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The reproductive cycle and transcription-level changes in the major yolk protein of wild northern sea urchin, *Mesocentrotus nudus*, in southern Hokkaido

Kazuhiro URA[1,*], Heng WANG[1], Tetsuo HORI[1], Shunsuke AIZAWA[1], Shiori TSUE[1], Mizuha SATOH[1], Narumi TAKEI[1], Kosuke HOSHINO[1], Ichiro HIGUCHI[1], Shun SANUKI[1], Tomoharu YUHI[1], Osamu NISHIMIYA[1] and Yasuaki TAKAGI[1]

Abstract: We examined the reproductive cycle and transcription-level changes of the major yolk protein (MYP) in gonads of both sexes during the different reproductive stages of wild northern sea urchin, *Mesocentrotus nudus*, collected in two areas of southern Hokkaido, Japan. Histological evaluation revealed mature gonads in both April and August, suggesting two annual spawning seasons at Otoshibe, whereas one annual spawning season was observed in autumn (September) at Usujiri. The transcription levels of MYP, which is synthesized and stored in the gonads, increased from stage 1 (recovering) to stage 2 (growth) and thereafter decreased, in both female and male sea urchins. The current results indicate that two annual spawning seasons may occur in wild populations of the northern sea urchin in southern Hokkaido.

Key words: Gonads; *Mesocentrotus nudus*; Reproductive cycle; Sea urchin; Yolk protein

Introduction

Sea urchins are commercially important animals in Japan, where their gonads (also known as the roe) are commonly eaten as a seafood. The sea urchin catch from Hokkaido comprises about 50% of the total Japanese catch, making sea urchins an important coastal fishery resource (Ministry of Agriculture, Forestry and Fisheries, Japan, 2015). The short-spined sea urchin *Strongylocentrotus intermedius* and the northern sea urchin *Mesocentrotus nudus* are the main targeted urchin species in Hokkaido's coastal fisheries. Only the immature gonads of males and females are consumed since the gonads of mature individuals taste bitter. Although most sea urchins spawn once per year, mature gonads have been detected in both spring and autumn in populations around Hokkaido (Agatsuma 2013), but the cause of this phenomenon is unclear.

Sea urchin gonads are made up of germ cells and nutritive phagocytes. The nutritive phagocytes store nutrients for gametogenesis in each of the sexes (Walker 1982). Gonad size increases before gametogenesis owing to an accumulation of nutrients, such as lipids, proteins and polysaccharides, in the nutritive phagocytes. During gametogenesis, the number of nutritive phagocytes gradually decreases as the lumen fills with mature ova or sperm (Walker et al. 2001; Unuma et al. 2003). Several previous investigators have described seasonal changes in sea urchin gonadal development using histological analysis (Fuji 1960; Sugimoto et al. 1982; Agatsuma et al. 1988; Unuma et al. 1996).

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[1] Laboratory of Comparative Physiology, Graduate School of Fisheries Science, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan.
[*] Corresponding author: Tel./Fax (+81)138-40-5551; E-mail, kazu@fish.hokudai.ac.jp (K. Ura).

The main protein contained in immature gonads of both sexes is a glycoprotein called the major yolk protein (MYP). In both sexes, the MYP is synthesized and stored in nutritive phagocytes before gametogenesis (Harrington and Easton 1982; Yokota and Kato 1988; Unuma et al. 1998, 2001). The MYP stored in female gonads is transferred to ripe ova, whereas in males the MYP stored in the nutritive phagocytes disappears during spermatogenesis in the testis. Therefore, it has been noted that MYP plays an important physiological role during gametogenesis in both sexes of sea urchin (Ozaki et al. 1986; Unuma et al. 1998, 2003). The cDNA encoding the MYP of gonad has been reported for several species of sea urchin, including *Pseudocentrotus depressus* (AB368934), *Hemicentrotus pulcherrimus* (AB097218), *Strongylocentrotus purpuratus* (AY090112), *Strongylocentrotus intermedius* (AB192414), *Mesocentrotus nudus* (DQ102372, LC175467), *Tripneustes gratillia* (AY026514), and *Lytechinus variegatus* (AY090113), where it is understood to be a transferrin-like protein (Unuma et al. 2001; Brooks and Wessel 2002; Yokota et al. 2003). MYP mRNA is expressed in the testes or ovaries, digestive organ, and coelomocytes, and the major synthesis sites are the gonads and digestive organs in both sexes of sea urchin (Yokota et al. 2003; Unuma et al. 2010).

To date, changes in MYP levels only during gonadal growth and gametogenesis have been reported for artificially stocked populations of sea urchins (Unuma et al. 2003), while the MYP expression profile during gonadal growth and gametogenesis in wild populations remains unclear. Thus, in the present study, we examined changes in transcript levels of MYP in gonads during gametogenesis in northern sea urchin (*M. nudus*) collected from southern Hokkaido, Japan. Furthermore, seasonal variations in the gonadal growth and gametogenesis were observed histologically. To our knowledge, this is the first report on changes in transcript levels of MYP during the gametogenic cycle of wild sea urchins.

Materials and Methods

Animals

The samples of northern sea urchin *Mesocentrotus nudus* were collected by diving at Otoshibe, Hokkaido, Japan, at a depth of approximately 5 m, from March to August 2012; the same species was collected at Usujiri, at approximately 10 m depth, from May 2015 to October 2016. Sea urchins were transported to and dissected at the Faculty of Fisheries Sciences of Hokkaido University, where the gonads were excised and weighed (Tables 1 and 2). 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 quickly frozen in liquid nitrogen and stored at -80°C until analysis.

The tissue samples were dehydrated through a graded ethanol series before being embedded in paraffin; 6-μm-thick serial sections were mounted on glass slides and stained with hematoxylin and eosin. The stained sections were observed with a differential interference contrast microscope (Eclipse E800, Nikon; Tokyo, Japan) connected to an LV-TV camera (Nikon). The gonadal maturity of each animal was classified according to five stages, following the methods of Unuma et al. (1996): stage 1 (spent and 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.

Quantitative real-time RT-PCR (RT-qPCR) of MYP genes in the gonads

Total RNA was extracted from the frozen gonad samples taken from Usujiri sea urchins, using ISOGEN, following the manufacturer's instructions. From total RNA, poly(A)$^+$ RNA (mRNA) was isolated by oligo(dT)-latex beads (Oligotex-dT30; Takara, Japan), according to the manufacturer's instructions. The concentration and quality of the extracted mRNA was

determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer; Thermo Fisher Scientific, USA). Quantified mRNA samples were used for RT-qPCR. Real-time RT-PCR was carried out using an ABI Prism 7300 system (Applied Biosystems). Reactions were performed in 25-µl volume with 1 ng of mRNA, using a one-step real-time RT-PCR kit according to the manufacturer's instructions (One Step SYBR PrimeScript RT-PCR Kit; Takara, Japan). Melting curve analysis was performed for each sample in order to check for the presence of a single amplicon. The qPCR primers were designed from the MYP of northern sea urchin (LC175467) as follows: sense, 5′-GTTCAAGGATCAGACCGGCA-3′; antisense, 5′-GCAGGTAACGTCCTTCACGA-3′ (product size, 100 bp).

Statistical analysis

Data on transcript abundance are presented as relative copy number/ng mRNA; values are reported as mean ± SD. Statistical differences between means across months were determined by one-way ANOVA and subsequent Tukey's multiple-range test.

Results

Test diameter, body weight, and gonad weight of the sea urchins

Table 1 shows the monthly variations in mean test diameter (TD), body weight (BW), and gonad weight (GW) of the sea urchins collected at Otoshibe, from March to August 2012; Table 2 shows the monthly variations in these means for the sea urchins collected at Usujiri, from May 2015 to October 2016.

GSI of the sea urchins

Mean GSIs during the survey period at each location are shown in Fig. 1. At Otoshibe, the GSI of sea urchins increased gradually from March (16.9 ± 3.1) to August (22.6 ± 4.4); the GSI increased significantly in June and August as compared with the value March (Fig. 1A). At Usujiri, the GSI of sea urchins increased from May (11.6 ± 3.9) to July 2015 (14.1 ± 2.2); in 2016, the GSI increased from March (10.0 ± 2.0) and peaked in July (13.1 ± 6.4), then gradually decreased by October. The GSI in July was a significant increase as compared with the mean in March 2016 (Fig. 1B).

Table 1. Biometrics of *Mesocentrotus nudus* at Otoshibe during the survey period

Month	Test diameter (TD: mm)	Body weight (BW: g)	Gonad weight (GW: g)	Number
March	82.95 ± 6.14	181.61 ± 41.80	30.77 ± 9.12	N = 32
April	77.80 ± 9.67	174.88 ± 59.28	25.74 ± 8.78	N = 82
June	79.96 ± 10.36	192.38 ± 65.38	38.11 ± 13.09	N = 103
August	75.34 ± 10.27	167.41 ± 58.74	38.09 ± 13.71	N = 87

Table 2. Biometrics of *Mesocentrotus nudus* at Usujiri during the survey period

Year	Month	Test diameter (TD: mm)	Body weight (BW: g)	Gonad weight (GW: g)	Number
2015	May	80.15 ± 7.38	179.65 ± 47.71	21.21 ± 7.83	N = 15
2015	July	74.87 ± 5.84	164.54 ± 39.24	22.78 ± 4.31	N = 15
2015	October	76.50 ± 9.75	161.64 ± 54.69	16.08 ± 6.14	N = 14
2015	December	74.15 ± 8.13	167.69 ± 51.35	18.61 ± 6.78	N = 30
2016	March	68.17 ± 7.56	136.56 ± 37.00	13.35 ± 4.58	N = 29
2016	July	75.52 ± 8.28	162.13 ± 53.65	23.43 ± 14.68	N = 29
2016	September	78.47 ± 6.43	184.70 ± 29.29	18.83 ± 7.27	N = 30
2016	October	71.17 ± 9.87	139.26 ± 47.49	6.01 ± 4.25	N = 30

Changes in gonadal development

Histological examinations were used to determine the monthly change in relative frequency of each gonadal stage among the sea urchins collected (Fig. 2). At Otoshibe, only stages 1 and 2 were present in March 2012, at 88% and 12%, respectively. In April, the relative frequency of stage 2 had increased, and stages 3 and 4 also appeared. By June, stage 2 became dominant (58%). Finally, in August, stage 1 was undetected, stage 2 was negligible, stage 3 became dominant (75%), and stage 4 also appeared (22%) (Fig. 2A).

At Usujiri, in 2005, stages 1 and 2 were dominant in May, at 33% and 60%, respectively. In July, stages 2 and 3 were dominant, at 40% and 53%, respectively. In October 2005, stage 3 was dominant (64%) and stage 4 was also observed (21%). By December, stage 1 was overwhelmingly dominant, at 90%. In March 2006, stages 1 and 2 were more equally present, at 59% and 41%, respectively. In July, stage 2 was most abundant at 48%. In September, stage 3 was dominant (77%) and stage 4 had appeared (13%), although stages 1 and 2 were

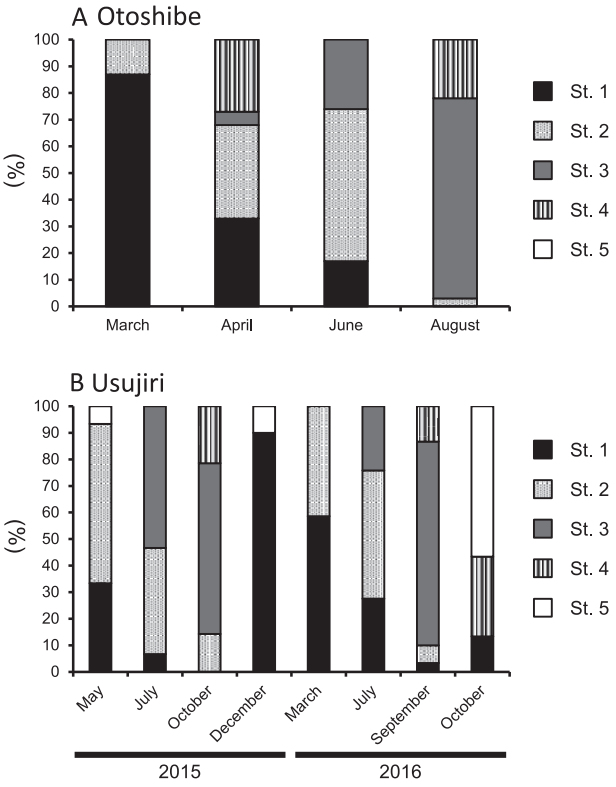

Fig. 2. Relative frequencies of the fine different gonadal maturation stages of wild northern sea urchins at Otoshibe in 2012 (A) and at Usujiri in 2015–2016 (B).

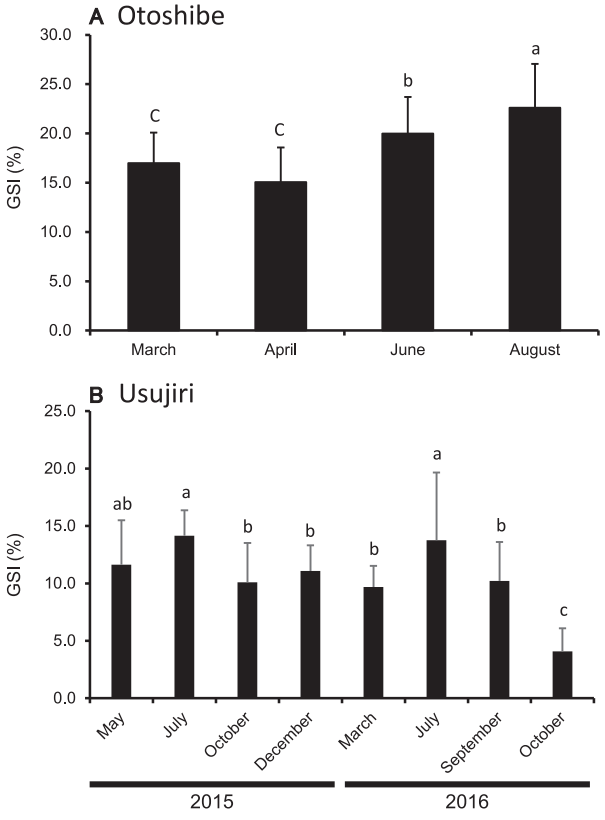

Fig. 1. Annual changes in the gonadosomatic index (GSI) of northern sea urchin at Otoshibe (A) and Usujiri (B), southern Hokkaido, Japan. Values are mean ± standard deviation. Different letters above the bars indicate significantly different means.

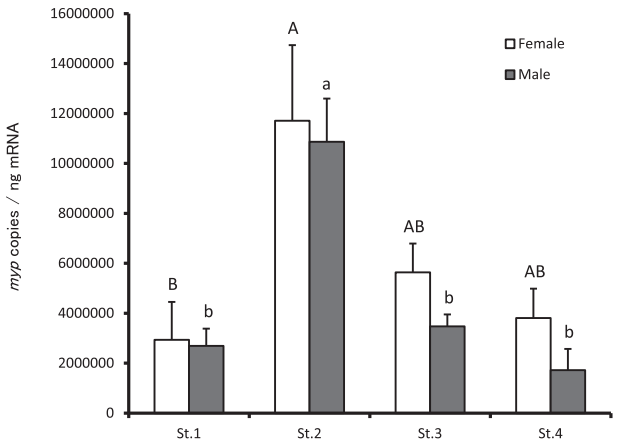

Fig. 3. Quantitative changes in major yolk protein (MYP) mRNA in gonads of male and female wild northern sea urchin collected in Usujiri at different gametogenic stages. Each value is the mean of 4 or 5 animals. Vertical lines indicate standard deviation. Different capital or small letters above the bars indicate significantly different means in the females or males, respectively.

still negligibly present. By October, stages 4 and 5 became dominant, at 30% and 59%, respectively (Fig. 2B), and the remainder belonged to stage 1.

Gonadal MYP levels

Levels of MYP mRNA at each gonadal stage in both sexes of the sea urchins collected at Usujiri are shown in Fig. 3. In males, the MYP mRNA level increased dramatically from stage 1 ($2.69 \times 10^6 \pm 0.69 \times 10^6$ copies, $n = 5$) to stage 2 ($10.86 \times 10^6 \pm 1.72 \times 10^6$ copies, $n = 4$), then drastically decreased again by stage 4 ($1.72 \times 10^6 \pm 0.84 \times 10^6$ copies, $n = 4$). In females, the MYP mRNA levels also increased significantly from stage 1 ($2.93 \times 10^6 \pm 1.51 \times 10^6$ copies, $n = 5$) to stage 2 ($11.71 \times 10^6 \pm 3.02 \times 10^6$ copies, $n = 5$), and thereafter decreased gradually at stage 3 ($5.63 \times 10^6 \pm 1.16 \times 10^6$ copies, $n = 5$) to stage 4 ($3.80 \times 10^6 \pm 1.17 \times 10^6$ copies, $n = 5$). For both females and males, no significant difference was found in the gonadal MYP mRNA levels at each stage of gametogenesis.

Discussion

Gonadal maturation in sea urchins is regulated primarily by water temperature, photoperiod, and feeding conditions (Pearse et al. 1986; Agatsuma et al. 1988; Yamamoto et al. 1988; Sakairi et al. 1989; McClintock and Watts 1990). Gonadal maturation in sea urchins is correlated with a greater GSI, which is highest just before spawning (Agatsuma et al. 1988; Unuma et al. 1996). In the present study, the GSI values increased from March to August, before spawning of wild populations in September at Otoshibe and Usujiri, southern Hokkaido. Moreover, the GSI of wild northern sea urchins captured at 10 m depth was lower than that of individuals captured at 2 m depth (Agatsuma et al. 1988). In the present study, average GSI values for sea urchins collected at Otoshibe were higher than that for sea urchins at Usujiri, which had been collected at deeper sites. Nonetheless, the mean GSI values increased gradually from spring to late summer among urchins from both study areas.

Agatsuma et al. (1988) reported that gonadal maturation (stage 4) peaks in July–September and that spawning among wild northern sea urchins in southern Hokkaido is complete by late September. In this study, at Otoshibe, mature gonads (stage 4) were observed mainly in August, indicating a September spawning season in this area, as reported by Agatsuma et al. (1988). However, in our study, mature gonads (stage 4) were also observed in April for Otoshibe sea urchins, suggesting there are two annual spawning seasons among populations there. This is the first report of this phenomenon for wild northern sea urchins in an area of southern Hokkaido. For Usujiri sea urchins, mature gonads (stage 4) were observed only in September–October, suggesting one annual spawning season there, as reported by Agatsuma et al. (1988). Thus, in the present study, mature gonads were uniquely detected in Otoshibe in springtime (April); this finding merits further investigation to determine the influences of this phenomenon among wild northern sea urchins in southern Hokkaido.

Gonad size begins to increase during stages 1 and 2, as nutrients, such as lipids, proteins, carbohydrates and polysaccharides, accumulate in the nutritive phagocytes. These stored nutrients are used for gametogenesis as the numbers of nutritive phagocytes begin to decrease in the gonads of both sexes (Walker et al. 2001; Unuma et al. 2003). Active protein synthesis occurs before gametogenesis in stages 1 and 2. MYP is the leading protein produced in sea urchin gonads (Unuma et al. 2003). As oogenesis proceeds in females, MYP is transferred from nutritive phagocytes to the ripe ova to form yolk granules (Ozaki et al. 1986); as spermatogenesis proceeds in males, most of the MYP disappears from the testis (Unuma et al. 1998). Therefore, it is generally accepted that MYP has a significant role as a nutrient for gametogenesis in sea urchins. Unuma et al. (2010) reported that levels of MYP mRNA in the gonad of cultured red sea urchin were higher at stage 1 than at stage 3; however, no information is available concerning changes in MYP transcript levels in the gonads of either

sex during gametogenesis in red sea urchins. Among wild northern sea urchins in the present study, the MYP transcript level began to increase during stage 1, peaked at stage 2, and then decreased during gametogenesis (stages 3 and 4) in gonads of both females and males. However, the MYP transcript level decreased gradually in females, while it decreased drastically in males. At the protein level, using cultured red sea urchin, Unuma et al. (2003) likewise reported that MYP decreased gradually as oogenesis proceeded in females, while it decreased rapidly as spermatogenesis progressed in males.

To our knowledge, this is the first study to examine changes in the MYP transcript level during gonadal growth and gametogenesis in wild sea urchins. Changes in the amounts of MYP at the protein level in wild sea urchin, however, are still unclear. Further work is needed to determine the relationship between changes in the transcript and protein levels during the different reproductive stages of wild sea urchins.

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北海道南部におけるキタムラサキウニ (*Mesocentrotus nudus*) の生殖周期と主要卵黄タンパク質 mRNA 量の変化

浦　和寛・Heng WANG・堀　哲郎・相澤俊介・津江志緒莉・佐藤瑞葉・武井成美
星野航佑・樋口一郎・讃岐　駿・由比智春・西宮　攻・都木靖彰

北海道南部の海域に生息するキタムラサキウニの生殖周期と生殖巣に含まれる主要卵黄タンパク質の mRNA 量の変化を調べた。落部海域に生息するキタムラサキウニでは，4 月と 8 月に成熟する個体が観察され，年に 2 回放卵・放精が行われている可能性が示された。一方，臼尻海域では秋にのみ成熟個体が観察された。また，生殖巣で合成・蓄積される主要卵黄タンパク質の mRNA 量は，雌雄共に回復期から成長期にかけ増加し，配偶子形成の進行に伴い減少した。以上の結果から，北海道南部の海域によっては本種の放卵・放精が年に 2 回行われている可能性が示された。