Vol. 25(3):249-256 | Ocean and Polar Research | September 2003

Article

Quantification of Reproductive Output of the Butter Clam, *Saxidomus purpuratus* (Sowerby, 1852) Using Enzyme-Linked Immunosorbent Assay (ELISA)

Kyung-Il Park[1†], Jin-Woo Choi[2], and Kwang-Sik Choi[1*]

[1]*School of Applied Marine Science, Cheju National University*
1 Ara 1-Dong, Jeju 690-756, Korea
[2]*Environmental Science Laboratory, South Sea Institute, KORDI*
391 Jangmok, Geoje 656-830, Korea
[†]*Current address: Institute of Aquaculture, University of Santiago de Compostela, Spain*

Abstract : An immunological method was developed in this study to quantify reproductive output of the female butter clam, *Saxidomus purpuratus*. A clam egg-specific polyclonal antibody was developed using the purified butter clam egg as an antigen. An indirect Enzyme-Linked Immunosorbent Assay (ELISA) was used in quantitative measurement of the eggs. Size of the butter clam eggs ranged from 70.81 ± 7.52 μm in histology or 88.56 ± 11.31 μm in intact eggs. The predominant egg constituent was protein (37.44%), followed by lipids (11.40%) and carbohydrates (9.68%). The SDS-PAGE showed that the egg proteins are composed of several peptides of molecular weights consisting of 247, 200, 99, 91, 54, and 47 kDa. ELISA indicated that the clams collected from Geoje Island in May 2002 produced 8.2 to 26.8% of their body weight as eggs or 9,307,309 to 31,156,333 with a mean of 16,931,893 eggs per individual clam. The results of this study thus suggest that indirect ELISA using rabbit anti-clam egg IgG as a primary antibody is a rapid, affordable and sensitive method to assess reproductive output of *S. purpuratus* and possibly other bivalves using a small amount of eggs.

Key words : *Saxidomus purpuratus*, immuno-assay, reproduction, reproductive output, egg, fecundity, ELISA, Korea

1. Introduction

The butter clam, *Saxidomus purpuratus* Sowerby 1852, is a highly valued shellfish species distributed in a subtidal silty-sand substrate on the south and west coasts of Korea. *S. purpuratus* is commercially exploited in small bays on the southeast coast supporting local shellfish industries. Annual landings of the clam in Korea in 1999 reached approximately 9,000 metric tons (Kim *et al.* 2001). For the past few years, the clam landings have been declining and intensive clam fishing is considered one of the reasons for the current decline.

To protect wild shellfish populations from heavy fishing pressure, seeds of marine bivalves are often artificially produced from a hatchery and released into their habitat to increase the density (Loosanoff and Davis 1963; South Sea Fisheries Institute 2002). For the development of an artificial breeding program, an intensive study of reproduction for the target species such as the annual reproductive cycle or estimation of its fecundity is essential (Galsoff 1964; Pillay 1990; Massapina *et al.* 1999). In particular, knowledge of the quantity of gametes produced during spawning (i.e., reproductive output or fecundity) is critical to the hatchery operation as well as to understanding the life history of the target species (Choi *et al.* 1993; Powell *et al.* 1994; Hyun *et al.* 2001; MOMAF 2001). A few studies have been conducted on the reproductive biology of clams in Korea. However, most studies are limited to the qualitative aspect

*Corresponding author. E-mail : skchoi@cheju.ac.kr

of reproduction, such as annual or seasonal changes in gonadal development (Kim 1971; Chung and Kim 1994; Ahn 2001; Kim *et al.* 2001).

Reproductive output of marine bivalves, which normally do not exhibit discrete gonads, has been measured through numerous methods. The number of gametes or areas occupied by the gametes is often measured from histological slides (Tirado and Salas 1998; Ceballos-Vazquez *et al.* 2000). Reproductive output of marine bivalves is often estimated by measuring the change in body weight of specimens before and after spawning (Deslous-Paoli and Heral 1988; Tirado and Salas 1998). Gravid females are also induced to spawn by various spawning stimulants, and the number of eggs released from each individual is directly counted (Galinou-Mitsoudi and Sinis 1994; Chung *et al.* 2001). However, such quantitative methods described above may underestimate the true quantity of gonad in an individual bivalve, as spawning is incomplete in most cases; spawning occurs several times at different intensities during reproductive seasons (Lucas 1982; Ahn 2001; Kang *et al.* 2003).

An immunological technique has been utilized in quantification of reproductive output of marine bivalves. Choi *et al.* (1993, 1994) developed an egg-specific polyclonal antibody in the American oyster, *Crassostrea virginica* and the quantity of egg protein in an oyster was then assessed using the egg-specific antibody as a primary antibody in indirect ELISA. The technique was sensitive enough to measure reproductive output from a very small quantity of the egg protein remaining in some oysters collected even in winter (Choi *et al.* 1993). Recently, Kang *et al.* (2003) also have developed specific antibodies in eggs of the Pacific oyster, *C. gigas* to quantify egg mass using ELISA. Due to the rapidity and high sensitivity, immunological methods have been recognized as a suitable method for studying bivalve reproduction (Choi *et al.* 1993; Kang *et al.* 2003).

We have developed an immunological method for quantification of the eggs of butter clams. The present study examines the technique and some measurements of the female clam reproductive output assessed from a population collected during the spawning season.

2. Materials and methods

Purification of the eggs

Ripe female *S. purpuratus* were collected from Geoje Island on the south coast of Korea in May 2002 for harvesting eggs, the source of an antigen for antibody development. Visceral mass containing eggs were excised from the clam tissue and gently squeezed to release eggs on a petri-dish. Crude egg extracts were diluted in filtrated seawater containing 0.02 mM ammonium hydroxide for preventing aggregation of the eggs. Diluted egg extracts were then filtered through 100 μm and 63 μm mesh screens to remove impurities. Filtered eggs were washed several times by centrifuging the eggs at 100 x g for 10 minutes. The number of eggs in the filtrate were counted under a microscope using a hemocytometer. A known number of eggs were freeze-dried and weighed to determine egg weight.

SDS-PAGE analysis

Egg protein homogenates were precipitated with acetone, and the quantity was determined using BCA Protein Assay Kit (Pierce, USA). Egg protein was loaded on 10% SDS-polyacrylamide gel and electrophorised (SDS-PAGE) according to Laemmli (1970). For SDS-PAGE, the egg protein was either untreated (i.e., natural) or treated with 5% β-mercaptoethanol by boiling (i.e. reducing condition).

Biochemical composition of the eggs

Protein concentration of purified egg extracts was determined with a BCA Protein Assay Kit using bovine serum albumin as a standard. The total carbohydrates in eggs was determined according to Taylor (1995) using dextrose anhydrose as a standard. The total lipid content of the eggs was measured using the sulfuric acid-charring technique of Marsh and Weinstein (1966) employing tripalmitin as a standard after extracting lipids from the eggs according to Bligh and Dyer (1959).

Development of Rabbit Anti-Clam egg Antibodies

A New Zealand White rabbit was selected as a host animal to raise antibodies against *S. purpuratus* egg protein. Table 1 summarizes the protocol used in the production of

Table 1. Summery of 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: Freunds adjuvant complete
FAI: Freunds adjuvant incomplete

an antiserum. The rabbit anti-serum initially showed a weak but recognizable cross-reaction to the somatic tissues in a double immuno-diffusion test (Ouchterlony and Nilsson 1978). The cross-reacting antibodies in the serum were then removed in an affinity column prepared according to Fuchs and Sela (1979). Immuno-adsorbents packed in the column were prepared by cross-linking clam somatic tissue extracts with glutaraldehyde. To remove the cross-reacting antibodies, the antiserum was added onto the column packed with the immuno-adsorbent and incubated in room temperature for a few hours. Specificity of the antibodies was then tested again using an indirect enzymelinked immunosorbent assay (ELISA) using alkaline phosphatase-labeled goat anti-rabbit IgG as a secondary antibody. A rabbit anti-clam egg IgG was then purified from antiserum using ammonium sulfate precipitation.

Sampling efforts

A total of 50 *S. purpuratus* averaging 86.94 mm ± 3.77 mm in shell length (SL) and 48.129 ± 5.507 g in tissue wet weight (TWWT) were collected from Geoje Island on the south coast of Korea in May 2002 (Fig. 1). After recording SL and TWWT, 24 sexually mature clams were selected and freeze-dried to determine their dry weight (TDWT), the rest of the clams were used in histological analysis. Surface sea-water temperature and salinity in May in this area were 16.2°C and 30.98 psu, respectively.

Quantification of the clam eggs using ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was used in quantification of the clam eggs. The rabbit anti-clam egg IgG developed in this study was used as a primary antibody and the alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) as a secondary antibody in ELISA. For quantification of the eggs, triplicates of 100 μl of each clam tissue extract dissolved in 0.15 M PBS (pH 7.5) and duplicates of the standard solution (i.e., *S. purpuratus* egg extract of 0.156 μg to 5 μg/ml in 0.15 M PBS, pH 7.5) were included in a 96-well ELISA microplate. Optical density (OD) of the antibody-antigen complex developed in ELISA was read at 405 nm using a micro-plate spectrophotometer. A standard regression curve was plotted based on the OD of the standard and their protein concentration. The concentration of egg protein in each clam was then estimated from the curve. Finally, the weight-based gonado -somatic index (GSI) and fecundity (i.e., number of eggs in a clam) were estimated from the following relationship:

1) GSI = quantity of egg protein estimated from ELISA × 2.67/TDWT,

where the constant 2.67 is a conversion factor of egg protein to egg dry weight.

2) Fecundity = quantity of eggs estimated from the ELISA /weight of single egg.

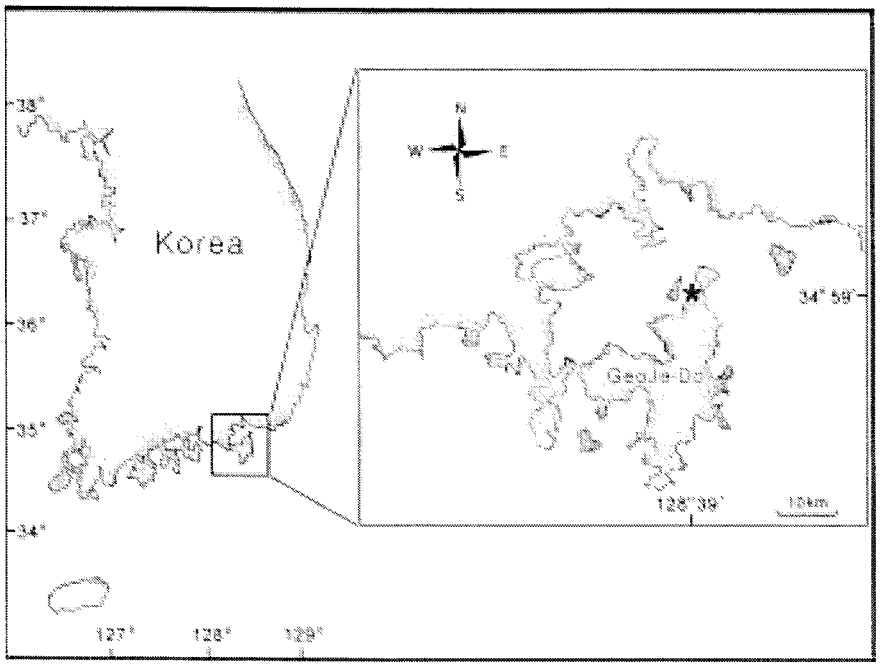

Fig. 1. Location of the studied area (*), Geoje-Do Island, Korea.

Histological analysis of gonadal tissues

Cross-sections cut in the middle of clam bodies containing gonads were fixed in Davidson's solution. After dehydration and clearing, each section was embedded in paraffin and cut to 6 μm. The sections were stained with Harry's hematoxylin and eosin Y, and gonadal tissues were examined under a light microscope.

3. Results and discussion

Biometric and biochemical characteristics of *S. purpuratus* eggs

Table 2 shows size, weight and biochemical composition of *S. purpuratus* eggs in comparison with other marine bivalve eggs. The mean egg diameter of *S. purpuratus* was 70.81 ± 7.52 μm in histology or 88.56 ± 11.31 μm in an intact condition during suspension. The dry weight of individual eggs was estimated at 95 ng. The size and weight of the *S. purpuratus* egg estimated in this study is somewhat larger and heavier than that of other marine bivalves. Dry weight of oyster eggs ranged from 12 to 13 ng (Choi *et al.* 1993; Kang *et al.* 2003). Park and Choi (in preparation) report the dry weight of *Ruditapes philippinarum* egg as being 22 ng. Compared to oyster and other clam eggs, the *S. purpuratus* egg is approximately 4 to 8 times heavier. Lee and Heffernan (1991) reported the dry weight of *Mercenaria mercenaria*, a clam species taxonomically close to *S. purpuratus*, as being 51 ng. Our data suggests that hard clams such as *S. purpuratus* and *M. mercenaria* produce relatively large and heavy eggs, which might sink easily when discharged in the water column during spawning.

Table 2 also shows that protein accounts for 37.44% of *S. purpuratus* eggs, followed by lipids, 11.40% and carbohydrates at 10.83%. Proximate compositions of *S. purpuratus* eggs is similar to those of other marine bivalves; a major constituent of oyster and clam eggs is protein, which accounts for 38 to 50% of egg weight, followed by lipids and carbohydrates.

SDS-PAGE of *S. purpuratus* egg protein

Fig. 2 shows the electrophoretogram of *S. purpuratus* egg proteins. In a natural condition (i.e., without β-mercaptoethanol treatment), the egg protein expressed two major bands of molecular mass of 163 and 95 kDa. In a reduced condition treated with β-mercaptoethanol, the egg protein consisted of six bands with molecular masses of 247, 200, 99, 91, 54, and 47 kDa, respectively. High molecular peptides observed in the clam eggs are believed to be vitellin, an egg-specific protein that plays an important function during development (Shafir *et al.* 1992). High-

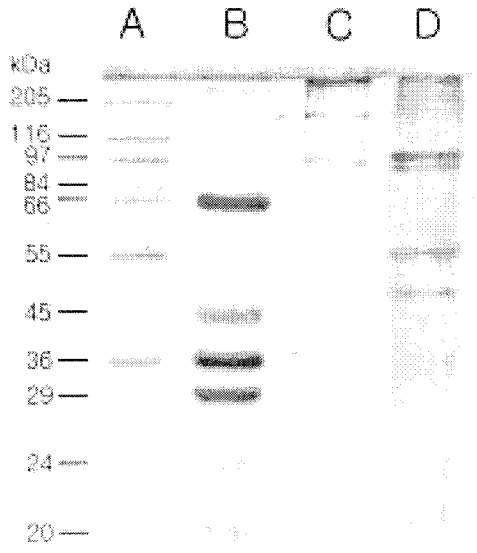

Fig. 2. 10% SDS-PAGE of *S. purpuratus* eggs. Eggs were purified and precipitated with 100% acetone then loaded onto wells. Lanes A and B: molecular weight standards. C: non-denaturing condition, D: denaturing condition.

Table 2. Biochemical composition of marine bivalve eggs.

Species	Egg dry-weight in ng	Percentage of Protein (%)	Percentage Lipids (%)	Percentage Carbohydrate (%)	Reference
Crassostrea virginica	12	50	21	9	Lee & Heffernan (1991)
Mercenaria mercenaria	51	40	14	8	Lee & Heffernan (1991)
Crassostrea gigas (Portuguese strain)	-	44-74	16-38	7-12	Massapina *et al.* (1999)
Crassostrea gigas	13	41	3.3 (25.5)	11.7	Kang *et al.* (2003)
Ruditapes philippinarum	22	41	-	-	Park & Choi (in preparation)
Saxidomus purpuratus	95	37.44	11.40	10.83	Present study

molecular egg-specific peptides are also observed in other marine bivalves. Lee and Heffernan (1991) reported that egg proteins of *M. mercenaria* are composed of several peptides of 98, 87, 68, 60, 56, 36 and 19 kDa. Park and Choi (in preparation) reported that egg proteins of *R. philippinarum* also consist of several peptides with a molecular mass of 330, 96, 64, 50 and 31 kDa.

Specificity of Antibody

The rabbit anti-clam egg IgG initially exhibited a minor but recognizable cross-reaction to non-gonadal somatic proteins in double immunodiffusion (Fig. 3). The antiserum and proteins extracted from somatic tissue formed weak precipitin lines in double-immunodiffusion, indicating that the antiserum contains non-specific antibodies (Fig. 3B). The cross-reacting antibodies were removed in the immuno-affinity column developed in this study. No further cross-reaction was detected in ELISA (Fig. 4) and in double immunodiffusion (Fig. 3C) after the immuno-adsorption. Kang *et al.* (2003) and Park and Choi (in preparation) also observed weak but noticeable cross-reactions of rabbit antisera developed from oyster and Manila clam eggs to non-gonadal protein extracted from gills and mantles. Such cross-reacting antibodies were successfully removed using immuno-adsorbents prepared with somatic tissues. The antigenic determinants of cross-reacting antibodies observed in this study may be peptides of smaller molecular sizes commonly occurring in both somatic tissues and eggs, which have also been observed in oyster eggs (Choi *et al.* 1993; Kang *et al.* 2003).

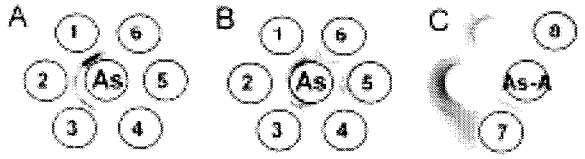

Fig. 3. Double immunodiffusion test used for confirmation of antibody titer and antibody specificity. A: Interaction between antiserum and twofold diluted egg extract, B: Interaction between antiserum and twofold diluted somatic tissue extract. C: Interaction between antiserum after immunoadsorption and egg and somatic tissue extracts. As: antiserum against *S. purpuratus* egg, As-A: antiserum after immunoadsorption, 1-6: 1/2-1/64 diluted, 7: egg extract, 8: somatic tissue extract. Note that A shows strong immunoreaction to egg, B shows a faint precipitant line between antiserum and somatic tissue, and C shows the specific interaction to egg extract.

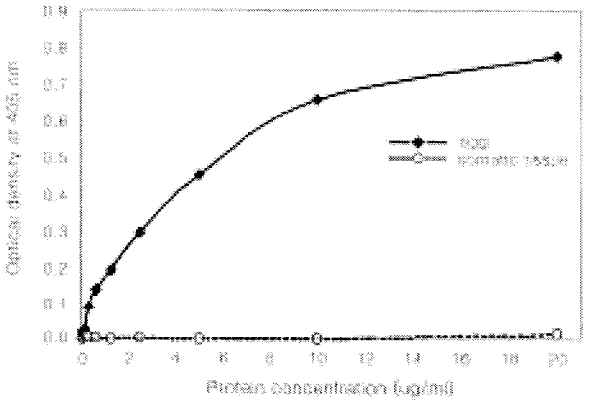

Fig. 4. Specificity of antibody against *S. purpuratus* egg protein.

Reproductive output of clams estimated from ELISA

The rabbit anti-clam egg IgG detects as little as 0.078 μg/m*l* of *S. purpuratus* egg protein in ELISA. Previous studies on the measurement of reproductive output of marine bivalves using ELISA reported similar detecting ranges of target protein concentrations; 0.2 to 10 μg/m*l* for the eastern oyster *C. virginica* (Choi *et al.* 1993), 0.01 to 0.3 μg/m*l* for the Pacific oyster *C. gigas* (Kang *et al.* 2003), and 0.23 to 15 μg/m*l* for the Manila clam, *R. philippinarum*, egg protein (Park and Choi, in preparation).

Table 3 shows reproductive output of female *S. purpuratus* measured by ELISA. Of the 24 clams collected in May, 13 clams showed strong positive reactions to the clam egg specific antibodies in ELISA, indicating that they are females. The weight of the total eggs found in each clam (dry weight) ranged from 0.884 to 2.362 g, with a mean value of 1.609 g. GSI, a ratio of egg weight to the total tissue weight varied from 0.082 to 0.268, with a mean of 0.154 ± 0.060. The fecundity of *S. purpuratus* collected from Geoje Island in May 2002 varied from 9,307,309 to 31,156,333 eggs per clam with a mean of 16,931,893. Microscopic examination on gonadal tissues of clams indicated that both female and male clams analyzed in this study were ripe and ready for spawning when they were collected (Fig. 5).

A number of studies conducted on the annual reproductive cycle of clams on the south coast of Korea indicated that *S. purpuratus* carries ripe eggs or sperm from March to November. However, the previous studies suggested that the main spawning of clams on the south coast occurs between June and July when surface water temperature reaches 22 to 25°C (Ahn 2001; Kim *et al.* 2001; South Sea Fisheries Institute 2002). Histological

Table 3. Size, GSI and fecundity of *S. purpuratus* collected from Geoje Island, Korea in May 2002. SL, Shell Length, TTWWT, Total Tissue Wet Weight, TDWT, Total Tissue Dry Weight, EDWT, Egg Dry Weight, GSI, Gonado-Somatic Index.

N	SL (mm)	TTWWT (g)	TTDWT (g)	EDWT (g)	GSI (EDWT/TTDWT)	Fecundity
1	92.1	54.791	12.297	1.703	0.138	17,923,639
2	86.7	47.254	10.733	-	-	-
3	88.7	59.006	13.824	-	-	-
4	82.7	40.943	9.523	1.308	0.137	13,765,220
5	88.3	53.213	12.213	-	-	-
6	87.6	48.211	11.025	2.960	0.268	31,156,333
7	88.3	49.597	11.047	-	-	-
8	89.8	44.919	10.264	-	-	-
9	87.2	43.239	9.211	2.362	0.256	24,864,841
10	92.2	60.870	13.128	1.513	0.115	15,927,443
11	81.5	39.330	8.857	-	-	-
12	85.4	48.910	11.362	-	-	-
13	91.5	56.371	13.286	-	-	-
14	87.6	49.363	10.932	-	-	-
15	94.4	46.575	10.429	1.478	0.142	15,560,395
16	77.6	37.194	8.423	-	-	-
17	81.6	43.788	10.488	-	-	-
18	91.7	43.940	10.560	1.865	0.177	19,629,799
19	90.5	43.497	9.429	0.886	0.094	9,327,884
20	84.8	49.196	10.741	0.884	0.082	9,307,309
21	90.5	45.974	9.595	1.788	0.186	18,818,360
22	84.8	46.909	9.869	1.790	0.181	18,840,573
23	83.3	54.222	11.535	0.885	0.077	9,313,589
24	92.2	50.393	10.502	1.490	0.142	15,679,228
Mean	87.5	48.238	10.803	1.609	0.154	16,931,893
STD	4.2	5.957	1.390	0.594	0.060	6,253,074

(STD = standard deviation)

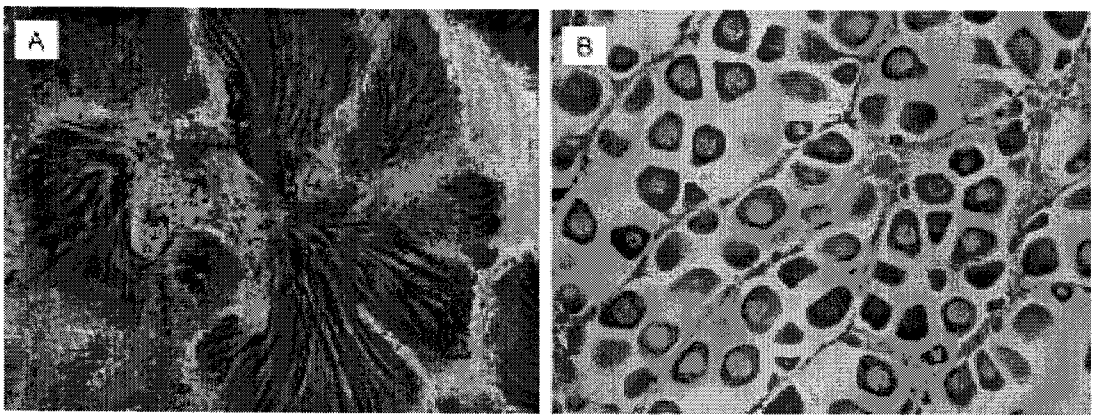

Fig. 5. Microscopic appearance of the male (A) and female (B) gonad of butter clam, *Saxidomus purpuratus*, collected in May 2002.

Table 4. Reproductive output of marine bivalves reported from various studies.

Species	Location	Shell length (mm)	Egg diameter (μm)	Methods	Highest monthly mean GSI	Fecundity	Author
Ruditapes philippinarum	Gomso Bay, Korea	21.11-43.50	89.57±8.66 μm (suspended) 61.19±5.66 (histology)	Immunological method (ELISA)	0.250	0.94-11×10^6	Park and Choi (in preparation)
Crassostrea virginica	Galveston Bay, USA	70-120	-	Immunological method (ELISA)	0.201	3.7-65.4×10^6	Choi *et al.* (1993)
Crassostrea gigas	Goseong Bay, Korea	74-91.6	-	Immunological method (ELISA)	0.423	4-196×10^6	Kang *et al.* (2003)
Saxidomus purpuratus	Taean, Korea	-	-	Direct egg counting after chopping off clam body	-	20×10^6	Dr. Choi (personal communication)
Mercenaria mercenaria	-	-	60-85 μm	-	-	30×10^6	Dietrich *et al.* (2002)
Saxidomus purpuratus	Geoje, Korea	86.94	88.56±11.31 μm (suspended) 70.81±7.52 (histology)	Immunological method (ELISA)	0.154	9-31×10^6	Present study

slides of the clams analyzed in this study indicated that none of the clams were in a spawning or spent stage in May. It is believed that the *S. purpuratus* of Geoje Island may spawn in June or July and the clams we used in ELISA were fully ripe and at a stage just prior to spawning, as observed in other studies (Kim 1971; Chung and Kim 1994; Ahn 2001). Therefore, the fecundity of the clams estimated in this study using ELISA must be a maximal value as normally clams spawn several times in a spawning period (Davis and Chanley 1956; Peterson 1983; Chung and Kim 1994; Ahn 2001).

Few studies have reported on the fecundity of clams measured by various methods. Dietrich *et al.* (2002) reported that the hard clam *M. mercenaria* releases approximately 30,000,000 eggs during the spawning season. The number of eggs in a ripe *S. purpuratus* gonad was estimated by excising the gonad and squeezing out eggs into a petri-dish and counting the number of eggs (Choi, Y.-S. of NFRDA, pers. comm.). According to Choi, a sexually mature *S. purpuratus* distributed on the west coast of Korea produces as many as 20,000,000 eggs during a spawning season (Table 4). The fecundity of the clam observed by Choi is somewhat comparable to the fecundity of clams estimated by ELISA in this study, which varied from 9,307,309 to 31,156,333 eggs per clam with a mean of 16,931,893.

In conclusion, the rabbit anti-clam egg IgG was successfully applied in the quantification of *S. purpuratus* eggs using ELISA; it was a rapid and affordable method for direct quantification of the clam eggs. Clams collected from Geoje Island in May 2002 accumulated 15.4% of their body as eggs or 16.9 million eggs per clam.

Acknowledgement

We are grateful to Dr. Yong-Suk Choi at NFRDI for his technical advice on the purification of clam eggs. We also wish to thank the staff of the Shellfish Research Laboratory at Cheju National University for their help in data acquisition. This study was supported by the Korea Research Council of Public Science and Technology (KORP) under the title, "Eco-environmental studies for the restocking and enhancement of bivalve resources on the south coast of Korea" (BSPG 337-00-1447-3).

References

Ahn, S.-H. 2001. Annual reproductive cycle of *Saxidomus purpuratus* (Sowerby, 1852) (Bivalvia: Veneridae) in Sacheon Bay, Korea. M.S. Thesis, Pukyung Nat'l Univ., Busan, Korea. (in Korean)

Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37, 911-917.

Ceballos-Vazquez, B.P., M. Arellano-Martinez, F. Garcia-Dominguez, and M. Villalejo-Fuerte. 2000. Reproductive cycle of the rugose pen shell, *Pinna rugosa* Sowerby,

1835 (Mollusca: bivalvia) from Bahia Concepcion, Gulf of California and its relation to temperature and photoperiod. *J. Shellfish Res.*, 19, 95-99.

Choi, K.-S., D.H. Lewis, E.N. Powell, and S.M. Ray. 1993. Quantitative measurement of reproductive output in the American oyster, *Crassostrea virginica* (Gmelin), using an enzyme-linked immunosrobent assay (ELISA). *Aquacult. Fish. Manage.*, 24, 375-398.

Choi, K.-S., E.N. Powell, D.H. Lewis, and S.M. Ray. 1994. Instantaneous reproductive effort in female American oysters, *Crassostrea virginica*, measured by a new immunoprecipitation assay. *Biol. Bull.*, 186, 41-61.

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

Chung, E.-Y. and B.-S. Kim. 1994. Histological and ultrastructural studies on gonadal development and germ cell development of the purplish Washington clam, *Saxidomus purpuratus* (Sowerby). *Bull. Coastal Res., Kunsan Nat'l Univ.* 6, 1-15.

Davis, H.C. and P.E. Chanley. 1956. Spawning and egg production of oysters and clams. *Biol. Bull.*, 110, 117-128.

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

Dietrich, A.M., T.M.C. LaBreche, and N. Shepherd. 2002. A flow-through chamber for toxicity testing of 80-200 μm organisms. *Water Res.*, 36, 2002-2010.

Fuchs, S. and M. Sela. 1979. Immunoadsorbents. p. 10.1-10.6. In: *Handbook of Experimental Immunology. Vol. 1, Immunochenistry*, ed. by D.M. Weir. Blackwell, Philadelphia.

Galinou-Mitsoudi, S. and A.I. Sinis. 1994. Reproductive cycle and fecundity of the Date mussel *Lithophaga lithophaga* (Bivalvia: Mytilidae). *J. Moll. Stud.*, 60, 371-385.

Galsoff, P.S. 1964. The American oyster *Crassostrea virginica* Gmelin. *US Fish and Wildlife Serv. Fisher. Bull.*, 64, 1-480.

Hyun, K.-H., I.-C. Pang, J.M. Klinck, K.-S. Choi, J.-B. Lee, E.N. Powell, E.E. Hofmann, and E.A. Bochenek. 2001. The effect of food composition on Pacific oyster *Crassostrea gigas* (Thunberg) growth in Korea: a modeling study. *Aquaculture*, 199, 41-62.

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

Kim, A.-Y. 1971. Ecological studies on the propagation of *Saxidomus purpuratus* (Sowerby). *Bull. Kor. Fish. Soc.*, 4, 92-98. (in Korean)

Kim, S.K., K.Y. Park, G.N. Jang, D.J. Kim, and H.C. Seo. 2001. Studies on the ecological aspect and gametogenesis of *Saxidomus purpuratus* (Sowerby) in Yellow Sea area. *Bull. NFRDI*, 59, 152-158. (in Korean)

Laemmli, V.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Lee, R.F. and P.B. Heffernan. 1991. Lipids and proteins in eggs of eastern oysters (*Crassostrea virginica* (Gmelin, 1791)) and northern quahogs (*Mercenaria mercenaria* (Linnaeus, 1758)). *J. Shellfish Res.*, 10, 203-206.

Loosanoff, V.L. and H.C. Davis. 1963. Rearing of bivalve mollusks. *Advances in Mar. Biol.*, 1, 1-136.

Lucas, A. 1982. Evaluation of reproductive effort in bivalve molluscs. *Malacologia*, 22, 183-187.

Marsh, J.B. and D.B. Weinstein. 1966. Simple charring method for determination of lipids. *J. Lipid Res.*, 7, 574-576.

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

MOMAF. 2001. Development of optimal technology for sustaining production in shellfish farm. (Final report) (in Korean)

Ouchterlony, Ö. and L.Å. Nilsson. 1978. Immunodiffusion and immunoelectrophoresis. p. 19.1-19.44. In: *Handbook of Experimental Immunology. Vol. 1*, ed. by D.M. Weir. Blackwell, Oxford, England.

Peterson, C.H. 1983. A concept of quantitative reproductive senility: application to the hard clam, *Mercenaria mercenaria* (L.). *Oecologia*, 58, 164-168.

Pillay, T.V.R. 1990. *Aquaculture principles and practices*. Cambridge Univ. Press, UK. 575 p.

Park, K.-I. and K.-S. Choi. (in preparation) Study on the reproduction of the Manila clam *Ruditapes philippinarum*. I. Quantification of the reproductive output using Enzyme Linked-Immunosorbent Assay (ELISA).

Powell, E.N., J.M. Klink, E.E. Hofmann, and S.M. Ray. 1994. Modeling oyster populations. IV: rates of mortality, population crashes, and management. *Fish. Bull.*, 92, 347-373.

Shafir, S., M. Ovadia, and M. Tom. 1992. *In vivo* incorporation of labeled methionine into proteins, vitellogenin, and vitellin in females of the shrimp *Penaeus semisulcatus* de Haan. *Biol. Bull.*, 183, 242-247.

South Sea Fisheries Institute, NFRDI. 2002. Fisheries resources. Available from WWW: <http://ssfri.nfrdi.re.kr/dt_resource.php> [cited 2003-04-30].

Taylor, K.A. 1995. A modification of the phenol sulfuric acid method and total sugar determination. *Appl. Biochem. & Biotechnol.*, 53, 207-214.

Tirado, C. and C. Salas. 1998. Reproduction and fecundity of *Donax trunculus* L., 1758 (Bivalvia: Donacidae) in the littoral of Malaga (Southern Spain). *J. Shellfish Res.*, 17, 169-176.

Received Jul. 25, 2003
Accepted Sep. 15, 2003