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FURTHER STUDIES ON THE CUTICULAR DAILY GROWTH LAYERS OF ANOPHELINE MOSQUITOS

by

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INTRODUCTION

A method of determining the calendar age by counting the daily growth layers in the cuticle of Diptera was described for a number of flies and for culicine mosquitos by Schlein & Gratz (1972). Previous methods, summarized by Detinova (1961), relied mainly on the observation of changes in the female reproductive system which indicated the physiological age.

The growth layers could also be counted after staining in the otherwise transparent thoracic phragma of Anopheles gambiae. In field-collected material the age could be determined in most specimens but the quality of the staining was dependent on both the method and the duration of preservation of the mosquitos. The daily lines could seldom be observed in laboratory-reared mosquitos.

As the age determination of vectors is important in the epidemiology of malaria and in ecological studies, anophelines from different regions of the world were examined in order to assess the applicability of the method to the various species. Different preservation methods were compared and alternative staining methods and variations thereof were tried in an attempt to improve the demonstration of the daily growth layers.

MATERIALS AND METHODS

Approximately 1500 specimens of Anopheles, including at least 20 per species, were examined from various countries as follows: A. gambiae: Botswana, Kenya, Malawi, Nigeria: A. funestus: Kenya, Nigeria; A. culicifacies: Sri Lanka; A. algeriensis: Israel; A. minimus flavirostris: Philippines; A. sacharovi: Iran, Israel; A. balabacensis: Thailand; A. sergenti: Israel; A. albimanus: El Salvador, Guatemala, Mexico; A. farauti: British Solomon Islands Protectorate; A. pseudopunctipennis: Mexico.

The majority of the mosquitos were kept in test-tubes in a dry state; some batches were dried with dehydrating agents (silica gel or calcium chloride, etc.) and others were preserved in 70% ethanol.

The thoracic phragma is the posterior area of insertion of the large longitudinal flight muscles. These and other muscles and tissues have to be removed and the cleared phragma has to be dissected, cleaned, stained and mounted in order to be able to observe the daily growth lines. The final result depends on the proper execution of these procedures. The sequential steps in the treatment of the mosquitos after capture are discussed below.

1. Preservation of mosquitos

The most successful method of preservation is dry-storing of the mosquitos in stoppered test-tubes. To ensure sufficient dryness, the specimens may be left in the sunshine or in front of an electric heater for two hours. Residual humidity inside stoppered test-tubes damages the cuticle and interferes with the staining. The staining properties of the cuticle were also impaired, and a precipitate formed around the phragmata, when a mordant was used on stored specimens that had been dehydrated with dehydrating agents such as silica gel or calcium chloride. This interference was most manifest when the mosquitos were actually in contact with these chemicals. This could be prevented by first fixing the preparations with picric acid as described in the staining method below.

Preservation of the material in 70% ethanol did not improve the results. Fragments of the tissues adhered to the cleared skeleton and were difficult to remove. They often remained as a layer attached to the phragma and masked the growth lines after staining.

2. Maceration of the tissues

Skeletons of mosquitos were cleaned routinely by macerating the tissues in 7% potassium hydroxide (KOH). The mosquitos floated in the KOH solution which was brought to boiling point, removed from the flame and left standing for a few minutes. For specimens of large species the KOH solution had to be kept gently boiling for about a minute in order to clean the phragma completely.

Attempts at removing the tissues by means of chloral hydrate solutions at various concentrations and lactophenol proved ineffective, and the growth lines did not stain after such treatment.

3. Dissection and staining

The parts of the mosquitos which were dissected in order to observe the thoracic phragma and the growth ring included the third thoracic segment, the postnotum and the scutellum. The mosquitos were dissected in embryo dishes, containing water and using watchmakers' forceps. The abdomen was removed from the thorax, and the thorax transversely split between the third and second pairs of coxae, towards the scutellum. The dissected preparations were cleaned of muscle fragments and tracheae in another change of water; the third coxae were separated from one another and the metapleurae, which normally partially cover the phragma, were laterally extended by pressing on the postnotum with the tip of the forceps.

For the staining of the dissected preparation a modification of the technique described by Schlein & Gratz (1973) was used. This modified technique makes allowance for the size of the mosquitos, for the species or, if need be, for different batches of a given species. The modified staining procedure was as follows:

(i) Fixation in saturated picric acid for 10 minutes.

(ii) Rinsing in water.

(iii) Oxidation in 1% potassium permanganate for 5 minutes.

(iv) Rinsing in water.

(v) Soaking in mordant, fresh solution of iron alum 1% (ammonium ferric sulfate) for 15 minutes (the solution deteriorates after a few days).

(vi) Rinsing in two changes of water for 20 minutes.

(vii) Staining in a ripe solution of 0.2% haematoxylin (Gurr's) in 70% ethanol for up to 1 minute(checked under the microscope, to avoid overstaining).

(viii) Rinsing in water.

(ix) Dehydration in absolute ethanol.

(x) Clearing in xylene and mounting in Canada balsam.

Fixation in picric acid greatly improves the results of staining and prevents the formation of a precipitate on the preparations when in the mordant solution. It is especially effective with preparations of mosquitos that have been kept dry for extended periods, or with mosquitos that have been stored with dehydrating agents. At this stage it is not known whether this technique of fixation is useful in the treatment of preparations of freshly collected mosquitos.

The quality of the final staining is a result of the interaction between potassium permanganate oxidation and mordant. Preparations do not stain if they are over-oxidized and, on the other hand, the growth lines are invisible after insufficient oxidation. The phragmata of small mosquitos were oxidized for 10 minutes in the potassium solution and immersed for only 10 minutes in the mordant. Preparations of large mosquitos were oxidized for a shorter period or were immersed in a 2% solution of the mordant. The iron alum could be replaced by a 1% solution of ferrous ammonium sulfate which was found to be a more active mordant. The right equilibrium between oxidant and mordant has been reached at the point when occurs a bleaching of the brown colour of the phragma (due to oxidation).

RESULTS

The above described technique was used to stain the phragmata of a large number of anophelines and produced the best results in comparison to the many different stains and conventional staining methods which were tried as well. In addition to the reading of the stained preparations, the cleared phragmata of A. gambiae were also examined using interference and fluorescence microscopy (Zeiss-Nomarski Differential Interference for transmitted light: Reichert Zetopan Fluorescent Microscope). These techniques yielded neither an indication of the growth lines on the surface of the phragma nor an autofluorescence of these lines.

Growth lines on the thoracic phragmata could be stained in all Anopheles species examined, provided the mosquitos were in good condition and properly preserved. The general pattern, except for variation in the intensity of the appearance, was as described for A. gambiae (Schlein & Gratz, 1973). In two species the arrangement of the growth layers did not conform with this general pattern although growth lines were observed. In A. sacharovi the upper dorsal area of the phragma looked thicker than the rest of the apodeme and was delineated by a conspicuous line formed at eclosion. The subsequent growth area was relatively narrow, the growth lines were closely packed and sometimes difficult to discern (Fig. 1). In A. balabacensis the width of the, apparently, daily lines was larger than in the other species examined. In all other species the first daily line was sometimes confused with the small striae that cover the area of the previous growth, particularly when the staining was not satisfactory. All subsequent lines were, however, relatively clear. This small striation covers the whole growth area of the phragma of A. balabacensis and it differs from the widely separated darker lines only in the depth of staining.

The specimens of A. albimanus, received from different countries, varied in size: very small ones from Guatemala, medium sized from El Salvador and large mosquitos from Mexico. The degree of oxidation with potassium permanganate required for staining differed according to the size of the mosquitos.

A gentle and correct oxidation was also necessary in the treatment of the large specimens of A. pseudopunctipennis from Mexico, in which the growth lines could easily be obliterated. In other species, especially in A. gambiae, the growth lines were readily and evenly stained in all the batches provided the staining technique was consistent. Even the phragmata of specimens of A. sacharovi, A. sergenti and A. algeriensis, that had been mounted on pins and kept in a collection for over 30 years, were adequately stained, A. sacharovi still showing the above-mentioned characteristics.

The results of staining and the aspects of the growth layers vary between different specimens of the same species and of the same batch, even when staining seems to be optimal. In some cases, they appear only as dark-stained dots or small cross lines, in a ladder-like arrangement on the longitudinal costae, whereas in others they are visible as clear transversal lines, circling part or all of the semilunar growth area of the phragma. In the majority of the species the growth lines could be counted in about 80% of the specimens examined.

Table 1 shows the age-grouping of batches of Anophelines of various species. Since the purpose of these studies was the verification of the applicability of a particular method of age determination to different species, no ecological data are presented. In most of the batches the largest groups are those aged two to five days. The growth of the phragma ceases at the age of 10-14 days and the last growth lines are sometimes difficult to discern. However, most of the mosquitos in the samples have an age of less than eight days so that in the majority of these populations the age may be conveniently and accurately assessed by counting the daily growth lines on the thoracic phragma.

DISCUSSION

The appearance of the growth layers was uniform in most of the species examined and a comparison of the growth layers seen in field-collected insects with those of laboratory reared mosquitos of the species A. gambiae, A. stephensi, and A. albimanus indicated that these are daily layers. Although the lines seen in the phragmata of A. balabacensis and A. sacharovi are apparently also daily lines, this has as yet to be confirmed by comparing them with those seen in laboratory reared mosquitos of known age. The rate of growth of the cuticle is dependent on temperature. Growth ceases altogether at low temperatures (Dingle et al., 1969; Schlein, 1972; Tyndale-Biscoe & Kitching, 1974). Inaccuracies in age determination of field-collected specimens could thus occur. Schlein & Gratz (1973) observed that temperature fluctuations emphasized the daily lines of laboratory bred A. gambiae, and Tyndale-Biscoe & Kitching (1974) stated that in Lucilia cuprina the formation of daily layers was exclusively dependent on this factor. From their experiments with fluorescent lamps they concluded that light does not affect the formation of the growth rings. However, Sarcophaga kept in darkness and at constant temperature, invariably showed growth lines, although these were weaker than normal. Alternation of ultraviolet light and darkness was observed to have an effect on the formation of the lines (Schlein, unpublished data). It may be assumed that in nature the growth lines in Anopheline mosquitos are enhanced by the combined effect of temperature fluctuations and the alternation of light and darkness.

Excluding technical factors, such as preservation, stain absorption, etc., the differences in the appearance of the daily lines in mosquitos of the same batch may be due to several variables. These are the various micro-climates in which the mosquitos have lived and the individual variation in their reaction to environmental conditions. These variations notwithstanding, it is safe to consider the lines seen on the phragmata of field-caught mosquitos as representing the actual age, in days, of the specimen.

RESUME

Il est important, dans les enquêtes épidémiologiques, de déterminer l'âge des moustiques vecteurs capturés sur le terrain. On a déterminé l'âge de spécimens de plusieurs espèces d'Anopheles recueillis sur le terrain dans plusieurs régions du monde en colorant et en dénombrant les couches quotidiennes de croissance dans la phragma thoracique. Diverses méthodes de coloration et de conservation ont été testées et comparées, et on décrit celle qui a permis d'obtenir les meilleurs résultats. Avec la technique en question, il a été possible de déterminer l'âge d'environ 80 % des spécimens de chacune des douzes espèces examinées.

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TABLE 1. AGE-GROUPING OF BATCHES OF ANOPHELINE SPECIES FROM DIFFERENT COUNTRIES

Species	Country	Date	Number of mosquitos of different ages in days													
			0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11	11-12	12-13	13-14
A. gambiae	Nigeria (North)	30.10.72				7	28	21	6	2		1				
A. gambiae	Nigeria, Kano	13.11.72	6	16	27	24	21	14	5	2	4	4				
A. gambiae	Kenya	11.72			3	6	22	10	7							
A. gambiae	Malawi	30.11.73	7	23	32	20	4	4	2	1						
A. gambiae*	Botswana (North)	2.74		22	14	5	4	2								
A. albimanus**	El Salvador	8.12.72		6	8	18	10	3		1						
A. albimanus*	" "	8.12.72	5	10	23	24	14	4	3	2						
A. albimanus	Guatemala	23. 2.73			10	11	12	6		2	2		2			
A. albimanus	Mexico	20.10.74	5	5	12	15	7	3								
A. balabacensis	Thailand	2. 8.73	10	31	23	19	7	1								
A. funestus	Nigeria (North)	13.11.72			7	15	18	6	3	1						
A. farauti	BSIP	24. 4.73		1		9	5	5	2	10	4	7		1	2	1
A. culicifacies	Sri Lanka	13. 3.73				1	3	5	4							
A. minimus flavirostris	Philippines	17. 4.73			3	8	5	3	2	1						
A. pseudopunctipennis	Mexico	29.10.74	12	25	16	9	6	4	4							
A. sacharovi	Iran	9. 7.73	3	6	3	8	13	18	8	8	1	2	1			
A. sacharovi	Israel	1948			2		3	2	1	1		1				
A. superpictus	Israel	1943-48		1	4	3	1									
A. claviger	Israel	1943-48		5	3	2										
A. sergenti	Israel	1943-48				2	4	1								

* Collected in houses.

** Man bait.

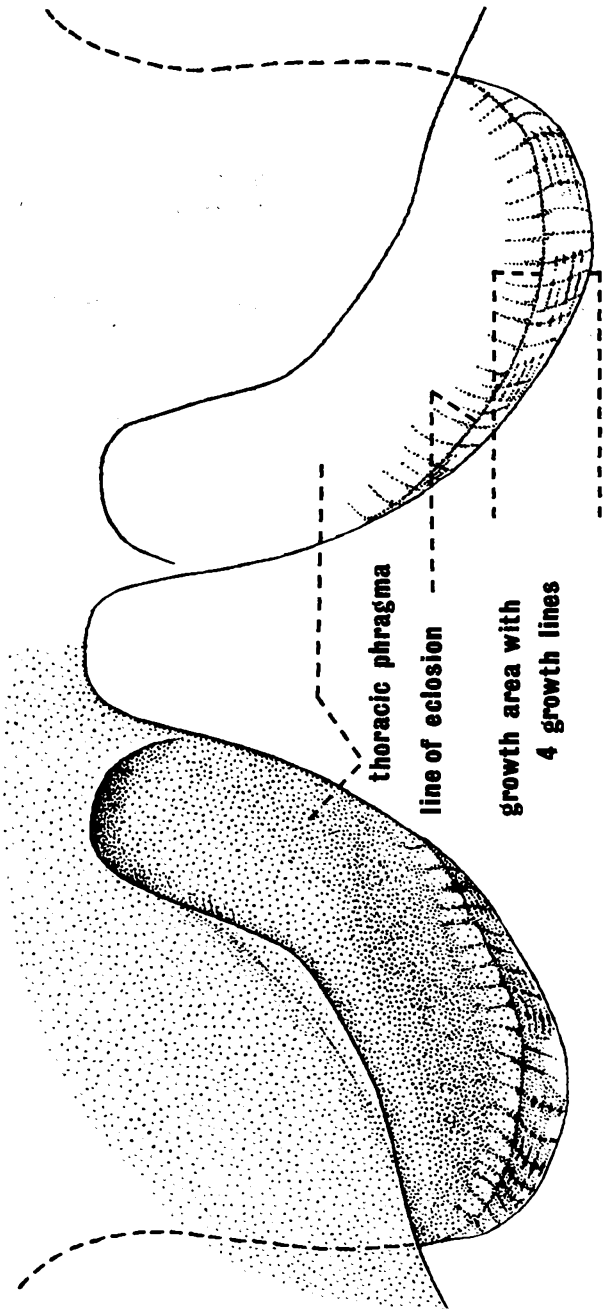

FIG. 1. GROWTH LINES ON A FLATTENED STAINED THORACIC PHRAGMA OF A. SACHAROVI

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