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Induction of vitellogenesis and reproductive maturation in tiger shrimp, *Penaeus monodon* by 17ß-estradiol and 17α-hydroxyprogesterone: *in vivo* and *in vitro* studies

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Ovarian explants of *Penaeus monodon* taken from different developmental stages (immature (transparent), immature (yellow), and vitellogenic (green)) were incubated for 24 h in medium 199 supplemented with either E_2 or 17α-OHP. Exogenous E_2 stimulate Vg mRNA synthesis in the immature (yellow) ovary only, while 17α-OHP stimulates mRNA synthesis only in vitellogenic ovary. However, *in vitro* treatment with both hormones resulted in significant increase in oocytes diameter at higher doses. *In vivo* assays were carried out by administrating these hormones alone or along with eyestalk ablation at a dose of 9×10^{-5} M per individual. After 7 days, significantly higher gonadosomatic index resulted in E_2 and 17α-OHP treatment along with eyestalk ablation. Further, Vg mRNA transcript level was significantly higher in the group received both 17α-OHP and eyestalk ablation. Progesterone and estradiol receptors were characterized using western blotting. Discrete immunoreactive bands of estrogen Receptor (ER) (~78 kDa) and progesterone Receptor (PR) (~70 kDa) were detected in the ovary of wild brood stock. These findings suggest a reproductive role for these steroidal hormones, which could possibly be used in the induction of maturation and spawning in captive female penaeids.

Keywords: vitellogenesis; reproductive maturation; *Penaeus monodon*; sex steroids; progesterone; estradiol

Introduction

The most acknowledged paradigm of crustacean reproductive endocrinology is that ovarian maturation or vitellogenesis is regulated by two antagonistic neuropeptides, gonad- or vitellogenesis-inhibiting hormone (G/VIH) synthesized by X organ of eyestalk and gonad-stimulating hormone synthesized by brain and thoracic ganglion (Van Herp & Soyez 1997). In addition to these neuropeptides, malacostracan crustaceans use a wide array of nonpeptidergic hormones in the proximate control of reproduction. Among these gonadotropic hormones, ecdysteroid and methyl farnesoate, vertebrate-type sex steroids play a key role on reproduction in crustaceans (Yano 1985; Quackenbush 1989; Tsukimura 2001; Rodriguez et al. 2002).

In order to expedite the domestication of commercially important shrimp, the identification of gonadotropic hormone is imperative, as the removal of inhibitory hormone by eyestalk ablation is only partially successful in producing healthy seeds. Sex steroids (for example estradiol, progesterone and testosterone) play pivotal physiological roles including growth, reproduction, and sex differentiation in vertebrates (Edwards 2005). Although sex steroids are reported among many invertebrates, their role in the control of female reproduction is far from clear (Scott 2012). Many authors have reported the presence of vertebrate-type sex steroids in crustaceans (Subramoniam 2000, 2011, for review). The first evidence for the presence of sex steroid in Crustacea was provided by Lisk (1961), who showed that eggs of American lobster, *Homarus americanus*, contained E_2. Subsequently, many researchers reported the existence of sex steroids and enzymes involved in their biosynthesis in crustacean reproductive tissues such as ovary and testis (Sarojini 1963; Kanazawa & Teshima 1971; Jeng et al. 1978; Couch et al. 1987; Sasser & Singhas 1992; Ghosh & Ray 1993; Quinitio et al. 1994; Shih & Liao 1998). Possible stimulatory or gonadotropic effect of vertebrate sex steroids has been reported in many malacostracan crustaceans such as crayfish (Rodriguez et al. 2002; Coccia et al. 2010), the crab, *Scylla serrata* (Warrier et al. 2001), the anomuran crab, *Emerita asiatica* and the freshwater prawn, *Macrobrachium rosenbergii*, and in marine shrimps (Kulkarni et al. 1979; Nagabhushanam & Kulkarni 1981; Yano 1985, 1987; Tsukimura & Kamemoto 1991; Scott Quackenbush 1992; Yano & Hoshino 2006). In vertebrates, steroid hormones mediate their action through receptors. Although reports on steroid receptors are limited in invertebrates, among crustaceans, progesterone and estradiol receptors have been detected in crayfish (Paolucci et al. 2002) and mud crab, *Scylla paramamosain* (Ye et al. 2008; Ye et al. 2010).

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The present study aims to investigate the steroidal control of vitellogenesis in the penaeid shrimp, *Penaeus monodon*, one of the top-most farmed shrimp globally, by focusing on the specific role of E_2 and17α-OHP. In order to credibly define the functions of sex steroids in relation to different phases of oogenesis, we used both *in vitro* and *in vivo* bioassay. In addition, the presence of specific receptors for both the hormones has also been studied in the ovary of *P. monodon*.

Materials and methods

Animals

Female and male broodstocks of *P. monodon* (200–250 g and 90–120 g for females and males, respectively) were obtained from the broodstock fishery along the coasts of Chennai, India. The experiment was carried out in the experimental station of Central Institute of Brackishwater Aquaculture, Muttukadu, Tamil Nadu (India). Animals were acclimatized for at least one week before being used for experimentation. They were kept in FRP tanks containing sea water with 28–30‰ salinity, at a temperature of 26–28 °C and continuous aeration. Shrimps were kept at a sex ratio of 2F:1 M, and maintained under natural photoperiod. They were fed clams and squids thrice a day. Approximately 90% of the sea water was replaced twice daily.

In vitro *hormone treatment*

For the present study, the ovarian development was classified into three major stages according to the previously characterized criteria with minor modifications: Immature – white (The ovary is white with hematoxylin- stained previtellogenic oocyte at early chromatin nucleolus stage), immature – yellow (ovary is yellow with eosinophilic oocyte at late chromatin nucleolus stage) and vitellogenic stage (Ovary is green with yolky acidophilic oocytes) (Tan-Fermin & Pudadera 1989; Minagawa et al. 1993; Abdu et al. 2000) (Figure 1). To study the *in vitro* effect of E_2, all the stages were used, whereas, for the study of the 17α-hydroxyprogesterone, only immature (white) and vitellogenic ovary were used.

The ovarian tissue collected from a single animal was cut into small pieces (100 mg each). The tissues were then rinsed twice with sterile filtered Medium 199 containing 2X antibiotic, anti-mycotic solution with 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL (Sigma). Four of them were placed in each well of the 12-well tissue culture plate containing 4 ml of Medium 199 containing 1X antibiotic, anti-mycotic solution with 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL (Sigma). Stock solutions of hormones were prepared in ethanol and serially diluted to appropriate working concentration with medium. Three doses of E_2 (5×10^{-5}, 9×10^{-5} and 1.4×10^{-5} M) and 17α-OHP ($5 \times 1\,0^{-5}$, 9×10^{-5} and 1.1×10^{-5} M) were prepared. Equal volumes of ethanol were added to the culture medium of the control group. Three wells were used for each treatment group. The explants were incubated at 37 °C in an Incubator (Jeio Tech) for 24 h, after which the tissues from each group were collected for analysis. From each treatment group, two explants were stored at 4 °C along

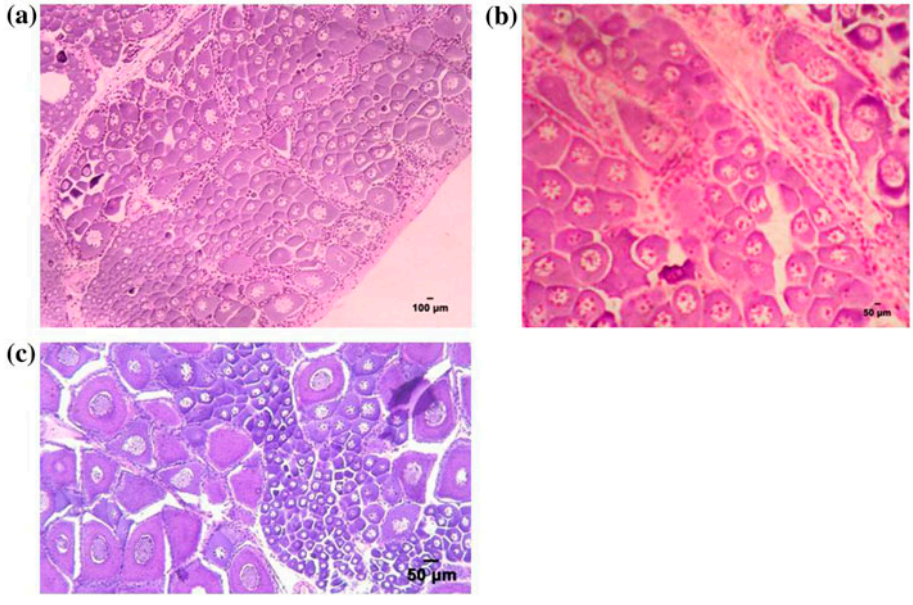

Figure 1. Ovarian maturation stages in *P. monodon*. (a) immature (white) ovary, (b) immature (yellow) ovary, (c) vitellogenic ovary.

with 1 mL of respective culture medium until oocyte diameter measurement and the remaining two pieces of ovarian tissues were stored in RNAlater (Sigma).

Oocyte measurement

Ovarian explant was kept on a clean glass slide along with few drops of their respective culture medium (M199 with the respective concentration of the hormone). The tissue was teased out and the individual oocytes were taken out. These oocytes were examined under microscope (Carl Zeiss Axiostar plus) and the oocyte measurement was taken using ProgRes® CapturePro software (v2.8.8). The mean oocyte diameter (MOD = major diameter + minor diameter/2) was measured.

In vivo *hormonal treatment*

Forty female shrimps with immature ovary were divided into four groups: Control, injected with crustacean saline solution (vehicle); unilateral eyestalk ablation; hormone treatment, injected either with 17α-hydroxyprogesterone or 17ß-estradiol (Sigma); hormone treatment along with unilateral eyestalk ablation. Five milligram of hormone was dissolved in ethanol and diluted to the required concentrations using crustacean saline solution. The hormone was injected intramuscularly between the first and second thoraco-abdominal segments at a dose of 9×10^{-5} M per animal. The injection volume was 200 µl. Ovary maturation and spawning in all groups was monitored for a week. At the end of the treatment, the animals were weighed, anesthetized on ice and killed. Subsequently, portion of ovary from each animal was removed and stored in RNAlater (Sigma) for RNA extraction. The middle portion of ovary from each animal was fixed for histological analysis.

Histological analysis

Histological procedures were carried out according to Bell and Lightner (1988). The ovary was fixed in Davidson's fixative for 24 h, dehydrated with ascending alcoholic series, cleared in xylene, and then embedded in paraffin wax. The sections were cut to 5 µm thickness and stained with hematoxylin and eosin.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRI Reagent (Sigma) from the ovary samples obtained after *in vitro* or *in vivo* treatment. The total RNA was treated with RQ DNaseI (Promega) and the resulting total RNA (2 µg) was used for the preparation of the template cDNA to ensure that there is no genomic DNA contamination. Reverse transcription was performed using Sensiscript Reverse Transcription kit (Qiagen). cDNAs were stored at −20 °C until analysis.

PCR amplification, cDNA cloning, and nucleotide sequencing

Oligonucleotide primers (Vg Forward- 5′GGACTTCAGGAAATTCTCTCG 3′; Vg Reverse-5′CAATGAGTGTGTCAGCTGTGA 3′) were designed on the sequence of the *P. monodon* Vg gene submitted in the GenBank (Accession No. DQ288843.1). PCR amplification was carried out in Veriti 96Well Thermal Cycler (Applied Biosystems) in a 25 µl reaction using 1.25 units of Taq DNA Polymerase, 1X Taq DNA Polymerase Buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, and 2 µg of template cDNA. PCR conditions consisted of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 40 s and extension at 72 °C for 1 min. A final elongation step was performed at 72 °C for 10 min. The PCR product was separated by 1.5% agarose gel electrophoresis with ethidium bromide and visualized with Gel documentation system (BioRad). From the gel obtained, PCR product was purified using HiYield Gel/PCR extraction kit (Real Genomics). The 500 bp long fragment was cloned using InsTA PCR Cloning kit (Thermo Scientific). The plasmid DNA was purified using HiYield Plasmid Mini kit (Real Genomics). The nucleotide sequencing was carried out by 1st base sequencing services.

Real Time PCR

The primers used for Real-Time PCR were designed using OligoArchitect™ Online v3.0 (Sigma). The sequences of primers and probes used for qPCR are given in Table 1. The quantitative assay of target (Vg) gene expression and the reference (β-actin) gene expression was set up using TaqMan Probe assay with QuantiFast Probe PCR + Rox Vial kit (Qiagen). The TaqMan probe was synthesized and labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5′ end and N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end. The PCR was performed using

Table 1. Sequences of primers and probes used in the present study for real-time quantitative polymerase chain reaction.

No	Gene	Sequence (5′–3′)
1	Vg Forward	CATGCCCCTGCCTTTGG
	Vg Reverse	GGAATGAAAGATCCAGGAGCAT
	Vg Probe	ATGCCGGCCTCGAATCGAACAT
2	ß-actin Forward	CTTCGAGCAGGAGATGAC
	ß-actin Reverse	GGAGTTGTAGGTGGTCTC
	ß-actin Probe	CTGCTTCCTCCTCCTCGCTG

a 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: an initial PCR heat activation at 95 °C for 3 min, followed by two-step cycling for 35 cycles: 95 °C for 3 s, 60 °C for 3 s, and 72 °C for 3 mins. All reactions were performed in duplicate to confirm the reproducibility of the results. The specificity of the assay was confirmed by sequencing the reaction product obtained from both target and reference genes. Primer amplification efficiencies were calculated from the slope of the standard curve, which was derived from a dilution series (1:10, 1: 100, 1:1000, 1:10,000 and 1:100,000). A NTC (No template control) which used Nuclease-free water instead of cDNA as templates was designed to confirm no primer dimmers existing. The reference gene β-actin was used to calibrate the cDNA template in the present study, as it showed relatively stable gene expression in all the experiments. The relative mRNA expression levels were calculated by $2^{-\Delta\Delta CT}$ method.

Western blotting

All the stages of ovary from the wild brood stock were dissected, minced and homogenized in Radio Immuno Precipitation Assay buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Sodium deoxycholate, 0.1% SDS, 1% Triton-X 100, pH 7.4) supplemented with the Protease Inhibitor cocktail (Sigma). 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 stored at −80 °C until analysis. Total protein concentrations in the ovary extracts were determined with the Bradford method using BSA as the standard (Bradford 1976). Ten microgram of ovarian extracts were electrophoretically separated by 10% SDS-PAGE (Laemmli 1970) and transferred onto a Immun-Blot® PVDF membrane (Bio-rad) in the 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 20% methanol at 100 V for 1 h. The membrane was blocked for 1hr at 37 °C with 5% BSA in 1 × Tris Buffer Saline-Tween-20 (TBST; 20 mM Tris–HCl, 50 mM NaCl, pH 7.6, 0.2% Tween-20) and then incubated with primary antibodies (Polyclonal Human anti-progesterone receptor and anti-estrogen receptor-α produced in rabbit) (Sigma) in 1:10,000 dilution at 37 °C overnight. Subsequently, the membrane was washed thrice with 1 × TBST (20 mM Tris–HCl, 50 mM NaCl, pH 7.6, 0.2% Tween-20) and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (GeNei) at 1:15,000 for 45 min. The membrane was then washed thrice with 1X TBST and once with 1X TBS (20 mM Tris–HCl, 50 mM NaCl, pH 7.6). The signals of immunostaining were visualized using BCIP/NBT (GeNei) as the substrate. The controls were obtained by omitting the primary antibody. The western blot image was captured by Olympus c5050 Zoom camera.

Statistical analysis

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

Results

In vitro *effect of sex steroids on vitellogenesis: 17ß-estradiol*

Exposure of E_2 facilitated significant oocyte enlargement in all the stages of ovary. A maximum response of 186% enlargement of oocyte was observed in the immature (yellow) ovary explants at the dose of 1.4×10^{-5} M E_2 ($p < 0.01$, overall ANNOVA, Figure 2). In immature (white) and vitellogenic ovary, significant enlargement of oocyte was detected only at a dose of 9×10^{-5} M E_2 (Figure 2). Significant Vg mRNA upregulation was detected in the immature (yellow) ovary explants treated with E_2 at 9×10^{-5} M and 1.4×10^{-5} M doses (Figure 3). On the contrary no significant Vg mRNA upregulation was detected in immature (white) and vitellogenic ovary treated with E_2.

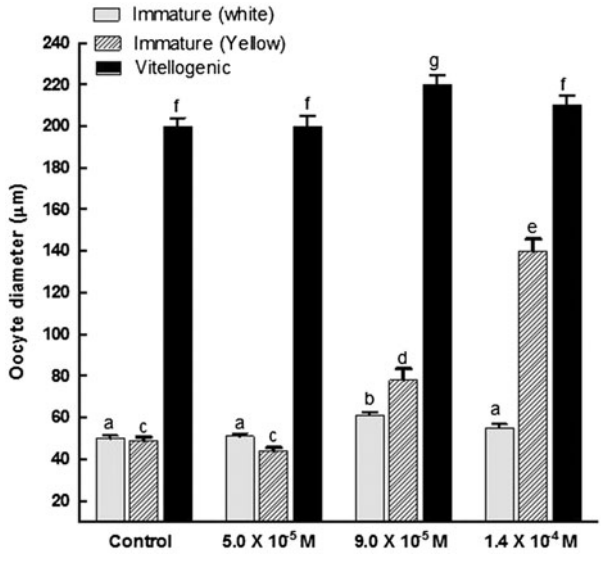

Figure 2. In vitro effect of 17ß-estradiol on oocytes diameter in the shrimp, *P. monodon*.
Note: The ovarian explants at different developmental stages were incubated with different concentration of 17ß estradiol. Significant differences ($p < 0.05$) between means were determined using ANOVA.

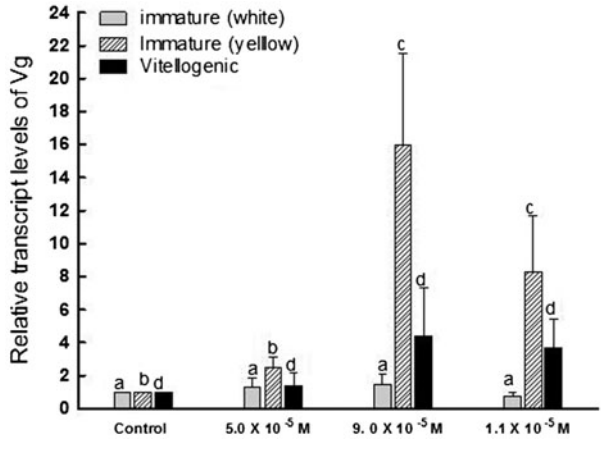

Figure 3. Changes in the transcript levels of Vg in the ovary explants after 24 h *in vitro* treatment with 17ß-estradiol measured by quantitative RT-PCR.
Note: Bars indicate mean relative levels of Vg for 5 individuals. Vertical lines on the bars indicate standard deviations. Vg values were normalized to ß actin and are expressed relative to the levels at control, which are set at one. Significant differences ($p < 0.05$) between means were determined using ANOVA.

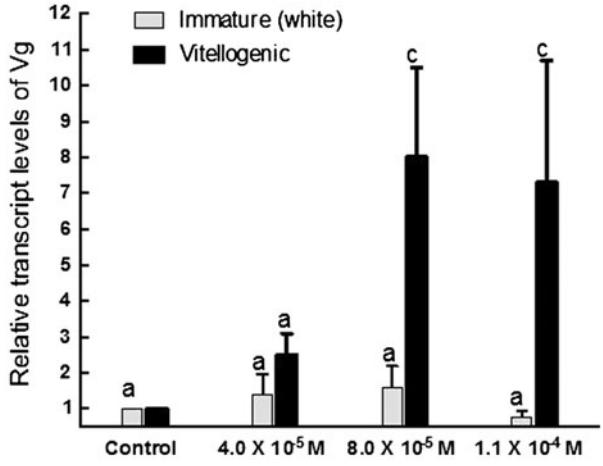

Figure 5. Changes in the transcript levels of Vg in the ovary explants after 24 h *in vitro* treatment with 17α-hydroxyprogesterone measured by quantitative RT-PCR.
Note: Bars indicate mean relative levels of Vg for 5 individuals. Vertical lines on the bars indicate standard deviations. Vg values were normalized to ß actin and are expressed relative to the levels at control, which are set at one. Significant differences ($p < 0.05$) between means were determined using ANOVA.

In vitro *effect of sex steroids on vitellogenesis: 17α-hydroxyprogesterone*

The process of vitellogenesis in *P. monodon* could be induced by direct exposure of ovarian explants to 17α-OHP. Significant enlargement of the oocytes and upregulation of vitellogenin mRNA were observed in immature (white) and vitellogenic ovarian explants (Figures 4 and 5). In immature (white) ovary, significant enlargement of oocytes compared to control was detected only at the dose of 1.1×10^{-4} M, whereas in vitellogenic ovary significant enlargement of oocytes was detected in 8×10^{-5} M and 1.1×10^{-4} M treatments ($p < 0.05$; overall ANNOVA, Figure 4). Further, no Vg mRNA upregulation was detected in the immature (white) ovary explants after hormonal treatment. However, significant Vg mRNA upregulation was detected in vitellogenic ovarian explants in dose-dependent way ($p < 0.05$; overall ANNOVA, Figure 5).

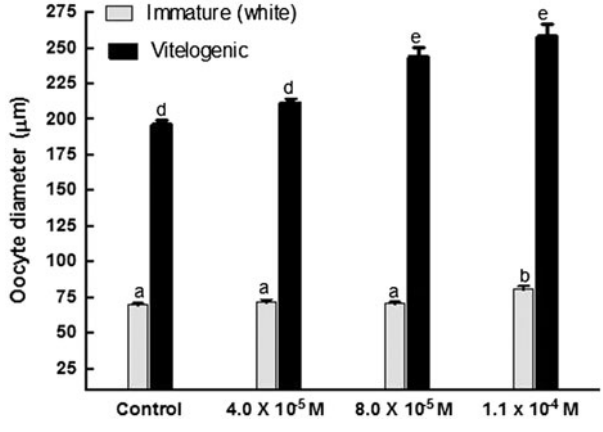

Figure 4. *In vitro* effect of 17α-hydroxyprogesterone on oocytes diameter in the shrimp, *P. monodon*.
Note: The ovarian explants at different developmental stages were incubated with different concentration of 17α-hydroxyprogesterone. Significant differences ($p < 0.05$) between means were determined using ANOVA.

In vivo *effect of sex steroids on vitellogenesis: 17ß-estradiol*

After one week of hormone treatment, the mean gonadosomatic index of shrimps that received E_2 and were eyestalk ablated (8.42 ± 3.8) was significantly higher than the gonadosomatic indices of the control (1.44 ± 0.28), eyestalk ablation (positive control) (4.89 ± 2.3), and E_2 alone (4.1 ± 2.73) (Figure 6). Further, percentage of females that reached ripe stage was higher in the groups received E_2 as well as eyestalk ablation (Figure 7).

In vivo *effect of sex steroids on vitellogenesis: 17α-hydroxyprogesterone*

After one week of administration of 17α-OHP, none of the females reached ripe stage, although gonadosomatic index of all the treatment groups were significantly higher than the vehicle-treated control group (Figure 8). Conversely, at molecular level Vg mRNA gene expression

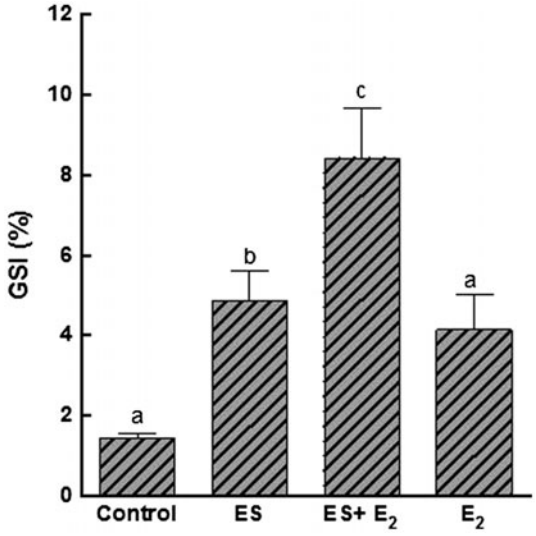

Figure 6. The *in vivo* effect of 17ß-estradiol on gonadosomatic indices in shrimp, *P. monodon*.
Note: Significant differences ($p < 0.05$) between means were determined using ANOVA.

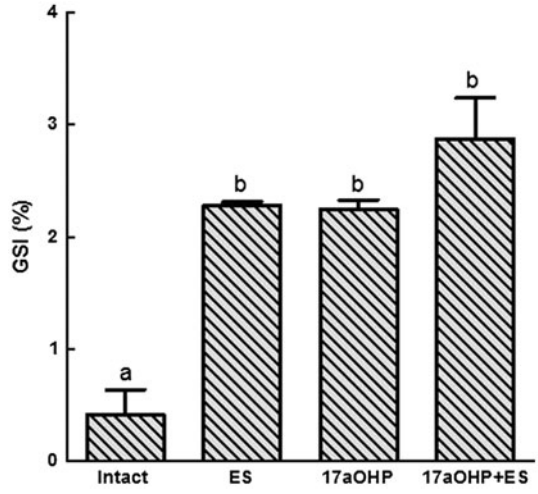

Figure 8. The *in vivo* effect of 17α-hydroxyprogesterone on gonadosomatic indices in shrimp, *P. monodon*.
Note: Significant differences ($p < 0.05$) between means were determined using ANOVA.

was significantly increased by the administration of 17α-OHP (Figure 9).

Western blot analysis of estrogen and progesterone receptor

Discrete immunoreactive bands of estrogen receptor (ER) and progesterone receptor (PR) were detected in the ovary of wild brood stock. The bands were detected around 78 and 70 kDa for ER and PR respectively. The immunological signals were greater in previtellogenic and early vitellogenic stages (Figures 10 and 11). Minimal immunoreactivity was found in later stages. No signals were observed in ripe stage.

Discussion

The present study provides evidence that sex steroids, E_2 and 17α-OHP, induce vitellogenesis in *P. monodon*. However, the effects of these ligands are found to vary in different phases of oogenesis: E_2 upregulates Vg mRNA expression of immature (yellow) ovarian explants, whereas 17α-OHP increases Vg mRNA expression only in vitellogenic ovary. Our study provides further evidence to the growing body of literature indicating

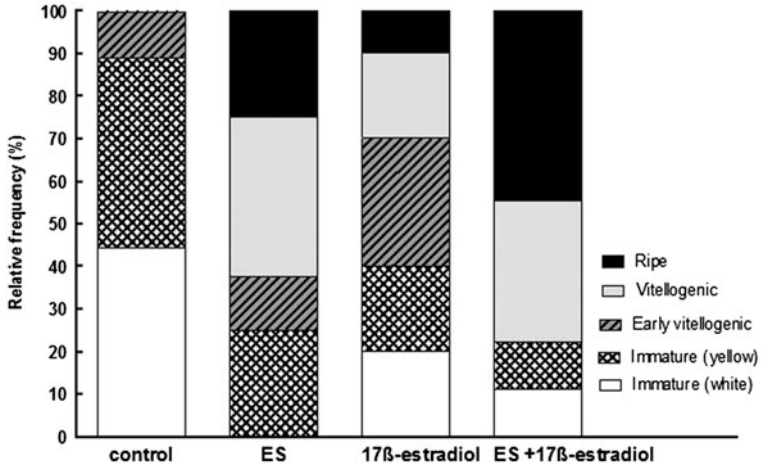

Figure 7. Relative frequency of maturation stages in different experimental groups.

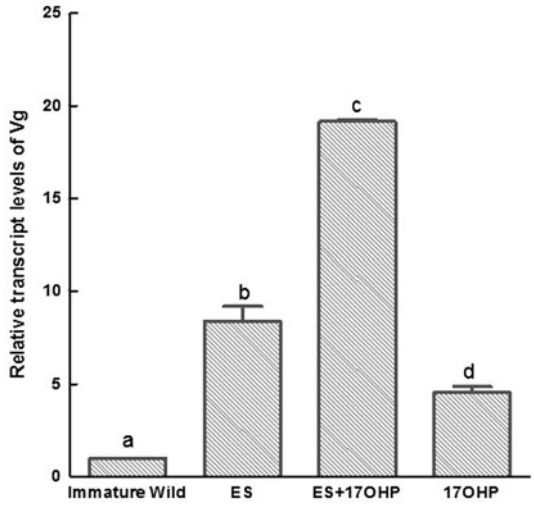

Figure 9. *In vivo* effect of 17α-hydroxyprogesterone on vitellogenesis in shrimp, *P. monodon*.
Note: Changes in the transcript levels of Vg in the ovary at different treatment group measured by quantitative RT-PCR. Bars indicate mean relative levels of Vg for 5 individuals. Vertical lines on the bars indicate standard deviations. Vg values were normalized to ß actin and are expressed relative to the levels at immature wild, which are set at one. Significant differences ($p < 0.05$) between means were determined using ANOVA.

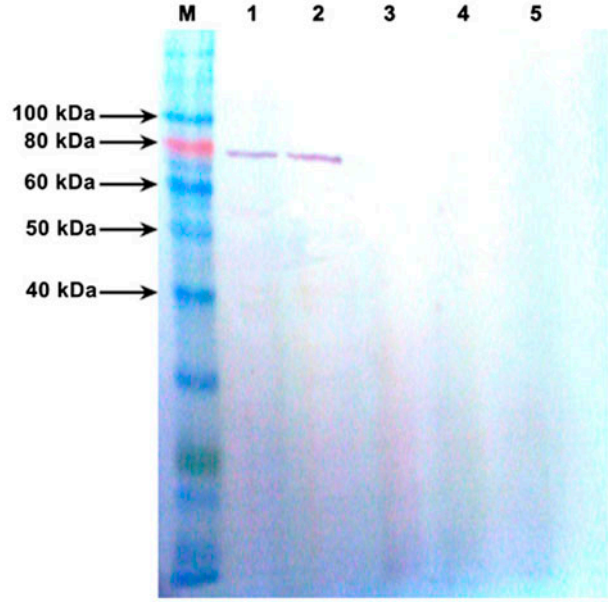

Figure 10. Western blot of ovarian samples of *P. monodon*. Samples were probed with polyclonal hER antibody.
Note: Immunoreactivity was found in the extract of ovarian sample 1 (Previtellogenic stage) and sample 2 (early vitellogenic stage).

that vertebrate-like sex steroids play a significant role in the control of reproduction in invertebrates. (Stout et al. 2010).

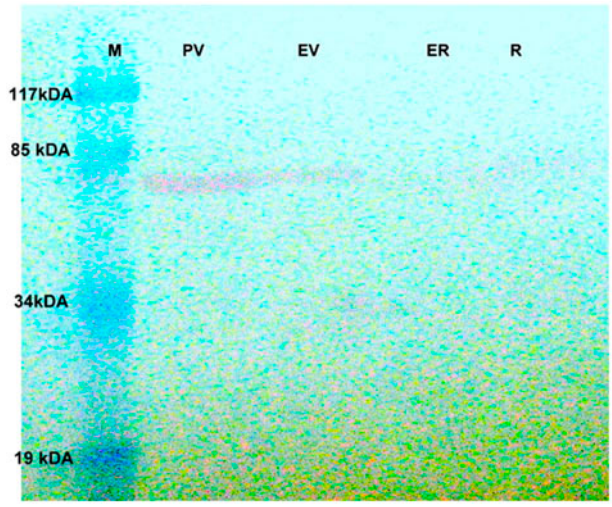

Figure 11. Western blotting results of PR with normal rabbit IgG anti-PR antibody in *P. monodon*.
Note: This immunoblot image shows a single immunoreactive band of PR with apparent molecular weight of about 70 kDa in ovary of *P. monodon.* (M = Molecular Marker; PV = Previtellogenic stage ovary; EV = early vitellogenic stage ovary; ER = early ripe stage ovary; R = ripe stage ovary).

Our data corroborate earlier studies in which treatment of E_2 and 17α-OHP stimulated ovarian growth and vitellogenesis in related penaeid shrimps. Yano (1987) reported 9.6-fold increase in serum vitellogenin in the female *M. japonicus* injected with 17α-OHP. Later, Yano and Hoshino (2006) reported significant increase in Vg in the media after incubating ovarian explants along with E_2 for three days. Similarly *Parapenaeopsis stylifera* spawned within five weeks after being injected with 17α-OHP (Nagabhushanam et al. 1980). Quinitio et al. (1994) reported a positive correlation between sex steroid titers and Vg concentration in the hemolymph of *P. monodon*. In *Pandalus kessleri* the level of estradiol corresponds to vitellogenin level in the hemolymph (Quinitio et al. 1991). Conversely, no correlation was detected between sex steroid concentration in hemolymph and ovarian development stages in *M. japonicus* (Okumura & Sakiyama 2004). The difference observed could be due to the species-specific hormone mechanism and variations in experimental condition or variation in the physiological status of the animal.

Present data also indicate that E_2 and 17α-OHP significantly enhance Vg mRNA synthesis in the ovary at specific ovarian phase of maturation: While E_2 stimulates Vg mRNA synthesis in the immature (yellow) ovary, 17α-OHP stimulates only in vitellogenic ovary. This is in agreement with the sex steroid levels in the hemolymph during reproductive cycle. It is reported that the E2 level was higher in the early ovarian stages in other related crustaceans (Shen et al. 2010). Similarly, highest

concentration of progesterone was reported in the hemolymph, ovary, and hepatopancreas of vitellogenic and late vitellogenic *S. serrata* females (Warrier et al. 2001). Coccia et al. (2010), further, reported a higher vitellogenin levels in progesterone-treated full vitellogenic female than early vitellogenic females. The presence of progesterone receptors in the vitellogenic female ovary could be related to the physiological role of maturational hormone played by progestins in vertebrates (Paolucci et al. 2002). Our *in vivo* experiment also supports this finding, as none of the females spawned or reached ripe stage after the progesterone treatment, although significant increase in GSI and Vg mRNA accumulation occurred after the treatment.

The effective concentration of sex steroids for *in vitro* actions in the present study is found to be higher than the actual steroid levels observed in crustaceans. In Crustacea, the hemolymph concentration of sex steroids is reported to be low, for example, the hemolymph concentrations of E_2 and progesterone are 23–104 pg per ml and 25–185 pg per ml, respectively, in the mud crab *S. serrata* (Warrier et al. 2001). The transportation of sex steroid in intact animals is thought to be facilitated by the steroid hormone-binding protein, whereas in the *in vitro* experiments sex steroids diffused into the tissue passively, and thus a higher concentration would be required (Wang 2000).

The classical mechanism of steroid hormone action involves the passage of hormone through the plasma membrane and binding with specific receptor. The activated hormone receptor complex then moves into the nucleus and binds with the response elements on hormone responsive gene to regulate transcriptional activity (Thornton et al. 2003). The present study provides evidence for the involvement of classical nuclear receptors, ER and PR. The anti-human ER and PR antibody cross reacted with immunoreactive protein of about 78 and 70 kDa in ovarian samples at the initial phases of oogenesis, previtellogenic, and early vitellogenic stages. Similar to our findings, PR of approximately similar molecular weights was identified by western blot in mud crab *S. paramamosain* (Ye et al. 2010) and in crayfish *Austropotamobius pallipes* (Paolucci et al. 2002). Nevertheless, Paolucci et al. (2002) could not identify ER immunoreactivity in the ovary of crayfish *A. pallipes*, although they identified ER immunoreactivity in the hepatopancreas. Recently, Coccia et al. (2010) confirmed that hepatopancreas is the site for synthesis of yolk protein in fresh water crayfish, and the possible absence of autocrine role of estradiol in these crustaceans. Contrastingly, our results suggest the presence of estradiol receptor in the ovary of *P. monodon*, in which ovary is the principal organ of vitellogenin synthesis (unpublished observations). This may be due to the variability in site of Vg synthesis in Crustacea. In many penaeid shrimps, ovary is the major site of vitellin synthesis, for example, *P. japonicus* (Yano & Chinzei 1987), *P. vannamei* (Rankin et al. 1989) and *P. semisulcatus* (Browdy et al. 1990).

In summary, present results clearly indicate that sex steroid, particularly E_2, offers a mean for controlling reproduction in captive females of *P. monodon*. But development of tools for hormonal therapy is the essential prerequisite to further explore the strategies towards the practical application. When considering the exogenous hormonal therapy, the first question to be addressed is the route of administration. While studying the induced maturation protocol for crabs, Zapata et al. (2003) proved that hormone-fortified formulated diet offers efficient strategy for the administration of hormone in brachyuran crabs. Further, in crustaceans, vitellogenin-inhibiting neuropeptides play an important role in reproduction. Therefore, removing this inhibitory control in combination with exogenous hormonal therapy with sex steroid could result more efficient way to control reproduction.

Disclosure statement

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