Vertebrate-type steroid profile in different tissues of wild and endocrinologically manipulated female brood stocks of *Penaeus monodon*

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The endocrine mechanism regulating reproduction of tiger shrimp, *Penaeus monodon*, is a field of topical interest, which is to be addressed in detail as control of reproduction in captivity is conditional for domestication. In this study, the titres of estradiol and progesterone in hemolymph, ovary and hepatopancreas during various reproductive phases of females were quantified using ELISA. Circulating concentration of hemolymph estradiol and progesterone in the endocrinologically-induced (eyestalk ablated) females was also quantified. Additionally, the evidence for estradiol and progesterone receptors was provided using immunohistochemistry. Levels of progesterone in hemolymph and ovary were highest in the vitellogenic and ripe phases ($P > 0.05$). Conversely, in hepatopancreas significantly higher progesterone level was detected at immature phase (184.3 ± 2.3 pg/ml). Estradiol was highest in hemolymph (65.97 ± 0.8 pg/ml) and ovary (58.47 ± 0.7 pg/ml) at the early vitellogenic phase. In hepatopancreas, however, lowest level of estradiol was detected at the early vitellogenic phase (121 ± 0.38 pg/ml). Progesterone and estradiol receptor protein signals were predominantly found in the pre vitellogenic oocytes. These results suggest the possible involvement of vertebrate-type steroid in the reproductive maturation of *P. monodon*.

Keywords: Eyestalk ablation, immunohistochemistry, *Penaeus monodon*, reproductive maturation, sex steroids.

IN vertebrates, sex steroid hormones like progesterone, estradiol and testosterone are synthesized in the gonads and play several physiological roles in reproduction, growth and development[1,2]. Changes in the levels of these sex steroids in vertebrates regulate gonad activity[3]. A hypothetical parallelism in the endocrine functions between invertebrates and vertebrates has been suggested, although the mechanism of hormonal action and its precise role in oocyte development are not unequivocally proved in invertebrates[4]. The existence of sex steroids in many invertebrates, including coelenterates[5], sponges[6], helminthes[7], annelids[8], molluscs[9], crustaceans[10] and echinoderms[3] has been reported. The most convincing evidence for the role of sex steroids in reproduction has been reported in molluscs[4].

Giant tiger shrimp, *Penaeus monodon* supports a vibrant aquaculture industry in the eastern hemisphere. Earlier, it was the most valuable aquacultured species globally. However, its importance is now lost due to constraints in the domestication and development of specific pathogen-free stock. The domestication and breeding of *P. monodon* is severely hindered owing to the reproductive dysfunction of pond-reared broodstock. The eyestalk neuroendocrine system has long been known to play an important role in the vitellogenesis of crustaceans[11,12]. Therefore, most researchers focused attention on eyestalk neurohormones and their role in maturation. As these neuropeptids are inhibitory in nature, the induced breeding of crustaceans is based on the releasing of this negative control by ablating the eyestalk. Thus eyestalk ablation has been the most common procedure in commercial hatchery production systems globally. In order to refine the induced maturation procedure and to manage reproduction under captivity more effectively, it is imperative to use hormones of 'gonadotropic' nature. In crustaceans, so far several hormones have been reported to have stimulating effect; for example, methyl farnesoate[13], neurotransmitters[14] and vertebrate-type sex steroids[15–17]. Our recent study suggests the role of 17-β-estradiol and 17-α-hydroxyprogesterone on vitellogenesis and reproductive maturation of *P. monodon*[17]. Establishment of concomitant changes in the concentration of sex steroids in the hemolymph and specific reproductive tissues is the primary source to confirm the regulatory role of individual hormones[18]. In penaeid shrimps, however, studies on quantification of sex steroids in relation to reproductive maturation phase are limited[19,20]. In this study, therefore, we quantified the titres of estradiol 17-β and

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progesterone in hemolymph, ovary and hepatopancreas during the progression of natural reproductive cycle of *P. monodon*. Additionally, we have quantified these hormones in endocrinologically induced females (eyestalk ablated) to further evaluate the role of these hormones. In addition to this analysis, the receptors for these hormones were localized immunologically in the ovary of *P. monodon*.

Materials and methods

Wild-caught brooders of *P. monodon* were obtained from the broodstock fishery along the Chennai coast (Tamil Nadu, India). The animals were divided into five groups (four animals/group) according to their apparent ovarian development stages: immature, pre-vitellogenic, early vitellogenic, late vitellogenic and ripe (D. L. Mohanlal, unpublished Ph D dissertation). The animals were housed in the aerated aquarium until the collection of tissues.

In order to analyse the changes in the level of progesterone and estradiol in the hemolymph of wild brood stock before and after eyestalk ablation, immature female brood stocks were obtained from Kasimedu Fish Landing Centre (Chennai, India). They were transported in oxygenated polyethylene bags to the Muttukadu Experimental Station of the Central Institute of Brackishwater Aquaculture (CIBA), Chennai and acclimatized under ambient salinity (28–30‰) and temperature (26–28°C) for approximately one week prior to the study. Shrimps were maintained at a stocking density of 6 animals/m^2 under natural photoperiod. They were fed with fresh clam meat and squid mantle thrice a day. About 90% of the sea water was replaced twice daily. Inter- molt and premolt females were unilaterally eyestalk-ablated for accelerating reproductive maturation and spawning. Hemolymph was obtained before and after 7 days of eyestalk ablation.

Collection of tissues

After a quick cold shock, the hemolymph was collected directly from the pericardial cavity with EDTA-rinsed needle and syringe. Samples were immediately placed in EDTA-rinsed Eppendorf tube and stored at –20°C until analysis. A fraction of ovary and hepatopancreas was immediately dissected out from each maturity stage. They were minced and homogenized in Radio Immuno Precipitation Assay buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxy cholate, 0.1% SDS, 1% Triton-X 100, pH 7.4) supplemented with the protease inhibitor cocktail (Sigma, USA). The samples were homogenized on ice using Teflon tissue grinders and centrifuged at 12,000 *g* for 20 min at 4°C. The supernatants were collected and the procedure was repeated twice. The final supernatant was kept at –80°C until analysis. The other portion of ovary and hepatopancreas tissues was fixed in 10% neutral buffered formalin for a period of 24 h and then transferred to 70% alcohol for histological and immuno-histochemical analysis.

Enzyme-linked immunosorbent assay (ELISA)

Assays were carried out using Progesterone EIA kit and Estradiol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the protocols supplied by the manufacturer. These assay kits are based on the competition between free hormone and a tracer linked to an acetylcholinesterase enzyme. The dried standards were dissolved in EIA buffer (0.1 M phosphate buffer, pH 7.4 containing 0.1% bovine serum albumin, 0.4 M NaCl, 1 mM EDTA, and 0.01% sodium azide) prior to the assay and stored in 4°C. To wells of a 96-well plate, 50 µl of sample or standard solution 50 µl of tracer solution and 50 µl of antiserum solution were added and the plate was incubated for 4 h at room temperature. After washing the plate five times, 200 µl of Ellman's reagent was added to each well and the plate was developed for 40–90 min. After development, absorbance of each well was read at 415 nm using a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Assays were done in duplicate.

Histology and immunohistochemistry

Histological analysis was carried out according to the procedure suggested by Bell and Lightner[21]. The samples were dehydrated progressively in ascending concentrations of ethanol solution and the dehydrated specimens were cleared with xylene before being embedded in paraffin wax (melting point 56–58°C). Serial sections of 5–7 mm were cut using a rotary microtome (Leica RM 2016; Leica Microsystems Inc., Bannockburn, IL) and attached to glass slides with egg white liquid and glycerin solution (v/v, 1 : 1). The slides were coated with gelatin using gelatin coating solution. Consecutively, the sections were mounted onto the slide and dried at room temperature overnight. The tissue sections were dewaxed in xylene (twice for 10 min) and dehydrated through a series of ethanol dilutions (50%, 70%, 95% and 100%). The slides were rinsed twice in running cold tap water. They were incubated in epitope retrieval buffer solution and boiled for 20 min in a microwave oven set to full power. For immunohistochemical staining, the sections were washed twice in TBS + 0.025% Triton-X 100 with gentle agitation for 5 min. The non-specific sites were blocked in 10% normal rabbit serum in 1% BSA in TBS for 2 h at room temperature. The slides were drained for a few seconds and wiped around the section with a tissue paper. The primary antibody of polyclonal human anti-progesterone receptor and human anti-estrogen receptor-α (1 : 500 dilution) were diluted in 1% BSA in TBS and

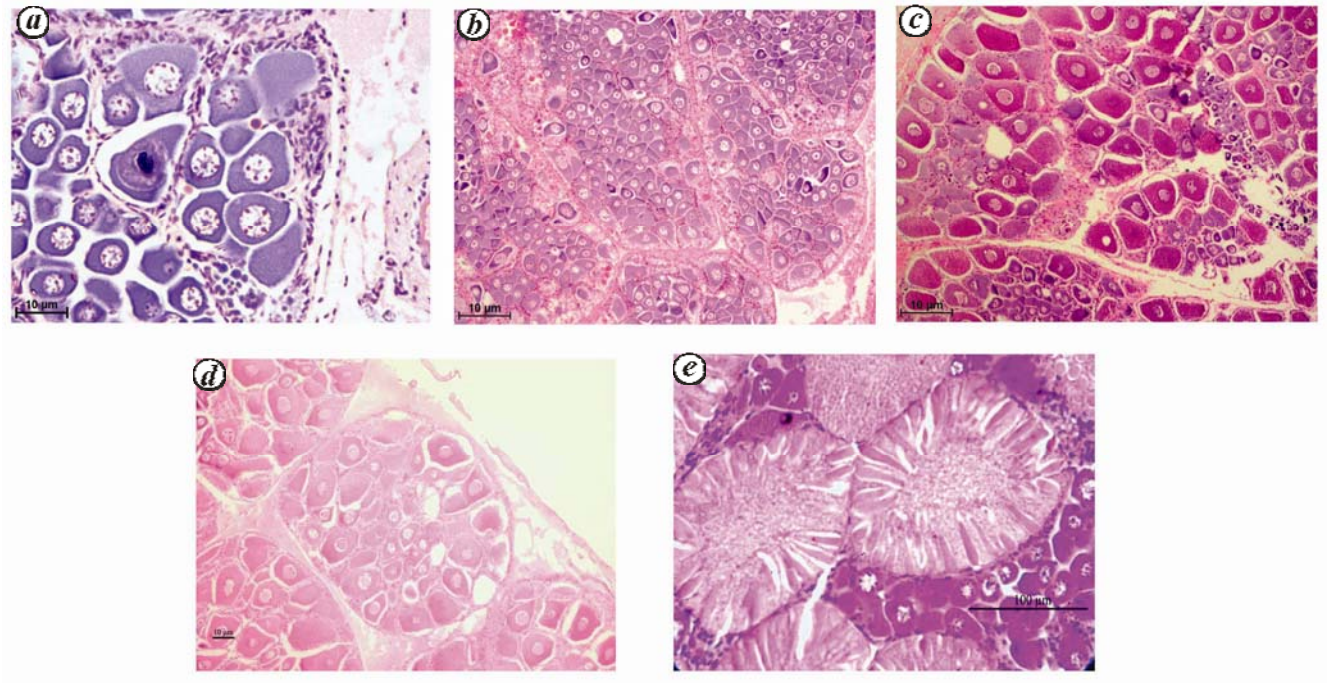

Figure 1. ***a***, Immature ovary of *Penaeus monodon*; ***b***, Previtellogenic ovary of *P. monodon*; ***c***, Early vitellogenic ovary of *P. monodon*; ***d***, Late vitellogenic ovary of *P. monodon*; ***e***, Ripe ovary of *P. monodon*.

added in separate slides. These slides were incubated overnight at 4°C. Simultaneously, the control slide was prepared using rabbit IgG omitting the specific primary antibody. The slides were washed twice in TBS + 0.025% Triton-X 100 with gentle agitation for 5 min. After washing, the slides were incubated in 0.3% hydrogen peroxide in TBS for 15 min to block endogenous peroxidase activity. The secondary antibody, conjugate of anti-sheep IgG–HRP (1 : 1000 dilution) was diluted in 1% BSA in TBS and added. The slides were incubated for 1 h at room temperature. After incubation, the slides were rinsed thrice with TBS for 5 min. Finally, they were stained with DAB chromogen and incubated for 10 min at room temperature. The slides were rinsed with running tap water for 5 min and counter-stained using hematoxylin. The tissue sections were dehydrated alcohol with ascending series. The slides were immersed in xylene, air-dried and mounted with mounting media (SRL) and observed in a bright field microscope (Olympus, Germany). The image was captured by Olympus c5050 zoom camera.

Statistical analysis

Data analysis of different hormone titres was performed using GraphPad Prism software version 5.00 (GraphPad, San Diego, CA, USA) by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Results were considered significant when $P < 0.05$.

Results

Ovarian development stages

Immature: In this stage the ovary was not discernible externally, and was thin and transparent. Ovarian lobe was dominated by basophilic primary oocytes (Figure 1 *a*). The mean diameter of oocytes ranged from 12 to 24 μm with a mean of 18 ± 0.19 μm. The smaller oocyte lay at the periphery of the ovarian lobe.

Pre-vitellogenic stage: The ovary was yellow to faint orange and discernible externally. Oocyte diameter ranged between 24 and 35 μm with a mean of 27 ± 0.18 μm and each nucleus had 5–10 nucleolii (Figure 1 *b*). In this stage number of follicle cells are found to be increased, and cells are stained with hematoxylin.

Early vitellogenic stage: In this stage, the ovary was found to be acidophilic (eosinophilic) and developed green colour owing to co-deposition of carotenoid pigment. The ovary absorbed light and a shadow was seen clearly when females were illuminated by torch light. The oocyte diameter ranged from 36 to 110 μm with a mean of 67 ± 2.1 μm (Figure 1 *c*).

Late vitellogenic ovary: Ovaries exhibited dark green colour and were clearly visible through the exoskeleton. Oocyte diameter ranged from 43 to 196 μm with a mean of 147.8 ± 2.6 μm. The cytoplasm of oocyte was granular

owing to the presence of yolk platelets. Follicle cells were elongated and formed a thin sheath surrounding each individual oocyte (Figure 1 *d*).

Ripe ovary: The most remarkable characteristic of this stage was the presence of cortical rods (cortical specialization) outside the periphery of the cytoplasm (Figure 1 *e*). These rods were club-shaped and lay within the crypts. The ovarian lobes showed an arrangement similar to the previous stage. Ovary was large and turgid, occupying all the space available in the body cavity. The oocyte diameter ranged from 125 to 248.7 μm with a mean of 194.5 ± 2.2 μm.

Steroids levels

Concentration of progesterone in hemolymph was found to increase along with the progression of ovarian maturation and highest level was observed at the late vitellogenic phase (76.57 ± 2.1 pg/ml) (Figure 2). While similar concentration of progesterone was found in the immature and pre-vitellogenic phases ($P > 0.05$), it was about four times lower than the early vitellogenic, late vitellogenic and ripe phases ($P < 0.05$). In the ovary, progesterone concentration was lower in immature stage (30.17 ± 1.4 pg/ml) than in all the remaining stages ($P < 0.05$), whereas maximum level was observed at the ripe ovarian stage (73.24 ± 2.4 pg/ml). In hepatopancreas, progesterone concentration was higher compared to all other tissues throughout the stages of ovary development (144.1 ± 3.9–184.3 ± 2.3 pg/ml). Significantly higher level of progesterone was found in the hepatopancreas at the immature ovarian stage (184.3 ± 2.3 pg/ml; $P < 0.05$).

Estradiol concentration in hemolymph showed a pronounced increase in the early vitellogenic stage (65.97 ± 0.8 pg/ml) (Figure 3), followed by a significant drop in the late and ripe stages of ovary. In the ovary, estradiol concentration level showed almost similar trend with highest concentration in the early vitellogenic ovary (58.47 ± 0.7 pg/ml). Relatively higher level of estradiol was found in the hepatopancreas (in the range of 144.3 ± 0.8 to

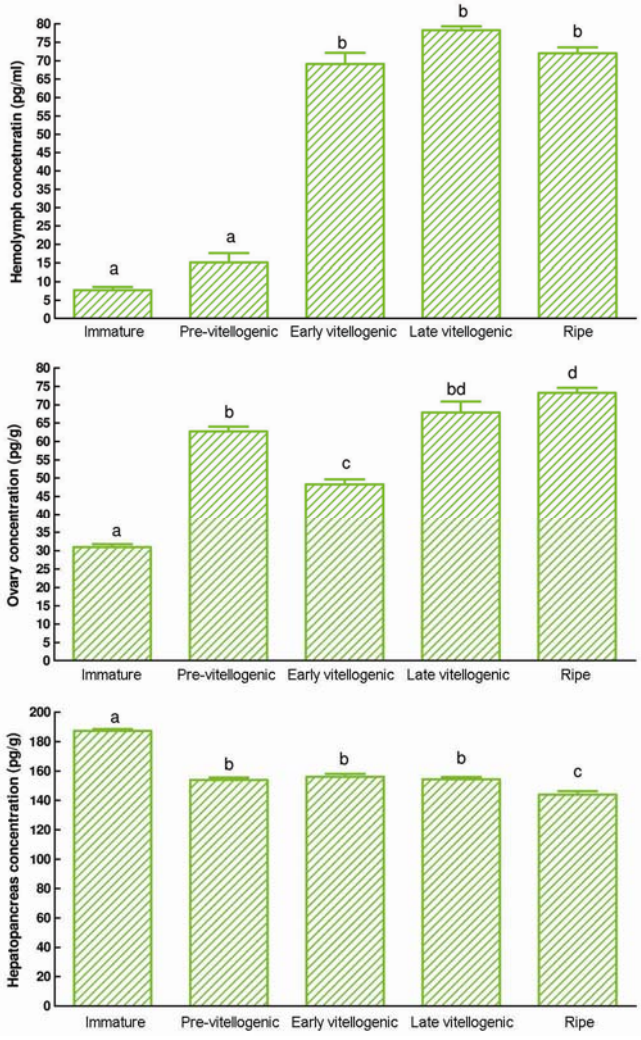

Figure 2. Concentration of progesterone in hemolymph, ovary and hepatopancreas during different stages of ovarian development of *P. monodon.*

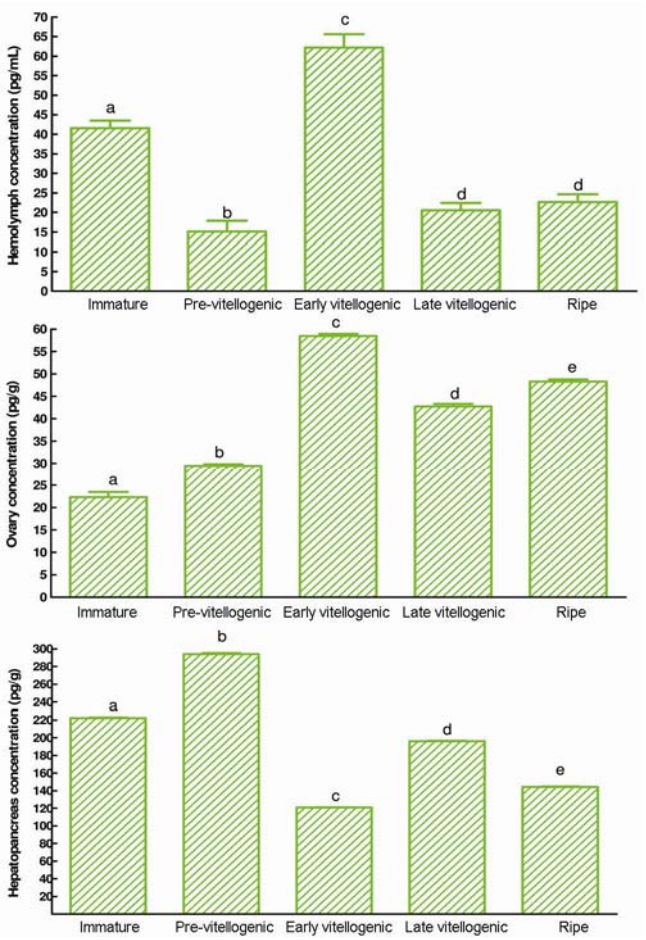

Figure 3. Concentration of estradiol in hemolymph, ovary and hepatopancreas during different stages of ovarian development of *P. monodon*.

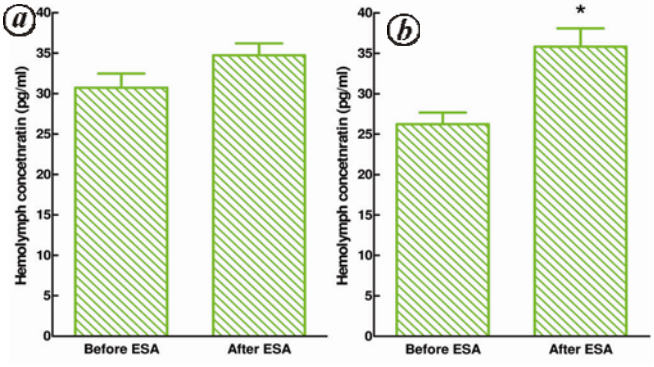

Figure 4. Concentration of estradiol (***a***) and progesterone (***b***) in hemolymph of immature wild brooders of *P. monodon* before and after eyestalk ablation (ESA).

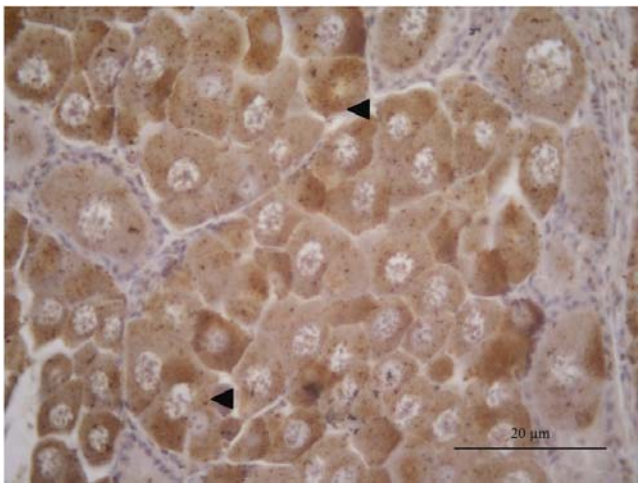

Figure 5. Immunolocalization of progesterone receptor in immature wild ovary tissue of *P. monodon*. Progesterone receptor immunoreactivity is localized predominantly in pre-vitellogenic and early vitellogenic oocytes. Arrowheads indicate strong positive immunoreactive signals.

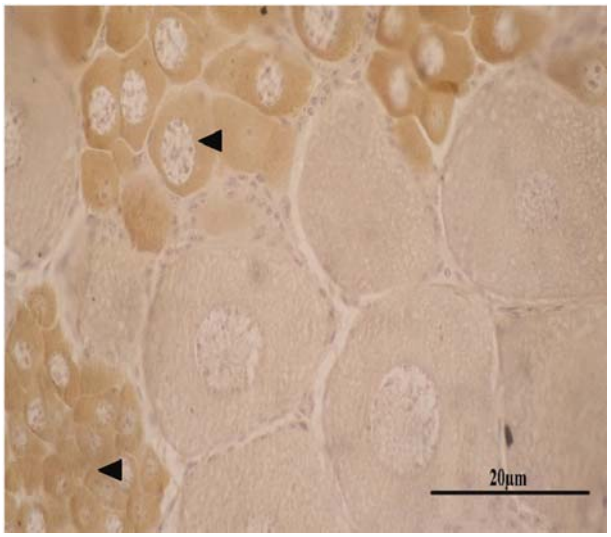

Figure 6. Immunolocalization of estradiol receptor in immature wild ovary tissue of *P. monodon*. Estradiol receptor immunoreactivity is localized predominantly in pre-vitellogenic oocytes. Arrowheads indicate strong positive immunoreactive signals.

296.4 ± 2.0 pg/ml) than ovary and hemolymph. Significantly higher level of estradiol was found at the pre-vitellogenic stage (296.4 ± 2.0 pg/ml; $P < 0.05$).

One week after eyestalk ablation, average progesterone concentration in the hemolymph increased from 26.25 ± 2.8 to 35.83 ± 4.4 pg/ml ($P < 0.05$) (Figure 4). Although hemolymph concentration of estradiol increased slightly following eyestalk ablation, it was not significantly different from the initial concentration (Figure 4).

Localization of progesterone receptor and estradiol receptor using immunohistochemistry

In immature ovary of *P. monodon*, immunohistochemistry revealed positive signals of progesterone receptor protein mainly in pre-vitellogenic oocytes (Figure 5) and also in vitellogenic oocytes, whereas immunoreactivity of estradiol receptor exhibited strong positive signals predominantly in pre vitellogenic oocytes (Figure 6). The negative control with rabbit IgG showed no immunoreactivity in the immature ovary.

Discussion

In crustaceans, two sets of hormones are reported. The first group comprises of hormones, which were originally isolated from crustaceans and proved to have specific physiological roles, for example, CHH family hormones and ecdysteroid. The second group includes hormones which have originally been discovered in vertebrates and later identified in crustaceans using modern techniques; for example, vertebrate-type steroids. While the first group of hormones is known as 'native hormones', the second group of hormones is known as 'naturalized hormones'[10]. The search for function of any hormone, most often relies on the establishment of correlation between hormone titres, and developmental and reproductive events[18]. In the present study, it is established that titres of vertebrate hormones in tissues show parallel fluctuation with the identified events in the reproductive cycle or ovarian maturation. The titres for estradiol and progesterone in the hemolymph, ovary and hepatopancreas are well within those reported by Quinitio *et al.*[19] for *P. monodon*, although these authors used radioimmunoassay (RIA) for determination of steroid hormones. We have used ELISA instead, and our results confirm that enzyme immunoassay can provide comparable data. Owing to the inherent problems in using radioactive material, presently, many laboratories prefer using ELISA[22].

Although levels of steroid hormones reported herein are similar to those reported by Quinitio *et al.*[19], our results concerning the dynamic changes of steroid titres

seem inconsistent with their data. This variation may largely be due to the difference in the criteria used for classification of ovarian stages. Quinitio *et al.*[19] followed a classification scheme in which juveniles and sub-adult shrimps were also included. However, we have used adult broodstock from the wild with body weight above 100 *g*. Unsurprisingly, the initial two phases of the classification scheme of Quinitio *et al.*[19] (primordial germ cell stage and chromatin-nucleolus stage) were not found in the catch from wild broodstock. When we adjust our classification protocol with their scheme of classification, steroid profile in the hemolymph and ovary is consistent. However, our hepatopancreas data are found to be different from their data. The plausible explanation for this may be the difference in the broodstock origin: Quinitio *et al.*[19] studied pond-reared broodstock, whereas we used broodstock caught in the wild. In the present study, relatively high levels of progesterone and estradiol were found in the hepatopancreas at all the ovarian stage. This may be due to the difference in the nutritional status between captive-reared and wild broodstock. It is also intriguing to note that recent microarray data of pond-reared and wild-caught shrimps show reduced expression levels for transcript-encoding lipid accumulation genes in the pond-reared animals. Recently, while investigating reproductive dysfunction of pond-reared *P. monodon*, Bradly *et al.*[23] reported that gene transcript for high-density lipoprotein in the hepatopancreas is differently expressed between wild-caught and pond-reared animals. This differential expression partly explains the reason for high titre of lipophilic hormones such as progesterone and estradiol in the hepatopancreas (present study).

Many authors have characterized ovarian histology of *P. monodon*[19,24] and other penaeid shrimps. However, there is no consistency among the various subdivisions, and classification of ovary is only a convenient way of labelling a continuous process. In the present study, we were able to classify ovary into five stages, mainly based on oocyte diameter and cellular characteristics of oocytes. Our study also indicates that the presentation of histological data is essential to compare the previous results owing to the wide difference in the classification of ovarian stages among researchers.

Remarkably high levels of progesterone and estradiol were found in hepatopancreas compared to hemolymph and ovary. This proposes that these hormones could be synthesized in the hepatopancreas and then transported into developing ovaries. The current findings are consistent with the hypothesis that hepatopancreas may be the primary site of steroid synthesis[25]. This finding is also consistent with our earlier findings on Vg mRNA expression profile in the hepatopancreas of *P. monodon*: the highest level of expression was found at the previtellogenic stage (D. L. Mohanlal, unpublished Ph D thesis). Our results are consistent with the earlier report in *Scylla serrata*[26].

Progesterone has a pivotal role in vertebrate reproduction by its influence on post meiotic oocyte maturation. The higher level of progesterone in the hemolymph and ovary suggests the functional role for these hormones in advanced vitellogenic phases in *P. monodon*. Our earlier studies on the *in vivo* treatment of *P. monodon* ovarian explant with 17α-hydroxy progesterone showed that this hormone was effective only in vitellogenic ovary[17]. In cray fish, Cocci *et al.*[27] also reported higher vitellogenin level in progesterone-treated females at advanced vitellogenic ovary.

The present results indicate that hepatopancreas is the major site of production of sex steroid hormones, and failure to production of these hormones may cause the reproductive dysfunction. Therefore, further studies are needed to localize and quantify the expression profile of steroidogenic enzymes in the hepatopancreas.

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ACKNOWLEDGEMENTS. We thank Dr A. G. Ponniah (CIBA, Chennai) for his keen interest and support throughout the study. We also thank Dr P. Ravichandran (Crustacean Culture Division, CIBA) for advice and support. This study was supported by a Department of Biotechnology grant (BT/PR15414/AAQ/03/555/2011).

Received 3 March 2016; accepted 3 June 2016

doi: 10.18520/cs/v111/i7/1194-1200