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Salinity effects on viability, metabolic activity and proliferation of three *Perkinsus* species

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ABSTRACT: Little is known regarding the range of conditions in which many *Perkinsus* species may proliferate, making it difficult to predict conditions favorable for their expansion, to identify conditions inducing mortality, or to identify instances of potential cross-infectivity among sympatric host species. In this study, the effects of salinity on viability, metabolic activity and proliferation of *P. marinus*, *P. olseni* and *P. chesapeaki* were determined. Specifically, this research examined the effects of 5 salinities (7, 11, 15, 25, 35‰), (1) without acclimation, on the viability and metabolic activity of 2 isolates of each *Perkinsus* species, and (2) with acclimation, on the viability, metabolic activity, size and number of 1 isolate of each species. *P. chesapeaki* showed the widest range of salinity tolerance of the 3 species, with high viability and cell proliferation at all salinities tested. Although *P. chesapeaki* originated from low salinity areas (i.e. <15‰), several measures (i.e. cell number and metabolic activity) indicated that higher salinities (15, 25‰) were more favorable for its growth. *P. olseni*, originating from high salinity areas, had better viability and proliferation at the higher salinities (15, 25, 35‰). Distinct differences in acute salinity response of the 2 *P. olseni* isolates at lower salinities (7, 11‰), however, suggest the need for a more expansive comparison of isolates to better define the lower salinity tolerance. Lastly, *P. marinus* was more tolerant of the lower salinities (7 and 11‰) than *P. olseni*, but exhibited reduced viability at 7‰, even after acclimation.

KEY WORDS: Salinity · *Perkinsus marinus* · *P. olseni* · *P. chesapeaki* · Viability · Metabolic activity · Proliferation

INTRODUCTION

Protozoan parasites of the genus *Perkinsus* have been reported widely in marine molluscs since Mackin et al. (1950) noted *Dermocystidium marinum* — later renamed *Perkinsus marinus* (Levine, 1978) — in the eastern oyster *Crassostrea virginica* (Andrews 1955, Goggin & Lester 1987, Perkins 1993, Villalba et al. 2004). Although 8 species of *Perkinsus* have been reported, 1 has been determined as not belonging to the genus *Perkinsus* (*P. karlssoni*; Goggin et al. 1996), while 2 species, *P. olseni* and *P. atlanticus*, have been found to be conspecific (Murrell et al. 2002). Recently, another 2 of these 8 species (*P. chesapeaki* and *P. andrewsi*) have also been demonstrated to be conspecific (Burreson et al. 2005). In general, little is known regarding the optimal conditions for proliferation of these *Perkinsus* species, making it difficult to predict conditions favorable for their dissemination, to identify conditions inducing their mortality, or to identify instances of potential cross-infectivity among sympatric species. With the expansion of mariculture and, in some cases, the proposed use of non-native species to restore past shellfish fisheries (e.g. *C. ariakensis* in Chesapeake Bay), basic information on the environmental factors influencing parasite viability and dispersion is needed.

Perkinsus marinus is the most studied of the *Perkinsus* species, with extensive research documenting tem-

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perature and salinity as the most important environmental factors affecting its interactions with its primary host *Crassostrea virginica* (e.g. Soniat 1985, Chu & Greene 1989, Soniat & Gauthier 1989, Chu & La Peyre 1993, Chu et al. 1993, Ragone & Burreson 1993, Burreson & Ragone Calvo 1996, Ragone Calvo et al. 2001). *P. marinus* is found on the coast of the Gulf of Mexico and along the east coast of the United States and causes significant mortalities in the eastern oyster *C. virginica* (Craig et al. 1989, Burreson & Ragone Calvo 1996, Ford 1996, Soniat 1996). Higher temperatures are usually associated with maximum *P. marinus* prevalence and infection intensities, as are salinities above 12‰ (Ray 1954, Andrews & Hewatt 1957, Chu et al. 1993, Ragone & Burreson 1993). Both acute and acclimated responses to lower salinity have been tested in *P. marinus*. Specifically, acute exposure of *in vitro* cultures to lower salinity resulted in high parasite mortality (70% at 6‰, >90% at 3‰ and lower; Burreson et al. 1994) while exposure of *in vitro* cultures to lower salinity after acclimation greatly reduced mortality (41% mortality at 2.5‰ after acclimation versus 100% mortality without acclimation; O'Farrell et al. 2000). A recent laboratory and field study of *P. marinus* survival *in vivo* found that lowered salinity (0 to 1‰) did not eliminate the parasite, but did lower infection intensities (La Peyre et al. 2003). The above research suggests that to understand the full potential of cellular proliferation and dissemination of disease the effects of lower salinity need to be tested both with and without acclimation periods.

Overlapping the distribution of *Perkinsus marinus*, is that of the recently identified *P. chesapeaki* (McLaughlin et al. 2000), which has been found to infect the softshell clam *Mya arenaria* and has been associated with recent declines in commercial landings (McLaughlin & Faisal 1998a, Dungan et al. 2002). A field survey showed that, similar to *P. marinus*, higher infection in *M. arenaria* was associated with higher temperatures but, in contrast to *P. marinus*, the salinity range in which *P. chesapeaki* infections have been reported is from 0 to 14‰ (McLaughlin & Faisal 2000), indicating that *P. chesapeaki* may be less salt tolerant than *P. marinus*, although its specific salinity range has not been explicitly tested. Most recently, there has been discussion regarding the ability of *P. marinus* to infect *M. arenaria* and for both *Perkinsus* species to concomitantly coinfect *M. arenaria* (Kotob et al. 1999a,b, Dungan et al. 2002), although no substantive evidence exists supporting either claim (Dungan et al. 2002). A first step in addressing these questions is to explicitly identify the salinity tolerances of the *Perkinsus* spp. trophozoites, the stage present in host tissues.

A third *Perkinsus* species that has recently attracted attention is *P. atlanticus* (now referred to as *P. olseni*) infecting the carpet shell clam *Tapes decussatus* in Portugal and Spain (Azevedo 1989, Casas et al. 2002a,b, Murrell et al. 2002). This species has been associated with elevated clam mortalities in areas with high temperatures (Azevedo 1989, Sagrista et al. 1996). Recent studies of zoosporulation *in vitro* determined that the optimum temperature ranged from 15 to 32°C and optimum salinity from 25 to 35‰, although zoosporulation did occur at salinities from 10 to 35‰ (Casas et al. 2002b). This species was first described in the blacklip abalone *Haliotis ruber*, and is known to infect a number of molluscan species in coastal waters of at least Australia, Portugal, Spain, Japan and Korea (Lester & Davis 1981, Azevedo 1989, Hamaguchi et al. 1998, Casas et al. 2002b, Murrell et al. 2002, Park et al. 2005). Hine (2001) suggested that *P. olseni* was introduced to Europe by the movement of the manila clam *Ruditapes phillipinarum* from Asia.

Explicit testing of environmental factors on the viability and growth of these *Perkinsus* species would provide basic information on how environmental factors may influence their proliferation and disease dynamics, data on which are for the most part lacking. In this study, the effects of salinity on metabolic activity, viability and cellular proliferation of *P. marinus*, *P. olseni* and *P. chesapeaki* were determined. We examined (1) the effects of 5 salinities (7, 11, 15, 25, 35‰), without acclimation, on the viability and metabolic activity of 2 isolates of the 3 *Perkinsus* species and (2) the effects of the same 5 salinities, with acclimation, on the viability, metabolic activity, size and proliferation of 1 isolate of each of the 3 species.

MATERIALS AND METHODS

Cultures and experimental design. We used 2 *Perkinsus marinus* isolates (GTLA-33, GTLA-34), 2 *P. olseni* isolates (PaHm-2f, PaHm-14f) and 2 *P. chesapeaki* isolates (CRMA-J44/E3, CRTP-9/F8/G5). *P. marinus* cultures were established from the hearts of infected *Crassostrea virginica* collected in Lower Barataria Bay (Grande Terre), Louisiana (USA) following the method of La Peyre et al. (1993) and using the culture medium JL-ODRP-2A (Casas et al. 2002a). Oysters off Grande Terre, Louisiana, are exposed to a wide range of salinities from seawater to near freshwater conditions (USGS continuous data recorder; see http://waterdata.usgs.gov/usa/nwis/uv). *P. olseni* cultures were established from the hemolymph of *Tapes decussatus* from Galicia (NW Spain) (Casas et al. 2002a), where salinities below 25‰ are rare (Beiras et al. 2003, Page & Lastra 2003). *P. chesapeaki* cultures were obtained from C. Dungan (Maryland Department of Natural Resources, Cooperative Oxford Laboratory) (Dungan et al. 2002). CRMA-J44/E3 (ATCC 50864) was isolated from the

softshell clam *Mya arenaria* and CRTP-9/F8/G5 was isolated from the razor clam *Tagelus plebeius*, both collected from the Choptank River estuary in Maryland's Chesapeake Bay where salinities rarely exceed 15‰ (Maryland Department of Natural Resources Continuous Data Recorder; see http://mddnr.chesapeakebay.net/eyesonthebay/ index.cfm). As Perkinsosis is a warm-water disease, all cultures were maintained at 28°C and subcultured every other month in the culture medium JL-ODRP-2A (775 mOsm kg^{-1}; Casas et al. 2002b). JL-ODRP-2A was selected because all 3 *Perkinsus* species grow vigorously in this culture medium and its salt composition and osmolality can be easily adjusted to any desired value, an attribute essential for studying the osmolality tolerance of *Perkinsus* spp. (La Peyre 1996). Moreover, no culture medium has yet been optimized for growth of any *Perkinsus* species. To prepare the JL-ODRP-2A medium, a balanced solution was made by dissolving (l^{-1}) 1.18 g $CaCl_2 \cdot 2H_2O$, 2.26 g $MgSO_4$, 3.5 g $MgCl_2 \cdot 6H_2O$, 1.03 g KCl, 20.05 g NaCl, and 0.34 g $NaHCO_3$ in tissue-culture-grade water to a final volume of 944 ml. The balanced salt solution was filter sterilized (0.2 µm) and the following solutions were added under sterile conditions: 1 ml of a solution containing 0.834 mg $FeSO_4 \cdot 7H_2O$ and 0.143 mg $ZnSO_4 \cdot 7H_2O$; 1 ml of a solution containing 0.249 mg $CuSO_4 \cdot 5H_2O$; 5 ml of minimal essential medium (MEM) amino acids without glutamine; 5 ml of MEM nonessential amino acids; 5 ml of a solution containing (ml^{-1}) 10 mg alanine, 5 mg glycine, 5 mg serine, 15 mg taurine and 5 mg glutamine, 2 ml of a solution containing (ml^{-1}) 0.5 mg each of adenosine 5′-monophosphate, cytidine 5′-monophosphate, uridine 5′-triphosphate and 0.5 mg Coenzyme A; 2 ml of MEM vitamin solution; 2 ml of yeastolate ultrafiltrate solution; 10 ml of a solution containing (ml^{-1}) 50 mg glucose, 10 mg trehalose and 10 mg galactose; 1 ml of lipid mixture; 10 ml of a solution containing 0.5 mg chloramphenicol ml^{-1}; and 10 ml of 1 M N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) hemisodium salt (HEPES) solution. All chemicals used to prepare JL-ODRP-2A were purchased from Sigma-Aldrich except yeastolate, which was purchased from Life Technologies.

Expt 1—Without acclimation: Four weeks prior to the experiment to address the effects of salinity without acclimation, each isolate was seeded in JL-ODRP-2A culture medium in 75 cm^2 flasks (Corning) at a density of 1×10^6 cells ml^{-1}. At the start of the experiment (Day 0), the parasites were harvested from the 75 cm^2 flasks. Cells were passed 5 times through a 23-gauge needle attached to a 10 ml syringe to remove clumps. Cells were rinsed with fresh medium (JL-ODRP-2A) 3 times and resuspended at a density of 2×10^5 cells ml^{-1} of media with osmolalities of 205, 320, 450, 775, 1025 mOsm kg^{-1}. The composition of the media was the same except for the salt concentrations (Table 1), which were extrapolated from a pericardial fluid composition of *Crassostrea virginica* subjected to fluctuating salinity (Hand & Stickle 1977). The osmolalities of the media were equivalent to the osmolalities of seawater (i.e. 35‰) diluted with freshwater to salinities of 7, 11, 15 and 25‰. For clarity, all media will be referred to by their seawater salinity equivalence. We added 100 µl of each *Perkinsus* sp. isolate (2 per species), suspended at 2×10^5 cells ml^{-1}, to wells of 96-well tissue culture plates (Costar) and held them at 28°C. From these tissue culture plates, the effects of salinity without acclimation on cell viability and metabolic activity were measured, as indicated in Table 2, over a 30 d period at 11 and 7‰ and over a 12 or 14 d period at 15, 25 and 35‰, depending on the parameter measured.

Table 1. Salt concentrations (mg l^{-1}) of media used in experiments, with osmolalities of 1025, 775, 450, 320 and 205 mOsm kg^{-1}. Osmolalities of media were equivalent to osmolalities of seawater at a salinity of 35‰ and seawater diluted with freshwater to salinities of 25, 15, 11, and 7‰

Salt	Osmolality/salinity equivalence				
	1025/35	775/25	450/15	320/11	205/7
$CaCl_2 \cdot 2H_2O$	1646	1176	1030	683	515
$MgSO_4$	3168	2258	1092	728	546
$MgCl_2 \cdot 6H_2O$	4916	3502	1638	1091	819
KCl	1788	1028	600	399	300
NaCl	28 076	20 052	11 104	8176	5256
$NaHCO_3$	336	336	336	336	336

Expt 2—With acclimation: To test the effects of salinity with acclimation, only 1 isolate of each species was used (GTLA-33, PaHm-2f, CRMA-J44/E3 of *Perkinsus marinus*, *P. olseni* and *P. chesapeaki*, respectively), since the response of the isolates of each species were relatively similar in Expt 1. Cells of each isolate were transferred from the 25‰ culture medium into the 35, 15, 11 and 7‰ culture media using a gradual procedure by which cells from the 25‰ medium were placed into the 35 or 15‰ culture media, from the 15 into the 11‰, and from the 11 into the 7‰ medium with stepwise transfer at intervals of 2 wk each. This procedure resulted in 2 flasks per isolate at each of the 5 salinities. All flasks were held at 28°C. After being transferred to their final salinities, the cells were subcultured 3 times prior to testing the effects of salinity. Cells were harvested and 100 µl of each *Perkinsus* sp. isolate, suspended at 2×10^5 cells ml^{-1}, were added to wells of 96-well tissue culture plates and held at 28°C. From these tissue culture plates, the effects of salinity with acclimation on cell viability, metabolic activity, density, size and percentage of cells in divi-

Table 2. Sampling schedule for Expt 1 and 2. Lower salinities (7 and 11‰) were sampled to Day 30 in Expt 1 and to Day 28 in Expt 2, while higher salinities (15, 25, 35‰) were sampled to Day 12 in Expts 1 and 2. MA: metabolic activity

Parameter	Day of experiment															
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
Expt 1																
Cell viability	x	x		x			x			x						x
Cell MA	x	x	x	x	x	x	x	x		x			x			x
Expt 2																
Cell viability	x		x		x		x		x				x		x	
Cell MA	x	x	x	x	x	x	x	x	x		x		x		x	
Cell density	x	x	x	x	x	x	x	x	x		x		x		x	
Cell size	x	x	x	x	x		x		x				x		x	

sion (i.e. schizogony) were measured, as indicated in Table 2, over a 28 d period at 11 and 7‰, and over a 12 to 28 d period at 15, 25 and 35‰, depending on the parameter measured.

Viability. The percent of viable cells was recorded for each isolate at each salinity. We added 10 µl of neutral red (50 mg l^{-1}) to wells. Plates were incubated at 28°C for 2 h. Following incubation, the number of live (stained) cells and the number of dead (unstained) cells were counted. At least 200 total cells per isolate were counted. Percent viability was calculated by dividing the number of live cells by the total number of cells counted. We sampled 2 replicate wells for each isolate and salinity combination following the timetable for each experiment (Table 2).

Density. The number of cells ml^{-1} was recorded for each isolate for each salinity on designated days (Table 2). Cells were passed 5 times through a 23-gauge needle attached to a 1 ml syringe to break-up clumps and ensure the culture was well-mixed. Cell densities were determined using Neubauer Bright-Line hemocytometers (Reichert). We sampled 2 replicate wells per isolate per salinity following the timetable for each experiment (Table 2).

Size. Diameters of 50 trophozoites per well were measured with an ocular micrometer at 400× magnification, using an inverted microscope (Zeiss) following the timetable for Expt 2 (Table 2). In addition, when division occurred, the diameters of 25 schizonts per well were measured; 2 replicate wells for each isolate by salinity combination were sampled.

Metabolic activity. Metabolic activity was measured for each *Perkinsus* sp. isolate for each salinity using the alamarBlue™ assay (Alamar Biosciences). This assay allows continuous monitoring of cell metabolic activity over extended periods and is routinely used to assay cell growth and survival (Fields & Lancaster 1993, Page et al. 1993, DeFries & Mitsuhashi 1995, Voytik-Harbin et al. 1998, Le Pape et al. 2002). The alamarBlue assay incorporates an oxidation-reduction indicator that changes from an oxidized (non-fluorescent, blue) form to a reduced (fluorescent, red) form in response to chemical reduction of the growth medium resulting from cell metabolic activity. Fluorescence was determined using a Cytofluor Multiwell Plate Reader (Series 4000, PerSeptive Biosystems) connected to a personal computer. An excitation wavelength of 530 nm (25 nm bandwidth filter) and an emission wavelength of 580 nm (50 nm bandwidth filter) were used. Measurements were expressed in fluorescence units (fu). Fluorescence measurements for each isolate were the result of substracting the readings of wells with the isolate from the background readings of wells with media without cells. We added 10 µl of alamarBlue reagent to wells of 5 plates seeded with *Perkinsus* sp. (1 plate for each salinity—7, 11, 15, 25, 35‰) for each isolate. These plates were incubated at 28°C and read every other day for 30 d, or earlier if all the redox indicator was essentially reduced (i.e. >10 000 fu); 6 replicate wells per isolate per salinity were measured following the timetable in Table 2.

Statistical analysis. Statistical analysis was performed using SAS Version 8.0 software (SAS Institute, Cary, North Carolina). Data collected from the experiment testing the effects of salinity without acclimation were analyzed separately from those for the experiment testing the effects of salinity with acclimation. Data at each salinity were analyzed with a 2-factor ANOVA (Expt 1 factors: day and isolate; Expt 2 factors: day and species). Least-square means with a Tukey adjustment was used following significant ANOVA results ($p < 0.05$) to examine the differences among treatments. All data are reported as mean ± SD. Data were log transformed (metabolic activity) or arcsine transformed (viability) to achieve normality and homogeneity of variance.

RESULTS

Expt 1

Viability

A significant isolate × day interaction for viability of all 3 *Perkinsus* species was found at all 5 salinities, as indicated by a 2-factor ANOVA. Overall, *P. chesapeaki* isolates had greater viability at lower salinities (7, 11‰) compared to *P. marinus* and *P. olseni* isolates (Fig. 1). At higher salinities (15, 25, 35‰), all 6 isolates

fared well, with small differences among species and isolates, as detailed below.

At 7‰, no cells survived for any of the *Perkinsus marinus* and *P. olseni* isolates. In contrast, both *P. chesapeaki* isolates survived and divided. Initially (Day 0), *P. chesapeaki* Isolate CRTP-9/F8/G5 (63.6 ± 0.6%) survived the initial hypo-osmotic shock better than Isolate CRMA-J44/E3 (16.5 ± 3.8%). By Day 30, both isolates had recovered (CRMA-J44/E3, 95.8 ± 8.7%; CRTP-9/F8/G5, 100 ± 0.7%).

At 11‰, *Perkinsus chesapeaki* isolates again had greater viability than *P. marinus* and *P. olseni* isolates. As in 7‰, there were significant initial (Day 0) differences in *P. chesapeaki* isolates (CRTP-9/F8/G5, 95.3 ± 6.0%; CRMA-J44/E3, 36.3 ± 3.9%), although by Day 30, both had 100% viability. *P. olseni* isolates also differed significantly at 11‰. Both isolates had low initial viabilities (PaHm-2f, 3.2 ± 0.7%; PaHm-14f, 13.7 ± 0.7%), however, no live PaHm-2f cells were found after Day 18, while dividing PaHm-14f was able to recover (Day 30: 79.4 ± 29.4%). Initially, both *P. marinus* isolates responded similarly to the hypo-osmotic shock at 11‰ (GTLA-33, 14.6 ± 0.4%; GTLA-34, 13.7 ± 1.7%). Both divided and viability increased; however, GTLA-33 had achieved a higher viability by Day 30 (88.8 ± 1.0%) than GTLA-34 (46.1 ± 2.7%).

At 15‰, viability of both *Perkinsus chesapeaki* isolates was initially (Day 0) lower than 100% (CRTP-9/F8/G5, 85.0 ± 4.4%; CRMA-J44/E3, 70.1 ± 7.4%), but both divided quickly and had achieved 100% viability by Day 2; this was maintained throughout the incubation period. Similarly, viability of both *P. marinus* isolates was lower than 100% at Day 0 (GTLA-33, 91.7 ± 1.8%; GTLA-34, 92.6 ± 5.3%). While GTLA-34 quickly recovered to 100% during the 12 d incubation period, GTLA-33 viability ranged between 93.9 ± 0.1% (Day 6) and 98.7 ± 1.4% (Day 12). Significant differences existed between *P. olseni* isolates. Isolate PaHm-2f had a low initial viability (62.1 ± 0.2%) and had only reached 85.3 ± 1% by the end of the incubation period, while PaHm-14f had high tolerance to the initial hypo-osmotic shock (Day 0, 99.8%) and 100% viability on Day 12.

At 25‰, all isolates were not initially statistically different. Viabilities on Day 0 varied from a low of 97.1 ± 6.9% (CRMA-J44/E3) to 100 ± 8.7% (PaHm-14f). For the remainder of the incubation period, *Perkinsus marinus* and *P. chesapeaki* isolates had 100% viability. The significant day × isolate interaction is explained by lower viability of *P. olseni* isolates on Day 2 (PaHm-2f, 91.1 ± 4.4%, PaHm-14f, 82 ± 3.8%), although both had recovered by Day 12 (PaHm-2f, 99.5 ± 1.2%; PaHm-2f, 100 ± 0.7%).

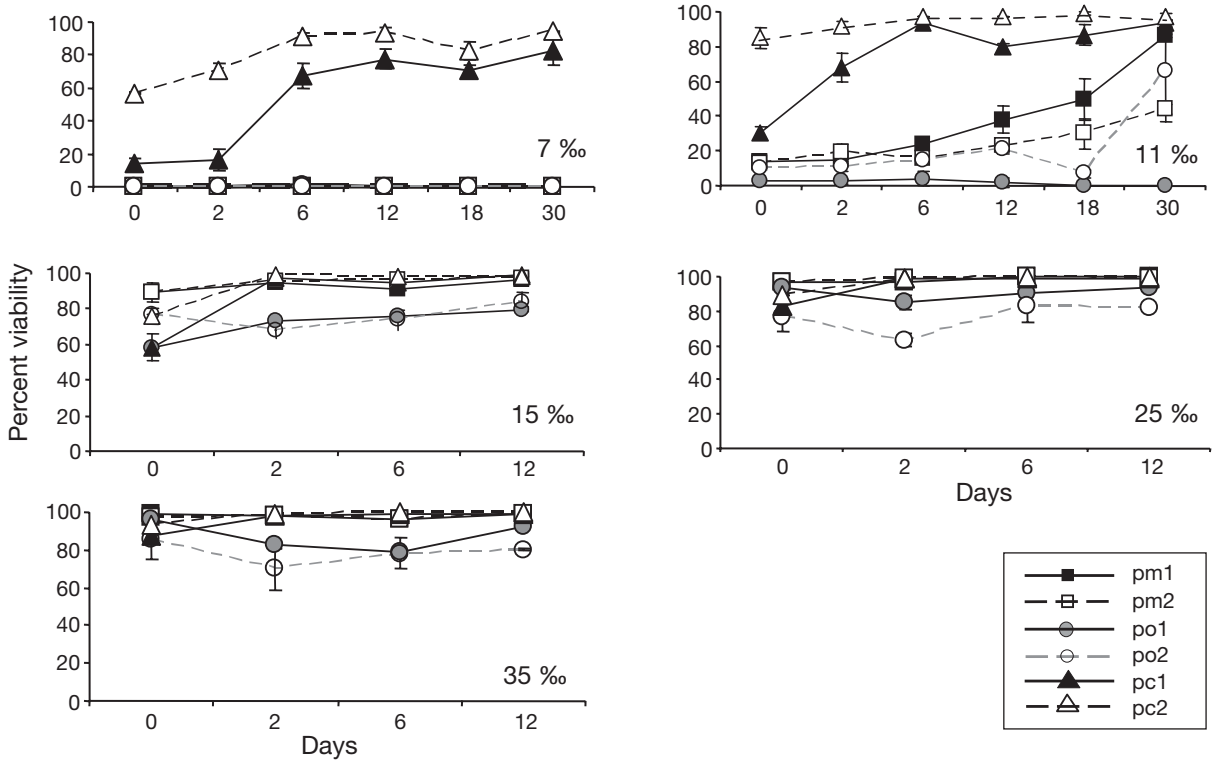

Fig. 1. *Perkinsus* spp. Mean ± SD percent viability over 30 d period of response of 2 isolates each of *P. marinus* (GTLA-33 [pm1], GTLA-34 [pm2]), *P. olseni* (PaHm-2F [po1], PaHm-14F [po2]) and *P. chesapeaki* (CRMA-J44/E3 [pc1], CRTP-9/F8/G5 [pc2]) exposed to 5 salinities without acclimation (Expt 1)

At 35‰, all *Perkinsus marinus* and *P. chesapeaki* isolates maintained 100% viability from Day 0 through the incubation period. As at 25‰, the significant day × isolate interaction was explained by lower viability of *P. olseni* isolates on Day 2 (PaHm-2f, 88 ± 2.0%; PaHm-14f, 90.4 ± 11.1%), despite a Day 0 viability of 100% for both isolates. By Day 12, both isolates had recovered (PaHm-2f, 98.5 ± 0.2%; PaHm-14f, 100 ± 0.4%).

Metabolic activity

A significant isolate × day interaction for metabolic activity was found for all 3 *Perkinsus* species at all 5 salinities. In general, throughout the incubation period, at all 5 salinities tested, both *Perkinsus chesapeaki* isolates had significantly higher metabolic activity than all 4 other isolates (2 species) examined (Fig. 2). Because of their high metabolic activity, both *P. chesapeaki* isolates had essentially reduced all redox-indicator molecules by Day 4 at 15, 25 and 35‰, and by Day 8 at 7 and 11‰.

At 7 and 11‰, the cumulative metabolic activity of *Perkinsus olseni* isolates differed on Day 0 from all other days, but no other significant differences were found during the 30 d incubation period (for both isolates at 7‰: Day 0, 2 ± 3.4 fu; Days 2 to 28, 46.5 ± 17.8; at 11‰: Day 0, 6.5 ± 4.7 fu; Days 2 to 28, 123 ± 31.4 fu). At 7‰, *P. marinus* isolates showed significant differences as a function of day, but cumulative metabolic activity remained at very low levels during the incubation period (Day 30 average of both isolates = 67.6 ± 26.8 fu). Higher metabolic activity was evident at 11‰ for *P. marinus* isolates, as it increased from Day 0 (0 fu for both isolates) to Day 30 (GTLA-33, 84 ± 23.5 fu; GTLA34, 51.3 ± 30.0 fu).

At 15‰, with the exception of *Perkinsus olseni* isolate PaHm-2f, all isolates had essentially reduced all redox-indicator molecules during the study period by Day 14. *P. olseni* isolate PaHm-2f cumulative metabolic activity was still increasing at the end of the incubation period on Day 14 (5781 ± 408.3 fu). In contrast, PaHm-14f had reduced all redox-indicator molecules by Day 6 (10 051 ± 243.2 fu). *P. chesapeaki* isolates had similarly reduced all redox-indicator molecules by Day 4 (CRMA-J44/E3, 11 617 ± 129.2 fu; CRTP-9/F8/G5, 11 814 ± 68.4 fu). *P. marinus* isolates had reduced all redox-indicator molecules by Day 14 (GTLA-33, 9452 ± 108.6 fu; GTLA-34, 10 171 ± 71.4 fu).

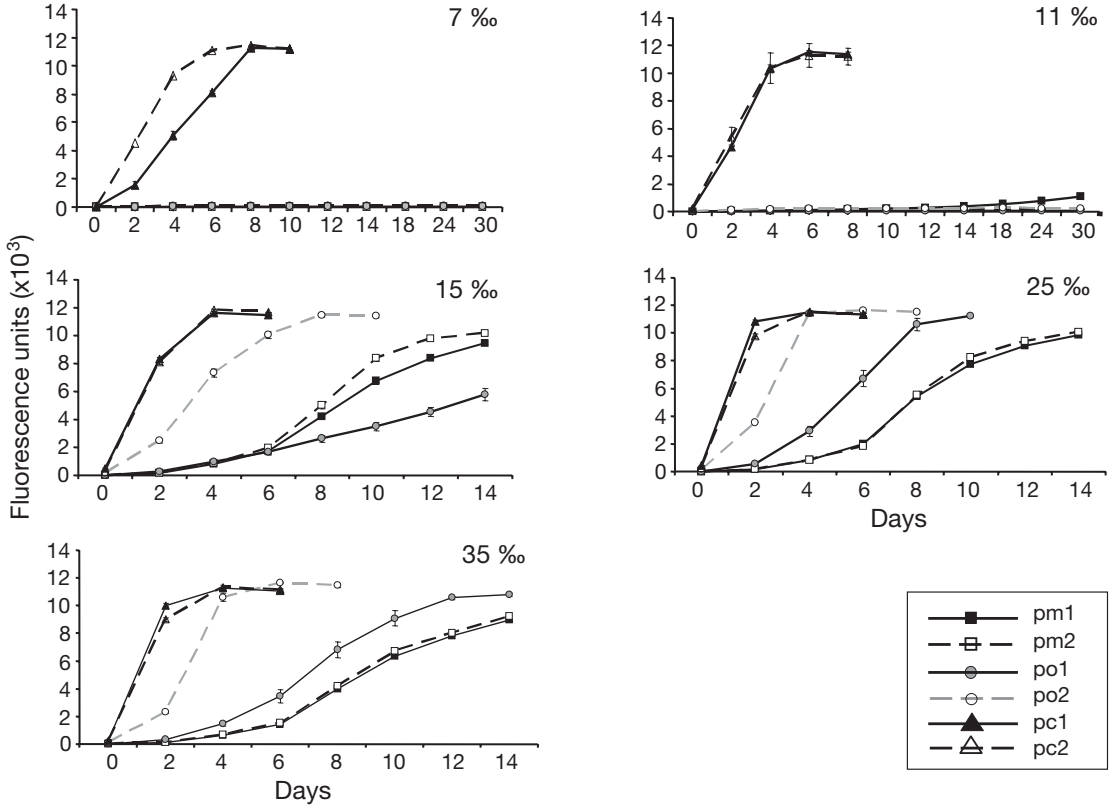

Fig. 2. *Perkinsus* spp. Mean ± SD cumulative metabolic activity over 30 d period of response of 2 isolates each of *P. marinus* (GTLA-33 [pm1], GTLA-34 [pm2]), *P. olseni* (PaHm-2F [po1], PaHm-14F [po2]) and *P. chesapeaki* (CRMA-J44/E3 [pc1], CRTP-9/F8/G5 [pc2]) exposed to 5 salinities without acclimation (Expt 1)

At 25‰, *Perkinsus chesapeaki* isolates differed during the first few days of the study, with CRMA-J44 isolate having higher metabolic activity. However, both *P. chesapeaki* isolates had reduced all redox-indicator molecules by Day 4 (CRMA-J44/E3, 11 522 ± 61.3 fu; CRTP9/F8/G5, 11 537 ± 101.9 fu). Both *P. marinus* isolates responded similarly, essentially reducing redox-indicator molecules by Day 14 (GTLA-33, 9853 ± 39.6 fu; GTLA-34, 9973 ± 85.3 fu). Again, *P. olseni* isolates differed significantly from each other, with PaHm-14f reducing all redox-indicator molecules earlier (PaHm-14f, Day 4, 11 397 ± 92.1 fu; PaHm-2f, Day 8, 10 631 ± 457 fu).

At 35‰, trends were very similar to those at 25‰. *Perkinsus chesapeaki* isolates differed in the first few days of the study, with the CRMA-J44/E3 isolate having higher metabolic activity. Again, both *P. chesapeaki* isolates had reduced all redox-indicator molecules by Day 4 (CRMA-J44/E3, 11 256 ± 92.3 fu; CRTP-9/F8/G5, 11 351 ± 111.4 fu). Both *P. marinus* isolate responses showed similar trends, reducing almost all redox-indicator molecules by Day 14 (GTLA-33, 8950 ± 97.3 fu; GTLA-34, 8812 ± 90.1 fu). Again, *P. olseni* isolates differed significantly, with PaHm-14f reducing all redox-indicator molecules earlier (PaHm14f, Day 4, 10 617 ± 270.1 fu; PaHm-2f, Day 14, 10 808 ± 122.8 fu).

Expt 2

Viability

In general, *Perkinsus chesapeaki* had high viability at all 5 salinities tested during the incubation periods and *P. marinus* had high viability at 11, 15, 25 and 35‰, and initial high viability at 7‰ that declined during the extended incubation period (Fig. 3). In contrast, *P. olseni* was not viable at 7‰, demonstrated low and rapidly declining viability at 11‰, and showed high viability at 15, 25 and 35‰.

At 7‰, a significant day × species interaction was found. No live *Perkinsus olseni* cells were found. In contrast, *P. chesapeaki* and *P. marinus* had similar initial high viabilities (CRMA-J44/E3, 85.2 ± 0.5%; GTLA-33, 86.0 ± 1.6%). While *P. chesapeaki* viability remained high, and actually increased (Day 28, 95.4 ± 2.2%), *P. marinus* viability declined after 28 d without being subcultured (Day 28, 63.6 ± 6.4%).

At 11‰, a significant species effect was found. *Perkinsus olseni* had very low viability from Day 0 (6.4 ± 2.1%) throughout the incubation period (Day 28, 0.6 ± 0.4%). In contrast, *P. chesapeaki* and *P. marinus* had high initial viabilities (Day 0: GTLA-33, 97.6 ± 1.3%;

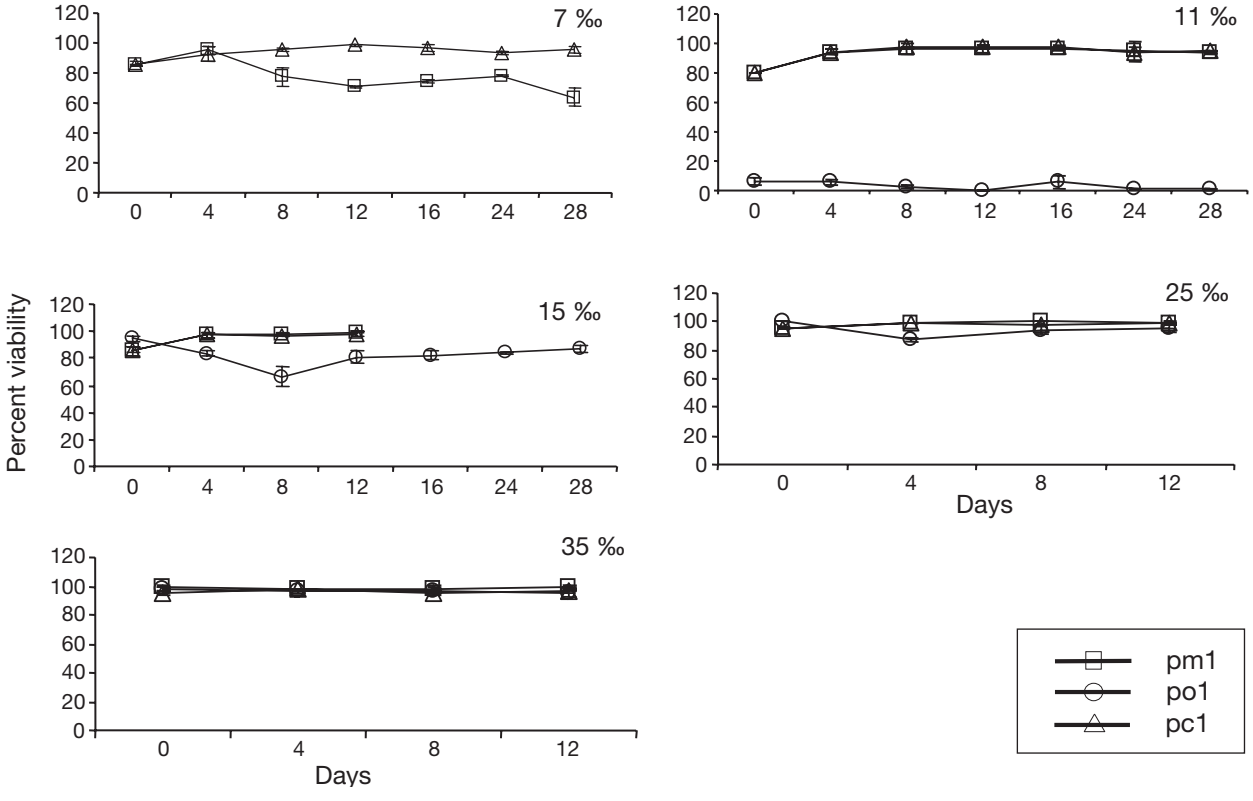

Fig. 3. *Perkinsus* spp. Mean ± SD percent viability over 28 d period of response of 1 isolate each of *P. marinus* (GTLA-33 [pm1]), *P. olseni* (PaHm-2F [po1]) and *P. chesapeaki* (CRMA-J44/E3 [pc1]) exposed to 5 salinities after acclimation (Expt 2)

CRMA-J44/E3, 80.1 ± 0.4%) and similar final viabilities (Day 28: GTLA-33, 95.6 ± 2.4%; CRMA-J44/E3, 95.0 ± 0.4%).

At 15‰, a significant day × species interaction was found. Again, *Perkinsus chesapeaki* and *P. marinus* displayed high viabilities (Day 0: GTLA-33, 86.2 ± 2.4%; CRMA-J44/E3, 85.6 ± 3.3%; Day 12: GTLA-33, 99.3 ± 0.4%; CRMA-J44/E3, 97.8 ± 1.8%). *P. olseni* initial viability was higher than for the other 2 isolates (Day 0: 94.8 ± 1.9%); however, viability declined, with a low on Day 8 (71.7 ± 0.5%), but was showing some recovery by Day 28 (86.8 ± 2.4%).

At 25‰, a significant species × day interaction was found. On Day 0, *Perkinsus chesapeaki* and *P. marinus* had similar (CRMA-J44/E3, 95.8 ± 1.0%; GTLA-33, 94.6 ± 1.4%) and lower viability than *P. olseni* (PaHm-2f, 100 ± 0%). However, while *P. chesapeaki* and *P. marinus* viabilities increased throughout the 12 d incubation period (Day 12: CRMA-J44/E3, 98.8 ± 1.0%; GTLA-33, 99.3 ± 0.3%), *P. olseni* viability had declined significantly by Day 4 (87.3 ± 1.8%), but again recovered to some extent by Day 12 (94.8 ± 2.7%).

At 35‰, only a significant difference between species was found. *Perkinsus marinus* had slightly higher viability (98.7 ± 1.5%) than either *P. olseni* (96.8 ± 2.0%) or *P. chesapeaki* (96.1 ± 1.4%) which had similar viability.

Metabolic activity

Significant differences were found for the species × day interaction at all salinities. Overall, the cumulative metabolic activity of *Perkinsus chesapeaki* was significantly greater than that of *P. marinus* (35, 25, 15, 11, 7‰) and *P. olseni* (35, 25, 15, 11‰) (Fig. 4). No cumulative activity data were obtained for *P. olseni* at 7‰ as no cells were viable at the end of the acclimation period. The cumulative metabolic activity of *P. chesapeaki* increased rapidly, as indicated by the complete reduction by Day 4 of the redox indicator added to each well. Additionally, at 35, 25, 15 and 11‰, the metabolic activity of *P. olseni* was significantly higher than that of *P. marinus*.

The cumulative metabolic activity of *Perkinsus chesapeaki* was similar at all salinities. In contrast, significant differences occurred as a function of salinity for *P. olseni* and *P. marinus*. The cumulative metabolic activity of *P. olseni* was generally highest at 11 and 35‰, and lowest at 15 and 25‰ throughout this experiment. In contrast, the cumulative metabolic activity of *P. marinus* was generally highest at 25‰, and lowest at 11‰ throughout this experiment. Cumulative metabolic activity of *P. marinus* was higher at 7‰ than at 11‰ from Day 0 to Day 16, and higher

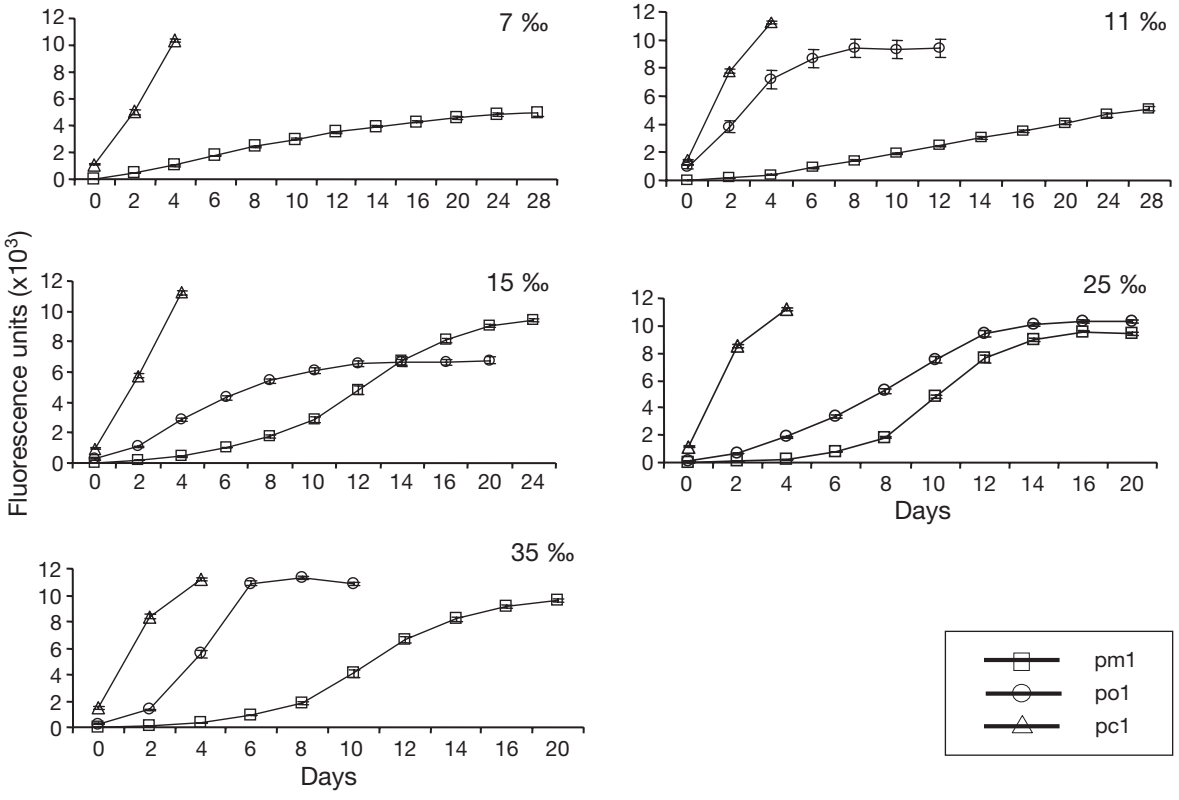

Fig. 4. *Perkinsus* spp. Mean ± SD cumulative metabolic activity over 28 d period of response of 1 isolate each of *P. marinus* (GTLA-33 [pm1]), *P. olseni* (PaHm-2F [po1]) and *P. chesapeaki* (CRMA-J44/E3 [pc1]) exposed to 5 salinities after acclimation (Expt 2)

than at 15, 25 and 35‰ from Day 0 to Day 8. The cumulative metabolic activity of *P. marinus* at 25 and 35‰ surpassed the cumulative activity of *P. marinus* at 7‰ on Day 10 and thereafter, while the cumulative metabolic activity of *P. marinus* at 15‰ surpassed the cumulative metabolic activity of *P. marinus* at 7‰ on Day 12 and thereafter.

Density

Significant differences were found for the species × day interaction for cell density. In general, *Perkinsus chesapeaki* cell density was greatest at 15 and 25‰, and lowest at 7, 11 and 35‰ (Fig. 5). *P. chesapeaki* cell density increased from Day 0 at all salinities, while *P. marinus* and *P. olseni* cell densities, when they increased, did not increase significantly until Day 8. *P. chesapeaki* cell density was greater than that of *P. marinus* at 7, 11 and 15‰, and lower than that of *P. marinus* at 25 and 35‰, after Day 8. *P. marinus* cell density was greatest at 15, 25 and 35‰ and lowest at 7 and 11‰. No cell density data were obtained for *P. olseni* at 7‰ as no cells were viable at the end of the acclimation period. Furthermore, *P. olseni* cell density increased by over 2.0×10^6 ml^{-1} only at 25‰.

At 7‰, *Perkinsus chesapeaki* cell density was significantly greater than that of *P. marinus* throughout this experiment. No cell density data were obtained for *P. olseni* at 7‰ as no cells were viable at the end of the acclimation period. *P. chesapeaki* density increased significantly from its initial density ($0.20 \pm 0.03 \times 10^6$ ml^{-1}) and its density the previous day on Day 4 ($1.87 \pm 0.65 \times 10^6$ ml^{-1}) to Day 28 ($2.46 \pm 0.05 \times 10^6$ ml^{-1}). *P. marinus* cell density did not differ significantly from Day 0 ($0.21 \pm 0.04 \times 10^6$ ml^{-1}) through Day 28 ($0.16 \pm 0.04 \times 10^6$ ml^{-1}).

At 11‰, *Perkinsus chesapeaki* cell density was significantly greater than *P. marinus* and *P. olseni* cell density throughout this experiment. *P. chesapeaki* cell density increased significantly from its initial density ($0.19 \pm 0.08 \times 10^6$ ml^{-1}) to Day 4 ($2.13 \pm 0.07 \times 10^6$ ml^{-1}), and was statistically similar from Day 4 to Day 28 ($3.25 \pm 0.16 \times 10^6$ ml^{-1}). In contrast, *P. olseni* cell density declined from Day 0 ($0.18 \pm 0.02 \times 10^6$ ml^{-1}) to Day 28 ($0.02 \pm 0.01 \times 10^6$ ml^{-1}). *P. marinus* cell density did not increase from initial cell density ($0.21 \pm 0.47 \times 10^6$ ml^{-1}) until Day 12 ($0.67 \pm 0.03 \times 10^6$ ml^{-1}). *P. marinus* cell density also increased from Days 12 to 16 ($1.53 \pm 0.3 \times 10^6$ ml^{-1}), and did not increase significantly for the remainder of this experiment.

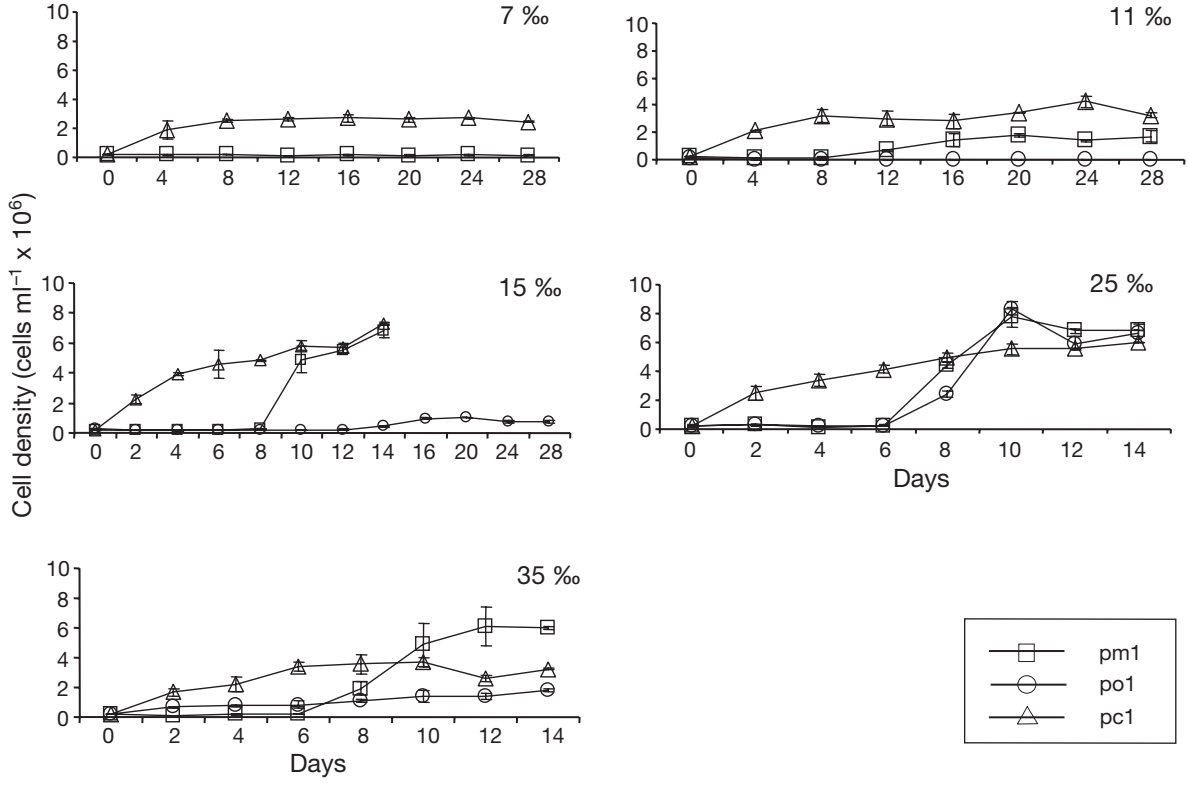

Fig. 5. *Perkinsus* spp. Mean ± SD cell density (cells ml^{-1}) over 28 d period of response of 1 isolate each of *P. marinus* (GTLA-33 [pm1]), *P. olseni* (PaHm-2F [po1]) and *P. chesapeaki* (CRMA-J44/E3 [pc1]) exposed to 5 salinities after acclimation (Expt 2)

At 15‰, *Perkinsus chesapeaki* cell density was significantly greater than that of *P. marinus* from Days 2 to 8, and was significantly greater than *P. olseni* cell density throughout the experiment. *P. chesapeaki* density increased significantly from its initial density ($0.20 \pm 0.04 \times 10^6$ ml^{-1}) and its density on the previous day on Days 2 ($2.24 \pm 0.30 \times 10^6$ ml^{-1}) to 14 ($7.26 \pm 0.08 \times 10^6$ ml^{-1}). *P. marinus* cell density increased significantly from Day 6 ($0.17 \pm 0.01 \times 10^6$ ml^{-1}) to Day 10 ($4.90 \pm 0.85 \times 10^6$ ml^{-1}) and was statistically similar to that of *P. chesapeaki* on Days 10 to 14. *P. olseni* cell density increased slightly, but remained generally low throughout the 28 d period. *P. olseni* cell density did not differ from its initial density ($0.25 \pm 0.05 \times 10^6$ ml^{-1}) from Days 2 ($0.19 \pm 0.01 \times 10^6$ ml^{-1}) to 12 ($0.23 \pm 0.06 \times 10^6$ ml^{-1}). From Day 14 ($0.45 \pm 0.04 \times 10^6$ ml^{-1}) to Day 28 ($0.79 \pm 0.18 \times 10^6$ ml^{-1}), *P. olseni* cell density remained below 1.00×10^6 ml^{-1}.

At 25‰, *Perkinsus chesapeaki* cell density was significantly greater than that of *P. marinus* and *P. olseni* on Days 0 to 6. *P. chesapeaki* density increased significantly from its initial density ($0.26 \pm 0.01 \times 10^6$ ml^{-1}) and its density on the previous day on Days 2 ($2.49 \pm 0.48 \times 10^6$ ml^{-1}) to 14 ($6.05 \pm 0.00 \times 10^6$ ml^{-1}). *P. marinus* cell density surpassed that of *P. chesapeaki* cell density from Days 10 ($7.77 \pm 0.67 \times 10^6$ ml^{-1}) to 14 ($6.83 \pm 0.04 \times 10^6$ ml^{-1}). In contrast, *P. olseni* cell density only significantly exceeded that of *P. chesapeaki* on Day 10 (PaHm-2f, $8.35 \pm 0.49 \times 10^6$ ml^{-1}; CRMA-J44/E3, $5.54 \pm 0.37 \times 10^6$ ml^{-1}), and both species' cell density was similar on Day 14 (PaHm-2f, $6.6 \pm 0.62 \times 10^6$ ml^{-1}; CRMA-J44/E3, $6.05 \pm 0.00 \times 10^6$ ml^{-1}).

At 35‰, *Perkinsus chesapeaki* cell density was significantly greater than that of *P. marinus* on Days 2 to 8, and greater than *P. olseni* density throughout the experiment. Specifically, *P. chesapeaki* density increased significantly from its initial density ($0.19 \pm 0.02 \times 10^6$ ml^{-1}) on Days 2 ($1.66 \pm 0.25 \times 10^6$ ml^{-1}) to 8 ($3.56 \pm 0.64 \times 10^6$ ml^{-1}), but stayed constant for the remainder of the study. *P. marinus* density increased significantly from Days 6 ($0.18 \pm 0.07 \times 10^6$ ml^{-1}) to 8 ($1.93 \pm 0.47 \times 10^6$ ml^{-1}), and again to Day 10 ($4.85 \pm 1.48 \times 10^6$ ml^{-1}). *P. marinus* density surpassed *P. chesapeaki* density from Days 10 to 14.

Size

Significant species × day interactions were found at all salinities. In general, *Perkinsus chesapeaki* had similar trophozoite diameters as a function of both salinity and day throughout the experiment. In contrast, at higher salinities (15, 25, 35‰), *P. marinus* and *P. olseni* trophozoite diameters increased from the day of seeding until substantial schizogony (i.e. >1% of cells dividing) was observed (Fig. 6; Table 3). At these higher salinities (15, 25, 35‰), *P. marinus* generally had the smallest trophozoite diameter. Substantial schizogony occurred much earlier (Day 2) in *P. chesapeaki* at 15, 25 and 35‰ than in *P. marinus* (Day 6) and *P. olseni* (Day 12 at 15‰, Day 8 at 25‰, and Day 6 at 35‰). At 11‰, *P. olseni* did not undergo schizogony, and the trophozoite sizes observed were significantly larger than at any of the higher salinities. Furthermore, at 11‰, substantial schizogony was delayed in *P. marinus* (Day 8). At 7‰, no viable *P. olseni* cells were found at the end of the acclimation period and *P. marinus* demonstrated a low level of schizogony (<1% cells dividing).

At 7‰, no cell size data were obtained for *Perkinsus olseni* as no viable cells were obtained at the end of the acclimation period. In contrast to higher salinities, *P. marinus* had significantly larger trophozoites than *P. chesapeaki* at the time of seeding (GTLA-33, 19.8 ± 2.3 µm; CRMA-J44/E3, 14.2 ± 4.7 µm) and at each sampling time except for Day 4. The mean diameter of *P. marinus* schizonts (27.8 ± 4.1 µm) was also significantly larger that of *P. chesapeaki* schizonts (18.9 ± 3.8 µm).

At 11‰, *Perkinsus olseni* trophozoites were significantly larger than those of both *P. marinus* and *P. chesapeaki* throughout the study. The lowest mean trophozoite diameter was 6.4 ± 1.7 µm for *P. marinus*, 11.0 ± 4.5 µm for *P. chesapeaki* and 32.0 ± 15.3 µm for *P. olseni*. Only *P. marinus* trophozoites increased in diameter from the time of seeding (6.4 ± 1.7 µm) to Day 8 (14.7 ± 3.4 µm) when schizogony was first observed. Both *P. chesapeaki* trophozoites (range 11.0 ± 4.5 µm to 12.0 ± 3.3 µm) and *P. olseni* trophozoites (range 32.0 ± 15.3 µm to 36.9 ± 8.3 µm) showed no significant variation in diameter. Mean diameter of both *P. marinus* (17.5 ± 2.5 µm; Day 12) and *P. chesapeaki* (17.2 ± 3.8 µm; Day 8) schizonts was similar. No *P. olseni* schizonts were observed.

At 15‰, *Perkinsus marinus* trophozoites were significantly smaller than those of *P. olseni* and *P. chesapeaki* at the beginning of the study and on Day 2. The lowest mean trophozoite diameter was 5.1 ± 0.8 µm for *P. marinus*, 9.3 ± 2.7 µm for *P. chesapeaki* and 10.8 ± 3.8 µm for *P. olseni*. The diameter of *P. marinus* and *P. olseni* trophozoites increased significantly from the time of seeding to Day 6. Schizonts were first observed on Day 6 for *P. marinus* and on Day 12 for *P. olseni*. In contrast, *P. chesapeaki* trophozoites showed no significant variation in diameter (range 9.2 ± 1.2 µm to 9.6 ± 2.4 µm), and schizogony was observed as early as Day 2. The mean diameter of *P. olseni* schizonts (34.2 ± 7.2 µm; Day 12) was significantly higher than that of both *P. marinus* (15.2 ± 2.1 µm; Day 8) and *P. chesapeaki* (16.5 ± 2.6 µm; Day 2), which were similar.

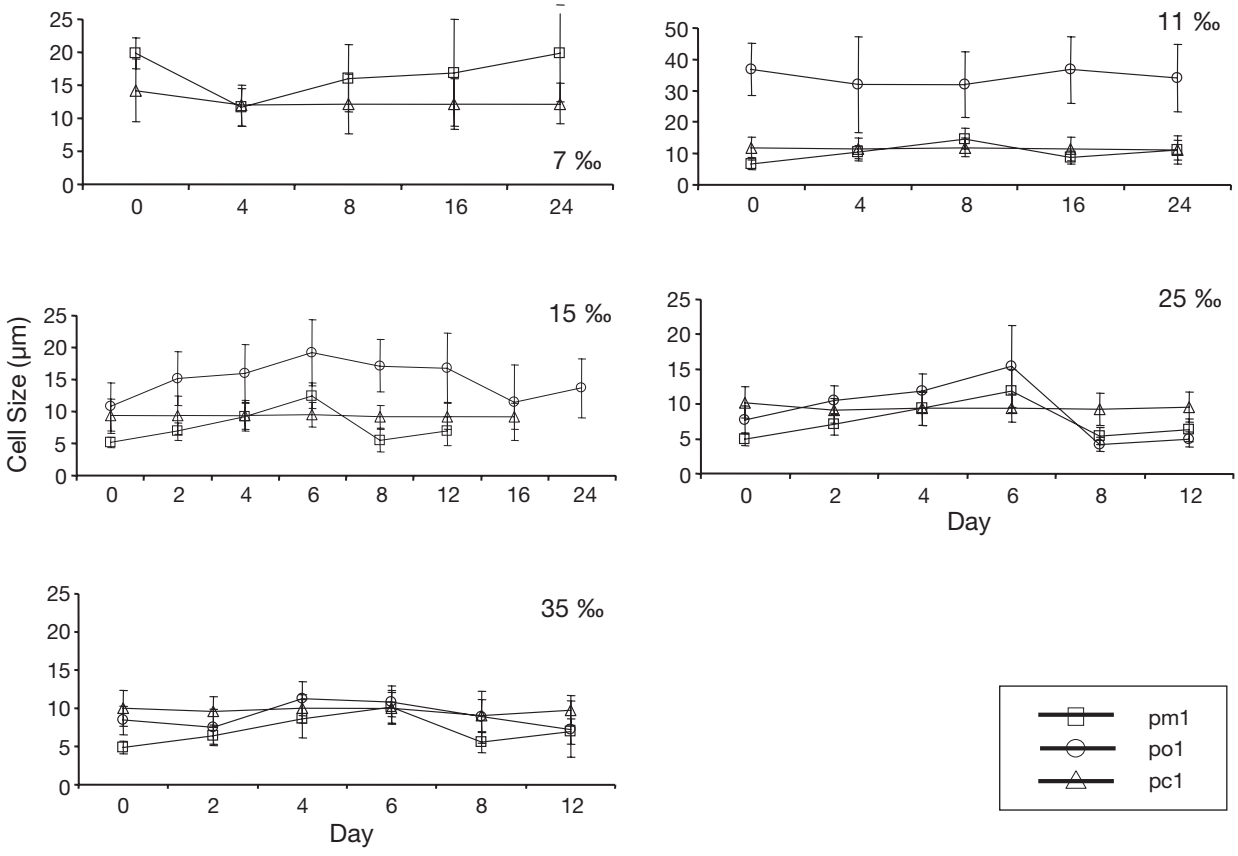

Fig. 6. *Perkinsus* spp. Mean ± SD trophozoite diameter (µm) over 28 d period of response of 1 isolate each of *P. marinus* (GTLA-33 [pm1]), *P. olseni* (PaHm-2F [po1]) and *P. chesapeaki* (CRMA-J44/E3 [pc1]) exposed to 5 salinities after acclimation period (Expt 2)

At 25‰, *Perkinsus marinus* trophozoites were significantly smaller than those of *P. olseni* or *P. chesapeaki* at the beginning of Expt 2 and on Days 2 and 4. Both *P. marinus* and *P. olseni* trophozoites diameters increased significantly from the time of seeding to Day 6 (*P. marinus*) and Day 8 (*P. olseni*), when schizonts were first observed. In contrast, *P. chesapeaki* trophozoites showed no significant variation in diameter during the study (range 9.1 ± 1.8 µm to 10.2 ± 2.3 µm) and schizogony was observed as early as Day 2. The mean diameter of *P. olseni* schizonts (22.6 ± 5.4 µm; Day 8) was significantly larger than those of either *P. marinus* (15.0 ± 2.1 µm; Day 6) or *P. chesapeaki* (15.4 ± 2.6 µm; Day 2).

Table 3. *Perkinsus marinus*, *P. olseni* and *P. chesapeaki*. Mean ± SD diameter (µm) of schizonts in Expt 2 at all 5 salinities (N = 25). *P. olseni* schizonts were not observed at 7 or 11‰

Species	Salinity (‰)				
	7	11	15	25	35
P. marinus	27.8 ± 4.1	17.5 ± 2.5	15.2 ± 2.1	15.0 ± 2.1	15.1 ± 2.2
P. olseni	–	–	34.0 ± 7.2	22.6 ± 5.4	15.3 ± 3.3
P. chesapeaki	18.9 ± 3.8	17.2 ± 3.8	16.5 ± 2.6	15.4 ± 2.6	15.3 ± 2.3

At 35‰, patterns were very similar to those at 25‰. There was a significant species × day interaction. *Perkinsus marinus* trophozoites were significantly smaller than those of *P. olseni* or *P. chesapeaki* at the beginning of the study, on Days 2 and 4, and following schizogony. The lowest mean trophozoite diameter observed was 4.9 ± 0.9 µm for *P. marinus* (beginning of Expt 2), 7.3 ± 3.7 µm for *P. olseni* (Day 12) and 9.1 ± 2.1 µm for *P. chesapeaki* (Day 8). Diameter of *P. marinus* and *P. olseni* trophozoites increased significantly from the time of seeding (GTLA-33, 4.9 ± 0.9 µm; PaHm-2f, 8.5 ± 1.9 µm) to Day 6 (GTLA-33, 10.2 ± 2.2 µm; PaHm-2f, 10.9 ± 2.0 µm) when schizonts were first observed. In contrast, *P. chesapeaki* trophozoites showed no significant variation in diameter (range 9.1 ± 2.1 µm to 10.0 ± 2.4 µm), as schizogony was observed as early as Day 2. The mean diameter of *P. marinus* (15.1 ± 2.2 µm; Days 6, 8, 12), *P. olseni* (15.3 ± 3.3 µm; Days 6, 8, 12), and *P. chesapeaki* (15.3 ± 2.3 µm; Days 2, 4, 8) schizonts were similar.

DISCUSSION

While there was significant overlap in the salinity tolerance of the 3 *Perkinsus* species studied, distinct salinity tolerances and responses to acute and acclimated salinity changes were noted. Overall, *P. chesapeaki* showed the widest range of salinity tolerance of the 3 species, with viability and cell proliferation at all salinities tested (7, 11, 15, 25, 35‰). *P. olseni*, originating from high salinity areas, had higher viability and proliferation at the higher salinities (15, 25, 35‰), with no viability at 7‰ and minimal viability at 11‰, even with acclimation. Lastly, *P. marinus* was more tolerant of the lower salinities (7 and 11‰) than *P. olseni*, but exhibited minimal growth and declining viability without subculture at 7‰, even after acclimation. While all 3 species had very distinct responses to the range of salinities tested, both with and without acclimation, there was significant overlap in their salinity tolerances, with all 3 displaying high viability and metabolic activity in the mid-to-high salinities tested (15, 25, 35‰).

Perkinsus chesapeaki

Perkinsus chesapeaki was more tolerant to low salinity (7, 11‰) than either *P. marinus* or *P. olseni*, both with and without acclimation. The percent of *P. chesapeaki* parasites surviving hypo-osmotic shock (e.g. 25‰ to 7 or 11‰) was much greater than for either *P. marinus* or *P. olseni*. Furthermore, *P. chesapeaki* was able to divide rapidly (by Day 2) after some initial mortality following hypo-osmotic shock in Expt 1, as indicated by a significant rise in percent viability at 7 and 11‰. McLaughlin et al. (2000) examined *P. chesapeaki's* (G-117) response to hypo-osmotic shock and, based on cell density measurements, also concluded that *P. chesapeaki* tolerates hypo-osmotic shock better than *P. marinus* (H-49) (McLaughlin et al. 2000). This conclusion, however, was based on a much smaller salinity change than we tested, i.e. from 756 (~25‰) to 372 mOsm kg^{-1} (~14‰). Using explicit viability measurements of *P. chesapeaki* undergoing more acute hypo-osmotic shock (from 25‰ to 11 or 7‰), our study was able to confirm and expand the suggestion of McLaughlin et al. (2000).

While *Perkinsus chesapeaki* was clearly tolerant of the lower salinities tested (i.e. 7, 11‰), several measures indicated that these lower salinities were suboptimal for its growth: cell density and its rate of increase were both greater at 15 and 25‰ than at 7 and 11‰. At the same time, metabolic activity was minimally affected by the lower salinities (all >10 000 U by Day 4). This finding is clearly different from that of McLaughlin et al. (2000), who found that the growth rate of *P. chesapeaki* (G-117) at 14‰ was significantly greater than at 24‰, and concluded that this might be the reason why *P. chesapeaki* is found in *Mya arenaria* in low salinity (<15‰) area. While the variability in the *P. chesapeaki* isolates response to salinity was not reported by McLaughlin et al. (2000), our Expt 1 used 2 isolates that responded very similarly to the hypo-osmotic shock at all salinities.

Perkinsus chesapeaki further differed from the other 2 *Perkinsus* species studied in that it divided rapidly upon subculture, whereas the lag phases of *P. marinus* and *P. olseni* lasted from days to weeks, depending on salinity. Interestingly, *P. chesapeaki* readily divides at low seeding density (<<10^5 cells ml^{-1}, La Peyre unpubl. data) and is thus readily cloned by limiting dilution (Dungan pers. comm.). This may be a result of the higher metabolic activity recorded for *P. chesapeaki* at all salinities compared to that of *P. marinus* and *P. olseni*. McLaughlin et al. (2000) reported low division of *P. chesapeaki* after 1 wk. This contradictory finding may be due to the fact that (1) McLaughlin et al. (2000) used the chemically defined medium JL-ODRP-3, which has less nutrients (e.g. lacks yeastolate and cod-liver oil) and has been found to support lower rates of division for *P. marinus* Isolate P-1 (La Peyre & Faisal 1996), and (2) they used a higher parasite seeding density than in our study (1×10^6 cells ml^{-1} versus 2×10^5 cells ml^{-1} in our studies). The high metabolic activity of *P. chesapeaki* raises questions about a possible relationship with infectivity and pathogenicity. It will be interesting to determine in future studies whether the number of parasites needed to establish an infection, and to cause pathological effects and mortality, is lower for *P. chesapeaki* in *Mya arenaria* than in *P. marinus* in eastern oysters or *P. olseni* in carpet shell clams or other molluscan hosts.

Division of *Perkinsus chesapeaki* in our culture media was almost exclusively by schizogony, and the percentage of cells zoosporulating was always very low (<<1% cells). This is in contrast to the finding of Burreson et al. (2005), who reported a high percentage of cells zoosporulating with *P. chesapeaki* Isolate ATCC PRA-65 (range 20 to 25%) and with the conspecific *P. andrewsi* Isolate ATCC 50807 (range 25 to 27%) in their culture medium DMEM/HamF12-3 (850 mOsm kg^{-1}). The elevated percentage of zoosporulation in Burreson et al.'s (2005) study may have been due to the higher levels of nutrients in their culture medium, DMEM/HamF12-3, which is composed of DME/Ham's 12 supplemented with 3% fetal bovine serum and various other nutrients.

While *Perkinsus chesapeaki* clearly grew well, and by some measures better at the higher salinities (15, 25‰), the *P. chesapeaki* cultures used in our study and

other published studies (McLaughlin et al. 2000, Dungan et al. 2002) all come from the upper Chesapeake Bay where salinities rarely exceed 15‰ (Maryland Department of Natural Resources Continuous Data Recorder: http://mddnr.chesapeakebay.net/eyesonthebay/index.cfm). Infections in *Mya arenaria* in the upper Chesapeake Bay (<15‰) were found to occur in 37 to 64% of this species tested in one study (McLaughlin & Faisal 2000), and in 30 to 100% of clam in samples from *M. arenaria* and *Tagus plebeius* populations in a second study (Dungan et al. 2002). The impact of *P. chesapeaki* on clam populations (e.g. *M. arenaria*, *Tagelus plebeius*, *Macoma balthica*) in the lower Chesapeake Bay needs to be investigated urgently, as higher salinity (15, 25‰) resulted in better growth of *P. chesapeaki* in our study. Unfortunately, little is known about the prevalence and intensity of *P. chesapeaki* infection in clam populations of the lower Chesapeake Bay despite the fact that their infection by *Perkinsus* spp. (probably *P. chesapeaki*) was described, more than 50 yr ago in Virginia's York River (Andrews 1955). There are no data to suggest that *P. chesapeaki* is pathogenic to oysters and, in fact, the vast majority of *Perkinsus* spp. cultured from eastern oysters have been *P. marinus*. Moreover, no *P. chesapeaki* cultures have been established from Chesapeake Bay oysters. In a recent study, eastern oysters were challenged with *P. chesapeaki* at 25‰ and became infected. However, the infections failed to progress, and in fact decreased over a 2 mo period (<1000 parasites g^{-1} wet tissue; Casas et al. 2005).

Perkinsus olseni

Perkinsus olseni isolates were the least tolerant of low salinity compared to *P. marinus* and *P. chesapeaki*, with optimum growth (in terms of parasite density) occurring at 25‰. No *P. olseni* survived at 7‰ even after acclimation. At 11‰, only 1 of the 2 isolates (PaHm14f) survived and grew after the initial hypo-osmotic shock, and Isolate PaHm2f (the least tolerant to hypo-osmotic shock) never acclimated and failed to grow. Interestingly, Casas et al. (2002b) reported zoosporulation in *P. olseni* occurring from 10 to 35‰, suggesting that the more hypo-osmotic-tolerant isolate (PaHm14f) used in this study may actually have been more representative of *P. olseni* in general. Still, the optimum salinity range for zoosporulation was reported as 25 to 35‰ (Casas et al. 2002b).

While *Perkinsus olseni* was clearly more tolerant of the higher salinities (i.e. 15, 25, 35‰), metabolic activity at 11‰ was higher than at 15‰. This may simply reflect the increase in parasite size at the lower salinity. Similar to the findings of enlarged *P. marinus* trophozoites at 7‰, and to some extent with *P. chesapeaki*, cell diameter increased at the lower salinities. These increases in cell size may be a stress response, since parasite enlargement and the formation of hypnospores can also be induced by rapidly changing the culture medium's physical and chemical properties such as pH, gas phase and metabolic constituents (Casas & La Peyre pers. obs.). The size of *Perkinsus* spp. cells have also been shown to vary substantially *in vivo*, depending on host (Goggin & Lester 1995, Burreson et al. 2005) and time of year (Ray & Chandler 1955, Bushek et al. 1994), and can be influenced *in vitro* by culture methodology (La Peyre 1996, Casas et al. 2002a). The osmolarity of the culture medium is therefore one more factor that can influence the size of cultured *Perkinsus* spp. cells and must be considered when comparing their sizes. It remains to be determined whether salinity has a similar effect on the size of *Perkinsus* spp. cells *in vivo*.

While clear differences in the response of the 2 isolates, especially to lower salinity (i.e. 11‰) occurred, *Perkinsus olseni's* salinity tolerance clearly ranges from 15 to 35‰, with optimal growth at 25‰. This range coincides with the natural conditions encountered by *P. olseni*. Along the western Atlantic coast of Spain, where *P. olseni* infections of clams have been recorded, salinities below 25‰ are rare (Casas et al. 2002b, Beiras et al. 2003, Page & Lastra 2003). While *P. olseni* infections in molluscs have been observed in the coastal waters of Australia, Japan and Korea, salinity data have seldom been reported. In Korea, Park & Choi (2001) measured salinities which rarely were below 25‰ at locations where Manila clams infected with a *Perkinsus* sp. (recently identified as *P. olseni*: Park et al. 2005), were collected. However, since there were distinct responses to the low salinities by the 2 isolates examined, comparisons of other *P. olseni* isolates are needed to determine which response is more typical. Specifically, as Expt 2 with acclimation used the isolate most sensitive to low salinity in Expt 1, determining which of the 2 isolates is more typical is critical in setting the lower salinity bounds for *P. olseni*. Further study with other isolates may indicate a better tolerance to lower salinity (i.e. 11‰). The distinct differences between the 2 isolates may also reflect the likelihood that *P. olseni*, which is a widely distributed species with a wide range of hosts, may have greater genetic variation than other *Perkinsus* species.

Similar to *Perkinsus chesapeaki*, which infects at least 3 species of clams (*Mya arenaria*, *Tagelus plebeius*, *Macoma balthica*) in Chesapeake Bay (McLaughlin & Faisal 2000, Coss et al. 2001, Dungan et al. 2002), *P. olseni* is known to infect various species of molluscs (e.g. *Haliotis ruber*, *Tapes decussatus*, *Venerupis philippinarum*, *Anadaria trapezia*, *Austrovenus*

stutchburyi) in various regions of the world (Goggin 1994, Hamaguchi et al. 1998, Murrell et al. 2002). Although there is some evidence that *P. chesapeaki* and *P. olseni* have been associated with mortalities of a few molluscan species (Lester & Davis 1981, Azevedo 1989, Goggin & Lester 1995, Sagrista et al. 1996, McLaughlin & Faisal 2000, Dungan et al. 2002), their pathological effects on most species they infect remain to be fully described.

Perkinsus marinus

Perkinsus marinus was found to be more tolerant of low salinity than *P. olseni*, but less tolerant than *P. chesapeaki*. The results of the *P. marinus* salinity-tolerance experiment agreed with the extensive epizootiological data available for *P. marinus* in oysters (reviewed in Burreson & Ragone Calvo 1996) as well as with the extensive experimental/laboratory data available (Chu & La Peyre 1993, Chu et al. 1993, Ragone & Burreson 1993, Ragone Calvo et al. 2001). In general, all measures (viability, metabolic activity, number) indicate that optimal *P. marinus* growth occurs at the higher salinities (15, 25, 35‰), and that after acclimation *P. marinus* tolerates lower salinities (i.e. 7‰). That 7‰ was stressful to *P. marinus* was reflected (similar to *P. olseni* at the higher 11‰ salinity) in an increase in metabolic activity associated with larger cell sizes, but a lower increase in cell numbers. Increased metabolic activity is simply a product of larger, and probably stressed, cells. While assays of metabolic activity (i.e. alamarBlue, MTS/PMS) have been used to measure *Perkinsus* spp. proliferation under various conditions (Dungan & Hamilton 1995, Elandalloussi et al. 2003), the results from such assays depend not only on cell numbers but also cell size and culture phase, and must be interpreted cautiously (La Peyre 1996).

Distinct differences in *Perkinsus marinus* viability were observed at the low salinities with and without acclimation. Without acclimation, no *P. marinus* cells survived hypo-osmotic shock (from 25 to 7‰); with 10 wk acclimation to 7 from 25‰, initial *P. marinus* viability was high (86 ± 1.6%) but declined after 28 d of monitoring (63.6 ± 6.4%). A similar trend of better survival at 11‰ after acclimation was also observed. This agrees in general with other studies which indicate that, while *P. marinus* can survive some hypo-osmotic shock, acclimation to low salinities clearly results in decreased mortality. O'Farrell et al. (2000) examined the effects of hypo-osmotic treatments (56 mOsm, ~2.5‰) on *P. marinus* cells and found that cells cultured at low osmolality (168 mOsm, ~6.5‰) experienced only 41% mortality, whereas cells cultured at higher salinities and experiencing more acute hypo-osmotic shock similar to that in our study (341 to 737 mOsm) experienced 100% mortality. Similarly, an earlier *in vitro* study with cultured *P. marinus* cells found that viability measured 24 h after transfer from a salinity of 22‰ (~660 mOsm kg^{-1}) to 9‰ (~270 mOsm kg^{-1}) was 57%, after transfer from 22 to 6‰ (~180 mOsm kg^{-1}) viability was 30%, and after transfer from 22 to 3‰ (~90 mOsm kg^{-1}; Burreson et al. 1994) it was 10%. Most recently, an *in vivo* study found that severe hypo-osmotic shock (>20 to 1‰) significantly reduced *P. marinus* infection intensity, but failed to eliminate the parasite (La Peyre et al. 2003).

As with the other 2 species, the findings coincide with the salinity range for *Perkinsus marinus*. The isolates used were from the Grande Terre area of Louisiana, which generally has a salinity range of 15 to 25‰, with occasionally (and not unusually) higher and lower salinity due to drought or rain. However, as most of the previous experimental/laboratory data which concur with our findings are for isolates from the Chesapeake Bay, these findings apply beyond Louisiana in areas where other *Perkinsus* species such as *P. chesapeaki* may overlap with *P. marinus*. Interestingly, isolates of each species (H49 pm, G117 pc) were isolated from the same host clam, *Mya arenaria* (McLaughlin & Faisal 2000), demonstrating cross-infectivity and, more importantly, raising questions related to the pathogenecity of each species to both clams and oysters. While cross-infectivity has been demonstrated, it is unlikely that it occurs frequently, as the large majority of *Perkinsus* species cultured from clams have been *P. chesapeaki* (Dungan, MDNR pers. comm.) and the vast majority of *Perkinsus* spp. cultured from oysters have been *P. marinus*. Moreover, H49 is the only *P. marinus* isolate acquired from *M. arenaria* (McLaughlin & Faisal 1998b). Based on the findings of our study, as well as previous work on *P. marinus* (e.g. Chu et al. 1993, Ragone & Burreson 1993) mortality is not likely to result at low salinities, regardless of host. The effects of *P. marinus* at high salinities in clam hosts remains to be determined empirically.

CONCLUSIONS

Using 2 isolates of 3 *Perkinsus* species, acute and acclimated salinity tolerances of each species were defined. While variation within species must be better characterized, our results do provide general trends on species tolerances that match existing data (i.e. *P. marinus*), and on the home ranges of host species. For *P. olseni*, distinct differences in acute salinity response of the 2 isolates failed to provide a clear indication of the lower salinity tolerance of *P. olseni* in general. Use

of more isolates to define the low end of the salinity range of *P. olseni* would provide better data on the likelihood of its expansion into lower salinity waters. In contrast, for *P. chesapeaki*, despite the fact that the isolates originated from the upper Chesapeake Bay (salinity <15‰), our data indicate that *P. chesapeaki* would do well at higher salinities (15, 25‰). This suggests that we might expect to see the range of *P. chesapeaki* expand in time to higher salinity areas. For *P. olseni* and *P. chesapeaki*, a more expansive comparison of isolates originating from different salinity regimes might further clarify their lower (*P. olseni*) and upper (*P. chesapeaki*) salinity bounds.

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