Cross-Tolerance in the Tidepool Sculpin: The Role of Heat Shock Proteins

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ABSTRACT

Cross-tolerance, or the ability of one stressor to transiently increase tolerance to a second heterologous stressor, is thought to involve the induction of heat shock proteins (Hsp). We thus investigated the boundaries of cross-tolerance in tidepool sculpins (*Oligocottus maculosus*) and their relationship to Hsp70 levels. Survival of sculpins exposed to severe osmotic (90 ppt, 2 h) and hypoxic (0.33 mg O_2/L, 2 h) stressors increased from 68% to 96%, and from 47% to 76%, respectively, following a +12°C heat shock. The magnitude of this heat shock was critical for protection. A +10°C heat shock did not confer cross-tolerance, while a +15°C heat shock was deleterious. Sculpins required between 8 and 48 h of recovery following the +12°C heat shock to develop cross-tolerance. There was no association between Hsp70 levels before the onset of the secondary stressor and cross-tolerance. However, branchial Hsp70 levels following osmotic shock were highly correlated with the time frame of cross-tolerance. Thus, Hsp70 induction by the priming stressor may be less important than the ability of the cell to mount an Hsp response to subsequent stressors. The time frame of cross-tolerance is similar to the interval between low tides, suggesting the possible relevance of this response in nature.

Introduction

Cross-tolerance, also known as cross protection, is the ability of one stressor to transiently increase the resistance of an organism to a subsequent stressor of a different nature (Kampinga et al. 1995; Sabehat et al. 1998). Li and Hahn (1978) were among the first researchers to document that cultured mammalian cells, preconditioned by exposure to a sublethal heat stress, acquire greater resistance to subsequent heat and chemical exposure. Studies in fish have shown that a mild heat shock can increase the tolerance of cells to subsequent thermal (*Oncorhynchus mykiss* RTG-2 cells; Mosser et al. 1987), chemical (*Pleuronectes americanus* renal epithelia cells; Brown et al. 1992; Renfro et al. 1993), osmotic (*Salmo salar*; DuBeau et al. 1998), and acid challenges (Martin et al. 1998). Studies conducted in model systems have expanded our understanding of the boundaries of stress-induced tolerance (Henle and Leeper 1976; Mosser et al. 1987; Hahn and Li 1990; Krebs and Feder 1998). In general, increasing the magnitude or duration of the preliminary stressor increases subsequent stress tolerance; however, once the preliminary stressor reaches a certain magnitude, organisms require a period of recovery between the two stressors for stress tolerance to develop. In addition, there appears to be substantial variation among organisms in the timing and duration of the response, although the phenomenon of cross-tolerance appears to be present in a diverse array of organisms (Lee and Hahn 1988; Krebs and Feder 1998).

Although the mechanisms underlying cross-tolerance are not fully understood, heat shock proteins (Hsps), particularly Hsp70, are thought to be involved. Heat shock proteins are an important component of the cellular stress response and play a critical role in the recovery of cells from stress (for review, see Lindquist 1986; Hightower 1991; Morimoto 1998; Feder and Hofmann 1999; for review in fishes, see Iwama et al. 1998; Basu et al. 2002). The cytoprotection afforded by the induction of Hsps is thought to accrue from their function as molecular chaperones in maintaining the integrity of the cellular protein pool (Parsell and Lindquist 1993; Sherman and Goldberg 1996; Hartl and Hayer-Hartl 2002). Studies in rainbow trout (*O. mykiss*) fibroblasts have shown that the appearance and decay of Hsps correlates with the induction and disappearance of thermotolerance (Mosser et al. 1987; Mosser and Bols 1988). In these studies, inhibition of Hsp synthesis by administration of actinomycin D prevented the development of thermotolerance, suggesting that Hsps are crucial mediators of this tolerance. Studies conducted in bacteria and plants provide evidence that stressors, such as high temperature, that induce a more generalized cellular stress response (i.e., a larger suite of Hsps) are more capable of conferring cross-tolerance than stressors that induce a more specific cellular stress response, such as

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heavy metals (Flahaut et al. 1996; Laplace et al. 1996; Sabehat et al. 1998). Hsps have also been implicated as mediators of cross-tolerance in fish. In the renal tissues of winter flounder (*P. americanus*), induction of Hsp28, Hsp70, and Hsp90 coincided with the protection of sulphate transport against the deleterious effects of a heat and chemical stressor (Brown et al. 1992). DuBeau et al. (1998) reported that heat-shocked Atlantic salmon (*S. salar*) with elevated levels of branchial and hepatic Hsp70 were better able to tolerate an osmotic challenge. Collectively, these studies suggest an important role for Hsps in cross-tolerance in fish; however, what sets the limits on cross-tolerance in fish and how Hsps relate to these parameters has yet to be examined.

The induction of Hsps is thought to have adaptive significance for organisms faced with environmental change (Feder and Hofmann 1999). However, most studies that have investigated the functional significance of Hsps in stress-induced tolerance from an environmental perspective have focused on thermotolerance (Hofmann and Somero 1996; Hofmann 1999; Tomanek and Somero 1999; Buckley et al. 2001; Nakano and Iwama 2002), with little attention to cross-tolerance. In natural environments, organisms seldom experience a single stressor; more commonly they experience multiple stressors simultaneously or in sequence. Therefore, it is possible that cross-tolerance is a critical feature of the cellular stress response in nature. The intertidal zone offers a particularly good environmental system in which to investigate the effects of short-term changes in environmental condition on the cellular response to stress and stress tolerance. This habitat is characterized by rapid changes in temperature, salinity, and oxygen that occur daily to an unpredictable degree with each tidal cycle, and therefore cross-tolerance could play a role in protecting fish from one low tide period to the next. Tidepool sculpins (*Oligocottus maculosus*) are widely distributed throughout the intertidal zones of the Pacific Northwest, most densely populating tidepools in the upper mid-intertidal region, where they experience dramatic daily changes in water quality (Green 1971). On a typical summer day, they experience temperature, salinity, and dissolved oxygen fluctuations as large as 11°–25°C, 21–37 ppt, and 1.5–19 mg O_2/L, respectively (A. E. Todgham, unpublished observations). Thus, this eurythermal and euryhaline tidepool fish is an excellent organism in which to investigate the extent of the phenomenon of cross-tolerance in fish, lending itself well to future studies to determine whether cross-tolerance is part of the adaptive mechanisms allowing these fish to thrive in such a variable and unpredictable habitat.

To determine if cross-tolerance is an important phenomenon in nature, it is important to first understand the extent of the preliminary stressor required to induce cross-tolerance and the duration of this increase in stress tolerance. Thus, the main goal of this study was to determine the limits of cross-tolerance in the tidepool sculpin and to examine the relationship between these boundaries and Hsp70 induction. To address this goal, we first determined whether a mild heat shock could increase the stress tolerance of tidepool sculpins to a subsequent more severe stressor of a different nature (osmotic shock and hypoxia). In addition, we investigated the magnitude of heat shock required to confer cross-tolerance and the recovery time needed following heat shock for cross-tolerance to develop. Third, we investigated whether an increase in Hsp70 concentration caused by the initial heat shock was necessary for increased stress tolerance to a subsequent stressor. Finally, we examined the relationship that these features of cross-tolerance have to the magnitude and periodicity of environmental change that characterizes this animal's natural habitat.

Material and Methods

Fish Collection in the Field

Tidepool sculpins (2.96 ± 0.3 g, 59.6 ± 1.7 mm) were collected using dip nets from tidepools on Wizard Rocks in Barkley Sound, Bamfield, British Columbia, Canada, during July 1999 and September 2000 and transferred to outdoor flow-through stock tanks at the Bamfield Marine Sciences Centre. Fish were held at 10°C and 32 ppt under natural photoperiod for 2 wk before experimentation. Fish were fed blue mussels, presented by cracking the shells, ad lib. daily. Feeding was stopped 48 h before experimentation. All experiments were conducted in accordance with an approved University of British Columbia Animal Care protocol (A01-0172).

Experimental Protocol

Two separate series of experiments were run to investigate cross-tolerance in the tidepool sculpin. The first set of experiments was conducted in July 1999 and was designed to determine (1) if a +12°C heat shock could confer cross-tolerance to a severe osmotic shock, (2) if the degree of cross-tolerance to osmotic shock is sensitive to the magnitude of the preliminary heat shock, and (3) if a +12°C heat shock could also confer cross-tolerance to severe hypoxia. The second experiment was conducted the following year (September 2000) and was designed to examine the length of time required at ambient temperature following a +12°C heat shock to provide cross-tolerance against a subsequent osmotic shock.

Experimental Series 1: Cross-Tolerance (July 1999)

Fish were netted from the outdoor stock tanks and transferred to indoor holding tanks, where they were held for 48 h. At this point, fish were randomly divided into four groups (of 32 fish each) and placed in 10-L aquaria and allowed a further 48-h acclimation period. The experimental protocol is outlined in Figure 1. At time zero, eight fish were randomly sampled from the four experimental tanks as a time 0 control. Fish were then transferred to similar tanks either at 22°C (12°C above ambient)

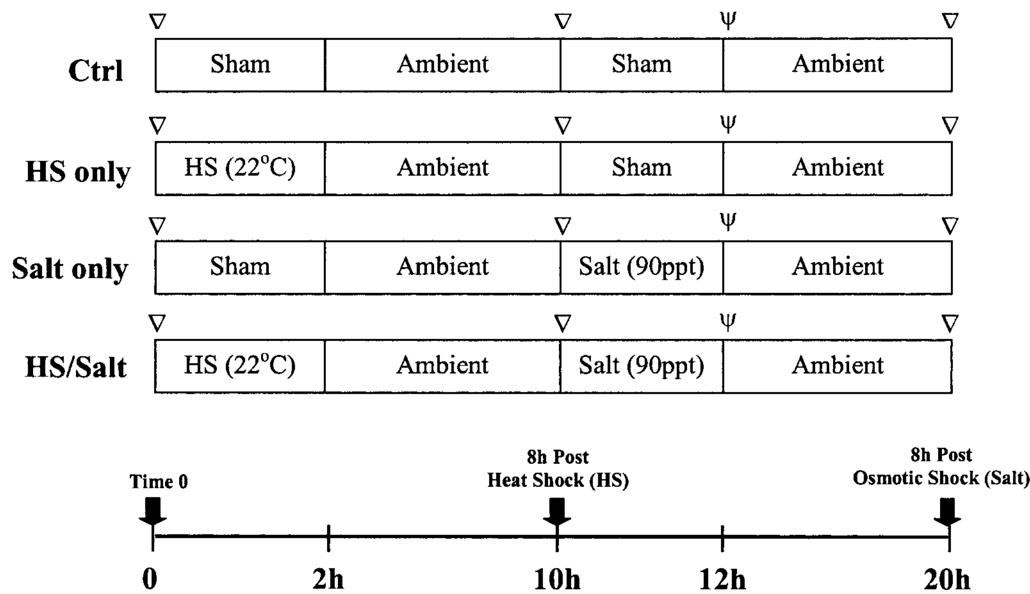

Figure 1. Experimental protocol for experimental series 1 (cross-tolerance) in tidepool sculpins. ∇ indicates sampling points for each treatment; ψ indicates when survival was assessed.

or at ambient temperature (10°C). After 2 h, all fish were transferred to tanks at ambient temperature (10°C). Eight hours later (10 h after the onset of the experiment), fish were transferred to similar tanks at either 90 ppt (salt) or 32 ppt (sham). Fish were held under these conditions for 2 h and then transferred back to tanks at ambient conditions (10°C, 32 ppt). This design resulted in four different experimental groups: control (ctrl; 10°C, 32 ppt), HS (heat shock) only (22°C, 32 ppt), salt only (10°C, 90 ppt), and HS/salt (22°C, 90 ppt). Eight fish were sampled from each tank at 10 h and 20 h, and morbidity was assessed immediately following osmotic shock (12 h, noted as ψ in Fig. 1). We chose this abrupt transfer protocol coupled with an extreme second stressor in order to best control the magnitude of heat shock, to maximize the heat shock response, and to push the mechanisms underlying cross-tolerance to their limits.

To assess the magnitude of the heat shock required to confer cross-tolerance, two additional experiments were performed. The experimental design was identical to that shown in Figure 1 except that fish were exposed to a 2-h 20°C or 25°C heat shock (+10°C and +15°C above ambient, respectively).

To assess whether a +12°C heat shock could confer cross-tolerance to stressors other than osmotic shock, an experiment was performed in which fish were exposed to hypoxia rather than the osmotic shock. The experimental design was as shown in Figure 1 except that fish were exposed to 4% air saturation (0.33 mg O_2/L) for 2 h as the second stressor. Hypoxia was achieved by bubbling N_2 into experimental tanks. The water surface was covered with plastic wrap to minimize aquatic surface respiration. N_2 flow was monitored throughout the experimental period to ensure that O_2 levels remained constant. O_2 levels were monitored with a Handy MK III OxyGuard probe (Point Four Systems, Richmond, British Columbia, Canada).

Experimental Series 2: Time Frame of Cross-Tolerance (September 2000)

To determine the recovery time at ambient temperature following a +12°C heat shock required to confer cross-tolerance, an additional experiment was conducted in September 2000. This experimental protocol was very similar to that described for experimental series 1, with the initial stressor being a +12°C heat shock (22°C absolute temperature) and the subsequent severe stressor being an osmotic stress of 85 ppt (in September 2000, tidepool sculpins could not tolerate 90 ppt). This experiment differed from the 1999 experiments in the amount of time the fish were allowed to recover from the initial heat shock before exposure to the osmotic shock. The experimental protocol is outlined in Figure 2. Fish were randomly divided into four experimental groups: control (10°C, 32 ppt), HS only (22°C, 32 ppt), salt only (10°C, 85 ppt), and HS/salt (22°C, 85 ppt). As shown in Figure 2, this experimental design resulted in seven groups of fish exposed to both a heat shock and an osmotic shock, each with different periods of time at ambient conditions between exposures to the two stressors. Fish exposed to the mild heat shock were either directly transferred into similar tanks at 85 ppt and held under these conditions for 2 h (0 h, no recovery time) or returned to ambient conditions for 4, 6, 8, 12, 24, or 48 h (32 fish in each group) before being transferred to tanks at 85 ppt for 2 h. Fish were then transferred

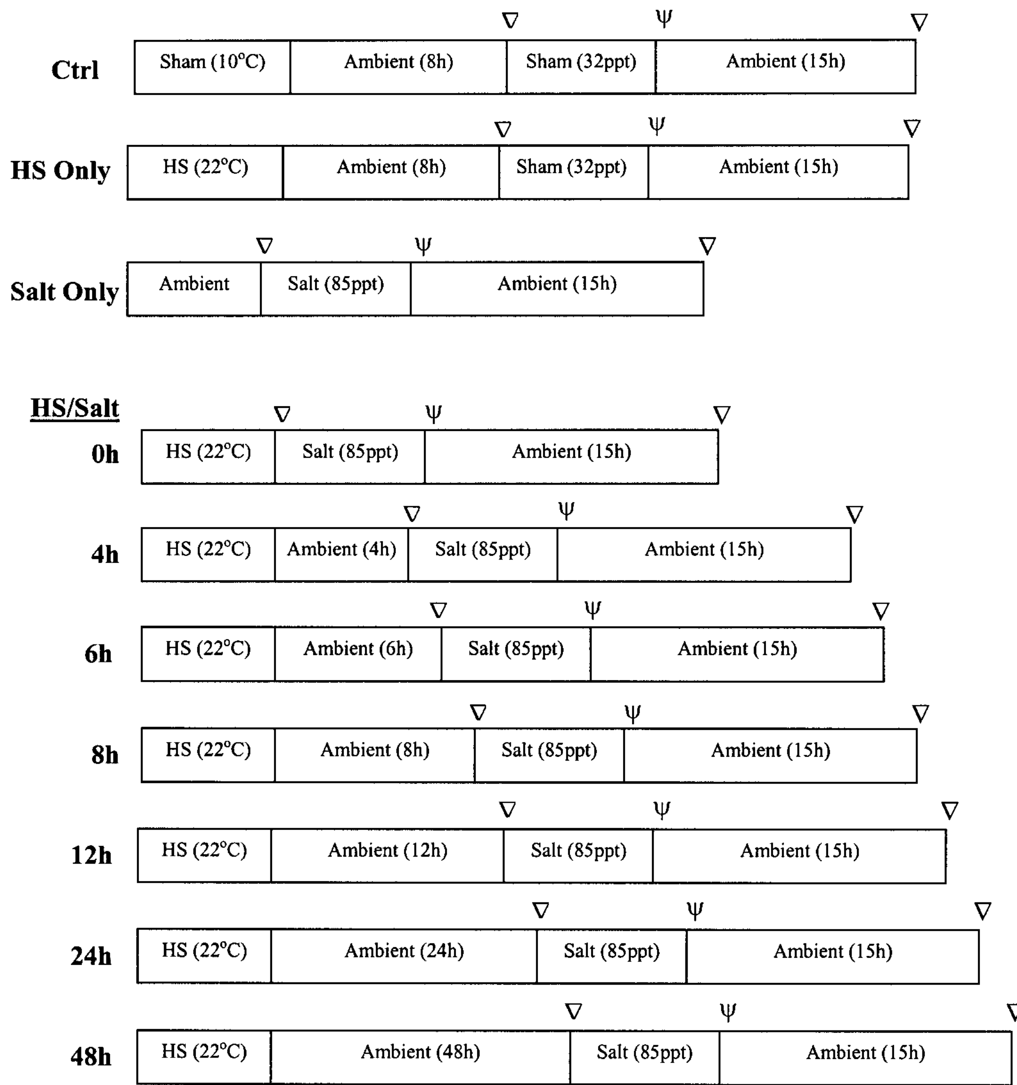

Figure 2. Experimental protocol for experimental series 2 (time frame of cross-tolerance). Times noted on the left indicate the amount of time the tidepool sculpins were returned to ambient conditions (10°C, 32 ppt) between exposure to a heat shock (*HS*; 2 h, 22°C) and exposure to a severe hyperosmotic challenge (2 h, 85 ppt). ∇ indicates sampling points for each treatment; ψ indicates when survival was assessed.

back to tanks at ambient conditions (10°C, 32 ppt) for 15 h. Eight fish were sampled from each group immediately before exposure to the osmotic shock (85 ppt) and 15 h following recovery from the osmotic shock (noted as ∇ on Fig. 2). Morbidity was assessed immediately following exposure to the osmotic shock (noted as ψ on Fig. 2).

Tissue Sampling

Fish were netted and rapidly anesthetized with a high dose of MS-222 (0.29 g MS-222/L of water), and following onset of anaesthesia, the spinal cord was severed. In July 1999, the liver was then rapidly excised, snap-frozen on dry ice, and stored at −80°C until further analysis. On analysis of these samples and with the development of subsequent experiments, we decided that in September 2000 gill tissues should also be sampled to discern differences in the heat shock response between an internal tissue and one that is closely associated with the external environment and that has an important role in ion regulation and oxygen uptake.

Sample Treatments for SDS-PAGE and Protein Analysis

Tissue samples were dispersed by sonication (Vibra Cell, Sonic and Materials) 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 11,600 *g* 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 it was 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 Hsp70

Levels of Hsp70 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 (Gibco-BRL, Burlington, Ontario, Canada) and liver homogenate samples from arsenite-induced coho salmon or gill homogenate from tidepool sculpins were added to every gel as an internal standard to standardize between gels (referred to as "standard" in figures). Proteins were separated by SDS-PAGE at 75 V for 15 min followed by 150 V for 1 h. Following electrophoretic separation, the proteins were transferred to nitrocellulose membranes for immunoblotting as detailed by Forsyth et al. (1997). 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 salmonid Hsp70 in 2% skim milk, 1 : 5,000; see "Antibodies" below for specifics) for 1 h. Following three 5-min washes in TTBS, membranes were soaked in a secondary antibody (goat antirabbit IgG in TTBS, 1 : 3,000) for 1 h. After three 5-min washes in TTBS and one 5-min wash in tris-buffered saline (TBS) 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; 167 μg/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.

Antibodies

Primary and secondary antibodies used for western blotting differed for the analysis of the samples from July 1999 and September 2000. This prevented us from making direct comparisons between hepatic Hsp70 levels between years but did not interfere with comparisons made within a year. Primary antibodies used were rabbit IgG for rainbow trout (RTG-2) Hsp70 (developed in collaboration with P. Candido's laboratory at the University of British Columbia, Canada) and rabbit IgG for chinook salmon Hsp70 (StressGen, Victoria, British Columbia, Canada) for the 1999 and 2000 analysis, respectively. The secondary antibodies used were alkaline phosphatase conjugated goat antirabbit IgG from Kirkegaard and Perry Laboratories (Gaithersberg, MD) and Sigma (St. Louis) for the 1999 and 2000 analysis, respectively.

Statistical Analyses

Differences in survival between the treatments were compared by χ^2 analysis. When over 20% of expected values in the contingency table were less than 5, a Fisher's exact test was run as an alternative to χ^2. For Hsp70 quantification, band intensities were obtained using SigmaGel software (Jandel Scientific), and values were standardized using band intensity values from either arsenite-induced coho salmon lysate or tidepool sculpin gill homogenate that were run concurrently on each gel (referred to as "standard" in figures). Results for Hsp70 are reported as mean ± SE. Two-way ANOVA was used to determine significant ($P \leq 0.05$) differences in Hsp70 levels. For two-way ANOVA, treatment and time were used as independent categorical variables, and Hsp70 levels were used as the dependent variable. 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

Experimental Series 1: Cross-Tolerance (July 1999)

Cross-Tolerance to Osmotic Shock. Exposure of tidepool sculpins to a severe hyperosmotic challenge (90 ppt) resulted in 68% survival; however, sculpins that were exposed to a +12°C heat shock (2 h) before the osmotic challenge (HS/salt) had significantly higher survival (96%) compared to fish that were not heat shocked (salt only; Fig. 3*A*). Sculpins exposed to a milder heat shock (+10°C, 2 h) did not demonstrate increased survival compared to non-heat-shocked fish when they were exposed to an osmotic challenge (Fig. 3*B*). If sculpins were exposed to a stronger heat shock (+15°C, 2 h) before the osmotic challenge, the fish experienced only 12.3% survival, a significantly

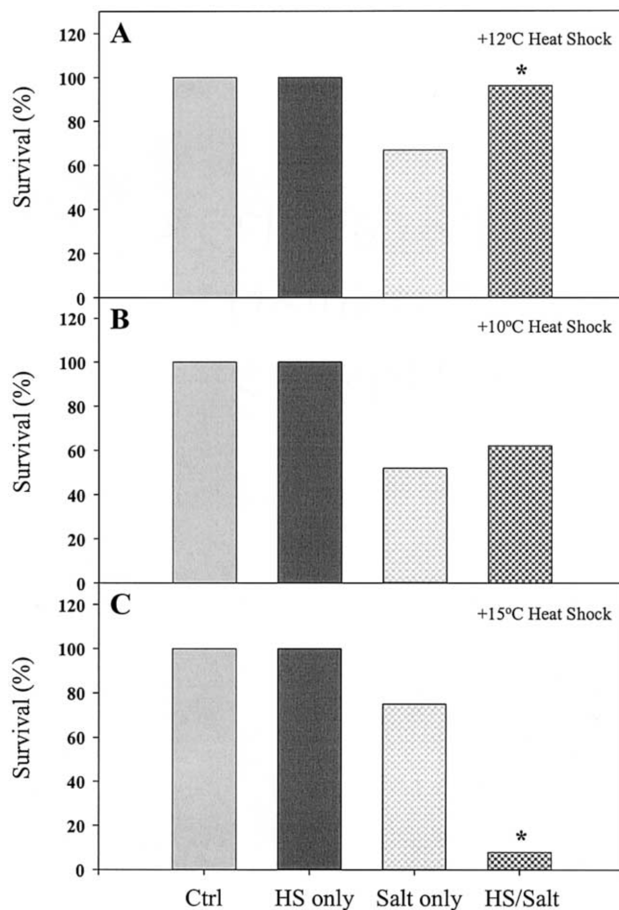

Figure 3. Survival (%) of tidepool sculpins following a 2-h heat shock. *A*, +12°C heat shock. *B*, +10°C heat shock. *C*, +15°C heat shock. Light gray bars (*ctrl*) are control fish that were handled identically but not exposed to heat shock or osmotic shock. Dark gray bars (*HS only*) are fish exposed to the heat shock alone. Crosshatched light gray bars (*salt only*) are fish exposed to a severe hyperosmotic shock (2 h, 90 ppt) only. Crosshatched dark gray bars (*HS/salt*) are fish exposed to both the heat shock and osmotic shock. An asterisk indicates a significant difference in survival between HS/salt and salt only ($P \leq 0.05$).

lower survival compared to fish exposed to the osmotic challenge without prior heat shock (Fig. 3*C*). Heat shock alone, at any of these temperatures, did not cause any mortality.

Exposure to a 2-h, +12°C heat shock did not increase hepatic Hsp70 levels after 8 h of recovery at ambient conditions following the heat shock (heat shock, 10 h; Fig. 4*A*). However, Hsp70 levels were significantly elevated in fish allowed to recover for 18 h at ambient temperatures, and this pattern was not altered by exposure to a 2-h secondary osmotic shock following 8 h of recovery (HS only and HS/salt, respectively, 20 h; Fig. 4*A*). Exposure to a +10°C heat shock did not increase hepatic Hsp70 levels in any group (Fig. 4*B*), while a +15°C heat shock significantly increased hepatic Hsp70 levels after 8 h recovery at ambient temperatures, and these levels remained elevated for 18 h following the heat shock (heat shock, 10 h; and HS only, 20 h, respectively; Fig. 4*C*). This pattern was not altered by exposure to a secondary osmotic stressor (HS/salt, 20 h; Fig. 4*C*). In these three experiments, the exposure to the severe osmotic stressor alone did not affect hepatic Hsp70 levels.

Cross-Tolerance to Hypoxia. Tidepool sculpins that were exposed to a 2-h, +12°C heat shock before exposure to severe hypoxia (2 h, 0.33 mg O_2/L) experienced significantly less mortality (71% survival) compared to fish that were exposed to the severe hypoxia without prior exposure to heat shock (47%

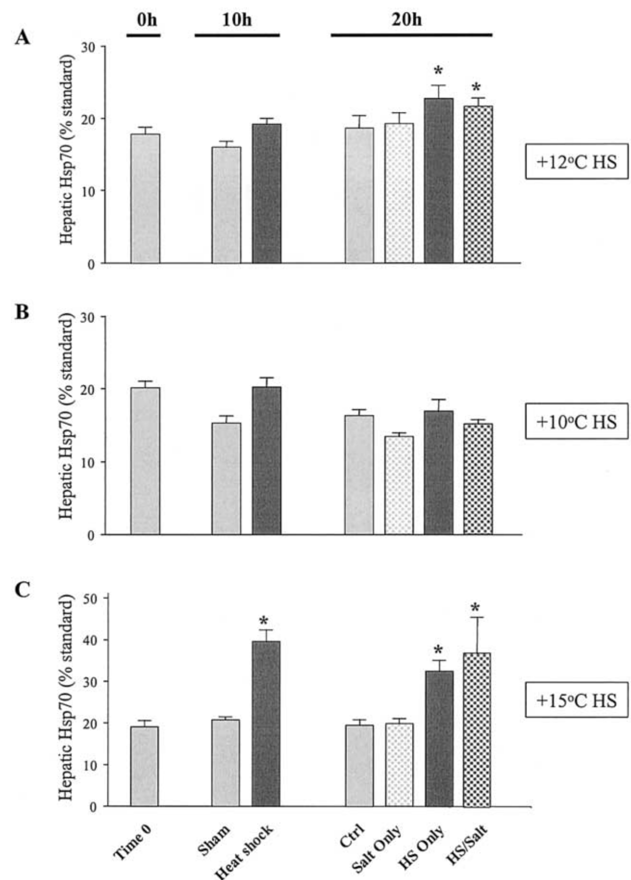

Figure 4. Hepatic Hsp70 levels of tidepool sculpins exposed to a mild heat shock (+12°C [*A*], +10°C [*B*], or +15°C [*C*]) and a severe hyperosmotic challenge (90 ppt). Hsp70 levels were measured before experimentation (time 0), 8 h following the mild heat shock, and 8 h following the hyperosmotic stressor. Sham and control fish were treated in an identical manner but without heat shock or hyperosmotic exposure. Hsp70 levels are shown as relative values based on band intensities standardized with the level of hepatic Hsp70 from arsenite-exposed coho salmon (means ± SE). Note difference in the *Y*-axis of *C* versus *A* and *B*. An asterisk indicates a significant difference in the level of Hsp70 from time 0 ($P \leq 0.05$). See Figure 1 for sampling details.

survival; Fig. 5). There was no mortality associated with the heat shock.

Fish exposed to a +12°C heat shock had significantly elevated hepatic Hsp70 levels 8 h following heat shock (heat shock, 10 h; Fig. 6), but these levels returned to control values by 18 h following heat shock (HS only, 20 h; Fig. 6). However, hepatic Hsp70 levels remained significantly elevated for up to 18 h following the primary stressor, when sculpins were exposed to hypoxia following the initial heat shock (HS/hypoxia, 20 h; Fig. 6). Exposure to hypoxia alone did not affect Hsp70 levels.

Experimental Series 2: Time Frame of Cross-Tolerance (September 2000)

Survival. Sculpins transferred directly from the +12°C heat shock to the severe osmotic challenge (85 ppt) experienced 100% mortality (Fig. 7), while fish allowed 4 or 6 h of recovery did not differ significantly in survival from those exposed to osmotic shock alone. However, tidepool sculpins returned to ambient temperature for 8 h before the osmotic shock had significantly increased survival compared to those exposed to the osmotic stressor alone. This increased survival extended at least 48 h after the initial heat shock. There was no mortality associated with exposure to the heat shock alone (data not shown).

Hepatic Hsp70 Levels. There was a transient increase in Hsp70 levels at 8 and 12 h following heat shock (Fig. 8*A*). Fifteen hours following exposure to the osmotic challenge, there were no significant differences in hepatic Hsp70 levels between any of the treatment groups, and these levels did not differ significantly from levels measured at the start of the experiment (Fig. 8*B*). Osmotic shock alone did not increase hepatic Hsp70 levels.

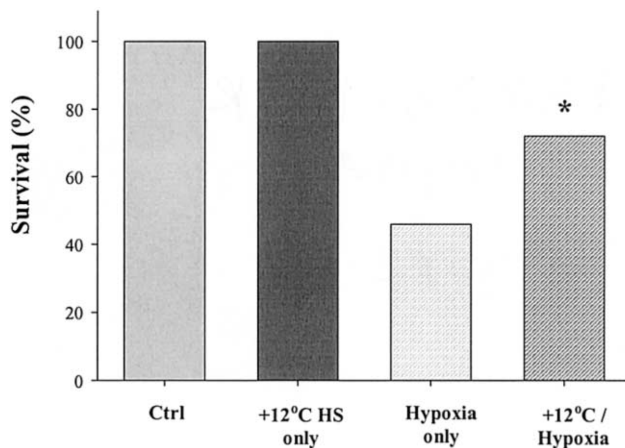

Figure 5. Survival (%) of tidepool sculpins following a 2-h heat shock (*+12°C HS only*), exposure to a severe hypoxia (4% saturation) for 2 h (*hypoxia only*), and following the combined exposure to the mild heat shock and hypoxia (*+12°C/hypoxia*). Control fish (*ctrl*) were treated in an identical manner but without heat shock or hypoxic shock. An asterisk indicates a significant difference in survival between +12°C/hypoxia and hypoxia only ($P \leq 0.05$).

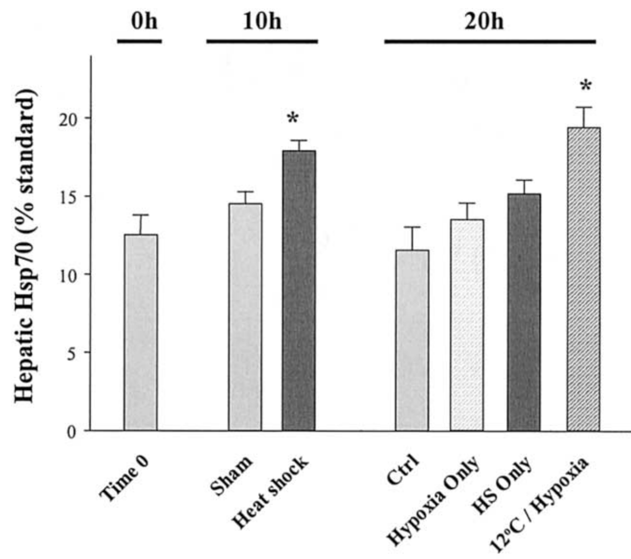

Figure 6. Hepatic Hsp70 levels of tidepool sculpins exposed to a +12°C heat shock and hypoxia (4% saturation). Hsp70 levels were measured before experimentation (time 0), 8 h following the mild heat shock, and 8 h following the hypoxic stressor. Sham fish were treated in an identical manner but without heat shock. Control fish (*ctrl*) were treated in an identical matter without heat shock or hypoxic exposure. Hsp70 levels are shown as relative values based on band intensities standardized with the level of hepatic Hsp70 from arsenite-exposed coho salmon (means ± SE). An asterisk indicates significant differences in hepatic Hsp70 levels from time 0 ($P \leq 0.05$). See Figure 1 for sampling details.

Branchial Hsp70 Levels. Branchial Hsp70 levels were only significantly elevated after 24 h of recovery at ambient temperature following a +12°C heat shock (Fig. 9*A*). In contrast, 15 h after the osmotic challenge, branchial Hsp70 levels were elevated in fish exposed to an initial heat shock and allowed to recover for 8, 12, 24, and 48 h between the stressors (Fig. 9*B*). Note that these times of elevated Hsp70 correlate well with times of increased osmotic tolerance (Fig. 7). Osmotic shock alone did not increase branchial Hsp70 levels.

Discussion

Cross-Tolerance in Tidepool Sculpins

This is the first study in aquatic organisms to demonstrate that the magnitude of the preliminary heat shock is critical for the development of cross-tolerance. Pretreatment with a +12°C heat shock increased stress tolerance, while a +10°C heat shock had no effect, and a +15°C heat shock was deleterious. These results suggest that the mechanisms underlying cross-tolerance

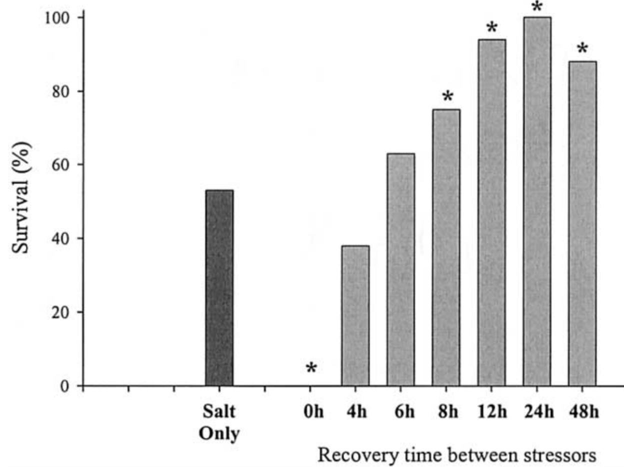

Figure 7. Survival (%) of tidepool sculpins exposed to a severe hyperosmotic shock (85 ppt; *salt only*) and exposed to a +12°C heat shock before hyperosmotic challenge with 0, 4, 6, 8, 12, 24, or 48 h of time at ambient water conditions between stressors. An asterisk indicates a significant difference in survival from the salt only group ($P \leq 0.05$).

are particularly sensitive to slight adjustments in temperature and that, as a result, cross-tolerance is only inducible over a narrow range of temperatures. In addition, this is the first study to document the time frame of thermally induced cross-tolerance in a fish. Tidepool sculpins required at least 8 h of recovery at ambient temperatures following a +12°C heat shock for that heat shock to be protective against a secondary stressor, and this protective window lasted for at least 48 h. This time period of recovery, which was essential for cross-tolerance, may reflect the time needed to initiate the appropriate cellular pathways to confer an increase in stress tolerance.

The Role of Hsp70 in Cross-Tolerance

Previous research has suggested that Hsp induction may be an important component of cross-tolerance in fish (Brown et al. 1992; Renfro et al. 1993; DuBeau et al. 1998; Martin et al. 1998). The strongest association between Hsp70 levels and cross-tolerance in our experiments is seen in the gills. Exposure to osmotic shock resulted in a significant elevation in branchial Hsp70, but only in fish that were given a prior heat shock (Fig. 9*B*). The dynamics of the branchial Hsp70 levels followed an identical time frame as the window of cross-tolerance, requiring 8 to 48 h of recovery at ambient temperatures following heat shock before being increased by exposure to osmotic shock. Osmotic shock alone was insufficient to increase branchial Hsp70 levels. These results provide good correlative evidence of the involvement of branchial Hsp70 in the osmotic tolerance conferred by a mild heat shock. In the liver, there was no consistent evidence to suggest a clear association between elevated levels of Hsp70 and cross-tolerance to a secondary stressor. However, taken together, the results in both the liver and gills provide some insights into the mechanisms underlying cross-tolerance.

Previous studies in fish, as well as those in model systems, have shown a strong association between elevated levels of Hsps before the second stressor and cross-tolerance (Lee and Hahn 1988; Flahaut et al. 1996; Laplace et al. 1996; Krebs and Feder 1998). In experimental series 1, Hsp70 levels were elevated before the secondary stressor in only one of the two experi-

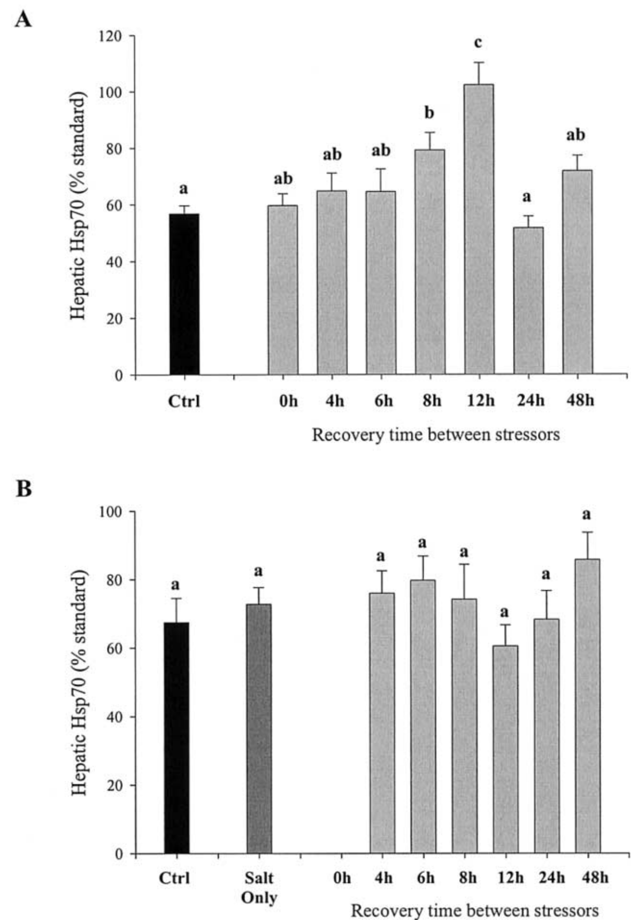

Figure 8. Hepatic Hsp70 levels of tidepool sculpins exposed to a severe hyperosmotic shock (85 ppt; *salt only*) and exposed to a +12°C heat shock before hyperosmotic challenge with 0, 4, 6, 8, 12, 24, or 48 h at ambient water conditions between stressors. Hsp70 levels were measured (*A*) following recovery at ambient conditions for 0, 4, 6, 8, 12, 24, or 48 h after heat shock immediately before exposure to a hyperosmotic challenge and (*B*) 15 h following exposure to the hyperosmotic challenge. Control fish (*ctrl*) were treated in an identical manner but without heat shock or hyperosmotic exposure. Hsp70 levels are shown as relative values based on band intensities standardized with the level of hepatic Hsp70 from arsenite-exposed coho salmon (means ± SE). A difference in letters denotes significant differences in hepatic Hsp70 levels ($P \leq 0.05$). See Figure 2 for sampling details.

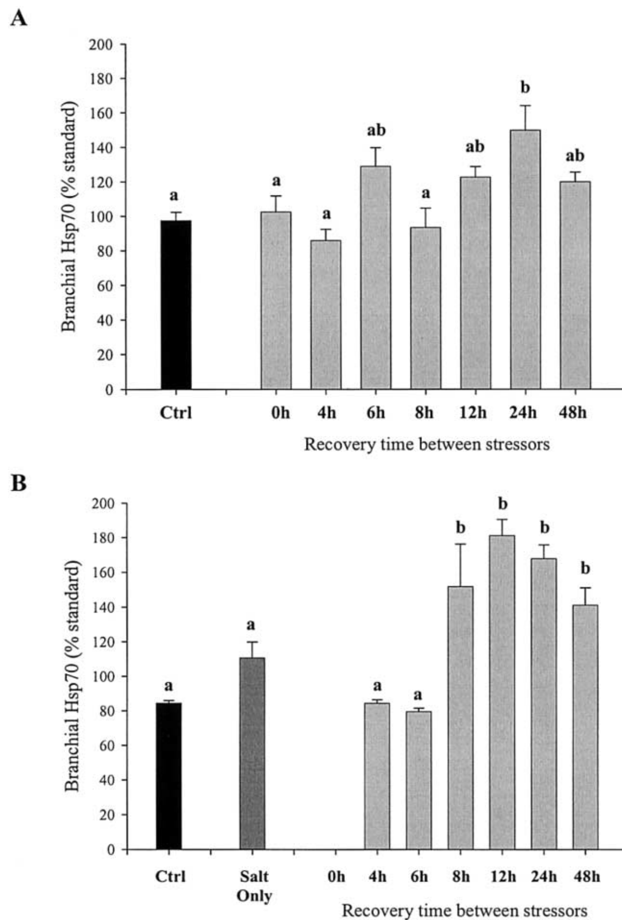

Figure 9. Branchial Hsp70 levels of tidepool sculpins exposed to a severe hyperosmotic shock (85 ppt; *salt only*) and exposed to a +12°C heat shock before hyperosmotic challenge with 0, 4, 6, 8, 12, 24, or 48 h at ambient water conditions between stressors. Hsp70 levels were measured (*A*) following recovery at ambient conditions for 0, 4, 6, 8, 12, 24, or 48 h after heat shock immediately before exposure to a hyperosmotic challenge and (*B*) 15 h following exposure to the hyperosmotic challenge. Control fish (*ctrl*) were treated in an identical manner but without heat shock or hyperosmotic exposure. Hsp70 levels are shown as relative values based on band intensities standardized with the level of branchial Hsp70 from tidepool sculpins (means ± SE). A difference in letters denotes significant differences in branchial Hsp70 levels ($P \leq 0.05$). See Figure 2 for sampling details.

ments (cf. Fig. 4*A* to Fig. 6; 10-h heat shock), despite the fact that cross-tolerance occurred in both cases. These experiments involved the same initial heat shock, suggesting that either the initial heat shock is sufficiently mild so that only a modest (sometimes nonsignificant) induction of Hsp70 occurs or that 8 h of recovery is a critical period in which induction is first detectable. This hypothesis is supported by the data shown in Figure 8*A*, in which Hsp70 levels increase gradually from 6 to 12 h following heat shock. Thus, our data suggest that elevated levels of Hsp70 protein are not required before exposure to the second stressor for the heat shock to confer cross-tolerance. Similarly, in experimental series 2, a +12°C heat shock induced cross-tolerance, but there was no association between elevated levels of either hepatic or branchial levels of Hsp70 before osmotic shock and increased osmotic tolerance (Figs. 8*A*, 9*A*). In fact, if hepatic Hsp70 levels were highly induced before the second stressor, as seen with the +15°C preconditioning heat shock, this increase corresponded with a decreased stress tolerance. Therefore, contrary to previous reports of cross-tolerance, there is no simple relationship between elevated Hsp levels before the onset of the second stressor and the development of cross-tolerance.

It is possible that the role of the preconditioning heat shock in cross-tolerance is to enable the cells to mount an Hsp70 response following exposure to a second stressor that alone would not induce Hsp70. Therefore, we would expect to see an association between elevated levels of Hsp70 following the second stressor and cross-tolerance. Our data provide mixed support for this hypothesis. In the liver, exposure to hypoxia following an initial heat shock resulted in increased Hsp70 levels relative to either heat shock or hypoxia alone (Fig. 6), consistent with the hypothesis that the initial heat shock primes the cell to mount an increased response to the secondary stressor. This increase in Hsp70 was associated with cross-tolerance. In contrast, exposure to a secondary osmotic shock following an initial heat shock did not increase hepatic Hsp70 levels relative to fish exposed to the heat shock alone (20 h; Fig. 4*A*) or else did not increase Hsp70 levels relative to controls (Fig. 8*B*). These results suggest that Hsp70 levels in the liver do not respond to osmotic shock and that preexposure to an initial heat shock does not prime the hepatic Hsp70 response to a secondary osmotic stressor. In the gills, Hsp70 levels increased following osmotic shock in all heat-shocked groups, when allowed at least 8 h of recovery between the two stressors (Fig. 9*B*), while osmotic shock alone did not increase Hsp70 levels. Since samples were taken either 8 or 15 h following exposure to a single severe osmotic or hypoxic shock alone, it is possible that in these treatments the apparent lack of an Hsp response could be due to a rapid and transient Hsp response in which Hsp70 levels peaked and then declined by the sampling time. Alternatively, it is possible that in these treatment groups the stressors were sufficiently severe that they inhibited the Hsp response entirely by damaging the transcriptional and translational machinery of the cell. Whatever the mechanism, however, these data suggest that an initial heat shock primes the gills to respond to a subsequent osmotic shock and the liver to respond to a subsequent hypoxic shock with an altered Hsp70 response. This altered Hsp70 response is then associated with an increase in survival.

The differences between the Hsp70 responses in the gills and liver and the development of cross-tolerance suggest that the involvement of Hsp70 in cross-tolerance may be tissue specific. In fish, the gills are essential osmoregulatory and ionregulatory

organs; therefore, it is not surprising that a mild heat shock would confer protection at the level of the gills against osmotic shock by increasing Hsp70 levels. The liver may not be a suitable tissue in which to examine cross-tolerance to osmotic shock, as it would not experience the same degree of osmotic change as an external tissue. Hypoxia, rather than osmotic shock, likely has a greater effect on liver metabolism, as the liver would directly experience this hypoxic shock. Therefore, if we take into account the most relevant stressor for a particular tissue when examining the involvement of Hsp70 in the development of cross-tolerance, it appears that in cross-tolerance the initial heat shock serves to prime the cell to increase Hsp70 levels in response to a subsequent stressor.

One possible mechanism by which a mild heat shock may prime the Hsp70 response is through posttranscriptional accumulation of *hsp70* mRNA. *Hsp* mRNAs are relatively unstable at normal temperatures (half-life = 15–30 min in *Drosophila*) but are stabilized by heat shock (half-life > 4 h in *Drosophila*; Lindquist and Petersen 1990). Many studies implicate Hsp70 in self-regulating these rapid increases in *hsp* mRNA stability by binding to their own mRNA (DiDomenico et al. 1982; Henics et al. 1999). Therefore, it is possible that a +12°C heat shock is sufficient to induce the expression of *hsp70* mRNA but is mild enough that translation of these messages is not required. With an accumulation of *hsp70* mRNA in the cytoplasm, these cells would be able to more rapidly synthesize the Hsp70 required to cope with the denaturing stress of the second insult. Unfortunately, sufficient tissue samples were not taken to examine *hsp70* mRNA levels in these experiments, but future studies will address this hypothesis. Another possible mechanism by which a mild heat shock may allow a cell to better cope with a subsequent stressor is through the protection of the translation machinery by the constitutively expressed heat shock cognate protein 70 (Hsc70) already present in the cell. In the unstressed cell, Hsc70 functions to maintain protein homeostasis by regulation of protein quality control (Hartl and Hayer-Hartl 2002). On heat shock, translation of preexisting mRNAs is repressed such that there is the preferential translation of *hsp* transcripts. Therefore, preexisting Hsc70 is no longer required for the proper folding and stabilization of normal cellular proteins and may be sequestered to protect Hsp synthesis following heat shock. Beck and De Maio (1994) determined in HepG2 cells that on heat shock Hsp72 transiently associated with the ribosomal subunits and thereby preserved translation of Hsp messages in the thermotolerant cell. By protecting the cell's ability to synthesize Hsps and/or priming the heat shock response through the buildup of *hsp70* mRNA, the cell is able to respond more rapidly and effectively to a subsequent protein-denaturing stress such that the cellular protein integrity is not compromised to such an extent as to affect the organism's survival.

From the data presented here, it appears that for cross-tolerance to develop, a delicate balance between damage and repair of the cellular protein pool and subsequent tolerance must be achieved. The magnitude of Hsp synthesis has been shown to be proportional to the severity of the heat stress (DiDomenico et al. 1982). The magnitude and speed of Hsp70 synthesis following a +15°C heat shock likely reflects the degree of protein repair required to restore protein homeostasis. Eight hours of recovery following this heat shock may have been insufficient time to restore critical protein function, leaving insufficient resources to protect against a second stressor, and this is reflected in the increased mortality in fish exposed to a subsequent severe stressor. However, a +12°C heat shock may have been mild enough to prime the cellular stress response, preparing the animal to respond more quickly to a subsequent stressor without causing irreparable cellular damage.

Implications for Stress Tolerance in the Intertidal Zone

In tidepool sculpins, cross-tolerance appears to be a finely tuned phenomenon sensitive to a narrow range of heat shock temperatures, requiring a distinct period of recovery between the two stressors to induce protection. It is possible that such a structured system may not be significant in nature where environmental conditions are variable; however, the time frame of cross-tolerance and its relationship to branchial Hsp70 indicate that cross-tolerance could be an important aspect of stress tolerance in intertidal fish. Tidepool sculpins live in an environment where tidal cycles result in substantial variations in water quality. There are approximately 8–12 h of high tide between low tide periods, depending on an animal's vertical location in the intertidal zone. This study demonstrated that tidepool sculpins required a "recovery period" at stable ambient ocean conditions following a mild heat shock for their osmotic tolerance to be enhanced and their Hsp70 response to be upregulated. The duration of this recovery period is similar in duration to the time period that tidepool sculpins are immersed by the ocean between low-tide periods. Therefore, we suggest that the time frame of the protective window and the concurrent branchial Hsp70 response may reflect the periodicity of environmental change that characterizes the intertidal zone. The correlation between the threshold for Hsp expression and the levels of stress that an animal naturally experiences is well established (reviewed by Feder and Hofmann 1999). In addition, Hsps have been shown to fluctuate in response to natural daily and seasonal temperature variations in the aquatic environment (Dietz and Somero 1992; Fader et al. 1994). Recently, a few studies investigating the functional significance of Hsps in nature have provided evidence suggesting that organisms living in the intertidal zone have a heat shock response that is structured to reflect the periodicity of the tidal cycle (Hofmann and Somero 1996; Tomanek and Somero 2000; Schill et al. 2002). Although the periodicity of environmental change within the intertidal zone is predictable, the magnitude of these fluctuations in temperature, salinity, and oxygen is not. The time

frame of the cross-tolerance window and the relationship with increased branchial Hsp70 levels may provide evidence of the tidepool sculpin's ability to invoke a protective mechanism from one low-tide period to prepare them for the unpredictable nature of subsequent ones.

Conclusions

Exposure to a +12°C heat shock confers increased tolerance to both a severe osmotic or hypoxic shock in the tidepool sculpin. The magnitude of this preliminary heat shock is critical for the development of cross-tolerance, and it appears that the degree of cross-tolerance conferred by the heat shock is sensitive to slight adjustments in temperature. Cross-tolerance was present in a defined temporal window, requiring 8–48 h of recovery at ambient temperatures following the +12°C heat shock before exposure to osmotic shock. The results from this study provide strong evidence that elevated levels of Hsp70 protein are not required before exposure to the second stressor for the heat shock to confer protection. Rather, this heat shock may prime the cell to mount an Hsp70 response following exposure to a second stressor that alone did not induce Hsp70, thereby facilitating a faster cellular stress response to a subsequent stressor. The transient nature of this cross-tolerance and the time frame of protection induced by heat shock suggest that fish in nature could be conditioned by one stressor to better tolerate a subsequent insult.

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