Effects of the Natural Tidal Cycle and Artificial Temperature Cycling on Hsp Levels in the Tidepool Sculpin *Oligocottus maculosus*

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ABSTRACT

The rocky intertidal zone is characterized by a predictable cycle of environmental change cued by the ebb and flow of the tides. Tidepools are thus an excellent environment in which to determine whether predictability of environmental change can entrain an endogenous rhythmicity in heat shock protein (Hsp) levels. In this study, we monitored changes in Hsp mRNA and protein levels that occurred over the tidal cycle in tidepool sculpins and investigated whether there was an endogenous tidal rhythm in Hsp expression that persisted once the sculpins were transferred to a stable environment. Fluctuations in the tidepool environment increased *hsc70*, *hsp70*, and *hsp90* mRNA levels, which translated into increased Hsc/Hsp70 and Hsp90 protein levels; however, this was not due to an endogenous tidal rhythm in Hsp levels because sculpins held under constant conditions did not show any rhythmicity in the expression of these genes. By exposing sculpins to an artificial temperature cycling regime that mimicked the temperature changes of a mid-intertidal pool, we were able to account for the direct role of temperature in regulating Hsp expression. However, there are additional extrinsic factors that likely integrate with temperature and result in differences between the *hsp* induction profiles that were observed in sculpins inhabiting their natural environment and those in cycling conditions in the laboratory.

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Introduction

The rocky intertidal zone is an interesting environment in which to examine the impact of environmental variability on organismal physiology. On the one hand, there is a predictable cycle in environmental change that is dependent on tidal cycle; on the other hand, many factors influence the degree of fluctuation in the environment (e.g., vertical location in the intertidal zone, weather, and wave action) such that the magnitude of these environmental changes is unpredictable. Organisms inhabiting the intertidal zone must be able to tolerate these daily fluctuations and may have strategies that allow them to take advantage of the predictable nature of their habitat to prepare them for the impending environmental change.

Many intertidal organisms have evolved mechanisms for timing their activities to predictable elements in their environments, and this enables them to anticipate tidal as well as solar day-night cycles (Green 1971*a*; Northcott 1991; Palmer 2000; Gibson 2003). It is thought that these rhythms are entrained by both mechanical (e.g., wave action and turbulence) and physical (e.g., temperature, pressure, and salinity) characteristics of the tidal cycle. Despite this substantial work at the organismal level, there have been very few reports of endogenous rhythms in physiological parameters in intertidal animals. There have been, however, studies in many organisms that have shown that acclimation to fluctuating or constant thermal environments results in different physiological phenotypes (Lowe and Heath 1969; Otto 1973; Feldmeth et al. 1974). In addition, photoperiod is known to have an important role in modulating physiological parameters in organisms that inhabit other highly variable environments such as estuaries and marshes (Weld and Meier 1983; Frick and Wright 2002). Taken together, these data suggest that some aspect of environmental variation is likely to play an important role in modulating physiological responses in intertidal animals.

There has been significant interest in characterizing the environmental regulation of heat shock protein (Hsp) gene expression. Much of this work has been done on intertidal organisms and has highlighted the plasticity of Hsp gene expression (Hofmann et al. 2002). Acting as molecular chaperones, Hsps are an important component of the cellular stress response and play a critical role in the recovery of cells from stress by maintaining the integrity of the cellular protein pool

(for review, see Lindquist 1986; Hightower 1991; Morimoto 1998; Hartl and Hayer-Hartl 2002; for review in fishes, see Iwama et al. 1998; Basu et al. 2002). From an organismal perspective, it is becoming clear that elevated levels of Hsps confer an increase in stress tolerance (Parsell and Lindquist 1993; Krebs and Feder 1998) and that Hsps likely have a protective role in an animal's natural tolerance to environmental change (Feder and Hofmann 1999).

Currently, there is little research documenting whether intertidal organisms are able to anticipate the changes in their natural environment by "presynthesizing " Hsps before a low-tide period. There is, however, accumulating evidence that organisms living in the intertidal zone have a heat shock response that reflects the periodicity of environmental change. Branchial Hsp70, Hsp90, and ubiquitin levels of the intertidal mussel *Mytilus trossulus* were significantly increased within the first few hours of recovery from tidal emersion, indicating that protein repair and degradation in this sessile animal occurs once it is submerged by high tide (Hofmann and Somero 1996). Subsequent studies in *Tegula funebralis* demonstrated that the time required to induce and complete Hsp synthesis in this intertidal snail is very rapid, and it has been suggested that *T. funebralis* might be able to repair protein damage incurred from one low-tide period before the onset of the next (Tomanek and Somero 2000). Schill et al. (2002) observed daily changes in Hsp70 protein levels in the foot muscle of an intertidal chiton *Acanthopleura granulata* in the field, which followed air temperature with a 4–8-h time lag. Collectively, these studies provide some initial evidence of the degree to which the kinetics of the heat shock response reflect the temporal scale of environmental change inherent in the ebb and flow of the tides; however, it is still unknown whether these differences are the results of an intrinsic diurnal rhythmicity in the Hsp response or whether this variation is simply elicited in response to an environmental variable, such as temperature.

Tidepool sculpins (*Oligocottus maculosus*) are widely distributed throughout the intertidal zones of the Pacific Northwest, most densely populating tidepools in the mid-intertidal region, where they routinely experience dramatic daily fluctuations in temperature, salinity, and oxygen availability (Green 1971*a*). Although there has been substantial work on the Hsp response of sessile intertidal invertebrates (Hofmann and Somero 1996; Roberts et al. 1997; Buckley et al. 2001), little is known about the Hsp response of intertidal vertebrates. Tidepool sculpins are known to home to the same tidepool over consecutive low-tide periods (Green 1971*b*) and therefore select for an environment that undergoes drastic fluctuations in environmental conditions on a daily basis. Because of their high propensity for natal homing and their ability to thrive in this highly variable habitat, tidepool sculpins are excellent organisms in which to investigate the rapid shifts in Hsp expression that occur over the tidal cycle and whether these predictable fluctuations in environmental conditions impart some degree of natural rhythm to endogenous levels of Hsps.

The main objective of this study was to characterize the changes in Hsp mRNA and protein levels that occur over the tidal cycle in tidepool sculpins and to investigate whether there is an endogenous diurnal rhythm in Hsp expression that persists once the sculpins are removed from the variable intertidal environment and held under constant conditions. In addition, we examined the effect of a fluctuating thermal environment in the laboratory that mimicked the temperature changes of a mid-intertidal pool in order to account for the direct role of temperature in modulating Hsp expression. The influence of the predictability of environmental change in the intertidal zone is still largely unknown, and while there is an accumulating body of evidence demonstrating the plasticity of the Hsp response of intertidal organisms, it is still unclear how environmental variation regulates this response. By monitoring changes in Hsp mRNA and protein that correspond to fluctuations in environmental conditions, we can begin to address the importance of a variable environment in modulating the Hsp response.

Material and Methods

Experiment 1: Endogenous Levels of Hsps in Response to Tidal Cycle

Fish Collection. Tidepool sculpins (2.1 ± 0.1 g, 5.5 ± 0.1 cm) were collected, using dip nets and minnow traps, from Wizard Rocks in Barkley Sound, Bamfield, British Columbia, during July 2003. Sculpins to be used for the acclimation component of this study were collected from a large mid-intertidal pool and transferred to outdoor flow-through stock tanks at the Bamfield Marine Sciences Centre. These fish were acclimated to a stable environment at ambient ocean conditions (11°C, 32 ppt) under a natural photoperiod for up to 1 wk. Fish were fed blue mussels, presented by cracking the shells, ad lib. daily. All experiments were conducted in accordance with an approved University of British Columbia Animal Care protocol (A01-0172).

Experimental Design. At the start of a midday low-tide period (July 19, 2003), all tidepool sculpins to be used for this experiment were collected from a single large mid-intertidal pool. All but 18 of these fish were transported back to the Bamfield Marine Sciences Centre and held in outdoor flow-through stock tanks (11°C, 32 ppt). Six fish were immediately sampled following collection (morning). In order to continue sampling fish throughout the next 12 h of tidepool exposure, two groups of six fish were placed in submerged screened containers that were secured to the bottom of the tidepool by anchoring them to a large rock with a cable tie. One of these groups of fish was sampled 6 h following the first sampling point, the time point that corresponded to the end of the midday low-tide

Table 1: Temperature, salinity, and oxygen levels of the large mid-intertidal collection tidepool

Sampling Period	Temperature (°C)	Salinity (ppt)	Oxygen (mg O_2/L)
Morning	16.0	30	4.9
Midday	21.9	30	10.1
Evening	18.9	25	18.2

Note. Morning = start of midday low-tide period, midday = end of midday low-tide period, evening = start of evening low-tide period.

period (midday). The second group was sampled 12 h following the first sampling point, and this time point corresponded to the start of the evening low-tide period (evening). At this point, sampling was discontinued. Sampling was not continued beyond this point because as the night progresses, these tidepools become extremely hypoxic (0.2 mg O_2/L; A. E. Todgham, personal observation), and sculpins must be allowed access to the surface of the pool for aquatic surface respiration and thus cannot be held in submerged containers. Temperature, salinity, and oxygen levels in the tidepool at the morning, midday, and evening sampling periods were recorded (Table 1). The lower salinity of the tidepool in the evening reflects that it started to rain lightly between the midday and evening sampling periods.

Sculpins that were returned to the Bamfield Marine Sciences Centre were acclimated to ambient ocean conditions (11.4° ± 0.3°C, 32.1 ± 0.2 ppt, 7.44 ± 0.2 mg O_2/L) in outdoor stock tanks and then sampled at several points after either 24 h or 1 wk of acclimation. In order to examine whether there was some degree of diurnal rhythm in the endogenous levels of Hsps in sculpins, six fish were sampled every 6 h over two tidal cycles. Following each acclimation period, sampling began at the time of day that corresponded to the start of the midday low-tide period specific for that day. This sampling protocol resulted in seven sampling points for each acclimation period (day 2 or day 7: morning, midday, evening, and night; day 3 or day 8: morning, midday, and evening).

Experiment 2: Endogenous Levels of Hsp Gene Expression in Response to Cycling Temperature

Fish Collection. Tidepool sculpins (0.8 ± 0.1 g, 4.4 ± 0.1 cm) were collected in November 2002 in an identical manner to the fish from experiment 1. Sculpins were initially transferred to outdoor flow-through stock tanks at the Bamfield Marine Sciences Centre and subsequently transported to the University of British Columbia. There, these fish were held in a large aerated 125-L glass aquarium (11°C, 32 ppt, and 12L : 12D) with biological filtration for 2 wk in order to acclimate to the laboratory conditions. Fish were fed blue mussels, presented by cracking the shells, ad lib. daily. Water was changed every 2–3 d depending on the fouling.

Experimental Design. To account for the direct role of temperature in regulating *hsp* expression, we examined the effect of a fluctuating thermal environment that mimicked the temperature changes of a mid-intertidal pool (Fig. 1*A*) on *hsp* mRNA levels. At the start of the experiment, fish were acclimated for an additional 2 wk in aerated and biologically filtered 75-L glass aquaria to one of two temperature regimes: (1) a cycling temperature regime that cycled daily between 11°C and 22°C and was designed to mimic the temperature profile of a mid-intertidal tidepool during an average summer day (cycling temperature; Fig. 1*B*) and (2) a constant temperature regime in which water temperature was held at 10.8° ± 0.2°C, which was

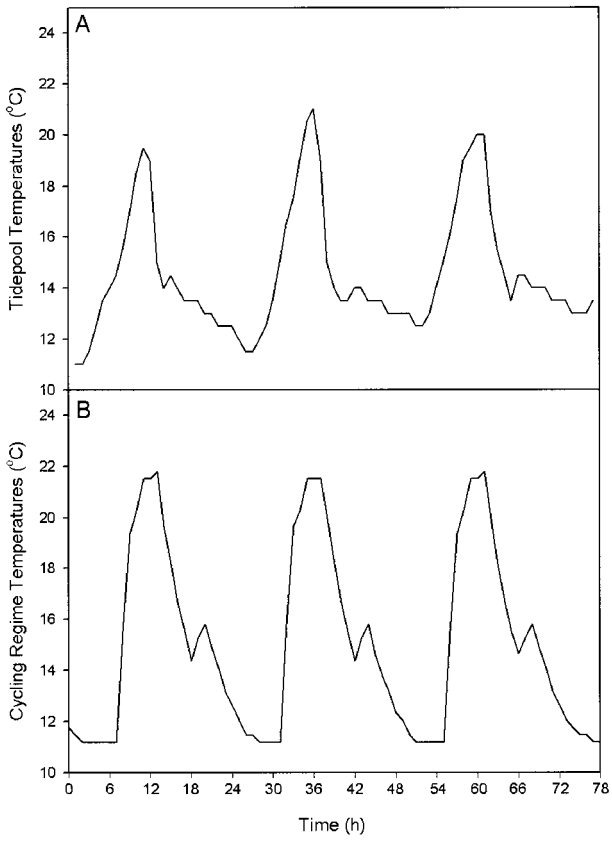

Figure 1. Water temperature (°C) profiles of a mid-intertidal tidepool (*A*) and an artificial temperature cycling regime (*B*) designed to mimic the temperature fluctuations of a mid-intertidal tidepool.

designed to mimic ambient ocean conditions (constant temperature). During summer, low temperatures in mid-intertidal pools vary between 11° and 15°C, and high temperatures vary between 18° and 26°C, depending on the day (A. E. Todgham, personal observation). We selected 11°C for the constant temperature regime as representative of the subtidal environment, and a 11°C change in temperature (11°–22°C) was selected for the cycling temperature regime as representative of the average temperature change experienced by tidepool sculpins during a summer day. In previous studies, we have observed that 2 wk of acclimation is sufficient to induce changes in whole organism thermal and osmotic tolerance (A. E. Todgham, personal observation). The cycling temperature regime was created using a combination of 250-W submersible aquarium heaters and a bench top water chiller (Lauda RM6) connected to timers as well as a Mag-Drive model 1.5 submersible pump to ensure complete mixing. In order to separate the effects of photoperiod and temperature, the temperature cycling regime was set up such that peak temperatures were reached at 1:00 a.m. as opposed to 1:00 p.m., as would be experienced under natural conditions in the tidepool. For the constant temperature regime, a bench top water chiller (Lauda RM6) was used to cool the water temperature below room temperature and maintain it at 10.8° ± 0.2°C. Temperature data loggers were deployed in each aquarium to record water temperatures throughout the 2-wk acclimation period.

Following the 2-wk acclimation period, six sculpins from each of the temperature regimes were sampled every 6 h over two full temperature cycles, resulting in seven sampling periods. The second and sixth sampling points coincided with the minimum temperatures in the cycling regime, immediately before temperature was increased, while the third and seventh sampling points coincided with peak temperatures. The first, fourth, and fifth sampling points were taken as temperature decreased (Fig. 1*B*).

Tissue Sampling

Fish were netted and rapidly anesthetized with a high dose of MS-222 (0.3 g MS-222/L water), and following onset of anesthesia, the spinal cord was severed. Gills were then rapidly excised, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

Sample Treatments for SDS-PAGE and Protein Analysis

Tissue samples were dispersed by sonication (Vibra Cell, Sonic and Materials, Danbury, CT) in homogenization buffer (0.1% SDS [w/v], 0.02 mg/mL PMSF, 0.25 mg/mL EDTA, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, and 1 μg/mL aprotinin in 100 mM Tris-HCl buffer, pH 7.5) at a ratio of 10 mg tissue to 100 μL of buffer. Homogenates were then centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to a tube containing an equal volume of 2 × Laemmli's sample buffer (4% SDS [w/v], 20% glycerol [v/v], 10% β-mercaptoethanol [v/v], and 0.0025% bromophenol blue [w/v] in 0.5 M Tris-HCl buffer, pH 6.8; Laemmli 1970) for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). These samples were then boiled for 3 min to denature all proteins and then stored at −20°C before electrophoresis (maximum 1 wk). The remaining supernatant was transferred to an empty tube and stored at −20°C until analyzed for total protein (within 2 d). Protein concentration of the tissue homogenate was determined using the bicinchoninic acid method (Smith et al. 1985).

SDS-PAGE and Western Blot Analysis for Total Hsc/Hsp70 and Hsp90

Levels of Hsc/Hsp70 and Hsp90 were measured using the discontinuous SDS-PAGE method of Laemmli (1970). Equal amounts of total protein (15 μg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). Prestained molecular markers (Invitrogen Life Technologies, Carlsbad, CA) and a pooled sample of gill homogenate from heat-shocked tidepool sculpins were added to every gel as an internal standard to standardize between gels (referred to as "internal std" in figures). Proteins were separated by SDS-PAGE at 75 V for 15 min followed by 150 V for 1 h. The separated proteins were transferred onto nitrocellulose (Bio-Rad, 0.2-μm pore size) at 17 V for 30 min with transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol [v/v], and 0.0375% SDS [w/v], pH 9.2) using a semidry transfer apparatus (Bio-Rad Trans-Blot). Transfer membranes were blocked in 2% skim milk in Tween-20 Tris-buffered saline (TTBS; 17.4 mM Tris-HCl, 2.64 mM Tris Base, 0.5 M NaCl, and 0.05% Tween-20 [v/v]) with 0.05% sodium azide for 1 h. Membranes were then rinsed once and soaked for 5 min in TTBS. Membranes were then soaked in primary antibody: rabbit IgG for chinook salmon Hsp70 (1 : 5,000, StressGen, Victoria, British Columbia) or rat IgG for human Hsp90 (1 : 250, StressGen) in 2% skim milk for 1 h. Following three 5-min washes in TTBS, membranes were soaked in an alkaline phosphatase conjugated secondary antibody: goat antirabbit IgG (1 : 5,000, Sigma, St. Louis, MO) or goat antirat IgG (1 : 5,000, StressGen) in TTBS for 1 h. After three 5-min washes in TTBS and one 5-min wash in Tris-buffered saline to remove Tween-20, the membranes were then developed in a nitro blue tetrazolium (NBT; 333 μg/mL)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 167μg/mL) solution in alkaline phosphatase buffer (0.01 M Tris-HCl, 0.1 M NaCl, and 21 mM $MgCl_2$, pH 9.5) for 5–7 min.

RNA Extraction and Reverse Transcription

Total RNA was extracted from gill tissues using the guanidine isothiocyanate method outlined by Chomczynski and Sacchi

(1987) using TRIzol Reagent (Invitrogen Life Technologies). Following isolation, RNA was quantified spectrophotometrically and electrophoresed on an agarose-formaldehyde gel (1% w/v agarose, 16% formaldehyde) to verify RNA integrity. RNA was stored at −80°C. First-strand cDNA was synthesized from 5 μg total RNA using oligo(dT_{18}) primer and RevertAid H Minus M-MuLV reverse transcriptase following the manufacturer's instructions (MBI Fermentas, Burlington, Ontario).

Real-Time PCR Analysis of hsc70, hsp70, *and* hsp90 *Gene Expression*

Partial sequences for *hsc70* (accession no. DQ013308) and *hsp70* (accession no. DQ013309) were kindly provided by K. Nakano in advance of publication. Partial *hsp90* sequence was obtained using primers determined from conserved regions of *Salmo salar* (accession no. AF135117), *Dicentrarchus labrax* (accession no. AY395632), and *Danio rerio* (accession no. AF042108). The forward primer was 5′-TGG KCA GTT TGG TGT KGG YTT YT-3′, and the reverse primer was 5′-TGG GRT GGT CMG GGT TGA TCT C-3′. Elongation factor 1 α (*EF-1α*) was used as a reference gene to quantify relative gene expression, and partial sequence was obtained using primers determined from conserved regions of *Carassius auratus* (accession no. AB056104), *D. rerio* (accession no. L23807), *Oncorhynchus mykiss* (accession no. AF498320), and *Oryzias latipes* (accession no. AB013606). The forward primer was 5′-GAA GGA AGC HGC TGA GAT GG-3′, and the reverse primer was 5′-CGG TCT GCC TCA TGT CAC GC-3′. Primers were designed with the assistance of GeneTool Lite software (BioTools, Edmonton, Alberta). Polymerase chain reactions (PCRs) were carried out in a PTC-200 MJ research thermocycler using 1.25 U Taq DNA polymerase (MBI Fermentas) and isolated gill cDNA. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide, and bands of appropriate size were extracted from the gels using the QIAEX II gel extraction kit (Qiagen, Mississauga, Ontario). The extracted PCR product was ligated into a T-vector (pGEM-T easy; Promega; Fisher Scientific, Nepean, Ontario), transformed into heat shock competent *Escherichia coli* (strain JM109; Promega; Fisher Scientific), and colonies were grown on ampicillin LB-agar plates. Several colonies containing the ligated PCR product were selected and grown overnight in double yeast tryptone bacterial growth medium. Plasmids were isolated from the liquid culture using GenElute Plasmid Miniprep kit (Sigma-Aldrich, Oakville, Ontario) and sequenced at the Nucleic Acid Protein Service Unit DNA Sequencing Facility at the University of British Columbia. Sequences were deposited into GenBank as follows: hsp90 (accession no. DQ013311) and EF-1α (accession no. DQ013310).

Gene-specific primers were designed using Primer Express software (ver. 2.0.0; Applied Biosystems, Foster City, CA). Primer sequences were as follows: hsc70 forward 5′-TGA TGC GGT TGT CCA GTC A-3′, hsc70 reverse 5′-CTT TAG GGC GCG TTG CA-3′, hsp70 forward 5′-AGT GGT GCA GGC GGA CAT-3′, hsp70 reverse 5′-TCT TGG GCT TCC CTC CAT CT-3′, hsp90 forward 5′-CGA TGG GCT CCG TCA TGT-3′, hsp90 reverse 5′-TGA GCG CGT CCG TAA GC-3′, EF1α forward 5′-CCC GGA CAC AGG AAC TTC AT-3′, and EF1α reverse 5′-GGC GCA GTC AGC CTG AGA-3′. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence detection system (Applied Biosystems). The qRT-PCR reactions were run with 2 μL cDNA, 4 pmol of each primer, and 2× SYBR Green Master Mix (Applied Biosystems) to a total volume of 22 μL. All qRT-PCR reactions were run as follows: 1 cycle of 50°C for 2 min, 1 cycle of 94°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. At the end of each PCR reaction, PCR products were subjected to a melt curve analysis to confirm the presence of a single amplicon, and representative samples were sequenced to verify that the appropriate gene fragments were amplified.

Statistical Analyses

For Hsc/Hsp70 and Hsp90 protein quantification, band intensities were obtained using SigmaGel software (Jandel Scientific, San Rafael, CA), and values were standardized using band intensity values from a pooled sample of tidepool sculpin gill homogenate that was run concurrently on each gel (referred to as "internal std" in figures). Results for Hsc/Hsp70 and Hsp90 protein are reported as mean ± SEM. To quantify *hsc70*, *hsp70*, and *hsp90* mRNA expression, one control sample was used to develop a standard curve for all primer sets relating threshold cycle to cDNA amount, and this standard curve was run on each qRT-PCR plate. All results were expressed relative to these standard curves, and mRNA values were normalized relative to *EF-1α*. *EF-1α* is a widely used control gene in qRT-PCR analysis, and specifically levels have been shown not to change in response to temperature (Nicot et al. 2005). This was verified in tidepool sculpins when examining data expressed relative to total RNA, and thus this gene is an appropriate internal control. Results for *hsc70*, *hsp70*, and *hsp90* mRNA expression are reported as mean ± SEM. Two-way ANOVA was used to determine significant ($P \leq 0.05$) differences in both Hsp protein and mRNA levels within a treatment group. For the two-way ANOVA, fish group (e.g., cycling temperature, constant temperature) and time of day were used as independent categorical variables, and Hsp protein or mRNA level was used as the dependent variable. Where significant interactions were detected, data were decomposed and one-way ANOVAs were performed as appropriate. Means were compared using the post hoc Student-Newman-Keuls (SNK) multiple comparison test ($P \leq 0.05$). All data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene median test). In cases where these assumptions were not met, values were log transformed, and the statistical analysis was repeated.

Results

Experiment 1: Hsp mRNA and Protein Levels in the Tidepool and Following Acclimation to a Constant Environment

Hsc70 *and* Hsp70. Results of two-way ANOVA on *hsc70* mRNA levels showed that there was a significant effect of group (field-acclimatized vs. 24-h lab-acclimated, $P < 0.001$), no significant effect of time of day, but a significant interaction between group and time of day ($P = 0.034$). In the two-way ANOVA comparisons of *hsp70* mRNA expression, there was a significant effect of group ($P < 0.001$) and time of day ($P < 0.001$) and a significant interaction between group and time of day ($P < 0.001$). There was a significant effect of time of day ($P = 0.005$) on Hsc70 and Hsp70 protein levels; however, there was no significant effect of group or an interaction between the two factors.

There was no significant increase in *hsc70* mRNA levels over the midday low-tide period (comparison of morning and midday levels, field-acclimatized) in sculpins sampled directly from the tidepool; however, *hsc70* mRNA levels were significantly elevated in the evening at the time that corresponded to the onset of the evening low-tide period (field-acclimatized; Fig. 2*A*). When fish were removed from the intertidal zone and held under ambient ocean conditions overnight (24-h lab-acclimated), *hsc70* mRNA levels returned to the basal levels measured in the field-acclimatized sculpins. There was no significant fluctuation in *hsc70* mRNA levels at any of the time points over the next 2 d (24-h lab-acclimated; Fig. 2*A*).

For *hsp70* mRNA levels, at the start of the low-tide period, there was a significant increase at midday compared with levels measured in the morning (field-acclimatized; Fig. 2*B*), and *hsp70* mRNA levels remained elevated into the evening in the field-acclimatized fish. When sculpins were removed from the intertidal zone and held under laboratory conditions, *hsp70* mRNA levels were close to the lower limits of detection. There was a slight but significant increase in *hsp70* mRNA levels over the lab-acclimation period, with levels being significantly elevated in the morning, midday, and evening of day 3.

For Hsc/Hsp70 protein levels there was a significant increase over the midday low-tide period, and these levels remained elevated into the evening (field-acclimatized; Fig. 2*C*). There was no significant change in Hsc/Hsp70 protein levels over the tidal cycle in sculpins that were removed from the tidepool and held under ambient ocean conditions in the laboratory; however, evening Hsc/Hsp70 protein levels in the 24-h lab-acclimated group were significantly lower than in the evening sample of the field-acclimatized group (Fig. 2*C*). Following 1 wk of acclimation to the more stable conditions of the ambient ocean, there were no significant daily changes in *hsc70* mRNA, *hsp70* mRNA, or Hsc/Hsp70 protein levels, and levels were not significantly different from the lowest levels measured in the field-acclimatized group (data not shown).

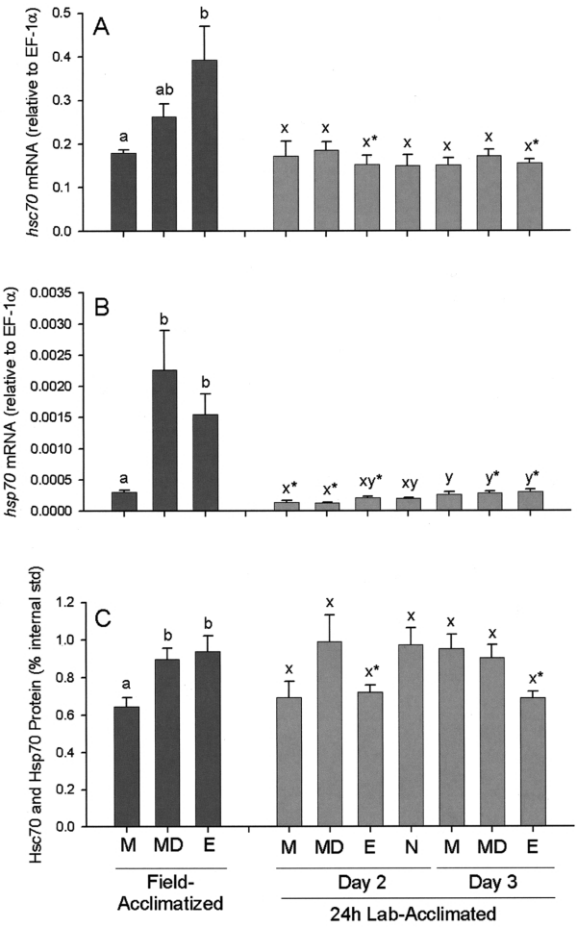

Figure 2. Branchial *hsc70* mRNA (*A*), *hsp70* mRNA (*B*), and Hsc70 and Hsp70 protein levels (*C*) of tidepool sculpins over a midday low-tide period in a mid-intertidal tidepool (field-acclimatized; *solid bars*) or following 24 h of acclimation to constant conditions (11°C, 32 ppt) in the laboratory (24-h lab-acclimated; *gray bars*). Hsp levels in the field-acclimatized fish were monitored at the start and end of the midday low-tide period as well as following high tide at the start of the evening low-tide period. These periods of time corresponded to morning (*M*), midday (*MD*), and evening (*E*), respectively. Hsp levels in the 24-h lab-acclimated fish were measured every 6 h over 2 d (days 2 and 3), and the exact sampling periods corresponded to morning, midday, evening, and night (*N*). *Hsc70* and *hsp70* mRNA levels are normalized to a control gene, *EF-1α* (mean ± SEM). Hsc70 and Hsp70 protein levels are shown as relative values based on band intensities standardized with the level of branchial Hsc70 and Hsp70 in a pooled sample of heat-shocked sculpins (mean ± SEM). Different letters denote significant differences within a group of sculpins (field or lab) over all sampling times ($P \leq 0.05$). An asterisk indicates a significant difference from the field-acclimatized group at a particular time of day ($P \leq 0.05$).

Hsp90. Results of two-way ANOVA showed that there was a significant effect of group (field-acclimatized vs. 24-h lab-acclimated) on both *hsp90* mRNA ($P = 0.002$) and Hsp90 protein levels ($P = 0.008$). There was no effect of time of day, nor

was there any interaction between group and time of day for the two-way ANOVA for either *hsp90* mRNA or Hsp90 protein.

There was no significant increase in branchial *hsp90* mRNA levels over the midday low-tide period; however, *hsp90* mRNA levels were significantly elevated by the start of the evening low-tide period (field-acclimatized; Fig. 3*A*). In the 24-h lab-acclimated group, there were modest fluctuations in *hsp90* mRNA levels, but these were not concordant with tidal cycle (24-h lab-acclimated; Fig. 3*A*). Consistent with this observation of relatively constant hsp90 mRNA levels, the hsp90 sequence that we cloned was most similar to that of the constitutive isoform (hsp90β) of other fish. We found no evidence for the expression of sequences similar to the inducible isoform (hsp90α) under the heat shock conditions employed in these experiments.

There was no significant increase in Hsp90 protein from the morning to the evening in sculpins sampled directly from the tidepool (field-acclimatized; Fig. 3*B*). In the 24-h lab-acclimated group, Hsp90 protein levels were significantly lower in the morning of day 2 compared with the morning in the field-acclimatized group. There was a slow increase in Hsp90 protein levels over the acclimation period such that levels were significantly elevated by midday of day 3 (24-h lab-acclimated; Fig. 3*B*). Hsp90 protein levels returned to basal levels by the evening of day 3. Following 1 wk of acclimation to the constant conditions of the ambient ocean, there was no significant change in *hsp90* mRNA or Hsp90 protein levels over the day, and levels were not significantly different from the lowest levels measured in the field-acclimatized group (data not shown).

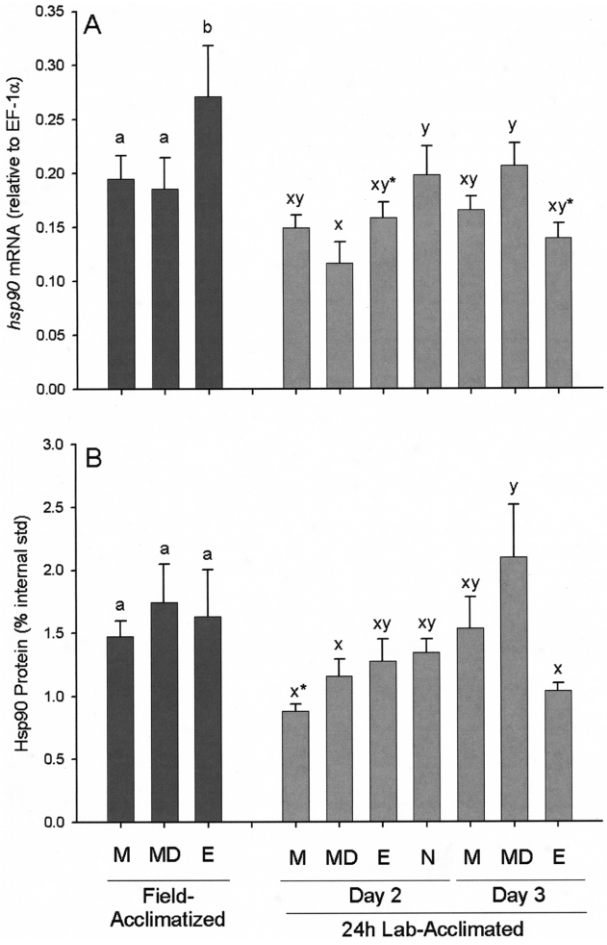

Figure 3. Branchial *hsp90* mRNA (*A*) and Hsp90 protein levels (*B*) of tidepool sculpins over a midday low-tide period in a mid-intertidal tidepool (field-acclimatized; *solid bars*) or following 24 h of acclimation to constant conditions (11°C, 32 ppt) in the laboratory (24-h lab-acclimated; *gray bars*). Hsp levels in the field-acclimatized fish were monitored at the start and end of the midday low-tide period as well as following high tide at the start of the evening low-tide period. These periods of time corresponded to morning (*M*), midday (*MD*), and evening (*E*), respectively. Hsp levels in the 24-h lab-acclimated fish were measured every 6 h over 2 d (days 2 and 3), and the exact sampling periods corresponded to morning, midday, evening, and night (*N*). *Hsp90* mRNA level is normalized to a control gene, *EF-1α* (mean ± SEM). Hsp90 protein levels are shown as relative values based on band intensities standardized with the level of branchial Hsp90 in a pooled sample of heat-shocked sculpins (mean ± SEM). Different letters denote significant differences within a group of sculpins (field or lab) over all sampling times ($P \leq 0.05$). An asterisk indicates a significant difference from the field-acclimatized group at a particular time of day ($P \leq 0.05$).

Experiment 2: Effects of a Cycling Temperature Regime on Hsp mRNA Expression

Results of two-way ANOVA on *hsc70* mRNA levels showed that there was a significant effect of group (cycling vs. constant temperature, $P < 0.001$), a significant effect of time of day ($P = 0.05$), and no significant interaction between group and time of day. In the two-way ANOVA of *hsp70* mRNA levels, there was a significant effect of group ($P < 0.001$) and time of day ($P < 0.001$) and a significant interaction between group and time of day ($P < 0.001$). There was a significant effect of group ($P = 0.021$) on *hsp90* mRNA levels; however, there was no significant effect of time of day or an interaction between the two factors.

Hsc70. Two weeks of acclimation to a cycling temperature regime (11°C ↔ 22°C) resulted in a modest but significant rhythm in branchial *hsc70* mRNA levels that cycled in phase with peak temperatures (Fig. 4*A*). The lowest levels of *hsc70* mRNA were measured in the evening immediately before the increase in water temperature, when water temperatures were at 11°C, whereas peak levels in *hsc70* mRNA were measured when maximum water temperatures were reached. Acclimation of sculpins to a constant temperature regime of 11°C for 2 wk resulted in no significant daily fluctuations in *hsc70* mRNA levels; however, overall *hsc70* mRNA levels were significantly higher than levels measured in the cycling temperature group in four of the seven sampling points (Fig. 4*B*).

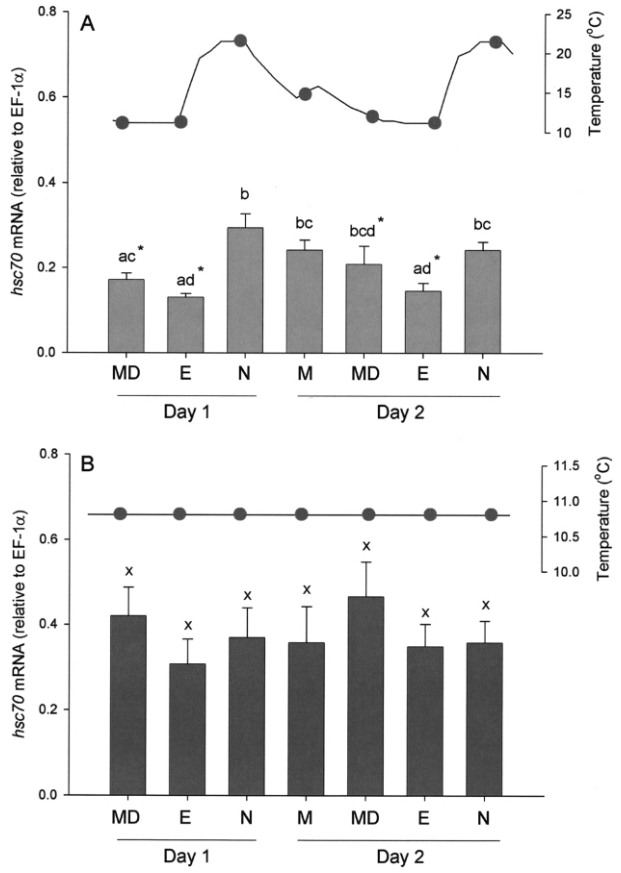

Figure 4. Branchial *hsc70* mRNA levels of tidepool sculpins acclimated for 2 wk to either a cycling temperature regime (*A*; 10.8° → 22°C, 32 ppt) that mimics a mid-intertidal tidepool or a constant temperature regime (*B*; 10.8°C, 32 ppt). Transcript level was monitored every 6 h over 2 d (days 1 and 2), and the exact sampling periods corresponded to morning (*M*), midday (*MD*), evening (*E*), and night (*N*). *Hsc70* mRNA level is normalized to a control gene, *EF-1α* (mean ± SEM). Different letters denote significant differences within a group of sculpins (cycling or constant temperature regime) over all sampling times ($P \leq 0.05$). An asterisk indicates a significant difference from the constant cycling regime group within a single sampling time ($P \leq 0.05$).

Hsp70. *Hsp70* mRNA levels peaked with peak water temperature in sculpins inhabiting the cycling temperature regime (Fig. 5*A*). At the time of peak water temperature, there was a 200-fold induction of *hsp70* mRNA in the cycling temperature group, whereas at all other sampling points, *hsp70* mRNA levels were barely above detection limits. Sculpins inhabiting the constant temperature regime did not experience significant changes in *hsp70* mRNA levels throughout the day, but *hsp70* mRNA levels in the constant temperature group were significantly elevated above what was measured in the cycling temperature group, except at the times of peak induction (Fig. 5*B*).

Hsp90. There was no significant daily fluctuation in *hsp90* mRNA levels in either the cycling or constant temperature groups (Fig. 6). In general, there was no significant difference in the *hsp90* mRNA levels between the cycling and the constant temperature groups; however, when peak temperatures were reached on day 1 of sampling in the cycling temperature group, *hsp90* mRNA levels were significantly elevated above the same sampling point in the constant temperature group.

Comparison of the Induction Profiles of Hsp Gene Expression within the Tidepool versus within a Cycling Temperature Regime

There were a number of significant differences in the induction profiles of *hsc70*, *hsp70*, and *hsp90* mRNA expression in re-

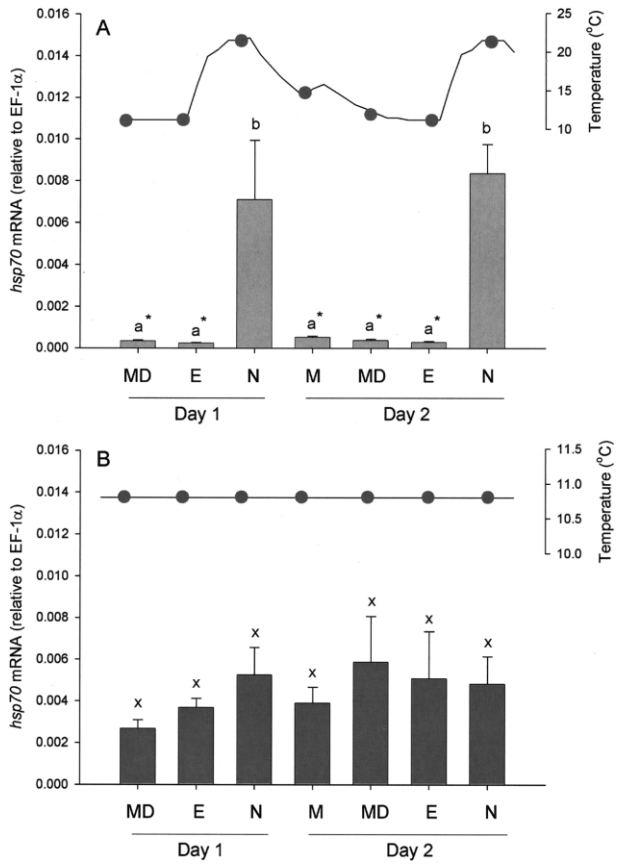

Figure 5. Branchial *hsp70* mRNA levels of tidepool sculpins acclimated for 2 wk to either a cycling temperature regime (*A*; 10.8° → 22°C, 32 ppt) that mimics a mid-intertidal tidepool or a constant temperature regime (*B*; 10.8°C, 32 ppt). Transcript level was monitored every 6 h over 2 d (days 1 and 2), and the exact sampling periods corresponded to morning (*M*), midday (*MD*), evening (*E*), and night (*N*). *Hsp70* mRNA level is normalized to a control gene, *EF-1α* (mean ± SEM). Different letters denote significant differences within a group of sculpins (cycling or constant temperature regime) over all sampling times ($P \leq 0.05$). An asterisk indicates a significant difference from the constant cycling regime group within a single sampling time ($P \leq 0.05$).

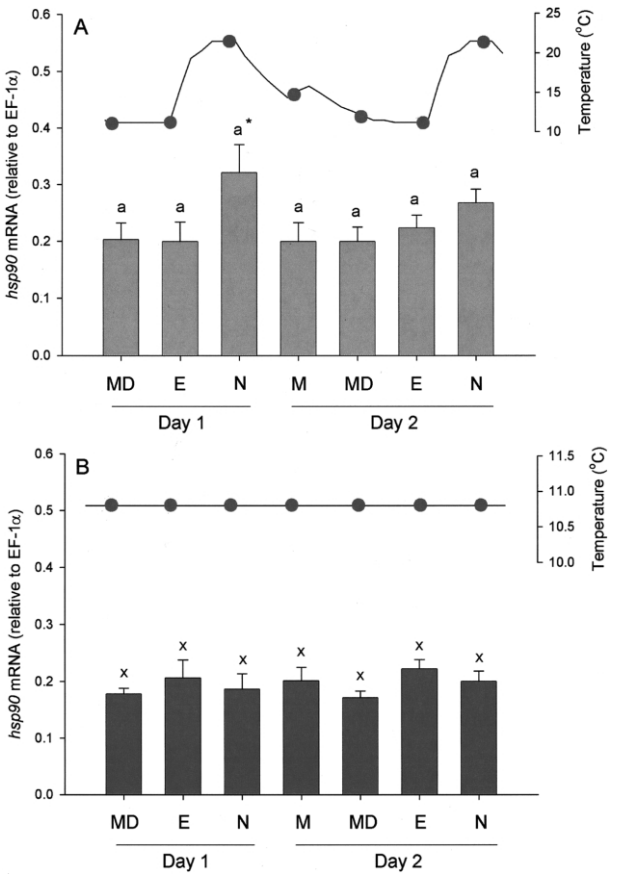

Figure 6. Branchial *hsp90* mRNA levels of tidepool sculpins acclimated for 2 wk to either a cycling temperature regime (*A*; 10.8°→22°C, 32 ppt) that mimics a mid-intertidal tidepool or a constant temperature regime (*B*; 10.8°C, 32 ppt). Transcript level was monitored every 6 h over 2 d (days 1 and 2), and the exact sampling periods corresponded to morning (*M*), midday (*MD*), evening (*E*), and night (*N*). *Hsp90* mRNA level is normalized to a control gene, *EF-1α* (mean ± SEM). Different letters denote significant differences within a group of sculpins (cycling or constant temperature regime) over all sampling times ($P \leq 0.05$). An asterisk indicates a significant difference from the constant cycling regime group within a single sampling time ($P \leq 0.05$).

sponse to natural changes in environmental conditions within the tidepool when compared with the response to an artificially controlled temperature cycling regime that mimicked daily temperature fluctuations of a mid-intertidal tidepool (Fig. 7). Within the tidepool-acclimatized group, *hsc70* mRNA levels were not significantly elevated until 6 h following peak tidepool temperature, whereas under the cycling temperature regime, *hsc70* mRNA levels were significantly elevated when peak temperatures were reached and remained elevated for an additional 6 h. *Hsp70* mRNA levels were significantly elevated in association with peak temperature in both groups of sculpins; however, induction was much greater in the cycling temperature group, while it was longer lasting in the tidepool-acclimatized group. There was no significant change in *hsp90* mRNA levels in the cycling temperature–acclimated sculpins, although there was a modest increase coincident with peak temperatures. In contrast, in the tidepool-acclimatized group, *hsp90* mRNA levels were significantly elevated but not until 6 h following peak tidepool temperature.

Discussion

The data presented here demonstrate that there is no endogenous diurnal rhythm in the Hsp response entrained to the tidal cycle in the tidepool sculpin; rather, both Hsp gene expression and protein levels are increased in response to the

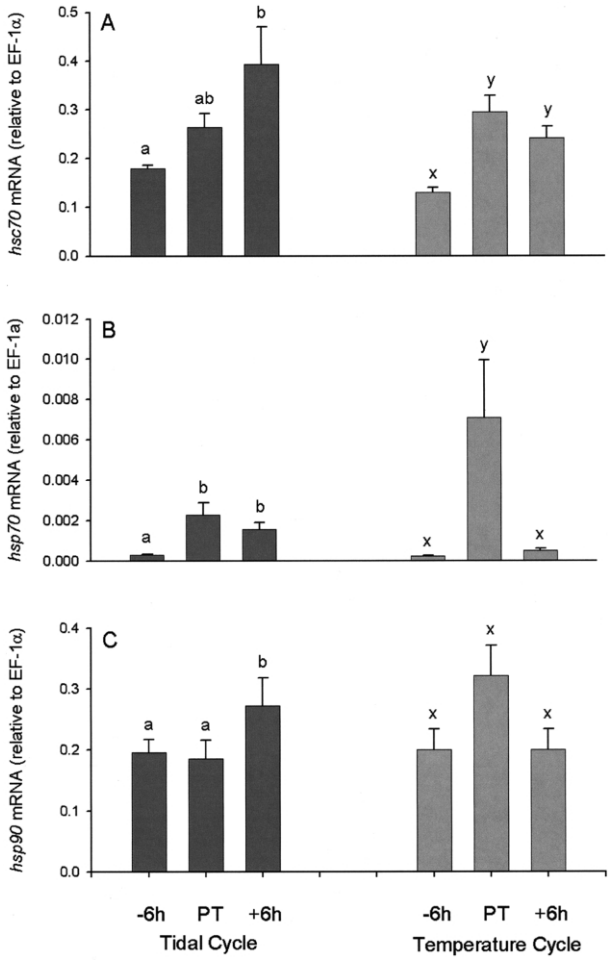

Figure 7. Comparison of the hsc70 (*A*), hsp70 (*B*), and hsp90 (*C*) mRNA profiles in response to temperature fluctuations in the natural tidepool (tidal cycle) and in an artificially manipulated temperature cycling regime (temperature cycle). Hsp mRNA levels are compared 6 h before peak temperature (−6 h), at peak temperature (*PT*), and 6 h following peak temperature (+6 h). Hsp mRNA levels are normalized to a control gene, EF-1α (mean ± SEM). Different letters denote significant differences within a group of sculpins (tidal cycle or temperature cycle) over all sampling times ($P \leq 0.05$).

environmental change that occurs over a midday low-tide period. Once sculpins are removed from this fluctuating environment and are held under constant ambient ocean conditions in a laboratory setting, Hsp mRNA and protein levels return to basal levels. The lack of any endogenous tidal rhythm in Hsp levels in the tidepool sculpins suggests that it is the unpredictable nature of the low-tide periods that ultimately structures the Hsp response such that it can be rapidly regulated and tailored to the immediate environmental conditions rather than the predictable timing of the tides. The Hsp response is costly in terms of both the energetic cost of synthesizing these proteins and the biochemical cost to the cell as a consequence of Hsps being preferentially synthesized at the expense of other cellular proteins (Lindquist and Petersen 1990; Krebs and Feder 1998). Therefore, it may not be a cost-effective strategy to entrain endogenous Hsp levels to the predictable diurnal cycle of the environmental change inherent of the intertidal zone when it is the unpredictable nature of this environmental change that has the greatest impact on an organism's physiology.

There has been very little work addressing the nature of Hsp expression and protein levels in organisms inhabiting a cyclic environment. From the results of our study, it is apparent that fluctuations in the tidepool environment can induce increased *hsc70*, *hsp70*, and *hsp90* mRNA levels and that this translated into increased Hsc/Hsp70 and Hsp90 protein levels. Since Hsp levels in this study were measured over only a single tidal cycle, we cannot say conclusively that this pattern of Hsp expression was repeatable over multiple tidal cycles or simply a response to a single exposure that was particularly stressful. However, the change in temperature the sculpins experienced over our sampling period was typical of a summer midday low-tide period, suggesting these fish routinely experience such fluctuations in Hsp expression. By exposing sculpins to an artificial temperature cycling regime, we have clearly demonstrated that temperature can regulate the Hsc/Hsp70 response in sculpins such that *hsc70* mRNA levels cycled in phase with peak temperature and *hsp70* mRNA was induced at peak temperature. In contrast, *hsp90* mRNA did not vary significantly in response to our thermal cycling regime. Taken together, these results provide novel insights into the complex nature of the environmental regulation of the Hsp response and the variation in significance of temperature in modulating different *hsp* genes. Podrabsky and Somero (2004) also examined the changes in *hsp* gene expression associated with daily temperature changes over a 2-wk period in the annual killifish *Austrofundulus limnaeus* under laboratory conditions but found no strong temperature-dependent cyclic patterns of gene expression. Instead, these researchers observed a significant induction of *hsp22*, *hsp27*, *hsc70*, and *hsp90* on transfer of these killifish into a temperature cycling environment, but the transcript levels of these chaperones returned to control levels within 2 wk, and no cycling was observed. In contrast, we still observed cycling of *hsc70* and *hsp70* mRNA levels in response to temperature cycling after 2 wk of acclimation in tidepool sculpins. It is unclear why a temperature cycling environment has different effects on the rhythmicity of the Hsp response of two eurythermal fish inhabiting highly variable environments, but it may reflect differences in the life-history strategies of these fish.

Within the tidepool, sculpins rapidly increased *hsp70* mRNA levels during a low-tide period, and *hsc70* and *hsp90* mRNA were significantly elevated within hours of the onset of high tide. These changes in gene expression were reflected at the protein level for Hsc/Hsp70 but not for Hsp90. Consistent with our data, previous work on intertidal invertebrates has demonstrated that these organisms have the capacity to regulate the Hsp response within the time frame of a single tidal cycle; however, these organisms induced the Hsp response and other protein repair mechanisms only once they were submerged by high tide (Hofmann and Somero 1996; Tomanek and Somero 2000; Schill et al. 2002). This is in contrast to the change in *hsp70* mRNA and Hsc/Hsp70 protein levels that occurred during the low-tide period in sculpins. During low-tide, sculpins are actively foraging in the tidepool, while many intertidal invertebrates, such as bivalves, gastropods, and polyplacophorans, are relatively inactive in order to minimize the impact of exposure to aerial conditions. Therefore, the differences in the kinetics of the Hsp response between these organisms may reflect differences in strategies used to tolerate the particular fluctuations in abiotic conditions encountered during low tide. In a poikilotherm that is active within a variable environment, the capacity to rapidly adjust Hsp levels in concert with changes in environmental conditions would permit maximization of the benefits of the Hsp response while minimizing overexpression of Hsps, which is known to impair normal cellular function. The exact mechanisms underlying how this is achieved in the tidepool sculpin remains to be elucidated; however, from the results of this study, one can begin to address the role of intrinsic as well as environmental regulation of Hsp expression in this eurythermal tidepool fish.

By comparing the expression profiles of different Hsps within a particular environment (e.g., within the tidepool), we are able to gain some insight into the factors affecting the Hsp response. There were some significant differences in the expression profiles between *hsc70*, *hsp70*, and *hsp90* within the tidepool as well as within the cycling temperature environment. Within the tidepool, *hsp70* mRNA levels increased rapidly and significantly during a low-tide exposure, whereas *hsc70* mRNA levels increased more gradually and were not significantly elevated until after the onset of high tide. In contrast, *hsp90* mRNA levels remained low during low-tide exposure and were significantly elevated only once high tide had occurred. Similarly, within the temperature cycling environment, *hsp70* mRNA expression showed a distinct on/off phenomenon in sync with peak temperatures, *hsc70* cycled mildly in phase with temperature, and there was no significant change in *hsp90* mRNA in response to temperature cycling. These dissimilarities in gene expression in

response to the same environmental conditions likely reflect differences in the mechanisms of Hsp regulation among *hsp* genes and may be a consequence of their differing roles as chaperones (Picard 2002; Mayer and Bukau 2005). There has been significant work investigating the transcriptional regulation of inducible *hsp* genes by heat shock transcription factor 1 (HSF1) in intertidal organisms, and these studies have demonstrated that there exists a certain degree of plasticity in the activation temperature of HSF1 (Buckley et al. 2001; Buckley and Hofmann 2002, 2004; Tomanek and Somero 2002; Lund et al. 2006). However, because of the differences in *hsp70* and *hsp90* mRNA expression over a low-tide period in the tidepool sculpins, it is clear that there are additional mechanisms that are important in transcriptional regulation of these inducible *hsp* genes. In *Drosophila melanogaster*, the 3′ and 5′ sequences of *hsc70* and *hsp70* are known to share little homology, and this has been shown to be partially responsible for their independent patterns of regulation (Lindquist and Petersen 1990). Although the full gene sequences of *hsc70* and *hsp70* are not available for tidepool sculpins, it is likely that similar mechanisms are involved. It is noteworthy that despite observing a significant increase in *hsp90* mRNA levels, there was no corresponding increase in Hsp90 at the protein level, suggesting that our sampling protocol may have missed the peak in Hsp90 protein, that Hsp90 protein turnover was relatively rapid, or that there are important posttranscriptional mechanisms of regulation of the Hsp90 response of the tidepool sculpin. This disparity between mRNA expression and protein induction of Hsp90 requires further investigation.

Through comparison of the gene expression profile of a particular *hsp* gene in response to the two different fluctuating environments in this study (tidepool vs. laboratory-induced temperature cycling regime), we have been able to address some of the environmental factors that affect its Hsp response. It is clear from this study that temperature has an important and direct role in structuring the Hsp response, particularly the Hsc/Hsp70 response, of organisms inhabiting a variable environment. However, the *hsc70*, *hsp70*, and *hsp90* induction profiles of sculpins in response to either the natural cycle of environmental change of a tidepool or a change in temperature that mimicked what was occurring in the tidepool were not identical (Fig. 7). These Hsp gene expression profiles differed in both kinetics and magnitude and therefore suggest that factors in addition to temperature in a sculpin's natural environment shape its Hsp response. Within the tidepool environment, there are significant daily fluctuations in both dissolved oxygen levels and salinity, and variability in these factors may integrate with temperature to regulate Hsp expression in the intertidal zone. In addition, the artificial temperature cycling regime that the sculpins were acclimated to in this study was somewhat larger than that experienced on the day of sampling in the field and on a reversed 12L : 12D photoperiod, which was very different from the natural photoperiod of these fish during the summer months. Because photoperiod is known to have a significant effect on physiological processes (Weld and Meier 1983; Filadelfi and Castrucci 1996; Frick and Wright 2002), it too may have a role in structuring Hsp gene expression in nature. Alternatively, the fish used for laboratory experiments were somewhat smaller and collected in the fall rather than the summer, and it is possible that age or seasonal effects could influence these *hsp* expression patterns. It is also interesting to note that fish held under a constant temperature regime that mimicked ambient ocean conditions had significantly higher levels of *hsc70* and *hsp70* mRNA when compared with fish in the temperature cycling regime at many of the sampling points. Rarely in their natural habitat would tidepool sculpins encounter absolutely constant conditions, and the high levels of mRNA in this group of fish suggest the possibility that this constant environment represents a stressor to sculpins. Finally, it should be noted that the magnitude and timing of the temperature fluctuations in the tidepool environment were much less consistent than the temperature cycling regime used in the lab, in which temperature cycled in an identical manner every day. Therefore, although there was no endogenous rhythm in Hsp levels entrained to tidal cycle, the differences in the timing of induction of *hsp* gene expression in relation to peak temperatures between the tidepool and the laboratory suggest that the complex combination of stressors in nature and the unpredictability of stressor magnitude are important for shaping patterns of *hsp* gene expression.

Conclusions

It has been well documented that thermal history plays a critical role in structuring the Hsp response of a wide variety of intertidal organisms. It has been unclear, however, how tightly environmental temperature regulates the Hsp response of these animals in their natural environment and whether the predictability of environmental change in the intertidal environment has entrained an endogenous rhythmicity to Hsp levels. The results of this study provide novel insights into our understanding of the environmental regulation of the Hsp response of intertidal organisms. It is clear that there is no endogenous tidal rhythm in Hsp mRNA or protein levels in the tidepool sculpin in nature. Rather, the rapid changes in Hsp gene expression that we observed within the tidepool were a direct response to fluctuations in environmental conditions and did not persist if the organism was removed from the intertidal environment. Fluctuations in environmental temperature have a critical role in regulating Hsp gene expression; however, there are additional extrinsic factors that likely integrated with temperature and resulted in the Hsp expression profiles that were observed in sculpins inhabiting their natural environment. To truly appreciate the significance and the nature of environmental regulation of the Hsp response, it is essential that we examine organisms within their natural environment and take

advantage of the wide variety of environments available for study that can allow us to isolate the particular extrinsic factors involved in this regulation.

Acknowledgments

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