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Vertebrate steroids and the control of female reproduction in two decapod crustaceans, *Emerita asiatica* and *Macrobrachium rosenbergii*

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Vertebrate steroids, estradiol-17β (E_2) and progesterone (P), have been estimated in the hemolymph, ovary and hepatopancreas of mole crab *Emerita asiatica* and freshwater prawn *Macrobrachium rosenbergii* during the reproductive and molt cycle stages by radioimmunoassay. The maximum level of E_2 in hemolymph, ovary and hepatopancreas was detected only during the intermolt stage, whereas the level gradually decreased during premolt and postmolt stages in *E. asiatica*. The E_2 level in the hemolymph was high in crabs with mature ovaries, while those with quiescent ovaries were low or undetectable. The trend in P level in all tissues during different molt and reproductive stages was remarkably similar to that of E_2. However, in *M. rosenbergii*, with two types of molt cycles, viz. reproductive and common (non-reproductive) molt, E_2 and P levels in hemolymph, ovary and hepatopancreas showed wide variation between them. During the reproductive molt cycle, the level of E_2 and P in all tissues peaked during intermolt, but declined drastically at premolt and postmolt stages. On the contrary, the level of E_2 in hemolymph was not detectable in any molt stage during the non-reproductive molt with the ovary containing undeveloped oocytes. However, the inactive ovary and hepatopancreas showed basal level of E_2 during non-reproductive molt cycle, whereas P was totally undetectable in the above tissues. Cumulatively, these studies suggest that the ovary may synthesize E_2 and release them into the hemolymph from where it may reach the hepatopancreas to stimulate vitellogenin synthesis in the two decapods. P may have a role in the post-vitellogenic meiotic maturation of the oocytes, as in vertebrates.

Keywords: Crustaceans, estradiol-17β, molting, progesterone, reproduction.

UNLIKE insects, most malacostracan crustaceans continue growth and molting with reproductive activities. Hormonal coordination of molting and reproduction in crustaceans is achieved by the combinatorial effects of eyestalk inhibitory neuropeptides and a variety of trophic hormones. The control of molting in crustaceans is accomplished by the common arthropodan molting hormone, ecdysteroid, the action of which is uniquely inhibited by the molt-inhibiting hormone[1]. Similarly, the inhibitory role of gonad-inhibitory hormone on the reproductive activities, especially on vitellogenesis, has been well documented[2,3]. Conversely, there are discordant results concerning the gonad stimulatory factors among various crustacean species. For example, earlier studies revealed the occurrence of gonad stimulatory neuropeptides in the brain and thoracic ganglia of some crustaceans[4]. Following this, several hormonal factors such as methyl farnesoate, a structural homologue of insect juvenile hormone, ecdysteroids as well as vertebrate steroids like estradiol-17β (E_2) and progesterone (P) have been implicated with inducement of ovarian maturation in different crustacean species (see ref. 5 for review). Apparently, crustaceans might employ more than one type of gonad stimulatory principles in the control of vitellogenesis, the central event of oogenesis.

In decapod Crustacea, physiological processes of both molting and female reproduction are linked and hence the temporal separation of reproductive and molting activities becomes a necessity for judicial apportioning of the organic storage materials for both these energy-demanding processes. Yet another complexity in the female reproduction of malacostracan crustaceans is that they carry the brood

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in the pleopods and hence, the developing brood henceforth exerts an inhibitory influence on the onset of molting. Our recent work on a mole crab *Emerita asiatica* has shown the importance of ecdysteroids in coordinating the molting and reproductive events which almost overlap in this crab[6–8]. Ecdysteroids not only influence the onset and accomplishment of adult molt, but also enhance embryonic molting and egg hatching in the attached brood, thereby facilitating successful molting without wastage to the brood. However, the hormonal regulation of vitellogenic activities completed within the extended intermolt period is elusive. A previous study from our laboratory has, however, revealed the accumulation of vertebrate steroids such as E_2 and P in the ovary of *E. asiatica* during vitellogenesis[9]. This finding along with several other studies[5] has indicated a causal role for these vertebrate hormones in the control of crustacean vitellogenesis. To see whether these steroids have an integrative role in ovarian maturation along with other reproductive processes like brood development, as well as molting, their fluctuation during the entire molt cycle stages of *E. asiatica* and a freshwater prawn *M. rosenbergii* is investigated in the present study. *M. rosenbergii* is chosen as it has two types of molting, namely reproductive and non-reproductive (common) molt[10]. This facilitated the delineation of specific role, if any, for the vertebrate steroids independently in the control of vitellogenesis during the two types of molt cycle in the freshwater prawn.

Mole crab, *E. asiatica*, the typical burrowing anomuran mole crab, ranging in size 23–33 mm carapace length (CL) was collected from the intertidal region of Elliot's beach at Besant Nagar, Chennai, India. The CL was measured from the posterior margin of the carapace along the mid-dorsal line to the tip of the rostrum. The females were identified by the occurrence of three pairs of pleopods. The molt stages of *E. asiatica* were determined based on microscopic observations of pleopodal setae such as epidermal retraction and new setagenesis. Four different ovarian stages were classified as stage I–IV based on the colour, oocyte diameter and gonadosomatic index (GSI = gonad weight/body weight × 100)[6]. The same method has been used to determine the molt and ovarian cycle stages of *M. rosenbergii*.

Five hundred microlitres of hemolymph was taken in a stoppered test tube and added triple distilled water to make up the volume to 2.0 ml. To this 5.0 ml of HPLC grade diethyl ether was added and vortexed for 3 min. Diethyl ether was aspirated and dried under N_2 gas. Later, 1.0 ml of phosphate buffered saline (PBS, 0.01 M, pH 7.0) was added and from this 500 µl was taken for the assay of E_2 and P after maintaining the tubes at 4°C for 30 min. E_2 and P from the ovary and hepatopancreas were extracted by following the procedure of Shih[11].

The levels of E_2 and P in the extracts of hemolymph, ovary and hepatopancreas were measured by radioimmunoassay following the method developed and validated by Lamba *et al.*[12]. Five hundred microlitre of reconstituted serum extract was taken in an assay tube and 100 µl of anti-estradiol or anti-progesterone (both gifted by Prof. G. D. Niswender, Colorado State University, USA) and approximately 10,000 counts per min (cpm) of H^3-estradiol (2,4,6,7-H^3) or H^3-progesterone (1,2,6,7-H^3) (both from New England Nuclear, USA; Sp. Act.: 71 Ci/mmol and 104 Ci/mmol for H^3-estradiol and H^3-progesterone, respectively) in 100 µl of PBS were added. The reaction mixture was vortexed and left in room temperature for 1 h and then it was incubated overnight at 4°C. Following the incubation, 200 µl of ice-cold dextran-coated charcoal was added and vortexed briefly before leaving for 20 min to adsorb the free steroids. The tubes were centrifuged at 2500 rpm for 10 min at 4°C and the supernatant was decanted to a scintillation vial containing 10.0 ml of scintillation fluid (Amersham, Hisafe 2). The vials were vortexed gently and counted for 1 min using a liquid scintillation counter (Wallace 1409 DSA) after keeping overnight in dark. The detectable limit of present assay system was 10 pg/ml. Standard curves were obtained by processing tubes containing 0–1000 pg of unlabelled E_2 or P (Sigma, USA) in a similar manner as described for unknown samples, after selecting the antibody titre for 50% binding. For non-specific binding, 100 µl of H^3-estradiol or H^3-progesterone (approx. 10,000 cpm) was added to an assay tube containing 0.6 ml of PBS and the tubes were processed in the same manner as the standards. Total counts were obtained by directly adding 100 µl of H^3-estradiol or H^3-progesterone to 10.0 ml of scintillation fluid. The level of hormones of unknown samples was calculated using the standard curve.

Quantification of free steroids in the hemolymph, ovary and hepatopancreas of *E. asiatica* and *M. rosenbergii* has been made separately during different molt and reproductive stages (Figure 1; Tables 1 and 2). Both E_2 and P follow a periodical pattern of fluctuation in the hemolymph. During the postmolt stage, the level of these vertebrate steroids is very low but gradually rises during the entire intermolt period to reach a peak at the late intermolt stage (C_3) of *E. asiatica*. Significantly, both the hormonal levels declined drastically during the ensuing premolt stages. Furthermore, the amount of hemolymph P is always higher than that of E_2 in all stages. An analysis of ovarian steroids in different molt cycle stages indicated a similar decline during the premolt stage. The level continued to be low up to spawning, occurring after ecdysis. The same trend of hormonal fluctuation is also seen in hepatopancreas during the molt cycle (Table 1). RIA determination of ovary in stages I and II (previtellogenic stage) revealed that the concentration of E_2 and P is too meagre to be detected. However, there is a sharp increase in the free steroids during stages III (E_2, 4410 ± 225 pg/g; P, 4215 ± 162 pg/g) and IV (E_2, 8021 ± 139 pg/g; P, 9684 ± 12 pg/g), representing active vitellogenesis. Similar increase in the steroids has also been registered in hemolymph and hepatopancreas during ovarian vitellogenesis. In *E. asiatica*,

the vitellogenesis is completed within the intermolt stage, but the spawning occurs only in the postmolt stage.

Unlike *E. asiatica*, the adult *Macrobrachium* exhibits two types of molt cycle, namely, common molt (non-reproductive molt) and reproductive molt. In the common molt, the prawn enters into successive molt without undergoing ovarian development in the intermolt stage. During the reproductive molts, the ovary undergoes maturation during the intermolt followed by next molting and spawning. The existence of these two types of molt cycle facilitated quantification of both E_2 and P during vitellogenic and non-vitellogenic intermolt stage.

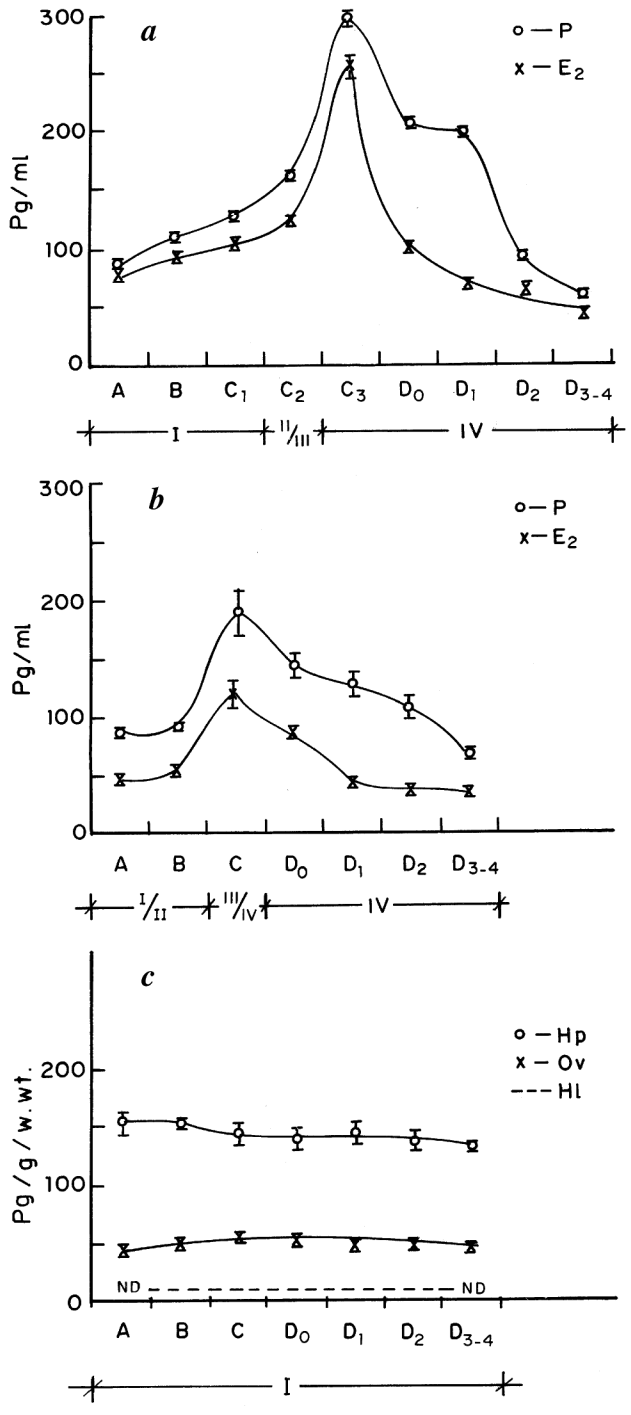

Figure 1 *a–c*. Level of estradiol-17β and progesterone in hemolymph during different molt cycle stages associated with reproductive stages of *E. asiatica* (*a*) and *M. rosenbergii* (*b*, reproductive; *c*, non-reproductive). *X* axis gives the molt cycle stages along with corresponding ovarian stages. Hp, Hepatopancreas; Ov, Ovary; Hl, hemolymph.

In the reproductive molt of *M. rosenbergii*, the level of E_2 and P in hemolymph, ovary and hepatopancreas increased from postmolt stage, to reach a peak in intermolt stage, when active vitellogenesis occurs in the ovary (Figure 1 *b*; Table 2). However, the level in the above tissues gradually decreased from intermolt to early premolt (D_0), and dropped precipitously to basal level at late premolt (D_{3-4}) stage. Further analysis indicated that the hemolymph steroidal level during early stage of ovarian development (stage I) was undetectable but exhibited a sharp increase during active vitellogenesis (E_2, 101 ± 11 pg/ml; P, 149 ± 7 pg/ml). In ovary and hepatopancreas, the levels of E_2 and P were low during early ovarian development but showed sharp increase during active vitellogenesis (E_2, 303 ± 8, 673 ± 7 pg/g; P, 358 ± 8, 639 ± 24 pg/g, respectively for ovary and hepatopancreas).

During non-reproductive molt of *M. rosenbergii*, the level of E_2 in the hemolymph was not detectable during entire molt cycle. However, the quiescent ovary and the hepatopancreas showed only a basal level of E_2 throughout the molt cycle stages. Surprisingly, P was totally undetectable in the hemolymph, ovary and hepatopancreas during the entire non-reproductive molt cycle stages.

In malacostracan crustaceans, molting and reproduction are the two competitive physiological processes in respect of utilization of energy reserves. During the extended intermolt period, all reproductive activities such as egg production in the ovary and brood development in the pleopod are accomplished, whereas during premolt period, the metabolism shifts to formation of a new cuticle to replace the old one in ecdysis. Such a temporal pattern of reproduction and molting is achieved by a precise hormonal coordination to control these physiological events. In the mole crab *E. asiatica* we have earlier demonstrated the role of ecdysteroids both in the control of adult molting and the embryonic molting and larval hatching of the pleopodal brood[8]. The results presented in the present study provide further insight into the role of the vertebrate sex steroids such as E_2 and P in controlling the female reproductive activities, as occurring during the entire intermolt period. To be specific, E_2 exhibits a fluctuation in the hemolymph correlative to vitellogenesis. However, free E_2 makes a drastic decline during the premolt period, which also represents the period of post vitellogenesis. Similar trend has also been noticed for P. The cyclical changes in the blood E_2 are highly correlative of vitellogenesis in lower vertebrates and, there is molecular evidence to indicate the transcriptional activation of vitellogenin

Table 1. RIA determination of estradiol and progesterone in ovary and hepatopancreas during different molt stages of *E. asiatica*. Values are expressed as mean ± SEM; number of replicates, $n = 6$

	Estradiol-17β		Progesterone	
Molt stages	Ovary	Hepatopancreas	Ovary	Hepatopancreas
A	2158 ± 103.0	3204 ± 121.8	2615 ± 117.5	3421 ± 155.4
B	2418 ± 37.0	3507 ± 100.1	2933 ± 74.6	3484 ± 51.0
C_1	2907 ± 114.5	3789 ± 52.0	3258 ± 156.2	3924 ± 115.5
C_2	4410 ± 115.5	4651 ± 65.3	4215 ± 201.3	5643 ± 84.1
C_3	8021 ± 98.8	9723 ± 104.5	8607 ± 114.5	10404 ±92.7
D_0	5574 ± 81.1	5988 ± 144.7	6426 ± 235.5	7477 ± 64.5
D_1	4603 ± 15.7	4529 ± 232.7	3254 ± 72.5	4557 ± 126.1
D_2	3405 ± 32.7	3943 ± 84.6	2901 ± 109.9	3909 ± 142.6
D_{3-4}	1975 ± 87.6	2856 ± 108.0	2288 ± 43.2	2915 ± 160.8

E_2 in ovary: Overall comparison within group: $p < 0.001$ (F = 1623.368). All are significant except A vs B and B vs C_1.
E_2 in hepatopancreas: Overall comparison within group: $p < 0.001$ (F = 873.428). All are significant except A vs B and B vs C_1.
P in ovary: Overall comparison within group: $p < 0.001$ (F = 701.499). All are significant except A vs B, B vs C_1, C_1 vs C_2, D_1 vs D_2 and D_2 vs D_{3-4}.
P in hepatopancreas: Overall comparison within group: $p < 0.001$ (F = 1309.00). All are significant except A vs B, B vs C_1, D_1 vs D_2 and D_2 vs D_{3-4}.

Table 2. RIA determination of estradiol and progesterone in ovary and hepatopancreas during different reproductive molt stages of *M. rosenbergii*. Values are expressed as mean ± SEM; number of replicates, $n = 6$

	Estradiol-17β		Progesterone	
Molt stages	Ovary	Hepatopancreas	Ovary	Hepatopancreas
A	80 ± 5.0	202 ± 7.2	95 ± 5.5	190 ± 9.1
B	84 ± 3.6	211 ± 19.2	103 ± 6.2	216 ± 10.1
C	341 ± 10.8	663 ± 24.2	360 ± 8.6	597 ± 7.0
D_0	276 ± 3.6	337 ± 19.6	320 ± 4.5	436 ± 8.0
D_1	121 ± 11.5	276 ± 20.0	260 ± 4.3	413 ± 7.0
D_2	97 ± 2.6	245 ± 5.0	209 ± 7.9	359 ± 12.2
D_{3-4}	67 ± 2.0	158 ± 2.6	76 ± 4.5	165 ± 9.5

E_2 in ovary: Overall comparison within group: $p < 0.001$ (F = 808.987). All are significant except A vs B and . D_1 vs D_2.
E_2 in hepatopancreas: Overall comparison within group: $p < 0.001$ (F = 332.679). All are significant except A vs B, D_0 vs D_1 and D_1 vs D_2.
P in ovary: Overall comparison within group: $p < 0.001$ (F = 1033.286). All are significant except A vs B.
P in hepatopancreas: Overall comparison within group: $p < 0.001$ (F = 885.089). All are significant except A vs B, D_0 vs D_1 and D_1 vs D_2.

gene[13–15] by E_2. Progesterone has also a role in vertebrate female reproduction by its influencing effect on post-meiotic egg maturation. Similar evidence to suggest functional roles for these steroidal hormones to crustacean vitellogenesis and post-meiotic maturation of oocytes come from our analysis of the same hormones in the freshwater prawn *M. rosenbergii*. Whereas the hemolymph level of these hormones makes a similar kind of fluctuation correlative to vitellogenesis, during the non-reproductive molt cycle, the hemolymph is totally devoid of these hormones during the entire intermolt period of this prawn with ovary only in a quiescent state. This clearly indicates that the hemolymph vertebrate steroids have a functional role exclusively in vitellogenesis and post-meiotic maturation. Nevertheless, in the freshwater prawn, *Palaemon serratus* and in insects like *Locusta migratoria*, ecdysteroids have also been attributed a role in meiotic reinitiation[16–18].

The hormonal accumulation in the ripe ovary may imply a future role during embryogenesis. Interestingly, the drastic decline in the ovarian steroids during the premolt stage may reveal their esterification with fatty acids of the lipoproteinous yolk, thereby annulling the RIA positivity. We have also recently indicated similar sequestration of the molting hormone ecdysteroids in the ovary of this crab *E. asiatica* and showed evidence that they have a morphogenetic hormonal role during embryogenesis in controlling embryonic molting and hatching[8,9,19]. Evidently, the vertebrate steroids have a putative role in embryogenesis of both *E. asiatica* and *M. rosenbergii*, if not similar to ecdysteroids. The steroidogenic ability of ovary and hepatopancreas of *M. rosenbergii* has also been demon-

strated by Ghosh and Ray[20]. Again, in the penaeid shrimp, *Penaeus monodon*, Quinitio *et al.*[21] found substantial rise in these vertebrate steroids during ovarian maturation. In analogy with oviparous vertebrate system, both E_2 and P might be synthesized in the ovarian follicle cells and then released into the hemolymph to reach the hepatopancreas to stimulate vitellogenin synthesis and lipid metabolism in crustaceans[22–24]. Molecular studies such as characterization of hormone receptors are needed to provide direct evidence in this regard. The periodical pattern found in the fluctuation of both ecdysteroids and vertebrate steroids in these two crustaceans is highly correlative to both molting and reproduction. Hence, mere determination of hormonal levels in the hemolymph would serve to predict the reproductive and molt cycle status of crustaceans of commercial importance.

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