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RESEARCH ARTICLE

STUDIES ON THE OVARIAN MATURATION STAGES AND ITS ASSOCIATED BIOCHEMICAL AND HISTOLOGICAL CHANGES IN THE MARINE SHRIMP *FENNEROPENAUES INDICUS* (MILNE EDWARDS, 1837)

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ABSTRACT

The present study was intended to determine the ovarian developmental stages and changes in the level of vitellogenin and vitellin as a biomarker of ovary maturation. Five different stages were classified based on the size and colour of the ovary. Histological studies clearly illustrate a gradual increase in oocyte development from stage I to IV based on yolk accumulation. Enzyme linked immunosorbent assay results also further confirmed gradual increase in the level of Vitellogenin and vitellin levels as the ovary maturation proceeds. The present study was intended to determine the ovarian developmental stages and changes in the level of vitellogenin and vitellin as a biomarker of ovary maturation. Five different stages were classified based on the size and colour of the ovary. Histological studies clearly illustrate a gradual increase in oocyte development from stage I to IV based on yolk accumulation. Enzyme linked immunosorbent assay results also further confirmed gradual increase in the level of Vitellogenin and vitellin levels as the ovary maturation proceeds.

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INTRODUCTION

In many decapod crustaceans, control of reproductive maturation is a major problem in developing commercial aquaculture programs. Vitellogenin (Vg) a precursor of egg yolk protein, is a necessary prerequisite for ovarian oocytes to reach full maturation. During maturation, the ovary exhibits size and colour changes those are macroscopically visible through the transparent carapace. These changes are due to the deposition of yolk material in the oocytes, which results in a rapid increase in oocyte diameter (Sagi *et al.,* 1995; Tsukimura, 2001) and colour changes due to the carotenoid components with specific colour changes each being related to a new maturation stage (Arculeo *et al.,* 1995).The source of yolk proteins in crustacean ovaries hasbeen a subject of controversy for many years. Whereas thehepatopancreas was considered the main site for VTG synthesis substantial intraovarian de novo synthesisof yolk protein has been demonstrated for several species, including *Penaeus semisulcatus* (Jean *et al*., 2003).

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Yano and Chinzei (1987) reported that Vt was synthesized by the follicle cells of *Penaeus japonicus*. Nevertheless, a protein that reacts immunologically to the antiserum prepared against purified Vt was detected in the hemolymph of vitellogenic females. This female- specific protein (FSP), known as vitellogenin (Vg), has been found in all the species studied so far (Shafir *et al*., 1992). Vg is one of the two lipoproteins recognized in crustacean hemolymph (Lee, 1990). A large increase in the concentration of Vg has been reported in the hemolymph during vitellogenesis. While some reports show that the appearance of Vg in the hemolymph is correlated with morphological changes in the ovaries precursor to egg release, few other reports show that Vg is at the highest concentrations prior to the maximum accumulation of yolk in the oocyte (Quackenbush, 1989b; Lee, 1990). In general, Vg expression may occur at multiple sites, but expression patterns still vary according to species. The same gene for Vitellogenin and vitellin can be expressed together both in the ovary and hepatopancreas was shown in *P. semisulcatus*. Multiple genes may also show tissue specific expression of Vg in the ovary and hepatopancreas as demonstrated in another penaeid shrimp.Reproductive studies of crustaceans require a thorough knowledge of the morphological, biochemical and physiological changes of the gonad (Emilia *et al*., 1992).Determination of oocyte diameter with histological tools

provides basic information on classification of ovarian development (Peixoto *et al.,* 2005; Revathi, 2010). Quantification of Vg and Vt are required for the investigation of the dynamics during vitellogenesis. Earlier studies have often relied on oocyte size or ovarian weight (Anikumar and Adiyodi, 1980). Being a commercially important species, *Fenneropenaeus indicus* (Milne Edwards) gains a major economic status in aquaculture. However a major constraint in the commercial culture of white shrimp is the lack of incessant supply of fry. A reliable source of seed can be obtained if the reproduction of this species is meticulous. Studies associated to ovarian cycle allied with vitellogenesis as well as biochemical changes in marine shrimp *Fenneropenaeus indicus* is scanty. Hence, the present study was carried out to document the ovarian development in female shrimp *F.indicus* with reference to morphological, histological, biochemical changes and vitellogenesis.

MATERIALS AND METHODS

Collection and maintenance of prawn

Fenneropenaeusindicus were collected from muttukadu hatchery Chennai, South India. The collected prawns were brought to laboratory with aerated habitat water and were transferred into FRP tanks with sufficient aeration. The water was changed daily and fed with *ad libitum* commercial pelletized food. They were maintained in the laboratory for 2-3 weeks for acclimatization.

Oocyte diameter

Oocyte diameter was measured using an ocular micrometer calibrated with a stage micrometer fitted in a light microscope (Labex,India). For each prawn, the diameters of as many as 30 oocytes were measured and mean oocyte diameter was calculated. The stage of oocyte development was characterized based on the maximum number of oocytes confined to a particular stage. Photomicrographs of various stages of oocyte development were taken using a Leica 2500 microscope (Germany).

Histology

For histological examinations, the ovary was dissected from different ovarian stages of shrimps. The isolated ovarian samples were fixed in Bouin s fixative for 24 h and washed with distilled water. The samples were dehydrated with different grades of an alcohol series and processed by routine procedure. Sections of 6-8 µm thickness were taken and stained with haematoxyline and eosin. The stained sections were mounted using DPX and photomicrographs of varying magnifications were taken using a Leica 2500 microscope.

Biochemical analysis Protein

Various reproductive tissue samples were dissected from different ovarian stages and used for protein estimation. The samplessuch as hemolymph (100 µl), ovary and hepatopancreas (100 mg) were taken individually, homogenized in 10% Trichloroacetic acid (TCA) and centrifuged for 10 min at 9000 Xg at 4 ºC. The supernatant, diluted with 0.15 M NaCl, was used to measure the protein concentration For each sample, the soluble protein concentration was determined spectrophotometrically at 595nm by Coomassie brilliant blue G–250 method described by Bradford (1976). Bovine Serum Albumin (BSA) was used as a standard.

Lipid

The total lipid content was analyzed using the Vanillin–Phosphoric acid method according to Folch *et al.* (1957). Hundred milligram of wet tissue of each sample was taken and homogenized with 0.5 ml of chloroform: methanol (2:1) and 0.5 ml of 0.9 % NaCl was added and kept in a separating funnel at room temperature for 12 h. The lower phase was collected, 0.5 ml of Conc. H_2SO_4 was added, heated in boiling water for 10 min, cooled to room temperature and then 1ml of phosphoric vanillin solution (13 mMol/l vanillin in 14 Mol/L phosphoric acid) was mixed immediately and held at room temperature for 30 min. The optical density was measured at 540 nm. Cholesterol was used as a standard.

Isolation of vitellogenin and vitellin

Vitellogenin and vitellin were isolated from the hepatopancreas, hemolymph and ovaries of *F. indicus*following the method of Tsukimura *et al.* (2000). Briefly, the reproductive tissues were homogenized in homogenization buffer (containing 0.1 M NaCl, 0.05 M Tris, 1mM ethylene diamine tetra acetic acid and 0.1 % Tween 20 with 10 mg/ml PMSF; pH 7.8) using an ice cold glass homogenizer. The homogenate was centrifuged at 4000 Xg for 5 min at 4 ºC. The resultant supernatant was again centrifuged at 20,000 Xg for 20 min at 4 ºC. To the supernatant, saturated ammonium sulphate (SAS) was added to produce 25 % SAS solution. After incubation for 1 h at 4 ºC, the solution was centrifuged at 20,000 Xg for 10 min at 4 ºC. The supernatant as collected and SAS was added to produce 40 %, 50 % and 60 % SAS solution sequentially. The pellets of 60 % SAS solution was suspended in appropriate volume of homogenization buffer and dialyzed thrice at 4 ºC for 12 h each against homogenization buffer. The isolated vitellogenin and vitellin were stored at - 20 ºC for further analysis.

Enzyme linked immunosorbent assay

Hundred milligrams of ovary, hepatopancreas and hemolymph (100 µl) samples were taken from different ovarian stages of shrimps. Tissues were homogenized with phosphate buffer and centrifuged at 13000 Xg for 10 min at 10 ºC, to remove cellular debris. The supernatant was collected and then coated on the 96-well plates for overnight at 4 ºC. Then after three washing with washings buffer, the wells were blocked with 200 µl of blocking buffer and incubated at 37 ºC for 1 h. Washing was followed by the addition of 100 µl of primary antibody (anti Vg at 1:2000), for 3 h at 37 ºC. After three times washing, the wells were coated with 100 µl of secondary-antibody enzyme conjugate (Anti rabbit IgG-Alkaline phosphatase) at 1:500 dilutions for 1h at 37ºC. Incubation was terminated by washing and wells were filled with 100 µl of substrate solution (1mg pNPP- paranitrophenyl phosphate/ml of substrate buffer).The reaction was stopped with the stop buffer after the required colour development was attained.

Absorbance at 405 nm was measured in an automated ELISA plate reader (Titertek Multiscan Plus, MK II, Denmark).

RESULTS

Description of the female reproductive system

In female shrimps, a pair of ovaries forms the reproductive system. The ovaries are positioned dorsally and extend from the anterior most region of the cephalothorax to the extreme posterior region of the abdomen. Ovarian development was classified based on the size, colour and texture of the ovary. As many as five stages of ovarian development were classified and salient features of each stage are detailed as follows.

Stage I (Immature)

Ovaries appears to be much transparent, thin and very difficult to distinguish externally due to lack of pigmentation. The ovaries do not have any lobules and restricted only to cephalothoracic regions.Average length of the shrimp ranging from the size of 120mm to 130mm belong to this group.

Stage II (Early pre-vitellogenic)

At this stage ovary looks more thich compared to stage I. Moreover, ovary appears to be transparent yellowish which can be distinguished upon dissection. The two lobes of the ovary is not fused in the cephalothoracic region Shrimps ranging from the size of 130mm to 140mm belong to this group.

Stage III (Pre-vitellogenic)

This stage represents the maturing stage. The ovary attains a pale green colour which can be identified externally through the external cuticle. The anterior region of the ovary attains a lobulated structure and the posterior region is divided into two arms and exhibits a greater degree of fusion in the cephalothoracic region. Females ranging from the size of 140mm to 160mm belong to this group.

Stage IV (Vitellogenic)

Ovary appears to be more prominent, occupying the whole thoracic and abdominal cavity as a thick mass, which could identified easily through the dorsal cuticle. The ovary is firm and granular in texture. Females measuring 160–220 mm in length belong to this stage.

Stage V (Spent)

Shrimps ranging from the size of 170-200mm belongs to spent stage. Ovary completely collapses with no apparent tissue formation. Ovary becomes slender and attains a thim strand like structure similar to that of immature stage. This stage consists of either immature or rejuvenating oocytes.

GSI and HSI Vs Ovarian development

Ovarian development was further assessed by calculating the gonado somatic index (GSI) and hepato somatic index (HSI) of the shrimp at various stages of development (Fig.1). It was clear from the results that GSI level increased gradually from immature to vitellogenic stages of development and drastically decreased at spent stage. The GSI values increased from immature (0.65±0.26 %) to vitellogenic stage (7.71± 0.58 %) indicating complete maturation of the ovary. On the other hand, HSI values varied significantly from immature to vitellogenic stage of ovarian development (Fig. 1). Statistical analysis indicated that GSI and HSI values differed significantly from stage I to IV of ovarian development ($P < 0.05$).

Oocyte diameter

Oocyte size measurement showed an increase in size during each stage of development. Gradual increase was observed in the ovarian index as well as oocyte diameter throughout the maturation stages. Oocyte diameter (204.04 ± 21.38 µm) were found to be greater at vitellogenic stage of ovarian development. Sudden decline in the oocyte diameter (25.71 ± 1.31µm) was observed in spent stage (Fig. 2).Statistical analysis revealed that the variation of oocyte growth during various stages in ovarian development is significant ($P<0.05$).

Histological studies of the ovarian developmental stages

Cross section of the stage I (Fig.3A) ovary showed immature oocytes without much yolk accumulation. Oocytes occupy the entire lumen surrounded by follicle cells while stage II(Fig.3B) ovary showed oocytes of larger size along with the younger oocytes. At this stage, follicle cells are also found to be scattered throughout the interstitial tissue mass. In stages III & IV (Fig.3 C&D) ovaries were filled with mature oocytes with abundant yolk granules. The follicle cells appeared as a narrow band of flattened cells encompassed with oocytes. The ovary is characterized by green colour and seen predominantly with vitellogenic oocytes and stage IVovary appears alrge in size, with more mature oocytes. Follicle cells are not so distinct as the fully mature oocytes occupy the entire ovary. Spent oocytes of stage V (Fig.3E) showed characteristic features similar to those of immature ovary with bare follicle cells, proliferating oocytes and empty follicle cells. Empty follicle cells showed hypertrophied texture. (A) Stage I ovary shows the presence of and immatureoocytes (IO). (B) Stage II ovary shows zone of proliferation (ZP), immatureoocytes (IO) and follicle cells (FC). (C) Stage III ovary shows developing oocytes(DO) with the presence of yolk granules surrounded by prominent follicle cells (FC). (D) Stage IV ovary shows vitellogenic oocytes with distinct ooplasm (OP)filled yolk granules (Yg). (E) Stage V ovary shows rejuvenating oocyte (RO) enveloped by thin row of follicle cells (FC). Bar: 50 µm.

Biochemical variations in tissues

Protein content in the hepatopancreas differed during various ovarian developmental stages (Fig 4). Marginal increase in protein content was observed in hepatopancreas in stage II (9.31± 0.60 mg/g) and it was comparatively high in stage V (11.41 ± 0.71 mg/g) (Fig. 4). Protein content in hemolymph increased during vitellogenic (37.27 ±1.28) stage, possibly reflecting accelerated release of protein from the hepatopancreas. Similarly, protein content in hemolymph increased progressively during ovarian developmental stages and decreased in spent stage (17.87 ±16.98).

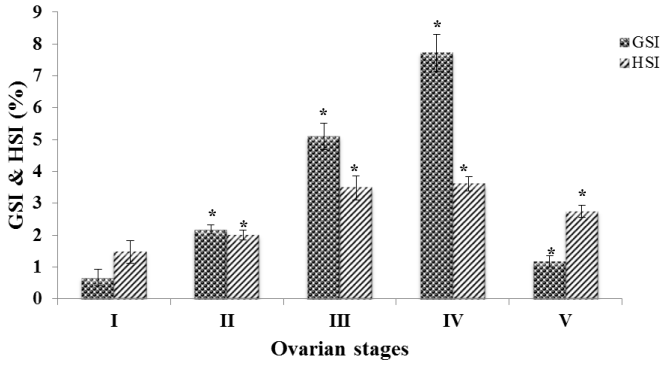

Fig. 1. Gonado somatic index and Hepato somatic index at different ovarian stages

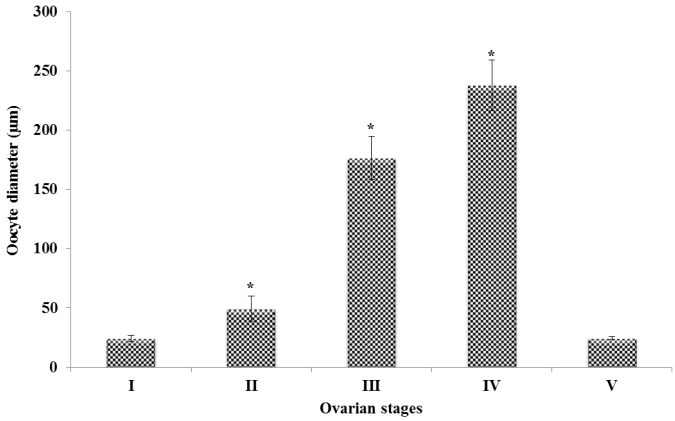

Fig. 1. Oocyte diameter during different stages of ovarian development in *F.indicus*

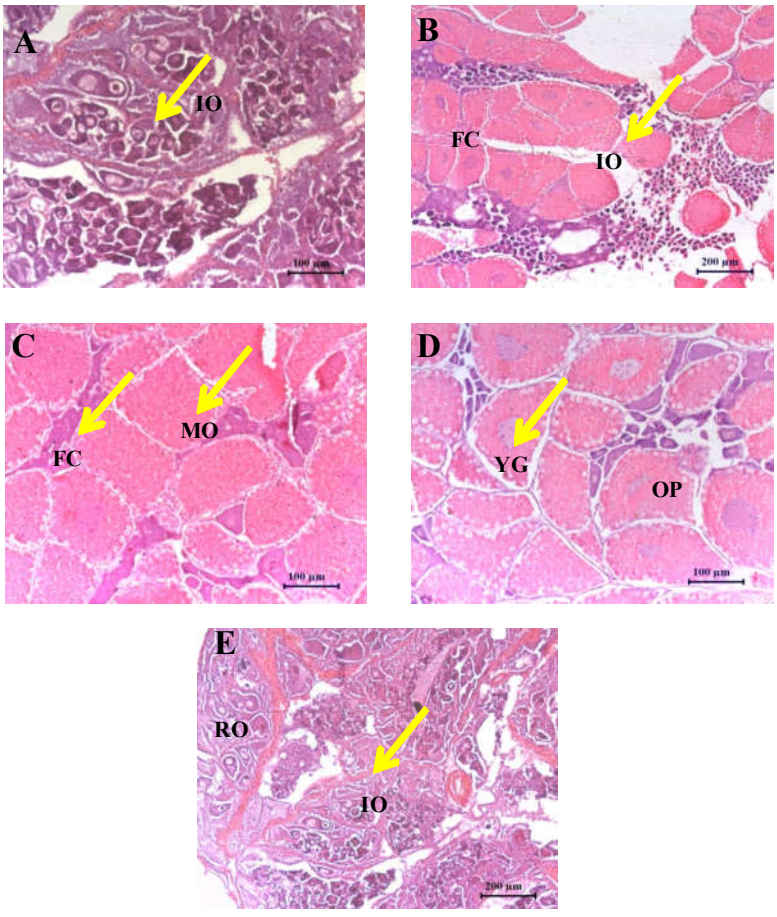

Fig.3. Photomicrographs through the section of ovary during different ovarian developmental stages

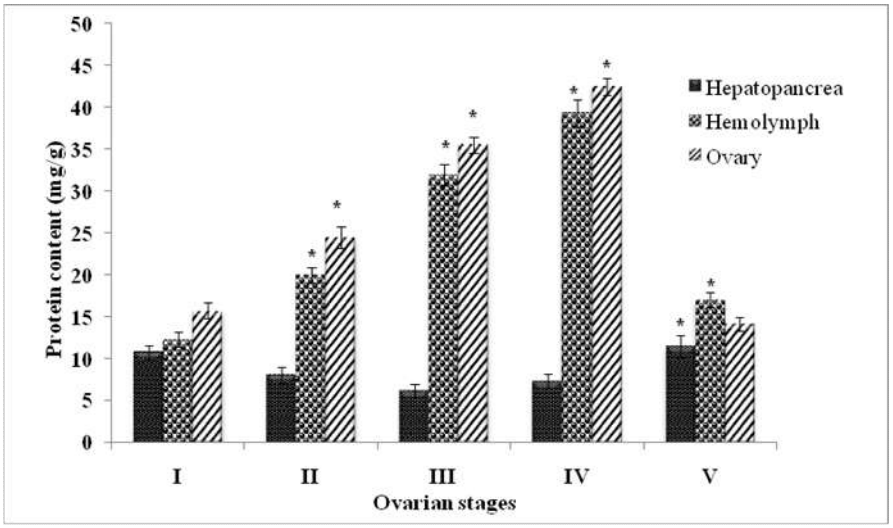

Fig.4. Protein content in various tissues at various ovarian maturation stages

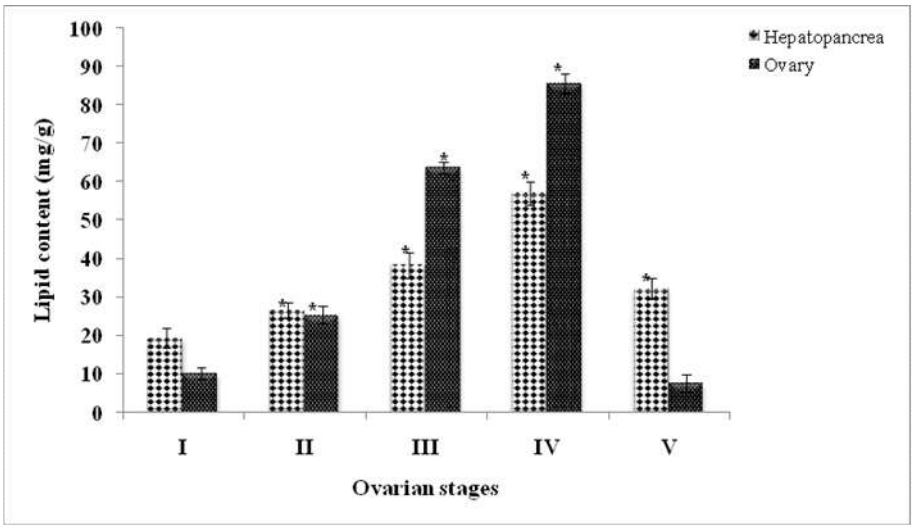

Fig.5. Lipid content in various tissues at various ovarian maturation stages

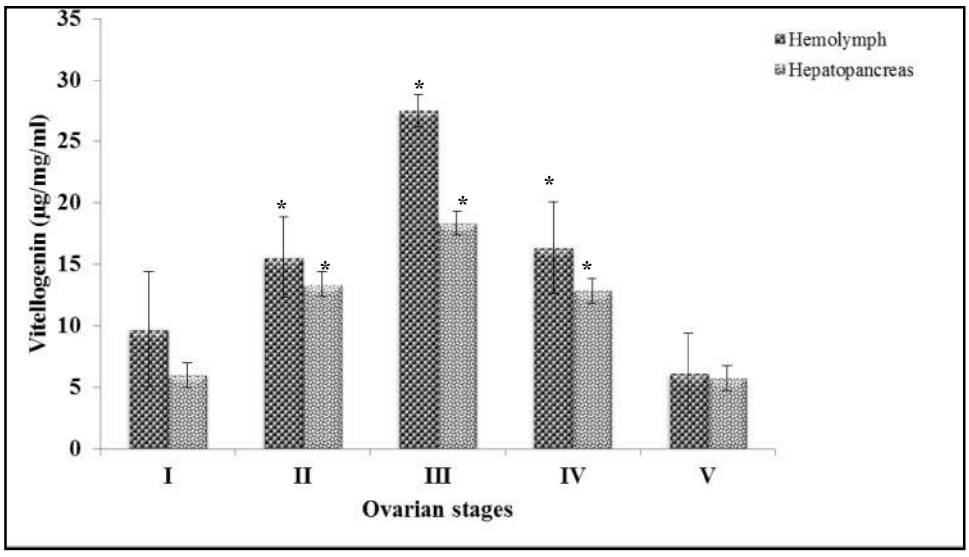

Fig.6. Vitellogenin content in various tissues at various at different maturation stages

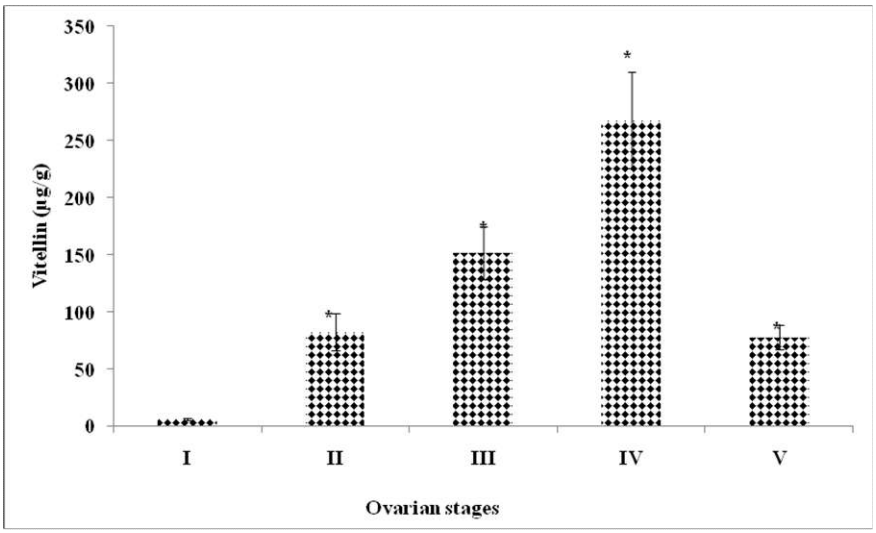

Fig.7. Vitellin content in various tissues during different ovary maturation stages

Protein content in the hemolymph varied in all five stages according to the maturation of the ovarian stages. These observations possible elucidate that the protein content increased in accordance with ovary maturation. The protein content increased gradually from stage I (15.73 ± 0.99 mg/g) to stage IV (42.42± 1.02 mg/g). The changes in the protein content of the ovary differed significantly during the ovarian development (P<0.05). Lipid levels were found to be high in hepatopancreas (57.06 ±2.98) and ovary (85.61 ±2.59) during the vitellogenic stage while the minimum was observed at spent stage (Fig. 5). Lipid content varied from stages I toV (Fig.5). Consequently, the lipid content in the hepatopancreas showed asharp decrease to 32.18 ± 2.61 mg/ g at V stage. The ovarian lipid content increased graduallyin all five stages of development. Lipid content in the ovary increased gradually from immatureto vitellogenic stage and rapidly decreased at spent stage. It increased from stage I(10.11 ± 1.58 mg/g) to stage IV (85.61 ± 2.59 mg/g). The variation in the lipid content of the ovary differed significantly during the ovarianstages (P<0.05).

Vitellogenesis during ovarian development

Vitellogenin content in both hepatopancreas and hemolymph varied during ovarian development (Fig. 6). Vitellogenin content in thehepatopancreas increased gradually from early pre vitellogenic stage to vitellogenic stage of ovarian development. However, the vitellogenin content decreased to5.77±3.41 µg/g at spent stage. The vitellogenin levelin the hemolymph showed a gradual increase from stage I to stage IV of the ovarian development and the maximum was observed in IV stage. Thereafter, the vitellogenin content decreased (6.13±3.24 µg/ml) at stage Vof ovarian development. The variations in thevitellogenin content in hepatopancreas and hemolymphdiffered significantly during ovarianstages (P<0.05).The vitellin content in the ovary increasedfrom immature to vitellogenic stage of ovarian development (Fig. 7). In stages I and II, the vitellin contentincreased gradually from 6.11 ± 0.69 µg/g to82.44 ± 15.94 µg/g. However, there was an sharpincrease in previtellogenic (151.62± 22.88µg/g) and vitellogenic (267.61 ± 42.17 µg/g) and drastically decreased at spent stage (77.9±10.33 µg/g) of ovarian development (Fig.). The changes in the vitellin content of the ovarydiffered significantly during the ovarian stages (P<0.05).

DISCUSSION

The present study clearly showed five different stages of ovarian development based on the histological and morphological obesrvations of the ovary in *F. indicus*. These stages of ovarian development are substantiated with measurements of GSI and HSI indices. ovarian index gradually increased from stages I to IV as well as oocyte growth also gradually increased during ovarian development. Similar observations have been made in crustaceans, especially colour changes during gonadal maturation in prawn (Martins *et al.,* 2007); shrimp (Dall *et al.,* 1990). In crustaceans, the mature ovary is known to have mature oocytes (Chen and Chen 19994) which was in accordance with the results of the present study. Developing oocytes have a uniform structural unit with the wall made up of thin layers of follicle cells in *Penaeus monodon* (Tan Fermin and Pandadera, 1989) and the deep sea shrimp *Aristaeo morpha* (Kao *et al.,* 1999). Follicle cells, surrounding mature oocytes, which is comparable with the present study where early maturing stages consisted of immature and developing oocytes with prominent follicle cells. From the present study it was clearly evident that protein and lipid levels varied during ovarian development, which was closely associated with the maturation stages. Besides, lipid content also fluctuated in the tested tissues during the ovarian development. The oocyte development interrelated to the hemolymph vitellogenin content. In agreement with the present results, Chang and Shih (1995) reported the accumulation of vitellin content in the ovary from stages I to IV. The transfer in protein and lipid contents from hepatopancreas to ovary, through hemolymph in *Crangon crangon* supports the hypothesis that organic reserves stored in the hepatopancreas are transported to the ovary through hemolymph during gonadal maturation. Vitellogenin content in the hemolymph was in peak at stage IV and declined at stage V in

F.indicus. A similar pattern has been reported in *H. americanus* (Byard and Aiken 1984). Similar results are reported from several crustacean species, with a substantial increase of vitellogenin content in the hemolymph during vitellogenesis (Lee, 1991; Okumura *et al.,* 1993; Quackenbush, 1989). Vitellogenesis as a biomarker of female reproductive activity, which indicate that the vitellin accumulation gradually increased in oocytes during ovarian development. Hence the present study provides a better understanding on the ovary maturation with a detailed report on the changes occurring during ovarian maturation in *F. indicus*.

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