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ARTICLE

Negative Impacts of *Perkinsus olseni* Infection in Manila Clam *Ruditapes philippinarum* Observed from Tidal Flats in Anmyeondo Island on the West Coast of Korea During Post-Spawning Period

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Abstract
The high load of protozoan parasites in marine bivalves often leads to mass mortalities of the hosts. On the west coast of Korea in the Yellow Sea, the protozoan parasite *Perkinsus olseni* has been identified as the agent causing mass mortality of Manila clam *Ruditapes philippinarum*. During August and September 2004, mass mortality of clam occurred at Hwangdo (HD) tidal flat in Anmyeondo Island on the west coast, resulting in a 50% reduction in the clam landings. Shortly after the mortality event, we examined pathology, and the fitness of the survived clams from HD to elucidate the impacts of *P. olseni* infection. Histology revealed that clams collected from HD in October 2004 were infected by *P. olseni.* In histology, *P. olseni* could be observed from all types of tissues of clams from HD, and severe inflammation was observed in the gills. Ray's fluid thioglycollate medium assay (RFTM) indicated that the infection intensity in clams from HD (1.738×10^6 cells/g gills in October and 1.476×10^6 cells/g gills in December) was significantly higher than the levels in clams from the neighboring tidal flats (0.001 to 0.622×10^6 cells/g gills, $P < 0.05$). Condition index (CI) and the total carbohydrate levels in clams from HD in October were significantly lower than those values in clams from other tidal flats ($P < 0.05$). In October, a negative correlation was observed between *P. olseni* infection intensity and CI in clams from HD, suggested that a high load of *P. olseni* causes substantial impacts on the host condition.

Keywords Fitness · *Ruditapes philippinarum* · *Perkinsus olseni* · *Parvatrema* · *Cercaria* · Condition index

1 Introduction

Occurring widely in low intertidal or shallow subtidal on the west coast of Korea, Manila clam *Ruditapes philippinarum* is one of the key species in the tidal flat ecosystem linking the primary production to the upper trophic level (Koh and Khim 2014). Manila clams are often cultured at a commercial scale at licensed tidal flats by sowing 1.5 to 2.5 cm in shell length (SL) juveniles. Clams are harvested 2 to 3 years after the sowing, as they reach 3 to 4 cm in SL (Park et al. 2006; Ahn et al. 2016). In 2004, Korea produced 27,570 MTs of Manila clam, and approximately 75% of the national landings were originated from Taean coast on the west coast. The Taean coast includes numerous sandy-mud tidal flats with high benthic primary production, which support the clam to grow (Lee et al. 2013; Park et al. 2014). In 2005, the Manila clam landings from the Taean coast dropped dramatically to 12,000 MT, approximately one-half of the landings recorded in the area in 2004 (KOSIS 2019). Such dramatic drops in the Manila clam production were linked to the mass mortalities of clams in tidal flats Taean in 2005 summer, where the mortality was recorded as high as 50% (NFRDI, unpublished data).

Numerous studies have demonstrated that the fitness of marine bivalves is often impaired by biotic and abiotic factors, including habitat destruction, oil spill, thermal stress, summer hypoxia, low food availability, harmful

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algal blooms, and a high burden of parasitism (Soletchnik et al. 2005; Mizuta et al. 2011; Burdon et al. 2014; Hong et al. 2016; Turra et al. 2016). Parasite infection has been identified as one of the leading causes responsible for deteriorating fitness, as high levels of parasitism often lead to mass mortalities of the host organisms. Various prokaryotic, eukaryotic, and metazoan parasites have been identified from cultured marine bivalves, including single-celled *Marteilia*, *Bonamia*, *Haplosporidium*, digenetic metazoan trematode, and crustacean sea spider as they induced lethal and/or sub-lethal impacts on the host organisms (Allam et al. 2002; Thieltges 2006; Miyazaki et al. 2010; Carrasco et al. 2015; de Montaudouin et al. 2016; Robledo et al. 2018). Thieltges (2006) reported that heavy infestation by trematode *Gymnophallus choledochus* induces mass mortality of the edible cockle *Cerastoderma edule* in the northern Wadden Sea. In Japan, the sea spider *Nymphonella tapetis* also caused the mortality of Manila clam in Tokyo Bay, resulting in a shutdown of the clam fishery in 2007 (Miyazaki et al. 2010; Yamada et al. 2017). In Korean waters, Manila clams are known to host protozoan and metazoan parasites, including the protozoan parasite *Perkinsus olseni* (Park and Choi 2001; Park et al. 2005; Kang et al. 2017) and the metacercaria stage of trematode *Parvatrema duboisi* on the mantle and *Cercaria* sp. in the gonad and the visceral mass (Ngo and Choi 2004; Le et al. 2015).

The epizootic protozoan *Perkinsus olseni* has been listed as notifiable mollusk disease by the World Organization for Animal Health (OIE), which is responsible for the decrease in clam populations in Europe and Asia (Allam et al. 2002; Pretto et al. 2014; Nam et al. 2018; Waki et al. 2018). According to Park et al. (2005) and Kang et al. (2017), *P. olseni* is the sole agent responsible for perkinsosis in Manila clams in Korean waters. Sub-lethal impact of *P. olseni* parasitism, such as low level of fitness, has been reported from clam culture grounds in Europe (see the review of Villalba et al. 2004). Slow growth and retarded gonad maturation as the sub-lethal impact of *P. olseni* infection in Manila clam in intertidal have been reported from the south coast of Korea (Lee et al. 2020).

In the summer of 2004, mass mortality of Manila clam occurred on Hwangdo (HD) tidal flat in Anmyeondo Island, where numerous adult and juvenile clams emerged from the sediment and perished. In an attempt to understand the mass mortality dynamics of clams, we examined pathologic condition, the energy reserve, and condition index in October and December, as the clams were in post-spawning.

2 Materials and Methods

2.1 Sampling Efforts

Anmyeondo Island (36°29′ N, 126°21′ E) encompasses well-developed tidal flats served as clam culture grounds (Fig. 1). In October and December 2004, clams with shell length ranging from 36.2 ± 3.2 mm (i.e., the longest axis of the shell, SL) were collected from Hwangdo (HD) tidal flat clam exhibited mass mortality in August and September 2004. For comparisons, clams were also collected from three other tidal flats in Anmyeondo Island (Nudong (ND), Gonam (GN), and Bangpo (BP), where no clam mass mortalities were observed in October and December in 2004 (Fig. 1). A total of 240 adult clams were collected from the four tidal flats and analyzed during the study (Table 1).

In the laboratory, the collected clams were acclimated in seawater at room temperature for 24 h to clear the sediments in the digestive tract. After measuring the SL, the soft body was removed from the shells, and excessive water on the tissue surface was removed, then weighed to mg using an electronic valance. Shells of each clam were dried at room temperature and weight to mg. Condition index (CI) was determined as the ratio of the wet tissue weight (g) to the dry shell weight (g).

2.2 Histology and Identification of Parasitic Organisms in Manila Clams

For histology, a 2–4 mm thick dorso-ventral section was made in the middle of the clam body. The body section containing the gonad, digestive gland, and gills was fixed in Davidson's solution for 24 h. The tissues were dehydrated in a series of ethanol, embedded in paraffin, sliced at 5 µm, then stained with Harris hematoxylin, and counterstained with eosin Y. The remained tissue was lyophilized and stored at − 70 °C for the total carbohydrate analysis. The histology slides were examined under a compound light microscope to identify parasitic organisms, such as *P. olseni* (Park and Choi 2001; Ngo and Choi 2004) and the larval trematode (Shimura et al. 1982; Ngo and Choi 2004; Le et al. 2015; Jung et al. 2021).

2.3 Quantification of *P. olseni* Using RFTM Assay

Ray's fluid thioglycollate medium (RFTM) assay and 2 M NaOH digestion (Ray 1966; Choi et al. 1989) were used to determine the density of *P. olseni* in each clam. For the assay, one part of the gill tissue was excised from each clam and added to 5 ml of FTM supplemented with

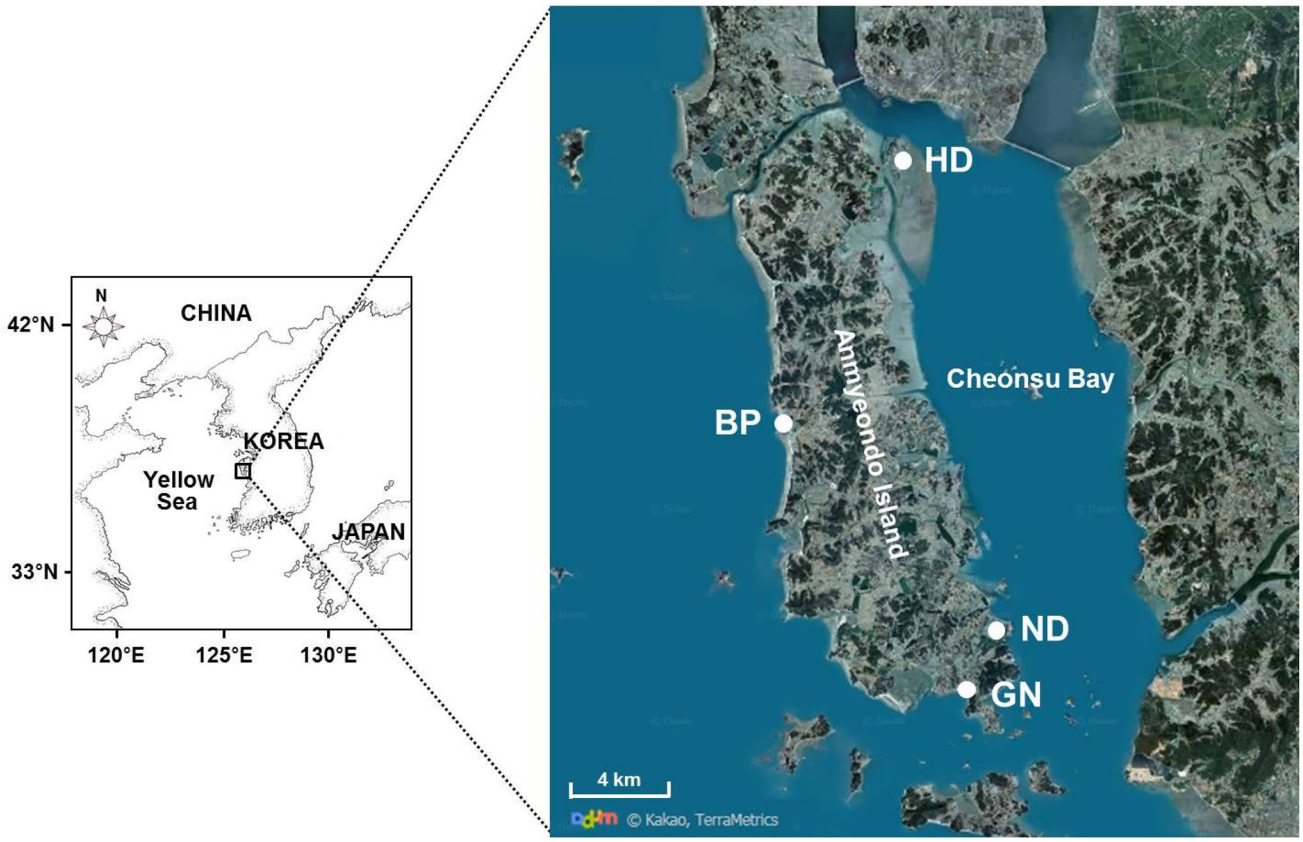

Fig. 1 Location of the study site. *Ruditapes philippinarum* was collected from HD (Hwangdo) tidal flat on the west coast of Korea, where mass mortality of clams occurred in October 2004. BP (Bangpo), ND (Nudong) and GN (Gonam); the comparison sites

Table 1 The number (*N*) and shell length (SL) of Manila clams collected in October and December 2004. The values represent the mean ± standard deviation (SD)

Sampling period		Site	*N*	SL (mm) ± SD
2004	OCT	HD	30	36.3 ± 2.3
		BP	30	37.9 ± 3.0
		GN	30	35.3 ± 2.5
		ND	30	35.4 ± 3.2
	DEC	HD	30	36.4 ± 3.4
		BP	30	36.2 ± 3.8
		GN	30	38.1 ± 2.5
		ND	30	33.8 ± 2.2

antibiotics (nystatin 200 unit/ml, chloramphenicol 100 ng/ml). After a week of incubation in the dark at room temperature, the gill tissues were digested in 2 M NaOH at 60 °C. The number of *P. olseni* hypnospores was then counted using a hemocytometer. Finally, the infection intensity was expressed as the number of *P. olseni* cells per gram gill tissue.

2.4 Biochemical Composition of the Manila Clams

The total carbohydrate in the tissue was determined using a phenol–sulfuric acid solution, according to Taylor (1995). Lyophilized clam tissue was pulverized, and 20–25 mg of subsample was taken and further homogenized in phosphate-buffered saline (PBS, 0.15 M NaCl, pH 7.3) using an ultrasonicator. The homogenized tissue was centrifuged, then the phenol–sulfuric acid solution was added to the supernatant. After recording the optical density (OD) at 480 nm using a spectrophotometer, the carbohydrate level was referred from the standard material, dextrose (anhydrous, SIGMA).

2.5 Statistical Analysis

Spatial variation in CI, *P. olseni* infection intensity among 4 populations of clams collected in October and December were compared using Kruskal–Wallis one-way analysis of variance (ANOVA) followed by Tukey's range test. Spatial variation in CI and the total carbohydrate content were also tested using one-way ANOVA and Tukey's range test. Statistical analysis was carried out using SAS statistical package program (SAS

Institute, NC, USA), and the significance level was set at alpha < 0.05.

3 Results

3.1 Identification of Parasitic Organisms Appeared in Histology

Histology revealed that in October, *P. olseni* infection was prevalent among the clams in the tidal flats in Anmyeo-ndo Island; clams from HD tidal flat showed numerous *P. olseni* trophozoites clusters in the connective tissues of the gills exhibiting excessive inflammation (Fig. 2A). The basophilic *P. olseni* trophozoites were appeared as a small spherical cell (5–15 μm in diameter) with a large vacuole in histology. Under a high magnification in a light microscope, the trophozoite could be identified by its characteristic nucleus, which appeared as a signet ring (McLaughlin and Faisal 1998; Villalba et al. 2004). The massive infection by *P. olseni* resulted in hemocyte infiltrations in the gills, which led to swollen connective tissue and deterioration in the gill structures (Fig. 2A).

Histology also demonstrated that clams in the study sites were infected by the metacercariae stage of trematode *Parvatrema duboisi* in the mantle epithelia, characterized by the oral suckers (Fig. 2B). The sporocysts stage *Cercaria* sp. was also commonly identified from the ovaries, where the sporocysts contained the germinal balls and mature metacercaria characterized by eyespot and tail (Fig. 2C). The sporocysts stage of *Cercaria* sp. also occurred in the testis (Fig. 2D). It was notable that the larval *P. duboisi* occurred limitedly in the mantle tissue, whereas the sporocysts of *Cercaria* sp. distributed in the gonad and the visceral mass. In most cases, Manila clams infected by *Cercaria* sp. and

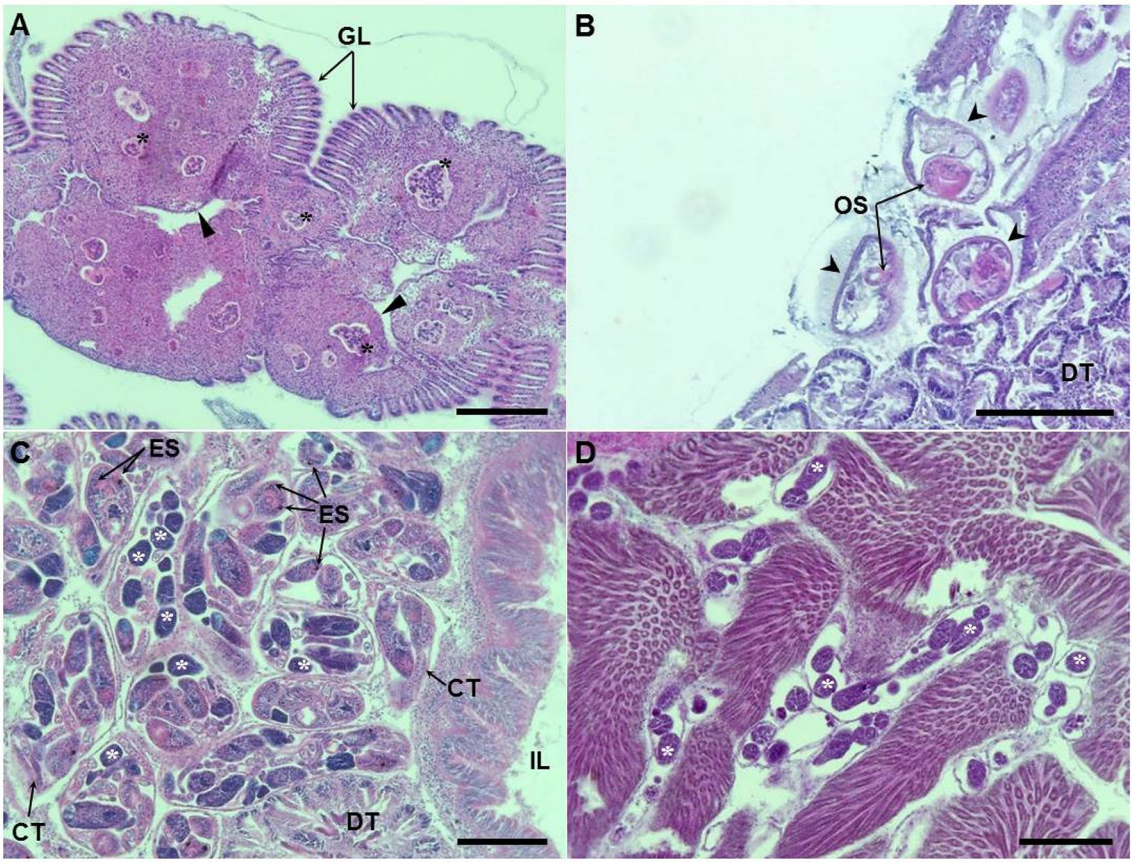

Fig. 2 Photomicrographs of different types of parasites observed from Manila clams. **A** *P. olseni* in the gill connective tissues causing inflammation. Severe hemocyte infiltration surrounding the clusters of *P. olseni* trophozoites (*, asterisk) resulted in granulomas (arrowheads) and gill deformation. GL, gill lamella. **B** Matacercaria of *Parvatrema* sp. (arrowheads) in the mantle epithelium. OS, oral sucker, DT, digestive tubule. **C** Gonad castration caused by heavy infection with *Cercaria* sp. in the the connective tissue of the ovary. The gonad was fully occupied by the sporocysts of *Cercaria* sp. consisting of mature *Cercaria* sp. and germ balls (*, asterisk). ES, eyespot, CT, *Cercaria* tail, IL, intestinal lumen, DT, digestive tubule. **D** Sporocyst of *Cercaria* sp. containing germ balls (*, asterisk) in the testis. Scale bar = 200 μm

Parvatrema sp. were co-infected by the protozoan parasite *P. olseni*.

In October, *P. olseni* infection prevalence of clams at HD tidal flat was 100%, and the prevalence remained 100% in December. At GN and ND, the infection prevalence ranged from 88.9 to 90.0% in October, and then it dropped to 40% in December at ND. Among the four sampling sites, BP showed the lowest *P. olseni* prevalence, ranging from 11.1% (December) to 53.3% (October), respectively. In October, *Parvatrema* sp. infection prevalence was highest at GN (40%), while the prevalence at other tidal flats ranged from 0.0 (BP) to 4.3% (HD) (Fig. 3). In December, the *Parvatrema* sp. infection prevalence ranged 5.6 (BP) to 26.7% (ND). The infection prevalence of *Cercaria* sp. was highest at ND (22.2%) in October, while the lowest prevalence was recorded at HD as 4.3%.

3.2 Quantification of *P. olseni* Infection Intensity

Figure 4 shows the mean and standard error of *P. olseni* infection intensity of clams determined using RFTM. In October, the infection prevalence ranged from 96.7% (ND) to 100% (HD, BP, and GN), indicating that most clams in tidal flats in Anmyeondo island were infected by *P. olseni*. RFTM indicated that *P. olseni* infection intensity of clams at HD tidal flat (1.739×10^6 cells/g gills) was significantly higher than the intensities measured from BP (0.134×10^6 cells/g gills), GN (0.622×10^6 cells/g gills), and ND (0.483×10^6 cells/g gills) ($P < 0.05$).

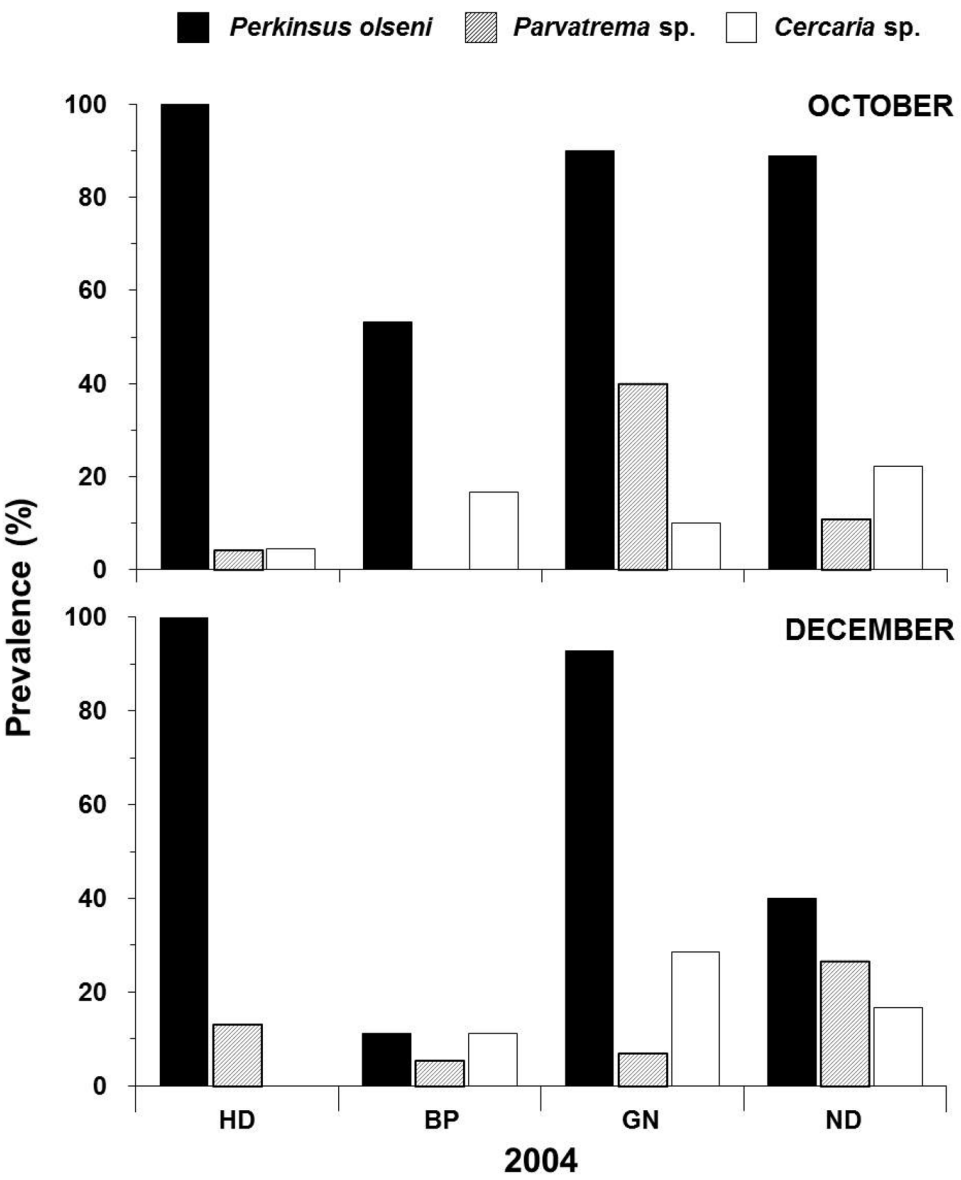

Fig. 3 Prevalence of *P. olseni* and the metazoan parasite *Parvatrema duboisi* and *Cercaria* sp. in Manila clams collected in October and December 2004

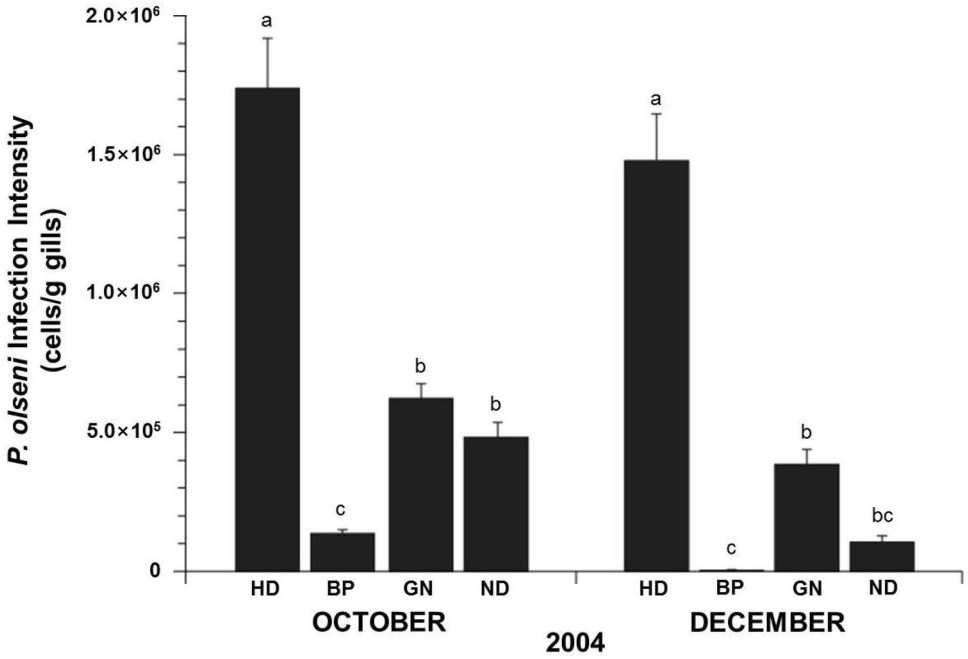

Fig. 4 Mean infection intensity of *P. olseni* in Manila clams recorded in October and December 2004. The error bars represent the standard error (SE), and the different superscript letters (a – c) indicate the significant differences among the means (ANOVA, $P < 0.05$)

In December, *P. olseni* prevalence remained 100% at HD and GN, and 16.7% and 83.3% at BP and ND, respectively. At HD tidal flat, the infection intensity remained high as 1.476×10^6 cells/g gills, which was significantly higher than the intensities recorded from BP (0.001×10^6 cells/g gills), GN (0.385×10^6 cells/g gills), and ND (0.104×10^6 cells/g gills) (Kruskal–Wallis test, $P < 0.05$).

3.3 CI and the Total Carbohydrate

CI of clams collected in October showed a spatio-temporal variation during the sampling (Fig. 5). In October, CI ranged from 0.51 (HD and ND) to 0.56 (BP and GN), and the one-way ANOVA indicated that CIs of clams in HD and ND tidal flats were significantly lower than clams in BP and

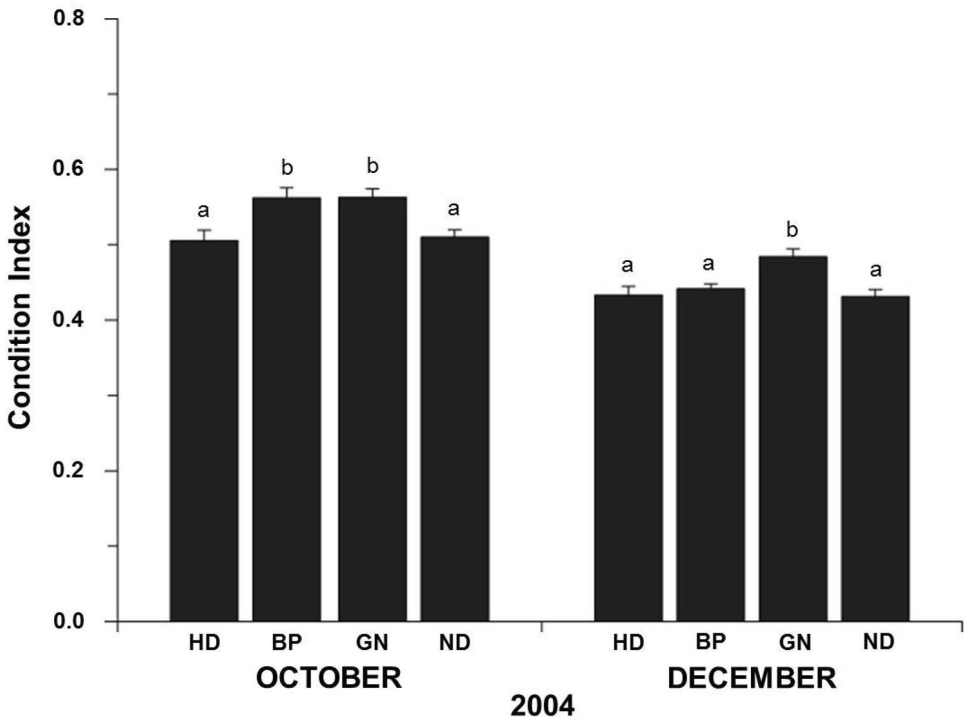

Fig. 5 Condition index (CI) of Manila clams collected in October and December 2004. The error bars represent the standard error (SE), and the different superscript letters (a, b) indicate significant difference among the means (ANOVA, $P < 0.05$)

GN ($P < 0.05$). In December, CI ranged from 0.43 (HD and ND) to 0.48 (GN). Compared to October, CI determined in December was significantly lower than CI recorded in October (Student's *t* test, $P < 0.05$).

Figure 6 shows the level of total carbohydrates in clams. In October, the mean total carbohydrate content varied from 7.34 (HD) to 17.86% (GN). ANOVA test indicated that in October, the total carbohydrate content of clams in HD was significantly lower than BP, GN, and ND ($P < 0.05$). In December, the total carbohydrate levels ranged from 8.00% (ND) to 13.18% (BP). In December, the total carbohydrate level recorded at ND was significantly lower than the levels determined from three other sites, including HD ($P < 0.05$).

The effect of *P. olseni* infection on host fitness was tested using a simple regression between the CI and *P. olseni* infection intensity determined by RFTM. In October, *P. olseni* infection intensity was negatively correlated with the CI at HD ($r^2 = 0.25$, $P < 0.01$, Fig. 7). In contrast, there was no significant correlation between CI and the infection intensities at the three sites (BP, $r^2 = 0.14$, GN, $r^2 = 0.001$, ND,

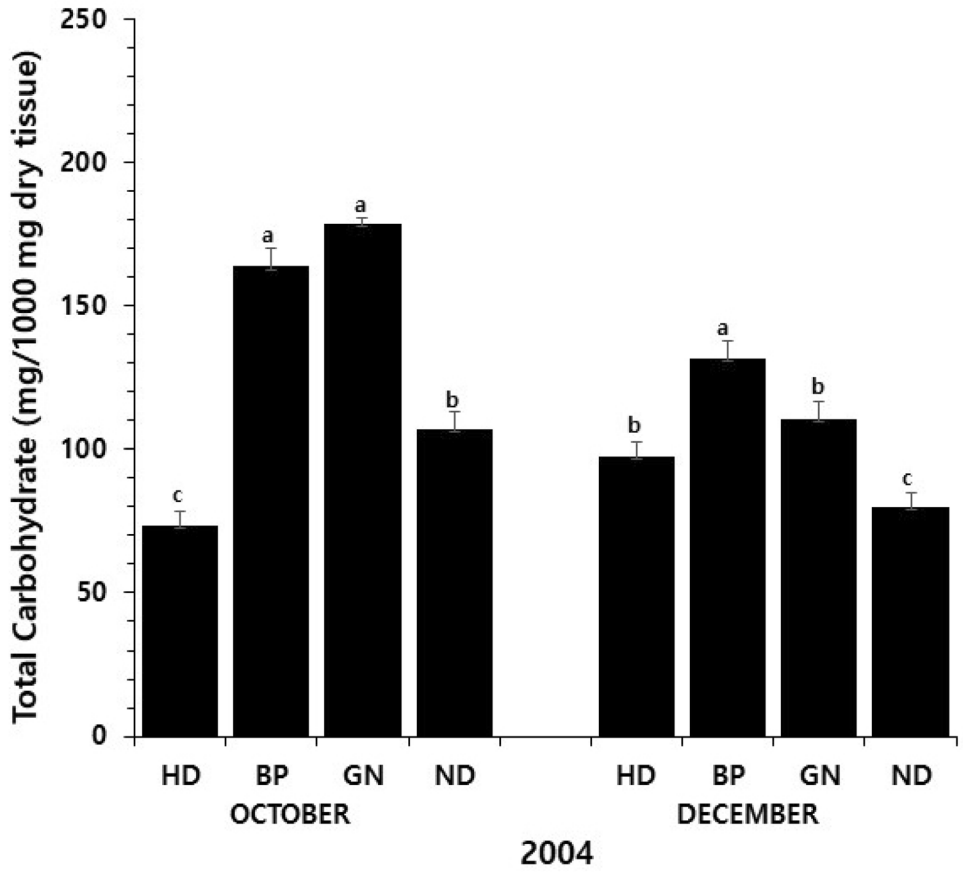

Fig. 6 The total carbohydrate concentration in Manila clams collected in October and December 2004. The error bars represent the standard error (SE), and the different superscript letters (a – c) indicate significant differences among the means (ANOVA, $P < 0.05$)

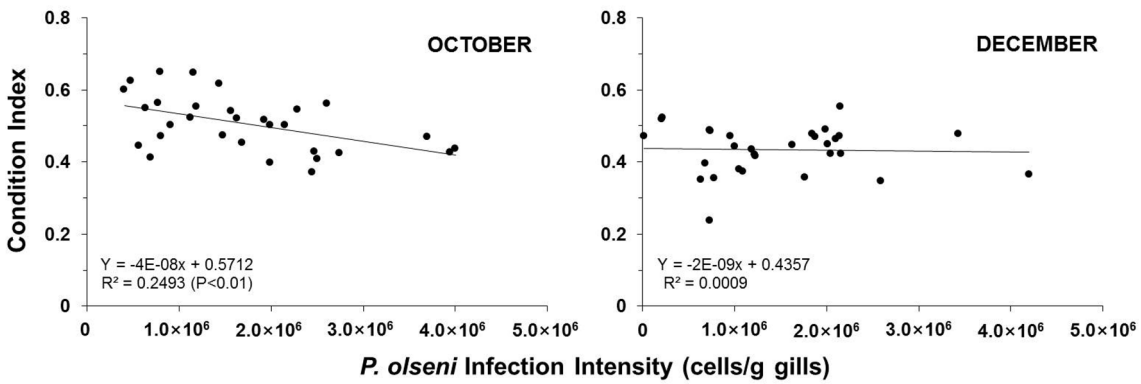

Fig. 7 Correlation between *P. olseni* infection intensity and CI of Manila clams from HD tidal flat in October and December 2004

$r^2 = 0.07$). In December, no clear correlation was observed between CI and *P. olseni* infection intensity among the four sampling sites, including HD (Fig. 7).

4 Discussion

4.1 Mass Mortality of Manila Clams

During the mass mortality incident at the HD tidal flat in 2004 summer, numerous clams emerged on the sediment surface. Such surfaced clams were exposed to the hot air temperature during low tide and possibly received a high degree of thermal stress. Histology revealed that the clams collected in October 2004, shortly after the mass mortality event, were infected by *P. olseni*, exhibiting inflammation and necrosis in the gills. It is believed that such a severe infection in the gills could disrupt the respiration and feeding while they were in the sediment. RFTM assay of HD clams indicated that *P. olseni* infection intensity reached 1.74 million cells per gram gills, which is considered to be a heavy infection (Park and Choi 2001). In contrast, *P. olseni* density was significantly lower in clams in three other tidal flats, where no apparent mass mortality occurred during the summer. It is believed that the high level of *P. olseni* is responsible, at least in part, for the mortality that occurred at HD tidal flat. According to the studies carried out in Japan, *P. olseni* infection leads lethal to sub-lethal impacts on juveniles to adult clams, especially when the infection intensity reaches over a million cells per gram host tissue (Shimokawa et al. 2010; Waki and Yoshinaga 2013; Waki et al. 2012, 2018). *Perkinsus olseni* infection-driven mass mortality of Manila clam also reported from clam culture grounds in Europe (Villalba et al. 2004; Pretto et al. 2014).

Nam et al. (2018) first examined a correlation between *P. olseni* infection and clam emerging phenomenon during a mass mortality occasion in late summer. In mid-August of 2015, numerous clams emerged on the sediment surface within a day in a tidal flat on the west coast of Korea. Most of the emerged clams remained on the surface and perished within a week, possibly due to desiccation and thermal stress while exposed to the atmosphere (Nam et al. 2018). Compared to the normal clams in the sediment, the surfaced clams exhibited a significantly low CI and low cell-mediated immune capacity. RFTM assay revealed that *P. olseni* infection level of the surfaced clams (1.98×10^6 cells/g wet tissue) was significantly higher than the level recorded from clam in the sediment (1.16×10^6 cells/g wet tissue). *Perkinsus olseni* infection intensity of clams determined from HD tidal flat in October was somewhat comparable to the level reported by Nam et al. (2018), suggesting that mortality of clams observed at HD tidal flat was associated with the high level of *P. olseni*. It is also believed that some other internal and external parameters, such as spawning activity and high air temperature, also exerted synergistic stresses with *P. olseni*. According to Yang (2011), Manila clams in HD tidal in August are mostly partially spawning or spent stages, and such stressful reproductive activity could deteriorate the cell-mediated immune capacity (Hong et al. 2016).

4.2 Impacts of *P. olseni* Infection

In October, a negative correlation between CI and *P. olseni* infection intensity was observed from clams in HD tidal flat, whereas clams in the neighboring tidal flats showed no significant association with the infection intensity. It was also noticeable that CIs of clams from HD in October were significantly lower than those of clams in other tidal flats, although such difference in CI was no longer observed in December. Such degraded fitness of clams infected by *P. olseni* was also reported from tidal flats in Incheon bay and Gomso bay on the west coast of Korea. Park et al. (2006) first reported a negative correlation between *P. olseni* infection intensity and CI of Manila clams in Gomso bay, where *P. olseni* infection prevalence stayed at 100% throughout the year, and the intensity reached 2.03×10^6 cells/g gills in late summer. Yang et al. (2012) also reported a high *P. olseni* infection and low CI in clams in Gomso bay.

Along with CI, clams in HD tidal showed a significantly low carbohydrate content level, suggesting that the carbohydrate metabolism of clams in HD tidal flat is substantially higher than clams in other tidal flats. Such a high level of carbohydrate metabolism is a symptom of physiological stress in marine bivalves (Scheurink and Steffens 1990; Mizock 1995). Robledo et al. (1995) reported that the carbohydrate content decreased in the mussel *Mytilus galloprovincialis* when they were severely infected by *Marteilia refringens*, a protozoan parasite of some commercially important marine bivalves. Coustaua et al. (1991) also reported trematode infection facilitated glycogen mobilization in *Mytilus edulis*, resulting in declined carbohydrate content. Therefore, we believe that the significantly low level of carbohydrate in clams in HD tidal flat is closely linked to the high level of *P. olseni* infection, as the parasite exerted a certain level of stress on the host metabolism. We also observed the larval trematode infection in Manila clams in this study, and the larval trematode infection may cause a certain level of negative impacts on the host health condition, as Coustaua et al. (1991) reported previously.

In conclusion, we surveyed parasite load and health condition of Manila clams in tidal flats in Anmyendo Island during late summer and early winter to understand the sublethal effects of the parasitism. In October 2004, clams collected from HD tidal flat on the west coast of Korea were heavily infected by *P. olseni*. Clams in HD tidal flat also demonstrated significantly low CI and total carbohydrate

levels, suggesting that the major sub-lethal impact of *P. olseni* is a decrease in fitness, as was reported from other marine bivalves heavily infected by *P. olseni*.

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