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Effects of *Perkinsus olseni* parasitism and environmental conditions on the gonad maturation and reproductive effort of female Manila clam (*Ruditapes philippinarum*) on the west and south coasts of Korea

Thatchaneshkanth Subramaniam [a,e], Hyun-Ki Hong [b,*], Kyung-Il Park [c], Young-Ghan Cho [d], Kwang-Sik Choi [a,*]

[a] Department of Marine Life Science (BK21 FOUR) and Marine Science Institute, Jeju National University, Jeju 63243, Republic of Korea
[b] Department of Marine Biology and Aquaculture, Gyeongsang National University, Tongyeong 53064, Republic of Korea
[c] Department of Aquatic Life Medicine, Kunsan National University, Gunsan 54150, Republic of Korea
[d] Tidal Flat Research Center, National Institute of Fisheries Science, Gunsan 54001, Republic of Korea
[e] Department of Animal Science, Faculty of Agriculture, University of Jaffna, Kilinochchi 44000, Sri Lanka

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ABSTRACT

In the intertidal zone, the reproductive dynamics of bivalves are influenced by both biotic and abiotic factors, including spatial and temporal fluctuations in sea surface temperature (SST), food availability, and diseases. Notably, disease proliferation is markedly enhanced under conditions favorable to pathogen entities, such as elevated SST and low food availability. This study examined the associative impacts of *Perkinsus olseni* parasites on the reproduction of the Manila clam *Ruditapes philippinarum*, across a latitudinal range covering four sampling sites along the west and south coasts of Korea, ranging approximately 400 km. In late June, we collected adult female clams to assess their condition index, reproductive output, and the prevalence of *P. olseni* infection. Histology indicated that 94 % of clams from SJ tidal flat, the cooler northwest coast were in the ripe stage. In contrast, clams from WD and GS, the warmer regions of the southwest and south exhibited more progressed stages of gonadal maturation, including the spent and resting phases. Notably, clams at the ripe stage in WD and GS demonstrated significantly lower gonad somatic index (GSI, $P < 0.05$) than their counterparts in the northwest. Moreover, the intensity of *P. olseni* infection was substantially higher ($P < 0.05$) in WD in the south compared to SJ in the northwest. The histopathological index (HPI) revealed minimal tissue damage and lower HPI scores at SJ, in stark contrast to the progressively severe tissue damage and elevated HPI scores observed moving southward along the latitudinal gradient. It was postulated that the combined effects of higher SST, low food availability, and high levels of *P. olseni* infection are key factors contributing to the reduced GSI and condition index (CI) in ripe clams in WD on the warm southern coast. Additionally, the lower CI observed in clams from WD potentially heightens their vulnerability to diseases by weakening their immune defenses during critical reproductive phases, such as spawning and post-spawning.

1. Introduction

The Manila clam *Ruditapes philippinarum*, commonly occurring on sandy or muddy tidal flats along the west and south coast of Korea, supports local shellfish industries and plays a vital role as a suspension feeder (Lee et al., 2020; Park and Choi, 2001; Park and Choi, 2004; Yang et al., 2010). Understanding the reproductive dynamics of such commercially valuable bivalves is crucial for devising effective management strategies. Consequently, numerous studies have explored the annual gametogenesis of Manila clams, aiming to pinpoint the spawning period in an annual reproductive cycle, and the magnitude of gamete production (Park and Choi, 2004; Drummond et al., 2006; Uddin et al., 2010, 2012; Matsumoto et al., 2014). Research across the distribution range of Manila clams has yielded diverse records detailing their reproductive cycle. For instance, along the west coast of Korea, gametogenesis typically initiates in February, with the first spawning females observed in July (Uddin et al., 2010, 2012). However, some environmental variables significantly influence bivalve gametogenesis and

* Corresponding authors.
E-mail addresses: hyunki@gnu.ac.kr (H.-K. Hong), skchoi@jejunu.ac.kr (K.-S. Choi).

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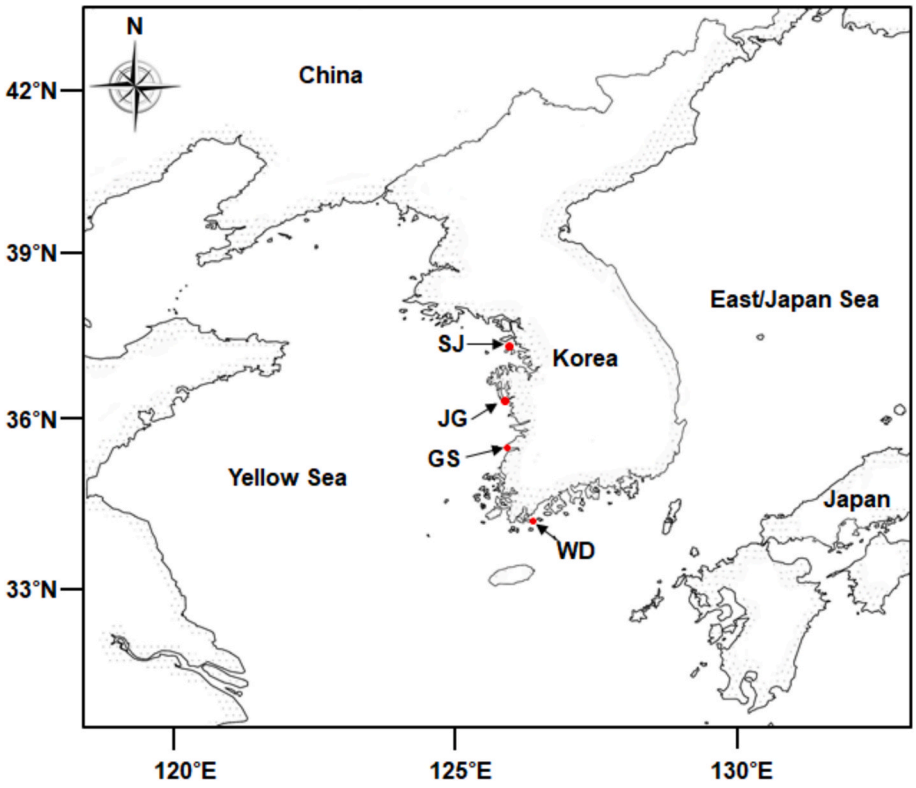

Fig. 1. Location of sampling sites in this study. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando.

spawning, chiefly SST, salinity and food availability, which can induce alterations in reproductive processes, complicating our understanding of these intricate mechanisms (Robinson and Breese, 1984; Xie and Burnell, 1994; Gosling, 2003; Drummond et al., 2006; Park et al., 2006; Baek et al., 2014).

Temperature, inherently linked to geographical locations, leads to anticipated variations in bivalve gametogenesis and spawning across different latitudes (Beninger and Lucas, 1984; Kang et al., 2010, 2015; Joaquim et al., 2023). Such latitudinal gradient in SST is often closely linked to spatiotemporal variation in the infection of *Perkinsus olseni*, the parasitic protist infecting Manila clams (see review of Villalba et al., 2004). Intense *P. olseni* infection has been linked to delayed gonad maturation and reduced reproductive effort, manifesting as a significantly lower reproductive effort in heavily infected clams (Park and Choi, 2004; Kang et al., 2015; Lee et al. 2021). This parasitic protist exhibits spatiotemporal variations in infection prevalence and intensity, particularly on the west and south coasts of Korea, which is closely associated with the latitudinal variations in SST (Park and Choi, 2001; Yang et al., 2010, 2012; Nam et al., 2018; Lee et al., 2020; Cho et al., 2022; Subramaniam et al., 2024). Environmental factors, notably SST and salinity, play pivotal roles in *P. olseni* physiology, distribution, and abundance, with higher temperatures correlating with increased parasite proliferation and intensified parasitic infestations in clams (Burreson and Calvo, 1996; Kang et al., 2015; Soudant et al., 2013). Similarly, salinity emerges as a critical determinant influencing the spatial and temporal distribution of *P. olseni*, with higher infection intensity and prevalence often aligning with elevated salinity levels (Park and Choi, 2001). Furthermore, larval trematode infections in Manila clams have been closely linked to reproductive abnormalities in Manila clams, such as reduced gonad area and inhibited gonad maturation, resulting in significant damage to their reproductive capacity (Ngo and Choi, 2004; Yang et al., 2021; Cho et al., 2022).

Histology has been adapted widely in monitoring the gonad development of clams, while condition indexes (CI), a simple ratio of the tissue to the shell weight serve as an indirect yet valuable means of approximating reproductive effort under specific environmental conditions (Park and Choi, 2004; Drummond et al., 2006; Uddin et al., 2012). Moreover, immune-detection methods have emerged as successful alternatives for quantifying egg proteins in marine bivalves, offering rapidity, cost-effectiveness, and high sensitivity (Park and Choi, 2004; Park et al., 2005; Jeung et al., 2014). The integration of histology and ELISA techniques allows for a stepwise assessment of gametogenic reproductive output in clams, enabling simultaneous determination of reproductive stage and egg quantity (Uddin et al., 2012).

While studies on bivalve reproduction often focus on individual factors, understanding the complexity of multiple interacting factors in the natural environment is crucial (Baek et al., 2014; Rato et al., 2022). Therefore, the histopathologic index (HPI) emerges as a valuable tool for effectively assessing the overall health status of marine bivalves for environmental monitoring purposes (Au, 2004; Costa et al., 2013; Joshy et al., 2022; Yavasoglu et al., 2016). HPI calculation involves a two-tiered approach: scoring the extent of histological changes and assigning an importance factor to denote the pathological significance of the alteration (Bernet et al., 1999). Despite its potential, limited research has focused on exploring histopathological changes in Manila clams concerning their reproductive processes, leaving a significant gap in our understanding of the intricate tissue alterations associated with reproduction (Cherel and Beninger, 2017; Sugiura and Kikuya, 2022). In this study, we integrated the HPI with other obtained results to comprehensively elucidate variations observed in the reproductive effort of Manila clams attributed to *P. olseni* parasitism along the latitude gradient on the west coast of Korea.

2. Materials and methods

2.1. Sampling efforts

On June 30, 2006, Manila clams were collected from four tidal flats: Sunjae (SJ), Jugyo (JG), Gomso (GS), and Wando (WD), located along the western and southern coastlines of Korea (Fig. 1). Approximately

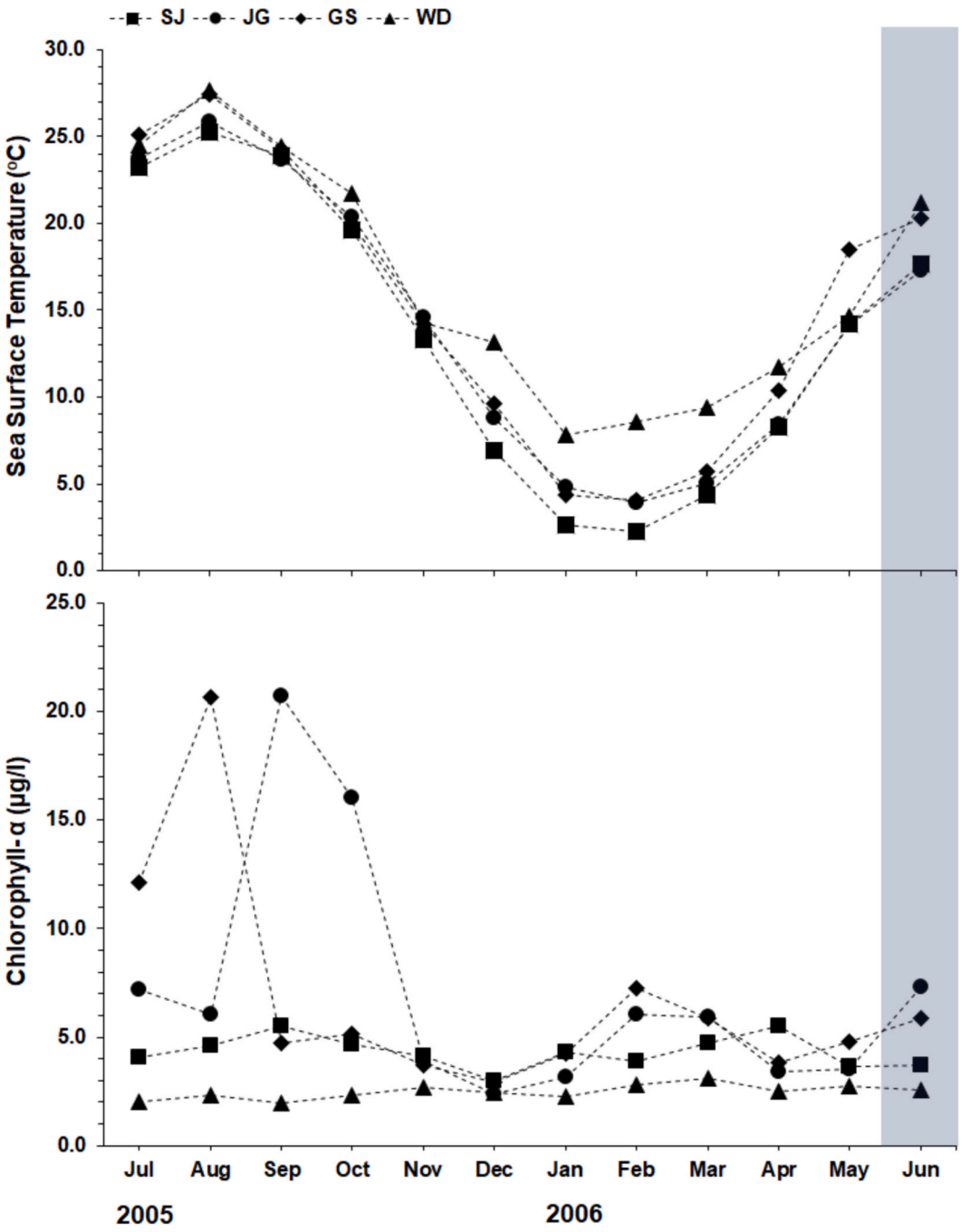

Fig. 2. Seasonal changes in the sea surface temperature (SST) and chlorophyll-a concentration in the study sites from 2005 July to 2006 June. The highlight in the figure (June) illustrates the SST and chlorophyll-a levels, marking the period during clam sampling.

200 clams with a shell length (SL) exceeding 30 mm were collected from each site for the study. Upon arrival at the laboratory, gonad smears were examined under a light microscope, with only female clams being selected and utilized in the subsequent analysis.

Monthly sea surface temperature (SST) and chlorophyll-a data for the study sites were obtained from the Giovanni online data system, accessible through NASA GES DISC (https://giovanni.gsfc.nasa.gov/). Fig. 2 depicts the seasonal dynamics of SST and chlorophyll-a level variations across all study sites, ranging from 2.3 °C in winter to 27.7 °C in summer, over the period from July 2005 to June 2006. During winter, WD area on the south coast exhibited the highest SST, reaching up to 8.5 °C, while the northernmost study site SJ recorded the lowest SST below 2.7 °C. Seasonal fluctuations in chlorophyll-a levels were evident across the study sites throughout the study period. The WD area on the south coast consistently showed the lowest chlorophyll-a levels, measuring below 3 µg/L, whereas other sites recorded chlorophyll-a levels of up to 20 µg/L.

2.2. Histology

After recording the shell weight, shell length (i.e, the longest axis of the shell), and wet tissue weight, a cross-section of the tissue, measuring 2–3 mm in thickness, was excised from the dorso-ventral region, encompassing the gonadal tissue, foot, and gill, and fixed in Davidson's solution. Approximately 5 µm thin sections were sliced from the paraffin-embedded blocks, stained with Haematoxylin and Eosin (HE),

Table 1
Importance weight (W) of the observed histopathology alterations and their scoring in the gonads of the *Ruditapes philippinarum* (W: 1, Minimal pathological importance; 2, Moderate pathological importance; 3, Marked pathological importance; Score: 0, No alteration; 2, Minimum alteration; 4, Moderate alteration; 6, Severe alteration).

Organ	Reaction pattern	Tissue Alteration	w	Score
Gonad	Connective tissues alteration	Hemocytic infiltration	1	0, 2, 4, 6
		Fibrosis	2	0, 2, 4, 6
		Granulocytomas	2	0, 2, 4, 6
	Follicular alteration	Necrosis	3	0, 2, 4, 6
		Hemocytic infiltration	2	0, 2, 4, 6
		Oocyte atresia	3	0, 2, 4, 6
	Parasitosis	*Perkinsus*	3	0, 2, 4, 6
		other parasites	2	0, 2, 4, 6

and examined under a light microscope. The remaining tissue was subjected to lyophilization and preserved at a temperature of −70 °C, and the egg quantity using ELISA and condition index (CI) was determined as CI = (dry tissue weight/dry shell weight) × 1000. Based on digitized microscopic images of the cross-section, the reproductive stages were classified into five categories: 1) developing, 2) ripe, 3) partially spawned, 4) spent/reabsorbing, and 5) resting. The percentage of gonad area (PGA), representing the ratio of gonadal area to the total cross-sectional area was also measured from the microscopic image according to Kang et al. (2003).

2.3. Histopathological condition indices

The histopathological condition index (HPI) was initially developed to evaluate environmental quality based on tissue alterations in fish (Bernet et al. 1999). The technique was later modified to assess clam tissues in environmental quality studies (Costa et al., 2013). Table 1 outlines the specific types of tissue alterations examined in Manila clam gonad. Accordingly, the tissue alterations were categorized into follicular alteration, alterations in connective tissues, and parasitosis; then, each identified tissue alteration was assigned an importance weight, ranging from 1 to 3, to signify its biological significance for the organism. Subsequently, a scoring system was applied to measure the severity of the impact of each tissue alteration, ranging from 0 (indicating no alteration) to 6 (indicating severe alteration), as detailed in Table 1. Accordingly, the HPI was calculated based on the assigned importance weight (w) and the extent of diffusion (score). The importance weights proposed in this study were derived from the current observations and partly learned from the literature on bivalve histopathology (Beninger, 2017; Costa et al., 2013; Donaghy et al., 2009; Joshy et al., 2022; Yang et al., 2012; Yavasoglu et al., 2016). Accordingly, necrosis, oocyte atresia, and *Perkinsus* infection were assigned the highest weight (w = 3), followed by fibrosis, granulocytomas, and other parasites with w = 2. Inflammation-related alterations, excluding haemocytic infiltration inside the follicular lumen (w = 2), received the lowest weight (w = 1). Notably, infections by protozoans, particularly *Perkinsus*, have been linked to significant Manila clam mortalities in Korean waters (Kang et al., 2015; Lee et al., 2021; Nam et al., 2018; Park and Choi, 2001). Hence *Perkinsus* parasites were assigned an importance weight of 3. Conversely, trematode infections were rare in the present study and were assigned a weight of 2 in clams.

Fig. 3 and Fig. 4 describes the scoring method applied to tissue alterations in the gonads. In calculating the HPI, the modified equation introduced by Costa et al. (2013) was employed.

$$I_h = \frac{\sum_1^k w_k a_{kh}}{\sum_1^k M_k}$$

Here, I_h represents the HPI for the gonad h^{th} individual, W_k is the weight of the k^{th} histopathological alteration, a_{kh} is the score value for the extent of diffusion of the k^{th} alteration in the h^{th} individual, and M_k is the attributable maximum (i.e., importance weight multiplied by the maximum score 6) for the k^{th} alteration. The denominator of the equation normalizes the final HPI value (*I*h) between 0 and 1, allowing for comparisons between distinct situations, such as clams from different locations. The average gonad HPI across sampling locations was then assessed, compared, and categorized as low (0.00–0.30), moderate (0.31–0.60), and high (0.61–1.00) based on the average HPI value (Joshy et al., 2022).

2.4. Quantification of *P. olseni* infection using RFTM assay

The infection intensity of *P. olseni* was determined using Ray's fluid thioglycollate medium (RFTM) assay and subsequent 2 M NaOH digestion (Choi et al., 1989; Ray, 1966). One part of the gill was dissected and placed in 5 ml of fluid thioglycollate medium (FTM) solution in a conical tube. Nystatin (200 units/mL) and chloramphenicol (100 ng/mL) were added in the solution to prevent bacterial growth. Following a one-week incubation in darkness at room temperature, the gill tissues underwent digestion in 2 M NaOH at 60 °C. After removing the NaOH solution, the number of *P. olseni* hypnospores in a subsample was counted, and the infection intensity was expressed as the number of *P. olseni* cells per gram of gill tissue (Park et al., 2006; Lee et al., 2021; Subramaniam et al, 2024).

2.5. Quantification of reproductive effort using ELISA

The indirect enzyme-linked immunosorbent assay (ELISA) along with the rabbit anti-clam egg protein antibody developed by Park and Choi (2004) was adapted to quantify the egg mass. Approximately 10 mg of the lyophilized clam tissue was homogenized in phosphate-buffered saline (PBS, pH 7.4) using an ultrasonifier, and diluted up to 1000-fold. A 100 µL aliquot of the diluted homogenate was loaded into a 96-well microplate, along with a positive control of homogenized clam eggs ranging from 0.1 to 5 µg. The rabbit anti-Manila clam egg protein IgG (Park and Choi, 2004) was used as the primary antibody and goat anti-rabbit IgG alkaline phosphatase-conjugate was used as the secondary antibody. A standard regression curve was constructed based on the optical density of a known quantity of the egg mass included in the plate. The concentration of egg protein in the tissue homogenate was then estimated from the regression curve and the dilution factor. Finally, to quantify eggs, the amount of egg protein measured by ELISA was multiplied by 2.44, representing the egg protein ratio to the total egg weight, as established by Park and Choi (2004).

3. Results

3.1. The condition index (CI)

Table 2 summarizes the biometry of clams collected from four tidal flats on the west and south coasts. The clams used in the study were believed to be over 3-year-old adults with SL ranging from 35.6 mm to 38.9 mm and WTW from 2.215 to 4.630 g. The mean CI of clams collected in late June varied from demonstrated variation across sites, ranging from 109 (WD) to 193 (JG) (Fig. 5). The ANOVA Duncan's range test indicated that the mean CIs of clams from SJ (188) and JG (193) on the northwest coast were significantly higher than those for the clams from GS (117) and WD (109) on the south coast ($P < 0.05$). The ANOVA test also revealed no significant difference between the mean CI of clams from JG and SJ, as well as between those from WD and GS (Fig. 5).

3.2. Reproductive condition

Fig. 6 illustrates the gonadal stages of clams along the various coastal sites. As of June 30th, the majority of clams collected from JG and SJ on the northwestern coast, 94 % and 95 % respectively, were in the ripe stage, characterized by fully mature eggs densely packed within the

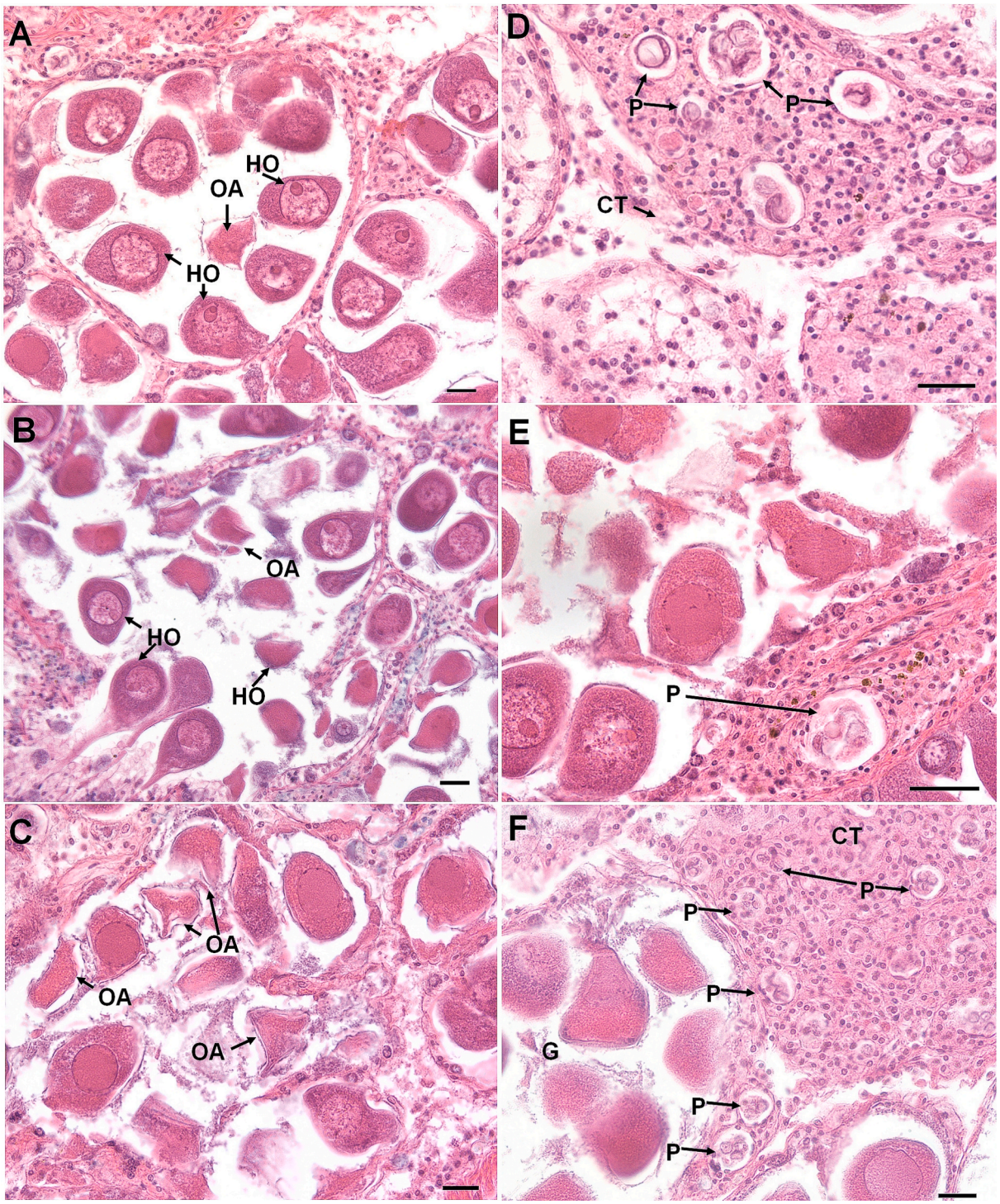

Fig. 3. A. Microphotographs depicting histopathological alterations in the gonad of *Ruditapes philippinarum* from various locations. A–C: Diffusion of oocyte atresia; A (score 2): Minimal tissue alteration due to oocyte atresia, where only a few oocytes among the healthy ones exhibit symptoms such as cytoplasmic retraction and altered shape; B (score 4): Moderate tissue alteration due to oocyte atresia, with a moderate number of oocytes displaying symptoms of oocyte atresia; C (score 6): Extensive tissue alteration due to oocyte atresia, wherein most oocytes in the gonad exhibit pronounced cytoplasmic retraction, shrinkage, and distorted forms, accompanied by a high prevalence of degenerated oocytes. D–F: Diffusion of *Perkinsus*; D (score 2): Minimal *Perkinsus* dissemination, with *Perkinsus* cells invading the connective tissue around the gonad; E (score 4): Moderate *Perkinsus* diffusion, characterized by *Perkinsus* cells invading the connective tissue and a few observed near the gonad wall; F (score 6): Heavy infestation of *Perkinsus* cells in the connective tissue around the gonad and near the gonad wall. G: gonad, HO: healthy oocyte, OA: oocyte atresia, CT: connective tissue, P: *Perkinsus*. (Scale bar = 20 μm).

follicles. Histological analysis revealed that a small fraction, 1.7 % from SJ and 1.9 % from JG, were actively spawning at the end of June. In contrast, the WD tidal flat on the south coast and the GS tidal flat on the southwest coast exhibited significantly higher spawning activity, with 48 % and 15 % of clams engaged in spawning, respectively. Additionally, a larger proportion of clams at WD (48 %) and GS (25 %) were still in the developing stage, compared to only 3.4 % at SJ and 3.7 % at SJ and JG on the northwestern coast.

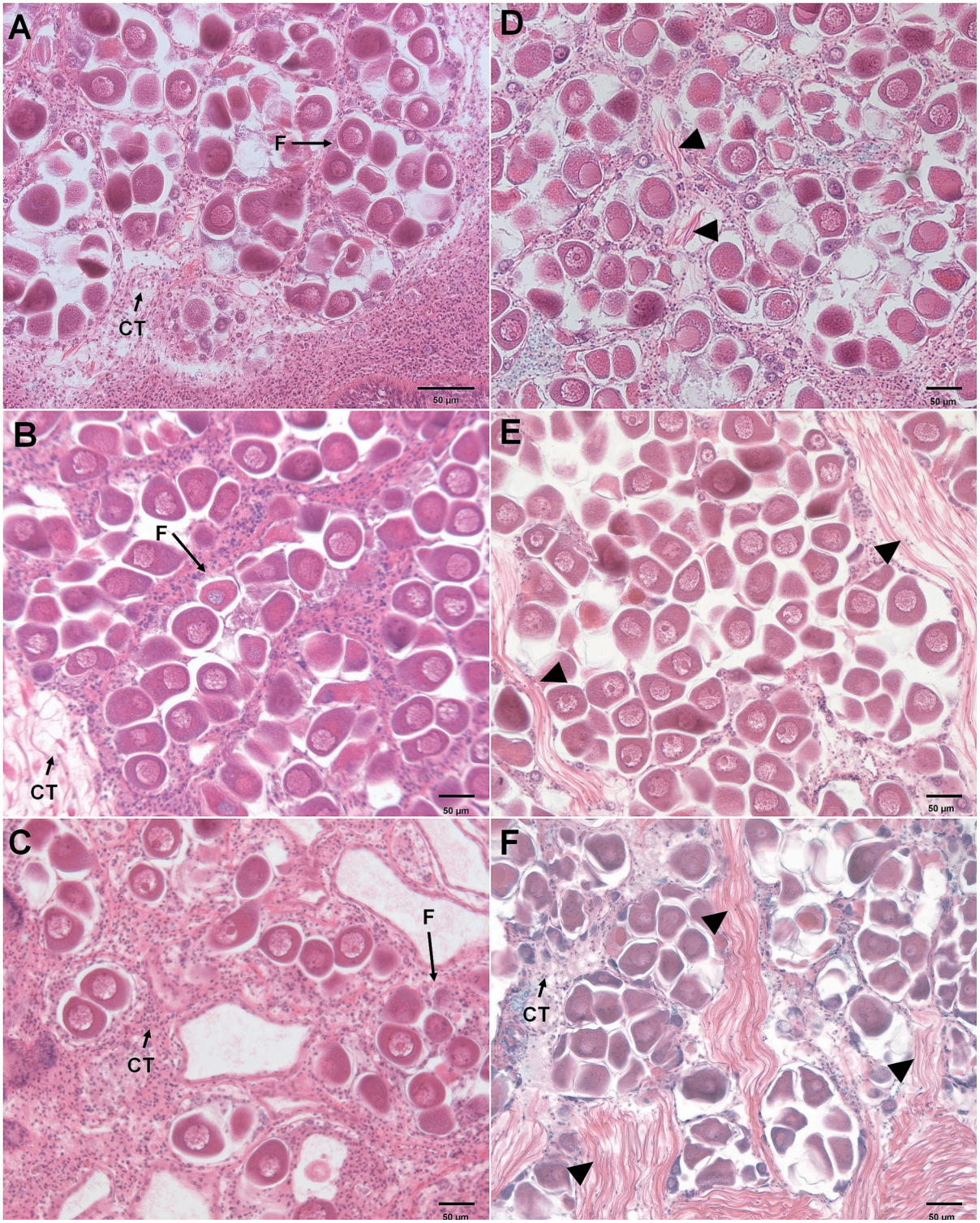

Fig. 4. Microphotographs depicting histopathological alterations in the gonad of *Ruditapes philippinarum*, including diffusion of hemocyte infiltrations (A–C) and fibrosis (D–F). A, Minimal impact of hemocyte infiltration (score 2). The hemocytes aggregate around the gonads; B, demonstration of moderate histopathological alterations reflecting a noticeable effect of hemocyte infiltration on gonadal tissues (score 4). Infiltration of hemocytes within the follicle could be noticed; C, Extensive and widely spread hemocytes inside and outside of the follicle, indicating a severe disruption in gonadal morphology (score 6). D, Minimal histopathological changes with less evidence of fibrosis. The tiny fibrosis around the connective tissues (CT) could be observed (score 2); E, Moderate histopathological alterations reflecting a noticeable fibrosis effect on CT. Consistency and prominent fibrosis could be noticed (score 4); F, Extensive and widely disseminated large fibrosis around gonads, indicating a severe disruption in gonadal morphology (score 6). F: follicle. (Scale bar = 50 µm).

Table 2
Summary of sampling efforts. The values represent the monthly mean ± the standard error. SJ, Sunjae; JG, Boryung; GS, Gomso; WD, Wando; N, number of clams used in the analysis; SL, shell length in mm (SL); WTW, wet tissue weight in gram.

Sites	N	SL (mm)	WTW (g)
SJ	57	38.0 ± 0.9	3.992 ± 0.100
JG	54	38.9 ± 0.8	4.630 ± 0.177
GS	57	35.6 ± 0.7	2.215 ± 0.061
WD	57	36.7 ± 0.7	2.757 ± 0.079

Fig. 7 presents the mean Gonadosomatic Index (GSI) and Percentage Gonadal Area (PGA) of clams in the ripe stage. The non-parametric Kruskal-Wallis H-test and subsequent post-hoc Tukey's range test showed significant differences in GSI, with clams from SJ (12.0, N = 55) and JG (15.0, N = 51) having higher means compared to those from GS (5.7, N = 15) and WD (5.3, N = 23), with a significance level of $P < 0.05$. Similarly, PGA measurements were significantly higher in clams from SJ (46.6) and JG (45.8) than those from GS (26.9) and WD (29.1), also significant at $P < 0.05$. These findings indicate that the reproductive efforts of clams at SJ and JG were markedly greater than those at GS and WD during the study period.

3.3. *P. olseni* prevalence and infection intensity

Fig. 8 plots the mean infection intensity and prevalence of *P. olseni* across four intertidal areas on the west and south coasts as measured on June 30th. On this date, the prevalence of *P. olseni* infections (i.e., the percentage of infected clams in a population) ranged from 89.5 % in SJ to 98.2 % in both GS and WD. The infection intensity, determined by the RFTM and subsequent counting of *P. olseni* hypnospores in gill tissues, varied significantly, ranging from 415,000 cells/g gill at SJ to 1,586,000 cells/g gill at WD. Statistical analysis using the non-parametric Kruskal-Wallis H-test, followed by post-hoc Duncan's range test, revealed significant differences in mean infection intensities. Specifically, clams from WD (1,586,000 cells/g gill) and GS (1,391,000 cells/g gill) had significantly higher infection intensities compared to those from JG (1,374,000 cells/g gill) and SJ (415,000 cells/g gill), with $P < 0.05$. Additionally, clams from JG exhibited a significantly higher infection intensity than those from SJ, also significant at $P < 0.05$.

3.4. *Histopathological condition indices (HPI)*

Fig. 9 shows common pathological features observed in Manila clams from different sampling sites. The normal microscopic anatomy of Manila clam gonads consists of a single layer of germinal cells lining the follicle, with the lumen densely filled with mature oocytes (Fig. 9A). A common pathological change is hemocyte infiltration (HI), characterized by a marked accumulation of hemocytes within the follicular lumen and the surrounding connective tissue regions between follicles (Fig. 9B), particularly noted in clams from the WD tidal flat. These clams often exhibited additional histopathological features, including fibrosis and granuloma formation in the spaces of the connective tissue around gonadal follicles (Fig. 9D). Atresic oocytes, indicative of degenerating oocytes, were frequently observed in clams from WD and GS; over 97 % of clams in the ripe and partially spawned stages showed signs of oocyte atresia (Fig. 9E). Necrosis of the germinal epithelium was also a frequent finding, particularly in clams from the WD tidal flat. Numerous trophozoites of *P. olseni* were identified in the connective tissues of the follicles (Fig. 9G), and occurrences of metacercaria and sporocysts of cercaria were sporadically reported in some clams (Fig. 9H).

As shown in Fig. 9, the mean HPI of Manila clams collected from the west and south coasts varied from 0.18 (SJ) to 0.47 (WD). One-way ANOVA test indicated that the mean HPIs exhibited a significant latitudinal gradient ($P < 0.05$), as the highest HPI recorded at WD (0.47) was significantly higher than GS (0.35), JG (0.30), and SJ (0.19). Conversely, the northernmost site, SJ, recorded a significantly lower HPI score of 0.18, indicative of fewer tissue abnormalities than observed at

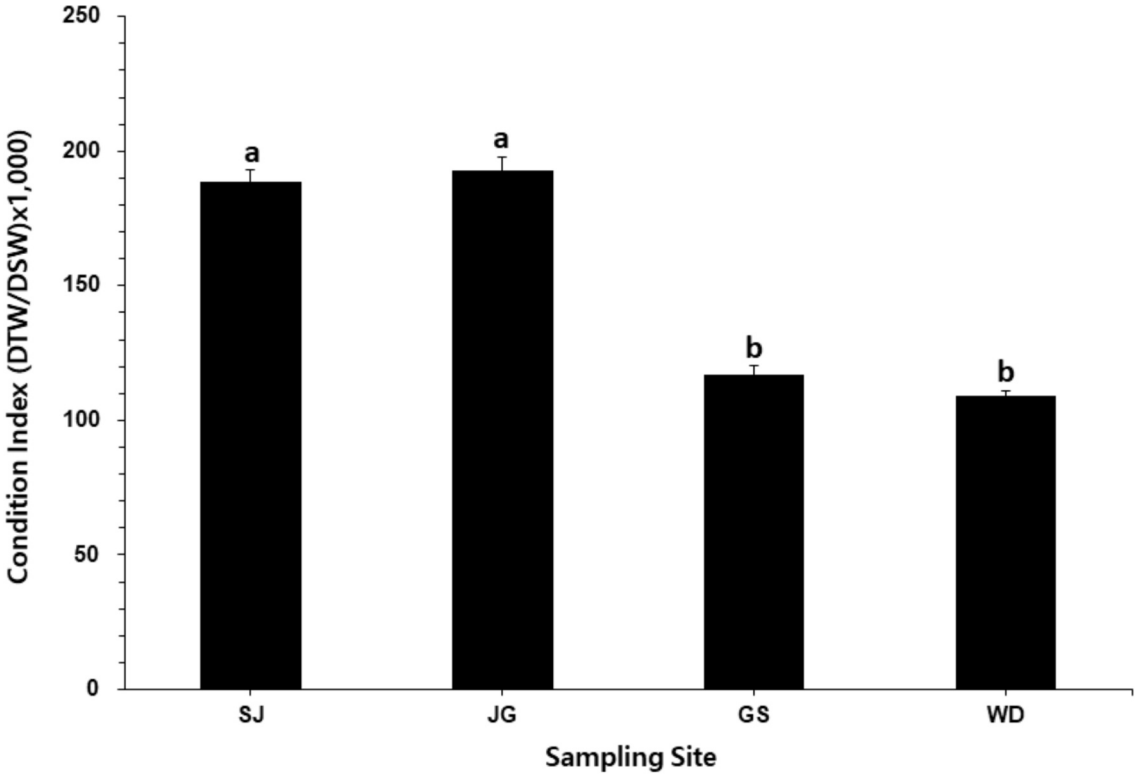

Fig. 5. Spatial variations of condition index (CI) of *Ruditapes philippinarum* collected in this study. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando. Different letters (a, b) in the mean values columns represent significant differences among sampling sites (ANOVA, $P < 0.05$).

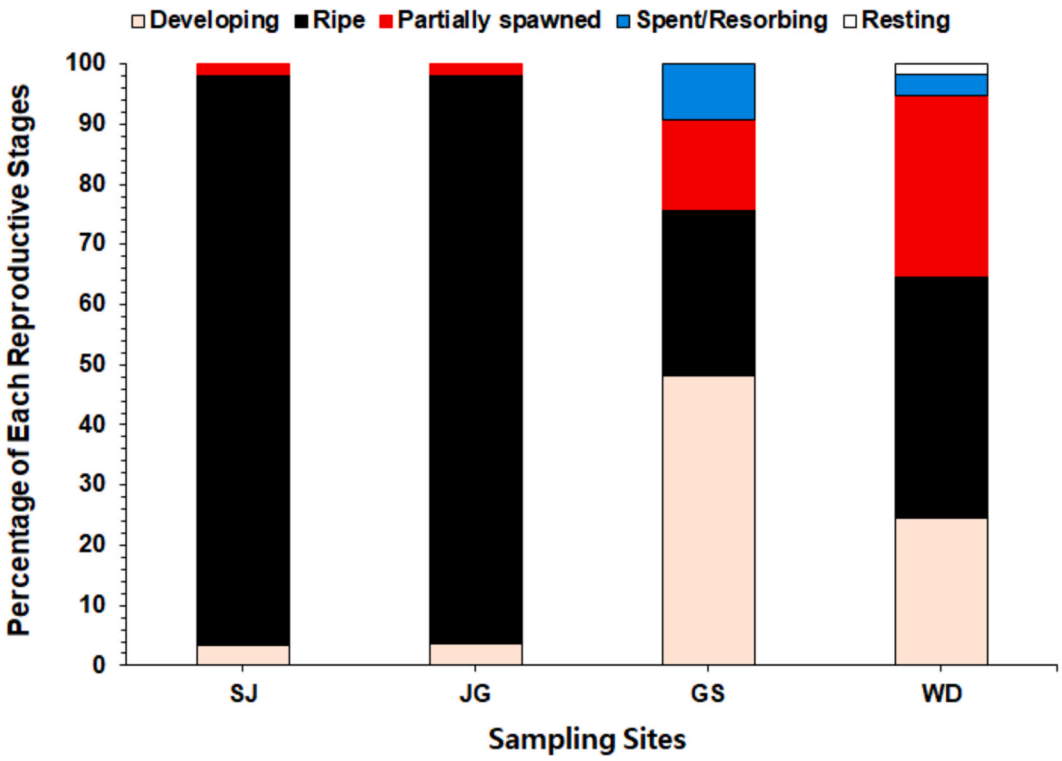

Fig. 6. Distribution of gametogenic stages of the female *Ruditapes philippinarum* collected in this study. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando.

other sites. The present study identified three principal histopathological patterns affecting Manila clams: follicular alterations, connective tissue alterations, and parasitosis (Fig. 9). These factors collectively influence the overall HPI in clams. Notably, clams from WD displayed elevated levels of connective tissue alterations at 0.17, follicular alterations at 0.21, and parasitosis at 0.09, relative to other sites. The significant increase in follicular alterations was particularly attributed to a high incidence of oocyte atresia and parasitosis, predominantly caused by *P. olseni*. It was noted that clams from SJ, which reported the lowest HPI, also showed lower levels of connective tissue alterations at 0.06, follicular alterations at 0.10, and parasitosis at 0.01 compared to other sites.

Fig. 10 also plots the percentage of clam HPI categories, as low (0.00–0.30), moderate (0.31–0.60), and high (0.61–1.00). Notably, 32 % of clams from WD were classified within the high HPI category, in stark contrast to only 3.1 % and 3.5 % from sites JG and GS, respectively. Although the proportions of clams in the high HPI category were similar for JG and GS, a significantly larger percentage of clams at GS were in the moderate HPI group, suggesting more pronounced histopathological alterations at this site compared to JG. Conversely, site SJ reported no clams in the high HPI category, with the majority over 86 % exhibiting low HPI, thus indicating a minimal pathological impact on clams at this location.

4. Discussion

In this study, Manila clams were systematically collected along a latitude gradient from the northernmost coast of SJ to the southernmost WD, covering a vertical distance of approximately 400 km, all on the same day. The simultaneous sampling approach was designed to elevate the quality of results by ensuring a precise assessment of the reproductive condition of the studied clams while mitigating potential variations that could arise from sampling on different days or weeks in this study. Consequently, the results of this study unequivocally highlight the apparent influence of latitude differences and *Perkinsus* parasitism on the reproductive dynamics of Manila clams. Studies have been conducted on the reproductive stage and reproductive output analysis along the latitudinal gradient (Clarke, 1987; Mahony et al., 2021), given the interdependence and influence of temperature gradient along the latitude.

The delayed maturation or different development of gonads in bivalves can be attributed to various factors, such as delay in reaching optimal SST (Park and Choi, 2004; Uddin et al., 2010; Kang et al., 2015), limited food availability (Delgado and Camacho 2005; Uddin et al. 2012), and parasitic infections (Choi et al., 1989; Burreson and Calvo, 1996; Lee et al., 2020). Our current investigation shows that study sites SJ and JG on the northwest coast predominantly reported clams in the ripe gonadal stage, with a small proportion in a partially spawning condition. Conversely, southernmost sites GS and WD exhibited a mix of gonadal stages with resting gonadal stage, a rare occurrence during the end of June (Park and Choi, 2004; Uddin et al., 2012). During the transition from winter to summer, the SST influences the timing of optimal gametogenesis differently in low-latitude and high-latitude regions. In low-latitude areas, the optimal SST for gametogenesis is achieved earlier, leading to the earlier development of ripe oocytes in the gonads by allocating more energy to reproduction rather than metabolism. Conversely, clams in high-latitude regions allocate more energy to metabolism, reducing energy allocation for reproduction (Clarke, 1987; Gosling, 2003). However, the presence of mixed gonadal stages and lack of ripe oocytes in the gonads raise speculation regarding additional factors influencing their reproductive dynamics other than SST in low-latitude study sites. As reported in previous studies, most Manila clams on the west coast of Korea initiate spawning during the summer, triggered by reaching the threshold SST of approximately 25–26 °C (Uddin et al., 2012), along with other factors such as adequate food. Even within a particular bivalve species, the timing of gametogenesis and spawning can vary temporally at specific geographic locations (Navarro et al., 1989; Mahony et al., 2021). Nevertheless, the unusual gonadal stage complications in Manila clams from Gomso and Wando were reported despite favorable SST conditions, prompting further discussion and investigation into the underlying factors influencing these anomalies.

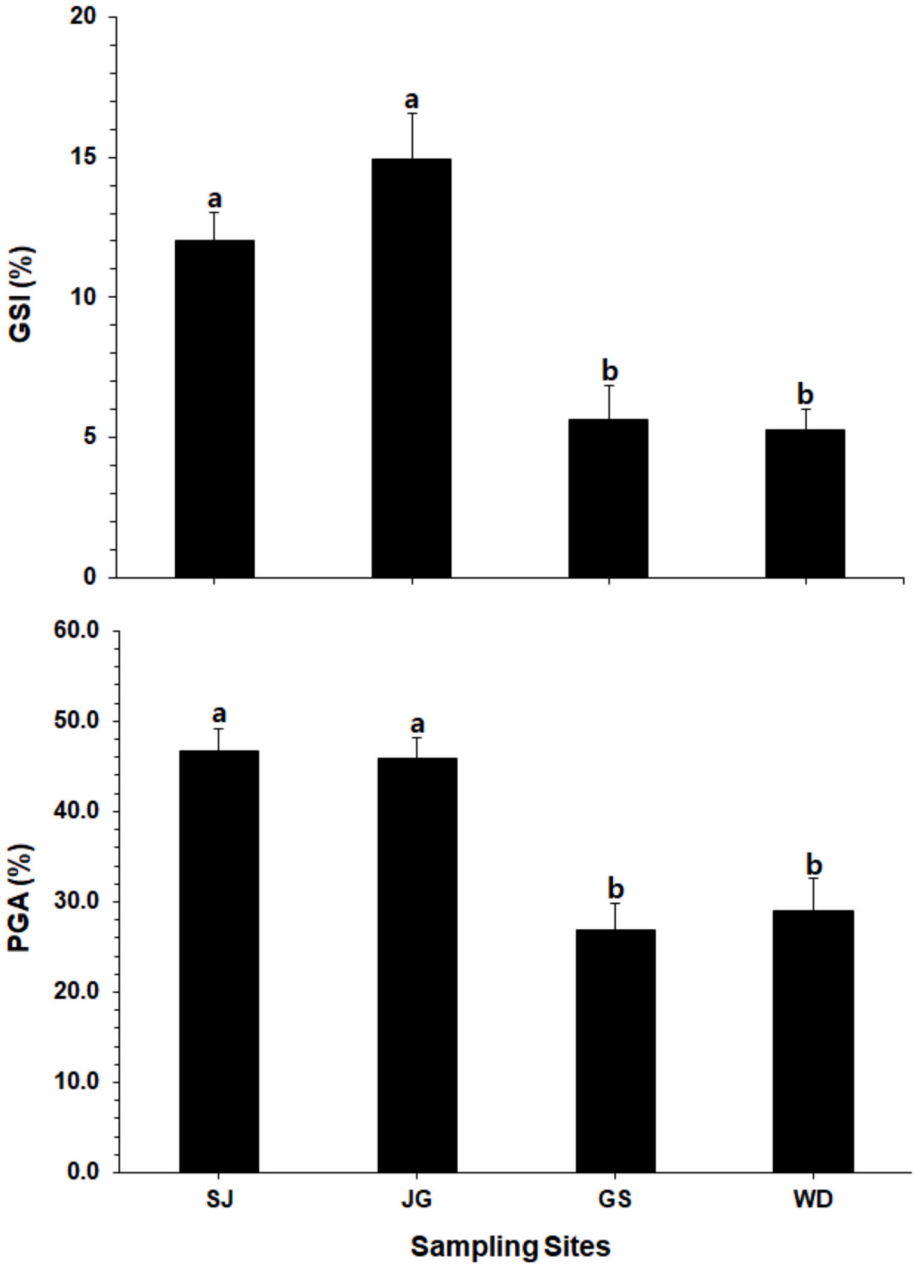

Fig. 7. Gonad somatic index (GSI, A) and percentage of gonad area (PGA %, B) of the female Manila clams in the ripe stage. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando. Different letters (a,b) in the mean values represent significant differences among sampling sites (ANOVA, $P < 0.05$).

Perkinsus infection in Manila clams leads to a range of consequences, including reduced reproductive output, decline in the condition index, diminished growth, and the occurrence of atrophy in host tissues, often culminating in mortality (Park et al., 2006; Donaghy et al., 2009; Soudant et al., 2013; Lee et al., 2020; Yang et al., 2021). The latitudinal gradient reveals an increase in *P. olseni* infection intensity from the north coast to the south coast, with SJ, the northernmost coast, showing significantly lower *P. olseni* infection intensity compared to the other three study sites. Interestingly, JG, GS, and WD exhibited similar infection intensities (Fig. 8). However, the intriguing absence of apparent impacts on the reproductive cycle and output observed in JG contrasts with observations in GS and WD. This discrepancy could be elucidated by considering the concurrent high infection intensity and abundant food availability during clams sampling in JG. The nutritional abundance in JG may facilitate energy expenditure associated with *P. olseni* infection without adversely affecting clam growth and reproduction (Choi et al., 1989; Yang et al., 2012; Subramaniam et al., 2024).

The SST plays a pivotal role in limiting the geographical distribution, abundance, and proliferation of *P. olseni* (Burreson and Calvo, 1996; Park and Choi, 2001). A positive correlation between SST and the proliferation rate of *P. marinus* was observed up to 35 °C (Dungan and Hamilton, 1995), suggesting that elevated SST in GS and WD along the latitudinal gradient may have led to increased *P. olseni* proliferation and activity against the host, consequently decreasing in reproductive output in these regions (Hofmann et al., 1992; Burreson and Calvo, 1996; Uddin et al., 2012). Therefore, the seasonal variations in SST critically influence the seasonal abundance of *P. olseni*, with the low SST (<5°C) during winter contributing to relatively minimal infection intensity and prevalence, leading to a decrease in infection levels in early spring (Park et al., 2006; Gleason et al., 2017; Lee et al., 2021). As SST progresses from spring, *P. olseni* infection intensifies, particularly when

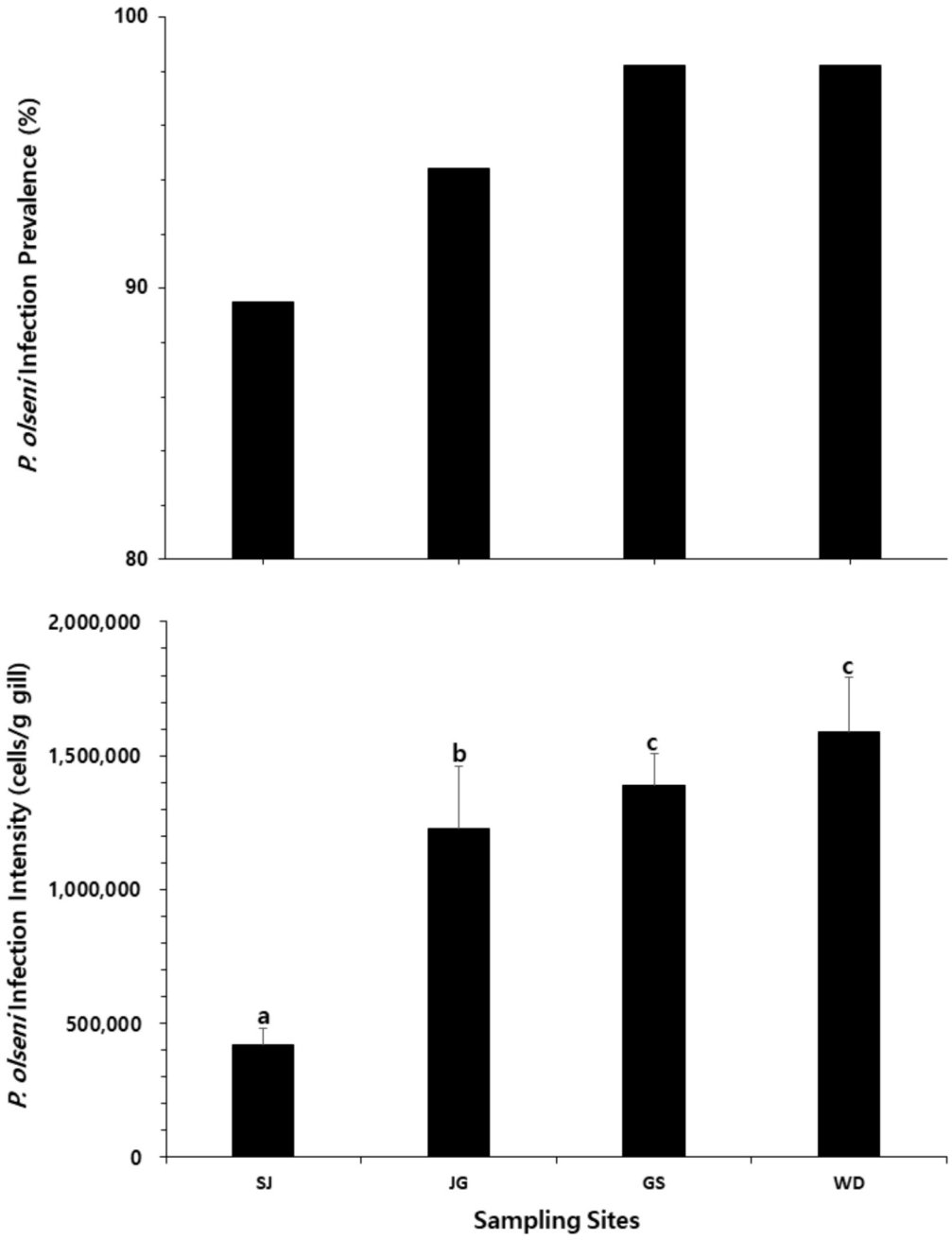

Fig. 8. Infection prevalence of *P. olseni* and total number of *Perkinsus olseni* cells per gram gill in *Ruditapes philippinarum* samples collected in this study. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando. Different letters (a,b) in the mean values columns represent significant differences among sampling sites (ANOVA, $P < 0.05$).

SST exceeds 20 °C, facilitating the proliferation (Dungan and Hamilton, 1995; Burreson and Calvo, 1996). Notably, in this study, winter SST ranged from 7.8 to 8.5 °C on the south coast, conducive to *P. olseni* sustaining normal physiological functions more effectively, resulting in peak infection intensities on the south coast. Additionally, the consequence of *P. olseni* infection tends to be higher when the host exhibits low immune capacity or the *P. olseni* expresses high virulence (Hasanuzzaman et al., 2016; Proestou and Sullivan, 2020). Hence, we assume that despite the study sites exhibiting similar infection intensity, variations in tissue damage may occur based on the virulence of *P. olseni*. Further investigation is necessary to elucidate the relationship between the virulence of *P. olseni*, infection intensity, and resulting host tissue damage by *P. olseni* parasitism.

We hypothesize that despite favorable SST conditions for clam gametogenesis and early spawning on the south coast, the observed mixed reproductive stage in clams may be influenced by additional factors such as elevated *P. olseni* infection intensity and limited food availability. According to Kang et al. (2015), previous studies across the west and south coasts consistently revealed a comparable frequency distribution of gametogenic stages in female Manila clams, with the south coast notably exhibiting approximately 50 % of clams in the spawning reproductive stage in May. Moreover, the south coast documented the highest *P. olseni* infection intensity, causing smaller follicle size and fewer ripe eggs in the follicle, negatively impacting clam reproduction by impeding gonad maturation and declining GSI. Interestingly, JG exhibited a normal reproductive stage and a high CI, similar to the northernmost study site SJ, despite experiencing high *P. olseni* infection intensity. This observation suggests a potential influence of food availability that aligns with the finding by Subramaniam et al. (2024). Their study indicated that Manila clams could sustain a higher

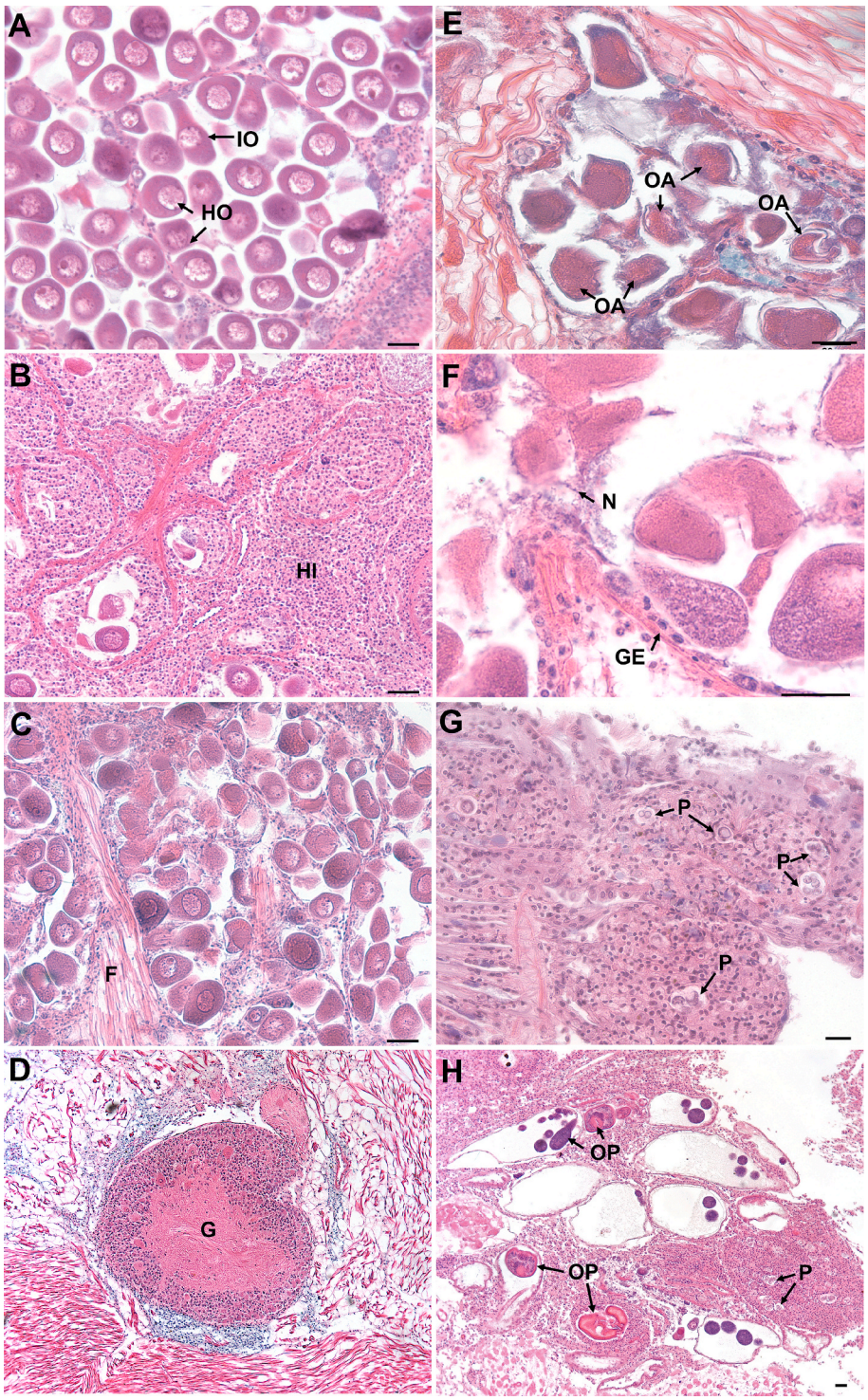

Fig. 9. Representative microphotographs of observed histopathological alterations in the gonads of *Ruditapes philippinarum* collected from the study locations. A, the normal structure of gonads with healthy oocytes (HO) and immature oocytes (IO); B, occurrence of hemocyte infiltration (HI) near the gonadal tubules and around the oocytes; C–D, presence of fibrosis (F) and a granuloma (G); E, evidence of oocyte atresia (OA); F, occurrence of necrosis (N) in the germinal epithelium (GE) of gonad; G, *Perkinsus* infection (P); H, occurrence of trematode infection (OP) along with *Perkinsus*. (Scale bar = 20 μm).

CI even in the presence of a high parasite load, potentially attributable to the abundance of nutrients and chlorophyll-*a* content in the water. Therefore, our hypothesis integrates these factors to provide a comprehensive understanding of the complex interactions affecting clam reproductive dynamics on the south coast.

Histology observation revealed apparent lesions and alterations in Manila clams across the sites may be related to considerable variation in SST, food availability, and *P. olseni* infection intensity. Unfavorable SST conditions, coupled with intense *P. olseni* infections, frequently coincide with disrupting various functions in bivalves, including the reproductive process (Choi et al., 1989; Burreson and Calvo, 1996; Uddin et al., 2012; Kang et al., 2015; Lee et al., 2020). Quantifying the impacts of various factors on bivalves remains challenging in field conditions, yet the HPI provides a comprehensive evaluation of bivalve health by assessing

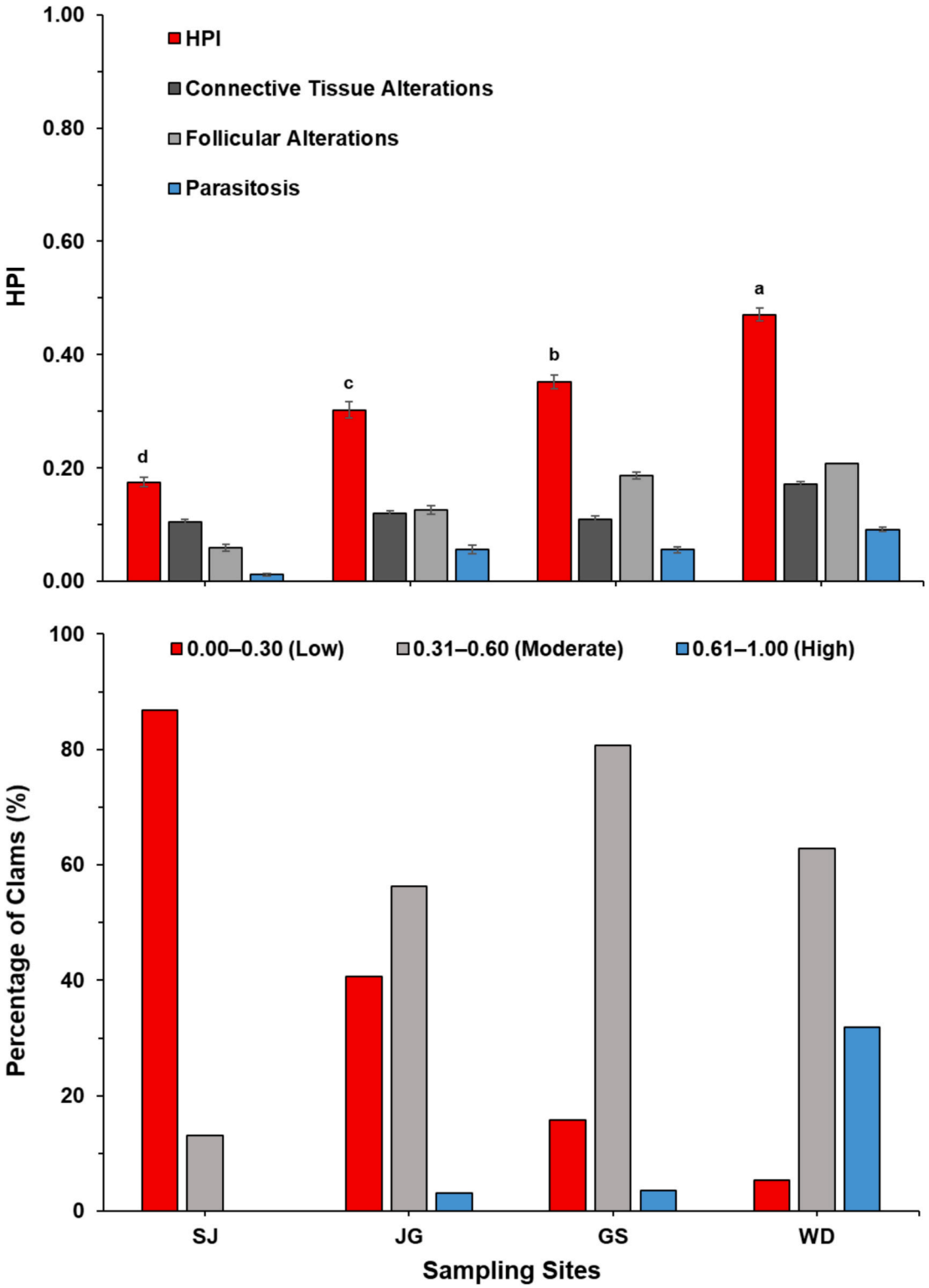

Fig. 10. HPI and the respective proportions of HPI categories, including connective tissue changes, follicular alterations, and parasitosis. The percentage of *Ruditapes philippinarum* is categorized into low, moderate, and high HPI groups across different locations in this study. SJ, Sunjae; JG, Jugyo; GS, Gomso; WD, Wando. Different letters (a, b, c, and d) in the mean values columns represent significant differences among sampling sites (ANOVA, $P < 0.05$).

tissue alterations, irrespective of the underlying causes (Costa et al., 2013; Yavasoglu et al., 2016; Bennion et al., 2022). In the present study, detailed histology of the female clams gonads revealed microscopic lesions such as hemocyte infiltration, fibrosis, granulocytomas, oocyte atresia, necrosis, and *P. olseni* infection at each site. Notably, oocyte atresia, necrosis, and hemocyte infiltration were more prevalent in WD and GS clams, while most SJ and JG clams were at the mature reproductive stage with a low level of tissue abnormality. Despite similar *P. olseni* infection intensity and prevalence reported at WD and GS sites, the HPI in WD is significantly higher than in GS, which may be attributed to pronounced alterations in connective tissues, leading to significant tissue impacts and elevating the HPI in WD. Still, the incidence of

follicular alteration was slightly higher in WD compared to GS, mainly attributed to oocyte atresia, with the presence of mixed gonadal stages observed in both WD and GS (refer to Fig. 6). Although, JG exhibited a low level of *P. olseni* infection intensity than GS, similar impacts induced by *P. olseni* between JG and GS, suggesting that even with low infection intensity, tissue damage could be increased due to the high virulence of parasites, coupled with the low immune capacity of the host, as well as limited food availability and unfavorable SST for the host while favorable conditions for parasites (Choi et al., 1989; Dungan and Hamilton, 1995; Burreson and Calvo, 1996; Hasanuzzaman et al., 2016; Proestou and Sullivan, 2020).

The histopathological condition indices of individual clams, derived from the formula, were categorized to establish a qualitative ranking system, defined as follows: low prevalence of histopathological alterations (0–0.30); moderate (0.31–0.60); and high (0.61–1.0) (Joshy et al. 2022). Accordingly, very few clams in SJ showed the moderately impacted category and no highly impacted clams, resulting in a low average HPI. Conversely, JG, GS, and WD reported a higher proportion of moderately impacted and highly tissue-damaged clams, leading to higher HPI scores in respective order. Despite clam from SJ and JG exhibiting well-developed gonads and ripe oocytes in the histological analysis (see Fig. 6), the HPI scores suggest that JG also experiences gonadal tissue damage, possibly due to a higher *P. olseni* infection rate compared to SJ (refer to Fig. 8). However, despite the tissue damage, JG reported high CI and GSI, indicating that multiple factors influence the overall reproductive success, fitness and health condition of bivalves (Uddin et al. 2012; Subramaniam et al. 2024).

Examining the features related to inflammatory responses, particularly hemocyte infiltration, has been extensively investigated in bivalves using histopathology (Ordas et al., 2001; Donaghy et al., 2009; Yang et al., 2012; Kim et al., 2021). Numerous studies have undertaken visual inspections to elucidate the morphological alterations associated with hemocyte infiltration induced by factors such as reproductive activity (Kim et al., 2022), parasitic infestations (Ordas et al., 2001; Donaghy et al., 2009; Bennion et al., 2022), and exposure to heavy metals (Yavasoglu et al., 2016; Joshy et al., 2022). Additionally, SST, food availability, and exposure to toxicants significantly influence hemocyte physiology and count in various studies exploring the impact on hemocyte dynamics (Ballina et al., 2022; Kim et al., 2022). Therefore, the comprehensive evaluation of these inflammatory changes, such as hemocyte infiltration, provides valuable insights into the intricate interplay between immune responses and unfavorable environmental conditions in bivalves (Donaghy et al., 2009). Hemocyte infiltration is generally seen in the latter phases of reproduction in all bivalves, where it functions to reabsorb leftover relict oocytes; yet, this process is universal in all bivalves (Beninger, 2017; Cherel et al., 2020). In our study, an unusually intense hemocyte infiltration was widely noted on the south and southwest coasts. This intensity was observed with hemocyte aggregation proximal to heavy *P. olseni* infestation, surrounding gonads, and even within gonadal tissues. In response to detecting foreign materials in clam tissues, hemocytes migrate to the affected sites, a process induced and regulated by soluble molecules known as chemotaxins, released either by the foreign agent or host cells, which is not exclusively associated with immune responses (Lopez-Cortes et al., 1999; Lee et al., 2001; Donaghy et al., 2009). In scallop *Pecten maximus*, Beninger et al. (2003) reported the migration of ferritin-bearing hemocytes from the digestive system to oocytes, providing ferritin for the development of oocytes. Given the many factors that can trigger hemocyte migration, such as those mentioned above, it is challenging to speculate on the specific reasons for the hemocyte infiltration observed in WD and GS.

Bivalves exhibit a phenomenon known as oocyte atresia, where they are unable to spawn all of their oocytes before the end of their reproductive period, regardless of their spawning schedule (Beninger, 2017; Sugiura and Kikuya, 2022). Consequently, macrophages reabsorb the leftover gametes, often known as “residual” or “relict” oocytes, degenerating inside the gonad (Beninger, 2017; Cherel and Beninger, 2017). Oocyte atresia is widely acknowledged as a common occurrence in the residual reproduction stage. Given the known reproductive behavior of Manila clams in Korea, where the average spawning period is throughout the summer, oocyte resorption and degeneration typically occur after the primary spawning event (usually late summer to fall) in the typical reproductive process (Park and Choi, 2004; Park et al., 2006; Uddin et al., 2012). Therefore, the reported incidence of oocyte atresia in WD and GS suggests distinctive pre-spawning oocyte atresia in Manila clam. Understanding the timing and prevalence of oocyte atresia in the pre-spawning phase is crucial, as it sheds light on the reproductive dynamics of these bivalves, potentially influencing fecundity estimates and resulting in overestimations of fecundity, reproductive output, and anticipated larval density and recruitment of bivalves (Beninger et al., 2021; Sugiura and Kikuya, 2022).

5. Conclusion

Based on the findings of this study, we conclude that employing a semi-quantitative HPI provides a visual means to assess the impact of adverse environmental conditions, *P. olseni* infections, and other stressors on Manila clam reproduction. The prevalence of mixed gonadal conditions and low CI were notably higher on the south and southwest coast, resulting in reduced reproductive output and fitness compared to the northern coast despite the presence of favorable environmental factors for reproduction. Furthermore, this investigation offers valuable insights into the potential interplay between environmental factors and the intensity of parasitic load, elucidating how these factors may compromise gonadal maturation and reproductive output in the Manila clam.

CRediT authorship contribution statement

Thatchaneshkanth Subramaniam: Writing – original draft, Methodology, Data curation, Conceptualization. **Hyun-Ki Hong:** Writing – original draft, Methodology, Investigation, Conceptualization. **Kyung-Il Park:** Writing – review & editing, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Young-Ghan Cho:** Writing – review & editing, Formal analysis, Data curation. **Kwang-Sik Choi:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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