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The combined influence of sub-optimal temperature and salinity on the in vitro viability of *Perkinsus marinus*, a protistan parasite of the eastern oyster *Crassostrea virginica*

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ABSTRACT

Perkinsus marinus is a major cause of mortality in eastern oysters along the Gulf of Mexico and Atlantic coasts. It is also well documented that temperature and salinity are the primary environmental factors affecting *P. marinus* viability and proliferation. However, little is known about the effects of combined sub-optimal temperatures and salinities on *P. marinus* viability. This in vitro study examined those effects by acclimating *P. marinus* at three salinities (7, 15, 25 ppt) to 10 °C to represent the lowest temperatures generally reached in the Gulf of Mexico, and to 2 °C to represent the lowest temperatures reached along the mid-Atlantic coasts and by measuring changes in cell viability and density on days 1, 30, 60 and 90 following acclimation. Cell viability and density were also measured in 7 ppt cultures acclimated to each temperature and then transferred to 3.5 ppt. The largest decreases in cell viability occurred only with combined low temperature and salinity, indicating that there is clearly a synergistic effect. The largest decreases in cell viability occurred only with both low temperature and salinity after 30 days (3.5 ppt, 2 °C: 0% viability), 60 days (3.5 ppt, 10 °C: 0% viability) and 90 days (7 ppt, 2 °C: 0.6 ± 0.7%; 7 ppt, 10 °C: 0.2 ± 0.2%).

1. Introduction

Perkinsus marinus, a protistan parasite, is the most studied of the *Perkinsus* species and causes significant mortalities of eastern oysters (*Crassostrea virginica*) in the major growing areas of the Gulf of Mexico and the Atlantic coasts of the United States (Soniat, 1996; Burreson and Ragone Calvo, 1996). As such, much effort has gone into both attempting to predict epizootics, and to control the distribution or expansion of *P. marinus* to new regions. This ability to predict epizootics and range expansion is ultimately dependent on understanding not just the optimal conditions for parasite growth and production, but also the extreme conditions in which the parasite can survive as well as the conditions that significantly reduce its viability.

Extensive research has identified temperature and salinity as the most important environmental factors affecting *P. marinus* and its interactions with its host *C. virginica* (Soniat and Gauthier, 1989; Chu and La Peyre, 1993; Chu et al., 1993; Ragone and Burreson, 1993; Burreson and Ragone Calvo, 1996; Ford, 1996; Ragone Calvo et al., 2000). As such, a fairly precise range of optimal environmental conditions for growth and proliferation of *P. marinus* has been defined (28–32 °C, 24–36 ppt) (Dungan and Hamilton, 1995; Gauthier and Vasta, 1995; La Peyre and Faisal, 1996; La Peyre, 1996). Despite this narrow range of optimal conditions, it has also been shown that *P. marinus* is highly resistant to a wide range of environmental conditions (Chu and Greene, 1989; O'Farrell et al., 2000; La Peyre et al., 2006, 2008) leading to more in-depth work trying to establish what extreme conditions might limit *P. marinus* epizootics or expansion. This research is of particular importance given concerns about species range expansion during a time of significant climate change (Burreson and Ragone Calvo, 1996; Ford, 1996; Cook et al., 1998; Ford and Chintala, 2006; Ford and Smolowitz, 2007).

A few studies have explored sub-optimal temperature and salinity limits on *P. marinus* viability. Specifically, lower salinity has been examined using acute exposure of in vitro cultures which found that lower salinity resulted in high parasite mortality (70% at 6 ppt, >90% at 3 ppt and lower; Burreson et al., 1994), however, when cultures were acclimated to lower salinity, mortality was greatly reduced (41% mortality at 2.5 ppt after acclimation versus 100% mortality without acclimation) (O'Farrell et al., 2000). More

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recently, a combined laboratory and field study of *P. marinus* survival in vivo found that lowered salinity (0–1 ppt) decreased infection intensities but failed to eliminate the parasite (La Peyre et al., 2003). For temperature, a study testing acute and acclimated response of *P. marinus* in vitro to low temperatures (2, 4 and 6 °C) found that some *P. marinus* survived up to 90 days at these lower temperatures (La Peyre et al., 2008). Combined, these studies suggest that neither sub-optimal salinity nor sub-optimal temperature alone will necessarily eliminate *P. marinus*, nor prevent its spread; however, the combined effects of sub-optimal salinity and temperature have not been explicitly tested. This study was therefore designed to test the combined effects of salinity (3.5, 7, 15, 25 ppt) and temperature (2, 10, 28 °C) on *P. marinus* viability.

2. Materials and methods

2.1. P. marinus isolate and acclimation

Perkinsus marinus isolate P-1 (La Peyre et al., 1993) was maintained at 28 °C, and sub-cultured every other month in the culture medium JL-ODRP-2A adjusted to osmolalities of 230, 450 and 775 mOsm per kg for over a year before the beginning of this study. The composition of the media was the same except for the salt concentrations (Table 1, La Peyre et al., 2006). The osmolalities of the media were equivalent to the osmolalities of a seawater salt solution (i.e. 35 ppt) diluted with freshwater to salinities of 7, 15, and 25 ppt. For clarity, all media will be referred to henceforth by their seawater salinity equivalence. All chemicals used to prepare JL-ODRP-2A culture media were purchased from Sigma–Aldrich Co. (St. Louis, Missouri) except yeastolate which was purchased from Life Technologies Inc. (Rockville, Maryland).

Four weeks prior to the start of the experiment, cells at each salinity were passed five times through a 25-gauge needle attached to a 10 ml syringe to remove clumps, rinsed three times with fresh media, and added to five 25 cm^2 flasks (Corning, Inc., Corning, New York) at a density of 1×10^6 cells per ml of corresponding medium. At each salinity, cells in one flask were maintained at 28 °C, cells in two of the flasks were acclimated from 28 °C to 10 °C by decreasing incubation temperature by 0.5 °C every day, over a period of 36 days and cells in the two remaining flasks were acclimated from 28 °C to 2 °C by decreasing incubation temperature by 0.5 °C every day, over a period of 52 days.

When temperatures reached 10 °C (i.e., day 36) and 2 °C (i.e., day 52), cell suspensions from each flask were pooled and prepared at a density of 1×10^6 viable cells per ml at each salinity (7, 15, 25 ppt) and temperature combination (2, 10 °C) as described above. Cells from flasks maintained at each salinity (7, 15, 25 ppt) and at 28 °C were harvested 36 days after subculture and prepared at a density of 1×10^6 viable cells per ml at their respective salinities. Cell viability was measured using neutral red as described by La Peyre and Chu (1994). In addition, cells harvested from flasks with 7 ppt medium at each temperature were rinsed twice and resuspended in a 3.5 ppt medium (110 mOsm per kg, Table 1) and their densities were adjusted to 1×10^6 viable cells per ml within 1 h of being transferred to the new medium. For *P. marinus* at each temperature (2, 10, 28 °C), 100 µl of cell suspension at each salinity (3.5, 7, 15 and 25 ppt) were then added to four replicate wells in four 96-well tissue culture plates (i.e., 12 plates total). The four plates which were maintained at each temperature in humidified chambers in incubators were used to measure cell viability and density after 1, 30, 60, and 90 days.

Table 1
Salt concentrations (mg/l) of media used in experiments with osmolalities of 775, 450, 230, and 205 mOsm/kg. Osmolalities of media were equivalent to osmolalities of seawater (35 ppt) diluted with freshwater to salinities of 25, 15, 7, and 3.5 ppt.

Salt	Osmolality (mOsm/kg)/Salinity equivalences (‰)			
	775/25	450/15	230/7	110/3.5
$CaCl_2{\cdot}2H_2O$	1176	1030	515	172
$MgSO_4$	2258	1092	546	182
$MgCl_2{\cdot}6H_2O$	3502	1638	819	273
KCl	1028	600	300	100
NaCl	20,052	11,104	5256	1752
$NaHCO_3$	336	336	336	336

2.2. Cell density and viability

On each sampling day, 10 µl of neutral red (50 mg/l) were added to each well of one of the plates that had been maintained at each temperature and the plates were incubated at 28 °C for 2 h to allow active uptake of neutral red by live cells. Cells from each well were then resuspended and passed five times through a 25-gauge needle after which 10 µl of cell suspension was added to each side of a Neubauer Bright-LineR hemocytometer (Reichert, Buffalo, New York). Cell density was determined by counting all live (stained) and dead cells (unstained) in a specific area of the hemacytometer containing a minimum of 200 cells. The percent viability was calculated by dividing the number of live cells by the total number of cells counted.

Cell density and percent viability data collected at each temperature were analyzed separately, by salinity using a one-way ANOVA (factor: day; $N = 192$). Cell density data were log transformed in order to achieve normality and homogeneity of variance. When ANOVA models were significant ($P < 0.05$), Student–Newman–Keuls method was used for pairwise comparisons. Statistical analysis was performed using Sigma Stat Version 3.5 (Systat software Inc., Chicago, Illinois). All data are reported as mean ± standard deviation (SD).

3. Results

3.1. Cell viability

Overall, cell viability at 3.5, 7, 15 and 25 ppt decreased significantly between most consecutive sampling days when the incubation temperature was 2 and 10 °C (Fig. 1). In contrast, at 28 °C, significant decreases in cell viability were only observed at 7 and 3.5 ppt (Fig. 1). The decreases in cell viability were greatest at the lowest salinity and temperature. After 52 days of acclimation to a final temperature of 2 °C, the viability of cells harvested from 7 ppt cultures was 74.1 ± 4.9% and rapidly decreased to 35.4 ± 2.2% after their transfer to 3.5 ppt medium and prior to seeding in 96 well plates (Fig. 1, 3.5 ppt). The viability of those cells further decreased to 19.1 ± 1.9% after 1 day and 0% after 30 days in 3.5 ppt medium at 2 °C. After 36 days of acclimation to a final temperature of 10 °C, the viability of cells at 7 ppt was 68.7 ± 3.7% and rapidly decreased to 32.4 ± 2.8% following their transfer to 3.5 ppt medium (Fig. 1, 3.5 ppt). The viability of those cells further decreased to 27.0 ± 1.8%, after 1 day, 5.6% after 30 days and 0% after 60 days in 3.5 ppt medium at 10 °C. In contrast, the viability of cells cultured at 7 ppt and 28 °C only decreased from 94.6 ± 2.4% to 91.2 ± 0.6% after their transfer to 3.5 ppt medium and to 90.4 ± 1.7% on day 1 (Fig. 1, 3.5 ppt). The viability of those cells further decreased to 45.3 ± 2.8% on day 30, 22.9 ± 7.7% on day 60 and 15.6 ± 0.6% on day 90.

The majority of cells cultured at 7 ppt and 2 °C died between day 1 (74.4 ± 4.9%) and day 30 (17.3 ± 3.4%) (Fig. 1, 7 ppt). Viability was further reduced to 3.5 ± 0.7% on day 60 and 0.6 ± 0.7% on day 90. At 10 °C, viability of cells cultured at 7 ppt was 68.7 ± 3.7% on day 1 and decreased to 40.6 ± 1.2% on day 30 and 9.2 ± 1.8% on

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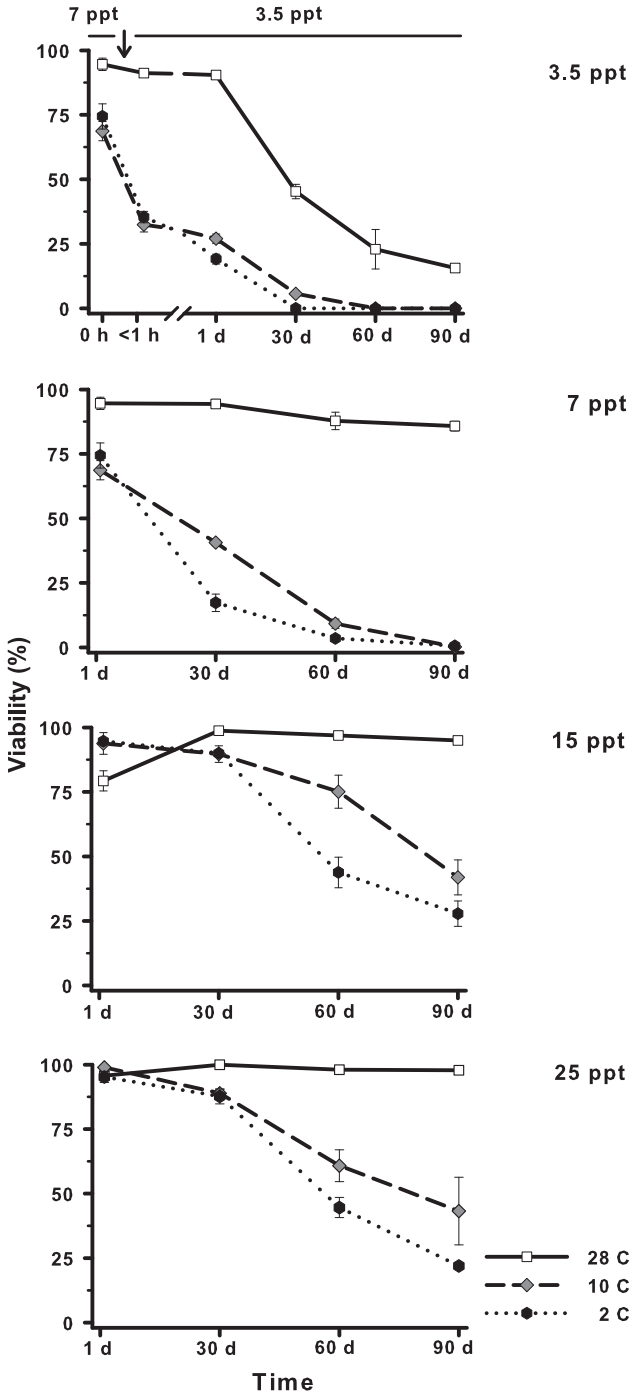

Fig. 1. Viability (±SD) of *P. marinus* cells from four replicate wells at 3.5, 7, 15 and 25 ppt after acclimation to 2 and 10 °C, and at 28 °C. The cells at 3.5 ppt were transferred from 7 ppt at the end of the temperature acclimation period. Viabilities of cells at 7, 15 and 25 ppt were measured on day 1, 30, 60 and 90. Viabilities of cells at 3.5 ppt were measured at 1 h and on day 1, 30, 60 and 90 after their transfer from 7 ppt.

day 60 from day 1. By day 90, the percentage of live cells at 7 ppt was only 0.2 ± 0.2%. Cell viability of cultures at 15 and 25 ppt at 2 °C followed a similar trend (Fig. 1, 15 and 25 ppt): a slight but significant decrease in viability of about 6% between day 1 and 30, a more pronounced decrease in viability of about 50% between day 30 and 60 to a final viability on day 90 of 27.8 ± 4.9% at 15 ppt (Fig. 1, 15 ppt) and 21.9 ± 0.7% at 25 ppt (Fig. 1, 25 ppt). At 10 °C, cell viability of 15 and 25 ppt cultures decreased significantly but more gradually than at 2 °C between each consecutive sampling time reaching 41.9 ± 6.8% at 15 ppt and 43.2 ± 13.1% at 25 ppt on day 90. At 28 °C, the viability of proliferating cells at 15 and 25 ppt was consistently above 90% throughout the incubation period while proliferating cells at 7 ppt experienced a slight but significant decrease in viability between day 1 (94.6 ± 2.4%) and 60 (87.8 ± 3.4%).

3.2. Cell density

Cell density differed significantly by day for all temperature and salinity combinations, with the exception of 3.5 ppt (10 °C) which showed no significant differences (Fig. 2). Overall, at 2 °C and 10 °C, cell density did not change drastically (<30%) and indicated that cell wall lysis after death was minimal. Differences that were significant were predominantly between day 1 and day 30 (for 2 °C), and between day 1 and day 60 (for 10 °C). At 28 °C, significant differences occurred predominantly between day 1 and day 30 when cell densities increased significantly at all salinities (Fig. 2, 3.5 ppt, 7 ppt, 15 ppt, 25 ppt) and the greatest increase in cell density was at 25 ppt.

4. Discussion

The largest decreases in cell viability occurred when *P. marinus* was exposed simultaneously to sub-optimal salinity (3.5 and 7 ppt) and temperature (2 and 10 °C). Specifically, *P. marinus* exposed to both low salinity and temperature resulted in almost no viability after 30 days at 3.5 ppt and 2 °C (0% viability), 60 days at 3.5 ppt and 10 °C (0% viability) and 90 days at 7 ppt and 2 °C (0.6 ± 0.7%) and at 7 ppt and 10 °C (0.2 ± 0.2%). Our results also demonstrated how much less affected *P. marinus* is to sub-optimal salinities (3.5 and 7 ppt) at high temperature (28 °C) or to sub-optimal temperatures (2 and 10 °C) at the higher salinities (15 and 25 ppt) and suggest that extended and simultaneous exposure to both low temperature and salinity are necessary to have a large impact on slowing or ending a *P. marinus* enzootic or epizootic event.

While past studies have found that low salinity and temperature alone limit *P. marinus* proliferation, none have succeeded in identifying conditions that would actually eliminate all parasites from individual oysters (Ray, 1954; Andrews and Hewatt, 1957; Perkins, 1966; Chu et al., 1993; Ragone and Burreson, 1993; Chu and La Peyre, 1993; La Peyre et al., 2003, 2006, 2008, 2009). Our current in vitro study demonstrated that (1) *P. marinus* was more limited by the synergistic effects of salinity and temperature than when exposed to sub-optimal temperature or salinity alone, (2) only the extreme low salinity and temperature (3.5 ppt, 2 °C) combination reduced viability to 0% within 30 days, (3) other sub-optimal temperature and salinity combinations (3.5 ppt, 10 °C; 7 ppt, 2 °C) required extended periods of exposure (60, 90 days) to reduce viability below 1%. These lethal conditions resulting from combined low salinity and temperature, for the time durations described in this research, however are likely rare combinations in nature where *C. virginica* survives and may explain the persistence of *P. marinus* infections in most oyster growing sites along the Gulf of Mexico and Atlantic coasts in average years. These data also help explain seasonal cycling of *P. marinus* infections that are observed, and help in interpretation of inter-annual variations driven by local weather events including extreme temperatures and flood events.

The degree and period of low temperature conditions to which *P. marinus* may be exposed differ greatly between the Gulf of Mexico, the mid-Atlantic and northeastern Atlantic coasts of the United States. The low temperatures that *P. marinus* may be exposed to in Louisiana and along the northern coast of the Gulf of Mexico, are generally just below 15 °C between November and February, with lows down to 5 °C for less than 10 days a year on average (La Peyre

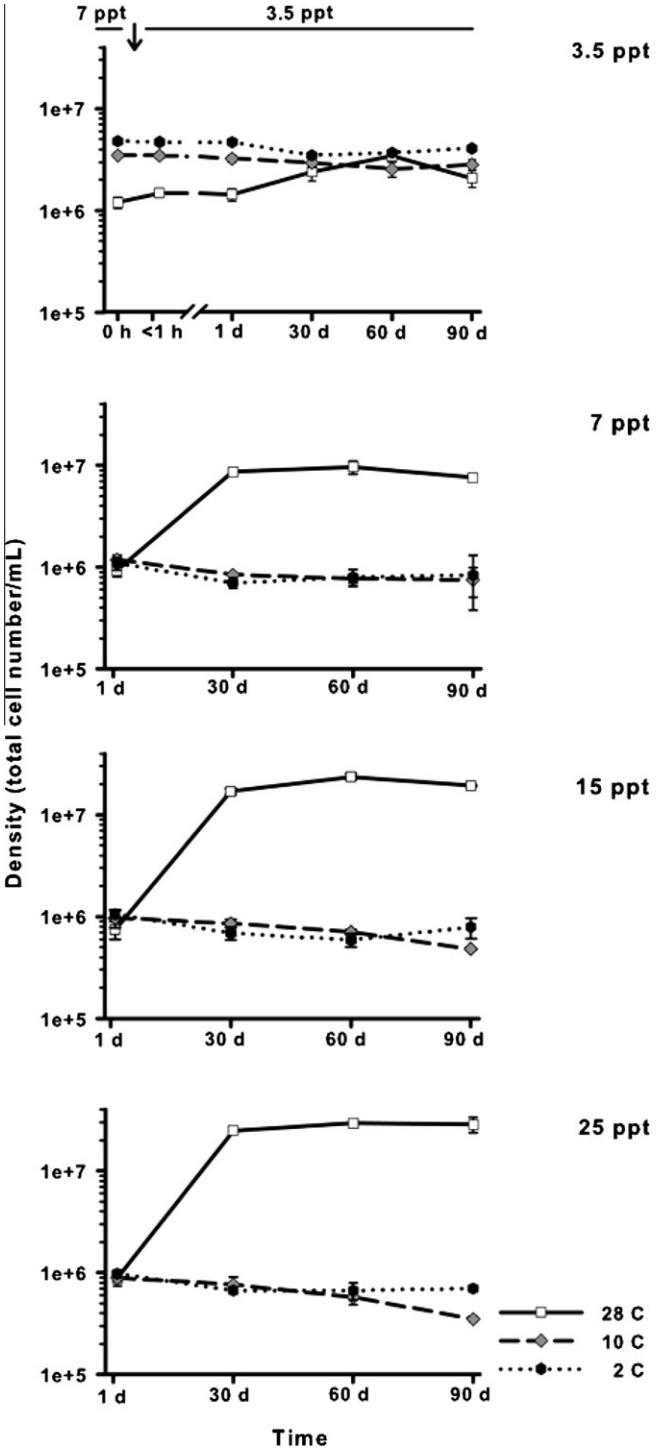

Fig. 2. Density (±SD) of *P. marinus* cells from four replicate wells at 3.5, 7, 15 and 25 ppt after acclimation to 2 and 10 °C, and at 28 °C.

et al., 2009). Even during an extreme year such as during this ongoing El Ni/o event which is characterized by colder and wetter winter and spring weather (Ropelewski and Halpert, 1987; USGCRP, 2009), temperatures were lower than 10 °C for less than 20 days and lower than 5 °C for less than 5 days in Black Bay, in Louisiana major oyster public ground between December 2009 and March 2010 (provisional data from USGS 07374526 continuous recorder in Black Bay near Snake Island near Pointe La Hache, LA). In contrast, water temperatures are generally below 5 °C for about 8 weeks in the Chesapeake Bay (Burreson and Ragone Calvo, 1996) and for 2–3 months (i.e., 1996–1997) in New England waters (Ford and Smolowitz, 2007). Oysters in estuaries along both coasts however are exposed to a similar range of salinities that is dependent on freshwater flow, local geography, and seasonal effects with most oyster production occurring in areas between 5 and 20 ppt. Although salinity at or below 3.5 ppt does occur in these productive areas, these extreme low salinity periods tend to be of short duration in most oyster growing areas of Louisiana (Chatry et al., 1985; Melancon et al., 1998). Salinities reached lower than 3.5 ppt during less than 5 days between December 2009 and March 2010 in Black Bay, Louisiana which is one of the major oyster public grounds (provisional data from USGS 07374526 continuous recorder in Black Bay near Snake Island near Pointe La Hache, LA). In the upper parts of mid-Atlantic estuaries such as the Chesapeake Bay, salinity does occasionally drop below 5 ppt, but this generally coincides with higher temperature periods (>5 °C) in the spring (Ragone Calvo and Burreson, 1994). Along the northeastern coast, salinity generally remains between 20 and 35 ppt for most of the year (Ford and Smolowitz, 2007).

Based on our in vitro results, field conditions do not appear to be extreme enough to eliminate all parasites from individual oysters in most oyster growing areas along the Gulf of Mexico and Atlantic coasts; this conclusion is also supported by field results (Bushek et al., 1994; Burreson and Ragone Calvo, 1996; Ragone Calvo et al., 2000; Oliver et al., 1998; La Peyre et al., 2009). Our in vitro results do partly explain differences in the extent of the decrease of parasite body burden observed latitudinally, between oysters located along the northern shore of the Gulf of Mexico and the mid-Atlantic and northeastern estuaries, where greater decreases in body burden coincide with the greater decreases in water temperature (Burreson and Ragone Calvo, 1996; Ragone Calvo et al., 2000; Oliver et al., 1998; La Peyre et al., 2009). At a more local level where temperature changes are consistent across salinity zones, our in vitro results partly explain the more pronounced decrease in parasite body burden with decreasing salinity observed for example in Louisiana oysters deployed at low (i.e., Cow Bayou), intermediate (i.e., Bay Gardene, Snake Island) and high (i.e., Grand Isle) salinity sites during low temperature periods (i.e., winter and early spring) (La Peyre et al., 2009). Similarly, epizootiological data from Chesapeake Bay also find that the prevalence and intensity of *P. marinus* infection decline much more rapidly during winter and spring in low salinity areas than in high salinity areas and during average years as opposed to drought years (Ragone Calvo and Burreson, 1994; Burreson and Ragone Calvo, 1996).

Our in vitro results generally agree with the findings of past in vivo laboratory studies examining *P. marinus* response to temperature and salinity (Fisher et al., 1992; Chu et al., 1993; Ragone and Burreson, 1993; Chu and La Peyre, 1993; Chu and Volety, 1997; La Peyre et al., 2003, 2009). These studies reported that the intensity of *P. marinus* infection decreases with decreasing salinities (Chu et al., 1993; La Peyre et al., 2003) and that the combination of low salinity with low temperatures reduces infection intensity to a greater degree (Fisher et al., 1992; Chu and Volety, 1997). Chu and Volety (1997) tested the interaction of three salinities (3, 10, 20 ppt) with three temperatures (10, 15, 25 °C) on weighted prevalence 60 days after challenging oysters with *P. marinus*. These authors found that either low salinity or low temperature prevented progression of the disease but that the lowest infection intensities occurred when the lowest salinity and lowest temperature were combined. Under their most extreme conditions tested (10 °C, 3 ppt) infections were not eliminated after 60 days. Although this in vivo result appears different than our in vitro results as we did not observe viable *P. marinus* cells after 60 days at 10 °C and 3.5 ppt (110 mOsm/kg), the plasma osmolalities of the oysters maintained at 3 ppt in the Chu and Volety (1997) study were not measured and may not have reached osmolarities

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equivalent to or lower than 3.5 ppt. While the plasma osmolalities of field oysters collected from an area where salinity was lower than 3 ppt for about 2 months were below 100 mOsm/kg (La Peyre et al., 2009), the plasma osmolalities of oysters exposed to 1 ppt in the laboratory had values ranging from 82 to 371 mOsm/kg regardless of whether they had been acclimated or not to the extreme salinity (La Peyre et al., 2003, 2009). To corroborate that the conditions found in vitro are close to the response of *P. marinus* cells in the host tissues, it is clear plasma osmolality in relation to water salinity needs to be measured.

In conclusion, the largest decrease in *P. marinus* viability occurred through the synergistic effect of low temperature and salinity. Since temperatures and salinities chosen for this study were selected to represent the extremes experienced by *P. marinus* in N. America, these data suggest that environmental conditions (defined by salinity and temperature) alone are not likely to limit *P. marinus* presence in the eastern oyster, as the lower range tolerance of *P. marinus* appears to exceed the salinity and temperature range where most eastern oyster production occurs. In assessing the control of temperature and salinity on *P. marinus* infections, the synergistic effects clearly need to be incorporated into models. Furthermore, in assessing the impacts of global climate change on *P. marinus* presence and infection levels, not just the effects of changes in temperature, but also the effects in terms of differences in rainfall, and potentially freshwater inflow into regions need to be accounted for in future models. This is especially relevant in areas such as in the Chesapeake Bay where increases in salinity associated with sea-level rise have been reported (Hilton et al., 2008), and in areas where freshwater flow is altered due to anthropogenic projects, and from altered regional rainfall patterns (Wilber, 1992; Wilber and Bass, 1998; Powell et al., 2003; Tolley et al., 2005; Wang et al., 2008).

Along the Gulf Coast, a significant means of restoring coastal ecosystems involves the manipulation of freshwater inflow; control of freshwater inflow could thus potentially be used to some degree to control *P. marinus* in oyster populations if the inflows can be managed such that they are increased during times of low temperature. Moreover, river water can be cooler than estuarine waters (Lane et al., 2007) further favoring the reduction of parasite load. The potential impact of freshwater diversions in decreasing *P. marinus* body burden along the Gulf of Mexico coast would be enhanced in years when water temperatures are lower than average such as during El Ni⁄o years. High freshwater flow to help keep salinity lower during period of warmer, drier La Ni⁄a phase of ENSO cycles that favor *P. marinus* proliferation and disease epizootics (Soniat et al., 2009) may also be very helpful to limiting oyster mortalities during such climate events (La Peyre et al., 2009).

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