

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

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19 reproductive cycle

20

21 **Abstract**

22

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

## 36 **Introduction**

37

38 In sea urchins, the major yolk protein (MYP) is the most abundant yolk protein, and is  
39 stored in the yolk granules of eggs as a nutrient source during early development (Ozaki,  
40 1980; Harrington and Easton, 1982; Kari and Rottmann, 1985), similar to the yolk  
41 proteins provided by vitellogenin in other species of oviparous animals (Sullivan et al.,  
42 2003; Hiramatsu et al., 2005). It has been reported that MYP is stored in nutritive  
43 phagocytes, which are versatile somatic cells, in the gonads of both sexes in sea urchins  
44 (Unuma et al., 1998). In males, the stored MYP is used as energy for spermatogenesis as  
45 gametogenesis proceeds. In females, some MYP is degraded and used as a nutrient  
46 source, while some is transported to the oocytes and actively packed into yolk granules  
47 through endocytosis via a dynamin-dependent mechanism (Unuma et al., 2003; Brooks  
48 and Wessel, 2004; Unuma et al., 2010).

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

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

63 Fish vitellogenin is a female-specific protein and MYP precursor synthesized in  
64 the female liver, and vitellogenin is transported into the growing oocytes via the blood.  
65 Consequently, vitellogenin is used as a biomarker of the onset of puberty and the  
66 progression of sexual maturity in female fish (Hiramatsu et al., 2005; Mushirobira et al.,  
67 2013). However, we are not aware of any report using an assay system to profile  
68 CF-MYP during the reproductive cycle in adult sea urchins. Although CF-MYP is  
69 transported from coelomic fluid to the nutritive phagocytes of gonads and then into the  
70 growing oocytes, it is still unclear whether CF-MYP represents a suitable biomarker of  
71 the progression of sexual maturity in sea urchins. Thus, in the present study, we  
72 developed an assay system to examine changes in the levels of MYP in coelomic fluid  
73 and gonad, and changes in the level of total proteins in the coelomic fluid during the  
74 reproductive cycle in both sexes of wild sea urchins (*Mesocentrotus nudus*) collected at  
75 southern Hokkaido, Japan.

76

77 **Materials and methods**

78

79 *Animals and sampling*

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

88 After removing the peristomial membrane, coelomic fluid was collected and  
89 combined with an equal volume of anticoagulant solution (20 mmol  $\text{l}^{-1}$  Tris-HCl, 500  
90 mmol  $\text{l}^{-1}$  NaCl, 30 mmol  $\text{l}^{-1}$  EDTA; pH 7.4) and then centrifuged at 900 g for 10 min at  
91  $4^{\circ}\text{C}$  to separate the coelomic fluid, according to the method of Schillaci et al. (2013).  
92 The coelomic fluids were stored at  $-30^{\circ}\text{C}$  until analysis.

93

94 *Histology of gonads*

95 The tissue samples were dehydrated through a graded ethanol series and embedded in  
96 paraffin; 6- $\mu\text{m}$ -thick serial sections were mounted on glass slides and stained with  
97 hematoxylin and eosin. The gonadal maturity of each animal was classified according to  
98 the five stages described by Unuma et al. (1996): Stage 1 (recovering), Stage 2 (growth),  
99 Stage 3 (pre-mature), Stage 4 (mature), and Stage 5 (spent). The gonads of 15–30  
100 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<sup>-1</sup>  
104 Tris-HCl (pH 8.0), containing 10 mmol l<sup>-1</sup> NaCl, 1 mmol l<sup>-1</sup> EDTA, and 0.1% NaN<sub>3</sub>,  
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<sup>-1</sup> 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<sup>-1</sup>) at 4°C. Fractions (100 mmol l<sup>-1</sup> NaCl) rich in the  
111 targeted protein were pooled and dialyzed against 20 mmol l<sup>-1</sup> KP at 4°C for overnight.  
112 The dialyzed sample was fractionated by hydroxylapatite chromatography, and MYP  
113 was eluted at 400 mmol l<sup>-1</sup> 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<sup>-1</sup> Tris-HCl buffer (pH  
116 7.5) containing 500 mmol l<sup>-1</sup> 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  $\mu$ g 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<sup>-1</sup>  
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<sup>-1</sup> 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  $\mu\text{g ml}^{-1}$ ) was used as the standard  
152 for quantitative SRID in the gonads. Purified MYP (20, 30, 60, 120 and 200  $\mu\text{g ml}^{-1}$ )  
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  $\pm$  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  $9.42 \pm 0.37\%$  at Stage 1 (recovering) to  $13.02 \pm 0.59$  at Stage 2 (growth) and  $12.18$   
172  $\pm 0.56$  at Stage 3 (pre-mature), and thereafter decreased at Stage 4 (mature) (Fig. 1A).  
173 In females, the mean value significantly increased from  $9.67 \pm 0.53\%$  at Stage 1 to  
174  $14.28 \pm 0.74\%$  at Stage 2, and thereafter gradually decreased (Fig. 1B). In males, the  
175 mean value significantly increased from  $9.06 \pm 0.48\%$  at Stage 1 to  $12.02 \pm 0.66\%$  at  
176 Stage 3, and then decreased to  $7.35 \pm 1.22\%$  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

190 fluids of both sexes appeared to generate a fuse against purified NP-MYP from male  
191 gonads; and a precipitin line of gonad extract protein of both sexes appeared to generate  
192 a fuse against purified NP-MYP.

193

#### 194 *Quantitative measurement of MYP in gonads*

195 Different dilutions of the antiserum to MYP were incorporated into the agarose gel used  
196 for SRID. A concentration of 2% antiserum gave the best quantitative results for  
197 measurement of MYP in the gonads of both sexes (Fig. 3A). Using this dilution, the  
198 squared diameter of a precipitate ring was directly proportional to the amount of the  
199 sample, in the range of 25, 50, 100, 200 and 400  $\mu\text{g ml}^{-1}$  standards, as shown in Fig. 3B.  
200 The diameter of precipitation rings produced by purified NP-MYP were directly  
201 proportional to the concentration of the MYP standard ( $R^2 = 0.9927$ ), and the serial  
202 dilutions of gonad samples ran parallel to the standard curve. The inter-assay coefficient  
203 of variation was 2.55% ( $n = 20$ ) and the intra-assay coefficient of variation was 0.87%  
204 ( $n = 4$ ). Recovery of various concentrations (25, 50, 100, 200 and 400  $\mu\text{g ml}^{-1}$ ) of  
205 purified NP-MYP standard added to gonad extract was from 93.8 to 98.3%.

206 The concentration of MYP in the gonads of both sexes of *M. nudus* was  
207 measured during the reproductive cycle (Fig. 3C, D). In females, the mean levels of  
208 MYP during Stages 1, 2, 3 and 4, respectively, were:  $106.5 \pm 5.2 \text{ mg g}^{-1}$  (dry weight) ( $n$   
209  $= 6$ ),  $145.1 \pm 13.1 \text{ mg g}^{-1}$  (dry weight) ( $n = 6$ ),  $84.6 \pm 9.9 \text{ mg g}^{-1}$  (dry weight) ( $n = 6$ ),  
210 and  $72.0 \pm 7.7 \text{ mg g}^{-1}$  (dry weight) ( $n = 6$ ) (Fig. 3C). In males, the mean levels of MYP  
211 were:  $126.4 \pm 7.0 \text{ mg g}^{-1}$  (dry weight) ( $n = 5$ ),  $169.0 \pm 8.8 \text{ mg g}^{-1}$  (dry weight) ( $n = 6$ ),  
212  $64.8 \pm 6.5 \text{ mg g}^{-1}$  (dry weight) ( $n = 6$ ), and  $48.8 \pm 13.6 \text{ mg g}^{-1}$  (dry weight) ( $n = 3$ ) (Fig.  
213 3D). Moreover, significant changes during the reproductive cycle were found for both

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

218

### 219 *Quantitative measurement of MYP in coelomic fluids*

220 A concentration of 0.5% antiserum gave the best quantitative results for measurement of  
221 MYP in the coelomic fluids of both sexes (Fig. 4A). Using this dilution, the squared  
222 diameter of a precipitate ring was directly proportional to the amount of the sample, in  
223 the range of the 20, 30, 60, 120 and 200  $\mu\text{g ml}^{-1}$  standards, as shown in Fig. 4B. The  
224 diameter of the precipitation rings produced by purified NP-MYP were directly  
225 proportional to the concentration of the MYP standard ( $R^2 = 0.993$ ), and the serial  
226 dilutions of coelomic-fluid samples ran parallel to the standard curve. The inter-assay  
227 coefficient of variation was 3.81% ( $n = 20$ ) and the intra-assay coefficient of variation  
228 was 1.79% ( $n = 4$ ). Recovery of various concentrations (20, 30, 60, 120 and 200  $\mu\text{g}$   
229  $\text{ml}^{-1}$ ) of purified NP-MYP standard added to coelomic fluid was from 96.9 to 99.9%.

230 The concentration of MYP in the coelomic fluids of both sexes of *M. nudus*  
231 was measured during reproductive cycle (Fig. 4C, D). In females, the mean levels of  
232 MYP for Stages 1, 2, 3 and 4, respectively, were:  $98.6 \pm 10.3 \mu\text{g ml}^{-1}$  ( $n = 10$ ),  $116.6 \pm$   
233  $25.0 \mu\text{g ml}^{-1}$  ( $n = 10$ ),  $190.6 \pm 37.8 \mu\text{g ml}^{-1}$  ( $n = 7$ ), and  $117.1 \pm 16.1 \mu\text{g ml}^{-1}$  ( $n = 10$ )  
234 (Fig. 4C). In males, the mean levels of MYP for Stages 1, 2, 3 and 4, respectively, were:  
235  $122.9 \pm 18.6 \mu\text{g ml}^{-1}$  ( $n = 10$ ),  $108.6 \pm 13.6 \mu\text{g ml}^{-1}$  ( $n = 10$ ),  $192.0 \pm 19.4 \mu\text{g ml}^{-1}$  ( $n =$   
236  $10$ ), and  $160.5 \pm 17.8 \mu\text{g ml}^{-1}$  ( $n = 3$ ) (Fig. 4D). In both females and males, the level of  
237 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  $0.337 \pm 0.015 \text{ mg ml}^{-1}$  ( $n = 20$ ) at Stage 1 to  $0.427 \pm 0.025 \text{ mg ml}^{-1}$  ( $n =$   
251  $17$ ) at Stage 3, and then decreased to  $0.362 \pm 0.021 \text{ mg ml}^{-1}$  ( $n = 13$ ) at Stage 4 (Fig.  
252 6A). In females, the mean levels of total protein were  $0.328 \pm 0.023 \text{ mg ml}^{-1}$  ( $n = 10$ ),  
253  $0.373 \pm 0.030 \text{ mg ml}^{-1}$  ( $n = 10$ ),  $0.431 \pm 0.038 \text{ mg ml}^{-1}$  ( $n = 7$ ) and  $0.342 \pm 0.022 \text{ mg}$   
254  $\text{ml}^{-1}$  ( $n = 10$ ) for Stages 1, 2, 3 and 4, respectively (Fig. 6B). In males, the mean levels  
255 of total protein were  $0.345 \pm 0.021 \text{ mg ml}^{-1}$  ( $n = 10$ ),  $0.354 \pm 0.034 \text{ mg ml}^{-1}$  ( $n = 10$ ),  
256  $0.424 \pm 0.033 \text{ mg ml}^{-1}$  ( $n = 10$ ) and  $0.427 \pm 0.037 \text{ mg ml}^{-1}$  ( $n = 3$ ) 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*

262 Figure 7 shows the correlation between GSI and total protein level in the coelomic  
263 fluids. In the wild population ( $n = 70$ ), the correlation coefficient was  $R^2 = 0.086$ , and a  
264 significant positive correlation was observed ( $P = 0.031$ ) (Fig. 7A). The correlation  
265 coefficient was  $R^2 = 0.382$ , and a significant positive correlation was observed ( $P =$   
266  $0.00014$ ) in females ( $n = 37$ ); however, a positive correlation was not observed ( $P =$   
267  $0.425$ ) between GSI and total protein level in males ( $n = 33$ ) (Fig. 7B, C).  
268

269 **Discussion**

270

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

279 The GSI significantly increased at Stage 2 (growth) and Stage 3 (pre-mature) in  
280 the wild population (Fig. 1A). The GSI increased from Stage 1 (recovering) to Stage 2  
281 or Stage 3 in females and males, and then that gradually decreased in Stage 4 (mature)  
282 (Fig. 1B, C). In wild sea urchins *Paracentrotus lividus* collected off southern France the  
283 GSI peaked at Stage 3 and thereafter decreased (Spirlet et al., 1998). Agatsuma et al.  
284 (1988) likewise examined wild *M. nudus* (previously known as *Strongylocentrotus*  
285 *nudus*) from southern Hokkaido, and similarly reported that GSI peaked around Stage 3,  
286 and then decreased during the spawning season.

287 To develop an assay system to measure MYP in sea urchin, we performed  
288 purification of NP-MYP and obtained a specific antiserum against NP-MYP. Purity of  
289 the NP-MYP was assessed by disc-PAGE and immunoelectrophoresis. The purified  
290 NP-MYP yielded one band in disc-PAGE and a single precipitin line when reacted  
291 against a polyvalent antiserum to gonad extract as well as when it was precipitated by  
292 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

317 digestive tract, gonads and coelomocytes (Unuma et al., 2001). The coelomocytes of sea  
318 urchins are classified as four types: phagocytes, vibratile cells, and red and white  
319 morula cells (Bertheussen and Seljelid, 1978; Gerardi et al., 1990; Unuma et al., 2010).  
320 Unuma et al. (2010) observed the expression of MYP mRNA mainly in vibratile cells  
321 and white morula cells in sea urchin. Without anticoagulant solution, vibratile cells  
322 become immediately broken *in vitro* (Matsutani, 1995). This suggested the importance  
323 of collecting the coelomic fluid with an anticoagulant solution to determine CF-MYP  
324 concentrations in sea urchins, as carried out in the present study. Accordingly, we  
325 examined changes in the concentration of CF-MYP during the reproductive cycle in  
326 both males and females. Furthermore, we were aware of no previous reports using an  
327 assay system for profiling CF-MYP during the reproductive cycle in adult sea urchins.  
328 In this study, changes in the concentration of MYP in the coelomic fluids of both sexes  
329 during the reproductive cycle were determined by SRID (Fig. 4), and significant  
330 changes were found for both female and male sea urchins. A positive correlation  
331 between CF-MYP levels and GSI was observed in females, but not in males (Fig. 5). In  
332 an exquisitely designed *in vivo* experiment, Unuma et al. (2007) found that CF-MYP is  
333 taken up by nutritive phagocytes in the gonads, and is finally transported into the  
334 growing oocytes in females. In teleost fishes, the level of serum vitellogenin (an MYP  
335 precursor) showed a significant positive correlation with GSI (Mushirobira et al., 2013).  
336 These results indicate that CF-MYP is suitable as a biomarker of the onset of puberty  
337 and the progression of sexual maturity in female sea urchins, similar to vitellogenin in  
338 fishes. Whereas, in males, the level of CF-MYP significantly increased and reached a  
339 peak at Stage 3, it did not positively correlate with GSI; therefore, CF-MYP is not  
340 suitable as a biomarker of the progression of sexual maturity in male sea urchins. It is



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

345 We also examined changes in the concentration of total proteins in the  
346 coelomic fluids of both sexes of wild sea urchin during the reproductive cycle. In the  
347 wild population, total protein concentration increased from Stage 1 and peaked at Stage  
348 3, and thereafter decreased to a low level at Stage 4 (Fig. 6). In a natural population of  
349 *Strongylocentrotus purpuratus* the concentration of total proteins in the coelomic fluid  
350 increased with an increasing GSI, and then decreased at a mature stage, with total  
351 protein concentration in the coelomic fluid reported as 0.20–0.45 mg ml<sup>-1</sup> (Holland et  
352 al., 1967). In this study, the concentration of total proteins in the coelomic fluids were  
353 0.17–0.65 mg ml<sup>-1</sup> in wild *M. nudus*, a range similar to that found for purple sea urchin  
354 as reported by Holland et al. (1967). However, the observed changes in the  
355 concentration of total proteins in the coelomic fluids of females and males were not  
356 significant. A significant positive correlation between total protein level and GSI was  
357 observed for females, but not for males. Moreover, the strong positive correlation  
358 between total protein level and GSI in females, and not between CF-MYP and GSI,  
359 suggests that CF-MYP and another protein increases as gametogenesis proceeds in  
360 females. However, the identification and characterization of proteins in the coelomic  
361 fluids of sea urchins before maturity of the gametes remains to be done.

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

365

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375

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457

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  $\pm$   
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  $\pm$   
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  $\pm$  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  $\pm$  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



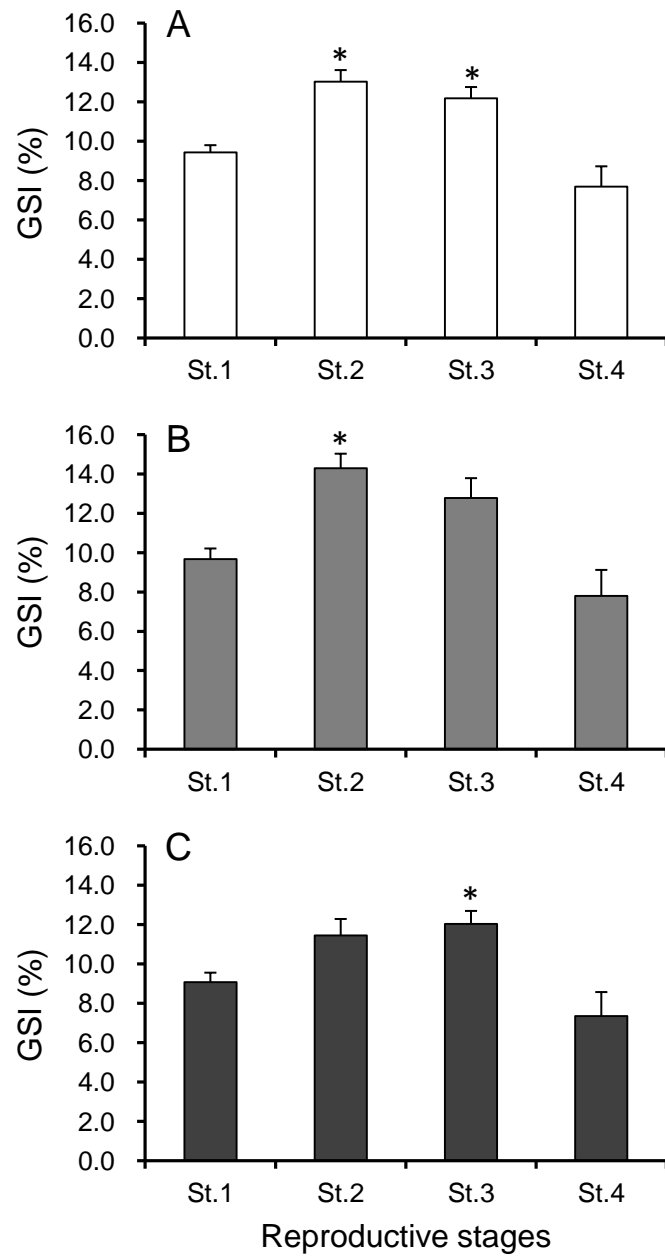


Fig. 1

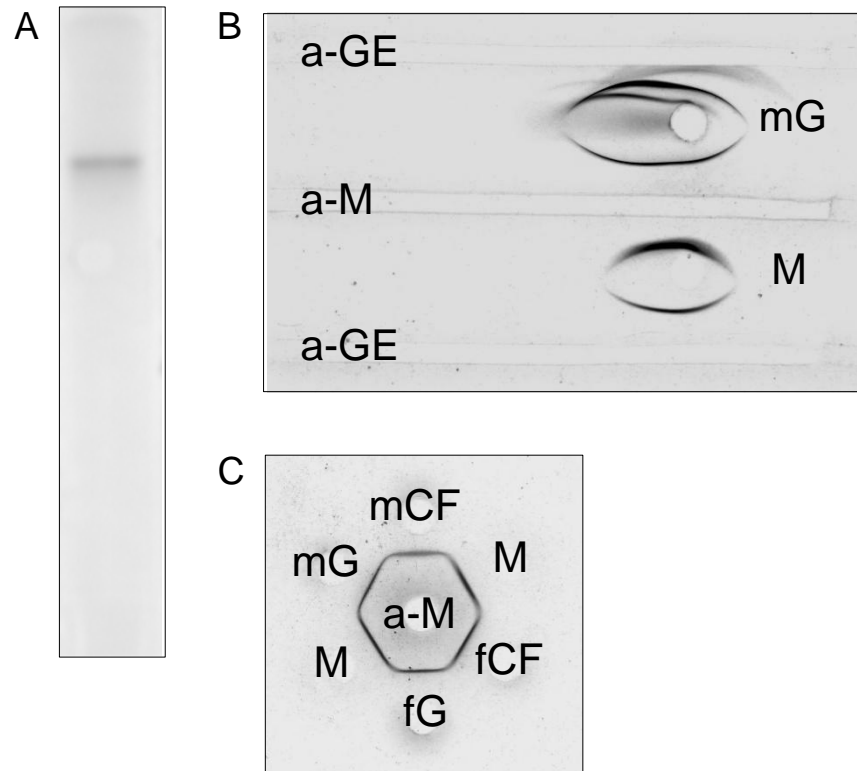


Fig. 2

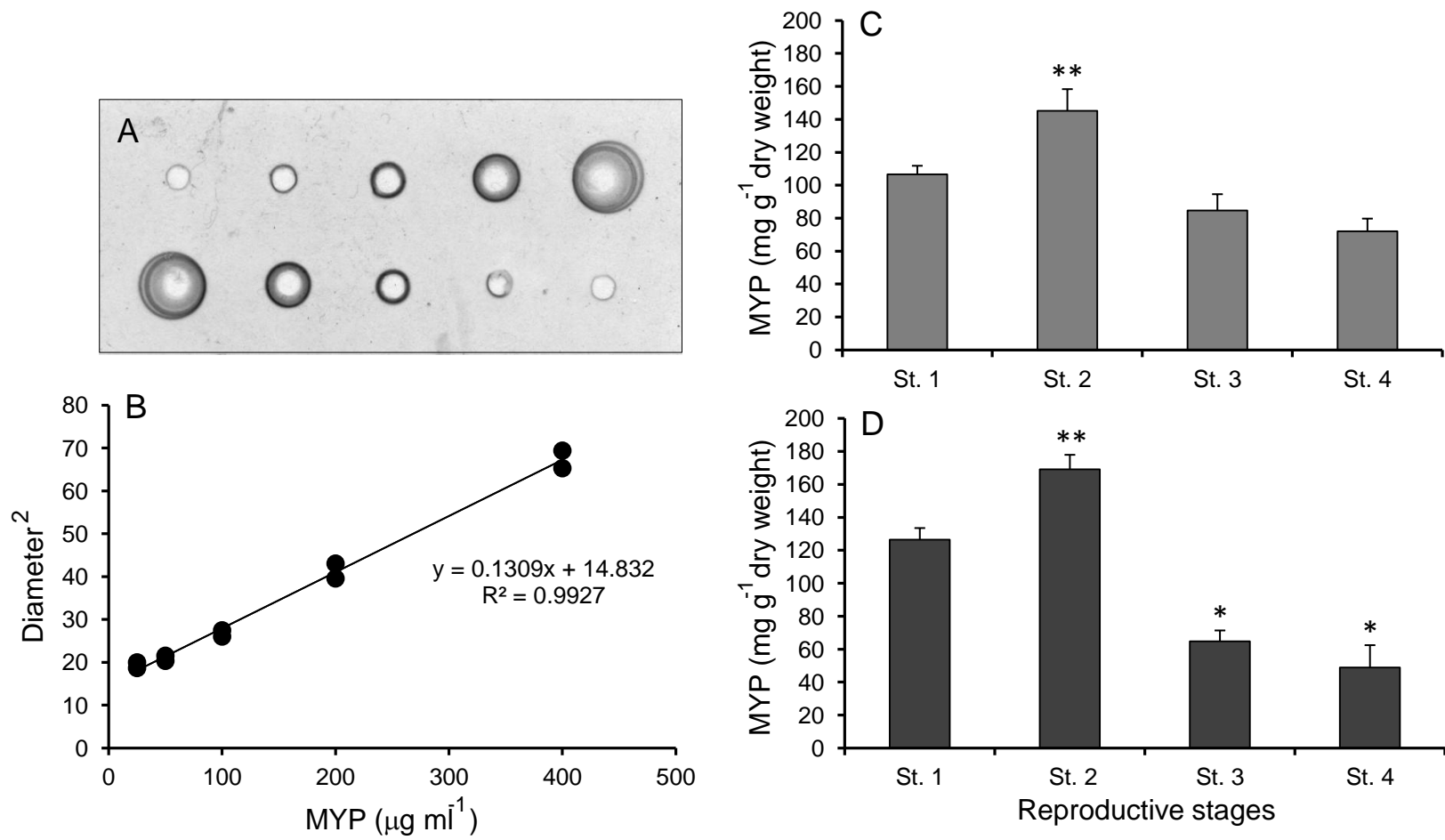


Fig. 3

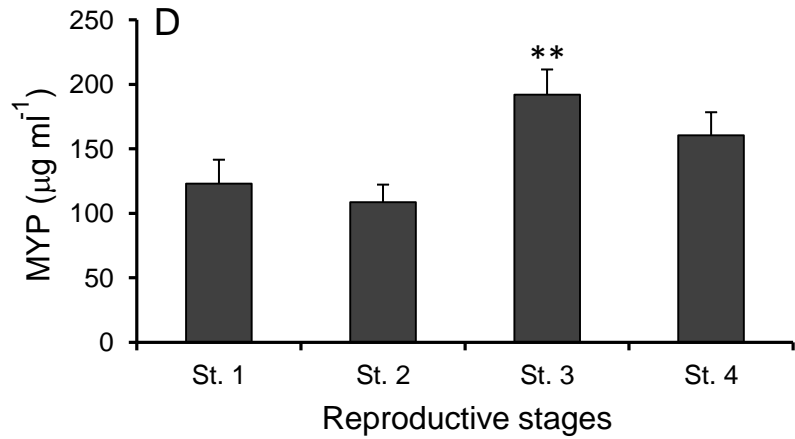
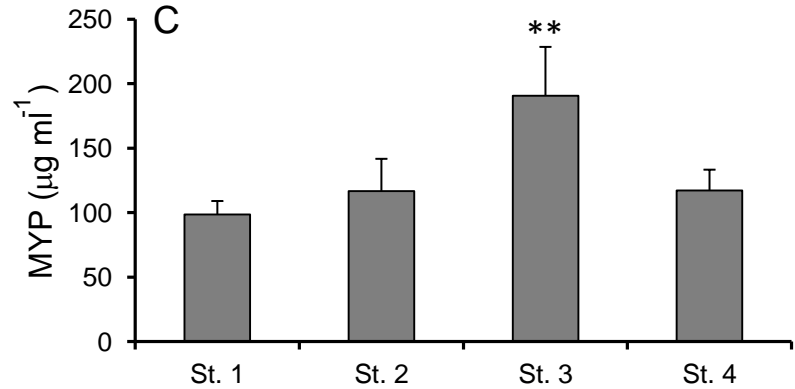
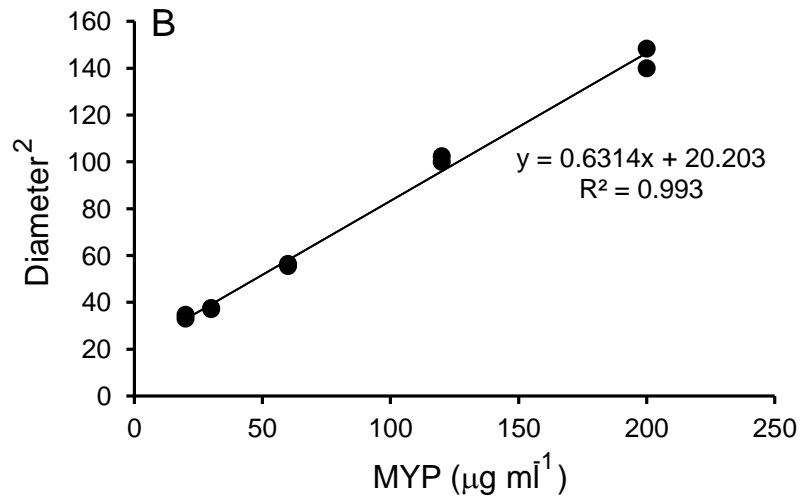
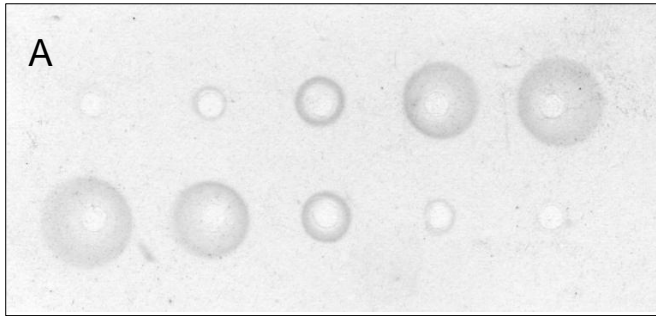


Fig. 4

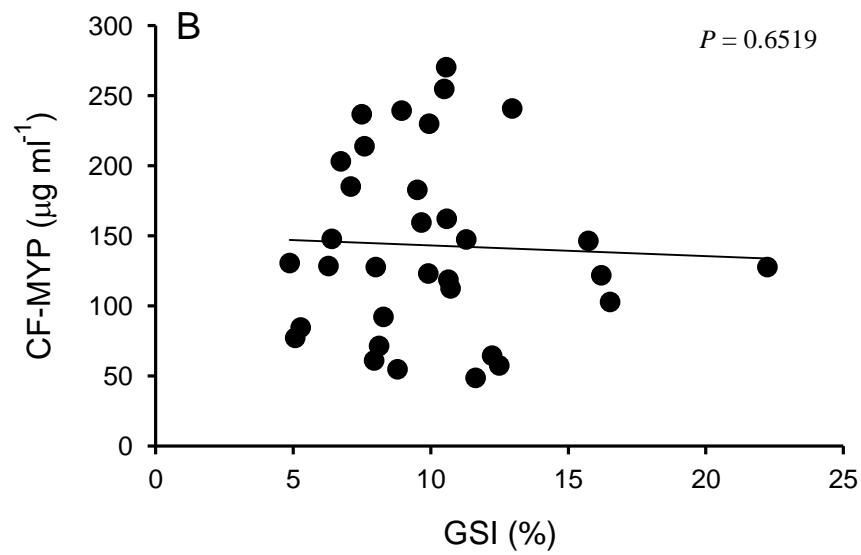
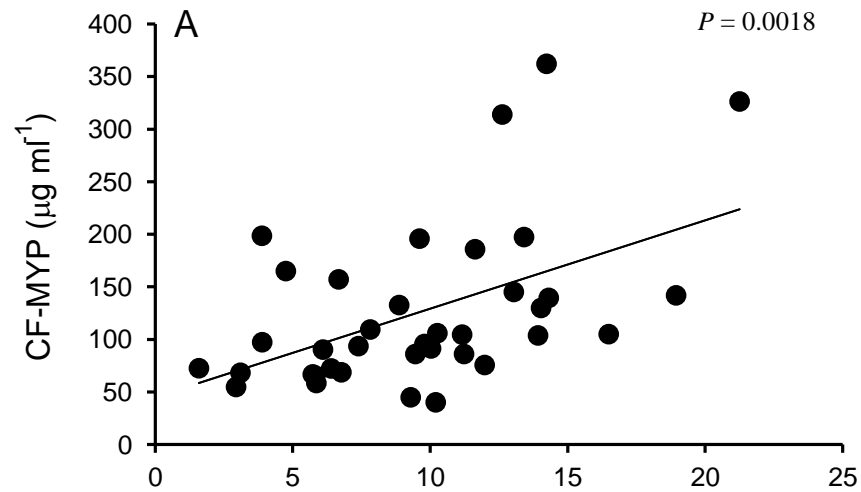


Fig. 5

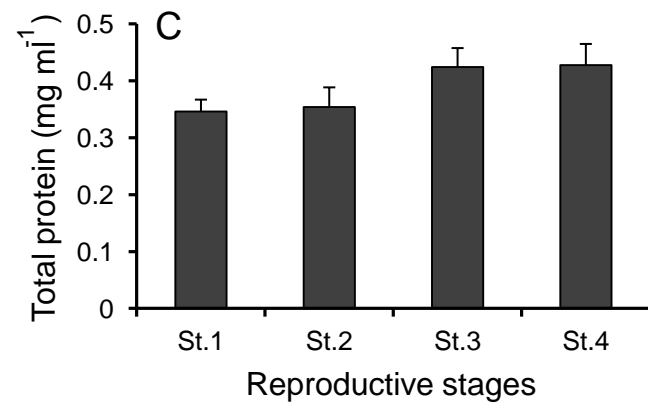
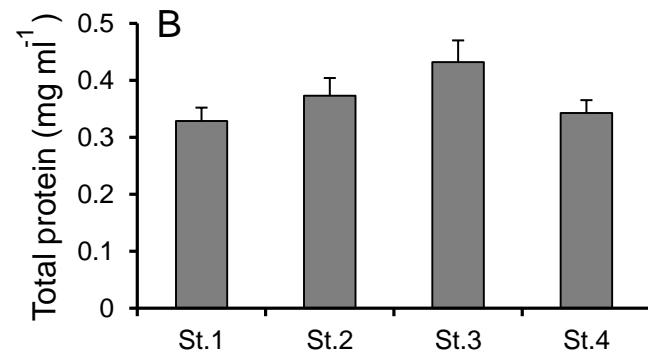
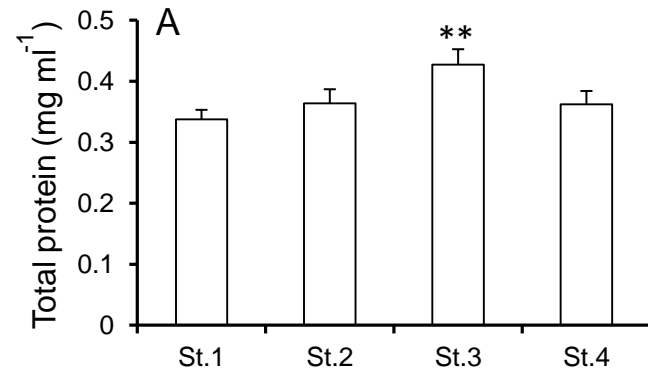


Fig. 6

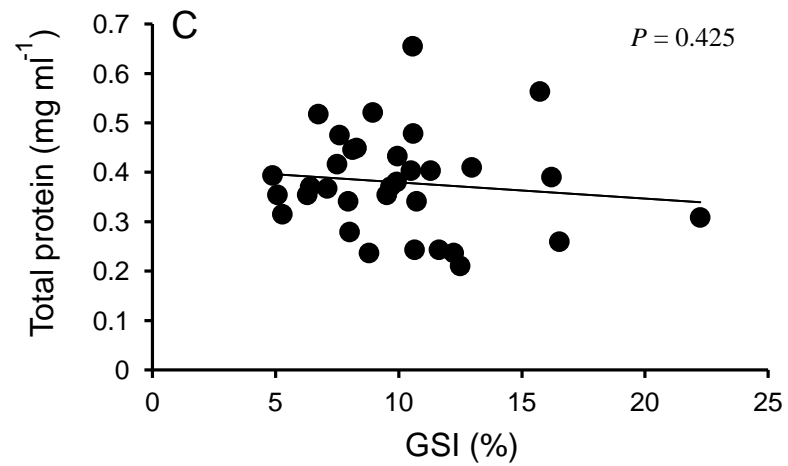
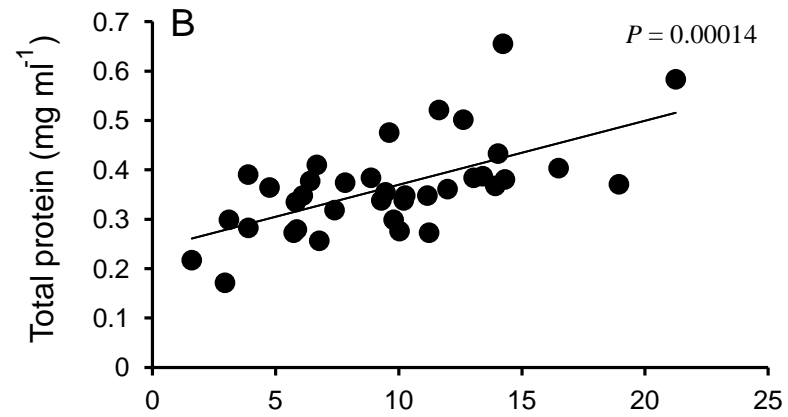
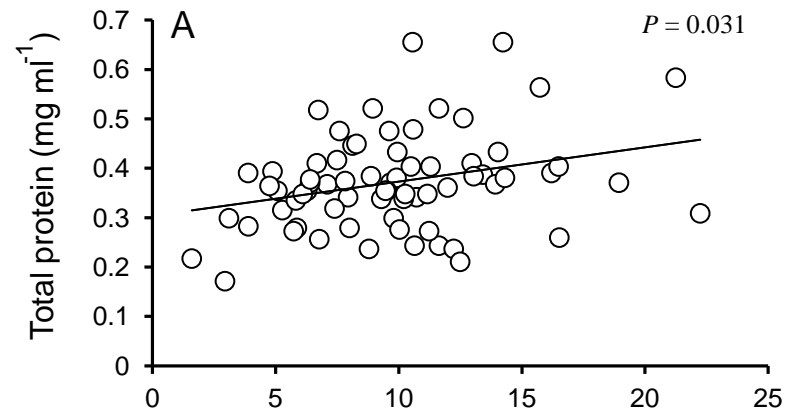


Fig. 7