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Annual reproductive cycle and reproductive efforts of the Manila clam *Ruditapes philippinarum* in Incheon Bay off the west coast of Korea using a histology-ELISA combined assay

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ABSTRACT

We investigated the reproductive effort of the Manila clam *Ruditapes philippinarum* during different gametogenic stages with combining histology and immune assay techniques. To determine the level of gonad maturation, 1- to 2-mm-thick slices of the dorso-ventral section were cut from the middle of clams for histology. The quantity of eggs in each clam was determined from the remaining tissue using rabbit anti-clam egg protein IgG in an indirect enzyme-linked immunosorbent assay (ELISA). At the Begmiri tidal flat in Incheon Bay off the west coast of Korea, clams commenced gametogenesis in February and the first spawning female was observed in July. Clams continued to spawn into October (18.5 °C). The monthly mean gonad-somatic index (GSI), the ratio of egg mass to body weight, ranged from 4.27 (April) to 20.63 (July). The GSI increased rapidly from April (4.3) to May (16.8), peaked in July (20.6), then dropped dramatically from August (14.0) to September (5.5), indicating that clams at the Begmiri tidal flat have a major spawning pulse during August and September. The histology–ELISA combined technique enabled us to assess gametogenic stepwise reproductive efforts of clams because we could simultaneously determine the reproductive stage and quantity of eggs. The GSI of mature females ranged from 20.9 (May) to 26.6 (July), while that of partially spawned clams ranged from 12.8 (June) to 7.2 (September), suggesting that clams discharge as much as 50% of their eggs during the major spawning pulse. Histology revealed that residual eggs in spent clams were resorbed, and that clams may restore approximately 4.6% of their body weight by this energy-recycling process. The histology–ELISA combined technique provided both quantitative and qualitative information about clam reproduction, which is crucial for clam fisheries and aquaculture.

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1. Introduction

Many studies have investigated the annual gametogenesis of marine bivalves to clarify the timing and duration of spawning and the quantity of gametes released during spawning; these data are crucial to manage natural populations and hatchery operations (Drummond et al., 2006; Gosling, 2003; Park and Choi, 2004; Toba and Miyama, 1991, 1994; Yang et al., 2011). To this end, histology has been used extensively, providing qualitative data about reproduction such as developmental conditions and the sizes of gametes (Heffernan et al., 1989; Kang et al., 2007, 2009; Limpanont et al., 2011; Park et al., 2008; Uddin et al., 2007, 2010). Fewer studies have investigated quantitative aspects of bivalve reproduction, because these are technically difficult to measure. In marine bivalves, with the exception of scallops, the gonads are an integral part of the visceral mass and usually cannot be separated from the body (Beninger and Lucas, 1984; Choi et al., 1993; Lucas, 1982; Thompson et al., 1996).

Several techniques have been used to measure the quantity of gametes in marine bivalves, such as measuring the difference in weight immediately prior to and after spawning (Deslous-Paoli and Heral, 1988; Kautsky, 1982; Pouvreau et al., 2000), and counting or weighing the number of gametes released after inducing spawning via chemical and/or physical stimulation (Chung et al., 2001; Massapina et al., 1999; Toba and Miyama, 1991, 1994). Stereological methods coupling histology and image analysis have also been widely used to quantify bivalve reproduction by enumerating gametogenic products (i.e., oocytes) by staging a defined number of oocytes (Hadfield and Anderson, 1988; Robinson and Breese, 1982) or by estimating the total number of oocytes per individual (Brousseau, 1978),

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the percentage of the lumen filled with oocytes (planimetry; Dinamani, 1987; Lango-Reynoso et al., 2000; Perdue and Erickson, 1984), or the size/frequency of oocytes (Grant and Tyler, 1983; Heffernan et al., 1989; Kanti et al., 1993; Keck et al., 1975). However, such techniques can only yield semi-quantitative estimations and often underestimate the actual quantity of gamates (Lucas, 1982; Thompson et al., 1996). Alternatively, immunological methods have been used successfully to quantify egg proteins in marine bivalves; these methods are fast, inexpensive, and highly sensitive (Choi et al., 1993, 1994; Kang et al., 2003; Park and Choi, 2004; Park et al., 2005). To estimate the quantity of eggs in an individual clam, Park and Choi (2004) developed a polyclonal antibody using purified Manila clam (*Ruditapes philippinarum*) egg proteins as an antigen. This was subsequently utilized as the primary antibody in an indirect enzyme-linked immunosorbent assay (ELISA). They lyophilized and homogenized the whole body of the clam, then extracted a 10–20 mg sample and used ELISA to determine the amount of egg protein in it, based on the known amount of egg protein included in the assay as standard material. This method was fast and sufficiently sensitive to detect minute quantities of egg protein, even in early stages of development (Park and Choi, 2004; Park et al., 2005).

Endemic to the Yellow Sea, the Manila clam *R. philippinarum* is common on tidal flats and sand beaches on Korea's west and south coasts (Park and Choi, 2001, 2004). Manila clams on the sandy mud tidal flats in Incheon Bay have been particularly extensively exploited: these areas have been licensed to local villagers and clam fishery cooperatives. Although Manila clams have been harvested from the bay for the past few decades, few studies have investigated their reproductive biology (Uddin et al., 2010). In an attempt to clarify their reproductive physiology, we investigated annual gametogenic patterns and reproductive efforts of Manila clams from a tidal flat in Incheon Bay.

2. Materials and methods

2.1. Sampling

The Begmiri tidal flat is located at the southern tip of Incheon Bay, off the west coast of Korea. This area has a high density of Manila clams, which have been extensively harvested for decades by a local clam cooperative (Fig. 1). Analyses were based on clams collected each month over a 12-month period (January–December 2007). Surface water temperature and salinity were recorded in situ during sampling. The chlorophyll-*a* level in the water, an indicator of phytoplankton biomass, was also determined monthly using the spectrophotometric method set out by Strickland and Parsons (1972).

Upon arrival at the laboratory, shell length (SL) was measured; only clams that were at least 2 years old and had an SL of 27 mm or longer were included in the analysis; 40 clams were selected for testing each month. After the soft tissue was weighed, a thin slice (1.5 mm) was cut dorso-ventrally in the middle of the body for histology. The remaining tissue was weighed, freeze-dried and stored at −70 °C. The shells were dried at 50 °C and weighed to obtain the condition index (CI), a ratio of tissue dry weight to shell dry weight (Fig. 2).

2.2. Histology

The dorso-ventral sections were fixed in Davidson's fixative, dehydrated in a graded series of alcohol, and embedded in paraffin. The paraffin blocks were sliced to 6 μm and stained with Harris's haematoxylin and counter-stained with eosin Y. After mounting, the sample's gametogenic condition was categorized based on microscopic examination. The reproductive maturity of the gonads was categorized into six stages using the maturity scale described by Drummond et al. (2006): 1) early developing, 2) late developing, 3) ripe, 4) partially spawned, 5) spent-resorbing, and 6) resting (i.e., sex not distinguishable). Analyses of annual gametogenesis included only samples from female clams and clams in the resting stage (i.e., unidentified sex), or a total of 20–25 clams.

2.3. Quantification of reproductive effort

Egg mass was assessed using the clam egg protein-specific rabbit antibody and quantification protocol developed by Park and Choi (2004). Based on histology, only individual clams with oocytes in the follicles were included in this analysis. For the assay, the lyophilized clam tissue was homogenized using a mortar and pestle. A 20 mg subsample of clam tissue was taken from each clam and dissolved in phosphate-buffered saline (PBS, pH 7.4). The tissue homogenate in PBS was further homogenized using an ultrasonifier. The homogenate was diluted 500– to 2000-fold for ELISA analysis (Park and Choi, 2004). A 100 μl aliquot of the diluted homogenate and control solutions (0.1–5 μg purified and homogenized clam eggs as a positive control and PBS as a negative control) were loaded into a 96-well microplate. Goat anti-rabbit IgG alkaline phosphatase-conjugate (1:1000, Sigma) was used as the secondary antibody along with ρ-nitrophenylphosphate (ρ-NPP) substrate. A standard regression curve was constructed from the optical density of the known quantity of standard material included in the plate (i.e., purified clam egg). The concentration of egg protein in the tissue homogenate was estimated from the regression curve and the dilution factor. The quantity of eggs was then estimated by multiplying the quantity of the egg protein measured by ELISA by 2.44, the ratio of egg protein to total egg weight (Park and Choi, 2004). Finally, reproductive effort was expressed as the gonad-somatic index (GSI), a ratio of the estimated total dry weight of the eggs to the total dry weight of the clam tissues in the form of a percentage.

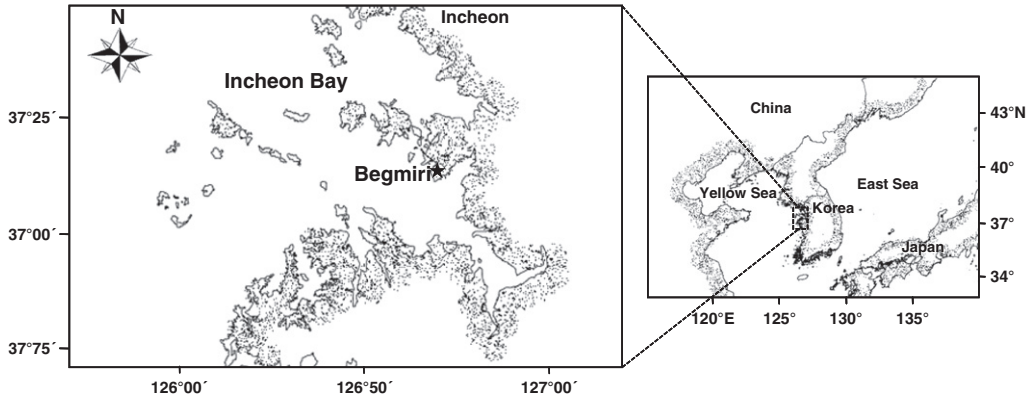

Fig. 1. Map showing the sampling site (▲).

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