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(II)
A TECHNIQUE FOR STAINING, DISSECTING, AND MOUNTING
THE MALE TERMINALIA OF MOSQUITOES

By W. H. W. Komp, Senior Medical Entomologist, United States Public Health Service

The classification of mosquitoes is based to a large extent on the characters of the sexual appendages of the male adult, which are collectively known as the terminalia. In a number of groups, notably the genera Anopheles and Culex, certain minute though definite differences in these structures have proved so far to be the only available characteristics for the separation of a number of closely related species. From a practical standpoint, the study of the male terminalia has become of prime importance in connection with the anopheline vectors of malaria. With increasing knowledge of the problems involved, it has become evident that the older methods of mounting mosquito terminalia do not yield suitable material for the critical analysis of minute differences, which is now known to be necessary.

Among certain medically important groups in the genus Anopheles, for example, the shape of the male generative organ, the mesosome, and of the claspette lobes, which lie behind the mesosome, is of paramount importance in the ultimate identification of species. In the usual whole mount of the male terminalia these parts are covered by the anal lobe, a large hood-like structure, which often obscures the view of the more characteristic parts. In some species only the presence or absence of very small lateral spines on the mesosome, called leaflets, separates one species from another. These leaflets are usually invisible if covered by the anal lobe, and often must be stained to make them visible.

The usual method of preparing the terminalia for study involves maceration of the whole terminalia in weak alkali, dehydration, clearing, and mounting in balsam on a 1 x 3 inch glass slide. This method gives specimens in which many of the characteristic structures are obscured by the parts which lie over them. Some method of staining and dissecting these parts, to render their minute details more easily seen, is required for the recognition and separation of closely related species. It is often desirable to examine both sides of the terminalia under high magnification, but if they are mounted on

1 From the Gorgas Memorial Laboratory, Panama, Republic of Panama.
the ordinary 1 x 3 inch glass slide only the upper side can be examined, as the working distance of a high-power objective is usually much less than the thickness of the slide. Some method of mounting the terminalia which will permit the examination of both sides of the parts is therefore an advantage.

The technique given below, in part devised by the author and in part adapted from other workers, is a combination of staining, micro-dissection and special mounting, which renders all parts of the male terminalia visible on both sides and permits accurate drawing or photographing of the component structures.

STAINING

Staining is done as one of the steps in preparing the material for dissection. These steps, including the staining, are as follows:

1. Under the low power of the dissecting microscope, cut off the tip of the abdomen, including the terminalia, with a small needle sharpened at one side to a knife-edge.

2. Place the severed portion in a drop of absolute ethyl alcohol in a small glass cell. The alcohol allows the immediate wetting of the specimen by the alkali used in step 3.

3. Cover with 20 percent sodium hydroxide (NaOH) solution in distilled water and let stand for 12 hours.

4. Remove alkali from cell with capillary pipette and replace with acetic alcohol (3 parts of 50 percent ethyl alcohol, 1 part acetic acid). Leave in acetic alcohol for 15 minutes.

5. Remove acetic alcohol with another capillary pipette and replace with Gage's stain, diluted 1 to 5 with distilled water. Gage's stain (1) has the following formula:

\[
\text{Acid fuchsin} \quad 0.5 \text{ gram} \\
\text{10 percent hydrochloric acid} \quad 25.0 \text{ cc.} \\
(\text{Add 10 cc. of concentrated HCl (sp. gr. 1.18) to 90 cc. distilled water.)} \\
\text{Distilled water} \quad 300.0 \text{ cc.}
\]

Place one drop of this stock solution of stain in the glass cell and add five drops of distilled water. Allow to remain for 12 hours.

6. Remove stain from glass cell with capillary pipette and replace with 95 percent ethyl alcohol. Let stand for 5 minutes.

7. Remove 95 percent alcohol and replace with absolute ethyl alcohol. Let stand for 15 minutes.

8. Remove absolute alcohol and replace with clove oil. The specimen may remain in the clove oil until thoroughly cleared, or it may be left for a longer time, as the stain does not fade if the oil is fresh. Long immersion in the oil makes the specimen brittle, which is sometimes an advantage. The specimen is now ready for dissection.
PREPARATION OF DISSECTING NEEDLES

No needles fine enough to dissect the minute structures of the male terminalia are obtainable commercially. Even the small "minuten nadeln" used for double mounts of small insects are much too coarse for the purpose. It has been found impossible to grind needle points to the required fineness by using a flat stone and manual grinding; the point breaks long before such fineness is reached. Mechanical grinding, using a small high-speed motor and a special grinding wheel, will give the necessary fineness.

No. 0 stainless steel insect-pins are the crude stock from which the fine needles are made. A pin is stuck into the tip of an ordinary wooden applicator, a thin stick of wood 6½ inches long and about one-sixteenth of an inch in diameter. The pin is driven well into the wood, using forceps to press it in, and the head end is cut off, leaving about an inch projecting from the end of the applicator. The grinding apparatus is a special stone, mounted on a small high-speed motor. Three coarse stones are sold with this motor, and may be used for the preliminary rough shaping of the needle. The special stone used for the final grinding is made to standard dimensions, of a composition used commercially in grinding safety-razor blades. The motor with the stone is held in the left hand, and the applicator with the needle inserted is held in the right hand. Grinding is done under visual control under the low power of the binocular dissecting microscope (fig. 1). Two opposite sides of the needle are ground first, making a long thin blade; the other two sides are then ground, making a fine point with a cutting edge. Considerable practice is necessary before a good point can be made. However, points half the thickness of a human hair can eventually be made without difficulty. It is well to make a number of needles at the same time so that dissecting need not be interrupted if one needle breaks.

DISSECTION

The stained and cleared terminalia are placed in a very small amount of clove oil in the depression of a hollow-ground slide. It is better to cut off one end of the slide, just beyond the depression, and orient it so that the long end points away from the operator. This is done so that the slide will not be touched and accidentally moved by the mounds of plasticine used to steady the needles during dissection. Two applicators with the prepared needles are imbedded in the tops of two mounds of plasticine (modeling clay): These mounds should weigh about 35 grams each, are roughly pyramidal, and about one inch in height. The applicators are set in the plasticine at an angle of about 30 degrees from the horizontal. The points of the needles are brought close together in the field of the microscope. The left-
hand needle is used to hold the specimen down on the slide, and the right-hand needle cuts and dissects out the parts of the terminalia. The arrangement of applicators and plasticine mounds is shown in figure 2.

Detailed instructions on the dissection procedure cannot be given here, as the procedure varies with the arrangement of the parts of the terminalia of the species being dissected. However, in *Anopheles* it is best to remove the ninth tergite and the anal lobe first, and then the mesosome, leaving the claspette lobes until the last. Considerable practice is required to make a perfect dissection so that all the parts are unbroken. However, no worthwhile technique requiring a considerable degree of muscular coordination can be acquired in a day.

It is well to examine the parts while they are still in clove oil so that various aspects may be brought into view by moving the parts about with a needle. Drawings of the parts in various aspects can now be made, using a compound microscope with a 20× objective and a 10× eyepiece. The part to be studied should be stranded in the edge of the drop of clove oil to avoid motion from currents in the oil.

**MOUNTING**

After dissection the parts are removed to the slide on which they are to be permanently mounted. The usual 1 x 3 inch glass slide may be used, but this allows only the upper side of the specimen to be viewed with a high-power objective, as the thickness of the glass slide is greater than the working distance of such an objective.

The special slides designed by the writer are a modification of the Cobb slide (3). They are made from 1 x 3 inch (25 x 75 mm.) aluminum blanks, 1.5 mm. in thickness, with a central circular hole 13 mm. in diameter; concentric with this hole is a shoulder 17 mm. in diameter and 0.3 mm. deep. An ordinary No. 1 circular cover-glass 15 mm. in diameter is cemented to the shoulder, covering the hole, using any one of a number of commercial cements, such as "Duco." The cement should be thinned with acetone and liberally applied to the shoulder of the slide with a small brush. The cover-glass is set into the cement immediately, before it has a chance to dry.

The various separate parts of the terminalia should be arranged in a systematic order on the slide. It is well to follow the order in which the parts occur in the undissected terminalia; the two side pieces are placed nearest 12 o'clock on the cover-glass; immediately beneath is the mesosome, then the claspette lobes, and, nearest 6 o'clock, the ninth tergite and anal lobe. Small drops of balsam, one for each part to be mounted, are placed on the lower cover-glass which has been affixed to the aluminum slide. These small drops are applied from the point of a capillary pipette which can be made by drawing out in a flame the
Figure 1.—Needle in applicator being sharpened against stone mounted on small electric motor, under visual control.

Figure 2.—Method of supporting dissecting needles in mounds of modeling clay. Note position of hollow-ground slides.
end of a pipette from a dropping bottle provided with a ground-in pipette. The balsam, which should be rather thin, is kept at a level in the dropping-bottle such that the fine tip of the pipette barely reaches below the surface. Using the rubber bulb of the pipette, the drops are carefully squeezed out onto the lower cover-glass under visual observation under the low power of the dissecting microscope. The separated pieces of the terminalia are picked up on the end of a needle, and each is transferred to one of the balsam drops. All this is done, of course, under the dissecting microscope. To make the transfer easy, the slide containing the parts in clove oil is placed parallel to the slide to which the parts are to be transferred, so that, using the left hand, both may be moved back and forth as a unit under the microscope.

After the parts have been transferred to the balsam drops and properly oriented, the slide is put away in a dust-proof box for a week or more. This allows the balsam to harden so that the parts imbedded in it cannot move about when the upper cover-glass is placed over them. At the end of this time a small drop of balsam is placed in the middle of a 12 mm. round cover-glass and inverted over the lower cover-glass. Just enough balsam should be used to spread evenly to the edges of the upper cover-glass. Both sides of the specimen may now be examined with the oil-immersion objective, since the material is covered on each side only by the thickness of a cover-glass.

The preparation may be labeled by scratching the data on the aluminum slide with a diamond pencil or the conventional gummed paper label may be used.

ILLUMINATION

The smallness of the parts to be dissected requires high illumination of the microscope field. The writer has used a Spencer Universal Microscope Lamp, No. 358, in his work, and finds it satisfactory if suitably adjusted. The lamp bulb must be drawn back as far as possible in the sleeve, so that the intensely brilliant image of the lamp filament is thrown on the field. The Nicholas Illuminator of Bausch & Lomb can also be used if the tube containing the lower lens is suitably lengthened so that an image of the filament may be obtained. A makeshift lamp, which may be very useful in the field, can be made from a two-cell focusing flashlight, focused so that the light is concentrated on the field, and supported on a stiff bent wire stand.

THE MICROSCOPE

Almost any type of binocular dissecting microscope capable of giving a wide field and a magnification of from 60 to 80 diameters may be used for dissection. The base of the microscope which carries the illuminating mirror must be removable, as the glass plate forming the stage of the microscope must rest on the table on a level with the operator’s hands. Some method of changing quickly from low to high
power is convenient, but not absolutely necessary. Eyepieces giving a magnification of 9\(\times\) and objectives giving a magnification of 6.8\(\times\), a total magnification of about 60 diameters, are used for the actual dissection, and a magnification of about 10 diameters is used for grinding the needles and putting the drops of balsam on the slide.

A good binocular compound microscope is a necessity for the examination of the parts of the terminalia after they have been mounted under the cover glass. A useful combination is a 20\(\times\) objective and a 7.5\(\times\) or 10\(\times\) ocular; but to show fine detail higher powers must be used.

Paired eye caps with perforated diaphragms are useful in obtaining a stereoscopic image. A simple expedient, giving the same effect as the eye caps, is to cut from thin black paper a circular disc which has the same diameter as the ocular. This disc is cut in half, and the halves are stuck lightly to the oculars so that the inner halves of each are covered, the straight edges of the half discs being vertical. By moving the lightly adhering half discs toward and away from each other a position will be found where an excellent stereoscopic image is obtained. The half discs are then glued securely to the oculars. A stereoscopic image is very useful in determining whether a certain part is below or above another part when making descriptions or drawings.

APPLICATIONS OF THE METHODS TO OTHER OBJECTS

The methods outlined above are applicable to other objects besides the terminalia of mosquitoes. Anyone working with insect genitalia which require dissection for proper demonstration will find the method of grinding the needles and supporting them during dissection of great assistance.

The methods described here have been taught to two coworkers who now make creditable preparations; the technique apparently does not require any special aptitude, although facility cannot be gained in a day. Dissection requires considerable patience and practice but the results are worth the time and effort expended.

One example of the advantages of dissection as applied to mosquito terminalia is the demonstration of the striking differences between two anopheline species found in the Caribbean region, A. neivai and A. bellator. Regarding the terminalia of these, Root (4) states: "The genitalia of these two species [bellator and kylephilus (=neivai)] agree in every detail with those of A. neivai, so far as I could see." The writer (5) has shown that the mesosome of A. neivai is without leaflets, while that of A. bellator has two very large reflexed leaflets, somewhat closely appressed to the mesosome, but easily visible when stained and dissected. The microphotographic illustrations in his article (5) are examples of the possibilities of the method in clearing up the specific identities of closely related species and in illustrating these differences by photography, thereby eliminating the personal equation inherent in entomological drawings.
SUMMARY

Methods of staining, dissecting, and mounting the male terminalia of mosquitoes are described. Procedure for preparing the material, a description of the method and apparatus used in grinding needles, the technique of dissecting, and a description of the modified slide-mount used, are given. The methods outlined give preparations which show both sides of the well-stained, dissected specimen, and which permit a thorough study of the parts to be made. Such preparations are superior to mounts made without dissection, in which some of the characteristic structures may be obscured by other parts which overlie them.

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REFERENCES