A DISSERTATION

FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY

MOLECULAR PHYLOGENETICS, IMMUNOLOGY, AND REPRODUCTION OF SUBTROPICAL OYSTERS *SACCOSTREA KEGAKI* (TORIGOE & INABA 1981), *OSTREA CIRCUMPICTA* (PILSBRY 1904), AND *HYOTISSA HYOTIS* (LINNAEUS 1758) OFF THE SOURTHERN COAST OF JEJU ISLAND, KOREA

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JEJU NATIONAL UNIVERSITY

FEBRUARY 2015

MOLECULAR PHYLOGENETICS, IMMUNOLOGY, AND REPRODUCTION OF SUBTROPICAL OYSTERS *SACCOSTREA KEGAKI* (TORIGOE & INABA 1981), *OSTREA CIRCUMPICTA* (PILSBRY 1904), AND *HYOTISSA HYOTIS* (LINNAEUS 1758) OFF THE SOURTHERN COAST OF JEJU ISLAND, KOREA

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A dissertation submitted in partial fulfillment of the requirement for the degree of

DOCTORATE OF PHILOSOPHY

February 2015

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ABSTRACT

Previous studies have reported on the occurrence of subtropical oysters in shallow subtidal in Jeju Island where benthic animal diversity is recorded to be the highest in Korea. These subtropical oysters are believed to be extending their distribution range from southern Asia to Jeju due to the global seawater temperature increase. Despite its abundance and popularity, extremely few studies have been carried out on the ecology of these oysters in Jeju.

Polymerase chain reaction (PCR) amplification, cloning and sequence determination of nuclear DNAs of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis* in Jeju Island were carried out to provide basic genetic information and phylogenetic relationship between species. The total genomic DNA was extracted from adductor muscles of each oyster species. For the analysis, 18S ribosomal DNA (18S rDNA), internal transcribed spacer (ITS), and histone H3 specific primers were designed based on conserved domains of each genes following the multiple alignments of published sequences of bivalves reported in the GenBank. The phylogenetic affiliation of the oyster species were analyzed by neighbor-joining (NJ) and maximum parsimony (MP) methods. The phylogenetic analysis allowed a clear distinction between Family Ostreidae and Family Gryphaeidae. Ostreidae oysters *S. kegaki* and *O. circumpicta* were group together and formed a sister group with genera *Crassostrea*. On the other hand, Gryphaeidae oyster *H. hyotis* formed a monophyletic clade as outgroup indicating a distant relationship between Ostreidae and Gryphaeidae. The results suggest that the nuclear DNA sequences including 18S rDNA, ITS, and H3 are useful tools in the identification of oyster species.

To understand the immune system of subtropical oysters, as the second study, morphology and immune-related activities of the hemocytes in *S. kegaki*, *O. circumpicta*, and *H. hyotis* were characterized using light microscopy and flow cytometry. The hemocytes of the 3 oyster species could be classified into three types: 1) granulocytes, 2) hyalinocytes, and 3) blast-like cells. The percentage of each hemocyte population was similar in all species; hyalinocytes were the most abundant cell in the hemolymph accounting for more than 59%, followed by granulocytes (23-31%) and blast-like cells (3-5%). The size of granulocytes of *S. kegaki* was smaller ($p < 0.05$) than those of *O. circumpicta* and *H. hyotis*. Light miscopy also allowed the description of vacuolated cells characterized by large vacuoles in the cytoplasm. Flow cytometry analysis confirmed that the granulocytes of 3 oyster species was the major hemocytes engaged in the cellular defense as the largest lysosome content, the most active phagocytosis activity and oxidative activity, as was previously reported in several marine bivalves. Immune-related activity of hemocytes was different between the species. Phagocytic activity was the lowest in *S. kegaki* hemocytes and PMA-stimulated oxidative activity was the lowest in *H. hyotis* hemocytes. Our results provide the basic information of hemocytes population of three subtropical oysters for further investigations associated with various environmental stress of disease.

Effects of seasonal changes in the environmental parameters on the hemocyte parameters and an annual reproductive cycle of the intertidal oyster *S. kegaki* and the subtidal oyster *H. hyotis* was investigated from March 2011 to February 2012. The hemocyte density strongly fluctuated with the surface seawater temperature in the intertidal oyster, while no clear seasonal pattern was observed in the subtidal oyster, possibly due to wide daily and seasonal temperature fluctuations and cyclic air exposure in the intertidal zone. Other environmental factors such as salinity and food availability had no direct influence on the

measured hemocyte parameters in both oysters. Immune-related hemocyte parameters (mortality and phagocytosis) of both intertidal and subtidal oysters were strongly correlated with an annual reproductive cycle of the oysters. The increase in hemocyte mortality and decrease in phagocytosis activity were observed when most of the oysters were in post-spawning condition. During post spawning period, levels of total protein and carbohydrate in the tissues were substantially lower than pre-spawning period, suggesting that oysters in post-spawning period may not hold enough energy reserves to be used in the maintenance of the cellular immune defense activity.

The results of this study have provided the basic information of molecular phylogenetics, immunology, and reproduction for efficient resource management of subtropical oyster that inhabits in Jeju Island, and it can be used as efficient biomarker for monitoring environmental change.

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I. General introduction

1.1. Physicochemical properties of Jeju waters

Jeju is a 1849 km^2 volcanic island situated on the continental shelf 100 km off the south of the Korean Peninsula. Jeju Island located downstream of Yellow Sea Warm Current, a branch of Kuroshio Current (Fig. 1-1). Because of the influence of Yellow Sea Warm Current, Jeju Island features high-latitude climate with the surface water temperature in the subtidal area during winter ranged from 13 to 18 °C (Ichikawa and Beardsley 2002; Kim et al. 2010; Wang et al. 2012).

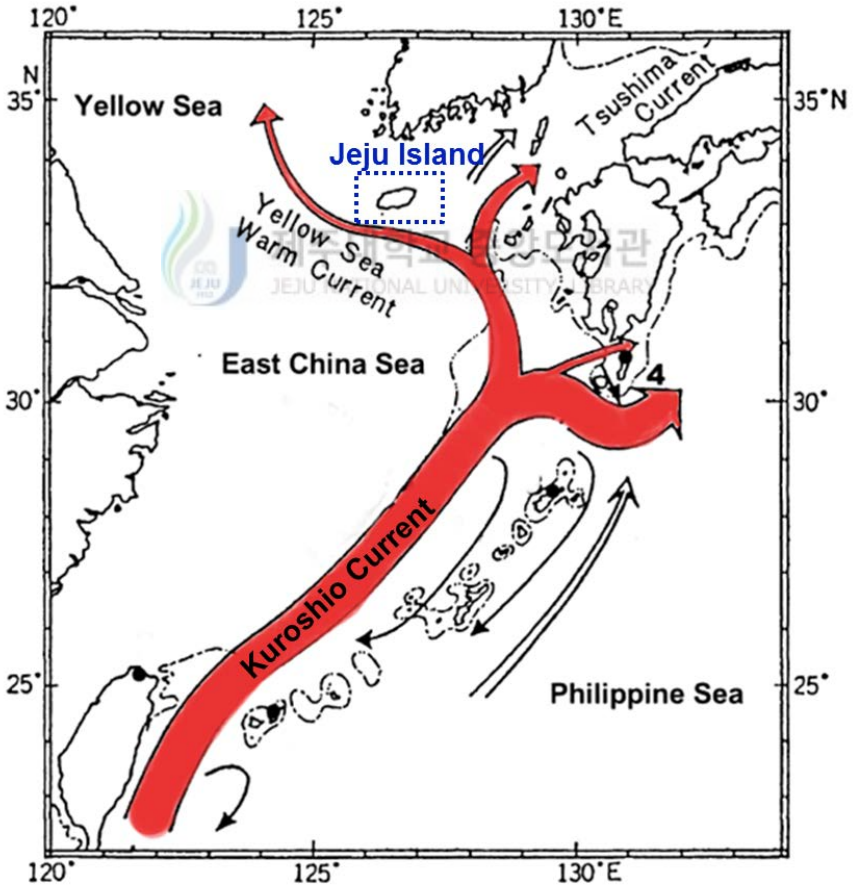


Fig. 1-1. Currents in the coastal waters of Jeju (Figure was modified from Ichikawa and Beardsley 2002).

High-latitude locations are expected to experience significant changes in environmental conditions (Denis et al. 2014). Temperature is projected to increase more than average in high-latitude locations and at high elevations. Animals living at lower elevations could migrate to higher elevations in response to climate change as temperature increase (Mortiz 2008). The East China Sea is one of the areas where the water temperature is rising rapidly (Belkin 2009). Therefore, animals can undertake long migratory movements likely to range shift (Laudy et al. 2010). For example, tropical fish (Kim et al. 2014), subtropical mollusks (Noseworthy and Choi 2010), and high latitude corals (Denis et al. 2014) species recently have been reported to thrive in Jeju waters.

1.2. Subtropical oysters in Jeju waters

Fourteen species of oysters belonging to the Families Ostreidae including *Crassostrea gigas*, *C. ariakensis*, *C. nippona*, *C. nigromarginata*, *C. pestigris*, *Ostrea denselamellosa*, *O. circumpicta*, *Saccostrea kegaki*, *Dendostrea crenlifera*, *D. folia*, and Family Gryphaeidea including *Hyotissa hyotis*, *Parahyotissa hyotis*, *P. chemnitzii*, *Neopycnodonte cochlear* have been reported in Korea (Min, 2004; Table 1-1). The oysters including *C. nigromarginata*, *O. circumpicta*, *S. kegaki*, *D. crenlifera*, *D. folia*, *H. hyotis*, *P. hyotis*, *P. chemnitzii* were limitedly distributed in Jeju Island. These oyster species are believed to be extending their distribution rage (range shifts) from tropic or subtropical area to Jeju (high-latitude area) due to the seawater temperature increase. Among the subtropical oysters, three species including *S. kegaki*, *O. circumpicta*, and *H. hyotis* widely distributed along the Jeju coast were used in this study.

S. kegaki is a small oyster with 2-6 cm in shell length and features black pip-like spines on its valves (Fig. 1-2A). The distribution of *S. kegaki* has been reported from

Japanese waters (Torigoe 1999; Lam and Morton 2006) and Jeju coast of Korea (Kim et al. 2010). In Jeju, *S. kegaki* heavily encrusts on rocks in the lower intertidal area along the coastline.

O. circumpicta is a medium-sized oyster with 5-8 cm in shell length. Surface of shell is characterized by tinged purplish brown, imbricated and irregularly divergent ribs (Okutani et al. 2000; Fig. 1-2B). *O. circumpicta* is brooding oyster, releasing embryo instead of gametes during spawning periods (Kang et al. 2004). The occurrence of *O. circumpicta* has been reported on rocky subtidal zones of southern Japan (Hirase 1930), China (Bernard 1993), and the southern coast of Jeju Island (Kang et al. 2004).

H. hyotis is a giant oyster with a maximum shell length of 30 cm and is characterized by thick and heavy shell with radial ribs (Fig. 1-2C). The distribution of *H. hyotis* has been reported from shallow subtropical and tropical coral reefs such as the Northern Red Sea (Titschack et al. 2010), Gulf of California (Duprat-Bertazzi and García-Domínguez 2005; Robríguez-Astubilo et al. 2005; Villalejo-Fuerte et al. 2005), Florida Keys (Kirkendale et al. 2004), Malaysia and Singapore (Lam and Morton 2009). In Jeju, this grypaeid oyster commonly occurs on subtidal rocky bottoms at depths of 5-15m.

Despite their abundance and popularity, very few studies related to larvae morphology of *O. circumpicta* (Kang et al. 2004) and annual reproductive cycle of *O. circumpicta* (Lim 2005) and *S. kegaki* (Kim et al. 2010) carried out on the ecology of oysters in Jeju.

Table 1-1 List of oyster species in Korean waters.

Family	Species	Korean Name (국명)	Habitat
Ostreidae	*Crassostrea gigas* Thunberg 1793	굴	Whole coastal area
	Crassostrea nippona Seki 1934	바윗굴	East coast, Southern coast, Jeju coast
	Crassostrea ariakensis Fujita 1913	강굴	Seomjin River of southern coast
	Crassostrea pestigris Hanley 1846	호랑이발굴	Southern coast
	Crassostrea nigromarginata Sowerby 1871	주름가시굴	Southern coast of Jeju
	Ostrea denselamellosa Lischke 1869	벗굴	West coast, Jeju coast
	Ostrea circumpicta Pilsbry 1904	태생굴	Southern coast of Jeju
	Dendostrea folia Linnaeus 1758	악어굴	Southern coast of Jeju
	Dendostrea crenulifera Sowerby 1971	톱니턱굴	Southern coast of Jeju
	Saccostrea kegaki Torigoe & Inaba 1981	가시굴	Jeju coast
Gryphaeidae	*Hyotissa hyotis* Linnaeus 1758	중국굴	Southern coast of Jeju
	Parahyotissa inermis GB Sowerby II 1871	겹지붕굴	Southern coast of Jeju
	Parahyotissa chemnitzii Hanley 1846	주홍굴	Southern coast of Jeju
	Neopycnodonte cochlear Poli 1795	주름꼬마굴	Southern coast

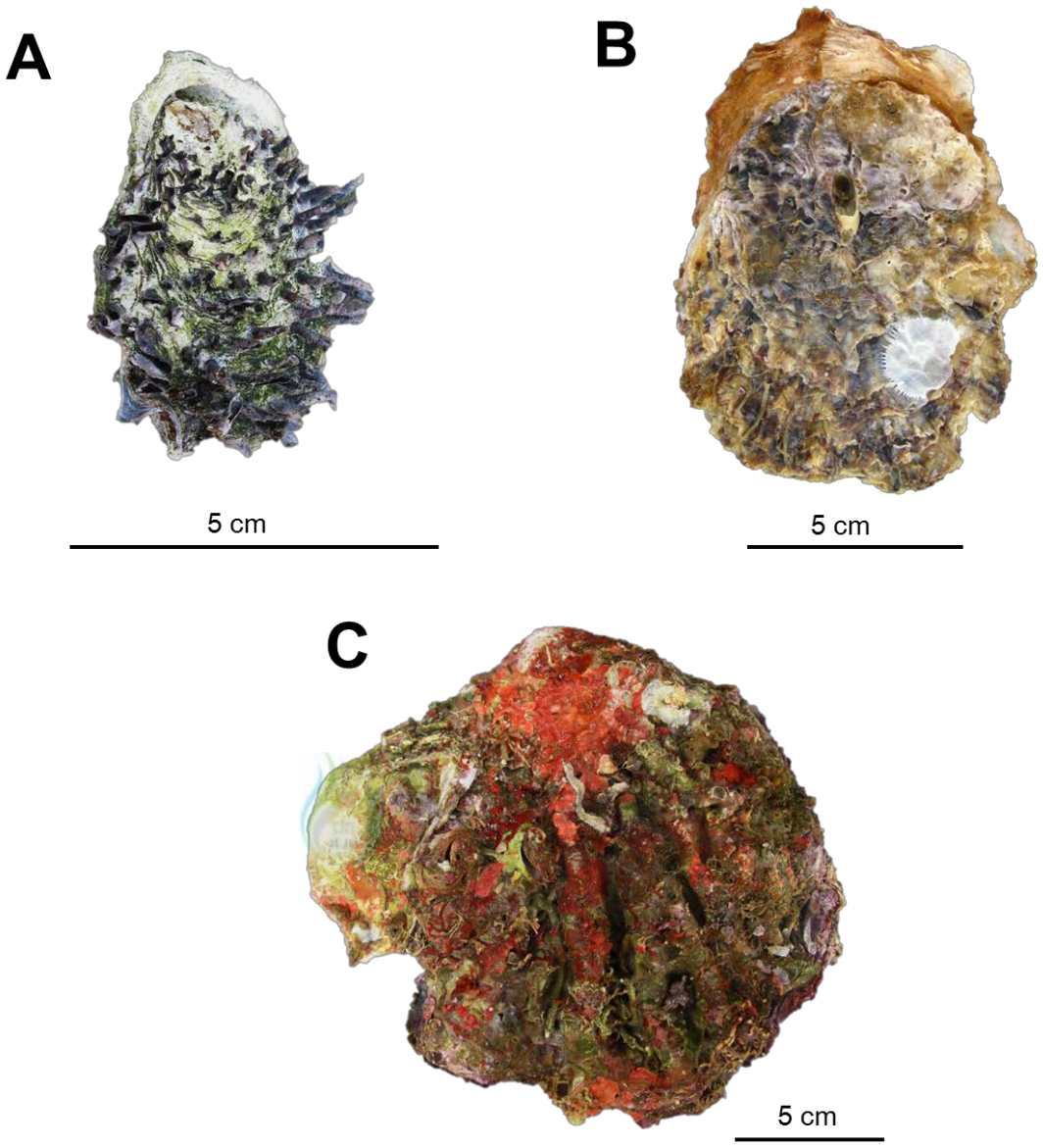


Fig. 1-2. External view of subtropical oysters *Saccostrea kegaki* (A), *Ostrea circumpicta* (B), and *Hyotissa hyotis* (C) collected from Jeju Island of Korea in this study.

1.3 Sentinel species for climatic change monitoring

Due to the effect of climate change, the interest in the physiological mechanisms that animals use to tolerate extreme heat and adapt to thermal fluctuations in their natural environment is increasing (Li et al. 2007). Sessile invertebrates are widely used as an indicator for environmental monitoring because they are exposed to environmental fluctuations and the effects are manifested through the physiology of the cells or tissues (Fig. 1-3). As sessile invertebrates, oysters and mussels are an important environmental bio-indicator species. For example, the “Mussel Watch Programme” is a world-wide project using mussels and oysters to assess environmental impacts in coastal waters (Cantillo 1998; Monirith et al. 2003; Kim et al. 2008; Kimbrough et al. 2008; Apeti et al. 2010; Choi et al. 2010; Sparks et al. 2014).

Fig. 1-3. Sentinel species of sessile invertebrate for climatic change study

1.4. Objectives of this study

To provide basic genetic information of 3 subtropical oyster species (*S. kegaki, O. circumpicta, and H. hyotis*) and their phylogenetic relationship between other oyster species, I developed nuclear DNA markers (18S rDNA, ITS, and histone H3) for each oyster species. I also characterized hemocytes and the immunological activities of the subtropical oysters using light microscopy and flow cytometry which is crucial to understand cell-mediated responses of oysters to environmental and biological stress. Finally, I investigated the influence of environmental factors and reproductive activity on hemocyte parameters of the oysters in the field. To compare the difference of habitat conditions, I compared the seasonal variation in reproductive activity and hemocyte parameters of intertidal oyster *S. kegaki* and subtidal oyster *H. hyotis* from the coast of Jeju. Through these basic studies, the subtropical oysters in Jeju Island can be use as efficient biomarker for environmental change monitoring.

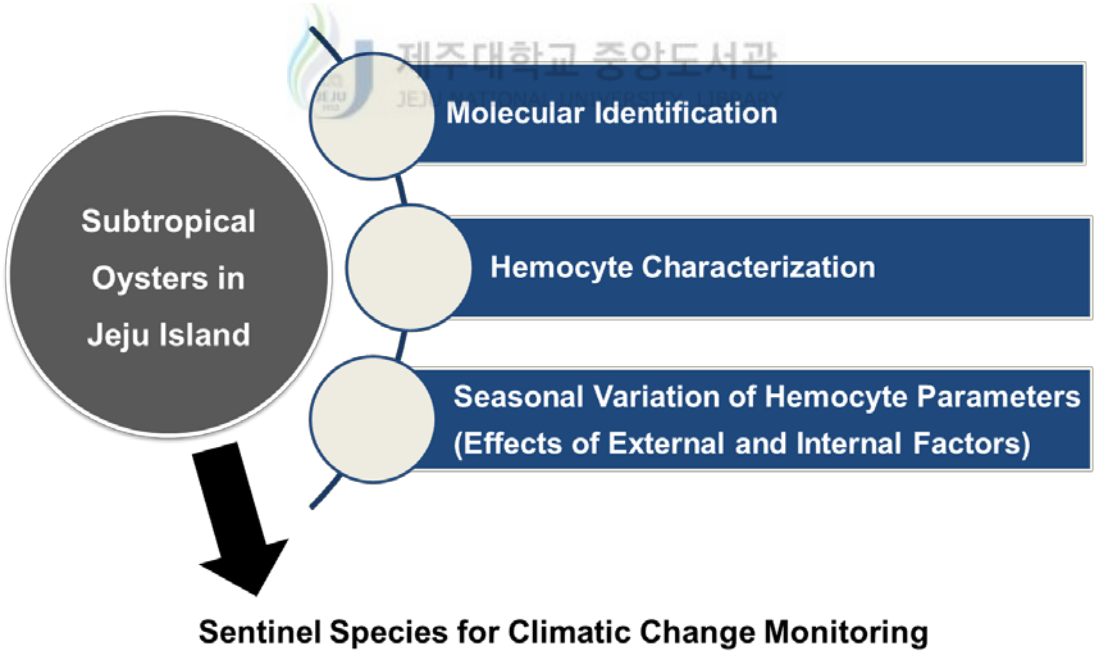


Fig. 1-4. The objectives of this study.

II. Molecular identification of subtropical oysters *Saccostrea kegaki* (Torigoe & Inaba, 1981), *Ostrea circumpicta* (Pilsbry, 1904), and *Hyotissa hyotis* (Linnaeus, 1758) from Jeju Island based on nuclear DNA

2.1. Introduction

Shell morphology (i.e. shell pattern, shape and color) and anatomical features are commonly used as a primary key to identify the oyster species, but these characters are faced with the problem to resolve taxonomic classification due to the plasticity of shell morphology depending on the environment (Varela et al. 2007; Reece et al. 2008; Cunha et al. 2011). Alternatively, various molecular markers have been applied to corroborate the taxonomical confusion and species identification of marine bivalves based on nuclear and mitochondrial genes (O'Foighil et al. 1995 and 1998; Reece et al. 2008).

In nucleus, ribosomal genes are most commonly used for diagnostics and phylogenetic studies because the regions of ribosomal DNA (rDNA) are comprised of several copies of similar sequences (Bendezu et al. 2005). The low rate of polymorphism in the rDNA transcription allows characterization of the rDNA of each species (Lourenço et al., 2003). For this reason, the sequence of rDNA has been considered as a good molecular marker for interspecific comparison. For example, in bivalve phylogeny, the development of primers and probes based on 18S rDNA region has been demonstrated to be a reasonable technique for diagnostics and phylogenetic studies (Steiner 1999; Distel 2000; Giribet and Wheeler 2002; Klinbunga et al. 2003 and 2005; Regina et al. 2011).

Internal transcribed spacer (ITS) region of rDNA has been widely used as a suitable molecular genetic identification tool, especially for congeneric species because its high degree of variation even between closely related species (Beltrame-Botelho et al. 2005). ITS

has been proven useful for elucidating relationships among closely related genera in *Crassostrea* (Cordes et al. 2008; Reece et al. 2008; Damasceno et al. 2013).

Histone H3 is one of the five main histone proteins involved in the structure of chromatin in eukaryotic cells. Histone H3 is highly conserved at the amino acid level and is easily amplifiable allowing evolutionary studies of many invertebrates (Lydeard and Lindberg 2003). However, molecular phylogeny of oysters using histone H3 has limitedly been reported in *Crassostrea* and *Ostrea* spp. (Bouilly et al. 2010; Tëmkin 2010).

The subtropical oysters including *Saccostrea kegaki* (Family Ostreidae), *Ostrea circumpicta* (Family Ostreidae), and *Hyotissa hyotis* (Family Gryphaeidae) had scarce distribution in Jeju Island (Min 2004). *S. kegaki* heavily encrusts on rocks in the lower intertidal area along the coastline while *O. circumpicta* and *H. hyotis* commonly occur on subtidal rocky bottoms at depths of 5-15m. Three subtropical oyster species were classified based on morphological characteristics (Min 2004) but there is no report about the molecular phylogeny of the subtropical oyster. Most genetic studies have been conducted in commercial oysters including *Crassostrea* (*C. gigas*, *C. ariakensis*, and *C. nippona*) and *Ostrea* (*O. denselamellosa*) species inhabiting in Korean peninsula (Park and Kim 1995; Kim et al. 1997; Lee et al. 2000; Kim et al. 2009).

In the present study, I performed the polymerase chain reaction (PCR) amplification, cloning and sequence determination of 3 subtropical oyster species (*S. kegaki*, *O. circumpicta*, *and H. hyotis*) collected from Jeju Island using three nuclear DNA (18S rDNA, ITS, and histone H3) to provide basic genetic information and phylogenetic relationship between rhe oyster species. To understand the genetic relationship of the subtropical oysters with commercial oyster species, five commercial oyster species including *C. gigas*, *C. ariakensis*,

C. nippona, and *O. denselamellosa* from Korea peninsula and *C. virginica* from United States were also analyzed in this study.

2.2. Materials and methods

Oysters

Table 2-1 lists the information of oyster samples used in this study. The subtropical oysters including *S. kegaki*, *O. circumpicta*, and *H. hyotis* were collected from the southern coast of Jeju Island. To compare the genetic relationship with Korean oysters, commercially important oyster species including *C. gigas*, *C. ariakensis*, *C. nippona*, and *O. denselamellosa* were obtained from south and east coast of Korea. The Eastern oyster *C. virginica* from United States was used to determine the phylogeny with the subtropical oysters.

Table 2-1 List of oyster samples used in this study

Species	N	Shell height (cm)	Collection site	Habitat	Collected year
Saccostrea kegaki	3	4-6	Jeju Island of Korea	Intertidal rocky shore	2006
Ostrea circumpicta	3	6-8	Jeju Island of Korea	Subtidal rocky bottom	2006
Hyotissa hyotis	3	15-18	Jeju Island of Korea	Subtidal rocky bottom	2011
Crassostrea ariakensis	3	20-22	South coast of Korea	River estuary	2007
Crassostrea gigas	3	7-8	South coast of Korea	Suspended long-line facility in the bays	2006
Crassostrea nippona	3	11-13	East coast of Korea	Subtidal rocky bottom	2006
Ostrea denselamellosa	3	13-15	South coast of Korea	Subtidal rocky bottom	2007
Crassostrea virginica	3	10-12	United States	Shallow saltwater bay	2006

DNA extraction

The adductor muscle was removed from the oysters and frozen immediately in liquid nitrogen and stored at -70°C until genomic DNA extraction. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen., Germany). About 25 mg of adductor muscle was lysed in lysis buffer and proteinase K at 56°C for overnight and the DNA was extracted following the manufacturer's instructions.

PCR amplification and sequencing

Oligonucleotide primers of SSU rDNA, ITS, and H3 were designed based on conserved regions extracted from multiple alignment of published completed genes from marine bivalve species (Table 2-2). For PCR amplification, 200 ng of DNA was added to a 50 µl PCR reaction mixture containing 10x Ex Taq buffer, 50 pmole of each primer, 0.2 mM of each dNTP, and 1.25 U of Takara Ex Taq polymerase (Takara, Japan). Amplification procedure of the 18S rDNA included pre-denaturation step at 95°C for 5 min followed by 30 cycles of denaturation step at 95°C for 30 sec, primer annealing step at 55°C for 30 sec, extension at 72°C for 1 min 50 sec followed by a final extension step at 72°C for 5 min (Table 2-2). For ITS and H3 amplification processes were same as the 18S rDNA except for annealing temperature 50°C and 55°C respectively and extension times for 1 min 10sec and 20sec respectively (Table 2-2).

The PCR amplicons were electrophoresed on a 1.2% agarose gel with a 100 bp Plus DNA ladder (Bioneer, Korea) to confirm the product sizes. The amplified SSU rDNA was purified using AccuPrep® PCR purification kit (Bioneer, Korea) and the amplified ITS and H3 genes were excised from the gel and purified using AccuPrep® Gel extraction kit (Bioneer,

Korea). Purified DNA were ligated into the pGEM-T easy vector (Promega, USA), according to the manufacturer's instructions, and transferred to *Escherichia coli* DH5α. The recombinant clones (N=3 for each species) were sequenced using the modified dideoxy chain-termination method.

Phylogenetic analysis

The sequences of SSU rDNA, ITS, and H3 from 8 oyster species were deposited in GenBank with accession numbers (KM460830-KM460893). The similarity of nucleotide sequences of three nuclear DNA from 8 oyster species against diverse database deposited in GenBank was conducted using a basic local alignment search tool (BLAST). To construct the phylogenetic trees, the genes of SSU DNA, ITS, and H3 obtained in this study and those of other *Crassostrea*, *Ostrea*, *Saccostrea*, and *Hyotissa* species available from GenBank were aligned using ClustalW (Thmpson et al. 1994). Neighbor-joining (NJ) and Maximum Parsimony (MP) analyses were performed using MEGA 6.0 (Tamura et al. 2013), with 1,000 bootstrap replicates. Pair-wise sequence divergences among oyster species were calculated using Kimura's two-parameter model (Kimura 1980).

Table 2-2 Sequence of oligonucleotide primers, annealing temperature, and extension time for polymerase chain reaction (PCR) amplification of small subunit ribosomal DNA (SSU rDNA), internal transcribed spacer (ITS), and histone 3 (H3) genes

Gene	Primer	Sequence (5′→3′)	Annealing temperature (°C)	Extension time
SSU rDNA	OS SSU-F	GATCCTGCCAGTAGTCATATGCTTG	55	1 min 50 sec
	OS SSU-R	GTACAGTTTGCCCTTCTTCCCGG		
ITS	OS ITS-F	CCGGGAAGAAGGGCAAACTGTAC	50	1 min 10 sec
	OS ITS-R	GTTAGTTTCTTTCCTGCCCTTAGT		
H3	OS H3-F	ATGGCTCGTACMAAGCAGACYGC	55	20 sec
	OS H3-R	ATATCCTTRGGCATRATRGTGAC		

2.3. Results and Discussions

The nucleotide length, ratio of GC content, accession number deposited in GenBank, and BLAST similarity of 3 different nuclear DNA (18S rDNA, ITS and H3) from 8 oyster species are summarized in Table 2-3. The length of the 18S rDNA of oyster species amplified using OS SSU-F/OS SSU-R primer set was 1746-1750 bp, and its GC content ranged from 49 to 51%. Primer sets OS ITS-F and OS ITS-R amplified partial 18S, ITS1, 5.8S, ITS2, and partial 28S. The sequence of the 5.8S rRNA gene was highly conserved, with a length of 158-159 bp in each species and a GC content of 56%. The length and GC contents of ITS1 (340-515 bp, 53-61%) and ITS2 (342-537 bp, 53-62%) were variable among oyster species. Finally, the sequences of H3 were identical with 374 bp in nucleotide length and 124 amino acid. The percentage of GC contents in H3 genes of 8 oyster species ranged from 51 to 56%. From now on, I will only focus on the results of three subtropical oysters including *O. circumpicta*, *S. kegaki*, and *H. hyotis* to understand their genetic information and phylogenetic relationship between oyster species.

Table 2-3 Size of base pairs (bp), percentage of Guanine-Cytosine (GC) content, GenBank accession numbers, and BLAST similarity of the small subunit ribosomal DNA (SSU rDNA), internal transcribed spacer (ITS), and histone 3 (H3) genes in 8 oyster species used in this study. N, number of oyster clone; aa, amino acid

Locus	Species	N	Length (bp)	GC content (%)	GenBank Accession number	BLAST similarity (Accession number: species)
SSU rDNA	*Crassostrea ariakensis*	3	1750	50	KM460873, KM460874, KM460875	99.8-99.9% (AB064942: *C. gigas*)
	Crassostrea gigas	3	1750	50	KM460876, KM460877, KM460878	99.8-99.9% (AB604942: *C. gigas*)
	Crassostrea nippona	3	1750	50	KM460879, KM460880, KM460881	99.4-99.8% (AB604942: *C. gigas*)
	Crassostrea. virginica	3	1749	50	KM460894, KM460895, KM460896	99.3-99.5% (X60315: *C. virginica*)
	Ostrea denselamellosa	3	1749-1750	50	KM460882, KM460883, KM460884	99.0-99.1% (L49052: *O. edulis*)
	Ostrea circumpicta	3	1749	51	KM460885, KM460886, KM460887	97.7-98.3% (L49052: *O. edulis*)
	Saccostrea kegaki	3	1746-1747	50	KM460888, KM460889, KM460890	98.6-98.7% (AB064942: *C. gigas*)
	Hyotissa hyotis	3	1746	49	KM460891, KM460892, KM460893	99.2-99.3% (AJ389633: *H. numisma*)
ITS (ITS1/5.8S/ITS2)	*Crassostrea ariakensis*	3	470-472/158/471-473	58-59/56/55	KM460830, KM460831, KM460832	99.2-99.6% (FJ356685: *C. ariakensis*)
	Crassostrea gigas	3	449-452/159/535-537	57/56/53-54	KM460833, KM460834, KM460835	99.4-99.7% (FJ356690: *C. gigas*)
	Crassostrea nippona	3	514-515/158/531	57/56/55	KM460836, KM460837	99.6% (FJ356678: *C. nippona*)
	Crassostrea. virginica	3	432-433/158/506-512	58/56/56-57	KM460849, KM460850, KM460851	98.6-99.5% (EU072460: *C. virginica*)
	Ostrea denselamellosa	3	425-426/158/418	61/56/61	KM460838, KM460839, KM460840	99.6-99.8% (FJ356688: *O. denselamellosa*)
	Ostrea circumpicta	3	400/158/399-400	59-60/56/62	KM460841, KM460842, KM460843	99.7-100% (EU072462: *O. circumpicta*)
	Saccostrea kegaki	3	426-429/158/475	55/56/59	KM460844, KM460845	98.7-99.8% (EU072464: *S. kegaki*)
	Hyotissa hyotis	3	340-343/158/342-343	53/56/55-56	KM460846, KM460847, KM460848	98.5-99.9% (EU072465: *H.hyotis*)
H3	*Crassostrea ariakensis*	3	374 (124 aa)	53-54	KM460852, KM460853, KM460854	93-93.3% (KC429170: *C. virginica*)
	Crassostrea gigas	3	374 (124 aa)	53-54	KM460855, KM460856	92.7% (HQ009488: *C. gigas*)
	Crassostrea nippona	3	374 (124 aa)	54-55	KM460857, KM460858, KM460859	96.8% (HQ009488: *C. gigas*)
	Crassostrea. virginica	3	374 (124 aa)	56	KM460871, KM460872	98.2-98.5% (KC429170: *C. virginica*)
	Ostrea denselamellosa	3	374 (124 aa)	53-54	KM460860, KM460861, KM460862	95.6-95.9% (AY070151: *O. edulis*)
	Ostrea circumpicta	3	374 (124 aa)	51-53	KM460863, KM460864	94.0-95.3% (AY070151: *O. edulis*)
	Saccostrea kegaki	3	374 (124 aa)	53	KM460865, KM460866, KM460867	No significant similarity found
	Hyotissa hyotis	3	374 (124 aa)	54	KM460868, KM460869, KM460870	97.4% (HQ329258: *H. hyotis*)

18S rDNA

The length of 18S rDNA of *S. kegaki* (1746-1747 bp) and *H. hyotis* (1746 bp) were relatively shorter than that of *O. circumpicta* (1749 bp) (Table 2-3). The sequences of 18S rDNA from *O. circumpicta*, *S. kegaki* and *H. hyotis* showed high similarity with *O. edulis* (L49052, 97.7-98.3% of similarity), *C. gigas* (AB604942, 98.6-98.7 %) and *H. numisma* (AJ389633, 99.2-99.3%) respectively (Table 2-3).

In GeneBank, however, the sequence information of 18S rDNA in oysters is limited to six species including *C. gigas* (AB064942, AN182263), *C. virginica* (KC429335, X60315), *S. cucullata* (AJ389634), *O. edulis* (L49052, U88709), *H. hyotis* (AJ389632), *H. mcgintyi* (KC429336), and *H. numisma* (AJ389633). In the present study, we revealed the sequence of 18S rDNA from 8 oyster species including *C. gigas*, *C. nippona*, *C. ariakensis*, *C. virginica*, *O. denselamellosa*, *O. circumpicta*, *S. kegaki*, and *H. hyotis*, allowing the comparison of genetic relationship of oysters based on 18S rDNA regions.

Phylogenetic tree of eight oyster species with other *Crassostrea*, *Ostrea*, *Saccostrea*, and *Hyotissa* oyster species deposited in GenBank based on 18S rDNA is shown in Fig. 2-1. The topologies of the trees obtained by NJ and MP were similar. Two major clades were identified as belonging to Family Ostreidae and Gryphaeidae. In the Family Ostreidae, 3 different genus including *Crassostrea* (*C. ariakensis*, *C. nippona*, and *C. gigas*), *Saccostrea* (*S. kegaki*), and *Ostrea* (*O. denselamellosa* and *O. circumpicta*) were clearly divided based on the SSU rDNA genes. *S. kegaki* grouped with *S. cuculleta* in NJ and MP analyses (bootstrap values: NJ = 100% and MP = 99%), whereas *O. circumpicta* joined with *Ostrea* species (bootstrap values: NJ = 99% and MP = 99%). *Saccostrea* and *Ostrea* species were tended to group together (bootstrap values: NJ = 65% and MP = 78%) and formed sister group with

Crassostrea species. The similarity of 18S rDNA nucleotide sequence between *S. kegaki* and *O. circumpicta* ranged from 98.2 to 98.5%. *H. hyotis* combined with *H. numisma* (bootstrap values: NJ = 98% and MP = 94%) and formed outgroup.

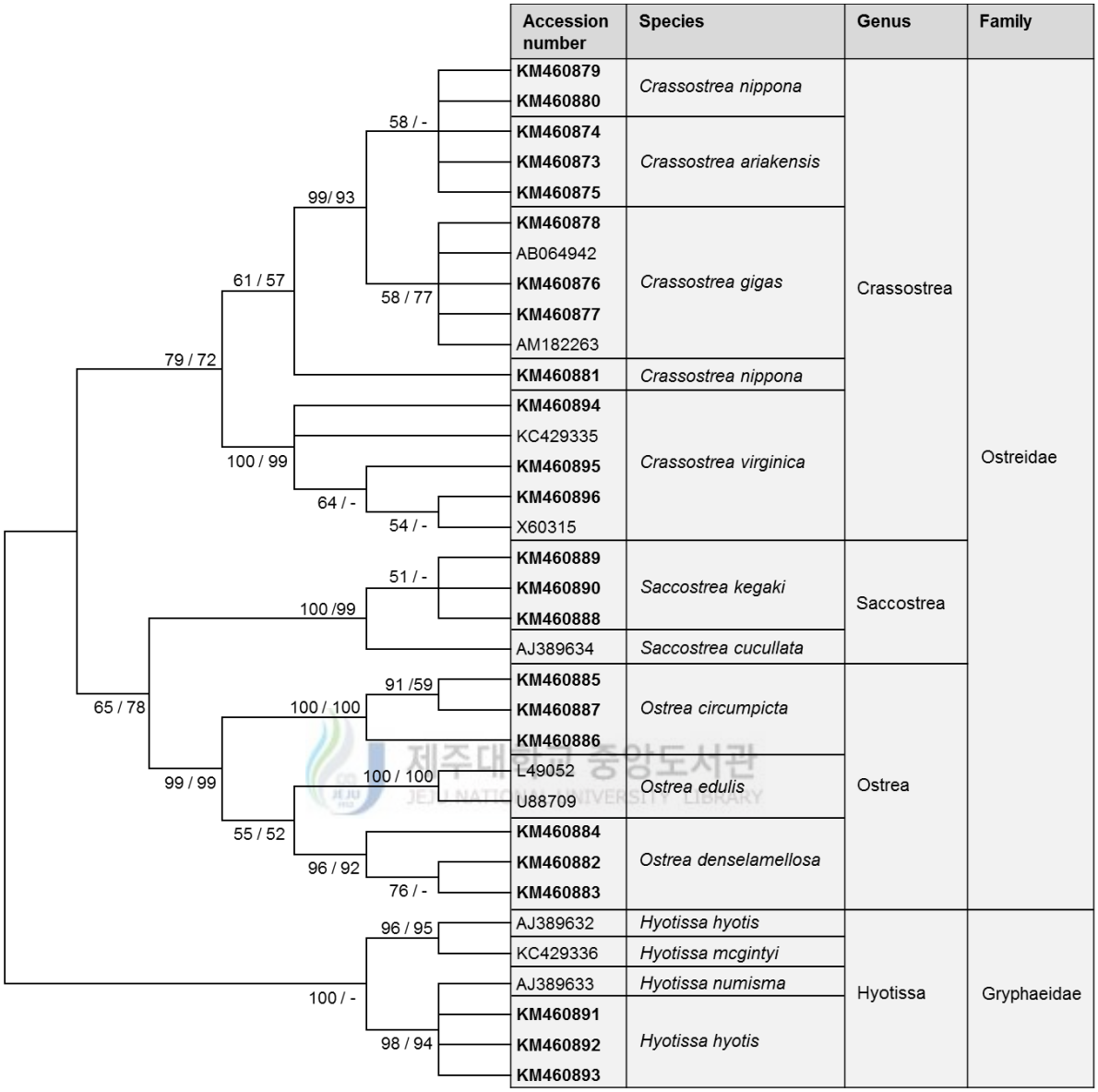


Fig. 2-1. Phylogenetic trees based on neighbor-joining analyses of SSU rDNA sequences of *Crassostrea*, *Saccostrea*, *Ostrea*, and *Hyotissa* species using Kimura two-parameter distances with 1000 bootstrap. Bootstrap values > 50% is shown at the nodes. Numbers above the branches are the bootstrap support values for NJ and Maximum Parsimony (MP), respectively.

The sequences of rDNA gene are easy to access due to highly conserved flanking regions allowing for the use of universal primer (Steiner 1999; Meyer et al. 2010). The 18S rDNA region is part of the ribosomal functional core and is exposed to similar selective force in all living beings. Thus, the sequence of 18S rDNA has been considered as a good molecular marker for investigation of bivalve mollusks, up to Family or Genus level (Winnepenninckx et al. 1996; Giribet and Carranza 1999). From this study, we provided the sequence information of 18S rDNA of oyster species including subtropical oysters where the 18S rDNA sequence data is limited. Therefore these sequence data can be used for efficient reconstruction of evolutionary relationship among oyster species as well as bivalve mollusk.

The sequence of 18S rDNA of *H. hyotis* from Red Sea in Egypt was reported (Steiner and Hammer 2000). The Gryphaidae oyster identified as *H. hyotis* based on shell morphology in this study shown the 99.3-99.4% similarity of 18S rDNA sequences with *H. hyotis* from Egypt. The distribution of *H. hyotis* has been reported from shallow subtropical and tropical coral reefs such as the Northern Red Sea (Titschack et al. 2010), Gulf of California (Duprat-Bertazzi and García-Domínguez 2005; Robríguez-Astubilo et al. 2005; Villalejo-Fuerte et al. 2005), Florida Key (Kirkendale et al. 2004), Malaysia and Singapore (Lam and Morton, 2009). The dissimilarity level of 0.6-0.7% may arise from the geographical difference of oyster habitat, such as high-latitude (Jeju Island) and subtropical (Red Sea) area. However, much more extensive and intensive sampling of oysters will be needed to gain additional confidence in the taxonomic relationship among *Hyotissa* species.

ITS

The ITS regions are particularly useful for comparing closely related groups, such as species within a genus because it has a high degree of variation even between closely related species (Fernández et al. 2001). The ITS-1 and ITS-2 regions of rDNA are widely used for phylogenetic analyses at and below the species levels due to the homogenous characteristics of nuclear ribosomal sequences within species (Morgan and Blair 1998; Insua et al. 2003; Cheng et al. 2006; Reece et al. 2008). In oyster phylogeny, the ITS-1 regions has been proven to be useful for identifying phylogenetic relationships among *Crassostrea* species (Reece et al. 2008)

The length between ITS1 and ITS2 regions of *O. circumpicta* (399-400 bp) and *H. hyotis* (340-343 bp) was similar while *S. kegaki* had long sequences in ITS2 (471-537 bp) than ITS1 (432-515 bp) regions (Table 2-3). The genetic similarity of ITS1-5.8S-ITS2 regions of three subtropical oyster species strongly matched with those of each oyster species deposited in GenBank (Table 2-3).

Because few data of ITS-2 region of oyster species were registered in GenBank, only ITS-1 region was used in phylogenetic analysis (Fig. 2-2). Like the result in 18S rDNA, two clades at Family level (Ostreidae and Gryphaeidae) and four clades at Genus level (*Crassostrea, Saccostrea*, *Ostrea*, and *Hyotissa*) were clearly divided based on ITS-1 region. Both NJ and MP trees indicated that *S. kegaki* grouped with *S. glomerata* (bootstrap values: NJ = 60% and MP = 63%) and formed sister group with *S. cucullata* (bootstrap values: NJ = 99% and MP = 99%). *O. circumpicta* strongly grouped with *O. denselamellosa* (bootstrap values: NJ = 99% and MP = 99%). *Saccostrea* and *Ostrea* species were grouped together (bootstrap values: NJ = 90% and MP = 90%) and formed sister group with *Crassostrea*

species. The similarity of ITS-1 nucleotide sequence between *S. kegaki* and *O. circumpicta* ranged from 73.6 to 74.1%. Because ITS-1 region of *Hyotissa* species were not reported in GenBank, *H. hyotis* formed monophyletic clade as outgroup.

The ITS-1 regions of *O. circumpicta* were only reported from Japan (AB377697). In the present study, we revealed the ITS-1 sequence of *O. circumpicta* from Jeju Island and it was 99.5-100% similar with the sequence of *O. circumpicta* from Japan, indicating that two oysters are same species.

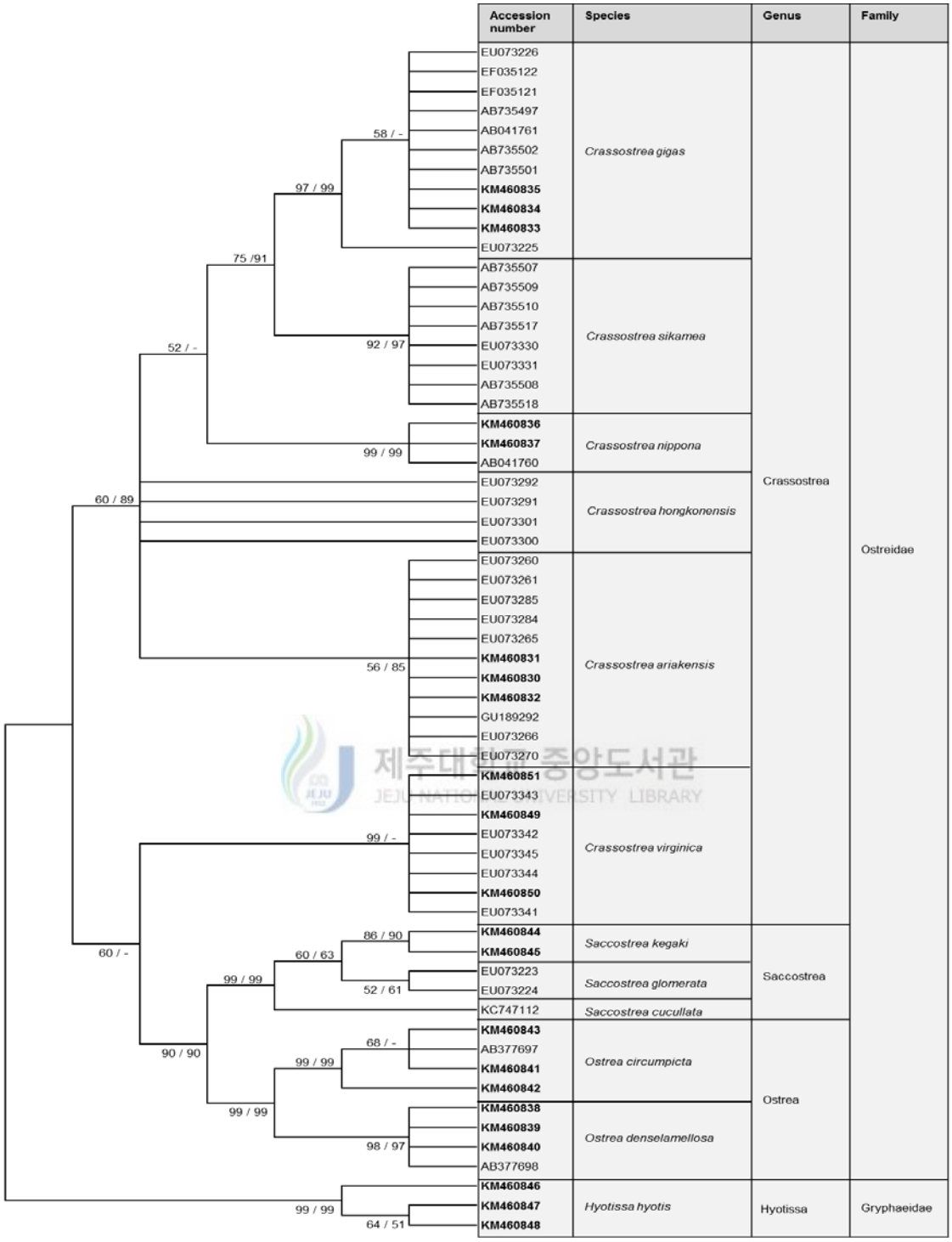


Fig. 2-2. Phylogenetic trees based on neighbor-joining analyses of ITS-1 sequences of *Crassostrea*, *Saccostrea*, *Ostrea*, and *Hyotissa* species using Kimura two-parameter distances with 1000 bootstrap. Bootstrap values > 50% is shown at nodes. Numbers above the branches are the bootstrap support values for NJ and Maximum Parsimony (MP), respectively.

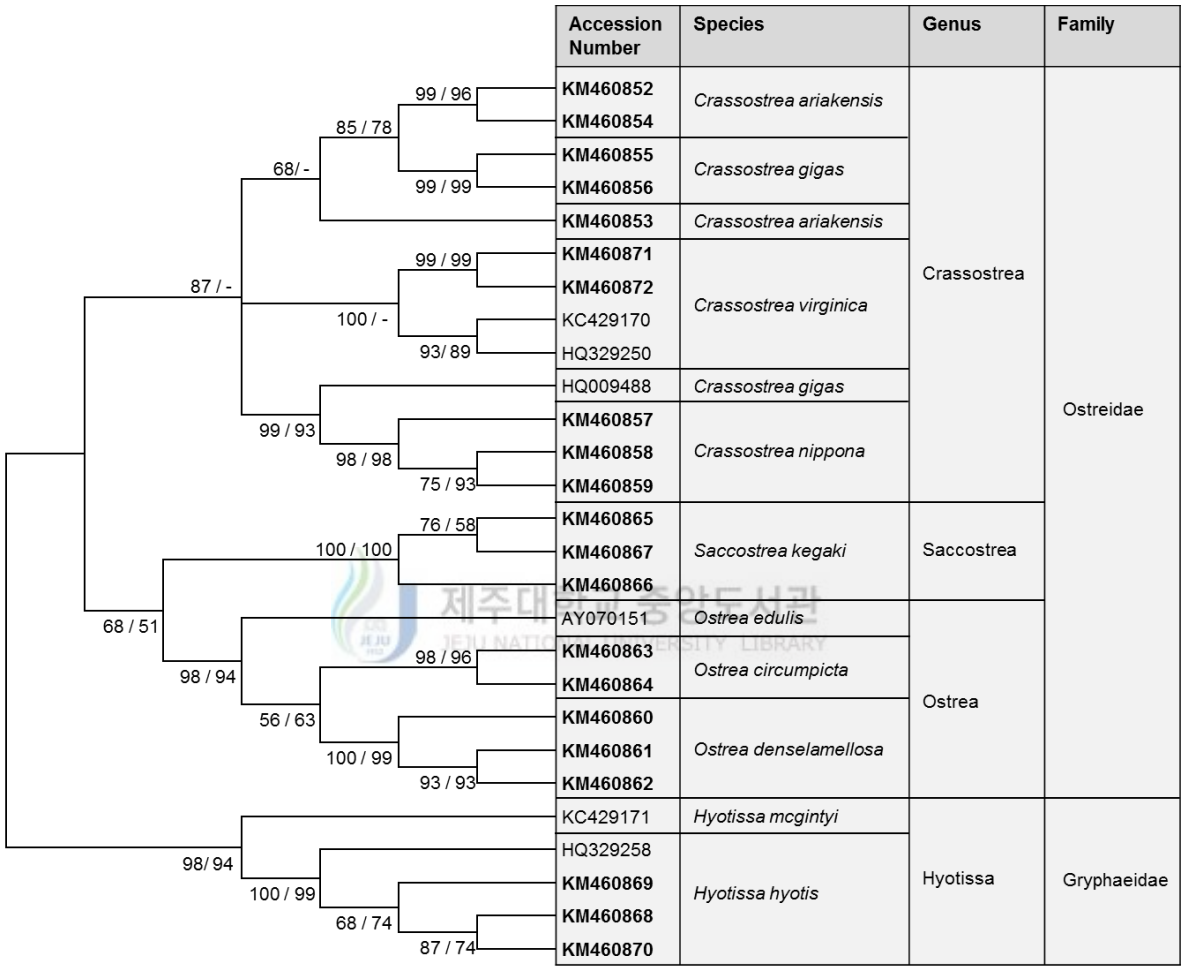
Histone H3

Histone H3 is highly conserved at the amino acid level and is easily amplified. Histone H3 has previously been used in phylogenetic studies of arthropod (Colgan et al. 1998; Giribet et al. 2001) and annelids (Brown et al. 1999). In bivalve mollusk, however, the genetic references of H3 are quite limited in some species such as *C. virginica* (KC429170, HQ329250), *C. gigas* (HQ009488), *O. edulis* (AY070151), *H. mcgintyi* (KC429171), and *H. hyotis* (HQ329258). In this study, we provided the H3 sequence of 8 oyster species allowing the analysis of phylogenetic relationship of subtropical oysters from Jeju Island with other oyster species based on H3 regions.

The sequences of H3 of three subtropical oyster species were identical with 374 bp in nucleotide length and 124 amino acid (Table 2-3). The H3 genes of *O. circumpicta* were shown the highest similarity (94-95.3%)with those of *O. edulis* (AY070151). For the *S. kegaki*, there were no similar sequence data for the H3 genes of *S. kegaki*. Finally, the sequence of H3 of *H. hyotis* represented 97.4% of similarity with those of *H. hyotis* (HQ329258).

Fig. 2-3 represents the phylogenetic tree of oyster species based on H3 sequences using NJ method. As expected, the Family Gryphaeidae formed a distinct group from the Family Ostreidae. In Family Ostreidae, *Saccostrea* species were genetically close to *Ostrea* species and formed sister branch with *Crassostrea* species. *O. circumpicta* joined with *O. denselamellosa* (bootstrap values: NJ = 56% and MP = 63%) and formed sister group with *O. edulis* (bootstrap values: NJ = 98% and MP = 94%). *Ostrea* species and *S. kegaki* were grouped together (bootstrap values: NJ = 68% and MP = 51%) and formed sister group with *Crassostrea* species. *H. hyotis* strongly combined with *H. mcgintyi* (bootstrap values: NJ = 98%

and MP = 94%) and formed outgroup.



Accession Number	Species	Genus	Family
KM460852	*Crassostrea ariakensis*	Crassostrea	Ostreidae
KM460854			
KM460855	*Crassostrea gigas*		
KM460856			
KM460853	*Crassostrea ariakensis*		
KM460871	*Crassostrea virginica*		
KM460872			
KC429170			
HQ329250			
HQ009488	*Crassostrea gigas*		
KM460857	*Crassostrea nippona*		
KM460858			
KM460859			
KM460865	*Saccostrea kegaki*	Saccostrea	
KM460867			
KM460866			
AY070151	*Ostrea edulis*	Ostrea	
KM460863	*Ostrea circumpicta*		
KM460864			
KM460860	*Ostrea denselamellosa*		
KM460861			
KM460862			
KC429171	*Hyotissa mcgintyi*	Hyotissa	Gryphaeidae
HQ329258	*Hyotissa hyotis*		
KM460869			
KM460868			
KM460870			

Fig. 2-3. Phylogenetic trees based on neighbor-joining analyses of histone H3 sequences of *Crassostrea*, *Saccostrea*, *Ostrea*, and *Hyotissa* species using Kimura two-parameter distances with 1000 bootstrap. Bootstrap values > 50% is shown at nodes. Numbers above the branches are the bootstrap support values for NJ and Maximum Parsimony (MP), respectively.

In the present study, the phylogenetic analyses by neighbor-joining (NJ) and maximum parsimony (MP) methods based on three different nuclear DNA primers (18S rDNA, ITS-1, and Histone H3) identically allowed a clear distinction between Family Ostreidae and Family Gryphaeidae. Ostreidae oysters *S. kegaki* and *O. circumpicta* were grouped together and formed sister group with *Crassostrea* species. On the other hand, Gryphaeidae oyster *H. hyotis* formed monophyletic clade as outgroup indicating a distant relationship between Ostreidae and Graphaeidae oysters. The result of my study suggests that the nuclear DNA sequences including 18S rDNA, ITS-1, and Histone H3 are useful tools for identifying oyster species.

III. Comparative study on the hemocytes of subtropical oysters *Saccostrea kega*ki, *Ostrea circumpicta*, and *Hyotissa hyotis* in Jeju Island: morphology and functional aspects

3.1. Introduction

Bivalves have an ‘open’ circulatory system in which the hemolymph, passing out of the open ends of arteries, bathes all the organs before returning to the heart by the way of sinuses and respiratory structures (gills) (Song et al. 2010). Hemocytes of marine bivalves are responsible for various physiological functions including internal defense, digestion and nutrient transport, wound healing, shell repair and excretion (see the review of Donaghy et al. 2009a). Like other marine invertebrate animals, hemocyte of marine bivalve is the main cellular mediator of the defense system, detecting and recognizing invading foreign substances (Ordás et al. 2000; Ottaviani et al. 2000; Kang et al. 2006; Takahashi et al. 2008). The hemocyte also produces reactive oxygen species (ROS) (Adema et al. 1991; Lambert et al. 2003), encapsulation and phagocytosis (Montes et al. 1995; Canesi et al. 2005), and subsequent degradation of the invading particles by various hydrolytic enzymes (López et al. 1997).

Based upon morphological and cytochemical features through microscopy and flow cytometry, bivalve hemocytes are categorized into two main hemocyte groups: granulocytes, which contain numerous granules within the cytoplasm, and agranulocytes, also called hyalinocytes, which are with no or fewer granules (Cheng 1981; Hinn 1999; Cima et al 2000; Aladaileh et al. 2007; Donaghy et al. 2009b; Song et al. 2010). Functionally, both granulocytes and hyalinocytes form pseudopodia, aggregate, phagocyte particles, and

produce ROS, although these immune parameters are highly variable among different bivalve species. Besides these two hemocyte types, undifferentiated cells characterized by small size, thin cytoplasm, and absence of immunological functions were classified in Manila clam *Ruditapes philippinarum* (Cima et al. 2000), Sydney rock oyster *Saccostrea glomerata* (Aladaileh et al. 2007), and Suminoe oyster *Crassostrea ariakensis* (Donaghy et al. 2009b). In the case of *R. philippinarum*, undifferentiated cells were positive for the anti-CD34 stem cell molecular marker (Cima et al. 2000).

Despite the abundance and popularity of subtropical oyster in Jeju Island, extremely few studies have been carried out on the ecology of oysters in Jeju (Kang et al. 2004; Kim et al. 2010). Effects of environmental changes on marine animals are often inferred from immunological activities of immune-related cells and molecules. The description of their immune systems is necessary to further assess the responses of oysters to environmental, toxic, and disease stresses.

In the present study, I first characterized hemocytes and the immunological activities of the oysters *S. kegaki*, *O. circumpicta*, and *H. hyotis* using light microscopy and flow cytometry which is crucial to understand cell-mediated responses of oysters to environmental, biological, and disease stresses.

3.2. Material and methods

Animals

To exclude the effect of the gametogenesis on hemocyte parameters, *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis* were collected during the winter period (January 2011 and 2012) when there is no gonadal development or spawning activity. In January, most of the oysters *S. kegaki* (Kim et al. 2010) and *H. hyotis* (data was shown Part 3 in this thesis) on the southern coast of Jeju were in spent or resting stage and *O. circumpicta* (Lim 2005) off southern Jeju exhibited early vitellogenic oocytes and spermatogonia. *S. kegaki* (4.2-5.9 cm in shell length) was collected from an intertidal rocky shore at Yeraedong on the southern coast of Jeju (Fig. 3-1). *O. circumpicta* (5.2-7.8 cm in shell length) and *H. hyotis* (14.3-17.8 cm in shell length) were collected from Supseom, southern Jeju, at depths of 5-15m by SCUBA diving (Fig. 3-1). The oysters were transported and acclimated in seawater (salinity 32-33 psu; temperature 15-16 °C) over 48 h in order for them to acclimate and recover from sampling and transportation stresses.

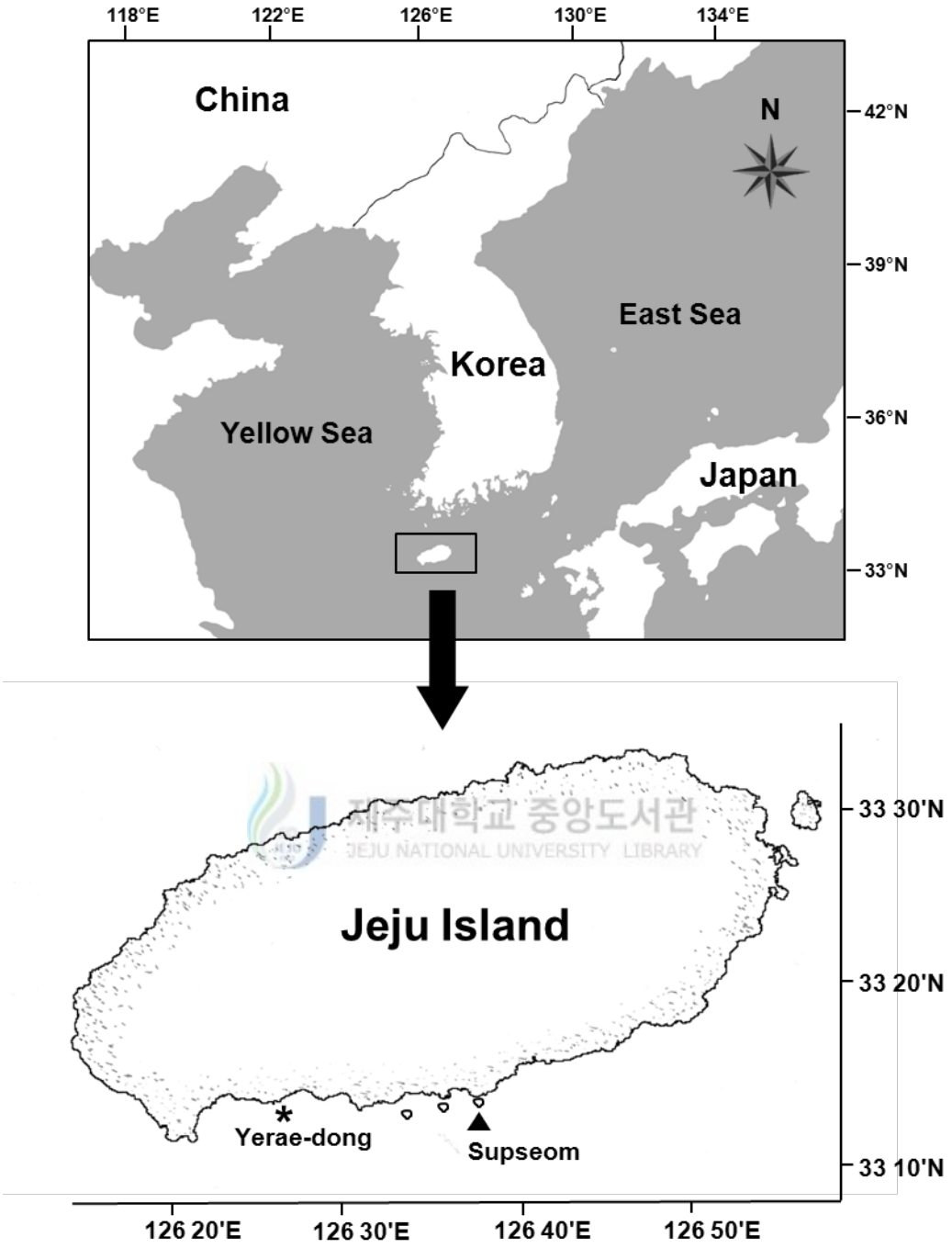


Fig. 3-1. Location map of the sampling sites. *Saccostrea kegaki* was collected from the inter-tidal area of Yerae-dong (∗). *Ostrea circumpicta* and *Hyotissa hyotis* were collected from Supseom (▲) at depth of 5-15 m by SCUBA diving.

Hemolymph collection

Using a syringe fitted with a 22Gx1 1/4″ needle, hemolymph was withdrawn from the adductor muscle through a previously ground notch on the anterior end of the shell. Collected hemolymph was filtered through an 60 μm nylon mesh and directly transferred into microtubes maintained on ice in order to minimize cell aggregation. Hemolymph from 3-5 oysters was pooled into one tube for the flow cytometry analyses.

Light microscope observation

Withdrawn hemolymph was placed onto glass slides coated with poly-L-Lysine, and hemocytes were allowed to adhere for 30 min in a humidity chamber at room temperature. The adherent hemocytes were fixed with absolute methanol and then stained with Hemacolor reagent (Merck, Darmstadt, Germany). A photograph of the hemocytes was taken under light microscopy with a digital camera. Size of pseudopodia, cytoplasm, and nucleus of hemocytes were measured using Image J 1.43u software (Fig. 3-2).

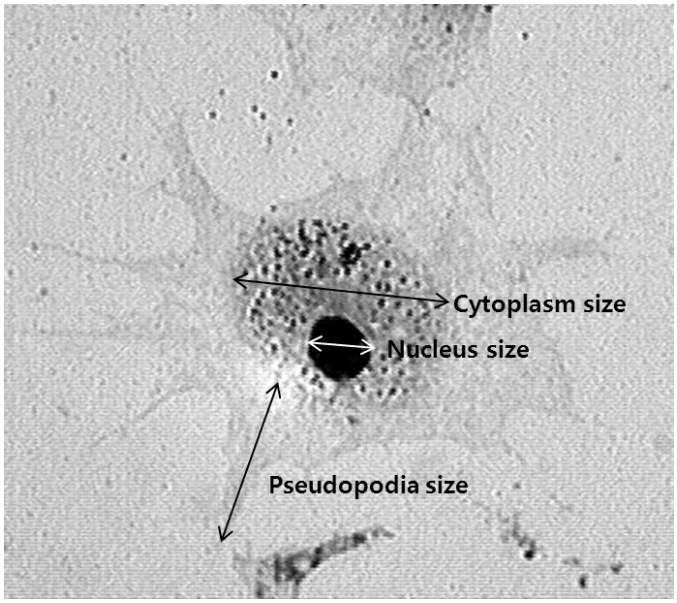


Fig. 3-2. Measurement of pseudopodia, cytoplasm, and nucleus size in oyster hemocytes.

Flow cytometry

Hemocyte population and concentration

Hemocyte population and concentration were determined using SYBR green I (Sigma-Aldrich, USA), a fluorescent dye that binds to double-stranded DNA. A 100 µL of hemolymph was fixed with an equal volume of 3% formalin solution. Fixed hemocytes were incubated with 1,000x SYBR green I (final dilution 10x) for 60 min in the dark at room temperature. After selecting hemocytes only stained by SYBR green I by FL1 detector of flow cytometry (FACSCalibur, Becton Dickinson, USA), hemocyte subtypes were discriminated based upon their relative internal complexity (granularity) and cell size (Side and Forward Scatter of the flow cytometry). Total hemocyte count (THC) was calculated as the number of cells mL^{-1} of hemolymph.

Lysosome quantification

The lysosome content in hemocytes subpopulations was determined using LysoTracker Red (Molecular Probes, Invitrogen, USA), that permeates the cell membrane and accumulates within lysosomal compartments. A 100 µL of hemolymph was diluted with an equal volume of antiaggregant solution (AASH; 2.5% NaCl and 1.5% EDTA in 0.1Mphosphate buffer, pH 7.4) and incubated LysoTracker Red (final dilution 1 mM) for 60 min in the dark at room temperature. Relative intracellular lysosomal quantity is expressed as the level of red fluorescence (FL3 detector of the flow cytometer) in arbitrary units (A.U.).

Phagocytosis capacity

Phagocytosis was induced using fluorescent latex beads (2.0 µm in diameter,

Polysciences Inc.). 100 µL of hemolymph was mixed with an equal volume of FSSW and fluorescent latex beads (final concentration at 2%). Phagocytosis activity of hemocytes was induced for 10, 30, 60, 120, and 180 min in the dark at room temperature. Phagocytosis capacity was expressed as the percentage of cells that engulfed more than three beads among all the hemocytes.

Oxidative capacity

Oxidative activity was evaluated using 2'7'-dichlorofluresceine diacetate (DCFH-DA; Molecular Probes, Invitrogen, USA). Penetrated DCFH-DA into the cells is oxidized to a highly fluorescent 2'7'-dichlorofluresceine (DCF) by reactive oxygen species (ROS) and reactive nitrogen species (RNS), and quantified by the FL1 detector in flow cytometer. Oxidative activity of hemocytes was stimulated by the addition of phorbol 1,2-myristate 1,3-acetate (PMA, Sigma–Aldrich, USA). A solution of DCFH-DA (final concentration 10 µM) was added to 100 µL of hemolymph diluted in the same volume of FSSW. The mixtures were then incubated for 10, 30, 60, 120, and 180 min in the dark at room temperature. Oxidative capacity was expressed as the level of green fluorescence (FL1 detector of the flow cytometer) in arbitrary units (A.U.)

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan’s test, when required, were conducted to compare the parameters of hemocyte populations between oysters. The percentage data were transformed as arc sine of the square root before ANOVA, but are presented in figures as untransformed percentage values. All results are expressed as mean ±

standard error (SE).

3.3. Results

Morphological and cytometric identification of hemocyte populations

Light microscopy

Four hemocyte populations were distinguished in three species of oysters by light miscopy based on the cell size, and presence or absence of granules in cytoplasm: granulocytes, vacuolated granulocytes, hyalinocytes, and blast-like cells (Fig. 3-3). Granulocytes contained numerous granules in the cytoplasm and formed many long pseudopodia. Vacuolated granulocytes were characterized by large vacuoles in the cytoplasm with some short pseudopodia. Hyalinocytes represented the cytoplasm containing no or a few granules, and formed many long pseudopodia. Blast-like cells were the smallest cells with very thin cytoplasm.

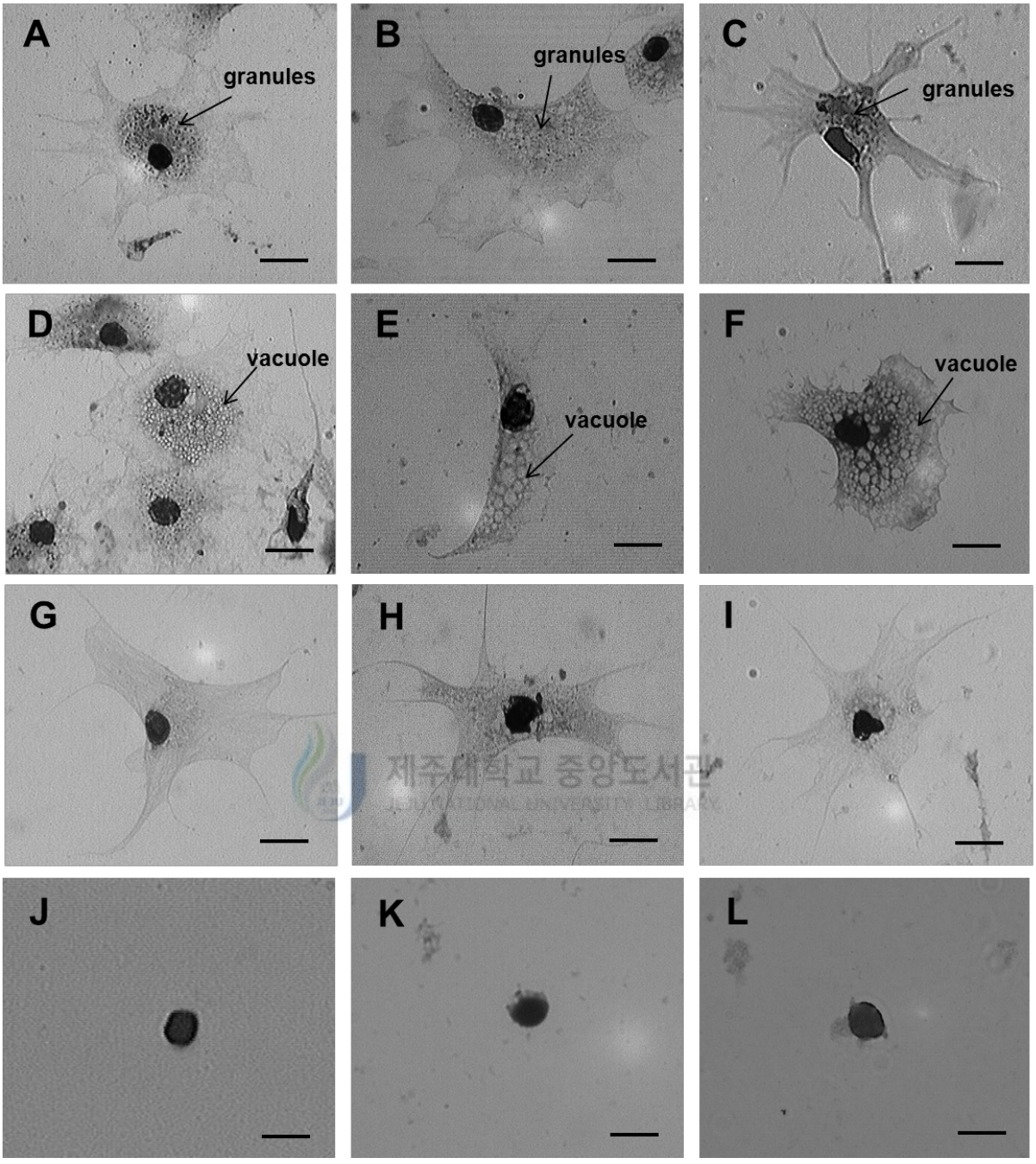


Fig. 3-3. Light micrographs of hemocytes of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis* stained with Hemacolor. Granulocytes in *S. kegaki* (A), *O. circumpicta* (B), and *H. hyotis* (C). Vacuolated granulocytes in *S. kegaki* (D), *O. circumpicta* (E), and *H. hyotis* (F). Hyalinocytes in *S. kegaki* (G), *O. circumpicta* (H), and *H. hyotis* (I). Blast-like cells in *S. kegaki* (J), *O. circumpicta* (K), and *H. hyotis* (L). Bar = 10 μm.

Table 3-1 summarized the size of pseudopodia, cytoplasm (C), and nucleus (N), as well as N/C ratio of each hemocyte populations. In all species, blast-like cells featured no pseudopodia and the thinnest cytoplasm (6.33 μm in *S. kegaki*, 6.50 μm in *O. circumpicta*, and 7.05 μm in *H. hyotis*) among hemocyte populations. Consequently, N/C ratio of the blast-like cells (0.77 in *S. kegaki*, 0.78 in *O. circumpicta*, and 0.76 in *H. hyotis*) was significantly higher than the ratio of granulocytes, vacuolated cells, and hyalinocytes (ANOVA, $p < 0.05$).

The length of the pseudopodia, cytoplasm, and nucleus varies depending on the species and cell types (Table 3-1). Pseudopodia of hyalinocytes were longer (ANOVA, $p < 0.05$) than those of granulocytes and vacuolated cells in *S. kegaki* and *O. circumpicta*. In contrast, *H. hyotis* was similar pseudopodia size in hyalinocytes and granulocytes. Cytoplasm of granulocytes was larger (ANOVA, $p < 0.05$) than hyalinocytes in *O. circumpicta* and *H. hyotis*, while the cytoplasm of two hemocyte populations of *S. kegaki* was similar in size. Between the three oyster species, the cytoplasm of *S. kegaki* granulocytes was smaller (ANOVA, $p < 0.05$) than those of granulocytes in *O. circumpicta* and *H. hyotis*.

Table 3-1 Microscopic characterization of the hemocyte populations of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis* stained with Hemacolor. Values are presented as mean ± standard error. n, number of analyzed cells. Different letters (a-f) in rows indicate significant (ANOVA, $p < 0.05$) differences among hemocyte populations.

	S. kegaki				*O. circumpicta*				*H. hyotis*			
	Granulocytes n=30	Vacuolated n=10	Hyalinocytes n=30	Blast-like n=20	Granulocytes n=30	Vacuolated n=10	Hyalinocytes n=30	Blast-like n=20	Granulocytes n=30	Vacuolated n=10	Hyalinocytes n=30	Blast-like n=20
Pseudopodia size (μm)	9.16^{e} ± 0.43	8.09^{e} ± 0.33	17.58^{bc} ± 0.76	-	12.21^{d} ± 0.41	4.43^{f} ± 0.55	16.43^{c} ± 0.57	-	19.42^{ab} ± 1.08	4.89^{f} ± 0.34	20.15^{a} ± 0.83	-
Cytoplasm size (C; μm)	14.79^{c} ± 0.52	16.95^{abc} ± 0.72	16.81^{abc} ± 0.56	6.33^{d} ± 0.25	18.83^{a} ± 0.55	16.75^{abc} ± 0.41	15.20^{c} ± 0.73	6.50^{d} ± 0.16	17.85^{a} ± 0.71	17.61^{ab} ± 0.45	15.53^{bc} ± 0.74	7.05^{d} ± 0.20
Nucleus size (N; μm)	5.29^{abc} ± 012	5.70^{ab} ± 0.0.25	5.45^{abc} ± 0.21	4.82^{c} ± 0.17	5.20^{abc} ± 0.28	5.17^{abc} ± 0.15	5.46^{abc} ± 0.14	5.08^{bc} ± 0.18	5.84^{a} ± 0.21	4.88^{c} ± 0.27	5.29^{abc} ± 0.25	5.32^{abc} ± 0.16
N/C ratio	0.37^{bc} ± 0.02	0.34^{bc} ± 0.01	0.34^{bc} ± 0.02	0.77^{a} ± 0.01	0.28^{c} ± 0.01	0.31^{bc} ± 0.01	0.40^{b} ± 0.03	0.78^{a} ± 0.02	0.33^{bc} ± 0.01	0.28^{c} ± 0.02	0.40^{bc} ± 0.07	0.76^{a} ± 0.02

Flow cytometry

Flow cytometry allows for the discrimination of three hemocyte type by relative cell size and internal complexity in three species: granulocytes, hyalinocytes, and blast-like cells (Fig. 3-4). Mean of THC in *S. kegaki* (491,861 cell mL^{-1}) and *O. circumpicta* (685,592 cells mL^{-1}) was statistically (ANOVA, $p < 0.05$) higher than those of *H. hyotis* (264,299 cells mL^{-1}). ANOVA indicated that the mean value of THC in *S. kegaki* and *O. circumpicta* was not significantly different.

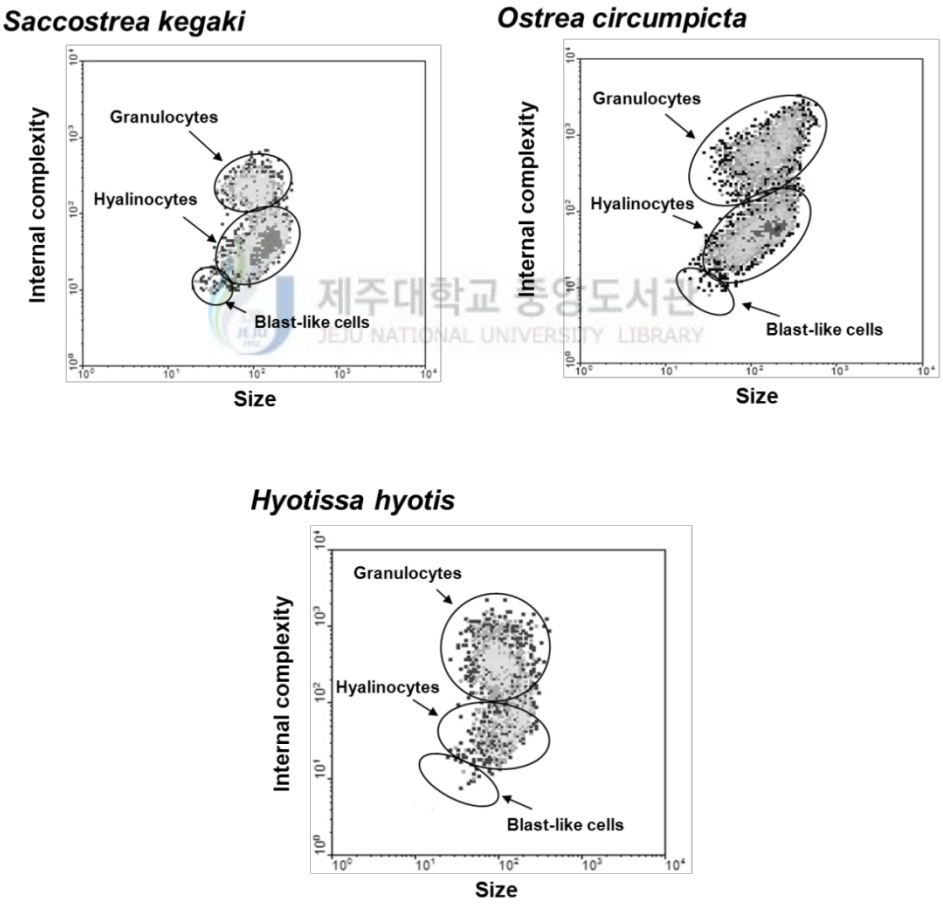


Fig. 3-4. Flow cytometric determination of the hemocyte population of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis*. Hemocytes were identically classified into three populations; granulocytes, hyalinocyte, and blast-like cells.

Table 3-2 summarized the percentage, size and internal complexity of each hemocyte populations determined by flow cytometry. In all species, hyalinocytes were the most abundant cells representing more than 59% of circulating hemocytes (61.7% in *S. kegaki*, 65.9% in *O. circumpicta*, 59.0% in *H. hyotis*). The second abundant cells in hemolymph were granulocytes: 29.2% in *S. kegaki*, 23.1% in *O. circumpicta*, and 30.5% in *H. hyotis*. Small and agranular cells accounted for less than 5% of circulating hemocytes (2.7% in *S. kegaki*, 3.0% in *O. circumpicta*, and 4.5% in *H. hyotis*) and were thought as blast-like cells. Granulocytes were the largest (ANOVA, $p < 0.05$) cells among hemocyte populations in *O. circumpicta* (154.4 A.U.) and *H. hyotis* (140.4 A.U.); whereas, the size of granulocytes (113.2 A.U.) and hyalinocytes (123.6 A.U.) in *S. kegaki* was not statistically different. Granulocytes displayed statistically higher (ANOVA, $p < 0.05$) internal complexity compared with hyalinocytes and blast-like cells in all species. Internal complexity of granulocytes was the highest in *O. circumpicta* (720.4 A.U.) and the lowest in *S. kegaki* (224.7 A.U.).

Table 3-2 Flow cytometric characterization of the hemocyte populations of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis*. The percentage, size and internal complexity of each hemocyte population from oysters were determined on SYBR green I positive cells fixed with 3% formalin. Size and internal complexity are expressed in flow cytometric arbitrary units (A.U.). Values are presented as mean ± standard error. n, number of analyzed cells. Different letters (a-d) in rows indicate significant (ANOVA, $p < 0.05$) differences among hemocyte populations.

	S. kegaki			*O. circumpicta*			*H. hyotis*		
	Granulocytes n=15	Hyalinocytes n=15	Blast-like n=15	Granulocytes n=15	Hyalinocytes n=15	Blast-like n=15	Granulocytes n=15	Hyalinocytes n=15	Blast-like n=15
Percentage	29.2	61.7	2.7	23.1	65.9	3.0	30.5	59.0	4.5
Size (A.U.)	113.2^{c} ± 3.3	123.6^{bc} ± 1.9	37.2^{e} ± 0.5	154.4^{a} ± 10.7	118.4^{c} ± 5.6	27.6^{e} ± 3.2	140.4^{ab} ± 7.5	119.0^{c} ± 4.9	55.9^{d} ± 5.3
Complexity (A.U.)	224.7^{c} ± 5.6	38.4^{d} ± 0.8	13.4^{d} ± 0.1	720.4^{a} ± 40.8	48.1^{d} ± 4.5	12.1^{d} ± 1.2	528.4^{b} ± 29.6	58.7^{d} ± 3.6	19.8^{d} ± 1.0

The presence of lysosomes in the hemocytes of three species is shown in Fig. 3-5. In all species, granulocytes contained higher lysosomal amounts than hyalinocytes. The lysosome contents in granulocytes were the highest in *O. circumpicta* (405.4 A.U., ANOVA, $p < 0.05$) and the lowest in *S. kegaki* (103.9 A.U., ANOVA, $p < 0.05$). In the case of hyalinocytes, the lysosome contents of *S. kegaki* were 26.3 A.U., which was significantly lower (ANOVA, $p < 0.05$) than *O. circumpicta* (57.4 A.U.) and *H. hyotis* (61.2 A.U.). The lysosome contents of hyalinocytes in *H. hyotis* and *O. circumpicta* were not significantly different.

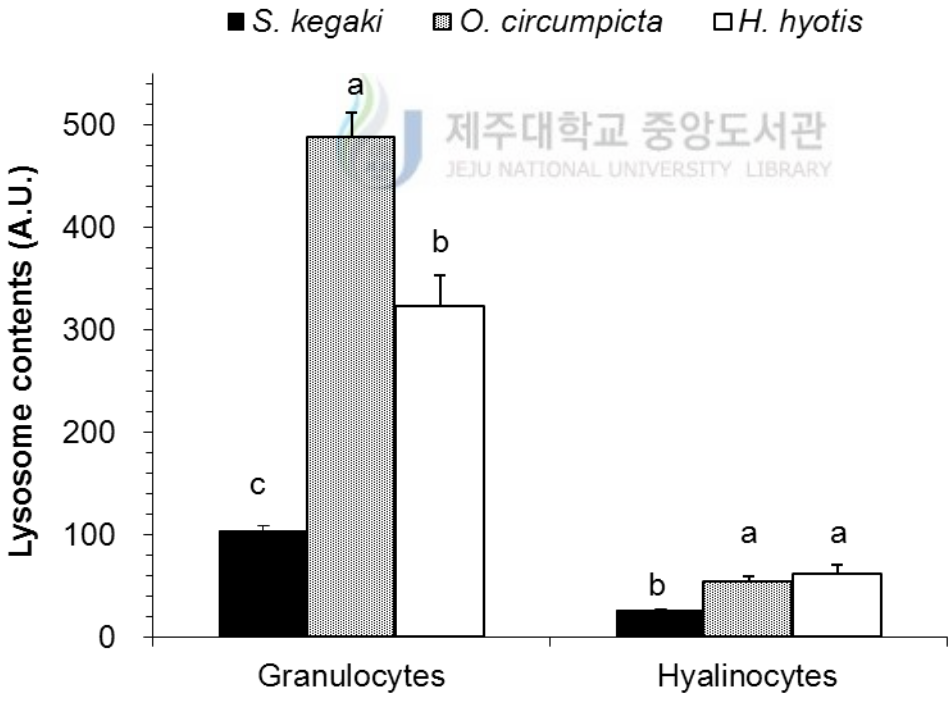


Fig. 3-5. Lysosome contents of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis*. Values are presented as mean ± standard error. For each species, n=10. Different letters (a-c) in columns represent significant (ANOVA, $p < 0.05$) difference among three species.

Flow cytometric characterization of the immune-related activities of hemocytes

Phagocytosis capacity

In all species, both granulocytes and hyalinocytes showed phagocytosis capacity while blast-like cells did not. The phagocytosis capacity of granulocytes was higher than those of hyalinocytes over time (10 to 180 min; Fig. 3-6). The development of phagocytosis index of granulocytes of *S. kegaki* and *O. circumpicta* was similar from 15 min to 30 min (Fig. 3-6). After 30 min, however, the mean value of phagocytosis capacity was higher (ANOVA, $p < 0.05$) in *O. circumpicta* granulocytes than in *S. kegaki* granulocytes; the phagocytosis capacity of granulocytes of *S. kegaki* and *O. circumpicta* was increased to approximately 30.9% and 45.0% at 180 min, respectively (Fig. 3-6). The granulocytes of *H. hyotis* showed a high phagocytosis capacity from 10 min (29.9%), which is three times higher (ANOVA, $p < 0.05$) than those of *S. kegaki* (10.6%) and *O. circumpicta* (8.4%, Fig. 3-6). The phagocytosis capacity of *H. hyotis* granulocytes then slightly increased to 36.2% at 180 min (Fig. 3-6).

The phagocytosis rate of hyalinocytes did not markedly increase over the time with low phagocytosis capacity of less than 10% in all species (Fig. 3-6). The range of phagocytosis rate of hyalinocytes for 180 min was 1.1-3.1% in *S. kegaki*, 2.3-9.3% in *O. circumpicta*, and 4.8-5.4% in *H. hyotis*. The development rate of phagocytosis index of the hyalinocytes of *O. circumpicta* was higher (ANOVA, $p < 0.05$) than those of *S. kegaki* and *H. hyotis* (Fig. 3-6).

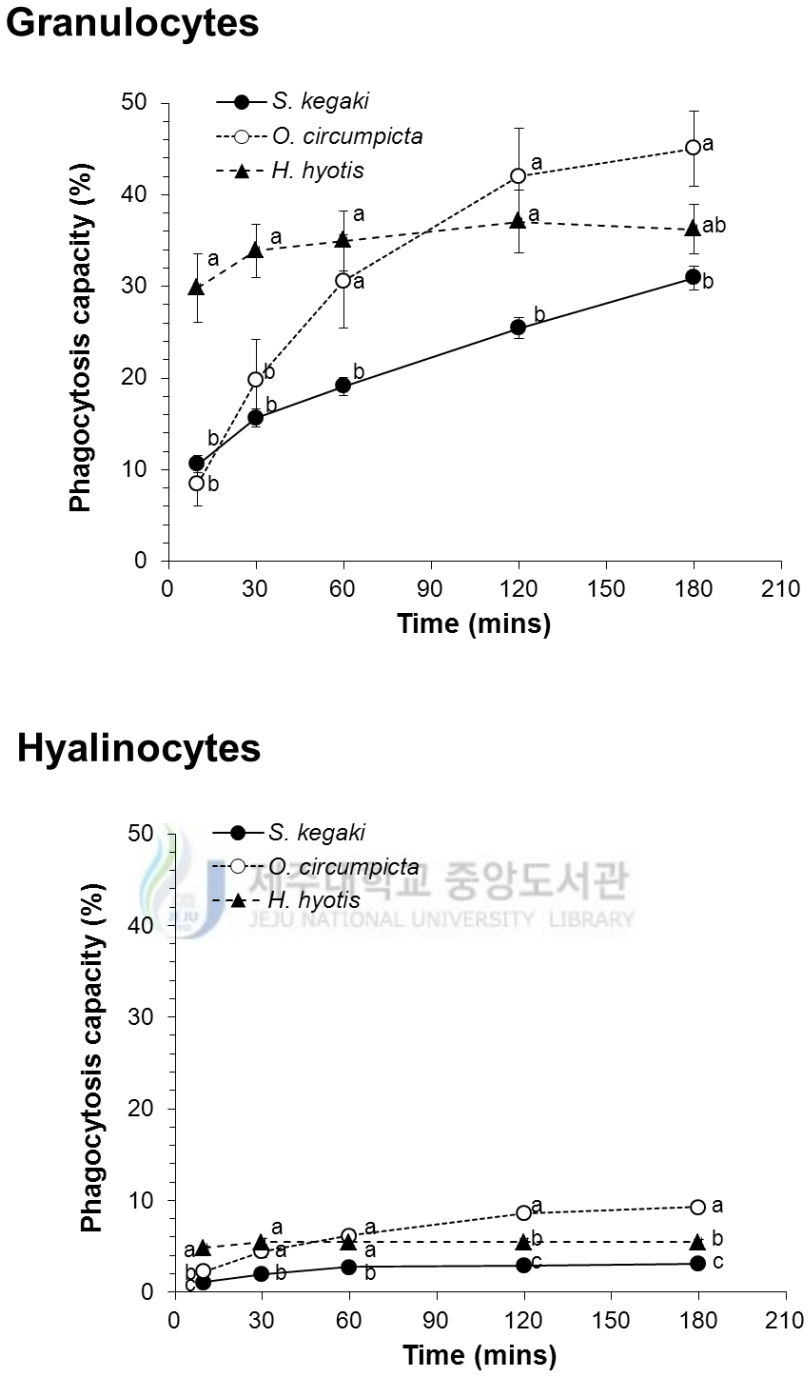


Fig. 3-6. Phagocytosis capacity of the granulocytes and hyalinocytes of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis.* Values are presented as mean ± standard error. For each time and each species, n=15. Different letters (a-c) in columns represent significant (ANOVA, $p < 0.05$) difference among three species.

Oxidative capacity

In all species, oxidative activity in each hemocyte population was confirmed. Because the amount of ROS and RNS production in blast-like cells was extremely low compared to other hemocyte population, blast-like cells were excluded from the measurements of oxidative activity. The relative amount of ROS and RNS of hemocytes had a different between species. Between granulocytes and hyalinocytes, oxidative capacity of granulocytes was higher than those of hyalinocytes over time (10 to 180 min) in all species (Fig. 3-7).

Oxidative activity in granulocytes from all species was similar from 15 min to 30 min (Fig. 2-7). *O. circumpicta* granulocytes generated the largest amount with 69.1 A.U. after 120 min, and *S. kegaki* granulocytes showed the 64.9 A.U. of generations after 180 min. On the other hand, the generations of ROS and RNS of *H. hyotis* granulocyte increased steadily up to 180 min and showed the lowest (ANOVA, $p < 0.05$) among three species (Fig. 3-7).

For the hyalinocyte, the mean value of oxidative activity in three species was not significantly different from 15min to 60 min (Fig. 3-7). *S. kegaki* hyalinocytes showed the highest (ANOVA, $p < 0.05$) oxidative activity among three species with 48.5 A.U. after 180 min (Fig. 3-7). Oxidative activity of hyalinocytes of *O. circumpicta* and *H. hyotis* slightly increased to 27.8 A.U. and 16.6 A.U. after 180 min, respectively, without significant difference (Fig. 3-7).

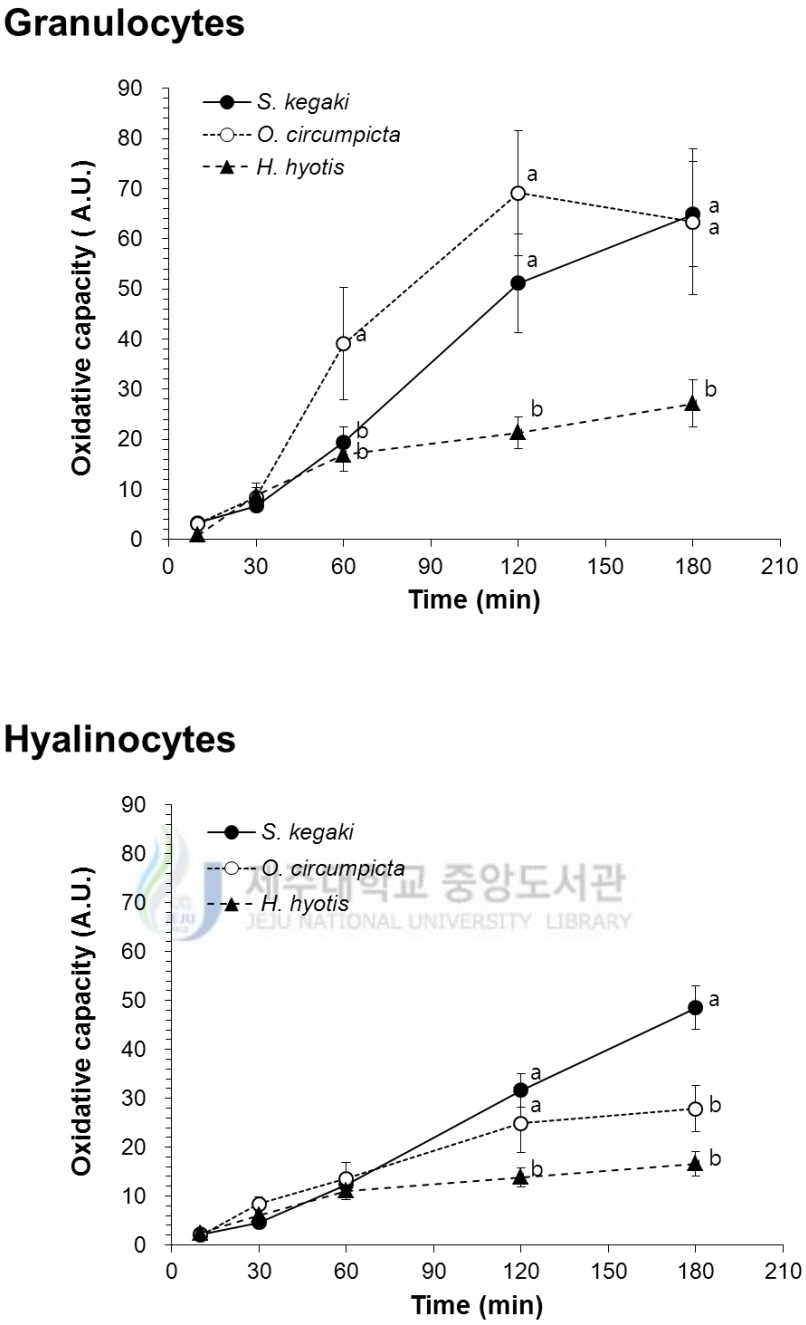


Fig. 3-7. Oxidative capacity of the granulocytes and hyalinocytes of *Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis*. Values are presented as mean ± standard error. For each time and each species, n=15. Different letters (a, b) in columns represent significant (ANOVA, $p < 0.05$) difference among three species.

3.4. Discussion

Like other marine bivalves, two major hemocyte types were identically classified into the oysters including *S. kegaki*, *O. circumpicta*, and *H. hyotis* based on the presence or absence of intra-cytoplasmic granules; granulocytes and hyalinocytes. Both hemocyte populations have been identified in the oysters *C. gigas* (Auffret 1989), *C. virginica* (Hégaret et al 2003a), *C. ariakensis* (Donaghy et al. 2009b), *S. glomerata* (aladaileh et al. 2007), *O. edulis* (Auffret 1989), the clams *R. philippinarum* (Cima et al. 2000), *R. decussatus* (López et al. 1997), *Mertrix lusoria* (Chang et al. 2005), *Mercenaria mercenaria* (Allam et al. 2002), the mussels *Mytilus edulis* (Pipe 1990), *M. galloprovincialis* (Carballal et al. 1997).

In addition to the two types of hemocytes, we discriminated the smallest agranular cells (6-7 μm diameters) with very thin cytoplasm and named them as blast-like cells. Flow cytometry allowed the blast-like cells did not show any phagocytosis or oxidative activity. Such morphological features and absence of the functionality are typical characteristics of undifferentiated cells (Donaghy et al. 2009b). These undifferentiated cells were also reported in the Sydney rock oyster *S. glomerata* (Aladaileh et al. 2007) and Sumino oyster *C. ariakensis* (Donaghy et al. 2009b). However, further studies will be needed to confirm those cells are really stem cells freely circulating in hemolymph. For example, Cima et al. (Cima et al. 2000) confirmed that small undifferentiated cells in the hemolymph of the Manila clam *T. philippinarum* were positive to the anti-CD34 stem cell molecular marker.

THC and percentage of each hemocyte population was similar between the three oyster species. Hyalinocytes were the most abundant cell in the hemolymph accounting for more than 59%, followed by granulocytes (23-31%) and blast-like cells (3-5%). Both light microscopy and flow cytometry revealed that the size of granulocytes is large ($p < 0.05$) than

hyalinocytes in *O. circumpicta* and *H. hyotis*, while two hemocyte types of *S. kegaki* were similar in size. Between the three oyster species, granulocytes of *S. kegaki* were smaller ($p < 0.05$) than those of *O. circumpicta* and *H. hyotis*. THC and size of hemocytes in marine bivalves varies with season and rearing sites, even in the same species (see the review of Donaghy et al. 2009a). In the present study, three oyster species were collected at the same time. Therefore the difference of hemocyte size between the species could be in part, explained by different environmental condition. The oysters *S. kegaki* analyzed in this study were from intertidal rocky area that is exposed in the air during low tide, whereas the oysters *O. circumpicta* and *H. hyotis* were collected from subtidal rocky bottoms at depth of 5-15m.

Apart from three types of hemocytes, hemocytes with big vacuoles within cytoplasm were found in light microscopy observation and were named as vacuolated granulocyte. Hemocytes with many vacuoles in cytoplasm were also reported in the Pacific oyster *C. gigas* (Chang et al. 2005). Besides marine bivalves, vacuolated hemocyte was described in tunicate *Holocynthia roretzi* (Amano and Hori 2008) and sea cucumber *Apostichopus japonicas* and *Cucumaria japonica* (Eliseikina and Magarlamov 2002) using light microscopy and transmission electron microscopy (TEM). It was reported that the vacuolated hemocyte does phaogyctosis in sea squirt *Phallusia mammillata* (Scippa et al. 1987) and involves in inflammatory response in sea squirt *Ciona intestinalis* (Parrinello 1981; Parrinello and Patricolo 1984; Parrinello et al. 1984 and 1990). In addition, some studies were reported that the frequency of vacuolated hemocyte increased in case of exposure of toxic substances. For instance, the number of vacuolated hemocyte was increased when exposing tributyltin (TBT) to tunicate *Styela plicata* (Radford et al. 2000). To understand the functions of vacuolated cells confirmed in this study, further analyses of the change of these cells should be

conducted under the conditions with various stresses or disease infections.

Lysosomes are organelles that can digest and decompose unnecessary substances as it exists within cytoplasm and contains various hydrolases (Luzio and Pryor 2007). Lysosome of marine organisms plays an important role in detoxification and defensive reaction (Moore 1980; Lowe et al. 1992 and 1995). In the present study, we confirmed that intracellular lysosomal content of granulocytes were 5-8 times more than those of hyalinocytes in all species. Between the oyster species, *S. kegaki* contained the fewest amount of lysosome in both granulocytes and hyalinocytes. These different quantities of lysosomes between hemocyte types or species might be related to different cellular functions. Cima et al. (2000) confirmed that different distribution of hydrolytic enzymes between granulocytes and hyalinocytes of Manila clam *T. philippinarum* resulted in different physiological and immune responses. In this study, therefore, the different content of lysosomes between cell types or species may be associated with functional differences.

Flow cytometry allowed that granulocytes of all species did phagocytosis 4-10 times more actively than hyalinocyte. This was also identical to the result of most active phagocytosis of granulocyte in bivalve, such as *C. ariakensis* (Donaghy et al. 2009b), *C. virginica* (Goedken and De Guise 2004), *M. mercenaria* (Tripp 1992), *R. decussatus* (López et al. 1997), and *Bathymodiolus azoricus* (Bettencourt et al. 2009). For all species, the hyalinocyte showed a low phagocyte rate with constant 10% after responding to fluorescent bead, while that of granulocyte increased at a different speed for each species according to time. The phagocytosis rate of *H. hyotis* granulocytes relatively high (29.9%) in 10 min and slightly increased to 37.1% at 120 min. In contrast, the phagocytosis rates of granulocytes of *S. kegaki* and *O. circumpicta* increased steadily according to elapse of time after showing low

phagocytosis rates with about 10% in 10 min. Between the three oyster species, the *O. circumpicta* granulocyte were the most active hemocytes engaged in phagocytosis among three species. In further studies, it should be conducted if the differences in phagocytosis capacity between the three species could be related to the phagocytic activities against actual pathogen.

The oxidative activity through production of ROS and RNS in the hemocytes of bivalves, such as the oysters *C. virginica* (Hégaret et al. 2003b), *C. gigas* (Lambert et al. 2003 and 2007), the clam *M. mercenaria* (Buggé et al. 2007), the mussel *M. galloprovincialis* (Torreilles and Guérin 1999) have been confirmed. In this study, we also detected the PMA-stimulated oxidative activity in three oyster species, and granulocyte in the all species was more active than hyalinocyte. This is identical to the result that granulocyte of *C. gigas* generated ROS and RNS more than that of hyalinocyte (Lambert et al. 2003). Therefore, it is thought that granulocyte and hyalinocyte possess the ability of generating each different ROS and RNS. The relative amount of ROS and RNS occurring due to PMA stimulus had a different between species. Both granulocytes and hyalinocytes of *H. hyotis* generated the smallest ($p < 0.05$) amount comparing to those of *S. kegaki* and *O. circumpicta*. Such difference of oxidative activity is thought to be specificity between species, which was controlled by each different pathway, and it is necessary to study the oxidative pathway through various inhibitor treatments to comprehend the pathway of oxidative activity. According to the result of inhibitor treatment by Donaghy et al. (2010), it was revealed that the oxidative activity of the hemocytes of the abalone *H. discus discus* and top shell *T. cornutus* is mostly oriented from mitochondria. Lambert et al. (2007) confirmed that both granulocytes and hyalinocytes possess NOX and NOX-like pathways to produce ROS/RNI

but the NOS pathway seemed more dominant in hyalinocytes whereas NOX was more active in granulocytes.

In this study, we first characterized the morphology and immune-related activities of hemocytes of subtropical oysters *S. kegaki*, *O. circumpicta* and *H. hyotis* inhabiting in Jeju Island using light microscopy and flow cytometry. In all oyster species, hemocytes in the hemolymph were identically classified into three major types: granulocytes, hyalinocyte, and blast-like cells. Granulocyte was identically the major hemocytes in charge of cell mediated immunity as the largest lysosome content, the most active phagocytosis activity and oxidative activity. The phagocytosis activity and oxidative activity were confirmed to have specificities between three oyster species. Phagocytic activity was the lowest in *S. kegaki* hemocytes and PMA-stimulated oxidative activity was the lowest in *H. hyotis* hemocytes. In the result of this study, we have provided the immunological basic data for efficient resource management of subtropical oyster that inhabits in Jeju Island, and it can be used as efficient biomarker for monitoring environmental change with understanding the changes of features and functions of hemocytes according to various environmental change or diseases.

IV. Effect of seasonal variation of environmental factors and reproduction on the hemocyte parameters in the subtropical oysters at Jeju Island: comparative study between intertidal oyster *Saccostrea kegaki* and subtidal oyster *Hyotissa hyotis*

4.1. Introduction

Environmental factors including temperature, salinity, nutrients, and toxicants modify the physiological condition of marine bivalve mollusks (see the review of Donaghy et al. 2009a). Cellular defense related parameters were suggested to be especially sensitive to variations in the environmental factors. Numerous laboratory-controlled studies have reported that extreme temperature and salinity generally induce the modifications of hemocyte parameters in marine bivalves (Fisher et al. 1987; Hégaret et al. 2003a,b; Reid et al. 2003; Liu et al. 2004; Chen et al. 2007; Matozzo et al. 2007; Monari et al. 2007; Yu et al. 2009). Cellular immune parameters are also changed by food quantity or quality (Delaporte et al. 2003, 2006, 2007a,b) as well as organic pollutants or heavy metals in the water column (Fisher et al. 1990 and 2000; Fournier et al. 2001; Matozzo et al. 2001; Oliver et al 2001; Gagnaire et al. 2004). Consequently, the health status of marine bivalves can be inferred from hemocyte immune parameters (see the review of Donaghy et al. 2009a).

Field surveys have also described that environmental variations induce alterations in hemocytes variables, but such variants are often inconsistent with no strong correlations between individual environmental conditions and hemocyte parameters (Soudant et al. 2004; Flye-Sainte-Marie et al. 2009). Internal factors such as reproduction may contribute to explain the high degree of variability of hemocyte responses and activities of individual

animals (Chu 2000; Donaghy et al. 2009a; Ellis et al. 2011). Reproductive investment in bivalves is usually associated with energy allocation for gametogenesis and spawning, at the expense of other physiological processes, including immune response (Cho and Jeong 2005; Li et al. 2010). Spawning is usually believed as one of the endogenous variables, through the necessary high energy investment resulting in an altered physiology of individuals (Soletchnik et al. 1997; Gosling 2003; Royer et al. 2008). Laboratory based studies indicate an influence of spawning on some hemocyte parameters. For instance, hemocyte concentration, phagocytosis activity and lysosomal membrane stability were reduced in post-spawning oysters *C. gigas* (Cho and Jeong 2005; Li et al. 2010). Field studies have also demonstrated seasonal variations of hemocyte variables concomitant with spawning periods (Soudant et al. 2004; Flye-Sainte-Marie et al. 2009; Fraser et al. 2013).

Bivalve mollusks growing in the intertidal zone are exposed to wide thermal fluctuations and cyclic air exposure while subtidal bivalve mollusks endure more stable thermal condition (Newell and Pye 1971; Carroll and Wells 1995; Dunphy et al. 2006). Therefore, intertidal bivalves are expected to show highly modified physiological response. The objective of this study was to understand the influence of environmental factors and reproductive activity on hemocyte parameters of the oysters in the field. To compare the difference of habitat conditions, we compared the seasonal variation in reproductive activity and hemocyte parameters of intertidal oyster *S. kegaki* and subtidal oyster *H. hyotis* from the coast of Jeju.

4.2. Materials and methods

Environmental parameters

Fig. 4-1 plots water temperature and salinity at the surface water and a depth of 10 m on the southern coast of Jeju recorded from March 2011 to February 2012. Surface water temperature was the lowest in March (14.8 °C) and the highest in September (22.9 °C) during a year. Throughout year except in June, the water temperature at a depth of 10 m was 0.4 to 2.8 degrees lower than the level at the surface water. The water temperature at a depth of 10 m ranged from 14.4 to 22.0 °C.

Salinity at the surface water ranged from 24.7 (August) to 31.2 psu (February) during the course of this study (Fig. 4-1B). At a depth of 10 m, the salinity was 3.3 to 6.7 degrees higher than the level of surface water during a year ranging from 31.3 to 34.5 psu (Fig. 4-1B).

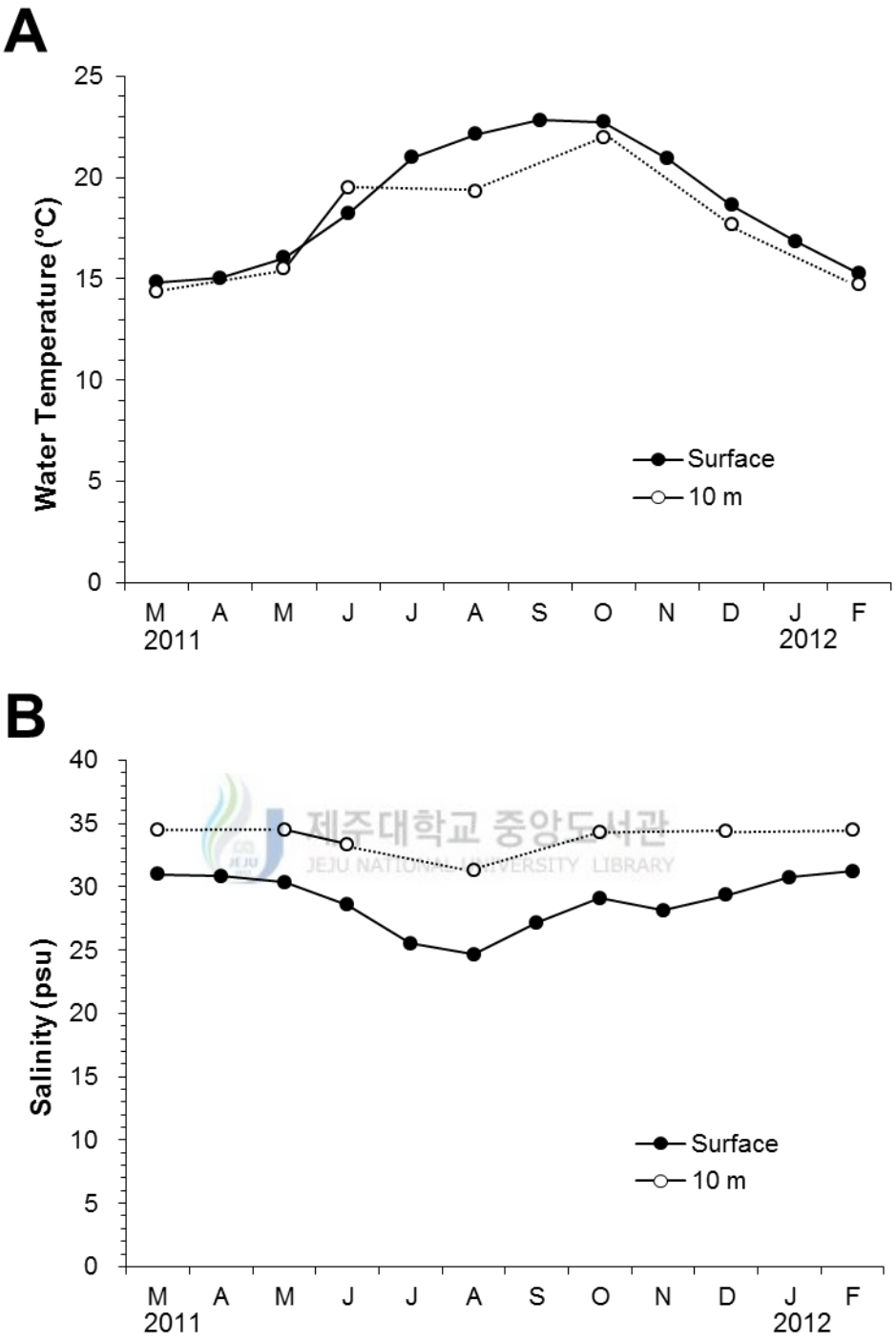


Fig. 4-1. Water temperature (A) and salinity (B) at the surface water and a depth of 10 m on the southern coast of Jeju.

The concentration of chlorophyll-*a* in the surface water and at depth of 10m tended to be higher when the water temperature was high (Table 4-1). Chlorophyll-*a* level in the surface water was relatively higher in August (2.23 μg/l) and November (1.68 μg/l) than May (0.87 μg/l) and February (0.87 μg/l). The level of chlorophyll-*a* at a depth of 10 m in August (1.01 μg/l) and November (1.10 μg/l) was lower than the value in the surface water. The concentration of chlorophyll-*a* at a depth of 10 m in May and February was 0.64 and 0.81 μg/l, respectively.

Table 4-1 Concentration of chlorophyll a at the sampling site during February 2011 to February 2012.

Year	Month	Chlorophyll-*a* (μg/l) concentration	
		Surface water	10 m depth
2011	May	0.87	0.64
	Aug	2.23	1.01
	Nov	1.68	1.10
2012	Feb	0.87	0.81

Sampling

Yerae-dong (33°14´N, 126°23´E) located at the southern coast of Jeju Island is a well-developed rocky intertidal area (Fig. 4-2). The oyster *S. kegaki* is heavily encrusting on lower rocky intertidal area along the coast of Yerae-dong and exposed to the air at low tide and submerged at high tide. Using chisel and hammer, the oysters *S. kegaki* attached on the rock were collected monthly from March 2011 to February 2012. Supseom (33°13´N, 126°35´E) is a small uninhabited island 500 m off the southern coast of Jeju (Fig. 4-1). Every month, from March 2011 to February 2012, the oyster *H. hyotis* were collected from subtidal rocky bottoms at depths of 5-15m of Supseom (33°13´N, 126°35´E), southern Jeju by scuba diving (Fig. 4-2).

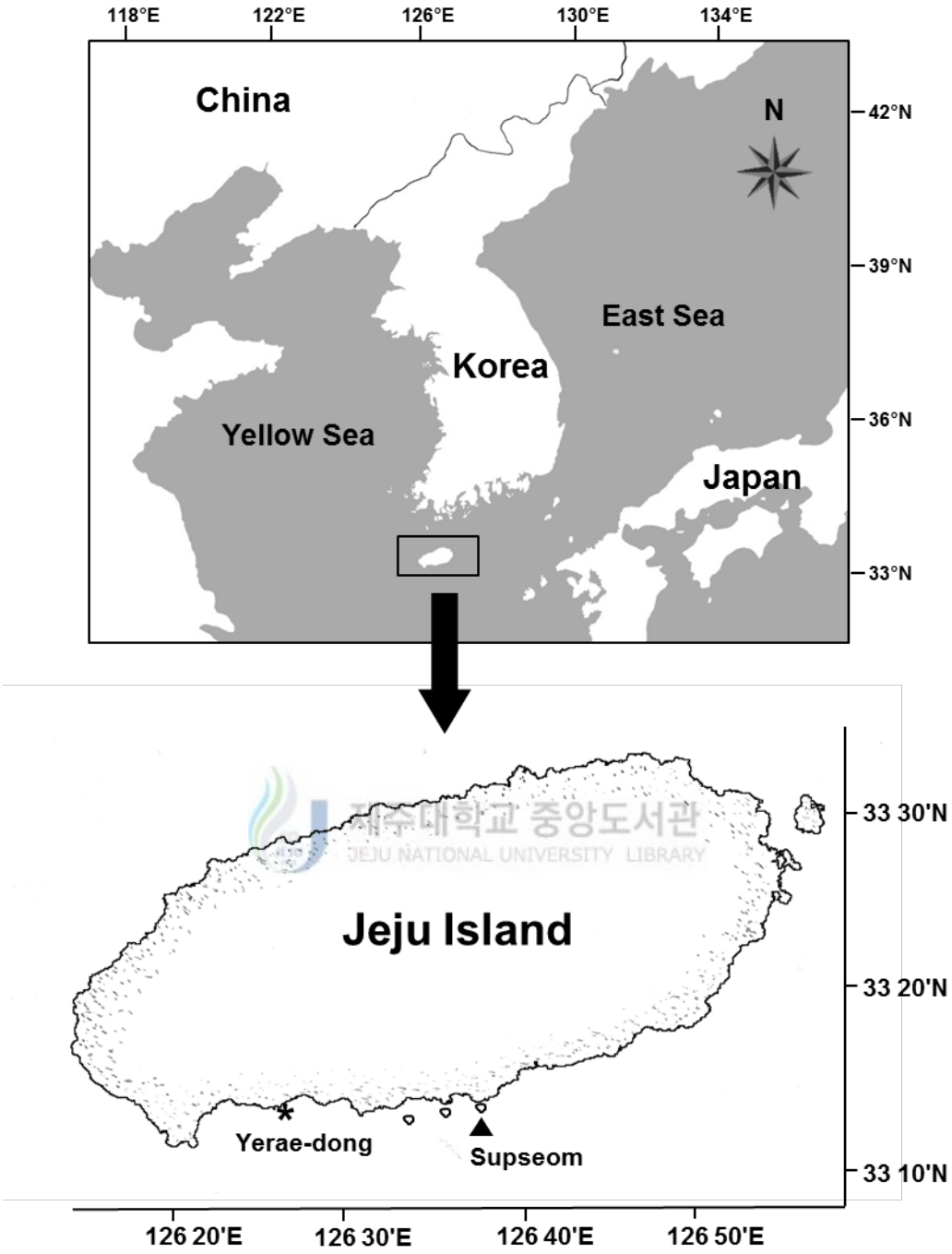


Fig. 4-2. Location map of the sampling sites. *Saccostrea kegaki* was collected from the inter-tidal area of Yerae-dong (⁎). *Hyotissa hyotis* were collected from subtidal rocky bottom at depth 5-15 m of Supseom (▲).

Oysters *S. kegaki* ranging from 4.6 to 5.4 cm in shell length (SL, the longest axis of the shell) and *H. hyotis* ranging from 6.6 to 13.6 cm in SL were selected for analysis each month (Table 4-2). In April 2011, *H. hyotis* could not be collected. Fig. 4-3 shows a flowchart of the experimental protocol used in this study. For hemocyte analyses, hemolymph was collected from the adductor muscle through a previously ground notch on the anterior end of the shell using a syringe fitted with a 22Gx1 1/4" needle. After the soft tissue was removed from shell, a thin slice (1.5 mm) was cut dorso-ventrally in the middle of the body for histological analysis. The remaining tissue was freeze-dried and used for further analyses of biochemical composition of oyster tissue. The shells were dried in air and weighted to determine the condition index (CI). The CI was calculated according to the following equation: CI = Tissue dry weight (TDWT, g) / Shell dry weight (SDWT, g) x 100.

Table 4-2 Shell length (SL), tissue wet weight (TWWT), and tissue dry weight (TDWT) of oysters *Saccostrea kegaki* and *Hyotissa hyotis* used in this study

Year	Month	*Saccostrea kegaki*				*Hyotissa hyotis*			
		N	SL (mm)	TWWT (g)	TDWT (g)	N	SL (mm)	TWWT (g)	TDWT (g)
2011	Mar	30	45.7 ± 0.8	1.142 ± 0.064	0.253 ± 0.014	19	66.2 ± 6.4	4.736 ± 0.994	1.012 ± 0.234
	Apr	30	50.1 ± 0.9	1.477 ± 0.093	0.304 ± 0.020	No sampling			
	May	30	54.0 ± 1.3	1.666 ± 0.059	0.387 ± 0.014	16	90.8 ± 13.4	10.144 ± 2.829	1.984 ± 0.556
	Jun	30	48.9 ± 0.8	1.562 ± 0.089	0.382 ± 0.023	30	131.6 ± 4.6	22.372 ± 1.267	5.553 ± 0.079
	Jul	30	54.0 ± 1.0	1.407 ± 0.065	0.330 ± 0.015	30	88.5 ± 15.5	5.302 ± 0.501	0.982 ± 0.093
	Aug	30	52.0 ± 1.1	1.189 ± 0.069	0.235 ± 0.013	18	118.1 ± 6.8	16.336 ± 1.779	3.472 ± 0.390
	Sep	30	50.4 ± 1.0	1.290 ± 0.076	0.224 ± 0.012	30	130.5 ± 3.2	19.451 ± 1.225	4.273 ± 0.277
	Oct	30	52.1 ± 1.1	1.371 ± 0.073	0.262 ± 0.013	30	123.7 ± 2.7	16.646 ± 0.690	3.516 ± 0.152
	Nov	30	53.0 ± 1.0	1.170 ± 0.056	0.240 ± 0.011	30	130.7 ± 2.1	17.924 ± 1.103	3.740 ± 0.248
	Dec	30	53.8 ± 1.0	1.780 ± 0.059	0.246 ± 0.013	30	131.3 ± 2.7	22.427 ± 1.253	4.699 ± 0.286
2012	Jan	30	50.6 ± 0.9	1.254 ± 0.077	0.257 ± 0.015	30	118.1 ± 2.2	16.879 ± 1.216	3.582 ± 0.271
	Feb	30	52.9 ± 0.9	1.357 ± 0.073	0.290 ± 0.018	30	131.3 ± 2.7	22.622 ± 1.383	4.718 ± 0.299

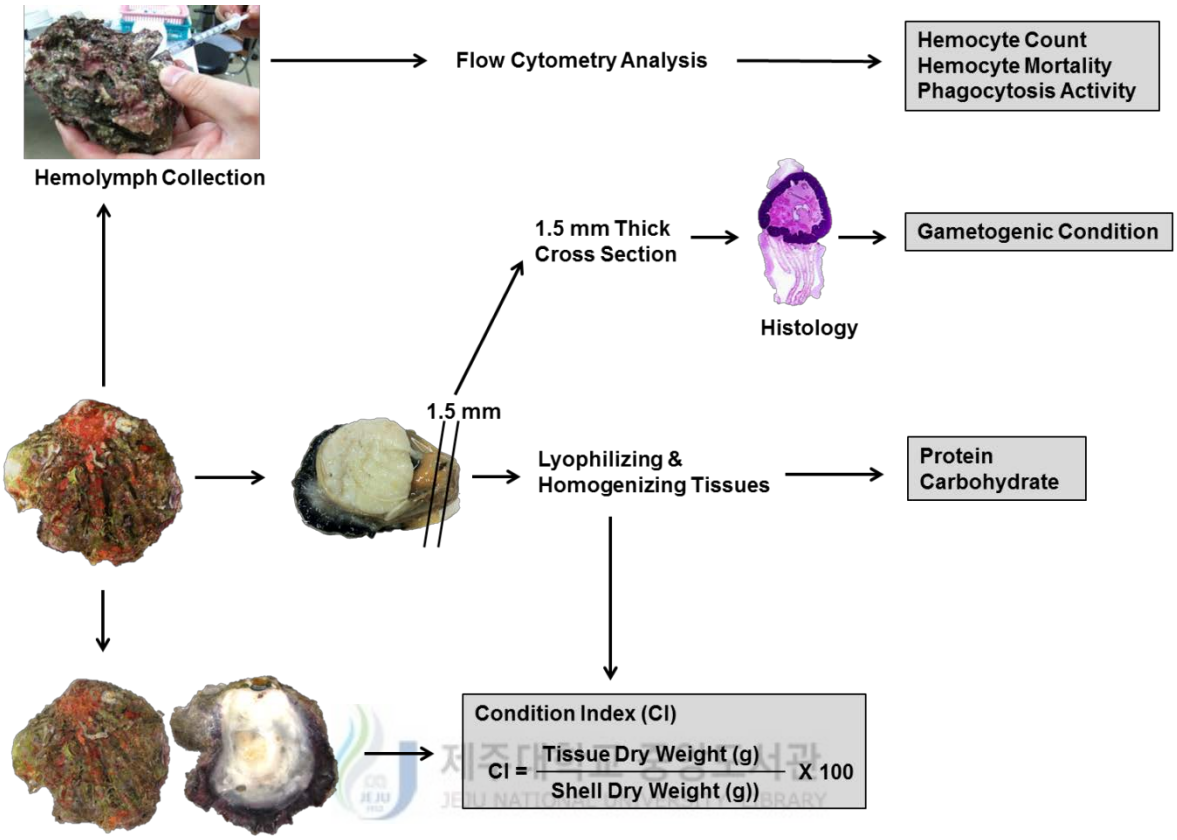


Fig. 4-3. Flowchart of the experimental protocol used in this study.

Hemocyte analyses

Hemolymph collection

Collected hemolymph was filtered through a 60 μm nylon mesh and directly transferred into microtubes maintained on ice in order to minimize cell aggregation. Hemolymph from six oysters was pooled in one tube to obtain sufficient amount of hemolymph for the flow cytometry analysis. Five replicates were prepared from each month. Hemocyte parameters including hemocyte count, mortality, and phagocytosis activity were analyzed using a FACS Calibur flow cytometry (Becton-Dickinson, USA) and protocol were adopted from Donaghy et al. (2009b).

Total hemocyte count and percentage of each hemocyte types

Total hemocyte count (THC) and percentage of each hemocyte types was determined using a SYBR green I (Sigma-Aldrich, USA), a fluorescent DNA binding dye. A 150 μl of hemolymph was fixed with an equal volume of 3% formalin. Fixed hemocytes were then stained with 3 μl of 1,000x SYBR green I for 90 min in the dark at room temperature. Hemocytes stained with SYBR green I was selected on green fluorescence detector of flow cytometer. Hemocyte subpopulation was classified based on their relative size and internal complexity. From the total hemocyte count obtained from the flow cytometer, percentage of the each hemocyte populations was calculated using the computer software Win MDI.

Hemocyte mortality

Hemocyte mortality was measured using a propidium iodide (PI; Sigma-Aldrich, USA), a fluorescent DNA binding dye, which selectively stains the nucleic acids of the dead

hemocytes. A 150 µl of hemolymph was mixed with an equal volume of an anti-aggregant solution (AASH, 2.5% NaCl, 1.5% EDTA in 0.1 M phosphate buffer, pH 7.4) to avoid the cell aggregation. The hemocytes were mixed with 3 µl of 2 mg/ml PI solution and incubated for 10 min in the dark at room temperature. Proportion of the dead and live hemocytes was then calculated to estimate the mortality, from the hemocyte cell counts obtained from the red fluorescence detector equipped in the flow cytometer.

Phagocytosis

Phagocytosis activity of hemocyte was evaluated using a fluorescent bead (2.0 µm in diameter, Polysciences Inc., USA). A 150 µl of oyster hemolymph was mixed with the equal volume of 2% fluorescent bead diluted in sterile seawater. Phagocytosis of hemocytes was induced for 2 h in the dark at room temperature. Phagocytosis activity was represented by the percentage of phagocytic hemocytes that engulfed at least three beads among the whole hemocytes on the green fluorescence detector of flow cytometer (Donaghy et al. 2009b).

Reproductive condition

The dorso-ventral section were fixed in Davidson's fixative, dehydrated in a graded series of alcohol, and embedded in paraffin. The paraffin blocks were sliced to 6 µm and stained with Harris's haematoxylene and eosin Y. After mounting, the gametogenic condition of each oyster was determined based on microscopic gonad appearance. The gonad maturity of oysters was categorized into six stages using the maturity scale; 1) indifferent stage, 2) early development, 3) late development, 4) ripe and ready for spawning, 5) partially spawning and 6) spent/resorbing (Fig. 4-4, 4-5, 4-6, 4-7).

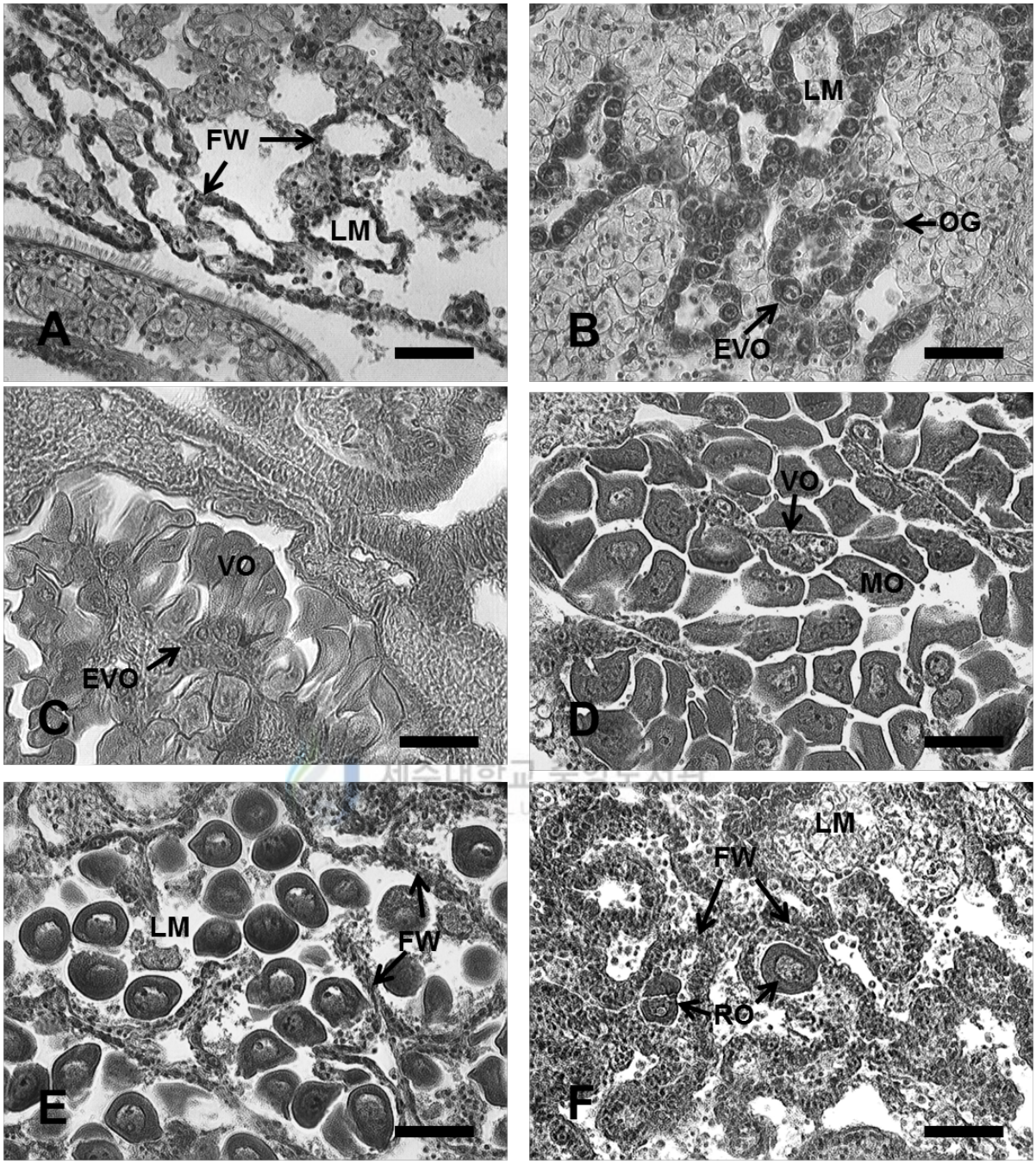


Fig. 4-4. Photomicrographs of ovaries of *Saccostrea kegaki*. (A) Indifferent stage: gonad with vacated lumen (LM) and thin follicle wall (FW). (B) Early developing stage: ovary containing small oogonia (OG) and early vitellogenic oocytes (EVO) occur along the follicle wall. (C) Late developing stage: growing ovary with early vitellogenic oocytes (EVO) and vitellogenic oocytes (VO). (D) Ripe stage: mature ovaries packed with ova and a thin layer along follicle wall. In germinal epithelium, very small primary oocytes are present. (E) Partially spawning stage: partly spawned ovaries with loosely packed ova and vacated space. (F) Spent stage: ovaries largely devoid of ova and growing ovary with vitellogenic oocytes and relict ova (RO). Scale bar: 50 μm.

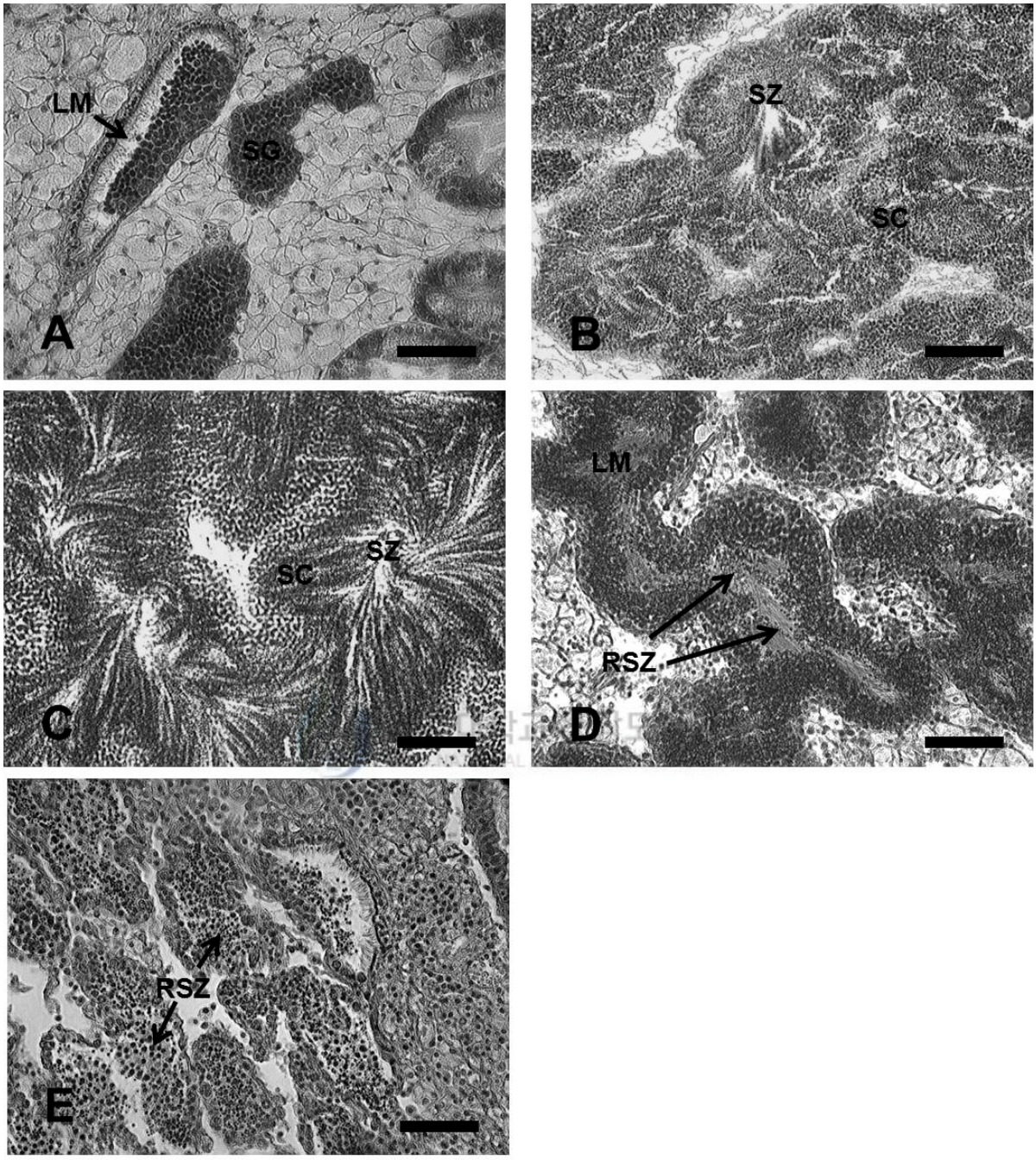


Fig. 4-5. Photomicrographs of testis of *Saccostrea kegaki*. (A) Early developing stage of testis exhibiting spermatogonia (SG). (B) Late developing stage: columns of SC and spermatozoa (SZ) move to the central part of growing testes. (C) Ripe stage: mature testes filled with SZ. (D) Spawning stage: partly spawned testes with vacated space. (E) Spent stage: partly devoid follicles with relict spermatozoa (RSZ). Scale bar: 50 μm. LM: lumen, FW: follicle wall

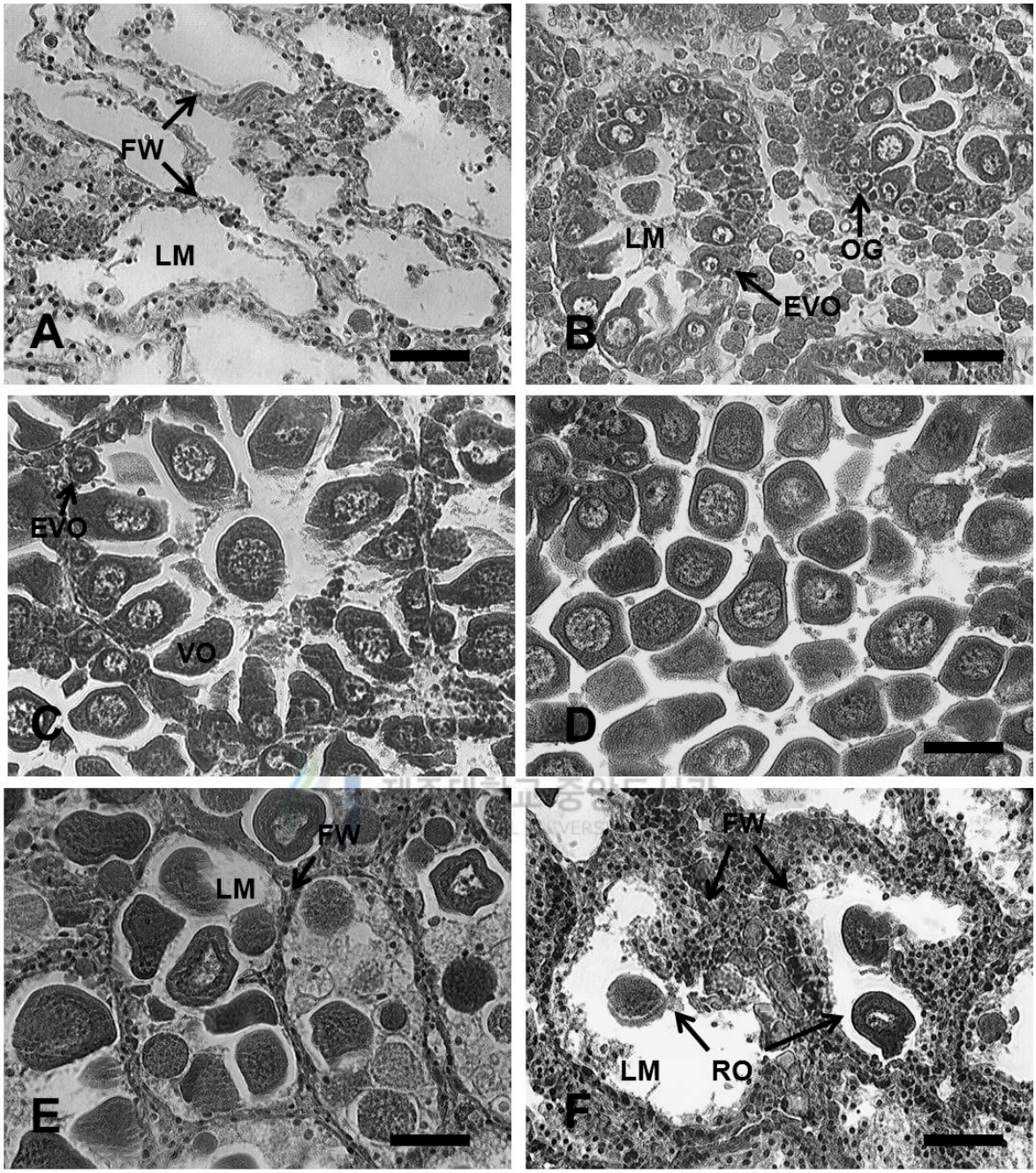


Fig. 4-6. Photomicrographs of ovaries of *Hyotissa hyotis*. (A) Indifferent stage: gonad with vacated lumen (LM) and thin follicle wall (FW). (B) Early developing stage: ovary containing small oogonia (OG) and early vitellogenic oocytes (EVO) occur along the follicle wall. (C) Late developing stage: growing ovary with early vitellogenic oocytes (EVO) and vitellogenic oocytes (VO). (D) Ripe stage: mature ovaries packed with ova and a thin layer along follicle wall. In germinal epithelium, very small primary oocytes are present. (E) Partially spawning stage: partly spawned ovaries with loosely packed ova and vacated space. (F) Spent stage: ovaries largely devoid of ova and growing ovary with vitellogenic oocytes and relict ova (RO). Scale bar: 50 μm.

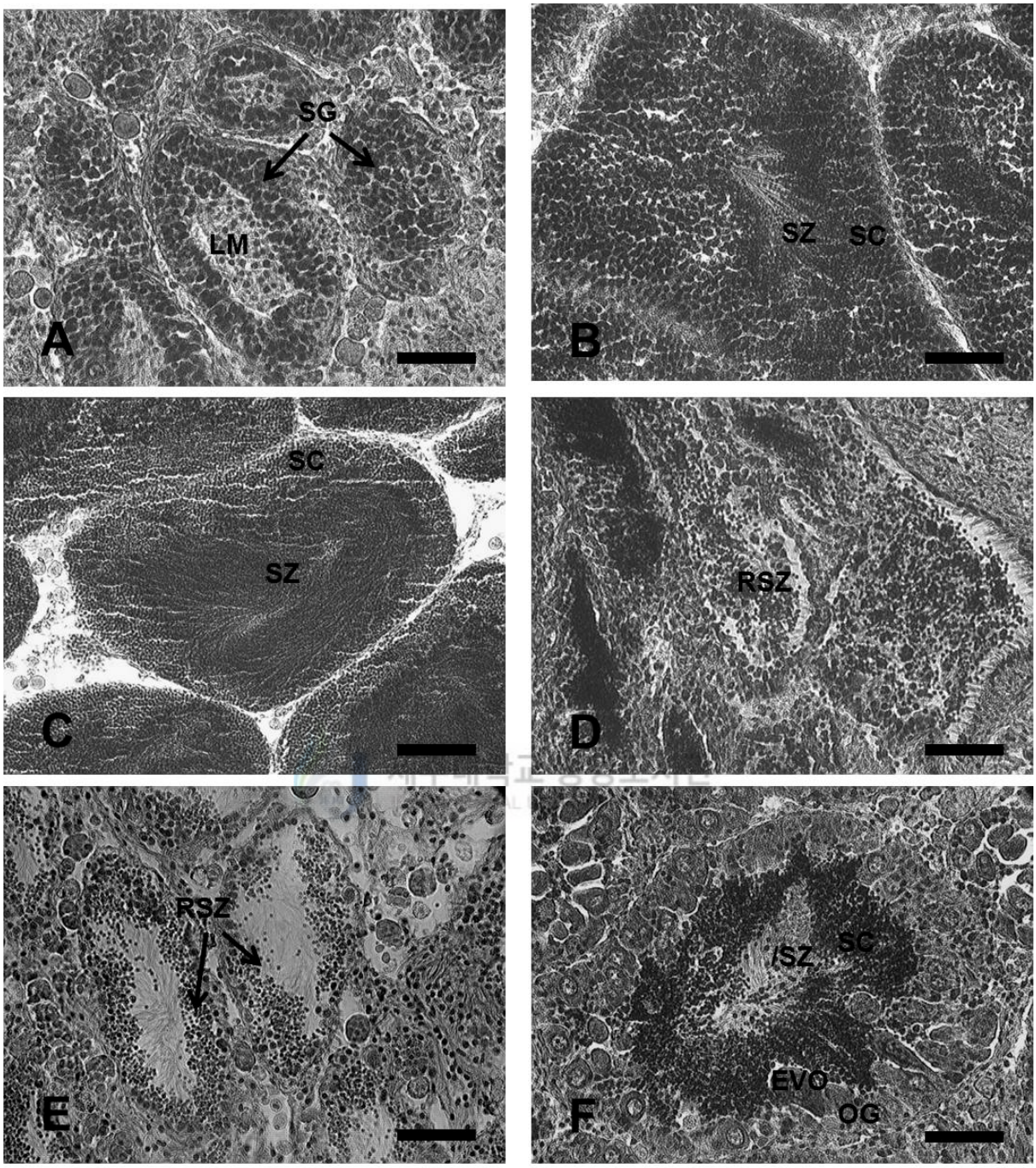


Fig. 4-7. Photomicrographs of testis of *Hyotissa hyotis*. (A) Early developing stage of testis exhibiting spermatogonia (SG). (B) Late developing stage: columns of SC and spermatozoa (SZ) move to the central part of growing testes. (C) Ripe stage: mature testes filled with SZ. (D) Spawning stage: partly spawned testes with vacated space. (E) Spent stage: partly devoid follicles with relict spermatozoa (RSZ). (F) Hermaphrodite. Scale bar: 50 um. LM: lumen, FW: follicle wall

Biochemical composition of tissue

Freeze dried oyster tissue was homogenized using mortar and pestle. Total protein in the oyster tissue was assessed using the colorimetric method described by Lowry et al. (1951), after extraction with 0.1 M NaOH at 37 °C for 3 h with bovine serum albumin as the standard material. Total carbohydrate level in tissue was determined using the phenol-sulfuric acid method as described by Dubois et al. (1956) with dextrose anhydrous as the standard material.

Statistical analysis

Statistical analysis was performed using SigmaPlot Version 12.5 (Systat Software Inc., Germany). Significance of variations of mean values among sampling month was tested using Kruskal-Wallis test. Relationships between environmental factors and hemocyte parameters were assessed by the mean of linear models. Significant was characterized as $P<0.05$.

4.3. Results

Hemocyte parameters

Total Hemocyte Count (THC)

Seasonal variation of hemocyte density in hemolymph of intertidal oysters *S. kegaki* and subtidal oysters *H. hyotis* from March 2011 to February 2012 is shown in Fig. 4-8. THC in hemolymph of intertidal oysters *S. kegaki* strongly fluctuated during a year. The level varied from 1.9 x 10^5 to 1.7 x 10^6 cells ml^{-1} along the sampling period and two major peaks were observed in May (9.3 x 10^5 cells ml^{-1}) and July (1.7 x 10^6 cells ml^{-1}).

In contrast, THC in hemolymph of subtidal oysters *H. hyotis* ranged from 1.4 x 10^5 to 5.9 x 10^5 cells ml^{-1} without remarkable seasonal fluctuation throughout the year (Fig. 4-8). The mean values of THC in *H. hyotis* hemolymph, except for October and November, were relatively lower than the levels in *S. kegaki* hemolymph.

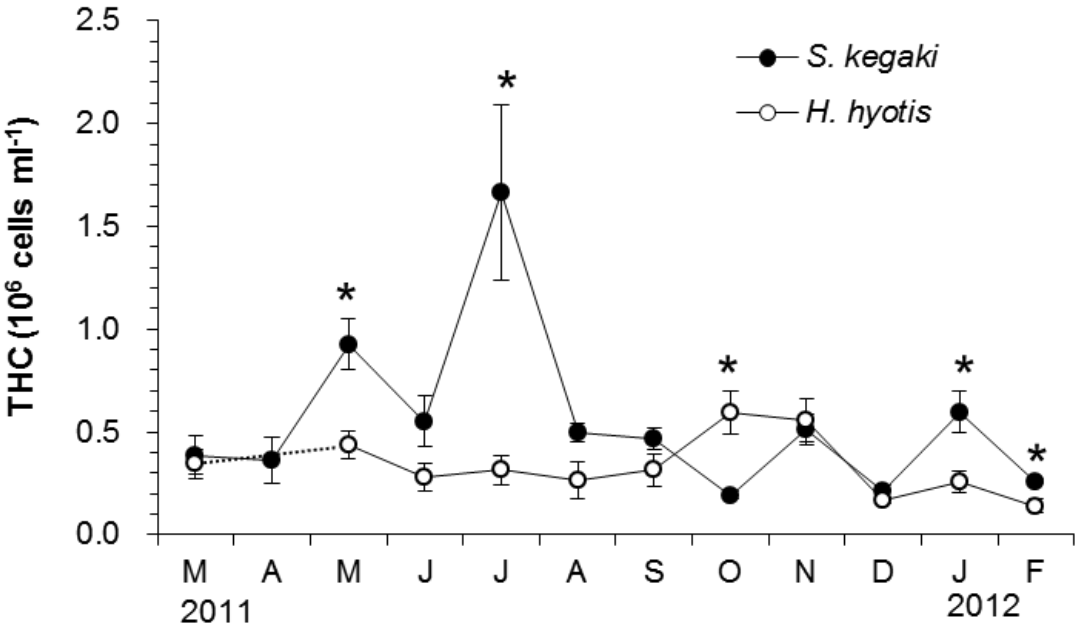


Fig. 4-8. Seasonal variation of total hemocyte count (THC) of *Saccostrea kegaki* and *Hyotissa hyotis* collected from the southern coast of Jeju. Data was expressed as mean ± SE. Asterisk (*) indicate statistically significant difference of THC between *S. kegaki* and *H. hyotis* each month ($P<0.05$).

Percentage of each hemocyte types

Fig. 4-9 shows the frequency of three hemocyte types including granulocytes, hyalinocyte, and blast-like cells of *S. kegaki* and *H. hyotis* from March 2011 to February 2012. In intertidal oyster *S. kegaki*, hyalinocytes were the most abundant cells in hemolymph accounting for from 47.6 to 81.4% (Fig. 4-9A). The proportion of hyalinocytes in hemolymph of *S. kegaki* was significantly ($P<0.05$) decreased from May (65.2%) to June (47.6%), then increased again to 62.2% in July. The highest proportion of hyalinocytes in hemolymph of *S. kegaki* was recorded 81.4% in September, and the level decreased to 51.1% in November. Proportion of granulocytes in hemolymph of *S. kegaki* was varied from 9.6 to 26.4% (Fig. 4-9A). The values were declined by half from 19% in June to 9.6% in July, and then increased more than two times to 21.6% in August. Blast-like cells were the lowest cell group accounting for from 5% in September to 15.8% in August except in June (34%), July (28.8%), and November (28.7%; Fig. 3-9A). The period that blast-like cells have an unusually high proportion in June, July and November coincided with decline in granulocytes or hyalinocytes. Pearson correlation test indicated that proportion between granulocytes and blast-like cells (Correlation coefficient=-0.41, $P<0.001$) and between hyalinocytes and blast-like cells (Correlation coefficient=-0.736, $P<0.0001$) were negatively correlated. These data suggested that stem cells (blast-like cells) proliferated in these periods and mature hemocytes such as granulocytes and hyalinocytes decreased.

Represented major group in *H. hyotis* was also hyalinocytes during a year (Fig. 4-9B). The percentage of hyalinocytes in *H. hyotis* hemolymph ranged from 64.4% (September) to 85.4% (January) without significant difference during a year. Granulocyte proportion in hemolymph of *H. hyotis* was constant within a range of 18 to 27% from March to August,

and significantly ($P<0.05$) increases to 30.5% in September (Fig. 4-5B). The percentage of granulocytes in *H. hyotis* hemolymph remained high from September (30.5%) to November (32.2%) then significantly ($P<0.05$) decreased in December (17.8%). Compared with *S. kegaki*, the proportion of blast-like cells in *H. hyotis* hemolymph did not strongly fluctuate and ranged from 1.8 (January) to 8.4% (May) during a year (Fig 4-9B). From the Person correlation test, positive correlation between granulocytes and hyalinocytes proportion (Correlation coefficient=0.37, $P<0.001$) and negative correlation between hyalinocytes and blast-like cell proportion (Correlation coefficient=-0.48, $P<0.0001$) was observed.

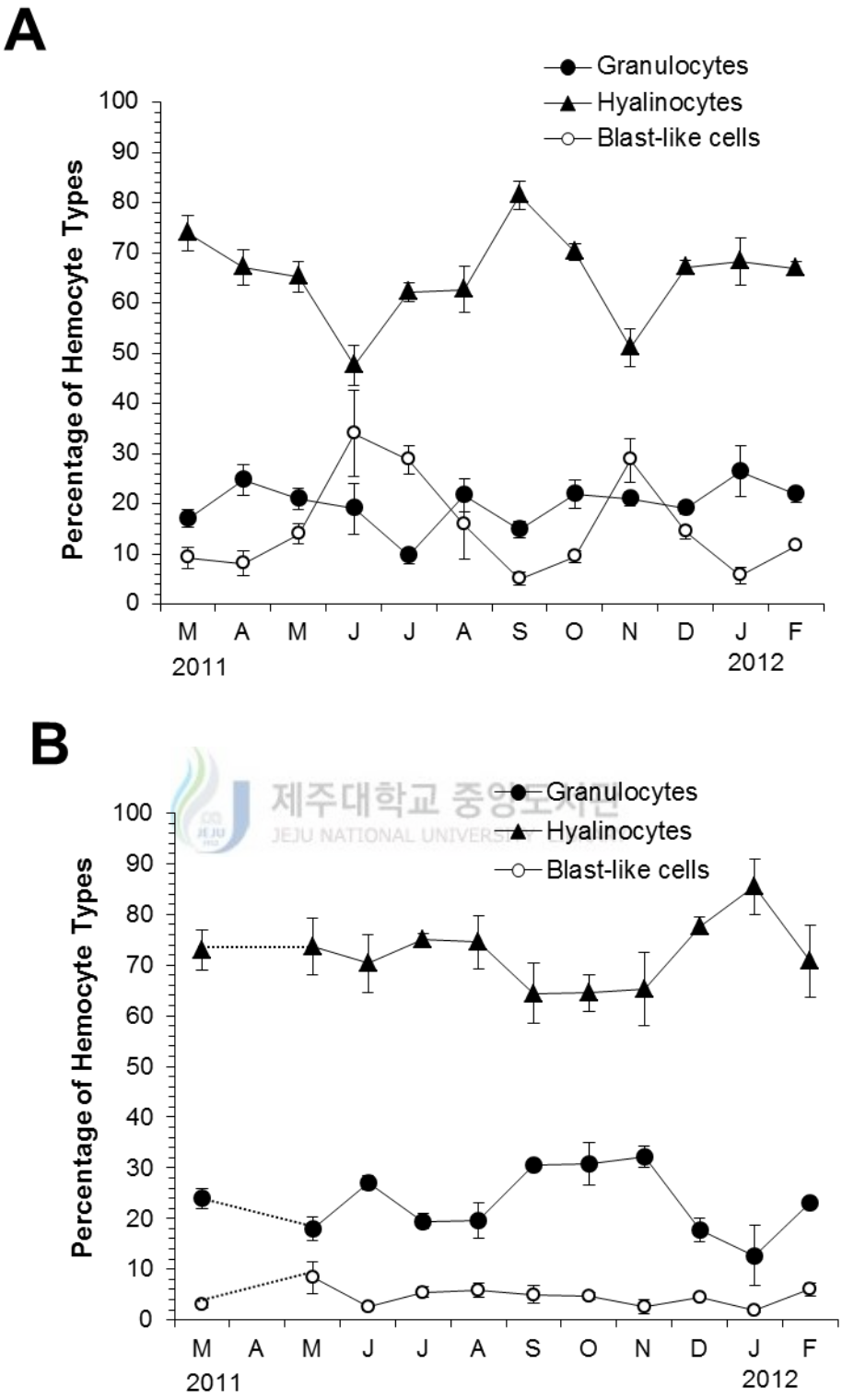


Fig. 4-9. Percentage of each hemocyte types of *S. kegaki* (A) and *H. hyotis* (B) collected from the southern coast of Jeju during a year. Data was expressed as mean ± SE.

Hemocyte mortality

Hemocyte mortality in oysters *S. kegaki* and *H. hyotis* varied with the sampling period (Fig. 4-10). The values in intertidal oyster *S. kegaki* varied between a minimum in May (2.7%) and a maximum in July (10.2%). A significant isolated peak value was observed in October (23%).

For subtidal oyster *H. hyotis*, the hemocyte mortality was stable from March (5.5%) to August (8.1%). The percentage of dead hemocytes of *H. hyotis* dramatically increased from August to October (25.8%) and then reduced to the level before the increase. In both oysters, the percentage of dead cells were dramatically increased in post-spawning periods (Fig. 3-12) in September and October (Fig. 4-10).

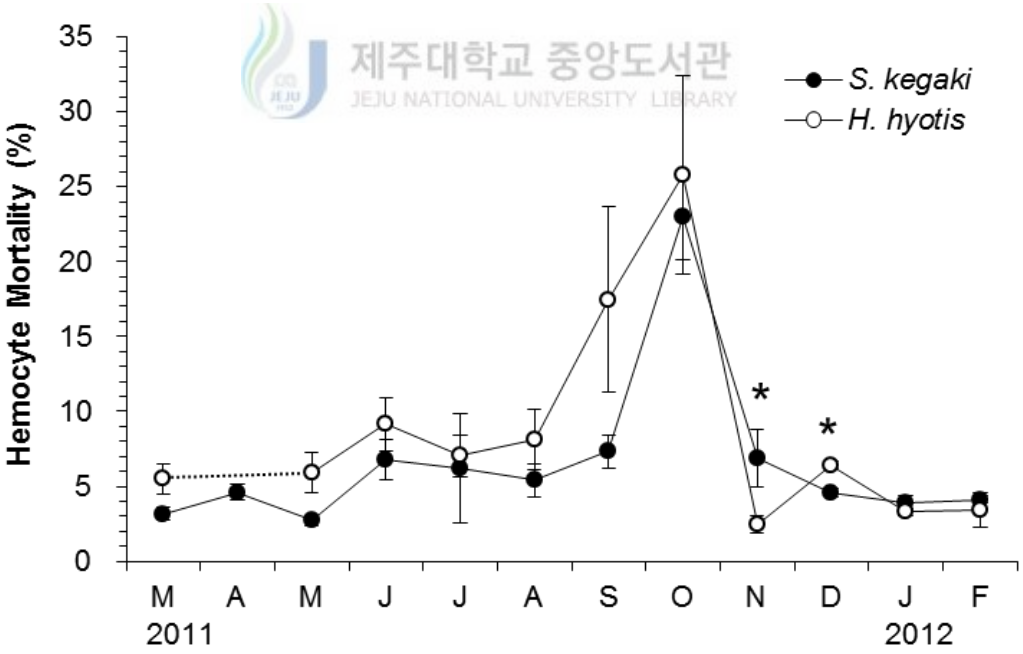


Fig. 4-10. Hemocyte mortality of *S. kegaki* and *H. hyotis* collected from the southern coast of Jeju during a year. Data was expressed as mean ± SE. Asterisk (*) indicate the statistically significant difference of the value between *S. kegaki* and *H. hyotis* each month ($P<0.05$).

Phagocytosis capacity

Fig. 4-11 plots phagocytosis capacity of hemocytes in intertidal oysters *S. kegaki* and subtidal oysters *H. hyotis* from March 2011 to February 2012. Phagocytosis capacity of *S. kegaki* hemocytes showed a broad seasonal variation. The values significantly ($P<0.05$) increased from April (43.6%) to May (65.9%), then remained high from May to September (72.8%). The level of phagocytosis capacity of *S. kegaki* hemocytes declined more than three times during post-spawning periods (Fig. 4-12) from September to February (23.1%).

The phagocytosis capacity of *H. hyotis* hemocytes represented similar pattern with that of *S. kegaki* hemocytes but it was less varying and not significantly different from March (45.9%) to September (55.6%). Same as the results of *S. kegaki*, the phagocytosis capacity of *H. hyotis* hemocytes decreased up to two-fold during post-spawning period (Fig. 4-12) from September to December (27.4%). From December to February, in contrast to the result of *S. kegaki* hemocytes, the phagocytosis capacity of *H. hyotis* significantly ($P<0.05$) increased up to 48.0%.

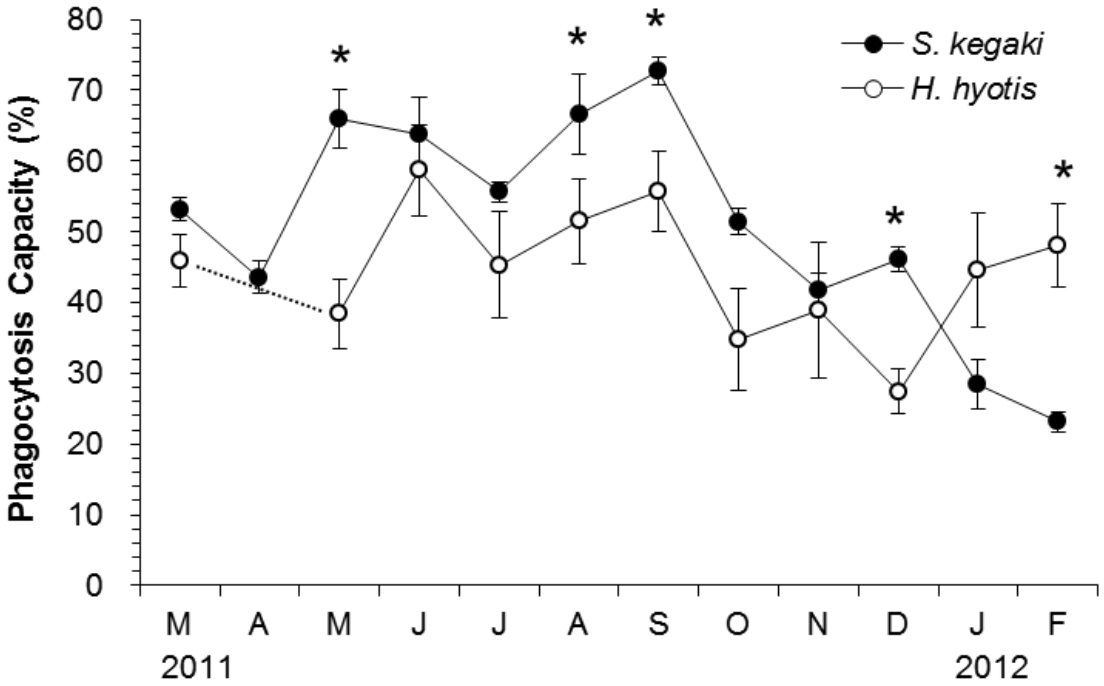


Fig. 4-11. Phagocytosis capacity of hemocytes of *S. kegaki* and *H. hyotis* collected from the southern coast of Jeju during a year. Data was expressed as mean ± SE. Asterisk (*) indicate the statistically significant difference of the values between *S. kegaki* and *H. hyotis* each month ($P<0.05$).

Gamotogenesis

Fig. 4-12 shows frequency distribution of the different gonad development stages in oysters *S. kegaki* and *H. hyotis* from March 2011 to February 2012. Histology indicated that gametogenesis of male and female intertidal oyster *S. kegaki* commenced in March and April respectively when the water temperature reached 14.8-15.1°C. *S. kegaki* in late development stage were dominant in June (63.6% male and 56.3% female). Ripe *S. kegaki* were first observed in June (23.1% male and 43.8% female), and the proportion of *S. kegaki* in the ripe stage increased from June to July (63.6% male and 100% female). Major spawning in both male and female of *S. kegaki* was observed in August (61.5% male and 82.4% female) when surface water temperature was 22.1 °C. Spawning activity of *S. kegaki* was continuous from August to November. Male *S. kegaki* in the spent/resorbing stage were observed in from September (5%) to February (6.7%) while female *S. kegaki* from September (23.1%) to November (13%) were in spent/resorbing stage. Sexually indeterminate individuals (resting stage) were evident during September to May.

For subtidal oyster *H. hyotis*, early developing testes could be observed from February (14.3%) when water temperature at a depth of 10m was 14.7 °C (Fig. 4-12). In contrast, the oogenesis tended to be late for about a couple of month compared with those of *S. kegaki* (Fig. 4-12). The number of small oocyte that developed on the follicle wall observed from June (25%) when water temperature reached 19.5 °C. The proliferation of spermatogonia and oogonia continued until August. The fully matured sperm and egg were dominant in August (59% male and 66.7% female) and September (69.2% male and 68.8% female). First spawning activity of *H. hyotis* was delayed a month compared with the spawning period of intertidal oyster *S. kegaki*. Male and female of subtidal oyster *H. hyotis*

initiated spawning in September (15.4% male and 25% female) at the same time when water temperature reached 21-22 °C and continued discharging spermatozoa and oocyte until December and November, respectively. The spent/resorbing stage was observed from September (5.9%) to March (42.9%) in male *H. hyotis* and October (15%) to March (14.3%) in female *H. hyotis*. In both male and female *H. hyotis*, resting stage were examined throughout the year even during the summer period (June to August).

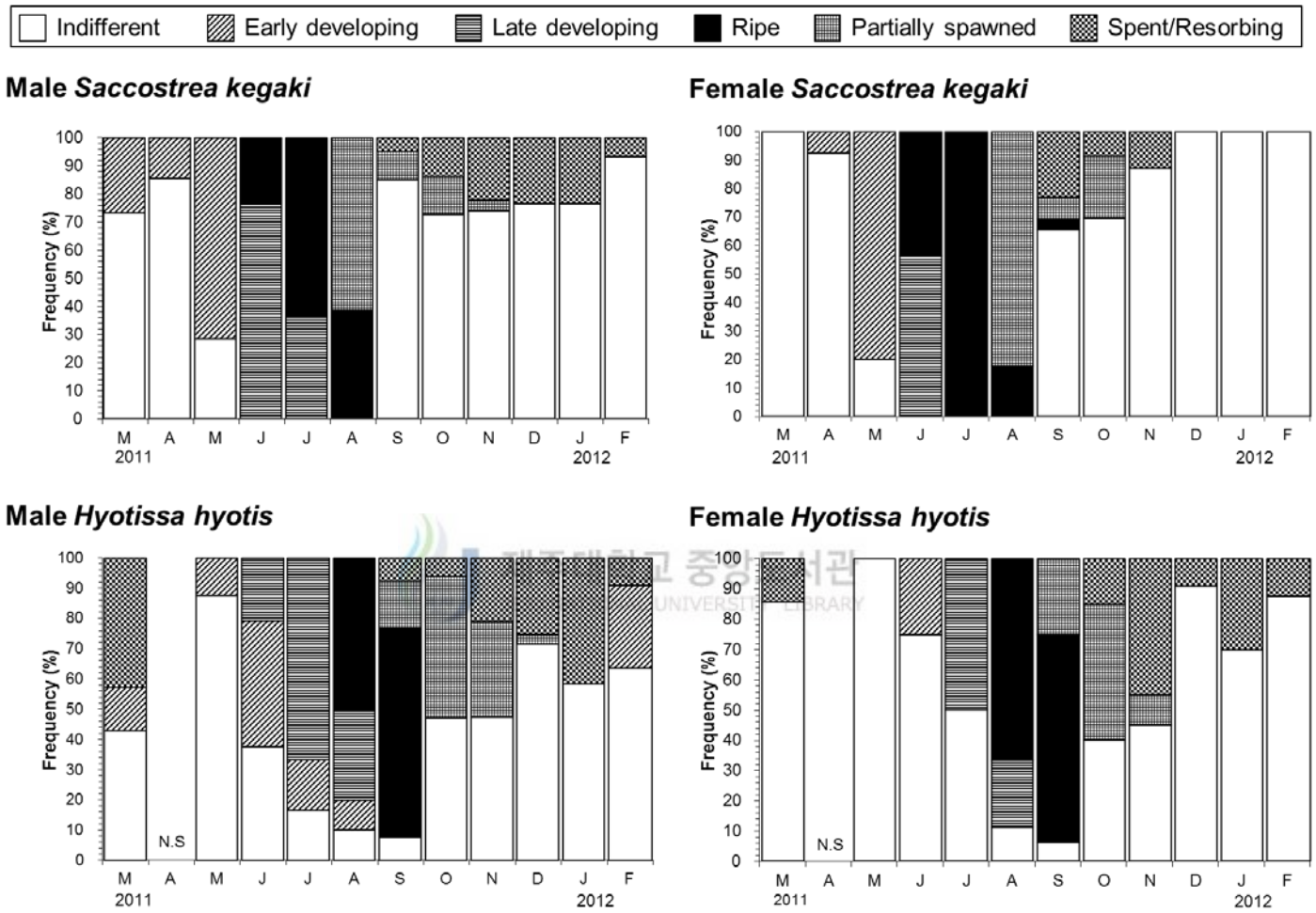


Fig. 4-12. Monthly variation in the frequency distribution of *Saccostrea kegaki* and *Hyotissa hyotis* at each gametogenic stage determined by histology. N.S., not sampled.

Biochemical composition

Total protein

Protein level in total dry tissue of *S. kegaki* tended to increase when gametes proliferated and ready for spawning (Fig. 4-13A). The level of protein in total dry tissue of *S. kegaki* continuously increased from May (284.3 mg/g TDWT; early developing stage) to September (387.7 mg/g TDWT; fully ripe and partially spawned stage). From October to May, in contrast, the protein level was considerably low in the range of 284.3-363.8 mg/g TDWT. This period coincided with most of oysters in resting stage after completion of spawning. On the other hand, the protein levels in *H. hyotis* tissue did not show clear seasonality and correlation with reproduction of oyster (4-13A). The mean values ranged from 383.8 mg/g TDWT in September to 439.8 mg/g TDWT in January.

Total carbohydrate

Total carbohydrate levels in total dry tissue of *S. kegaki* were strongly fluctuated during a year (4-13B). The levels sharply increased from January (61.1 mg/TDWT) to April (225.0 mg/g TDWT), then declined dramatically from April to August (21.9 mg/g TDWT) when gametes of oysters *S. kegaki* were developing and fully matured. Carbohydrates in soft tissue of *S. kegaki* kept constant at low levels from August to December with ranging from 21.9 to 49.6 mg/g (4-13B). For subtidal oyster *H. hyotis*, total carbohydrate contents in total dry tissue was stable without clear variation ranging from 6.8 to 21.9 mg/g TDWT (4-13B).

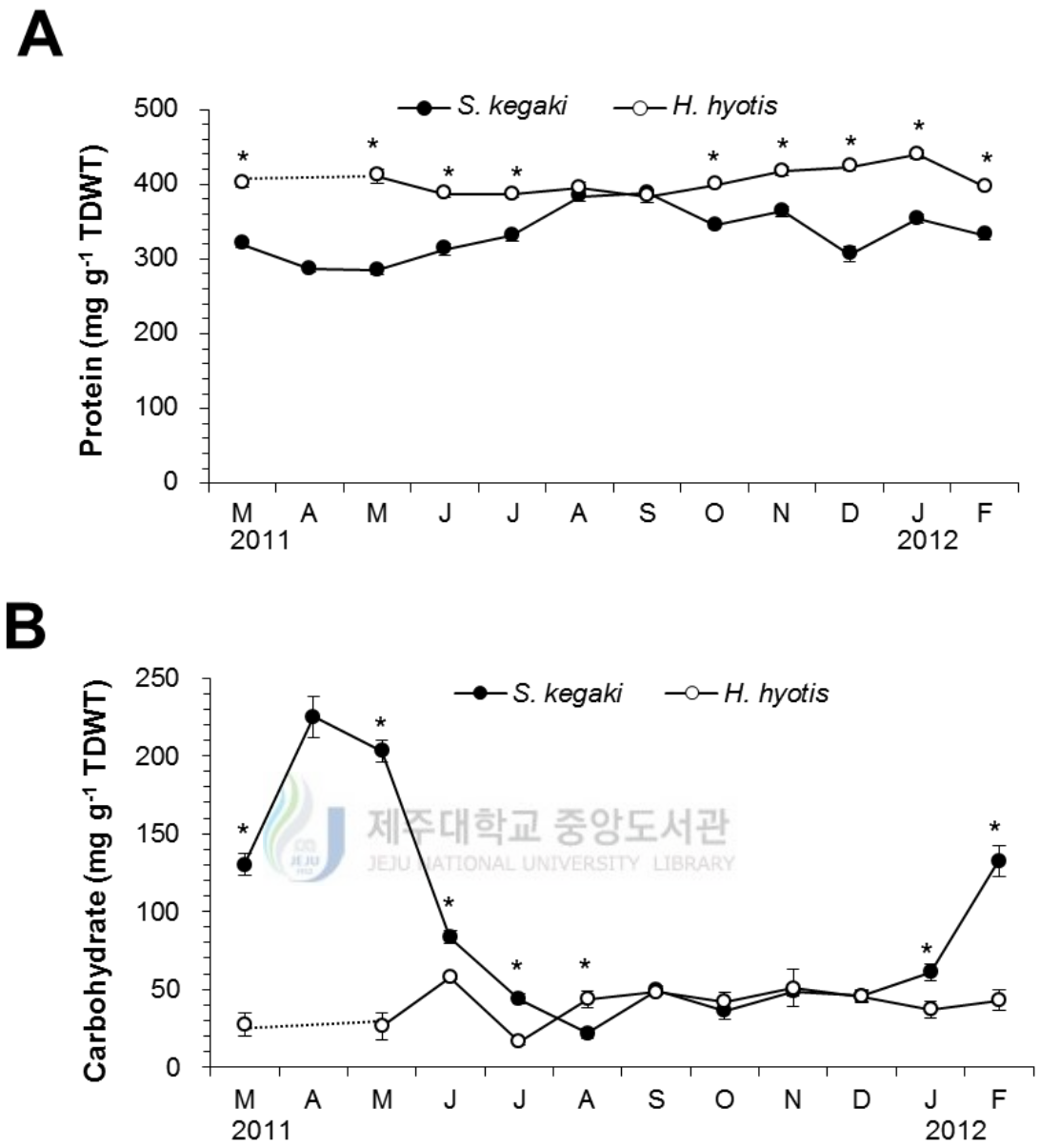


Fig. 4-13. Total protein (A) and carbohydrate (B) contents in dry tissue of *Saccostrea kegaki* and *Hyotissa hyotis* collected from the southern coast of Jeju during a year. Data was expressed as mean ± SE. Asterisk (*) indicate the statistically significant difference between *S. kegaki* and *H. hyotis* (P<0.05).

Condition Index (CI)

CI of the *S. kegaki* exhibited a clear seasonal variation, increasing progressively from March (2.8) to June (3.9) then declined dramatically from June to August (2.0; Fig. 4-14). From August to February, the CI of *S. kegaki* was stable without statistically significant different ranging from 2.0 to 2.4.

In contrast, the CI of *H. hyotis* displayed considerably (P<0.05) low values than that of *S. kegaki*, ranging from 1.1 to 1.9 throughout the year excluding August (Fig. 4-14). A significant (P<0.05) isolated peak value was observed in August (1.9) when most of *H. hyotis* gametes were in late developing and fully matured.

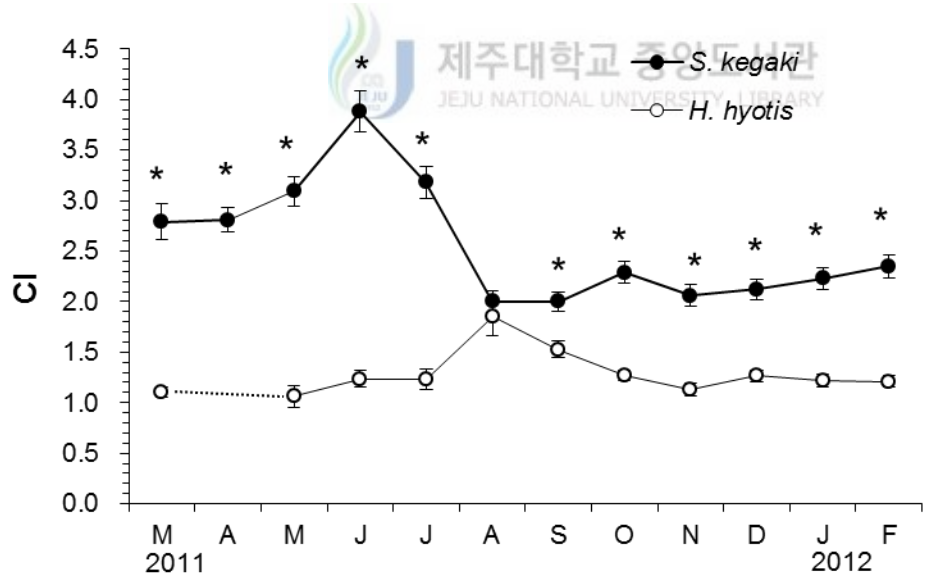


Fig. 4-14. Monthly variation of Condition index (CI) of *Saccostrea kegaki* and *Hyotissa hyotis* collected from the southern coast of Jeju during a year. Data was expressed as mean ± SE. Asterisk (*) indicate the statistically significant difference between *S. kegaki* and *H. hyotis* (P<0.05).

4.4. Discussion

Effect of environmental factors on hemocyte parameters

In the present study, the hemocyte density and proportion of each hemocyte types strongly fluctuated in intertidal oyster *S. kegaki* while it was stable in subtidal oyster *H. hyotis* during a year (Fig. 4-8 and Fig. 4-9). Temperature has usually been the main environmental factor modulating hemocyte parameters of marine bivalves (see the review of Donaghy et al. 2009a). Numerous studies have reported the positive effect of temperature on hemocyte concentration in hemolymph in various bivalves including oyster *Crassostrea virginica* (Chu and La Peyre 1993; Fisher et al. 1996), clam *Chamelea gallina* (Monari et al. 2007), *Mactra veneriformis* (Yu et al. 2009) and scallop *Chlamys farreri* (Lin et al. 2012). This phenomenon was also observed in the field studies of oyster *Crassostrea virginica* (Fisher et al. 1996), mussel *Mytilus galloprovincialis* (Carballal et al. 1998), and clam *Ruditapes philippinarum* (Soudant et al. 2004; Flye-Sainte-Marie et al. 2009). In this study, a linear model described that the water temperature positively affected on THC of intertidal oyster *S. kegaki* (Table 4-3) while no significant effect on subtidal oyster *H. hyotis*. Especially, the THC of intertidal oyster *S. kegaki* was considerably higher during summer period from May to July (Fig. 4-8). For the intertidal species, even during high tide, water column is short and water temperature increase quickly during summer. Actually, the air temperature in study site during summer period increased up to 35.1°C which is 10°C higher than water temperature. Several studies have reported that the circulating hemocytes of marine bivalves significantly increase by sudden increase in water temperatures (Monari et al. 2007; Yu et al. 2009; Lin et al. 2012) as well as air exposure (Park et al. 2012).

Table 4-3. Relationships between environmental factors and hemocyte parameters of oysters tested using linear models. NS, not significant (P-value > 0.05)

	Hemocyte parameters	Temperature (°C)			Salinity (‰)			Chlorophyll a (μg L^{-1})		
		slope	r^2	*p*-value	slope	r^2	*p*-value	slope	r^2	*p*-value
S. kegaki	THC (cell mL^{-1})	**2.6×10^4**	**0.024**	**<0.05**	-1.1×10^5	0.221	NS	-7.7×10^4	0.034	NS
	Granulocytes (%)	-0.52	0.130	NS	1.09	0.283	NS	0.04	0.005	NS
	Hyalinocytes (%)	-0.04	0.0002	NS	0.82	0.039	NS	-5.07	0.224	NS
	Blast-like cells (%)	0.67	0.046	NS	-1.97	0.193	NS	5.05	0.193	NS
	Mortality (%)	1.24	0.405	NS	-1.02	0.135	NS	1.92	0.513	NS
	Phagocytosis (%)	2.31	0.221	NS	-4.08	0.337	NS	1.3 x 10	0.171	NS
H. hyotis	THC (cell mL^{-1})	2.5×10^4	0.201	NS	2.1×10^4	0.026	NS	2.2×10^5	0.061	NS
	Granulocytes (%)	0.87	0.259	NS	0.55	0.018	NS	2.1 x 10	0.489	NS
	Hyalinocytes (%)	-0.70	0.234	NS	-0.56	0.026	NS	-1.1 x 10	0.306	NS
	Blast-like cells (%)	-0.17	0.059	NS	0.01	0.00001	NS	-1.0 x 10	0.801	NS
	Mortality (%)	2.2	0.674	NS	0.06	0.0001	NS	-2.06	0.028	NS
	Phagocytosis (%)	-0.16	0.002	NS	-4.68	0.277	NS	7.14	0.050	NS

THC increase in hemolymph is generally considered as a consequence of proliferation or movement of cells from tissue into hemolymph (Flye-Sainte-Marie et al. 2009). The proliferation of bivalve hemocytes is regulated by stem cell factor (Betti et al. 2008). The blast-like cells, a stem cell, of intertidal oyster *S. kegaki* was significantly increased from May to July when THC considerably increased (Fig. 4-8 and Fig 4-9A), indicating that the hemocyte proliferation of *S. kegaki* with high temperature was occurred. On the other hand, the water temperature at the subtidal zone varies slowly compared with air and surface water temperature and may remain cool even during summer period. Therefore, I think that the water temperature did not modify the THC of subtidal oyster *H. hyotis*.

Salinity is also one of the environmental factor influencing hemocyte parameters in marine bivalves (see the review of Donaghy et al. 2009a). Acute salinity changes have been displayed to induce variation of THC (Chu and La Peyre 1991; Reid et al. 2003; Matozzo et al. 2007), immune activity (Fisher et al. 1987; Reid et al. 2003) in several bivalve species. However, generally variation of salinity within the life range of the species does not affect hemocyte parameters (Matozzo et al. 2007). In this study, I could not find a clear correlation between salinity and hemocyte parameters. In this study, the correlation between salinity and hemocyte parameters were not observed in linear model test (Table 4-3), indicating that natural seasonal variation of salinity might not alter the hemocyte parameters in both oyster species.

Food availability also influence the hemocyte variables in marine bivalves. For example, the low food level and starvation have been shown to reduce THC in oysters (Delaporte et al. 2006). In the field study by Flye-Sainte-Marie (2009), there was no effect of food quantity (particulate organic matter) and quality (particulate organic carbon and nitrogen)

on hemocyte parameters of clam *R. philippinarum*. In this study, the level of chlorophyll-*a* in water column did not appear to affect any of the measured hemocyte parameters (Table 4-3). This suggests that in such natural conditions food level and quality were not low enough to induce any modification of hemocyte parameters during the study period.

All hemocyte parameters in both oysters significantly varied during the study period (Kruskal-Wallis test, $P<0.05$). Nevertheless, as shown in Table 4-3, there were few significant relationships between hemocyte parameters and measured environmental factors (Table 4-3). This result appears contradictory with laboratory experiments which showed that hemocyte parameters varied with environmental factors (Chu and La Peyre 1993; Reid et al. 2003; Monari et al. 2007). The reason for this difference remains unclear but this emphasizes the difficulty in extending laboratory results to the field.

Effect of reproduction on hemocyte parameters

Gonadal cycle in marine bivalves is major endogenous factors influencing the hemocyte system (Oliver and Fisher 1999; Delaport et al. 2006; Flye-Sainte-Marie et al. 2009; Lin et al. 2012). Especially, spawning is energy demanding process resulting in immune depression (Li et al. 2010). In agreement with this hypothesis, in this study, the immune-related hemocyte parameters of both intertidal oyster *S. kegaki* and subtidal oyster *H. hyotis* were depressed due to spawning stress. Significantly increased hemocyte mortality and decreased phagocytosis capacity of both oysters were observed during active spawning period (Fig. 4-10 and Fig. 4-11). This is accordance with previous studies. For example, hemocyte density, phagocytosis activity and lysosomal membrane stability were reduced in post-spawning oysters *C. gigas* (Cho and Jeong 2005; Li et al. 2010). Field studies also

showed seasonal variations of hemocyte variables concomitant with spawning periods (Flye-Sainte-Marie et al. 2009; Fraser et al. 2013; Soudant et al. 2004). Reproductive investment in bivalves is usually associated with energy allocation for gametogenesis and spawning, at the expense of other physiological processes, including immune response (Cho and Jeong 2005; Li et al. 2010). In the present work, energetic materials including protein and carbohydrate level in post-spawning (spawning or spent) oysters tended to be low (Fig. 4-13). Li et al. (2010) suggested that the energy shortage due to spawning is likely to reduce the required energetic supply for cell-mediated immune responses. Therefore, post-spawning oysters may not hold enough energy reserves for expenditure on cellular immune defense.

In marine bivalves, hemocyte infiltration in gonadal tissue of spent and sexually indifferent stage for gamete resorption and restructuration has commonly been suggested to cause change in circulating hemocyte count in hemolymph (Cajaraveille et al. 1996; Duchemin et al. 2007; Wendling and Wegner 2013). Massive migration of cells to gonads may then decrease the concentration of hemocytes in other tissues, as well as in circulating hemolymph. In the present study, THC in hemolymph inclined to decrease in both intertidal and subtidal oysters when most of animals were spent and sexually indifferent condition (Fig. 4-8). Furthermore, the large hemocyte infiltrations were observed in gonad tissue of spent and indifferent oysters (data no shown), and could explain the decrease in THC.

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국문요약

제주 연안은 겨울철 수온이 13-18°C의 따뜻한 수온으로 인하여 다양한 저서생물들이 분포하고 있으며, 최근 들어 다양한 아열대 외래종이 유입되고 있다. 특히 과거 20년간 제주연안에서 다양한 아열대성 굴이 분포하고 있음이 확인되고 있으나 이들에 관한 연구는 미진한 실정이다. 이 연구는 제주 연안에 서식하고 있는 가시굴 (*Saccostrea kegaki*), 태생굴 (*Ostrea circumpicta*), 중국굴 (*Hyotissa hyotis*) 세 종을 대상으로 DNA marker를 이용하여 굴들의 유전 정보를 밝히고 분류학적 위치를 확인하였다. 또한 굴들의 면역 시스템을 이해하기 위하여 면역 세포의 특성을 규명하고 환경변화와 굴의 번식활동에 의한 면역 세포기능의 계절적 변화를 연구하였다.

1. Nuclear DNA 유전자를 이용한 제주도 연안에 서식하는 아열대성 굴 세종의 분자유전학적 분류 연구

굴의 분류학적 분석은 외형적 특징을 바탕으로 패각의 형태 및 색, 육질부의 해부학적 특징 연구가 주를 이루고 있으나, 외형적 특징은 환경적 인자에 매우 가변적이고 민감하여 분류학적 정보를 제공하는데 한계점이 있다. 이를 보완하기 위하여 분자생물학적 방법으로 핵에 존재하는 유전자의 염기서열 분석에 기초한 분자적 계통 분류 및 종 분류 연구가 활발히 이루어지고 있다.

이 연구에서는 제주도 연안에 서식하는 아열대성 굴인 가시굴 (*Saccostrea kegaki*), 태생굴 (*Ostrea circumpicta*), 중국굴 (*Hyotissa hyotis*)의 분자생물학적 분류를 위하여 핵내 단백질 합성기관인 ribosome의 구성단위의 하나인 18S ribosomal RNAs (18S rRNA), rRNA 사이에 위치하여 전사가 일어나지만 기능을 갖지 않는 영역으로 종간 낮은

상동성을 보이는 internal transcribed spacer (ITS) 및 진핵세포 내 염색체를 구성하는 chromatin의 구성원의 하나인 Histone 3 (H3)의 염기서열 분석을 하였다. 기존에 밝혀진 이매패류의 18S rDNA, ITS-1, H3 유전자들을 이용하여 multiple alignment를 수행하고 잘 보존된 영역의 서열을 비교 분석하여 nuclear gene을 증폭하기 위한 각 유전자에 대한 primer를 제작하였다. 굴의 패각근으로부터 total DNA를 추출 후 제작된 primer를 이용하여 PCR을 통해 유전자를 증폭, cloning 및 염기서열을 분석하였다. 결정된 염기서열은 neighbor-joining (NJ)와 maximum parsimony (MP) 방법을 이용하여 굴들의 계통학적 유연관계를 분석하였다. 세 종의 유전학적 분류 위치를 확인하기 위하여, 우리나라에 서식하는 참굴 (*Crassostrea gigas*), 강굴 (*C. ariakensis*), 바윗굴 (*C. nippona*), 벗굴 (*O. denselamellosa*) 및 미국의 대서양굴 (*C. virginica*)를 함께 비교 분석하였다.

세 가지 유전자는 동일하게 굴류를 Ostreidae 과 (Family)와 Gryphaeidae 과로 (Family)로 명확하게 구분이 되었다. 가시굴은 *Saccostrea* 속 (Genus), 태생굴은 *Ostrea,* 중국굴은 *Hyotissa* 속에 포함되었다. 가시굴과 태생굴은 유전적으로 유연관계가 가까우며, *Crassostrea* 종들과 sister branch를 형성하였다. 중국은 Ostreidae 과의 굴들과는 독립된 단일 계통군을 형성하고 있었다. 이번 연구 수행의 결과로 제주도 연안에 서식하는 아열대성 굴들에 대한 18S rDNA, ITS, H3의 유전정보를 새롭게 획득하였으며, 이는 굴류를 비롯한 해산 이매패류의 유연관계 분석에 유용한 정보로 이용될 것이다.

2. 제주도 연안에 분포하는 가시굴, 태생굴, 중국굴의 면역학적 비교연구

해산 이매패류의 혈구는 모든 조직세포 사이를 자유롭게 순환하며 내부 면역 외에도 영양분의 소화와 이동, 패각과 조직의 수선, 분비 등에 관여를 하며 생리적 안정상태를 유지한다. 또한 혈구 세포의 형태와 기능은 환경인자의 영향에 따라 다양하게 변화하기 때문에 해양환경 변화 모니터링 및 저서생물의 건강도 측정 등에 이용되고 있다. 이

매패류의 혈구의 형태와 기능을 분석하는데 일반적으로 현미경이 사용되었으나, 이는 분석 속도가 느리고 혈구의 면역기능을 측정하고 정량적으로 분석하기에 한계가 있는 반면, 유세포 분석기 (flow cytometry)는 신속 정확한 방법으로 다양한 세포의 기능들을 정량적으로 측정할 수 있어 해산 이매패 혈구의 세포성 면역기작 측정에 매우 유용하다.

이 연구에서는 광학현미경과 유세포 분석기를 이용하여 가시굴 (*Saccostrea kegaki*), 태생굴 (*Ostrea circumpicta*), 중국굴 (*Hyotissa hyotis*) 세 종의 혈구세포의 집단을 분류하고, 이들의 면역 관련 기능들을 정량적으로 측정하여 비교 분석하였다. 번식활동이나 산란이 혈구 형태와 기능에 미치는 영향을 최소화하기 위하여 산란 후 성적으로 미분화기인 겨울철에 굴들을 채집하여 분석하였다. 굴의 패각근으로부터 혈림프액을 채혈 후, Hemacolor 염색 후 광학현미경 하에서 혈구의 형태를 관찰하였다. 혈구의 핵을 SYBR green I 으로 염색 후 유세포분석기를 이용하여 혈구 세포만을 선택한 후 세포의 형태에 따라 집단을 분류하고 세포의 수를 측정하였다. Propidium iodide (PI)에 염색된 혈구 세포들의 비율을 측정하여 혈구 사망률을 측정하였다. LysoTracker Red 를 이용하여 혈구 세포 내 lysosome 함량을 측정하였다. 마지막으로 형광 bead 를 이용하여 혈구 세포의 식세포 능력 (phagocytosis)을 측정하였으며, 2'7'-dichlorofluorescein diacetate (DCFH-DA)를 이용하여 세포 내 비 유동성 활성산소 (reactive oxygen species, ROS)와 활성질소 (reactive nitrogen speices, RNS) 생산력을 측정하였다.

가시굴, 태생굴, 중국굴의 혈구는 세포질 내 과립 (granule)이 많은 과립구 (granulocyte), 과립이 없거나 적은 무과립구 (hyalinocyte or agranulocyte) 및 세포의 크기가 작으며 세포질이 얇은 blast-like cell의 세 가지 집단으로 동일하게 분류되었다. 혈림프 내 세포 집단 별 구성 비율은 세 종 모두 비슷하였으며, hyalinocytes가 59%로 가장 많으며 granulocyte가 23-31%, blast-like cell은 3-5%의 순으로 구성되었다. 광학현미경 관찰에서는 세포질 내 공포 (vacuole)들이 많은 vacuolated granulocyte도 관찰되었다. 유세포 분

석기를 이용한 혈구 면역 기능 분석 결과, 세 종 모두 granulocytes가 세포질 내 lysosome 함량이 가장 많을 뿐만 아니라 세포성 면역 활동인 식세포 작용과 세포 산화 능력이 가장 활발한 것으로 확인되어 granulocyte가 주된 면역 세포임을 확인하였다. 식세포 능력은 가시굴이 가장 낮았으며, 세포 산화능력은 중국굴이 가장 낮았다. 세포성 면역활동 능력에 있어서 종간의 특이성이 확인되었으나, 이들의 면역 시스템 경로에 대한 보다 구체적인 연구가 요구된다. 이번 연구에서 가시굴, 태생굴, 중국굴의 혈구 집단 구조 파악 및 면역력 측정 결과는 제주 연안에 서식하는 아열대성 굴들의 병원성 생물 및 다양한 스트레스에 대한 면역 반응 연구에 중요한 기초 자료로 이용될 것이다.

3. 연중 환경변화와 번식활동이 굴 혈구세포에 미치는 영향: 조간대에 서식하는 가시굴과 조하대에 서식하는 중국굴의 비교 연구

해산 이매패의 혈구세포는 다양한 수온, 염분, 먹이 및 오염원 등의 외부적인 환경 변화뿐만 아니라 번식활동 등의 내부적인 변화에 따라 다양하게 변화하기 때문에 혈구의 구조적 특징과 기능들의 변화 조사는 대상 종의 생리학적 상태를 대표할 수 있는 좋은 마커 (marker)이다. 또한, 조석 차이에 의하여 일정 기간 공기 중에 노출되는 조간대와 항상 수층에 잠겨있는 조하대의 서로 다른 환경 조건에 의하여 서로 다른 생리적 변화를 나타낼 수 있을 것이다.

이 연구에서는 조간대에 서식하는 가시굴 (*Saccostrea kegaki*)와 조하대에 서식하는 중국굴 (*Hyotissa hyotis*)를 대상으로 혈구세포 수, 혈구 집단 별 구성비율의 변화, 혈구 사망률 및 식세포율의 연중 변화를 유세포 분석기를 이용하여 측정하였다. 채집 지역의 수온, 염분 및 먹이원인 chlorophyll-a의 함량의 정보를 채집하여 환경변화가 굴 혈구세포에 미치는 영향을 알아보았다. 조직학적 방법을 이용하여 굴의 연중 번식주기를 규명하여 번식활동이 혈구 세포 기능에 미치는 영향을 조사하였다. 또한, 조간대와 조하대의

서식환경에 따른 굴 혈구 세포 변화의 차이를 알아보았다.

조간대 굴의 혈구 수는 수온 변화와 양(+)의 상관관계를 보이며 연중 큰 변화를 보인 반면, 조하대 굴은 혈구 수와 수온변화와의 상관성이 관찰되지 않았다. 특히, 조하대 굴의 혈구 수는 기온과 수온이 높은 여름철에 급격하게 증가하는 모습을 보였다. 염분과 먹이량 (chlorophyll-a 농도)가 혈구세포의 미치는 영향은 관찰되지 않았다. 굴의 산란 및 산란 이후 혈구 사망률이 급격히 증가하였으며 식세포 능력이 상당히 저하되었다. 또한 이 시기에는 굴 체내 에너지원인 단백질과 탄수화물 함량이 매우 낮았다. 그러므로 에너지원을 많이 요구되는 산란활동에 의하여 면역 시스템에 충분한 에너지원이 공급되지 못함으로써 혈구 세포의 기능이 저하됨을 알 수 있었다. 면역학적, 조직학적 방법을 병합한 모니터링 한 이번 연구는 기존의 군집 차원의 연구에서 벗어나 개체군의 생리, 번식, 면역 등의 기능적인 특성을 분석한 결과로, 제주 연안에 서식하는 아열대성 굴들의 자원 관리 및 종 보존 차원뿐만 아니라 연안의 환경오염 및 수온 상승 등의 장기적 모니터링 연구에 널리 활용될 수 있을 것으로 기대된다.

감사의 글

이 논문이 완성되기까지 깊은 관심과 도움을 주신 최광식 교수님께 진심으로 감사를 드립니다. 바쁘신 중에도 저의 논문을 심사해주신 강창근 교수님, 이경준 교수님, 정석근 교수님, 박상율 교수님께도 감사를 드립니다. 그리고 학위과정 동안 많은 가르침을 주신 송춘복 교수님, 이제희 교수님, 허문수 교수님, 전유진 교수님, 여인규 교수님, 이영돈 교수님, 김기영 교수님, 정준범 교수님, 이승헌 교수님께도 깊은 감사를 드립니다.

실험실 생활의 시작부터 지금까지도 늘 따끔한 충고와 용기를 불어넣어주시는 한국해양과학기술원 강도형 박사님과 군산대학교 박경일 교수님께 감사의 말씀을 전합니다. 지난 학부, 석사, 박사과정의 동안 무척추동물양식 실험실에서 함께 동고동락하며 가족같이 지내왔던 김대경 박사님, Dr. Ludovic Donaghy, Dr. Md. Jasim Uddin, Dr. Yanin Limpanont, Dr. Md. Mostafizur Rahman Mondol, Le Thanh Cuong, 강현실 박사님, 양현성 박사님, 김태호 형, 임나래 누나, 이지연 누나, 정희도, 이희중, 박아름이, 김영민, 조영관에게 감사의 마음을 전합니다. 그리고 이 연구 주제를 저에게 선물해 준 지금은 하늘나라에서 있는 너무나 보고 싶은 김봉규 형에게 정말 감사 드립니다.

그리고 하늘나라에서 항상 저를 지켜주시는 할아버지, 할머니, 외할아버지께 감사의 말씀을 전합니다. 또한 저를 위해 항상 기도해주시고 응원해주시는 외할머니와 친척 분들께도 감사의 마음을 전합니다. 마지막으로 이 길을 걸어올 수 있도록 믿어주시고 격려해주신 부모님, 누나, 매형에게 감사의 말을 전하고 싶습니다.