ABSTRACT

Although there are a large number of studies on the role of heat shock proteins (Hsps) in stress tolerance, this literature consists mainly of studies conducted in the laboratory, making a comprehensive understanding of the ecological significance of Hsps in an animal’s natural stress tolerance difficult to obtain. The rocky intertidal is an ideal environment in which to examine the physiological and biochemical responses of organisms to changing abiotic factors and to investigate mechanisms underlying an organism’s ability to thrive in such a variable environment. My PhD thesis explores the role of environmental variation in modulating both the stress tolerance and the Hsp response of tidepool sculpins (Oligocottus maculosus).

Stress tolerance in tidepool sculpins displayed a significant amount of plasticity that was sensitive to both short and longer-term changes in the thermal environment. A mild heat shock conferred a transient increase in tolerance to both subsequent osmotic and hypoxic stressors. Similarly, exposure to the daily fluctuations in environmental conditions inherent in living in the intertidal zone also imparted a certain degree of enhanced stress tolerance to sculpins. There was no endogenous diurnal rhythm in the Hsp response entrained to the tidal cycle in the tidepool sculpin; rather hsp gene expression was rapidly induced in response to routine fluctuations in environmental conditions. The Hsp response of sculpins was sensitive to slight differences in environmental temperature that occurred over differing temporal (different times during a month) and spatial (tidepool vs. subtidal) scales. Tidepool sculpins maintained a large and relatively constant standing pool of Hsc70 to cope with the daily variability of their natural environment, but had the capacity to rapidly regulate hsp expression when environmental conditions approach their tolerance thresholds.

These results highlight the importance of an animal’s thermal history in structuring it’s Hsp response to natural environmental fluctuations and demonstrate that the variable intertidal
environment is able to module Hsp levels at both the transcriptional and posttranscriptional levels. Taken together, the research presented in this thesis provides novel ecological insights into the significance of Hsps in allowing organisms to cope with large and frequent fluctuations in their natural environment.
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CTM</td>
<td>critical thermal maximum</td>
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<tr>
<td>Ctrl</td>
<td>handling control</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DYT</td>
<td>double yeast-tryptone bacterial growth medium</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>EF-1α</td>
<td>elongation factor 1 alpha</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
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<td>human hepatoma cell line G2</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>LB</td>
<td>Luria Bertani bacterial growth medium</td>
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<tr>
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<td>magnesium chloride</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MS-222</td>
<td>tricaine methanesulfonate</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>-----------</td>
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<td>sodium chloride</td>
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<td>NBT</td>
<td>nitro blue tetrazolium</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>ppt</td>
<td>parts per thousand</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
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<tr>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>RTG-2</td>
<td>rainbow trout gonadal cell line</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SNK</td>
<td>Student-Newman-Keuls</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>$T_{on}$</td>
<td>threshold induction temperature for gene expression</td>
</tr>
<tr>
<td>TTBS</td>
<td>tween-20 tris-buffered saline</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>V</td>
<td>volts</td>
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CO-AUTHORSHIP STATEMENT

CHAPTER 2: CROSS-TOLERANCE IN THE TIDEPOOL SCULPIN: THE ROLE OF HEAT SHOCK PROTEINS

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CHAPTER 3: ENVIRONMENTAL VARIABILITY, STRESS TOLERANCE AND THE HEAT SHOCK PROTEIN RESPONSE IN TIDEPOOL SCULPINS

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CHAPTER 4: EFFECT OF THE NATURAL TIDAL CYCLE AND ARTIFICIAL TEMPERATURE CYCLING ON HSP LEVELS IN TIDEPOOL SCULPINS

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CHAPTER 5: CHANGES IN HSP LEVELS OVER CONSEQUETIVE LOW TIDE PERIODS: THE ROLE OF ACUTE AND CHRONIC THERMAL ENVIRONMENTS ON THE HSP RESPONSE OF TIDEPOOL SCULPINS

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CHAPTER 1. GENERAL INTRODUCTION

Overview

Organisms that inhabit a variable environment are generally more tolerant of a wide variety of environmental conditions than organisms that inhabit more stable environments (Hochachka and Somero 2002). Much of our understanding of these issues has come from experiments performed under controlled laboratory conditions where a limited number of variables are manipulated. In the work presented in my PhD thesis, I used a combination of laboratory experiments and measurements of animals in their natural environment to gain a deeper understanding of the mechanisms underlying an animal’s ability to cope with environmental fluctuations and the role of the environment in modulating an animal’s natural tolerance to environmental change. My overall thesis is that stress tolerance is central to successful adaptation to natural variations in the aquatic environment, and that heat shock proteins (Hsps) play a key role in this tolerance. This general introduction will briefly examine aspects of the cellular stress response to provide a better understanding of the importance of Hsps in recovery of cells from stress. The current state of knowledge regarding the role of Hsps in organismal stress tolerance will also be reviewed with emphasis on the potential ecological significance of Hsps in order to bring the hypotheses examined in this thesis into context.

The Stress Response in Fish

Fish are constantly being exposed to changes in their natural environment. Whether it is fluctuations in temperature or dissolved oxygen levels, or exposure to contaminants or pathogens, these changing environmental factors can impose considerable stress on the physiology of fishes (Wedemeyer et al. 1984; Wendelaar Bonga 1997). In response to stress,
animals attempt to establish and maintain homeostasis through behavioural, physiological and biochemical adjustments, and these changes occur from the whole animal to the cellular level of organization. The success of fish populations in tolerating these changes in their aquatic environment depends largely on their ability to effect and maintain the appropriate compensatory responses.

Classical studies of stress in fish have focused on the organismal stress response. As a pioneer in stress research, Selye (1950) defined stress as “the sum of all the physiological responses by which an animal tries to maintain or reestablish a normal metabolism in the face of a physical or chemical force”. These physiological responses have been broken down into primary, secondary and tertiary changes that involve progressively higher levels of biological organization (Wedemeyer et al. 1990; Barton and Iwama 1991). The primary stress response involves the production and release of stress hormones (e.g. catecholamines and cortisol) into the bloodstream, which results in secondary blood chemistry and tissue alterations, such as hyperglycemia, diuresis, and depletion of liver glycogen, to mobilize energy stores for the “fight or flight” reaction. The tertiary stress response involves long-term effects on individuals and populations such as reduced growth, disease resistance and reproductive success. These responses can represent a significant bioenergetic cost to the animal and therefore if the duration or severity of the stress exceeds a fish’s tolerance limits, these compensatory responses may ultimately reduce performance.

From this conceptual framework of stress one can begin to understand the complexity of responses required by fish to adjust to changes in their natural environment. Many descriptive studies have outlined the correlations between exposure of fish to stressors and the wide variety of physiological changes that occur at both the whole animal and cellular levels of organization in response to stress (for review see Wendelaar Bonga 1997). We know that fish have adapted to
cope with the potential stressors associated with living in a changing environment; however, it remains unclear what mechanisms are involved in their tolerance to stress and what signals initiate these responses.

The Cellular Stress Response

In addition to organismal stress response described above, a generalized stress response system exists at the cellular level. Much of the research in the last few decades has focused on the role of this cellular stress response in environmental physiology and the ability of heat shock proteins (Hsps) to assist in the recovery of the cell from stress (for review see: Lindquist 1986; Hightower 1991; Sanders 1993; Morimoto 1998; Feder and Hofmann 1999. For review in fish see: Iwama et al. 1998; Basu et al. 2002). Hsps are one important component of the cellular stress response, which also includes other molecular chaperones, antioxidases, proteases and DNA repair systems. While the term "heat shock protein" arose from early observations on *Drosophila* exposed to a severe heat stress (Ritossa 1962; Tissieres et al. 1974), Hsps can be up-regulated in cells that are exposed to a wide variety of stressors, particularly those that denature proteins (Welch 1993; Freeman et al. 1999).

Several families of Hsps have been identified and are named according to their molecular mass in kDa. The major Hsp families are: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsps (sHsps ≤30-kDa). Each family consists of one to several closely related genes, and their products can differ in inducibility, intracellular location and function (Gething 1997). The genes encoding for Hsps are highly conserved and have been found in every species studied to date. For example, for one of the most conserved Hsps, inducible Hsp70, amino acid similarity between *Escherichia coli* and *Homo sapiens* is around 50%, with some domains sharing 96% homology (Schlesinger 1990). The low variation between species in *hsp* genes and their
ubiquity suggests an important evolutionary significance of these proteins in protecting protein homeostasis.

*Heat shock proteins as molecular chaperones*

Hsps and their molecular co-chaperones are known to serve very important "house­keeping" roles in the unstressed cell in assisting in the folding, assembly and translocation of newly synthesized proteins, and the degradation of misfolded proteins (Gething and Sambrook 1992; Hartl 1996; Zimmerman 1998; Hartl and Hayer-Hartl 2002). Therefore, despite being first identified as stress proteins, Hsps have a very significant role in the unstressed cell as molecular chaperones and are expressed constitutively (e.g. heat shock cognate 70, Hsc70). Under stress, Hsps play a key role in the recovery of the cell following stress by maintaining the integrity of the cellular protein pool by interacting with other proteins that have begun to unfold and minimizing their potential to interact inappropriately with each other (Parsell and Lindquist 1993; Sherman and Goldberg 1996). In general, the consensus signal for the activation of inducible *hsp* genes is the presence of elevated levels of abnormal proteins (Ananthan et al. 1986; Lee and Goldberg 1998). Hsps target the hydrophobic patches of denatured proteins; stabilizing structure and restoring function of some proteins while speeding up the breakdown of irreversibly damaged proteins. Therefore, Hsps play a critical role in regulating the recovery of denatured proteins and returning them to the protein pool (Wickner et al. 1999). Proteins are arguably the most energetically expensive biomolecules that organisms synthesize and as a result, a substantial portion of the energy budget of an organism is dedicated to maintaining the protein pool (Houlihan et al. 1995). Therefore, having a cellular mechanism committed to minimizing the cost of environmentally induced protein damage and maintaining protein homeostasis would be of adaptive value.
Intrinsic regulation of hsp gene expression

The Hsp response is one of the most highly regulated inducible cellular responses, and is under the control of numerous transcriptional as well as posttranscriptional mechanisms, which allow for the rapid and preferential synthesis of Hsps under protein denaturing conditions (DiDomenico et al. 1982b, Yost et al. 1990, Wu et al. 1990). For example, Hsp70 is virtually absent in unstressed cells but within minutes can account for the bulk of protein synthesis during stress (Velazquez et al. 1983; Palter et al. 1986).

Inducible hsp gene expression is transcriptionally regulated by heat shock factor-1 (HSF1), which itself is regulated at multiple levels and is activated in response to stress (for review see Wu 1995; Voellmy 2004). In general, the transcriptional activation of stress-inducible hsp genes involves the trimerization of the monomeric HSF1 giving HSF1 DNA-binding ability, hyperphosphorylation and transactivation of HSF1 and the translocation of the HSF1 trimer into the nucleus (Sarge et al. 1993). Analysis of hsp genes and a comparison of their promoter sequences from a variety of organisms led to the identification a domain called the heat shock element (HSE) which consists of three inverted repeats of the motif 5'-nGAAn-3' (Pelham 1982). It has been demonstrated that hsp induction results primarily from the binding of an activated HSF to HSEs upstream of hsp genes (Morimoto et al. 1992). Hsps themselves are known to inhibit their own synthesis through a negative feedback loop, mediating the activity of HSF1 (Morimoto 1998).

At the posttranscriptional level, there are several mechanisms that promote the preferential synthesis of Hsps in the presence of protein denaturing conditions. Some of these include: the absence of introns in the case of hsp70 (Ingolia et al. 1980), special sequences in the 3' UTR of the gene that promote hsp mRNA stability (Petersen and Lindquist 1988), as well as sequences in the 5' UTR of the gene that promote translational efficiency (McGarry and
Lindquist 1986). Once organisms are returned to normal temperatures, the repression of Hsp synthesis is tightly regulated by multiple mechanisms (Petersen and Mitchell 1981; Petersen and Lindquist 1989).

These multiple levels of regulation at both the transcriptional and posttranscriptional level provide the cell with a considerable amount of control over the initiation and attenuation of the inducible Hsp response. The necessity of such tight regulation of a response that is extremely effective at maintaining the integrity of the cellular protein pool is intriguing; why not simply maximize the expression of Hsps? One reason is that Hsps are most effective as molecular chaperones at low to moderate concentrations. If these proteins are allowed to reach the high extremes of physiological levels they can bind proteins inappropriately, interfere with their cellular localization and negatively affect the organism’s growth, development and thermotolerance (Dorner et al. 1992; Krebs and Feder 1997, 1998). Another reason is that the Hsp response is costly both in terms of the energetics of synthesizing these proteins and biochemically to the cell as a consequence of Hsps being preferentially synthesized at the expense of other cellular proteins (Koehn and Bayne 1989; Lindquist and Petersen 1990; Hoffmann 1995). Therefore, due to their potential negative impacts, it is critical that these proteins are tightly regulated at effective concentrations to maximize their benefits as molecular chaperones, while minimizing their cost to organismal fitness. This level of control results in tightly regulated and tissue specific threshold temperatures for Hsp production in homeotherms (Sarge et al. 1995) while granting the flexibility in the same thresholds to poikilotherms, where environmental temperature is thought to modulate hsp gene expression.

*Heat shock proteins and stress tolerance in the organism*

It has been well documented that there is a correlation between the expression of Hsps and thermotolerance (an increased resistance to heat; Li and Hahn 1978; Hahn and Li 1990).
Studies in fish have shown that the synthesis and decay of Hsps share a close temporal relationship with the induction and disappearance of thermotolerance (Mosser et al. 1987; Mosser and Bols 1988). The role of Hsps in thermotolerance appears to be crucial, since the inhibition of Hsp synthesis prevents the development of thermotolerance in rainbow trout fibroblasts (Oncorhynchus mykiss: Mosser and Bols 1988). Studies in transgenic Drosophila that over-express hsp70 support the idea that Hsps are responsible, at least in part, for induced thermotolerance (Feder and Krebs 1998).

In addition to conferring thermotolerance, a preconditioning heat shock has been shown to confer protection to subsequent exposure to stressors other than heat (Li and Hahn 1978; Kampinga et al. 1995). This phenomenon is commonly known as cross-tolerance, although it is also referred to as cross protection, and has been defined as a transient increase in stress tolerance conferred by exposure to a preliminary mild heterologous stressor. Studies on cross-tolerance in bacteria and plants have expanded our understanding of inducible stress tolerance by demonstrating that stressors other than heat are able to induce this increased resistance to subsequent stressors (Lewis et al. 1995; Flahaut et al. 1996; Laplace et al. 1996; Sabehat et al. 1998). Descriptive studies performed in bacteria provide evidence that stressors that induce a more generalized cellular stress response (i.e. a larger suite of Hsps) such as high temperature are more capable of conferring cross-tolerance than stressors that induce a more specific cellular stress response such as heavy metals (Flahaut et al. 1996). Research on cross-tolerance in plants expands on this suggesting that there is likely sufficient overlap in the function of different Hsps to explain cross-tolerance between heterologous stressors (for review see Sabehat et al. 1998).

The earliest studies of cross-tolerance in fish were performed in vitro in the winter flounder (Pleuronectes americanus). Exposure of the renal epithelium to a heat stress protected these cells against the deleterious effects of a subsequent extreme temperature or chemical challenge (Brown et al. 1992). These researchers showed that protection afforded by a mild heat
shock coincided with increased levels of Hsp28, Hsp70, and Hsp90 in the cell. Subsequent work demonstrated that low levels of zinc had similar protective effects (Renfro et al. 1993). DuBeau et al. (1998) reported that heat stressed Atlantic salmon (Salmo salar) with elevated levels of branchial and hepatic Hsp70 were better able to tolerate an osmotic challenge, relative to control fish. This cross-tolerance was only observed during the two month period coinciding with parr-smolt transformation, suggesting a role for Hsps in ionic and osmotic adaptation.

Whether cross-tolerance is a significant aspect of natural stress tolerance and whether fish can mediate changes in Hsp levels in anticipation of predictable changes in their environment is an interesting aspect of Hsp biology that needs further study. Most studies that have investigated the functional significance of Hsps in an environmental context have focused on the effects of heat stress; however, natural environments are highly complex and organisms are often exposed to multiple stressors. Therefore cross-tolerance, in addition to thermotolerance, may be important to consider when examining both the features underlying the natural stress tolerance of organisms inhabiting a variable environment as well as the ecological significance of Hsps.

**Ecological significance of Hsps**

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 this may implicate a functional role for Hsps in an animal’s natural tolerance to environmental change. Much of our understanding of the adaptive significance of Hsps in allowing organisms to cope with environmental variation, particularly extreme temperature, has arisen from detailed studies of Drosophila (for review see Hoffmann et al. 2003). These studies have been invaluable in outlining the regulation of inducible Hsps (DiDomenico et al. 1982a,b; Lindquist and Petersen 1990), confirming the central role of Hsps in conferring thermotolerance (Krebs and Feder 1998), and detailing how natural selection has acted on hsp genes (Otsuka et al. 1997;
Bettencourt et al. 2002; Frydenberg et al. 2003). A limitation of this work, however, is that many of these Drosophila lines have been held in the laboratory for hundreds if not thousands of generations and therefore cannot adequately address the relevance of Hsps to an organism within its natural environment. To assess the ecological significance of Hsps, it is essential that we extend our investigations to species outside of the laboratory.

Recently, the focus of Hsp research on non-model species has progressed from descriptive laboratory studies documenting the Hsp response of a variety of organisms to a range of stressors and has moved to experiments using ecologically relevant stressors (Feder and Hofmann 1999). Correlation between Hsp expression and the level of stress an animal naturally experiences is well established, providing strong evidence that these proteins have an ecological significance to organisms in their natural environment. In general, the threshold environmental temperature for Hsp induction is correlated with the typical temperatures at which a species lives, with organisms inhabiting warmer thermal environments having higher threshold temperatures for Hsp induction (Dilorio et al. 1996; Roberts et al. 1997; Tomanek and Somero 1999; Nakano and Iwama 2002). It has also been shown that translation itself may have an upper thermal maximum that varies among species adapted to different thermal environments (Gehring and Wehner 1995). In addition, Hsp levels have been shown to fluctuate on both a seasonal and daily basis in association with changes in ambient temperature (Dietz and Somero 1992; Fader et al. 1994; Hofmann and Somero 1995; Buckley et al. 2001; Schill et al. 2002). From these studies, the importance of Hsps as a source of phenotypic plasticity in an animal's natural environment is becoming apparent; however, the mechanistic underpinnings of the regulation of Hsp gene expression and the significance of this environmental regulation for natural stress tolerance at the whole organism level are currently very poorly understood.
Intertidal Zone

The intertidal zone is a unique environment characterized by rapid changes in temperature (11°- 30°C), salinity (10-45 ppt) and oxygen (0.5-19 mg O₂/L) that occur with each tidal cycle. In spite of these harsh environmental conditions, rocky intertidal habitats are home to numerous organisms as they are highly productive and rich in nutrients (Horn et al. 1999). The rocky intertidal has proven to be an ideal environment in which to examine the physiological and biochemical responses of organisms to changing abiotic factors and to investigate what mechanisms are in place that allow these organisms to thrive in such a variable habitat. The vertical zonation of species’ distribution within the rocky intertidal clearly reflects the ability of organisms to tolerate the fluctuations in environmental conditions associated with tidal cycle and temperature specifically plays a pivotal role in establishing the upper limits of this zonation (Connell 1972; Newell 1979). For decades, physiologists have studied intertidal animals in order to understand the environmental parameters that set limits on an organism’s physiological responses and the associated underlying mechanisms (For review see Somero 2002).

The challenge faced by eurythermal organisms inhabiting variable thermal environments such as the intertidal zone is the maintenance of biochemical and physiological performance in a constantly changing environment. Although these organisms have evolved many mechanisms that provide them with impressive resistance to thermal stressors, they still expend a considerable amount of energy in the repair of thermally sensitive biochemical components of the cell (Hochachka and Somero 2002). Protein denaturation and disturbances to overall protein homeostasis are well-understood consequences of thermal stress. For ectotherms living in highly variable and unpredictable environments, such as the intertidal zone, Hsps could be particularly important as they represent a mechanism by which an organism can buffer the impact of fluctuations in environmental conditions on the protein pool.
It might be considered beneficial for intertidal organisms to possess proteins that are sufficiently rigid to avoid denaturation at the highest temperatures encountered in nature; however, these proteins would likely be too rigid to function appropriately at the lower range of environmental temperatures (Somero 1995). As a result, the need to have proteins that can undergo conformational changes necessary to maintain function at low temperatures implies a balance between protein stability and flexibility in intertidal ectotherms must be achieved (Fields and Somero 1998). To accommodate this, most proteins are inherently unstable at the upper temperatures that these organisms encounter in their natural environment and as a result they are routinely subject to thermal denaturation. By having a mechanism in place to handle modest amounts of protein denaturation, organisms would not have to employ specialist protein isoforms that are functional at the extreme ranges of environmental conditions that these organisms are likely to encounter. Therefore, it is easy to appreciate the importance of having a cellular stress response dedicated to restoring the integrity of the cellular protein pool in intertidal organisms.

It has been suggested that in order to appreciate the importance of Hsps and their relevance to adaptation and stress tolerance it is essential that research be moved to the field and studies must be conducted on natural populations (Feder and Hofmann 1999). There is still uncertainty as to how often Hsps are induced in nature, and therefore how important they are ecologically in an organism's capacity to tolerate environmental change has yet to be fully explored. The intertidal zone represents an excellent environment in which to address these questions.

Tidepool sculpins

Tidepool sculpins (Oligocottus maculosus) are widely distributed throughout the intertidal zones of the Pacific Northwest, from the Gulf of Alaska to central California (Green 1971a). These fish are widely distributed at all elevations in the intertidal zone, most densely
populating tidepools in the upper mid-intertidal region where they routinely experience dramatic daily fluctuations in temperature, salinity and oxygen availability. Tidepool sculpins are known to home to the same tidepool over consecutive low tide periods (Green 1971b) and therefore select for an environment that undergoes drastic fluctuations in environmental conditions on a daily basis. Because of their ability to thrive in such a variable habitat, tidepool sculpins are an excellent organism in which to investigate the plasticity of stress tolerance in a natural fish population and examine the role of Hsps in this plasticity.

**Thesis Objectives**

The main objectives of my PhD thesis were to investigate the role of environmental variation in modulating both the stress tolerance and the Hsp response of tidepool sculpins, and in turn provide novel insight into the ecological significance of Hsps in an animal’s natural environment. The intertidal zone is characterized by a predictable cycle of environmental change that is dependent on the ebb and flow of tides; however, the magnitude of environmental change encountered by intertidal inhabitants is unpredictable and dependent on factors such as weather and season. The research in my thesis exploits this variability of environmental conditions to explore the plasticity of stress tolerance and the Hsp response in a natural environment. This approach allows for the assessment of the implications of inhabiting an environment that is characterized by both predictable and unpredictable environmental fluctuations over a variety of temporal scales.

**Strategy for sequence of experiments**

Chapter 2 addresses whether cross-tolerance can be induced in tidepool sculpins in a laboratory environment. These experiments establish the magnitude of heat shock necessary to
induce an increase in stress tolerance as well as the time frame of induced tolerance and determine whether these are relevant to the natural conditions of environmental change encountered by tidepool sculpins in the intertidal zone. Lastly, I investigated whether there was evidence for a role of Hsps in this inducible stress tolerance.

In Chapter 3, I investigated the importance of inducible stress tolerance in field-acclimatized sculpins to determine whether living in a variable environment confers a natural increase in stress tolerance and whether this stress tolerance was generalized or limited to thermostolerance. I compared the thermal and osmotic tolerance of sculpins captured directly from the tidepool environment to those of fish maintained under constant thermal conditions for 2 weeks. In order to examine what abiotic factors were important in conferring this stress tolerance, I examined both subtidal and tidepool groups of sculpins to determine how differences in the nature of environmental variation affected stress tolerance. Similar to the objectives of Chapter 2, I also examined the role of Hsps in the sculpin's natural stress tolerance and how changes in the amount of environmental variation modulated the Hsp response. By monitoring the Hsp response at both the transcript and protein levels, I was able to investigate how the variable environment regulated the Hsp response at both the transcriptional and posttranscriptional levels.

In Chapter 4, I addressed whether there was a natural diurnal rhythm in Hsp expression that was entrained by tidal cycle. It is unclear whether intertidal organisms are able to take advantage of the predictable timing of environmental change in their environment such that they can anticipate periods of stress that occur to an unpredictable degree on a daily basis. I monitored changes in Hsp mRNA and protein levels over a low tide period as well as following transfer to a constant environment to determine whether an endogenous diurnal rhythm in Hsp expression persisted once organisms were removed from the variable environment. Finally, by
setting up an artificial temperature cycling regime that mimicked the temperature profile of a mid-intertidal tidepool, I examined the role of temperature alone in providing structure to the Hsp response of sculpins.

In Chapter 5, I documented Hsp levels in tidepool sculpins in their natural environment over consecutive low tide periods. This was the first time that Hsp levels were monitored on a daily basis in the intertidal zone and this allowed me to directly address whether routine fluctuations in environmental conditions impact the Hsp mRNA and protein levels. Laboratory studies demonstrated that the Hsp response of organisms adjust to the thermal environment; however, it is unclear whether this phenomenon exists in complex environments in nature. It is clear that the regulation of heat shock proteins has both an intrinsic and an environmental component. In Chapter 5, I investigated this complex and highly integrated relationship, and examined the relative importance of recent and long-term thermal history in regulating the Hsp response.

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CHAPTER 2. CROSS-TOLERANCE IN THE TIDEPOOL SCULPIN: THE ROLE OF HEAT SHOCK PROTEINS

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 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 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, 21-37ppt, and 0.5-19mg O₂/L, respectively. 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 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. Thirdly, 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.

Materials and Methods

Fish collection in the field

Tidepool sculpins (3.0 ± 0.3g, 5.7 ± 0.2cm) were collected using dip nets from tidepools on Wizard Rocks in Barkley Sound, Bamfield, BC, Canada during July 1999 and September 2000 and transferred to outdoor flow-through stock tanks at the Bamfield Marine Station. Fish were held at 10°C and 32ppt under natural photoperiod for 2 weeks prior to experimentation. Fish were fed blue mussels, presented by cracking the shells, ad libitum daily. Feeding was stopped 48h prior to 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 were conducted in July 1999 and were designed to determine if 1) a +12°C heat shock could confer cross-tolerance to a severe osmotic shock, 2) the degree of cross-tolerance to osmotic shock is sensitive to the magnitude of the preliminary heat, and 3) 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 at ambient temperature following a +12°C heat shock required 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 48h. At this point, fish were randomly divided into 4 groups (of 32 fish each) and placed in 10L aquaria, and allowed a further 48-h acclimation period. The experimental protocol is outlined in Figure 2-1. At time zero, 8 fish were randomly sampled from the 4 experimental tanks as a time zero control. Fish were then transferred to similar tanks at either 22°C (HS, 12°C above ambient) or at ambient temperature (10°C). After 2h, all fish were transferred to tanks at ambient temperature (10°C). Eight hours later (10h after the onset of the experiment), fish were transferred to similar tanks at either 90ppt (Salt) or 32 ppt (Sham). Fish were held under these conditions for 2h and then transferred back to tanks at ambient conditions (10°C, 32ppt). This design resulted in 4 different experimental groups: Ctrl (10°C, 32ppt), HS only (22°C, 32ppt), Salt only (10°C, 90ppt) and HS/Salt (22°C, 90ppt). Eight fish were sampled from each tank at 10h and 20h and morbidity was assessed immediately following osmotic shock (12h, noted as ψ in Figure 2-1). 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 2-1 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 2-1 except that fish were exposed to 4% air saturation (0.33mg O₂/L) for 2h as the second stressor. Hypoxia was achieved by bubbling N₂ into experimental tanks. The water surface was covered with plastic wrap to minimize aquatic surface respiration. N₂ flow was monitored throughout the experimental period to ensure that O₂ levels remained constant. O₂ levels were monitored with a Handy MK III OxyGuard® probe (Point Four Systems, Richmond, BC, 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 85ppt (in September 2000, tidepool sculpins could not tolerate 90ppt). 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 2-2. Fish were randomly divided into 4 experimental groups: Ctrl (10°C, 32ppt), HS only (22°C, 32ppt), Salt only (10°C, 85ppt) and HS/Salt (22°C, 85ppt). As shown in Figure 2-2, this experimental design resulted in 7 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 2 stressors. Fish exposed to the mild heat shock were either directly transferred into similar tanks at 85ppt and held under these conditions for 2h (0h, no recovery time), or returned to ambient conditions for 4h, 6h, 8h, 12h, 24h or 48h (32 fish in each group) before being transferred to tanks at 85ppt for 2h. Fish were then transferred back to tanks at ambient conditions (10°C, 32ppt) for 15h. Eight fish were sampled from each group immediately prior to exposure to the osmotic shock (85ppt) and 15h following recovery from the osmotic shock (noted as $\triangledown$ on Figure 2-2). Morbidity was assessed immediately following exposure to the osmotic shock (noted as $\psi$ on Figure 2-2).

**Tissue sampling**

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.3g 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. Upon 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 Inc., USA) in homogenization buffer (0.1% SDS [w/v], 0.02mg/mL PMSF, 0.25mg/mL EDTA, 1µg/mL
pepstatin A, 1μg/mL leupeptin and 1μg/mL aprotinin in 100mM Tris-HCl buffer, pH 7.5), at a ratio of 10mg tissue to 100μL of buffer. Homogenates were then centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to a tube containing an equal volume of 2x 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 0.5M 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 prior to electrophoresis (maximum 1 week). The remaining supernatant was transferred to an empty tube and stored at −20°C until analyzed for total protein (within 2 days). 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 (15μg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular markers (Gibco-BRL, Burlington, ON, 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 75V for 15 min followed by 150V for 1h. 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, 0.2μm pore size) at 17V for 30 min with transfer buffer (48mM Tris, 39mM glycine, 20% methanol [v/v] and 0.0375% SDS [w/v]. pH 9.2) using a semi-dry transfer apparatus (Bio-Rad Trans-Blot). Transfer membranes were
blocked in 2% skim milk in Tween-20 Tris-buffered saline (TTBS) (17.4mM Tris-HCl, 2.64mM Tris Base, 0.5M NaCl and 0.05% Tween-20 [v/v]) with 0.05% sodium azide for 1h. Membranes were then rinsed once and then soaked for 5 min in TTBS. Membranes were then soaked in primary antibody (rabbit IgG for salmonid Hsp70 in 2% skim milk, 1:5000, See Antibodies below for specifics) for 1h. Following three, 5 min washes in TTBS, membranes were soaked in a secondary antibody (goat anti rabbit IgG in TTBS, 1:3000) for 1h. 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) (333µg/mL), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (167µg/mL) solution in alkaline phosphatase buffer (0.01M Tris-HCl, 0.1M NaCl and 21mM MgCl₂, 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 Dr. Peter Candido’s laboratory at the University of British Columbia, Canada) and rabbit IgG for chinook salmon Hsp70 (StressGen, Victoria, BC, Canada) for 1999 and 2000 analysis respectively. The secondary antibodies used were alkaline phosphatase conjugated goat anti rabbit IgG from Kirkegaard and Perry Laboratories (Gaithersberg, MD, USA) and Sigma (St. Louis, MO, USA) for 1999 and 2000 analysis, respectively.
Statistical analyses

Differences in survival between the treatments were compared by Chi-square 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 Chi-square. For Hsp70 quantification, band intensities were obtained using SigmaGel software (Jandel Scientific, USA) 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 ± SEM. Two-way analysis of variance (ANOVA) was used to determine significant (p≤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≤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 (90ppt) resulted in 68% survival; however, sculpins that were exposed to a +12°C heat shock (2h) prior to the osmotic challenge (HS/Salt) had significantly higher survival (96%) compared to fish that were not heat shocked (Salt only; Figure 2-3A). Sculpins exposed to a milder heat shock (+10°C, 2h) did not
demonstrate increased survival compared to non-heat shocked fish when they were exposed to an osmotic challenge (Figure 2-3B). If sculpins were exposed to a stronger heat shock (+15 °C, 2h) prior to the osmotic challenge, the fish experienced only 12.3% survival, a significantly lower survival compared to fish exposed to the osmotic challenge without prior heat shock (Figure 2-3C). 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 8h of recovery at ambient conditions following the heat shock (Heat shock, 10h, Figure 2-4A). However, Hsp70 levels were significantly elevated in fish allowed to recover for 18h at ambient temperatures, and this pattern was not altered by exposure to a 2-h secondary osmotic shock following 8h of recovery (HS only and HS/Salt respectively, 20h, Figure 2-4A). Exposure to a +10°C heat shock did not increase hepatic Hsp70 levels in any group (Figure 2-4B), while a +15°C heat shock significantly increased hepatic Hsp70 levels after 8h recovery at ambient temperatures, and these levels remained elevated for 18h following the heat shock (Heat shock,10h, and HS only, 20h, respectively, Figure 2-4C). This pattern was not altered by exposure to a secondary osmotic stressor (HS/Salt, 20h, Figure 2-4C). 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 (2h, 0.33mg O₂/L) experienced significantly less mortality (71% survival) compared to fish that were exposed to the severe hypoxia without prior exposure to heat shock (47% survival, Figure 2-5). There was no mortality associated with the heat shock.
Fish exposed to a +12°C heat shock had significantly elevated hepatic Hsp70 levels 8h following heat shock (Heat shock, 10h, Figure 2-6), but these levels returned to control values by 18h following heat shock (HS only, 20h, Figure 2-6). However, hepatic Hsp70 levels remained significantly elevated for up to 18h following the primary stressor, when sculpins were exposed to hypoxia following the initial heat shock (HS/Hypoxia, 20h, Figure 2-6). 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 (85ppt) experienced 100% mortality (Figure 2-7), while fish allowed 4 or 6h of recovery did not differ significantly in survival from those exposed to osmotic shock alone. However, tidepool sculpins returned to ambient temperatures for 8h before the osmotic shock had significantly increased survival compared to those exposed to the osmotic stressor alone. This increased survival extended at least 48h 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 8h and 12h following heat shock (Figure 2-8A). 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 (Figure 2-8B). Osmotic shock alone did not increase hepatic Hsp70 levels.
Branchial Hsp70 levels

Branchial Hsp70 levels were only significantly elevated after 24h of recovery at ambient temperature following a +12°C heat shock (Figure 2-9A). In contrast, 15h 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 48h between the stressors (Figure 2-9B). Note that these times of elevated Hsp70 correlate well with times of increased osmotic tolerance (Figure 2-7). 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. Pre-treatment 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 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 8h 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 48h. 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 was 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 (Figure 2-9B). The dynamics of the branchial Hsp70 levels followed an identical time frame as the window of cross-tolerance, requiring 8h to 48h 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 prior to the second stressor and cross-tolerance (Lee and Hahn 1988; Flahaut et al. 1996; Laplace et al. 1996; Krebs and Feder 1998). In contrast, our data suggest that elevated levels of Hsp70 protein are not required prior to exposure to the second stressor for the heat shock to confer cross-tolerance. Hsp70 levels were elevated prior to the secondary stressor in only one of the three experiments (severe hypoxia experiment, Figure 2-6). Specifically, in the first experiment, hepatic Hsp70 levels were not elevated prior to osmotic shock despite the initial +12°C heat shock being able to confer cross-tolerance (Figure 2-4A). Similarly in experiment 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 prior to
osmotic shock and increased osmotic tolerance (Figures 2-8A, 2-9A). In addition, if hepatic Hsp70 levels were highly induced prior to 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 prior to 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 (Figure 2-6) 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 correlated with cross-tolerance. In the 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 (20h, Figure 2-4A) or else did not increase Hsp70 levels relative to controls (Figure 2-8B). These results suggest that Hsp70 levels in the liver do not respond to osmotic shock and that pre-exposure to an initial heat shock does not prime the hepatic Hsp70 response to a secondary osmotic stressor. In the gills, Hsp70 level was increased following osmotic shock in all heat shocked groups allowed at least 8h of recovery between the two stressors (Figure 2-9B), while osmotic shock alone did not increase Hsp70 levels. Taken together, 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
increased Hsp70 response. This increased Hsp70 response is then correlated 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 > 4h 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 (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). Upon heat shock, translation of pre-existing mRNAs is repressed such that there is the preferential translation of hsp transcripts. Therefore, pre-existing 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 upon heat shock Hsp72 transiently associated with the ribosomal subunits and thereby preserves 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 build-up 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. On the other hand, 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 and 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 hours 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 temperature. Cross-tolerance was present in a defined temporal window, requiring 8h to 48h 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 prior to 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.
References


Tomanek, L. and Somero, G.N. 1999. Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal

**Figure 2-1:** Experimental protocol for experimental series 1 (cross-tolerance) in tidepool sculpins. \(\triangledown\) indicates sampling points for each treatment. \(\psi\) indicates when survival was assessed.
### Figure 2-2: 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, 32ppt) between exposure to a heat shock (HS: 2h, 22°C) and exposure to a severe hyperosmotic challenge (OS: 2h, 85ppt). ▽ indicates sampling points for each treatment. ψ indicates when survival was assessed.
Figure 2-3: Survival (%) of tidepool sculpins following a 2-h heat shock. Panel A: +12°C heat shock. Panel B: +10°C heat shock. Panel C: +15°C heat shock. Light grey bars (Ctrl) are control fish that were handled identically but not exposed to heat shock or osmotic shock. Dark grey bars (HS only) are fish exposed to the heat shock alone. Hatched light grey bars (Salt only) are fish exposed to a severe hyperosmotic shock (2h, 90ppt) only. Hatched dark grey bars (HS/Salt) are fish exposed to both the heat shock and osmotic shock. * indicates a significant difference in survival between HS/Salt and Salt only (p≤0.05).
**Figure 2-4**: Hepatic Hsp70 levels of tidepool sculpins exposed to a mild heat shock [+12°C (A), +10°C (B) or +15°C (B)] and a severe hyperosmotic challenge (90ppt). Hsp70 levels were measured prior to experimentation (Time 0), 8h following the mild heat shock and 8h 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 (mean ± SEM). Note difference in the y-axis of panel C versus panels A and B. * indicates significant difference in the level of Hsp70 from Time 0 (p≤0.05). See Figure 2-1 for sampling details.
Figure 2-5: Survival (%) of tidepool sculpins following a 2-h heat shock (+12°C, HS only), exposure to a severe hypoxia (4% saturation) for 2h (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. * indicates a significant difference in survival between +12°C/Hypoxia and Hypoxia only (p≤0.05).
Figure 2-6: Hepatic Hsp70 levels of tidepool sculpins exposed to a +12°C heat shock and hypoxia (4% saturation). Hsp70 levels were measured prior to experimentation (Time 0), 8h following the mild heat shock and 8h following the hypoxic stressor. Sham fish were treated in an identical manner but without heat shock. Ctrl fish 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 (mean ± SEM). * indicates significant differences in hepatic Hsp70 levels from Time 0 (p<0.05). See Figure 2-1 for sampling details.
Figure 2-7: Survival (%) of tidepool sculpins exposed to a severe hyperosmotic shock (85ppt) (Salt only) and exposed to a +12°C heat shock prior to hyperosmotic challenge with either no (0h), 4h, 6h, 8h, 12h, 24h, or 48h of time at ambient water conditions between stressors. * indicates significant difference in survival from Salt only (p≤0.05).
Figure 2-8: Hepatic Hsp70 levels of tidepool sculpins exposed to a severe hyperosmotic shock (85ppt) (Salt only) and exposed to a +12°C heat shock prior to hyperosmotic challenge with 0h, 4h, 6h, 8h, 12h, 24h, or 48h at ambient water conditions between stressors. Hsp70 levels were measured [A] following recovery at ambient conditions for 0h, 4h, 6h, 8h, 12h, 24h, or 48h after heat shock, immediately prior to exposure to a hyperosmotic challenge and [B] 15h following exposure to the hyperosmotic challenge. Ctrl 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 (mean ± SEM). A difference in letters denotes significant differences in hepatic Hsp70 levels (p<0.05). See Figure 2-2 for sampling details.
Figure 2-9: Branchial Hsp70 levels of tidepool sculpins exposed to a severe hyperosmotic shock (85ppt) (Salt only) and exposed to a +12°C heat shock prior to hyperosmotic challenge with 0h, 4h, 6h, 8h, 12h, 24h, or 48h at ambient water conditions between stressors. Hsp70 levels were measured [A] following recovery at ambient conditions for 0h, 4h, 6h, 8h, 12h, 24h, or 48h after heat shock, immediately prior to exposure to a hyperosmotic challenge and [B] 15h following exposure to the hyperosmotic challenge. Ctrl 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 branchial Hsp70 from tidepool sculpins (mean ± SEM). A difference in letters denotes significant differences in branchial Hsp70 levels (p≤0.05). See Figure 2-2 for sampling details.
A

Recovery time between stressors

B

Recovery time between stressors
CHAPTER 3. ENVIRONMENTAL VARIABILITY, STRESS TOLERANCE, AND THE HEAT SHOCK PROTEIN RESPONSE IN TIDEPOOL SCULPINS

Introduction

The rocky intertidal has proven to be an ideal environment in which to examine the impact of environmental variation on an organism’s physiology. The vertical zonation of species’ distribution within the rocky intertidal clearly reflects the ability of organisms to tolerate the fluctuations in environmental conditions associated with tidal cycle (Connell 1972; Newell 1979). For decades, physiologists have studied intertidal animals in order to understand the environmental parameters that set limits on an organism’s physiology and the associated underlying mechanisms (For review see Somero 2002). It is clear that some aspect of environmental variation plays an important role in establishing the stress tolerance of eurythermal species such as those inhabiting tidepools. For example, it has long been known that eurythermal fish sampled directly from a fluctuating environment have a higher upper temperature tolerance (measured as critical thermal maximum, CTM) than after lab-acclimatization to a constant environment (Cyprinodon macularius, Lowe and Heath 1969; Gambusia affinis affinis, Otto 1973). Temperature appears to be one of the critical environmental factors involved in this phenomenon (Feldmeth et al. 1974; Otto 1974); however, it is still unclear how the environment transduces this effect. Given the potential significance of heat shock proteins (Hsps) as one of the mechanisms underlying the tight link between environmental temperature and species distribution in the intertidal zone (Hofmann et al. 2002), it is possible that these proteins could play a role in the plasticity of thermotolerance in intertidal species.

Heat shock proteins are an important component of the cellular stress response and 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 (Lindquist 1986; Hightower 1991; Sherman and Goldberg 1996; 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 this may translate into a functional role for Hsps in an animal’s natural tolerance to environmental change (Feder and Hofmann 1999). Organisms that inhabit distinct thermal niches differ in the temperature at which Hsps are induced, with organisms inhabiting warmer thermal environments having higher threshold temperatures for Hsp induction (Roberts et al. 1997; Tomanek and Somero 1999; Nakano and Iwama 2002). From these studies, the plastic nature of the Hsp response is becoming apparent; however, the mechanistic underpinnings of the regulation of Hsp gene expression and the significance of this environmental regulation on natural stress tolerance at the whole organism level are still largely unknown.

Most studies that have investigated the functional significance of Hsps in nature have focused on thermotolerance (Hofmann and Somero 1996; Tomanek and Somero 1999; Buckley et al. 2001; Nakano and Iwama 2002), with little attention being paid to a more generalized increase in tolerance to other stressors. In natural environments, organisms seldom experience a single stressor; more commonly they experience multiple stressors simultaneously or in sequence. However, there is little information on whether inhabiting a variable environment confers a generalized increase in stress tolerance or whether this acquired tolerance is stressor specific and confined to temperature tolerance. Based on the extensive literature on inducible stress tolerance, showing that exposure to a mild stressor can increase the tolerance of organisms to a subsequent severe stressor of a similar (e.g. thermotolerance, Hahn and Li 1990; Krebs and
Feder 1998) or different (e.g. cross-tolerance, Kampinga et al. 1995; Sabehat et al. 1998) nature, we hypothesized that organisms living in a fluctuating environment might utilize this phenomenon to cope with the unpredictable changes that accompany each low tide. We have previously shown that a mild heat shock, typical of a daily change in water temperature over a low tide period, can confer increased tolerance to both osmotic and hypoxic stressors in tidepool sculpins (Oligocottus maculosus, Todgham et al. 2005). Although the mechanisms underlying such cross-tolerance are not fully understood, there is a clear association between increased levels of Hsps and acquired stress tolerance (Brown et al. 1992; Kampinga et al. 1995; Laplace et al. 1996; Sabehat et al. 1998; Todgham et al. 2005).

The present study was conducted to examine the role of the Hsp response in the plasticity in stress tolerance associated with living in a variable environment in the tidepool sculpin. We investigated whether inhabiting a variable environment confers a generalized increase in stress tolerance or simply increases thermal tolerance. We examined sculpins from a mid-intertidal tidepool and the subtidal zone (environments that are both variable, but to differing degrees and in different environmental conditions) and compared their stress tolerance to that of sculpins held under constant conditions for two weeks. Secondly, we investigated whether transfer to a constant environment would modify the sculpins' Hsp induction profiles (mRNA expression and protein levels) in response to stressors that varied in nature and magnitude. Finally, we discuss whether the changes to the Hsp response that result from sculpins being held under constant conditions may be in part responsible for the differences in stress tolerance that we see between these fish.
Materials and methods

Fish collection

Tidepool sculpins (*O. maculosus* Girard) 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 environmental conditions (Green 1971). The fish used in this study (2.4 ± 0.1g, 5.6 ± 0.1cm) were collected using dip nets and minnow traps from Wizard Rocks in Barkley Sound, Bamfield, BC, Canada during July 2003. Two weeks prior to experimentation sculpins were collected from a large mid-intertidal pool and transferred to outdoor flow-through stock tanks at the Bamfield Marine Sciences Centre. This group of fish was held in ambient ocean conditions (11°C, 32ppt) under natural photoperiod for 2 weeks and was referred to as the Lab-Acclimatized group. Fish were fed blue mussels, presented by cracking the shells, *ad libitum* daily. During this 2-week acclimatization period, there was a submerged SmartButton data logger (ACR Systems Inc., Surrey, BC, Canada) deployed in the large mid-intertidal collection tidepool recording water temperature in the field (Figure 3-1). One day before experimentation, sculpins were collected from the same large mid-intertidal pool as the Lab-Acclimatized group, and from the subtidal zone below the tidepool. These fish were referred to as the Tidepool-Acclimatized and Subtidal-Acclimatized groups respectively. Tidepool- and Subtidal-Acclimatized fish were held in identical outdoor stocks at ambient ocean conditions overnight before experimentation. All experiments were conducted in accordance with an approved University of British Columbia Animal Care protocol (#A01-0172).
Experimental design

The experimental design was identical for each of the three groups of sculpins (Lab-Acclimatized, Tidepool-Acclimatized, and Subtidal-Acclimatized). Fish were netted from the outdoor stock tanks and randomly divided into four treatments: a mild heat shock, a severe heat shock, a severe osmotic shock and a control treatment. A mild heat shock (22°C, 32ppt for 2 hours) was chosen to monitor differences in the fish’s ability to respond to a stressor. Severe heat (28°C, 32ppt for 3 hours) and osmotic (11°C, 91ppt for 2 hours) shocks were chosen to investigate differences in the fish’s ability to tolerate a near lethal stressor. For the control treatment, fish were moved into experimental tanks held under control conditions (11°C, 32ppt for 2 hours) to account for handling stress.

At the time of day that corresponded to the onset of the midday low tide period, eight fish were sampled as a time zero reference. The remainder of the fish were exposed to one of the four treatments outlined above. Immediately following treatment, eight fish were sampled (0h sample) and morbidity was assessed. The remainder of the fish from each treatment were returned to the outdoor stock tanks (11°C, 32ppt), placed in separate floating baskets and sampled 8 and 24h following treatment.

Tissue sampling

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.3g MS-222/L of water) and following onset of anaesthesia 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 Inc., USA) in homogenization buffer (0.1% SDS [w/v], 0.02mg/mL PMSF, 0.25mg/mL EDTA, 1μg/mL
pepstatin A, 1µg/mL leupeptin and 1µg/mL aprotinin in 100mM Tris-HCl buffer, pH 7.5), at a ratio of 10mg tissue to 100µL of buffer. Homogenates were then centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to a tube containing an equal volume of 2x 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 0.5M 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 prior to electrophoresis (maximum 1 week). The remaining supernatant was transferred to an empty tube and stored at -20°C until analyzed for total protein (within 2 days). 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 and Hsp90

Levels of the constitutively expressed 70-kDa heat shock cognate (Hsc70), the inducible 70-kDa heat shock protein (Hsp70) and the 90-kDa heat shock protein (Hsp90) were measured using the discontinuous SDS-PAGE method of Laemmli (1970). Equal amounts of total protein (15µg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular markers (Invitrogen Life Technologies, Carlsbad, CA, USA) and a pooled sample of gill homogenate from heat shocked tidepool sculpins were added to every gel as an internal standard to allow comparison between gels (referred to as “Internal Std” in figures). Proteins were separated by SDS-PAGE at 75V for 15 min followed by 150V for 1h. The separated proteins were transferred onto nitrocellulose (Bio-Rad, 0.2µm pore size) at 17V for 30 min with transfer buffer (48mM Tris, 39mM glycine, 20% methanol [v/v] and 0.0375% SDS [w/v], pH 9.2) using a semi-dry transfer apparatus (Bio-Rad Trans-Blot). Transfer membranes were blocked in 2% skim milk in Tween-20 Tris-buffered saline (TTBS) (17.4mM Tris-HCl, 2.64mM Tris Base, 0.5M NaCl and
0.05% Tween-20 [v/v]) with 0.05% sodium azide for 1h. Following blocking, membranes were rinsed once and soaked for 5 min in TTBS. Membranes were then soaked in primary antibody: rabbit IgG for chinook salmon Hsp70 (1:5000, StressGen, Victoria, BC, Canada) which recognizes both Hsc70 and Hsp70, or rat IgG for human Hsp90 (1:250, StressGen, Victoria, BC, Canada), in 2% skim milk for 1h. Following three 5 min washes in TTBS, membranes were soaked in an alkaline phosphatase conjugated secondary antibody: goat anti rabbit IgG (1:5000, Sigma, St. Louis, MO, USA), or goat anti rat IgG (1:5000, StressGen, Victoria, BC, Canada), in TTBS for 1h. 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) (333μg/mL)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (167μg/mL) solution in alkaline phosphatase buffer (0.01M Tris-HCl, 0.1M NaCl and 21mM MgCl₂, 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 5μg total RNA using oligo(dT₁₈) primer and RevertAid H Minus M-MuLV reverse transcriptase following the manufacturer’s instructions (MBI Fermentas Inc., Burlington, ON, Canada).

Real-time PCR analysis of hsc70, hsp70 and hsp90 gene expression

Partial sequences for hsc70 and hsp70 were kindly provided by Dr. 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 alpha (*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), *Danio 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 Inc., Edmonton, AB, Canada). Polymerase chain reactions (PCRs) were carried out in a PTC-200 MJ Research thermocycler using 1.25U 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 Inc., Mississauga, ON, Canada). The extracted PCR product was ligated into a T-vector (pGEM-T easy; Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat shock competent *Escherichia coli* (strain JM109; Promega; Fisher Scientific, Nepean, ON, Canada) and colonies were grown on ampicillin LB-agar plates. Several colonies containing the ligated PCR product were selected and grown overnight in DYT liquid culture. Plasmids were isolated from the liquid culture using GenElute Plasmid Miniprep kit (Sigma-Aldrich, Oakville, ON, Canada) and sequenced at the NAPS Unit DNA Sequencing Facility at the University of British Columbia (Vancouver, BC, Canada). All partial sequences were deposited into GenBank as follows: hsc70 (Accession No. DQ013308), hsp70 (Accession No. DQ013309), hsp90 (Accession No. DQ013311) and EF-1α (Accession No. DQ013310).
Gene specific primers were designed from these sequences using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA) and are reported in Table 3-1. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence detection system (Applied Biosystems Inc.). qRT-PCR reactions were run with 2μL cDNA, 4pmoles of each primer and 2X SYBR Green Master Mix (Applied Biosystems Inc.) to a total volume of 22μL. 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 sec, 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

Differences in survival between the treatments were compared by Chi-square 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 Chi-square. For Hsp90, Hsc70 and Hsp70 protein quantification, band intensities were obtained using SigmaGel software (Jandel Scientific, USA) and values were standardized using band intensity values from a pooled sample of tidepool sculpin gill homogenate that were run concurrently on each gel (referred to as “Internal Std” in figures). Results for Hsp90, Hsc70 and Hsp70 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 levels did not change significantly in response to treatment (when 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 analysis of variance (ANOVA) was used to determine significant (p<0.05) differences in both Hsp protein and mRNA levels within a treatment group. For the two-way ANOVA, fish group and sampling time were used as independent categorical variables, and Hsp protein or mRNA levels were used as the dependent variable. Means were compared using the post-hoc Student-Newman-Keuls (SNK) multiple comparison test (p<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

Water temperature data

Water temperatures were recorded in the mid-intertidal tidepool (Figure 3-1) from which both the Lab- and Tidepool-Acclimatized sculpins were collected in order to describe the thermal conditions in the pool in the 2-week period between collection of these two groups of sculpins, and therefore the thermal history of the Tidepool-Acclimatized group. During this 2-week period, the mean daily temperature change was 8.3 ± 0.6°C, the daily mean average and high temperatures were 17.0 ± 0.1°C and 22.1 ± 0.6°C respectively and the maximum temperature reached in the tidepool was 31.5°C. In addition to temperature, salinity and oxygen levels within the tidepool also varied from as much as 21 – 39ppt and 0.3 to 19.4mg O2/mL respectively over this 2-week period. No temperature data logger was deployed in the subtidal zone, but at the time of collection water temperature was recorded at 24.2°C.
Survival

Exposure of Lab-Acclimatized tidepool sculpins to a severe heat shock (28°C, 3h) resulted in only 43% survival; however, tidepool sculpins collected directly from the field (Tidepool- and Subtidal-Acclimatized groups) experienced significantly higher survival, 98% and 100% respectively, when exposed to the same heat shock (Figure 3-2A). No Lab-Acclimatized fish survived to 24 hours following the severe heat shock. Lab-Acclimatized sculpins exposed to a severe hyperosmotic shock (92ppt, 2h) showed only 50% survival and there was no significant difference in the level of survival observed in the Subtidal-Acclimatized group (44.4%) when these fish were exposed to the same stressor (Figure 3-2B). Exposure of Tidepool-Acclimatized fish to the same severe osmotic shock resulted in 73% survival and this was significantly higher than both the Lab- and Subtidal-Acclimatized groups.

Gill Hsp90 mRNA and protein levels

Prior to being exposed to any of the treatments (Time 0), hsp90 mRNA levels in the Tidepool-Acclimatized group were significantly higher than both the Lab- and Subtidal-Acclimatized groups (Figure 3-3A,C); whereas Hsp90 protein levels were significantly higher in the Subtidal-Acclimatized group compared to the other two groups (Figure 3-3B,D). Exposure to a mild heat shock (25°C for 2h) resulted in similar patterns of hsp90 mRNA expression in all groups (Figure 3-3A). Hsp90 mRNA levels increased immediately and significantly following heat shock and remained elevated throughout the recovery period. There were no differences in the levels of hsp90 mRNA following the mild heat shock between any of the groups with the exception of levels being significantly lower in the Subtidal-Acclimatized fish when compared to the Lab-Acclimatized group immediately following heat shock (0h, Figure 3-3A). Exposure to a mild heat shock resulted in a 3-fold increase in Hsp90 protein levels in Lab-Acclimatized sculpins 24 hours following heat shock (Figure 3-3B). Tidepool-Acclimatized sculpins
experienced a modest but significant elevation in Hsp90 protein levels at both 8h and 24h
following a mild heat shock; however, there was no similar increase in Hsp90 protein levels in
the Subtidal-Acclimatized group. Both the Tidepool- and Subtidal-Acclimatized fish had
significantly lower Hsp90 protein levels at 24h recovery when compared to the Lab-
Acclimatized group.

A severe heat shock (28°C for 3h) significantly increased \( hsp90 \) mRNA levels in all 3
groups at all sampling points following heat shock (Figure 3-3C); however, the increase in \( hsp90 \)
mRNA was significantly greater in the Tidepool-Acclimatized group when compared to Lab-
Acclimatized fish. Exposure to the severe heat shock did not increase Hsp90 protein levels in
the Lab-Acclimatized group despite the large increase in \( hsp90 \) mRNA. In contrast, both the
Tidepool- and Subtidal-Acclimatized groups experienced a significant immediate (0h recovery)
and prolonged (8h and 24h recovery) increase in Hsp90 protein levels following severe heat
shock and these levels were significantly elevated above the Lab-Acclimatized group at all time
points following exposure to the severe heat shock.

There was no significant increase in either \( hsp90 \) mRNA or Hsp90 protein in response to
exposure to a severe osmotic stressor in any of the groups at any of the sampling points (Table 3-
2). Handling stress caused a slight but significant increase in \( hsp90 \) mRNA levels in the Lab-
Acclimatized group at 0h recovery; but this was not reflected at the protein level (Table 3-3).
There was no increase in \( hsp90 \) mRNA or Hsp90 protein as a result of handling in either the
Tidepool- or Subtidal-Acclimatized groups.

*Gill Hsc70 and Hsp70 mRNA and protein levels*

Prior to being exposed to any of the treatments (Time 0), Hsc70 and Hsp70 protein levels
were significantly higher in the Subtidal-Acclimatized group when compared to both the Lab-
and Tidepool-Acclimatized groups (Figure 3-4C,F). Exposure to a mild heat shock significantly
increased \textit{hsc70} mRNA levels in Lab-Acclimatized sculpins and these levels were increased 7, 5 and 2-fold immediately (0h), 8h, and 24h following heat shock respectively when compared to levels before heat shock (Figure 3-4A). The same mild heat shock also increased \textit{hsc70} mRNA levels in both of the Tidepool- and Subtidal-Acclimatized groups but these levels were significantly lower than the Lab-Acclimatized group at all sampling points and had returned to pre-heat shock levels by 24h recovery from heat shock. A mild heat shock resulted in a significant increase in \textit{hsp70} mRNA levels in all groups (Figure 3-4B). \textit{Hsp70} mRNA levels peaked immediately following heat shock and declined over the remainder of the recovery period with levels still being significantly elevated 24h following heat shock. At all recovery points \textit{hsp70} mRNA levels were significantly lower in both the Tidepool- and Subtidal-Acclimatized groups when compared to the Lab-Acclimatized group. Exposure to a mild heat shock (Figure 3-4C) significantly increased Hsc70 and Hsp70 protein levels in Lab-Acclimatized sculpins at all sampling times following heat shock with peak levels at 8h recovery. Tidepool-Acclimatized sculpins exposed to the same mild heat shock had elevated Hsc70 and Hsp70 protein levels at both 8h and 24h following heat shock; however, these levels were significantly lower immediately following (0h) and 24h following the mild heat shock when compared in the Lab-Acclimatized fish. Sculpins from the Subtidal-Acclimatized group did not experience an increase in Hsc70 and Hsp70 protein levels in response to the mild heat shock.

Exposure to a severe heat shock increased \textit{hsc70} mRNA levels in the Lab-Acclimatized group (Figure 3-4D), with peak levels reached immediately following heat shock. In the Tidepool-Acclimatized group, \textit{hsc70} mRNA levels were also increased following exposure to the same severe heat shock but maximal levels were not reached until 8h following heat shock. These elevated levels were maintained for the duration of the experiment. In the Subtidal-Acclimatized group, exposure to the severe heat shock increased \textit{hsc70} mRNA levels significantly higher than Lab-Acclimatized fish immediately following the heat shock and these
levels were maintained 8h into recovery. By 24h recovery, hsc70 mRNA levels in the Subtidal-Acclimatized group had begun to decrease but still remained significantly elevated above Time 0 levels. Exposure to a severe heat shock resulted in a massive induction of hsp70 mRNA expression in all groups (Figure 3-4E). In Lab-Acclimatized sculpins, hsp70 mRNA levels remained elevated 8h into recovery; whereas, in both the Tidepool- and Subtidal-Acclimatized groups these levels declined throughout recovery and were significantly lower than the Lab-Acclimatized group by 8h. Exposure to the severe heat shock (Figure 3-4F) resulted in a significant increase in Hsc70 and Hsp70 protein levels in all 3 groups, at all sampling times, and with peak levels reached 8h following heat shock. There was no significant difference in the magnitude of Hsc70 and Hsp70 protein increase between the groups.

Exposure to a severe osmotic shock (91ppt for 2h, Table 3-2) resulted in slight but significant increases in hsc70 mRNA levels in both the Lab- and Tidepool-Acclimatized groups 8h following the osmotic shock. Hsp70 mRNA levels were below the limits of detection following severe osmotic shock in all groups (data not shown). The severe osmotic shock resulted in very slight but significant increases in Hsc70 and Hsp70 protein levels in both Lab- and Tidepool-Acclimatized sculpins at all sampling points following osmotic shock. In contrast, the same osmotic shock did not increase Hsc70 and Hsp70 protein levels in the Subtidal-Acclimatized fish. Handling did not alter hsc70 mRNA levels in the Lab-Acclimatized group; however there were slight and variable changes in hsc70 mRNA levels in both the Tidepool- and Subtidal-Acclimatized groups (Table 3-3). There was no effect of handling on hsp70 mRNA levels (data not shown). Handling alone resulted in a modest but significant increase in Hsc70 and Hsp70 protein levels in the Lab and Tidepool-Acclimatized sculpins but not in the Subtidal-Acclimatized fish.
Discussion

\textit{Stress tolerance of lab- and field-acclimatized sculpins}

Two weeks under constant ambient ocean conditions is sufficient time for tidepool sculpins to lose some of their natural stress tolerance. Lab-Acclimatized sculpins were less tolerant of both severe thermal and osmotic shocks than sculpins collected from the same tidepool (Tidepool-Acclimatized) and challenged directly. During the two week period that the Lab-Acclimatized sculpins were held under constant conditions, the Tidepool-Acclimatized fish were experiencing water quality changes, including temperature changes that varied from as little as 5°C (15-20°C) to as much as 18.5°C (13-31.5°C) over a single day. In addition to temperature, salinity and oxygen levels within the tidepool also fluctuated with tidal cycle. It is difficult to determine which of the variable factors imparted the increased stress tolerance to the sculpins or whether it was a combined effect of multiple factors. Sculpins collected from the sandy subtidal zone directly below the tidepool (Subtidal-Acclimatized) were as thermotolerant as the Tidepool-Acclimatized fish but they were not as tolerant of osmotic shock as these tidepool fish, more closely resembling the Lab-Acclimatized sculpins in this respect. The shallow sandy subtidal zone where these sculpins were collected undergoes similar fluctuations in both temperature and oxygen levels with tidal cycle as a mid-intertidal tidepool; however the salinity remains relatively constant (A.E. Todgham, personal observation). Therefore, although subtidal and tidepool inhabiting sculpins likely have a similar thermal history, accounting for their similarities in thermotolerance; these two groups of fish live in different osmotic environments and this may be reflected in their osmotic tolerance.

There is substantial evidence to support the phenomenon of cross-tolerance under laboratory conditions, demonstrating that exposure to a mild heat shock can confer an increased tolerance to a number of secondary severe stressors other than heat (Kampinga et al. 1995;
Sabehat et al. 1998). Indeed, we have shown that a mild heat shock can confer an increased tolerance to both subsequent osmotic and hypoxic shock in tidepool sculpins within the laboratory (Todgham et al. 2005). There has been no research to document the phenomenon of cross-tolerance in the natural environment; but from the differences in stress tolerance between Tidepool- and Subtidal-Acclimatized sculpins observed in this study, it appears that cross-tolerance is not as easily induced in nature.

**Effect of laboratory-acclimatization on the Hsp response**

By removing tidepool sculpins from the fluctuating intertidal environment and acclimatizing them to relatively stable ambient ocean conditions we observed not only a decrease in stress tolerance but also a shift in the induction profiles of Hsps in response to heat shock within a two-week period. The most obvious differences between the Lab- and Tidepool-Acclimatized fish were observed in the heat shock-induced Hsp90 response. A mild heat shock was strong enough to induce \( hsp90 \) mRNA expression in both groups of fish; however, the Lab-Acclimatized group experienced a significantly larger increase in Hsp90 protein. In contrast, in response to the severe heat shock both groups induced \( hsp90 \) mRNA but only the Tidepool-Acclimatized group experienced a large and significant increase in Hsp90 protein. This lack of an Hsp90 protein response in the Lab-Acclimatized group following a severe heat shock could represent a generalized impairment of the translational machinery of the cell or a generalized increase in protein degradation; however, this group of fish was able to mount an Hsc70 and Hsp70 protein response to this severe stressor. Therefore, this suggests a specific inhibition of \( hsp90 \) translation or a decrease in Hsp90 protein stability upon severe heat shock in sculpins inhabiting a constant environment. The combination of the observed Hsp90 response to a mild heat shock and the inability to mount an Hsp90 protein response following a severe heat shock in
the Lab-Acclimatized group represents a downward shift in the lower and upper bounds (functional window) of the Hsp90 response upon removal from a naturally variable environment.

Acclimatization to constant conditions also led to a sensitization of the Hsc70 and Hsp70 response to temperature change. Although both groups increased hsc70 and hsp70 mRNA expression as well as Hsc70 and Hsp70 protein levels following a mild heat shock, the magnitude of induction was significantly higher and more prolonged in the Lab-Acclimatized group when compared to the fish sampled directly from the intertidal zone. These results suggest that the acclimatization of sculpins to the constant conditions of the ambient ocean resulted in an increase in the sensitivity of the Hsc70 and Hsp70 response to a mild heat shock. This increase in temperature sensitivity of the Lab-Acclimatized fish was also apparent when examining the Hsc70 and Hsp70 response to a more severe heat shock. A severe heat shock resulted in a more rapid induction of hsc70 mRNA and more prolonged induction of hsp70 mRNA in the Lab-Acclimatized sculpins; however, these sculpins were unable to mount as large an Hsc70 and Hsp70 protein response as the Tidepool-Acclimatized group. Therefore, in sculpins that are removed from the variable intertidal environment and acclimatized to stable conditions there is either a decrease in the translational efficiency of hsc70 and hsp70 mRNA or an increase in Hsc70 and Hsp70 protein degradation when exposed to a severe heat shock.

It is well documented that cellular levels of Hsps are subject to acclimation and that the threshold temperature for Hsp induction is lower in organisms acclimated to lower temperatures (Roberts et al. 1997; Tomanek and Somero 1999; Nakano and Iwama 2002). Therefore, it is not surprising that acclimatizing sculpins to a constant environment at the lower end of their environmental temperature range increased the sensitivity of their Hsp response to heat shock. Buckley et al. (2001) also observed a downward shift in the threshold induction temperatures of Hsps in intertidal mussels with lab acclimation to 10°C for 6 weeks. The present study confirms
that there is a significant amount of plasticity in the temperature sensitivity of the Hsp response and provides a shorter time frame of the effect of environmental history on the Hsp response.

The mechanism by which environmental history is able to structure the Hsp response is still unknown; however the downward shift in the functional window of Hsp90 and in the lower bounds of the Hsp70 response with acclimatization to stable conditions provides novel insights into what components of the Hsp response the environment may modulate. The shift in the functional window of both Hsp90 and Hsp70 in the Lab-Acclimatized fish suggests that being held for two weeks under constant conditions is sufficient to lower the temperature at which heat shock gene expression is induced ($T_{on}$). Inducible Hsp gene expression is transcriptionally regulated in part by heat shock factor-1 (HSF1) (for review see Wu 1995). The activation state of HSF1 is regulated by cellular levels of Hsps, and this autoregulatory scheme of Hsp induction is referred to as the “cellular thermometer” model of gene expression (Craig and Gross 1991). The “cellular thermometer” model predicts two primary mechanisms by which $T_{on}$ can be lowered: 1) lower endogenous levels of Hsps (Hsp40, Hsp70 and Hsp90) or 2) elevated concentrations of HSF1. It has been shown previously that organisms sampled in the field have higher endogenous levels of Hsps compared to organisms held under constant conditions in the lab (Buckley et al. 2001; Buckley and Hofmann 2002; Tomanek and Somero 2002). In the present study there was no consistent evidence to suggest that the Lab-Acclimatized sculpins had lower basal levels of Hsps. Therefore, if the environment is transcriptionally modulating the Hsp response of sculpins through the “cellular thermometer” model it must be doing so by directly acting on HSF1. A few studies have demonstrated that intertidal organisms that are held under constant laboratory conditions have either significantly elevated levels of HSF1 or HSF-DNA binding activity compared to organisms sampled in the field (Buckley et al. 2001; Buckley and Hofmann 2002; Tomanek and Somero 2002); however, there is little information on the mechanism by which the environment modulates HSF1.
Effect of differing field conditions on the Hsp response

In addition to the substantial differences in the Hsp responses between the Lab- and Tidepool-Acclimatized sculpins, there were some subtle differences in the Hsp responses to heat shock between the Tidepool- and Subtidal-Acclimatized groups. These differences provide insights into the subtle effects that slight variations in environmental history have on the Hsp response. There was no clear difference in the sensitivity of the Hsp90 or Hsc70 and Hsp70 response to temperature between these two groups of field-acclimatized fish. We did observe a slight difference in the hsc70 mRNA response to severe heat shock; however, the physiological effects of this change are hard to predict because of our inability to distinguish between Hsc70 and Hsp70 at the protein level. The most striking difference between the Tidepool- and Subtidal-Acclimatized fish was the difference in endogenous levels of Hsp mRNA and protein in these fish. Subtidal-Acclimatized fish had higher endogenous levels of both Hsp90 protein and the combined Hsc70 and Hsp70 protein when compared to sculpins collected from tidepools. In contrast, Tidepool-Acclimatized sculpins had significantly higher endogenous hsp90 mRNA expression. Therefore, there appears to be a general trend that Subtidal-Acclimatized fish maintain higher endogenous protein levels while mRNA expression is low, whereas, Tidepool-Acclimatized fish maintain higher hsp mRNA expression and lower endogenous levels of Hsp protein.

These differences in Hsp profiles resulted in different Hsp responses to temperature. Upon exposure to a mild heat shock there was no or little increase in Hsp90 and Hsc70 and Hsp70 proteins respectively in the Subtidal-Acclimatized group. On the other hand, Tidepool-Acclimatized sculpins increased Hsp protein levels significantly upon exposure to a mild heat shock to the levels found endogenously in the subtidal fish. Since induction of hsp90, hsc70, and hsp70 mRNA expression following a mild heat shock was similar in these two groups of fish, the
translational differences may reflect that subtidal sculpins have sufficient endogenous levels of Hsps to cope with a mild heat shock and therefore do not require de novo Hsp protein synthesis.

These posttranscriptional differences between the Subtidal- and Tidepool-Acclimatized fish are also apparent in the Hsp responses to a severe heat shock. A severe heat shock induced a 4-fold increase in hsp90 mRNA expression in both groups of fish; however, ultimately there was a larger increase in Hsp90 protein in the Tidepool-Acclimatized fish when compared to the Subtidal-Acclimatized fish, 4.2-fold vs. 3-fold increases, respectively. Similarly, Tidepool-Acclimatized sculpins experienced a 4.6-fold increase in Hsc70 and Hsp70 proteins levels upon severe heat shock, whereas Subtidal-Acclimatized fish only increased Hsc70 and Hsp70 levels 2.8-fold in response to the same stressor. While the induction profiles of hsc70 and hsp70 mRNA expression in response to severe heat shock were not similar between these two groups of fish, they did not account for the differences we saw at the protein level. Therefore, although there were no significant differences in the absolute levels of Hsps reached in both groups of field-acclimatized fish in response to severe heat shock, the larger increases in the induction of Hsps in the Tidepool-Acclimatized group suggests that the tidepool fish may have had a better translational capacity for hsp mRNA.

Taken together with the results of lab-acclimatization, it appears that the environment can modulate the Hsp response of sculpins at both the transcriptional and posttranscriptional level. We observed significant transcriptional regulation of the Hsp response upon transfer of sculpins to a constant environment, whereas, we observed subtle differences in the Hsp response of the field-acclimatized fish at the posttranscriptional level. These results imply that the mechanism by which the environment modulates the Hsp response could depend on the scale of variability in the environment. Exactly how this is achieved requires further investigation.
Decreases in stress tolerance and the associated changes in the Hsp response

There was a clear association between an increase in the temperature-sensitivity of organismal tolerance and the cellular Hsp response. Within 2 weeks of being removed from the variable environment of the intertidal zone, tidepool sculpins not only experienced a decrease in their stress tolerance but also a significant downward shift in the functional window of their Hsp response. Therefore the results of this study provide some evidence that Hsps may be one mechanism underlying the plasticity of thermotolerance in sculpins. It is difficult to draw similar conclusions with respect to the role of Hsps in the differences in osmotic tolerance observed between the groups of sculpins. The lack of any pronounced Hsp response to osmotic shock in any of the groups of fish could indicate that these sculpins do not respond to an osmotic challenge with an Hsp response; however, in a previous experiment we did observed a significant Hsp90, Hsc70 and Hsp70 response to a 2-h, 85ppt shock (Todgham et al. 2005). This suggests that the lack of Hsp response in the present experiment likely reflected an inability of the sculpins to mount an Hsp response to the magnitude of the imposed osmotic shock.

Conclusions

The short time frame within which we observed a restructuring in the Hsp response to temperature in this experiment is remarkable. Within 2 weeks of acclimatization to a constant ambient ocean conditions there was a significant decrease in stress tolerance of tidepool sculpins along with a significant decay in the influence that a variable thermal history had on the Hsp response of sculpins. In addition, the differences between the Subtidal- and Tidepool-Acclimatized groups suggest that even subtle differences in the variability of environmental history within nature can alter the Hsp response of sculpins from different microhabitats. The Hsp response is energetically costly not only in terms of the cost to synthesize Hsps but also in
terms of the 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, having a regulatory scheme that permits the continual readjustment of the Hsp induction set point such that this response could be tailored to the immediate thermal conditions would be important for poikilotherms living in fluctuating and unpredictable environments. This would allow these organisms the flexibility necessary to cope with the potential threat of environmental conditions approaching their tolerance thresholds while maintaining enough energy to accommodate other energetically demanding processes such as growth and reproduction.

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Physiology 44:1091-1101.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of

Laplace, J.-M., Boutibonnes, P. and Auffray, Y. 1996. Unusual resistance and acquired tolerance


Table 3-1: Gene specific qRT-PCR primers to quantify *hsc70*, *hsp70*, *hsp90* and elongation factor 1 alpha (*EF-1α*) mRNA expression in gills of the tidepool sculpin (*Oligocottus maculosus*).  

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsc70</td>
<td>5'-TGA TGC GGT TGT CCA GTC A-3'</td>
<td>5'-CTT TAG GGC GCG TTG CA-3'</td>
</tr>
<tr>
<td>hsp70</td>
<td>5'-AGT GGT GCA GGC GGA CAT-3'</td>
<td>5'-TCT TGG GCT TCC CTC CAT CT-3'</td>
</tr>
<tr>
<td>hsp90</td>
<td>5'-CGA TGG GCT CCG TCA TGT-3'</td>
<td>5'-TGA GCG CGT CCG TAA GC-3'</td>
</tr>
<tr>
<td>EF-1α</td>
<td>5'-CCC GGA CAC AGG AAC TTC AT-3'</td>
<td>5'-GGC GCA GTC AGC CTG AGA-3'</td>
</tr>
</tbody>
</table>
Table 3-2: Hsp mRNA expression and protein levels in response to a 3-h, 91ppt severe osmotic shock in Lab-, Tidepool-, and Subtidal-Acclimatized sculpins. mRNA expression is normalized to a control gene, $EF-1\alpha$ (mean ± SEM). Protein levels are shown as relative values based on band intensities standardized with the level of the particular branchial Hsp in a pooled sample of heat-shocked sculpins (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins over all sampling times ($p \leq 0.05$). An asterisk (*) indicates a significant difference from the Lab-Acclimatized group within a single sampling time ($p \leq 0.05$). An $\dagger$ indicates a significant difference between the Tidepool- and Subtidal-Acclimatized groups within a sampling time ($P \leq 0.05$).

<table>
<thead>
<tr>
<th>Time</th>
<th>hsp90 mRNA</th>
<th>Hsp90 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab</td>
<td>Tidepool</td>
</tr>
<tr>
<td>Time 0</td>
<td>0.23 ± 0.013$^a$</td>
<td>0.39 ± 0.022$^m\dagger$</td>
</tr>
<tr>
<td>0h Recovery</td>
<td>0.27 ± 0.013$^a$</td>
<td>0.30 ± 0.006$^m$</td>
</tr>
<tr>
<td>8h Recovery</td>
<td>0.25 ± 0.029$^a$</td>
<td>0.38 ± 0.025$^m$</td>
</tr>
<tr>
<td>24h Recovery</td>
<td>0.27 ± 0.036$^a$</td>
<td>0.32 ± 0.061$^m$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>hsc70 mRNA</th>
<th>Hsc70 &amp; Hsp70 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab</td>
<td>Tidepool</td>
</tr>
<tr>
<td>Time 0</td>
<td>0.18 ± 0.016$^a$</td>
<td>0.13 ± 0.012$^m\dagger$</td>
</tr>
<tr>
<td>0h Recovery</td>
<td>0.25 ± 0.047$^{ab}$</td>
<td>0.16 ± 0.015$^{mn}$</td>
</tr>
<tr>
<td>8h Recovery</td>
<td>0.29 ± 0.022$^{ab}$</td>
<td>0.21 ± 0.022$^*$</td>
</tr>
<tr>
<td>24h Recovery</td>
<td>0.19 ± 0.020$^a$</td>
<td>0.17 ± 0.020$^{mn}$</td>
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Table 3-3: Hsp mRNA expression and protein levels in response to handling stress in Lab-, Tidepool-, and Subtidal-Acclimatized sculpins. mRNA expression is normalized to a control gene, EF-1α (mean ± SEM). Protein levels are shown as relative values based on band intensities standardized with the level of the particular branchial Hsp in a pooled sample of heat-shocked sculpins (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Lab-Acclimatized group within a single sampling time (p≤0.05). An † indicates a significant difference between the Tidepool- and Subtidal-Acclimatized groups within a sampling time (P≤0.05).

<table>
<thead>
<tr>
<th>Time</th>
<th>hsp90 mRNA</th>
<th></th>
<th>Hsp90 Protein</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab</td>
<td>Tidepool</td>
<td>Subtidal</td>
<td>Lab</td>
</tr>
<tr>
<td>Time 0</td>
<td>0.23 ± 0.01a</td>
<td>0.39 ± 0.02m*†</td>
<td>0.27 ± 0.03x</td>
<td>95.80 ± 10.43y</td>
</tr>
<tr>
<td>0h Recovery</td>
<td>0.32 ± 0.02b</td>
<td>0.40 ± 0.02m*†</td>
<td>0.23 ± 0.03x</td>
<td>49.75 ± 17.09y</td>
</tr>
<tr>
<td>8h Recovery</td>
<td>NR</td>
<td>0.29 ± 0.01n</td>
<td>0.28 ± 0.02x</td>
<td>NR</td>
</tr>
<tr>
<td>24h Recovery</td>
<td>0.28 ± 0.01ab</td>
<td>0.38 ± 0.02m*†</td>
<td>0.30 ± 0.02x</td>
<td>66.89 ± 13.82y</td>
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<table>
<thead>
<tr>
<th>Time</th>
<th>hsc70 mRNA</th>
<th></th>
<th>Hsc70 &amp; Hsp70 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lab</td>
<td>Tidepool</td>
<td>Subtidal</td>
</tr>
<tr>
<td>Time 0</td>
<td>0.18 ± 0.02a</td>
<td>0.13 ± 0.01m*†</td>
<td>0.21 ± 0.02x</td>
</tr>
<tr>
<td>0h Recovery</td>
<td>0.26 ± 0.11a</td>
<td>0.11 ± 0.01m</td>
<td>0.15 ± 0.01y*</td>
</tr>
<tr>
<td>8h Recovery</td>
<td>NR</td>
<td>0.08 ± 0.01m</td>
<td>0.15 ± 0.01y*</td>
</tr>
<tr>
<td>24h Recovery</td>
<td>0.18 ± 0.08a</td>
<td>0.19 ± 0.02m*†</td>
<td>0.10 ± 0.01x</td>
</tr>
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</table>
Figure 3-1: Water temperatures (°C) recorded in the mid-intertidal tidepool during the 2-week period prior to the start of the experiment. Lab-Acclimatized sculpins were collected from this tidepool at the start of this 2-week period; whereas, Tidepool-Acclimatized sculpins were collected from this tidepool at the end of this 2-week period.
Figure 3-2: Survival (%) of tidepool sculpins exposed to a 3-h, 28°C severe heat shock [A] or a 2-h, 91ppt hyperosmotic stressor [B]. Open bars (Lab) represent Laboratory-Acclimatized fish that were collected from a mid-intertidal tidepool and then held under constant conditions (11°C, 32ppt) for 2 weeks prior to experimentation. Light grey bars (Tidepool) represent Tidepool-Acclimatized fish that were collected from a mid-intertidal tidepool one day prior to experimentation. Dark grey bars (Subtidal) represent Subtidal-Acclimatized fish that were collected from the subtidal zone one day prior to experimentation.
Figure 3-3: Branchial $hsp90$ mRNA and Hsp90 protein levels of tidepool sculpins in response to either a mild 2-h, 25°C heat shock [A,B] or a severe 3-h, 28°C heat shock [C,D]. The Hsp90 response was measured prior to experimentation (Time 0), and immediately (0h), 8h and 24h following heat shock. Open circles represent Lab-Acclimatized fish that were collected from a mid-intertidal tidepool and then held under constant conditions (11°C, 32ppt) for 2 weeks prior to experimentation. Closed circles represent Tidepool-Acclimatized fish that were collected from a mid-intertidal tidepool one day prior to experimentation. Closed triangles represent Subtidal-Acclimatized fish that were collected from the subtidal zone one day prior to experimentation. $Hsp90$ mRNA expression is normalized to a control gene, $EF-1\alpha$ (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). A difference in letters denotes significant differences within a group of sculpins over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Lab-Acclimatized group within a single sampling time (p≤0.05). An † indicates a significant difference between the Tidepool- and Subtidal-Acclimatized groups within a sampling time (p≤0.05).
Figure 3-4: Branchial hsc70 mRNA, hsp70 mRNA and Hsc70 and Hsp70 protein levels of tidepool sculpins in response to either a mild 2-h, 25°C heat shock [A,B,C] or a severe 3-h, 28°C heat shock [D,E,F]. The Hsp70 response was measured prior to experimentation (Time 0), and immediately (0h), 8h and 24h following heat shock. Open circles represent Lab-Acclimatized fish that were collected from a mid-intertidal tidepool and then held under constant conditions (11°C, 32ppt) for 2 weeks prior to experimentation. Closed circles represent Tidepool-Acclimatized fish that were collected from a mid-intertidal tidepool one day prior to experimentation. Closed triangles represent Subtidal-Acclimatized fish that were collected from the subtidal zone one day prior to experimentation. Hsc70 and hsp70 mRNA expression is 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). A difference in letters denotes significant differences within a group of sculpins over all sampling times (p<0.05). An asterisk (*) indicates a significant difference from the Lab-Acclimatized group within a single sampling time (p<0.05). An † indicates a significant difference between the Tidepool- and Subtidal-Acclimatized groups within a sampling time (p<0.05).
CHAPTER 4. EFFECT OF THE NATURAL TIDAL CYCLE AND ARTIFICIAL TEMPERATURE CYCLING ON HSP LEVELS IN TIDEPOOL SCULPIN

Introduction

The rocky intertidal zone is an interesting environment in which to examine the impact of environmental variability on organismal physiology. On 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 (Gibson, 1971a; Northcott 1991; Palmer 2000; Gibson 2003). It is thought that these rhythms are entrained by both mechanical (e.g. wave action and turbulence) as well as physical (e.g. temperature, pressure and salinity) characteristics of the tidal cycle. To date 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; Feldmeth et al. 1974, Otto 1973) suggesting that some aspect of environmental variation itself plays an important role in modulating physiological responses.
Given that temperature is one factor known to entrain biological processes and that an animal's thermal history has been shown to structure its cellular response to stress, endogenous heat shock proteins (Hsp) levels may be tightly regulated by environmental temperature in a variable environment such that they have a natural rhythm. 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 no research to document whether intertidal organisms are able to anticipate the changes in their natural environment by “pre-synthesizing” Hsps prior to 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). 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 rhythmicity in the Hsp response, or whether this variation is simply elicited in response to some environmental variable.

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 routinely experience dramatic daily fluctuations in temperature, salinity and oxygen availability (Green 1971b). Because of their ability to thrive in such a variable habitat, tidepool sculpins are an excellent organism 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 investigate whether there was an endogenous diurnal rhythm in Hsp expression that persisted once the sculpins were 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 regulating Hsp expression.
Materials and Methods

Experiment 1: Endogenous levels of Hsps in response to tidal cycle

Fish collection

Tidepool sculpins (2.1 ± 0.1g, 5.5 ± 0.1cm) were collected using dip nets and minnow traps from Wizard Rocks in Barkley Sound, Bamfield, BC, Canada during July 2003. Sculpins to be used for the acclimatization 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 acclimatized to a stable environment at ambient ocean conditions (11°C, 32ppt) under natural photoperiod for up to 1 week. Fish were fed blue mussels, presented by cracking the shells, ad libitum 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 19th, 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, 32ppt). Six fish were immediately sampled following collection (morning). In order to continue sampling fish throughout the next 12 hours of tidepool exposure, two groups of 6 fish were placed in submerged screened containers which 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 6h following the first sampling point, the time point that corresponded with the end of the midday low tide period (midday). The second group was sampled 12h 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. During the summer evening low tides these tidepools get extremely hypoxic 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 4-1). 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 acclimatized in to ambient ocean conditions (11.4 ± 0.3°C, 32.1 ± 0.2ppt, 7.44 ± 0.2mg O₂/L) outdoor stock tanks and then sampled at several points after either 24 hours or 1 week of acclimatization. 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 6h over two tidal cycles. Following each acclimatization period, sampling was begun 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 7 sampling points for each acclimatization period (day 2 or day 7: morning, midday, evening, night, and 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 ± 0.1g, 4.4 ± 0.1cm) were collected using dip nets and minnow traps from Wizard Rocks in Barkley Sound, Bamfield, BC, Canada 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 (UBC). At UBC these fish were held in a large aerated 125 litre glass aquarium (11°C, 32ppt and 12L:12D) with biological filtration for 2 weeks in order to acclimatize to the
laboratory conditions. Fish were fed blue mussels, presented by cracking the shells, *ad libitum* daily. Water was changed every 2-3 days 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 (Figure 4-1A) on *hsp* mRNA levels. At the start of the experiment, fish were acclimated for an additional 2 weeks in aerated and biologically filtered 75 litre 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 a mid-intertidal tidepool during an average summer day (Cycling Temperature, Figure 4-1B) and 2) A constant temperature regime in which water temperature was held at 10.8 ± 0.2°C, which was designed to mimic ambient ocean conditions (Constant Temperature). The cycling temperature regime was created using a combination of 250W 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 insure 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 1:00AM as opposed to 1:00PM 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-week acclimation period.

Following the 2-week acclimation period, six sculpins from each of the temperature regimes were sampled every 6h over two full temperature cycles resulting in 7 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 (Figure 4-1B).

Tissue sampling

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.3g MS-222/L of water) and following onset of anaesthesia 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 Inc., USA) in homogenization buffer (0.1% SDS [w/v], 0.02mg/mL PMSF, 0.25mg/mL EDTA, 1μg/mL pepstatin A, 1μg/mL leupeptin and 1μg/mL aprotinin in 100mM Tris-HCl buffer, pH 7.5), at a ratio of 10mg tissue to 100μL of buffer. Homogenates were then centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to a tube containing an equal volume of 2x 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 0.5M 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 prior to electrophoresis (maximum 1 week). The remaining supernatant was transferred to an empty tube and stored at -20°C until analyzed for total protein (within 2 days). Protein concentration of the tissue homogenate was determined using the bicinchoninic acid method (Smith et al. 1985).
Levels of Hsc70, Hsp70 and Hsp90 were measured using the discontinuous SDS-PAGE method of Laemmli (1970). Equal amounts of total protein (15μg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular markers (Invitrogen Life Technologies, Carlsbad, CA, USA) 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 75V for 15 min followed by 150V for 1h. The separated proteins were transferred onto nitrocellulose (Bio-Rad, 0.2μm pore size) at 17V for 30 min with transfer buffer (48mM Tris, 39mM glycine, 20% methanol [v/v] and 0.0375% SDS [w/v], pH 9.2) using a semi-dry transfer apparatus (Bio-Rad Trans-Blot). Transfer membranes were blocked in 2% skim milk in Tween-20 Tris-buffered saline (TTBS) (17.4mM Tris-HCl, 2.64mM Tris Base, 0.5M NaCl and 0.05% Tween-20 [v/v]) with 0.05% sodium azide for 1h. 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 (1:5000, StressGen, Victoria, BC, Canada), or rat IgG for human Hsp90 (1:250, StressGen, Victoria, BC, Canada), in 2% skim milk for 1h. Following three 5 min washes in TTBS, membranes were soaked in an alkaline phosphatase conjugated secondary antibody: goat anti rabbit IgG (1:5000, Sigma, St. Louis, MO, USA), or goat anti rat IgG (1:5000, StressGen, Victoria, BC, Canada), in TTBS for 1h. 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) (333μg/mL)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (167μg/mL) solution in alkaline phosphatase buffer (0.01M Tris-HCl, 0.1M NaCl and 21mM MgCl2, 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 5μg total RNA using oligo(dT18) primer and RevertAid H Minus M-MuLV reverse transcriptase following the manufacturer’s instructions (MBI Fermentas Inc., Burlington, ON, Canada).

Real-time PCR analysis of hsc70, hsp70 and hsp90 gene expression

Gene specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA) and are reported in Chapter 3. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence detection system (Applied Biosystems Inc.). qRT-PCR reactions were run with 2μL cDNA, 4pmoles of each primer and 2X SYBR Green Master Mix (Applied Biosystems Inc.) to a total volume of 22μL. 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 sec, 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 Hsp90, Hsc70 and Hsp70 protein quantification, band intensities were obtained using SigmaGel software (Jandel Scientific, USA) and values were standardized using band intensity values from a pooled sample of tidepool sculpin gill homogenate that were run concurrently on
each gel (referred to as “Internal Std” in figures). Results for Hsp90, Hsc70 and Hsp70 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. We have previously shown that EF-1α levels do not change significantly in response to heat shock (Chapter 3) and thus this gene is an appropriate internal control. Results for hsc70, hsp70 and hsp90 mRNA expression are reported as mean ± SEM. Two-way analysis of variance (ANOVA) was used to determine significant (p≤0.05) differences in both Hsp protein and mRNA levels within a treatment group. For the two-way ANOVA, fish group (eg. Cycling Temperature, Constant Temperature) and time of day were used as independent categorical variables, and Hsp protein or mRNA levels were used as the dependent variable. Where significant interactions were detected, data was decomposed and one-way ANOVAs were performed as appropriate. Means were compared using the post-hoc Student-Newman-Keuls (SNK) multiple comparison test (p≤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 acclimatization to a constant environment

Hsc70 and Hsp70

Results of the 2-way ANOVA on hsc70 mRNA expression showed that there was a significant effect of group (Field- vs. 24h Lab-Acclimatization, p<0.001), no significant effect of time of day but there was a significant interaction between group and time of day (p=0.034). In the 2-way ANOVA comparisons of hsp70 mRNA expression, there was a significant effect of group (p<0.001), 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 expression over the midday low tide period (comparison of morning and midday levels, Field-Acclimatization) in sculpins sampled directly from the tidepool; however hsc70 mRNA levels were significantly elevated in the evening, at the time that corresponded with the onset of the evening low tide period (Field-Acclimatization, Figure 4-2A). When fish were removed from the intertidal zone and held under constant environmental conditions overnight (24h Lab-Acclimatized), hsc70 mRNA levels returned to the basal levels measured in the Field-Acclimatized sculpins. There was no significant fluctuation in hsc70 mRNA expression at any of the time points over the next 2 days (24h Lab-Acclimatization, Figure 4-2A).

For hsp70 mRNA levels, there was a significant increase at midday compared to levels measured in the morning, at the start of the low tide period (Field-Acclimatized, Figure 4-2B)
and \textit{hsp70} mRNA levels remained elevated into the evening in the Field-Acclimatized fish. When sculpins were removed from the intertidal zone and held under constant conditions, \textit{hsp70} mRNA levels were close to the lower limits of detection. There was a slight but significant increase in \textit{hsp70} mRNA expression over the lab-acclimatization period, with levels being significantly elevated in the morning, midday and evening of day 3.

For Hsc70 and Hsp70 protein levels there was a significant increase over the midday low tide period and these levels remained elevated into the evening (Field-Acclimatized, Figure 4-2C). There was no significant change in Hsc70 and Hsp70 protein levels over the tidal cycle in sculpins that were removed from the tidepool and held under constant conditions in the laboratory; however, evening Hsc70 and Hsp70 protein levels in the 24h Lab-Acclimatized group were significantly lower than the evening sample of the Field-Acclimatized group (Figure 4-2C). Following 1 week of acclimatization to the constant conditions of the ambient ocean there were no significant daily changes in \textit{hsc70} mRNA, \textit{hsp70} mRNA or Hsc70 and Hsp70 protein levels and levels were not significantly different from the lowest levels measured in the Field-Acclimatized group (data not shown).

\textbf{Hsp90}

Results of the 2-way ANOVA showed that there was a significant effect of group (Field-vs. 24h Lab-Acclimatization) on both \textit{hsp90} mRNA expression (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 2-way ANOVAs run for either \textit{hsp90} mRNA expression or Hsp90 protein.

There was no significant increase in branchial \textit{hsp90} mRNA expression over the midday low tide period; however there was a significant induction of \textit{hsp90} mRNA levels by the start of the evening low tide period (Field-Acclimatized, Figure 4-3A). In the 24h Lab-Acclimatized
group there were modest fluctuations in hsp90 mRNA levels, but these were not concordant with tidal cycle (24h Lab-Acclimatized, Figure 4-3A).

There was no significant increase in Hsp90 protein from the morning to the evening in sculpins sampled directly from the tidepool (Field-Acclimatized, Figure 4-3B). In the 24h Lab-Acclimatized group, Hsp90 protein levels were significantly lower in the morning of day 2 compared to the morning in the Field-Acclimatized group. There was a slow increase in Hsp90 protein levels over the acclimatization period such that levels were significantly elevated by midday of day 3 (24h Lab-Acclimatization, Figure 4-3B). Hsp90 protein levels returned to basal levels by the evening of day 3. Following 1 week of acclimatization 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 the lowest levels measured in the Field-Acclimatized group (data not shown).

Experiment 2: Effects of a cycling temperature regime on hsp mRNA expression

Results of the 2-way ANOVA on hsc70 mRNA expression 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 2-way ANOVA of hsp70 mRNA expression, there was a significant effect of group (p<0.001), 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 expression; 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 expression that cycled in phase with
peak temperatures (Figure 4-4A). The lowest levels of \textit{hsc70} mRNA expression were measured in the evening immediately before the increase in water temperature when water temperatures were at 11°C; whereas, peak levels in \textit{hsc70} mRNA expression were measured when maximum water temperatures were reached. Acclimation of sculpins to a constant temperature regime of 11°C for 2 weeks resulted in no significant daily fluctuations in \textit{hsc70} mRNA expression; however, overall \textit{hsc70} mRNA levels were significantly higher than levels measured in the cycling temperature group in 4 of the 7 sampling points (Figure 4-4B).

\textbf{Hsp70}

\textit{Hsp70} mRNA expression peaked with peak water temperature in sculpins inhabiting the cycling temperature regime (Figure 4-5A). At the time of peak water temperature there was a 200-fold induction of \textit{hsp70} mRNA in the Cycling Temperature group, whereas at all other sampling points \textit{hsp70} mRNA levels were barely above detection limits. Sculpins inhabiting the constant temperature regime did not experience significant changes in \textit{hsp70} mRNA levels throughout the day but \textit{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 (Figure 4-5B).

\textbf{Hsp90}

There was no significant daily fluctuation in \textit{hsp90} mRNA expression in either of the Cycling or Constant Temperature groups (Figure 4-6A,B). In general, there was no significant difference in the \textit{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, \textit{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 vs. within a cycling temperature regime

There were a number of significant differences in the induction profiles of hsc70, hsp70 and hsp90 mRNA expression in response to natural changes in environmental conditions within the tidepool when compared to the response to an artificially controlled temperature cycling regime that mimicked daily temperature fluctuations of a mid-intertidal tidepool (Figure 4-7). Within the Tidepool-Acclimatized group, hsc70 mRNA levels were not significantly elevated until 6h 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 6h. 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. Peak hsp90 mRNA expression in the Cycling Temperature-Acclimatized sculpins coincided with peak temperatures; whereas, in the Tidepool-Acclimatized group, hsp90 mRNA levels were not significantly elevated until 6h 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 degree of 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, Hsp mRNA and protein levels return to basal levels. The lack of any endogenous diurnal 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 both in terms of 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 gene expression and that this translates into increased Hsc70, Hsp70 and Hsp90 protein levels. Because Hsp expression in this study was measured only over 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. We have, however, clearly demonstrated that temperature can regulate the Hsp70 response in sculpins such that hsc70 gene expression cycles in phase with peak environmental temperature and hsp70 gene expression peaks with peak temperature and this provides novel insights into the environmental regulation of the Hsp response. Schill and colleagues (2002) also observed a cyclical pattern of Hsp70 levels in the foot muscle of an intertidal chiton, Acanthopleura granulata, in the field, which followed the daily air temperature curve with a 2-h time lag. However, similar to our study, these results were over a single tidal cycle and did not demonstrate the cyclic nature of Hsp expression over multiple tidal cycles. Podrabsky and Somero (2004) examined the changes in Hsp gene expression associated with daily temperature changes over a 2-week 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 upon transfer of these killifish into a temperature cycling environment but the transcript levels of these chaperones returned to control levels within 2 weeks and no cycling was observed. In contrast, we still observed cycling in response to temperature cycling after two weeks 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.

One of the most remarkable findings of the present study is the speed at which the Hsp response is regulated in tidepool sculpins in their natural environment. Within the tidepool, sculpins rapidly induced *hsp70* gene expression during a low tide period and *hsc70* and *hsp90* gene expression was significantly elevated within hours of the onset of high tide. 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, their findings suggest that they induce the Hsp response and other protein repair mechanisms only once they are submerged by high tide (Hofmann and Somero 1996; Tomanek and Somero 2000). This is in contrast to the change in *hsp* expression that occurred during the low tide period in sculpins. During a low tide period, sculpins are actively foraging in the tidepool while many intertidal invertebrates, such as bivalves and gastropods, 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 their particular fluctuations in abiotic conditions encountered during low tide. In a poikilotherm inhabiting a variable environment, the capacity to rapidly adjust Hsp levels in concert with changes in environmental conditions would permit organisms to maximize the benefits of the
Hsp response while minimizing over expression 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 induction profiles of different Hsps within a particular environment (e.g. within the tidepool), we are able to shed some insight into the intrinsic regulatory mechanisms of the Hsp response. There were some significant differences in the induction profiles between \( hsc70 \), \( hsp70 \) and \( hsp90 \) mRNA expression 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 only significantly elevated 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 intrinsic mechanisms of Hsp regulation of different \( hsp \) genes. 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; Tomanek and Somero 2002; Buckley and Hofmann 2004). Due to 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 complete for tidepool sculpins, it is likely that similar differences exist due to the conserved nature of Hsps.

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 mechanisms by which an organism's environment is able to regulate its Hsp response. It is clear from the present study that temperature has an important and direct role in structuring the Hsp response, particularly the *Hsc70* and *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 to a change in temperature that mimicked what was occurring in the tidepool were not identical (Figure 4-7). These Hsp gene expression profiles differed in both the kinetics and the magnitude of the response and therefore suggest that factors in addition to temperature in a sculpins 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 a reversed 12L:12D photoperiod, which was very different from the natural photoperiod of these fish during the summer months. As photoperiod is known to have a significant effect on physiological processes (Filadelfi and Castrucci 1996), it too may have a role in structuring Hsp gene expression in nature. It is noteworthy that fish held under a constant temperature regime that mimicked ambient ocean conditions had significantly higher levels of *hsc70* and *hsp70* mRNA expression when compared to 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 therefore the high levels of mRNA expression in this group of fish may reflect 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 compared to 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 in induction of hsp gene expression in relation to peak temperatures in either the tidepool or the laboratory may suggest that the degree of environmental predictability has a role in fine tuning the timing 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 temperature change in the intertidal environment has entrained an endogenous diurnal 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 organism. It is clear that there is no endogenous diurnal 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 in response to fluctuations in environmental conditions that did not persist if the organism was removed from the intertidal environment. Fluctuations in environmental temperature have a critical role in regulating the hsp gene expression; however, there are additional extrinsic factors that likely integrated with temperature
and resulted in the hsp induction 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.

References


Table 4-1: Temperature, salinity and oxygen levels of the large mid-intertidal collection tidepool at the morning, midday and evening sampling periods.

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Salinity (ppt)</th>
<th>Oxygen (mg O₂/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning</td>
<td>16.0</td>
<td>30</td>
<td>4.9</td>
</tr>
<tr>
<td>Midday</td>
<td>21.9</td>
<td>30</td>
<td>10.1</td>
</tr>
<tr>
<td>Evening</td>
<td>18.9</td>
<td>25</td>
<td>18.2</td>
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</tbody>
</table>
Figure 4-1: 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. Gray circles (●) indicate sampling points for the temperature cycling experiment.
**Figure 4-2:** 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, dark grey bars) or following 24h of acclimatization to constant conditions (11°C, 32ppt) in the laboratory (24h Lab-Acclimatization, light grey 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 with morning (M), midday (MD) and evening (E), respectively. Hsp levels in the 24h Lab-Acclimatized fish were measured every 6h over two days (Day 2, Day 3) and the exact sampling periods corresponded with morning (M), midday (MD), evening (E) and night (N).

*Hsc70* and *hsp70* mRNA expression is 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). A difference in letters denotes significant differences within a group of sculpins (Field or Lab) over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Field-Acclimatized group within a single sampling time (p≤0.05).
Figure 4-3: 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, dark grey bars) or following 24h of acclimatization to constant conditions (11°C, 32ppt) in the laboratory (24h Lab-Acclimatization, light grey 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 with morning (M), midday (MD) and evening (E), respectively. Hsp levels in the 24h Lab-Acclimatized fish were measured every 6h over two days (Day 2, Day 3) and the exact sampling periods corresponded with morning (M), midday (MD), evening (E) and night (N). \( Hsp90 \) mRNA expression is normalized to a control gene, \( EF-1\alpha \) (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). A difference in letters denotes significant differences within a group of sculpins (Field or Lab) over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Field-Acclimatized group within a single sampling time (p≤0.05).
Figure 4-4: Branchial hsc70 mRNA expression of tidepool sculpins acclimated for 2 weeks to either a cycling temperature regime [A] (10.8 → 22°C, 32ppt) that mimics a mid-intertidal tidepool or a constant temperature regime [B] (10.8°C, 32ppt). Transcript expression was monitored every 6h over 2 days (Day 1, Day 2) and the exact sampling periods corresponded with morning (M), midday (MD), evening (E) and night (N). Hsc70 mRNA expression is normalized to a control gene, EF-1α (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins (Cycling or Constant Temperature regime) over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Constant Cycling regime group within a single sampling time (p≤0.05).
hsc70 mRNA (relative to EF-1α)

Day 1
- MD
- E
- N

Day 2
- MD
- E
- N

Temperature (°C)

A

B

hsc70 mRNA (relative to EF-1α)

Day 1
- MD
- E
- N

Day 2
- MD
- E
- N

Temperature (°C)
Figure 4-5: Branchial hsp70 mRNA expression of tidepool sculpins acclimated for 2 weeks to either a cycling temperature regime [A] (10.8 → 22°C, 32ppt) that mimics a mid-intertidal tidepool or a constant temperature regime [B] (10.8°C, 32ppt). Transcript expression was monitored every 6h over 2 days (Day 1, Day 2) and the exact sampling periods corresponded with morning (M), midday (MD), evening (E) and night (N). Hsp70 mRNA expression is normalized to a control gene, EF-1α (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins (Cycling or Constant Temperature regime) over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Constant Cycling regime group within a single sampling time (p≤0.05).
Figure 4-6: Branchial hsp90 mRNA expression of tidepool sculpins acclimated for 2 weeks to either a cycling temperature regime [A] (10.8 → 22°C, 32ppt) that mimics a mid-intertidal tidepool or a constant temperature regime [B] (10.8°C, 32ppt). Transcript expression was monitored every 6h over 2 days (Day 1, Day 2) and the exact sampling periods corresponded with morning (M), midday (MD), evening (E) and night (N). Hsp90 mRNA expression is normalized to a control gene, EF-1α (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins (Cycling or Constant Temperature regime) over all sampling times (p≤0.05). An asterisk (*) indicates a significant difference from the Constant Cycling regime group within a single sampling time (p≤0.05).
Figure 4-7: Comparison of the hsc70 [A], hsp70 [B] and hsp90 [C] mRNA expression 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 6h prior (-6h) to peak temperature, at peak temperature (PT) and 6h following (+6h) peak temperature. Hsp mRNA expression is normalized to a control gene, EF-1α (mean ± SEM). A difference in letters denotes significant differences within a group of sculpins (Tidal Cycle or Temperature Cycle) over all sampling times (p≤0.05).
`hsp90 mRNA (relative to EF-1α)`

-6h

PT

+6h

-6h

PT

+6h

`hsp70 mRNA (relative to EF-1α)`

-6h

PT

+6h

-6h

PT

+6h

`hsc70 mRNA (relative to EF-1α)`

-6h

PT

+6h

-6h

PT

+6h
CHAPTER 5. CHANGES IN HSP LEVELS OVER CONSECUTIVE LOW TIDE PERIODS: THE ROLE OF ACUTE AND CHRONIC THERMAL ENVIRONMENTS ON THE HSP RESPONSE OF TIDEPool SCULPINS

Introduction

Eurythermal organisms inhabiting variable thermal environments such as the intertidal zone face the challenge of maintaining biochemical and physiological performance in a constantly changing environment. Although these organisms have many mechanisms that provide them with impressive resistance to thermal stressors, they still expend a considerable amount of energy in the repair of thermally sensitive biochemical components of the cell (Hochachka and Somero 2002). Protein denaturation and disturbances to overall protein homeostasis are well-understood consequences of thermal stress and organisms have a cellular stress response dedicated to restoring the integrity of the cellular protein pool - the heat shock protein (Hsp) response. Acting as molecular chaperones, Hsps have a fundamental role in mitigating the effects of environmental stressors on protein structure and restoring protein homeostasis following stress (Lindquist 1986; Hightower 1991; Iwama et al. 1998; Morimoto, 1998; Hartl and Hayer-Hartl 2002). It has been well documented that Hsps have a critical role in stress tolerance (Parsell and Lindquist, 1993; Krebs and Feder, 1998) and that these proteins likely have a protective role in an animal’s natural tolerance to environmental change (Feder and Hofmann, 1999).

The Hsp response is one of the most highly regulated inducible cellular responses. This response has numerous transcriptional as well as posttranscriptional control mechanisms, allowing for the rapid and preferential synthesis of Hsps (Yost et al. 1990, Wu et al. 1990). However, in spite of the extensive examination of the intrinsic mechanisms of regulation of the
Hsp response in model organisms, the mechanisms underlying the plasticity of this response in the natural environment are not well understood.

To fully appreciate the functional significance of Hsps in protecting the integrity of the cellular protein pool in the face of constantly changing environmental conditions, an understanding of how this environment is able to modulate the Hsp response of an organism is essential. The intertidal zone has proven to be an excellent environment in which to address questions regarding the environmental regulation of the Hsp response (Hofmann et al. 2002). From laboratory investigations into the Hsp response of congeners that inhabit distinct thermal niches as well as organisms of a single species acclimated to different temperatures, it has become clear that thermal history of an organism affects its ability to respond to environmental change (Roberts et al. 1997; Tomanek and Somero 1999; Buckley et al. 2001; Nakano and Iwama 2002). These studies highlight the importance of considering both acute and chronic temperature changes in the environment in order to understand the complexity of the relationship between the thermal environment and the Hsp response.

Despite the accumulating number of laboratory studies that have investigated the Hsp response of natural populations of organisms exposed to ecologically relevant stressors, at present there has been little research that has directly monitored the Hsp response of an organism over consecutive days within their natural environment. Therefore, how thermal history integrates with immediate thermal conditions to structure the Hsp response of an organism to natural fluctuations in its environment is largely unknown. From research that has documented basal Hsp levels in organisms within their natural environment over both spatial (ex. crevice vs. exposed rock, mid-intertidal vs. subtidal at one time point) and temporal scales (monthly sampling), there is preliminary evidence to suggest that Hsp levels in nature vary across environmental gradients and with thermal habitat (Helmuth and Hofmann 2001; Halpin et al.
2002; Hofmann et al. 2002). However, it is still unclear how often Hsps are induced in nature and which Hsps are important ecologically. Previous reports in Drosophila demonstrate that natural fluctuations in environmental temperature can induce hsp70 expression; however, it appears that these organisms likely behaviourally thermoregulate to minimize exposure to hsp inducing temperatures (Feder et al. 1997, 2000). In an earlier study, we demonstrated that tidepool sculpins (Oligocottus maculosus) induce transcripts for both the constitutive (Hsc70) as well as inducible (Hsp70) isoforms of the 70 kDa family of Hsps and increase Hsc70 and Hsp70 protein levels within the tidepool in response to exposure to a midday low tide period (Chapter 4). However, since Hsp expression in this study was measured only over a single tidal cycle, we could not say conclusively that this pattern of Hsp expression was repeatable over multiple tidal cycles or how prior thermal exposures integrated into this response.

The objective of this study was to investigate the Hsp response of tidepool sculpins to the temperature fluctuations of a mid intertidal tidepool over multiple low tide periods and to provide insights into the mechanisms underlying environmental regulation of Hsp gene expression. Specifically, we monitored hsc70 and hsp70 mRNA expression as well as Hsc70 and Hsp70 protein levels over five consecutive midday low tide periods and conducted this sampling regime at two separate times during the summer (mid-July and early August). 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 routinely experience dramatic daily fluctuations in temperature, salinity and oxygen availability (Green 1971). Because of their ability to thrive in such a variable habitat, tidepool sculpins are an excellent organism in which to investigate the rapid shifts in hsp expression that occur over the tidal cycle and examine how previous thermal history may affect how an organism responds to an acute change in environmental conditions.
Material and Methods

Fish Collection

Tidepool sculpins (4.7 ± 0.1 cm) were collected using dip nets from a large mid-intertidal tidepool in Scott’s Bay, Bamfield, BC, Canada. Over five consecutive days, six sculpins were netted at the beginning (L) as well as at the end (H) of the each midday low tide period. In order to examine the effects of thermal history on the Hsp response of animals in their natural environment, this sampling regime was conducted at two intervals that were two weeks apart: July 14th to July 19th (mid-July) and from July 30th to August 3rd, 2003 (early August). These specific times of month were chosen because environmental temperatures in Bamfield, BC increase during the month of July and therefore the early weeks of July are typically 1-2°C cooler than the later ones (Environment Canada, Weather Office, Online Climate Data). Water temperatures within this tidepool were recorded by submerged SmartButton data loggers (ACR Systems Inc., Surrey, BC, Canada) over both the mid-July and early August collection periods (Figure 5-1).

Tissue sampling

Fish were netted and rapidly anaesthetized with a high dose of MS-222 (0.3g MS-222/L of water) and following onset of anaesthesia 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 Inc., USA) in homogenization buffer (0.1% SDS [w/v], 0.02mg/mL PMSF, 0.25mg/mL EDTA, 1μg/mL
pepstatin A, 1µg/mL leupeptin and 1µg/mL aprotinin in 100mM Tris-HCl buffer, pH 7.5), at a ratio of 10mg tissue to 100µL of buffer. Homogenates were then centrifuged at 13,000 rpm for 3 min. Supernatant was transferred to a tube containing an equal volume of 2x 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 0.5M 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 prior to electrophoresis (maximum 1 week). The remaining supernatant was transferred to an empty tube and stored at −20°C until analyzed for total protein (within 2 days). 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 and Hsp90**

Levels of Hsc70 and Hsp70 were measured using the discontinuous SDS-PAGE method of Laemmli (1970). Equal amounts of total protein (15µg) were resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Prestained molecular markers (Invitrogen Life Technologies, Carlsbad, CA, USA) 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 75V for 15 min followed by 150V for 1h. The separated proteins were transferred onto nitrocellulose (Bio-Rad, 0.2µm pore size) at 17V for 30 min with transfer buffer (48mM Tris, 39mM glycine, 20% methanol [v/v] and 0.0375% SDS [w/v]. pH 9.2) using a semi-dry transfer apparatus (Bio-Rad Trans-Blot). Transfer membranes were blocked in 2% skim milk in Tween-20 Tris-buffered saline (TTBS) (17.4mM Tris-HCl, 2.64mM Tris Base, 0.5M NaCl and 0.05% Tween-20 [v/v]) with 0.05% sodium azide for 1h. 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 (1:5000, StressGen, Victoria, BC, Canada) in 2% skim milk for 1h. Following three 5 min washes in TTBS, membranes were soaked in an alkaline phosphatase conjugated secondary antibody: goat anti rabbit IgG (1:5000, Sigma, St. Louis, MO, USA) in TTBS for 1h. 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) (333μg/mL)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (167μg/mL) solution in alkaline phosphatase buffer (0.01M Tris-HCl, 0.1M·NaCl and 21mM MgCl₂, 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 5μg total RNA using oligo(dT₁₈) primer and RevertAid H Minus M-MuLV reverse transcriptase following the manufacturer’s instructions (MBI Fermentas Inc., Burlington, ON, Canada).

**Real-time PCR analysis of hsc70, hsp70 and hsp90 gene expression**

Gene specific primers were designed using Primer Express software (version 2.0.0; Applied Biosystems Inc., Foster City, CA, USA) and are reported in Chapter 3. Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc.). qRT-PCR reactions were run with 2μL cDNA, 4pmoles of each primer and 2X SYBR Green Master Mix (Applied Biosystems Inc.) to a total volume of 22μL. 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 sec, 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 Hsc70 and Hsp70 protein quantification, band intensities were obtained using SigmaGel software (Jandel Scientific, USA) and values were standardized using band intensity values from a pooled sample of tidepool sculpin gill homogenate that were run concurrently on each gel (referred to as “Internal Std” in figures). Results for Hsc70 and Hsp70 protein are reported as mean ± SEM. To quantify hsc70 and hsp70 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. We have previously shown that EF-1α levels do not change significantly in response to heat shock (Chapter 3) and thus this gene is an appropriate internal control. Results for hsc70 and hsp70 mRNA expression are reported as mean ± SEM. Three-way analysis of variance (ANOVA) was used to determine significant (p≤0.05) differences in both Hsp protein and mRNA levels between sampling groups. For the three-way ANOVA, time of month (e.g. mid July, early August), day (days 1 through 5), and time of day (start of low tide or start of high tide) were used as independent categorical variables, and Hsp protein or mRNA levels were used as the dependent variable. Where significant interactions were detected, data was decomposed and two-way or one-way ANOVAs were performed as appropriate. Means were compared using the post-hoc Student-Newman-Keuls (SNK) multiple comparison test (p≤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

Tidepool temperatures

Over the five consecutive sampling days in mid-July, tidepool temperatures fluctuated as little as 3.5°C (day 1) and as much as 10°C over the midday low tide period (day 4) such that there was a generalized increase in temperature fluctuations over the 5 day period (Figure 5-2A). In early August, the magnitude of temperature fluctuations in the tidepool did not vary as much as mid-July, being lowest on day 2 (+4.5°C) and highest on day 5 (+8.5°C) (Figure 5-3A). The rates at which tidepool temperature increased over a low tide period also varied substantially over each day and ranged from 0.7 to 1.4°C/hr and from 0.8 to 1.2 °C/hr during the mid-July and early August sampling periods, respectively (Table 5-1).

Sculpin Hsc70 and Hsp70 response over consecutive midday low tide periods

Three-way analysis of variance revealed that there was a significant effect of time of month (p=0.003), time of day (p=0.001), no effect of day (p=0.07) and a significant interaction between time of month and day (p=0.001) on hsc70 mRNA expression. Results of the 3-way ANOVA on hsp70 mRNA expression showed that there was no effect of time of month (p=0.277), a significant effect of time of day (p=0.007) and a significant effect of day (p<0.001). There were three significant interactions with respect to the hsp70 mRNA results: time of month x time of day x day (p=0.005), time of month x day (p=0.022) and time of day x day (p=0.002). Three-way ANOVA of Hsc70 and Hsp70 protein levels revealed that there was a significant
effect of time of month (p<0.001), time of day (p=0.003), day (p<0.001) and a significant interaction between time of month and day (p<0.013).

The data was decomposed and two-way analysis of variance was conducted for each time of month independently. Results from the 2-way ANOVA of the mid-July sampling period indicate that there was a significant effect of day (p=0.001) and a significant interaction between time of day and day (p=0.016) for hsc70 mRNA expression. For hsp70 mRNA expression, there was a significant effect of time of day (p=0.008) and a significant interaction between time of day and day (p=0.047). For Hsc70 and Hsp70 protein, there was a significant effect of time of day (p<0.001), effect of day (p=0.05) and there was no significant interaction between these two factors.

In mid-July, hsc70 mRNA levels measured at the start of the low tide periods were significantly higher on days 1 and 2 compared to the other 3 days, whereas there was no significant daily variation in hsc70 mRNA expression at the start of the high tide periods (Figure 5-2B). Hsc70 mRNA expression was significantly induced over the midday low tide periods on days 4 and 5. Hsp70 mRNA expression at the start of each low tide period did not vary significantly between days (Figure 5-2C). There was a significant induction in hsp70 mRNA expression above starting levels following low tide exposure on days 4 and 5, similar to what was observed with hsc70 mRNA expression, and these levels were significantly higher than hsp70 mRNA levels measured following exposure to the low tide periods on days 1, 2 and 3. Hsc70 and Hsp70 protein levels measured at the start of the midday low tide periods on days 3, 4, and 5 were significantly lower than day 1 (Figure 5-2D). There were no significant differences in Hsc70 and Hsp70 protein levels at the start of high tide on any of the 5 days in mid-July and there was no significant increase in Hsc70 and Hsp70 protein levels in response to low tide.
Results from the 2-way ANOVA of the early August sampling period indicate that for hsc70 mRNA expression there was a significant effect of time of day (p=0.003), effect of day (p=0.044) and no significant interaction between time of day and day. For hsp70 mRNA expression, there was a significant effect of time of day (p<0.001), effect of day (p<0.001) and a significant interaction between these two factors (p<0.001). For Hsc70 and Hsp70 protein, 2-way ANOVA revealed that there was a significant effect of both time of day (p=0.025) and day (p=0.001) but no significant interaction between time of day and day.

Over five consecutive low tide periods in early August, there were only 2 days (days 3 and 5) when there was a significant induction of hsc70 mRNA expression in response to low tide exposure (Figure 5-3B). Hsc70 mRNA levels measured at either the start of the low tide or the start of the high tide periods, however, did not vary significantly between days. Hsp70 mRNA expression was significantly lower at the start of the low tide period on day 2 when compared to starting levels on the other 4 days (Figure 5-3C). There was a significant induction of hsp70 mRNA expression upon exposure to low tide on days 2, 3 and 5; however, only on day 5 was hsp70 mRNA expression significantly elevated above levels measured following low tide on the four previous days. Hsc70 and Hsp70 protein levels measured at the start of the midday low tide on day 4 were only significantly lower than protein levels measure on day 1 (Figure 5-3D). Hsc70 and Hsp70 protein levels following low tide were lowest on day 4 but were only significantly lower than levels measured on day 5. There were no significant increases in Hsc70 and Hsp70 levels in response to low tide on any of the 5 days in early August.

Discussion

This is the first study to document the dynamics of the Hsp response over several consecutive low tide periods in an intertidal organism. The results of this study provide novel
insights into what aspects of the thermal signal the organism may be responding to as well as the
temporal scale of thermal history that has an important role in structuring the Hsp response in
nature. In addition to highlighting the plastic nature of the Hsp response, this study begins to
address what components of the Hsp response have a functional significance for eurythermal
organisms inhabiting a variable environment.

Despite the accumulating body of literature documenting the tight link between
environmental temperature and the Hsp response (Feder and Hofmann 1999, Hofmann et al.
2002), it is still unclear what aspect of the thermal signal the Hsp response is cued to in nature.
Most of the thermal acclimation studies conducted in the laboratory have documented threshold
induction temperatures for Hsp under various conditions, but few studies have investigated
whether the magnitude or rate of temperature change may have a role in structuring the Hsp
response of organisms. Based on the results of the mid-July sampling period, it appears that
there was a threshold temperature (>21.5°C), temperature change (>7.5°C), as well as rate of
temperature change (>1.2°C/h) above which there was significant induction of both \( hsc70 \) and
\( hsp70 \) mRNA expression. Within the early August sampling period there was no consistent
effect of environmental temperature on the Hsp response of sculpins. There was no clear
threshold temperature for \( hsp \) gene induction, nor was there any correlation between the
magnitude or rate of temperature change over a low tide period and the induction of \( hsp \) gene
expression. For example, on day 1 of the early August sampling period, sculpins were exposed
to high tidepool temperatures (22°C) and a rapid rate of temperature increase (1.1°C/h) but there
was no induction of \( hsc70 \) or \( hsp70 \) mRNA expression. On day 3, however, when tidepool
temperatures reached 20.5°C, at a rate of 1.0°C/h, there was a significant induction of \( hsp \) gene
expression. Therefore, it is clear that it is not simply the temperature profile of a given day that
influences the Hsp mRNA response of tidepool sculpins. The differences in the Hsp response to
similar fluctuations in environmental temperature between the different times of month highlight
the plasticity of this response and suggest that thermal history, in addition to the immediate temperature change, modulates the Hsp response of sculpins in their natural environment.

It has been well documented that ectotherms synthesize Hsps near the upper temperatures that they encounter in their natural environment and that congeners that occupy distinct thermal niches differ in both their threshold induction temperatures of Hsps as well as the magnitude of the Hsp response (Hofmann and Somero 1995; Roberts et al. 1997; Tomanek and Somero 1999; Nakano and Iwama 2002). This study adds a novel perspective on how thermal history regulates Hsp levels in an organism when environmental temperature is increasing during the summer months. Over the 2-week period between the sampling cycles, there was a significant accumulation in overall levels of both hsc70 mRNA expression and Hsc70 and Hsp70 protein. It is possible that slight changes in basal Hsp mRNA and protein levels could account for the differences that were observed in the relationship between immediate thermal conditions and the Hsp response in the mid-July compared to the early August sampling period. As the summer progresses and water temperatures become higher, tidepool sculpins may become dependent on a larger standing pool of Hsps to tolerate the daily fluctuations in their environment and this may reduce the need to mount an Hsp response to daily changes in temperature.

From investigations into the adaptive significance of Hsps in thermal acclimation in Drosophila, it appears that populations that are regularly exposed to thermal stress select for higher basal levels of Hsps and do not rely heavily on inducible levels of Hsps to tolerate thermal stress (for review see Hoffmann et al. 2003). It is thought that this is an evolutionary response to reduce the costs (in terms of fecundity and reproduction) of repeated heat exposure. It was not possible to distinguish between Hsc70 and Hsp70 protein using Western blotting. The total amounts of these two proteins remained relatively stable throughout the course of the each 5-day sampling period, with levels increasing slightly over the month. In contrast, there was a
significant and sometimes substantial induction of \textit{hsp70} mRNA expression upon exposure to low tide; however, at no point did this induction culminate in a significant increase at the protein level. This observation could be explained in two ways: either the \textit{hsp70} mRNA is not translated into protein, or the amount of Hsp70 protein that is produced is small compared to the high basal levels of Hsc70 protein and thus the induction is not detectable by Western blotting. We favour the second of these hypotheses. The magnitude and nature of \textit{hsc70} mRNA expression in tidepool sculpins suggests that these fish maintain high levels of Hsc70 protein. \textit{Hsc70} mRNA levels are 50-fold greater than those for \textit{hsp70}, even under conditions of maximal induction. Southern platyfish (\textit{Xiphophorus maculatus}) have also been shown to maintain high basal levels of Hsc70 and 2D gel electrophoresis indicated that induced levels of Hsp70 represented a small fraction of the total Hsc70 and Hsp70 pool (Yamashita et al. 2004). This level of induction would not be detectable on a 1D gel.

The constitutive and inducible isoforms of the 70-kDa family of Hsps have the same function in the cell: to act as molecular chaperones and maintain the integrity of the cellular protein pool (Lindquist 1986). One major difference between these proteins is in their intrinsic mechanisms of regulation and that inducible isoforms are preferentially synthesized under protein denaturing conditions at the expense of all other important cellular proteins (Lindquist and Petersen 1990) and this can ultimately impact organismal fitness (Krebs and Feder 1998). For a poikilothermic organism that inhabits a highly variable environment, inducing Hsp70 protein and thus inhibiting the translation of other housekeeping protein transcripts on a daily basis would represents a significant cost to the organism. Therefore it may be a better strategy to maintain higher levels of the constitutive isoform, Hsc70, in order to deal with the majority of the protein damage that occurs on a daily basis and only increase Hsp70 protein levels when conditions approach the upper tolerance limits of the organism.
Conclusions

By monitoring Hsp levels of tidepool sculpins in response to the natural fluctuations in environmental conditions associated with consecutive midday low tide periods, we have gained novel perspective of the role of environmental temperature in modulating the Hsp response. From the comparison of the Hsp response of sculpins to virtually the same acute temperature change over a low tide period at two different times during the month, it is clear that Hsp mRNA and protein levels do not respond to immediate thermal conditions alone. Rather, thermal history or the chronic environmental temperature likely integrates with the acute thermal environment to structure the Hsp response of sculpins. There was a significant induction in hsp gene expression in response to multiple low tide periods, suggesting that routine fluctuations in environmental conditions can be severe enough to impact on the cellular protein pool. However, despite significant and sometimes substantial changes in hsp70 gene expression over multiple low tide periods there were no corresponding changes detectable at the protein levels. These results raise the question as to the role of the inducible mRNA response. Further studies are necessary to address this question and examine the reliance of sculpins on maintaining higher standing pools of Hsc70 protein to buffer the impact of environmental change within tidepools.

References


Table 5-1: Water temperatures of a mid-intertidal tidepool over a 5-day sampling schedule in both mid-July (July 14\(^{th}\)-July 18\(^{th}\), 2003) and early August (July 30\(^{th}\)-August 3\(^{rd}\), 2003).

<table>
<thead>
<tr>
<th>Mid July</th>
<th>Day</th>
<th>Start Temp</th>
<th>End Temp</th>
<th>Δ Temp</th>
<th>Rate of Temp Increase ((^{\circ})C/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>17.5</td>
<td></td>
<td>3.5</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>21.5</td>
<td></td>
<td>7.5</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>21</td>
<td></td>
<td>6</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>13.5</td>
<td>23.5</td>
<td></td>
<td>10</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>15.5</td>
<td>24</td>
<td></td>
<td>8.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Early August</td>
<td>Day</td>
<td>Start Temp</td>
<td>End Temp</td>
<td>Δ Temp</td>
<td>Rate of Temp Increase ((^{\circ})C/h)</td>
</tr>
<tr>
<td>1</td>
<td>15.5</td>
<td>22</td>
<td></td>
<td>6.5</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>19.5</td>
<td></td>
<td>4.5</td>
<td>0.8</td>
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<tr>
<td>3</td>
<td>14.5</td>
<td>20.5</td>
<td></td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>13.5</td>
<td>21</td>
<td></td>
<td>7.5</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>23.5</td>
<td></td>
<td>8.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Figure 5-1: Water temperatures (°C) recorded in a mid-intertidal tidepool from July 14th to August 9th, 2003. The 5-day sampling period in Mid-July was from July 14th-July 18th, 2003, and the sampling period in Early August was from July 30th-August 3rd, 2003.
Figure 5-2: Tidepool water temperature [A], branchial $hsc70$ mRNA [B], $hsp70$ mRNA [C] and Hsc70 and Hsp70 protein levels [D] of tidepool sculpins at the beginning (L) and end (H) of the midday low tide period over five consecutive days in mid-July. $Hsc70$ and $hsp70$ mRNA expression is normalized to a control gene, $EF-1\alpha$ (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). A difference in letters denotes significant differences within a time of day (start or end of low tide) over all the 5 days (p≤0.05). An asterisk (*) indicates a significant difference between the start and the end of the midday low tide period for a given day (p≤0.05).
Figure 5-3: Tidepool water temperature [A], branchial hsc70 mRNA [B], hsp70 mRNA [C] and Hsc70 and Hsp70 protein levels [D] of tidepool sculpins at the beginning (L) and end (H) of the midday low tide period over five consecutive days in early August. Hsc70 and hsp70 mRNA expression is 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). A difference in letters denotes significant differences within a time of day (start or end of low tide) over all the 5 days (p≤0.05). An asterisk (*) indicates a significant difference between the start and the end of the midday low tide period for a given day (p≤0.05).
CHAPTER 6. GENERAL DISCUSSION

Much of the research presented in this thesis was motivated by a pivotal review written by Martin Feder and Gretchen Hofmann (1999) that outlined many outstanding questions regarding the ecological significance of Hsps and their impact on an organism's natural stress tolerance. They emphasized the need to build on foundational data obtained under controlled conditions in the laboratory and begin to place such results in the context of an animal's natural environment. Organisms in nature seldom encounter one stressor at a time and therefore it is essential that we begin to address the complex properties (multiple stressors, variation in temporal scale of environmental change) of the natural environment and their role in Hsp expression and stress tolerance. Feder and Hofmann (1999) specifically outlined several major gaps in knowledge: 1) whether wild organisms routinely, occasionally, or seldom experience Hsp induction; 2) the degree of natural variation in Hsp expression patterns; and 3) whether the higher levels of Hsps that have been measured in the field result in greater stress tolerance at the organismal level.

The central focus of my PhD thesis was to address these outstanding questions and in doing so, contribute novel insights into the ecological significance of Hsps in allowing organisms to cope with large and frequent fluctuations in their natural environment. I specifically investigated the role of the variable intertidal environment in modulating both the stress tolerance and the Hsp response of tidepool sculpins and examined which aspects of environmental change were significant in providing physiological plasticity to these eurythermal fish.
Research Summary and Perspectives

Environmental variability and organismal stress tolerance

There is substantial evidence to support the phenomenon of cross-tolerance under laboratory conditions, demonstrating that exposure to a mild heat shock can confer an increased tolerance to a number of secondary severe stressors other than heat (Kampinga et al. 1995; Sabehat et al. 1998). In contrast, however, there has been little research that has documented the significance of cross-tolerance in the natural environment. One could imagine that in an environment in which there are routine fluctuations in environmental conditions, such as the intertidal zone, inducible stress tolerance could be a significant feature of an organism's natural stress tolerance. I showed that a mild heat shock could confer an increased tolerance to both subsequent osmotic and hypoxic shock in tidepool sculpins within the laboratory (Todgham et al. 2005). Cross-tolerance in tidepool sculpins appears to be a finely tuned phenomenon sensitive to a narrow range of heat shock temperatures and 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 transient nature of this cross-tolerance and the fact that the time frame of protection induced by heat shock is consistent with the length of the period between tides indicated that cross-tolerance could be an important aspect of adaptive processes in intertidal fish. These findings led me to hypothesize that the sub-lethal heat shocks that occur during low tide periods may invoke a protective mechanism to prepare sculpins for the unpredictable nature of subsequent low tide periods.

In a subsequent experiment, I showed that exposure to the daily fluctuations in environmental conditions inherent in living in the intertidal zone impart a certain degree of enhanced stress tolerance to tidepool sculpins. Two weeks held under constant ambient ocean conditions was sufficient time for tidepool sculpins to lose some of their natural stress tolerance
to both severe thermal and osmotic shocks. These results suggest that there is some aspect of the variable intertidal environment that imparts a high level of stress tolerance to tidepool sculpins. Through comparison of the stress tolerance of sculpins collected from a tidepool to those collected from the subtidal zone, it became apparent that subtle variations in the degree and nature of environmental variability could affect stress tolerance. Subtidal sculpins were as thermotolerant as those from a mid-intertidal tidepool; however, they were not as tolerant of osmotic shock as the tidepool inhabiting fish. The sandy subtidal zone where these fish were collected underwent similar daily fluctuations in environmental temperature as the tidepool, but likely did not undergo significant changes in salinity. Therefore, although subtidal and tidepool-inhabiting sculpins likely have a similarity in their experience of thermal variations, accounting for their similarities in thermotolerance, these two groups of fish live in different osmotic environments and this may be reflected in the difference in osmotic tolerance.

One would predict that the mild heat shocks that tidepool and subtidal inhabiting sculpins experience routinely would confer a generalized stress tolerance to both severe thermal and osmotic shock. In contrast, the experiments conducted on fish collected from the field (presented in Chapter 3) suggest that other environmental factors were important in conferring osmotic tolerance and that the cross-tolerance observed in Chapter 2 was not a significant phenomenon in natural populations. The difference in inducible stress tolerance observed in the laboratory-controlled study (Chapter 2) compared to the field-based study (Chapter 3) highlights the importance of placing results obtained in the laboratory in the context of an animal's natural environment.

*Environmental variability and the Hsp response*

Since the initiation of my thesis research there has been substantial progress made in understanding the plasticity of the Hsp response, including investigations of the mechanisms
underlying the thermal regulation of the onset temperature for hsp gene expression (Buckley et al. 2001; Buckley and Hofmann 2002, 2004; Tomanek and Somero 2002), and the variation in the Hsp response of a species over their geographical distribution (Hofmann et al. 2002; Halpin et al. 2004; Sorte and Hofmann 2004). However, little work has addressed how environmental variation at both acute and chronic temporal scales modulates the Hsp response. The rocky intertidal zone is becoming a significant model system for examining the impact of physical factors and environmental variation on the physiology of its inhabitants. The influence of the predictability of environmental change in the intertidal zone is still largely unknown and it is uncertain if any natural rhythmicity exists in hsp expression. In addition, there have been no studies that have monitored Hsp levels over consecutive low tide periods to document the Hsp response to routine environmental change in the field.

I addressed how acute changes in temperature affected the stress tolerance of tidepool sculpins, and demonstrated that exposure to an initial, sub-lethal heat shock was sufficient to alter the Hsp70 response of sculpins to subsequent more severe osmotic or hypoxic stressors. These findings highlight the short time frame over which an acute variation in environment can modulate how the sculpin responds to a stressor. The extent of the thermal modulation of the Hsp response was dependent on the magnitude of the heat shock, such that a +10°C heat shock did not increase Hsp levels, a +12°C heat shock increased Hsp levels moderately and a +15°C heat shock resulted in a substantial increase in Hsp levels. These results indicate that the Hsp response of this eurythermal intertidal fish was sensitive to acute exposure to slight variations in environmental temperature.

Results of Chapter 3 highlight the significance of longer-term environmental variation in modulating the Hsp response of sculpins to both mild and severe heat shock. Removal of sculpins from the intertidal zone to a constant environment resulted in a rapid (within 2 weeks)
downward shift in both the Hsp90 and the Hsc70/Hsp70 responses such that these lab-acclimatized sculpins were more sensitive to thermal stress. By monitoring both mRNA and protein levels, I was able to gain some novel insights into the mechanisms of environmental regulation of hsp gene expression. The results suggest that the environment can modulate the Hsp response of sculpins at both the transcriptional and posttranscriptional levels. Since there was significant transcriptional regulation of the Hsp response upon transfer of sculpins to a constant environment, whereas there were more subtle differences in the Hsp response of the field-acclimatized fish at the posttranscriptional level, the mechanism by which the environment modulates the Hsp response could depend on the scale of variability in the environment. Exactly how this is achieved requires further investigation.

There has been no research to address whether the predictable timing of environmental change inherent with the ebb and flow of the tides directly imparts a natural rhythmicity to the Hsp response. The results of presented in Chapter 4 demonstrate that there was no endogenous diurnal rhythm in the Hsp response entrained to the tidal cycle in the tidepool sculpin; rather hsp gene expression was induced in response to fluctuations in environmental conditions. These results suggest that the unpredictable nature of the water quality of a tidepool from one low tide to the next has a more important role in ultimately modulating the Hsp response rather than the predictable cycle of tides. The cueing of the Hsp response to acute environmental conditions allows for the response to be rapidly regulated and tailored to the immediate environmental conditions.

Within the tidepool, sculpins rapidly induced hsp70 gene expression during a low tide period and hsc70 and hsp90 gene expression was significantly elevated within hours of the onset of high tide. Similarly, the experiments reported in Chapter 5 demonstrated that there were significant and rapid increases in hsc70 and hsp70 gene expression over multiple low tide
periods. The significance of these findings is two-fold. First, these are the first comprehensive reports of wild animals routinely expressing inducible hsp transcripts in response to natural fluctuations in environmental conditions over multiple days. Previous work (e.g. on Drosophila, Feder et al. 1997, 2000) indicates that organisms can use behavioural thermoregulation to minimize exposure to heat shock temperatures. In contrast, tidepool organisms are somewhat constrained from using this strategy. These results provide direct evidence that daily variations in environmental conditions within the tidepool are severe enough to affect protein homeostasis. Further studies are necessary to determine the extent of the possible damage to the cellular protein pool and whether there are corresponding changes in ubiquitin levels that would indicate more severe protein denaturation. Second, the speed at which the Hsp response was regulated in tidepool sculpins in their natural environment was remarkable compared to that observed in other studies (Hofmann and Somero 1996; Tomanek and Somero 2000). During a low tide period, sculpins are actively foraging in the tidepool and therefore these organisms must have the capacity to deal quickly with the cellular stress associated with inhabiting this variable environment. By taking sculpins into the laboratory and exposing them to an artificial temperature cycle regime that mimicked the temperature profile of a mid-intertidal pool, I was able to show that fluctuations in environmental temperature are one of the key modulators of hsp gene expression in sculpins.

The experiments presented in Chapter 5 begin to dissect which aspects of temperature change were modulating the Hsp response. In nature, chronic environmental conditions, such as thermal history, are thought to play a role in determining the Hsp response to immediate temperature change (Feder and Hofmann 1999). From laboratory-based acclimation and acclimatization studies investigating the differences in the Hsp response of organisms from different thermal niches, we have gained considerable insight into the importance of environmental history in structuring an animal's cellular response to stress (Dietz and Somero
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1992; Roberts et al. 1997; Tomanek and Somero 1999; Buckley et al. 2001; Nakano and Iwama 2002). However, at the start of my thesis, it was still unclear how acute and chronic changes in environmental temperature integrate to influence the Hsp response of organisms to daily fluctuations in natural environmental conditions. From the comparison of the Hsp response of sculpins to virtually the same acute temperature change over a low tide period at two different times during the month, it is clear that Hsp mRNA and protein levels do not respond to immediate thermal conditions alone. Rather, thermal history or chronic environmental temperatures likely integrate with the acute thermal environment to structure the Hsp response of sculpins. This is the first study to document that both acute and chronic changes in the thermal environment modulated the Hsp response of organisms within their natural environment.

Although the breadth of Hsps that have been monitored in the thermal acclimation studies of intertidal organisms has increased, it is still unclear which Hsps play vital roles in enabling animals to cope with the daily variations in environment conditions that they experience in nature. Nakano and Iwama (2002) observed in nature higher basal levels of Hsc70 and Hsp70 in the tidepool sculpin compared to its congener, the fluffy sculpin (O. snyderi) that inhabits a less variable environment of the lower tidepool. They proposed that these differences might account for the ability of tidepool sculpins to inhabit the more thermally challenging environment of the upper, more isolated tidepools. I demonstrated that tidepool sculpins accumulated high and relatively constant standing levels of Hsc70 during the summer. This strategy may reduce the need to mount an inducible Hsp protein response to daily changes in temperature. By limiting the induction of Hsp70 in response to routine fluctuations in environmental temperature, sculpins may minimize the negative impact on the translation of other housekeeping protein transcripts that accompanies hsp70 gene expression (Dorner et al. 1992; Lindquist and Petersen 1990). It is important to note that I did observed significant induction in hsp gene expression in response to multiple low tide periods in the experiments reported in both Chapters 4 and 5; however, these
did not seem to correspond to detectable changes at the protein level. These results raise the question as to the role of the inducible mRNA response in tidepool sculpins. Further studies are necessary to address this question and examine the reliance of sculpins on maintaining higher standing pools of Hsc70 protein to buffer the impact of environmental change within tidepools.

**Ecological significance of the Hsp response: Role of Hsps in natural stress tolerance**

Previous studies in fish, as well as those in model systems, have shown a strong association between elevated levels of Hsps prior to exposure to a second stressor and cross-tolerance (Lee and Hahn 1988; Flahaut et al. 1996; Laplace et al. 1996; Krebs and Feder 1998). In contrast, I provided strong evidence that elevated levels of Hsp70 protein were not required prior to exposure to the second stressor for a mild heat shock to confer protection. Rather, this heat shock may have primed the cell to mount an Hsp70 response following exposure to a second stressor that alone could not induce Hsp70, thereby facilitating a faster cellular stress response to a subsequent stressor. Results reported in Chapter 3 placed some environmental context to what we observed in the laboratory, suggesting that the variable nature of intertidal environment is also able to prime tidepool sculpins to rapidly mount an Hsp90 response to severe heat shock. Sculpins that were acclimatized for 2 weeks to constant conditions in the laboratory were unable to mount an Hsp90 response to the same heat shock and this correlated with a significant decrease in thermotolerance. It may be of interest to note that the Hsp70 response to severe heat shock was similar for the lab- and field-acclimatized groups despite the differences in stress tolerance. These findings offer interesting insight into the different effects of environmental temperature on the upper thermal threshold for translation of different Hsps. It was difficult to draw any conclusions with respect to the role of Hsps in the differences in osmotic tolerance observed between the lab and field-acclimatized groups of sculpins due to a general lack of any pronounced Hsp response to osmotic shock.
As environmental temperatures increased over the month of July, there was a significant accumulation of Hsc70 protein in tidepool sculpins. These results suggested that in their natural environment, sculpins are sensitive to slight changes in their thermal environment and are able to modulate their Hsc70 levels accordingly. Although I was unable to demonstrate that these changes in Hsc70 protein levels corresponded to an increased stress tolerance, the tight link between environmental temperature and Hsp levels observed in the chapters of this thesis provides strong evidence for an ecological significance of Hsps in a sculpin’s natural stress tolerance.

Hoffmann and colleagues (2003) highlighted in a recent review that populations that are regularly exposed to thermal stress select for higher basal levels of Hsps and do not rely heavily on inducible levels of Hsps to tolerate thermal stress. It was suggested that this is an evolutionary response to reduce the costs (in terms of fecundity and reproduction) of repeated heat exposure. The results from experiments in Chapter 5 provide preliminary evidence that this may be a strategy used by tidepool sculpins to maintain high natural thermotolerance. To appreciate the ecological significance of large standing pools of Hsps in a diversity of intertidal organisms, a comprehensive monitoring of Hsp levels in a variety of organisms over multiple tidal cycles will be necessary.

**General Conclusions**

An animal’s natural environment is highly complex and varies considerably on a daily as well as a seasonal basis. Organisms inhabiting variable environments such as the intertidal zone face the challenge of maintaining biochemical and physiological performance in a constantly changing environment. Through the research presented in this thesis, I have attempted to understand how environmental variability affects organismal stress tolerance and specifically
addressed how variations in environmental conditions on both acute and more long-term scales modulate the natural stress tolerance of tidepool sculpins. Stress tolerance in tidepool sculpins displays a significant amount of plasticity that was sensitive to both short and long-term changes in the thermal environment. The ecological significance of such plasticity in stress tolerance is unclear but likely reflects plasticity of the cellular mechanisms underlying stress tolerance. From the results presented in this thesis, it is evident that Hsps play a key role in providing tidepool sculpins the flexibility necessary to handle the impacts of a variable environment on the integrity of the cellular protein pool and as a result have an important ecological role in the tolerance of these eurythermal fish to natural fluctuations in their environment.

The work presented in this thesis offers a unique perspective on the environmental regulation of *hsp* gene expression. While many research groups have been interested in determining the cellular mechanisms underlying how an animal senses changes in temperature and then transduces this effect through genotype to ultimately affect genotype (Hofmann et al. 2002; Tomanek and Somero 2002; Hoffmann et al. 2003), I have taken a different approach and closely monitored changes in *hsp* gene expression and Hsp protein levels in response to differing degrees of environmental variability in attempts to understand the aspects of the thermal signal to which the animal is responding. While the mechanistic approach taken by other research groups has been fruitful, it has been an arduous task identifying what aspects of the stress-signaling cascade to examine under laboratory-acclimation studies and then to take this one piece of the puzzle and place it in the context of the complicated nature of a variable environment. By using a qualitative approach to assess the plasticity of the Hsp response in the natural environment, my thesis research has provided novel ecological insights into the regulation of Hsps in response to routine fluctuations in environmental conditions (i.e. that there is both a transcriptional and posttranscriptional component). In addition, the results of this thesis have provided an ecological context for the significance of thermal history in structuring an animal's cellular response to
stress and has shown that the Hsp response in tidepool sculpins is sensitive to slight differences in environmental temperature that occur over both temporal (different times during a month) and spatial (tidepool vs. subtidal) scales. It will likely be through the integration of mechanistic and ecological approaches that we will gain a better understanding of how environmental temperature is transduced through the genotype to ultimately enable adaptation of the organism to its environment.

References


