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Gonad Maturation Cycle of the Sea Urchin *Strongylocentrotus nudus* Population Inhabiting an Artificial Seaweed Forest, Samchuk, Korea

Su Kyoung Kim[1]*, Young Dae Kim[2], Qtae Jo[2], Jong Ha Lee[2], Chu Lee[1] and Chae Sung Lee[2]

[1]*Aquaculture Management Division, NFRDI, Busan 619-705, Korea*
[2]*East Sea Fisheries Research Institute, NFRDI, Gangneung 210-861, Korea*

We determined the seasonal gonad maturation in *Strongylocentrotus nudus* sea urchins inhabiting an artificially enhanced seaweed forest along the Samchuk Coast of Korea from April 2006 to March 2007. A total of 30 sea urchins per month were collected from the study area, and gonadosomatic index (GSI), gonad index (GI), egg diameter, and RNA/DNA variation were measured for each specimen. GSI values of female and male urchins achieved maximums of 17.6 and 17.0, respectively, in June. Based on histological studies, maximum GI values occurred in July (4.6 for females and 4.8 for males). A mean ovarian egg diameter of 73.7 ± 14.2 μm was measured in August; during the main spawning period in September, mean egg diameter reached a maximum of 74.2 ± 17.8 μm. The RNA/DNA ratio and RNA content for both males and females showed a distinct peak during the ripe stage in July, but another peak occurred in the spring season from March to April, when urchins deposit protein into the nutritive phagocytes of immature gonads prior to gametogenesis. The reproductive cycle of *S. nudus* is divided into five stages: early active (December-May), late active (March-July), ripe (July-September), spent and degenerative (August-November), and inactive (October-February). Our continuous removal of sea urchins from the study area did not influence the reproductive cycle, as populations quickly recovered, and achieved normal gonad development cycle in the site.

Key words: Sea urchin, *Strongylocentrotus nudus,* Gonad maturation, RNA/DNA, GSI, GI

Introduction

Seaweeds are important primary producers that act as CO_2 sinks. Although they cover only 0.1% of the ocean bottom, seaweeds account for approximately 5% of global primary production (Smith, 1981). Recently, however, the macroalgal contribution to primary production has decreased because of the invasion of crustose coralline algae, a competitor for space (Ayling, 1981). Higher feeding pressure by herbivores has provided another force driving reductions in coastal macroalgae (Lawrence, 1975; Ichiki et al., 2000).

In many areas of coastal Korea, crustose corallines dominate the algal community. Visibly present seaweeds are annual and small, as herbivory keeps perennial macroalgae small and unnoticeable. The destruction of commercially important algae on coasts and expansions of barren ground have also induced a decline in marine production. For this reason, artificial reef installations and seaweed forest rebuilding are in progress, both locally and nationally. One threat to seaweed forests is the echinoid *Strongylocentrotus nudus*, which is a dominant herbivore that chiefly inhabits intertidal rocky habitats and subtidal areas to depths of 150 m. To promote the development of marine forests, control of sea urchin populations around artificial reefs is extremely important. Physical interventions (e.g., removal) should ideally be guided or supported by knowledge of the biology and physiology of a threatening organism. For example, the seasonal maturation cycle and spawning patterns of each local sea urchin population will vary according to geographical position, climate change, temperature, photoperiod, barren ground enlargement, and reduction of algae (Gonor, 1973; King et al., 1994; Kelly, 2001).

Researchers have paid particular attention to

*Corresponding author: sk6333@korea.kr

extensive grazing on various kelps and other large brown algae in algal communities over a broad geographical range (Lawrence, 1975; Schiel, 1982). Many contemporary studies of herbivores, especially sea urchins and abalone, have focused on estimating their grazing effects, food preferences (Lawrence, 1975; Ogden, 1976; Lawrence and Sammarco, 1982; Wessels et al., 2006; Yoo et al., 2004; Kim et al., 2007), and population control by predation (Hagen, 1995). Several researchers have studied annual gonad maturation, egg development, and larval development of the sea urchin *Strongylocentrotus intermedius* (Yoo et al., 1982; Lee et al., 2003). Studies have also examined age determination in *Anthocidaris crassispina* and growth of *Pseudocentrotus depressus* (Hong and Chung, 1998; Chung et al., 2005) in Korea. However, little information is available for local populations of *S. nudus*, either from natural habitats or barren grounds.

The purpose of this study was to determine the maturation cycle of *S. nudus* in Samchuk (where seaweeds are continuously replanted in barren ground areas) using histological, RNA/DNA, and protein analyses.

Materials and Methods

Seaweed sampling and analysis

Fig. 1 shows the study area (200 m^2 area, 10 m deep) in Samchuk, Korea, in which an artificial seaweed forest of *Ecklonia carva* and *Ecklonia stolonifera* has been maintained since 2005. Monthly samples were taken from three randomly placed quadrats (each 1 m^2) for the determination of total algal species and biomass (wet weight) around the artificial reefs from February to November 2006. All sampled algae were identified to the species level.

Sampling of sea urchin

Monthly sampling of sea urchins (100-200 individuals per month) started in April 2006. Thirty individuals per month were randomly sampled from the total collection for biometric measurements, such as diameter, length, height, and weight. Gonads were removed and measured individually, then weighed to the nearest 0.1 g with a Sartorius electric balance (Goettingen, Germany). Gonads were immediately freeze-dried for RNA/DNA and histological analyses. To determine spawning characteristics, five parameters were estimated or measured: gonadosomatic index (GSI), gonad index (GI), nucleic acid variation in the gonad, monthly change in egg size, and developmental stage.

Histological analysis

Gonads were fixed in Bouin's solution for histological analysis and processed using standard histological procedures to determine their development stage. Samples were embedded in paraffin (Automatic Tissue Processor: LEICA TP1020, Leica Instruments GmbH, Nussloch, Germany). The resulting paraffin blocks were sectioned at 6 μm into successive layers using a rotary microtome (LEICA 2125RT, Nussloch, Germany) and dyed using Harris's hematoxylin-eosin stain. Determination of histological gonad developmental stage was facilitated using an Image analyzer (Image Pro Plus 2.0; Image & Graphics) for classification into: early active stage (I), late active stage (II), ripe stage (III), spent and degenerative stage (IV) and inactive stage (V). The GSI was determined using the formula GSI=gonad weight/total weight ×100. GI determination followed Choi et al. (2006): GI=(number of stage I observed specimens×3)+(stage II×4)+(stage III×5)+(stage IV ×2)+(stage V×1)/total number of samples. To determine the relative frequency distribution of egg diameter, approximately 100 eggs were examined each month. Only eggs with nuclei cut through the center were included in the sample.

RNA/DNA analysis

Nucleic acid content was determined fluorometrically, as described by Belchier et al. (2004). Gonads were freeze-dried (-70°C, 24 h), weighed, and homogenized on ice in Tris-ethylenediaminetetraacetic acid (TE; 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) buffer solution (400 μL) with a hand pestle (duration 60 s). After centrifugation (10 min, 6000 rpm), nucleic acids were extracted and purified from the homogenate, and the total content was determined using ethidium bromide (EtBr, Sigma, Steinheim, Germany) dye. The RNA was then digested with RNAse (Roche, Mannheim, Germany), and the amount of DNA remaining was determined using EtBr.

Protein analysis

A Bio-Rad protein assay kit II (Bio-Rad, Hercules, CA, USA) was used for protein analysis. From samples homogenized in TE buffer for RNA and DNA analysis, 10 μL homogenate + 190 μL distilled water + 50 μL Coomassie Brilliant Blue (G-250) were mixed in a 96 well microplate and measured at 595 nm (UV spectrophotometer, Bio-Rad). A standard curve was prepared for a range of 1 to 32 μL/mL bovine serum albumin (BSA, 0.5 mL 2 mg/mL solution in water containing 2 mM sodium azide).

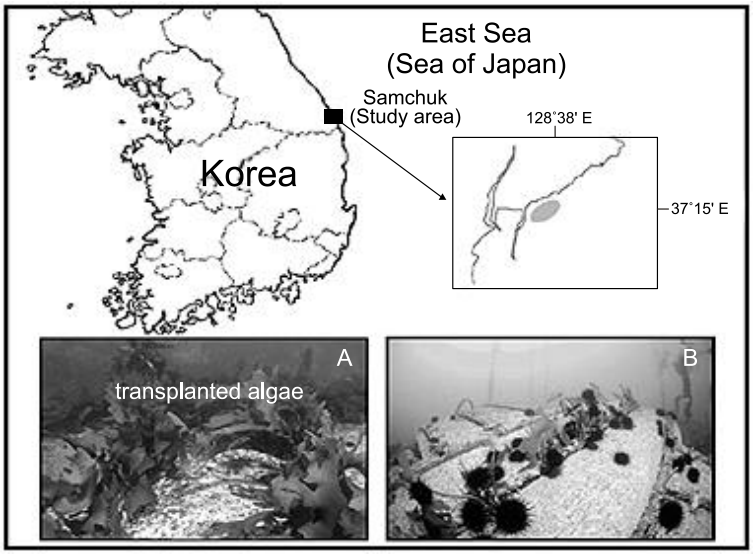

Fig. 1. Study area of Samchuk in East Sea (Sea of Japan), Korea. A; Transplanted *Ecklonia cava* (Kjellman) on the rock, where the barren ground was expanded. B; Aggregation of sea urchin on the transplanted algae.

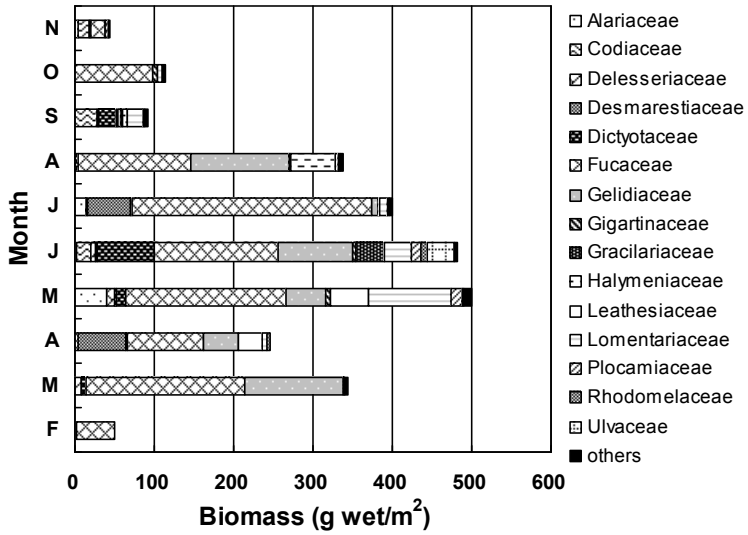

Fig. 2. Mean biomass of seaweeds around artificial seaweed forest area from February to November, 2006.

Statistical analysis

RNA and DNA concentrations, and their ratio in different gonad developmental stages, were subjected to ANOVA (STATISTIKA, StatSoft Inc., Tulsa, OK, USA). If significant differences were indicated at the 0.05 level, then Duncan's multiple range test was used to identify statistical significance.

Results

Seaweed population in the study area

A total of 54 algal species were identified: 32 species belonged to Class Rhodophyceae, 17 to Class Phaeophyceae, and 5 to Class Chlorophyceae. Of these, 38 species were categorized as small to mid-sized annual seaweeds (Fig. 2).

Seaweed biomass varied seasonally, highest in May (500.0 g), with *Sargassum horneri* accounting for 134.7 g. The dominant species differed slightly from month to month (Fig. 2). The most consistently dominant species were *S. horneri* and *S. confusum*. *Laminaria japonica* was recorded, but remained a minor species in the overall biomass.

Size and weight

Fig. 3 shows the monthly distribution of sea urchin

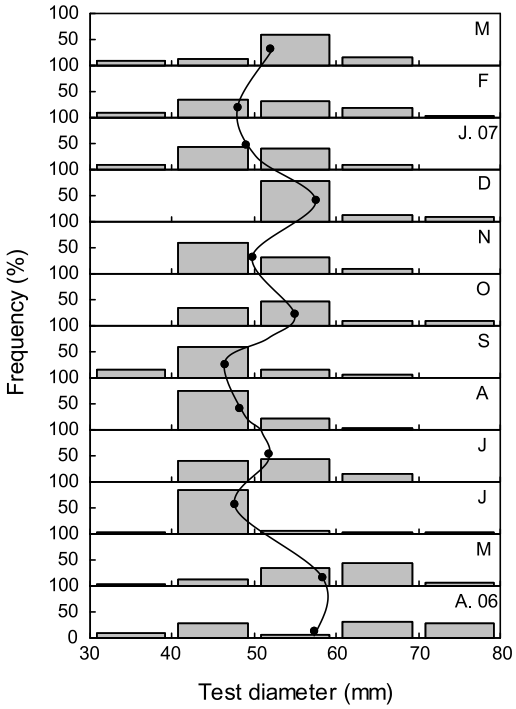

Fig. 3. Relative frequency distribution of test diameter (μm) of sea urchins. Line on the figure indicates the mean of test diameter.

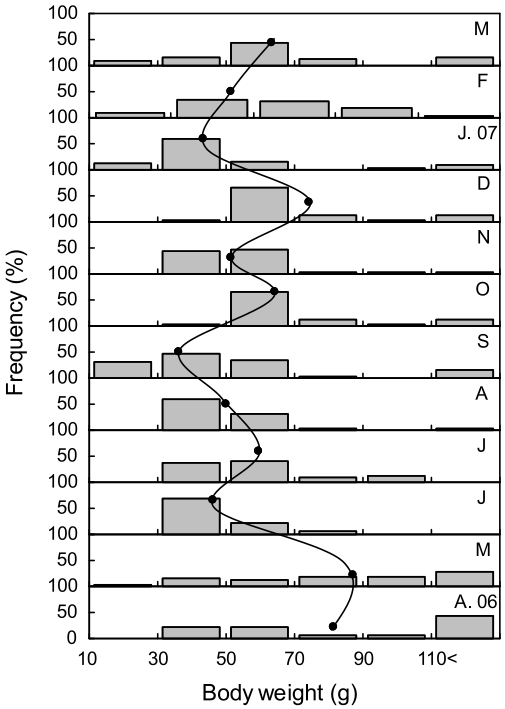

Fig. 4. Relative frequency distribution of body weight (g) of Sea urchins. Line on the figure indicates the mean of body weight.

test diameter. From April to June, all test diameter sizes were observed, but 50.7% were more than 60 mm. The largest test size group, measuring >70 mm, made up 3.3% of the June sample. Thereafter, no specimens belonging to this size group were found until September. Over the course of 10 months, mean test diameter increased and decreased repeatedly.

In April and May, all weight groups between 10 and 110 g were present. Mean wet sea urchin body weight was 81.7±41.7 g and 83.3±31.0 g in April and May, respectively (Fig. 4). Thereafter, it varied between 38.9±18.5 g in September and 73.9±26.3 g in December.

GSI and GI

Of the 360 individuals that were sectioned histologically, 48.1% were female and 51.9% were male (sex ratio 0.92:1). Fig. 5 shows the trend in monthly GSI variation for *S. nudus*. The GSI values for males and females were almost identical over time, with clear peaks in July. Those of females in April, May, and June were 10.1±3.5 (S.D), 9.8±2.7, and 12.1±3.1, respectively. Thereafter, it increased to 17.6±0.8 in July, started to decrease in August, drastically decreased in September (to 4.9±1.9), then remained relatively constant, from 2.2±2.7 to 4.9±2.8, until the following March. For males, spring and early

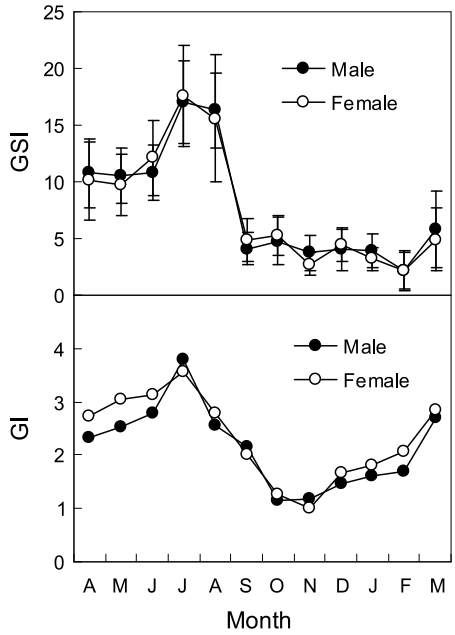

Fig. 5. Monthly change in males and females gonadosomatic index and gonad index of sea urchin, *Strongylocentrotus nudus*, collected at Samchuk in the east sea of Korea (April 2006-March 2007). Error bar=standard deviation.

summer GSI values remained relatively constant at 10.8±3.0, 10.6±2.5, and 10.8±2.5 in April, May,

and June, respectively, but the value increased to 17.0±0.7 in July, decreased rapidly to 4.1±0.3 in September, and thereafter remained low, between 2.2±1.6~5.8±3.4.

Based on the histological analyses, the highest female GI value of 4.6 also occurred in July. The lowest value of 1.2 was observed in November, and then it increased to 3.8 by March. Variation in male GI values was similar to that of females, showing an increase from 3.7 in April to 4.8 in July. The lowest was 1.6, observed in November. Thereafter, the value increased continuously to 3.7, which was reached in March.

Egg size

Monthly egg size frequencies are shown in Fig. 6. Oocytes varied from 10 to 50 μm in February (mean size, 23.5±6.3 μm), and the highest frequency obtained was 52.8% for the 20-30 μm size group. The 30-40 μm size group dominated (49.4%) in March. Oocyte diameter varied between 10 and 70 μm in April and May. In June, the 40-50 μm oocyte size group was the most frequent category, at 44.8%. Frequencies for August and September were dominated by large oocytes (above 70 μm), with percentages reaching 65.6% and 53.5%, respectively.

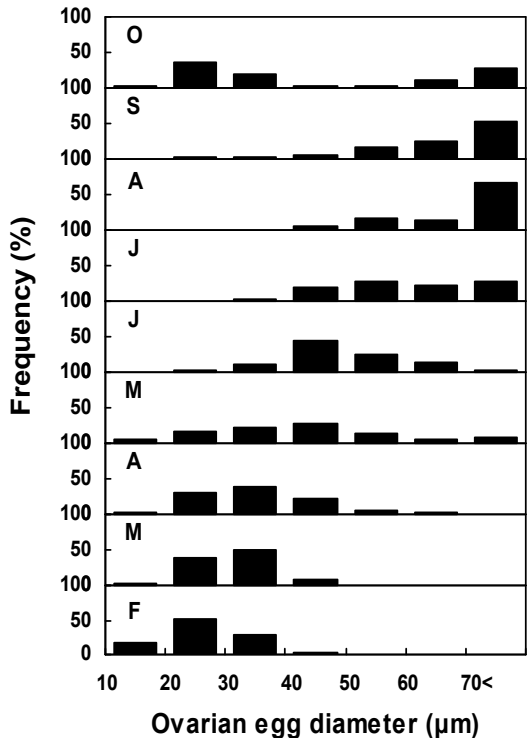

Fig. 6. Relative frequency distribution of the ovarian egg diameter of sea urchin, *Strongylocentrotus nudus*, from March 2006 to February 2007.

Gonad development

Fig. 7 shows histological sections of female and male gonad follicles in different developmental stages. For females, the oogonia begin to develop along the follicular germinal epithelium. In the early active stage, the nucleus and nucleolus are clearly seen in the oogonia along the gonad sac wall; nutritive phagocytes also occur throughout the gonad (Fig. 7-I, left). In the late active stage, the epithelial wall gets thinner, but oocytes increase in volume. During this stage, oocytes are released into the lumen of the sac (Fig. 7-II, left). During the ripe stage, mature eggs are connected by a slender yolk stalk and are separated from the epithelium, having migrated to the inner lumen. The nutritive phagocytes are obviously reduced in volume at this point (Fig. 7-III, left). By the time the spent and degenerative stages are reached, mature oocytes have been extruded and the gonad sac has been refilled with nutritive phagocytes (Fig. 7-IV, left). The gonad sacs keep their form and the few remaining oocytes are reabsorbed within a

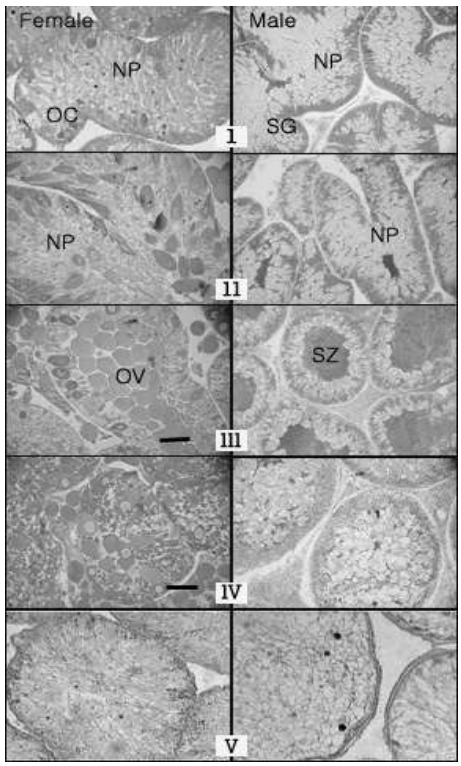

Fig. 7. Histological sections of female and male gonad of sea urchin, *Strongylocentrotus nudus*. I, early active stage; II, late active stage; III, ripe stage; IV, spent and degenerative stage; V, inactive stage; NP, nutritive phagocyte; OC, oocyte; OV, ripe ovum; SG, spermatogonium and spermatocyte, SZ, spermatozoon. Scale bar=100 μm.

short period; they can no longer be observed in the inactive stage (Fig.7-V, left).

For males, nutritive phagocytes, spermatogonium, and spermatocytes can be recognized along the wall during the early active stage (Fig. 7-I, right). In the late active stage, spermatogonium and spermatocytes fill the wall of sacs in high numbers (Fig. 7-II, right). Metamorphosed sperms totally fill the lumen in the mature stage (Fig. 7-III, right). Matured sperm spawn completely within a short time (Fig. 7-IV, right). During the inactive stage, gonad sacs maintain their size and are rapidly refilled with somatic cells and nutritive phagocytes (Fig. 7-V, right).

Frequency of gonad development stage

Monthly gonad development status and the frequency of each developmental stage in the sampled population are shown in Figure 8. For males (Fig. 8, top), the early active stage persisted from December to the following July; in February, 70.6% of the observed animals were in the early active stage. The late active stage was observed from March to September; 77.8% of the males in June belonged to this stage. The mature stage persisted from July to September, with frequencies of 80% in July and 44.8% in August. The spawning stage lasted from August to December, with 55.6% of urchins being in this stage in September and 42.1% in December. Inactive stages were observed from October to the following February, with values of 52.6% in October and 58.8% in December.

For females (Fig. 8, bottom), the early active stage was noted from December to May; 80% of females were in this stage in January. The late active stage was observed from February to July, with frequencies of 82.4% and 86.4% observed in May and June, respectively. The mature stage occurred from May to December, with the highest frequency (60%) observed in August. The spent and degenerative (spawning) stage was observed from August to December and the highest frequency recorded was 66.7%, in September. Inactive stages were found from October to the following January, with frequencies peaking at 76.9% in December and 54.5% in October.

RNA/DNA

Fig. 9 shows RNA/DNA ratios for gonad tissues. For males, higher ratios occurred in July (7.34±0.95) and March (7.0±0.67). The RNA concentration in the male gonad showed no distinct variation from April to June (2.8±0.4 [SE] μg/mg). However, it increased rapidly to 6.4±1.3 μg/mg in August and decreased again to 2.9±0.7 μg/mg in September. The DNA concentration decreased continuously from 0.58±1.10 μg/mg in May to 0.3±0.06 μg/mg in June. Another DNA concentration peak was observed in October (0.54±0.04 μg/mg), after which the concentration decreased slowly until March of the following year (0.27±0.02 μg/mg).

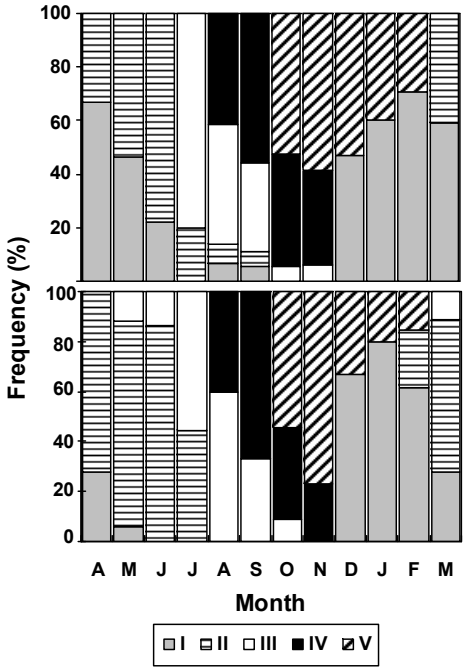

Fig. 8. Monthly changes in frequency of gonad development stages of sea urchins, *Strongylocentrotus nudus,* from April 2006 to March 2007. Top. Male; bottom, Female; I, early active stage; II, late active stage; III, ripe stage; IV, spent and degenerative stage; V, inactive stage.

The RNA concentration and the RNA/DNA ratio in the female gonad were consistently twice as high as that of males, except in October and November. The RNA/DNA ratio for female gonads also showed two obvious peaks with small fluctuations; one peak occurred in July (13.3±0.86) and the other was in March (11.2±0.83). The highest RNA concentration observed was in July (3.75±0.34 μg/mg), after which it decreased to 1.89±0.20 μg/mg in October. Another RNA content peak of 3.53±0.37 μg/mg was observed in March. DNA content showed only one peak in October (0.53±0.05 μg/mg).

Fig. 10 shows RNA and DNA contents, and their ratio, depending on gonad developmental stage. In both sexes, the highest RNA content was recorded during mature stages. The RNA content in testis increased from 1.6 μg/mg in stage I (early active stage) to 2.4 μg/mg in stage III (mature stage); the

RNA content in ovaries increased from 3.0 to 3.4

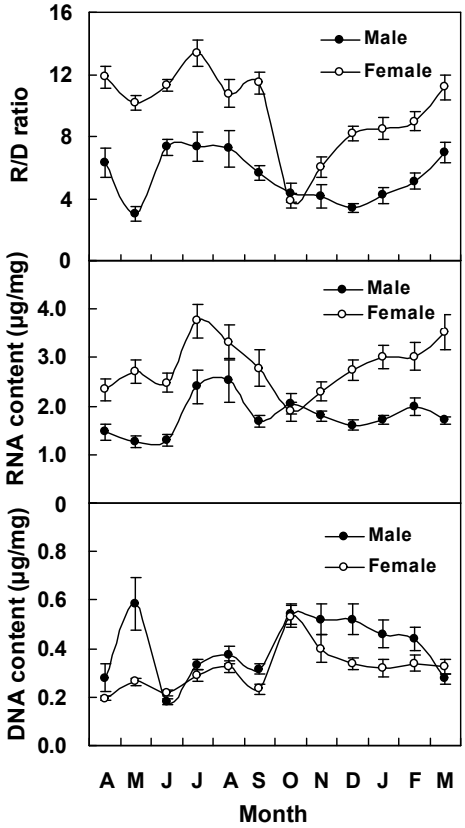

Fig. 9. Monthly variation of nucleic acid contents and ratios in gonads of sea urchins, *Strongylocentrotus nudus,* from April 2006 to March 2007. Error bar indicate standard error.

μg/mg between stages I and III. Only RNA/DNA ratios for mature stage males and late active stage females differed significantly from the other gonad stages ($P<0.05$). DNA concentrations in males remained unchanged throughout the gonad stages. In females, DNA concentrations in mature and late active stages were significantly higher than in the other stages. However, it was during the mature stage that females showed a significant difference in RNA concentration.

Protein content

Seasonal variation in gonad protein content showed two obvious peaks, in July and February (Fig. 11). Protein content was slightly higher in female gonads than in male gonads. The highest values recorded were 124.5±5.2 μg/mg for females and 100.3±6.9 μg/mg for males; both values were recorded in February. The lowest values were recorded in October, with concentrations reaching 32.2±3.7 μg/mg for males and 32.8±5.5 μg/mg for females.

Discussion

The influence of algal beds on *S. nudus* gonads is well documented (Agatsuma, 1997; Nabata et al., 1999; Sano et al., 2001; Tsuda et al., 2006; Thompson 1983; Briscoe and Sebens, 1988; Munk, 1992). In particular, gonad size in *Strongylocentrotus* spp. in Laminariales-dominated beds is significantly larger than in barren grounds, because of a lack of food in the latter habitat (Meidel and Scheibling, 1998). Most of the algae encountered in our study were annual seaweeds, with *E. carva* appearing as a majority species and *Laminaria* spp. as a minority. Without determining food consumption, sustainability, and population growth, we could not determine if the amount of algae is sufficient to sustain local herbivores or support gonad increases. Furthermore, because the area was open to its surroundings, the resident sea urchin population appeared to be affected by the movement of sea urchins from other areas. This could explain the reductions and fluctuations in mean test diameter and body weight that are shown in Figs. 3 and 4. A large number of new sea urchin populations immigrated into the study area each month, as all (approximately 100-200) individuals were regularly removed. Interestingly, regular removal of all sea urchins did not seem to pose a significant threat to population sustainability and reproduction in this study area, as was illustrated by the persistence of certain size (diameters 40-60 mm) and weight classes (30-70 g).

The histological sections showed that gonad development was normal and egg size increased with gonad maturation. But the GSI of *S. nudus* during the mature stage was 2-3% lower than that of *S. nudus* in southern Korea (Park and Son, 1998). Also, it was quite unique that the GSI value in this study decreased sharply from August to September, as other sea urchins show a slow GSI decrease during the spawning season (Agatsuma, 1997). The decrease in GSI corresponded to a drastic decrease in algal biomass in the study area in September (Fig. 1). Minor and Scheibling (1998) reported that algal food ration and the feeding regime can influence the GI and wet weight of *Strongylocentrotus droebachiensis*. Despite its great potential effect on gonad size, feeding treatment did not influence the egg size distribution in females, indicating that egg size may not influence larval development and survival (George et al., 1990). Because of a lack of algal biomass in our study area in autumn (September-November), the gonad was qualitatively replenished

in terms of RNA content, RNA/DNA ratio, and

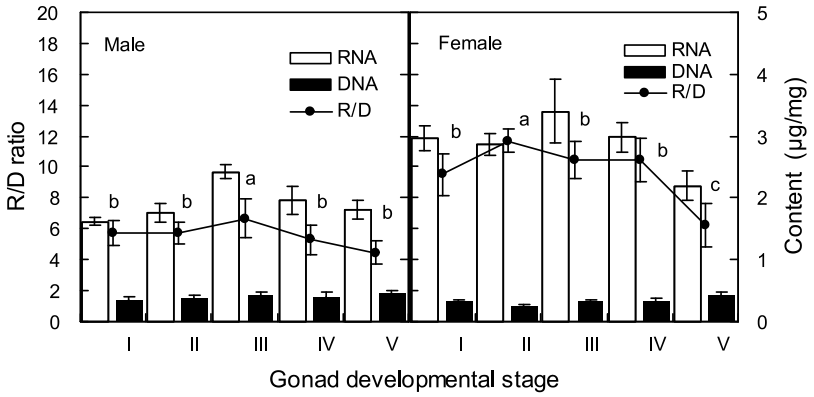

Fig. 10. Variations of nucleic acid content and ratio in gonads of sea urchin, *Strongylocentrotus nudus* according to the gonad developmental stages. Error bars indicate standard error. Alphabet=statistical analysis of RNA/DNA ratio with ANOVA ($P<0.05$).

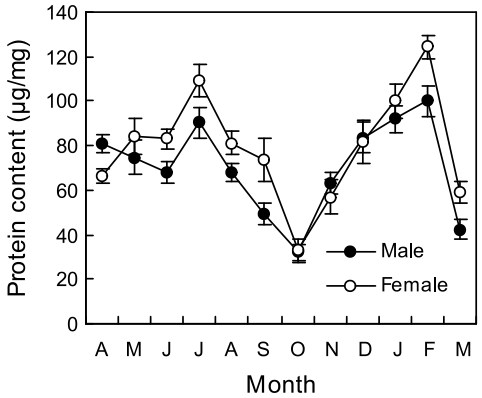

Fig. 11. Monthly variation of protein contents in gonads of sea urchin, *Strongylocentrotus nudus*. Error bars indicate standard error.

protein during the early and late active stages. However, the replenished gonad failed to recover in terms of GSI.

The remaining factors examined showed tendencies similar to those reported in other studies (i.e., GSI, GI, egg size, and histological section). However, some of the detailed results differed in that partial spawning did not occur until September (in GSI), October (in GI), or November (in histological sections). Nevertheless, GSI, GI, protein values, and the RNA/DNA ratio indicated that the peak in gonad maturation occurred in July. In contrast, Park and Son (1998) observed the highest number of mature stage individuals one month later, in August, for the same sea urchin species but at a study site located further south. Such early gonad maturation in a marine bivalve was also reported in another recent study (Kim et al., 2009), probably because of the effects of global warming in the study area.

In female sea urchins, vitellogenin is incorporated into the nutritive phagocytes (accessory cells) in the previtellogenic ovary for temporary storage, and is later transported to the oocytes to be accumulated as a major yolk protein (Ozak et al., 1986; Unuma et al., 1998; 2003). The gonads of male sea urchins also contain nutritive phagocytes for nutrient storage (Walker, 1982). Thus, the biochemical characteristics of sea urchin gonads differ from those of other marine animals. Our biochemical analysis of the gonads (seasonal RNA/DNA ratio, content and protein variation) showed a distinct peak in the summer season that seems to reflect high synthetic activities associated with gametogenesis; a second distinct peak in the winter seems to be associated with somatic growth fueled by nutrient storage in the gonad.

In this study, gonad protein content varied consistently with seasonal variation in the RNA/DNA ratio and RNA content, showing fast replenishment after spawning, from November to February. Protein content also coincided well with variation in nucleic acids. Generally, male marine animals have a higher DNA content in their gonads than do females during the maturation stage. A study of *Pseudocentrotus depressus* (Unuma et al., 2003) showed that the DNA content in males could be as much as four times greater than in females during the mature stage. In the present study, DNA content during the mature stage was only 0.45 μg/mg in males and 0.31 μg/mg in females. High DNA concentrations generally reflect small cell size and a large number of cells per unit weight of tissue (Bulow, 1970); in males, this could be interpreted as a large number of spermatozoa. Regardless of the seemingly normal development of

male gonads, the quality of the sperm and their potential for successful fertilization is still unknown. In contrast to trends in DNA content, we found that RNA content and the RNA/DNA ratio was higher in females than in males.

It remains unknown, however, how well the lowering of GSI values and the relatively low content of DNA in male sea urchin gonads might be explained in terms of the lack of food in the study area or by the population control (removal) efforts. However, the scale of population control for *S. nudus* used in this study, applied to ensure the successful growth of the artificial seaweed forest, still allowed the sea urchin population to show a normal pattern of gonad development and spawning potential (based on histological and biochemical analyses).

Acknowledgements

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