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Role of juvenile hormone and allatotropin on nutrient allocation, ovarian development and survivorship in mosquitoes

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Abstract

Teneral reserves are utilized to initiate previtellogenic ovarian development in mosquitoes. Females having emerged with low teneral reserves have reduced juvenile hormone (JH) synthesis and previtellogenic development. We investigated what role JH, allatotropin (AT) and other head-factors play in the regulation of previtellogenic ovarian development and adult survivorship. Factors from the head are essential for corpora allata (CA) activation and reproductive maturation. We have shown that decapitation of females within 9–12 h after adult ecdysis prevented normal development of the previtellogenic follicles; however maximum previtellogenic ovarian development could be induced in decapitated females by topically applying a JH analog. When females were decapitated 12 or more hours after emergence nutritional resources had been committed to ovarian development and survivorship was significantly reduced. To study if allatotropin levels correlated with teneral reserves, we measured AT titers in the heads of two adult phenotypes (large and small females) generated by raising larvae under different nutritional diets. In large mosquitoes AT levels increased to a maximum of 45 fmol in day 4; in contrast, the levels of allatotropin in the heads of small mosquitoes remained below 9 fmol during the 7 days evaluated. These results suggest that only when nutrients are appropriate, factors released from the brain induce the CA to synthesize enough JH to activate reproductive maturation.

Keywords: *Aedes aegypti*; Mosquito; Juvenile hormone; Allatotropin; Nutrition

1. Introduction

Oogenesis in mosquitoes is a nutrient-limited process, initiated only if sufficient nourishment is available (Briegel, 1990; Wheeler, 1996). The ovaries of *Aedes aegypti* are undifferentiated at adult emergence (Gwadz and Spielman, 1973), and their previtellogenic phase of development is initiated only when nutrients are appropriate (Briegel, 1990). Allocation of these nutritional reserves for ovarian development is life-threatening if the resources are not sufficient. Therefore females must have some mechanism to restrain ovarian development, and a hormonal control system that is activated by suitable nutritional stimuli is appropriate.

Juvenile hormone (JH) is the key hormone regulating previtellogenic ovarian development in mosquitoes (Hagedorn, 1994; Klowden, 1997). JH is synthesized and released from the corpora allata (CA), a pair of endocrine glands with nervous connections to the brain. Decapitations, CA removal and abdominal ligations were previously used to prove that the growth of the previtellogenic follicles is under control of factors from the brain and CA. (Lea, 1963; Gwadz and Spielman, 1973; Hagedorn et al., 1977).

In this study, we further investigated the role of teneral reserves, decapitations and JH on the previtellogenic development of the ovaries. Our results indicate that there is a critical period after emergence during which factors from the head direct the allocation of nutritional resources for reproductive maturation or survivorship. One of these factors might be allatotropin (AT), a peptide that

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stimulates JH synthesis (Kataoka et al., 1989; Li et al., 2003b); therefore we studied the levels of AT in the heads of females having emerged with low and high teneral reserves.

2. Materials and methods

2.1. Chemicals

The juvenile hormone analog methoprene was obtained from Zoecon Co (Palo Alto, CA).

2.2. Insects

Ae. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light: 8 h dark. Large mosquitoes were reared as described by Zhuo et al. (2004). Small mosquitoes were raised following the protocol previously described by Caroci et al. (2004). Virgin adult females were offered a cotton wool pad soaked in water.

2.3. Mosquito decapitations and analysis of follicle length and survivorship

Mosquitoes were immobilized by brief exposure to ice and decapitated with a blade at different times after adult eclosion (0, 6, 9, 12 and 24 h). Mosquitoes without heads were kept at room temperature in a humid chamber. Follicle lengths were measured under a dissecting microscope using an ocular micrometer. 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 phosphate buffer saline (PBS, 19 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.0). Survivorship was recorded every day for a total of 5 days.

2.4. Primary antibody against allatotropin

Rabbit polyclonal antisera against *Ae. aegypti* AT was produced using a synthetic peptide (Veenstra and Costes, 1999) conjugated to Keyhole limpet hemocyanin by Genemed Synthesis, Inc. (San Francisco, CA).

2.5. ELISA

Mosquito heads were collected by decapitation. Samples were homogenized in 80% acetronitrile and centrifuged at 14,000 g for 10 min at 4 °C. Supernatants were recovered and stored at −80 °C. Standard curves were produced using synthetic *Ae. aegypti* AT synthesized by Biopeptide Company (San Diego, CA).

An indirect ELISA was performed following the protocol described by Audsley et al. (1998) with some modifications. Mosquito samples and standards were dissolved in 20 µl of 80% CH_3CN, and coated onto wells by drying at 37 °C for 90 min, then incubating overnight at 4 °C with 100 µl of a 0.1 M sodium carbonate/sodium bicarbonate buffer (coating buffer, pH 9.6). After washing three times with 100 µl of 10 mM phosphate buffer pH 7.4/0.1% TWEEN-20 (PBS-T), 150 µl of blocking solution was added to each well (1% BSA in PBS), and the plates were incubated for another 90 min at 37 °C.

After washing 3 times with 100 µl of PBS-T, 100 µl of primary antiserum (1:5000 dilution in PBS) was added to each well, and the plate was incubated for another 1.5 h at 37 °C. Plates were washed three times with PBS-T and 100 µl of secondary antibody (1:3000 dilution in PBS of goat anti-rabbit monoclonal antibody conjugated to horseradish peroxidase) was added to each well. Plates were then incubated for 60 min at 37 °C.

After a final PBS-T wash (three times), 100 µl of substrate mixture of tetramethyl benzidine (TMB) and H_2O_2 (Pierce Technology, Inc.) was added to each well and incubated for 20 min at room temperature. The reaction was stopped by addition of 100 µl of 2 M H_2SO_4 to each well and optical density was read at 450 nm on an Elx 808 Ultra Microplate Reader (Bio-Tek Instruments, Inc. Winooski, VT).

2.6. Statistical analysis

Statistical analysis of the data was performed by *t*-test using GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA). The results were expressed as mean ± SEM, and considered significantly different at $P < 0.05$.

3. Results

3.1. Effect of decapitation and topically applied methoprene on ovarian development

Follicles from large newly emerged females were 41.5 ± 1.1 µm long ($n = 29$) and gradually increased in size to double their length by 72 h after emergence. Decapitation of females within one hour after emergence prevented the previtellogenic development of the ovaries (Fig. 1).

Humoral or nervous factors from the head are necessary for ovarian maturation, and this effect is mediated by JH. Methoprene, a JH analogue (JHA), when topically applied immediately after decapitation induced the development of previtellogenic ovary in decapitated females to levels comparable to those observed in control females (Fig. 1).

The critical period for the release of factors from the head necessary for ovarian maturation was evaluated by measuring follicle length from 72 h old females that were decapitated at different times after emergence. Factors from the head were necessary only during the first 9–12 h post-emergence, after this period, decapitation did not prevent the normal previtellogenic development of the ovaries (Fig. 2).

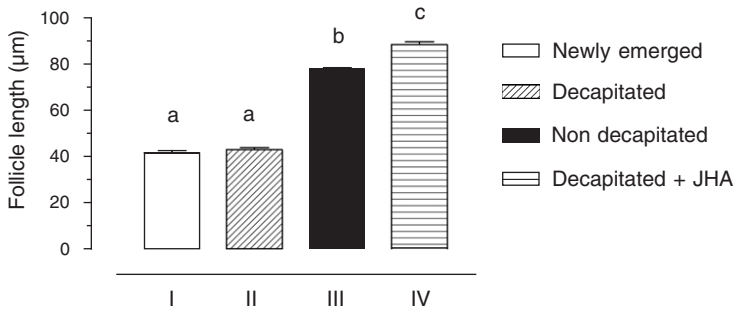

Fig. 1. Ovarian development in normal and decapitated females. The length of the terminal follicle of ovaries was measured from newly emerged females (I), and compared with the ovaries of 72 h old-females that were subjected to one of these three treatments: (II) Females were decapitated at emergence, (III) Females were not decapitated and treated with 0.5 µl of acetone, and (IV) Females were decapitated at emergence and immediately treated with methoprene (JHA) (500 ng/0.5 µl acetone). Each bar represents the mean ± SEM of at least 100 independent determinations of follicles dissected from three females. Values labeled with different letters are significantly different by Tukey's test after ANOVA at $P<0.05$.

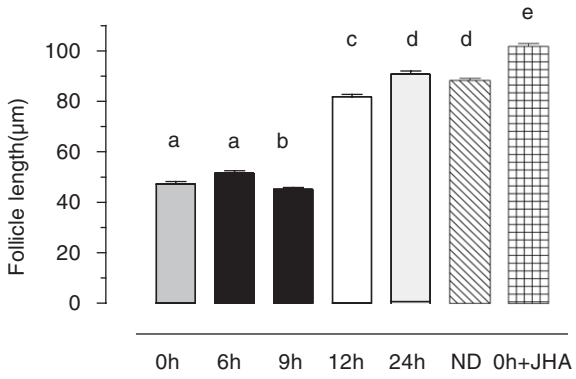

Fig. 2. Time of decapitation and ovarian development. The length of the terminal follicle of ovaries was measured in 72 h old-females after the following treatments: 0–24 h: decapitated at different hours after emergence; ND: non-decapitated; 0h + JHA: decapitated immediately after emergence and treated with methoprene (JHA) (500 ng/0.5 µl acetone). Controls (non-decapitated) were treated with 0.5 µl of acetone. Each bar represents the mean ± SEM of at least 100 independent determinations of follicles dissected from three females. Values labeled with different letters are significantly different by Tukey's test after ANOVA at $P<0.05$.

3.2. Effect of decapitation on survivorship

Decapitated females were unable to feed or drink, and therefore had to survive on the nutritional resources stored at the time of decapitation. There was a significant decrease in survivorship when females were decapitated 12 h or more post-emergence, a time when head factors have been already released to direct the allocation of nutritional resources for ovarian development (Fig. 3).

3.3. Allatotropin levels in brains of mosquitoes with different teneral reserves

We studied the levels of AT in the brains of females having emerged with low and high teneral reserves. Adult females emerged from larvae raised under a low diet protocol (small females) were approximately 74.1% smaller than females having emerged from larvae reared under a high diet protocol (large females) (wing length: small = 2.54 ± 0.05 nm, $n = 86$ vs. large = 3.42 ± 0.06 nm, $n = 84$).

We developed an indirect ELISA that showed a linear relationship and allowed detection of peptide at the fmol level (Fig. 4).

The ELISA was used to measure the changes in AT levels in heads of water-fed small and large females at different times after emergence. In large mosquitoes AT levels were between 8 and 6 fmol during the first 2 days after emergence, increased after the third day to a maximum of 45 fmol in day 4, and decreased again to 8 fmol on day 7. In contrast, the levels of AT in the heads of the small mosquito remained below 9 fmol during the 7 days evaluated (Fig. 5).

4. Discussion

Differences in the amount of reserves accumulated during the larval stages affect egg maturation in mosquitoes (Briegel, 1990). Growth of the primary follicle to the resting stage in *Ae. aegypti* is linear and reaches maximum development about 3 days after adult eclosion (Hagedorn et al., 1977). There are several reports describing correlations between nutritional reserves at adult emergence (teneral reserves), JH activity and previtellogenic egg maturation in mosquitoes (Lea, 1963; Gwadz and Spielman, 1973; Hagedorn et al., 1977; Feinsod and Spielman, 1980).

JH is the key hormone regulating previtellogenic ovarian development in mosquitoes (Hagedorn, 1994; Klowden, 1997). JH levels in *Ae. aegypti* are low at eclosion, increase during the first day after adult emergence and remain high in sugar-fed females (Shapiro et al., 1986). This initial rise in JH is essential for female's reproductive maturation (Klowden, 1997). Rates of JH biosynthesis by the CA in vitro closely reflect the levels of JH in mosquito; biosynthesis of JH is very low in newly emerged females and increases dramatically during the first 24 h after adult

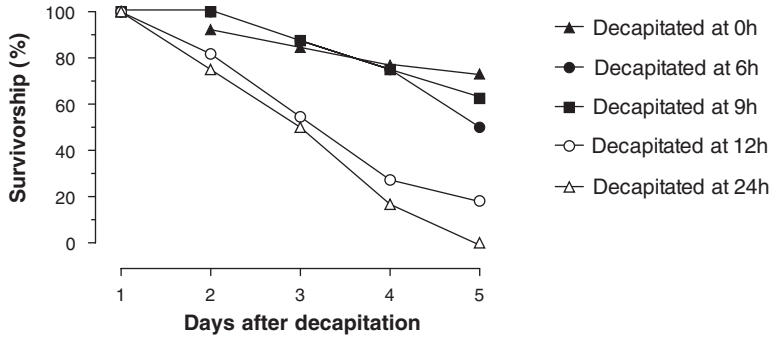

Fig. 3. Survivorship of decapitated females. Groups of 10–25 females were decapitated at different times after emergence and survivorship was evaluated every day. Each data point represents the percentage of insects alive.

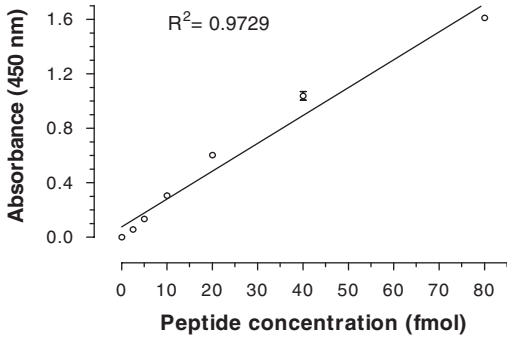

Fig. 4. ELISA standard curve. Standard curve for *Ae. aegypti* allatotropin. Each data point represents the mean ± SEM of three independent determinations of individual samples.

eclosion (Li et al., 2003a). There is a relationship between teneral reserves and CA activity; we previously described that the biosynthetic activity of the *Ae. aegypti* CA is significantly reduced in females emerged with low teneral reserves (Caroci et al., 2004).

Mosquito CA activity is controlled by factors present in the head (Li et al., 2004), and nutritional signals might affect their release resulting in the activation or inhibition of JH synthesis. Decapitation within 1 h of emergence or CA removal soon after eclosion prevents ovarian previtellogenic growth. Topical application of a JH analog stimulates normal growth of previtellogenic ovaries in decapitated or CA-ablated teneral females (Gwadz and Spielman, 1973; Hagedorn et al., 1977). We confirmed the role of the brain and JH on ovarian development by topically applying a JH analogue which stimulated previtellogenic ovarian development in decapitated mosquitoes. Follicles of these methoprene-treated decapitated females attained normal previtellogenic growth.

Factors from the head seem to be necessary only during the first 9–12 h; after this period decapitation did not prevent the normal previtellogenic development of the ovaries. Removal of the medial neurosecretory cells (mnc) from *Ae. aegypti* immediately after adult emergence suppresses egg maturation; in older females mnc ablation has little effect (Lea, 1967).

We have previously described that the CA of a newly emerged mosquito needs to be exposed to *Ae. aegypti* AT before it is capable of synthesizing JH (Li et al., 2003b), in addition, studies using antibodies against *Ae. aegypti* AT showed that this peptide is present in cells of the brain of *Aedes* and *Anopheles* mosquitoes (Hernández-Martínez et al., 2005). It is reasonable to hypothesize that AT is one of the factors from the head that are essential for reproductive maturation. We studied AT levels in the brains of normal and nutrient-deficient mosquitoes. In large females, AT levels in the head increased several folds during the first 4 days after emergence. This increase was not observed in the small nutrient-deficient mosquitoes.

Our decapitation experiments suggest that the nutritional activation of the CA occurs during the first 9−12 h after emergence. Nutritional deficient mosquitoes have reduced CA activity during the first day after the imaginal molt (Caroci et al., 2004); however AT levels were not significantly reduced in the heads of small mosquitoes during this period of time. The reduced CA activity in small mosquitoes can be explained by reductions of the release of peptides by the brain or alternatively, a decrease in the responsiveness of the CA to the peptide.

The physiological significance of the increase of AT levels at days 3 and 4 remains to be explained, and might be related to other regulatory functions of the peptide. Allatotropin is a cardioacceleratory peptide in *Manduca sexta* (Veenstra et al., 1994) and *Pseudaletia unipuncta* (Koladich et al., 2002). Veenstra and Costes (1999) reported that *Ae. aegypti* allatotropin stimulates oviduct contractions in the blowfly *Phormia sp*. In addition, *M. sexta* allatotropin stimulates hindgut contractions in *Leucophaea maderae* (Rudwall et al., 2000), and gut motility in *Heliothis virescens* (Oeh et al., 2003).

While mosquitoes reared in the laboratory can be provided an optimal diet; in the field, mosquitoes often encounter suboptimal conditions that result in a great variability in size and reproductive potential. Females must have some mechanism to control ovarian development and hormonal regulation activated by suitable nutritional stimuli is likely to be responsible. Females that do not

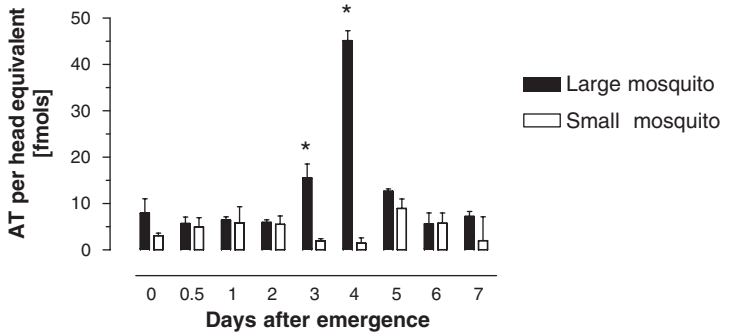

Fig. 5. Effect of nutrients on allatotropin levels. Allatotropin levels were evaluated by ELISA. Heads were dissected at different times after emergence. Values are expressed as fmol of AT in one head equivalent. Each data point represents the mean ± SEM of three independent determinations of individual samples of five heads. Asterisk above each bar indicates the value is significantly different by Tukey's test after ANOVA at $P<0.05$.

develop their ovaries relocate these resources and survive longer (Fig. 3).

In summary, there seems to be a mechanism involving a minimum threshold of nutrients that elicit the release of factors from the brain during the first 9–12 h after emergence. Inadequate larval nutrition might prevent the release of AT, the early peak of JH biosynthesis and the previtellogenic development of the ovaries.

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