MANUAL ON PRACTICAL ENTOMOLOGY IN MALARIA

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WHO Division of Malaria and Other Parasitic Diseases

PART II
Methods and Techniques

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CONTENTS

PART I. VECTOR BIONOMICS AND ORGANIZATION OF ANTI-MALARIA ACTIVITIES

PART II. METHODS AND TECHNIQUES

Contents (continued)

PART II (continued)

PART II

METHODS AND TECHNIQUES

INTRODUCTION

Details regarding the application of methods and techniques are given below. It is to be well understood that each method has its own limitations in relation to environmental factors and the vector's natural or induced behaviour. As adequate sampling and processing of material are essential for obtaining significant information, the epidemiologist and the entomologist have to decide together the extent of the entomological investigations to be undertaken in each situation in order to achieve fully the objectives for which the studies were planned. Where and when necessary, the investigations should be adjusted to the local requirements.

SAMPLING METHODS

The collecting methods are employed for two types of study:

(a) Qualitative studies, when we need to study the presence, distribution, type of behaviour of different mosquito species in different macro- and micro-environmental conditions, etc.

(b) Quantitative studies, when collections are made in order to measure in different situations the value and variability of vector relative density and abundance, longevity, infectivity, impact of antivector measures on the vector population, impact on the transmission (vectorial capacity), etc.

There are several methods for sampling adult mosquitos but the selection and application, alone or in combination with others, as well as the limitations of each method are imposed by:

- the objective of the study for which the collection is made;
- biology and behaviour of vectors (if this is already known);
- human behaviour and housing conditions;
- environmental conditions (natural or modified by man) in which the collections are made (temperature, percentage humidity, winds, rains, absence of breeding places, position of breeding places, insecticide spraying, smoke, introduction of animals inside houses, etc.).

It should be stressed from the beginning that the real significance of the data obtained by collecting the adult mosquitos for quantitative studies will vary, depending on the way in which the above-mentioned factors (which have an important influence on the efficiency of each method of collection) have been taken into account at the time of collection.

1. HAND COLLECTION

(a) Principle of the method

Mosquitos resting on different surfaces (indoor or outdoor) are collected by using a sucking tube, or a test tube.

(b) Specific objectives of hand collection

- To study the resting habits and density, indoor density and distribution on different surfaces of endophilic mosquitos, or of exophilic mosquitos remaining indoors for some period during the night, in situations when other methods are not applicable.
- To collect outdoor resting mosquitos.

- To collect mosquitos coming to bite.

- To collect live mosquitos for the study of susceptibility, bio-assay tests, obtaining the eggs or to observe survival rates.

- To collect mosquitos for precipitin tests.

- To collect mosquitos for taxonomic studies.

The method is not applicable as a routine investigation for the evaluation of the density where pyrethrum spray collection is efficient. It is only necessary to use this method in conjunction with pyrethrum spray collection when studying the indoor resting habits by performing hand collection first and then pyrethrum spray collection.

1.1 MATERIAL

- Test tubes, full length or short Pyrex tubes 100 mm or 60 mm x 10 mm (small tubes are useful when collecting a single specimen per tube and when specimens are kept for some time inside the tubes before being examined or processed).

- Sucking tubes.

- Electric torch.

- Cotton wool.

- Paper cups or small mosquito cages.

1.2 PROCEDURE

1.2.1 Use of the test tube

When collecting the mosquitos in test tubes the collector should apply the mouth of the test tube perpendicularly over the wings of the mosquito in such a way that when the mosquito is disturbed and attempts to fly, it will fly into the tube (Fig. 1, A and B). Care should be taken not to collect too many mosquitos in a single tube; about five is the average number for a normal length test tube and they should be transferred immediately to a paper cup or cage. Particular care should be taken not to damage the mosquitos when they are being collected for susceptibility or irritability tests. In this case they should be collected one by one and transferred immediately to the cage. When collections are carried out for taxonomic studies it is desirable to kill the mosquito immediately and a special tube (Fig. 2) is prepared as follows (see also section 8.1.1).

Place at the bottom of a normal test tube with a diameter of 15 mm a layer of about 30 mm of small pieces of rubber tubing. On top of this, place a layer of cotton wool, about 3-4 mm deep, covered with a circle of white cardboard or filter paper. Pour chloroform into the tube taking care that it penetrates the layer of rubber. Leave overnight and pour off excess chloroform the following day. The test tube should be tightly closed with a rubber cork to prevent the rapid loss of chloroform when the tube is not used for a long period. The tube can be used for several weeks. When the killing effect of the chloroform has disappeared the rubber should be soaked in chloroform again. In the absence of chloroform, ether or ammonium hydroxide (NH_4OH) may be used.

1.2.2 Use of the sucking tubes

In Fig. 3, A, B and C, three types of collecting sucking tubes are shown. Fig. 3 A represents the most commonly used type, composed of a glass or plastic tube (t) 15 mm in diameter, a rubber or plastic tube (pt) and a mouthpiece (mp).

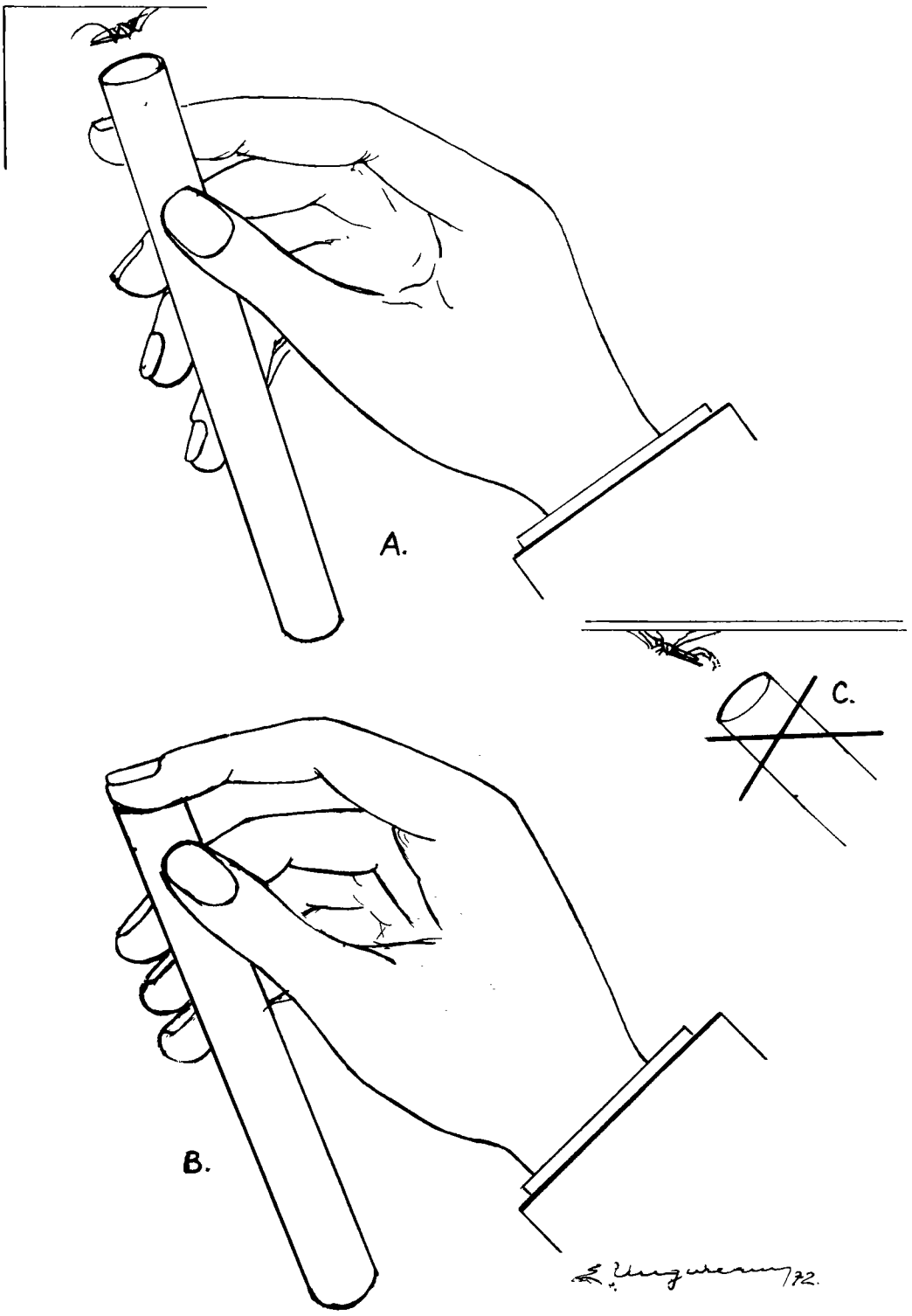

Fig. 1. Use of the test tube for mosquito collection.

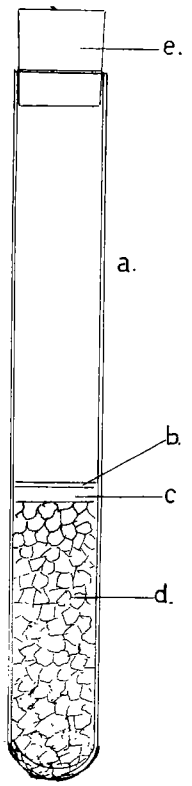

Fig. 2. Killing tube.

a = test tube; b = filter paper; c = cotton wool;
d = pieces of rubber; e = rubber stopper.

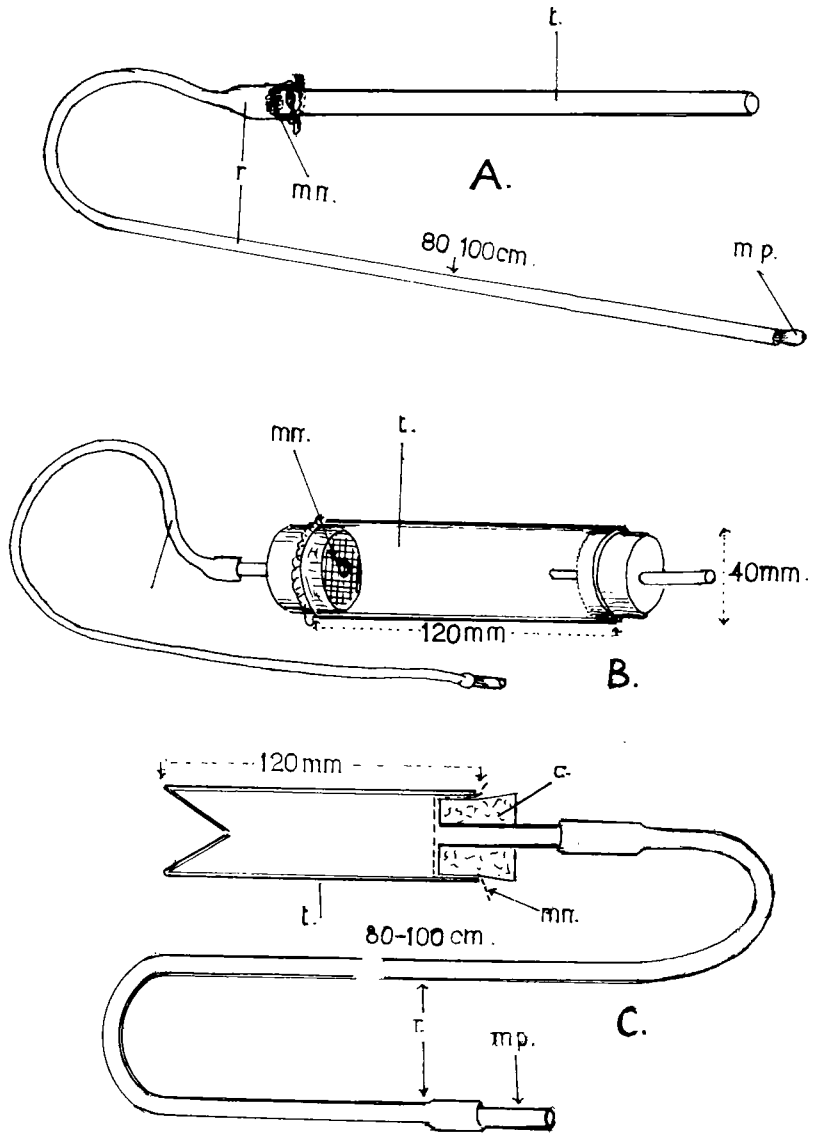

Fig. 3. Three types of aspirator.

t = glass or plastic tubing; mn = mosquito netting; mp = mouthpiece.

Fig. 3, B and C represent the Italian type of collecting tube, with details of the component parts and dimensions.

1.2.3 Remarks

When collecting mosquitos from surfaces sprayed with water-dispersible powder, care should be taken not to inhale particles of insecticide and the sprayed surfaces should not be touched with the mouth of the sucking tube. In such situations a test tube should be used or a cotton wool filter should be placed at the end of the collecting tube in order to retain the particles. The contamination of the collecting tube with insecticide particles might produce mortality among the collected mosquitos (see also Fig. 4).

1.3 MECHANICAL DEVICES FOR COLLECTING RESTING MOSQUITOS

1.3.1 CDC sweeper (Fig. 6)

The same motor and collecting cages as those used for the CDC miniature light trap are attached to a metal handle about 70-80 cm in length.

1.3.2 Modified small electric hand aspirator (Fig. 5)

This is made by using a small hand aspirator, the brush of which is replaced by a small plastic chamber (a). The bottom of the chamber is covered with mosquito net and a small tube 15 mm in diameter is put in the top for collecting the mosquitos (b). Two batteries of 1.5 volts are used for this apparatus. It is practical for collecting mosquitos from sprayed surfaces but it is not commonly used in the field. A wider field evaluation of the efficacy of this apparatus for collecting malaria vectors could be carried out. It has proved useful in collecting mosquitos from outdoor resting places in vegetation, roots of plants, or holes. When collecting mosquitos from holes, a tube of appropriate length should be fitted to the collection cage of the CDC sweeper (Fig. 6 (d)), or to the hand electric aspirator (Fig. 5 (b)).

2. SPRAY SHEET COLLECTION

2.1 PRINCIPLE OF THE METHOD

This method consists of the collection of indoor resting mosquitos on white cotton sheets after knock-down by space spraying of a pyrethrum solution.

2.2 GENERAL OBJECTIVE

To collect indoor resting mosquitos for quantitative and qualitative studies.

2.3 SPECIFIC OBJECTIVES

(a) to study quantitatively and qualitatively the indoor resting density, and to obtain material for the study of physiological stages;

(b) to determine the human blood index; longevity of the mosquitos; infection and infectivity of indoor resting mosquitos;

(c) to study the residual density after trap collection, or hand collection, or after night bait collection (only when necessary as a special study).

2.4 TIME OF APPLICATION

The method is applied during the daytime, usually early in the morning between 06.30 hours and 10.00 hours, depending on the situation and the objective.

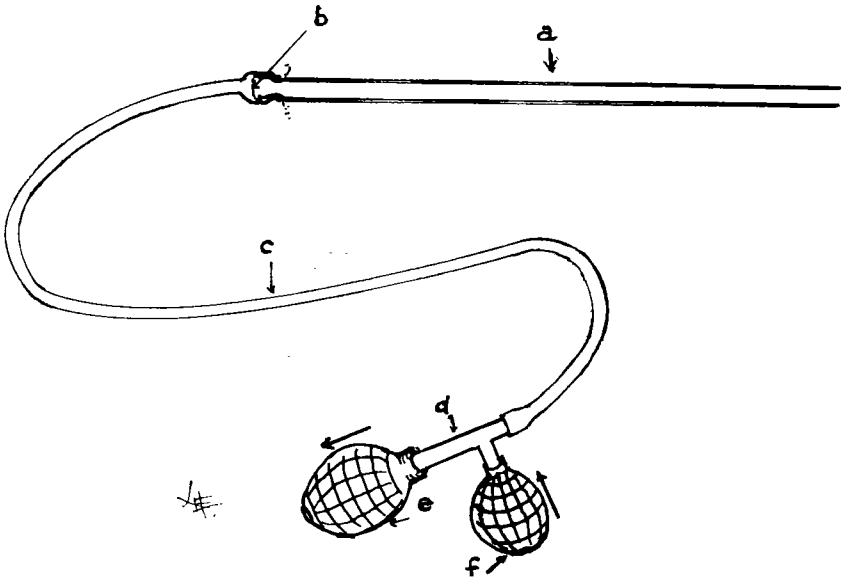

Fig. 4. Tube for collecting mosquitos from contaminated or dusty surfaces.

a = glass or plastic tubing, 1.5 cm in diameter;
b = nylon gauze inserted between glass and rubber tubing;
c = rubber tubing; d = metal or plastic T-piece;
e = aspirating rubber bulb, with valve; f = blowing rubber bulb, with valve.

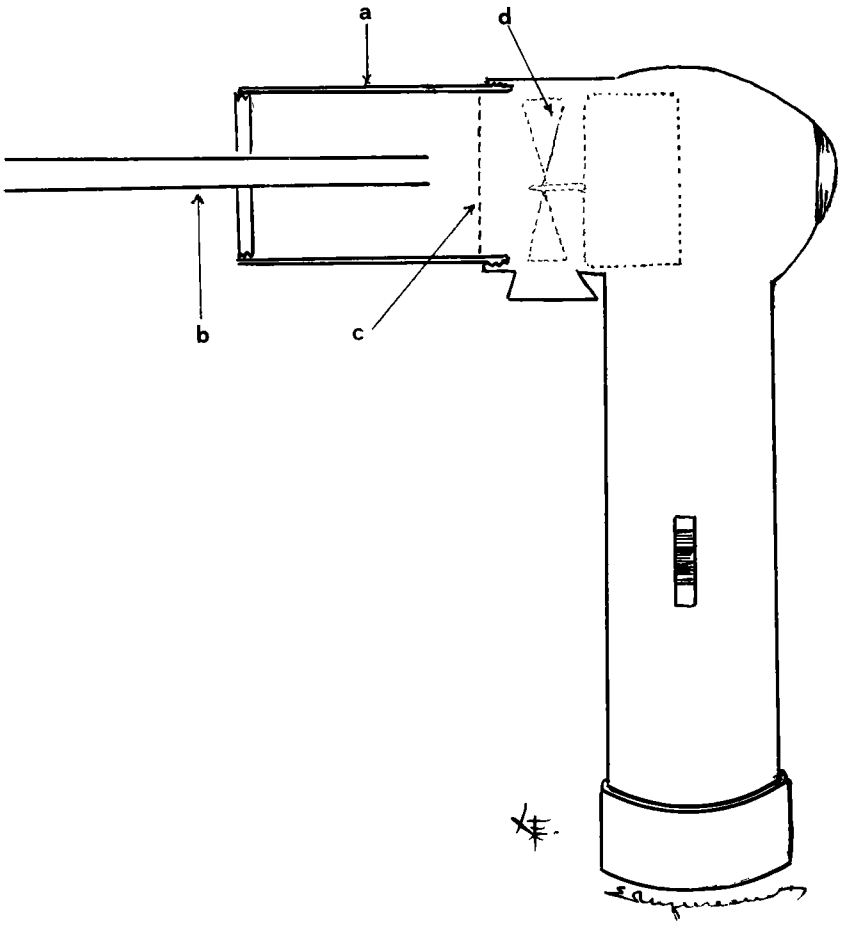

Fig. 5. Modified hand aspirator for collecting mosquitos.

a = plastic chamber; b = plastic tube 15 mm in diameter;
c = mosquito net; d = 3-volt electric fan.

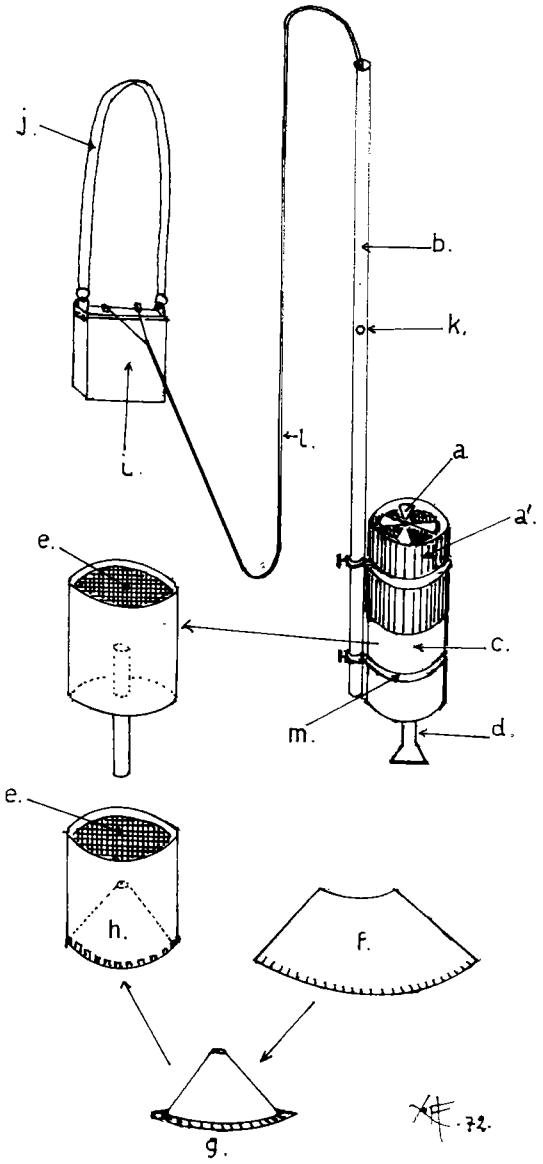

Fig. 6. "CDC" sweeper.

a = fan; a' = electric motor; b = handle;
c = container for mosquitos; d = inlet;
e = mosquito netting; f,g,h = formation of inlet cone;
i = battery; j = shoulder strap; k = switch on/off; l = cable.

2.5 MATERIALS

- White sheets, 2 x 2 m and 2 x 1 m specially made (standard hospital bed sheets are also appropriate).

- Hand atomizers (spray cans or spray pumps of minimum capacity).

- Pyrethrum solution (pyrethrin 0.1-0.2% in kerosene). Pyrethrum solution in kerosene ready for use is prepared from a concentrated solution of pyrethrum which might contain 2-5% of pyrethrin.

- Small plastic boxes or Petri dishes or, in the absence of these, small metal boxes.

- Entomological forceps.

- Hand lens, necessary for classification of mosquitos when this is done on the spot.

- Large thermos flask to keep the boxes with mosquitos on ice. This is necessary only when the mosquitos are brought to the laboratory more than four hours after collection, or for special studies in which the slightest modification of the stomach content must be avoided.

2.6 STAFF

Each collection team is composed of three men, one supervisor and two collectors, working together.

2.7 APPLICATION OF THE METHOD

The method consists of four steps:

(1) After the inhabitants have been invited to empty the house, the floor surfaces, as well as beds and any other areas are completely covered from wall to wall with white sheets. No space should be left between the walls and the surface covered by the sheets so that no mosquitos are lost. Fig. 7 A shows the sheets arranged inside the house. If the eaves are very open, they are closed with mosquito netting, the upper side is attached to the roof and the other to the wall, as shown in Fig. 7 B. Two pieces of nylon gauze about 5 m x 0.5 m will prove satisfactory for closing open eaves in many cases (for a hut with a diameter of 3 m); an extra piece of nylon gauze might be advisable for bigger houses. Banana or palm leaves may be employed where appropriate.

(2) After the floors of the hut have been completely lined with sheets, the collectors (one inside and one outside when the eaves have not been closed with nylon gauze) start to spray the pyrethrum solution along the eaves each starting from opposite ends. (Commercial aerosols of pyrethrum, when available, can be used but these are more expensive although more practical). After filling the room with the insecticidal mist, the collector leaves the hut and closes the door. (When the eaves have been closed with a nylon gauze, only one man sprays pyrethrum inside).

(3) Ten minutes later the door is opened and the mosquitos are collected, starting at the door and moving to the interior of the room. A torch is needed for this. A more practical method is to remove the white sheets carefully, lifting them by the four corners and moving them gently so that the mosquitos collect in the middle of the sheets. The sheets are then transferred outside and examined in daylight.

(4) The mosquitos are collected with entomological forceps and put in the small boxes or tubes which are prepared to ensure enough humidity for the mosquitos. If a vacuum flask with ice is available, then the mosquitos can be collected in test tubes which can be kept on ice until the mosquitos are processed. Precautions should be taken against trapping the specimens in condensation droplets.

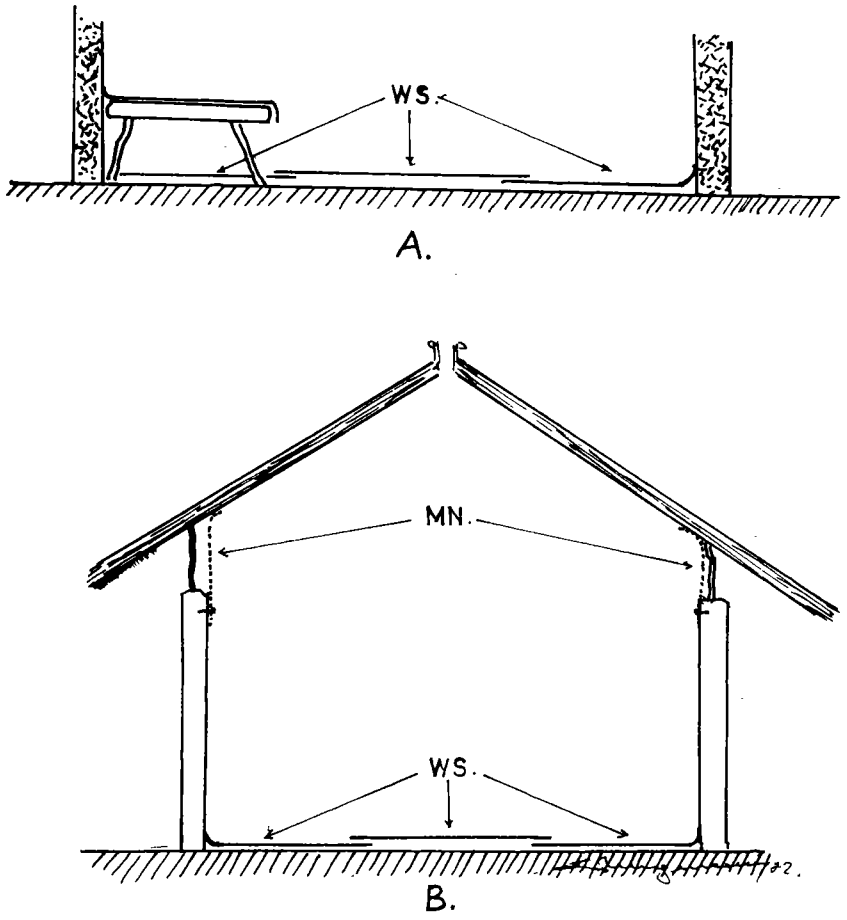

Fig. 7. Preparation of hut for pyrethrum spray catch.
mn = mosquito netting or banana leaves, etc.; ws = white sheets.

The classification of mosquitos might be done in the field (species, abdominal appearance) but a hand lens should be used. However, it is more practical for the mosquitos to be transferred to the laboratory so that the technicians may classify them and study different aspects more accurately.

2.8 REMARKS

While waiting for the mosquitos to be knocked down, the team can prepare and spray another hut. The number of people used for pyrethrum spray collection depends on the number of houses or huts which have to be investigated each time. A group of 9-10 people (three teams) can usually cover 10 houses in two hours using this method.

The method should not be repeated in the same huts at too short intervals as some repellent effect of pyrethrum extract might persist for two to three days. The minimum interval is therefore estimated at three days.

The method is very practical for the study of endophilic vectors before spraying, but is not satisfactory if used alone for the assessment of the impact of spraying on the vector relative density. It should be used in conjunction with bait collection and trap collection for the assessment of the expected change in transmission potential.

3. CATCHES OFF BAIT

3.1 PRINCIPLES OF THE METHOD

Mosquitos are collected while they land on the host to bite or while in the process of biting a human or an animal host.

3.2 OBJECTIVES

(a) To evaluate the man-mosquito contact (by establishing the man-biting rate) or contact with other hosts, and the place and time of biting.

(b) To collect live mosquitos for study of:

- parity rates;
- infection and infectivity of biting mosquitos;
- host attractiveness;
- susceptibility tests;
- duration of gonotrophic cycle, longevity (age grading), etc.

The above data are used to assess the impact of insecticides on the mosquito population and local transmission.

This method is one of the most important for collecting partially or entirely exophilic mosquitos or for collecting endophilic mosquitos where other sampling methods of adult densities are only partially efficient, as in areas with rudimentary houses or after insecticide spraying.

3.3 MATERIAL

- Torch
- Suction tube

- Test tubes full length or short Pyrex tubes 100 mm x 10 mm (small tubes are useful when collecting a single specimen per tube and when specimens are kept for some time inside the tubes before being examined or processed).

- Paper cups prepared for holding the mosquitos

- Cotton wool

- Rubber bands for keeping test tubes in groups.

3.4 DESCRIPTION OF THE METHOD

Catches using bait can be classified as follows:

- catches using human baits

- catches using animal baits.

3.4.1 Human baits are most commonly employed in epidemiological evaluation. There are two variations:

(a) The collector himself can act as bait; he sits on a small stool with bare legs on which he catches the mosquitos as they come to bite.

(b) The collector can catch the mosquitos on a local inhabitant or a member of the collecting team, who acts as bait. In this case the person acting as bait may lie down and even sleep. In some situations when the attack of mosquitos is very intensive, two collectors might be used for catching mosquitos from a single bait. The torch is used to see the mosquitos. This is switched on when a bite is felt in the case of the collector catching the mosquitos on himself. When the mosquitos are caught on another individual the torch is used to make periodical inspections at about two minute intervals. The light should not be too bright; green or blue filters are recommended if available, otherwise tracing paper or a piece of cloth may be used to reduce the intensity of the light.

3.4.2 Catches using animal baits

(1) Objectives

(a) To collect information on the feeding habits of anophelines on domestic animals.

(b) To establish more precisely the degree of availability of vectors in a sprayed area when the catches on human bait are very meagre or negative. (Collections on animal bait are not carried out regularly in malaria programmes since the density data collected are not used for epidemiological evaluation.)

(c) To collect mosquitos for susceptibility or bio-assay tests or for obtaining eggs, etc.

Local animals - donkeys, buffalo, cows, etc. - could be used for the collection of exophilic or partially exophilic mosquitos in addition to other methods of collection.

(2) Procedures

The collections employing animal baits can be made using one or both of the following techniques depending on the objective of collection:

(a) Mosquitos are collected directly off the animals (more easily off the legs and other places where the hair is short) with the aid of a sucking tube.

(b) Mosquitos are collected while resting in the vicinity of the bait, either before or after feeding. They may easily be found resting on vegetation, branches of small trees, etc., generally at a level varying from 15 cm to 2 m above the ground. The maximum concentration is in the proximity of the animals, varying from a few centimetres to a few metres away from the animals.

It is therefore recommended that, where possible, night bait collection on animals be performed where there is some suitable low vegetation.

3.5 CAPTURE OF MOSQUITOS

Mosquitos in the process of biting are collected by sucking tubes or test tubes. The former are widely employed and are very practical for collection and for the transfer of mosquitos into the cups. Test tubes are used when mosquitos are kept for some time in tubes before transfer to paper cups or small mosquito cages. When five or six mosquitos are collected in a normal test tube each one should be separated by cotton wool if it is intended to keep them in the tube for several hours; a piece of filter paper 1 cm wide and of the same length as the tube is put inside the tube before starting collection, in order to absorb the humidity.

3.6 ORGANIZATION OF ALL-NIGHT CATCHES

When night catches are used for epidemiological evaluation the method, timing, and duration of collection should not be changed during the longitudinal study. The exact procedure for organizing the work of the team will depend on the objectives, local conditions, and staff available. Since all-night collection is very tiring the collectors have to work in shifts of two hours. When collectors are also used as baits, alternating periods of sleep and duty should be arranged. When collections are made on local inhabitants the collectors can be changed at three to four-hour intervals. Collections may take place alternately inside and outside the huts but if the biting cycle is studied in detail the same collectors should collect either inside or outside throughout the night.

3.6.1 Remarks

Whole-night bait collection should start at sunset and stop at sunrise. For longitudinal studies the normal biting behaviour of the local vectors should be taken into account. In tropical areas the period between 18.00 (18.30) hours and 06.00 (06.30) hours represents the main period of biting activity and movement of the majority of the local vectors. Some of them, such as *A. albimanus*, have an early biting peak, whereas other species like *A. gambiae* generally start to bite in important numbers after 22.00 hours.

The duration of the night bait collection will depend on the objectives of the investigation; when studying the man/mosquito contact, it is necessary to make indoor collections all night while outdoor collection on human bait is necessary only during the periods when people remain outside. When studying the trends of density under the impact of vector control measures a practical method is the night bait collection during the main biting periods, supplemented from time to time by the whole-night bait collection. The behaviour of the local human population should also be taken into account.

The environmental conditions at the time of the collection are very important. Winds, rain, moonlight, temperature, and humidity all have an influence on the movements of mosquitos and therefore on the biting activity.

The collectors and the human bait should not smoke during the period of collection. Some human baits are less attractive to mosquitos than others; local people and children may be more attractive than the members of the entomological team. Therefore, the mosquitos should, if possible, be collected on the local people. The number of mosquitos per man per night obtained from baits who are not moving and who are more uncovered than the local people during the sleeping hours might be higher than the number biting a local inhabitant, who would be well covered during a cool night.

It is obvious from the above remarks that the figures obtained only indicate trends and do not entirely reflect all situations.

Indoor-biting densities should be established for different types of houses and situations. Bait collection carried out on a verandah cannot be considered as indoor collection. Outdoor collection should be carried out in spots where the local people are at risk.

The mosquitos collected are placed in paper cups, grouped according to the place and time when caught. Mosquitos collected for dissection are given 2-5% sugar solution and kept in a relatively cool humid micro-environment. Insulated picnic bags may be used, if available, for the transport of mosquitos from the field to the laboratory; otherwise a simple box is made by using a cardboard or wooden box and lining the inside with a thin plastic sheet fixed with a staple or by adhesive bands or other means. Paper cups or cages containing mosquitos are arranged inside and covered with a thin plastic sheet. A wet layer of cotton wool or a wet towel is then put on the top to ensure humidity. Such devices are necessary mainly during the dry season and should be kept out of direct sunlight. If mosquitos are collected to obtain eggs they should be allowed to feed completely before being collected from the bait. Cotton wool soaked in a 2% sugar solution or water should not be placed in the cages of these mosquitos as this might produce early elimination of the blood before digestion.

3.7 RECORDING OF DATA

A tentative form for recording the results of the night bait collection is given in (Part I, Chapter 4, Form 3); on this form details are given of the techniques of collection and environmental data during the collection period.[1] The mosquitos collected at each spot by each collector are placed each hour in a separate paper cup, on which are mentioned: place of collection, the number of the collector, the period of collection (in general, all mosquitos collected during one hour from the same spot are put together, but mosquitos collected during periods of less than one hour or more than two hours could be put in the same cup according to the objectives of the collection, number of mosquitos, etc.).

At the laboratory the number and species of mosquitos collected each hour is inscribed on the form as well as the results of processing.

The man/mosquito contact is estimated by establishing the man-biting rate on the basis of whole-night bait collections. It is expressed as an average number of bites per man per night. It is not possible to obtain an approximate average of mosquitos per man per night from the results of a few hours of collection by calculating an average per man per hour and multiplying by 12. The results of collection carried out over a period of a few hours can be compared only with the results obtained during similar periods of time and with the same techniques and calculated in this case as mosquitos per man hour. Therefore, it is very important to mention the exact duration of collection and the number of baits used during each period of time. A checking of the efficacy of baits is desirable if the same individual is used regularly.

4. TRAP COLLECTION

4.1 GENERAL OBJECTIVES

- To study the relative prevalence of mosquitos.
- To study mosquito behaviour.
- To study the effect of residual insecticides and/or other anti-vector measures.

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[1] Temperature (°C), relative humidity, wind, rain, moonlight (moon phase), presence of people outside, domestic animals nearby, etc.

Principles

Trap collections are used extensively for collecting mosquitos which are flying in search of food, shelter, or egg laying sites, i.e., which are changing from one place to another during their normal movement or due to some external unfavourable influences, whether natural (wind, change of humidity, temperature, light) or produced by man (e.g., smoke, insecticide, artificial ventilation, hosts, etc.).

4.2 CATEGORIES OF TRAPS

There are several types of traps which can be classified arbitrarily in three categories (the different types of trap and their uses are summarized in Table 1 on p. 41):

(a) fixed traps, attached to existing structures;

(b) portable traps enclosing bait, used independently of the existing structures;

(c) mechanical devices for the collection of outdoor or indoor mosquitos.

4.2.1 Fixed traps

Fixed traps are the most frequently employed for collecting mosquitos in a malaria programme. They are placed in the path of flying mosquitos and may be used with or without an attractant.

There are two types of trap in this category:

(a) Exit traps (window, door, eaves, wall, and verandah trap).

(b) Entry traps.

4.2.1.1 Exit traps

(a) Objectives of using exit traps

To study:

- the normal daily movements of mosquitos and their degree of normal or induced exophily;
- the effects of insecticides on normal movement, indoor density and, in part, the inhibition of feeding, i.e., degree of excito-repellency;
- the toxic effect on mosquitos leaving a house sprayed with insecticide (by counting the mortalities in the trap and the 24-hour survival rate of mosquitos found alive in the trap)

Determination of the toxic effect forms part of the estimation of overall room-kill. In Figs 8, 9, 10 and 11 details are shown of exit trap construction and mounting.

(b) Use of the exit trap

The use of exit traps is of considerable importance during any study of the effect of insecticides on endophilic species and house-frequenting exophilic species. Exit traps can be fitted to the windows, door eaves, or walls of the local house or hut or to an experimental one, depending on the stage of the study and type of local houses.

In general one trap is applied to one hut. Two traps per hut will offer better possibilities of collecting a more representative sample.

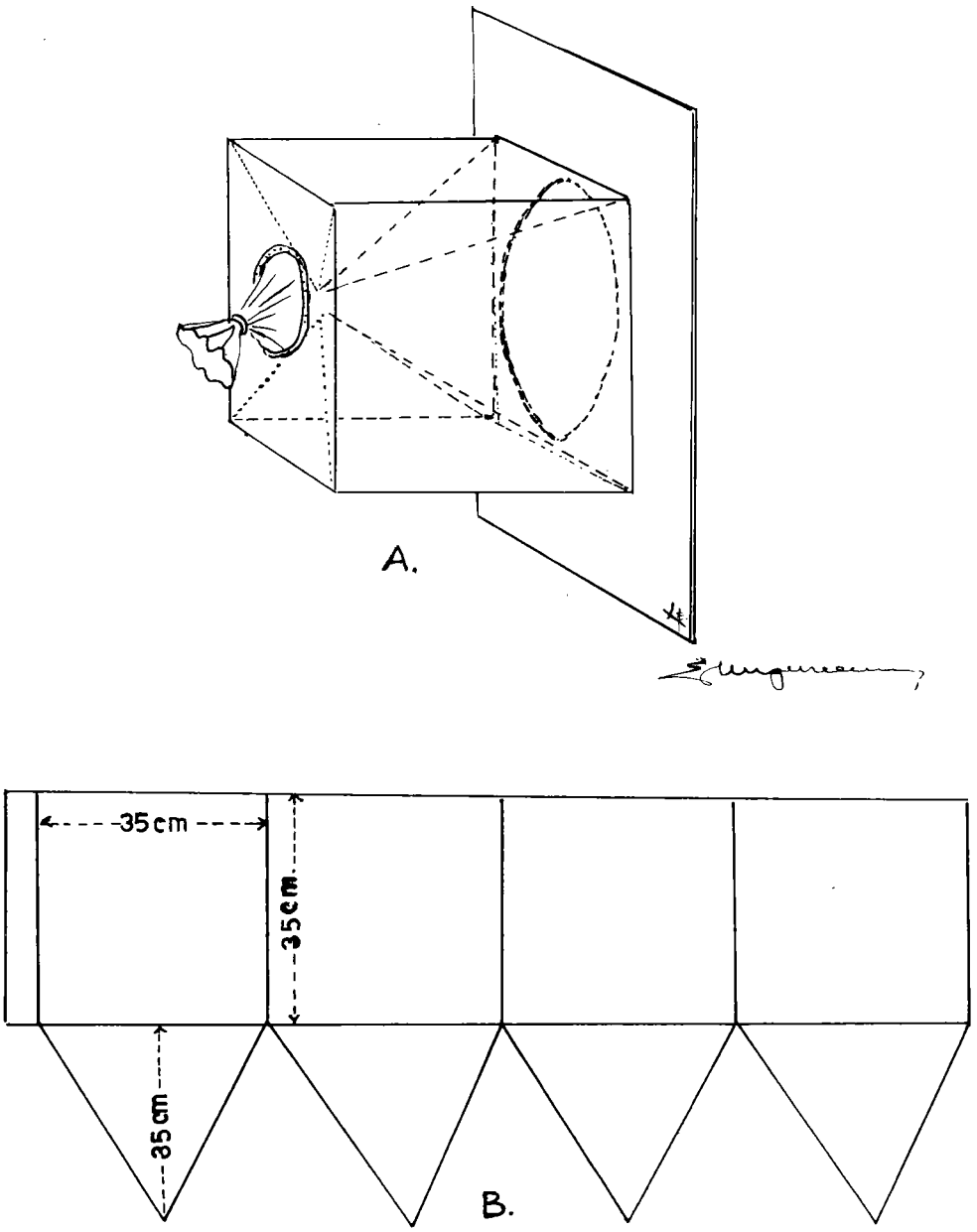


Fig. 8. Construction of an exit trap.

A. Completed trap; B. Pattern for cutting mosquito netting.

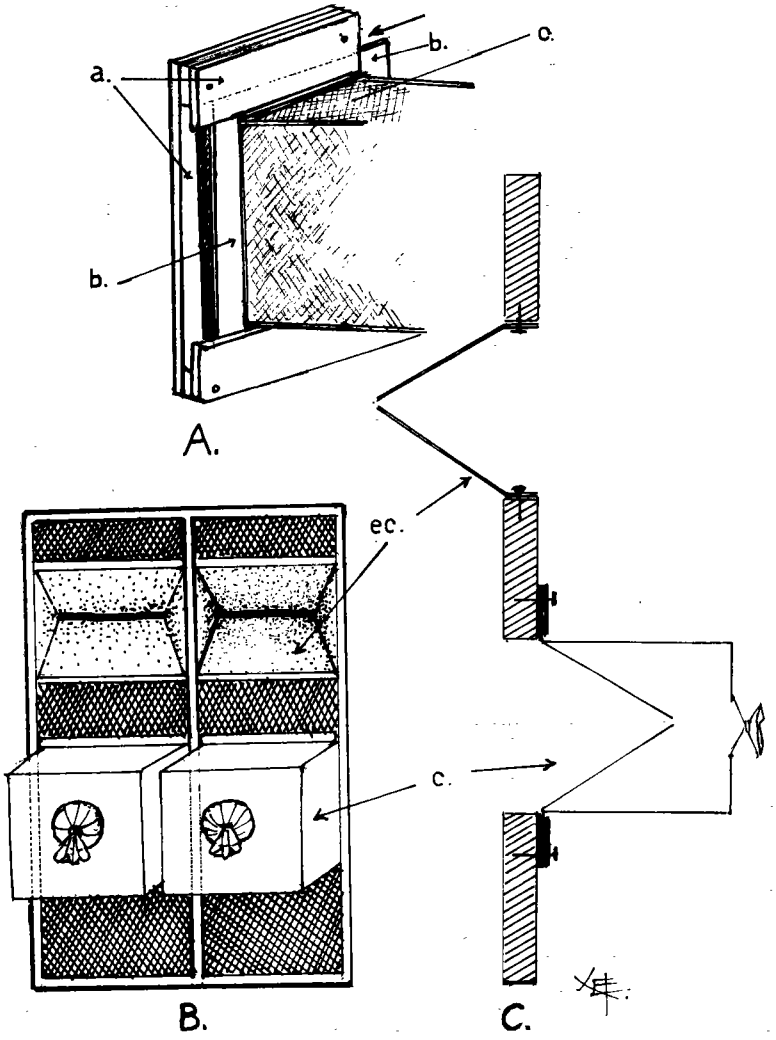
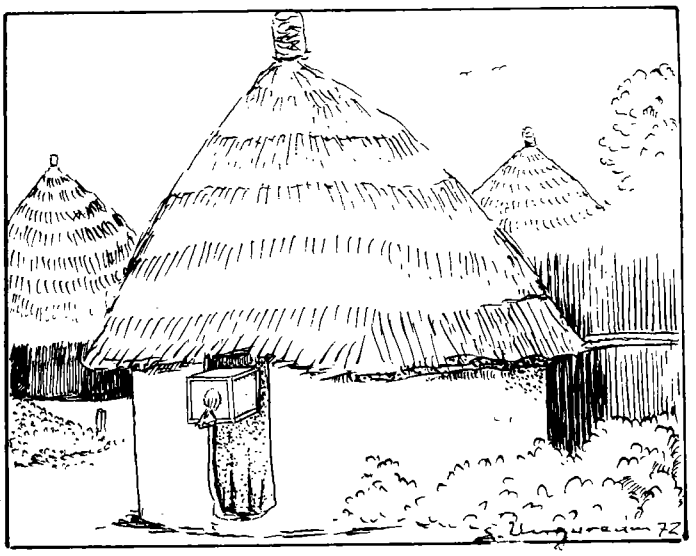


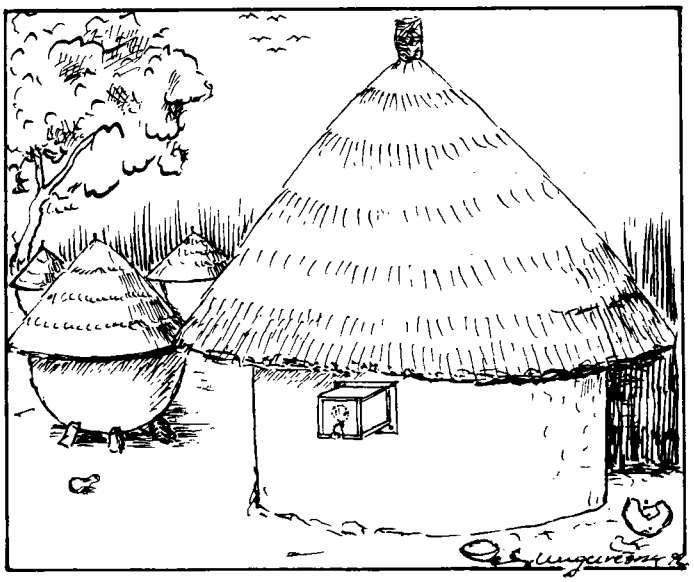
Fig. 9. Fitting of exit and entry traps.

A. Fitting trap to wall: a = wall fixture with groove; b = base plate of trap; c = trap

B, C. Exit trap units complete with entry slot: ec = entry slot; c = exit trap.



A.



B.

Fig. 10. Door and window traps in position.

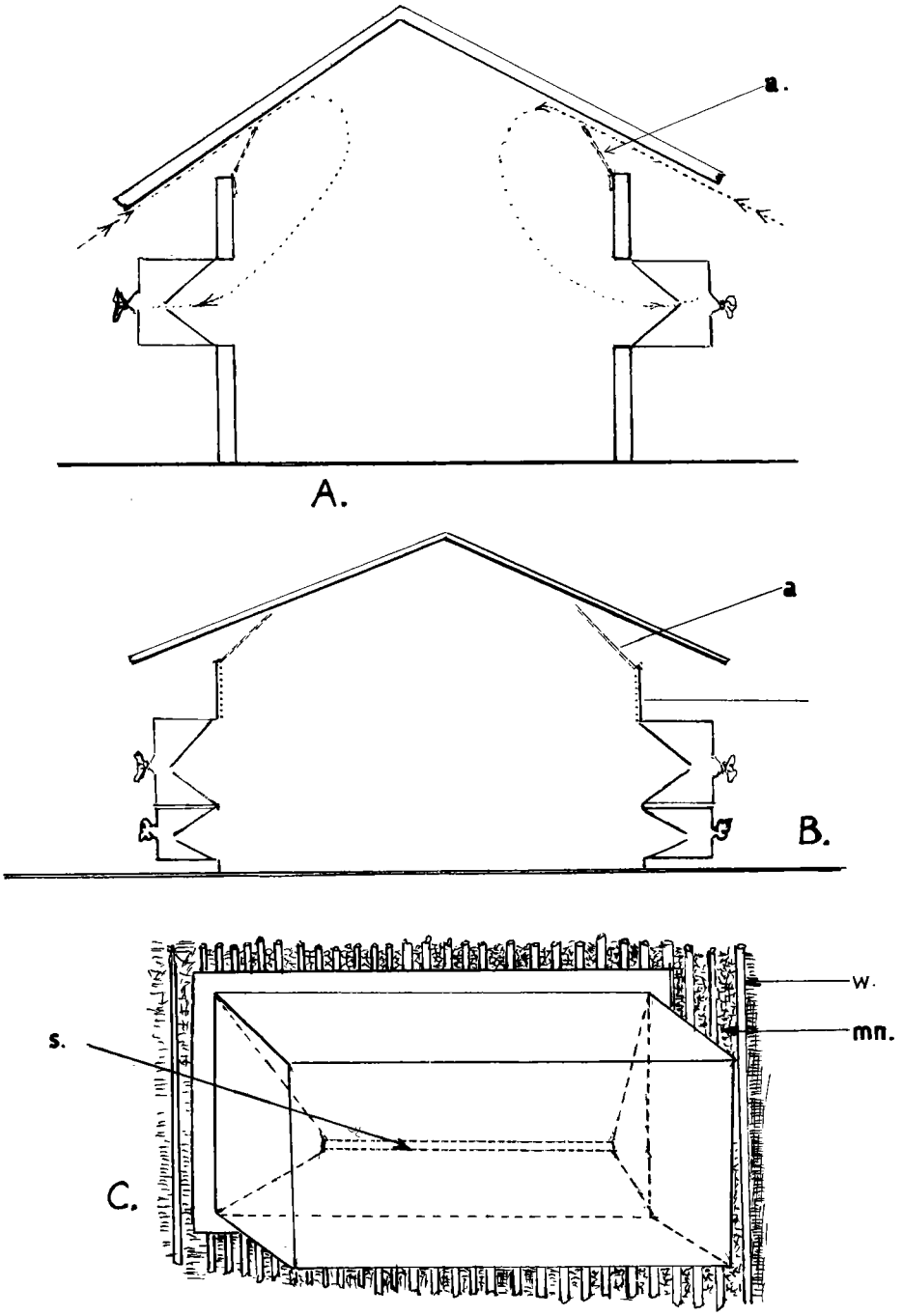


Fig. 11. Use of window traps and wall traps.

A, B. Partial blockage of eaves when using window traps or wall traps
C. Wall trap.

a = netting to prevent egress; mn = mosquito netting blocking gaps in wall;
s = entry slot; w = wall material.

The use of the exit trap in local huts is a very convenient additional method for study of the objectives mentioned under 4.1. The efficacy of this means of sampling the mosquito population depends on the way in which it is used.

A number of aspects should be considered:

First, verify that the huts used are frequented by a relatively high number of mosquitos. Carry out a preliminary study of the indoor resting density in the case of endophilic mosquitos, or see whether the huts are situated in environmental conditions that suggest the likelihood of a relatively high mosquito density (proximity of breeding places, attractiveness for mosquitos, e.g., relatively high number of hosts, presence of children, etc.).

Secondly, the huts should not have large spaces under the eaves or elsewhere. If the space between the walls and the roof is too large, it is recommended that this space be reduced by fixing mosquito netting or a piece of cotton sheet, leaving a space of only 3-4 cm between the roof and the roof poles. This will allow the mosquitos to enter the hut but will hinder them from leaving. When the human habitations have compact walls, window or door traps are easily applied. In areas where the walls are made of woven bamboo, sticks, or planks with spaces between them, the use of window traps is more difficult.

In this case the walls should be partially covered as well as the large spaces; in such conditions a good supply of cotton sheets is needed and the investigation should be limited to a small number of houses. Paper sheets could be used instead of cotton, e.g., packing paper which is available in strips of several metres long or nylon mosquito netting.

The window traps are fixed before sunset and removed at sunrise before pyrethrum spray collection starts. Pyrethrum spray collection in huts with traps should not be repeated at shorter intervals than seven days in order to avoid the repellent effect of pyrethrum spray.

Door traps are frequently used in huts without windows. The trap is fixed to a piece of dark cotton material longer and wider by about 20 cm than the opening of the door. The trap is fixed in the upper-half of the cotton sheet (Fig. 10 A). The piece of cotton is attached to the upper and lateral inside border of the wall; one lateral part and the lower part are not fixed, so that people may enter and leave the hut. The door trap is applied in the evening before sunset and removed in the morning. It needs more education of the inhabitants of the hut and closer supervision than does a window trap. A collection for special investigations - such as comparative studies of the efficiency of door and window traps - should be carried out during the night bait collections in the same village, in order to check from time to time during the night that the cloth on which the trap is fixed is in place. Each time a minimum of five huts should be investigated, but whenever possible an increased number of huts should be used. When using door traps, make the necessary arrangements with the local people to avoid disturbing the trap while entering or leaving the hut; education of people is indispensable.

The exit trap may be applied not only to the windows but also to the walls (see Fig. 11, B and C). The wall trap is built on the same principle as a window trap but is much higher and in the form of a rectangular prism, about 35 cm deep, 1-2 m long, and 40-50 cm high, and an entry slit is provided instead of an entry cone.

Several wall traps can be applied on the same wall. The spaces between the wooden sticks are covered on the inside, so that mosquitos can only enter the house through the eaves (Fig. 11 B (a)).

(c) Significance of exit trap collections

Exit trap collections give indirect information on the circulation of mosquitos in different physiological stages from outside to inside, and direct information on their circulation from inside to outside. When applied to sprayed houses the exit traps indicate

the degree of irritability and the toxic effect on the mosquito population leaving the treated space. The deterrent effect of an insecticide cannot be directly measured by exit traps; experimental huts should be used to investigate such a phenomenon.

If only freshly fed and unfed females of a certain species are found regularly in the exit traps (in non-sprayed areas) and none or only an occasional few are present inside the huts, this indicates that the species is purely exophilic but enters houses during the night to feed on the hosts indoors. The example of A. maculatus is well known.

After spraying, the immediate and/or delayed 24-hour mortality in the trap catch is normally high if the insecticide is active. Live mosquitos collected in exit traps are fed sugar water and kept for 24 hours in paper cups to observe the delayed mortality. The data reflect the impact of combined toxic and irritant effect.

In a non-sprayed area a higher proportion of unfed and gravid females than fed ones indicates an endophilic species (the indoor resting density also shows a high proportion of fed and fully gravid females). If endophilic fed mosquitos are found in the morning in the exit trap it does not mean that all these females remain during the daytime in outdoor resting places; some of them, if free, will enter other indoor resting habitats during the same night, so that the degree of facultative exophily of endophilic species might be overestimated if based only on the interpretation of the exit traps. Confirmatory data on indoor daytime resting mosquitos are obtained by pyrethrum spray collection in the morning. This is necessary to provide a more complete picture of the resting pattern.

The relative degree of exophily is indicated by the index $\frac{\text{gravid}}{\text{fed}}$. If the ratio of gravid to fed mosquitos found in the trap is constantly less than one then the species has exophilic habits (natural or induced by insecticide), if it is over one the species has endophilic habits. The closer the index is to one, the smaller is the differentiation in resting preferences so far as endophily or exophily are concerned.

4.2.1.2 Verandah traps

In order to provide a larger natural type of exit for mosquitos an alternative design dispenses with the window. In its place a door is fitted on the eastern wall, pierced by three horizontal slits, measuring about 4 x 20 cm, leading into a screened verandah about 1 metre across, 2.5 metres wide and 1.9 metres high. A ceiling is fitted inside the verandah and some form of roof is needed as a protection against rain (Fig. 12 A and B, and Fig. 14 A). In the early morning, after the catch has been made inside the hut, the collector enters the verandah via the door and completes the catch there.

4.2.1.3 Entry traps (Fig. 9)

Entry window or door traps are placed indoors with the aperture of the entry cone fixed in the space through which the mosquitos are entering.

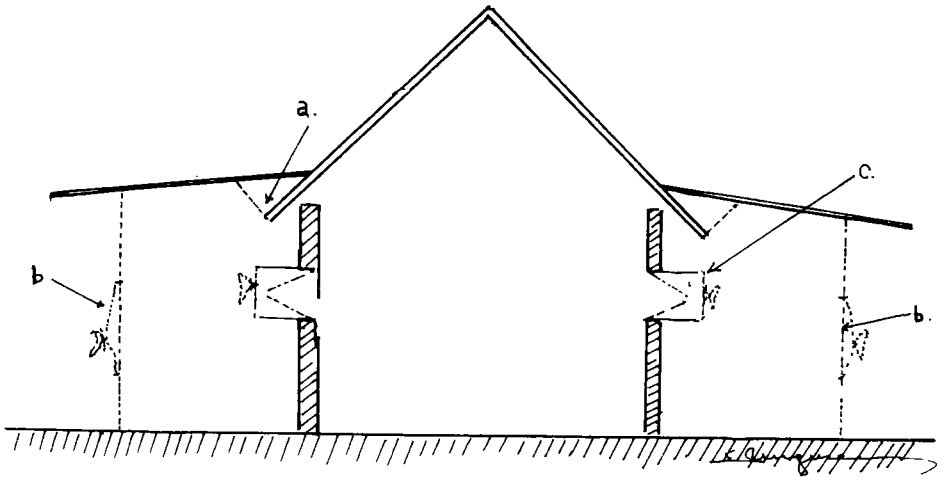
The use of trapping devices to catch mosquitos mainly for the study of entering has obvious advantages. At first sight it might be thought that this could be achieved easily by blocking up the eaves of an ordinary experimental hut and simply reversing the window-traps so that the funnel-shaped entrance faced outwards. In practice, however, this simple device is generally most unproductive, as few mosquitos are found in the traps in the morning.

4.2.2 Portable traps enclosing bait

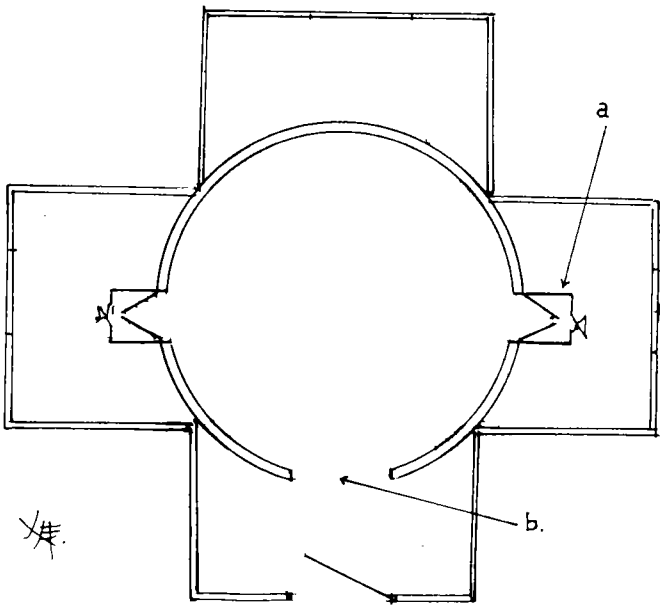
4.2.2.1 Bed-net traps

(a) Principle

Mosquitos are attracted under a big mosquito net covering a bed on which a human bait is sleeping unprotected or protected by a closer inner net (Fig. 13 A).



A.



B.

Fig. 12. Verandah traps.

A. Elevation: a = netting to prevent egress; b = access for collector; c = window trap

B. Plan: a = window trap; b = door of hut.

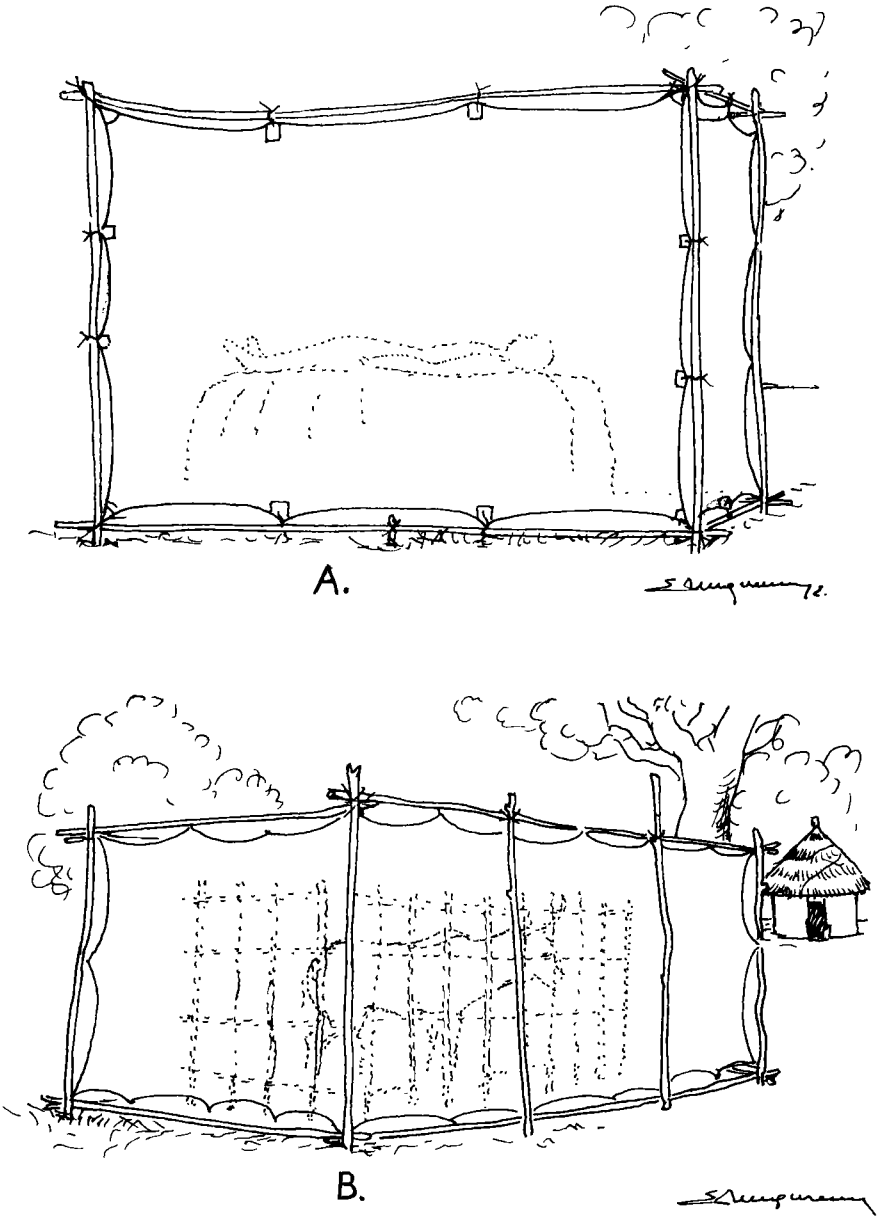


Fig. 13. Net traps.

A. Bed-net trap, with human bait
B. Trap net with animal bait (and human bait if desired).

(b) Objectives

- To study prevailing host preference of mosquitos, or trends in this over a period.

- To obtain additional information on the mosquito availability in the area.

(c) Description

The bed-net trap consists of a large mosquito net suspended from four poles, the beams of a house, or the branches of a tree. An efficient method of preparing the trap, which has been extensively used in the African region, is shown in Fig. 13 A. The net should be larger than the bed, hanging round it, and leaving a space of about 15-20 cm between the floor and the bottom of the net, through which the mosquitos will enter. The amount of free space should be determined by experience with local mosquitos. The trap net should be made as tight and as rigid as possible, the edge of the net being tied with string and pegged into the ground. This prevents the net from flapping in the wind, which deters and disturbs the mosquito. This precaution is necessary for outdoor collections. Bed-net traps have been used for indoor collections but not as frequently as indoor catching on human baits. Observations made with bed-net traps are useful, although this method cannot replace catching on human baits which yields data of greater epidemiological significance.

(d) Remarks

If a bed-net trap has wide orifices a number of mosquitos, including males, may enter while searching for a shelter; their presence does not, therefore, necessarily indicate that they have an anthropophilic tendency. This is shown mainly when females are captured unfed in a bed-net in which the bait is sleeping unprotected. When the human bait is sleeping under a protective net inside the bed-net trap, some mosquitos may leave the trap if the openings are too big, whereas when the mosquitos have the opportunity of feeding on the bait they are more likely to remain inside the net. To prevent hungry mosquitos escaping and to establish the number of mosquitos at different periods during the night, the sleeper may be woken at intervals by an alarm clock to collect the mosquitos. This usually results in a bigger catch. An interval of two hours is reasonable, and a torch with a blue light should be used.

4.2.2.2 Animal-baited traps

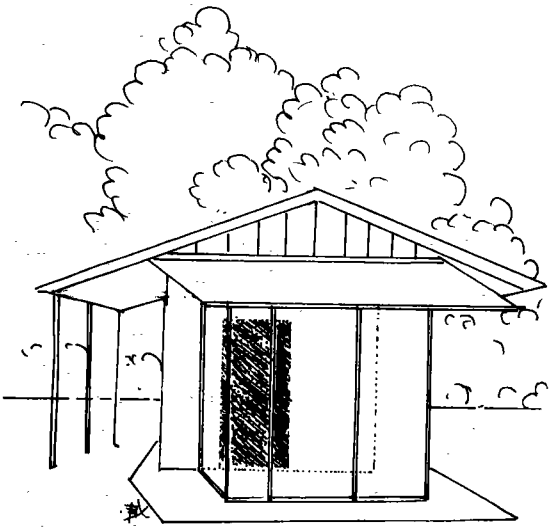
Objectives:

- To detect the presence and the relative density of mosquitos biting animals in a given area before and after spraying.

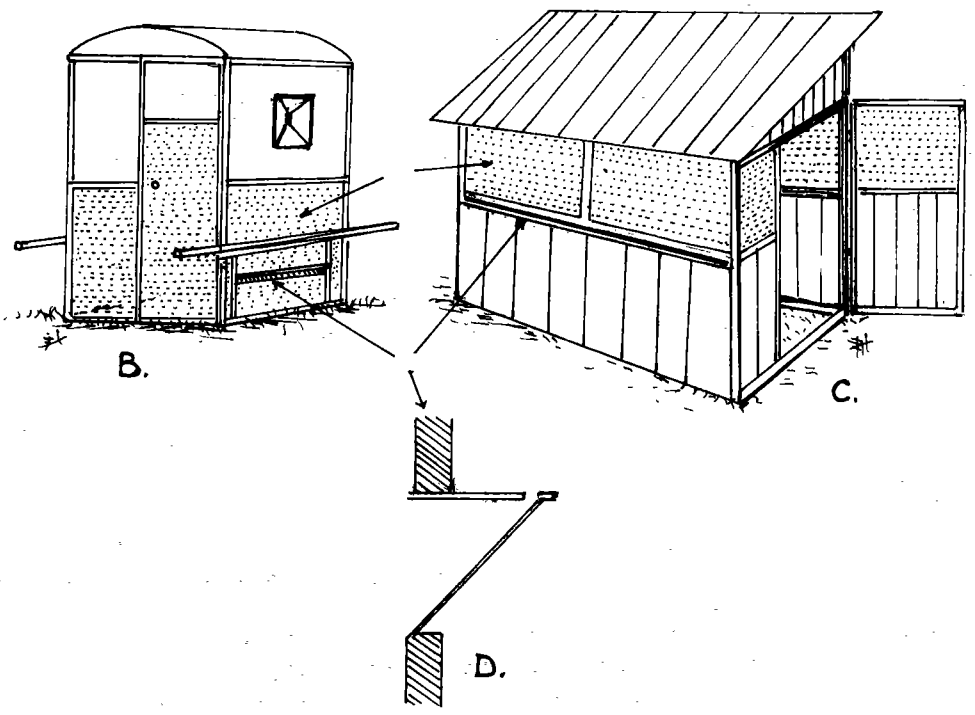
- To collect material for observations such as susceptibility tests or bio-assay tests.

(a) Magoon traps

These are portable wooden huts made of panels in which the upper half of the walls is removed and replaced by gauze netting and an entry slit is provided all the way round (Fig. 14, B and C). A convenient size is 2 metres by 1 metre; the hut should be high enough for the collector to be able to stand up inside. An internal ceiling of cotton or similar material should be fitted and some form of thatching provided. The various parts can be dismantled and bolted together again, so that the moving of the trap from one spot to another or from one locality to the next is very simple. The trap is baited with a calf or other suitable animal. Large numbers of mosquitos may be caught in a single trap, but catches will tend to vary with the exact location, and possibly with the individual host as well, and unless many traps are used exact quantitative data are hard to obtain.



A.



B.

C.

D.

Fig. 14. Verandah traps and magoon traps.

A. Type of verandah trap; B, C. Magoon traps;
D. Entry slot in section of magoon trap.

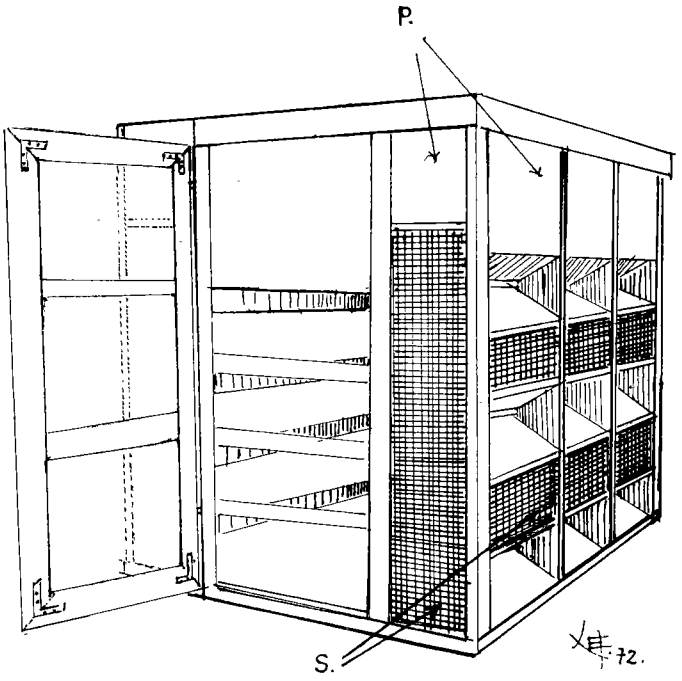


Fig. 15. Steer-baited trap.

p = polyethylene sheets;
s = mosquito netting (metallic).

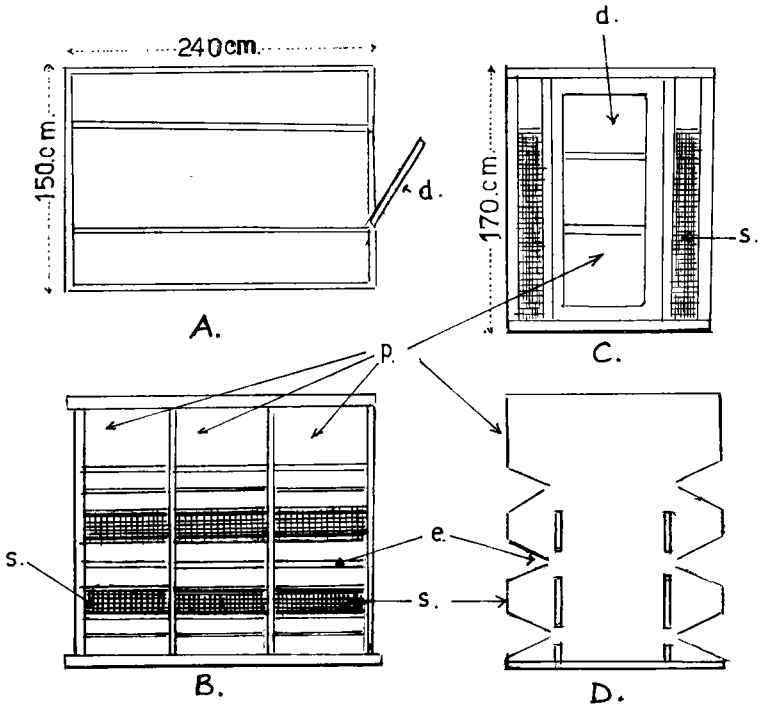
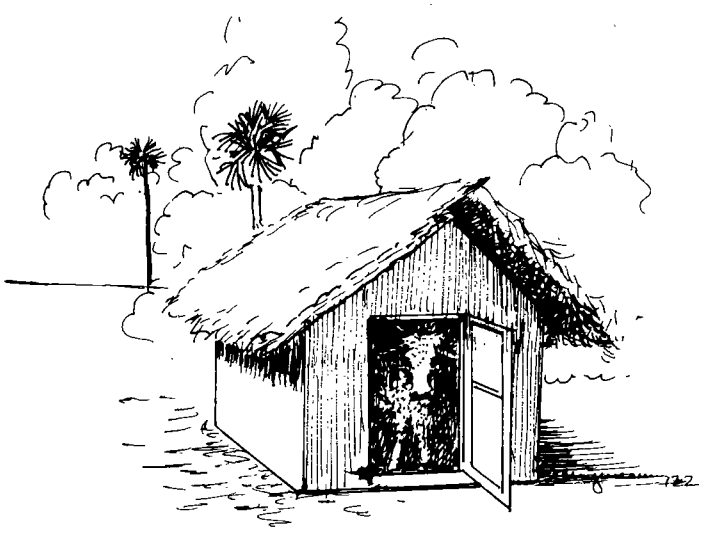
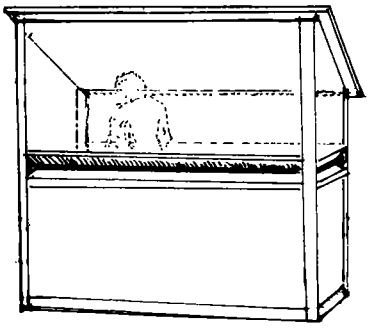


Fig. 16. Steer-baited trap.

A. Plan; B. Side elevation;
C. End elevation; D. Section
d = door; e = entry slot;
p = polyethylene; s = screen mesh.



A.



B.

Fig. 17. Modifications of magoon-type trap.

A. Local hut adapted as animal-baited trap

B. Highly portable magoon-type trap for man or small animal as bait.

(b) Steer-baited trap (Figs 15 and 16)

This is a more refined trap based on the same principle of collection as the magoon trap but the access for mosquitos is facilitated by a greater number of slits on both sides. Other possible modifications are shown in Fig. 17.

(c) Trap nets

These are made of ordinary mosquito netting supported on a metal or wooden skeleton and are big enough to enclose a large animal together with a human bait if desired (Fig. 13 B). The mosquitos can be given a choice of host under these circumstances; precipitin testing of the blood meals of engorged adults caught next morning will reveal the host preference. These trap nets are similar to the human-baited bed-net trap (Fig. 13 A), but are larger.

4.2.3 Mechanical devices for the collection of outdoor and/or indoor flying mosquitos

Principle

The general principle of all mechanical devices is that mosquitos are caught with the aid of a mechanically produced current of air.

Types of device

The mechanical devices used for the collection of mosquitos can be classified into two groups:

(a) Fixed traps using as an attractant light alone, light and a small animal, or light and carbon dioxide (New Jersey light trap, Monks Wood light trap, CDC miniature light trap). All these traps are based on the same principle; the mosquitos, after being attracted by the light (ultraviolet being the most attractive) are blown into a small reservoir (cage or bottle) by a current of air produced by an electric fan. Light traps may be employed in conjunction with human bait protected by a net.

Practically all light traps are constructed on the same principle (see Fig. 18), but CDC miniature light traps and Monks Wood light traps are much smaller than New Jersey traps (power is supplied by a battery of 6 or 12 volts).

(b) Mobile traps mounted on a vehicle.

4.2.3.1 Fixed traps (light traps)

The New Jersey Mosquito Light Trap, evolved in 1932, was, until recently, the most widely used light trap for mosquito work. Several modifications were made, including the Monks Wood light trap and the CDC miniature light trap, which seems to be the most appropriate for use in a malaria programme. The mosquitos are sucked into a funnel made of a 16-mesh screen terminating in a cyanide jar or killing bottle (Fig. 18). For special investigations in which live-caught mosquitos are required, the killing jar is replaced by a holding cage, which can be changed several times during the same night, as required. Larger insects are excluded by means of a quarter-inch (6 mm) wire-mesh screen over the mouth of the trap tube (New Jersey type) or round the tube and top supports (American type). This last arrangement of the screen is more appropriate in areas where large insects are very numerous.

In the CDC model the funnel-shaped screen is fastened in such a way that it can be removed for cleaning and maintenance purposes. Mechanical performance is also improved by means of a heavy-duty type motor and fan built with an enclosed pool for oil for lubrication purposes (and also to prevent dry dust, dirt or insects from penetrating to the interior of the motor). The fans are 22 cm in diameter and require a 25-cm diameter trap tube. With this design the air flow capacity is about 5 m^3 per minute.

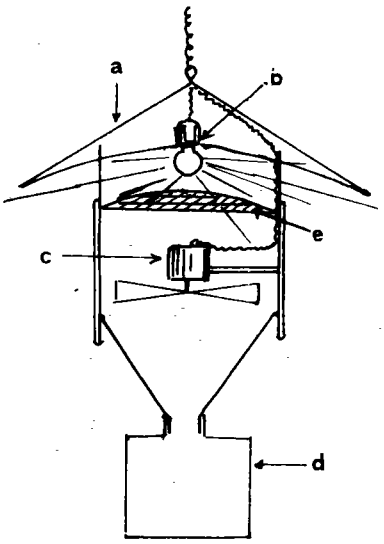


Fig. 18. General diagram of a light-trap.

a = shield; b = source of light;
c = electric fan; d = collecting reservoir;
e = metallic grill to retain butterflies and other large insects.

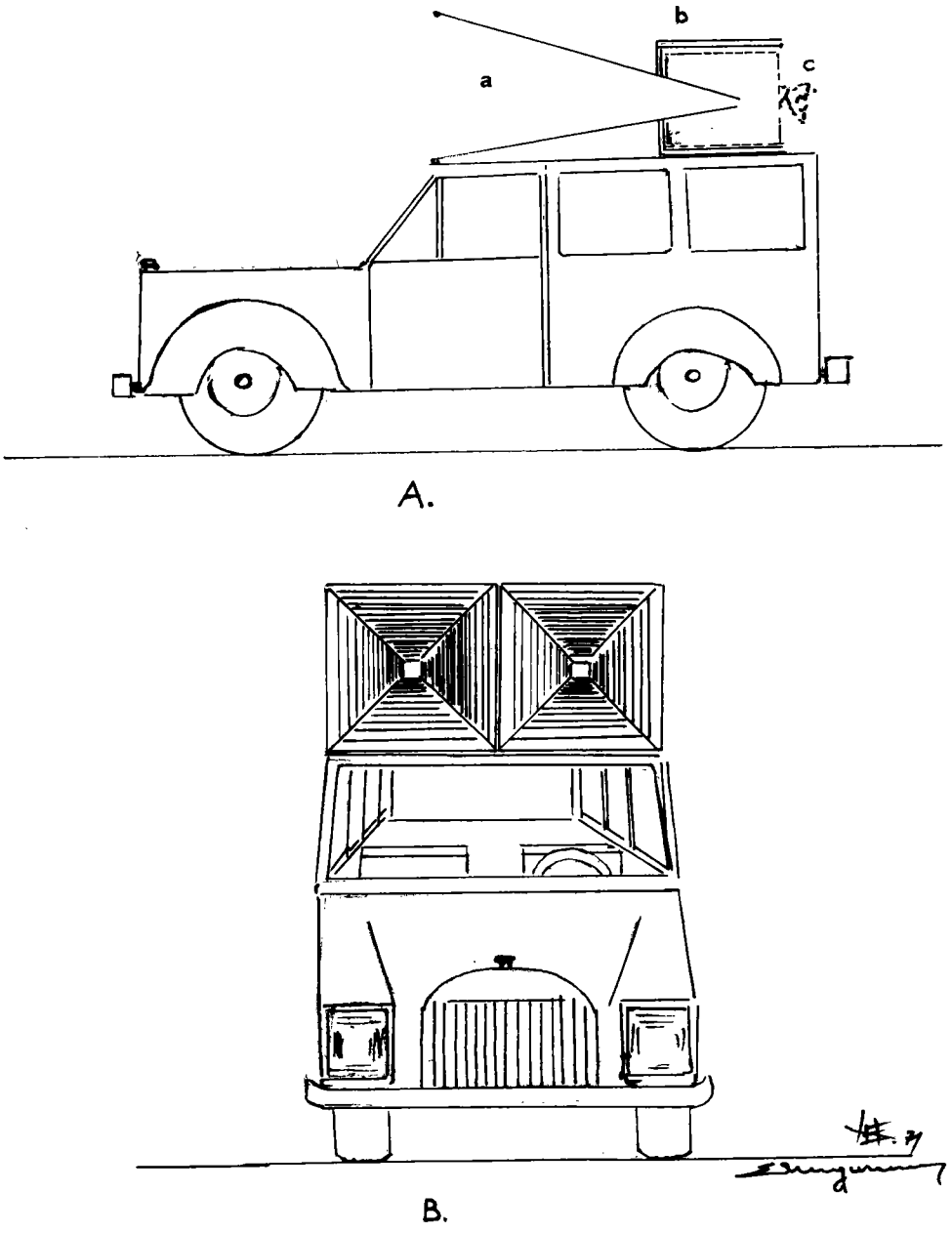


Fig. 19. Mobile traps mounted on top of a car.

A. Side view: a = nylon entry cone;
b = protective box; c = cage.
B. Front view.

New Jersey traps are equipped with a 25-watt white bulb, frosted inside. An automatic electric timer is adjusted to operate the trap from 18.00 to 06.00 hours each night.

Models are usually designed for 110-volt operation and for use with available power lines. In the absence of such facilities, power for the traps can be generated by portable petrol driven generators. Models are also available for use with 6-volt and 12-volt batteries, but these are rather heavy, e.g., 7 kg for the trap and 28 kg for batteries.

A miniature New Jersey trap has been developed which weighs only 700 g and needs only 2 kg of dry-cell batteries to operate the light and the motor. Several modifications of the source of light are now in use. Bulbs producing blue light or "black light" are on trial in order to find the optimum wavelengths for attracting mosquitos. From up-to-date experiments, it appears that "black light" attracts more anopheline species than other types of light.

Light traps have been widely used for routine sampling of culicine mosquito populations and for the study of culicine vectors of virus diseases. In the course of that work a considerable amount of information has accumulated about their efficiency with regard to anopheline mosquitos, especially A. quadrimaculatus, A. albimanus, A. hyrcanus, and others. In Africa, miniature light traps have been used for collecting A. gambiae s.l.

However, light traps have not generally been adopted in malaria eradication studies, and it appears that this method has certain limitations in that different species are not attracted to an equal extent to the light traps. Further critical work would be required before any decision or forecast could be made about the potentialities of this technique in malaria eradication programmes. An assessment of the efficiency of light traps compared with other methods of collection (human bait collections) still needs to be evaluated. The light traps cannot replace the collection from human baits for the direct estimation of the man-mosquito contact.

Light traps have mostly been used for collecting outdoor flying mosquitos. In recent years, miniature light traps have been used for indoor collection. From up-to-date experience it appears that before using the light traps routinely, the investigator has to make preliminary observations to find the most appropriate position for the trap. In principle, this should be placed in the way of flying mosquitos, e.g., indoors at the level of the eaves, near the host, near a door. The productivity of a light trap will depend on:

- position;
- type of light used (from up-to-date observations it appears that black light attracts a larger number of mosquitos than other types of light);
- species of mosquitos (some species are more attracted than others);
- physiological conditions (unfed mosquitos are more attracted than fed or gravid mosquitos);
- environmental conditions during the collection (meteorological, type of site, availability of hosts).

The appropriate time for investigating the comparative efficacy of the light trap is during a period of high mosquito density, but investigations during the low-density season have also to be carried out, in order to verify the efficiency of such a device compared to other methods of collection during different seasons.

Light traps are useful devices for obtaining additional material, but if used alone are not sufficient for sampling mosquito populations in a malaria eradication programme.

The siting of light traps is important and each investigator must find the most suitable position (in principle in the path of mosquitos) before using the device for longitudinal studies.

The attractiveness of light traps for different species of mosquitos should also be investigated before deciding to use the traps, in addition to other methods to be used simultaneously, for sampling a population of mosquitos under local conditions. The direction of the wind, height at which mosquitos are normally flying, temporary resting habits, and availability of hosts near the place of collection should be considered.

The use of light traps in malaria control or eradication programmes should be organized as follows:

All inhabitants of the rooms where the light traps are applied should sleep under a mosquito net. Ideally one light trap should be placed at the eaves, and one light trap should be placed at the level at which the bait is sleeping. Exit traps (door or window traps) should be applied to the same room.

The collecting bag should be changed as required for fractional sampling during the night.[1]

Indoors, light traps should be used without the shield, especially with black light.

4.2.3.2 Mobile traps mounted on the top of a vehicle (car, lorry or bicycle)

The trap consists of a large cone made of nylon netting, with an aperture of about 120-150 cm x 100 cm at the base and a small aperture at the tip opening into a mosquito cage from which the mosquitos can be easily collected. The cage can be protected by a wooden box in which the terminal part of the net is inserted. Details of the construction and mounting are shown in Fig. 19. A current of air is produced by the movement of the car. Flying mosquitos enter the nylon cone (a) at the end of which a cage is attached which retains the mosquitos and other insects. This mobile trap has been employed successfully for collecting anophelines during the evening and early in the night.

4.2.3.3 Comments on the use of mechanical devices

Of the mechanical devices mentioned above, light traps are used most frequently. The efficacy of light traps, especially miniature light traps, using different sources of light should be investigated, if possible, in all projects. Such observations should include:

- Use of light traps for collecting mosquitos indoors.
- Use of light traps for collecting mosquitos outdoors: (a) near houses; (b) away from houses, near source areas and very close to the most favourable sources.
- Exit trap and pyrethrum spray collections should be made in the morning and comparative studies should be carried out on the proportion of different abdominal stages and of parous females.
- Simultaneous night bait collections in huts of similar attractiveness and collections in the same huts on alternate nights should be carried out in order to make a comparison between the data obtained from light trap collections and those obtained from baits, e.g., one night bait collection, next night light trap and repeat alternate collections for six to eight days.

The disadvantage of light traps for sampling malaria vectors is that many species other than anophelines are collected and that very often the ornamentation of the wings and other parts of the body is damaged making identification more difficult. Some anophelines are not attracted in representative numbers, although they are abundant.

[1] Fractional sampling is necessary when studying the variation of the night-time movements of mosquitos and to reduce the mortality of mosquitos collected which might be produced by the whole night captivity in the collecting bag of the light trap.

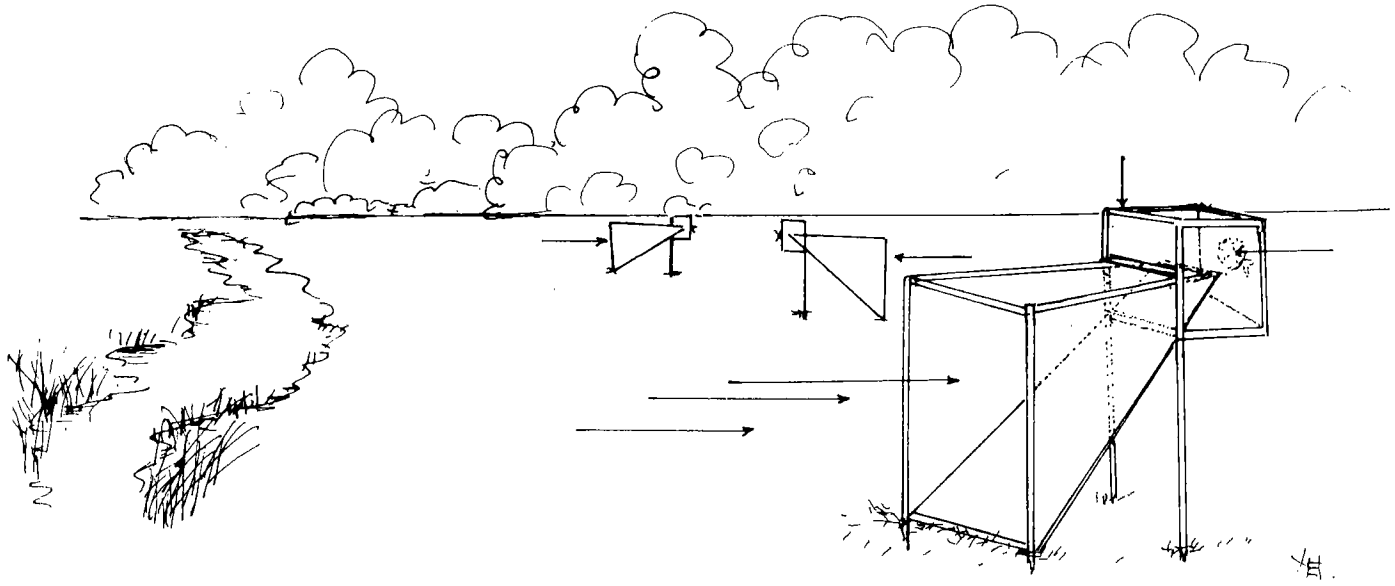


Fig. 20. Ramp trap.

The advantages are that less work is involved than for night bait collection and that collections can be made for several consecutive nights if necessary. The use of light traps as an additional method for the study of the phenology of some culicines in preliminary investigations for the evaluation of residual spraying and/or larviciding programmes might be practical with some species, but this still needs more investigation before these traps are used routinely in malaria programmes.

4.2.4 Other types of traps used for the collection of outdoor flying mosquitos

Apart from the traps mentioned above, a number of traps have been devised for the collection of mosquitos in flight outside. These are likely to be effective only under conditions where mosquitos are extremely abundant and concentrated in a small area. They find their main application in special investigations.

Two types deserve particular attention:

(a) Ramp traps

A simple tunnel of mosquito netting, supported on poles, 1.8 m high by 1.8 m wide by 2.7 m long, open at one end (Fig. 20). Mosquitos enter it in the course of normal flight and are trapped and collected at intervals by closing the flap over the entrance. Information on the movements of mosquitos flying at low levels, in the presence or absence of bait, can thus be readily obtained. Such traps can be placed at different distances in the area between breeding places and the inhabited area.

(b) Malaise trap

The method of collection with Malaise traps is based on the fact that mosquitos have a tendency to fly upwards when an obstacle is in their way.

There are several modifications of the original method published by R. Malaise (1937) but the structure remains the same. The trap is composed of the following main parts (Figs 21 and 22).

The bottom baffles (Fig. 21 A (a) are composed of four vertical walls made of two pieces of black netting sewn together down the middle and then arranged in the form of an X when they are mounted, as shown in Fig. 22 C. At each extremity of the arms of the X, two side baffles (Fig. 21 A (b)) made of the same material as the bottom baffle, are attached, as shown in Fig. 22 D.

The top panel has the form of a rectangular pyramid and is made of white netting. On the inner part of the top panel, extensions of the bottom baffles are fixed by tying in the manner shown in Fig. 21 A (c) and Fig. 22 A and B. A plastic or metal cylinder (Fig. 21 B) is glued over the aperture left at the top of the pyramid; this is attached to the centre vertical pole (Fig. 21 A (d)) which supports the top panel and holds the frame of the collecting cage.

The collecting apparatus is composed of the following pieces:

(a) A wooden frame in to which the cage can be slid (Fig. 21 C);

(b) A plastic or metal cylinder, mounted underneath the frame and of such dimension that it can be inserted inside the plastic cylinder of the top panel. The collecting cage is constructed like a normal window trap but can be smaller.

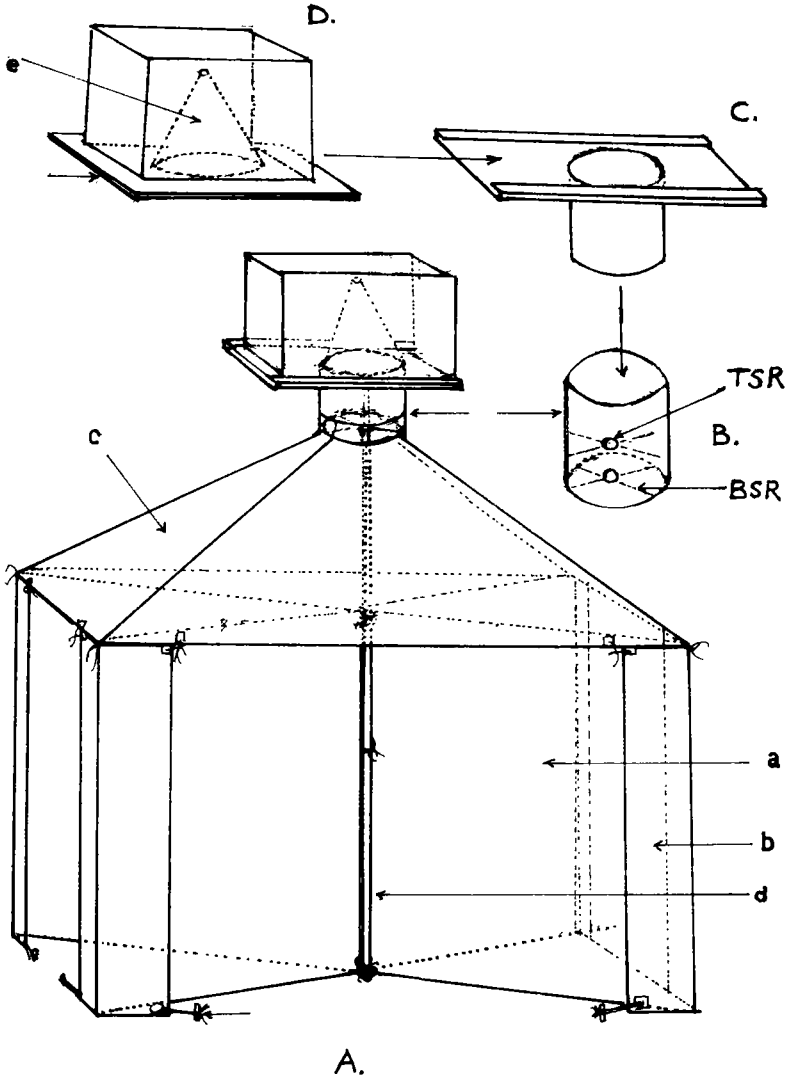


Fig. 21. Malaise trap.

a = bottom baffle; b = side baffle;
c = top panel; d = supporting pole; e = cage
TSR = top supporting ring; BSR = bottom supporting ring.

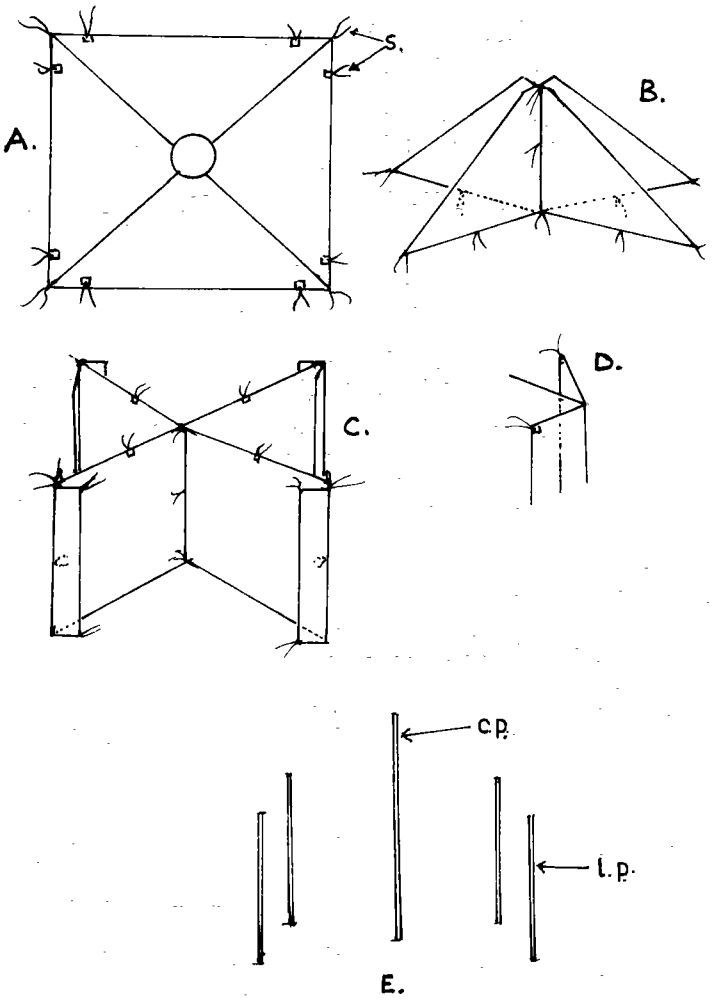


Fig. 22. Malaise trap: details of assembly.
s = string; c.p. = centre pole; l.p. = lateral pole.

Procedure for assembling the trap:

- fix the middle pole in the ground;
- fix the plastic cylinder of the top panel on the middle pole with the aid of two metal rings (Fig. 21 B, TSR and BSR);
- insert the tube of the collecting cage in the tube of the top panel;
- fix the four sticks at equal distances (Fig. 22 E) and attach the lower corners of the top panel to the corresponding sticks with string or cotton tape;
- mount the bottom baffles by fixing them on the poles at the upper, middle and lower parts with cotton tape (Fig. 22 C);
- tie on the bottom baffle extensions by means of the cotton tapes;
- fix the side baffles in position with cotton tape, tying the upper part to the border of the top panel and the lower part to a small wooden peg stuck in the ground (Fig. 21 A).

The trap can be used for collecting mosquitos without using baits, but before employing this routinely the efficiency for collecting different vector species should be carefully tested. It is efficient for mosquitos flying at low level, but should be used as an additional method only after field trials have shown it to be useful.

(c) Acoustic traps

These have been tried experimentally using a sound signal as an attractant, but they have not been developed for field use.

5. EXPERIMENTAL HUTS

5.1 OBJECTIVES

(a) To evaluate the normal behaviour of house-entering mosquitos.

(b) To evaluate the reaction of mosquitos to insecticides: deterrent, excito-repellent, and toxic effects; inhibition of feeding in endophilic mosquitos or in those exophilic mosquitos coming inside to bite.

5.2 USE OF THE EXPERIMENTAL HUTS

In general the experimental huts are used during stage IV and V trials of insecticides.[1] In exceptional cases experimental huts can be used (a) when a stage VII trial of an insecticide has started without complete information, (b) in a malaria pilot project to complete observations on the mosquito's normal behaviour, (c) to study the mosquito's behaviour under the influence of an insecticide, or (d) at a later stage of a programme when a change in behaviour is suspected. Such observations cannot entirely replace those carried out in local huts or houses, but if properly used give a controlled approximation.

To evaluate the effect of insecticide on a malaria vector, the experimental hut should, in principle, resemble the local huts as closely as possible, with the necessary modifications to ensure the collection of dead or alive mosquitos. The building materials should be the same as those used for the local huts. The main difference is that the experimental hut should be ant-proof when used for studying the global toxic effect of a residual insecticide.

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[1] See: Wright, J. W. (1971) Bull. Wld Hlth Org., 44, 11-22.

TABLE 1. TYPE AND USE OF VARIOUS TRAPS FOR COLLECTING MOSQUITOS[a]

Types of trap		Place to be used	General objectives	Specific objectives
Fixed	Portable			
Window trap	-	Houses (huts) local or experimental	To study the egress or ingress of mosquitos under normal or modified conditions. Investigation of mosquito behaviour related to human habitations under normal or modified conditions	1. To study in part the effect of insecticides on mosquitos entering treated huts 2. To study (in part) mosquito availability and density
Door trap	-	Houses without windows	"	"
Verandah trap	-	Houses (huts) local or experimental	"	"
Wall trap	-	"	" As above. For use where poorly constructed walls allow mosquito circulation	"
Eaves	-	"	"	"
Bed-net	Bed-net	Indoor or outdoor	To collect mosquitos attracted to a particular human bait	Detection of species, attracted to man. Collection of live mosquitos
-	Trap net (baited)	"	To collect mosquitos attracted to human and/or animal bait	Examination of availability of anopheline species. Collection of vectors for various investigations (susceptibility tests, infectivity, longevity, etc.). Indication of feeding preferences (when supplemented by precipitin tests)
-	Malaise	Outside	Outside collection of flying mosquitos	Availability of species. Collection of vectors for various investigations (susceptibility tests, infectivity, longevity, etc.). As above
-	Ramp trap	Outside	"	Availability of species, direction of flight
-	Magoon trap Stable trap	Outside	To collect mosquitos attracted to contained baits usually animals as combine protability with robustness	Availability of species. Collection of vectors for various purposes
-	Light trap	Inside and outside	To detect mosquito species attracted by normal or black light	Availability of species
-	Car trap or bicycle trap	Outside	To collect mosquitos in flight by active sampling	Availability of species

[a] The terms fixed or portable are relative. Exit traps are also portable, but should be always fixed to a specific type of entry or exit space of a room. None of these traps can be used alone for the study of relative seasonal prevalence etc., each type having its limitations; they therefore should be used in combination with other appropriate methods of collection.

A cement floor, extending below but not beyond the walls, should be provided in mud huts to enable an ant-proof drain to be fitted (Fig. 23 A). Thin plastic sheets may be used instead of cement and are considerably cheaper (Fig. 23 B). The access of ants to wooden huts built on poles may be prevented by placing a sticky or greasy band on the poles (Fig. 23 C).

The collection of mosquitos in experimental huts can be carried out using different methods. Generally a combination of methods is used, depending on the objective of the study, e.g., window trap, hand collection, pyrethrum spray collection.

The windows of the experimental hut are provided with exit traps. In some instances verandah traps can be used to collect mosquitos flying out from the huts.

5.3 OPERATION OF EXPERIMENTAL TRAP HUTS

Little standardization of design has been achieved. Ideally a trap hut should have the same conditions of attraction as exist in local huts. Two or more people should be employed to sleep in each hut, bearing in mind that the greater the number of hosts the larger the catch. They should be encouraged to use the house regularly, regardless of whether catches are being made or not, since it appears from experience that a lived-in house, possibly because it is more permeated with human smells, is more attractive to mosquitos than a clean, newly-built one. Cooking and the use of mosquito nets are normally forbidden.

5.3.1 Collection of resting mosquitos

Mosquitos should be collected by hand-catching carried out on a systematic basis from all parts of the hut. Particular attention should be paid also to the roof, and a short ladder or pair of steps may be provided to enable this to be carried out effectively. At the end of the catch the collector should poke the corners of the roof with a wooden stick to disturb the mosquitos which have escaped observation. As discussed above, the risk of a residual irritant effect from the repeated use of pyrethrum in kerosene prohibits the daily application of knock-down sprays. However, spray-sheet collections at, say, two-week intervals can give a most useful check on the effectiveness of the hand-catching. In this case, before starting the spray-catch, it is necessary to remove the window-traps and to block the windows with shutters.

The collection should follow the following steps:

- at sunrise, collect mosquitos from the exit traps and the dead mosquitos on the floor;
- put live mosquitos in paper cups for determination of 24-hours survival rate;
- between 14.00 and 16.00 hours, collect the dead mosquitos.

Indoor resting mosquitos could also be collected by hand; this depends on the type of observation.

5.4 USE OF EXPERIMENTAL HUTS FOR ASSESSING THE EFFECTS OF AN INSECTICIDE

A series of experimental huts with window or verandah traps is built for this purpose and suitably treated with different dosages of insecticide, one or more huts being left for control purposes. In the evening on the day before an assessment is to be made, the window-exit traps are placed in position and white sheets are suitably disposed on the floor of the hut. The following morning, before or at sunrise, the window-trap is removed and the window closed with a shutter. The mosquitos in the trap are counted and divided into living and dead, the former are then kept for 24 hours to determine mortality. The sheets are removed from the floor of the hut and placed outside and the number of dead mosquitos counted. The collection of indoor resting mosquitos is carried out between 14.00 and 16.00 hours in order to give enough time for the mosquitos to become intoxicated; the hut is carefully searched

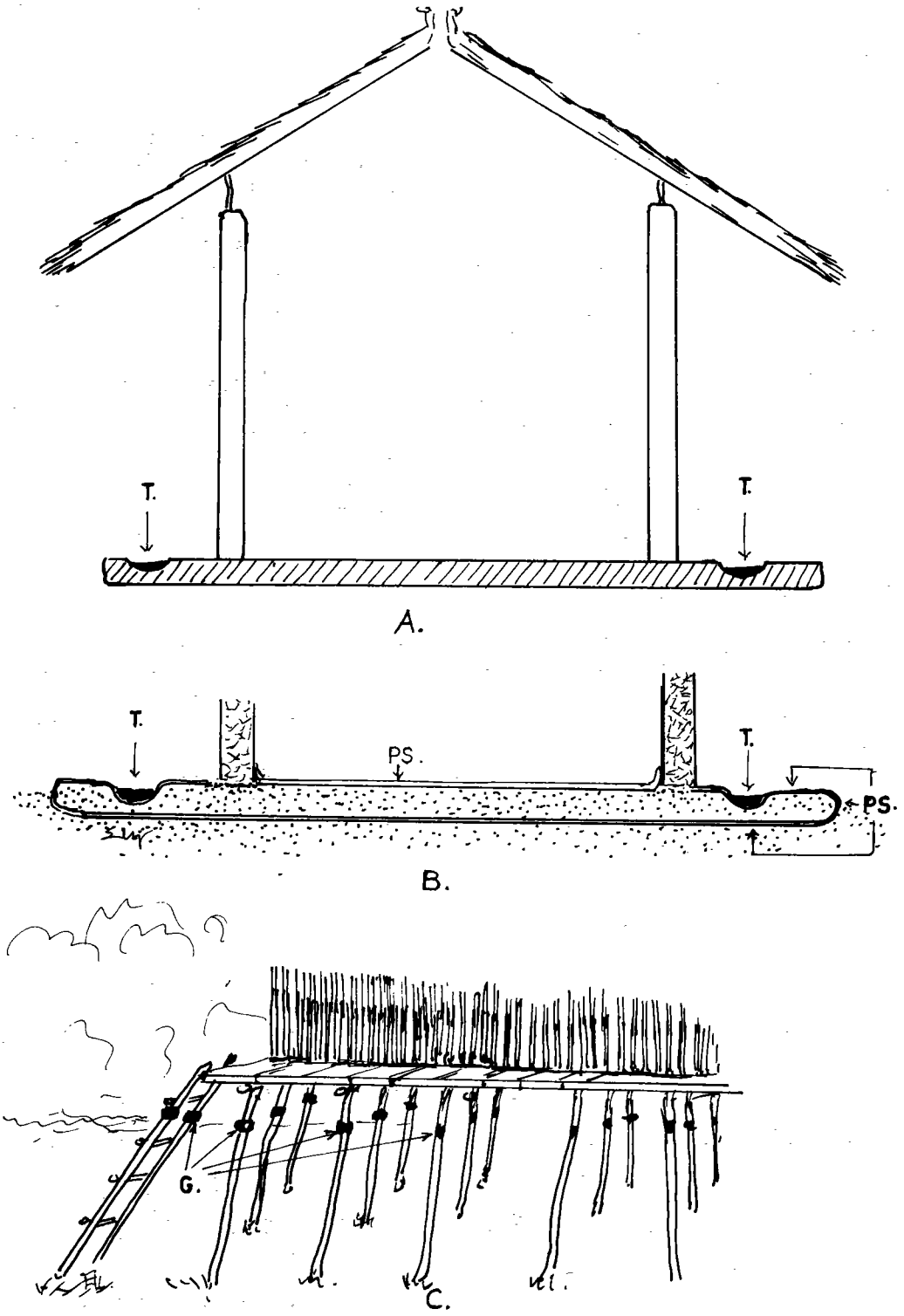


Fig. 23. Experimental hut design.

A. Built on a cement platform (T = trough with water and thin layer of oil).
B. Isolated by a plastic sheet (PS).
C. On poles, with grease rings (G) against ants.

and the mosquitos are hand-caught, placed in cages, and the 24-hour mortality determined. If there is not enough time or labour to make hand-catches, the experimental huts may be space-sprayed with pyrethrum between 14.00 and 16.00 hours, after removing the mosquitos dead on the floor. This makes it impossible to calculate the survival rate of indoor resting mosquitos after 24 hours; nevertheless a good impression of the killing power of the insecticide will be gained. If space-spraying is employed it is necessary that the intervals between observations should be not less than a week because of possible repellent effects of pyrethrum space-sprays used at shorter intervals.

5.4.1 The calculation of the mortality produced by insecticide (room kill)

The percentage mortality produced by an insecticide on mosquitos entering the experimental huts can be determined as follows:

a = number dead in exit-trap

b = number alive in exit-trap

c = number of "b" still alive after 24 hours

d = number dead on floor of hut (collected in the morning and afternoon)

e = number alive caught by hand in hut (in the afternoon)

f = number of "e" still alive after 24 hours

then $a + (b - c) + d + (e - f)$ = total dead = x

and $a + b + d + e$ = total mosquitos caught = y

then $\frac{x}{y} \times 100$ = % mortality.

Another and more satisfactory way is to carry out the observations for several consecutive days without collecting the live mosquitos resting inside (except the last day) since the mosquitos still alive during the day-time will be found dead on the floor and in the traps next morning if the insecticide is efficient. The collection in the traps can be carried out at several intervals during the night if special observations about the time of exit are needed.

5.4.2 Estimation of irritant effect

Experimental huts with exit-traps may also give valuable information on the repellent action of insecticides. If window-traps or other exit-traps of treated huts contain a far greater number of mosquitos than those of check huts, in relation to the total number which entered both types of huts, then irritant action can be assumed. The classification of the abdominal appearance of female mosquitos found in traps is very important since this indicates the effect of irritability on the feeding behaviour. Trap-collected females from well sprayed huts, surviving 24 hours, indicate that the mosquitos left the sprayed structure before picking up a toxic dose.

5.4.3 Estimation of deterrent effect

One can assess whether insecticides discourage the mosquitos from entering a sprayed structure by comparing the total number entering a sprayed hut (alive, dead inside exit-traps and inside the hut) with the situation before spraying and with the situation in an unsprayed hut.

5.5 REMARKS

Mosquitos collected from untreated huts as well as from treated experimental huts should be classified into their respective abdominal stages: unfed, freshly fed, late fed, half gravid, sub-gravid and gravid. The first group (unfed) are usually a mixture of virgin females that have entered for shelter only and of those multiparous and parous females that entered to feed but failed to complete the act. Thus without extensive dissections to distinguish these two groups, little can be deduced from data on unfeds. Of most interest is the movement of fed females, particularly in relation to possible changes in behaviour following spraying.

Floor and window-trap collections (when correctly carried out) in huts treated with residual insecticides correspond to those entering and dying and those leaving within 12 hours of entering. Although one is primarily interested in the fate of mosquitos after feeding, it may be found that with very active deposits and high kills few of the mosquitos will survive unaffected long enough to feed, so that the majority of the catch will be composed of unfed females. Consequently, the data will have to be analysed so that two estimates can be made; first, the percentage of females feeding and surviving for 24 hours (by excluding the unfeds), and secondly the total survival rate of all the mosquitos that entered the hut (unfed and fed).

In the use of experimental huts the following aspects should be considered:

(a) The design of the hut. Although this may be perfect from the point of view of collecting specimens on the walls or ceilings or of preventing the exit of specimens except through the window-trap or verandah, it may yet prove to be so unnatural for the mosquito that poor results are obtained. On the other hand, the ordinary local hut may be quite unsuitable because the mosquitos find so many exits and entrances that window traps catch hardly anything. When the anopheline population is very high, this problem loses some of its importance.

(b) The next point to consider is the accidental contamination of check huts, window-traps, holding tubes and other apparatus by personnel riding in, or transporting material in, contaminated vehicles or wearing contaminated boots and clothing. When visiting the experimental huts one should always go to the check huts first and then the sprayed huts in order of increasing dosage. Window (door) traps and other materials should never be interchanged between sprayed and unsprayed huts and should be washed regularly. The check huts should be kept as far away from sprayed huts as is possible and convenient. Where contamination is suspected, wild-caught mosquitos may be exposed to the hazard and observed for 24 hours.

(c) Experimental huts and window-traps of classical design have given good results in some places - in others they have been less successful. The reasons for this are not clearly understood but there is no doubt that the design of huts and traps is far from satisfactory. A further development of the "verandah-trap" seems to offer opportunities. Consideration might also be given to the enclosing of an ordinary hut, typical of the area, in a large screened cage in which daily mortalities would be calculated from mosquitos picked off sheets both inside and outside the hut. The contamination of traps should be checked periodically.

A recent modification of the entry space through the eaves proposed by Smith (1970) seems to increase the possibility of collecting a higher number of mosquitos attempting to leave the huts. The eaves are modified in such a way that the possibility of entry through the eaves remains high but the exit of mosquitos through the eaves is low, so that the mosquitos leaving are forced to fly into the window-traps. The eaves of the huts fitted with window-traps are modified as follows:

A piece of metallic or rigid plastic netting is fixed to the interior parts of the wall all round the eaves (Fig. 24 A), leaving a space of about 4-5 cm at the top through which the mosquitos will enter. The net is about 30-40 cm wide depending on the aperture of the eaves. The top of the net is fixed to the wooden pole of the roof in order to leave a space of about 4-5 cm (Fig. 24 B). In the absence of the above-mentioned materials, nylon mosquito netting, or pieces of white or dark sheets of convenient size, could be used for reducing the space between the roof and the wall to 2-4 cm.

It is reported that this method increases considerably the numbers caught in window-traps and can be used with door traps in huts with relatively large eaves.

6. CATCHES IN OUTDOOR SHELTERS

6.1 PRINCIPLE

Mosquitos resting outdoors are caught by hand or with the aid of an aspirator.

6.2 OBJECTIVES

(a) To study the outdoor resting habits of mosquitos resting naturally outdoors or as a consequence of the deterrent and irritant effects of insecticides.

(b) To collect blood samples for precipitin tests from blood-fed mosquitos resting outdoors.

(c) To collect samples for other qualitative studies (species present, age, infection).

Note

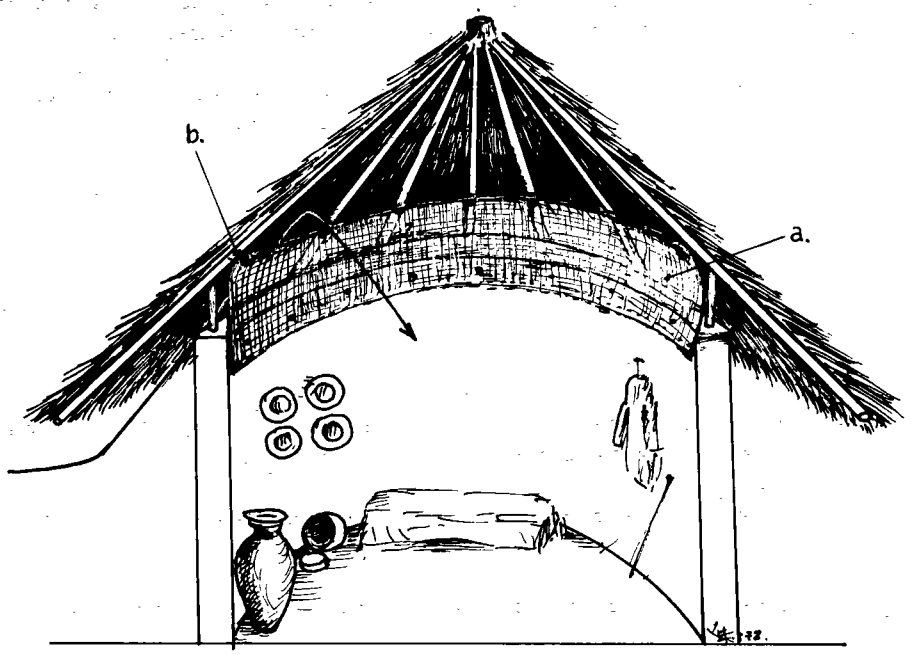
The collection of resting Anopheles in outside situations now occupies a position of some importance in malaria campaigns. Much time and ingenuity can be spent in making such catches more productive. Since the locating of natural resting sites is an arduous and frequently unprofitable occupation, artificial outdoor shelters are commonly employed. The distinction between natural and artificial is itself an arbitrary one, since "artificial" shelters may range from slight modification of existing harbourages to the setting up of traps or boxes made in a workshop. For certain studies, such as outdoor resting behaviour, it is essential to concentrate on natural resting sites only; but in assessing the effect of antimosquito measures on the outdoor resting population, artificial shelters have many advantages, not least among them being the provision of a standard type of catch which can be used for quantitative work.

6.3 MATERIALS AND METHODS OF COLLECTION

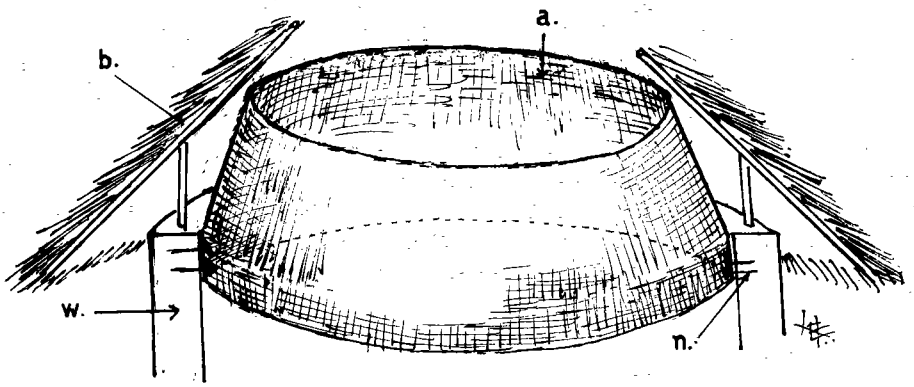
One of the following devices can be used:

- Suction tube and a torch for dark places
- Mechanical aspirator (sweeper) for collecting mosquitos from vegetation, holes, etc. (Fig. 6)
- Artificial resting shelters (pit shelter, barrels, boxes, etc.)
- Drop net for collecting mosquitos from grass or low vegetation (Fig. 25 A and B)

When using a drop net, the mosquitos may be driven out of their resting places by disturbing them mechanically, using a stick or an irritant such as citronella or a very dilute solution of pyrethrum. The mosquitos are easily collected by a man inside the drop net.

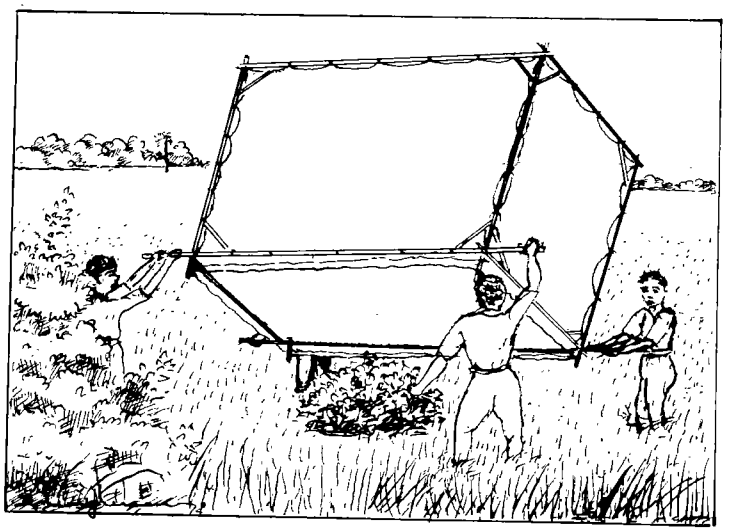


A.

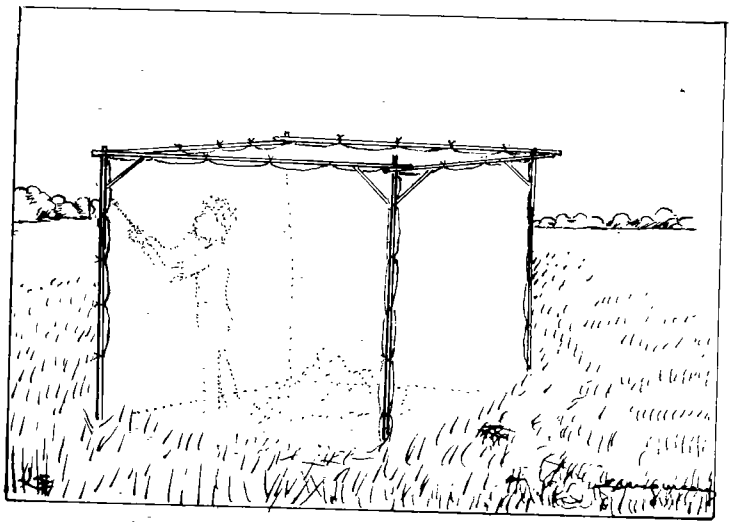


B.

Fig. 24. Modification of eaves of huts fitted with window traps. a = mosquito netting; b = roof pole; n = nail or peg; w = wall.

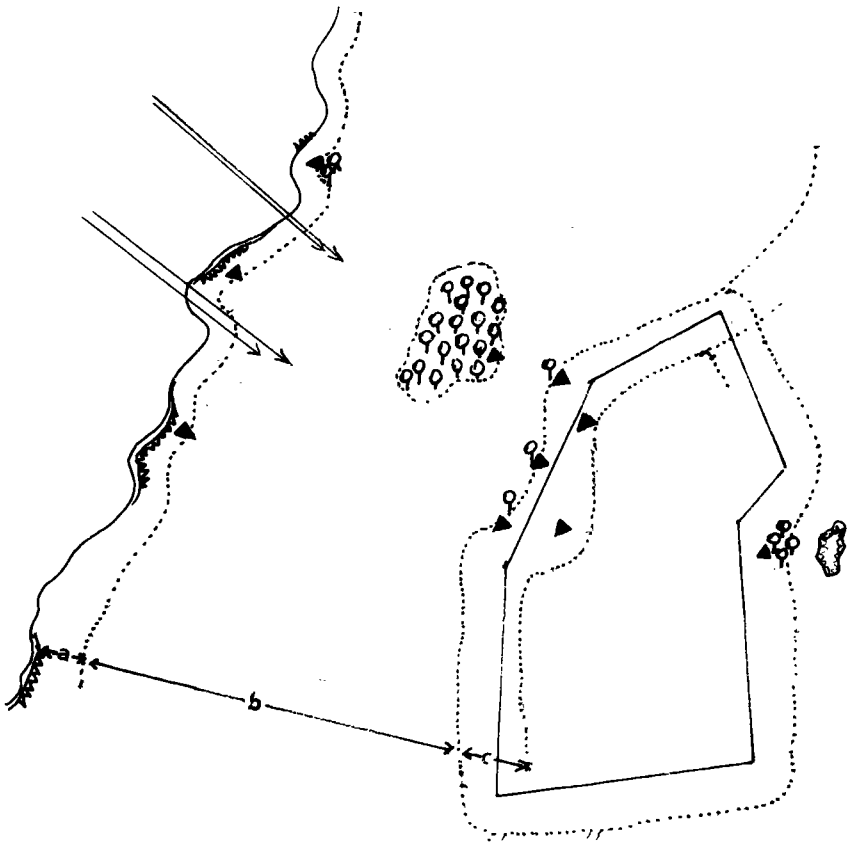


A.



B.

Fig. 25. Drop net for collecting mosquitos from grass or low vegetation.
A. Net being lowered; B. Net in place with collector inside.



= Breeding places
= Bush
= Artificial resting places
= Area of the locality
= Predominant wind direction

Fig. 26. Siting of artificial resting places.

A hand net may also be used to catch mosquitos in flight or resting on vegetation (Fig. 30).

6.4 NATURAL HARBOURAGES

The outdoor resting sites of mosquitos fall into two main groups, those actually within vegetation, and those, such as cavities in banks, under bridges, tree bases and rock hollows, in which there is some free space in the immediate vicinity of the insect and some continuity in the walls or overhang of the shelter. Most vector Anopheles fall into the second category, e.g., A. gambiae, all the A. funestus group, A. nili and a number of other species. They should be sought particularly in well-shaded earth banks, in the sides of deep gulleys, pits or river banks, under fallen logs, at the base of banana trees where these are grouped together, in open termite mounds and in shaded cavities among rocks and stones. Both A. melas and A. merus may be found in large numbers resting at the base of solitary trees and on the aerial roots of mangrove trees in situations which are often quite exposed to light but not sun. A. sergenti, A. superpictus and A. pulcherrimus are good examples of species which use caves to a considerable extent as resting places.

The group of vegetation resters includes A. albimanus, A. hyrcanus, A. sinensis, the A. coustani complex, A. pharoensis, A. squamosus, A. aquasalis, A. aconitus, A. sundaicus, A. balabacensis and A. farauti. They may be found amongst tall grasses at the base of growing rice stems, in reed beds, and in bushes very close to the ground. It should be mentioned that even domestic species such as A. maculipennis or A. sacharovi can often be found resting outside houses in natural shelters (under bridges, natural excavations, and caves, sometimes outside the village in spots where hosts are available near the natural resting places).

6.5 ARTIFICIAL SHELTERS

Artificial resting sites can be used to simulate the natural harbourages of the non-vegetation resting groups. They are designed with the object of competing with natural sites so as to cause concentrations of the mosquitos resting outside at a few, relatively accessible points (Fig. 26). Apart from the elementary procedure of hollowing out or modifying existing cavities, two types of shelter have been found to attract A. gambiae and A. funestus.

6.5.1 Box shelters

These are intended to provide relatively cool, dark shelters of a semi-permanent nature. They consist of boxes covered with a plastic sheet and then buried in the earth with the entrance partially closed with a dark cloth leaving a space below for the entry of mosquitos (Fig. 27). A useful size of box is 0.9 m high by 0.6 m wide by 0.9 m deep. The whole box is covered with a plastic sheet and then buried in a mound of earth, leaving the entrance clear. A curtain of some dark, tough, light-proof cloth is hung over the open front of the box, leaving a space of 20-30 cm below it. The cloth is fixed with rings and hooks or string at the bottom, so that it can be rolled up out of the way when the interior of the box is being examined for mosquitos.

Disadvantages

Box-shelters suffer from two disadvantages. Firstly, they are occasionally destroyed by termites, although this seems to happen less rapidly than might be expected. Secondly, they need a certain amount of routine maintenance. In particular, the earth covering them needs periodic reinforcing, especially after heavy rain. In some places goats and chickens may constantly lay bare the boxes, and it may be necessary to pile rocks and stones round them as well as earth. The main function of the earth is to insulate the box from the heat of the sun, and a partly exposed box tends to be avoided by mosquitos.

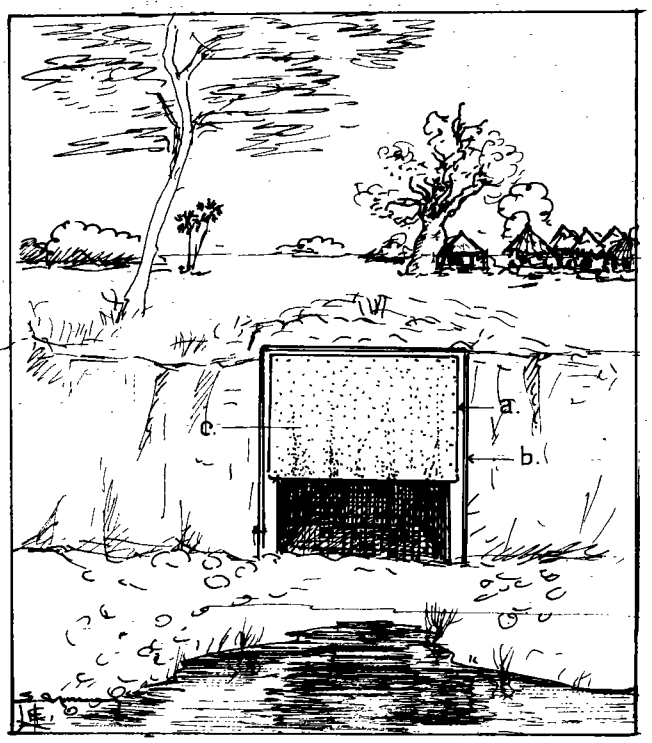
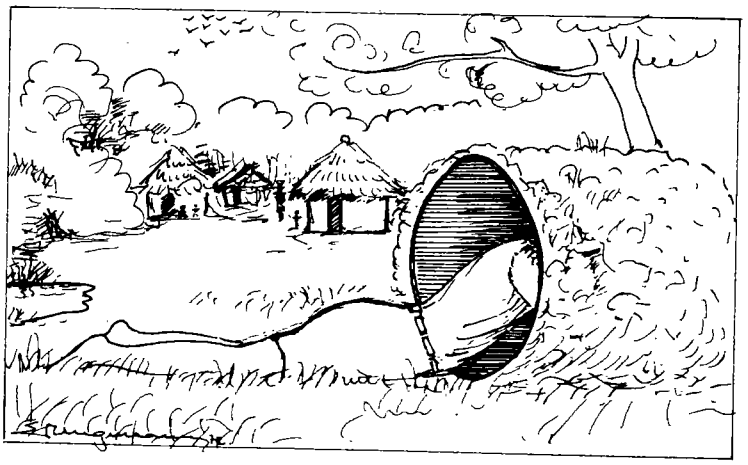


Fig. 27. Box shelter.

a = box; b = plastic cover;
c = curtain.

A.



B.

Fig. 28. Barrels as artificial resting places.

6.5.2 Barrels

Barrels or any other ready-made container, as for example large earthenware pots which are preferable to barrels during the dry season (Fig. 28 A and B) may be used in the same way as boxes, provided they are sufficiently large - not less than about 0.6 m in diameter - and are put up in shaded positions. The latter provision is particularly important if they are to be used without curtains. Metal barrels and earthenware pots may, with advantage, be buried in vertical banks or ravines, rivers or other suitable sites, like the box shelters.

6.5.3 Pit shelters

Deep pits are dug into the ground 1.5-2 m deep, 1.2-1.5 m long and 1 m wide. The site chosen should be well shaded by trees or by some form of artificial roofing. Small cavities are then hollowed out to a depth of about 0.3 m in the sides of the pit from 0.5-0.6 m above the bottom (Fig. 29 A and B). Mosquitos may be caught on the sides of the pit or inside the cavities. Pit shelters are relatively permanent and require only unskilled labour for their construction. They are also highly productive in some areas. It should be remembered that mosquitos only rest in the pit shelter if they find the same favourable conditions as those offered by the usual habitats of a given species. Therefore, pit shelters might not be useful for the collection of species which normally rest on vegetation. On the other hand, some vegetation-resters, e.g., A. aconitus, enter pit shelters readily.

Disadvantages

Pit shelters cannot be used where the water table is near the surface or the site is liable to flood, nor where the soil is sandy and friable or too humid.

It must be remembered that pit shelters are potentially dangerous and the entomologist's work may be greatly hampered by the accidental trapping of a cow, horse, or goat. Another matter to guard against is the tendency of local inhabitants to use the pit shelter as a latrine. To avoid these annoyances pit shelters should always be fenced in and their objective clearly explained. Even with these precautions it is still possible for the occasional snake to be accidentally trapped in these pits.

6.6 SELECTION OF THE TYPE OF SHELTER TO BE USED

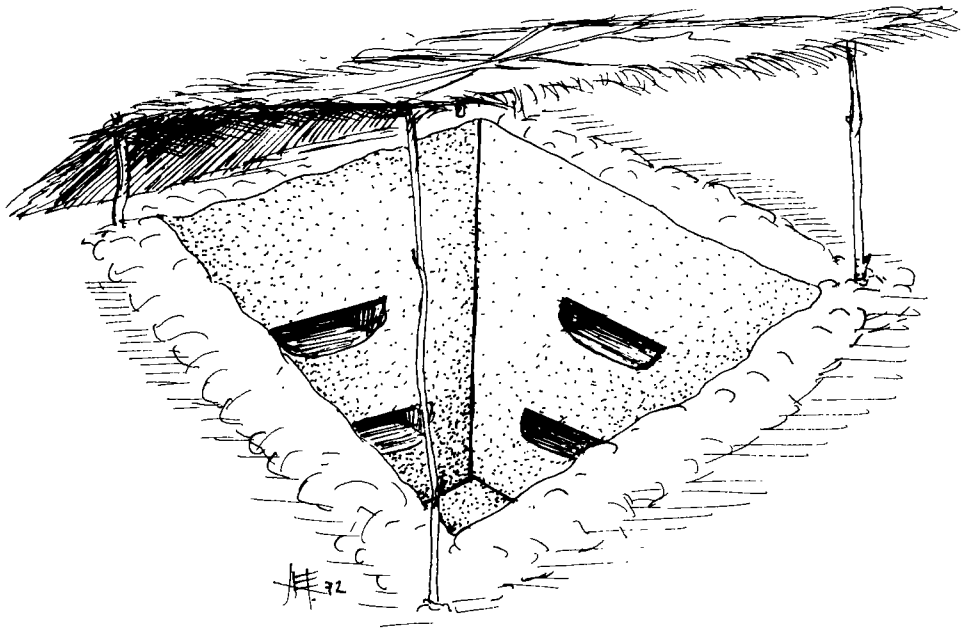
The entomologist must experiment in order to find out which is the best shelter to use in his particular area and with a given species. In some places, as for example some equatorial forests, outdoor resting vectors are very difficult to find and the best shelters appear to be small erections of leaves and sticks put up on the forest floor.

6.7 SIGNIFICANCE OF DATA COLLECTED

It is important to remember that seasonal changes in the densities in the artificial shelters may not necessarily correspond exactly with density changes in the whole population, since the availability and attraction of natural shelters may also vary seasonally. This effect is particularly marked in the northern savannas of Africa, where fires in the dry season drastically reduce the amount of shade available and the humidity, so that catches in artificial shelters are disproportionately high. It should be remembered that an outdoor artificial resting shelter represents a habitat close to the normal indoor conditions (light, humidity, etc.).

6.8 HAND NET

Collection of outdoor resting or flying mosquitos may also be carried out by use of a hand net. Details of construction and use are shown in Fig. 30.



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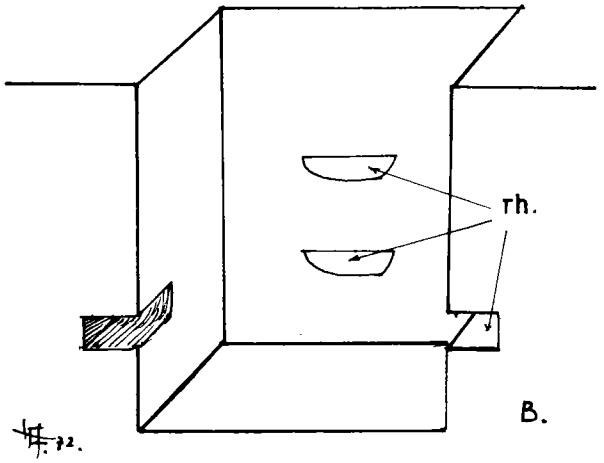


Fig. 29. Pit shelter.

rh = resting hole or niche.

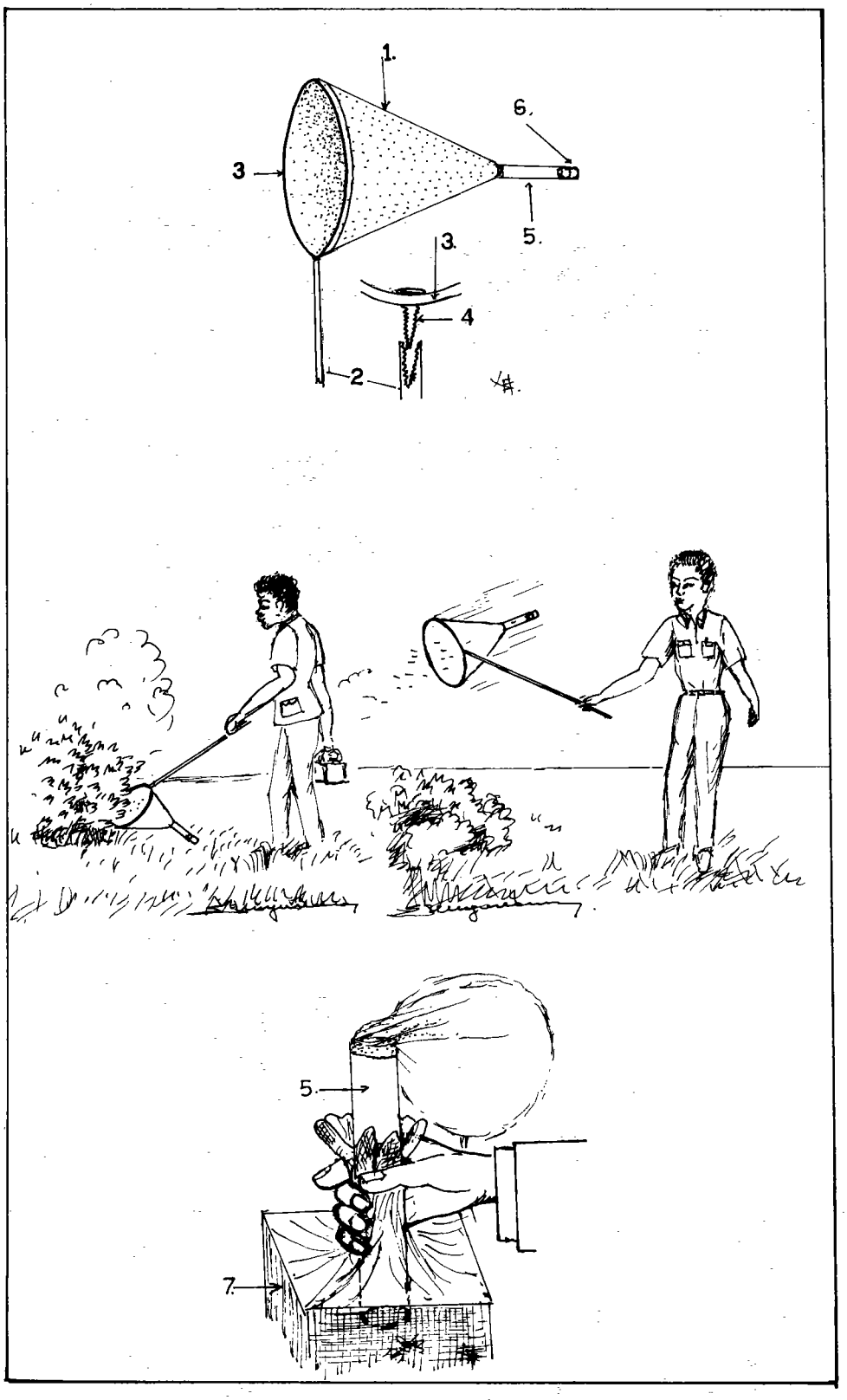


Fig. 30. Construction and use of a hand net.

1. Nylon gauze cone; 2. Handle; 3. Metal ring
4. Screw; 5. Plastic tube, open both ends
6. Cotton wool or cork stopper.

7. LARVAL SURVEYS

The first essential for making a larval survey is an adequate sketch map of the area, or at least something that shows the major topographical features, so that the approximate location and extent of permanent sources of water can be marked on it (Fig. 31). To get a good preliminary idea of an area one can hardly do better than to follow the traditional practice of the malariologist, namely to set out on foot and to make two traverses at right angles to each other across the whole extent of the district. In many tropical countries, the distribution of human settlements is partly determined by the availability of water, so the next step is the locating of domestic sources of water, such as wells, dams, and tanks. Exhaustive surveys of such sites in the later stages of an eradication scheme may be particularly important in defining residual pockets of anopheline breeding.

An experienced observer soon gets to know the usual characteristics of vector breeding sites and to learn the association between different species and different types of water - its permanence, flow and cleanliness, the nature of the substrata, the vegetation, whether emergent, trailing or floating, and the amount of high shade. However, he will also find out that there are very few types of water, with the possible exception of tree-holes, that will not at some time or other be found to harbour vectors. Consequently, when concentrated surveys are being made of a particular area, no pool or collection, however unlikely, may be left unsearched.

Surveys should be carried out at intervals throughout the year, since the results will obviously vary markedly according to the season. Many species tend to disappear almost completely during the dry season, probably because they persist in very low numbers in large or diffuse areas of permanent water. On the other hand, those that occupy small pools are sometimes more readily found at this time of year owing to the limited number of sites to be searched. When the rainy season sets in, the position is often reversed and when A. gambiae, for example, has been greatly reduced by spraying it may be almost impossible to locate its breeding sites. It should also be pointed out that larval surveys should not be carried out immediately after a period of heavy rain. The effect of this is simply to wash out the larvae from small pools and streams, while in larger collections the small numbers of insects originally present become scattered over wide areas of water and are correspondingly difficult to find. In many areas larval surveys will be most productive towards the end or just after the wet season, when most species will have built up to a peak and the limits of the breeding sites have temporarily stabilized.

Exact details of the sites from which larvae have been collected should always be recorded and a notebook carried for the purpose. A separate tube should be kept for each site, and this should be labelled appropriately. A solid block of wood with wide holes bored in it may be found useful for standing the tubes in while the actual collections are being made. In transporting larvae it is obvious that elementary precautions should be taken to prevent undue shaking or exposure to extremes of heat. When carried over long distances the principal causes of death are drowning, resulting from inability of the larvae to maintain themselves at the surface, and overheating. The first risk can be reduced if narrow tubes are used, with a diameter not greater than 2.5 cm. Overheating can be avoided by placing them in vacuum flasks or insulated in "picnic bags". It is also sometimes advisable to carry a supply of water from the breeding site in a separate container so as to have sufficient for rearing the larvae in the laboratory. Another method of transport is to strain the water with the larvae through a sieve lined with soft material, and to support this in a wide-mouthed container just above the surface of the water. The cloth is kept saturated by the water splashing up each time the container is shaken, and the larvae may survive quite long journeys under bumpy conditions. If large numbers of larvae are to be transported by this method, a satisfactory carrier can be made from a four-gallon (15-litre) kerosene can, containing a nest of perforated metal trays fitting one above the other and lined with cloth. The top tray is covered with grass and small quantities of water are poured on to it at intervals, so as to drip down through the trays and to keep them moist.

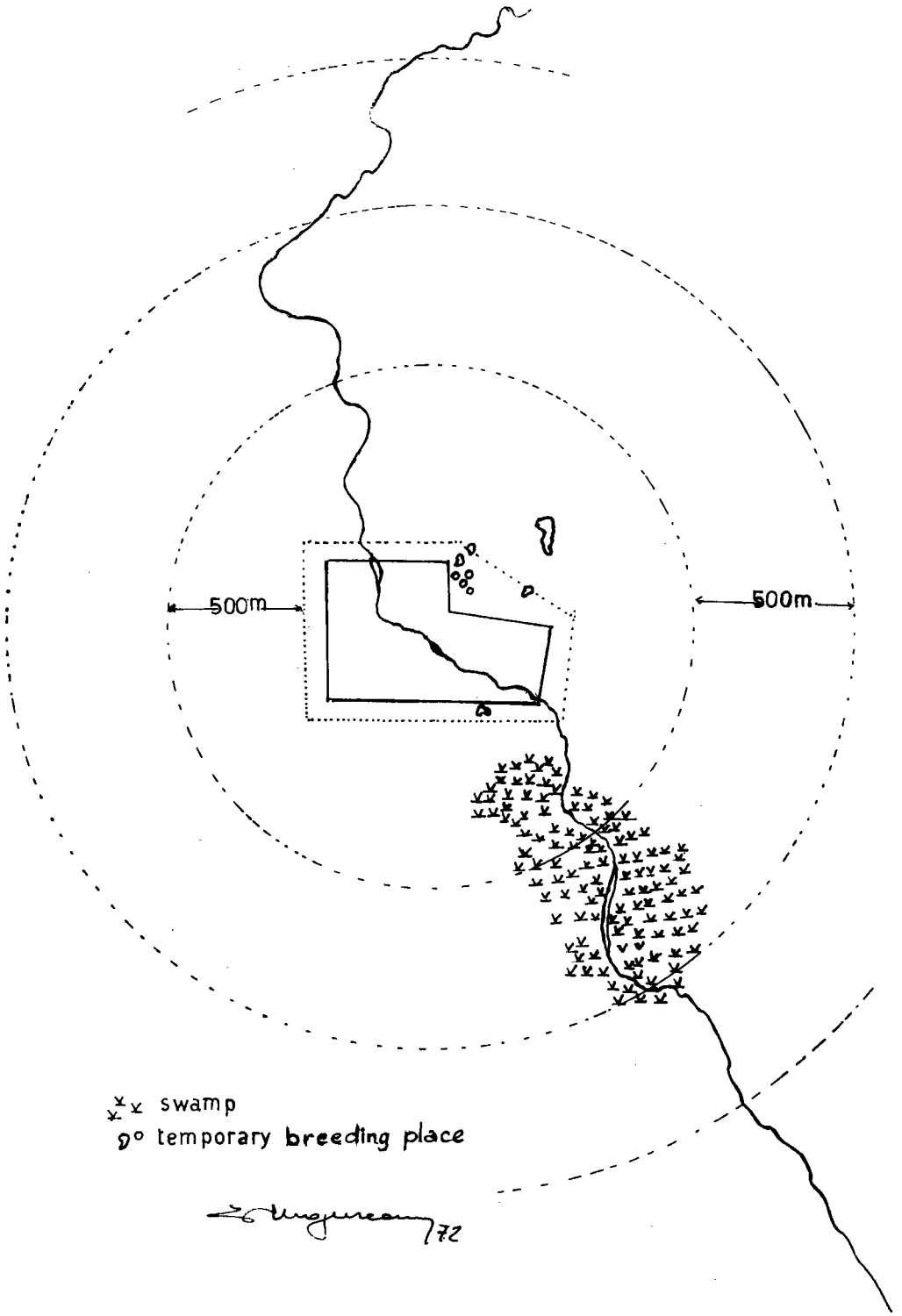


Fig. 31. Sketch map for surveying larval sites at increasing distances from a village.

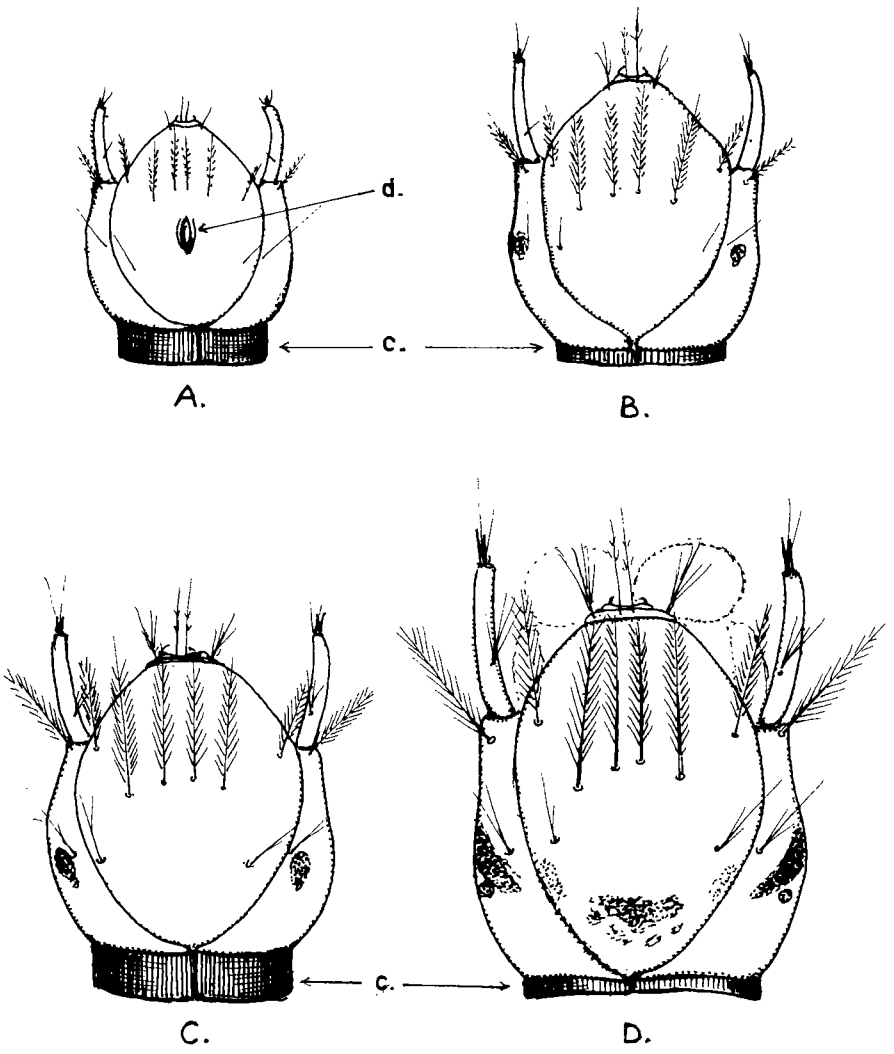


Fig. 32. Larval stages.

A. First stage; B. Second stage;
C. Third stage; D. Fourth stage
d = hatching spine (egg breaker); c = collar.

Larval instars can be recognized as shown in Fig. 32. Keys for identification of species give the diagnostic characters of the fourth instar larva.

Identification of the catch can be carried out either when the larvae are alive or after they have been killed. The former method must be used, of course, if it is desired to obtain adults. In case of doubt about the identity of larvae it is advisable to rear them singly and to preserve the pelts for study. Certain characters are also much more easily recognized in live specimens, for example, the long pleural and clypeal hairs and pigmented markings on the head. If the larvae are very active while under examination, they may be quietened temporarily by pouring a little chloroform vapour (not the liquid) into the tube before placing on the slide. Cavity slides should be used for preference. While it is sometimes possible to guess the identity of a larva from its macroscopic appearance, the tendency to examine a few specimens and to assume that the remainder from the same collection belong to the same species must be guarded against, particularly when the routine identification is delegated to assistants.

7.1 OBJECTIVES OF LARVAL COLLECTION

(a) To establish the breeding habits of different species.

(b) To establish the geographical distribution of the vectors.

(c) To establish the active breeding places.

(d) To evaluate the dynamics of development of aquatic stages of mosquitos.

(e) To evaluate the impact of antilarval measures on larval density.

(f) To evaluate the impact of adulticides on the larval density or to demonstrate the persistence or absence of a species that cannot be found as adults.

(g) To collect samples of larvae for rearing adults for taxonomic studies or biological observations (bio-assay tests, susceptibility tests, etc.).

The application of larval collection methods in time and space will depend on the objective. Investigations for fulfilling objectives (a) and (b) are carried out at the beginning of a programme during the season of vector prevalence. Once the information is complete they are not needed further. Investigations to meet objectives (c), (d) and (e) should be undertaken repeatedly throughout a malaria programme in order to collect the necessary base-line data for planning, organization, and direct evaluation of antilarval measures (see Part I, Chapter 2, section 6).

7.2 LARVAL SAMPLING PROCEDURES

Quantitative sampling of larval density with the available devices is far from accurate for the following reasons:

(a) Larvae are not distributed at random in the breeding places, but are often crowded in concentrated sites. Therefore, it is difficult to extrapolate from the data on larvae in the site sampled to all the surface area of each type of breeding place.

(b) Breeding places vary in size and shape and their surface area is apt to fluctuate with seasonal environmental changes. Therefore, it is difficult to fix a standard surface area for each type of breeding place. The amount of aquatic vegetation also varies, which increases the difficulty of reliable sampling of larval density.

(c) The behaviour of larvae in the breeding habitats varies with the species, and the collectors must be carefully trained as to what to expect with the different species existing in the area.

Therefore, sampling of larval density should aim at roughly estimating the relative density for qualitative evaluation. For this, the relative abundance of larvae of a particular species at different times of the year and in different places can be determined, provided that the methods of sampling are standardized as far as possible.

For the purpose of evaluating larval control measures, the following precautions should ensure reasonably standardized samples:

(a) Sampling should be directed to sites where there are larval concentrations in the breeding places. This can be ascertained after a wide search for larvae. Those sites having a high density should serve as fixed capture stations.

(b) Depending on the local conditions, sampling should be made with a ladle for small water collections and with a net dipper for larger collections.

From the experience gained, the number of dips should be standardized as units of 10 dips or multiples of 10 dips (i.e., 20, 30, etc.) per capture station depending on the density of the larvae.

As described above, a net dipper of standard size can cover 0.1 m^2 of water surface. This forms one dip after which the edge of the net should be slightly lifted above the surface of the water. Ten such dips (totalling 1 m^2) should be made to cover as far as possible the different aspects of each capture station.

Multiples of 10 dips may need to be made in each capture station if the density is low. This can be determined by experience during the preliminary surveys and a standardized unit of 10 dips or multiples of 10 should then be maintained at each capture station for regular or spot-check observations.

7.3 LARVAL COLLECTING METHODS

Three methods of collection are in common use:

- Dipping
- Netting
- Pipetting

Their respective value depends on the way in which each method is applied in different types of breeding place, the last method being of much more limited use than the first two. To some extent the behaviour of the species to be investigated has to be taken into account while carrying out larval collections. Those carrying out collections should be well-trained and enthusiastic. A degree of standardization is necessary and the same method and instruments of collection should be applied when carrying out longitudinal studies.

7.3.1 Dipping

The collecting device will depend upon the type and size of breeding places to be investigated:

- a white enamel bowl may be used to collect larvae from the edge of swamps, ditches and streams, rice fields or other large bodies of water;

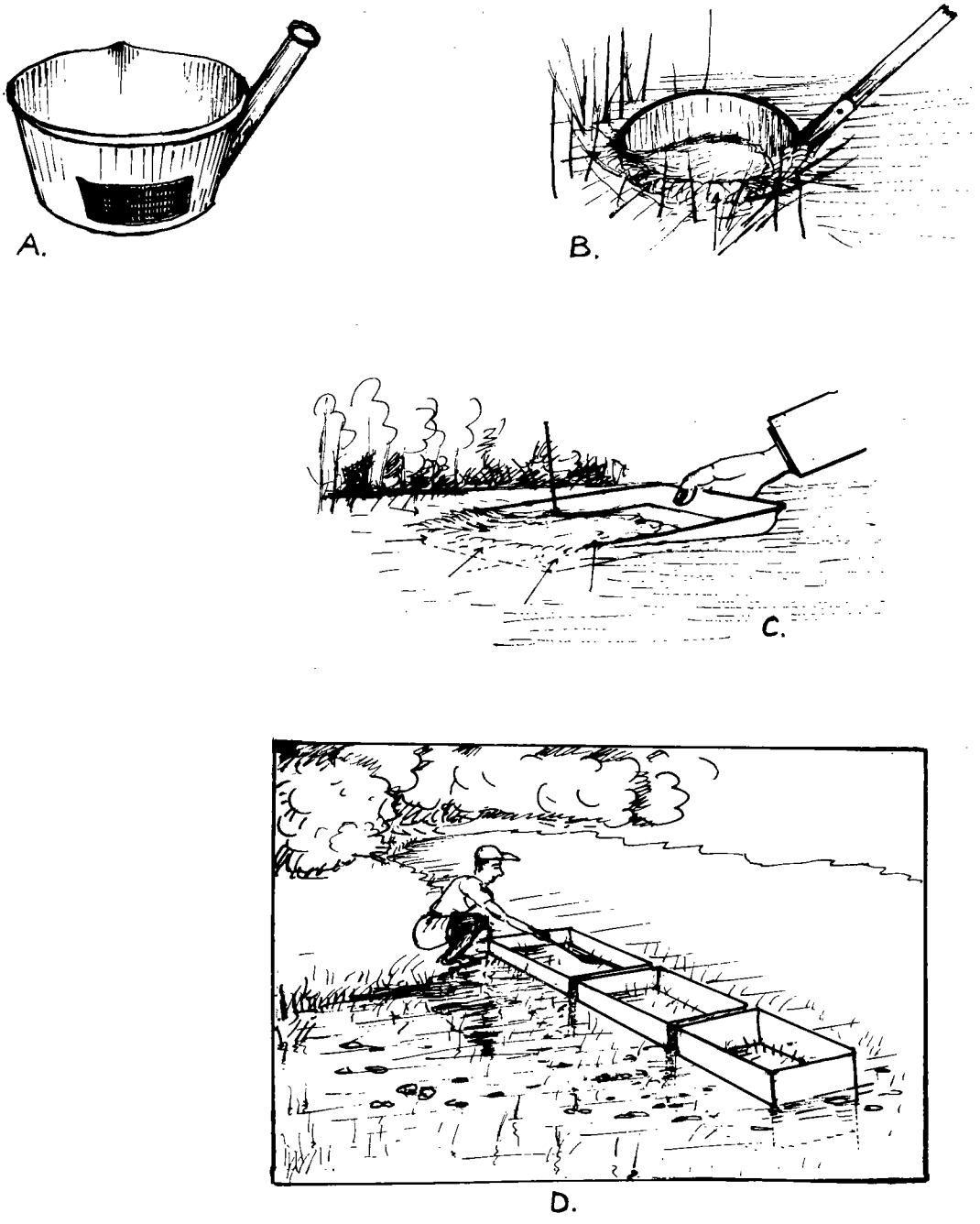


Fig. 33. Methods of larval sampling.

A. Dipper made from frying pan; B. Use of dipper
C. Use of photographic developing tray; D. Quadrats for larval density sampling.

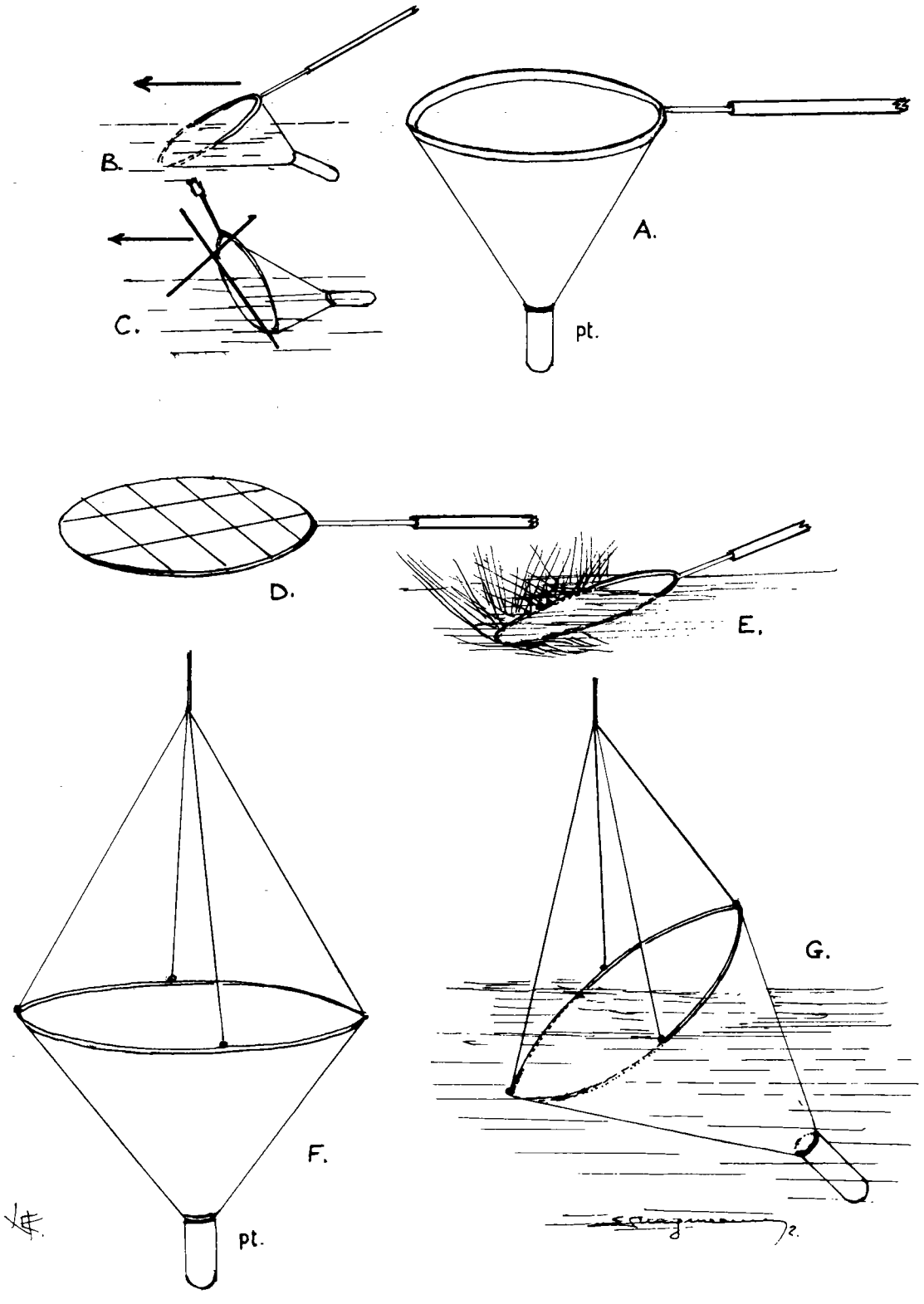


Fig. 34. Larval collecting methods.

A. Larval net; B. Correct method of use;
C. Incorrect method; D. Palette;
E. Use of palette; F, G. Well net;
pt = plastic tube.

- a rectangular or round frying pan, about 25 cm in diameter, with a long handle, is useful for collecting larvae from more inaccessible parts of the above-mentioned breeding places;

- a round palette made of a wire ring, 25 cm in diameter, to which 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; when investigating very small breeding places, such as hoof-prints the diameter can be reduced to 5-10 cm;

- ladles with a diameter of 5-10 cm should be used only for collecting larvae from very small breeding places and from tree holes.

The dipping method is the most frequently used for collection of mosquito larvae. The collecting instrument (enamel bowl, frying pan, ladle) should be immersed in the breeding places at an angle of 45° (Fig. 33 A, B and C). The surface water will flow into the cavity but care should be taken not to fill this completely as otherwise some larvae will be washed out. The palette should be used as shown in Fig. 34 E, and immersed with a quick movement. Larvae can be collected from a surface area equal to the size of the dipping instrument or from a larger surface area by moving the palette around a surface area 4-5 times that of the collecting instrument. If the dipper is immersed too slowly the larvae are disturbed and go to the bottom with the result that many escape collection. Often even the shadow of the hand of the collector approaching will disturb the larvae. Therefore, the site should be approached carefully. There should be an interval of 2-3 minutes between each dip to allow Stage III and IV larvae and pupae to return to the surface. When the water is cool (20°C or less) larvae may remain deep within the breeding place for several minutes and not many will be collected through repeated dipping at short intervals. The larvae of A. wellcomei climb up the stems of reeds and emerging vegetation and keep themselves slightly above the water surface. In this instance the water should be agitated or the dipper inserted in such a way that the vegetation is submerged as shown in Fig. 35 A. Some A. funestus larvae live under the vegetation and may remain there for long periods thus escaping collection. The colder the water, the longer will the larvae remain under the surface. When the surface of the water is covered with dense floating vegetation or organic debris, the water surface should be agitated to cause the larvae to sink; clear away the vegetation and then wait for 3-5 minutes for larvae to come to the surface once more.

After the removal of the sampling devices the water is examined carefully and the larvae coming to the surface are collected by a pipette or with a small nylon palette 2-3 cm in diameter. The larvae are transferred into a large bottle half full of water from the breeding place.

7.3.2 Netting

Using this method, the larvae are collected by sweeping the surface layer of the water with a net.

Larvae may be collected from large stretches of water along the edge of streams, wells, and other situations. A pond net can be constructed very simply using a ring of iron wire, 20-25 cm in diameter, to which a nylon bag is attached (do not use cotton material). 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, 3-1/2 cm x 10 cm, is attached. A long cane or bamboo handle is attached to the ring. During collection the larvae are washed into the tube which can then be emptied either directly into a bottle or into a white enamel dish. When used without the cylinder, the bag is washed in a white enamel bowl with clean water after each dipping. When collecting larvae, the net is held at an angle and skimmed rapidly through the surface water near emerging or floating vegetation or pushed along very slowly allowing the surface water to float into the net (this technique is not recommended for collecting A. funestus larvae). Alternatively, the net may be used as a ladle, a series of quick dips being made. The net is inverted and washed out in a bowl of water and the larvae

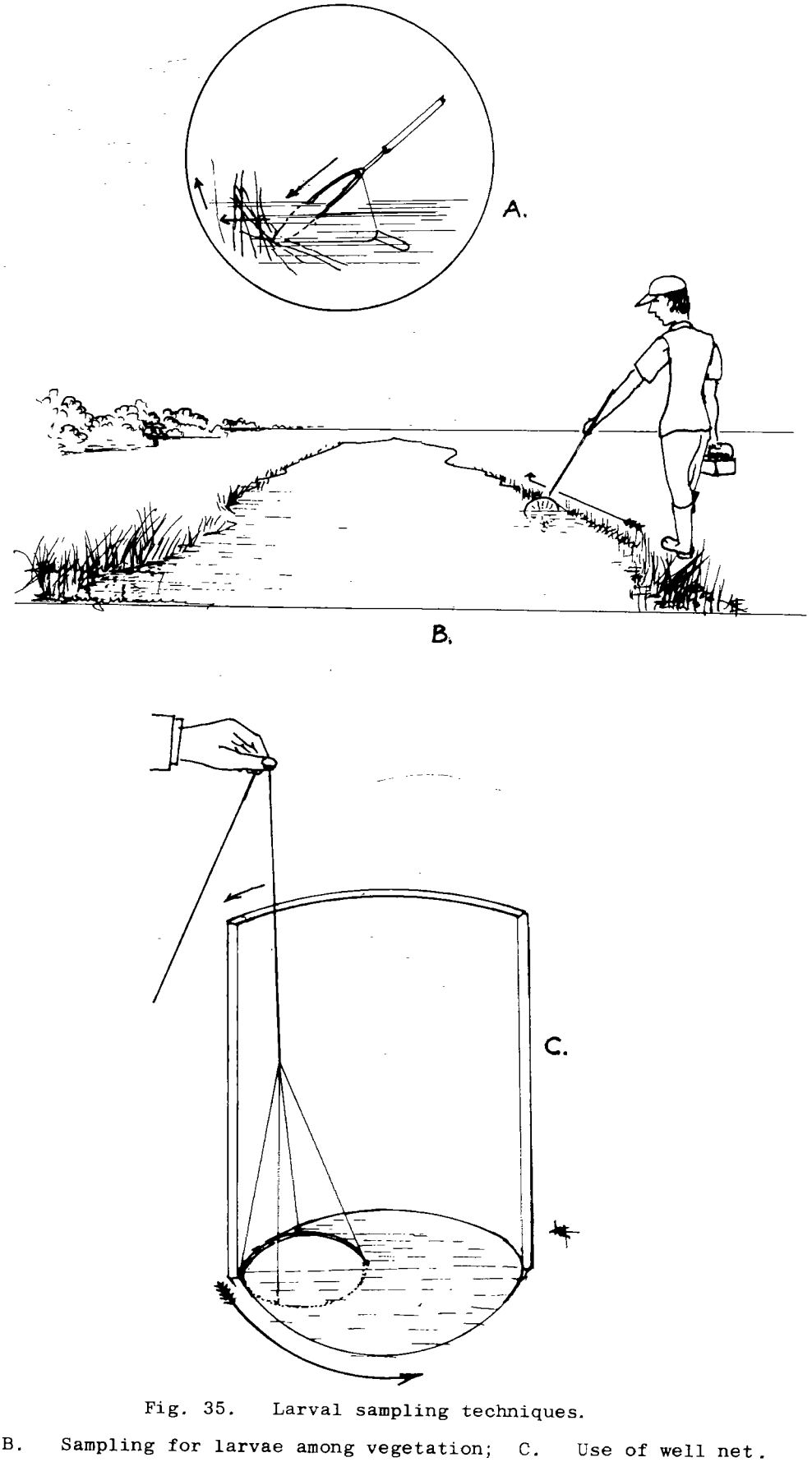


Fig. 35. Larval sampling techniques.

A, B. Sampling for larvae among vegetation; C. Use of well net.

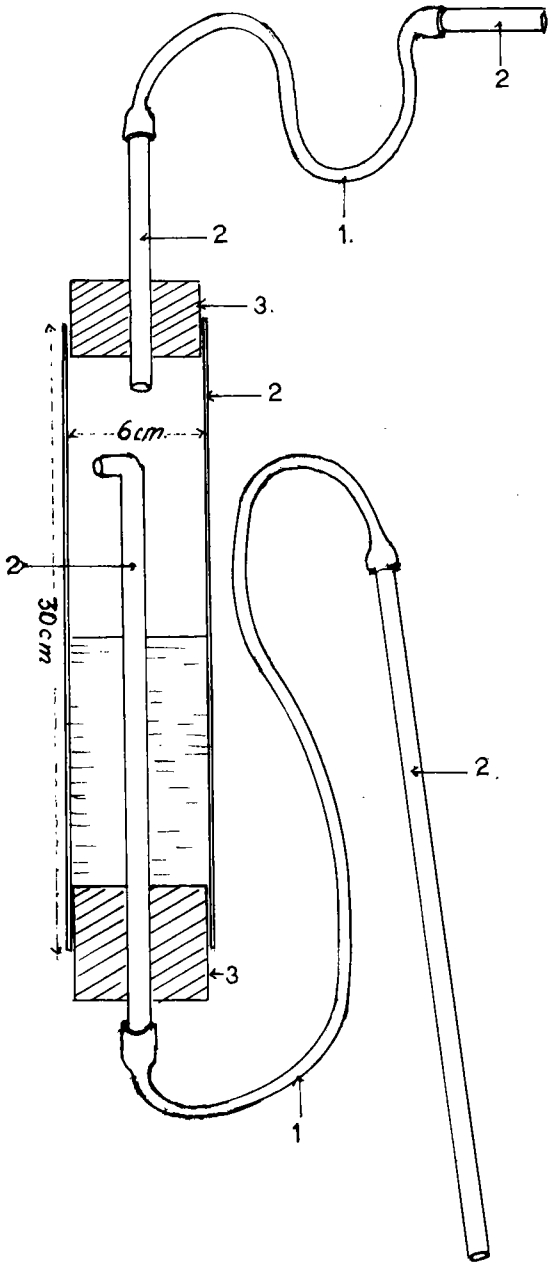


Fig. 36. Siphon for sampling in tree holes.

1. Rubber tube; 2. Glass or plastic tubing;
3. Cork or rubber stopper.

collected with a pipette or small palette. The use of nets permits the sampling of large areas of water in a relatively short time. The long handle facilitates collection from under high banks and other inaccessible situations. The use of pond nets is very practical and should be employed mainly in areas with larviciding programmes where the detection of positive breeding places is of great importance.

7.3.2.1 Collection of larvae from wells

Collections can be made from shallow, disused wells not more than 1-1½ m deep, with a long ladle or the pond net described above. A special net is used for deeper wells.

How to make a well net

Make a conical bag with the nylon netting; insert an iron ring in an inclined position of about 30° from the upper border of the bag (Fig. 34 G). Fix the nylon around the ring, cutting off the excess material, and reinforce the upper border of the bag around the iron ring. Attach the nylon string to four points on the ring at equal distances (Fig. 34 F). Join the four pieces of string in such a way that the ring is at an angle of about 30° and attach this to the rope, then fit a plastic tube of about 4 cm diameter and 10-12 cm long to the bottom of the net. Fix a piece of lead or a small stone weighing about 50 g to keep the bottom of the net under the water surface.

Use of the well net

The net is dipped slowly into the well keeping half the border above the water (Fig. 35 C). After waiting 2-3 minutes to allow the disturbed larvae to return to the water surface the net is dragged slowly and as quietly as possible around the edge of the well keeping the net at the initial depth. Dexterity in using the well net is acquired with practice. When the net has been moved around the border of the well two or three times it is withdrawn and inverted in a white enamel basin containing water. Wait for 2-3 minutes then repeat. The larvae are collected with a pipette or a small palette.

7.3.3 Pipetting

Small pipettes (glass tubes with a rubber bulb) may be used for collecting larvae from the surface of breeding places, etc.

7.4 COLLECTION OF LARVAE FROM TREE HOLES AND/OR AXILS OF LEAVES

Objective: Survey of tree-hole mosquito fauna in an area (very few malaria vectors are known to breed in such habitats).

Method

A ladle or small net can be used for quantitative studies of large, deep tree holes, or the water can be siphoned off with a piece of rubber tubing depending on the diameter of the tree holes. The holes may be washed two or three times with extra water. Wide pipettes or a special siphon (Fig. 36) can be used to collect larvae in small, narrow holes or from the axils of leaves. Dry tree holes can be investigated for eggs, either by scraping the deposits from the bottom or by filling them with water, agitating with a stick for 10-15 minutes and then siphoning the contents. Eggs of tree hole Culicidae can be identified as such or incubated in a laboratory to obtain the hatching of larvae. Bromeliads are carefully removed from the tree, inverted and rinsed out over a basin.

7.5 TRANSPORT OF LARVAE AND PUPAE

It is often necessary to move the larvae alive over long distances from the field to the laboratory. Larvae are placed in large bottles with corks through which a piece of glass or plastic tube is inserted to ensure aeration (Fig. 37), or else covered with nylon gauze. Some small pieces of vegetation may be left on the surface of the water. Care should be taken to ensure that none of the larvae's natural predators, e.g., dragon flies, water beetles, small fish, etc., are placed with them. Direct sunlight and excessive shaking in trucks or cross-country vehicles should be avoided. A cushioning effect may be achieved by placing the jars in a large plastic bucket half full of water (Fig. 37). Larvae may also be transported on damp trays.

7.6 PRESERVATION OF LARVAE AND PUPAE IN THE FIELD

Larvae can be preserved in fixative solutions. The ones most often employed are:

Formula A

Alcohol 70%	95 ml
Glycerol	5 ml

Formula B

Water	94 ml
Formalin (neutralized)	6 ml
Borax	0.5 g

Mosquito larvae can be mounted on slides in permanent preparations (see Fig. 44).

7.7 STUDY OF LARVAL DENSITY (QUANTITATIVE STUDY)

Estimation of the density of larvae in breeding sites is relative and is only valid if the same procedure and collecting device are used each time in the same type of breeding place. Seasonal variation in density may be very marked and this must be taken into consideration. The methods of collection described above cannot give the true picture, especially in the case of very large breeding places. If carefully carried out under strictly standardized conditions in selected breeding sites, they may give an approximate idea of relative changes in the numbers of larvae. Estimates of rather greater accuracy can be made by several methods.

In one method known areas of the breeding site are closed off by means of rectangular frames (quadrats) and the larvae inside them counted. It is customary to make the sampling area of some convenient size, such as 0.1 m^2 and to distribute the samples across the whole area of the breeding site in a semi-random fashion. An enclosure of 1 m^2 made of nylon gauze and thin poles of iron or wood can be constructed and used for the delimitation of the surface area of breeding places when a more accurate estimate of larval density is needed (Fig. 33 D). A tin frame of 0.5 m^2 may be used (such as an empty four-gallon (15-litre) kerosene can) which is pushed firmly into the mud, and the larvae collected from within it by dipping. The method is, of course, only suitable for shallow breeding sites of moderate size in which there are no rigid emergent plants or obstructions to interfere with the insertion of the frames. Theoretically this method can be used to estimate with considerable accuracy the total larval population of small and shallow breeding places. But in practice, this can seldom be done with any degree of confidence unless observations are undertaken to calculate the statistical errors of the sampling technique.

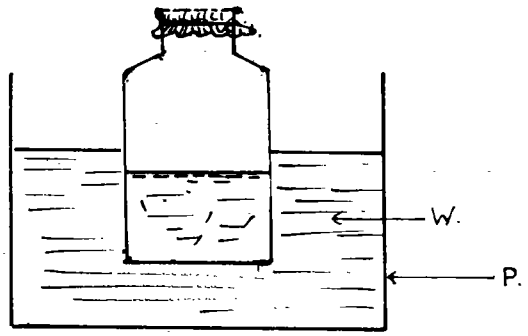


Fig. 37. Transport of larvae and pupae.

W = water; P = plastic bucket.

An alternative method, applicable only for special studies, is to evacuate all the water from a small breeding site and to pass it through a graduated series of sieves, from which the larvae are collected and counted. The water is removed with a hand-operated pump or bucket. The sieves are taken out at intervals, inverted and washed out into enamel bowls for collection of the larvae. When no more water can be removed from the pool it is washed down with 10-15 litres of water, which is then baled out and passed through the sieves again. If two such washing processes are carried out, almost complete counts can be made of the whole macroscopic fauna of the pool. It goes without saying that this method is limited to very small breeding sites, such as water-holes or borrow-pits containing not more than about 100-200 litres of water. It is particularly suited to the experimental study of A. gambiae breeding sites, since the water and the larvae can be returned after counting, with or without other elements of the fauna, so as to evaluate the role of predators as controlling agents.

Another possible method is the introduction into the breeding site of known numbers of larvae labelled with radioisotopes and the observation of the ratio of marked to unmarked larvae in subsequent samples. Provided the larvae are introduced in such a way as to ensure their rapid mingling with the whole natural population of the pool, and provided the loss of labelled larvae from predators or other causes is negligible during the interval between introduction and recapture, the total population may be estimated from the following formula:

$$\frac{\text{Total population}}{\text{Total numbers marked and introduced}} = \frac{\text{Numbers unmarked in sample}}{\text{Numbers marked in sample}}$$

The total production of adult mosquitos from small breeding sites may be estimated by erecting nets or screened cages over known areas of the pool, in the same way as for sampling larvae, and by making daily counts of new emergences in the nets. As with any of the sampling methods described, the accuracy of the estimate is dependent on proper randomization of the traps and on the possibilities of appropriate statistical analysis of the results.

7.8 METHOD FOR INVESTIGATING THE PRODUCTIVITY OF BREEDING PLACES OF ADULT MOSQUITOS UNDER FIELD CONDITIONS

Objectives

(a) To study the daily productivity of breeding places per unit of surface.

(b) To study the rhythm of emergence of mosquitos.

(c) To study under field conditions the number of adult mosquitos produced by a known number of eggs.

For the study of the first two objectives, the following steps should be followed:

- delimit a breeding place with high larval density;

- cover the breeding place or part of it with a demountable cage;

- collect the mosquitos at the desired interval for several days.

For study of objective (c), the breeding place or part of it is covered and left like this until no more adults are produced for at least three days. Then introduce into the breeding place a known number of eggs, and collect the adults when they start to appear until no more are produced.

Method

The cage is shown in Fig. 38. It is made of rectangular or square wooden frames covered with nylon mosquito netting. The frames can be assembled easily in the field and can cover up to 2-3 m^2 of breeding place.

The cage is fixed to the ground by attaching it to four poles. Small breeding places can also be covered by using a cage of appropriate dimensions. The big cage has a round hole of about 50 cm diameter, closed with a sleeve similar to those used for small mosquito cages, through which the collector enters. The small cages are provided on two walls with sleeves through which the mosquitos are collected.

In breeding places where the bottom is irregular the space between the border of the cage and the bottom is covered with nylon mosquito netting in order to prevent the escape of larvae and existing natural predators (Fig. 38 A). When the bottom of the breeding place is very deep there is no need to close the space below the base of the cage for a greater depth than 100 cm.

The mosquitos are collected at different intervals during the night, e.g., two hours after sunset, in the middle of the night, two hours before dawn, at 8 o'clock in the morning, in the middle of the day, or at any other desired intervals.

The method should be used only for special studies of short duration, during different seasons.

8. PRESERVATION OF MATERIAL

Specimens may be preserved in the dry state after pinning, or they may be mounted on slides or otherwise kept in a preservative fluid.

8.1 DRY PRESERVATION OF ADULT MOSQUITOS

Preservation of adult mosquitos includes three steps: killing, pinning, and mounting in boxes.

8.1.1 Killing

There are several simple devices for killing mosquitos, the main types being the killing tube and the killing bottle.

Killing tube (Fig. 2). This can be prepared easily by the field worker. It consists of a test tube about 16-18 cm long and 1.5-2 cm in diameter. Small pieces of rubber cut from a tube should be placed at the bottom of the test tube so that it fills about one-fifth of it. One or two layers of filter paper should be placed on top of the rubber, chloroform should then be added to soak the rubber. After a few minutes the excess chloroform should be poured off and the tube closed with a rubber stopper. The tube should always be kept closed when not in use.

Killing bottle. Special bottles for killing insects may have different dimensions. A killing bottle of moderate size might have the following characteristics: 7 cm height, 5-6 cm diameter with a tight-fitting screw top. At the bottom of the bottle is placed a layer of about 1-2 cm of plaster of Paris mixed with potassium cyanide - this emanates HCN vapour which is very toxic. The plaster of Paris is covered with a disc of cotton wool and then with filter paper. The bottle should be kept covered when not in use and care should be taken to avoid inhaling the vapour. The mixture of plaster of Paris/potassium cyanide can be replaced by pieces of rubber soaked in chloroform, carbon tetrachloride, or ammonia, as used in the killing tubes described above. The advantage of using potassium cyanide is that the mosquitos do not become as rigid as when they are killed by other chemicals. In the field, in the absence of any ready-made killing devices, the mosquitos can be easily killed by tobacco smoke.

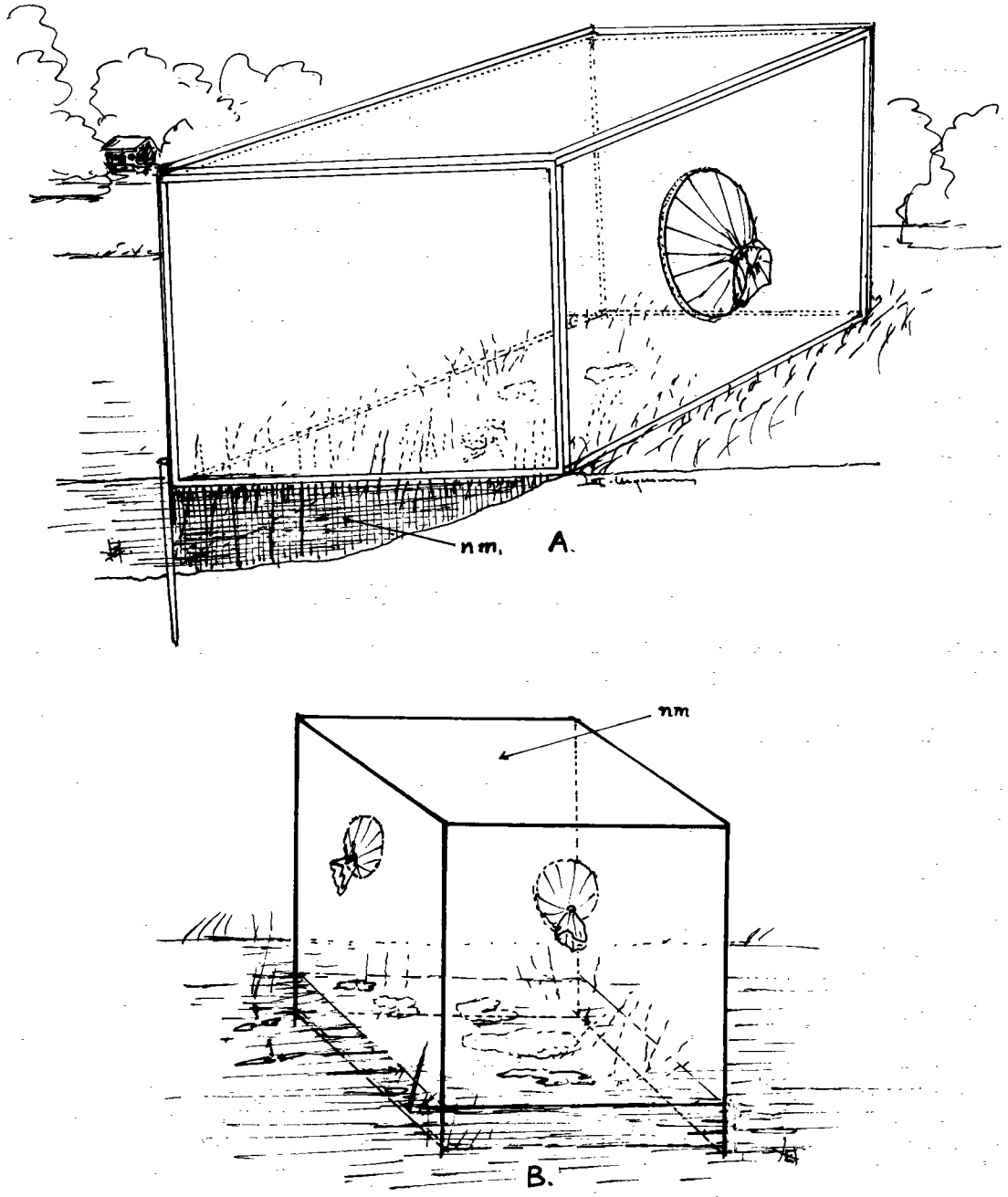


Fig. 38. Cages for investigating productivity of breeding sites.
mn = nylon mosquito netting.

Temporary storage of killed insects

Killed insects can be kept in a dry state for morphological identification in a cardboard box with layers of cotton wool wadding, with the date and place of collection written on a label. A fumigant insecticide (HCH, dichlorvos, camphor, or naphthalene) is added as preservative. When keeping killed insects in a dry state for dissection they should be stored in a small plastic or glass tube filled at the bottom with calcium chloride and then well closed in order to eliminate the humidity. Mosquitos kept in storage temporarily can be prepared for permanent storage using the same methods as for freshly collected mosquitos.

Recently killed mosquitos can be kept in a refrigerator at 0°C for dissection after 2-5 days, or frozen at -10°C to -18°C or lower for dissection after longer intervals (up to several months).

8.1.2 Pinning

Pinned mosquitos can easily be examined for external morphological characteristics. The mosquitos to be pinned may be:

(a) recently killed mosquitos which can be pinned without any other preparation; or

(b) dry mosquitos which have to be softened in a humid chamber before pinning.

Materials

- Large stainless pins 35-44 mm
- Small pins ("Minuten" pins)
- Narrow strips of cork or Polyporus
- Small labels of about 25 mm x 5-8 mm
- Boxes for storage of insects (special entomological boxes, cigar or biscuit boxes, etc.) which are large enough to hold pins, or glass tubes 25 mm x 100 mm as used in the laboratory for keeping gravid mosquito females for egg laying.
- One of the following substances to avoid damage produced by pests or fungi: naphthalene, creosote, or paradichlorbenzene.

Procedure (Fig. 39, stages 1-10)

(a) Place the killed mosquito on its back on a white sheet of paper (stage 1).

(b) Take a piece of cork or Polyporus 2 cm in length and pierce it with a small pin (stage 2).

(c) Insert this pin in the central part of the thorax of the mosquito in between the coxae (stage 3).

(d) Place the extremity of the strip of cork or Polyporus on a support of expanded polystyrene (stage 4).

(e) Pierce the strip with a large pin and arrange the strip of cork with the fixed mosquito in such a way that the thorax is about 1 cm below the head of the pin (stage 4).

(f) Write the date and place of collection on the label and pierce it with the large pin. On a second label write the name of the species and the person who identified it (stage 5).

(g) Fix the mosquito in the storage box.

(h) Place an appropriate quantity of one of the above-mentioned preservatives inside the box to protect the mosquitos against pests.

8.1.3 Remarks

Mosquitos can be pinned on a piece of cork covered with a white piece of paper (Fig. 39, stages 7-10). They can be kept individually in boxes or several mosquitos can be pinned on the same piece of cork and kept in a glass tube (Fig. 39, stage 11).

8.1.4 Setting of wings and legs

The mosquitos are pinned on a piece of cork or board covered with white paper. The wings can be arranged so that the dorsal part of the abdomen is exposed and the legs can be arranged more or less in their normal position. Sometimes it is necessary to fix the wings in the desired position for a few hours by using small entomological pins which are then withdrawn after the insect becomes dry (in general the next morning).

8.1.5 Method for softening dry mosquitos for pinning

Pinning of dry mosquitos is done in the same way but, as mentioned previously, it is necessary to relax (soften) them before pinning (see also Ungureanu, 1972). The relaxing is easily done by using a humid vessel which can be prepared easily by using plastic or plasticised paper cups or Petri dishes. One of the most practical is a double-bottomed device (Fig. 40 A) using two cups. At the bottom of the first cup a layer of cotton wool, about 1 cm thick, is placed and soaked with water and then a piece of camphor or dichlorbenzene is added in order to avoid the formation of fungus. The bottom of the second cup is perforated with holes of about 2 mm diameter and then inserted into the other one. Mosquitos are placed directly in the cup with the perforated bottom and then the cup is covered with a piece of plastic or Petri dish, etc. Dry mosquitos should be kept about 24 hours before pinning. They can be kept without any damage for several days owing to the presence of the preservatives.

If no cups are available, a humid chamber can be made using a Petri dish. The inside of the upper part is covered with a pad of cotton wool 2-3 mm thick covered on both sides with filter paper. The mosquitos are put on the lower part of the Petri dish out of contact with the wet part (Fig. 40 B) on a dry filter paper.

Another method of preparing a humid chamber is to use a Petri dish or any other recipient which can be well covered, in which a layer of sand 2-3 cm deep is placed and dampened with water (Fig. 40 C). This sand is covered with filter paper and the insects are placed on it. In order to prevent the mosquitos from getting wet, a circle of metal mosquito netting is placed about 1 cm from the surface of the humid sand and then the mosquitos are placed on it.

8.2 Preservation of mosquito larvae, pupae, and adults in mounting media

For the detailed microscopic examination of external or internal structures, mosquitos may be preserved on slides in mounting media, of which the following are recommended: chloralhydrate medium gelatine, glycerol, lactophenol, euparal, Apathy syrup, polyvinyl alcohol, Canada balsam. When mounting parts of a mosquito, or the entire mosquito if necessary, the specimen is first cleared. There are several methods for clearing specimens. One involves the use of a 10% solution of potassium or sodium hydroxide. Two procedures can be used for softening and clarifying the insects:

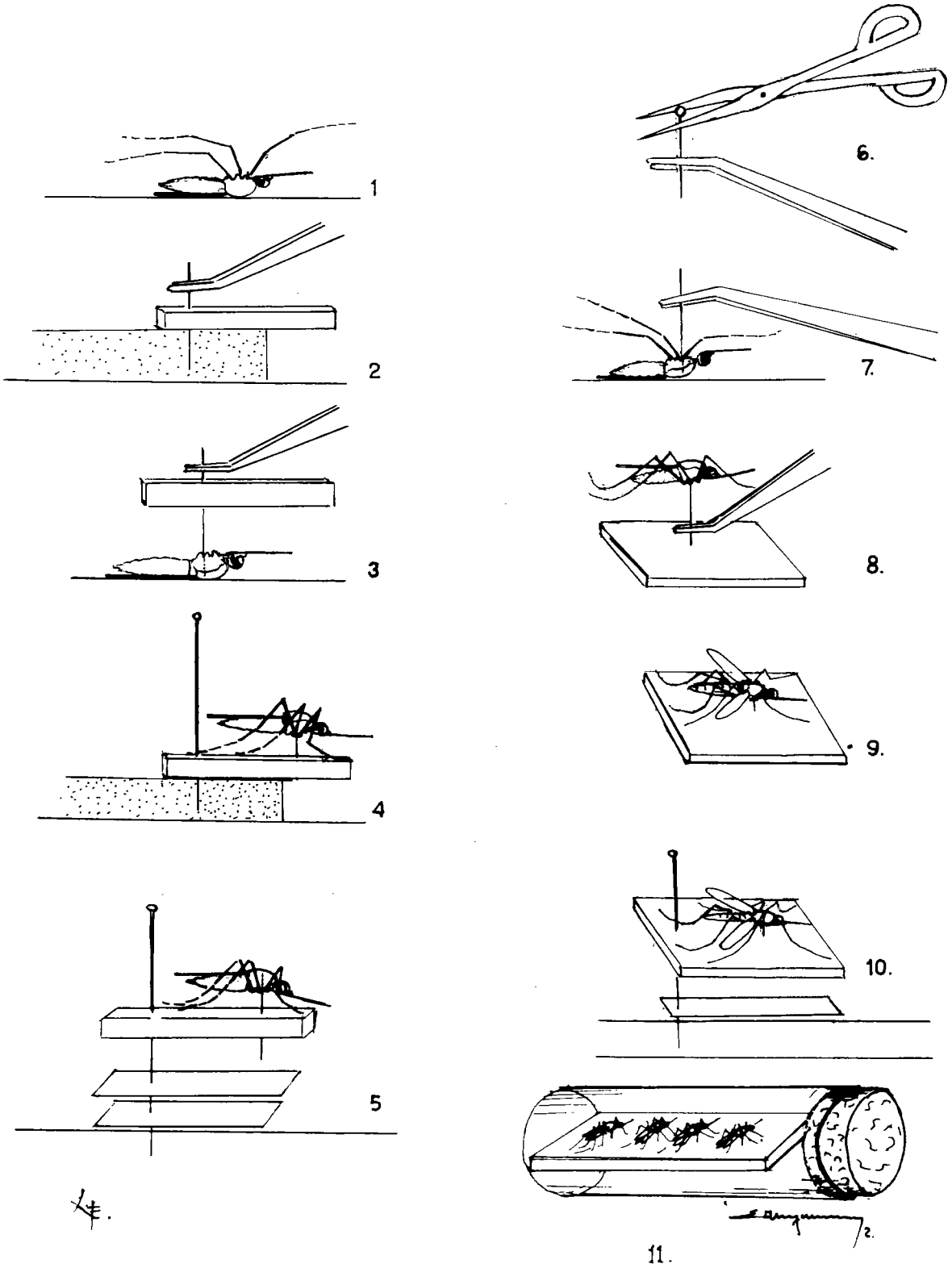


Fig. 39. Pinning methods.

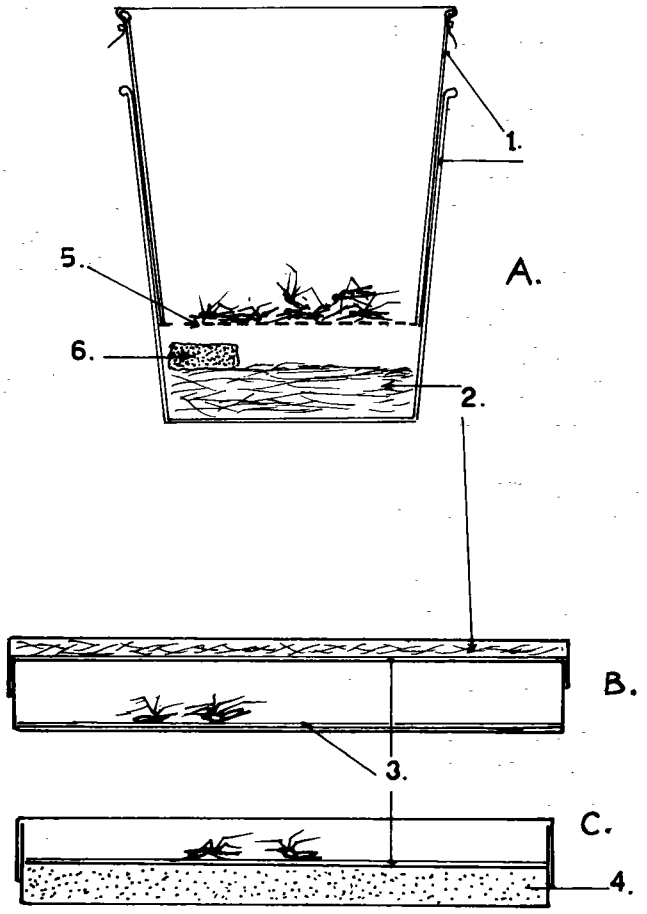


Fig. 40. Relaxing chambers.

1. Plastic cups. 2. Moist cotton wool.
3. Filter paper. 4. Moist sand.
5. Perforations. 6. Camphor or dichlorbenzene.

(a) slow procedure: keeping the insects for 24 hours in the above mentioned solution;

(b) fast method: consisting of the following steps:

- put about 5 ml of 10% sodium or potassium hydroxide in a test tube, add mosquitos
- heat for 5-10 minutes according to the degree of depigmentation required
- wash with tap water.

When mounting the mosquitos in a mounting medium with water there is no need for dehydration. Larvae if mounted in euparal have to be dehydrated with methanol or if mounted in Canada balsam a more complicated procedure of dehydration is necessary with alcohol, and toluene or xylol.

Important note: Before starting the process of mounting larvae from fixative solutions, the larvae should be washed in water in order to eliminate the fixative. This step is not necessary if the larvae are mounted in the same solution as the fixative.

The formulae of a few mounting media are given which can easily be prepared by field workers:

8.2.1 Gum arabic/chloral hydrate

Distilled water	35 ml
Gum arabic	35 g
Chloral hydrate	20 g
Glycerol	10 ml

Dissolve the gum arabic first (after placing the gum in a nylon gauze bag) and then add the other ingredients. In this medium, larvae can be mounted directly from water.

8.2.2 Levulose syrup

Levulose	50 g
Distilled water	50 ml

Mix well and leave for about 24 hours at a constant temperature of 56°C in order to obtain a thick syrup. Small pieces can be fixed in this syrup directly from water. After 24 hours it is necessary to seal the coverslip with a thin lacquer or special bitumen.

8.2.3 Gelatine/glycerine medium

Gelatine	10 g
Distilled water	50 ml
Glycerol	40 ml
Phenol	0.5 g

(a) Put the gelatine in the distilled water for at least two hours so that it absorbs as much water as possible.

(b) Add the glycerol: heat gently over a water bath and mix well till the gelatine is perfectly melted.

(c) Add the phenol, mix well.

(d) Filter on glass or cotton wool. Keep the medium in a bottle with a large mouth.

8.2.4 Process of mounting in gelatine/glycerine media (Fig. 41)

(a) Place the fixed object in glycerol 50%.

(b) Transfer the object on to the slide.

(c) Melt the medium in a water bath.

(d) Put a drop of medium on the slide on the top of the specimen to be mounted.

(e) With a warm needle arrange the specimen in the desired position.

(f) Leave to solidify.

(g) Place a coverslip on the top of the solidified jelly after moistening the coverslip slightly by breathing or by smearing with glycerine.

(h) Heat a small weight or hammer and apply gently to the coverslip until the jelly melts and spreads under the coverslip, or heat slide directly.

(i) With a razor blade remove the excess solidified mountant.

(j) Varnish around the edge of the coverslip.

After 24 hours seal the coverslip border with a synthetic lacquer or special bitumen.

Remarks

In order to avoid the re-melting of the medium under the influence of high temperatures, add a small drop of formalin 10% to the drop of medium on the slide, mix well and then transfer the object to be mounted.

8.2.5 Apáthy syrup

Gum arabic	30 g
Cane sugar	30 g
Distilled water	30 ml
Thymol	0.03 g

(a) Place the gum in a nylon gauze bag in about 20 ml of water.

(b) After complete solution of the gum, make a solution of the cane sugar in the distilled water.

(c) Mix both solutions.

(d) Add 2 ml of formalin or a few crystals of Thymol as a preservative.

(e) Place in a bottle with a rubber or glass stopper.

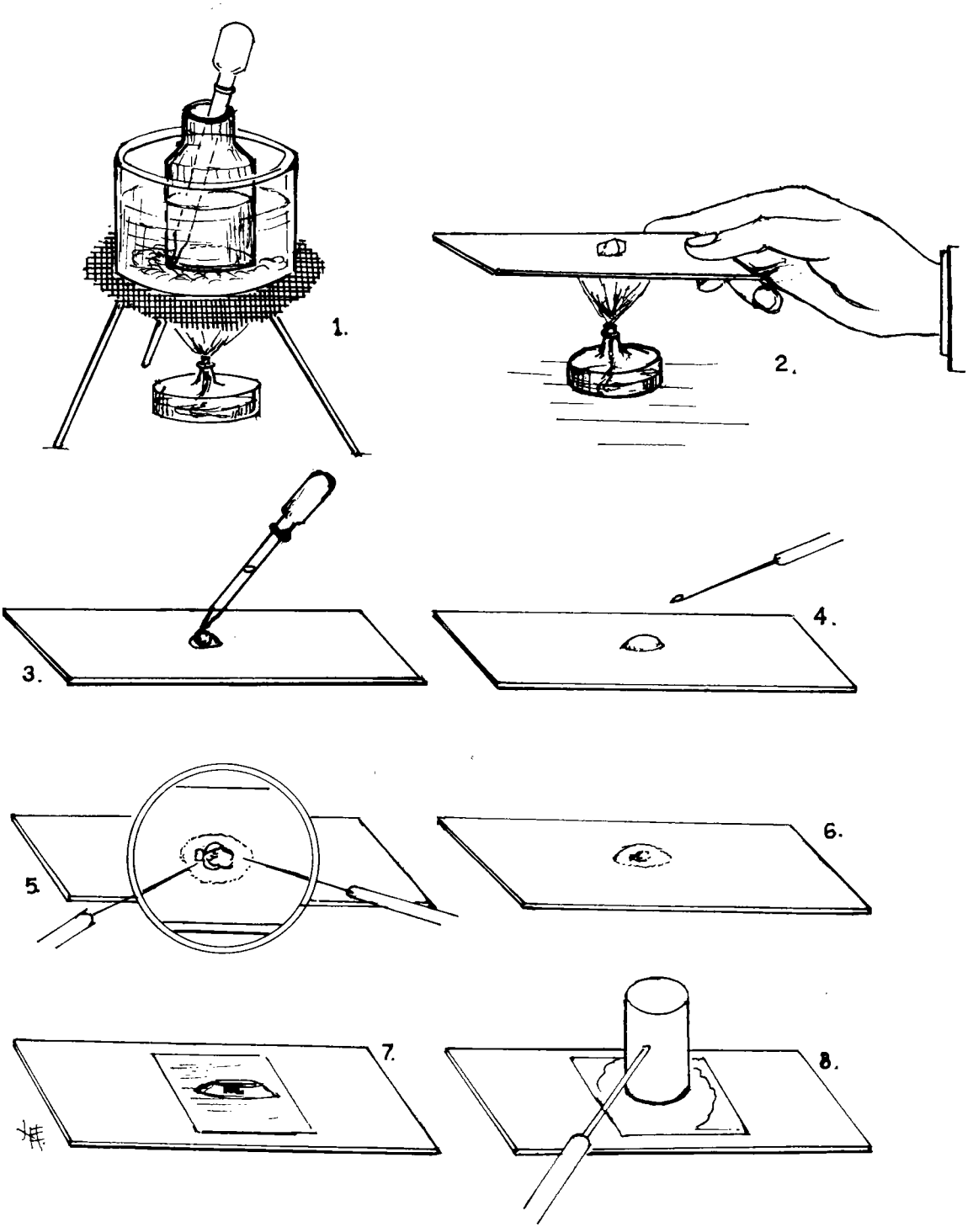


Fig. 41. Mounting a specimen in gelatine/glycerine media.

Use

In this medium all objects can be mounted directly from water. In order to avoid shrinkage with delicate objects use a 1:4 dilution of this syrup with distilled water and leave to evaporate slowly on the slide and then add the concentrated syrup.

8.2.6 Lactophenol solution (after Ammann)

Lactic acid	10 ml
Phenol	10 g
Glycerol	20 ml
Distilled water	10 ml

Use pure phenol and keep the solution in a brown bottle.

This solution is extremely useful for fixing small specimens. The objects become clear and all structures can be easily observed. The specimens can be mounted directly or, after desired clarification in this solution, can be mounted in gelatine-glycerine medium or Apáthy syrup.

8.2.7 Polyvinyl alcohol

Polyvinyl alcohol	15 g
Cold water	100 ml

Place in a water bath and mix continuously till the mixture becomes a thick syrup. Place in a bottle. The solution will become clear in a short time. It can be used directly for mounting specimens from water or may be combined in the following mixture:

Polyvinyl alcohol	50 g
Lactic acid	20 g
Phenol	20 g

8.2.8 Paraffin oil

Objects to be mounted in paraffin oil should be dehydrated as for mounting in Canada balsam. Dry, stained, thin films may be preserved for a long time in this medium. The slide is sealed with Apáthy syrup. This mounting medium preserves the basic blues well.

8.2.9 Formalin

10% formalin can be used for fixing objects on a slide under a coverslip which is then sealed with synthetic lacquer. A water soluble stain may be added to the formalin if desired.

Use

This method of preservation in formalin is extremely useful for preserving unstained internal organs of mosquitos and various internal parasites; for the preservation of mosquito eggs, 2% formalin is used (Fig. 42, stages 1-5).

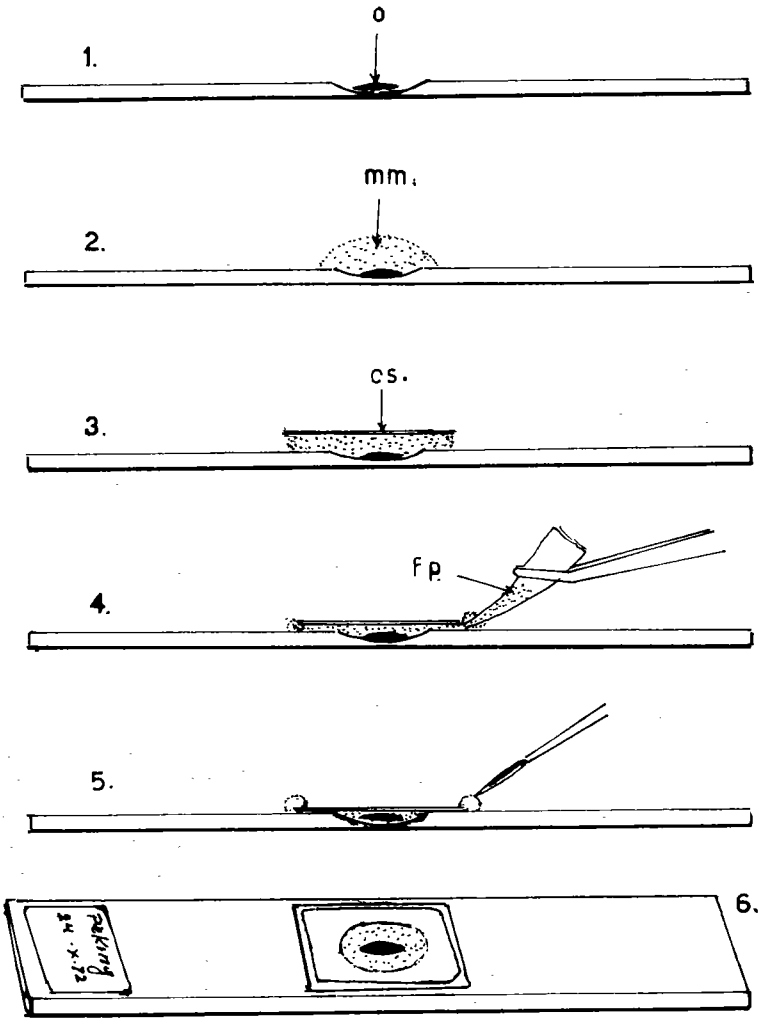


Fig. 42. Mounting a specimen in formalin and sealing with synthetic lacquer.
o = object; mm = mounting medium;
cs = coverslip; fp = filter paper.

8.2.10 Alkaline hydrating solution

When dry insects are to be mounted they should first be softened in a detergent solution or in one of the following:

Gage formula (1896)	Water	99.5 ml
	Formaldehyde (40%)	0.5 ml
	Sodium chloride	0.5 g
Gage formula (1897)	Water	100.0 ml
	Potassium dichromate	0.25 g
	Sodium sulfate	0.1 g
	Sodium chloride	0.9 g

After dipping the dry insects in 75% alcohol for one second, put the insects in one of the above-mentioned solutions till they become softened.

8.2.11 Other mounting methods

Other mounting methods are shown in Figs 43-45.

9. PROCESSING THE COLLECTED SAMPLES OF A MOSQUITO POPULATION

The processing of collected material represents a very important activity and for the accomplishment of this a number of successive steps have to be carried out.

9.1 Identification of species

(a) Identification of species based on the external morphology. This is the first step to be accomplished either under field conditions or in the laboratory. In the majority of cases a simple hand lens (X 5-10) is sufficient to distinguish the species which possess clear cut external morphological characteristics. Some species, especially the large ones can be recognized with the naked eye, the smaller species of mosquitos with few distinguishing characteristics or those partially damaged need to be examined with the hand lens. Field staff should always use a hand lens, which is necessary not only for species identification but also for classification of physiological stages.

(b) Identification of species by the cytogenetic method. This method is used in special circumstances when the morphological characteristics are not easily identified, as is the case when some groups of species have similar external morphology, e.g., the fresh water A. gambiae complex. The cytogenetic method consists of examining the polytene chromosomes of the ovum of the ovarial follicle in stage III larvae or the chromosomes of the salivary glands of the stage IV larvae.

(c) Cross mating. The identification of sibling species by mating and examination for sterility or fertility of the first generation resulting from the crossing. This method is a more laborious and time-consuming process than the examination of chromosomes. Artificial mating is used for this purpose (see Part II section 21). As this method is time consuming it is only used for special studies.

9.2 Classification of abdominal appearance using a hand lens (X 5-10) or a dissecting microscope

This method was described for the first time by Sella (1921) and in general this classification is performed with mosquitos collected by hand, pyrethrum spray and traps.

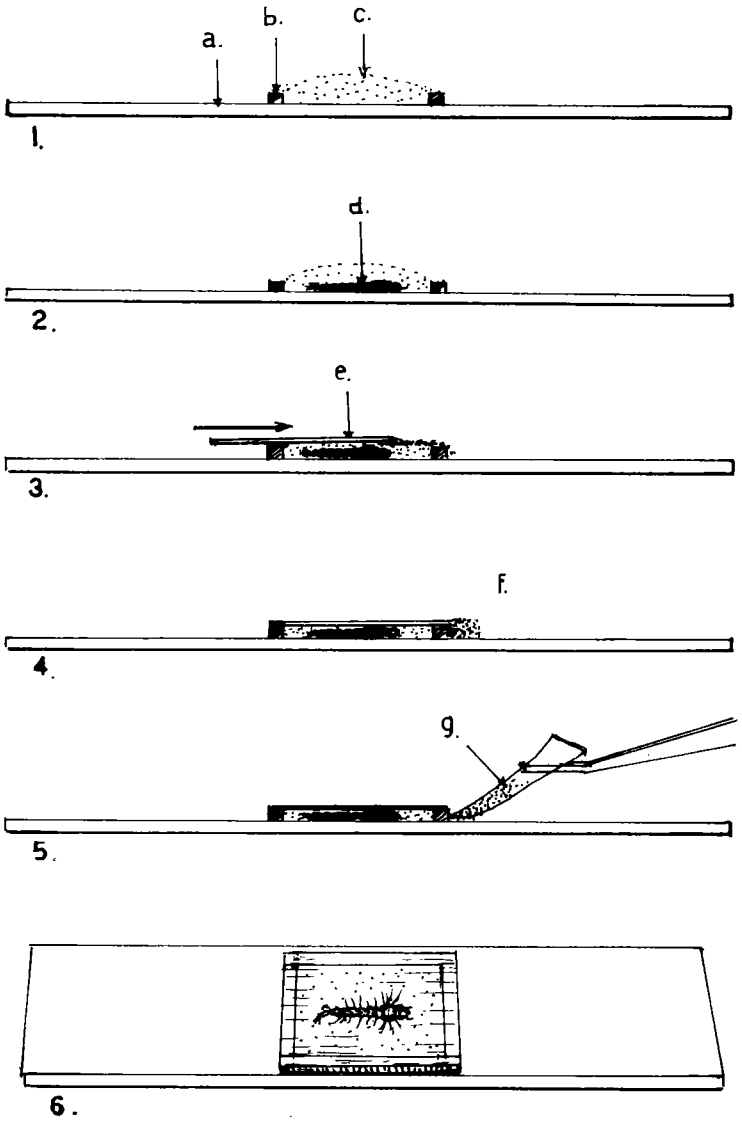


Fig. 43. Mounting a thick object such as a larva.
a = slide; b = glass strip to support coverslip;
c = mounting medium; d = larva;
e = coverslip; f = coverslip in place;
g = filter paper removing excess mountant.

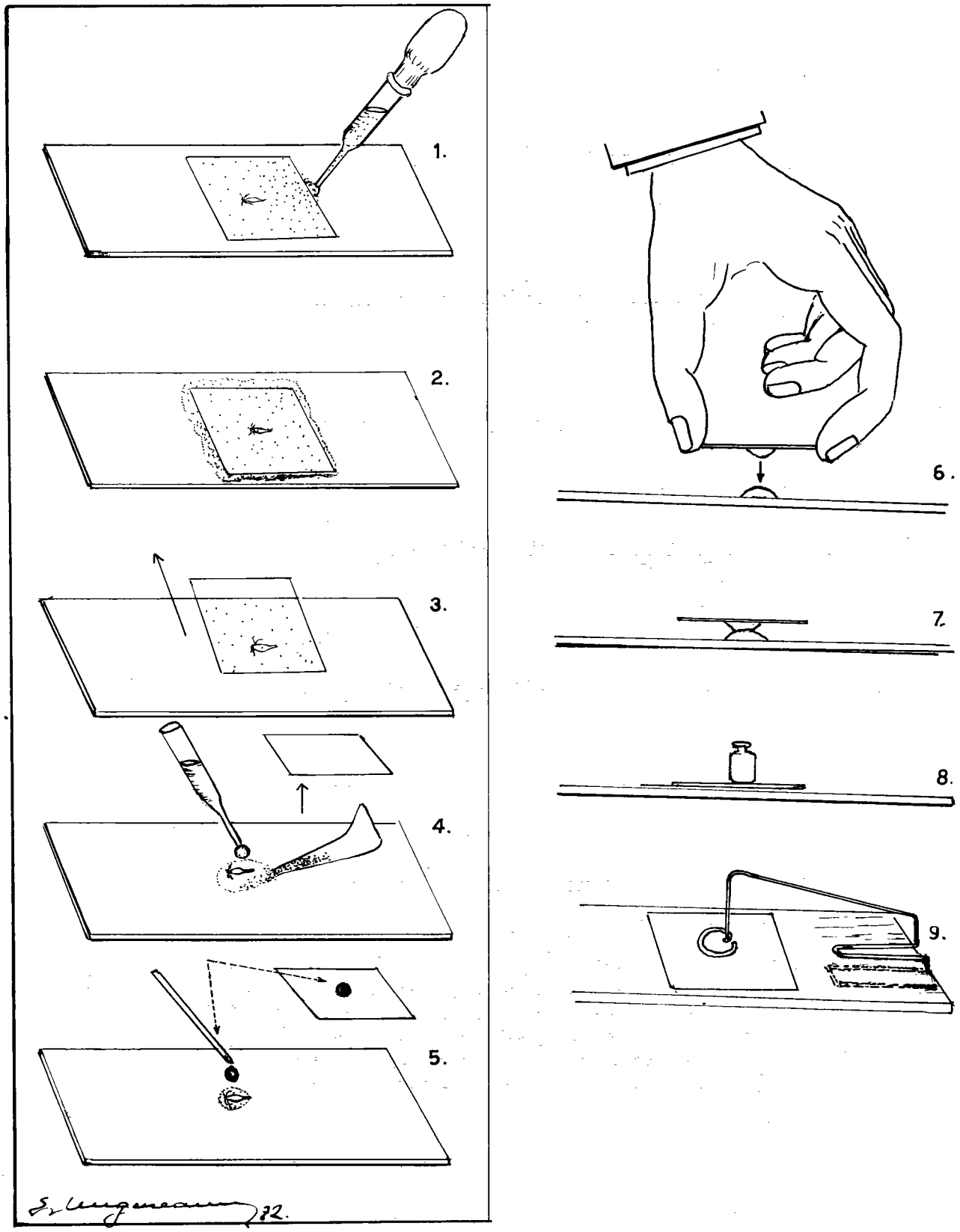


Fig. 44. Fixing and dehydrating a specimen by irrigation on a slide, and mounting in Canada balsam or euparal.

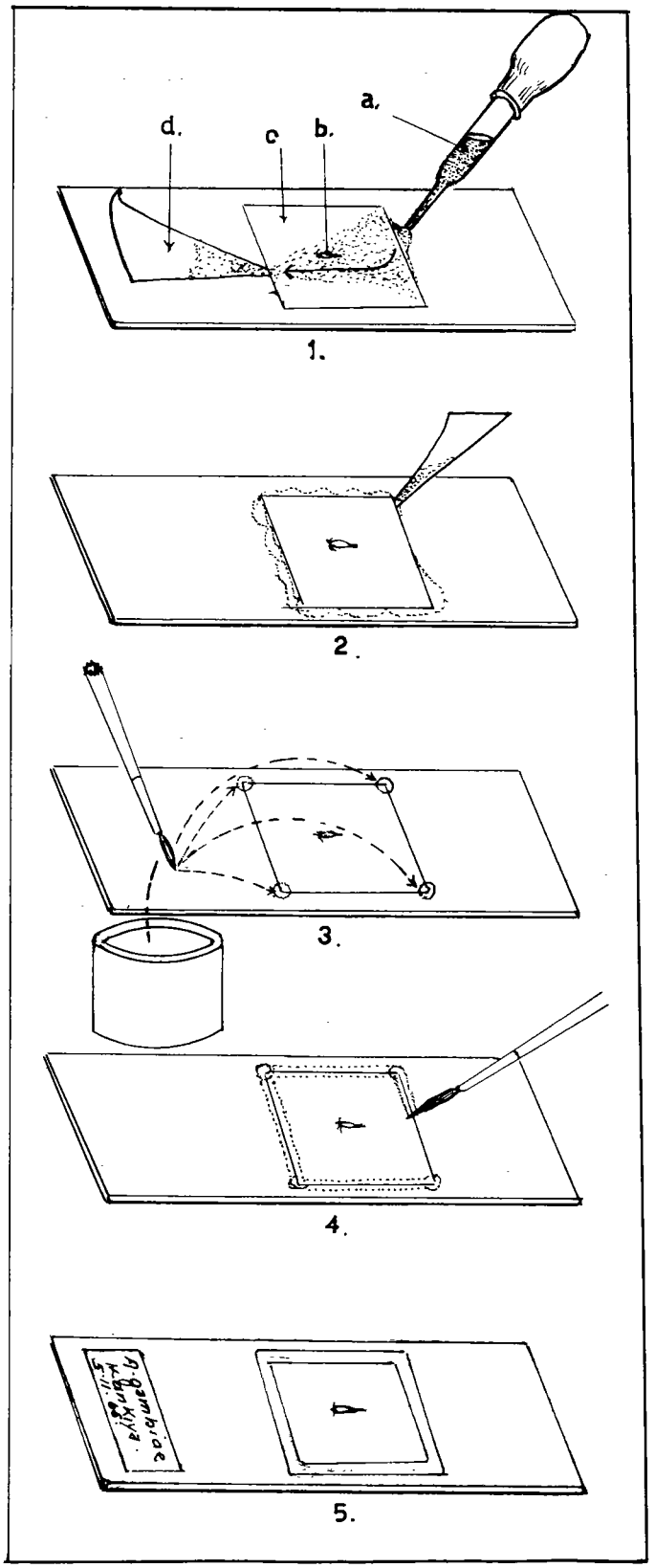


Fig. 45. Semi-permanent mount sealed with wax.

9.2.1 Principle of the method

External examination of the abodmen in order to see the feeding and ovary development status.

9.2.2 Objectives

- To obtain information on the movement and resting habits of mosquitos of different physiological stages.
- To study the duration of the gonotrophic cycle.
- To check on refeeding of gravid females.

9.2.3 Stages of abdominal appearance (Fig. 46)

Abdominal appearance is classified in the following stages:

Unfed

The abdomen is collapsed, the stomach empty and the ovaries occupy only one third or less of the abdomen (ovaries in stage I or II Christophers, Sella's stage I).

This group includes:
- newly emerged nulliparous females (pregravid)
- parous females which have not yet taken a blood meal (stage II Christophers).

Freshly fed

Stomach with red blood, ovaries occupying not more than 2-3 segments ventrally and up to 4 dorsally (Sella's stage II, stage I or II Christophers, see Fig. 47).

Late stage fed

Blood dark red, ovaries occupying 2-1/2 segments ventrally and 5 dorsally, ovaries in stage II-III Christophers.

Half gravid (Sella's stage III-IV)

Blood dark red, ovaries at late III-IV Christophers stage occupying 4-5 segments ventrally and 6 dorsally.

Sub-gravid (Sella's stage V)

Blood greatly reduced, dark in colour, ovaries stage IV or intermediate between IV and V Christophers, occupying most of the abdomen.

Gravid (Sella stage VI and VII)

Blood only a black trace as a narrow blackish line or completely digested. Ovaries in stage V Christophers.

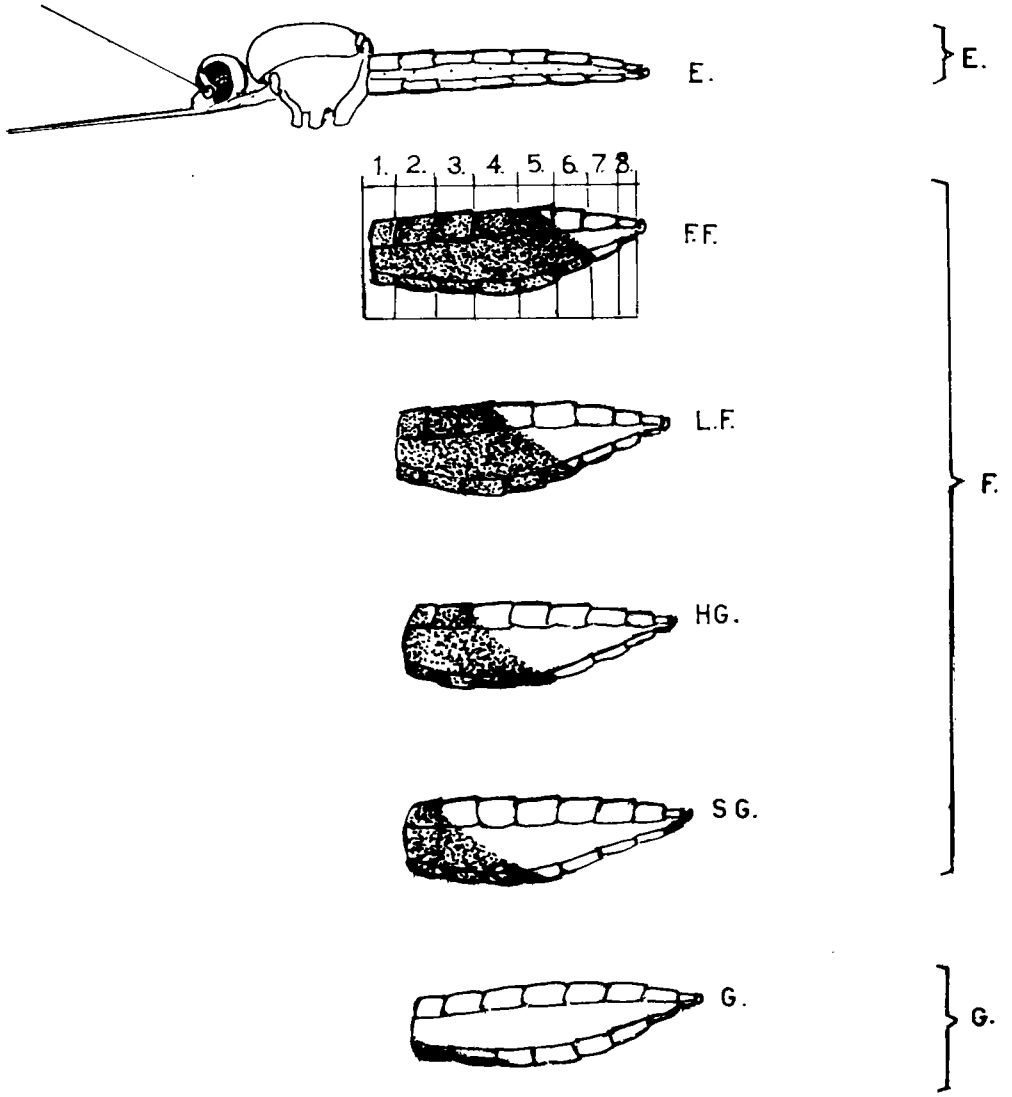


Fig. 46. Classification of abdominal appearance.
E = empty; FF = freshly fed; LF = late stage fed;
HG = half gravid; SG = sub-gravid; G = gravid.

The number of segments occupied by the stomach and ovaries during different stages might vary slightly especially with some small species such as A. funestus. During dry periods some gravid females, mainly in the species A. gambiae s.l. might have the abdominal appearance of half-gravid or sub-gravid females but upon dissection the ovaries are found to contain fully mature eggs (stage V Christophers). Therefore, ovary development is more accurately established by dissection and identification of the ovarian stages according to Christophers mainly during the dry period of the year or when examining small species with very dark cuticle. (By using a hand lens X10, refed gravid females can be easily identified since the eggs are fully formed.)

It should be mentioned that under field conditions when the gonotrophic cycle lasts only two days, and particularly when dealing with small species, the classification of all Sella stages is difficult, and therefore a simplified classification is used for the abdominal appearance of female mosquitos as follows: unfed; freshly fed (stomach with red blood); late fed or half-gravid (stomach with dark blood), and gravid (ovaries fully developed, blood completely digested or only a dark trace).

It should be stressed that when using this four-stage classification, only those females with fully developed ovaries having the characteristics mentioned for the Sella stages VI-VII should be included under gravid and all female mosquitos with the abdominal appearance corresponding to the Sella stages III-V should be included under late fed (Fig. 46).

9.3 Classification of ovarian stages

The system of classification was devised by Christophers and is used universally (Fig. 47 (1)-(5)).

9.3.1 Principle of the method

Extraction of the ovaries and microscopic examination of the degree of development of the ovarian follicles.

9.3.2 Objectives

To identify the stages of the development of ovarian follicles for a precise analysis of the gonotrophic conditions. Such a study is essential for the application of age-grouping techniques.

Christophers has divided the ovarian development into five main stages:

Stage I

Egg follicle round, yolk granules absent. The oocyte is small and not distinguishable from the nurse cells Fig. 47(1). The stage is characteristic for newly emerged females.

Stage II

Egg follicle oval, yolk granules present, the oocyte occupying up to half of the follicle, the nucleus of the ovum is visible (2, 2', 2'').

Stage II - early

A few fine granules of yolk around the nucleus of the ovum, visible only under high-power objective X40 (2).

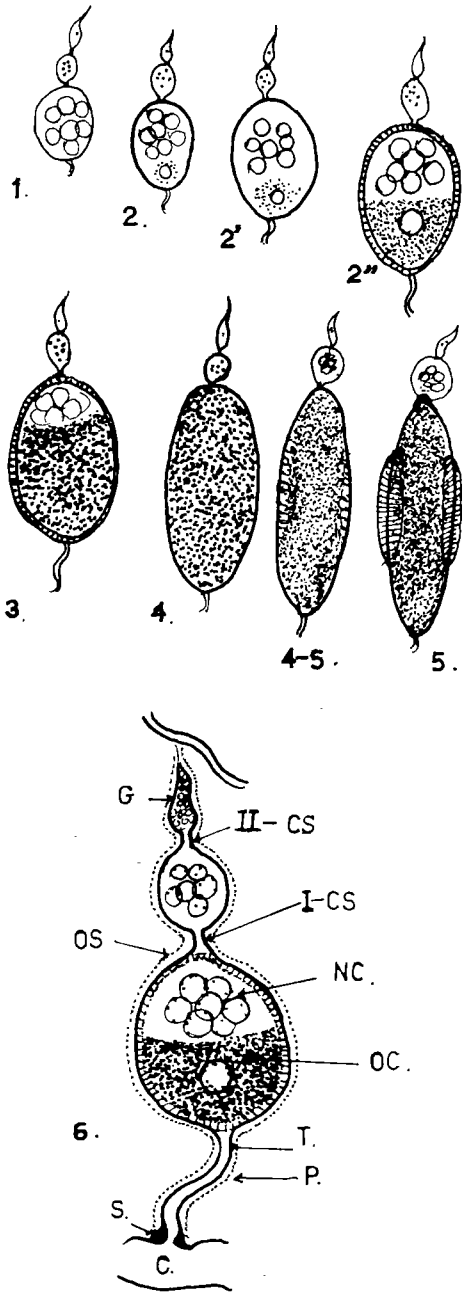


Fig. 47. (1)-(5). Development of an oocyte. (6). Structure of an ovariole
s = sphincter; p = pedicle; T = tunica;
C = calyx; OC = oocyte; OS = ovariole sheath;
NC = nurse cells; I-CS = first connecting stalk;
II-CS = second connecting stalk.

Stage II - mid

Yolk granules easily visible under low power X10, occupying about 1/4 of the follicle (2').

Stage II - late

Yolk granules very abundant occupying about half of the follicle and nucleus visible (2''). (Stage II, early and mid, are only encountered in nulliparous females where II-late is found in fertilized nulliparous as well as in unfed or very recently fed parous females. For distinction between the nulliparous and parous unfed or recently fed females, the morphology of the Malpighian tubes is also used. The Malpighian tubes in the recently fed parous females are not too opaque, showing clear excretion ducts.

Stage III

Yolk occupying about 3/4 of the follicle, nucleus of the ovum not visible but the nurse cells occupy about 1/4 of the follicle, (Fig. 47-3).

Stage IV

Egg follicle sausage-shaped, yolk granules completely filling follicle (4).

Stage V

Ova fully formed with well developed floats (5).

Remarks

An intermediate stage before IV-V stage is very often found (4-5).

Stage IV-V transition

Ova pointed at both ends, floats not well developed or not easily distinguishable.

9.4 Dissection of mosquitos

This is carried out routinely for establishing the parity, sporozoite and oocyst rate and in special circumstances for establishing the degree of fertilization, identification of polytene chromosomes or presence of different parasites which may be found in the abdominal organs. Internal anatomy of the female mosquito is illustrated in Fig. 48.

9.4.1 Objectives

- Ovary dissection for establishing Christophers' stages, the parous rate or the physiological age or to detect infection with Coelomomyces spores, chromosome examination, etc.

- Stomach dissection for establishing the oocyst rate or to identify recently emerged unfed mosquito females by the presence of meconin, or a tracheolar system.

- Salivary gland dissection to establish the sporozoite rate.

- Dissection of the spermatheca to establish the presence or absence of spermatozoa.

Dissection can be performed on:

(a) Fresh mosquitos recently dead or killed by ether, chloroform, carbon dioxide, etc.

(b) Frozen mosquitos (held at a few degrees below zero if to be dissected soon after collection (4-10 days), or frozen at about -18°C when dissected after a longer period).

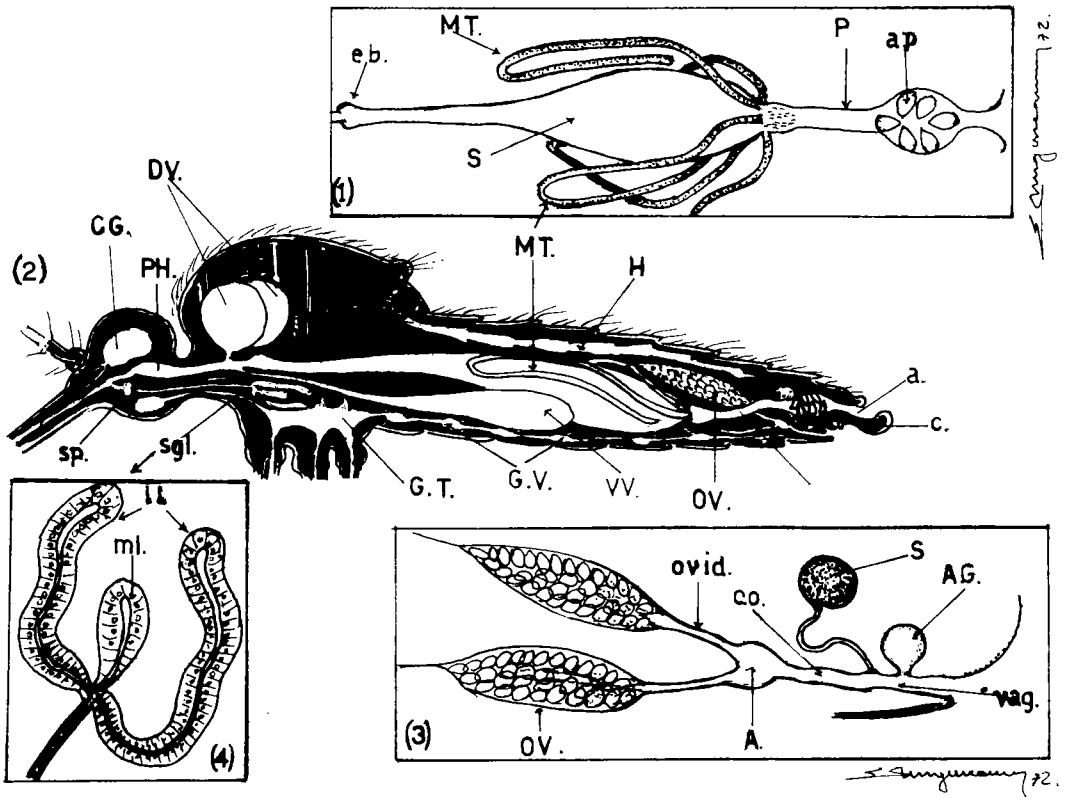


Fig. 48. Internal anatomy of female mosquito,
(1) Gut: eb = cardiac sphincter S = stomach (mid-gut)
MT = Malpighian tubes P = small intestine
AP = rectal papillae
(2) Longitudinal section through body: CG = cerebral ganglion
SP = salivary pump PH = pharynx Sgl = salivary glands
DV = dorsal diverticula GT = thoracic ganglia
GV = abdominal ganglia VV = crop MT = Malpighian tubes
OV = ovary H = heart A = anus C = cercus
(3) Female reproductive system: OV = ovary Ovid. = oviduct
A = ampullae CO = common oviduct S = spermatheca
AG = accessory gland Vag. = vagina
(4) Salivary gland: ML = middle lobe LL = lateral lobe.

(c) Dry mosquitos such as those found in traps and at the end of various laboratory observations, etc., after they have been rehydrated.

(d) Mosquitos preserved in fixatives (formalin, alcohol, etc.).

Remarks

Fresh and frozen mosquitos are dissected by applying the classical method. Dry mosquitos or those preserved in fixatives are dissected according to the method described by Ungureanu (1972).

9.4.2 Materials

- Dissecting microscope.
- Binocular or monocular microscope.
- Dissecting needles (Shute dissecting needles) or fine needles made by the investigator by inserting a very fine steel entomological pin (No. 000) into a wooden or plastic handle.
- Physiological saline for invertebrates, 0.65%.
- Small Petri dish or small round plastic box about 5 cm in diameter and at least 1.5 cm deep, or watch glass.
- Ordinary glass slides, 75 mm x 25 mm.
- Square coverslips about 15-19 mm (round coverslips are also suitable).
- Entomological forceps.
- Chloroform, ether or ammonia.

The physiological saline solution can be slightly stained with a solution of 0.1 g of methylene blue per litre of water.

When dissecting dry or partially dry mosquitos, it is necessary to have the following additional items: liquid detergent without enzymes, or any other substance with emollient properties.

9.4.3 Preparation of the mosquitos for dissection

Live mosquitos can be killed either with chloroform or ether or carbon dioxide: another simple method is to collect the mosquito in a normal test tube. When the insect is at the bottom, rap the end of the tube sharply against the palm of the hand to stun the mosquito. (This is not easy to do with blood-fed mosquitos). After immobilization, hold the insect by one wing and remove the legs one at a time and afterwards pull off the other wing. Return the mosquito to the slide, and cut off the remaining wing with the dissecting needle. The insect is then placed on a dry slide and arranged in a more suitable position for dissection of the gland or stomach, as described in the following chapters.

In order to avoid contamination of the slide by scales caused by wings or part of the leg, these are removed before dissection.

Remarks

Infected organs may be found during dissection. A method for their rapid fixation and preservation is illustrated in Figs 44 and 45. Alternatively, diseased insects may be sent to a WHO Collaborating Centre for Diseases of Vectors.

9.5 Age-grading

9.5.1 Principle of the method

Extraction and dissection of the ovaries of female mosquitos and their direct examination for the presence or absence of coiled tracheolar skeins or dilation of the ovarioles (Figs 49, 50, 51).

9.5.2 Simple age-grading technique

9.5.2.1 Principle

To study the absence or presence of the coiled tracheolar skeins in fresh and dry ovaries.

9.5.2.2 Objective

To establish the parous/multiparous proportion as a parameter necessary for the estimation of mosquito longevity.

9.5.2.3 Description of the method

The ovaries of unfed or freshly fed females (with abdominal appearance stage II-III) with ovaries not more advanced than Christophers' stage II are extracted. The procedure is as follows:

(a) Extraction of the ovary

There are several methods of extraction but the most commonly employed is the following (Fig. 49 (1)-(3)):

- place the anaesthetized mosquito on its side or back,
- add a drop of 0.65% saline near the extremity of the abdomen,
- place or insert the one dissecting needle in the thorax muscle and using the second one make a small cut between the VI and VII sternite (1).
- move the second needle gently to extract the ovaries. These come out, if in stage I-II, before the Malpighian tubes and stomach are extracted.
- once the ovaries have appeared, cut the hindgut (3) if you do not wish to examine the midgut for oocysts, and separate the ovaries.

(b) Identification of tracheolar skeins in dry ovaries

Once the ovaries have been extracted they can be detached and transferred to another slide in a drop of distilled water and allowed to dry. The slide is numbered for identification purposes. Drying is necessary because the tracheolar skeins can be seen more easily when filled with air. Once dry the ovaries are examined with an ocular X5-X7 and objective X40. This technique has the advantage that the slides can be examined or re-examined at a convenient time since the preparations have already been made. The ovaries can be preserved for months in boxes with a disinfectant (camphor, naphthalene, dichlorbenzene, etc.) which prevents degradation of the organic matter by fungi.

(c) Identification of tracheolar skeins in fresh material

A practical method for the identification of the tracheolar skeins is the examination of the freshly dissected ovaries. This is recommended when the work is carried out by a qualified entomologist and in research programmes when parous mosquitos are dissected for infection or infectivity or for other observations. The ovary extracts, in a drop of physiological saline

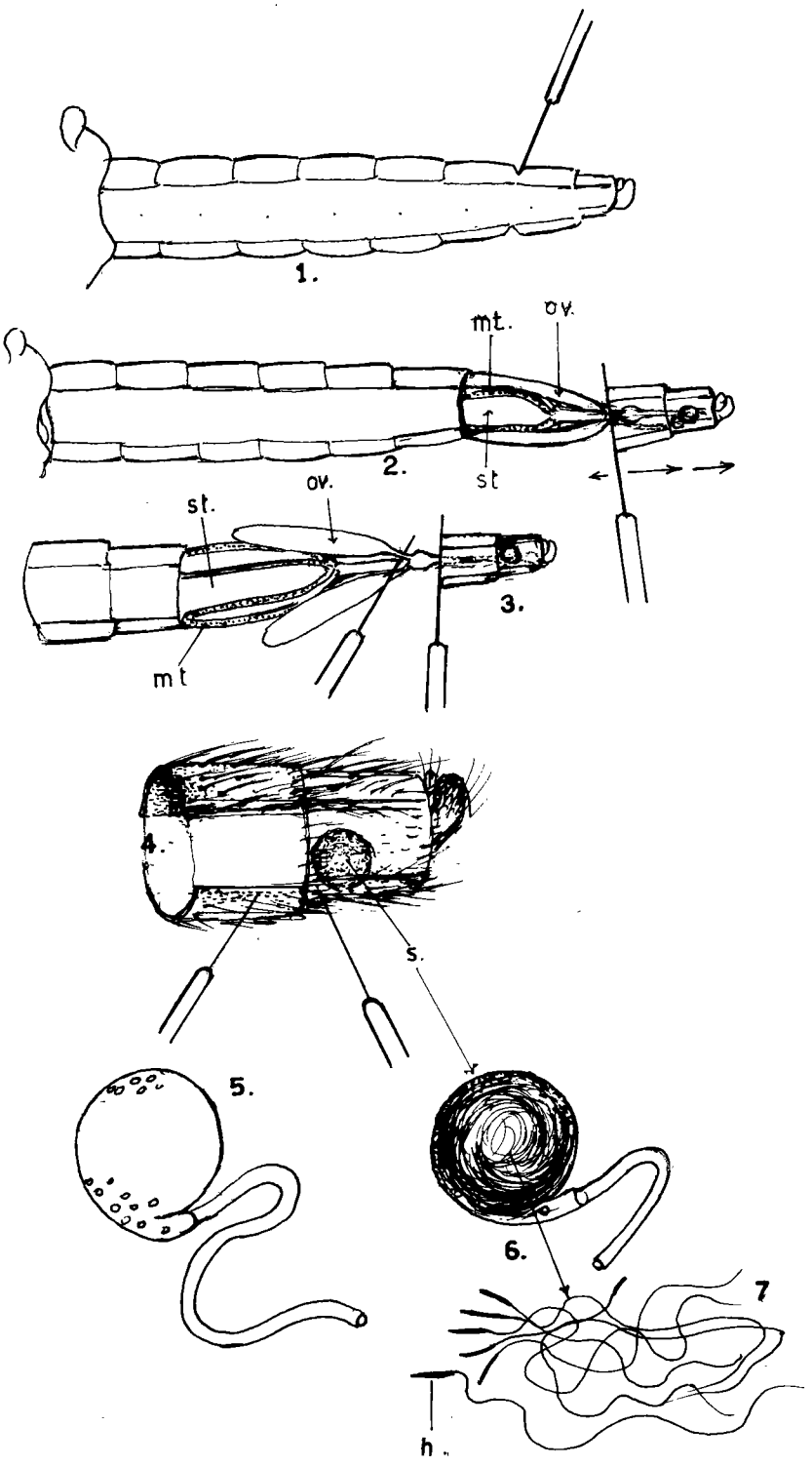


Fig. 49. Extraction of ovary and spermatheca.
mt = Malpighian tubes; st = stomach;
ov = ovary; s = spermatheca;
h = "head" of spermatozoon.

(0.65%), are covered with a coverslip and examined immediately. The coiling of the tracheoles can be easily seen. The addition of a drop of glycerol helps.

This method has an important advantage over the previous one because at the same time it permits the identification of Christophers' stages and eventually a more detailed examination of the ovarioles for dilations.

When in doubt about the tracheolar skeins, one ovary should be allowed to dry while the other is being examined for dilation of ovarioles (Fig. 50).

9.5.2.4 Identification of nulliparous females

The identification of nulliparous females, using freshly dissected material, can be established by observing one or more of the following aspects:

- Presence of coiled tracheolar skeins in the ovary in Christophers' stage I and and (Fig. 51(4)) early and middle stage II in fed mosquitos.
- Ovary in stage I or early stage II in unfed or fed mosquitos.
- Presence of meconium in the gut (ovaries are in Christophers' stage I or early II), and presence of coiled tracheoles on the surface of the gut of unfed females (Fig. 55).
- Unfertilized females, and ovaries in stage I or early II and mid II.
- Presence of hydracarine mites (Part I, Fig. 7).
- Absence of ovariole sac or dilation.

9.5.2.5 Identification of parous females

Parous females can be identified as follows:

- Presence of retained eggs.
- Ovaries in Christophers' stage II late, unfed or freshly fed (stomach with red blood) females and Malpighian tubes partially emptied or completely without granules of secretion.
- Presence of uncoiled tracheolar skeins in the ovary and stomach (Fig. 51(5) and Fig. 55).
- Presence of ovariole sac or dilation; ovaries in late stage II or III can be dissected and ovariole separated and examined with an ocular X5 or X7, objective X40 (see technique of dissection of ovariole). (Ovaries in all stages can be dissected and examined for the presence of degenerated follicles.)

9.5.3 Detailed (advanced) age-grading technique (Figs 52 and 53)

9.5.3.1 Principle of the technique

Dissection of the ovary for the isolation (stretching) of ovarioles so that the stalks may be straightened making possible the examination for the presence of dilations, follicular sacs and follicular relics (or degenerated follicles).

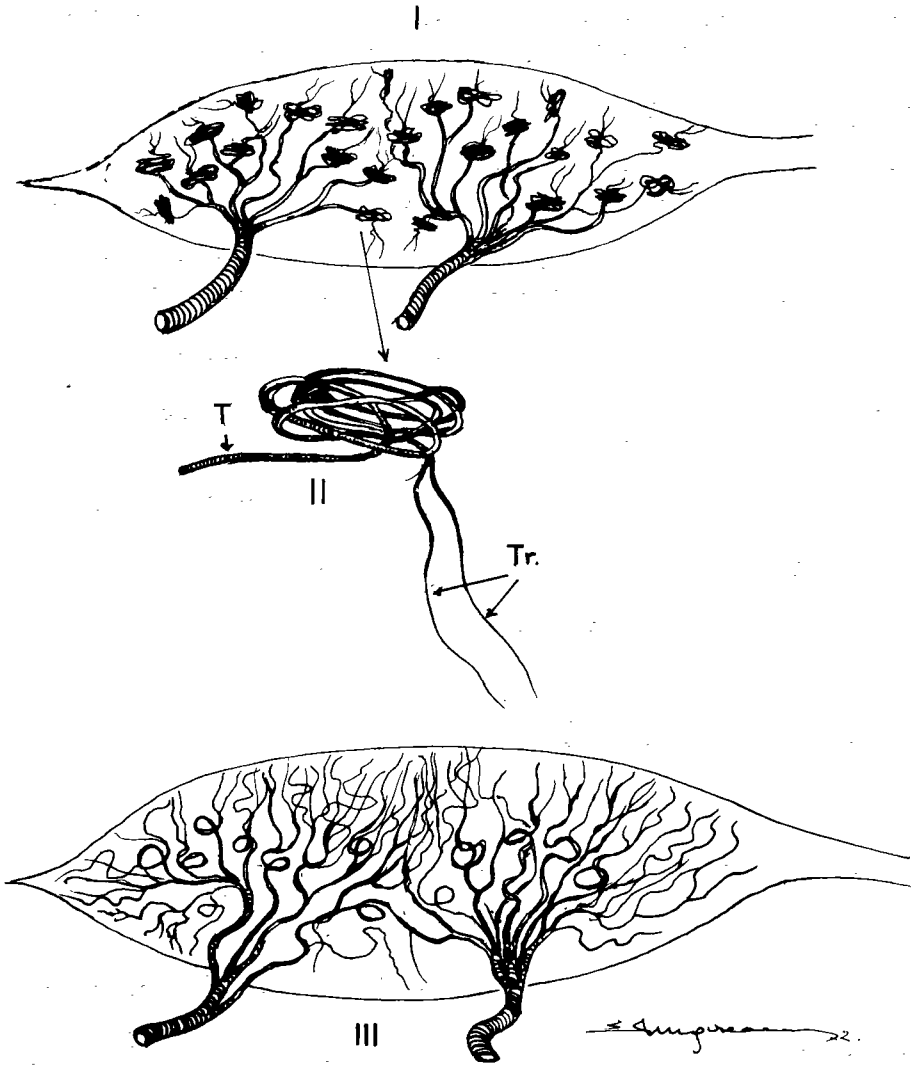


Fig. 50. I. Ovary of a nulliparous female: tracheolar system forming skeins.

II. End of a fine trachea forming skein

T = trachea; Tr = tracheoles.

III. Ovary of a parous female - note the characteristic tracheolar system without skeins.

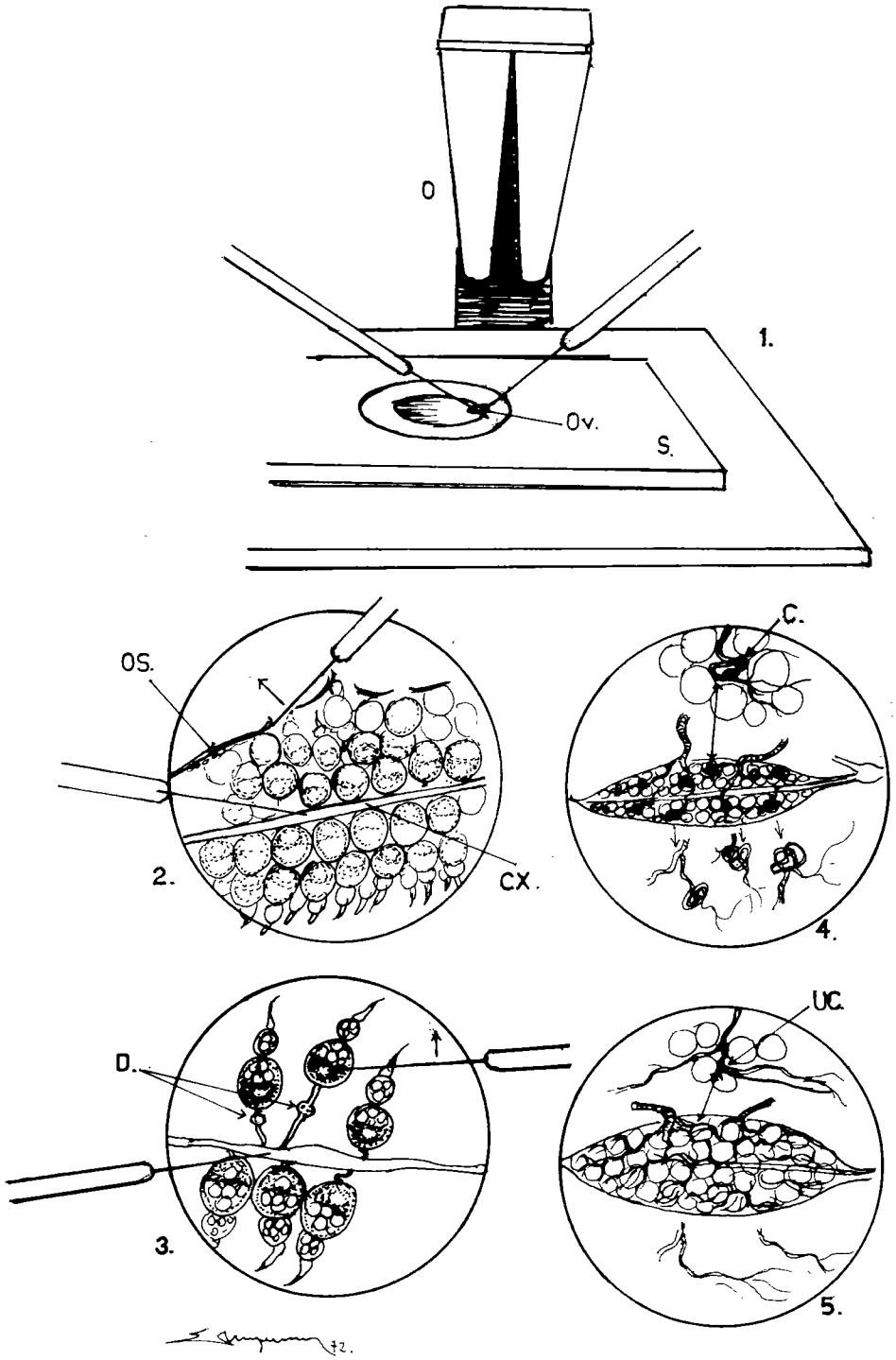


Fig. 51. Dissection of ovary.

(1) Cavity slide under dissecting microscope.
(2) Dissection, os = ovariole sheath , cx = calyx.
(3) Isolated ovariole, d = dilatation on the pedicle.
(4) Ovary of nulliparous female.
(5) Ovary of parous female.

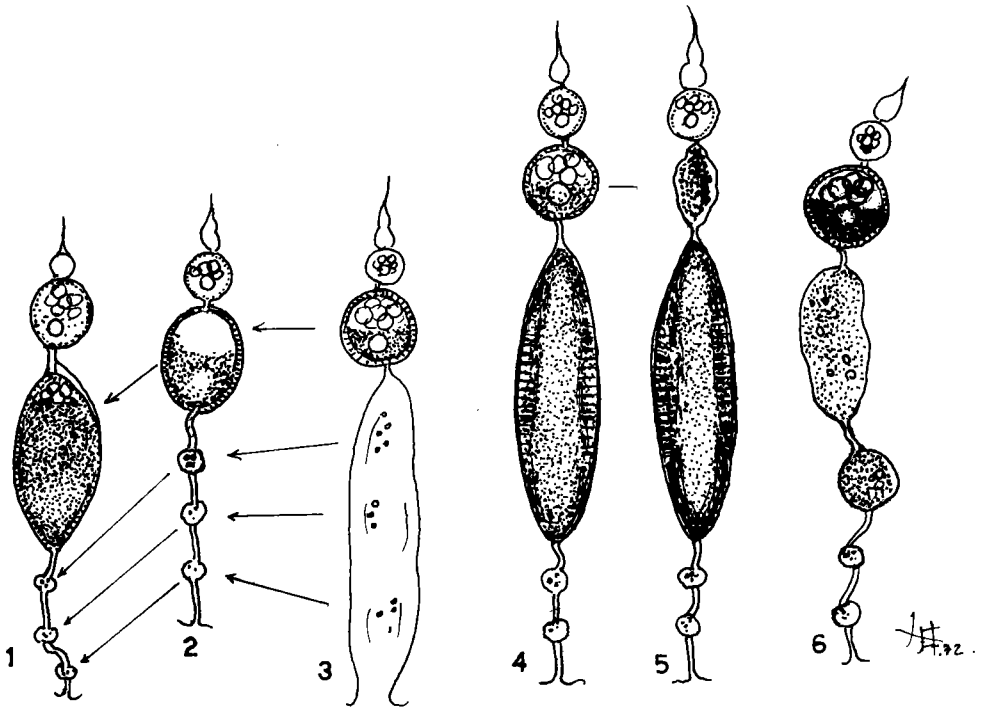


Fig. 52. Ovarioles in various states.

(1)-(2) = ovarioles with three dilatations.
(3) = the impressions of the three dilatations left on the ovariolar sac.
(4)-(6) = degenerating follicles.

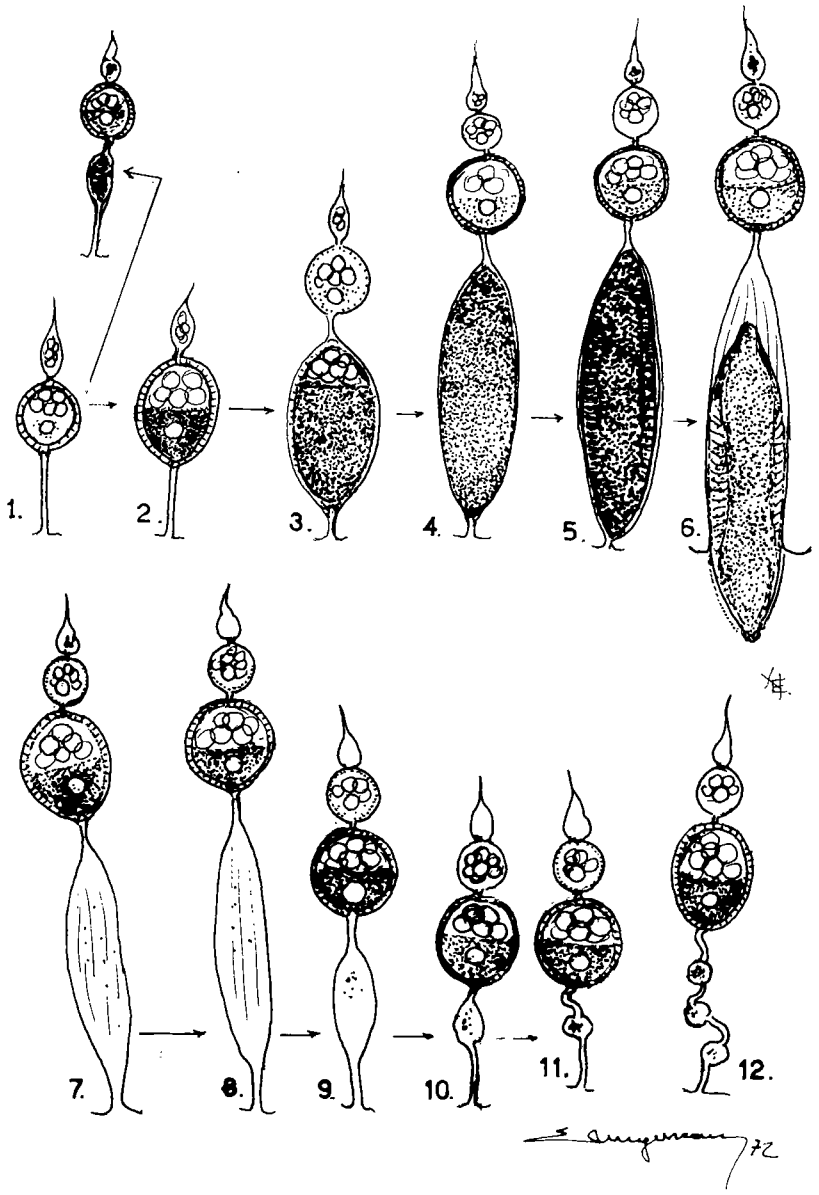


Fig. 53. Various stages of development of the ovarian follicle.

(1)-(5) = Christophers' stages I-V;
(7)-(10) = ovariolar sac in different stages of contraction;
(11) = ovariole with one dilatation;
(12) = ovariole with three dilatations.

9.5.3.2 Objectives

To establish the physiological age of female mosquitos (number of batches of eggs layed by a given female mosquito).

9.5.3.3 Application of the technique

Routinely the technique is applied for the examination of the ovaries of unfed, freshly fed or even late fed female mosquitos.

The method can be applied in special investigations to mosquitos in all physiological stages (including gravid) collected by various means. In ovaries with the ova at a more advanced stage than Christophers' stage III, observations should be made for the presence of degenerated follicles.

The ovaries are dissected under a high-power dissecting microscope (X50 magnification). Two very fine dissecting needles are necessary. These are easily prepared by inserting entomological pins No. 000 into wooden or plastic handles. The plastic section of a used ball-point pen may also be used. The pointed plastic extremity is heated, the section previously holding the metal part of the ball-point starts to close and at this juncture a "minute" steel pin is inserted.

The ovary is dissected in distilled water and careful attempts should be made to isolate ovarioles (at least 6 ovarioles) from three parts of the ovary (anterior, middle and posterior part).

The dissection of the ovaries is carried out as follows (Fig. 51) on normal slides or on slides with a concavity:

- Cut the wall of the ovary in several places as in (2).
- Try to detach the wall of the ovarial sheath leaving the ovariole free (2).
- With the right needle inserted in the follicle of the egg, try to extend the stalk of the ovariole which is inserted in the calyx, in order to see the number of dilations or presence of sac (3).

The number of dilations can be accurately established only when the ovariole is still attached to the wall of the calyx. In some instances when the ovariole is detached a part of the wall of the calyx may remain attached, giving the impression of a dilation which might be taken as such by an inexperienced observer.

9.5.4 Use and limitation

(a) Simple age-grading technique

This is the easiest method applied practically in all malaria projects in order to identify the parous females for sporozoite dissection and for establishing the parous rate of different samples of mosquito populations necessary for the estimation of longevity.

The data obtained are not significant for the establishment of the parous rate when investigations are carried out on a small number of specimens or without taking into account the following: (i) the relative output from the breeding places at the time of investigation, (ii) the density trends (increasing or decreasing density), various causes producing high mortality among field populations of mosquitos. It is necessary to carry out repeated observations in the same place on a weekly basis and on large samples.

(b) Study of the physiological age

This is the most accurate method at the present time (but see the note on daily incremental cuticular layers below) for establishing the age of mosquitos but it is time-consuming and needs a well trained technician capable of carrying out the dissection of the ovarioles and of identifying the dilations left after each batch of eggs. It is easier to apply with some species (e.g., A. maculipennis, A. albimanus) than with others (e.g., A. gambiae, A. funestus).

(c) Study of the calendar age

The data concerning the physiological age of female mosquitos are used for calculating more accurately the calendar age: such calculations require a correct evaluation of the physiological age and of the duration of the gonotrophic cycle. The method employed for the study of the duration of the gonotrophic cycle is given in Part II, section 11. The calculation of the calendar age is obtained by multiplying the number of dilations with the average number of days of the gonotrophic cycle. This is a relative estimate which serves the practical purpose of calculating the vectorial capacity but this does not exactly reflect reality in all cases. The duration of the gonotrophic cycle might vary among nulliparous and parous females between limits which might vary from one to several days.

9.6 Daily incremental cuticular layers

One very promising recent development in the field of calendar age determination, not connected with the female reproductive system, is that dealing with growth layers in the cuticle. In preparations of certain flies and mosquitos, killed in daily progression after emergence, daily deposition of cuticular layers has been detected.

Such layers appear to be most readily seen on the apodemes (portions of the cuticle invaginated for muscle attachment) especially those in the thorax. In the anophelines, growth layers can be observed on the mesosternal apodeme and on the thoracic phragma. Up to 13 daily layers have been counted on the latter (Schlein & Gratz, 1973).

9.6.1 Preparation

A 10% solution of potassium hydroxide containing the mosquitos is brought to boiling point, removed from the flame and allowed to stand for 10 minutes. Wash the mosquitos with water. They are then dissected in water using watchmakers forceps. The abdomen is separated from the thorax and the latter cut transversely between the second and third pairs of coxae and up to the scutellum. The separated hind part of the thorax is rinsed in water, cleaned of tracheae and the ventral part (metasternum and coxae) cut off. Staining procedure is as follows:

- Oxidation in 1% potassium permanganate for 5 minutes
- Rinse in water
- Mordant, 1% iron alum, 15 minutes
- Rinse in water, 10 minutes
- Change water and rinse for another 10 minutes
- Stain in a ripe solution of 0.2% haematoxylin in 70% ethanol for 1-2 minutes (under microscopic control to avoid overstaining; about 10 preparations can be stained and observed at a time).
- Rinse in water
- Counterstain for 20 minutes in 0.2% Congo red in water

- Dehydrate in absolute ethanol

- Clear in xylol and mount in Canada balsam.

9.6.2 Remarks

It appears that in anophelines the amount of growth of the thoracic phragma on the first day is determined by the nutritional condition of the larva, while the rest of the growth is dependent on the environment of the adult. Up to 85% of the specimens collected can be age graded if properly prepared.

Development of this technique is required, and also elucidation of the mechanism governing the deposition of fairly discrete incremental layers. The epidemiological importance of such a method would be of the highest significance as it would enable the age structure of a given vector population to be determined with a high degree of accuracy.

9.7 Stomach dissection

9.7.1 Principle of the method

Extraction of the stomach of unfed female mosquitos.

9.7.2 Objectives

- To estimate the oocyst rate (rate of infection of mosquitos).

- To estimate the speed of development of the sporogonic cycle and of the different oocyst stages.

- To determine the very young (newly) emerged unfed female by detecting the presence of a meconium or of tightly coiled tracheoles skeins on the surface of the empty stomach.

9.7.3 Description of the method (Fig. 54)

After preparing the mosquito as described in the previous chapter, the following steps are taken:

(a) Place the mosquito on a microscope slide with the apex of the abdomen to the right.

(b) Separate the abdomen from the thorax by a cut, leaving part of the metanotum attached to the abdomen (1).

(c) Fix the left dissecting needle in the attached part of the thorax (2).

(d) With the right needle, make a small cut in the integument on each side of the seventh abdominal segment (care should be taken not to cut the internal organs). Put a small drop of physiological saline at the tip of the abdomen (2).

(e) Holding the abdomen with the left needle, exercise gentle traction on the apex of the abdomen until the ovaries, Malpighian tubes and the stomach are gradually drawn out (3)-(4).

(f) When the stomach is partially extracted, it is recommended to detach the Malpighian tubes from around the stomach and to cut them as close as possible to their insertion without tearing the gut wall.

(g) The gut is then drawn out completely from the abdomen and the rectum cut off from the stomach just below the pyloric ampulla (5).

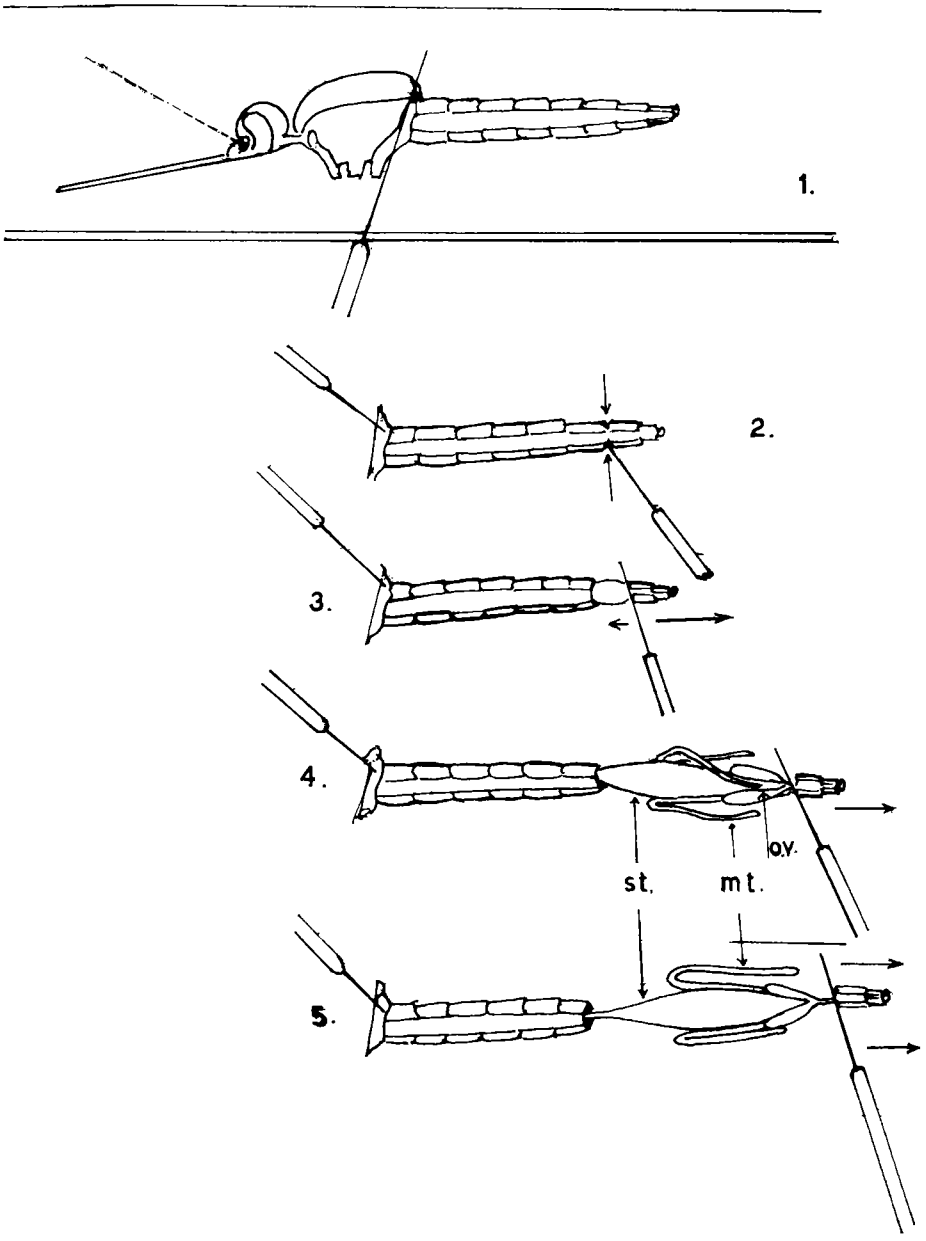


Fig. 54. Extraction of stomach.
st = stomach; mt = Malpighian tubes;
ov = ovary.

(h) The stomach may be removed and transferred to a cleaner part of the slide but, in general, it is left in the same place. Another drop of 0.65% physiological saline is added if necessary and the specimen is covered with a coverslip. If there is too much saline this can be absorbed with a small piece of filter paper.

9.7.4 Examination of the stomach for oocysts

Begin the examination of the stomach from the posterior end moving field by field to the anterior part. In general, in low or moderate infections the oocysts are located in the posterior half of the stomach (Fig. 56). First examine with a low power, in order to detect the large and medium size oocysts. The following magnifications are recommended for small oocysts (Shute & Maryon, 1960):

For oocysts of 3-4 days, 2 mm (1/12") objective and No. 6 eyepiece

For oocysts of 5-7 days, 4 mm (1/6") objective and No. 6 eyepiece

For oocysts of 8-11 days, 16 mm (2/3") objective and No. 6 eyepiece

Examine the stomach from the posterior part, field by field, as with a blood slide. If no oocysts are found in the posterior half of the stomach, then there is no chance of finding them on the anterior half; they would be present in the anterior half only in heavy infections.

Young oocysts are easy to detect because of their refractiveness and the presence of characteristic malaria pigment (Fig. 57). The pigment immediately attracts attention.

Both sides of the stomach should be examined. Some workers recommend that the stomach should be slowly rolled over by pushing the coverslip along the slide, so that the cysts appear along the moving edge of the stomach (this, however, is time-consuming and it is not recommended for normal practice). A very practical method is to examine the other side of the stomach by transparency, by modifying the depth of the microscopic field and focusing on the surface of the other side of the stomach. This is easily done by focusing on the stomach tracheae. When these appear clearly it indicates that the depth of the microscope field is at the stomach surface level. The young oocysts or even ookinetes just about to penetrate the surface of the stomach can be identified, as they appear as corpuscles more refringent than the stomach walls, containing melanic pigments. In many instances, pigment granules can be seen at the surface of the stomach; they have nothing to do with the parasites.

After some practice, there is no risk of confusing oocysts with fat cells or epithelial cells of the stomach.

9.8 Dissection of the salivary gland of an adult mosquito

9.8.1 Objectives

- To incriminate the vector,
- To establish the sporozoite rate,
- To estimate the onset, peak and termination of transmission.

9.8.2 Procedure I (Fig. 59)

(a) The mosquito is prepared in the same way as for stomach dissection.

(b) Arrange the mosquito on the slide with the head pointing to the right.

(c) Place or insert the left needle gently on the thorax just below the region where the glands lie (1).

TABLE 2. MORPHOLOGICAL CHARACTERISTICS OF THE OOCYSTS OF THE HUMAN PLASMODIA (see also Fig. 58)

Species	Dimensions of the oocysts		Dimensions at 4 days	Colour	Texture	No. of granules of pigment	Pattern (4-6 days)	Days when the pattern is more or less characteristic	Duration of sporogony at 25°C
	Min	Max							
P. vivax	8 µm	50 µm	12-14 µm	Greenish brown	Fine	50-100	Linear, suggesting x or y	4th-7th day	8-10 days
P. ovale	9 µm	37 µm		Dark brown	Medium coarse	50-60	Similar to P. vivax but more often in form of x or y in double rows	3rd-4th day	10-15 days
P. falciparum	8 µm	60 µm	10-17 µm	Blackish	Very coarse	10-20	Single or double rows often in arch form at the periphery	3rd-7th day	10-18 days
P. malariae	5 µm	45 µm	5-6 µm	Dark brown	Medium coarse	25-35	More uniformly distributed in the cysts, some granules are clumping	3rd-11th day	15-21 days

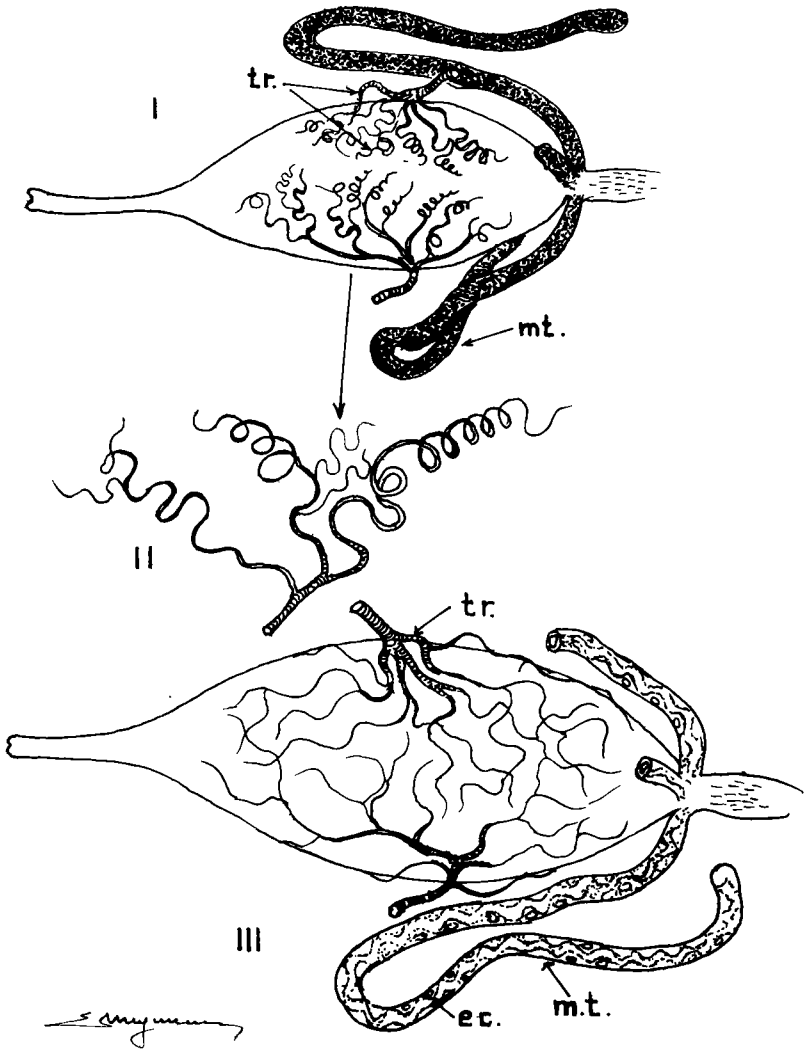


Fig. 55. I. Stomach of a nulliparous Anopheles female.

tr = tracheae; mt = Malpighian tubes filled with excretory granules (uric acid).

II. Enlarged trachea with terminal coiling from stomach of a nulliparous female.

III. Stomach of a parous female with the stomach much distended after the blood meal and the tracheae correspondingly stretched.

mt = Malpighian tubes at end of digestion phase; granules have been eliminated and excretory canal is plainly visible (ec).

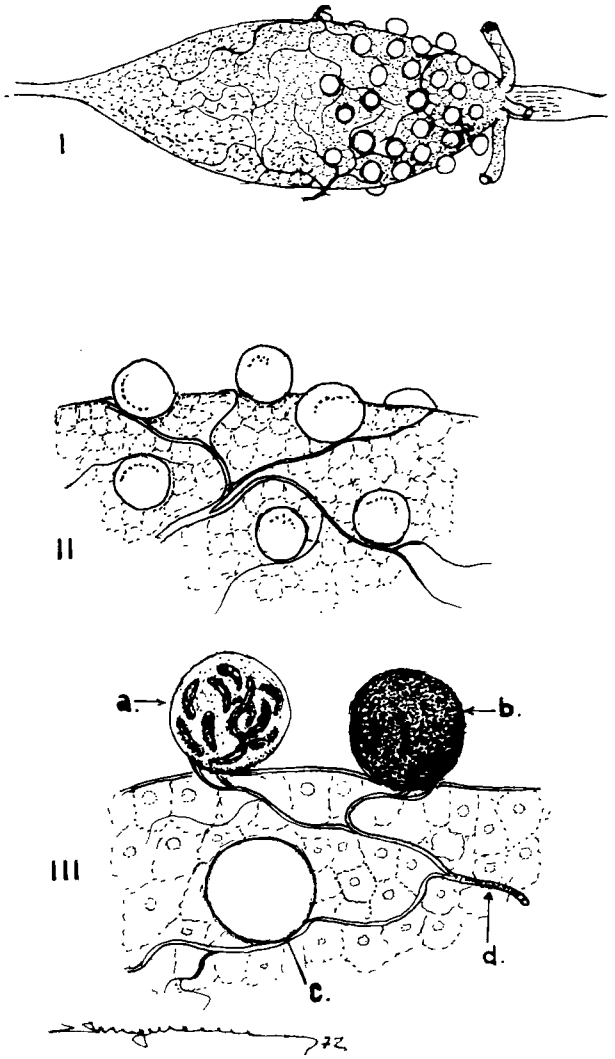


Fig. 56. Examination of stomach for oocysts.

I. Stomach with fully developed oocysts.

II. Young oocysts, 3-4 days old (P. falciparum).

III. a = black spores of Ross; b = black spores of Ross; entirely chitinised oocyst; c = normal oocyst; d = trachea.

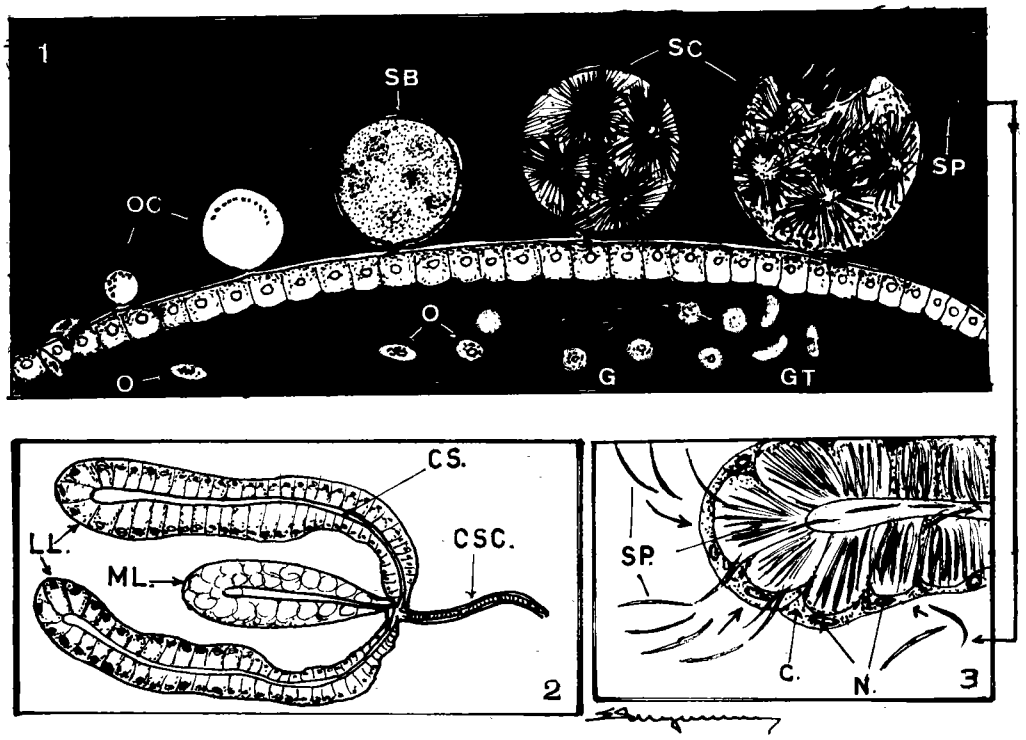


Fig. 57. (1) Sporogony: GT = gametocytes; G = gametes; O = zygotes and ookinetes
OC = oocysts; SB, SC = late stages in sporogony
SP = sporozoites.

(2) Salivary gland: ML = middle lobe; LL = lateral lobes
CS = salivary duct; CSC = common salivary duct.

(3) Sporozoites entering salivary gland: C = salivary gland cell
N = nucleus of gland cell; SP = sporozoites.

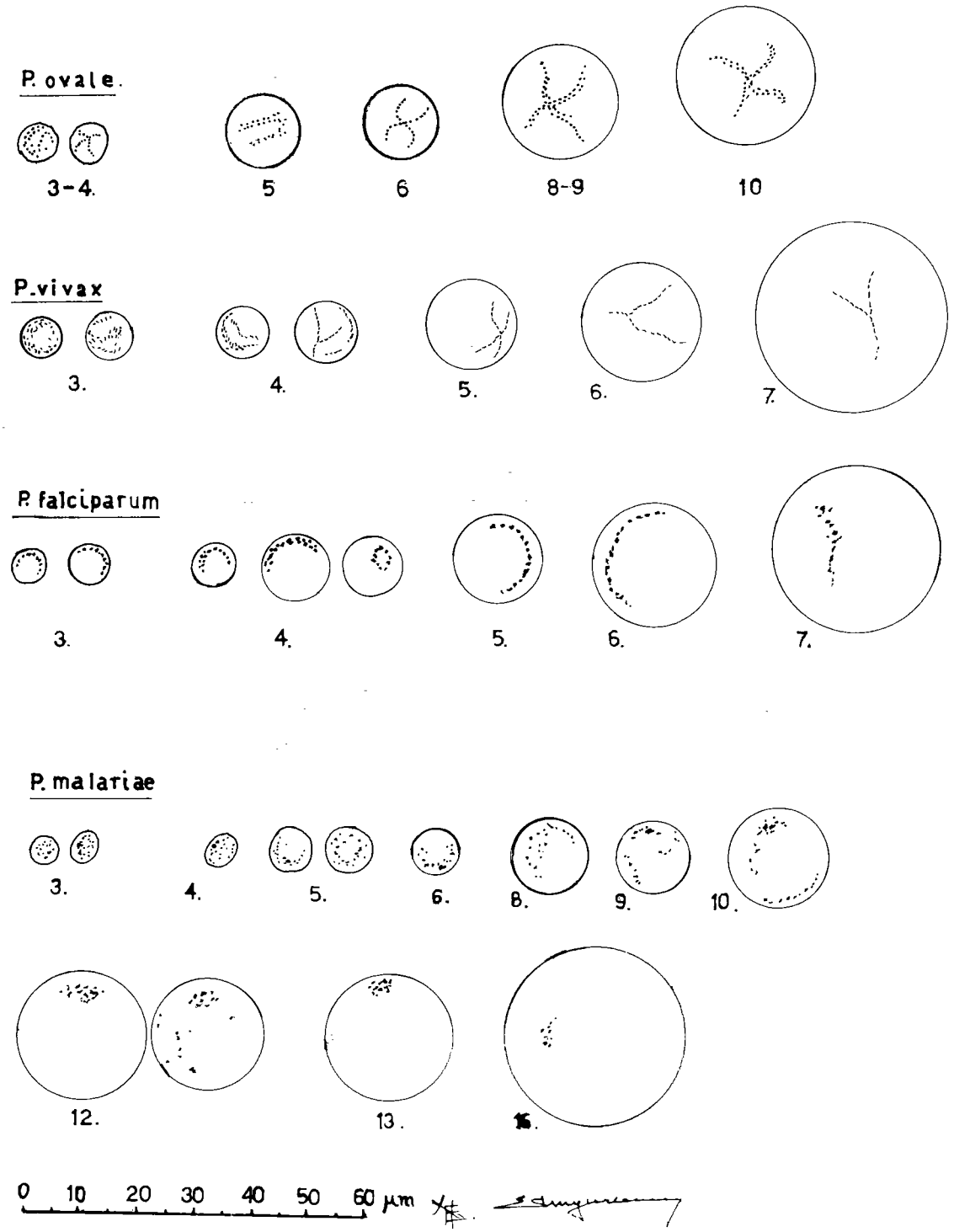


Fig. 58. Pigment pattern and dimensions of the oocysts of the human malaria parasites, the numbers giving the age in days.

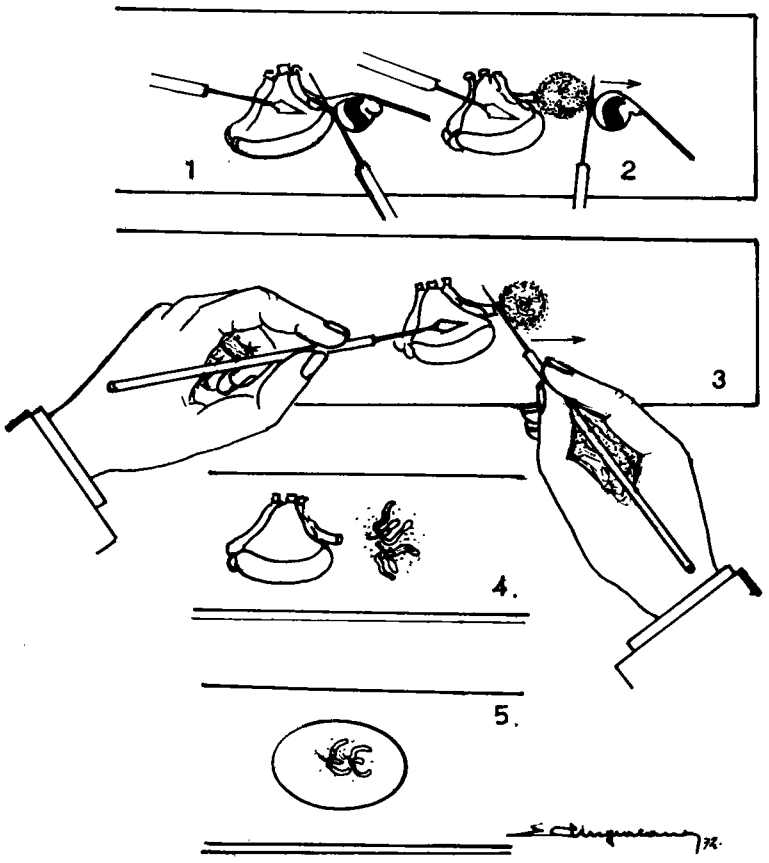


Fig. 59. Extraction of salivary glands - first method.

(d) Cut the neck close to the head with the right needle (2).

(e) Place a drop of saline (the size of a pinhead) close to the neck section.

(f) With the right needle press gently on the thorax a little above the left-hand needle in order to express the gland from the thorax. As soon as this occurs dip the point of the right-hand needle into the saline and bring it into contact with the glands - (3) and (4).

(g) Transfer the mosquito to the watch-glass or Petri dish containing the saline to await further dissection if necessary. Transfer the slide containing the salivary glands to the platform of the microscope and examine the specimen with a low-power lens, (if the dissection is carried out under a dissecting microscope or lens the presence of the glands is seen during the dissection).

(h) Place the coverslip on the glands (5) and examine with a 4 mm lens. The quantity of saline (0.65%) should not be excessive, just enough so that the coverslip presses the glands to rupture the cells but not to displace them. The sporozoites will start to come out from the gland cells if the gland is infected. When the gland cells are not ruptured by the pressure of the coverslip, press gently with the needle on the coverslip in order to disrupt the cells and to free the sporozoites. The sporozoites can be easily recognized (even inside the salivary gland cells) as very minute needle like forms (Fig. 61). They come out from the glands isolated or in clusters and they separate in saline solutions. On some occasions they might show slight movement at 26-27°C.

9.8.3 Procedure II (Fig. 60)

Steps (a), (b), and (c) as in procedure I. Then:

(d) Place the right needle on the neck of the mosquito without cutting it; then with light movements gently detach the head. The glands will come out from the thorax, attached to the head.

(e) Press the right needle in order to detach the glands from the head, and put aside the head and the thorax; verify if both glands were extracted entirely. (If the glands were not extracted by this procedure then press on the thorax as in procedure I.)

For the examination of the glands proceed as mentioned under procedure I (h).

If the sporozoites need to be stained for further confirmation etc., gently remove the coverslip after crushing the glands, leave to dry; fix with methanol for 1 minute and then stain for 40 minutes with Giemsa stain (or a similar one) and examine with an immersion objective.

9.9 Dissection of spermatheca

The spermatheca can be extracted when performing the extraction of the ovaries for Christophers' stages.

9.9.1 Objectives

- To identify the fertilized and unfertilized females and to establish the insemination rate.

- To identify some mosquito species.

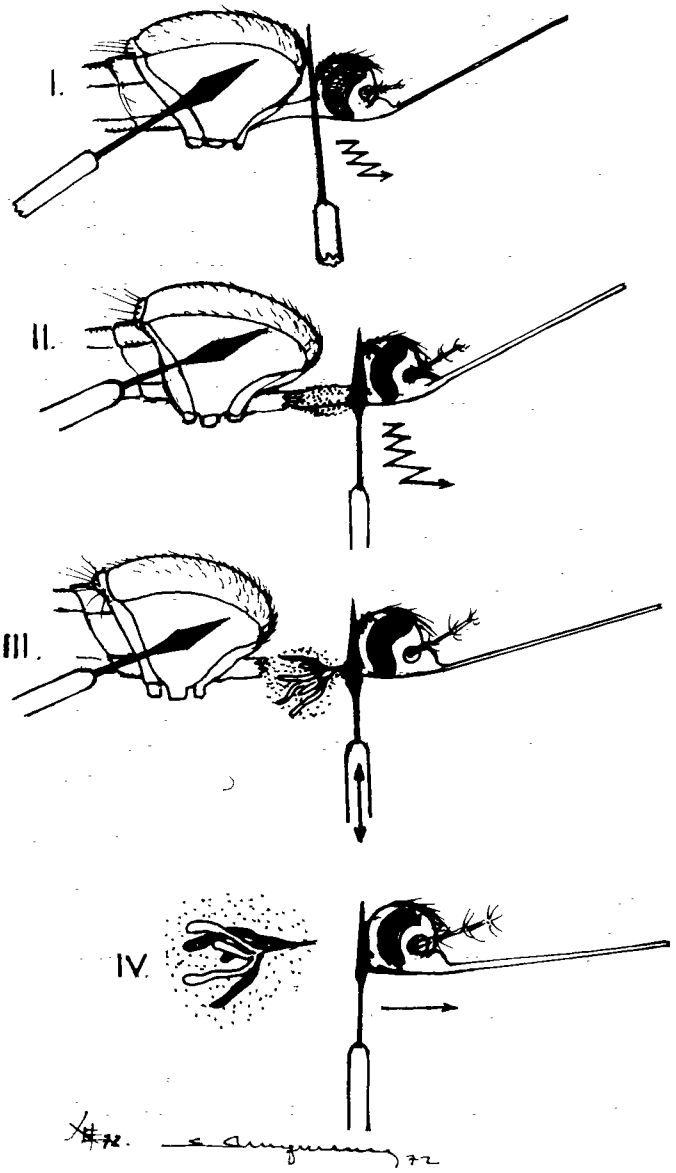
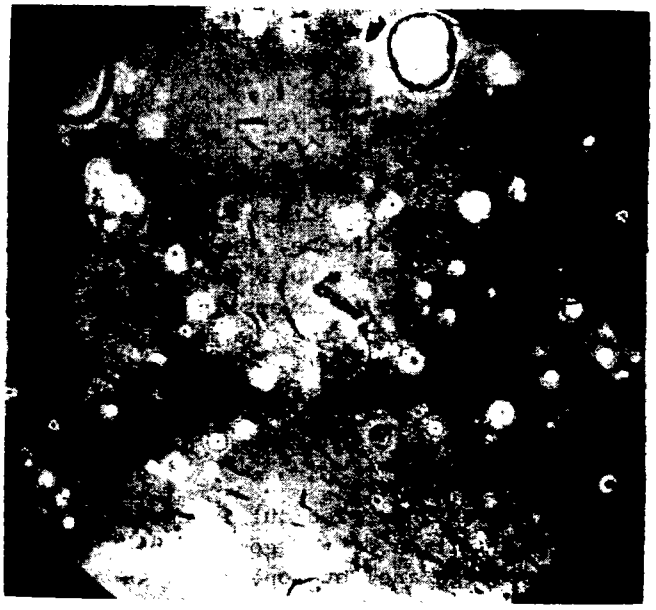


Fig. 60. Extraction of salivary glands, alternative method.



Photomicrograph of sporozoites in saline (X 600 approximately)

Photomicrograph of gregarine-like bodies seen in preparation of salivary glands (X 700 approximately)

Fig. 61. Sporozoites of a malaria parasite.

9.9.2 Extraction of spermatheca

The spermatheca in anophelines is a single, spherical, chitinous, perforated reservoir situated in the seventh abdominal segment, it can easily be recognized owing to its dark colour, varying according to species from light to dark brown, and it is extracted by dissecting the seventh segment - see Fig. 49 (4), (5) and (6). The spermatheca should be examined with an objective X40 and an ocular X5 or X7.

If the spermatheca is full of spermatozoa they appear as a packet of very long threads coiled inside. In fresh (unfixed) spermathecae, the mass of spermatozoa shows a rotating movement. If the mosquito was fixed, for example in formalin, or dried, the mass of spermatozoa does not move. By pressing the coverslip you can crush the spermatheca and the spermatozoa will emerge. The morphology of the spermatozoa is characteristic, - Fig. 49 (7). They possess a small head and a long mobile tail. An empty spermatheca is clear with no characteristic striated structure as described above (5).

9.9.3 Use

The examination of spermathecae is carried out in checking experimental mating; and when studying experimentally the duration of the gonotrophic cycle (females not developing eggs in spite of feeding, when no eggs are developed in a laboratory colony, etc.).

9.9.4 Interpretation of results

In general unfertilized females found during the active period of development of mosquitos are nulliparous. An unfertilized female can feed several times without ovary development even when kept at the optimum temperature. In an experimental study of the duration of gonotrophic cycle such females should be excluded.

10. THE STUDY OF VECTOR INFECTION AND INFECTIVITY

10.1 Introduction

Vector infection is established by demonstrating the presence of oocysts or sporozoites.

Vector infectivity is estimated by establishing the proportion of mosquitos found with normal sporozoites. The degree of natural infectivity of a vector species depends on:

- susceptibility of the vector to becoming infected when feeding on an infective host;
- degree of infectivity of the host (number of mature infective gametocytes circulating in the blood of the host at the time of feeding);
- contact with man;
- longevity of the mosquitos;
- climatic conditions (temperature of the mosquitos' resting place).

10.2 Objectives

- to incriminate the main or secondary vectors;
- to define the season of transmission and its variation;
- to evaluate the effect of mass drug administration on mosquito infectivity.

The study of infectivity can be carried out in two ways:

(a) Under natural conditions, by establishing the sporozoite index of mosquito populations collected in the field in different environmental conditions.

(b) Under experimental conditions, by laboratory observations being carried out for the following main purposes:

- to establish the duration of sporogony;
- to establish the susceptibility of different species of Anopheles to becoming infected with malaria parasites.

These might be carried out as follows:

- laboratory observations under constant temperature and humidity;
- laboratory observations with simulated field conditions;
- observations under field environmental conditions of experimentally infected mosquitos, kept in different containers (paper cups, nylon mosquito net cages, etc.).

It should be mentioned that experimental infections do not clearly indicate the real role of a species as a vector in nature even when experimentally the species concerned becomes infective with malaria parasites, because under field conditions the free contact of mosquitos with man and longevity determine the degree of transmission. This explains why some species which show experimentally a relatively high susceptibility to infection with malaria parasites may be important as vectors under natural conditions or may not transmit malaria at all.

10.3 Investigations for the incrimination of vectors

10.3.1 Objectives

To find the suspected vectors infected with sporozoites of human origin.

Suspicion is raised that an anopheline species might be a vector when the following aspects are found:

- the contact with man is high;
- the longevity is high enough for the completion of sporogony;
- infection with first stages of oocyst of human Plasmodia are found (immediate or delayed oocyst infection);
- the species is susceptible to infection under laboratory conditions.

Confirmation of suspected vectors is based entomologically on the detection of the oocysts and sporozoites of human origin, as well as on broader epidemiological grounds (persistence of transmission in the absence of vectors other than the suspected one).

10.3.2 Method of investigation

(a) Select a small human group with high parasite index (based on investigations of the population of the village) where contact between the suspected vector and man is high.

(b) Identify the houses with positive cases.

(c) Collect as many mosquitos as possible (by all efficient methods according to the local situation) from positive houses and, if they are widespread, start investigating the positive houses next to the area of the breeding places.

(d) Dissect the stomach and glands of all parous mosquitos (total processing of the sample). When the suspected vector is found infective with sporozoites, experimental infection with human malaria parasites might also be carried out if the possibility exists in the area that mosquitos may become infected with malaria parasites from other mammals.

10.3.3 Duration and timing

Investigations for the confirmation of vectors is not a routine study but a concentrated intensive study of a limited duration. Investigations should be carried out just after the density peak and the capture of mosquitos should be carried out daily or at a maximum of three-day intervals.[1] If the study is carried out well it should only need to be for one or two months and it is not necessary for it to be continued after the mosquitos have been found with sporozoites of human origin.

10.4 Investigations to define the transmission season

Seasonal malaria incidence in a local population, and mainly in infants, indicates indirectly the transmission season. It is a fact that the peak of transmission precedes by about one month the peak of malaria incidence. A more precise definition of the transmission season can be made by interpreting the significance of the malaria incidence in the light of entomological information on the following points:

- identification of vectors or vector;

- seasonal vector(s) density, mainly the seasonal incidence of the contact of vector(s) with man;

- climatological data;

- sporozoite index (only if accurately carried out) and mosquito infectivity.

10.4.1 Method of investigation

Such investigations are carried out in indicator localities (medium or small size) having a high parasite index (which should, if possible, be evaluated at 3-month intervals by examining infants and children from 2-10 years old. The selection of the capture stations for exit-trap and pyrethrum spray collections and for collection on human baits is made on the same criteria as those mentioned for the investigations for the seasonal density of vectors.

10.4.2 Duration and timing

Such observations have to be carried out for 1 year as a minimum, including two transmission seasons. Since the study is carried out routinely by dissecting the mosquitos collected during the study of the vector seasonal density, the dissection will be carried out at 2-week intervals for operational reasons.

10.4.3 Processing

The salivary glands of all parous females, plus females which are unidentifiable for parous rate, should be dissected. When the identification of parous females is more time-consuming than the dissection of all collected vector females, glands should be dissected without waiting for the results of identification of parous/nulliparous status.

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[1] The proportion of infective mosquitos is in general higher after than during the peak density; prolonged life of mosquitos and smaller daily influx of nulliparous mosquito females which normally occurs after the period of peak density are the main causes.

10.5 Study of the susceptibility of an anopheline species to human plasmodia

The susceptibility of mosquitos to infection with malaria parasites seems to be influenced by several factors:

- genetic constitution of mosquitos which confer on each species some morphophysiological characteristics which might be favourable or unfavourable to infection with plasmodia;
- size of blood meal;
- number and infectivity of gametocytes;
- temperature.

Another important aspect which merits mention with regard to the susceptibility of anophelines to human malaria parasites is the adaptation of a local strain of malaria parasite to develop more readily in a local vector than in a vector from a remote area. It is well known that some strains of P. falciparum from India and Africa do not infect A. (L) atroparvus; whereas the Italian strain infects this species in a very high proportion.

The susceptibility of an anopheline to human plasmodia can be assessed with accuracy only by experimental infection.

10.5.1 Experimental infection of Anopheles species with malaria parasites

10.5.1.1 Principle

Feeding of mosquitos on a parasite reservoir whose blood contains mature gametocytes and keeping them at the optimum temperature and humidity necessary for sporogony.

10.5.1.2 Objectives

- To study the susceptibility of a mosquito species to malaria infection.
- To study the speed of development of the different stages of malaria parasites in mosquitos.
- To ascertain the infectivity to local vectors of the inhabitants in an endemic area.
- To provide infective material for the study of exoerythrocytic cycle of malaria parasites.
- To provide infective material for malaria therapy or for various laboratory studies.

10.5.1.3 Materials required

(a) A colony of anopheline mosquitos, or, in absence of a colony, mosquitos can be bred out in the laboratory from larvae and pupae collected in nature.

(b) Suitable jars (9 cm in diameter) or small cages for feeding purposes (8 x 8 x 15 cm) made of nylon gauze with an iron frame.

(c) Test tubes 127 mm x 12 mm or sucking tubes.

(d) Cages with an iron frame, 30 x 30 x 30 cm or 20 x 20 x 20 cm, with all the sides covered with mosquito netting and the front provided with a netted sleeve through which the mosquitos are introduced after feeding.

10.5.1.4 Procedure

(a) Verify that the suitable host has numerous mature male gametocytes by making the exflagellation test. A suitable host for a heavy infection of vector should have not less than 200 exflagellating male gametocytes per mm^3 (approximately 5-10 micro-gametocytes per 100 leucocytes in a host showing about 6000 leucocytes per mm^3). It should be noted however that mosquitos have been infected by infected subjects in whom gametocytes were not detectable in normal thick blood films.

(b) After a suitable host has been selected, place 50 females of the species to be tested (3 days old) in a glass jar or 100 females in a small cage. Apply this to the body of the host allowing the mosquitos to feed for about 20 minutes (Fig. 62). Use not less than 150-200 females for each trial.

(c) Remove the jar or the cage from the host and separate the fed females into a bigger cage in which they will be kept during the experiment.

Under field conditions when the biting density is high, mosquitos biting on an infective host during the night may be collected and kept for the study of susceptibility to infection under given field conditions but care should be taken to leave the mosquitos to have a complete blood meal before collecting them from the host. This method could also be used for studying the infectivity of different human hosts under field conditions. For the study of the infectivity of a human host and of the susceptibility of mosquitos to infection, the mosquitos should be dissected starting on the second day after the infection; a preliminary investigation concerning the presence of gametocytes should be made.

(d) Two days after the infective meal, feed the mosquitos on 5% sugar solution until the end of the experiment. The advantage of this method is that the females feed readily and practically in totality on sugar solution and mortality in the batch is in general very low. The oocysts can be detected easily in all stages. One disadvantage is that the sugar solution might be infected with fungus. This can be avoided by changing the solution frequently (every 2 days) and the cotton wool before the fungus appears (usually in the form of dark brown points on the cotton wool). They should be kept at a favourable temperature of 25-27°C and at about 75-80% R.H. or under field conditions.

The observations are then carried out by dissecting all the mosquitos which had a meal on an infective host. The oocyst index will be established by dissecting the stomach and the sporozoite index by dissecting the glands. In two days the infective meal is usually digested and the young oocysts can be observed on the surface of the stomach, using a X40 or X70 objective lens. In order to establish accurately the number of mosquitos with oocysts it is preferable to dissect daily the dead or dying mosquitos found after the blood meal has been digested. Oocysts of 6-7 days will be seen more easily than younger oocysts of 2-3 days as they are bigger. The total oocyst rate should be calculated by adding together the number of mosquitos found with oocysts in different stages as well as the number of mosquitos found with sporozoites in the glands. When only the infections with oocysts are studied, the whole batch of mosquitos can be dissected 7-8 days after the infective meal (during which period 50-70% of the mosquitos will survive - Shute & Maryon, 1966). To establish the number of infective mosquitos, glands will be dissected at the end of the estimated interval necessary for the appearance of the sporozoites in the gland. This interval depends on temperature and on the species of Plasmodium (see Tables 3 and 4). The whole batch of mosquitos can be dissected for sporozoites at 3 days after the detection of the first infected mosquitos with mature oocysts on the stomach and sporozoites in the glands, in order to establish the sporozoite rate.

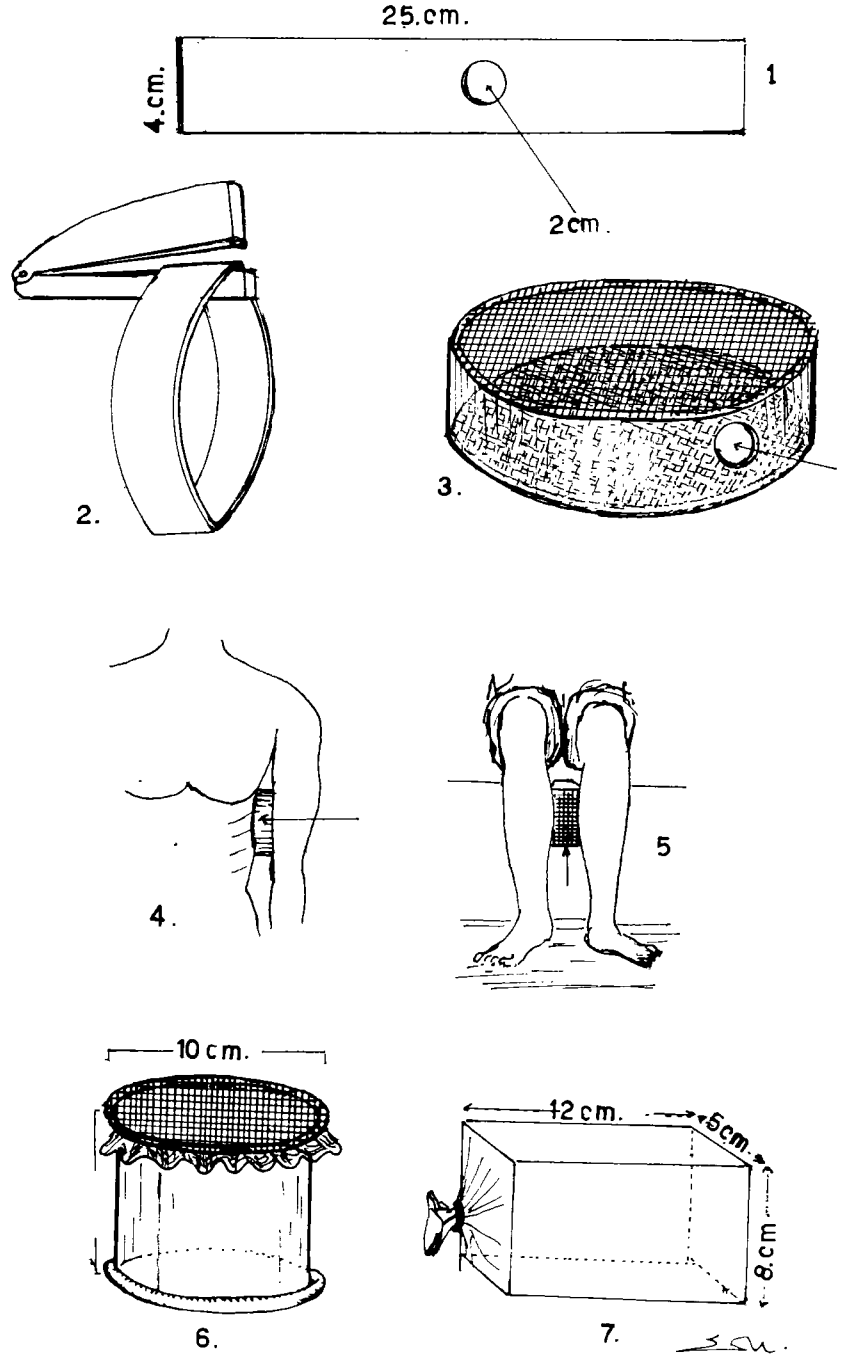

Fig. 62. Construction and use of jars or small cages for feeding purposes.

(1) Strip of cardboard or plastic,
(2) Stapling to form ring,
(3) Completed feeding cage,
(4)-(5) Use of feeding cage,
(6)-(7) Glass jar or cage may also be used.

10.5.1.5 Artificial feeding of mosquitos on defibrinated blood infected with mature gametocytes, through a membrane or from capillary tubes

The use of an organic membrane has proved very practical for artificial feeding of mosquitos. Animal membranes have been used and one of the most satisfactory appears to be the baudruche membrane (a piece of the intestine of cattle or sheep). This membrane, after having been cleaned and softened in physiological saline, is placed at one of the open ends of a glass or plexiglass tube about 3 cm in diameter x 10 cm high around which copper tubing 4-5 mm in diameter is coiled in several spirals. To both ends of the copper tubing a rubber tube is attached through which water is circulated at about 38-39°C. The glass tube is covered with a thin sheet of copper before being inserted between the copper spirals. This apparatus is shown in Fig. 63; an alternative method is illustrated in Fig. 64. About 2-3 cm of defibrinated blood is put in the tube and a thermometer is inserted in order to check that the blood is at a temperature of about 37°C.

10.6 Estimating the duration of sporogony

10.6.1 Direct estimation

This can be done under experimental conditions, following the methods described in the chapter dealing with experimental infection of anopheline species with human plasmodia.

(a) This study can be performed under constant temperature conditions in order to observe the speed of development of the different sporogonic stages, or under the variable temperature and humidity conditions that occur under field conditions.

(b) The mosquitos, after feeding on an infective host, are placed in small glass jars, paper cups or nylon gauze cages, protected against the attack of ants, and fed with sugar solution two days after the infective meal. All dying or dead mosquitos are dissected daily for stomach infections (dry mosquitos are dissected after softening them in detergent). In addition a few live mosquitos should be dissected every second day.

The presence of full mature oocysts on the surface of the stomach and with glands still negative, will indicate that in about 24 hours the sporozoites will penetrate the glands, if the temperature is about 25-26°C; if full mature oocysts are found, the number of mosquitos dissected for gland infection should be increased on the following day, and the whole batch of mosquitos can be dissected 3 days later, after the detection of the first mosquito infected with sporozoites.

The duration of sporogony is represented by the number of days which have elapsed between the infective meal and the date when the first salivary gland containing sporozoites was found.

The temperature and relative humidity inside the microhabitat where the infected mosquitos are kept should be recorded with a thermohygrograph.

TABLE 3. AVERAGE DURATION OF SPOROGONY OF HUMAN PLASMODIA

Parasite species	Temperature	No. of days	Temperature	No. of days
P. falciparum	20°C	22-23	25°C	12-14
P. vivax	20°C	16-17	25°C	9-10
P. malariae	20°C	30-35	25°C	23-24
P. ovale	-	-	25°C	15-16

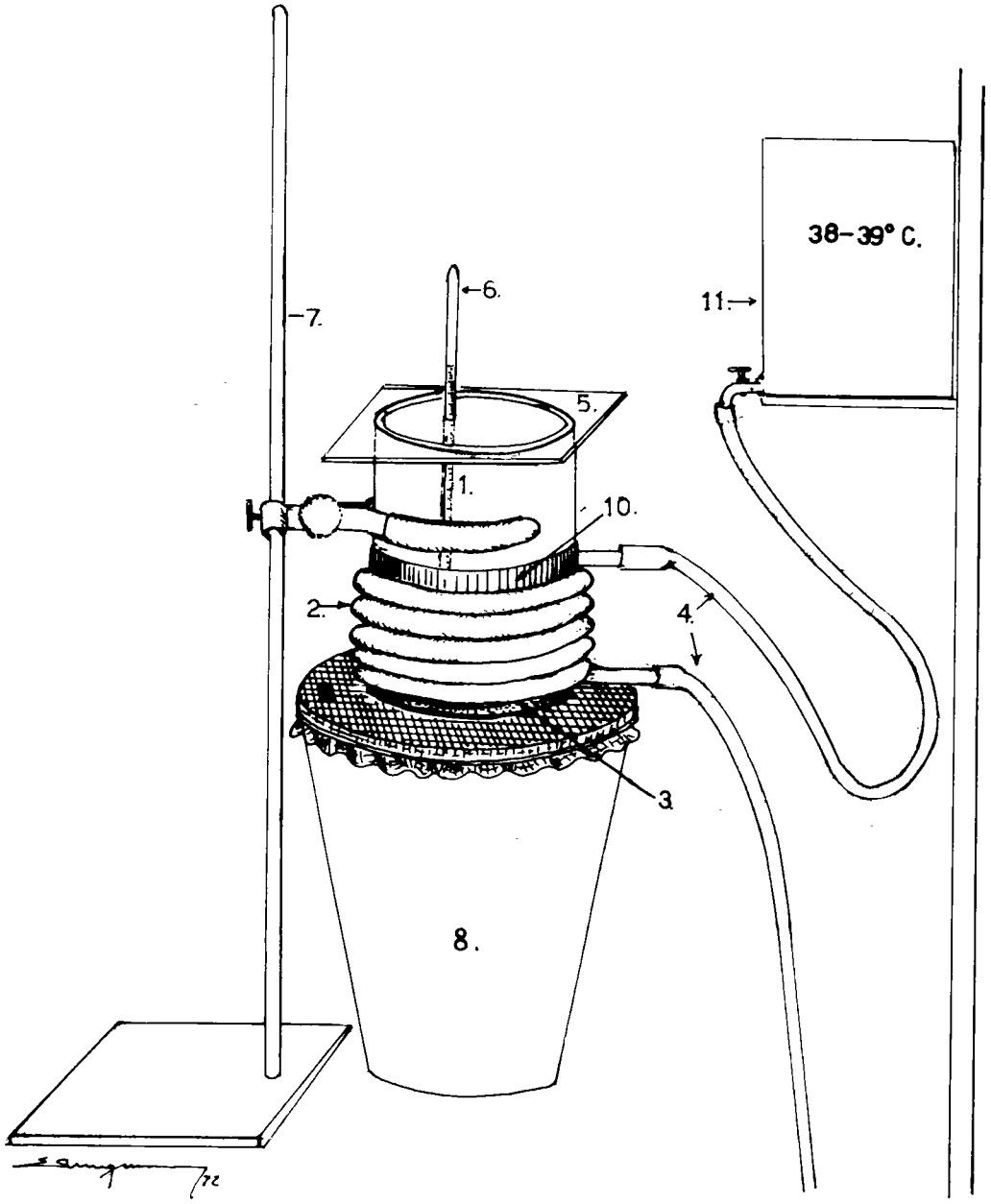

Fig. 63. Membrane feeding apparatus.

(1) glass or plastic container for blood; (2) copper heating coil (3) membrane; (4) rubber tubing; (5) glass plate; (6) thermometer; (7) stand; (8) paper cup with mosquitos; (9) mosquito net; (10) copper foil; (11) water tank.

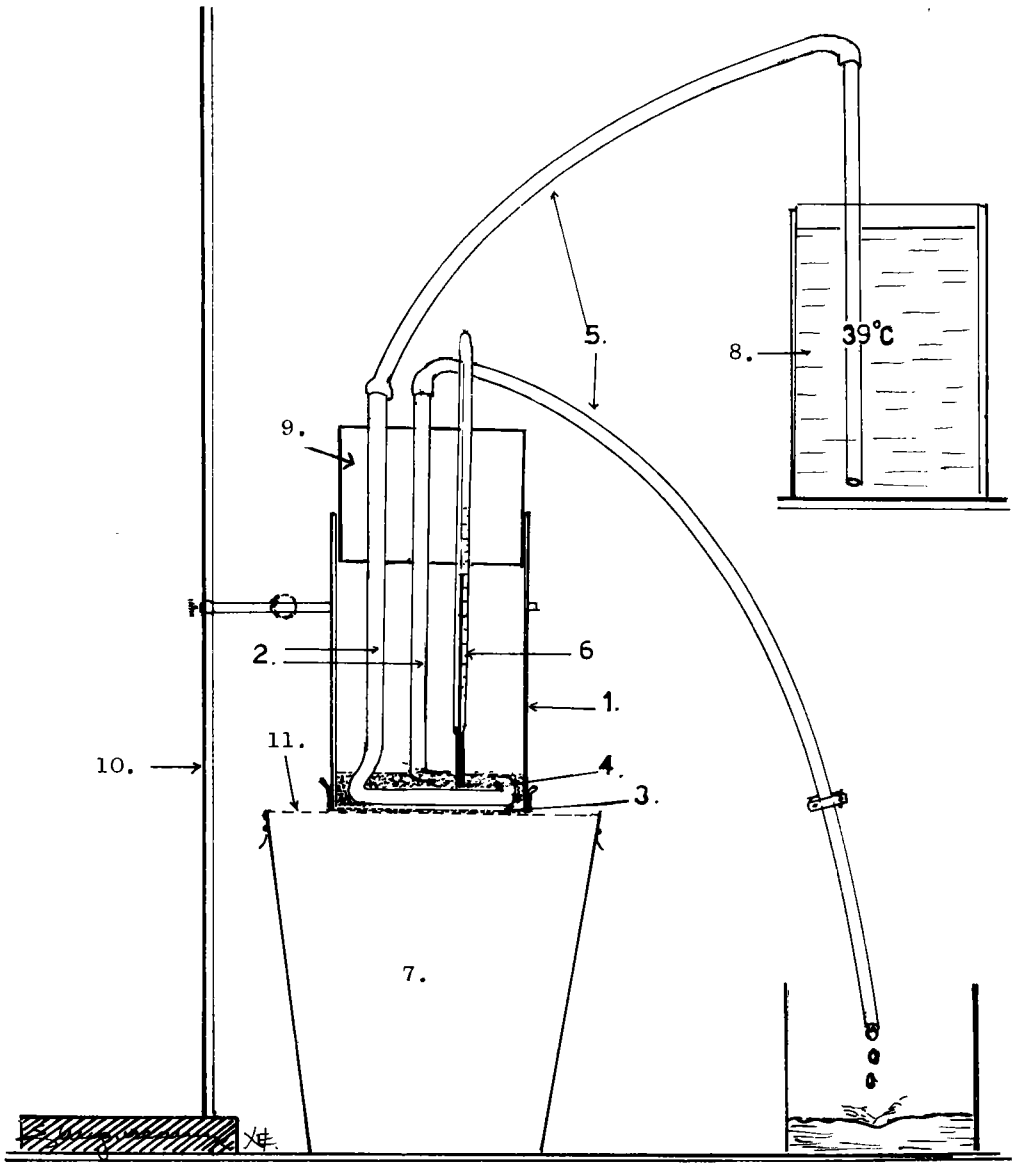

Fig. 64. Membrane feeding apparatus, alternative method.

(1) glass container; (2) heating coil; (3) membrane;
(4) blood; (5) rubber tubing; (6) thermometer;
(7) paper cup with mosquitos; (8) water reservoir;
(9) rubber or cork stopper; (10) stand; (11) mosquito net.

10.6.2 Indirect estimation

Another method is the indirect estimation of the duration of sporogony in natural habitats, based on studies of the temperature in such habitats. The determination of the time required for sporogony in the natural habitats is of considerable importance from the point of view of malaria epidemiology. It is well known that changes in environmental temperature have a direct effect on the development of plasmodia during the extrinsic part of their cycle and that the sporogony of P. vivax and P. falciparum does not take place at temperatures lower than 14.5°C and 16°C, respectively. During cold weather or at high altitudes an important difference in temperature can exist between indoor and outdoor resting places (microclimatic effects).

Russian malariologists have recommended two methods for indirect estimation of the duration of sporogony under natural conditions.

10.6.2.1 Moshkovsky's method. This is based on the calculation of the "sum of heat" represented by the total number of degree-days in a given period. The "degree-day" is the number of degrees by which the mean temperature of the day concerned exceeds the lower threshold temperature for the development of a given species of Plasmodium.

The estimated lowest development threshold in 24 hours was considered as being 14.5°C for P. vivax and 16°C for P. falciparum. The degree-day is obtained by subtracting the value of the lowest 24-hour threshold temperature from the daily mean temperature recorded in a given resting place. If the mean temperature is 25°C, the degree-day for P. vivax will be 25-14.5 = 9.5°C. The sum of heat in degree-days necessary for the sporogonic cycle is 105°C for P. vivax, 111°C for P. falciparum and 144°C for P. malariae. The duration of sporogony is represented by the number of days necessary to obtain the sum of heat required by a given species, as mentioned above (see Table 4).

10.6.2.2 Oganov-Rayevsky method. This method is based on the calculation of the daily percentage of the development of the complete sporogonic process at different temperatures. The number of days necessary to reach 100% of the development represents the duration of sporogony. This is obtained by measuring the mean daily temperature of the resting places, and reading the proportion of the daily development of the Plasmodium in Table 5. This type of calculation is illustrated in Table 6.

In temperate zones the calculation of duration of sporogony of P. vivax and P. falciparum begins when the average temperature reaches 16°C and 18°C, respectively. Sporogony is considered to stop when the daily average temperature falls below these temperatures.

It should be mentioned that the parameters for the indirect calculation of the duration of the sporogony of malaria parasites have been calculated on the basis of direct experimental observations.

11. DETERMINATION OF THE DURATION OF THE GONOTROPHIC CYCLE

11.1 Definition

The gonotrophic cycle is defined as a physiological process consisting of digestion of food and development of the ovaries.

When the digestion of blood is accompanied by the development of the ovaries this is known as gonotrophic concordance; when the blood is digested and the ovaries do not develop such a phenomenon is known as gonotrophic discordance. Gonotrophic discordance is typically seen in hibernating (non-active) females during the winter, due to exposure to a short photoperiod, but can also occur in some species under hot dry conditions. This condition may last several months.

TABLE 4. THE MOSHKOVSKY METHOD FOR CALCULATING THE DURATION OF SPOROGONY[a]

Date	Mean diurnal temperature		Degree-days			
			Plasmodium vivax		Plasmodium falciparum	
	External air	Day resting-place	External air	Day-resting place	External air	Day resting-place
20 June	19.0	20.0	4.5	5.5	3.0	4.0
21 June	21.5	21.0	7.0	6.5	5.5	5.0
22 June	22.5	22.5	8.0	8.0	6.5	6.5
23 June	23.0	22.5	8.5	8.0	7.0	6.5
24 June	22.5	22.0	8.0	7.5	6.5	6.0
25 June	22.0	21.0	7.5	6.5	6.0	5.0
26 June	24.5	22.5	10.0	8.0	8.5	6.5
27 June	25.0	23.0	10.5	8.5	9.0	7.0
28 June	25.5	21.0	11.0	6.5	9.5	5.0
29 June	24.0	22.5	9.5	8.0	8.0	6.5
30 June	24.5	22.0	10.0	7.5	8.5	6.0
1 July	25.0	22.0	10.5	7.5	9.0	6.0
2 July	25.0	23.0	105.0	8.5	9.0	7.0
3 July	26.0	23.0		8.5	10.0	7.0
4 July	23.0	24.0		105.0	7.0	8.0
5 July	26.0	25.0			111.0	9.0
6 July	27.0	26.0				10.0
						110.0

[a] After Moshkovsky & Rashina (1951).

TABLE 5. DURATION OF SPOROGONY OF PLASMODIUM VIVAX IN ANOPHELES MOSQUITOS AT VARIOUS TEMPERATURES[a]

Temperature in °C	Development per day as a percentage of the completed process	Duration of the whole process in days	Temperature in °C	Development per day as a percentage of the completed process	Duration of the whole process in days
16	1.82	55	19.5	4.55	22
16.1	1.89	53	19.6	4.65	21.5
16.2	1.96	51	19.7	4.76	21
16.3	2.04	49	19.8	4.88	25
16.4	2.13	47	19.9	5	20
16.5	2.22	45	20	5.26	19
16.6	2.27	44	20.1 - 20.3	5.4	18.5
16.7	2.35	42.5	20.4 - 20.6	5.55	18
16.8	2.44	41	20.7 - 20.9	5.74	17.5
16.9	2.5	40	21	5.8	17
17	2.6	38.5	21.1 - 21.3	6.06	16.5
17.1	2.7	37	21.4 - 21.5	6.25	16
17.2	2.78	36	21.6 - 21.8	6.45	15.5
17.3	2.86	35	21.9	6.66	15
17.4	3.03	33			
17.5	3.12	32	22	6.66	15
17.6	3.17	31.5	22.1- 22.2	6.9	14.5
17.7	3.22	31	22.3 - 22.4	7.14	14
17.8	3.33	30	22.5 - 22.8	7.7	13
17.9	3.39	29.5	22.9	8	12.5
18	3.45	29	23	8	12.5
18.1	3.51	28.5	23.1 - 23.2	8	12,5
18.2	3.57	28	23.3 - 23.6	8.33	12
18.3	3.64	27.5	23.7 - 23.9	8.7	14.5
18.4	3.85	26	24	9.09	11
18.5	3.85	26	24.1 - 24.4	9.09	11
18.6	3.92	25.5	24.5 - 24.9	9.52	10.5
18.7	3.92	25.5	25 - 25.5	10	10
18.8	4	25	25.6 - 25.9	10.52	9.5
18.9	4	25	26 - 26.4	11.11	9
19	4.08	24.5	26.5 - 26.9	11.8	8.5
19.1	4.16	24			
19.2	4.26	23.5	27 - 27.4	12.5	8
19.3	4.35	23	27.5 - 27.9	13.3	7.5
19.4	4.44	22.5	28	14.2	7

[a] After Detinova, 1962. Calculated by the method of Oganov-Rayevsky (Oganov, 1947).

TABLE 6. METHOD OF DETERMINING UNDER FIELD CONDITIONS THE DURATION OF SPOROGONY OF PLASMODIUM VIVAX

Date in July	Mean diurnal temperature in °C	Development of sporogony during one day as a percentage of the completed process
15	23.0	8.0
16	24.0	9.09
17	25.5	10.00
18	24.9	9.52
19	23.5	8.33
20	23.3	8.33
21	22.9	8.0
22	22.7	7.7
23	21.0	5.8
24	23.0	8.0
25	24.5	9.52
26	23.0	8.0
		100.29
27	19.5	4.55
28	19.7	4.76
		101.6

Duration of sporogonic cycle

Duration of sporogonic cycle

The normal development of the ovaries requires three favourable factors - temperature, a full blood meal and fertilization. A full blood meal appears to stimulate the excretion of a gonadotrophic hormone by the corpora allata[1] and neurosecretory cells of the brain, and this influences the development of the ovaries. A female mosquito has several gonotrophic cycles during her active life.

The duration of each gonotrophic cycle in gono-active females depends on:

- time required to find and feed on a host,
- time required for the digestion of the blood and ovary development,
- time until oviposition.

Temperature has an important influence on the duration of both blood digestion and ovary development, these being slowed up at low temperature. Low humidity appears to increase the duration of these processes.

In tropical areas where the temperature is constant for long periods the duration of the gonotrophic cycle varies less than in the temperate zones. Differences in the duration of the gonotrophic cycles occur in the tropical areas during the dry and wet seasons. There are some instances when females might take two blood meals before laying eggs, therefore the duration of the gonotrophic cycles will be increased in such cases.

11.2 Need for the study of the gonotrophic cycle

Such a study is needed for the estimation of the frequency of mosquito contact with man. If the gonotrophic cycle is 2 or 3 days it means that the mosquito will feed on average every 2 or 3 days; this has an important effect on the transmission of the disease. Therefore the duration of the gonotrophic cycle is one of the parameters needed for the calculation of the transmission potential (vectorial capacity). It is also needed for the calculation of the calendar age of mosquito females.

11.3 Estimation of the average duration of the gonotrophic cycle

11.3.1 Indirect method. This consists of establishing the proportion of fed-gravid resting mosquitos. This method can only be applied with endophilic species. In an average 2-day gonotrophic cycle, the abdominal appearance should be as follows:

- With a 2-day gonotrophic cycle it is expected that in the morning at 07.00-08.00 hours only late fed and sub-gravid and gravid females will appear.
- With a 3-day cycle an important proportion of half gravid females will be found in the morning, 07.00-08.00 hours, and more or less equal numbers of fed and gravid mosquitos.

It should be stressed that the first gonotrophic cycle lasts at least half a day longer than the subsequent gonotrophic cycles in parous females because in parous females the ovaries are already in the middle or late Christophers' stage II. If we add to this interval a minimum of half a day till the newly emerged female will feed, the first gonotrophic cycle will be longer, by at least one day, than the gonotrophic cycle in parous females. Since the time of egg laying and feeding might vary according to several ecological factors, it would appear reasonable to admit that the duration of subsequent gonotrophic cycles in the same female mosquitos will present some variations. There will be at the same time in the same population of mosquitos some females with a gonotrophic cycle of 3 days, some with a cycle of 2 days and

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[1] Corpora allata are small neurosecretory glands located behind the brain (which also secrete the juvenile hormone).

some with a cycle of 4 days duration due to the variability of the duration of the first and third periods mentioned above. When mosquitos rest in different habitats with different temperatures (e.g., with a variation of 4-5°C between one resting habitat and another), the duration of the gonotrophic cycle will also be influenced and will vary accordingly. Another difficulty in establishing with accuracy the significance of the abdominal stages in the determination of the duration of the gonotrophic cycle is the fact that the female mosquitos do not feed only for a short period of the night; a few species feed in high proportions during the first hours of the night, some feed all night and in many species there is normally a second biting peak early in the morning.

The dependence on factors mentioned above implies that we only obtain an approximate figure from these indirect estimations, which indicate the trend of the duration of the gonotrophic cycle but do not give a precise figure that may be applied to the whole population of mosquitos at a given time.

11.3.2 Direct method. Direct estimation of the duration of a single gonotrophic cycle not including the time between the complete maturation of eggs and egg laying.

An indirect estimation of the duration of a single gonotrophic cycle should be completed with direct observations which could be carried out as follows:

11.3.2.1 Laboratory observations

One or two batches of 100 freshly fully fed nulliparous females (laboratory bred and fertilized)[1] should be isolated in paper cups or paper cages (5-10 females per cup) and kept in the laboratory under known temperature and humidity conditions. Every 12 hours the abdominal stages should be recorded and a few females dissected every time to observe the Christophers' stages. Such an experiment should be repeated with parous laboratory bred females.

11.3.2.2 Field observations

(a) Several batches of about 25 freshly fully fed females are placed in paper cups or cages and suspended inside and outside in a normal outdoor resting place of mosquitos in the vegetation.

Three batches of mosquitos should be used in the same spot - the first batch of fully freshly fed mosquitos should be placed early in the evening during the first hours of biting, the second batch in the middle of the night and the third batch early in the morning during the second peak of biting, observations should be made every 12 hours.

The same field observations should be carried out with wild freshly fully fed mosquitos at the same times as previously mentioned. Observations on the abdominal appearance should be carried out at 06.30 hours and 18.30 hours. At the end of the observations, the whole batch should be dissected in order to establish the proportion of unfertilized females, primiparous and multiparous females.

Remarks. Wild collected mosquito females will include a variable proportion of fertilized nulliparous and parous females and a number of nulliparous unfertilized females which in some cases might reach 10-16% of a sample. Unfertilized females will develop eggs much slower than the fertilized females or will not develop them at all, even after several feeds. Parous females will reach the gravid stage faster since the ovaries of the females which feed immediately after laying eggs will have the ovaries in the Christophers' stage II. All females not developing eggs or developing eggs very slowly should be examined for insemination by examining the spermatheca for the presence or absence of spermatozoa.

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[1] Nulliparous females could be obtained from laboratory colonies or bred out from larvae collected in nature and mated artificially.

(b) The average duration of successive gonotrophic cycles in nature can be studied by using marked, newly emerged females released in nature and then collected and examined for ovary development. This could be carried out in an area with limited breeding and a small isolated group of houses far away (more than 2 km) from other human habitations.

Collection of mosquitos will start on the third day after release and will continue every day until no more marked females can be found for 3-4 successive days. All parous females should be dissected to observe the number of dilatations. The average duration of the gonotrophic cycle will be obtained by dividing the number of days since the release of mosquitos by the number of dilatations.

(c) Another method for direct observation is to release newly emerged nulliparous females and males of stenogamous species in a big cage with an attractive host and an attractive breeding place. Starting three days after release a number of female mosquitos should be dissected every second day, or all unfed or freshly fed females could be dissected 10 or 15 days after release, in order to count the dilatations of the ovariole. This second type of observation is less time-consuming and sufficiently accurate for the direct study of the average duration of the gonotrophic cycle.

The big cage could cover a small part of a natural breeding place from which the larvae will be removed daily. Apart from a source of food for mosquitos, an artificial resting shelter should also be provided (see Fig. 65).

The mosquito resting place communicates with the cage; this might be a barrel or a wooden box covered with earth, to be protected from the sun and rain.

The temperature and humidity should be carefully recorded inside the resting place, and inside the cage.

Remarks

The calendar age of mosquitos is calculated by multiplying the number of dilatations of the ovariole by the average duration of a gonotrophic cycle.

12. IDENTIFICATION OF BLOOD MEALS

12.1 Principle

The identification of the source of the blood meal by precipitin tests based on the serological characteristics of plasma or in some cases by microscopic examination (for detecting the blood of birds and reptiles).

12.2 Objectives

(i) To evaluate the actual feeding or the feeding preference on different hosts.

(ii) To establish the human blood index (necessary for the estimation of biting frequency on man).

12.3 Examination of red blood cells

In practice this method is restricted to establishing the presence or absence of nucleated red blood cells, in order to distinguish avian and reptilian (or amphibian) hosts from mammals. The undigested blood meal is smeared directly onto a slide and stained in the standard way with Giemsa stain. It can also be examined directly in a drop of 0.9% physiological saline, the blood cells of birds or reptiles being easy to identify (nucleated).

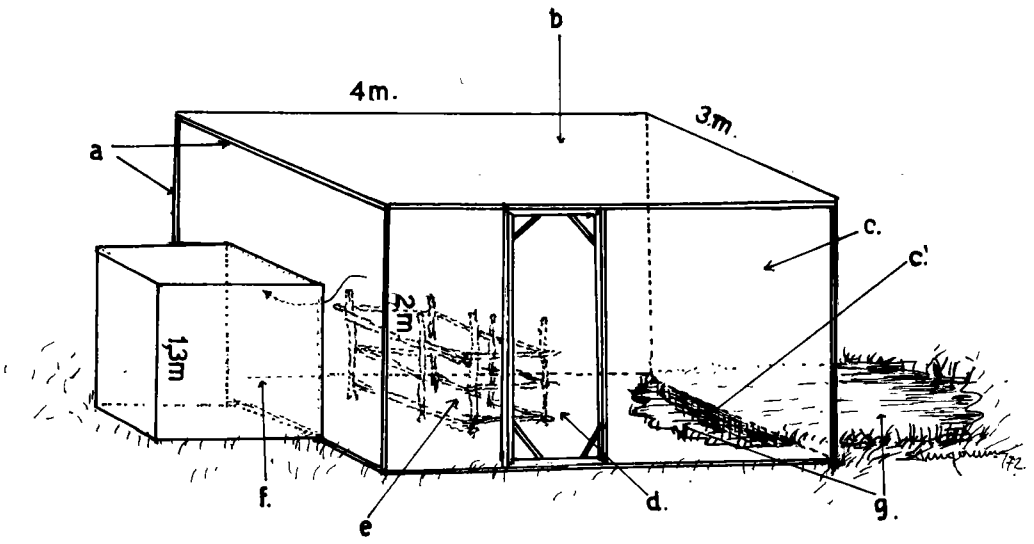

Fig. 65. Cage for study of duration of gonotrophic cycle under field conditions.

a = wooden frame; b = netting roof; c = netting wall

c' = extension of netting to seal off part of breeding site

d = door; e = pen for host; f = shelter for mosquitos

g = breeding site.

12.4 Study of the serological characters of blood meals

This study is possible due to the fact that when the serum of the blood ingested by a mosquito is put in contact with a specific antiserum, a precipitation occurs at the place of contact.

12.4.1 Collection of blood smears for precipitin tests

Such a study may be carried out in two ways:

- by collecting a standard number of about 50 blood samples from a mosquito species, or
- by collecting blood samples at short intervals of 2-3 days, 3-4 times during a representative period (choosing transmission periods) from all available mosquitos collected from representative resting places, such as houses, animal shelters and mixed dwellings with different animals species, outdoor resting places, traps, etc. At least 100 samples should be collected at a time and place.

The second method is recommended. Nevertheless in both cases the following information should accompany the collection:

(a) Place of collection and identification number.

(b) Number of human beings at the place of collection.

(c) Number of animals and their species existing inside the place of collection.

(d) Number of animals by species in the immediate vicinity of the place of collection (2-15 m).

(e) Number of animals more than 15 m away (specify the distance).

(f) The date of collection.

(g) The name of the locality and the number of each smear is written on each filter paper.

Code letters can be used in addition to numbers.

Only fresh fed or late stage fed mosquitos collected by PSC, hand or traps are used for precipitin tests. If the digestion of the blood is too advanced, the test will be negative.

12.4.1.1 Materials

- Filter papers 10 cm in diameter.
- Round cellophane sheets, the same size as the filter paper.
- Ordinary pins.
- Forms for precipitin tests.

12.4.1.2 Procedure

(a) The mosquito, after identification,[1] is placed on a filter paper at a distance of about 1 cm from the edge.

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[1] If the mosquito is not identified, the blood is extracted from the stomach and the mosquito is kept under a number for further identification.

(b) The abdomen is squashed with a pin (or other instrument) care being taken to avoid contamination between one specimen and the next. (A clean pin should be used for each sample.) Another technique is to squash the abdomen with the corner of a microscope slide, a different corner being used for each insect and the slide discarded after four specimens. Each blood sample should receive a number which will correspond with the number of mosquitos on the forms. The papers are left to dry, each being separated by a cellophane paper (or non-absorbent white paper) and then stored in a desiccator, or in a plastic bag containing a small bag of silica gel, where they are safe from the depredations of ants and run no risk of becoming wet in a humid climate. When they are sent off for testing, the papers should be packed in a waterproof container, such as a polythene bag, which can be sealed by running a hot iron along the open end.

12.4.1.3 Remarks

Locality, project reference code, date of collection, place of collection and mosquito species can be written on the centre of filter paper. Alternatively each filter paper should be marked with a roman number (I, II, III, etc.) or a letter or combination of letters, and the details should be entered on a form. Mosquitos from different sources or different localities should not be smeared on the same paper, likewise different species should be put on separate papers. Specimens of a single species from one locality may be grouped together over a maximum period of 1 calendar month.

12.4.2 Precipitin tests

12.4.2.1 Principle

The appearance of a whitish insoluble precipitate at the zone of contact of two solutions; antigen (extract of protein contained in the blood from the stomach of a mosquito) - antibody (human or animal antiserum).

12.4.2.2 Remarks

The ring test is commonly used in which a saline extract of the gut contents of the insect is layered above the specific antiserum in a narrow tube. The antigen and the antibody combine in such a way that a whitish insoluble ring is produced at the place of contact of the two solutions.

The value of the test depends on the specificity of the antiserum. Ideally this should react only with homologous sera, i.e., with those from the particular animal against which the antiserum has been produced. It is prepared by the repeated intramuscular injection of serum into a rabbit. The antiserum so produced contains antibodies that will react not only with serum from the animal concerned but also with that from other groups. Its specificity is then increased, by absorption of the antibodies responsible for non-specific reactions, by treatment with a mixture of (heterologous) sera from other animals. After treatment the antiserum is highly specific at a group level. However, since only a limited number of "unwanted" antibodies can be absorbed in this way without seriously affecting the sensitivity of the method, the precipitin test in practice can only be used for distinguishing human blood from that of domestic animals; anti-human serum will still react with sera from other primates, such as chimpanzees or monkeys.

Blood smears from squashed arthropods will retain their antigenic character for at least 5 years, if the filter papers are kept in a cold dry place or in a refrigerator. The protein from the blood meal is extracted with saline and the ring test carried out in the manner described.

12.4.3 Explanatory notes on the attached information sheet

When the smears are sent to the testing laboratory[1] each batch should be accompanied by an information sheet in triplicate together with the key sheets (one copy to be marked for WHO headquarters). The information required to be collected on this sheet indicates:

(a) The geographical/epidemiological situation in the area of the investigation for establishing a human blood index. The recording of the geographical situation, including the general ecological conditions, may lead to defining the variations which may occur in the feeding habits of vectors over its range of distribution. The recording of the epidemiological situation is of great importance in determining the reliability of the sampling procedures for obtaining bloodmeal smears, particularly in areas with malaria transmission, and consequently in utilizing the human blood index in combination with other entomological factors.

(b) The breakdown of the man:animal ratio into a ratio of man:cattle:other animals. This is valuable as it indicates the availability of cattle, a host which often attracts certain vectors from among other animal hosts.

(c) The availability of man and animal hosts in the indoor and outdoor resting sites during the night before the morning sampling. This will enable accurate estimation of the numbers of human and animal hosts, which may not be possible if it is done simultaneously with the collection of smears in the morning. Generally, inhabitants tend to take their animals with them to the fields early in the morning.

(d) The distances between the human and animal hosts and the indoor and outdoor sites. These distances have a bearing on the results of the precipitin tests. If, for example, cattle congregate near the resting shelters from which the smears are collected a large proportion may be expected to give a positive reaction to bovid. Such bias will not be evident if this information is not collected.

(e) The density of blood-fed female vectors in the day-time resting places and the proportion of specimens used for collecting bloodmeal smears. This will be valuable in providing information on the relationship between the size of the sample taken and the number of vectors and fed females found at the time of collection and may eventually lead to the setting of standards for the minimum proportion of smears to be collected from certain densities. However, this information cannot be interpreted on its own; information on other factors, i.e., the man:cattle:animal ratio and their location in relation to the collection sites should also be employed.

(f) An estimate of the biting rates of the vectors in the locality of collection of bloodmeal smears. This is valuable when interpreting the results of precipitin tests and may indicate the need for further enquiries regarding sampling bias if great discrepancies are noted.

By means of a sketch map show the approximate location of man and animal hosts in relation to the sites of collection of bloodmeal smears. This will be a useful aid in interpreting the above-mentioned information.

12.5 Interpretation of results

The results of the precipitin tests will give group reactions, which will be largely specific provided the only probable hosts are domestic animals. The only non-specific results will be those positive for sheep/goat and, in some areas, for the Equidae (horse/donkey). The precise significance of these results is indicated in Table 7.

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[1] Smears should be sent to: Department of Zoology, Imperial College of Science and Technology, Silwood Park, Sunninghill, Ascot, Berkshire, England.

FORM 1

WORLD HEALTH ORGANIZATION

I.C.S.T. Reference No. _____

RECORD OF MOSQUITO BLOODMEALS FOR PRECIPITIN TEST

Collector _____ Project reference _____

Country _____ Dispatch date _____

Estimated ratio of persons to cattle _____ : _____

Please test the enclosed bloodmeal smears for the presence of the blood of MAN (or other primate), and of the animal groups indicated:[1] ox/buffalo ..... sheep/goat ..... horse/ass ..... pig ..... dog ..... cat ..... rodent ..... bird ..... others (specify) .....

Serial No. of paper						
Mosquito species						
Locality (and district)						
Spray history[2]						
Resting place[3]						
Smear No.	Date of collection	Space for result	Date of collection	Space for result	Date of collection	Space for result
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
Space for office use						

[1] Number, in order of importance, up to four animal groups.

[2] State "never sprayed", or name insecticide and years of first and last indoor applications (e.g. "DDT, 62-67").

[3] DO NOT MIX ON ONE PAPER DIFFERENT BIOTOPES, LOCALITIES OR SPECIES. Designate biotopes as follows: H = human habitation, A = animal shed or shelter, M = mixed habitation, V = vacant structure, store, etc., E = outdoor shelter (show type if required). Add "/Tr." for collections from outlet traps. Add "/Kd." for collections from floors. In case of night collection, use "H/N", "E/N", etc. For all special collections (e.g. baited traps and cages) show "C (special)".

BACK OF FORM MAY BE USED BY INVESTIGATOR TO GIVE OTHER RELEVANT INFORMATION, OR BY TESTING INSTITUTE FOR REMARKS ABOUT THE SAMPLES

FORM 2

INFORMATION SHEET

Bloodmeal smears despatched on: Project:

Locality, species and serial No. of filter paper	Date	Geographical/ epidemiological situation[1]	Man/ cattle/ animal ratio[2]	Resting place	Distance of human and animals from site of collection	Daytime capture[3]			Date	Biting density[4]				Inhabitants night-time outdoor sleeping habits activities[5]	
										Man		Animal			
						Total catch	Number of smears	Percentage of smears		In	Out	In	Out	In the night before collection[5a]	In general[5b]

[1] Define geographical feature, phase of the programme, malaria endemicity and type of foci.

[2] Define the ratio as Man:Cattle:Other animals (define types).

[3] Total catch of bloodfed females in premises from which smears have been taken; the number of females used for making the smears and their proportion.

[4] From any record available that can represent the period of collection of smears (average bites per bait per night).

[5] Indicate:

(a) whether outdoor human sleeping and/or activities occurred; also whether night-biting observations were organized near the outdoor site of collection.

(b) general habit of staying out at night and/or sleeping outdoors.

TABLE 7. INTERPRETATION OF RESULTS OF PRECIPITIN TESTS

Precipitin test positive for:	Source of blood meal	
	Domestic	Wild
Man	Man	Monkeys, chimpanzee
Bovid	Ox	Buffalo, antelope, giraffe
Sheep/goat	Sheep, goat	Some antelopes
Equidae	Horse, donkey, mule	Zebra
Pig	Pig	Bush-pig, wart-hog, etc.
Avian	Fowl	Other birds
Carnivore	Dog, cat	Lion, leopard, hyena, etc.
Negative	Indicates poor quality of blood smear, or reptilian blood	

If more exact identification of feeds is required, such as in areas where game is abundant in the vicinity of a village, the inhibition test can be used after the determination of the precipitin reactions.

12.5.1 Use and limitations

The study indicates the actual feeding which might reflect preferences for different hosts as well as the influence exercised by the nearest host. However, the precipitin tests as carried out routinely under field conditions do not exactly reflect host preference, but indicate the feeding incidence on different hosts at a given moment and under particular environmental conditions, the availability of different hosts being important. The use of such a test is not indicated as a routine study. It should be carried out a maximum of 2-4 times per year during different seasons, but mainly during the main transmission period. Once the relative feeding preferences are established there is no need for such tests to be repeated frequently with well recognized vectors and in the absence or scarcity of domestic animals.

12.6 Study of feeding preference

The feeding preferences need a specially planned study. The principle of such a study is to offer to the same population of mosquitos a choice between the existing hosts. For such an experiment it is necessary to use three or more identical big net traps. A man is put into one, a cow in another and a pig in another.[1] The positions of the traps (and hosts) are interchanged for a minimum of 5 nights.

A second type of experiment is to put under a big tent trap a pig, a man and a cow. The three baits should be separated by a distance of at least 1-1/2 m. In the morning the blood fed mosquitos are collected and the contents of the stomach prepared for precipitin tests. The experiment should be repeated for several consecutive days and the positions of the hosts should be changed each time. The number of human baits might be increased from 1 to 4 or 5 in order to study the effects on the proportion of mosquitos feeding on man.

[1] The distance of different hosts from the place of collection is of great importance. There are situations where the human blood index inside houses is very low if there are animals outside only a few tens of metres away, whereas the same mosquito population might show a high human blood index if the animals are much further away.

12.6.1 Remarks

No domestic animals should be within a radius of at least 100 m during the experiments. When changing the position of hosts, the tents should be moved onto a clean surface.

The above methods are of an experimental nature.

It is possible to evaluate the feeding preference on a given host by establishing the host preference index (HPI) (sometimes called the Forage Ratio) which is calculated as follows:

$$HPI = \frac{\% \text{ of mosquitos found with blood of a given host A}}{\% \text{ of host A among the total hosts present}}$$

If the index is 1, this indicates no preference, if it is higher than 1 it indicates preference for host A, if it is lower this indicates a degree of avoidance (or less attractiveness) of the host. The results should be interpreted with care. Repeated observations are necessary in the same area and a significant number of mosquitos should be processed. In malaria epidemiological evaluation it is interesting to know the relative proportion of mosquitos feeding on human beings and the proportion feeding on animals. Therefore, in areas where monkeys are absent, when the basic preferences of a species are known, routine investigation of the human blood index could be carried out by using only human antiserum which is available everywhere. The study of the host preference is not a routine one, it is carried out only for special investigations, when the feeding preferences are not known or when environmental changes have occurred in the area which might have an important influence on the feeding preference (or on the availability of different hosts).

13. TECHNIQUES AND PROCEDURES FOR SQUASH PREPARATIONS OF THE POLYTENE CHROMOSOMES[1]

The original technique for making preparations of the polytene chromosomes of larval salivary glands of certain Diptera (Nicoletti, 1960) has proved readily applicable to anopheline larvae (Frizzi, 1953; French et al., 1962; Coluzzi & Sabatini, 1967) and, most fortunately, to the nurse cells of anopheline ovaries (Coluzzi, 1968).

The introduction of cytogenetics to the investigation of vector anophelines, particularly the sibling species, demands that large numbers of mosquito specimens should be used in order to obtain chromosomal preparations that show clearly the chromosomal banding pattern. In field activities, it is therefore important that specimens intended for chromosome examination should not be wasted through faulty planning or technique.

The following are the procedures and methods described by French et al. (1962) and Coluzzi (1968) with some modifications that will enhance the development of proficiency in performing the technique. The intention throughout is to guide non-specialists who may be interested to undertake cytogenetical studies. They should undertake this work with extreme caution, liaising with a known specialist in this field in order to check their preparations and identifications. It is easy for a beginner to lose heart and be defeated by technical obstacles that more experienced workers could help them avoid.

13.1 Material

13.1.1 Special equipment

- Microscope with phase contrast, illumination and magnification up to X 1000
- Dissecting microscope
- Dissecting needles

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[1] Based on "Review of Cytogenetic Studies on Anopheline Vectors of Malaria" by White, G. B., Coluzzi, M. & Zahar, A. R. (to be issued in the WHO/MAL series of documents in 1975).

- Dropping bottles
- Slide with one concavity for dissecting mosquitos
- Grease-free and dust-free microscope slides
- Coverslips (to be siliconed with the silicone GE SC-78 or "Repelcote")[1]
- Filter paper or blotting paper.

The type of coverslip used for ovarian squash preparations is of considerable importance. If too thin it tends to crack easily when tapped, and if it is too small there is a danger that much ovarian tissue may squeeze out around the edges. A size of 22 mm square is ideal and the thickness should be 0.15-0.20 mm to allow momentary denting in the middle when it is tapped to spread the follicles and chromosomes. Thicker cover glasses tend to be too rigid and may also be too deep for the focal depth of the high-power objective.

To coat the coverslips with silicone, 3-4 ml of the silicone solution will be sufficient for a large number of coverslips. The procedure is as follows:

- Dip dry coverslips in the silicone solution for 1-2 minutes with GE SC-87 or a few hours with Repelcote;
- Wash the coverslips in soapy water;
- Dry them thoroughly with tissue paper.

The coverslips can be washed and used again about five or six times.

13.1.2 Fixative and stain

Fixative:

(a) Concentrated Carnoy fixative

2 parts ethanol

1 part glacial acetic acid

(a 50% solution of acetic acid in water also gives satisfactory results)

(b) Dilute fixative

2-5% solution of concentrated fixative in distilled water

(c) Concentrated acid fixative

Glacial acetic acid diluted 1:1 with distilled water

Stain: Aceto-lactic orcein stain:

(a) Concentrated stain

2% by weight of synthetic orcein powder dissolved in

1 part of 85% lactic acid and

1 part of glacial acetic acid

Mix the orcein first in the acetic acid and then add the lactic acid. Nowadays some suppliers list two types of orcein as being for "connective" and "elastic" tissue. The latter gives a less satisfactory result with chromosome preparations.

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[1] Available, respectively, from, General Electric Corporation, United States of America, and Hopkins and Williams, Ltd., England.

(b) Diluted stain

1 part of the above stain mixed with 1-3 parts of 45% acetic acid or with 1-3 parts of the mixture of lactic acid and acetic acid (1:1).

13.1.3 Material for mounting

(a) Einschlussmittel L15 or L25

For permanent mounting.[1]

(b) Other optional chemicals

"Euparal" mounting medium

Freezer aerosol

Solid carbon dioxide ("dry ice") or liquid nitrogen

(c) Clear fingernail varnish

For ringing coverlsips. Obtainable from pharmacies almost anywhere in small bottles having a convenient applicator brush under the cap.

13.2 Procedures

13.2.1 Collection and handling of specimens

Healthy late 3rd or early 4th instar larvae from the field are best for preparation of salivary chromosomes. If pre-pupal trumpets are present within the thorax, then the larva is too old. Unhealthy larvae, especially those from weak or crowded colonies, do not give good preparations. Each larval salivary gland consists of two distinct lobes: a strong sphere containing cells with excellent chromosomes, and a more pliant bag of cells with smaller nuclei and chromosomes. It is useful to make a special spatulate or minutely curved needle for picking up the spherical section of the gland since this is the most desirable part and it often separates from the rest.

Under average tropical conditions, a mid-morning pyrethrum spray catch indoors should yield freshly fed female anophelines with Christophers' stage III ovaries in excellent condition for chromosome preparation. In cool climates, when the catch is made from houses before about 09.00 hours, or when minimum night temperatures outdoors falls below 12°C, the ovaries of fed specimens caught are likely to be underdeveloped, the chromosomes being small and straggly and their banding pattern ill-defined. Similarly, freshly fed anophelines from outdoor resting sites or outdoor traps may need to be held alive at 25-30°C until at least mid-day before an optimum stage of chromosome differentiation is reached in the ovarian nurse cells. The same applies to engorged females that have been captured biting outdoors at night and have been kept at outside temperatures until the catch is brought to the laboratory the following morning.

With female anophelines that have fed inadequately for ovarian development to proceed (Detinova, 1962), or those which will undergo an initial pregravid phase of ovarian growth leading only to stage II of development after the first blood meal (Gillies, 1955), it is not feasible to make readable preparations of the polytene chromosomes. The best approach to cytogenetical study with these specimens is to recognize them in advance, keep them alive, re-feed them and hold them for about 3 and 12 hours (depending on ambient temperature). Dissection will then show whether the ovaries have developed to stage III.

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[1] Manufactured by Carl Zeiss Ltd., Oberkochen, Federal Republic of Germany.

13.3 Killing of specimens

Mosquitos killed with pyrethrum, such as females knocked down by a spray catch, suffer no overt chromosomal damage. But the use of killing agents such as chloroform, ether or ethyl acetate may damage the nuclei containing the polytene chromosomes, causing them to remain as a dense nonspreading mass if such vapours have penetrated the ovaries during excessive exposure of the insect. It is usually safer, therefore, to kill female anophelines by stunning them in a tube. Polytene chromosomes do not deteriorate immediately after death of the mosquito, so that reasonable preparations can be made from specimens kept in a refrigerator (but not frozen) overnight or kept for some hours on damp cotton wool.

13.4 Dissection, fixation, staining and examination procedures

With fresh material it is essential to dissect stage III ovaries or larval salivary glands into dilute fixative. It is necessary to follow the advice of French et al. (1962) with regard to blotting the water from larvae before dissection. Chromosomes will be irreparably damaged and stay as clumped masses if exposed to saline or water during dissection.

13.4.1 Dissection of larvae

Dissection of larvae may be done in dilute fixative on a flat slide or on a siliconized coverslip. Jensens (1955) described how to find the salivary glands which lie antero-laterally in the thorax. The larval abdomen should be cut off and discarded. The thorax should then be slit open dorsally, which may be done by inserting one needle from the rear and running another needle over it to sever the layers between. Gentle traction on the head then usually pulls the glands free. They often remain attached to the head by the salivary ducts. The large white mass of brain may be squeezed from the head capsule and used for mitotic preparations according to the same schedule as described below for polytene chromosomes.

13.4.2 Dissection of adult female mosquitos

Adult females for ovarian dissection should first have legs and wings removed. They should then be dissected on a cavity slide. The last 3 abdominal segments should be pulled off in such a manner as to immerse the ovaries immediately in dilute fixative. If it is desired to use the mosquito stomach for oocyst examination or bloodmeal identification, then momentary exposure of the stomach to dilute Carnoy's fixative (5%) during dissection will do little harm. Practice even enables the ovaries and spermatheca to be extracted in body fluids alone in which case one ovary may be placed in normal saline and examined for parity while the other ovary is transferred to fixative and used for chromosome preparation. When ovaries have developed to advanced stage III, or even early stage IV, it is best to reduce the amount of tissue beneath the coverslip by making a separate preparation from each ovary. This is also a wise precaution when dealing with an especially important specimen. When sporozoite examination is required, it is obviously sensible to remove the salivary glands of adult females by dissecting them anteriorly into saline before the ovaries are removed posteriorly into fixative.

The following is a general outline of the standard procedures used by French et al. (1962) and Coluzzi (1968):

(a) Dissect the tissues in a drop of dilute fixative (Carnoy's or acetic acid) in the concavity of the slide.

(b) Place a small drop of fixative (about 2 mm in diameter) away from the centre of a siliconed cover slide and place a larger drop of the diluted stain next to it in the centre.

(c) Transfer the tissues with the point of a needle to the fixative and leave for 30 seconds, macerating them with the point of the needle; then mix with stain and leave for 3 minutes or more.

(d) Turn the coverslip over on to a clean (degreased) slide.

(e) Tap the coverslip with the butt of the needle or a finger several times.

(f) Wrap the preparation in absorbent or blotting paper and press firmly with the palm of the hand. Do not allow the coverslip to move during squashing.

The preparation can be kept unsealed for at least 2 weeks at a normal temperature, and may be preserved for much longer in a refrigerator at 5-6°C.

Inadequate fixing causes chromosomes to remain sticky and stretchy during staining and squashing. This happens when the tissues are not immersed in a sufficiently large droplet of full-strength concentrated modified Carnoy's fixative for at least one minute. To avoid carrying over a quantity of dilute fixative between the blades of forceps, the tissues should be transferred to concentrated fixative on the point of a needle. Needles of some sorts (including some of "stainless steel") corrode rapidly in Carnoy's fixative, causing two problems: (i) tissues adhere strongly to the corroded areas of metal, and (ii) the corrosion flakes off and contaminates the preparation.

When large numbers of specimens are being processed rapidly in the field, it is probably better to use the neat 2% stain and to reduce the staining period from 3 minutes to 30 seconds. A good safeguard, time permitting, is to check each slide at 100x magnification (a normal field microscope will suffice) to ensure that optimal spreading of chromosomes has been achieved before the preparation is sealed. The squashing of the preparation is rather a delicate procedure demanding some practice. It involves tapping the coverslip with a forefinger or needle-butt, followed by firm pressure to spread the chromosomes of the ruptured nucleus. Some workers prefer tapping with a "patella hammer". This is particularly good for larval chromosomes. An improvised hammer can be made by fixing a pointed rubber eraser on a strong wire handle.

Chromosomes continue to take up the stain for some hours after squashing, so it is not necessary to examine the freshly made preparations immediately. Slides may be accumulated over days, weeks or even months of field work and kept for later study, for instance, during the dry season.

Polytene chromosome preparations may be scanned at 100x or 250x magnification and read well at 250x or 400x. For detailed examination of banding patterns, 1000x magnification is essential. Phase contrast illumination is very desirable, perhaps with a green filter to counteract the red stain. The gross features of the chromosomes may be seen quite well using only a standard lighting system. With practice, for instance, it is possible with a normal compound microscope to identify the various types of X-chromosome that are diagnostic of the sibling species of the A. gambiae complex with standard illumination and 250x or 400x magnification.

13.5 Preservation before chromosome preparation

Hunt (1973) has developed a simple way of storing suitable anopheline females and preparing their ovarian chromosomes at a convenient later date. Apart from relieving the field-worker of the necessity to process his catch when the material is fresh, this method actually improves the quality of the chromosome preparations.

The technique employs Carnoy's fixative (3 parts ethanol with 1 part glacial acetic acid) as preservative for whole adult females. After preservation at room temperature for 24 hours, the material should be held at about 4°C in a refrigerator. To prepare slides, the ovaries are separated and each is placed on a slide in a droplet of 50% aqueous propionic acid for 1-2 minutes. This causes the tissues to swell and they should then be macerated. A droplet of diluted aceto-lactic orcein (2% aceto-lactic orcein diluted 1:10 with 50% propionic acid) is then added and mixed with the tissues. Excess stain should be removed after half a minute and the tissues washed with successive droplets of 50% propionic acid. The preparation may then be squashed under a siliconized coverslip in the usual way.

13.6 Permanent mounting

For preserving the squash preparation, it is very useful to seal it with transparent or translucent finger-nail varnish. The varnish may be diluted with acetone if it thickens or dries in the bottle. A good application of nail varnish is sufficient to keep a chromosome preparation usable for over a year, though some drying out inevitably does occur. The great advantage of the transparent kinds of nail varnish is that the microscope can focus down through the varnish to examine right up to the edge of the coverslip where some of the better chromosomes may lie. If an important slide happens to dry out, it is often possible to make it readable again by allowing a mixture of equal parts of concentrated lactic and acetic acid to infiltrate under the cover glass.

Few field entomologists in tropical countries have access to liquid nitrogen for making permanent chromosome preparations according to the method of Conger & Fairchild (1953) as recommended by French et al. (1962). Solid carbon dioxide ("dry ice") is a possible substitute but is equally difficult to obtain in many areas. As alternatives for freezing the squash while mounting fluid is added, either use (i) a deep frozen block of metal or ice from a very cold refrigerator, or (ii) a freezing jet directed under the slide from a "freezer aerosol" container (e.g., "Electrolube"). A much simpler approach (Hunt, 1973) is to remove the nail varnish carefully from a temporary preparation using a scalpel or acetone, and then to place the slide in absolute ethanol in a covered staining dish, changing the ethanol twice at 24-hour intervals, then to ring with "Einschlussmittel L15" and allow to dry. The "Einschlussmittel" permeates under the coverslip and serves as a permanent mountant with an ideal refractive index.

14. VECTOR SUSCEPTIBILITY TO INSECTICIDES

It is well known that the use of insecticides on a mosquito population exercises a selection pressure on the individuals which have the ability to survive contact with insecticides by different mechanisms. Such individuals are considered resistant. This resistance is now generally recognized as being of three types:

(a) Vigour tolerance

In general, a slight increase in tolerance to one or several insecticides (slight decrease of susceptibility) results from the continued selection of a population of insects that do not have specific genes for resistance to that particular insecticide or its chemical group. Tolerance may also be due to seasonal variations of some morpho-physiological characteristics, such as larger size, thicker cuticle, increased fat content, which seem to play a role in the phenomenon of non-specific resistance. This type of mechanism might explain seasonal variation in the susceptibility of the same species to several insecticides.

(b) Physiological resistance

Insect populations may be selected to survive insecticide pressure of a given insecticide by different physiological mechanisms (detoxifying enzymes, storage of insecticide in fat body, etc.). In some instances the degree of resistance and the number of resistant individuals may be so high that the use of the insecticide or a closely related chemical might have to be interrupted. True resistance may be specific or there may be cross-resistance to other chemicals as well. Cross-resistance is produced by insecticides belonging to the same group, e.g., dieldrin causes resistance to HCH and vice versa. DDT-resistant populations may also be resistant to analogues of DDT. Thorough investigations on resistant and susceptible populations of genetically pure strains have shown that specific resistance is as a rule controlled by a single-gene mechanism (as is the case with the resistance to dieldrin). This type of resistance is reversible when the insecticide pressure is removed but susceptibility seldom regains its previous value and decreases again rapidly once use of the insecticide recommences. Dieldrin resistance is restored to a very high level after a period of 1 year or at the most, 2 years. Specific resistance has been produced under field conditions not only by the use of

insecticides in malaria programmes but also by insecticides used in agriculture. From up-to-date data it appears that foci of resistance to insecticides were in many cases created by the use of insecticides in agriculture rather than by residual spraying in malaria programmes.

(c) Behaviouristic resistance

This is the ability of the mosquito population to escape the effect of insecticide because of their natural behaviour or a modification of their behaviour (induced behaviour by the presence of insecticide). This is achieved by avoiding the treated surface or shortening the period of contact with it (natural avoidance or induced avoidance due to the deterrent or repellent effects). Irritability might play an important role in interrupting the contact of insects with the sprayed surfaces before the insects have picked up a lethal dose of insecticide. Under laboratory conditions it was possible to select populations with higher irritability to DDT. This phenomenon still awaits clarification in nature, but from a theoretical point of view it is possible that under strong pressure mosquito populations with a higher irritability might be selected and then will not be easily controlled by indoor spraying in spite of the fact that the species remain susceptible to the insecticide. Reversibility of such populations to their normal status might also be expected; as in the case of physiological resistance.

14.1 Adult mosquito susceptibility tests

14.1.1 Principle

Exposure of mosquitos in a confined space to surfaces treated with a standard concentration of insecticide for a given standard time, or by dropping insecticide on each mosquito (topical application), and recording the ensuing knock down and mortality at the end of exposure and at the end of a 24-hour holding period.

14.1.2 Objectives

(a) To establish the baseline susceptibility level before commencing a programme of anti-vector measures with residual insecticide.

(b) To detect any change in the susceptibility level of vectors at different intervals after the start of the programme with residual spraying, or to assess the effect on the susceptibility of a vector population of insecticides used in agriculture in areas where no residual insecticides have been used against mosquitos.

(c) To evaluate the modification of the susceptibility level of the laboratory populations of mosquitos submitted, or those not submitted, to insecticide selective pressure.

14.1.3 Use and limitations of susceptibility tests

The adult susceptibility tests are used in the following situations:

(a) In unsprayed areas before deciding on the application of a residual insecticide, in order to obtain basic data on the susceptibility level necessary for:

- selection of the appropriate insecticide;
- to have comparative basic data for the monitoring of the susceptibility level in a sprayed area.

(b) When a decrease in susceptibility is suspected, based on the following factors: persisting, moderate or high vector relative density or a rapid increase of relative density at short intervals after spraying (4-6 weeks in localities and premises which were correctly sprayed and where the residual deposits were not disturbed or inactivated by human activity).

(c) To check the level of susceptibility of laboratory colonies of vectors used for bioassay tests.

(d) To evaluate the changes in susceptibility level of laboratory populations of vectors submitted to insecticide selective pressure.

Before a decision can be made to carry out the susceptibility tests in areas covered by residual spraying, the following information is necessary:

- the relative density of vectors,
- the quality of spraying (operational causes),
- whether the sprayed premises have been disturbed by natural events, rains washing the residual deposits etc., or by human activity (environmental causes).

Susceptibility tests are not indicated as a routine activity but as special investigations. In a field programme these have to be applied only when the above mentioned aspects indicate that the density of vectors is important in spite of the persistence of satisfactory residual deposits of insecticide. If it is desired to check the susceptibility of a vector population in previously sprayed areas, this should be carried out in general once a year only during the previously known peak of vector density.

If genetic resistance appears in a vector population, then the vector density will increase in spite of satisfactory spraying. In general, in a sprayed area when the mosquito density is very low, a number of mosquitos may appear to possess a certain amount of resistance when tested but this may be of unspecific vigour tolerance which can be proved by testing the F1 descendants. In such situations a carefully repeated investigation of the susceptibility level should be done and the significance of the results should be interpreted in the light of the local epidemiological data. The epidemiological significance of the resistance is given only by the evaluation of malaria in the area with vector resistance.

14.1.4 Performance of the WHO standard susceptibility test

Material and method. The WHO standard susceptibility test kit is used: a full description of materials and methods for performing susceptibility tests on various types of insecticide are described in Wld Hlth Org. techn. Rep. Ser., No. 443, 1970, Annexes 1A and 1B

The materials and the steps to be performed are illustrated in Fig. 66 and Fig. 67. In practice it is known that exposure for 1 hour of a given species of mosquitos to a single concentration normally results in 99-100% mortality. It is only when a reduction in mortality from this discriminating dose occurs that the species are exposed to a full range of concentrations (at least three concentrations).

14.1.5 Discriminating concentrations

After initial establishment of the baseline susceptibility level of a population of a particular species by means of a full test against several insecticide concentrations it is usual, in subsequent checks on susceptibility level, to use only the lowest insecticide concentration which consistently produced 100% mortality on 1-hour exposure in the original test. This discriminating concentration (discriminating dose) must be determined for individual species and areas, but in general with the chlorinated hydrocarbons, 4.0% DDT and 0.4% (or in some cases 0.8%) DLN are used. Other discriminating concentrations used are: malathion 3.2%, fenthion 2.5%, fenitrothion 1.0% and propoxur 0.1%; all at 1 hour.

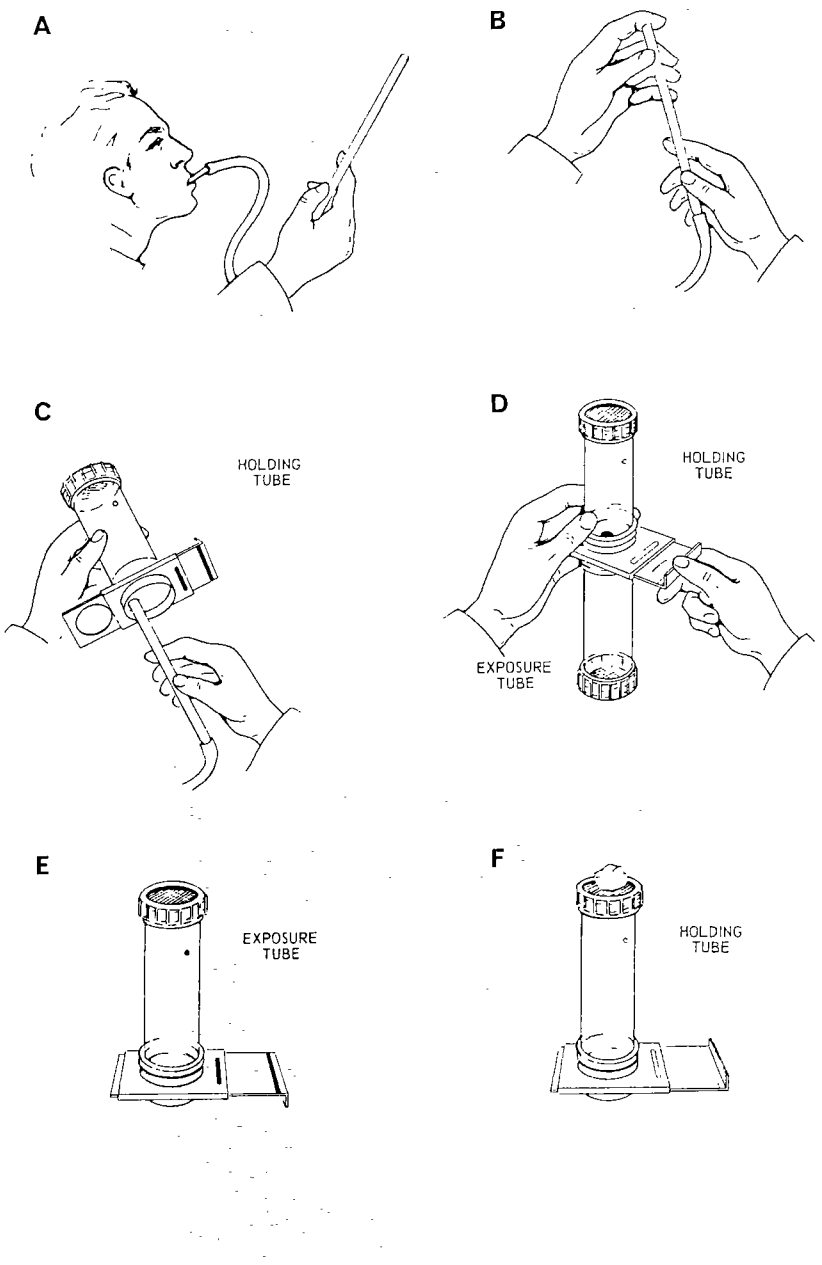

Fig. 66. Method for determining the susceptibility or resistance of adult mosquitos (Reproduced from WHO Technical Report Series, No. 443, 1970).

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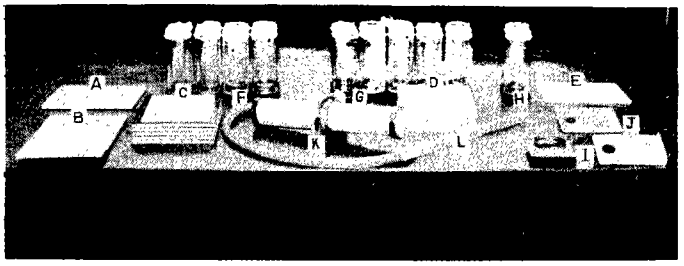

A. Risella oil-impregnated papers for use as controls or checks
B. Insecticide-impregnated paper removed from plastic case
C. Dieldrin-impregnated papers of varying concentrations
D. DDT - impregnated papers of varying concentrations
E. Unimpregnated papers for lining holding tubes
F. 8 exposure tubes (with red dots)
G. 10 holding tubes (with green dots) for pre-test sorting and post-exposure observation
H. 2 control or check tubes (with green dots)
I. Metal slide unit with screw cap on either side and slide with 20 mm hole
J. Alternative cardboard slide
K. 2 tubes joined with slide unit as used in the test
L. Aspirator

An alternative WHO tube with a slide unit with screw cap on one side only. These can be used with paper cups as recovery chambers, and the illustration shows the method of transfer from tube to cup

2

Fig. 67 Equipment required for the adult mosquito susceptibility test.

When survivors are obtained after a discriminating exposure, the test must be repeated using other concentrations in order to obtain a regression line and examine its shape. Alternatively, as a preliminary check, the time of exposure to the discriminating concentration may be doubled. If there are still survivors there is a high probability that physiological resistance is present.

14.1.6 Interpretation of the results of the susceptibility tests

For the interpretation of the significance of the susceptibility tests the following aspects should be taken into account:

- Physiological condition of insects, unfed, fed, gravid (and age when using laboratory bred mosquitos).
- Place of collection. If the mosquitos were collected from sprayed or unsprayed premises in a sprayed or unsprayed area.
- Microclimatic conditions. Temperature and percentage of humidity during the exposure periods and during observation periods.
- Season. A slight decrease in susceptibility might be due to seasonal variations.
- Number tested (whether large enough to be significant).
- Mortality in controls.
- Feeding status and type of food.

14.1.7 Processing and interpretation of results

The 24-hour mortality of mosquitos exposed to three standard concentrations of insecticide should be plotted on logarithmic probability paper to construct the dosage-mortality regression line or to construct the time-mortality regression line when different batches of mosquitos are exposed to the same concentration of insecticide, but for different periods of time (60 min, 120 min, 180 min) or shorter intervals depending on the standard concentration of indsecticide used.

The following steps are performed:

(a) Read the 24-hour mortality for each test and calculate in percentages. If the mortality in the control groups is over 5% but less than 20% a correction of mortality is made by applying the Abbot formula.

$$\frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

When the mortality in controls is over 20% then tests are discarded.

(b) Calculate an average of the mortality obtained to the same concentration in different triplicates.

(c) Construct a regression line (Fig. 68). If the regression line shows a drift to the right but remains parallel with the baseline data, this indicates an increase in tolerance only. If the regression line has a tendency to form a plateau at the upper part, this indicates resistance.

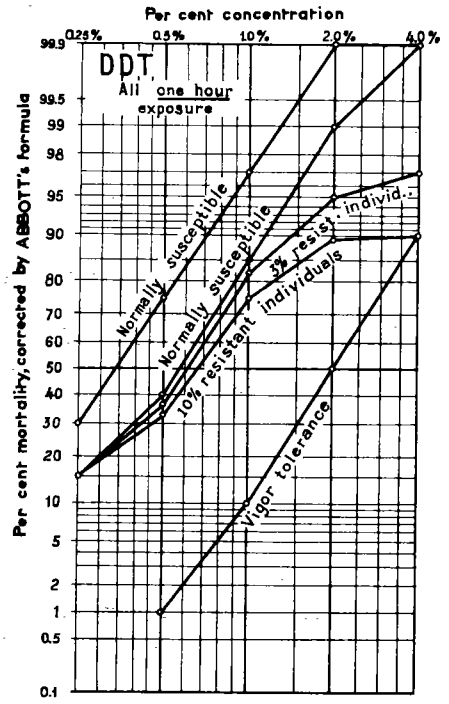

Fig. 68. Sample regression lines illustrating susceptibility, resistance and vigour tolerance.

The proportion of resistant individuals present in the population can be established by repeating the exposure at double the time of the initial standard exposure.

If after exposure for 2 hours a straight regression line is obtained, this indicates vigour tolerance, if the line has a plateau where an increase in dosage gave no increase in kill, then true resistant individuals are present in the population from which the samples have been collected.

The detection of a decrease of susceptibility, tolerance or even resistance does not automatically indicate that this is of epidemiological significance. When vector resistance is confirmed the following studies should be undertaken to determine the epidemiological significance:

- Studies of vector density (trap collection, biting densities, resting densities) in the presence of reliable operations activities.
- Parity rate.
- Dynamics and origin of malaria cases.

Resistance in sprayed areas, if associated with reduced density and absence of cases, does not have any immediate operational implications on the spraying programme, however the possible results of an increase in density and/or introduction of cases must be considered in contingency planning.

14.1.8 Operational implication of resistance (See Part I, Chapter 1, Section 7)

When it has been established that resistance is the cause of persistence of transmission, a change in attack measures is urgently required. The following measures could be taken:

(a) When resistance is low, spraying at shorter intervals or with an increased dosage and better case detection and treatment might be satisfactory but this is usually a short-term expedient.

(b) When resistance is high and transmission continues, a change of insecticide is indicated, supplemented with other attack measures where possible.

14.1.9 Susceptibility tests by topical application

14.1.9.1 Principle

Topical application of an insecticide dilution in small doses (of the order of 0.01-0.02 ul per individual) by using a small loop or micropipette. There are several devices available for delivering such small quantities.

14.1.9.2 Method

The mosquitos (all of the same physiological stage - and of the same age) are anaesthetized by carbon dioxide or by ether. The method, using ether, is as follows:

- Collect about 10 mosquitos in a normal laboratory test tube.
- Place 0.2 ml of ether on a piece of cotton wool.
- Leave the mosquitos for 2 minutes.
- Empty the mosquitos into a Petri dish.
- Arrange them for testing on a microscope slide.
- Apply the established dose of insecticide solution on the back of the thorax.

- Introduce the mosquitos into a cage or cup after about 1 minute. If the insecticide solution is in a non-volatile oil, introduce the slide with the mosquitos into the cage, taking care to avoid touching the resting surface (or other mosquitos) with the thorax of the tested mosquitos.

Such investigations are used only in special cases and by well-trained staff.

The results are read after a 24-hour holding period.

14.2 Larval mosquito susceptibility test

14.2.1 Principle

Exposure by immersion in water solutions or emulsions of insecticides[1] of mosquito larvae at a certain stage (usually 3rd-4th) and counting the mortality after a standard interval of time.

14.2.2 Objectives

- To establish the baseline susceptibility level of larvae in areas where larviciding has to be applied.
- To detect any change in the susceptibility level of larvae in areas where larviciding is applied correctly but larvae are still found.
- To isolate the homozygous - resistant individuals or strain or mosquito - from a mixed population where a single gene is responsible for resistance.

Such investigations are necessary only when antilarval measures with a given insecticide will be or are being carried out; they are not necessary when residual spraying is applied against adults. Modifications of susceptibility may be produced by:

- the wide use of insecticides in agriculture,
- the use of larvicides or adulticides in a malaria programme.

The susceptibility of larvae varies with their stage of development. Young larvae (I and II stage) are more susceptible than older ones (III and IV stage). Pupae are far less susceptible than the larvae and cannot be used for susceptibility tests since they do not feed and it appears that the mechanism of action of insecticides used as larvicides is both by penetration through the cuticle and by ingestion of particles of insecticides and of micro-organisms which are themselves covered with the insecticide.

There are several procedures for carrying out a susceptibility test of mosquito larvae. The WHO susceptibility test is recommended. It should be mentioned that, as in the case of other tests where longitudinal observations are carried out, the tests should be repeated each time under exactly the same conditions and using a satisfactory number of larvae.

14.2.3 Materials and methods

The WHO standard susceptibility test kit is used: a full description of materials and methods for performing susceptibility tests with various types of insecticides is given in WHO Technical Report Series, No. 443, 1970, Annexes 2A and 2B.

Note. When the tests tend to indicate resistance, they should be repeated before a final conclusion is made. The tests do not reflect directly the real consequences in the field of the resistance detected. The degree of resistance found in larvae does not necessarily reflect the degree of resistance in adults.

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[1] The duration of exposure might vary from 1 hour to 24 hours according to different methods.

14.2.4 Organization of a survey of larval susceptibility

Identify the different types of breeding place around the inhabited area, and inside the area, paying particular attention to those which have been treated with insecticide for agricultural purposes or for larviciding. Select two spots for each representative breeding place.

Collect some hundreds of larvae in stages III and IV and transport them with care to the field laboratory.

If the area which has been subjected to treatment for agriculture is very large (hundreds of km^2) the sampling of larvae should be carried out in several spots of each such treated area.

14.3 The Elliot larval test

14.3.1 Principle

Mosquito larvae are exposed for 1 hour in relatively concentrated solutions of insecticide and then transferred to clean water to observe mortality after 5 hours.

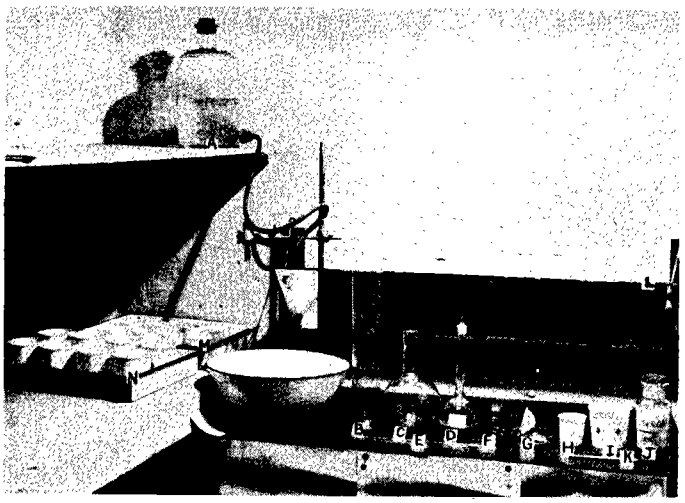
14.3.2 Materials

The original method has been modified slightly to eliminate the need to clean glass or porcelain vessels.

- 1%, 0.1% and 0.01% etc., solutions of the insecticide in absolute alcohol.
- Unwaxed paper cups to hold about 100 ml of insecticide solution used for exposure of larvae.
- Unwaxed paper cups to hold 500 ml of water used for the recovery of larvae.
- 1-ml blood pipettes or 1-ml graduated pipettes fitted with micropipette fillers (one pipette for each concentration of insecticide).
- Small nets made of iron wire and sand-fly netting, 5 cm in diameter and 2.5 cm deep (one for each concentration of insecticide).
- Thermometer for measuring temperature of the water.
- Glass pencil or adhesive labels to mark on the paper cup the concentration of the insecticide solution, number of larvae, species of larvae, date of test, etc.
- Half Petri dishes to hold the nets while not in use.

14.3.3 Procedure (Fig. 69 and Fig. 70)

- Place 25 larvae in a small amount of water in each recovery cup.
- Pour the premixed insecticide dilution (see Table 8) into an exposure cup.
- Pour the water containing the larvae from the recovery cup into the net.
- Immediately transfer the larvae from the net into the exposure cups containing prepared insecticide dilution (one net for each concentration must be used).
- Leave the larvae for 1 hour.
- Pour the solution containing the larvae into the net, the larvae will remain in the middle of the net.
- Wash the larvae and net thoroughly but gently with tap water to remove the insecticide.
- Transfer each batch of larvae into a large recovery cup in 100 ml of water. Sprinkle some larval food and leave for a period of 5 hours before reading.



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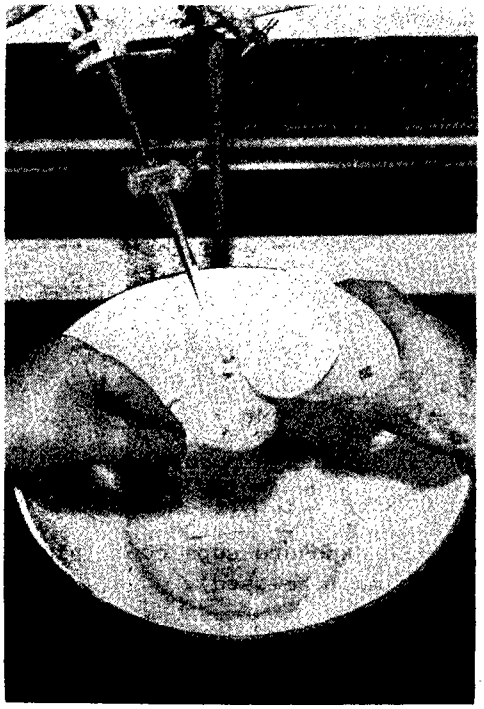
A. Water supply for washing larvae during transfer
B. Measuring cylinder
C. Flask for mixing insecticide dilutions
D. Stock solution of insecticide in ethanol
E. 1 ml graduated pipette
F. Micro-pipette filler
G. Small net for transfer of larvae
H. Exposure cup
I. Recovery cup
J. Larval food
K. Pen for distributing larval food
L. Inclined mirror for reading results
M. Tray of replicate exposure cups
N. Tray of replicate recovery cups

Transferring larvae into net

2



Washing larvae

3

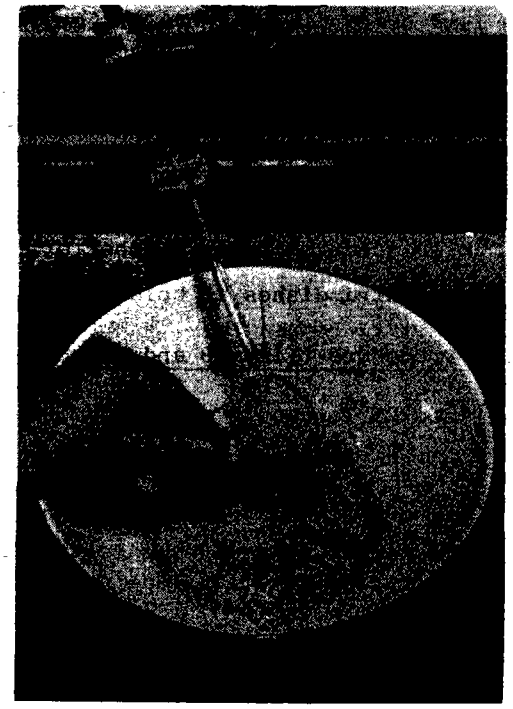


Fig. 69 The Elliot larval susceptibility test.

Immersing larvae in insecticide-alcohol-water mixture in exposure cup

Reading the results through an inclined mirror

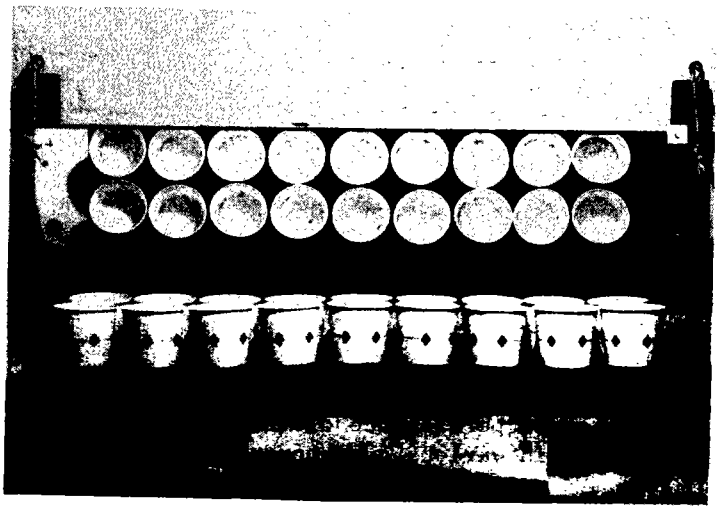


Fig. 70 The Elliot larval susceptibility test (continued).

TABLE 8. SOLUTIONS USED FOR ELLIOT LARVAL TEST

Concentration (mg/litre)	Insecticide stock solution to be used	Volume of stock solution in ml	Volume of water in ml
200	1%	20 or 2	1 000 or 100
100	1%	10 or 1	1 000 or 100
50	1%	5 or 0.5	1 000 or 100
10	1%	1 or 0.1	1 000 or 100
1	0.1%	1	1 000
0.1	0.01%	1	1 000
0.01	0.001%	1	1 000

14.3.4 Reading of the results

Before reading the mortalities all the larvae are disturbed and made to go below the surface by agitating the water with a pipette or dissecting needle. Some of the dead or dying larvae tend to float on the surface. Push these down and wait for a few minutes to allow the larvae which are able to swim to come up to the surface. Those remaining at the bottom or those not capable of coming up to the surface are counted as dead. An inclined mirror can be used for counting the larvae, thus avoiding their being disturbed by shadows (Fig. 70).

14.3.5 Interpretation of the results

The results indicate the level of susceptibility or of resistance of the larvae. However, since the mortality is not necessarily in proportion to the dosage, the form of the regression line cannot be relied on automatically to measure the degree of resistance. This method can be used to make a mass selection for resistance from a mixed population exposed to a discriminating dosage which has been determined from small-scale exposure. In this case 300-400 larvae can be exposed in 1 litre of insecticide mixture. The handling and the time of exposure to the insecticide is the same. The mortality is calculated as a percentage and the same precautions should be taken as those recommended for the calculation of the mortalities of the results obtained in the standard WHO larval test.

15. BIOASSAY TEST

15.1 Using adult mosquitos

15.1.1 Principle

Exposure of mosquitos of uniform physiological condition to the action of the residual deposits.

15.1.2 Objectives

(a) To assess the duration of persistence of the toxic effect of different insecticide deposits on different surfaces or in various environmental conditions.

(b) To assess the quality of operations. The toxic effect of the residual deposits may be of two types:

- by contact
- at a distance by vapour or particles.

This depends on the chemical structure and physical properties of the insecticide - DDT and dieldrin, have practically no toxic fumigant effect owing to low volatility, whereas HCH, malathion, fenitrothion, propoxur, and dichlorvos have an important fumigant effect.

Thus, there are two types of bioassay:

- Contact bioassay
- Bioassay for fumigant effect

15.1.3 Contact bioassay (Fig. 71)

15.1.3.1 Principle

To expose mosquitos of uniform physiological status in confined places, on sprayed surfaces for a standard period of time (minimum 30 min) and then to establish the mortality at the end of 24 hours' observation.

15.1.3.2 Objective

- To evaluate the duration of the residual effect on different surfaces of a given concentration of an insecticide under known laboratory conditions, or under known field environmental conditions.
- To assess the quality of spraying operations in terms of coverage when the expected efficacy of the insecticide and formulation is well known for specific environmental conditions.
- To assess the persistence of the residual effect at a given period after application.

15.1.3.3 Use and limitations

The application of the bioassay tests depends on the objective. When studying the duration of the residual effect we should know the quality of spraying, average concentrations used and whether the sprayed surfaces have been disturbed. Such investigations are widely used in stage III and stage IV insecticide trials, in experimental trap huts and in local huts.

For the first objective, the tests are applied on the same surface. For the third objective, some tests should be carried out on the same surface, some on other surfaces, at random, in order to check the degree of variation of the toxic effect. The surfaces where the test is made should be marked in order that they may be easily found. For the second objective, the test is carried out at random on various surfaces.

The tests should normally be carried out during the morning and not in the afternoon when mosquitos are more restless.

15.1.3.4 Remarks

Avoid contaminating mosquitos with insecticide before the test. The test should be carried out on the species against which the measures are applied.

Clean carefully, each time they are used, the collecting tubes and the tubes for transferring mosquitos. Particles of insecticide might be absorbed and contaminate the mosquitos in the tube. The sucking tube, therefore, should not come too close to the sprayed surface. Collect with test tubes wherever possible.

The bioassay tests are used as a special and not as a routine investigation, in order to fulfil the objectives mentioned at the beginning of the chapter.

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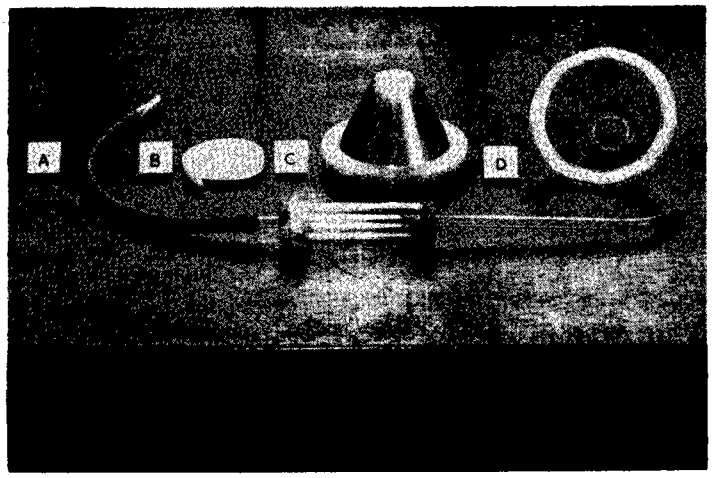

A. Aspirator tube with glass arm specially bent to aid the removal of the mosquitos from the conical exposure chamber
B. Adhesive sponge plastic for lining rim of exposure chamber
C. Conical exposure chambers made of transparent polished plastic
D. Exposure chamber with rim lined with sponge plastic ready for application to uneven surface

Introduction of mosquitos into conical chamber fixed in position on wall with adhesive tape

2

Fig. 71 Adult bioassay test. The World Health Organization conical exposure chamber outfit for the bioassay of insecticide deposits on wall surfaces.

15.1.3.5 Reading of results

The mortality is counted after the 24 hours' holding period for each individual test; if the control mortality is over 20% this test should be discarded. If the control mortality is more than 5% the Abbott formula for the correction of the mortality should be applied and the results should be inserted on the reporting form.

15.1.4 Bioassay test for fumigant effect of insecticides.

15.1.4.1 Principle

Mosquitos are exposed in a treated space in small cages, for a standard period of time, from 4-8 hours up to a maximum of 12 hours, at different distances from the sprayed surfaces. The mosquitos are then kept for 24 hours' observation having been transferred to clean cages.

15.1.4.2 Materials

The seventh report of the WHO Expert Committee on Insecticides[1] recommended the use of cages with dimensions of 12.5 cm x 6.5 cm x 6.5 cm made from a wire frame and mosquito netting for walls.

Walls made of metallic mosquito net should be used since these have a smaller electrostatic effect than nylon mosquito netting.

Flat cages made of a rectangular metallic frame, 15 cm high x 8 cm wide x 3 cm deep, can also be used (See Fig. 72).

15.1.4.3 Procedure

(a) Number of mosquitos per cage: 20-25 mosquitos (laboratory bred, 3-day-old blood-fed females or wild collected blood-fed females, belonging to the species against which the insecticide is being used in the field) are introduced into the cage before the cage is put in the space where the fumigant effect is to be tested.

(b) Siting of cages: the cages are suspended 50 cm from the wall at three levels - upper part (30 cm from eaves level), middle part and lower part of the wall. Another cage is suspended in the middle of the hut.

(c) Time of exposure: the same standard exposure time should be used for comparison purposes. In general, a minimum of 4 hours and a maximum of 12 hours (6-8 hours can also be used if found necessary during the preliminary tests).

(d) Reading of mortality: dead and knocked down mosquitos are counted at the end of the exposure periods, and then after the 24-hour observation period.

At the end of the exposure period, mosquitos are transferred to clean cages and given 5% sugar solution on cotton wool and kept for 24 hours. A thermohygrograph is desirable for recording the temperature and the relative humidity. If a thermohygrograph is not available a maximum and minimum thermometer might be sufficient for the recording of the temperature.

15.1.4.4 Remarks

Other types of exposure cage could be used (cylindrical cages or very flat cages as shown in Fig. 72). The material used for covering the cages should preferably be metallic mesh mosquito net. It should always be kept in mind that an easy penetration of air from the room into the exposure cage is necessary. It is often difficult to distinguish between a particulate effect and a fumigant plus sublimation effect.

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[1] WHO Technical Report Series, No. 125, 1957.

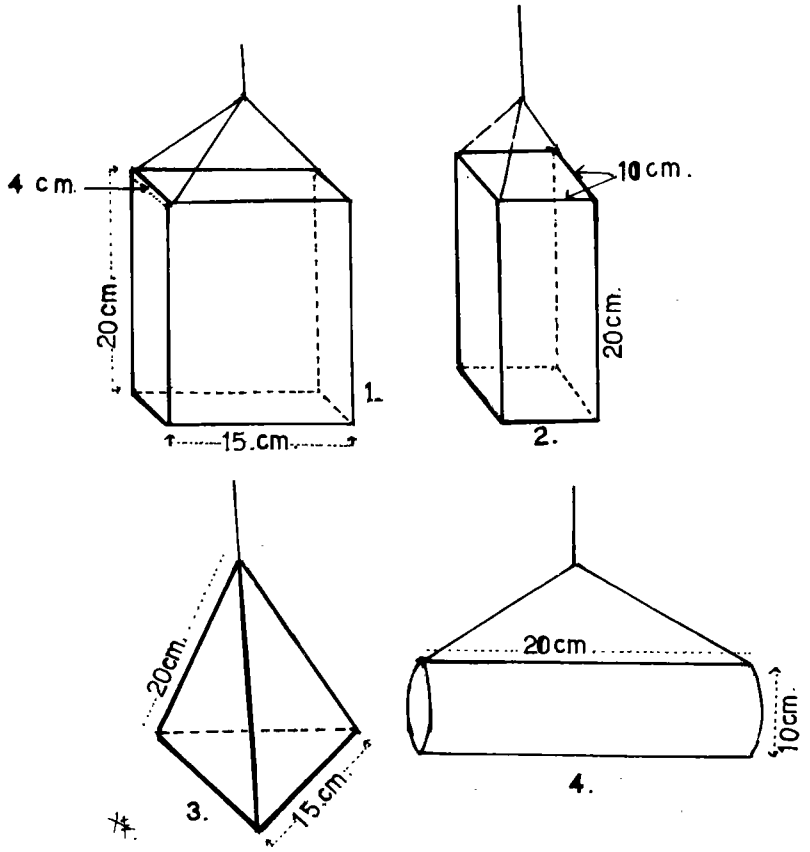

Fig. 72. Different shapes of cage for bioassay of fumigant effect.

15.2 Bioassay test using larval mosquitos (Fig. 73)

15.2.1 Principle

Immersion of laboratory-bred susceptible larvae for a standard period of time in the treated breeding places.

15.2.2 Objectives

- Direct evaluation of the immediate or of the residual effect of insecticide (larvicide) in treated breeding places.
- To evaluate the efficacy of larviciding operations in breeding places where mosquito larvae are found and where it is claimed that the breeding place has been correctly sprayed.

15.2.3 Materials

Several cylinders about 10 cm in diameter and 20 cm in height, made of metallic or rigid nylon mosquito net. The bottom of the cylinder being closed by a disc of the same material which is stapled on the cylinder. The dimensions of the net mesh should be such that the larvae cannot escape from the cylinder, normal mosquito netting is satisfactory for stage III and IV larvae. In order to avoid young larvae escaping (when the use of such stages is required) the cylinder should be covered inside with nylon gauze. The cylinder is fixed on a wooden stick and then immersed in the breeding place for about 13 cm. The top of the cylinder should be about 7 cm above the surface of the breeding place. The water enters easily through the gauze. After immersing the cylinder, put a known number of larvae inside and observe the effect after a period of not longer than 24 hours. Observations at shorter intervals of 8-12 hours can be made, depending on the expected toxicity to mosquito larvae of the water in the breeding place.

16. BEHAVIOURAL REACTION OF VECTORS TO INSECTICIDES

Irritability is one of the general properties of all living beings and is one of the responses to external stimuli which produces a certain degree of discomfort. The first sign of irritability in a mosquito is an increase in its movements and, finally, an attempt to get away from the source of irritation. Thus the repellent effect is a result of the irritant effect, which is thus exercised not only on contact with the sprayed surfaces but also at a distance from it. The intensity of the response to the same irritant agent is not always the same, but varies with the physiological status of the individuals at the moment of action of the irritant element. This explains why some individuals, having the same morphological structure, respond in different ways to the same irritant stimuli. In general, mosquitos are much more sensitive when unfed. Changes in light intensity, humidity and temperature have an important influence on irritability. The chemical stimuli are sensed by different receptors, some distributed at the periphery of the insect's body and some located at the level of the ganglia. Among the insecticides used as residual sprays in malaria programmes, only DDT is highly irritant to the majority of vectors. HCH is less irritant than DDT and dieldrin shows experimentally a low irritant effect. Most of the organophosphorus insecticides have some irritant effect.

It is one of the characteristics of the mode of action of DDT that the toxic effect is preceded by a very marked irritant effect, which obliges the mosquito to leave a DDT-treated surface, very often before absorbing a toxic dose.

From recent experimental observations on the irritability produced by DDT, the following main aspects emerge:

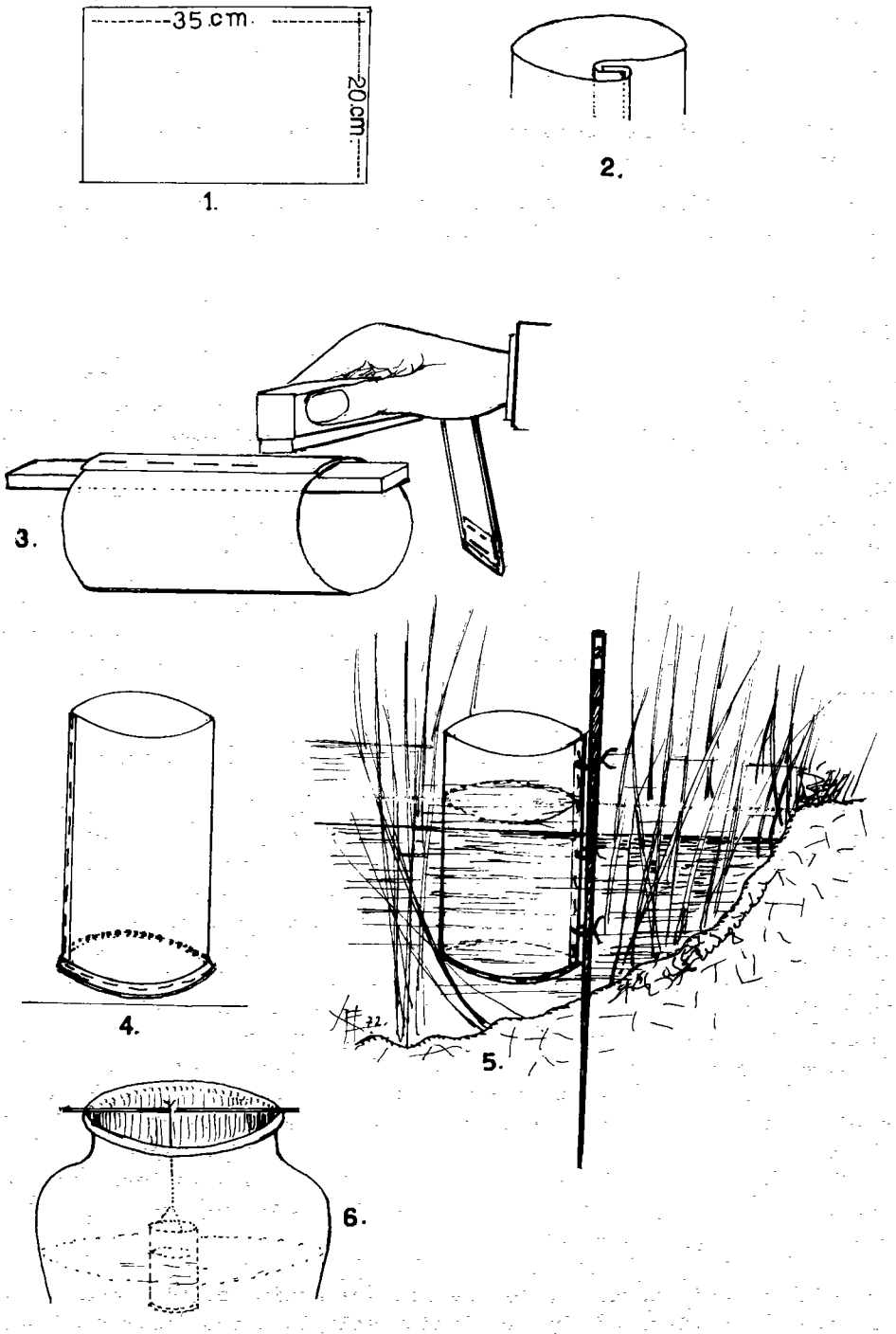

Fig. 73. Bioassay using larvae.

(a) Unfed mosquitos are more irritated by DDT than sugar-fed mosquitos and that blood-fed mosquitos are less irritable than unfed or sugar-fed mosquitos.

(b) That there are some slight differences in the degree of irritability of different mosquito species.

(c) That repeated contact with DDT-treated surfaces leads to a reduction of irritability.

(d) That, in general, mosquitos which show physiological resistance to DDT are less irritated by this insecticide. The experiments have been in some measure contradictory, due to the fact that the irritability has very often been evaluated on the basis of the average movements of all mosquitos observed in groups and not on the average number of mosquitos showing very high, high, moderate and low degree of movement when in contact with DDT.

In a mosquito population the change in the proportion of the number of mosquitos in the four categories mentioned above is more evident than the change in the average number of movements of the total number of mosquitos in the experiment, as assessed by the present standard methods. A single mosquito showing a very high number of movements might have an important influence on the average.

(e) Under experimental conditions it has been demonstrated that a susceptible mosquito population showing higher irritability can be selected by insecticide pressure (Gerold & Laarman, 1964), but such a phenomenon has not yet been clearly demonstrated in the field, although from a biological point of view it is possible.

16.1 Operational implications

The operational implications of the irritability might be negative or positive for an antimalaria programme. Transmission will not be interrupted if the contact with the insecticide occurs after the vector has fed on man and flies away after a short contact with the insecticide due to the irritant effect, survives and returns to feed on man, or if the vectors, being irritated, bite outside. However, interruption of transmission will be achieved if a large proportion of the mosquitos fly outside before feeding on man and if those that feed inside and are repelled outside are destroyed by adverse environmental conditions. Deviation to animals may also take place.

In general, the epidemiological importance of irritability is very low in temperate zones and with endophilic mosquitos. In areas where the mosquitos are more "zoophilic", and where animal shelters offer a relatively favourable resting place for the survival of the mosquitos, the fact that the mosquitos are irritated and forced to fly outside, very often before feeding inside the houses, indicates that the irritant effect of DDT is favourable for the reduction of the man-mosquito contact and does not constitute any problem for eradication. This situation has been encountered in Europe, the United States, Mauritius, Cuba and elsewhere.

Irritability might have a very important negative effect on the toxicity of DDT for those endophagic-exophilic species which enter and rest inside during the night for some periods before and/or after feeding, e.g., A. maculatus, A. balabacensis. The time of contact with DDT might be shortened and the mosquitos will leave the resting surface before picking up a toxic dose. The negative effect of irritability is more pronounced in very open houses than in well-constructed ones, because the mosquitos once irritated can easily find a way out. In a savannah area, endophilic mosquitos such as A. gambiae when irritated by DDT will survive outside and continue to transmit malaria. During the rainy season, the outdoor ecological conditions are very suitable for the survival of A. gambiae and for the extrinsic development of the malaria parasites.

16.2 Methods of investigating irritability

The irritability of a vector species to a given insecticide can be evaluated in two ways:

- direct evaluation by laboratory methods (applied under laboratory or field conditions),
- indirect observation, by using exit traps in experimental huts in order to collect the mosquitos flying out after being irritated by the insecticide.

16.2.1 Laboratory irritability test

16.2.1.1 Principle

Mosquitos are released and allowed to come into contact with a surface sprayed with a given amount of insecticide of a particular formulation in a confined space, and the time to first take-off and the number of movements during a standard time (usually 15 minutes), are recorded.

16.2.1.2 Objective

To evaluate directly the irritant effect produced by different insecticide concentrations and formulations.

This is not a routine investigation and it is performed mainly during preliminary investigations on the effects of insecticides (stage III-IV trials) or to check or evaluate the basic irritability level of different mosquito species when required. The procedure includes two steps:

- The determination of the length of time to the first take-off from the insecticide-impregnated surface.
- The determination of the number of take-offs in a given period of time (15 minutes has been adopted as a standard time, preceded by 3 minutes of adaptation).

16.2.1.3 Use and limitations

This test is used only for special studies.

WHO has produced a standard irritability test kit which is provided with all instructions on the manipulation and procedures.[1] The test is carried out using single mosquitos and is, therefore, time consuming. Experience has shown that unfed mosquitos are more irritated than sugar-fed, and blood-fed are less irritated than unfed or sugar-fed mosquitos. The age and duration of adaptation in the laboratory may influence the results.

The laboratory direct method of estimating irritability is in practice of limited use and restricted only to special investigations as mentioned above.

16.2.2 Excito-repellency test

Irritability can be measured by the excito-repellency test box (Fig. 74). This method measures the irritant effect as well as the toxic effect exercised by an insecticide used in a formulation similar to that used in the field.

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[1] WHO Technical Report Series, No. 443, 1970, Annex 17.

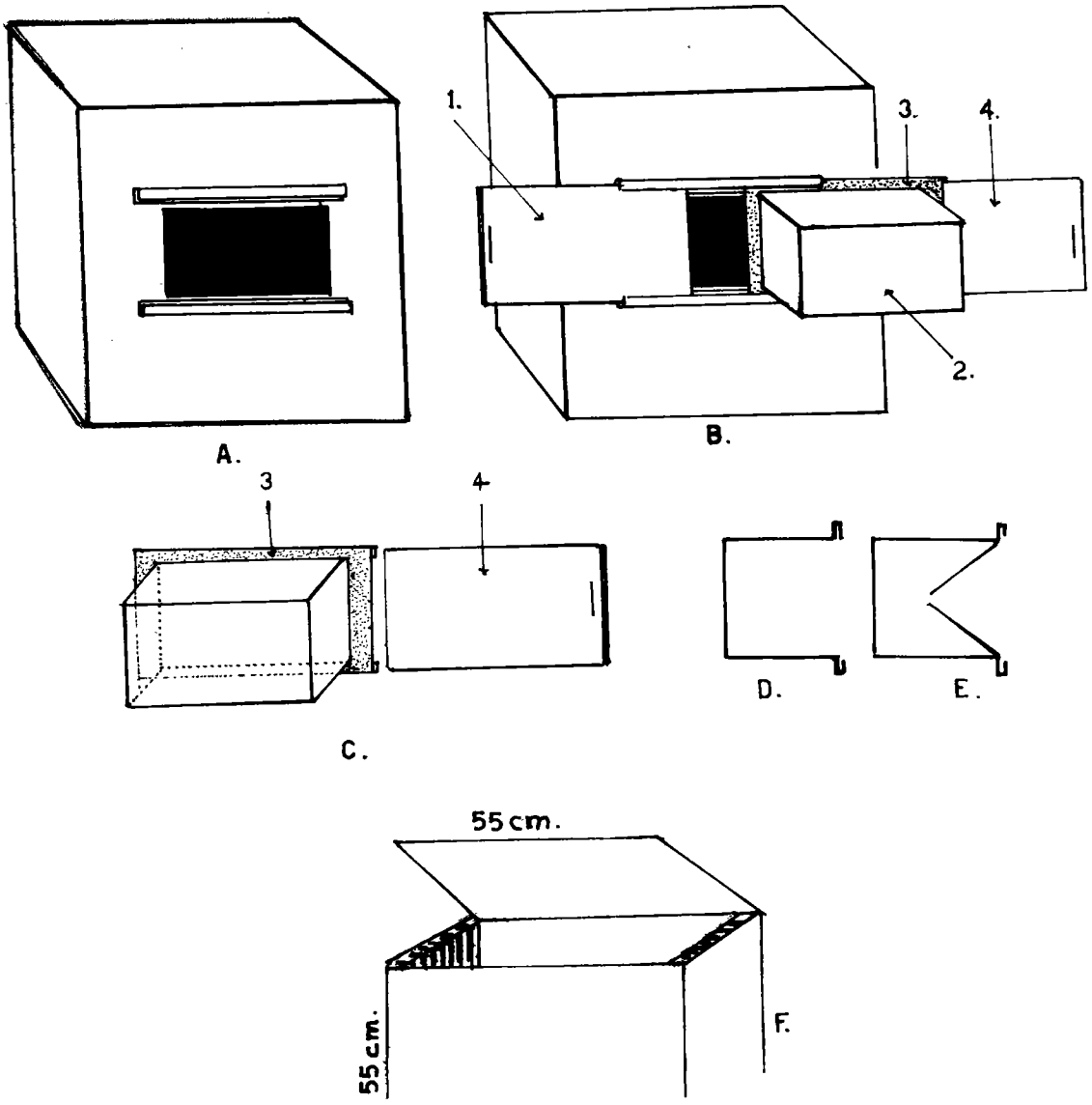


Fig. 74. Excito-repellency test box.

A = box without trap attached

B = box showing trap and slides: 1. box slide. 2. trap.
3. frame of trap which receives slide 4

C = trap detached, showing slide

D,E = trap, open and with entry cone

F = box for transporting the detachable walls of the test box.

16.2.2.1 The principle of the method is to release the mosquitos into a cage of about 0.125 m^3, lined with sprayed paper, with an exit leading to a small trap cage attached to one of the holes of the experimental cage, into which the mosquitos can fly.

16.2.2.2 The excito-repellency box is a demountable plywood cube of 50 cm x 50 cm x 50 cm and fitted with a trap from which mosquitos can fly back into the box if they do not like to stay in the trap. The opening in the wall of the box, which corresponds to the opening in the trap, can be closed by a sliding door of plywood. The trap is closed also by a sliding piece of plywood so that it can be changed at desired intervals (5-10 min) in order to count the mosquitos which have left the box after short periods. The internal part of the box is lined with pieces of insecticide treated paper, or sheets of cardboard; when the box is dismantled for transport the pieces can be easily stored in a special container (Fig. 73 F). One box is lined inside with insecticide-sprayed paper or cardboard and one is lined with untreated (non-contaminated) paper or cardboard and used as a control.

16.2.2.3 Use of the box. The box is used for studying the irritant effect of the insecticide and the effect of this on the toxic effect. The box is like a miniature room fitted with an exit trap; it is useful for the study of various insecticides and formulations on different surfaces. The advantage is that the insecticide can be used in concentrations and formulations similar to those used under field conditions.

16.2.2.4 Remarks. It should be mentioned that these tests do not replace the WHO standard susceptibility tests but offer realistic information on the expected standard performance of an insecticide and formulation used under field conditions.

The spraying of the test paper can be carried out by using a small atomizer or a small hand sprayer. A standard sprayer could also be used; the paper or other substrate to be used in the box being attached to the walls of a house, for example, and sprayed with the routine technique used in the field by a well-trained sprayman under the supervision of the entomologist or preferably by the entomologist himself. The spraying should be uniform.

The amount of insecticide sprayed for a given surface should be measured and the average quantity per square metre should be calculated.

The use of freshly fed females will indicate what proportion of mosquitos are irritated after feeding. The study of the exit trap mortality (at the time when mosquitos are collected from the trap and the 24 hours' mortality of the survivors found in the trap) indicates the effect of irritability and the degree of toxic effect of the insecticide.

16.2.3 Indirect estimation of the irritability

This is carried out by using exit traps and has been discussed in the sections on experimental huts, and trap collection (Part II, Sections 4 & 5).

17. PHYSIOLOGICAL TEST FOR THE TOLERANCE OF ANOPHELINE LARVAE TO SALINITY

17.1 Principle of the method

Newly emerged first stage larvae are put into contact with water containing different concentrations of sodium chloride.

17.2 Objectives

- To test the level of tolerance of some mosquito species to the salt content of natural breeding places, e.g., A. atroparvus, A. sacharovi.
- To identify salt water form of the A. gambiae complex (A. melas, A. merus). (The salt water form of A. gambiae can easily be separated from fresh water forms by this method.)

17.3 Material

The test can be carried out by using one of the following solutions.

- 3 parts of sea water to 1 part ordinary water or, in the absence of sea water, use the following solution:
- Water 1000 ml - sodium chloride 23.5 g.

17.4 Procedure

(a) Each batch of eggs (obtained from gravid females isolated in individual tubes - Fig. 75) is placed in a very small container in a few millilitres of distilled water. Immediately after hatching, the young larvae are transferred to a glass jar or other small container filled with about 10 ml of one of the above solutions.

(b) Read the results after 2 hours.

Note. All larvae of fresh water A. gambiae will be killed, whereas those of the salt water form will survive.

18. DETERMINATION OF SALINITY

18.1 Objectives

To determine the salt content in mosquito breeding places.

This special study is only carried out when studying the breeding habits of mosquitos. For this purpose salinity is usually expressed in terms of chloride.

18.2 Method

Two methods are commonly used:

(a) Direct estimation of total chloride content by chemical reaction using a silver nitrate solution which reacts with the chloride in the water when the latter is not too acid.

(b) Indirect estimation which is carried out by using a hydrometer (salinometer). The results can be read to two degrees of salinity with the aid of a conversion table (Table 9) or directly on the instruments (some instruments are calibrated in parts of chloride per 1000, in which case the salinity is read directly). All hydrometers measure the total salt content of water.

18.2.1 Determination of salt content by chemical reaction

18.2.1.1 Materials

- Graduated cylinders of 10 ml graduated in tenths of a millilitre.
- Weak silver nitrate (analytical grade) solution 9.58 g/litre.
- Strong silver nitrate (analytical grade) solution 47.9 g/litre.
- Potassium chromate solution, 5%.
- Brown bottles with droppers for silver nitrate solution.

18.2.1.2 Procedure

- 4 ml of water from the breeding place is measured in the graduated tube.
- Add 2-3 drops of potassium chromate solution and mix.
- Pour the silver nitrate solution (weak or strong solution according to salinity - see below) in the tube in small quantities and mix well with the water after each addition by closing the opening with a rubber stopper and inverting the tube 3-4 times.
- A red precipitate persisting after shaking indicates the end point of the reaction.
- Read on the graduations of the tube the amount of reagent added.

Remarks

1 ml of the weak silver nitrate solution to reach the end point of 4 ml of water tested indicates 0.5 g of chloride/litre: 1 ml of strong solution indicates 2.5 g of chloride/litre.

18.2.1.3 Calculation of chloride content

This is obtained by multiplying by 0.5 or 2.5 (as the case may be) the number of millilitres of silver nitrate required to reach the end point with a 4 ml water sample.

Example 1

4.75 ml of weak solution was required to reach the end point with 4 ml of the water under test:

0.5 x 4.75 = 2.375 g of chloride/litre

Example 2

1.2 ml of strong solution was required with 4 ml of water:

2.5 x 1.2 = 3 g of chloride/litre

Example 3

If any volume other than 4 ml of water is used for tests then the above equivalents are multiplied by $\frac{4}{x}$. x = being the quantity of water used in the reaction. Supposing that 8 ml of water had been used in example 2 instead of 4 ml of water, the calculation would be:

$2.5 \times 1.2 \times \frac{4}{8} = 1.5$ g of chloride/litre.

To convert sodium chloride into chloride equivalent, multiply the weight of sodium chloride by 0.606.

To convert chloride into weight of sodium chloride equivalent: multiply by the weight of chloride 1.65, e.g., 19 g of chloride/litre is equivalent to 31.35 g of sodium chloride/litre.

Sea water = approximately 19 g of chloride/litre, or 3.13% sodium chloride.

Note. The specially graduated tube is prepared as follows:

An ordinary 10-ml pipette graduated in tenths of a millilitre is modified as follows:

- the delivery end is cut off,
- the mouthpiece is cut off and the end shortened and sealed in a flame in such a way that the volume from the bottom of the tube to the zero mark is 4 ml.

TABLE 9. SPECIFIC GRAVITY OF SEA WATER AT 25°C IN RELATION TO CHLORIDE CONTENT[a]

Chloride (‰)	Specific gravity	Chloride (‰)	Specific gravity
2	0.9998	14	1.0162
4	1.0027	16	1.0190
6	1.0055	18	1.0215
8	1.0081	20	1.0244
10	1.0107	22	1.0271
12	1.0134	24	1.0298

[a] Data extracted from Harvey (1945).

At a water temperature of 30°C, the above values of specific gravity should be reduced by 0.0014.

19. BREEDING AND MAINTENANCE OF MOSQUITOS UNDER LABORATORY CONDITIONS

19.1 Principle

Breeding and maintaining mosquitos under artificial or simulated natural conditions by ensuring the basic needs of different species for development and survival, such as temperature, food, humidity and light.

19.2 Objectives

- To provide a constant supply for various biological observations (study of longevity, susceptibility to infection with malaria parasites, longevity, feeding habits, etc.).
- To study the effect of insecticides under various conditions by using populations of mosquitos of known age and physiology.
- To provide material for training.
- To identify sibling species (genetic studies).
- Mass production of sterile hybrid males.

These objectives can be easily attained in a research institution as well as under field conditions. Although some facilities may be lacking under field conditions, with some ingenuity all the basic needs can be met and a mosquito colony can be maintained without undue difficulty.

19.3 The insectary

The insectary is the base for breeding and maintaining mosquitos under laboratory conditions. It may consist of one small room, in which both larvae and adults are reared, or several rooms, some for breeding larvae and one or more for maintaining adult mosquitos. Plans for insectary rooms or boxes for use in the field are shown in Fig. 79. One of the characteristics of these rooms is the low ceiling, the height of the walls should not exceed 220 cm. The floor should be made of cement. The larval room should have large windows, if this is not possible artificial light can be used for about 12 hours per day.

19.4 Materials necessary for breeding mosquitos

- Glass or plastic tubes for egg-laying, 2 cm in diameter x 8 cm high (Fig. 75).
- Egg-laying dishes or Petri dishes of about 10 cm diameter.
- White, yellow or green polythene bowls, about 30 cm in diameter, 10-15 cm deep, or unglazed earthenware pans of the same dimensions, or enamel photographic trays. The selection of the material for rearing larvae depends on availability and price.
- Small air pump for aerating the water (if possible); if not a rubber bulb attached to a glass tube can be used for this purpose.
- Small nets about 5 cm in diameter made of stainless wire on nylon gauze.
- Eye droppers for the collection of larvae and pupae.
- A simple apparatus for collecting pupae (optional: Fig. 76).
- Thermometer for measuring the water temperature in the artificial breeding places.
- Maximum-minimum thermometer.
- An electric convection heater fitted with a thermostat set at 25°-27° (where electricity is available).
- Humidifier fitted with hygrostat to ensure optimum relative humidity between 70-80%. This can be adapted to the optimum requirements of a given species.
- Glass jars with screw cap.
- Cages with wire frame, 30 x 30 x 30 cm, and covered with mosquito netting.
- Cotton wool.
- Pentobarbital sodium
- Hypodermic syringes, 2-ml and 5-ml.
- Test tubes.
- Glass jars 10 x 7 cm for holding pupae.
- Long-sleeved gloves.
- Dimethyl-phthalate used as a repellent to prevent mosquito bites.
- A few small penicillin bottles.
- Dry yeast or bran, Farex, etc., to feed larvae.
- Sugar or glucose to make solution to feed adult mosquitos.

19.5 Rearing of larvae

19.5.1 Collection of eggs

Individual batches of eggs are obtained by isolating gravid females in glass or plastic tubes in which a wet support or water is provided (Fig. 75).

Collection of eggs from cages with gravid females (Fig. 77):

- Place a thick layer of damp cotton wool in a Petri dish and cover it with a piece of filter paper, or
- Place sterilized damp mud from a favourable breeding place in a Petri dish and cover it with thin sheets of filter paper, or
- Use a Petri dish or a bowl with water if it is not possible to prepare the collecting dishes as above.

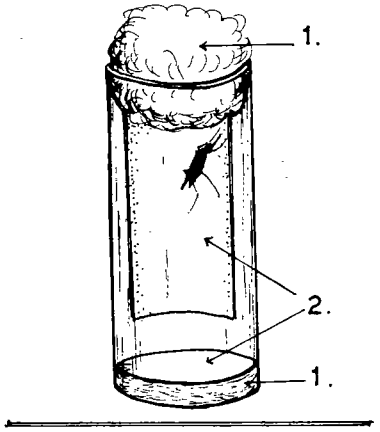


Fig. 75. Tube for mosquito egg-laying:

(1) Cotton wool; (2) Filter paper.

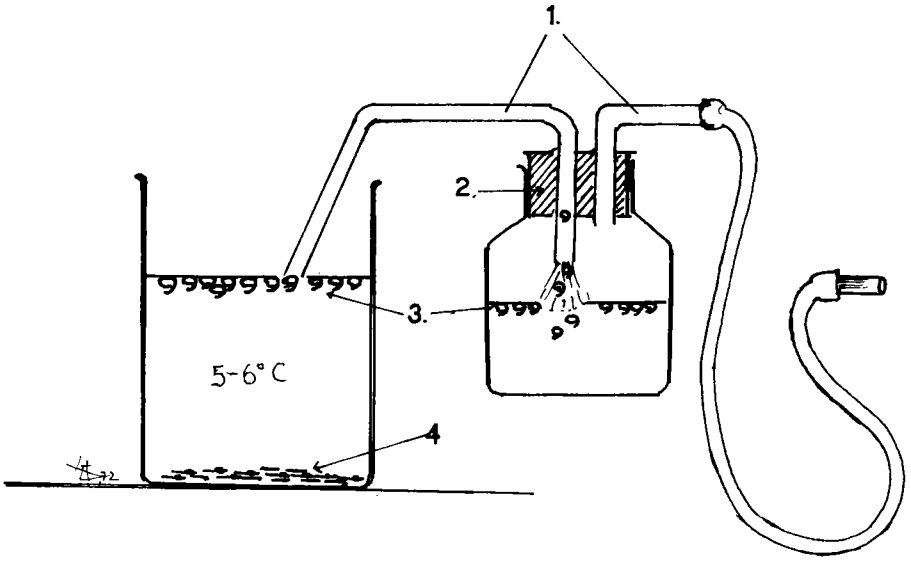


Fig. 76. Separation of larvae and pupae by cold water "shock".

(1) Glass tubing, 8 mm diameter; (2) Cork ;

(3) Pupae; (4) Larvae.

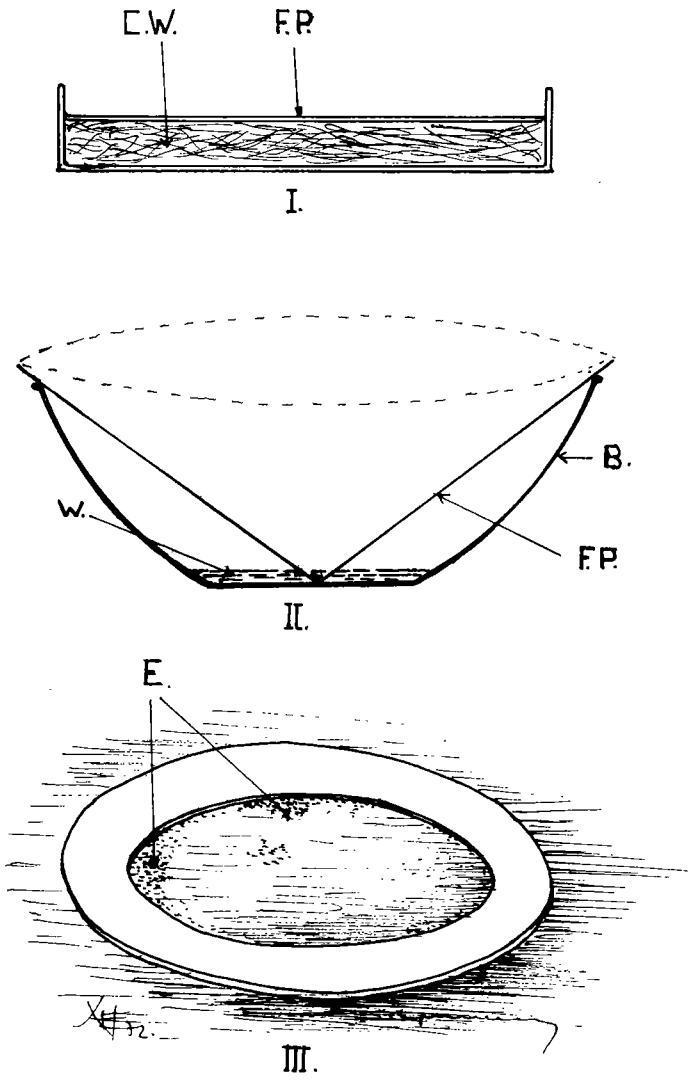


Fig. 77. Simple devices for obtaining and incubating mosquito eggs:

I. Petri dish: CW = wet cotton wool; FP = filter paper;

II. Glass, plastic or metal bowl (B), 10-15 cm in diameter, FP = filter paper; W = water (not too much water);

III. Cardboard ring impregnated with paraffin wax. This prevents the stranding of eggs on walls of container.

19.5.2 Preparation of the artificial rearing pans

White polythene bowls or other suitable recipients are filled with chlorine-free rainwater or fresh distilled water. In the absence of these, water from the breeding places can be used; this should be filtered to prevent the introduction of natural enemies. The rearing pans are filled to a depth of about 4 cm. Tap water should be put in the bowl 24 hours before the young larvae are introduced to allow the water to reach room temperature.

The eggs are allowed to hatch in the container in which they were collected: 12-24 hours after hatching, the larvae are transferred to the artificial breeding places, 100-200 larvae to each bowl. Overcrowding is unfavourable for satisfactory development and results in higher mortality and small larvae which produce weak pupae and adults.

19.5.3 Hatching of larvae

The incubation period lasts about 2 days at 25°C. The eggs are left until the larvae hatch, and then the young larvae are transferred to breeding pans; or the eggs, in a known number of about 100-200 - depending on the size of the artificial breeding place - are placed on the surface of the water inside a ring made of a waxed piece of board. The eggs float inside the ring and therefore do not touch the side of the breeding pan: this avoids their becoming attached to the pan and, in the case of the water evaporating rapidly, being killed.

19.5.4 Feeding of larvae

Newly hatched larvae do not need feeding during the first 24 hours. After their transfer to the breeding pans they are given food in the form of an infusion or in the form of dry powder.

Solid food in the form of a very fine powder is used for breeding most species. Several types have been used such as finely ground dry yeast powder, Farex or a mixture of 10% Bemax (wheat germ product with added vitamins and minerals) and 90% dog biscuit, fine powdered bran free of insecticide and suet passed through a close mesh sieve. The powder is placed in a glass jar with a screw ring to retain the cap. A fine white nylon gauze or a wire mesh 60 x 60 x 37 S.W.G. is fixed over the top of the jar, being retained by screwing. When not in use the top of the jar should always be covered with an inverted Petri dish to prevent the food from becoming damp. The quantity of dry food and the frequency of application depend on the number of larvae in the breeding pans. First-stage larvae will need much less food than third or fourth stage. If an excess of food is given, fermentation and the development of fungus and bacteria will take place, resulting in a high death rate amongst the larvae. The larvae should be fed with small quantities at a time, at least three times a day. However, larvae can be moved to another breeding pan should the condition of the water alter. If a pellicle forms on the surface, this can be broken by aerating the water with the aid of a pump or a hand aerator in the form of a large pipette. In general, the water is topped up but otherwise left unchanged. Liquid food is recommended for breeding anopheline species whose larvae feed on the bottom as well as the surface of the breeding pans (A. gambiae, A. stephensi, A. funestus, etc.). Larvae which feed mainly on the surface should be given solid food in the form of a fine powder which floats on the surface. Some authors recommend placing a piece of grass turf, about 25 cm square, in the breeding pans (24 hours before putting in the larvae) thus providing a permanent source of food. This will provide micro-organisms and protozoa from the soil on which the very young larvae will feed. Care should be taken to ensure that the soil is not contaminated with insecticide or other harmful substances or pathogens.

The speed of the larval cycle depends on the temperature and this should be approximately the optimum; for example, A. gambiae larvae require a higher temperature than A. funestus or A. balabacensis. The temperature of the breeding place should be around 28°, but temperatures of 30-32°C will not be harmful for species which can breed in nature in small breeding places exposed to the sun.

Under field conditions, when a special insectary or larval breeding room is not available, the artificial breeding place can be placed outside in a partially shaded spot in order that the temperature of the breeding place should not exceed 32-33°. In tropical areas, the larval breeding places can be exposed directly to sunlight in the early morning hours, up to about 9.30-10.00 hours. The temperature of the water should not be higher than 32-33°C for A. gambiae. It is then covered with thick wooden planks or other materials to prevent sunlight getting through and overheating the water. The larval breeding pans can be moved inside at night when there is a great difference between day and night temperature. If they are left outside at night they should be covered with pieces of nylon gauze fitted with small beads of lead to prevent them from being blown away and to stop wild mosquitos laying eggs (Fig. 78).

19.6 Rearing adult mosquitos

Pupae are collected from the breeding pans with an eye dropper or vacuum pump connected to a collecting bottle (Fig. 76). With this device it is possible to collect a large number of pupae in a short time. The pupae are transferred from the collecting bottle to glass jars or small enamel bowls, and placed in the breeding cages. The majority of adults will emerge in about two days at 25-27°C. To maintain the colony, the females have to be fertilized by natural or artificial mating. With stenogamous species mating takes place in cages of any size, and those mentioned above are suitable. Mosquito species which do not mate readily in captivitiy, e.g., eurygamous species, are artificially mated (see Section 21 on artificial mating). Adult mosquitos need an optimum temperature of about 25-26°C and a relative humidity of 75-85% which can be provided by automatic apparatus. If this is not available, a damp cloth can be fixed around the top of the rearing cages. Under field conditions the temperature can easily be maintained in a simple cupboard made of wood and plastic (Fig. 79).

19.6.1 Feeding of adult mosquitos

To maintain a mosquito colony, the adult females should be given blood meals at intervals of about 2-3 days. Males are fed with 5-10% sugar or glucose solution contained in small glass bottles in which cotton wool is placed (Fig. 78). About 2 cm of the cotton wool is left sticking out of the solution and on this the males and females will readily feed. Female mosquitos can feed on human arms or legs, rabbits, guineapigs, pigeons or chickens. When animals are used, the skin on the thorax, abdomen or sides can be shaved, depending on the species of animal and on the way in which it is placed on, or in the cage. The animals should be anaesthetized by an intravenous, intramuscular or intraperitoneal injection of pentobarbital. About 2 ml per kilogram of body weight is recommended for rabbits and this should be adapted for the species of animal used, some of them having a lower or higher tolerance for the barbiturates. In the majority of cases, anaesthesia is complete in 10-15 minutes and will last 1-2 hours. Care should be taken not to inject too much anaesthetic as, when this is absorbed by the mosquito, with the blood meal, it might cause mortality. Usually anaesthetized guineapigs are merely placed on the top of the cages where they lie as on a hammock. Rabbits or guineapigs can be placed in a special compartment of a large cage and left overnight, but active animals may kill some mosquitos while these are feeding. Pigeons can be immobilized by tying up the wings and legs. When a large number of mosquitos have to be fed, the anaesthetized animals are placed inside the cage on their backs and the mosquitos feed from their legs, head, ears, etc. When egg development is not required, feeding with 2%-10% sugar solution will keep the mosquitos alive for long periods.

The following precautions should be taken in maintaining a mosquito colony:

- avoid contamination with insecticide,
- protect the larvae from natural enemies and the adult mosquitos from ants,
- provide the optimum temperature and light for larvae,
- provide the necessary humidity, temperature, food and shade for adults.

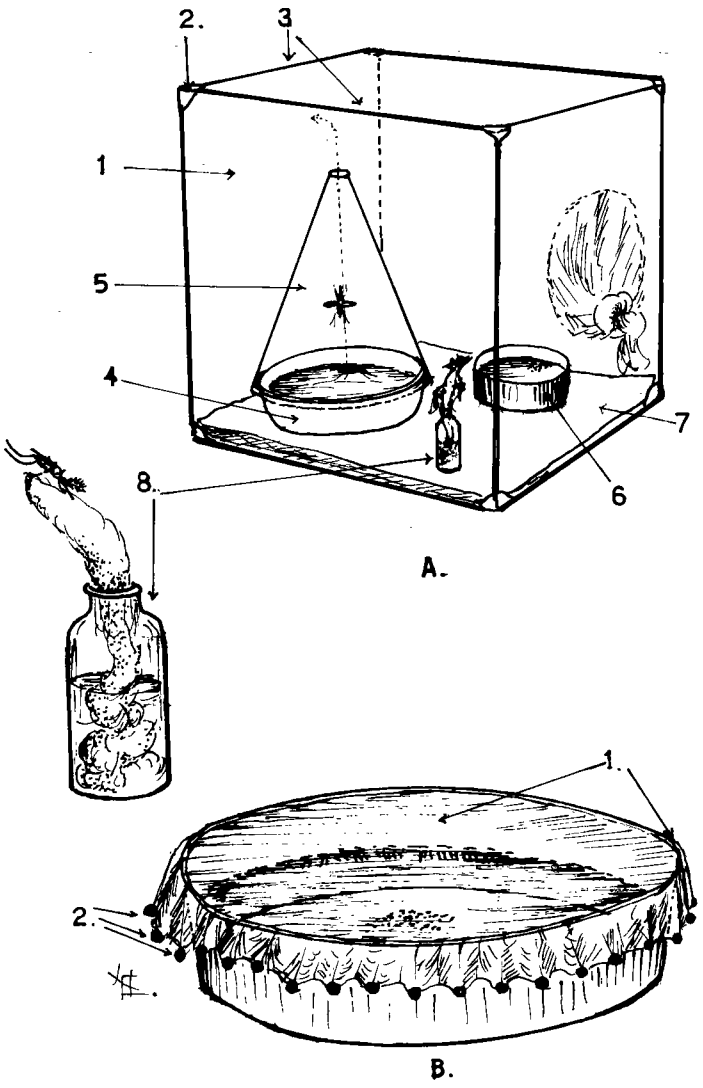


Fig. 78.

A. Laboratory colony cage:

(1) mosquito netting; (2) reinforced corners; (3) metal or wooden frame;

(4) bowl with pupae covered with cone;(5) to avoid egg-laying but which ; allows emerging mosquitos access to the cage;

(6) bowl for egg-laying; (7) white paper sheet;

(8) bottle with sugar solution (mainly for males, the females being fed on an anaesthetized guineapig laid on top of cage.

B. Larval bowl covered with nylon gauze weighted with beads. Ordinary netting should not be used as loose gravid females may drop eggs through it.

20. MOSQUITO REARING UNDER FIELD CONDITIONS

20.1 General

Mosquito rearing under field conditions is not difficult in many tropical areas as the appropriate temperature and humidity required either exist in any shaded room or can easily be obtained by slightly modifying the existing rooms, or by constructing special compartments using cheap materials.

There are three possibilities for the rearing of mosquitos in the field:

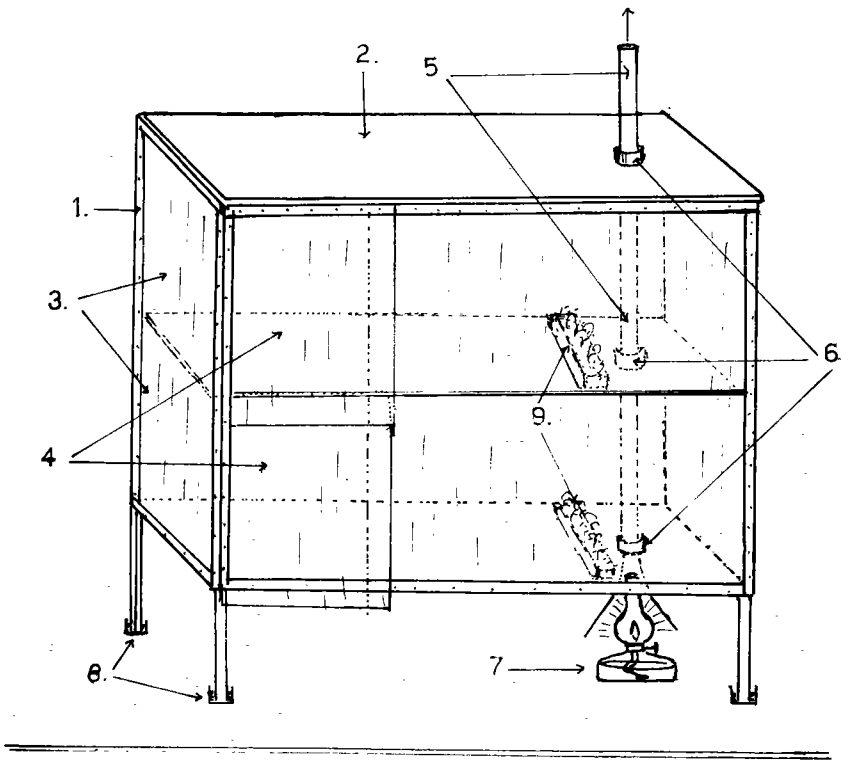
- Breeding mosquitos from larvae and pupae collected in natural breeding places.
- Breeding mosquitos from eggs laid in captivity by wild-caught females, the required number of adult mosquitos always originating from the wild population.
- A self-perpetuating colony which is automatically maintained in the case of stenogamous mosquitos or by artificial mating in the case of eurygamous mosquitos.

20.2 Maintenance of adult mosquitos under field conditions

Adult mosquitos can be kept in a local room, uncontaminated by insecticide, if a special insectary cannot be built. The most important modification to be made is a low ceiling, 220 cm high, using local materials. This reduces ventilation to a minimum, makes it easier to achieve the necessary humidity and temperature and facilitates collection of mosquitos which might escape and rest on the ceiling. During dry periods it is difficult to maintain the necessary humidity. The following humidity chamber can easily be constructed according to the specification given in Fig. 79. The top, bottom and intermediate levels are made of wood fixed to a wooden frame. The back and side walls are covered with a single or double layer plastic sheet. One sheet of plastic is fixed on the outside of the frame and another inside, with drawing pins. The front part is covered with small pieces of plastic, one sheet for each level, fixed at the top but free at the bottom to form a flap. Each sheet should be large enough to overlap the neighbouring sheet by about 10 cm. Small cages, paper cups or even cages of 30 x 30 x 30 cm can be used in this chamber. The chamber is placed on a table with the legs in tins containing mineral or edible oil to prevent ants reaching it. A maximum/minimum thermometer and a hygrometer are used for recording the temperature and the humidity at each level; however, if there is free circulation of air, these can be placed at the upper level only. Sufficient circulation of air can be obtained by separating the three levels with wire netting instead of wood. Humidity can be ensured by placing inside the cage a tray, or several trays, filled with cotton wool saturated with water, or by suspending in a vertical position some cotton gauze or filter paper dipped in a narrow tray filled with water. In areas where the nights are cold and a higher temperature is required, and provided that electricity is available, an electric bulb inside a metal tube or covered with aluminium foil is placed in the lower part. The wattage necessary in relation to the volume and the ambient temperature has to be established by preliminary trials. If it is necessary to keep the inside of the chamber dark the exterior can be covered with black cloth or paper. Where electricity is not available the cage can be heated with a paraffin or oil lamp with a metal tube to transfer the heat (Fig. 79 (5)). The required temperature is obtained by regulating the flame of the lamp. The maximum and minimum thermometer records the variation in temperature. With practice, this type of heating is suitable for larval breeding or for keeping adults at the required temperature for physiological activity.

These cupboards can be constructed in different sizes and may be used for keeping adult mosquitos in excellent condition for observations on longevity, duration of gonotrophic cycle, survival rates, etc. A separate and relatively small humidity chamber can be constructed to study the 24-hour survival rate of mosquitos exposed to insecticide.

Note. If available, a wooden bookcase or similar support can easily be transformed into a miniature mosquito chamber by covering the front and the back with plastic sheets.



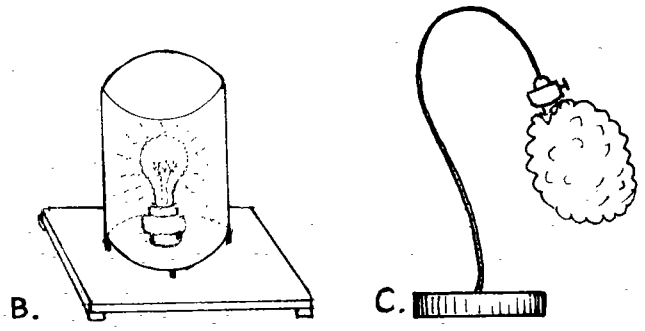


Fig. 79.

(A) Improvised insectary cabinet: (1) wooden frame; (2) wood; (3) wall of plastic sheets; (4) plastic flaps for access; (5) tin tube; (6) asbestos rings; (7) oil or kerosene lamp; (8) ant-traps; (9) plastic trays with cotton wool and water to keep humidity high.

(B) Electric bulb covered with metal cylinder.

(C) Electric lamp covered with aluminium foil.

When expanded polystyrene sheets 2-3 mm thick are available, the upper part, the sides and the back of the miniature humidity chamber can be made of this material, which has good insulating properties thus making it easier to maintain the desired temperature.

21. ARTIFICIAL MATING

21.1 Principle

Anaesthetized, recently blood-fed unfertilized female mosquitos are inseminated by induced copulation with decapitated males.

21.2 Objectives

- To maintain laboratory colonies of eurygamous species.
- To identify sibling species.
- To study the pattern of inheritance of different characteristics.
- To produce sterile males in large numbers.

21.3 Techniques

Two techniques used for artificial mating:

- microscopic technique.
- macroscopic technique.

21.3.1 Microscopic technique (Fig. 80)

Copulation is obtained by the manipulation of females and is observed under a dissecting microscope, the decapitated males being fixed with the back of the thorax on the rim of a Petri dish and the females being held by a small suction tube.

21.3.1.1 Materials

- Dissecting microscope
- Petri dish
- A small suction (vacuum) pump
- A thick mollet capillary tube
- Tube for connecting the capillary tube to a suction pump
- Ether or carbon dioxide
- Test tubes for anaesthetizing mosquitos
- Paper cups
- Fine scissors
- Entomological forceps
- Glue for fixing the males on the rim of Petri dish
- Males of about 3 days old
- Females 12-14 hours old (too young females will not easily feed).

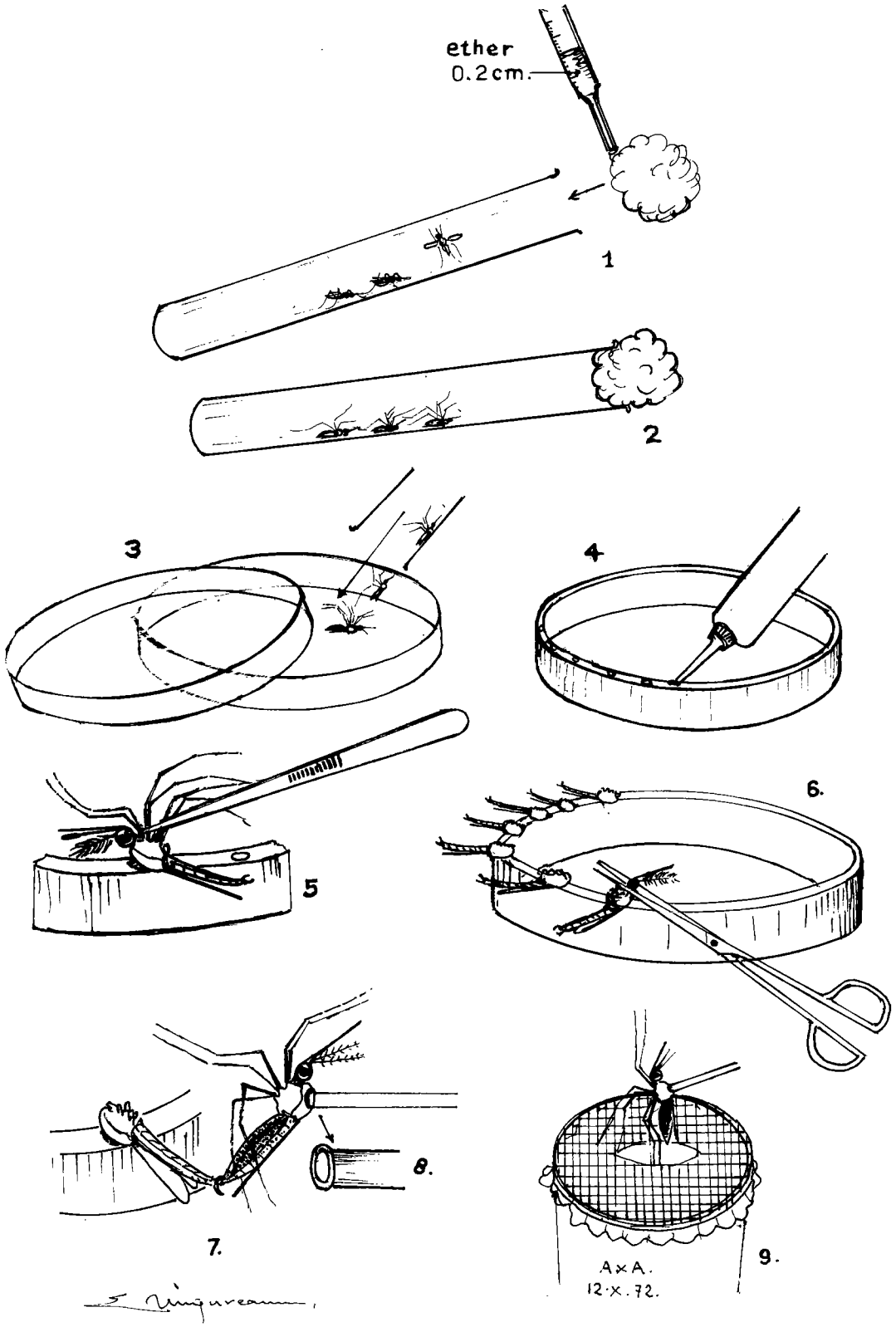


Fig. 80. Procedures for artificial mating.

21.3.1.2 Procedure

- Feed the females on an appropriate host.
- Collect and anaesthetize the males.
- Fix the males with the forceps on the border of the Petri dish at an angle of less than 45°.
- Remove the heads of males with fine scissors.
- Place the Petri dish under the dissecting microscope and adjust the magnification to x6 or x10.
- Hold females by the thorax by applying the tube connected to the suction pump.
- Touch the terminalia of the males with the extremity of the abdomen of the female at an angle of about 45° observing the moment of copulation under the microscope.
- After copulation the female is transferred to a paper cup and kept at a temperature of 25-26°C and about 80% RH to allow the ovaries to mature.

21.3.2 Macroscopic technique (Fig. 81)

The copulation is observed with the naked eye, by the manipulation of the male pinned laterally through the thorax using fine pins fixed in the end of wooden handles.

21.3.2.1 Materials

- Wooden needle holders with Fine pins with wooden handles.
- Ether or carbon dioxide and test tubes.
- Petri dishes to cover the anaesthetized females.
- Fine scissors.
- Paper cups.
- Mosquitos of the same condition as mentioned under 21.3.1.1 above.

21.3.2.2 Procedure

- Anaesthetize the males.
- Pass a pin laterally through the thorax of the male.
- After fixing a given number of males on pins, cut their legs and the heads off.
- Anaesthetize the females.
- Put the females on a white sheet of paper and arrange them with the ventral surface uppermost.
- Manipulate, using the method proposed above, with the male ventral side down and touch the female terminalia at an angle of about 45°.
- When the male is firmly attached to the female transfer the couple to a paper cup, leaving the female to recover inside.

  The couple can be removed from the pin by brushing the male against the edge of the hole in the mosquito netting. The mosquitos will separate after the completion of mating.

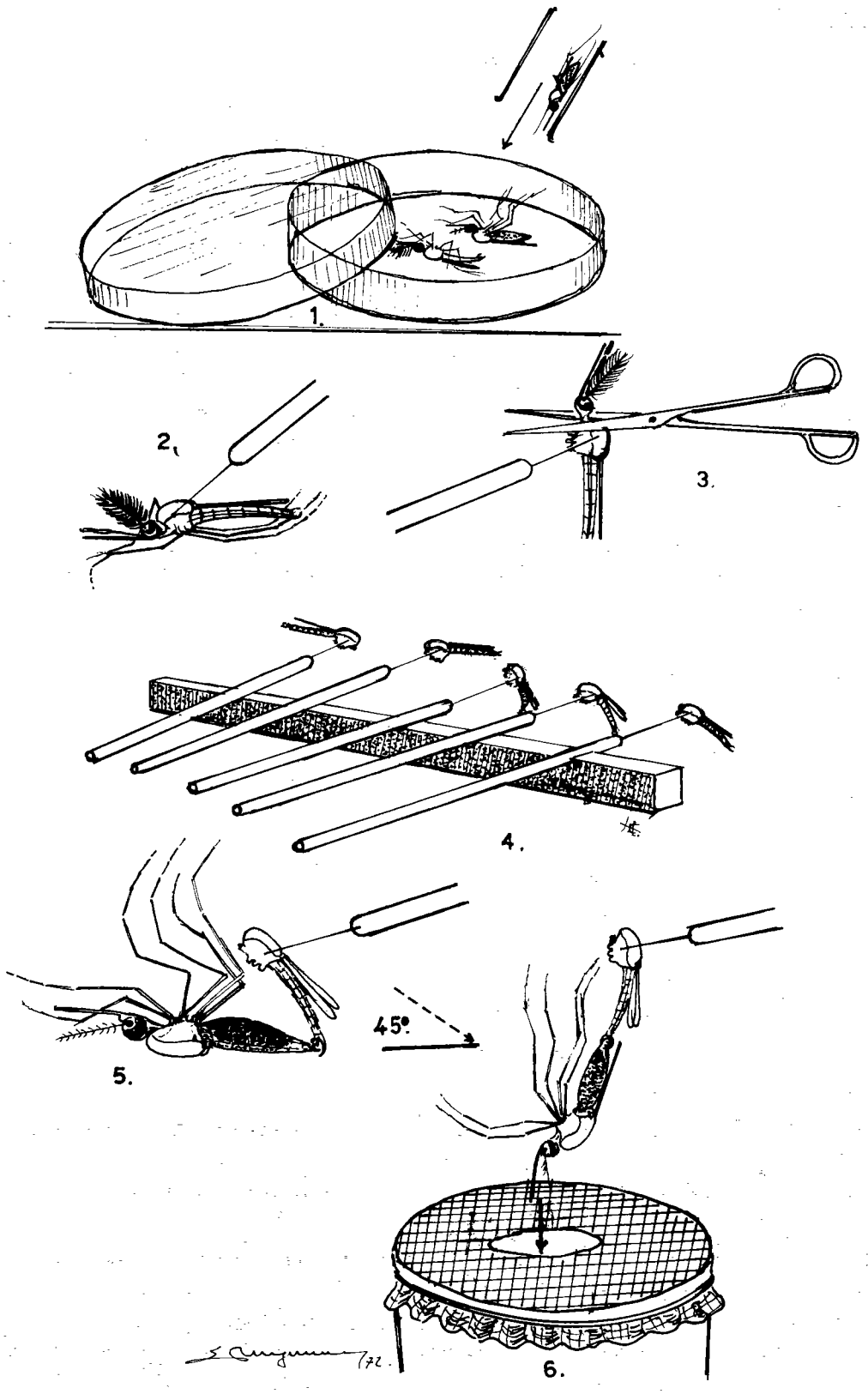


Fig. 81. Artificial mating - alternative procedure.

22. METHODS FOR MARKING MOSQUITOS

22.1 Principle

Application of stains or metallic dust to adult mosquitos, or feeding larvae or adults with a food which includes a radioisotope.

22.2 Objectives

- To study the dispersion of mosquitos.
- To study the longevity.
- To study calendar age, and the average duration of the gonotrophic cycle.

22.3 Method

The method is not carried out routinely in a malaria programme. Three alternative techniques may be used: (a) dusting with powders; (b) painting; (c) labelling with radio-isotopes.

22.3.1 Dusting with powders (Fig. 82)

The use of coloured or fluorescent dusts is the simplest way of marking mosquitos. It is suitable for experiments in the laboratory and for field releases.

Two types of dust have been successfully used, namely metallic dusts and those showing fluorescence when exposed to ultraviolet light. The former group consists of powders used in printing, known as "Printers' Gold", which are readily obtainable from the appropriate suppliers. A number of colours can be bought. The only apparatus required is a small insufflator, which can be made in any laboratory. A quantity of gold powder is placed in the bottom of the tube, from whence it is blown into a nylon gauze cage 15 x 10 x 10 cm containing the mosquitos and covered by a transparent plastic bag. The dust sticks mainly to the inferior part of the thorax in between the coxae and can be readily detected in light with or without the aid of a hand lens. Gold, silver or copper dust should be used when three batches of mosquitos are released at short intervals.

Fluorescent dusts have two particular advantages. Firstly, they fluoresce brightly when illuminated with a broad spectrum ultraviolet lamp (of the order of 407-313 nm) and can be detected at a range of up to 3 m. Thus individual handling of the specimens is unnecessary. Secondly, mosquitos are relatively insensitive to some ultraviolet spectral ranges and the behaviour of marked specimens can be watched at night without disturbing them. The dusts are applied to the mosquitos in the same way as for gold dusts. They may be made up in the laboratory from dyes mixed with gum in a little water, which is subsequently evaporated to dryness and ground up to form a light powder. Three different colours can be obtained, all of which are easy to distinguish under ultraviolet light. They are prepared from the following constituents.

- 1 part of anthracene to 2 parts of gum arabic to give a blue fluorescence,
- 1 mg of rhodamine B to 3 g of gum arabic to give a red fluorescence,
- 1 mg of water-soluble fluorescein to 3 g of gum arabic to give a green fluorescence.

A more convenient method is to use commerically prepared fluorescent dusts, which may be obtained from certain manufacturers. These powders give four different colours when illuminated by an ultraviolet lamp, and are supplied ready for use.

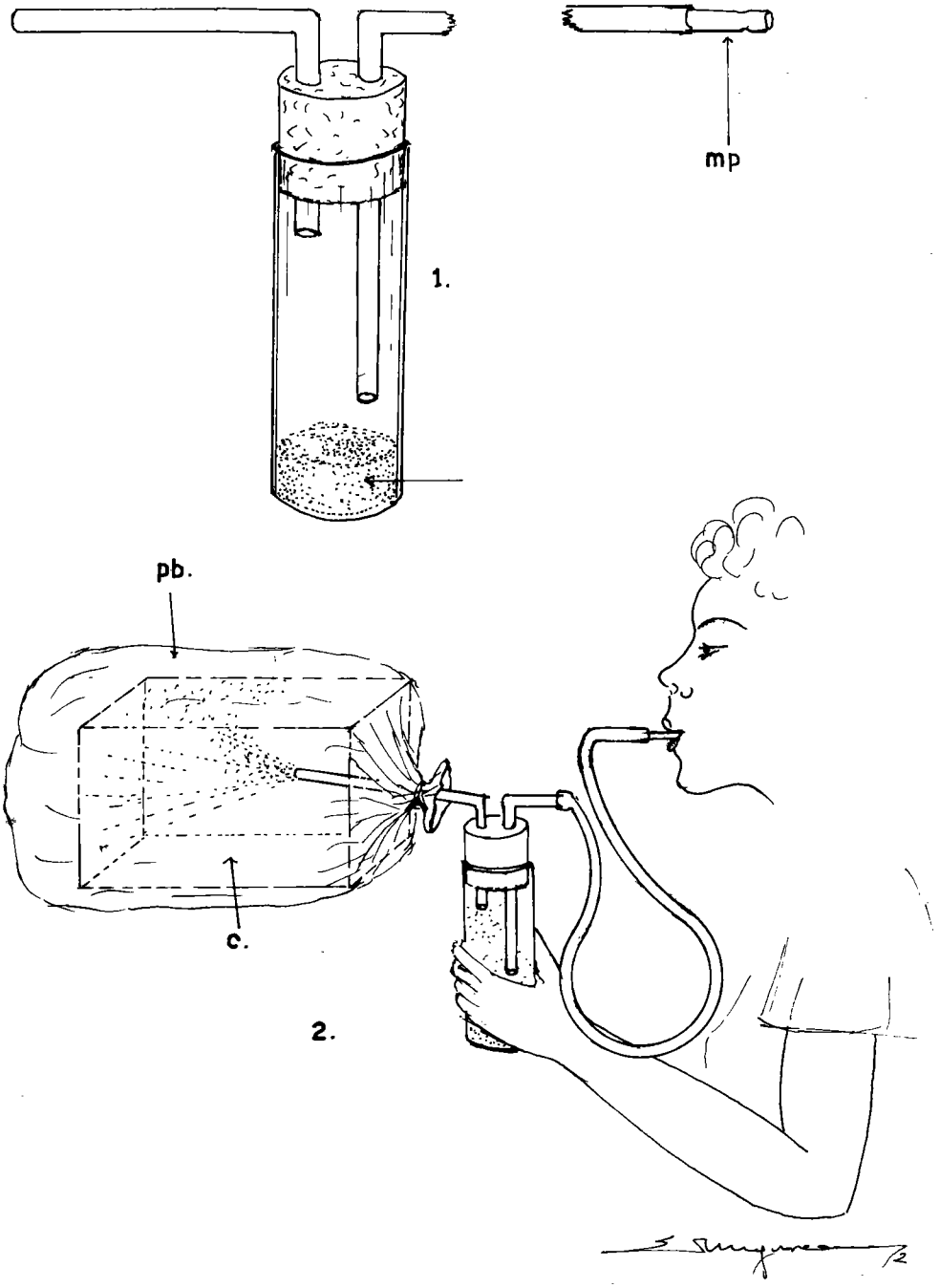


Fig. 82. Marking mosquitos with dust.

22.3.2 Marking with paints

In certain types of investigation the release of relatively small numbers of mosquitos may be adequate, but a greater variety of labels is demanded than can be provided by other methods. To meet this requirement, the topical application of small spots of paint to the thorax has been used. The paint, a water miscible artist's powder paint, can be obtained in a range of colours which can be blended at will.

It is applied with a microloop, made from plated copper wire (about gauge 0024) attached to a small splinter of wood or matchstick. The mosquitos are lightly anaesthetized a few at a time, with ether or carbon dioxide, if available, tipped out onto a piece of cork, and gently flipped over with a dissecting needle so as to lie dorsal side up. The cork is then placed under a wide-field low-power magnifier ready for painting. By gently touching each mosquito on the thorax with the loop they are lifted up just up off the cork. A slight tap of the hand holding the loop causes the insect to fall back again, leaving a discrete spot of paint at the site of contact. If the loop is dipped into a dilute solution of detergent, such as Teepol, a smaller spot of paint remains on the insect than if the paint alone is used. It should be possible to mark 150-200 specimens an hour. Individual numbering can be achieved by this method, on the basis of a positional code.

In sorting recaptures, the mosquitos are laid out in rows, dorsal side up, and each one examined with a hand lens. If colours in the cream or buff range are employed, some confusion can arise with spots of excreta accidentally transferred to the thorax from other specimens. For this reason such colours should be avoided. For checking the paint on recaptured specimens, it is necessary to keep a pinned example showing the exact blend and pattern used for each day's releases, and to use this as a reference for matching.

22.3.3 Radioactive labelling

22.3.3.1 Choice of isotope

The marking of mosquitos with radioisotopes is a relatively simple procedure, and provided elementary precautions are observed in handling concentrated radioactive solutions, there should be no hazards. From a practical point of view the only isotopes suitable for use in the field are those emitting beta-particles. Phosphorus-32, which produces "hard" (high energy) radiation, has been widely used, although sulphur-35, which produces "soft" (low energy) radiation, may be useful in some circumstances. Both of these are cheap, readily obtainable and of established value in field investigations. The characteristics of these, and of some other isotopes that have been used for labelling mosquitos, are shown in Table 10.

TABLE 10. CHARACTERISTICS OF SOME RADIOISOTOPES SUITABLE FOR LABELLING MOSQUITOS

Isotope	Type of radiation	Half-life	Energy
Phosphorus-32	beta	14.3 days	Hard (1.71 Mev)
Strontium-89	beta	51 days	Hard (1.46 Mev)
Sulphur-35	beta	87.2 days	Soft (0.17 Mev)

22.3.3.2 Equipment

For detecting or measuring radioactivity, ordinary counting equipment is required. No details are given here since expert advice and practical instruction on the ordering and use of such instruments are necessary. With autoradiography no electronic equipment is required for testing recaptured mosquitos. However, it is necessary to use a simple monitoring instrument, of the type used by prospectors (fitted with a beta-window), for monitoring hands

and clothes after handling radioactive solutions. With the isotopes recommended, and with the small quantities needed for labelling, one can dispense with special garments such as gowns and rubber gloves. However, rigid rules should be enforced to prevent radioactive material being ingested by mouth, and the hands should always be washed and monitored before leaving the insectary. The only other items required for labelling are a set of graduated 5-10 ml syringe-pipettes for measuring and delivering solutions containing isotopes. If large numbers of bowls have to be treated, automatic delivery pipettes can be most useful.

Any type of photographic film is sensitive to radiation, but X-ray film is normally used for autoradiography, since it is coated with emulsion on both sides and the image is enhanced accordingly. Industrial X-ray film is particularly suitable and can be obtained in narrow strips (29 x 280 mm), which are especially convenient for the exposure of strips of mosquitos. The only accessories required for the dark-room are a dark green "safe-light" and light-proof film holders for exposing films to mosquitos.

For developing films the following routine works well.

- Develop in Ilford's ID 19 developer (or equivalent) for 7-8 minutes.
- Wash in acid stop solution (1% acetic acid).
- Wash in distilled water.
- Fix in any standard fix-hardener for 15 minutes.
- Wash in running water for half an hour.

22.3.3.3 Labelling procedure

Phosphorus-32 is commonly supplied as a solution of sodium dihydrogen phosphate, containing 1 millicurie of radioactivity per ml. For small- or moderate-scale work this solution should be diluted down as soon as possible by a factor of about 100, in order to minimize the risk of contamination and to enable the very small quantities of active material required for marking purposes to be accurately measured. For labelling Anopheles gambiae the diluted solution of isotope is introduced into the breeding pans when the larvae are in the late third or early fourth instar, or two or three days before overt pupation. A dosage of 5 microcuries of ^{32}P per litre of water in the breeding pans is sufficient to give clear autoradiographs up to 5-6 weeks after marking, or of counts of several hundred per minute when tested with a counter. The dosage can, however, be increased without harm to the insects if resources permit. With sulphur-35 a dose of 15-20 microcuries per litre is required in order to obtain good autoradiographs. The pupae are transferred to small cups or bowls placed in cages for emergence. It is easier to construct these cages with a removable lid, so that after emergence of the adults, they can be transported to the field and the mosquitos released without further handling. Sugar solution should be provided if there is a delay of more than 12 hours between emergence and release. Releases are best made at dusk, so that the mosquitos can disperse naturally.

Mosquitos can also be marked as adults by providing them with sugar solutions containing isotopes. This is less satisfactory than the standard method owing to the variation in the quantity of solution imbibed, but it could be used to label wild-caught mosquitos for special investigations.

22.3.3.4 Release of mosquitos

Mosquitos can be released in their normal resting places, or in the area of the breeding place.

22.3.3.5 Capture of mosquitos

The mosquitos should be sampled in different capture stations at different distances from the place of release. Daily capture should be performed. The duration of observations will depend on the objective. For dispersion and movements of mosquitos 6-10 days will be enough; for the study of longevity, a period up to 20-30 days may be required. Observations on the longevity of marked mosquitos should be carried out in very small localities.

22.3.3.6 Detection of recaptures

Marked mosquitos may be recognized either by the use of a Geiger counter or by auto-radiography. The former method enables quick sorting of large numbers of mosquitos. Furthermore, the specimens can be recognized while still fresh so that detailed dissection and examination can be made. On the other hand, it requires electronic equipment facilities for the maintenance of which need to be assured, and it is probably not suitable for the detection of mosquitos labelled with ^{35}S when the activity has fallen to a low level. The use of auto-radiography obviates the need for any special equipment beyond normal photographic darkroom facilities. It is also more sensitive and makes it possible to employ two different isotopes at the same time without risk of confusion when sorting the recaptured mosquitos. On the other hand the process of monitoring catches is a good deal more involved, and radioactivity cannot be detected in fresh specimens.

When monitoring catches with a counter, it is simply necessary to bulk all the mosquitos from a particular sector and test them together. Any negative batches are discarded, and the others then carefully sorted in order to locate the labelled specimen or specimens.

In testing catches by autoradiography it is convenient to spread them out onto cellulose tape to which details of the catch can also be attached. If only ^{32}P had been used, the mosquitos can be covered with a thin layer of cellophane before testing. However, with ^{35}S they must be left in direct contact with the film and heavy weights must be placed on top of the film holders. In order to identify individual mosquitos it is necessary to adopt some system of reference that can be applied both to the strips of mosquitos and to the film after developing. One convenient method is to pin the tape, sticky side up, over a numbered grid, and place the mosquitos on the tape in such a way as to cover each number exactly. If the developed film indicates the presence of any radioactive insects, it can be placed over the grid and the number of the specimen read off. If there is any doubt about the result, a small piece of film can be cut out and placed over the mosquito thought to be labelled. An exposure time of 24-48 hours is normally sufficient but if there is any reason to expect that the amount of radioactivity may be low, this period may be extended up to several days or even longer. Fig. 83 shows some typical autoradiographs of labelled mosquitos.

If overlapping releases have been made with two different isotopes, it is possible to differentiate between them by using double layers of X-ray film. The top layer of film then acts as an absorber which screens off the soft radiation of an isotope such as ^{35}S while allowing the relatively hard radiation from ^{32}P to pass through. Thus in one instance the fogging is confined to the layer of film in contact with the mosquito while in the other an image is formed on both layers. To distinguish between the two isotopes the mosquitos are routinely exposed to a double layer of film; or, more simply, only those individuals found to be radioactive are re-exposed on two layers of film.

22.4 Use and limitations of different methods for marking mosquitos

Metallic dust powder can be successfully employed for most kinds of observations. The marked mosquitos can be easily detected immediately after the collection by using an electric light or a hand lens, and observing them directly in the glass test tube in which they are collected. It is recommended that a glass test tube be used, in the bottom of which a piece of cotton wool has been placed; a few drops of chloroform are put on the cotton wool just before using the tube. After collecting four to five mosquitos separate them with a piece

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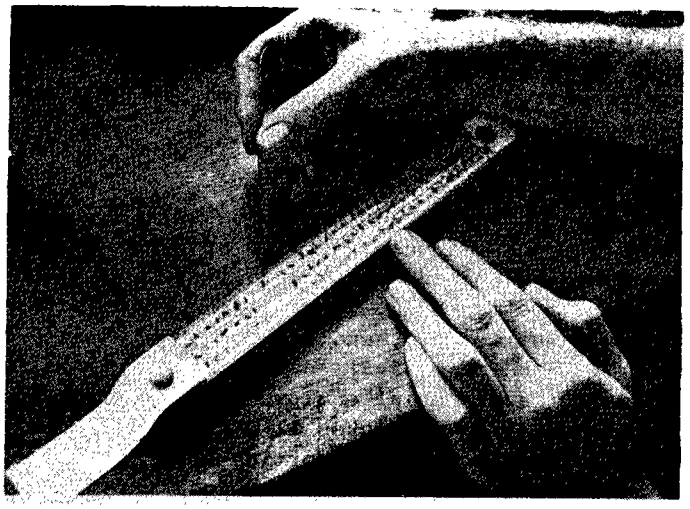

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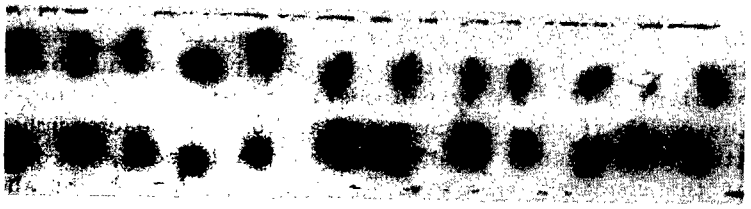

Fig. 83 Radioactive labelling - autoradiography.

1. Reference grid for matching up autoradiographs with the orginal catch.
2. Autoradiograph of A. gambiae labelled with ^{32}P (above) and ^{35}S (below).

of cotton wool and collect another group of five mosquitos; repeat the operation till the collecting tube is full. The marked mosquitos collected in this way do not contaminate each other with the metallic powder, and can be examined directly in the tube, the metallic powder particles should be present in between the coxal segments. Contaminated mosquitos may have a few particles of metallic dust on parts other than the coxal area.

This is the oldest method of marking mosquitos and the easiest to apply as long as the dusting is carefully done according to the instructions given.

The use of the other methods mentioned above are more laborious and time-consuming, and in the case of mosquitos marked with isotopes, the identification takes much longer.

PART II

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