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Sequestration of ecdysteroid hormone into the ovary of the mole crab, *Emerita asiatica* (Milne Edwards)

V. Gunamalai*, R. Kirubagaran[#] and T. Subramoniam*,[†]

*Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Chennai 600 025, India
[#]National Institute of Ocean Technology, Pallikarnai, Chennai 600 302, India

Ecdysteroids are the principal steroid hormones of arthropods, regulating different physiological functions such as growth, metamorphosis and reproduction. Radioimmunoassay indicates the accumulation of ecdysteroids into the ovary during maturation of a sand crab *Emerita asiatica*. These ecdysteroids are sequestered from the hemolymph during the entire period of intermolt stage. However, the ovarian ecdysteroids drastically declined during the premolt stage corresponding to a steep rise in the hemolymph ecdysteroids level. The possible role of ecdysteroids in vitellogenesis and embryonic development is discussed.

ECDYSTEROIDS, a group of polyhydroxy steroids, occur in all arthropod groups serving the main function of molting hormones[1]. In the holometabolous insects, during larval stages, ecdysteroids produced by the prothoracic glands control the molting and metamorphic activities; in the reproductive adulthood, ovarian ecdysteroids are thought to play a role in vitellogenesis, as demonstrated in the higher dipteran flies[2]. Embryonic ecdysteroids, derived both from maternal contribution during vitellogenesis as well as the *de novo* synthesis by larval prothoracic gland, function as morphogenetic hormones, controlling vital activities such as the secretion of embryonic envelopes and embryonic molting[3].

Crustaceans differ from insects in that they continue to molt and grow even after attainment of sexual maturity. In the large decapod crustaceans such as crabs and lobsters, the female reproductive cycle is completed within the protracted intermolt period and the molting is initiated only after reproductive arrest. Conversely, in many soft-shelled shrimps and prawns, the molting is permitted to occur during the course of the oogenic cycle, thus revealing a close synchronization between the molting and reproductive processes (see ref. 4 for a review). This kind of synchronized activity of these two rather energy-demanding physiological processes may suggest that molting and reproductive cycles are regulated in a coordinated manner by common endocrine means. The anomuran mole crab, *Emerita asiatica* not only molts and breeds throughout the year, but also exhibits synchronization of these two processes to bring about the continued body growth even in the egg-laying adults[5]. In these crabs, the ovarian development, commencing in the early intermolt stage, continues well into the premolt stage, when the preparatory processes for molting occur. Such an overlapping of reproductive and molting activities found in *E. asiatica* is similar to that described in a freshwater prawn *Macrobrachium rosenbergii*[6]. Furthermore, radioimmunoassay of the active molting hormone, 20-hydroxyecdysone (20E) revealed a premolt surge in the hemolymph of *E. asiatica*[7], as shown already in other crustaceans[8]. In this communication, we report on the sequestration of hemolymph ecdysteroids into the ovary during a specific period in the molt cycle of *E. asiatica*.

E. asiatica is a typical burrowing decapod, found on the wave-washed sandy beaches of Madras coast. *E. asiatica*, ranging in size from 18 to 33 mm carapace length (CL), were collected from the intertidal region of Elliots Beach, Besant Nagar, Chennai. The size was measured from the posterior margin of the carapace along the middorsal line to the tip of the rostrum. At least 50 to 100

[†]For correspondence. (e-mail: tsbl71@hotmail.com)

females per collection were hand-picked and brought to the laboratory. The females were identified by the occurrence of three pairs of pleopods. The ovarian stages were classified as stage I to IV, based on the colour change and gonado somatic index (GSI = gonad wt × 100/body wt). The molt-cycle stages were determined by the sequential changes in the setae and epidermal retraction in the pleopod, as detailed in Gunamalai and Subramoniam[5].

Extraction of ecdysteroid from the ovary was performed according to Rotland *et al.*[9]. Ovarian tissues were dissected out and rinsed with crustacean physiological saline to wash-off the adhering hemolymph. Approximately 200 to 300 mg ovary from 5 to 10 individual crabs at each ovarian stage was taken and stored at –20°C until ecdysteroid extraction. Ovarian tissue of each stage was homogenized in 80% methanol (100 mg wet wt/ml) and centrifuged at 10000 *g* for 10 min. The supernatant was decanted and saved, the pellet was re-extracted twice in the same way. The supernatants were pooled and evaporated under a gentle stream of nitrogen and the resultant residue was dissolved in a known volume of borate buffer (0.1 M, pH 8.4) for radioimmunoassay.

The polyclonal ecdysteroid antiserum (2A series) used in this study was produced by W. E. Bollenbacher (Univ. of North Carolina, Chapel Hill) and distributed by E. S. Chang (Bodega Marine Laboratory, Bodega Bay, CA). ^{3}H-ecdysone {α-(23, 24-^{3}H(N)} was procured from NEN Ltd, USA. Also, 20-hydroxyecdysone was used as the standard. Radioimmunoassay of ecdysteroids was carried out according to Soumoff *et al.*[10]. Ten microlitres of borate buffer (0.1 M, pH 8.4) reconstituted samples was added to 100 µl of borate buffer containing approximately 24,000 dpm ^{3}H-ecdysone and mixed well, in order to reach adequate equilibration of labelled and unlabelled ecdysone. Following this, 100 µl antisera in borate buffer was added and thus a final dilution of 1 : 4000 was maintained for antisera at the time of incubation. The tubes were again mixed thoroughly and allowed to incubate overnight at 4°C. The incubation was terminated by precipitation of the antibody–hapten complex upon the addition of 200 µl of cold saturated ammonium sulphate solution. After 20 min, the samples were centrifuged for 15 min at 5000 rpm. The supernatant was removed by vacuum aspiration. The pellet was washed with 400 µl of 50% saturated ammonium sulphate in borate buffer. The washed pellets were centrifuged as previously described. Following the removal of supernatants, the pellets were dissolved in 25 µl of water and then 600 µl of universal cocktail (Hisafe, Amersham Pharmacia Biotech) was added. The tubes were mixed thoroughly and the contents transferred to 20 ml standard scintillation vials before allowing to equilibrate in the dark overnight. The following day, the vials were measured for radioactivity using Wallac 1400 DSA liquid-scintillation system. The statistical significance of differences among means was determined using one way analysis of variance (ANOVA), following Zar[11].

Changes in the ovarian ecdysteroid titre were quantified at four stages of ovarian maturation in crabs undergoing first maturation (18–22 mm CL) and repetitive reproduction (23–33 mm CL), and the result is presented in Table 1. During ovarian maturation, the gonadosomatic index increased from 1.06 in the first stage to 4.12 in the fully mature ovary (stage IV; Figure 1). The colour of the ovary also changed from whitish yellow to bright orange.

In females maturing for the first time in the size group of 18–22 mm CL, the amount of ovarian ecdysteroids was found to be the lowest in stage-I ovary. This level gradually increased to succeeding ovarian stages II and III, and then reached a maximal level in stage IV (Table 1). In this size group, the completion of ovarian maturation is intervened by at least three molts[12].

An analysis of different ovarian stages in the repetitively reproducing females in the size class of 23–33 mm CL also revealed the same trend in the ecdysteroid level (Table 1). However, the absolute quantity of ecdysteroid in all stages was considerably lower than that of the corresponding stages in the ovary of the females maturing for the first time. Figure 2 shows the ovarian accumulation of ecdysteroids during different molt-cycle stages of the repetitively breeding females. As seen from the figure, the ovarian ecdysteroid level is low (56.15 ng/g) in C_1 stage, when the ovary is in stage I. The level goes on increasing in the subsequent intermolt stages to reach a maximum (236.25 ng/g) in C_3 stage corresponding to ovarian stage IV. When the crab enters the early premolt stage, ovarian development is almost completed, but the level of ecdysteroids begins to decline in the subsequent premolt stages reaching a lowest value of 8.32 ng/g in D_{3-4} stage. However, in the postmolt stage (A/B), there is a slight increase in the ecdysteroid level in the ovary before spawning. The level of ecdysteroids in the spent ovary is also low (Figure 2).

Crustacean ovary has been known to accumulate significant quantities of this steroidal hormone during maturation[13–17]. In insects, follicle cells surrounding the growing oocytes are known to synthesize ecdysteroids and transfer them to the oocytes[3]. On the contrary, in the crustaceans, the Y-organ in the adult actively synthesizes

Table 1. Ovarian ecdysteroid level in females maturing for the first time and repetitively reproducing females of *E. asiatica*

Ovarian stage	Females maturing for the first time (ng/g)	Repetitively reproducing females (ng/g)
Stage I	164.15 ± 9.52	56.22 ± 5.80
Stage II	288.21 ± 14.52*	106.51 ± 8.51*
Stage III	611.58 ± 27.10*	172.28 ± 12.15*
Stage IV	764.45 ± 23.65*	267.15 ± 15.63*

*$P < 0.001$.

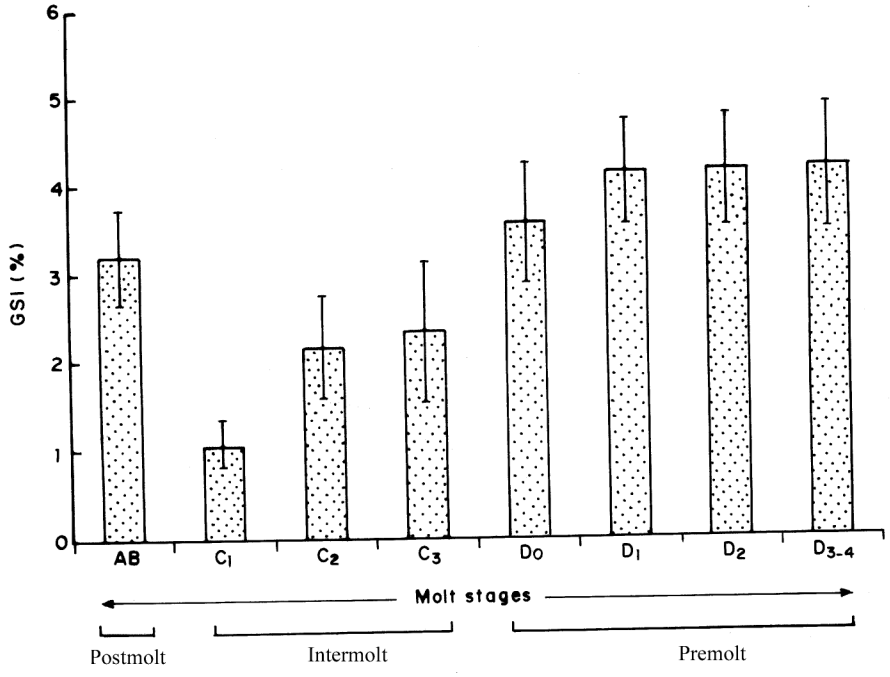

Figure 1. Gonadosomatic index in different molt-cycle stages.

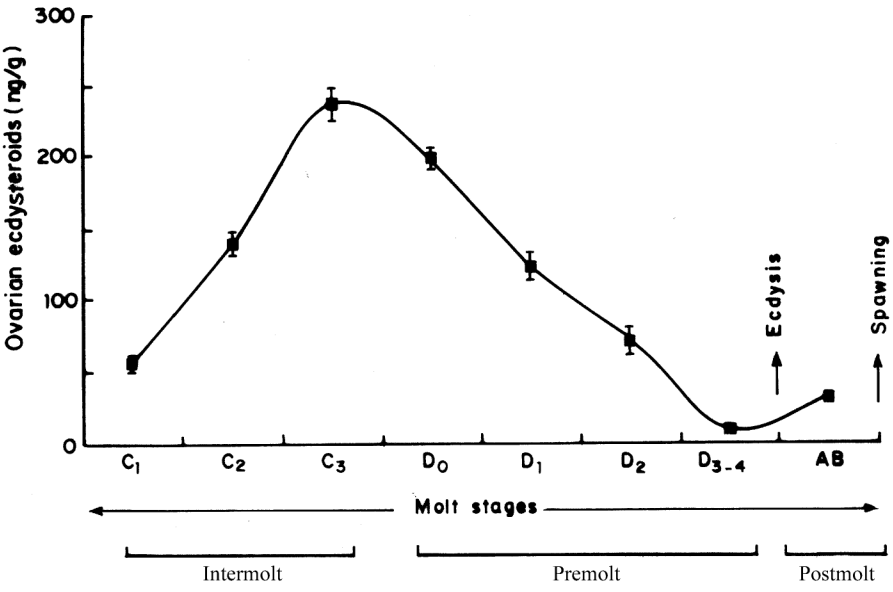

Figure 2. Ecdysteroid titres in different reproductive stages of repetitively reproducing females associated with molt-cycle stages. (Bars represent mean ± SE). Data were analysed by ANOVA ($P < 0.001$).

and secretes ecdysteroids into the hemolymph[13,18], suggesting a possible entry of the same into the ovary for sequestration into the growing oocytes. The titre of hemolymph ecdysteroids also changes from a low level of 3.486 ng/ml in the postmolt stage to a high level (247.402 ng/ml) during premolt stage (D_2), but drops precipitously prior to ecdysis in *E. asiatica*[7]. The present results indicate a gradual accumulation of ecdysteroids within the growing ovary during the entire intermolt stage. However, by the onset of premolt, the ovarian ecdysteroid level falls drastically to reach a low level just before ecdysis. The decline in the ovarian ecdysteroid level during the premolt stage is thus reciprocal to the steep rise in the hemolymph ecdysteroids. Apparently, there is release of ecdysteroids from the ovary back into the hemolymph, causing its rise in the circulating ecdysteroids. Alternatively, a reduction in the ovarian ecdysteroids may also be caused by esterification of

ecdysteroids with the long-chain fatty acids of the lipovitellin, giving rise to the storage forms of conjugated ecdysteroids. Our previous study on this crab also revealed the occurrence of significant quantities of polar and apolar conjugated ecdysteroids in the freshly-laid eggs[19], suggesting their bioconversion from the free active ecdysteroids, such as 20E. Evidently, these metabolic conversions of ecdysteroids may bring about a decrease in detectable ecdysteroid RIA activity in the ovary. Nevertheless, a genuine accumulation of ecdysteroids from the hemolymph into the ovary is still possible by virtue of the ability of the crustacean ovary to convert the ecdysone into the active 20E, using 20-hydroxylase enzyme, as reported in the crab *Cancer antennarius*[20].

In the freshwater prawn *Macrobrachium rosenbergii*, Wilder *et al.*[16] observed continued vitellogenic activity during premolt, resulting in the increase of ecdysteroids level in the ovary even during premolt stages, with a corresponding increase in the gonadosomatic index. Apparently, the hemolymph ecdysteroids are transported to the ovary along with the yolk precursor protein, vitellogenin. This study, however, did not reveal any decline in the ovarian ecdysteroids in the premolt stage.

The accumulation of ecdysteroids within the maturing ovary does not *per se* suggest a role in vitellogenesis. Crustacean ecdysteroids are very polar and hence circulate freely in the hemolymph to enter cells by simple diffusion. However, Spindler *et al.*[21] reported an energy-dependent and carrier-mediated process of ecdysteroid entry into the epidermis of the crayfish. Several recent studies have recognized the presence of ecdysteroid receptors in various tissues. More particularly, Chung *et al.*[22] found the co-expression of both ecdysteroid receptor and retinoid-X-receptor in the epidermis during the molt cycle of the fiddler crab, *Uca pugilator*. Recently, Durica *et al.*[23] found expression of these two receptors (UpEcR and UpRXR) in the ovary of *U. pugilator* during ovarian cycle, suggesting that the ovary is a potential target tissue for ecdysteroid hormonal control. In the dipteran insect, *Aedes aegypti*, the regulatory region of vitellogenic gene contains an ecdysteroid response element. Ecdysteroid receptor after binding with 20E, dimerizes with another transcription factor, USP to initiate transcription of vitellogenin[24]. In the light of the above information on the ecdysteroid regulation of gene activity in the ovary, it may be suggested that the ovary of *E. asiatica* may also be involved in protein synthesis under the influence of ecdysteroid hormones. In an earlier study, Gunamalai[7] found evidence that there is increase in protein synthetic activities in the hepatopancreas, hemolymph and ovary of *E. asiatica* after receiving injections of exogenous ecdysteroids. In Crustacea, although the majority of the yolk proteins are derived from the vitellogenin, ovarian synthesis of both yolk and non-yolk proteins involved in the enzyme activity or synthesis of embryonic envelope could still occur under the influence of ecdysteroids. Further studies on molecular pathways leading to the expression of vitellogenin genes under ecdysteroid control would unravel the hormonal regulation of vitellogenesis in crustaceans.

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