CRITERIA OF THE GROWTH STAGES OF ANOPHELINE LARVAE

A MORPHOLOGICAL STUDY ON ANOPHELES HYRCANUS VAR. SINENSIS

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In anopheline larvae the criteria of age are not only of morphological interest but also of practical value. Theoretically, the age is defined as the period of time elapsed since the hatching of larvae. In most instances, however, especially in ecological odservations, the time of the hatching of larvae is unknown. Moreover, the duration of the larval period is subjected to a great variation according to environmental conditions, epspecially the temperature, and hence the estimation of the time of hatching of individual larvae is practically impossible. It seems, therefore, better to gain criteria of the age indirectly from the "larval growth stages", artificially set up according to the number of times of ecdysis which the larvae have passed through in their development.

The anopheline larvae become pupae after passing through three times of moulting, by which the larval period is divided into four instars, the first, second, third and fourth. Komiya and Ohuchi (1942)[2] classified the larval stages of *Anopheles hyrcanus var. sinensis* into four groups according to the body length: larvae smaller than 2 mm, the first instar; 2–4 mm larvae, second instar; 4–6 mm lavrae, third instar; and larvae greater than 6 mm, fourth instar. But such a simple classification of larval stages is unreliable, because the developmental stage of the larva is so much influenced by various evironmental conditions (food, temperature, and others) that the larvae of the same size are not always in the same instar. According to Christophers (1933)[1] the first instar larva of anophelines is distinctly different from the larvae of the other growth stages. Namely, the first instar larva possesses (1) an egg-breaker, (2) simple foliate spines on the abdominal segments, (3) two long lateral hairs, No. 6 and No. 7, on the third abdominal segment, (4) two pairs of pectens, and (5) ventral caudal hair composed of short spines; whereas the larvae of the other growth stages lack (1) the egg-breaker, but have (2) palmate hairs composed of several or more leaflets, (3) only one set of long lateral hair No. 6 (hair No. 7 being short), (4) only one pair of pectens, and (5) ventral caudal fan composed of feathered hairs. Since these five morphological units, as mentioned above, undergo extremely prominent alterations at the first moulting, similar striking changes may be expected to occur at

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every succeeding ecdysis.

Two possible modes of approach, direct and indirect, may be considered: first, to rear the larvae in the laboratory so as to observe the changes at every eclosion; and second, to seek some morphological features characteristic of each larval stage during the observation of a large number of larvae caught from the natural breeding places. In the present study the hair No. 6 of the third abdominal segment and the breadth of head were selected as the chief objects of observation, for both are to be readily examined with a microscope. The description will begin with observations of larvae collected from the field, followeld by those of the rearing experiments.

Materials and Methods

1. As material *Anopheles hyrcanus var. sinensis* was used. The larvae were caught from their natural breeding places (ponds and paddy fields of Nanking and Wanshiching, Central China) and killed in hot water. After dehydration and clarification with alcohol and xylol, they were mounted on slides and sealed with canada balsam. Their body length was measured.

2. Two hundred and thirty six larvae collected from the field were mounted on slides with Gater's fluid. Then the number of ramifications of their hairs No. 6 on the third abdominal segment was counted under low-power magnification of the microscope.

3. Two hundred and twenty larvae used in counting hair No. 6 were brought under microscope to measure the width (or the broadest diameter) of their heads

Table I

Course of rearing (A. hyrcanus var. sinensis)

Day		Weather	Temperature, °C			Ecdysis	Water
			Max.	Min.	Aver.		
Sept	10.	○	47.0	22.0	33.5	oviposition	
	11.	○			33.5		
	12.	○	47.0	22.0	33.5		
	13.	○	46.0	23.0	34.5	hatched	changed
	14.	○→●	48.0	22.0	35.0	first E.	
	15.	○	48.5	22.5	36.0	first E.	
	16.	○→●	42.0	21.0	31.5		changed
	17,	▲	29.0	19.0	24.0	second E.	
	18.	○	44.0	18.0	31.0	second E.	changed
	19.	○	45.0	18.0	31.5		
	20.	○	49.0	18.0	33.5	third E.	changed
	21.	○	47.0	17.0	32.0	third E.	
	22.	○	49.0	18.0	33.5		changed
	23.	▲	46.0	21.0	33.5		
	24.	▲	45.0	21.0	33.0		fed with spirogyra
	25.	▲→●	31.0	19.0	30.0	fourth E.	
	26.	▲→●	37.0	17.0	27.0	fourth E.	
	27.	▲	35.0	18.0	26.5		

N.B. ○ : clear, ▲ : cloudy, ● : rainy.
Tap water was used for rearing, so that the larvae were meagre and small.

with an ocular micrometer.

4. Female imagines of *Anopheles hyrcanus var. sinensis* bred with human blood were brought into a glass bottle, the bottom of which was covered by a sheet of blotting paper moistened with water. The opening of the bottle was closed by a piece of gauze. The eggs which had been deposited on the blotting paper after a few days were set afloat on the surface of tapwater in a petri-dish to rear the egg. The course of rearing is summarized in Table I. At each ecdysis which could be readily recognized by the appearance of larval skins casted away in water, a small number of larvae was dipped up into McGregor's fluid and mounted on slides with Gater's fluid. The specimens thus prepared were observed under the microscope.

Results and Discussion

The results concerning the larval size are summarized in Fig. 1, where curves N and W represent the frequency-distributions of the body length of larvae caught in two different districts, i. e. in Nanking and Wanshiching respectively. Both are alike in showing three peaks, which, however, are less distinct in the W than in the N curve. If they are considered in relation to the "larval growth stages", the first, second and third peak, enumerated from the left to the right, may be regarded as the second, third and fourth larval instar respectively, because it can be expected that the first instar larvae are too small to be caught, resulting in the lack of a peak in the frequency distribution curve.

Two modes of growth seem to be involved in the development of a larval body. The one is an abrupt and periodical growth at every eclosion, and the other a continuous growth throughout the life. The predominance of the latter type of growth over the former can be seen in Fig. 1. When the two curves in Fig. 1. are compared, the modes of their peaks are in the order: In N curve 3.3 mm (the second instar), 4.7 mm (the third instar), and 6.3 mm (the fourth instar); while in W curve, 2.3, 3.3, and 6.3 mm. From this comparison it will be seen that 3.3. mm in the second instar of the N curve corresponds closely to the third instar of the W curve. This indicates that the larval size alone cannot serve as a criterion for the classification of the larval growth stages.

On the contrary, the ramification of hair No. 6 on the third abdominal segment seems to be more useful, as shown in Table II and Fig. 2. Fig. 2 shows a frequency distribution curve, the number of ramifications being classified in the order: 0–1, 2–3, 4–5, and so forth, till 26–27 branched groups. Four distinct peaks are found in this curve, although the most left one is somewhat small. The three valleys are so sharply cut down that the independence of each peak can hardly be doubted. Inasmuch as the peaks are equal in number with the larval instars, they, enumerated from the left to the right, seem to represent the first, second, third and fourth instar, respectively. For the sake of clearer separation, the

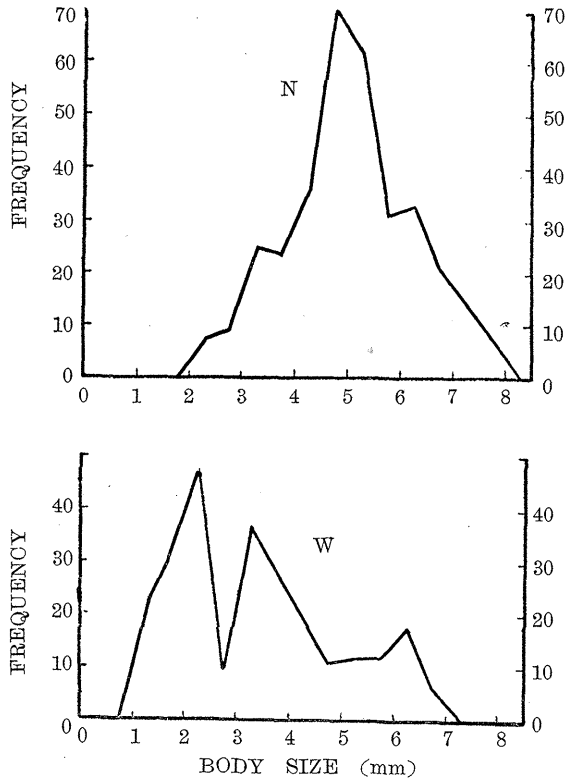

Fig. 1. Frequency distribution of body length of the larvae of *Anopheles hyrcanus var. sinensis* (N: caught in Nanking; W: caught at Wanshiching)

descending and ascending lines of the valleys in Fig. 2 are devided into two equivalent parts with reference to Table II. If we take up as an example the columnn of the eight-and nine-branched group in Table II, 2 eight-branched are alloted to the second peak and 6 nine-branched to the third peak. Thus, four groups of larvae can be classified according to the number of ramifications of hair No. 6, as follows: Groups I, ramification 0–2; Group II, ramification 3–8; Group III, ramification 9–16; and group IV, ramification 17–27. No significant correlation between the number of ramifications of hair No. 6 and the body size is demonstrated, so far as the examination is limited to the same group; whereas a remarkable correlation is found when larvae are viewed as a whole, including all the afore-mentioned groups. (The coefficients of correlation are 0.470, 0.169, 0.410, 0.184, and 0.936 for Group I, II, III, and "larvae as a whole" respectively). The author believes that this proves an abrupt increase of the number of ramifications of the hair No. 6 at every eclosion, and therefore the ramification of the hair No. 6 can serve as an excellent measure for the determination of larval gro-

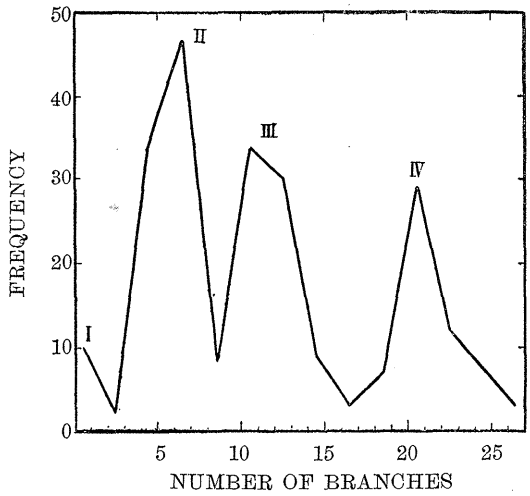

Fig. 2. Frequency distribution of the number of ramifications of hair No. 6 on the third abdominal segment of the larvae of *Anopheles hyrcanus var. sinensis*.

TABLE II

Correlation between the ramification of hair No. 6 and the body size

Body size in mm. \ Number of branches	0~1	2~3	4~5	6~7	8~9	10~11	12~13	14~15	16~17	18~19	20~21	22~23	24~25	26~27
~ 1.40	9	1	8	3										
~ 2.00	1	1	16	21	1									
~ 2.60			9	23	1	3	2	5						
~ 3.20			1			3	19	4	1					
~ 3.80							8	14	5					
~ 4.40							5	5	2		1	3		1
~ 5.00								2	1	1	1	5		4
~ 5.60										1	4	8	4	1
~ 6.20										1	1	5	6	2
~ 6.80												7	2		3
~ 7.40												1		
GROUP	I		II			III				IV				

wth stages. The rearing experiments which will be described later substantiated this claim.

The larva's head undergoes as striking changes as those of hair No. 6 at every ecdysis. The data of the head changes are summarized in Fig. 3, where four prominent peaks are noticed in the frequency distribution curve of head diameter, each of which seems to represent, from the left to the right, the first, second, third, and fourth instar respectively. The individual peaks can be separeted with the aid of Table III, in the same way as mentioned above, into four groups according to the head diameter as follows: Group I, less than 0.24 mm

TABLE III

Correlation between head diameter and body size
(Larva of A. hyrcanus var. sinensis)

Head diameter / Size of body	0.14	0.18	0.22	0.26	0.30	0.34	0.38	0.42	0.46	0.50	0.54	0.58	0.62	0.66	0.70	0.74	0.78	0.82 mm.
~1.40 mm.	8	7	2	3	1													
~2.00	3	1	1	10	16	2												
~2.60			1	10	13				2	4	1							
~3.20									8	16	4							
~3.80									10	10	11							
~4.40									5	6	4				1	1	2	
~5.00										2			1	3	3	2		
~5.60													1	2	9	4		2
~6.20														2	8	7		
~6.80															5	5		
~7.40																1		
GROUP	I		II					III					IV					

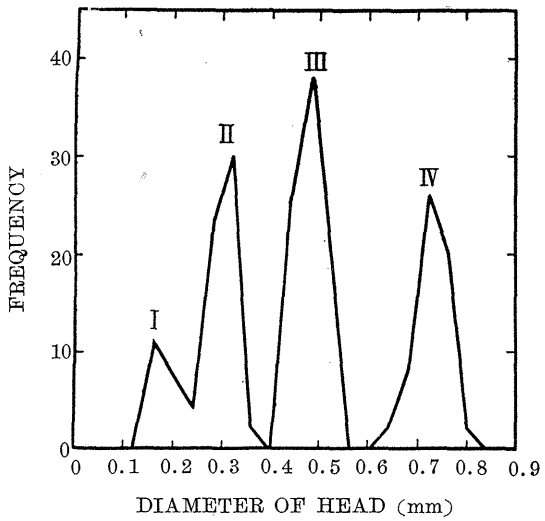

Fig. 3. Frequency distribution of head diameter of the larvae of *Anopheles hyrcanus var. sinensis.*

in width; Group II, 0.24 to 0.40 mm; Group III, 0.40 to 0.60 mm; Group IV, more than 0.60 mm. And 0.197, 0.320, 0.498, and 0.736 mm are calculated as the average head-diameter for the Groups I, II, III and IV respectively, i. e. a geometrical progression with a common ratio $r=1.55$. This interesting relation and the clear-cut appearance of peaks with deep valleys seem to indicate the usefulness of the head-diameter as a criterion for the determination of larval instar. This was also proved by the rearing experiments.

The results of the experimental observations of the larva's rearing are presented in Table IV, in which the ramification of hair No. 6 and the diameter of

TABLE IV

Results of the measurement upon reared Larvae (A. hyrcanus var. sinensis)

Instar	Body size (mm.)	Head diameter (mm.)	Ramification of hair No. 6	
			Right side	Left side
First	0.80	0.194		
	0.88	0.216		
	0.91	0.208		
	0.96	0.194		
	0.99	0.208		
	1.04	0.208		
	1.17	0.200		
Second	1.52	0.296	6	6
	1.57	0.264	7	5
	1.59	0.272	6	6
	1.62	0.296	6	7
Third	2.27	0.432	10	10
	2.58	0.424	12	11
	2.66	0.448	12	12
	2.66	0.472	14	12
	2.80	0.456	10	11
Fourth	3.44	0.672	19	?
	3.60	0.688	20	18
	3.65	0.688	20	18
	3.97	0.672	22	19
	4.06	0.656	20	?
	4.42	0.720	17	20
	4.55	0.736	22	?

head are listed in association with the larval size. In this table a certain degree of variation is seen in each larval instar, as follows:

Larval stage	Head diameter	Ramification of the hair No. 6
First instar	0.194–0.216 mm	no ramification
Second instar	0.264–0.296 mm	5–7 branches
Third instar	0.424–0.472 mm	10–14 branches
Fourth instar	0.656–0.736 mm	17–22 branches

The corresponding values from the larvae caught in the natural breeding places can be summarized from data given in Tables II and III, as below:

Group	Head diameter	Ramification of the hair No. 6
Group I	0.140–0.240 mm	0–2 branches
Group II	0.240–0.400 mm	3–9 branches
Group III	0.400–0.600 mm	9–16 branches
Group IV	0.600–0.820 mm	17–27 branches

These two sets of values accord very well, especially when the meager nutritional condition of the rearing experiments due to the lack of food is taken into

consideration. Therefore, the identity of the instars of larvae either from the rearing experiments or from the natural breeding places seems unquestionable. The fact that the ramification of hair No. 6 and the diameter of head, irrespective of the evironmental conditions, undergo definite abrupt changes at each ecdysis, offers a strong evidence for the usefulness of these morphological units as criteria for the determination of larval instars

However, some additional remarks should be made of the head diameter. Our subsequent study which has been conducted with the collaboration of Mizukawa (1948)[3] in Kurashiki City, Okayama Prefecture, revealed that the head diameter of larvae of *Anopheles hyrcanus var. sinensis* seems to show some differences according to the different districts where the larvae lived. In Kurashiki the demarcation values for each larval instar were somewhat larger than those described above (observations in Nanking and Wanshiching, Central China), as follows:

	First instar→	Second instar→	Third instar→	Fourth instar
Demarcation values	0.315 mm.	0.450 mm.	0.660 mm.	

The ramification of hair No. 6, on the other hand, showed no differences according to the different districts.

As to the availability of the morphological units other than those mentioned above, the hair No. 17 on the head, hair No. 5 on the metathorax, and hair No. 6 and No. 7 of the first and second abdominal segments were studied, but all those were found less useful on account of the difficulties involved in their examination. The data are listed in Table V and Plates I–III, where the nomenclature of larval hairs is given according to Christiphers (1933)[1].

TABLE V

Number of ramifications of principal hairs (Larva of Anopheles hyrcanus var. sinensis)

Hair \ Instar	First	Second	Third	Fourth
Head, No. 17	2	4–8	11–15	20–30
Metathorax, No. 5	1–2	8–11	14–23	23–34
First abd. seg., No. 6	l, s, f	6–9	12–16	19–26
No. 7	l, s, f	5–8	10–15	19–25
Second abd. Seg., No. 6	l, s, f	7–12	15–19	22–31
No. 7	l, s, f	6–9	14–18	20–29

(N.B. l. s. f = long, simple and frayed hair)

SUMMARY

Two series of morphological observations, based upon the collections of larvae from different natural sources and the rearing experiments in the laboratory, have been carried out in *Anopheles hyrcanus var. sinensis*, in an attempt to obtain some

clues for the determination of the larval instar. The results obtained can be summarized as follows:

1. The number of ramifications of the hair No. 6 on the third abdominal segment can serve as the most reliable criterion for the determination of the larval growth stages, because the ramification shows no variations according to the different districts.

2. The maximum transverse diameter of the head seems also useful in this respect, but because of a greater degree of variations according to the different districts its usefulness is limited to the larvae collected in the same district.

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Explanation of Plates

Morphology of the larva of *Anopheles hyrcanus var. sinensis.*

Dorsal views : (α)—The fourth instar, (β)—The first instar, (γ)—The second instar, (δ)—The third instar. The left half of each figure refers to the larvae collected in Central China, and the right half to those caught in Japan.

Ventral views : (α′)—The fourth instar, (β′)—The first instar, (γ′)—The second instar, (δ′)—The third instar. The left half of each figure refers to the larva collected in Japan, and the right half to that caught in Central China.

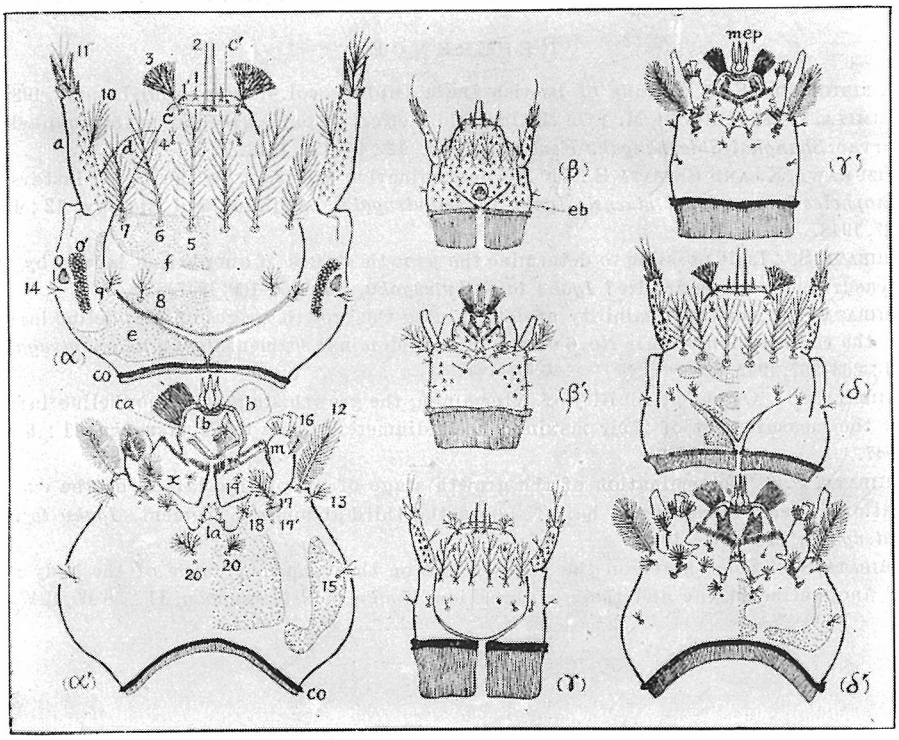


Plate I. The head

a : antenna, b : mouth brush, c : clypeus, c′ : preclypeus, ca : chitinous appendages of oral cavity, d : mandible, e : epicranium, eb : egg-breaker, f : front, la : labium, lb : labrum, m : maxillary palp, mep : median process of preclypeus, o : ocellus, o : oculus, x : maxillary plate. The numbers denote the bristles according to Christophers (1933).

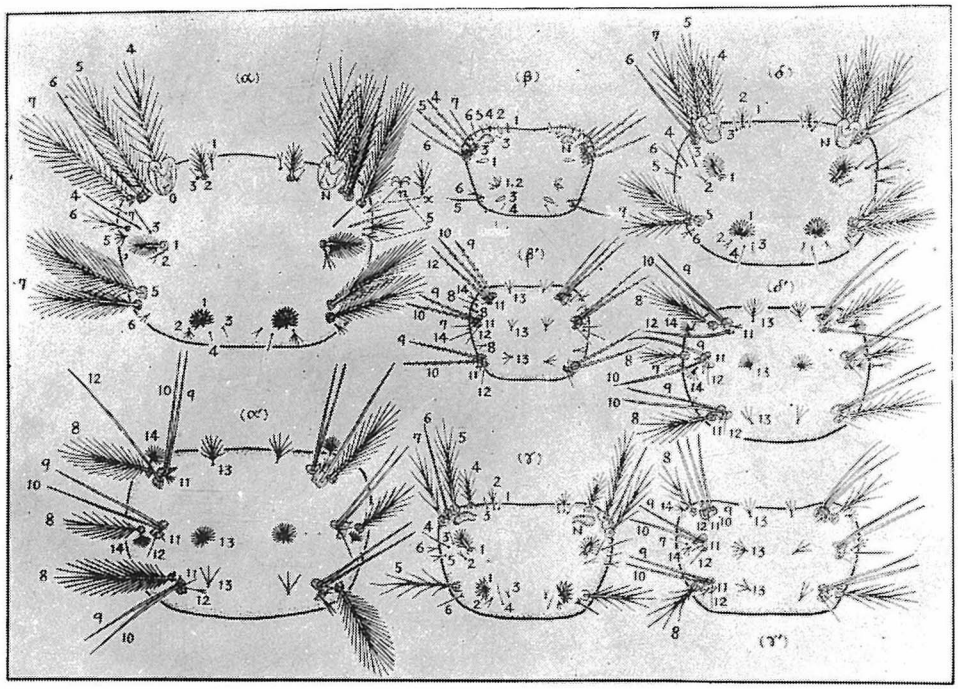
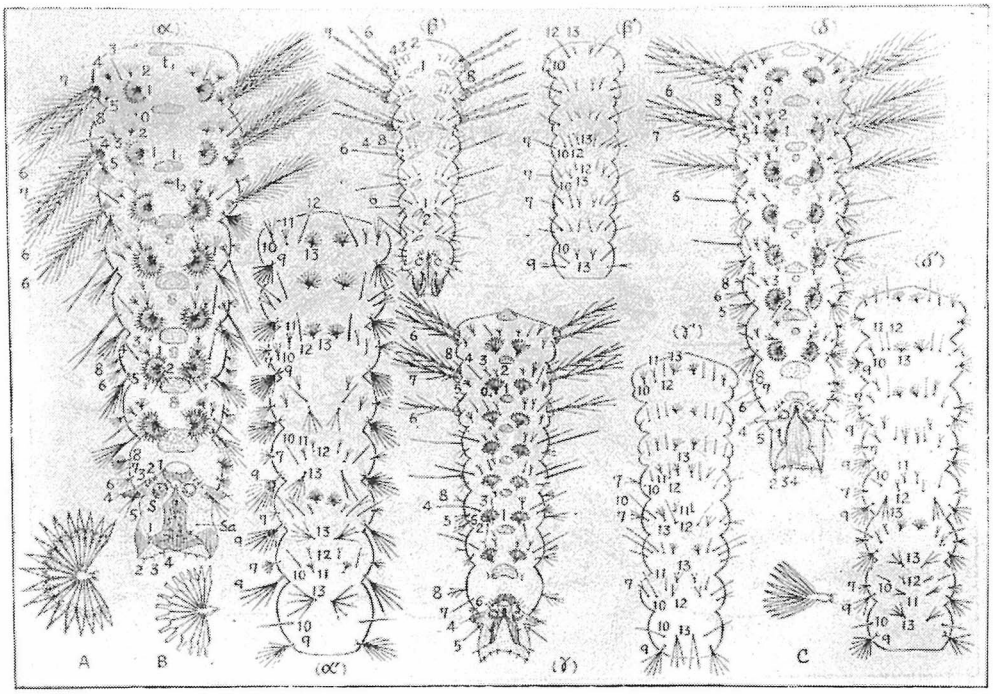


Plate II. The thorax

o : notched organ of Nutall and Shipley, n : hair No. 5 on the mesothorax of the larva of *Anopheles hyrcanus var. nigerrimus.* x : the same one of *Anopheles hyrcanus x.* The numbers denote the bristles (Christophers, 1933)



. Plate III. Abdomen

(excluding the terminal segment)

A : perfect palmate hair of the fourth instar larva, B : palmate hair of the third instar larva, C : palmate hair of the second instar larva. S : spiracle, Sa : spiracular apparatus, t_1 : anterior tergal plate, t_2 : posterior tergal plate. The numbers denote the bristles (Christophers, 1933).