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Application of enzyme-linked immunosorbent assay for studying of reproduction in the Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia) I. Quantifying eggs

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Abstract

A polyclonal antibody specific to *Ruditapes philippinarum* egg protein (ME-ab) was developed to quantify clam eggs using an enzyme-linked immunosorbent assay (ELISA). Western blots revealed that ME-ab reacted with egg proteins of molecular masses 475, 84, and 40 kDa under non-reducing conditions and 330, 96, 64, 50, and 31 kDa under reducing conditions. With ELISA, ME-ab detected between 0.23 and 15 μg ml^{-1} of clam egg protein; the number of eggs per clam was quantified by dividing the weight of the total egg protein by the average weight per egg. Reproductive output, expressed as the gonadosomatic index (GSI), was calculated as the ratio of the egg weight to the total tissue weight. Seasonal changes in reproductive output were measured in clams collected on a monthly basis from Gomso Bay, Korea. Clam egg protein was detected during all months except January. The monthly mean GSI varied from 0 (January) to 0.25 (August), and the highest GSI (0.389) was recorded from a clam collected in late July. ELISA indicated that clams in Gomso Bay spawned when the gonad accounted for 20% of the total tissue weight. The fecundity estimated from individual clams before spawning ranged from 0.94 to 11.79 million eggs, with a mean of 4.15 million. In conclusion, the ELISA used in this study was a sensitive and rapid method

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for quantifying eggs in individual clams and is considered the method of choice for quantifying the reproductive output.

Keywords: Reproductive output; *Ruditapes philippinarum*; Fecundity; ELISA; Gonadosomatic index; Korea

1. Introduction

Ruditapes philippinarum, the Manila clam (=Japanese littleneck clam), is widely distributed on sandy or muddy tidal flats along the coast of Korea. The clam is one of the most important shellfish in the Korean fisheries industry. Manila clams are endemic to the western Pacific and have been introduced to the Pacific coast of North America and European waters for commercial purposes (Cesari and Pellizzato, 1990; Goulletquer, 1997). In 2000, aquaculture and fisheries produced 1,700,000 metric tons (MT) of Manila clams worldwide, and more than 95% of the landings originated from the Yellow Sea, mainly from China, Korea, and Japan (FAO 2003). In 2000, a total of 39,000 MT of clams were produced in Korea by aquaculture and fisheries, constituting the second largest shellfish production after oysters.

Quantitative reproduction information about marine bivalves, such as the biomass of the gonads or the fecundity, is crucial for understanding their life histories, as well as for successful management (Braley, 1982; Kautsky, 1982; Thompson et al., 1996). Despite the importance of bivalves, relatively few studies have quantified bivalve gonads owing to the technical difficulties involved in the measurement (Honkoop and van der Meer, 1998). Unlike in other marine invertebrates, in most marine bivalves the gonads are an integral part of the visceral mass and cannot be separated from the body (Lucas, 1982; Thompson et al., 1996). Consequently, the direct assessment of gonad weight is unfeasible in most cases, except scallops. Instead, the gonad mass is often estimated from histological preparations of gonadal tissues using stereology or planimetry (Morvan and Ansell, 1988; Tirado and Salas, 1998; Ceballos-Vazquez et al., 2000; Kang et al., 2003a). The mass can also be estimated by determining the difference in body weight before and after spawning (Kautsky, 1982; Pouvreau et al., 2000). To weigh or count the number of eggs produced, sexually mature bivalves are often induced to spawn (Galinou-Mitsoudi and Sinis, 1994; Chung et al., 2001). However, these techniques often underestimate the true gonad biomass because spawning is often incomplete and occurs continuously with differing intensity throughout the spawning season.

Immunological methods have been used to study the reproductive biology of aquatic organisms. In an immunoassay, target molecules can be visualized or quantified using antibodies raised against different molecules. In particular, the enzyme-linked immunosorbent assay (ELISA) is the method of choice for quantifying egg proteins owing to its speed, low cost, and high sensitivity (Johnsen et al., 1999). ELISA has been used successfully to quantify the egg proteins of marine bivalves (Choi et al., 1993, 1994; Kang et al., 2003b; Park et al., 2003), shrimp (Tsukimura et al., 2000), and fish (Johnsen et al., 1999; Glasser et al., 2003).

The quantity of *R. philippinarum* eggs produced annually was assessed using ELISA with an antibody against clam yolk proteins. In this study, we report the development of

the antibody and the seasonal variation in the reproductive output measured using ELISA.

2. Materials and methods

2.1. Antigen preparation and antibody development

To obtain ripe eggs as an antigen for antibody development, ripe clams were collected in June from Gomso Bay on the west coast of Korea. The eggs were extracted from the gonad and diluted in 0.6 M phosphate-buffered saline (PBS). The egg extracts were filtered through 100- and 63-μm mesh screens sequentially to remove impurities. The eggs were then sedimented by gravity in a 50-ml conical tube at 4 °C, and the supernatant was discarded. This cleaning procedure was repeated three to five times. The eggs in the tube were counted using a hemocytometer. To determine the mass of a single egg, a known number of eggs was freeze-dried and weighed. The protein concentration in an egg was then determined using a BCA protein assay kit (Pierce) with bovine serum albumin (BSA) as the standard.

A New Zealand White rabbit was selected as the host animal in which to develop egg-specific antibodies; Table 1 summarizes the immunization protocol used to produce antiserum. Four weeks after the first injection, 10 ml of blood was withdrawn, and the antiserum titer was determined using Ouchterlony's double immunodiffusion test (Ouchterlony and Nilsson, 1978). Ten days after the final injection, 20 ml of blood was withdrawn from the rabbit, when the antiserum titer was sufficiently high.

2.2. Preparation of clam egg-specific antibody

The specificity of the antiserum was tested by the double immunodiffusion test using the egg homogenate as a positive control and somatic tissue proteins prepared from the gills, mantle, and foot as negative controls. The rabbit antiserum proved to be very specific for the clam egg proteins, although the antiserum also exhibited a weak, but recognizable, cross-reaction to the somatic proteins extracted from the gills, mantle, and foot. The antibodies in the antiserum that cross-reacted with the somatic tissue proteins

Table 1
Summary of the immunization protocol

Time	Procedure	Dose of antigen
1st week	Initial injection	1 mg antigen in 500 μl+500 μl FAC
2nd week	Booster	500 μg antigen in 500 μl+500 μl FAI
3rd week	Booster	500 μg antigen in 500 μl+500 μl FAI
4th week	Bleeding	
5th week	Booster	500 μg antigen in 500 μl+500 μl FAI
6th week	Booster	500 μg antigen in 500 μl+500 μl FAI
7th week	Bleeding	

FAC: Freund's adjuvant complete.
FAI: Freund's adjuvant incomplete.

were removed using an immunoadsorbent prepared according to Fuchs and Sela (1979). The immunoadsorbent was prepared by using glutaric dialdehyde to polymerize somatic tissue protein extracted from a male clam. Twenty milliliters of antiserum was mixed with the same volume of immunoadsorbent and incubated for 3 h at room temperature; non-specific antibody in the antiserum bound to the surface of the immunoadsorbent particles, while the egg-specific antibody remained unbound. The Manila clam egg-specific antibody (ME-ab) was isolated by centrifugation to remove the immunoadsorbent, which was eluted with 0.2 M glycine–HCl buffer (pH 2.2) to isolate the cross-reacting antibody. Then, the rabbit ME-ab in the supernatant was purified by saturated ammonium-sulfate precipitation. The specificity of the ME-ab was retested using gel diffusion, immunofluorescence, and ELISA, and no further cross-reaction with somatic proteins was demonstrated.

2.3. Quantifying clam eggs using ELISA

For the analysis, lyophilized whole clam tissue was homogenized using a mortar and pestle. Ten milligrams of clam homogenate was dissolved in 10 ml PBS and further homogenized using a sonicator. The clam homogenates were diluted up to 1000-fold for ELISA. Indirect ELISA was used for the quantification according to Kang et al. (2003b). A 100-μl aliquot of clam homogenate to be analyzed or a control was added to a polystyrene 96-well microplate and incubated at 4 °C overnight. After incubation, the plate was washed with PBS containing 0.05% Triton X-100 (PBST) four times, and 150 μl of 1% BSA in PBST was added as a blocking agent. After a 1-h incubation and washing, ME-ab primary antibody was added in 100-μl aliquots to each well and incubated another 1 h at room temperature. Goat anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:1000) was then added as a 100-μl aliquot to each well, incubated for another 1 h, and washed. Finally, 100 μl of *p*-nitrophenylphosphate (*p*-NPP) substrate dissolved in 0.1 M glycine buffer was added as a coloring agent. The optical density of the end product was measured at 405 nm using a microplate reader.

The quantity of egg protein in the clam homogenate was estimated from a standard regression curve constructed from the optical density and the concentration of purified clam egg protein in the plate. Finally, the quantity of eggs in an individual clam was estimated by multiplying the quantity of egg protein measured using ELISA by 2.44, the ratio of the weight of protein to the total egg weight, as the protein accounts for 41.0% of the egg weight. The gonadosomatic index (GSI), the ratio of the dry weight of clam eggs to the dry weight of all the clam tissues, was used to investigate the seasonal variation in clam reproduction. The number of eggs produced (i.e., the fecundity) by each clam during spawning was estimated by dividing the mass of eggs measured using ELISA by 22 ng, the mean weight of a clam egg determined in this study.

2.4. Immunofluorescence assay

An immunofluorescence assay was used to localize the immunological reaction between ME-ab and the egg protein. Female clam gonadal tissue containing ripe eggs was embedded in paraffin, sliced at 6-μm thickness, deparaffinized, and rehydrated. The

sections were incubated for 30 min in 5% (w/v) BSA in PBST as a blocking agent. After blocking, the tissues were reacted with ME-ab (1:200 dilution) at room temperature for 1 h. The tissues were washed three times with PBST; fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:400 dilution, Sigma) was added, and the tissues were again incubated for 1 h. The FITC-stained gonadal tissues were washed again with PBST, mounted in glycerol–PBS solution (1:1), and observed under a fluorescence microscope.

2.5. Characterization of Manila clam egg-specific protein

Western blotting was used to characterize the egg protein involved in antibody development. The egg protein was extracted from the egg with lysis buffer (nNtRon Technology) and precipitated with trichloroacetic acid. We loaded 8.5 μg of purified egg protein onto a 10% SDS–polyacrylamide gel for electrophoresis (SDS–PAGE). The egg peptides separated on SDS–PAGE under reducing and non-reducing conditions were blotted onto a polyvinyl difluoride (PVDF) membrane. After blocking with 0.15 M Tris-buffered saline containing 1% Tween 20 (TBST) supplemented with 5% BSA solution, the membrane was incubated in ME-ab solution (1:5000 dilution) for 1 h. After three washes with TBST, the membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) for 30 min. Then, the membrane was washed again with TBST, and the immunoreactive bands were visualized using the ECL enhanced chemiluminescence detection reagent.

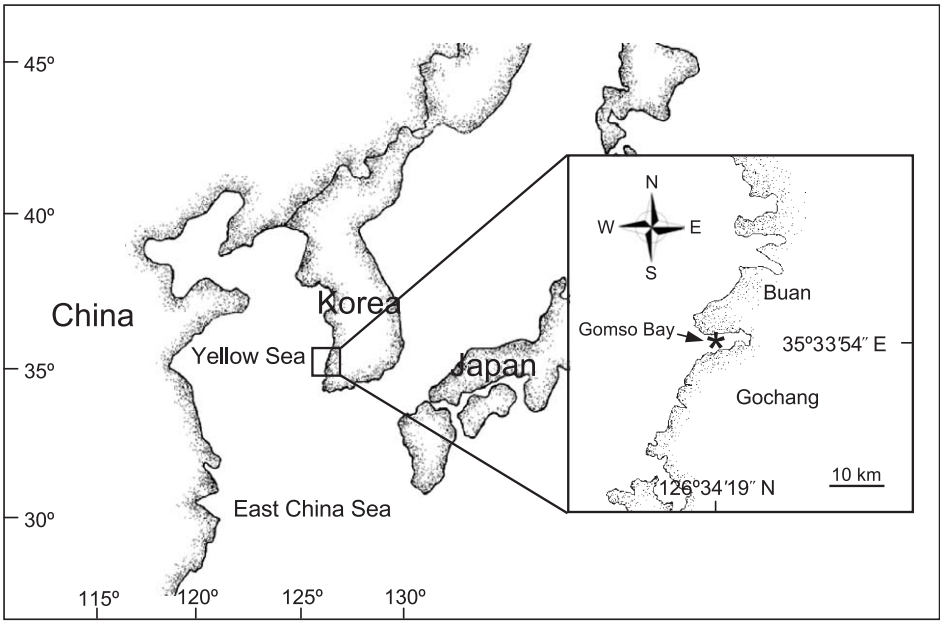

Fig. 1. Sampling site (*) in Gomso Bay, Korea.

Table 2
Classification of gonadal development in the reproductive cycle of the Manila clam

Developmental stage	Description
Indifferent	Little or no gonadal tissue visible
Early developing	Follicles are expanded, no mature gametes present
Late developing	Follicles are expanded, mature gametes first appear
Ripe	Most gametes mature
Spawning	Reduced number of gametes
Spent	Few gametes visible, some gonadal tissue undergoing cytolysis

2.6. Quantifying the eggs produced annually using ELISA

The variation in the annual reproductive output of clams was investigated in a clam population in Gomso Bay, on the west coast of Korea (35°33′ 19″N and 126°32′ 54″E, see Fig. 1). In the bay, clams are cultivated commercially on the sandy-mud tidal flat. For analysis, 30–50 market-sized clams were collected each month from March 1999 to February 2000 and biweekly during the spawning period in July and August. After recording shell length, height, thickness, and tissue wet-weight, the tissues were lyophilized and kept at −70 °C until analyzed. Monthly changes in the surface water temperature and salinity of the study area were recorded. Food availability in the water column (i.e., the chlorophyll *a* concentration) during the sampling period was inferred from a study conducted in 1999 and 2000 by the National Fisheries Research and Development Institute of Korea (NFRDI, 2001).

2.7. Gametogenesis of clams

The annual variation in the gametogenic development of clams was monitored using histology. A 5-mm-thick longitudinal section was cut from the middle of each clam fixed in Bouin's fixative. The sections were dehydrated in ethanol, embedded in paraffin, sliced at 6-μm thickness, stained with Harris hematoxylin, and counter-stained in eosin Y. The reproductive states of the clams were categorized into six stages according to the microscopic appearance of the gonad (Table 2). The egg diameter and the area occupied by the gonad in randomly selected microscopic fields (percent gonad area, PGA) were also measured from the histological preparation using an image analyzer, to follow the seasonal changes in reproductive conditions.

3. Results

3.1. Specificity of the antigen–antibody reaction

The antiserum raised from whole clam egg protein initially demonstrated a minor, but recognizable, cross-reactivity to the non-gonadal tissue proteins in the double immunodiffusion assay. After the cross-reaction was successfully eliminated with the immunoadsorbent assay, a strong antibody–antigen reaction was observed between egg protein and

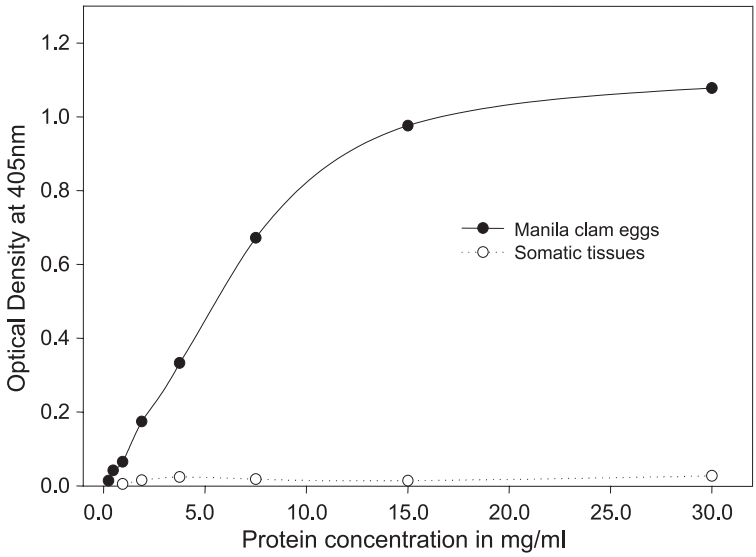

Fig. 2. Specificity of the antibody to Manila clam egg and somatic tissues.

the antibody purified using the immunoadsorbent, while no noticeable reaction was observed between the antibody and the somatic tissue protein, in an ELISA (Fig. 2). The antibody (i.e., ME-ab) developed in this study detected between 0.23 and 15 μg ml^{-1} of clam egg protein in the indirect ELISA. In the immunofluorescence assay, ME-ab bound specifically to the yolk granules of the eggs, while ME-ab did not bind to either connective tissue in the gonad or the nucleus in each egg (Fig. 3). It was believed that ME-ab reacts with vitellins, egg-specific proteins in animal eggs.

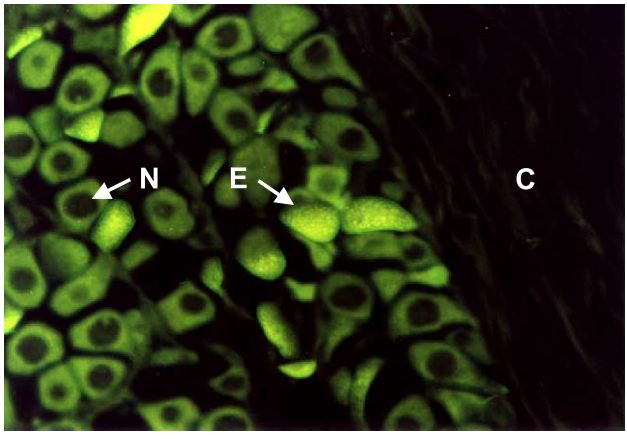

Fig. 3. Fluorescence pattern of immunostained histological sections of mature eggs. N=Nucleus, E=egg, C=connective tissue.

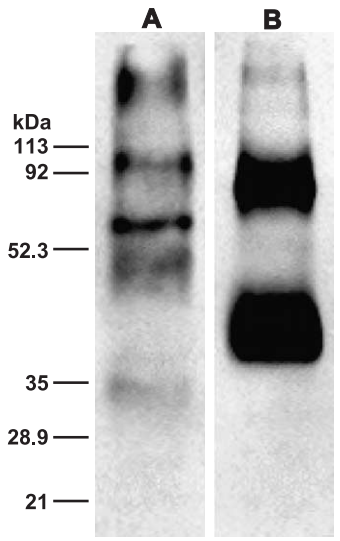

Fig. 4. Western immunoblot of the extract from Manila clam eggs. Lane A: reducing conditions; Lane B: non-reducing conditions.

3.2. Characteristics of Manila clam egg-specific protein

SDS–PAGE showed that clam eggs were composed of numerous peptides ranging in size from 6.5 to 500 kDa. In the Western blot assay, two major bands with molecular masses of 84 and 40 kDa, and one minor 475-kDa band, immunoreacted specifically with the antibody under non-reducing conditions. After samples treatment with β-mercaptoe-

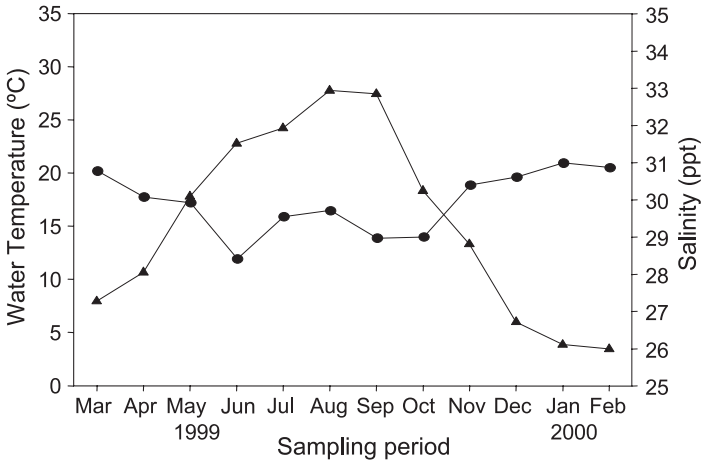

Fig. 5. Fluctuations in the surface water temperature and salinity in Gomso Bay in 1999–2000. (▲) Water temperature; (●) Salinity.

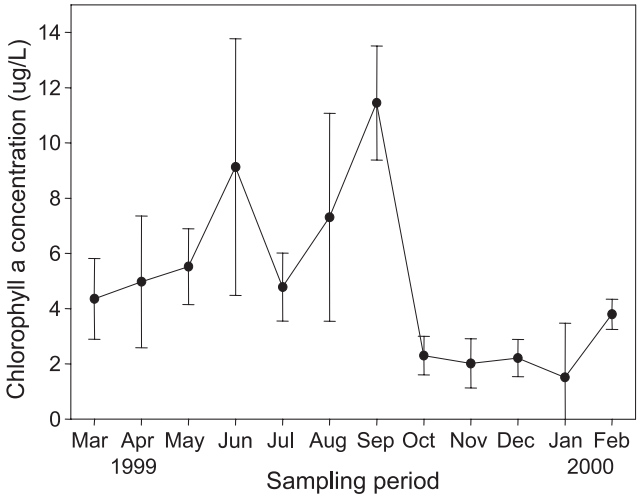

Fig. 6. Seasonal changes in the chlorophyll *a* concentration in Gomso Bay in 1999–2000 (adopted from NFRDI of Korea).

thanol, a protein-reducing agent, ME-ab-specific peptides were observed as five bands of approximately 330, 96, 64, 50, and 31 kDa (Fig. 4).

3.3. Seasonal change in environmental conditions

The seasonal variation in the surface water temperature is plotted in Fig. 5. The temperature varied from 3.43 °C in February to 27.8 °C in August. The salinity ranged

Table 3
Biometric data and gonadosomatic index GSI, (mg dry egg/mg dry clam) of the Manila clam measured using ELISA

Period	Mean shell length (mm)±S.D.	Mean tissue wet weight (g)±S.D.	Mean tissue dry weight (g)±S.D.	Mean GSI±S.D.	Highest GSI	Lowest GSI
March 20, 1999	34.09±3.50	2.219±0.711	0.462±0.165	0.009±0.007	0.024	0.004
April 17, 1999	36.74±3.58	3.547±1.171	0.820±0.231	0.089±0.051	0.105	0.032
May 15, 1999	37.76±2.49	3.617±0.564	0.751±0.173	0.187±0.083	0.281	0.032
June 30, 1999	34.10±2.62	2.394±0.584	0.524±0.138	0.122±0.079	0.248	0.043
July 18, 1999	36.26±3.71	2.846±0.816	0.553±0.190	0.134±0.071	0.290	0.071
July 27, 1999	31.18±2.47	1.389±0.371	0.313±0.079	0.250±0.063	0.389	0.167
August 18, 1999	30.80±2.88	1.377±0.305	0.228±0.043	0.107±0.078	0.252	0.015
August 29, 1999	37.56±1.82	2.696±0.711	0.411±0.128	0.178±0.132	0.386	0.064
October 4, 1999	34.52±2.69	1.261±0.263	0.187±0.039	0.050±0.053	0.158	0.016
November 23, 1999	29.84±1.64	1.268±0.223	0.200±0.037	0.001[a]	–	–
December 23, 1999	31.72±5.77	1.452±0.857	0.284±0.167	0.038[a]	–	–
January 15, 2000	32.32±1.53	1.327±0.258	0.250±0.053	–	–	–
February 23, 2000	33.53±1.35	1.518±0.303	0.294±0.082	0.018[a]	–	–

SD-standard deviation.
[a] Only one sample detected.

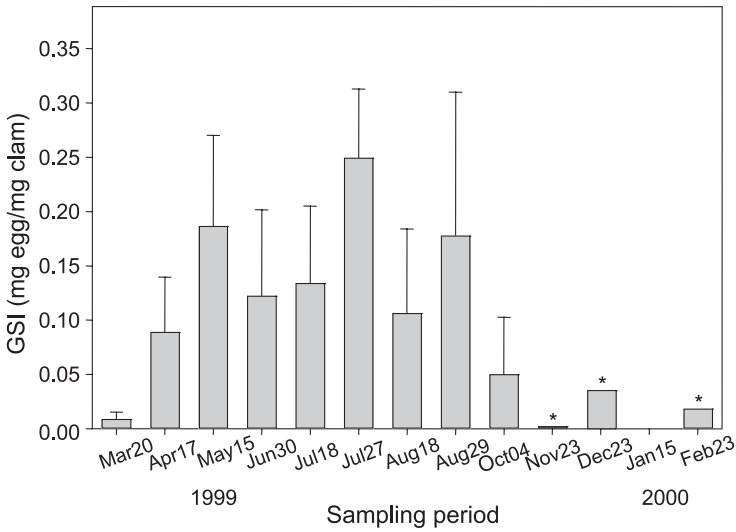

Fig. 7. Changes in the GSI of Manila clam measured using ELISA. *Only one sample detected.

from 28.5 ppt in June to 31.3 ppt in March. The salinity in the bay was relatively low during the summer monsoon period owing to heavy rainfall and freshwater input from rivers (Fig. 5). The chlorophyll *a* concentration in the water column during the study is plotted in Fig. 6. The concentration increased gradually from January to May and peaked in June and September, indicating that there were spring and fall plankton blooms in the bay.

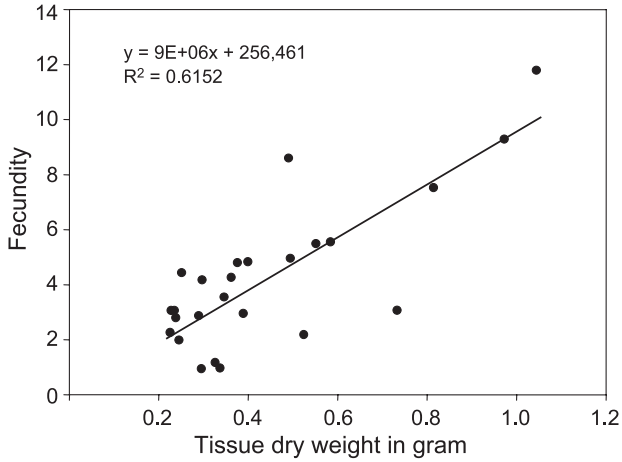

Fig. 8. Correlation between clam dry-tissue weight and fecundity during the peak of GSI.

3.4. Reproductive output of clams measured by ELISA

The sizes of the clams analyzed in this study ranged from 27.25 to 42.45 mm in shell length and from 1.112 to 4.284 g in wet-tissue weight. The reproductive output of a clam expressed as the weight-normalized GSI is summarized in Table 3. Indirect ELISA using ME-ab as the primary antibody was sensitive enough to detect the small quantity of egg protein remaining in some clams collected in December and February, a time during which most clams are in the resting or undifferentiated stage. In practice, ELISA could measure as little as a few milligrams of egg protein present in several hundred milligrams of clam tissue, or a GSI less than 0.004 (Table 3).

Fig. 7 plots the monthly GSI measured using ELISA from March 1999 to February 2000. The GSI increased at a faster rate from March to May and dropped in late June, indicating that spawning occurred between mid-May and late June in the bay. The GSI was again elevated from late June to mid-July; it peaked in late July and then dropped in mid-August, suggesting a second spawning pulse during this period. ELISA also detected a small quantity of egg protein in clams collected from late fall to late winter. The GSI

Table 4
Individual GSI (GSI, mg dry egg/mg dry tissue) and estimated fecundity of clams measured during the period of peak GSI in 1999

Period	Shell length (mm)	Tissue wet weight (g)	Dry tissue weight (g)	Estimated egg dry weight (mg)	GSI	Estimated fecundity
May 15	37.30	3.719	0.814	165.56	0.203	7,525,000
	38.80	4.168	0.972	204.32	0.210	9,287,000
	39.10	3.884	0.861	25.73	0.030	1,170,000
	42.00	4.592	1.044	259.47	0.249	11,794,000
	37.10	3.881	0.376	105.63	0.281	4,802,000
	33.50	2.841	0.551	120.89	0.219	5,495,000
	37.20	3.316	0.733	67.60	0.092	3,073,000
	36.70	2.929	0.584	122.18	0.209	5,553,000
July 27	31.30	1.742	0.362	93.96	0.260	4,271,000
	30.95	1.238	0.251	97.58	0.389	4,435,000
	32.00	1.610	0.346	78.16	0.226	3,553,000
	35.95	2.381	0.494	109.02	0.221	4,956,000
	30.45	1.100	0.228	67.39	0.296	3,063,000
	29.45	1.130	0.245	43.77	0.179	1,990,000
	31.90	1.342	0.238	61.56	0.259	2,798,000
	32.00	1.473	0.297	91.91	0.309	4,178,000
	30.85	1.129	0.225	49.78	0.221	2,263,000
	31.20	1.312	0.289	63.22	0.219	2,874,000
	36.10	1.751	0.389	65.11	0.167	2,959,000
August 29	36.55	3.228	0.524	48.13	0.092	2,188,000
	38.95	2.276	0.326	25.85	0.079	1,175,000
	37.70	2.890	0.490	189.24	0.386	8,602,000
	34.20	2.125	0.295	20.78	0.070	945,000
	35.85	2.127	0.337	21.42	0.064	973,000
	36.25	2.558	0.399	106.41	0.267	4,836,000
	36.80	1.811	0.235	67.53	0.287	3,070,000

Table 5
Percentage of gonadal maturation of the Manila clam in Gomso Bay, Korea, measured histologically

Period	March 20, 1999	April 17, 1999	May 15, 1999	June 30, 1999	July 18, 1999	July 27, 1999	August 18, 1999	August 29, 1999	October 4, 1999	November 23, 1999	December 23, 1999	January 15, 2000	February 23, 2000
Indifferent		7.7								75.0	75.0	52.9	25.0
Early developing	100.0	7.7											75.0
Late developing		84.6											
Ripe			100.0	16.7	21.4	52.9	66.7	60.0					
Spawning				75.0	71.4	47.1	33.3	40.0					
Spent				8.3	7.1				100.0	25.0	25.0	47.1	

values of clams collected in November, December, and February were 0.001, 0.038, and 0.018, respectively. When the GSI peaked in mid-May, late July, and late August, the fecundity of individual clams was estimated by dividing the weight of the eggs in each clam by 22 ng, the estimated weight of a single egg. The mean fecundity of a spawning clam was estimated as 6,087,000 eggs in May, 3,394,000 eggs in late July, and 3,113,000 eggs in late August. There was a positive correlation between the dry-tissue weight of a clam and fecundity (R^2=0.615), suggesting that larger clams produced more eggs (Fig. 8). After the fecundity or GSI was normalized for weight, no correlation was observed between the size and reproductive output (R^2=0.060) (Table 4).

3.5. Clam annual gametogenic cycle

Table 5 summarizes the temporal variation in the developmental stages of clams as determined from histological preparations. Female and male clams showed synchronized gametogenic development and spawning during the course of the study. In March and April, most clams were at the early or late developing stages, although 7.7% of the clams investigated were still at the indifferent stage. In May, all the clams were at the ripe stage. Spawning clams were common from June to August. More than 70% of the clams were at the spawning stage in June and mid-July, and 33–47% of the clams continued to spawn until late August. Spent clams were observed in late June and mid-July, and all of the clams collected in early October were at the spent stage. Clams were sexually indifferent from November through February. Some of the clams collected in February were at the developing stage, indicating that gametogenesis starts as early as February.

Fig. 9 shows the seasonal changes in the percent egg area (PEA). The PEA increased greatly from April (4.8) to May (34.9) and then was lower from late June (20.8) to mid-July (26.7). It increased again from mid-July to late July (44.2), remained stable until the end of August (36.8), and then dropped in early October (4.2).

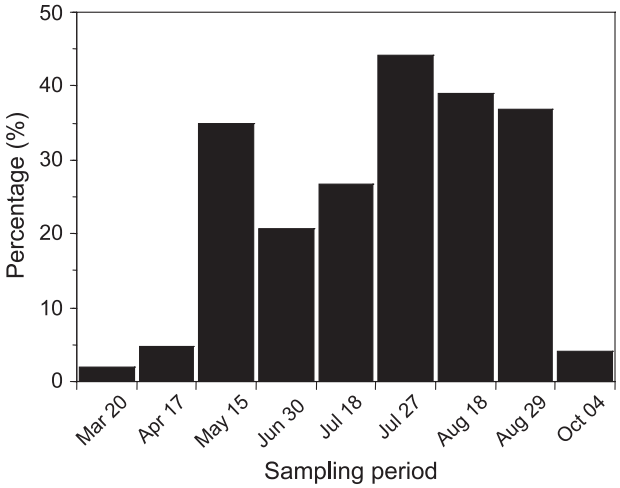

Fig. 9. Change in the percent egg area (PEA) in the gonads of Manila clams measured using image analysis.

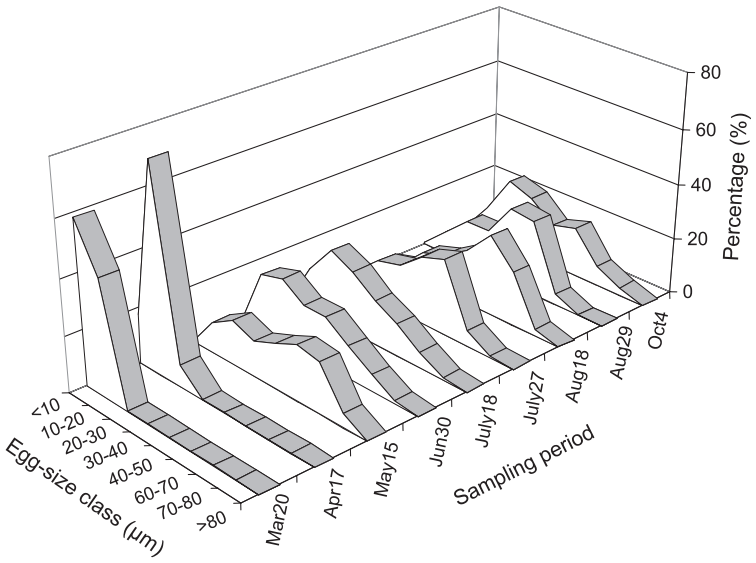

Fig. 10. Size-class distribution of Manila clam eggs measured using image analysis (n=7515).

A seasonal change in gonadal maturation was also inferred from the monthly variation in egg size (Fig. 10). Early developing eggs smaller than 20 μm in diameter were dominant in March and April. Fully mature eggs (50–70 μm) began to appear in May, and a wide range of egg sizes was recorded in May. The proportion of mature eggs decreased in late June as a result of the discharge of mature eggs during the first spawning pulse, while the proportion of small developing eggs increased slightly from May to late June. The proportion of mature eggs increased from late June to late August and then decreased in early October, suggesting that clams spawned during this period. Mature eggs were dominant in October, when no developing eggs smaller than 20 μm were observed.

4. Discussion

4.1. Development of clam egg-specific antibody

The rabbit anti-clam egg IgG developed in this study initially showed a weak, but recognizable, reaction to non-gonadal protein, including proteins extracted from the gills, mantle, and foot. The cross-reacting antibodies were eliminated using the affinity column packed with the immunoadsorbent. After the removal of the cross-reacting antibody, ME-ab showed strong immuno-specificity for clam egg protein in ELISA and immunofluorescence (Fig. 3). Choi et al. (1993) and Kang et al. (2003b) also observed that polyclonal antisera raised against oyster eggs cross-reacted with somatic tissues. Combined, these results suggest that a common protein is present in eggs and somatic tissues, although it has not yet been characterized.

SDS–PAGE (Fig. 4) shows that clam egg protein is composed of peptides with sizes ranging from 40 to 475 kDa. The Western blot of the reduced egg protein revealed that clam egg protein is composed of 330, 96, 64, 50, and 31 kDa subunits. Suzuki et al. (1992) reported egg-specific 105-, 85-, 66-, 64-, 60-, 45-, and 41-kDa peptides in Pacific oysters. These egg-specific peptides are vitellins, a major constituent of invertebrate eggs, and are used as an energy source during development inside the egg (Shafir et al., 1992; Suzuki et al., 1992). The immunofluorescence assay (Fig. 3) showed that the ME-ab–egg protein interaction is limited to the yolk granules inside the egg. The Western blot and immunofluorescence assays suggest that our ME-ab was raised against vitellins in the yolk granules of clam eggs, as observed in the Pacific oyster (Suzuki et al., 1992; Kang et al., 2003b).

4.2. Quantifying clam eggs using ELISA

The ME-ab developed in this study is sensitive enough to measure very small amounts of clam egg protein. Fig. 2 shows that ME-ab detected 0.23–15 μg ml^{-1} of egg protein in an ELISA. The lowest GSI in this study was 0.001, which was measured in November; this indicates that ME-ab can detect as little as a few milligrams of egg protein contained in clams with dry-tissue weights of 0.1–1.1 g. Several other studies have developed egg-specific antibodies for quantifying eggs using ELISA. Choi et al. (1993) produced a polyclonal antibody to the egg protein of the American oyster, *Crassostrea virginica*, which detected 0.2 to 10 μg ml^{-1} of egg protein in an ELISA. The rabbit anti-*Crassostrea gigas* egg protein antibody reported by Kang et al. (2003b) was also sensitive enough to measure a few milligrams of egg protein contained in 2–2.5 g of freeze-dried oyster tissue. Park et al. (2003) developed polyclonal antibody against the egg protein of the butter clam, *Saxidomus purpuratus*; this clam antibody detected as little as 0.078–20 μg ml^{-1} of the egg protein in an ELISA, which is comparable to our results. ELISA is also rapid and economical; samples from 10 to 15 clams can be loaded on a 96-well microplate and analyzed within 24 h, which is very fast compared with conventional gonad assays using chemical or physical stimulation to induce spawning.

ELISA detected no or very low levels of egg protein from clams collected in January and February (Table 3). The histology indicated that most clams collected in January and February are sexually undifferentiated or at the early developing stage (Table 5). However, the monthly mean GSI increased dramatically from April (0.089) to May (0.187); this coincided with an increased egg size as well as an increase in the percentage of the follicular area occupied by eggs in histological preparations (Figs. 9 and 10). The eggs present in clams collected from February to April were mostly pre-vitellogenic eggs, which probably contained no or low levels of yolk protein. The low GSI measured during this period is probably associated with the low level of vitellin in early developing eggs, as ME-ab was raised against vitellin-like egg proteins present primarily in ripe eggs. By contrast, clams collected from May to August contained ripe post-vitellogenic eggs at the spawning or spent stages, resulting in a high GSI during this period. The histology indicated that vitellogenesis, the process of converting reserve materials into yolk granules in the oocyte, occurred between April and May when the water temperature increased from 10 to 17 °C (Fig. 5). The GSI measured during each of the three spawning pulses observed

during the course of this study was approximately 0.2, suggesting that spawning is triggered when the egg mass reaches 20% of the total tissue weight. Powell et al. (1992) demonstrated that the frequency of spawning over an entire spawning season is in part determined by the cumulative reproductive biomass. Choi et al. (1993) reported that oysters in Galveston Bay initiate spawning when the egg mass accounts for 20% of the body weight. According to Kang et al. (2003b), the spawning of oysters is triggered when the egg mass becomes 40% of the body mass.

4.3. Clam fecundity estimated from ELISA data

Fecundity, which is defined as the number of gametes produced by an individual clam during the spawning season, was deduced from the ELISA data. The fecundity estimation was limited to clams just before spawning, when the eggs are fully mature (Kang et al., 2003b). The ELISA data were converted to the number of eggs by dividing the egg mass estimated from ELISA by the estimated weight of a single mature egg (22 ng), resulting in a calculated fecundity of from 0.94 to 11.8 million eggs per clam, with a mean of 4.1 million. We also estimated the fecundity of clams during the spawning period by counting the eggs directly. To assess the numbers, the body containing eggs was excised from clams collected during the spawning season, placed on a Petri dish, and squeezed to release the eggs. The discharged eggs were harvested and counted using a hemocytometer. The direct counting method yielded fecundities of 2 to 2.5 million eggs per clam; these numbers are considered minimum estimates, because the entire egg mass could not be separated from the body and not all of the eggs were released from the excised tissue. However, the fecundity estimated from direct counting is within the range of the fecundity estimated from ELISA, suggesting that our ELISA method is a reliable method for measuring the fecundity.

A numbers of studies have reported the fecundity of clams estimated using various methods (Table 6). Morvan and Ansell (1988) estimated the number of eggs in *Tapes* (=*Ruditapes*) *rhomboids* (shell length range 35–45 mm), using stereology and reported that the potential fecundity of the clam was 50×10^4 eggs. Toba and Miyama (1991) estimated the fecundity of *R. philippinarum* (shell length range 34.1–36.0 mm) in Tokyo Bay, Japan, by counting the number of eggs released from clams induced to spawn by ammonia stimulation; they obtained an estimate of 33.1×10^4 eggs per clam. The reported fecundities of *R. philippinarum* in Hawaii (USA) and Fujian (China) were $0.43–2.35\times10^6$ and $0.19–1.50\times10^6$, respectively (Yap, 1977 and Shi et al., 1984 cited from Ponurovsky and Yakovlev, 1992). Nell et al. (1995) estimated the fecundity of *Tapes dorsatus* by counting the eggs released from clams after serotonin stimulation and found that clams collected from Sydney Harbor, Australia, discharged 1.11×10^6 eggs. Chung et al. (2001) measured the fecundity of clams in Gomso Bay, Korea, by raising the water temperature to induce spawning and counting the number of eggs released; they found that the clams (shell length range 20.2–46.9 mm) in Gomso Bay released $2.01–17.9\times10^5$ eggs at the first spawning and $3.2–17.2\times10^5$ eggs at a second spawning, 15 days after the first.

Our estimate of the fecundity of clams is somewhat higher than previous estimates. We believe that the fecundity deduced from the ELISA data is the maximum fecundity, as an entire clam is homogenized and the total gonadal protein in the clam is measured. By

Table 6
Fecundity of marine bivalves reported from various studies

Species	Location	Shell length (mm)	Egg diameter (μm)	Estimation method	Fecundity	Author
Tapes rhomboides	Bay of St. Malo, France	35–45	35–45 (histology)	Stereology	$0.3–70.0\times10^4$, 50×10^4 (potential)	Morvan and Ansell (1988)
R. philippinarum	Tokyo Bay, Japan	34.1–36.0	63–70 (intact)	Induced spawning by ammonia	$0.24–1.35\times10^6$	Toba and Miyama (1991)
T. dorsatus	Sydney Harbor, Australia	–	67.7±3.6 (intact)	Induced spawning by serotonin	1.11×10^6	Nell et al. (1995)
R. philippinarum	Gomso Bay, Korea	20.2–46.9	55–62 (histology)	Induced spawning by exposing to air, feeding stimulus, and thermal shock	$0.20–1.79\times10^6$	Chung et al. (2001)
R. philippinarum	Gomso Bay, Korea	21.11–43.50	89.57±8.66 (intact) 61.19±5.66 (histology)	Immunological method (ELISA)	$0.94–11\times10^6$	Present study
C. virginica	Galveston Bay, USA	70–120	–	Immunological method (ELISA)	$3.7–65.4\times10^6$	Choi et al. (1993)
C. gigas	Goseong Bay, Korea	74–91.6	–	Immunological method (ELISA)	$4–196\times10^6$	Kang et al. (2003b)
Mercenaria mercenaria	–	–	60–85	–	30×10^6	Dietrich et al. (2002)
S. purpuratus	Geoje, Korea	86.94	88.56±11.31 (intact) 70.81±7.52 (histology)	Immunological method (ELISA)	$9–31\times10^6$	Park et al. (2003)

contrast, the fecundities reported in other studies typically estimate the number of eggs by direct counting after the treatment of ripe clams with various chemical or physical stimuli to induce spawning. As many studies have indicated, the spawning of marine bivalves is often partial and discontinuous (Choi et al., 1993; Chung et al., 2001; Kang et al., 2003a). Therefore, the fecundity of clams and other bivalves estimated from induced spawning must be a minimum estimate (Choi et al., 1993; Chung et al., 2001; Kang et al., 2003a).

We also calculated the differences in the GSI before and after spawning to estimate the probable number of eggs released during spawning. We estimated that Manila clams in Gomso Bay released eggs equivalent to 6.5%, 14.3%, and 12.8% of the total dry-tissue weight. Converting these quantities into numbers reveals that the clams may discharge 2.9, 2.3, and 2.7 million eggs, respectively; these numbers are similar to the numbers reported in other studies (Table 6). These values are comparable to the estimates for other veneriid clams. According to Honkoop et al. (1999), *Macoma balthica* releases eggs accounting for up to 38% of its total ash-free dry biomass during spawning. Park et al. (2003) reported that the reproductive effort of the butter clam *S. purpuratus* during the spawning season was approximately 17 million eggs, which is equivalent to 26.8% of its body weight.

4.4. Seasonal change in gonad development

Many studies have shown that water temperature is the environmental factor that regulates the gametogenic process of clams in the annual reproductive cycle (see the review of Ponurovsky and Yakovlev, 1992). Clams in warmer regions, such as Hawaii, Israel, and other tropical or sub-tropical areas, often spawn year-round or for a long period each year (Yap, 1977; Shpigel and Fridman, 1990). In contrast, the spawning period of clams in temperate areas is limited to late spring and summer (Kulikova, 1979, as cited in Ponurovsky and Yakovlev, 1992). According to Chung et al. (1994, 2001), the clams in Gomso and an adjacent bay initiate vitellogenesis in April to May and spawn from July to early October when the water temperature remains above 20 °C. The seasonal changes in gametogenesis we observed match the seasonal changes in water temperature; vitellogenesis begins between April and May, and clams spawn from early June to late August, with two major spawning pulses during this period, as observed previously (Chung et al., 2001; Table 5).

The cyclic change we observed in the gametogenesis of clams in Gomso Bay coincides with the seasonal variation in the abundance of food in the water column. The rapid increase in the chlorophyll *a* concentration from May to June was followed by the first spawning by clams in June, indicating that the abundance of food in the water column is crucial for reproduction, as well as for growth (Figs. 6 and 7). The third spawning pulse in late August (Fig. 7) also matched a chlorophyll *a* maximum during August and September in the bay (Fig. 6). Our data clearly indicate that the standing stock of phytoplankton in the bay controls the spawning pulses of clams, along with other physicochemical factors such as temperature and salinity. Soniat and Ray (1985) demonstrated that the gametogenesis of oysters in Galveston Bay, Texas, is synchronized with the seasonal fluctuation in food availability in the water column. Using a computer simulation, Hofmann et al. (1992) demonstrated that a change of a few degrees in water temperature or a small shift (2–4 weeks) in the timing of the spring and fall phytoplankton blooms significantly alters the

duration of spawning and the amount of gametes produced. A decline in the standing stock of phytoplankton in a coastal bay was also responsible for a long-term reduction in the oyster population (Powell et al., 1995).

5. Conclusion

In this study, we successfully quantified the reproductive output of the Manila clam *R. philippinarum* using ELISA. The immunological assay provided a variety of reproductive data on Manila clams in Gomso Bay, and this information will be used for clam management in the region. Our results show that an indirect ELISA using rabbit anti-clam egg serum as the primary antibody is a practical, affordable, and sufficiently sensitive assay, and allows the expeditious testing of a large number of samples to assess the reproductive output of *R. philippinarum* quantitatively.

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