Current Status of *Perkinsus* Infection in Korean Waters

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ABSTRACT

Perkinsosis is a shellfish disease caused by parasitic protozoan in some commercially important mollusks including oysters, clams, abalones and scallops. Heavy infection with *Perkinsus* often results in mass mortalities and commercial loss. Currently *Perkinsus* is classified as a molluscan disease by the OIE. In Korean waters, *Perkinsus* sp. has been found in the Manila clam, *Ruditapes philippinarum. Perkinsus* is also believed to be responsible for the decline in clam landings for the past decade in Korea. *Perkinsus* trophozoites are distributed commonly in gills, digestive glands, mantle and gonadal connective tissues. They are relatively scarce in foot and siphons. Heavy infection with *Perkinsus* often caused white nodule formation on gills and mantle, as well as massive concentration of haemocytes around the infected tissues. Microscopic features of different life stages and DNA sequences from the non-transcribed spacer and internal transcribed spacer indicated that *Perkinsus* sp. discovered in the clam in Korea could be *P. atlanticus (=P. olsoni)* described from clams in European waters. Ray's fluid thioglycollate medium (RFTM) with Choi's NaOH lysis method was used in the detection and quantification of *Perkinsus* in the clams. The prevalence was mostly 80 to 100% in commercial clam beds on the west and the south coasts of Korea. The infection intensity was found to be highest in October when most clams completed spawning and mass mortalities where observed in the beds. Quantification of clam eggs using enzyme-linked immunosorbent assay with rabbit anti-clam eggs protein IgG demonstrated that the amount of eggs produced during spawning was negatively correlated with infection intensity of *Perkinsus*. In conclusion, high level of *Perkinsus* infection in the clam could precipitate reduced growth and reproduction, as well as mass mortalities in the bed, resulting in decreases in clam harvesting in Korean and possibly in other Asian waters.

INTRODUCTION

In the year 2000, global fisheries production rose to 130,433,800 MT and shellfish production accounted for 14.2% (18,525,140 MT) of the global production (FAO, 2002). It is note worthy that 80% of the world shellfish production in year 2000 originated from Asia, mostly from China, Japan and Korea. Oysters, clams, cockles, scallops and mussels were the main molluscan species that comprised over 60% of the total shellfish production in Asia in 2002. Currently these species are cultured in high density using intensive culture systems, which requires relative less area for the culture. However, the mass culture of those species in limited space often cause outbreak of diseases. Shellfish disease outbreaks are increasingly recognized as a significant constraint to aquaculture production and trade, affecting both

Choi, K-S. and K-I. Park 2005. Current status of *Perkinsus* infection in Korean waters. *In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 263-274. Fish Health Section, Asian Fisheries Society, Manila.

the economic development and socio-economic revenue. To date, several shellfish diseases have been reported from some commercially important marine bivalves including Bonamiosis, Marteiliosis, Haplosporidiosis, Marteilioidosis and Perkinsosis (see Bondad-Reantaso *et al*., 2001).

SPECIES AND PATHOLOGIC FEATURES OF PERKINSUS

Since the first report of P. marinus (= *Dermocystidium marinum*) in the Gulf of Mexico (Mackin *et al*., 1950), several species of Perkinsus has been reported from various marine mollusks including oysters, scallops, clams and abalones in the world (Lester and Davis, 1981; Azevedo, 1989; Navas et al., 1992; Blackbourn *et al*., 1998; Canestri-Trotti *et al*., 2000; Choi and Park, 1997; Hamaguchi *et al*., 1998; Liang *et al*., 2001, see Table 1). In particular, *P. atlanticus* has been associated with mass mortalities of the venerid clams of the genus *Ruditapes* (i.e., *Tapes* or *Venerupis*) inhabiting all along the Mediterranean and Atlantic coasts of Europe (Da Ros and Cansonier, 1985; Chagot *et al*., 1987; Sagrista; *et al*., 1995; Canestri-Trotti *et al*., 2000). Slow growth, gaping and mass mortalities are typical symptoms of *Perkinsus* infection in the clam and oyster populations (Mackin, 1962; Park *et al*., 1999; Park and Choi, 2001). In the Manila clams, *Perkinsus* trophozoites are mostly aggregated in gills, digestive diverticulars and mantle while they are less common in the foot, adductor muscle and siphons (Fig. 1). *Perkinsus* cells are also found in connective tissues of female and male gonadal tissues (Park and Choi, 2001; Choi *et al*., 2002). Heavily infected clams often exhibit white spots (i.e., nodules) on their mantle, foot and gills due to inflammatory reaction of the clams (Fig. 1B, 1D). Fig. 1C shows typical Perkinsus trophozoites displaying a *Perkinsus*-specific "ring" structure (i.e., large vacuole and that displaces the nucleus to the periphery of the cell, Azevedo, 1989; Azevedo *et al*., 1990; Auzoux-Bordenave *et al*., 1995; Perkins, 1996; Park and Choi, 2001). Size of the trophozoite isolated from Korean water in terms of diameter was similar to the size reported by Hamaguchi *et al*. (1998) and Maeno *et al*. (1999) in Japan. Trophozoite diameters reported by Park and Choi (2001) varied from 7.73 to 15.80 μm, with a mean of 10.98 μm. Hamaguchi *et al*. (1998) reported 5.3 to 32.5 μm, with a mean of 14.8 μm, as diameter of trophozoites in Manila clams, and Maeno *et al*. (1999) reported 5.7 to 11.4 μm. Sizes of trophozoites of *Perkinsus* sp. measured from *R. philippinarum* distributed on the northern coast of China varied from 2 to 10 μm, which were somewhat smaller than the sizes reported from Korea and Japan (Liang *et al*., 2001). The size of trophozites estimated by Park and Choi (2001) is also similar to the size of *P. olseni* discovered in the Australian black-ribbed abalone, *Haliotis rubra,* (Lester and Davis, 1981).

Some heavily infected clams exhibited numerous clusters of trophozoites on their gill plicae and connective tissue of the digestive tubules with severe hemocytic infiltration (Fig. 1E, 1F). Such a heavy infection in gill tissues may lower filtration efficiency and, in turn, cause retarded growth. However, the effects of *Perkinsus* infection on filtration activity of the clams have not been experimentally proven. Infestation of *Perkinsus* in digestive tubules would cause digestive tubule atrophy and exert deleterious effects on the food digestion of the clams, as reported by Lee *et al*. (2001). *Perkinsus* was also observed among the connective tissues of female and male gonads (Fig. 1G), indicating that *Perkinsus* infection may disturb reproductive activity of the clams in some way.

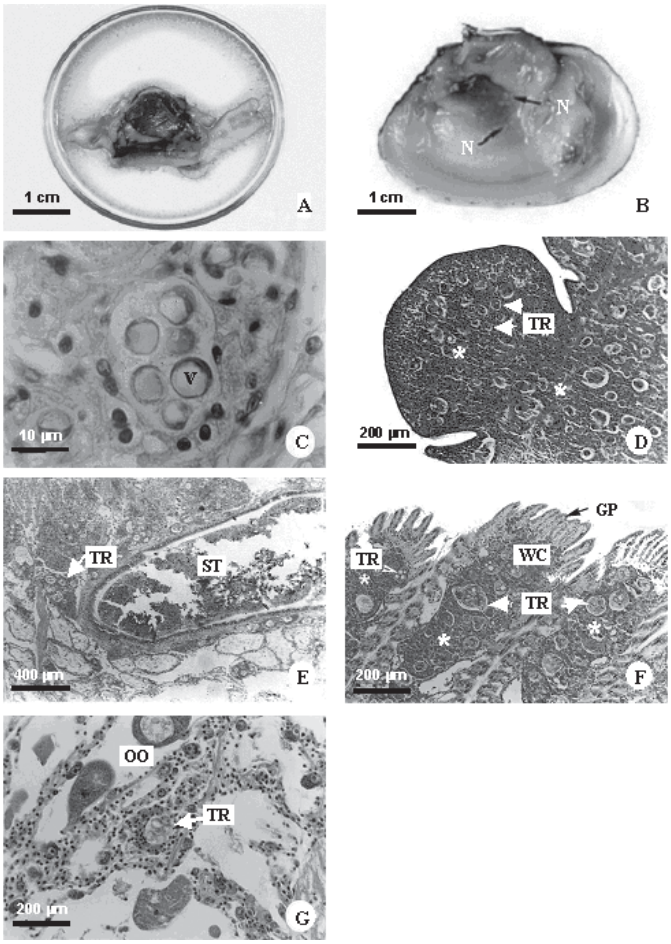

Figure 1. Internal and external features of *Perkinsus* infection in the Manila clam. A: Lugol's iodine stained *Perkinsus* hypnospores after RFTM incubation. B: Nodules on the clam body caused by inflammatory reaction against the parasite. C: Trophozoites of *Perkinsus* in clam tissues. D: A nodule showing the infiltration of host's hemocytes around *Perkinsus* cells. E: Trophozoites parasitizing around the stomach. F: Gills infiltrated with trophozoites and clam hemocytes. G: Trophozoites in the connective tissues of the female gonad. Nodule (N): Eccentric vacuole (V): Trophozoite (TR): Stomach (ST): Gill plica (GP): Water chamber (WC): Oocyte (OO): Asterisk-infiltrated clam hemocytes (Park and Choi, 2001).

As reported by Auzoux-Bordenave (1995) and Perkins (1996), several life stages could be identified from *Perkinsus* sp. found in the Manila clams in Korea (Park *et al*., in preparation). In host tissues, *Perkinsus* trophozoite occurs as a single cell or tomont of multi-nucleated form. Once the trophozoite or tomont are placed in an anaerobic condition such as in fluid thioglycollate medium (FTM) or necrotic tissues, they develop a dormant form of hypnospores or prezoosporangia, which are characterized as enlarged cell size and thick cell wall that stain dark blue or brown with iodine. When the hypnospores are placed in

aerated seawater, they undergo zoosporulation (Fig. 2). Two to three days after initial incubation at room temperature, a pore which later forms a discharge tube, is observed in the cell wall of hypnospores as early as in 2-celled stage (Fig. 2B-F). Successive bipartition of the nuclei results in 4, 8, 16 to 64 cell-stages. After two to three days of incubation, bi-flagellated motile zoospores are released from mature hypnospores via the discharge tube (Fig. 2G).

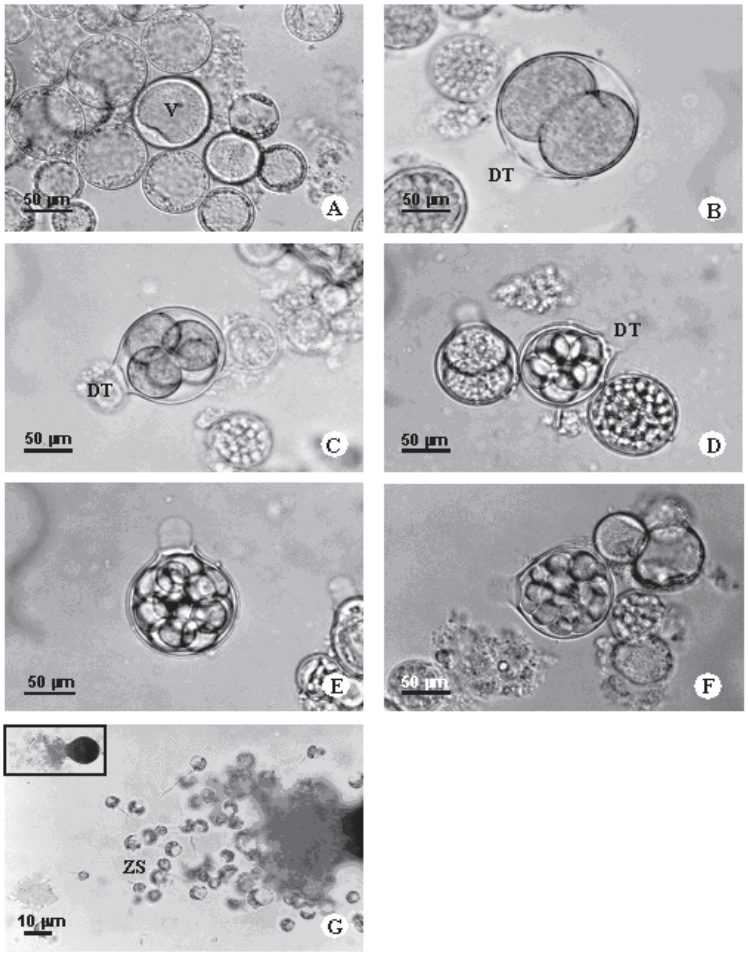

Figure 2. *In vitro* sporulation of *Perkinsus* sp. in GF/C filtered seawater. A: Beginning of ecentric vacuole subdivision, B: 2 cell-stage, C: 4 cell-stage, D: 8 cell-stage, E: 16 cell-stage, F: 32 cell-stage, G: Discharge of mortile zoospores. Scale-bar = 25 mm. Vacuole (V): Discharging tube (DT): Zoospore (ZS) (Park *et al*., in preparation).

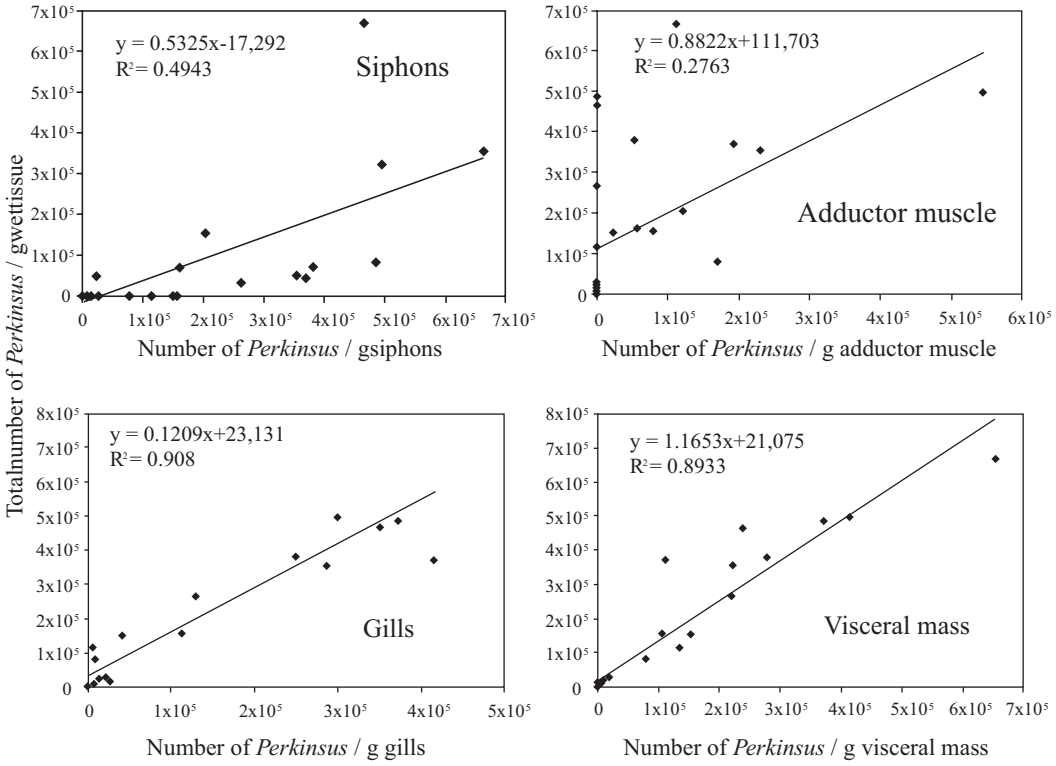

Figure 3. Correlation between the total number of *Perkinsus* per gram tissue wet weight and the number of *Perkinsus* per gram in siphons, adductor muscle, gills and visceral mass (Choi *et al*., 2002).

DIAGNOSTICS OF PERKINSUS INFECTION

Numerous methods have been applied in the diagnosis of *Perkinsus* infection including histology and electron microscopy (Mackin, 1951; Perkins and Menzel, 1966; Azevedo, *et al*., 1990; Navas *et al*., 1992; Montes *et al*., 1996; Bower *et al*., 1998), the fluid thioglycollate medium (FTM) technique (Ray, 1953, 1966; Choi *et al*., 1989; Bushek *et al*., 1994; Rodriguez and Navas, 1995; Fisher and Oliver, 1996; Almeida *et al*., 1999; Ford *et al*., 1999), immunology (Choi *et al*., 1991; Dungan *et al*., 1993; Romestand *et al*., 2001) and PCR assays (Marsh *et al*., 1995; Penna *et al*., 2001; Park *et al*., 2002). Among these methods, FTM is the most widely used, regardless of the species of *Perkinsus*. Although FTM assay was initially designed for the investigation of *P. marinus* infection in oysters, the technique has been successfully applied in the study of *Perkinsus* spp. infection in clams (Rodriguez and Navas, 1995; Auzoux-Bordenave *et al*., 1995; Choi and Park, 1997; Cigarria *et al*., 1997; Almeida *et al*., 1999). For quantitative assessment of *Perkinsus* infection, the total number of *Perkinsus* cells in a clam (i.e., total body burden) was counted by dissolving a whole clam with 2 M NaOH after incubation in FTM (Choi *et al*., 1989; Almeida *et al*., 1999; Park *et al*., 1999; Park and Choi, 2001). The NaOH digestion assay is sensitive enough to detect only a few cells in the entire clam (Park and Choi, 2001). Rodriguez and Navas (1995) also suggested that the total body burden analysis with FTM followed by 2 M NaOH digestion method is highly recommended for accurate diagnosis of *Perkinsus* infection.

Choi *et al*. (2002) found that the best positive correlation between the total number of *Perkinsus* cells in clams and the number of Perkinsus cells in gill tissues of the clams collected from Isahaya Bay, Japan (Fig. 3). They recommended the gill assay for the routine diagnostic of *Perkinsus* infections in clams, instead of whole clam assay which requires more time and cost for the analysis.

INCIDENCE OF PERKINSUS IN ASIAN WATERS

For the past few years, Perkinsus disease has been reported from the Manila clam, R. philippinarum (= *Tapes philippinarum, Venus philippinarum, Venus semidecussata, Tapes japonica, Tapes semidecussata*) distributed in Korea, Japan and China. *R. philippinarum* is one of the most important shellfish species that supports the shellfish industry of China, Japan and Korea. The clams are commonly cultured in an intensive manner on tidal areas of these countries.

Choi and Park (1997) first report the occurrence of *Perkinsus*-like organisms in *R. philippinarium* on the west and south coast of Korea. Park *et al*. (1999) investigated prevalence and infection intensity of *Perkinsus* in the Manila clams in Gomsoe Bay, one of the major clam culture ground on the west coast, in late summer when a mass mortality of the clams occurred. They indicated that the mass mortalities of the clams observed in Gomsoe Bay were closely associated with the extremely high level of *Perkinsus* infection in the clam population. Park and Choi (2001) also investigated spatial distribution of *Perkinsus* in Korean waters. They reported that water temperature and salinity are two major environmental parameters that regulate the prevalence and infection intensity. Phylogenetic identity of Korean *Perkinsus* was studied by Park *et al*. (in preparation) by analyzing ribosomal DNA sequences of *Perkinsus* isolated from clam serum. They sequenced NTS, ITS1, ITS2 and 5.8S rRNA of *Perkinsus* sp. isolated from the Manila clams in Korea, which has 1147, 183, 371 and 159 nucleotides, respectively. The NTS sequence of Korean *Perkinsus* was 99.9% identical to that of *P. atlanticus*, and showed 97.3% and 71.2% identity to those of P. olseni and P. marinus, respectively. The ITS 1, 2 and 5.8S rRNA sequences of Korean *Perkinsus* were 100% identical to *P. atlanticus* and *Perkinsus* sp. found in the Manila clam in Japan (Hamaguchi *et al*., 1998) and showed 99.9% and 89.1% similarities to those of *P. olseni* and *P. marinus*, respectively (Table 2, 3).

Table 1. Distribution of Perkinsus species in the world.

Species	Host species	Location	Author
P. marinus	Crassostrea gigas	Gulf of Mexico Atlantic coast of USA	Mackin *et al*. (1950)
P. olsoni	*Haliotis rubra*	Australia	Lester and Davis (1981)
P. atlanticus	*Ruditapes philippinarum* *R. dicussatus*	Portugal, Spain, France, Italy, possibly in Korea	Azevedo (1989)
P. qugwadi	*Patinopecten yessoensis*	Pacific coast of Canada	Blackbourn *et al*. (1998)
P. andrewsi	*Macoma baltica*	Atlantic coast of USA	Coss *et al*. (2001)
P. chesapaeki	*Mya arenaria*	Atlantic coast of USA	McLaughlin *et al*. (2000)

Table 2. Percentage similarities among *Perkinsus* spp. NTS gene sequences obtained from Gene Bank. KOR, an isolate from Korea, ATL, *P. atlantics*, MAR, *P. marinus*, OLS, P. *olseni*, AND, *P. andrewsi* (Park *et al*., in preparation).

Organism	KOR	ATL	OLS	MAR	AND
KOR		99.9	96.7	71.9	51.7
ATL			96.8	71.9	51.8
OLS				70.8	51.3
MAR					51.3
AND					

Table 3. Percentage similarities among *Perkinsus* spp. ITS-1, ITS-2 and 5.8S rRNA gene sequences obtained from Gene Bank. KOR, an isolates from Korea, ATL, *P. atlantics*, MAR, *P. marinus*, OLS, *P. olsoni*, KMA, *Perkinsus* isolated from Kumamoto, HRO, *Perkinsus* isolated from Hiroshima Japan, QUG, *P. qugwadi*, AND, *P. andrewsi* (Park *et al*., in preparation).

Perkinsus strain	KORATL	MAR	OLS	KMA	HRO	QUG	AND
KOR	100	89.1	99.9	100.0	100.0	62.9	84.9
ATL		89.1	99.9	100.0	100.0	62.9	84.9
MAR			89.2	89.1	89.1	59.8	80.8
OLS				99.9	99.9	62.8	85.1
KMA					100	62.9	84.9
HRO						62.9	84.9
QUG							61.3
AND							

Perkinsus-like pathogen was also reported from the clam populations in Hiroshima, Kumamoto and Nagasaki areas in Japan (Hamaguchi *et al*., 1998; Maeno *et al*., 1999; Choi *et al*., 2002). Hamaguchi *et al*. (1998) compared DNA sequences of *Perkinsus* found in Hiroshima and Kumamoto with the sequences of *Perkinsus* previously reported elsewhere. *Perkinsus* found in Kumamoto and Hiroshima Japan was taxonomically very close to *P. atlanticus* and *P. olseni* reported from Portugal and Australia (Hamaguchi *et al*., 1998). Choi *et al*. (2002) also reported *Perkinsus* infection in a clam population distributed in Isahaya Bay, Nagasaki Japan. Microscopic appearance of the trophozoites in the histological section and hypnospores formed in FTM were very similar to *P. atlanticus* reported from Portugal, in terms of size (Azevedo, 1989).

Perkinsus was also identified from *R. philippinarum* populations distributed on intertidal areas of northern coast of the Yellow Sea along the Liaodung Peninsula, China. Liang *et al*. (2001) surveyed the prevalence and infection intensity of *Perkinsus* in clams using RFTM assay. As high as 4,391,732 *Perkinsus* cells per clam or 2,271,883 cells per gram of tissue were observed in their study and a similar high infection was observed from places where mass mortalities of the clams were recently reported. They believed that the high level of *Perkinsus* infection observed in their study would implicate *Perkinsus* as a possible cause of the current mortalities in clams on the northern coast of China. Recently, *Perkinsus*-like organism was also isolated from the undulated clam, *Paphia undulata* distributed in Thai Bay, Thailand (Leethochavalit *et al*., personal communication). The nucleotide sequences

of NTS and ITS-5.8S rRNA of Thai *Perkinsus* showed a 99.85% similarity to *P. olseni*, indicating that *Perkinsus* found in Thailand must be taxonomically very close to *P. olseni*.

EFFECTS OF PERKINSUS PARASITISM ON CLAM REPRODUCTION

Several studies have reported negative effects of Perkinsus parasitism on reproduction of the host organisms. Choi *et al*. (1989) suggested that high level of *P. marinus* in the American oysters continuously drain out net energy of the oysters that supposedly was needed for growth and reproduction of the oysters, resulting in reduced growth and reproductive output. From oysters in Galveston Bay, Choi *et al*. (1994) observed a negative correlation between infection intensity of *P. marinus* and instantaneous rate of reproduction assessed using an immunoprecipitation assay; the heavier the infection, the longer it took the oyster to become ready for spawning. Dittman *et al*. (2001) also reported *P. marinus*-induced reduction in relative gonadal size and proportions of gametogenic tissue and gametes of the female oysters collected from Delaware Bay.

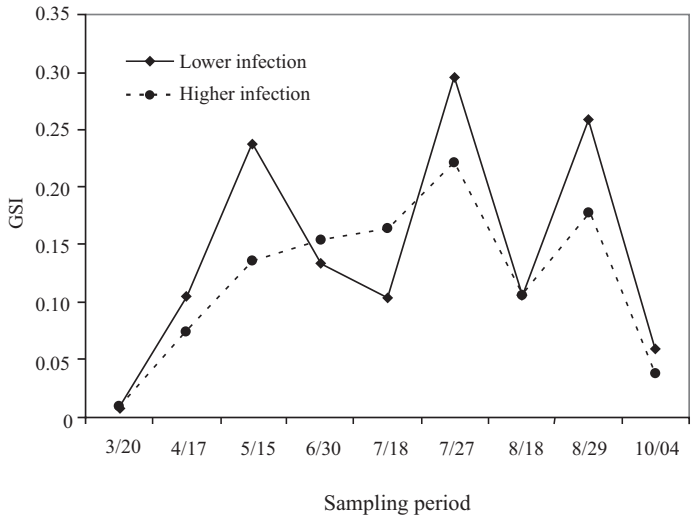

Figure 4. Seasonal variation in the gonad-somatic index (mg egg/ mg dry-tissue) of the clams. Higher infection; clams with the infection intensity higher than that of the monthly mean, and lower infection, clams with the infection intensity lower than that of the monthly mean (Park and Choi, in preparation).

Park and Choi (in preparation) investigated level of *Pekinsus* infection and reproductive output (i.e., the quantity of egg) of the clams in Gomsoe Bay, Korea using immunoassay and FTM technique. For quantification of eggs, they developed polyclonal antibody against the clam egg protein and the quantity of eggs in clams was assessed using enzyme-linked immunosorbent assay (ELISA). Level of *Perkinsus* infection in each clams used in the quantification was determined from their gills using Ray's FTM (Ray, 1966) and Choi's 2 M NaOH digestion assay (1989). Finally, the quantity of the eggs produced from clams was expressed as gonado-somatic index (GSI, percentage weight of eggs in total clam tissue

weight) and the infection level was expressed as total number of *Perkinsus* cells per gram tissue weight. Figure 4 shows the monthly changes in reproductive output and *Perkinsus* infection level in the clams collected during an annual reproductive cycle. The quantity of eggs could be assessed from March to October 1999 and the effect of *Perkinsus* on the egg production measured. The GSI measurements of clams in each month were separated into two groups; those of clams with infection intensity higher than the monthly mean intensity (i.e., higher infection in Fig. 4), and the other with their infection intensity lower than the monthly mean (i.e, lower infection in Fig. 4). Three distinct peaks of GSI could be identified from clams with light infection during the survey, indicating that the clams spawned at least three times in an annual reproductive cycle; the clams spawned in mid May, late July and late August. During each spawning peaks, the clams produced 25 to 30% of their total tissue weight as eggs. In contrast, quantity of eggs produced from the clams with heavy infection was much smaller, approximately two-fold smaller than that of the clams with light infection during the first spawning peak in May. The heavily infected clams also exhibited lower GSI values in late July and August relative to the less heavily infected clams. The data clearly indicated that high level of *Perkinsus* infection exerts negative effects on the clam reproduction, such as reduced egg production and delayed spawning.

In conclusion, *Perkinsus* has been reported from Asian waters, including Korea, China, Japan and Thailand. Heavy infection with *Perkinsus* in the Manila clams resulted in various pathologic symptoms such as white spots on the surface of gills and mantle tissues due to the infiltration of haemocytes. Heavily infected Manila clams also showed reduced egg production and delayed spawning. DNA sequence analysis of the *Perkinsus* isolates from Manila clams in Korea indicated that *Perkinsus* sp. discovered in Korean waters was most closely related to *P. atlanticus* (= *P. olseni*) from clams distributed in European waters.

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