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Quantitative changes in yolk protein and other components in the ovary and testis of the sea urchin *Pseudocentrotus depressus*

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Summary

Both male and female sea urchins accumulate the major yolk protein (MYP; the most abundant yolk granule protein in sea urchin eggs) in the nutritive phagocytes of immature gonads before gametogenesis. In this study, quantitative changes in MYP as well as in other biochemical components in the ovary and testis were examined in the course of gametogenesis in *Pseudocentrotus depressus*. Before gametogenesis, both the ovary and testis contained large quantities of proteins, lipids and polysaccharides. MYP reached about 80% of total protein in both sexes. In the testis, MYP decreased rapidly as spermatogenesis proceeded, and the fully mature testis contained little MYP; the levels of lipids and polysaccharides also decreased. In contrast, the levels of nucleic acids and proteins other than MYP increased markedly. In the ovary, MYP decreased gradually as oogenesis proceeded, and the fully mature ovary contained less than half of the initial amount of MYP. Polysaccharides also decreased, whereas proteins other than MYP increased. These results, taken together with those from other studies, suggest that MYP serves as a protein reserve that accumulates before gametogenesis and is used as material for synthesizing new substances constituting gametes in both male and female sea urchins.

Key words: sea urchin, *Pseudocentrotus depressus*, yolk protein, ovary, testis, gametogenesis, nutritive phagocyte, protein reserve, nutrition.

Introduction

Gonadal growth and maturation of sea urchins are characterized by intra-gonadal nutrient storage and its use for gametogenesis (Walker et al., 2001). Sea urchin gonads contain two main types of cells: germ cells and nutritive phagocytes, somatic cells common in both sexes that store nutrients necessary for gametogenesis (Walker, 1982). Before the initiation of gametogenesis, the gonads increase in size by accumulating nutrients such as proteins, lipids and carbohydrates into the nutritive phagocytes that fill the gonadal lumina in both sexes. After gametogenesis begins, nutritive phagocytes gradually decrease in size, supplying nutrients to the developing germ cells, and finally the lumina are filled with ova and sperm (Walker et al., 2001).

The major protein contained in immature gonads is a glycoprotein of molecular mass of 170 kDa (Unuma et al., 1998), which was originally identified as the most abundant component of yolk granules in sea urchin eggs and termed the major yolk protein or major yolk glycoprotein (MYP; Harrington and Easton, 1982; Kari and Rottmann, 1985; Yokota and Kato, 1988; Scott and Lennarz, 1989). Unlike other oviparous animals, where the yolk protein is female-specific, both male and female sea urchins synthesize MYP (Shyu et al., 1986; Unuma et al., 2001) and store it in nutritive phagocytes before gametogenesis (Ozaki et al., 1986; Unuma et al., 1998). As oogenesis proceeds, MYP is transferred from nutritive phagocytes to the ripe ova to form yolk granules (Ozaki et al., 1986). As spermatogenesis proceeds, most of the MYP disappears from the testis (Unuma et al., 1998). Considering the consensus among researchers that nutritive phagocytes are the main storage site of nutrients for spermatogenesis as well as for oogenesis (Walker, 1982), it has been postulated that male MYP serves as a nutrient source for spermatogenesis (Unuma et al., 1998). The nutritional role of MYP in sea urchin gametogenesis is, however, still not evident because MYP and other biochemical components in the gonads have so far not been quantitatively analyzed.

In the present study, we examined the changes in MYP content and the biochemical composition of the gonads in the course of gametogenesis in *Pseudocentrotus depressus*, in order to clarify the definite role of MYP.

Materials and methods

Animals

6-month-old juvenile *Pseudocentrotus depressus* A. Agassiz 1863, hatched and reared at the Fukuoka Prefectural Fish

Farming Center, were transferred to the Coastal Station of the National Research Institute of Aquaculture, raised in 1000 liter tanks, and reared on kelp, *Eisenia bicyclis*. About 2 years later, 10–20 individuals (59.6±4.1 mm test diameter and 74.0±13.8 g wet body mass, mean ± S.D.) were randomly collected twice a month from September to January and used for analyses.

The gonads were excised and weighed; the gonad index was then calculated for each animal as follows:

$$\text{Gonad index} = 100 \times \text{wet gonad mass} / \text{wet body mass}\,.$$

A small portion of the gonad was fixed in Bouin's solution for histological observation, and the remainder was stored at –80°C for biochemical analysis. Paraffin sections 6 µm thick were prepared and stained with Hematoxylin and Eosin. The gonadal maturity of each animal was classified into the six stages of Fuji (1960) with some slight modifications as described (Unuma et al., 1996). Gonads at stages 1–4 only were used for biochemical analysis. 3–6 individuals of each sex and at each stage were analyzed. The representative morphology of the analyzed gonads is shown in Fig. 1. The eggs were obtained from gravid females by a coelomic injection of 20% KCl and stored at –80°C.

Purification of standard MYP

MYP used as a standard protein for enzyme-linked immunosolvent assay (ELISA) was purified from immature testis (stage 1), using the method of MYP purification from eggs (Unuma et al., 1998) with slight modifications. Testes at stage 1 (1 g) were homogenized with 15 ml of 10 mmol l^{-1} Tris-HCl buffer, pH 8.0, containing 10 mmol l^{-1} NaCl (TBS) in a Polytron (Kinematica, Switzerland). The homogenate was centrifuged at 25 000 $\boldsymbol{g}$ for 20 min at 4°C, and the supernatant was applied to a HiLoad 16/10 Q Sepharose Fast Flow column (Amersham Pharmacia LKB Biotechnology, Sweden) equilibrated with TBS. The retained proteins were eluted with a 10 mmol l^{-1} to 1 mol l^{-1} linear NaCl gradient (300 ml in total). A protein peak eluted at 280 mmol l^{-1} NaCl was collected and

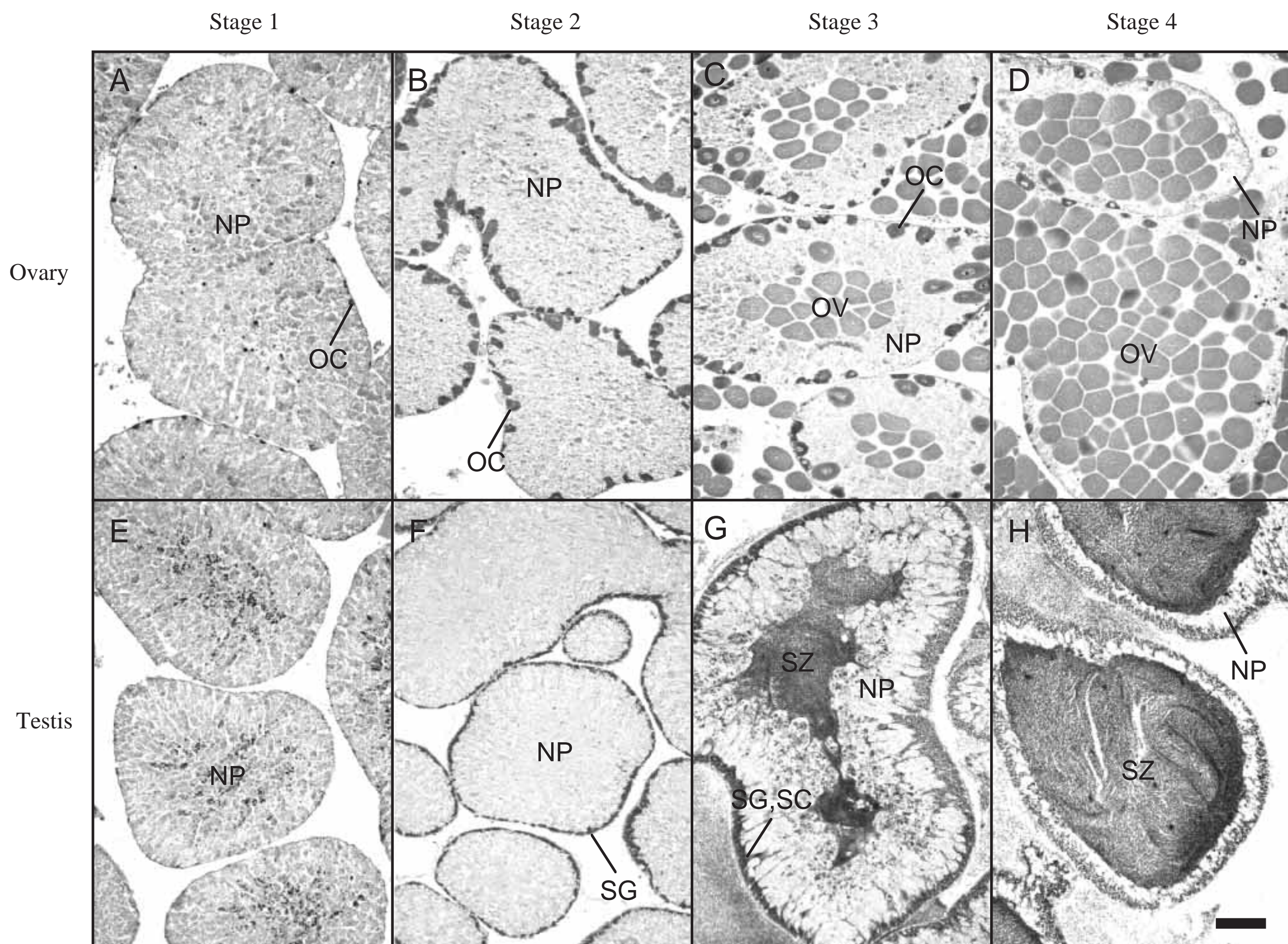

Fig. 1. Representative morphology in the ovary (A–D) and testis (E–H) of *P. depressus* at different stages. Paraffin-embedded sections were stained with Hematoxylin and Eosin. At stage 1 (A,E), the gonadal lumina are filled with nutritive phagocytes. At stage 2 (B,F), many developing oocytes or clusters of spermatogonia are present at the periphery. At stage 3 (C,G), nutritive phagocytes are replaced with ripe ova or spermatozoa in the center of the lumina. At stage 4 (D,H; fully mature gonad), the lumina are filled with ripe ova or spermatozoa. NP, nutritive phagocyte; OC, oocyte; OV, ripe ovum; SG, spermatogonium; SC, spermatocyte; SZ, spermatozoon. Scale bar, 100 µm.

applied to a HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated with 10 mmol l^{-1} Tris-HCl buffer, pH 8.0, containing 150 mmol l^{-1} NaCl. The peak of MYP at 50 ml elution volume was collected. The concentration of this MYP standard was determined by the Bradford method (Bradford, 1976) using the Bio-rad protein assay (Bio-rad, USA) with gamma globulin as a standard.

ELISA procedures

The polyvalent antiserum against MYP (anti-MYP) was raised by immunizing a rabbit as described (Unuma et al., 1998). The IgG was purified from the antiserum using a protein G column (MAbTrap kit; Pharmacia). Most of the purified IgG was dialyzed against 10 mmol l^{-1} phosphate buffer, pH 7.4, containing 150 mmol l^{-1} NaCl (PBS) for coating the microtiter plates. The rest of the purified IgG was dialyzed against 100 mmol l^{-1} $NaHCO_3$ for biotinylation. The dialysate was mixed with the biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, USA), incubated on ice for 8 h, and dialyzed against PBS containing 0.1% NaN_3.

Sandwich ELISA was carried out according to the method of quantitating eel vitellogenin (Okumura et al., 1995) with some modifications as follows.

Antibody coating: a quantity of 100 µl IgG solution (40 µg IgG ml^{-1} PBS) was dispensed into each well and incubated for 24 h at 4°C.

Blocking: a quantity of 250 µl blocking buffer (PBS containing 3% skimmed milk, 1% rabbit normal serum and 0.1% NaN_3) was dispensed into each well and incubated for 8 h at 4°C.

Sample and standard incubation: the gonads were homogenized with 20× 10 mmol l^{-1} Tris-HCl buffer, pH 7.4, containing 2% NaCl, in a Polytron. The homogenate was centrifuged at 25 000 ***g*** for 20 min at 4°C, and the supernatant was used as the gonadal extract. The gonadal extract or standard MYP (100 µl) diluted with the dilution buffer (PBS containing 0.1% BSA, 0.1% polyoxyethylene sorbitan monolaurate and 0.002% thimerosal) was dispensed into each well and incubated for 16 h at 4°C.

Incubation with biotinylated IgG: biotinylated IgG (100 µl) diluted at 1:1000 with the dilution buffer was dispensed into each well and incubated for 8 h at 4°C.

Incubation with streptavidin: streptavidin–peroxidase conjugate (100 µl; Nichirei, Japan) diluted at 1:1000 with the dilution buffer was dispensed into each well and incubated for 1 h at 23°C.

Enzymatic color reaction: TMB One-step Substrate System (100 µl; DAKO, USA) was dispensed into each well and incubated for 30 min at 23°C. The absorbance at 450 nm was measured with a microplate reader (Bio-rad 2550, USA). All standard and sample measurements were made in duplicate.

Validation of assay: the absorbance curves of the assays of the serial dilutions of purified MYP and of the gonadal extract are shown in Fig. 2. A steep standard curve covering 0.05–3.0 µg ml^{-1}, parallel to the curve for the gonadal extract, was obtained. The intra- and inter-assay coefficients of variation were 8.6% (*N*=10) and 7.5% (*N*=3), respectively.

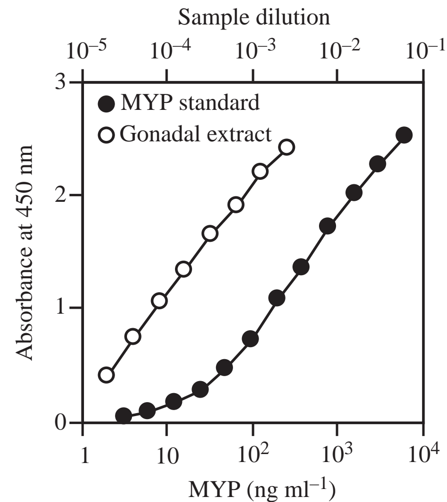

Fig. 2. Standard curves of sandwich ELISA for serial twofold dilution of the purified major yolk protein (MYP) and the gonadal extract in *P. depressus*.

Biochemical composition

The water content was measured by drying the sample at 110°C for 8 h and the lipid content determined by the gravimetric method using the Soxhlet device with diethyl ether as a solvent (Yamamoto et al., 2000).

To measure the protein content, 0.2 g of the gonad was homogenized with 8 ml of 10% trichloroacetic acid (TCA) and centrifuged at 3000 ***g*** for 10 min. To remove lipids and nucleic acids, the precipitate was incubated with 5 ml ethanol-diethylether (1:1, v/v) at 50°C for 15 min, and then with 5 ml of 10% TCA at 100°C for 15 min. After centrifugation at 3000 ***g*** for 10 min, the precipitate was dissolved in 4 ml of 1 mol l^{-1} NaOH. Protein in the solution was measured by the Biuret method (Gornall et al., 1949), with bovine serum albumin as a standard.

The polysaccharide content was quantitated as follows. To solubilize the tissue, 1 ml of 30% KOH was added to 0.3 g of the gonad and incubated at 100°C for 20 min. Ethanol (1.25 ml) was added and the solution was centrifuged at 3000 ***g*** for 10 min. The precipitate was dissolved in 100 ml distilled water and neutralized with HCl. Polysaccharide in the solution was measured according to the anthrone–sulfuric acid method (Koehler, 1952), with glucose as a standard.

The nucleic acid content was measured by the Schmidt–Thannhauser–Schneider method (Schneider, 1957) with some modifications (Nakano, 1988). The gonad (0.5 g) was homogenized with 14 ml of 5% perchloric acid and centrifuged at 3000 ***g*** for 10 min. To remove lipids, the precipitate was mixed with 10 ml ethanol-diethylether (1:1, v/v) and incubated at 50°C for 15 min. After centrifugation at 3000 ***g*** for 10 min, the precipitate was dissolved in 10 ml of

0.3 mol l^{-1} KOH, incubated at 37°C for 18 h, and then neutralized with HCl. Perchloric acid (60%; 1 ml) was added to the solution and centrifuged at 3000 g for 10 min. The supernatant was obtained as RNA solution. The precipitate was mixed with 10 ml of 5% perchloric acid and incubated at 100°C for 15 min. After centrifugation at 3000 g for 10 min, the supernatant was obtained as DNA solution. The absorbance at 260 nm of DNA and RNA solutions was then measured.

SDS-PAGE and western blotting

Gonads or eggs were homogenized with 20× 125 mmol l^{-1} Tris-HCl buffer, pH 6.8, containing 2% sodium dodecylsulphate (SDS) and 5% 2-mercaptoethanol. The homogenate was used as a sample for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To measure the protein concentration in the sample, a portion of the homogenate was mixed with TCA, and the precipitated protein quantitated by the method described above. SDS-PAGE was performed using a 5–20% gradient gel, according to Laemmli (1970). The samples containing 10 μg of total protein were applied to each lane. Protein bands were visualized with Coomassie Brilliant Blue R-250.

The separated proteins were subsequently subjected to western blotting with anti-MYP as described (Unuma et al., 1998).

Results

Gonadal maturity and gonad indices

Gonads were observed at stage 1 in all the animals in early September, at stage 2 in 60% of the animals in early October, and at stage 4 in 90% of the animals in late November.

Changes in gonad indices from stages 1 to 4 are shown in Fig. 3. At stage 1, the indices were 9.6% in females and 11.3% in males. They increased gradually and reached the highest value of 13.8% at stage 4 in females and 12.5% at stage 3 in males.

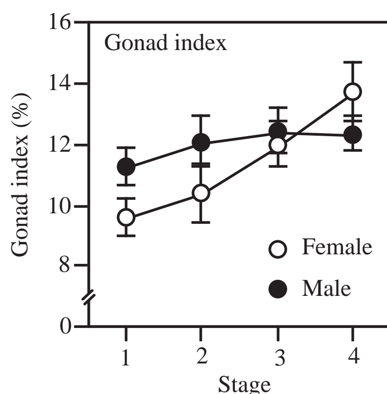

Fig. 3. Changes in the gonad indices in male and female *P. depressus* at different stages during gametogenesis. Values are means ± S.E.M. obtained from 6 to 36 individuals.

MYP in gonads

Changes in the content of MYP in the gonads are shown in Fig. 4A. At stage 1, the ovary and the testis contained 85.4 and 87.3 mg g^{-1} wet mass of MYP, respectively. In the testis, MYP content decreased rapidly as spermatogenesis proceeded and reached only 3.3 mg g^{-1} wet mass at stage 4. In the ovary, it also decreased during oogenesis although not as rapidly as in males. At stage 4, the ovary contained 28.4 mg g^{-1} wet mass of MYP, one-third of the initial value.

Biochemical composition

Quantitative changes in the content of water, protein, lipid, polysaccharide and nucleic acids from stages 1–4 are shown in Fig. 4B–G.

In the ovary and the testis, the content of water increased gradually from 61.9% to 72.6% and from 61.4% to 75.6%, respectively. In contrast, the content of protein, lipid and polysaccharide decreased with the progress of gametogenesis: proteins, from 106.6 to 70.2 mg g^{-1} wet mass (ovary) and from 114.9 to 62.4 mg g^{-1} wet mass (testis); lipids, from 94.9 to 72.3 mg g^{-1} wet mass (ovary) and from 91.0 to 67.0 mg g^{-1} wet mass (testis); polysaccharides, from 77.8 to 29.0 mg g^{-1} wet mass (ovary) and from 71.7 at stage 1 and 73.8 at stage 2 to 30.4 mg g^{-1} wet mass (testis). Nucleic acids (DNA+RNA) increased from 2.5 to 6.4 mg g^{-1} wet mass (ovary) and from 1.6 to 25.4 mg g^{-1} wet mass (testis). In the ovary, most of the nucleic acids was RNA. In the testis, the content of RNA was larger than that of DNA at stages 1 and 2, and then DNA increased rapidly while RNA decreased gradually.

To understand the quantitative change of each component in the gonads during gametogenesis, changes in the size of gonads need to be considered. Fig. 5 shows changes in the biochemical composition of ovaries and testes, standardized to g 100 g^{-1} body mass by multiplying the level of each component in Fig. 4 by the gonad indices in Fig. 3. Before the initiation of gametogenesis (stage 1), MYP in the ovary and testis was 80.1% and 76.0% of the total protein, respectively. As spermatogenesis proceeded, the amount of MYP rapidly decreased, as did the amount of polysaccharide. In contrast, the amounts of nucleic acids and proteins other than MYP increased markedly. In the ovary, the MYP amount did not decrease as drastically as it did in the testis. In the fully mature ovary (stage 4), however, the MYP amount was less than half (47%) of the initial value, and the amount of polysaccharide also decreased, whereas that of proteins other than MYP increased.

Protein composition

Total protein was extracted from the gonad with buffer containing SDS and 2-mercaptoethanol and then subjected to SDS-PAGE and western blotting (Fig. 6). In the immature ovary and testis (stage 1), MYP was obviously predominant. As spermatogenesis proceeded, the level of MYP decreased drastically and that of other proteins of lower molecular mass, which were not reactive with the anti-MYP, increased. A

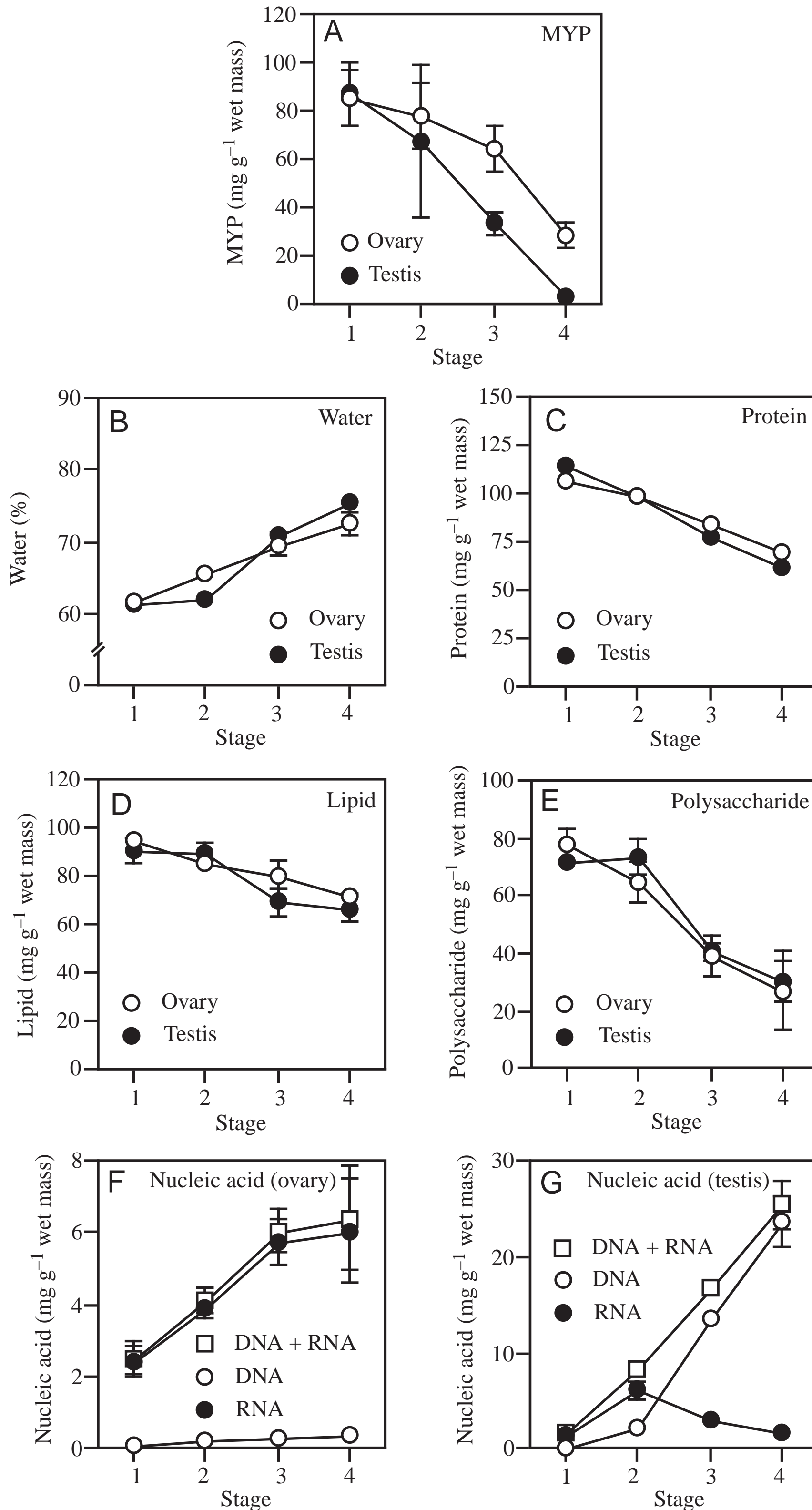

Fig. 4. Quantitative changes in the content of major yolk protein (MYP) (A), water (B), protein (C), lipid (D), polysaccharide (E) and nucleic acids in ovary (F) and testis (G) in the gonad of *P. depressus* at different stages during gametogenesis. Values are means ± S.E.M. obtained from 3–6 individuals, except for the testes at stage 2 for protein (2 individuals). In some graphs, the S.E.M. values are smaller than the size of the symbols.

decrease in the level of MYP was also observed in the ovaries, whereas the level of other proteins with lower molecular mass, which were not reactive with the anti-MYP, increased. The level of MYP was much lower in both the fully mature ovary and the egg than in the immature ovary.

Discussion

In *P. depressus*, most of the MYP stored in the immature testis is lost in the course of spermatogenesis (Unuma et al., 1998). Moreover, this study revealed that a considerable amount of MYP stored in the immature ovary was also lost during oogenesis.

Both the immature ovary and testis (stage 1) contained a similarly abundant amount of MYP (about 9% of wet gonad mass), constituting about 80% of total protein. As gametogenesis proceeded, however, the MYP content decreased rapidly in males and gradually in females. In the fully mature gonad (stage 4), the amount of MYP was very low in males and less than half of the initial amount in females. In a previous study (Unuma et al., 1998), a decrease in MYP during spermatogenesis was demonstrated by SDS-PAGE and western blotting without revealing quantitative changes in insoluble proteins such as histones, because the buffer used for extraction (Tris-HCl without detergent) could not solubilize such proteins. In the present study total protein, including insoluble protein, was efficiently extracted from the gonad with buffer containing SDS and 2-mercaptoethanol and then analyzed by SDS-PAGE and western blotting (Fig. 6). Levels of proteins that did not react with anti-MYP increased in both sexes in the course of gametogenesis. These newly emerging proteins are assumed to include various proteins that are synthesized as gametogenesis proceeds. A protein with a molecular mass of 30 kDa, abundant in the fully mature ovary (stage 4) and in the egg, is probably YP30, which accumulates specifically in yolk granules in the egg (Wessel et al., 2000). Some of the clear bands observed in the fully mature testis (stage 4) are probably histones, which constitute chromatin in sperm (e.g. Puigdomenech et al., 1987; Poccia et al., 1989). The nucleic acid content also increased as gametogenesis proceeded in both sexes, although their

increase in females was not so large as in males. Most of the nucleic acids in the fully mature gonad was DNA in males and RNA in females, consistent with the well-known facts that the major content of sperm is genomic DNA and that eggs accumulate abundant amounts of RNA required for immediate protein synthesis after fertilization. The immature ovary and testis also contained a large quantity of polysaccharides, most of which is probably glycogen in the form of granules (Verhey and Moyer, 1967; Marsh and Watts, 2001). The polysaccharide content decreased as gametogenesis proceeded in both sexes, possibly having been consumed as an energy source, as suggested for *Strongylocentrotus intermedius* (Marsh and Watts, 2001). Compared with other components, the lipid content was rather stable throughout this period, although it would tend to be underestimated to some extent because of the inefficiency of the diethyl ether used in the Soxhlet method to extract the phospholipids that constitute the biomembrane or the lipid bodies of *P. depressus* spermatozoa (Mita and Nakamura, 1993).

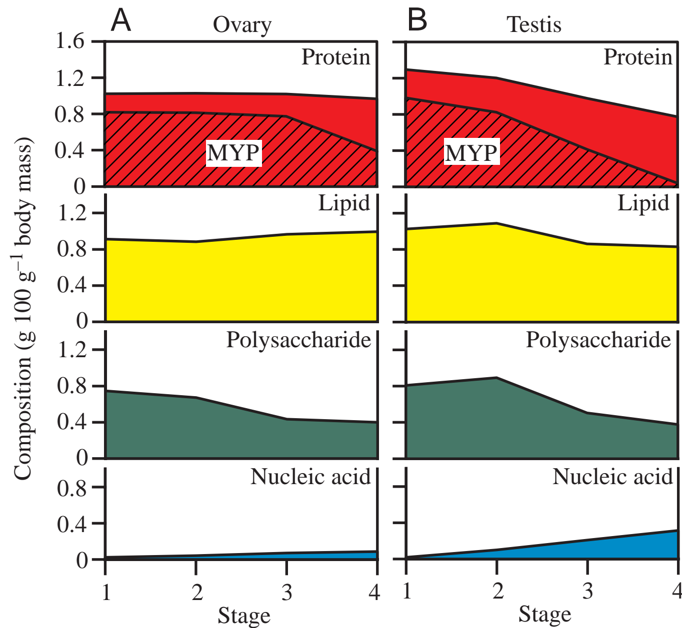

Fig. 5. Changes in the biochemical composition of the gonad of *P. depressus* during gametogenesis. To standardize the change in the gonadal size, the level of each component shown in Fig. 4 was multiplied by the average gonad index at each stage shown in Fig. 3. Thus the values represent the amount of each component of the gonad in animals at a body mass of 100 g. (A) Ovary, (B) testis. Protein includes major yolk protein (MYP), denoted by diagonal stripes, and other proteins.

In animals that produce enormous numbers of eggs and sperm, gametogenesis is a process requiring abundant amounts of nutrients such as proteins, lipids and carbohydrates (including polysaccharides). Protein is essential for supplying amino acids, which are utilized to synthesize new proteins and other nitrogen-containing substances including nucleotides and nucleic acids (for the biosynthetic pathway of nucleotides, see Lehninger et al., 1993). To meet the enormous demand for protein during gametogenesis, some oviparous animals store protein reserves in some organs of their body prior to gametogenesis. In scallops, proteins are stored in the adductor muscle and then mobilized into the gonad for gametogenesis (Mathieu and Lubet, 1993). In salmonids, proteins stored in the white muscle are mobilized into the gonad to produce gametes (Mommsen et al., 1980; Martin et al., 1993). Muscle protein can be considered to serve as a protein reserve for gametogenesis in these animals. Sea urchins do not have an extra-gonadal site for nutrient storage that is obviously involved in supplying nutrients to germ cells, although the gut can act as an immediate storage site for excess nutrients (Lawrence et al., 1966; Walker, 1982). Considering these reports and the data presented above, we assume that MYP stored in nutritive phagocytes is a protein reserve in both male and female *P. depressus*, and is probably utilized mainly as material for synthesizing new substances contained in eggs and sperm. We also observed an accumulation of MYP in the immature ovary and testis and its decrease during gametogenesis in *Anthocidaris crassispina* and in *Scaphecinus mirabilis*, by SDS-PAGE and western blotting (T. Unuma, T. Yamamoto, T. Akiyama, M. Shiraishi and H. Ohta, unpublished data). Intra-gonadal

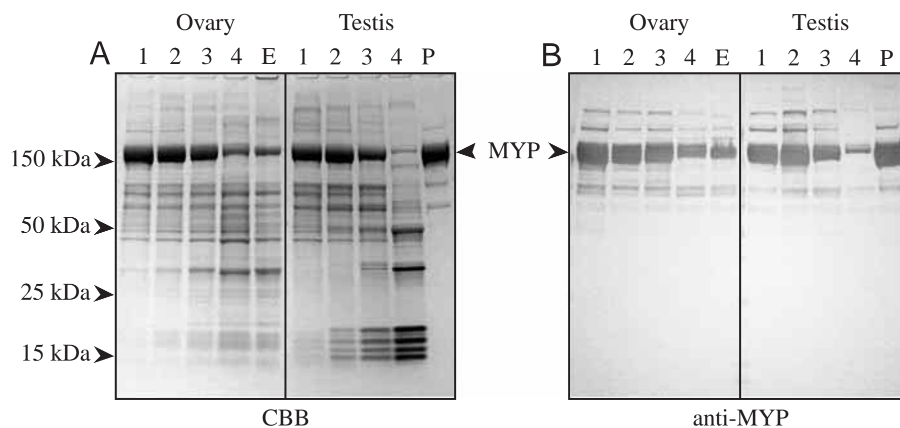

Fig. 6. Gonadal homogenates, egg homogenate (E), and the purified major yolk protein (P) in *P. depressus*, subjected to (A) SDS-polyacrylamide gradient (5–20%) gel electrophoresis, stained with Coomassie Brilliant Blue (CBB), and (B) western blot analysis, immunostained with anti-MYP. Samples containing 10 µg of protein were applied to each lane. Numbers indicate the gonad stages. Molecular mass values on the left are indicated by the migration positions of marker proteins. In the western blots, higher molecular mass bands above the main bands are probably aggregations of MYP. Lower molecular mass bands below the main bands are cleavage products of MYP (e.g. Scott and Lennarz, 1989).

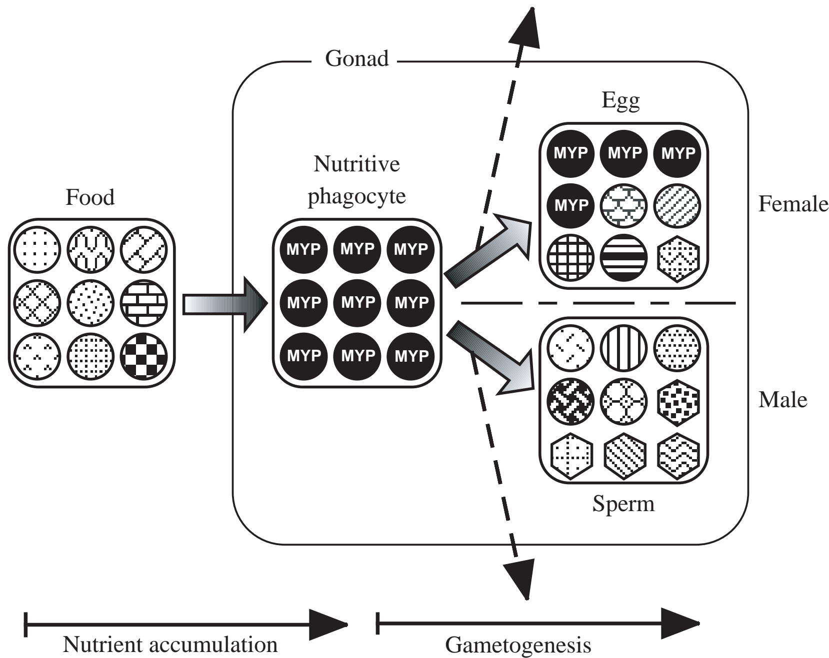

Fig. 7. Hypothetical diagram of the nutritional role of major yolk protein (MYP) in sea urchin reproduction. Circles, proteins; hexagons, other molecules such as nucleic acids, lower molecular mass substances, etc. Broken lines with arrows indicate the loss caused by metabolism as an energy source.

storage of MYP and its subsequent utilization for gametogenesis in both sexes may be a common phenomenon in sea urchins.

Based on this premise, we propose our hypothesis on the nutritional role of MYP in sea urchin reproduction (Fig. 7). Food for sea urchins contains various proteins. Both male and female sea urchins ingest these proteins and convert some of them to MYP, which accumulates in nutritive phagocytes. Synthesis of MYP occurs mainly within the nutritive phagocytes themselves (Unuma et al., 2001). As gametogenesis proceeds, the MYP stored in both ovarian and testicular nutritive phagocytes is utilized as material for synthesizing new substances (proteins, nucleic acids, etc) that constitute eggs and sperm. Some of the stored MYP may be metabolized as an energy source during this period. MYP synthesis probably continues even in the course of gametogenesis, since weak expression of MYP mRNA has been detected in the mature gonads (Shyu et al., 1986). Nevertheless, MYP synthesis would be considerably less than its consumption during gametogenesis. In males, most of the stored MYP is consumed by the end of spermatogenesis. In females, on the other hand, a portion of the stored MYP is transported to the ova and finally forms yolk granules (Ozaki et al., 1986). The MYP in the yolk granules degrades after fertilization and serves as a nutrient source, possibly for the larval stage (Scott et al., 1990), and as a cell adhesion molecule (Noll et al., 1985; Cervello and Matranga, 1989).

In terms of its role as a protein reserve, MYP in sea urchins may be equivalent to the muscle protein in salmonids and scallops. One unique feature in sea urchins, however, is that MYP has two different working stages as a nutrient source: for gametogenesis before spawning and for larval development after fertilization, the latter being the classic role of a yolk protein.

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