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Non-cerebral ecdysiotropic and gonadotropic activities from the mosquito *Aedes aegypti* (Diptera: Culicidae)

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Abstract. The head is traditionally considered to be the site of production and release of the egg development neurosecretory hormone (EDNH), involved in ovarian maturation in dipterans. We find, however, that the thorax and abdomen of the mosquito *Aedes aegypti* each possess factors which resemble EDNH in both physiological and biochemical properties. Extracts of thoraces or abdomens each stimulated the dose-dependent appearance of ecdysteroid in incubations of *A. aegypti* ovaries in vitro. In addition, each extract is capable of stimulating ovarian maturation in vivo in decapitated *Aedes atropalpus*. The head contains higher levels of activity, absolute and specific, than either the thorax or abdomen, but extracts of all three body portions yield similar chromatographic patterns of activity. Gonadotropic activity in all three extracts elutes in two molecular weight ranges, large (approx. 6,600–7,700 MW) and small (approx. 4,150–4,250 MW). In each tissue, the small molecular weight component is more active than the large component. The roles of these non-cerebral ecdysiotropins and gonadotropins are not clear, but their similarities to cerebral EDNH suggest a function in ovarian maturation.

INTRODUCTION

Ovarian maturation in dipterans is dependent upon the integration of a number of regulators from a variety of sources. In the mosquito *Aedes aegypti*, ovaries undergo an obligatory, pre-vitellogenic development initiated by juvenile hormone (JH) from the corpus allatum (CA; Gwadz & Spielman, 1973). This JH-directed development is necessary for the subsequent ovarian response to the ecdysiotropin, egg development neurosecretory hormone (EDNH; Lea, 1972; Shapiro & Hagedorn, 1982). Ecdysteroids are essential hormonal components of normal mosquito reproductive development (Hagedorn, 1985) and are synthesized and released under the influence of peptide hormones, including EDNH, produced by cerebral neurosecretory cells (Hanaoka & Hagedorn, 1980; Lea, 1967). EDNH is released from its storage in the cerebral corpus cardiacum (CC) after stimulation of the CC by a hemolymph-borne factor from the ovary (Borovsky, 1982; Lea & Van Handel, 1982). Release of this factor follows blood feeding in *A. aegypti*. In the autogenous mosquito *Aedes atropalpus*, which produces mature follicles without blood feeding, the head is also essential for ovarian maturation (Masler et al., 1980). Decapitation, following eclosion, prevented follicle development when performed within a critical period (Masler et al., 1980). After the critical period, decapitation became increasingly less effective, suggesting that there is a timed release of a cerebral signal necessary for normal ovarian maturation (Masler et al., 1980). Evidence that *A. aegypti* EDNH would stimulate

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ovarian maturation in decapitated *A. atropalpus* was provided by the studies of Fuchs et al. (1980). Attempts at purification of EDNH have logically focussed on the head as source of the peptide (Masler et al., 1983; Wheelock & Hagedorn, 1985; Matsumoto et al., 1989; Wheelock et al., 1991). However, corpus cardiacum-associated neurosecretory structures appear to extend into the thorax of *A. aegypti* (Clements et al., 1985), EDNH activity has been reported in the thorax of *Musca domestica* (Masler & Adams, 1986), and preliminary evidence suggested the presence of non-cerebral EDNH-like activity in *A. aegypti* (Masler et al., 1991). It seemed appropriate, then, to further explore the situation in *A. aegypti*. We report here the existence and partial characterization of non-cerebral ecdysiotropic (NCE) and non-cerebral gonadotropic (NCG) factors in the thorax and abdomen of *A. aegypti*.

MATERIALS AND METHODS

Animal rearing and tissue preparation

A. aegypti (USDA Gainesville strain) and *A. atropalpus* (Bass Rock strain) were reared as described (Birnbaum et al., 1984, Masler et al., 1980). *A. aegypti* used for tissue preparation were mass-reared males and non-blood-fed females, collected 3–10 days after adult eclosion as a 1 : 1 male : female sample and stored at –20°C. *A. aegypti* were further chilled on dry ice and body parts obtained by fracture and sieving (Masler et al., 1983). Separated body parts were lyophilized and stored frozen (–20°C) until used. All body parts (heads, thoraces, abdomens) were examined prior to extraction to assure non-contamination with other body parts.

Tissue extraction

Tissues were extracted in *Aedes* saline (150 mM NaCl, 1.4 mM $CaCl_2.2H_20$, 2 mM KCl, 1.2 mM $NaHCO_3$, pH 7.2; Hayes, 1953) in an all glass homogenizer on ice. Extracts were centrifuged (1 min, 26°C, 8,000 × g) and the supernatants used for bioassay. In some experiments, homogenates were heated in a boiling water bath for 5 min., chilled on ice, centrifuged as above, and the supernatants collected for bioassay.

Bioassays

For bioassay in vivo, *A. atropalpus* females were decapitated within 2 hours of emergence to inhibit ovarian development (Masler et al., 1980) and kept from dessicating by holding in 9 × 50 mm polystyrene culture dishes (Gelman Sciences, Ann Arbor, MI) lined with moist filter paper covered with Parafilm (American National Can, Greenwich CT). The dishes were then held at 26°C, 70 percent relative humidity, and 16 hr light : 8 hr dark photoperiod. At 18–24 hours after emergence, test animals were injected intrathoracically with 1 µl of the extract. Control animals were injected with saline only. Injected animals were held in the above conditions, and examined for the presence of yolk deposition 24 hr after injection. The percent of females depositing yolk and the degree of yolk deposition were used as a measure of the gonadotropic potency of the extract. Ovarian follicles in normal, control females at 24 hours after eclosion have a mean length of approximately 0.2 mm and are nearly completely filled with yolk (vitellogenin) (Kelly et al., 1984). Only females in which all ovarian follicles are of this morphology are considered to be at the 24 hour level of development. Females in which ovarian follicles contain vitellogenin but none or only some are at the 24 hour level are considered to be intermediate in development. Quantification was done by visually scoring ovarian follicles 24 hours after injection and comparing with follicles from normal, intact females 24 hours after eclosion. Females with vitellogenic follicles (i.e., deposition of vitellogenin clearly visible) and which had developed to the 24 hour level were scored as 2. Females with no vitellogenic deposition were scored 0, and females showing intermediate follicle development (i.e., partial deposition of vitellogenin) were scored 1. The percent vitellogenic development (%Vg) was computed using the following formula:

$$\%Vg = [(N_f \times 2) + (N_i \times 1)/(N_f + N_i + N_o) \times 2] \times 100,$$

where N is the number of females showing full (f), intermediate (i) or no (o) development.

For bioassay in vitro, ovaries from 3–7 day old, non-blood-fed *A. aegypti* were dissected and rinsed in saline and incubated in 25 µl of test solution (10 ovaries/25 µl, 6 hr, 26°C, with shaking). Post-incubation medium was removed and assayed by ecdysteroid radioimmunoassay (RIA). The titer of ecdysteroid was used as a measure of ecdysiotropic activity. Details of the bioassays are found in Kelly et al. (1984) and Masler et al. (1983, 1991).

Inhibition assays

Decapitation and ligation: Females were anaesthetized on ice or with ether, and decapitated using fine iridectomy scissors. The neck was severed close to the thorax taking care not to disturb the thorax, thus removing the head (brain and CC) while leaving the thorax (CA) and abdomen intact. Ligatures were applied to the neck using human hair so as to impede humoral communication between the head and the thorax-abdomen, while leaving them physically intact. All ligatures were examined prior to scoring to assure that they were secure. Injection: intact *A. atropalpus* females were collected within 2 hours of emergence, held for 3 hours at 26°C, 70 percent relative humidity, then injected at 5 hr ± 1hr after emergence with 1 µl of crude or heated test extract. Controls were injected with saline. Animals were held as above, and scored for the presence of yolk deposition 24 hr after injection.

Chromatography and molecular weight estimation

Tissues were extracted in *Aedes* saline, on ice, at 10 mg tissue (dry weight) per 1 ml of saline using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) equipped with a PT-10 generator. Homogenates were centrifuged at 48,000 × g, 20 min., 4°C and the supernatants processed via Sep Pak C_{18} (Millipore Corp., Milford, MA) cartridge chromatography as previously described (Masler et al., 1983; 1991). The cartridge, loaded with tissue supernatant, was eluted sequentially with 4 ml each of 0.1% aqueous trifluoroacetic acid (TFA, Aldrich Chemical Co., Milwaukee, WI), 10% acetonitrile (CH_3CN, Fisher Scientific, Springfield, VA) in 0.1% TFA and 60% CH_3CN in 0.1% TFA. The 60% eluant was dried on a Speed Vac concentrator (Savant Instruments, Farmingdale, NY). The dry extract was then fractionated by high-performance size-exclusion chromatography (HP-SEC), using two Protein-Pak I-125 HP-SEC columns connected in series (7.8 × 600 mm) with an I-125 guard column attached (3.9 × 50 mm). The mobile phase was 40% CH_3CN/0.1% TFA with a flow rate of 1 ml/min. Samples were dissolved in the mobile phase, centrifuged at 8,000 × g for 1 min at room temperature, and injected onto the column. Peak detection was at 214 nm. Fractions were collected every 30 seconds from 12–24 minutes, dried in the Speed Vac, and held at –20°C until use. For bioassay, dried fractions were dissolved in *Aedes* saline. Molecular weights were estimated by comparing the elution volumes of biological activity with the elution volumes of standards of known molecular weight. Standards used were carbonic anhydrase (29, 000), cytochrome C (12,300), aprotinin (6,500), insulin chain B (3,496), all from Sigma Chemical Co. (St. Louis, MO), and locust adipokinetic hormone-I (1,159) from Peninsula Laboratories (Belmont, CA). Total protein was estimated using the BioRad protein assay kit (BioRad Laboratories, Richmond, CA). Bovine serum albumin (Sigma) was used as standard.

Radioimmunoassay

The ecdysteroid RIA was performed as described (Kelly et al., 1984; Masler & Adams, 1986). Labeled ecdysone ([23,24]-^{3}H(N); 63.5 Ci/mmol) was from New England Nuclear (Boston, MA). 20-OH-ecdysone (Sigma) was used as the unlabeled ligand.

RESULTS

In vitro kinetics

Ovaries respond in a dose-dependent fashion to increasing concentrations of crude thorax saline extract (Fig. 1a, filled circles). Stimulation occurs at concentrations > 0.005 tissue eq/µl and response is essentially linear ($r^2 = 0.986$) from 0.005 through 0.1 tissue eq/µl. Maximum response is observed at 0.1 tissue eq/µl with an average of 271 ± 30 pg of 20-OH-ecdysone equivalents detected in post-incubation medium. The 50 percent

response (135 pg) is at a dose of 0.030 tissue eq/µl. At two doses of abdomen extract (0.05 and 0.1 tissue eq/µl, open circles, Fig. 1a), ecdysteroid levels in post-incubation medium are each lower than the levels for the corresponding doses of thorax extract. Response to heated thorax extract (Fig. 1b, filled circles) is similar to the response to crude extract. The response was nearly linear (r^2 = 0.996) from 0.01 to 0.1 tissue eq/µl. Maximum response is again at 0.1 eq/µl (261 ± 37 pg) and the 50 percent response is at 0.032 eq/µl (130 pg). Heated abdominal extracts (Fig. 1b, open circles) tested at 0.05 and 0.1 eq/µl yield somewhat lower responses than the corresponding thorax preparations (Fig. 1b, solid circles). Crude head extract (Fig. 1c, solid circles) results in post-incubation ecdysteroid levels of 503 ± 48 pg at 0.1 eq/µl and 344 ± 36 pg at 0.05 eq/µl. Response levels to heated extract are consistently lower than to crude.

Ecdysteroid is detected after 1 hour of incubation in the presence of 0.1 eq/µl of heated thorax extract (Fig. 2, filled circles). The level of ecdysteroid increases linearly (r^2 = 0.989) through 12 hours, then declines through 18 hours. Ovaries incubated in saline also result in some increase in levels of ecdysteroid through 12 hours (Fig. 2, open circles).

Fractionation and in vitro assay

Gonadotropic activity is detected in preparations from heads, thoraces and abdomens (Fig. 3) fractionated on the size exclusion system. Activity from the thorax elutes at 15–15.5 ml and 16.5–18 ml, corresponding to molecular weights of 7700 ± 600 and 4150 ± 350, respectively. Activity from head extract preparations elutes from 13.5 ml through 18 ml with peaks of activity at 15–15.5 ml and 16.5–18 ml corresponding to molecular

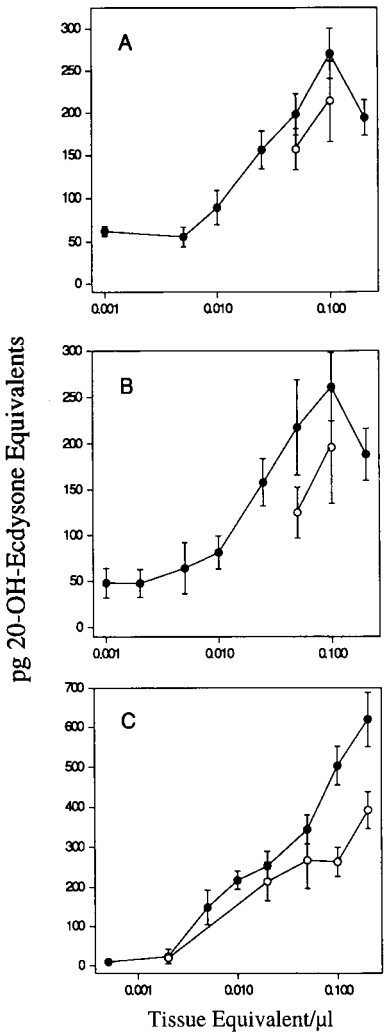

Fig. 1. In vitro dose responses of *A. aegypti* ovaries to tissue extracts. Ovaries were obtained from non-blood-fed *A. aegypti*, and saline extracts of thoraces, abdomens or heads were prepared as described in Materials and methods. Incubation conditions and RIA were as described in Materials and methods. Each data point is the mean of 3 to 46 observations ± SEM. Units are pg of 20-OH-ecdysone equivalents/6 hr/10 ovaries. Doses are tissue equivalent/µl in *Aedes* saline of either crude (A) or heated (B) extracts of thoraces (filled circles) or abdomens (open circles), or head extracts (C) which were crude (filled circles) or heated (open circles).

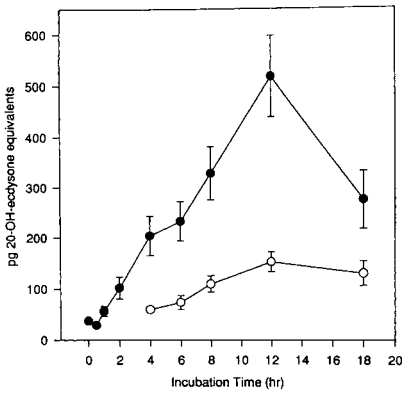

Fig. 2. Kinetics of ecdysteroid output by *A. aegypti* ovaries exposed in vitro to thorax extracts. Ovaries were obtained from non-blood-fed *A. aegypti* and incubated in vitro as described in Materials and methods. Incubation medium was collected at the times indicated and ecdysteroid quantified by RIA. Each data point represents the mean ± SEM of total ecdysteroid detected at the indicated time. Each data point represents 4–16 observations. Ovaries were incubated in either heated thorax extract (filled circles) or *Aedes* saline (open circles). All extract doses were 0.1 tissue equivalent/µl.

weights of 7700 ± 600 and 4250 ± 940, respectively. Activity from abdomen extracts elutes from 15.5–16 ml (6574 ± 506 MW) and 17–17.5 ml (4150 ± 350 MW). Specific activities for the large and small molecular weight species of each extract fractionation are given in Table 1. Within each extract type, the small molecular weight component is more active than the large component. In each molecular weight range, the head extract preparations are the most active. The large molecular weight component from the head is 13.1 times more active than the corresponding component from the thorax, and 16.7 times more active than the large abdomen component. The small molecular weight component from the head is 7.2 times and 26.4 times more active than those from the thorax and abdomen, respectively.

TABLE 1. Specific activities of large and small molecular weight gonadotropic fractions from HP-SEC of *A. aegypti* head, thorax and abdomen.

Source	Fraction	Specific activity[1]	R(S)[2]	R(L)[3]	R(S/L)[4]
Head	L	160.5	–	1	8.2
	S	1315.9	1	–	
Thorax	L	12.3	–	13.1	14.9
	S	183.2	7.2	–	
Abdomen	L	9.6	–	16.7	5.2
	S	49.9	26.4	–	

[1] The percent vitellogenic response (%Vg; see Materials and methods for formula) to a 1 µl injection of column fraction preparation was divided by the amount of protein (µg) in the injected sample; units are % Vg/µg.

[2] Ratio of the specific activity of the small component from the head to the small component from the thorax or abdomen.

[3] Ratio of the specific activitiy of the large component from the head to the large component from the thorax or abdomen.

[4] Ratio of the specific activities of the small vs large components of each source.

Inhibition in vivo

Crude and heated thorax extracts inhibit the development of vitellogenic follicles in otherwise intact females (Fig. 4). Inhibition increases with increasing doses of either

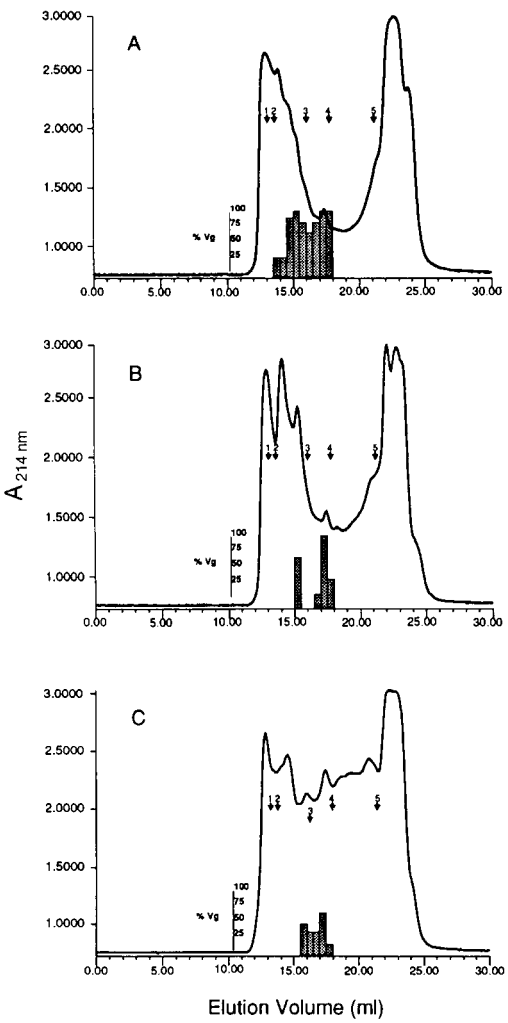

Fig. 3. Fractionation of *A. aegypti* tissue extracts and detection of vitellogenic activity in vivo. Purified heads, thoraces and abdomens were extracted in *Aedes* saline (10mg dry wt/ml), processed on the Sep Pak and fractionated by HP-SEC as described in Materials and methods. Fractions were tested in the in vivo bioassay. Chromatographic conditions were as described in Materials and methods. UV detection was at 214 nm, 3 absorbance units full scale. Biological activity (filled bars) is expressed as the percent of decapitated *A. atropalpus* females showing vitellogenic ovarian development after injection with preparations of the indicated fractions (see Materials and methods for methods of calculation). Extracts fractionated were from heads (A), thoraces (B) and abdomens (C). Numbered arrows indicate elution volumes of molecular weight standards (1, carbonic anhydrase; 2, cytochrome C; 3, aprotinin; 4, insulin chain B; 5, locust adipokinetic hormone-I).

extract, but is more pronounced with crude extract. Inhibition is detected with as little as 0.25 tissue eq/µl and increases to 30% at 0.75 eq/µl for heated extract (Fig. 4, filled circles) and to over 60% for crude extract (Fig. 4, open circles). Inhibition shows no further increase at the highest dose (1.25 eq/ul) of heated extract, but does increase to over 80% with crude extract at that dose.

Decapitation up through 7 hours post-emergence inhibits ovarian development essentially 100% (Fig. 5, D2–D7). Neck ligation inhibits development by as much as 75% (Fig. 5) when the ligation is applied by 2 hours (L2) after emergence. Inhibition decreases as the ligature is applied at later hours (Fig. 5).

DISCUSSION

Thorax extracts from *A. aegypti* contain both ecdysiotropic (Figs 1, 2) and gonadotropic (Fig. 3) activities. Ecdysiotropic activity is detected in both crude and heated extracts (Figs 1, 2) whereas gonadotropic activity is undetectable in these whole extracts (data not shown). This is in contrast with extracts of heads which show both ecdysiotropic and gonadotropic activities in crude (Masler et al., 1991) as well as more purified (Matsumoto et

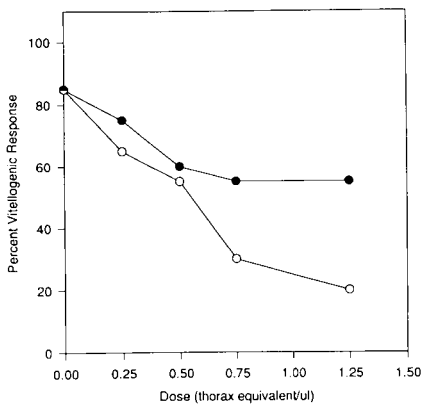

Fig. 4. Inhibition of ovarian maturation in *A. atropalpus* by thorax extracts. Intact *A. atropalpus* females were injected with *Aedes* saline (zero value) or the indicated doses of crude (open circles) or heated (filled circles) thorax extracts. Injections were done at 5 hr ± 1 hr post-emergence. Percent vitellogenic response was calculated as specified in Materials and

al., 1989; Wheelock et al., 1991) preparations. Following fractionation by HP-SEC, thorax extracts (Fig. 3b) and abdomen extracts (Fig. 3c) yield gonadotropic activity. Possibly, inhibitory materials present in these extracts are separated, by size, from the gonadotropins. This observation is similar to that made with *A. aegypti* head extracts and ecdysiotropic activities. Crude extracts and extracts fractionated by bulk, step-wise, elution showed some inhibition of activity at high doses whereas extracts fractionated by reverse phase HPLC showed no such inhibition (Masler et al., 1983). In our usual in vivo assay procedure, females are decapitated to inhibit ovarian maturation, then injected with extract in an attempt to stimulate ovarian maturation. This works regularly when head extract is used (Masler et al., 1991) but fails when either crude or heated thorax extract is used (Masler et al., unpublished observations). Indeed, when intact females are injected with thorax extract, ovarian maturation is clearly inhibited (Fig. 4). The inhibition is dosage dependent and is more pronounced with crude rather than heated extract. In contrast, neither crude nor heated head extracts show any inhibition up to 2 eq/μl (data not shown). The depression of ovarian maturation by thorax extract is probably responsible for masking the effect of gonadotropic factors in non-fractionated preparations such as ours and those of Whisenton et al. (1987). This depression may be due to specific inhibitors such as the trypsin modulating oostatic factor (Borovsky et al., 1993), non-specific inhibition resulting from a high concentration of a variety of materials in the extracts, or an inadequate level of gonadotropic factor. Nevertheless, it is clear that both ecdysiotropic and gonadotropic activities are present in the thorax and the abdomen.

The stimulation of ecdysteroid production and ovarian maturation by thorax- and abdomen-derived factors raises the question of why such activity is present in these locations. The head has long been considered as the source of ecdysiotropic and gonadotropic activities (Hagedorn, 1985), and does possess higher levels of each activity than either the thorax or abdomen (Figs 1A–C; Table 1). Nonetheless, significant levels of each activity are present in both thoraces and abdomens. This is a somewhat surprising observation, since only tissues from males and non-blood fed females are used as sources for extracts. One expects that in such animals, EDNH is not released from the cerebral corpus cardiacum storage site. However, an examination of anatomical and histological studies begins

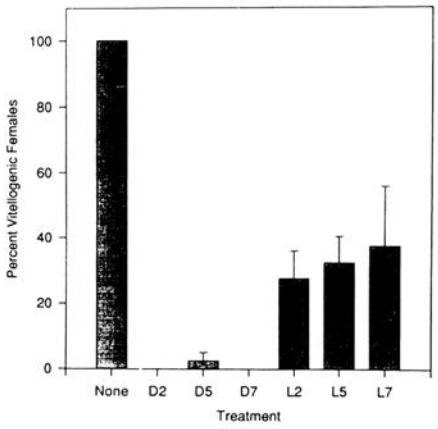

Fig. 5. Effect of decapitation and neck ligation upon ovarian maturation. *A. atropalpus* females were decapitated (D) or neck-ligated (L) at 2, 5 or 7 hr post-emergence. Controls were not treated. Vitellogenic response is expressed as the percent ± SEM of females with vitellogenic follicles at 48 hr post-emergence. Data were obtained from 10 to 40 females per treatment.

to provide some insight. Neuroanatomical observations indicate that the corpora cardiaca of mosquitos comprise a dorsal mass above the aorta in the head, and a cellular contribution to a thoracic endocrine complex, which includes the corpora allata (Burgess & Rempel, 1966). Such cells contributed by the corpus cardiacum appear to be secretory (Meola & Lea, 1972), and form part of the retrocerebral endocrine complex (Normann, 1983). The complex is described as consisting of two parts, a neurohemal organ in the head, dorsal to the aorta, and a gland complex, in the thorax, with corpora cardiaca neurosecretory cells associated with the corpora allata (Normann, 1983). Clements et al. (1985) further defined thoracic endocrine complexes in *A. aegypti* as consisting of corpora allata and groups of neurosecretory cells termed cardiacal neurosecretory cells. Neurochemical evidence is indirect. Brown & Lea (1989) reported the presence of FMRF-amide immunoreactivity throughout the central nervous system of *A. aegypti*, and AKH immunoreactivity in all abdominal as well as cerebral ganglia. Thus, neurosecretory structures distributed throughout the insect can possess the same neurochemical factors. FMRF-amide and AKH, in this example, are neuropeptides not limited to cerebral locations. The distribution of ecdysiotropic and gonadotropic activities in non-cerebral locations may, in fact, be unexceptional. That is, they may represent a widespread phenomenon of multiple locations for peptides with biochemical or immunochemical identity. However, their presence raises interesting questions, including one of function. Decapitation during a critical period immediately following eclosion prevents ovarian maturation (Masler et al., 1980). If the non-cerebral ecdysiotropic and non-cerebral gonadotropic factors in the thorax and abdomen participate in ovarian maturation and are independent of the head, one could speculate that some development might occur. Decapitation through 7 hours after eclosion, however, eliminates development (Fig. 5). If the humoral connection between the head and the thorax/abdomen is removed but the neural connection is not severed, by substituting ligation for decapitation, then some development occurs even when the ligature is applied within 2 hours of eclosion (Fig. 5). This suggests that the presumptive release of NCE or NCG may be under the influence of neural, rather than humoral, signals from the head. The distinct possibility exists that the ecdysiotropic and gonadotropic activities which we have found outside of the head are not involved with ovarian maturation in situ, but have some other purpose of

which we are not yet aware. Homogenates and extractions can reveal activity but not function. Isolation and characterization of these factors are necessary for the full exploration of their functions. Clearly, our results show that the head is not the sole province of ecdysiotropic and gonadotropic factors in *A. aegypti*. The elucidation of their roles will be the focus of future research.

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