THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Impacts of reproduction and parasite on the physiological and immunological parameters of Manila clam, *Ruditapes philippinarum*

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February 2010

Impacts of reproduction and parasite on the physiological and immunological parameters of Manila clam, *Ruditapes philippinaum*

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

February 2010

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February 2010
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CONTENTS

국문요약 iii

LIST OF FIGURES iv

LIST OF TABLES v

INTRODUCTION 1

MATERIALS AND METHODS 3

1. Sampling effort 3

2. Flow cytometric analyses of hemocyte parameters 3

2.1. Hemolymph collection 3

2.2. Flow cytometry system 3

2.3. Total hemocye count (THC) and hemocyte mortality (MOT) 5

2.4. Phagocytosis capacity (PHG) 6

2.5. DNA damage (DDM) oh hemocyte.. 6

3. *Perkinsus* 10

4. Histology 10

5. Condition index (CI)... 12

6. Biochemical analysis 12

RESULTS 13

1. Biometry data.. 13

2. Biochemical composition.. 13

3. Hemocyte parameters 13

4. Perkinsus 16

5. Reproductive stage 16

Collection @ jeju

6. CI, biochemical composition, and immunological parameters in different reproductive stage …………………………………………………............................ 16

7. Correlation among parameters ………………………………………………….……. 25

DISCUSSION ………………………………………………………………………………. 34

SUMMARY ………………………………………………………………………………... 37

REFFERENCES …………………………………………………………………………… 38

Collection @ jeju

국 문 요 약

이 연구는 바지락 (*Ruditapes philippinarum*)의 개체 별 번식 활동과 기생충 감염이 생리학적 및 면역학적 요인에 미치는 영향을 측정하기 위해 실시되었다. 전라남도 고흥에서 200 개체의 바지락을 채집하여 개체 별 비교를 실시 하였다. 생리학적 요인으로는 바지락의 비만도 (condition index), 단백질과 탄수화물 함량을 측정하였고, 면역학적 요인으로는 바지락의 주된 세포면역을 담당하는 혈구를 유세포 분석기 (flow cytometry)를 이용하여 총 혈구 수, 혈구 사망률, 식세포율 및 혈구 DNA 손상도를 측정하였다. 조직학적 관찰을 통해 바지락의 번식단계를 관찰하였다. 바지락의 주요 기생충인 *Perkinsus olseni*의 감염률과 감염 정도를 Ray's fluid thioglycollate medium (RFTM) 방법과 조직병리학적 방법으로 확인하였다. 바지락의 번식 단계는 대부분이 산란 중이거나 산란을 마친 상태로 완숙기 (3.5%), 산란기 (24%), 산란후기 (60.5%) 및 휴지기 (12%)로 분류되었다. 번식 단계 별로 나누어 비교 결과 비만도, 단백질 함량, 혈구 사망률 및 식세포율에서 유의적인 차이가 나타났다. 비만도와 단백질 함량은 완숙기 때 유의적으로 가장 높았고 산란기, 산란후기, 휴지기기로 갈수록 낮아져 바지락의 생식소의 발달 상태를 잘 반영하였다. 탄수화물과 비만도의 강한 양의 상관관계를 미루어 보아 주요 에너지 저장원인 탄수화물이 산란기 이후부터 축적이 되는 것을 유추할 수 있었다. 생리적으로나 영양학적으로 좋은 상태인 완숙기 단계에서 가장 낮은 혈구 사망률과 가장 높은 식세포율을 보였다. 따라서 혈구 사망률과 식세포율은 혈구의 생리적 상태나 건강도 판정에 유용하게 이용 될 수 있을 것으로 사료된다. *P. olseni*는 97%의 높은 감염률을 보였지만, 감염 정도와 면역학적 요인 간의 상관관계는 보이지 않았다. 이는 *P. olseni* 감염 정도가 바지락의 생식소 활동이나 면역 시스템에 치명적인 영향을 줄 수 있는 만큼의 높은 감염도가 아니었다고 사료된다.

Collection @ jeju

LIST OF FIGURES

Fig. 1. Location of sampling site, Goheung………… …………………………….... 4

Fig. 2. Flow cytometric analyses of total hemocyte count (THC) and hemocyte mortality (MOT)……..……………………………………………………… 7

Fig. 3. Flow cytometry count of hemocytes associated with fluorescent beads. Phagocytosis capacity was defined as the percentage of hemocytes that have engulfed more than three beads (M1). ………………………………………… 8

Fig. 4. Measurement of the DNA damage of hemocytes by flow cytometry. ………… 9

Fig. 5. Regression of *Perkinsus olseni* infection intensity determined by RFTM and histology………………………………. …………………………………..... 17

Fig. 6. Frequency of reproductive stage of Manila clam from Goheung ……………… 18

Fig. 7. Condition index of Manila clam grouped by reproductive stage……………… 21

Fig. 8. Total protein contents of Manila clam grouped by reproductive stage……….... 22

Fig. 9. Hemocyte mortality of Manila clam grouped by reproductive stage………….... 23

Fig. 10. Phagocytosis capacity of Manila clam grouped by reproductive stage……….. 24

Collection @ jeju

LIST OF TABLES

Table 1 Semi-quantitative scale of infection intensity for *Perkinsus olseni* (Ngo, 2004)... 11

Table 2 Biometry data of Manila clam from Goheung 14

Table 3 Mean of hemocyte parameters of Manila clam from Goheung 15

Table 4 Mean of parameters grouped by gonad development stage (GS)................. 20

Table 5 Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in all clams 26

Table 6 Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in spawning clams................................. 27

Table 7 Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in spent clams...................................... 28

Table 8 Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in resting clams.. 29

Table 9 Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in all clams.. 30

Table 10 Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in spawning clams.. 31

Table 11 Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in spent clams.. 32

Table 12 Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in resting clams... 33

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1. Introduction

Marine bivalves have open-blood vascular system. Hemocytes are cells freely circulating in hemolymph and infiltrating in tissues of marine bivalves. Hemocytes are involved in various functions including digestion and nutrient transport, wound healing, shell repair, excretion, and internal defense (Cheng, 1996). Internal defense system of marine bivalves possesses various cellular and humoral components. It is well known that the hemocytes are the main cellular mediators of the defense system in bivalves, responsible for chemotaxis (Ottaviani, 2000), recognition (Ordás et al., 2000; Kang et al., 2006; Takahashi et al., 2008), encapsulation (Montes, 1995), phagocytosis (Canesi, 2002), and degradation of invading foreign particles by various hydrolytic enzymes (López, 1997) and reactive oxygen species (ROS) production (Lambert et al., 2003).

Cellular immune parameters of marine bivalves are affected by environmental factors such as salinity, temperature (Hégaret et al., 2003ab; Chen et al., 2007ab), food supply (Delaporte et al., 2006) and toxicants (Brousseau et al., 1999; Fisher et al., 2000; Fournier et al., 2001; Matozzo et al., 2001; Gagnaire et al., 2004), internal factor such as reproduction (Delaporte et al., 2006), and invading pathogen (Anderson et al., 1995; La Peyre et al., 1995; Ordás et al., 2000; Lambert et al., 2003; Labreuche et al., 2006ab). Cellular defense-related hemocyte parameters were highly sensitive to variation of external and internal factors. Consequently, they were suggested to successfully reflect the physiological and health status of bivalves (Chu, 2000).

Recently, flow cytometry has been spotlighted as a powerful tool in the study of cellular defense-related bivalve hemocytes at the single cell level because of the rapid, accurate and quantitative analyses. Immune mechanisms of marine bivalves such as hemocyte

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concentration, mortality, phagocytosis, ROS production have been recently measured by using a flow cytometry (Choquet et al., 2003; Hégaret et al., 2003ab; Lambert et al., 2003; Goedken and De Guise, 2004; Soudant et al., 2004; Delaporte et al., 2006; Gagnaire et al., 2006; Labreuche et al., 2006ab; Chen et al., 2007ab; Delaporte et al., 2007; Hégaret et al., 2007; Lambert et al., 2007; da Silva et al., 2008; Donaghy et al., 2009; Flye-Sainte-Marie et al., 2009; Hégaret et al., 2009). Detection of DNA damage level of bivalve hemocytes using a flow cytometry is also suggested as successful biomarkers to assess marine pollution and stress (Bihari et al., 2003; Park et al., 2006b; Park et al., 2009).

Goheung is one of the commercial Manila clam beds of Korea. The reproductive status of *R. philippinaru*m of Korea in October has been reported as partially spawning or spent stage (Kang et al., 2004; Choi et al., 2005; Chung et al., 2005; Son and Kim, 2006; Kang et al., 2007). During spawning processes, the clams exhaust the reserved energy and become physiologically weak in terms of low energy budget and immunity. As a result, the weakened clam is even more susceptible to opportunistic pathogen and environmental stress (La Peyre et al., 1996).

Perkinsus were reported as the main parasites in Manila clam population of Korea (Kim et al., 1995; Park and Choi, 2001; Ngo et al., 2004). Infection of parasites trigger host defense and take up energy from the host (Park et al., 2006a).

A multi-parametric study was designed to assess the relative effect of reproduction and parasite infection on physiological and immunological (hemocyte immunity) parameters of *R. philippinarm* in the field. Flow cytomeric assessments were applied to determine total hemocyte count, mortality, and phagocytosis in the cellular level and DNA damage of hemocyte in molecular level.

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2. Materials and methods

2.1. Sampling effort

In October 2009, *Ruditapes philippinarum* were collected from the commercial intertidal mud flat of Goheung, southern coast of Korea (Fig .1). Surface water temperature and salinity of sampling site was recorded as 21.4 ℃ and 31.0 psu (Korea Hydrographic and Oceanographic Administration, KHOA). After arrival in laboratory, clams were placed into a seawater tank of recirculation system with a temperature of 21.5 ℃ and salinity of 33.2 psu. Calms were kept in the tank over 60 hrs to acclimate and recover from the stress induced by transportation. Total two hundred clams (shell length 35.5 ± 2.1 mm) were chosen at random from the tank and used in this study.

2.2. Flow cytometric analyses of hemocyte parameters

2.2.1. Hemolymph collection

Approximately 1 mL of hemolymph were withdrawn from the adductor muscle of individual clams using a syringe fitted with a 26Gx1/2″ needle and immediately transferred into micro-tubes held on ice to minimize cell clumping. All subsequent analyses were performed on individual samples.

2.2.2. Flow cytometry system

All hemocyte parameters were analyzed using a BD FACScalibur™ flow cytometer (Becton-Dickinson) equipped with an argon-ion laser emitting light at 488 nm. The diffracted light on the cell is detected and represented the relative cell size through forward scatter (FSC)

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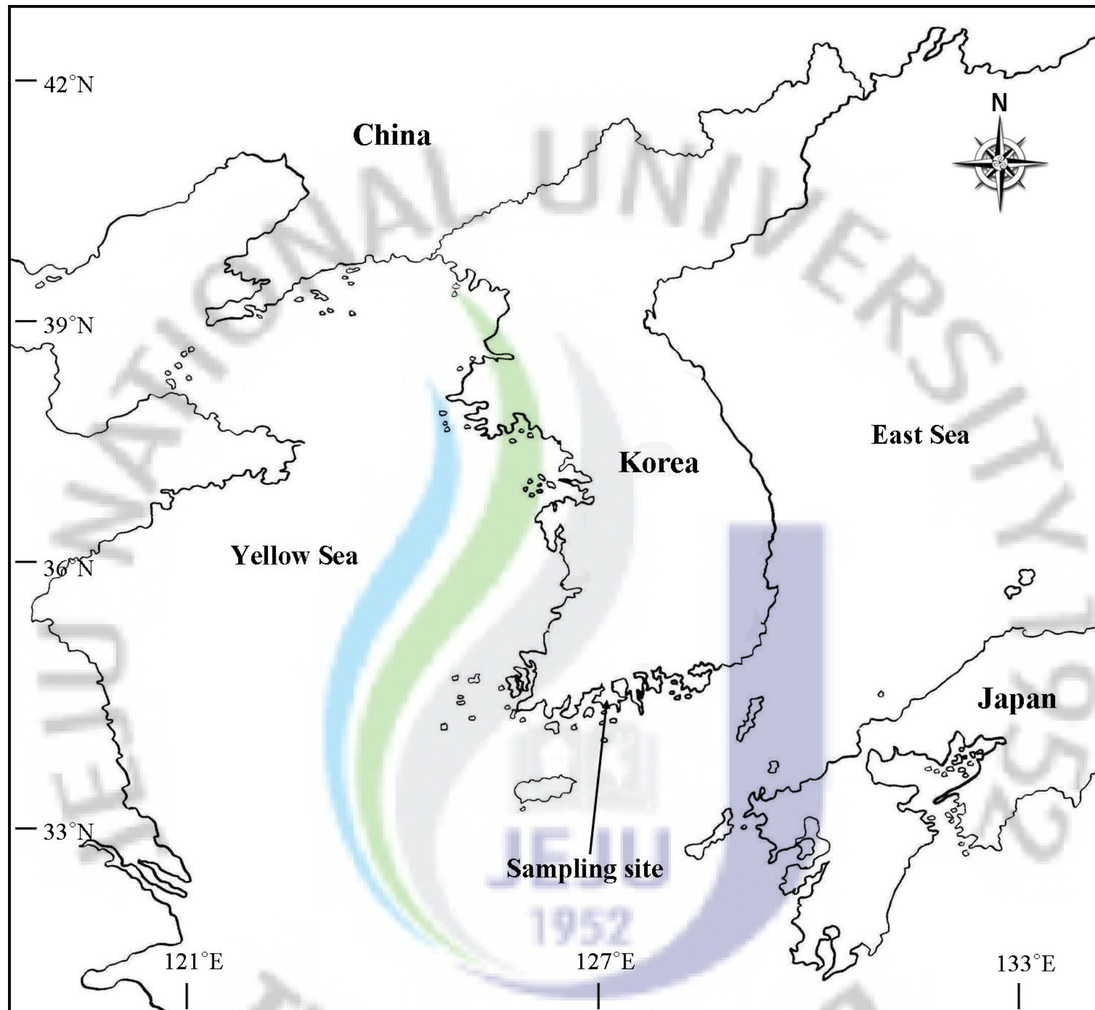

Fig. 1. Location of sampling site, Goheung.

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detector and internal complexity of cells thorough side scatter (SSC) detector. The flow cytometer is equipped with three specific fluorescence sensors: FL1 (500-530 nm), FL2 (550-600 nm) and FL3 (>630 nm) allowing the detection of autofluorescence or fluorescent dyes. Data analyses were used a Win MDI Ver. 2.8 software.

2.2.3. Total hemocye count (THC) and hemocyte mortality (MOT)

Hemocyte concentration in hemolymph (Total hemocyte count = THC) and hemocyte mortality (MOT) were determined by double staining with SYBR Green Ⅰ (Sigma-Aldrich, Saint Louis, USA) and propidium iodide, (PI, Sigma-Aldrich) which is both fluorescent dye that binds to the double-stranded DNA. PI selectively stains only dead hemocytes because PI cannot penetrate the membrane of viable cells. A 150 µl of hemolymph was quickly added to a mixture of 150 µl of antiaggregant solution (AASH, containing 2.5% NaCl and 1.5% EDTA in 0.1 M phosphophate buffer, pH 7.4) and 3 µl of 10x SYBR Green Ⅰ (final dilution 1/1000 of the DMSO commercial solution). The tubes were then incubated for 60 min at room temperature in the dark. PI (final dilution 20 µg ml^{-1}) was added 10 min prior to flow cytomery analysis.

Hemocytes stained with SYBR green Ⅰ can visualized and separated from the particles in the hemolymph on the FL1-SSC density plot (Fig. 2A). THC was calculated by the formula:

THC = 10,000 / (A x B) x 2

10,000: Total number of counted hemocytes, A: Counting time of 10,000 hemocytes (min), B: Flow rate (mL min^{-1}) of flow cytometer, 2: Dilution factor

Live and dead cells stained with SYBR Green Ⅰ are detected by the FL1 detector of the flow cytometer, and dead cells stained with PI is detected by the FL3 detector. By

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visualization on the FL3-FL1 density plot, it was possible to estimate precisely the percentage of dead cells in each sample (Fig. 2B).

2.2.4. Phagocytosis capacity (PHG)

A 150 μL of hemolymph was incubated with a mixture of 150 μl of filtered sterile seawater (FSSW) and 30 μl of fluorescent latex beads (2.0 μm in diameter, Polysciences Inc) for 2 hrs at room temperature in the dark. The tubes were transferred on the ice to stop phagocytosis reaction until flow cytometry analysis. The hemocytes containing fluorescent beads can detect using a FL1 detector. According to Delaporte et al. (2003) and Hégaret el al. (2003b), the percentage of phagocytic cells was defined as the percentage of cells that had internalized three or more of the fluorescent beads (Fig. 3).

2.2.5. DNA damage (DDM) of hemocyte

DNA damage (DDM) of clam hemocyte was measured following the protocol described in Park et al. (2006b). A 100 μl of hemolymph was permeabilized with 900 μl of cold ethanol at -20 ℃ overnight. The cells were washed two times by centrifuging (3000 rpm for 10 min) with 0.1 M phosphate buffer (pH 7.4) and incubated with 3 μL of RNase A (10 mg ml^{-1}, Sigma) for 30 min at room temperature. Hemocytes were then stained with PI (final dilution 50 μg ml^{-1}) for 30 min in the dark before flow cytomety analysis. The percentage of cells presenting DNA fragmentation (sub-G0/G1) was then determined (Fig. 4).

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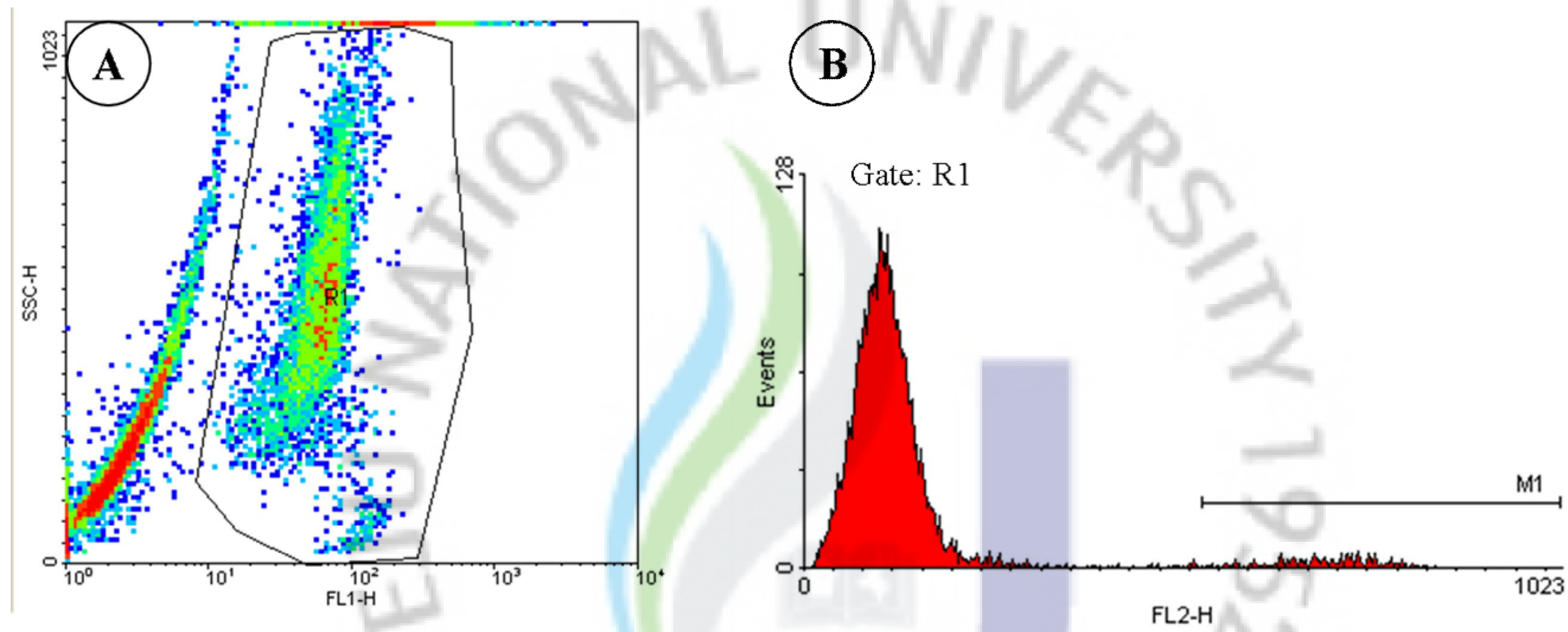

Fig. 2. Flow cytometric analyses of total hemocyte count (THC) and hemocyte mortality (MOT). A: Gate R1 on the FL1-H Log/SSC-H Lin density plot used to select from clam hemocytes containing DNA strained by SYBR Green Ⅰ, fluorescent dye that binds to double-stranded DNA. B: FL2-H Log/events histogram of dead hemocytes stained by propidium iodide, under marker M1, and unstained living hemocytes represented by the large peak to the left.

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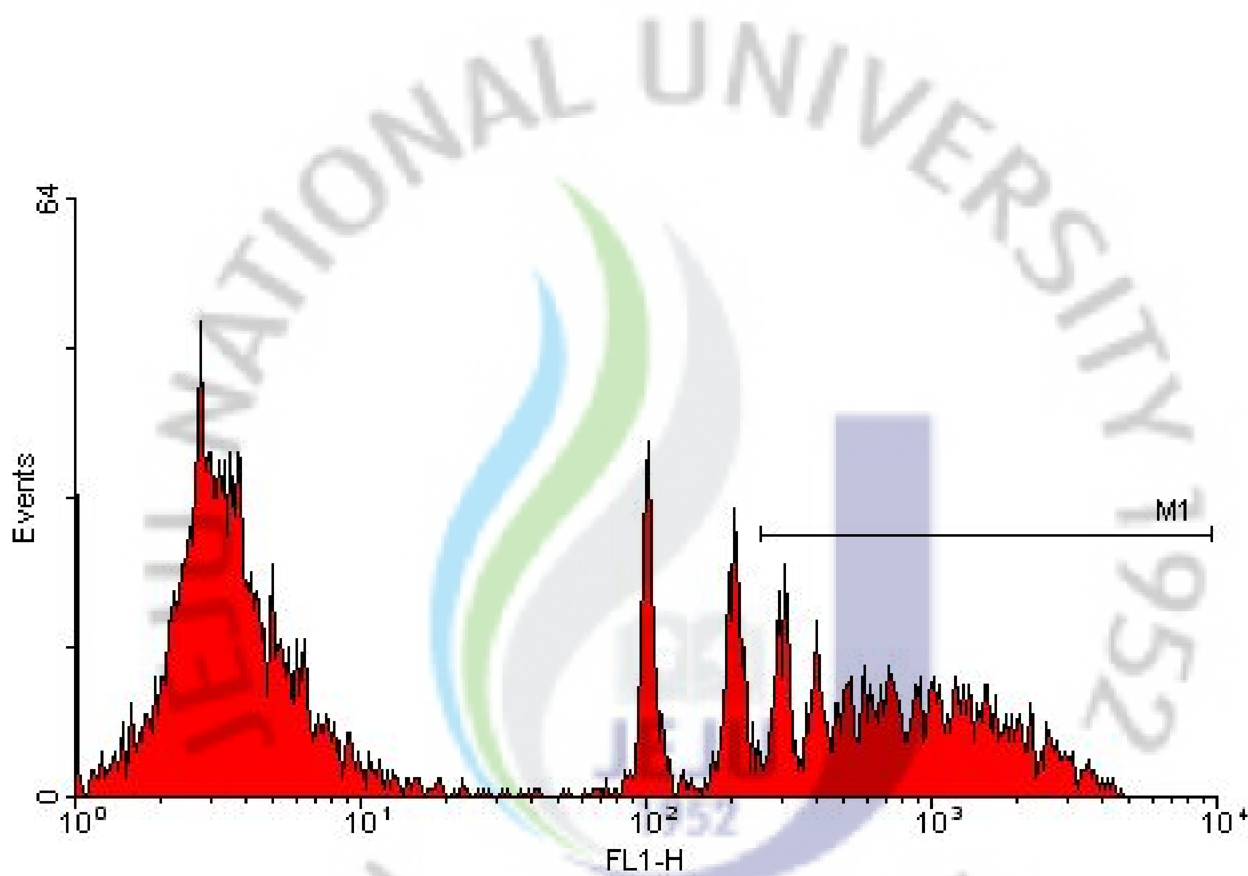

Fig. 3. Flow cytometry count of hemocytes associated with fluorescent beads. Phagocytosis capacity was defined as the percentage of hemocytes that have engulfed more three beads (M1).

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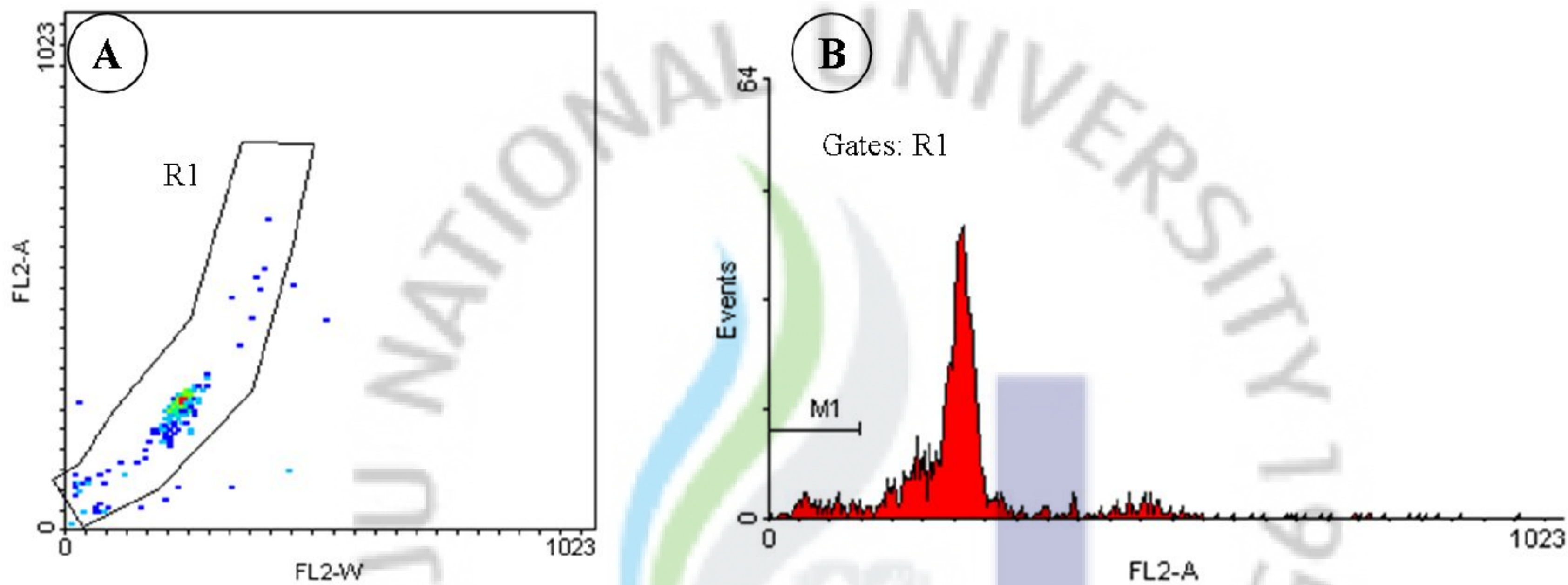

Fig. 4. Measurement of DNA damage of clam hemocytes by flow cytometry. A: Density plot representinf the FL2-width and the FL2-Area of fluorescence signals related to propidium iodide stained DNA. Single cells are gathered in region R1. B: Histogram of the FL2 area of fluorescence signals related to propidium iodide stained DNA of singles cells gated on R1.The cell cycle distribution and quantification of DNA fragmentation (M1) were determined by the fluorescence of individual cells.

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2.3. *Perkinsus*

After withdrawal hemolymph, clams were dissected from the shell and measured total tissue wet weight (TWWT). To detection and quantification of *Perkinsus olseni* infection using a Ray's fluid thioglycollate medium (RFTM), one side of gill was removed from the tissue and weighted. Gill tissues were placed in 5 ml FTM in the dark for 1 weeks at room temperature and digested in 2 M NaOH (Park and Choi., 2001). Then hypnospores were counted using a haemocytometer. *P. olseni* infection intensity was represented by the total number of *P. olseni* cells in an individual gill tissue weight (g).

2.4. Histology

For observation of gonad development, digestive gland atrophy, parasites burden, and necrotic digestive gland of clams, a cross-sectioned tissue (5 mm) was fixed in Davidson's solution for 48 hours. After fixation, the tissue was dehydrated and embedded in paraffin. Paraffin blocks were sectioned at 6 μm thickness and were stained with Harris's haematoxylene and Eosin Y. After mounting, the histological preparations were examined under a light microscope to determine gametogenic stage, digestive gland atrophy, parasites burden, and necrosis of the clams.

The gonad maturity was categorized into six stages using the maturity scale described by Park and Choi (2004). The stages are 1) early developing; 2) late developing; 3) ripe; 4) partially spawning; 5) spent; and 6) resting.

Infection intensity of protozoan parasite *Perkinsus* in *R. philippinarum* was scored on a semi-quantitative scale according to Table 1.

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Table 1

Semi-quantitative scale of infection intensity for *Perkinsus olseni* (Ngo, 2004).

Score	Description
0	No *Perkinsus* detected in any tissue
1	*Perkinsus* was limited to the mantle and gills
2	*Perkinsus* was limited to mantle, gill filaments and digestive tubules
3	*Perkinsus* was found in mantle, gill filaments, digestive tubules and gonads
4	*Perkinsus* was found in all types of tissue.

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2.5. Condition index (CI)

The residual body of the clams which is removed gill and cross-sectioned tissue were lyophilized for 60 hrs and the remaining-tissue dry weight (R-DWT) was measured. As gills were removed for quantification of *P. olseni* infection and cross-sectioned for histology, dry tissue weight (DTWT) was calculated by multiplying the TWWT with the ratio of R-DWT and R-WWT. Shell were dried and weighted as shell dry weight (SDW). Condition index (CI) was calculated by dividing DTWT (g) by SDW (g).

2.6. Biochemical analysis

For biochemical analysis, lyophilized clams were homogenized using a mortar and a pestle. Protein content was determined using the method of Lowry et al. (1951) after alkaline hydrolysis with 0.1 M NaOH at 37 ℃ for 2 hrs using bovine serum albumin as standard. Carbohydrates were determined as glucose by the phenol-sulfuric acid method (Dubois et al., 1956) using dextrose anhydrate as standard. Results are expressed as milligram of carbohydrate and protein per gram of DTWT.

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3. Results

3.1. Biometry data

Table 2 summarized biometry datas of Manila clam. Similar size of clams with average shell length 35.5 ± 2.1 mm was used in this study. Mean of dry tissue weight (DTWT) was 0.3461 g, ranged from 0.1218 to 0.7759 g. Mean of shell dry weight (SDT) was 4.4087 g, ranged from 2.8589 to 7.4892 g. Consequently, condition index (CI, *i.e.* DTWT/SDW) of clams varied from 0.0386 to 0.1625 with a mean 0.0786.

3.2. Biochemical composition

Protein content of clams varied from 211.4 to 469.6 mg/g DTWT with a mean 372.3 mg/g DTWT. Carbohydrate content of clams varied from 28.1 to 180.8 mg/g DTWT with a mean 83.1 mg/g DTWT.

3.3. Hemocyte parameters

Total hemocyte count (THC), hemocyte mortality (MOT), phagocytosis capacity (PHG), and hemocyte DNA damage (DDM) of Manila clam were summarized in Table 3. Every parameter was quite variable 1) THC: ranging from 46,101 to 3,056,212 cells ml^{-1} with a mean of 1,110,114 cells ml^{-1}; 2) MOT: ranging from 0.36 to 32.30% with a mean of 5.70%; 3) PHG ranging from 12.75 to 58.55% with a mean of 32.09%; 4) DDM ranging from 1.97 to 39.48% with a mean of 12.17%.

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Table 2

Biometry data of Manila clam from Goheung. SL, shell length; DTWT, dry tissue weight; SDT, shell dry weight; CI, condition index (DTWT/SDT), N, sample number; SD, standard deviation; Min, minimum value; Max, maximum value

Parameters	N	Mean	SD	Min	Max
SL (mm)	200	35.5	2.1	31.0	42.8
DTWT (g)	200	0.3461	0.1037	0.1218	0.7759
SDT (g)	200	4.4087	0.8121	2.8589	7.4892
CI	200	0.0786	0.0191	0.0386	0.1625

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Table 3

Mean of hemocyte parameters of Manila clam from Goheung. THC, total hemocyte counts in hemolymph; MOT, percentage of hemocyte mortality; PHG, percentage of phagocytosis capacity; DDM, percentage of hemocyte DNA damage; SD, standard deviation; Min, minimum value; Max, maximum value

Parameters	N	Mean	SD	Min	Max
THC (cells ml^{-1})	200	1,110,114	623,536	46,101	3,056,212
MOT (%)	200	5.70	5.33	0.36	32.30
PHG (%)	200	32.09	7.97	12.75	58.55
DDM (%)	200	12.17	7.26	1.97	39.48

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3.4. Perkinsus

Detection and intensity of *P. olseni* infection were assessed by both quantitative and semi-quantitative methods. Most of clams were infected with *P. olseni* (prevalence 98%). Infection intensity of *Perkinsus*, a number of *P. olseni* in gill tissue weight (g) was ranged from 0 to 1,974,098 cells/g gill tissue with a mean of 486,254 cells/g gill tissue. Infection intensity of *P. olseni* was also categorized from 0 to 4 depend on the infection distribution in histological slides. The regression of *P. olseni* infection intensity determined by RFTM and histology was showed in Fig. 5. We found that at score (0), the *Perkinsus* cells in g gill tissue was 0; score (1), 65,570 ± 69,599; score (2), 193,262 ± 134,737; score (3), 460,031 ± 218,803; and score (4), 1,045,622 ± 383,208.

3.5. Reproductive stage

The gonad development stages of the clams were examined into four stages (Fig. 6). Most of clams were in spent stage (121 clam, 60.5%), followed by spawning stage (48 clams, 24.0%). Resting stage, predominantly composed of connective tissue in gonad after spent, was observed in 24 clams (12.0%). Seven clams (3.5%) were fully matured and were ready for spawning.

3.6. CI, biochemical composition, and immunological parameters in different reproductive stage

Because the reproductive processes demanded the high energetic cost, it could be hypothesized that the reproductive cycle strongly affects condition, biochemical composition, and immunological parameters of Manila clam. Therefore, Manila clams were analyzed after

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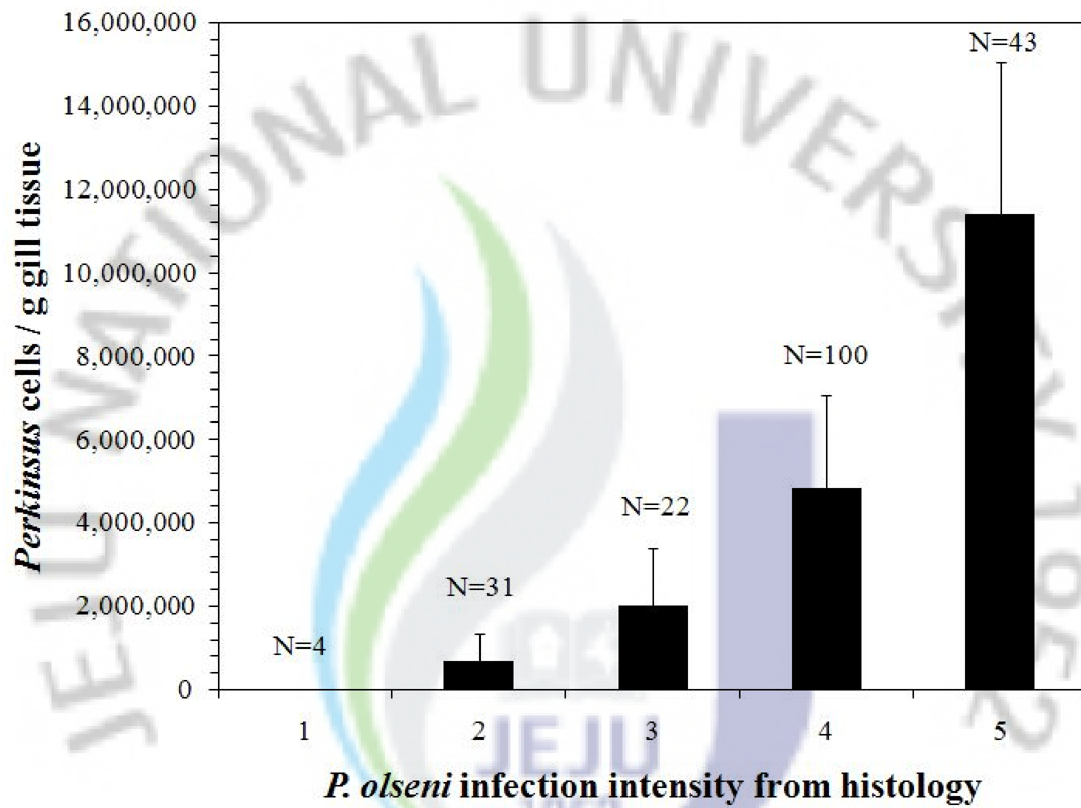

Fig. 5. The regression of *Perkinsus olseni* infection intensity determined by RFTM and histology. N, sample number.

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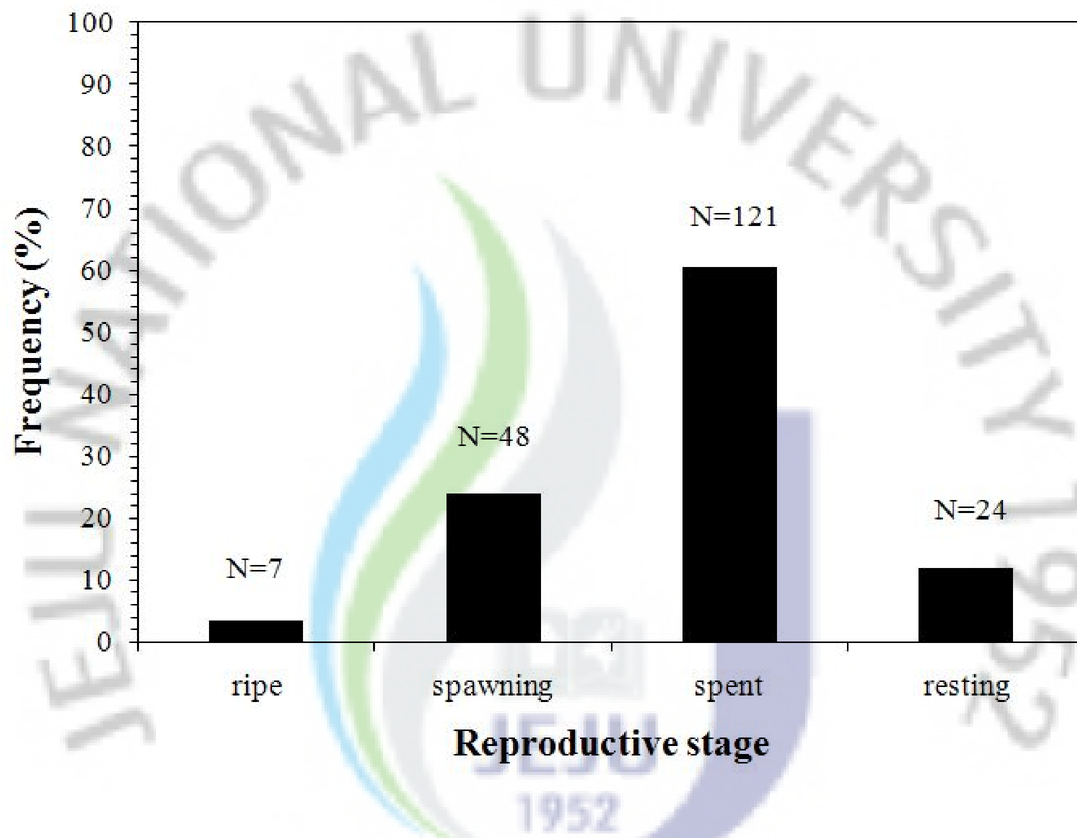

Fig. 6. Frequency of reproductive stage of Manila clam from Goheung.

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grouping the clams by gonad development stage which summarized in Table 4 (ripe, spawning, spent, and resting stage). The mean of CI and protein content closely related with gonad maturity. The highest value was observed in ripe stage followed by spawning, spent, and resting stage. Carbohydrate content was lowest in resting stage. Regarding to the hemocyte parameters, THC and PHG was highest in ripe stage and the lowest was detected in resting stage. On the other hand, MOT and DDM was lowest in ripe stage. To compare the statistical difference of each parameters among different gonad development stage, nonparametric multiple comparisons was conducted in this study.

As a result of nonparametric ANOVA test, significant difference in parameters including CI, PROT, MOT, and PHG was observed. The CI in ripe stage (0.1149) was significantly higher than spawning (0.830), spent (0.766), and resting (0.694) stage (Fig. 7). The variation of protein content was similar to the CI (Fig 8). The highest of protein content (435.4 mg/g DTWT) was found in ripe stage followed by spawning (385.3 mg/g DTWT) and spent stage (368.0 mg/g DTWT). The Manila clam in resting stage showed the significant lowest protein content (349.8 mg/g DTWT). The significant highest (6.4%) and lowest (4.1%) hemocyte mortality was detected in spawning stage and ripe stage respectively (Fig. 9). The Manila clam during ripe stage showed the highest phagocytosis capacity at 36.8% followed by the clam in spent stage (32.9%) and spawning stage (31.1%). The phagocytosis capacity of resting clam was the lowest which 28.9% phagocytosis was observed (Fig. 10).

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Table 4

Mean of parameters grouped by gonad development stage (GS). MT, mature (ripe) stage; SP, spawning stage; ST, spent stage; RS, resting stage; N, sample number; CI, condition index; PROT, milligram protein in gram dry tissue ; CARBO, milligram carbohydrate in gram dry tissue; GPK, *Perkinsus* infection intensity in gill tissue (cells/g); THC, total hemocyte count in hemolymph (cells/ml); MOT, percentage of hemocyte mortality; PHG, percentage of phagocytosis capacity; DDM; percentage of hemocyte DNA damage; SD, standard deviation

GS	N	CI		PROT		CARBO		GPK		THC		MOT		PHG		DDM	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MT	7	0.1149	0.0218	435.4	26.6	85.1	35.6	555,739	319,014	1,265,423	481,648	4.0	5.2	36.8	5.9	10.4	5.9
SP	48	0.0830	0.0201	385.3	28.4	90.1	34.9	555,819	404,411	1,096,203	578,765	6.4	5.9	31.1	9.4	13.8	8.9
ST	121	0.0766	0.0169	368.0	29.2	81.9	31.8	477,706	420,542	1,137,089	634,728	5.5	5.3	32.8	7.4	11.1	6.4
RS	24	0.0694	0.0133	349.8	44.2	74.5	37.0	369,961	370,660	956,636	693,386	5.7	4.6	28.9	6.9	15.0	7.1

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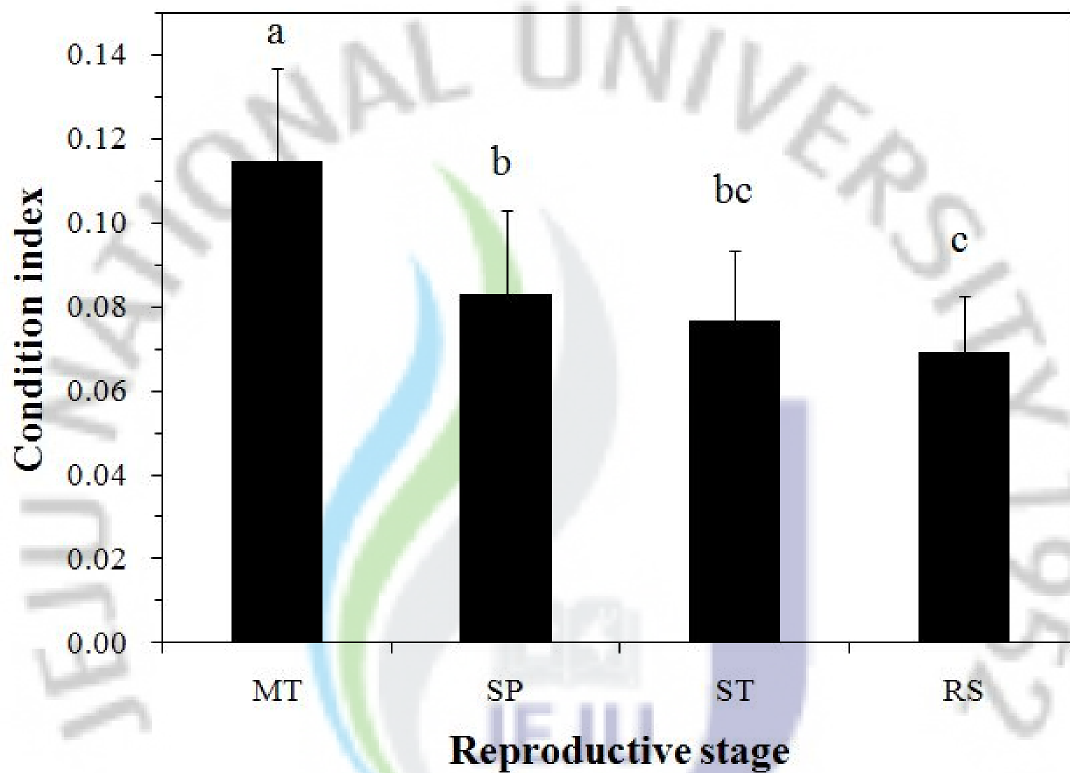

Fig. 7. Condition index of Manila clam grouped by reproductive stage. Different letters (a-c) represent significant differences among gonad stage (rank ANOVA, $p<0.05$). MT, mature (ripe) stage; SP, spawning stage; ST, spent stage; RS, resting stage.

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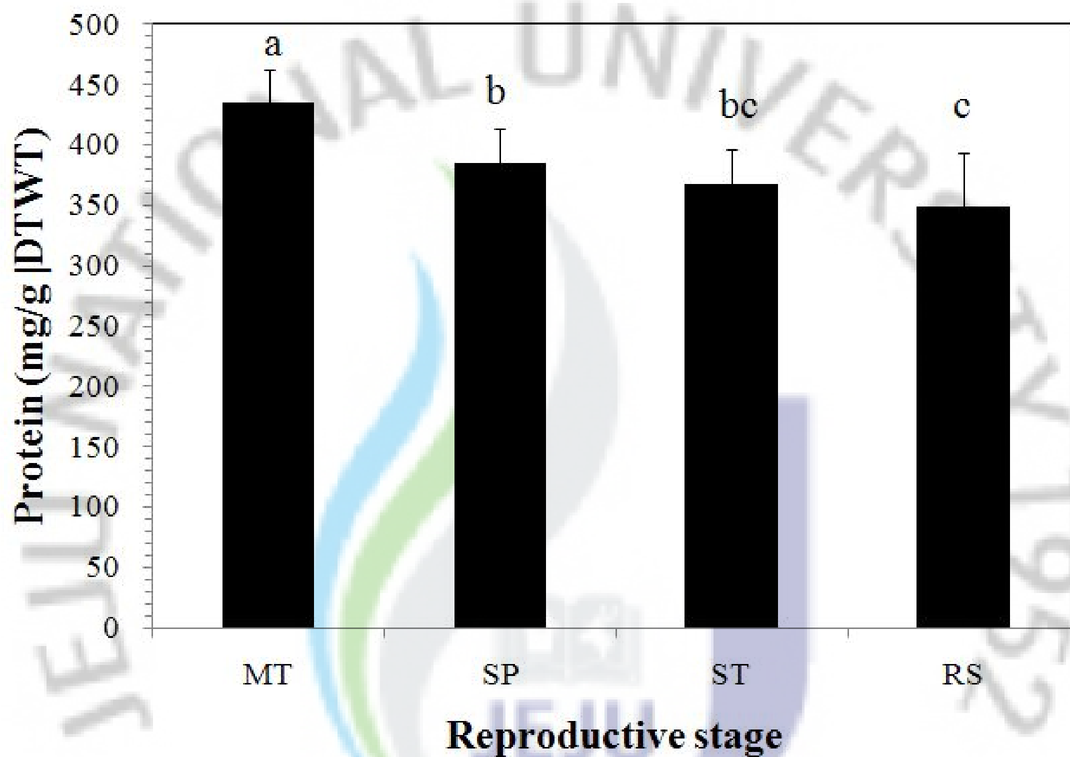

Fig. 8. Total protein contents of Manila clam grouped by reproductive stage. Different letters (a-c) represent significant differences among gonad stage (rank ANOVA, p<0.05). MT, mature (ripe) stage; SP, spawning stage; ST, spent stage; RS, resting stage; DTWT, dry tissue weight.

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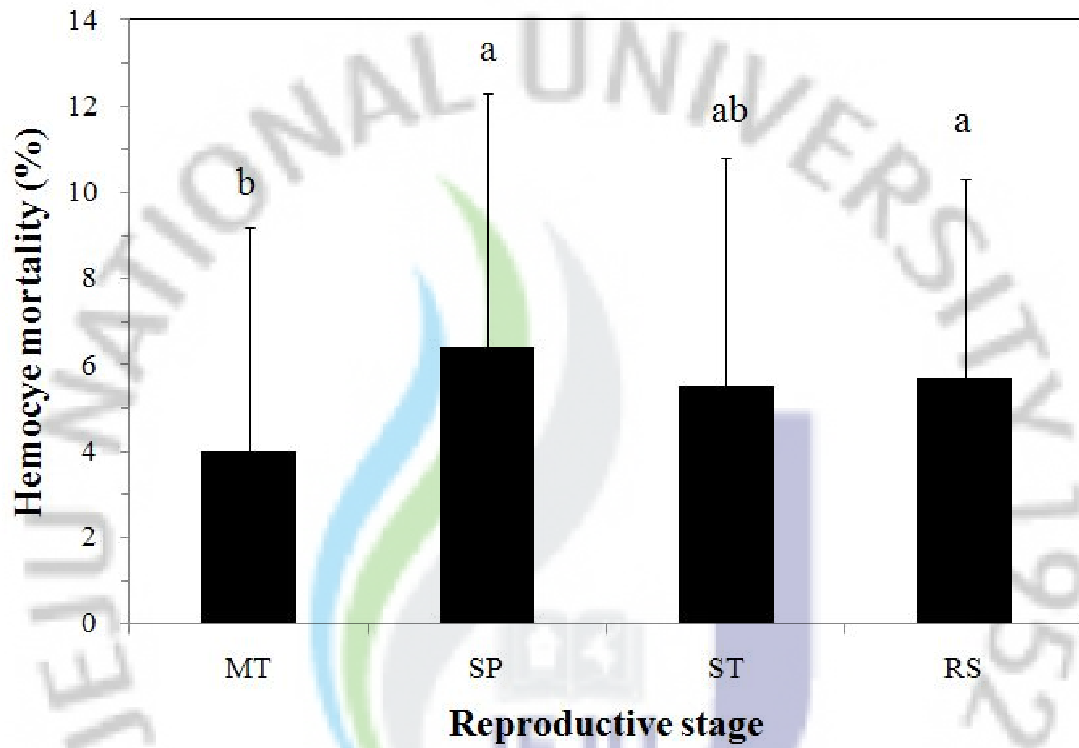

Fig. 9. Hemocyte mortality of Manila clam grouped by reproductive stage. Different letters (a-c) represent significant differences among gonad stage (rank ANOVA, $p<0.05$). MT, mature (ripe) stage; SP, spawning stage; ST, spent stage; RS, resting stage.

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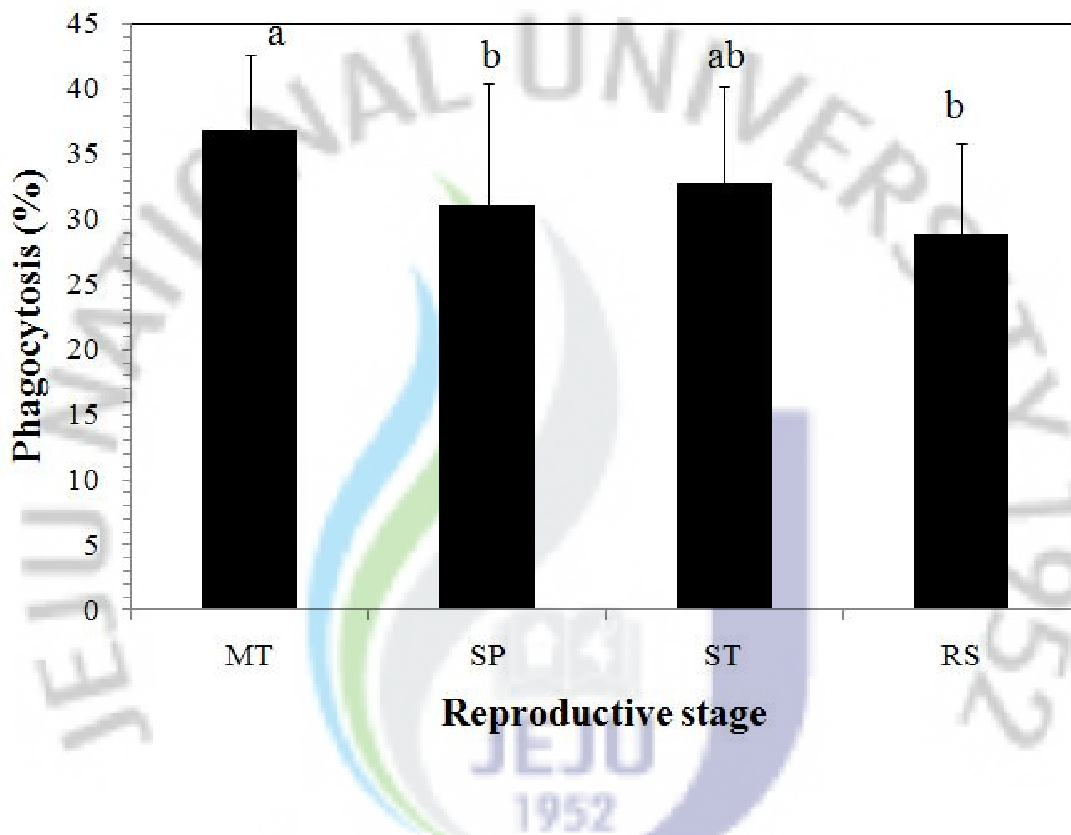

Fig. 10. Phagocytosis capacity of Manila clam grouped by reproductive stage. Different letters (a-c) represent significant differences among gonad stage (rank ANOVA, p<0.05). MT, mature (ripe) stage; SP, spawning stage; ST, spent stage; RS, resting stage.

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3.7. Correlation among parameters

To observe the relation among parameters, Pearson correlation coefficient test was performed in this study. Table 5 shows the correlation coefficient among CI, PROT, CARBO, and GPK in all clams. There was the strong positive correlation between CI and PROT (p<0.01) and between CI and CARBO (p<0.01). To reflect the gonad development stage, we grouped the parameters by GI and tested the correlation among parameters. Due to the small number of ripe clams, the statistic analysis of the correlation among the parameters in ripe clam group was not possible to establish. The strong positive correlation between CI and CARBO was observed in spawning (Table 6, p<0.01), spent (Table 7, p<0.01) and resting clams (Table 8, p<0.01). The positive correlation between CI and PROT also observed in spent clams (Table 7, p<0.05).

Table 9 shows the correlation among CI and immune parameters in all clams. There was positive correlation between CI and THC (p<0.05). Among the immune parameters, there was strong negative correlation between THC and MOT (p<0.01) and between MOT and PHG (p<0.01). Immune parameter also tested the correlation after grouping by gonad development stage (Table 10, Table 11, and Table 12). THC was negatively correlated with MOT in spawning (p<0.01), spent (p<0.01), and resting clams (p<0.01). MOT was negatively correlated with PHG in spawning (p<0.01), spent (p<0.01), and resting (p<0.05) clams.

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Table 5

Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in all clams. *, $p<0.05$; **, $p<0.01$

	CI	PROT	CARBO	GPK
CI	1.0000			
PROT	0.3724**	1.0000		
CARBO	0.4105**	-0.0092	1.0000	
GPK	0.0649	0.2099	-0.0424	1.0000

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Table 6

Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in spawning clams. *, $p<0.05$; **, $p<0.01$

	CI	PROT	CARBO	GPK
CI	1.0000			
PROT	0.2230	1.0000		
CARBO	0.5546**	-0.0479	1.0000	
GPK	0.03623	0.2339	-0.1278	1.0000

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Table 7

Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in spent clams. *, $p<0.05$; **, $p<0.01$

	CI	PROT	CARBO	GPK
CI	1.0000			
PROT	0.2244*	1.0000		
CARBO	0.3330**	-0.1476	1.0000	
GPK	0.0243	0.2011	-0.0855	1.0000

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Table 8

Result of Pearson correlation coefficient test among condition index (CI), protein content (PROT), carbohydrate content (CARBO), *Perkinsus* infection intensity in gill tissue (GPK) in resting clams. *, p<0.05; **, p <0.01

	CI	PROT	CARBO	GPK
CI	1.0000			
PROT	0.2203	1.0000		
CARBO	0.6995**	0.2011	1.0000	
GPK	0.0227	0.1547	0.1324	1.0000

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Table 9

Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in all clams. THC, total hemocyte count; MOT, hemocyte mortality; PHG, phagocytosis capacity; DDM, hemocyte DNA damage; *, $p<0.05$; **, $p<0.01$

	CI	THC	MOT	PHG	DDM
CI	1.0000				
THC	0.1805*	1.0000			
MOT	-0.0243	-0.5187**	1.0000		
PHG	-0.1349	0.0389	-0.4304**	1.0000	
DDM	0.0064	0.0435	-0.0347	-0.0760	1.0000

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Table 10

Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in spawning clams. THC, total hemocyte count; MOT, hemocyte mortality; PHG, phagocytosis capacity; DDM, hemocyte DNA damage; *, $p<0.05$; **, $p<0.01$

	CI	THC	MOT	PHG	DDM
CI	1.0000				
THC	0.2094	1.0000			
MOT	0.0897	-0.6027**	1.0000		
PHG	-0.3232	-0.0190	-0.4698**	1.0000	
DDM	0.0100	0.0699	-0.0734	-0.2343	1.0000

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Table 11

Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in spent clams. THC, total hemocyte count; MOT, hemocyte mortality; PHG, phagocytosis capacity; DDM, hemocyte DNA damage; *, $p<0.05$; **, $p<0.01$

	CI	THC	MOT	PHG	DDM
CI	1.0000				
THC	0.1760	1.0000			
MOT	-0.0543	-0.4950**	1.0000		
PHG	-0.1772	0.0059	-0.4068**	1.0000	
DDM	0.0119	0.0531	-0.1120	-0.0507	1.0000

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Table 12

Result of Pearson correlation coefficient test among condition index (CI) and hemocyte parameters in resting clams. THC, total hemocyte count; MOT, hemocyte mortality; PHG, phagocytosis capacity; DDM, hemocyte DNA damage; *, $p<0.05$; **, $p<0.01$

	CI	THC	MOT	PHG	DDM
CI	1.0000				
THC	0.1466	1.0000			
MOT	-0.0149	-0.5310**	1.0000		
PHG	-0.0899	0.1373	-0.4202*	1.0000	
DDM	0.0713	0.0755	-0.0195	-0.1318	1.0000

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4. Discussion

Many studies have been revealed that CI and biochemical composition had a close relationship with reproductive cycle in bivalves (Marin et al., 2003; Ojea et al., 2004; Kang et al., 2007). According to Ojea et al. (2004), the CI of *R. decussates* in Spain was closely related with its gonad maturity index. The gradual increase of CI coincided with gonad maturation. The CI peaks were observed when the clams are ready to spawn and drops dramatically after spawning caused by weight loss through gonadal discharge. Although our study is not seasonal, we found that the CI reflected reproductive status of *R. philippinarum*.

Protein is a major organic component of gametes of marine bivalves (Kang et al., 2007). In this study, protein contents in clams were reflected the gonad maturity. Protein content was the highest in ripe clams that filled with fully matured gametes in gonad area and the lowest in resting clams that predominantly composed of connective tissue in gonad area.

The CI was closely related with total carbohydrate content in every reproductive stage excepting the ripe clam cause of small sample number for statistic analysis. Carbohydrate, particularly glycogen, has been considered to the main energy reserve for both the formation of gametes and the maintenance of bivalves (Kang et al., 2007). Therefore the positive correlation between carbohydrate content and CI in our study may conclude that the clam accumulated the glycogen as main reserve energy in spawning, spent and resting stages.

Regarding to hemocyte activities, the lowest MOT and the PHG were observed in ripe stage when clams were in good condition and sufficient nutrition status. When the food availability limited or spawning completed, MOT was higher and PHG decreased in *R. philippinarum* (Soudant et al., 2004). It is possible that the effective cellular defense mechanism

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of Manila clam was depended on the good condition. Moreover the strong negative correlation between MOT and PHG was observed. We suggested that the measurement of MOT and PHG can be use to reflect the physiological and health status of Manila clam.

A positive correlation between CI and THC was observed in all clams. Fly-Sainte-Marie et al. (2009) explained that the positive relation between the CI and total THC of Manila clam could link to the amount of stored energy reserves. Besides internal defense, hemocyte was involved in nutrition transport (Cheng, 1996). Therefore, the positive relationship between CI and THC in *R. philippinarum* could be explained by the involvement of hemocyte in nutrient mobilization.

P. olseni infection intensity was any significant effect on CI and hemocyte parameters. Heavy infection of *P. marinus* and *P. olseni* had significant negative effects on CI of oyster and clam (Crosby and Roberts, 1990; Chu and Volety, 1997; Dittman et al., 2001; Park et al., 2006a). In present study *P. olseni* infection intensity was categorized into 5 groups (score 0-4) for comparison the CI of Manila clam, however, *P. olseni* infection intensity was no significant influence on CI. Several studies are shown that heavy *Perkinsus* spp. infection affected on various immunological parameters of bivalves. For example, Ordás et al. (2000) reported a decrease total number of circulating hemocytes in Carpet shell clam infected with *P. atlanticus*, while oysters infected with *P. marinus* showed a trend of increasing the total hemocyte number (Anderson et al., 1992, 1995; La Peyre et al., 1995ab). One of the most important cellular defense mechanisms, phagocytosis, has been found to decrease in clams heavily infected with *P. atlanticus* (Ordás et al., 1999, 2000) or *Perkinsus* sp. (Flye-Sainte-Marie et al., 2009) and oyster infected with *P. marinus* (La Peyre et al., 1995b). On the other hand, Hégaret et al. (2007, 2009) and da Silva et al. (2008) showed that *P. olseni* had no significant impacts on the immunological

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and physiological parameters of Manila clams.

In this study prevalence of *P. olsni* infection was quite high (98%), however, infection intensity was moderate (average 486,254 cells/g gill tissue) in comparison with the report of *Perkinsus* infection in various places of Korea by Park and Choi (2001). Park et al. (2006a) explained that the reason of no effect of the *Perkinsus* spp. on host is the low-level infection of *Perkinsus*. Therefore, it is possible that the infection level of *P. olseni* in Goheung is low to interfere with reproduction or immunity of Manila clam.

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SUMMARY

A multi-parametric study was designed to assess the relative effect of reproduction and parasite infection on physiological and immunological parameters of Manila clam, *Ruditapes philippinarum,* in the field. Two hundred clams were sampled from a population in Goheung, Korea and analyzed individually. For physiological parameters, condition index (CI), protein content (PROT), and carbohydrate content (CARBO) were measured. For immunological parameters, total hemocyte count (THC), hemocyte mortality (MOT), phagocytosis (PHG), and hemocyte DNA damage (DDM) were measured individually using a flow cytometry. Reproductive stage was determined by histology. Infection intensity of protozoan parasite *Perkinsus olseni* was estimated by Ray's fluid thioglycollate medium (RFTM) and histology. CI and PROT were closely related with reproductive status of *R. philippinarum*. The positive correlation between CARBO and CI may conclude that the clam accumulated the glycogen as main reserve energy in spawning, spent and resting stage. The lowest MOT and the highest PHG was observed in ripe stage when clam was in good condition and sufficient nutrition status. Therefore, measurement of MOT and PHG can be use to reflect the physiological and health status of Manila clam. *P. olseni* had no significant impacts on the physiological and immunological parameters of Manila clams. Infection level of *P. olseni* in Goheung may low to interfere with reproduction or immunity of Manila clam.

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감사의 글

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

실험실 생활의 시작부터 지금까지도 늘 따끔한 충고와 용기를 불어넣어주시는 군산대학교 박경일 교수님과 해양연구원 강도형 박사님께 감사의 말씀을 전합니다. 무척추동물양식 실험실 생활 동안 많은 격려와 실험을 도와준 김대경 박사님, Dr. Ludovic Donaghy, Dr. Mausum Adhya, Dr. Md. Jasim Uddin, Yanin Limpanont, 현성이 형, 봉규 형, 태호 형, 나래 누나, 규성이, 김경훈 선배님, 강경표 선배님, Md. Mostafizur Rahman Mondol, Le Thanh Cuong 그리고 석사동기 지연누나, 희도, 희중에게 감사의 마음을 전합니다. 또한, 실험에 필요한 샘플 채집을 해주신 서해수산연구소 박광재 연구사님께 감사를 드립니다.

그리고 하늘나라에서 기도해주시는 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 부모님과 누나에게 감사의 말을 전하고 싶습니다.

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