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Serotonin induced changes in the expression of ovarian gene network in the Indian white shrimp, *Penaeus indicus*

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ABSTRACT

The potential effect of serotonin (5-hydroxytryptamine, 5-HT) to induce oocyte maturation in the white shrimp, *Penaeus indicus* was examined in the present study. Serotonin treatment was given to intact and unilaterally eyestalk ablated females at 50 μg/g body weight and the differential expression of ovarian genes involved in vitellogenesis [vitellogenin (vg), vitellogenin receptor (*vgr*)] and meiotic maturation [cyclin-dependent kinase 2 (*cdc2*), *cyclin B* and thrombospondin (*tsp*)] was examined. Quantitative real-time PCR indicated that the levels of *vg*, *cdc2 and cyclin B* increased with advancement in ovarian maturation. Significant increase in the expression of these genes in the eyestalk ablated (ESA) and 5HT + ESA group indicated post-vitellogenic meiotic resumption and maturation of oocytes. It was also observed that for effective stimulation of ovarian maturation by serotonin, the levels of inhibitory hormone should be removed. The significant increase in *cdc2* and *cyclin B* levels in 5HT + ESA compared to ESA suggests that serotonin acts synergistically with other stimulatory hormones during eyestalk ablation to stimulate ovarian maturation. The present investigation suggests a possible dual regulatory role of serotonin on both vitellogenesis and indirect stimulation of post-vitellogenic meiotic resumption and oocyte maturation in penaeid shrimps.

Statement of relevance: Ovarian maturation and associated changes in the expression profile of ovarian genes in intact and unilaterally eyestalk ablated females treated with serotonin confirms the regulatory role of serotonin on ovarian maturation in *Penaeus indicus*. Significant increases in the expression of genes were observed in ablated females treated with serotonin. The possible dual regulatory role of serotonin on both vitellogenesis and indirect stimulation of oocyte maturation in penaeid shrimps is suggested.

1. Introduction

Female reproduction in crustaceans is an energy driven process, consisting of oogenesis, vitellogenesis, maturation and spawning. The primary oocytes entering the vitellogenic phase is accompanied by the accumulation of vitellogenin from the follicle cells surrounding oocytes and also from hepatopancreas (Subramoniam, 2011). Vitellogenin, transported through the haemolymph to developing oocytes, is sequestered into the developing oocytes by the vitellogenin receptor through receptor-mediated endocytosis (Warrier and Subramoniam, 2002) and is processed to vitellin, the major nutrient source during embryogenesis. At the termination of vitellogenesis, the post-vitellogenic oocytes enter the maturation phase characterized by resumption of meiotic division, germinal vesicle breakdown and migration away from the zone of proliferation towards the peripheral cytoplasmic membrane of the ovary. The complex process of oocyte maturation is orchestrated by the maturation-promoting factors (MPF, a complex of cyclin-dependent kinase 2 (cdc2) and Cyclin B) which regulates meiotic resumption as the oocytes mature making them possible marker genes of oocyte developmental competence (Han et al., 2012). Germinal vesicle breakdown (GVBD) and formation of cortical rods in oocytes (Kim et al., 2005; Yamano et al., 2004) are considered signs of oocyte maturation in penaeid shrimps.

A complex multi-hormonal cascade signalling system regulates ovarian development in Crustacea. Manipulation of reproduction in captive brood stock poses a major hurdle in crustacean aquaculture. Among the known neuroendocrine factors regulating crustacean vitellogenesis, gonad inhibiting hormone (GIH), secreted from the neurosecretory cells of the X-organ sinus gland complex (XO-SG) in the eyestalks, exerts negative regulation on vitellogenesis. Unilateral eyestalk ablation (ESA) commonly practised in hatcheries reduces the GIH levels and induces ovarian maturation of captive broodstock, but results in the production of inferior quality seeds and high mortality with successive spawning (Benzie, 1998; Browdy and Samocha, 1985). Developing alternative techniques to eyestalk ablations for captive broodstock development is challenging, as the

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molecular mechanisms concerned with maturation are not yet fully understood (Benzie, 1998).

Serotonin (5-hydroxytrptamine, 5-HT), functions as neurotransmitter, neuromodulator and neurohormone and hence act as "activity promoting agent" in several species (Beltz, 1999; Cooper et al., 2003). This gonadotrophic hormone has confirmed receptor mediated regulatory role in several physiological and behavioural processes in both vertebrates and invertebrates (Lee et al., 2001; Mattson and Spaziani, 1985; Sarojini et al., 1995). It has been reported to act as a regulator of follicular growth in fishes (Cerda et al., 1998) and oocyte maturation in penaeid shrimps (Alfaro et al., 2004; Fingerman, 1997; Santhoshi et al., 2009; Vaca and Alfaro, 2000; Wongprasert et al., 2006) mainly by regulating the release of gonad enhancing factors in brain and thoracic ganglions. Gonad maturation requires precisely co-ordinated expression of specific genes and the role of serotonin on these genes to regulate ovarian maturation is inconclusive.

The present study was designed to evaluate the effects of serotonin on ovarian maturation in Indian white shrimp *Penaeus indicus* (the genus names of penaeid shrimps will be referred to as *Penaeus* according to Ma et al. (2011). We hypothesise that treatment of pre-vitellogenic females with serotonin alone or together with eyestalk ablation may display differential gene expression patterns, whose functional products may have potential implication on oocyte development and thus help in understanding the molecular mechanism underlying impaired ovarian maturation in female shrimps.

2. Materials and methods

2.1. Animals

Live adult females of *P. indicus* at different stages of maturity (n = 10 per stage; size range of 60–90 g) collected from Chennai coast, Tamil Nadu, India were dissected and ovarian tissues collected for gene expression analysis. Classification of ovarian development was done on a five point scale (immature, early maturing, late maturing, ripe and spent) based on their relative size, outline and colour of ovaries as seen through the transparent dorsal exoskeleton (Primavera, 1982). To analyse the effect of serotonin treatment on ovarian maturation, previtellogenic females (size range of 60–90 g) and males (size range of 30–55 g) from the wild were transported to the quarantine section of Muttukadu Experimental Station of CIBA, Chennai (Tamil Nadu, India). The shrimps tested negative for white spot syndrome virus (WSSV) by nested PCR (Kimura et al., 1996) were acclimated to the experimental tank condition with aerated seawater of salinity (salinity of 28–30 ppt; temperature of 26–28 °C) and a light:dark cycle of 2:22 h for at least one week before being used for experimentation. The shrimps were stocked at a sex ratio of 2 females:1 male and were fed with live polychaete and clam meat ad libitum. Approximately 90% of the seawater was replaced twice daily.

2.2. Experimental design

Previtellogenic impregnated female *P. indicus* in intermoult stage were randomly assigned into four experimental groups (n = 10 shrimps per treatment group/sampling point):

1) 5-HT group: shrimps injected with serotonin (5-HT creatinine sulfate, Sigma, St. Louis, USA) at 50 μg/g body weight (BW) dissolved in crustacean saline (the dose that was found to be effective for ovarian induction in *Penaeus vannamei* (Vaca and Alfaro, 2000) and *Penaeus monodon* (Wongprasert et al., 2006)).
2) ESA group: shrimps were unilaterally eyestalk ablated and injected with crustacean saline devoid of serotonin.
3) 5-HT + ESA group: shrimps administered with same concentration of serotonin as in 5-HT group and were unilaterally eyestalk ablated.
4) Control group: Eyestalk intact shrimps injected with crustacean saline devoid of serotonin.

The shrimps were injected intramuscularly between the 1st and 2nd thoraco-abdominal segments on Days 0 and 7 of the experiment, and the volume of injection was adjusted to 100 μl/shrimp. The females were sampled on 7 and 14th day and vitellogenic stages were studied at cellular and molecular level.

2.3. Histological observations

Ovarian maturation from three representative experimental shrimps on Days 0 (excluding the experimental shrimps), 7 and 14 were examined histologically according to standard protocols (Bell and Lightner, 1988). The ovary tissue was fixed in Davidson's fixative for 24 h, dehydrated with ascending alcoholic series, cleared in xylene, and embedded in paraffin wax. The sections were cut to a thickness of 5 μm and stained with haematoxylin and eosin. Histological sections from three shrimps of each treatment group were examined and photographed by a Nikon light microscope (DSFI-2) equipped with a digital camera E600.

2.4. RNA extraction, cDNA synthesis and cloning of reproductive genes in *P. indicus*

Total RNA was extracted from the ovaries of the experimental shrimps and also from wild shrimps at different maturity stages (n = 10 shrimps per stage) using TRIzol (Invitrogen), and was reverse transcribed to the first-strand cDNA using Superscript III First-Strand Synthesis (Invitrogen) with the oligo(dT)12-18 primers which was used for gene expression analysis. Primers for the genes were designed based on the sequence of *P. monodon* to amplify them in *P. indicus*. The PCR reaction was performed in a final volume of 25 μl containing 1 × reaction buffer, 2 mM of dNTPs, 2 mM $MgCl_2$, 0.2 μM of each primer, 1.25 unit of Taq polymerase (Promega, Madison, WI, USA) and 2 μg of template cDNA. The PCR reaction consisted of initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C, 1 min; 55 °C, 40 s; and 72 °C, 1 min and a final elongation at 72 °C for 7 min. The products were cloned into the pGEM-T Easy Vector (Promega) and sequenced. Nucleotide sequence analysis of genes was performed by BLASTN (http://www.ncbi.nlm.nih.gov/blast).

2.5. Quantification of genes by real-time PCR analysis

The abundance of the mRNA transcripts of vitellogenin (*vg*), the biomarker for maturation in penaeid shrimp, vitellogenin receptor (*vgr*), components of maturation promoting factor (*cdc2* and *cyclin B*) and thrombospondin (*tsp*) were measured by quantitative real-time PCR analysis (qPCR) using ABI StepOne Plus thermocycler (Life Technologies Corporation) with 1 × Power SYBR green PCR master mix (Applied

Table 1
Specific primers used for quantitative real-time PCR analyses (S: sense strand; AS: antisense strand).

Gene	Orientation	Sense
vg	S	5′-caggcgttccatcgattctt-3′
vg	AS	5′-cggcgtggacatgtgatct-3′
vgr	S	5′-actgctggcttggctttgac-3′
vgr	AS	5′-tttggatcatgcagtggtgagt-3′
cdc2	S	5′-atggaggattacttacgtatagaaaa-3′
cdc2	AS	5′-ttaattcttggctggaagagtgg-3′
cyclin B	S	5′-ggatggcaataactggagtgatac-3′
cyclin B	AS	5′-gtacttctgtcttacagcctgtt-3′
tsp	S	5′-tgcatcaataggccaaattc-3′
tsp	AS	5′-atcagcatccgagtcacaag-3′
ef1-α	S	5′-cgctcattgtcagtgctcttgt-3′
ef1-α	AS	5′-aggttcctgctgccaagct-3′