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Application of enzyme-linked immunosorbent assay (ELISA) for the study of reproduction in the Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia): II. Impacts of *Perkinsus olseni* on clam reproduction

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Abstract

We investigated the effects of infection by the protozoan *Perkinsus olseni* on the reproduction of female Manila clams, *Ruditapes philippinarum*, from a population in Gomso Bay, Korea. The reproductive effort of the clams was assessed by ELISA using a clam egg-specific antibody and was expressed as a weight-based gonadosomatic index (GSI). The number of *Perkinsus* infecting each clam was estimated from the gills using Ray's fluid thioglycollate medium (RFTM) along with a NaOH digestion assay. We found that reproductive effort was negatively correlated with the intensity of the *Perkinsus* infection: more heavily infected clams produced fewer eggs during the spawning period from May to August. Frequency of spawning was also negatively correlated with the level of *Perkinsus* infection; heavily infected clams (HIC) exhibited a single spawning pulse in late July, whereas lightly infected clams (LIC) showed three spawning peaks in mid-May, late July, and late August. Egg production of HIC was only 30–75% of LIC during spawning. The level of total protein in LIC was also higher year round than in HIC. In conclusion, our investigation demonstrates that a high level of *Perkinsus* infection affects spawning frequency and reduces egg production, which may have long-term impacts on clam recruitment and population growth.

Keywords: Reproductive output; *Ruditapes philippinarum*; *Perkinsus olseni*; ELISA; Gametogenesis; Korea

1. Introduction

Protozoan parasites of the genus *Perkinsus* have caused mass mortalities of several commercially important shellfish, including oysters, clams, and abalone. High levels of *Perkinsus* infection often result in slow growth, poor condition, and retarded reproduc-

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tion in host animals (see review by Ford and Tripp, 1996). In eastern Asia, *Perkinsus* sp. has been identified in the Manila clam, *Ruditapes philippinarum*, which is very common in Korea (Choi and Park, 1997; Lee et al., 2001; Park and Choi, 2001; Elston et al., 2004), Japan (Hamaguchi et al., 1998; Maeno et al., 1999), and China (Liang et al., 2001). Leethochavalit et al. (2003) reported incidences of *Perkinsus* infection in the undulate clam, *Paphia undulate*, in Thai Bay, Thailand. Park et al. (in press) recently showed that sequences of the non-transcribed spacer (NTS), internal spacer 1, 2 (ITS 1, 2), and 5.8S rRNA of *Perkinsus* sp. in Korean waters are identical to those of *Perkinsus atlanticus*, and given that *Perkinsus olseni* has taxonomic priority (Murrell et al., 2002), the Korean *Perkinsus* sp. is identified as *P. olseni*.

A high level of parasitism can interfere with host reproduction through a number of processes, including sterilization (Bollache et al., 2002), manipulation of behavior (Klein, 2003), reduced reproductive effort, and reduced mating success (Córdoba-Aguilar et al., 2003). Oysters heavily infected with *Perkinsus marinus*, which is responsible for mass mortality among the American oyster *Crassostrea virginica*, also showed slow growth and retarded gonad maturation (Mackin and Ray, 1954; Choi et al., 1993, 1994; Dittman, 1993; Dittman et al., 2001). Reduced reproductive output or slow gonad maturation observed in oysters heavily infected with *P. marinus* can be explained in part by insufficient net energy dedicated to reproduction, resulting from the continuous drain on net production by *P. marinus* (Choi et al., 1989, 1994).

Immunological assays have proven to be a method of choice for the quantification of reproductive effort in marine bivalves, which do not exhibit discrete gonads in most cases. Choi et al. (1993) first developed egg-specific antibodies and used them in quantifying the eggs of *C. virginica* with an enzyme-linked immunosorbent assay (ELISA). Kang et al. (2003) also reported the development and application of egg-specific antibodies from the Pacific oyster *Crassostrea gigas* for quantification purposes. In our previous study (Park and Choi, 2004), we developed an *R. philippinarum* egg-specific antibody and successfully measured the reproductive output of individual clams using ELISA. Quantification of clam eggs using ELISA was sufficiently sensitive to measure even the small quantity of egg protein present in clams during the non-reproductive season.

In this study, we investigated the effects of *P. olseni* infection on the reproductive output of Manila clams. We re-evaluated the ELISA assessment of reproductive output of the clams in the previous study (Park and Choi, 2004), with respect to the infection intensity in each clam.

2. Materials and methods

2.1. Sampling

Manila clams were collected between March 1999 and February 2000 from Gomso Bay on the western coast of Korea (Fig. 1). Gomso Bay is one of the largest commercial clam beds in Korea, producing approximately one-tenth of the annual clam landings in Korea. Clams were collected bi-weekly during the spawning season from July to August and monthly during the non-spawning period. Shell length and tissue wet weight of each clam used in the analysis were recorded. Condition of each clam was assessed by a condition index (CI), where CI = tissue dry weight (g)/shell length $(\mathrm{mm})^3 \times 10{,}000$. Fig. 2 shows a flowchart of the experimental protocol used in this study.

2.2. Prevalence and infection intensity of Perkinsus

The infection intensity of *Perkinsus* in each clam was estimated from the gills. Gill tissues of individual clams used in the measurement of egg production were excised and incubated in FTM fortified with antibiotics (Choi et al., 2002). After incubation in the dark at room temperature for one week, the gill tissues were digested with 2 M NaOH based on the protocol used by Choi et al. (1989), and the number of *Perkinsus* hypnospores was counted using a hemocytometer. *Perkinsus* infection intensity–represented by the total number of *Perkinsus* cells in an individual clam–was then estimated using the empirical equation $Y = 2.0417\,X + 186{,}572$, where Y is the total number of *Perkinsus* in a clam and X is the number of *Perkinsus* cells counted in the gill tissue (Park, 1999). The number of *Perkinsus* cells in each clam was then