THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Temporal variation of *Perkinsus olseni* infection among Manila clams (*Ruditapes philippinarum*) transplanted from low infection area to high infection area on the western coast of Korea

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국문요약

*Perkinsus olseni*는 우리나라 서해안에 서식하는 바지락에서 주로 관찰되는 원충류로 2004년 이후로 발생 빈도가 증가하고 있는 바지락 대량 폐사의 주요 원인 생물로 인식되고 있다. 이번 연구에서는 *Perkinsus* 저감염 지역인 파도리와 고감염 지역인 황도 그리고 저감염 지역에서 고감염 지역으로의 이식을 통하여, 바지락의 감염 정도 그리고 이식된 바지락의 건강도 지수 및 서식환경 조사를 실시하여 생물 이식을 통한 생물 대상종인 바지락과 질병 발생 간의 관련성에 관한 연구를 실행하였다.

실험은 2008년 6월부터 9월까지 107일간 이루어졌고, 감염 측정은 Ray's fluid thioglycollate 배양, 바지락 개체의 건강도 지수와 조직 및 생화학적 분석, 서식환경 측정인 수온, 염분 그리고 해수에서의 클로로필 농도 측정을 하였다. 그 결과, 파도리 바지락은 35%의 감염률을 황도의 바지락은 실험기간 내내 100%를 나타냈고, 이식 개체의 감염률은 이식 후 점차 증가하여 49일째에 100%의 감염률을 보였다. 이와 유사한 결과로 감염도 역시 파도리의 감염도는 실험기간 내내 낮은 값 (5,074 cells/WTWT (g))을 보인 반면에, 이식 후 34일째부터 증가하여, 107일째는 고감염 지역인 황도의 감염도 값 (5,327,451 cells/WTWT (g))과 유사한 값까지 증가함을 보였다. 또한, 실험기간 동안 두 지역의 서식환경 변화는 황도가 파도리 보다 수온이 2.4도가 높았고, 클로로필 양도 높게 관찰되었다. 특히, 겨울철부터 봄철에 높은 값을 보인 고감염 지역의 황도에 이식한 개체들의 건강도 지수는 산란 기간이라는 것을 감안하여 보면 증가되었음을 관찰할 수 있었다. 이는 황도의 환경 조건, 특히 풍부한 먹이 조건이 감염의 증가에도 불구하고 개체의 건강도 지수를 증가시킬 수 있는 역할을 하였음을 관찰할 수 있었다. 바지락 개체내의 기관별 (foot, gill, mantle, adductor muscle, siphon, digestive gland)로 *Perkinsus* 감염을 관찰한

결과에서는, 다른 기관에 비하여, gill에서의 감염이 가장 먼저 나타났고, 이 후, mantle, siphon, digestive gland, adductor muscle 그리고 foot 순으로 관찰되었다. 이는 여과와 먹이 섭취와 관련된 기관의 감염이 먼저 일어남을 확인할 수 있었으며, 폐사 개체나 높은 감염을 보이는 개체에서 *Perkinsus*가 배설물 혹은 분비물에 의해서 저질로 나와 해수에 부유되어 여과를 통해서 다른 바지락으로의 이동을 추정할 수 있었다.

따라서, 이번 연구는 저감염 지역으로부터 고감염 지역으로의 바지락 이식 실험을 통해 바지락의 *Perkinsus*에 대한 감염정도를 관찰함으로써, 바지락 이식을 통한 병원체의 유입경로와 바지락 이식을 통한 양식 방법에 대한 기초 연구자료로 이용될 것으로 사료된다.

1. Introduction

Manila clam, *Ruditapes phillippinarum* is one of the most important shellfish resources which inhabits all along the coast of Korea for a commercial culture. Being a high annual product, yielded up to 74,581 ton in 1990 (FAO 2000), this had been a key role in the economic income of fishermen. However it has been declined dramatically ever since, as well as mass mortality of manila clam has occurred since 2004 up to thirty times in 2006 (NFRDI 2007). Factors of the product reduction and mass mortalities were considered as high culturing density, clam disease, pollution or atmosphere changes in culturing area (NFRDI 2007). One of protozoan parasites, the genus *Perkinsus* is disputable as primary element of clam disease associated with mass mortality. The existence of *Perkinsus* sp. in manila clam was confirmed by Choi and Park (1997) and this *Perkinsus* sp. was defined as *Perkinsus olseni* (2001). *P. olseni* was conspecific with *Perkinsus atlanticus*, the causative agent of mass mortality in Europe based on molecular data (Murrell et al. 2002). The infection prevalence was reported over 50% in most of west and south coast areas through the national survey of 24 areas

along the entire coast in Korea (Park and Choi, 2001).

The genus *Perkinsus* is a protozoan parasite that belongs to phylum Perkinsozoa (Noren et al. 1999), class Perkinsea, order Perkinsida, and family Perkinsidae (Levine, 1978) and so far 9 species have been described in it. Over the year since the first discovery of *P. marinus* in the eastern oyster, *Crassostrea virginica* in the Gulf of Mexico (Mackin et al. 1950), *Perkinsus* sp. is given an attention as a causative agent of mass mortalities in various marine molluscs. The life cycle of *Perkinsus* sp. has 3 main stages (Villalba et al. 2004). The first step, the trophozoite is the stage of vegetative proliferation occurring in living host tissue. In this stage, the trophozoite passes through bipartitioning to product daughter cells inside wall (Perkins, 1966). By bursting the wall, the cells come out, then grow and mature. However, under the improper condition, the trophozoite gets enlarged and makes a thicker wall for a new stage named the hypnospore (Villalba et al. 2004). The hypnospore is pre-zoosporangum (Perkins, 1966) and it is durable without progressing to the zoosporulation until to be in proper conditions. This stage can be prolonged depending on the condition and be a dormant stage to withstand under the improper condition

(Casas et al. 2002a). When the condition turns to be proper, the hypnospore goes through zoosporulation (Chu and Greene 1989; Casas et al. 2002a) then discharges zoospores which are able to infiltrate to the host (Villalba et al. 2004). Transmission of *Perkinsus* sp is not involved with intermediate hosts (Ray 1954; Goggin and Lester 1995; Chu 1996; Blackbourn et al. 1998) and the cell is unable to be dispatched with host death. Because the cell is discharged from dying host (Ray 1954, Andrews 1988) or with output, such as feces or pseudofeces (Bushek et al. 1997, Bushek et al. 2002) and most cells survive in vivo at lethal temperature of cold winter. (Andrews 1988, Ragone Calvo and Burreson 1994, Burreson and Ragone Calvo 1996). Symptoms shown on heavily infected host is a cyst on the host tissue externally and perkinsosis retards the host growth, interference energy flux and deteriorates the host fecundity with hemocyte infiltration of the gonad increase internally (Villalba et al. 2004).

Temperature and salinity take a profound role in proliferating and spreading *Perkinsus* sp.. Temperature to restrain the *Perkinsus* cell multiplication is below 20 ℃ (Ford 1996) and to make them the most active, 25 to 30 ℃ is needed. In salinity, *Perkinsus* sp. prefers to high salinity above 25 psu than low salinity and below 10 psu,

the cell division is particularly decreased (Soniat 1996).

Manila clam is more considered as a commercial cultured species and already culturing in the western coast of Korea because of the well growth. However, *Perkinsus* sp. was partly responsible for the recent decrease in the clam product. This study aims to observe effects on the transplanted clam on the occasion of exposure to *Perkinsus olseni* in high infected habitat with time series and to understand a distribution of *Perkinsus* transmission, moreover, to know effects of the clams by habitat condition and its economic value.

2. Materials and Methods

2.1 Experimental design

Observation of 4 areas in the western coast and national survey of 23 areas along the entire coast of Korea have been performed about *Perkinsus* infection of manila clam since 2007 at NFRDI. According to these reports, Padori showed a light infection which was over 5,000 cells/g of infection intensity and 30.8% of prevalence

and Hwangdo was a high infection which was over 1,000,000 cells/g of infection intensity and 99.8% of prevalence. Based on reported data of NFRDI in 2007 and 2008, Padori and Hwangdo were used for low infection area and high infection area (Fig. 1). For the transplanted group, original clams at Padori where infected lightly were collected and measured to sort out clams below 25 mm at shell length (Mean: 33.94 mm±1.81). And each clam was marked with white or black color in order to distinguish transplanted clams from original ones in Hwangdo, then clams were transplanted into Hwangdo area where infected heavily on June 4th 2008.

In the experimental field, 3 squares with 25 m interval were installed (Experiment 1, 2 and 3) and each experiment was given different densities of transplanted clam and original clam (Table 1). After transplanting, 60 transplanted clams from each experiment in the transplanted were sampled bi-monthly and also 70 original clams in Padori and Hwangdo were sampled monthly from June to September 2008.

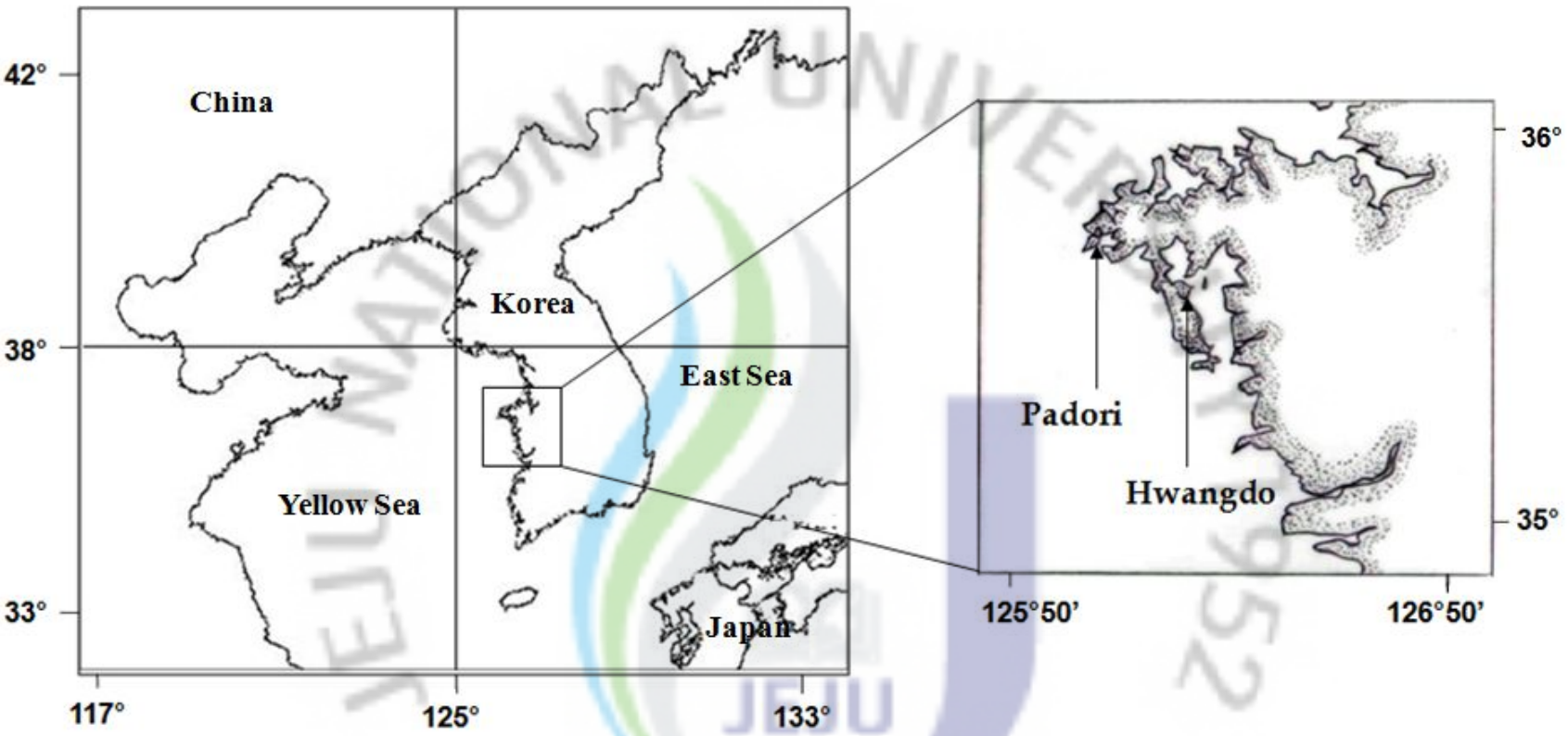

Fig. 1. Location of the sampling areas, Padori and Hwangdo.

Table 1. Experimental design of the transplanted clams. Size of experimental square = 5m x 5m, unit: individuals/m^2, initial day of density: Padori and Hwangdo on May 29 2008, the transplanted on June 4 2008, the transplanted includes Exp 1, 2 and 3.

	Padori	Hwangdo	Transplanted		
			Exp. 1	Exp. 2	Exp. 3
Transplanted	-	-	4,600 ind./ 25m^2 (184 ind./m^2)	2,300 ind./ 25m^2 (92 ind./m^2)	4,600 ind./ 25m^2 (184 ind./m^2)
Original	4,300 ind./ 25m^2 (172 ind./m^2)	7,600ind./ 25m^2 (304 ind./m^2)	-	7,600 ind./ 25m^2 (304 ind./m^2)	7,600 ind./ 25m^2 (304 ind./m^2)
Total	4,300 ind./ 25m^2 (172 ind./m^2)	7,600 ind./ 25m^2 (304 ind./m^2)	4,600 ind./ 25m^2 (184 ind./m^2)	9,900 ind./ 25m^2 (396 ind./m^2)	12,200 ind./ 25m^2 (488 ind./m^2)

2.2 Sampling effort

At laboratory, biometric parameters of each individual (shell length (SL), wet tissue weight (WTWT), dry tissue weight (DTWT), dry shell weight (DSWT)) were measured. Among sampled clams, gill tissue of 40 clams in Padori, Hwangdo and 30 transplanted clams from each 3 experiments were taken for a quantification of *Perkinsus* cells in gill and rests were used for a histological observation and an analysis of biochemical composition.

Thirty clams in Padori and Hwangdo were used for total body burden assay which is the quantification of *Perkinsus* cells in whole body tissue and 30 transplanted clams from each experiment in the transplanted were the quantification of cells in 6 main parts (Foot, Gill, Mantle, Adductor muscle, Siphon and Digestive gland). And this data was also used for total body burden assay by summing up all parts.

2.3 Detection and quantification of *Perkinsus* infection

The tissue (gill, whole body burden or the 6 main parts) from each individual was placed in 5 ml FTM at room temperature in the dark for a week (Ray, 1966). The

addition of 200 units of mycostatin (nystatin) and 2 mg of chloromysetin (chloramphenicol) dissolved in 50 ul was needed for restraining a further bacterial growth. Then, it was dissolved with 2 M NaOH and the number of *Perkinsus* cells at a proper dilution was counted (Choi et al. 1989). After counting, infection intensity was calculated from the number of cells divided by tissue wet weight and prevalence ratio was shown by the number of infected ones among total clams.

2.4 Histological preparation

For histological examination, the middle part of sample tissue which contained the gonad, the digestive gland and the foot was fixed in Davidson's solution for 48 hours and then was dehydrated in ethanol series, cleared in xylene, embedded in paraffin, sliced to 7 μm and stained with Harris hematoxylin and eosin Y. The histological preparation was examined under a light microscope to determine the gonad development stage of the manila clam using the maturity scale described by Park and Choi (2004).

2.5 Proximate composition of the tissue

Remaining samples after taking the middle part for histological preparation were determined in protein and carbohydrate. Samples were freeze-dried for 60 hours and were homogenized in an ultrasonifier. Protein content determination was based on the method of Lowry et al. (1951) using bovine serum albumin as a standard. Carbohydrates were analyzed using the phenol-sulfuric acid method and were measured at 490nm by Spectrophotometer as described by Taylor (1995).

2.6 Mortality and Condition index

To calculate mortality, alive and dead clams among marked clams were separately counted each time in 50cm×50cm quadrate in 3 replicates from each experiment in the transplanted. Only necessary clams were used and rests were put back in order to reduce a variation of the density.

However, there popped up dead marked shells. Each square had no barrier to protect clam getting in/out and to maintain a natural sediment condition. The rope on the land was only taken a role as a boundary, therefore, this study on mortality indicates

minimum index.

Mortality was calculated by ratio of number to dead clams in the transplanted clams and Condition Index (CI) was calculated by ratio of WTWT to DSWT from Padori, Hwangdo and 3 experiments.

2.7 Hydrography

Water temperature in sampling areas was measured every hour using water temperature logger, minilog (HOBO, USA) and was calculated by a daily average. Salinity was taken at each sampling by YSI 85 DO & Conductivity Meter during the period. Chlorophyll-a concentration was measured from collected water at each sampling. The collected water was filtered on a glass fiber filter and added 90% Aceton 10ml in it. And stored for 20 hours in a dark, centrifuged then was determined by the analysis using SCOR/UNESCO calculation (Strickland and Parsons, 1972). This analysis was committed to Environment Research Division in National Fisheries Research and Development Institute (NFRDI).

3. Results

3.1 Hydrography of the sampling areas

3.1.1 Temperature and salinity

The water temperature at Hwangdo ranged from 18.4 to 27.1 ℃ while Padori ranged from 16.0 to 24.9 ℃ (Fig. 2). In water temperature, Hwangdo showed higher values than Padori throughout the period and the value difference was 2.4 ℃. However, the salinities showed similar values of both sampling areas and the values were ranged from 30.1 to 32.9 psu while Hwangdo were from 30.0 to 31.0 psu except the value in July (Fig. 2).

3.1.2 Trophic resource (chlorophyll-a)

Fig. 3 shows the values of chlorophyll-a concentration in the water samples in 2007 and 2008. Padori had a peak value in September while Hwangdo had in May and the other in September. In May at Hwangdo, the peak was not shown in sudden, but it has a pattern which increased gradually due to enough amount of chlorophyll-a in the

water in winter.

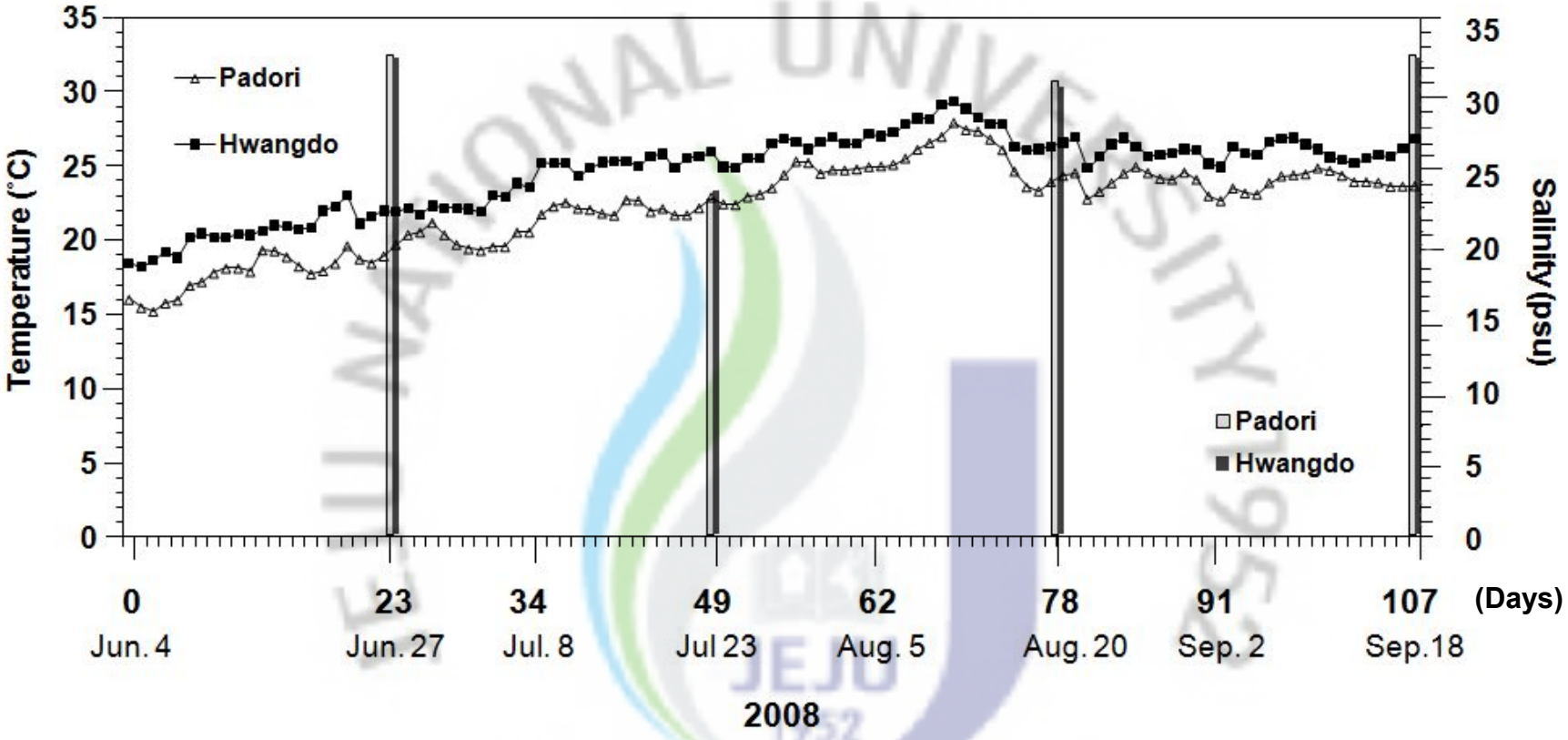

Fig. 2. Variations in water temperature and salinity in Padori and Hwangdo during the course of experiment

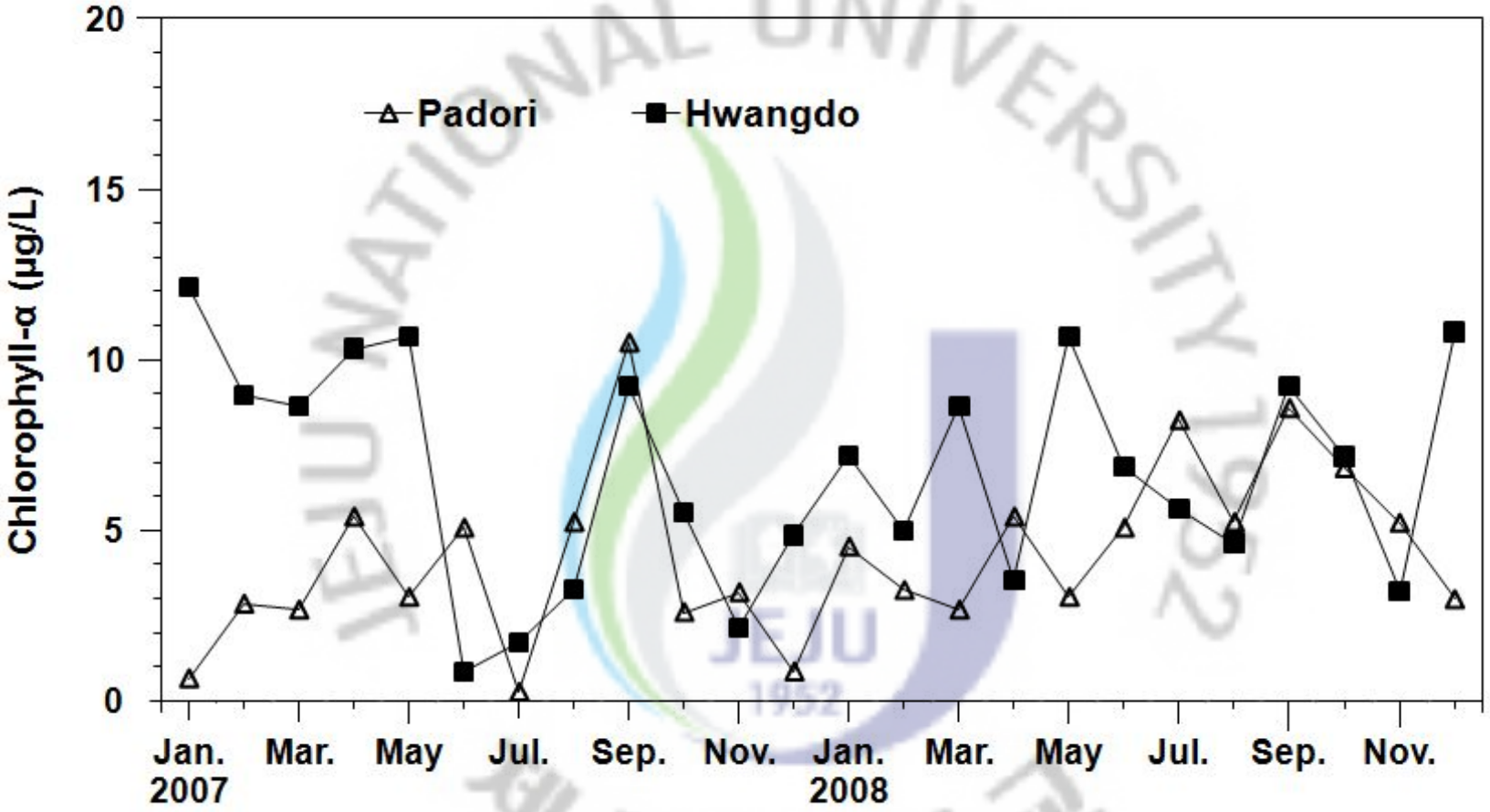

Fig. 3. Monthly changes in chlorophyll-a concentration in Padori and Hwangdo

3.2 *Perkinsus* infection

3.2.1 Infection prevalence

Comparison of Padori, Hwangdo and 3 experiments on *Perkinsus* infection prevalence is shown in Fig. 4. In 2007, the extensive difference was manifested between Padori and Hwangdo. Infection prevalence of Padori was mostly below 50% while Hwangdo was 100% through out the year. Also during the sampling period, Padori had a range from 22.5% to 52.5%, while Hwangdo was 100% in all samplings. In the transplanted group, the former term is when the prevalence of *Perkinsus* infection increased rapidly and reached up to 100% that was from the first sampling to 49th day. And the latter term is when the prevalence had been maintaining 100% as the value of Hwangdo that was 49th day henceforth.

In comparison of 3 experiments on *Perkinsus* infection prevalence (Fig. 4), significant differences are not shown. All the patterns of increments were similar and also show near 100% infection prevalence on 49th day.

3.2.2 Infection intensity

Perkinsus infection intensities in gill and total body burden are shown as Fig. 5 and Fig. 6. From whole body burden assay, infection intensity at Padori and Hwangdo showed a similarity to the intensity in 2007. Padori had below 40,000 cells /g while Hwangdo ranged from 2,902,603 *Perkinsus* cells / g to 5,074,419 cells/g wet weight of gill. 34th day was a significant point for the infection intensity of the transplanted. Before 34th day, intensity value was similar to that of Padori and after 34th day, it gradually reached up to the value of Hwangdo. Likewise, the result of total body burden assay showed its significance on 34th day. However, on 109th day, the last day of sampling, *Perkinsus* infection reached up to the half value of Hwangdo while low and gradual increase of the value were shown on 49th day as the gill.

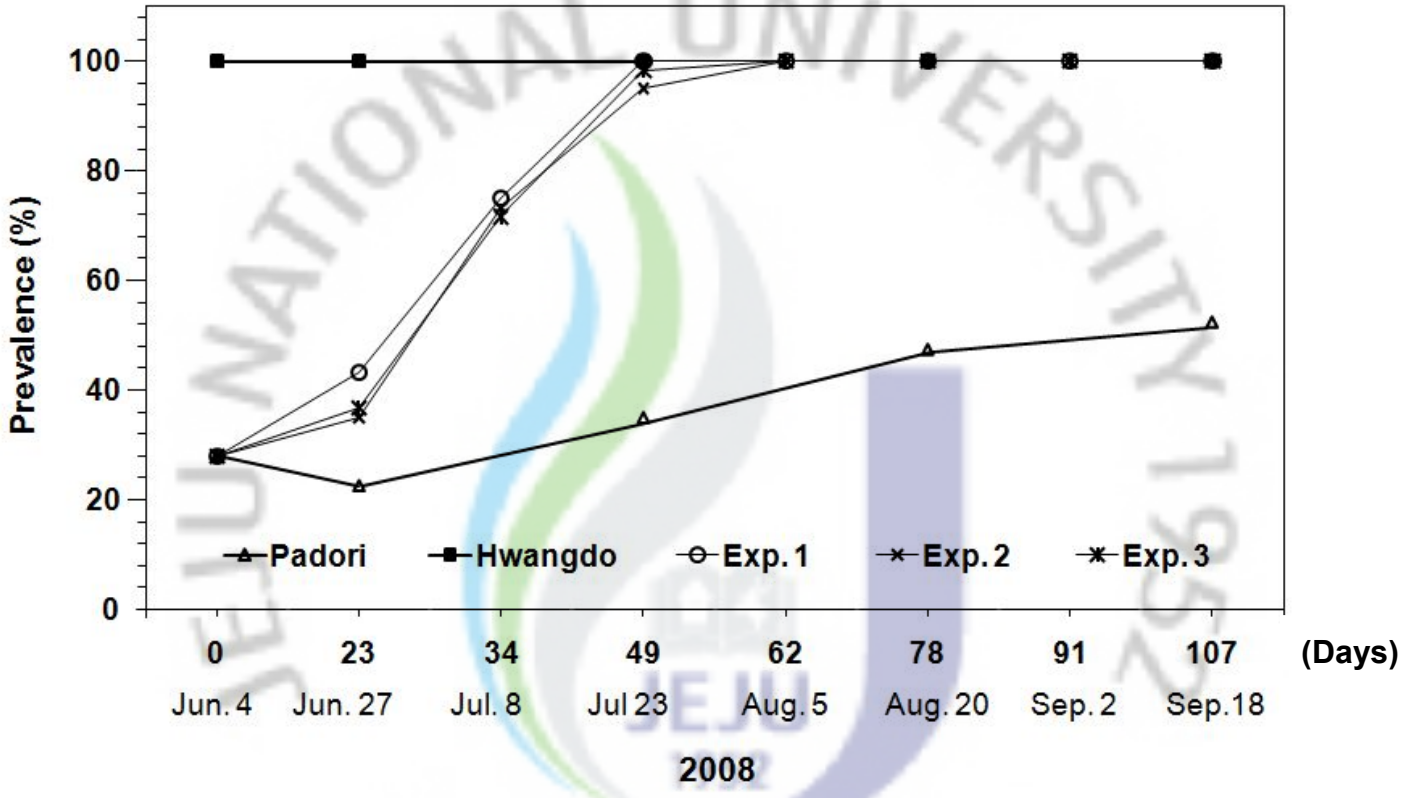

Fig. 4. *Perkinsus* prevalence in Padori, Hwangdo and 3 experiments

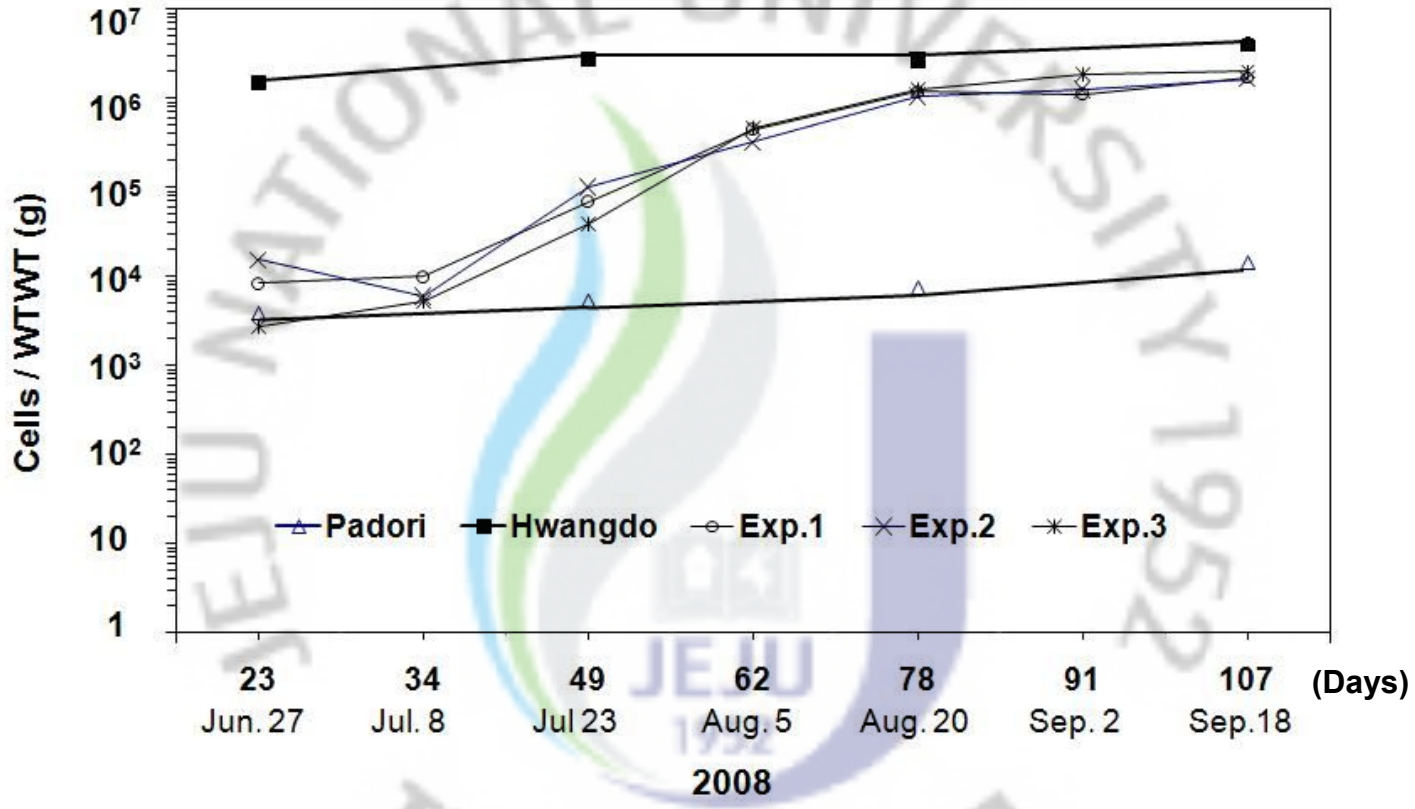

Fig. 5. *Perkinsus* intensity of total body burden in Padori, Hwangdo and 3 experiments

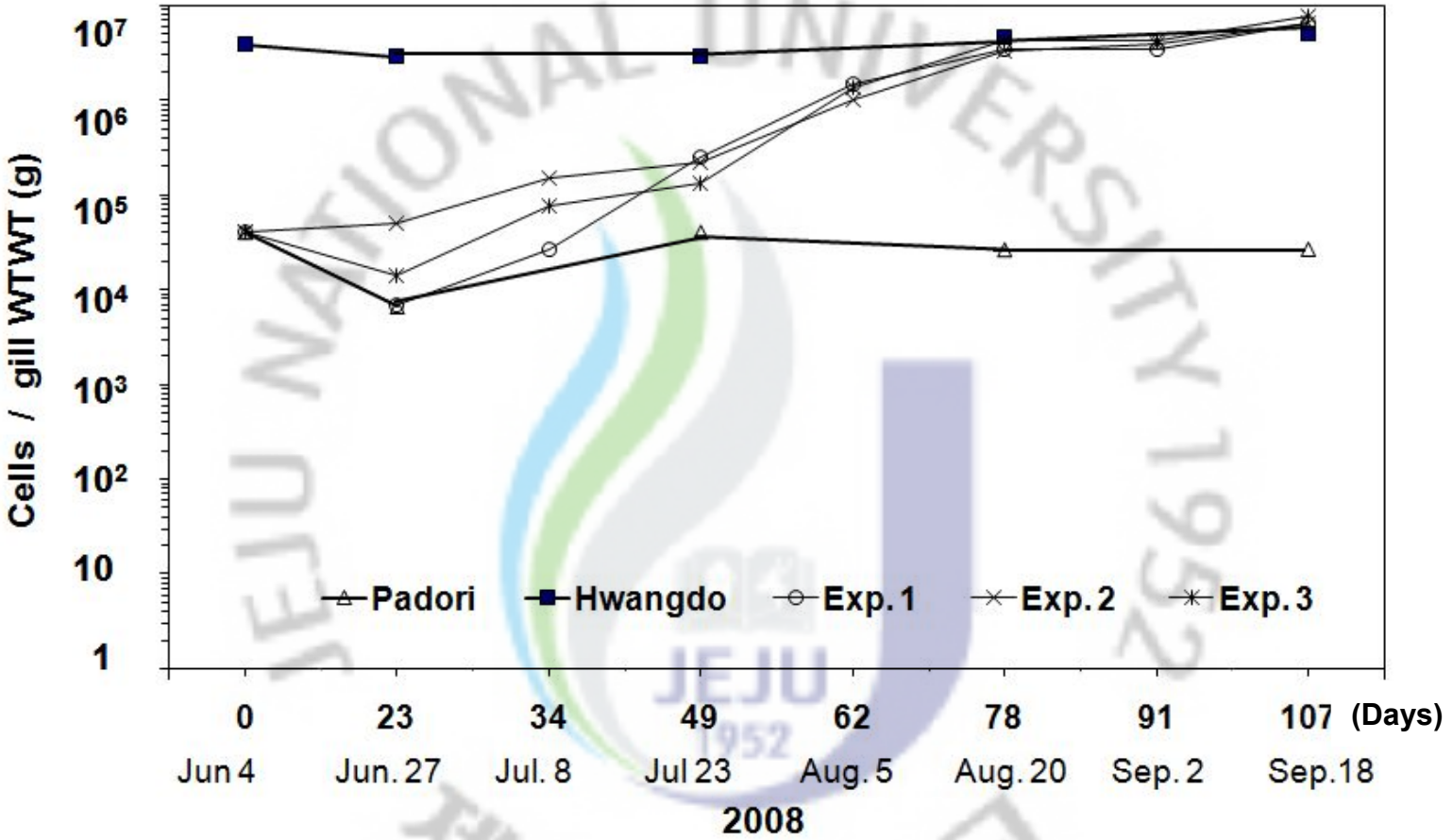

Fig. 6. *Perkinsus* intensity of gill tissue in Padori, Hwangdo and 3 experiments

3.2.3 Infection of 6 main parts

To observe *Perkinsus* infection and its progress in organs, each individual was calculated by infection prevalence (Fig.7) and intensity of 6 parts (Fig. 8). The infection started first in Gill (47.2%), Digestive gland (47.2%) and Mantle (24.7%) on 23rd day, and their intensity reached nearly up to 100% on 49th day, while 3 other parts began to show a remarkable infection on 49th day (73.3% in Siphon, 44.4% in Adductor muscle and 18.0% in Foot) and it progressed towards Siphon, Adductor muscle and Foot, subsequently.

3.3 Gonad development

In the sampling period, gonad development showed a similarity in Padori, Hwangdo and the transplanted. In general, clams in western coast of Korea spawn from July to October; so do those of Padori and Hwangdo. Also, the transplanted clams showed a similar pattern (Fig. 9). Except the first sampling (30.8%) in early June, spawning stage was observed over 70% in the sampling period. In August, the clams in all 3 groups reached a peak value of spawning shown as Fig. 9, however there was no

significant difference compared with other sampling months.

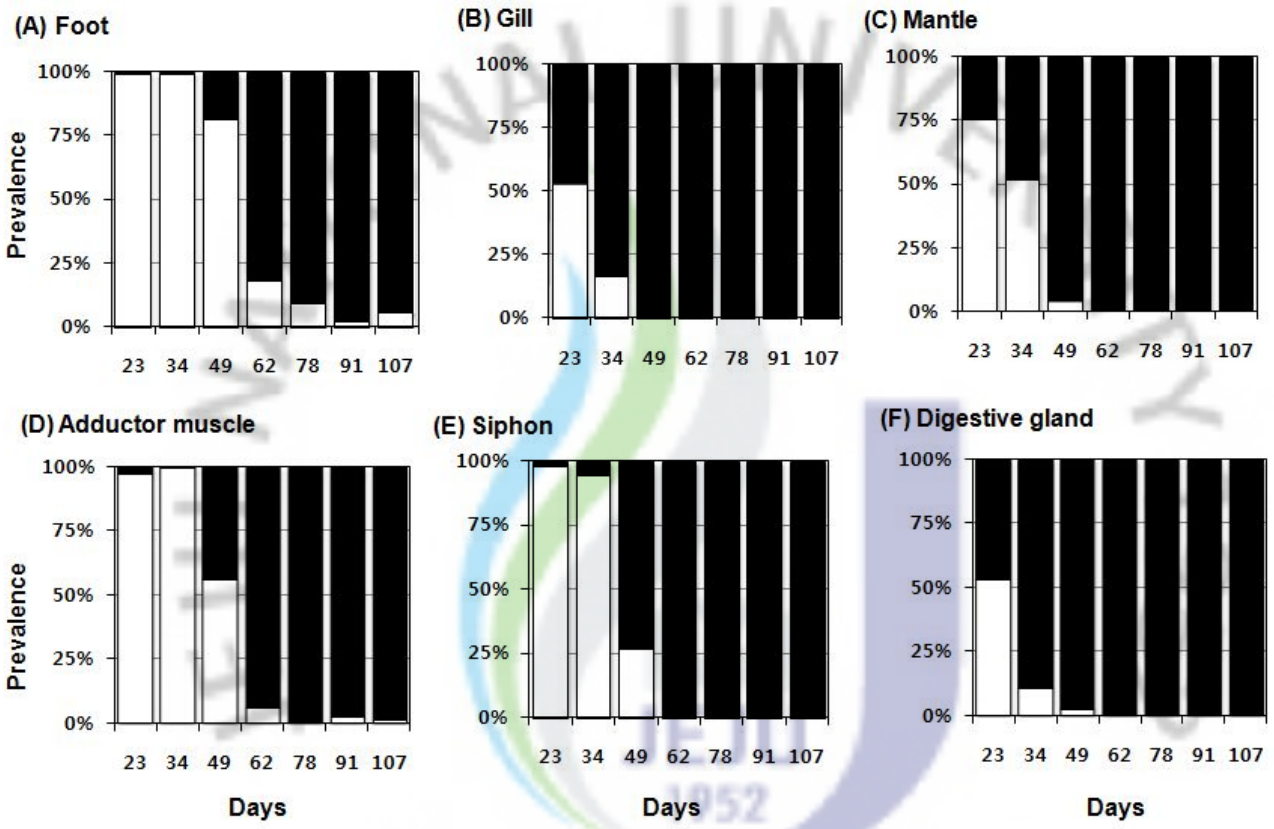

Fig. 7. *Perkinsus* prevalence in 6 organs of individual clams in the transplanted (□ : non-infected, ■ : infected)

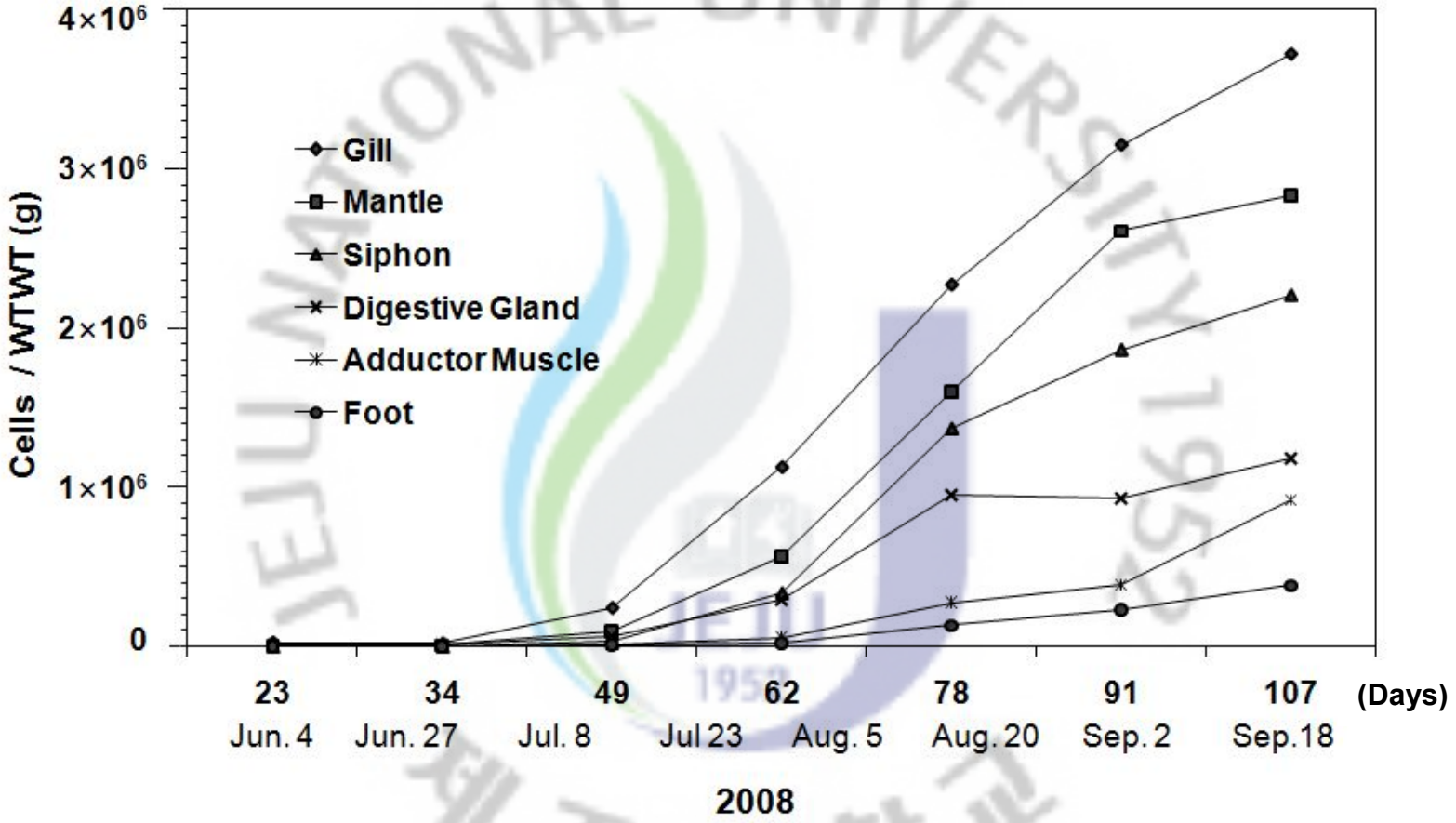

Fig. 8. *Perkinsus* intensity in 6 organs of individual clams in the transplanted

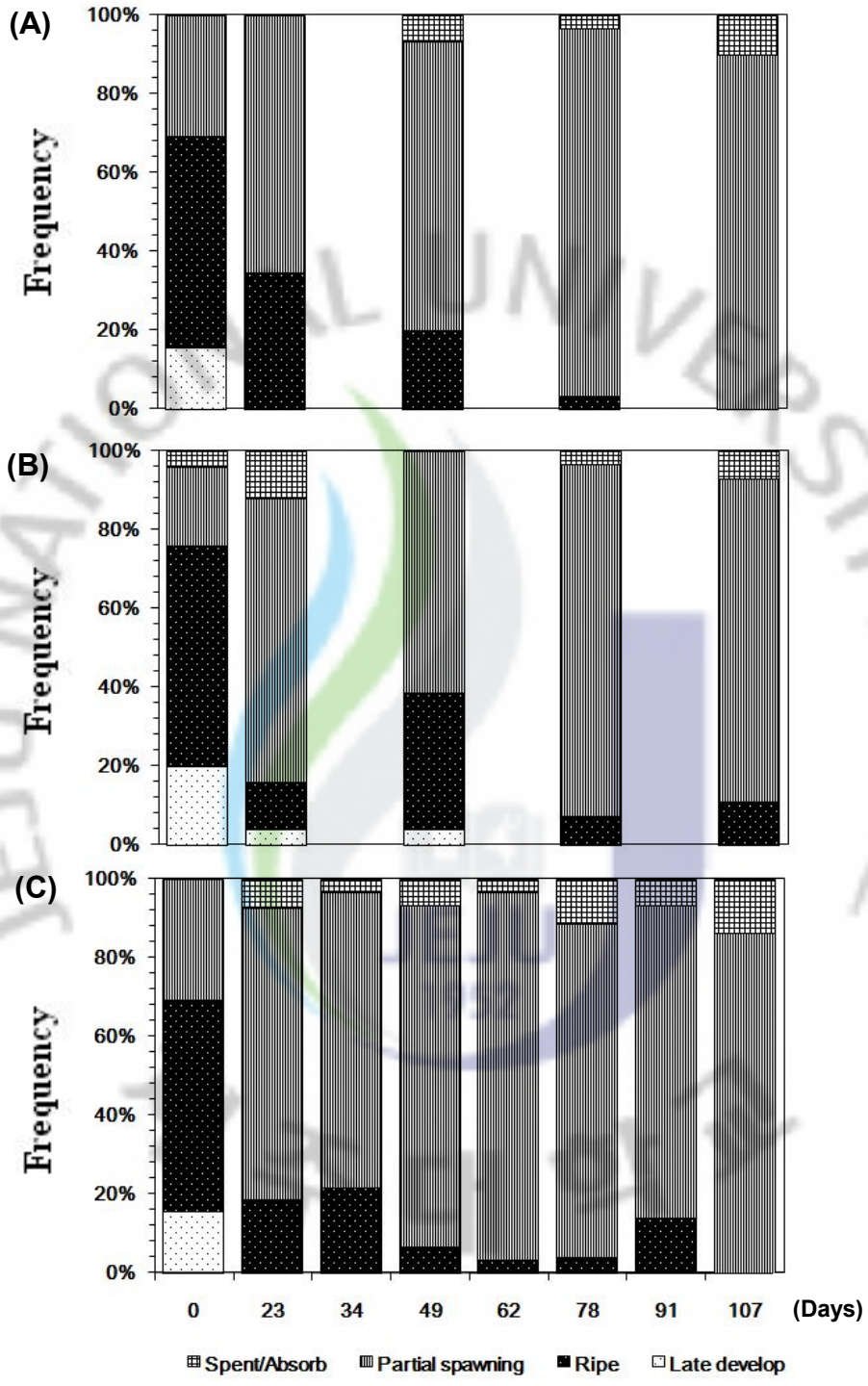

Fig. 9. Frequency of the reproductive stage in Padori (A), Hwangdo (B) and the transplanted (C), no samplings in Padori and Hwangdo on day 34, day 62 and day 91.

3.4 Proximate composition of the tissue

3.4.1 Protein

The variations of total protein content in Padroi, Hwangdo and 3 experiments are shown in Table 2. The values of Padori, Hwagndo and the transplanted clams were all shown similar patterns and no significant increasing or decreasing was existed. And they were all ranged from 255.4mg/g to 435.7mg/g in this study.

3.4.2 Carbohydrate

Table 2 shows the comparison of carbohydrate content in Padori, Hwangdo and 3 experiments. In this figure, nevertheless the value of the transplanted clams started as that of Padori, at the last sampling, they show almost the same value of Hwangdo. All groups showed declining patterns in the sampling, however the transplanted clams decreased to 178.7mg/g of an initial value while the clams of Padori and Hwangdo have fallen off below 178.7mg/g and 263.5mg/g of initial values.

Table 2. Mean values ± SD of the biochemical composition of protein and carbohydrate in the tissue in Padori, Hwangdo and 3 experiments.

	Protein (mg / DTWT (g))					Carbohydrate (mg / DTWT (g))				
Day	Padori	Hwangdo	Exp.1	Exp.2	Exp.3	Padori	Hwangdo	Exp.1	Exp.2	Exp.3
0	324.9±21.8	305.0±30.6	-	-	-	178.7±40.2	263.5±55.0	-	-	-
23	414.5±54.5	317.7±59.5	340.7±31.4	332.4±38.3	361.7±12.7	129.7±24.8	237.5±33.4	150.8±25.7	161.4±22.7	138.8±23.1
34	-	-	285.0±25.9	284.5±21.3	293.3±31.2	-	-	167.9±50.1	129.4±38.2	196.3±44.8
49	371.6±29.3	346.1±42.9	279.5±41.2	254.0±21.9	279.4±42.3	127.4±27.0	282.9±31.2	164.2±42.5	190.6±24.5	142.1±48.9
62	-	-	275.0±44.8	238.3±27.4	269.0±41.1	-	-	141.0±30.6	148.7±29.4	136.8±34.4
78	435.7±21.7	365.2±30.3	349.3±18.8	338.8±21.6	351.0± 9.7	77.9±26.6	228.8±59.1	130.2±34.9	127.5±25.7	149.7±48.1
91	-	-	336.6±35.8	337.2±34.0	319.1±36.4	-	-	110.1±44.4	95.4±28.3	115.1±30.1
107	433.1±36.9	398.9±32.7	285.6±49.0	327.1±30.3	255.4±40.5	50.1±18.6	108.0±32.8	102.5±33.1	86.4±25.9	129.5±29.0

3.5 Biometry and condition index

The biometric measurements of the analyzed clams are shown in Table 3. Each SL of the clams in Padori ranged from 31.01 to 36.53 mm, Hwangdo was from 35.25 to 40.31 mm and the transplanted clam was from 31.01 to 34.71 mm. The mean WTWT of the clams in Padori ranged from 1.55 to 2.34 g, Hwangdo was from 2.77 to 3.38 g and the transplanted clams was from 1.81 to 2.47 g. In general, clams in Hwangdo had higher values of SL and WTWT and Hwangdo and the transplanted were shown similar values in both measurements.

The variations of condition indices among groups are shown Fig. 10. These results were similar patterns to those of total carbohydrates content. The value of the transplanted clams showed a stable pattern while those of Padori and Hwangdo decreased gradually in all samplings although they all spawned in this study.

3.6 Mortality

The mortality of the transplanted clams is shown in Fig 11. The mortality rate was observed nearly 0 % before 62nd day and then slightly increased up to 7.68 % at the

last sampling. This observation was to get information regarding mortality due to transplantation. Rare occasions of the mortality till 62nd day indicated that mortality due to transplantation was not taken place at all. Also, this result was not to be excluded from correlating with *Perkinsus* infection because though the mortality was not occurred till 62nd day, then it occurred since 62nd day that showed 100% of the prevalence and the intensified infection.

Table 3. Mean values ± SD of shell length (SL) in millimeters and wet tissue weight (WTWT) in grams in Padori, Hwangdo and the transplanted included Exp. 1, 2 and 3.

	SL (mm)					WTWT (g)				
Day	Padori	Hwangdo	Exp.1	Exp.2	Exp.3	Padori	Hwangdo	Exp.1	Exp.2	Exp.3
0	33.94±1.81	35.25±3.52	-	-	-	2.34±0.40	3.17±1.21	-	-	-
23	31.01±1.95	37.44±3.06	33.30±1.63	31.41±2.11	31.01±3.02	1.55±0.30	3.38±1.02	2.13±0.35	1.70±0.39	1.65±0.55
34	-	-	33.05±2.18	32.68±1.73	32.22±1.90	-	-	2.18±0.51	2.00±0.38	2.08±0.47
49	35.68±1.66	39.14±4.55	34.61±1.45	32.87±1.60	33.52±1.90	2.31±0.42	3.55±0.76	2.47±0.39	2.06±0.38	2.39±0.47
62	-	-	34.71±1.86	33.85±1.92	32.75±1.75	-	-	2.33±0.41	2.22±0.39	1.95±0.35
78	35.50±2.35	37.94±3.38	33.84±1.96	33.09±1.61	33.59±2.24	1.90±0.46	2.77±0.82	1.81±0.38	1.81±0.33	1.90±0.41
91	-	-	34.34±1.61	31.08±2.07	34.24±1.97	-	-	2.13±0.47	1.89±0.41	2.58±0.59
107	36.53±2.11	40.31±2.76	33.63±2.25	33.10±1.77	33.11±1.85	2.01±0.46	3.21±0.83	2.12±0.50	2.07±0.44	1.93±0.44

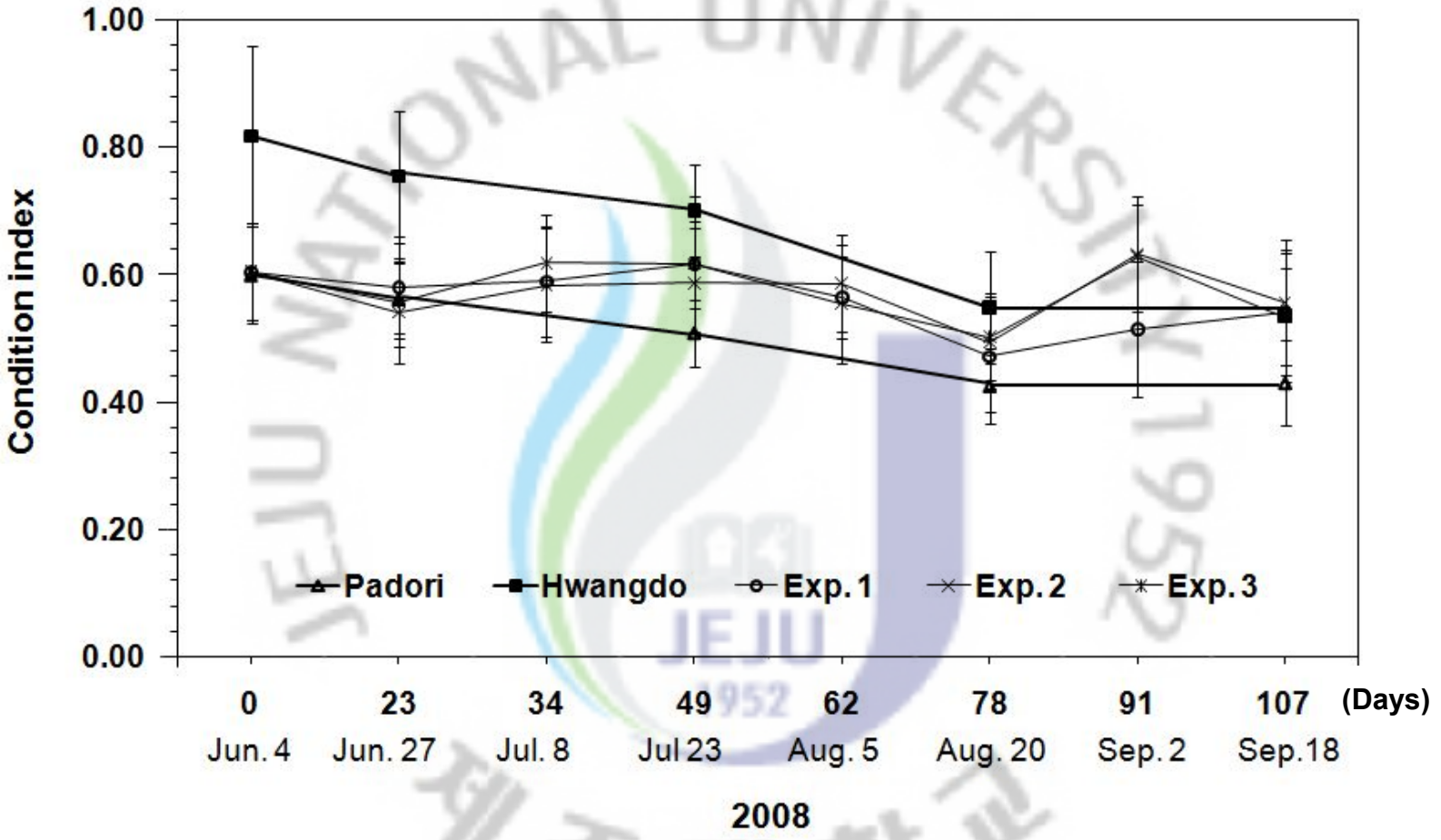

Fig. 10. Variations in condition index in Padori, Hwangdo and 3 experiments

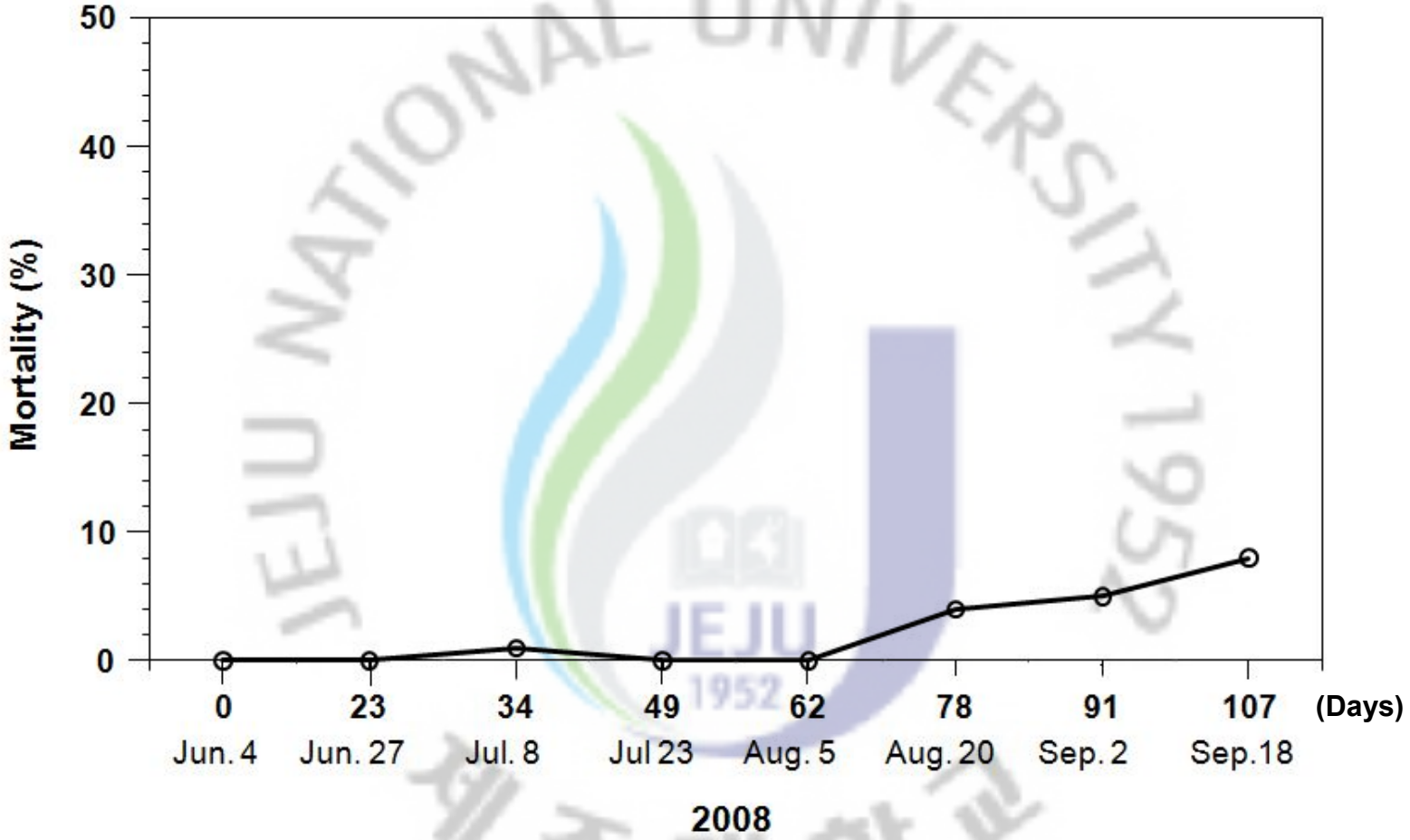

Fig. 11. Mean mortality rate of the transplanted (9 replicates)

4. Discussion

4.1 Effects on *Perkinsus* infection

Environmental factors are profoundly correlated to living organisms and its changes have much influence on organisms. Particularly, marine organisms were affected by factors such as current flow, temperature, salinity and food supply (Ray and Mackin, 1954; Andrews and Hewaatt, 1957; Hewatt, 1956; Soniet, 1996; Burreson and Ragone Calvo, 1996; Cigarria et al, 1997) and parasites lived upon host organism were also affected by them. Thus in this study, temperature, salinity and chlorophyll-a concentration were measured to observe influence of habitat change so to say changes of environmental condition on *Perkinsus* infection of manila clam. Observing local conditions of both sampling areas, Padori and Hwangdo have only 25 km intervals. However, Padori and Hwangdo are shown differences in observation of hydrograph in this study. Hwangdo had 2.4 °C higher than Padori throughout the sampling period (Fig. 2). The higher temperature affected not only the outbreak of *Perkinsus*, but also growth conditions of host clam. Because of the fact that temperature is positively correlated with the proliferation rate of *Perkinsus* cells (Chu, 1996, Soniet, 1996, Park and Chio,

2001) and the fact that temperature may also affect metabolic processes like oxygen consumption or cilia activity of gill.

The outbreak of *Perkinsus* infection is determined by how it goes through the winter. Ford (1996) reported that high winter temperature is more decisive to the proliferation of *Perkinsus* than high summer temperature for oyster. Spreading of *Perkinsus* infection of oyster in the north-eastern U.S.A is consistent with increasing winter water temperature (Cook et al. 1998). It is affected by not only increasing winter temperature, but also by availability of food. Although reduction of food supply was not influential to the *Perkinsus* cell division rate (Powell et al. 1996), it can influence sufficiently to undergo in winter and it is able to control matters after passing critical winter season.

In this study, temperature in Hwangdo showed 2.4 ºC higher than that of Padori (Fig. 2) and salinity was not significantly different during the sampling (Fig. 2). Annual patterns of chlorophyll-a concentration in both areas were similar and the value of Hwangdo was slightly higher (Fig. 3). However, a noticeable point was that Hwangdo had a significantly higher value than that of Padori in winter. It was the fact that the

clams at Hwangdo are able to accumulate more energy with sufficient food supply furthermore it might affect incident time of mass mortality. Therefore, it is likely to be related that Hwangdo has infrequency of mass mortality in early spring, unlikely mass mortality usually occurs early spring in other areas. The reason why sufficient food supply in early spring generates less mortality in the population is that more energy from food intake is used for host growth and less is used for reproduction (Hofmann et al. 1992). In addition, at the results of CI (Fig. 10), the effect was shown by change of food condition. Performing this study in spawning period, at the clam transplanted into Hwangdo, the habitat where got a better food condition, its CI showed more stable value while the original clams in both areas showed a decline caused by spawning. Above results were out from the clams in which infection was intensified rapidly at spawning period and these indicate that habitat condition affected CI which used to decrease by spawning or heavy infection. Though these results were not to be excluded from correlating to a decline in spawning ability due to transplantation, considered the increment of intensified infection, it could be said that habitat condition has a possibility to affect CI of infected clam.

4.2 *Perkinsus* transmission, intensification and mortality

One of the widely studied parts of *Perkinsus* infection is the physiological effects appeared infected host such as the reduction in growth rate, interference in energy flux and the host death (Ford and Tripp. 1996, Park et al., 2006b). As these reasons, *Perkinsus* infection is noted taking a decisive role to conduct the host mortality. However, *Perkinsus* infection does not directly affect host death if correlations with other factors are considered. Mostly, mass mortalities occur in early spring after persisting of winter season, or in late autumn after spawning period of host. The reason why it happens in early spring is correlated to the weaken condition of host caused by low energy accumulation throughout winter combined with *Perkinsus* infection. And mass mortalities in late autumn are also correlated to exhausted condition of host caused by consummation of most energy for spawning combined with *Perkinsus* infection. Therefore, *Perkinsus* infection takes a role to generate host death under the lethal condition. In this study, *Perkinsus* infection prevalence and intensity showed significant changes on 49^{th} day for the transplanted clams (Fig 4, Fig 5). After transplanting, on 49^{th} day, infection prevalence attained 100% and it was also the starting point of intensifying

infection. Therefore, the transplanted clams were completely infected at first, since then the infection was intensified rapidly. It suggests that the incubation time of *Perkinsus* infection was indicated on the 49th day and obvious increasing of infection prevalence (intensity) was detected at this time.

As it is mentioned above, *Perkinsus* heavy infection finally leads host to death (Villalba et al. 2004). At transplantation study in field, mortality due to transplantation can occur in early period after transplanting. However, that mortality rate showed its significance since 62nd day in this study (Fig. 11) was not related to transplantation, because the distance and specific character of clam habitat condition of both areas were considered in order to reduce stress. Therefore, it points out that mortality of the transplanted clams is related to dramatically intensified infection.

4.3 The distribution of *Perkinsus* transmission

Perkinsus sp. is known as an infectious parasite which transmits without intermediate host (Ray 1954). As above, a new host clam can be infected by the infected clam directly and this transmission is performed by filtration process. That is, they use

dying host or fecal discharge of infected host for the route of transmission from infected host to a new host, then disperse into water, and get into other host by filtration process (Bushek et al. 1997). After entering new host through filtering of gill, the cells attach to the cilia of the gills and then distribute to other organs inside the new host (Chinatala et al. 2002). Considered infection prevalence of 6 main parts in this study, the gill and the digestive gland were infected earlier and then it proceeded to mantle, siphon, adductor muscle and finally to foot (Fig. 7). It indicates that the gill which plays a part for entrance and the digestive gland which is the track of food particles are easy to be infected because they have frequency of exposure for incoming water and sediments. Besides, as the results of infection intensity, the gill was intensified most dramatically than other organs and next was the mantle which is also easy to be exposed and be infected directly without passing by other organ (Fig. 8). Afterward, the siphon was next instead of the digestive gland because the siphon takes a role for inhaling and exhaling and the digestive gland had lower intensity in this result showed quantification of *Perkinsus* cells per 1g caused of diluting by weight. Adductor muscle and Foot showed low value of infection intensity because these organs are the last contaminated parts by

being carried in hemocytes and they have fewer cavities due to consisting of the muscular tissue. Of total *Perkinsus* cells in heavy infected clam, the gill accounts for 34.05%, the mantle does 24.42%, digestive gland is 31.05% and other organs account for below 5%. That is, most exposure parts from water, the gill by filtering and the mantle by the shell cavity fluid and the part taken food particles with sediment, digestive gland were distributed most of *Perkinsus* cells.

4.4 Spawning, biochemical compositions and *Perkinsus* infection

Spawning is the prime work for marine organisms and it makes organisms to consume lots of energy. This is why it is led to be under unhealthy condition and weakens the immunity defense function. For that reason, post spawned clams are exposed to the attacks from invaders such as parasite and ultimately these attacks bring about its mass mortality of clams because the majority of the clams spawn concurrently (Park et al. 2006). In this study period performed at spawning season of all groups (Fig. 9), the clams showed the reaction against exposure of *Perkinsus* infection, therefore every infection index manifested an increasing pattern. However total carbohydrate

contents showed a similar pattern as that of CI (Tab. 2). Based on the report that glycogen is used to supply for mobilization of energetic reserves and gametogenesis (Ruiz et al., 1992; Takuji et al. 2002), the fact that the transplanted clams in the transplanted showed a stable patterns at spawning while the original clams of both areas decreased gradually indicates that even the clams under unfavorable condition can be under control by other condition such as habitat or environmental factors.

4.5 Effect on *Perkinsus* infection by different densities

To observe the effects of the density on *Perkinsus* infection, 3 experiments were given each different density of transplanted clam and original clam (Table 1). As the results, there was no significant difference in 3 experiments. That reason is inferred from the route of transmission by the fecal discharge. Even though the transmission of *Perkinsus* infection is directly performed from clam to clam, they are used through the transmission via output such as the feces or pseudo feces as mentioned above. Therefore, because original clams inside of experiment 1 were discarded all just the day before the transplantation, there might be still remaining the fecal discharges of infected clams and

a possibility to come in from outside of site by current flow due to no barrier. In addition, observing *Perkinsus* infection intensity of Hwangdo in 2007 to 2009 from unpublished data in our lab, the average value kept increasing every year. In 2007, 1.8 million *Perkinsus* cells were observed in whole body burden, 2.3 million cells in 2008 and 3.0 million cells in 2009. Bushek et al. (2002) reported that chronically dosed oysters were discharged *Perkinsus* cells in the feces 4 times more than dosed oysters at once since 28th day. It indicates that the ground of Hwangdo has already contained sufficient *Perkinsus* cells in the sediment and these cells keep circulating between clam and sediment and finally these amounts also keep increasing by proliferation of cells.

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Summary

Perkinsus olseni is a protozoan parasite which is observed in Manila clam along the western coast of Korea, varies spatially and temporally. This parasite is responsible for mass mortality associated with clam disease in heavy infected clam. In the present study, we attempted to monitor the infection intensity among clams transplanted from Padori, where the prevalence and intensity recorded as lowest to Hwangdo, the area known as the highest prevalence and infection intensity. After transplantation, clams originally existing in the low infection area, Padori, clams already in the high infection area, Hwangdo and the transplanted clams from Padori to Hwang-do were monitored bimonthly or monthly from June to September 2008 using Ray's fluid thioglycollate medium assay, proximate compositions of the tissue and histology. From the result of *Perkinsus* infection, after 49 days, Hwangdo and the transplanted showed 100% prevalence compared with the prevalence was remained low (35%) in Padori. On 107 days, an infection intensity of 5,328,451 and 5,074,419 *Perkinsus* cells/gram gill tissue wet weight were observed in Hwangdo and the transplanted. In contrast, Padori still

showed a low infection intensity of 5,074 *Pekinsus* cells. The total body burden among transplanted clam increased gradually increased after the transplantation and become equal or higher to Hwangdo after 2 months. However, as results of condition index, the value of the transplanted is more stable value while the original clams in both areas showed a decline caused by spawning. It might be affected by environmental factors, especially in chlorophyll-a concentration in winter. According to results of the hydrograph that observed water temperature, salinity and chlorophyll-a concentration, temperature of Hwangdo was 2.4 C higher and salinity was shown similar. Chlorophyll-a concentration had significant different values. Therefore, sufficient food supply affects to clam condition and also to improve the preventing against *Perkinsus* in the host. Result of the experiment that quantified *Perkinsus* cells of each part divided by 6 organs (foot, gill, mantle, adductor muscle, siphon and digestive gland) showed the gill to be noticeably increased the prevalence and infection intensity than other organs. Following were that of mantle, siphon, digestive gland, adductor muscle and foot. Therefore, it is recommended that the sediment condition has considerable influence upon a transmission of the parasite and that the parasite which may be released to sediment

from the fecal discharges of infected clams or accumulated in dead clam tissues are transmitted to uninfected clam by filtering system of the gill and then distributed to other organs.

감사의 글

이 논문이 완성되기까지 늘 부족한 저를 이끌어 주신 최광식 교수님께 진심으로 감사를 드립니다. 또한, 미흡한 저의 논문을 심사해주신 허문수 교수님과 군산대학교 박경일 교수님께도 감사를 드리고, 학위과정 동안 많은 가르침을 주신 이기완 교수님, 송춘복 교수님, 이제희 교수님, 여인규 교수님, 전유진 교수님, 이경준 교수님, 김기영 교수님, 이영돈 교수님, 정준범 교수님께도 깊은 감사를 드립니다.

실험실 생활의 시작부터 지금까지도 늘 따끔한 충고와 용기를 불어넣어주시는 해양연구원 강도형 박사님께 감사의 말씀을 전합니다. 무척추동물양식 실험실 생활 동안 많은 격려와 실험을 도와준 Yanin Limpanont, 양현성, 강현실, 김봉규, 김태호, 임나래, 강경표, Md. Mostafizur Rahman Mondol, Le Thanh Cuong, 박아름이, 김영민 그리고 석사동기 홍현기, 정희도, 이희중에게 감사의 마음을 전합니다. 또한, 제주도가 아닌 지역에서의 실험을 도와주신 서해수산연구소 증식연구팀의 송홍인 연구관님, 박광재 연구사님, 백상호 박사님, 윤혜영, 한성기, 김경미, 김수경, 김수진, 정진영에게도 감사 드립니다.

그리고 먼 곳에서 항상 지켜봐 주시는 아버지께 감사 드리고, 또한 저를 위해 항상 기도해주시고 응원해주시는 우리 가족에게 감사의 마음을 전합니다. 마지막으로, 끝없이 믿어주시고 격려해주신 어머니께 감사의 말을 전하고 싶습니다.

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