A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GROWTH, BIOCHEMICAL COMPOSITION AND REPRODUCTIVE ACTIVITY OF PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG, 1793) IN DIFFERENT ENVIRONMENTS AND CULTURE CONDITIONS

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국문요약

Part I. Effects of grow-out transplantation timing on growth and reproduction of juvenile Pacific oyster, *Crassostrea gigas*, in Gamakman Bay, off the south coast of Korea

우리나라 참굴 양식 산업은 자연 채묘에 의한 종패 확보에 의존하고 있으며, 최근 몇 년동안 만 (Bay)에 서식하는 모패의 건강도가 좋지 않아 안정적인 치패 공급에 어려움을 겪고 있다. 일반적으로 5-10개월 단련된 참굴 치패를 5월에 양성장으로 이식하는 방법을 이용하고 있으나, 이 방식은 참굴 양식에 있어 낮은 생산력이 보고되고 있다. 이 연구에서는 단련된 참굴 치패를이식시기를 구분하여 2009년 1월과 5월에 남해안의가막만에 이식하고 참굴 치패의 성장과 번식를 조사하고, 이식 시기에 따른 참굴 치패의 양성 효과를 비교하였다. 연구기간 동안, 참굴 각장 (Shell Length)의 변화는 1월에 이식한 참굴 치패 (JTO)가 2월에 33.2 mm에서 11월 97.3 mm로, 5월에 이식한 참굴 치패 (MTO)는 6월에 20.0 mm에서 11월에 80.5 mm로 증가하였다. 탄수화물과 단백질 함량은 JTO가 MTO보다 유의하게 높았다 ($p<0.05$). 조직학적 관찰시, JTO에서 빠른 배우자 형성과 생식소 성숙이 이루어졌고, 7월과 9월 사이에 산란이 일어났다. 반면에, MTO에서는 배우자 형성 활동이 지연되는 경향을 보였으며, 산란은 8월과 10월 사이에 일어났다. 효소면역학적 방법으로 측정된 JTO의 포란수 (fecundity)는 평균 2.7×10^7 egg로 추산되었으며, 이 수치는 MTO 포란수의 약 6배 정도 높은 수치였다. 산란시, JTO에서는 상당히 많은 배우자 방출이 일어나는 반면, MTO에서는 지연되는 경향을 보였다. 위의 결과를 종합하면, 1월 이식 (January transplantation)이 치패 확보와 생산력 증대에

더 유용한 방법임을 시시한다. 또한, 현재 이용되고 있는 참굴의 5월 이식시기의 재평가가 필요하다; 최소한 일부 단련된 참굴 치패를 가능하면 1월초에 이식하여, 만에서 자연 치패 확보와 더불어 안정적인 참굴 양식 발달을 위한 산란군 (spawner)로양성해야 한다.

Part II. Early growth and reproduction of hatchery-produced juvenile Pacific oyster, *Crassostrea gigas*, in Gamakman Bay, off the south coast of Korea

최근 우리나라 굴 양식 산업은 자연산 치패의 불안정한 공급을 보완하기 위해, 인공종묘를 통한 치패의 생산에 대한 연구가 시도되고 있으나, 인공종묘로 생산된 참굴 치패의 성장과 번식에 관한 연구가 빈약한 실정이다. 이 연구는 인공종묘를 통해 생산된 참굴 치패의 초기 성장과 번식 특성을 조사하기 위해, 인공종묘로 생산된 치패를 2009년 7월부터 11월까지 남해안의 가막만에 수하식으로 양성하였다. 참굴 치패의 각장 (Shell Length)은 27.4 mm (2009년 7월)에서 이식 4개월 후인 2009년 10월 82.5 mm로 성장하였고, 습중량 (tissue weight)은 0.2 g에서 5.2 g으로 증가하여, 이 시기의 치패가 단기간에 상품 규격으로 성장하였음을 알 수 있었다. 조직학적 관찰 결과, 생식소 발달이 빠르게 이루어지면서 8월에서 10월 사이에 산란을 하는 것이 관찰 되었고, 11월에 휴지기 (resting phase)가 관찰되었다. 알의 양을 정량하기 위해 실시된 효소면역측정법 (enzyme-linked immunosorbent assay)의 결과, 참굴 치패는 체중의 5.1-8.8%의 알을 생성하며, 이는 성체 굴에 비해 상대적으로 적은 양의 알을 생성하고 있었다. 8월과 9월에 감소하는 체내 탄수화물량은 산란 활동의 에너지로 사용되었을 것으로 사료되며, 9월의 최대 단백질 함량은 최대 생식소 지수 (gonad-somatic index) 와 일치하였다. 이 연구 결과는 인공 종묘를 통

해 생산된 참굴 치패의 초기 성장과 번식에 대한 기초적인 정보를 제공함과 동시에 앞으로의 참굴 양식에 있어서 인공 종묘를 통해 생산된 치패가 양식 산업에 유용하게 이용될 것으로 기대된다.

Part III. Monitoring of biological activities of the wild Pacific oyster, *Crassostrea gigas*, two-years after the *Hebei Spirit* oil spill at Taean, off the west coast of Korea

2007년 12월 우리나라에서 가장 큰 해양 사고인 *Hebei Spirit* 유류 유출이 황해의 태안 인접 지역에서 발생하여, 해양 생물체에 막대한 영향을 주었다. 유류 유출의 생물학적 영향을 밝혀내기 위해, 2010년 4월부터 2011년 4월까지 중유 (heavy oil)의 영향을 받은 구례포와 대조구 지역인 인천 종현에서 자연산 참굴 (*Crassostrea gigas*)을 채집하여 성장, 에너지 저장과 번식 활동의 특성을 조사하였다. 연구 기간 동안 구례포 참굴은 종현 참굴보다 체중의 성장이 유의하게 높았다. 두 지역 참굴의 체중의 계절적 변동은 비슷하였고, 이 변동은 비만도 (condition index)의 변동과 일치하였다. 탄수화물 수치는 두 지역의 참굴에서 비슷한 수준으로 유지되었으나, 오염 지역 참굴의 단백질 수치가 대조구 지역보다 유의하게 높았다. 조직학적 관찰시 두 지역 참굴 모두 6월에 완숙하였고, 수온이 증가하는 7월부터 10월까지 산란이 일어났다. 최대 산란 활동으로, 8월부터 9월까지 참굴의 모든 생물학적 요소들의 수치가 감소하였다. 산란기의 오염 지역 암컷 참굴 개체에서 생성되는 알의 수는 대조구 지역보다 유의하게 높았다. 이 결과는 이매패류에대한 유류 유출의 영향이 사고 2년이 지난 시점에 더 이상 지속되지 않고 있으며, 이는 참굴의 생리학적 상태가 정상 수준으로 회복되었음을 의미한다.

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GENERAL INTRODUCTION

1. Biology and reproduction of oyster

The Pacific oyster, (*Crassostrea gigas*, Thunberg, 1793) also known as Pacific cupped oyster or Japanese oyster is a molluscan bivalve under the family of Osteridae. Pacific oysters are native to the Pacific coast of Asia, and became an introduced species in North America, Europe, Australia and Africa (FAO, 2011). This is an estuarine species and found to attach on rocks, debris and shells from the lower intertidal zone to 40 m deep sub-tidal regions. They are filter feeders, consume a variety of species of phytoplankton (or microalgae), bacteria and detritus from the surrounding water. They can tolerate a wide range of temperatures from -1.8 to 35℃ and salinities about 10 to 35 psu (FAO, 2011).

Pacific oysters are protandrous hermaphrodite, starting life as male and then changes to female later in life (Coe, 1934; Ventilla, 1984). They spawn as broadcast method, releasing gametes into the water column. Spawning is regulated by the environmental parameters, predominantly by the seawater temperature and food availability (Ruiz et al., 1992; Kang et al., 2003; Ngo et al., 2006; Kang et al., 2010). In the Korean waters, the gametogenesis of the oysters initiated around 10℃ and spawning occurred above 20℃ and rarely at 15-18℃ (Choi, 2008). The oysters spawn during June to October with a single or multiple spawning peaks during the spawning season (Kang et al., 2003; Ngo et al., 2006; Kang et al., 2010).

The life cycle of the Pacific oysters divided into a planktonic larval stage and sessile benthic stage. After the fertilization a non-feeding trocophore stage develops and

then changes to “veliger” or “D-shape”stage within 15-28 hours of the fertilization. The pedivelliger stage begins after the foot develops and the larvae begin crawling on surface and searching for a suitable substrate for their settlement. Once cemented on a suitable substrate, oysters are called “spat (juvenile oyster). Spat are sessile; they begin losing their larval organs (velum, foot and eyespot) and begin structure of adult oysters (Pauley et al., 1988).

2. Ecological and economic importance of oyster

Oysters are important species in keeping the diverse and healthy ecosystem. They improve the water quality through filtering the plankton and pollutants out of the water column. Adult Pacific oyster may filter 10L of water per hour (Pauley et al., 1988). In a wild environment the oysters cement themselves each other and creating underwater reef, which provide a food source and habitats for numerous species of crabs, birds, snails, and fish. Oyster reefs also help to stabilize shorelines, reduce erosion and water quality maintenance (Piazza et al., 2005).

The shellfish industry is an important economic activity in Korea, where the Pacific oysters playing a vital role. In 2005, more than 250,000 tons of oyster landing (worth US $ 128, 269) were recorded from Korea (Choi, 2008). The majority of the oysters produced in Korea are exported to the United States and Japan. The jobs that are produced from the culture, harvest and processing and marketing of Pacific oysters are a valuable asset, especially for the people of the south coast, the most important oyster aquaculture zone of Korea. In a report by Korean oyster long-line culture cooperation in 2005, estimates about 22,000 full-time employees engaged in the oysters industry from

farming to processing (Choi, 2008).

3. Oyster aquaculture in Korea

The Pacific oyster aquaculture method includes seed collection (from natural resources or hatchery), hardening, grow-out rearing and harvesting. In Korean oyster farming industries, about 90 percent of the national seed demand are supplied from the wild, naturally occurring in water (Choi, 2008). From the natural resources the oyster spats are collected during late June to August using 2 to 3 m long oyster culture string tied with numerous oysters shell and placed in the intertidal zone for hardening (Choi, 2008). Hardening is a common practice in Korea as well as in Japan, which provides the oysters higher survival rate, improved meat quality and greater adaptability to varying environmental conditions than non-hardened oysters in the grow-out field (Ventilla, 1984; Arakawa, 1990). After 9 to 10-months hardening, using the suspended long-lines the oysters are transplanted into the grow-out area located in the middle of the bays in May or early June for growing. Harvesting of the suspended culture oysters started as early as September and continued up to the following April (Choi, 2008).

4. Decline of oyster production in Korea

In Korea, the Pacific oyster production is decreasing in recent years. The decline in oysters landing has been associated with the overall environmental deterioration of the oyster culture ground due to intensive oyster farming, insufficient supply of healthy spat, effects of pollution and/ or pathogenic infection along the oyster culture grounds (Kang

et al., 2000; Park et al., 1999a, 1999b; Park and Chun, 1989; Choi et al., 1997).

Marine pollution is one of the major concerns to the bivalve industry which threatens the productivity of bivalve farms in coastal waters (Jeong and Cho, 2007). Bivalves are sessile filter-feeder, passively affected by the environmental changes and used as the sentinel organisms for the assessment of environmental pollution (Wade et al., 1998; Peteiro et al., 2006; Jeong and Cho, 2007). In exposure to pollutants various sub-lethal effects have been documented in different species of bivalve. A decline in scope for growth in Pacific oysters, *Crassostrea gigas* is documented from a laboratory experiment by Jeong and Cho (2007). Scope for growth represents an integration of major physiological responses, and specifically the balance between the process of energy acquisition and energy expenditure (Widdows and Donkin, 1991). Bayen et al. (2007) observed a reduced shell and somatic tissue growth in the Pacific oyster (*Crassostrea gigas*) from Singapore marine environment in exposure to chemical contaminants. Lee (2010) reported a rapid decline in growth and reproductive activity in farmed Pacific oysters immediately after the *Hebei Spirit* oil spill at Taean off the western coast of Korea. *Hebei Spirit* oil spill is considered as one of the major oil tanker accident of recent years and heavily impacted the west coast environment of Korea (ITOPE, 2008).

5. Confinement of this thesis

In this thesis, Part I compares the growth and reproduction of hardened juvenile Pacific oyster, *Crassostrea gigas*, transplanted into the Gamakman Bay, off the south coast of Korea in two different times to determine the effects of grow-out transplantation

timing on growth and reproduction of the oysters. Part II investigates the early growth and reproduction of hatchery-produced juvenile Pacific oysters in Gamakman Bay, off the south coast of Korea to know the aquaculture potentiality of the hatchery-seed oysters. Part III monitors the biological activities of the wild Pacific oysters two years after the *Hebei Spirit* oil spill at Taean, off the west coast of Korea to know their physiological condition and recovery status of the oysters after the oil accident. During this study along with histology, an indirect immunosorbant-assay (ELISA) was used according to the method of Kang et al. (2003) to quantify the reproductive effort of the oysters. Kang et al. (2003) developed a polyclonal antibody and using indirect ELISA they successfully quantified the reproductive effort of the Pacific oysters from the Gosung Bay, off the southern coast of Korea. In shellfish research ELISA is the more recent technique for the quantification of egg protein and many authors have been successfully used this method due to its high speed, low cost and high sensitivity (Choi et al., 1993; Kang et al., 2003; Park and Choi, 2004; Ngo et al., 2006; Royer et al., 2008).

Part I

Effects of grow-out transplantation timing on growth and reproduction of juvenile Pacific oyster, *Crassostrea gigas*, in Gamakman Bay, off the south coast of Korea

Abstract

Korean Pacific oyster industry is dependent on wild-harvested seeds but in recent years facing an unstable seed supply as a consequence of the unhealthy brood stock in the bays. Traditionally, the oyster spats are transplanted into the grow-out area in the month of May after 9 to 10-months hardening, and these oysters have a less reproductive activity during their rearing period. This study evaluated the traditional grow-out transplantation timing and investigated the growth and reproduction of the hardened juvenile Pacific oysters, *Crassostrea gigas*, differently transplanted in the Gamakman Bay, Korea in January and May (traditional transplantation time), 2009. During this monitoring shell length increased from 33.2 mm (February) to 97.3 mm (November) for the January transplanted oysters (JTO) and from 20.0 mm (June) to 80.5 mm (November) for the May transplanted oysters (MTO). The carbohydrate and protein level was significantly higher for the JTO than that of the MTO ($P<0.05$). Histology revealed an earlier gametogenesis and progressive gonad maturation for the JTO and they spawned during June and September. While, a delayed gametogenic activity exhibited for the MTO and they spawned during August and October. An ELISA estimated mean 27.92 million fecundity for the JTO, which was about 6-times higher than that of the MTO. During the spawning a substantial gamete releasing documented for the JTO, while

gamete releasing was retarded for the MTO. Results indicate that January transplantation could significantly contribute to spat recruitment as well as more oyster production. This study also suggest that the traditional grow-out transplantation timing needs to be re-evaluated; at least certain proportion of hardened spats should be transplanted as early as January; and/or reared in the bays as spawners for the natural seed stock improvement as well as for the sustainable development of oyster aquaculture in Korea.

1. Introduction

The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), is the most important shellfish resource in Korea and has been widely cultured in numerous semi-enclosed bays along the south coast. Using the suspended long-line culture technique, the south coast bays are currently contributing about 90 percent of the annual oyster production in Korea (Choi, 2008). Traditionally, the Korean oyster farming industry is using the wild-caught oyster spat from bays as seed. The wild oyster spats (seeds) are collected during late June to August and placed in the intertidal zone, where they periodically exposed to air with the tidal fluctuation and became hardened (Kang et al., 2010). Hardening is a common practice in Korea as well as Japan, as provides a higher survival rate, improved meat quality, and greater adaptability to varying environmental conditions than non-hardened oysters in the grow-out field (Ventilla, 1984; Arakawa, 1990; Kang et al., 2010). After 9 to 10-months intermediate rearing in the hardening ground, the oysters are transplanted into the grow-out areas located in the middle of the bays in May or early June for growing. The oysters are then harvested during winter and mid-spring, 6-11 months after grow-out rearing at reaching more than 70 mm shell length (Kang et al.,

2000; Hyun et al., 2001; Kang et al., 2010). Over a recent decade, Korean oyster aquaculture industry has been suffering from an unstable supply of healthy natural oyster seeds. Several studies have been carried out to understand the problems associated with oyster aquaculture of Korea (Park et al., 1999a, 1999b; Kang et al., 2000; Oh et al., 2002; Kang et al., 2010). Park et al. (1999a) reported that reproductively unhealthy brood stock is the main reason for the decline in wild spat collection in the south coast bays of Korea. Kang et al. (2010) investigated the growth and reproduction of farmed Pacific oyster transplanted into the Gamakman Bay off the south coast of Korea in May after 10-months of hardening. They observed only one spawning season during the production cycle when the oysters produce a less quantity of gametes and suggested that hardened oysters use more of their net energy for shell and somatic growth rather than reproduction during their first spawning period.

Understanding the annual gametogenesis and the quantitative estimation of reproductive effort are crucial for the proper management as well as development of aquaculture practices of marine bivalves (Gosling, 2003; Kang et al., 2003; Park and Choi, 2004; Yang et al., 2011). Reproductive activity of marine bivalves includes the initiation of gametogenesis, gonad maturation, spawning and egg fertilization and successive larval development. These reproductive processes are internally influenced by genetic factors and externally by the environmental parameters, like water temperature, salinity and food availability (Kennedy et al., 1996; Gosling, 2003). The food availability is the main factor which determines the seasonal energy storage and gametogenic development of bivalves (Bayne, 1976; Arakawa, 1990; Kang et al., 2000). Numerous studies have been carried out in relation to the reproductive cycle and biochemical composition of Pacific oyster and suggested that reproductive activity of

oyster are closely related to the seasonal cycle of biochemical composition (Whyte and Englar, 1982; Whyte et al., 1990; Ruiz et al., 1992; Park et al., 1999a; Ngo et al., 2006; Dridi et al., 2007). In general, prior to gametogenesis, reserves are accumulated in the form of glycogen, lipids, and protein, when food is available. The specific importance of these substrates and the timing of consumption in relation to gametogenesis vary between species as well as among populations of the same species (Bayne, 1976; Barber and Blake, 1981).

Histology has been widely used for the gametogenic study of bivalve molluscs, since it provides only the visual information of the gonad and does not provide precise information on the gonad biomass. Bivalves gonads are integrated part of the visceral mass (except in scallops) and cannot be separated from the soft body (Beninger and Lucas, 1984; Choi et al., 1993; Thompson et al., 1996). Several studies also have used the stereological methods coupling histology and image analysis for the quantitative estimation of bivalve's gonad (Kanti et al., 1994; Park and Choi, 2004; Royer et al., 2008). In recent years, immunological methods have been successfully used for the quantification of reproductive effort of bivalves (Choi et al., 1993; Kang et al., 2003; Park and Choi, 2004; Ngo et al., 2006; Royer et al., 2008). To estimate the quantity of eggs in an individual oyster Kang et al. (2003) developed a polyclonal antibody using purified Pacific oyster, *Crassostera gigas,* egg proteins as an antigen and subsequently utilized as the primary antibody in an indirect enzyme-linked immunosorbent assay (ELISA). This was a rapid and sensitive enough method to detect a less quantity of egg protein, even in early stages of development (Kang et al., 2003; Park and Choi, 2004; Ngo et al., 2006).

Until now, a few studies have examined the growth and reproduction of the

hardened oysters from the south coast of Korea (Kang et al., 2000; Oh et al., 2002; Kang et al., 2010) during their initial growing year, but no attention has been paid on the growth and reproduction of hardened juvenile Pacific oysters in relation to grow-out transplantation timing. In an attempt this study investigated the growth and reproduction of hardened juvenile Pacific oysters differently transplanted in January and in May (traditional transplantation time), 2009 into the grow-out area in the Gamakman Bay, a predominated oyster culture area on the south coast of Korea.

2. Materials and methods

2.1. Study area and sampling

Gamakman Bay (34°40′ N, 127°42′ E), is a major oyster farming area, located on the south coast of Korea, and encompassing a 112 km^2 surface area with a mean 9 m depth (Lee et al., 1995; Kang et al., 2010; Fig. 1-1). The oyster spats used in this study were initially collected from the sub-tidal area of Gamakman Bay in July, 2008 and then placed in the intertidal zone of the bay for hardening. Using a suspended long-line method, the hardened spats were transplanted into the middle of the bay at two different time periods, in January, 2009 (12.0 mm initial shell length with 6-months hardening) and another one in May, 2009 (19.9 mm initial shell length with 10-months hardening), and reared until January, 2010. For the January transplanted oysters (JTO), monthly 30-40 specimens were collected from February, 2009 to January, 2010 from the grow-out site for various analyses. While for the May transplanted oysters (MTO), monthly 30-40 specimens were collected from the grow-out site during June, 2009 and January, 2010.

Additionally, sampling also performed from the hardening site during February and May, 2009, to determine the growth and reproductive activity of the MTO during their additional hardening period compared to JTO. The environmental parameters, water temperature, salinity and chlorophyll *a*, were recorded at two-week intervals and averaged monthly during the study period.

2.2. Biometric measurements and histology

Upon arrival at the laboratory the specimens were cleaned of adherent epifauna and the shell length of the oysters was recorded to the nearest 0.1 mm length using vernier calipers. The soft tissues were separated from the shells, weighed and sectioned (2mm) dorsoventrally in the middle of the body and preserved in Davidson's fixative for histology. The remaining tissue was weighed, freeze dried and dry weight was measured. Tissue dry weight was calculated for the total wet weight (TWT) of the soft body using the dry weight of freeze dried remaining tissue (TWT-sliced tissue weight) and assuming that the water content was similar in all tissues. The lyophilized tissues were ground separately by mortar and pestle and mixed properly and then used for biochemical analysis and to quantify the egg mass (i.e., reproductive effort). The histology tissue sections were dehydrated with an ascending alcohol series, embedded in paraffin, sectioned at 6 µm thicknesses, and stained with Harri's hematoxylin and counter stained with eosin Y. The stained slides were examined under a light microscope to identify the sex and to evaluate the level of gonad maturation as: 1. resting, 2. early developing, 3. late developing, 4. ripe, 5. partially spawning, and 6. spent/ absorbing (Kang et al., 2010). Analyses of annual gametogenesis included only samples from female oysters and

oysters in the resting stage (i.e., unidentified sex). The condition index (CI) was measured as the ratio of the tissue dry weight (g) to the shell length (mm) (Lucas and Beninger, 1985).

2.3. Biochemical analysis

Homogenized tissue samples, from 20 to 25 mg, were weighed separately but a pooled method was applied for the MTO during February and June, 2009 due to insufficiency of tissue powder for the biochemical assay. Total carbohydrate in the tissue was measured using the phenol-sulfuric acid method (Taylor, 1995) with dextrose (anhydrous) as the standard material. Total protein was determined using the method of Lowry et al. (1951), after extraction with 0.1 N sodium hydroxide at 37℃ for 2 hours and bovine serum albumin used as the standard material. The concentrations of total protein and carbohydrate were expressed as mg carbohydrate or protein per gram tissue dry weight (TDW).

2.4. Quantification of reproductive effort by ELISA

Reproductive effort (i.e., egg mass) of female oysters was estimated using an indirect enzyme-linked immunosorbant assay (ELISA) and the rabbit anti-oyster egg protein antibody developed by Kang et al. (2003) was used as the primary antibody. For quantification, 20–25 mg lyophilized and pulverized tissue powder per female oyster (pooled method use for the MTO in the month of June due to insufficiency of tissue

powder) was measured, dissolved in 1 ml phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.4) and further homogenized using an ultrasonicator. The 100μl aliquots of oyster tissue homogenate (i.e., diluted 500 to 5,000-fold) and purified oyster egg protein as standard (i.e., diluted with a serial dilution from 5.8 μg/ml to 0.045 μg/ml) were added to the wells of a 96-well polystyrene micro-plate. Subsequent ELISA steps were performed as described by Kang et al. (2003) and Park & Choi (2004). Goat anti-rabbit IgG alkaline phosphate-conjugate (1:1000, Sigma) was used as the secondary antibody and ρ-nitrophenylphosphate was used as the coloring agent. A log polynomial standard curve was constructed from the optical density of the known quantity of standard material included in the plate (i.e., purified oyster eggs). The concentration of egg protein in the tissue homogenate was estimated using the standard curve and the dilution factor of the homogenate. The amount of egg in each oyster was determined by multiplying the amount of egg protein measured by ELISA by 2.5, the ratio of protein to whole egg weight (Kang et al., 2003). The gonad-somatic index (GSI) was calculated as the ratio of the total dry weight of eggs to that of whole oyster tissue. Fecundity was estimated from the ratio of total egg weight measured by ELISA to the weight of an individual egg (i.e., 13 ng, Kang et al., 2003).

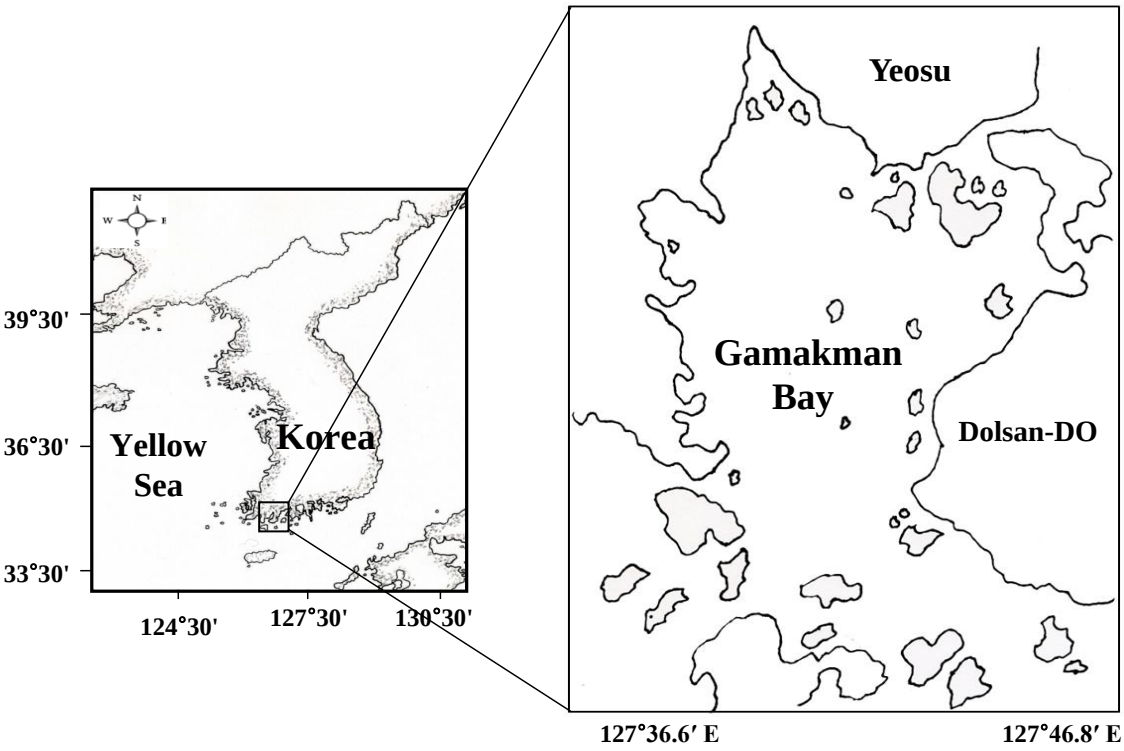

Fig. 1-1. Study area the Gamakman Bay, on the south coast of Korea.

2.5. Statistical analysis

All statistical analyses were performed using SPSS (version 18.0). Data were tested for normality and homogeneity of variance to meet the assumptions of parametric statistics. If these assumptions were not satisfied, data were arcsine-transformed or log-transformed. Significant differences in shell length, tissue dry weight, condition index, carbohydrate and protein content of *C. gigas* between treatments (JTO vs. MTO) and among sampling times were tested using a two-way ANOVA. A two-way ANOVA with time as a block was performed to test significant differences in gonad somatic index and egg mass of *C. gigas* between treatments (JTO vs. MTO) and among sampling date. When significant differences among treatments were observed, a Student–Newman–Keuls (SNK) *post hoc* test was performed. Statistical significance was set at the alpha < 0.05 level.

3. Results

3.1. Seasonal variation of environmental parameters

At the beginning of the study the sea water temperature was 8.1℃, and increased rapidly, reaching 25.0℃ during August-September, and then decreased to the end of the study (Fig. 1-2). Salinity remained stable, ranging from 30.6 to 35.6 psu during the study period (Fig. 1-2). Chlorophyll *a* exhibited a clear picture of seasonality with two peaks, one in spring (March to May) and another one in late summer to early autumn (August and September). During the monitoring the chlorophyll *a* ranged from 0.9-6.8 μg/L (Fig.

1-2).

3.2. Growth and condition index

After the grow-out transplantation into the bay, a gradual shell and tissue growth was observed in both of the oyster population (Table 1-1& Fig. 1-3). For the JTO, the SL increased linearly from February (33.2±0.6 mm) to November (97.3±2.6 mm) and then remained stable during the winter period (Table 1-1 & Fig. 1-3). Tissue weight increased from February to June, decreased during July and October and then again increased during November and January (Table 1-1& Fig. 1-3). These seasonal changes in tissue weight were coincided respectively with the gonad maturation, spawning, and sexually inactive condition of the oysters. An intermittent decline in growth of the oysters in the month of April is not clearly understood; it may be due to a sampling artifact. Concurrently, the MTO showed a stagnant shell and tissue growth during February and May, the additional hardening period compared to the JTO (Table 1- 1 & Fig.1-3). After being transferred into the grow-out area the SL increased exponentially from June (20.5±0.6 mm) to November (80.5±2.2 mm) and then remained stable onwards (Table 1-1 & Fig.1-3). Tissue weight gradually increased from June to the end of the experiment without any seasonal fluctuations relating to spawning (Table 1-1 & Fig. 1-3). During this monitoring both the shell and tissue growth were significantly higher for the JTO than that of the MTO ($P<0.05$, Table 1-2).

Figure 1-3 shows the seasonal changes of the CI of the oysters during the study period. The seasonal changes in CI for the JTO were similar with the tissue weight, increased from February to June, decreased during July and October, and then again

increased from November and January. For the MTO, the CI did not clearly correspond with gametogenesis, and increased throughout the study period without any seasonal fluctuation relating to spawning. During this monitoring the MTO showed a significantly lower monthly CI than that of the JTO ($P< 0.05$, Table 1-2).

3.3. Gametogenesis

Histology revealed that more than 90% oysters of the JTO were in early gonad development condition in February, indicating that the oysters initiated gametogenesis as early as February (Fig. 1-4). The oysters matured rapidly, became ripe in May, and spawned during June and September. Most of the oysters became reproductively inactive from October onwards and initiated a new gametogenic cycle in December (Fig. 1-4). Concurrently, the MTO initiated gametogenesis at the hardening ground during the month of March but the gonad maturation seemed to be retarded up to May. After being grow-out transplanted into the bay the oysters mature rapidly and more than 90% oysters collected in July were in ripe condition and the oysters spawned during August and October. Both of the groups became in resting condition from October onwards and reinitiated gametogenesis in December (Fig. 1-4).

3.4. Biochemical composition

Figure 1-5 shows the seasonal variations in biochemical composition of the oysters. The total carbohydrate level in the tissues of the JTO increased in spring with the highest level in March (332.3±6.3 mg/g), and dramatically decreased in summer with the lowest level in August (139.3±13.8 mg/g), indicating a high energy catabolism of the oysters for

spawning during the summer (Fig. 1-5). A progressive recovery of carbohydrate reserve was observed during the autumn-winter period. In contrast, the MTO represented a depleted level of total carbohydrate, especially during February and May, when their reserve level was about 2 times lower than the JTO (Fig. 1-5). The lowest carbohydrate level 79.4±5.8 mg/g observed in July and then gradually increased from August onwards, reaching the highest level 306.6±9.9 mg/g in January, 2010

The protein content for the JTO showed clear seasonal fluctuations; gradually increased during April and October and then decreased onwards up to the end of the study. A higher protein level observed during the July and October was coincided with spawning of the oysters (Fig. 1-5). Concurrently, for the MTO the protein content fluctuated many times and the higher level observed during July and August was attributed with gonad maturity and spawning of the oysters (Fig. 1-5). The monthly protein levels ranged from 273.1±3.1 to 386.3±6.1 mg/g in the JTO and from 284.2±19.6 to 368.3±6.1 mg/g in the MTO (Fig. 1-5).

3.5. Reproductive effort and fecundity

The gonad-somatic index in females of the JTO estimated by indirect ELISA rapidly increased from March to June, at reaching the highest mean 27.7±1.2% GSI (i.e., egg mass, Fig. 1-6). The GSI dropped dramatically during July and September, indicated a synchronous spawning of the oysters during this period. After a sexual inactivity from October to December, 1.3±0.3% GSI was estimated in January, 2010 when the oysters were in pre-vitellogenic stages. During this study the absolute egg mass calculated from the ELISA results also represented similar seasonal cycle as

percentage GSI (Fig. 1-6). Concurrently, for the MTO the monthly mean GSI slowly increased from June to July (11.1±2.6%) and then slowly decreased during August and October with a little increase in September, indicated a spawning asynchrony of the oysters (Fig. 1-6). The absolute egg mass of the MTO indicated different trend than percentage GSI, increased during June and September and then decreased in October. Both the percentage GSI and absolute egg mass of the MTO was significantly lower than that of the JTO ($P<0.05$, Table 1-2).

During our study we used the same female individuals for the histology and to quantify the reproductive effort which enabled us to estimate the monthly gametogenic stage-wise reproductive effort as reported in Fig. 1-7. The early developing females represented a very less amount of egg mass (up to 2.1±0.2% GSI). The egg mass rapidly increased from early to late developing stage and the ELISA estimated up to 11.4±1.3% GSI for the JTO and 7.1±0.2% for the MTO during the late developing condition. The ripe stage females represented 28.4±1.3% GSI for the JTO and 11.1±2.6% for the MTO. The spawning stage GSI result of the JTO showed a synchronous decrease during June and September from 24.8±2.7% to 11.5±1.1%. While for the MTO, the spawning females GSI decreased in August, again increased in September and then again decreased during October, indicating an asynchrony of gamete release (Fig. 1-7). In both of the groups the spent stage oysters represented up to 5.3 % GSI (Fig. 1-7).

Using the absolute egg weight we also calculated the fecundity of the females and highest 27.92±1.78 million (mean ± standard error) fecundity was estimated in June for the JTO, when most of the oysters were in ripe condition and ready for spawning. While for the MTO only 1.13±0.23 (mean ± standard error) million fecundity estimated

in July when the oysters were in ripe condition but the highest fecundity 4.88±0.76 million (mean ± standard error) was calculated in September when the oysters were engaged in spawning.

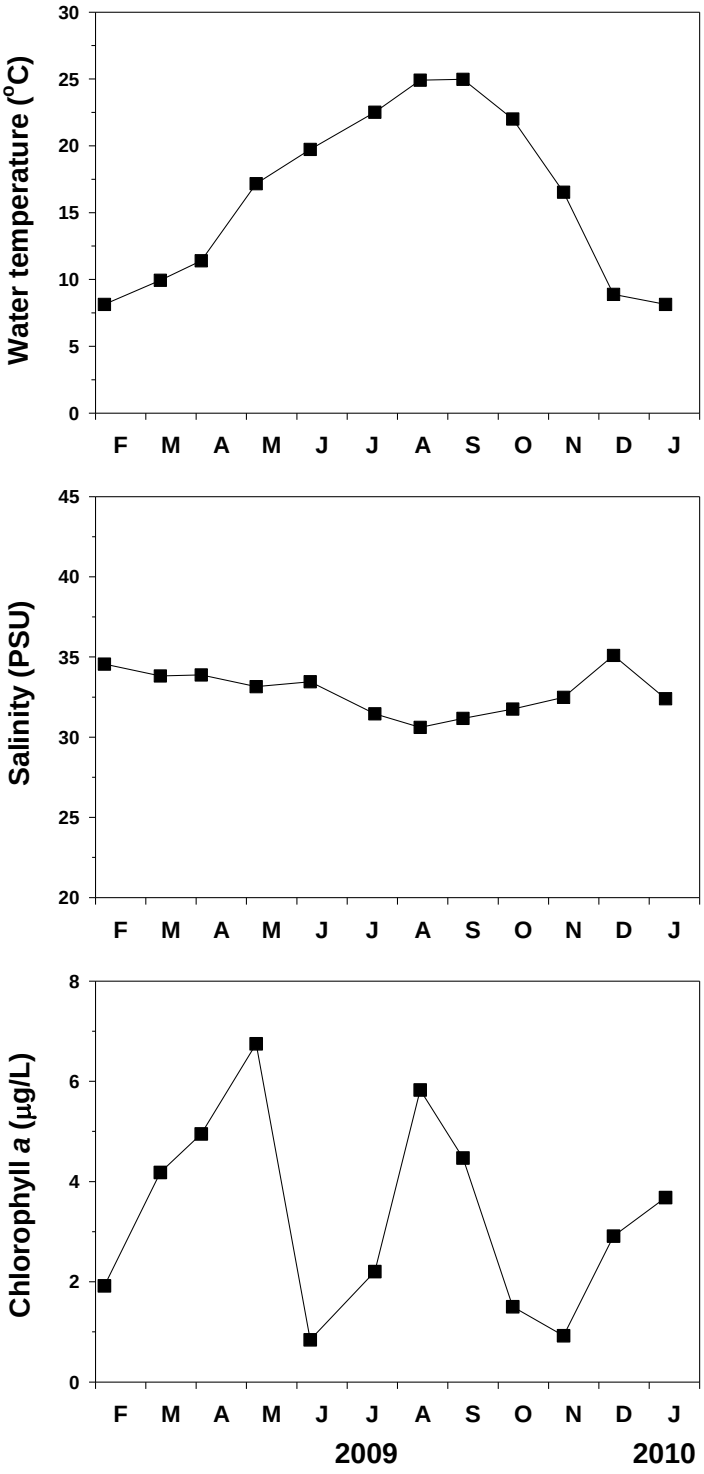

Fig. 1-2. Seasonal variations in water temperature, salinity and chlorophyll *a* concentration in Gamakman Bay from February 2009 to January 2010.

Table 1-1. Monthly analyzed oyster number (N), shell length (SL), tissue wet weight (TWT) of the JTO and the MTO from February, 2009 to January, 2010. The values are mean ± standard error, except the number of oysters.

Sampling	January transplanted oyster			May transplanted oyster		
periods	N	SL (mm)	TWT (g)	N	SL (mm)	TWT (g)
2009, Feb	40	33.2 ± 0.6	0.61 ± 0.03	40	13.3 ± 0.4	0.04 ± 0.00
Mar	40	45.9 ± 0.9	1.81 ± 0.08	40	15.7 ± 0.8	0.08 ± 0.01
Apr	40	42.7 ± 0.9	1.55 ± 0.10	40	18.8 ± 0.6	0.10 ± 0.01
May	40	48.6 ± 0.8	1.62 ± 0.08	30	19.9 ± 0.5	0.10 ± 0.01
Jun	40	64.4 ± 1.5	5.25 ± 0.29	30	20.5 ± 0.6	0.17 ± 0.01
Jul	40	70.0 ± 2.1	4.33 ± 0.22	40	35.5 ± 0.7	0.69 ± 0.04
Aug	40	75.5 ± 1.5	4.31 ± 0.18	40	51.5 ± 0.8	2.02 ± 0.08
Sep	40	85.9 ± 1.9	6.11 ± 0.26	40	65.4 ± 1.2	3.09 ± 0.13
Oct	40	91.2 ± 2.1	5.55 ± 0.31	40	77.6 ± 2.1	4.40 ± 0.26
Nov	30	97.3 ± 2.4	8.68 ± 0.50	30	80.5 ± 2.2	5.01 ± 0.18
Dec	40	95.8 ± 1.5	10.34 ± 0.41	40	70.3 ± 1.6	5.97 ± 0.36
2010, Jan	30	87.5 ± 1.9	9.80 ± 0.29	30	76.2 ± 1.4	8.97 ± 0.24

Table 1-2. Results of two-way ANOVA for the effects of date (month) and treatments (JTO vs. MTO) on shell length, tissue dry weight, condition index, carbohydrate, protein, gonad somatic index and egg mass. Time as blocked for the effects of date (month) and treatments in case of gonad somatic index and egg mass.

Variables	Source	df	MS	F-ratio	P-value
Shell length	Date (D)	11	4.034	932.157	< 0.001
	Treatment (T)	1	14.464	3342.275	< 0.001
	D × T	11	0.520	120.097	< 0.001
Tissue Dry weight	Date (D)	11	1.596	496.481	< 0.001
	Treatment (T)	1	3.814	1186.189	< 0.001
	D × T	11	0.130	40.461	< 0.001
Condition index	Date (D)	11	1.408	358.824	< 0.001
	Treatment (T)	1	4.749	1210.644	< 0.001
	D × T	11	0.300	76.604	< 0.001
Carbohydrate	Date (D)	11	0.955	34.347	< 0.001
	Treatment (T)	1	3.237	116.424	< 0.001
	D × T	11	0.322	11.587	< 0.001
Protein	Date (D)	11	0.057	32.225	< 0.001
	Treatment (T)	1	0.015	8.578	0.004
	D × T	11	0.021	11.959	< 0.001
Gonad somatic index	Treatment (T)	1	0.0000075	31.194	< 0.001
	Date (T)	9	0.0000102	42.514	< 0.001
	Error	191	0.0000007		
Egg mass	Treatment (T)	1	0.037	55.823	< 0.001
	Date (T)	9	0.026	39.405	< 0.001
	Error	191	0.001		

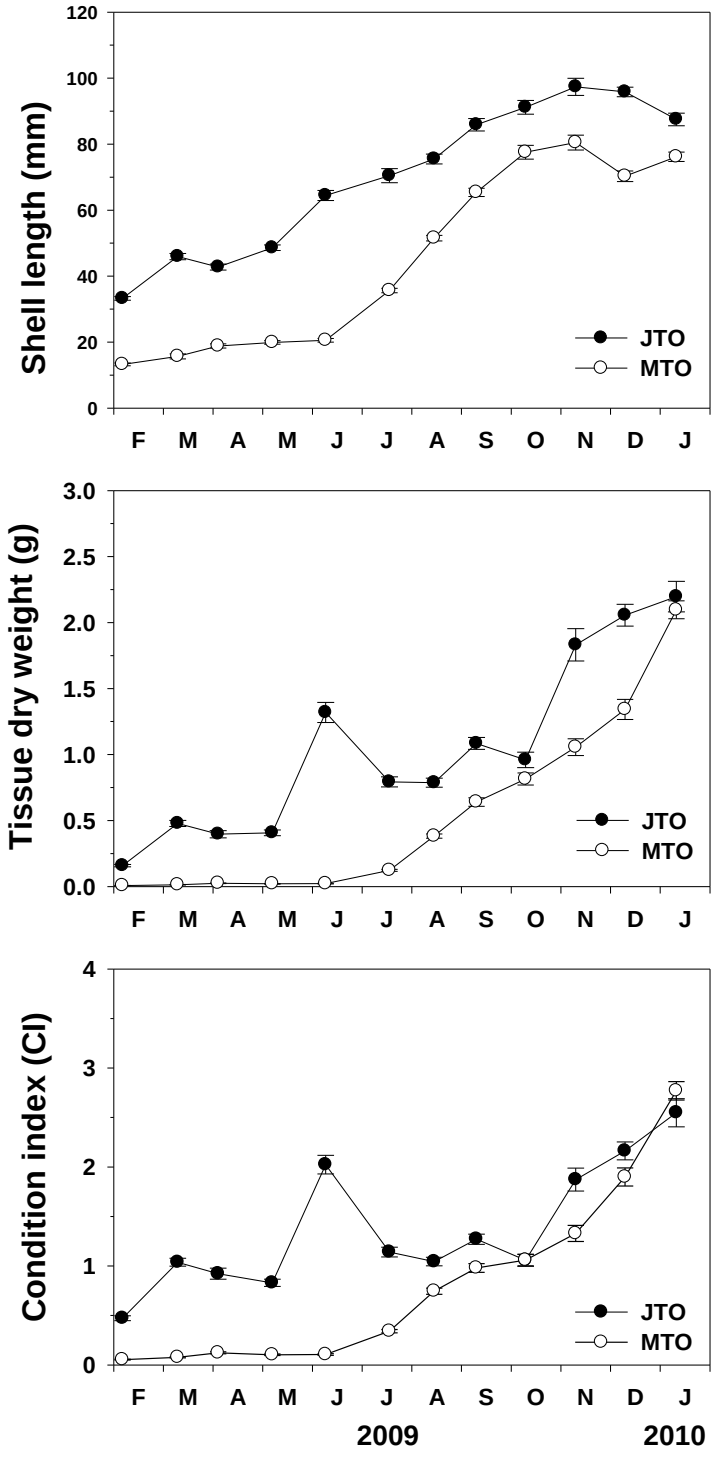

Fig. 1-3. Monthly variations in shell length, tissue dry weight and condition index (CI) of *Crassostrea gigas* transplanted in January and May 2009. Values represent mean ± standard error (n = 30-40).

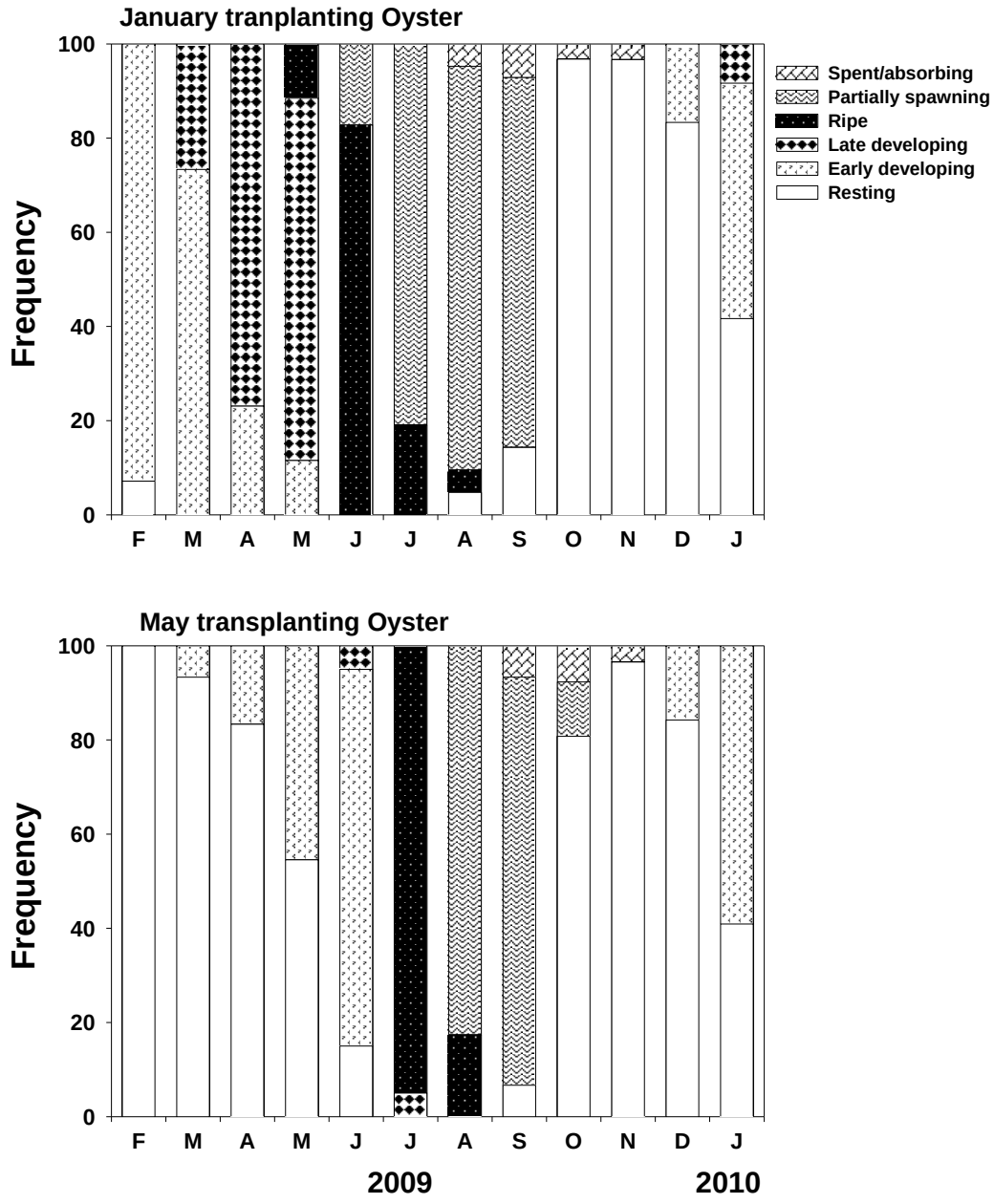

Fig. 1-4. Monthly percentage distribution of *Crassostrea gigas* at different gametogenic stages transplanted in January and May, 2009.

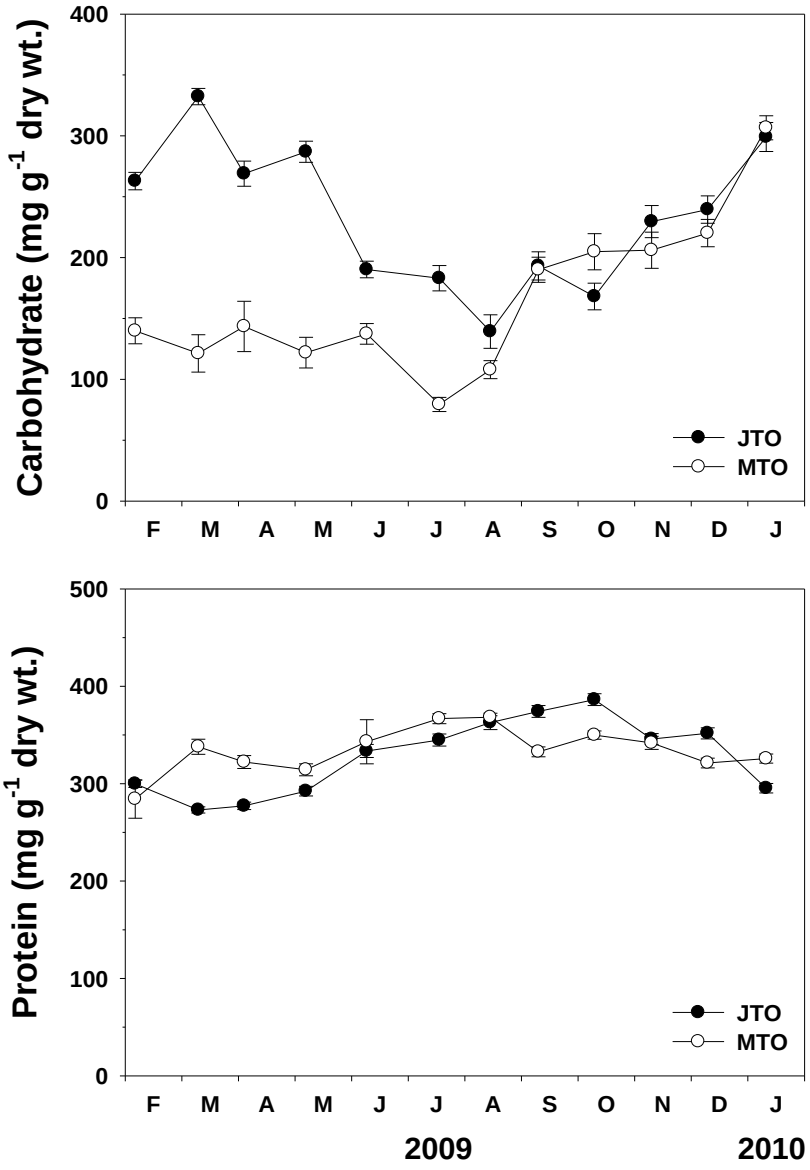

Fig. 1-5. Seasonal variations in carbohydrate and protein content of *Crassostrea gigas* transplanted in January and May 2009. Values are mean ± standard error (n = 20-40).

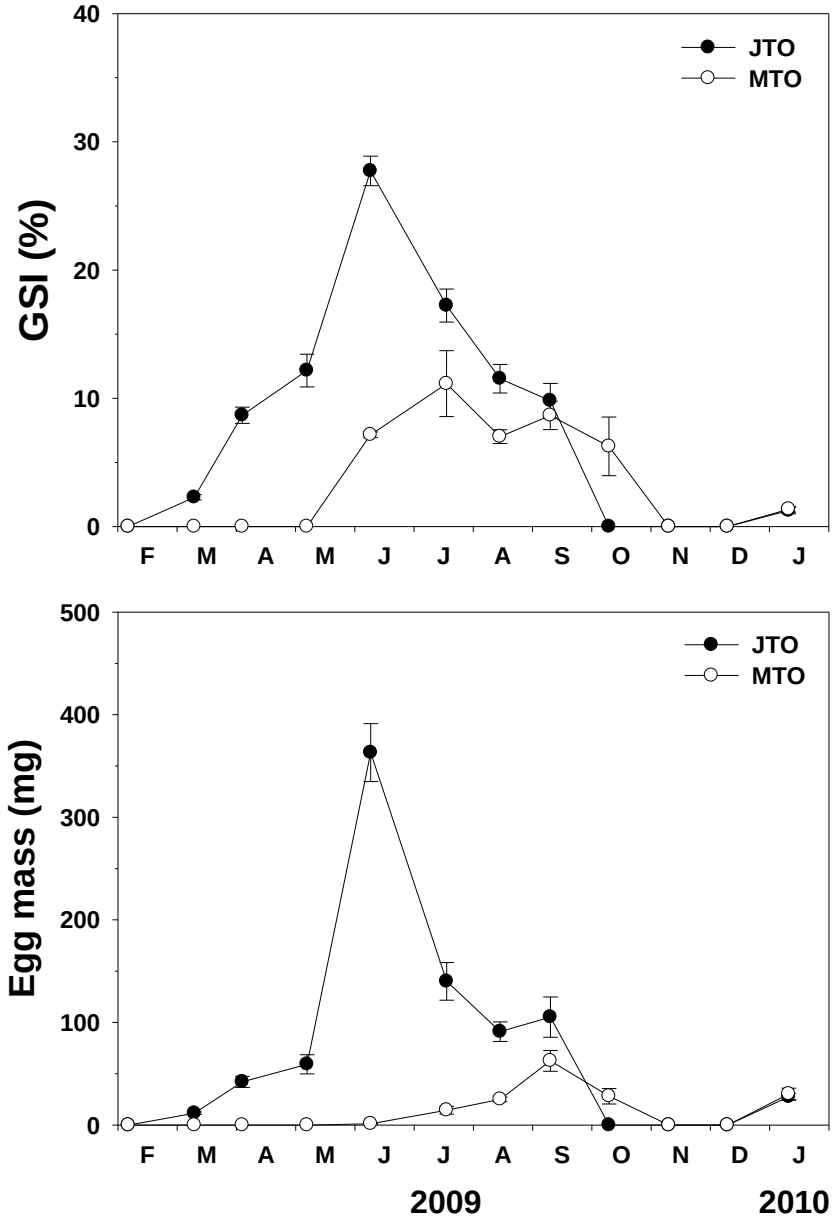

Fig. 1-6. Seasonal variations in gonad somatic index (GSI) and egg mass of *Crassostrea gigas* transplanted in January and May 2009. Values are mean ± standard error (n = 5-27).

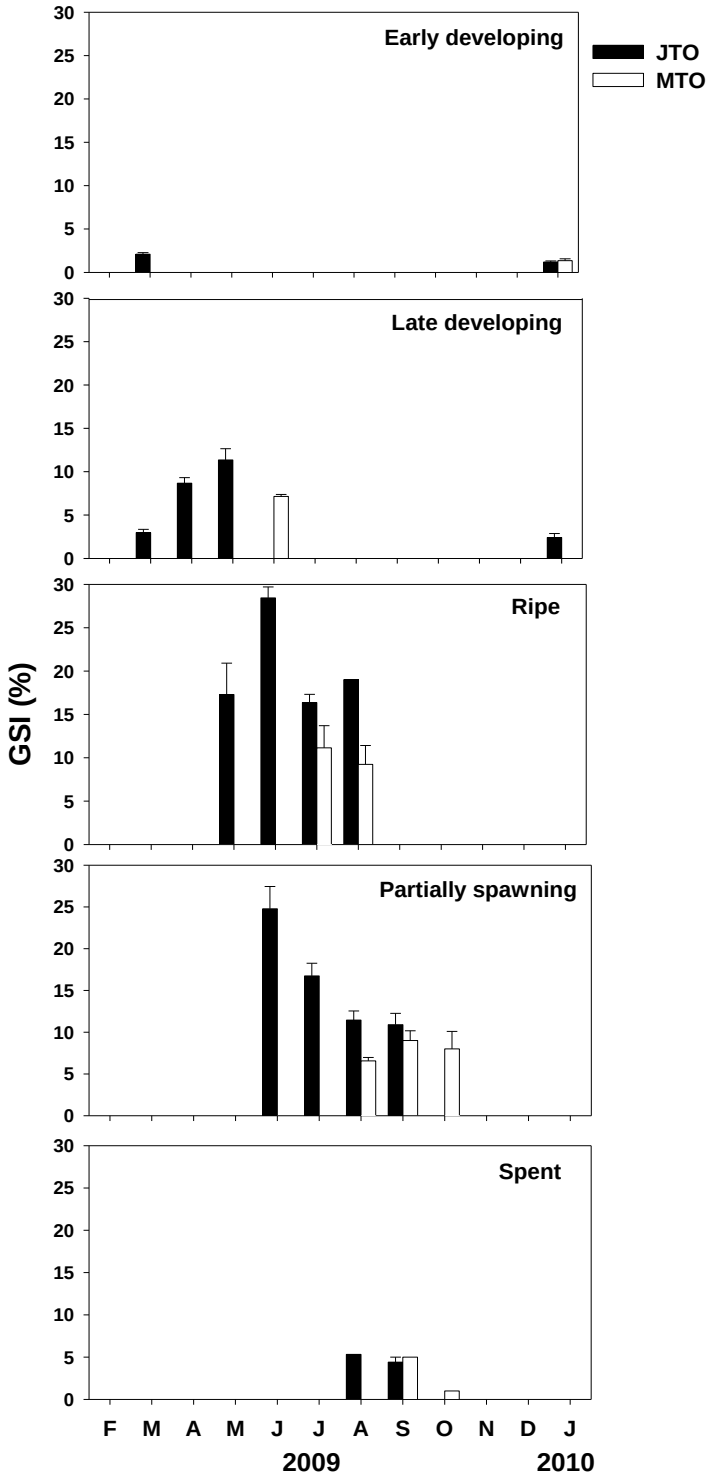

Fig. 1-7. Gametogenic stage-wise GSI (%) of *Crassostrea gigas* transplanted in January and May 2009. Values represent mean ± standard error (n = 5-20).

Table 1-3. GSI (%) of Pacific oyster, *Crassostrea gigas*, reported by different authors estimated by using an immunological probe (ELISA). TDW, tissue dry weight.

Location	TDW	Oysters	GSI (%)	Authors
Gosung Bay, Korea	0.49-5.4 g	Adult	42.3.	Kang et al. (2003)
Gosung Bay, Korea	2.6 -3.1 g	Adult	41.1-49.5	Ngo et al. (2006)
Normandy, France	0.68 g	Spat	36.0	Royer et al. (2008)
	3.74 g	Half grown	61.0	
	5.95	Marketable	59.6	
Gamakman Bay,	1.32 g	JTO	27.7	Present study
Korea	0.38 g	MTO	11.1	

4. Discussion

4.1. Growth and condition index

In the present study, the hardened juvenile oysters showed a rapid growth in the grow-out field. Most shell growth occurred during spring and autumn months and then remained stable in winter (Table 1-1 & Fig. 1-3). These seasonal shell growth variation of the oysters can be explained as the variation of environmental parameters, particularly water temperature and food availability (Brown and Hartwick, 1988; Hofmann et al., 1992; Powel et al., 1995; Kang et al., 2000; Kang et al., 2010). In the Gamakman Bay, the marked increase of shell length of the oysters during the spring and autumn months were coincided with the higher water temperature and higher food availability (reflected in chlorophyll *a* result in Figure 1-2). While the colder water temperature and less food availability during the winter period stabled the shell growth of the oysters. Kang et al. (2010) also investigated the annual growth of hardened oysters in Gamakman Bay, into which they transplanted 10-month-stunted spat in May and raised them using a suspended long-line culture system. They observed rapid shell growth from May to October corresponding with higher water temperature and chlorophyll *a* level, and growth stability during the winter period. From Normandy France, Royer et al. (2007) reported that shell growth of Pacific oyster occurred over spring and summer months with a plateau in the winter. They also reported that tissue growth occasionally decreased in summer due to spawning. In this study we also observed a summer decline in tissue weight for the JTO, attributed with the spawning of the oysters but the MTO showed a continuous tissue weight increase without any seasonal fluctuation relating to spawning.

During this monitoring, the SL increased from 10 mm to 97.3 mm for the JTO and from 20 mm to 80.5 mm for the MTO, after 10-months and 6-months of grow-out rearing respectively (Table 1-1and Fig. 1-3). Several studies also have reported the annual shell growth of the traditionally transplanted stunted juvenile oysters from Korea. Kang et al. (2010) recorded a SL increase from 15-20 mm to 74.2 mm in May transplanted stunted juvenile oysters, after 12-months of grow-out rearing in Gamakman Bay. A study by Oh et al. (2002) on May transplanted hardened juvenile oysters reported a SL increase, from 20 mm to 85.0 mm after 11-months of rearing from Gosung Bay, off the southern coast of Korea. The observed shell growth variations of the oysters in the present study and those reported by Kang et al. (2010) and Oh et al. (2002) may be related to differences in environmental parameters, mainly water temperature and food availability of the oyster culture grounds. During this study, we recorded a water temperature of 8.1 to 25.0℃ and 0.9 to 6.8 µg/L chlorophyll *a* from Gamakman Bay (Fig. 1-2). Kang et al. (2010) reported 7.5-26.1℃ water temperature and 0.1-3.5 µg/L chlorophyll *a* from Gamakman Bay and Oh et al. (2002) reported 4-25℃ water temperature and 0.2-6.2 µg/L chlorophyll *a* from Gosung Bay. The water temperature and chlorophyll *a* results indicate somewhat similar hydrodynamic conditions at the oyster culture grounds; thus, the higher shell growth of the JTO may not be associated with the differences in environmental conditions. It is reasonable to consider that the observed shell growth variations of the oysters could be associated with the differences in grow-out transplantation timing. Grow-out transplantation in January provides a scope to the oysters for better nourished using the spring season available food plankton, and this represented higher growth. However, the oysters transplanted into the bays in May, at the traditional time, faced a long period of food deprivation at the hardening ground,

and also could not obtain a suitable environment in their first spring season for their growth and energy storage. The favorable water temperature and chlorophyll *a* levels during the spring season also suggest that grow-out transplantation before the spring is better for better oyster production in Korea.

As indicated in Figure 1-3 the condition index of the JTO clearly followed the gametogenic cycle, increased with the advance of gonad maturation up to June, and then decreased from July to October in association with the spawning of the oysters. During the spawning season a sharp decline of CI also coincided with the decline of tissue weight and egg mass, indicating a concentrated spawning of the JTO. However, a linear increase of CI, together with tissue weight, indicated the more partial spawning as well as resorption of unspawned gametes of the MTO (Steele and Mulcahy, 1999). Park et al. (1999a) investigated the reproductive condition of wild seed oysters in Tongyong and Goje, Korea. They observed a dramatic decline of CI during the spawning period which suggested stronger spawning activities in Tongyong oysters than those of Goje. During their monitoring they also measured both the abundance and lipid content of D-shaped larvae which were much higher at Tongyong than at Goje and concluded that the Tongyong spawners are physiologically healthier than those of Goje. Such a difference in the fluctuations in CI observed between the JTO and the MTO also indicated that the JTO are physiologically healthier to perform a successful reproduction as well as spat recruitment in the Gamakman Bay.

During the autumn-winter period the CI again increased, when most of the oysters were sexually inactive, and accumulated more reserve materials in their tissues in the form of carbohydrate, which makes the oysters tasty in flavor and ultimately enhance the marketability. This finding also has an important implication for the oyster industry of

Korea, as the greatest market demand is in winter (Park et al., 1999a) and the oysters in our study, especially the JTO, achieved marketable size with sufficient meat content and tasty flavor before winter, earlier than those raised by traditional methods. Therefore, the Korean oyster industry can be benefitted by transplanting the oysters into the grow-out area as early as traditional transplantation time.

4.2. Gametogenesis and environmental consequences

The timing and period of gametogenesis in the Pacific oyster varies with geographic locations (Ventilla, 1984), and is predominantly influenced by both water temperature and food availability (Ruiz et al., 1992; Park et al., 1999a; Kang et al., 2000; Kang et al., 2010). In the present study, gametogenesis was initiated during February and March when the bay water temperature was about 10.0℃. In association with food availability and increasing water temperature the oysters matured rapidly and became ripe during May and June. Spawning occurred during June and October, when the bay was warmer (19.7-25.0℃). Most of the oysters became reproductively inactive from October onwards when both the water temperature and chlorophyll *a* level decreased (Fig. 1-4). Several studies also reported a similar seasonal cycle of gametogenesis of oysters in Korean waters. As mentioned previously, Kang et al. (2010) investigated the annual gametogenesis of stunted juvenile oysters transplanted into Gamakman Bay. The stunted oysters transplanted in May become ripe in June, and exhibited spawning from June to September when the bay water temperature ranged from 21 to 26℃. The oysters become reproductively inactive from October onwards. Kang et al. (2003) investigated the annual reproductive cycle of adult oysters from Gosung Bay, off the southern coast

of Korea. The adult oysters in Gosung Bay, initiated gametogenesis in March at 10-12℃ and after rapid gonad maturation spawning occurred from May to October at 20-27℃. Most oysters became reproductively inactive in November when water temperature dropped below 17℃.

During this study the microscopic observation revealed an earlier gametogenesis, gonad maturation and spawning activity for the JTO than that of the MTO (Fig. 1-4). The rapid gonad maturation and spawning of the JTO could be due to their earlier grow-out transplantation into the bay. Before spring grow-out transplantation provides the oysters a better opportunity for food ingestion as well as storage accumulation (observed in carbohydrate results) using the plankton available in spring season. However, a long period of hardening up to the month of May results in less storage accumulation for successful gametogenic activity for the MTO. Our results also consistent with the suggestion of Deslous-Paoli and Héral (1988) that, spring disturbance affecting the reproductive cycle and delaying spawning, can also induce the failure of spat settlement. Consistent with our finding, Liu et al. (2010) also reported a delay in gonad maturation of starved oysters compared to fed oysters from Weihai, China, and suggested that gametogenesis depends on reserve materials accumulated by *C. gigas* prior to gonad development.

4.3. Storage metabolism in relation to reproduction

Storage and reserve mobilization in bivalve molluscs is closely linked to the annual reproductive cycle (Gabbot, 1975; Ruiz et al., 1992; Kang et al., 2003; Ojea et al., 2004; Ngo et al., 2006; Yang et al., 2011). Carbohydrates (in the form of glycogen) are

considered as the main energy source and its variation is inversely related with the gonadal maturity (Gabbott, 1975; Camacho et al., 2003). In the present study, oyster gonad development seemed to occur along with the utilization of reserve carbohydrate originating from food consumed previously. The seasonal cycle of total carbohydrate content followed by the gametogenic cycle, the higher reserve observed during February and May when most of the oysters were in early and late gonad maturation condition (i.e. pre-vitellogenic stage). And then carbohydrate contents rapidly decline during the summer period as because of spawning, indicating that the oysters transformed the carbohydrate reserves into gonad particles during this period as a consequence of vitellogenesis (Deslous-Paoli and Héral, 1988; Marin et al., 2003; Ojea et al., 2004; Yang et al., 2011). After completion of spawning, the carbohydrate level again increased during the autumn-winter periods, when most of oysters were sexually inactive and some of them also initiated a new gametogenic cycle (Fig. 1-5). Ngo et al. (2006) also reported a higher carbohydrate reserve in adult Pacific oysters in Gosung Bay, on the south coast of Korea during winter and spring, as compared to summer. This seasonal variation in the carbohydrate level of oysters in Gosung Bay was associated with seasonal reproductive activity, including gonad maturation and subsequent spawning.

January transplantation provides a scope to the oysters to store significantly higher carbohydrate reserves in their tissues, especially during the spring than that of the traditional one ($P< 0.05$; Fig. 1-5). The spring inclination of the carbohydrate levels of the JTO indicated an active feeding during this nutrient-rich period and the accumulation of storage materials. This finding is also consistent with several other previous studies (Marin et al., 2003; Camacho et al., 2003; Dridi et al., 2007). Concurrently, the limited carbohydrate reserve of the MTO presumably relates to the insufficient food intake as

well as less storage accumulation as because of negative energy balance in the hardening ground. When the amount of food ingested is insufficient to meet the energy demand (i.e., the negative energy balance), the glycogen and other carbohydrate content in bivalve rapidly decrease to almost a quarter of their initial values paralleling the process of sexual maturation (Camacho et al., 2003). A study by Liu et al. (2010) also reported lower glycogen content in starved oysters than in fed individuals, and the glycogen reserve was quickly mobilized and depleted because of food deprivation. They also suggested that gonad maturation depends on reserve materials accumulated by the oyster prior to gonad development.

Seasonal changes in protein reserves are closely linked with gonad maturation and may reflect the protein accumulation in oocytes; in addition, protein contributes to energy maintenance during the periods of reduced glycogen levels (Berthelin et al., 2000; Dridi et al., 2007). In this study, protein level increased with the advance of gonad maturation and the higher content observed during June and October, when the oysters were in maximum ripeness and spawning condition with a lower carbohydrate level (Fig. 1-5). These higher protein levels also coincided with higher egg content of the oysters as reflected in the reproductive effort results in Figure 1-6. The protein level slowly decreased from October onwards when the spawning was already completed. Ngo et al. (2006) also reported a similar seasonal trend in protein composition, as in the present study, from Gosung Bay, Korea. They observed an elevation of protein reserves during and at the end of spawning, and the summer protein maxima coincided with the GSI peaks. They postulated that the seasonal changes of protein reserves are closely linked with the annual reproductive cycle. They measured 114-428 mg/g tissue protein in the oysters, which is also somewhat comparable to our results as shown in Figure 1-5.

4.4. Reproductive effort

Reproductive effort in marine bivalves has been estimated using different methods, and the ELISA is the more recent one (Choi et al., 1993). Compared to other methods ELISA has proven to be highly sensitive, rapid and affordable method in quantification of reproductive effort of bivalves (Choi et al, 1993; Kang et al., 2003, Park and Choi, 2004; Ngo et al., 2006). In the present study, we applied a histology–ELISA combined technique to determine the gonad development stages and the quantity of eggs per female oysters. A dorsoventral section of tissue (15–20% of the total tissue weight) was cut from each oysters and used in histology to determine the gonad maturation stages and the remaining tissue was lyophilized and the amount of eggs in the tissue was assessed using ELISA. During our investigation ELISA successfully quantified the egg mass from the early gonad developing to spent stage having some inter-individual variability in each developments stage. As documented in Figure 1-7 the egg mass (i.e., reproductive effort) of the oysters increases with maturation of the gonads. For the JTO the highest 28.4% GSI were recorded in June when the females were in ripe condition and ready for spawning. Whereas, in the same months the spawning stage females of the JTO represented 24.8% GSI, indicated that the spawning was more partial in the months of June. A gradual GSI decreased in the spawning females during July and August indicating a synchronous spawning during this period for the JTO. Concurrently, for MTO only 11.1% GSI was estimated from the ripe female in the months of July but the spawning stage females represented an asynchronous gamete release during August and October. In both of the groups we estimated about 5% GSI from the spent stage females; indicating a resorption of the unspawned gametes in the body tissue. Generally, the

residual eggs of the marine bivalves are resorved at the end of spawning cycle (Thompson et al., 1996; Park and Choi, 2004; Ngo et al., 2006; Drummond et al., 2006; Yang et al., 2011). Ngo et al. (2006) estimated the reproductive effort of Pacific oyster using ELISA from the Gosung Bay, Korea and reported that eggs produced during the first spawning (i.e., in June spawning) could significantly contribute to the larval recruitment in the bay, but larval development is uncertain for the eggs produced at the end of August. According to the method of NGO et al. (2006), we also estimated the egg release per female by calculating the difference before and after the peak spawning. The JTO released 16.78 million eggs per individual during the June-July spawning when the bay environmental parameters (reported in Fig. 3-2) were suitable for larval development. While, the MTO showed a retarded spawning, and only 2.72 million eggs released per individual was estimated during September-October spawning when both the water temperature and food availability decreased in the Gamakman Bay. Based on these results we can also speculate that eggs produced by the JTO could significantly contribute to spat recruitment as well as seed supply in Gamakman Bay, but spat recruitment is quite uncertain for the MTO.

As reported in Figure 1-6 the reproductive effort of the JTO is significantly higher than that of the MTO ($P<0.05$). This asynchrony of the reproductive effort of the two transplanted oysters could be associated with differing of energy allocations for growth and reproduction. Marine bivalves rapidly transfer their assimilated food from the digestive gland to the gonadal tissue when food supply is sufficient during gametogenesis (Gabbott, 1976; Ngo et al., 2006), but under the stress of starvation they use their reserves for the maintenance of metabolism rather than gonad growth and gamete production (Sastry, 1975). In the present study the higher reproductive effort of

the JTO seemed to be associated with the utilization of higher storage material accumulated during the spring when food is available. Concurrently, the MTO may use their reserve energy for maintenance metabolism during their hardening stress period. After being transplanted into the bay they preferably used their net energy for shell and somatic tissue growth rather than reproduction. Kang et al. (2010) also suggested that stunted oysters transplanted in May utilize more net energy for shell and somatic growth rather than reproduction during their first spawning after a hardening period and produce less quantity of gametes.

Using immunological probe (ELISA) several authors also have estimated the reproductive effort of the Pacific oyster and reported a mean of about 40% GSI, the egg mass, in regular size adult females (see reviews in Table 1-3). In comparison with regular size oysters, the juveniles of the present study contributed about 1.5 times (for the JTO) and 4 times (for the MTO) lower reproductive efforts, indicating that early grow-out transplantation favors the juveniles for better reproduction. The lower GSI in our study, compared to regular size adult oysters, could be due to age-specific variation in reproductive effort. The juvenile oysters devote their energy metabolism for growth, but adults use their energy, during the summer period preferably for reproduction (Héral and Deslous-Paoli, 1983). Royer et al. (2008) reported an age-specific variation in reproductive effort in Pacific oysters from Normandy, France. Using an indirect ELISA, they measured GSIs of 36% in spat, 61% in half-grown specimens, and 59.6% in marketable oysters, which were 13, 25, and 41 months old, respectively.

Part II

Early growth and reproduction of hatchery-produced juvenile Pacific oyster, *Crassostrea gigas*, in Gamakman Bay, off the south coast of Korea

Abstract

Early growth and reproduction of hatchery-produced juvenile Pacific oysters, *Crassostrea gigas*, raised in a suspended long-line facility in Gamakman Bay, off the south coast of Korea, were investigated from July to November, 2009. In October, 4 months after transplanting, the shell length increased from 27.4 mm (July, 2009) to 82.5 mm (October, 2009) and the tissue weight increased from 0.2 g to 5.2 g, suggesting that the oyster reached a marketable size in this month. Histology indicated rapid gonad maturation and the oyster spawned during August and October, with a peak in September. Oysters collected in November were in the resting phase. An enzyme-linked immunosorbent assay used to quantify egg biomass indicated that juvenile oysters produced a relatively small quantity of eggs compared to adults, ranging from 5.1% (August) to 8.8% (September) of their body weight. The low total carbohydrate reserve in the tissue recorded in August and September coincided with the intense energy utilization of spawning, while the protein maxima in September coincided with the peak in the gonad-somatic index. Our results suggest that hatchery-produced seed could supply a portion of the spat required in Gamakman Bay as well as in other oyster culture grounds of Korea, where the oyster industry is facing a shortage of natural spat supply.

1. Introduction

The Pacific oyster, *Crassostrea gigas*, is the most commonly cultured shellfish in small bays off the south coast of Korea using a suspended long-line culture system (Choi, 2008). In 2009, the Korean oyster farming industry produced 241,000 tons of oysters, which accounted for 70% of the total shellfish landings. Traditionally, the Korean oyster farming industry has utilized wild-caught oyster spat from bays as seed. During the post-spawning period in late summer, oyster spats are harvested using 5- to 6-m-long oyster culture strings tied with numerous oyster shells (Choi, 2008). For the past two decades, the Korean oyster aquaculture industry has been suffering from an unstable supply of healthy natural oyster seeds. Accordingly, numerous studies have been carried out to understand the problems associated with oyster aquaculture along the south coast (Park et al., 1999a, 1999b; Kang et al., 2000; Kang et al. 2003). To compensate for the shortage, oyster seeds are also supplied by private hatcheries located on the south coast, although these account for only 3% of the total oyster seed demanded by the industry (Choi, 2008).

Numerous studies have investigated the growth and reproduction of naturally supplied seed oysters to establish a successful aquaculture industry on the south coast (Park et al., 1999a; Kang et al., 2000; Kang et al., 2003; Ngo et al., 2006; Kang et al., 2010). In general, natural oyster spats are harvested during July and September, and the seed oysters undergo 9–10 months of hardening before they are transplanted to the long-line culture system in May. According to Kang et al. (2010), natural seed oysters grow rapidly after transplantation from the intertidal hardening ground to the suspended long-line grow-out system, which is mostly located in the middle of the bay. In late fall and

early winter, the natural seed oysters raised in the suspended culture system reach over 70 mm in shell length (SL) and are ready to be harvested for marketing (Choi, 2008). In contrast, hatchery-produced oyster spats are directly transplanted to the suspended culture system 3–4 months after hatching. Currently, oyster farmers prefer to use hatchery-produced spats as seeds, because they grow faster with low mortality compared to natural seed oysters. Despite the popularity and increasing demand of using hatchery-produced seeds, no studies have investigated the early growth and reproduction of hatchery-produced seeds during the grow-out period.

We investigated the early growth and reproductive activity of hatchery-produced oyster spats transplanted to a suspended long-line system in Gamakman Bay, off the south coast of Korea. This is the first report of the shell and tissue growth, changes in tissue proximate biochemical composition, and reproductive effort of hatchery-produced oysters during growth

2. Materials and Methods

2.1. Sampling site

Gamakman Bay (34°40′N, 127°42′E), with a surface area of 112 km^2 and mean depth of 9 m, is a major oyster-farming area located off the south coast of Korea (Lee et al., 1995; Kang et al., 2010; Fig. 1-1.). The oysters used in this study were produced by a private hatchery located on the south coast, Korea in late February, 2009. In the first week of June 2009, hatchery-produced juvenile oysters with a mean shell length (SL) of

about 10 mm were suspended on a long-line facility located in the middle of Gamakman Bay, and monitored until November, 2009. Monthly 30-40 oysters were collected from July to November, 2009 for different analyses. The water temperature in the Gamakman Bay was 8.1℃ in February, reached to 25.0℃ in September and then decreased onward (Fig. 1-2). The monthly chlorophyll *a* level ranged 0.9-6.8 µg/L with two peaks one in May (6.8 µg/L) and another one in August (5.8 µg/L) (Fig. 1-2).

2.2. Biometry and histology

After recording SL, the soft tissue was separated from the shell and weighed. For histology, a dorsoventral section (2 mm) was cut in the middle of the body and preserved in Davidson's fixative. The remaining tissue was weighed, freeze-dried, and homogenized for biochemical analysis of total carbohydrate and protein and to quantify the egg mass (i.e., reproductive effort). The preserved tissue sections were dehydrated in an ascending alcohol series, embedded in paraffin, sectioned (6 µm thickness), stained with hematoxylin, and counter-stained with eosin Y. The stained tissue sections were examined under a light microscope to identify the sex and evaluate the level of gonad maturation. Based on the microscopic appearance of the gonad, reproductive condition of individual oysters was categorized into 1) resting, 2) early developing, 3) late developing, 4) ripe, 5) partially spawning, and 6) spent and/or absorbing (Kang et al., 2010). The daily shell growth rate was calculated for each measurement interval using the following formula: daily shell growth rate (mm/day) = (average SL at t_1-average SL at t_0) / (t_1-t_0), where SL is in mm and (t_1-t_0) is the interval (days) between the two

samplings. The condition index (CI) was calculated as the ratio of the tissue wet weight (g) to the shell length in mm (Lucas and Beninger, 1985).

2.3. Quantification of reproductive effort

Reproductive effort (i.e., egg mass) of female oysters was estimated using an indirect enzyme-linked immunosorbant assay (ELISA) and the rabbit anti-oyster egg protein antibody developed by Kang et al. (2003) was used as the primary antibody. For quantification, 20–25 mg lyophilized and pulverized tissue powder per female oyster was measured, dissolved in 1 ml phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.4) and further homogenized using an ultrasonicator. The 100μl aliquots of oyster tissue homogenate (i.e., diluted to 1,000-fold) and 0.1–6.0 μg/ml purified oyster egg protein as standard were added to the wells of a 96-well polystyrene micro-plate. Subsequent ELISA steps were performed as described by Kang et al. (2003) and Park and Choi (2004). A log polynomial standard curve was constructed from the optical density of the known quantity of standard material included in the plate (i.e., purified oyster eggs). The concentration of egg protein in the tissue homogenate was estimated from the standard curve and the dilution factor. The amount of egg in each oyster was determined by multiplying the amount of egg protein measured by ELISA by 2.5, the ratio of protein to whole egg weight (Kang et al., 2003). The gonad-somatic index (GSI) was calculated as the ratio of the total dry weight of eggs to that of whole oyster tissue.

2.4. Biochemical analysis

From the lyophilized and pulverized tissue powder 20–25 mg subsample/oyster was measured separately for the total carbohydrate and protein assay. Total carbohydrate in the tissue was measured using the phenol-sulfuric acid method (Taylor, 1995) with dextrose (anhydrous) as the standard material. Total protein was determined using the method of Lowry et al. (1951), after extraction with 0.1 N sodium hydroxide and bovine serum albumin used as the standard material. The concentrations of total protein and carbohydrate were expressed as mg carbohydrate or protein per gram tissue dry weight (TDW).

2.5. Statistical analysis

Data were tested for normality and homogeneity of variance to meet the assumptions of parametric statistics. If these assumptions were not satisfied, data were arcsine-transformed or log-transformed. Significant differences in shell length, tissue wet weight, condition index, carbohydrate and protein of *C. gigas* were tested using an One-way analysis of variance (ANOVA) followed by Duncan's multiple range test. The level of significance was set at $P<0.05$ and the statistical program SAS was used for the analyses.

3. Results

3.1. Shell and somatic growth

After being transplanted into the bay, the oysters grew rapidly; SL increased linearly from July (27.4±0.8 mm) to October (82.5±1.9 mm) and remained stable in November (84.7±1.9 mm, Fig. 2-1, Table 2-1, $P<0.05$). In October, the hatchery-produced oysters were of a marketable size, after 4 months of grow-out rearing corresponding to 7 months from fertilization (Fig. 2-1). The faster daily shell growth rate observed in August (0.88 mm/day, Fig. 2-1) coincided with a higher water temperature and chlorophyll a level in the bay. The somatic tissue growth pattern was similar to that of shell growth, exhibiting a dramatic increase in tissue weight from July (0.24±0.02 g) to October (5.11±0.23 g) and remained stabled in November (Fig. 2-1, Table 2-1,$P<0.05$). Figure 2-2 shows the monthly change in CI from July to November. As was observed for shell and somatic growth, CI increased linearly from July to October, and stabilized during October and November.

3.2. Gonad condition and reproductive effort

Figure 2-3 shows monthly changes in the frequency distribution of gonad development stages. In July, all the sexually differentiated individuals were male, 46% in early and 30% in late development stage. During July and August, the hatchery-produced juveniles reproductively matured rapidly, coincided with the higher water

temperature and chlorophyll *a* level in the bay. The oysters spawned during August and October, with a peak in September, when the surface water temperature reached 25℃. In October, 6% of male oysters were in spawning condition, indicating that spawning is asynchronous and that males spawn 1 month prolonged. During this study the mean GSI of the female oysters estimated using ELISA was 5.1±1.0% in August and 8.8±1.1% in September, when oysters were 5–6 months old (Fig. 2-4).

3.3. Proximate biochemical composition

The total carbohydrate level dropped from July (116.1±11.5 mg/g) to August (45.9±4.9) and then increased dramatically from August to October (200.3±13.3 mg/g, Fig. 2-5). Monthly changes in total carbohydrate level were closely linked to the gonad maturation and subsequent spawning of oysters. Contrary to the total carbohydrate, the tissue protein content showed less variation, ranging from 309.1±11.6 to 373.6±5.3 mg/g. The significant increase in total protein from August to September coincided with the GSI peak of female oysters (Fig. 2-5).

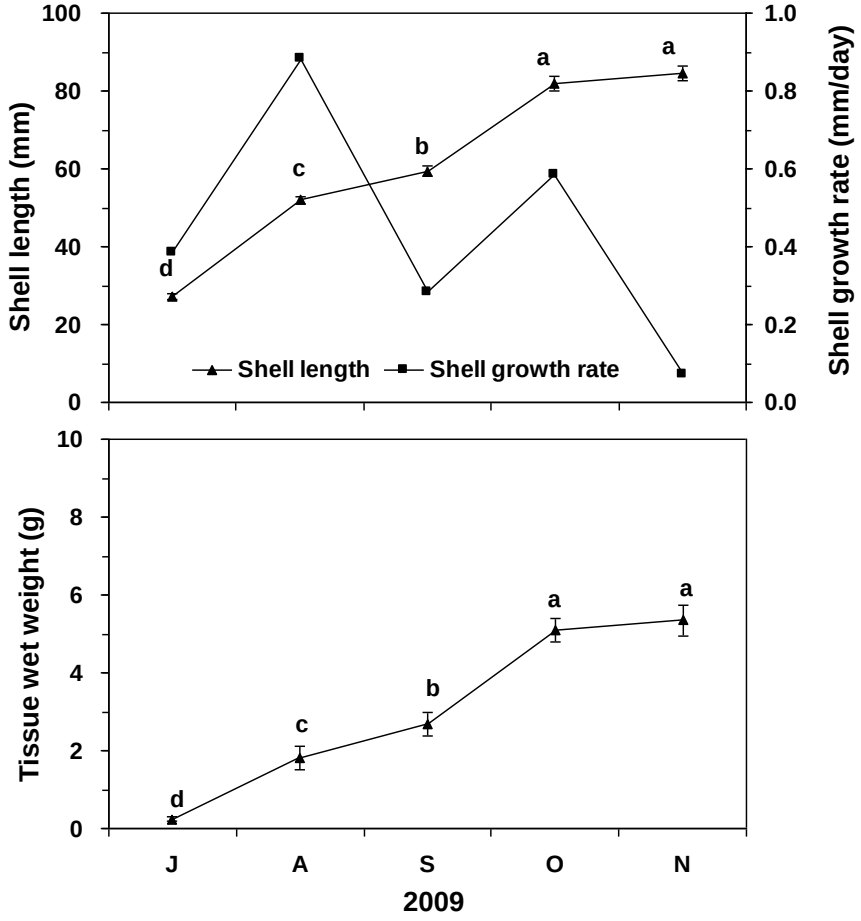

Fig. 2-1. Monthly variation of shell length, daily shell growth rate and tissue wet weight of the oysters from July to November, 2009. The vertical bars represent standard error and different alphabets indicate the significant variation (ANOVA, Duncan's, P <0.05).

Table 2-1. Monthly analyzed oyster number (N), shell length (SL) and tissue wet weight, (TWT) of the oysters from July to November, 2009 in the Gamakman Bay, Korea. Values are mean ± standard error, except the number of oysters.

Period	N	SL (mm)	TWT (g)
2009, Jul	30	27.1 ± 0.8	0.24 ± 0.02
Aug	40	52.1 ± 0.9	1.81 ± 0.07
Sep	37	59.5 ± 1.2	2.68 ± 0.10
Oct	30	82.5 ± 1.9	5.11 ± 0.23
Nov	30	84.7 ± 1.9	5.34 ± 0.30

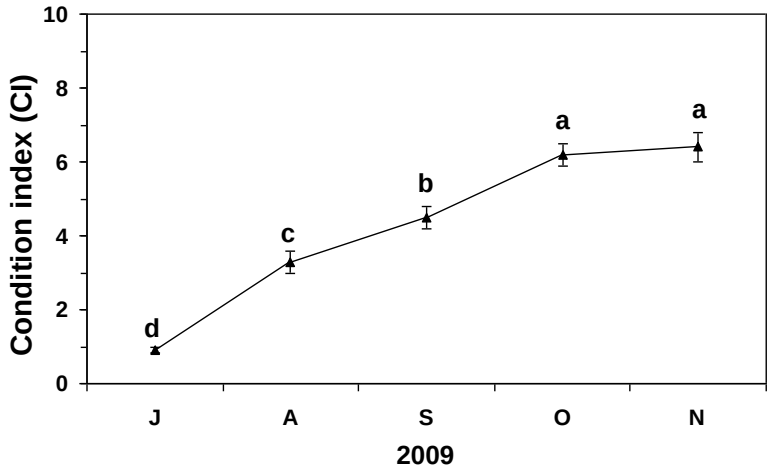

Fig. 2-2. Monthly variation of condition index (CI) of the oysters during the study period. The vertical bars represent standard error and different alphabets indicate the significant variation (ANOVA, Duncan's, P <0.05).

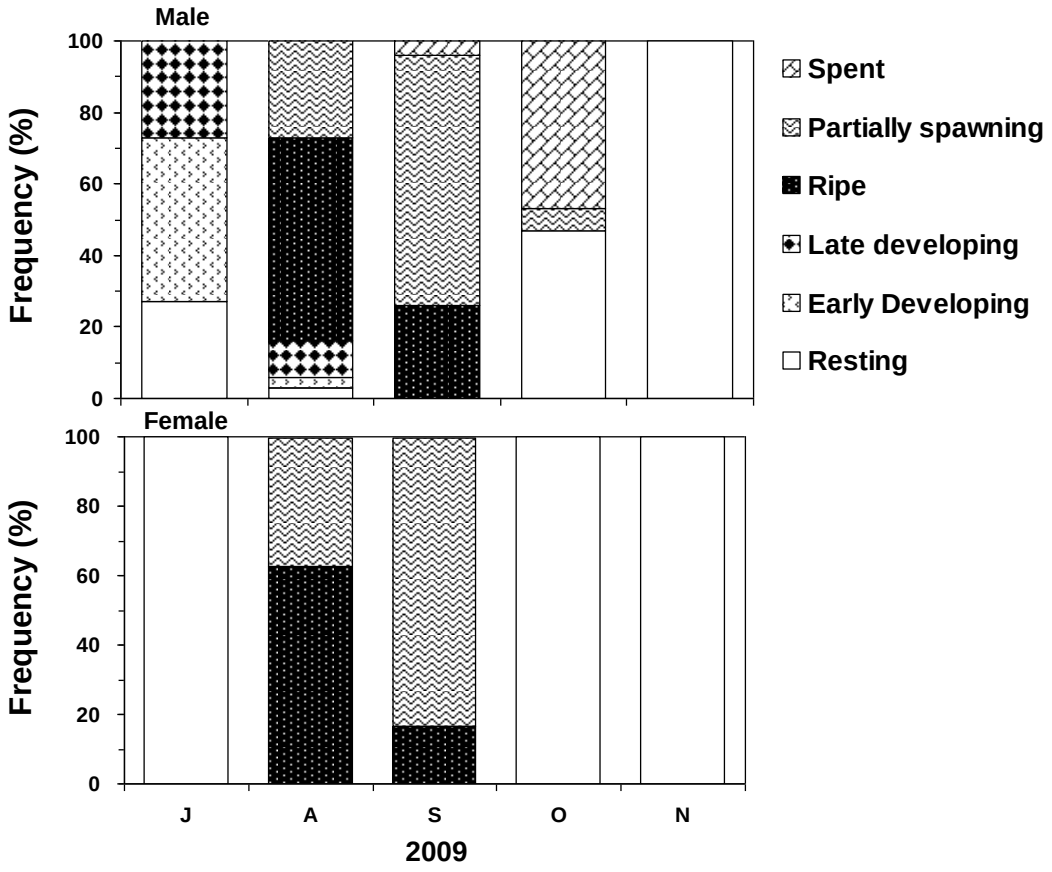

Fig. 2-3. Monthly percentage distribution of male and female oysters at different gametogenic stages during the study period.

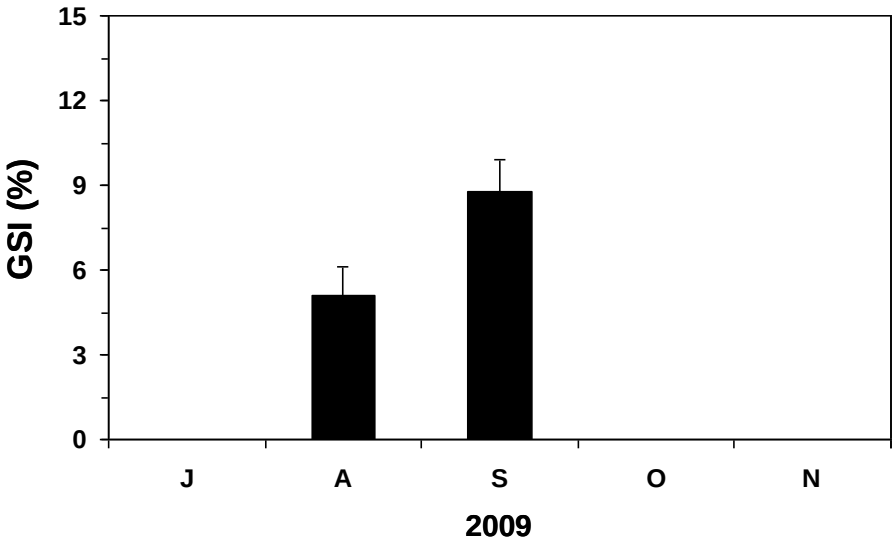

Fig. 2-4. Gonad-somatic index, GSI (%) of the oyster during the study period. The vertical bars represent standard error.

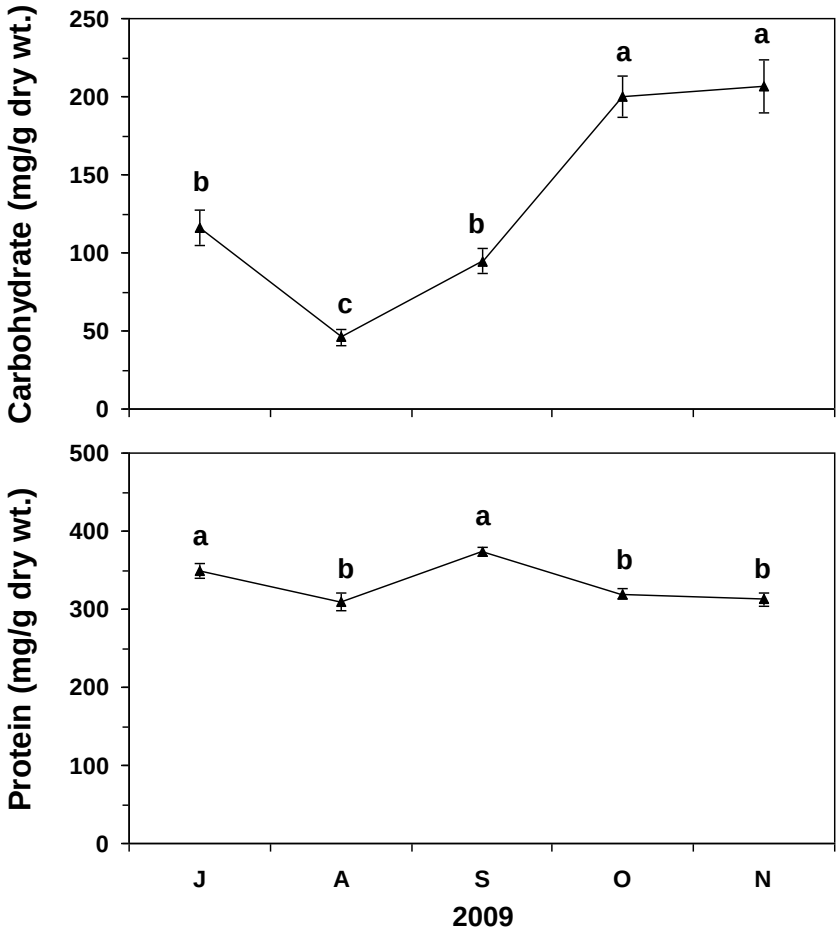

Fig. 2-5. Monthly variation of tissue carbohydrate and protein content of the oysters during the study period. The vertical bars represent standard error and different alphabets indicate the significant variation (ANOVA, Duncan's, $P < 0.05$).

4. Discussion

4.1. Early growth of hatchery-produced juvenile oysters

In the Korean oyster farming industry, oyster spats harvested from bays during the post-spawning season are hardened for 9–10 months in the inter-tidal zone before they are transplanted into a bay for grow-out. Grow-out normally takes 6–11 months, and juveniles transplanted in May or June become a marketable size at the end of the year, reaching more than 70 mm in SL (Hyun et al., 2001; Kang et al., 2010). Several studies have reported the annual growth and reproduction of suspended cultures of stunted juvenile oysters in Korea (Hyun et al., 2001; Oh et al., 2002; Kang et al., 2010). In contrast, no studies have investigated the early growth and reproduction of hatchery-produced juvenile oysters.

Kang et al. (2010) investigated the annual growth of stunted oysters in Gamakman Bay, into which they transplanted 10-month-stunted spat and raised them using a suspended long-line culture system. They observed rapid shell growth from May to October 1997, SL increasing from 15–20 mm to 55.6 mm. Shell growth then slowed during the rest of the period, reaching 74.2 mm at the end of monitoring in May, 1998. Kobayashi et al. (1997) also reported the growth of stunted oysters from the Hinase waters of Japan. Using a hanging raft culture system, they raised 10-month-hardened spats from May, 1990, to January, 1991. Juvenile oyster SL increased gradually, reaching 75.0 mm in October, 1990, and 93.1 mm at the end of the study in January, 1991. In the present study, 3-month-old hatchery-produced oyster spats with an SL of

about 10 mm reached 84.7±1.9 mm SL after 5 months of grow-out in November (Fig. 2-1). Compared to the reports of Kang et al. (2010) and Kobayashi et al. (1997), shell growth of hatchery-produced oysters raised in Gamakman Bay was more rapid, suggesting that a hatchery seed supply is a viable alternative to the traditional natural seed supply from bays, if survival of the hatchery-produced seeds is acceptable during grow-out compared to the stunted oysters.

As indicated in Figure 2-2, the CI of the transplanted oysters increased linearly from July to October, despite a spawning pulse in September. The insignificant drop in CI observed in this study is likely closely related to the less quantity of gametes released (reported in Fig. 2-4) during the spawning. Royer et al. (2008) investigated the gametogenic cycle and reproductive effort of three age classes of Pacific oyster from Normandy, France. After spawning, the CI of oyster spats decreased by 20%, while that of half-grown and marketable size individuals dropped by 40% and 37%, respectively. Such decreases in CI were also closely related to the quantity of eggs produced and released during spawning; the mean GSI of spats and half-grown and marketable-size individuals was 36%, 61%, and 59.6%, respectively. Kang et al. (2000) also reported remarkable drops in CI in Osu (50%) and in Jaran (60%) oysters on the south coast of Korea after spawning when the oyster's gonad maturity index (GMI) was at its maximum. In October and November, when the oysters were sexually inactive, CI remained high, which coincided with the highest carbohydrate level (Fig. 2-5). This has an important implication for the oyster industry in Korea, because the highest demand occurs during winter, and our experimental oysters reached a marketable size before winter, with a higher nutrient content and tasty flavor.

4.2. Reproductive effort

Reproductive effort was estimated using an indirect ELISA and a rabbit anti-oyster egg IgG as the primary antibody (Choi et al., 1993; Kang et al., 2003). The ELISA technique has previously been used to quantify the reproductive effort of Pacific oysters, and was sufficiently sensitive to measure the small quantities of egg present even during the early reproductive stage (Kang et al., 2003; Ngo et al., 2006; Royer et al., 2008). The mean GSI of 6-month-old juvenile oysters measured in September was 8.8±1.1%, which is remarkably lower than that of adult oysters (30–60%, Kang et al., 2003; Ngo et al., 2006; Royer et al., 2008; Fig. 2-4). Such a low GSI level was also observed in 14-month-old stunted juvenile oysters during their first spawning period. In Gamakman Bay, Mondol et al. (submitted) estimated the GSI of stunted oysters to be 11.1±2.6% by ELISA. The relatively low level of reproductive effort observed in juvenile oysters may account for the age-specific variation in reproductive effort. Young oysters devote more of their energetic metabolism to growth, while adults use their energy during the summer period preferably for reproduction (Héral and Deslous-Paoli, 1983). Royer et al. (2008) reported the age-specific variation in reproductive effort of Pacific oysters from Normandy, France. Using an ELISA, they measured GSIs of 36% in spat, 61% in half-grown oysters, and 59.6% in marketable oysters, which were 13, 25, and 41 months old, respectively. Deslous-Paoli and Héral (1988) also reported the age-specific reproductive effort of Pacific oysters in Marenes-Oleron Bay, France. In their study, the reproductive effort varied from 43.1% in 1-year-old and 61.9% in 3-year-old females.

The gametogenic pattern of hatchery-produced juveniles observed in this study is somewhat comparable to the pattern of stunted oysters transplanted into bays. Kang et al. (2010) investigated the annual gametogenesis of stunted juvenile oysters transplanted into Gamakman Bay. The stunted oysters transplanted in May attained gonadal maturation in late summer, and exhibited only one spawning pulse during August and September, although the quantity of eggs produced and released during spawning is unknown. In contrast, adult Pacific oysters (i.e., those that are more than 2 years old) on the south coast of Korea spawn continuously during summer with multiple spawning pulses (Kang et al., 2003).

4.3. Storage metabolism in relation to reproduction

Sexual maturation is an energy-demanding process that requires the mobilization of nutrients from ingested food or the storage and subsequent utilization of reserves in body tissue (Ruiz et al., 1992). Carbohydrates (in the form of glycogen) are the main energy reserve in marine bivalves (Beninger and Lucas, 1984; Mathieu and Lubet, 1993) and decrease during the spawning season (Marin et al., 2003; Camacho et al., 2003; Ojea et al., 2004; Ngo et al., 2006). In the present study, oyster gonad development seemed to occur concomitantly with utilization of the reserve carbohydrate originating from food consumed previously. Carbohydrate content followed gametogenic development, in that the higher reserve in July coincided with earlier gonad maturation. Carbohydrate content significantly decreased in August and September with advancing gonad maturation and spawning (Fig. 2-5, P <0.05). Carbohydrate level again increased in October and November when the oysters completed spawning. Ngo et al. (2006) also reported a

higher carbohydrate reserve in adult Pacific oysters in Gosung Bay off the south coast of Korea during winter and spring, compared to summer. This seasonal difference in carbohydrate level was associated with the seasonal reproductive activity of oysters, including gonad maturation and subsequent spawning.

Carbohydrate level in oyster tissues recorded during the spawning period (50–120 μg/mg tissue) was lower than that (100–200 μg/mg tissue) measured during the summer spawning period by Ngo et al. (2006). Such a difference in total carbohydrate level is believed to be associated with different grow-out rearing periods and ages. In the present study, the hatchery-produced spats were transplanted into the bay in June, and spawned only 2 months later, when the oysters may have had insufficient time to accumulate a reserve (i.e., carbohydrate), in their bodies. Concurrently, Ngo et al. (2006) conducted their study on market-size oysters (shell length >70 mm), when the oysters already stored sufficient carbohydrate reserve in their tissue for future expenditure on gonad development and spawning.

Several studies have reported that protein is the major component of the oocytes of marine bivalves, accounting for 40–50% of egg weight (Choi et al., 1993; Kang et al., 2003; Park and Choi, 2004; Ngo et al., 2006; Yang et al., 2011). Ngo et al. (2006) reported that the summer protein maxima coincided with GSI peaks in Gosung Bay, Korea, and postulated that seasonal changes in protein reserve were closely linked to the annual reproductive cycle of the oyster. Protein represented a high level during this monitoring and the monthly highest protein level observed in September, corresponded with the highest GSI, and may have been associated with protein-rich eggs (Fig. 2-5).

Part III

Monitoring of biological activities of the wild Pacific oyster, *Crassostrea gigas*, two-years after the *Hebei Spirit* oil spill at Taean, off the west coast of Korea

Abstract

The *Hebei Spirit* oil spill occurred in December, 2007 on the Yellow Sea, near at Taean was the largest oil tanker accident in Korea. After the oil accident a recovery of the oil impacted sites have already been discussed but the potential biological impacts of the oil spill remain largely unknown. Two-years after the oil accident we investigated the growth, energy storage and reproductive activity of wild Pacific oyster, *Crassostrea gigas*, collected from a heavily oil impacted site of Taean and compared with those of a control population to know the physiological condition as well as recovery status of the oysters from the oil stress. Along with histology an enzyme-linked immunosorbant assay was used to know the gonad development and reproductive effort of the oysters. During this study the contaminated site oysters exhibited a significantly higher ($P< 0.05$) growth by tissue weight than that of the control population. Both of the groups performed a similar carbohydrate reserve level but protein level was significantly higher ($P < 0.05$) for the contaminated site population. Histology revealed that most oysters collected in April were in earlier gonad maturation condition, became ripe in June and spawned during July and October when the bays were warmer. As a consequence of peak spawning, all biometric components levels decreased during August and September in both of the population. During the spawning season the contaminated site females produced a significantly higher ($P< 0.05$) quantity of egg mass than the control

population. This study results, indicate that the effects of oil spill could no longer be sustained after two years of the accident and the oysters might be recovered their physiological status to normal level.

1. Introduction

The collision of the *Hebei Spirit* oil tanker with a crane barge in December, 2007 was the largest navigation catastrophe in the Republic of Korea. The collision caused the spill of 12,547 kl of crude oil into the Yellow Sea about 10 km off the coast of Taean, and this spill extended along >70 km of Taean shoreline within several weeks of the accident (MLTM, 2008). Immediately after the oil spill an extensive cleanup operation was conducted, which removed the visual oil from most of the affected beaches within a month of the accident. The majority of the oil impacted sites is sandy beaches and rocky shores, and is fully exposed to wave action. A rapid decline in water column crude oil concentration was observed as a consequence of the rapid "dilution, degradation and weathering" of spilled oil (ITOPF, 2008). Several environmental monitoring studies also reported that the total petroleum hydrocarbon (TPH) concentration both in seawater and pore water was very high immediately after the *Hebei Spirit* oil accident and then decreased onwards at reaching the Korean seawater quality standard levels (10 ppb), after 10-months of the oil accident at most of the oil impacted sites (Kim et al., 2009; MLTM, 2008). The *Hebei Spirit* oil spill ranks as one of the largest oil spill of recent years (ITOPF, 2008) and may have certain long-term biological and environmental consequences.

Marine bivalves are often used as sentinel organisms for the monitoring of

environmental pollution (Ji et al., 2006; Peteio et al., 2006; Soniat et al., 2011). Their worldwide distribution, sedentary mode of life, filter feeding habits, and susceptibility to the bioaccumulation of pollutants make them ideal species for the assessment of environmental pollution (Wade et al. 1998; Peteio et al., 2006, 2007; Soniat et al., 2011). Bivalves are likely to be a stable indicator of pollutants, and are more contaminated than fish and other mobile species (Milan and Whelan, 1978; Soniat et al., 2011). Various eco-physiological disturbances caused by exposure to petroleum hydrocarbons have been reported in different species of bivalves (Peteiro et al., 2007). A marked decline in scope for growth (SFG) as a consequence of the reduction in feeding rates and increases in metabolic expenditures is documented for the turkey wing mussel (*Arca zebra*) collected along contaminant gradients in Bermuda (Widdows et al., 1990). SFG represents an integration of major physiological responses, and specifically the balance between the process of energy acquisition and energy expenditure (Widdows and Donkin, 1991). A reduced shell and somatic tissue growth was reported in the Pacific oyster (*Crassostrea gigas*) from the Singapore marine environment after exposure to chemical contaminants (Bayen et al., 2007). Stress related to pollution can also alter the biochemical composition especially the carbohydrate or protein level of bivalves (Stekoll et al., 1980; Patel and Eapen, 1989; Smolders et al., 2004; Peterio et al., 2007). Several studies have reported a marked decline in the glycogen (major proportion of the total carbohydrate) concentration, which is the most important reserve material in marine bivalves (Patel and Eapen, 1989; Smolders et al., 2004; Peterio et al., 2007). Furthermore, the reproductive activity of marine bivalves is closely linked with the energy storage cycle (Gabbott, 1975; Whyte and Englar, 1982; Ruiz et al., 1992; Park et al., 1999a; Ojea et al., 2004) and petroleum contamination can interrupt the gonad

maturation as well as spawning of bivalves (Bayne, 1982; Chu et al., 2003). In exposure to petroleum hydrocarbons a loss of reproductive output of marine bivalves is also documented (Lowe and Pipe, 1986; Mcdowell et al., 1999; Chu et al., 2003).

Despite the harmful impacts of petroleum hydrocarbon a few studies have investigated the biological impacts of the *Hebei Spirit* oil spill (Lee et al., 2009; Donaghy et al., 2010; Lee, 2010; Jung et al., 2011; Lee et al., 2011). Immediately after the oil accident a significant growth decline (by tissue weight) and less reproductive activity were documented for the oil impacted Pacific oysters (Lee, 2010). As a consequence of the adverse effect of the oil contaminants a loss of immunocompetence of the Pacific oyster also reported a-year after the *Hebei Spirit* oil accident (Donaghy et al., 2010). To know the physiological condition as well as recovery status of the oysters two-years after the *Hebei Spirit* oil spill, this study investigated the seasonal variations of somatic tissue growth, energy storage and reproductive activity of the wild Pacific oysters, *Crassostrea gigas,* collected from a heavily oil contaminated site at Taean, and compared those with a population at a control site nearby, in the Incheon area. The Pacific oyster, is the most important bivalve species in Korea, and is widely cultured in numerous semi-enclosed bays along the south coast (Choi, 2008). The Taean coast is more productive than the Incheon coast, and in recent years getting importance for the expansion of the shellfish industry (Yang, 2011). Based on the higher productivity of the Taean area we also hypothesized that, if the oysters recovered their normal physiological state from the oil stress, the contaminated site population would show better biological performances than that of the control population. A better understanding of the biological functions will provide new insights about the adaptive mechanisms and recovery status of the oyster population after the oil contamination.

2. Materials and methods

2.1. Study area and sampling

The present study was conducted at Guryepo (36°53' N and 126°11' E), near Taean, which was heavily contaminated by the *Hebei Spirit* oil spill and a neighboring control site Jonghyun (37°13'N and 126°34'E) near Incheon, on the west coast of Korea (Fig. 3-1). The control site is located about 60 km north from the oil spill site and was not affected, as the prevailing northwesterly winds and coastal currents after the spill carried the *Hebei Spirit* oil mostly in a southeast direction and contaminated the coastline of Korea (ITOPF, 2008; Lee et al., 2009; Kim et al., 2010). The oil concentration at the contaminated site (Guryepo) reached 58.2 (μg/L) in sea water and 1110.0 (μg/L) in pore water in February, 2008, two months after the accident, and decreased to 1.79 (μg/L) and <0.13 (μg/L) respectively in October, 2008, but the control area always exhibited the oil concentration lower than the background level, (10 μg/L), of the marine water quality standard of Korea (Kim et al., 2009). Monthly 30 wild Pacific oysters, *Crassostrea gigas*, were collected from both sites from April, 2010 to April, 2011 to check the physiological status of the oysters after the oil spill. The oysters were about 2-years old at the time of first sampling on April, 2010. During this monitoring, water temperature was recorded from both stations. Salinity and chlorophyll *a* data were gathered from the regular environmental monitoring data of Korea Hydrographic and Oceanographic Administration (KHOA) and Korean Marine Environment Management Corporation, (KOEM) respectively, using the website http://www.meis.go.kr/.

2.2. Biometry and histology

Upon arrival at the laboratory, all oysters were washed to remove mud adhering to the surface, and also encrusting barnacles and other epibiota, and then shell length (SL) was recorded to the nearest 0.1 mm using vernier calipers. Total volume was measured by displacement of water nearest to 0.5 ml, and soft tissue was separated from the shell, blotted and weighed, and sectioned at 2 mm dorsoventrally for histology. The remaining tissue was weighed, and freeze dried, and the tissue dry weight (TDW) was measured. The tissue was then pulverized separately with mortar and pestle, and used for biochemical analysis and for measuring the reproductive effort, the egg mass. Shell volume was measured and cavity volume was calculated by subtracting shell volume from total volume. Condition index (CI) was measured as the ratio of tissue dry weight (g) to shell cavity volume (ml) (Lucas and Beninger, 1985).

The histology tissue sections were fixed in Davidson's solution, dehydrated with an ascending alcohol series, and embedded in paraffin. The embedded tissues were sliced at 6 μm thickness and stained with Harri's hematoxylin and eosin Y. The stained slides were examined under a light microscope to identify the sex and to evaluate the gonad development stages as: 1. resting, 2. early developing, 3. late developing, 4. ripe, 5. partially spawning, and 6. spent/ absorbing (Kang et al., 2010). Analyses of annual gametogenesis included only samples from female oysters and oysters in the resting stage (i.e., unidentified sex).

2.3. Biochemical analysis

Twenty to 25 mg lyophilized and pulverized tissue powder was measured separately per individual for the carbohydrate and protein assay. Total tissue carbohydrate of the oyster was measured by the phenol-sulfuric acid method with dextrose as a standard (Taylor, 1995). Protein was estimated using the method described by Lowry et al. (1951), after alkaline hydrolysis with 0.1M sodium hydroxide at 37℃, and bovine serum albumin was used as a standard.

2.4. Quantification of reproductive effort by ELISA

The reproductive effort of female oysters was measured by indirect ELISA using *C. gigas* egg-specific antibody previously developed by Kang et al. (2003) as a primary antibody. Twenty-25mg of lyophilized and pulverized female oyster tissue was dissolved in 1ml phosphate buffered saline (PBS, 0.15M NaCl, pH 7.4) and then homogenized using an ultrasonicator. One hundred μl aliquots of the tissue homogenate (i.e., 500 to 5000-fold) and 0.1-6 μg/ml purified egg protein as standard were added to the wells of a 96-well polystyrene micro-plate. Subsequent ELISA steps were performed as described by Kang et al. (2003) and Park and Choi (2004). A log polynomial standard curve was constructed from the optical density of the known quantity of standard material, included in the plate. The concentration of egg protein in the tissue homogenate was estimated from the standard curve and the dilution factor. The amount of egg in each specimen was determined by multiplying the amount of egg protein measured by ELISA by 2.5, the ratio of protein to whole egg weight (Kang et al., 2003).

The gonad-somatic index (GSI) was calculated as the ratio of the total dry weight of eggs to that of whole oyster tissue.

2.5. Standardized measurements

For the better evaluation of the physiological status of the contaminated and control site oysters, the tissue dry weight, biochemical components, and egg mass were standardized to a 44.3 mm SL (mean SL of all specimens analyzed), for each sampling date. Regression analyses were performed after logarithmic transformation (base 10) of tissue dry weight and shell length for each sampling date. The allometric equation $Y=aL^b$ was used, where Y=tissue dry weight, L=shell length, a=intercept and b=slope of the regression equation. The same analysis was conducted for the gross weight of the biochemical components and egg weight to tissue dry weight. Gross biochemical composition and egg weight was then calculated for a given shell length by substituting the appropriate values of dry weight in the regression equations. The results of tissue dry weight, biochemical composition, and egg weight were expressed as mg per standard animal (Beninger and Lucas, 1984; Navarro et al., 1989).

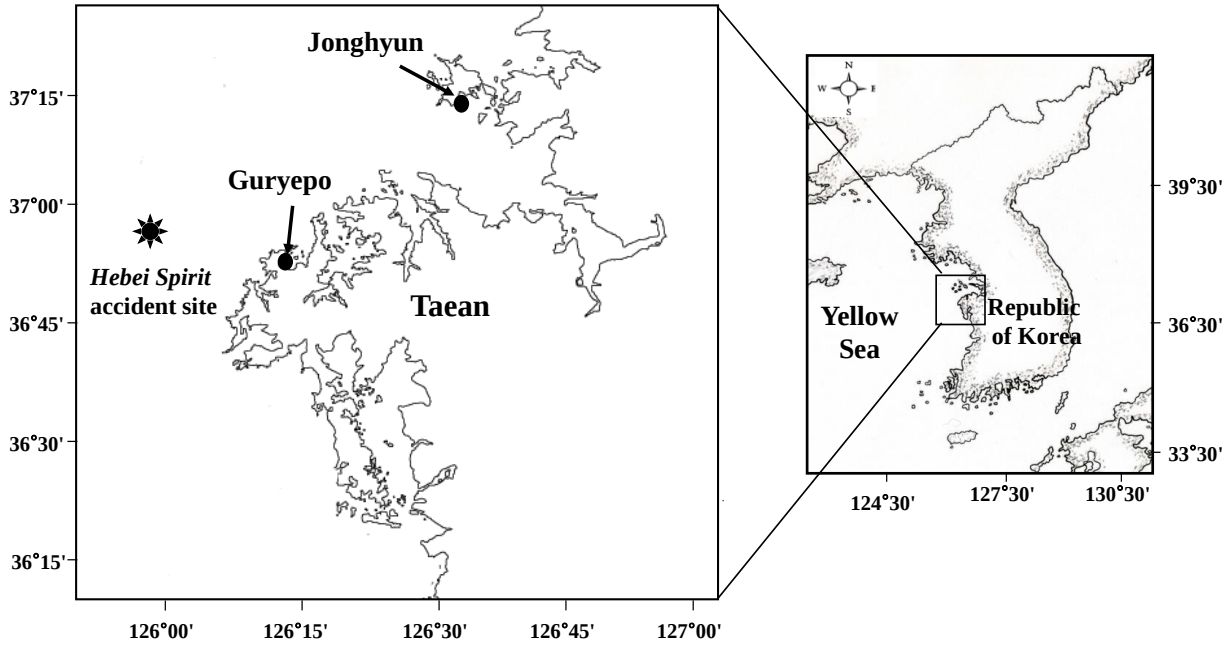

Fig. 3-1. Location of study areas on the west coast of the Korea. Guryepo and Jonghyun are the oil contaminated and the control site, respectively.

2.6. Statistical analysis

All statistical analyses were performed using SPSS (version 18.0). Data were tested for normality and homogeneity of variance to meet the assumptions of parametric statistics. If these assumptions were not satisfied, data were arcsine-transformed or log-transformed. Significant differences in condition index, tissue dry weight, protein, carbohydrate, gonad somatic index, and standardized egg mass of *C. gigas*, between treatments (oil contaminated vs. control) and among sampling times, were tested using a two-way ANOVA. When significant differences among treatments were observed, a Student Newman–Keuls (SNK) *post hoc* test was performed. Statistical significance was set at the alpha< 0.05 level.

3. Results

3.1. Environmental parameters

At the start of the study sea water temperature was 6.4℃ at the oil contaminated site and 8.1℃ at the control site. Temperature increased rapidly, reaching 23.8℃ in September at the contaminated site and 25.7℃ in August at the control site. The water temperature decreased from September onwards, reaching the lowest level during January and February (Fig. 3-2). Salinity remained stable and ranged 22.5-28.8 psu at the contaminated site and 22.6-28.9 psu at the control site during the study period (Fig. 3-2). Figure 3-2 also shows the seasonal data of chlorophyll *a*. The chlorophyll *a* level ranged from 1.4-4.8 (μg/L) at the contaminated site and 0.3-4.6 (μg/L) at the control site.

3.2. Growth and condition index

In both groups the specimens were similar in size in most of the sampling months; ranging 37.9-50.7 mm SL for the oil contaminated site and 39.8-47.6 mm SL for the control site (Table 3-1). For a better comparison of somatic growth, when we converted the gross TDW to standard 44.3 mm SL (see above: materials and methods), the standardized TDW of specimens at the contaminated site was significantly higher than that of the control population ($P<0.05$; Fig. 3-4, Table 3-2). In both groups the TDW showed similar seasonal fluctuations, increasing during spring and summer periods with the advance of gonad maturation, and then decreasing after spawning during August-September. Specimens at both sites recovered their tissue weight after spawning during the winter season.

The condition index showed similar seasonal fluctuations, and the population at the contaminated site exhibited significantly higher values than the control population ($P<0.05$; Fig. 3-3, Table 3-2). In both populations the CI was higher from April to July and then decreased during August and September, coincided with the spawning of the oysters. After spawning the oysters recovered their CI from October onward. During this monitoring a sudden decrease of CI in June and November was not clearly understood.

3.3. Biochemical composition

The seasonal cycle of standardized carbohydrate content was similar in both oyster populations (Fig. 3-4). The carbohydrate content increased in spring and the highest level was observed in May when 40.4 mg carbohydrate/standard animal was measured

for the contaminated population and 46.8 mg/standard animal for the control population. In both groups the carbohydrate level decreased from June to September, coinciding with the advance of gonad maturation and spawning. The oysters recovered their carbohydrate levels from October onwards, when they were in resting condition and initiated their new reproductive cycle. During this monitoring there were no significant differences in carbohydrate levels between the populations (Table 3-2).

In contrast to carbohydrate, the standardized protein levels of the oyster populations showed fewer seasonal fluctuations, increasing as gonad maturation progressed, reaching the highest level in August (137.9 mg/ standard animal) for contaminated site specimens and in July (96.3 mg/ standard animal) for the control population (Fig. 3-4). In accordance with spawning, the protein level decreased during September in contaminated oysters and from August to September in the control population (Fig. 3-4). During this study the protein content of contaminated oysters was statistically higher than that of the control oyster population ($P<0.05$; Table 3-2).

3.4. Gametogenesis and reproductive effort

In Figure 3-5, we plotted the gonad development of female oysters at the contaminated and control sites. Histology revealed similar seasonal trends in overall gonad development of females between the populations. At the first sampling in April, 2010 more than 90% of females were in early gonad maturation stage in both groups (Fig. 3-5). The oysters become ripe in June, and spawned from July to October, with a peak during August and September. In both groups most of the oysters became reproductively inactive from October onward, and initiated a new gametogenic cycle in

November; however, gonad maturation did not progress until April, 2011.

In contrast to gonad development, the reproductive effort of female oysters, measured by indirect ELISA, showed similar seasonal fluctuation for both groups (Fig. 3-6). The GSI (i.e., the egg mass) gradually increased from April to July with the advance of gonad maturation, and decreased from August to October, indicating spawning (Fig. 3-6). When we converted the percentage of GSI to the standard animal egg content, it also showed the similar seasonal pattern as GSI, but a significantly higher quantity of egg mass was documented for the females at the contaminated site ($P<0.05$; Fig. 3-6, Table 3-2). The highest egg content was calculated in July, when females at the contaminated site produced 136.0 mg eggs/standard animal and the control population produced 120.5 mg eggs/standard animal. Both of the population showed a gradual decrease of egg mass during their spawning. After the first spawning cycle, when the oysters again initiated a new gametogenic cycle, the ELISA also quantified a less amount of egg mass for both of the populations (Fig. 3-6).

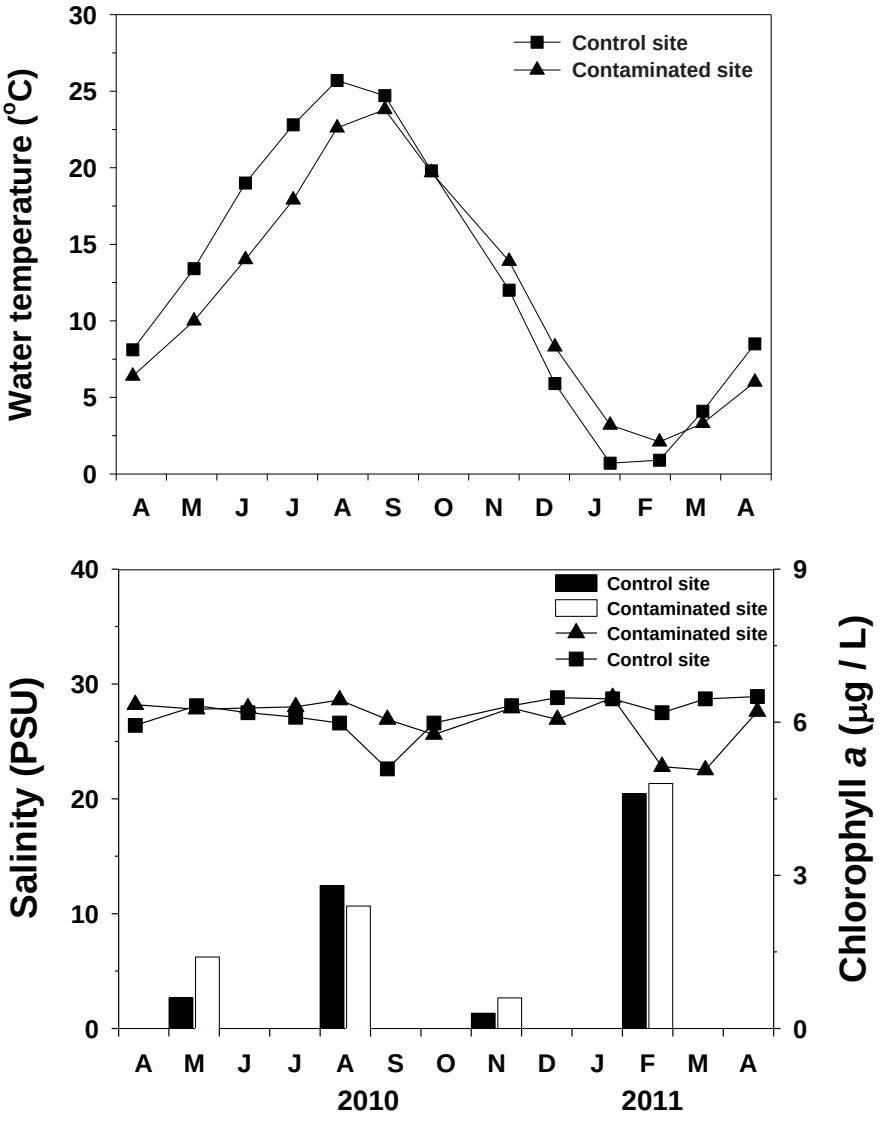

Fig. 3-2. Seasonal variations in water temperature, salinity and chlorophyll *a* in the oil contaminated and the control site from April 2010 to April 2011.

Table 3-1. Monthly analyzed oyster number (N), shell length (SL), tissue wet weight, (TWT) of the oil contaminated and the control site oysters from April, 2010 to April, 2011. The values are mean ± standard error, except the number of oysters.

Sampling Periods	N	Contaminated site		Control site	
		SL (mm)	TWT (g)	SL (mm)	TWT (g)
2010, Apr	30	49.8±0.9	1.96±0.13	47.6±1.2	1.37±0.07
May	30	50.7±0.5	2.31±0.17	44.2±1.3	1.33±0.08
Jun	30	42.9±0.9	1.37±0.06	45.1±0.9	1.23±0.08
Jul	30	42.1±1.0	1.52±0.06	45.2±1.1	1.34±0.08
Aug	30	43.8±1.0	1.86±0.11	43.4±0.9	1.17±0.08
Sep	30	38.2±0.7	1.29±0.07	44.8±1.4	1.16±0.10
Oct	30	45.2±0.8	1.29±0.06	42.8±1.1	0.90±0.07
Nov	30	43.1±0.9	1.30±0.06	48.0±1.7	1.59±0.11
Dec	30	47.0±1.1	1.61±0.09	44.3±0.7	1.16±0.05
2011, Jan	30	42.5±1.0	1.53±0.11	43.4±0.9	1.29±0.08
Feb	30	50.3±1.1	1.98±0.11	47.7±1.2	1.38±0.12
Mar	30	37.9±0.9	0.87±0.05	39.8±1.1	0.95±0.09
Apr	30	40.2±0.9	1.01±0.06	42.4±1.3	1.20±0.15

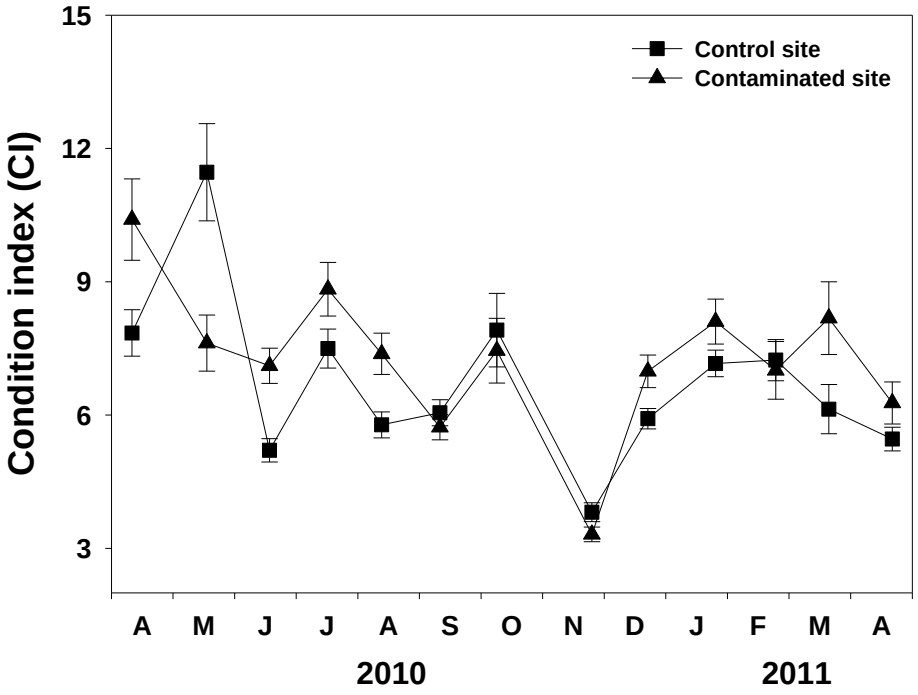

Fig. 3-3. Monthly variations in condition index of *Crassostrea gigas* in the oil contaminated and the control site from April 2010 to April 2011. Values represent mean ± standard error (n = 25-30).

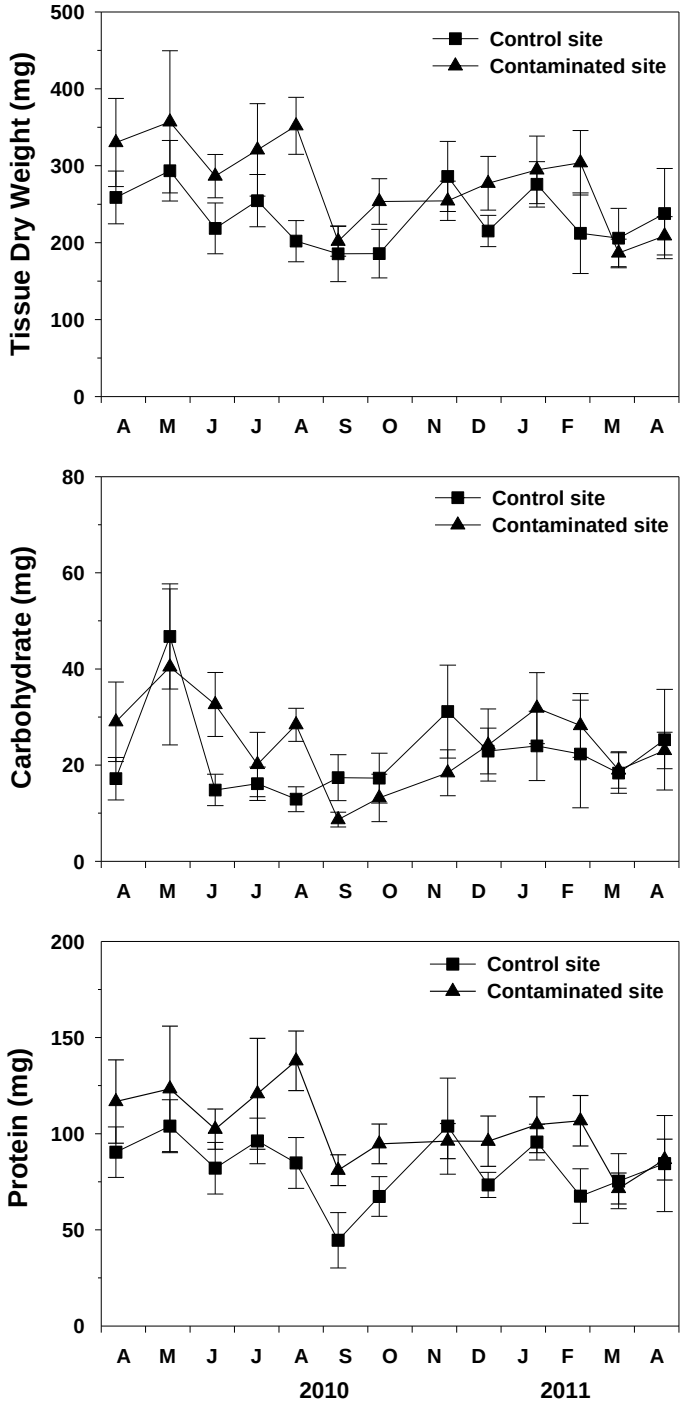

Fig. 3-4. Seasonal variations in tissue dry weight, protein and carbohydrate in a standard animal with a shell length of 44.3 mm in the oil contaminated and the control site. Vertical bars represent 95% confidence of intervals.

Table 3-2. Results of two-way ANOVA for the effects of date (sampling time) and treatments (control site vs. oil contaminated site) on, condition index, tissue dry weight, protein, carbohydrate, gonad somatic index and standardized egg mass.

Variables	Source	df	MS	F-ratio	P-value
Condition index	Date (D)	12	0.014	15.249	< 0.001
	Treatment (T)	1	0.005	5.988	0.015
	D × T	12	0.004	4.614	< 0.001
Tissue Dry weight	Date (D)	12	0.025	20.717	< 0.001
	Treatment (T)	1	0.058	48.354	< 0.001
	D × T	12	0.010	7.961	< 0.001
Protein	Date (D)	12	0.618	21.853	< 0.001
	Treatment (T)	1	1.979	70.010	< 0.001
	D × T	12	0.127	4.497	< 0.001
Carbohydrate	Date (D)	12	1.607	21.688	< 0.001
	Treatment (T)	1	0.112	1.518	0.218
	D × T	12	0.738	9.959	< 0.001
Gonad somatic index	Date (D)	8	0.00005	58.433	< 0.001
	Treatment (T)	1	0.0000001	0.017	0.897
	D × T	8	0.000003	4.172	< 0.001
Egg mass	Date (D)	8	6.375	76.887	< 0.001
	Treatment (T)	1	0.468	5.640	0.019
	D × T	8	0.399	4.810	< 0.001

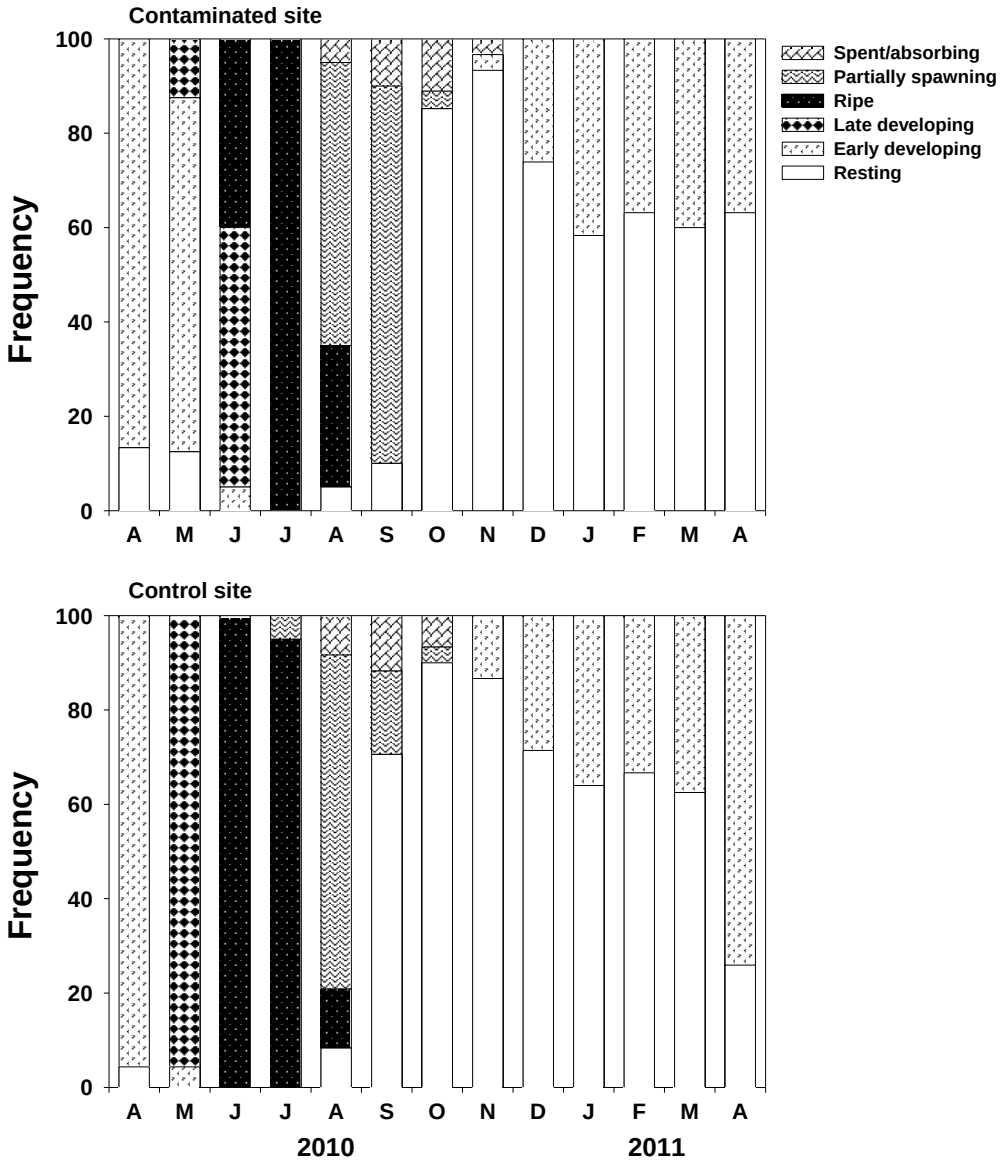

Fig. 3-5. Monthly percentage of distribution of *Crassostrea gigas* at different gametogenic stages in the oil contaminated and the control site from April 2010 to April 2011.

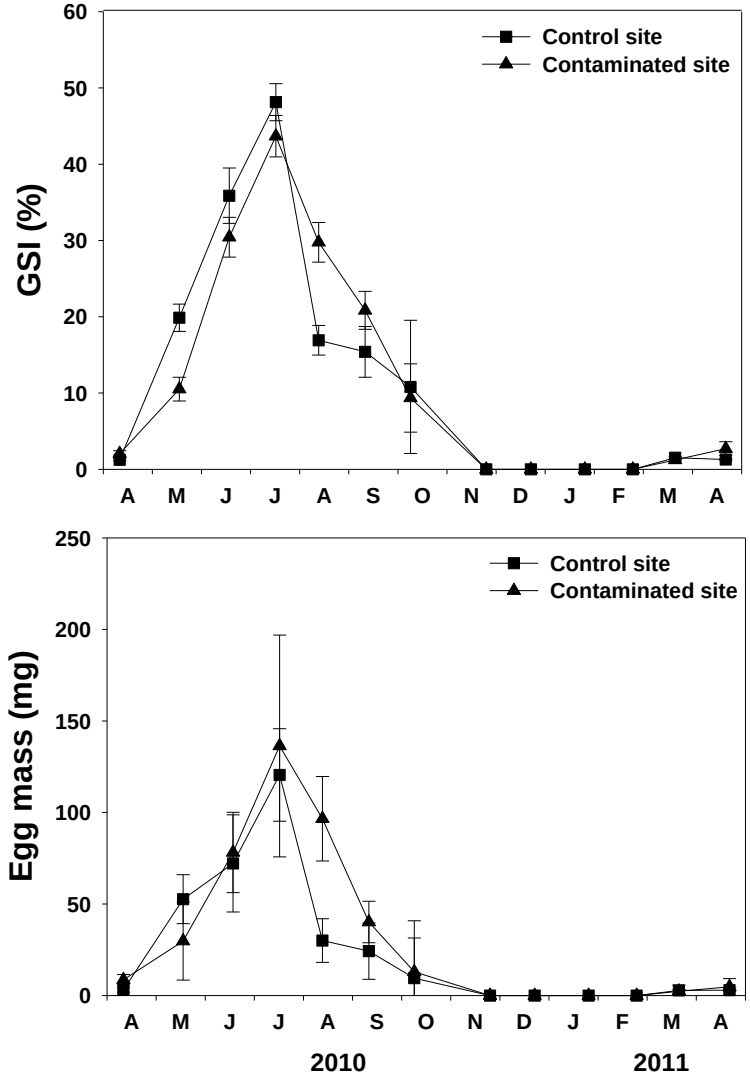

Fig. 3-6. Seasonal variations in gonad somatic index (GSI) and standardized egg mass of *Crassostrea gigas* in the oil contaminated and the control site from April 2010 to April 2011. For GSI values are mean ± standard error (n = 5-21) and for standardized egg mass vertical bars represent 95% confidence of intervals.

4. Discussion

In recent years, oil spills are the main reason for marine environmental pollution, and adversely impacts species biodiversity and economic resources (Laffon et al., 2006). Marine organisms can be severely impacted by the uptake of petroleum hydrocarbons (Livingstone, 1993). Several studies have reported the deleterious impact of hydrocarbon contamination on the scope for growth (SFG), somatic growth, proximate biochemical composition, and reproduction in marine bivalves (see the reviews in introduction). In the present study we evaluated the impact of the *Hebei Spirit* oil spill two-years after the incident by monitoring growth, condition index, biochemical composition, and reproduction of wild Pacific oysters, *Crassostrea gigas*, collected from an intensively oil-affected site and compared with those of a control site for a 13-month period.

Growth of bivalve molluscs represents an integrated physiological status and affected by pollution and varying environmental conditions (Dame et al., 1996; Peteiro et al., 2006). Marine bivalves commonly show slow growth rate when exposed to pollutants and tissue growth is extremely sensitive to hydrocarbon contamination (Soto et al., 2000; Le Floch et al., 2003). Peteiro et al. (2006), 4 months after the *Prestige* oil spill, reported a significantly lower growth rate (by weight) and smaller yield of harvest in *Mytilus galloprovincialis* population intensively affected by the spilled oil than those from a control population. After 15-months of the *Prestige* oil spill, Peteiro et al. (2008), from another investigation, reported no differences in growth performance (shell length and total tissue dry weight) of oil-exposed mussels in comparison with a control population, and postulated signs of recovery of the contaminated population from the oil stress. Lee (2010) investigated the impacts of the *Hebei Spirit* oil spill on commercially

raised Pacific oysters at Taean, Korea and observed a depressed somatic tissue growth of the oil affected oysters during January 2008 and July 2008, which was much lower than that of a control population. From July to onwards the oil affected oysters slowly increased their tissue weight. In the present study, two-years after the *Hebei Spirit* oil spill, we observed a significantly higher tissue growth of the wild Pacific oysters at the oil contaminated site than those of the control population ($P<0.05$; Fig. 3-4, Table 2-2). Oil contamination has been known to cause reduced growth rates; therefore, the higher tissue growth of the oysters at the contaminated site, indicates a sign of recovery of the physiological status of the oysters from the oil stress. Several environmental studies have reported that bivalves molluscs can successfully recover their physiological status to normal level when oil concentrations return to background levels (Lowe and Pipe, 2003; Laffon et al., 2006; Cajaraville et al., 2006; Soniat et al., 2011). In materials and methods, according to Kim et al. (2009), we reported that our contaminated site reached a background level in oil concentration within 10-months of the accident. An extensive environmental monitoring program conducted after the *Hebei Spirit* oil spill by the Ministry of Land, Transport, and Maritime affairs of Korea (MLTM) also pointed out a recovery in most of the oil impacted sites, including our treatment site, within 10-months of the oil accident (MLTM, 2008). The environmental survey (MLTM, 2008) also revealed that oil concentration in Pacific oyster tissue rose to 10-1000 times higher than the pre-spill levels within a few days after the spill, and the residual oil contaminants in oyster tissues decreased dramatically reaching pre-spill state in October, 2008, 10-months after the accident. The MLTM (2008) report and Kim et al. (2009) study also allowed us to make a recovery hypothesis. The oysters might be reverted their physiological status to normal level when the oil concentrations return to background

level or they already developed their physiological tolerance to oil contaminants within two-years of the accident (Thomas et al. 1999). After 3-4 years of the *Exxon Valdez* crude oil spill in Prince William Sound, Thomas et al. (1999) observed a lack of physiological responses (byssal thread production, condition index, clearance rate and glycogen content) between chronic oil exposed and reference site *Mytilus trossulus* population instead of high level of tissue PAH of the contaminated site population and suggested a physiological tolerance (either innate or acquired) of the chronic oil exposed mussels population to the elevated PAH.

The observed tissue growth variations of the specimens between the populations can be explained as the site specific variations of environmental parameters. Among the environmental parameters, food availability is the most important factor in growth variations of oysters (Powel et al., 1995; Hyun et al., 2001; Paterson et al., 2003). Several studies have reported that the Taean coast is a dynamic ecosystem and more productive than the Incheon area (Yang, 2011 and other references therein). The seasonal dynamics of chlorophyll *a* concentration (reported in Figure 3-2) also indicate better food availability at the oil contaminated site than at the control site. This higher food availability at the contaminated site may provide a better opportunity for growth for the oysters.

During our investigation, both oyster populations showed similar seasonal fluctuations in tissue dry weight (Fig. 3-4). The TDW increased during spring and summer, coincided with the progress of gonad maturation and higher CI values. In association with peak spawning, TDW decreased from August to September. From October onwards, most oysters were inactive or in early gonad maturation condition, and again increased the TDW in association with the increase of reserve storage. Such a

seasonal cycle of the TDW in Pacific oysters is usually observed in different natural environments (Kang et al., 2000, Kang et al., 2003; Ngo et al., 2006; Kang et al., 2010).

The condition index of marine bivalves has been widely used in growth studies as well as in research related to pollution (Lucas and Beninger, 1985; Pridmore et al., 1990; Dame, 1996; Peteiro et al., 2006). In general CI variability depends on the seasonal changes of food availability or the gametogenic cycle of bivalves (Lucas and Beninger, 1985; Amiard et al., 2004; Peterio et al., 2006). In the present study oysters at both sites showed a similar seasonal cycle in CI with higher values during April and July, and then decreasing from August to September in association with peak spawning (Fig. 3-3). After spawning the oysters recovered their CI during the autumn-winter period. The autumn-winter higher CI also coincided with higher reserve storage, indicating that the oysters accumulated their reserve materials during this period for their future expenditure on gonad development and spawning (Pridmore et al., 1990). McDowell et al. (1999) reported significantly lower condition index values during the pre-spawning period in a transplanted *Mytilus edulis* population from New Bedford Harbor, contaminated with PCBs and PAHs, than that in reference populations. In association with spawning, the condition index and lipid content in mussel's populations decreases, as lipid is incorporated in the production of new gametes. A lack of seasonal variability in the flesh condition index (FCI) of the *Mytilus galloprovincialis* population as a consequence of the deterioration of environmental quality after the *Prestige* oil spill is documented in a study by Marigomez et al. (2006). In the present study, a similar seasonal cycle with significantly higher CI of the contaminated site oysters ($P< 0.05$; Fig. 3-3, Table 3-2) also indicated their better health condition than the control population.

The biochemical composition of the oysters in this study showed that the storage

and reserve mobilization, mainly carbohydrate, are closely linked to the annual reproductive cycle as reported by several authors for different species of bivalves (Gabbot, 1975; Ruiz et al., 1992; Kang et al., 2003; Ngo et al., 2006; Ojea et al., 2004; Yang et al., 2011). Tissue carbohydrate levels were higher during the spring season when the oysters were in earlier gonad maturation condition and then decreased during summer to early autumn in association with the intense energy utilization for gonad maturation and spawning (Fig. 3-4). Both the groups again increased the carbohydrate levels during the remainder of the period when they were sexually inactive or in earlier gonad maturation condition. In contrast to carbohydrate, the protein content represented the major part of the tissue for both of the groups (Fig. 3-4). Higher protein levels observed during July and August also coincided with the ripe gonads of the oysters. In association with massive release of egg mass, the protein levels suddenly dropped during September, and both of the population again increased their protein levels after the spawning from October onwards.

The level of biochemical composition often used as an indicator of environmental contamination (Patel and Eapen, 1989; Peteiro et al., 2008). Peteiro et al. (2007) investigated the variability of the biochemical composition in *Mytilus galloprovincialis* 4-months after the *Prestige* oil spill. Proteins were the main biochemical components during most of the culture time, and the mussel population of the heavily contaminated site represented significantly lower total tissue carbohydrate and protein levels than the control population. Fifteen-months after the *Prestige* oil spill, Peteiro et al. (2008) observed no differences in the biochemical composition level between the control site and the contaminated area, and postulated a recovery of the species' health from the oil stress. Stekoll et al. (1980) investigated the sub-lethal effects of chronic oil exposure on

the intertidal clam *Macoma balthica* at Seward, Alaska. They recorded a marked decline in absolute values of total carbohydrate and protein content of the clams with the inclination of oil contaminants level. In the present study, a similar seasonal cycle with similar carbohydrate levels and significantly higher protein levels in the contaminated oyster population also indicated their better physiological status than that of the control population.

Gametogenesis of marine bivalves is an energy dependent process, affected by varying environmental conditions (Ruiz et al., 1992; Park et al., 1999a; Kang et al., 2000; Ojea et al., 2004). In the present study the gonad maturation in both populations progressed during spring and summer in accordance with spring season storage utilization, which was documented in the carbohydrate result, and the increasing water temperature of the bays. Most were in early gonad maturation condition in April, 2010 when the bays were colder (6.4-8.1℃). With the increasing surface water temperature, the oysters become ripe in June and spawned during July and October when the bays became warmer (22.0-26.0℃, Fig. 3-5). From August to September, spawning coincided with a significant decline in TDW, CI, and GSI, indicating concentrated spawning activity during this period for both oyster populations. This spawning pattern is similar to Kang et al. (2010). Kang et al. (2010) investigated the annual gametogenesis of Pacific oysters from Gamakman Bay, off the south coast of Korea. They observed only one spawning peak during August and September at 22-26℃, when the oysters decreased their percentage of gonad area (PGA) from 40% to 19%. Not in agreement with our findings, Kang et al. (2003) reported two major spawning, one in late June and another from late July to mid-August, in adult Pacific oysters. They monitored the monthly changes in egg masses in oysters from Gosung Bay, off the southern coast of

Korea, using an immunological method.

It is generally believed that, petroleum contamination can interrupt the annual gametogenic cycle of marine bivalves, and results in asynchronous spawning activity (Bayne et al., 1982). Chu et al. (2003) from their laboratory experiment on American oysters (*Crassostrea virginica*) fed with PCBs contaminated diet observed a slower gonad maturation and lower reproductive success in contaminated oysters than that of the control population. Immediately after the *Hebei Spirit* oil spill a reproductive impairment was documented for the Pacific oyster, *Crassostrea gigas* by Lee (2010). He observed asynchronous spawning during July to late September with less quantity of gametes release for the oil spilled site oysters, while the control population synchronously spawned during late June to early August with substantial gamete releasing. In the present study histology revealed a similar seasonal trend of gonad maturation and synchronous spawning activity for both oyster populations, but an earlier partial spawning in July accounted for only 5% of the control population (Fig. 3-5). The earlier partial spawning of the control group in July is not supported by the seasonal changes in GSI. The GSI level tended to increase with the maturation of gonads up to July and then started to decrease from August, indicating that both oyster populations effectively spawned from August onwards. This study also suggests that, along with histology, ELISA could be a good alternative for the gametogenic study of bivalves

Although there is a lack of sufficient research information on the relationship between petroleum exposure and reproductive impairment, it has been suggested that the reproductive effort of oysters is negatively correlated with their PCB body burden (Wade et al., 1992; Chu et al., 2003). McDowell et al. (1999) reported a significantly lower reproductive effort (RE) in PCBs and PAHs-exposed *Mytilus edulis* than that in control

populations. They calculated the RE as the proportion of energy allocated to reproduction through the collection of released gametes in relation to the total amount of energy assimilated and partitioned to growth and respiratory demands. A deleterious oil impact was documented on the reproductive effort of the Pacific oyster after the *Hebei Spirit* oil spill (Lee, 2010). Using ELISA, Lee (2010) estimated the highest GSI 47.3 %, in early June for the females of the control oyster population; but only 29.9% GSI in late June for oil contaminated females. Using the indirect ELISA we estimated about 40% mean GSI for both of the populations and the standardized egg content was significantly higher for the contaminated site oysters ($P<0.05$; Fig. 3-6, Table 3-2). The higher reproductive effort also indicated a better physiological condition of the contaminated site oysters. Between-site variation of the reproductive effort of the oysters can be explained as the site specific variation of environmental parameters, particularly the food availability (MacDonald and Thompson, 1985; Bayne et al., 1983). The higher food availability of the contaminated site enabled the oysters to store more energy in their tissues (observed in protein and carbohydrate results) and subsequently a better reproductive effort than the control population. The recovery of water column crude oil concentration also supports the above details concerning the variation of reproductive effort of the oysters. Soniat et al. (2011) investigated the physiological status of *Crassostrea virginica* from oil-exposed sites in Louisiana, USA, and they observed no oil impact on the oysters 6 months after the oil spill; the contaminated site oysters performed with a better condition index and reproductive activity than the control population. They also concluded that variation in condition and reproductive state are consistent with the natural variation of environmental parameters, such as salinity gradients, not the impacts of PAHs contaminants.

GENERAL CONCLUSIONS

Grow-out transplantation in the month of January favors the oysters in better growth, physiological condition and energy storage than that of the traditionally transplanted (i.e., transplanted in May) oysters. January transplanted oysters represented an earlier gametogenesis and synchronous spawning activity during June-September, while a delayed gametogenic activity with asynchronous spawning (August-October) exhibited for the May transplanted oysters. An ELISA estimated mean 27.92 million fecundity for the January transplanted oysters, which was about 6-times higher than that of the traditionally transplanted oysters, indicating that January transplantation could significantly contribute to natural seed stock improvement in Korean waters.

In the Gamakman Bay, the hatchery-produced oyster showed high aquaculture potential, grew rapidly after transplantation into the Bay and became 84.3 mm in shell length after 5 months of grow-out rearing corresponding to 8 months old from the fertilization. During the first spawning season the hatchery-seed oysters produced a less quantity of eggs compared to the adults.

Two years after the *Hebei Spirit* oil spill we observed a recovery in physiological status of the Pacific oysters from the adverse effects of petroleum contamination. The contaminated site oysters represented significantly higher somatic tissue growth, condition index, protein level, and egg mass than the control population. Both of the groups showed similar seasonal cycle in the biometric components and followed the reproductive cycle. Histology revealed one spawning peak during August and September for both of the populations.

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