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Determination of the age of some anopheline mosquitos by daily growth layers of skeletal apodemes

Y. SCHLEIN[1] & N. G. GRATZ[2]

Earlier work has shown that it is possible to determine the calendar age of Culex *and* Aedes *mosquitos by counting the daily layers of cuticular growth on the inner apodemes. In view of the epidemiological importance of age grouping in* Anopheles *species, the applicability of the method to this genus was studied in both laboratory-reared and field-collected specimens. In the four species examined, it was found possible to distinguish daily growth layers for periods of up to 10–13 days provided that proper staining procedures are used.*

A method for determining the age of certain Diptera including *Culex* and *Aedes* mosquitos by counting the number of growth layers on the skeletal apodemes was described by Schlein and Gratz (5). In view of the epidemiological importance of age-grouping in anopheline mosquitos, several malaria vector species of *Anopheles* were examined to determine the applicability of this method to that genus.

The age assessed by counting the daily layers is the actual calendar age, whereas the previous methods, summarized by Detinova (3), rely mainly on observations of changes in the female reproductive system which indicate the physiological age.

The daily rhythmicity of cuticle deposition, first described by Neville (4), has since been studied in many insect species. In Diptera, the growth of the cuticle other than the growth of the skeletal apodemes seems to be limited; indeed, these apodemes are apparently the only parts of the skeleton where daily growth layers can be observed. The thoracic apodemes are the sites of attachment of the thoracic muscles and their length is directly related to the size of these muscles. Thus the length of the apodemes and the amount of daily growth reflect the amount of growth of the thoracic muscles under different environmental conditions.

MATERIAL AND METHODS

The specimens examined were a series of laboratory-reared adults of *A. gambiae*, *A. stephensi*, and *A. albimanus* killed at different known ages and field-collected *A. gambiae* and *A. funestus*. All the specimens were dry-stored because the cuticle is damaged and its staining properties deteriorate if the material is preserved under humid conditions after collection. To ensure that the specimens are sufficiently dry, the mosquitos may be placed in sunshine for 2 hours or before en electric heater for 1–2 hours. The apodeme showing daily growth layers in anophelines is the thoracic phragma (Fig. 1 and 2). After the mosquitos had been macerated in potassium hydroxide (KOH), it was excised with the adjacent sclerites and cleaned as follows: A 7% solution of potassium hydroxide containing the mosquitos was brought to boiling point, removed from the flame, and left standing for a few minutes. The mosquitos were then dissected in water with watchmaker's forceps; the abdomen was first separated from the thorax and then the thorax was cut transversely between the second and third pairs of coxae and up to the scutellum. The separated rear part of the thorax (Fig. 1) was rinsed in water and cleaned of the tracheae and its ventral part (metasternum and coxae) was cut off. These preparations were stained by means of the following procedure.

1. Oxidation in 1% potassium permanganate for 5 min.
2. Rinsing in water.
3. Soaking in mordant, 1% iron alum, 15 min.[a]

[1] Department of Medical Entomology, Hebrew University Medical School, Jerusalem, Israel.

[2] Vector Biology and Control, World Health Organization, Geneva, Switzerland.

[a] If a deposit is precipitated on to the preparations when they are transferred to the iron alum, acidulated water (0.1 ml of hydrochloric acid per litre) must be used for washing them (steps 2, 4, and 5). In addition, the preparations should be washed in acidulated water for 30 min prior to step 1; this procedure also causes them to stain faster in the haematoxylin.

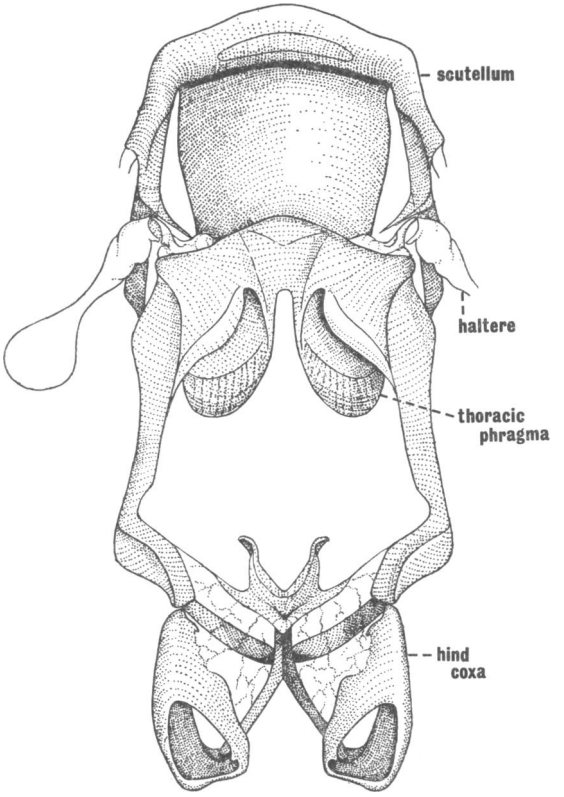

Fig. 1. Posterior view of thoracic skeleton of *A. gambiae*.

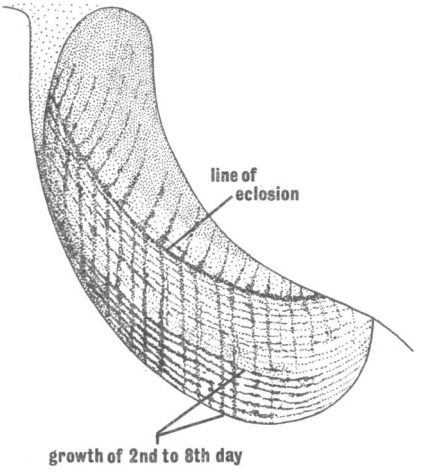

Fig. 2. The daily lines of growth on the thoracic phragma of field-collected *A. gambiae*.

4. Rinsing in water, 10 min.

5. Changing of water and rinsing for a further 10 min.

6. Staining in a ripe solution of 0.2% haematoxylin (Gurr's) in 70% ethanol for 1–2 min. (under microscopic control, to avoid over-staining; about 10 preparations can be stained and observed at a time).

7. Rinsing in water.

8. Counterstaining for 20 min. in 0.2% Congo red in water.

9. Rinsing in water and flattening of preparation.

10. Dehydration in absolute ethanol.

11. Clearing in xylene and mounting in Canada balsam.

Preparations of *A. funestus*, which is smaller than the other species and has a thinner cuticle, were treated and stained as above, except that they were left in potassium permanganate for 10 min. and in iron alum for 5 minutes.

RESULTS

The duration, degree, and form of cuticle deposition varies between different areas for the same species of mosquito. Growth layers can be observed only on the mesosternal apodeme and the thoracic phragma. The striation on the mesosternal apodemes stains faintly and no more than 5–6 growth layers can be counted, whereas up to 13 daily layers can be observed on the thoracic phragma.

A distinct line on the thoracic phragma marks the extent of the area of the phragma at eclosion. To this is added a wide layer representing the first day's growth and then narrower bands for the subsequent days (Fig. 2). The daily bands can be distinguished from one another when stained: they appear in alternating light and dark colours. However, this picture may sometimes be less clear when the daily layers do not stain deeply enough and, in fact, they can then be confused with another striation present within the first day's growth. When this first-day striation is confluent with the daily layers, it is impossible to count them. In most of the field-collected mosquitos, however, the beginning of the second-day growth was fairly distinct and the age could be determined in more than 85% of the batches of field-collected *A. gambiae* (Fig. 3).

There was a difference between the results with laboratory-reared and field-collected mosquitos; the

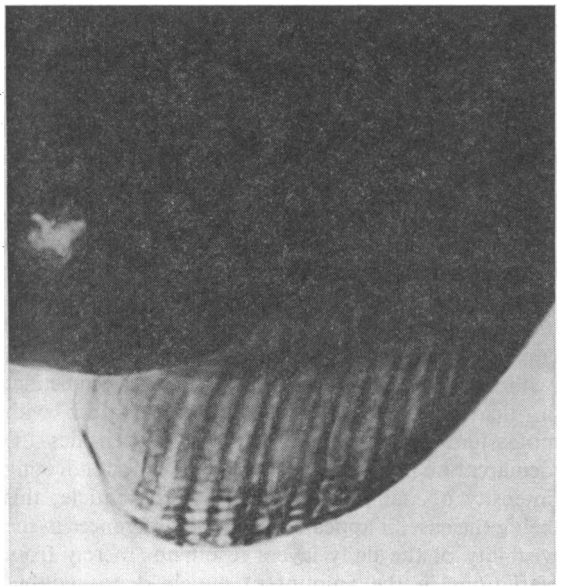

Fig. 3. Thoracic phragma of 5-day-old *A. gambiae* from northern Nigeria.

daily layers failed to stain in most of the laboratory-reared anophelines of all the above-mentioned species, and the age could be determined in only approximately 25% of the specimens examined. The appearance of the layers varied from one batch to another in the same species and in many specimens the daily layers were visible only as interruptions on the longitudinal ridges of the thoracic phragma.

These mosquitos had been kept in a constant temperature of 27°C, with light and darkness alternating every 12 hours. It was easier to distinguish the stained growth bands in mosquitos that had been reared at room temperature and exposed to temperature fluctuations, but even in these specimens the daily layers were far less distinct in appearance than in the field-collected material.

Some measurements were taken to estimate the rate of growth and to try to account for the variation in the visibility of the daily layers. The width of the cuticle and the amount of growth in length of the thoracic phragma were measured in both field-collected and laboratory-bred *A. gambiae*; 15 laboratory-bred females of the same size and of different known ages were compared with 15 selected field-collected females of the same size and corresponding age. The thickness of the cuticle was measured in the anterior midline of the mesonotum and the anterior upper part of the fore femur after the mosquitos had been macerated in potassium hydroxide. The average thickness of the femur cuticle in the field-collected mosquitos was 4 μm, compared with 5.25 μm in the laboratory-bred mosquitos. The thickness of the cuticle of the mesonotum of the field-collected mosquitos was 4.7 μm on average, compared with an average of 5.4 μm in the laboratory mosquitos.

The range of variation of cuticle thickness of the mesonotum and femur in mosquitos of the same age and batch reared in the laboratory was the same as the range measured in the field-collected mosquitos (all mosquitos were more than 1 day old). The growth in length of the thoracic phragma in the first day in laboratory-reared mosquitos was 38 μm on average, compared with 36 μm in the field-collected mosquitos.

There appears to be little or no relation between the amount of growth on the first day and that on subsequent days. The first-day growth in a given mosquito might be 42 μm, and 20 μm in the following 9 days or, in another specimen, 22.5 μm in the first day and 20 μm in the subsequent 9 days. The first day's growth is apparently independent of the feeding of the adult, since a first-day cuticular band of normal size was deposited even in mosquitos that were not allowed to feed after eclosion. The variation measured in the growth between the second and tenth day was much smaller than that of the first day and ranged between 20 μm and 27 μm. In contrast, the first day's growth may range between 22.5 μm and 42 μm, as mentioned above.

DISCUSSION

The number of growth layers in the *Anopheles* spp. bred in the controlled conditions of the laboratory was found to correspond with the age of the mosquitos in days whenever the layers were distinct enough to be counted. The same correlation was observed in mosquitos reared under variable conditions in the laboratory. It may therefore be assumed that the layers of growth on the apodemes in the field-collected material are also deposited daily, i.e., each layer represents the amount of growth in one day. This makes it possible to assess the age of field-collected anophelines and relate the chronological age to the physiological changes that take place over a period of up to 10–13 days. In most of the mosquitos of all the species examined it was possible to count up to 10 growth layers, corresponding to 10

days of calendar age. In a small number of specimens, up to 13 layers could be distinguished. In less than 15% of the field-collected specimens the growth layers could not be counted, either because the preparations failed to stain or because the daily layers were not distinct; therefore, although the procedure is not successful in the case of every individual mosquito, as indicated above, up to 85% of the specimens collected can be age-graded if properly prepared.

As the thoracic phragma is the attachment area of the longitudinal flight muscles, its growth in length would be directly related to the growth of these muscles and could apparently be used in estimating the degree of muscle development, the daily layers indicating that a certain amount of growth has occurred each day. Estimation of the degree of muscle development by measuring the residual dry weight had been suggested by Bursell (1) as a method for age determination in *Glossina*. This method was later used (2) to establish the differences in the rate of growth between field and laboratory populations of *Glossina*.

It appears that in the anophelines the amount of growth in the first day is determined by the nutritional condition of the larva, whereas the rest of the growth is dependent on the environment of the adult, but further investigations are necessary to confirm this possibility.

The appearance of the daily layers differs markedly between mosquitos reared in the laboratory and those collected in field. The cuticle in *A. gambiae* reared in the laboratory was found to be thicker than that of field-collected mosquitos of the same species. It does not seem likely that the thickness of the cuticle could account for the absence of daily growth layers in most of the laboratory material. Alternations of cuticle thickness within the apodeme suggest a partial answer, because if the apodemes are stained without oxidation the daily layers are very indistinct in field-collected mosquitos. The oxidation with potassium permanganate selectively intensifies the demarcation lines of the daily layers without affecting intensity of staining in the rest of the cuticle; this being the case, it appears that these differences in the visibility of the daily layers result not merely from variations in the amount of cuticle deposited but rather from properties of the cuticle itself, dependent upon physiological differences between the anophelines in the field and those reared in the laboratory.

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RÉSUMÉ

DÉTERMINATION DE L'ÂGE CHEZ CERTAINS MOUSTIQUES ANOPHÉLINÉS D'APRÈS LES COUCHES DE CROISSANCE QUOTIDIENNE SUR LES APODÈMES DU SQUELETTE

Une étude précédente avait montré que l'on pouvait déterminer l'âge effectif de *Culex pipiens* et d'*Aedes aegypti* en comptant le nombre des couches de croissance quotidienne sur les apodèmes du squelette thoracique. La présente investigation visait à déterminer si la méthode était applicable aux anophèles.

On s'est servi, d'une part d'*Anopheles gambiae*, d'*A. funestus* et d'*A. albimanus* adultes élevés en laboratoire et tués à différents âges connus, d'autre part d'*A. gambiae* et d'*A. funestus* collectés dans la nature. Au moyen de techniques de coloration spéciales, on a pu observer les couches de croissance quotidienne sur le phragma thoracique. Ces couches étaient moins nettement décelables chez les insectes élevés en laboratoire que chez ceux que l'on avait capturés dans la nature. Dans le cas des *A. gambiae* récoltés dans la nature, l'âge effectif a pu être déterminé chez environ 85% des individus examinés, contre 25% seulement pour les spécimens élevés en laboratoire. Parfois, les couches quotidiennes ne prenaient pas suffisamment la coloration, de sorte qu'on les confondait avec un autre type de striure qui, chez certains spécimens, apparaissait dès le premier jour de la croissance. Chez la plupart des moustiques recueillis dans la nature, on a pu compter jusqu'à 10 couches de croissance correspondant à 10 journées d'âge et chez un petit nombre d'individus, on a pu dénombrer jusqu'à 13 couches. On espère qu'après avoir été suffisamment éprouvée sur le terrain cette méthode pourra être introduite dans des études épidémiologiques portant sur la longévité du vecteur.

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