Effects of Temperature and Salinity on the *in vitro* Proliferation of Trophozoites and the Development of Zoosporangia in *Perkinsus olseni* and *P. honshuensis*, Both Infecting Manila Clam

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ABSTRACT—We evaluated the effects of temperature and salinity on *in vitro* trophozoite proliferation and zoosporangial development in *Perkinsus olseni* and *P. honshuensis*, infecting Manila clam. Trophozoite proliferation was highest at 28°C for both species and at 18–21‰ and 21–33‰ for *P. olseni* and *P. honshuensis*, respectively. Zoospore release rates, as the indicator of zoosporulation, were highest at 25–30°C and 25–35‰ for both species. However, these results seem not to explain the previously observed phenomena, i.e. lower infection prevalence in estuarine tidal flats (the two species not distinguished) and higher infection prevalence of *P. olseni* than *P. honshuensis*.

Key words: *Perkinsus olseni*, *Perkinsus honshuensis*, temperature, salinity, *Ruditapes philippinarum*

In Japan, the annual harvest of Manila clam *Ruditapes philippinarum* has decreased significantly since the mid-1980s and remains low because of a concurrent decline in the abundance of wild Manila clam populations¹⁾. Overfishing, degeneration of sediments, mass depletion of larvae, predators and some other factors have been suggested as the causes of the decline in population abundance¹⁾. Although the exact causes remain unclear, infection with *Perkinsus* species has recently shown up as a major contributor to the depletion of Manila clam resources^{2,3)}. *Perkinsus* infections have been reported in Manila clams throughout Japan, including areas other than northern and eastern Hokkaido^{4,5)}. The prevalence and intensity of infection with *Perkinsus* species varies considerably among different populations of Manila clams^{4,5)}. Manila clams in

Japan have been thought to be infected only with *Perkinsus olseni*. Recently, Dungan and Reece (2006) described a new species, *P. honshuensis*, in Manila clams collected from Mie Prefecture, Japan⁶⁾. However, our prior research suggests that *P. olseni* is the dominant species infecting Manila clams in Japan⁷⁾.

The variation in infection levels among different Manila clam populations and between the two *Perkinsus* species suggests that environmental factors may affect *Perkinsus* infection in Manila clams. We evaluated whether temperature and salinity affected the proliferation of trophozoites and the development of zoosporangia *in vitro* in *P. olseni* and *P. honshuensis*.

Materials and Methods

Manila clams

We purchased uninfected Manila clams (shell length: 35–45 mm) from the Akkeshi Fisheries Cooperative Association. The clams were collected from the tidal flat at Akkeshi, Hokkaido, Japan, an area that is free of *Perkinsus* spp.

Trophozoites and prezoosporangia

We purchased trophozoites of *P. olseni* (PRA-181) and *P. honshuensis* (PRA-177) from the American Type Culture Collection (ATCC) and subcultured them in *Perkinsus* broth medium (PBM) (ATCC medium 1886), 745 mOsm (equivalent to the seawater at a salinity of 22‰), as follows. We added 1 mL of the old culture to 10 mL of fresh medium at 2–3 wk intervals and incubated the culture at 25°C in the dark.

Prezoosporangia were obtained by culturing the soft tissue of clams injected with the trophozoites. Two tiny holes were drilled in the anterior and posterior side of the shell of each uninfected clam. Through the holes, each adductor muscle was injected with 0.1 mL culture of trophozoites. The injected clams were held in a tank containing 40 L aerated seawater at 20°C for a week. The gill and mantle tissues of the clam (0.7–1.0 g) were homogenized, centrifuged at 300 ×g for 5 min, and incubated individually in 20 mL Ray's fluid thioglycollate medium (RFTM), which is commonly used to develop *Perkinsus* trophozoites infecting host tissues into prezoosporangia^{8,9)}, supplemented with antibiotics (500 IU/mL penicillin G potassium and 500 µg/mL streptomycin sulfate) at 25°C for 4 d. After incubation, the media were removed by centrifuging (300 ×g for 5 min), and the clam tissue homogenates were digested in 0.25% trypsin solution in 20 mL filter-sterilized seawater (FSSW, 30‰ salinity) for 90 min at 25°C to obtain prezoosporangia. After serial filtrations through 200, 100, and 50 µm nylon meshes for *P. olseni* or 300, 200, and 50 µm for *P. honshuensis*, the prezoosporangia trapped on the 50 µm mesh were collected and washed twice with FSSW by centrifuging at 300 ×g for 5 min.

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We used filter-sterilized artificial seawater (FASW, 30‰ salinity) (Instant Ocean Sea Salt, Aquarium Systems) supplemented with antibiotics (200 IU/mL penicillin G potassium and 200 µg/mL streptomycin sulfate) for the production and incubation of prezoosporangia throughout the present study, unless otherwise stated.

Effect of temperature and salinity on trophozoite proliferation

Temperature. Cultures of *P. olseni* and *P. honshuensis* were filtered in a sterile environment through 10 µm nylon mesh to remove aggregated trophozoites. The filtrates were centrifuged at 275 ×g for 5 min to remove the old culture medium. The pelleted cells were resuspended in fresh PBM to adjust the cell densities to 4.0×10^5 cells/mL for *P. olseni* and to 2.0×10^5 cells/mL for *P. honshuensis*. Cell densities were determined using a hemocytometer. We pipetted 100 µL of each suspension into wells of each 96-well culture plate in triplicate and 100 µL of PBM was used as a blank sample. The plates were individually placed in incubation moisture chambers and incubated at 16°C, 19°C, 22°C, 25°C, 28°C, 31°C, 34°C, or 37°C. The number of cells in each well was quantified on day 0, 2, 3, 4, 5, 6, and 7 using a commercial cell proliferation assay kit [Cell Counting Kit-8 (CCK-8), Dojindo Laboratories]. Identical trials were repeated twice using different batches of cultures.

Salinity. We evaluated the effect of salinity by rearing *P. olseni* and *P. honshuensis* in culture media of different osmolalities of 300 mOsm (9‰), 405 mOsm (12‰), 510 mOsm (15‰), 605 mOsm (18‰), 700 mOsm (21‰), 890 mOsm (27‰), 1080 mOsm (33‰), or 1270 mOsm (39‰) (the value in each parenthesis represents salinity equivalent to each osmolality). The osmolality was adjusted by varying the amount of artificial seawater salts in original PBM, and measured using a vapor pressure osmometer (Vapro Osmometer 5520, Wescor). Cultures of *P. olseni* and *P. honshuensis* were filtered through a 10 µm nylon mesh to remove aggregated trophozoites. The cell densities of the filtrates were adjusted to 1.0×10^6 cells/mL for both species. The cell suspensions were dispensed into 3 mL aliquots and pelleted by centrifugation (275 ×g for 5 min) then resuspended in 3 mL fresh media at different osmolalities. We incubated the suspensions at 25°C in 96-well culture plates, and the cell densities were determined using CCK-8 at intervals similarly to the experiment for temperature-dependent proliferation. Identical trials were repeated twice using different batches of cultures.

Cell proliferation assay. Ten microliters of CCK-8 solution supplemented with antibiotics (200 IU/mL of penicillin G potassium and 200 µg/mL of streptomycin sulfate) was added to the wells containing the cell suspensions. The plate was incubated for 3 h

(temperature) or 2.5 h (osmolality) at 25°C in the dark to allow the reaction and colorization. The absorbance (ABS) of each well was read at 450 nm using a microplate reader (model MPR-A4, Tosoh). The incubation periods and temperature were optimized during preliminary experiments, and the ABS was proportional to the living cell number between 5.0×10^5 – 1.0×10^7 cells/mL (*P. olseni*) or 1.0×10^5 – 5.0×10^6 cells/mL (*P. honshuensis*) for this assay, which corresponded to ABS 0.04–0.80 or ABS 0.04–2.00, respectively.

Effects of temperature and salinity on development of zoosporangia

The release of zoospores from prezoosporangia can be an indicator of zoosporangial development. Therefore, we measured the effect of temperature and salinity on the rate of zoospore release. Zoospore release rates were arcsine-transformed, and then analyzed by Tukey-Kramer test.

Temperature. The prezoosporangia of the two *Perkinsus* species were incubated in FASW at 20°C, 25°C, 30°C, or 35°C. Prezoosporangia (30–70 cells) were placed in 1 mL FASW in each well of 24-well plates. They were observed under an inverted microscope every day for a week. Zoosporangia that were releasing zoospores at the time of observation or those that had already completed releasing were counted to obtain zoospore release rates.

Salinity. Prezoosporangia (30–70 cells) were placed in 1 mL FASW with different salinities (10‰, 15‰, 20‰, 25‰, 30‰, 35‰, or 40‰) in each well of 24-well plates. The culture plates were incubated at 25°C. Zoospore release rates were measured as described above.

Experiments were carried out in triplicates for both temperature and salinity.

Results

Effects of temperature and salinity on trophozoite proliferation

We repeated identical trials twice, and described below the trends observed commonly in the two trials.

Temperature. The proliferation of both *P. olseni* and *P. honshuensis* was highest at 28°C (Fig. 1A and B). Although high temperatures had a similar effect on proliferation between the two species, the effects of lower temperatures slightly differed. Both the species proliferated at 34°C but did not at 37°C. However, whereas the proliferation rate of *P. olseni* decreased gradually as temperature declined, that of *P. honshuensis* decreased drastically below 22°C.

Salinity. The proliferation of *P. olseni* was highest between 600 mOsm (18‰) and 890 mOsm (27‰) (Fig. 1C). Osmolalities outside this range clearly suppressed the proliferation of *P. olseni*. On the other

Fig. 1. Effects of incubation temperature (A, B) and media salinity (C, D) on *in vitro* proliferation of *P. olseni* (A, C) and *P. honshuensis* (B, D). Each point represents the mean of absorbance $\pm$ SD. BASE represents the mean of absorbance $\pm$ SD on day 0.

Fig. 2. Effect of temperature (A, B) and salinity (C, D) on zoosporangial development in *P. olseni* (A, C) and *P. honshuensis* (B, D). The points represent the mean of cumulative zoospore release rates at different treatment temperatures and salinities.

hand, the proliferation of *P. honshuensis* was highest within a wider range of osmolalities (700–1,080 mOsm, 21–33‰) (Fig. 1D). For both species, trophozoite proliferation was strongly inhibited at 300 mOsm (9‰).

Effects of temperature and salinity on the development of zoosporangia

Temperature. The cumulative zoospore release rate of *P. olseni* prezoosporangia was highest (15–25%) between 25°C and 30°C on day 7. At 35°C, zoosporulation was observed in some prezoosporangia but zoospore release was not (Fig. 2A). For *P. honshuensis*, zoospore release was highest (65–75%) between 25°C and 30°C (Fig. 2B), but zoospore release was observed at all temperatures.

Salinity. The zoospore release rate was higher between 25‰ and 35‰ on day 7 in *P. olseni* (Fig. 2C). Zoosporulation occurred at a low rate but no zoospore release was observed at 10‰. For *P. honshuensis*, zoospore release rate was high (65–75%) at 20–35‰. Zoospores were released at a low rate (< 10%) at 15‰, but they looked abnormal (i.e. heterogeneity of size and abnormal shape). There was no zoosporulation and zoospore release at 10‰ (Fig. 2D).

Discussion

Temperature and salinity conditions play a large role in determining the distribution of aquatic organisms. Thus, understanding the effects of these factors on trophozoite proliferation and zoosporangial development are critical to estimate the potential geographical distribution of *Perkinsus* species. Our prior research suggests that *P. olseni* was dominant over *P. honshuensis*^{7,10}. We hypothesized that temperature and salinity prefer-

ences may have played a role in this dominance and in their distribution.

The trophozoites of *P. olseni* and *P. honshuensis* vary considerably in size and frequently exhibit aggregation, thereby rendering conventional cell counting or turbidimetric analysis of little use for measuring cell proliferation. Moreover, viable trophozoites are not distinguishable from nonviable cells. As an alternative, Dungan and Hamilton (1995) successfully used a tetrazolium-based cell proliferation assay to measure the effects of *in vitro* conditions on *P. marinus* proliferation¹¹. Thus, we used a tetrazolium-based cell proliferation assay kit (CCK-8) to evaluate trophozoite proliferation in *P. olseni* and *P. honshuensis*.

Our results suggest that the effects of temperature on proliferation are similar between these two species, although lower temperatures (< 22°C) appeared to suppress the proliferation of *P. honshuensis* more than *P. olseni*. Proliferation was highest for both species at 28°C and both were able to proliferate at temperatures as high as 34°C, although 37°C was lethal for both the species. The development of zoosporangia was highest between 25°C and 30°C for both the species. The zoospore release rate was significantly lower (Tukey-Kramer test, $p < 0.01$) at 35°C than at 30°C for *P. honshuensis*, and we did not observe zoospore release at 35°C for *P. olseni*. We recently measured water temperature (5 cm above the mud surface) and ground temperature (at 5 cm depth) continuously in a habitat occupied by Manila clams on a tidal flat in the Ariake Sea. Our data suggest that the ground temperature peaked at 35°C during summer and declined to 5°C during winter (data not shown). The highest and lowest temperatures coincided with low tides when the sediment was exposed to air, and did not continue all day

long. In the present study, trophozoites of both species proliferated even at 34°C. Thus, in Manila clams on tidal flats, both species are likely to be able to survive even in summer.

P. olseni and *P. honshuensis* were able to proliferate within the range of 400–1,270 mOsm (12–39‰). Given the salinities at which Manila clams can survive ($\geq 15\text{‰}$)¹²⁾, trophozoite proliferation and zoospore release can likely occur in both species in the majority of Manila clam beds. Trophozoite proliferation was highest at 600–890 mOsm (18–27‰) and 700–1,080 mOsm (21–33‰) for *P. olseni* and *P. honshuensis*, respectively. The zoospore release rates were highest between 25‰ and 35‰ and were suppressed at levels $\leq 15\text{‰}$ for both *P. olseni* and *P. honshuensis*. Temperature and salinity ranges optimum for the development of *P. olseni* (= *P. atlanticus*) have been previously reported: roughly 20–30°C and 25–35‰^{13,14)}. Our present results were not inconsistent with the previous observations.

The infection prevalences and intensities of *P. olseni* were much higher than those of *P. honshuensis* in all Manila clam populations that we previously examined for *Perkinsus* infection¹⁰⁾. Furthermore, Hamaguchi *et al.* (2002) demonstrated that prevalences of *Perkinsus* sp. were generally lower in estuarine tidal flats than in non-estuarine tidal flats, suggesting that lower salinities suppress the infection of the parasite⁴⁾. However, either temperature or salinity appears not to limit the proliferation and zoospore release of these two species at the majority of Manila clam beds, based on the present results. We remain unable to explain the observed differences in infection levels between the two species and those between the types of tidal flats demonstrated by Hamaguchi *et al.* (2002). Lowered oxygen, acidic pH, and increased nutrient levels induced trophozoite differentiation into hypnospore (= prezoosporangium) in *P. chesapeaki* and *P. olseni* in a previous report¹⁵⁾. Such factors may influence the production of prezoosporangium and zoospore release for *P. olseni* and *P. honshuensis*, which may result in their difference

in infection levels. Furthermore, we have yet to identify the environmental factors affecting the infection level in different areas. To determine why *P. olseni* are dominant over *P. honshuensis*, other factors, such as specific defenses against *Perkinsus* species and host-parasite interactions, should also be considered. In addition, we suggest that more rigorous monitoring of the physical environment throughout the range of Manila clams might yield insight into the factors contributing to difference in infection level. The effects of environmental conditions on other developmental stages, including the transformation from trophozoites to prezoosporangia, and the survival of zoospore should also be evaluated.

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References

- 1) Matsukawa, Y., N. Cho, S. Katayama and K. Kamio (2008): *Bull. Jpn. Soc. Sci. Fish.*, **74**, 137–143.
- 2) Shimokawa, J., T. Yoshinaga and K. Ogawa (2010): *J. Invert. Pathol.*, **105**, 347–351.
- 3) Waki, T., J. Shimokawa, S. Watanabe, T. Yoshinaga and K. Ogawa (2012): *J. Invert. Pathol.*, **111**, 50–55.
- 4) Hamaguchi, M., M. Sasaki and H. Usuki (2002): *Jpn. J. Benthol.*, **57**, 168–176.
- 5) Nishihara, Y. (2010): *Sci. Rep. Hokkaido Fish. Exp. Stn.*, **77**, 83–88.
- 6) Dungan, C. F. and K. S. Reece (2006): *J. Eukaryot. Microbiol.*, **53**, 316–326.
- 7) Takahashi, M., T. Yoshinaga, T. Waki, J. Shimokawa and K. Ogawa (2009): *Fish Pathol.*, **44**, 185–188.
- 8) Ray, S. M. (1952): *Science*, **116**, 360–361.
- 9) Ray, S. M. (1966): *Proc. Natl. Shellfish Assoc.*, **54**, 55–69.
- 10) Umeda, K. and T. Yoshinaga (2012): *Dis. Aquat. Org.*, **99**, 215–225.
- 11) Dungan, C. F. and R. M. Hamilton (1995): *J. Euk. Microbiol.*, **42**, 379–388.
- 12) Matsuda, M., A. Shinagawa, J. Higano, A. Fujii, K. Hirano and A. Ishimatsu (2008): *Aquaculture Sci.*, **56**, 127–136.
- 13) Casas, S. M., A. Villalba and K. S. Reece (2002): *Dis. Aquat. Org.*, **50**, 51–65.
- 14) Ahn, K. J. and K. H. Kim (2001): *Dis. Aquat. Org.*, **48**, 43–46.
- 15) Casas, S. M. and J. F. La Peyre (2012): *Eur. J. Protist.*, (in press)