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# Neuroendocrine factors affecting the steady-state levels of early trypsin mRNA in *Aedes aegypti*

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## Abstract

Transcription of the early trypsin gene occurs in the midgut after adult emergence under control of juvenile hormone (JH). We tested the hypothesis that factors that affect the steady-state levels of early trypsin mRNA do so by influencing the levels of JH. We investigated the effect of ingesting different meals on early trypsin mRNA levels as well as on JH levels. We also studied how early trypsin mRNA levels changed when the midgut was isolated from different components of the neuroendocrine system by abdominal ligation and decapitation. Early trypsin transcripts levels are high in unfed females; feeding different meals had three distinct effects on the changes of steady-state levels of early trypsin mRNA: (1) blood and protein meals caused the level to decrease drastically and remained low for at least 24 h; (2) amino acid meals caused a transient decrease in the mRNA level, but it returned to high levels after 12–18 h; and (3) sugar, latex and saline meals had no effect on the early trypsin mRNA steady-state levels. The changes in JH levels after ingesting blood and amino acid meals show profiles resembling the changes in early trypsin mRNA levels for the corresponding meal. Decapitation at 1, 2 and 3 days after emergence does not affect the steady-state levels of early trypsin in unfed females. In contrast, 24 h after feeding, transcript levels were significantly higher in decapitated females when compared with non-decapitated fed females. We propose that the changes in the steady-state levels of early trypsin mRNA observed after the ingestion of different meals, ligations and decapitations are generated by changes in the levels of juvenile hormone. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Mosquito; Trypsin; Gene and juvenile hormone

## 1. Introduction

Ingestion of a blood meal by the female mosquito, *Aedes aegypti*, induces the synthesis of two female-specific trypsins: early and late trypsin (Noriega and Wells, 1999). Early trypsin is synthesized in small amounts within the first 4–6 h following a blood meal and is responsible for the first phase of trypsin activity described by Felix et al. (1991) and Barillas-Mury et al. (1995). The enzymatic activity of early trypsin is an obligatory component of the signaling system that activates the synthesis of late trypsin, the major endoprote-

ase responsible for blood protein digestion (Barillas-Mury et al., 1995).

Transcription of the early trypsin gene begins shortly after adult emergence under the control of juvenile hormone (JH) (Noriega et al., 1997; Edgar et al., 2000). Abdominal ligation within 1 h of emergence completely prevents the transcription of the early trypsin gene (Noriega et al., 1997). Low doses of a JH analogue (methoprene) or higher doses of JH III restore the expression of the early trypsin gene in ligated abdomens (Noriega et al., 1997). Despite the high levels of early trypsin mRNA in sucrose-fed or starved females, translation of the mRNA occurs only after a blood or protein meal (Noriega et al., 1996b). After the ingestion of a blood meal there is a rapid decrease in early trypsin mRNA levels, and the transcript levels remain low for the next 2 days (Noriega et al., 1996b).

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In this study we tested the hypothesis that factors that affect the steady-state levels of early trypsin mRNA do so by influencing the levels of juvenile hormone. We investigated the effect of ingesting different meals on early trypsin mRNA levels as well as on JH levels. We also studied how early trypsin mRNA levels changed when the midgut was isolated from different components of the neuroendocrine system by abdominal ligation and decapitations.

Juvenile hormone levels might play a central role in establishing a connection between the composition of the meal, the activity of some components of the neuroendocrine system and the regulation of digestive and reproductive physiology of the female mosquito. We propose that the level of early trypsin mRNA is a molecular marker for JH levels.

## 2. Materials and methods

### 2.1. Insects

*Ae. aegypti* of the Rockefeller strain were reared at 30°C and 80% relative humidity under a 16 h light:8 h dark photoperiod regime.

### 2.2. Materials

Guanidinium isothiocyanate and sodium *N*-lauroylsarcosine were purchased from Fluka (Buchs, Switzerland); 2-mercaptoethanol and phenol from Amresco (Solon, OH); ultra-pure agarose and RNA electrophoresis markers were obtained from GIBCO-BRL (Gaithersburg, MD); RNA-binding glass powder (RNAid™ Kit) was purchased from BIO 101 (La Jolla, CA).

### 2.3. Mosquito meals

Mosquitoes were fed using an artificial feeder as described by Kogan (1990). Pig blood was supplemented with 2 mg/ml of isoleucine and ATP. Protein meals were 100 mg/ml solutions of pig albumin (fraction V), pig  $\gamma$ -globulin or pig hemoglobin. Grace's amino acid solution (a 20 natural amino acid mix) (21 mg/ml), latex beads (0.460  $\mu$ m diameter) and sucrose (100 mg/ml) were from Sigma. All meals were prepared as 100 mM NaHCO<sub>3</sub> and 100 mM NaCl, pH 7.0 solutions, equilibrated to 37°C, and ATP was added to a final concentration of 1 mM immediately before use.

### 2.4. Stage of ovarian development

The ovaries were isolated by tearing the soft cuticle between the fifth and sixth abdominal sternites, pulling off and placing the terminal segments in a drop of saline.

Ovaries were examined under a dissecting microscope using an ocular micrometer.

### 2.5. Measurement of juvenile hormone levels by radioimmunoassay (RIA)

The RIA was performed, with some modifications, as described by Goodman et al. (1995). Briefly, after organic solvent extractions, samples were purified using silica Sep-Pak cartridges (Waters Corp, Mildford, MA) and normal phase silica thin-layer chromatography plates. Sudan Black B (Sigma, St Louis) was added as an internal marker, and recoveries were estimated by spectrometry at 600 nm.

### 2.6. Abdominal ligations, decapitations and enemas

Females were anaesthetized on ice and abdominal ligations were performed by tying a fine thread at the base of the abdomen and removing the anterior portion as described by Hagedorn et al. (1977). The wound was sealed with tackiwax (Boekel Industries, Philadelphia, PA). Ligated abdomens were kept in a humidified chamber (Hagedorn et al., 1977). Decapitations were performed in a similar fashion by tying a fine thread around the neck, removing the head and sealing the wound with tackiwax (Briegel and Lea, 1979). Immobilized females received enemas using a glass needle (Briegel and Lea, 1975).

### 2.7. RNA isolation and characterization

Total RNA was isolated from females using RNA-binding glass powder (Noriega and Wells, 1993). RNA was separated by electrophoresis on 1.5% agarose gels under denaturing conditions using the formaldehyde method (Fourney et al., 1988). The RNA was transferred to Nytran (Schleicher & Schuell, Keene, NH) and hybridized with an early trypsin cDNA (Noriega et al., 1996a) or a late trypsin cDNA (Barillas-Mury et al., 1991) labeled using a Random Primers Kit (BRL) and ( $\alpha$ -<sup>32</sup>P-)dATP (ICN, Irvine, CA). Hybridizations were done under high stringency conditions (Noriega and Wells, 1993). The amount of radioactivity bound to individual samples on the filter was quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) (Edgar et al., 2000).

## 3. Results

### 3.1. Effect of ingestion of different meals on the early trypsin mRNA steady-state levels

In these experiments we investigated how the composition of the meal affected the steady-state levels of early

trypsin mRNA. In addition, we evaluated the effect of the ingested meals on late trypsin mRNA steady-state levels and oocyte development. All artificial meals were readily ingested. Only insects fully engorged were used.

We observed three distinctive patterns of changes in the steady-state levels of early trypsin mRNA (Fig. 1).

### 3.1.1. Pattern 1

Feeding blood or any of several single protein meals (100 mg/ml of albumin, hemoglobin or  $\gamma$ -globulin) resulted in a rapid decrease in the steady-state levels of early trypsin mRNA. Transcript levels remained low for more than 24 h. These protein meals induced translation of early trypsin (Noriega et al. 1996b, 1999), as well as transcription (Fig. 2) and translation of late trypsin (Barillas-Mury et al. 1991, 1995; Noriega et al., 1994).

Oocyte development was evaluated from groups of 20 females every 24 h for 4 consecutive days. Each day the development of the ovaries was staged according to Christophers' stages as described by Clements and Boocock (1984). The incorporation of yolk was evident in blood-fed females at 24 h, and oocytes that contained yolk were scored as "matured oocytes". After 96 h all the females fed on blood had mature oocytes; and 98% of the females fed on protein (albumin) had mature oocytes (Fig. 3).

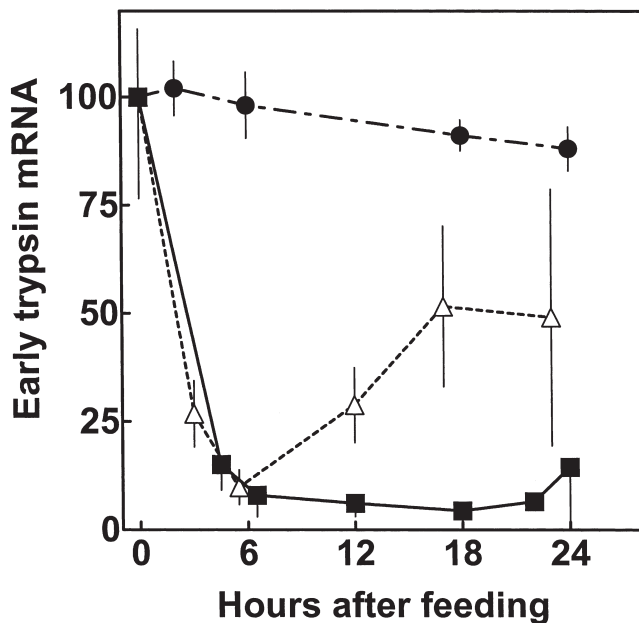


Fig. 1. Effect of feeding different meals on early trypsin mRNA levels. Time-course of early trypsin mRNA levels after feeding mosquitoes on: saline (100 mM  $\text{NaHCO}_3$  and 100 mM  $\text{NaCl}$ , pH 7.0) (●); an amino acid solution (21 mg/ml) (△) or albumin (100 mg/ml) (■). Each point represents the mean  $\pm$ SD of three independent assays of three groups of five mosquitoes. Early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (unfed mosquito).

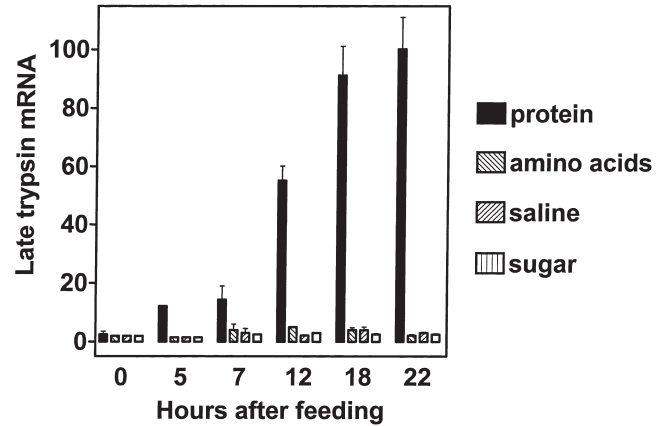


Fig. 2. Effect of feeding different meals on late trypsin mRNA levels. Time-course of late trypsin mRNA levels after feeding mosquitoes on: protein (albumin, 100 mg/ml), amino acid solution (21 mg/ml), saline or sucrose (100 mg/ml). Values represent the mean  $\pm$ SD of three independent assays of three groups of five mosquitoes. Late trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of late trypsin mRNA after background subtraction, multiplied by 100/maximum value (protein 22 h).

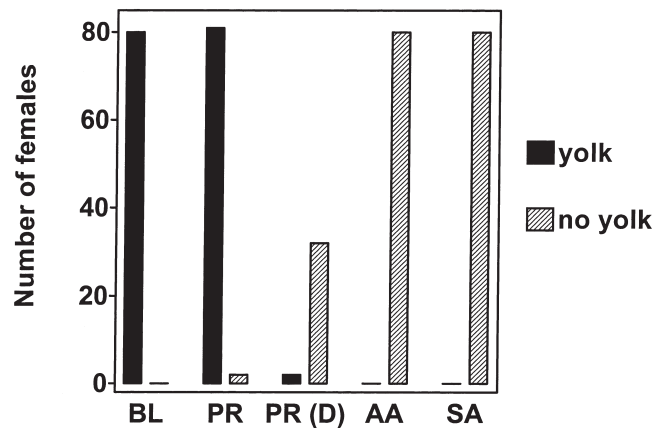


Fig. 3. Effect of feeding different meals and decapitation on yolk deposition in the oocyte. Females were fed on blood (BL), albumin (100 mg/ml) (PR), amino acid solution (21 mg/ml) (AA) or saline (SA). Oocyte development was evaluated from groups of 20 females every 24 h for 4 consecutive days. An additional group of females was fed on protein, decapitated within 5–10 min after feeding and the development of oocytes was examined 24 h later (PR (D)). Results are expressed as accumulative number of females with yolk (solid bars) or without yolk (hatched bars).

### 3.1.2. Pattern 2

Feeding an amino acid meal resulted in a rapid decrease in early trypsin transcript levels, but 12–18 h after feeding early trypsin mRNA levels significantly increased again. This meal induced only the early phase of trypsin synthesis (Noriega and Wells, 1999). There was translation of early trypsin mRNA (Noriega et al., 1999), but late trypsin transcription was not activated (Fig. 2). Ninety-six hours after feeding, none of the 80 females studied had incorporated yolk in their oocytes (Fig. 3).

### 3.1.3. Pattern 3

Feeding saline, sugar solutions (100 mg/ml of sucrose, trehalose or lactose) or latex beads solutions ( $1.8 \times 10^8$  particles/ml) did not cause a significant decrease in the steady-state levels of early trypsin mRNA. These meals did not induce translation of early trypsin (Noriega et al., 1999). Late trypsin transcription was not induced by saline or sucrose (Fig. 2) and oocytes were not developed after feeding saline (Fig. 3).

### 3.2. Effect of feeding different meals on juvenile hormone levels

Juvenile hormone levels were measured by RIA. Sudan Black B was added as an internal marker, and recoveries were estimated by spectrometry at 600 nm. Using [ $^3\text{H}$ ]-JH we found that there was a linear relation between the amounts of dye and JH recovered. Using whole mosquito samples, the average recovery of dye was 33% (most values were between 30 and 40%). The working range of the assays was between 10 and 300 pg per tube. Most of the values obtained were between 30 and 40 pg, which, after correction for recovery, were in the range of values published by Shapiro et al. (1986). Triplicate samples of 50 females were analyzed at several times after feeding a blood or an amino acid meal (Fig. 4A). Six hours after feeding blood, JH levels decreased drastically and remained low for the first 24 h. Six hours after feeding amino acids, JH levels were reduced significantly but they increased by 24 h (Fig. 4A). Hormone levels and early trypsin levels were measured from different pools of insects. When we analyzed the relationship between early trypsin mRNA levels (Fig. 1) and JH levels (Fig. 4A) from insects at similar times after blood or amino acid meals, we found a direct correlation ( $r^2$  0.78,  $P < 0.001$ ).

### 3.3. Effect of abdominal ligation and decapitation on early trypsin mRNA steady-state levels

We isolated the midgut from different components of the neuroendocrine system. Decapitation removes the cerebral neurosecretory system but leaves the corpora allata (CA) intact. Isolation of the abdomen by severing the body between the metathorax and the first abdominal segment removes both the CA and the brain.

Abdominal ligation of unfed females at 1, 2 and 3 days after emergence resulted in a significant decrease in early trypsin mRNA levels (Fig. 5A), implying that the CA is essential at all times for the maintenance of the high steady-state levels of early trypsin mRNA.

To confirm a role for JH, abdomens from 3-day-old unfed females were ligated and methoprene (a JH analogue) was applied topically. Twenty-four hours later, early trypsin mRNA levels from methoprene-treated abdomens were significantly higher than from

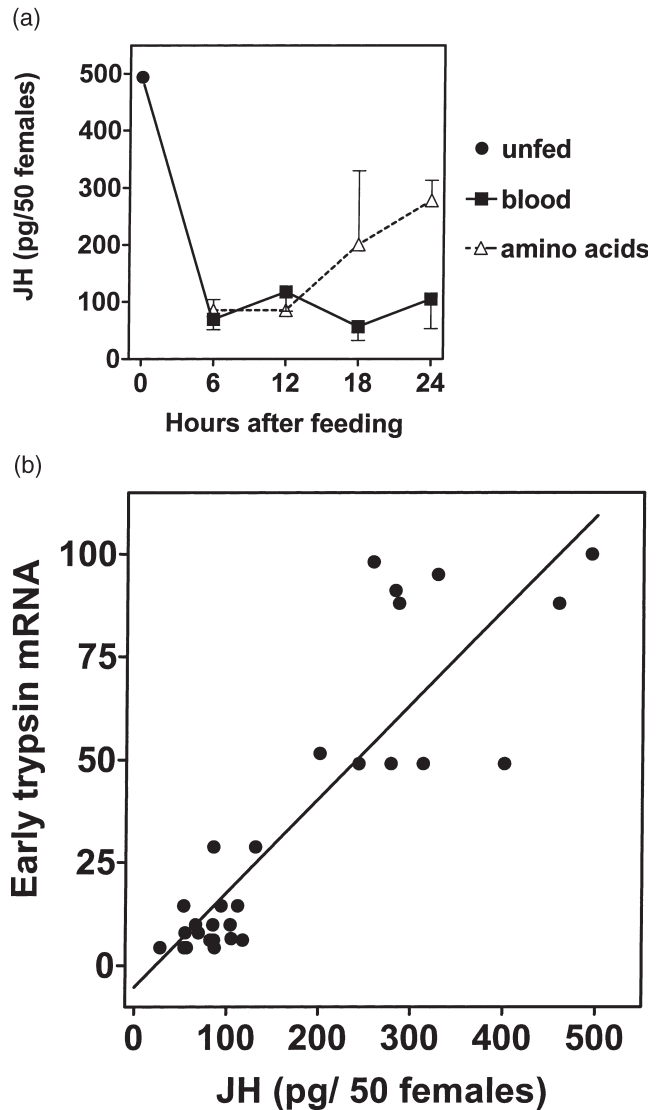


Fig. 4. (a) Effect of feeding different meals on juvenile hormone (JH) levels. JH levels were measured by RIA. Time-course of JH levels after feeding mosquitoes on: an amino acid solution (21 mg/ml) ( $\Delta$ ) or albumin (100 mg/ml) ( $\blacksquare$ ). Each point represents the mean  $\pm$  SD of three independent assays of groups of 50 mosquitoes. JH levels are expressed as picograms of JH per 50 females. (b) Relationship between the early trypsin mRNA levels and the JH levels. Early trypsin mRNA levels were measured by Northern blot and JH levels were measured by RIA from pools of insects at similar times after blood or amino acid meals. The line is a linear regression line ( $r^2$  0.78,  $P < 0.001$ ).

abdomens that were applied topically with acetone ( $P < 0.01$ ) (Fig. 5B).

Fig. 6 shows the effect of decapitation of unfed females on the early trypsin mRNA levels. Decapitation as early as 1 h after emergence did not prevent the normal increase in early trypsin mRNA. When females were decapitated at 1, 2 or 3 days after emergence, early trypsin mRNA levels were always similar to those of the non-decapitated controls.

We also explored the role of the "head" in the regulation of early trypsin mRNA steady-state levels after

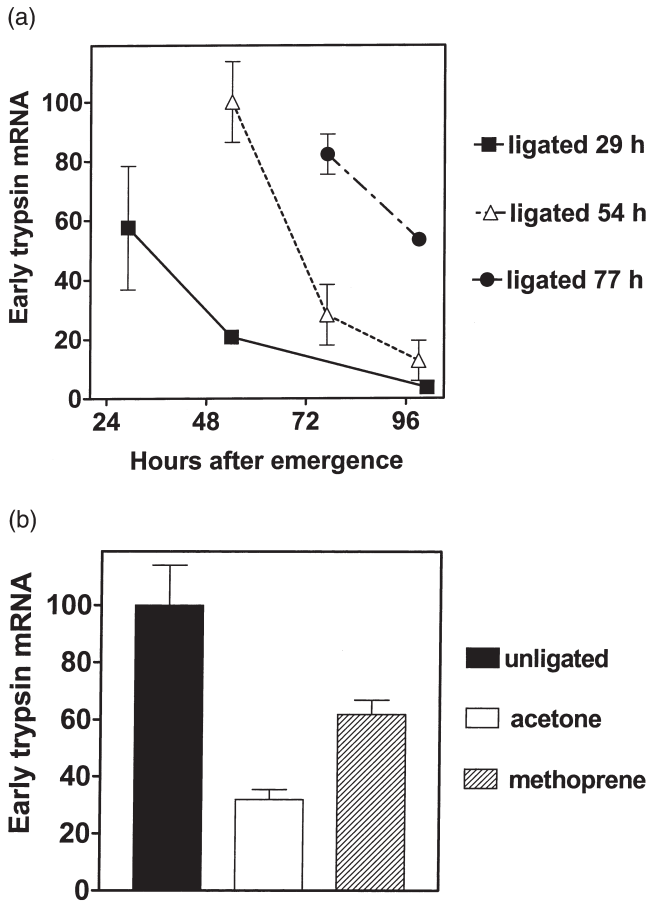


Fig. 5. (a) Effect of abdominal ligation on early trypsin mRNA levels. Abdomens were ligated at different times after emergence: (■), ligated at 29 h; (△), ligated at 54 h; (●), ligated at 77 h. Early trypsin mRNA levels were assayed at different times after ligation. Each point represents the mean±SD of three independent assays of three groups of five abdomens. Early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (54 h control). (b) Effect of methoprene on the early trypsin mRNA level after abdominal ligation. Abdomens of 3-day-old females were ligated and topically applied with methoprene (500 ng) or acetone (1 µl). Twenty-four hours after the ligations the levels of early trypsin mRNA were measured by Northern blot hybridization. Each value represents the mean±SD of three independent assays of three groups of five mosquitoes. Early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (unligated females).  $P < 0.01$ .

feeding. Fig. 7 shows three experimental conditions: (1) controls were fed a protein meal, (2) females were decapitated within 5 min after feeding a protein meal, and (3) females were decapitated, and then provided a protein meal by enema. Early trypsin mRNA steady-state levels decreased during the first hours after feeding in all three treatments, but 24 h after feeding, transcript levels were significantly higher in decapitated females than in controls.

Fig. 8 shows the effect of decapitation on late trypsin

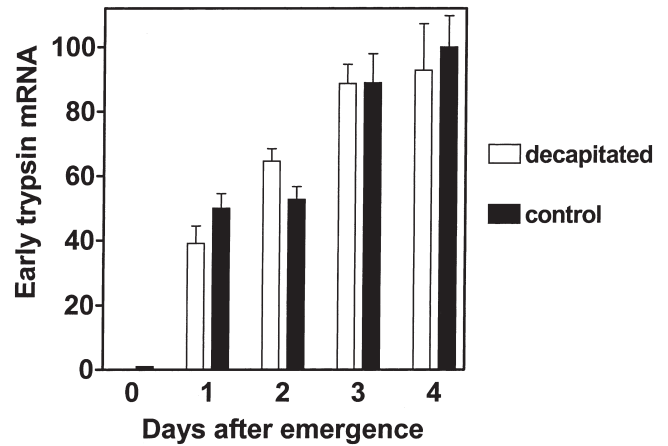


Fig. 6. Effect of decapitation on early trypsin mRNA levels in females before feeding. Females were decapitated at different times after adult emergence. Early trypsin mRNA was analyzed 24 h after decapitation (open bars). Controls were not decapitated (filled bars). Each point represents the mean±SD of three independent assays of three groups of five mosquitoes. Relative early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (4-day-old controls).

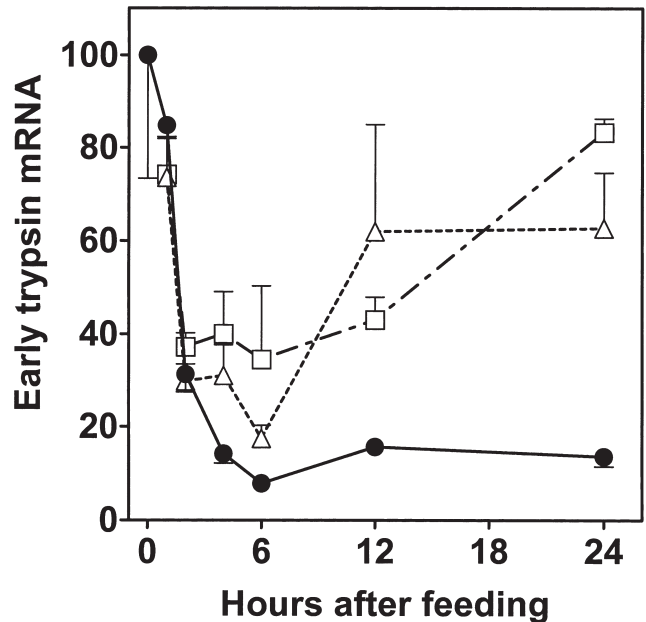


Fig. 7. Effect of decapitation on early trypsin mRNA levels in females after feeding. Levels of early trypsin mRNA were analyzed after feeding under three experimental conditions: (●) controls were fed a protein meal (100 mg/ml albumin); (△) females were decapitated within 5 min after feeding a protein meal; and (□) females were decapitated, and then provided a protein meal by enema. Each point represents the mean±SD of three independent assays of three groups of five mosquitoes. Early trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (unfed females).

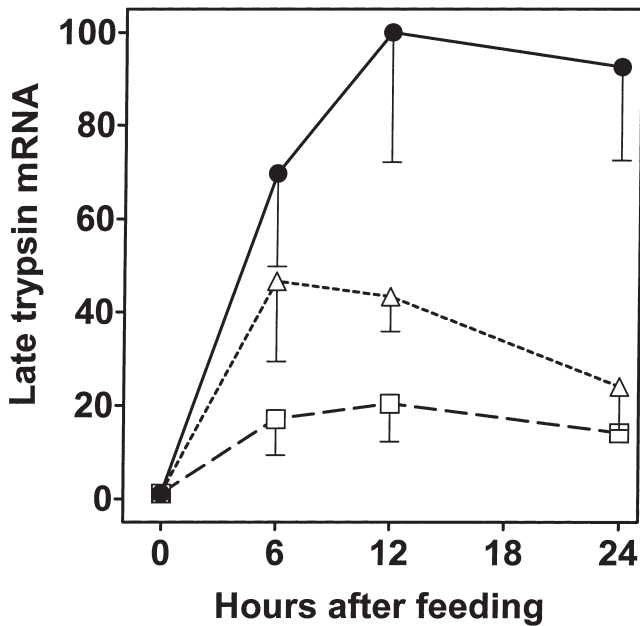


Fig. 8. Effect of decapitation on late trypsin mRNA levels in females after feeding. Levels of late trypsin mRNA were analyzed after feeding under three experimental conditions: (●) controls were fed a protein meal (100 mg/ml albumin); (△) females were decapitated within 5 min after feeding a protein meal; and (□) females were decapitated, and then provided a protein meal by enema. Each point represents the mean  $\pm$  SD of three independent assays of three groups of five mosquitoes. Late trypsin mRNA levels are expressed as proportion of the maximum value observed, and are presented as cpm of early trypsin mRNA after background subtraction, multiplied by 100/maximum value (females 12 h after feeding).

mRNA steady-state levels under the three experimental conditions described above. Late trypsin mRNA levels 24 h after feeding were significantly lower in decapitated animals when compared to controls.

Decapitation within 5–10 min after feeding a protein meal prevented the normal development of oocytes (Fig. 3).

#### 4. Discussion

Juvenile hormone titers must be modulated to permit the normal progress of development and reproduction. In the female mosquito, juvenile hormone signals that ecdysis to the adult has been completed and reproductive processes should begin, inducing simultaneous changes in the expression of many genes in different tissues.

Juvenile hormone regulates the expression of the early trypsin gene in the *Ae. aegypti* midgut (Noriega et al., 1997; Edgar et al., 2000). The changes in the steady-state levels of early trypsin mRNA after a blood meal (Noriega et al., 1996b) follow exactly those changes described for JH levels (Shapiro et al., 1986). When a female mosquito takes a blood meal, both the JH and early trypsin mRNA levels fall rapidly during the first 3

h and reach their lowest point 24 h after the blood meal. By 48 h after the blood meal, levels start to rise again, and after 96 h are equivalent to the pre-blood meal value (Shapiro et al., 1986; Noriega et al., 1996b).

In the experiments presented in this paper we showed that feeding different meals had three distinct effects on the changes of steady-state levels of early trypsin mRNA: (1) blood and protein meals caused the level to decrease drastically and remained low for at least 24 h, (2) amino acid meals caused a transient decrease in the mRNA level, but it returned to high levels after 12–18 h, and (3) sugar, latex and saline meals had no effect on the early trypsin mRNA steady-state levels.

In addition to their effect on the early trypsin mRNA levels, the three groups of meals also had different effects on three important post-feeding physiological processes: (1) the translation of early trypsin mRNA, (2) the transcription of the late trypsin gene and (3) the deposition of yolk in the oocytes.

Translation of early trypsin is one of the first detectable events after the ingestion of a blood meal and it is a sign that the first phase of digestion has been activated (Graf and Briegel, 1989; Felix et al., 1991; Noriega and Wells, 1999). The interaction between the enzymatic activity of early trypsin and the protein meal leads to the transcriptional activation of the late trypsin gene (Barillas-Mury et al., 1995). The increase in late trypsin mRNA levels is a good molecular marker for the activation of the second phase of digestion (Noriega and Wells, 1999). Digestion of the blood proteins by midgut proteases leads to the development of the ovaries. The deposition of yolk in the oocytes indicates that the vitellogenic phase of oocyte development is progressing normally (Clements and Boocock, 1984).

Blood and protein meals activate translation of early trypsin mRNA, transcription of late trypsin and promote oocyte yolk deposition. In contrast, ingestion of meals such as saline, sugars or latex beads, which do not modify early trypsin mRNA steady-state levels, do not stimulate translation of early trypsin mRNA, transcription of late trypsin and oocyte development.

Feeding amino acid solutions activates translation of the early trypsin mRNA (Noriega et al., 1999) but fails to induce either late trypsin transcription or yolk deposition. Concurrently with translation there is a decrease in early trypsin mRNA levels, but by 12–18 h mRNA levels return to pre-feeding values.

Based on earlier studies (Noriega et al., 1997; Edgar et al., 2000) we presumed that the effects of the meals on early trypsin mRNA levels were mediated through changes in the levels of juvenile hormone. To confirm this hypothesis we measured the changes in JH levels after ingestion of a blood or an amino acid meal. In both cases the changes in JH levels corresponded with the changes observed for early trypsin mRNA steady-state levels.

We suggest that the changes in JH levels are primarily the result of changes in the biosynthetic activity of the corpora allata in response to the ingestion of different meals. Feeding is known to modify JH synthesis in cockroaches (Osorio et al., 1998). Blood feeding causes a major reduction in the biosynthetic activity of the corpora allata of *Ae. aegypti* (Borovsky and Carlson, 1992; Borovsky et al., 1992; Noriega et al., unpublished results), as well as that of *Culex pipiens* (Radio et al. 1988, 1999). The effect of other type of meals on CA activity has not been investigated.

The abdominal ligation experiments also indicate that the steady-state level of early trypsin mRNA in unfed females is dynamic and requires continual transcriptional activation by JH. This result confirms other experiments in which it was shown that injection of recombinant juvenile hormone esterase into unfed females 3 days after emergence resulted in a large but transient reduction of the early trypsin mRNA steady-state levels (Edgar et al., 2000). Additionally, a pattern of decrease of early trypsin mRNA levels followed by a rapid increase, as observed after ingesting an amino acid meal, was also observed when methoprene was applied after feeding a protein meal. This JH analogue caused the amount of early trypsin mRNA in the midgut to reach, within 12 h, levels normally observed in controls at 40–60 h (Noriega et al., 1997).

There seems to be a critical period after emergence during which the factors from the head are essential for normal development. Decapitation of *Ae. aegypti* females 20 min after adult ecdysis prevented normal development of the previtellogenic follicles (Feinsod and Spielman, 1980); however, treatment with JH analogues caused the follicles of decapitated females to develop normally. In addition, removal of the medial neurosecretory cells in *Ae. aegypti* females within 1 h after emergence prevented the maturation of eggs after a blood meal (Lea, 1967).

We previously described a critical period of 6 h after emergence during which abdominal ligations prevented the transcription of the early trypsin gene (Noriega et al., 1997). In the present studies we showed that females decapitated 1 h after adult emergence had a normal increase in early trypsin mRNA levels. Because of the delay between emergence and decapitation, we cannot exclude the possibility that factors from the head are important for stimulation of the corpora allata during the first hour after adult emergence. However, we can conclude that the continuous presence of factors from the head is not required for the post-emergence activation of transcription by juvenile hormone nor the maintenance of the steady-levels of early trypsin mRNA in unfed mosquitoes.

In contrast, factors from the head seem to play an important role in the regulation of the steady-state levels of early trypsin mRNA after blood feeding. The normal

progress of digestion and oogenesis depends on head factors. Thus, insects decapitated prior to 30 min after a blood meal showed no ovarian development (Larsen and Bodenstern, 1959). In addition, trypsin synthesis was reduced to less than half its normal output when decapitations were performed prior to, or immediately after, a blood meal (Graf et al., 1998). Our experiments showed that factors from the head are involved in maintaining the low steady-state levels of early trypsin mRNA observed 24 h after feeding. Decapitations also prevented the normal expression of the late trypsin gene and the initiation of yolk deposition in the oocyte.

In summary, there is a relationship between the composition of the meal, the regulation of JH levels, and the activation of digestion and oogenesis. We propose that (1) the effects of the meals on the changes of the steady-state levels of early trypsin mRNA are mediated by changes in the levels of JH, (2) the changes in JH levels are primarily the result of changes in the biosynthetic activity of the corpora allata in response to the ingestion of different meals, and (3) factors from the head are involved in maintaining the low steady-state levels of early trypsin mRNA observed 24 h after feeding.

A simple hypothesis based on these three observations would be that the “factors” from the head are allatregulatory molecules that are affecting the synthesis of JH by the CA and these factors are released from the head only after ingesting specific meals.

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