A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Quantitative Reproductive Ecology of Manila Clam, *Ruditapes philippinarum* in Korean Waters

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GRADUATE SCHOOL

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Quantitative Reproductive Ecology of Manila Clam, *Ruditapes philippinarum* in Korean Waters

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CONTENTS

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DEDICATION

This dissertation is dedicated to
my wife, Fatima Sultana Tanvi (Lisa), my daughter, Labiba Homayra,
my parents and my mother-in-law

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국문 요 약

1. 항체면역효소측정법 (ELISA)을 이용한 제주도 성산 연안에 분포하는 바지락(*Ruditapes philippinarum*)의 번식량 측정

1.1. 2006년 6월부터 11월까지 제주도 성산에 서식하는 바지락의 번식량을 효소면역흡착법 (ELISA)과 조직학적 방법을 이용하여 측정하였다.

1.2. 성산 지역 표층 해수의 chlorophyll-a 농도는 7월 초와 8월 중순에 높게 나타났으며, 바지락 조직에서 측정된 chlorophyll-a 농도는 해수와 유사한 7월 하순경 최고 값을 나타냈고 이후 서서히 감소하였다. 이는 바지락의 생식소 발달이 활발한 시기와 산란이 일어나는 시기에 해수와 체내 모두에서 chlorophyll-a 농도가 증가함을 나타내었다.

1.3. 전체 조직 건조중량에 대한 생식소 건조중량의 비로 나타낸 생식소지수 (GSI, gonadosomatic index) 는 생식소 발달기인 6월 초 0.9%였으나 급격이 증가하여 8월초에 19.7%로써 최대 값을 나타냈다. 8월초 이후 GSI는 감소하였으며 이는 조직학적 검사 결과 산란활동과 밀접한 관계가 있음이 확인 되었다. 9월 말 평균 GSI는 4.9%로 감소하였으며 이때는 대부분의 바지락이 산란 후기

상태였다.

1.4. 성산지역 바지락의 최대 월평균 GSI는 19.7%였고 이후 감소하였으며 이는 생식량이 전체 중량의 약 20%에 도달한 이후 산란이 일어남을 의미 하였다.

1.5. 위 사실을 바탕으로 산란이 임박한 시기라고 가정된 GSI가 20.0%이상의 개체들의 포란도를 측정한 결과 2,420,350~8,967,785 개의 알을 포함하고 있었으며 이들은 평균 4,955,706±1,075,854 개로 나타났다.

1.6. 이상의 결과는 우리나라 서해안의 곰소 지역에서 수행된 바지락의 생식소 발달에 관한 연구와 비교할 때 성산지역 바지락의 산란 시기가 2 달 정도 늦게 시작되며 GSI 또한 낮음이 확인 되었다. 이 같은 지역적 차이는 성산 지역의 낮은 먹이 함량 때문인 것으로 판단된다.

2. 서해안 백미리 연안에 서식하는 바지락의 연중 생식주기에 대한 번식량 측정

2.1. 2007년 서해안 백미리 지역 바지락 양식장에서 채집된 암컷 바지락의 생식주기와 번식량을 조직학적 관찰과 ELISA를 이용하여 측정하였다.

2.2. 본 연구를 위하여 암컷 바지락의 중앙부를 두께 3 mm의 단면으로 적출하여 이를 조직학적 관찰에 이용하고 나머지 부분은 생식량과 생화학적 정량에 이용하였다.

2.3. 암컷 바지락의 번식량 정량을 위하여 New Zealand white rabbit을 이용하여 바지락 알에 대한 특이 항체를 개발하고 면역흡착법을 이용하여 Rabbit anti-clam egg IgG를 분리하였다. 개발된 항체는 바지락 알 단백질에 매우 특이적 반응을 보였고 효소면역흡착법 (ELISA)를 이용하여 측정한 결과 최소 177 ng/ml의 알 단백질이 측정 가능함을 확인하였다. 바지락의 번식량은 바지락 전체 건중량에서 생식소 건중량을 나타내는 생식소중량지수 (GSI)로 나타내었다.

2.4. 암컷 바지락의 월평균 GSI는 생식소 초기 발달기에 3.5±2.2%였으며 후기발달기에는 10.8±6.5%, 성숙기에는 24.9±8.2%, 산란기에는 12.0±8.8%, 산란후기에는 5.2±4.6%를 나타내었다. 본 연구에서 수행된 ELISA를 이용한 바지락의 번식량 측정법은 바지락의 생식소 발달기인 후기발달기, 성숙기 및 산란기에는 번식량 측정에 매우 효과적이지만 산란후기와 생식소 초기 발달기에는 측정효율이 감소하였다.

2.5. 조직학적 관찰을 통해 생식소가 성숙기로 확인된 바지락의 포란수를 ELISA로 확인한 결과 바지락 1 개체 당

2,544,553~13,839,452 개의 알을 포함하고 있으며 이는 평균 7,140,354±2,881,586 개의 알을 보유하고 있는 것으로 측정되었다.

2.6. 생식소 발달에 따른 번식량과 성숙한 바지락의 포란수 측정은 이매패류의 번식량 연구에 효과적으로 사용할 수 있을 것으로 사료된다.

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SUMMARY

1. Quantification of reproductive effort of Manila clam, *Ruditapes philippinarum* from Seong San, east coast of Jeju, Korea by enzyme-linked immunosorbent assay (ELISA)

1.1. The reproductive effort of Manila clam, *Ruditapes philippinarum* was estimated by enzyme-linked immunosorbent assay (ELISA) along with histology. The clams were collected from a natural habitat at Seong San, off the east coast of Jeju, Korea during the active gametogenic period from June to November 2006. A polyclonal antibody developed against Manila clam egg-specific protein was used for the quantification of Manila clam egg protein by indirect ELISA.

1.2. Chlorophyll-a level in the water column exhibited two peaks; first during early July coinciding with the ripening of the clams and second timing mid August when majority of the clams were engaged in spawning activities. The food intake was considerably higher during ripe and spawning stages than rest of the gametogenic phases as evidenced from the chlorophyll-a concentration in the clam tissues.

1.3. The gonadosomatic index (GSI), a ratio of egg dry weight to tissue dry weight varied from 0.9% (early June when most of the analyzed clams were in early developing stage) to 19.7% (early August when most of the clam were ripe) by mean. A declining inclination in GSI after early August confirmed the major

spawning period of the clams in this habitat which was consistent with the histological observations. GSI dropped to 4.9±2.4% during late September when clams were in spent phase as evidenced from histology.

1.4. The GSI peaked only once during early August when the mean value was 19.7% indicating that the clams underwent spawning when the gonad occupied around 20% of the total tissue weight.

1.5. We assumed that the clams were ripe and ready for spawning when the gonad occupied over 19.7% of the body weight and the potential fecundity was estimated in those clams having individual GSI over 19.7%. The potential fecundity, as obtained from ELISA data, ranged from 2.42 to 8.97 million, averaging 4.97±1.08 million (n=28).

1.6. Spawning of this clam was delayed by two months and the mean GSI was somewhat lower than those reported from Gomso Bay, west coast of Korea. The remarkable difference in timing of spawning and quantity of reproductive materials might be associated with difference in the availability of foods between those two habitats.

2. Reproductive efforts in relation to annual gametogenic cycle of Manila clam, *Ruditapes philippinarum* collected from Begmiri, west coast of Korea

2.1. The gametogenic stage-wise reproductive efforts of Manila clam, *Ruditapes philippinarum* was quantified collected monthly during 2007 from a commercial bed at Begmiri, west coast of Korea using combined histology and

enzyme-linked immunosorbent assay (ELISA).

2.2. A thin slice (3 mm) was cut through the gonad of each soft clam by a special knife for histological preparations to confirm the gametogenic stage and the remaining part was lyophilized and used for the quantification of reproductive effort by ELISA as well as for the quantification of proteins and carbohydrates.

2.3. Rabbit anti-clam egg IgG was developed in New Zealand white rabbit following the standard immunization protocol. The immunized rabbit IgG was purified from the blood using immunoadsorbent and 50% ammonium sulfate treatment followed by dialysis. The antibody was highly specific to clam egg protein and was sensitive enough to detect minute quantity of the egg protein by ELISA.

2.4. The mean GSIs were 3.35±2.15% in early developing females, 10.82±6.49% in late developing females, 24.92±8.21 in ripe females, 12.01±8.79% in spawning females and 5.23±4.59% in case of spent females. This method could successfully detect reproductive effort in late developing, ripe and spawning clams however, the eggs could be detectable in most of the spent clams and not sensitive enough to detect reproductive effort in most of the early developing clams.

2.5. The potential fecundity was estimated only for ripe clams as confirmed from histology. The potential fecundity varied from 2,544,553 to 13,839,542 with an average of 7,140,354±2,881,586.

2.6. The new approach for the estimation of gametogenic-stage wise GSI and potential fecundity of precisely selected ripe clams can be used successfully in

quantitative study of reproduction in bivalves.

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SINCE 1952

LIST OF FIGURES

CHEJU NATIONAL UNIVERSITY · SINCE 1952 ·

LIST OF TABLES

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BACKGROUND

The quantity of reproductive output and the time and duration of spawning are crucial for understanding life history, population dynamics, as well as for proper management of that particular species. Numerous techniques have been used to assess the reproductive condition of marine mollusks (Deslous- Paoli and Heral, 1988; Choi et al., 1993; Kanti et al., 1993; Chung et al., 2001; Park and Choi, 2004). Histological methods have been the most widely and frequently used, since histology provides visual information about gonadal tissues. The gametogenic status can be graded based on the microscopic appearance of the gonadal tissues from the histological preparations (Lango-Reynoso et al., 2000). Histology is an indispensable technique for studying reproduction because it has the ability to detect disease agents, pathological lesions and morphological features, which can be used to provide important information to the overall condition of the animal, in addition to reproductive status (Diggles et al., 2003). Some quantitative methods have also been developed for the assessment of bivalve gonads by coupling histology and image analysis. It involves enumeration of gametogenic products either by percent gonad area or percent oocyte area (Eversole et al., 1980; Heffernan and Walker, 1989; Kanti et al., 1993; Mann et al., 1994; Park and Choi, 2004; Royer et al., 2008), by gonadal weight and gonadosomatic index (Royer et al., 2008), by mean egg number per field of view (Walker and Heffernan, 1994), or by oocyte size/frequency distribution (Kanti et al., 1993; Mann et al., 1994; Walker and Heffernan, 1994; Park and Choi, 2004). Though histology is widely used for

the study of bivalve reproductive biology but it does not provide precise information on bivalve gonad biomass.

The accumulation of energy reserves and the allocation of reserves into somatic growth and/or gametogenesis are directly linked with the sexual maturity of the mollusk species and the availability of foods in that particular habitat (Sastry, 1979; Camacho et al., 2003). Seasonal changes in the biochemical composition of bivalve mollusks have been extensively studied in bivalves (Marin et al., 2003; Camacho et al., 2003; Kang et al., 2007). The gametogenic cycle of bivalves involves the storage of energy reserves to support gametogenesis, the production and accumulation of gametes by cell proliferation and differentiation, the release of ripe gametes and the recovery or resting period (Giese and Pearse, 1974; Thompson et al., 1996). For the explanation of above events, generally six stages of reproductive maturity of bivalves including the indifferent stage have been used by most authors for studying the reproductive biology using histology (Kanti et al., 1993; Xie and Burnell, 1994; Park and Choi, 2004; Drummond et al., 2006). Annual gametogenic cycle, and timing and duration of the spawning of marine invertebrates are strongly influenced by exogenous factors (Sastry, 1979; Meneghetti *et al.*, 2004; Drummond *et al.*, 2006). Temperature is influential for the initiation of gametogenesis and spawning, whereas food availability determines the extent and duration of gonadal development (Delgado and Camacho, 2007). Numerous studies have reported that gonad development and subsequent spawning of marine bivalves is synchronized with seasonal changes in available food in the water column (Grant and Creese, 1995; Kang et al., 2000; Park and Choi, 2004).

Reproductive output or fecundity of marine bivalves has been estimated by measuring the difference in weight just prior to and after spawning (Deslous-Paoli and Heral, 1988; Pouvreau et al., 2000), counting or weighing the gametes released after inducing animals to spawn using various chemicals and thermal shock (Toba and Miyama ,1991; Chung et al., 2001; Massapina et al., 1999), or estimating the quantity of eggs from histological preparation of the gonad using steriology or planimetry (Perdue and Erickson, 1984; Dinamani, 1987; Lango-Reynoso et al., 2000). These methods often underestimate the true gonads, as reproduction is not always complete and occurs at various intensities throughout the spawning period (Lucas, 1982; Thompson et al., 1996). Immunological methods have been used successfully for quantification of egg proteins of marine bivalves due to their high speed, low cost and high sensitivity (Choi et al. 1993; 1994; Kang et al., 2003; Park et al. 2003; Park and Choi, 2004; Park et al., 2005). However, the seasonal changes in reproductive output were focused in the above reports, though inter-individual variations in reproductive stages at any sampling date are often common in bivalves as evidenced from the histology. Therefore, it is necessary to quantify the dynamics of gametogenic materials in relation to reproductive cycle of the bivalves independent of season.

Fecundity is the total number of eggs released per female in a year (Hunter et al., 1992). Usually, the fecundity is determined either experimentally by counting the eggs after spawning (actual fecundity), or by measuring the potential fecundity in the pre-spawning gonad. Potential fecundity is defined as the total number of matured oocytes per female in a year in the pre-spawning gonad at the

time when no more oocytes are assumed to be recruited to the seasons maturing stock, uncorrected for atretic losses (Macer, 1974; Kjesbu et al., 1991; Hunter et al., 1992). As the spawning of Manila clam is often incomplete and occurs continuously throughout the spawning season (Park and Choi, 2004), it makes difficult to monitor the reproductive activity of mature individuals over an extended period of time. Due to determinate fecundity, the standing stock of the yolk oocytes of the Manila clam prior to the onset of spawning is considered to be equivalent to the potential annual fecundity. Within a given species, fecundity may vary as a result of different adaptations to environmental habitats (Witthames et al., 1995).

The potential fecundity was estimated by ELISA in case of American oyster, *Crassostra virginica* (Choi et al. 1993), Pacific oyster, *C. gigas* (Kang et al. 2003) and Manila clam, *R. philippinarum* (Park and Choi 2004). However, in the above reports, the fecundity was estimated for all females when the GSI peaked assuming that all the individuals were ripe and did not release any egg yet. In all cases, they reported more than one peaks and accordingly they estimated fecundity GSI peak-wise. As the spawning of Manila clam is often asynchronous, it is difficult to capture all the mature clams in a specific sampling period. The precise selection of ripe clams is most important in estimating potential fecundity as it is the estimate of total number of ripe eggs in the pre-spawning gonad.

On the above context, the present study was undertaken with the following specific objectives:

i) To determine the annual gametogenic cycle of Manila clam

collected from a natural habitat at Seong San, east coast of Jeju, Korea

ii) To quantify the reproductive efforts of female clams during active gametogenic condition by an enzyme-linked immunosorbent assay

iii) To estimate the potential fecundity of this clam in this habitat

iv) To develop a new approach for the quantification of reproductive outputs of female Manila clams in relation to gametogenic cycle collected from a commercial bed at Begmiri, west coast of Korea

v) To estimate the potential fecundity of precisely ripe clams in this habitat.

Part I

Quantification of reproductive output of Manila clam, *Ruditapes philippinarum* from Seong San, east coast of Jeju, Korea by enzyme-linked immunosorbent assay (ELISA)

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ABSTRACT

The reproductive effort of Manila clam, *Ruditapes philippinarum* was estimated by an enzyme-linked immunosorbent assay (ELISA) along with histology. The clams were collected from Seong San off the east coast of Jeju, Korea during the active gametogenic period. A polyclonal antibody developed against Manila clam egg specific protein was used in the quantification of egg protein using indirect ELISA. The mean gonadosomatic index (GSI) varied from 0.9% (early June) to 19.7% (early August). The mean GSI was 0.9±0.6% during early June when the analyzed clams were principally in the early developing stage. Mean GSI then increased rapidly, exhibiting only one peak during early August (19.7±11.0%) when majority of the clams (48.3%) were ripe and ready for spawning. A declining inclination in GSI after early August confirmed the major spawning period of the clams in this habitat which was in consistent with the histological observations. Chlorophyll-a concentration in the water column as well as in the tissues of the clams was relatively higher during spawning period. GSI dropped to 4.9±2.4 during late September when all the clams used for ELISA were in spent phase as evidenced from histology. The potential fecundity, as converted from ELISA data, ranged from 2.42 to 8.97 million, averaging 4.97±1.08 million (N=28). The process of gonad development corresponded to an absolute increase in total proteins and carbohydrates. After evacuation of gametes, carbohydrates decreased to a quarter whereas, proteins declined to a half of their maximum

values at peak. In conclusion, the delayed spawning time and the lower mean peak GSI values than those at west coast of Korea might be associated with the lower food availability in this habitat.

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SINCE 1952

1. INTRODUCTION

Studies on reproduction, including the assessment of size at maturity, fecundity, duration of spawning season, daily spawning behavior and spawning fraction, permit quantification of the reproductive capacity of individual female. Establishment of extensive data bases on reproductive parameters with corresponding data on abiotic factors enables the study of causal relationships between reproductive potential and environmental variations (Murua et al., 2003). In the previous study of the Manila clam on the east coast of Jeju, Korea, Kang et al. (2004) reported that gametogenic activity was evident from late March with over 50% of females ripe by late June and a single spawning peak in early August. In contrast, gametogenesis of this clam commenced in February with 100% ripe clams in May and major spawning occurred from June to August(Chung et al., 2002; Park and Choi, 2004; Choi et al., 2005). Park and Choi (2004) reported three spawning pulses of this clam timing after mid May, late July and late August off the west coast of Korea.

Studies have shown that gametogenic cycle in marine invertebrates are influenced by the exogenous factors of which water temperature (Xie and Burnell, 1994; Drummond et al., 2006), food availability (Soniat and Ray, 1985; Hofmann et al., 1992; Park and Choi, 2004) and pollution are the most important. Previous observations revealed that in bivalves, the number of spawning per year and the duration of spawning increase with increasing temperatures and movement towards the equator (Yap, 1977; Ponurovsky and Yakovlev, 1992; Chung et al., 1994;

Drummond et al., 2006; Park and Choi, 2004). In spite of higher temperature and shorter distance, the onset of gametogenesis and spawning of Manila clams are delayed remarkably on the east coast of Jeju than those on the west coast of Korea.

The suitability of the methods for the estimation of reproductive output principally related to the reproductive strategy of the species (Murua and Saborido-Rey, 2003). Consequently, prior knowledge of the reproductive biology of the species is essential. As gonads are an integral part of the visceral mass in Manila clam like most other bivalves, its gonads can not be separated from the body (Lucas, 1982; Thompson et al., 1996). Reproductive output or fecundity of marine bivalves has often been estimated from the histological preparations using stereology or planimetry (Perdue and Erickson, 1984; Dinamani, 1987; Lango-Reynoso et al., 2000), though the major limitation remained due to irregular structure of the gonad. The reproductive output or fecundity can also be estimated by counting or weighing the number of gametes released after inducing animals to spawn using various chemicals and thermal shock (Toba and Miyama, 1991; Chung et al., 2001; Massapina et al., 1999). However, the estimate would be biased if the female spawned ova prior to capture, if oocytes remained in the ovary after spawning, or if some of the standing stock of the oocytes were lost because of atresia (Laptikhovsky, 2000). The gonad mass can also be weighed by measuring the difference in weight just prior to and after spawning (Deslous-Paoli and Heral, 1988; Pouvreau et al., 2000). The reproductive output is often underestimated by the above methods, as reproduction is not always complete and occurs at various intensities throughout the spawning period (Lucas, 1982; Thompson et al., 1996).

The value of accurate and precise estimates of reproductive output has motivated a search for alternative methods that are accurate, efficient, and economical.

Immunological methods have been used successfully to quantify egg proteins of marine bivalves due to their high speed, low cost and high sensitivity (Choi et al., 1993, 1994; Kang et al., 2003; Park et al., 2003; Park and Choi, 2004). Park and Choi (2004) developed an anti-Manila clam egg specific antibody and quantified the reproduction of this clam from a commercial bed at Gomso Bay, Korea. But no attempt has yet been made for the quantification of reproductive output of this clam from natural habitat in Korean waters. Therefore, the present study was designed to quantify the reproductive output of Manila clam from a natural habitat at Seong San, on the east coast of Jeju, Korea by using ELISA along with histology. The study also aimed to know the temporal variation in total proteins and carbohydrates in relation to gametogenesis; and to ascertain if there are differences in the availability of foods between this habitat and west coast of Korea.

2. MATERIALS AND METHODS

2.1. Sampling efforts

Manila clam, *Ruditapes philippinarum* samples were collected from Seong San, on the east coast of Jeju, Korea during active gametogenic period from June to November 2006 (Fig. 1). After arrival in laboratory, the shell length of the clams was measured. The clams were then dissected and the weight of the soft body was taken. The soft tissues of sixty clams were processed for histology. In case of the rest of the dissected clams, sexes were identified by microscopic observation of the smears taken from the gonadal portion. Twenty female clams were lyophilized at each sampling date and kept at -70°C until use for ELISA. The shells of all the dissected clams were allowed to dry for calculation of condition index and condition index was calculated by dividing the wet tissue weight by dry shell weight.

Surface seawater temperature and salinity were monitored during sampling time. The availability of chlorophyll-a in the seawater at sampling site was also monitored during sampling. For chlorophyll-a analysis, 2 liters of seawater was filtered through a filter paper (GF/C, 0.47 μm mesh size). The filter paper along with the filtrate was put onto the conical tube containing N,N-dimethylformamide and incubated overnight. After centrifugation, the chlorophyll-a concentration was measured from the optical density at 750, 664 and 647 nm wave length using the N,N-dimethylformamide as control.

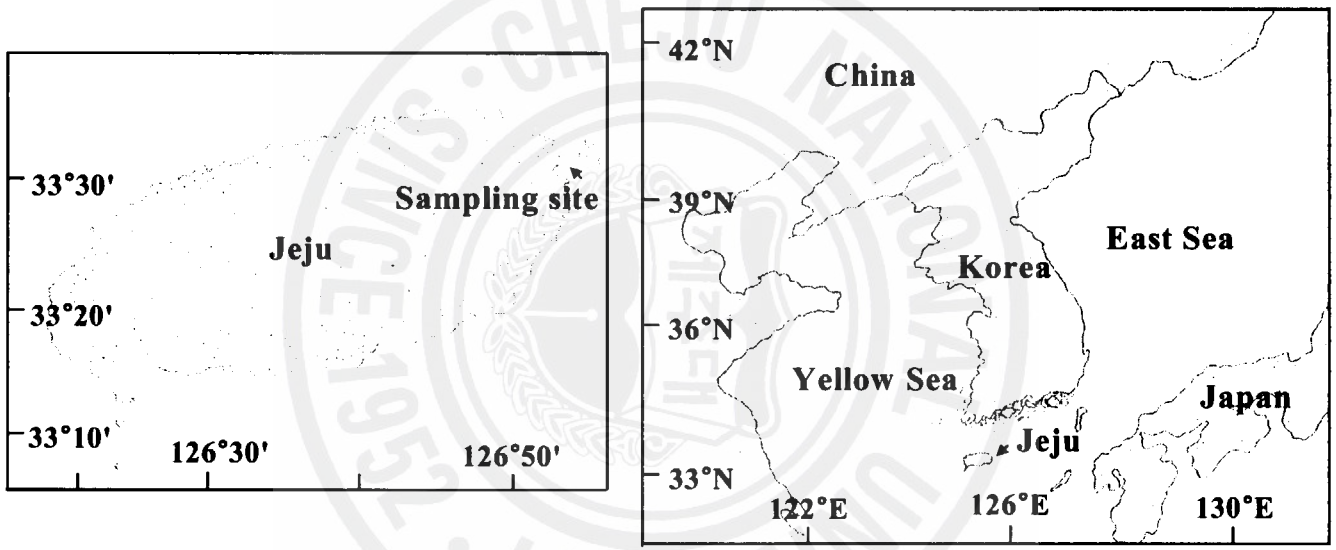

Fig. 1. Map showing sampling site

2.2. Histology

The soft tissue of the clams was fixed in Davidson's solution. After fixation, a transverse section was cut in the middle of the body which contained gonad, digestive gland, mantle and muscular foot tissue. The tissue was subsequently dehydrated and embedded in paraffin. Paraffin blocks were sectioned at 6 μm thickness and were stained with Harris's haematoxylene and counter-stained in eosin Y. After mounting, the histological preparations were examined under a light microscope to determine sex and gametogenic stage of the clams. The reproductive maturity of the gonads observed in the histological preparations 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) spawning; 5) spent; and 6) indifferent. To follow the seasonal changes in egg size, the egg diameter was measured from the histological preparations using an image analyzer.

2.3. Quantification of clam eggs using ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to determine the quantity of Manila clam egg specific protein in the lyophilized clams. For analysis, lyophilized whole clams were homogenized using a mortar and a pestle. Around 25 mg clam homogenate was dissolved in 900 μl PBS and further homogenized using a sonicator. The homogenate was diluted up to 2000 folds to get the optical density within the acceptable range of the standard curve. A 100 μl aliquot of standard (prepared from purified clam eggs), negative controls

(PBS and male clam homogenate) and clam homogenate to be analyzed was added to a polystyrene 96-well microplate and was incubated for 3 hours at room temperature. After incubation, the plate was washed with PBS and blocked with 150 μl 1% bovine serum albumin. After 1 hour incubation and subsequent washing, primary antibody previously developed and characterized by Park and Choi (2004) was added in each well at a volume of 100 μl and incubated further 1 hour at room temperature. Goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000) was then added as a 100 μl aliquot at each well, incubated for another 1 hour, and washed. p-nitrophenylphosphate (p-NPP) substrate dissolved in 0.1M glycine buffer was added as a coloring agent and the optical density of the end product was measured at 405 nm using a 96-well micro-plate reader.

The quantity of the egg protein in the clam homogenate was estimated from the standard regression curve constructed from the optical density and concentration of the purified clam egg protein using the same plate. The quantity of the eggs in an individual clam was then estimated by multiplying the quantity of the egg protein measured by ELISA by 2.44, the ratio of the egg protein to total egg weight. The reproductive output was expressed as gonadosomatic index (GSI), a ratio of the estimated total dry weight of the eggs to total dry weight of the clam tissues multiplied by 100.

2.4. Estimation of potential fecundity

The potential fecundity was estimated in case of matured clams. I assumed that the clams were full with mature oocytes when the individual GSI exceeded the

mean peak GSI. Therefore, I estimated the potential fecundity in those clams having GSI above the sample mean during peak irrespective to sampling period. The potential fecundity was calculated by dividing the estimated total weight of eggs using ELISA data by the weight of an individual egg. The mean dry weight of a mature egg of Manila clam was 22 ng as reported in the previous study (Park and Choi, 2004) was used in the present study.

2.5. Biochemical analysis

Protein content was determined using the method of Lowry et al. (1951) after alkaline hydrolysis with 0.1M NaOH at 37°C for 2 hrs using bovine serum albumen as standard. Carbohydrates were determined as glucose by the phenol-sulfuric acid method (Dubois et al., 1956) using dextrose enhydrase as standard. Chlorophyll-a was extracted by dissolving the dried clam tissues into the solvent N,N-dimethylformamide (Moran and Porath, 1980). The concentration of chlorophyll-a in the extracted solution was calculated from the optical density at 750, 664 and 647 nm.

2.6. Standard animal

To present the absolute value for protein, carbohydrate and chlorophyll-a, the composition of a standard animal of 33.0 mm shell length was calculated for each sampling date. The standard animal shell length was the mean shell length of all the clams analyzed for the study. This was done because, a variation in weight with no variation in size should correspond principally to the accumulation or loss

of reproductive tissue. Allometric equations of $\log_{10}$ dry tissue weight against $\log_{10}$ shell length at each sampling date were determined by linear regression analysis. Proteins and carbohydrates were then expressed in mg per standard animal and total chlorophyll-a was also expressed in μg per standard animal.

2.7. Statistical analysis

All the data were analyzed statistically and expressed as mean (±SD) using SAS statistical package.

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3. RESULTS

3.1. Environmental parameters

Surface water temperature and salinity were monitored during sampling to know their relationship with the annual reproductive cycle of the clams as shown in Fig. 2. Over the study period, the surface water temperature ranged from 12.0 to 16.2°C with the maximum in August and the minimum in November. The salinity of the water did not vary greatly fluctuating from 27.4 to 32.5 psu during the study period. The lowest salinity was recorded in July during monsoon when freshwater from land drainage considerably diluted the seawater due to heavy rainfall. In contrast, the salinity was highest timing November owing to minimum rainfall at that time. The concentration of chlorophyll-a varied considerably ranging from 0.52 to 2.54 μg/l of seawater (Fig. 3). Two distinct peaks in the concentration of chlorophyll-a were evident, one in early July and the other during mid August. The lowest level of chlorophyll-a was noted in early June and the highest concentration was recorded in mid August.

3.2. Condition index (CI)

The inclination in CI was illustrated in Fig. 4 and Table 1. At the beginning of the study during early June, CI was 0.369±0.075. The CI then increased rapidly, peaked in mid August as 0.529±0.168. A sudden drop in the index from mid August to early September indicated most clams spawned during this period. The

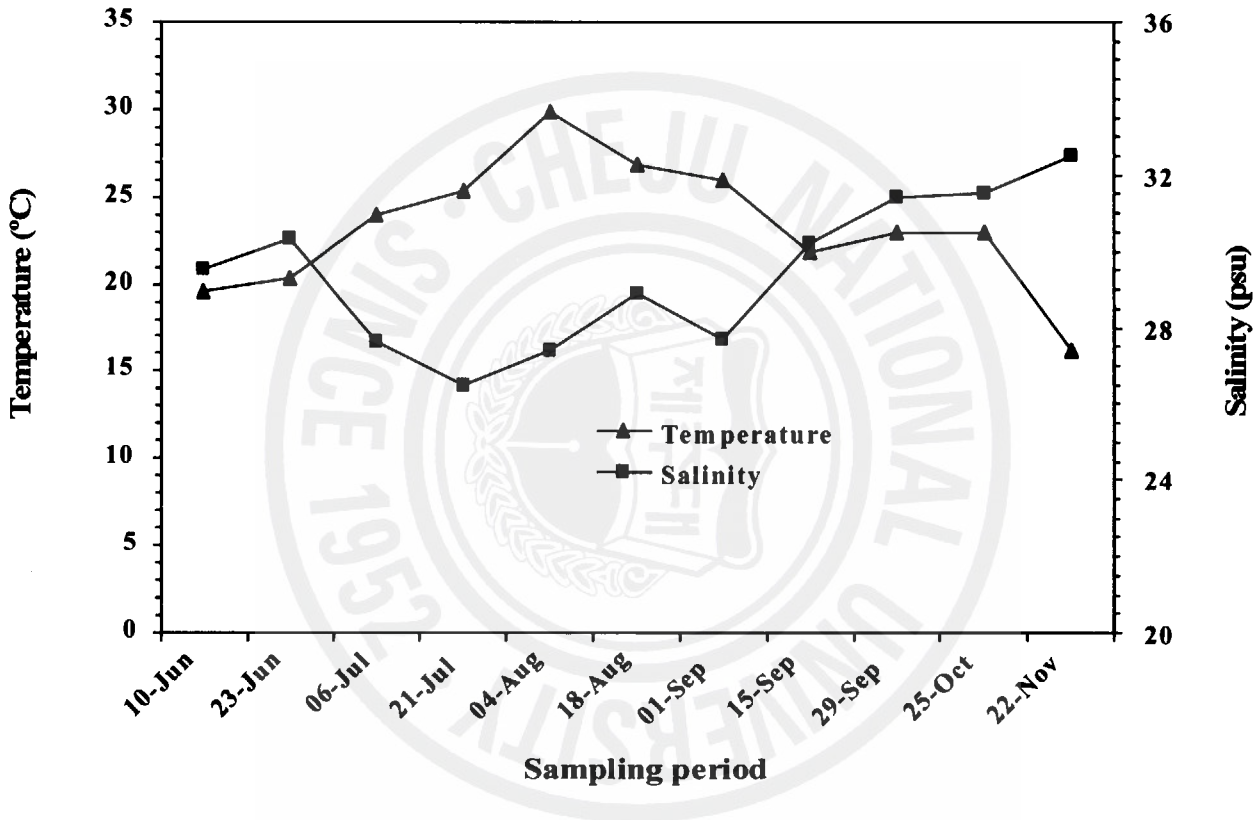

Fig. 2. Variations in surface water temperature and salinity at sampling site.

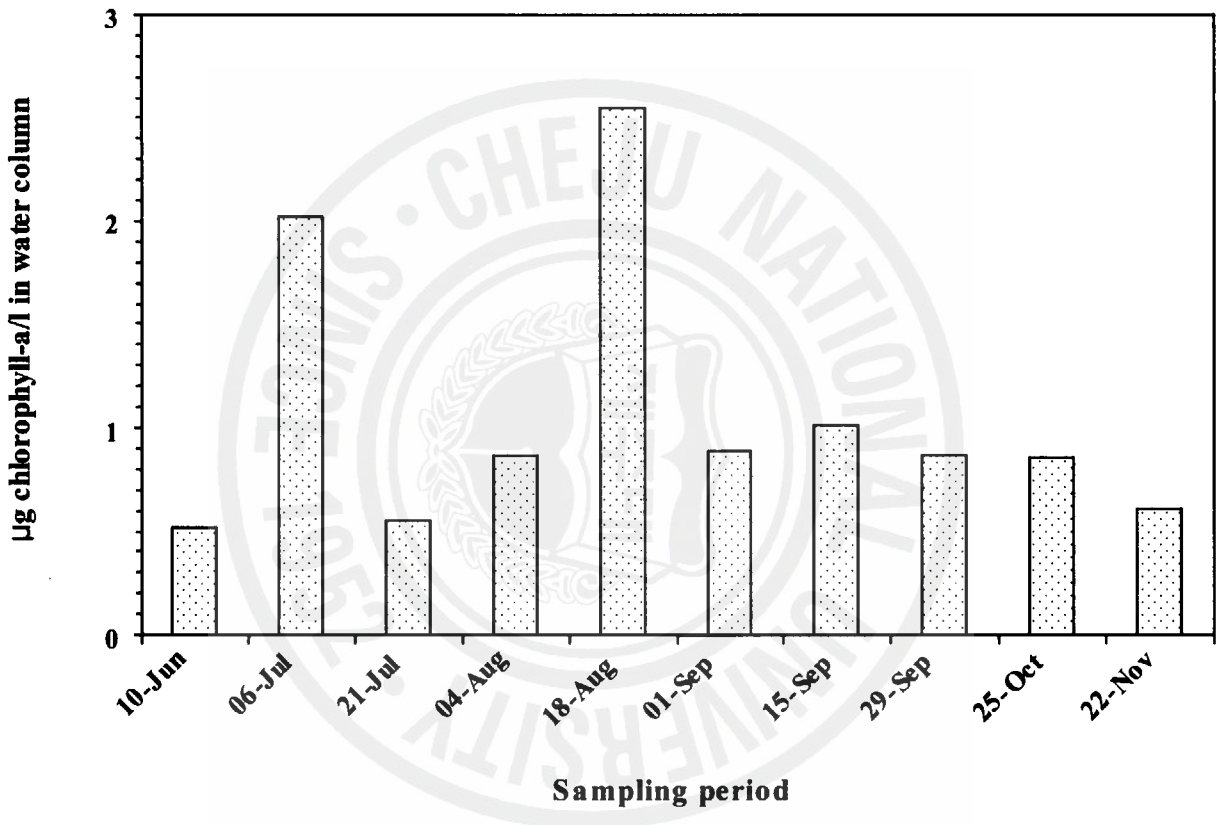

Fig. 3. Variations in chlorophyll-a concentration in the seawater at sampling site.

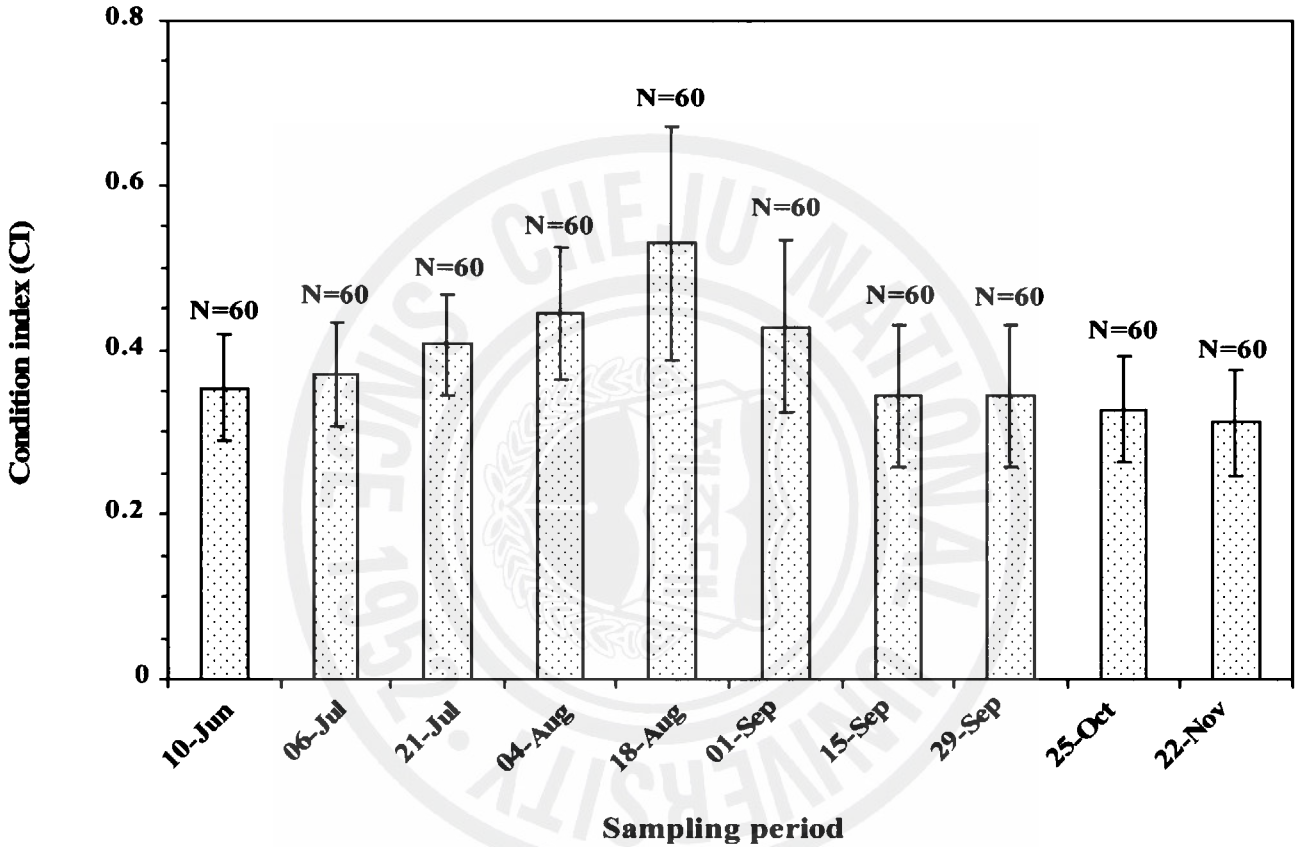

Fig. 4. Variations in condition index of *Ruditapes philippinarum.*

Table 1. Biometric data of the analyzed clams collected from the Seong San, east coast of Jeju, Korea. SL, shell length in mm ±SD; TWWT, tissue wet weight in g ±SD; CI, condition index ±SD.

Sampling period	N	SL	TWWT	CI
10-June	80	30.5±3.1	1.30±0.62	0.369±0.075
06-July	80	33.2±4.2	1.72±0.65	0.377±0.062
21-July	80	32.7±4.3	1.87±0.79	0.412±0.071
04-August	80	33.7±5.0	2.20±1.01	0.455±0.088
18-August	80	33.8±4.7	2.40±1.12	0.529±0.168
01-September	80	35.0±4.4	2.24±0.95	0.420±0.110
15-September	80	34.0±5.1	1.66±0.72	0.351±0.078
29-September	80	34.3±6.1	1.77±0.90	0.350±0.094
25-October	80	36.0±4.7	2.01±0.78	0.314±0.060
22-November	80	34.3±5.7	1.56±0.77	0.297±0.063

lowest CI was noted in November (0.297±0.063) when most of the clams terminated their spawning as reflected by the presence of spent and indifferent clams in the histological preparations.

3.3. Gametogenesis of the clams

We attempted to cover the active gametogenic stages from early developing to the spent condition of the clam using histology when the reproductive effort can be quantifiable by ELISA. Fig. 5 shows the temporal distribution of gametogenic stages of female *R. philippinarum* over the study period. Here I used half of the number of indifferent clams as female during each sampling date for the construction of the graph considering the male:female as 1:1. At the beginning of the study during early June, most of the clams were in indifferent stage (79.3%). By this time 17.2% clams were in early developing stage and only 3.5% clams exhibited late developing stage. Late developing clams were principally detected in July, and ripe clams dominated from late July to mid August. Spawning commenced in late July at a small scale when only 10.5% clams were detected in spawning condition. Majority of the clams spawned during early August to mid September accounting 38 to 92% of the clams in spawning stage during this time. Spent females first appeared in mid September with majority of the clams in this stage timing late September, while only 13.3% clams were graded as spent stage during October. The sexually indifferent clams were prominent from late September (60%) to the end of the study during November when all the clams were in this stage.

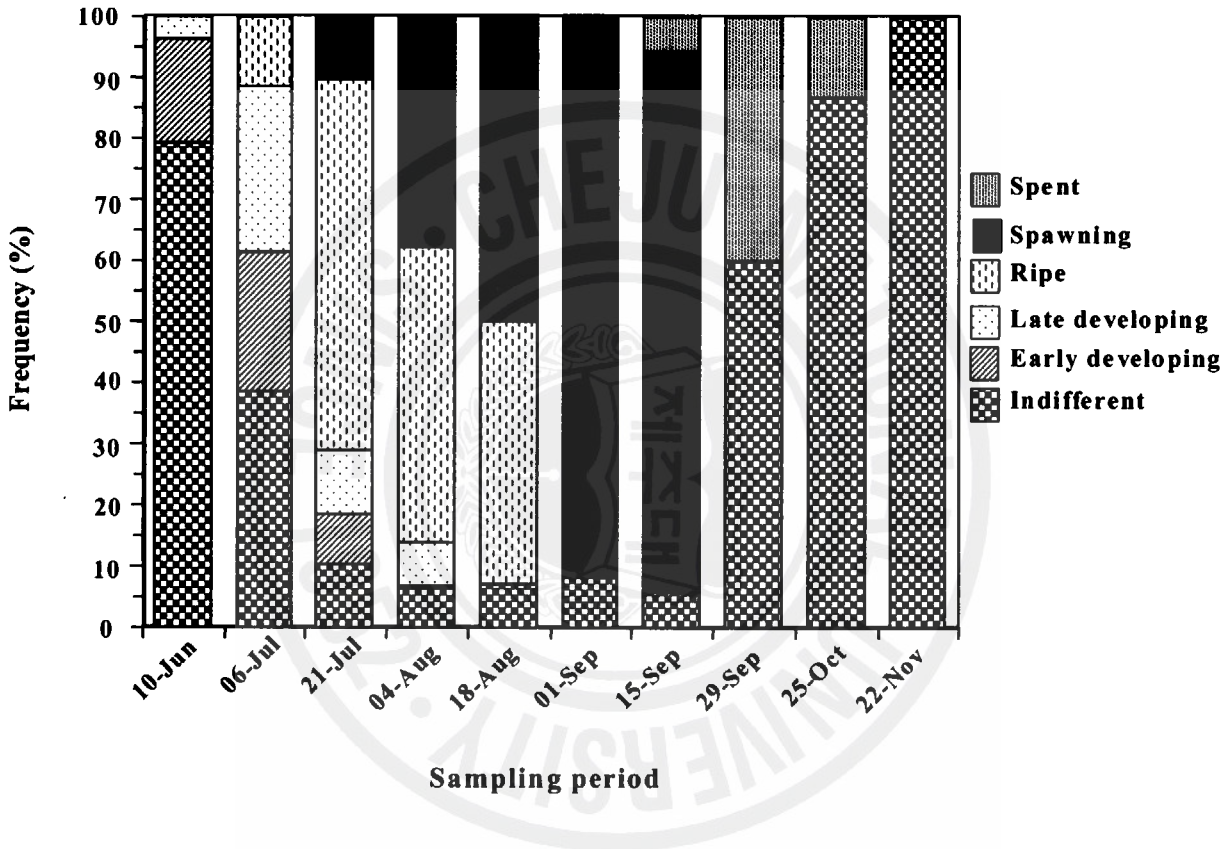

Fig. 5. Temporal distribution of gametogenic stages of female *Ruditapes philippinarum.*

Fig. 6 shows the seasonal changes in egg diameter over the study period when the eggs appeared in the histological preparations. The mean egg diameter was 28.0±8.18 μm at the beginning of the study during early June when most of the female clams were in early developing stage. The egg size increased greatly from early June to early July and the maximum proportion of eggs matured by early August. The appearance of ripe eggs was confirmed by the presence of more or less similar sized eggs till the end of the spawning activity. The matured eggs dominated from early August to late September having a mean diameter from 57.7 to 61.5 μm. Only a few relict eggs were detected during late October in 3 clams out of 60 and the mean egg diameter was 52.1±3.3 μm.

3.4. Reproductive effort of clams measured by ELISA

The clam egg-specific antibody was sensitive enough to detect very small amount of egg protein during the early stage of gametogenic development or even when clams were in spent stage having some residual eggs. The reproductive output of the clams measured by ELISA was expressed as the weight-based gonadosomatic index (GSI) as shown in Fig. 7. The size of the analyzed clams varied from 21.6 to 43.3 mm in shell length. Out of 20 female clams examined collected during early June, the antibody successfully detected the egg specific protein in only 6 clams having an average GSI of 0.9±0.6%. As we ran ELISA only for female clams confirming by microscopic observation of the smears, majority of the clams analyzed were in early developing stage. The GSI increased rapidly with the progression of gametogenesis and reached 6.8±5.6 by early July when majority

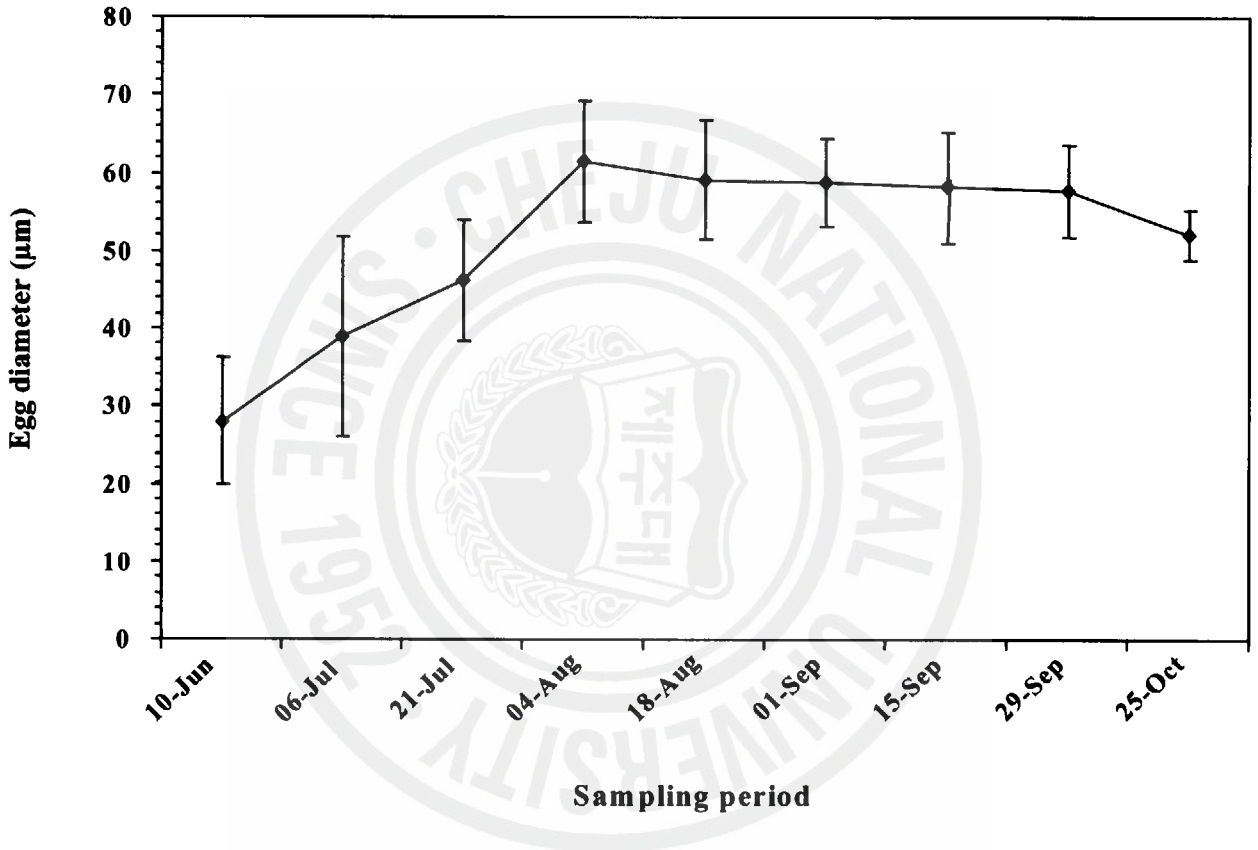

Fig. 6. Mean (±SD) egg diameter of *Ruditapes philippinarum.*

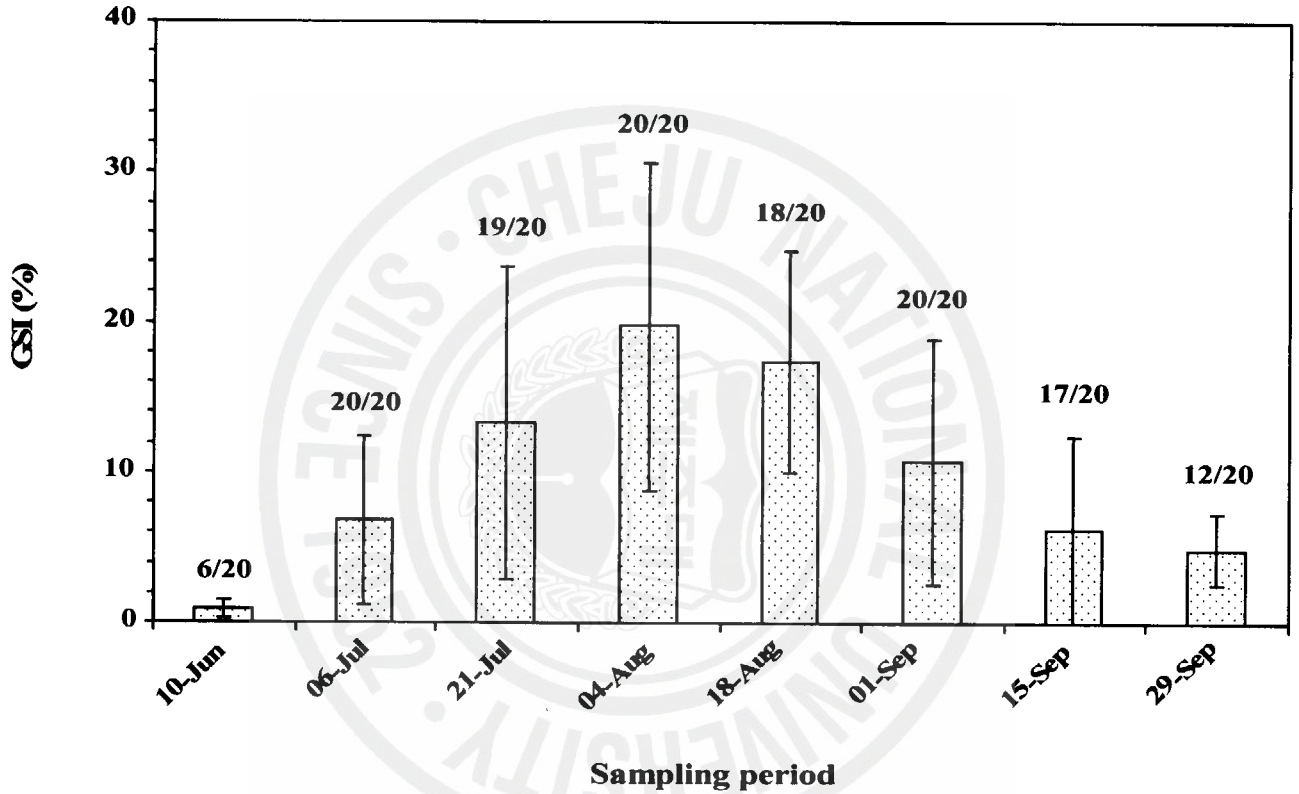

Fig. 7. Variations in mean (±SD) GSI of *Ruditapes philippinarum* measured by ELISA. Vertical line represents the standard deviation and the figures above the bar indicate the number of clams in which egg protein was within detectable range/total no. of females analyzed by ELISA.

of the clams were either in early developing or late developing stage. The GSI doubled within 15 days from 06-July to 21-July when most of the individuals became matured. Only one peak was evident during early August when the GSI was 19.7±11.0%. A drop in GSI was detected by mid August, indicating the spawning event and 12% gametes were discharged from 04-August to 18-August determined from the differences in mean GSI between the two sampling dates. A further decline in GSI to 10.7±8.1% by early September from the preceding sampling date implied that about 34% gametes were released by the clams during this time interval. Twenty two percent ovulation was noted during the first-half of September and only 6.6% eggs were released in the last-half of September. ELISA was not performed after September as no more females was detected by microscopic smears during this time. The high inter-individual variation in GSI during each sampling date indicated that the spawning of this clam was asynchronous and occurred over a long time from late July to late September exhibiting different gametogenic condition over the entire spawning period.

We considered mean GSI during peak as the triggering point to initiate spawning. The individual GSI was higher than 19.7% (mean GSI during peak) in 10 clams out of 20 clams analyzed during early August indicated that 50% clams were ready for spawning by this time. Though the mean GSI was increasing from early June to early August, but some clams exhibited individual GSI over 19.7% as early as early July. Only 4.7% clams were ready for spawning during early July and 26% of the clams prepared themselves for spawning by late July. Similarly, some clams exhibited higher individual GSI than the mean value at peak after early

August indicated that some clams delayed their spawning even until mid September. About 44% clams were ready for spawning during 18 August, whereas, 14% and 6% clams prepared themselves for spawning timing early September and mid September respectively.

3.5. Estimation of potential fecundity

The potential fecundity was estimated based on the mean peak GSI (19.7%). I assumed that the clams did not release any egg and became ready for spawning when the individual GSI exceeded 19.7% as the fecundity is the measure of total number of ripe eggs produced by an individual female from the start of the spawning period to the termination of the spawning season. I detected 28 clams having GSI above mean GSI at peak, and I estimated the potential fecundity of those clams as shown in Table 2. The fecundity ranged from 2,420,350 to 8,967,785 eggs having a mean of 4,955,706±1,075,854.

3.6. Tissue weight of a standard animal

Fig. 8 illustrates the temporal variations in dry tissue weight for a standard animal of 33.0 mm in shell length. There were remarkable seasonal variations in the dry tissue weight of the clams and the trends were similar to those of condition index. The absolute dry tissue weight of a standard animal closely followed the gametogenic cycle, having an increasing inclination with the gametogenesis, expressing its peak during mid August. A decreasing trend after mid August

Table 2. Individual GSI and potential fecundity of *Ruditapes philippinarum* having GSI higher than the mean GSI during peak (19.7%). SL, shell length in mm ±SD; TWWT, tissue wet weight in g ±SD; TDWT, tissue dry weight in g ±SD; GSI, gonadosomatic index in percentage ±SD.

Sampling period	% ripe clams	SL	TWWT	TDWT	GSI	Fecundity ±SD
06-July	5.0	34.4	1.73	0.33	23.5	3,569,411
21-July	25.0	32.7±3.3	1.80±0.59	0.34±0.10	28.5±4.1	4,232,675±1,808,552
04-August	50.0	34.0±3.3	2.04±0.60	0.39±0.10	28.7±6.2	5,114,965±1,701,943
18-August	40.0	34.6±3.6	2.58±0.83	0.50±0.16	24.2±4.8	5,595,634±2,199,096
01-September	15.0	36.7±4.6	2.33±0.60	0.46±0.11	23.8±5.0	5,145,274±2,059,855
15-September	5.0	36.3	1.93	0.35	20.1	3,182,812
Overall	-	34.4±3.4	2.17±0.69	0.42±0.13	26.3±5.5	4,973,791±1,075,854

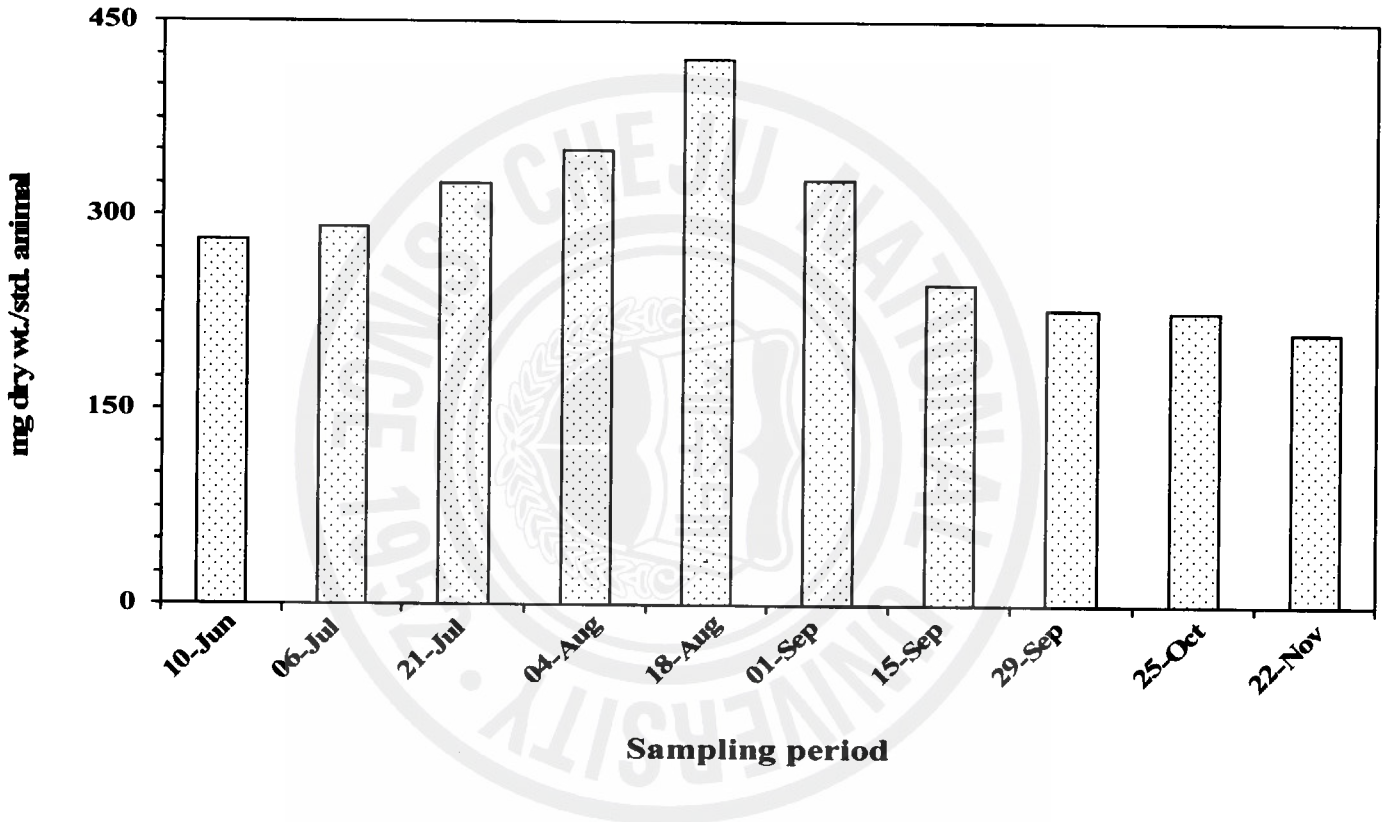

Fig. 8. Temporal variation in dry tissue weight of the standard animal (33.0 mm in shell length).

implied the major spawning period of this clam in this habitat by this time. The lowest tissue dry weight was measured in November when all the clams were in indifferent stage.

3.7. Variations in gross biochemical composition

3.7.1. Proteins

The variations in the absolute values of total proteins showed clear seasonal trend as shown in Table 3 and Fig. 9. Total protein content in standard clam increased from early July to mid August synchronizing with the condition indices in direct relation with the gametogenesis. Protein content per standard animal decreased after mid August coinciding with the gamete evacuation due to spawning activity of the clams as evidenced from the histological preparations. The available total protein in a standard animal decreased as late as November and the values were almost half of that of the peak during mid August. However, the percentage of protein in relation to total dry weight increased from early June to late July and it then remained constant till early September. The proportion of protein decreased rapidly by mid September when most of the clams were in spent condition and then increased again with the onward of post spawning recovery time.

Table 3. Temporal variations in total proteins, carbohydrates and chlorophyll-a in the standard animal. All the analyzed clams were female except in October and November when females were not detectable in microscopic smears.

Period	N	Total proteins		Total carbohydrates		Chlorophyll-a	
		mg/std. animal	% dry wt.±SD	mg/std. animal	% dry wt.±SD	μg/std. animal	ng/mg dry wt.±SD
10-Jun	20	100.15	35.78±4.06	32.06	11.45±3.18	7.64	27.3±19.9
06-Jul	20	101.20	34.80±2.63	29.41	10.11±3.08	2.53	8.7±5.2
21-Jul	20	132.44	40.80±5.92	30.11	9.28±1.47	34.73	107.0±31.9
04-Aug	20	137.89	39.35±5.22	34.35	9.80±2.68	23.37	66.7±43.9
18-Aug	20	165.41	39.35±5.19	38.79	9.23±2.96	22.21	52.8±24.9
01-Sep	20	132.53	40.45±3.91	28.12	8.58±2.05	3.97	12.1±6.2
15-Sep	20	88.62	35.98±4.79	16.77	6.81±1.73	5.32	21.6±8.0
29-Sep	20	84.81	37.21±6.45	14.37	6.31±1.50	5.29	23.2±9.2
25-Oct	20	89.49	39.51±2.54	13.01	5.74±0.99	3.51	15.5±7.1
22-Nov	20	80.40	38.30±2.66	11.33	5.40±2.01	4.43	21.0±9.2

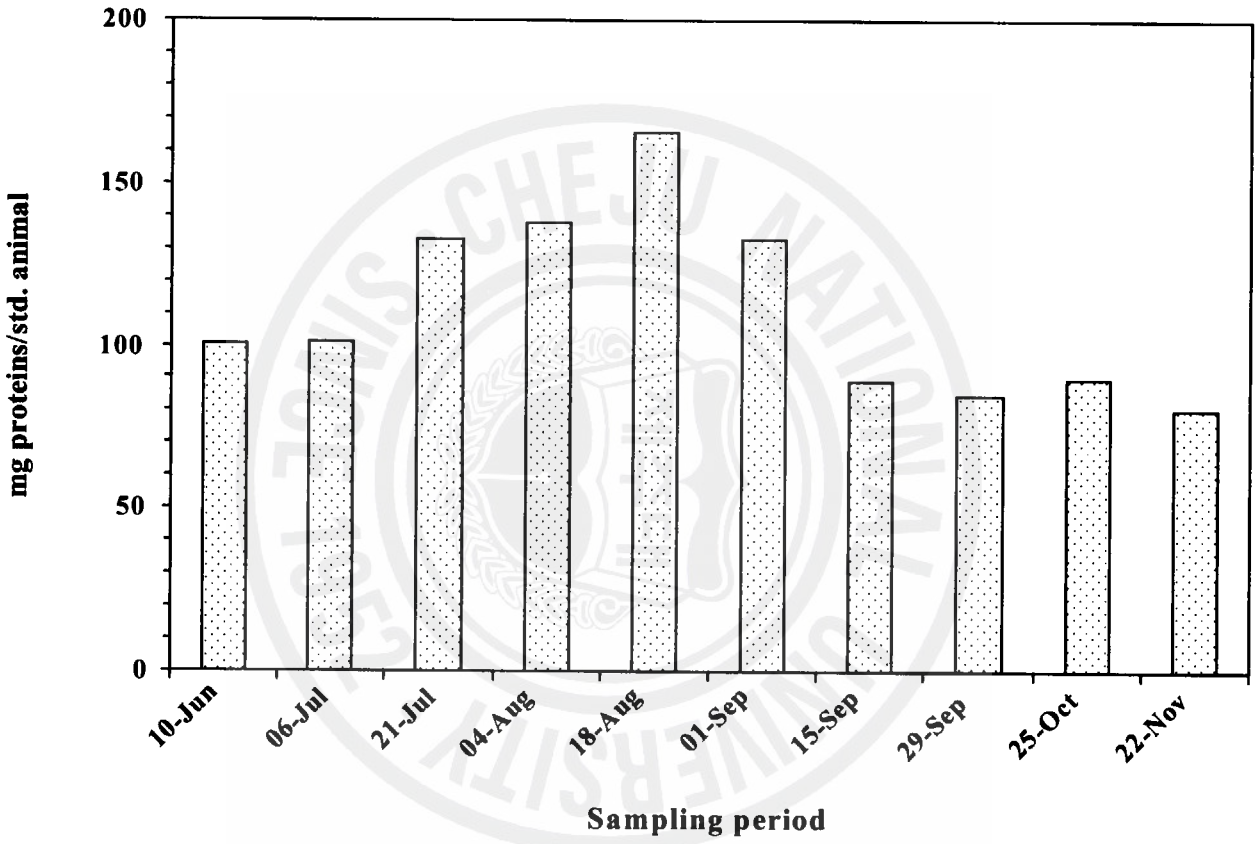

Fig. 9. Temporal variations in total proteins in the standard animal (33.0 mm in shell length).

3.7.2. Carbohydrates

Total carbohydrate content in standard animal varied remarkably over the study period as shown in Table 3 and Fig. 10. Similar to proteins, total carbohydrate increased from late July, and the highest content reached in mid August when the condition of the clams was the best. Gross carbohydrate content declined after mid August and this trend was maintained till the remainder of the study period. Total carbohydrate content per standard animal reduced to one-fourth in November in comparison to the maximum value exhibited in mid August. Expressing the biochemical composition as a percentage of dry weight, the carbohydrate content decreased consistently from early June to end of the study period during November.

3.7.3. Chlorophyll-a

The chlorophyll-a in the lyophilized tissues of standard clam varied markedly during the study period as shown in Table 3 and Fig. 11. The chlorophyll-a content per standard animal decreased sharply from early June to early July and then increased rapidly, exhibiting its peak in late July. The values remained high till mid August and then declined by early September. Chlorophyll-a in the clam tissues remained more or less stable during the remainder of the study period. It was remarkable that the spawning activity of the clams well-matched with the higher chlorophyll-a content in the clams. Chlorophyll-a was shown as a measure of food intake and this parameter was monitored as an indicator of food availability in the water-body indirectly.

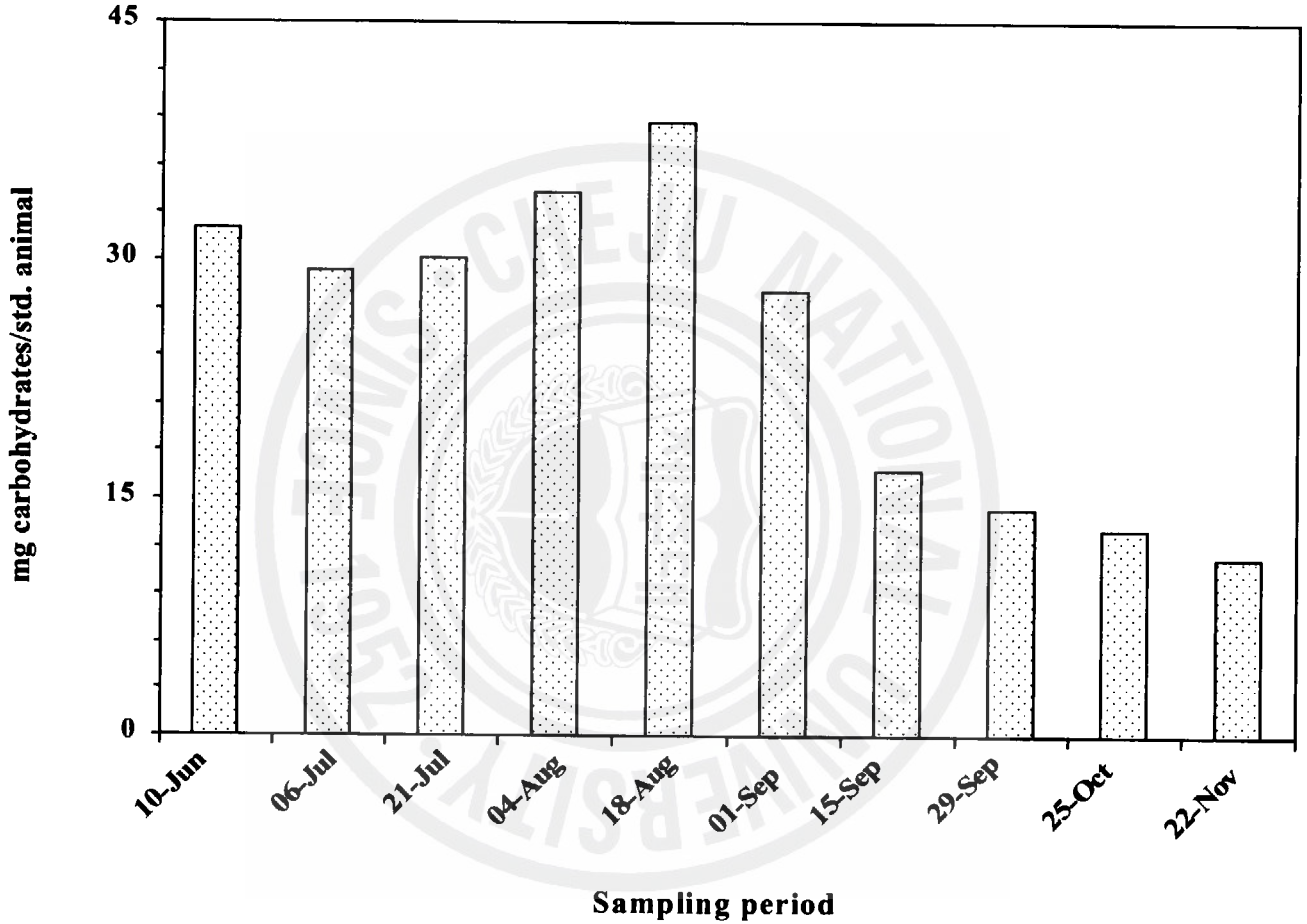

Fig. 10. Temporal variations in total carbohydrates in the standard animal (33.0 mm in shell length).

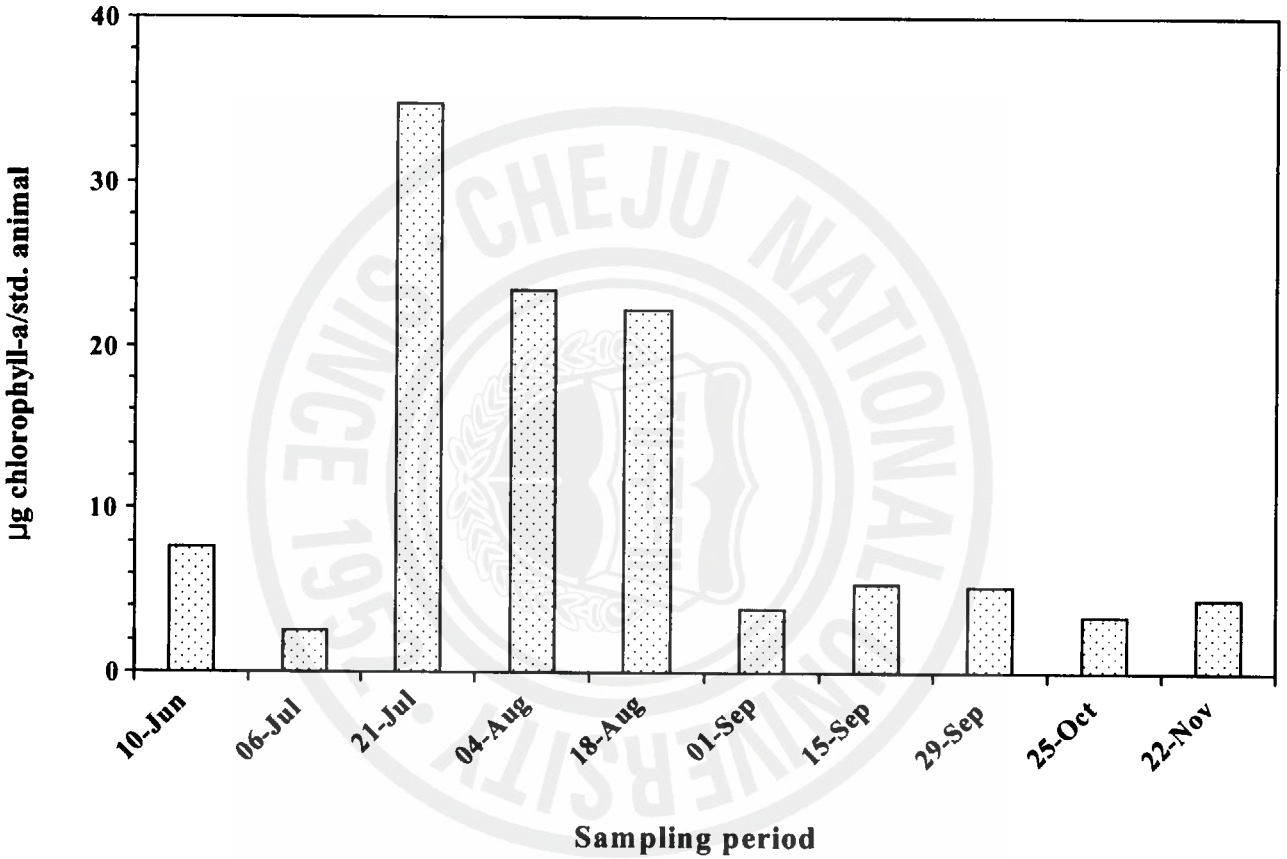

Fig. 11. Temporal variations in chlorophyll-a in the standard animal (33.0 mm in shell length).

4. DISCUSSION

4.1. Gametogenic cycle

I employed histology to confirm the active gametogenic stages of the clams during which eggs can be quantifiable by ELISA. Histology revealed that gametogenesis initiated in early June and spawning commenced in small scale during late July and terminated by mid September. The spawning of this clam was reported to occur earlier timing from early July to August in the same habitat (Kang et al., 2004). The little difference in spawning time between our study and the previous report might be caused by inter-annual variation in gametogenesis, caused by various exogenic factors such as temperature, salinity, and food availability (Barber and Blake, 1991; Arellano-Martinez et al., 2004; Dukeman et al., 2005). In contrast, the clams undergo spawning much earlier timing principally during June-July in Gomso Bay off the west coast of Korea (Park and Choi, 2004; Choi et al., 2005). Spawning of this clam occurs timing late spring to early summer in temperate waters (Xie and Burnell, 1994; Chung et al., 2002; Park and Choi, 2004; Choi et al., 2005; Drummond et al., 2006) is broadly in agreement with the present study. The gametogenesis and spawning of clams synchronized with the seasonal fluctuations in food availability in the water column. The rapid increase in the chlorophyll-a concentration in early July followed by the initiation of spawning by late July and the major spawning pulse during mid August also matched the relatively higher chlorophyll-a concentration, suggesting that the abundance of food in the seawater is vital for reproduction and growth. Hofmann et al. (1992)

reported that a few degrees change in temperature or a small (2 to 4 weeks) shift in timing of the spring or fall bloom can considerably alter the duration of spawning and the seasonal spawning patterns of American oyster, *Crassostrea virginica*.

Spawning of this clam started in late July in the present site whereas, spawning of this species commenced as early as May (Choi et al., 2005) and June (Park and Choi, 2004) on the west coast of Korea. In spite of close distance (200～400 km), the considerable difference in timing of spawning might be due to the difference in the availability of food in the water column. The chlorophyll-a concentration in the water column reported from various locations in Korea are summarized in Table 4. The mean chlorophyll-a concentration in the water column was remarkably lower at the present site than that reported from the south and west coasts of Korea. At present site, the chlorophyll-a peaked in mid August having the value as 2.6 μg/ml whereas, the values peaked as 11.8 μg/ml in Gomso Bay, 8.5 μg/ml at Seonjaedo, west coast of Korea and 4.5 μg/ml at South coast of Korea. The comparison clearly indicated that the delayed spawning time of the clams in Seong San more likely to be associated with lower food availability in this habitat than that in south and west coasts of Korea.

The gametogenic development was inferred by the remarkable increase in egg diameter from early developing to ripe stage of the clams. The mean mature egg diameter measured in the present study is almost similar with that reported in clams collected from Gomso Bay, west coast of Korea (Chung et al., 2001; Park and Choi, 2004) as shown in Table 4. The increment in egg diameter matched with

Table 4. Concentration of chlorophyll-a in the water column reported from various locations in Korea.

Location	Range (μg/l) (Mean±SD)	Peak (μg/l), Month	Author
Gomso Bay, west coast of Korea	2.0 to 11.8 (4.7±2.99)	8.4, June, 11.8, September	Park and Choi (2004)
Josan, south coast of Korea	0.4 to 4.5 (2.48±1.23)	4.5, May	Kang et al. (2000)
Osu, south coast of Korea	0.4 to 2.8 (1.43±0.79)	2.8, April	Kang et al. (2000)
Seonjaedo (east), west coast of Korea	0.9 to 4.5 (2.66±1.17)	4.5, April	Kim (2005)
Seonjaedo (west), west coast of Korea	1.0 to 8.5 (3.87±2.33)	8.5, April	Kim (2005)
Seong San, east coast of Jeju, Korea	0.5 to 2.6 (1.1±0.7)	2.0, early July 2.6, mid August	Present study

the corresponding increase in GSI.

4.2. Condition index (CI)

Condition indices are generally regarded as useful measurements of nutritive status of bivalves. These indices are often used for commercial quality of bivalves as they provide a useful means of assessing the overall health status of a population and hence its suitability for harvest (Crosby and Gale, 1990). Though the CI is related to many factors, such as gametogenic cycle, food availability, space, infections and diseases but its general inclination often matches with the annual gametogenic cycle. The condition index was increasing from the beginning of the study during early June coinciding with the gametogenic development of the clams and peaked in mid August when 43% clams were in ripe stage. A major spawning event was inferred by the massive drop in CI timing mid August to mid September when its value decreased from 0.529 to 0.350 and this was consistent with the histology. In previous study conducted by Ngo and Choi (2004), the CI of the clams peaked in early August and accordingly the spawning period was earlier than that of the present study as expected. The condition index was remarkably higher and the values peaked much earlier in clams collected from Seonjaedo Island, west coast of Korea as reported by Uddin et al. (unpublished) than those obtained in the present study. They reported the peak CIs of the clams as 0.888±0.015 during May 2003 and 0.876±0.029 in May 2004. However, the mean minimum values were almost similar between the two sites.

4.3. Reproductive effort

Quantification of reproductive effort is necessary to study life history and population dynamics. The number of eggs and hatchlings produced by an individual female in a calendar year is also a useful message for the management of brood stock in a hatchery. Unlike most other marine invertebrates, clam gonads are an integral part of the visceral organs which makes it difficult to isolate completely from the somatic tissues. Owing to this difficulty, Park and Choi (2004) developed a polyclonal antibody against purified clam egg protein in New Zealand white rabbit. They successfully quantified the reproductive effort of Manila clam collected from a commercial bed in Gomso Bay, west coast of Korea using the antibody by an indirect ELISA. That was the first report on quantitative assessment of clam egg using immunological method, carried out from this laboratory. In the present study, the reproductive effort of Manila clam collected from a natural habitat on Seong San, east coast of Jeju, Korea, was quantified using the remaining antibody after reexamining the sensitivity of the antibody.

Very low level of egg protein was detected during early June when 14 female clams out of 20 remained undetectable by ELISA. The female clams analyzed collected during this time were mostly in early developing stage as evident from histology. The vitellin protein during this time remained very low or undetectable because the early developing eggs were mostly pre-vitellogenic eggs which probably contained no or low levels of yolk proteins as outlined by Park and Choi (2004). A massive increase in mean GSI from early July (6.6%) to early August (19.7%) coincided with the rapid increase in egg diameter resulting from

the mobilization of the reserved substances into yolk granules.

ELISA indicated that the GSI peaked in early August with an average of 19.7%. This value was considered as the triggering point for spawning which means that the clams were supposed to be ready for spawning when their egg mass accounted for ~20% of the total tissue weight in this location. When the accumulation of gametogenic material reaches certain percentage of the body, then the animal undergoes for spawning. The American oyster *Crassostrea virginica* initiate spawning when the egg mass accounts 20% of the body weight in Galveston Bay (Choi et al. 1993), whereas the Pacific oyster *Crassostrea gigas* undergoes spawning in Goseong Bay, south coast of Korea when the egg mass becomes 40% of the total dry weight (Kang et al., 2003).

The frequency of spawning over an entire spawning season is in part determined by the cumulative reproductive biomass (Powell et al., 1992). Park and Choi (2004) detected three spawning pulses of Manila clam collected from the Gomso Bay, west coast of Korea using an ELISA. They reported that the GSI of this clam peaked in mid May (18.7%), late July (25.0%) and late August (17.8%) in that habitat. In the present study, I detected only one peak in GSI (19.7%) timing early August but a number of clams exhibited GSI values above the mean during peak from early July to mid September. Therefore, more spawning pulses might be evident in the current stock which I could not detect due to the remarkable variation in individual GSI resulting from presence of different gametogenic stages of the clams in each sampling date. Though the two locations have ~235 km distance maintaining commercial clam culture bed in Gomso Bay and natural

habitat in the present study site but the triggering point to initiate spawning is almost similar, however, the mean maximum GSI was remarkably higher in Gomso Bay (25.0%) than that in the present location (19.7%). This discrepancy was at least partly contributed by the differences in food level between the two sites as I reported in the present study. It was well documented that the abundance of food in the water column is crucial for gametogenesis, reproduction, as well as for growth (Soniat and Ray, 1985; Powell et al., 1995; Park and Choi, 2004).

4.4. Estimation of potential fecundity

Park and Choi (2004) developed an antibody against Manila clam egg and they used the antibody for the quantification of reproductive effort of this clam. Due to high speed, low cost and high sensitivity of the method, I estimated the potential fecundity of the matured clams from the ELISA data just before the spawning commenced. Park and Choi (2004) reported that the GSI peaked in mid May, late July and late August in clams collected from Gomso Bay, west coast of Korea and the fecundity was estimated for the corresponding three times. In the present study, I confirmed that the GSI peaked only once during early August in clams collected from Seong San, east coast of Jeju, Korea. Here I considered the mean GSI (19.7%) during peak as the triggering point for spawning. I hypothesized that all the clams analyzed collected during early August were not ripe; at least some of the clams still maturing and some of the clams already commenced spawning by this time. An inter-individual variation in gametogenic condition at every sampling date was also evident from the histological

preparations. Hence the fecundity was estimated in those clams exhibited GSI over 19.7% during the study period, not restricted on the early August. The presence of gravid clams over a long time reflects that their spawning is asynchronous and they may have more than one spawning peak in different time which might be overlapped.

Table 5 shows the fecundity of *R. philippinarum* estimated using various methods collected from different habitats. In the present study, the potential fecundity of Manila clam ranged from 2.42 to 8.97 million (n=28) having an average of 4.97±1.08 million collected from Seong San, east of Jeju, Korea. The estimates of the present study is within the range of the potential fecundity reported by Park and Choi (2004) where they reported that the fecundity of this clam varied from 0.94 to 11.8 million in Gomso Bay, west coast of Korea. However, the fecundity estimated in the present study is much higher than the number reported by Toba and Miyama (1991) and Chung et al. (2001) as 0.24 to 1.35 million in Tokyo Bay and 0.20 to 1.79 million in Gomso Bay respectively by counting the number of released eggs using inducing agent to spawn. As the spawning of marine bivalves is often partial and occurs over a prolonged period, therefore counting of released eggs using inducing agent must be a minimum estimate (Choi et al., 1993; Chung et al., 2001; Kang et al., 2003).

4.5. Seasonal changes in biochemical composition

The accumulation of energy reserves and the allocation of reserves into somatic growth and/or gametogenesis are directly linked with the sexual maturity

Table 5. Fecundity of *Ruditapes* spp. reported from various studies.

Species	Location	Shell length (mm)	Egg diameter (μm)	Estimation method	Fecundity	Author (s)
Tapes rhomboides	Bay of St. Malo, France	35-45	35-45	Stereology	0.3 to 70.0 x 10^4 50 x 10^4 (Potential)	Morvan and Ansell, 1988
R. philippinaum	Tokyo Bay, Japan	34.1 to 36.0	63 to 70 (intact)	Induced spawning by ammonia	0.24 to 1.35 x 10^6	Toba and Miyama, 1991
R. largillierti	Launceston, Tasmania	-	-	Induced spawning by thermal shock	0.5 to 0.9 x 10^6	Kent et al., 1999
R. philippinaum	Gomso Bay, Korea	20.2 to 46.9	55 to 62 (histology)	Induced spawning by exposing to air, feeding stimulus, and thermal shock	0.20 to 1.79 x 10^6	Chung et al., 2001
R. philippinaum	Gomso Bay, Korea	21.11 to 43.5	89.57±8.66 (intact) 61.19±5.66 (histology)	Immunological method (ELISA)	0.94 to 11 x 10^6	Park and Choi, 2004
R. philippinaum	Seong San, Jeju, Korea	21.6 to 43.3	58.93±6.87 (histology)	Immunological method (ELISA)	2.42 to 8.97 x 10^6	Present study

of the mollusk species and the availability of foods in that particular habitat. Seasonal changes in the biochemical composition of bivalve mollusks have been extensively studied in species in their natural habitat (Marin et al., 2003; Camacho et al., 2003; Kang et al., 2007). Camacho et al. (2003) showed a clear sexual differentiation in the biochemical composition in *R. decussatus*. They reported that during active gametogenic phases, males had consistently higher protein level than in females; females had higher lipid level than in males; carbohydrates remained similar in both sexes. They interpreted that the differentiation might be associated with the gonadal development of the clams. To overcome the effect of sex on biochemical composition, in the current study, sexes were determined by gonadal smear under a microscope. Only female clams were analyzed except samples collected in October and November when sexes were not identifiable in the smears. The protein contents in a standard clam fluctuated consistently linked to the gametogenic state. The total protein content per standard animal increased with gonad maturation process. The increasing protein level during this period might be contributed by the positive energy balance due to the higher food availability as evidenced from the chlorophyll-a contents in the tissues. Conversely, the decrease recorded after mid August may mostly be due to spawning. The positive and negative energy balance have been reported in many bivalves, such as *Mytillus galloprovincialis* from the Lagoon of Venice (Bressan and Marin, 1985), *Ostrea puelchana* from the Argentinean coast (Fernandez Castro and de Vido Mattio, 1987), *R. philippinarum* from the Lagoon of Venice (Marin et al., 2003) and from west coast of Korea (Kang et al., 2007), and *R. decussatus* (Camacho et al., 2003).

I observed that total carbohydrates simultaneously increased with gametogenesis which implied that carbohydrates may not be converted into egg lipid during gametogenesis rather than they used as a source of energy during spawning and post-spawning periods. However, to draw conclusion about the dynamic changes of biochemical components, the gametogenic status of the analyzed clams needs to be confirmed as the inter-individual variations in gametogenic condition is often common at any sampling date. It was also evident from our findings as the total carbohydrates in standard clam declined with the initiation of spawning and reached about one-fourth of its mean maximum values during post-spawning period. Camacho at al. (2003) reported that glycogen and other carbohydrates in *R. decussatus* were rapidly consumed after the onset of spawning and reduced to almost a quarter of just before spawning.

I measured the chlorophyll-a from the dried tissues as an index of food intake in the form of phytoplankton. To our knowledge, this is the first instance to measure the chlorophyll-a level from the clam tissues. The seasonal variations in the chlorophyll-a level in tissues are most likely associated with the availability of food in the sediment surface and the filtration rate of the clams. Hornbach et al. (1984) reported that seasonal filtration rates for adult clams peaked during periods of greatest reproduction. The standing stock of phytoplankton biomass in the water column often synchronizes with the gametogenesis and spawning of bivalves (Soniat and Ray, 1985; Park and Choi, 2004). Our data clearly showed that the chlorophyll-a was remarkably higher during the major spawning period than that of other seasons, suggesting that this method could be used successfully as an index

of food intake by clams.

In conclusion, I quantified the reproductive effort of female Manila clam in terms of GSI and potential fecundity collected from a natural stock along the east coast of Jeju, Korea using ELISA. Our data clearly showed that the clams were ready for spawning when the gonad accounted for 20% of the total tissue weight and the potential fecundity ranged from 2.42 to 8.97 million. Histology and ELISA data indicated that the spawning period of this clam timing from late July to mid September with major spawning activities during August to mid September in this area. The positive correlation between total protein and total carbohydrates ($r=0.85$) implied that in clams carbohydrates may not be used as a reserve for gametogenesis rather than it is used as a source of energy during spawning and post-spawning period. The observed difference in mean maximum GSI of the clams between the present study and previous study conducted in Gomso Bay, west coast of Korea might be associated with the difference in availability of foods in the water column.

Part II

Reproductive efforts in relation to annual gametogenic cycle of Manila clam, *Ruditapes philippinarum* collected from Begmiri, west coast of Korea

ABSTRACT

The gametogenic stage-wise reproductive efforts of Manila clam, *Ruditapes philippinarum* was quantified collected monthly during 2007 from a commercial bed at Begmiri, west coast of Korea using combined histology and enzyme-linked immunosorbent assay (ELISA). Forty clams were randomly selected from monthly samples. After dissection, a thin slice of 3 mm was cut through the gonad for histology and the remaining tissues were lyophilized for the quantification of reproductive efforts and biochemical analysis. Manila clam egg-specific antibody was raised in New Zealand white rabbit following standard protocol. An indirect ELISA confirmed that the antibody raised can successfully detect egg-specific protein during late developing, ripe and spawning condition. The presence of egg protein was detectable in most of the spent females; however, the egg protein in most of the early developing clams remained undetectable by ELISA. The mean gonadosomatic index (GSI) was 3.35±2.15% in early developing clams, 10.82±6.49% for late developing clams, 24.92±8.21% for ripe clams, 12.01±8.79% for spawning individuals and 5.23±4.59% for spent clams. The potential fecundity of the clams ranged from 2.54 to 13.84 million with a mean of 7.14±2.88 million. Seasonal and gametogenic stage-wise variations in proteins and carbohydrates were prominent and carbohydrates were more likely to be utilized as major energy source or converted to lipid before the clam became sexually mature. The new approach for gametogenic stage-wise reproductive effort

and estimation of potential fecundity of precisely ripe clams could be used successfully in all bivalves.

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1. INTRODUCTION

Qualitative and quantitative information on bivalve reproduction is essential for understanding their life history, population dynamics and for the development of aquaculture industry. The quantitative data on bivalve reproduction is scarce due to the technical difficulties involved in its measurement (Choi et al., 1993; Thompson et al., 1996; Park and Choi, 2004). In bivalves, the gonads are an integral part of the visceral mass except scallops (Lucas, 1982; Thompson et al., 1996) makes it difficult to quantify the actual gonad (Choi et al., 1993; Park and Choi, 2004). Reproductive effort or fecundity of marine bivalves has been estimated by measuring the difference in weight just prior to and after spawning (Deslous-Paoli and Heral, 1988; Pouvreau et al., 2000), counting or weighing the number of gametes released after inducing animals to spawn using various chemicals and thermal shock (Toba and Miyama, 1991; Chung et al., 2001; Massapina et al., 1999). Stereological methods coupling histology and image analysis have also been widely used for the quantification of bivalve reproduction by enumerating the gametogenic products (i.e., oocytes) either by staging a defined number of oocytes (Robinson and Breese, 1982; Hadfield and Anderson, 1988), by estimating the total number of oocytes per individual (Brousseau, 1978), by the percentage of the lumen filled with oocytes (planimetry, Eversole et al., 1980; Perdue and Erickson, 1984; Dinamani, 1987; Lango-Reynoso et al., 2000), or by oocyte size/frequency (Keck et al., 1975; Grant and Tyler, 1983; Heffernan et al.,

1989; Kanti et al., 1993). These methods often underestimate the true gonads, as reproduction is not always complete and occurs at various intensities throughout the spawning period (Lucas, 1982; Choi et. al., 1993; Thompson et al., 1996).

Immunological methods have been used successfully for the quantification of egg proteins of marine bivalves due to its high speed, low cost and high sensitivity (Choi et al., 1993, 1994; Kang et al., 2003; Park et al., 2003; Park and Choi, 2004; Park et al., 2005). In the above studies, the temporal variations in reproductive efforts were quantified, but most importantly, the inter-individual variations in gametogenic condition at every sampling date were overlooked. It is obvious that the spawning of Manila clam is asynchronous and the individuals are often composed of different gametogenic stages in each sampling date as evidenced from the histological data (Kang et al., 2003; Park et al., 2003; Park and Choi, 2004). Another limitation of the existing methods was that the potential fecundity was estimated in all females when the mean GSI peaked using ELISA assuming that all the individuals were ripe and not spawned yet by that time (Kang et al., 2003; Park et al., 2003; Park and Choi, 2004). The ripe clams need to be ascertained to estimate the potential fecundity as it is the total number of mature oocytes in the pre-spawning gonad. Therefore, the present study was undertaken with the following objectives: (1) to quantify the dynamic changes in the reproductive effort of Manila clam, *Ruditapes philippinarum* with its reproductive cycle; (2) to estimate the potential fecundity of this clam in this habitat and (3) To quantify the seasonal and gametogenic stage-wise changes in proteins and carbohydrates using standard animal model.

2. MATERIALS AND METHODS

2.1. Sampling efforts

Clams were obtained monthly from a commercial bed at Begmiri, west coast of Korea during 2007 (Fig. 12). Forty adult clams (shell length >27 mm) were randomly selected from the monthly samples. The schematic diagram of sample treatments is outlined in Fig. 13. The shell length of the clams was measured and the clams were dissected. After weighing the soft body, the gills were isolated for the quantification of *Perkinsus olseni* by fluid thioglycollate medium (FTM) assay. A thin slice (3 mm) was cut through the gonad for histological preparations and the remaining tissues were lyophilized for quantification of gonadosomatic index (GSI) and other biochemical analysis. The lyophilized clams were homogenized using mortar and pestle and were kept in the refrigerator at -70 ℃ until use. Shells of the clams were dried and weighed. The condition index as a measure of general health status of the clams was calculated by dividing the tissue wet weight by shell dry weight.

2.2. Histology

The thin slices were fixed in Davidson's solution and dehydrated in graded series of alcohol. The tissue slice was subsequently embedded in paraffin. Paraffin blocks were sectioned to 6 μm, stained with Harris's haematoxylene and counter-stained with eosin Y. Gametogenic condition was categorized based on the visual examination of the histological preparations under microscope. The reproductive

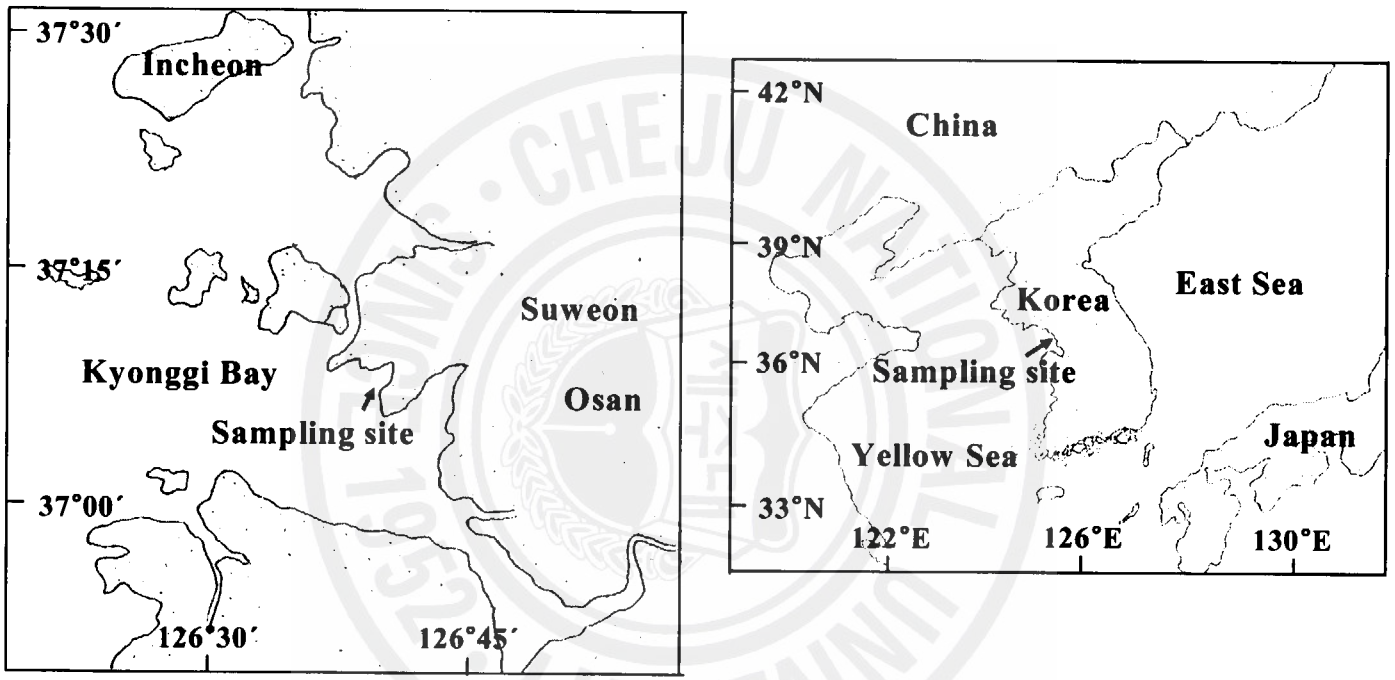

Fig. 12. Map showing sampling site

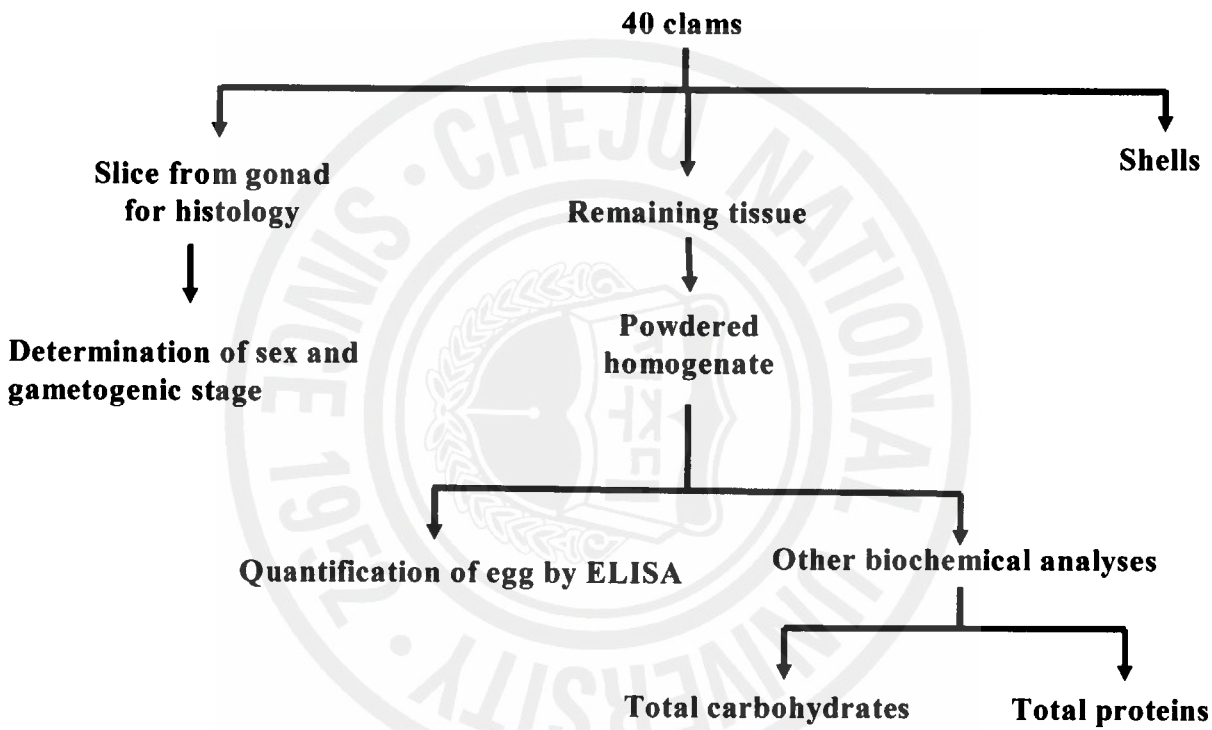

Fig. 13. Schematic diagram of sample treatment applied in the present study.

maturity of the gonads observed in the histological preparations was categorized into six stages using the maturity scale described by Park and Choi (2004), including 1) early developing; 2) late developing; 3) ripe; 4) spawning; 5) spent; and 6) sexually indifferent.

2.3. Development of Manila clam egg-specific antibody

A New Zealand white rabbit was selected for the development of Manila clam egg protein-specific antibody. The rabbit anti-Manila clam egg protein-specific antibody was raised following the protocol outlined by Park and Choi (2004). The antibody developed in the present study was highly specific to clam egg protein though some cross reactivity was recognizable with somatic tissue proteins. The cross reacting antibody was eliminated using immunoadsorbent according to Fuchs and Sela (1973). In brief, clam somatic tissue proteins were polymerized with glutaric dialdehyde to form the immunoadsorbent. The somatic protein-specific antibodies were bound to the immunoadsorbent whereas, egg protein-specific antibodies remained unbound due to the absence of egg protein in the immunoadsorbent. After centrifugation, the supernatant containing clam egg protein-specific antibody was isolated and precipitated using 50% saturated ammonium sulfate. The rabbit anti-clam egg protein-specific IgG was purified by dialysis and the sensitivity was tested by ELISA. No further cross reactivity was evident by the purified IgG with somatic tissues.

2.4. Quantification of gonadosomatic index (GSI)

An indirect enzyme-linked immunosorbent assay was used for quantification of egg-specific protein as well as for testing the specificity of the IgG. Only female clams were analyzed for this purpose. For analysis, around 20 mg clam homogenate was dissolved in 900 µl PBS and further homogenized using a sonicator The homogenate was diluted up to 2000 folds to get the optical density within the acceptable range of the standard curve. Here I used purified clam eggs as positive control and clam somatic tissues and PBS as negative control.

A 100 µl aliquot of clam homogenate to be analyzed and controls were added to the well and incubated at 4°C overnight or at 37°C for 3 hrs. After incubation, the plate was washed with PBS containing 0.05% Triton X-100 (PBST-100) and 150 µl of 1% bovine serum albumin was added as blocking buffer. The plate was incubated again for 1 hr at room temperature and washed with PBST-100. Primary antibody diluted in blocking buffer (1:2000 dilution; 9.4 µg protein/ml) was added in each well at a volume of 100 µl and incubated further 1 hour at room temperature. Goat anti-rabbit IgG alkaline phosphatase conjugate (1:1000) diluted in blocking buffer was then added as a 100 µl aliquot at each well, incubated for another 1 hour, and washed. Finally, 100 µl of p-nitrophenylphosphate (p-NPP) substrate dissolved in 0.1M glycine buffer (1 mg/ml) was added as a coloring agent and the optical density of the end product was measured at 405 nm using a 96-well micro-plate reader. A standard regression curve was constructed from the optical density and the standard prepared from the clam egg protein included in the plate.

The concentration of egg protein in the clam homogenate was estimated from the regression curve and dilution factor. The quantity of the eggs in an individual clam was then estimated by multiplying the quantity of the egg protein measured by ELISA by 2.44, the ratio of the egg protein to total egg weight. The reproductive effort was expressed as gonadosomatic index (GSI), a ratio of the estimated total dry weight of the eggs to total dry weight of the clam tissues in percentage.

2.5. Estimation of potential fecundity

I selected the ripe clams as evidenced from the histological observations for the estimation of potential fecundity. The potential fecundity of the ripe clams was calculated by dividing the estimated total dry weight of eggs measured by ELISA, by the dry weight of an individual egg. The mean dry weight of a mature egg of this clam collected from the west coast of Korea was 22 ng as reported in the previous study carried out from this laboratory by Park and Choi (2004) was used for calculation in the present study.

2.6. Measurement of total proteins and carbohydrates

Total proteins and carbohydrates were assessed from the dried clam tissues of the females to express their seasonal changes in connection with gametogeneic activity. Total proteins were determined by colorimetric method of Lowry et al. (1951) after extraction with 0.1M NaOH using bovine serum albumen as standard. Total carbohydrates were analyzed using phenol-sulfuric acid method as described by Dubois et al. (1956) using dextrose enhydrase as standard.

2.7. Standard animal

Total proteins and carbohydrates of a standard animal of 36.6 mm shell length were calculated for each sampling date. The standard animal length was found out by averaging the shell length of all the analyzed clams. This was done to eliminate the effect of size and to express the total quantity of proteins and carbohydrates in absolute values as their seasonal changes often related with the gametogenic cycle in bivalves. Allometric equations of $\log_{10}$ dry tissue weight against $\log_{10}$ shell length for each population at each sampling date was determined by linear regression analysis. All regressions were statistically highly significant ($P < 0.001$). This technique was used for the clam, *Tapes decussatus* (Beninger and Lucas, 1984), *T. philippinarum* (Beninger and Lucas, 1984; Kang et al., 2007) and for the oyster, *Cressostrea gigas* (Ruiz et al., 1992; Kang et al., 2000).

2.8. Statistical analysis

All the data were analyzed statistically and expressed as mean (±SD) using SAS statistical package.

3. RESULTS

3.1. Biometry and condition index

The biometric measurements of the analyzed clams are shown in Table 6. The individual shell length of the clams ranged from 27.7 to 42.8 mm and the mean shell length over the sampling period was 36.6 mm. Mean wet and dry tissue weight showed a clear seasonality with two peaks, during May and July. Mean wet tissue weight and dry tissue weight varied consistently in relation to gametogenic stages having an increasing tendency with the progression of gametogenesis, peaking in ripe stage and then declining abruptly during spawning stage, exhibiting the lowest values during spent condition.

The seasonal variations in CI are very clear as shown in Table 6. CIs increased rapidly after February with two maxima as such in tissue weight, one in May and the other during July. A slight decrease was noted in June but an abrupt decline was observed between July and August. The values remained constant from October to December. However, the gametogenic stage-wise changes in CIs of females were more distinct having an increasing tendency from early developing (0.499±0.068) to late developing clams (0.569±0.082). The indices reached maximum during ripe stage (0.640±0.083). A sudden drop was identified in spawning clams when the indices remarkably declined to 0.472±0.111, while the lowest CI was noted in spent stage of the clams (0.390±0.099).

Table 6. Monthly mean (±SD) shell length in mm (SL), tissue wet weight in g (TWWT), tissue dry weight in g (TDWT), condition index (CI) and gonadosomatic index in percentage (GSI) of *Ruditapes philippinarum* collected from Begmiri, west coast of Korea. GSI was estimated only for females.

Month	N	SL	TWWT	TDWT	CI	N (females)	GSI
January	40	44.0±53.7	1.867±0.450	0.394±0.116	0.459±0.083	0	-
February	40	36.9±2.4	2.059±0.426	0.433±0.089	0.431±0.059	4	Undetectable
March	40	34.1±1.0	1.766±0.263	0.369±0.069	0.509±0.065	13	Undetectable
April	40	37.1±2.1	2.515±0.574	0.589±0.164	0.518±0.103	11	4.27±1.98
May	40	36.7±2.6	2.758±0.583	0.634±0.144	0.587±0.094	24	16.77±8.19
June	40	36.6±2.2	2.666±0.610	0.576±0.152	0.563±0.134	20	17.62±13.05
July	40	36.9±2.1	2.686±0.609	0.636±0.099	0.615±0.147	21	20.63±10.15
August	40	36.3±2.0	2.045±0.486	0.425±0.113	0.452±0.070	18	14.00±9.82
September	40	36.2±1.8	1.812±0.372	0.361±0.086	0.415±0.079	15	5.48±4.52
October	40	30.5±2.3	1.514±0.397	0.308±0.081	0.334±0.041	9	4.58±3.80
November	40	37.2±2.0	1.688±0.340	0.328±0.070	0.347±0.052	1	Undetectable
December	40	36.3±2.2	1.570±0.275	0.373±0.056	0.326±0.056	1	Undetectable

3.2. Gametogenic cycle

Temporal variations in the percentage of female animals in each reproductive stage are shown in Fig. 14. In case of indifferent clams, I considered 50% animals supposed to be female for the convenience of the description and subsequent interpretation. All the individuals collected in January were composed of sexually indifferent clams. Gametogenesis commenced in February and the early developing clams occupied the major proportion during March and April. Late developing clams appeared in April and May while the majority of the clams (50.0%) were ripe by May. Spawning commenced in May when only one clam out 24 females analyzed were in spawning. Ripe clams dominated from May to July and spawning clams were evident from May to September. Spent clams appeared mainly from August to October and the sexually indifferent clams occupied principally from November to the end of the study in December. Some unusual indifferent clams were identified all through the study period and most of them were heavily infected by trematodes.

3.3. Quantitative measurement of GSI

The seasonal changes in reproductive effort of the female clams measured by ELISA were expressed as weight-normalized GSI as shown in Table 6 and Fig. 15. The reproductive effort was undetectable from February to March when the analyzed clams were in early developing stage. The eggs were quantifiable in clams collected during April when five clams out of 13 females were successfully detected by indirect ELISA having a mean GSI of 4.27±1.98%. The GSI increased

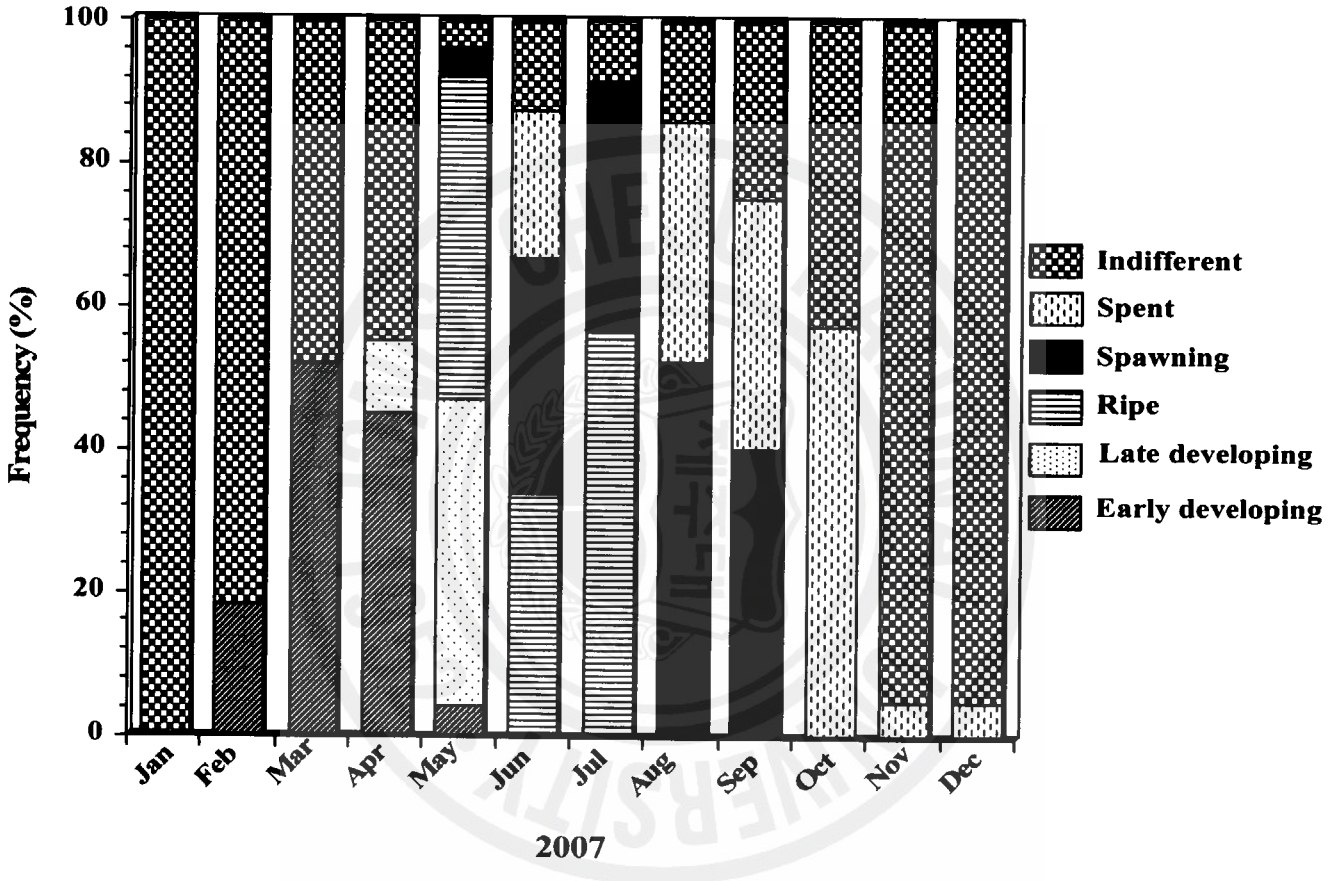

Fig. 14. Temporal distribution of gametogenic stages of female *Ruditapes philippinarum.*

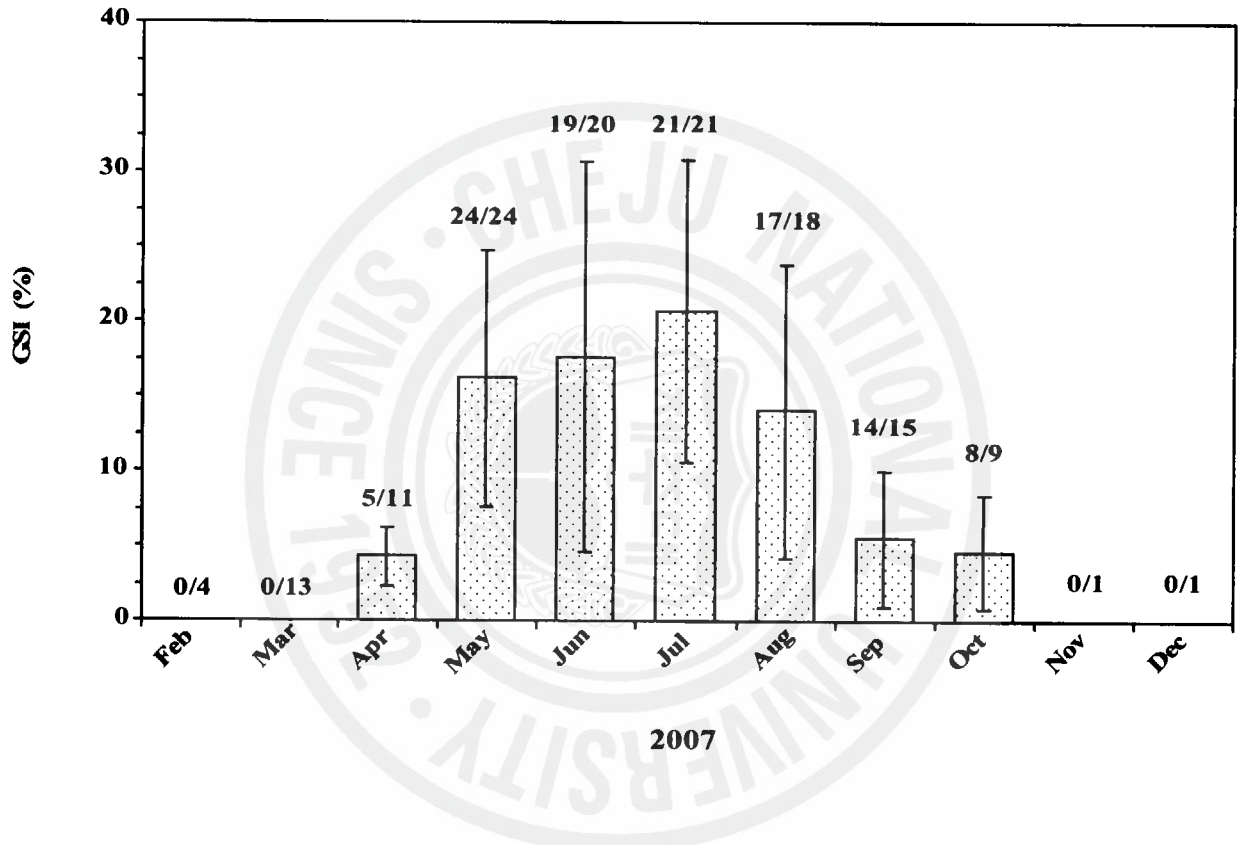

Fig. 15. Temporal variations in gonadosomatic index (GSI) of Manila clam collected from Begmiri, west coast of Korea. Vertical line represents the standard deviation and the figures abobe the bar indicate the number of clams in which egg protein was within detectable range/total no. of females analyzed by ELISA.

rapidly by May during which the reproductive efforts increased by four times in comparison to that obtained in April. The GSI remained almost same between May (16.77±8.19%) and June (17.62±13.05%) when some of the females already commenced spawning as evidenced from the histology. The high standard deviation in GSI during this time reflected the presence of clams in several reproductive categories during June. An increase in GSI during July (20.63±10.15%) indicated the major spawning pulse of the clams in this habitat. A massive drop in GSI during July to August, confirmed the major spawning activity of the clams during this time. Most of the clams continued their spawning event till September (5.48±4.52%) and the reproductive effort was detectable till October. The egg proteins could not be detected by ELISA in clams collected during November and December when the 2 analyzed females were in spent condition, one in either month.

In the present study I used the same clam for histology as well as for the quantification of reproductive effort. The new technique allowed me to quantify the gametogenic stage-wise GSI as shown in Table 7 and Fig. 16. The quantity of eggs remained very low during early developing stage when the GSI of three clams out of 27 females were detected by ELISA performed in the current study. The mean GSI of the detected early developing clams was 3.35±2.15%. The GSI increased at a faster rate from early developing to late developing stage during which it increased by three-folds by mean. The reproductive effort of all the female clams analyzed was detectable at this stage. The GSI increased with the proliferation of eggs exhibiting a mean GSI of 24.92±8.21% for ripe females. The

Table 7. Gametogenic stage-wise changes in total proteins, carbohydrates and reproductive efforts of female *Ruditapes philippinarum* collected from Begmiri, west coast of Korea.

Gametogenic stage	N	Shell length	Std. animal wt. (mg)	Proteins		Carbohydrates		Condition index	GSI	Fecundity (Million)
				mg/std. animal	%	mg/std. animal	%			
Early developing	27	36.0±2.8	506.0	191.5	37.9±3.4	50.40	9.96±4.3	0.499±0.068	3.35±2.15	-
Late developing	12	37.1±3.4	626.2	219.7	35.1±3.1	100.68	16.1±3.3	0.569±0.082	10.82±6.49	-
Ripe	33	36.3±1.8	635.3	243.6	38.4±5.1	74.43	11.7±3.5	0.640±0.083	24.92±8.21	7.14±2.88
Spawning	37	36.9±2.1	444.4	173.8	39.1±3.7	35.7	8.03±4.3	0.472±0.111	12.01±8.79	-
Spent	28	34.0±3.4	373.8	139.8	37.4±5.9	20.33	5.44±4.2	0.390±0.099	5.23±4.59	-

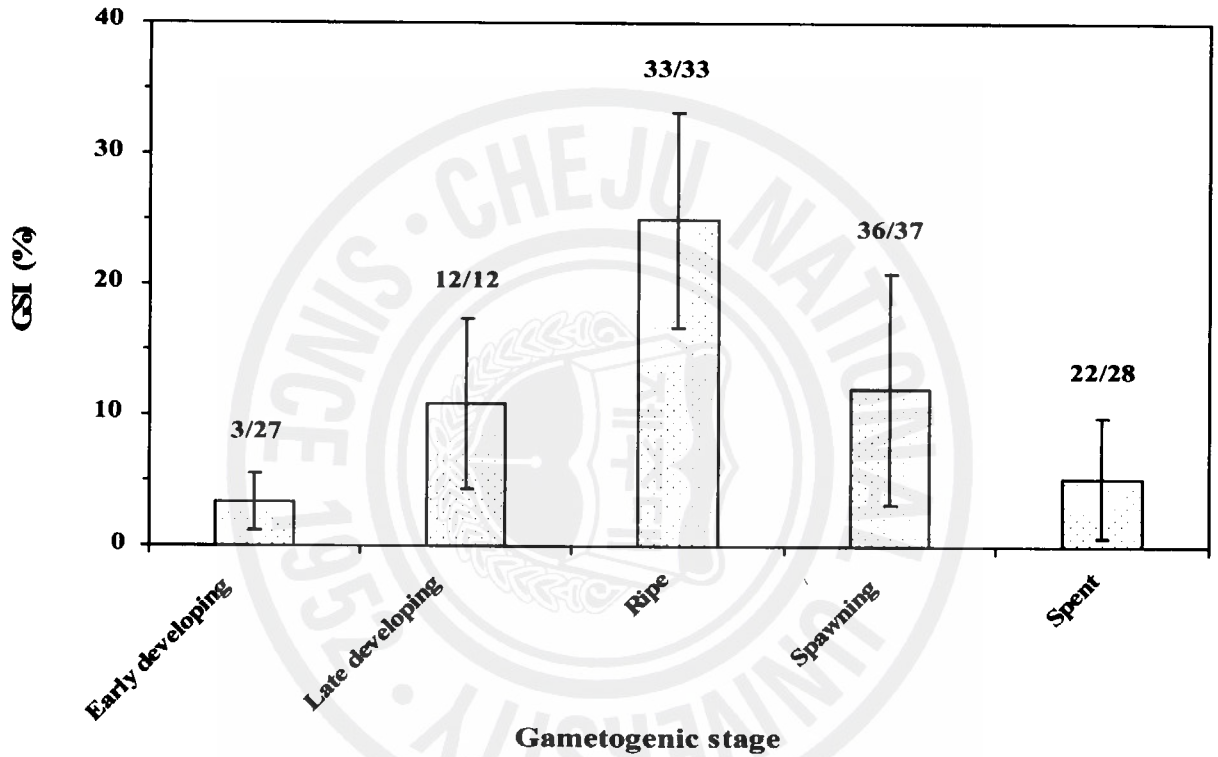

Fig. 16. Gametogenic stage-wise variations in gonadosomatic index (GSI) of Manila clam collected from Begmiri on the west coast of Korea. Vertical line represents the standard deviation and the figures above the bar indicate the number of clams in which egg protein was within detectable range/total no. of females analyzed by ELISA.

GSI of all the ripe clams were successfully detected by ELISA and the individual GSI varied from 9.09 to 43.20%. The mean increment was almost two-folds from late developing to ripe stage. Spawning clams showed a mean GSI of 12.01±8.79% ranging from 1.90 to 35.11%. Out of 37 spawning females analyzed, the egg protein was undetectable in only one clam. The GSI remained 5.23±4.59% in spent clams when the eggs considered being unviable by histological observations due to the presence of haemocytes with deformed eggs. Among the spent females, the reproductive effort was efficiently detected in 22 clams out of 28 by ELISA.

3.4. Potential fecundity estimated from ELISA data

I used only the ripe clams ascertained from the histological observations for the estimation of potential fecundity as it is the total number of mature eggs in the pre-spawning gonad. Among the analyzed clams, 33 individuals were ripe, obtained during May, June and July. High inter-individual variations in GSI of the ripe clams were noted. The potential fecundity varied from 2,544,553 to 13,839,542 with an average of 7,140,354±2,881,586 (Table 7).

3.5. Tissue weight of a standard animal

The seasonal variations in tissue dry weight for a standard animal of 36.6 mm shell length are shown in Fig. 17A. A remarkable seasonal variation in tissue dry weight of the clams was evident and the trend was similar with that of the condition index. The standard animal dry weight increased slowly from January, rapidly from March with the first peak in May and the second peak in July seemed

to track the condition index of the clams. The lowest tissue dry weight of the standard animal was noted in November. The tissue dry weight of a standard animal matched with the reproductive cycle of the clams. The weight clearly increased from early developing to late developing stage, exhibiting its highest value in case of ripe clams. Tissue dry weight decreased markedly in spawning stage while the lowest value was noted in spent clams (Table 7).

3.6. Total proteins and carbohydrates

Seasonal variations in total proteins and carbohydrates are shown in Table 8. No clear seasonality was evident in the percentage of proteins in dry tissues and the mean values ranged from 32.1% in November to 42.2% in September. However, the percentage of carbohydrates varied consistently in analyzed females collected in different months. The proportion of carbohydrate increased from January, exhibiting its peak during May. The percentage of carbohydrates declined from June and reached less than one-sixth of its peak during October. The ratio of carbohydrates then increased from November to December.

Seasonal changes in the absolute values of proteins and carbohydrates for a standard animal, calculated from the percentage composition and the dry tissue weight are presented in Table 8. Both components reflected a clear seasonal changes tracking with the gametogenic pattern of the clams as illustrated in Figs. 18B and 18C. The quantity of total proteins in standard animal increased from January and peaked in May. A second peak was noted in July while the quantity decreased rapidly after July sowing the lowest value in December. About 50%

Table 8. Seasonal changes in total proteins and carbohydrates in female *Ruditapes philippinarum* collected from Begmiri, west coast of Korea.

Month	N	Shell length	Wt. of std. animal (mg)	Proteins		Carbohydrates	
				mg/std. animal	%	mg/std. animal	%
January	0	-	-	-	-	-	-
February	4	35.1±1.8	417.6	153.5	36.8±1.29	35.9	8.6±2.36
March	13	34.1±1.2	427.2	159.9	37.5±4.11	33.1	7.7±3.76
April	11	37.7±2.6	547.7	211.4	38.6±3.16	75.5	13.8±3.90
May	24	37.0±2.8	617.0	218.9	35.4±4.04	84.5	13.7±3.61
June	20	35.9±2.1	557.0	214.5	38.5±5.06	65.5	11.8±3.77
July	21	37.4±1.8	584.0	231.0	39.6±3.55	65.9	11.3±4.14
August	18	36.8±1.9	424.3	167.7	39.5±6.71	25.1	5.9±2.26
September	15	35.8±1.7	367.3	155.2	42.2±3.79	17.3	4.7±1.33
October	9	30.0±2.5	441.3	152.2	34.5±2.93	9.8	2.2±0.46
November	1	36.6	307.6	98.8	32.1	7.9	2.6
December	1	34.5	326.5	111.2	34.06	22.6	6.9

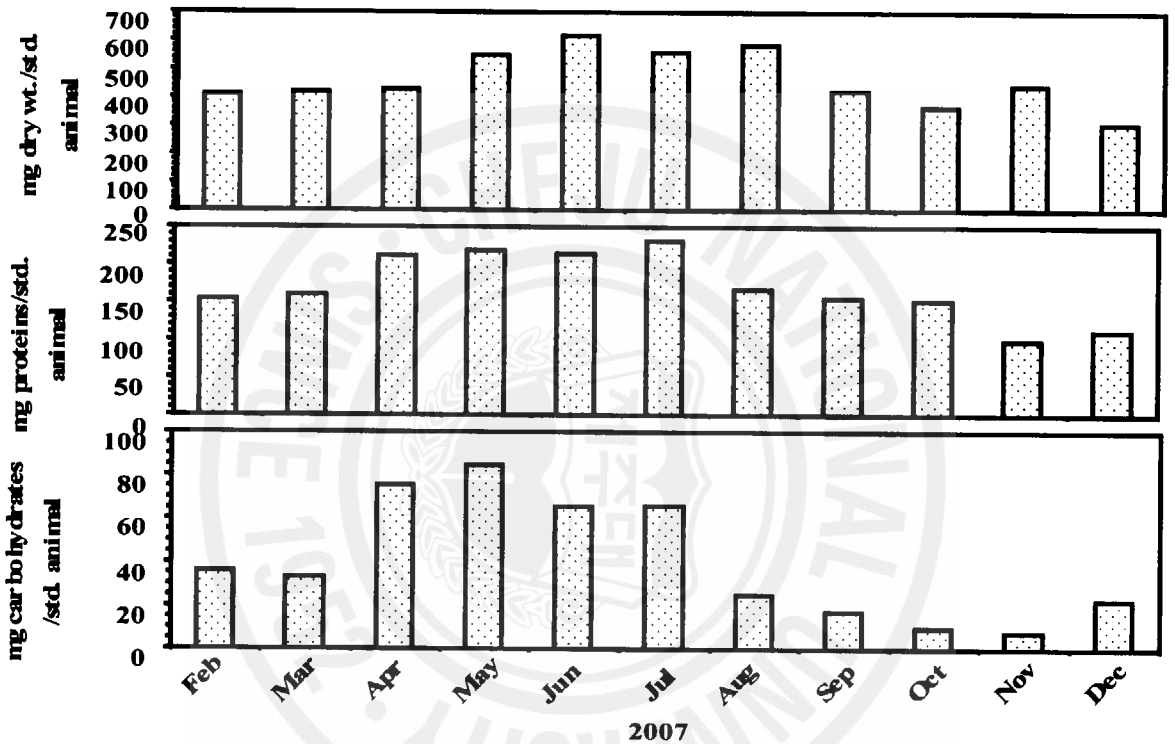

Fig. 17. A. Temporal variations in the tissue dry weight of the standard animal of 36.6 mm in shell length;

B. Temporal variations in total proteins of the standard animal;

C. Temporal variations in total carbohydrates of the standard animal.

protein was used during spawning as gametes and energy. Total carbohydrates showed the similar trend as in proteins but the rate of utilization was much higher than proteins, leaving only one-ninth of its maximum quantity during reproduction.

The gametogenic stage-wise protein percentage remained more or less stable however, total carbohydrate percentage varied markedly as shown in Table 7. Total carbohydrates increased from indifferent to early developing stage and peaked in late developing stage. Interestingly, the proportion of carbohydrates decreased from late developing to ripe stage and this trend was maintained till the termination of spawning. The gametogenic stage-wise absolute quantity of proteins increased from early developing to late developing stage and reached its peak in ripe stage. Total proteins dropped suddenly during spawning and exhibited its lowest quantity in spent stage. The absolute values of carbohydrates increased from indifferent to early developing stage, having its peak during late developing stage. Total carbohydrates then decreased in ripe stage and consistently declined till spent condition of the clams.

4. DISCUSSION

4.1. Gametogenic cycle

The reproductive maturity was categorized into six stages that have been used by most authors for studying the reproductive biology of Manila clam (Kanti et al., 1993; Xie and Burnell, 1994; Park and Choi, 2004; Drummond et al., 2006). The study broadly agrees the observations of Park and Choi (2004) on the gametogenic cycle of Manila clam in the west coast of Korea, with the initiation of gametogenesis in February. However, an early spawning was evident in the present site than that reported from other locations on the west coast of Korea. The continual large scale spawning from June to September was somewhat longer than that reported from June to August by Park and Choi (2004) for this clam on the west coast of Korea. This discrepancy might be due to the difference in sampling frequency as they did not sample during September. However a delayed spawning period was reported by Chung et al. (1994, 2001) timing from July to October on the west coast of Korea. The differences in timing and duration of spawning are more likely due to the inter-annual variation in gametogenesis, as the exogenous factors controlling gametogenesis typically vary from year to year, such as water temperature, salinity, and food availability (Barber and Blake, 1991; Arellano-Martinez *et al.*, 2004; Dukeman et al., 2005).

4.2. Gonadosomatic index

The combined histology and ELISA using the same clam enabled me to

quantify the gametogenic stage-wise reproductive effort. It is obvious that the dynamics of gametogenic materials follows the reproductive cycle of bivalves but no gravimetric method has yet been developed for bivalves to investigate it. However, some quantitative staging methods were developed using histological preparations which involves enumeration of gametogenic products either by staging a defined number of oocytes (Robinson and Breese, 1982; Hadfield and Anderson, 1988), by estimating the total number of oocytes per individual (Brousseau, 1978), by percentage of the lumen filled with oocytes i.e., planimetry (Eversole et al., 1980; Park and Choi, 2004), or by oocyte size/frequency (Keck et al., 1975; Grant and Tyler, 1983; Heffernan et al., 1989; Kanti et al., 1993). But all of the above methods lack the weight based gonadosomatic index though it is crucial for the quantification of reproduction in any animal. Several methods have also been applied for the quantification of reproductive effort of bivalves, including weight loss before and after spawning (Kautsky, 1982; Pouvreau et al., 2000), weighing or counting the released gametes after spawning using inducing agent (Chung et al., 2001). The estimate of the above methods is often lower than the actual values as spawning is not always complete and occurs over a prolonged spawning period (Park and Choi, 2004).

The immunological method developed by Choi et al. (1993) opened a new dimension for the quantification of GSI of American oyster, *Crassostrea virginica* due to its high speed and sensitivity. The method subsequently used for the quantification of reproductive effort in Pacific oyster, *C. gigas* (Kang et al., 2003), in butter clam, *Saxidomus purpuratus* (Park et al., 2003) and in Manila clam, *R.*

philippinarum (Park and Choi, 2004). However, in the above studies, the temporal variation in reproductive effort of the aforesaid bivalves was emphasized. In addition to ELISA, they used histology which revealed that the spawning of bivalves is often asynchronous and the inter-individual variations in gametogenic stages are common in almost every sampling date. In the present study, I observed three or more gametogenic staged clams at every month when the gametigenic activity was more prominent (April to September). The new approach for the quantification of gametogenic stage-wise GSI could be useful for better understanding the gametogenic cycle in Manila clams as well as in other bivalves. I prepared the standards using somatic tissue solutions as solvent. I used same dilutions for eggs as well as somatic tissues having exactly similar dry tissue weight as I used for the unknown clam samples. The egg solution of known protein concentration was then diluted by somatic tissue solutions to use as the standards. The modification allowed me to eliminate the effect of dilution by keeping a constant dilution for the standards as well as samples. The mixture of eggs and somatic tissues as standards was also comparable with those present in unknown clam samples.

The study confirms the observations of the previous study on the seasonality of reproductive efforts of Manila clam on the west coast of Korea. The egg proteins remained undetectable by ELISA till March though some early developing clams were present as indicated by histology. ELISA detected no or very low level of egg protein in April. The clams collected from February to April were predominantly composed of pre-vitellogenic eggs, which contained no or

very low level of vitellin as outlined by Park and Choi (2004). As the antibody was raised against ripe eggs which were thought to be composed of vitellin, it did not react with vitellogenin, the precursor of vitellin, mostly present in the early developing eggs. The egg protein of some early developing clams was detected in April might be due to the conversion of some vitellogenin into vitellin. The mean GSI increased rapidly from April (4.27%) to May (16.77%) and this was in agreement with the findings of Park and Choi (2004). The major peak in GSI was noted in July (20.63%) and the timing was in consistent with the previous study conducted on the west coast of Korea (Park and Choi, 2004). The major spawning pulse broadly agrees the previous study timing from June to September. However, the mean peak GSI was markedly lower in the present study than the previous study which might be associated with the food availability and perkinsosis, an epidemic in this coast.

The gametogenic stage-wise GSI from the histology and ELISA data introduced a new approach for the better explanation of quantitative reproductive cycle of this clam. The seasonal changes in the reproductive effort of some bivalves have been reported by Choi et al. (1993), Kang et al. (2003), Park et al. (2003) and Park and Choi (2004). It is obvious that the quantity of gametogenic materials changes with the gametogenic status of the clams which is not always a seasonal phenomenon. This is because, spawning of most of the bivalves is asynchronous and inter-individual variation in gametogenic condition is often common in any sampling date as reported in the previous studies. This method could successfully detect reproductive effort in late developing, ripe and spawning

clams however, the eggs could be detectable in most of the spent clams and not sensitive enough to detect reproductive effort in most of the early developing clams. The gametogenic stage-wise GSI revealed the quantitative gametogenic cycle with approximate mean viable egg weight per female of the stock by deducting the mean egg weight during spent stage from the mean egg weight during ripe stage. I considered that the gametes observed during spent stage were resorbed into the gonad. This was suggested by the presence of haemocytes, and broken appearance of the follicles leads the process of atresia mediated by apoptosis as evidenced from the histology. The presence of haemocytes in spent clams is common to resorb the residual eggs (Drummond et al., 2006). As I obtained the mean GSI of spent clams of 5.23%, I considered that these eggs were not released and supposedly resorbed by the clams. Therefore, this method can be used for the quantification of atretia in bivalves.

4.3. Estimation of potential fecundity

The potential fecundity was estimated in case of ripe clams as confirmed by histology. The precise selection of ripe clams is an important tasks in estimation of potential fecundity as it the total number of ripe eggs in the pre-spawning gonad. In the previous methods, the fecundity was estimated in all case of all females during mean peak GSI assuming that all the individuals were ripe and ready for spawning during that time (Kang et al., 2003; Park et al., 2003; Park and Choi (2004). However, histological preparations indicated that the individuals are often composed of different gametogenic stages during each sampling period. Therefore,

the current estimate is more accurate and remarkably higher than that reported by Park and Choi (2004).

4.4. Seasonal changes in proteins and carbohydrates

The seasonal changes in dry tissue weight of standard animal tracked the gametogenic cycle as the gametogenesis and evacuation of gametes often related with the changes in weight with constant seize of the bivalves. Although there was a clear tendency that the seasonal changes in proteins and carbohydrates paralleled with those of dry tissue weight. Proteins fluctuated with non regular seasonal trend perhaps due to transformation of somatic tissue proteins to egg proteins during the proliferation of the gametes as outlined by Adachi (1979) and Marin et al. (2003). However, the seasonal changes in absolute values of proteins in standard animal distinctly related to the progressive gametogenesis are in agreement with the findings of Kang et al. (2007) for this species collected from Korean coast. A slight decrease in absolute values of proteins in June and August might be associated with spawning activity of the clams by this time. The abrupt decline in proteins due to spawning was also reported in many bivalves, such as *Mytillus galloprovincialis* from the Lagoon of Venice (Bressan and Marin, 1985), *Ostrea puelchana* from the Argentinean coast (Fernandez Castro and de Vido Mattio, 1987), *R. philippinarum* from the Lagoon of Venice (Marin et al., 2003) and from west coast of Korea (Kang et al., 2007), and *R. decussatus* (Camacho et al., 2003).

Carbohydrates mainly in the form of glycogen are the main energy reserve in adult bivalves, being used during gametogenesis and in conditions of nutritional

stress (Marin et al., 2003). Manila clam is an opportunistic species, whose gametogenesis occurs when there is an abundance of food in the environment, and sexual maturing parallels the accumulation of nutrients (Drummond, 2006). Marked seasonal fluctuation in the percentage of carbohydrates in dry tissues occurred indicated the importance of carbohydrates in reproductive cycle of this clam. An increasing trend of carbohydrates with the intensity of gamete proliferation and then decreasing suddenly with the decreasing CI was reported by Marin et al., (2003) as I observed in the present study.

The gametogenic-stage wise proportion of proteins remained more or less constant but carbohydrates increased from indifferent to late developing stage and then decreased consistently until the termination of spawning. This indicated that carbohydrates may be transformed into lipid for the gamete formation or it was utilized more for energy production than the other biochemical components. Marin et al. (2003) reported that glycogen can be converted to lipids during gametogenesis of *Tapes decussatus*. Unfortunately I couldn't quantify the seasonal changes of lipids for this species in the present study.

5. CONCLUSIONS

I quantified the reproductive efforts of Manila clam using combined histology and ELISA. Temporal variation in GSI indicated a distinct peak during July when the mean value was 20.63% however, spawning commenced as early as May. Major spawning event occurred from June to September. The present technique allowed me to quantify gametogenic stage-wise GSI showing the intensity of mobilization of gametogenic materials during annual reproductive cycle. The GSI of most of the early developing clams remained undetectable. The mean GSI of late developing, ripe, spawning and spent clams were 10.82%, 24.92%, 12.01% and 5.23% respectively. The potential fecundity was estimated precisely for ripe clams having a mean value of 7.14 million ranging from 2.54 to 13.84 million. The temporal changes in proteins and carbohydrates per standard animal showed similar trend but the gametogenic stage-wise carbohydrates peaked in late developing stage while the proteins peaked in ripe stage. The combined histology and immunological method can be used successfully for the quantification of reproductive efforts in terms of gametogenic stages of the Manila clam and other bivalves.

REFERENCES

Adachi, K. 1979. Seasonal changes of the protein level in the adductor muscle in the clam, *Tapes philippinarum* (Adams and Reeve) with references to the reproductive seasons. Comp. Biochem. Physiol. A 64:85-89.

Arellano-MDrtnez, M., Ceballos-9 i zquez, B.P., Villalejo-Fuerte, M., GDrcta-DoP tnguez, F., Elorduy-Garay, J.F., Esliman-Salgado, A., Racotta, I.S. 2004. Reproduction of the lion's paw scallop *Nodipecten subnodosus* Sowerby, 1835 (Bivalvia: Pectinidae) from Laguna Ojo de Liebre, B.C.S., Mpxico. J. Shellfish Res. 23:723-729.

Barber, B.J., Blake, N.J. 1991. Reproductive physiology. In: Shumway, S.E. (Ed.), Scallops: Biology, ecology and aquaculture. Elsevier, pp. 1095.

Beninger, P.G., Lucas, A. 1984. Seasonal variation in condition, reproductive activity and gross biochemical composition of two species of adult clam reared in a common habitat: *Tapes decussatus* L. (Jeffreys) and *Tapes philippinarum* (Adams and Reeve). J. Expt. Mar. Biol. Ecol. 79:19-37.

Bressan, M., Marin, M.G. 1985. Seasonal variations in biochemical composition and condition index of cultured mussels (*Mytilus galloprovincialis*) in the Lagoon of Venice (North Adriatic). Aquaculture 48:13-21.

Brousseau, D.J. 1978. Spawning cycle, fecundity and recruitment in a population of soft shelled clam, *Mya arenaria*, from Cape Ann, Massachusetts. Fish. Bull. 76:155-166.

Camacho, A.P., Delgado, M., Fernandez-Reiriz, M.J., Labarta, U. 2003. Energy balance, gonad development and biochemical composition in the clam *Ruditapes decussatus*. Mar. Ecol. Prog. Ser. 258:133-145.

Choi, K.-H., Park, G-M., Chung, E.-Y. 2005. Ovarian maturation in female *Ruditapes philippinarum* on the west coast of Korea. Dev. Reprod. 9:123-134.

Choi, K.-S., Lewis, D.H., E.N. Powell, Ray, S.M. 1993. Quantitative measurement of reproductive output in the American oyster, *Crassostrea virginica*, using enzyme-linked immunosorbent assay (ELISA). Aquacult. Fish. Mgmt. 24:299-322.

Choi, K.-S., Powell, E.N., Lewis, D.H., Ray, S.M. 1994. Meausrement of instantaneous reproductive effort in the American oyster, *Crassostrea virginica*, using a Protein A immunoprecipitation assay. Biol. Bull. 186:41-61.

Chung, E.-Y., Ryou, D.-K., Lee, J.-H. 1994. Gonadal development, age and growth of the shortnecked clam, *Ruditapes philippinarum* (Pelecypoda: Veneridae), on the coast of Kinje, Korea. Korean J. Malacol. 10:38-54.

Chung, E.-Y., Hur, H.-B., Hur, Y.-B., Lee, J.-S. 2001. Gonadal maturation and artificial spawning of the Manila clam *Ruditapes philippinarum* (Bivalvia: Veneridae), in Komso Bay, Korea. J. Fish. Sci. Technol. 4:208-218.

Chung, E.-Y., Lee, J.-S., Lee, C.-H., Hur, S.B. 2002. Reproductive cycle of natural population and artificial control of gonadal development of *Ruditapes philippinarum* by the conditions of water temperature-feeding and

starvation. Korean J. Malacology 18:83-91.

Crosby, M.P., Gale, L.D. 1990. A review and evaluation of bivalve condition index methodologies with a suggested standard method. J. Shellfish Res. 9:233-237.

Delgado, M., Camacho, A.P. 2007. Influence of temperature on gonadal development of *Ruditapes philippinarum* (Adams and Reeve, 1850) with special reference to ingested food and energy balance. Aquaculture 264:398-407.

Deslous-Paoli, J.M., Heral, M. 1988. Biochemical composition and energy value of *Crassostrea gigas* (Thunberg) cultured in the Bay of Marennes-Oleron. Aquat. Living Resour. 1:239-249.

Diggles, B.K., Cochennec-Laureau, N., Hine, P.M. 2003. Comparison of diagnostic techniques for *Bonamia exitiosus* from flat oysters *Ostrea chilensis* in New Zealand. Aquaculture 220:145-156.

Dinamani, P. 1987. Gametogenic patterns in populations of Pacific oyster, *Crassostrea gigas*, in Northland, New Zealand. Aquaculture 64:65-76.

Drummond, L., Mulcahy, M., Culloty, S. 2006. The reproductive biology of the Manila clam, *Ruditapes philippinarum*, from the north-west of Ireland. Aquaculture 254:326-340.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

Dukeman, A.K., Blake, N.J., Arnold, W. 2005. The reproductive cycle of the flame

scallop, *Ctenoides scaber* (Born 1778) from the lower Florida keys and its relationship with environmental conditions. J. Shellfish Res. 24:341-351.

Eversole, A.G., Michener, W.K., Eldridge, P.J. 1980. Gametogenesis and spawning in North American clam populations: implications for culture. Vol. 19. pp. 111-125. In: Developments in aquaculture and fisheries Science, Clam mariculture in North America. J.J. Manzi and M. Castagna (eds.). Elsevier, Amsterdam.

Fernandez Castro, N., de Vido de Mattio, N. 1987. Biochemical composition, condition index and energy value of *Ostrea puelchana* (D'Orbigny): relationships with the reproductive cycle. J. Exp. Mar. Biol. Ecol., 108:113-126.

Fuchs S., Sela. M. 1973. Immunoadsorbents. In: Handbook of Experimental Immonology, 2nd edition. Ed. Weir, D.M. Blackwell Scientific Publications, pp. 10.1-10.6.

Giese, A.C., Pearse, J.S. 1974. Introduction: general principles. Pages 1-49 in A.C. Giese and J.S. Pearse (eds.). Reproduction of Marine Invertebrates, Vol. 1. Academic Press, New York.

Grant A., Tyler, P.A. 1983. The analysis of data in studies of invertebrate reproduction. II: The analysis of oocyte size/frequency data, and comparison of different types of data. Int. J. Invertebr. Reprod. 6:271-283.

Grant, C.M., Creese, R.G. 1995. The reproductive cycle of the Tuata-*Paphies subtriangulata* (Wood, 1828) in New Zealand. J. Shellfish Res. 14:287-292.

Hadfield, A.J., Anderson, D.T. 1988. Reproductive cycle of the bivalve mollusks *Anadara trapezia* (Deshayes), *Venerupis crenata* Lmark and *Anomia descripta* Iredale in the Sydney region. Aust. J. Freshwater Res. 39:649-660.

Heffernan, P.B., Walker, R.L. 1989. Gametogenic cycles of three bivalves in Wassaw Sound, Georgia. III. *Geukensia demissa* (Dillwyn, 1817). J. Shellfish Res. 8:327-334.

Heffernan, P.B., Walker, R.L., Carr, J.L. 1989. Gametogenic cycles of three bivalves in Wassaw Sound, Georgia: I. *Mercenaria marcenaria* (Linnaeus, 1758). J. Shellfish Res. 8:51-60.

Hofmann, E.E., Powell, E.N., Klinck, J.M., Wilson, E.A. 1992. Modelling oyster populations III. Critical feeding periods, growth and reproduction. J. Shellfish Res. 11:399-416.

Hornbach, D.J., Way, C. M., Wissing, T.E., Burky, A.J. 1984. Effects of particle concentration and season on the filtration rates of the freshwater clam,*Sphaerium striatinum* Lamarck (Bivalvia: Pisidiidae). Hydrobiologia 108:83-96.

Hunter, J.R., Macewicz, B.J., Lo, N.C.H., Kimbrell, C.A. 1992. Fecundity, spawning, and maturity of female Dover Sole, *Microstomus pacificus*, with an evaluation of assumptions and precision. Fish. Bull., U.S. 90:101-128.

Kang, C.-K., Kang, Y.-S., Choy, E.-J., Kim, D.-S., Shim, B.-T., Lee, P.-Y. 2007. Condition, reproductive activity, and gross biochemical composition of the Manila clam, *Tapes philippinarum* in natural and newly created sandy

habitats of the southern coast of Korea. J. Shellfish Res.

Kang, K.-K., Park, M.-.S., Lee, P.-Y., Choi, W.-J., Lee, W.-C. 2000. Seasonal variation in condition, reproductive activity, and biochemical composition of the Pacific oyster, *Crassostrea gigas* (Thunberg), in suspended culture in two coastal bays of Korea. J. Shellfish Res. 19:771-778.

Kang, S.-G, Choi, K.-S., Bulgakov, A.A., Kim, Y., Kim, S.-Y. 2003. Enzyme-linked immunosorbent assay (ELISA) used in quantification of reproductive output in the pacific oyster, *Crassostrea gigas*, in Korea, J. Exp. Mar. Biol. Ecol. 282:1-21.

Kang, D.-H., Ngo, T.T.T., Koh, K.-J., Choi, K.-S. 2004. Seasonal changes in gonadal development of Manila clam, *Tapes philippinarum* from Shi-Heung-Ri, Jeju, Korea. J. of Aquaculture 17:81-88.

Kanti, A., Heffernan, P.B., Walker, R.L. 1993. Gametogenic cycle of the southern surf clam, *Spisula solidissima* similes (Say, 1822) from St. Catherines Sound, Georgia. J. Shellfish Res. 12:255-261.

Kautsky, N. 1982. Quantitative studies on gonad cycle, fecundity, reproductive output and recruitment in a Baltic *Mytilus edulis* population. Mar. Biol. 68:143-160.

Keck, R.T., Maurer, D., Lind, H. 1975. A comparative study of the hard clam gonad developmental cycle. Biol. Bull. (Woods Hole). 148:243-258.

Kim, H.-J. 2005. A comparative study on the growth, spawning and recruitment of the Manila clam, *Ruditapes philippinarum* according to their environmental factors in Seonjaedo, Incheon, Korea. MS thesis, Inha

University, 63p.

Kjesbu, O.S., Klungsøyr, J., Kryvi, H., Witthames, P.R., Greer Walker, M. 1991. Fecundity, atresia, and egg size of captive Atlantic cod (*Gadus morhua*) in relation to proximate body composition. Can. J. Fish. Aquat. Sci. 48:2333–2343.

Lango-Reynoso, F., Chávez-Villalba, Cochard, J.C., Pennec, M.L. 2000. Oocyte size, a means to evaluate the gametogenic development of the Pacific oyster, *Crassostrea gigas* (Thunberg). Aquaculture 190:183-199.

Laptikhovsky, V. 2000. Fecundity of squid *Loligo vulgaris* Lamarck, 1978 (Myopsida, Loliginidae) off northwest Africa. Scientia Marina 64:275-278.

Lowry, O.H., Rosebrough, N.J., Lewis Farr, A., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Lucas, A. 1982. Evaluation of reproductive effort in bivalve mollusks. Malacologia 22:183–187.

Macer, C.T. 1974. The reproductive biology of the horse mackerel (*Trachurus trachurus* L.) in the North Sea and English Channel. J. Fish. Biol. 6:415-438.

Mann, R., Rainer, J.S., Morales-Alamo, R. 1994. Reproductive activity of oysters, *Crassostrea virginica* (Gmelin, 1971) in the James River, Virginia, during 1987-1988. J. Shellfish Res. 13:157-164.

Marin, M.G., Moschino, V., Deppieri, M., Lucchetta, L. 2003. Variations in gross biochemical composition, energy value and condition index of *T. philippinarum* from the Lagoon of Venice. Aquaculture 219:859-871.

Massapina, C., Joaquim, S., Matias, D., Devauchelle, N. 1999. Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Porugal. Aquat. Living Resour. 12:327-333.

Meneghetti, F., Moschino, V., Da Ros, L. 2004. Gametogenic cycle and variations in oocyte size of *Tapes philippinarum* from the Lagoon of Venice. Aquaculture 240:473-488.

Moran, R., Porath, D. 1980. Chlorophyll determination in intact tissues using N,N-dimethylformamide. Plant Physiol. 65:478-479.

Morvan, C., Ansell, A.D. 1988. Stereological methods applied to reproductive cycle of *Tapes rhomboids*. Mar. Biol. 97:355–364.

Murua, H., Kraus, G., Saborido-Rey, F., Withames, P.R., Thorsen, A., Junquera, S. 2003. Procedures to estimate fecundity of marine fish species in relation to their reproductive strategy. J. Northw. Atl. Fish. Sci. 33:33-54.

Murua, H., Saborido-Rey, F. 2003. Female reproductive strategies of marine fish and their classification in the North Atlantic. J. Northw. Atl. Fish. Sci. 33:23-31.

Ngo, T.T.T., Choi, K.-S. 2004. Seasonal changes of *Perkinsus* and *Cercaria* infection in the Manila clam *Ruditapes philippinarum* from Jeju, Korea. Aquaculture 239:57-68.

Park, K.-I., J.-W. Choi, Choi, K.-S. 2003. Quantification of reproductive output of the butter clam, *Saxidomus purpuratus* (Sowerby, 1852) using enzyme-linked immunosorbent assay (ELISA). Ocean Polar Res. 25:249–256.

Park, K.-I., Choi, K.-S. 2004. Application of enzyme-linked immunosorbent assay

for studying of reproduction in the Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia): I. Quantifying eggs. Aquaculture 241:667-687.

Park, K.-I., Choi, J.-W., Choi, K.-S. 2005. Development of a *Saxidomus purpuratus* (Mollusca: Bivalvia) egg-specific antibody for the quantification of eggs using an enzyme-linked immunosorbent assay. J. Shellfish Res. 24:1079-1086.

Perdue, J.A., Erickson, G. 1984. A comparison of the gametogenic cycle between the Pacific oyster *Crassostrea gigas* and the Suminoe oyster *Crassostrea rivularis* in Washington State. Aquaculture 37:231-237.

Ponurovsky, S.K., Yakovlev, Y. 1992. The reproductive biology of the Japanese littleneck, *Tapes philippinarum* (A. Adams and Reeve, 1850) (Bivalvia: Veneridae). J. Shellfish Res. 11:265-277.

Pouvreau, S., Gangnery, A., Tiapari, J., Lagarde, F., Garnier, M., Bodoy, A. 2000. Gametogenic cycle and reproductive effort of the tropical blacklip pearl oyster, *Pinctada margaritifera* (Bivalvia: Pteriidae), cultivation in Takapoto atoll (French Polynesia). Aquat. Living Resour. 13:37-48.

Powell, E.N., Hofmann, E.E., Klinck, J.M., Ray, S.M. 1992. Modeling oyster populations I. A commentary on filtration rate. Is faster always better? J. Shellfish Res. 11:387-398.

Powell, E.N., Klinck, J.M., Hofmann, E.E., Wilson-Ormond, E.A., Ellis, M.S. 1995. Modeling oyster populations. V. Declining phytoplankton stocks and the population dynamics of American oyster (*Crassostrea virginica*) populations. Fish. Res. 24:199-222.

Robinson, A.M., Breese, W.P. 1982. The spawning season of four species of clams in Oregon. J. Shellfish Res. 2:55-57.

Royer, J., Seguineau, C., Park, K.-I., Pouvreau, S., Choi, K.-S., Costil, K. 2008. Gametogenic cycle and reproductive effort assessed by two methods in 3 age classes of Pacific oyster, *Crassostrea gigas*, reared in Normandy. Aquaculture (Accepted article).

Ruiz, C.M.A., Sedano, F., Garcia-Martin, L.O., Sanchez Lopez, J.L. 1992. Influence of seasonal environmental changes on the gamete production and biochemical composition of *Crassostrea gigas* (Thunberg) in suspended culture in El Grove, Galicia, Spain. J. Expt.Mar. Biol. Ecol. 155:249-262.

Sastry, A.N. 1979. Pelecypoda (excluding Ostreidae). p. 113-292. In: Reproduction of marine invertebrates, ed. by Giese, A.C. and J.S. Pearse, Academic Press, New York.

Soniat, T.M., Ray, S.M. 1985. Relationships between possible available food and the composition, condition and reproductive state of oyster from Galveston Bay, Texas. Contrib. Mar. Sci. 18:109-121.

Thompson, R.J., R.I.E. Newell, V.S. Kennedy, Mann, R. 1996. Reproductive process and early development In: V.S. Kennedy, R.I.E. Newell and A.F. Eble, Editors, The Eastern Oyster *Crassostrea Virginica*, A Maryland Sea Grant Book, Maryland, USA (1996), pp. 335–370.

Toba, M., Miyama, Y. 1991. Gonadal development and spawning induction in artificially conditioned Manila clams *Ruditapes philippinarum*. Nippon

Suisian Gakkaishi 57:1269-1275.

Uddin, M.J., Kim, H.-J., Hong, J.-S., Yang, H.-S., Choi, K.-S. (unpublished). Seasonal changes in *Perkinsus olseni* infection and gametogenesis in Manila clam, *Ruditapes philippinarum* from Seonjaedo Island in Incheon, off the west coast of Korea.

Walker, R.L., Heffernan, P.B. 1994. Temporal and spatial effects of tidal exposure on the gametogenic cycle of the northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758), in coastal Georgia. J. Shellfish Res. 13:479-486.

Witthames, P.R., Greer Walker, M., Dinis, M.T., Whiting, C.L. 1995. The geographical variation in the potential annual fecundity of Dover Sole *Solea solea* (L.) from European shelf waters during 1991. Neth. J. Sea Res. 34:45-58.

Xie, Q., Burnell, G.M. 1994. Comparative study of gametogenic cycles of the clams *Tapes philippinarum* (Adams & Reeve 1850) and *Tapes decussatus* (Linnaeus) on the south coast of Ireland. J. Shellfish Res. 13:467-472.

Yap, W.G. 1977. Population biology of the Japanese little-neck clam, *Tapes philippinarum* in Kaneoche Bay, Oahu, Hawaiian Islands. Pac. Sci. 31:223–244.

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List of Scientific Papers

1. **Uddin, M.J.**, Park, K.-I., Kang, D.-H., Park, Y.-J., Choi, K.-S. 2007. Comparative reproductive biology of Yezo scallop, *Patinopecten yessoensis*, under two different culture systems on the east coast of Korea. Aquaculture 265:139-147.
2. **Uddin, M.J.**, Dewan, S., Haque, M.R. 2006. Effect of fish culture in rice fields on the availability of nutrients in soil. Bangladesh J. Progress. Sci. Tech. 4:155-158.
3. Dewan, S., Yeasmine, S., **Uddin, M.J.**, Hossain, M.S. 2004. Performance of soybean as a replacer of fish meal in the supplementary feed on the growth of carps. Bangladesh J. Fish. 28:63-70.
4. Kunda, M., Dewan, S., **Uddin, M.J.**, Yousuf, A.M., Uddin, M.S. 2004. Length-weight relationship, condition factor and relative condition factor of *Barbodes gonionotus*, *Amblypharyngodon mola*, and *Cyprinus carpio* in rice fields. Progress. Agric. 15:159-169.
5. Dewan, S. **Uddin, M.J.**, Hossain, M.M.M., Das, D.R., Hossain, M.S. 2002. Studies on production potentials of 'Kua' fisheries of o aktodaha Beel, Bogra, Bangladesh. Pak. J. Biol. Sci. 5:221-224.
6. **Uddin, M.J.**, Dewan, S., Ashrafuzzaman, M., Haque, M.M. 2001. Growth and yield of GIFT (*Oreochromis niloticus*) and Thai silver barb (*Barbodes gonionotus* Bleeker) in rice fields and their effects on the yield of rice. Bangladesh J. Fish. Res. 5:29-35.

7. **Uddin, M.J.**, Dewan, S., Hossain, S.M.A., Islam, M.N. 2000. Effect of fish culture in rice fields on the yields of rice and nutrients uptake by rice grain and straw. Bangladesh J. Fish. 23:89-97.

8. Chowdhury, M.T.H., Dewan, S., Wahab, M.A., **Uddin, M.J.,** Thilsted, S.H. 2000. Water quality parameters of the rice fields for rice cum fish culture. Bangladesh J. Fish. 23:25-29.

Papers Presented in International Conferences

1. **Uddin, M.J.,** Choi, K.-S. 2008. Quantification of reproductive output of Manila clam, *Ruditapes philippinarum* from the east coast of Jeju, Korea by enzyme-linked immunosorbent assay (ELISA). Proceedings of the World Aquaculture 2008 Conference, 19-23 May, Busan, Republic of Korea, p. 712.

2. **Uddin, M.J.,** Park, K.-J., Park, Y.-J., Choi, K.-S. 2008. Estimation of potential fecundity of Manila clam, *Ruditapes philippinarum* using an enzyme-linked immunosorbent assay (ELISA). Proceedings of the World Aquaculture 2008 Conference, 19-23 May, Busan, Republic of Korea, p. 713.

3. Yang, H.-S., Kim, B.-K., **Uddin, M.J.,** Limpanont, Y., Park, K.-J., Park, Y.-J., Choi, K.-S. 2008. Comparative survey on pathologic condition of clams *Ruditapes philippinarum* distributed along the Korean coast. Proceedings of the World Aquaculture 2008 Conference, 19-23 May, Busan, Republic of Korea, p. 750.

4. Choi, K.-S., Yang, H.-S., Kim, B.-K., **Uddin, M.J.,** Limpanont, Y., Park, K.-J., Park, Y.-J. 2008. Spatial variation of pathologic condition of Manila clam, Ruditapes philippinarum surveyed in spring and autumn 2007. Proceedings of the World Aquaculture 2008 Conference, 19-23 May, Busan, Republic of Korea, p. 125.

5. Limpanont, Y., Yang, H.-S., Hong, H.-K., Kim, B.-K., Chong, H.-D., Choi, K.-S., Lee, H.-C., **Uddin, M.J.,** Park, K.-J., Park, Y.-J., Choi, K.-S. 2008. Comparative study on annual gametogenesis of Manila clam (*Ruditapes philippinarum*) collected from eight locations on the west coast of Korea in 2007. Proceedings of the World Aquaculture 2008 Conference, 19-23 May, Busan, Republic of Korea, p. 447.

6. **Uddin, M.J.,** Choi, K.-S. 2007. Comparative annual gametogenic pattern of Manila clam (*Ruditapes philippinarum*) from a commercial bed and a natural habitat on Seonjae Island, Incheon, west coast of Korea. Proceedings of the Sixth International Workshop on the Oceanography and Fisheries Science of the East China Sea (2007 ECS Workshop), 3-5 November, Nagasaki, Japan, p. 23.

7. Yang, H.-S., Kim, B.-K., **Uddin, M.J.,** Limpanont, Y., Park, K.-J., Park, Y.-J., Lee, K.-W., Choi, K.-S. 2007. Report on the pathologic condition of Manila clam, *Ruditapes philippinarum* surveyed in spring 2007. Proceedings of the Sixth International Workshop on the Oceanography and Fisheries Science of the East China Sea (2007 ECS Workshop), 3-5 November, Nagasaki, Japan, p. 33.

8. **Uddin, M.J.,** Choi, K.-S. 2007. Quantification of reproductive output of Manila clam, *Ruditapes philippinarum* collected from the east coast of Jeju, Korea by enzyme-linked immunosorbent assay (ELISA). Proceedings of Asian-Pacific Aquaculture, August 5-7, Hanoi, Vietnam, p. 304.

9. **Uddin, M.J.**, Yang, H.-S., Park, K.-I., Choi, K.-S. 2006. Temporal and spatial distribution of brown ring disease (BRD) and trematode infection in Manila clam from five different locations of Korea. IX International Congress on Medical and Applied Malacology, October 17-20, Qindao, China, p.50.

10. Choi, K.-S., **Uddin, M.J.** 2006. Shellfish Aquaculture: Current status and related problems in Korea. IX International Congress on Medical and Applied Malacology, October 17-20, Qindao, China, p.40.

Papers Presented in Domestic Conferences

1. **Uddin, M.J.,** Park, K.-J., Park, Y.-J., Choi, K.-S. 2007. Survey on brown ring disease and trematode infection in Manila clam, *Ruditapes philippinarum* in eight different locations of Korea using traditional shell valve analysis method. Proceedings of the 2007 Joint Meeting of the Korean Societies on Fisheries Science, Busan, p. 384.71.

2. Limpanont Y., **Uddin, M.J,** Yang, H.-S., Kim, B.-K., Hong, H.-K., Jeung, H.-D., Choi, K.-S., Park, K.-J., Park, Y.-J., Choi, K.-S. 2007. Histological observation of the Manila clam, *Ruditapes philippinarum* in different

clam beds of Korea observed in 2007 winter. Proceedings of the 2007 Joint Meeting of the Korean Societies on Fisheries Science, Busan, p. 371.

3. Yang, H.-S., K.-Y. Park, **Uddin, M.J.,** Choi. K.-S. 2007. Seasonal dynamics of perkinsosis, brown ring disease (BRD) and trematode infection in the Manila clam, *Ruditapes philippinarum* from Hwangdo, Anmyeon Island. Proceedings of the 2007 Joint Meeting of the Korean Societies on Fisheries Science, Busan, p. 383.

4. Uddin, M.J., Hong, J.-S., Kim, H.-J., Choi, K.-S. 2006. Effects of protozoan parasite, *Perkinsus olseni* and trematode parasite, *Cercaria tapidis* on the reproductive pattern of Manila clam, *Ruditapes philippinarum* from Seonjae island, west coast of Korea. The 2006 Joint Meeting of the Korean Societies on Fisheries Science, Busan, p. 43.