The Journal of Experimental Biology 210, 854-864
Published by The Company of Biologists 2007
doi:10.1242/jeb.02715

Larval feeding duration affects ecdysteroid levels and nutritional reserves regulating pupal commitment in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae)

Aparna Telang*, Laura Frame and Mark R. Brown

Department of Entomology and Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA 30602, USA

*Author for correspondence at present address: University of Richmond, Department of Biology, Gottwald Science Center, Richmond, VA 23173, USA (e-mail: atelang@richmond.edu)

Accepted 11 January 2007

Summary

What little is known about the endocrine regulation of mosquito development suggests that models based on Lepidoptera and *Drosophila* may not apply. We report on basic parameters of larval development and the commitment to metamorphosis in the yellow fever mosquito *Aedes aegypti* that are affected by varying the length of feeding time for last instar larvae. A critical mass for pupal commitment was achieved after 24 h of feeding by last instars, also the age at which tissue production and hemolymph titers of ecdysteroids are increasing. A greater proportion of last instars successfully pupated and eclosed as adults as the length of their feeding time increased. Less than 24 h of feeding time resulted in last instars that were developmentally arrested; these larvae tolerated starvation conditions for up to 2 weeks and retained the capacity to pupate if re-fed. Starvation tolerance may be a common trait among container-inhabiting species, and this period is an important factor to be considered for vectorial capacity and control measures. To distinguish cues for metamorphosis related to a larva's nutritional status *versus* its age, newly molted last instars were fed for different periods of time but sampled at the same age; ecdysteroid levels, body mass and nutrient reserves were then measured for each group. Our data suggest that metamorphic capacity is dependent on a larva's nutritional condition and not just the age at which ecdysteroid titers increase. Last instars that have fed for a particular length of time may initiate their metamorphic molt when both threshold levels of nutrient reserves and ecdysteroid titer have been met. Future studies will lead to a conceptual model specific for the nutritional and hormonal regulation of mosquito post-embryonic development. This model should facilitate the exploitation of current and novel insect growth regulators that are among favored strategies for vector population suppression.

Key words: larval nutrition, larval-pupal molt, metamorphosis, development, hormone.

Introduction

Insect larval nutrition is important in provisioning growth and development, and hormones play a major role in regulating these processes. Post-embryonic development of larval insects consists of periods of growth that stop when the larva molts, and growth finally ceases at the metamorphic molt to the pupal (in holometabola) or adult (in hemimetabola) stage. Both endocrine and non-endocrine physiological processes control the onset of insect metamorphosis.

Data on the endocrine and non-endocrine mechanisms underlying insect metamorphosis have been collected mainly from lepidopteran species, especially the tobacco hornworm *Manduca sexta* and the silkmoth *Bombyx mori*, and these data have come to form a central dogma of insect endocrinology (Nijhout, 1994): larvae grow to a certain mass before molting to the next larval stage, and a critical mass must be attained prior to committing to a metamorphic molt. Both the larval and metamorphic molts are mediated by the release of a neuropeptide from the larval brain, now considered to be prothoracicotropic hormone (PTTH), which then stimulates the prothoracic glands (PG) to synthesize and release ecdysteroids that cause the larva to stop feeding and commit to pupation. However, the metamorphic molt only occurs when ecdysteroids are produced in the absence of juvenile hormone (JH) during a critical period in the last larval stage. If JH is present during the critical period, the insect molts into another larval stage and retains larval characteristics. In the absence of JH, the larva commits to its first metamorphic molt into its pupal stage.

Using model insects such as *M. sexta* and *Drosophila melanogaster*, progress has lead to an understand of the neural events involved in the release of PTTH and the biochemical

events of PTTH signal transduction (Rybczynski, 2005). Likewise, the entire ecdysteroid biosynthetic pathway is close to being characterized in larval insects (Gilbert, 2004; Gilbert et al., 2002). Unfortunately, little more is known about what other than the 'critical mass' triggers the metamorphic molt. Nijhout stated that the '*critical size is merely a symptom of the underlying physiology and as such provides no information on what factors are of importance to the animal in question, nor is the critical size straightforwardly related to the size at which the molt will ultimately occur*' (Nijhout, 1981). So, what is the underlying physiological mechanism(s) and what are the factors an animal uses to make molting decisions? Physical mass may be a general parameter for the larva, but some correlate of mass, like nutrient reserves, is surely relevant.

Past and recent studies implicate larval feeding and nutrient intake as factors to cue metamorphic readiness. For example, nutrient intake and accumulation of mass is important for two phases during the last larval instar of *M. sexta*. In the first phase, nutritional input and growth allow a larva to reach a critical mass for metamorphic commitment (Nijhout, 2003). In the second phase, after reaching its critical mass, additional larval growth occurs only if food is available. If nutrient intake is reduced and the larva grows only a little after meeting its critical mass, it will metamorphose at a smaller size and subsequently form a smaller sized adult. This second larval growth phase has major consequences for the individual during its adult stage because a larger adult body size, especially for females, is often correlated with greater reproductive success (Bronson, 1985; Iyengar and Eisner, 1999; Tu and Tatar, 2003). Additional evidence comes from studies in which *M. sexta* were starved during the last larval stage, resulting in either death or a delay in metamorphosis, depending on when in the last instar starvation was initiated (Nijhout and Williams, 1974b). Feeding the larvae after a period of starvation usually result in a supernumery larval molt (Bhaskaran and Jones, 1980; Cymborowski et al., 1982; Jones et al., 1980). This delay in metamorphosis affords larvae an additional period of time to feed, grow and meet a critical mass to commit to pupation. Recently, pupal commitment by last instar *M. sexta* was shown to depend on sugar intake, whereas the proliferation and growth of imaginal discs, cells destined to be adult-specific structures, required both dietary sugar and protein (MacWhinnie et al., 2005). Juvenile animals ingest food not only for energy production and maintenance of biochemical processes, but also to accumulate larval nutrient reserves. Nutrient reserves acquired during the juvenile stage play a large role for the adult toward its reproductive success (Boggs, 1997; Briegel, 1990a; Briegel, 1990b; O'Brien et al., 2000; Rivero et al., 2001; Telang et al., 2006; Telang and Wells, 2004). Threshold levels of larval nutrient stores are likely candidates that inform the larva it is ready for metamorphosis. Recent studies using *M. sexta* and *D. melanogaster* have further elaborated how larval nutrition and hormones affect growth and metamorphosis in these model insects (Colombani et al., 2005; Truman et al., 2006).

Given the medical importance of mosquitoes, it is surprising how little is known about the nutritional and hormonal regulation of their post-embryonic development. What we do know indicates that endocrine regulation of molting and metamorphosis in mosquitoes may differ from the model developed for Lepidoptera and applied to higher dipterans. In Lepidoptera, the PG secretes ecdysteroids, and in higher dipterans, such as the fleshfly *Neobellieria* (formerly *Sarcophaga*) *bullata* and *D. melanogaster*, the larval ring gland has the PG as one of its components and secretes ecdysteroids (Bollenbacher et al., 1976; Dai and Gilbert, 1991; Parvy et al., 2005; Redfern, 1983). Contrary to this classical view, the tissue identified as the PG complex in fourth (last) instar *Aedes aegypti* does not release ecdysteroids but, instead, tissues in the thorax and abdomen synthesize and release ecdysteroids *in vitro* (Jenkins et al., 1992). Although a few current studies have begun examining the endocrine regulation of larval mosquito metamorphosis (Lan and Grier, 2004; Margam et al., 2006; Nishiura et al., 2003), the role of larval nutrition in regulating metamorphosis has not been addressed.

In the present study, we have examined the development and metamorphic readiness of larval *Aedes aegypti* in response to larval nutrition. We manipulated the length of time that fourth instars had access to food and measured its effect on larval mass, nutritional reserves and profiles of ecdysteroid production and hemolymph titers. Our data suggest that the timing of ecdysteroid release is not necessarily critical to initiate the larval–pupal molt, but both the ecdysteroid titer and the nutritional status of the fourth instar are crucial factors in initiating this first metamorphic molt in mosquito larvae. These findings will allow us to eventually construct a conceptual model specific for the nutritional and hormonal regulation of mosquito post-embryonic development.

Materials and methods

Animals

Aedes aegypti (UGAL strain) were maintained at 27°C with a photoperiod of 16 h:8 h light:dark. Larvae were reared in shallow, 34 cm×21 cm×5 cm pans at a density of 200 in 500 ml distilled water and fed a mixture of ground rat chow/lactalbumin/brewers yeast (1:1:1 w/w) daily. Under standard rearing conditions, individuals of this strain completed larval development in 6–7 days and were pupae on day 7 or 8 (hatch day being day 1). The following masses (mg) of food were dispensed into each larval rearing pan per day for optimal colony rearing: 100 (day 1), 140 (day 2), 260 (day 3), 260 (day 4), 470 (day 5) and 0 (day 6). Pupae and remaining late fourth instars were collected on day 7, and the pupal stage lasted roughly 48 h until adult eclosion. Adults were offered 10% sucrose solution on the third day post-eclosion and provided distilled water all other days. For egg production, females were fed on an anesthetized rat until engorged and allowed to lay eggs on moistened paper towel strips.

Larval staging

Under our standard colony rearing conditions, a majority of *A. aegypti* larvae are late third instars 4 days post-hatching. For

experiments, in the late morning of day 4, late third instars were chosen based on the presence of darkly tanned head capsules. Selected larvae were then housed individually in 24-well cell culture plates with each well containing 1.6 ml of distilled water. Third instar larvae were not given food during this period. The next morning, newly molted fourth instars were transferred individually to new wells containing fresh 1.6 ml distilled water.

Measurement of growth

To monitor increase in mass when optimally fed, individual fourth instars were fed *ad libitum* on a 2% (w/v) solution of *A. aegypti* diet that was easily dispensed to individual larvae. In previous trials, this feeding regimen supported the growth and metamorphosis of experimental larvae at the same degree and schedule as that of larvae mass reared for the colony. Wet mass of fourth instars was measured at different times during this stadium (newly molted, 12, 24, 36 and 48 h) and in pupae (12 h after larval–pupal ecdysis). Groups of three larvae were removed from their feeding wells, rinsed in fresh water and blotted dry prior to weighing. Each larval group was dried on a heat block at 90°C to determine dry mass. Both fresh and dry masses were obtained using a microbalance (Mettler MT5, Columbus, OH, USA. Three replicates were collected using different cohorts (N=18).

Hemolymph ecdysteroid titer, in vitro ecdysteroid secretion and the ecdysteroid radioimmunoassay

To verify ecdysteroid release from both the thorax and abdomen (Jenkins et al., 1992), triplicate preparations of thorax and abdomen and hemolymph samples were collected at different times during their fourth stadium (newly molted, 6, 12, 24, 30, 36, 48 and 52 h) and in pupae (12 h after larval–pupal ecdysis). Dissections and tissue incubations were conducted in a buffered medium (139 mmol l^{-1} NaCl, 4.05 mmol l^{-1} KCl, 1.85 mmol l^{-1} $CaCl_2$, 12.5 mmol l^{-1} Hepes, 2.5 mmol l^{-1} trehalose, 0.3 mmol l^{-1} $MgCl_2$ and 0.9 mmol l^{-1} $NaHCO_3$; pH 6.5, adjusted with NaOH) (Riehle and Brown, 1999) containing phenylthiourea (0.5 mg ml^{-1} buffered medium), added to minimize melanization. Hemolymph was collected from the same set of larvae prior to body region separation for the *in vitro* bioassay. To collect hemolymph, groups of four larvae were decapitated and hemolymph was allowed to flow into 75 μl of buffered medium with phenylthiourea, assisted by gentle pressure applied to each carcass. After 5 min incubation, 50 μl of the hemolymph solution were collected and assayed for ecdysteroids using a radioimmunoassay (RIA). Hemolymph ecdysteroid titers are reported on a larva equivalent basis.

For isolation of body regions, the terminal abdominal segments (including segment 8, the anal lobe, anal papillae and the respiratory siphon) were discarded. Thoraces and abdomina were separated from each other to facilitate removal of the alimentary canal from each section. Removal of PGs from thoraces was not confirmed since it has already been determined that PGs are not the source of ecdysteroids (Jenkins et al., 1992). To measure the amounts of ecdysteroids released, groups of four thoraces and four abdomina were incubated together in 60 μl buffered medium with phenylthiourea in a 0.6 ml polypropylene tube lid at 27°C for 6 h. After incubation, 25 μl of medium was collected and analyzed for ecdysteroid content using RIA. It was previously determined that the amount of ecdysteroids secreted from body region incubations into 50 μl of medium was beyond the linear range of the standard curve (see below), so these samples were diluted by half to stay within the quantitation range (10–250 pg). Ecdysteroid secretion by tissues is reported on a single thorax and abdomen set basis.

The RIA uses an ecdysteroid antiserum (AS 4919, a gift from P. Poncheron, Université P. et M. Curie, Paris, France) that recognizes ecdysone and 20-hydroxyecdysone equally (Porcheron et al., 1989). Full details of the RIA were previously described (Sieglaff et al., 2005). Values for each tissue sample type are reported as 'mean pg ecdysteroid', because the secreted ecdysteroid species are unknown. For this experiment, triplicates of four thorax plus abdomen preparations and four body hemolymph collections were analyzed for all the time points. Values reported are means of triplicate tissue preparations for larval age groups taken from one cohort (N=27).

Critical feeding period and scoring of metamorphosis

Experimental fourth instars were allowed to feed on a 2% (w/v) solution of *A. aegypti* standard diet for the following time periods only: 12, 18, 24, 30, 36, 42 or 48 h. After the allocated time period, larvae were transferred into new wells containing fresh water only and scored for pupation and adult eclosion. A control group consisted of larvae with access to food for the entire fourth stadium. For both experimental and control animals, we recorded duration of fourth instar development time (N=261), pupation (N=335), and adult eclosion in response to food access period (N=335). We did not determine the sex of larvae but recorded gender of each individual at adult eclosion only. Lastly, wing length was used to assess body size of females successfully emerging from each treatment and was measured from the point of attachment to the wing tip, not including fringe, under a dissecting microscope using an ocular micrometer (N=116). Three replicates were set up using different cohorts.

For larvae that did not pupate, the duration of time they remained alive during their fasting period was monitored (N=49). To examine whether larvae retain their capacity to molt after a period of fasting, newly molted fourth instars were allowed to feed for 12 h, fasted for 7 days, and then given additional opportunities to feed for defined periods of time. Larvae were then scored for pupation and adult eclosion (N=140). Lastly, wing length was used to assess body size of females successfully emerging from each supplementary feeding treatment (N=37). Two replicates were examined using different cohorts.

Quantification of nutritional reserves

Groups of larvae, either newly molted fourth instars or larvae from the three feeding regimens, were immediately

frozen for analysis of nutritional reserves. Dry mass was obtained by drying each larval group on a heat block at 90°C until no further water loss was detected. Whole-body homogenates of four larvae per treatment were made to extract storage lipid, glycogen and proteins using a published procedure (Van Handel, 1965) modified for *A. aegypti* (Zhou G. et al., 2004), except that the isolation of amino acids and sugars was omitted. Fractions of lipid, glycogen and protein were frozen until they could be quantified using colormetric-based assays. The amount of storage lipid, triacylglycerol, was determined by a modified vanillin reagent assay (Van Handel, 1985b). Total amount of glycogen was determined using a modified anthrone-based assay (Van Handel, 1985a). Protein was quantified using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Complete details of our assay procedures were previously described (Telang and Wells, 2004). All nutrients are reported on a per larva basis and three replicates of all feeding regimens were collected (N=12).

Data analyses

Increase in wet and dry mass of larvae during their fourth stadium was analyzed using analysis of variance (ANOVA). Both *in vitro* ecdysteroid release by thoraces and abdomina and hemolymph titer during the fourth larval stage and in pupae were analyzed by ANOVA. Likelihood of pupation and successful male or female eclosion in response to periods of food availability were examined using contingency analysis and the χ^2 statistic. Wing length was analyzed using ANOVA, with periods of food availability as the explanatory variable. For the feeding regimen experiment, amount of lipid, glycogen and protein per individual larva was analyzed using analysis of covariance (ANCOVA), using larval dry mass as a covariate. *In vitro* ecdysteroid release by thoraces and abdomina and hemolymph titer for larval feeding regimen were analyzed by ANOVA. When necessary, differences between means were further analyzed using the Tukey–Kramer HSD test. All data were statistically analyzed using JMP IN (version SAS Institute Inc.). Least square means (± s.e.m.) were obtained from statistical models and used in all graphical illustrations.

Results

Relationship of growth, in vitro ecdysteroid secretion and hemolymph ecdysteroid titer to fourth instar development and metamorphosis

Under our standard colony rearing conditions, the fourth larval stadium of *A. aegypti* lasts about 48 to 52 h. Although fourth instar larvae were housed and fed individually, their development matched the schedule of colony animals. Larvae were monitored for increase in mass over the course of their fourth instar when fed optimally. Both wet and dry mass of individual larvae increased from the time of their fourth instar molt to 48 h later (Fig. 1), and both measures were positively correlated with each other (r^2=0.96). The

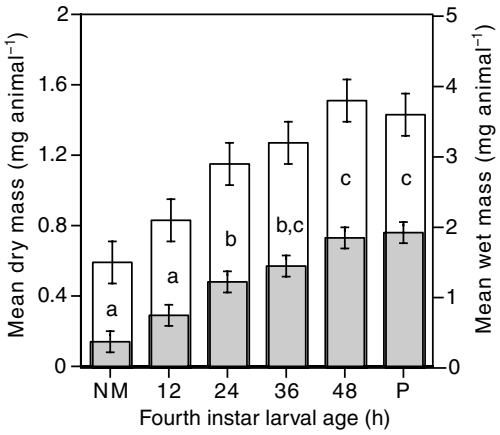

Fig. 1. Mean wet mass (white bars) and dry mass (gray bars) of *A. aegypti* at different times during their fourth larval stadium and in 12 h old pupae (P). Values represent least square means ± s.e.m.; N=18. Within both dry and wet mass values, columns with different letters are significantly different from each other (Tukey–Kramer HSD, $P \leqslant 0.05$). The relationship between wet and dry mass was analyzed by regression analysis: (dry mass larva^{-1})=–0.23+0.257(wet mass larva^{-1}), r^2=0.96, P<0.0001. NM, newly molted fourth instars.

post-hoc Tukey–Kramer test of means indicates that 24 h into the fourth instar is the first time point at which increase in mass is statistically different compared to earlier time points.

An *in vitro* bioassay was used to determine the capacity for ecdysteroid production by thoraces and abdomina dissected from larvae at different times during their fourth stadium and into their pupal stage. In addition, hemolymph was collected from the same set of larvae prior to tissue dissection for the *in vitro* bioassay. In an earlier study (Jenkins et al., 1992), ecdysteroid production by these tissues and hemolymph titers were measured only for the first 36 h of the fourth instar, even though the duration of the stadium was reported to be 36–52 h. In the present study we clarified the ecdysteroid profile of the entire last larval and early pupal stage of *A. aegypti*. Levels of ecdysteroid production by thorax and abdomen preparations differed over the course of the fourth larval stage, including the pupal stage (one-way ANOVA, P<0.0001) (Fig. 2). Thoraces and abdomina of newly molted fourth instars produced a detectable level of ecdysteroids (Fig. 2). During the fourth instar, this capacity increased over the period of 12–30 h to peak at about 90 pg before falling to a basal level at 48 h (Tukey–Kramer HSD, $P \leqslant 0.05$). Ecdysteroid release by thoraces and abdomina again increased in 12 h old pupae (Tukey–Kramer HSD, $P \leqslant 0.05$) (Fig. 2). Hemolymph ecdysteroid titer also differed over the course of the fourth larval stage (one-way ANOVA, P=0.009), with high levels in 30 h old larvae and higher still in 12 h old pupae (Fig. 2). Hemolymph ecdysteroid levels correlated well with that of tissue production (r^2=0.52), and both showed a similar rise and fall pattern (Fig. 2).

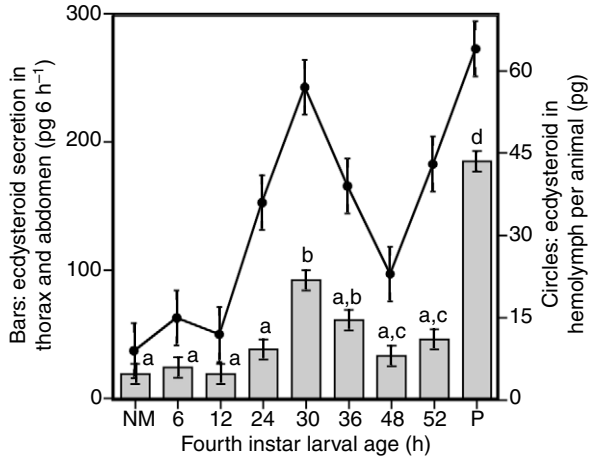

Fig. 2. Hemolymph ecdysteroid titers in a single larva (filled circles) and ecdysteroid secretion by a single set of thorax and abdomen (bars) at different times during *A. aegypti* fourth larval stadium and in 12 h old pupae (P). Each point or bar represents least square means ± s.e.m.; *N*=27. For ecdysteroid secretion values, columns with different letters are significantly different from each other (Tukey–Kramer HSD, $P \leqslant 0.05$). NM, newly molted fourth instars never given access to food.

Critical feeding period for pupation and adult eclosion

For these experiments, we manipulated nutrient input by giving fourth instars access to food for defined time periods, followed by a period of fasting. Ingestion was not measured in this experiment, but larvae most likely maintained high ingestion rates when food was present. Earlier studies indicate that an *A. aegypti* larva fills its gut after just 1 h of feeding on nutritive substances (Rashed and Mulla, 1989; Sneller and Dadd, 1977) and continues to ingest and move food through its gut as long as additional nutritive substances are present (Merritt et al., 1992). The larval diet for colony rearing and for these experiments includes brewers yeast, and yeast has been found to ensure high ingestion rates by larval *A. aegypti* and other species (Aly, 1985; Rashed and Mulla, 1989). Under our rearing conditions, the probability of pupation was influenced by feeding period ($P<0.0001$, χ^2 test), greater than 50% of fourth instars successfully pupated when they had at least 24 h to feed (Fig. 3A). For larval groups that had access to food for 24 h or longer, we found no evidence that feeding period affected the development time fourth instars took to pupate ($P=0.210$). On average, fourth instars spent 2.6 days until pupation. Under our rearing conditions, mortality was minimal for these groups, and only 14 of 288 total (<5%) died as either larvae or pupae when given access to food for 24 h or longer. Most fourth instars were developmentally arrested if given less than 24 h to feed (Fig. 3A). There was no statistical difference in mean survival time between developmentally arrested larvae that were allowed to feed for 12 or 18 h ($P=0.833$). Both groups of larvae survived on average for 14 days prior to death.

When given only 12 h to feed as newly molted fourth instars, five males and only one female out of a total of 30 larvae eclosed (Fig. 3A). When fourth instars were allowed to feed for

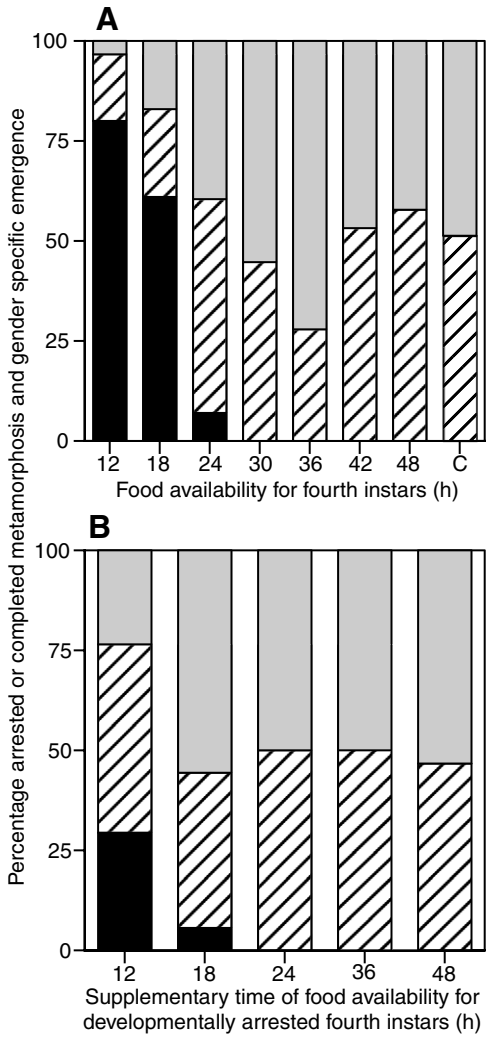

Fig. 3. (A) Effect of duration of food access on metamorphosis of *A. aegypti* fourth instars. Shown are percentages of individuals that remained as fourth instars (black bars) or that underwent metamorphosis and subsequently emerged as males (striped bars) or females (gray bars) in response to a period of access to food. Control (C) group of larvae were given food in excess and never underwent a period of time in which food was withheld. *N*=335. (B) Effect of supplementing food to fourth instars initially fed for 12 h followed by a 7 day fast. Shown are percentages of individuals that remained as fourth instars (black bars) or underwent metamorphosis and subsequently emerged as males (striped bars) or females (gray bars) in response to supplementary feeding. *N*=80.

24 h, more than 50% of the individuals successfully eclosed as males and around 40% did so if they were females. Presumably, the fourth instars that were developmentally arrested when fed for 24 h or less were females; however, we did not determine the sex of larvae. When fourth instars were allowed to feed for 30 h and longer, all individuals pupated and eclosed as adults, and at least 50% of the individuals successfully eclosed as females (Fig. 3A). Feeding period greatly influenced female body size, as assessed by wing length (Fig. 4). Females fell

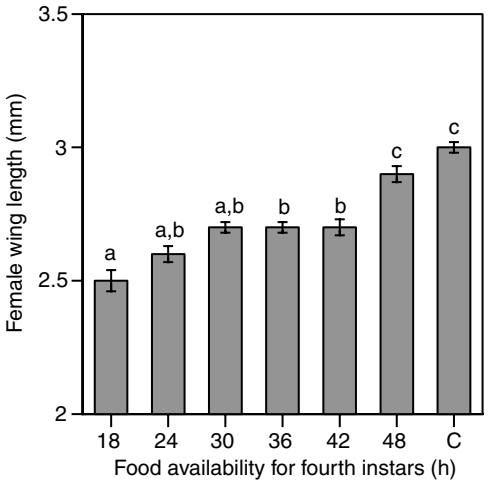

Fig. 4. Effect of food access period on wing length of newly emerged *A. aegypti* females. Values represent least square means ± s.e.m.; *N*=116. Columns with different letters are significantly different from each other (Tukey–Kramer HSD, $P \leq 0.05$).

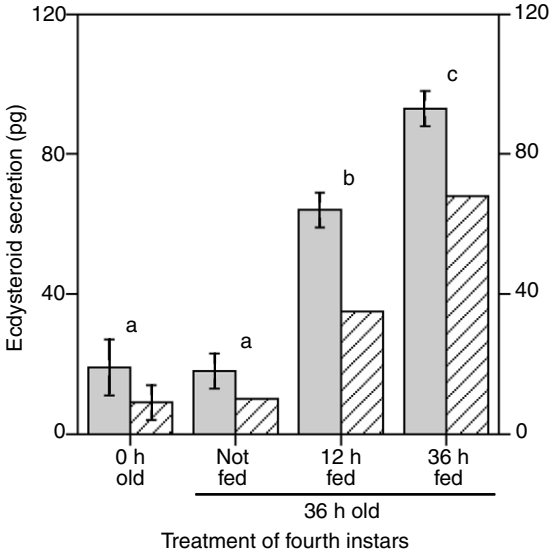

Fig. 5. Hemolymph ecdysteroid titers in a single larva (striped bars) and ecdysteroid secretion by a single set of thorax and abdomen (gray bars) in groups of 36 h old fourth instar *A. aegypti* given different periods of time to feed or not. Newly molted fourth instars were either deprived of food for 36 h, or fed for 12 h or 36 h prior to tissue collection at 36 h for the ecdysteroid bioassay. Values represent least square means ± s.e.m.; *N*=12. For both ecdysteroid secretion and hemolymph values, columns with different letters are significantly different from each other (Tukey–Kramer HSD, $P \leq 0.05$). NM, newly molted fourth instars never given access to food. No bar indicates that the s.e.m. is smaller than column scale.

roughly into three classes of wing length measurements: 2.5 mm wing length (18 h food availability level), 2.7 mm (36 h food availability level), and 3.0 mm (48 h food availability level or *ad libitum* feeding).

We also examined whether developmentally arrested larvae retained the capacity for metamorphosis. Another group of newly molted fourth instars were allowed to feed for only 12 h and fasted for 7 days. When fed *ad libitum* after fasting, 100% of larvae successfully pupated and eclosed as adults (data not shown). However, when given only 12 h to feed after fasting, only 70% of fourth instars successfully pupated, and a greater proportion of the eclosed individuals were males (Fig. 3B). A 50/50 ratio of male and female emergence is observed when developmentally arrested larvae are given supplementary periods of feeding of 18 h or longer (Fig. 3B). Female body size, as assessed by wing length, increased with a longer supplementary period of feeding (data not shown).

The influence of larval age and nutritional condition toward metamorphosis

This experiment was carried out to distinguish cues for metamorphosis related to a larva's nutritional state *versus* its age. For this experiment, newly molted fourth instars were set up in specific feeding period regimens, and tissues were collected from these larvae after 36 h. Since thoraces and abdomina produced peak levels of ecdysteroids around 30–36 h (Fig. 2) and all individuals pupated and eclosed as adults if fed for at least 30 h (Fig. 3A), we focused our feeding regimen experiments on 36 h old fourth instars. In one feeding regimen, newly molted fourth instars were deprived of food for the entire 36 h period. In a second regimen, fourth instars were given access to food for 12 h but fasted until they were 36 h old. In the third regimen, fourth instars were given access to food for the entire 36 h period. Specific correlates of larval development, such as ecdysteroid levels, body mass and nutrient reserves, were measured for each larval group.

Levels of ecdysteroid production by thorax and abdomen preparations differed among the fourth instar groups given different periods of time to feed or not (one-way ANOVA, $P<0.0001$) (Fig. 5). Thorax and abdomen tissues of fourth instars that were 36 h old, but deprived of food during that period, did not increase ecdysteroid production compared to newly molted fourth instars (Fig. 5). However, thoraces and abdomina of fourth instars that were 36 h old, but had access to food for 12 h, secreted significantly greater levels of ecdysteroids compared to tissues from the food deprived group but significantly less than the tissues in fourth instars that were 36 h old and had access to food during that period. Tissues in the fourth instar group that fed for 36 h produced the highest levels of ecdysteroids overall (Fig. 5). Hemolymph ecdysteroid titer also differed among the fourth instar groups given different periods of time to feed or not (one-way ANOVA, $P<0.0001$), with the highest level found in 36 h old fourth instars that had access to food for the entire 36 h period (Fig. 5).

Fourth instar larvae that were given access to food for 36 h had a significantly higher body mass compared to newly molted larvae and larval groups given only 12 h to feed or not (Tukey–Kramer HSD, $P \leq 0.05$) (Fig. 6A). Levels of nutrient reserves in fourth instars, corrected for larval dry mass, differed

among the feeding treatment groups (ANCOVA, $P<0.0001$) (Fig. 6B–D). Compared to newly molted fourth instars, levels of lipid, glycogen and protein decreased significantly in fourth instars that were deprived of food for 36 h (Fig. 6B–D), although no such decrease in dry mass was measured (Fig. 6A). Fourth instars 36 h old that had access to food for 12 h had significantly greater lipid levels compared to newly molted fourth instars (Fig. 6B) but similar levels of glycogen and protein (Fig. 6C,D). Overall, fourth instars that were 36 h old and had access to food for that length of time were the heaviest and contained significantly higher levels of all nutrient reserves.

Discussion

We began our study with the premise that nutrient reserves sequestered by mosquito larvae serve as a likely correlate of critical body mass and a candidate for cueing pupal commitment. Our earlier studies indicated that larval nutrient reserves (protein, lipid and glycogen) were important for egg production and the endocrine regulation of egg development in autogenous and anautogenous female mosquitoes (Telang et al., 2006). Females of mosquito species that must ingest blood meals for all egg cycles are considered anautogenous, whereas, females of autogenous species can produce at least their first egg batch without a blood meal. When larval mosquitoes were fed high or low amounts of food, both autogenous and anautogenous females emerged with high or low levels of nutritional reserves, respectively (Telang et al., 2006; Telang and Wells, 2004). In those studies, larvae restricted to a poor diet emerged with low reserve levels, but they committed to pupation nonetheless. In our current study, we wanted to explore the importance of larval nutrition in more detail and specifically toward its importance for metamorphic readiness. To begin examining the readiness of mosquito larvae to pupate, we measured basic parameters of larval development such as critical mass, nutritional reserves, and ecdysteroid production with hemolymph titers during the fourth instar and early pupal stage of *A. aegypti*.

In mosquitoes, the nutritional environment experienced by a female when a larva dictates her adult body size (Briegel, 1990a; Briegel, 1990b; Telang et al., 2006; Telang and Wells, 2004), as for many insects (Calvo and Molina, 2005; Nijhout, 2003; Slansky and Scriber, 1985; Stern, 2003). An important physiological determinant of adult body size is a minimum or critical mass that must be gained during the larval stage to trigger the commitment to metamorphose. For the model insect *M. sexta*, the critical mass has been defined as '*the minimal weight in which further feeding and growth are not required for a normal time course to metamorphosis and pupation*' (Davidowitz et al., 2003; Nijhout and Williams, 1974b). This same definition was used to determine the critical mass for pupariation in last instar *D. melanogaster* (Mirth et al., 2005; Zhou X. et al., 2004). For the yellow fever mosquito *Aedes aegypti*, the critical mass has been defined as the mass at which 50% of larvae pupate when they are starved subsequent to being weighed (Chambers and Klowden, 1990). Using both these definitions, we estimated the critical mass to be 2.7–3.2 mg wet mass for larvae reared at 27°C (Fig. 1). This critical mass was achieved by 24 h old fourth instar larvae. This is also the minimum length of time these larvae needed to

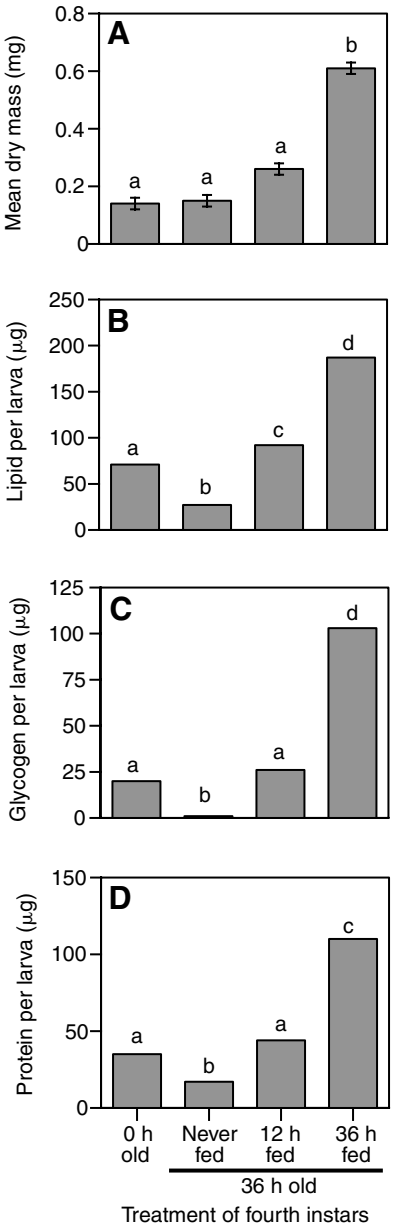

Fig. 6. Dry mass and nutrient reserves in groups of 36 h old fourth instar *A. aegypti* given different periods of time to feed or not. Newly molted fourth instars were either deprived of food for 36 h, or fed for 12 h only, or fed for 36 h prior to weighing and analyses. (A) Dry mass of fourth instars, (B) lipid amounts in fourth instars, (C) glycogen amounts in fourth instars, and (D) protein levels in fourth instars. All values represent least square means ± s.e.m.; N=12. All nutrient values were obtained from an ANCOVA model and are corrected for larval dry mass (covariate). Error bars represent s.e.m.; no bar indicates that the s.e.m. is smaller than column scale.

access food so that at least 50% of them pupated and emerged as adults (Fig. 3A), and the age at which ecdysteroid production and titer begins to rise (Fig. 2). Our measurement of critical mass is greater than previously reported for *A. aegypti* strain Segemaganga (Chambers and Klowden, 1990). In that study, the critical mass for *A. aegypti* varied from 1.9–2.6 mg when larvae were reared at 22°C to 1.6–1.9 mg when larvae were reared at 32°C (Chambers and Klowden, 1990). Differences in larval critical mass reported in both studies most likely reflect genetic differences among the various strains of *A. aegypti* currently under long-term colonization.

According to the model developed for *M. sexta*, when larvae reach the critical mass, the corpora allata (CA), glands responsible for JH synthesis and secretion, are turned off and JH levels begin to decrease (Nijhout and Williams, 1974a), with the elimination of JH facilitated by the catabolic enzyme JH esterase (Browder et al., 2001). The absence of JH for a metamorphic molt is important, and it is thought to render the larval brain competent to secrete PTTH (Truman, 1972; Truman and Riddiford, 1974). PTTH stimulates secretion of ecdysteroids by the PG that then causes larvae to stop feeding and commit to pupation (Nijhout, 1994). Therefore, the size that a larva attains at its metamorphic molt determines the size of its adult body. In our current study, fourth instar mosquito larvae reach their critical mass 24 h post-ecdysis (Fig. 1), and we measured a single peak of ecdysteroid secretion by tissues and hemolymph titers 30–36 h post-eclosion (Fig. 2). For mosquitoes, it is not known if a brain factor with PTTH-like activity is responsible for stimulating ecdysteroid production by larval tissues, thereby initiating the metamorphic molt. Whether JH plays a regulatory role in mosquito metamorphosis is also not known. However, larvae treated with the JH mimic, methoprene, exhibited delayed pupation, suggesting that a critical 'JH sensitive' period exists in mosquito larval development (Lan and Grier, 2004), as has been shown in some some lepidopteran studies (Nijhout, 1994).

Among past studies on *A. aegypti* metamorphosis, two main areas of discrepancy exist (Chambers and Klowden, 1994; Fournet et al., 1995; Jenkins et al., 1992; Lan and Grier, 2004; Margam et al., 2006). First, the length of time fourth instars take to pupate differs greatly among the studies (51–72 h). This development time is affected by several factors such as temperature, food quantity and rearing densities. Unfortunately, the studies have employed different larval rearing protocols or have not adequately described conditions to facilitate experimental duplication. To assist future experiments, it is important to report relevant larval rearing parameters such as temperature, rearing densities, container dimensions and feeding regimen, as herein. Second, there is disagreement about the number of ecdysteroid peaks that occur during the last instar. In some studies, two or three peaks in ecdysteroid titer are reported for the last instar (Fournet et al., 1995; Lan and Grier, 2004; Margam et al., 2006; Westbrook and Russo, 1985). Different ecdysteroid antisera and immunoassays (RIA *versus* enzyme immunoassay) are used in these studies, and more notably, whole body extracts taken at different times were assayed. The amount of ecdysteroids in such extracts is greater than that in hemolymph taken at similar time points (Jenkins et al., 1992) (see also this study), and from personal experience, there is a high degree of non-specific binding of ecdysteroid antisera to such extracts that can only be resolved by the assay of serial dilutions of each extract to identify its appropriate dilution for accurate quantification in the linear range of the immunoassay. This important information is not to be found in studies using whole body extracts of mosquito larvae. These studies do discuss a 'commitment peak' that occurs 14–28 h (Lan and Grier, 2004) or 24 h (Margam et al., 2006) post ecdysis to the last instar larvae, which in this last instance is not a significant increase in comparison to the larger peak occurring 30–36 h post ecdysis. Among studies, there is a consensus that an ecdysteroid peak occurs 24–36 h later in fourth instar development (Fournet et al., 1995; Lan and Grier, 2004; Margam et al., 2006; Westbrook and Russo, 1985) and a larger one occurs during the early part of the pupal stage (Fournet et al., 1995; Margam et al., 2006; Whisenton et al., 1989). Similarly in a previous study (Jenkins et al., 1992) and the present one, a significant increase in ecdysteroid secretion by tissues in thoraces and abdomina of 30–36 h old fourth instar larvae corresponded to an ecdysteroid increase in hemolymph titer, which we believe is the pre-molt increase that triggers larval–pupal apolysis. Ecdysteroid secretion by tissues and hemolymph titer decline in 48–52 h old fourth instars, which are nearing pupation, and greatly increase in pupae 12 h after the larval–pupal molt.

A more thorough examination of ecdysteroid titers at lower temperatures (thereby prolonging larval development) or at shorter time intervals in last instar larvae, may reveal an earlier peak between 6 and 9 h post last instar ecdysis that would initiate the rise in gene expression for a variety of ecdysteroid-regulated genes, such as the isoforms of the ecdysteroid receptor and ultraspiracle recently reported (Margam et al., 2006). It should be noted, however, that a recent and exhaustive study of ecdysteroid titer and related biosynthetic enzyme levels in whole bodies of last instar *Drosophila melanogaster* failed to substantiate such an early commitment peak (Warren et al., 2006). This study did find surprisingly low whole body ecdysteroid peaks at 20 and 28 h post ecdysis to the last instar that were correlated with an increase in biosynthetic enzyme levels in this same study, along with a sevenfold increase in ecdysteroid titer during the early phase of pupation. For now, it appears that the timing of major ecdysteroid peaks during the last instar and early pupae required for metamorphosis by these two dipteran species is similar, but the tissue sources of the ecdysteroids are different, as may be the regulation of ecdysteroid secretion.

For metamorphosis to occur, nutrient intake is necessary to facilitate growth during the last instar (Nijhout, 2003). When fourth instars were given longer periods of time to feed, a greater proportion of them successfully pupated and eclosed as adults. However, most fourth instars allowed to feed for only 12 h and then starved were developmentally arrested (Fig. 3A).

Similar results were reported using a different experimental design (Nishiura et al., 2007). Larvae can live for up to 2 weeks when starved and still retain the capacity to pupate if re-fed (Fig. 3B). Our study is not the first to observe the ability of *A. aegypti* to withstand starvation for this period of time (Rasnitsyn and Yasyukevich, 1989; Wigglesworth, 1942). The ability of larval *A. aegypti* to tolerate starvation is believed to depend on stored energy, primarily lipids (Gilpin and McClelland, 1979; Wigglesworth, 1942). Starvation tolerance over a long period of time is thought to be common among container-inhabiting species in general (Barrera, 1996; Barrera and Medialdea, 1996), and this period may be an important factor to be considered for control measures and the study of their vectorial capacity.

When fed optimally during their fourth instar, the increase of ecdysteroids to cue metamorphic commitment is seen in 30–36 h old fourth instar larvae. When newly molted fourth instars were starved at ecdysis and deprived of food for 36 h, they show no increase in ecdysteroid secretion or body mass compared to newly molted fourth instars (Figs 5, 6A). These starved fourth instars maintained similar body mass but had lower levels of lipid, glycogen and protein compared to newly molted fourth instars (Fig. 6A–D). When fourth instars face starvation conditions, we expect them to catabolize storage nutrients. Given that fourth instars remain alive for 14 days without food, it is not surprising that we see no decrease in mass after just a 36 h fasting period. Another study observed a major decrease in *A. aegypti* pupal mass only when they were starved for at least 4 days as fourth instars (Rasnitsyn and Yasyukevich, 1989). Lastly, our biochemical analyses do not account for sugars, amino acids or storage excretion, but the dry mass of starved fourth instars may include these materials. It is thought that the larval fat body can function in storage excretion, and large deposits of uric acid have been observed in fat body cells of starved *A. aegypti* larvae, presumably as a product of deamination of amino acids or proteins (Wigglesworth, 1942; Wigglesworth, 1987). If accumulation of uric acid occurred in starved fourth instars in our study, this would undoubtedly contribute toward their mass maintenance. Overall, fourth instar *A. aegypti* allowed to feed for the entire 36 h period accumulated the highest levels of all three storage nutrients (Fig. 6B–D). The fat content of insect larvae of many species increases from early to late instars (Slansky and Scriber, 1985). Mosquito larvae may be no exception, but this awaits further study.

Our data suggest that metamorphic capacity is dependent on a larva's nutritional condition, not just the chronological age at which ecdysteroid increase occurs. Tissues in 36 h old fourth instars that had fed for 12 h secreted higher levels of ecdysteroids compared to newly molted larvae or larvae deprived of food for 36 h (Fig. 5). The level of ecdysteroids measured in the 12 h fed/36 h old fourth instars (65 pg per thorax and abdomen set per 6 h) falls at the lower end of the 60–100 pg range, which appears to commit metamorphosis in 30–36 h old fourth instars fed *ad libitum* (Fig. 2). However, that level of ecdysteroid secretion is clearly not sufficient to initiate pupation since we know fourth instars are developmentally arrested if given only a 12 h feeding period (Fig. 3A). Only in 36 h old fourth instars that had access to food for 36 h do we observe significant accumulation of nutrients and the necessary ecdysteroid increase, indicating that both conditions must be met for larvae to initiate metamorphosis (Fig. 5, Fig. 6A–D).

Strong positive correlations have been found between body mass and caloric reserves of lipid and carbohydrates (Chambers and Klowden, 1990), and it was concluded that carbohydrates accumulated by larvae strongly influenced readiness to pupate. According to our data, it is during the 12 h and 36 h time spent feeding that the lipid, glycogen and protein thresholds are met to trigger the metamorphic molt (Fig. 6B–D). Nutrients accumulated by larvae have to serve both body-building and energy needs. Thus, the threshold for metamorphic readiness must encompass all three nutrient reserves. To the best of our knowledge, data concerning nutritional reserves accumulated by larvae during development have not been collected for the model insects *M. sexta* or *D. melanogaster*.

Thus, the timing of ecdysteroid release may not be critical to initiate the larval–pupal molt for mosquito larvae, but both the ecdysteroid titer and the nutritional condition of fourth instars are crucial factors in initiating the first metamorphic molt. *De novo* synthesis of ecdysteroids by larval abdomen has been documented in the housefly *Musca domestica* (Studinger and Willig, 1975), the mealworm beetle *Tenebrio molitor* (Romer et al., 1974) and in *A. aegypti* (Jenkins et al., 1992). Currently it is not known what cells common to both the thorax and abdomen of *A. aegypti* larvae are ecdysteroidogenic. Ecdysteroid release from epidermal cells and oenocytes, which are associated with the fat body in some insects (Chapman, 1998), are dispersed throughout the thorax and abdomen and have been implicated in these past studies. Future studies of larval mosquitoes will discover how the fat body and associated tissues convey information about nutrient levels to the nervous system so that PTTH, JH and ecdysteroid production by non-prothoracic gland cells are coordinated to induce metamorphosis.

Mosquitoes such as *A. aegypti* and *Anopheles gambiae* are responsible for vectoring pathogens that cause serious human diseases, such as dengue fever and malaria. A complimentary aspect of effective disease intervention is suppression of the vector. Effective mosquito population control is best targeted at the larval and pupal stages because these stages are confined by their aquatic habitats relative to the highly mobile aerial adult stage. We focus our work on the yellow fever mosquito *A. aegypti* in the hope that studies regarding its larval development will contribute to knowledge regarding its biology and control. Traditional vector control methods have employed chemical pesticides. However, concerns over non-target effects and target insect resistance have led to a shift in control towards more biorational approaches, such as the bacterium *Bacillus thuringiensis israelensis* (Bti), and insect growth regulators (IGRs), such as JH mimics (methoprene) and ecdysone agonists. The normal larval physiology of metamorphosis is disrupted by IGRs (Beckage et al., 2004;

Nishiura et al., 2003; Palli et al., 2005), but the mechanisms by which they do so are not well understood. Full exploitation of current and novel IGRs would be aided by an understanding of the regulation of postembryonic development of mosquitoes.

The authors would like to thank Judy Willis for critically reading our manuscript and Danny Fendley for technical assistance. This collaborative work was funded by NIH/NIAID postdoctoral fellowship1 T32 AI060546-01 (Center for Tropical and Emerging Global Diseases, University of Georgia) to A.T. and NIH grant AI33108 to M.R.B.

References

Aly, C. (1985). Feeding rate of larval *Aedes vexans* stimulated by food substances. *J. Am. Mosq. Control Assoc.* **1**, 506-510.

Barrera, R. (1996). Competition and resistance to starvation in larvae of container-inhabiting *Aedes* mosquitoes. *Ecol. Entomol.* **21**, 117-127.

Barrera, R. and Medialdea, V. (1996). Development time and resistance to starvation of mosquito larvae. *J. Nat. Hist.* **30**, 447-458.

Beckage, N. E., Marion, K. M., Walton, W. E., Wirth, M. C. and Tan, F. F. (2004). Comparative larvicidal toxicities of three ecdysone agonists on the mosquitoes *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*. *Arch. Insect Biochem. Physiol.* **57**, 111-122.

Bhaskaran, G. and Jones, G. (1980). Neuroendocrine regulation of corpus allatum activity in *Manduca sexta*: the endocrine basis for starvation induced supernumerary larval moult. *J. Insect Physiol.* **26**, 431-440.

Boggs, C. L. (1997). Dynamics of reproductive allocation from juvenile and adult feeding: radiotracer studies. *Ecology* **78**, 192-202.

Bollenbacher, W. E., Goodman, W., Vedeckis, W. V. and Gilbert, L. I. (1976). The *in vitro* synthesis and secretion of alpha-ecdysone by the ring glands of the fly, *Sarcophaga bullata*. *Steroids* **27**, 309-324.

Briegel, H. (1990a). Fecundity, metabolism, and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria. *J. Med. Entomol.* **27**, 839-850.

Briegel, H. (1990b). Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *J. Insect Physiol.* **36**, 165-172.

Bronson, F. H. (1985). Mammalian reproduction: an ecological perspective. *Biol. Reprod.* **32**, 1-26.

Browder, M. H., D'Amico, L. J. and Nijhout, H. F. (2001). The role of low levels of juvenile hormone esterase in the metamorphosis of *Manduca sexta*. *J. Insect Sci.* **1**, 11.

Calvo, D. and Molina, J. M. (2005). Fecundity–body size relationship and other reproductive aspects of *Streblote panda* (Lepidoptera: Lasiocampidae). *Ann. Entomol. Soc. Am.* **98**, 191-196.

Chambers, G. M. and Klowden, M. J. (1990). Correlation of nutritional reserves with a critical weight for pupation in larval *Aedes aegypti* mosquitoes. *J. Am. Mosq. Control Assoc.* **6**, 394-399.

Chambers, G. M. and Klowden, M. J. (1994). Nutritional reserves of autogenous and anautogenous selected strains of *Aedes albopictus* (Diptera: Culicidae). *J. Med. Entomol.* **31**, 554-560.

Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge: Cambridge University Press.

Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carré, C., Noselli, S. and Léopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. *Science* **310**, 667-670.

Cymborowski, B., Bogus, M., Beckage, N. E., Williams, C. M. and Riddiford, L. M. (1982). Juvenile hormone titres and metabolism during starvation-induced spernumerary larval moulting of the tobacco hornworm, *Manduca sexta* L. *J. Insect Physiol.* **28**, 129-135.

Dai, J.-D. and Gilbert, L. I. (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Dev. Biol.* **144**, 309-326.

Davidowitz, G., D'Amico, L. J. and Nijhout, H. F. (2003). Critical weight in the development of insect body size. *Evol. Dev.* **5**, 188-197.

Fournet, F., Sannier, C., Moriniere, M., Porcheron, P. and Monteny, N. (1995). Effects of two insect growth regulators on ecdysteroid production in *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **32**, 588-593.

Gilbert, L. I. (2004). Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* **215**, 1-10.

Gilbert, L. I., Rybczynski, R. and Warren, J. T. (2002). Control and biochemical nature of the ecdysteroidogenic pathway. *Annu. Rev. Entomol.* **47**, 883-916.

Gilpin, M. E. and McClelland, G. A. H. (1979). Systems analysis of the yellow fever mosquito *Aedes aegypti*. *Fortschr. Zool.* **25**, 355-388.

Iyengar, V. K. and Eisner, T. (1999). Heritability of body mass, a sexually selected trait, in an arctiid moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. USA* **96**, 9169-9171.

Jenkins, S. P., Brown, M. R. and Lea, A. O. (1992). Inactive prothoracic glands in larvae and pupae of *Aedes aegypti*: ecdysteroid release by tissues in the thorax and abdomen. *Insect Biochem. Mol. Biol.* **22**, 553-559.

Jones, D., Jones, G. and Bhaskaran, G. (1980). Induction of supernumerary molting by starvation in *Manduca sexta* larvae. *Entomol. Exp. Appl.* **28**, 259-267.

Lan, Q. and Grier, C. A. (2004). Critical period for pupal commitment in the yellow fever mosquito, *Aedes aegypti*. *J. Insect Physiol.* **50**, 667-676.

MacWhinnie, S. G. B., Allee, J. P., Nelson, C. A., Riddiford, L. M., Truman, J. W. and Champlin, D. T. (2005). The role of nutrition in creation of the eye imaginal disc and initiation of metamorphosis in *Manduca sexta*. *Dev. Biol.* **285**, 285-297.

Margam, V. M., Gelman, D. B. and Palli, S. R. (2006). Ecdysteroid titers and developmental expression of ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae). *J. Insect Physiol.* **52**, 558-568.

Merritt, R. W., Dadd, R. H. and Walker, E. D. (1992). Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annu. Rev. Entomol.* **37**, 349-376.

Mirth, C., Truman, J. W. and Riddiford, L. M. (2005). The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Curr. Biol.* **15**, 1796-1807.

Nijhout, H. F. (1981). Physiological control of molting in insects. *Am. Zool.* **21**, 631-640.

Nijhout, H. F. (1994). *Insect Hormones*. Princeton: Princeton University Press.

Nijhout, H. F. (2003). The control of body size in insects. *Dev. Biol.* **261**, 1-9.

Nijhout, H. F. and Williams, C. M. (1974a). Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.* **61**, 493-501.

Nijhout, H. F. and Williams, C. M. (1974b). Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last-instar larva and the decision to pupate. *J. Exp. Biol.* **61**, 481-491.

Nishiura, J. T., Ho, P. and Ray, K. (2003). Methoprene interferes with mosquito midgut remodeling during metamorphosis. *J. Med. Entomol.* **40**, 498-507.

Nishiura, J. T., Burgos, C., Aya, S., Goryacheva, Y. and Lo, W. (2007). Modulation of larval nutrition affects midgut neutral lipid storage and temporal pattern of transcription factor expression during mosquito metamorphosis. *J. Insect Physiol.* **53**, 47-58.

O'Brien, D. M., Schrag, D. P. and Martinez del Rio, C. (2000). Allocation to reproduction in a hawkmoth: a quantitative analysis using stable carbon isotopes. *Ecology* **81**, 2822-2831.

Palli, S. R., Tice, C. M., Margam, V. M. and Clark, A. M. (2005). Biochemical mode of action and differential activity of new ecdysone agonists against mosquitoes and moths. *Arch. Insect Biochem. Physiol.* **58**, 234-242.

Parvy, J. P., Blais, C., Bernard, F., Warren, J. T., Petryk, A., Gilbert, L. I., O'Connor, M. B. and Dauphin-Villemant, C. (2005). A role for *B*FTZ-F1 in regulating ecdysteroid titers during post-embryonic development in *Drosophila melanogaster*. *Dev. Biol.* **282**, 84-94.

Porcheron, P., Moriniere, M., Grassi, J. and Pradelles, P. (1989). Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. *Insect Biochem.* **19**, 117-122.

Rashed, S. S. and Mulla, M. S. (1989). Factors influencing ingestion of particulate materials by mosquito larvae (Diptera: Culicidae). *J. Med. Entomol.* **26**, 210-216.

Rasnitsyn, S. P. and Yasyukevich, V. V. (1989). On the ability of mosquito larvae (Diptera, Culicidae) to endure starvation. *Entomol. Rev.* **68**, 143-151.

Redfern, C. P. F. (1983). Ecdysteroid synthesis by the ring gland of

Drosophila melanogaster during late-larval, prepupal and pupal development. *J. Insect Physiol.* **29**, 65-71.

Riehle, M. A. and Brown, M. R. (1999). Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **29**, 855-860.

Rivero, A., Giron, D. and Casas, J. (2001). Lifetime allocation of juvenile and adult nutritional resources to egg production in a holometabolous insect. *Proc. R. Soc. Lond. B Biol. Sci.* **268**, 1231-1237.

Romer, F., Emmerich, H. and Nowock, J. (1974). Biosynthesis of ecdysones in isolated prothoracic glands and oenocytes of *Tenebrio molitor in vitro*. *J. Insect Physiol.* **20**, 1975-1987.

Rybczynski, R. (2005). Prothoracicotropic hormone. In *Comprehensive Molecular Insect Science*. Vol. 3 (ed. L. I. Gilbert, K. Iatrou and S. S. Gill), pp. 61-123. Oxford: Elsevier.

Sieglaff, D. H., Duncan, K. A. and Brown, M. R. (2005). Expression of genes encoding proteins involved in ecdysteroidogenesis in the female mosquito, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **35**, 471-490.

Slansky, F. and Scriber, J. M. (1985). Food consumption and utilization. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Vol. 4 (ed. G. A. Kerkut and L. I. Gilbert), pp. 87-163. Oxford: Pergamon Press.

Sneller, V.-P. and Dadd, R. H. (1977). Requirement for sugar in a chemically defined diet for larval *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **14**, 387-392.

Stern, D. (2003). Body-size control: how an insect knows it has grown enough. *Curr. Biol.* **13**, 267-269.

Studinger, G. and Willig, A. (1975). Biosynthesis of alpha- and beta-ecdysone in isolated abdomens of larvae of *Musca domestica*. *J. Insect Physiol.* **21**, 1793-1798.

Telang, A. and Wells, M. A. (2004). The effect of larval and adult nutrition on successful autogenous egg production by a mosquito. *J. Insect Physiol.* **50**, 677-685.

Telang, A., Li, Y., Noriega, F. G. and Brown, M. R. (2006). Effects of larval nutrition on the endocrinology of mosquito egg development. *J. Exp. Biol.* **209**, 645-655.

Truman, J. W. (1972). Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. Exp. Biol.* **57**, 805-820.

Truman, J. W. and Riddiford, L. M. (1974). Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.* **60**, 371-382.

Truman, J. W., Hiruma, K., Allee, J. P., MacWhinnie, S. G. B., Champlin, D. T. and Riddiford, L. M. (2006). Juvenile hormone is required to couple imaginal disc formation with nutrition in insects. *Science* **312**, 1385-1388.

Tu, M.-P. and Tatar, M. (2003). Juvenile diet restriction and the aging and reproduction of adult *Drosophila melanogaster*. *Aging Cell* **2**, 327-333.

Van Handel, E. (1965). Microseparation of glycogen, sugars, and lipids. *Anal. Biochem.* **11**, 266-271.

Van Handel, E. (1985a). Rapid determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 299-301.

Van Handel, E. (1985b). Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 302-304.

Warren, J. T., Yerushalmi, Y., Shimell, M. J., O'Connor, M. B., Restifo, L. L. and Gilbert, L. I. (2006). Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: correlations with changes in gene activity. *Dev. Dyn.* **235**, 315-326.

Westbrook, A. and Russo, R. (1985). Ecdysone titers during the fourth larval instar of three species of *Toxorhynchites*. *Proc. N. J. Mosq. Control Assoc.* **72**, 63-70.

Whisenton, L. R., Warren, J. T., Manning, M. K. and Bollenbacher, W. E. (1989). Ecdysteroid titres during pupal-adult development of *Aedes aegypti*: basis for a sexual dimorphism in the rate of development. *J. Insect Physiol.* **35**, 67-73.

Wigglesworth, V. B. (1942). The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of mosquito larvae. *J. Exp. Biol.* **19**, 56-77.

Wigglesworth, V. B. (1987). Histochemical studies of uric acid in some insects. 2. Uric acid and polyphenols in the fat body. *Tissue Cell* **19**, 93-100.

Zhou, G., Flowers, M., Friedrich, K., Horton, J., Pennington, J. and Wells, M. A. (2004). Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. *J. Insect Physiol.* **50**, 337-349.

Zhou, X., Zhou, B., Truman, J. W. and Riddiford, L. M. (2004). Overexpression of broad: a new insight into its role in the *Drosophila* prothoracic gland cells. *J. Exp. Biol.* **207**, 1151-1161.