Molting and metamorphosis in mosquito larvae : a morphometric analysis

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Molting and metamorphosis in mosquito larvae: a morphometric analysis

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Insect body size is determined by the larval growth period and developmental instars of insects can be characterized by structural parameters of their exoskeleton. Two morphometric traits were followed throughout the whole larval development of six mosquito species: *Aedes aegypti*, *Ae. vexans*, *Culex pipiens* and three *Anopheles* species. The constant rearing temperature was 27 °C; only for *Ae. vexans* it was 22 °C. The diameter of the head capsule as a sclerotized structure allowed to clearly distinguish each instar, according to Dyar's rule, and no overlaps exist. The thorax diameter grew exponentially, but with tremendous overlaps. For both parameters the cubic values were applied as an estimate for the respective volumes. Thorax dimensions reflect continuous growth during the intermolt and therefore are a valid estimate of growth in biomass. Mean growth rates for biomass, measured over the whole larval period, were lowest in Anophelinae with 0.011 mm^3/h, contrasting with 0.027 mm^3/h in Culicinae.
Starvation experiments with fourth instar larvae of *Ae. aegypti* revealed a certain flexibility in growth rates. Previously well-fed larvae exposed to starvation at the onset of the final instar, extended their phagoperiod, followed by death or, in a minority by tissue degradation and metamorphosis, but no size reduction. Conversely, starved larvae fed surplus during the final instar could largely compensate their body size to nearly normal values.
Pupation and eclosion occurred continuously in Culicinae, primarily on days 7–9, and days 9–11, respectively. In Anophelinae metamorphosis was clearly gated: pupation during the photophases of days 9–11 and eclosion during the scotophases of days 11–13.

Key words: Anophelinae, Culicinae, larvae, morphometry, growth, gating.

INTRODUCTION

Molting is a fascinating problem of arthropod biology and insects growing by molting have conquered our planet in space, in time, and in number. But even more fascinating is the process of metamorphosis, as evolved by the endopterygote insects. This dramatic change in form and physiology allows the insect to colonize different and additional ecological niches. Depending on the nature of these niches and their food supply, not only the external morphology requires redesigning, but equally so the physiology and metabolism. In the case of the mosquitoes, larvae and pupae are fully aquatic, feeding on so-called detritus while imagines in alteration seek carbohydrates or vertebrate blood. All these processes and their adaptations ultimately are governed by the neuroendocrine system (NIJHOUT, 1994).

In arthropods growth processes basically follow two modes. In sclerotized parts of the exoskeleton, such as the head capsule, size increase occurs during the molting, before the new cuticle hardens. Hence, the sclerotized head capsule increases in a discontinuous, saltatory pattern. The other possibility for growth occurs during the intermolt period, characterized by continuous growth (WILLIAMS, 1980). Linear measurements of the larval head capsule diameter are often used to

identify larval instars. This criterion was first recognized by DYAR (1890) as a geometric progression of growth rate in caterpillars; the head capsule increased with each molt by a constant and species-specific ratio. Subsequently, many workers have used this technique in different insect orders to classify instars (GHENT, 1956; FRAMPTON, 1986). It has also been successfully applied to larvae of a few mosquito species (ABDEL-MALEK & GOULDING Jr., 1948; JONES, 1953; DE OLIVEIRA & DURAND, 1978; DESLONGCHAMPS & TOURNEUR, 1980; NEMJO & SLAFF, 1984; LARDEUX & TETUANUI, 1995).

The reproductive physiology of mosquitoes is affected largely by female body size, which in turn is a result of larval rearing and feeding conditions (VAN HANDEL, 1986, 1988; BRIEGEL, 1990a, 199b; TIMMERMANN & BRIEGEL, 1996). Despite their tremendous significance as vectors of diseases, the larval period of the mosquitoes has received only limited attention by insect physiologists, except for field investigations (HARAMIS, 1985; LAIRD, 1988).

Under natural conditions mosquitoes rarely attain maximal body sizes (MOORE & FISHER, 1969; MORI, 1979; SULEMAN, 1982). Larval stress is caused by limitations of space and/or food, and by the food quality (TIMMERMANN & BRIEGEL, 1993, 1996). In our previous studies *Ae. aegypti* appeared to be the most adaptive species in coping with such unfavorable larval conditions; *Anopheles* species were much more sensitive to increasing water depths, crowding conditions and unbalanced food supplies, and thus restricted in successful development, imaginal body sizes and teneral reserves (TIMMERMANN & BRIEGEL, 1993, 1996).

Upon reaching a certain size larvae start to pupate. The manifestation of an endogenous rhythm has been observed in a few mosquitoes. Rhythmic pupation in the black saltmarsh mosquito *Aedes taeniorhynchus* was found by PROVOST & LUM (1967) and NAYAR (1967a, 1967b) and in *Culex nigripalpus* by NAYAR (1968). GOMA (1959) and JONES & REITER (1975) have shown circadian rhythms of pupation in *An. gambiae*. The Zeitgeber for the eclosion rhythm was the change from light to darkness (REITER & JONES, 1975).

In this study we compare larval growth among six species of the three major genera, *Anopheles*, *Aedes*, and *Culex*, known as vectors of diseases. This comparative approach was necessary because Anophelinae eclose with very limited reserves in contrast to the Culicinae (BRIEGEL, 1990a, 1990b). It will be demonstrated that these Diptera follow a different strategy for molting and metamorphosis than the Lepidoptera, independent of critical thresholds, although the nature of the stimulus for pupal molts still remains enigmatic.

We present morphometric parameters to characterize the larval instars and their different growth patterns depending on the dietary constraints during their developmental period, providing the basis for a physiological investigation of the larval development (TIMMERMANN & BRIEGEL, unpubl.).

MATERIALS AND METHODS

The following species were used in this study: *Aedes aegypti* (L.) (strain UGAL), *Ae. vexans* (MEIGEN) from the upper Rhine valley (kindly provided by Dr. R. KUHN, Mainz, Germany) and from the Greyerzersee (kindly provided by Prof. P. LÜTHY, Zürich ETH, Switzerland), *Culex pipiens* L. (strain Colorado), *Anopheles albimanus* WIEDEM. (strain El Salvador), *An.* (*Cellia*) *gambiae* GILES, strain 16c55 from Nigeria, and an Indian strain of *An.* (*Cellia*) *stephensi* LISTON, obtained through NIH (Washington DC). Larvae and imagines were routinely colonized at 27±0.5°C,

85±5 % r.h., under long-day conditions (14L:10D); larval food was Tetramin® according to our standard regime (BRIEGEL, 1990a, 1990b; TIMMERMANN & BRIEGEL, 1993). All imagines had free access to 10 % sucrose solution. The native species *Ae. vexans* was always kept at 22 °C. Bloodmeals for stock maintenance were given weekly on two consecutive days to *Anopheles* while *Aedes* or *Culex* were fed once a week, all on a restrained guinea-pig.

To study larval development under standard conditions, all animals were reared with optimal densities and constant water depths for each species; in some experiments crowding conditions were arranged, all as described before (TIMMERMANN & BRIEGEL, 1993). For measuring larval body sizes, every 12 h random samples of 50-100 larvae were taken, depending on the different rates of development, each time from another pan. Therefore, up to 24 rearing pans were set up simultaneously for each species, in order to have available at any one time undisturbed rearing pans for each instar. After sampling, these pans were discarded. This arrangement guaranteed samples from standardized conditions.

To determine instar and body size of a larva, we measured the width of the head capsule and the width of the thorax under a stereo-microscope fitted with an ocular micrometer at 25x (LIII, LIV) or 100x (LI, LII) magnification. The larvae were quickly immobilized on ice-cooled glass slides and adhering water was removed to a minimum with strips of blotting paper prior to measurements.

The width of the head capsule was read on the dorsal or ventral side by measuring the longest distance from eye to eye between their outer margins. The thorax was viewed dorsally or ventrally and its widest distance determined. For scaling physiological data, cubic values are more suitable than linear dimensions (SCHMIDT-NIELSEN, 1984). Therefore, all linear measurements were converted to millimeters and then cubed (mm^3) as an estimate of body volume, comparable to our treatment of wing length data (BRIEGEL, 1990a, 1990b).

In *Ae. aegypti* the fourth instar larvae were sexed by examination of external male genitalia. The developing gonocoxites were recognized in the 8th abdominal segment of a larva at 100x magnification. In Anophelinae this structure could not be reliably resolved.

Upon appearance of the first teneral pupae, they were collected in 2-3 h intervals until the whole population had pupated. Eclosion times of males and females were recorded in equally narrow time intervals.

RESULTS

Larval period

For six species from three genera the morphometric changes of head capsule and thorax diameter were recorded during the whole larval period: *Aedes aegypti*, *Anopheles albimanus*, and *Anopheles gambiae*, representing tropical or subtropical species, and compared with three holarctic species, representatives of temperate regions, *Aedes vexans*, *Anopheles quadrimaculatus*, and *Culex pipiens*.

The mean cubic values of the head capsule measurements in relation to the instar always increased exponentially. Therefore, the logarithms of the linear values when plotted against instar number revealed highly significant linear regressions for each species (Fig. 1; all $p<0.001$). There was never any overlap in head capsule sizes among the instars. As a consequence, based on head capsule readings, larval growth in these mosquitoes follows exactly Dyar's rule. Correspondingly, the

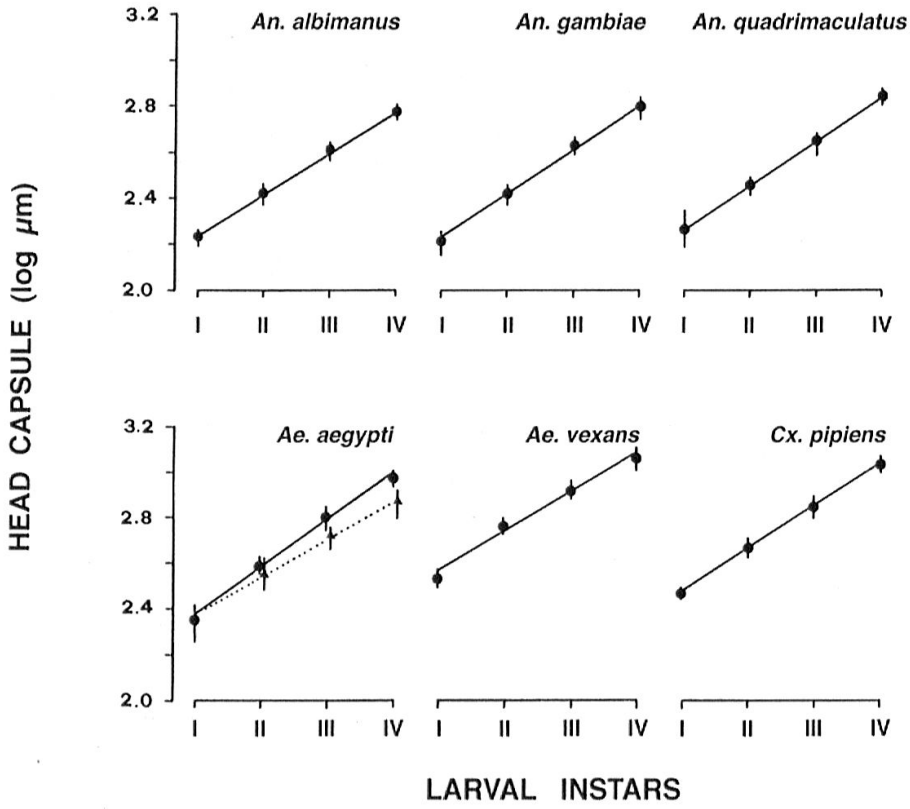

Fig. 1. Measurements of head capsule width in the four larval instars of six mosquito species. The exponential growth is presented in semilogarithmic plots of the means; vertical bars comprise the absolute range of the measurements. All the growth coefficients, i.e. the size increments from instar to instar, were between 1.06 and 1.09 for all species, thus clearly following Dyar's rule. For *Ae. aegypti* data from a crowded population are also included as a dotted line. All regression lines are significant ($p<0.001$).

postmolt size/premolt size or growth coefficients were remarkably constant, varying between 1.06 and 1.09 for all instars and all species.

The mean cubic values of the larval thorax basically show an identical growth pattern, i.e. an exponential increase from one instar to the next as revealed again by linearity in a logarithmic plot against instar number (Fig. 2). But there was a tremendous overlap among the various instars in all species. The relative growth patterns of mean thorax volumes are presented in Tab. 1, where the thorax volume of the first instar larva arbitrarily was set 1. In this way we found a remarkable similarity in relative enlargement of the thoraces among the instars and the species. By and large, the increments for each molt varied between 4-6 (*Ae. aegypti*), 2-6 (*Ae. vexans* and *Cx. pipiens*), or 3-6 (all *Anopheles*).

The thorax volumes of all species are also plotted against absolute developmental times in Figs 3 and 4. This illustrates the growth along the time axis. Figs 3 and 4 further provide the time requirements for each instar as well as for larval development until pupation at 27 °C under standardized, optimal dietary, and density conditions. The developmental period was shortest in *Ae. aegypti* (144h) and longest in *An. albimanus* or *An. quadrimaculatus* (216h). The duration of the larval period was correlated with the larval body size attained at the end of its development, i.e. in the pharate pupae for each species (Tab. 1) by dividing the latest thoracic values (mean mm^3) by the duration (hours). This revealed an average growth rate and two groups

were clearly recognized, pertaining to the two subfamilies: means of 0.011 mm^3/h for three Anophelinae versus 0.027 mm^3/h for the three Culicinae.

We have also analyzed the effects of body size of an instar on consecutive instars. Larvae of various sizes were measured shortly before and after their molts. This procedure was carried out for each molt of *Ae. aegypti* from the second instar until eclosion of the imagines. For pupae the length of the cephalothorax was taken along the ventral midline, and for imagines the wing length. Linear proportions were observed with each molt (not shown); this allows to approximately predict the size of a next instar from measurements of a given larva or pupa, but each molt and each sex revealed a different relationship.

Effect of starvation and crowding on growth

Larvae of *Ae. aegypti* growing under extreme crowding and starvation were measured and the data compared with animals under standard conditions. Plotting the thoracic volumes against their individual head capsule volumes for both rearing regimes, demonstrates the strong effect of crowding on both morphometric parameters (Fig. 5). The clustering of the head capsule readings was still characteristic for

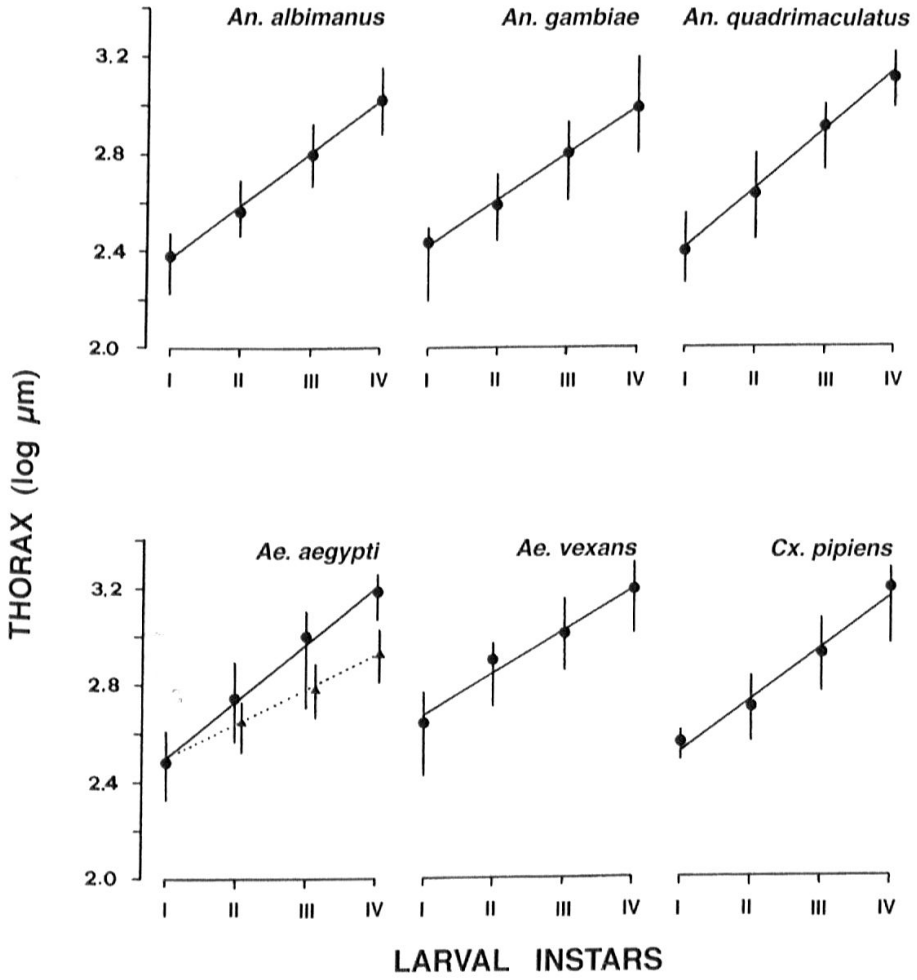

Fig. 2. Measurements of thorax diameter in the four larval instars of six mosquito species reared under standard conditions. The exponential growth curves are presented in semilogarithmic plots of the means; vertical bars cover the absolute range of measurements. For *Ae. aegypti* data from a crowded population are also included as a dotted line. All regression lines are significant ($p<0.001$).

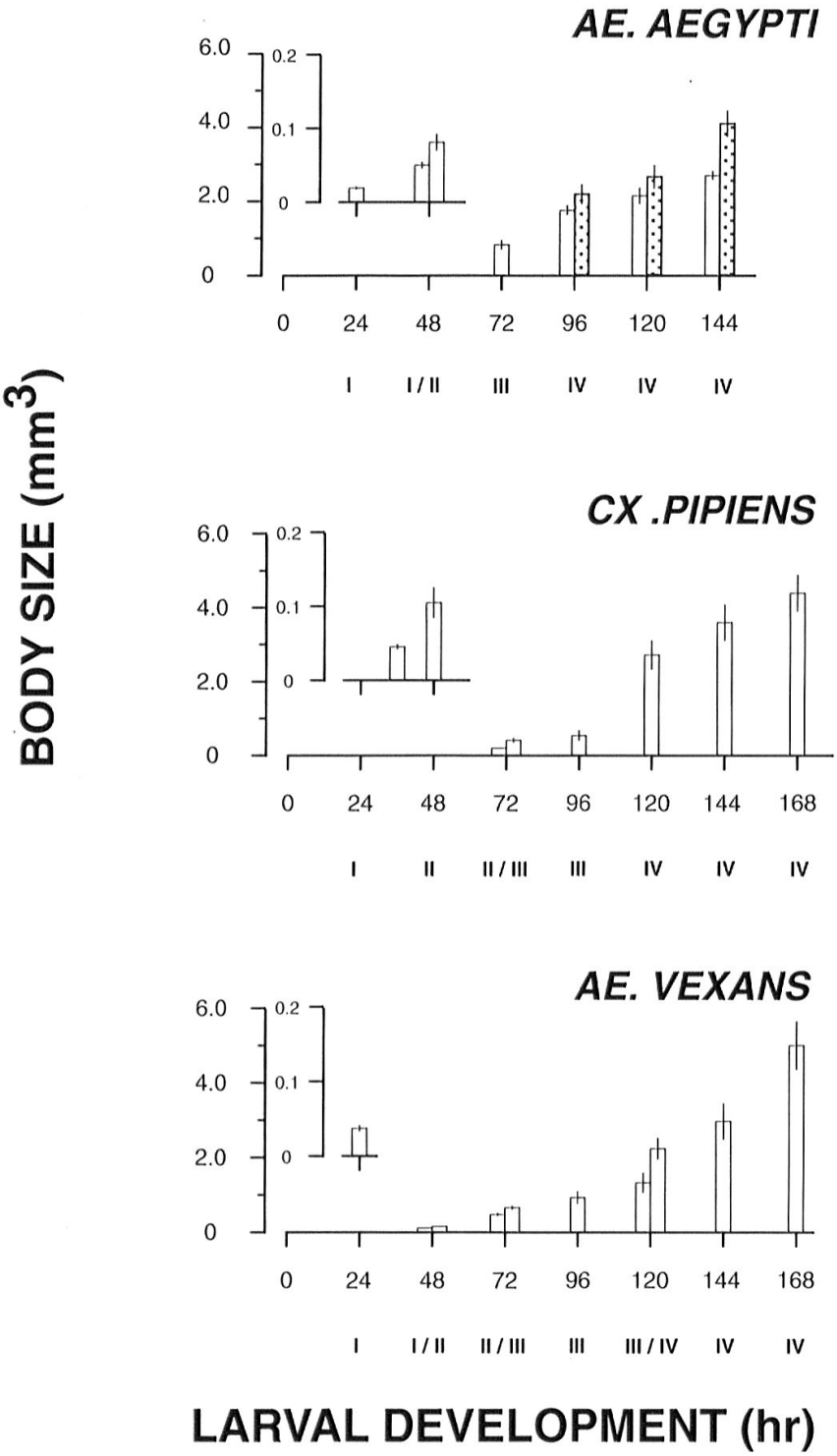

Fig. 3. Time of larval development until pupation for three culicine species and their increase in body size, given as the cubic value of thorax diameters (means±SD, N=83–415). Larval rearing followed our standard procedure at 27 °C, with the exception of *Ae. vexans* raised at 22 °C. Fourth instars of *Ae. aegypti* were sexed (stippled bars are female larvae).

each instar, while thorax volumes showed considerable overlaps. In fourth instars particularly, thorax values showed over 4-fold variations in their volumes, even among individuals of identical head capsule size (Fig. 5). Therefore, in mixed populations, segregation of the instars based on their morphometry is unreliable.

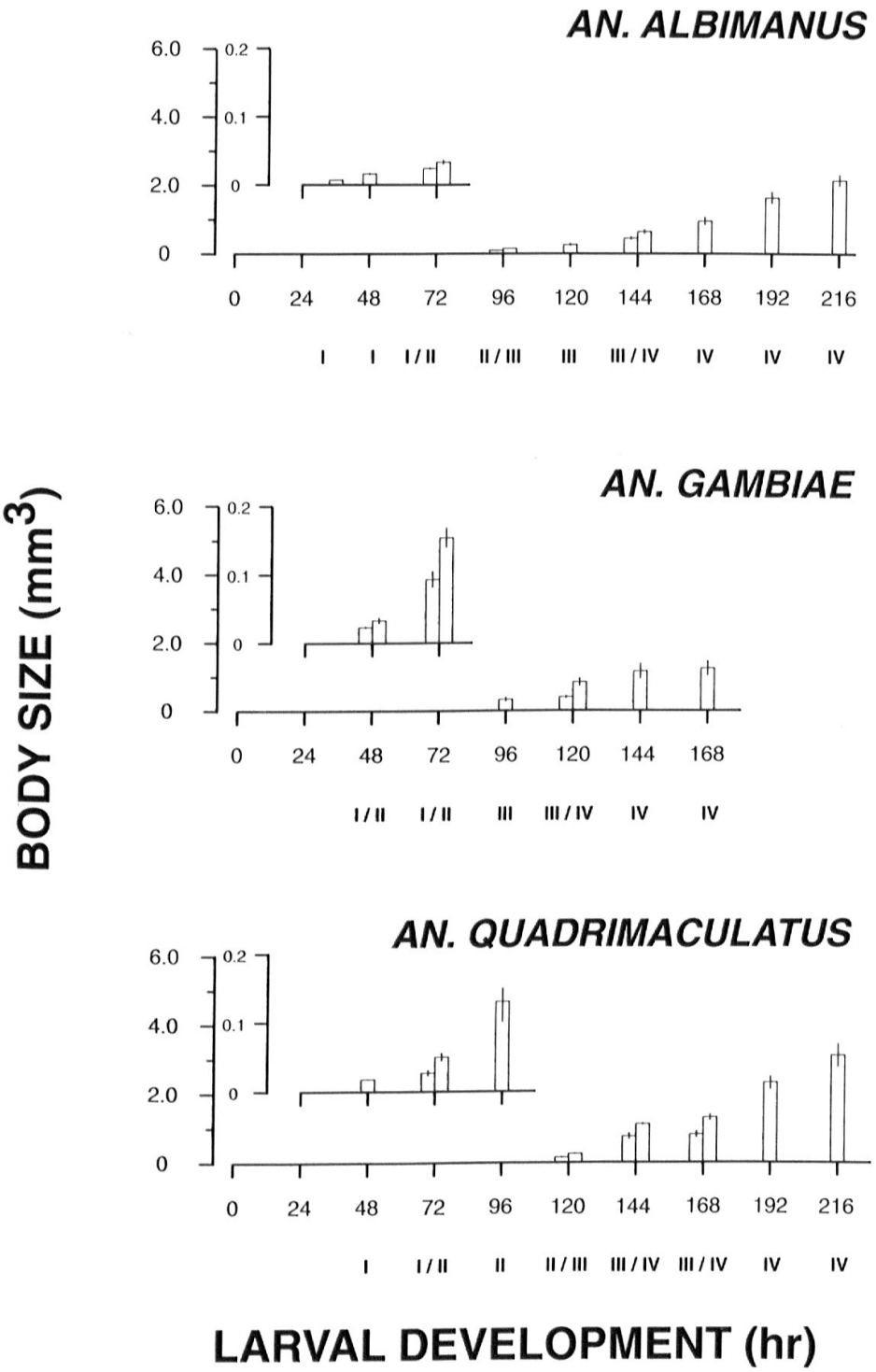

Fig. 4. Time of larval development until pupation for three *Anopheles* species and their increase in body size, given as the cubic value of thorax diameters (means ± SD, N=99–574). Larval rearing followed our standard procedures at 27 °C.

To further analyze the effect of nutritional stress on larval growth, well-fed *Ae. aegypti* were subjected to starvation for 10-19 days as soon as they molted into the fourth instar. This led to a high mortality, accompanied by translucent appearance of their abdominal tissue. Only 54 starved larvae out of 200 went through metamorphosis, but their body size was not affected, i.e. size did not regress. The exper-

Tab. 1. Relative and absolute growth rates of cubic thorax dimensions of the four larval instars (I–IV) in six mosquito species reared on standard conditions. The mean thoracic volumes of the first instars were arbitrarily set 1, and the volumes of subsequent instar larvae are given in multiples thereof, indicating an estimate of the relative growth rates. The duration of the developmental period from hatching until pupation is given in hours; together with the thorax volumes (mm^3) of the fourth instars, measured shortly before pupation, they provide the basis for the mean absolute growth rates (mm^3/h).

	relative growth				absolute growth		
	I	II	III	IV	mm^3	hr	mm^3/h
Ae. aegypti *	1	6	36	133	3.49	144	0.024
	1	3	8	22	0.55		
Ae. vexans	1	6	13	44	5.03	168	0.030
Cx. pipiens	1	3	13	80	3.50	168	0.026
An. albimanus	1	4	18	88	1.19	216	0.010
An. gambiae	1	3	14	50	0.97	168	0.008
An. quadrimaculatus	1	5	33	130	2.21	216	0.014

* Second line are data for extreme crowding conditions

imental alternative was to raise larvae under extreme crowding, and shortly after ecdysis of the fourth instar, they were boosted with surplus quantities of optimal food until pupation. The thorax was measured the day of molting to the final instar and shortly before pupation, when the abdominal tissue had turned opaque. The thorax volumes increased significantly from 0.72±0.24 (N=36) to 1.59±0.32 (N=30) in males, and from 0.79±0.25 (N=43) to 2.32±0.45 (N=39) in females (for both, $p<0.001$). These latter values are within the range of at least average size animals; obviously the deleterious effects of starvation on body size could largely be recuperated.

Pupation and eclosion

As soon as the first pupae appeared, they were sampled in narrow time intervals to trace the temporal pattern of pupation and eclosion (Figs 6, 7). In *Ae. aegypti* 50% of the larval population pupated between 03-06 hr of day 8 when reared under optimal conditions. Subsequently, 50% of the males eclosed between 0-03 hr during night 10, while 50% female eclosion was between 08-10 hr, i.e. 7-8 h later than males. The average duration for the pupal period was 50±7 h for both sexes.

In *Cx. pipiens* 50% pupation occurred between 20-22 hr on day 8 (Fig. 6). Fifty percent eclosion of males was until 08 hr on day 10, followed by the females in the evening of the same day, 20-22 hr. Since pupae were sexed the average duration of the pupal period was determined with 40±2 h for males and 38±2 h for females.

In both species, pupation and eclosion followed a sigmoid curve with no clear preference of the photophase or the scotophase, except that the first pupae always appeared during the photophase of day 7. When *Ae. aegypti* was exposed to crowding and starvation, the developmental time was extremely extended. The sigmoid pupation curve was stretched from day 15 to day 40 post hatching. The 50% pupation time was day 25 (day 8 in standard), and the 90% pupation time was day 34-35 (day 9 in standard).

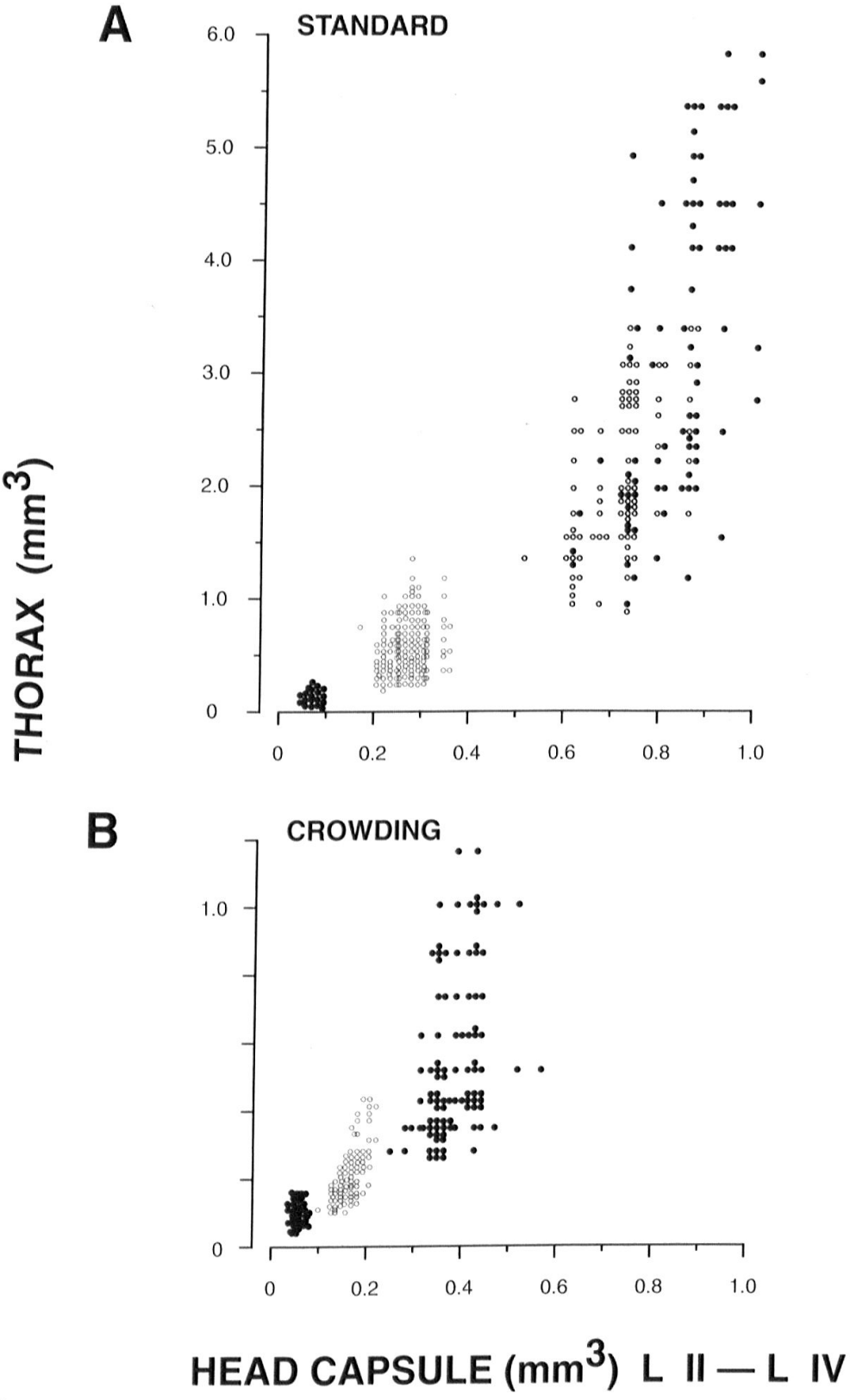

Fig. 5. Growth of larval instars of *Ae. aegypti* reared under standard (A) or crowding (B) conditions. Cubic thorax values are plotted against those of the head capsules. First instar larvae were not considered. In both graphs, the black dots clustered to the left are larvae II, the open circles in the center larvae III. Fourth instar larvae were sexed when grown under standard conditions. In A the open symbols are for male larvae IV, filled symbols for female larvae IV. To facilitate evaluation of the distributions, data points have been slightly spread around their means, particularly with L II and L III.

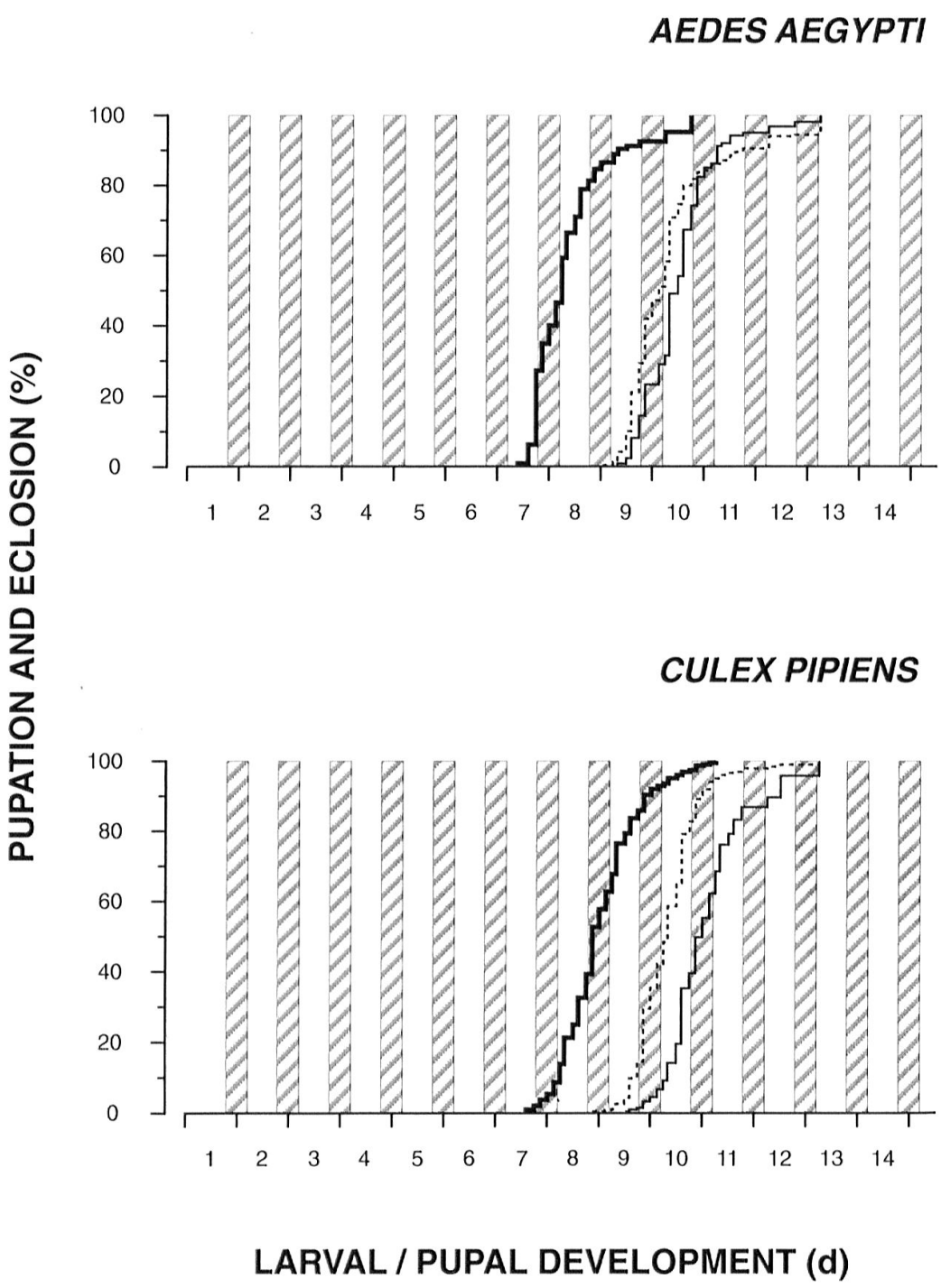

Fig. 6. Pupation and eclosion of *Ae. aegypti* and *Cx. pipiens* in relation to time (days) after hatching. Grey bars represent scotophases (7.30 pm – 5.30 am). Cumulative percentages are plotted for pupation (thick line), for eclosion of males (dotted line) and females (solid thin line).

The Anophelinae clearly differed from the Culicinae by pupating primarily during the photophases of day 9-11, while pupations during the scotophases were marginal (Fig. 7). In *An. albimanus* 22 % of the larval cohort pupated at day 10, 30 % on day 11, and 20 % on day 12. In *An. gambiae* 30 % pupated during day 9 and 52 % during day 10; in *An. stephensi* 24 % pupated at day 9 and 70 % at day 10. In contrast, total pupation during scotophases was only 28 % in *An. albimanus*, 18 % in *An. gambiae*, and 6 % in *An. stephensi*.

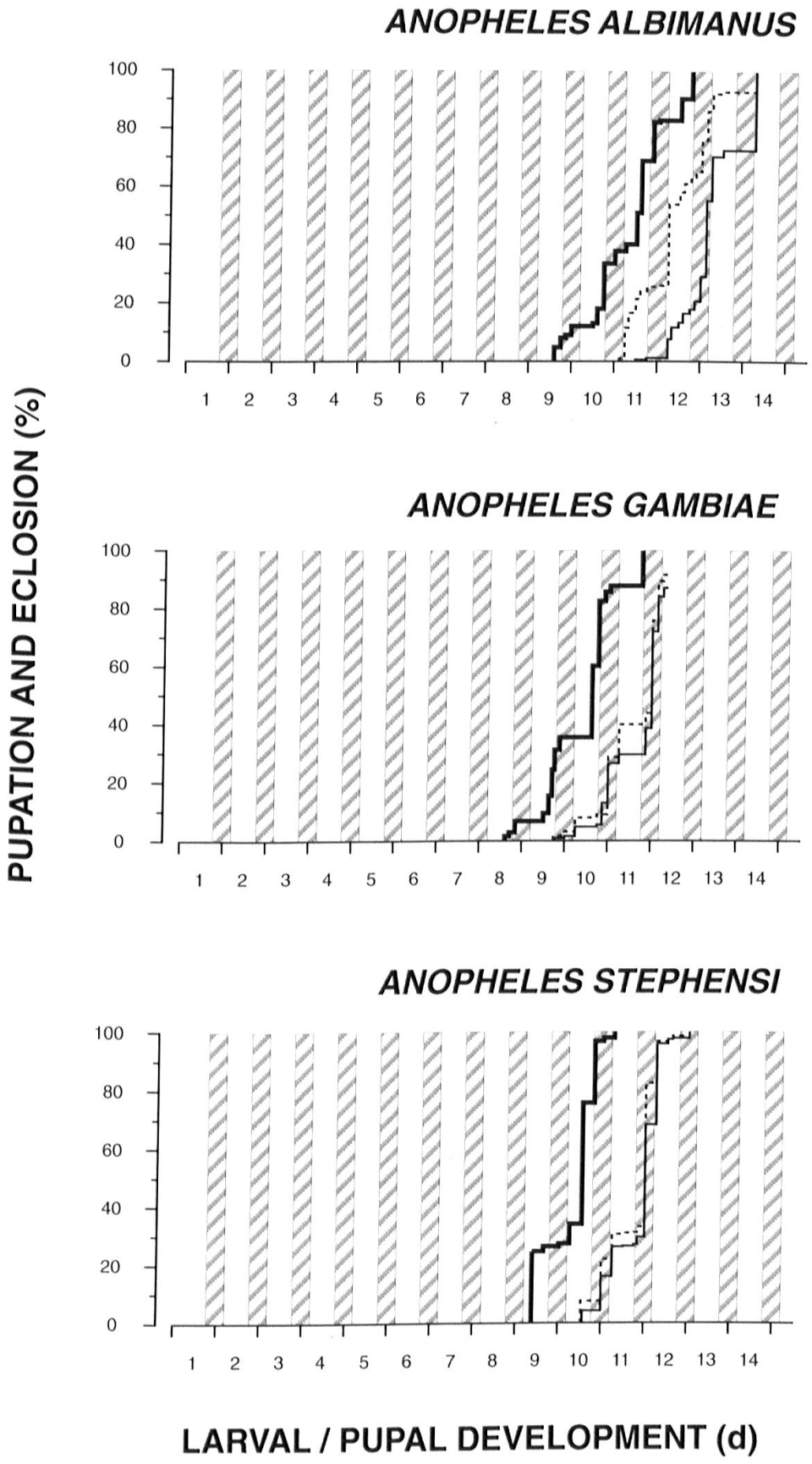

Fig. 7. Pupation and eclosion of *An. albimanus*, *An. gambiae*, and *An. stephensi* in relation to time after hatching (days). Symbols as in Fig. 6.

The 50% pupation time for the whole population of *An. albimanus* was 09–12 hr on day 11, and for *An. gambiae* and *An. stephensi* 12-16 hr on day 10. Since the pupae were not sexed in these experiments, the duration of the pupal period showed considerable variability: 40±10 h for *An. albimanus* and for *An. gambiae* and *An. stephensi* it was 32±4 h. As will be shown below, in *An. albimanus* males eclose one night before the females, while in the other two species they eclose synchronously. This probably is the reason for the broader variability of pupal times in *An. albimanus*.

Eclosion was confined to the scotophases in all three *Anopheles* species and in both sexes (Fig. 7). In *An. albimanus* 28% of males eclosed during night 11 and 26% during night 12, while females eclosed mainly during night 12 (38%). In *An. gambiae* eclosion of both sexes was observed predominantly at nights 11 and 12 (32% males, 25% females). In *An. stephensi* the majority of both sexes eclosed synchronously during night 11 (64% males, 66% females).

At any rate, the Anophelinae showed definitely shorter pupation periods than *Ae. aegypti*, whereas *Cx. pipiens* with its longer pupal period resembled more the *Aedes*.

DISCUSSION

DYAR (1890) was the first to provide a morphometric description of larval instars. Working with Lepidoptera, he recognized that with each molt increasingly larger head capsules appeared. The ratio of increase was said to be constant and species-specific and this statement is referred to as Dyar's rule in the literature (WILLIAMS, 1980; NIJHOUT, 1994).

In our experiments mean head capsule values consistently followed exponential curves, and the growth coefficients were constant in Culicinae and in Anophelinae, as opposed to data reviewed by CLEMENTS (1992). Based on our results Dyar's rule is applicable to mosquito larvae with a generalized growth coefficient of approximately 1.1 for each species and instar. Consequently, in mosquitoes head capsule measurements can indeed be useful for reliable identification of larval instars, because there were no overlaps between successive instars as long as reared under optimal conditions. Under field conditions however, this is not applicable because under suboptimal conditions the head capsule measurements revealed no distinct categories among the different instars. Similar limitations were also reported for *Manduca* (SAFRANEK & WILLIAMS, 1984).

The growth of the thorax diameter was also exponential, but with tremendous overlap between the instars, even under optimal conditions. This is in clear contrast to the sclerotized structures, reflecting a continuous growth pattern throughout every instar. Therefore, thorax dimensions provide an acceptable estimate of growth of biomass, i.e. biosynthesis, whereas the sclerotized exoskeleton provides an estimate of the instar.

GHENT (1956) did a critical study of Dyar's rule in sawfly species, and he concluded that it was more of a descriptive value rather than a law. With respect to mosquitoes, DE OLIVEIRA & DURAND (1978) and DESLONGCHAMPS & TOURNEUR (1980) also found exponential growth in two species of *Culex*, while LARDEUX & TETUANUI (1995) presented linear regressions for head capsule growth in two *Aedes* species. Similar to our results, ABDEL-MALEK & GOULDING Jr. (1948) for *Ae. aegypti* and JONES (1953) for *An. quadrimaculatus* reported exponential growth.

Under optimal growth conditions, most insects undergo a species-specific number of larval molts and metamorphose into imagines of a species-specific size.

Under continuously suboptimal conditions, such as crowding and starving, the larval period was prolonged considerably and the body size of larvae, pupae, and eclosing imagines is strongly reduced (TIMMERMANN & BRIEGEL, 1993; 1996). In this study, this was pushed to the extreme for *Ae. aegypti*, where pupation occurred 3- to 4-times later than with standard conditions. Continuous starvation affected the size and larval survival and caused the appearance of a translucent body tissue but there was never an additional molt. Well-fed larvae, exposed to complete starvation only during their final fourth instar could wait for 10–19 days, either they died or a small number metamorphosed. Conversely, larvae starved during the first three instars could be rescued by optimal feeding during their fourth and last instar, and finally eclosed as imagines of nearly normal size. Obviously the last instar is the most important phagoperiod, and as RIDDIFORD (1996) stated for higher Diptera, the number of larval instars is determinate and cannot be changed by humoral or nutritional alterations, contrary to Lepidoptera (NIJHOUT, 1975). In view of the fixed number of molts, the mosquito's flexibility along the temporal axis of the larval period might be of adaptive value. The duration of the pupal period on the other hand, never differed significantly.

Studies on the endocrine regulation of molting in *Manduca* revealed a critical body size that is required for initiating metamorphosis, i.e. a pupal molt (NIJHOUT, 1975). By manipulating the dietary conditions of larvae, supernumerary larval molts could be induced before pupation occurred (NIJHOUT & WILLIAMS, 1974). This result is in sharp contrast to our mosquito data. Obviously, there have evolved quite different endocrine regulations for the larval-pupal transition between Lepidoptera and nematoceran Diptera, in the latter largely independent of body size.

The temporal distribution of pupation and eclosion of the three *Anopheles* species studied (Fig. 7), followed dial rhythms, confirming results of GOMA (1959) and COLUZZI (1972) for *An. gambiae* and *An. stephensi*. A gating mechanism in the endocrine system has to be assumed, comparable to *Manduca* or other insects (TRUMAN & RIDDIFORD, 1974). Interestingly, pupations occurred mainly in the photophases while eclosion prevailed in the scotophases. This might be of adaptive value in reducing predation by water bugs or spiders.

Culicinae however, pupated and eclosed continuously (Fig. 6), supporting experiments with *Ae. aegypti* by HADDOW *et al.* (1959) or NAYAR & SAUERMAN (1970). It is surprising that in *Ae. aegypti* and some other East African species molting occurs continuously, while later in their life circadian patterns prevail, such as blood feeding or oviposition (GILLETT, 1971). This can be explained in behavioral terms: approaching a host for blood meal is always dangerous and circadian patterns undoubtedly are of advantage for avoiding defense behavior of the host. This equally applies for the usually nocturnal host-seeking by *Anopheles*.

The fact that eclosion is gated in *Anopheles* but not in *Aedes* and *Culex* might reflect basic ecological differences between these two groups. *Ae. aegypti* or *Cx. pipiens* often breed in biotopes associated with human settlements, coinciding with the habitats of their future blood donors. Therefore, a circadian timing of blood feeding seems more important for avoiding host-defense reactions than a timing of eclosion. In contrast, *Anopheles* larvae often breed in pools away from human dwellings and therefore, female *Anopheles* might require long-distance migrations in search of blood donors for which darkness appears to be preferred by these tropical species for climatic reasons.

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