J. Fish. Sci Tech.. 6(2), 51-58, 2003

JOURNAL OF

FISHERIES SCIENCE
AND TECHNOLOGY
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Gametogenic Cycle and Fine Structure of Ripe Germ Cells in the Pacific Oyster, *Crassostrea gigas* on the South Coast of Korea

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The gonadal development and the gametogenic cycle and the fine structure of ripe germ cells of the cultured Pacific oyster, *Crassostrea gigas* were investigated using oysters monthly collected from the southern coast of Korea from October 2000 to September 2001. Monthly changes in the condition index were similar to that of meat weight rate and the highest value was observed in between April and May, and the lowest value in August. The external colors of the testis and the ovary were milky white and yellowish, respectively. The spawning period of the Pacific oyster was continued from May to September, with a peak in July. The gametogenic cycle could be classified into five successive stages: multiplicative stage (December to March), growing stage (March and April), mature stage (April to June), spawning stage (June to August) and resting stage (August to January). Variety of egg yolk granules, lipid granules, mitochondria, and endoplasmic reticula were observed in cytoplasm of ripe oocyte. The spermatozoon consisted of the head, middle piece and tail; including cap-shaped acrosome with domed structure, elliptical shaped nucleus, four mitochondria, two centrioles and flagellum.

Key words: Pacific oyster, *Crassostrea gigas*, Germ cell, Fine structure, Gametogenic cycle

Introduction

The Pacific oyster, *Crassostrea gigas* is one of the important commercial bivalves in Korea. Recently, the quality of oysters declined and its production has been gradually reduced because of the coastal pollution, aging of oyster farms, declining of planktonic larvae, slow growth, increasing mortality and expression of recessive characters. As the seeds of the natural populations have been declined, the seed collections were decreased and also the physiological activity of the larvae and the spats declined. Therefore, the year-round seed production is necessary to resolve these problems. One approach to consistent artificial seed production is to cryopreserve a large quantity of developmental embryos from a superior mother oyster. Recently, the cryopreservation of embryos has been investigated in various bivalves including the Pacific oyster (Chang et al., 1999; Choi and Chang, 1999).

The most studies on artificial seed production of the Pacific oyster have been investigated larvae rearing and environment (Kim et al., 1995; Min et al., 1995). They considered only artificial seed collection in the natural Pacific oyster but not mentioned its gametogenic cycle. The gametogenic cycle is very important basic data to find spawning season in the cultured oysters.

Therefore, the present study investigated the gametogenic cycle through histological method and the fine structure of ripe germ cells through electron microscopic observation in the cultured Pacific oyster. The results of this study could be applied to determine the optimum sampling period of eggs and sperm for the seed production, and to be utilized to obtain the proper developmental stage for the cryopreservation.

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Materials and Methods

The 30 Pacific oysters were collected monthly from October 2000 to September 2001 at the coast of Namhae, Korea. Shell length (SL), shell height (SH) and shell width (SWi), total wet weight, meat weight (MW) and shell weight (SW) were measured (Table 1). Condition index (CI; TW(g)/{SL(mm)×SH(mm)×SWi(mm)}×1000) and the meat weight rate (MWR; MW(g)/{MW(g)+SW(g)}×100) were calculated using the modified method of Momoyama and Iwamoto (1979).

To observe gonadal development, gonadal tissues were fixed in Bouin's solution for 24 hr. and then they were preserved in 70% ethanol. Subsequently the tissues were washed for 24 hr. dehydrated through a graded series of ethanol, and embedded in paraffin. The embedded tissues were sectioned to 5 μm in thickness with a microtome and then were stained with Harry's hematoxylin and 0.5% eosin. Gonad developmental stages were classified into five stages according to Chang and Lee (1982). Oocyte diameters were sampled and calculated at random from the oocytes with clear a nucleus and a nucleolus using computer picture analysis program (Matrox Electronic Systems Ltd., Canada).

For TEM observations, excised pieces of gonads were cut into small pieces and prefixed immediately in 2.5% glutaraldehyde solution buffered by 0.1 M phosphate buffer solution (PBS, pH 7.2). Washing with PBS for 10 minutes, the sample was post-fixed in 1% osmium tetroxide (OsO_4) for 2 hours at 4℃. The sample was washed again with PBS, then serially dehydrated with ethanol from 50 to 100% and embedded in Epon 812. First, 0.5 μm thickness sections were semithin sectioned with ultramicrotome (LKB, Nova, Sweden), and then stained with toluidine blue to determine an observation region. After that, 70 nm thickness sections (ultra-thin section) were cut again. The sections were double-stained with uranylacetate and lead citrate solution and examined with TEM (JEM 1200 E-XⅡ, 60-80 Kv, JEOL, Japan).

Data from the frequency distribution of oocyte diameter experiments were expressed as arithmetic mean and SD. The significance of differences among the means of oocyte in each month was tested by a post hoc, Duncan's multiple range test. Differences with a probability value (P) of 0.05 were considered significant.

Results

Water temperature, salinity, condition index and meat weight rate

Maximum surface water temperature and salinity were recorded with a maximum of 26.5℃ and 36.2 practical salinity units (psu), respectively in July 2001, and a minimum of 8.0℃ and 32.3 psu, respectively in January 2001 (Fig. 1).

Monthly changes in CI and MWR of the Pacific oyster were as shown in Fig. 1. CI of 0.06 in October increased to 0.10-0.11 in April and May, and was rapidly decreased to 0.06 in June and showed the minimum value of 0.04 in August. MWR changed with CI, showed the minimum value of 11.1% in August and the maximum value of 29.5% in May. Therefore, the monthly changes of CI were associated with those of MWR.

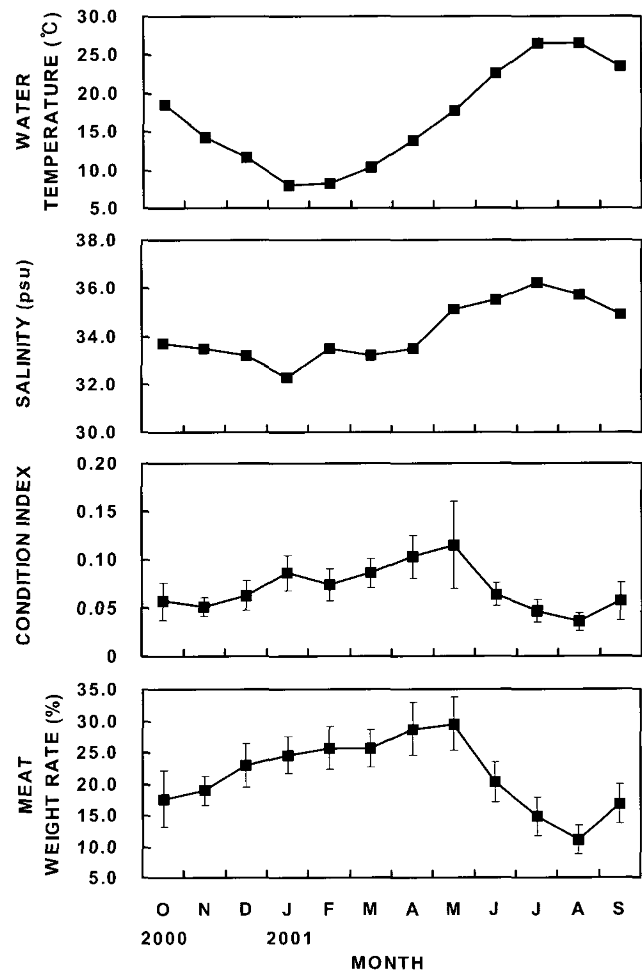

Fig. 1. Monthly changes of condition index and meat weight rate of the Pacific oyster, *Crassostrea gigas*.

Gonadal development

Gonad of the Pacific oyster was situated around digestive diverticula. External colors of the testis and the ovary were milky white and yellowish, respectively.

The gametogenic cycle could be divided into five stages based on morphological observations: multiplicative, growing, mature, spawning and resting stages (Fig. 2).

Multiplicative stage (Mu): Oogonia and spermatogonia appeared along the germinal epithelium in the ovary and testis, respectively. Ovarian sacs were irregular in shape and supported by intertubular connective tissue. Oogonia and spermatogonia had commenced to proliferate.

Growing stage (G): As gametogenesis proceeds, the connective tissues were reduced and extents of the gonad were enlarged. In the ovary, many oocytes attaching to the germinal epithelium developed. Nucleus and cytoplasm of the oocyte were gradually increased in quantity, and oocyte became polygon in shape. In the testis, primary and secondary

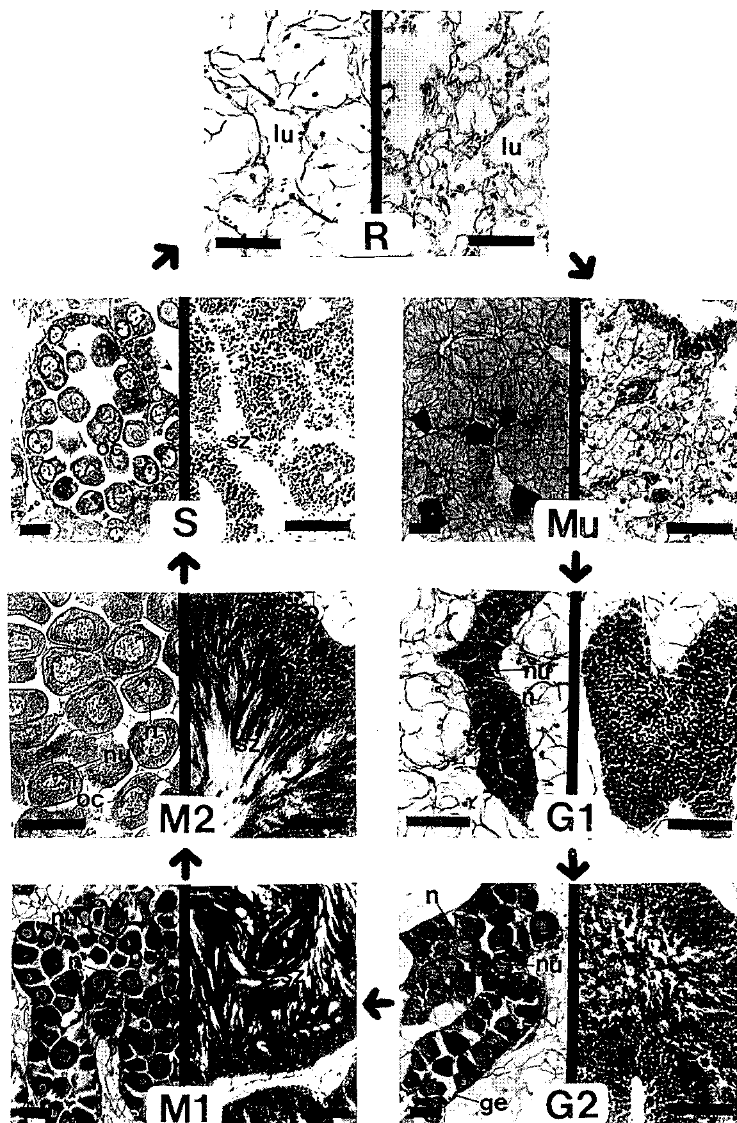

Fig. 2. Histological identifications of the gonad development phase of the Pacific oyster, *Crassostrea gigas*. Left side of each photograph shows ovary and right side testis. Mu: multiplicative stage, G: growing stage, M: mature stage, S: spawning stage, R: resting stage. ge: germinal epithelium, lu: lumen, n: nucleus, nu: nucleolus, oc: oocyte, og: oogonium, sc: spermatocyte, sg: spermatogonium, sz: spermatozoon. Scale bar = 50 μm.

spermatocytes proliferated rapidly on the germinal epithelium. The spermatogenic cells were stained with hematoxylin.

Mature stage (M): Connective tissues had been reduced to a small and thin layer in the distal regions of the gonad. In the ovary, polygonal oocytes became partly round in shape and filled up ovarian sac. In the testis, numerous spermatozoa occurred in the center of the lumen of the testicular tubule.

Spawning stage (S): In the ovary, the ripe oocytes were released and a few ripe oocytes were not released. The lumen of ovarian sac became enlarged. In the testis, spermatozoa were released and the lumen of the testicular tubule became empty. However, a few undischarged spermatozoa as well as spermatids and spermatocytes remained in the testicular tubule.

Resting stage (R): After spawning, the ovarian and testicular tubules became contracted. The lumen was filled with residual degenerating oocytes or spermatozoa, representing signs of regression. Then volume of gonad was shrunken and the tubules were emptied. There were no histological changes during definite periods.

Fine structure of the ripe germ cells

Mature oocyte stage: Lipid and yolk droplets were accumulated in the cytoplasm, and then they were mixed and became larger in size. A jelly coat surrounded the vitelline envelope with the microvilli of a mature oocyte. Nucleus of mature oocyte was approximate 6 μm in diameter (Fig. 3, A-C).

Spermatozoon stage: The spermatozoon was composed of the head, middle piece and tail. The nucleus of spermatozoon showed elliptical in shape approximate 1.5 μm in major axis and contained dense chromatins. The anterior part of the sperm head was a domed acrosome. The nucleus had both an anterior nuclear fossa and a posterior nuclear fossa. Axial rod showed clearly in lumen formed by invagination of acrosomal membrane. The mass of the mitochondria was located in the middle region of the spermatozoon. Four spherical mitochondria formed the paranucleus behind the nucleus and they surrounded 2 centrioles (proximal and distal centriole) oriented

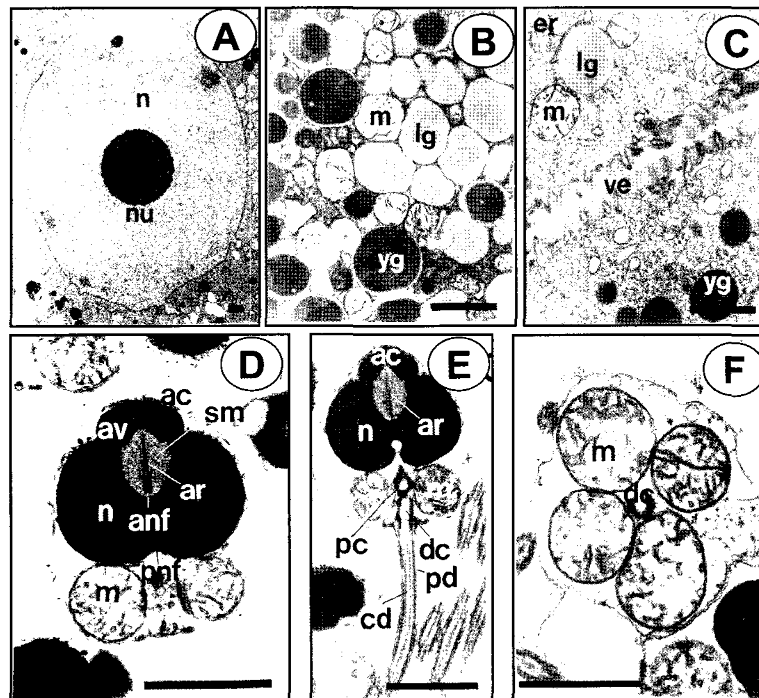

Fig. 3. Electron micrographs of ripe germ cells of the Pacific oyster, *Crassostrea gigas*. A-C: Mature oocyte. D-F: Spermatozoon. ac: acrosome, ag: acrosomal granule, anf: anterior nuclear fossa, ar: axial rod, av: acrosomal vesicle, cd: central doublets, dc: distal centriole, er: endoplasmic reticulum, g: Golgi body, ge: germinal epithelium, ld: lipid droplet, lg: lipid granule, m: mitochondrion, n: nucleus, nu: nucleolus, pc: proximal centriole, pd: peripheral doublets, pnf: posterior nuclear fossa, sm: subacrosomal material, t: tail, ve: vitelline envelope, yg: yolk granule. Scale bar = 1 μm.

at 90° to each other. The flagellum of tail showed typical 9+2 structure (Fig. 3, D-F).

Monthly frequency of oocyte diameter

None of oocytes were observed in October. Size of oocytes showed average 4.3 μm in diameter in November and slowly grew to 18.3 μm in March. Continuously oocytes were rapidly grew to 29.2 μm in April. In May, part of oocytes which had grown up to 40-45 μm in diameter were partially spent into surrounding environment. In June and July, oocytes of 40-45 μm (average 42.2-43.5 μm) in diameter were occupied over 50% in all oocytes, and then began to spawn. Therefore, oocyte diameter was rapidly decreased and newly formed oocytes were also begun to degenerate. Diameter of oocytes were showed 5.3 μm in August and volume of oocytes gradually became larger (Fig. 4).

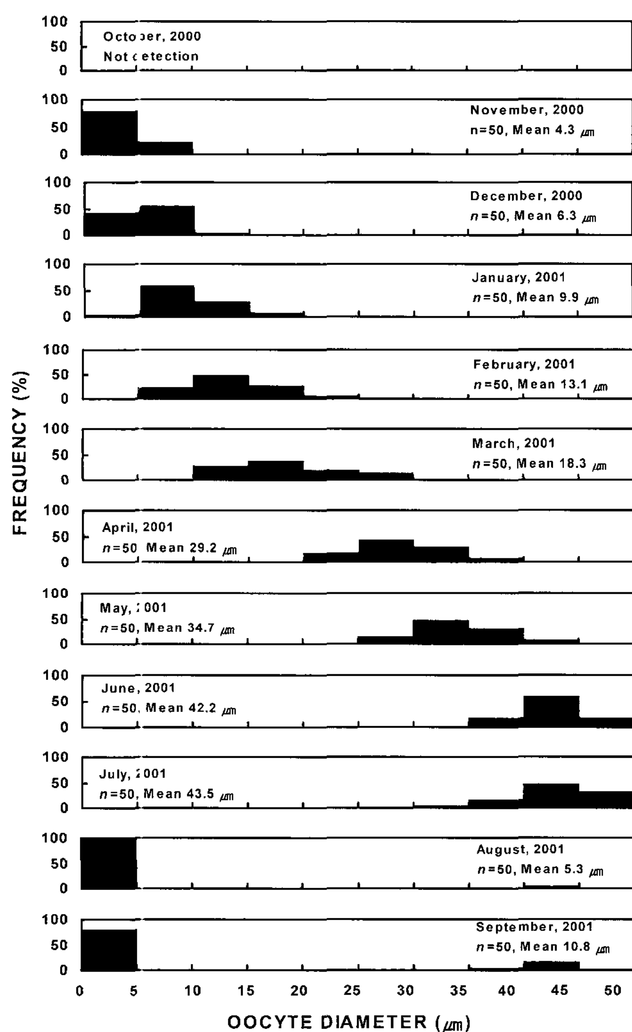

Fig. 4. Monthly frequency of oocyte diameter distribution of the Pacific oyster, *Crassostrea gigas*.

Gametogenic cycle

The annual gametogenic cycle of the Pacific oyster is summarized in Fig. 5.

In the ovary, most of oocytes were resting stage from August 2000 to January 2001 (70-96% of the oocytes). The oogonium in the multiplicative stage and oocytes in the spawning stage, resting stage were observed in the ovarian sacs in September. Histological phases in the growing stage were showed in February 2001 and most of the phases in the multiplicative and growing stages appeared in between March and April 2001. The ovaries in the mature stage were predominated in May and June 2001 (50-80% of the oysters), and spawning began in May 2001 (20% of the oysters). All of oocytes were spawned in July 2001, the individuals in the spawning stage were found in September. It was indicated that the ovaries in the resting stage is observed continuously except the spawning season.

The developmental phases of the testis were similar to those of ovary, but histological phases of the testis

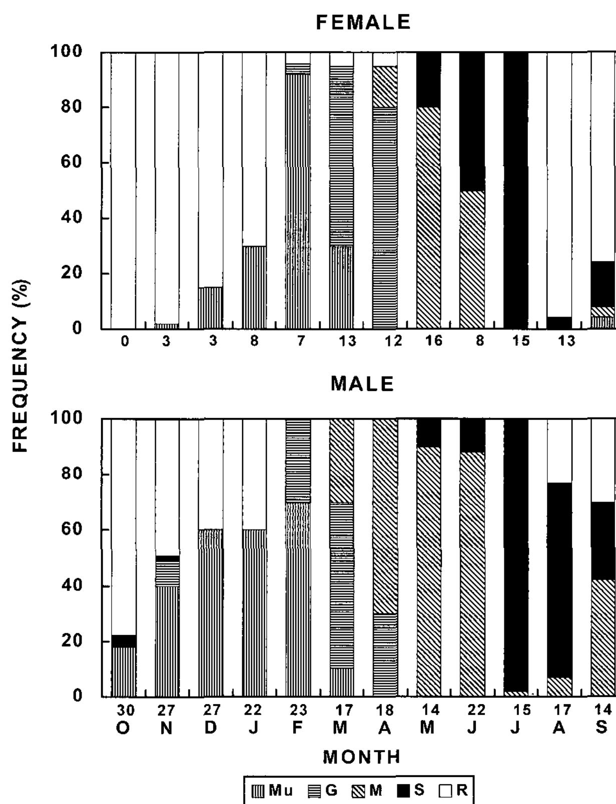

Fig. 5. Gonad developmental phases of the Pacific oyster, *Crassostrea gigas*. Mu: multiplicative, G: growing, M: mature, S: spawning, and R: resting stage. The Arabic numerals under X-axis are number of animals.

Table 1. Measurement of the Pacific oyster, *Crassostrea gigas* used in the present study[1]

Sex	Shell length (mm)	Shell height (mm)	Shell width (mm)	Total weight (g)	Meat weight (g)	Shell weight (g)
Female	62.7±10.3	127.4±15.7	30.7±5.9	109.5±37.0	17.8±6.9	66.9±23.3
Male	56.2±10.1	123.1±17.9	28.5±6.5	83.4±35.7	14.0±9.2	50.7±22.5
Average	58.2±10.5	124.4±17.4	29.1±6.4	91.4±38.0	15.1±8.7	55.7±23.9

[1]Data presented as mean±SD.

showed more complex than those of the ovary. The testis in the multiplicative stage appeared from October 2000 to March 2001 (10-70% of the oysters) and over 60% of the individuals in the multiplicative stage were found from December to February. The spermatogonium grew to the spermatocyte in February. Some of individuals matured in March, and then that in the mature stage appeared 70-90% in April to June. Also some of individuals showed 42% in September, the releasing rate of the spermatozoa was 98% in July, 70% in August, and 28% in September, and even in October and November the spermatozoa partially released.

According to the results gametogenic cycle of the Pacific oyster was divided into five stages; multiplicative stage (December-February), growing stage (March-April), mature stage (April-June), spawning stage (June-August) and resting stage (August-January).

Discussion

Temperature is an important environmental factor in the regulation of bivalve reproduction (Sastry, 1979). In this study, water temperatures ranged from 8.0 to 26.5℃ during the period of sampling. Releasing of matured oocytes began during a period of rapidly increasing water temperature (May), when temperature rose 17.7℃. The maximum spawning peak was from June to July, when water temperature rose to 22.6-26.5℃. Besides, a short spawning had occurred in September (Fig. 5) by decreasing water temperature. In this study, we considered that the cultured Pacific oyster spawned once because of short spawning period and small spawning populations.

CI and MWR showed a maximum value in May in which was started spawning. During spawning season water temperature increased whereas CI and MWR rapidly decreased, namely the relation between spawning and CI were negative. Searcy-Bernal (1984) reported that the fluctuations of the CI are associated with the reproductive or nutritional conditions of the mollusks.

Robinson (1992) described that maximum frequency of sexually matured Pacific oyster in Oregon occurred in August and September and declined rapidly between October and November, thereafter reached the minimum in March. Dinamani (1987) reported that spawning of the Pacific oyster in New Zealand was found in September. Differences in the timing of gametogenesis and spawning within a species over a latitudinal range occur because critical temperatures are attained at different times (Hesselman et al., 1989). Brousseau (1995) compared spawning pattern of intertidal and subtidal population of the eastern oyster, *Crassostrea virginica*. Most of the intertidal populations spawned in August and early September, and the subtidal populations spawned in June and July. However, this was very different from that of the Indian oyster, *C. madrasensis* in India. Its spawning season had two peaks from December to May (December-January and April-May) (Joseph and Madhyastha, 1984), whereas Stephen (1980) reported that spawning activity of the Indian oyster in India had two peaks from April to November (from mid-April to June and until the end of November). Like this, oysters are known to show considerable variation in their reproductive habits (Stephen, 1980). The environmental factors that could effect the gametogenic cycle in bivalves might be the differences in environment such as temperature and food availability, and especially temperature may be the most important (Wada et al., 1995).

Gonadal developmental stage of the *C. gigas* can be classified into 3-6 stages as reported by the previous authors (Stephen, 1980; Dinamani, 1987; Robinson, 1992; Brousseau, 1995). Number of the developmental stages varies according to the classification method they applied.

The sperm of bivalves generally has the primitive or basic forms; a spermatozoon usually consists of

an ellipsoid or conical nucleus, an acrosome of variable complexity, a middle piece consisting of an aggregation of usually 4-5 mitochondria surrounding a pair of centrioles, and a flagellum or sperm tail (Retzius, 1905). The spermatozoon of the Pacific oyster consisted of a cap shaped acrosome with domed structure, an elliptical nucleus, four spherical mitochondria and tail. The nucleus had both an anterior nuclear fossa and a posterior nuclear fossa. An axial rod also existed. Presence of an anterior nuclear fossa can be correlated with the presence of an axial rod in the acrosome. The occurrence of a posterior nuclear fossa appears to be correlated with the presence of the satellite body of the proximal centriole (Popham, 1979). The axial rod in the bivalve sperm may act as a support about which the inner acrosomal membrane is folded into a small radius of curvature, a factor promoting fusion of cell membranes (Pethica, 1961). In some species, there is no axial rod and in those species having an axial rod it may be long and penetrated deeply into an anterior nuclear fossa as in *Mytilus* spp. (Niijima and Dan, 1965) and the eastern oyster (Galtsoff and Philpott, 1960). It is possible that in those sperm having an axial rod it may play a role in the fertilization of the bivalve oocyte (Niijima and Dan, 1965). The mitochondria have been found to be intimately associated with the nuclear envelope, spherical to ovoid in shape and 4-5 in number in the eastern oyster (Galtsoff and Philpott, 1960) and the mussel, *M. edulis* (Longo and Dornfield, 1967). The sperm morphology of the Pacific oyster is similar to that of the ark shell, *Anadara trapezia* reported by Popham (1979). An acrosome with domed structure at the anterior part was found in the Pacific oyster but not shown in the eastern oyster (Galtsoff and Philpott, 1960).

Acknowledgements

This work was supported by grant No. R01-2000-000-214-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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(Received September 2002, Accepted June 2003)