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Ovarian Development and Hemolymph Vitellogenin Levels in Laboratory-maintained Protandric Shrimp, *Pandalus hypsinotus*: Measurement by a Newly Developed Time-resolved Fluoroimmunoassay (TR-FIA)

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ABSTRACT—Most pandalid shrimps exhibit protandric hermaphroditism, and detailed information on ovarian development of pandalid species is important for a better understanding of vitellogenesis in crustacean species. In the present study, we characterized ovarian development under light and electron microscopy and examined the hemolymph vitellogenin levels in the coonstriped shrimp, *Pandalus hypsinotus* under laboratory conditions. To measure vitellogenin levels, a time-resolved fluoroimmunoassay (TR-FIA) was developed after purification of vitellin and production of the anti-vitellin antiserum. The TR-FIA showed wide assay range (0.98–2000 ng/ml), high sensitivity (0.5 ng/ml), and low assay variability (0.9–6.4% of intraassay coefficients, 1.4–5.1% for interassay coefficients). Female *P. hypsinotus* had non-vitellogenic ovaries in March after the eggs attached to the abdomen hatched, and started yolk accumulation in the ovaries during April–October. During yolk accumulation, yolk globules appeared and increased in the ooplasm. After yolk accumulation, gonadosomatic index (GSI) reached 8.3–8.5 just before oviposition. Females spawned and were ovigerous during June–July of the next year. Hemolymph vitellogenin levels were low (0.006 ± 0.008 mg/ml, mean \pm SD) before the yolk accumulation, and became significantly higher (2.66 ± 0.93 mg/ml) during yolk accumulation (GSI, 2–8). Just before oviposition, levels declined to low levels (0.040 ± 0.012 mg/ml). Vitellogenin levels were significantly correlated to GSI during the yolk accumulation. The obtained results show that the process of vitellogenesis during the female phase of *P. hypsinotus* is similar to other crustacean species that do not change sex.

Key words: Crustacea, ovarian development, *Pandalus hypsinotus*, time-resolved fluoroimmunoassay, vitellogenin

INTRODUCTION

In crustaceans, the ovary develops rapidly during the process of vitellogenesis as in other oviparous animals (reviewed by Meusy and Payen, 1988; Wilder *et al.*, 2002). In this process, vitellogenin, the precursor of the major yolk protein, vitellin, is synthesized in the hepatopancreas or ovary/hepatopancreas, and is taken up by the oocytes. In the oocytes, vitellogenin is processed and accumulated as vitellin, and the accumulation of vitellin results in a rapid

increase of oocyte diameter. Vitellin is utilized as a nutritional source during embryogenesis.

Vitellogenin is characterized as a large molecule of lipoprotein (molecular weight, 290–700 kDa), and its hemolymph levels are used as an indicator of female reproductive status in crustaceans (reviewed by Wilder *et al.*, 2002). Several studies have revealed that hemolymph vitellogenin levels change in relation to ovarian development in crustacean species, however most studies have been carried out using species that do not change sex during the course of their lives.

Pandalid shrimps are a unique and scientifically interesting group, because most species exhibit protandric hermaphroditism (reviewed by Bergström, 2000). During the

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male phase, the gonad is an ovotestis, but only the testicular tissues are functional; on the other hand, during the female phase, the gonad contains only ovarian tissues after the degeneration of the testicular tissues during sexual transformation. Detailed information on ovarian development of pandalid shrimps is important for a better understanding of vitellogenesis in crustacean species.

The coonstriped shrimp, *Pandalus hypsinotus*, used in the present study, is distributed in the North Pacific Ocean and the Japan Sea. In the north Japan Sea, shrimp mature as males at an age of 1–2 years and as females at an age of 3 years after changing sex (Kurata, 1957). In the Japan Sea, the depth distribution of this shrimp is from 200 to 350 m. Thus, it is difficult to consistently obtain live shrimp from wild stocks by fisheries catch throughout the year. The present study was carried out using female shrimp under laboratory conditions.

The main purpose of the present study was to characterize details of the ovarian development in *P. hypsinotus*. First, we developed a time-resolved fluoroimmunoassay (TR-FIA) for *P. hypsinotus* vitellogenin using the antibody raised against purified vitellin. Second, we examined details of ovarian development under light and electron microscopy. Third, we determined hemolymph vitellogenin levels during ovarian development, and examined the relationship between hemolymph vitellogenin levels and ovarian development.

MATERIALS AND METHODS

Animals

Ovigerous female (body weight, 46–95 g) and male (body weight, 30–45 g) *P. hypsinotus* which were caught in the offshore waters of Ishikawa Peninsula, the Japan Sea were obtained from local fishermen in February 1999. They were kept in recirculating tanks of the Obama National Center for Stock Enhancement at 1–3°C under natural photoperiod conditions and were fed moist pellets. After the eggs of ovigerous females hatched larvae during February–March 1999, 103 female shrimp were taken for the sampling experiment.

Sampling

Sampling was carried out in March, April, May, June, August, October, and December 1999, and February, April, June, August, and November 2000. At each sampling, female shrimp (2–12 animals) were randomly chosen and sacrificed. Hemolymph samples were taken from the pericardial cavity using a syringe with a needle, and were stored at –80°C until analysis. Ovaries were dissected and weighed, and pieces of the midportion of the ovaries were fixed for histological observation. From some female shrimp, pieces of the ovaries were quickly frozen in liquid nitrogen and stored at –80°C until analysis. Gonadosomatic index (GSI) was calculated as the gonad weight (g) × 100/body weight (g). During the experiment period, March 1999–November 2000, 20 animals died.

Histological procedures

For light microscopy, the ovarian pieces were fixed with Bouin's solution. After dehydration with an ethanol and xylene series, the fixed samples were embedded in paraffin, and sectioned at 4 μm thickness. Sections were stained with hematoxylin-eosin or hematoxylin-periodic acid Schiff (PAS).

For transmission electron microscopy, the ovarian pieces were fixed with ice-cold 2.25% paraformaldehyde, 2% glutaraldehyde, and 6.5% sucrose buffered with 0.1 M sodium cacodylate, pH 7.5. The fixed ovaries were post-fixed with 1% osmium tetroxide, and embedded in Epon 812 after dehydration in a series of ethanol, propylene oxide, and QY-1. Ultra-thin sections were stained with uranyl acetate and lead citrate. The sections were observed under a Hitachi H-7100 electron microscope at 75 kV or a JEOL JEM-100CX electron microscope at 80 kV.

Classification of developing stages of oocytes and ovary

According to criteria previously reported for the giant freshwater prawn, *Macrobrachium rosenbergii* (Okumura and Aida, 2000), the developmental stages of the oocyte were classified into four stages: previtellogenic oocyte (oocytes with homogeneously hematoxylin-stained cytoplasm), endogenous vitellogenic oocyte (oocytes with PAS-positive but eosin-negative vesicles in the cytoplasm), exogenous vitellogenic oocyte (oocytes with eosin-positive yolk globules in the cytoplasm), and maturing oocyte (nucleus is not observable in the center of the cytoplasm due to germinal vesicle break down (GVBD)). For naming cytoplasmic components, we followed the terminology for *M. rosenbergii* (Okumura and Aida, 2000)

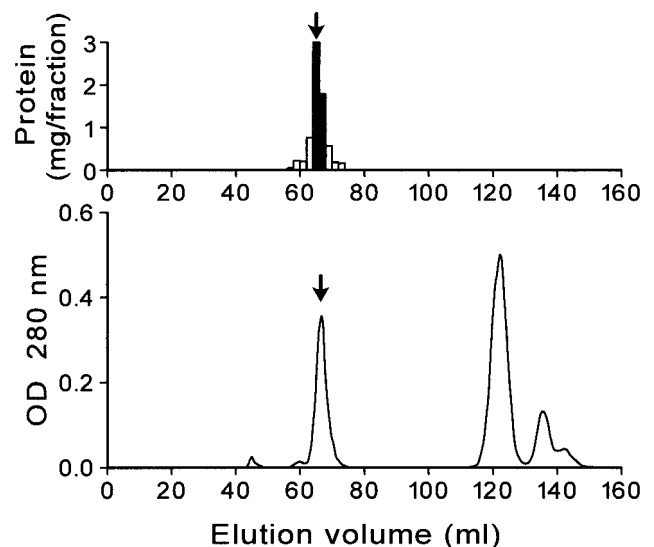


Fig. 1. Gel filtration chromatogram of vitellogenic ovarian extract of *Pandalus hypsinotus* on a HiPrep 16/60 Sephacryl S-300 HR column. Flow rate, 12 ml/h; fraction size, 2 ml. Arrows indicate the vitellin peak. Closed fractions were subjected to ion-exchange chromatography.

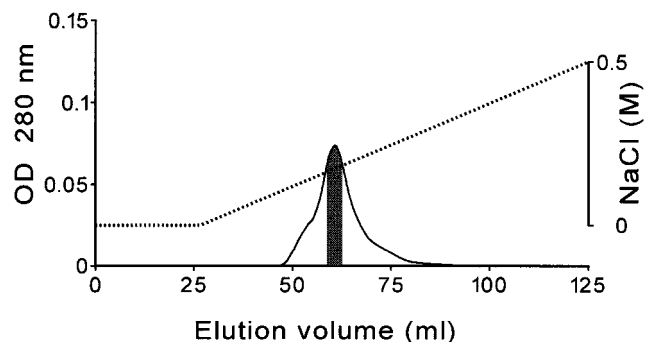


Fig. 2. Ion-exchange chromatogram of gel-filtration fractions of *Pandalus hypsinotus* ovarian extract. Dotted line indicates NaCl gradient elution. Closed peak was taken as the purified vitellin.

and the spiny lobster, *Panulirus japonicus* (Minagawa and Sano, 1997).

The ovarian developmental stage was determined according to the relative abundance of the most advanced type of oocyte in the ovary. We followed the terminology for the spiny lobster (Minagawa and Sano, 1997), and the ovarian developmental stages were classified into five stages: inactive stage (ovaries without exogenous vitellogenic oocytes), early developing stage (ovaries containing exogenous vitellogenic oocytes, $GSI < 2$), late developing stage (ovaries containing exogenous vitellogenic oocytes, $GSI \geq 2$), ripe stage (ovaries containing maturing oocytes), and spawned stage (ovaries after oviposition).

Purification of vitellin

Vitellogenic blue/green ovaries from a female ($GSI: 3.2$) were homogenized in 0.02 M Tris-HCl buffer, pH 8.7, containing 0.15 M NaCl using a HG-30 homogenizer (Hitachi, Tokyo, Japan). The homogenate was centrifuged at 10000 g for 10 min at 4°C, and the interphase was carefully taken so as not to contaminate the upper lipid phase and the precipitate. The interphase was subjected to gel filtration on a HiPrep 16/60 Sephacryl S-300 HR column (1.6×60 cm, 120 ml in bed volume, Amersham Biosciences, Piscataway, NJ) and eluted by the same buffer at a flow rate of 12 ml/h. The absorbance at 280 nm was monitored during the elution, and the eluted fractions were collected every 2 ml. Molecular weight of the eluted peak was estimated using a HMW gel filtration calibration kit (Amersham Biosciences). Protein content of each fraction was determined using an Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO) with bovine γ -globulin as a standard.

The major protein peak fractions were pooled and concentrated using the water-absorbing beads Ms. Btaury-kn (Atto, Tokyo, Japan). After desalting on a PD-10 column (Amersham Biosciences), the major protein fractions were subjected to ion-exchange chromatography on a HiPrep 16/10 DEAE FF column (1.6×10 cm, 20 ml in bed volume, Amersham Biosciences) for further separation. Elution was done at a flow rate of 5 ml/min with a linear gradient from 0.02 M Tris-HCl buffer, pH 8.7 to 0.02 M Tris-HCl buffer, pH 8.7, containing 0.5 M NaCl in 100 ml elution. Fractions were collected every 4 ml. The fraction with the highest protein content was taken as the purified vitellin.

Preparation of antiserum against vitellin

Polyvalent antiserum against the purified vitellin was raised in a rabbit. An emulsion of the purified vitellin fraction and Freund's complete adjuvant was injected intradermally into the back of the rabbit twice in two weeks. Blood was collected 8 days after the second injection. Antiserum was stored at -80°C.

Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of 2-mercaptoethanol

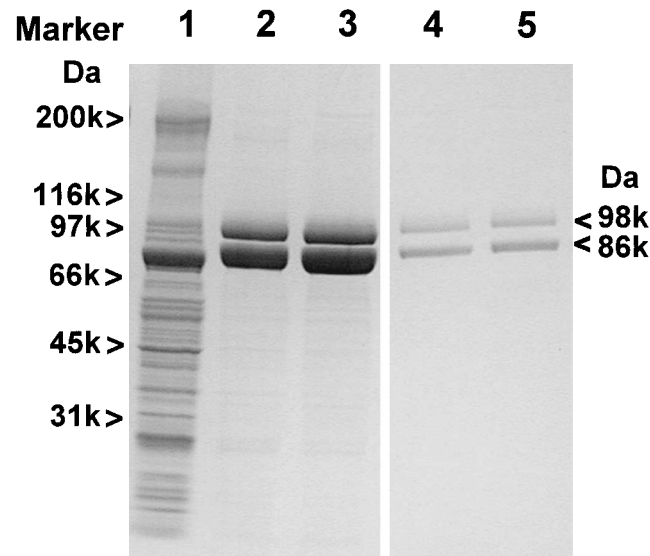


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5–20% gradient gel) of ovarian extracts (lanes 1–3) and purified vitellin (lanes 4 and 5) of *Pandalus hypsinotus*. Lane 1, ovary at the inactive stage ($GSI: 0.78$); lane 2, ovary at the late developing stage ($GSI: 3.23$); lane 3, ovary at the ripe stage ($GSI: 8.53$); lane 4, fraction of gel-filtration chromatography; lane 5, fraction of ion-exchange chromatography.

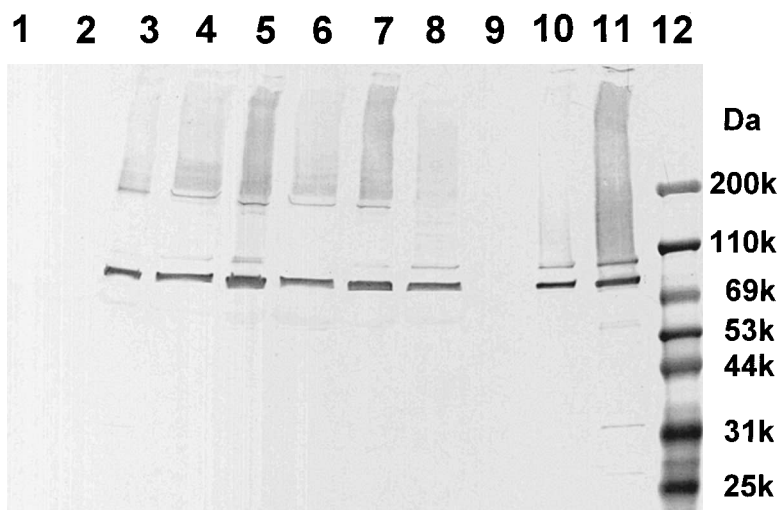


Fig. 4. Western blotting analysis of hemolymph (lanes 1–8) and ovarian extracts (lanes 9–11) of *Pandalus hypsinotus* stained with anti-vitellin antiserum. Lane 1, male ($GSI: 0.33$); lane 2, immature female ($GSI: 0.63$); lanes 3–8, vitellogenic females ($GSI: 2.92, 3.70, 4.12, 4.76, 6.51,$ and 9.76 , respectively); lane 9, immature female ($GSI: 0.63$); lanes 10 and 11, vitellogenic females ($GSI: 4.12$ and 6.51), lane 12, molecular weight marker.

on precast 5–20% acrylamide gradient slab gels (Bio-Rad, Hercules, CA) using routine procedures. The gels were stained with Coomassie Brilliant Blue R-250. Molecular weights of bands were estimated using Bio-Rad broad molecular weight standards.

After SDS-PAGE, proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (immune-blot PVDF membrane, Bio-Rad) using a wet transfer apparatus (Mini trans-blot cell, Bio-Rad). The blotted membrane was blocked with a blocking solution of Block Ace (Snow Brand Milk Products, Sapporo, Japan) for 1 h at room temperature. After washing the membrane in 0.01 M phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS), the membrane was incubated in the raised anti-vitelin antiserum in a Block Ace solution (1:1000) for 1 h at room temperature. Immunoreactive bands were made visible by a Histofine Simple Stain PO (MULTI) (Nichirei, Tokyo, Japan) and a peroxidase substrate solution (0.02% 3,3'-diaminobenzidine, 0.06% H₂O₂ in 0.1 M phosphate buffer, pH 6.4).

Immunohistochemistry

After deparaffinization, sections of vitellogenic ovary were incubated for 5 min in 3% H₂O₂. After washing in PBS, sections were incubated for 10 min in a Block Ace solution, and further incubated in the raised anti-vitelin antiserum diluted in a Block Ace solution (1:5000) for 30 min. After washing in PBS, sections were incubated in a Histofine Simple Stain PO (MULTI) solution for 30 min, and developed in the substrate solution.

Eu-labeling of anti-vitelin immunoglobulin G

The anti-vitelin antiserum was desalted and eluted in 0.02 M phosphate buffer, pH 7 using a PD-10 column. The elution was subjected to a HiTrap rProtein A 1 ml column (Amersham Biosciences), and anti-vitelin immunoglobulin G (IgG) was eluted in 0.1 M sodium citrate buffer, pH 3.6. After changing the buffer from 0.1 M sodium citrate buffer to 0.05 M sodium carbonate buffer, pH 9.0, the anti-vitelin IgG was labeled with isothiocyanatophenyl-EDTA-Eu chelate (Eu-labeling reagent) using a DELFIA Eu-labeling kit (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer's protocol. Briefly, 0.04 mg of Eu-labeling reagent was added to 0.05 ml of sodium carbonate buffer which contained 0.23 mg of IgG, and incubated for 27 h at room temperature. The Eu-labeled IgG was separated from free Eu-chelate and eluted in an elution buffer (0.05 M Tris-HCl buffer, pH 7.8, containing 0.9% NaCl and 0.1% NaN₃) using a PD-10 column. The labeling yield (Eu³⁺/IgG) was 15.

Vitellogenin time-resolved fluoroimmunoassay

Vitellogenin TR-FIA was carried out using a sandwich method. The wells of a DELFIA Strip Plate 8x12-well (PerkinElmer Life and Analytical Sciences) were coated with the protein A-purified anti-vitelin IgG by physical adsorption. The anti-vitelin IgG in sodium carbonate buffer, pH 9.0 (0.2 ml) was put into wells, and allowed to stand at room temperature overnight. After three washes with wash buffer (5 mM Tris-HCl buffer, pH 7.8, containing 0.05% Tween 20 and 0.1% NaN₃), the wells were blocked with 0.5% bovine serum albumin in 0.05 M Tris-HCl buffer, pH 7.8, which contained 0.9% NaCl and 0.1% NaN₃ at 4°C overnight. The blocking solution was discarded, and the plates were briefly dried before being stored at 4°C.

Hemolymph samples were diluted in assay buffer (0.05 M Tris-HCl buffer, pH 7.8, containing 0.9% NaCl, 0.5% bovine serum albumin, 0.05% NaN₃, 0.01% Tween 40, and 0.02 mM diethylene-triaminepentaacetic acid) (1000–20000 fold dilutions). The purified vitellin was dissolved in assay buffer as standard (0.98–2000 pg/ml). The hemolymph samples or standards (0.2 ml) were put into the anti-vitelin IgG-coated wells, and incubated at 4°C overnight. After four washes with wash buffer, 0.2 ml of the Eu-labeled anti-vitelin IgG in assay buffer (250 ng/ml) was put into the wells, and

incubated for 4 h at room temperature. After five washes with wash buffer, 0.2 ml of enhancement solution (0.1 M acetate-phthalate buffer, pH 3.2, containing 0.1% Triton X-100, 0.015 M 2-naphthoyl-trifluoroacetone, and 0.05 mM tri-*n*-octylphosphine oxide, Perkin-Elmer Life and Analytical Sciences), and the plates were slowly shaken for 5 min at room temperature. The Eu fluorescence in the wells was measured by a time-resolved fluorometer (Wallac 1420 ARVOsx-d, PerkinElmer Life and Analytical Sciences).

Statistical analysis

The quantitative data are presented as mean±SD (standard deviation). Nonparametric statistics were used because the data exhibited non-normal distributions. For multiple comparisons, overall analysis was done by the Kruskal-Wallis test, followed by the Dunn test when overall significance was detected. The Spearman rank correlation test was done for correlation analysis. Probability values of $P < 0.05$ were considered statistically significant.

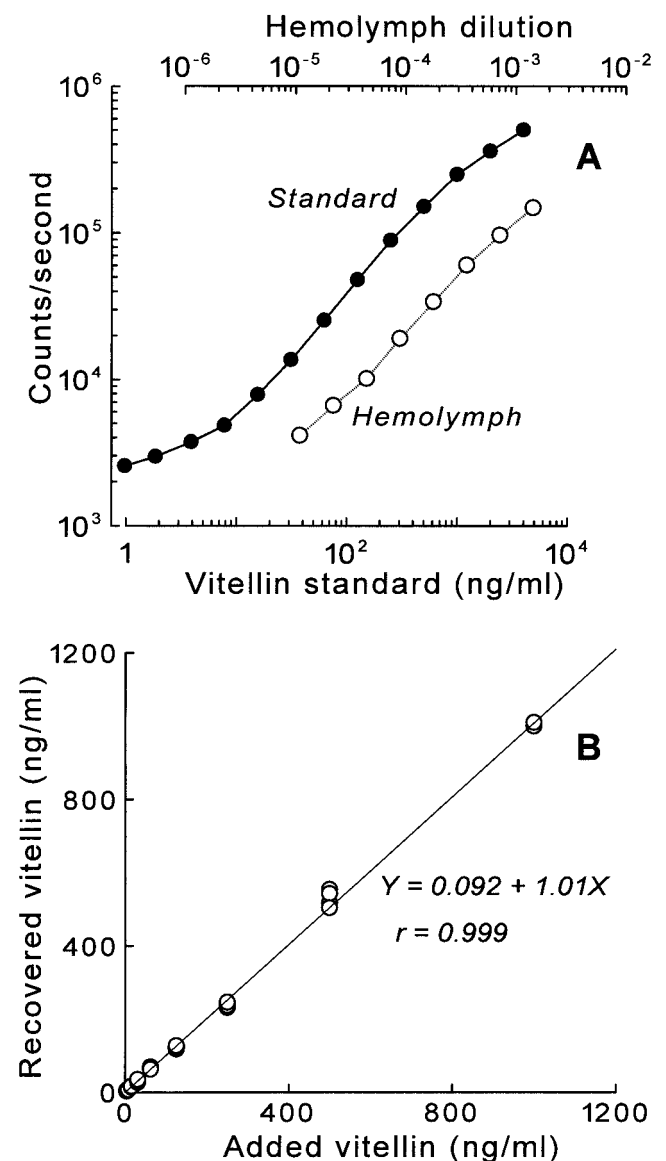


Fig. 5. Validation of time-resolved fluoroimmunoassay for *Pandalus hypsinotus* vitellogenin. A, parallelism between standard curve and hemolymph serial dilution; B, recoveries of standard vitellin added to hemolymph.

RESULTS

Purification of vitellin

Six peaks were detected by gel filtration chromatography, and the third peak (65 ml-elution volume) was the major protein peak (Fig. 1). This peak was successively purified by DEAE ion-exchange column chromatography, and the major peak was taken as purified vitellin (Fig. 2). The molecular weight of the purified vitellin was estimated as 295 kDa on gel filtration chromatography.

The purified vitellin was separated into two bands of 86 and 98 kDa in SDS-PAGE (Fig. 3). These two bands were also observed as major bands in the gel-filtrated fraction and crude extracts of vitellogenic ovary and matured ovary (after GVBD), but the bands were not observed in the crude extract of immature ovary.

Characterization of vitellin and vitellogenin

By Western blotting using the antibody raised against the purified vitellin, the 86 and 98 kDa bands were detected

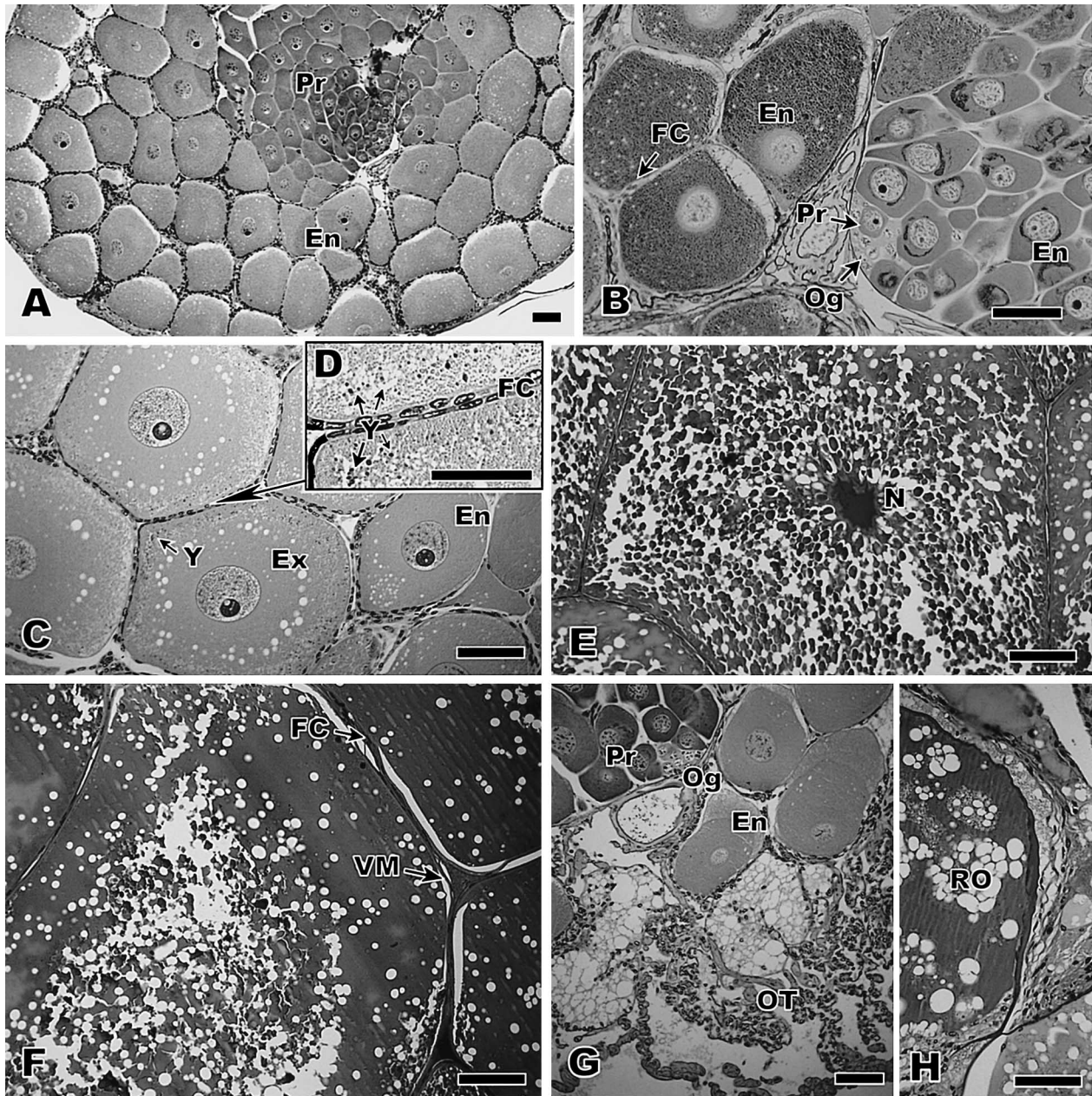


Fig. 6. Cross sections of *Pandalus hypsinotus* ovaries. A, B, inactive stage after releasing the hatched larvae (GSI: 0.62); C, D, beginning of yolk accumulation at the early developing stage (GSI: 1.00); E, late developing stage (GSI: 6.12); F, ripe stage (GSI: 8.52); G, spawned stage (GSI: 0.28); H, regressing mature oocytes in ovary at the spawned stage (GSI: 3.05). A, C–H, hematoxylin-eosin staining; B, hematoxylin-periodic acid Schiff staining. Bars: 0.1 mm. En, endogenous vitellogenic oocytes; Ex, exogenous vitellogenic oocytes; FC, follicle cells; N, nucleus; Og, oogonia; Pr, previtellogenic oocytes; OT, ovulation traces; RO, regressing mature oocytes; VM, vitellin membrane; Y, yolk globules.

in extracts of vitellogenic ovaries as the major immunoreactive bands, but not detected in the extracts from immature ovaries (Fig. 4). This result indicates that the antibody is specific to the vitellin. In addition to the two major bands, minor immunoreactive bands were detected at 26, 38, and 57 kDa in extracts of vitellogenic ovaries. In the hemolymph of vitellogenic females, the 86 kDa band was detected as the major band, and 98 and 190 kDa bands were detected as minor bands (Fig. 4).

Validation of time-resolved fluoroimmunoassay

A standard curve for vitellogenin TR-FIA was obtained in the range of 0.98–2000 ng/ml (Fig. 5A). In this range, the coefficient of variation for each standard was less than 6%. A dilution series of hemolymph samples (700–89600 fold

dilutions) were parallel to the standard curve (Fig. 5A). Analytical sensitivity was 0.5 ng/ml (0.1 ng/well), which was calculated as the value, 2 SD above the mean of blank measurement values.

Intraassay and interassay variation was examined by measuring hemolymph samples of immature and mature females. Intraassay coefficients were 0.9–6.4% (6 replications for each sample) for the concentrations of 2.6–1000 ng/ml, and interassay coefficients were 1.4–5.1% (6 replicated assays for each sample) for the concentrations of 14.7, 65.5, 248, and 993 ng/ml.

Recovery was examined by measuring the hemolymph samples that were prepared by adding varying levels of standard vitellin (7.8–1000 ng/ml) to immature female hemolymph (Fig. 5B). Recoveries were in the range of 95–

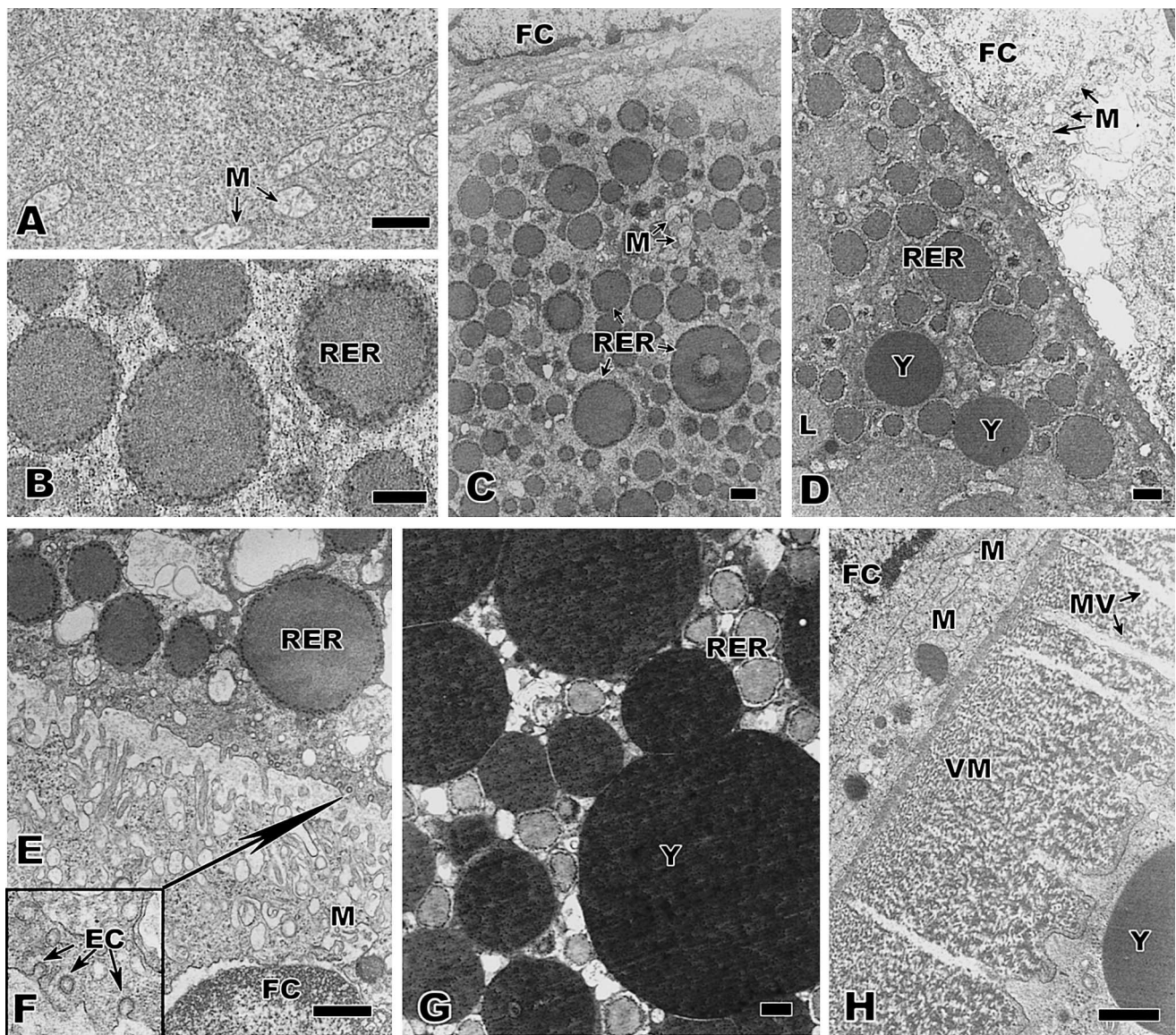


Fig. 7. Transmission electron micrographs of *Pandalus hypsinotus* ovaries. A, previtellogenic oocyte (GSI: 0.60); B,C, endogenous vitellogenic oocyte (GSI: 0.55); D, exogenous vitellogenic oocyte and follicle cell (GSI: 1.17); E, F, endocytosis in the exogenous vitellogenic oocyte (GSI: 1.12); G, exogenous vitellogenic oocyte (GSI: 2.25); H, maturing oocyte (GSI: 8.52). Bars: 1 μ m. EC, endocytosis; FC, follicle cells; L, lipid droplets; M, mitochondria; MV, microvilli; RER, rough endoplasmic reticulum; VM, vitellin membrane; Y, yolk globules.

111% (102.2±5.1%, mean±SD, 4 replications for each sample).

Ovarian developmental stages

In the inactive stage of the ovary, the ovary contains oogonia, previtellogenic oocytes, and endogenous vitellogenic oocytes (Fig. 6A, B). The previtellogenic oocytes are present in the center, and the endogenous vitellogenic oocytes are at the periphery. The previtellogenic oocytes are 35–60 µm in diameter, and in electron microscopy observations, the ooplasm contains ribosomes and mitochondria (Fig. 7A). The endogenous vitellogenic oocytes are 60–280 µm in diameter, and larger oocytes (120–280 µm in diameter) are enveloped by follicle cells (Fig. 6B). There are unstained vesicles in the cytoplasm of the endogenous vitellogenic oocytes, which are considered to be lipid droplets. In electron microscopy observations (Fig. 7B,C), the ooplasm is filled with rough endoplasmic reticulum (0.5–3 µm in diameter) which contains relatively dense homogeneous material. The rough endoplasmic reticulum also contains small electron-dense granules at the periphery.

In the early developing stage of the ovary, the exogenous vitellogenic oocytes are present at the periphery, and the endogenous vitellogenic oocytes are present in the center. At the beginning of this stage, small eosin-positive yolk globules are observed in the cytoplasm of the oocytes (300–340 µm in diameter, Fig. 6C, D), and at the end of this stage, yolk globules fill the ooplasm. The exogenous vitellogenic oocytes were positively stained by the anti-vitellin antibody, but the previtellogenic oocytes, endogenous vitellogenic oocytes, oogonia, and follicle cells were not stained (Fig. 8). In electron microscopy observations, the ooplasm contains electron-dense yolk globules (0.8–2.6 µm) in addition to mitochondria, lipid droplets and rough endoplasmic reticulum (Fig. 7D). The follicle cells have mitochondria but no developed rough endoplasmic reticulum in the cytoplasm. Active endocytosis was observed at the oocyte surface (Fig. 7E, F).

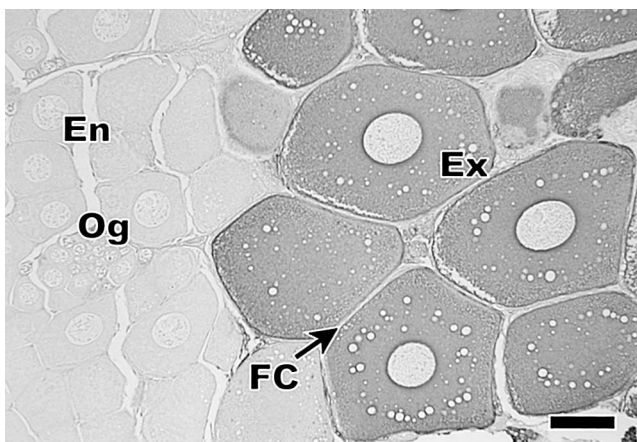


Fig. 8. Immunohistochemistry of an ovarian section (GSI: 1.00) with anti-vitellin antiserum. En, endogenous vitellogenic oocyte; Ex, exogenous vitellogenic oocytes; FC, follicle cells; Og, oogonia.

In the late developing stage of the ovary, the exogenous vitellogenic oocytes become larger and are filled with yolk globules (Fig. 6E). Oogonia, the previtellogenic oocytes, and the endogenous vitellogenic oocytes are present in the center of the ovaries. The diameter of the oocytes reaches 875–950 µm at the end of this stage. In electron microscopy observations, yolk globules become larger (2–15 µm), and fill the ooplasm (Fig. 7G). Among the accumulated yolk globules, rough endoplasmic reticulum is present.

In the ripe stage of the ovary, the maturing oocytes are present at the periphery (Fig. 6F). The maturing oocytes (875–975 µm) are still enveloped by the follicle cells, and vitellin membrane develops on the oocyte surface. The vitellin membrane is 4 µm thick, and the oocytes project microvilli toward the follicle cells (Fig. 7H).

In the spawned stage of the ovary, the ovulation traces are present at the periphery of the ovary, and oogonia, the previtellogenic oocytes, and the endogenous vitellogenic oocytes are present in the center (Fig. 6G). The ovary often

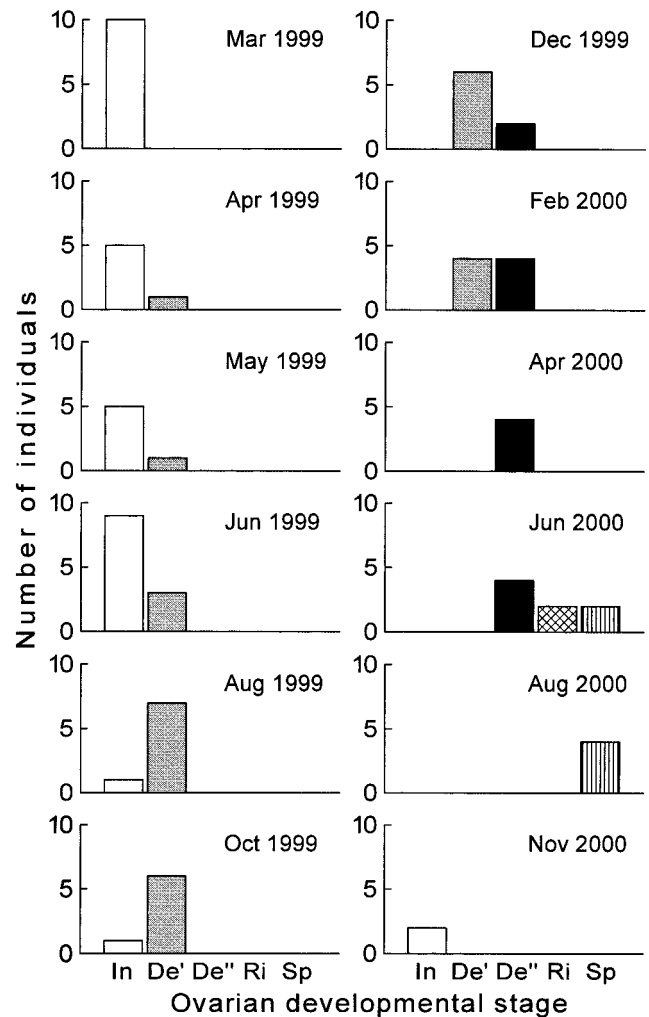


Fig. 9. Seasonal frequency of the ovarian developmental stages in *Pandalus hypsinotus*. In, inactive stage; De', early developing stage; De'', late developing stage; Ri, ripe stage; Sp, spawned stage.

contains regressing mature oocytes (Fig. 6H).

Ovarian development and hemolymph vitellogenin levels

Seasonal changes in ovarian development and vitellogenin levels are shown in Figs. 9 and 10. In March 1999, all the sampled females had ovaries at the inactive stage, and GSI and hemolymph vitellogenin levels were low (0.55 ± 0.04 and 0.010 ± 0.011 mg/ml, respectively, mean \pm SD). In April and May 1999, females had ovaries at the early developing stage appeared, but most females had ovaries at the inactive stage. The number of females with ovaries at the early developing stage increased during June-October 1999. In October 1999, GSI became significantly higher (1.00 ± 0.21 , $P < 0.05$) than in March 1999, but vitellogenin levels (0.25 ± 0.52 mg/ml) were not significantly different. During December 1999-April 2000, all the sampled females had ovaries at the early or late developing stages, and GSI was high (2.0–3.2). Vitellogenin levels were significantly higher in February and April 2000 (1.30 ± 1.40 and 2.82 ± 0.62 mg/ml, respectively) than in March 1999. In June 2000, females with ovaries at the ripe stage appeared, and some females had spawned. GSI and vitellogenin levels were high but showed high variances (5.6 ± 2.1 and 2.26 ± 2.94 mg/ml, respectively). In August 2000, all the sampled females had ovaries at the spawned stage. Two to the four sampled females carried embryos on the abdomen, but the other two females did not carry the embryos, probably due to a failure to fertilize the eggs. In August 2000, GSI significantly declined (1.36 ± 1.93 , $P < 0.05$) due to oviposition; on the other hand, vitellogenin levels highly varied (2.24 ± 3.31 mg/ml) due to the occurrence of shrimp having unspawned mature oocytes. In November 2000, females brooded embryos on the abdomen and had ovaries at the inactive stage. GSI and vitellogenin levels were low (0.34 ± 0.06 and 0.001 ± 0.000 mg/ml, respectively).

All the female shrimp molted once during May-August

1999 (the first ecdysis). The second ecdysis occurred during November 1999-February 2000, although some female shrimp did not molt during this period. Before oviposition, female shrimp molted (the second or third ecdysis).

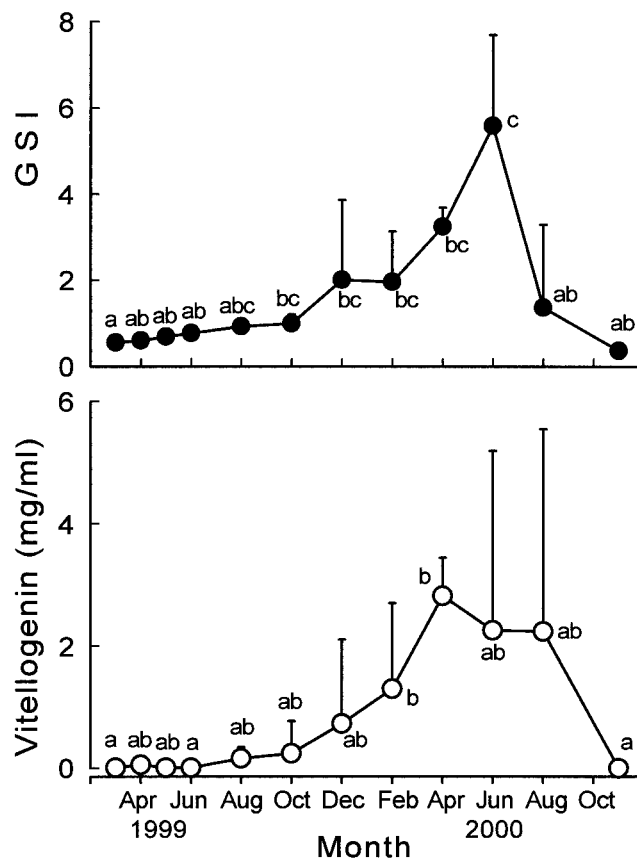


Fig. 10. Seasonal changes in GSI and hemolymph vitellogenin levels in *Pandalus hypsinotus*. Points and bars indicate the mean and SD. Points without common letters indicate significant differences ($P < 0.05$).

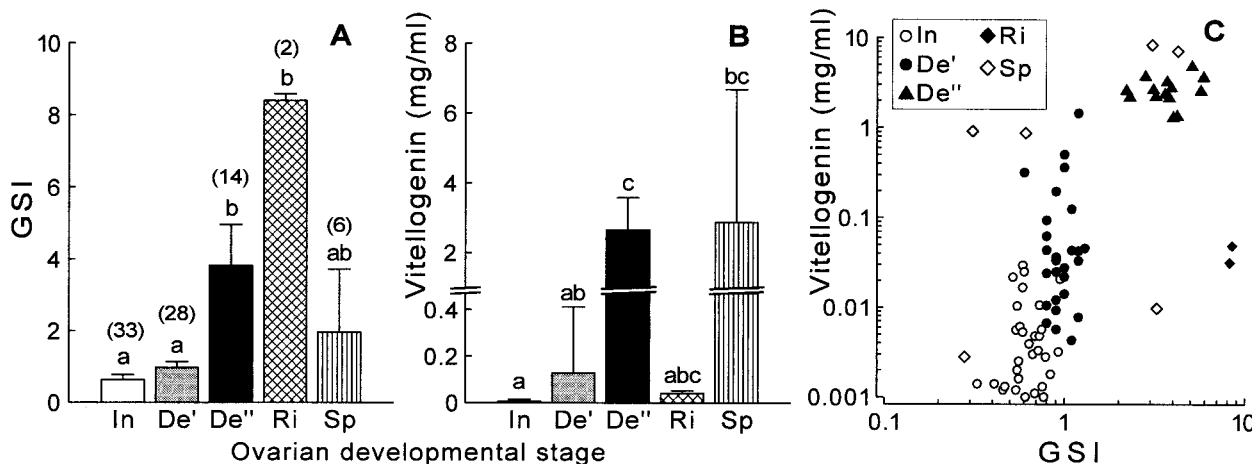


Fig. 11. GSI (A) and hemolymph vitellogenin levels (B) at ovarian developmental stages and relationship between GSI and hemolymph vitellogenin levels (C) in *Pandalus hypsinotus*. Bars indicate the mean and SD. Bars without common letters indicate significant differences ($P < 0.05$). Numbers in parenthesis indicate the number of animals sampled for each ovarian developmental stage. Abbreviations are shown in Fig. 9.

GSI and vitellogenin levels were compared between the ovarian developmental stages (Fig. 11). In the females with ovaries at the late developing stage, GSI was significantly higher (3.82 ± 1.13 , $P < 0.05$) than at the inactive stage (0.63 ± 0.14), and vitellogenin levels was significantly higher (2.66 ± 0.93 mg/ml, $P < 0.05$) than at the inactive stage (0.006 ± 0.008 mg/ml). In the females with ovaries at the ripe stage, GSI peaked (8.40 ± 0.19), but vitellogenin levels became low (0.040 ± 0.012 mg/ml, not significant mainly due to the small number of samples). Vitellogenin levels and GSI (after logarithmic transformation) were significantly correlated ($P < 0.05$, Fig. 11C).

DISCUSSION

In the present study, vitellin of *P. hypsinotus* was purified and its molecular weight was estimated as 295 kDa by gel filtration chromatography. Among caridean species, molecular weight of the vitellin is 560 kDa in the pandalid shrimp, *Pandalus kessleri* (Quinitio *et al.*, 1989), 330 kDa in *M. rosenbergii* (Derelle *et al.*, 1986), and 350 kDa in the freshwater prawn, *Macrobrachium nipponense* (Han *et al.*, 1994). The molecular weight of the purified vitellin in *P. hypsinotus* is close to those in *Macrobrachium* species. The major subunits of the purified vitellin were determined as 86 and 98 kDa in *P. hypsinotus*. This result is the same as the previous report in *P. hypsinotus* (85 and 100 kDa, Tsutsui *et al.*, 2004) and similar to previous reports in other caridean species: two subunits (81 and 110 kDa) in *P. kessleri* (Quinitio *et al.*, 1989), two subunits (84–90 and 92–102 kDa) in *M. rosenbergii* (Derelle *et al.*, 1986; Wilder *et al.*, 1994), and two subunits (90 and 102 kDa) in *M. nipponense* (Han *et al.*, 1994). By immunoblotting analysis, subunits of hemolymph vitellogenin were determined as 86, 98, and 190 kDa, similar to *M. rosenbergii* (90, 102, and 199 kDa) (Wilder *et al.*, 1994).

A sandwich TR-FIA for vitellogenin was developed in the present study, and to our knowledge this is the first TR-FIA for crustacean vitellogenin. The developed TR-FIA showed advantages over commonly used EIA. Several types of EIA have been used for crustacean vitellogenin assays: a direct EIA (Quackenbush, 1989; Okumura *et al.*, 1992; Lee and Watson, 1994; Chang and Shih, 1995; Sagi *et al.*, 1999; Jasmani *et al.*, 2000), a sandwich EIA (Derelle *et al.*, 1986), and a competitive EIA (Mendoza *et al.*, 1993; Tsukimura *et al.*, 2000, 2002). Sensitivity of the TR-FIA (0.5 ng/ml, 0.1 ng/well) is higher than that of EIA (3–148 ng/ml, 0.3–15 ng/well). The assay variability of the TR-FIA at the middle standard range (2.4% for intraassay coefficient, 2.8% for interassay coefficient) is lower than that of EIA (3.0–7.2% for intraassay coefficient, 3.0–20.6% for interassay coefficient). The standard curve of this TR-FIA produced low interassay coefficient (< 10%) over the assay range (0.98–2000 ng/ml). This assay range (2040 times) is wider than in EIA (24–90 times). The developed TR-FIA exhibited high sensitivity, low variability, and a wide assay range.

The ovarian development in *P. hypsinotus* was characterized under light and electron microscopy. In pandalid shrimps, ovarian development has been studied under light microscopy (Aoto, 1952; Allen, 1959; Hoffman, 1972) but has not been studied under electron microscopy.

The ovaries at the inactive stage contain the previtellogenic oocytes and the endogenous vitellogenic oocyte. The observed accumulation of ribosomes in the previtellogenic oocyte cytoplasm is similar to a previous report on the spider crab, *Libinia emarginata* (Hinsch and Cone, 1969). The endogenous vitellogenic oocytes are characterized by the accumulation of rough endoplasmic reticulum containing small electron-dense granules at the periphery. A similar phenomenon has been also observed in the crayfish (Beams and Kessel, 1962), *L. emarginata* (Hinsch and Cone, 1969), *M. nipponense* (Han, 1988), the spiny lobster (Minagawa and Sano, 1997), the hermit crab, *Coenobita clypeatus* (Komm and Hinsch, 1987) and penaeid shrimps (Hong, 1977; Rankin and Davis, 1990; Yano *et al.*, 1996; Carvalho *et al.*, 1998). The accumulation of rough endoplasmic reticulum indicates that proteins are endogenously synthesized and accumulated in the oocytes. The contents of the rough endoplasmic reticulum are most likely not vitellin, because the cytoplasm of the endogenous vitellogenic oocytes is not stained with anti-vitellin antiserum. The rough endoplasmic reticulum appears in the ooplasm concomitantly with PAS-positive granules. Relation of both should be further examined.

In the ovaries at the early and late developing stages, the exogenous vitellogenic oocytes accumulate the yolk globules in the cytoplasm. The yolk globules most probably contain vitellin and are formed by uptake of vitellogenin from hemolymph. This is also supported by the active endocytosis observed at the oocyte surface. Similar observations of active endocytosis have been reported in several crustacean species (Beams and Kessel, 1962; Hong, 1977; Han, 1988; Yano *et al.*, 1996; Minagawa and Sano, 1997; Carvalho *et al.*, 1998).

In the ovaries at the ripe stage, the nucleus of the maturing oocytes is not visible in the center of the cytoplasm due to GVBD. The vitellin membrane is formed on the surface of the oocytes. These characteristics are similar to other crustacean species (Hong, 1977; Han, 1988; Yano *et al.*, 1996; Minagawa and Sano, 1997; Carvalho *et al.*, 1998). The cortical vesicles observed at the oocyte maturation stage in some penaeid shrimps (Hudinaga, 1942) were not observed as in other caridean species (Han, 1988; Okumura and Aida, 2000).

During ovarian development, the exogenous vitellogenic oocytes synchronously accumulate yolk globules and are spawned after GVBD. After oviposition, the oogonia, previtellogenic oocytes and endogenous vitellogenic oocytes remain, and the remaining non-vitellogenic oocytes will start to develop in the next ovarian developmental cycle. This process is similar to other crustacean species which carry the eggs on the abdomen until the larvae hatch (e.g.,

Han, 1988; Minagawa and Sano, 1997; Okumura and Aida, 2000; Okumura, 2003). This ovarian developmental cycle appears to be a common feature among the ovigerous crustacean species.

During carrying the eggs, female *P. hypsinotus* do not start yolk accumulation, and the ovaries contain previtellogenic oocytes and endogenous vitellogenic oocytes. On the other hand, most other ovigerous species develop the ovaries during carrying the eggs, and after the release of the hatched larvae, females spawn eggs. This difference is probably because *P. hypsinotus* inhabits cold water and needs a long period for ovarian development.

The vitellogenin synthesis site in *P. hypsinotus* has been determined as the hepatopancreas, based on the expression of vitellogenin mRNA (Tsutsui *et al.*, 2004). Hepatopancreas is also the vitellogenin synthesis site in *M. rosenbergii* (Chen *et al.*, 1999; Yang *et al.*, 2000) and the crayfish *Cherax quadricarinatus* (Abdu *et al.*, 2002). On the other hand, in penaeid shrimps, follicle cells in the ovary are the synthesis site as well as the hepatopancreas (Tsutsui *et al.*, 2000; Tseng *et al.*, 2002; Avarre *et al.*, 2003). In the present study, the follicle cells in the ovaries did not contain developed rough endoplasmic reticulum. This result indicates that the follicle cells are not highly active in protein synthesis and that the follicle cells in the ovaries are not the synthesis site in this species.

In female *P. hypsinotus*, high hemolymph vitellogenin levels (> 1 mg/ml) were concomitant with yolk accumulation in the oocytes (GSI \geq 2), while vitellogenin levels were low (< 0.1 mg/ml) at the ovarian inactive stage. This result indicates that vitellogenin levels are an indicator for vitellogenesis in this species. At the ovarian ripe stage, vitellogenin levels declined to low levels. This result indicates completion of vitellogenesis at this stage. These changes in vitellogenin levels resemble those in *P. kessleri* (Quinitio *et al.*, 1989) and other crustacean species (Quackenbush, 1989; Okumura *et al.*, 1992; Shafir *et al.*, 1992; Quinitio *et al.*, 1994; Chang and Shih, 1995; Okumura and Aida, 2000; Jasmani *et al.*, 2000). Although pandalid shrimps change the functional sex from male to female, the process of vitellogenesis during the female phase is similar to other crustacean species that do not change sex.

Under laboratory conditions, female *P. hypsinotus* developed ovaries after releasing the hatched larvae and spawned in June–July of the next year as reported previously (Murakami 1992). On the other hand, under natural conditions, the rematuration cycle of the ovary has not been reported in this species. In the middle Japan Sea, another *Pandalus* species, the northern shrimp, *Pandalus eous* shows a two-year spawning cycle under natural conditions (Sadakata 2000) similar to *P. hypsinotus* under laboratory conditions.

In the present study, we examined details of ovarian development histologically and hemolymph vitellogenin levels during the ovarian development using a newly developed vitellogenin TR-FIA, and found that high vitellogenin

levels are related to ovarian development. The obtained results show that process of vitellogenesis during the female phase of *P. hypsinotus*, a protandric hermaphrodite shrimp, is similar to other crustacean species that do not change sex. The TR-FIA exhibited high sensitivity, low variability, and wide assay range. This assay is a useful method to determine hemolymph vitellogenin levels.

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