CORRELATION OF NUTRITIONAL RESERVES WITH A CRITICAL WEIGHT FOR PUPATION IN LARVAL *AEDES AEGYPTI* MOSQUITOES

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ABSTRACT. The relationships between the readiness of fourth instar *Aedes aegypti* to metamorphose and their weight, nutritional reserves and sex were examined. Both the critical larval weight required for metamorphosis and the potential weight were influenced by the temperature at which the larvae were reared, and were greater for females than for males. Caloric levels of soluble carbohydrates and soluble lipids correlated strongly with weight. The accumulations of both carbohydrates and lipids were sex-dependent and were increased in both sexes at the higher temperature, but caloric levels of glycogen at the critical weights were all similar. We suggest that weight *per se* is not a critical factor in determining readiness to pupate, and that carbohydrate levels may be one important causal variable.

INTRODUCTION

In several species of insects, a minimum or critical weight must be achieved during the larval stage in order for development to continue (Nijhout and Williams 1974a, Nijhout 1975, Lounibos 1979, Safranek and Williams 1984). Larvae that are starved after reaching their critical weight generally will metamorphose, but if they are starved before they reach this weight, they eventually die without initiating metamorphosis. These critical weights may be considerably less than the weights that are attained when the larvae are provided with an excess of food. The physiological mechanisms by which larvae are able to monitor their critical weight have never been determined, although stretch receptors that evaluate size increases have been suggested (Nijhout 1979), and *Manduca sexta* (Linn.) larvae that are starved during early instars will undergo supernumerary larval molts if their head capsules are small (Nijhout 1975).

Within the limits of tolerance, the rate at which mosquito larvae develop increases with temperature (Bar-Zeev 1958, Nayar 1968), whereas the resulting size of the adult varies inversely with temperature (van den Heuvel 1963, Nayar 1969, Hien 1975). We observed that the timing of metamorphosis by larval *Aedes aegypti* (Linn.) mosquitoes is influenced both by the availability of adequate food and by temperature. It appeared, therefore, that the ability of these larvae to undergo metamorphosis might be related to a temperature-dependent accumulation of a minimum level of nutritional reserves, which would be manifested in a critical weight. In this study, we examined the relationships between nutritional reserves, critical weight and metamorphosis in female and male *Ae. aegypti* larvae by manipulating rearing temperatures.

MATERIALS AND METHODS

Rearing and weight measurement: Mosquitoes from a laboratory colony of *Ae. aegypti*, Segemaganga strain, were used. The pupal molt of *Ae. aegypti* does not show a diel periodicity (Haddow et al. 1959, Nayar and Sauerman 1970a), but occurs following a fairly definite, temperature dependent interval measured from the hatching time of the eggs. This eliminated the complication of a circadian gate in determining requirements for pupation in our experiments. Larvae were reared in shallow, 30 × 21 × 5.5 cm pans at a density of 200 in 450-ml water, at 22°C or 32°C (±1°C). They were fed a diet consisting of pulverized rat chow, brewer's yeast and lactalbumin hydrolysate (1:1:1 by weight) according to the schedule shown in Table 1, which provided nourishment in excess of that required for maximal growth. The larvae began to pupate on the day following the last feeding. Under these conditions the larvae achieved the maximum weights attainable at either of the 2 temperatures. Sex was determined by examining the larvae for the presence or absence of developing male gonocoxites and ejaculatory ducts within the anal segment (Christophers 1960). For weighing, fourth instar larvae were rinsed in clean water to remove the rearing medium, transferred individually in a drop of water to a weighing pan, and blotted dry. Their weight was determined to an accuracy of 1 μg using a Cahn G-2 electronic balance (Ventron Instruments Corporation, Paramount, CA). The larvae then were floated off the weighing pan into individual 30-ml cups containing 15-ml of clean water.

Determination of critical weight: The critical weight was defined for this study as the weight at which 50% of the larvae pupated when they were starved after being weighed. As controls, every fourth larva was fed. The remaining larvae were starved until day 4 (22°C) or day 6 (32°C) following the pupation of the last control larva in each group. These cutoff dates, after which no further pupation would occur, were determined earlier using larvae that were starved until they either pupated or died. To minimize microbial growth on fecal material as a source

Table 1. Larval feeding schedules for the 2 rearing temperatures (mg diet/cohort/day).

Day	22°C	32°C
1	40	50
2	70	150
3	70	400
4	250	500
5	250	
6	250	
7	250	

of nourishment, starved larvae were transferred daily to clean cups containing clean water. Control larvae similarly were drawn into a pipette, but were released back into the same medium. Dead larvae were examined for initiation of metamorphosis, as evidenced by the development of pupal characters visible through the larval cuticle. Larvae that pupated before the cutoff date were scored as positive for pupation. Those remaining were scored as negative. The probability of pupation as a function of larval weight, and 95% confidence intervals for the critical weight, were calculated using probit analysis (Statistical Analysis System, SAS Institute Inc., Cary, NC). The confidence intervals were used to determine the significance ($P < 0.05$) of the critical weights. Goodness of fit was determined by the chi-square test.

Determination of potential weight: In contrast to the critical weight, which indicates the minimum amount of growth required for pupation, the potential weight is a measure of the maximum growth that may be achieved when food is not limiting. Larvae that were well-nourished throughout the larval stage were used. Because the larvae continued to feed into the late prepupal stage, those prepupae with well-developed respiratory trumpets and float setae, visible through the larval cuticle, were selected for weighing. These advanced prepupae were presumed to have achieved their potential larval weight. Statistical differences between mean weights were determined by the *t*-test.

Determination of nutritional reserves: Fourth instar larvae were individually weighed and homogenized. Soluble carbohydrates were extracted in hot 2% Na_2SO_4 and were separated into sugar and glycogen fractions using methods adapted from Van Handel (1965a, 1965b, 1985a). Glycogen was precipitated twice with 100% ethanol. Following centrifugation, the supernatants from the 2 separations, containing the dissolved sugars, were decanted into 1 test tube. The glycogen precipitate was redissolved in 0.6-ml H_2O, and after centrifugation a 0.5-ml aliquot was transferred to another test tube, leaving behind cuticular and other solid components. The 2 carbohydrate fractions were measured colorimetrically using the anthrone reaction (Van Handel 1985a). Soluble lipids were extracted twice in chloroform-methanol (1:1) and measured colorimetrically (sulfophospho-vanillin reaction) using the method of Van Handel (1985b). The extractions included components from the gut contents, but analysis showed these comprised less than 10% of the respective fractions. Because only a small portion of the protein component consists of nonstructural energy reserves, proteins were not measured. The values obtained for carbohydrates and lipids were converted to calories and regressed on live weight. The slopes of the regression lines were compared using the *t*-test. The statistical significance between the caloric levels of reserves in experimental groups was determined using 95% confidence intervals ($P < 0.05$).

RESULTS

Relationship between temperature and critical weight: The critical weight is a measure of the minimum amount of growth required before larvae can undergo metamorphosis. Larvae that have exceeded this minimum requirement are able to complete development even when they are subsequently starved, although the resulting pupae and adults may be much smaller than if adequate food were available throughout the larval feeding period. All of the control larvae pupated. Figure 1 shows the percentages of starved larvae that pupated as a function of their weights at the onset of starvation. The chi-square test indicated a good fit for all probit curves ($P > 0.10$). The weight at which 50% of the larvae pupated (0.5 probability of pupation) represents the critical weight (Table 2). Critical weights for male larvae were significantly lower ($P < 0.05$) than for female larvae at each temperature, and the critical weights for females and males at 32°C were, respectively, significantly lower ($P < 0.05$) than for females and males at 22°C. The difference between the critical weights for females and males was less at the higher temperature, suggesting that there was a greater temperature dependency among females as compared with males.

Relationship between temperature and potential weight: The ultimate size of the adult mosquito is dependent upon the amount of larval growth in excess of that represented by the critical weight. The potential weight is a measure of the upper limit for larval growth. The potential weights of both females and males were greater at 22°C (Table 2); and, as with the critical weight, the temperature effect was apparently greater among females. The potential weights for males were only 61–67% those of females. The critical weights for females were

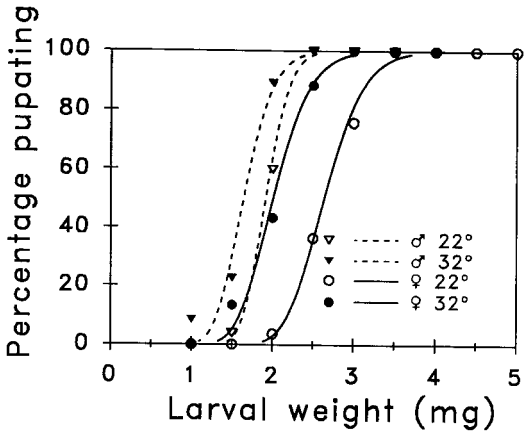

Fig. 1. Percentages of starved female and male *Aedes aegypti* larvae undergoing metamorphosis as a function of their weights at the onset of starvation. Curves were generated from prediction values obtained using probit analysis (chi-square test for goodness of fit: $P > 0.10$ for all curves). Critical weights and sample sizes for each curve are shown in Table 2.

Table 2. Effect of rearing temperature on the critical weights for metamorphosis and potential larval weight.

Temp. (°C)	Critical weights mg ± 95% CL (*n*)	Potential weights mg ± SEM (*n*)
Females		
22	2.63 ± 0.13 (121)	4.80 ± 0.07 (34)
32	1.99 ± 0.13* (191)	3.93 ± 0.04 (58)
Males		
22	1.91 ± 0.09* (113)	2.94 ± 0.03 (53)
32	1.66 ± 0.10 (183)	2.62 ± 0.03 (53)

* Critical weights followed by an asterisk did not differ significantly ($P > 0.05$).

All means for potential weight were significantly different ($P < 0.005$).

51–55%, and for males 63–65%, of their respective potential weights.

Relationship between nutritional reserves and weight: Both the critical weight and the potential weight were influenced by temperature. To ascertain whether there was a relationship between nutritional status and weight that might be similarly temperature dependent, the caloric levels of soluble carbohydrates and soluble lipids of fourth instar larvae were determined and regressed on body weight. Because their relationships to weight were curvilinear, the values for lipids first were transformed to their $\log_{10}$ values. The caloric levels for carbohydrates, measured separately as glycogen (Fig. 2) and sugars (Fig. 3), showed strong positive linear correlations with body weight. Lipids, which comprised 74–88% of the caloric reserves, showed strong positive correlations between caloric levels ($\log_{10}$) and body weight (Fig. 4).

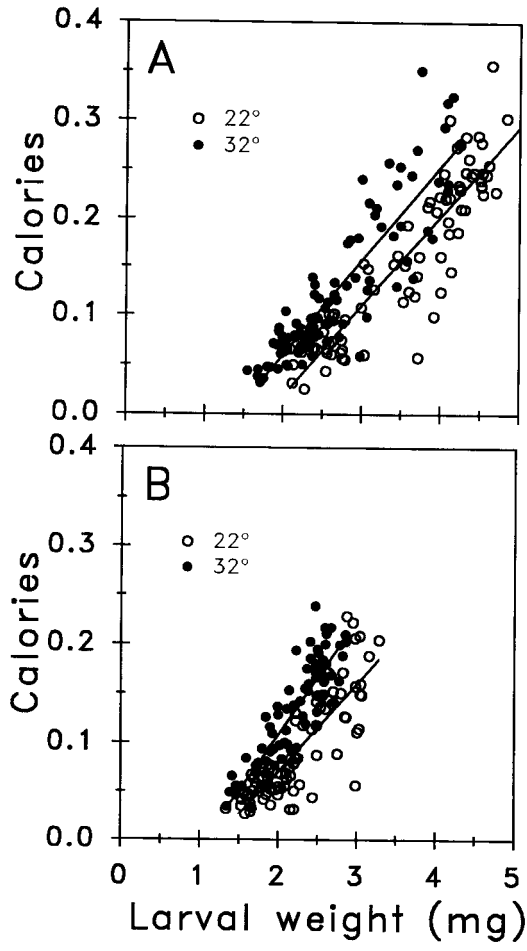

Fig. 2. Caloric values of glycogen as a function of body weight in *Aedes aegypti*. Regressions for females (A): 22°C, $y = -0.16589 + 0.09203x$, $r = 0.91$, $n = 81$; 32°, $y = -0.13342 + 0.09675x$, $r = 0.89$, $n = 93$. Regressions for males (B): 22°C, $y = -0.10720 + 0.08886x$, $r = 0.83$, $n = 81$; 32°C, $y = -0.12237 + 0.11511x$, $r = 0.88$, $n = 80$. All regressions were significant ($P < 0.005$).

Differences between the slopes of the regression lines indicated that temperature influenced the rate at which storage nutrients were accumulated. Confidence intervals showed that their levels relative to body weight were significantly higher in males than in females ($P < 0.05$). During the fourth instar, glycogen (Fig. 2) increased at higher rates in males at 32°C than at 22°C ($P < 0.005$). Glycogen levels in early fourth instar females were higher relative to weight at 32°C than at 22°C ($P < 0.005$), differences that were maintained throughout the fourth instar by similar rates of accumulation ($P > 0.10$) at the 2 temperatures. Sugar caloric levels (Fig. 3) increased at higher rates in relation to weight in

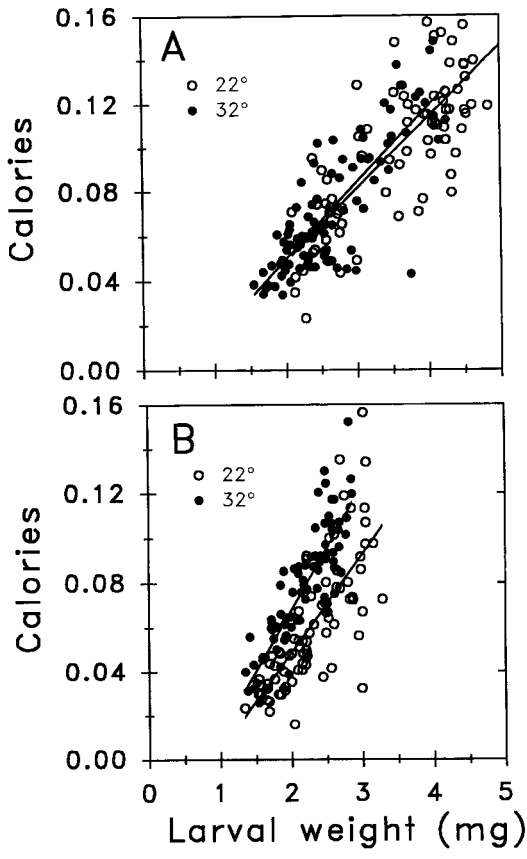

Fig. 3. Caloric values of sugars as a function of body weight in *Aedes aegypti*. Regressions for females (A): 22°C, y = −0.01623 + 0.03251x, $r = 0.79$, $n = 82$; 32°C, y = −0.01962 + 0.03461x, $r = 0.83$, $n = 93$. Regressions for males (B): 22°C, y = −0.03954 + 0.04411x, $r = 0.75$, $n = 80$; 32°C, y = −0.04319 + 0.05550x, $r = 0.84$, $n = 80$. All regressions were significant ($P < 0.0005$).

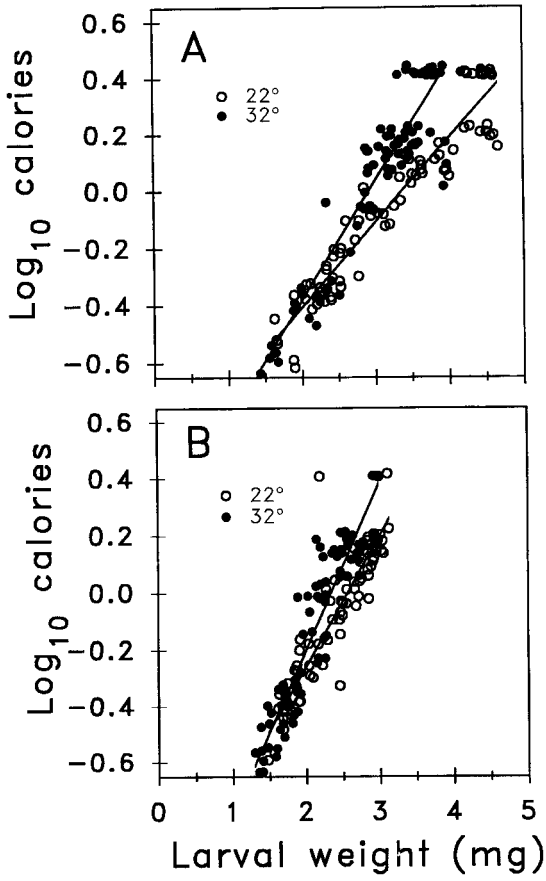

Fig. 4. Caloric values ($\log_{10}$) of soluble lipids as a function of body weight in *Aedes aegypti*. Regressions for females (A): 22°C, y = −1.00113 + 0.29674x, $r = 0.95$, $n = 79$; 32°C, y = −1.21533 + 0.41647x, $r = 0.93$, $n = 86$. Regressions for males (B): 22°C, y = −1.17144 + 0.45694x, $r = 0.91$, $n = 78$; 32°, y = −1.36796 + 0.58413x, $r = 0.94$, $n = 83$. All regressions were significant ($P < 0.005$).

males than in females at the respective temperatures ($P < 0.005$) and at higher rates in males at 32°C than at 22°C ($P < 0.005$). Both the caloric levels and rates of accumulation of sugars were the same in females at the two temperatures ($P > 0.10$). Lipid caloric levels (Fig. 4) increased at higher rates ($P < 0.005$) in relation to weight at 32°C than at 22°C in both females and males, and at higher rates ($P < 0.005$) in males than in females at the respective temperatures.

Both carbohydrates and lipids increased as a percentage of total body weight during the last instar. When percentages-by-weight of glycogen, sugars or lipids were regressed on larval weight, the slopes of all regressions were significant (% glycogen and % lipids, $P < 0.005$; % sugars, $P < 0.025$).

Correlation between nutritional reserves and pupation: Because nutritional status and pupation were both related to weight, the above data were examined for a possible relationship between nutritional reserves and the ability of larvae to pupate. Using the regression equations, the caloric levels of storage nutrients at the critical weights were estimated (Table 3); the estimates for lipids were transformed to their antilog values. Although the relationship between weight and nutrient accumulation varied with temperature, the estimated caloric levels of glycogen at the critical weights for both females and males at the 2 temperatures were not significantly different. There was no consistent statistical correlation between the levels of the other 2 nutrient components at the critical weights. In comparison, confidence intervals for the critical weight were lower for males than for females, and were lower for both females and

Table 3. Estimated caloric values of nonprotein reserves (±95% confidence limits) at the critical weights.

°C	Glycogen	Sugars	Lipids
Females			
22	0.08 ± 0.01*	0.07 ± 0.01	0.61 ± 0.07
32	0.06 ± 0.01*	0.05 ± 0.01*	0.42 ± 0.04*
Males			
22	0.06 ± 0.01*	0.04 ± 0.01*	0.50 ± 0.04
32	0.07 ± 0.01*	0.05 ± 0.01*	0.40 ± 0.03*

* Caloric values within a column followed by an asterisk were not significantly different ($P > 0.05$).

males, respectively, at 32°C than at 22°C (Table 2). These relationships suggest that weight, *per se*, is not a critical factor in pupation, and that the levels of carbohydrate reserves, primarily in the form of glycogen, may be instrumental in determining the readiness of larvae to initiate metamorphosis.

DISCUSSION

Larval *Ae. aegypti* mosquitoes must achieve a critical weight before they can undergo metamorphosis. This critical weight was higher for female larvae than for males, and both female and male larvae reared at the higher temperature initiated metamorphosis at lower weights, with the temperature-dependent decrease in the critical weight being greater among females.

In addition to its influence on the critical weight, temperature also affected the proportion of body weight attributable to storage nutrients. Carbohydrate and lipid reserves constituted a greater proportion of the total live weight of male larvae as compared with females. In both females and males, this proportion was increased at the higher temperature. The one common factor in both female and male larvae at the 2 temperatures was the level of glycogen reserves at the critical weights. Although an inherent aspect of correlation is the difficulty in distinguishing between cause and effect, one possible hypothesis for the difference in the critical weights is that male larvae and the larvae reared at the higher temperature have accumulated a critical level of glycogen at a lower weight, and therefore are able to initiate metamorphosis at a reduced body size.

A possible regulatory role for carbohydrate in insect development is suggested by an inverse relation between hemolymph trehalose levels and juvenile hormone (JH) titers (Jones et al. 1981). According to a model proposed by Bollenbacher (1988) for *M. sexta*, a drop in the JH titer triggers the cascade of endocrine events involved in the initiation of metamorphosis, in particular the release of prothoracicotrophic hormone (PTTH) (Nijhout and Williams 1974b, Rountree and Bollenbacher 1986, Kikukawa and Tobe 1986) and its subsequent stimulation of the prothoracic glands to produce ecdysone. At the same time, with the absence of JH the responsiveness of the prothoracic glands is irreversibly altered, rendering them competent to respond to PTTH (Watson et al. 1987, Watson and Bollenbacher 1988). The attainment of sufficient caloric reserves, with the concomitant increase of certain carbohydrate components such as hemolymph trehalose to critical levels, might initiate the processes by which the JH titer is reduced, resulting in a state of developmental competence to undergo metamorphosis that is not reversed by subsequent starvation. This hypothesis is further supported by the earlier pupation and smaller size of male *Ae. aegypti* as compared with females: the earlier ecdysteroid peak in male *Ae. aegypti* during pupal-adult development (Whisenston et al. 1989) may be due to the sex-related difference in larval nutrient metabolism seen here, and similar to that suggested by Brust (1967) for *Aedes vexans* (Meigen) and *Culiseta inornata* (Williston). Because of the strong correlations of carbohydrate and lipid caloric levels with body weight, the critical weight might be regarded as one easily measurable indicator of the attainment of developmental competence. The requirement for a minimum head capsule size before metamorphosis can occur in *M. sexta* larva (Nijhout 1975) may be rationalized similarly as a reflection of nutritional status, because the size of a larval insect's head capsule is directly related to the weight the larva attains during the previous instar (Beck 1950, Nijhout 1975).

The decision to pupate occurred early in the last instar, considerably before the mosquito larvae had reached their potential weight. This early decision represents a probable trade-off between maximal caloric attainment and the achievement of reproductive maturity. The small female mosquitoes resulting from less than optimal conditions of larval density and nutrition (Terzian and Stahler 1949, Nayar and Sauerman 1970b) produce fewer eggs per reproductive cycle (Barlow 1955, Bar-Zeev 1957, Steinwascher 1982, Hawley 1985). It would be to the insect's advantage, therefore, to maximize its caloric gain. However, when this is prevented by competition for resources, the ability of larvae to metamorphose at lesser weights would enable at least some individuals to mature.

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