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Determination of Physiological Age in Anophelines and of Age Distribution in Anopheline Populations in the USSR

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A technique is described whereby it is possible to make an accurate determination of the physiological age of anopheline females, i.e., the number of gonotrophic cycles through which the female has passed. This technique is based on counts of the dilatations left in the ovarioles subsequent to each maturation of follicles and oviposition. The number of dilatations found on dissection usually corresponds exactly to the number of gonotrophic cycles.

By dissection of a large number of female anophelines the age composition of the mosquito population and any changes of it in the course of the transmission season may be determined, and a number of problems of population biology may be elucidated. With a knowledge of the temperature in mosquito shelters, it is possible to establish the number of gonotrophic cycles coinciding with a single sporogonic cycle of the malaria parasite and thus the physiological age at which the salivary glands of females may contain sporozoites. Dissection also makes it possible to determine the proportion of potentially dangerous females at various periods of the season. The data thus obtained are used in drawing up an epidemiological picture of a locality and in evaluating the effectiveness of anti-malaria measures.

A simple technique is also described for distinguishing between parous and nulliparous female anophelines by examination of the tracheoles of the ovaries.

INTRODUCTION

The study of age distribution in anopheline populations is of great interest for understanding the population biology of the mosquitos and the epidemiology of the diseases transmitted by them, particularly malaria. The widespread use of residual insecticides has greatly increased the importance of determination of the age of malaria vectors. Complete coverage with insecticides of all the resting-places of anophelines results in such high mortality during each gonotrophic cycle that the proportion of females reaching the age at which sporozoites may appear in their salivary glands is practically insignificant so far as the transmission of the infection is concerned. Thus the main objective of insecticide treatment is to affect the mean longevity of the vocal vector population. The most direct way of checking the efficiency of imagicidal measures is the study of the age composition of the vector population treated with insecticides, and a comparison on the one hand with the age composition of untreated populations and on the other hand with the age of populations so successfully treated with DDT that malaria transmission has been interrupted.

The age grouping of populations of *Anopheles maculipennis* has been studied in the Soviet Union since the 1930's, but conclusive data were obtained

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only in 1947, when a technique was worked out for determining the number of ovipositions by an individual female (Polovodova, 1947, 1949). As there is a close connexion between the number of blood meals and the development of ovaries in mosquitos as well as in other blood-sucking Diptera, suborder Orthorrhapha (Heleidae, Simuliidae, Phlebotominae, Tabanidae), the number of previous blood meals can be deduced from the number of egg batches laid. This makes it possible to estimate the importance of each anopheline female in the transmission of malaria infection. The older the female, the greater is her epidemiological significance, as each blood meal provides an additional chance of the vector's being infected by the host or of the host's acquiring infection from a vector. Several authors have commented on the value of a technique for determining exactly the age of female mosquitos (Muirhead-Thomson, 1951 ; van Thiel, 1953 ; Macdonald, 1956), and a review of the age determination technique used in the USSR has recently been made by Gillies (1958).

In the present paper we propose to describe our method for determining the age of female mosquitos and its possible application in evaluating the effectiveness of insecticidal measures and in problems of the population biology of malaria vectors.

Several methods have been suggested in the past to determine the age of insects : Perry (1912) used the wear of the wing scales in females, Mer (1932) the changes in the size of the ampullae of the common oviduct, Polovodova (1941) the morphological changes in the ampullae, and Detinova (1942, 1945) the changes in the tracheal system of the ovaries. These methods have undoubtedly made a contribution to the study of the population biology, but they all give a very approximate picture of the age composition of the population. The method introduced by Mer (1932), which was widely used by Soviet entomologists in the 1930's, makes it possible to distinguish, more or less accurately, between females with at least one oviposition and nulliparous females, and to distinguish with some approximation between those with many ovipositions and those with few. It has been proved by Polovodova (1941), and confirmed by van Thiel (1953) that more cannot be obtained by means of this method. The technique introduced by Detinova (1945) easily and accurately differentiates between parous and nulliparous females, but does not allow of further determination of the number of gonotrophic cycles.

We consider it more important and convenient to determine the physiological age—i.e., the number of gonotrophic cycles—of adult female anophelines than the number of days since their emergence. Indeed, although at every gonotrophic cycle a certain percentage of females perish, the chances of infection of the surviving females increase with each successive cycle. The number of gonotrophic cycles of each female is therefore epidemiologically more important than the number of days she has lived.[1]

However, the knowledge of temperatures at which the insect has lived and of some of the main traits of its biology make it possible to calculate the age of the female, if the physiological age is known. Thus, for *Anopheles maculipennis messeae,* the duration of the period of blood digestion and of the maturation of the ovaries, which depend on the environmental temperature and humidity, have been definitely established (Shlonova, 1938). Thus the length of this most important period of the gonotrophic cycle can be easily calculated. In the tropics the length of the period of digestion and of egg maturation is more or less constant at 48 hours (Muirhead-Thomson, 1951).

According to a study of *A. maculipennis* populations in the Moscow region (Detinova, 1953a) and in Kirghizia (unpublished data, 1954), the remaining part of the gonotrophic cycle, from the end of egg maturation to the next blood meal, lasts 24 hours on the average. The length of this period can be established in two ways : first, from the time elapsed between the first appearance in day shelters of females of the first spring-summer generation completing the digestion of the first meal and the appearance of females with one oviposition after their first meal ; and secondly, from the aspect of the distal end of the follicular tubes in dissected insects (see below).

DETERMINATION OF PHYSIOLOGICAL AGE IN FEMALE ANOPHELINES ACCORDING TO NUMBER OF OVIPOSITIONS

Observations made in the late 1930's led us to conclude that the whole genital apparatus of anopheline females undergoes changes (Beklemi-

[1] An exception is gonotrophic dissociation, but in that case the epidemiological importance of each female is determined by the number of digested blood meals.

shev, 1944). Age changes in the ampullae of the common oviduct were studied in detail by Polovodova (1941). Age changes in the tracheoles of the ovaries were discovered in females of several species of *Aëdes* (*Ochlerotatus*) by Detinova (1942) and later studied in detail in anopheline females (Detinova, 1945). The suggestion was made in our laboratory by Kuzina (1942) that in *Stomoxys calcitrans* the presence of corpus luteum in the distal part of the internal oviduct might be a sign that the female had laid eggs; the possibility of applying this criterion to the differentiation of parous from nulliparous anopheline females has been proved by Beklemishev and Polovodova, and on our advice this method was used by Yaguzhinskaya (1945) in the analysis of the biology of a population of *A. m. sacharovi* and *A. superpictus* near Samarkand. Later it proved possible to describe the changes taking place in the follicular tube during the process of egg maturation and at the first and subsequent ovipositions (Polovodova, 1947, 1949; Detinova, 1949, 1952b, 1953a). These changes are so pronounced that it is easy to determine the number of egg batches laid by each female.

The structure of the ovaries has been well described by many authors, including Kulagin (1901), Kozhevnikov (1905), Christophers (1911, 1913), Nicholson (1921), Mer (1932), Beklemishev (1944) and Polovodova (1941, 1947). Each ovariole has a germinal zone at its proximal end, after which come two follicles, the foremost often not being completely differentiated in newborn females. The whole follicular tube, as was shown by Nicholson (1921), is covered with an intimal sheath closely adhering to the follicles; at the distal end it continues into the oviduct, which does not change until the follicles are fully developed. The follicle increases in length throughout the various developmental stages of Christophers-Mer. Simultaneously with this growth of the follicle, distension of the surrounding intimal sheath takes place, and at the moment of oviposition the end of the follicular tube through which the mature egg passes into the inner oviduct is also greatly distended. Immediately after the developed egg is laid, the follicular tube with its duct presents the following picture: the penultimate follicle is distinct from the germinal zone; above it lies the zone of growth with the next detached or separating follicle; where the egg had previously been, the greatly distended intimal sheath of the follicular tube forms a sac with thin folded walls, containing the remains of the nurse cells and of the follicular epithelium, which form the so-called "corpus luteum" (Mechnikov, 1867). This sac contracts so that instead of a large distended sheath there remains a narrow duct with a small dilatation where the first follicle was. The average diameter of this dilatation is 0.027 mm, corresponding to the diameter of the follicle detached from the germinal zone. The intimal sheath is thus able both to contract and to distend. In the remaining dilatation are usually found a variable number of refractile grains, which are the products of the degeneration of the nurse cells and follicular cells.

In the next gonotrophic cycle, the whole process is repeated. The intimal sheath becomes distended with the growth of the follicles, and the mature egg distends not only the end of the duct, but also the whole distal portion together with the dilatation caused by the development of the first follicle. If a female is dissected immediately after oviposition, it is impossible to conclude from the shape of the tubes whether she has laid eggs previously or not, as the tubes are sac-like in appearance. The tubes then begin to contract and two dilatations are formed at their distal ends, one where the first follicle had developed and the other at the site of the second follicle. Subsequent gonotrophic cycles lead to the development of the next follicles and as each mature egg is ejected a new dilatation is formed. After each gonotrophic cycle the length of the distal end of the tube increases correspondingly.

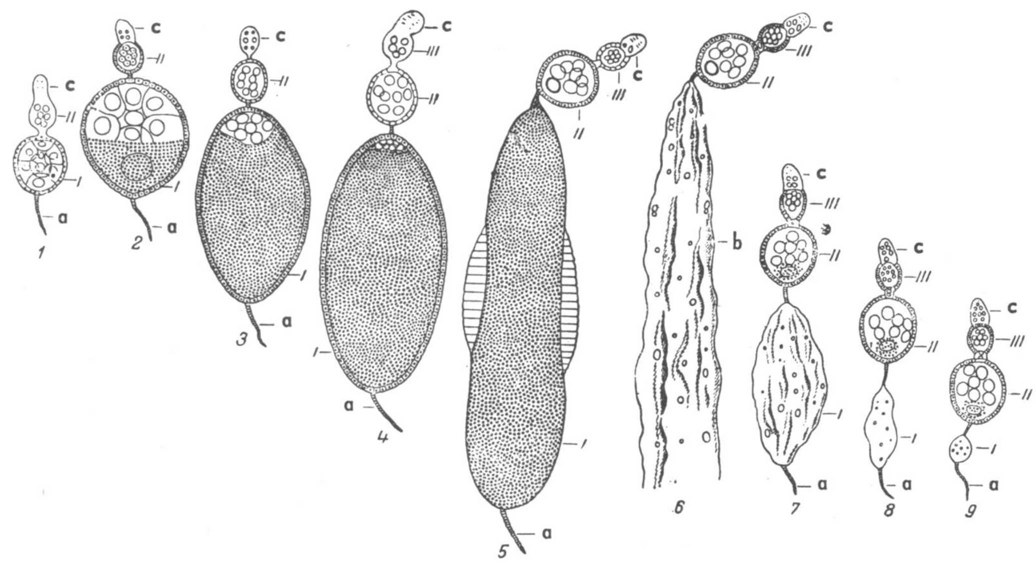
Under field conditions in various parts of the Soviet Union where the mosquito population had not been subjected to DDT treatment, females of *Anopheles maculipennis messeae* were found in the 13th gonotrophic cycle. L. V. Ivanova (unpublished data) has discovered in Tadjikistan a female of *A. superpictus* passing through the 14th cycle.

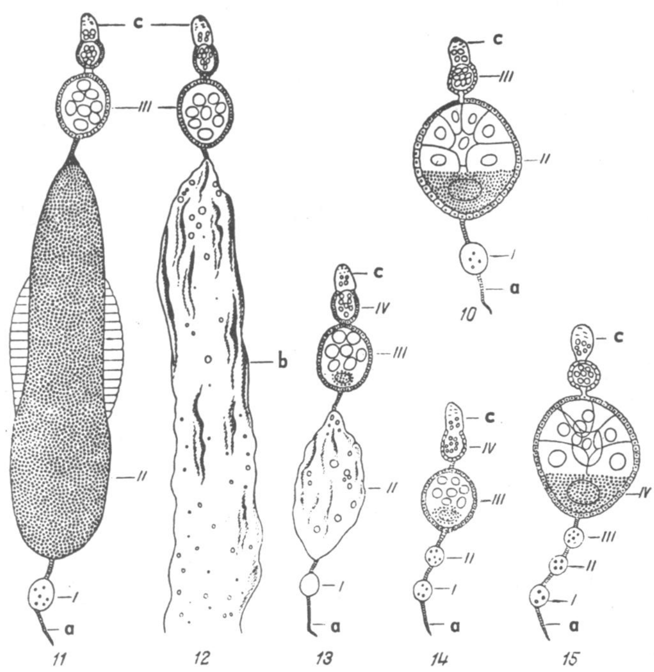
The described changes in the ovarioles during the gonotrophic cycles are shown in Fig. 1.

The size of the dilatations may vary considerably and the length of the distal end of the tube may be different in specimens of the same physiological age. In passing through the distal end of the tube the mature egg pushes the cell remains forward; thus, the greatest number of inclusions is found in the dilatation left by the last gonotrophic cycle.

FIG. 1

OVARIOLAR CHANGES AND FORMATION OF DILATATIONS IN FOLLICULAR TUBES OF ANOPHELINE FEMALE





1-5. Development of first follicle into mature egg.

6-9. Formation of follicular sac after ovulation, contraction of the sac, and subsequent pigmented dilatation marking the site of its development.

10-15. Subsequent ovulations and formation of "rosary" indicating the number of past gonotrophic cycles.

I. First follicle and remaining dilatation.

II. Second follicle and remaining dilatation

III. Third follicle and remaining dilatation

IV. Fourth developing follicle

a. Follicular tube

b. Intimal sheath distended after ovulation (the "sac")

c. Germinal zone

Our observations have shown that not all follicular tubes behave similarly during each gonotrophic cycle. In most tubes, especially in females with few ovipositions, the follicles pass through the normal process of oogenesis, which leads to the formation of mature eggs. In other tubes, the last follicles may begin to develop and then stop and undergo granular degeneration. This degeneration may occur at any stage in the development of the follicle but takes place mostly during the 2nd or 3rd stage (Fig. 2). This degeneration is seen more frequently as the age of the female increases. In a few cases the last follicle may not develop at all during the cycle.

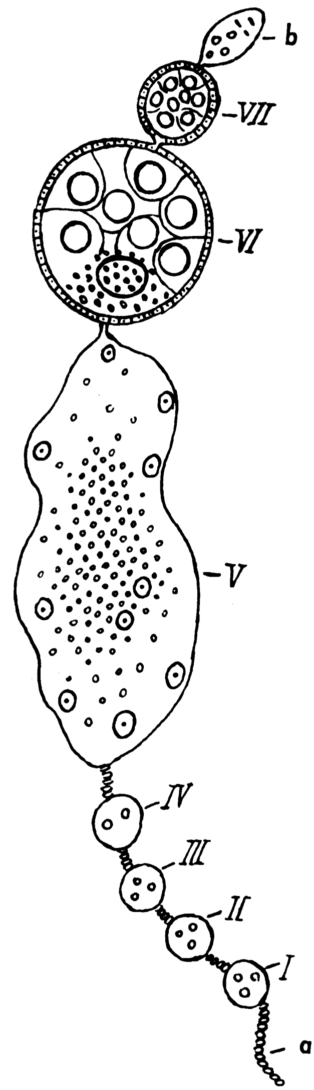
As has been pointed out above, after the completion of the cycle a normally developed follicle leaves a dilatation in the distal end of the follicular tube which shows that the ovariole in question has produced a mature egg. The tube in which the follicle has not developed remains unchanged, and thus the cycle passed through by the female leaves no traces. Finally, tubes in which the follicles have degenerated form dilatations larger than the normal, filled with the degenerated products of the egg. Frequently, at the end of the cycle, the degenerating egg takes on a brown, red or yellow coloration. The size of the dilatation depends on the stage of egg development in which degeneration took place. In some of the older females some tubes react to a new gonotrophic cycle only by the detachment of a new follicle from the germinal zone; these follicles, however, do not grow, and when the process is repeated the follicular tube assumes the shape of a thin beaded chain. The same phenomena have been observed in females of *A. bifurcatus* in our laboratory by N. Y. Markovich (1951).

The degenerated follicle does not prevent the normal development of the subsequent follicle during the next gonotrophic cycle. During the oviposition, the mature egg pushes out the degenerated products from the dilatation below, which then contracts and no longer differs in size from a normal one.

It should be emphasized that both normal and abortive oogeneses leave traces in the follicular tubes which make it possible accurately to determine the number of gonotrophic cycles through which the female has passed. Determination of the duration of the process of contraction of the distal end of the follicular tube and of the formation of dilatations after ejection of a mature

FIG. 2

DEGENERATED FOLLICLE IN FOLLICULAR TUBE AT FIFTH GONOTROPHIC CYCLE OF ANOPHELINE FEMALE



I-IV. Pigmented dilatations left by four previous follicles
V. Degenerated fifth follicle
VI. Sixth fo licle about to develop
VII. Undifferentiated seventh follicle
a. Terminal stalk
b. Germinal zone

egg makes it possible to determine the period between oviposition and a fresh blood meal. These data, obtained by dissecting empty or freshly fed insects, allow the length of the whole gonotrophic cycle to be determined, whereas formerly only the duration of one phase — that of blood digestion and egg development—could be calculated (see above).

DISSECTION AND AGE DETERMINATION OF ANOPHELINE FEMALES

The female is slightly anaesthetized with chloroform or ether, moistened with alcohol and placed on a slide in a drop of physiological saline. Dissection of the ovaries is carried out as follows.

With the left dissecting needle the insect is secured to the slide by piercing its thorax, and the last two segments of the abdomen are nicked from both sides with the right needle held at an angle. The whole genital apparatus is then slowly pulled out. The isolation and counting of dilatations in the follicular tubes is carried out on the slide under a binocular microscope, ocular 7, object lens 8. For examination, one of the ovaries is secured to the slide with the left needle in a small drop of saline, while with the right needle the outer membrane of the ovary is gradually torn away. The ovary still being secured with the left needle, the right needle is then introduced at a sharp angle under the follicular tube, which is carefully drawn away from the internal oviduct. The dilatations are counted on the isolated follicular tube which is still attached to the internal oviduct at its distal end. If the ovaries are developed (5th stage of Christophers), the intimal sheath is torn as the tube is being isolated and the mature eggs fall out; this, however, does not prevent the correct determination of physiological age. When the outer membrane of the ovary has been removed, the tubes are pulled aside with both needles simultaneously, and those which have degenerated during the last cycle are sought out. If the ovaries are in the 3rd to 5th stage of Christophers, the degenerated follicle corresponds to normal ones in other ovarioles and the number of dilatations is related to the number of ovipositions. Age determination in females with degenerated follicles can also be checked in specimens having ovaries in the early stages of development. It should be borne in mind that no degeneration of follicles can have occurred in the early stages, as this process is simultaneous with the development of eggs in other tubes, and the degenerations must therefore be ascribed to the preceding cycle. In the determination of age of such females, therefore, dilatations filled with products of degeneration must be included in the total number of dilatations determining physiological age. For instance, if a female in the 2nd stage of oogenesis (according to Christophers) has the majority of ovarioles with three normal dilatations, then a tube in which the preceding oogenesis ended abortively will have two normal dilatations and one larger one filled with degeneration products.

To avoid mistakes in determining physiological age a number of follicular tubes should be examined in each ovary. It should be borne in mind that the follicular tubes are easily torn and require very careful dissection. Counting should be performed only in tubes which are normally connected with the internal oviduct.

PRACTICAL APPLICATION OF AGE-DETERMINATION METHOD

Since 1947 Soviet entomologists using the technique developed by us have been carrying out an extensive study of the age distribution of mosquitos in various parts of the USSR. The data obtained have made it possible to evaluate the effectiveness of insecticidal measures and to establish the time when the insect population is at the peak of the transmission period, thus allowing for intensification of antimalaria measures at that time. The data have also revealed a number of interesting features in the life of the insect population, depending upon a variety of conditions; these features include the rates of aging, ability to survive, age differences in females from shelters of different types, and so on.

The data show that, for all subspecies of *A. maculipennis* and *A. superpictus*, the mosquitos which enter houses after careful treatment of an inhabited district with residual insecticides are mostly newly emerged, with a few females showing evidence of one or two ovipositions. In such cases the density of the mosquito population is renewed by females remaining in the open near the breeding-places.

To determine the age distribution of the anopheline population in a particular settlement, capture stations are selected; but as the number of insects saught often exceeds the number that can be dissected, only a certain number of females from each capture station are taken. These females may be taken at any stage of blood digestion. The number of dissections is obviously dependent on the personnel available; one worker can dissect and count dilatations of the follicular tubes in 100-120 specimens per working-day. On the basis of the age of the dissected females and the total number of mosquitos collected, it is possible to determine the proportion of each age-group in the locality under observation.

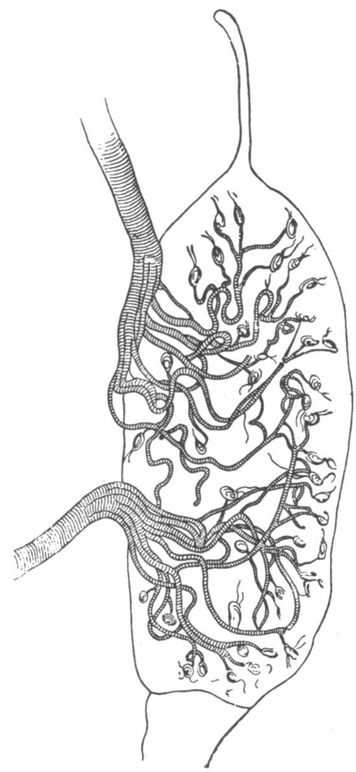
The data obtained are used to establish the total mosquito density and the proportion of epidemiologically dangerous mosquitos at each period of the season, i.e., those whose age suggests that they may contain sporozoites in their salivary glands. Calculation is based on the following factors: the duration of sporogony, calculated on the basis of experimental data obtained at various temperatures and the microclimate of the shelter (Rayevski, 1942); the duration of the gonotrophic cycles (Shlonova, 1938; Detinova, 1953b); the number of gonotrophic cycles of a female during one sporogonic cycle, and eventually the age of the potentially dangerous females. All the older females are considered potentially dangerous. The proportion of dangerous females among those dissected makes it possible to establish the absolute size of the epidemiologically important group in capture stations, if the total number of females in the shelter is known.

Studies of the age distribution of mosquito populations, both intact and those subjected to treatment with insecticides, have been described by Soviet entomologists in a number of papers (Almazova et al., 1957; Bandin, 1953; Detinova, 1952a, 1952b, 1953a; Zorin, 1957; Kozhevnikova, 1953; Korzhenevskaya, 1957; Markovich, 1951; Selens, 1955; Strelkov, 1953; Sukhomlinova, 1956; Chernyshova, 1955; Chubkova & Ambartsumyan, 1954; Shevchenko, 1953).

DISTINGUISHING PAROUS FROM NULLIPAROUS FEMALES BY STATE OF TRACHEAL SYSTEM OF OVARIES AND USE OF THIS METHOD FOR EVALUATION OF MOSQUITO-CONTROL MEASURES

In practical mosquito-control work and in the evaluation of the effectiveness of measures adopted, it is possible to use a time-saving method for establishing the proportion of parous and nulliparous females under investigation. This method is based on the changes in the tracheal system of the ovaries during the first gonotrophic cycle. Each ovary is connected with two branching tracheae: the large branches penetrate the ovary, entwine the internal oviduct and branch off again; small branches spreading in every direction from the oviduct form tracheoles, which in young females are coiled, forming distinct skeins (Fig. 3). These tracheoles are situated between individual ovarioles, and their distal threads are attached in the germinal zones of the tubes. With the development of the follicles, the tracheoles gradually straighten, and are completely uncoiled by the end of the maturation period of the ovaries, having entwined the tubes with the mature eggs. This uncoiling process is irreversible. Therefore a female with one oviposition possesses a tracheal system in the shape of a very thin net of tracheoles (Fig. 4).

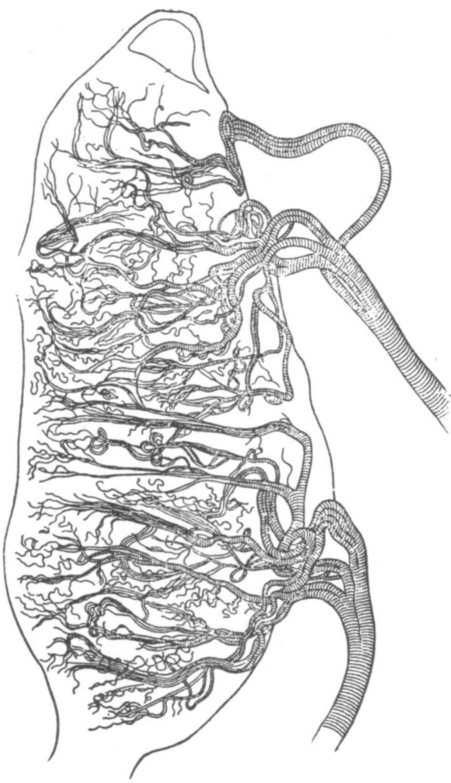
FIG. 3
OVARY OF NEWLY EMERGED ANOPHELINE FEMALE, WITH TERMINAL TRACHEOLES SHOWING CHARACTERISTIC TIGHT SKEINS INDICATING THAT FEMALE IS NULLIPAROUS



(After Detinova, 1954)

When the state of the tracheal system is being investigated, the ovaries are dissected in pure

FIG. 4
OVARY OF ANOPHELINE FEMALE AFTER OVIPOSITION, WITH TERMINAL TRACHEOLES SHOWING NET-LIKE FORMATION



(After Detinova, 1945)

water and left on the slide in a drop of pure water to dry. In the course of drying, air penetrates into the tracheae and tracheoles, which become dark and are clearly visible under the microscope. The ovaries must not be dissected in saline, as crystals of salt cover up the tracheoles and render them invisible.

This technique for distinguishing parous from nulliparous females is very simple and enables the investigator to examine a large number of mosquitos. No great skill is required for the operation. It should be noted that such examination of the tracheal system of the ovary can be performed only on empty or freshly fed females, while the ovaries are still transparent; the state of the tracheae is then clearly visible.

As has been said, this technique can also be used for evaluating the effectiveness of mosquito-control measures. In anti-larval operations, the presence, even in small numbers, of females with tracheolar skeins shows that mosquitos are breeding and that the control measures are not sufficiently effective. Similarly, with imagicidal operations, there should be a total absence, or a very insignificant number of parous females; the presence of even a relatively small number of such females indicates ineffective control.

Similar methods for age determination and for evaluating the effectiveness of control measures have been worked out in our institute for Muscidae and Hippoboscidae (Kuzina, 1942, 1950; Lineva, 1953a, 1953b; Derbenova-Ukhova, 1952; Detinova, 1955), for horseflies (Bey-Bienko — in preparation), and for blackflies (Detinova & Beltyukova, 1958). An effective technique has also been worked out for sandflies (Dolmatova, 1942). Thus, in the females of a number of Diptera the state of the ovaries indicates similar changes connected with maturation and egg laying, which make it possible to establish the age of those females. It may be that analogous techniques could be used to determine the number of ovipositions in many other species of insect.

Finally, it may be said that all the data on the effectiveness of the treatment of dwellings with residual insecticides against all arthropod vectors correspond closely to the epidemiological control data, i.e., to the data on the rate of infection. A decrease in the number of vectors and a lowering of the age of the vector population always go hand in hand with a decrease in the transmission of the infection.

RÉSUMÉ

Les techniques permettant d'établir l'âge des moustiques ont pris une importance particulière avec les mesures imagicides de lutte antipaludique. Le but de ces mesures est de détruire les femelles de moustiques à un taux tel qu'aucune n'atteigne l'âge où les sporozoïtes peuvent se développer dans les glandes salivaires. La méthode la plus directe est d'évaluer l'action de l'insecticide dans la population des moustiques, par « groupes d'âge ». La détermination de l'âge physiologique de chaque femelle, soit le nombre de cycles

trophogoniques, est plus important que le nombre de jours qu'elle a vécus, car plus l'âge physiologique est élevé plus le risque d'infection augmente.

L'appareil génital des femelles se modifie avec l'âge. Cependant, la plupart de ces changements permettaient seulement de différencier les nullipares de celles qui ont déjà pondu. En 1947, une technique a été proposée, permettant d'établir, par la dissection, le nombre de cycles trophogoniques de chaque femelle. Il apparut qu'après la ponte, l'ovariole subit une dilatation à la place laissée vide par l'œuf. Ce processus se répétant à chaque cycle, le nombre des dilatations indique le nombre d'ovogenèses. Si l'œuf subit un défaut de maturation ou dégénère, ce qui est souvent le cas, la dilatation se produit tout de même.

Les auteurs décrivent la technique de dissection et de numération des dilatations dans les tubes folliculaires. La dissection de très nombreuses femelles au cours de la saison, dans la même localité, permet de calculer le taux de vieillissement de la population, la mortalité et la distribution par groupes d'âge, les changements physiologiques dus à l'âge, le choix des abris d'après l'âge, et, s'il s'agit d'une saison brève, la succession des générations. (On a constaté, par exemple, que c'est au cours du premier cycle trophogonique que *Anopheles maculipennis* est le plus fortement exophile.)

Virtuellement dangereuses sont les femelles qui ont atteint l'âge où elles peuvent héberger des sporozoïtes dans leurs glandes salivaires. Pour établir cet âge, connaissant la température des abris, il faut déterminer la durée de la sporogonie et celle des cycles trophogoniques. Le nombre de ces derniers au cours de la dite période indique l'âge où la femelle représente un danger virtuel de transmission. Cet âge étant connu, ainsi que la répartition par groupes d'âge dans une population de moustiques, on peut calculer le pourcentage de femelles virtuellement dangereuses dans une période donnée.

Le traitement minutieux des bâtiments par les insecticides abaisse fortement l'âge de la population des moustiques, et son succès entraîne la disparition presque totale des femelles virtuellement dangereuses. Leur persistance est un indice de l'échec partiel du traitement.

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