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Phenotypic Plasticity of HSP70 and HSP70 Gene Expression in the Pacific Oyster (*Crassostrea gigas*): Implications for Thermal Limits and Induction of Thermal Tolerance

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Abstract Pacific oysters, *Crassostrea gigas,* living at a range of tidal heights, routinely encounter large seasonal fluctuations in temperature. We demonstrate that the thermal limits of oysters are relatively plastic, and that these limits are correlated with changes in the expression of one family of heat-shock proteins (HSP70). Oysters were cultured in the intertidal zone, at two tidal heights, and monitored for changes in expression of cognate (HSC) and inducible (HSP) heat-shock proteins during the progression from spring through winter. We found that the "control" levels (*i.e.*, prior to laboratory heat shock) of HSC77 and HSC72 are positively correlated with increases in ambient temperature and were significantly higher in August than in January. The elevated level of HSCs during the summer was associated with moderate, 2–3 °C, increases in the upper thermal limits for survival. We measured concomitant increases in the threshold temperatures (T_{on}) required for induction of HSP70. Total *hsp70* mRNA expression reflected the seasonal changes in the expression of inducible but not cognate members of the HSP70 family of proteins. A potential cost of increased T_{on} in the summer is that there was no extension of the upper thermal limits for survival (*i.e.,* induction of thermotolerance) after sublethal heat shock at temperatures that were sufficient to induce thermotolerance during the winter months.

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Introduction

Marine invertebrates tolerate a remarkable array of natural and anthropogenic stresses. Although the nearshore environment supports great invertebrate diversity, it is physiologically challenging. Littoral invertebrates routinely encounter anoxia, osmotic stress, and wide thermal fluctuations. Withstanding these conditions usually requires the initiation of coordinated cellular responses to stress. These responses are particularly important in the adaptive response of sessile intertidal invertebrates such as mussels and oysters.

Many of these natural stresses can directly alter protein conformation and stability. Because proteins must be sufficiently labile to interact with their substrates, they are often highly susceptible to protein-denaturing stresses such as reduced intracellular pH or elevated temperature (Somero, 1995). Protein-denaturing stressors such as heat can result in the revealing of hydrophobic domains of proteins and cause nonspecific protein interactions. Under these conditions, several families of proteins known as heat-shock proteins (HSP), or molecular chaperones, perform critical protein-stabilizing functions (Lindquist, 1986; Gething and Sambrook, 1992; Morimoto *et al.,* 1994). Under "non-stress" conditions, molecular chaperones are required to stabilize hydrophobic protein domains exposed to aqueous cellular environments during protein synthesis and translocation (for review, see Hartl and Hayer-Hartl, 2002). Stress-induced expression of HSPs contributes dramatically to tolerance of otherwise lethal conditions (Parsell and Lindquist, 1994), known as "induced thermotolerance."

Natural variation in the heat-shock response appears to be correlated with distribution along environmental gradients of stress (Hofmann and Somero, 1996; Tomanek and Somero, 1999). Threshold induction temperatures for HSPs are positively correlated with ambient environmental temperatures (Dietz and Somero, 1992; Buckley *et al.,* 2001). In the marine mussel *Mytilus,* threshold temperatures can be higher in animals living at higher tidal heights (Roberts *et al.,* 1997). In congeneric species of the marine snail *Tegula,* threshold temperatures are positively correlated with the maximal tidal height at which each species is found. In *Tegula,* these differences are maintained even after laboratory acclimation, suggesting a role for gene regulatory factors that establish set points for HSP induction or interspecific variation in the stability of cellular proteins (Tomanek and Somero, 1999).

The basic features of the heat-shock response in the Pacific oyster, *Crassostrea gigas,* have been well characterized (Clegg *et al.,* 1998). HSP70 has been shown to be the primary family of HSP that is responsive to thermal stress. Three isoforms of HSP70 family protein are resolved after one-dimensional electrophoresis and western blotting. Two proteins, HSC77 and HSC72, are constitutively expressed, and their level of expression and accumulation increases after acute thermal stress. In contrast to other bivalve species such as *Mytilus, C. gigas* can express a third protein, HSP69, but it is typically only detectable after acute thermal stress. Several days after heat shock, the levels of HSP69 account for roughly one-third of total HSP70. In the absence of additional stress, HSP69 completely disappears within 7–10 days of heat shock. Two genes encoding mRNAs that correspond to the constitutively expressed (cognate) and inducible forms of oyster HSP70 family have been sequenced and characterized. Distinct gene products encode a cognate protein of about 72 kDa (Gourdon *et al.,* 2000) and an inducible protein of about 68–70 kDa (Boutet *et al.,* 2003). In Pacific oysters, a single sublethal heat shock sufficient to cause significant HSP69 expression also results in induced thermotolerance for a remarkably prolonged period of up to 2 weeks (Clegg *et al.,* 1998).

In this study, we attempted to determine the extent and the mechanism of adaptive plasticity of the heat-shock response in a group of sibling Pacific oysters planted at two tidal heights (+0.3 m and +1.2 m) at Totten Inlet, Southern Puget Sound, Washington State. We measured the seasonal changes in HSP70 and *hsp70* gene expression in these oysters, and we examined these findings in the context of the observed, concomitant changes in control and inducible upper thermal limits for survival. Our findings suggest that phenotypic plasticity of the heat-shock response is a significant component of adaptation to natural thermal stress.

Materials and Methods

Animals and study site

A commercial oyster bed in Totten Inlet, south Puget Sound, Washington, was selected as an experimental study site. The juvenile "seed" (about 5–10 cm in length) oysters for this experiment, 1999 age class, were obtained in late June 1999 and planted at two tidal elevations: a low-intertidal plot at 0.3 m mean lower low water (MLLW) and a high-intertidal plot at 1.2 m MLLW. The seed were initially placed in rectangular, 0.5-cm mesh, plastic grow-out bags set on top of metal racks at each tidal height. The oysters were later transplanted to larger, 2–4-cm mesh, grow-out bags. Oysters were sampled monthly during low tide events starting in April 2000 and continuing until January 2001.

External site temperature data were recorded every 15 min using Hobo temperature sensors. Internal temperature data were recorded using Hobo 4-channel external data loggers and TMC6-HA temperature sensors. Temperature data were recorded at each tidal height from three oysters with internally mounted sensors and one externally mounted sensor (TMC6-HA sensor), which was placed next to the oysters either in a grow-out bag or on the ground. The internal temperature sensors were placed inside the shell of live oysters through a 6mm opening. The sensors were secured and sealed by using marine epoxy.

Heat shock

Immediately after collection oysters were shipped overnight in chilled (12–15 °C) coolers to Bodega Marine Laboratory, Bodega Bay, California, for use in experiments. After 24 h of recovery in flow-through tanks with raw Bodega Bay seawater (RSW; 13°C, ± 2°C), animals were placed in fine mesh bags and heat-shocked at a variety of temperatures (see Results) by being submerged in preheated, aerated filtered seawater baths for 65 min. Animals were returned to the flow-through seawater tanks immediately after heat shock. Five to six oysters were heat-shocked at each temperature for sampling of gills for northern and western blots. After heat shock, oysters were returned to the RSW tanks for 48 h before gills were sampled for protein and mRNA analysis; 48 h has previously been shown to be the point at which accumulation of HSP70 protein is maximal, and *de novo* synthesis of HSP70 is still elevated relative to control specimens of *C. gigas* (Clegg *et al.,* 1998). This protocol allowed us to compare the level of HSP70 protein and mRNA from identical gill sections. It is important to note that HSP70 levels remain elevated in oysters for at least one week following heat shock. Thermal limits were determined by heat-shocking three groups of seven animals (in fine-mesh bags) at each temperature. Survival was determined after 10 d of recovery at 13°C (± 2°C) in RSW tanks.

To ensure that shipping and brief holding did not alter the profile of HSP70 expression, we compared samples of gill from freshly collected oysters (prior to shipping), from oysters that had just arrived at the laboratory, and from oysters that had been held for 2 days in ambient RSW. No difference in HSP70 protein levels was observed between any of these groups (data not shown).

Tissue sampling and sample preparation

Oysters were opened and gills were dissected and divided into two sections for protein and RNA isolation. Gill samples for western blots were prepared according to Clegg *et al.* (1998). Briefly, they were rinsed in double-distilled H_2O, blotted dry, weighed, and flash-frozen in liquid nitrogen. These samples were stored at −70°C and later homogenized on ice in glass tissue grinders containing potassium gluconate buffer (5 m*M* $MgSO_4$, 5 m*M* NaH_2PO_4, 40 m*M* HEPES, 70 m*M* gluconic acid, 150 m*M* sorbitol; pH 7.5) at a concentration of 100 milligrams of tissue per milliliter of buffer. Homogenates were prepared for electrophoresis by mixing 1:1 in 2× sample buffer and boiling for 5 min.

Gill sections for RNA isolation were immediately preserved in 5 volumes of RNAlater (Ambion) solution, frozen in liquid nitrogen, and stored at −70°C. Gills were later homogenized in 3 ml guanidine thiocyanate denaturing solution; 900 μl of this homogenate was mixed with 100 μl of 2 *M* sodium acetate and incubated on ice for 5 min. Samples were next mixed 1:1 with ice-cold phenol/chloroform (pH 4.3; Sigma) and incubated on ice for 20 min. After centrifugation for 25 min at 10,000 × *g*, the aqueous layer was removed, and RNA was precipitated overnight in an equal volume of isopropyl alcohol. The next day the RNA was pelleted, washed, and resuspended in DEPC H_2O. Purity and concentration were checked using a spectrophotometer.

cDNA cloning and probe synthesis

Total RNA was isolated from heat-shocked oysters and used to synthesize cDNA with Superscript Reverse Transcriptase (Gibco-BRL) according to manufacturer's instructions. A 660-bp fragment of oyster HSC72 cDNA was amplified from this template using degenerate HSP70 primers (Cochrane *et al.,* 1994). PCR products were checked for size and yield on 1% agarose gel prior to TA cloning using the dual promoter pCRII-TOPO vector (Invitrogen) and cloning kit. Aliquots of plasmid minipreps (Qiagen) of positive clones were sent to Davis Sequencing (Davis, California) for sequencing. Results were analyzed by using the BLAST program at the National Center for Biotechnology Information to compare existing sequences. A 660-bp fragment was cloned that was identical to a portion of a previously submitted sequence of Pacific oyster HSC72 mRNA (Gourdon *et al.,* 2000).

An antisense RNA probe for total HSP70 probe was synthesized from minipreps (confirmed to be positive for the *hsc72* gene product by sequencing). Briefly, a digoxigenein-labeled UTP (Roche) probe was synthesized by *in vitro* transcription (Maxiscript; Ambion) of the antisense strand. Because of the high homology in this region of the known oyster HSP70 genes, we expected this probe to hybridize with all of the known gene products of the HSP70 family. The yield of resulting probe was determined by using a dot blot of the probe *versus* a digoxigenein-RNA probe standard (Roche) according to manufacturer's instructions.

Western blotting

Western blots of oyster HSP70 family were prepared according to Clegg *et al.* (1998) and 10 μl of each solubilized sample was resolved on a 10% SDS-polyacrylamide gel. To normalize between blots, multiple aliquots of a single sample of heat-shocked oyster gill were made. Each time gels were run, an aliquot of this sample was loaded on the center lane of each gel. The internal control sample was arbitrarily chosen from a previous experiment and was known to have all three isoforms of HSP70 family protein. Therefore, levels of each HSP70 family member were normalized relative to the level of the corresponding band from the internal standard (the maximal variation in standard level between gels was less than 10-fold). To ensure that the large differences in HSP70 protein levels between summer and winter controls were not attributable to blot variation, the summer and winter samples were run on a single blot and their values relative to heat-shocked oysters were adjusted according to the level of the corresponding standard sample run on that blot. Control levels of HSP70 mRNA were compared from samples run on a single northern blot.

Proteins were transferred onto nitrocellulose and probed using a monoclonal rat anti-HSP70 antibody (Affinity Bioreagents, MA3-001), and an HRP-conjugated goat secondary antibody (Sigma). Signal was detected by chemiluminescence using enhanced detection reagents (Supersignal, Pierce) on a Bioimaging Systems digital gel scanner or by exposing blots to photographic film (Kodak, Biomax MR). Relative levels of band were intensified using gel plotting macros available on Scion Image (ver. 1.61) software.

Northern blotting

For each northern blot, 4 μg of denatured total RNA was loaded onto each lane of 1% agarose (in 1× MOPS), 6.66% formaldehyde gels and run for 3–5 h at 50 V. RNAs were transferred overnight onto nylon membranes (Hybond) using standard capillary methods in 10× SSC buffer. After transfer, RNA was cross-linked to the blots (Stratalinker), and blots were stored at −20°C until probed. DIG-labeled RNA probes were prepared at a final concentration of 50

ng/ml in high SDS buffer (all buffers in RNA detection were prepared according to instructions from DIG applications manual, Roche). Blots were incubated overnight at 50°C in probe solution with gentle rotation. The next day, nonspecific hybridization was removed using four 15-min stringency washes at 60°C in decreasing concentration of SSC solution (2×–0.5×). Blots were then washed twice in maleic acid buffer containing 0.3% Tween 20, and blocked for 30 min using 1× blocking buffer (Roche). Blocked blots were incubated for 1 h with gentle rotation at room temperature in a 1:10,000 solution of anti-DIG AP Fab fragments (Roche) in blocking buffer. CSPD solution (Roche) was used 1:100 in detection buffer to detect hybridized probe. Chemiluminescence was enhanced by 15-min incubation of the blot at 37°C, followed by detection using a Bioimaging Systems digital gel scanner.

Statistics

Differences in protein and mRNA level were analyzed for statistical significance using one-way ANOVA, and multiple pairwise comparisons were performed according to Dunn's method; all tested data conformed to equal variance and normality assumptions. All analyses were conducted using Sigmastat software ver. 2.03.

Results

Animals and study site

Temperatures recorded in living oysters generally matched those recorded by external data temperature loggers placed alongside the oysters in the culture bags (Fig. 1). This suggests that despite its thickness, oyster shell is a relatively poor insulator against thermal stress. Oysters at 1.2 m MLLW generally experienced higher temperatures and longer durations of exposure to elevated temperature than oysters at 0.3 m. To estimate the duration of exposure to elevated (>20°C) temperatures, measurements from data loggers were sorted into 5°C intervals, and measurements in each class were summed together. Each data point was assumed to represent about 15 min of exposure to the recorded temperature. In nearly all cases the approximate duration of exposure to warm temperatures was longer at the high tidal height (Fig. 2) for the 2 weeks preceding the summer sampling. During the summer 2000 period, the total exposure to temperatures in excess of 20°C was about 67 h at the high tidal height compared to 44 h at the lower tidal height. The thermal regime based on mean emersion is summarized in Figure 3. The majority (>70%) of the time (not shown), oysters at both tidal heights were submerged and encountered temperatures in the 15–20°C range. Oysters encountered elevated (>20°C) and reduced (<15°C) temperatures at or above the point of emersion.

No significant difference in mortality was recorded in animals at either site during the experiment. Total mortality at both sites was less than 20% annually. Animals at the high tidal height (1.2 m) showed a cumulative mortality of 16.5% (62 out of 375 animals); those at the low tidal height (0.3 m) experienced an 18.4% (69 out of 375 animals) mortality. Most of the mortalities occurred during the warmest months (June–September).

HSP70 protein and hsp70 *mRNA levels*

The levels of HSC70 (HSC77 and HSC72) in control (non-heat-shocked) Pacific oysters were associated with the level of environmental thermal stress (Fig. 4). At both study sites the control levels of HSC77 and HSC72 were as much as 100-fold greater in summer than in winter ($P < 0.001$). These differences were not attributed to differences in total protein levels, as mean control protein levels were 41.5 mg/g wet weight and 46.2 mg/g wet weight in winter and summer, respectively (Bradford protein assay, BioRad), and these differences were not significantly different ($P > 0.07$). During the winter months there was no statistically significant difference in the control levels of HSC70 between the high and low tidal height sites ($P = 0.917$).

The threshold temperature for induction of HSP69 appeared to be associated with the level of environmental thermal stress (Figs. 1–4). In January, HSP69 accumulation was induced in oysters from both sites after heat shock at 37°C. In contrast, limited or no induction of HSP69 and HSC72 was observed following heat shock at 37°C in August oysters living at high or low tidal height (Fig. 4; see also Fig. 6A). Full induction (*i.e.*, HSP69 expression at levels approximating those of HSC77 or HSC72) of HSP69 was not observed in August until after heat shock at 40–43°C. Thus, both the threshold temperature for HSP69 induction and the control levels of HSC70 exhibited significant seasonal plasticity.

The level of *hsp70* mRNA in summertime high tidal height oysters increased dramatically relative to control after heat shock at 40 and 43°C, but not after heat shock at 33 and 37°C (Fig. 5). This result reflected the observed increases in HSP69 protein after heat shock at these temperatures in the same animals.

A subset of oysters from each site was sampled and heat-shocked at 37°C monthly during the summer, beginning in April and ending in September (not shown). Based on the results of these observations, the greatest change in threshold temperature for HSP69 induction and control level of HSC77/HSC72 temperature occurred between our June and July sampling dates and winter (January) (Fig. 6A, B; Fig. 7). In June, oysters at the high tidal height completely induced HSP69 after a 37°C heat shock. In contrast, by July, induction of HSP69 was much more limited in these oysters. Control levels of HSC70 also increased markedly during this period. Although there were slight increases

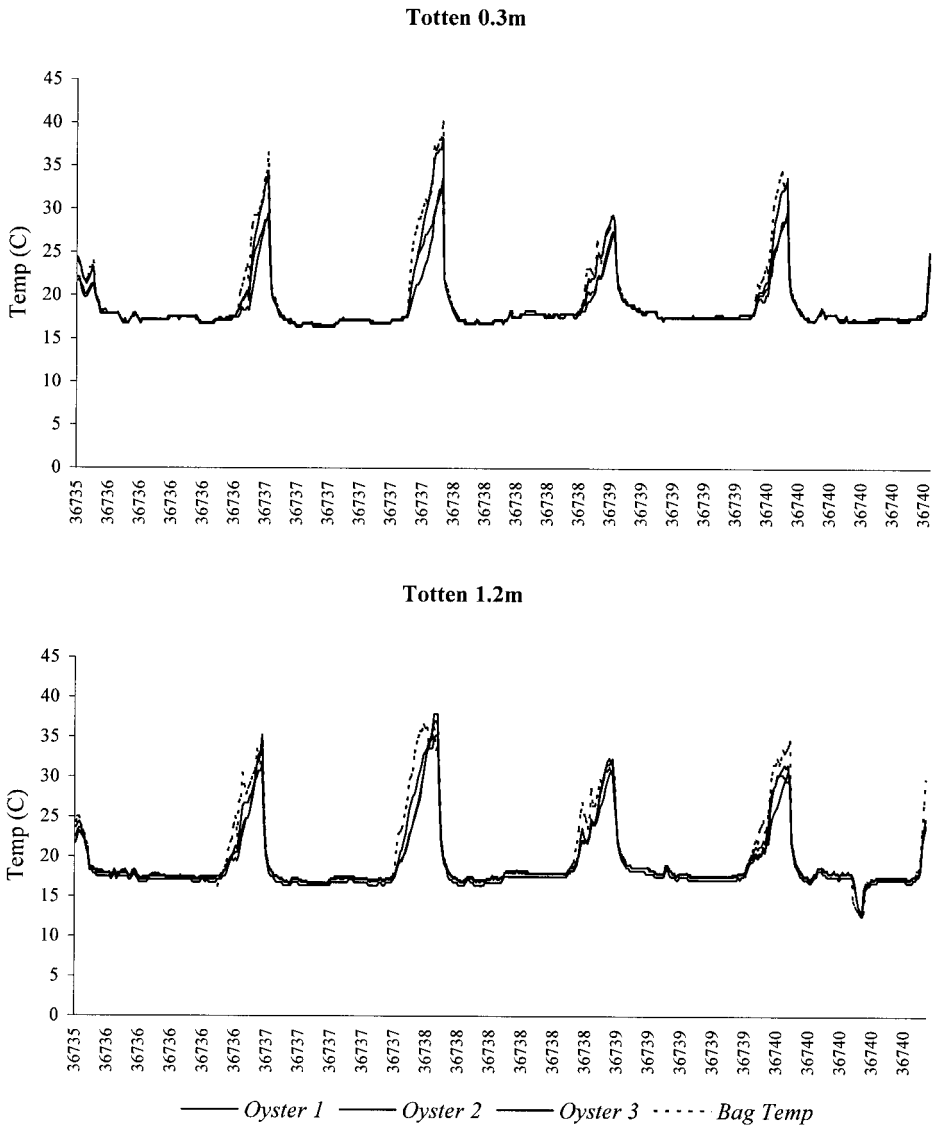

Figure 1. Internal temperatures of three living oysters (solid lines) at two tidal heights (1.2 m and 0.3 m at Totten Inlet, Washington) and external temperatures from a temperature probe placed alongside the oysters (dotted lines). Internal temperatures closely track external temperatures.

in mean levels of control *hsp70* mRNA in summer relative to winter oysters, the differences were not significant (P = 0.13; Fig. 7).

Thermal limits and induction of thermal tolerance

Laboratory heat shock of oysters at both tidal heights suggested both seasonal and tidal height influences on the upper thermal limits for survival (Fig. 8). In January there was no significant difference in lethal temperatures between oysters at the two tidal heights. However, by August there were significant increases in the baseline thermal limits of oysters at both study sites. Moreover, oysters at the high tidal height appeared to exhibit elevated thermal limits compared to their low tidal height counterparts during the summer. August survival of high tidal height oysters after 44°C heat shock was significantly higher than that of the low tidal height oysters (P = 0.03). These differences occurred in the absence of HSP69 expression, suggesting that control thermal limits in Pacific oysters are likely to be more closely associated with cognate HSC70 expression than to inducible HSP70 expression. In contrast, induced thermal tolerance appeared to be associated with inducible HSP70 expression (Fig. 9). Oysters at the high tidal height that were heat-shocked at 37°C during the winter were able to completely withstand a subsequent lethal treatment of 44°C. Heat shock at 37°C did not induce thermotolerance

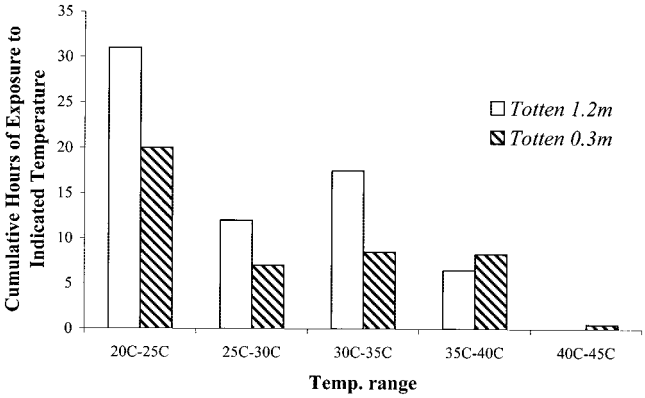

Figure 2. The approximate hours of exposure to elevated (>20°C) air temperatures in oysters at high and low tidal heights for the 2 weeks prior to the summer sampling. The vast majority of the time, oysters experienced temperatures (15–20°C) below the range shown.

(to 46°C) during the summer. However, when August oysters were heat-shocked at 40°C, they were able to withstand heat shock at 46°C. These results suggest that induction of thermal tolerance is more closely associated with inducible HSP70 family expression.

Discussion

Our results demonstrate significant phenotypic plasticity in the heat-shock response of Pacific oysters. Massive changes (10- to 100-fold increases) in the amounts of constitutively expressed HSP70 proteins were measured in summer as compared to winter. These changes are associated with concomitant increases in thermal limits and set point for HSP69 induction. In oysters at both high and low tidal height, the threshold for complete (*i.e.*, equivalent to levels of HSC77 and HSC72) induction of HSP69 increased

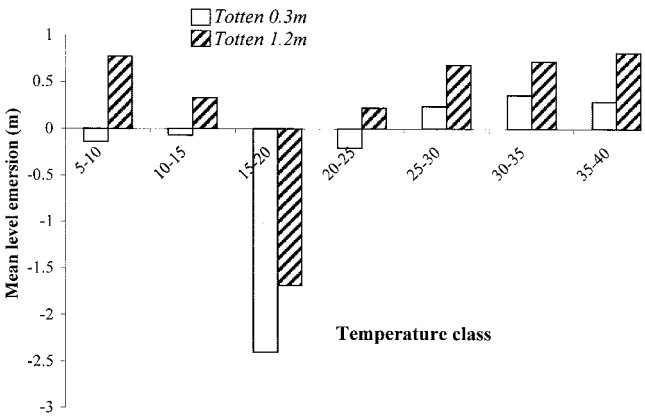

Figure 3. Mean level of emersion for 5°C temperature intervals encountered during the month of August. For the vast majority of time (> 70%), temperatures were in the range of 15–20°C. At this temperature range, oysters were completely submerged. At temperatures outside of this range, oysters were at or above the point of emersion.

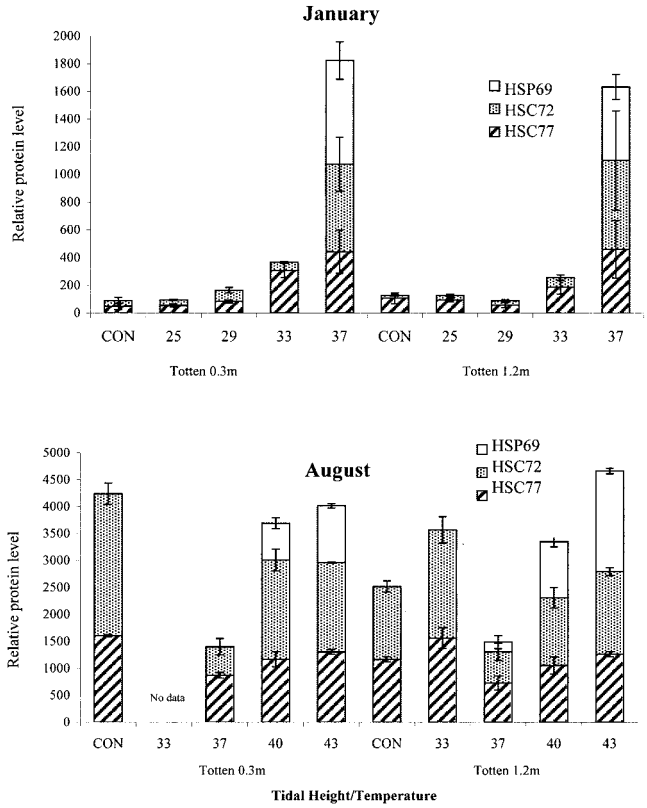

Figure 4. Relative levels of HSP70 as determined from western blots for animals sampled in August and later in January. Bars represent the mean values of total HSP70 for 5–6 animals (± s.e.m. for each isoform). The relative amount of each isoform is indicated within the total amount. Three isoforms were resolved by one-dimensional electrophoresis: two cognate forms (HSC77 and HSC72) and one inducible isoform (HSP69). The *x*-axis of each graph indicates the heat-shock temperature series for the low tidal height (0.3-m) group, followed by the temperature series for the high tidal height (1.2-m) group.

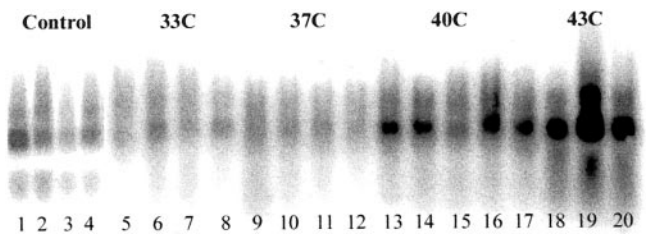

Figure 5. Northern blots of *hsp70* mRNA in high tidal height (1.2-m) oysters 48 h after heat shock (HS) in August. Lanes are loaded with equivalent amounts (4 μg) of total mRNA. HSP70 was analyzed with an antisense digoxygenein-labeled UTP probe corresponding to a 660-bp portion of oyster HSC72. Each lane represents mRNA from one oyster. Four oysters were subjected to each treatment (1–4 are Controls, 5–8 are 33°C heat shock (HS), 9–12 are 37°C HS, 13–16 are 40°C HS, 17–20 are 43°C HS).

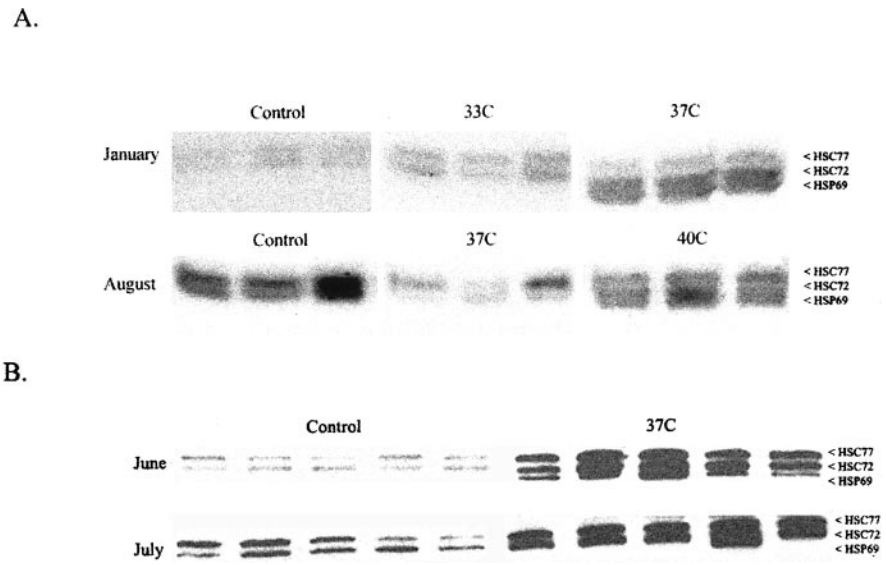

Figure 6. (*A*) Western blots, captured on digital scanner, of HSP70 family expression in high tidal height (1.2-m) oysters (3 individuals per treatment) in August and in January. HSC77 and HSC72 are dramatically elevated in control oysters sampled in August. (*B*) Western blots, exposed to film, of HSP70 protein family in oysters (5 individuals per treatment) living at high tidal height (1.2-m). In June, a single 1-h heat shock (37°C) completely induces HSP69 expression. In July, HSP69 expression is absent in most animals sampled, but the control levels of HSC77 and HSC 72 are elevated relative to June.

from about 33–37°C in winter to 37–40°C in summer. In high tidal height oysters, the temperature required for induction of thermal tolerance also increased from 37°C in winter to 40°C in summer.

Although we did not determine the changes to the absolute limits for control and inducible thermal tolerance, the observed changes suggest potential costs for plasticity of the heat-shock response. It is clear that seasonal increases in thermal limits are associated with concomitant increases in the temperatures required for induction of tolerance to oth-

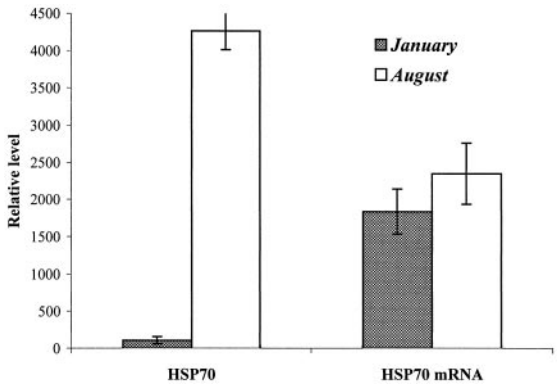

Figure 7. Control levels of total *hsp70* mRNA ($n = 4$) and total HSP70 ($n = 5$) prior to heat shock in summer and winter from oysters from the high tidal height (1.2 m). Summer and winter protein samples were run on the same blot, as were mRNA samples, and relative levels were measured in arbitrary units using Scion Image software.

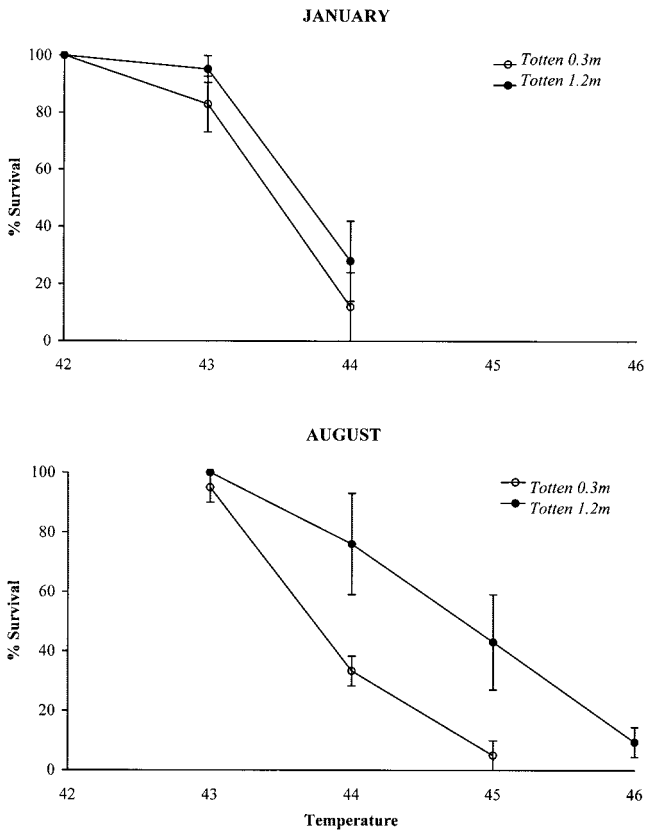

Figure 8. Survival of oysters after 1 h of submerged heat shock in summer and winter. Points are the means of three groups of seven oysters each (± s.e.m.).

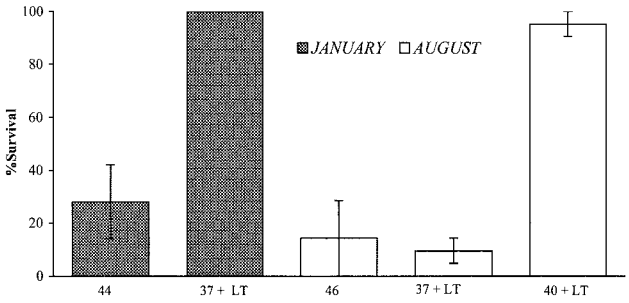

Figure 9. Induced thermal tolerance in oysters from the high tidal height (1.2 m). Oysters were either heat-shocked directly at lethal temperature or shocked for 1 h at a sublethal temperature and then allowed to recover for 2 days before lethal temperature treatment. Numbers below bars indicate the initial treatment temperature, followed by the temperature of the subsequent treatment (LT = lethal temperature as determined for each group of oysters 1 week prior to the start of the experiments). LT was 44°C in the winter and 46° C in the summer. Bars represent means of three groups of seven oysters each (± s.e.m.).

erwise lethal temperatures. However, induction of thermal tolerance is a laboratory phenomenon and may not directly relate to changes in thermal limits in nature. Clearly most of the temperatures encountered in nature are far below those required to measure changes in thermal limits. Thus the consequences of changes in inducible thermal tolerance may be most significant during periods when elevated temperatures are extreme or when they occur in concert with other stresses.

Over-expression of HSPs is known to have deleterious consequences. Initiation of heat-shock responses usually results in a concomitant reduction in the synthesis of all other proteins (Parsell and Lindquist, 1994). During stress, HSPs can account for a large fraction of cellular protein; thus it is hypothesized that their synthesis represents a significant energetic cost. In *Drosophila,* artificially high levels of HSP70 can reduce thermotolerance and slow the development of larvae (Krebs and Feder, 1998). Thus it is possible that the observed plasticity of the heat-shock response in Pacific oysters represents a trade-off between the costs of maintaining elevated control thermal tolerance and the cost of the resting state in which the heat shock proteins are maintained at lower levels.

In the laboratory, other bivalve species such as *Mytilus* exhibit significant plasticity in various features of the heat-shock response (Buckley *et al.,* 2001). However, two factors confound the interpretation of results from experiments with adult bivalves collected from the field. First, significant selection occurs at settlement (Launey and Hedgecock, 2001), and it is likely to correspond with distribution along gradients of stress (Michener and Kenny, 1991). Second, large-scale mortality may occur within adult bivalve beds during warm seasons (Cheney *et al.,* 2000). Because there was no significant differential mortality during our experiments, we can downgrade the likelihood of a strong site-specific effect due to selection. Moreover, by planting sibling juvenile oysters at each site, we limited any confounding effect of selection for thermal tolerance at settlement. Thus, we conclude that our results and perhaps those previously observed in *Mytilus* (Roberts *et al.,* 1997; Buckley *et al.,* 2001) are probably the consequence of significant phenotypic plasticity in the heat-shock response rather than of fixed genotypic differences between bivalves living along gradients of stress.

Further experiments using genetically characterized families of bivalves, and other model organisms, may be the most useful in differentiating components of the heat response that are genetically fixed from those that exhibit adaptive plasticity or intra-specific variability. Oysters may be an ideal system for these studies. Because oysters are a commercially valuable species, recent studies (*e.g.,* Launey and Hedgecock, 2001) have focused on characterizing the genetics of Pacific oysters from known lineages. The possibility of producing oysters with characterized genotypes and improved culture performance under a wide range of environmental conditions is being explored (Langdon *et al.,* 2003). A major challenge facing oyster growers is reducing mortality during the warmest summer months (Cheney *et al.,* 2000). It is not yet known if the improved performance of these characterized families of oysters is related, at least in part, to genetically fixed differences in their ability to mount functional heat-shock responses and withstand thermal stress.

Our results suggest that both accumulation of HSP and rates of gene expression are responsive to environmental stress, but that changes in gene expression alone are not sufficient to account for the differences in protein accumulation. Remarkably, *hsp70* mRNA levels were elevated 2 days after heat shock in high tidal height oysters that were heat-shocked at 40 and 43°C. It is possible that elevated mRNA levels would have also been measured at lower temperatures if tissues were sampled sooner than 48 h after heat shock. Our results suggest that cognate HSP70 protein expression in oysters is not directly controlled at the transcriptional level. It is possible that changes in the rate of HSP70 protein synthesis and degradation may be important regulatory components of the long-term modulation in control HSP70 level. Consistent with this hypothesis, Clegg *et al.* (1998) demonstrated that levels of HSC77 and HSC72 remain elevated long after total HSP70 synthesis has returned to control levels.

Other authors (Tomanek and Somero, 2002) have proposed that level of cognate HSP70 isoforms may be an index of protein synthesis capacity, whereas the levels of inducible isoforms may be more accurate indices of adaptation to heat stress. In our study it was the threshold for HSP69 expression that reflected adaptation to thermal stress associated with seasonal changes. The seasonal plasticity of the heat-shock response was reflected most dramatically in

the large seasonal changes in the control levels of HSC77 and HSC72, suggesting that these isoforms may also play important roles in mediating the effects of thermal stress.

In laboratory selection experiments using *Drosophila* (Lerman and Feder, 2001), there is evidence that the heat-shock response may be regulated by genetic factors that are fixed during evolution in a particular environment. However, in sessile intertidal species, several key features of the heat-shock response (*e.g.,* control levels of HSP and threshold temperatures of HSP induction) appear to be sensitive to acclimation to laboratory and natural seasonal conditions (Roberts *et al.,* 1997; Buckley *et al.,* 2001). In sessile species such as oysters or mussels, behavioral adaptation plays a more limited role in regulating exposure to environmental stress. Thus, we hypothesize that the heat-shock response, and particularly the levels of HSC70 in Pacific oysters, must exhibit sufficient phenotypic plasticity to allow organisms to cope with a wide array of environmental conditions.

Most studies of the ecological physiology of stress proteins have two key limitations (Feder and Block, 1991; Feder and Hofmann, 1999). First, most relevant stressors initiate responses from a variety of cellular and systemic stress-response systems. Second, single stresses are rarely encountered in the environment. More typically, organisms encounter multiple stresses that interact with a variety of stress-responsive systems. In the case of sessile bivalves, a critical stressor that has been overlooked is the simultaneous exposure to anoxia or hypoxia during periods of thermal stress. Since most periods of elevated ambient temperatures occur during emersion (Fig. 3), it will be important to address the question of whether anoxic and hypoxic conditions interact with heat-shock responses in intertidal bivalves.

Plasticity of the heat-shock response is apparently a common feature in sessile intertidal invertebrates but not in other marine invertebrates (Tomanek and Somero, 1999; Spees *et al.,* 2002). This suggests that the potential costs of adaptive plasticity of the heat-shock response outweigh the benefits in species and life-history stages for which behavioral regulation of thermal environment is possible. Perhaps more significantly, these results could indicate that although heat-shock proteins and the genes that encode them are highly conserved, the composition and expression of the regulatory components that collectively constitute the cellular thermostat may exhibit significant interspecific variability. Thus oysters, mussels, and other species that exhibit significant adaptive plasticity of the heat-shock response may have evolved the most complex and robust regulatory mechanisms. An unresolved question is the mechanism by which control or resting levels of HSP70 are regulated during chronic stress exposure. It is not yet clear if these mechanisms provide distinct regulatory pathways that might be adaptive in specific tolerance of acute *versus* chronic stress.

This study has shown that a change in the threshold temperature for HSP70 induction, in response to chronic stress, is associated with a concomitant change in the threshold temperature required to induce thermotolerance. We have shown that the changes in threshold temperature for induction of thermal tolerance match the seasonal changes in threshold temperature for HSP70 induction and expression. Of particular ecological significance is the finding that an increase in threshold temperature narrows the range of temperatures at which HSP induction can contribute to the acquisition of thermotolerance in oysters. Although this suggests a cost for adaptive plasticity of the heat-shock response, the impact of this cost on survival in the field is not clear.

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