ORIGINAL ARTICLE

Biology

Experimental challenges of juvenile and adult Manila clams with the protozoan *Perkinsus olseni* at different temperatures

Tsukasa Waki · Tomoyoshi Yoshinaga

Abstract Infection with *Perkinsus* species, primarily *P. olseni*, is thought to be a major cause of the decline of Manila clam populations in Japan since the 1980s. However, the pathogenicity of the infection has not been sufficiently evaluated to estimate the impact of infection on wild Manila clam populations. We experimentally challenged juvenile (3- to 6-mm shell length) and adult (18- to 22-mm shell length) Manila clams with *P. olseni* at 18, 23, 28, and 30 °C. Mortality was significantly higher in challenged groups than in control groups. The difference in mean mortality between the challenged and control groups (all life stages and temperatures) was only significant above a threshold of infection intensity $\sim 10^6$ cells/g soft wet tissue (SWT). As temperature increased, the onset of mortality occurred more rapidly. The increase in mortality occurred earlier in juveniles than adults at 28 °C and lower. Our results suggest that the pathogenicity of *P. olseni* is higher in juveniles than in adults and at higher water temperatures. Given the infection intensities (ca. 10^6 cells/ g SWT) previously reported in wild Manila clams, the parasite likely has considerable impact on wild Manila clam populations, particularly juveniles during periods of high temperature.

Keywords *Perkinsus olseni* · Manila clam · *Ruditapes phillipinarum* · Pathogenicity · Temperature · Host size

T. Waki · T. Yoshinaga (✉)
Department of Aquatic Bioscience, Graduate School of Agriculture, The University of Tokyo, Tokyo 113-8657, Japan
e-mail: atyoshi@mail.ecc.u-tokyo.ac.jp

Introduction

Manila clams, *Ruditapes philippinarum*, form the basis of one of the most important molluskan fisheries in the coastal waters in Japan. In addition, researchers have pointed to their ability to filter large quantities of water as being of benefit to improving water quality [1–3]. However, the annual catch of Manila clams has decreased since the mid-1980s because of a decrease in the population size of several Manila clam beds [4]. The primary cause of this decrease remains unclear, despite numerous studies. Although a variety of conservation measures (e.g., restricted harvest, expansion of conservation areas, and cultivation and soil dressing of intertidal flats) have been implemented to recover the resource, there has been little success to date [4–8].

Recent research suggests that infection with protozoan *Perkinsus* species may be playing a major role in the decrease in Manila clam abundance. This is based on detection of high infection levels of *Perkinsus* species in several Japanese and Korean populations of Manila clams that have suffered declines in population size [9–11]. *Perkinsus* species are parasitic protozoans that infect mollusks and belong to Alveolata [12, 13]. They generally have two phases in their life cycles: an asexual propagation phase in the host tissue and a zoosporulation phase in seawater [14]. Uninucleate trophozoites propagate by repeated cell divisions in host tissue and, when exposed to anaerobic conditions after the death of the host, they enlarge and transform into prezoosporangia and subsequently into zoosporangia, in which infective zoospores are generated and released into the surrounding seawater.

Within the genus *Perkinsus*, two species, *P. olseni* and *P. honshuensis*, are known to infect Manila clams in Japan [11, 15, 16]. The two species are also known to co-infect

Manila clams [11, 15]. Umeda and Yoshinaga [11] demonstrated that *P. olseni* was dominant in all Manila clam populations they examined in Japan; the prevalence and infection intensity of *P. olseni* ranged from 45 to 100 % and from 10^2 to 10^7 cells/g soft tissue, respectively, and those of *P. honshuensis* ranged from 0 to 40 % and from 10^2 to 10^4 cells/g soft tissue, respectively. This suggests that the impact of *P. olseni* is larger than that of *P. honshuensis*, which likely has smaller effect on Manila clam populations. Shimokawa et al. [17] demonstrated that a cultured strain of *P. olseni* became lethal to juvenile Manila clams when infection intensities exceeded 10^7 cells/g soft tissue at 22 °C. Waki et al. [18] challenged Manila clams held at 22 °C with wild *Perkinsus* species (unidentified but presumed to be *P. olseni*) isolated from naturally infected wild Manila clams and demonstrated that the infection was lethal. A number of recently deceased juveniles were infected with $>10^7$ *Perkinsus* trophozoite cells/g soft tissue. Interestingly, however, the maximum infection intensity of *Perkinsus* species was $\sim 10^6$ cells/g soft tissue in Manila clam populations with the highest infection levels, such as those in Ariake Bay [11, 19–21]. Moreover, Yoshinaga et al. [21] reported few negative effects of *Perkinsus* infection on the physiological condition of commercially sized Manila clams naturally infected at 10^3–10^7 cells/g soft gill tissue. Thus, the results of experimental studies are often inconsistent with observations in naturally infected Manila clams, particularly with respect to the pathogenicity of *Perkinsus* infection. However, it is reasonable to assume that the pathogenicity of the infection is influenced by ambient conditions and host size. For example, Waki et al. [18] reported that approximately 80 % of experimentally challenged juveniles died within 48 days at 22 °C, whereas mortality was not observed until 49 days after challenge in adults. Such inconsistencies may be explained by differences in pathogenicity under different experimental conditions and in hosts of different sizes. We conducted a challenge experiment at a range of temperatures using adult and juvenile Manila clams to determine the effect of temperature and host size on the pathogenicity of infection with *P. olseni*.

Materials and methods

Manila clams

We obtained *Perkinsus*-free [22, 23] Manila clams (32- to 41-mm shell length) of commercial size from Akkesi Bay, Hokkaido Prefecture. The clams were subsequently used to produce *P. olseni* prezoosporangia. We obtained uninfected juvenile Manila clams (3- to 6-mm shell length) and adult Manila clams (18- to 22-mm shell length) produced

in a *Perkinsus*-free hatchery at the Fisheries Research Institute, Oita Prefectural Agriculture, Forestry and Fisheries Research Center, located in Bungotakada City, Oita Prefecture. These clams were used in the challenge experiment. Prior to the challenge, we tested 50 juveniles and 50 adult Manila clams for *Perkinsus* infection using the RFTM method (see below) and found no evidence of infection.

Production of prezoosporangia

We produced prezoosporangia by incubating the tissues of Manila clams injected with the cultured trophozoites of a strain of *P. olseni*. The trophozoite strain was purchased from the American Type Culture Collection (ATCC#: PRA-181) and routinely cultured in *Perkinsus* broth medium (ATCC medium 1886; PBM) at 25 °C. After culture for 7 days, the trophozoites were concentrated to 1.0×10^7 cells/ml by centrifugation ($120 \times g$, 5 min) and injected into uninfected Manila clams as described below.

Two holes (2 mm diameter) were drilled into the shells of 40 Manila clams near the anterior and posterior adductor muscles. Into each of the anterior and posterior adductor muscles, 100 μl of the concentrated trophozoite suspensions was injected (2.0×10^6 cells/individual), through the holes. The injected Manila clams were maintained unfed in a recirculating seawater aquarium equipped with a sand biological filter with aeration at 22 °C for 26 days. At the end of the experiment, the gill and mantle tissue was removed from each of the injected Manila clams and cut into fine pieces. The tissues (20 g) were divided into four tubes containing 50 ml Ray's fluid thioglycollate medium (RFTM) supplemented with 500 iu/ml penicillin G potassium and 500 μg/ml streptomycin sulfate, and incubated at 25 °C for 4 days to transform trophozoites into prezoosporangia [24]. The incubated tissues containing prezoosporangia were dissociated in seawater by dissolving with 0.25 % trypsin for 90 min at 22 °C, then washed with filtered seawater by centrifugation ($300 \times g$, 5 min) according to Casas et al. [25]. Prezoosporangia were isolated from the pellets consisting of prezoosporangia and Manila clam tissue by serial filtration through 200-, 100-, and 50-μm mesh sieves. The prezoosporangia and tissue residues trapped on the 50-μm sieve were collected and resuspended in 20 ml of filtered seawater. The density of prezoosporangia in this suspension was determined using a Burker-Turk hemocytometer. Last, we obtained 4.7×10^7 prezoosporangia cells from the tissues for use in the challenge experiment. Uninjected Manila clams were maintained in another aquarium separate from the injected Manila clams and processed in the same way as the injected Manila clams. The residual tissues of uninjected

Manila clams trapped on a 50-μm sieve were used as a negative control in the challenge experiment.

Challenge experiment

We challenged juvenile and adult Manila clams by immersing them in suspensions of prezoosporangia. All the obtained prezoosporangia were incubated in 40 l seawater in an aquarium for 4 days (25 °C, salinity 30), then divided into two aquaria, each containing 20 l seawater. Juvenile ($N = 620$) and adult Manila clams ($N = 620$) were immersed in one of the two aquaria for 24 h (juveniles and adults held separately). The timing of the immersion was determined based on our preliminary observation that the highest daily zoospore release ratio occurred between the 4th and 5th day during incubation. The zoospore release ratio was calculated by collecting triplicate ~ 400 μl samples of seawater daily from the bottom of aquaria. The number of prezoosporangia and zoosporangia in each sample were counted under an inverted light microscope. The negative controls consisted of juvenile and adult Manila clams ($N = 620$ of each) that were immersed (life stages held separately) in the residual tissues of uninjected Manila clams suspended in 40 l seawater for 24 h.

We maintained the challenged and control clams in floating baskets ($30 \times 15 \times 15$ cm L $\times$ W $\times$ D for adults and $15 \times 15 \times 15$ cm for juveniles) divided among four 1-ton recirculating seawater aquaria equipped with a biological filter and a temperature controller. Twelve baskets were prepared for each of the four treatment groups (challenged juveniles, challenged adults, control juveniles, and control adults). Fifty Manila clams were placed in each basket. Each recirculating aquarium accommodated 12 baskets (four treatments in triplicate). At the time the Manila clams were introduced into the aquaria, water temperature and salinity were 25 °C and 30 ppt, respectively. Within 1 day after the introduction, the water temperature in each aquarium was gradually changed to the appropriate target temperature (18, 23, 28, or 30). The clams in each aquarium were fed with 10 ml of a commercially produced diatom (cell density, approximately 600,000 cells/l; Sunculture, Nissin Marintech, Aichi, Japan). One clam was sampled from each basket at 0, 1, 3, 5, 7, 12, or 14 dpc (days post challenge), and then every 7 days until 42 dpc. The experiment with juvenile clams was terminated 15 and 26 dpc for challenged and control groups, respectively, at 30 °C; 22 and 30 dpc for challenged and control groups, respectively, at 28 °C; 32 dpc for both groups at 23 °C; and 43 dpc for both groups at 18 °C. The experiment with adult clams was terminated 34, 38, 42, and 44 dpc for both the challenged and control groups maintained at 30, 28, 23, and 18 °C, respectively. All surviving Manila clams were also sampled at the time

the experiment was terminated. Each individual was weighed (soft tissue weight) and measured (shell length) at the time of sampling or soon after death. The entire soft tissue of each individual was then used to determine the infection intensity by the RFTM method. We plotted Kaplan–Meier survival curves to assess survival in each experimental basket.

Ray's fluid thioglycollate medium (RFTM) assay

We used the RFTM method to quantify infection intensity and prezoosporangia production [26, 27]. The whole soft tissue of each Manila clam was cultured in 5 ml RFTM supplemented with antibiotics for 4–7 days at 25 °C. After the incubation, the tissue was pelleted by centrifugation ($300 \times g$, 5 min) and lysed in 2 M sodium hydroxide solution at 60 °C until the tissue was dissolved. Prezoosporangia in the dissolved tissue were collected by centrifugation ($300 \times g$, 5 min), washed 2 times in deionized water, and counted in 96-well plates (following dilution and staining with Logol's solution).

Results

Zoospore production

During incubation in seawater, 42 % of the zoosporangia released zoospores. The release of zoospores occurred between 3–7 days after transfer from RFTM to seawater and peaked on the 5th day. During the period when Manila clams were exposed to the suspension, approximately 25 % of zoosporangia released zoospores.

Challenge experiment

Challenged Manila clams began to die earlier and had significantly lower survival than controls, regardless of the developmental stage (juvenile or adult) or temperature with the exception of the adult stage held at 18 °C. However, we also observed mortality in control groups at most temperatures (Table 1 and Fig. 1). The difference in survival was greater in juveniles than in adults and was apparent earlier in the experiment. As temperature increased, we were able to detect a significant difference in survival at an earlier stage.

All challenged juveniles and adults became infected with _P. olseni._ The infection intensity in 12 juveniles and 12 adults examined soon after the challenge (three juveniles and three adults from each of the four temperature groups) varied considerably and ranged from 10^3 to $10^{5.5}$ and 10^2 to 10^6 cells/g soft tissue, respectively (Fig. 1). The geometric mean infection intensity was between 10^4 and

Table 1 Time to first mortality [days post challenge (dpc)] in the triplicate groups for each treatment group and time to a significant increase in mortality relative to control treatments

Water temperature	Life stage	Treatment	Time to initial mortality (dpc)	Time to a significant increase in mortality relative to the control group (U-test, $p < 0.05$)
30 °C	Juveniles	Challenged	10, 10, 11	11 dpc
		Control	15, 15, 18	
	Adults	Challenged	4, 7, 10	10 dpc
		Control	12, 13, 15	
28 °C	Juveniles	Challenged	11, 11, 11	11 dpc
		Control	25, 28, *	
	Adults	Challenged	9, 12, 18	18 dpc
		Control	10, 15, 20	
23 °C	Juveniles	Challenged	19, 19, 19	19 dpc
		Control	28, *, *	
	Adults	Challenged	21, 25, 32	32 dpc
		Control	31, 41, 42	
18 °C	Juveniles	Challenged	30, 37, 41	41 dpc
		Control	*, *, *	
	Adults	Challenged	43, *, *	No significant difference
		Control	44, *, *	

* No clams died during the experiment

10^5 cells/g soft tissue in both juveniles and adults. Although the infection intensity gradually increased with time in all challenged groups, the geometric mean infection intensity peaked at $\sim 10^7$ cells/g soft tissue in juveniles and only $\sim 10^6$ cells/g soft tissue in adults. As temperature increased, we observed an earlier increase in the geometric mean infection intensity in both life stages (Fig. 1). At the end of the experiment, the infection intensity in challenged juveniles and adults ranged from 10^3 to 10^9 and 10^2 to 10^7 cells/g soft tissue, respectively (Fig. 1).

We observed a significant difference in survival only after the geometric mean infection intensity reached $\sim 10^6$ cells/g soft tissue in any of the treatment groups, except the adult groups held at 18 °C (Table 1 and Fig. 1). After reaching this infection level, survival began to decrease rapidly, other than in challenged adults held at 23 °C. In this latter group, survival began to decrease 2 weeks after the geometric mean infection intensity reached $\sim 10^6$ cells/g soft tissue at 14 dpc.

We were able to quantify the infection intensity in adults soon after death, as their soft tissue remained viable for some time. The intensities ranged from 10^5 to 10^7 cells/g soft tissue and exceeded 10^6 cells/g soft tissue in a number of individuals (36 of 42 adults) (Fig. 1). In contrast, we

were unable to determine infection intensity in juveniles after death as the soft tissue was quickly lysed. We found no evidence of *P. olseni* infection in any control juveniles. However, we observed low levels of infection in adult control groups 14 dpc (Fig. 2). The prevalence of infection at the end of the experiment was 6.1, 7.7, 7.8, and 4.3 % in control adults held at 30, 28, 23, and 18 °C, respectively. The intensity of infection appeared to increase over time. The maximum infection intensity in control adults was $\sim 10^1$ and $\sim 10^3$ cells/g soft tissue at 30 and 28 °C, respectively, and 10^5 cells/g soft tissue at both 23 and 18 °C. These levels were significantly lower than in challenged adults.

Discussion

We obtained prezoosporangia from the gill and mantle tissue, the primary sites of *P. olseni* infection in Manila clams [28]. Approximately 40 % of the prezoosporaniga developed into zoosporangia and released zoospores. Furthermore, all challenged Manila clams were infected with *P. olseni*. Shimokawa et al. [17] conducted a challenge experiment using prezoosporangia obtained from the whole tissue of Manila clams injected with *P. olseni* trophozoites. However, this approach suffers from a decrease in zoospore release rates caused by contamination of seawater with residual Manila clam tissue. We minimized contamination by using only gill and mantle tissue.

We observed mortality in challenged juveniles and adults when the geometric mean infection intensity reached $\sim 10^6$ cells/g soft tissue. Moreover, the majority of recently moribund, challenged adults were infected with 10^6–10^7 cells/g soft tissue. Our results suggest that *P. olseni* infection can be lethal when the infection intensities exceed $\sim 10^6$ cells/g soft tissue in juvenile and adult Manila clams. Interestingly, some surviving Manila clams had much higher infection intensities than 10^6 cells/g soft tissue, suggesting that there is variability among individuals in the sensitivity to infection. Shimokawa et al. [17] reported that hatchery-raised juvenile Manila clams (3- to 10-mm shell length) challenged with *P. olseni* began to die when the infection intensity exceeded $\sim 10^7$ cells/g soft tissue in the majority of recipient Manila clams at 22 °C. In other challenge experiments using wild juvenile Manila clams (3- to 15-mm shell length) and wild *Perkinsus* isolates from naturally infected wild Manila clams, mortality was observed at infection intensities above 10^6 cells/g soft tissue [18]. Despite differences in rearing conditions, the history and size of the host, and the source of *P. olseni*, the combined evidence from these studies and our own suggests that an infection intensity of $>10^6$ cells/g soft tissue can be lethal to Manila clams.

Fig. 1 Kaplan–Meier survival curves and infection intensities in regularly sampled juvenile (*left panels*) and adult (*right panels*) Manila clams maintained at 30 (**a**), 28 (**b**), 23 (**c**), and 18 °C (**d**). The *numbers 1* and *2* represent the *survival curves* and *3* and *4* represent the infection intensities of juveniles and adults, respectively. In *1* and *2*, *open* and *closed circles* represent challenged and control groups, respectively. In *3* and *4*, *open circles* represent infection intensities in the regularly sampled Manila clams, with the *solid line* representing the geometric mean infection intensity. The *crosses* in **4** represent the infection intensities in recently deceased individuals
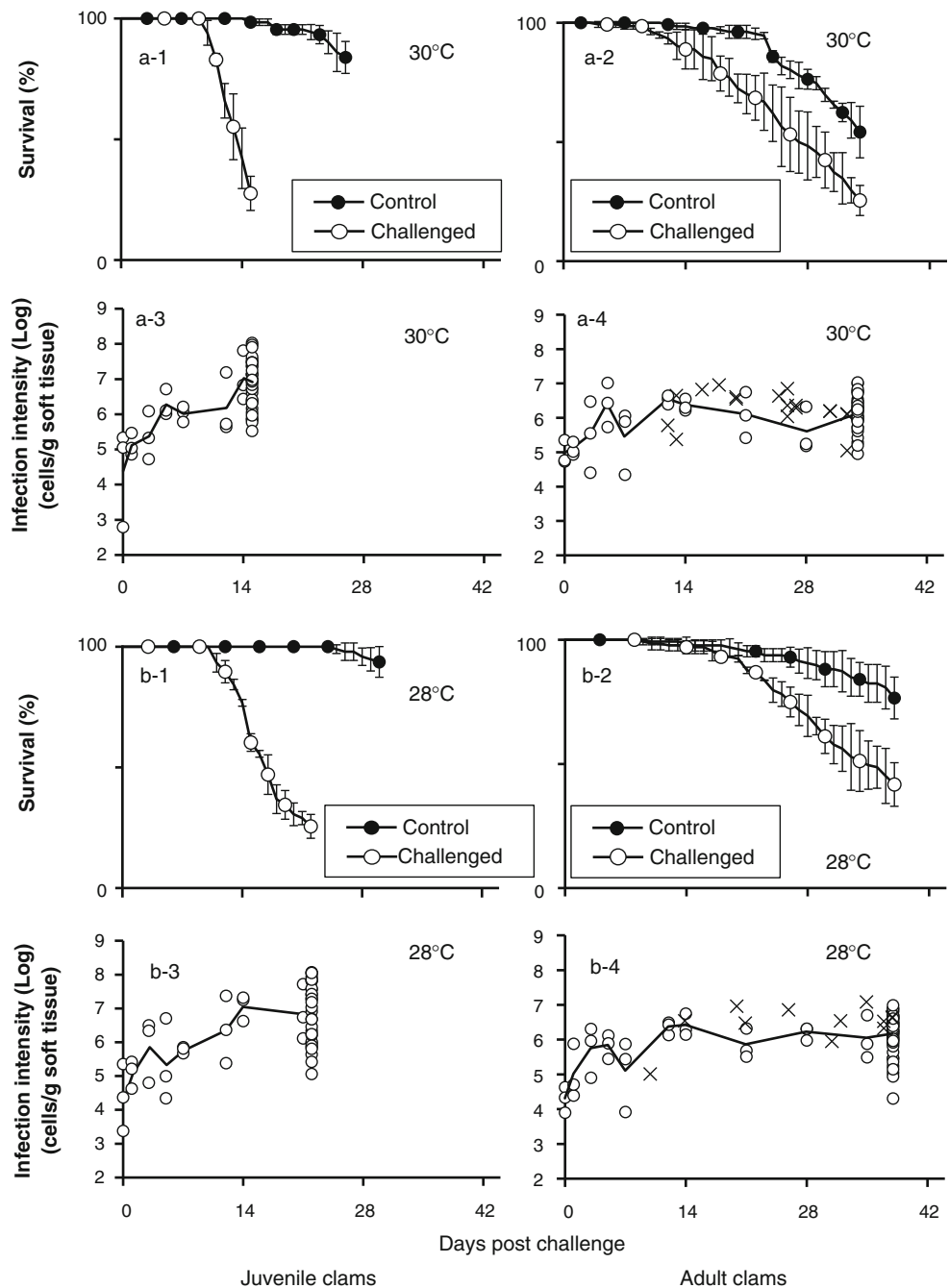
Prior reports concluded that the maximum infection intensity was $\sim 10^6$ cells/g soft tissue in Manila clam populations on the coast of Ariake Bay in Japan [19, 20]. *Perkinsus* infection appears to have had a significant effect on the survival of wild Manila clams in the area, likely because the infection intensity is close to the experimental lethal level. Researchers have tested for *Perkinsus* infection throughout Japan and documented a number of incidences in wild Manila clam populations [11, 21, 22, 29–32]. However, the impact of these infections is unclear as the reports only document prevalence or infection intensity in

the gills. Thus, these data cannot be compared with the experimental lethal infection intensity. Moreover, the study periods were short and there was no consideration to the temporal change in infection intensity. Additional research into the temporal changes in *Perkinsus* infection and wild Manila clam abundance is needed to determine the impact of the parasite on wild Manila clam populations.

In general, juveniles died earlier than adults following the challenge with *P. olseni*. Moreover, the peak geometric mean infection intensity was much higher in juveniles than adults (10^7 versus 10^6 cells/g soft tissue, respectively). We
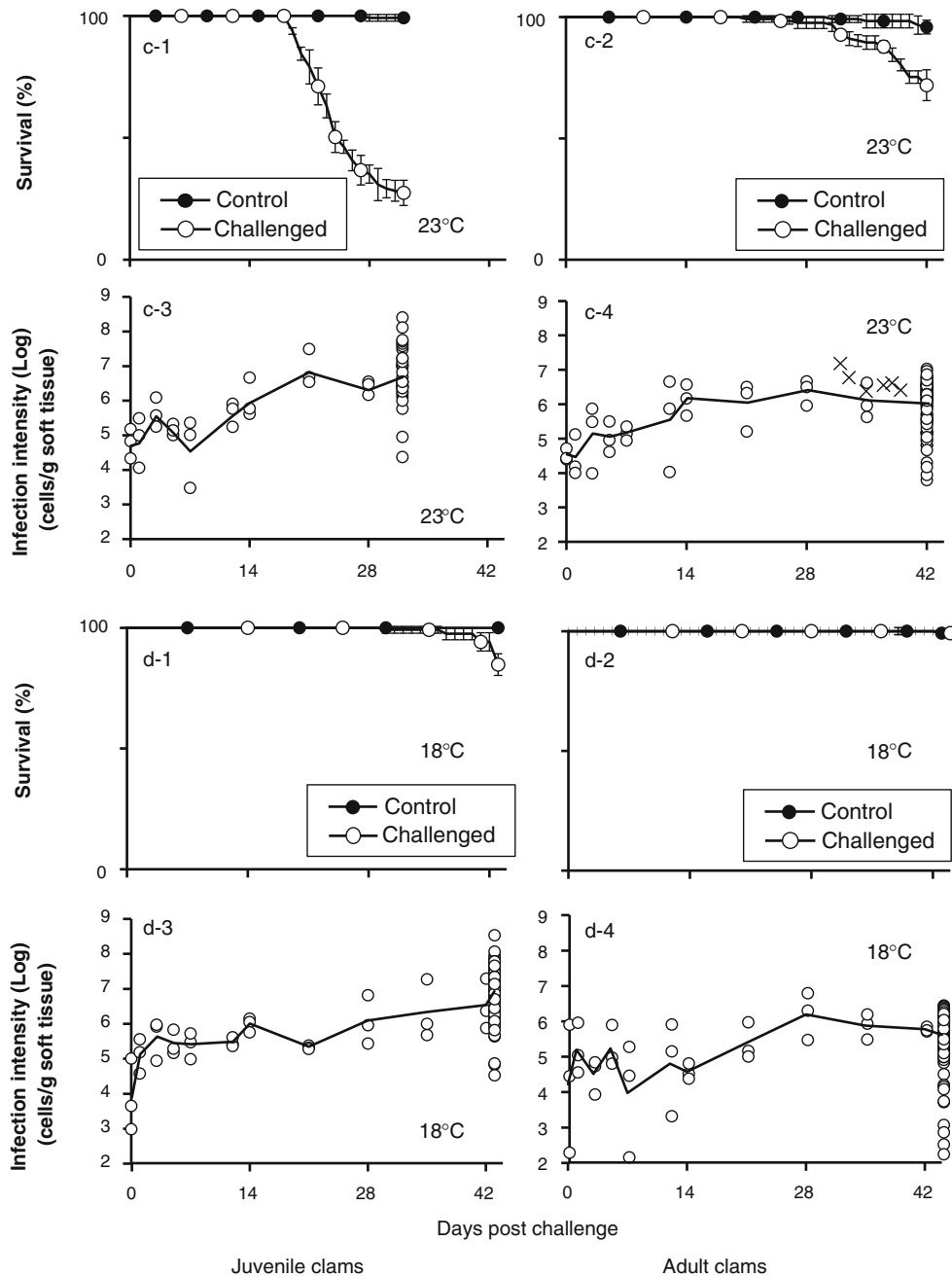
Fig. 1 continued

speculate that the propagation of trophozoites was more rapid in juveniles than in adults, resulting in earlier death in the juveniles. Furthermore, adult clams appear to be able to limit the propagation of trophozoites to levels around 10^6 cells/g soft tissue. Regardless, persistence of infection at the lethal level still resulted in mortality in adults.

Increases in temperature were associated with a more rapid increase in the rate of mortality. Peyre et al. [33] demonstrated that in vitro propagation of *P. olseni* trophozoites was promoted by increasing temperature within a range of 4–28 °C. Umeda et al. [34] concluded that the optimal temperature for the in vitro propagation of *P. olseni* was between 28 and 34 °C. Our results also suggest that trophozoite propagation is more rapid at 28 and 30 °C than at 18 and 23 °C. Increasing temperature is known to stimulate hemocyte immune activity in Manila clams within the range 8–21 °C [35]. However, further studies are needed to determine the nature of the interaction between trophozoite propagation and the immune response of hosts at high temperatures. Interestingly, we observed higher
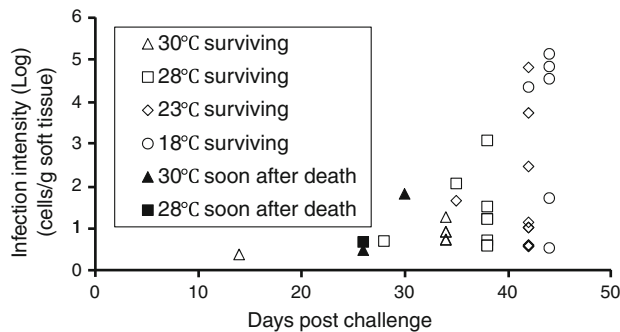
Fig. 2 Infection intensities in control adults infected with *P. olseni*. *Open triangles, squares, diamonds,* and *circles* represent infection intensities in the regularly sampled surviving adults maintained at 30, 28, 23, and 18 °C, respectively. *Closed triangles* and *closed squares* represent recently deceased adults held at 30 and 28 °C, respectively

mortality in control groups held at higher temperature, suggesting that thermal stress alone is sufficient to cause mortality in Manila clams.

Only one challenged adult died in the group that was held at 18 °C, even though the infection intensity exceeded 10^6 cells/g soft tissue in several individuals within this group. This suggests that *P. olseni* infection may have less of an effect on adults at this temperature. Lester and Davis [36] reported that blacklip abalones *Haliotis ruber* infected with *P. olseni* died at 20 °C, whereas abalones held at 15 °C encapsulated *P. olseni* trophozoites and killed them. Similarly, the mortality of cockles, *Anadara trapezia*, was higher in a challenged group maintained at 27–30 °C than in a group maintained at 20 °C [37]. Taken together, these results suggest that the pathogenicity and propagation of *P. olseni* is suppressed by low temperature.

We observed infection with *P. olseni* in some control adults. Zoospores are thought to be the primary source of infection with *P. olseni* [14]. However, trophozoites in the feces and pseudofeces of infected Manila clams may also be a source of infection [38]. We speculate that the control clams were infected via zoospores released from zoosporangia formed in the tissue of dead hosts and trophozoites released from live hosts. However, because the infection intensity remained low in control clams ($<10^{5.2}$ cells/g soft tissue), we do not believe infection contributed to the death of the control adults. The absence of infection in control juveniles may be explained by the lower filtration rate of juveniles, which decreases the probability of invasion by the parasite. Previous reports suggest that the absence of *Perkinsus* infection in juveniles smaller than 15- or 19-mm shell length in highly infected populations of Manila clams in Korea and Spain can be explained by their low filtration rates [39, 40].

In contrast to our results, Yoshinaga et al. [21] demonstrated that *Perkinsus* infection intensity did not affect tolerance to high water temperature stress, clearance rate,

or boring activity in naturally infected wild large adults (34- to 44-mm shell length). In light of our current observations, the adults in the prior study may have been of sufficient size that they were less sensitive to *Perkinsus* infection.

In conclusion, *P. olseni* infection had a greater impact on juvenile survival than adult survival in Manila clams. Furthermore, increased temperature resulted in a more rapid decrease in survival. Our results suggest that *Perkinsus* infection can be a major cause of mortality among wild Manila clams in heavily infected populations, particularly in juveniles during periods of high temperature.

References

1. Aoyama H, Suzuki T (1997) In situ measurement of particulate organic matter removal rates by tidal flat macrobenthic community. Bull Jpn Fish Oceanogr 61:265–274 (in Japanese)
2. Isono R, Nakamura Y (2000) Comparative studies of the water filtering rate in marine bivalves, with particular reference to thermal effects. J Jpn Soc Water Environ 23:683–689 (in Japanese)
3. Kohata K, Hiwatari T, Hagiwara T (2003) Natural water-purification system observed in a shallow coastal lagoon: Matsukawa-ura. Jpn Mar Pollut Bull 47:148–154
4. Matsukawa Y, Cho N, Katayama S, Kamio K (2008) Factors responsible for the drastic catch decline of the Manila clam *Ruditapes philippinarum* in Japan. Nippon Suisan Gakkaishi 74:137–143 (in Japanese)
5. Tsutsumi H, Ishizawa R, Tomishige M, Moriyama M, Sakamoto K, Montani S (2002) Population dynamics of a clam, *Ruditapes philippinarum*, on an artificially created sand cover on the tidal flats at the river mouth of Midorikawa River. Jpn J Benthol 57:177–187 (in Japanese)
6. Toba M (2007) Stock decline and the attempts to restore the local populations of Manila clam in Tokyo Bay. Mon Oceanogr 39:268–273 (In Japanese)
7. Watanabe S (2007) Causes of decline in Manila clam production and attempts for recovery in Japan. Mon Oceanogr 39:229–233 (In Japanese)
8. Hirano K, Higano J, Nakata H, Shinagawa A, Fujiya T, Tokunaga M, Kogo K (2010) An experiment for preventing mass mortality of cultured short-neck clams due to hypoxia formation during summer in Isahaya Bay. Fish Eng 47:53–62 (In Japanese)
9. Park KI, Choi KS (2001) Spatial distribution of the protozoan *Perkinsus* sp. found in the Manila clams, *Ruditapes phillippinarum*, in Korea. Aquacul 203:9–22
10. Hamaguchi M, Sasaki M, Usuki H (2002) Prevalence of a *Perkinsus* protozoan in the clam *Ruditapes philippinarum* in Japan. Jpn J Benthol 57:168–176 (in Japanese)
11. Umeda K, Yoshinaga T (2012) Development of real-time PCR assays for two *Perkinsus* spp. in the Manila clam *Ruditapes philippinarum*. Dis Aquat Org 99:215–225
12. Siddall ME, Reece KS, Graves JE, Burreson EM (1997) 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicompplexa. Parasitology 115:165–176

13. Saldarriaga JF, McEwan ML, Fast NM, Taylor FJ (2003) R, Keeling PJ. Mutipule protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. Int J Syst Evol Microbiol 53:355–365

14. Bordenave SA, Vigario AM, Ruano F, Domart-Coulon I, Doumenc D (1995) In vitro sporulation of the clam pathogen *Perkinsus atlanticus* (Apicomplexa, Perkinsea) under various environmental conditions. J Shellfish Res 14:469–475

15. Dungan CF, Reece KS (2006) In vitro propagation of two *Perkinsus* spp. parasites from Japanese Manila clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. J Eukaryot Mircobiol 53:316–326

16. Takahashi M, Yoshinaga T, Waki T, Shimokawa J, Ogawa K (2009) Development of PCR-RFLP method for distinction of *Perkinsus olseni* and *P. honshuensis* infecting Manila clam *Venerupis philippinarum*. Fish Pathol 44:185–188

17. Shimokawa J, Yoshinaga T, Ogawa K (2010) Experimental evaluation of the pathogenicity of *Perkinsus olseni* in juvenile Manila clams *Ruditapes philippinarum*. J Invertebr Pathol 105:347–351

18. Waki T, Shimokawa J, Watanabe S, Yoshinaga T, Ogawa K (2012) Experimental challenges of wild Manila clams with *Perkinsus* species isolated from naturally infected wild Manila clams. J Invert Pathol 111:50–55

19. Choi KS, Park KI, Lee KW, Matsuoka K (2002) Infection intensity, prevalence, and histopathology of *Perkinsus* sp. in the Manila clam, *Ruditapes philippinarum*, in Isahaya Bay. Jpn. J. Shellfish Res 21:119–125

20. Park KI, Tsutsumi H, Hong JS, Choi KS (2008) Pathology survey of the shortneck clam *Ruditapes philippinarum* occurring on sandy tidal flats along the coast of Ariake Bay, Kyushu. Japan J Invertebr Pathol 99:212–219

21. Yoshinaga T, Watanabe S, Waki T, Aoki S, Ogawa K (2010) Influence of *Perkinsus* infection on the physiology and behavior of adult Manila clam *Ruditapes philippinarum*. Fish Pathol 45:151–157

22. Hamaguchi M, Suzuki N, Usuki H, Ishioka H (1998) *Perkinsus* protozoan infection in short-necked clam *Tapes* (= *Ruditapes*) *philippinarum* in Japan. Fish Pathol 33:473–480

23. Nishihara Y (2010) Infection of protozoan *Perkinsus* in the short-necked clam (*Ruditapes philippinarum*) on the Hokkaido coastal region and the infection examination. Sci Rep Hokkaido Fisheries Exp Stn 77:83–88 (In Japanese)

24. Ray SM (1966) A review of the culture method for detecting *Dermocystidium marinum* with suggested modifications and precautions. Proc Natl Shellfish Assoc 54:55–69

25. Casas SM, Antonio V, Kimberly SR (2002) Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I. Identification of the aetiological agent and in vitro modulation of zoosporulation by temperature and salinity. Dis Aquat Org 50:51–65

26. Choi KS, Wilson EA, Lewis DH, Powell EN, Ray SM (1989) The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. J. Shellfish Res 8:125–131

27. Almeida M, Berthe F, Thébault A, Dinis MT (1999) Whole clam culture as a quantitative diagnostic procedure of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams *Ruditapes decussates*. Aquaculture 177:325–332

28. Nakatsugawa T (2007) Improvement of a PCR detection method for *Perkinsus olseni* isolated from Manila clam, *Ruditapes philippinarum*. Bull Kyoto Inst Ocean Fish Sci 29:17–21 (in Japanese)

29. Hamaguchi M, Suzuki N, Usuki H, Ishioka H (1998) *Perkinsus* protozoan infection in short-necked clam *Tapes* (=*Ruditapes*) *philippinarum* in Japan. Fish Pathol 33:473–480

30. Ikeura S (2002) Retention of *Perkinsus* spp. in Japanese short-necked clam at Buzen sea. Bull Fukuoka Fish Mar Technol Res Cent 12:127–129 (In Japanese)

31. Momoyama K, Taga S (2005) Detection of a parasitic protozoa *Perkinsus* sp. in the clam *Ruditapes philippinarum* collected from the tidal flats along the Seto Inland Sea in Yamaguchi Prefecture. Bull Yamaguchi Pref Fisheries Res Cent 3:111–117 (in Japanese)

32. Sakai K, Onodera J (2006) Epizootiological investigation on *Perkinsus* Protozoan (Apicomplexa) infection in Manila Clam *Ruditapes philippinarum* in Miyagi Prefecture. Jpn Miyagi Pref Rep Fish Sci 6:77–81 (in Japanese)

33. Peyre ML, Casas SM, Villalba A, Peyre JL (2008) Determination of the effects of temperature on viability, metabolic activity and proliferation of two *Perkinsus* species, and its significance to understanding seasonal cycles of perkinsosis. Parasitology 135:505–519

34. Umeda K, Shimokawa J, Yoshinaga T. 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. Fish Pathol (In press)

35. Paillard C, Allam B, Oubella R (2004) Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. Dis Aquat Org 59:249–262

36. Lester RJG, Davis GHG (1981) A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. J Invertebr Pathol 37:181–187

37. Goggin CL, Lester RJG (1995) *Perkinsus*, a protistan parasite of abalone in Australia: a review. Mar Freshwater Res 46:639–646

38. Park KI, Yang HS, Kang HS, Cho M, Park KJ, Choi KS (2010) Isolation and identification of *Perkinsus olseni* from feces and marine sediment using immunological and molecular techniques. J Invertebr Pathol 105:261–269

39. Choi KS, Park KI (1997) Report on the occurrence of *Perkinsus* sp. in the Manila clams *Ruditapes philippinarum* in Korea. Aquacult 10:227–237

40. Villalba A, Casas SM, López C, Carballal MJ (2005) Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality. Dis Aquat Org 65:257–267