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Vitellogenesis during the ovarian development in freshwater female prawn *Macrobrachium rosenbergii* (De Man)

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Abstract: In the present investigation, vitellogenesis during ovarian development in the freshwater prawn *Macrobrachium rosenbergii* was assessed. Ovarian development was classified into five stages based on size, colour and texture of ovary. Interestingly, histological results clearly indicate that the oocyte development gradually increased from stages I to V based on the yolk material accumulation. Besides, the biochemical changes associated with ovarian development was also analyzed. On the other hand, vitellogenin (Vg) and vitellin (Vt) content during ovarian development in female prawn was quantified as a measure of reproductive activity.

Key Words: *Macrobrachium rosenbergii*, Ovarian development, Reproductive activity, Biomarker, Vitellogenesis

Introduction

In crustaceans, substantial quantities of yolk accumulation within the developing oocytes serve to meet the basic requirement of embryonic and larval development (Adiyodi and Subramonium, 1983). 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 main constituents of yolk are protein and lipid

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content; vitellin is the major yolk protein that accumulates with in the ovary during vitellogenesis (Chen *et al.,* 1999).

Lipoglycoprotein and vitellin are the major components of yolk and vitellogenin is a protein that reacts immunologically to the antiserum prepared against the purified vitellin from the hemolymph of vitellogenic females. This FSP, known as vitellogenin (Vg) is reported in all organisms studied so far (Dehn *et al.,* 1983; Fyfee and O'Connor, 1974; Susuki, 1987). In decapods, the transformation of vitellogenin to vitellin revealed that a vitellin subunit highest in molecular mass disappeared during embryo-genesis (Chang and Bradley, 1983). During these processes, vitellogenin and vitellin are modified through cleavage, glycosylation, lipidation and phosphorylation (Raikhel and Dhadialla, 1992). They serve as storage protein providing amino acids, carbohydrates, lipid and phosphates to the developing embryo (Byrne and Gruber, 1989). Determination of oocyte diameter with histological tools provides basic information on classification of ovarian development (Peixoto *et al.,* 2005; Revathi, 2010).

Synthesis, secretion and processing of vitellogenin differ among phyla (Chen *et al.,* 1997). Hemolymph vitellogenin concentration is a good indicator of the onset of vitellogenesis during early maturation, rapidly increasing until reaching a plateau in mid-maturing females. After being internalized into the ovary by a receptor mediated endocytotic process, they undergo proteolytic processing to give rise to major yolk protein, namely vitellin which is considered to be phosphorylated glycoprotein (Fyffe and O'Connor, 1974). In general, vitellogenin is synthesized by extra ovarian tissues like liver in vertebrates (Byrne and Gruber, 1989) and fat body in insects. In decapod crustaceans, the hepatopancreas (Eastman- Reks and Fingerman, 1985; Khayat *et al.,* 1994; Lui and O'Connor, 1976) and subepidermal adipose tissues (Rani and Subramoniam, 1997) have been reported as vitellogenin synthetic sites. 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).

Studies related to ovarian cycle associated with vitellogenesis as well as biochemical changes in freshwater female prawn *Macrobr-achium rosenbergii* is scarce. Hence, the present study has been under taken to document the ovarian development in female *M. rosenbergii* with reference to morphological, histological, biochemical changes and vitellogenesis.

Material and methods

Collection and maintenance of prawn

Freshwater prawn, *Macrobrachium rosenber-gii* were collected from the Aqua Nova hatchery in Kannathur, Chennai, South India. The collected prawns were brought to the laboratory

in a plastic cover with aerated habitat water and were transferred into plastic tanks with sufficient aeration. The water was changed daily and they were fed *ad libitum* with commercial pelletized food. They were maintained in the laboratory for 2-3 weeks for acclimatization.

Experimental design

Five months old female prawn, weighing 16 ± 2 gm were taken for experimental studies. A total of 50 prawn were used for this study and divided into 5 groups each with 10 prawns.

Ovarian developmental stages

Ovarian developmental stages were determined according to the type, size and frequency of the germinal cells (Chaves and Magalhaes, 1993; Htun-Han, 1978; Martins *et al*., 2007; Okumura and Aida, 2000).

Gonado Somatic Index and Hepato Somatic Index

The prawns were weighed, gonads removed and the weight of the gonads were recorded. The Gonado Somatic Index (GSI) and Hepato Somatic Index (HSI) were calculated following the procedure outlined by Zhang *et al.* (2007).

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 prawns. 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 taken from different ovarian stages of prawns and used for protein estimation. The samples such as hemolymph (100 µl), ovary and hepatopancreas (100 mg) were taken individual-lly, 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 spectro-photometrically 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 chlorof-orm: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 547 nm. Cholesterol was used as a standard.

Isolation of vitellogenin and vitellin

Vitellogenin and vitellin were isolated from the hepatopancreas, hemolymph and ovaries of prawn *M. rosenbergii* following the method of Tsukimura *et al.* (2000). 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 was 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 homo-genization 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 prawns. 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- paranit-rophenyl 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

In the present study the reproductive activity of the adult freshwater female prawn *M. rosenbergii* was assessed based on the morphological variation of ovary, Gonado Somatic Index and Hepato Somatic Index, oocyte diameter, cellular level changes in ovary and quantification of female specific protein at different ovarian stages.

Morphological observation of the ovary

In *M. rosenbergii*, ovarian development was classified based on the size, colour and texture of the ovary. Stage I (spent stage) ovary is thin strand-like structure, very small in size, transparent, with no apparent ovarian tissue formation. Stage II (early previtellogenic stage) ovary is slightly larger in structure, transparent and elongated. Stage III (late previtellogenic stage) ovary is further thickened, became dark yellow. Besides, Stage IV (early vitellogenic stage) ovary is characterized by orange colour. Stage V (late vitellogenic stage) ovary is dark green in colour and enlarged. At this stage of development the ovaries fill up the entire dorsal region of the prawn. Mature oocytes are visible with naked eyes (Fig. 1).

GSI & HSI

GSI and HSI are two of the indicators of ovarian development. The GSI level was increased gradually from stages I to V (Fig. 2). Whereas the HSI values declined gradually from I^{st} to V^{th} stages of development. The GSI values increased from stage I (0.24 ± 0.04 %) to stage IV (3.79 ± 0.25 %) and at stage V of ovarian development, the GSI value increased to 8.49 ± 0.39 % indicating complete maturation of the ovary. On the other hand, HSI values varied significantly from stage I to V with a marginal increase at stage II of ovarian development. GSI and HSI values differed significantly from stages I to V of ovarian development ($P<0.05$).

Oocyte diameter

The oocyte development was evident by the measurement of oocyte diameter. Gradual increase was observed in the oocyte diameter throughout the maturation stages. Oocyte diameter (400 ± 81.1 µm) was found to be greater at stage V of ovarian development. However, it was drastically decreased to 60 ± 11.3 µm at stage I, representing the spent stage. There exists a gradual increase in oocyte

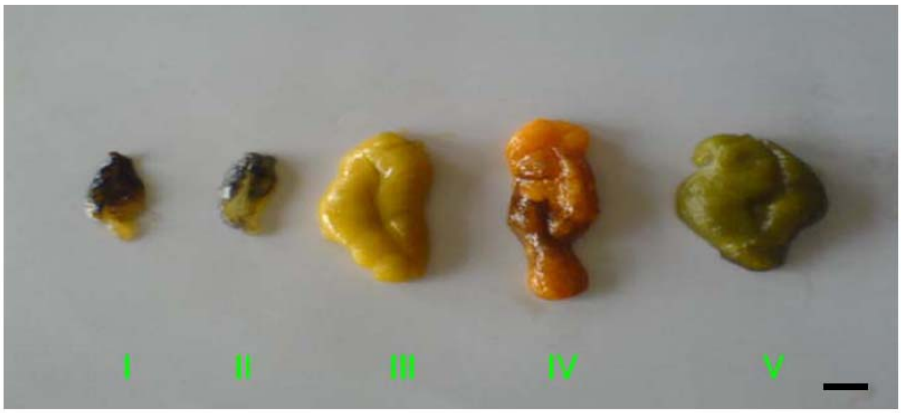

Fig. 1: Photographs showing the gross morphology of ovaries depicting different stages of ovarian development in *M. rosenbergii.* (A) Spent stage, (B) Previtellogenic stage (early previtellogenic stage), (C) Previtellogenic stage (late previtellogenic stage), (D) Vitellogenic stage (early vitellogenic stage), (E) Vitellogenic stage (late vitellogenic stage). Note the variation in size and colour of the ovary during different stages of development. Bar: 50 mm.

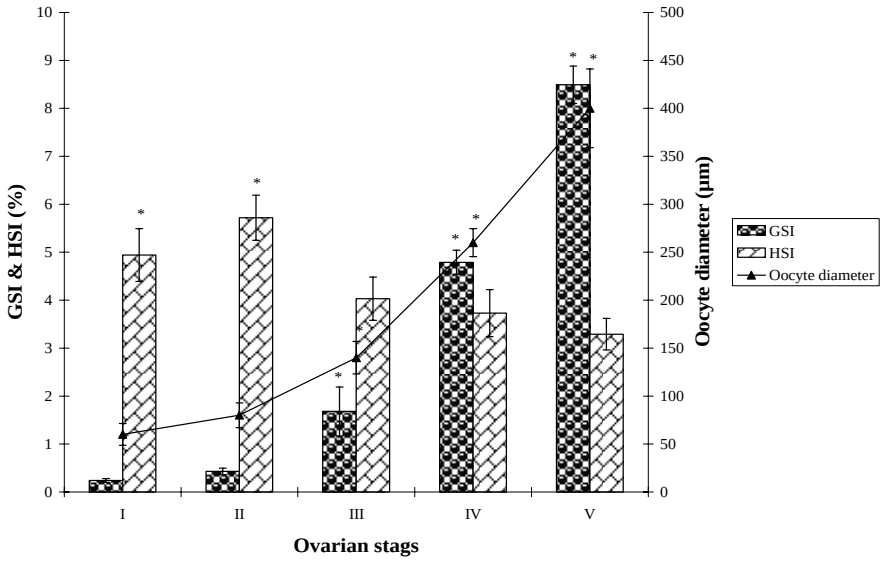

Fig. 2: GSI, HSI level and oocyte diameter at different ovarian stages (* F test P<0.05)

diameter from stage I to V which reflects oocyte growth leading to vitellogenesis in the final stage of ovarian development V (Fig. 2). Statistical analysis revealed that the variation of oocyte growth during various stages in ovarian development is significant ($P<0.05$).

Histological variations in the ovary

In spent stage, ovary was devoid of mature oocytes but seen with a few primordial germ cells measuring around 60 ± 11.3 µm in diameter. Cross section of the ovary at this stage clearly shows the domination of interstitial connective tissues and immature oocytes with very few rejuvenating oocytes. At this stage, follicle cells are also found to be scattered throughout the interstitial tissue mass (Fig. 3A). In early previtellogenic stage, the ovary exhibited the presence of previtellogenic oocytes measureing around 80 ± 13.0 µm in diameter. In stages I and II, the prominent cells are oogonia, primary oocytes and previtellogenic oocytes because these stages correspond to early development of ovary. Here each oocyte has a clear nucleus with sparse yolk globules in the ooplasm and a few immature oocytes are also seen which are opaque and devoid of a nucleus (Fig. 3B). In the late previtellogenic stage, the ovary was found to be large in size as it accumulated more yolk globules. The nucleus is smaller and the radial zone is broad with the deposition of yolk globules. The follicle cells are seen enveloping the pre-vitellogenic oocytes. Some of the oocytes are opaque and without nucleus (Fig. 3C). During the early vitellogenic stage, ovary illustrated the vitellogenic oocytes containing predominantly yolk globules. Three types of vitellogenic oocytes are recorded as small, opaque and large oocytes based on the yolk material accumulation. At this stage, the ovary is characterized by orange colour and seen predominantly with vitellogenic oocytes increased in size (260 ± 14.5 µm in diameter), arranged compactly and clearly visible (Fig. 3D). At late vitellogenic stage, the ovary was large in size, with more mature oocytes. The follicle cells are not as distinct as the fully mature oocytes fill up the entire ovary (Fig. 3E).

Biochemical variations in tissues

Protein content in the hepatopancreas showed variation during the different stages of ovarian development. A marginal increase in protein content was noticed in hepatopancreas at stage II (20 ± 6.08 mg/g) and it was comparatively lower at stage V (7.95 ± 1.08 mg/g) (Fig. 4).

Protein content in hemolymph was relatively consistent throughout the ovarian development. There was a slight elevation during the previtellogenic and vitellogenic stage, possibly reflecting accelerated release of protein from the hepatopancreas. Hemolymph protein content increased gradually during ovarian development and decreased following spawning. Protein content in the hemolymph varied in all five

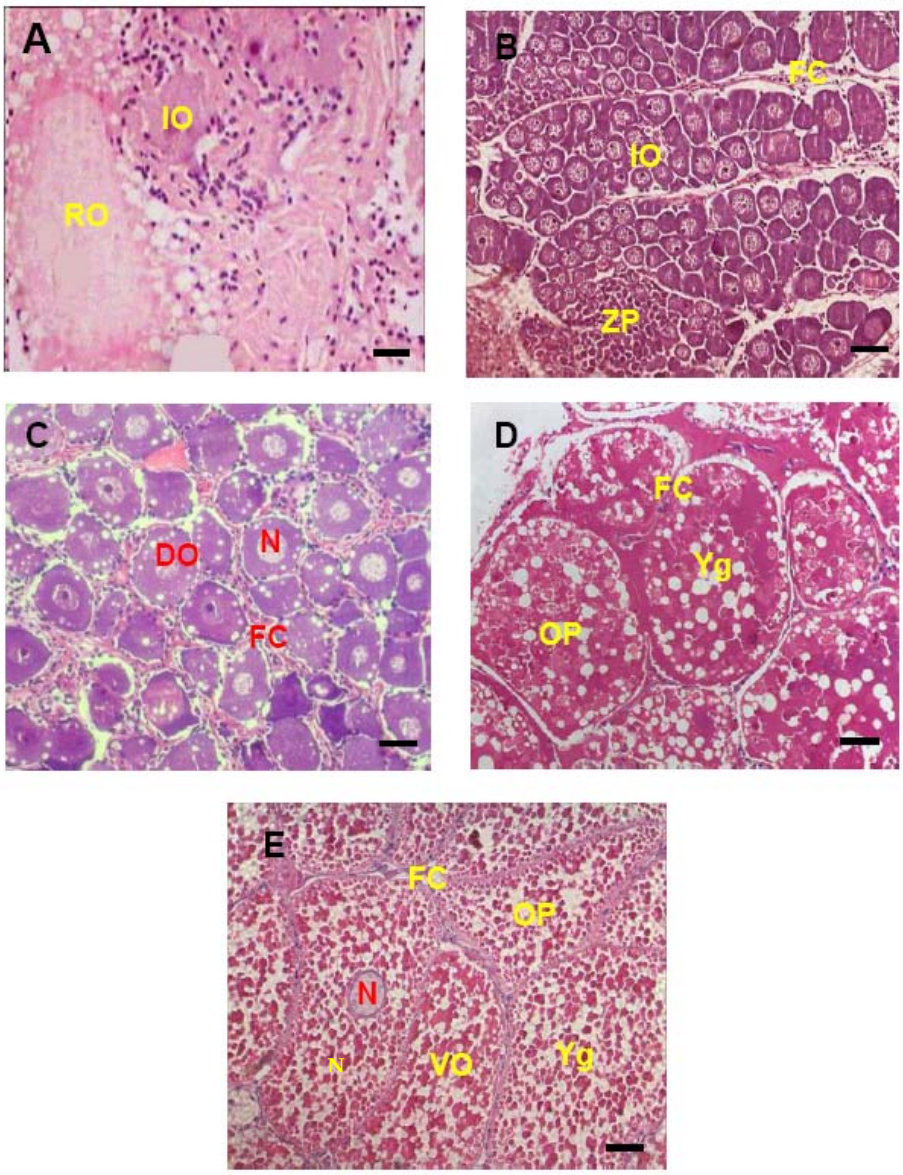

Fig. 3: Cross- section of ovary of different ovarian stages of prawns. (A) Stage I ovary showing the presence of rejuvenating oocytes (RO) and immature oocytes (IO). (B) Stage II ovary showing zone of proliferation (ZP), immature oocytes (IO) and follicle cells (FC). (C) IIIrd stage ovary showing developing oocytes (DO) with sparse yolk globules in the ooplasm, clear nucleus (N) and follicle cells (FC). (D) Stage IV ovary showing vitellogenic oocytes with distinct ooplasm (OP) filled yolk globules (Yg). Note oocytes are enveloped by a row of follicle cells (FC). (E) Stage V ovary showing the vitellogenic oocyte (VO), oocytes are enveloped by a row of follicle cells (FC). Note the accumulation of yolk globules (Yg) in the ooplasm (OP) and prominent nucleus (N). Bar: 50 µm.

stages and ranged from 20.16 ± 0.97 mg/ml to 27.32 ± 1.28 mg/ml.

During ovarian development (stages II and III), the protein content increased steadily from the previtellogenic stage, and remained relatively constant through the vitellogenic stage. These observations clearly indicated that the protein content increased as the development advanced. The protein content increased gradually from stage I (5.65 ± 0.25 mg/g) to stage V (208.09 ± 1.52 mg/g). The protein content of the ovary in stage V showed 40 fold increase compared to stage I. The changes in the protein content of the ovary differed significantly during the ovarian development ($P<0.05$).

The lipid content in the hepatopancreas and ovary reached a maximum level during the vitellogenic stage (Fig. 5). Hepatopancreatic lipid content increased gradually through the previtellogenic stage and decreased at the vitellogenic stage. Finally, the hepatopancreas lipid content was increased to its initial level at stage V. The lipid content varied from stages I to V. Thereafter, in stage IV, there was a drastic fall in lipid content to 65 ± 0.21 mg/g. Subsequently, the lipid content in the hepatopancreas showed a steep increase to 190 ± 0.94 mg/ g at V stage.

The ovarian lipid content increased gradually in all five stages of development. Lipid content in the ovary increased gradually from early stages of the previtellogenic stage and rapidly during the vitellogenic stage. It increased from stage I (20.9 ± 0.97 mg/g) to stage V (56.5 ± 1.28 mg/g). The variation in the lipid content of the ovary differed significantly during the ovarian stages ($P<0.05$).

Assessment of vitellogenesis

Vitellogenin content in both hepatopancreas and hemolymph varied during ovarian development (Fig. 6). Vitellogenin content in the hepatopancreas increased gradually from stages I to III with marginal increase at stage IV (1.39±0.29 µg/g) of ovarian development. However, the vitellogenin content decreased to 0.69±0.14 µg/g at stage V. The vitellogenin level in the hemolymph showed a gradual increase from stage I to stage III of the ovarian development. Thereafter, the vitellogenin content decreased (2.60±0.32 µg/ml) at stage IV and reached 2.51±0.36 µg/ml at stage V of ovarian development. The variations in the vitellogenin content in hepatopancreas and hemolymph differed significantly during ovarian stages ($P<0.05$).

The vitellin content in the ovary increased from early to final stages of ovarian development (Fig. 6). In stages I and II, the vitellin content increased gradually from 0.05 ± 0.01 µg/g to 0.27 ± 0.10 µg/g. However, there was an abrupt increase observed from stage III (9.40 ± 1.53 µg/g), IV (117.18 ± 15.85 µg/g) and V (286.34 ± 24.93 µg/g) of ovarian development. The final stages (IV^{th} and V^{th}) represented the vitellogenic stage of the ovary with fully mature oocytes. The details of this are described elsewhere. The

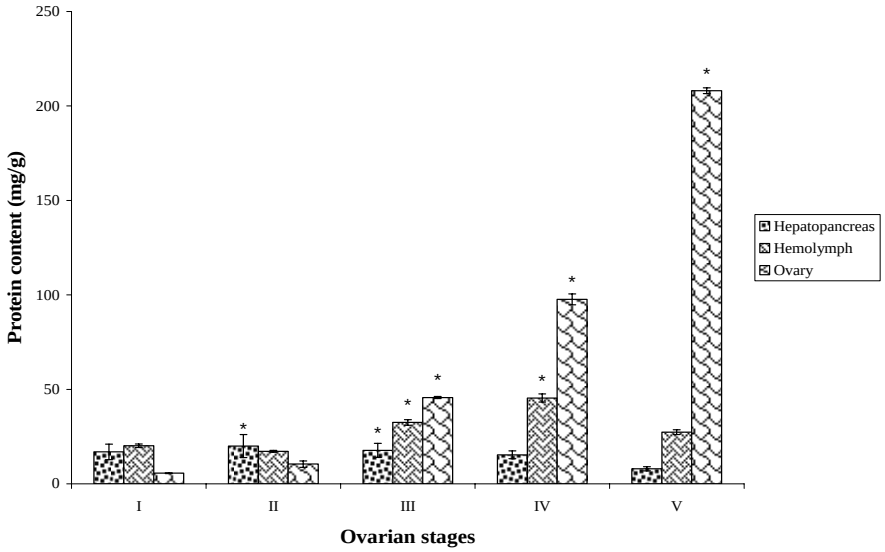

Fig. 4: Protein content in different reproductive tissues during different ovarian Stages (* F test P<0.05)

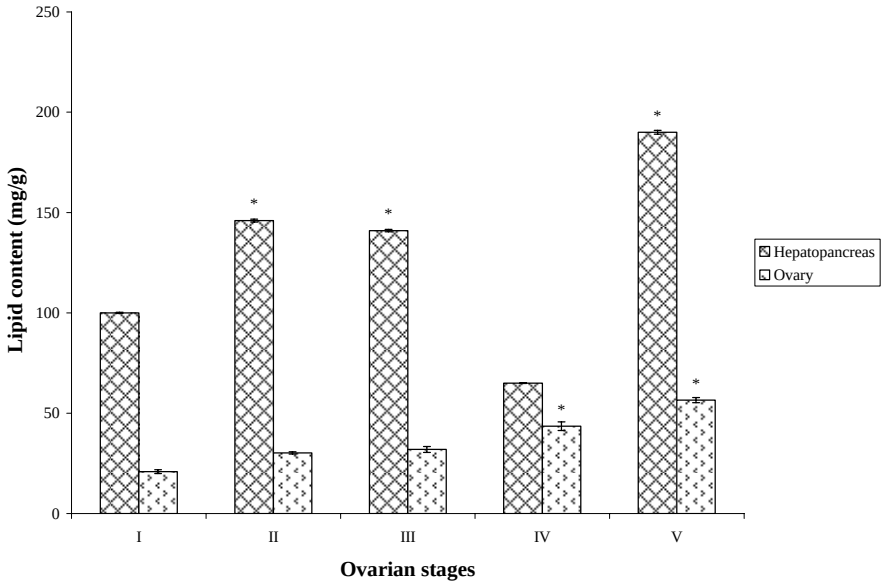

Fig. 5: Lipid content in different reproductive tissues during different ovarian stages (* F test P<0.05)

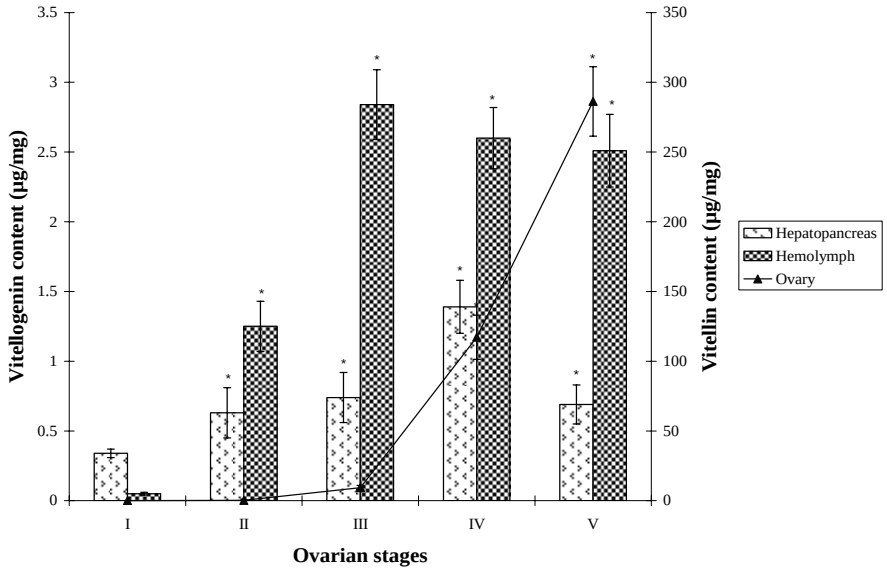

Fig. 6: Vitellogenin and vitellin content in different reproductive tissues during different ovarian stages (* F test P<0.05)

changes in the vitellin content of the ovary differed significantly during the ovarian stages (P<0.05).

Discussion

Our results clearly explain that ovarian development can be classified into five different stages based on colour, size and texture of the ovary in *M. rosenbergii*. These stages of ovarian development are substantiated with measurements of GSI and HSI indices. The average ovarian index level increased from stages I to V 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). Likewise, classification of the ovarian development based on the observation of external characteristics has been proposed (Chang and Shih, 1995; Damrongphol *et al.,* 1991). In crustaceans, the mature ovary is known to have mature oocytes, formatting a single unit by itself (Chang and Shih, 1995; Chen and Chen, 1994; Dall *et al.,* 1990; Tan Fermin and Pandadera, 1989; Yano, 1988).

From the present study cellular level changes were obtained in oocytes during ovarian development. Stages I, II and III showed oogonia, primary oocytes and previtellogenic oocytes because these stages correspond to the early development of the ovary. The follicle cells are so distinct because immature oocytes lack yolk material. Besides, IV and V stages illustrated prominent of vitellogenic oocytes which indicate the mature stage of ovary. The

follicle cells are not so distinct because fully mature oocytes fill up the entire ovary. Similar observation has been reported in crustaceans, oocyte cellular changes during gonadal maturation in prawn (Martins *et al.,* 2007). 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, are visible during the initial vitellogenesis (stage III), as mentioned by other authors (Chang and Shih, 1995). In stages IV and V, follicle cells do not appear to be enlarged, although this fact may result from the stretching of its cytoplasm due to a substantial increase of its cytoplasm (Van Herp and Payen, 1991).

The present results clearly explain that the biochemical contents in tested reproductive tissues varied during ovarian development. The ovarian vitellin content and total protein content were closely associated in the ovarian developmental stages. Besides, lipid content also fluctuated in the tested tissues during the ovarian development. The oocyte development correlated to the hemolymph vitellogenin content in *M. rosenbergii.* In agreement with the present results, Chang and Shih (1995) reported the accumulation of vitellin content in the ovary from stages I to V. 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. Vitellogenesis involves the synthesis of numerous components in the oocytes of crustaceans (Krol *et al.,* 1992). Protein and lipid contents synthesized into more complex molecules variously called vitellogenin (Croisille *et al.,* 1974), lipovitellin (Paulus and Laufer, 1982) and high-density lipoproteins (Lee and Puppione, 1988). They originate from ingested food either directly or after storage in the hepatopancreas and must be transported via the hemolymph as lipoproteins (Allen, 1972).

Our results clearly indicated that the appearance of vitellogenin in immature female hemolymph prior to gonadal development indicates that an extra ovarian site may be involved in vitellogenin synthesis. Increased hemolymph vitellogenin levels at early vitellogenic stage could also attribute vitellogenin synthesis and release from the extra ovarian site. Vitellogenin content in the hemolymph increased gradually to reach a peak at stage III and declined at stage IV in *M .rosenbergii*. The vitellogenin content increased from 0.05 ± 0.01 µg/ml at the beginning of the reproductive cycle to a maximum level of 2.84 ± 0.35 µg/ml in stage III and then declined sharply before spawning in *M. rosenbergii*. A similar pattern has been reported in *M. nipponense* (Vg range 1-9 mg/ml) (Okumura *et al.,* 1993) and *H. americanus* (Vg range 0-12 mg/ml) (Byard and David, 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; Vafopoulou and Steel, 1995). Although the site of vitellogenin synthesis is still controversial, some evidence indicates that the hepatopancreas is one of the possible sites (Castille and Lawrence, 1989; Paulus and Laufer, 1987). Increase in carbohydrate, protein and lipid content of the hepatopancreas during gonadal maturation is an indicator of the extent of glycogen and lipoprotein synthesis (Castille and Lawrence, 1989; Quackenbush, 1989). However, vitellin has been shown to be synthesized in the ovary during stages of ovarian maturity in invertebrates (Paulus and Laufer, 1987; Quackenbush, 1989).

This study demonstrated that the classification of ovarian development based to the colour, size and texture of the ovary and the identification of the extra ovarian synthesis site. On the other hand, it was evidenced that biochemical constituents are also closely associated with the ovarian development. Vitellogenesis as a biomarker of female reproductive activity, which indicate that the vitellin accumulation gradually increased in oocytes during ovarian development. Besides, the vitellogenin content fluctuated in different reproductive tissues during the ovarian development in *M. rosenbergii*.

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