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## Embryonic ecdysteroids in a mole crab, *Emerita asiatica* (Milne-Edwards)

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Using radioimmunoassay (RIA) and high performance liquid chromatography (HPLC), the presence of a complex mixture of free and conjugated ecdysteroids is reported in the embryonated eggs of a mole crab, *Emerita asiatica*. From an initial low value of 6.5 ng/g egg wet weight in stage I, the total ecdysteroids increased in concentration to 15.2 ng/g egg wet weight in stage III. This was followed by a sharp fall in stage IV, but again increased to 15.0 ng/g egg wet weight in stage VI. After a further decline in stage VII, the total ecdysteroids registered the highest value of 36.2 ng/g egg wet weight in stage VIII. This value, however, declined to a low level in the prehatching stage (IX). The concentration of the free ecdysteroids always predominated over the conjugated ones. The HPLC analysis of free ecdysteroids demonstrated the presence of 20-hydroxyecdysone and ecdysone in the ratio of 2.5. Purified lipovitellin II also contained free and conjugated ecdysteroids. The functional significance of the embryonic ecdysteroids as well as their nature of synthesis and storage within the eggs is discussed in the light of the information available on insect embryogenesis.

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### 1. Introduction

Ecdysteroids, a group of polyhydroxy steroids, occur in all arthropod groups serving the function of moulting hormones or morphogenetic hormones (Bückmann 1989). They are also known to exist in other invertebrate groups such as the helminths (Nirde *et al* 1983; Mendis *et al* 1983, 1984), leeches (Sauber *et al* 1983; Welter *et al* 1986) and even in coelenterates (Sturaro *et al* 1982; Guerriero and Pietra 1985), where their functions are not definitely known. However, extensive studies on the occurrence and physiological functions of ecdysteroids in insects have suggested an additional role in reproduction (Hoffmann *et al* 1986; Hagedorn 1989) and embryogenesis (Sall *et al* 1983). Although the prothoracic gland forms the primary source of ecdysone secretion, other sites such as the ovary (follicle cell epithelium),

epidermis and even the oenocytes of the insects have been reported as sources (Delbecque *et al* 1990).

The role of ecdysteroids in crustacean moulting is well-documented (Jegla 1990; Chang 1993). The ectodermally derived Y-organ forms the site of ecdysteroid synthesis in all crustaceans studied so far. The role of ecdysteroids in reproduction has also been pointed out in the prawn *Palaemon serratus* (Lanot and Clédon 1989) and in an amphipod *Orchestia gammerella* (Blanchet *et al* 1979). Supportive evidence for the reproductive functions of ecdysteroids as suggested by these authors is, however, lacking in other crustaceans. Nevertheless, there are reports of the occurrence of embryonic ecdysteroids in several decapod crabs (Chaix and De Reggi 1982; Lachaise and Hoffmann 1982; Okazaki and Chang 1991) and shrimps (Spindler *et al* 1987; Wilder *et al* 1990; Chang 1993), as also in several insect species

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where they influence the developmental programme during embryonic exuviation (Hoffmann *et al* 1986; Lanot *et al* 1989). The source of ecdysteroids present during embryogenesis of crustaceans as well as their function are still controversial.

The present paper reports on the extraction, separation and quantification of different ecdysteroid species occurring in the developing embryos of the mole crab *Emerita asiatica*. The objective of this study is also to indicate the source and functions of the ecdysteroids in crustacean embryogenesis.

## 2. Materials and methods

### 2.1 Experimental animal

The mole crabs *E. asiatica* were collected from Elliot's beach, Chennai. At least 80 crabs per collection were hand-picked and brought to the laboratory. The females were identified by the occurrence of three pairs of pleopodal hairs. Embryos belonging to the different stages of development were identified based on the criteria given by Subramoniam (1991).

### 2.2 Sample preparation

The crabs were washed with filtered sea water to remove the adhering sand particles. Eggs were removed from the pleopodal hairs using a fine forceps. Embryos belonging to the different stages of development were blotted with a filter paper to remove water and weighed. Two grams of the first stage eggs were homogenized with 10 ml of Tris-HCl buffer (20 mM Tris, pH 7.4, 5 mM EDTA). After centrifuging the homogenate at 8000 *g* for 15 min at 4°C, the clear supernatant was collected and used for the chromatographic purification of the major yolk protein (lipovitellin).

### 2.3 Purification of lipovitellin

The egg supernatant (3 ml) was separated on Sephadex G-200 gel filtration column (60 × 2 cm, Pharmacia, Sweden) and purified using the procedure described earlier (Tirumalai and Subramoniam 1992). Lipovitellin II (Lv II) was identified based on its electrophoretic mobility, biochemical nature, subunit pattern on SDS-PAGE (Tirumalai and Subramoniam 1992), its cross reactivity with *E. asiatica* antivitellinogen antibodies in immunodiffusion and immunoelectrophoresis (Tirumalai 1996).

### 2.4 Extraction and quantification of ecdysteroids

Total lipid was extracted (Folch *et al* 1957) from a known quantity of embryos and also from the purified Lv II using chloroform : methanol (2 : 1, v/v). The extract was filtered through Whatman No. 1 filter paper and

the non-lipid residue was re-extracted 2–3 times with chloroform : methanol (2 : 1, v/v). The extracts were pooled and dried by flushing with gaseous nitrogen and the dried lipid residues were weighed gravimetrically.

To extract the ecdysteroids from the total lipids, a known quantity of the lipid residue was treated twice with 88% methanol in water (v/v). An equal volume of *n*-hexane was added to each methanolic extract and mixed on a vortex mixer. After standing for 10 min at 4°C, it was centrifuged at 6000 *g* for 5 min and the hexane phase was discarded. The methanolic fractions were again extracted 2–3 times with an equal volume of *n*-hexane and the hexane phase was discarded. Finally, the methanolic phases were dried in a rotary SpeedVac vacuum concentrator (Savant, USA) and stored at –18°C until use.

Fractionation of ecdysteroids was carried out on Sep-Pak C18 HPLC column (Waters/Millipore, Germany) according to Espig *et al* (1989). Polar and apolar ecdysteroid conjugates were completely hydrolyzed with porcine liver esterase for 16 h at 37°C and again subjected to Sep-Pak C18 fractionation (Thiry and Hoffmann 1992). Radioimmunoassay (RIA) was used for quantification of the ecdysteroid following the procedure detailed in Hoffmann *et al* (1981), using the antiserum prepared according to the method of Soumoff *et al* (1981). Values of cross-reactions of antiserum were ecdysone = 1.0; 20-hydroxyecdysone, makisterone, inokosterone = 0.9; 3-dehydroecdysone ≥ 0.1, cholesterol = 0. Intact ecdysteroid conjugates did not bind to the antiserum.

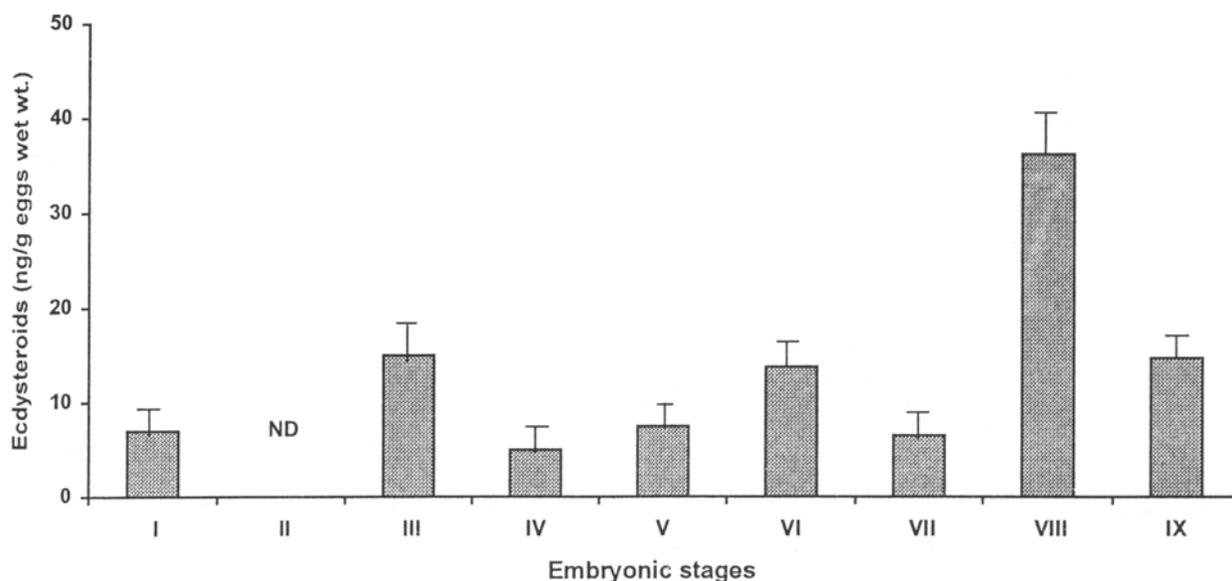
### 2.5 Analysis of free ecdysteroids by HPLC

To separate the free ecdysteroids, aliquots of 60% methanol eluents from the Sep-Pak fractionation were pooled and flushed with nitrogen, dissolved in 100 µl of 18% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) and further fractionated on a supersphere RP-18 column (250 × 4 mm; Merck, Germany). The following operating conditions were used: a linear gradient of 18–40% acetonitrile in water with 0.1% TFA for 40 min at a flow rate of 1 ml/min. All ecdysteroids were monitored at 254 nm. Fractions were collected every 30 s and were analysed by RIA.

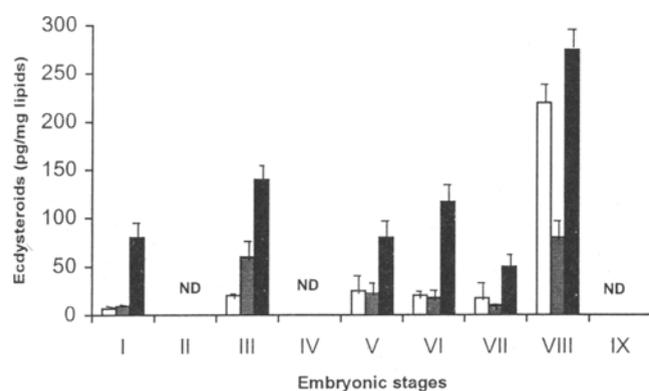
The data obtained were subjected to analysis of variance to test for significance.

## 3. Results

Figure 1 shows the quantitative determination of total ecdysteroids in different stages of embryonic development in *E. asiatica*. In stage I, the total ecdysteroid level was found to be 6.5 ng/g of egg wet weight. In this stage, the free ecdysteroids (80.7 pg/mg lipid) predominated over the polar and apolar conjugates (figure 2). In stage



**Figure 1.** Changes in total ecdysteroid concentrations in the developing embryos of *E. asiatica* (mean  $\pm$  SD;  $n=4$  except in stage IX). ND, Not done.



**Figure 2.** Separate determination of the titres of conjugated and free ecdysteroids during embryonic development of *E. asiatica*. (□), Apolar conjugate; (▨), polar conjugate; (■), free ecdysteroids.

III, there was a rise in the total ecdysteroid amounting to 15.2 ng/g egg wet weight with a proportionate increase of polar conjugate (61.8 pg/mg lipid) and free ecdysteroids (146.7 pg/mg lipid). The total ecdysteroids registered a distinct fall in stage IV. However, in stage V and VI, the total ecdysteroids increased to 15  $\mu$ g/g egg wet weight. This corresponds with an increase in the free ecdysteroid level in stages V and VI. Following this, in stage VII, there was a small decrease in the total as well as individual ecdysteroids. Most interestingly, in stage VIII, representing the fully formed embryos within the hatching envelope, a remarkable rise ( $P < 0.01$ ) in

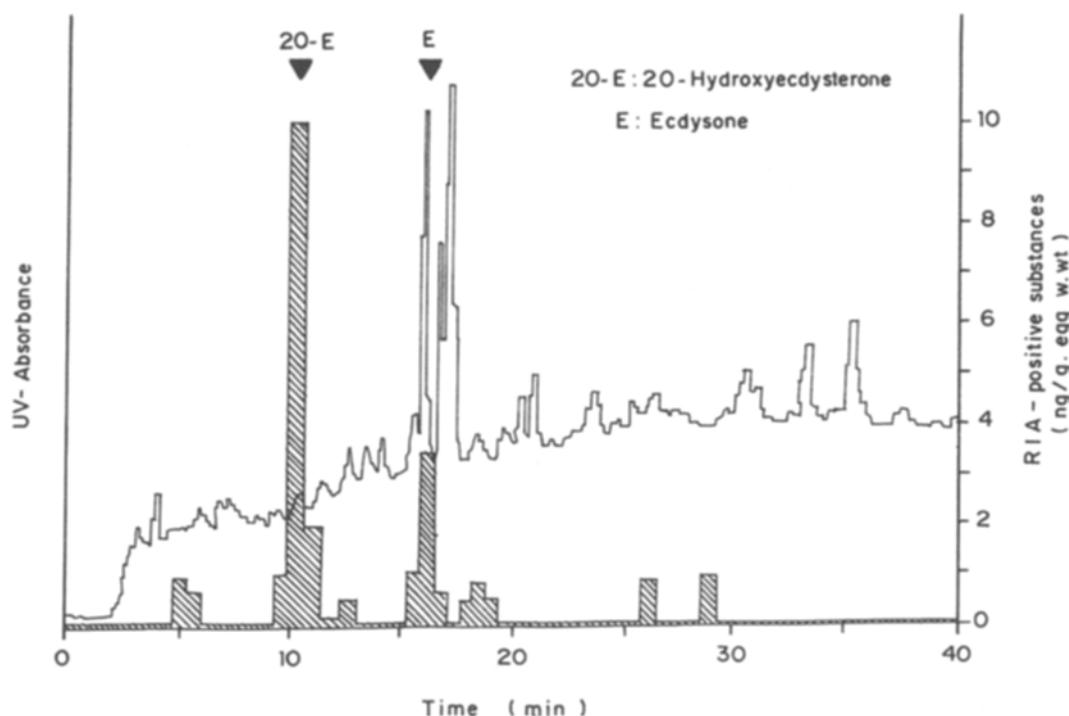
both total and individual ecdysteroid classes was observed (figures 1 and 2). However, only low amounts of total ecdysteroids were found at the end of the egg development, shortly before hatching (stage IX; figure 1). Variation in total and individual ecdysteroid levels in the different stages of embryonic development was highly significant ( $P < 0.01$ ).

The purified lipovitellin of the stage I eggs also contained considerable amount of free (64.1 pg/mg lipid) as well as polar (22.3 pg/mg lipid) and apolar (41.8 pg/mg lipid) conjugated ecdysteroids (not shown in the figure).

Free ecdysteroids from the various embryonic stages of *E. asiatica* were pooled and subjected to HPLC analysis using a C18 reversed phase column. There were two major peaks of immunopositive eluents at fractions 19–21 (9.5–10.5 min) and fractions 32–34 (16.0–17.0 min), which exactly corresponds to 20-hydroxyecdysone ( $R_f$  for standard – 9.5 min) and ecdysone ( $R_f$  for standard – 15.6 min) respectively (figure 3). Calculating the amount of ecdysteroids in the various fractions resulted in 13.4 ng of 20-hydroxyecdysone and 5.3 ng of ecdysone, which means a ratio of 20-OHE/E of 2.5. There may also have been a small amount of polar free ecdysteroids in fractions 36–38 and 54–58 respectively.

#### 4. Discussion

The results obtained from this study reveal the presence of a complex mixture of ecdysteroids in the embryonated eggs of *E. asiatica*. Among the polar, apolar conjugated and free ecdysteroids determined by the RIA technique, the free ecdysteroids always constitute a very high



**Figure 3.** Reverse phase HPLC/RIA of the free ecdysteroids from the eggs of *E. asiatica*.

proportion of total ecdysteroids in the developmental stages analysed. Only in late embryogenesis, considerable amount of polar and apolar conjugates accumulate. That a majority of total ecdysteroids found in the eggs is present in the Lv II fraction is also demonstrated by RIA. Possibly, these ecdysteroids are linked to the lipovitellin through non-covalent interactions. The presence of ecdysteroids in Lv II fractions of newly deposited eggs suggests that they are maternally derived as the Lv II itself is the sequestered form of vitellogenin in this crab (Tirumalai and Subramoniam 1992). The predominant free ecdysteroids are 20-hydroxyecdysone ( $\beta$ -ecdysone, crustecdysone) and ecdysone. Similar results are observed in the eggs of *Palaemon serratus* (Spindler *et al* 1987), the crabs *Cancer magister* and *C. anthonyi* (Okasaki and Chang 1991) and in the spider crab *Acanthonyx lunulatus* (Chaix and De Reggi 1982), whereas in *Carcinus maenas*, ponasterone A is the primary ecdysteroid (Lachaise and Hoffmann 1982).

In insects, the majority of ecdysteroids stored in the eggs are found in conjugated forms. They are conjugated either to long chain fatty acids (Slinger *et al* 1986; Witing and Dinan 1988; Espig *et al* 1989) through ester linkages or undergo sulphation or phosphorylation (Modde *et al* 1984; Rees and Issac 1984; Thompson *et al* 1985). At least in some species, ecdysteroids are tightly bound to the vitellin (Lagueux *et al* 1981; Bownes *et al* 1988).

The studies on insects have further provided experimental evidence for embryonic degradation of ecdysteroid conjugates and of yolk protein ecdysteroid complexes to release the free functional ecdysteroids at the appropriate time during embryonic development (Hoffmann *et al* 1986). In *E. asiatica* eggs, the neutral lipid fraction of the purified Lv II contains long chain fatty acids such as linolenic, linoleic and stearic acids (Tirumalai 1996). Possibly, these fatty acids might be esterified, at least in part to ecdysteroids at the C<sub>22</sub> carbon atom, as has been suggested for insects (Slinger *et al* 1986). Again, non-specific esterase activity during yolk utilization, especially the breakdown of lipovitellin, occurs during embryogenesis of *E. asiatica* (Subramoniam 1991). Evidently, the conjugated ecdysteroids appear to be the storage form which the active ecdysteroids can be released at the appropriate time during embryogenesis.

The periodic fluctuation in the total ecdysteroid level throughout embryogenesis of *E. asiatica*, with the highest amount found in stage VIII of embryonic development, indicates their involvement in the regulation of embryonic development as well as their well known role in cuticular synthesis. The first rise in RIA active ecdysteroids occurred in stage III, when the blastoderm extends. Another increase in ecdysteroids was seen in stage VI, when the embryonic eyes are fully developed and the first limb buds become apparent. The sharp rise of

ecdysteroids towards the prehatching period may be correlated with the deposition of the embryonic cuticle of the zoea larva (Goudeau *et al* 1990).

Unlike in insects, where the ovary is demonstrated to synthesize ecdysteroids, which accumulate, at least in part, in the eggs (see Wilder *et al* 1991), the source of ecdysteroids in Crustacea is still debatable. In the caridean shrimp *Palaemon serratus* and the giant freshwater prawn *Macrobrachium rosenbergii*, the ovarian accumulation of ecdysteroids coincides with premoult activity during which the ecdysteroid level in the haemolymph as well as the ecdysteroid synthesis in the Y-organ is very high (Lanot and Clédon 1989; Wilder *et al* 1990, 1991). This may suggest a direct derivation of ecdysteroids from the haemolymph in these shrimps and prawns. However, in the brachyuran crabs such as *Carcinus maenas* (Lachaise and Hoffmann 1982), the ovarian development and hence the ecdysteroid accumulation within the maturing oocytes occur during the intermoult stage, when the titre of ecdysteroids in the haemolymph is very low. In *E. asiatica*, moulting alternates with reproduction (Subramoniam 1977). Here the female gametogenic cycle starts during the intermoult period, and the ovarian maturation goes right into the premoult stage. Hence, in comparison with the freshwater prawns and caridean shrimps, ecdysteroid accumulation in the ovary may occur by sequestration from the haemolymph of *E. asiatica*. The question of the ultimate source of embryonic ecdysteroids in the crustaceans would be resolved only when *in vitro* incubation studies on the ovarian cells are made, to see its steroidogenic capabilities, as in insects. The highest level of total ecdysteroids in stage VIII embryo, representing the fully formed zoea larva, implies that the embryonic Y organ or some other tissue capable of ecdysteroid synthesis develops at this time.

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