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REPRODUCTIVE CYCLE, BIOCHEMICAL CHANGES AND PARASITIC INFECTIONS IN PACIFIC OYSTER, *CRASSOSTREA GIGAS* AND MANILA CLAM, *RUDITAPES PHILIPPINARUM*

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국 문 요 약

고성만에서 양식하고 있는 참굴, *Crassostrea gigas* 의 배우자 형성은 체내 에너지축적과 수온에 따라 동시에 일어났다. 산란은 수온이 20-27°C 인 5 월부터 10 월까지 일어났다. 주산란은 6 월과 8 월에 관찰됐고, 6 월이 더 높았다.

서로 다른 두 수심에서 채집한 참굴은 생식소성숙 기간인 4 월과 산란 후인 8 월의 조단백질과 조지방 함유량에 있어 가장 큰 차이점을 보였다. 탄수화물은 휴지기인 1 월과 11 월, 첫 산란정점인 6 월과 7 월 사이에 가장 큰 차이점을 보였다. 세가지 조성분을 비교하여 분석한 결과, 표층에서 채집한 참굴의 조성분간 관계가 저층에서 채집한 참굴의 관계보다 연관성이 더 높았다.

첫 산란정점에서는 표층에서 채집한 참굴이 저층에서 채집한 참굴보다는 생식소성숙지수와 포란도가 좋았으나, 두번째 산란정점에서는 반대의 경향을 보였다. 표층에서 채집한 참굴이 번식을 위한 에너지축적과 생화학적 전환이 보다 좋은 것으로 보인다. 그러므로 고성만 참굴의 수심에 따른 양성은 생리적인 지표와 번식에 분명한 영향이 있는 것으로 보여진다.

난소 기생충인 *Marteilioides chungmuensis* 는 발달중이거나 완숙한 난이 형성되는 6 월부터 1 월까지는 관찰되지만 2 월부터 5 월까지는 관찰되지 않았다. 감염정도는 첫 산란정점인 6 월말에 매우 높았고, 8 월말부터 산란이 대부분 끝나는 11 월까지 지속됐다. 한편 11 월과 12 월에 채집된 참굴 난소에서는 감염된 상태의 완숙난을 많이 관찰할 수 있었는데, 이는 산란이 지연되거나 파괴된 난에 감염이 이루어졌다고 볼 수 있다.

시흥리에서 채집한 바지락, *Ruditapes philippinarum* 의 배우자 형성은 수온이 13°C 에 이르는 3 월에 초기 상태를 이룬다. 생식소지수는 수온과 더불어 5 월부터 8 월초까지 매우 빠르게 증가하였다. 바지락의 산란성기는 7 월 초순부터 8 월말까지 지속적으로 이어졌다.

평균 조단백질 함량은 32.6-51.2%의 범위를 보였고, 조지방함량은 9.3-22.2%, 탄수화물 함량은 12.1-30.1%의 범위를 나타냈다. 바지락 조성분의 변화는 번식주기와 관계가 있었다. 생식소성숙지수는 산란이 시작되는 6 월말에 높았다(18.9%). 바지락 암컷의 포란수는 6 월 첫 산란정점에서 2,470,000 개, 8 월말 두번째 산란정점에서 2,020,000 개로서 첫번째 산란정점에서 더 많았다. 이 연구에서 나타난 시흥리 바지락의 낮은 생식소성숙지수와 짧은 산란시기는 시흥리 조간대의 먹이가용성과 관계가 있는 것으로 생각되며, 이는 번식량에 영향을 주는 것으로 보인다.

바지락에 있어서의 *Perkinsus* 감염 빈도는 연평균 32.9%로서 2001 년 9 월에 가장 낮았고(6.0%), 2002 년 3 월에 가장 높았다(86.0%). *Perkinsus* 의 감염도 또한 평균 0.63 으로서 2001 년 9 월에 가장 낮았고(0.11), 2002 년 3 월에 가장 높았다(2.08). *Cercaria* 의 출현빈도는 8 월초에 가장 높았으나(12.0%), 대부분의 채집기간 동안에는 관찰되지 않았다. 산란기 동안 *Cercaria* 에 감염된 개체들의 생식소에서는 난모세포들의 퇴행과 위축된 여포들을 관찰할 수 있었다. 이 연구에서는 *Perkinsus* 와 *Cercaria* 에 의한 바지락의 사망률은 관찰하지는 못했지만, 기생충 감염이 바지락의 번식에는 영향을 준다는 것을 조직학적 관찰을 통해 확인하였다.

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SUMMARY

In Gosung Bay (south coast of Korea), the gametogenesis of Pacific oysters, *Crassostrea gigas* occurred concurrently with the accumulation of energy reserves and temperature; spawning presented when water temperature increased from 20 to 27^{o}C and continuously from May to October. Two spawning peaks were observed in June and August, with higher magnitude in June.

Surface temperature was always higher than the bottom temperature (2-5^{o}C) from April to August. Salinity of surface water showed abruptly decreased during monsoon period (June to August) while it was more stable at the bottom layer. Chlorophyll *a* and seston concentration presented a few different levels between surface (0-2m) and bottom (3-5m) water layer. Oysters from 2 depth intervals showed major different levels of protein and lipids during gonad development in April and after second spawning in August. The differences of carbohydrates were found in resting phase (January and November) and during first spawning peak (June to July). There were stronger correlations between biochemical compositions in oysters from surface layer compared to those from the bottom layer. At the first spawning peak, surface-oysters showed higher gonad somatic index and fecundity than the bottom-oysters, however the reversion was observed after second spawning. Oysters in surface layer could possess better bioconversion and investment energy reserves for their production. Therefore, the depth culture could contribute certain effects on physiological state and reproductive effort of oysters in Gosung Bay.

Prevalence and infection intensity of the ovarian parasite *Marteilioides chungmuensis* in oysters showed that the parasite occurred in developing and fully mature eggs of spawning oysters collected in June to January; but was not observed from February to May. The infection intensity was high in late June when most oysters had their first spawning period; and was also high in late August and November when

oysters were spawning or had completed spawning. Some of oysters collected in November and December carried a large quantity of ripe but *Marteilioides*-infected eggs, suggesting that the infection also causes spawning failure by delaying spawning and destroying ripe oocytes.

In Shi-Heung-Ri Beach (Jeju Island, Korea), gametogenesis of Manila clam *Ruditapes philippinarum* was initiated as early as March when the surface water temperature reached to 13^{o}C. Gonad index increased at a faster rate from May to early August, as the water temperature rose rapidly. Major spawning was observed in the clams in early July and continuously until the end of August. Average percent of protein in clam tissues was 32.6-51.2% by weight, total lipid ranged 9.3-22.2% and carbohydrate varied 12.1-30.1%. These biochemical changes related to the reproductive cycle of clams. The gonad somatic index was highest in late June (18.9%) when clams began to spawn. Female clams produced 2,470,000 eggs in first spawning peak in June and 2,020,000 eggs in the second, in late August. Low gonad somatic index and short spawning period of clams in studied area suggests that food availability may limit reproductive output of the population.

Prevalence of *Perkinsus* infection in Manila clam varied seasonally, it was lowest in late September 2001 (6.0%) and highest in March 2002 (86.0%), with an annual mean of 32.9%. The infection intensity of *Perkinsus* changed from 0.11 (September 2001) to 2.08 (March 2002), with a mean of 0.63. The prevalence of *Cercaria* was highest in early August (12.0%), while none of the clams was infected with *Cercaria* in several months. Degenerated oocytes and castrated follicles were observed in clams that were severely infected with *Cercaria* during the spawning period. Although *Perkinsus*- and *Cercaria*-related clam mortalities were not observed, the histological findings clearly show that the parasitism impacts clam reproduction.

INTRODUCTION

The Pacific oyster, *Crassostrea gigas* (Fig.1), is one of the most important commercial species supporting the Korean fisheries industry. In Korea, the oyster culture uses naturally available spat as seeds, which are collected on spat collectors made of oyster shells attached to a plastic string 4 to 6 m long during summer. The seeds settled on the oyster shells are then hung on a longline suspended in the water column by numerous buoys for grow out. For successful seed production and ultimately for oyster production, studies on seasonal changes in gonadal maturation and impacts of environmental parameters on reproduction are essential for the Korean oyster industry. Over 170,000 metric tons of oysters were produced in Korea in the year 2000 from the intensive suspended long line culture system which places Korea as one of the world's largest oyster exporters (Ventilla, 1984; FAO, 1999).

Oyster landings in Korea have declined for the past decade. Several theories have been proposed to explain the decrease in oyster production, such as slow growth in highly intensive culture systems (Kang et al., 2000, Oh et al., 2002) and insufficient supply of healthy seed oysters (Park et al., 1999a, b). The current decline may be due to the effects of pollution and outbreaks of pathogenic infection along oyster culture grounds (Park and Chun, 1989; Choi et al., 1997).

Protozoan parasites in the phylum Paramyxea have been extensively studied where oysters and mussels are commercially raised (Becker and Pauley, 1968; Wolf, 1977; Figueras and Montes, 1988; Villalba et al., 1993; Fuentes et al., 1995; Renault et al., 1995; Camacho et al., 1997; Berthe et al., 2000; Hine and Thorne, 2000). The presence of the ovarian parasite, *Marteilioides chungmuensis* (Comps et al., 1986) has been reported from oyster culture grounds in Korea since 1970's (Chun, 1970, 1972, 1979) and in Japan (Imai et al., 1968; Matsusato and Masumura,

1981). Chun (1970, 1972, 1979) initially reported on the occurrence and pathologic features of an unidentified amoeba-like pathogen in the gonad of oysters collected along the south coast of Korea. Later, the ameboid pathogen was named *Marteilioides chungmuensis*, a new genus and a new species by Comps et al. (Comps et al., 1986, 1987). *Marteilioides chungmuensis* infected Pacific oysters develop lumps or nodule-like gonads on their body during spawning season. Such infected oysters with abnormal appearance are unacceptable in the market, resulting in serious economic losses to oyster farmers. Microscopic examination of an infected oyster indicates that *M. chungmuensis* are mostly distributed inside the ovary, within oocytes. Anderson and Lester (1992) also have reported a second species of the genus, *M. branchialis* from Sydney rock oysters, *Saccostrea commercialis*. More recently, Lee et al. (2001) also have reported a *Marteilioides*-like organism from *Tapes philippinarum* distributed on the southern coast of Korea, although its impacts on the clam industry is unknown.

The interannual or local differences in the cycles of energy storage and reproduction of marine bivalves are often associated with changes in environmental conditions, in particular available food in the water column (Newell et al., 1982; Rodhouse et al., 1984; Bricelj et al., 1987; Navarro et al., 1989; Kang et al., 2000). In coastal area, available food for suspension feeders such as bivalves decreases as depth increases. The poor feeding conditions associated with greater water depths resulted in reduced growth and reproductive output. Barber et al. (1988) observed that gonad weight and gonad index of *Placopecten magellanicus* in shallow-water (13 to 20 m) were significantly greater than those from deep water (170 to 180 m).

Kang et al. (2000) suggested that food availability could be a major factor to determine gonad proliferation in *C. gigas* in Jaran Bay and Hansan-Koje Bay on the southern coast of Korea. The author also indicated that differences in physiological status of oysters were strongly related to site-dependent variation in the storage-utilization cycle of energy reserves (particularly glycogen), depending on food availability. In *C.*

virginica, fecundity was highly variable within and among locations (Cox and Mann, 1992; Mann et al., 1994). The variation was attributed to differences in oyster size, asynchrony and variation in time since previous spawning, prevalence of parasites and different salinity regimes. Choi et al. (1993) used an enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of reproductive output in *C. virginica* and detected the positive correlation between the number of eggs produced and oyster size. Kang et al. (2003) have reported an ELISA technique to assess egg production of the Pacific oysters, *C. gigas* and obtained the same result. However, Hofmann et al. (1994) suggested that adult size and reproductive effort are determined by the allocation of net production to somatic or reproductive tissue development and the rate of food acquisition. The author concluded that the variations in temperature and food supply affect reproductive effort more than adult size.

The Manila clam, *Ruditapes philippinarum* (Fig.2.), is one of the most common marine bivalves in coastal areas of Jeju, Korea. Clams are often exploited commercially by local villagers or by tourists visiting beaches. In Jeju, clam populations have been declining for the past few years due to over exploitation without proper management. Previous studies conducted in Jeju have reported that clams are often infected with various types of parasites, although no parasite-induced mortalities have been reported to date (Park and Choi, 2001; Choi and Park, 2001). Several protozoan and metazoan pathogens have been identified from *R. philippinarum* in Korean waters. *Perkinsus* has been identified from clams on the west and south coasts of Korea, and heavily infected clams exhibited pathologic symptoms such as digestive epithelium atrophy, hemocyte infiltration, and tissue inflammation (Choi and Park, 1997, 2001; Park and Choi, 2001; Lee et al., 2001). Park and Choi (2001) suggested that *Perkinsus* is the agent responsible for the current decline in clam landings in Korea.

Along with the fluid thioglycollate medium assay invented by Ray (RFTM, Ray, 1953, 1966), histology has been used in *Perkinsus* diagnosis

(Perkins and Menzel, 1966; Navas et al., 1992; Sagrista et al., 1995; Bower et al., 1998; Hine and Thorne, 2000). Histology enables investigators to examine *Perkinsus* infection visually in a microscopic field and provides valuable pathologic information. Despite its advantages, histology also has a certain drawback in *Perkinsus* diagnosis; it does not provide quantitative data and is not sufficiently sensitive to detect a low level of infection (Bushek et al., 1994; Fisher and Oliver, 1996; Almeida et al., 1999).

The infection intensity of parasites in marine bivalves is often determined from histological preparations. Several studies have categorized the infection intensity of MSX (*Haplosporidium nelsoni*) disease in American oysters based on histological examination of oyster tissues (Ford, 1985, 1986; Ford and Figueras, 1988; Barber et al., 1988). Ellis et al. (1998) developed histology-based numerical scales as measures of the intensity of *Bucephalus* infection in mussels. Recently, Ngo et al. (2003) also developed a numerical scale for diagnosis of *Marteilioides chungmuensis* infection in the Pacific oyster, *Crassostrea gigas*. Although these scales are not truly quantitative, they are affordable and sensitive enough to differentiate the effects of different levels of infection on the host organisms.

Bivalves often serve as a first intermediate host for digenea. In particular, trematode species belonging to the family Bucephalidae use a variety of marine bivalves as intermediate hosts (Lauckner, 1983). *Cecaria tapidis* is a bucephalid trematode that is commonly found in marine bivalves such as *Meretrix lusoria* (Chun and Lee, 1976) and *Ruditapes philippinarum* (Kim and Chun, 1981, 1983; Shimura et al., 1982). A high level of bucephalid parasitism often results in slow growth and reduced fecundity of host organisms (Taskinen and Valtonen, 1995; Taskinen, 1998). Khamdan (1998) reported that *Bucephalus* destroyed female gonads of the pearl oyster *Pinctada radiata*, resulting in reproductive failure. Lee et al. (2001) also observed that sporocyst and cercaria of the trematode parasite were mostly distributed in female gonads of *R. philippinarum* disturbing the reproductive processes of the clam.

The nutritional and energy demands of marine animals are not constant, but are affected by exogenous factors such as food availability and temperature, and by endogenous factors such as requirements for reproduction. Metabolic reserves accumulated in tissues may be used in energy production or converted into various biochemical components (Gabbott, 1983; Martinez, 1991). Seasonal variation in the biochemical composition of bivalve tissues thus results from complex interactions between environmental factors and metabolic processes. Variations of biochemical compositions are closely related to the reproductive cycle, in various species of bivalves, including *R. philippinarum* and *Tapes decussatus* (Beninger and Lucas, 1984; Robert *et al*., 1993). Laruelle et al. (1994) suggested that the diffused muscle of the visceral mass might be associated with gamete development in *R. philippinarum*. Recently, Chung *et al*. (2003) indicated that adductor muscle and visceral mass tissues are important energy storage and nutrient supply organ and their variation in biochemical compositions were correlated with gonadal energy requirements.

The objectives of this study were to investigate the seasonal variation in reproduction of *Crassostrea gigas* population in suspended culture systems in Gosung, Korea, and describe the prevalence and infection intensity of *Marteilioides chungmuensis* parasitism concomitant with the reproductive activity of oysters. In previous study (Kang et al. 2003), the authors have reported development of an immunological technique for quantitative evaluation of reproductive evaluation of reproductive effort of oysters as well as seasonal change in the reproductive effort in Gosung Bay. This study is part of a continuing effort to improve the quantitative descriptions of the relationships between depth of culture, energy storage and reproductive effort of oysters in Gosung Bay, Korea. For that purposes, we compare (1) biochemical composition of oysters from different depths, i.e. a surface (0 to 2 m) and bottom (3 to 5m) of suspended line culture, and (2) to determine depth-dependent variation in the role of energy storage relative to reproductive effort of oysters in Gosung Bay.

On the other hand, this study reports monthly changes in the prevalence and infection intensity of *Perkinsus* and *Cercaria* in a clam population distributed on Shi-Heung-Ri Beach on the east coast of Jeju Island, Korea. We also investigate the seasonal variation of biochemical compositions and fecundity during the reproductive cycle to evaluate the condition and reproductive effort of *Ruditapes philippinarum* at studied area.

Fig. 1. Pacific oyster, *Crassostrea gigas*

Fig. 2. Manila clam, *Ruditapes philippinarum*

Part I

Prevalence and infection intensity of the ovarian parasite *Marteilioides chungmuensis* during an annual reproductive cycle of the Pacific oysters *Crassostrea gigas*

1. ABSTRACT

Seasonal variation in reproductive condition of the Pacific oyster, *Crassostrea gigas* was investigated from a suspended cultured oyster population in Gosung Bay, Korea using histological techniques. Gametogenesis of oysters initiated in February when water temperature reached 11 to 13^{o}C. Increase in oocytes size and the number resulting in expansion was observed from March to May when surface water temperature reached 22 to 25 °C. Spawning activity extended from mid June to late September with two marked spawning peaks in June and August. Most oysters collected from October to December exhibited few residual eggs in packed follicles showing a typical spent condition. No gametes were observed from December to February from oysters collected in the Bay. Gonadal development of oysters in Gosung Bay seemed to follow a seasonal fluctuation in environmental conditions such as water temperature and food availability in the water column. Spawning of oysters in late June was in part associated with sudden drop in salinity due to vast amount of freshwater input in the Bay after the summer flooding. Sex ratio of oysters was 59.5% male and 39.8% female. Less than 1 percent (0.6%) of the oysters examined were hermaphrodite; few eggs were observed in testis.

Occurrence of *Marteilioides chungmuensis,* a protozoan paramyxean parasite in the reproductive system of the oysters was observed at Gosung Bay, Korea. *M. chungmuensis* occurred in developing and fully mature eggs of spawning oysters collected in late June to January; but were not observed from February to May. Monthly mean infection intensity was

high in late June when most oysters had their first spawning period. The infection level was also relatively high in late August and November when oysters were spawning or had completed spawning. Several oysters collected in November (11.4%) and December (16.3%) carried a large quantity of ripe but *Marteilioides*-infected eggs, suggesting that the infection also causes spawning failure by delaying spawning and destroying ripe oocytes.

2. MATERIALS AND METHODS

2.1. Study area and oyster sampling

Gosung Bay is located on the south coast of Korea, where several semi-enclosed bays and islands are located (Fig. 3). The bay has $2,165 \times 10^4$ m^2 surface area with an average depth of 7 m. In this bay, oysters are cultured intensively by suspending oyster ropes hanging from longlines. In the year 2000, 16,230 metric tons of oysters including shells were produced from Gosung Bay, which is 10% of the total oyster production in Korea.

Adult Pacific oyster, *Crassostrea gigas* with mean shell length over 7cm were collected from 3 different sampling locations in Gosung Bay, Korea. From each sampling site, an oyster suspended rope was randomly taken and oysters were collected. Sampling continued from January to December 2000 on a monthly basis and from June to August, oysters were sampled on a biweekly basis in order to follow spawning activity of oysters. Water temperature and salinity were recorded *in situ* when oyster sampling was made. Upon arrival laboratory, shell length of oysters was recorded in mm using a caliper. Flesh of oysters was then removed from the shell and wet tissue weight was measured after removing excessive water with absorbent tissue paper.

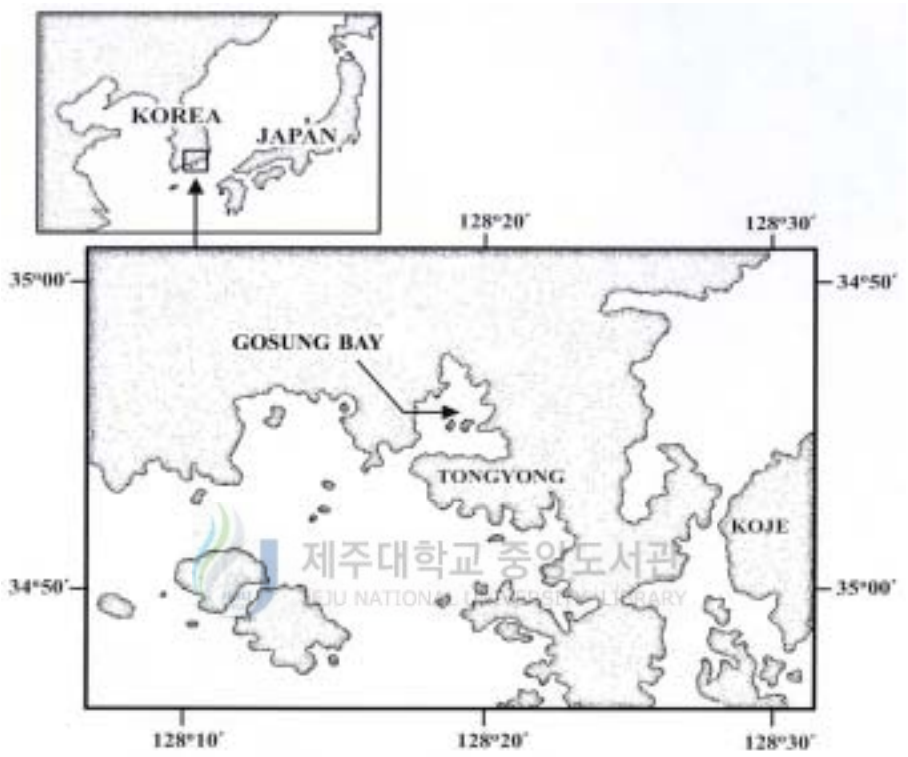

Fig. 3. Map showing the location of Gosung Bay, on the south coast of Korea.

Thirty to 45 oysters were collected from each oyster string for histological preparation. A transverse cut was made in the middle of the oyster body and a 3 mm-thick section was fixed in Bouin's solution. Tissue samples were then dehydrated in an ethanol series of progressive concentrations, cleared in xylene, and embedded in paraffin. Four μm-thick serial sections were cut with a rotary microtome and they were stained with Harris' haematoxilyn and eosin Y.

2.2. Gonad development

Based upon microscopic examination of histology, gonad development of *C. gigas* was categorized into five stages as described by Heffernan et al. (1989). Gonad development of each oyster was then scored on a 0 to 4 scale; 0, undifferentiated stage; 1, spent stage; 2, developing stage; 3, ripe stage; 4, spawning. The monthly gonad index (GI) for both sexes was determined by multiplying the number of specimens ascribed to each category by the category score, summing all such values and dividing this figure by the total number (Heffernan et al. 1989).

2.3. Diagnosis of *Marteilioides chungmuensis*

Prevalence and infection intensity of *M. chungmuensis* was determined by examining the gonad slides under a light microscope according to Park and Chun (1989). Infection intensity of each oyster was then rated according to Villalba et al. (1993): no infection when no parasite was detected from the entire microscopic field (0); a light infection when the parasites were confined to the follicle wall or occurred in primary oocytes (1); a moderate infection when only some part of gonad was occupied by the parasite (2); and a heavy infection when a whole gonad was infiltrated with *M. chungmuensis* (3).

3. RESULTS

3.1. Sampling effort and environmental conditions

Monthly mean salinity and temperature of water column are given in Fig. 4. Surface water temperatures varied seasonally with a maximum of 30.4°C in July and minimum of 4.3°C in February. Highest salinity was recorded in May, 34.3 ‰. From June to August, salinity dropped abruptly and reached minimum value at 27.6 ‰. From August onward, salinity fluctuated around 31 ‰ and increased to 32.4 ‰ at the end of the year.

Fig. 5 summarizes size frequency distribution of oysters analyzed in this study. Size class of 80 to 90 mm in shell length was most common, accounting for 28 to 32% of total oyster analyzed. The ratio of males (59.5%) was higher than females (39.8%) during sampling period. Hermaphrodic individuals were also found in June and November although they were only 0.6% of total oyster examined.

3.2. Microscopic appearance of gonad development

Photomicrographs of various developmental stages of male and female oysters are presented on Fig. 6 and 7. In undifferentiated stage, little or no gonad tissue is visible, sex of oyster cannot be distinguished at this period (Fig. 6A and 7A). During developing stage, number of follicles increased and follicles became expanded (Fig. 6B and 7B). As the developing stage progressed, the follicles continued to expand and coalesce. No mature gametes were observed during this period (Fig. 6C and 7C). Late gonad developmental stage was characterized with greatly expanded and coalesced follicles with some connective tissues remained unfilled. During this period, the lumen was filling with growing and ripe oocytes. Fig. 6D shows spawning female gonad, exhibiting free mature oocytes filling the follicles. Free gametes were also visible in gonoduct during this period. In spawning male, the follicles were almost filled with spermatozoa (Fig. 7D).

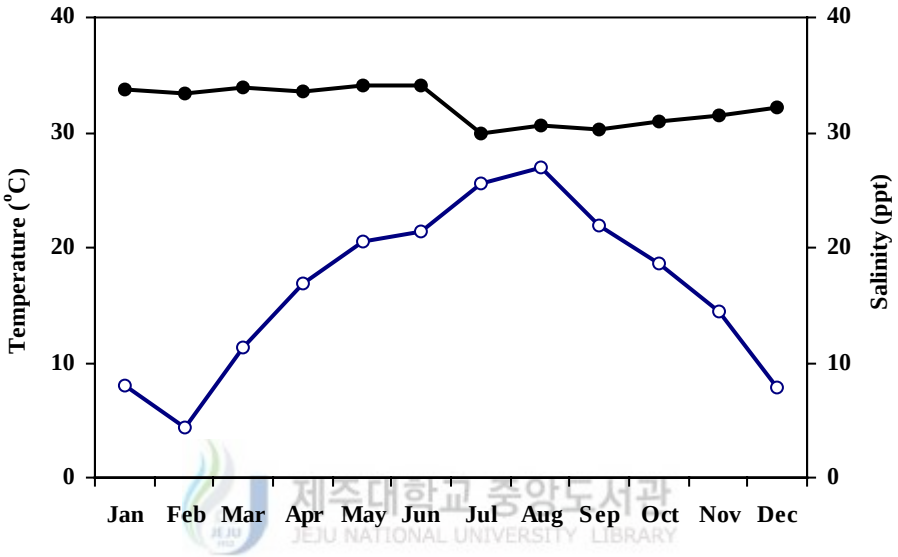

Fig. 4. Seasonal variation of water temperature and salinity in Gosung Bay during January to December 2000.

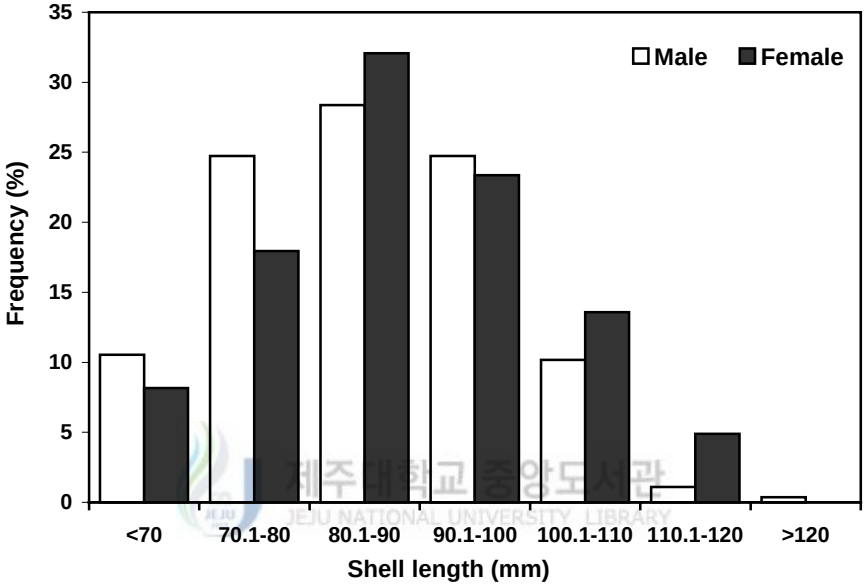

Fig. 5. Shell length frequency distribution of male and female oysters during sampling period.

After completion of spawning, gonads were considerably shrunken in volume and contain a small number of residual gametes (Fig. 6E and 7E). Fig. 6F shows a typical female spent gonad, displaying empty follicles with a few numbers of free oocytes. For males, the follicles are collapsed and decreased in size. A few follicles contain a small amount of residual unspent spermatozoa (Fig. 7F). Hermaphroditic oysters are shown on Fig.8, exhibiting developing testis and ovary in a follicle (Fig. 8A and B).

3.3. Reproductive pattern

Fig. 9 shows changes in GI of oysters in the Bay during the course of study. Microscopic observation of the histological slides indicated that gametogenesis of oysters started as early as in December or January. Monthly mean GI increased at a fast rate from February to April as the surface water temperature elevated. In mid June, mean GI of oysters indicated that most oysters were ready for spawning or in partial spawning.

Relatively lower GI observed in late June indicated that first spawning of oysters in the bay occurred from mid June to late June. Increase in GI from mid July to mid August then dropped in late August suggested that another mass spawning of oyster population occurred from mid to late August. Table 1 also summarizes monthly changes in percent composition of various developmental stages. The data indicated that spawning was quite synchronous in the first spawning peak in June (64.3 - 68.1%) relative to those in August (53.3 - 58.3%). Sexually indifferent oysters were dominant during December and February. Fig. 10 also illustrates cyclic changes in gonad development of oysters in the bay.

3.4. Occurrence of *Marteilioides chungmuensis*

In the year 2000, 40 out of 608 (7%) oysters collected from Gosung Bay were infected with *Marteilioides chungmuensis*. Highest prevalence was recorded in December (16.3%); no infection was observed from February to early June (Fig. 11).

There was very low correlation between the infection prevalence of parasite and mean GI of oysters during year cycle (R^2=0.0208). The infection was limited to female oysters and gonad was only tissue targeted. The prevalence of *M. chungmuensis* in various reproductive stages is summarized in Fig. 12. In a total 40 of infected oysters, low prevalence was observed at developing (15%) and ripe (10%) stages, with higher prevalence in the spawning (35%) and spent (40%) stages. Fig. 13 shows seasonal changes in the infection intensity *M. chungmuensis* in female oysters. Infection intensity was high in late June (intensity rate = 2.0) when most oysters were spawning and also high in mid August (rate = 2.0) and November (rate = 2.0) when some oysters were still spawning or were spent. There was no clear correlation between the infection intensity and mean GI of the observed oysters at both depth intervals (R^2=0.0023).

Histological sections of the oyster gonad revealed different developmental stages of *Marteilioides chungmuensis*. Several different vegetative stages, including one or more secondary cells were observed within follicles (Fig. 14). Heavily infected oysters often exhibited large accumulations of haemocytes within or around the follicle wall. *M. chungmuensis* displaying 2 to 4 nuclei, 10 to 20 μm in diameter, were also found in fully mature eggs (Fig. 15). Some female oysters, collected from November to January, exhibited fully mature intact eggs in their follicles when almost all oysters in this period were reproductively inactive. These infected female oysters exhibited several lump-like egg masses in their mantle, a typical sign of *M. chungmuensis* infection.

Table 1: Percentage of oysters at each reproductive stage during sampling period

Gonad stages	Sampling period (from January to December 2000)														
	Jan	Feb	Mar	Apr	May	14. Jun	28. Jun	14. Jul	28. Jul	14. Aug	28. Aug	Sep	Oct	Nov	Dec
Indifferent	100	75.6	8.9												51.1
Developing		24.4	77.4	60.6	14.4			13.8		2.2	4.9				
Ripe			13.7	39.4	78.9	31.9	29.9	47.6	48.9	33.3	25.7	2.4			
Spawning					6.7	68.1	52.6	31.9	39.0	53.9	38.2	33.5	26.3	11.3	
Spent							17.6	6.7	12.1	10.6	31.1	64.2	73.7	88.7	48.9

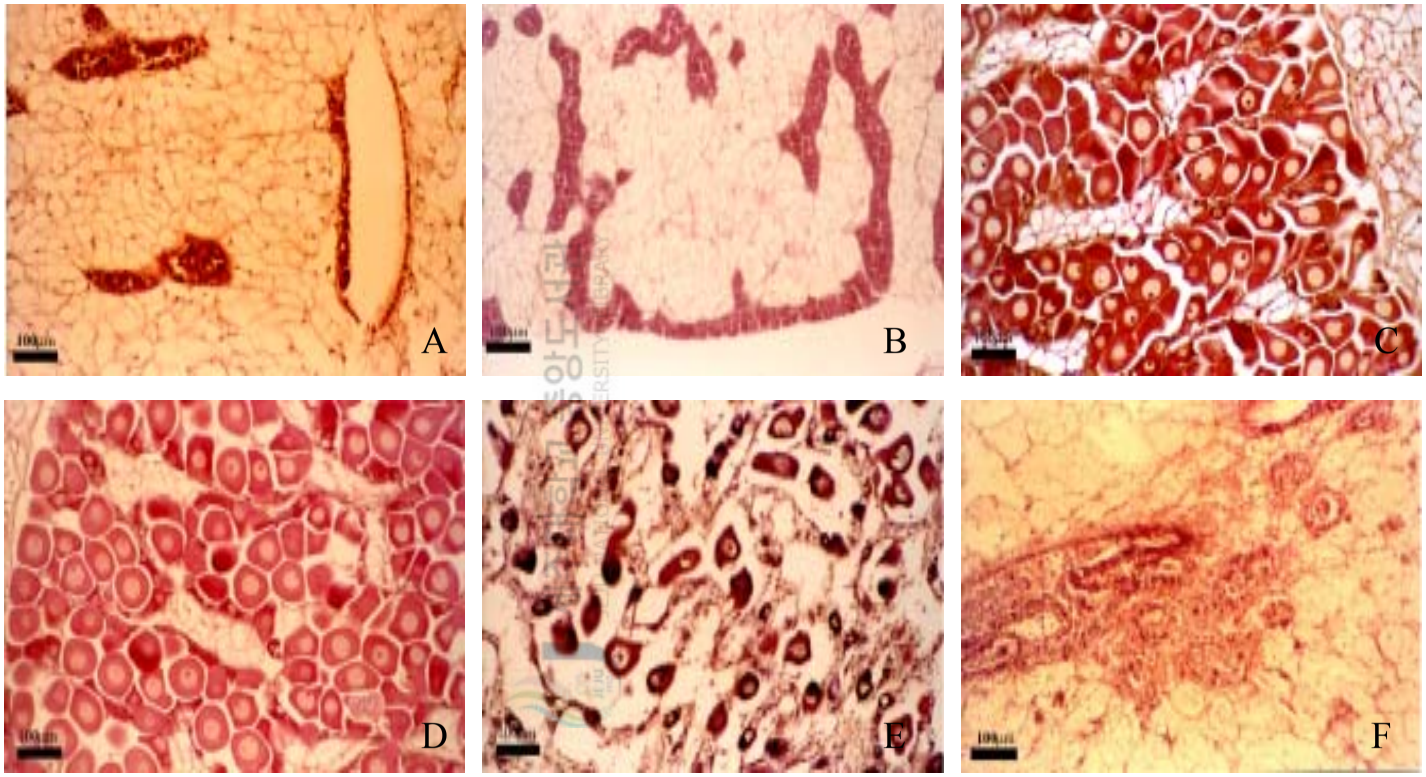

Fig. 6. Gonad development in female oysters. (A) Sexually undifferentiated stage, (B) Early development stage, (C) Late development stage, (D) Ripe stage, (E) Spawned stage, (F) Gonadal tissue atrophy.

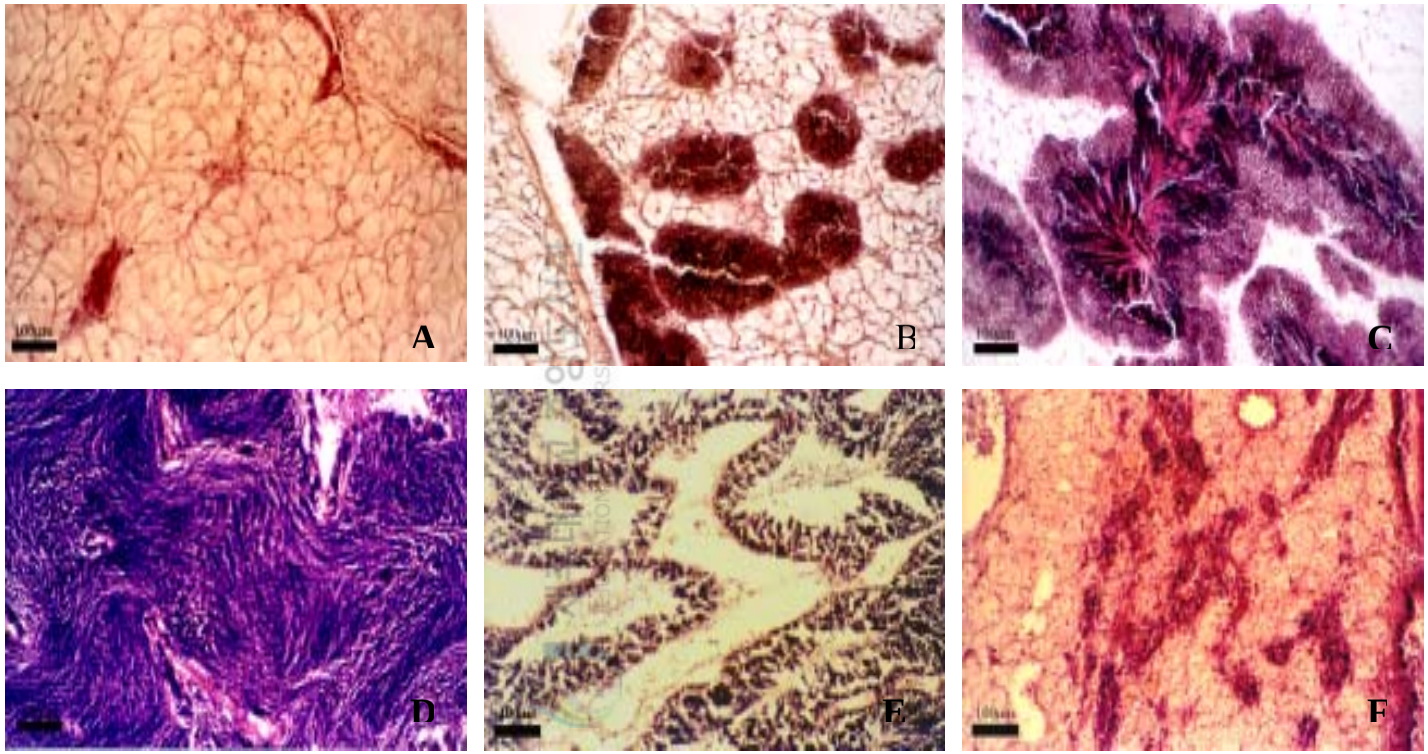

Fig. 7. Gonad development in male oysters. (A) Sexually undifferentiated stage, (B) Early development stage, (C) Late development stage, (D) Ripe stage, (E) Spawned stage, (F) Gonadal tissue atrophy.

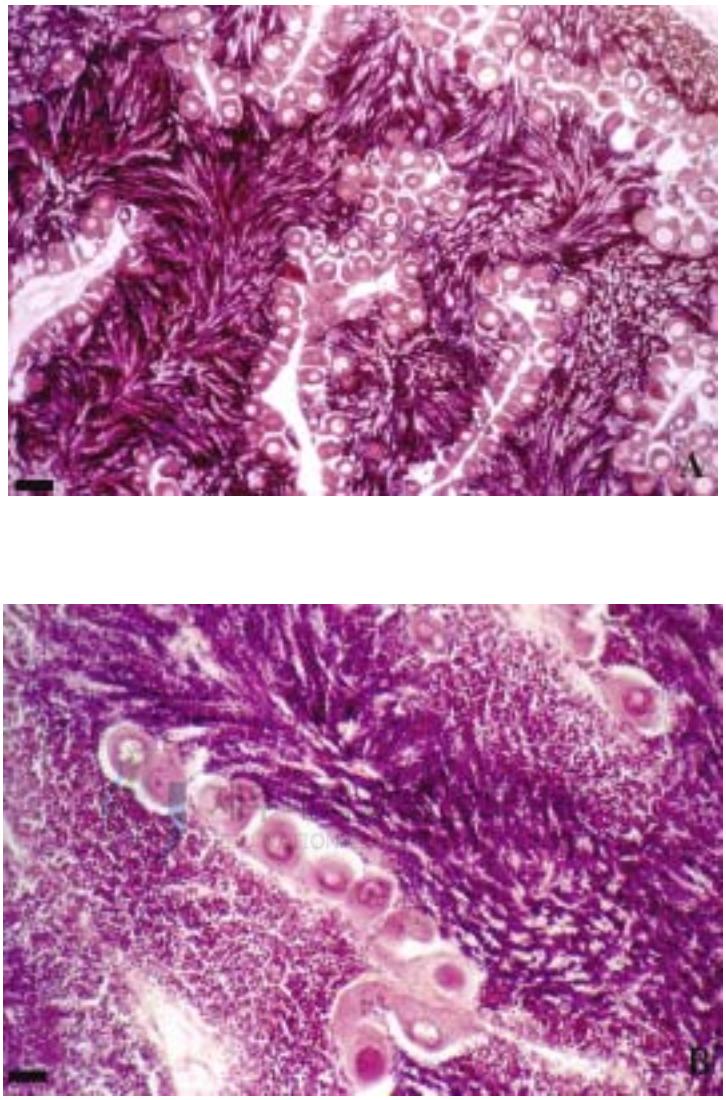

Fig. 8. Hermaphroditic oysters. (A) Developing oocytes in periphery of follicle wall with sperm in follicular lumen, (B) In the same follicles, both sex are developed (bar = 50μm).

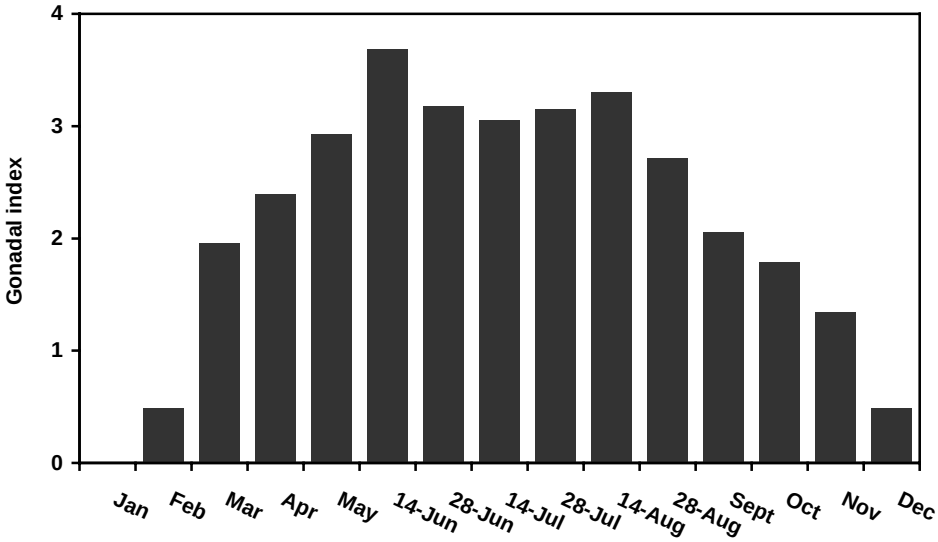

Fig. 9. Seasonal variation of gonad index of oysters during year 2000.

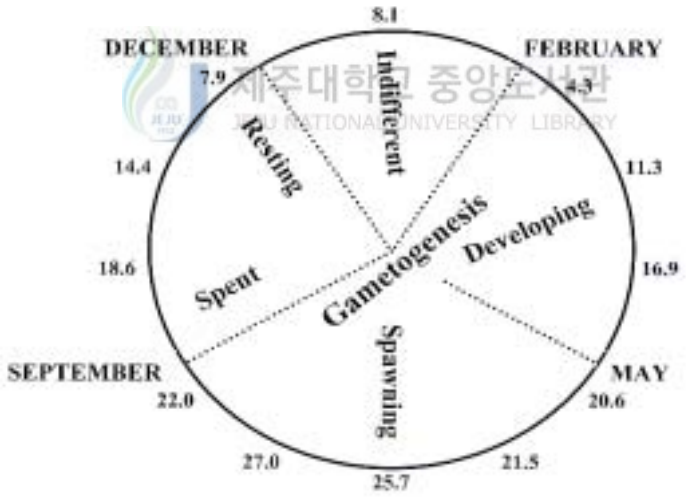

Fig. 10. Cyclic change in gametogenesis of oysters in Gosung Bay. The numbers represent monthly mean value water temperature (^{o}C).

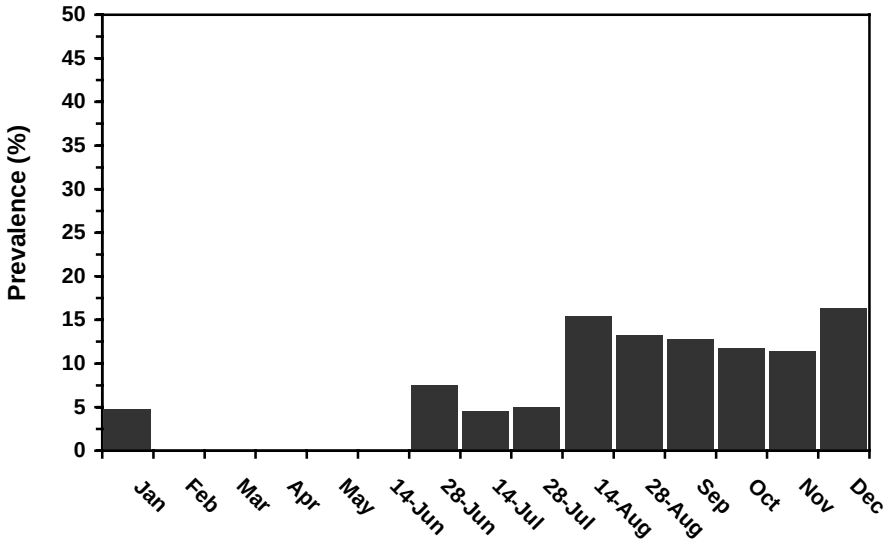

Fig. 11. Prevalence of *Marteilioides chungmuensis* in oysters during year 2000.

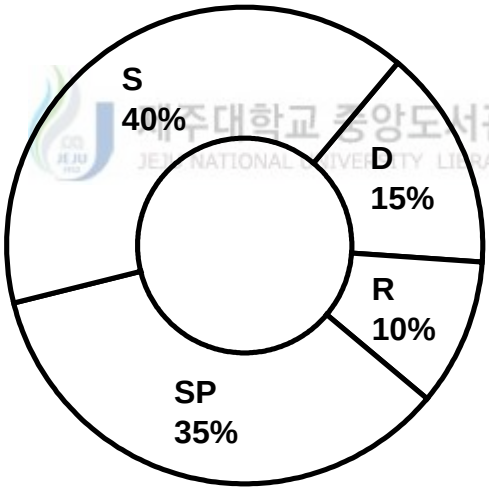

Fig. 12. Prevalence of *Marteilioides chungmuensis* in various reproductive stages of oysters. (D) Developing; (R) Ripe; (SP) Spawning; (S) Spent.

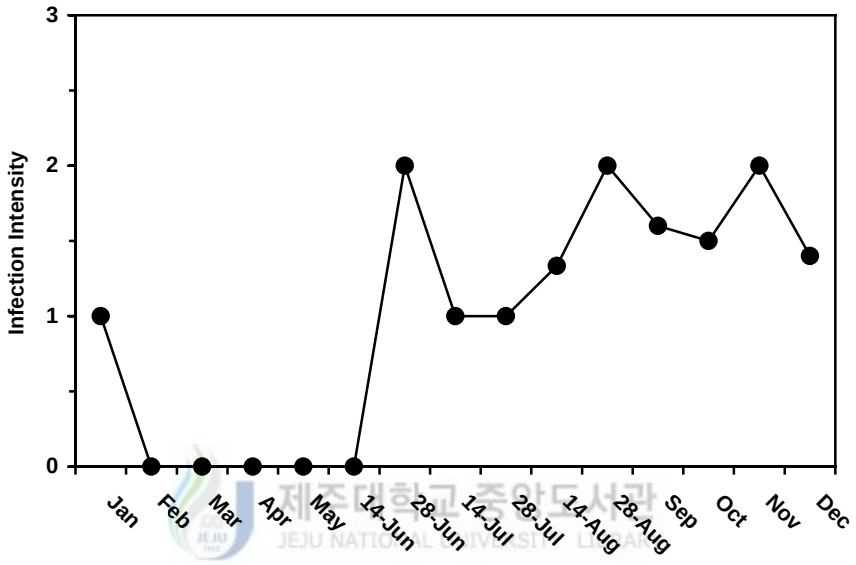

Fig. 13. The infection intensity of *Marteilioides chungmuensis* measured from January to December 2000 in oysters from Gosung Bay, Korea.

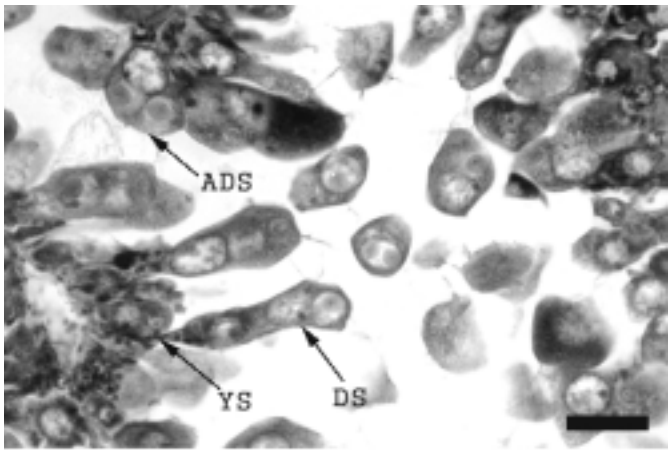

Fig. 14. Different stages of *Marteilioides chungmuensis* in oyster gonad. (YS) Young stage; (DS) Developing stage and (ADS) Advanced developmental stage (bar = 50 μm).

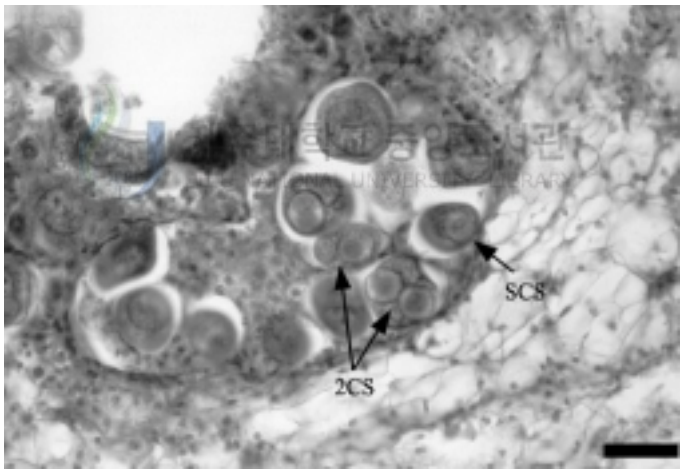

Fig. 15. Different life stages of *Marteilioides chungmuensis* present in mature oyster oocytes. (SCS) Single-celled stage; (2CS) Two-celled stage (bar = 50 μm).

4. DISCUSSION

Several studies have reported that reproductive process of marine bivalves is mainly governed by two major external parameters, temperature and food availability (Bayne 1976; Ruiz et al. 1992). In general, temperature accelerates or decelerates the rate of gamete production, while food availability determines quantity as well as quality of reproductive output (Galtsoff 1964; Thompson et al. 1996). In temperate area, cyclic changes of gonad development in bivalves often matches with seasonal changes in water temperature and phytoplankton blooms which provide sufficient food for both broodstock and larvae (See review of Galtsoff 1964). Studies previously conducted on gonad development of *C. gigas* in small bays on the south coast of Korea also confirmed that seasonal changes in temperature and food supply in the water column are coincided with cyclic changes in reproductive condition of oysters (Bae and Han 1998; Park et al. 1998; Hyun et al. 2001; Kang et al. 2000).

Oysters in Gosung Bay begin oogenesis in March when water temperature reached 10 to 12 ^{o}C. The oocytes grow at a faster rate from April to early June as surface water temperature increase also at a faster rate. Histological data indicate that spawning occurs as early as May and it continues until the end of October while water temperature fluctuates from 20 to 27^{o}C. During this period, two major spawning peaks are observed, one in mid June and the other in mid August. Most oysters complete spawning from September to October and become reproductively inactive in November when water temperature drops below 17^{o}C. Spawning of oysters often occurs at a certain water temperature (Galtsoff 1964; Kang et al. 2000; Thompson 1996). Mann (1979b) suggested that a minimum temperature of 18 to 20^{o}C is necessary to induce spawning in *C. gigas*. In contrast, Kennedy and Krantz (1982) reported that some oysters initiate spawning even when water temperature is below 20^{o}C. In Gosung Bay, oysters started spawning when the surface water temperature reached 18 to

20°C, indicating that 18 to 20°C is a minimum temperature to initiate spawning. Kang et al. (2000) also reported that oysters on the south coast of Korea may start spawning when water temperature rise to 18 to 20°C.

Continuous or multiple spawning of oysters has been reported from oysters distributed on the south coast of Korea. Bae and Han (1998) reported that oysters in Gosung Bay initiate spawning in late June when water temperature reached 23 to 25°C. Park et al. (1999) also investigated gametogenic cycle of *C. gigas* at two oyster culture grounds near Gosung Bay. In their study, suspended culture oysters in Tongyoung and Koje started spawning in early May and the spawning continued to October. The oysters in those areas also exhibited two distinct spawning peaks, one in late June to early July and the other in late August. Kang et al. (2000) observed two major spawning peaks of oyster populations at Osu and Hansan-Koje Bay in southern coast of Korea. Such a continuous spawning in *C. gigas* also has been reported in Japan. Spawning activity of oysters in Hiroshima continues from May to September with a peak in July-August (Arakawa, 1990). Arakawa (1990) reported that *C. gigas* in Hiroshima spawn when water temperature are 21 to 30°C although an optimal temperature for spawning is considered to be 25°C. Kobayashi et al. (1997) also reported similar pattern of oyster spawning in Hinase water near Hiroshima; gonad development occurs at water temperature above 23°C and spawning occurs at water temperature above 27-28°C.

According to Bayne (1976), *C. gigas* is classified as an opportunistic species whose gametogenesis occurs concurrently with the accumulation of energy reserves, resulting in spawning in the summer after spring phytoplankton bloom so that larvae and adults can access the abundant food supplies. Successful spawning and consequent larval development would maximize the probability of successful recruitment. During the course of study in Gosung Bay, the D-shaped oyster larvae first observed in late June and the presence of larvae in the water column continued until the early September (MOMAF 2001). However, the larval abundance in the water column was highest in mid July and then in late August (MOMAF

2001). It is believed that the first peak of larval occurrence was consequence of the first spawning peak occurred in late June.

The present study has documented the prevalence and infection intensity of *Martelioides chungmuensis* over the annual reproductive cycle of oysters in Korea. Interestingly, none of the oysters collected from February to mid June were infected by *M. chungmuensis.* In contrast, infected oysters were detected continuously from June to February, with highest prevalence in the fall. Park and Chun (1989) also reported the prevalence of this parasite in *Crassostrea gigas* populations in the Hwado and Och'on areas on the south coast of Korea. The degree of infection in the summer and fall seasons was 5.3 and 4.2% in 1986, 6.7 and 2.8% in 1987, respectively. The prevalence of *M. chungmuensis* in Gosung Bay surveyed in the present study in 2000 was as much as 16%, somewhat higher than the values reported previously. At Mie, Japan, Imanaka et al. (2001) also observed high prevalence (18 to 20%) from autumn through spring, when oysters completed spawning, as in the present study.

The first incidence of *M. chungmuensis* in this study occurred in late June, when most oysters were partially spawned or ready for spawning. An earlier occurrence of *M. chungmuensis* in early reproductive stages of oysters cannot be ruled out, since our observation was only based on a light microscopic examination of histological slides. In contrast, Itoh et al. (2002) observed immature stages of *M. chungmuensis* in the lining of follicles, although most of the parasites occurred in mature ova in the follicle or genital canal of the infected oysters. In this study, it is possible that *M. chungmuensis* in early developing ova could have been missed during microscopic examination due to their small sizes. According to Itoh et al. (2002), the growth of *M. chungmuensis* in Japanese oysters is highly correlated with the maturation of oyster ova, with the parasite increasing in diameter and becoming easier to identify in a microscopic field. Consequently, low prevalence or no infection recorded in spring could reflect a lack of sensitivity of the microscopic examination, especially on early stages of the infection. The use of DNA probes during

in situ hybridization could clarify this point (Le Roux et al. 1999, Kleeman et al. 2002)

Temperature is considered as main parameter governing the life cycle and sporulation process of another paramyxean parasite, *Marteilia refringens* (Berthe et al. 1998, Audemard et al. 2001). High infection intensity and prevalence of *M. refringens* in European flat oysters, *Ostrea edulis* in France was correlated with high water temperature (Berthe et al. 1998). In the present study, infection intensity of *M. chungmuensis* was highest in June (Fig. 5), when water temperature was high, although some oysters collected in December and January, when temperature was as low as 8 ^{o}C, exhibited mature eggs infected with the parasite. Our data shows that *M. chungmuensis* can survive in such low water temperature, as long they are enclosed in the eggs. It is noticeable that no phagocytosis of these infected ova was observed in oysters collected in winter, while uninfected oyster eggs that remain in normal oysters in winter were phagocytized and absorbed by hemocytes. Perhaps *M. chungmuensis* inhibits phagocytic activity of oyster hemocytes in order to survive in the eggs, although the mechanism has yet to be proven.

Based on microscopic appearance and size, Imanaka et al. (2001) described 8 different life stages of *M chungmuensis* in *C. gigas* in Japanese waters while Itoh et al. (2002) described only 4 life stages. According to Imanaka et al. (2001), *M. chungmuensis* undergoes intracellular divisions in the eggs and progressively moves to the lumen of the genital tubules. However, the life stages proposed by Imanaka et al. (2001) and Itoh et al. (2002) are limited to the stages presented inside the eggs. The life stage of *M. chungmuensis* in the water column and the mechanism by which it enters the oyster eggs are still unknown. Since *M. chungmuensis* are not observed from oysters in reproductively inactive stage in February and March, it is postulated that the parasites are distributed in the water column and may enter oysters during the early developing stage in April or May. As gonad development progresses, *M. chungmuensis* grow and reproduce inside the eggs. Infection probably begins in spring, as observed in Japan,

and proceeds through multiplication and sporulation stages during high water temperature period from summer to early fall. Stressful environmental conditions such as high temperature and sudden decrease in salinity could weaken the cellular defense activity of oysters and possibly accelerate multiplication of the parasite. The poor physiological condition of oysters after spawning could provide relatively favorable conditions for *M. chungmuensis*, resulting in higher infection intensity and prevalence in late summer and fall. Villalba et al. (1993) also illustrated that high prevalence of a heavy *Marteilia refringens* infection in mussels *Mytilus galloprovincialis* is more frequent when pre-spawning and post-spawning stages are co-occurring in a population of mussels. They suggested that *M. refringens* inhibits gonad development of the mussels mainly after the spring spawning, and that, subsequently, the parasite interferes with metabolic activity of the mussels, resulting in poor condition index for infected individuals. The massive haemocytic infiltrations observed in infected gonads of *C.gigas* in this study suggest that the infected oysters allocate a certain amount of energy to combating the infection. Villalba et al. (1993) demonstrated that there is a negative correlation between *M. refringens* infection and gonad development in mussels they examined. Moreover, Bayne et al. (1982) showed that mussels *Mytilus edulis* infected by parasites also allocate a high portion of energy for cellular defense mechanisms, resulting in reallocation of their energy reserves.

Part II

Seasonal variation and effect of different depths on the biochemical composition and reproduction of Pacific oyster, *Crassostrea gigas*

1. ABSTRACT

Seasonal variations in biochemical composition and reproduction of the oysters, *Crassostrea gigas*, from Gosung Bay, Korea were determined at two different depths of suspended line culture. Protein of surface oysters showed significantly lower levels in April ($P<0.01$) and September ($P<0.05$) and remained similar to that of bottom oysters in other months. Lipid levels of oysters from 2 depths were not different except in April ($P<0.05$), late August and September ($P<0.001$). Carbohydrates in surface oysters were significantly higher in January, mid July ($P<0.05$), late July, and in November ($P<0.01$), however it was lower than in bottom oysters in mid June ($P<0.05$). Change of biochemical compositions, especially carbohydrates followed the reproductive cycle of oysters in studied area. At an early stage of gonad development, gonad maturation of oysters in the bottom water layer was slower than that of oysters in the surface layer. However, spawning activity was fairly synchronized in the 2 different depth intervals during the first spawning peak in June. Mean fecundity varied from 26 to 107 million eggs/female in surface oysters and from 20 to 99 millions eggs/female in bottom oysters. At the first spawning peak, surface oysters showed higher gonad somatic index and fecundity than the bottom oysters, however, the significant reversion was observed after second spawning ($P<0.05$). Results from the present study suggest that surface oysters possessed better nutritional conditions and invested more energy content for their gametogenesis. Our findings also indicate the possible effect of cultured depths on the biochemical compositions and reproduction of oysters in Gosung bay.

2. MATERIALS AND METHODS

2.1. Oyster sampling

This study was carried out at Gosung Bay on the southern coast of Korea (Fig.3). For analysis, adult oysters with shell length over 7 cm were collected from 3 different sampling locations. From each sampling site, an oyster rope was randomly taken and oysters were collected by depth from the surface (0 to 2 m) and the bottom layer (3 to 5 m). Sampling continued monthly from January to December 2000. From June to August, oysters were sampled biweekly to follow spawning activity of this species. At the laboratory, the sizes of oysters were recorded as shell length (mm) using calipers. Soft tissues of oysters were then removed from the shell and wet tissue weight was determined after removing excessive water with absorbent tissue paper. After 24 hours of freeze-drying, the dry tissue weight (g) of each oyster was measured by using a microbalance.

2.2. Environmental conditions

Water temperature and salinity were recorded in situ when oyster sampling occurred. Phytoplankton biomass (chlorophyll *a* concentration) was collected at the water depth of 1 and 5 m with a 3-L van Dorn water sampler. The water was passed through a 250 μm mesh net to remove zooplankton and large particles. Chlorophyll *a* concentration was determined on acetone extracts using the fluorometric method as modified by Parsons et al. (1984) with a 10 AU Fluorometer (Turner Designs). Seawater with suspended solids (SS) and seston was sampled vertically from the surface and the bottom with 10-L plastic bottle. The seawater was transferred to the laboratory, the SS were removed by filtration with a

Millipore membrane filter, and seston was then collected by filtration with a glass-fibre filter (0.47 μm-GF/F). After freeze-drying, seston was weighed and used for biochemical analysis. The freeze-dried seston was homogenized into phosphate buffer solution (0.15M NaCl, pH 7.4, PBS) using an ultrasonifier, and the tissue was then centrifuged to collect the supernatant. Water-soluble fraction of protein in supernatant was determined using the BCA Protein Assay (Pierce, USA). Bovine serum albumin (BSA) was used as a standard for the protein quantification. The lipid fraction in the filtrate was determined gravimetrically after chloroform-methanol extraction (Bligh and Dyer, 1959). Total carbohydrate level in the filtrate was determined with the phenol-sulfuric acid method and agar was used as a standard according to Dubois et al. (1956).

2.3. Biochemical measurements of oyster tissues

The dry tissue of 30 individual was ground separately after freeze-drying for 24 hours. To determine biochemical composition, a known quantity of dry tissue was homogenized in phosphate buffer solution (PBS) using an ultrasonifier. Total protein in the tissue was measured by BCA Protein Assay. Total carbohydrates were quantified photometrically by phenol-sulphuric acid method and dextrose was used as a standard according to Taylor (1995). Extraction for total lipid was performed in a mixture of chloroform and methanol (Bligh and Dyer, 1959) and charred following the method of Marsh and Weinstein (1966). The optical density was red at 375 nm and tripalmitin was used as standard to estimate lipid concentration. The calculation of total protein, lipid and carbohydrates was based on dry tissue weight of each individual and presented as percentage (%).

2.4. Histological preparation and gonad development

Histological slides were prepared as in Part I. Oysters were separated into 2 groups depending on the depth intervals, i.e. surface (0 to 2m) and bottom layer (3 to 5 m). Gonad development of each oyster was categorized into five stages with a numerical code assigned based on microscopic appearance of the gonad tissues, modified from Heffernan et al. (1989), Rose et al. (1990) and Powell et al. (1993). The stages of gonad development are undifferentiated (1), developing (2), ripe (3), spawning (4) and spent (5). Gonad index (GI), a monthly mean of the numerical code of oysters, was then calculated for each sampling period to follow cyclic changes of the reproductive tissues. Since there was no time lag observed in gonad maturation between male and female oysters, the numerical codes of the female and male of each month were then combined for calculating GI.

2.5. Reproductive effort of oyster

Kang et al. (2003) has reported reproductive effort of oysters used in the biochemical analysis in this study. To investigate effect of depth, reproductive effort of oysters reported by Kang et al. (2003) was grouped according to the depth. In brief, reproductive effort of oyster was measured using the egg-specific antibody in enzyme-link immunosorbent assay (ELISA). Total weight of eggs in an oyster was finally expressed as gonad-somatic index (GSI), which is a ratio of egg weight to the total tissue weight.

2.6. Statistical analyses

Non-parametric *t*-test was used to compare the biochemical components and reproductive effort of oysters by month from two depth levels (Sokal and Rohlf, 1995).

3. RESULTS

3.1. Environmental conditions

Monthly mean salinity and water temperature measured from two different depths of the suspended longline are presented in Fig. 16. Water temperature on the surface varied from 4.3°C (February) to 30.4°C (July) seasonally while water temperature at 5m depth varied from 4.2°C (February) to 26.6°C (August). There was a strong stratification of water temperature between the surface and bottom of the oyster string in the Bay from April to September (Fig. 16A). Maximum salinities of 34.3 and 34.2 ‰ were recorded in May in the surface and in the 3-5 m layer respectively. From late June to early July, salinity of the surface water dropped abruptly from 34.0 to 27.6 ‰ due to a large freshwater inflow into the Bay caused by the seasonal monsoon. In contrast, salinity dropped less dramatically in the 3 to 5 m depth layer from June to July (from 34.0 to 32.3 ‰).

Chlorophyll *a* concentration was high in April (4.9 μg/L), July (3.6 μg/L) and September (1.9 μg/L) in surface water layer. In bottom layer, chlorophyll *a* peaked in April (4.2 μg/L), June (3.3 μg/L) and September (3.9 μg/L). From August to September, the chlorophyll *a* level in bottom layer was higher than in the surface however, it decreased abruptly from October to December and reached the lowest level in December (Fig. 17A).

In surface water layer, seston increased from January (3.8 mg/L) and peaked in March (12.7 mg/L). It showed lightly fluctuation from April to May, decreased from June to August (8.0 mg/L) and presented highly fluctuation from September to December. In bottom water layer, seston showed high variation from January (8.1 mg/L) to February (4.0 mg/L) and increased steady from March (9.0 mg/L) to May (11.7 mg/L). Highest seston concentration was recorded in June (14.2 mg/L) and it showed high fluctuation from July to December (Fig. 17B).

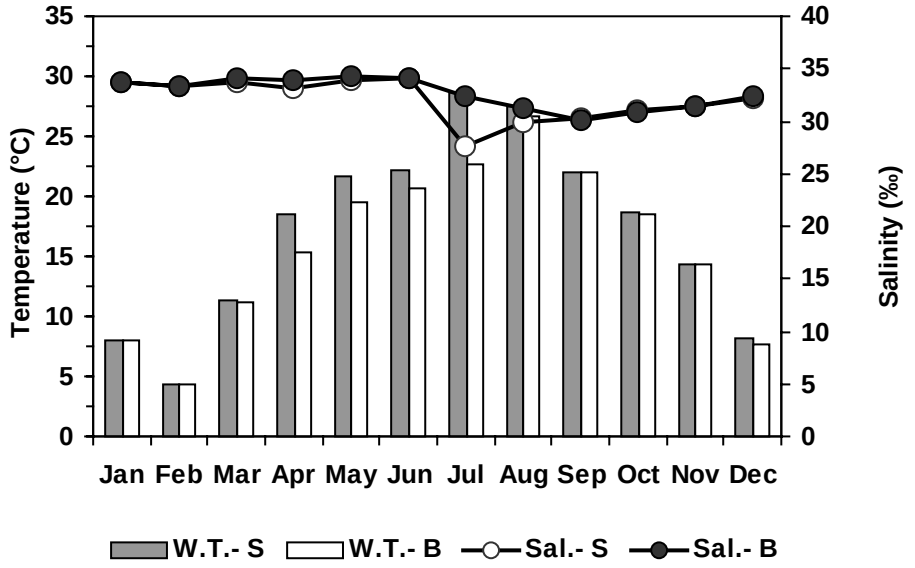

Fig. 16. Seasonal variation of water temperature and salinity in the water column measured from January to December 2000 at Gosung Bay, Korea. (W.T.-S), water temperature on the surface, (W.T.-B), water temperature on the bottom, (Sal.-S), salinity on the surface, (Sal.-B), salinity on the bottom.

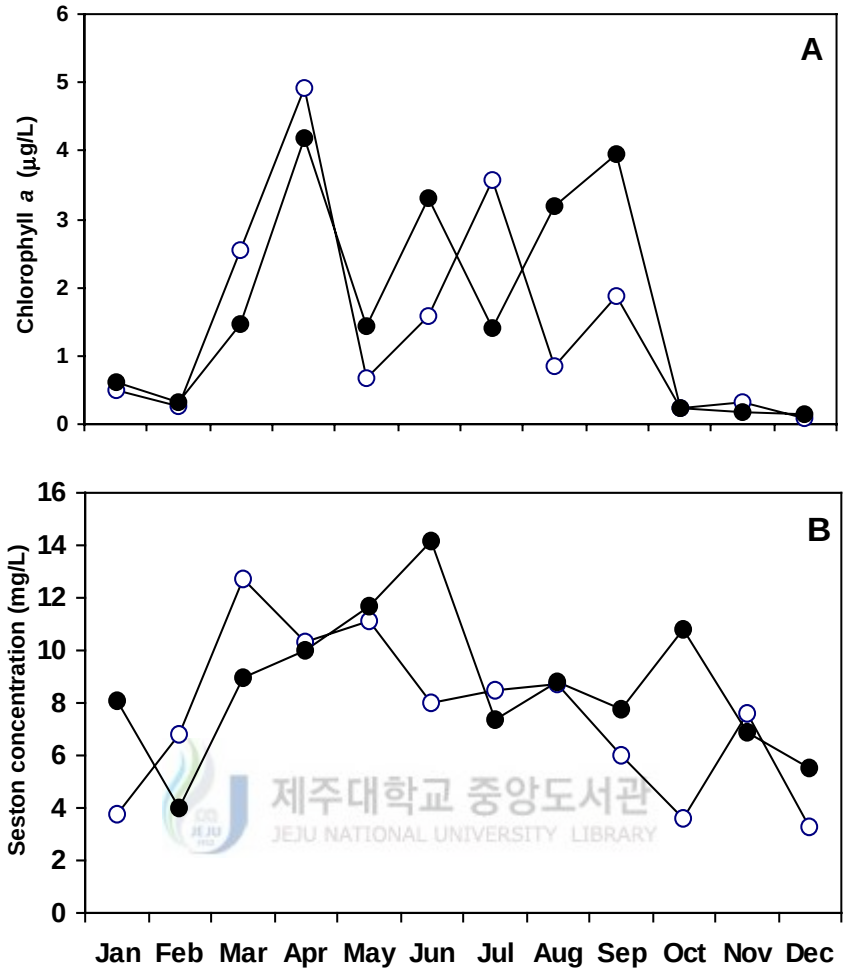

Fig. 17. Seasonal variation of chlorophyll *a* (A) and seston concentration (B) from surface water layer (empty circle) and bottom water layer (solid circle) during sampling period

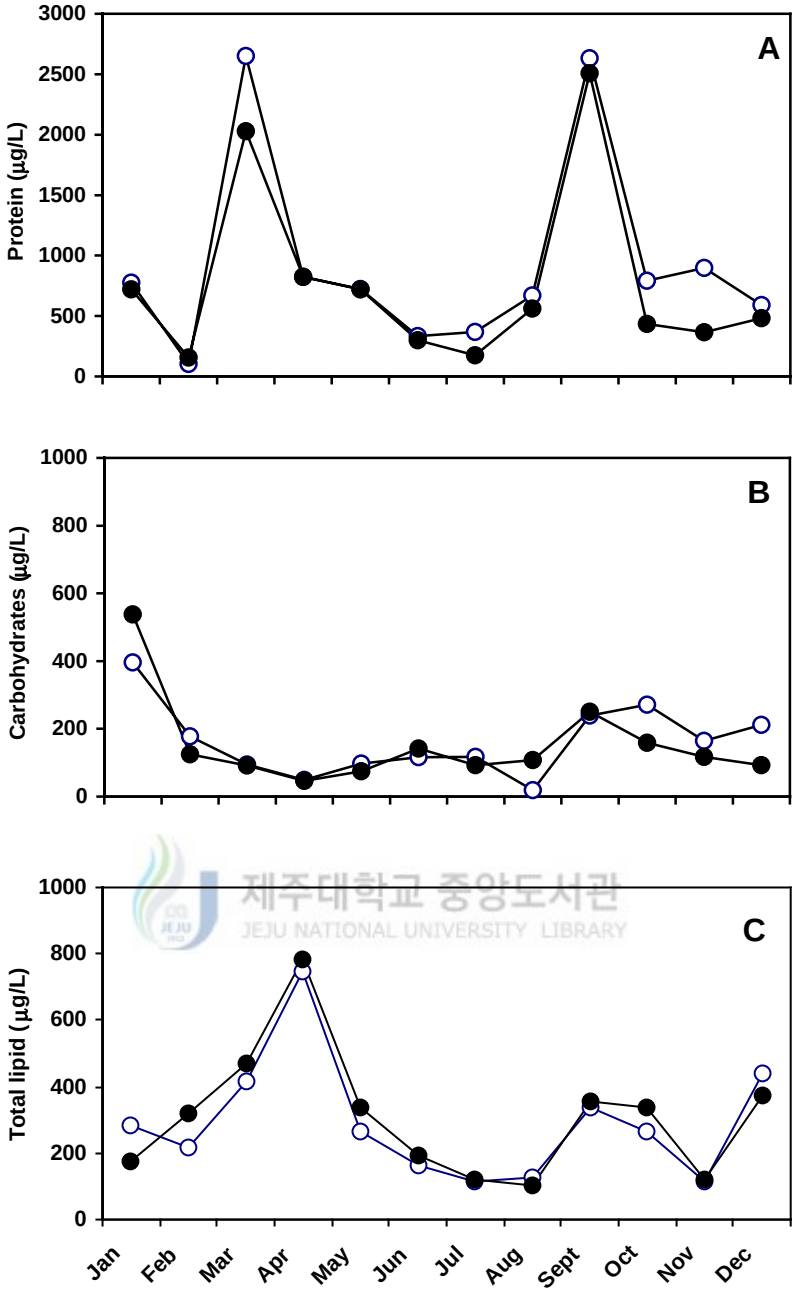

Fig. 18. Seasonal variation of protein (A), carbohydrates (B) and total lipids (C) in seston from the surface water layer (empty circle) and bottom water layer (solid circle) during sampling period.

Figure 18A illustrates the seasonal fluctuation of the protein extracted from seston in seawater samples. In surface layer, the protein level peaked in March (2650 μg/L) and in September (2633 μg/L). High protein levels of seston from bottom layer occurred in March (2029 μg/L) and in September (2507 μg/L). Regardless of depth, high variation of protein occurred in early spring and from August to October. Seston of surface layer showed highly fluctuation of carbohydrates with the peaks in January (396 μg/L) and in October (272 μg/L). Carbohydrates increased steady during phytoplankton bloom from April to June and then decreased to the lowest level in August (19 μg/L); during winter season, carbohydrates maintained higher levels than previous period (Fig. 18B). In seston of bottom layer, highest carbohydrate level occurred in January (538 μg/L) and peaked again in September (251 μg/L). It increased from July to September and decreased from September to the end of year. Lipid of seston from both depths showed 3 peaks in April (750 and 780 μg/L), in September (338 and 355 μg/L) and in December (438 and 372 μg/L). Regardless of depth, lipid levels were low from May to late August and more variation during winter period (Fig. 18C). Seasonal fluctuation of chlorophyll *a* was not completely coincided with the variation of seston. However, high concentration of protein and lipid in seston was observed in March and December corresponding with the high levels of chlorophyll *a* in the water column.

3.2. Variation of dry- wet tissue weight ratio (DWR)

Fig. 19 illustrated the variation of DWR (%) in oysters from 2 depth levels. Oysters in surface layer showed highest DWR in March and April (25 %), DWR peaked in March (27 %) in oyster from bottom layer. Regardless of depth, oysters showed other DWR peak (24 %) in mid June and mid July; then high and low DWR alternated in consecutive months

during April to mid August (corresponding to the spawning period). After spawning period, DWR decreased and showed slightly fluctuation in oysters from surface layer, lowest value was recorded in September (16 %). In oysters from bottom layer, DWR decreased continuously after second spawning in mid August (18 %) to the lowest value in November (15 %). From late June to December, DWR of oysters from surface layer were slightly higher than that from bottom layer. Significant difference of DWR of oysters between 2 depths was detected before first spawning (in February and April); after first spawning (in mid and late July); after second spawning (late August) and in November ($P<0.05$, *t*-test).

3.3. Biochemical composition of oyster tissue

Oysters in surface layer showed three peaks of protein concentration in mid June (39.4%), mid August (37.9%) and October (40.4%). In bottom layer, oysters showed the protein peaks in late June (40.0%), mid August (36.3%) and in September (42.8%). From September to December (post spawning period), the change of protein was similar among oysters from two depth levels (Fig. 20A). In April and in September, surface oysters presented significantly lower protein level than those from the bottom layer ($P<0.05$, *t*-test).

Carbohydrates changed seasonally among oysters from two depth intervals coinciding with reproductive cycle. The highest carbohydrate levels were recorded during early spring and then decreased to minimum values in the second spawning period. Oysters in surface layer showed carbohydrate peak in February (48.2%) and this level decreased to a minimum in late June (12.4%). Oysters in bottom layer also presented highest carbohydrate level in February (44.7%) and decline in late June (9.2%). After spawning, oysters from both depths presented a recovery in carbohydrate level. However, the process seemed more rapid in oysters from the surface layer compared to those from the bottom layer (Fig. 20B).

Statistical analysis indicated the significant difference in carbohydrate levels between oysters from two depths in January; mid June; mid and late July; and in November ($P<0.05$, *t*-test).

In oysters from the surface, lipid level was high in May (24.7 %) and increased to major peak in mid June (31.9%). In oysters from bottom layer, lipid levels reached the maximum value in May (28.4%), varied during spawning period and decreased to the lowest level in late August (10.1%). After second spawning period, lipid levels were low among oysters from two depths and showed high variation at the end of the year (Fig. 20C). Lipid levels of oysters in surface seemed higher than that in the bottom, however the statistical analysis detected the significant in late August and September ($P<0.01$, *t*-test). On the contrary, in April, oysters from bottom layer showed higher lipid level ($P<0.05$, *t*-test).

3.4. Seasonal changes in reproductive condition

Seasonal changes in gonad index (GI) for the 2 depth intervals at which oysters were collected are summarized in Fig. 21. In Gosung Bay, gametogenesis of oysters started as early as January when water temperature reached 8.0°C. The GI of oysters in the surface layer increased rapidly from February to April as the surface water temperature rose from 4.4 to 18.5°C. The first major spawning of oysters was observed in mid-June regardless of the depth, when water temperature reached 20.7 to 22.3°C. A second spawning was also observed in late August, although its intensity was somewhat lower than the peak in mid June. The GI declined dramatically from late August to September and October as the surface water temperature dropped from 27.5 to 18.5°C. Sexually undifferentiated oysters were dominant during December and February at all depths (Table 2).

The effect of culture depth on gonad development of oysters was obvious at an early stage of gonad development; gonad maturation of

oysters in the bottom water layer was slower than that of oysters in the surface layer (Fig. 21). From March to May, when most oysters progressed from early developing stage to fully mature, the monthly mean GI of oysters in the bottom layer was somewhat lower than that of oysters in the surface layer, indicating that gonad development in the bottom layer is slower. Despite the different gonad development rate between oysters in the bottom and surface layers, spawning activity was fairly synchronized in the 2 different depth intervals during the first spawning peak in June, when water temperature on the surface is approximately 2^{o}C higher than in the bottom water layer.

3.5. Gonad somatic index and egg reproduction

Figure 22 illustrated the seasonal changes of GSI in oysters from both depths during sampling period. In surface layer, monthly mean GSI increased rapidly from March to April (0 - 16.4 %) and reached the highest value of 49.5 % in mid June corresponding to the gametogenesis and the first spawning of oysters. Mean GSI decreased abruptly in late June (10.4 %) and increased again in late July up to 26.7 % (before second spawning). Mean GSI decreased from mid to late August (18.8-22.0 %) after second spawning peak. In oysters from bottom layer, mean GSI also increased continuously from March to April (0-15.6 %) and highest value was recorded in mid June (41.1 %), however, the magnitude was somewhat lower than the surface oysters. The spawning process of oysters from bottom layer was completed slower than those from the surface water with the mean GSI decreased slowly from mid August to late August (19.5-12.6 %).

Table 3 showed the variation of fecundity of oysters from each depth during the spawning period. In May, mature egg numbers were recorded as 39.4 million and 28.2 million in oysters from surface and bottom layer, respectively. In mid June, the highest fecundity was recorded as 107

million in surface oysters and 99 million in bottom oysters. After the first spawning, egg numbers of oysters from both depths decreased and then increased slightly to reach the second peak in late July. At the second spawning, the fecundity was much lower than in the first; with 26 million eggs/female (in oysters from surface layer) and 20 million eggs/female (in oysters from bottom layer). At the first spawning peak in mid June, surface oysters showed higher GSI and fecundity than the bottom oysters ($P<0.05$, *t*-test), however, the reversion was observed after second spawning in late August.

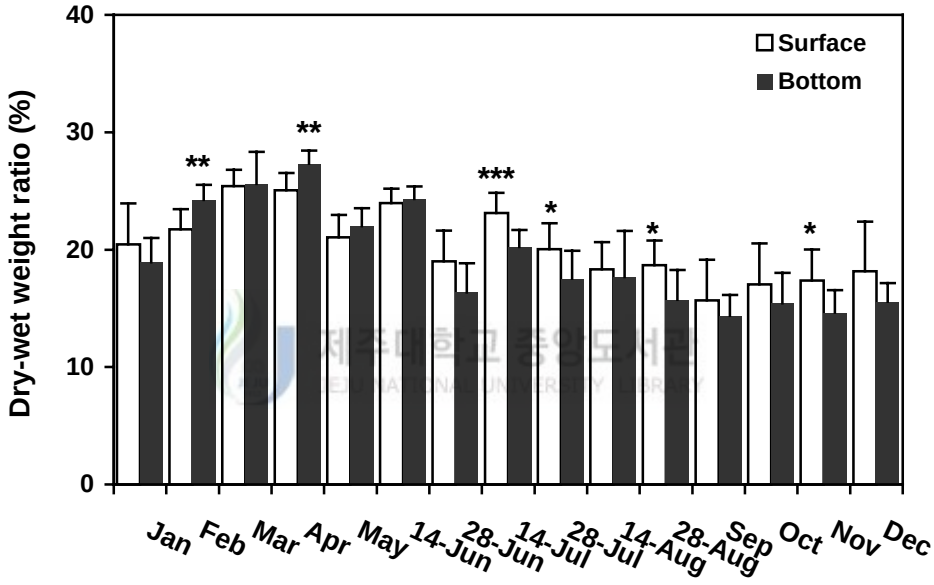

Fig. 19. Seasonal variation of dry-wet tissue weight ratio (%) in oysters from different depths during year 2000 in Gosung Bay. Each bar represents the monthly mean value with the standard deviation as a vertical line. Significant difference levels with *t*-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

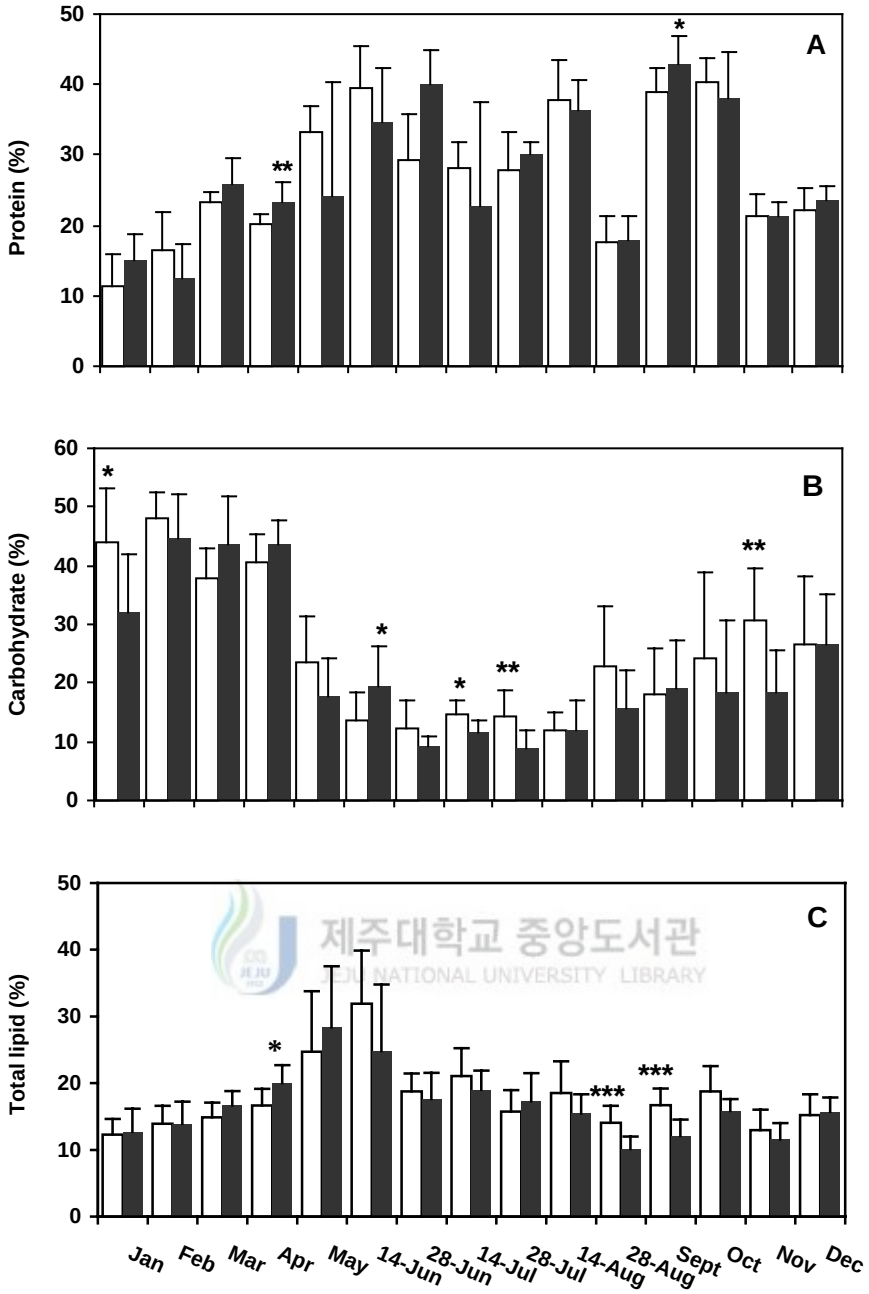

Fig. 20. Seasonal variation of protein (A), carbohydrates (B) total lipids (C) in oysters from surface water layer (empty bar) and bottom water layer (solid bar) during sampling period. Vertical lines indicate the standard deviations. Significant difference levels with *t*-test: *, P<0.05; **, P<0.01; ***, P<0.001.

Table 2. Percentage composition of various development stages of oysters collected from January to December 2000 in Gosung Bay, Korea

Gonad stages	Sampling period (from January to December 2000)														
	Jan	Feb	Mar	Apr	May	14. Jun	28. Jun	14. Jul	28. Jul	14. Aug	28. Aug	Sep	Oct	Nov	Dec
Surface															
Undifferentiated	100	73.3													76.9
Developing		26.7	66.7	53.8	6.7										
Ripe			33.3	46.2	80.0	26.7	30.8	46.7	50.0	41.7	25.0				
Spawning					13.3	73.3	53.8	33.3	35.7	50.0	33.3	33.3	23.1	7.1	
Spent							15.4	20.0	14.3	8.3	41.7	66.7	76.9	92.9	23.1
Bottom															
Undifferentiated	100	80.0	26.7												23.1
Developing		20.0	73.3	63.6	16.7			21.4			7.1				
Ripe				36.4	83.3	35.7	35.7	42.9	42.9	25.0	21.4				
Spawning						64.3	50.0	14.3	42.9	58.3	42.9	38.5	30.8	20.0	
Spent							14.3		14.3	16.7	28.6	61.5	69.2	80.0	76.9

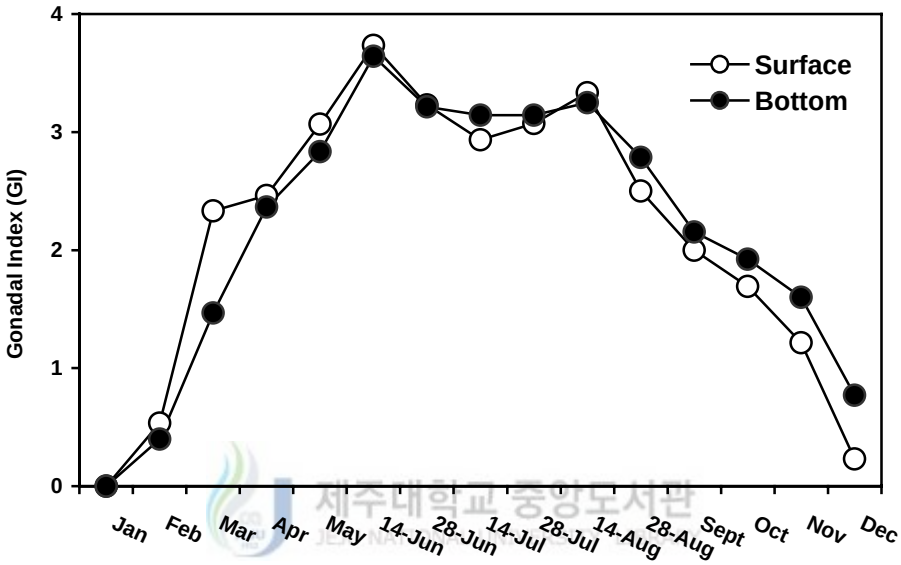

Fig. 21. Seasonal variation in gonad index of oysters collected from 2 different depth intervals during year 2000 at Gosung Bay.

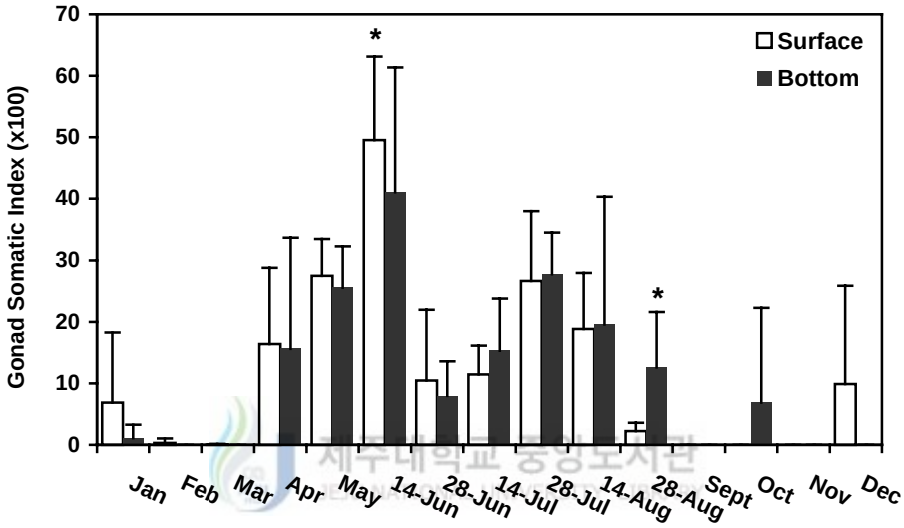

Fig. 22. Seasonal variation in gonadosomatic index of the oysters from different depths during year 2000 in Gosung Bay. Each bar represents the monthly mean value with the standard deviation as a vertical line. Significant difference with t-test: *, $P<0.05$.

Table 3. Mean shell length (mm), dried tissue weight (g) and fecundity (million of eggs/female) of oysters from different depths during spawning period in Gosung bay, Korea.

Sampling date	Surface oysters				Bottom oysters			
	SL	DTW	Fecundity	Range	SL	DWT	Fecundity	Range
May	90.3 (10.5)	1.8(0.5)	39.4 (15.2)	19.2-66.3	74.4 (10.3)	1.4(0.3)	28.2 (9.0)	12.4-42.7
14.Jun	81.0 (6.4)	2.6(0.6)	106.5 (50.7)	49.9-196.3	88.2 (12.7)	3.1(1.0)	99.1 (61.9)	28.6-165.2
28.Jun	74.5 (12.8)	1.4(0.2)	10.0 (11.8)	8.7-28.8	64.6 (6.9)	0.8(0.3)	5.4 (4.3)	0.5-10.7
14.Jul	89.4 (9.1)	1.4(0.4)	11.0 (2.9)	6.7-15.4	72.2 (3.4)	0.9(0.2)	10.1 (5.2)	7.7-18.0
28.Jul	82.3 (15.2)	1.2(0.2)	26.1 (15.7)	10.1-51.1	74.6 (8.7)	0.9(0.4)	19.9 (11.8)	8.7-51.8
14.Aug	95.2 (13.4)	1.2(0.5)	16.4 (10.9)	5.4-37.9	84.2 (6.5)	0.7(0.3)	7.8 (11.3)	7.9-34.6
28.Aug	82.3 (15.7)	0.9(0.1)	1.6 (0.7)	0.8-2.7	79.8 (4.4)	0.6(0.1)	4.9 (2.4)	2.5-8.3

SL, shell length of oysters (mm); DTW, dried tissue weight; data in parentheses indicate standard deviation.

4. DISCUSSION

Regardless of depth, high concentration of chlorophyll *a* in March and in September was concomitant with high concentration of protein and lipid in seston. It was suggested that during early spring and autumn, the phytoplankton contributed major nutrients in water column. That concomitance was not found in December, therefore during winter period other food sources might contribute the main proportion. Ren and Ross (2001) observed the growth of the Pacific oyster is strongly regulated by the phytoplankton concentration, while detritus has little contribution. However, Hyun et al. (2001) mentioned that the growth rates of Pacific oysters require an alternate, substantial food source, besides phytoplankton. Our findings showed that before or during spawning period, the high levels of protein, carbohydrates and lipids occurred in oysters after those components were rich in seston for 1 to 3 months. However, in winter season, the high levels of biochemical compositions in oysters from both depths closely matched with those from the seston. The observation could lead to the suggestion that the assimilation of energy from other food source in seston continued during winter while phytoplankton was limited.

Water temperature and food availability in the water column are the two main environmental parameters that regulate the reproductive process of oysters. Water temperature accelerates or retards the rate of gonadal development while food availability mainly determines quality and quantity of reproductive output (Hayes and Menzel 1981; Soniat and Ray 1985; Hofmann et al. 1992, Choi et al. 1994; Kang et al. 2000). Previous studies on gonad development of *C. gigas* in small bays on the southern coast of Korea have suggested that cyclic changes in reproductive condition of oysters follows seasonal changes in water temperature and food supply in the water column (Bae and Han 1998; Kang et al. 2000; Hyun et al. 2001).

Our data have shown that a few oysters collected in May spawned when water temperature reached 20^{o}C. As Kang et al. (2000) suggested, water

temperature over 18 to 20°C could be the minimum required to induce spawning in *C. gigas* in this area, with major spawning events occurring at water temperature between 23 and 25°C in both depth intervals. The spawning activity of oysters in Gosung Bay continued from May to September, when seawater temperature exceeded 20°C. Such continuous spawning of oysters in summer has also been reported from several studies along the bays of the southern coast of Korea (Bae and Han 1998; Park et al. 1999a, b).

Fig. 16 indicates that water temperature in the water column was stratified from the surface to the bottom from April to July, with surface temperature 2 to 5°C higher than the bottom temperatures. This stratification of water temperature could be reflected in gonad maturation of the oysters. As shown in Table 2, gonad development rate in the surface waters was faster than in the bottom waters from March to May, indicating that water temperature is one of the crucial factors in gonad maturation. Synchronous spawning of oysters in June regardless of the depth, could partly be due to a temperature threshold for spawning (Galtsoff 1964; Kang et al. 2000). In June, water temperature at the bottom level exceeded 20°C, high enough to induce spawning although 2°C lower than the temperature of the surface water layer. Cáceres-Martinez and Figueras (1998) also found no difference in gonad development of the mussel *Mytilus galloprovincialis* between 2 and 5 m depths. Deslous-Paoli and Heral (1988) demonstrated that seasonal variations in reproductive condition and biochemical composition of the oyster *Crassostrea gigas* could be affected by cultivation density (i.e. in relative food availability), in addition to temperature. Loosanoff (1965) observed that oysters, *Crassostrea virginica* living in shallow waters (10 feet) developed much larger quantities of spawn than those living at greater depths (20 or 30 feet). The oysters may have accumulated more spawn in shallow water because the phytoplankton they use as food were more abundant at that depth. There is no information from our study showing the relationship between food availability and reproductive effort in oysters from Gosung Bay. In

addition, the synchronous spawning and gamete production for depths was not significantly different. Loosanoff and Engle (1947) confirmed that within the depth range of 10 to 30 feet, the spawning of the oysters began at the same time.

DWR reflected the variation of condition of oysters during year cycle, especially during gametogenesis from March to September. Regardless of depths, high DWR occurred in April, mid June and mid July and it coincided with the intensive accumulation of energy reserves to build up the gametes. DWR decreased after each spawning peak, especially when most of oysters completed their spawning in September. In our study, oysters showed slow recovery after spawning and the lowest DWR occurred during winter season. Kang et al. (2000) observed a rapid recovery in November in oysters from the Jaran bay whereas the recovery was slow in February from the Hansan-Koje bay at the southern coast of Korea. Part of the differences in condition index and recovery were most likely due to differences in food availability. In Gosung bay, high levels of seston were observed in March (surface water) and in June (bottom water) somewhere later than in southern coast however, high variation and low levels were observed earlier during July to October. After spawning, the energy reserves were depleted in oyster bodies and the food availability was limited in the environment, which could explain the prolonged recovery state in studied area.

Seasonal increase in the protein level in June and early August is concomitant with a period of intense energetic demand for gamete development. In Gosung bay, oysters from different depths also showed increase in protein levels during and at the end of the spawning period. It was in accordance with previous suggestions that protein could serve as an energy reserve during and at the end of gametogenesis (Gabbott and Bayne, 1973, Barber and Blake, 1981, Ruiz et al.1992). From mid June onward, especially during the second spawning period, the protein in bottom oysters were somewhere higher than in surface ones indicating the important role of this component when carbohydrate and lipid reserves were limited.

Whyte et al. (1990) mentioned that protein contributed more than carbohydrate to maintenance energy in oysters under conditions of extended food deprivation, even carbohydrate was apparently available in sufficient quantity. Furthermore, Beninger and Lucas (1984) observed the important role of protein when bivalves were in an imbalanced energy condition. On the other hand, protein levels of oysters from both depths were high in September and October that coincided with high levels of chlorophyll *a* also high concentrations of protein and lipid from the seston. Oyster is considered as the oppoturnistic species and it may speed up the accumulation of energy storage when the food availability is abundant in ambient environment.

During recovery status (in November) and accumulative period (in January), especially before second spawning peak (from mid to late July), the surface oysters presented higher carbohydrate levels than those from the bottom layer. Our findings were in accordance with Lodeiros et al. (2001) who showed that carbohydrate content decreased markedly with depth, especially during the phase of limited reproductive investment. In addition, the accumulation and utilization of carbohydrates in surface oysters was highly correlated to protein and lipid levels. The natural gametogenic cycle in bivalve mollusks is closely linked with cycles of glycogen storage and subsequent de novo synthesis of lipid during vitellogenesis at the expense of stored glycogen (Gabbott, 1975). Gallager and Mann (1986) mentioned that the limitation of this process might lead to the production of either fewer eggs or eggs of sub optimal quality. In the present study, surface yielded a higher reproductive output than the oysters from greater depth during the first spawning. Limited accumulation of carbohydrates presumably relates to insufficient food availability and this might affect the energy allocation to form the gametes in the bottom-layer oysters.

During gametogenesis, the bioconversion between protein-carbohydrate and lipid-carbohydrate were clearly observed in surface oysters not in the deeper oysters. These results could lead to the suggestion that oysters in

surface water possibly possessed better bioconversion of the energy reserves for their gametogenesis. In *C. gigas*, Ruiz et al. (1992) observed the stored glycogen was used during gametogenesis, proteins and lipids were utilized during winter when available food is scarce. In Gosung Bay, the seasonal changes of the biochemical composition of *C. gigas* were similar to that observation. Robledo et al. (1995) detected that reducing proteins were higher in haemolymph of mussels kept at 5-m depth compared to 2-m depth. The author suggested a possible rhythmic mobilization of the reserves, i.e. in function of the environmental differences carbohydrate accumulation occurs at one point and protein at another.

At early stage of gonad development, our findings indicated that gametogenesis of oysters in bottom water layer was slower than that in the surface. Kang et al. (2003) found the positive correlation between oyster size (i.e. total dry weight) and egg number. In the present study, bottom oysters presented slower growth performance consequently the mature size was smaller than those from the surface. It could be one of reasons to explain the lower fecundity occurred at the first spawning. However, Barber et al. (1988) mentioned that a lack of available energy would best account for the lower gonad production, greater decrease of resorption and lower fecundity exhibited by the deep-water scallops. In addition, Loosanoff (1965) observed that *Crassostrea virginica* living in shallow water (3 m) developed much larger quantities of spawn than oysters living at greater depth (6 or 9 m). The oysters may have more spawn in shallow water because the phytoplankton they use as food were more abundant. The pattern of accumulation and transfer of energy in deeper-water oysters from the present study could be accordance with that observation. On the other hand, in late August, GSI and fecundity of oysters in bottom layer was higher than in the surface. It could lead to the suggestion that oysters might speed up their energy conversion and immediately use for gametogenesis when higher food availability together with higher temperature occurred.

Several studies mentioned the rapid transfer of assimilated food from the digestive gland to the other tissue when sufficient food was available during gametogenesis (Gabbott, 1976; Urratia et al., 2001). According to Thompson (1972), this transfer may occur over a period of about seven days in *Mytilus edulis*. Furthermore, Hofman et al. (1994) mentioned that variations in temperature and food supply affect reproductive effort more than adult size because the rate of energy flow through the oyster is higher in warmer months when most net production is allocated to reproduction. On the recruitment aspect, surface oysters could take some advantages when most of their energy invested for earlier spawning. Larvae might avoid food competition during the early planktonic larval stages and might avoid the high predation pressure of zooplankton-eating predators. Other study showed that D-shaped oyster larvae occurred with highest density in late July (MOMAF, 2001). Although oysters in deeper water layer might produce higher number of eggs during second spawning peak, their larvae could face to the nutrient shortage when the high competition occurred with larger size and higher density of larvae from first spawning period.

This study is the first attempt to correlate the biochemical composition and reproductive effort in the Pacific oyster, *C. gigas* in Korea. The depth levels of suspended culture possibly related to the variations in biochemical compositions and reproduction of oysters. The faster gonad development and faster recovery of the oysters in surface layer offer good indications that they are in a better nutritional condition with much greater energy contents to invest for their gametogenesis. Our findings indicate that the cultivated depth of oysters in Gosung Bay should be better considered in order to reach the best conditions for growth and for reproduction.

Part III

Seasonal changes of *Perkinsus* and *Cercaria* infections in the Manila clam *Ruditapes philippinarum* from Jeju, Korea

1. ABSTRACT

Seasonal changes in the infection intensity of *Perkinsus* and *Cercaria* were investigated in Manila clams *Ruditapes philippinarum* collected from a sand beach on the east coast of Jeju, Korea. *Perkinsus* prevalence and infection intensity were determined from histological preparations of clams. The prevalence varied seasonally; it was lowest in late September 2001 (6.0%) and highest in March 2002 (86.0%), with an annual mean of 32.9%. The infection intensity of *Perkinsus* varied from 0.11 (September 2001) to 2.08 (March 2002), with a mean of 0.63. Tissue inflammation caused by massive hemocyte infiltration was commonly observed in the gills of clams that were heavily infected with *Perkinsus*. Degenerated oocytes and castrated follicles were observed in clams that were severely infected with *Cercaria* during the spawning period, indicating that *Cercaria* interferes with the reproductive process of clams. The prevalence of *Cercaria* was highest in early August (12.0%), while none of the clams were infected with *Cercaria* in some months. Although *Perkinsus*- and *Cercaria*-related clam mortalities were not observed in this study, the histological findings clearly show that the parasitism impacts clam reproduction, at least during part of the annual reproductive cycle.

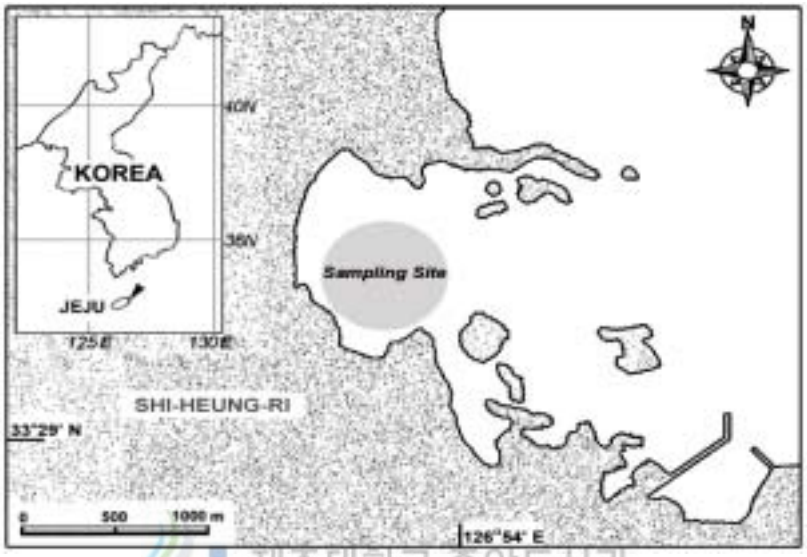

Fig.23. Location of the sampling area, Shi-Heung-Ri Beach on the east coast of Jeju Island.

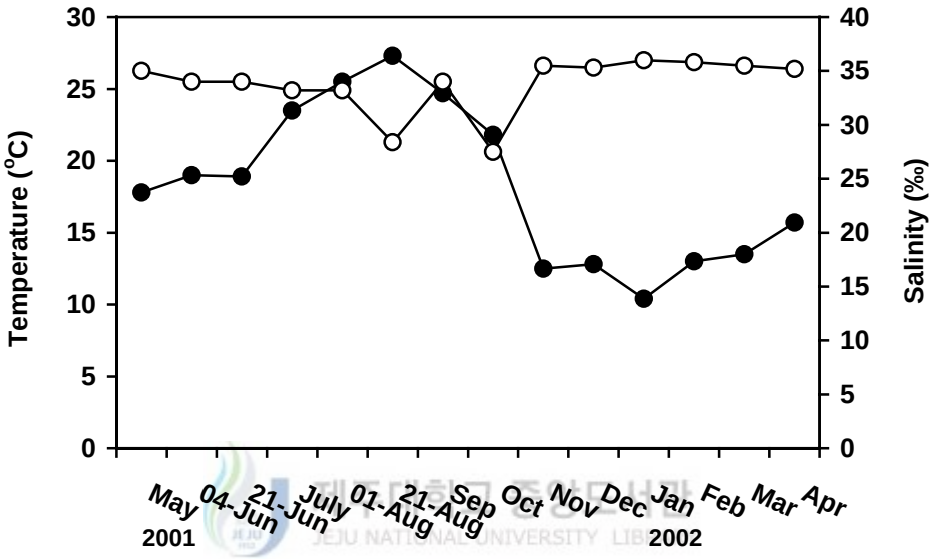

Fig. 24. Monthly change of surface water temperature (•, °C) and salinity (○, ‰) during sampling period.

2. MATERIALS AND METHODS

2.1. Clam sampling and histological preparation

Shi-Heung-Ri Beach is located on the east coast of Jeju Island (Fig. 23). Clams from a natural population were collected monthly or biweekly during the spawning season from May 2001 to April 2002. At the laboratory, the shell lengths of clams were measured using a Vernier caliper. The tissues were then removed, and their weight was recorded. For histological preparation, a transverse section was cut in the middle of the body and fixed in Davison's fixative. Tissue samples were then dehydrated in ethanol, embedded in paraffin, and sliced to 5 μm. The histological sections were stained with Harris' haematoxilyn and eosin Y and examined under a light microscope.

2.2. Annual reproductive cycle of clam

The condition of gonad maturation observed in the histological preparations was categorized according to the gonad index (GI) of Walker and Heffernan (1994). GI varies from 0 to 4: 0, inactive; 1, spent; 2, developing; 3, ripe; and 4, spawning. The mean GI of each sampling period was calculated in order to follow the annual reproductive cycle of the clams.

2.3. Prevalence and infection intensity of *Perkinsus* and *Cercaria*

Prevalence (the percentage of clams infected) and infection intensity of *Perkinsus* and *Cercaria* were determined from histological slides. A numerical scale was developed to describe the infection intensity of *Perkinsus*. The infection was ranked as follows: 0 = no *Perkinsus* detected in any tissue; 1 = *Perkinsus* was limited to the mantle and gills; 2 = limited to mantle, gill filaments and digestive tubules; 3 = found in mantle, gill filaments, digestive tubules, and gonads; and 4 = *Perkinsus* was found in

all types of tissue. The effect of *Perkinsus* infection on gonad maturation was tested for each sampling period using non-parametric ANOVA and a range test. For *Cercaria* infection, only prevalence was determined for each sampling period.

3. RESULTS

3.1. Environmental conditions and sampling effort

The salinity and temperature of surface water from May 2001 to April 2002 are plotted in Fig. 24. Water temperature varied from 10.4 (January) to 27.3°C (August). The highest salinity was recorded in January (36‰), and the lowest was observed in October (27.5‰). A total of 686 clams was used in the analysis, with a mean shell length of 32.4 ± 4.1 mm and a mean wet tissue weight of 1.30 ± 0.60 g.

3.2. Annual reproductive cycle of *Ruditapes philippinarum*

Fig. 25 and 26 illustrate the different stages of gonad development in female and male clams. In early developing stage, the number of follicles increased and the follicles became expanded with the present of early vitellogenic oocytes or spermatogonia (Fig. 25B and 26B). Follicles continuously expanded and exhibited growing oocytes or spermatocytes in late developing stage (Fig. 25C and 26C). Fully ripe gonads were filled with ripe oocytes or active spermatozoa (Fig. 25D and 26D). After spawning, gonads were considerably decreased in volume and contained a small number of residual gametes (Fig. 25E and 26E). Fig. 25F shows a female spent gonad with empty and shrunken follicles. In male gonad, a few follicles contain residual unspent spermatozoa (Fig. 26F).

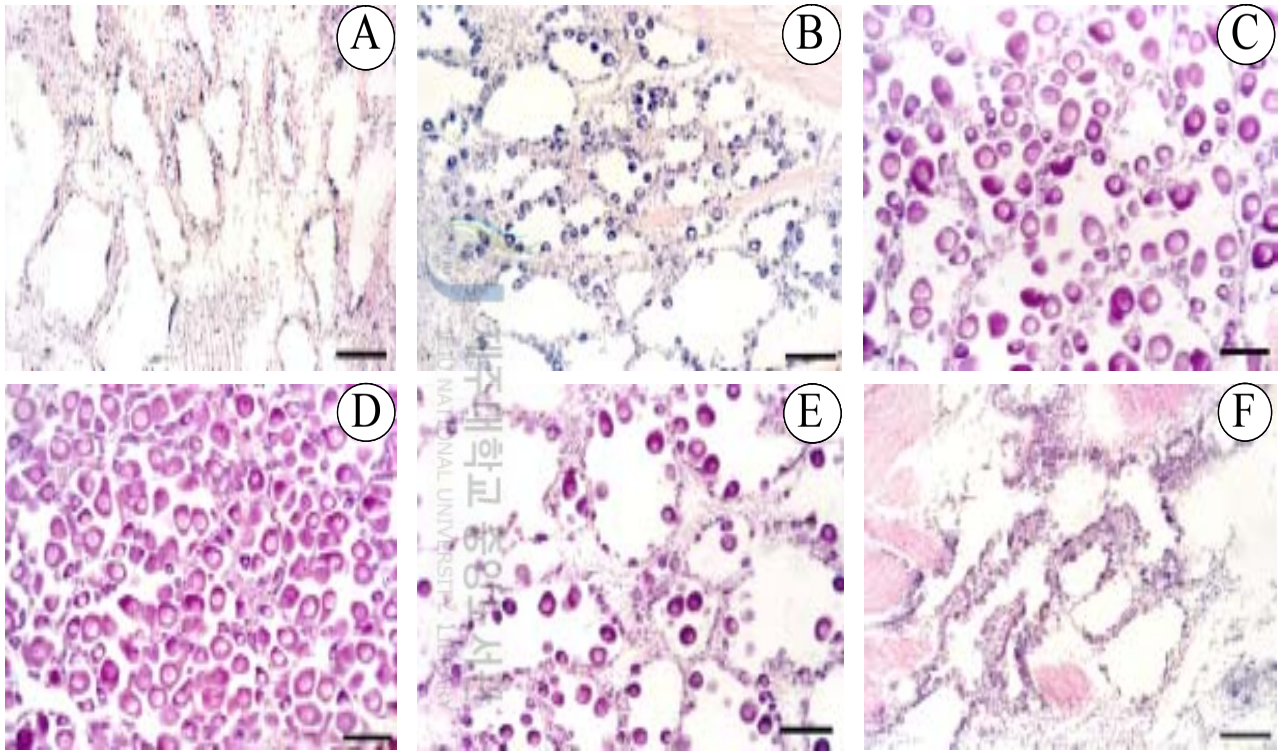

Fig. 25. Gonad development in female clams. (A) Sexually indifferentiated stage; (B) Early development stage; (C) Late development stage; (D) Ripe stage; (E) Spawned stage; (F) Gonad tissue atrophy.

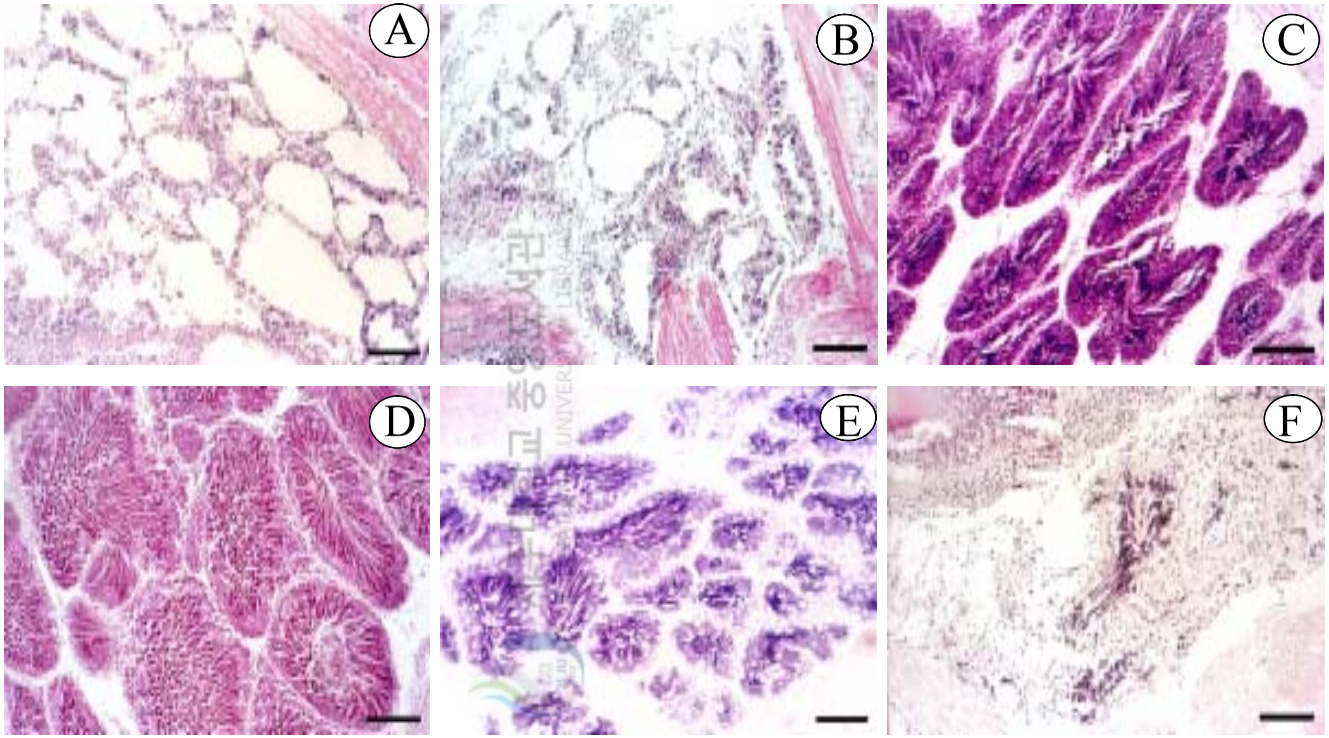

Fig. 26. Gonad development in male clams. (A) Sexually indifferentiated stage; (B) Early development stage; (C) Late development stage; (D) Ripe stage; (E) Spawned stage; (F) Gonad tissue atrophy.

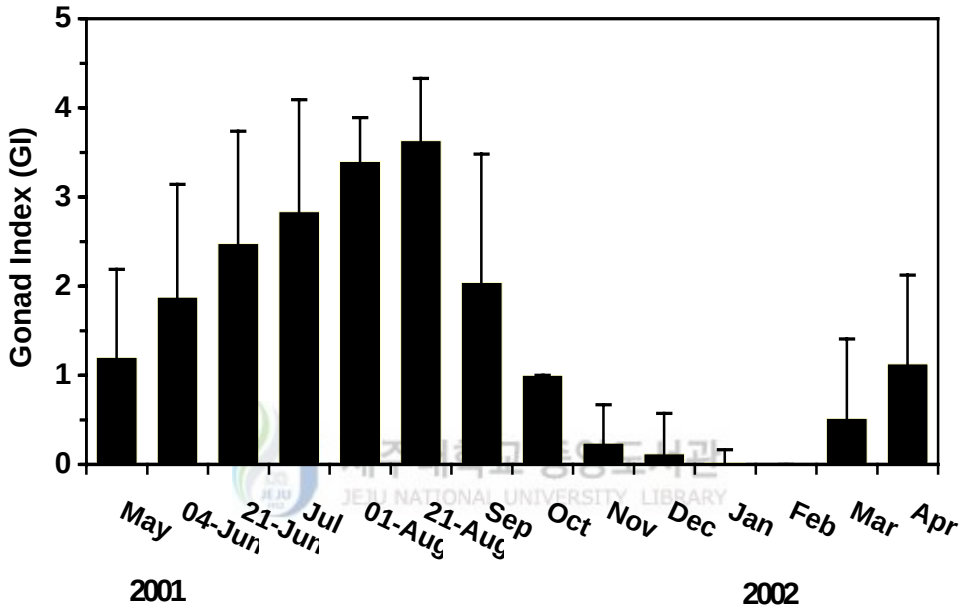

Fig. 27. Monthly mean gonad index of clams collected from Shi-Heung-Ri beach from May 2001 to April 2002.

Fig. 27 shows the mean GI for each month. Gametogenesis was initiated in clams as early as March when the surface water temperature reached to 13°C. GI increased at a faster rate from May to early August, as the water temperature rose rapidly. Major spawning was observed in the clams in early July, when the surface water temperature was 23°C, and continued until the end of August. Clams at the spent stage were commonly observed from September to November. Sexually inactive clams were dominant during winter (December to February).

3.3. Seasonal changes of *Perkinsus* infection

Table 4 shows the prevalence and infection intensity of *Perkinsus*. The prevalence was relatively high in winter to spring, and the highest prevalence was recorded as 86.0% in March 2002. In contrast, *Perkinsus* prevalence was lower in summer (6 - 26% from June to late September) than in winter and spring. Fig. 28 shows the monthly mean infection intensity of *Perkinsus*. The average infection intensity was less than 1 (i.e., *Perkinsus* infection was limited to gill tissues) in almost all sampling periods, except March 2002 (2.08). *Perkinsus* infection intensity was higher in winter and early spring than in summer. No statistically significant difference in gonad development was observed among the different levels of *Perkinsus* infection (0 to 4 in this study) for each sampling period, except in March 2002. In this month, less heavily infected clams showed more advanced gonad development (non parametric ANOVA, $p < 0.001$). *Perkinsus* was most common in gill filaments and digestive tubules, whereas they were rare in the foot and adductor muscle (Fig. 29). Hemocytes were often aggregated around the trophozoites, although the inflammation of clam tissues was not observed in this study.

Table 4. Mean shell length, wet tissue weight and monthly infection prevalence of *Perkinsus* and *Cercaria* in Manila clam collected during sampling period in Shi-Heung-Ri, Jeju Island.

Sampling date	Number of samples	SL (mm)	WTW (g)	*Perkinsus* infection			*Cercaria* infection	
				N	Prevalence (%)	Intensity	N	Prevalence (%)
11-May-2001	50	31.5 (3.2)	1.22 (0.39)	21	42.0	0.59 (0.81)	1	2.0
04-Jun	50	33.6 (4.2)	1.71 (0.81)	13	26.0	0.65 (1.19)	4	8.0
21-Jun	50	33.2 (2.9)	1.47 (0.42)	12	24.0	0.43 (0.89)	4	8.0
11-July	50	31.0 (2.1)	1.16 (0.37)	13	26.0	0.40 (0.80)	1	2.0
01-Aug	50	28.7 (3.1)	1.18 (0.46)	5	10.0	0.16 (0.55)	6	12.0
21-Aug	50	33.7 (5.3)	1.92 (0.99)	15	30.0	0.68 (1.27)	0	0.0
29-Sep	50	32.6 (4.2)	1.44 (0.59)	3	6.0	0.11 (0.48)	5	10.0
31-Oct	49	32.3 (4.5)	1.09 (0.47)	18	36.7	0.58 (0.99)	4	8.2
31-Nov	50	35.1 (2.9)	1.50 (0.38)	14	28.0	0.49 (0.82)	4	8.0
30-Dec	50	32.5 (3.3)	1.10 (0.38)	18	36.0	0.73 (1.16)	0	0.0
24-Jan-2002	48	31.6 (4.5)	1.04 (0.57)	17	35.4	0.73 (1.16)	3	6.0
25-Feb	50	32.1 (4.5)	1.09 (0.51)	17	34.0	0.58 (1.05)	1	2.0
28-Mar	50	32.7 (3.4)	1.09 (0.37)	43	86.0	2.08 (1.38)	0	0.0
29-Apr	50	31.5 (4.0)	1.16 (0.56)	20	40.0	0.71 (1.01)	2	4.0

SL, shell length; WTW, wet tissue weight; N, number of infected clams; number in parentheses indicate the standard deviation.

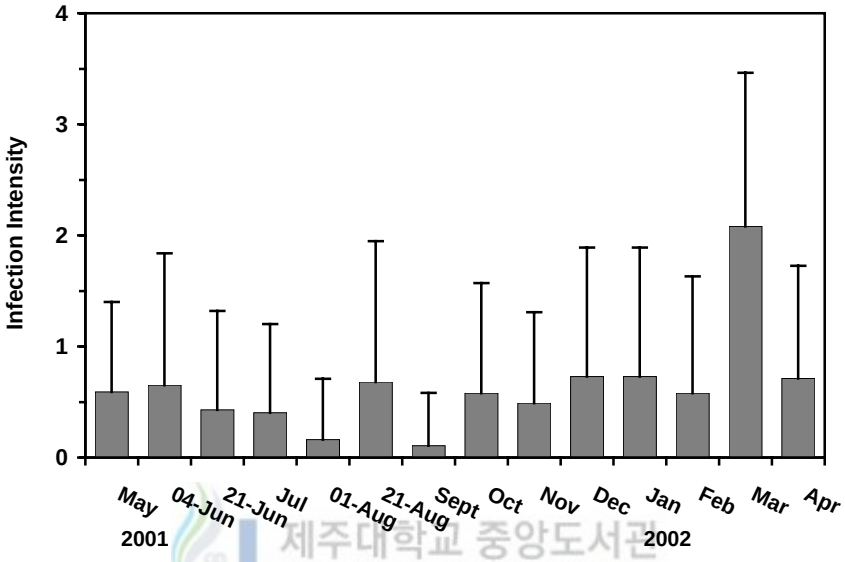

Fig. 28. Monthly mean of *Perkinsus* infection intensity measured from histological slides.

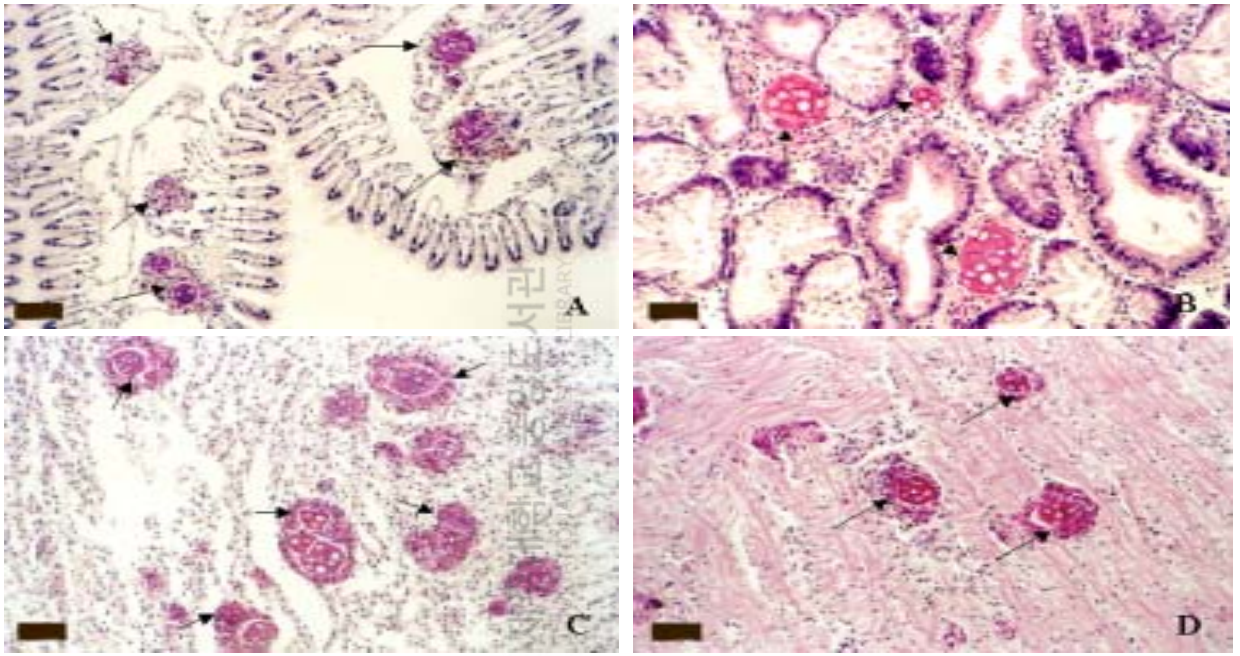

Fig. 29. Histopathologic features of *Perkinsus* infection in *Ruditapes philippinarum.* (A) Severe hemocyte infiltration (arrow head) around the *Perkinsus* trophozoites in gills; (B) Trophozoites of *Perkinsus* (arrow head) in connective tissues of digestive glands. Inflammation of clam hemocytes; (C) Encapsulated *Perkinsus* trophozoites (arrow head) in clam gonad and massive hemocyte aggregation in the follicle; (D) *Perkinsus* trophozoites in the foot, arrow indicates trophozoites and concentrated hemocytes around *Perkinsus* (bar = 50μm).

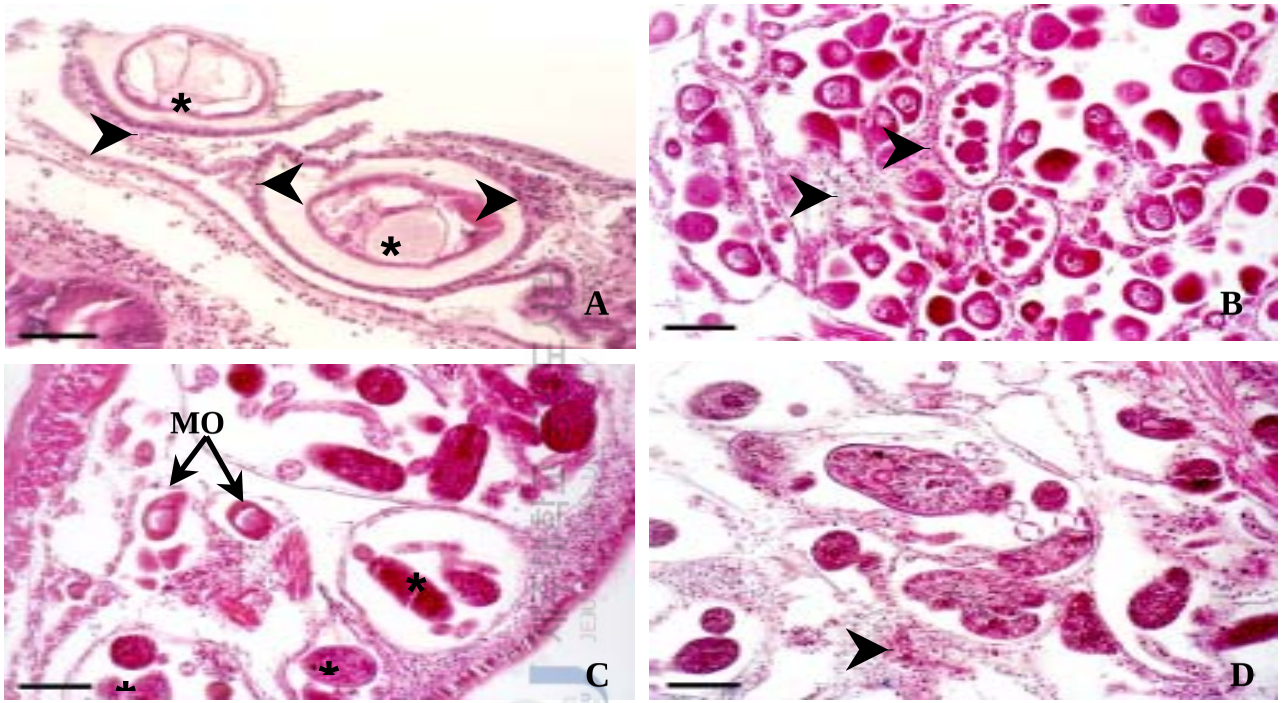

Fig. 30. Histopathology of *Cercaria* in infected-clam. (A) Cross section of parasite in mantle tissues (∗) (bar = 50 µm); (B) Sporocysts of parasite (∗) present in clam gonad; (C) Sporocysts expand in most of follicles (*), few mature oocytes present (MO); (D), Sporocysts and young *Cercaria* (*) in the degenerating gonad (bar = 100 µm for B, C and D). Arrow heads indicate hemocyte infiltration of the clam.

3.4. Prevalence of *Cercaria*

The prevalence of *Cercaria* at each sampling period varied from 0% to 12%. No apparent seasonal change was observed in the incidence of *Cercaria* (Table 4). *Cercaria* was common in the mantles and gonads of female clams (Fig. 30A and B). In particular, heavy infestation of the trematode resulted in gonad castration and deterioration of the connective tissues, indicating that the female gonad is the main target of *Cercaria* (Fig. 30C and D).

4. DISCUSSION

The monthly prevalence of *Perkinsus* observed in clams from Shi-Heung-Ri beach was usually less than 40%, except in March, which is somewhat similar to the previous report for this area. Choi and Park (2001) surveyed the spatial distribution of *Perkinsus* along the coast of Jeju Island and reported the prevalence as 33% near the Shi-Heung-Ri beach area. However, the prevalence in this study is much lower than that level from clam beds along the west coast of Korea or in China and Japan where the prevalence was generally 70% - 100% (Choi and Park, 1997; Hamaguchi et al., 1998; Liang et al., 2001; Park and Choi, 2001; Choi et al., 2002). Perkinsosis is an epidemic disease, and often infection intensity, as well as prevalence, is positively correlated with the density of host organisms (Da Ros and Canzonier, 1985; Auzoux-Bordenave et al., 1995). The low infection intensity and prevalence of *Perkinsus* at Shi-Heung-Ri beach could be associated with a low density of clams, given that clam density at Shi-Heung-Ri beach is much lower than that reported for commercial clam beds in Korea. Choi and Park (2001) and Park and Choi (2001) have also mentioned that clams distributed on Jeju Island beaches and along the east coast of Korea were mostly *Perkinsus*-free or exhibited very low prevalence and infection intensity.

A numerical scale from 0 (no infection) to 4 (*Perkinsus* in all tissues) was developed in this study to grade the infection intensity of *Perkinsus*, based on the occurrence of this parasite in clam tissues. Several studies have indicated that the gill is the portal for *Perkinsus* infection, based on findings that *Perkinsus* is limited to gill tissues in the early phase (Mackin, 1962; Azevedo, 1989; Rodriguez and Navas, 1995). As the infection advances, *Perkinsus* spreads from the gill to other tissues using host hemolymph as a way of dispersal (Azevedo, 1989; Navas et al., 1992; Rodriguez and Navas, 1995). At a high level of infection, *Perkinsus* is found in all types of tissue, including adductor muscle and gonadal connective tissues (Rodriguez and Navas, 1995; Choi et al., 2002). In the present study, *Perkinsus* was confined to gill tissues in most of the clams, suggesting that the level of infection in clams at Shi-Heung-Ri is low or at an early stage. Choi and Park (2001) also assessed the infection intensity of clams in this area by counting of *Perkinsus* hypnospores from FTM cultivation and reported the number of 4,290 *Perkinsus* cells per gram of tissue. The infection intensity was much lower than those in commercial clam beds along the west and south coasts of Korea (Park et al., 1999; Park and Choi, 2001).

A negative correlation was observed between the gonadal index of clams and the infection intensity of *Perkinsus* in March. The histology indicated that gametogenesis of clams at Shi-Heung-Ri initiated in March. Less heavily infected clams were in the early stage of gametogenesis, with small developing oocytes in the follicles; more heavily infected clams were still inactive. However, this negative correlation was limited to March, and no further correlation was observed between the level of infection and the gonadal index at other sampling times. The prevalence and infection intensity were unusually high in March, but the reason for this is unclear.

The prevalence of *Cercaria* in clams varied from 0 to 12.0% in this study; there was no obvious seasonal trend, although the prevalence was somewhat higher in the fall. Kim and Chun (1983) also surveyed the

prevalence of *Cercaria tapidis* in clams from the south of Korea, and the percentage of infected clams (2%) was much lower than the level observed in this study. Kim et al. (1995) also investigated the prevalence of *Cercaria tapidis* and *Cercaria harengulae* in *Ruditapes philippinarum* and *Solen strictus* from Kum Kang Estuary on the west coast of Korea. The authors observed that the prevalence of *Cercaria tapidis* in clams varied from 4 to 48%, while *Cercaria harengulae* in *Solen strictus* varied from 1 to 20%. There was no obvious seasonality in the occurrence of trematode *Cercaria*; the prevalence of this parasite was high in certain months, and was completely absent in other months. Such irregularity is not understood, although the sudden appearance of large numbers of the final hosts (for example, fish or birds) or a second intermediate host has been assumed (Khamdan, 1998).

Histology also clearly demonstrated that a high level of *Cercaria* infection cause gonad castration of female clam. The gonads of several clams collected during the spawning season were completely occupied by sporocysts of *Cercaria*, and no eggs were present in the follicles. Several studies have also reported the detrimental effects of trematode parasitism on reproduction in marine molluscs. Degenerated gonads with smaller ova were observed in oysters that were heavily infected with a trematode parasite. Cheng and Burton (1965) reported that oysters heavily infected with *Bucephalus* produced eggs that were significantly smaller than those of healthy individuals. Khamdan (1998) also reported that *Bucephalus* destroyed the gonads of pearl oysters and that heavily infected oysters could not initiate gametogenesis. An inverse correlation between the number of eggs produced and trematode infection were also observed in the freshwater clam *Anodonta piscinalis* infected with *Rhipidicotyle fennia* (Taskinen and Valtonen, 1995). These studies suggest that high levels of trematode parasites disturb the reproductive processes of host organisms, possibly retarding gonadal development or destroying gametes, as was observed in this study.

In conclusion, *Perkinsus* and *Cercaria* were the two main parasites found in the clam population of the study area. Although the prevalence and infection intensity of *Perkinsus* and *Cercaria* were lower than in other areas, the parasites did disturb gametogenesis in the clams during the annual reproductive cycle.

Part IV

Seasonal changes in biochemical composition and reproduction of the Manila clam, *Ruditapes philippinarum* from Jeju, Korea

1. ABSTRACT

Condition index, biochemical composition and reproductive output of the Manila clam *Ruditapes philippinarum* was determined from May 2001 to April 2002 at Shi-Heung-Ri, east coast of Jeju, Korea, to detect seasonal variation. The condition index of clams varied from 0.09 during winter-early spring to 0.16 in early August. Average percent of protein in clam tissues was 32.6- 51.2% by weight, total lipid ranged 9.3-22.2% and carbohydrate, 12.1-30.1%. These biochemical changes relate to the reproductive cycle of clams. A polyclonal antibody specific to *Ruditapes philippinarum* egg protein was applied to quantify clam eggs using an enzyme-linked immunosorbent assay (ELISA). The mean gonad somatic index was highest in late June (19.5%) when clams began to spawn. Female clams produced up to 2,470,000 eggs in the first spawning peak in June and 2,020,000 eggs in the second, in late August. The low gonad somatic index and short spawning period of clams in studied area suggests that food availability may limit reproductive output of the population.

2. MATERIALS AND METHODS

2.1. Collecting and preparing clams

Shi-Heung-Ri Beach is located on the east coast of Jeju Island (Fig. 23). Thirty clams from a natural population was sampled monthly from May 2001 to April 2002, except that, in June and August, clams were sampled biweekly to follow spawning activity. Water temperature and salinity were recorded *in situ* at the time of sampling. After sampling, clams were transported to the laboratory and placed in filtered seawater for 24 h in order to purge their pseudofaeces and stomach contents. Shell length (SL), width (SW), thickness (ST) and wet tissue weight (WTW) were recorded. Condition index (CI) was calculated to evaluate the condition of clams followed Won and Hur (1993):

$$CI = [WTW/(ST \times SW \times SL)] \times 1000$$

2.2. Biochemical measurements

Monthly, the dry tissues of 30 individuals were weighed and then ground separately after freeze-drying for 24 hours. To determine biochemical composition, a known quantity of dry tissue was homogenized in phosphate buffer solution (PBS) using an ultrasonifier. Water-soluble protein was measured by BCA Protein Assay (Pierce, USA) with bovine serum albumin was used as standard. Carbohydrates were quantified by phenol-sulphuric acid method modified by Taylor (1995) and dextrose anhydrous was applied to obtain the standard curve. Total lipid was extracted in a mixture of chloroform and methanol (Bligh and Dyer, 1959) and lipid solution was charred with sulfuric acid at 180^{o}C for 15 minutes. The absorbance was red in a spectrophotometer at 375nm following the method of Marsh and Weinstein (1966) and lipid content of samples was calculated by using tripalmitin as standard.

2.3. Gonad somatic index and fecundity of clams

An indirect enzyme-link immunosorbent assay (ELISA) was used in quantification of the clam eggs. The rabbit anti-clam egg developed by Park and Choi (2004) was used as a primary antibody and the alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) as a secondary antibody in an indirect ELISA. For quantification of the eggs, triplicates of 100 µl of each clam tissue extract dissolved in 0.15M PBS (pH 7.5) and duplicates of the standard solution (purified clam eggs) were included in a 96-well ELISA microplate. Optical density (OD) of the antibody–antigen complex developed in ELISA was red at 405 nm using a micro-plate spectrophotometer. A standard regression curve was plotted to estimate the concentration of egg protein in each clam. The weight – based gonadosomatic index (GSI, dry weight eggs/dry weight tissues) and fecundity (i.e. number of eggs in a clam) were estimated from the following formula:

1) GSI = quantity of egg protein estimated from ELISA ×2.44/total dry tissue weight

where the constant 2.44 is a conversion factor of egg protein to egg dry weight.

2) Fecundity = quantity of eggs estimated from the ELISA/weight of single egg (i.e. 22ng)

3. RESULTS

3.1. Environmental conditions and sampling effort

Seasonal changes of water temperature and salinity were mentioned in Part 3 (see Fig. 24).

A total of 417 clams with mean shell length of 32.4±4.1mm and mean wet tissue weight of 1.3±0.6g were collected for analysis. The 30-35mm shell length class was most common, accounting for 45% of all clams analyzed. The condition index (CI) increased in late June (0.12) and peaked during spawning period in early August (0.15). After late August, CI decreased gradually to its lowest level in January (0.09) and remained stable during winter to early spring (Fig. 31).

3.2. Biochemical composition

Protein level was highest in late June (51.2%), remained generally stable during the spawning period, and then decreased abruptly in December to 35.5%; the lowest level was recorded in January (32.6%) (Fig. 32A).

Carbohydrate reached a maximum value in May (30.0%), varied widely, and declined during the spawning period from May to a minimum of 15.9% in early August 2001 (Fig. 32B), increased to 29% in September, then declined to its lowest level in December (12.1%) and remained low until spring.

Lipid content varied less seasonally than protein or carbohydrate. It peaked in early August at 22.2%. After the spawning season, lipid values decreased continuously to the minimum in December (9.26%), then increased steadily from January to April (Fig. 32C).

The convert strategy between carbohydrates and lipid was mainly observed during early June to early August and less pronounced from January to March. Those periods were coincided with the energy storage and gametogenesis of Manila clam from studied area. There was unclear relationship between protein-carbohydrate or protein-lipid during year cycle.

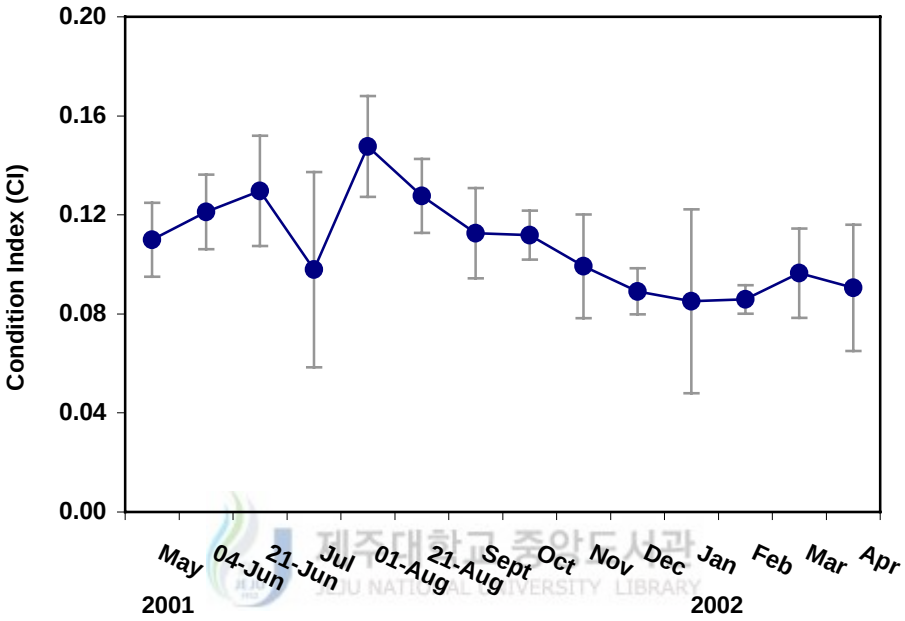

Fig. 31. Variation of condition index of Manila clams during sampling period. Vertical bars denote the standard deviation.

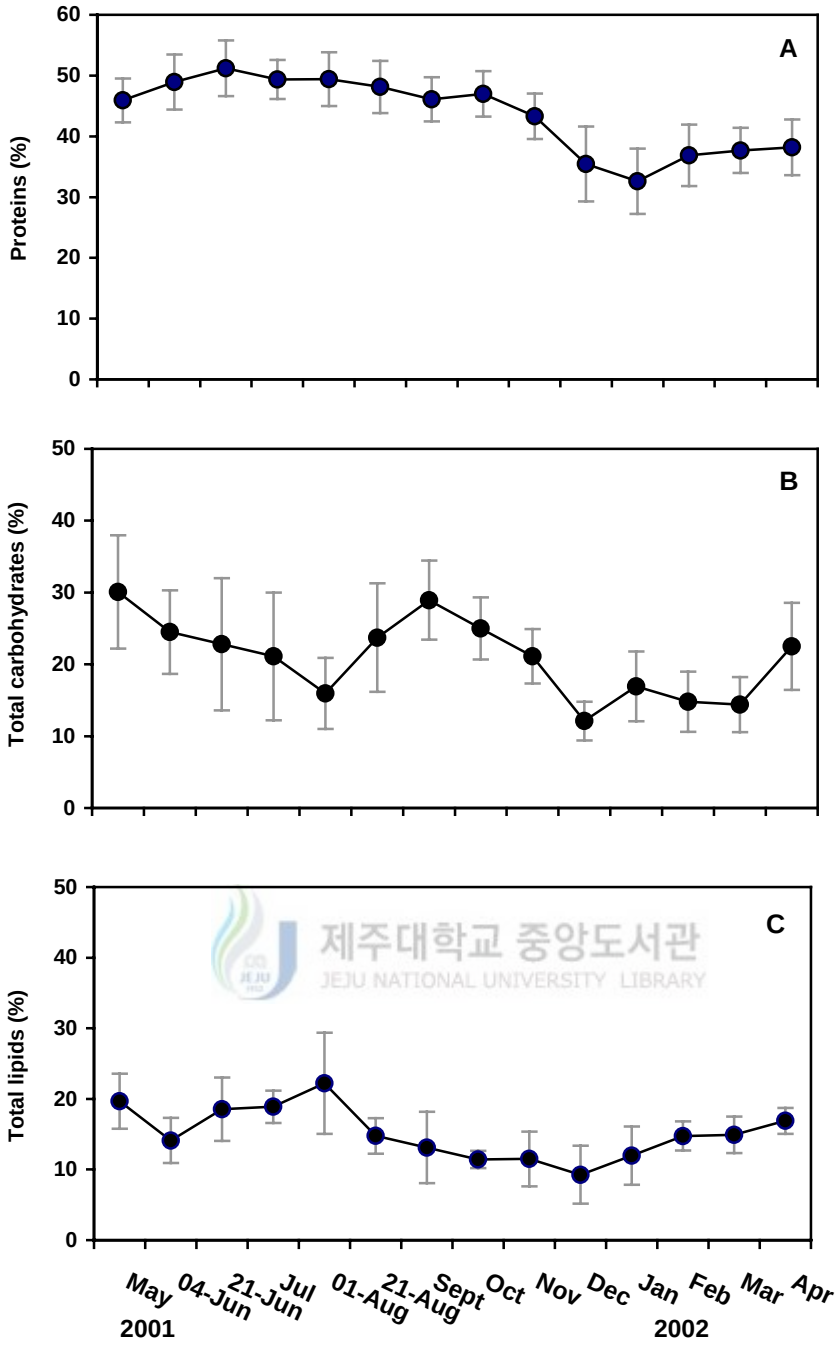

Fig. 32. Monthly variation of protein (A), total carbohydrates (B) and total lipids (C) in clam *Ruditapes philippinarum* during sampling period. Vertical bars denote the standard deviations.

3.3. Gonad somatic index and fecundity of clams

Gonad somatic index (GSI) of female clams increased dramatically from May (2.5%) and was highest in late June (18.9%). GSI was stable during spawning period from July (15.1%) to late August (15.5%) and decreased in September (4.3%). The highest level of GSI at 36.3% was observed in one female clam in July (Fig. 33).

Mean fecundity of clams increased from May (310,000 eggs/female) to a maximum value in late June of 2,470,000 eggs/female (Table 5). Mean fecundity decreased slightly from July to early August and increased in late August (2,020,000 eggs/female). In July, one clam produced the maximum number of 5,010,000 eggs observed in the studied population. After the main spawning season, clams produced fewer eggs with a mean of 540,000 eggs/female in September 2001.

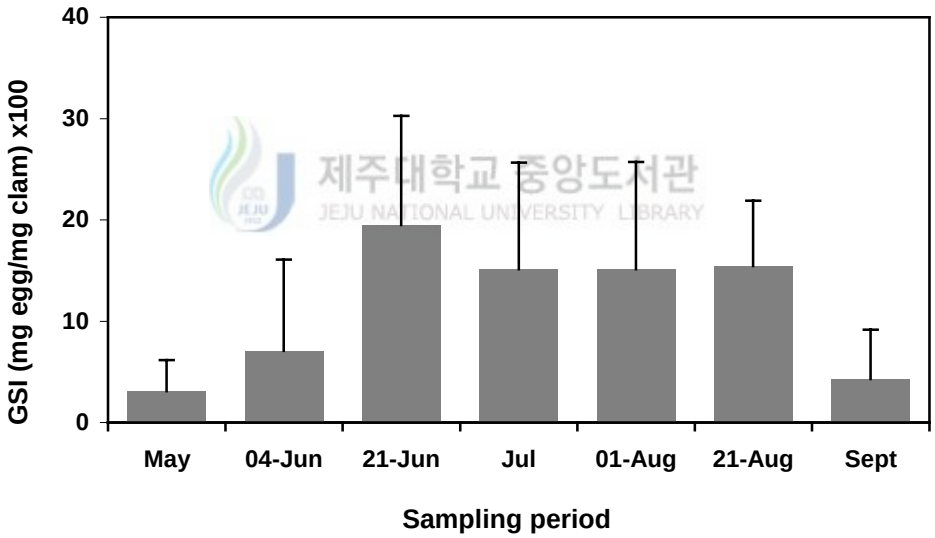

Fig. 33. Changes of gonad somatic index (×100) in clam *Ruditapes philippinarum* during spawning period from May to September 2001. Vertical bar denotes the standard deviations.

Table 5. Biometric data, gonadosomatic index (GSI, percentage of dry egg weight /dry tissue weight) and mean fecundity (million of eggs/female) during sampling period from May to September 2001.

Sampling dates	SL ± SD	TDW± SD	GSI ±SD	Range	Fecundity ± SD	Range
May	31.57 ± 3.24	0.24 ±0.10	2.5 ± 2.7	0.9-10.1	0.31 ± 0.32	0.11-1.19
04.Jun	33.69 ± 4.27	0.31 ±0.15	7.1 ± 8.9	1.8-29.6	0.63 ± 0.82	0.16-2.60
21.Jun	33.26 ± 2.90	0.26 ±0.08	18.9 ±11.5	5.8-32.4	2.47 ± 1.55	0.76-4.51
Jul	31.03 ± 2.13	0.20 ±0.05	15.1 ±10.5	1.2-36.3	1.96 ±1.40	0.15-5.01
01.Aug	28.78 ± 3.13	0.35 ±0.17	15.1 ±10.6	2.5-34.4	1.93 ± 1.44	0.32-4.96
21.Aug	33.78 ± 5.38	0.27± 0.10	15.5 ± 6.4	8.7-27.3	2.02 ± 0.85	1.11-3.76
Sep	32.66±4.29	0.19± 0.09	4.3 ± 4.9	1.5-16.9	0.54 ± 0.62	0.21-2.14

SL, shell length (mm); SD, standard deviation; TDW, total dry weight

4. DISCUSSION

The relationship of weight of bivalve mollusks to reproductive cycles and environmental conditions is well documented (Thompson, 1977; Jaramillo and Navarro, 1995). The present results showed that condition index (CI) of the Manila clam *Ruditapes philippinarum* in Jeju varied little except during the spawning period. CI declined abruptly after the clams completed spawning in late August. During winter, CI of clams was lower than that in late spring and summer while the clam size was not different. In southern Jeju Island, Lee et al. (2000) observed the primary productivity was lowest from December to February compared to another months of year. We suggest that low temperature and food availability during winter season might reduce the condition index of clams in studied area.

Whyte et al. (1990) indicated that protein contributed more than carbohydrate to maintenance energy in oysters under conditions of extended food deprivation, even when carbohydrate was apparently available in sufficient quantity. In the present study, seasonal increase observed in the protein level from May to late June was concomitant with a period of intense energetic demand in the gonad due to gamete development.

Increased carbohydrates in February related to the absorption of nutrients from the food. Clam is able to take advantage of particulate food matter resuspended by tidal movement, waves and winds. In the present study, carbohydrates together with lipids increased from February to May in clams observed. It was suggested that nutrients from phytoplankton were rapidly transferred as maintain energy, for growth and to sustain incipient gametogenesis. Mathieu and Lubet (1993) reviewed that muscular and glycogen cells are storage tissues in Veneridae and Cardiidae family. Gabbott (1976) mentioned that when sufficient food was available during gametogenesis, there was rapid transfer of assimilated food from the digestive gland to the other tissues.

Maguire and Burnell (2001) mentioned that declining carbohydrate levels indicates metabolism of carbohydrates. The considerable decrease of carbohydrate we observed from May to early August agrees with that observation. Carbohydrates are used as the energy–rich fuel for the build-up of gametes and vitellogenesis in bivalves via the conversion into lipids reserves (Gabbott 1975, 1976, 1983). Our results showed little conversion between carbohydrates and lipid during spawning period from late June to early August and not in the gonad development stage. Gallager and Mann (1986) mentioned that the limitation of this process might lead to the production of either fewer eggs or eggs of suboptimal quality.

Lipid has been considered one of the principal energy sources for gametogenesis of adult bivalves (Gabbott, 1983) and an energy reserve in conditions of either imposed or natural nutritional stress, particularly in autumn-winter when water temperature and food availability decrease (Beninger and Lucas, 1984). Marin et al. (2003) observed that lipids increased in October and January from *R. philippinarum* in the Lagoon of Venice. They suggested that lipids represent an important metabolic reserve for winter maintenance energy and for spring gametogenetic processes. However, in the present study, lipids decreased from late autumn to winter season and only increased in early spring. Clams could use their energy reserves to maintain growth, and subsequently gametogenesis might be retarded until late spring when food becomes more availability.

Gonad somatic index (GSI) of clams increased from April to May and reached its highest value in late June when temperature reached 19^{o}C. The high GSI was associated with the highest temperature period in the year (from 19.0^{o}C to 27.3^{o}C). Our results were in accordance with Mann (1979) who indicated the direct role of temperature for the maturation process in Manila clams. On the other hand, several studies suggested that the reproductive strategy of *R. philippinarum* is governed by two main environmental factors, temperature and nutritional conditions (Bayne, 1976; Ruiz et al., 1992). In the present study, synchrony between

temperature and food availability in the environment could favor maturation of Manila clams. These optimal conditions may induce the rapid increase of GSI and mass spawning of clam population in late June. Ansell and Trevallion (1967) reported that dry body weight of *Tellina tenuis* could increase by 44% when food was abundant and the gonad was developing. Honkoop et al. (1999) found that the amount of eggs spawned ranged from 0 to 33% of the total ash-free dry mass in *Macoma balthica.* In our study, the GSI of clams varied from 0 to 36% of total DTW, however, mean GSI reached only 18.9% at the spawning peak.

In *R. philippinarum* from Tokyo Bay, Toba et al. (1993) reported that highly synchronized gonad maturation occurred in spring spawning but not in the summer spawning. Our finding showed that clams at Jeju invested major energy reserves for the first spawning but maturation was also highly synchronized in the second. After the first spawning peak, GSI of clams remained stable until late August (15%) and then decreased rapidly in September (4%). We consider it likely that the gonad development was limited and GSI could not reach a higher level as suggested in other location in Korea (Park and Choi, 2004). Tirado and Salas (1998) suggested that high levels of chlorophyll *a* due to upwelling might explain the long reproductive cycle February to October in *Donax trunculus*. Furthermore, Delgado and Camacho (2003) indicated that the extent of gonadal development is directly related to the amount of food available, with smaller rations leading to a lower rate of increase in the gonadal occupation index and the percentage of ripe oocytes in *Ruditapes decussatus*.

Several studies have reported the fecundity of Manila clams measured using different methods. Induce clams spawning by ammonia, Toba and Miyama (1991) obtained the number of 0.24-1.35x10^6 eggs/clam. Chung et al. (2001) applied the several methods to induce spawning as exposing to the air, feeding stimulus, and thermal shock and reported the fecundity of 0.20-1.79×10^6 eggs/clam. Fecundity of clams from Shi-Heung-Ri was higher than the numbers mentioned above, however, it was lower than the

result from the study of Park and Choi (2004). The authors applied ELISA and showed the range of fecundity was 944,584 - 11×10^6 eggs/clam during spawning period in Gomso Bay, Korea. Several studies mentioned that the fecundity of clams estimated from induced spawning must be underestimation due to partial and discontinuous activity (Choi et al., 1993; Chung et al., 2001; Kang et al., 2003). It could explain the higher fecundity of clams in studied area with the results from previous methods not ELISA. Park and Choi (2004) found the positive correlation between the size and fecundity of Manila clam. The larger size of clam in Gomso Bay might explain the higher fecundity. However, Park and Choi (2004) suggested the gametogenesis of clams in Gomso Bay coincides with the variation of food abundance in water column. The authors observed the third spawning peak in late August matched a maximum level of chlorophyll *a* during August and September. Lee at al. (1998) measured chlorophyll *a* in north Jeju and recorded the range of 0.04-0.77 μg/L, it has been much lower than those from Gomso bay (2-12μg/L).

According to Laruelle et al. (1994), in Morbihan Gulf (France), *R. philippinarum* spawned completely three times (in early June, in early July and in early September) and once partially (in early October). Park and Choi (2004) also recorded three GSI peaks in May, late July and late August in Manila clams from Gomso Bay. In Shi-Heung Ri, clams showed two GSI peaks in late June and in late August indicating the long period to locate energy reserve however short period for spawning activity. Spawning of *Tapes philippinarum*, can occur either once or twice each year depending on location and environmental conditions such as temperature (review of Ponurovsky and Yakovlev, 1992). The temperature, however, does not explain the less intensive reproductive activity of clams in Shi-Heung Ri compare to Gomso Bay since the studied area showed higher temperature during year around. The lower food availability could greatly limit the reproductive period and fecundity of *R. philippinarum* in present study.

GENERAL CONCLUSIONS

In Gosung Bay, oysters spawned continuously from May to October. Two spawning peaks occurred in June and August with the remarkable peak in June.

Marteilioides chungmuensis was not observed from February to May, however the high prevalence and infection intensity occurred after spawning season. Heavy infection might disturb the reproductive cycle and cause spawning failure

Biochemical compositions of oysters from 2 depths showed that storage phase and spawning timing are different between surface and bottom. Surface-oysters showed high accumulation and transfer of reserves for reproduction.

Surface-oysters showed higher fecundity than bottom-oysters during spawning period; average fecundity was 107 million eggs/female in mid June.

Culture depth affected both biochemical composition and reproduction of oysters in Gosung Bay and surface culture was found to be more favorable than the bottom.

In Shi-Heung Ri, Manila clam spawned from May to August with major peak in late June.

Perkinsus prevalence and infection was low in summer than in winter. *Cercaria* showed no seasonal pattern, however the infection interfere the reproductive process of clams.

Protein played major role in energy reserves of Manila clam in studied area.

Mean fecundity of clam was 2.47 million eggs/female in late June. Limited food availability might cause the low fecundity and short spawning period.

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List of publications

1. Thao T.T. Ngo, Sang-Gyun Kang and Kwang-Sik Choi. 2002. Seasonal changes in reproductive condition of the Pacific oysters *Crassostrea gigas* (Thunberg) from suspended culture in Gosung Bay, Korea. Korean Journal of Environmental Biology. Vol. 20 (3): 268-275.

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5. Thao T.T. Ngo, Sang-Gyun Kang, Do-Hyung Kang, Yoon Kim, Patrick Sorgeloos, Kwang-Sik Choi (in preparation). Seasonal variation and effect different depths on the biochemical composition and reproduction of Pacific oyster, *Crassostrea gigas*.

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