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Laboratory Growth of *Anopheles gambiae* (Diptera: Culicidae) and Morphological Determinism of Moulting

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Abstract

Growth in arthropods in general and in insects in particular, is supposed to be discontinuous and occurs during moulting. In Culicidae in general and *Anopheles* in particular, the number of moults is five with the fourth which gives the pupae. It is known that moulting in insects is a genetic and physiological phenomenon. Most physiological reactions are triggered by chemical or physical stimuli. The pressure exerted by the growth of the larval body on the exocuticle is one of the triggers of moulting. The objective of this work was therefore to determine the exact timing of the first three moults that determine the growth of *An. gambiae* larvae from egg hatch to pupation to highlight the role of increased larval size in the stimulation of moulting. We therefore, undertook to rear larvae of this anopheline species in the laboratory under conditions close to their natural environment from hatching to pupation. The length and width of the head, thorax and abdomen were recorded daily. Data analysis showed that the size of the head and thorax remained constant for the first three days (D0 to D2) of development and abdomen's length for the first two days and then increased daily until day seven (D7) when it stopped. These observations led us to say that the M1 moult occurs at end of the third day of development and the M3 moult at end of the eighth day; the M2 moult could not be determined. All these observations led to the conclusion that the larval growth of *An. gambiae* has a continuous regimen and the growth of the head and thorax of the larva plays a crucial role in the onset of moulting.

Keywords

Anopheles gambiae, Larva, Growth, Determinism, Body Sizes, Moulting

1. Introduction

Anopheles gambiae Giles, 1902 is an insect belonging to the Order Diptera, Family Culicidae. Insects are arthropods which according to [1], constitute the largest phylum in the animal kingdom in terms of the number of species identified, their biomass and their impact on ecosystems. They are animals that colonise all environments and are important in various domains, particularly in medical aspects. Thus, many studies have shown that mosquitoes in general and *Anopheles* in particular, are vectors of many human diseases such as filariasis and especially malaria [2] [3]. According to the [4], since 2000 there have been about 1.5 billion cases of malaria with about 7.6 million deaths. In 2019, there was 229 million new cases of malaria with about 409 thousand deaths. Africa, especially the sub-Saharan region, counts about 92% of these malaria cases. Entomological studies carried in this part of the African continent show that *An. gambiae* is the main malaria vector in this region [5] [6]. This fact is a sufficient evidence of the impact of *An. gambiae* on public health whose impact on Malaria, especially in sub-Saharan Africa, is undisputed.

Anatomically, arthropods are characterised by the structure of their tegument. Studies have shown that it has an exoskeleton that is sometimes very rigid, the cuticle, which in some groups like insects, crustaceans, myriapods (centipedes and millipedes), and chelicerates (spiders, the horseshoe crab, and sea spiders) constitutes a real carapace [7]. According to [1] and [3], the cuticle contains chitin, which gives it this rigidity. The stiffness of the cuticle prevents larval growth. This only occurs during moulting, which is the replacement of the old cuticle by a new one that gradually hardens by impregnation with chitin [7] [8]. The direct consequence of this is that for these animals, growth is discontinuous. To highlight the importance of this phenomenon, it has been established that moulting marks the passage of one stage of development to the next one. In the case of Culicidae, a larva undergoes four moults to produce a pupa. The first M1 moult transforms the L1 larva into the L2 larva, the second transforms the L2 larva into the L3 larva, the third gives L4 larva from the L3 larva, the fourth moult transforms L4 larva into the pupa, and a final moult transforms the pupa into an adult [9].

Much of the current studies on anopheline species focus on their importance in the transmission of diseases through bites and on their control in order to reduce populations and thus minimise disease transmission. In recent studies, most larvicidal tests are done on specific stages. In order to allow a more precise choice of the larval stage, the present work aims not only to determine the morphological modalities of growth in *An. gambiae* but also the duration of each larval stage before pupation. In addition, there is still some doubt as to the determinism of moulting. It is accepted that the growth of the pre-imaginal stages of insects as well as their moulting is a phenomenon whose regulation is essentially hormonal, in particular the role of the juvenile hormone [7] [8] and [10] and ecdysone [11]. The latter was able to establish the role of the prothoracic gland in the production of ecdysone and especially the action of this hormone in triggering moulting. However, the factor triggering the production of ecdysone remains to be elucidated. This work contributes to clarifying this aspect of *An. gambiae* in particular.

2. Material and Methods

Study of *An. gambiae* larval growth was carried out at the laboratory of the Biotechnology Centre of the University of Yaounde located at Nkolbisson locality. The eggs and larvae came from a strain of *An. gambiae* maintained at the Laboratory of Zoology of Higher Teacher's Training College of the same university. These experiments were carried out at room temperature (25.5˚C ± 2.6˚C). The spring water used is water that flows out of a rock at Nkolbisson and has a pH of 6.7 ± 0.8. In order to minimise the effects of food ration, density, water pH and water depth on larval growth, we conducted preliminary studies, some of which have already been published, including the effect of larval density [12] [13] and effect of water depth of larval habitat [13] [14] on its development. Based on these studies, the following conditions were retained: a daily food supply of 20 mg of manufactured Baby Fish Food (TetraMin® per 100 larvae (quantity of food allowing a harmonious development of *An. gambiae* larvae with a low risk of inducing environmental pollution), a larval density of 1 larva/cm^2, water depth of 3 cm and pH of 6.7 ± 0.8. Every day, 30 larvae were taken from the tanks and introduced into 70 % ethyl alcohol. Then the length and width of the head, thorax and abdomen were measured. These measurements were made using a Wild® binocular magnifying glass with a micrometer in one of its objectives. Conversion of micrometer units to millimeters was done by using a graduated calibration blade.

Larval growth of *An. gambiae* was assessed over a period of 10 days. Indeed, day 10 corresponds to the date on which we obtained the transformation of two-thirds of the larvae into pupae [15]. We excluded the pupa from the study because it does not feed [3] [16] and therefore does not grow.

Statistical Analysis

The ANOVA test was used to compare the average daily length and width of *An. gambiae* larvae. When the difference was significant, we performed a multiple comparison using Tukey's test. We also determined correlations between the daily changes in length and width of the head, thorax and abdomen of the larvae and between the length and the width of each part. The correlation test used was the Pearson test. The Wilcoxon test allowed the size of the different parts of the body of the larva to be compared with each other.

3. Results

Growth of the head

Length of the larval head of *An. gambiae* increased daily except for the first three days (D0, D1 and D2) and the last three days (D7, D8 and D9) of development. It varied from 0.187 mm at hatching to 0.685 mm on day 10. Width did not vary during the first four days (D0 to D3) and the last three days. It went from 0.160 mm at hatching to 0.685 mm at D10 (Table 1). The length and the width differ significantly ($Z = -11.466$, $P < 0.0001$) from each other. At hatching the head was longer than it was wide. But towards the end of larval development (D8 and D9), the head became as long as its width (Table 1).

Growth of the thorax

Anopheles gambiae larval thorax length increases significantly from one day to another except for the first three days and the last two days. It varies from 0.163 mm at D1 to 0.973 mm at D9. The width of the thorax follows rigorously the same evolution pattern as the length (Table 1). It increased from 0.160 mm at hatching to 1.209 mm at D9. As for the head, the two parameters differ significantly from each other ($Z = -12.023$, $P < 0.0001$). During the first three days of development, the thorax is longer than it is wide. But from the 4th day onwards, width dominated over length (Table 1).

Growth of the abdomen

Length of the abdomen of *An. gambiae* larvae increase significantly daily, except for the first two days and the last two days of development. This length increases from 0.544 mm at hatching to 4.403 mm at D9. Width remains constant

Table 1. Daily's variations in the size (in mm) of different parts of the body of *An. gambiae* larvae and comparisons in each parameter.

	Lh	Wh	Lth	Wth	Lab	Wab
D0	0.187 ± 0.019^{a}	0.160 ± 0^{a}	0.163 ± 0.010^{a}	0.160 ± 0^{a}	0.544 ± 0.041^{a}	0.062 ± 0.002^{a}
D1	0.195 ± 0.014^{a}	0.160 ± 0^{a}	0.163 ± 0.010^{a}	0.160 ± 0^{a}	0.619 ± 0.047^{a}	0.071 ± 0.002^{a}
D2	0.205 ± 0^{a}	0.160 ± 0^{a}	0.164 ± 0.012^{a}	0.160 ± 0^{a}	0.909 ± 0.048^{b}	0.097 ± 0.001^{a}
D3	0.240 ± 0^{b}	0.160 ± 0^{a}	0.199 ± 0.007^{b}	0.245 ± 0.013^{b}	0.995 ± 0.044^{c}	0.157 ± 0.010^{b}
D4	0.352 ± 0.054^{c}	0.280 ± 0^{b}	0.240 ± 0^{c}	0.320 ± 0^{c}	1.475 ± 0.094^{d}	0.205 ± 0.014^{c}
D5	0.352 ± 0.054^{d}	0.312 ± 0.054^{c}	0.301 ± 0.036^{d}	0.357 ± 0.038^{d}	1.637 ± 0.095^{e}	0.229 ± 0.023^{c}
D6	0.483 ± 0.058^{e}	0.432 ± 0.022^{d}	0.483 ± 0.055^{e}	0.627 ± 0.098^{e}	2.571 ± 0.300^{f}	0.392 ± 0.079^{d}
D7	0.663 ± 0.058^{f}	0.680 ± 0.018^{e}	0.712 ± 0.054^{f}	0.933 ± 0.102^{f}	3.435 ± 0.328^{g}	0.684 ± 0.076^{e}
D8	0.680 ± 0^{f}	0.681 ± 0.007^{e}	0.949 ± 0.071^{g}	1.200 ± 0.089^{g}	4.309 ± 0.184^{h}	0.824 ± 0.041^{f}
D9	0.6853 ± 0.014^{f}	0.685 ± 0.014^{e}	0.973 ± 0.035^{g}	1.209 ± 0.089^{g}	4.403 ± 0.108^{h}	0.825 ± 0.045^{f}
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Same letter in columns mean not significantly different at $P < 0.05$. Lh = length of head, Wh = width of head, Lth = length of thorax, Wth = width of thorax, Lab = length of abdomen and Wth= width of abdomen.

for the first three and the last two days (Table 1). It increases from 0.062 mm at hatching to 0.825 mm at D9. The two parameters differ significantly ($Z = -15.013$, $P < 0.0001$). As in the large majority of insects, the abdomen is always longer than it is wide.

Evolution of larval size and occurrence of moulting

It is evident from the evolution of the length of different parts of the body of *An. gambiae* larvae that the total length of the larva also increases significantly on a daily basis except for the first two days and the last two days of development (Table 1). Total larval length increases from 0.893 mm at hatching to 6.061 mm at D9 (Table 1).

Size of different parts of the body of *An. gambiae* larvae differ significantly from one to another (Table 2). Thus, the abdomen is longer than the head and thorax (Figure 1). As for the width, it appears from (Figure 2) that width does not differ between the three parts of the body until day 5 when the thorax becomes wider than the other two parts. However, it can be seen from the correlation test that there is a certain synchronisation in the growth of all parts of the larval

Table 2. Comparison between the different body measurements of *An. gambiae* larvae by the Wilcoxon test.

	Lh-Lth	Lh-Lab	Lth-Lab	Wh-Wth	Wh-Wab	Wh-Wth
Z	−5.296	−15.015	−15.015	−12.265	−3.732	−14.879
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

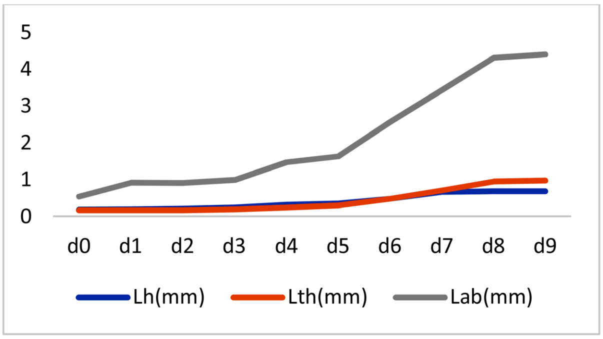

Figure 1. Variation of length of body parts of *An. gambiae* larvae.

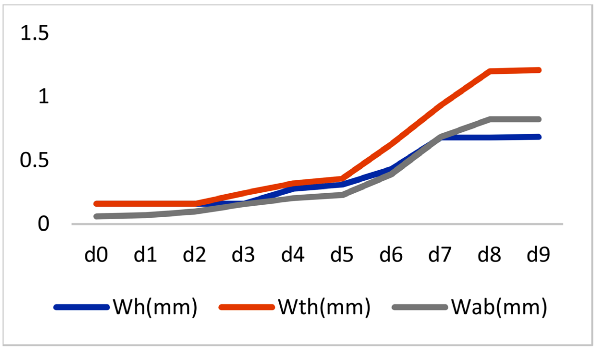

Figure 2. Variation of width of body parts of *An. gambiae* larvae.

Table 3. Pearson correlations between different body measurements of *An. gambiae* larvae.

	Lh-Wh	Lh-Wh	Lab-Wab	Lh-Lth	Lh-Lab	Lth-Lab	Wh-Wab	Wth-Wab
R	0.987	0.989	0.983	0.956	0.975	0.983	0.969	0.982
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

body (Table 3). The head and thorax have more or less the same evolutionary pattern (Table 1).

It has been established that the larvae of anopheline species, like those of Culicidae in general, undergo four moults during development to give the pupa. In this work, we wanted to see if it is possible to highlight the occurrence of the moults mentioned in the introduction using biometric data. Data analysis from this study reveals that it is possible to identify the occurrence of the M1 moult three days after hatching which allows the transition from the L1 larva to the L2 larva, as well as the M3 moult on the eighth day that transforms the L3 larva into the L4 larva. Thus, we can say that the M1 moult would probably occur at the end of the 3rd day of development while the M3 moult would occur on the 8th day (Table 1). The M2 moult that produces the L3 larva from L2 is not biometrically detectable.

4. Discussion

The evolution of the length and the width of some parts of animal's body could highlights the phenomenon of growth for any young organism. Thus, the larva of *An. gambiae* grows both in length and width. This growth affects the three parts of the body. The growth of an individual reflects the accumulation of material brought by feeding. It is mainly an accumulation of protein and lipid reserves [17] [18]. Thus, we can say that the gain in material is made at approximately the same rate in the three parts of the body of the *An. gambiae* larva because their growth is synchronous. However, we found that the abdomen's length increased more rapidly than that of the other two parts of the larval body. This would imply that this part accumulates more reserves. However, when we take into account the physical aspect of the three parts of the body of the mosquito larva and even of the adult, we notice that the tegument of the abdomen is less rigid than that of the head and thorax (personal observation). In fact, segments of the abdomen of Culicidae larvae, like those of the adult abdomen (during metamorphosis, the abdomen does not present any profound changes like the head and thorax) are made up of rigid tergites and sternites, whereas pleurites are flexible [3] like the intersegmental membranes of this same part. This observation implies that the abdomen would be more extensible both in length and width than the head and thorax and would justify the data obtained in the study. In fact, it is observed that the length of the abdomen increases after two days of development, whereas the head and thorax take three days.

Matter which accumulated in the body comes from the food that the individual consumed. Thus, the speed of growth of an individual depends on the availability of food on the one hand and on the other hand on the capacity of the individual to digest it. As far as the availability of food was concerned, arrangements were made to make it available. In this case, the factor on which the individual's growth rate would depend is its ability to consume it. The study data showed that the growth rate of *An. gambiae* larva remained null during the first three and last two days of larval development. The facts observed for the last two days obviously reflect the end of larval growth and preparation for pupal development. Therefore, we can state that L3 larvae transform into L4 at the end of the eighth day of development. On the other hand, lack of growth observed during early development could be attributed to the inability of larvae to consume the available food. According to [19], first instar larvae are less able to feed because their oral apparatus is not well developed to feed on food in granular form. Based on all the above, we can state that transformation of the L1 larva into L2 occurs at the end of the third day of development. The time of transformation of L2 to L3 larva remains to be determined because between the fourth and eighth day, growth of *An. gambiae* larvae are continuous as the size differs from one day to the next. This observation reopens the debate on the determinism of moulting in insects in general and anopheline species in particular.

It is established that moulting plays a crucial role in the growth of the juvenile stages of arthropods in general and insects in particular. Indeed, according to [8], insect growth only takes place for a few hours corresponding to the impregnation of chitin by the new cuticle. While this work has made it possible to locate with some accuracy the occurrence of the M1 and M3 moult, the occurrence of the M2 moult could not be determined by studying the variations in the larval size of *An. gambiae* because the data do not indicate any interruption of growth either in length or width of the larva between the fourth and eighth day of development. These observations highlight the continuous nature of *An. gambiae* larval growth, especially since lack of growth observed during the first three days would be justified by an anatomical limit of L1 larva. This result calls into question the discontinuous character of growth of arthropods in general and insects in particular as described by [3] [8] [20] and many others. It becomes clear that the cuticle of *An. gambiae* larvae would be less rigid and therefore extensible. The question that arises at this level is what triggers hormone production responsible for moulting in this larva. Would it be of genetic origin, *i.e.* inscribed in the genome, or would it be morphological? In the first case, this would imply that each species has a fixed number of moults and that these moults would occur at regular time intervals [20] independently of factor and consequently duration of each larval stage would be fixed. The first aspect is verifiable. Indeed, each insect species presents a fixed number of moults [8] [20] and [3] confirming genetic control of the number of developmental stages of species. However, the duration of each developmental stage is beyond genetic control because it is largely influenced by the environment. Indeed, numerous studies have shown that factors such as temperature [3], larval density [12] [13] and water depth in the deposit [13] [14] modify duration of the larval stage of *An. gambiae*. In terms of larval density and water depth, these two factors directly affect larval growth. The first acts through intraspecific competition due to overpopulation [12] [13] and the other through the loss of energy due to movement of larvae which goes to the bottom of the water to feed (personal observation) and must return to the surface to breathe [13] [14]. Morphology and, more precisely, the size of the individual thus appears to be a determining factor in the onset of moulting. To this end, we believe that although growth is continuous, the extensibility of the cuticle has a critical threshold beyond which moulting process is triggered. Numerous studies have highlighted the role of the insect's brain in regulating the production of the moulting hormone, ecdysone. Thus, although produced by the insect's prothoracic gland, production's regulation of this hormone is under the control of a neurohormone produced by *pars intercerebralis* [11]. This reflects the nervous component of moult control. In this case, we are forced to admit that the initiation of moulting has an anatomical base. Indeed, it has been established that moulting is a phenomenon that frees the body of an arthropod, which has become compressed by an inextensible shell. In this case, we can assume that *pars intercerebralis* would be sensitized by tensio-receptors or baroreceptors which detect the pressure exerted by the growing body on the cuticle. Based on the results of the study, we can state that the growth of the head and thorax plays a major role in the initiation of moulting in *An. gambiae* larvae. Indeed, the integument of segments of these two parts of the body is more rigid because all plates (tergites, pleurites and sternites) which compose it are sclerified and limits of the three thoracic segments are completely erased. The integument thus forms capsules, unlike the abdomen whose pleurites are flexible and which have intersegmental membranes [3]. Although we have accepted that integument of the *An. gambiae* larva is more or less flexible, it should be considered that extensibility of this cuticle has a critical threshold beyond which moulting is triggered on one hand, and on the other hand that moulting is fairly rapid (of the order of hours).

5. Conclusion

When we initiated this work, we were certain that *An. gambiae* larvae grow discontinuously after moult like all other arthropod larvae. In addition, the number of moults to obtain the adult from egg hatching is established at five in Culicidae, four between hatching and pupation. Although, the occurrence of the fourth moult is very obvious to detect, pupa being morphologically different from larva, the first three moults are difficult to detect. We therefore, thought that by daily recording the evolution of length and width of larvae, we could accurately determine the occurrence of the first three moults and thus reveal the relationship between variation in larval size and onset of moulting. In this work, the end of the third day of development was determined as the date of the M1 moult occurrence and that of the eighth day for M3 and it was established that the growth of head and thorax of *An. gambiae* larvae could be responsible for the production of the moult hormone ecdysone. On the other hand, the fact that we could not determine the occurrence of the M2 moult led us to conclude that the growth of *An. gambiae* from L2 to L4 larvae is continuous, as lack of growth observed during the first three days of development is simply due to the inability of L1 larvae to feed.

Data Availability

The datasets used during the current study are available from the corresponding author on reasonable request.

Author's Contributions

THG designed the research and conducted the study and assays, ANP co-directed the research work as well as the statistical analysis**,** MEAL, TT and MM did the proofreading of the work.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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