

# Heat shock protein (hsp70) expression and thermal tolerance in sublethally heat-shocked eastern oysters *Crassostrea virginica* infected with the parasite *Perkinsus marinus*

V. G. Encomio<sup>1</sup>, F.-L. E. Chu<sup>2,\*</sup>

<sup>1</sup>Florida Gulf Coast University, 10501 FGCU Blvd. South Fort Myers, Florida 33965, USA

<sup>2</sup>Virginia Institute of Marine Science, 1208 Greate Road Gloucester Point, Virginia 23062, USA

**ABSTRACT:** To investigate whether sublethal heat shock protects *Perkinsus marinus* (Dermo)-infected oysters *Crassostrea virginica* from lethal heat stress, and the effects of *P. marinus* infection on sublethal heat shock response, oysters were first experimentally challenged with *P. marinus*. Then, when infections in oysters progressed to moderate levels (parasite burden =  $10^4$  to  $10^5$  cells g<sup>-1</sup> wet tissue weight), oysters were treated with a sublethal heat shock at 40°C for 1 h (heat shock + Dermo challenge). Other treatment groups included heat-shocked, unchallenged (non-*P. marinus* challenged) oysters and non-heat-shocked, *P. marinus*-challenged and -unchallenged oysters. Thermal tolerance was compared among these treatments by administering a lethal heat treatment at 44°C for 1 h, 7 d after sublethal heat shock. Sublethal heat shock enhanced survival to lethal heat treatment in both *P. marinus*-challenged and -unchallenged oysters. Although levels of hsp70 isoforms (hsp69 and hsp72) did not vary significantly by heat shock or infection with *P. marinus*, responses due to these treatments were apparent when comparing hsp70 levels within infected and uninfected oysters. Infection enhanced expression of hsp69, regardless of whether oysters were heat shocked or not. In uninfected oysters, hsp72 increased due to heat shock 2 and 7 d post heat shock. Overall, this study demonstrates that heat shock can improve survival in oysters, even in oysters infected with *P. marinus*. Expression of hsp70 varied among isoforms after sublethal and lethal heat shocks and in infected and uninfected oysters. The heat shock response was not negatively affected by *P. marinus* infection.

**KEY WORDS:** *Crassostrea virginica* · Heat shock protein · Hsp70 · *Perkinsus marinus* · Thermal tolerance

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## INTRODUCTION

Dermo disease, caused by the parasitic protozoan *Perkinsus marinus*, has devastated populations of the eastern oyster *Crassostrea virginica* along the Atlantic and Gulf of Mexico coasts of the United States since the 1950s (Andrews 1988). The disease is particularly problematic because *P. marinus* persists over a broad range of temperatures and salinities (Chu & LaPeyre 1993, Burreson et al. 1994). Mortalities caused by *P. marinus* increase at temperatures >25°C, as the parasite proliferates rapidly, causing

extensive damage to host cells and tissues (Andrews 1965, Fisher et al. 1992, La Peyre 1996). Although eastern oysters can tolerate high temperatures, the interactive effect of disease and thermal stress may be intense, leading to death. Dermo-resistant oysters have been identified and selectively bred, but mechanisms contributing to Dermo resistance are currently unknown (Maers 1993, Calvo et al. 2003, Encomio et al. 2005). Mechanisms such as heat shock proteins, which provide protection for cells and tissues, may be important in counteracting the dual stresses of temperature and disease in oysters.

\*Corresponding author. Email: chu@vims.edu

Heat shock proteins act as molecular chaperones, facilitating proper folding, assembly and intracellular transport of proteins. They are highly conserved, occurring in all organisms (Feder & Hofmann 1999). Heat shock proteins in the 70 kDa family are the most commonly expressed heat shock proteins in response to stress (Lindquist 1986). Their activity aids in protecting organisms from thermal or other stress-induced damage (Lindquist & Craig 1988, Morimoto et al. 1990, Gething & Sambrook 1992, Gupta & Golding 1993). Enhanced tolerance to stress, resulting in improved survival and condition, has been correlated with increased levels of heat shock proteins (Bosch et al. 1988, Sanders 1988).

In a wide variety of organisms, thermal tolerance can be enhanced experimentally via exposure to a sublethal heat shock (Lindquist 1986). Over-expression of heat shock proteins is associated with this phenomenon, known as acquired or induced thermal tolerance (Sanchez & Lindquist 1990). Furthermore, application of sublethal heat shock has been shown to increase resistance to other stressors besides high temperature. For example, sublethal heat shock increased cadmium resistance in mussels (Tedengren et al. 1999). Increased expression of heat shock proteins due to sublethal heat shock and the acquisition of enhanced tolerance to stress, be it thermal or otherwise, are distinct characteristics of the heat shock response.

Oysters are estuarine species that are impacted by various stressors (e.g. diseases, hypoxia, and toxicants or pollutants) and wide fluctuations in temperature. An understanding of the stress response in oysters may offer insights on how to improve their survival and production. The heat shock response has been studied in several species of oysters (Shamseldin et al. 1997, Clegg et al. 1998, Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003, Brown et al. 2004). Increased levels of heat shock proteins were associated with improved thermal tolerance in Pacific oysters (Clegg et al. 1998). Levels of hsp70 increased after heat shock treatments of eastern oyster hemocytes. Tirard et al. (1995) proposed a potential mechanism by which the immune function in oysters could be improved. However, no study has determined whether sublethal heat shock would protect *Perkinsus marinus*-infected oysters from lethal thermal stress and whether the heat shock response of oysters is affected by *P. marinus* infection.

The objectives of the present study were (1) to determine whether sublethal heat shock improves the survival of *Perkinsus marinus*-infected and -uninfected oysters to later lethal thermal stress and (2) to characterize the hsp70 expression in *P. marinus*-infected and -uninfected oysters after sublethal and lethal heat shocks.

## MATERIALS AND METHODS

**Oysters.** Adult eastern oysters *Crassostrea virginica* originated from the Damariscotta River, Maine (Pemaquid Oyster Company), where oysters are rarely infected by *Perkinsus marinus* (Kleinshuster & Parent 1995). Oysters shipped from Maine were screened initially for *P. marinus* infection ( $n = 25$ ) prior to experiments following Ray's (1966) method. Results were negative. Oysters were acclimated to and then maintained at temperatures of 20 to 22°C and salinities of 15 to 18 for 2 wk, until experiments were performed.

**Experiments. Experimental infections with *Perkinsus marinus*:** Oysters were challenged with *P. marinus* meronts ( $\sim 10^6$  cells oyster<sup>-1</sup>) freshly isolated from infected oyster tissues, as described by Chu & Volety (1997). Briefly, infected oyster tissues were mechanically homogenized in 0.22  $\mu\text{m}$  filtered York River water (YRW). The suspension was then passed through a series of filters (100, 50, 35 and 20  $\mu\text{m}$  mesh sizes). The filtrate was then centrifuged and washed several times to remove any remaining tissue debris. Meronts were counted in a hemacytometer, and the cell density was adjusted with YRW to  $10 \times 10^6$  cells ml<sup>-1</sup>. Oysters were inoculated with 100  $\mu\text{l}$  ( $\approx 1 \times 10^6$  cells oyster<sup>-1</sup>) of the meront suspension. Inoculation was performed by shell cavity injection through a notch made on the dorsal axis of the oyster shell. Non-challenged control oysters were inoculated with a sham treatment of 100  $\mu\text{l}$  YRW (Chu & Volety 1997). To monitor progression of infection in *P. marinus*-challenged oysters, 3 oysters from 2 tanks (24 oysters per tank) were sampled ( $n = 6$  oysters per sampling) weekly for *P. marinus* analysis. *P. marinus* infections were assessed by the body burden enumeration technique (Choi et al. 1989, Bushek et al. 1994). Moderate to high densities of infection have typically been obtained 60 d post-challenge with  $2.5 \times 10^4$  cells (meront stage) per oyster (Chu & Volety 1997).

**Sublethal and lethal heat shock treatments:** When *Perkinsus marinus* (Dermo)-challenged oysters attained moderate to high infections ( $\sim 10^4$  to  $10^5$  cells g<sup>-1</sup> wet tissue weight), 60 d after *P. marinus* inoculation, both *P. marinus*-challenged and -unchallenged oysters were exposed to a sublethal heat shock (40°C, 1 h), to enhance hsp70 expression and induce thermal tolerance. This heat shock temperature was chosen because heat shocks up to 42°C for 1 h did not kill oysters (Encomio 2004, Encomio & Chu 2004). In eastern oysters, after a 1 h heat shock at 37°C, total hsp70 increased 7 and 8 d post sublethal heat shock and remained elevated up to 14–15 d after heat shock (Encomio 2004).

Four experimental treatments were arranged in a 2  $\times$  2 factorial design, with 3 to 4 replicate holding tanks

for each of the treatments (18 to 20 oysters per tank). The 4 treatment groups were: (1) sublethal heat-shocked, Dermo-challenged oysters (HS + DC); (2) non-sublethal heat-shocked, Dermo-challenged oysters (N + DC); (3) sublethal heat-shocked, unchallenged oysters (HS + UC); and (4) non-sublethal heat-shocked, unchallenged oysters (N + UC). After sublethal heat shock at 40°C for 1 h, 3 oysters from each replicate tank of each treatment group (n = a total of 9 to 12 oysters per treatment) were sampled at 0 (immediately after sublethal heat shock), 2 and 7 d after sublethal heat shock for analysis of hsp70 and *Perkinsus marinus* infection.

After oysters were sampled on Day 7, all treatment groups were subjected to a lethal heat treatment (LHT) of 44°C for 1 h, and mortality was monitored for 1 wk. The chosen temperature of 44°C as the LHT was based on our previous observation that mortality occurred in oysters after heat shock at 43 to 45°C for 1 h (Encomio 2004, Encomio & Chu 2004). The number of oysters remaining in each tank at the time of the LHT ranged between 8 and 11 individuals per tank. Surviving oysters were sampled for analysis of hsp70 and *Perkinsus marinus* infection 1 wk after LHT (14 d after sublethal HS).

#### **SDS-PAGE and Western blot detection of hsp70:**

Gill tissues from oysters were excised, freeze-dried for 48 h, weighed and stored at -80°C. Gill tissues were then homogenized on ice in 2 ml of buffer (66 mM Tris pH 7.2, 3% Igepal CA-630, 0.1 mM phenylmethane-sulfonyl fluoride [PMSF]). The homogenate was centrifuged at 10000 × g for 30 min at 4°C, and the supernatant (gill extract) was collected. Total protein concentration was determined using a modified version of the Lowry assay (Bio-Rad DC Protein Assay; Lowry et al. 1951).

A portion of the gill extract was diluted 1:2 in Laemmli sample buffer (BioRad) for SDS-PAGE. Samples were boiled for 5 min, and 10 µg of total protein per sample were electrophoresed on 8% polyacrylamide gels (150 V, 90 min). In addition to experimental gill samples, 10 µg protein from a 'reference sample' of gill tissue, obtained by exposing an oyster to a 1 h heat shock at 40°C, was run on every gel. Separated proteins were then transferred onto nitrocellulose membrane at 100 V for 1 h in transfer buffer (192 mM glycine, 24 mM Tris base and 20% methanol). After transfer, nitrocellulose blots were processed for immunodetection of hsp70 isoforms. The blot was blocked with 5% bovine serum albumin (BSA) in Tween-Tris-buffered saline (TTBS; 0.05% Tween, 30 mM NaCl, 24 mM Tris pH 7.5) for 30 min followed by 2 washes in Tris-buffered saline (TBS; 30 mM NaCl, 24 mM Tris pH 7.5) for 5 min each. Primary monoclonal antibody against hsp70 (Clone 3A3; Catalog MA3-006,

Affinity Bioreagents) was applied for 90 min (1:5000 dilution), followed by two 5 min washes with TBS. A secondary antibody (goat anti-mouse, alkaline phosphatase conjugated) was applied for 90 min (1:1000 dilution), washed once in TBS, and placed in a developing solution containing NBT (*p*-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate). Protein bands developed between 30 and 60 min. The blot was then stored in deionized water until analysis. Densitometric analysis of developed slot blots was performed using an Enprotech scanner and software. Data are expressed as mean units (relative amounts) of hsp70 µg<sup>-1</sup> protein ± the standard error of the mean (SEM). Units are the sample density divided by the reference sample density per microgram of protein (Cruz-Rodríguez & Chu 2002).

#### **Diagnosis of *Perkinsus marinus* (Dermo) infection:**

Prevalence and intensity of *P. marinus* infection in oysters were determined using total body burden assessment (Choi et al. 1989, Bushek et al. 1994). Oyster tissue was weighed and mechanically homogenized in 0.1 M sodium phosphate buffer (Ultrapurax). A 1.0 ml tissue aliquot was incubated in alternative fluid thioglycollate medium (AFTM) for 5 to 7 d at 20°C. Tissue suspensions were then centrifuged at 800 × g for 10 min. Tissue pellets were re-suspended in 2.0 M NaOH and incubated overnight at 60°C. Tissue pellets were washed, centrifuged and re-suspended in distilled water. Aliquots of 100 µl from each sample were added to a 96-well plate and stained with 1 to 2 drops of Lugol's solution (1:9 dilution). Stained *P. marinus* cells were counted under an inverted microscope at 400× magnification. Results are expressed as the log of the number of *P. marinus* cells g<sup>-1</sup> wet tissue weight (ww).

**Statistical analyses.** *Perkinsus marinus* infections were compared by ANOVA between Dermochallenged and -unchallenged oysters (DC vs. UC), sublethally heat-shocked and non-sublethally heat-shocked oysters (HS vs. N), and over time (0, 2, 7 and 14 d).

Proportional survival data were arcsine-square-root transformed and analyzed by ANOVA for effects of heat treatment (heat-shocked vs. non-heat-shocked oysters; HS vs. N), infection status (Dermo-challenged vs. -unchallenged oysters; DC vs. UC) and their interactions. In addition to ANOVA, mortalities were compared by survival analysis using the product-limit (Kaplan-Meier) method (Cox & Oakes 1980, Newman 1994). Analysis was performed using the LIFETEST procedure in SAS. This procedure calculates log-rank and Wilcoxon statistics, which test if time-to-death is the same for each treatment.

Units of hsp70 isoforms and *Perkinsus marinus* infection data were log transformed to meet assumptions of normality and homogeneity of variance. Because of the

presence of infected oysters in the UC treatments (see 'Results; Experimental infections with *Perkinsus marinus*'), infected individuals from these treatment groups were pooled statistically with the DC oysters, which had 100% prevalence of infection, and were designated as infected (I) oysters for hsp70 analyses. For hsp70 data, treatments were then designated as follows: (1) HS + I (sublethally heat-shocked, infected oysters), (2) N + I (non-sublethally heat-shocked, infected oysters), (3) HS + UI (sublethally heat-shocked, uninfected oysters) and (4) N + UI (non-sublethally heat-shocked, uninfected oysters). Hsp70 data were analyzed by repeated measures ANOVA for the factors treatment (HS or N), infection status (I or UI), day (0, 2, 7 and 14 d post sublethal heat shock) and their interactions. Infection status and aquaria sampled over time in each treatment were treated as a within-subjects random factor. To examine the effect of infection levels on heat shock protein expression, body burden counts were classified into 4 categories: 0 (negative for *P. marinus* infection), 1 ( $1$  to  $10^2$  cells  $g^{-1}$  ww), 2 ( $10^3$  to  $10^4$  cells  $g^{-1}$  ww) and 3 ( $10^5$  to  $10^6+$  cells  $g^{-1}$  ww). The effect of infection level on hsp70 isoform expression was analyzed by ANOVA, with infection level as a random factor.

## RESULTS

### Experimental infections with *Perkinsus marinus*

*Crassostrea virginica* oysters experimentally challenged with *P. marinus* had mean ( $\pm$ SEM) infection densities of  $7.76 \times 10^3$  ( $\pm 0.70$ ) cells  $g^{-1}$  ww 60 d after inoculation of freshly isolated *P. marinus* meronts. *P. marinus* infection was also detected in UC oysters, but infections were significantly lower than in DC oysters ( $p < 0.0001$ ,  $n = 93$ ) (Fig. 1). Prevalence of infection among UC oysters was 32.7%, and mean infection densities were  $9.23 \pm 0.84$  cells  $g^{-1}$  ww over the course of the experiment. Prevalence among the DC oysters was 100%, and mean infection densities were  $6.38 \times 10^4$  ( $\pm 3.85$ ) cells  $g^{-1}$  ww. No significant changes in *P. marinus* infection were found in comparisons of sublethally heat-shocked versus non-sublethally heat-shocked oysters (Fig. 2) or over time.

### Survival after lethal heat treatment

Induced thermal tolerance was observed in oysters subjected to a sublethal heat shock (HS) ( $40^\circ\text{C}$ ) prior to a lethal heat treatment (LHT) of  $44^\circ\text{C}$ . Survival was significantly elevated in HS treatments compared to non-HS (N) treatments (ANOVA,  $p = 0.013$ ,  $n = 3$  to 4

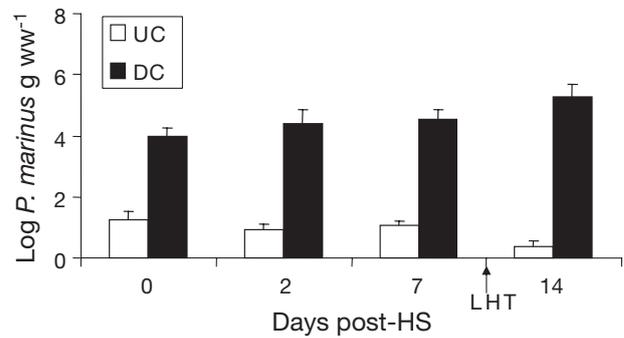


Fig. 1. *Perkinsus marinus* infecting *Crassostrea virginica*. Burden (log of the number of *P. marinus* cells  $g^{-1}$  wet tissue weight [ww]  $\pm$  SEM;  $n = 93$  oysters per treatment) in Dermo-challenged (DC) and -un-challenged (UC) oysters in relation to induced thermal tolerance. Oysters were subjected to a lethal heat treatment (LHT) for 1 h at  $44^\circ\text{C}$ , immediately after the Day 7 sampling. *P. marinus* infection was evident in UC oysters, albeit at significantly lower levels than in DC oysters (ANOVA,  $p < 0.0001$ ) HS: heat shock

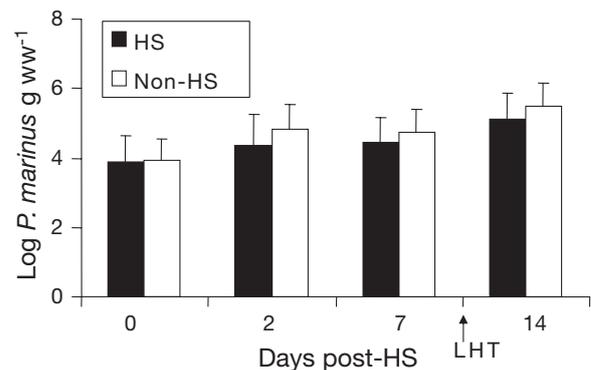


Fig. 2. *Perkinsus marinus* infecting *Crassostrea virginica*. Burden (log of the number *P. marinus* cells  $g^{-1}$  wet weight [ww]  $\pm$  SEM;  $n = 12$ ) in sublethally heat-shocked (HS) and non-sublethally heat-shocked (non-HS) oysters. A lethal heat treatment (LHT) was applied for 1 h at  $44^\circ\text{C}$

replicate tanks of 8 to 11 oysters each; total  $n = 138$  oysters. See Fig. 3 legend for specific  $n$ -values per treatment). Effects of Dermo challenge were not significant, and survival was similar between DC and UC oysters (Fig. 3).

Survival analysis also showed that sublethal heat shock significantly improved survival compared to survival in oysters that were not heat shocked (log-rank test,  $p < 0.0001$ ; Wilcoxon test,  $p < 0.0001$ ) (Fig. 4). Challenge status (either DC or UC treatments) did not affect survival after lethal heat shock. When survival data in DC and UC treatments were pooled statistically, sublethal heat shock (HS) significantly improved survival in oysters compared to oysters that were not sublethally heat shocked (N treatment) (log-rank test,

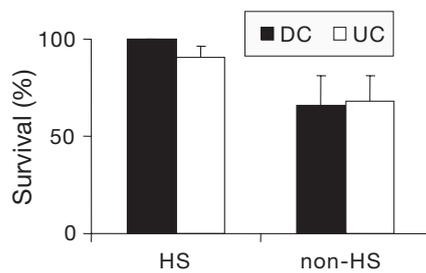


Fig. 3. *Crassostrea virginica*. Survival (%) of oysters in sublethally heat-shocked (HS) and non-sublethally heat-shocked (non-HS), Dermo-challenged (DC) and -unchallenged (UC) oysters, 7 d after a lethal heat treatment of 44°C, 1 h. Data shown are percent survival  $\pm$  SEM of 3 to 4 tanks per treatment, with each tank containing 8 to 11 oysters per tank. For HS + DC treatment:  $n = 36$  oysters; non-HS + DC treatment:  $n = 28$ ; HS + UC treatment:  $n = 30$ ; non-HS + UC treatment:  $n = 44$ . Effects of sublethal heat shock significantly enhanced survival (ANOVA,  $p = 0.013$ ). Challenge treatments (DC or UC) did not significantly affect survival

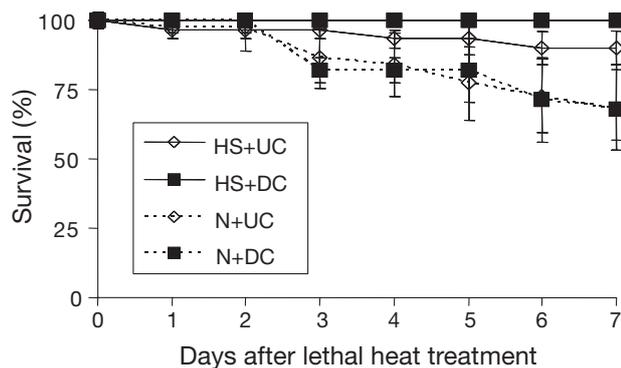


Fig. 4. *Crassostrea virginica*. Survival (mean % survival/treatment  $\pm$  SEM at each day) in oysters over 7 d after a lethal heat treatment (LHT) of 44°C for 1 h. HS + UC: sublethally heat-shocked, unchallenged oysters; HS + DC: sublethally heat-shocked, Dermo-challenged oysters; N + UC: non-sublethally heat-shocked, unchallenged oysters; N + DC: non-sublethally heat-shocked, Dermo-challenged oysters ( $n = 3$  to 4 tanks per treatment with 8 to 11 oysters per tank). For HS + UC treatment:  $n = 30$ ; HS + DC treatment:  $n = 36$  oysters; N + UC treatment:  $n = 44$ ; N + DC treatment:  $n = 28$ . Time-to-death analyses resulted in  $p < 0.0001$  for both log-rank and Wilcoxon tests

$p = 0.0002$ ; Wilcoxon test,  $p = 0.0003$ ). Sublethal heat shock also improved survival in DC oysters alone (log-rank test,  $p = 0.033$ ; Wilcoxon test,  $p = 0.035$ ). The effect of Dermo challenge within HS and N treatments was also compared. The effect of Dermo challenge was not significant, although a trend was apparent in HS oysters ( $p = 0.054$  for both log-rank and Wilcoxon tests). In that comparison, DC oysters exhibited higher survival than UC oysters (100% vs.  $90.3 \pm 5.8\%$ ,  $n = 32$  to 44 for DC oysters and  $n = 24$  to 33 for UC oysters).

### Hsp70 expression

Western blot analyses showed variation in expression of the hsp70 isoforms. Two molecular weight isoforms were detected at 69 and 72 kDa, as previously described (Fig. 5 in the present study, Encomio & Chu 2005). The 69 kDa isoform (hsp69) increased significantly after sublethal heat shock ( $p = 0.0303$ ; Fig. 6A), and the levels of hsp69 increased over time, although not significantly ( $p = 0.0718$ ; Fig. 6B). The increase of hsp69 over time accounted for most of the increase in total hsp70 over time, although the increase in total hsp70 was not significant. Levels of hsp69, however, did not significantly change with infection status (I or UI). Interactions between infection status and day were significant ( $p = 0.0495$ ) in hsp69. Changes in hsp72 and total hsp70 were not significant, either due to heat shock, infection status, or day. Furthermore, infection levels (classified as 0, 1, 2, or 3; see 'Materials and methods; Statistical analyses') did not significantly affect the expression of either hsp isoform detected in the present study.

Although ANOVA did not detect significant differences due to sublethal heat shock or infection, there were apparent differences in isoform expression of hsp70 when comparing infected (combined HS and N oysters) and uninfected (combined HS and N) treatments separately. Among infected oysters hsp69 increased over time in non-sublethally heat-shocked (N) oysters (Fig. 7A). At Day 14, levels of hsp69 in the N + I group were similar to those of the HS + I oysters, although this was not surprising as these were the oysters that survived the LHT. In uninfected oysters hsp69 was higher in sublethally HS individuals up to Day 7, and it decreased 7 d after LHT (Day 14) (Fig. 7B). Expression of hsp69 also increased with time (up to 7 d) in the N + UI oysters. Overall expression of hsp72 occurred less frequently and at lower levels than that of hsp69. At Day 0, when oysters were sampled to measure hsp70 immediately after sublethal heat shock and

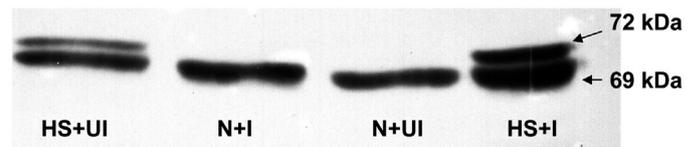


Fig. 5. *Crassostrea virginica*. Western blot showing expression of hsp70 isoforms (hsp69 and hsp72) in oysters. Samples shown are representative of 4 different treatments, i.e. HS + UI: sublethally heat-shocked, uninfected oysters; N + I: non-sublethally heat-shocked, infected oysters; N + UI: non-sublethally heat-shocked, uninfected oysters; HS + I: sublethally heat-shocked, infected oysters. Uninfected or infected oysters denote actual infection status regardless of whether oysters were initially challenged with *Perkinsus marinus* or not

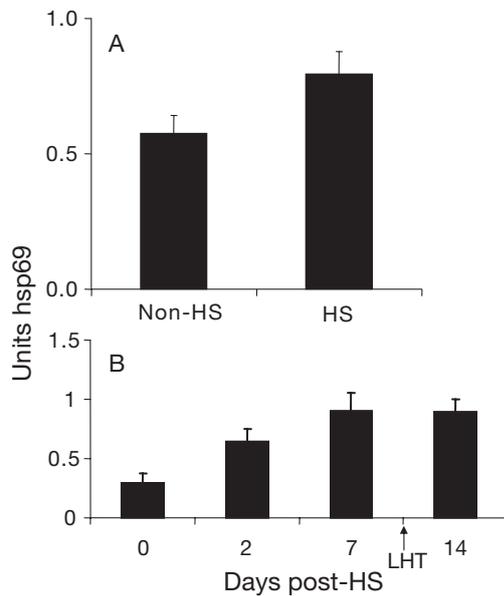


Fig. 6. *Crassostrea virginica*. (A) Units of hsp69 as analyzed by Western blots. Data presented are means  $\pm$  SEM,  $n = 61$  for sublethal heat shock (HS) and  $n = 52$  for non-sublethal heat shock (non-HS) treatments collapsed across effects of day and infection status. HS significantly elevated hsp69 over the levels in non-HS oysters (ANOVA,  $p = 0.0303$ ). (B) Changes in the hsp69 isoform 0, 2, 7 and 14 d post-HS. Data are presented as mean units of hsp69  $\pm$  SEM ( $n = 13$  to 14) collapsed across factors of HS treatment and infection status. A lethal heat treatment (LHT) of 1 h at 44°C was administered immediately after the Day 7 sampling

infected oysters that were heat shocked had the highest levels of hsp72 (Fig. 8A). At Days 2 and 7 expression of hsp72 was similar between HS + I and N + I oysters (Fig. 8A). Expression of hsp72 was higher at Day 14 (7 d after LHT) in the HS + I oysters compared to the N + I oysters. In UI oysters, sublethal heat shock increased expression of hsp72 (Fig. 8B). However, levels of hsp72 decreased at Day 14 in HS + UI oysters, while expression of hsp72 was very low in the N + UI oysters overall, with hsp72 expression absent to nearly absent at Days 2 and 14 (Fig. 8B). Infection (I) alone appeared to have an effect on non-HS oysters (N + I) as expression of hsp72 was more frequent and at higher levels compared to N + UI oysters (Fig. 8A,B). A similar trend was seen in hsp69, but the differences between N + I and N + UI oysters were slight (Fig. 7A,B).

## DISCUSSION

Similar to a previous study (Chu & Volety 1997), in the present study inoculation of *Crassostrea virginica* oysters with a dose of  $\sim 1 \times 10^6$  freshly isolated meronts per oyster resulted in 100% prevalence with moderate

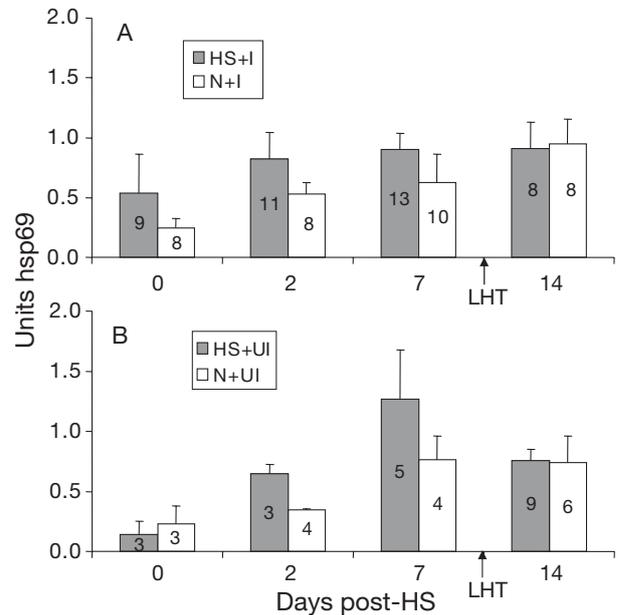


Fig. 7. *Crassostrea virginica*. (A) Levels of hsp69 at 0, 2, 7 and 14 d after sublethal heat shock (post-HS) in sublethally heat-shocked (HS + I) and non-sublethally heat-shocked (N + I) oysters infected with *Perkinsus marinus*. (B) Levels of hsp69 in uninfected sublethally heat-shocked (HS + UI) and non-sublethally heat-shocked (N + UI) oysters at 0, 2, 7 and 14 d post-HS. Data are presented as mean units of hsp69  $\pm$  SEM ( $n$ -values for each treatment are shown within their respective bar). A lethal heat treatment (LHT) of 1 h at 44°C was administered immediately after the Day 7 sampling

to high infections in oysters after 60 d. Light infections found in the unchallenged treatment groups likely originated from Maine. The Ray tissue assay (Ray 1966) used for the initial screening of *Perkinsus marinus* infection is less sensitive than the body burden assay and has been cited as being prone to false negatives at light infection levels (Bushek et al. 1994). Although infections in Maine oysters are still rare, the northward extension of *P. marinus* has been documented and is likely currently occurring (Ford 1996). The infections found in the unchallenged oysters were unlikely to have caused much physiological stress. Calculations estimate that the energy budget of an oyster is only significantly impacted by moderate to very high *P. marinus* infections (Choi et al. 1989).

A sustained, elevated expression of heat shock proteins after exposure to acute thermal stress is an almost universal characteristic of the heat shock response (Lindquist 1986, Lindquist & Craig 1988, Feder & Hofmann 1999). The length of the response may be dependent on the severity of the heat stress (Lindquist 1986). In *Crassostrea virginica*, we observed that total hsp70 remained elevated in gill tissues for 2 to 4 wk after heat shock (Encomio 2004, Encomio & Chu 2004).

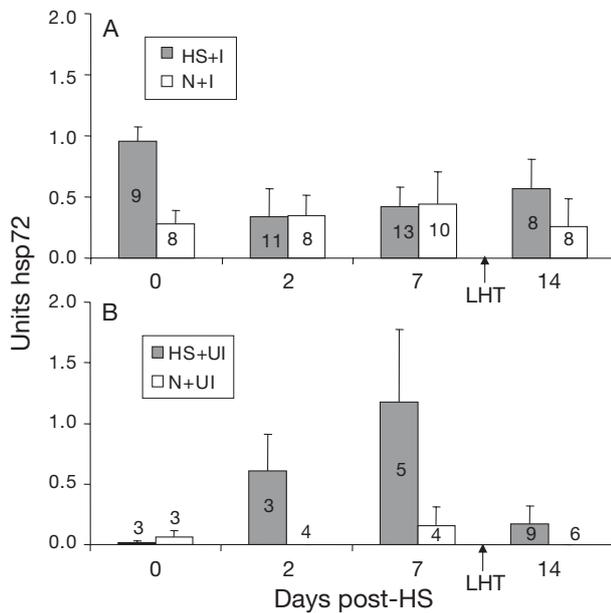


Fig. 8. *Crassostrea virginica*. (A) Levels of hsp72 as analyzed by Western blot in sublethally heat-shocked (HS + I) and non-sublethally heat-shocked (N + I) oysters infected with *Perkinsus marinus* at 0, 2, 7 and 14 d post-HS. (B) Levels of hsp72 in uninfected sublethally heat-shocked (HS + UI) and non-sublethally heat-shocked (N + UI) oysters at 0, 2, 7 and 14 d post-HS. A lethal heat treatment (LHT) of 1 h at 44°C was administered immediately after the Day 7 sampling, as shown in both (A) and (B). Data are presented as mean units of hsp72  $\pm$  SEM (n values for each treatment are shown within their respective bar). Bars that are not visible denote means either close to or at 0 units hsp72

This also occurred in *C. gigas* and the flat oyster *Ostrea edulis*, in which elevated levels of hsp70 persisted for 2 wk (Clegg et al. 1998, Piano et al. 2002). In several bivalve species increased levels of heat shock proteins are seen after a distinct period of time (~48 to 96 h after heat shock). This occurred in the mussels *Mytilus edulis* and in several species of oysters, e.g. *C. gigas*, *O. edulis* and *C. virginica* (Tirard et al. 1995, Chapple et al. 1997, Clegg et al. 1998, Cruz-Rodriguez & Chu 2002, Piano et al. 2002). During the period of increased hsp70 levels, thermal tolerance is enhanced. Pre-treatment of *M. edulis* with high temperature resulted in increased heat resistance after 3 d, and induced thermal tolerance was highest 2 d after heat shock in *C. gigas* (Huppert & Laudien 1980, Clegg et al. 1998). The elevated expression of hsp70 in *C. gigas* was associated with an enhanced thermal tolerance that lasted up to 14 d (Clegg et al. 1998). In the present study we showed that a sublethal heat shock improved tolerance to thermal stress in *C. virginica* for at least 7 d, with increased survival in HS oysters. The implication for elevation of hsp70 and thermal tolerance is that both eastern and Pacific oysters can resist multiple bouts of

thermal stress over prolonged periods. Although the reasons for sustained elevation of heat shock proteins are unknown, but it is speculated that heat shock may disrupt protein synthesis and thus the mechanisms controlling the regulation of hsp synthesis, resulting in reduced turnover of heat shock proteins (Hochachka & Somero 2002). Additionally, hsp70 mRNA may be stable for several days (Hofmann 1999).

The present study demonstrated that sublethal heat shock improved thermal tolerance in *Crassostrea virginica*, in a manner similar to *C. gigas* (Shamseldin et al. 1997, Clegg et al. 1998, Hamdoun et al. 2003). Initially, it was expected that *Perkinsus marinus* infections would increase susceptibility to lethal heat stress. In oysters infected with *Haplosporidium nelsoni*, acute elevation of temperature from 20 to 30°C increased oxygen consumption and ammonia production compared to that in uninfected oysters, implying that diseased oysters were more susceptible to heat stress (Littlewood & Ford 1990). Challenge with the bacterium *Nocardia* reduced thermal tolerance in *C. gigas* (Friedman et al. 1999). Other parasites, such as trematodes, reduce the resistance of gastropods to extreme changes in temperature and salinity (Lauckner 1980). In the present study, however, infection did not reduce thermal tolerance. The level of protection conferred by sublethal heat shock may have overridden any deleterious effects of infection on thermal tolerance. The sublethal heat shock temperature also may have affected function in *P. marinus*. Heat treatments of 40°C and higher for 30 min reduced the viability of cultured *P. marinus* (Soudant et al. 2005). High temperatures that oysters are capable of withstanding may limit the growth of *P. marinus*. Infection did not reduce condition, as there were no differences in the condition index between infected and uninfected oysters (data not shown). Physiological condition in oysters is only significantly reduced in advanced infections (Dittman et al. 2001). Infection intensities did reach levels ( $10^4$  to  $10^5$  cells  $g^{-1}$  ww) that would be expected to affect oyster physiology. However, physiological effects of *P. marinus* infection have been difficult to demonstrate, and are strongly influenced by season, environment and reproductive state (Craig et al. 1989, Newell et al. 1994, Dittman et al. 2001, Encomio et al. 2005).

As shown previously (Encomio & Chu 2005), hsp isoform expression varied and was not identified strictly as inducible (hsp69) or constitutive (hsp72), as demonstrated in previous studies (Clegg et al. 1998, Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003, Brown et al. 2004). In the present study, hsp69 was expressed in a constitutive manner, and was present in almost all samples of all treatments. Differences in hsp69 between treatments were due to an increase in levels, and not induction of expression, which implies

de novo synthesis of this isoform. Furthermore, hsp69 was predominantly expressed over hsp72 (Figs. 5, 7 & 8). Differences in patterns of hsp70 expression between our study and previous studies may be due to differences in antibody specificity. The antibody clone (7.10, Affinity Bioreagents) used in other studies recognized 3 isoforms of hsp70 (69, 72 and 77 kDa), with the 69 kDa isoform characterized as inducible upon sublethal heat shock (Clegg et al. 1998, Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003, Brown et al. 2004). Although the antibody used (Clone 3A3, Affinity Bioreagents) in the present study recognized a 69 kDa isoform, it is possible this isoform is still different from the inducible isoform identified in the preceding studies (Clegg et al. 1998, Piano et al. 2002, Boutet et al. 2003, Hamdoun et al. 2003, Brown et al. 2004). Although similar in molecular weight, isoforms may have different isoelectric points, resolvable only by 2-dimensional electrophoresis. If hsp69 is more heat sensitive, predominant expression may be a result of using Maine oysters in our experiments, which would be expected to be more sensitive to temperature than Chesapeake Bay oysters. Control temperatures in our experiments (20 to 22°C) approach the maximal aquatic temperatures (25°C, C. Davis, Pemaquid Oysters, pers. comm.) that Maine oysters experience in their native habitat.

Infection with *Perkinsus marinus* may have also contributed to the increase in hsp72 in both heat-shocked and non-heat-shocked oysters. Pathogens can induce expression of heat shock proteins in various host species. Levels of hsp70 increased in livers and kidneys of coho salmon *Oncorhynchus kisutch* when artificially infected with *Renibacterium salmonarum* (Forsyth et al. 1997). House martins *Delichon urbica* parasitized by triatoid bugs and trypanosomes had increased levels of hsp60 in their blood (Merino et al. 1998). The nematode *Trichinella spiralis* induced elevated expression of hsp25 and hsp70 in rats (Martinez et al. 1999). The increase in hsp72, however, was not indicative of an increase in thermal tolerance, as non-sublethally heat-shocked (N treatments) oysters experienced greater mortalities than those that were heat shocked, regardless of differences in heat shock protein expression. The increase in hsp72 observed in infected oysters may not substantially contribute to thermal tolerance and may be an indicator of stress, but not stress resistance. Additionally, heat shock protein expression and thermal tolerance are not always tightly coupled (Easton et al. 1987). Other heat shock proteins and other mechanisms, such as changes in membrane fluidity may contribute to thermal tolerance.

In previous studies we demonstrated that the heat shock response can be sustained for >2 wk (Encomio 2004, Encomio & Chu 2004). Prolonged over-expres-

sion of heat shock proteins may be indicative of how long oysters can remain tolerant to stress, and whether that resistance can be artificially enhanced. The present study shows that sublethal heat shock improved survival in oysters. Thermal tolerance was enhanced, even in oysters infected with *Perkinsus marinus*, showing that the heat shock response was not negatively affected by disease. This is important as heat shock could be utilized as a way to improve survival in diseased oysters. Techniques to harden animals, such as intertidal culture, are often employed to improve survival of oysters and reduce fouling (Ventilla 1984, Handley & Bergquist 1997). An understanding of the heat shock response and its role in thermal and pathogenic stress is important to improve survival in cultured bivalves.

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*Editorial responsibility: Richard Cawthorn,  
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