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| Citation         | 北海道大学水産科学研究彙報, 69(1), 29-36   |
| Issue Date       | 2019-08-07  |
| DOI              | 10.14943/bull.fish.69.1.29  |
| Doc URL          | <a href="http://hdl.handle.net/2115/75196">http://hdl.handle.net/2115/75196</a>   |
| Type             | bulletin (article)  |
| File Information | bull.fish.69.1.29.pdf   |



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## Quantitative Changes of Major Yolk Protein in the Coelomic Fluid and Gonads of the Sea Urchin, *Mesocentrotus nudus*, during the Reproductive Cycle

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(Received 4 April 2019, Accepted 9 May 2019)

### Abstract

Both female and male sea urchins accumulate the major yolk protein (MYP) in the nutritive phagocytes of immature gonads before gametogenesis. Additionally, the most abundant protein in the coelomic fluids of both sexes is MYP. In females, MYP in the coelomic fluid is taken up by the nutritive phagocytes and then transported into growing oocytes. In this study, quantitative changes of MYP in the coelomic fluid of both sexes were examined during the reproductive cycle of the wild sea urchin, *Mesocentrotus nudus*. In females but not males, positive correlation between MYP level and the gonadosomatic index was observed. These results indicate that MYP in the coelomic fluid is suitable as a biomarker of the onset of puberty and progression of maturity in female sea urchins.

**Key words** : Sea urchin, *Mesocentrotus nudus*, Major yolk protein, Coelomic fluid, Gonad, Reproductive cycle

### Introduction

In the sea urchin, the major yolk protein (MYP) is the most abundant yolk protein and is stored in yolk granules of eggs as a nutrient source during early development (Ozaki, 1980 ; Harrington and Easton, 1982 ; Kari and Rottmann, 1985), as are the yolk proteins provided by vitellogenin in other species of oviparous animals (Sullivan et al., 2003 ; Hiramatsu et al., 2005). It was reported that MYP is stored in the nutritive phagocytes, which are somatic cells, in the gonads of both sexes of sea urchins (Unuma et al., 1998). In males, the stored MYP is used as energy for spermatogenesis during gametogenesis. In females, some of the MYP is degraded and used as a nutrient source, and some is transported to the oocytes and actively packed into yolk granules through endocytosis via a dynamin-dependent mechanism (Unuma et al., 2003 ; Brooks and Wessel, 2004 ; Unuma et al., 2010).

It is well known that MYP mRNA is ubiquitously expressed in the digestive tract, coelomocytes, and gonads (Unuma et al., 2001), and major MYP production sites include the digestive tract and gonads of both sexes in adult sea urchins (Unuma et al., 2010). In the gonads of both sexes, MYP mRNA level increases during gonadal growth, and that level decreases as gametogenesis proceeds in the wild sea urchin, *Mesocentrotus nudus* (Ura et al., 2017).

Although MYP is abundant in the coelomic fluid of male and female sea urchins and is thought to be secreted from the digestive tract, there is no histological evidence of this. However, Unuma et al. (2007) thoroughly examined MYP by *in vivo* experiment ; MYP in the coelomic fluid is taken up by the gonadal nutritive phagocytes in the gonads and is then transported into the growing oocytes of female sea urchins. To avoid confusion, we refer to the MYP in coelomic fluid as CF-MYP, as reported by Unuma et al. (2007). Additionally, we refer to the MYP that is synthesized in the gonads of both sexes as NP-MYP, and we use the term MYP in a broad sense when type is not specified.

It is generally accepted that fish vitellogenin, which is a female-specific protein and MYP precursor, is synthesized in the liver, and vitellogenin is transported into the growing oocytes from the blood. Therefore, vitellogenin is used as a biomarker of the onset of puberty and progression of maturity in females (Hiramatsu et al., 2005 ; Hara et al., 2016). However, we are not aware of any report on the profile of CF-MYP during the reproductive cycle using an assay system in adult sea urchins. Therefore, although CF-MYP is transported into the nutritive phagocytes of gonads and growing oocytes from the coelomic fluid, it is still unclear if CF-MYP is suitable as a biomarker of the progression of maturity in sea urchins. Thus, in this study, we developed an MYP assay

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system and examined changes in MYP levels in the coelomic fluids and gonads, and changes in levels of total protein in the coelomic fluids of both sexes during the reproductive cycle in wild sea urchins (*Mesocentrotus nudus*) collected from southern Hokkaido, Japan.

## Material and Methods

### *Animals and sampling*

*Mesocentrotus nudus* sea urchins were collected by diving at Usujiri in Hokkaido from May 2015 to October 2016 (Ura et al., 2017). Sea urchins were transported to and dissected at the Faculty of Fisheries Sciences of Hokkaido University, where the gonads were excised and weighed. The gonadosomatic index (GSI) was calculated for each animal as follows:  $GSI (\%) = 100 \times \text{wet weight of gonad} / \text{total wet body weight}$ . A small portion of each gonad sampled was fixed in Bouin's solution for histological examination of reproductive stage, and the remainder was stored at  $-30^{\circ}\text{C}$  until analysis.

After removing the peristomial membrane, coelomic fluid was collected into anticoagulant solution (20 mM Tris-HCl, 500 mM NaCl, 30 mM EDTA, pH 7.4) with equal volume, and centrifuged at 900 *g* for 10 min at  $4^{\circ}\text{C}$  to collect the coelomic fluid by the method of Schillaci et al. (2013). The coelomic fluids were stored  $-30^{\circ}\text{C}$  until analysis.

### *Histology of gonads*

The tissue samples were dehydrated through a graded ethanol series and embedded in paraffin; 6- $\mu\text{m}$ -thick serial sections were mounted on glass slides and stained with hematoxylin and eosin. Gonadal maturity of each animal was classified into the five stages according to Unuma et al. (1996) as follows: stage 1 (recovering), stage 2 (growth), stage 3 (pre-mature), stage 4 (mature), and stage 5 (spent). The gonads of 15–30 individuals were collected and analyzed each month.

### *Purification of NP-MYP*

The immature male gonads were homogenized with five volumes of 10 mM Tris-HCl (pH 8.0) containing 10 mM NaCl, 1 mM EDTA, and 0.1%  $\text{NaN}_3$  using a Teflon homogenizer. The homogenate was centrifuged at 17,000 *g* for 20 min at  $4^{\circ}\text{C}$ , and supernatant was collected. Ion-exchange chromatography was performed on the gonad extract using a  $2.5 \times 8$  cm column of DEAE cellulose (TOYOPEAL DEAE) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The retained proteins were eluted in step-wise fashion using the same buffer at various molar concentrations of NaCl (0, 100, 200, and 300 mM) at  $4^{\circ}\text{C}$ . Fractions (100 mM NaCl) rich in the targeted protein were pooled and dialyzed against 20 mM KP at  $4^{\circ}\text{C}$  overnight. The dialyzed sample was fractionated by hydroxylapatite

chromatography, and MYP was eluted at 400 mM KP. This fraction was concentrated by ultrafiltration to 2 ml and fractionated twice by a Superose 6 10/300GL gel filtration column (GE Healthcare Life Sciences, Little Chalfont, UK) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl. The single protein peak was the target MYP. At each purification step, electrophoresis and immunological procedures were used to ensure that the target protein was present. Purified MYP concentration was measured as described by Lowry et al. (1951), with bovine serum albumin (BSA; Bio-Rad Laboratories, Inc., Hercules, CA, USA) as the standard.

### *Preparation of antisera*

A polyvalent antiserum against gonad extract proteins (anti-Gonad) was raised in rabbits. The specific antiserum to purified NP-MYP (anti-MYP) was obtained from a rabbit immunized with 1 ml of solution containing 300  $\mu\text{g}$  of purified NP-MYP mixed with an equal volume of Freund's complete adjuvant (Wako Pure Chemical Industries, Osaka, Japan). The rabbit received four such immunizations at about 7 day intervals. After four injections, blood was collected from the ear vein of the rabbit. The blood was allowed to clot for about 1 h at room temperature and was then stored overnight at  $4^{\circ}\text{C}$ . The blood was centrifuged at 17,000 *g* for 15 min at  $4^{\circ}\text{C}$ , and the supernatant was collected as antiserum.

### *Electrophoresis and immunological procedure*

Disc-polyacrylamide gel electrophoresis (disc-PAGE) was carried out in 5% polyacrylamide gel using a Tris-glycine buffer system, as described by Davis (1964). The gel was stained with 1% Amido black 10B in 7% acetic acid and destained with 7% acetic acid. Immunoelectrophoretic analysis was performed with 1% agarose in 190 mM Tris-HCl buffer (pH 8.6). The immunoprecipitates were stained with 1% Amido black 10B in 7% acetic acid and destained with 7% acetic acid in the dried gel. Double immunodiffusion using anti-MYP was performed in 1% agarose gel by the method of Ouchterlony (1953).

### *Assay of MYP*

Single radial immunodiffusion (SRID) was carried out according to the procedure of Mancini et al. (1965). Antiserum to purified MYP was diluted at  $56^{\circ}\text{C}$  in a solution of 1% (w/v) agarose (Nacalai, HGT) in 190 mM Tris-HCl buffer (pH 8.6). Fifteen milliliters of the hot solution was then layered onto a  $10 \times 10$  cm GelBond film (GE Healthcare Life Sciences, Little Chalfont, UK). The SRID plate was incubated in moist chamber at room temperature for 2 days. After incubation, it was washed with 0.9% NaCl, dried on filter paper, stained with 1% Amido Black 10B in 7% acetic acid, and destained with 7% acetic acid. Purified MYP (25,

50, 100, 200, and 400 µg/ml) was used as the standard for quantitative SRID in the gonads. Purified MYP (20, 30, 60, 120, and 200 µg/ml) was used as the standard for quantitative SRID in the coelomic fluid.

**Measurement of total protein concentration in the coelomic fluids**

The total protein concentrations in the coelomic fluids were measured as described by Lowry et al. (1951), with BSA (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as the standard.

**Statistical analysis**

Data are expressed as mean ± SE. Statistical differences between means across months were determined by one-way ANOVA and subsequent Tukey’s multiple-range test. Correlation was determined by Spearman’s rank correlation test.

**Results**

**GSI changes during the reproductive cycle**

GSI changes during the reproductive cycle in sea urchins are shown in Fig. 1. The mean value significantly increased from 9.67 ± 0.53% (stage 1 : recovering) to 14.28 ± 0.74% (stage 2 : growth), and then that gradually decreased in females (Fig. 1A). In males, the mean value significantly increased from 9.06 ± 0.48% (stage 1 : recovering) to 12.02 ± 0.66% (stage 3 : pre-mature), which then decreased to 7.35 ± 1.22% (stage 4 : mature) during the reproductive cycle (Fig. 1B).

**Purity of the isolated NP-MYP**

The purity of the isolated preparation was assessed by disc-PAGE and immunoelectrophoresis using a polyvalent antiserum to gonad extract and a specific antiserum to NP-MYP. One homogenous band was observed on disc-PAGE when stained with Amido black 10B (Fig. 2A). In immunoelectrophoresis, the purified NP-MYP produced a single precipitin line against the polyvalent antiserum to gonad extract proteins. Conversely, the antiserum raised against the purified NP-MYP developed only a single precipitin line with male gonad extract proteins, purified NP-MYP in sea urchin (Fig. 2B).

**Antigenic relationships between MYP in coelomic fluids and gonads**

The result of double immunodiffusion of coelomic fluids and gonad extract protein using antiserum against NP-MYP is shown in Fig. 2C. A precipitin line of coelomic fluids of both sexes appeared to generate a fuse against purified NP-MYP from male gonads. Additionally, a precipitin line of gonad extract protein of both sexes appeared to generate a fuse against purified NP-MYP.

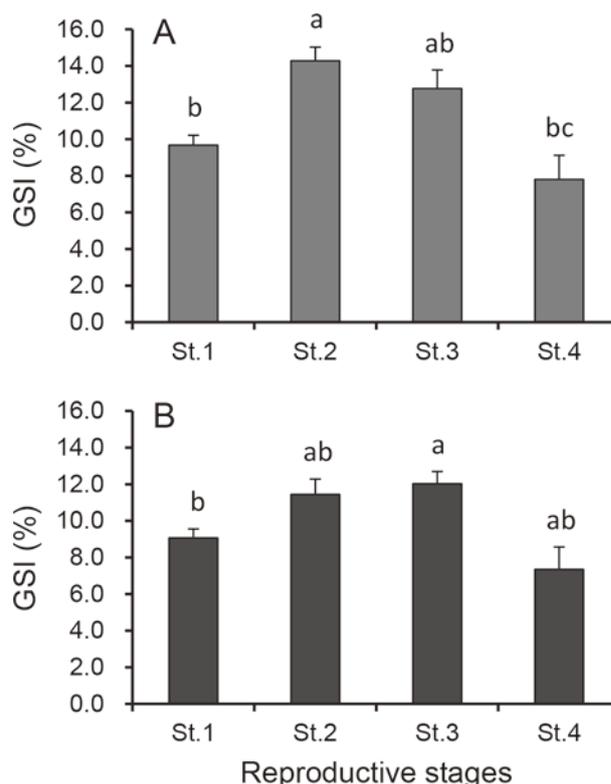


Fig. 1. Changes in the gonadosomatic index (GSI) of female (A) and male (B) sea urchins at different reproductive stages. Values are means ± SE. Different letters above the bars indicate significantly different means.

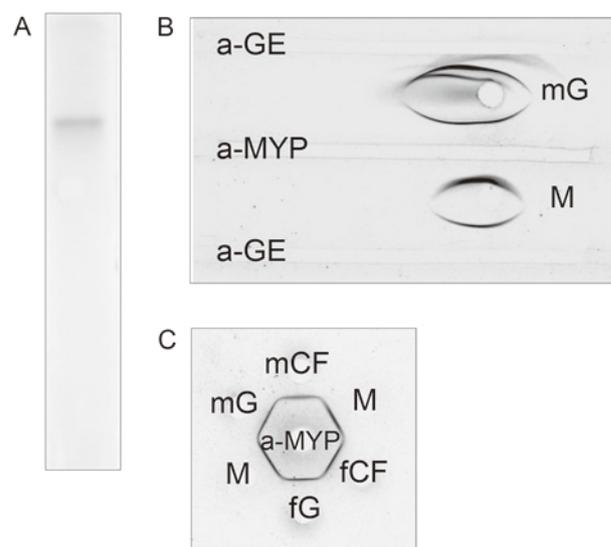


Fig. 2. Disc electrophoresis (A) and immunoelectrophoresis (B) of purified NP-MYP from immature male gonads. Precipitin reaction of MYP in double immunodiffusion (C). Disc electrophoresis was stained with Amido black 10B; a-GE : polyvalent antiserum to gonad extracts ; a-MYP : specific antiserum to purified NP-MYP ; M : purified NP-MYP ; mG : male gonad extract ; fG : female gonad extract ; mCF : male coelomic fluid ; fCF : female coelomic fluid.

### Quantitative measurement of MYP in gonads

Different dilutions of the antiserum to MYP were incorporated into the agarose gel used for SRID. A concentration of 2% antiserum produced the best quantitative results for measurement of MYP in the gonads of both sexes (Fig. 3A). Using this dilution, the squared diameter of a precipitate ring was directly proportional to the amount of the sample, in the range of 25, 50, 100, 200, and 400  $\mu\text{g/ml}$  standards, as shown in Fig. 3B. The diameter of precipitation rings produced by purified NP-MYP were directly proportional to the concentration of the MYP standard ( $R^2 = 0.9927$ ), and serial dilutions of gonad samples ran parallel to the standard curve. The interassay coefficient of variation was 2.55% ( $n = 20$ ) and the within-assay coefficient of variation was 0.87% ( $n = 4$ ). Recovery of various concentrations (25, 50, 100, 200, and 400  $\mu\text{g/ml}$ ) of purified NP-MYP standard added to gonad extract ranged from 93.8 to 98.3%.

The concentration of MYP in the gonads of both sexes was measured in *M. nudus* during the reproductive cycle (Fig. 3C, D). In females, the mean levels of MYP were  $106.5 \pm 5.2$  mg/g (dry weight) ( $n = 6$ ),  $145.1 \pm 13.1$  mg/g (dry weight) ( $n = 6$ ),  $84.6 \pm 9.9$  mg/g (dry weight) ( $n = 6$ ),  $72.0 \pm 7.7$  mg/g (dry weight) ( $n = 6$ ) for stage 1, 2, 3, and 4, respectively (Fig. 3C). In males, the mean levels of MYP were  $126.4 \pm 7.0$  mg/g (dry weight) ( $n = 5$ ),  $169.0 \pm 8.8$  mg/g (dry weight) ( $n = 6$ ),  $64.8 \pm 6.5$  mg/g (dry weight) ( $n = 6$ ),  $48.8 \pm 13.6$  mg/g

(dry weight) ( $n = 3$ ) for stages 1, 2, 3, and 4, respectively (Fig. 3D). The significant changes were observed during the reproductive cycle in female and male sea urchins. In females, the level of MYP increased from stage 1 to stage 2 and then significantly decreased at stage 4. In males, the level of MYP significantly increased from stage 1 to stage 2, and then drastically decreased during the reproductive cycle.

### Quantitative measurement of MYP in coelomic fluids

A concentration of 0.5% antiserum produced the best quantitative results for measurement of MYP in the coelomic fluids of both sexes (Fig. 4A). Using this dilution, the squared diameter of a precipitate ring was directly proportional to the amount of the sample, in the range of the 20, 30, 60, 120, and 200  $\mu\text{g/ml}$  standards, as shown in Fig. 4B. The diameter of precipitation rings produced by purified NP-MYP were directly proportional to the concentration of the MYP standard ( $R^2 = 0.993$ ) and serial dilutions of coelomic fluid samples ran parallel to the standard curve. The interassay coefficient of variation was 3.81% ( $n = 20$ ), and the within-assay coefficient of variation was 1.79% ( $n = 4$ ). Recovery of various concentrations (20, 30, 60, 120, and 200  $\mu\text{g/ml}$ ) of purified NP-MYP standard added to coelomic fluid varied from 96.9 to 99.9%.

MYP concentration in the coelomic fluids of both sexes was measured in *M. nudus* during the reproductive cycle (Fig. 4C, D). In females, the mean levels of MYP were 98.6

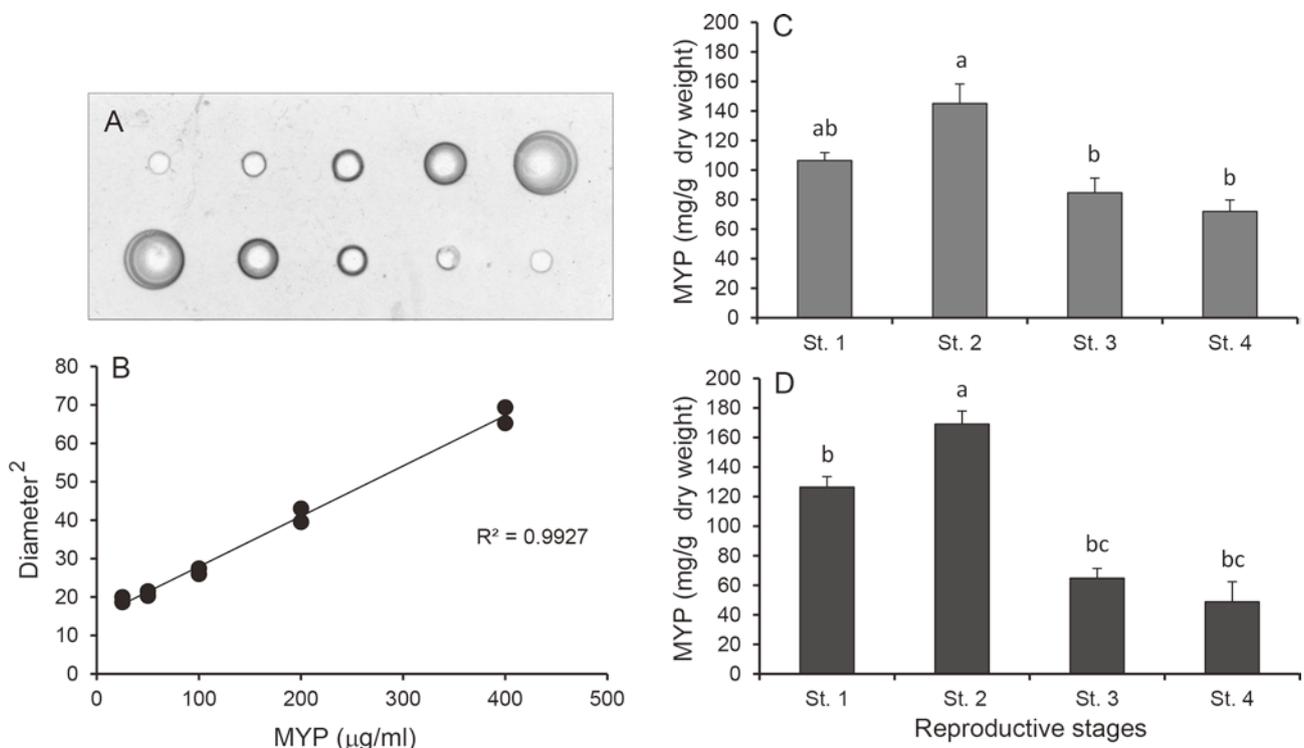


Fig. 3. SRID plate with 2% concentration of antiserum to NP-MYP (A) and their standard curve (B) for MYP measurement in the gonads of both sexes. Quantitative changes in MYP content in female (C) and male (D) gonads at different reproductive stages. Values are means  $\pm$  SE. Different letters above the bars indicate significantly different means.

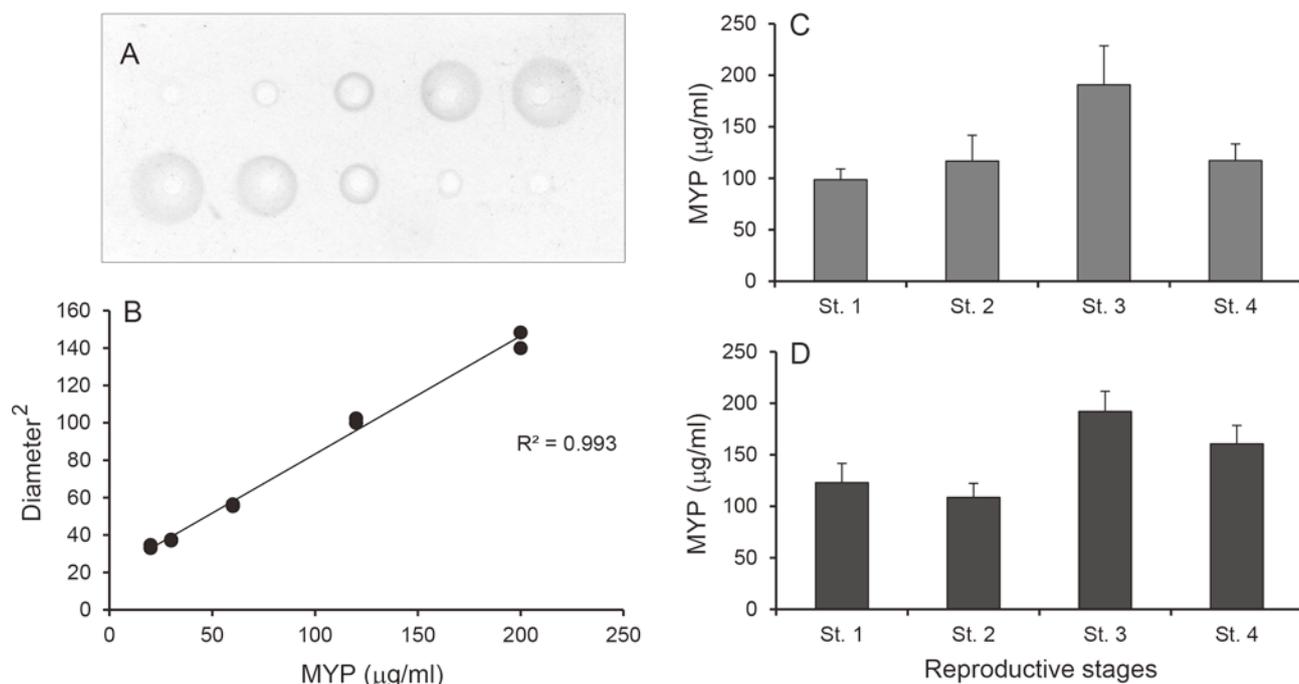


Fig. 4. SRID plate with 0.5% concentration of antiserum to NP-MYP (A) and their standard curve (B) for MYP measurement in the coelomic fluids of both sexes. Quantitative changes in CF-MYP content in female (C) and male (D) gonads at different reproductive stages. Values are means  $\pm$  SE.

$\pm 10.3 \mu\text{g/ml}$  ( $n = 10$ ),  $116.6 \pm 25.0 \mu\text{g/ml}$  ( $n = 10$ ),  $190.6 \pm 37.8 \mu\text{g/ml}$  ( $n = 7$ ),  $117.1 \pm 16.1 \mu\text{g/ml}$  ( $n = 10$ ) for stages 1, 2, 3, and 4, respectively (Fig. 4C). In males, the mean levels of MYP were  $122.9 \pm 18.6 \mu\text{g/ml}$  ( $n = 10$ ),  $108.6 \pm 13.6 \mu\text{g/ml}$  ( $n = 10$ ),  $192.0 \pm 19.4 \mu\text{g/ml}$  ( $n = 10$ ),  $160.5 \pm 17.8 \mu\text{g/ml}$  ( $n = 3$ ) for stages 1, 2, 3, and 4, respectively (Fig. 4D). The level of CF-MYP increased from stage 1 to stage 3, but significant changes were not observed in both females and males during the reproductive cycle.

**Correlation between GSI and CF-MYP level**

Figure 5 shows the correlation between GSI and CF-MYP in female and male in sea urchin. In females ( $n = 37$ ), the correlation coefficient was  $R^2 = 0.2483$ , and significant positive correlation was observed ( $P = 0.0018$ ). In males ( $n = 33$ ), the correlation coefficient was  $R^2 = 0.0019$ , and significant positive correlation was not observed ( $P = 0.6519$ ).

**Changes of total protein level in coelomic fluids during the reproductive cycle**

The concentration of total protein in the coelomic fluids was measured during the reproductive cycle in sea urchins (Fig. 6). In females, the mean levels of total protein were  $0.328 \pm 0.023 \text{ mg/ml}$  ( $n = 10$ ),  $0.373 \pm 0.030 \text{ mg/ml}$  ( $n = 10$ ),  $0.431 \pm 0.038 \text{ mg/ml}$  ( $n = 7$ ), and  $0.342 \pm 0.022 \text{ mg/ml}$  ( $n = 10$ ) for stages 1, 2, 3, and 4, respectively (Fig. 6A). In males, the mean levels of total protein were  $0.345 \pm 0.021 \text{ mg/ml}$  ( $n = 10$ ),  $0.354 \pm 0.034 \text{ mg/ml}$  ( $n = 10$ ),  $0.424 \pm 0.033 \text{ mg/ml}$  ( $n = 10$ ), and  $0.427 \pm 0.037 \text{ mg/ml}$  ( $n = 3$ ) for stages 1,

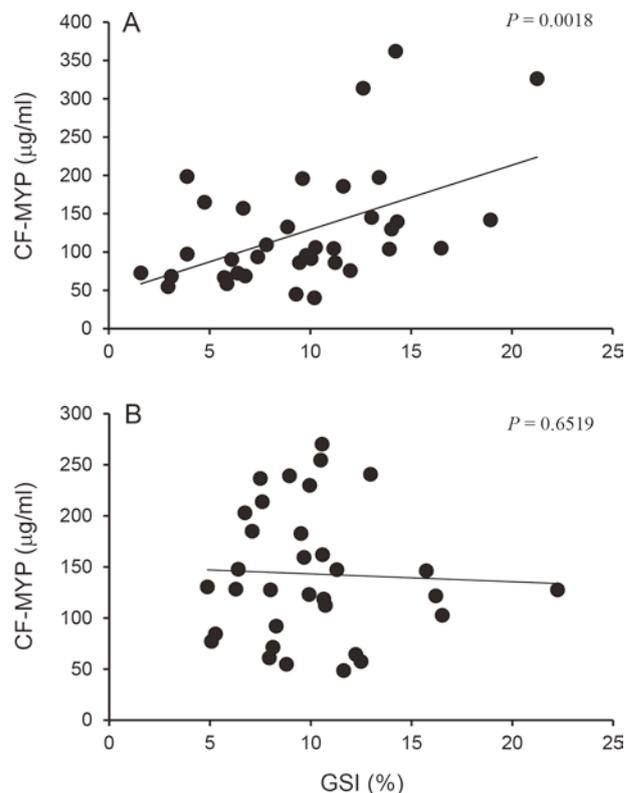


Fig. 5. Correlation of gonadosomatic index (GSI) with MYP in the coelomic fluid of female (A) and male (B) sea urchins.

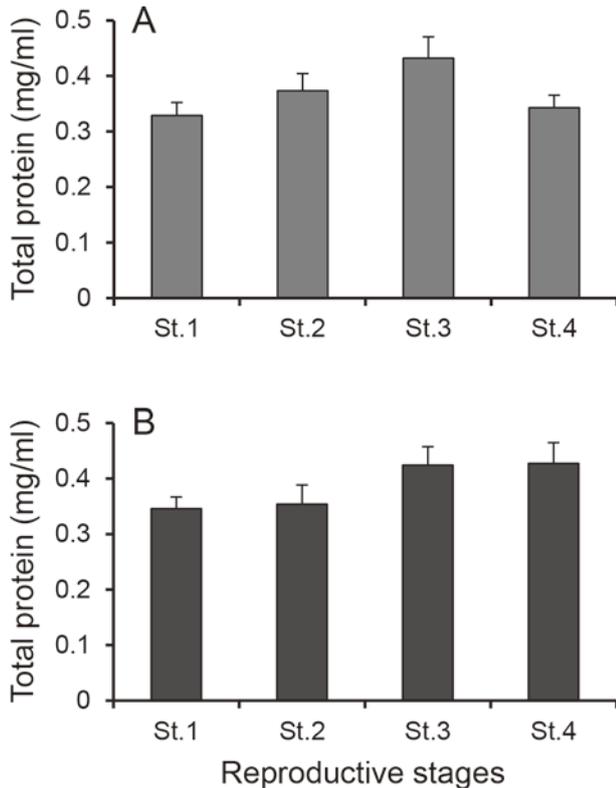


Fig. 6. Quantitative changes in the content of total protein in the coelomic fluids of females (A) and males (B) at different reproductive stages. Values are means  $\pm$  SE.

2, 3, and 4, respectively (Fig. 6B). Although significant changes were not observed during the reproductive cycle in females and males, the mean levels slightly increased from stage 1 to stage 3 in both sexes.

#### Correlation between GSI and total protein level in coelomic fluid

Figure 7 shows correlation between GSI and total protein levels in coelomic fluids. Although the correlation coefficient was  $R^2 = 0.382$  and significant positive correlation was observed ( $P = 0.00014$ ) in females ( $n = 37$ ), positive correlation was not observed ( $P = 0.425$ ) between GSI and total protein level in males ( $n = 33$ ) (Fig. 7A, B).

#### Discussion

The aim of this study was to identify if CF-MYP can be used as a biomarker of the progression of maturity in the sea urchin, *M. nudus*; therefore, we examined MYP concentration changes in the gonads and the coelomic fluids of male and female sea urchin during the reproductive cycle. In this study, we used wild sea urchins collected from southern Hokkaido in Japan, because we previously examined the reproductive cycle and transcription-level changes of the MYP in gonads of both sexes during the different reproductive stages

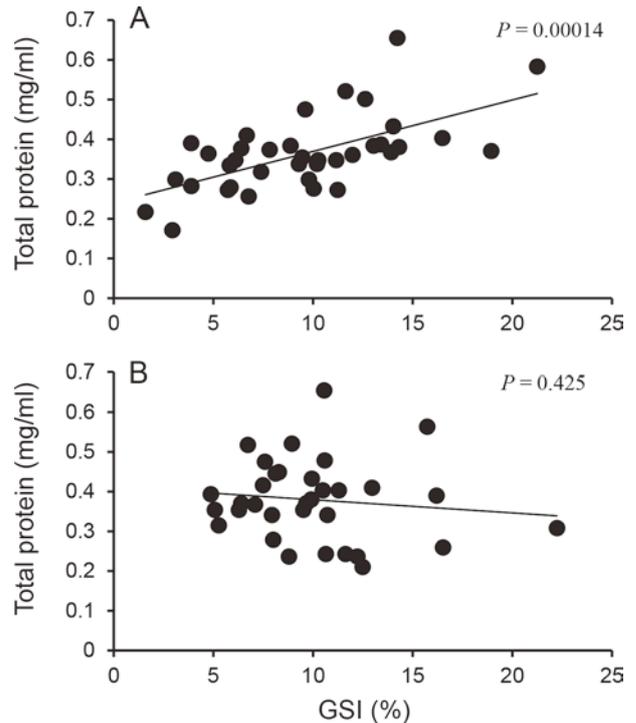


Fig. 7. Correlation of gonadosomatic index (GSI) with total protein levels in the coelomic fluids of females (A) and males (B).

(Ura et al., 2017).

The GSI increased from stage 1 (recovering) to stage 2 (growth) and stage 3 (pre-mature) in females and males, respectively, and then gradually decreased in stage 4 (mature) during the reproductive cycle in sea urchins (Fig. 1A, B). To date, in the wild sea urchin *Paracentrotus lividus*, it was reported that the GSI reached a peak at stage 3 (pre-mature) and then decreased during the reproductive cycle (Spirlet et al., 1998). Agatsuma et al. (1988) reported that the GSI reached a peak around stage 3 (pre-mature) and then decreased during spawning season in wild *Strongylocentrotus nudus* (*M. nudus*) from southern Hokkaido, similar to this study.

In this study, to develop the MYP measurement assay system, we performed purification of NP-MYP and obtained a specific antiserum against NP-MYP. NP-MYP purity was assessed by disc-PAGE and immunoelectrophoresis. The purified NP-MYP yielded one band in disc-PAGE and a single precipitin line when reacted against a polyvalent antiserum to gonad extracts and when it was precipitated by specific antiserum to NP-MYP. These results indicate that the NP-MYP preparation was electrophoretically and immunologically pure (Fig. 2A, B). It is well known that the MYP stored in the nutritive phagocytes of sea urchin gonads is a glycoprotein (Ozaki et al., 1986). Furthermore, based on the antigenic relationship between gonad extracts and coelomic fluids of both sexes in double immunodiffusion, it was indi-

cated that the antigenicity of MYP in gonads was immunologically the same as MYP in coelomic fluids of both sexes (Fig. 2C). These results reveal that this specific antiserum against NP-MYP is usable for measurement of MYP level in the gonads and coelomic fluids in female and male sea urchins.

In this study, MYP concentration changes in the gonads of both sexes during the reproductive cycle were determined by SRID using the specific antibody raised NP-MYP (Fig. 3). In the female gonads, MYP concentration reached a peak at stage 2 (growth) and gradually decreased as gametogenesis proceeded. In males, concentration of MYP reached a peak at stage 2 (growth) and rapidly decreased to stage 4 (mature). This profile of MYP content in the gonads of both sexes is similar to that previously reported in cultured *P. depressus* by Unuma et al. (2003). In our previous report, MYP mRNA expression level significantly increased from stage 1 and reached a peak at stage 2, and then gradually decreased in females; however, the level drastically decreased in male *M. nudus* sea urchins (Ura et al., 2017). From those results, it was suggested that MYP in the gonads of both sexes are synthesized and stored at stage 2 (growth). Thereafter, the part of MYP stored in the nutritive phagocytes is transported into growing oocytes in females, whereas MYP is used as nutrients for gametogenesis in males.

It is well known that MYP is also abundant in coelomic fluids of both sea urchin sexes (Giga and Ikai, 1985; Unuma et al., 1998), and MYP mRNA is expressed in the digestive tract, gonads, and coelomocytes (Unuma et al., 2001). The coelomocytes of sea urchins were classified into four types: phagocytes, vibratile cells, and red and white morula cells (Bertheussen and Seljelid, 1978; Gerardi et al., 1990; Unuma et al., 2010). Unuma et al. (2010) reported that MYP mRNA was mainly expressed in mainly vibratile and white morula cells within coelomocytes in sea urchins. It is generally accepted that vibratile cells are immediately broken *in vitro* without anticoagulant solution (Matsutani, 1995); therefore, it is important to collect coelomic fluid with anticoagulant solution to determine CF-MYP concentration in sea urchins. In this study, we collected the coelomic fluids with anticoagulant solution and examined the CF-MYP concentration changes during reproductive cycles in sea urchins. We found no reports of the profile of CF-MYP during the reproductive cycle using an assay system in adult sea urchins.

In this study, MYP concentration changes in the coelomic fluids of both sexes during the reproductive cycle were determined by SRID (Fig. 4). Although, significant changes in CF-MYP level were not observed during the reproductive cycle in female and male sea urchins, the CF-MYP levels increased from stage 1 to stage 3. Moreover, a positive correlation was observed between CF-MYP level and GSI in females but not in males (Fig. 5). Unuma et al. (2007) revealed by *in vivo* experiment that CF-MYP is taken up by

the gonadal nutritive phagocytes in the gonads, and is finally transported into the growing oocytes in females. In teleosts, the level of serum vitellogenin, which is an MYP precursor, showed significant positive correlation with GSI (Hara et al., 2016). These results indicated that CF-MYP is suitable as a biomarker of the onset of puberty and progression of maturity in females, similar to vitellogenin in fish. Alternatively, in males, although the CF-MYP level increased and reached a peak at stage 3 (premature), there was not a positive correlation between CF-MYP level and GSI. Therefore, CF-MYP is not suitable as a biomarker of the progression of maturity in males.

Moreover, we examined total protein concentration changes in the coelomic fluids of both sea urchin sexes during the reproductive cycle. Significant changes in total protein concentration were not observed during the reproductive cycle in female and male sea urchins (Fig. 6). In natural *S. purpuratus* populations, total protein concentration in the coelomic fluid increased with increasing of GSI, and then decreased at the mature stage (Holland et al., 1967). Holland et al. (1967) reported that the total protein concentrations in coelomic fluids were 0.20–0.45 mg/ml in wild *S. purpuratus*. The total protein concentrations in the coelomic fluids were 0.17–0.65 mg/ml in wild *M. nudus* in this study, which is similar to the range previously reported by Holland et al. (1967). However, significant total protein concentration changes were not observed in the coelomic fluids of females and males. Additionally, a significant positive correlation between total protein level and GSI was observed in females but not males. Moreover, a strong positive correlation was observed between total protein level and GSI rather than between CF-MYP and GSI, which indicates that CF-MYP and another protein concomitantly increases while gametogenesis proceeds in female coelomic fluid. The increasing protein in the coelomic fluid before maturity of gametes in sea urchins still needs to be identified and characterized.

In conclusion, CF-MYP level significantly changed concomitantly with changes in GSI during the reproductive cycle in sea urchins. Moreover, significant positive correlation was observed between CF-MYP level and GSI in females. Therefore, CF-MYP is suitable as a biomarker of the onset of puberty and progression of maturity in female sea urchins. These results indicate that CF-MYP is a part of MYP that is stored in sea urchin eggs.

#### Acknowledgments

We thank Dr Hiroyuki Munchara (Usujiri Fisheries Station, Field Science Center for the Northern Biosphere, Hokkaido University, Japan) for providing sea urchin gonad samples and helpful advice. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Educa-

tion, Culture, Sports, Science and Technology, Japan (No. 25660159) and the science and technology research promotion program for agriculture, forestry, fisheries and food industry. We thank Mallory Eckstut, PhD, from Edanz Group ([www.edanzediting.com/ac](http://www.edanzediting.com/ac)) for editing a draft of this manuscript.

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