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Effect of temperature and salinity on *in vitro* zoosporulation of *Perkinsus* sp. in Manila clams *Ruditapes philippinarum*

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ABSTRACT: The effects of temperature and salinity on *in vitro* development of *Perkinsus* sp. prezoosporangia isolated from cultured Manila clams in Korea were investigated, and the difference in resistance to low temperature between prezoosporangia collected in winter and those collected in summer was compared. Temperature and salinity had significant effects on the development of prezoosporangia, and the developmental rates increased with increasing temperature and salinity. Prezoosporangia isolated in winter sporulated and released motile zoospores at 10°C, although the rates were significantly lower than those at 20 and 30°C. However, no prezoosporangia collected in summer sporulated at 10°C. Low salinities (≤10‰) had a significant negative effect on the development of prezoosporangia. A small number of prezoosporangia sampled in summer did sporulate at 5‰, but further developments including formation and release of zoospores were not observed. However, prezoosporangia sampled in winter and incubated at 5‰ released motile zoospores, although the rates were significantly lower than those at higher salinities.

KEY WORDS: *Perkinsus* sp. · *Ruditapes philippinarum* · Manila clam · Prezoosporangia development · Temperature · Salinity

INTRODUCTION

Perkinsus species are parasites of shellfish, affinities having been suggested both with the Apicomplexa (Perkins 1996) and with the dinoflagellates (Perkins 1996, Siddall et al. 1997). The species have been associated with mass mortalities of commercially important molluscs (Andrews & Hewatt 1957, da Ros & Canzonier 1985, Goggin & Lester 1987). Six species of this genus — *P. marinus*, *P. olseni*, *P. atlanticus*, *P. qugwadi* (*insertae sedis*), *P. chesapeaki* and *P. andrewsi* — have been described in molluscs from various parts of the world (Mackin et al. 1950, Lester & Davis 1981, Azevedo 1989, Blackbourn et al. 1998, McLaughlin et al. 2000, Coss et al. 2001). Recently, unidentified *Perkinsus* sp. infections in Manila clams *Ruditapes philippinarum* were reported from Korea, and were considered to be a cause of recent mass mortalities of cultured Manila clams (Choi & Park 1997, Choi et al. 1998, Park et al. 1999).

Effects of natural environmental factors on the epizootiology of the disease caused by *Perkinsus* sp., especially *P. marinus*, have been extensively studied, and it is concluded that temperature and salinity are the 2 most important controlling factors (Mackin et al. 1950, Ray 1954, Andrews 1955, Crosby & Roberts 1990, Chu & La Peyre 1993, Chu et al. 1993, Ragone Calvo & Burreson 1994). A few studies have been done on the effects of temperature and salinity on *in vitro* development of prezoosporangia (Perkins 1966, Chu & Greene 1989) and cultured cells (Burreson et al. 1994) of *P. marinus*, and prezoosporangia of *P. atlanticus* (Auzoux-Bordenave et al. 1995). Goggin et al. (1990) reported that prezoosporangia and trophozoites of *Perkinsus* spp. showed high tolerance to a range of

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temperatures, salinities and chlorine concentrations. There are no data, however, concerning differences in tolerance of prezoosporangia to temperature and salinity as a function of the seasons at which the cells were sampled.

In this study, therefore, the effects of temperature and salinity on *in vitro* development of *Perkinsus* sp. prezoosporangia isolated from cultured Manila clams in Korea were investigated, and the difference in tolerance to low temperatures between prezoosporangia collected in winter and in summer was compared.

MATERIALS AND METHODS

Clams. Manila clams *Ruditapes philippinarum* were collected from cultured clam beds in an inner bay of the southern part of Korea in February and July 2000. The temperature and salinity in February were 9.8°C and 32.8‰, and in July were 25.0°C and 32.3‰, respectively. The experiments were conducted immediately after transferring the clams to the laboratory. The same experimental regimen was applied to both winter and summer samples.

Preparation of prezoosporangia. Whole tissues of clams were incubated in fluid thioglycollate medium (FTM; Sigma) for 5 d in the dark at 30°C. After incubation, the tissues were minced and washed by centrifugation 4 times in sterile artificial seawater (ASW) adjusted to 30‰ and containing 200 units ml^{-1} penicillin/streptomycin (Sigma) and 500 units ml^{-1} nystatin (Sigma). The pellet was resuspended in ASW and filtered serially with sterilized gauze. Prezoosporangia were isolated from the filtrate with micropipettes under a stereomicroscope and inoculated into 24-well plates.

Experimental regimen. The temperatures tested were 10, 20 and 30°C, and were controlled using an automatic temperature controlling (±0.5°C) multiroom incubator. Six salinity groups (5, 10, 15, 20, 25 and 30‰) were combined with each temperature. The salinities were adjusted by adding distilled water to ASW and measured daily by a refractometer. The purified prezoosporangia were inoculated into sterile 24-well plates and incubated in each experimental condition. The inoculated number of prezoosporangia in each well was 20, and all experimental cultures were performed in triplicate.

The plates were placed in the dark and examined daily under an inverted phase contrast microscope for 15 d. The following 4 criteria were used for evaluation of prezoosporangia development: the first day at which the discharge tube appeared; the day at which free zoospores were found; cumulative sporulation rates [SR = (number of sporulated zoosporangia/number of all zoosporangia) × 100] at Day 15; and cumulative release rates of zoospores [ZR = (number of zoosporangia with released zoospores/number of all zoosporangia) × 100] at Day 15.

Statistics. Differences in SR and ZR between prezoosporangia collected in winter and in summer were examined at each temperature and salinity using unpaired Student's *t*-test, and $p < 0.05$ was considered statistically significant.

RESULTS

The times for appearance of the discharge tube in prezoosporangia, collected in both winter and summer, were shortened in proportion to the increase in temperature and salinity (Table 1). However, no prezoosporangia sampled in summer formed discharge tubes at any salinities when they were maintained at 10°C.

The times for appearance of free zoospores were also negatively correlated with temperature and salinity in prezoosporangia sampled in both winter and summer (Table 2). However, no free zoospores were observed earlier than 15 d from prezoosporangia that were sam-

Table 1. First appearance (in d) of discharge tubes of *Perkinsus* sp. prezoosporangia isolated from Manila clams collected in winter and summer. –: not observed in the 15 d test period

Salinity (‰)	Temperature (°C)					
	Winter sample			Summer sample		
	10	20	30	10	20	30
5	8–9	5–6	4–5	–	5–6	2–3
10	8–9	5–6	1–2	–	4–5	2–3
15	8–9	4–5	1–2	–	4–5	1–2
20	7–8	3–4	1–2	–	4–5	1–2
25	7–8	3–4	1–2	–	4–5	1–2
30	7–8	3–4	1–2	–	4–5	1–2

Table 2. First appearance (in d) of free zoospores from *Perkinsus* sp. prezoosporangia isolated from Manila clams collected in winter and summer. –: not observed in the 15 d test period

Salinity (‰)	Temperature (°C)					
	Winter sample			Summer sample		
	10	20	30	10	20	30
5	12–13	9–10	10–11	–	–	–
10	12–13	8–9	6–7	–	–	–
15	12–13	7–8	1–2	–	9–10	4–5
20	11–12	6–7	1–2	–	6–7	3–4
25	9–10	5–6	1–2	–	5–6	2–3
30	9–10	5–6	1–2	–	5–6	2–3

Table 3. Cumulative sporulation rates (SR; %) of *Perkinsus* sp. prezoosporangia exposed to various temperatures and salinities during the 15 d test period. Values are mean ± standard deviation. *p < 0.05; **p < 0.01, winter versus summer samples in the same conditions

Salinity (‰)	Temperature (°C)					
	Winter sample			Summer sample		
	10	20	30	10	20	30
5	8.3 ± 2.2*	18.3 ± 2.2	36.7 ± 5.6**	0	10.0 ± 3.3	8.3 ± 2.2
10	10.0 ± 3.3*	86.7 ± 8.9	100.0 ± 0**	0	81.7 ± 2.2	71.7 ± 4.4
15	15.0 ± 6.7*	96.7 ± 4.4	100.0 ± 0	0	96.7 ± 4.4	95.0 ± 3.3
20	28.3 ± 2.2**	100.0 ± 0	100.0 ± 0	0	91.7 ± 7.8	100.0 ± 0
25	33.3 ± 4.4**	100.0 ± 0	100.0 ± 0	0	96.7 ± 4.4	100.0 ± 0
30	43.3 ± 7.8*	100.0 ± 0	100.0 ± 0	0	96.7 ± 4.4	100.0 ± 0

Table 4. Release rates of free zoospores (ZR; %) of *Perkinsus* sp. prezoosporangia exposed to various temperatures and salinities. Values are mean ± standard deviation. *p < 0.05; **p < 0.01, winter versus summer samples in the same conditions

Salinity (‰)	Temperature (°C)					
	Winter sample			Summer sample		
	10	20	30	10	20	30
5	8.3 ± 2.2	11.7 ± 2.2*	15.0 ± 3.3**	0	0	0
10	6.7 ± 2.2	41.7 ± 8.9*	65.0 ± 6.7**	0	0	0
15	8.3 ± 2.2**	60.0 ± 3.3	86.7 ± 2.2*	0	36.7 ± 14.4	53.3 ± 1.1
20	18.3 ± 2.2**	71.7 ± 8.9	76.7 ± 5.6	0	60.0 ± 10.0	81.7 ± 2.2
25	13.3 ± 7.8**	71.7 ± 4.4	86.7 ± 2.2	0	76.7 ± 7.8	91.7 ± 7.8
30	13.3 ± 3.8	73.0 ± 4.4	88.3 ± 7.8	0	86.7 ± 11.1	93.3 ± 4.4

pled in summer and incubated either at 10°C or at 20 and 30°C combined with 5 and 10‰.

Both SR and ZR were positively related to temperature and salinity (Tables 3 & 4). At 10°C, development of prezoosporangia was somewhat inhibited in the winter sample and completely inhibited in the summer sample. Prezoosporangia sampled in winter and incubated at 5 and 10‰ at 30°C showed significantly higher SR than those sampled in summer and incubated in the same conditions. ZR in the winter sample were significantly higher than those in the summer sample at the lower salinities.

DISCUSSION

The present results clearly showed that temperature had a significant effect on *in vitro* zoosporulation of *Perkinsus* sp. prezoosporangia, and the developmental rates increased with increasing temperature. Temperature-dependent *in vitro* development of prezoosporangia has been observed, also, in other *Perkinsus* species. It was reported that optimum temperature for sporulation of *P. marinus* prezoosporangia was 28°C, and no sporulation was observed at temperatures below 18°C (Perkins 1966, Chu & Greene 1989). According to the results of Auzoux-Bordenave et al. (1995), prezoosporangia of *P. atlanticus* sporulated only at 24 and 28°C; sporulation did not occur at 7 and 15°C although the viability of prezoosporangia was not affected. In the present study, however, *Perkinsus* sp. prezoosporangia isolated in the winter sporulated and released motile zoospores at 10°C, although the rates were significantly lower than those at 20 and 30°C. In contrast, no prezoosporangia collected during the summer sporulated at 10°C. The difference in developmental ability at low temperatures between winter and summer samples in this study suggests that prezoosporangia of *Perkinsus* sp. sampled in winter probably have an innate adaptation or tolerance to low temperatures and, therefore, can trigger their zoosporulation more readily at low temperatures than those sampled in summer. Since the water temperature of the present sampling area in winter ranges from 8 to 12°C, zoosporulation of *Perkinsus* sp. might be possible even in winter.

In the present study, low salinities (≤10‰) did have a strong negative effect on the development of *Perkinsus* sp. prezoosporangia. *In vitro* development of *P. marinus* prezoosporangia has also been reported to be significantly affected by salinity (Perkins 1966, Chu & Greene 1989). Salinity lower than 6‰ inhibited the sporulation of *P. marinus* prezoosporangia (Chu & Greene 1989). Salinities ranging between 25 and 35‰ promoted the best development of *P. atlanticus* prezoosporangia, but no sporulation occurred within 5 d at 5‰ (Auzoux-Bordenave et al. 1995). A small number of *Perkinsus* sp. prezoosporangia sampled in summer in this study did sporulate at 5‰, but further development including formation and release of zoospores was not observed. However, prezoosporangia sampled in winter and incubated at 5‰ released motile zoospores, though the rates were considerably lower than those at higher salinities. The reasons for this difference are not known, but prezoosporangia sampled in winter seemed to have more tolerance to low salinity and low temperature than those sampled in summer.

Although the biological significance of zoosporulation in *Perkinsus* species is not clear because zoosporangia are rarely found in host tissue, zoospores

must have some function or their production would not have evolved (Burreson & Ragone Calvo 1996). Auzoux-Bordenave et al. (1995) proposed that the prezoosporangial stage of *P. atlanticus* obtained in artificial conditions in FTM happens in natural conditions when the host dies, due to anaerobic conditions created inside tissues. When released in seawater, *P. atlanticus* sporangia sporulate quickly and in 2 to 3 d produced hundreds of biflagellated zoospores, which are an infective and dispersive stage of the parasite. In the present study, the role of zoospores in the early infection of *Perkinsus* sp. was not investigated. However, the high tolerance of prezoosporangia collected in winter to low temperatures might explain the high prevalence (100%) and infection intensity (2.87 according to Mackin's scale) of *Perkinsus* sp. in Manila clams collected from a southern bay of Korea in winter (Park et al. 1999).

In conclusion, temperature and salinity have a significant effect on the *in vitro* development of *Perkinsus* sp. prezoosporangia isolated from Manila clams, and further research is needed to elucidate why prezoosporangia collected during different seasons showed different developmental responses to low temperatures and low salinities.

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