ANATOMICAL INDICATORS FOR ASSESSING AGE OF MOSQUITOES: DISSECTION TECHNIQUES AND FIELD APPLICATION OF METHODS

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In areas of the world where mosquitoes are a major factor in public health programs, the estimation of the physiological age of mosquitoes has been important to evaluate control measures and to assess the epidemiological significance of vector populations (Detinova 1968). In the U.S.A., age-grading of mosquito populations by the Polovodova method (Detinova, 1962) has been primarily used for behavioral studies (Carpenter and Nielsen, 1965; Hitchcock, 1968; Romney, 1968). Operational applications of age assessment have been of value in locating unknown breeding sites (McFarland and Magy, 1962; Rosay and Frolli, 1968).

Some of the anatomical characters used for estimating physiological age are difficult to find. The locating of dilatations, in particular, has presented special problems, a reality that has prevented wider acceptance of the Polovodova technique for mosquito studies. Irreversible physiological changes in female mosquitoes that occur during subsequent gonotrophic cycles have been fully explained by Detinova (1962). An excellent account of dissection difficulties has been given by Gillies and Wilkes (1965).

For our studies, a series of anatomical changes was used to determine the age of natural populations of mosquitoes. Females were dissected and examined for insemination, presence or absence of muscle remnants, midgut contents, ovarian developmental stages, and number of ovarian cycles completed. Males were inspected for degree of rotation of terminalia. The purpose of this paper is to describe dissection techniques as they were used in our studies, difficulties encountered in locating age characters, and application of the methods to field projects. *Aedes nigromaculis* (Ludlow) was of principal concern during these studies, but the dissection techniques have been used without modification for other Culicidae.

DISSECTION OF FEMALE MOSQUITOES. Mosquitoes were transported alive from the field in small cages placed in ice chests to retard further aging. Dissections could be accurately performed only on freshly killed specimens, so collections were refrigerated at 4° C until they could be processed. Refrigeration was effective for up to 48 hours, after which time deterioration of the tissues occurred with mortality of the specimens.

Dissections were made in a drop of 0.675 percent NaCl in distilled water on a glass slide. Tools for dissection were size 0 or 1 insect pins fastened to small diameter glass rods. Some workers have found jeweler's forceps and/or minuten nadeln easier to control. All dissections were made under a binocular dissecting microscope, at 24 diameters magnification, and at 48 diameters magnification for finer work and confirmation of details. The use of transmitted rather than reflected light was sometimes helpful.

Mosquitoes were anesthetized by brief exposure to chloroform. After identifying the species, the abdomen, which was cleanly severed from the thorax, was saved for further dissection.

INSEMINATION. Since the spermathecae and bursa copulatrix were easily lost during dissections, these were examined first. They were removed from the eighth abdominal segment by pulling out the ovipositor with the internal appendages attached. Sperm, if present, were seen as a mass of threads in the spermathecae, or as an opaque cloud in the otherwise clear

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bursa of culicines or in the atrium of anophelines. If either the spermathecae or bursa were crushed or opened, the sperm were released into the saline solution in a bluish mass and were recognized individually as undulating threads.

MUSCLE REMNANTS. The abdomen was opened by tearing along the pleural membrane and then lifting away the integument to inspect for muscle remnants. These appeared as varying amounts of translucent tissue of characteristic muscle appearance located between the gut and the abdominal wall. In newly-emerged mosquitoes, the entire immature musculature was present, but as the remnant was autolyzed, lesser amounts of it were found until it was no longer evident. If the separation of the abdomen from the thorax was not clean, wing muscles sometimes were included with the abdominal structures. These muscles as well as the ovipositor muscles in the apical abdominal segment could be confused with the muscle remnant.

MIDGUT CONTENTS. The ovaries and gut were withdrawn from the opened abdomen and separated from each other. The midgut was examined for the presence of meconium and for indications of blood. Meconium was seen as a semi-solid yellow, green, or brown mass. States of blood digestion were classified as follows: "fresh blood" was bright red; "digested blood" was a bluish or brownish clot with a red core; and "old blood" was brown throughout and small in quantity. Combinations of these terms, such as fresh and old, were used to indicate multiple feedings. However, evaluation of multiple feedings had to be done with caution. At temperatures above 30° C. digestion of the first particles of blood to reach the midgut began immediately while the rest of the meal remained undigested for several hours. This resulted in the appearance of both fresh and old blood in the midgut after a single feeding (Rosay 1969). Note was also made of an empty stomach.

OVARIES. Ovarian development was classified according to Christophers' stages (1911). These were: Stage I, follicle without yolk granules; Stage II, yolk granules occupying less than one-half of the follicle; Stage III, yolk granules occupying more than one-half of the follicle; Stage IV, follicle elongating; and Stage V, oöcyte fully developed with final external chorionic structures. Any doubt about divisions between stages was resolved in favor of the more advanced.

By working at the edge of a drop of saline, the ovaries were supported on the slide but covered by a film of the solution. The ovarian sheath was removed by gently stroking it with a dissecting pin, and as many ovarioles as possible were loosened. The entire ovariole from the germarium to its attachment to the internal oviduct was exposed. The purpose of this step was to orient the dissector with respect to the location of dilatations which were found on the follicular pedicel between the oöcyte and the pedicel attachment to the internal oviduct. If the lengths of a number of pedicels were noted, an approximate pedicel length was recognized which was representative of the ovary being dissected. By knowing the approximate length of pedicels and the position of dilatations on the pedicels, confusion of dilatations with bits of tissue torn away from the internal oviduct during dissections was reduced.

The pedicels were composed of a series of small swellings that gave the appearance of a rope. Dilatations were two or three times larger than these swellings, usually about the same size as the germarium. As the oöcytes grew larger, the dilatations, by contrast, looked smaller, so that a dilatation on a pedicel attached to a Stage IV or V oöcyte was still comparable in size to the germarium which was distal to the oöcyte, although both the germarium and the dilatation appeared minute in comparison with the bulk of the oöcyte.

Dilatations were usually of the same unpigmented color that characterized most of the tissues in the abdomen, but sometimes they were yellow or red. Pigmented dilatations were found in the same ovaries as unpigmented ones.

In addition to the difficulties in recognizing dilatations by location, size, and color, and in confusing them with bits of tissue torn away from the internal oviduct, the pedicels were easily broken during dissection which contributed to a possible loss of dilatations. In order to make an accurate judgment of the number of ovarian cycles completed by the female being dissected a number of pedicels had to be examined until a constant maximum number of dilatations per pedicel was accepted by the dissector. This was also necessary when no dilatations were found.

In females that had recently oviposited, the pedicels were found in a stretched condition. Terms used to describe the progressive contraction of a pedicel were: stretched, one-third shrunken, and two-thirds shrunken. A fully shrunken pedicel possessed the formed dilatation.

Sometimes while Stage V eggs were present in the ovarioles and immediately after oviposition when the pedicels were stretched, dilatations were difficult to find. At these times a search had to be made carefully for follicles that did not mature during the current or just-past ovarian cycle. These follicles were found among the mature oöcytes or among the stretched pedicels. They had either not developed at all beyond being simply formed by the germarium or they had started normal development with the rest of the eggs and were later resorbed. The dilatations resulting from such arrested development corresponded to the batch of eggs just produced.

Ovariole dissection was less difficult in parous females because stretching of the tissues during ovarian development effected a generalized looseness of the ovarioles. In multiparous females, dilatations sometimes coalesced thus somewhat obscuring the individual dilatations. In these it was especially important to look at many ovarioles to estimate accurately the maximum number of dilatations.

AGE DETERMINATION OF MALES. After identifying the species of mosquitoes, the degree of rotation of the terminalia was classified as unrotated, partially rotated, or completely rotated. The males were considered to be mature when the terminalia were completely rotated, and there has been no need for further specific determination of age. As in the females, meconium and muscle remnants were present in young males. It has not been necessary to use these characters in field studies because the terminalia complete rotation at about the same time as internal indicators of immaturity disappear. (Rosay 1961).

FIELD APPLICATION OF AGE DETERMINATION METHODS. Age determination techniques have been conveniently applicable to studies of activity and behavior of natural populations of adult mosquitoes. Since the rate of change of the anatomical characters used in age assessment is directly related to temperature, the duration of each phase was determined in the laboratory for species that would be observed in field studies. Several constant temperatures were used against which average

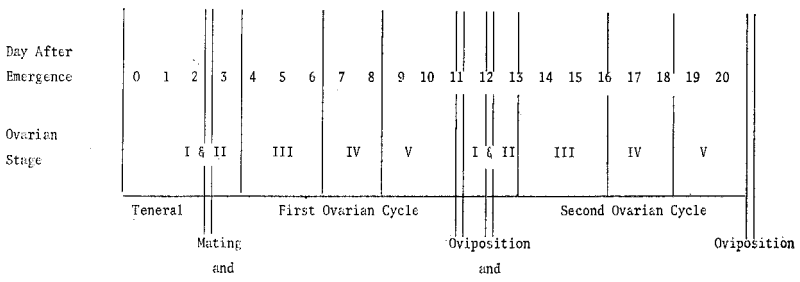

FIG. 1. Sequence to estimate chronological age on basis of physiological age with mean duration of stages at 17±1° C.

AGE DETERMINATION STUDIES

PAGE ________
OF ________

DAY OF STUDY ________ STUDY ________ DATE ________ TIME ________

COLLECTION METHOD ________ STATION NUMBER ________

TEMPERATURE ________ HUMIDITY ________ EVAPORATION RATE ________

SPECIES	TERMINALIA ROTATION			MUSCLE REMNANTS		INSEMINATION		GUT CONTENTS					OVARIAN STAGES					PEDICELS				OVARIAN CYCLE	OTHER
	0	±	+	+	−	−	+	MEC.	EMPTY	FR.	DIG.	OLD.	I	II	III	IV	V	STR.	1/3	2/3	SHR.		

OTHER SPECIES AND NUMBER:

COLLECTOR: ________
DISSECTOR: ________

FIG. 2.—Form used for tabulation of dissections.

field temperatures could be compared to obtain an estimate of physiological age. A time sequence relating physiological age to chronological age is shown in Fig. 1 for *A. nigromaculis* females at 17±1° C. The original data for the teneral stage and the first gonotrophic cycle were given in previous papers (Rosay, 1961, 1969). The second cycle is presented hypothetically although no divergence is expected in actuality.

Tabulations of dissections during age determination studies were made on a form similar to the one shown in Fig. 2.

Routine dissections of large numbers of individual mosquitoes provided a reasonable picture of the age composition of a population and its change with time.

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