# THE HEAT SHOCK PROTEIN RESPONSE AND PHYSIOLOGICAL STRESS IN AQUATIC ORGANISMS.

by

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#### Abstract

The experiments described in this thesis were performed to examine the relationship between heat-shock proteins (hsp) and the hormonal stress response in aquatic animals. Cell lines were first exposed to selected stressors to determine which heat shock proteins are induced by environmental stressors. The hsp70 and hsp30 responses were then measured in the tissues of live fish pre-exposed to a 45 s handling stress, intraperitoneal cortisol implants or heat shock to test the effect physiological stress and cortisol on the heat shock protein response. Heat shock protein levels were quantified by densitometric scanning of Western blots probed with salmonid-specific rabbit polyclonal antibodies. The stressors used for these experiments were 10 and 15°C heat shocks, pH 4.5 to pH 10, 10 mM L-azetidine, and 45 s physical handling.

Autoradiographs of one and two dimensional SDS-PAGE gels showed, with few exceptions, that new 70 and 30 kDa proteins were synthesized by cell lines exposed to the selected stressors. A further investigation of radiolabeled proteins in chinook salmon embryonic (CHSE) cell line exposed to a wide pH range, from pH 4.5 to pH 10, showed a prominent 70 kDa protein in response to either low or high pH. A novel 80 kDa protein was observed 1 h and 3 h after exposure of the cells to pH 10.0, and a 32 kDa protein band was observed in cells exposed to pH 5.5.

The physiological stress response was induced in live fish by physical handling. Circulating plasma cortisol levels were increased alone by intraperitoneal slow-releasing cortisol implants. Handling and cortisol implants resulted in elevated plasma cortisol and glucose levels. Plasma sodium, chloride and potassium ion levels were essentially unaffected by any of the treatments. Gill, anterior kidney, liver and skeletal muscle samples were taken at regular intervals heat shock treatments and showed elevated hsp70 levels in

the gill, liver, and kidney tissues 1 h after a 2-h 10°C step change heat shock. The four tissues, including skeletal muscle, had elevated hsp70 concentrations 48 h after heat shock. Only the gill tissues had elevated hsp30 levels 1 h after heat shock and no tissues had elevated hsp30 levels 48 h after the heat shock. Hsp70 levels were elevated in gills tissues for three weeks after the fish were exposed to a 2-h 15°C step change heat shock and returned to ambient temperature. Physical handling did not induce increased levels of hsp70 in gill, liver or kidney tissues. Handling the fish prior to heat shock suppressed the typical hsp70 level increase observed in the gills of heat-shocked fish, however, elevated plasma cortisol concentrations alone did not attenuate the gill hsp70 increase caused by heat shock. Gill tissues from fish which were handled or cortisol-injected prior to heat shock showed no increase in hsp30 concentration either 1 or 48 h after the heat shock.

Primary cultures of trout hepatocytes were exposed to physiological  $(10^{-7})$  and pharmacological  $(10^{-5})$  concentrations of adrenaline to test for its effect on heat shock protein levels *in vitro*. Propranolol was used to test the effect of blocking ß-receptors during exposure of hepatocytes to adrenaline. These hepatocyte exposures showed that cells exposed to physiological or pharmacological concentrations of adrenaline had elevated levels of hsp70. Cells exposed to equimolar pharmacological  $(10^{-5})$  concentrations of both adrenaline and the  $\beta$ -blocker propranolol had the same hsp70 concentrations as those found in control cells. The blocking effect of propranolol on the  $\beta$ -receptors may therefore block the intracellular alkalosis known to be caused by adrenaline, and which may have induced the hsp70 in adrenaline exposed primary hepatocytes.

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#### **Chapter 1: General introduction**

#### General thesis objective

The purpose of this study is to examine the relationship between the hormonal stress response and the heat shock protein response in aquatic animals and cell lines. Despite a large body of literature on the heat shock protein response and the extensive work done to date on the hormonal stress response of salmonids, little is known about the relationship between these two adaptive responses to environmental stress.

This general introduction is written with three specific objectives. The first is to discuss the heat shock protein response in general terms, giving information and a brief review of the mechanisms involved in the regulation and synthesis of heat shock proteins. The role they play as chaperones, in protein folding and transmembrane transport is also discussed. The second goal is to describe the physiological stress response of fish, with emphasis on the hormonal stress response which occurs when organisms are faced with suboptimal environmental conditions. The third segment of this chapter outlines the studies and the organization of the experiments described in this thesis.

#### The heat shock protein response

Heat shock proteins (hsp) are synthesized in the cells of most organisms, in response to various environmental and pathophysiological stressors. The heat shock response was first observed as "puffing" in the polytene chromosomes of *Drosophila* salivary glands exposed to heat shock (Ritossa 1962). The products formed were later named heat shock proteins (Tissières et al., 1974). Although some of these proteins have also been called stress proteins (SP), the traditional hsp nomenclature will be used throughout this thesis. Some hsps have been found to increase in concentration when organisms are exposed to environmental stressors such as ultraviolet light, heavy metals, xenobiotics, ethanol, anoxia, amino acid analogues, viral infections and pH change (Hightower 1980; Kothary and Candido 1982; Misra et al. 1989; Sanchez et al. 1992; Welch 1993;) and provide a measure of protection against stress insult. Other hsps facilitate the degradation of denatured proteins. Many heat shock proteins also participate in a number of essential metabolic pathways in normal cells, such as the synthesis, assembly and transport of cellular proteins, as well as regulating interactions between steroid hormones and their receptors (see Nover, 1984; Lindquist, 1986; Lindquist and Craig, 1988; Nagao et al., 1990; Welch et al., 1991; Welch, 1993).

The hsp response allows organisms to survive stressors that might otherwise result in cell damage or death (Hightower 1980). Cells exposed to different chemical, physical or physiological stressors respond by expressing these evolutionarily conserved proteins (Schlesinger et al., 1982), which have been found in organisms as diverse as bacteria; yeast, mollusks, fish and humans (Margulis et al. 1989; Schlesinger 1986; Zafarullah et al. 1992). Hsp70 is among the most highly conserved proteins known and is found in nearly all organisms (Gupta and Golding 1993); only *Hydra oligactis* has been found not to synthesize this protein upon temperature elevation (Bosch et al. 1988). The genes for hsp70 are more than 50 percent similar between bacteria, yeast and *Drosophila* (Craig et al. 1979). Such conservation suggests that hsps may play a role in the generalized stress response of whole animals described by Selye (1950). Acquired tolerance, or the ability to survive subsequent and more serious environmental stress, correlates with the accumulation of hsps (Subjeck et al., 1982; Lindquist 1986).

The expression of heat shock genes, which are common among nearly all organisms, is involved in the survival of cells exposed to harsh environments (Morimoto et al., 1993). Figure 1.1 shows the main factors which stimulate the heat-shock response, also referred to as the cellular stress response. As Figure 1.1 outlines, this response is involved in protecting organisms from damage which may be caused by environmental or, pathophysiological stressors and in non-stressful conditions such as development and differentiation (Morimoto, 1992).

Hsps have been grouped into four families according to similarities in their molecular weights and primary structures. The four major families are: (1) 90kDa, (2) 70kDa, (3) 60kDa and (4) the 16-30 kDa families. These are usually referred to as hsp90, hsp70, hsp60 and the low molecular weight (LMW) hsps, respectively (Schlesinger et al., 1982).

The hsp family most commonly induced in organisms exposed to suboptimal physiological conditions is composed of proteins of relative molecular mass about 70,000 (hsp70). This family of hsps is synthesized by both prokaryotic and eukaryotic cells.

#### **Regulation of the heat shock response**

#### Transcriptional regulation of hsp70 gene expression:

The hsp70 gene has been well studied as a classical heat shockresponsive gene. Certain growth hormones, mitogens, serum stimulation, and viral infections lead to the expression of the human hsp70 gene at the G1/S boundary of the cell cycle. The promoter of the human hsp70 gene contains multiple heat shock elements (HSE), which are inverted repeats of the pentameric sequence nGAAn. These confer stress inducibility and have a basal promoter region comprised of a complex array of cis-binding sites (Sp1, CCAAT,

ATF), which are multiple cis-regualtory elements. These elements confer a range of constitutive and inducible transcriptional responses to the hsp70 gene. Similar elements are also common to the promoters of other heat shock genes (Morimoto et al., 1992). Induction of heat shock genes in higher eukaryotes is mediated by the activation of a pre-existing heat shock factor (HSF) that binds to a HSE, which is a target gene sequence located in the promoters of heat-inducible genes (Pelham 1982; Wu et al., 1990).

Heat shock factor structure and regulation: Two adjacent regions near the amino terminus of HSF proteins are required for high-affinity binding of this protein to the HSE. Deletion of a region bearing conserved leucine zipper motifs abolishes the multimeric association and high-affinity binding to the HSE (Westwood et al., 1991). The native molecular mass of active and inactive forms of HSF has been determined using western blotting after pore exclusion limit electrophoresis on nondenaturing gels. Analysis of unshocked Drosophila tissue culture cells revealed a single complex migrating at a native relative molecular mass of about 220,000. This complex reacts with polyclonal antibodies raised to bacterially produced HSF protein ( $\alpha$ -HSF). Heating this complex in vitro results in a partial depletion of the 220K species and the appearance of a 690K HSF species, in addition to a small amount of higher Mr material. Analysis of both small and large HSF complexes for DNA-binding activity (by mobility shift technique using pore exclusion limit electrophoresis) revealed that the 690K species, but not the 220K species, showed specific binding to <sup>32</sup>P-labeled HSE. The 220K HSF species therefore represents the inactive form of HSF, and heat treatment converts it to the active 690K species (Westwood et al., 1991). Figure 1.2 is a model of HSF regulation which has been proposed by Morimoto (1993) but has yet to be proven.

Monomeric HSF is thought to be present in both the cytoplasm and nucleus of unstressed cells and has no DNA binding activity. Upon oligomerization, the HSFs are redistributed to Drosophila chromosomal puff sites. The HSF also bind to as many as 150 other chromosomal sites, such as developmental loci, which are repressed during heat shock. This binding suggests a repressor role of normal gene activity for HSF during heat stress (Westwood et al., 1991). HSF assembles into a trimer and accumulates within the nucleus in response to heat shock and other physiological stressors. HSF trimers bind to the HSEs, located in the 5'-flanking sequences on heat shock responsive genes. These genes are activated within minutes of temperature elevation. HSF-DNA binding activity, however, does not always correlate with transcriptional activity. This suggests that there are multiple steps in the activation process. HSF exhibits a stress-dependent phosphorylation which might affect its transcriptional activity. The heat shock-activated transcriptional response is attenuated if the cells are exposed to prolonged intermediate heat shock. Under these conditions, the trimeric form of the HSF is thought to be converted back to the DNA non-binding monomeric form and the distribution returns to the normal subcellular compartments. Under prolonged heat shock, however, sustained heat shock gene transcription and HSF DNA binding activity is observed (Morimoto, 1993). Heat shock proteins themselves may regulate heat shock gene expression via an autoregulatory loop (Morimoto, 1993). One proposed hypothesis is that as the increased levels of misfolded proteins induced during heat shock or other forms of stress bind to Hsp70, HSF becomes activated as it is released from Hsp70. Morimoto et al. (1992) showed that HSF associates with hsp70 and that the association between the two increases as the hsp70 levels increase in cells exposed to heat. This HSF-hsp70 complex is disrupted upon addition of ATP or other hydrolyzable nucleotides while the

addition of non-hydrolyzable nucleotide analogues do not disrupt the complex. Negative regulation of HSF by hsp70 is suggested, as exogenous recombinant wild-type hsp70 can associate with activated HSF and disrupt its DNA-binding ability. Amino-terminal or carboxy-terminal deletion mutant hsp70 cannot bind to HSF and block binding to DNA.

The inhibitory effect of hsp70 on HSF-DNA complex binding is abolished by ATP. The oligomeric nature of the heat shock factor protein after exposure of cells to stress may explain how these factors can bind to the structurally distinct 5 bp units and to heat shock elements with more than 5 bp unit. Both arrangements of the 5 bp unit, head-to-head and tail-to-tail, have the same affinity for HSF. The length of the HSF footprint increases with the addition of other 5 bp units to form longer arrays. The electrophoretic mobility of HSF-DNA complexes decreases markedly with the addition of three 5 bp units, suggesting that binding of the HSF trimers to DNA subunits is determined by the number of 5 bp recognition site units that are correctly positioned with the array (Perisic et al., 1989). Sorger and Nelson (1989) also suggested that the oligomerization of the yeast HSF from Saccharomyces cervisiae leads to the formation of trimers in solution and bound to DNA. The trimerization of HSF is proposed to be mediated by the occurrence of hydrophobic amino acids at every 7 residues, like the leucine zipper. This multimerization of HSF is thought to be essential for affinity binding of HSF to DNA. A marked difference exists between the HSF of yeast cells and HSF of human cells. Yeast cell HSF binds to DNA at all temperatures and the oligomerization state of these HSF appears not to change. In human (HeLa) and Drosophila cells, however, HSF binds to DNA only after heat shock. It is assumed that a transition between the monomer and trimer state of these HSF increases their ability to bind DNA.

Despite widespread conservation of heat-shock proteins and the HSE sequence in eukaryotes, the predicted amino-acid sequences of the cloned HSFs are highly divergent, except for the DNA-binding domain and several heptad repeats of hydrophobic residues, or leucine-zipper motifs. For *Drosophila* and *S. cervisiae* HSFs, deletion of a region bearing conserved leucine zipper motifs abolishes the multimeric association and high-affinity binding to HSE (Sorger and Nelson, 1989).

The regulatory involvement of heat shock factors is different for eukaryotes and prokaryotes. In higher eukaryotes, HSF is maintained in a non-DNA binding state in unstressed cells and in response to heat shock acquires an oligomeric state and binds to the HSE in the promoters of heat shock-responsive genes. In S. cervisiae, HSF constitutively binds DNA and undergoes heatinduced phosphorylation with an increase in transcriptional activity. Phosphorylation of HSF is not essential for oligomerization, acquisition of DNA binding activity, or translocation of HSF into the nucleus. However. phosphorylation may be important for attaining maximal inducible transcriptional activity or for attenuation of the heat shock response. HSFs have two highly conserved segments; the amino-terminal localized DNA binding domain, which is approximately 100 amino acids long, and a motif of hydrophobic heptad repeats, which mediates the oligomerization of HSF (Morimoto et al., 1992). Abravaya et al. (1992) performed gel mobility-shift assays with an oligonucleotide containing the HSE sequence from the human hsp70 promoter and whole-cell extracts from human K562 cells preincubated with the anti-hsp70 monoclonal antibody C92 to determine if hsp70 associates with HSF. They showed that the addition of this anti-hsp70 antibody to extracts from cells heat-shocked for 3 h results in the appearance of supershifted complexes while the addition of equivalent amounts of anti-mouse IgG had no effect on the mobility of the HSF-HSE complex. This

suggests that in cells heat-shocked for 3 h, some of the activated HSF is complexed with hsp70. Hsp70 therefore prevents the activation of HSF by inhibiting its transformation from the non-DNA-binding state to the DNA-binding form; ATP abolished this inhibitory effect.

*DNA recognition:* Eukaryotic heat shock gene induction in response to a temperature upshift is mediated by the binding of heat shock factor (a transcriptional activator) to a highly conserved short DNA sequence known as heat shock element. These are best described as contiguous arrays of variable numbers of the 5 bp sequence nGAAn arranged in alternating orientation (n denotes less strongly conserved nucleotides that nevertheless may be involved in important DNA-protein interactions). Two or more nGAAn units are required for high affinity binding of heat shock factor *in vitro*. The orientation of these units can be either head-to-head (nGAAnnTTCn) or tail-to-tail (nTTCnnGAAn) Sorger (1991).

In addition, a region necessary for efficient transcription and translation of the *Drosophila* heat-shock gene for hsp22 has been identified downstream of the transcription start site. The expression of P-factor-mediated mutant gene insertions show that deletions within the first 26 nucleotides blocks the preferential translation of hsp22 mRNA at high temperatures. If this region is intact, up to 86% of the leader can be deleted without affecting transcription or translation (Hultmark et. al., 1986).

#### Translational control of the heat shock response:

Less is known about the translational control of the heat shock response compared to the transcriptional control of the response. One of the best examples of translational regulation of the heat shock response is seen in *D. melanogaster* cells. Translation of pre-existing mRNAs is repressed by more than 95% within 10 min when cells from this organism are shifted from 25° to

37°C. When newly transcribed mRNAs for hsps appear in the cytoplasm the 5' end of their untranslated leader sequence mediates their preferential translation, to the virtual exclusion of other proteins as long as the cells are maintained at elevated temperatures (Petersen and Lindquist, 1988). Cells returned to 25°C continue to synthesize hsps for a time that is proportionate to the severity of the preceding stressor. Hsp70 synthesis is invariably repressed when the translation of pre-existing mRNAs is restored (DiDomenico et al., 1982). Unusually long A-rich leader sequences are found in all Drosophila heat-shock genes induced in response to elevated temperatures. Induction of heat-shockprotein synthesis is due both to an induced transcription of the heat-shock genes and to the selective translation of these transcripts (Hultmark et al., 1986). The protein synthetic machinery of heat-shocked cells is modified such that heatshock messengers are preferentially translated (Scott and Pardue, 1981; Kruger and Benecke, 1982). The repression of normal message translation is due to the inactivation or modification of a factor that is required for the translation of normal cellular mRNAs, possibly the cap-binding factor. Maroto and Sierra (1988) showed with lysates from heat-shocked Drosophila embryos that translation of heat-shock mRNA is basically unaffected by the addition of cap analogues, while the residual synthesis from normal cellular mRNAs is repressed. An antibody against the Drosophila cap-binding protein blocks the translation of the bulk of normal cellular mRNAs, but not the translation of hsp70 mRNA (Lindquist and Petersen, 1990). Hsp70 is associated with translating ribosomes and this association can be disrupted by the drug puromycin, which causes the release of nascent polypeptides from the ribosome. Hsp70 may therefore aid in the passage of nascent polypeptides through the ribosome channel to the cytosol (Nelson et al., 1992).

#### Functions of heat shock proteins

Heat shock proteins are not only present when organisms are exposed to stressful environmental conditions, but are also synthesized by cells under normal conditions (Craig et al., 1983). The constitutively expressed heat shock proteins, often referred to as cognates (hsc) appear to be important for the regulation of protein homeostasis. Molecular chaperones are a ubiquitous group of hsps which include the hsp90, hsp70 and hsp60, also referred to as chaperonin. This group of proteins directs the folding and assembly of other cellular proteins, and is also involved in regulating the kinetic partitioning between protein folding, translocation reactions and protein aggregation (Sanders et al., 1992). Protein denaturation resulting in misfolding and aggregation is caused by the weakening of polar bonds and exposure of hydrophobic groups, often the result of exposure of proteins to adverse physical and chemical conditions (Wedler, 1987). The term proteotoxicity was coined by Hightower (1991) to underscore this important target for stressor-induced Members of the hsp70 and hsp90 families are involved in the damage. assembly and disassembly of proteins and protein-containing structures (Pelham, 1986).

#### <u>Chaperoning</u>:

Molecular chaperones have recently been defined as a family of unrelated classes of proteins that mediate the correct assembly of other polypeptides but that are not components of the functional assembled structures. The molecular chaperone concept suggests that self-assembly is not the predominant process of protein assembly *in vivo;* observations have shown that some proteins will not assemble correctly unless assisted by other proteins. The chaperone function appears to be required because many cellular processes that involve protein assembly carry an inherent risk of malfunction due to the number, variety and flexibility of the weak interactions that hold proteins in their functional conformations. It appears to be the role of molecular chaperones to combat the problem of incorrect interactions which generate nonfunctional structures (Ellis and van der Vies, 1991).

hsp 60: The 60K hsp family of 'chaperonins' includes mitochondrial hsp60, chloroplast Rubisco-subunit binding protein and GroEL in Escherichia coli. Transport intermediates of precursor proteins have been used to show that hsp60 interacts with imported proteins in an ATP-dependent reaction, and promotes the (re)folding and assembly of proteins. This is an indication that hsp60 has the function of a 'foldase' (Kang et al., 1990). Unlike hsp70, binding of chaperonin seems to involve side-chain hydrophobicity (Flynn et al., 1991). Furthermore, chaperonin (cpn60) forms an oligomeric complex that is arranged as a single toroidal ring of seven 60-kDa subunits (Viitamin et al., 1992). Seven identical binding sites are created by this "donut" structure. This complex binds incompletely folded proteins and directs the folding peptide to the correct conformation, in an ATP-dependent fashion (Buchner et al., 1991). The complex also prevents aggregation of incompletely folded proteins until they are competent for oligomer assembly (Cheng et al., 1989; Martin et al., 1991). Chaperonin synthesis increases under adverse environmental conditions which cause protein denaturation. Unlike hsp70, however, chaperonin is unable to break up existing protein aggregates (Caltabiano et al., 1988). These differences in conformational binding suggest that chaperonin and hsp70 perform different folding functions.

*hsp* 70: Hsp70 is a ubiquitous and highly conserved family of stress proteins. At least 21 proteins belonging to this multigene family have been characterized. Hsp70 DNA and RNA from mammals, fish, nematodes and mollusks cross-hybridize, indicating a high degree of similarity in nucleotide sequence from

these diverse phyla (Nover and Scharf, 1991). Antigenic and ATP-binding domains on hsp70 from organisms as divergent as mollusks and humans are similar, further indicating the similarity of hsp70 from these divergent organisms (Margulis et al., 1989). Hsp70 synthesis increases during adverse environmental conditions, and protects the cells from proteotoxicity. Cytoplasmic hsp70 migrates to the nucleus, binds to pre-ribosomes and other protein complexes to help prevent their denaturation (Lindquist 1986; Gething and Sambrook 1992). There is also evidence that hsp70 can prevent the formation of insoluble aggregates and break up existing aggregates that can be damaging to the cell. These functions may allow damaged proteins to refold and resume their normal biological activity (Ellis 1990; Gaitanaris et al. 1990; Skowyra et al., 1990). Damaged proteins are also vectored to lysosomes for degradation by hsp70 (Chiang et al., 1989).

Hsp70 is also a molecular chaperone, if it is assumed that the primary effect of stress is to cause the appearance of interactive surfaces that are recognized by chaperones. The hsp70 proteins are produced by prokaryotes (DnaK in E. coli) and several cellular compartments in eukaryotes (cytosolic clathrin-uncoating ATPase and BiP in the endoplasmic reticulum). Nonfunctional structures appear to be prevented from forming when polypeptides interact with hsp70 during the assembly process. Examples of these interactions are: (1) the association of BiP protein with nascent unfolded polypeptides as they emerge in the lumen of endoplasmic reticulum during cotranslational protein transport, (2) the association of hsp70, encoded by the SSA1 and SSA2 genes of yeast, with precursor polypeptides destined to enter the endoplasmic reticulum or mitochondrion, which maintains the polypeptides in a translocation-competent conformation. An hsp70 member is also required in the mitochondrial matrix for protein translocation and correct folding. Hsp70 also

promotes the disassembly of proteins which have been damaged as a result of stress. Heat-shock proteins associate with the interactive surfaces of damaged proteins, and the ATP-mediated release of the denatured proteins appears to favor correct reassembly of the proteins structures. DnaK from *E. coli* causes the reappearance of RNA polymerase activity when incubated with heat denatured inactive polymerase and MgATPase (Ellis and van der Vies, 1991).

*hsp90*: Members of the hsp90 family of proteins are molecular chaperones involved in signal transduction by steroid receptors. The steroid receptor is bound to hsp90 in the absence of steroid hormones, and is unable to activate transcription of steroid-controlled genes. Steroid hormones displace the bound hsp90, which results in a steroid-receptor complex capable of activating transcription. Yeast studies suggest that this effect is not one of steric hindrance of the DNA binding site by hsp90. Receptors can be activated by the hormone only if they are initially bound to hsp90, which appears to bind to the receptor and maintain it in an activatable conformation (Ellis and van der Vies, 1991).

Wiech et al. (1992) have shown that Hsp90 suppresses the formation of protein aggregates by binding to the target proteins at a stoichiometry of one Hsp90 dimer to one or two substrate molecule(s). It may be that Hsp90 uses a novel molecular mechanism to assist protein folding *in vivo* as this action of Hsp90 is not dependent on the presence of nucleoside triphosphate.

*Small hsps*: The low molecular weight stress proteins (LMW) are a diverse group of heat-inducible proteins in the 20-kDa range. These proteins are more species-specific and are less conserved than the other major stress protein families. These proteins are, however, all homologous to  $\alpha$ -crystallin and have the ability to form higher order structures of approximately 500,000 kDa. They are not synthesized under normal conditions, as hsp90, hsp70 and the chaperonins are. The synthesis of these proteins is developmentally regulated

and is induced by adverse environmental conditions (Sanders, 1993). Although little is known of the mechanisms in their thermotolerance role, presence of LMW heat shock proteins correlates with acquired thermal tolerance in *Drosophila* and rodent cells (Berger and Woodward, 1983; Landry et al., 1989).

#### Protein folding

*Chaperonin:* As was mentioned previously, hsp60 interacts with imported proteins in an ATP-dependent reaction, and promotes the (re)folding and assembly of proteins (Kang et al., 1990). Binding of hsp60 to proteins appears to involve side-chain hydrophobicity (Flynn et al., 1991). Chaperonin (cpn60) forms an oligomeric complex that is arranged as a single toroidal ring of seven 60-kDa subunits (Viitanen et al., 1992). The ring results in seven identical binding sites created by the "donut" structure. The complex binds incompletely folded proteins and directs the folding peptide to the correct conformation, in an ATP-dependent fashion (Buchner et al., 1991).

*Hsp70*: Hsp70 is present in the mitochondrial matrix and is involved in the (re)folding of proteins which are unfolded during their translocation from the cytosol through the mitochondrial membrane to the mitochondrial matrix. Cytosolic hsp70, called Ssa1-4p in yeast, confers transport-competence to protein precursors, as they stimulate translocation in mitochondria and endoplasmic reticulum (Kang et al., 1990). Hsp70 is found in several subcellular compartments and binds primarily to aliphatic residues (probably through hydrogen bonding with the peptide backbone, hydrophobic contacts, and salt bridges with side chains) on target proteins (Flynn et al., 1991; Landry et al., 1992) to modulate protein folding, transport and repair. Cognate hsc70 facilitates the folding of new peptides by stabilizing the peptide chains in a loosely folded state until synthesis has been completed, and then prevents incorrect folding of the peptide (Beckmann et al., 1990; Gething and Sambrook,

1992). ATP hydrolysis appears to be necessary for the dissociation of hsp70 from the protein during the folding process.

Ssc1p (mitochondrial hsp70) has been shown to be required in the mitochondrial matrix of yeast (*S. cervisiae*) for translocation and (re)folding of proteins within the matrix (Koll et al., 1992). Proteins that remain in the matrix after translocation are transferred from hsp70 to the chaperonin hsp60, which is involved in their ATP-dependent folding and assembly (Cheng et al., 1989).

#### Membrane transport

Compelling genetic and biochemical evidence suggests that hsp70 proteins aid in protein translocation. Proteins translocating into the mitochondria must unfold on the cytoplasmic side and then refold on the matrix side (this process is promoted by the hsp60 protein). Unfolding of the mitochondrial precursor proteins to reach translocating-competent conformation requires extra-mitochondrial ATP (Rothman 1989). Figure 1.3 is a model of protein import and sorting proposed by Fölsch et al. (1996).

Cytoplasmic hsc70 stimulates the import of proteins into the mitochondria and may explain the dependence on ATP for the import of proteins (Murakami et al., 1988). Cytosolic hsp70, called Ssa1-4p in yeast stimulates translocation of precursor proteins into mitochondrial and endoplasmic reticulum (ER), suggesting that these hsp70s are involved in conferring transport competence to precursors (Kang et al., 1990). Hsp70 has been shown to hold some proteins in an unfolded configuration, which is necessary for targeting and translocation to cellular compartments such as mitochondria, chloroplasts and endoplasmic reticulum (Sanders et al., 1992). Some pathways of protein sorting between the four mitochondrial subcompartments: (1) the outer membrane, (2) the intermembrane space, (3) the inner membrane, and (4) the matrix, have been conserved throughout evolution (Hartl and Neupert, 1990). Koll et al. (1992) used the translocation of cytochrome b2 across the mitochondrial membrane to elucidate the antifolding role of hsp60. Their results show that cytochrome b2 reaches the intermembrane space of mitochondria after being transported into the matrix and then exported across the inner membrane prior to arriving at the inner membrane space. Hsp60 prevents the folding of cytochrome b<sub>2</sub> while the protein is in the matrix. This allows the protein to be exported to the inner membrane space while still in the unfolded state. The export sequence in precytochrome b<sub>2</sub> functions by inhibiting the ATP-dependent release of the protein from hsp60. In addition to ATP, the release for export apparently requires the interaction of the signal sequence with a component of the export machinery in the inner membrane. If a critical length of the polypeptide chain has been translocated into the matrix, export of the polypeptide can begin, even though import into the matrix is not completed. Hsp60 is therefore involved in two activities: (1) catalyzing the folding of proteins destined for the matrix, and (2) facilitating the channeling of proteins between the machineries for import and export across the inner membrane. This is accomplished by maintaining the proteins in an unfolded state. Hydrophobic export sequences in cytochrome b2 may act as antifolding signals to switch hsp60 from one function to the other.

The heat-shock protein response has been studied extensively recently, and much is known about the roles of hsps in regulating the hsp response, protein folding and transmembrane protein transport. New insights into gene function and regulation in the face of environmental stressors, such as heat shock, are beginning to emerge with numerous laboratories now investigating the molecular biology of this response. An area of investigation that has, however, received little attention has been the effect of the hormonal stress response on hsp abundance in stressed cells or whole organisms.

#### Physiological stress in fish

The concept of stress is one that is not unfamiliar. Organisms can become stressed when physiological or psychological conditions and patterns are extended beyond their normal range. Fish are no exception; they are exposed to stressors both in their natural environment and in aquaculture facilities' operations. The intensity and the duration of exposure to the stressor may, to a large extent, determine if the animal is capable of coping with the stress; many negative impacts, especially on health, can appear when fish are subjected to long-term stress.

There are many definitions of stress in fish (see Barton and Iwama 1991). However, the definition proposed by Brett (1958), which states that stress is "a state produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that in either case, the chances of survival are significantly reduced" will be used as a conceptual framework here. While this definition excludes any adaptive aspects of stress, it does provide the researcher with some firm criteria by which stressed states can be defined. In this regard it stands out as a very functional definition, compared to others which are somewhat vague by trying to encompass all considerations.

The following is a brief overview of the generalized stress response in fish. The data are heavily dominated by salmonids, as many studies regarding stress physiology in fish have been conducted in temperate areas of the world where this family of fishes has prevailed as the most commonly consumed as well as studied species. This overview consists of (1) a summary of some of the common factors that can cause stress in fish, (2) some of the commonly studied physiological responses of fish to stress, (3) some of the indicators of stressed states in the field, and (4) the applications of such knowledge. Some important,

but little studied areas, such as the behavioral aspects associated with stress in fish are only mentioned and not discussed. Most physiological studies carried out with fish subject the animal to a certain degree of stress. In reading this overview the reader should become aware of the stress experienced by the fish used for the studies presented in this thesis.

#### Common fish stressors

The selection of the stressors discussed here is by no means meant to exclude other possible stressors unique to certain species or geographic areas. While the categories of environmental, physical, and biological stressors are rather arbitrary terms, they help to group the diverse possible stressors into a few themes. The susceptibility of fish to different stressors also involves genetic components. There are differences in the stress responses among species (see Barton and Iwama, 1991; Vijayan and Moon, 1994), and differences among stocks of the same species in tolerance to applied stressors. It is not the intention of this review to discuss in any detail the extensive list of known stressors to fish. The different types of potential stressors that may affect fish have simply been summarized. Environmental stressors are mainly associated with adverse chemical conditions of the water. Although pollutants are common environmental stressors, extreme conditions or changes in water quality parameters such as dissolved oxygen, ammonia, hardness, pH, gas content and partial pressures, and temperature can stress the fish. Table 1.1 lists water quality variables found in pristine waters, which are suitable for rearing of salmonids such as trout and salmon in a healthy and unstressed state (Iwama et al. 1996).

Metals such as copper, cadmium, zinc, and iron can cause stress and death in fish. Contaminants such as arsenic, chlorine, cyanide, various phenols, and polychlorinated biphenyls are potent stressors for all salmonids. Other potential environmental stressors include insecticides, herbicides, fungicides, and defoliants. Industrial, domestic, and agricultural activities certainly add such contaminants to the environment, which may affect fish at all life stages (Heath, 1995).

Physical stressors include those that are involved in handling, crowding, confinement, transport, or other forms of physical disturbance. Chasing fish to exhaustion has been a common protocol in some physiological studies. Angling also stresses fish in this way. Biological stressors can be manifest in dominance hierarchies which develop between individuals within confines such as experimental tanks or possibly in natural environments. Disease pathogens can also be considered as biological stressors.

#### The physiological stress response of fish

In response to a stressor such as handling or crowding, a fish will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it, and thereby cope with the stress. The stress response in fish has been broadly categorized into the primary, secondary and tertiary responses (Mazeaud et al. 1977; Wedemeyer et al. 1990) In addition to the whole animal changes that have been widely studied, recent reports indicate cellular adjustments in response to different stressors which are characterized by the expression of stress proteins (Welch, 1993).

#### Primary response.

This initial response represents the perception of an altered state and initiates a neuroendocrine response that forms part of the generalized stress response in fish (Gamperl et al. 1994). This response includes the rapid release of stress hormones, catecholamines and cortisol, into the circulation. Catecholamines (CATS) are released from the chromaffin tissue situated in the

head kidney of teleosts, and also from the endings of adrenergic nerves (see Randall and Perry, 1992). Cortisol is released from the interrenal tissue, located in the head kidney, in response to several pituitary hormones, but most potently to adrenocorticotropic hormone (ACTH) (see Balm et al. 1994). A recent study showed that ACTH may also stimulate epinephrine release, and that chronic cortisol treatment may affect catecholamine storage and release in trout (Reid et al. 1996). As both the chromaffin tissue and the interrenal tissue lie in close proximity in fish, these results suggest to the possibility that a paracrine control for stress hormone regulation exists in fish (Reid et al. 1996). The physiological actions of both hormones are dependent on appropriate receptors on target tissues.

#### Secondary response:

This response consists of the various biochemical and physiological effects associated with stress, and may be mediated to a large extent by the above stress hormones. The stress hormones activate a number of metabolic pathways such as the glycolytic and gluconeogenic pathways, which result in alterations in blood chemistry and hematology (see Maule et al. 1989; Barton and Iwama, 1991; Randall and Perry, 1992; Vijayan et al., 1994b).

#### Tertiary response:

This response represents whole animal and population level changes associated with stress (Wedemeyer et al., 1990). If the fish is unable to acclimate or adapt to the stress, whole animal changes may occur as a result of energy-repartitioning by diverting energy substrates to cope with the increased energy demand associated with stress and away from anabolic activity such as growth and reproduction. Thus, chronic exposure to stressor, again depending on the intensity and duration of the stressor, can lead to decreases in growth, disease resistance, reproductive success, smolting and swimming performance.

At a population level, decreased recruitment and productivity may alter community species abundance and diversity.

#### **Detection of Stressed States**

The detection of stressed states in fish depends upon the measurement of the responses described above. There are a number of methods available for evaluating the effects of stress on fish (Adams 1990; Wedemeyer et al. 1990), but many of these techniques are only appropriate for research or clinical laboratories, because they involve relatively sophisticated procedures and require expensive equipment to perform. Plasma adrenaline concentrations can be measured by HPLC (see Woodward 1982), and cortisol concentrations can be measured by radioimmunoassay (e.g. Kits- Clinical Assay, Massachusetts) or by ELISA (Barry et al. 1993). Table 1.2 gives estimates of adrenaline, cortisol and glucose concentrations in the plasma of resting and stressed salmonids. These values should serve as general guidelines as individual conditions of species, including prior rearing history and local environment and state of development will modify the plasma values for control and stressed states (Barton and Iwama, 1991).

As well as the measurement of these stress hormones, the measurement of plasma glucose concentration has been used as an indicator of stressed states in fish. It is probably the most commonly measured secondary change that occurs during the stress response in fish (Wedemeyer et al. 1990). In addition to the measurement of the stress hormones and glucose, the definition of the stressed state in fish used here implies that monitoring of any physiological or behavioral variable could be used as an indicator of stress. This is certainly true provided that good control, or resting, values data are available for comparison. Hematological variables such as hematocrit. hemoglobin concentration, red and white cell number, and clotting time can be used for such purposes. Plasma concentrations of ions and protein can also be useful indicators of physiological status. A semi-quantitative protocol for assessing the physical condition of fish has been developed and used successfully by fishery biologists for the determination and monitoring of fish health and quality (see Goede and Barton 1990).

Temporal changes in any of these variables are vital in such assessments. A routine monitoring program that incorporates several of these variables can be used to study fish populations, rather than relying on a single indicator reflecting only one level of biological organization (Adams, 1990). Clinical as well as field methods for the determination of other physiological variables that may be useful in such assessments have been described (see lwama et al. 1995).

#### **Objective of the thesis:**

The main objective of this thesis was to establish the relationship between the hsp and hormonal stress responses, as no studies to date have investigated how the two stress responses interact in salmonids or fish cells exposed to stressful environmental conditions. The primary objective was then to examine the heat shock protein response within the context of the physiological stress response in fish. The following basic questions were investigated: (1) which heat shock proteins are induced when whole aquatic organisms, or the cells of aquatic organisms, are exposed to various environmental stressors, and (2) what effects do the hormonal changes associated with the physiological stress response have on the heat shock-protein response in whole animal tissues or fish cells cultured *in vitro* ? The studies presented in this thesis were conducted to test two hypotheses, the first being that a generalized heat shock protein response would be observed in fish or fish cell lines exposed to various environmental stressors. The second hypothesis was that the hormonal stress response, observed in physically stressed fish, would affect the levels of heat shock proteins found in heat shocked fish. These two hypotheses were tested in the studies described in Chapters 3 to 7.

In Chapter 3, novel proteins induced in four fish cell lines exposed to heat shock, pH change, L-azetidine and bleach Kraft pulp mill effluent were identified by one and two dimensional SDS-PAGE of radiolabeled proteins. The experiments conducted for this chapter showed that novel proteins of 70 and 30 kDa were, with few exceptions, consistently synthesized in response to the various stressors tested.

The experiment described in chapter 4 further investigated the hsp response of the chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line exposed to a wide pH range. This cell line was chosen for further investigation because it had been found to exhibit a strong hsp response in previous heat shock trials. Results from this chapter showed that a 70 kDa protein was synthesized in CHSE cells exposed to low and high pH, with a strong response induced when cells were exposed to decrease in pH, from pH 7.5 to pH 7.0.

Experiments in Chapter 5 described the time course and tissue-specific hsp response of salmonids exposed to heat shock. Results from these experiments showed that hsp70 and hsp30 levels are consistently elevated in the fish tissues of fish exposed to heat shock. The heat shock protein response was detectable 1 h after heat shocked fish were returned to ambient temperature and were elevated for as long as three weeks after the heat shock treatment.

Fish gills, liver, anterior kidney, muscle and red blood cells from heat shocked whole fish all responded with elevated hsp70 levels. Skeletal muscles showed a delayed onset of hsp70 elevation compared to the other tissues. Although hsp30 levels did increase in response to heat shock, the response was not as pronounced as that of hsp70.

The second hypothesis tested was that interaction occurs between the hormonal-stress and the hsp responses of salmonids exposed to environmental stressors. Experiments in Chapter 6 described the effects of handling and artificial elevation of plasma cortisol (by intraperitoneal cortisol implants) on the heat shock protein levels of cutthroat trout (O. clarki) exposed to heat shock. The plasma cortisol, glucose and ion concentrations, as well as the heat shock protein levels, in tissues from handled or cortisol-injected fish were measured to determine if these treatments would affect the heat shock protein response. A separate experiment was performed to determine if physiological or pharmacological concentrations of epinephrine elicited a hsp response in isolated rainbow trout (O. mykiss) hepatocytes maintained in primary culture. Results from the experiments show that a handling stress prior to heat shock exposure causes hsp70 and hsp30 levels in the gill tissues of cutthroat trout to be suppressed relative to the levels observed in gill tissues of fish which were only heat shocked. Elevated plasma cortisol levels, caused by intraperitoneal cortisol implants, suppressed hsp30 but not hsp70 levels in the gill tissues of cutthroat trout exposed to heat shock. These results suggest that the hsp30 response is downregulated by corticosteroids while hsp70 is downregulated by other factors which are part of the handling stress response such as the adrenaline response. Results from the hepatocyte epinephrine exposure experiment showed that hsp70 levels increase when these cells are exposed to physiological or pharmacological levels of epinephrine in vitro.
Experiments in described in Appendix 1 examined the effect of crude oil on the hsp response of two benthic fish species and two shellfish species chronically exposed to a controlled marine environment contaminated with "Fortis" crude oil and a new crude oil anti-adhesive compound developed for crude oil spill remediation in arctic environments. Tissues from each of the two fish and the two shellfish species were examined for the presence of heat shock proteins by Western blot, using fish-specific, anti-hsp70 and anti-hsp30 polyclonal antibodies. No change in hsp concentration was measured in fish or shellfish tissues sampled from crude oil or anti-adhesive exposed animals.

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Table 1.1 Water quality standards necessary for rearing unstressed salmonids (from Iwama et al., 1996).

Variable	Range	Comments
Temperature	4-15°C	
Total Gas Pressure	< 103 % saturation	
Dissolved O <sub>2</sub>	6.0 mg/L	at the outflow
Ammonia	< 10.0 ug/L as NH3	
рН	6.5 - 8.5	
CO <sub>2</sub>	< 20.0 mg/L	
Suspended Solids	< 3.0 mg/L	for incubation of eggs
	< 25.0 mg/L	for general rearing
H <sub>2</sub> S (hydrogen sulfide)	< 2.0 ug/L	as undissociated form
NO <sub>2</sub> - (nitrite)0.015 @ [Cl-]	< 2.0 mg/L	toxicity affected by [CI-]

	Resting	Stressed	
Adrenaline (nmoles/L)	< 3	20 - 70	
Cortisol (ng/mL)	< 10	40 - 200	
Glucose (mmol/L)	<4	> 5	

Table 1.2 Common physiological variables measured to determine stress levelsin salmonids (Iwama et al., 1996).

Figure 1.1 The cellular stress response and its inducers. Elevated levels of stress proteins are known to result from exposure of cells to three general classes of stressors: 1. environmental and physiological stressors, 2. pathophysiological states including disease and 3. non-stressful conditions such as cell growth and development. Some of these stressors cause heat shock factors (HSF) to become activated, enter the nucleus and bind to heat shock elements (HSE). HSE is the promoter of heat shock gene expression. Adapted from Morimoto et al. (1992).



The cellular stress response and its inducers. (Adapted from Morimoto et al., 1992).

Figure 1.2 Proposed model of the heat shock factor cycle. When cells are not stressed, heat shock factor (HSF) is thought to maintained in monomeric, non-DNA binding form by its interaction with hsp70 (1). As heat shock, or other stressor are applied, HSF monomers trimerize (2). This trimer then binds to specific sequence elements in heat shock gene promoters (3), and becomes phosphorylated (4). Increased levels of hsp70, resulting from the transcriptional activation of the heat shock genes, leads to the formation of HSF-hsp70 complexes (5). HSF then dissociates from the DNA and is converted to non-DNA binding monomers (6) (hypothetical model from Morimoto, 1993).



Figure 1.3 Proposed model of import and sorting of the BCS1 protein. A positively charged segment of amino acids which is located immediately C-terminal to the transmembrane domain acts as an internal targeting signal. In order to function, this sequence is postulated to cooperate with the transmembrane domain to form a tight hairpin loop structure. This loop is translocated across the inner membrane via the MIM/mt-Hsp70 machinery in a membrane potential-dependent manner. The novel mechanism of import and sorting of the BCS1 protein is proposed to represent a more general mechanism used by a number of inner membrane proteins. IM, inner membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane import machinery; 70, mt/Hsp70; the transmembrane domain (amino acids 45068) is indicated by a black box; the internal targeting sequence (amino acids residues 69-83) is represented by a zigzag line with positive charges (from Fölsch et al., 1996).



# Chapter 2

#### **General Materials and Methods**

This chapter is included as guide to the materials and methods which were common to chapters 5 and 6 as well as the experiments described in appendix 1.

# Fish holding tanks, water quality and rearing conditions

The holding tanks for all fish were supplied with dechlorinated Vancouver city tap water (6.2-14.1 °C, pH 6.1, total hardness 4.2 mg/L;  $O_2$  10 mg/L). The fish were held in 800-, 200- or 50-L tanks at a stocking density of ranging from 16.5 to 19.5 18 g/L and were fed to satiation once daily. Food was withheld for 48 h prior to all sampling periods.

## Fish anesthesia

All fish used for these studies were anesthetized by gently dipnetting the them from their holding tank and transferring them to a bath containing 100 mg/L of tricaine methanesulfonate (TMS) buffered with an equal weight of sodium bicarbonate (NaHCO<sub>3</sub>). The anesthetic bath was buffered to avoid the stressful effects of low pH which occur when TMS is added to soft freshwater (Summerfelt and Smith, 1990)

## Blood sampling

Blood sampling was performed on anesthetized fish or fish which were sacrificed with a blow to the head. Length and weight measurements were taken on all fish and then 0.5 mL of blood was drawn into heparinized tuberculin syringes fitted with 20G needles. The needles were inserted at a 45° angle, at the ventral midline, just posterior to the anal fin (see Houston, 1990). The needles were inserted until they rested against the backbone, at which point they penetrate the caudal vessels which lie between adjacent hemal arches. All samples were collected within 2 min of removing the fish from the water. Each blood sample was then transferred to 1.5-mL conical Eppendorf tubes and centrifuged for 2 min at 11,600 g in a Micro-Centaur Centrifuge (MSE Scientific Instruments, Sussex, England). The plasma fraction was then aspirated from the red blood cell pellets, the pellets were frozen on dry ice and then stored at -20°C until they were processed for BCA protein determination and SDS-PAGE. Plasma fractions were similarly frozen and stored until plasma cortisol, glucose and ion determinations.

## Plasma cortisol determination

Plasma cortisol concentrations were determined using a solid-phase coat-A-Count® <sup>125</sup>I-radioimmunoassay (Diagnostic Products Corporation, Loa Angeles, CA). In this procedure, <sup>125</sup>I-labeled cortisol competes for a fixed time with cortisol in the sample, for antibody sites which are immobilized on the wall of a polypropylene tube. Decanting the supernatant is sufficient to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled cortisol. Counting the tube in a gamma counter then yields a number which can be converted, by way of a calibration curve, to a value for the cortisol concentration in the sample. This procedure was modified from Foster and Dunn (1974) and validated for use with fish plasma by Redding et al. (1984) who showed that crossreactivity with cortisone, the other major corticosteroid in teleostean fishes, is 7.0%.

All frozen plasma samples in heparinized microcapillary tubes were thawed and 10  $\mu$ l of plasma was transferred into each antibody-coated polypropylene tube directly, without sample preparation. 1.0 mL of <sup>125</sup>I cortisol

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was added to every tube which was then mixed and incubated at 37°C for 45 min. Each tube was then decanted thoroughly to stop the reaction and counted for 1 min in a 1282 Compugamma CS universal gamma counter (LKB Wallace, Turku, Findland).

# Cell lines

The four fish cell lines used in Chapters 3 and 4 experiments were: 1) rainbow trout gonadal cells (RTG-2; ATCC,CRL55), 2) rainbow trout hepatoma cells (RTH-149; ATCC,CRL1710), 3) chinook salmon embryonic cells (CHSE-214; ATCC,CRL1681) and 4) carp (*Cyprinus carpio*) epithelial cells (EPC; Fijan et al., 1983). The cell lines were obtained from the Pacific Biological Station, Department of Fisheries and Oceans, Nanaimo, B.C., Canada. They were maintained in Minimum Essential Medium (MEM) with Earle's salts, supplemented with 10% fetal calf serum, 15 mM HEPES (GIBCO) and 2.2 mg/mL NaHCO<sub>3</sub> in air at 20°C. The cells were cultured in 75 cm<sup>2</sup> tissue culture flasks (Nalgene). They were subcultured as they approached confluence and were used in the present experiments as they became confluent.

#### Radiolabeling

All cells were radiolabeled by removing the growth medium from the culture flasks and adding 3 mL of methionine-free medium containing 50 or 100 µCi of [<sup>35</sup>S]-methionine per mL (New England Nuclear) per mL of medium. Following each experimental treatment (Table 1) and radiolabeling, the [<sup>35</sup>S]-methionine medium was removed from each flask and the cells were gently washed twice for 5 min with complete medium. The washes were discarded and 3 mL of fresh complete medium was added to each flask. The cells were then scraped off of the bottom of the flasks using a cell scraper and the suspension

was transferred to a sterile culture tube. The cell suspension was mixed and aliquoted into 1.5-mL Eppendorf tubes. The samples were frozen and stored at -80°C until they were run on SDS-PAGE gels.

## Radiolabeled cell sample preparation

The labeled cells were thawed and boiled for 5 min with an equal volume of Laemmli sample buffer (60 mM Tris-HCl, 10% glycerol, 2% SDS, 1% ßmercaptoethanol and 0.001% bromophenol blue; pH 6.8). The cell suspension was cooled to room temperature, and, if viscous, was extruded three of four times through a 27G needle fitted to a 1-mL syringe. The suspension was then clarified by centrifugation for 1 min at 15,000 g.

#### Tissue sample preparation

All fish and shellfish tissues were prepared for the bicinchoninic acid (BCA) assay and SDS-PAGE as follows. The 1-g tissue samples were thawed on ice, from -20°C at which they were stored, and rinsed in 10 mL of 500-mM Tris-HCI buffer (pH 7.5). A 0.5-g sample of tissue was added to 1.0 mL of hypotonic solution (100mM Tris-HCI (pH 7.5); 0.1% SDS; 0.1mM PMSF) and 250 µL of a 5X protease inhibitor cocktail ( 5.0 mM PMSF; 7.5 mM EDTA; 5 µM pepstatin A; 5 µM leupeptin; 1.5 µM aprotinin in 10 mM Tris-HCI (pH7.5)). Each sample was homogenized on ice for 30 s using a teflon tipped homogenizer (TRI-R Instruments, Jamaica, NY) mounted in a 10-mm Type 6 hand held electric drill (Black & Decker, Brockville, Ont.) revolving at 100-125 rpm with speed regulated by an ADJUST-A-VOLT voltage regulator (Standard Electric Product Co., Dayton OH.). The homogenized tissues were transferred to 1.5 mL conical Eppendorf tubes and centrifuged at 17,000 g for 45 min at 4°C. The supernatant was separated into two fractions; 150 µl was used directly for the

BCA protein assay and a 650-µl fraction was mixed 1:1 with 2X Laemmli sample buffer (120 mM Tris-HCI, 20 % glycerol, 4 % SDS, 2 % ß-mercaptoethanol and 0.002 % bromophenol blue; pH 6.8), boiled for 3 minutes and stored at -20°C until they were run on SDS-PAGE gels.

# Bicinchoninic acid protein assay

The bicinchoninic acid protein assay combines the reduction of Cu+2 by protein to Cu<sup>+1</sup>, in an alkaline medium, with the cuprous (Cu<sup>+1</sup>) ion detecting property of BCA (Smith et al., 1985). The purple-colored product of this assay is formed by the interaction of two molecules of BCA with one cuprous ion. The structure of the protein, the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) and the number of peptide bonds are responsible for color formation in protein samples. The Cu<sup>+1</sup>-BCA complex is water soluble and exhibits a strong absorbance at 562 nm. This BCA assay was used in a 96-well microplate format, which allowed the use of only 10 µl of sample supernatant per quantitation. The working solution of BCA / copper sulfate (50:1) was prepared fresh daily by mixing 50 parts of bicinchoninic acid solution (Sigma, Mississauga, Ont.) with one part of 4% w/v copper sulfate (Cu<sub>2</sub>SO<sub>4</sub>·5H<sub>2</sub>O). Protein standards were prepared from standard bovine serum albumin (BSA) (Sigma, Mississauga, Ont.). Ten microliters of each standard were added to the first three columns on the plate and standards were all done in triplicate. Serial dilutions of the sample supernatant were quantified to ensure that one dilution level would lie within the 50- to 400-µg/mL range of the standards. The top of each well was sealed with 6-cm wide Tuck Tape© transparent packing tape (Canadian Technical Tape Ltd., Montreal, Qué.) and the plates were floated in a 60°C water bath for 15 min. The absorbance at each well was read at 562 nm with a model 3550 microplate reader (Bio-Rad, Laboratories, CA).

Absorbance values from the standards, measured at 570 nm, were used to generate a regression equation for standard protein concentration.

# SDS-PAGE (one dimensional gels)

The labeled cells were thawed and boiled for 5 min with an equal volume of Laemmli sample buffer (30 mM Tris-HCI (pH 6.8), 100 mM glycerol, 70 mM SDS, 0.7 M ß-mercaptoethanol and 20 µM bromophenol blue). The solution was cooled to room temperature, and was extruded three or four times through a 27G needle fitted to a syringe if the solution was viscous. The solution was then clarified by centrifugation for 1 min at 15,000 g. One dimensional SDS-PAGE was performed according to the discontinuous system of Laemmli (1970) using a Mini-Protean II Dual Slab Cell (Bio-Rad) with a 4% stacking gel and a 12.5% resolving gel. Bromophenol Blue (0.001%) was added to the stacking gel preparation to help visualize the well septa while loading the samples. A lane on each gel was loaded with 20-30 µg of prestained molecular weight markers (Gibco-BRL Inc., Burlington, Ont.), which were used to estimate the molecular mass of each protein band separated by electrophoresis. Note that the molecular weights of unknown proteins are only estimates as their position relative to the known molecular weight markers was estimated. Each well received 50 µg of protein, as determined by the Bichinconinic acid protein assay. Clarified sample supernatants were thawed from -20°C and were loaded directly to the stacking wells after the protein concentrations of all were adjusted to the same level by diluting the concentrated samples with Learnhi's sample buffer. This practice enabled loading the same volume into each well and helped prevent band distortions which can result from uneven ionic content causing electric field distortions within the gel. Electrophoresis was performed at 200 V until the dye front reached the bottom of the resolving gel.

# Two dimensional gels

Two dimensional gel electrophoresis using isoelectric focusing followed by SDS-PAGE was done according to the O'Farrell (1975) method, as described by Welch and Feramisco (1985). Clarified sample supernatants were incubated with an equal volume of isoelectric sample buffer (10 M urea, 1.6 % NP-40, 5.0 % ampholyte (pH 5-7), 100 mM dithiothreitol) at 37°C for 15 min, mixed, and centrifuged for 30 s at 15,000 g prior to loading onto the tube gels. The supernatant was then applied to the top of the tube gels with care, to avoid mixing the sample with the upper tank buffer. The samples were run sequentially at 400 V for 3 h, 600 V for 2 h, 800 V for 15 h and 1000 V for 5 h. After a run was completed, the tubes were removed from the apparatus and the gels were carefully extruded onto the stacking portion of the slab gels (5% stacking gel, 12.5% resolving gel). The tube gels were secured by sealing them into position onto the stacking gel with 0.5% agarose dissolved in Laemmli sample buffer. Electrophoresis was performed at 80 V until the dye front reached the stacking/resolving gel interface and then at 180 V until the dye front reached the bottom of the resolving gel.

# Coomassie staining

Each tissue sample supernatant was loaded onto at least two identical gels to allow for one gel to be stained with Coomassie Blue R-250 to visually verify that the protein loading was equal among all wells. Coomassie staining was performed by soaking the gels in the staining solution (0.1% coomassie blue R-250, 10% acetic acid, 40% methanol 50% H<sub>2</sub>O) on an orbital shaker for 30 min, destaining the gels in destain solution (10% acetic acid, 40% methanol 50% H<sub>2</sub>O) and then drying them between two sheets of hydrated cellophane paper.

# Autoradiography & Fluorography (full size gels)

Autoradiography of the one-dimensional SDS-PAGE mini-gels was done by drying the gels using a Bio-Rad gel dryer and applying the dried gels to AGFA CURIX X-Ray film for a 5- to 8-day exposure at -80°C.

Fluorography was done on large slab gels as follows: the gels were submerged for 20 - 30 min in three changes of fresh dimethyl sulfoxide (DMSO). The gels were then transferred to a 15% diphenyloxazole (PPO)/DMSO solution and soaked for 1 h on a rotary shaker. The PPO in the gel was precipitated by soaking the gels in distilled water, with three changes of water over a 1-h period. The gels were then dried and applied to film for 24 h at -80°C.

# Autoradiography (minigels)

The mini-gels (one-dimensional electrophoresis) were dried using a Bio-Rad gel dryer and applied to AGFA CURIX X-Ray film. The film was exposed at -80°C for 5 to 8 days and then developed.

# Transferring proteins from minigels to membranes

All proteins were transferred from the gels to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) for Western blotting. The semi-dry blotting method, first reported by Kyhse-Anderson (1984), was used to transfer proteins from all acrylamide gels to nitrocellulose. These transfers were performed using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA). Prior to transferring the proteins to nitrocellulose, the electrophorized gels were equilibrated for 15 min in Bjerrum and Schafer-Nielsen (1986) transfer buffer (48 mM Tris; 39 mM glycine, 20 % methanol (pH 9.2)). Equilibration facilitates the removal of electrophoresis buffer salts and detergents, and reduces the amount of heat generated during the

transfer. It also allows the gel to shrink to its final size prior to electrophoretic transfer. The nitrocellulose membranes were soaked in transfer buffer for 15-30 min prior to transfer to ensure proper binding of the acrylamide gel to the membrane, care was taken not to entrap air bubbles during the wetting process as these can block transfer of molecules. The gel and nitrocellulose were sandwiched between two 0.33-mm thick sheets of Whatman chromatography paper (Balston Ltd., England) presoaked in transfer buffer. Air bubbles were rolled out at each step of applying the bottom filter paper and gel/membrane sandwich and top filter paper onto the bottom platinum anode. The cathode was then added onto the stack and the current applied. All gels were transferred for 15 min with 15 volts, supplied by the model 200/2.0 power supply (Bio-Rad Laboratories, Richmond, CA). Transfer efficiency was verified for each gel by using pre-stained molecular weight markers (BRL) and noting for their presence on the nitrocellulose membrane following transfer, and also by staining the gel with Coomassie blue R-250 protein stain after the transfer was completed.

#### Primary antibodies

Rabbit polyclonal anti-hsp70 and anti-hsp30 antibodies used in the study were produced in Dr. E.P.M Candido's laboratory in the Department of Biochemistry, University of British Columbia. For production of hsp70, rainbow trout RTG-2 cells were cultured in roller bottles in Eagle's MEM with 10% fetal bovine serum. Before reaching confluence, cells were induced with 50 mM sodium arsenite for 15-24 h, allowed to recover in fresh medium for 2-3 h, harvested by trypsinization, washed in PBS and stored at -70°C.

For preparation of rainbow trout hsp70, frozen RTG-2 cell pellets were homogenized with a stainless steel Dounce homogenizer in a lysis buffer consisting of 10 mM Tris-acetate pH 7.5, 10 mM NaCl and 0.1 mM EDTA to

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which was added PMSF to 120 mM immediately before use. The lysate was centrifuged at 12,000 *g* for 15 min at 4 °C, and the hsp70 was purified from the resulting supernatant by ATP-agarose affinity chromatography, essentially as described by Welch and Feramisco (1985). New Zealand White rabbits were initially immunized with purified hsp70 emulsified in Complete Freund's Adjuvant, and boosted with the antigen in Freund's Incomplete Adjuvant. Antiserum was precipitated with 50% saturated ammonium sulfate, dialyzed against PBS and stored at -20C in 0.02 % sodium azide.

Rabbit polyclonal anti-hsp30 antibodies were made against recombinant chinook salmon hsp30 produced in *E. coli* from a cDNA clone provided by Dr. L. Weber, University of Reno, Reno Nevada.

# Western blotting

Nitrocellulose membranes with transferred proteins were probed for hsp70, hsp60 and hsp30. The membranes were first blocked at room temperature for 1 h or under refrigeration with blocking solution of 2% skim milk and 0.1% NaN<sub>3</sub> in TTBS, which is made with 0.05% Tween-20 in TBS (50mM tris-base, 200 mM NaCl, pH 7.5). The membranes were washed for 5 min in TTBS and then incubated for 1 h in primary antibody solution diluted 1:500 with blocking solution to which was added 0.57 mM phenolmethylsulphonoflouride (PMSF), 5 µM leupeptin and 0.3 µM Aprotinin and 1 % Nonidet. Primary antibodies specific to rainbow trout hsp70 and chinook salmon hsp30 were developed in the laboratory of Dr. E.P.M. Candido and were made available through a cooperative agreement with Stress*gen* Inc. (Victoria, BC). Hsp60 was probed for using anti-hsp60 antibodies raised against either *H. virescens* (moth) hsp60 or PCC 7942 (cyanobacteria). The membranes were then washed twice for 5 min with TTBS and incubated in conjugated secondary antibody (goat anti-

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rabbit or anti-mouse with conjugated phosphatase (Gibco-BRL Inc., Burlington, Ont.) diluted to recommended concentration with TTBS) for 2 h with gentle agitation. The membranes were then washed twice for 5 min with TTBS and once for 5 min with TBS to remove residual Tween-20.

Color development was performed by decanting the TBS and equilibrating the membranes with substrate buffer (100 mM tris-base, 100 mM NaCl, 50 mM MgCl<sub>2</sub>) for 10 min before development. The membranes were then developed in alkaline phosphatase developer [100  $\mu$ l of NBT reagent (Nitro blue tetrazolium: 50 mg/mL dissolved in 70 % DMF (dimethylformamide), 8 % methanol and 22 % ddH<sub>2</sub>O) and 100 $\mu$ l of BCIP reagent (5-bromo-4-chloro-3-indolyl phosphate; in 100% DMF) in 15 mL of the alkaline phosphatase substrate buffer]. The membranes were immersed in the color development solution for 1-5 min, depending on the color development rate. The development was stopped by decanting the dish and flooding the membrane with distilled water. The membranes were then dried on filter paper and stored between polyester sheets to minimize fading.

### Densitometry measurements

All Western blot band intensities were determined using the SigmaGel densitometry software program (Jandel Corporation., San Rafael, CA). The Gaussian model (specified percent of the height of the peak used to integrate the area under the curve created by the intensity of the scanned band), the threshold level (pixel intensity) and threshold percent (user defined percentage of all pixels used to integrate the area under the curve) were all set at the same levels when data sets were created to compare the tissue heat shock protein response to different experimental treatments. A linear relationship between the log of antigen concentration and the area under the peaks produced by

densitometry has been reported (Blake et al. 1984). The concentration vs. band intensity relationship observed in our trials agreed with this relationship.

## Chapter 3

Enhanced proteins in fish cell lines exposed to environmental stressors.

#### Introduction

The objective of this study was to determine if any proteins commonly increased in concentration when different fish cell lines were exposed to four environmental stressors. Several studies document the expression of stress proteins in aquatic organisms, focusing on the effects of heat shock on whole animals or in cell lines (Misra et al., 1989; Parsell et al., 1993; Sanders and Martin, 1993; Flanagan et al., 1995). While novel proteins have appeared with a range of stressors, there has been a lack of comprehensive studies where fish or fish cell lines are exposed to a variety of stressors in an attempt to describe a generalized response. Studies on the heat shock protein response, as it relates to the whole stress response in general and the stress hormone response in particular, are needed to better understand the relationships between the várious facets of these adaptive stress responses.

Four fish cell lines were exposed to four different stressors, and were analyzed for the expression of heat shock proteins by one and two dimensional SDS-PAGE and autoradiography or fluorography.

#### Materials and Methods

Materials and methods describing the cell lines, radiolabeling, radiolabeled cell sample preparation as well as one and two dimensional gel electrophoresis used for the experiments in this chapter are presented in the General Materials and Methods section (Chapter 2).

## Stressors

The stressors used for this series of experiments are summarized in Table 2.1. Heat shock, pH change, exposure to L-azetidine, and exposure to bleached Kraft mill effluent (BKME) were applied to all cell lines. The heat shock was applied by transferring the flasks from room temperature (20°C) to a 28°C water bath. Control cells were maintained at 20°C. pH change and L-azetidine was performed by aspirating the normal growth medium (pH 7.5) from each flask and replacing it with medium adjusted to pH 5.7, 6.7, 8.7 or 9.7, or medium containing 10 mM L-azetidine. Tissue culture medium for the BKME exposure trials were prepared by dissolving powdered, methionine-free MEM with Earl's salts, 10% FBS, 15 mM HEPES and 2.2 mg/mL NaHCO<sub>3</sub> in one quarter, one half and full strength BKME effluent, which corresponded to organochlorine concentrations shown in Table 3.1, respectively. Control cells for each of these experiments had a concurrent normal growth medium change to control for handling effects.

# Results

#### Heat Shock

One-dimensional SDS-PAGE gels showed a prominent band of approximate molecular mass 70 kDa in heat shocked RTG-2 and CHSE-214 cells. The controls of these cell lines had only a faint band at this locus (Figure 3.1). Only a faint band at 70 kDa was seen in the heat shocked RTH-149 cells. No difference was observed between the banding patterns of heat-shocked and control carp epithelial cells (EPC) (data not shown).

In two dimensional electrophoresis, heat-shocked RTG-2 cells revealed enhanced expression of two isoforms of proteins of approximately 70 kDa. In

addition, 200 kDa and 30 kDa proteins showed enhanced expression in the heat shocked samples, compared to controls (Figure 3.2: 1a & 1b). Heat-shocked RTH-149 cells showed increased production of three proteins with approximate molecular masses of 80, 70 and 30 kDa compared to controls (Figure 3.2: 2a & Heat-shocked CHSE-214 cells showed massive diffused spots at 2b). approximately 90 and 70 kDa indicative of large scale protein production. Individual proteins (i.e., number and isoforms) of these two protein groups could not be differentiated due to the large autoradiograph area exposed by these proteins. The heat shocked CHSE cell line also showed increased concentrations of three proteins with approximate molecular masses of 50, 28 and 25 kDa, which were not evident in the control cells. Two proteins of approximate molecular masses of 30 and 28 kDa, showed enhanced expression in heat shocked cells compared to controls (Figure 3.2: 3a & 3b). EPC, upon heat shock, synthesized 13 new proteins, four of these (28, 27, 20 and 15 kDa) were in the usual slightly acidic to near-neutral pH range of heat shock proteins. Ten other proteins induced by heat shock in the EPC cells were in the highly alkaline range and had approximate molecular weights of 60, 50, 47, 44, 42, 26, 25, 23 and 22 kDa (Figures 3.2: 4a & 4b).

# L-Azetidine

A protein with approximate molecular mass of 70 kDa was highly induced in L-azetidine-treated RTG-2 cells and was present at a low level in the control cells. The RTG-2 fluorograph suggests a generalized reduction of protein synthesis as a result of L- azetidine exposure (Figure 3.2: 1c). L-azetidinetreated RTH-149 also showed a generalized attenuation of protein synthesis (Figure 3.2: 2c). A protein with approximate molecular mass of 50 kDa, absent in the control, was expressed in CHSE-214 in response to this amino acid analogue (Figure 3.2: 3c). L-azetidine treatment of EPC induced the synthesis of two proteins with approximate molecular masses of 80 and 70 kDa as well as a new protein with an approximate molecular mass of 40 kDa in the highly alkaline range (Figure 3.2: 4c).

## pH change

No difference was observed between the protein profiles of RTG-2 control cells and cells exposed to pHs of 5.7 to 9.7. (data not shown). RTH-149 cells revealed increased production of a protein of approximate molecular weight of 68 kDa in cells exposed to pH 9.7 relative to controls cells. CHSE showed an enhanced production of a 30 kDa protein in all of the experimental pH regimes. In EPC, the following bands were more pronounced in the experimental treatments, compared to controls: >200, 100, 90, 65, 60, 44, 43, 40, 30, 25, and 23 kDa, with the most prominent band appearing at approximately 65 kDa in the cells exposed to environmental pHs of 8.7 and 9.7 (Figure3.3).

## Bleached Kraft pulp mill effluent

Despite technical problems causing streaking of the proteins during electrophoresis of the SDS-PAGE gels, RTG-2, RTH-149 and CHSE exposed to the BKME appeared to show induction of novel proteins of approximate molecular weights of 100 kDa, 70 kDa and a 50 kDa, while effluent-treated EPC cells did not show induction of any new proteins. Data are not shown due to quality of the autoradiographs.

# Discussion

The small proteins synthesized by heat shocked CHSE and EPC cells are similar in size to the low molecular weight (LMW) heat shock proteins implicated in thermotolerance (Landry et al., 1989; Subjeck et al., 1982). These LMW heat shock proteins are known to be more species specific and less conserved than the 70 kDa and 90 kDa families of hsps (Sanders, 1993).

Three salmonid cell lines consistently expressed proteins of 70 and 30 kDa in response to heat shock. The fading in intensities of bands other than the newly induced proteins on the one dimensional gels is consistent with the knowledge that during heat shock there is a reduction in the synthesis of most proteins other than heat shock proteins (see Lindquist, 1986). The induction of a 70 kDa and a 30 kDa heat shock protein has been reported for heat-shocked RTG-2 (Kothary and Candido, 1982). Misra et al. (1989) reported the increase of a prominent 70 kDa protein in heat-shocked RTH-149 and CHSE-214 in addition to proteins of 95, 84, 74 and 28 kDa. Although the number and molecular masses of the proteins induced in the present study varied from those of the above workers, the results are in general agreement with their findings.

Heat shocked EPC cell line showed increased concentrations of a number of highly alkaline proteins. Highly alkaline heat shock proteins are rare; one is a heat-induced 47-kDa protein in chicken fibroblasts with a pl of approximately 9.0 (Nagao et al., 1986). The occurrence of such a large number of proteins with highly alkaline pl in the cyprinid cell line calls for further study.

Amino acid analogues such as canavanine, ethionine and L-azetidine have been shown to induce heat shock proteins in *Tetrahymena* and HeLa cells (Wilhelm et al., 1982; Hightower, 1980; Thomas and Mathews, 1982). The generalized reduction of protein synthesis observed in the fish cell lines used in this study was perhaps due to the use of an excessive concentration of Lazetidine.

Although whole fish show a stress response (i.e., elevated plasma concentration of cortisol and glucose; see Barton and Iwama, 1991) when they

are exposed to blood pH changes of fractions of a pH unit, RTG-2 cells did not show an increased heat shock protein induction when exposed to pH changes of 1 or 2 units above or below normal growth medium pH. Cell lines cultured for many generations are known to lose surface receptors and therefore respond differently than normal cells to environmental stressors. These cellular changes lead us to studies examining tissues from whole animals in chapters four to six.

# Conclusion

The present study showed that various novel proteins were synthesized in three salmonid and one cyprinid cell line exposed to four stressors. Although only a few stressors were investigated, novel proteins of approximately 70 and 30 kDa appeared to be generally induced, while a 100 kDa protein may be a specific indicator of BKME exposure in salmonids.

## Acknowledgments

The collaboration of Dr. Robert Devlin, West Vancouver Laboratory, Department of Fisheries and Oceans, B.C., Canada, in providing the cell lines, equipment and space to do preliminary work on hsp response is gratefully acknowledged.

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Stressor	Treatment (duration)	Radiolabeling	(post-
stress)			
heat shock	20→28°C	1 h	· ·· _
pH change	pH 6.7, pH 8.7 (1 h)	4 h	
L-azetidine	10 mM (1 h)	4 h	
BKME	3.75, 7.5, 15.0 mg/mL	4 h	

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Table 3.1Treatments used to induce the synthesis of novel proteins in thefour fish cell lines.

Figure 3.1 Hsp70 synthesis in two fish cell lines; RTG-2 and CHSE-214 exposed to a temperature increase of 8°C. Control samples (C) were grown at 20°C while experimental samples (E) were exposed to 28°C for 1 h, and returned to 20°C for radiolabeling with 100  $\mu$ Ci per mL [<sup>35</sup>S]-methionine. Samples were run on 12.5% acrylamide SDS-PAGE gels. Arrowheads indicate bands of protein that were induced by the heat shock treatment.



Figure 3.2 Two-dimensional SDS-PAGE of fish cell lines showing synthesis of novel proteins in response to heat shock and L-azetidine. Fluorographs labeled 1,2,3 and 4 are; RTG-2, RTH-149, CHSE-214 and EPC, respectively. The subscripts a, b, and c denote control, heat shocked (20 to 28°C) and L-azetidine (10 mM) exposure respectively. Control cells were grown at 20°C. Heat shocked cells were exposed to 28°C for a 1 h period. The L-azetidine treatment was a 4-h exposure to 10mM L-azetidine. Cells were labeled for 1 h with 100  $\mu$ Ci [<sup>35</sup>S]-methionine. Samples were loaded with equal counts and run on 12.5% acrylamide SDS-PAGE gels. Arrowheads indicate protein spots which were enhanced relative to those observed in control cells.



Figure 3.3 pH change causes synthesis of novel 25 and 69 kDa proteins in three fish cell lines. RTH-149, CHSE-214 and EPC cells labeled with [35S]methionine (1 h with 100  $\mu$ Ci / mL) and run on 12.5% acrylamide gels. RTH-149 exposed to pH 9.7 synthesized enhanced levels of a 68 kDa protein. CHSE-214 show increased production of a low molecular weight protein of approximately 25 kDa. EPC cells exposed to pHs of 8.7 and 9.7 showed increased synthesis of a 68 kDa protein compared to cells maintained at pH 7.7. Arrowheads indicate bands which were enhanced relative to controls.



## Chapter 4

pH change induces increased levels of novel proteins of 80, 70 and 32 kDa in chinook salmon embryonic (CHSE) cells

## Introduction

Information on the effects of environmental pH on many forms of aquatic life is accumulating and shows a variety of cellular responses in organisms or cells exposed to pH change (Morris et al., 1989). In chicken embryo cells, low extracellular pH induces the synthesis of glucose regulated proteins while high extracellular pH induces heat shock proteins (Whelan and Hightower 1985). *Histoplasma capsulatum*, an intracellular pathogenic fungus, which multiplies inside macrophages and resists macrophage microbiocidal mechanisms, survives exposure to pH 4.0 and increases the synthesis of eight constitutive proteins some of which may be heat-shock proteins (Kamei et al. 1992). At least sixteen polypeptides are induced in *E. coli* after an external pH shift down from pH 6.9 to pH 4.3; four of these polypeptides correspond to the well known hsps GroEL, DnaK, HtpG and HtpM (Heyde and Portalier, 1990). Khandjian (1990) showed that primary mouse kidney cell cultures exposed to acidic medium (pH 5.5) synthesized a 70 kDa protein, possibly hsp70.

Changes in water pH are potent stressors of fish (see Barton and Iwama, 1991). The experiments presented here were performed to investigate the stress response of a chinook salmon cell line (CHSE-214; ATCC CRL1681) experimentally exposed to a wide pH range. The hypothesis tested was that salmon embryonic cells exposed to a shift in pH would synthesize novel proteins and that different proteins might be induced at the acidic and the basic ends of the tested pH range. Investigating which proteins are induced by these pH shifts

may help to elaborate the effect that external pH change has on cellular metabolism and on the stress response at the organismal level.

#### Materials and Methods

### Cell line

The chinook salmon embryonic (CHSE-214) cell line (ATCC CRL 1681) was used for this experiment. The media and conditions used for normal cell maintenance are described in the General Materials and Methods (Chapter 2).

The radiolabeling, SDS-PAGE minigel and autoradiography protocols are also described in Chapter 2.

#### Stressors

The cells were exposed to experimental medium adjusted from 4.5 to 10.0, in 0.5-pH increments. The cells were grown to confluence at pH 7.5 in control growth medium prior to all trials and were stressed with the experimental medium for a period of 1 h. Each experimental medium was buffered with 20 mM of the buffers (Sigma, St. Louis, MO) as outlined in Table 3.1. Control cells were maintained in medium adjusted to pH 7.5 and buffered with 15 mM HEPES. The medium of the control cells was changed concurrently with the other experimental medium to control for handling effects. The pH of all media were measured using a standard pH meter, (Beckman, Fullerton, CA) calibrated with reference buffer solutions (Sigma, St. Louis, MO). The pH of each medium remained constant during the cell exposure trials; variations in pH (0.01-0.05) were within the measurement error of the instrument.
## Results

Decreasing the pH of the growth medium from the control pH of 7.5 to pH 4.5, or increasing it to pH 10.0 for a period of 1 h resulted in the induction of a set of novel proteins with relative molecular weights of 80, 70 and 32 kDa (Table 4.2).

Cells exposed to pH 7.0 medium for 1 h expressed elevated levels of a novel 70 kDa protein 1 h and 3 h after the stress, compared with control cells maintained at pH 7.5. Cells labeled during the 1-h pH-7.0 exposure did not show elevated stress protein expression (Figure 4.1). Cells exposed to pH 4.5 showed the same pattern and time course of elevated 70 kDa protein expression (Figure 4.2) while the cells exposed to pH 5.5 showed enhanced synthesis of 70 and 32 kDa proteins 1 h and 3 h after exposure to the stressor medium (Figure 4.3). Cells labeled during the 1-h acidic pH exposures did not show elevated stress protein synthesis. Cells exposed to the pH 10.0 medium synthesized a 70 and 80 kDa protein 1 h after return to the control growth medium. Synthesis of the 80 kDa protein remained elevated 3 h after the cells were returned to the control medium, whereas expression of the 70 kDa protein returned to control levels in that period. Cells labeled during the 1-h alkaline pH exposure did not show elevated stress protein synthesis (Figure 4.4).

## Discussion

This study shows that CHSE cells exposed to acidic or alkaline growth medium synthesized elevated levels of novel proteins. Enhanced synthesis of 80, 70 and 32 kDa proteins were observed in cells exposed to a wide pH range. Increased synthesis of the most prominent protein, with an approximate molecular weight of 70 kDa, was observed 1 and 3 h after the cells were exposed to either acidic and alkaline pH, but not during the 1-h pH challenge.

This high level of a novel 70 kDa protein 1 h after the pH stress exposure is similar to the high level of hsp70 seen 1 h after *Drosophila* are exposed to heat shock (Feder et al. 1995). Craig and Gross (1991) and Lindquist (1993), have reported that, in *Drosophila*, RNA polymerase is constitutively bound to the hsp70 promoter, and that the hsp70 mRNA contains no introns and can therefore be transcribed, processed and exported to the nucleus very rapidly (as it requires no splicing, which is time consuming). Assuming that the novel 70-kDa proteins that were observed are hsp70, the mechanisms described by those authors may explain the rapid increase of the novel 70-kDa protein.

At present, one can only speculate on the mechanisms of stress protein induction by pH change in the medium. Erythrocytes of fish and some other vertebrates resemble somatic cells in that they possess nuclei, mitochondria, endoplasmic reticulum, and various other organelles (Schweiger 1962; Weber 1982). In situ regulation of rainbow trout erythrocyte intracellular pH during extracellular acidosis occurs upon adrenergic stimulation. The adrenergic stimulation causes a rise in cyclic AMP, which activates the Na<sup>+</sup>-H<sup>+</sup> exchanger leading to intracellular alkalinization (Boutilier and Ferguson, 1989). Lack of adrenergic stimulation in the cultured cells may have rendered the CHSE cells in the present experiment unable to adjust their intracellular pH when exposed to acidic growth media. A decrease in intracellular pH may have caused the synthesis of the observed novel proteins. Udelsman et al. (1993) postulates that hsp synthesis is controlled by glucocorticoid-catecholamine interactions and that a1-adrenergic receptors interact with glucocorticoids to maintain homeostasis. If CHSE cells behave similarly to cultured mammalian cells, in which the relationship between internal and external pH has been investigated (Gillies et al. 1982), it is likely that reducing the extracellular pH resulted in a decrease in intracellular pH of at least several-tenths of a pH unit.

Alkalinization (pH 10.0) of the extracellular medium induced the synthesis of new 80-, 70- and 32-kDa proteins. Hammond et al. (1982) showed that the intracellular pH of mammalian cells exposed to alkaline medium may have approached the extracellular pH of 8.7. The CHSE cells exposed to pH 10.0 in the present study may also have experienced significant intracellular pH increases.

As important pH-induced structural transitions occur, even within the biological pH range (Hochachka and Somero, 1984), and improper protein folding, leading to protein aggregation, is thought to initiate a heat shock-like response (Georgopoulos and Welch, 1993), the observed hsp70 increase seen in CHSE exposed to pH changes was expected. One may conclude that pH change has a dramatic effect on protein synthesis in CHSE cells grown in culture and that these cells show enhanced synthesis of new proteins when they are exposed to pHs above or below normal physiological pH. It is therefore imperative that investigators who work with CHSE cells buffer the growth medium to ensure that the pH does not drift, as this change in culture condition will elicit the synthesis of novel proteins that could confound experimental results.

Results from Chapters 2 and 3 showed that novel proteins are synthesized when fish cells are exposed to stressful growth medium conditions. These new proteins were detected by radiolabeling, followed by one or two dimensional SDS-PAGE and then autoradiography, which separated the proteins by molecular weight or isoelectric point. These methods were useful for showing the variety of novel proteins synthesized in cultured cells exposed to various changes *in vitro*.

The next line of investigation was to examine the response of whole aquatic animals exposed to environmental changes and to establish if proteins

induced in the tissues of whole animals might be the same or similar to those observed in the stressed fish cell lines. The technique that was used to pursue this line of inquiry was to use primary antibodies against the proteins of interest that had been observed to date. Polyclonal antibodies against 70- and 30-kDa salmonid proteins were developed for Western blotting protein samples collected from the tissues of whole animals exposed to environmental stressors.

#### Acknowledgments

The collaboration of Dr. R. Devlin, Department of Fisheries and Oceans, Canada, who for provided the cell lines, equipment and space to do preliminary work on the hsp response is gratefully acknowledged. The laboratory assistance of Mr. R. Forsyth and the collaboration of Dr. B. Finlay is gratefully acknowledged.

pН	Growth medium buffers		
4.5	20 mM 2-[-Morpholino]ethanesulfonic acid (MES)		
5.0	20 mM MES		
5.5	20 mM MES		
6.0	20 mM MES		
6.5	10 mM MES and 10 mM 2-[bis(2Hydroxyethyl)amino]ethanesulfonic acid (BES)		
7.0	10 mM BES and 10 mM (N-[2-Hydroxyethyl]piperazine- N'-[2 ethanesulfonic acid] (HEPES)		
7.5	15 mM HEPES		
8.0	10 mM HEPES and 10 mM tris[Hydroxymethyl]aminomethane (Trizma®)		
8.5	10 mM Trizma® and 10 mM 3-[(1,1-Dimethyl-2 -hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO)		
9.0	20 mM AMPSO		
9.5	10 mM AMPSO and 10 mM 3-[Cyclohexylamino]-1 -propane sulfonic acid (CAPS)		
10.0	20 mM CAPS		

TABLE 4.1. Experimental media adjusted to pH 4.5 to 10.0.

	Proteins (kDa) at elevated levels		
pН	During exposure	1 h after exposure	3 h after exposure
4.5	none	70	70
5.0	none	70	70, 32
5.5	none	70, 32	70, 32
6.0	none	70	70
6.5	none	70	70
7.0	none	70	70
7.5 (control)	none	none	none
8.0	none	70	70
8.5	none	70	70
9.0	none	70	80
9.5	none	70	80
10.0	none	70, 80	80

Table 4.2. Novel proteins induced in CHSE cells exposed to a broad pH range and labeled either during exposure, 1 h, or 3 h after return to control medium (pH 7.5).

Figure 4.1. Novel proteins synthesized in CHSE cells exposed to pH changes. CHSE cells exposed to pH 7.0 (a) or maintained in control medium (pH 7.5) (b). The cells were labeled with [<sup>35</sup>S]-methionine for 1 h either during the pH change or 1 h and 3 h after return to control pH. Cells exposed to pH 7.0 synthesized elevated levels of a 70-kDa protein 1 h and 3 h after return to the control medium. Control cells at pH 7.5 showed no elevated protein synthesis.



Figure 4.2. Novel proteins synthesized in CHSE cells exposed to pH 4.5 and pH 5.5. The first lane marked 0 h refers to cells which were radiolabeled during the pH challenge. CHSE cells exposed to pH 4.5 (a) synthesized elevated levels of a 70 kDa protein 1 h and 3 h after return to the control medium (pH 7.5). CHSE cells exposed to pH 5.5 (b and c) expressed elevated levels of 70 and 32 kDa proteins at 1 h and 3 h after return to the control medium (pH 7.5).



Figure 4.3. CHSE cells synthesize novel 70- and 80- kDa proteins when exposed to pH 10.0. CHSE cells exposed to pH 10.0 synthesized elevated levels of a 70 kDa protein 1 h after return to control medium (pH 7.5). A newly formed 80-kDa protein was observed 1 h and 3 h after the cells were returned to control medium.



0h 1h 3h

#### Chapter 5

# Time course and levels of heat shock proteins in the tissues of heat shocked cutthroat trout

### Introduction

The physiological responses that enable organisms to tolerate temperature changes encountered in their environment demonstrate that the higher the level of biological organization, the more thermally sensitive are the processes involved (Lagerspetz, 1987). Although heat shock proteins are ubiquitous components of all living systems (Nover and Scharf, 1991), their physiological roles as part of the organismal stress response are only now becoming clearer. At the cellular level, all organisms respond to a hyperthermic stress by synthesizing a highly conserved set of proteins known as heat shock proteins. Depending on the organisms or cell type, the heat-shock, or cellular stress response, can be expressed within minutes of exposure to adverse environmental conditions (see Lindquist, 1986). It is generally agreed that the heat-shock response is an immediate and transient response to heat as well as other types of cellular injury (Skidmore et al., 1995; Flanagan et al., 1995). Among the different hsps induced when cells are stressed, the 70 kDa hsp has the highest correlation to the magnitude and duration of a thermal stress (Mizzen and Welch, 1988). A sublethal heat shock has pleiotropic effects on cells: There is an inactivation of the "normal" pattern of genetic expression at different levels (transcription, splicing, and translation of mRNAs into proteins) as well as alterations in cellular morphology (Nguyen et al., 1989). Synthesis of hsps is closely correlated with the phenomenon of "tempering" the cells or thermotolerance; a conditioning heat shock confers the ability to survive a subsequent, more severe heat shock that otherwise would be lethal to the organism (Gedamu et al. 1983; Mosser et al., 1987). Differences in survival between naive and conditioned organisms are often in the range of 100- to 1000-fold. Conditioning heat treatments also provide tolerance to many other types of stress, such as exposure to ethanol and sodium arsenite. Conversely, exposure to moderate concentrations of ethanol and sodium arsenite induce tolerance to both higher concentrations of ethanol and sodium arsenite, as well as to high temperatures (Lindquist, 1986; Nover and Scharf, 1991). As these stressors also induce the synthesis of hsps, it has long been postulated that hsps are responsible, at least in part, for induced tolerance (see Lindquist, 1986).

The synthesis of heat shock proteins in aquatic organisms may be useful as an indication of aquatic environment degradation. A primary mechanism of toxicity involves protein denaturation which results in protein aggregation and misfolding (Hightower, 1991) and the synthesis of heat shock proteins. Heat shock protein synthesis occurs as intracellular protein denaturation causes hsp70 proteins to release monomeric heat shock factor (HSF) proteins, which then trimerize and bind to gene promoter, resulting in the transcription of the heat shock protein genes. Heat shock proteins then facilitate the "repair" of the denatured proteins. As the denatured proteins are repaired, release of the HSF declines, slowing the hsp induction process, thereby completing the autoregulatory feedback loop (Morimoto et al., 1994a; Welch, 1993).

The hsp response of salmonids exposed to heat shock was further investigated by measuring the duration and levels of hsp70 found in different cutthroat trout tissues after the fish were exposed to step change heat shock and returned to normal environmental conditions. Five different tissues were excised and analyzed to determine tissue-specific hsp 70 and hsp 30 concentrations during recovery from the heat shock treatments.

## Materials and Methods

*Fish:* Cutthroat trout were used for all heat shock experiments. Cutthroat trout of both sexes, weighing  $54.3 \pm 11.5$  g (Mean  $\pm 1$ SD) were raised from eggs at the University of British Columbia aquaculture facility and were held in 200-L oval fiberglass tanks. The holding tanks for the fish were supplied with dechlorinated Vancouver city tap water (6.4-14.1 °C, pH 6.1, total hardness 4.2 mg/L; O<sub>2</sub> 10 mg/L).

Core Temperature measurements: To assess the actual temperature of cutthroat trout during exposure to a rapid water temperature change, the fish were fitted with a core temperature probe as follows. The fish were anesthetized by gently dipnetting them from the tank and transferring them to a bath containing 100 mg/L of tricaine methanesulfonate (TMS), buffered with an equal weight of sodium bicarbonate (NaHCO3) to avoid the stressful effects of low pH (Summerfelt and Smith, 1990), and fitted with the remote probe of a smart2™ LCD indoor / outdoor digital thermometer (InterTAN, Barrie, Ont.; temperature resolution of 0.1°C). The probe was inserted through the esophagus, lodged in the stomach and held in place by suturing the signal wire to the lower jaw using size 0 non-absorbable silk surgical suture (Ethicon Inc.) The fish were allowed to recover from this procedure for 1 h in ambient temperature water and were then transferred to a separate tank with water heated to 17.5°C. Core temperatures of the fish were measured before, and every 2 min after the fish were transferred from ambient temperature (6.6°C) to the heated water, maintained at 17.5°C by two thermal standard A-758 immersion aguarium heaters (Hagen Inc., Toronto, Ont.). Core temperatures were again recorded at the same intervals when the fish were transferred back to ambient temperature water. The ambient and heated water were monitored for temperature and

oxygen content using a handy mkll portable temperature and dissolved oxygen meter (OxyGuard, Birkerod, Den.). Dissolved oxygen level was maintained above 80% of saturation throughout the experiment by aerating each tank with a fine bubble aeration diffuser.

Four gill arches were excised from all fish when the core temperatures reequilibrated with the ambient water temperature (24 min after the initiation of step change heat shock) to determine if the gill tissues had elevated hsp70 levels after 12 min of recovery at ambient temperature. Control fish were transferred from ambient water temperature to a different tank maintained at the same temperature to control for handling effects. A second group of control fish were treated with anesthesia only, to control for the effect of anesthesia in the fish fitted with a core temperature probe. These fish were anesthetized with buffered TMS and kept out of water for 3 min, the time required to perform the probe insertion and suturing, were then returned to ambient water temperature and sampled after 24 min of recovery. The gill arches from all fish were frozen on dry ice immediately after excision and were stored at -20°C until they were processed by SDS-PAGE and Western blot analysis for hsp level determination.

To determine which tissues show a heat shock protein response to a rapid temperature elevation, cutthroat trout where exposed to a rapid 10°C temperature elevation (13.2 to 23.2°C), maintained at this elevated temperature for 2 h and then returned to ambient 13.2°C water (Figure 5.1). Gill, liver, anterior kidney, and skeletal muscle samples were excised from these fish 1 h and 48 h after they were returned to ambient temperature.

In a separate experiment, cutthroat trout were exposed to a rapid 16.2°C temperature elevation (from 6.2 to 22.4°C) to measure heat shock protein levels in the circulating red blood cells. These fish were kept at 22.4°C for 2 h and

then returned to ambient 6.2°C water. Blood samples were taken 1 h and 5 days after the fish were returned to ambient temperature.

To determine the duration of the heat shock protein response, cutthroat trout were subjected to the following three experimental temperature elevation protocols: (1) a step change 10°C temperature elevation, accomplished by transferring the fish from ambient water temperature (7.4°C) to 17.4°C water, leaving the fish at this elevated temperature for 2 h and then returning the fish to ambient water temperature; (2) a step change 15°C temperature elevation (7.4°C to 22.4°C water), again leaving the fish at this elevated temperature for 2 h and returning them to ambient water temperature; or (3) a step change 10°C (7.4°C to 17.4°C) temperature elevation, maintained for 2 h, followed by a 1 h recovery period at ambient temperature (7.4°C), a 2 h 15°C step change temperature elevation (7.4°C to 22.4°C) and then returning the fish to ambient temperature (Figure 5.2). Four gill arches were excised from all treatment groups at 1 h, 96 h, and then at 1, 2, 3 and 4 weeks after the fish were returned to ambient temperature. Control fish were transferred from ambient temperature water to a separate tank with water at the same temperature. All gill arches were immediately frozen on dry ice and stored at -20°C until they were analyzed for hsp content by SDS-PAGE and Western blot.

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*Tissue excisions:* Gill arches, anterior kidney, liver and skeletal muscle were excised by removing the fish from the ambient temperature water tank, quickly pulling the temperature probe out through the esophagus (core temperature trial fish) and administering a blow to the head with a plastic baton to kill the fish. The fish were measured and weighed and then four gill arches, the anterior kidney, 1 g of liver and 1 g of skeletal muscle were excised with surgical scissors and forceps. The tissues were immediately placed inserted into 1.5 mL conical

Eppendorf tubes and frozen on dry ice. The entire excision procedure was normally performed in less than 2 min. The tissues were stored at -20°C until they were processed for BCA protein determination, SDS-PAGE and Western blotting. Refer to the General Materials and Methods (Chapter 2) for the standard protocols used for blood sampling, plasma cortisol determination, tissue sample preparation, the bicinchoninic acid protein assay, SDS-PAGE, coomassie staining, transferring proteins from gels to nitrocellulose membranes, Western blotting and densitometry measurements.

#### Statistical analysis

Data were statistically analyzed either by *t*-test or one-way analysis of variance (ANOVA), Student-Newman-Keuls or Dunn's Multiple Range Test where appropriate. In cases where the data set failed the normality test required to run the *t*-test, a Mann-Whitney rank sum test was used as a nonparametric alternative. If a data set failed to pass the normality test prior the performing a multiple comparisons test, a Kruskall-Wallis ANOVA on ranks was performed, rather than a standard one way ANOVA. Significant differences were established at the p=0.05 level for all tests.

# Results

The preliminary study documented the core body temperature during exposure to a rapid temperature elevation. Surprisingly few data are available on the rate of internal body temperature change in fish exposed to environmental temperature changes. Figure 5.3 shows how the internal body temperature of these fish equilibrated to the elevated water temperature in approximately 10 min. When the fish were returned to ambient 6.6°C water, their core temperature again equilibrated to ambient temperature in approximately 10 min.

Gill samples taken from these fish as their body temperature re-equilibrated to ambient 6.6°C, i.e., 12 min after their return to 6.6°C water, showed no increase in hsp70 levels, compared with control fish that had been transferred from 6.6°C to a separate tank at 6.6°C and sampled 24 min after the transfer. Anesthesia control fish, which were anesthetized and kept out of water for 3 min, also showed no difference in hsp70 levels when sampled 24 min after anesthesia, compared to control fish.

These results show that the hsp70 response had not occurred 24 min after the onset of a step change 10.9°C temperature elevation (from 6.6 to 17.5°C) and 12 min after the fish had been returned to ambient 6.6°C water (Figure 5.4).

Tissue samples from a separate experiment, where fish were exposed to a 2-h step change 10°C heat shock (from 12.4 to 22.4°C) show that all tissues, except for the skeletal muscle, had elevated hsp70 concentrations 1 h after the fish were returned to ambient temperature. All tissues, including the skeletal muscles, had elevated hsp70 levels 48 h after the fish were returned to ambient temperature (Figure 5.5). The tissue samples were also probed for hsp30 and showed that only gill tissue sampled 1 h after the fish were returned to ambient temperature had elevated hsp30 levels. Tissue levels of hsp30 were either nondetectable or unchanged 48 h after the fish were returned to ambient temperature (Figure 5.6).

In a separate trial, cutthroat trout were exposed to a 2-h step change 16.2°C heat shock (from 6.2 to 22.4°C). Blood samples showed hsp70 concentrations in the red blood cells were unchanged 1 h after return to ambient temperature and significantly higher 5 days after the heat shock (Figure 5.7).

The experiments described here therefore show that heat shock proteins are synthesized in a tissue selective-manner in salmonids exposed to elevated temperatures.

Results from the three different heat shock treatments on gill tissue hsp70 concentrations, during a one month recovery period, showed that a 15°C - 2 h step change heat shock treatment (7.4 to 22.4°C) caused hsp70 levels to be elevated above control levels for three weeks (Figure 5.8). Fish that received a 10°C heat shock had elevated hsp70 levels for one week following the treatment while fish exposed to a 10°C heat shock followed by a 15°C heat shock had elevated hsp70 levels for three weeks after return to ambient temperature.

## Discussion

The results showed that the *in situ* body temperature of 55-g cutthroat trout equilibrated to the environmental water temperature within 12 min after returning to ambient temperature were highly repeatable. The rate of *in situ* temperature change was rapid, as would be predicted for poikilotherms, which are environmental temperature conformers. The rapid equilibration demonstrates the low heat retention of these fish. Their normal calm behavior observed during the rapid temperature transition suggests that a rapid temperature change, within the upper and lower lethal temperature limits for this species, does not cause behavioral distress, and that the fish do not perceive this rapid temperature change as life threatening.

Results showing that hsp70 concentrations in the gill tissue samples were not elevated 24 min after the onset of a sudden 10.9°C temperature elevation suggest that the hsp70 concentration increase in these fish occurs at a slower rate than the "immediate" response seen by researchers investigating the effect of heat shock on mammalian cells (Cairo et al., 1983; Li et al., 1983; Ryan

et al., 1991). The fact that the fish in these trials had low body temperatures, compared to homeotherms, probably explains the slower response time and longer duration of the heat shock protein response observed in these fish. Decreasing temperature slows the rate at which chemical transformations occur, and small temperature changes have a large effect on reaction rates (Hochachka and Somero, 1984).

The present study reveals that accumulation of hsp70 is generally uniform across the tissues sampled; however, skeletal muscle showed a slower response time than the other tissues sampled. This observation is consistent with the findings of Flanagan et al. (1995), who found that the quadricep muscles of male Sprague-Dawley rats (250-300g) required greater thermal stress than that necessary to induce hsp70 synthesis in the rat liver, small intestine or kidney tissues. It is noteworthy that the other tissues sampled, and that had elevated hsp70 concentrations 1 h after the fish were returned to ambient temperature, were perfused with blood when sampled. Although red blood cell samples taken 1 h after the fish were returned to ambient temperature had variable hsp70 concentrations compared to the levels found in control fish red blood cells, it is possible that the elevated hsp70 concentrations measured in the liver, anterior kidneys and gill tissues were caused by elevated hsp70 levels in the red blood cells in those tissues.

The results of the time course experiment, performed to determine the duration of a heat shock response, in fish exposed to a 2 h 15°C step change heat shock, showed that hsp70 proteins can remain elevated for up to three weeks after the fish are returned to ambient 7.4°C water. Although a pattern of elevated hsp70 concentration has previously been shown in *Mytillus* gill tissue for up to 8 weeks (Sanders et al., 1992), that pattern of elevated hsp70 was observed in mussels maintained at a high (27°C) temperature for the duration of

the sampling period. The present findings demonstrate that hsp70 concentrations can remain elevated for a prolonged period after fish are returned to colder ambient temperature. As temperature has sharp effects on the reaction velocity constants of chemical reactions, changes in temperature frequently have substantial effects on the equilibrium constants of biochemical reactions. It was Svante Arrhenius who, in the 1880s, developed an equation to determine the effect of temperature on the fraction of a population of molecules that is reactive at any given temperature. His equation reveals that only a small fraction of the total population of molecules possess enough energy to react at a certain temperature, and that changes in temperature lead to marked increases or decreases in the size of this reactive population (Hochachka and Somero.)

1984). Variations in body temperature impact fish physiology in two primary ways: (1) Temperature determines the rate of chemical reactions, and even modest reductions in temperature (e.g. 10°C within the biological temperature range, corresponding to a change of only 3% in average kinetic energy) markedly depress reaction rates (typically 2- to 3-fold); (2) temperature dictates the point of equilibrium between the formation and disruption of the noncovalent forces which stabilize both macromolecular (e.g. biological membranes and subunit interactions between proteins) and molecular (e.g. proteins and nucleic acids) structures of physiological importance, and the binding of ligands to proteins (e.g. hormone-receptor and enzyme substrate interactions). Houlihan et al. (1995) states that the maximum value for whole fish fractional rates of protein turnover, which is equal to the rate of protein synthesis and protein degradation under maintenance conditions, is approximately 4-5% per day in rainbow trout less than 200 g. Average rates have been measured at 2-4% per day, depending on the experimental methods Such degradation rates are consistent with the three week elevation of used.

hsp70 observed in the gill tissues of heat shocked cutthroat trout in these trials. The duration of the observed hsp elevation is therefore in the correct time range and is consistent with the protein turnover rates reported by Houlihan et al. (1995).

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Figure 5.2 Three step change heat shock treatments use to determine the duration of the heat shock response. These are the three step change heat shock treatments used to establish the duration of the heat shock response in the gill tissues of cutthroat trout. The fish were exposed to the different water temperatures by gently transferring them from the control tank maintained at ambient temperature (7.4°C) to tanks heated to either 17.4°C or 22.4°C as shown in graphs A, B and C. All treatment groups were sampled at 1h, 48 h, 1, 2, 3, and 4 weeks after the fish were returned to ambient temperature.



Sampling time (days) after return to ambient temperature

Figure 5.3 Core temperatures of heat shocked cutthroat trout. Core temperature of cutthroat trout exposed to a 12-min step change 10.9°C heat shock and then returned to ambient (6.6°C) water. Temperature measurements were taken every 2 min while the fish were acclimating to the new temperature water. (p < 0.05, N=4, mean  $\pm$  1SE).



Time after transfer (min)

Figure 5.4 Gill tissue hsp70 levels 12 min after a 12-min 10.9°C step change heat shock. Densitometry measurements of Western blots of cutthroat trout gill samples probed with anti-trout hsp70 rabbit polyclonal antibodies. Control, TMS anesthetized fish and fish exposed to a 12 min step change 10.9°C heat shock, were all sampled 24 min after the start of the treatments. (p< 0.05, N=4, mean  $\pm$  SE).



Figure 5.5 Tissue hsp70 levels after a step change heat shock. Densitometry measurements of Western blots of cutthroat trout gill, liver, anterior kidney and skeletal muscle samples probed with anti-trout hsp70 rabbit polyclonal antibodies 1 h and 48 h after the fish were returned to ambient water temperature. Asterisks denotes treatments with significantly different hsp70 band intensities (p< 0.05, N=4, mean  $\pm$  SE).



Time after heat shock (h)

Figure 5.6 Tissue hsp30 levels after a step change heat shock. Densitometry measurements of Western blots of cutthroat trout gill, liver, anterior kidney and skeletal muscle samples probed with anti-trout hsp30 rabbit polyclonal antibodies 1 h and 48 h after the fish were returned to ambient water temperature. Asterisks denotes treatments with significantly different hsp30 band intensities (p < 0.05, N=4, mean  $\pm$  SE).



Time after heat shock (h)

Figure 5.7 Red blood cell hsp70 levels after a step change heat shock. Densitometry measurements of Western blots of cutthroat trout red blood cell samples probed with anti-trout hsp70 rabbit polyclonal antibodies 1 h and 5 days after the fish were returned to ambient water temperature. Asterisks denotes treatments with significantly different hsp70 band intensities (p < 0.05, N =4, mean ± SE).


Time (h) after heat shock

Figure 5.8 Gill hsp70 levels showing the duration of the heat shock response. Densitometry measurements of Western blots of cutthroat trout gill samples probed with anti-trout hsp70 rabbit polyclonal antibodies 1 h, 96 h, and 1, 2, 3 and 4 weeks after the fish were returned to ambient temperature water. Bars not sharing a letter are significantly different (p < 0.05, N=4, mean ± SE). *Note*: Asterisk on 10°C heat shock data in 2 week samples denotes that the data are shown for illustration only and are not included in the statistical analysis due to the large variation within this group.



Hsp band intensity (arbitrary units)

#### Chapter 6

The relationship between heat shock protein levels and stress hormones.

# Introduction

Physical stressors of fish such as handling (Barton et al., 1980), crowding and confinement (Pickering and Pottinger, 1989) cause increases in plasma cortisol concentration, which have been shown to result in both lymphocytopenia (Pickering, 1984) and susceptibility of salmonids to various diseases and infections (Pickering and Duston, 1983). The importance of environmental stressors and the association of hsps with steroid receptors, and hsp 90 overexpression in steroid responsive malignancies has generated considerable interest (Parsons, 1993; Jameel et al., 1993) and prompted further investigation into the association between environmental stress and the heat shock protein response in this thesis. An interesting observation on the activation of heat shock genes in whole animals was made by Blake et al. (1991). These investigators demonstrated that the stress imposed by physical restraint of the rat was sufficient to induce the expression of select heat shock genes in the adrenals and vascular tissues. More recent evidence (Holbrook and Udelsman 1994) now indicate that restraint stress induces HSF1 (the same transcription factor that is induced by heat shock) and that the stress response was endocrine-regulated by adrenocorticotropic hormone (ACTH). Hypophysectomized rats did not exhibit this restraint-related heat shock gene induction, whereas the acute treatment of hypophysectomized rats with ACTH showed induction of hsp70 expression in the adrenal glands (Blake et al. 1991). These observations point to a link between the neuroendocrine pathways and

the models proposed for HSF activation by the presence of unfolded or misfolded proteins.

If the heat shock protein response in aquatic organisms is to be used as a reliable indicator of physiologically stressed states, it is necessary to establish what, if any, effect the handling and sampling procedures have on heat shock protein levels in the tissues of organisms which have been exposed to stressful environments. To investigate the relationship between stress hormones and hsp concentration in the tissues of rainbow trout and cutthroat trout, both responses were measured in fish stressed with handling and heat shock. The relationship between adrenaline and hsps was investigated with experiments using trout hepatocytes in primary culture. Cells were exposed to heat and physiological or pharmacological levels of adrenaline *in vitro*. The heat shock protein levels in these cells were measured and compared to control cells in normal growth medium.

#### Materials and Methods

*Fish:* Rainbow trout and cutthroat trout were used for these experiments. Rainbow trout of both sexes, weighing  $51.1 \pm 11.1$  (mean  $\pm$  SD) were obtained from the West Creek trout farm, Aldergrove, British Columbia, and acclimated in 200-L oval fiberglass tanks for 2 weeks before the handling, cortisol injection and heat shock experiments. Cutthroat trout of both sexes, weighing  $54.5 \pm$ 11.11 (Mean  $\pm$  SD) were also used for separate handling and heat shock experiments. One large rainbow trout weighing approximately 200 g was used to isolate hepatocytes for primary culture. The larger rainbow trout was maintained in a 800-L oval fiberglass tank prior to the hepatocyte isolation and culture procedure.

# Handling and heat shock

To determine the effect of handling, heat shock and the combination of handling and heat shock on plasma cortisol and heat shock protein levels in rainbow trout gill tissues, fish were exposed to the three following experimental treatments: (1) a 45-s aerial exposure; (2) a 10°C heat shock (from 12.4 to 22.4°C) with the fish maintained at this elevated temperature for 2 h; or (3) a 45-s aerial exposure followed by the same heat shock regime. All fish were sampled at 1 and 48 h after they were returned to ambient 12.4°C. Control fish for these treatments were maintained at ambient 12.4°C and sampled concurrently with the treated fish. Plasma cortisol, glucose, sodium, potassium and chloride concentrations were measured to determine the physiological effects of each treatment.

# Cortisol injections

To determine the effect of elevated cortisol levels and heat shock on heat shock protein levels in cutthroat trout gill tissues, fish were exposed to the following experimental treatments: (1) cortisol implants; (2) a 10°C heat shock (from 12.4 to 22.4°C) with the fish maintained at this elevated temperature for 2 h, or (3) cortisol implants followed by the same heat shock regime. All fish were sampled at 1 and 48 h after they were returned to ambient 12.4°C. Control fish for these treatments were maintained at ambient 12.4°C and sampled concurrently with the treated fish.

# Epinephrine exposure

To determine the effect of epinephrine on cultured liver cells, hepatocytes from a 200-g rainbow trout were cultured and exposed to physiological and pharmacological concentrations of epinephrine with and without a prior 2-h step change 15°C heat shock (from 15 to 30°C). The cells were allowed to recover from the heat shock and / or epinephrine exposure for 1 h and were then harvested, washed, frozen on dry ice and stored at -70°C until SDS-PAGE and Western blotting. A second experiment was performed to test the effect of blocking  $\beta$ -receptors during exposure to epinephrine on hsp70 levels in trout hepatocytes. Cultured hepatocytes were exposed to a pharmacological concentration (10<sup>-5</sup> M) of epinephrine either with and without the  $\beta$ -blocker propranolol. The cells were incubated in these media for 3 h and then harvested for SDS-PAGE and Western blotting probed with the trout specific anti-hsp70 rabbit primary antibody.

# Primary hepatocyte isolation and culture:

Primary hepatocyte isolation and culture was performed essentially as described by Mommsen et al. (1994). The fish were first anesthetized with a lethal dose of TMS (0.5 g TMS and 1 g NaHCO<sub>3</sub>/L). All procedures were performed under aseptic conditions. The fish skin was swabbed with alcohol, all surgical instruments, nylon filters and solutions were sterilized. The anesthetized fish was then cut from the vent to the peritoneum along the ventral midline with care, to avoid nicking the liver. The posterior intestinal vein was teased free from the surrounding fat about 8 cm from the liver and two loops of 3-0 surgical thread were tied around the gut and vessel. Scissors were used to nick the vessel and a PE-90 cannula was slipped into the vessel with slight rolling until a slight resistance was felt, indicating that the tip of the cannula was about 5 mm from the liver. The cannula was then fixed in position by tightening the surgical threads and securing them with a double knot. A multichannel peristaltic pump was set to minimum flow rate to infuse modified Hank's medium [136.9 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO4, 0.44 mM KH2PO4, 0.33 mM

Na<sub>2</sub>HPO<sub>4</sub>, 5.0 mM NaHCO<sub>3</sub>, 10 mM HEPES, (pH 7.63 at room temperature)] during cannulation. The flow rate was increased gradually to about 2 mL/min (about 0.5 to 1.0 mL per g liver per min) after successful cannulation while the liver blanched. The heart was exposed and removed during the blanching period to allow free drainage of the perfusate and to prevent pressure build-up in the liver. The liver was massaged lightly for 5 to 8 min to assist in clearing all of the blood from the liver. When all of the blood had cleared, the perfusate was switched to collagenase, [17.5 mg collagenase (Sigma type IV), a total of 75,000 units was dissolved in modified Hank's medium, made fresh just before use] taking care not to introduce any air into the perfusion line. The liver was again massaged lightly for about 5 min after the switch to collagenase to increase cell yield. The liver was perfused in situ with collagenase at room temperature for 20 to 25 min, at which time the liver had some fluid flowing freely from areas on the surface. The liver was then carefully removed from the cavity, taking care not to puncture the gall bladder or to drain bile into the liver. The vena cava and peritoneal lining were cut and the liver was lifted by slipping two fingers underneath both sides of the organ. The gall bladder was cut off while the liver was supported between the fingers and the liver was transferred to a small glass petri dish on ice containing 2 mL of modified Hank's medium. The liver was then chopped finely with a razor blade and the tissue suspension was poured into coarse 253-µm plankton netting fused under heat into the bottom of a 200mL plastic beaker. The suspension of cells flowed from the coarse filter onto a finer 52- or 73-µm plankton net which was heat sealed onto the bottom of a second plastic 200-mL beaker and finally into a beaker sitting on ice. Cells plus debris were gently massaged through the coarse filter but allowed to drain freely through the second filter. The cells were flushed through the screens using about 50 mL of ice-cold, Ca2+-free modified Hank's solution. The cells were

then centrifuged at slow speed (60 g) for 3 to 4 min. The supernatant was discarded, the resulting pellet was gently swirled to break up the cell clusters and then taken up in washing and incubation medium [2% defatted bovine serum albumin (BSA) plus 1.5mM CaCl<sub>2</sub> made up in modified Hank's medium], total volume about 5 times the weight of the liver. The resulting suspension was mixed gently by swirling and re-centrifuged. This washing procedure was repeated four or five times. The final cell pellet was taken up in culture medium.

The cells were cultured in L-15, diluted to a concentration of 1.0 X 10<sup>6</sup> cells/mL and plated at a density of about 0.3 X 10<sup>6</sup> cells/cm<sup>2</sup> in Primaria flat bottom six-well plates (Becton Dickson Labware, Franklin Lkes, New Jersey). The cells settled to the bottom of the wells and started adhering immediately. The cells were cultured in ambient air at 15°C and the culture medium was changed every 2 days. The cells formed a confluent monolayer within 24 to 48 h and were used for the trials three days after they were isolated.

# Epinephrine and heat shock exposures

The cultured hepatocytes were exposed to physiological (10<sup>-7</sup> M) and pharmacological (10<sup>-5</sup> M) (Gamperl et al., 1994) concentrations of epinephrine (Sigma, Mississauga, Ont.). The epinephrine concentrations were established by adding the appropriate volume of a stock (10<sup>-3</sup> M) solution of epinephrine dissolved in water. The epinephrine was added to the cell culture and the cells were then either incubated for 2 h at ambient 15°C or transferred to a 30°C incubator for 2 h. The ambient temperature cells were harvested 3 h after the addition of epinephrine to the growth medium and the heat shocked (30°C) cells were allowed to recover at ambient temperature for 1 h prior to harvesting, resulting in equal epinephrine exposure time for the two treatment groups.

# Hepatocyte harvesting from 6 well plates

The L-15 medium from each well of the six-well culture plates was removed from each well. The cells were then washed once with about 1 mL of 0.05 M Tris buffer (pH 7.5) being careful not to disturb the cells (i.e. pour the Tris buffer down the side wall of the well rather than directly onto the cells to avoid dislodging the cells from the bottom of the well). The plates were swirled gently to wash the cells and the Tris was then aspirated off and discarded. 750  $\mu$ l of fresh buffer was added to each well and the cells were scraped off the bottom of the wells with a rubber policeman. The cell suspensions were harvested with a Pasteur pipette and transferred to a set of labeled 1.5-mL tubes. The tubes were spun at low speed (1,100 g) for 2 min, the supernatant was aspirated and discarded and the pellets were frozen on dry ice and stored at -70°C until SDS-PAGE and Western blotting.

# $\beta$ -blocking of epinephrine receptors

The  $\beta$ -receptors of cultured trout hepatocytes were  $\beta$ -blocked by adding propranolol (10<sup>-3</sup> M) to the culture medium at the same time as a pharmacological dose of epinephrine (10<sup>-5</sup> M) was added. Control cells had epinephrine added to the culture medium only. The exposure time for both groups of cells was 3 h. The cells were harvested at the end of the 3 h exposure period, frozen on dry ice and stored at -70°C until SDS-PAGE and Western blotting.

#### Hepatocyte sonication

1.5-mL conical Eppendorf tubes containing frozen hepatocyte pellets received 150 µl of Tris buffer (0.05 M; pH 7.5). The cells were mixed and sonicated on ice for 30 s with a Vibracell sonicator (Sonics and Materials Inc.,

CT., USA). 10  $\mu$ I of the sonicated cell suspension was added to 90  $\mu$ I of Tris buffer (0.05 M, pH 7.5) in a 1.5 mL conical Eppendorf tube and used to determine protein concentration by BCA assay. 75  $\mu$ I of sonicated cell suspension was added to a second tube containing 75  $\mu$ I of 2X Laemmli's sample buffer, boiled for 3 min and then frozen at -20°C until SDS-PAGE and Western blotting.

# Cortisol injections

Slow-releasing intraperitoneal cortisol implants were injected into the rainbow trout using the method described by Specker et al. (1994). The cortisol (hydrocortisone; Sigma, St. Louis MO) was dissolved in a 1:1 mixture (w/w) of coconut oil (Sigma, St. Louis, MO) and canola oil (Proctor and Gamble, Inc., Toronto, Ont.) heated to liquefaction (42°C), at a concentration of 15 mg cortisol / mL of oil. The injections were done while the mixture was still fluid (25°C) and the implant solidified in the colder trout. The injections were made using a 1-mL plastic syringe attached to a 21 G, 2.5-cm needle. The insertion point into the peritoneal cavity was on the ventral body surface, midway between the pelvic and pectoral fins.

To achieve plasma cortisol concentrations similar to those seen in stressed fish, the fish were implanted with a dose of 75  $\mu$ g cortisol / g body weight. The fish were lightly anesthetized prior to injection and weighed to determine the injection volume (5  $\mu$ l per g of fish).

### Blood sampling

Blood sampling was carried out by severing the caudal peduncle with a scalpel and drawing approximately 100 µl of blood into two separate heparinized microhematocrit capillary tubes. The hematocrit tubes were then centrifuged in

an IEC Model MB microhematocrit centrifuge (International Equipment Co., Needham Hts., MA). The red blood cell fraction of the blood was separated from the plasma fraction by cutting the tubes with a diamond scribe. The plasma fraction was sealed in the tube with CRITOSEAL and stored at -20°C until used for cortisol, glucose and ion determinations.

### Plasma glucose determination

Plasma glucose concentrations were determined using the a modified Trinder (1969) enzymatic determination method available in kit form (Sigma, Mississauga, Ont.). In this assay, glucose is oxidized to gluconic acid and hydrogen peroxide, in the reaction catalyzed by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneamine dye, with an absorbance maximum at 505 nm. The intensity of the color produced is directly proportional to the glucose concentration in the sample. The glucose (Trinder) reagent (0.5 mM 4-aminoantipyrine; 20 mM p-hydroxybenzene; 15,000 U/L glucose oxidase (Aspergillus niger); 10,000 U/L peroxidase (horseradish) at pH 7.0) was used in a 96-well microtiter plate format. A standard curve was generated for each assay by adding 2.5 µl of standards of known glucose concentration to 250 µl of Trinder reagent at room temperature and the reaction allowed to proceed for 18 min. The absorbance at 505 nm was read with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). Glucose concentrations in unknown samples were calculated from the regression equation derived from absorbance values vs. known standard concentrations.

Plasma ion determinations

Plasma chloride concentrations were measured using a Haake Buchler digital chloridometer (Haake Buchler Instruments, Inc., Saddlebrook, N.J.) which is a coulometric titrator designed to determine the chloride ion concentration of solutions such as serum, urine and other aqueous solutions. The principle of operation is the quantifiable reaction which results when silver and chloride ions combine to form insoluble precipitates of silver chloride (AgCI). This reaction is carried out at a constant rate by passing a fixed direct current between a pair of silver electrodes immersed in an acid solution (0.64% nitric acid and 10% glacial acetic acid in distilled water). The anode, which is consumed in this reaction is a continuous spool of silver wire. As the equivalence point of the reaction is reached, the current flowing between a pair of separate indicator electrodes increases and is detected by the sense circuitry. At a preset indicator current, the instrument automatically stops the incremental counter. Since the generator current is constant, the total titration time is directly proportional to the number of chloride ions present in the sample vial. The instrument displays their relative time in units of milliequivalents chloride per liter. The reproducibility and accuracy of the instrument are both  $\pm$  0.5% and the resolution is 1 mEg/L. Chloride content of each plasma sample was measured by adding 10 µl of plasma to 4 mL of acid solution. The titration time for each sample was about 20 s. Each sample was analyzed in duplicate.

Plasma sodium and potassium measurements were performed using an ion chromatograph. The samples were processed by adding 5  $\mu$ l of plasma and 5  $\mu$ l of acetonitrile (a protein precipitator) to 180  $\mu$ l of distilled and deionized water NANO*pure* Ultrapure water system (Barnstead, Duburque, Io.) and then centrifuging for 2-3 min at 11,600 *g* in a Micro-Centaur centrifuge (MSE Scientific Instruments, Sussex, England). 100  $\mu$ l of the supernatant was then pipetted directly into an autosampler vial fitted with a 200- $\mu$ l polypropylene

insert. The samples were injected into the detector with a SIL-9A auto injector and pumped through the system using an LP-6A pump. The conductivity of each sample was detected by a CDD-6A conductivity detector which was temperature stabilized using a CTO-6AS column oven. The output signal from each sample, as it passed through the detector, was analyzed by a CR-501 integrator (all components by Shimadzu Scientific Instruments, Inc., Columbia, Md).

### Gill excision

Gill arches were excised after removing the fish from the ambient temperature water tank, and quickly pulling the temperature probe out through the esophagus (in the case of fish which had been fitted with a temperature probe) administering a blow to the head with a plastic baton, and measuring and weighing each fish. Four gill arches, the whole anterior kidney, 1.0 g of liver and 1.0 g of skeletal muscle were excised with surgical forceps and scissors, immediately placed in 1.5 mL conical Eppendorf tubes and frozen on dry ice. The entire excision procedure was normally performed in less than 2 min. The tissues were stored at -20°C until they were processed for BCA protein determination and SDS-PAGE.

#### Gill sample preparation

All fish gills were prepared for the bicinchoninic acid (BCA) assay, to determine protein concentration, and SDS-PAGE as follows. The gills were thawed on ice, from -20°C at which they were stored, and rinsed in 10 mL of 500 mM Tris-HCI buffer (pH 7.5). A 0.5-g sample of tissue was added to 1.0 mL of hypotonic solution [100 mM Tris-HCI (pH 7.5); 0.1% SDS; 0.1 mM PMSF) and 250 µl of a 5X protease inhibitor cocktail ( 5.0 mM PMSF; 7.5 mM EDTA; 5 µM pepstatin A; 5 µM leupeptin; 1.5 µM aprotinin in 10 mM Tris-

HCI (pH7.5)] and homogenized on ice for 30 s using a Teflon tipped homogenizer (TRI-R Instruments, Jamaica, NY.) mounted in a 10-mm Type 6 handle held electric drill (Black & Decker, Brockville, Ont.) revolving at 100-125 rpm with speed regulated by an ADJUST-A-VOLT voltage regulator (Standard Electric Product Co., Dayton OH.). The homogenized tissues were transferred to 1.5-mL conical Eppendorf tubes and centrifuged at 17,000 *g* for 45 min at 4°C. The supernatant was separated into two fractions; 150 µl was used directly for the BCA protein assay and a 650-µl fraction was mixed 1:1 with 2X Laemmli sample buffer (120mM Tris-HCl, 20% glycerol, 4% SDS, 2% ß-mercaptoethanol and 0.002% bromophenol blue; pH 6.8), boiled for 3 min and stored at -20°C until they were run on SDS-PAGE gels. Standard protocols for plasma cortisol determination, the bicinchoninic acid protein assay, SDS-PAGE, coomassie staining, transferring proteins from gels to nitrocellulose membranes, Western blotting, and densitometry measurements can be found in the General Materials and Methods section (Chapter 2).

#### Statistical analysis

Data were either statistically analyzed by *t*-test or one-way analysis of variance (ANOVA) followed or Student-Newman-Keuls or Dunn's Multiple Range Test where appropriate. In cases where the data set failed the normality test required to run the *t*-test, a Mann-Whitney rank sum test was used as a nonparametric alternative. If a data set failed to pass the normality test prior the performing a multiple comparisons test, a Kruskall-Wallis ANOVA on ranks was performed, rather than a standard one way ANOVA. Significant differences were established at the p=0.05 level for all tests.

Results

The handling protocol was effective in elevating plasma cortisol and glucose levels. Plasma cortisol concentrations peaked at 116  $\pm$  11.2 ng/mL (mean  $\pm$  SE) 1 h after the fish were returned to the holding tanks. The glucose values peaked at 224  $\pm$  6.4 mg/dL (mean  $\pm$  SE) 3 h after the stressful handling event (Figure 6.1). Plasma sodium, potassium and chloride values were unchanged after the 45 s handling stress (Figure 6.2) Gill, liver and kidney concentrations of hsp70 measured in tissue samples taken 1 h after the 45-s handling stress showed no differences between stressed fish and control fish (Figure 6.3). A time course trial performed to measure gill hsp70 concentrations during a 48-h recovery period after a 45-s handling stress also showed no differences between the handled fish and the undisturbed control fish (Figure 6.4). A 45-s handling stress therefore did not induce the expression of hsp70 in any of the tissues that were surveyed during the 48-h period following exposure to that stressor.

Plasma cortisol and glucose measurements from fish exposed to a 10°C heat shock with and without a prior 45-s handling stress showed that the fish sampled 1 h after the single or double stressor exposures had elevated plasma cortisol and glucose levels, which returned to control levels 48 h after exposure to the stressors (Figure 6.5) Plasma sodium, potassium and chloride measurements taken 1 and 48 h after the fish were exposed to handling, heat shock or both generally showed no effect of either handling or heat shock on these variables 1 h after the fish had been exposed to the stressors. The only observed differences were between the plasma sodium levels of fish 48 h after one group had been both heat shocked and handled compared to fish that had been handled only (Figure 6.6). Figure 6.7 shows typical Western blots of fish proteins transferred to ntirocellulose and probed with the salmonid-specific

hsp70 and hsp30 rabbit polyclonal antibodies. The sample shown is of rainbow trout gill tissue proteins from control and heat-shocked fish. Gill tissue hsp70 and hsp30 concentrations measured in tissue samples taken 1 h after the fish were either handled, or both handling and heat shock showed that hsp70 and hsp30 levels were significantly lower in fish handled before being exposed to a 10°C heat shock protocol (Figures 6.8 and 6.9).

Results from the cortisol injected and heat shocked fish were similar to the results obtained from fish that had been handled and heat shocked, except for one observation. Hsp70 concentrations in gill tissues following a 10°C heat shock were unaffected by high circulating cortisol concentrations. Fish that received the cortisol implants had significantly higher plasma cortisol and glucose compared to control fish that received sham implants with cortisol carrier only. The elevated cortisol and glucose levels were observed at both 1 h and 48 h after the fish were returned to ambient water temperature (Figure 6.10). Plasma sodium, potassium and chloride concentrations again remained unchanged after the cortisol injection or heat shock treatments. The only plasma ion differences were observed 48 h after the fish were returned to ambient temperature water. Plasma sodium levels at this time were significantly lower in fish that had received a cortisol implant and had been heat shocked, compared with control fish, or fish that had only been heat shocked. Similarly, fish that had received a cortisol implant and were heat shocked had lower plasma chloride levels than the control fish and fish only cortisol injected or heat shocked (Figure 6.11). The most striking results from these experiments are those shown in figures 6.12 and 6.13. Figure 6.12 shows that gill tissue hsp70 levels were unaffected by high circulating plasma cortisol concentrations. Cortisol-implanted and sham-implanted fish subjected to a 10°C step change heat shock had the same hsp70 concentration 1 h after the heat shock treatments. Figure 6.13 shows that gill tissue hsp30 concentrations were significantly lower in fish that had intraperitoneal cortisol implants compared with fish sham implanted with the cortisol carrier only. The results showed that fish with elevated plasma cortisol concentrations due to handling stress or cortisol implant have the same or lower gill hsp70 and hsp30 concentrations in response to a heat shock.

When plasma cortisol concentration was artificially elevated (by slowreleasing intraperitoneal cortisol implants) the fish gill tissues did not have the normal increased hsp30 concentration but did show a normal increase of hsp70, after a 10°C step change heat shock.

Results from the primary hepatocyte cultures exposed to epinephrine showed that when the cells are maintained at 15°C, a 2-h exposure to either a physiological (10<sup>-7</sup> M) or a pharmacological (10<sup>-5</sup> M) dose of epinephrine caused a significant elevation in hsp70 concentration (Figure 6.14a) When the same experimental concentrations of epinephrine were applied to cells before a 15°C heat shock (from 15°C to 30°C), all cells, including the control cells not exposed to epinephrine, had the same elevation in hsp70 concentration (Figure 6.14b). When primary hepatocyte cells were exposed to the β-blocker propranolol together with a pharmacological dose of epinephrine (10<sup>-5</sup>M), the elevation of hsp70 previously observed did not occur (Figure 6.15).

### Discussion

Although much is known about the role of heat shock proteins in regulating the function, folding and trafficking of glucocorticoid receptors (Pratt, 1993), little study has been made of the effects of circulating levels of corticosteroids on heat shock protein synthesis. The present results show that the plasma cortisol and glucose concentrations of cutthroat trout exposed to a 45-s handling stress were elevated to levels similar to previously reported values

in salmonids measured after handling or confinement stress (Barton et al. 1980; Barton et al. 1985; Barton et al., 1986; Barton and Iwama, 1991). The data from the present studies suggest that the stress experienced by these fish was a normal hormonal stress response to physical handling. The fish were and measured 0.5, 1, 2, 3, and 48 h after the fish were returned to their holding tanks, were normally elevated and typical of fish adapting to an acute handling stress. The unaffected plasma sodium, potassium and chloride levels measured in these fish demonstrated that the fish were able to adjust to the handling stress and did not suffer from ionoregulatory impairment. The normal gill, liver and anterior kidney hsp70 concentrations in these fish, 1 h after the handling stress, showed that a standard 45-s handling stress did not induce the synthesis of hsp70 in any of these tissues. The hsp70 concentrations were unaffected in the gill tissues of these fish for 48 h after the handling stress and these consistent levels suggest that the lack of a measured response to elevated plasma cortisol is unlikely due to inappropriate sampling schedule. These findings are interesting, as a hsp70 elevation has clearly been demonstrated in some tissues in rats exposed to a restraint stress (Blake et al. 1991).

The lower hsp70 and hsp30 concentrations in the gill tissues of cutthroat trout exposed to a 45-s handling stress immediately before a 2-h heat shock, compared with fish not exposed to a handling stress prior to heat shock, has not previously been reported. Plasma cortisol, glucose and ion levels measured in these fish indicate that the fish were experiencing a typical hormonal stress response to the handling stress (see Barton and Iwama, 1991).

Plasma cortisol and glucose concentrations in fish with slow releasing cortisol implants were as expected with high circulating plasma cortisol concentrations in the cortisol implanted fish. The data obtained from cortisol implanted and heat shocked fish suggest that high circulating plasma cortisol levels do not affect the synthesis of hsp70 but may suppress the synthesis of hsp30 in the gill tissues of live fish exposed to heat shock.

A direct link between steroid hormones and heat shock proteins has been known for some time. It is well documented that an association exists between hsps and the glucocorticoid receptors (Pratt, 1993). The steroid receptors are direct signal transduction systems in which the receptor requires heat shock proteins to form a steroid-compatible heterocomplex of steroid receptor and four or more known heat shock proteins. This complex binds steroid hormones, translocates to the nucleus, binds to enhancer sequences in the genome and alters the transcription rates of specific genes (Yamamoto, 1985). Separate domains of the receptors are responsible for signal reception and subsequent DNA binding. Deletion of the hormone binding domain (HBD) in the COOH terminus yields receptors that are constitutive activators of transcription (Evans, 1988). It is known that steroid binding induces a conformational change within the receptor protein and that this alteration renders a fragment of the human mineralocorticoid receptor (MR) resistant to proteolysis (Trapp and Holsboer, 1995). Although hsp70 is known to be required for the assembly of the glucocorticoid receptor into a heterocomplex with hsp90 (Hutchison et al., (1994), little information about the effects of glucocorticoids on heat shock protein synthesis is available. Two studies, Holbrook and Udelsman (1994) and Brunt et al. (1990) show heat shock protein expression in response to physiological stress and steroid hormones, respectively. Although these two studies showed an induction of hsps in response to stressors or exposure to steroid hormones, they were performed on rats and a fungus respectively and may not accordingly be relevant to the present findings.

Acknowledgment

The collaboration of Stressgen Biotechnology Corp. and Dr. P. Candido in providing the monoclonal and polyclonal antibodies used for this work is gratefully acknowledged. The laboratory assistance of R. Forsyth, G. Cho, J. Morgan, S. Babich and E. Teng is sincerely appreciated. The liberal use of Dr. B. Finlay's laboratory equipment throughout this study was greatly appreciated. Figure 6.1 Plasma cortisol (A) and glucose (B) concentrations in rainbow trout exposed to a 45-s handling stress. Asterisks denotes significant differences between the handled (filled circle) and control (open circle) fish sampled at the indicated times after the fish were returned to their recovery tanks. (p < 0.05, N=8, mean ± SE).



Time post handling (h)

Figure 6.2 Plasma sodium (A), potassium (B) and chloride (c) concentrations in rainbow trout exposed to a 45-s handling stress. No significant differences were found between the handled fish (filled circle) and the control fish (open circle), at any of the sampling times after the fish were returned to their recovery tanks. (p < 0.05, N=8, mean ± SE).



45s handling

Figure 6.3 Tissue hsp70 levels 1 h following a 45-s handling stress. Densitometry analysis data of Western blots of cutthroat trout gill, liver and anterior kidney samples probed with anti-trout hsp70 rabbit polyclonal antibodies. Samples were collected 1 h after the fish were returned to their respective recovery tanks. Bars sharing a letter are not significantly different (p< 0.05, N=4, mean ± SE).



Hsp70 band intensity (arbitrary units)

Figure 6.4 Time course of gill hsp70 elevation following a 45-s handling stress. Densitometry analysis data of Western blots of cutthroat trout gill probed with anti-trout hsp70 rabbit polyclonal antibodies. Samples were collected 1, 3, and 48 h after the fish were returned to their respective recovery tanks. Bars within a sampling period sharing a letter are not significantly different (p< 0.05, N=4, mean  $\pm$  SE).



(lontration (% of control)

125

Time after 45s handling (h)

Figure 6.5 Plasma cortisol (A and B) and glucose (C and D) concentrations in rainbow trout exposed to a 10°C step change heat shock (solid bars), a 45-s handling stress (horizontal lines) or both handling and heat shock (vertical lines). Controls are blank bars. Data shown in graphs A and C are from plasma samples collected 1 h after the fish were returned to the recovery tanks. Graphs B and D show data from plasma samples collected 48 h after the fish were returned to the recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=8, mean  $\pm$  SE).



Figure 6.6 Plasma sodium (A and B), potassium (C and D) and chloride (E and F) concentrations in rainbow trout exposed to a 10°C step change heat shock (solid bars), a 45-s handling stress (horizontal lines) or both handling and heat shock (vertical lines). Controls are blank bars. Data shown in graphs A, C and E are from plasma samples collected 1 h after the fish were returned to the recovery tanks. Graphs B, D and F show data from plasma samples collected 48 h after the fish were returned to the recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=8, mean  $\pm$  1 SE).

(Mm) noits trans noi smeal



Figure 6.7 Example of Western blots of gill tissue proteins probed with salmonid-specific anti-hsp70 and anti-hsp30 rabbit polyclonal antibodies. The four control samples were sham injected with the cortisol carrier and the heat shocked samples were injected with the same carrier then exposed to a 2 h - 10°C step change heat shock and sampled after 1 h of recovery from the heat shock.



Figure 6.8 Gill hsp70 levels in handled and heat shocked rainbow trout. Densitometry analysis data of Western blots of gill tissue samples probed with anti-trout hsp70 rabbit polyclonal antibodies. The samples were collected 1 h after the sham implanted then heat shocked (solid) or the handled then heat shocked (crosshatched) fish were returned to their recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=4, mean ± SE).


Figure 6.9 Gill hsp30 levels in handled and heat shocked rainbow trout. Densitometry analysis data of Western blots of gill tissue samples probed with anti-trout hsp30 rabbit polyclonal antibodies. The samples were collected 1 h after the sham implanted then heat shocked (solid) or the handled then heat shocked (crosshatched) fish were returned to their recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=4, mean  $\pm$  SE).



Figure 6.10 Plasma cortisol (A and B) and glucose (C and D) concentrations in rainbow trout exposed to a 10°C step change heat shock (solid bars), cortisol implants, (horizontal lines) or both cortisol implants and heat shock (vertical lines). Controls are blank bars. Data shown in graphs A and C are from plasma samples collected 1 h after the fish were returned to the recovery tanks. Graphs B and D show data from plasma samples collected 48 h after the fish were returned to the recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=8, mean  $\pm$  SE).



Figure 6.11 Plasma sodium (A and B), potassium (C and D) and chloride (E and F) concentrations in rainbow trout exposed to a 10°C step change heat shock (solid bars), cortisol implants, (horizontal lines) or both cortisol implants and heat shock (vertical lines). Controls are blank bars. Data shown in graphs A, C and E are from plasma samples collected 1 h after the fish were returned to the recovery tanks. Graphs B, D and F show data from plasma samples collected 48 h after the fish were returned to the recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=8, mean  $\pm$  SE).



Time after heat shock (h)

Figure 6.12 Gill hsp70 levels in cortisol implanted and heat shocked rainbow trout. Densitometry analysis data of Western blots of gill tissue samples probed with anti-trout hsp70 rabbit polyclonal antibodies. The samples were collected 1 h after the sham implanted then heat shocked (solid) or the cortisol implanted then heat shocked (crosshatched) fish were returned to their recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=4, mean ± SE).



Figure 6.13 Gill hsp30 levels in cortisol implanted and heat shocked rainbow trout. Densitometry analysis data of Western blots of gill tissue samples probed with anti-trout hsp30 rabbit polyclonal antibodies. The samples were collected 1 h after the sham implanted then heat shocked (solid) or the cortisol implanted then heat shocked (crosshatched) fish were returned to their recovery tanks. Bars not sharing a letter are significantly different (p < 0.05, N=4, mean  $\pm$  SE).



Figure 6.14 Hsp70 levels in rainbow trout hepatocytes exposed to physiological and pharmacological concentrations of epinephrine *in vitro*. Densitometry analysis data of Western blots of rainbow trout hepatocytes probed with anti-trout hsp70 rabbit polyclonal antibodies. In (A), the cells were maintained at ambient 15°C. Control cells were grown in normal L-15 medium (blank), a separate treatment group of cells were exposed to a physiological concentration  $(10^{-7} \text{ M})$  of epinephrine (hatched) and a third group was exposed to a pharmacological concentration  $(10^{-5} \text{ M})$  of epinephrine (solid). In (B), The cells were exposed to a 15°C step change heat shock before the epinephrine was added to the medium. Bars not sharing a letter are significantly different (p < 0.05, N=3, mean ± SE).





Figure 6.15 Hsp70 levels in rainbow trout hepatocytes exposed to a pharmacological concentration of epinephrine and propranolol. Densitometry analysis data of Western blots of rainbow trout hepatocytes probed with anti-trout hsp70 rabbit polyclonal antibodies. Control cells were grown in normal L-15 medium (blank), a group of cells were exposed to  $10^{-5}$  M propranolol (crosshatch) and the third group was exposed to a pharmacological ( $10^{-5}$  M) concentration of epinephrine and  $10^{-5}$  M propranolol (solid). Bars not sharing a letter are significantly different (p < 0.05, N=3, mean ± SE).





## Chapter 7

## **General discussion and Conclusions**

Most of our knowledge concerning the homeostatic role of heat shock proteins has come from studies using cultured cells. Although much less is known about their expression *in situ*, heat shock proteins are induced acutely in intact animals in response to localized tissue injury, as well as systemically following heat stress (Vas et al. 1988; Gower et al. 1989; Blake et al. 1990; Morimoto et al. 1990).

The salmonid stress hormone response observed in fish exposed to various environmental stressors (see Wedemeyer et al., 1990; Barton and Iwama, 1991; Iwama et al., 1996) and the heat shock protein response, studied extensively in gene regulation experiments (see Welch, 1993; Georgopoulos and Welch, 1993; Morimoto et al., 1994) have both been well documented. Few investigators (Udelsman et al., 1993; Holbrook and Udelsman, 1994; Pratt, 1993), however, have examined the interaction between the two responses and none of these studies had been performed on fish. The experiments described in this thesis investigated the heat shock protein response, within the context of the physiological stress response, in intact salmonids and fish cell lines. The experiments focused on two major stress hormones (epinephrine and cortisol) and the effect they have on hsp70 and hsp30 concentrations measured in fish cell lines and the tissues of whole salmonids.

The general experimental approach was to induce one or both responses, by exposing the organisms to known stressors which induce the responses i.e. handling stress for the hormonal response and heat shock for the hsp response, and then measure both responses in the stressed organisms. My results clearly show that the hsp response can be detected in salmonid tissues, both *in situ* and *in vitro*. These results are consistent with the results of Gedamu et al. (1983), Kothary and Candido (1982) and Dyer et al. (1991) who showed that fish cells and tissues isolated from whole organisms have a detectable heat shock protein response when exposed to heat shock or other environmental toxicants.

The hsp response is thought to be induced by the denaturation of endogenous proteins, which release hsfs that are normally bound to hsp70. This triggers a cascade of events that causes the synthesis of additional hsp70, which can bind and inactivate free hsf thus completing the feedback loop and terminating the synthesis of more hsp70 (Morimoto et al., 1994).

Results from the present show that, depending on the severity of the treatment, elevated hsp70 levels can be detected for up to three weeks after the fish are returned to ambient temperature in the gill tissues of fish exposed to a brief heat shock treatment. The temperatures necessary to induce a hsp response in various fish tissues had previously been reported (Dyer et al., 1991; Dietz and Somero, 1993) and the results shown here demonstrate, for the first time, the long duration of the response.

A function of protein turnover under normal circumstances is to eliminate abnormal and potentially 'toxic' polypeptides that result from nonsense or missense mutations, errors in gene expression or post-synthetic damage (see Hawkins, 1991). In both animal and bacterial cells, such proteins are selectively hydrolyzed in a form of 'cellular sanitation system', which mainly involves ubiquitin-dependent pathways (Goldberg et al., 1987; Goff, Voellmy and Goldberg, 1988). The duration of elevated hsp70 reported here suggests that hsp70 present in the gill tissues of salmonids is not perceived as 'toxic' by these tissues. The observation that the heat shock treatment which resulted in the

longest lasting hsp70 elevation was the treatment that brought the fish closest to their normal maximal environmental temperature is consistent with the concept that protein denaturation and loss increases with increased temperature (see Somero, 1995). The duration of the response is consistent with the protein turnover times reported for salmonids reared at temperate temperature (Houlihan et al., 1995).

The results shown here are the first to demonstrate the effect of cortisol on hsp concentration in the tissues of intact salmonids. My results show that elevated plasma cortisol concentration, caused by physical handling, do not affect hsp70 concentration in the gill, anterior kidney or liver of cutthroat trout. These results are consistent with rat experiments which show that restraint stress does not induce hsp70 gene expression in 12 of 14 tissues tested (Udelsman et al. 1993). The inner cortical adrenal tissue and thoracic aorta tissues from those rat trials were the only tissues to show an hsp70 gene expression followed by elevated hsp70 protein concentration, with maximum expression occurring 3 and 6 h after restraint. The present results showed no hsp70 induction in either the liver or anterior kidney tissue 1 h after handling, or in the gill tissues sampled 1, 3 or 48 h after the handling stress.

The steroid response is known to affect energy metabolism in stressed organisms and to cause circulating glucose levels to increase. Cells are known to possess extremely high intracellular protein concentrations in all cellular compartments (100-150 mg/mL) and fish are known to utilize proteins as an energy source for the production of glucose via the gluconeogenic pathway (see Gamperl et al, 1994). The attenuation of some protein synthesis in the face of increased circulating steroid may therefore have been caused by the allocation of resources to energy mobilization rather than normal protein synthesis.

The cortisol and glucose measurements taken after the cutthroat trout were exposed to a 45-s handling stress indicate that these fish experienced a normal physiological stress response, as previously described in similarly handled fish (Barton et al. 1980; Barton et al. 1985; Barton et al., 1986; Barton and Iwama, 1991). The measured plasma sodium, potassium and chloride ions were not affected by any of the treatment groups and showed that the fish were not suffering from ionoregulatory impairment.

The lower hsp70 and hsp30 concentrations in the gill tissues of cutthroat trout exposed to a 45-s handling stress immediately before a 2-h heat shock, compared with fish that were not exposed to a handling stress prior to heat shock, has not previously been reported.

Results from the present study demonstrate that salmonid liver and kidney tissues do not show an increase in hsp70 in response to handling stress. This is similar to results obtained from liver and kidney tissues of restrained rats (Udelsman et al., 1993). The increase in thoracic aorta and adrenal hsp70 concentrations observed in the restrained rat were linked to activation of both the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. Fish vascular tissues may also have elevated hsp70 levels caused by adrenergic stimulation but these tissues were not sampled in the present experiment.

A direct link between steroid hormones and heat shock proteins has been known for some time. An association between hsps and glucocorticoid receptors is well documented (Pratt, 1993). The steroid receptors are direct signal transduction systems in which the receptor, which requires heat shock proteins to form a steroid-compatible heterocomplex of steroid receptor and four or more known heat shock proteins, binds steroid hormones, translocates to the nucleus, binds to enhancer sequences in the genome and alters the transcription rates of

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specific genes (Yamamoto, 1985). Separate domains of the receptors are responsible for signal reception and subsequent DNA binding. Deletion of the hormone binding domain (HBD) in the COOH terminus yields receptors that are constitutive activators of transcription (Evans, 1988). It is known that steroid binding induces a conformational change within the receptor protein and that this alteration renders a fragment of the human mineralocorticoid receptor (MR) resistant to proteolysis (Trapp and Holsboer, 1995). Although hsp70 is known to be required for the assembly of the glucocorticoid receptor into a heterocomplex with hsp90 (Hutchison et al., 1994), little information about the effects of glucocorticoids on the heat shock protein synthesis is available. Two studies, Holbrook and Udelsman (1994) and Brunt et al. (1990) show heat shock protein expression in response to physiological stress and steroid hormones, respectively.

The present results show that cultured hepatocytes respond to epinephrine in a  $\beta$ -receptor dependent manner suggest that the hypothalamic-pituitary-interrenal axis mediates the hsp70 response in some fish tissues as it does in some rat tissues, with the distinction that the fish tissue may be  $\beta$ -receptor mediated as opposed to the  $\alpha_1$ -receptor mediation reported for rat aorta tissue (Holbrook and Udelsman, 1994).

The first two sets of experiments, discussed in chapters 3 and 4, showed that hsp70 and hsp30 were the hsps most commonly synthesized in cell lines exposed to environmental stressors. These results show that, as had previously been demonstrated in the hsp literature on non-fish species, hsp70 is the most commonly represented hsp family synthesized when cells or whole organisms are exposed to various adverse environmental conditions. The present results therefore confirm that salmonids and salmonid cell lines generally respond to environmental stressors as do non-salmonid species. These findings are consistent with the conserved nature of hsp70 genes and the wide diversity of organisms previously shown to synthesize this family of proteins in response to heat shock and other inducers of the hsp response.

The finding that the normal hsp70 and hsp30 elevation seen in the gill tissues of heat shocked fish is attenuated by a 45-s handling stress prior to the heat shock demonstrates that the physiological stress response has an influence on the hsp response of salmonid tissues. The observation that the normal hsp30 response is attenuated by artificially elevated circulating cortisol levels (by intraperitoneal cortisol implants) while the hsp70 response following heat shock is unaffected by the same treatment suggests that these two hsps are regulated by different mechanisms.

Further studies on the induction of the response in various fish tissues exposed to both elevated cortisol and adrenaline *in vitro* may help to elucidate some of these mechanisms. Further work on the expression of hsps *in situ* with more tissues examined, and with the blood component of the tissues separated from the structural cells prior to tissue homogenization will surely also lead to interesting new insights on the interaction between the hormonal and heat shock protein response in salmonids.

## General conclusion

The general conclusions which can be drawn from the experiments conducted here are that hsp70 and hsp30 may be used as reliable indicators of environmental stress.

Physical handling, which results in elevated plasma corticosteroid levels, does not increase hsp70 or hsp30 levels in fish tissues and was found to *suppress* the induction of hsp70 in the gill tissues of heat shocked cutthroat trout.

Artificially elevated cortisol levels, from intraperitoneal implants, did not affect the normal elevation of hsp70 observed in the gill tissues of heat shocked cutthroat trout but it did suppress the induction of hsp30 in these same gill tissues.

The handling procedures necessary for sampling fish from the environment should not, therefore, by themselves, result in elevated heat shock proteins in salmonid liver, gill, kidney or skeletal muscle tissues. Further studies on the interaction of the hormonal and heat shock protein responses of salmonid tissues and cells lines, and the cell specific hsp responses of salmonid tissues need to be performed. The use of salmonid-specific anti-hsp antibodies in immunohistochemistry will surely yield new insights into these interactions.

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#### Appendix 1

# Exposure of Winter Flounder, Sculpins, Mussels and Oysters to crude oil and a crude oil anti-adhesive

## Introduction

Hydrocarbons are ubiquitous environmental contaminants that originate from industrial effluents and the diverse by-products of petroleum exploitation. The composition of crude oil is complex and varies from region to region. The major components are aliphatic hydrocarbons, cyclic paraffin hydrocarbons, aromatic hydrocarbons, naphtha-aromatic hydrocarbons, resins, asphaltenes, heteroatomic compounds, and metallic compounds. The aromatic and naphthano-aromatic hydrocarbons are considered to be the most toxic components in oil (Heath, 1995). The prevalence and persistence of these compounds in the environment is of major global concern for the environment.

The hsp response is involved in protecting organisms from damage due to exposure to a wide variety of stressors. This stress response entails the rapid synthesis of a set proteins known as heat shock proteins named as such because they are heat-inducible. Acquired tolerance to environmental change correlates with the accumulation of these proteins, as exposure to a mild stress regime confers the ability to survive a subsequent and more severe stress which would otherwise be lethal to the organism (see Lindquist, 1986). Protection by heat shock proteins also appears to involve common targets of environmentally induced damage, as tolerance is enhanced as long as stress proteins are elevated and is independent of the specific chemical or physical properties of the stressor (Sanders, 1993). This study investigated the effect of crude oil and a crude oil anti-adhesive compound developed to neutralize crude oil spilled in arctic marine environments on the levels of heat shock proteins found in the tissues of two benthic fish species and two shellfish species sampled from protected mesocosms.

### Materials and Methods

# Animals

The two fish species used for these trials were: (1) one year old winter flounder (*Pseudopleuronectes americanus*) and (2) one-year-old sculpins (*Myoxocephalus aenaeus*). These fish were chosen because they are non-migratory and therefore would be cotinuously exposed to toxicants in the vicinity of pollution sources. The two mollusks in these trials were: (1) the long neck clam (*Mya arenaria*) and (2) the blue mussel (*Mytilus edulis*). These organisms are endogenous to Point-au-Père, Québec, and were readily obtained from local fishers.

## Mesocosms

The protected mesocosms used for these trials are those described by Siron et al. (1993). These habitats are a group of five double-walled stainless steel tanks (internal dimension : height = 3 m; diameter = 1.4 m; volume =  $3.5 \text{ m}^3$ ) shown in Figure 4.1. Thermoregulated coolant was circulated between the two walls of each tank, allowing control of water temperatures for experiments conducted at near-natural conditions. Ambient air was blown over the seawater surface of each tank, to simulate weathering processes and conditions similar to those found in a natural subarctic environment. Sampling and temperature measurements were performed by means of two sampling ports located on the side wall of each tank, at 1 m and 2 m depths. These exterior tanks were exposed to natural photoperiod. Aeration of the water column was provided by a continuous stream of air bubbles entering through the 2-m sampling port of each tank. The bubbling induced a turbulent mixing sufficient to homogenize the

entire water column. Seawater supplied to the tanks was drawn from the estuary at Pointe-au-Père, at a depth of 5 m. This seawater was filtered through a gravel bed as it was pumped to the tanks. Salinity of the seawater varied between 26.5 and 27.1 ‰ and the temperature varied from 6.1 to 8.9 °C.

Each of the four mesocosms housed free-swimming winter flounder and sculpins, 30 of each species. Rectangular polypropylene baskets, measuring 40 cm by 50 cm and 20 cm deep containing sediment collected from the intertidal area adjacent to the research station at Pointe-aux-Père, were seeded with 20 mussels and 20 clams and suspended 1 m from the bottom of each mesocosm. These baskets were pulled up to the surface to sample the mussel and clams.

To determine if the tissues of winter flounder, sculpins, mussels or clams had a heat shock protein response when chronically exposed to crude oil, crude oil anti-adhesive or the combination of both crude oil and anti-adhesive, the fish and mollusks were held in four separate mesocosms receiving estuarine seawater and exposed to four different treatments. One mesocosm received 500 ppm of crude oil "Fortis", a type of crude oil that is regularly transported by tanker along the St. Laurence Seaway and that periodically reaches the rocky shores of the seaway due to spills or tanker bilge discharge. The second mesocosm received 500 ppm of the crude oil anti-adhesive compound, which was developed to neutralize crude oil floating in arctic marine environments. The third mesocosm received 500 ppm of crude oil and 500 ppm of the antiadhesive compound. The fourth mesocosm was the control and received ambient seawater from the estuary.

#### Tissue sampling

All fish were pithed prior to excising the tissues. Gill arches, liver, and skeletal muscle samples were excised from 10 fish that had been held in the

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mesocosms for 3 months. Each fish was measured for length and weight and then four gill arches, approximately 1 g of liver and 1 g of skeletal muscle were excised from each fish. Gill and mantle tissues were collected from the mollusks by separating the valves and excising the two tissues together, taking care not to contaminate samples with debris or adductor muscle tissue. All tissues were immediately placed in 1.5-mL conical Eppendorf tubes and were frozen on dry ice. The entire excision procedure was normally performed in less than 2 min. The tissues were stored at -20°C until they were processed for SDS-PAGE and Western blot.

The protocols for tissue sample preparation, the bicinchoninic acid protein assay, SDS-PAGE, Coomassie staining, transferring proteins to membranes and Western blotting are described in the General Materials and Methods section (Chapter 2).

## Results

The results from these trials showed that none of the three treatments had significant effects on any heat shock protein concentrations found in tissues of the fish or shellfish sampled during these trials (selected data showing lack of difference between treatment groups are omitted and will be included in final thesis draft).

# Discussion

The lack of difference in heat shock protein levels found in the tissues of fish and shellfish exposed to the different treatments suggests that the organisms exposed to these different treatments did not experience sufficient stress to induce the protein denaturation, proposed as the trigger for activation of heat shock genes (Hightower, 1980; Anathan et al., 1986).

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Figure App.1 Diagrams of a single and a bank of five protected marine mesocosms. Protected experimental mesocosms at the Institut National de la Recherche Scientifique (INRS) wet laboratory are located along the St. Laurence Estuary at Rimouski, Quebec. Seawater drawn from a depth of 5 m had typical estuarine characteristics with salinity and temperature ranging from 26-27 ‰ and 8-9°C, respectively.





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