-06—.22", etc., bore) may be needed for medium or large mammals from long range.

Various types of traps are used for collecting mammals, but different sizes of rat- or mouse-traps, both the "breakneck" and "cage" types, will cover most needs of trapping small mammals. There are, of course, various special traps for capturing medium and large mammals. Before setting traps carefully reconnoitre to find spots frequented by mammals for shelter, food and water. Set traps in or about those spots in a line, semicircular or straight. Secure each light weight trap (*e.g.*, "breakneck" type) by fixing it with a piece of wire to a nearby boulder or tree trunk.

Trapped mammals may be killed by shooting or by exposure to chloroform vapour, depending on its size, before preservation.

II. ARTICLES REQUIRED FOR PRESERVING MAMMALS

1) A metric rule, folding or non-folding type, about 30 cm. long, graduated in millimetres. 2) A metric rule, metal tape or folding type, about 2 metres long, graduated in millimetres. 3) A pair of fine-pointed dividers, about 12 cm. long. 4) A scalpel with about 4 cm. long blade. 5) A stout scalpel with about 6-7 cm. long blade. 6) A "Butcher's knife" 7) A pair of dissecting scissors with about 6 cm. long blades, one of which is with pointed end and the other with rounded end. 8) A pair of dissecting forceps with

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pointed ends, about 12-13 cm. long. 9) A pair of long forceps with rounded ends, about 25 cm. long. 10) A pair of bone cutters. 11) A pair of cutting pliers. 12) Galvanized iron wire, 18 and 20 or 22 gauges. 13) A small triangular file. 14) A nail- or tooth-brush. 15) Sewing needles, Nos. 2-8. 16) Cotton sewing thread, Nos. 8, 60 and 90. 17) A hoghair brush (rounded), No. 8, as used by artists. 18) A 10 or 20 ml. metal-covered glass (or all glass) hypodermic syringe with stout (Nos. 1, 2, 8, etc.) needles. 19) A medium-sized carborandum hone. 20) Common salt. 21) Heavy Magnesium carbonate (Magnesium carbonate pond) or magnesium oxide. 22) Chloroform. 23) Formalin (38-40% solution of Formaldehyde). 24) Rectified spirit. 25) Arsenical soap. (Melt 1 kg. of soft soap, such as "Sunlight", cut up into small bits in a little water over a low fire. Add 230g of white arsenic (Arsenic trioxide), 125g of Borax. Boil for a few minutes, stirring all the time. Remove from fire, add 63g of Camphor, and continue stirring till it cools. The consistency of arsenical soap depends on the amount of water initially used for melting soap. By adjusting it, the arsenical soap may be made thick enough to form cakes. 26) Teased non-absorbent cotton wool. 27) Absorbent cotton wool. This must be of very good quality, with long fibres, that can be easily peeled into thin layers. 28) Large pins, preferably "Hair" or "Hat" pins — long steel pins with rounded glass, plastic or metal heads. 29) Soft wooden board. 30) Old newspaper for carrying dead specimens and for wrapping prepared skins.

III. MEASUREMENTS AND DETERMINATION OF SEX

a) *Measurements*

Always take measurements of mammals before preservation. Straighten out dead specimen and place it on its back on a wooden board. Set pins vertically, one at the tip of snout and another at the fleshy tip of tail (Fig. 1a). Remove specimen and measure distance between pins to get the total length. Bend tail at right angles to dorsal side of body and measure distance from base to fleshy tip of tail (Fig. 1b). Subtract tail length from total length to obtain head-and-body length. Measure hindfoot from heel to fleshy tip of the longest toe by placing it directly against a ruler or with a pair of dividers (Fig. 1c). Measure ear from intertragal notch to farthest point on its edge excluding hairs (Fig. 1d).

b) *Determination of sex*

Determine sex of specimen by examining its external genitalia. In a few cases, however, it may be necessary to examine internal reproductive organs to determine sex. Note position, condition and number of mammae of female specimens.

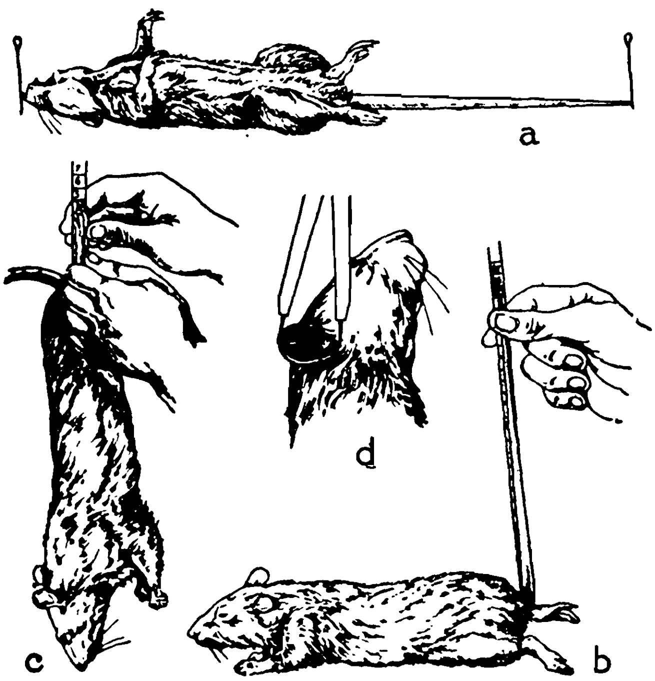

Fig. 1. — Method of taking measurements of a small mammal. (*a*) Total length ; (*b*) tail length ; (*c*) length of the hind foot ; (*d*) length of the ear.

IV. PRESERVATION

a) *Preparation of study skins*

Small mammals (up to the size of Palm Civet or Hare) : Place specimen on its back. Part the fur along midline of the abdomen, and with a scalpel or knife cut the skin longitudinally from about the breast bone to base of tail (Fig. 2). Loosen the skin from flesh with fingers or handle of scalpel until knee-joint is exposed. Loosen

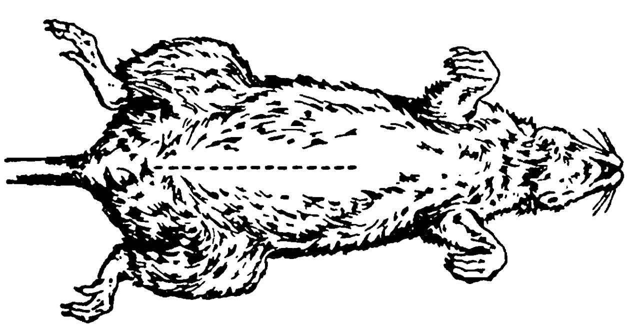

Fig. 2. Line of initial incision (dotted) on the ventral side of a small mammal.

skin round knee and sever the joints one after another. Separate skin round to base of tail and cut through. Continue forward freeing skin from body, turning it inside out. Sprinkle Magnesium carbonate on the inner surface of skin to facilitate gripping. On forelegs, free skin up to elbow-joint and sever the joint. Continue rolling back the skin forward. Cut ears as close to skull as possible, without cutting any bone. Over the eyeballs cut the eyelids free with the point of scalpel, but do not cut the lids. Further anteriorly, cut the skin free at nose by slicing it through the nasal cartilage, but do not cut delicate bones of nose. Cut skin free from body at the base of lips.

The skin is now completely inside out. Skin out legs (fore and hind) as far as possible and clean off flesh from bones. Separate skin round base of tail till it can be grasped with two fingers (Fig. 3). Hold the skin at base of tail between thumb and forefinger and steadily

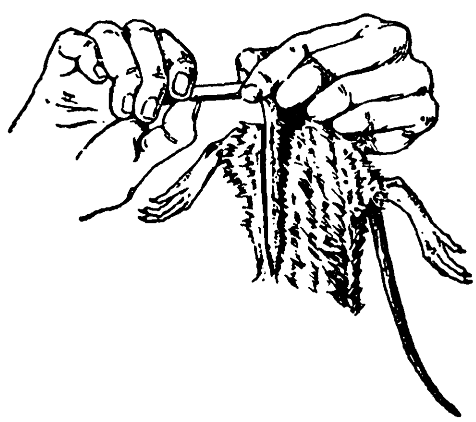

Fig. 3. Skinning the tail of a small mammal.

pull the base, using the fingernails to strip skin, till the whole tail comes out of skin. In some cases the tail cannot be stripped this way, when the skin of tail should be cut lengthwise so as to remove flesh and bones. Scrape off as much fat and flesh from skin as possible, paying special attention to the base of ears. Sew up lips by a few stitches (Fig. 4).

Turn skin fur side out. Clean mud and blood stains, if any, with cotton wool soaked in water. For removing very large or thick patches

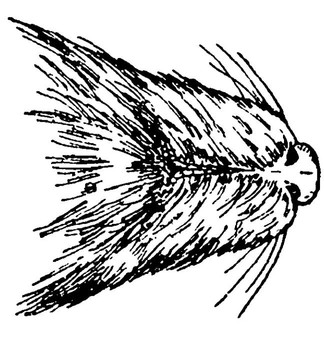

Fig. 4. Sewed up lips of a small mammal.

of blood, wash skin in cold water ; and if skin is too dirty or too greasy, wash it in cold water and soft soap. Wash thoroughly and squeeze out water, but do not wring or stretch. Dry fur by a liberal use of magnesium carbonate. Shake off powder and turn skin inside out. Paint inner surface of skin all over with arsenical soap, and pass some down into tail skin with a wire. Make up leg muscles with cotton wool wound round leg bones. Turn skin right side out. For larger small mammals, such as civets, hares, etc., remove as much soft tissue from soles of feet as possible through a slit made down the middle or along one edge of sole. Introduce some arsenical soap through the opening and close by a stitch or two.

Take a piece of straightened wire (use No. 22 for very small mammals like mice, otherwise use No. 18 or 20) about equal to the length of tail plus the opening on skin. If the tail tapers to a point, file down an inch or two at one end of wire. Twist a thin wisp of cotton wool about wire until it approximates the tail taken out of the skin. Smear a little arsenical soap on the artificial tail and insert it into tail skin. Slip the other end of wire through cut on belly into skin cavity. With cotton wool prepare an artificial body somewhat similar in size and shape to the body removed. Insert it within skin. Work it into head and nose, and shape them. Draw skin smoothly over artificial body, inserting little wisps of cotton wool wherever required to give the skin a good shape. Sew up cut on skin.

Brush off any dirt from finished skin with the nail brush. Tie the label on right hindleg above heel. Wrap in a thin layer of cotton wool from nose to neck so that the whiskers (vibrissae) lie backward along sides of head, and ears lie on head. Pin specimen on a soft wooden board keeping legs close and parallel to body (Fig. 5), and lay aside in a shady airy place for drying. Take special care so that ants and other insects, rats, mice, dogs, cats, etc., have no access to specimen.

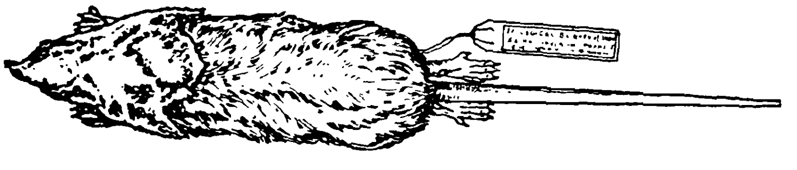

Fig. 5. A completed study skin of a small mammal pinned up and with a thin layer of cotton wool wound round the head and neck.

Medium and large mammals are prepared flat in field and are made up, if necessary, in laboratory.

The lines of cut of such a specimen is shown in text-figs. 6*a* and *b*. Take out skin by separating it from body starting from edges of cuts. Disjoint legs at base of foot.

For mammals with horns and antlers, give the additional Y-cut shown in Fig. *6b*. Work the skin loose round nape through the longitudinal arm of Y, and through the oblique cuts free the skin

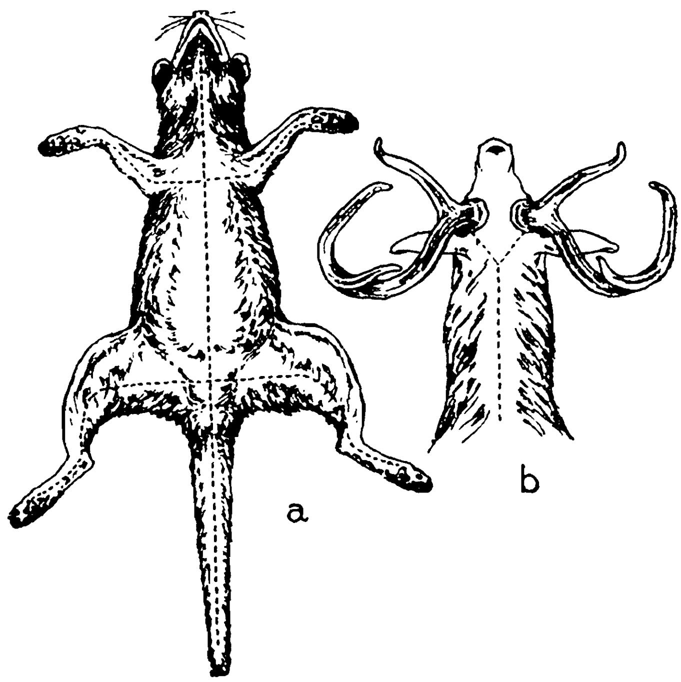

Fig. 6. Lines of cut (dotted) for medium and large mammals. (*a*) Ventral view of a medium mammal, showing the lines of cut ; (*b*) dorsal view of the head and neck of a deer showing the Y-cut.

around bases of horns. Sever head from neck and free the skin from head. For all large mammals skin ear cartilage from inside, remove all flesh and leave cartilage attached to skin. For hoofed mammals, remove all soft tissue from the base of hoof. For mammals with fleshy soles, skin up to the last digits of toes and remove all soft tissue.

Spread skin with raw side up. Rub in common salt thoroughly all over skin, in and around ear cartilage, and the skin and bones of feet up to bases of claws. Turn head and ears right side out, and fold them on body skin. Fold in skins of legs, and roll up skin compactly with the hair side out. Keep in a shady place. After about 24 hours, unroll skin, shake off salt and moisture. Salt again and keep it rolled up for another 24 hours or so. Then unroll and hang it over a line or pole, raw outside, in a shady place to dry. If weather is very humid, apply arsenical soap on skin around ear cartilage and on inner suface of skin of soles. When the skin is almost, but not quite dry, fold it to a convenient size with fur side in.

b) *Preparation of skull*

Each study skin of mammal should also have its skull available for study. Cleaning of skulls, except for very delicate and large ones, should be done in field.

Cut off head from body at neck. For larger small mammals remove larger muscles ; for smaller mammals remove no muscle. Boil skull in water on a low flame until flesh can be easily removed from bone with forceps. Remove from fire, replace hot water by cold. With forceps remove all flesh from bones. Remove brain through opening at back of skull (foramen magnum) with a piece of wire. Wash skull clean, and dry it. Tie a label bearing the same collection number as its skin. Keep it in a cloth bag.

Delicate skulls if cleaned out in field are liable to get damaged in transit. Drop such skulls, without cleaning, in 75-80% alcohol, with a label. They should be cleaned in the laboratory.

For large skulls cut off as much flesh as possible. Take out as much brain as possible through foramen magnum. Preserve it in alcohol or dry it thoroughly in sun. It should also be cleaned in the laboratory.

c) *Preservation of whole specimens*

Use 75-80% alcohol (80 parts of rectified spirit 95-96% strength diluted with 20 parts of clean water) or formalin solution (1 part of strong formalin 38-40% strength diluted in 9-14 parts of water and three tablespoonfuls of common salt added per litre of solution) may be used as preservative. Inject a liberal dose of preservative into abdominal cavity of specimen (or make an incision on abdominal wall large enough to expose viscera), tie a label, and drop specimen in preservative. If alcohol is used, replace with fresh alcohol after two or three days ; for large and fatty specimen change alcohol two or three times. The volumes of preservative should be about 10 times that of specimen.

V. LABELS

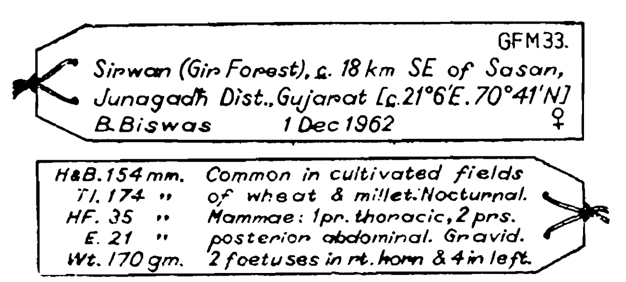

Fig. 7. Front (upper fig.) and back (lower fig.) of an ideal label for a mammal skin.

Every specimen must bear a label made up of good, strong, durable paper, approximately 7 × 1.5 cm. in dimensions, with a cotton string (No. 8 or so) attached at one end. The following are the minimum data that should be entered on each label :

1. *Locality* of collection, followed by names of district and State.

2. *Date* of collection : the day of the month followed by first three letters of the name of month and the year in full. Example : 14 Mar 1962, but *not* 14. 3. 62.

3. *Name of collector.*

4. *Sex* of specimen : ♂ for male, ♀ for female, and '—' for doubtful cases and for specimens whose gonads could not be examined.

5. *Measurements*, in millimetres, of head-and-body (H & B), tail (Tl), hindfoot (HF) and ear (E).

Additional data, such as on number and position of mammae, their condition (if nursing), number of foetuses, if any, in each horn of uterus and their stage of development, food habits, etc., are also desirable.

All entries on the label should be made with waterproof black Indian ink.