Changes in levels of major yolk protein in the coelomic fluid and gonad during the reproductive cycle in wild sea urchins, *Mesocentrotus nudus*

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Abstract

Both female and male sea urchins accumulate the major yolk protein (MYP) in the nutritive phagocytes of immature gonads before gametogenesis, and MYP is the most abundant protein in the coelomic fluid of both sexes. In females, MYP in the coelomic fluid is taken up by the nutritive phagocytes and transported to the growing oocytes. This study examined quantitative changes of MYP in the coelomic fluid of both sexes during the reproductive cycle of wild sea urchins, *Mesocentrotus nudus*. Levels of MYP in the coelomic fluid of females increased and reached a peak at the histological pre-mature stage of gonad activity (i.e. Stage 3), and positive correlation between the MYP level and the gonadosomatic index (GSI) was observed. In male sea urchins the level of MYP in the coelomic fluid increased at the pre-mature stage, though positive correlation between the MYP level and GSI was not observed. These results indicate that MYP in the coelomic fluid is suitable as a biomarker of the onset and progression of sexual maturity in female sea urchins.

Introduction

In sea urchins, the major yolk protein (MYP) is the most abundant yolk protein, and is stored in the yolk granules of eggs as a nutrient source during early development (Ozaki, 1980; Harrington and Easton, 1982; Kari and Rottmann, 1985), similar to the yolk proteins provided by vitellogenin in other species of oviparous animals (Sullivan et al., 2003; Hiramatsu et al., 2005). It has been reported that MYP is stored in nutritive phagocytes, which are versatile somatic cells, in the gonads of both sexes in sea urchins (Unuma et al., 1998). In males, the stored MYP is used as energy for spermatogenesis as gametogenesis proceeds. In females, some MYP is degraded and used as a nutrient source, while 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).

MYP mRNA expression is ubiquitous in the digestive tract, coelomocytes and gonads of sea urchins (Unuma et al., 2001), while the major production sites of MYP are the digestive tract and gonads in adults of both sexes (Unuma et al., 2010). In both sexes of *Mesocentrotus nudus*, the MYP mRNA expression level increased during gonadal growth, and then decreased as gametogenesis proceeded in wild sea urchins (Ura et al., 2017). Whereas MYP is abundant in the coelomic fluid of male and female sea urchins, and is thought to be secreted in the digestive tract, as yet no histological analysis has provided evidence of this. Nonetheless, an *in vivo* experiment by Unuma et al. (2007) determined that MYP in the coelomic fluid is taken up by nutritive phagocytes in gonad, and this MYP is transported into the growing oocytes in females. To avoid confusion, we refer to MYP in coelomic fluid as CF-MYP, as originally

reported by Unuma et al. (2007); we denote MYP synthesized in the gonads of both sexes as NP-MYP; finally, we use the term MYP in a broad sense when the type is not specified.

Fish vitellogenin is a female-specific protein and MYP precursor synthesized in the female liver, and vitellogenin is transported into the growing oocytes via the blood. Consequently, vitellogenin is used as a biomarker of the onset of puberty and the progression of sexual maturity in female fish (Hiramatsu et al., 2005; Mushirobira et al., 2013). However, we are not aware of any report using an assay system to profile CF-MYP during the reproductive cycle in adult sea urchins. Although CF-MYP is transported from coelomic fluid to the nutritive phagocytes of gonads and then into the growing oocytes, it is still unclear whether CF-MYP represents a suitable biomarker of the progression of sexual maturity in sea urchins. Thus, in the present study, we developed an assay system to examine changes in the levels of MYP in coelomic fluid and gonad, and changes in the level of total proteins in the coelomic fluid during the reproductive cycle in both sexes of wild sea urchins (*Mesocentrotus nudus*) collected at southern Hokkaido, Japan.

Materials and methods

Animals and sampling

Sea urchins *Mesocentrotus nudus* were collected by diving at Usujiri in southern Hokkaido, Japan, from May 2015 to October 2016 (Ura et al., 2017). Sea urchins were transported to 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 × wet weight of gonad/total wet body weight. A small portion of each gonad sampled was fixed in Bouin's solution for histological examination of the reproductive stage, and the reminder was stored at −30°C until analysis.

After removing the peristomial membrane, coelomic fluid was collected and combined with an equal volume of anticoagulant solution (20 mmol l^{-1} Tris-HCl, 500 mmol l^{-1} NaCl, 30 mmol l^{-1} EDTA; pH 7.4) and then centrifuged at 900 *g* for 10 min at 4°C to separate the coelomic fluid, according to the method of Schillaci et al. (2013). The coelomic fluids were stored at −30°C until analysis.

Histology of gonads

The tissue samples were dehydrated through a graded ethanol series and embedded in paraffin; 6-μm-thick serial sections were mounted on glass slides and stained with hematoxylin and eosin. The gonadal maturity of each animal was classified according to the five stages described by Unuma et al. (1996): 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.

101

102 *Purification of NP-MYP*

103 The immature male gonads were homogenized with five volumes of 10 mmol l^{-1}
104 Tris-HCl (pH 8.0), containing 10 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, and 0.1% NaN_3,
105 using a Teflon homogenizer. The homogenate was centrifuged at 17,000 *g* for 20 min at
106 4°C, and supernatant was collected. Ion-exchange chromatography was performed on
107 the gonad extract using a 2.5 × 8-cm column of DEAE cellulose (TOYOPEAL DEAE)
108 equilibrated with 50 mmol l^{-1} Tris-HCl buffer (pH 7.5). The retained proteins were
109 eluted in stepwise fashion using the same buffer at various molar concentrations of
110 NaCl (0, 100, 200 and 300 mmol l^{-1}) at 4°C. Fractions (100 mmol l^{-1} NaCl) rich in the
111 targeted protein were pooled and dialyzed against 20 mmol l^{-1} KP at 4°C for overnight.
112 The dialyzed sample was fractionated by hydroxylapatite chromatography, and MYP
113 was eluted at 400 mmol l^{-1} KP. This fraction was concentrated by ultrafiltration to 2 ml
114 and fractionated twice by Superose 6 10/300 GL gel filtration column (GE Healthcare
115 Life Sciences, Little Chalfont, UK) equilibrated with 50 mmol l^{-1} Tris-HCl buffer (pH
116 7.5) containing 500 mmol l^{-1} NaCl. The single protein peak was the target MYP. At
117 each purification step, electrophoresis and immunological procedures were used to
118 ensure that the target protein was present. Purified MYP concentration was measured as
119 described by Lowry et al. (1951), with bovine serum albumin (BSA) (Bio-Rad
120 Laboratories Inc., Hercules, CA, USA) as the standard.

121

122 *Preparation of antisera*

123 A polyvalent antiserum against gonad extract proteins (anti-gonad) was raised in rabbits.
124 The specific antiserum to purified NP-MYP (anti-MYP) was obtained from a rabbit

125 immunized with 1 ml of solution containing 300 μg of purified NP-MYP mixed with an
126 equal volume of Freund's complete adjuvant (Wako Pure Chemical Industries, Osaka,
127 Japan). The rabbit received four such immunizations at about 7-day intervals. After four
128 injections, blood was collected from the ear vein of the rabbit. The blood was allowed to
129 clot for about 1 h at room temperature, and was then stored overnight at 4°C. The blood
130 was centrifuged at 17,000 *g* for 15 min at 4°C, and the supernatant was collected as
131 antiserum.
132
133 *Electrophoresis and immunological procedure*
134 Disc-polyacrylamide gel electrophoresis (disc-PAGE) was carried out in 5%
135 polyacrylamide gel using a Tris-glycine buffer system, following Davis (1964). The gel
136 was stained with 1% Amido black 10B in 7% acetic acid and destained with 7% acetic
137 acid. Immunoelectrophoretic analysis was performed with 1% agarose in 190 mmol l^{-1}
138 Tris-HCl buffer (pH 8.6). The immunoprecipitates were stained with 1% Amido black
139 10B in 7% acetic acid and destained with 7% acetic acid in the dried gel. Double
140 immunodiffusion using anti-MYP was performed in 1% agarose gel, using the method
141 of Ouchterlony (1953).
142
143 *Assay of MYP*
144 Single radial immunodiffusion (SRID) was carried out according to the procedure of
145 Mancini et al. (1965). Antiserum to purified MYP was diluted at 56°C in a solution of
146 1% (w/v) agarose (Nacalai, HGT) in 190 mmol l^{-1} Tris-HCl buffer (pH 8.6). Next, 15
147 ml of the hot solution was layered onto a 10 × 10 cm sheet of GelBond film (GE
148 Healthcare Life Sciences). The SRID plate was incubated in a moist chamber at room

149 temperature for 2 days; after incubation, it was washed with 0.9% NaCl, dried on filter
150 paper, stained with 1% Amido black 10B in 7% acetic acid, and destained with 7%
151 acetic acid. Purified MYP (25, 50, 100, 200 and 400 μg ml^{-1}) was used as the standard
152 for quantitative SRID in the gonads. Purified MYP (20, 30, 60, 120 and 200 μg ml^{-1})
153 was used as the standard for quantitative SRID in the coelomic fluids.
154
155 *Measurement of total protein concentration in the coelomic fluid*
156 The total protein concentrations in the coelomic fluids were measured as described by
157 Lowry et al. (1951), with BSA as the standard.
158
159 *Statistical analysis*
160 Data were expressed as mean ± SE. Statistical differences between means across the
161 stages of gonad activity were determined by one-way ANOVA and subsequent
162 Dunnett's test, where Stage 1 (recovering) was used as a control group. Statistical
163 significance is denoted at the level $P < 0.01$ (*) or $P < 0.05$ (**). Correlation was
164 determined by Spearman's rank correlation test.
165

166 **Results**

167

168 *Changes in GSI during the reproductive cycle*

169 The change in the GSI during the reproductive cycle of *Mesocentrotus nudus* is shown
170 in Fig. 1. In the wild population of sea urchins, the mean value significantly increased
171 from 9.42 ± 0.37% at Stage 1 (recovering) to 13.02 ± 0.59 at Stage 2 (growth) and 12.18
172 ± 0.56 at Stage 3 (pre-mature), and thereafter decreased at Stage 4 (mature) (Fig. 1A).
173 In females, the mean value significantly increased from 9.67 ± 0.53% at Stage 1 to
174 14.28 ± 0.74% at Stage 2, and thereafter gradually decreased (Fig. 1B). In males, the
175 mean value significantly increased from 9.06 ± 0.48% at Stage 1 to 12.02 ± 0.66% at
176 Stage 3, and then decreased to 7.35 ± 1.22% at Stage 4 (Fig. 1C).

177

178 *Purity of the isolated NP-MYP*

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

186

187 *Antigenic relationships between MYP in coelomic fluid and gonad*

188 The result of double immunodiffusion of coelomic fluids and gonad extract protein
189 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; and a precipitin line of gonad extract protein of both sexes appeared to generate a fuse against purified NP-MYP.

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 gave 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 μg ml^{-1} 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 the serial dilutions of gonad samples ran parallel to the standard curve. The inter-assay coefficient of variation was 2.55% (n = 20) and the intra-assay coefficient of variation was 0.87% (n = 4). Recovery of various concentrations (25, 50, 100, 200 and 400 μg ml^{-1}) of purified NP-MYP standard added to gonad extract was from 93.8 to 98.3%.

The concentration of MYP in the gonads of both sexes of *M. nudus* was measured during the reproductive cycle (Fig. 3C, D). In females, the mean levels of MYP during Stages 1, 2, 3 and 4, respectively, were: 106.5 ± 5.2 mg g^{-1} (dry weight) (n = 6), 145.1 ± 13.1 mg g^{-1} (dry weight) (n = 6), 84.6 ± 9.9 mg g^{-1} (dry weight) (n = 6), and 72.0 ± 7.7 mg g^{-1} (dry weight) (n = 6) (Fig. 3C). In males, the mean levels of MYP were: 126.4 ± 7.0 mg g^{-1} (dry weight) (n = 5), 169.0 ± 8.8 mg g^{-1} (dry weight) (n = 6), 64.8 ± 6.5 mg g^{-1} (dry weight) (n = 6), and 48.8 ± 13.6 mg g^{-1} (dry weight) (n = 3) (Fig. 3D). Moreover, significant changes during the reproductive cycle were found for both

females and males. In females, the level of MYP significantly increased from Stage 1 to Stage 2 ($P < 0.05$) and then gradually decreased at Stage 4. In males, the level of MYP significantly likewise increased from Stage 1 to Stage 2 ($P < 0.05$), but then drastically decreased during the rest of the reproductive cycle.

Quantitative measurement of MYP in coelomic fluids

A concentration of 0.5% antiserum gave 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 μg ml^{-1} standards, as shown in Fig. 4B. The diameter of the precipitation rings produced by purified NP-MYP were directly proportional to the concentration of the MYP standard ($R^2 = 0.993$), and the serial dilutions of coelomic-fluid samples ran parallel to the standard curve. The inter-assay coefficient of variation was 3.81% (n = 20) and the intra-assay coefficient of variation was 1.79% (n = 4). Recovery of various concentrations (20, 30, 60, 120 and 200 μg ml^{-1}) of purified NP-MYP standard added to coelomic fluid was from 96.9 to 99.9%.

The concentration of MYP in the coelomic fluids of both sexes of *M. nudus* was measured during reproductive cycle (Fig. 4C, D). In females, the mean levels of MYP for Stages 1, 2, 3 and 4, respectively, were: 98.6 ± 10.3 μg ml^{-1} (n = 10), 116.6 ± 25.0 μg ml^{-1} (n = 10), 190.6 ± 37.8 μg ml^{-1} (n = 7), and 117.1 ± 16.1 μg ml^{-1} (n = 10) (Fig. 4C). In males, the mean levels of MYP for Stages 1, 2, 3 and 4, respectively, were: 122.9 ± 18.6 μg ml^{-1} (n = 10), 108.6 ± 13.6 μg ml^{-1} (n = 10), 192.0 ± 19.4 μg ml^{-1} (n = 10), and 160.5 ± 17.8 μg ml^{-1} (n = 3) (Fig. 4D). In both females and males, the level of CF-MYP significantly increased from Stage 1 to Stage 3 ($P < 0.05$) and then gradually

238 decreased at Stage 4.

239

240 *Correlation between GSI and CF-MYP level*

241 Figure 5 shows the correlation between GSI values and CF-MYP levels in female and
242 male sea urchins. In females (n = 37), the correlation coefficient was $R^2 = 0.2483$, and a
243 significant positive correlation was observed ($P = 0.0018$). In males (n =33), the
244 correlation coefficient was $R^2 = 0.0019$, but no significant positive correlation was
245 observed ($P = 0.6519$).

246

247 *Changes in levels of total proteins and the ratio of MYP in coelomic fluids*

248 The concentration of total proteins in the coelomic fluids was measured during the
249 reproductive cycle (Fig. 6). In the wild population, the mean value significantly
250 increased from 0.337 ± 0.015 mg ml^{-1} (n = 20) at Stage 1 to 0.427 ± 0.025 mg ml^{-1} (n =
251 17) at Stage 3, and then decreased to 0.362 ± 0.021 mg ml^{-1} (n = 13) at Stage 4 (Fig.
252 6A). In females, the mean levels of total protein were 0.328 ± 0.023 mg ml^{-1} (n = 10),
253 0.373 ± 0.030 mg ml^{-1} (n = 10), 0.431 ± 0.038 mg ml^{-1} (n = 7) and 0.342 ± 0.022 mg
254 ml^{-1} (n = 10) for Stages 1, 2, 3 and 4, respectively (Fig. 6B). In males, the mean levels
255 of total protein were 0.345 ± 0.021 mg ml^{-1} (n = 10), 0.354 ± 0.034 mg ml^{-1} (n = 10),
256 0.424 ± 0.033 mg ml^{-1} (n = 10) and 0.427 ± 0.037 mg ml^{-1} (n = 3) for Stages 1, 2, 3 and
257 4, respectively (Fig. 6C). Although significant changes were not observed during the
258 reproductive cycle in either the females or males, the mean levels increased slightly
259 from Stage 1 to Stage 3 in both sexes.

260

261 *Correlation between GSI and total protein level in coelomic fluids*

Figure 7 shows the correlation between GSI and total protein level in the coelomic fluids. In the wild population (n = 70), the correlation coefficient was $R^2 = 0.086$, and a significant positive correlation was observed ($P = 0.031$) (Fig. 7A). The correlation coefficient was $R^2 = 0.382$, and a significant positive correlation was observed ($P = 0.00014$) in females (n = 37); however, a positive correlation was not observed ($P = 0.425$) between GSI and total protein level in males (n = 33) (Fig. 7B, C).

Discussion

This study aimed to identify whether CF-MYP is useful as a biomarker of the progression of sexual maturity in sea urchins. Thus, we examined changes in the concentration of MYP in the gonads and coelomic fluids of male and female *Mesocentrotus nudus* over the course of the reproductive cycle. We used wild sea urchins collected from southern Hokkaido, Japan, since we previously conducted a detailed study of the species' reproductive cycle and examined transcription-level changes of the MYP in gonads of both sexes during the different reproductive stages (Ura et al., 2017).

The GSI significantly increased at Stage 2 (growth) and Stage 3 (pre-mature) in the wild population (Fig. 1A). The GSI increased from Stage 1 (recovering) to Stage 2 or Stage 3 in females and males, and then that gradually decreased in Stage 4 (mature) (Fig. 1B, C). In wild sea urchins *Paracentrotus lividus* collected off southern France the GSI peaked at Stage 3 and thereafter decreased (Spirlet et al., 1998). Agatsuma et al. (1988) likewise examined wild *M. nudus* (previously known as *Strongylocentrotus nudus*) from southern Hokkaido, and similarly reported that GSI peaked around Stage 3, and then decreased during the spawning season.

To develop an assay system to measure MYP in sea urchin, we performed purification of NP-MYP and obtained a specific antiserum against NP-MYP. Purity of the NP-MYP 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 extract as well as when it was precipitated by specific antiserum to NP-MYP. These results indicate that the NP-MYP preparation was

293 electrophoretically and immunologically pure (Fig. 2A, B). The MYP stored in the
294 nutritive phagocytes of sea urchin gonads is understood to be a glycoprotein (Ozaki et
295 al., 1986). Furthermore, the antigenic relationship between gonad extracts and coelomic
296 fluids of both sexes in double immunodiffusion indicated that the antigenicity of MYP
297 in gonads was immunologically the same as MYP in the coelomic fluids of both sexes
298 (Fig. 2C). These results demonstrate that this specific antiserum against NP-MYP can
299 be used to measure MYP levels in the gonads and coelomic fluids of female and male
300 sea urchins.

301 In the present study, changes in the concentration of MYP in the gonads of both
302 sexes during the reproductive cycle were determined by SRID using the specific
303 antiserum raised against the purified NP-MYP (Fig. 3). In female gonad, concentrations
304 of MYP peaked at Stage 2 and gradually decreased as gametogenesis proceeded. In
305 male gonad, concentrations of MYP likewise peaked at Stage 2 but decreased to rapidly
306 Stage 4. This profile of MYP content in the gonads of both sexes is similar to that
307 previously reported for cultured *Pseudocentrotus depressus* (Unuma et al. 2003). In our
308 previous study of *M. nudus* (Ura et al., 2017), MYP mRNA expression levels
309 significantly increased from Stage 1 and reached a peak at Stage 2; thereafter the levels
310 gradually decreased in females, but drastically decreased in males. These results suggest
311 that MYP in the gonads of both sexes of sea urchin is synthesized and stored at Stage 2;
312 thereafter, a part of the MYP stored in the nutritive phagocytes is transported into
313 growing oocytes in females, while MYP functions as a nutrient for gametogenesis in
314 males.

315 MYP is an abundant protein in the coelomic fluid of both sexes in sea urchins
316 (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 are classified as 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) observed the expression of MYP mRNA mainly in vibratile cells and white morula cells in sea urchin. Without anticoagulant solution, vibratile cells become immediately broken *in vitro* (Matsutani, 1995). This suggested the importance of collecting the coelomic fluid with an anticoagulant solution to determine CF-MYP concentrations in sea urchins, as carried out in the present study. Accordingly, we examined changes in the concentration of CF-MYP during the reproductive cycle in both males and females. Furthermore, we were aware of no previous reports using an assay system for profiling CF-MYP during the reproductive cycle in adult sea urchins. In this study, changes in the concentration of MYP in the coelomic fluids of both sexes during the reproductive cycle were determined by SRID (Fig. 4), and significant changes were found for both female and male sea urchins. A positive correlation between CF-MYP levels and GSI was observed in females, but not in males (Fig. 5). In an exquisitely designed *in vivo* experiment, Unuma et al. (2007) found that CF-MYP is taken up by nutritive phagocytes in the gonads, and is finally transported into the growing oocytes in females. In teleost fishes, the level of serum vitellogenin (an MYP precursor) showed a significant positive correlation with GSI (Mushirobira et al., 2013). These results indicate that CF-MYP is suitable as a biomarker of the onset of puberty and the progression of sexual maturity in female sea urchins, similar to vitellogenin in fishes. Whereas, in males, the level of CF-MYP significantly increased and reached a peak at Stage 3, it did not positively correlate with GSI; therefore, CF-MYP is not suitable as a biomarker of the progression of sexual maturity in male sea urchins. It is

generally understood that MYP stored in the male gonad decreases with maturation, a phenomena observed in the present study. However, further investigation is needed to explain the high levels present in males at Stage 3, and to exactly determine the role of CF-MYP in male sea urchins.

We also examined changes in the concentration of total proteins in the coelomic fluids of both sexes of wild sea urchin during the reproductive cycle. In the wild population, total protein concentration increased from Stage 1 and peaked at Stage 3, and thereafter decreased to a low level at Stage 4 (Fig. 6). In a natural population of *Strongylocentrotus purpuratus* the concentration of total proteins in the coelomic fluid increased with an increasing GSI, and then decreased at a mature stage, with total protein concentration in the coelomic fluid reported as 0.20–0.45 mg ml^{-1} (Holland et al., 1967). In this study, the concentration of total proteins in the coelomic fluids were 0.17–0.65 mg ml^{-1} in wild *M. nudus*, a range similar to that found for purple sea urchin as reported by Holland et al. (1967). However, the observed changes in the concentration of total proteins in the coelomic fluids of females and males were not significant. A significant positive correlation between total protein level and GSI was observed for females, but not for males. Moreover, the strong positive correlation between total protein level and GSI in females, and not between CF-MYP and GSI, suggests that CF-MYP and another protein increases as gametogenesis proceeds in females. However, the identification and characterization of proteins in the coelomic fluids of sea urchins before maturity of the gametes remains to be done.

In conclusion, CF-MYP appears suitable as a biomarker of the onset of puberty and the progression of sexual maturity in female sea urchins, but not in males. This finding indicates that CF-MYP is a part of the MYP stored in the eggs of sea urchins.

365

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458 **Figure legends**

459

460 Figure 1. Changes in gonadosomatic index (GSI) in the wild population (A) and in
461 female (B) and male (C) sea urchins at different reproductive stages. Values are mean ±
462 SE. Significant differences between means at the level $P < 0.01$ (*) were determined
463 using Dunnett's test, where Stage 1 (St. 1) gonad activity (i.e. recovering) was used as a
464 control group.

465

466 Figure 2. Observations of disc electrophoresis (A) and immunoelectrophoresis (B) of
467 purified NP-MYP from immature male gonads, and the precipitin reaction of MYP in
468 double immunodiffusion (C). Disc electrophoresis was stained with Amido black 10B;
469 a-GE: polyvalent antiserum to gonad extracts; a-M: specific antiserum to purified
470 NP-MYP; M: purified NP-MYP; mG: male gonad extract; fG: female gonad extract;
471 mCF: male coelomic fluid; fCF: female coelomic fluid.

472

473 Figure 3. A single radial immunodiffusion (SRID) plate with 2% concentration of
474 antiserum to NP-MYP (A) and their standard curve (B) for measurement of MYP in the
475 gonads of both sexes of sea urchin. Quantitative changes in the content of MYP in the
476 gonad of females (C) and males (D) at different reproductive stages. Values are mean ±
477 SE. Significant differences between means at the level $P < 0.01$ (*) or $P < 0.05$ (**)
478 were determined using Dunnett's test, where Stage 1 (St. 1) gonad activity (i.e.
479 recovering) was used as a control group.

480

481 Figure 4. SRID plate with 0.5% concentration of antiserum to NP-MYP (A) and their

482 standard curve (B) for measurement of MYP in the coelomic fluids of both sexes of sea
483 urchin. Quantitative changes in the content of CF-MYP in the gonads of females (C)
484 and males (D) at different reproductive stages. Values are mean ± SE. Significant
485 differences between means at $P < 0.05$ (**) were determined using Dunnett's test,
486 where Stage 1 (St. 1) gonad activity (i.e. recovering) was used as a control group.
487
488 Figure 5. Correlation of gonadosomatic index (GSI) with MYP in the coelomic fluid of
489 female (A) and male (B) sea urchins.
490
491 Figure 6. Quantitative changes in the content of total proteins in the coelomic fluid in
492 the wild population (A), and in females (B) and males (C) at different reproductive
493 stages. Values are mean ± SE. Significant differences between means at $P < 0.05$ (**)
494 were determined using Dunnett's test, where Stage 1 (St. 1) gonad activity (i.e.
495 recovering) was used as a control group.
496
497 Figure 7. Correlation of gonadosomatic index (GSI) with total protein levels in the
498 coelomic fluid of the wild population (A) and in female (B) and male (C) sea urchins.
499

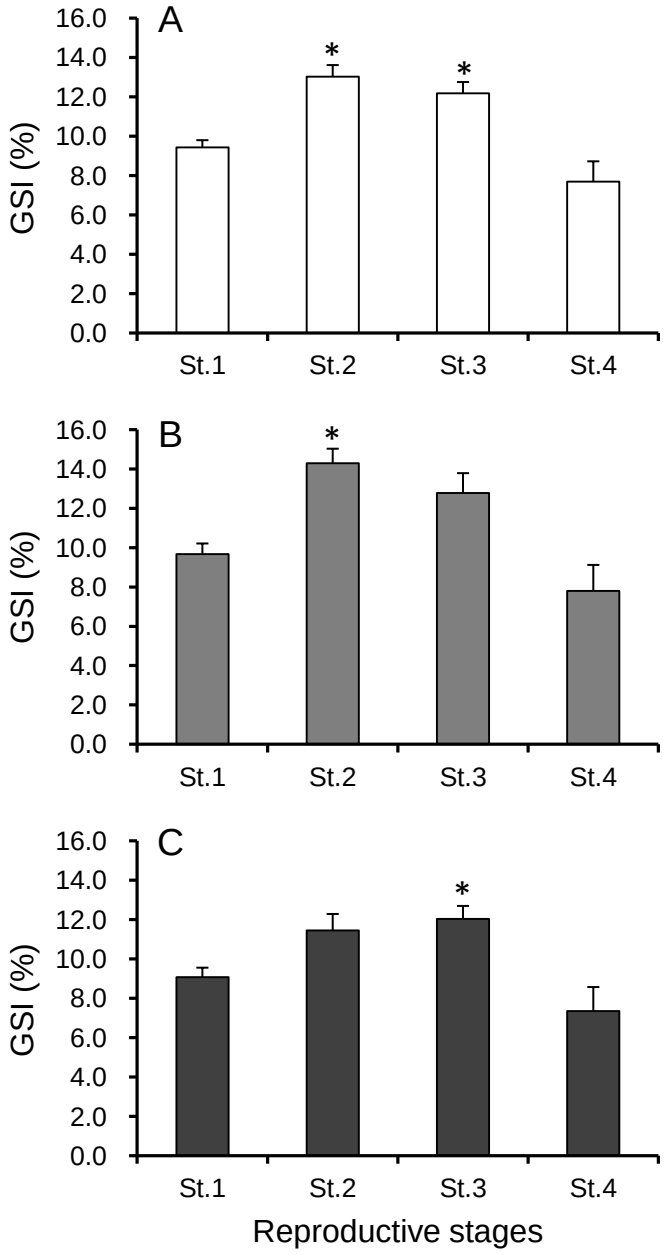

A
B
C
GSI (%)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
St.1
St.2
St.3
St.4
Reproductive stages

Fig. 1

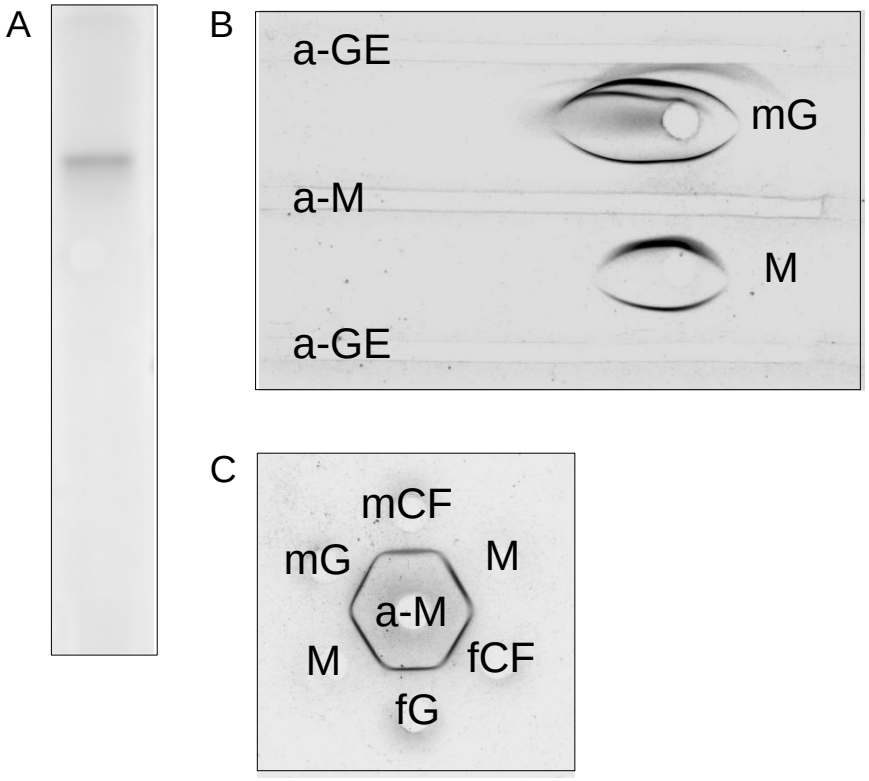
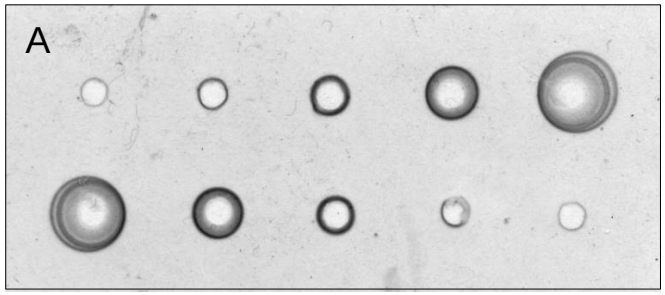
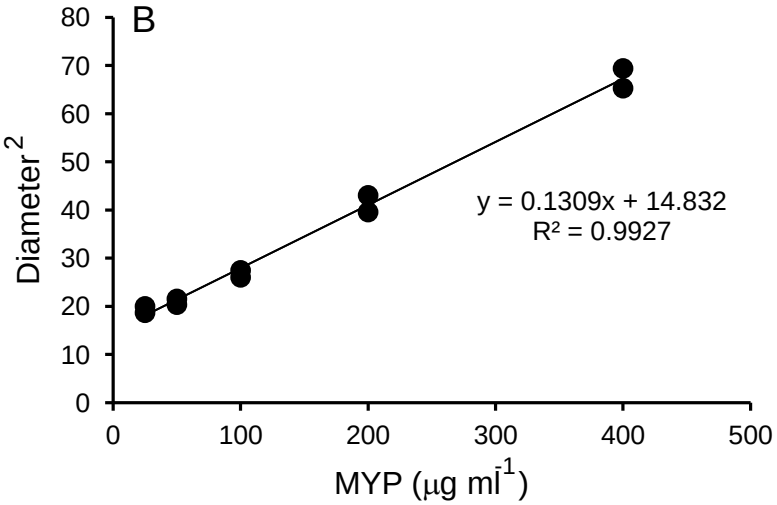
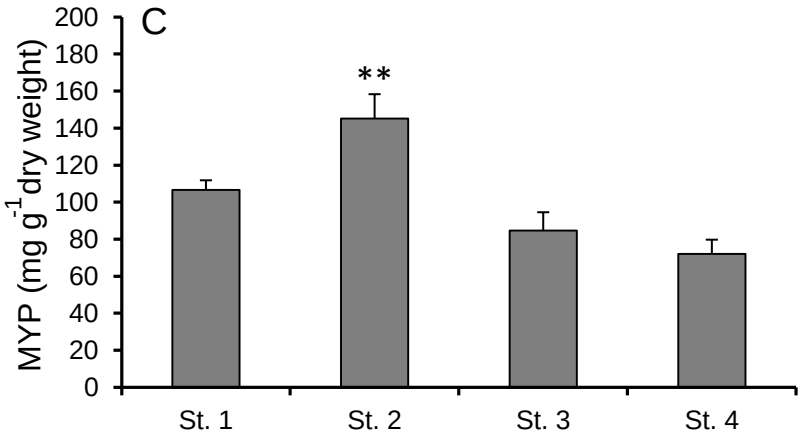


Fig. 2


A


B
Diameter 2
MYP (μg ml-1)
y = 0.1309x + 14.832
R² = 0.9927
0
10
20
30
40
50
60
70
80
0
100
200
300
400
500


C
MYP (mg g-1dry weight)
St. 1
St. 2
St. 3
St. 4
**
0
20
40
60
80
100
120
140
160
180
200

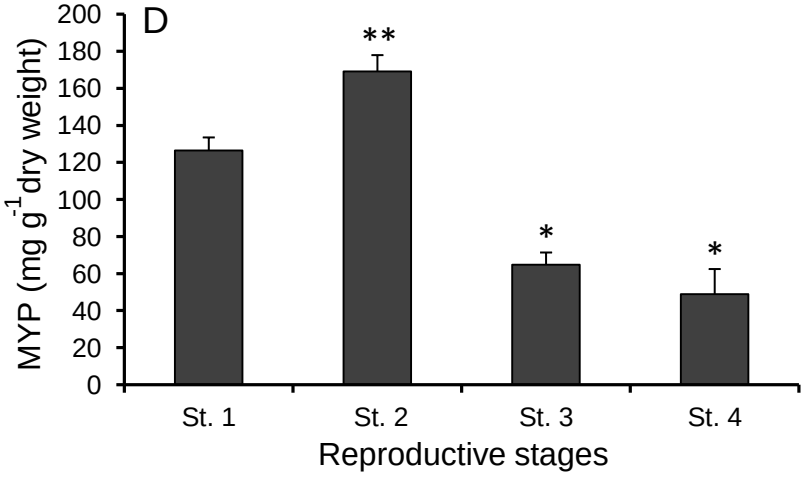
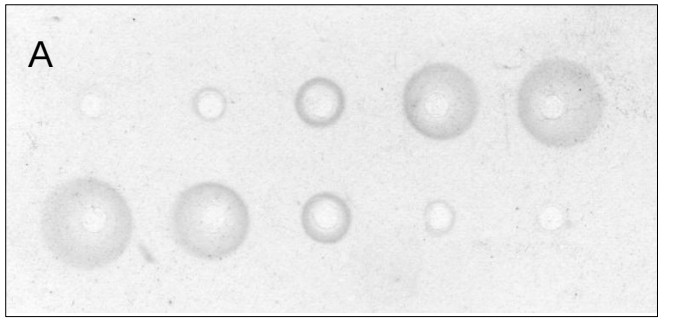
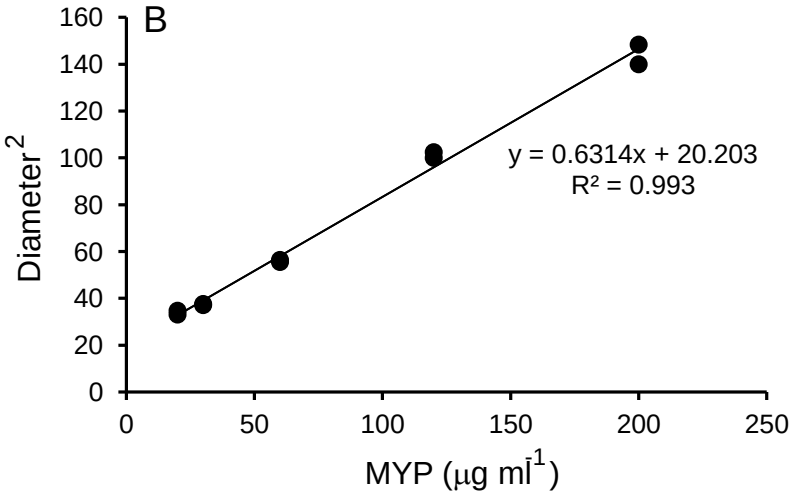
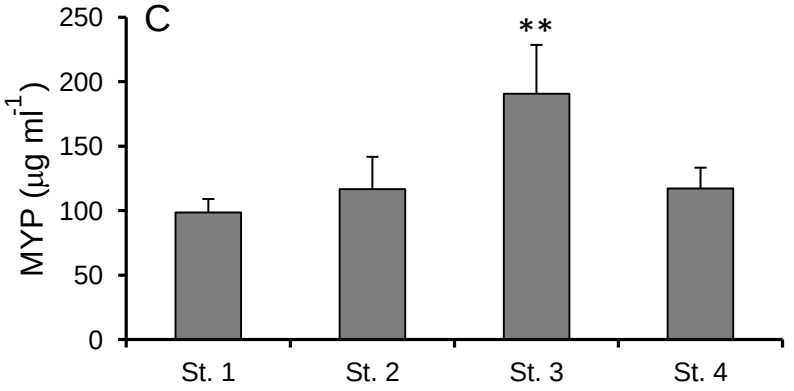

D
MYP (mg g-1dry weight)
Reproductive stages
St. 1
St. 2
St. 3
St. 4
**
*
*
0
20
40
60
80
100
120
140
160
180
200

Fig. 3


A


B
Diameter^2
MYP (µg ml^{-1})
y = 0.6314x + 20.203
R² = 0.993
0
20
40
60
80
100
120
140
160
0
50
100
150
200
250


C
MYP (µg ml^{-1})
**
0
50
100
150
200
250
St. 1
St. 2
St. 3
St. 4

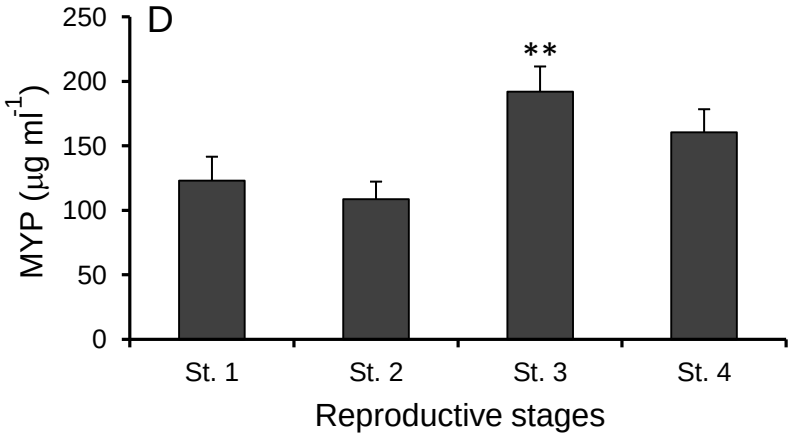

D
MYP (µg ml^{-1})
**
0
50
100
150
200
250
St. 1
St. 2
St. 3
St. 4
Reproductive stages

Fig. 4

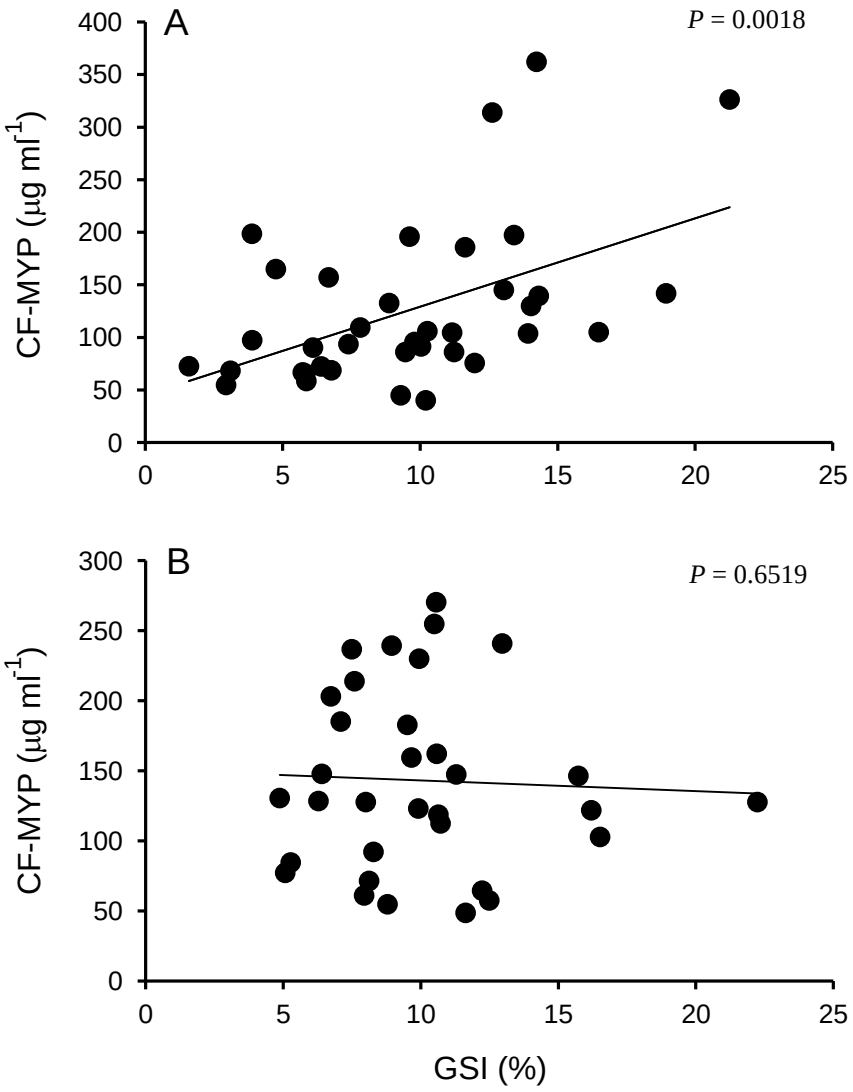

A
P = 0.0018
CF-MYP (μg ml-1)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
B
P = 0.6519
CF-MYP (μg ml-1)
300
250
200
150
100
50
0
0
5
10
15
20
25
GSI (%)

Fig. 5

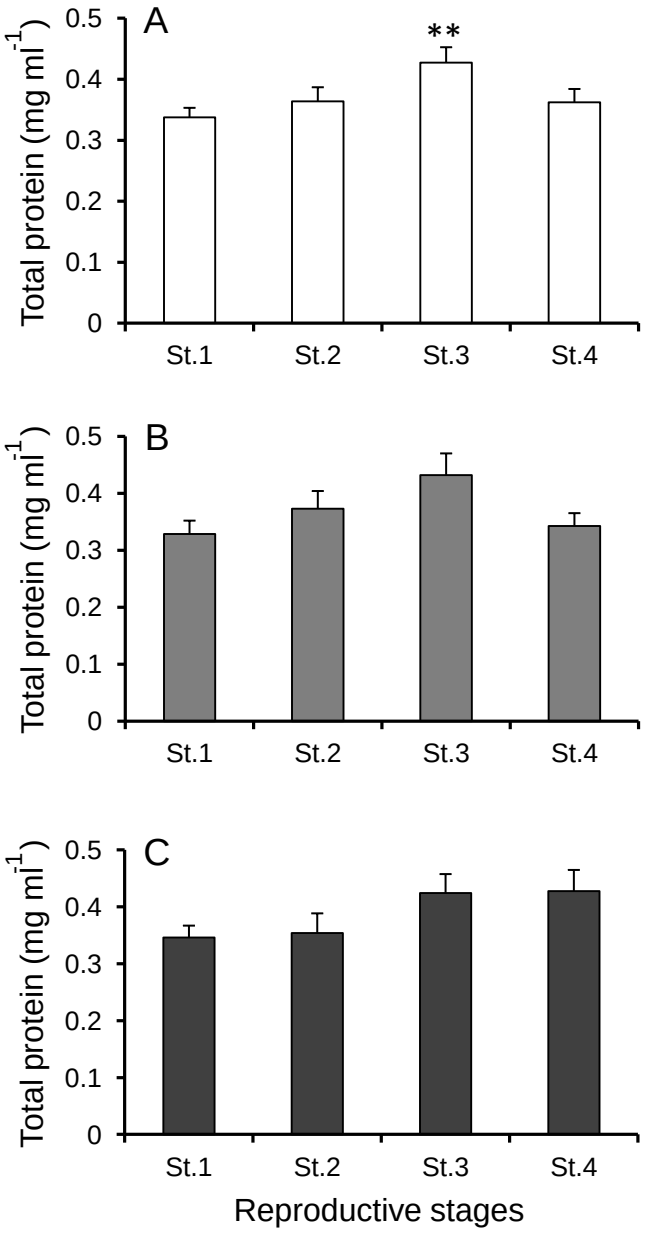

A
B
C
**
Total protein (mg ml-1)
0.5
0.4
0.3
0.2
0.1
0
St.1
St.2
St.3
St.4
Reproductive stages

Fig. 6

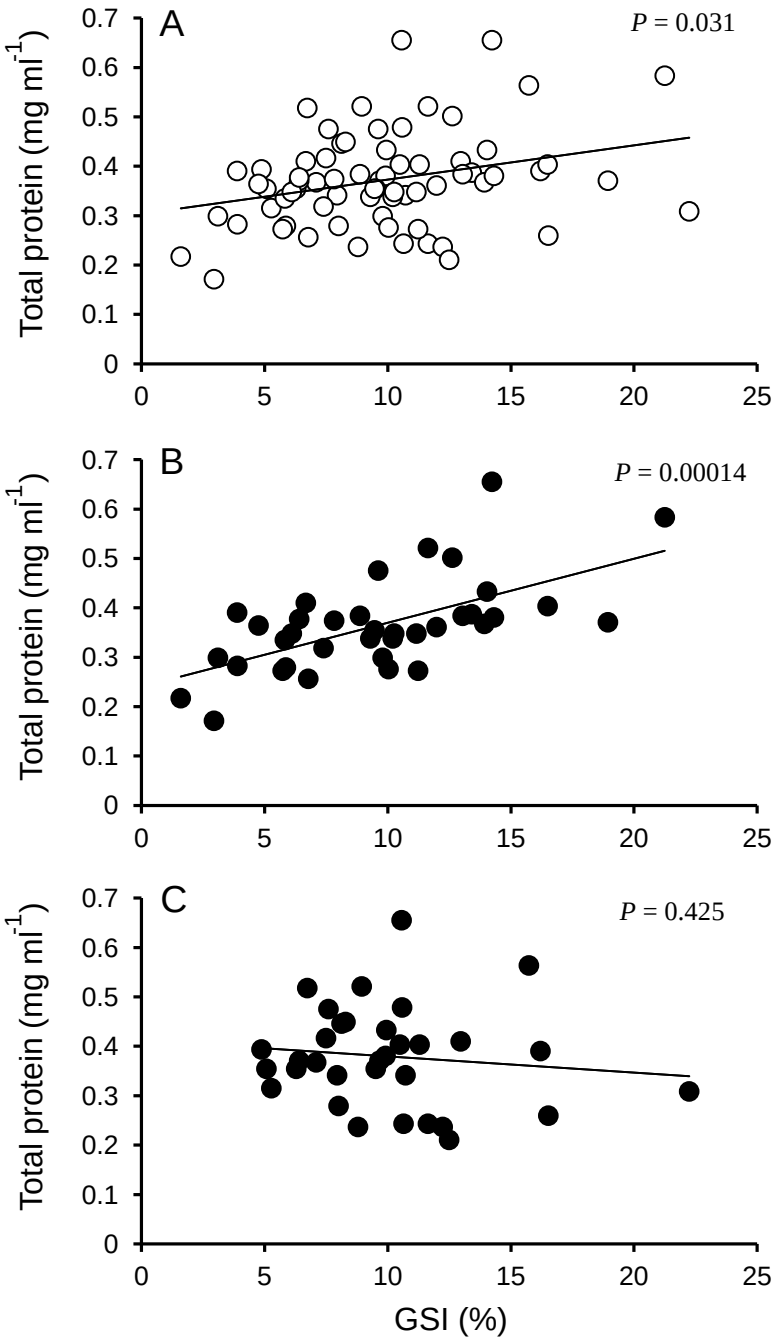

A
P = 0.031
B
P = 0.00014
C
P = 0.425
Total protein (mg ml-1)
GSI (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
5
10
15
20
25

Fig. 7