Regulation of Heat Shock Protein 70 Levels in Red Blood Cells of Rainbow Trout

by

Lynsi Henrickson

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The physiological responses to stressor exposure in animals can be broadly grouped into the organismal and the cellular stress responses. During the organismal stress response, two key hormones, cortisol and epinephrine, are released into the general circulation. These hormones have widespread action, including elevation of glucose and lactate levels that are important in the metabolic adjustments to stress in fish. The cellular stress response involves the synthesis of a suite of proteins, the most important being the heat shock proteins (HSPs). These play a very important role in maintaining cellular function during stressor exposure. In fish, the expression of the inducible 70 kDa family of HSPs (HSP70) has been widely studied in various tissues and in response to a variety of stressors. HSP70 is synthesized in response to other protein disruptions and plays a key role in maintaining protein homeostasis. Consequently, HSP70 is used as a reliable indicator of cellular stress effects in fish.

Although elevated HSP70 expression in response to stressors have been demonstrated in trout (*Oncorhynchus mykiss*) red blood cells (RBCs), the utility of this protein as a marker of cellular stress has not been explored. The ease of repeated sampling of blood without euthanasia, suggests the possibility of using this tissue as a non-lethal marker of cellular stress in fish. This study tested the hypothesis that acute and chronic stressor exposure will elevate HSP70 expression in RBCs of rainbow trout. Also, studies were carried out to examine the role of stress hormones, cortisol and epinephrine, in modulating HSP70 expression in trout RBCs.

Acute heat shock exposure (+12^{o}C above ambient temperature for 1 h) significantly elevated plasma cortisol, glucose and lactate levels compared to the control fish over a 24 h

period. There was a tissue-specific induction of HSP70, but not HSC70 expressions in response to heat shock and recovery in trout. While liver and brain tissues showed enhanced HSP70 expression at 4 and 24 h post-heat shock, gill and RBCs had elevated HSP70 expression even at 1 h after a heat shock and this remained elevated over the 24 h post-exposure period. Overall, the results support the utility of using HSP70 expression in RBCs as an indicator of cellular stress effects in trout.

In order to quantify HSP70 levels in RBCs, a competitive antibody-capture enzyme-linked immunosorbent assay (ELISA) was developed using a commercially available rabbit anti-salmon HSP70 and a recombinant chinook salmon (*Oncorhynchus tshawytscha*) HSP70 protein. This ELISA enabled the measurement of HSP70 concentrations in RBCs. The specificity and reliability of this ELISA was confirmed by showing a temporal elevation in HSP70 levels in the RBCs in response to an acute heat shock exposure in trout. To determine effects of chronic exposure, two studies were conducted. First, fish were exposed to either a low (0.75 µg/L) or high concentrations (2.0 µg/L) of cadmium over a 28 d period. There was no significant effect of chronic sublethal cadmium exposure on HSP70 levels in trout RBCs. Also, a second study showed that exposure of trout to sublethal concentrations of municipal wastewater effluents (MWWE) at 0, 20 or 90% did not elicit a significant HSP70 response over a 14 d period. These results suggest that HSP70 levels in RBCs may be a reliable marker of acute cellular stress effects, but may not be a sensitive indicator of chronic effects associated with sublethal concentrations of contaminants in trout.

To test the effect of stress hormones in modulating HSP70 levels, RBCs were exposed to either cortisol (10 and 100 ng/mL) or epinephrine (10 nM) *in vitro* and the basal

and heat-induced HSP70 levels assessed. Heat shock elevated HSP70 content in trout RBCs at 4 and 24 h after the stressor exposure. However, this heat shock response was not modulated by either cortisol or epinephrine treatment. Interestingly, the results demonstrate for the first time that HSP70 content is released from RBCs into the medium in response to an acute heat shock. This heat-induced HSP70 release measured in the medium was in the absence of any changes in RBC viability, suggesting extracellular secretion of this protein. This HSP70 release from RBCs was attenuated in the presence of cortisol and epinephrine pointing to a key regulatory role for these stress hormones in the extracellular secretion of this protein.

Overall, measuring HSP70 levels in RBCs has the potential to be a reliable non-lethal marker of acute cellular stress effects in fish. The novel observation of measurable increases in heat shock-mediated medium HSP70 levels, mimicking this protein response in the RBCs, leads to the hypothesis that HSP70 may also have an extracellular role in fish, and warrants further study.

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Chapter 1
General Introduction

1.1 Introduction

The heat shock proteins (HSPs) are a family of highly conserved and ubiquitous intracellular molecules that are critical for cellular functions in unstressed as well as in stressed cells (Kregel, 2002). Families of heat shock proteins are classified based on their molecular mass. These families range in size from the very large HSP110 family, consisting of proteins around the 110 kDa range, to the small HSP family (sHSP), made up of proteins with a molecular mass less than 40 kDa. There is a high level of conservation among HSPs between species. For instance, HSP70 found in drosophila have a 72% amino acid identity with yeast (Hackett & Lis, 1983), while human HSP70 amino acid sequence has a 73% similarity with drosophila HSP70 (Hunt & Morimoto, 1985). Five to ten percent of the total proteins found in the cell during unstressed periods are made up of constitutively expressed HSPs (Pockley, 2003). Some of these HSPs are synthesized acutely in response to cellular stress (Ulrich Hartl, 1996; Welch & Feramisco, 1984). While not only assisting with oligomeric assembly, activation and transport of proteins (Eustace & Jay, 2004), HSPs, in general, also offer cytoprotection during periods of stress, including folding and refolding of proteins, maintaining structural proteins, protein translocation across membranes, prevention of protein aggregation and degradation of unstable proteins (Kregel, 2002). They also play an important role in thermotolerance, the extent of which is dependent upon the severity of the initial stress (Kregel, 2002).

Generally, HSP activation is triggered by a variety of stressors, including hyperthermia (Burdon et al., 1982; Flanagan et al., 1995; Kregel et al., 1995; Kregel & Moseley, 1996; Parsell & Lindquist, 1993; Skidmore et al., 1995), oxidative stress (Parsell & Lindquist, 1993; Wallen et al., 1997), UV radiation, chemicals, viral infection, nutritional deficiencies (Parsell & Lindquist, 1993), amino acid analogues (Thomas & Mathews, 1984), ischemia-reperfusion injury (Cairo et al., 1985; Li & Shrieve, 1982; Marber et al., 1995; Parsell & Lindquist, 1993), energy depletion (Sciandra & Subjeck, 1983) and acidosis (Weitzel et al.,1985). As my objectives will focus on the HSP70 family, only this group will be discussed hereafter.

1.1.1 Role of HSP70 Family Proteins

The HSP70 family is the most temperature sensitive and most highly conserved HSP family (Kregel, 2002), with a 60-80% base identity among eukaryotes (Craig, 1985). The two main members of this family include the inducible isoform, HSP70, and the constitutively expressed isoform, HSC70 (Pockley, 2001) . While these two molecules are similar, they do not have identical peptide maps (Welch & Feramisco, 1984) nor are they triggered by the same stimuli (Kregel, 2002). At normal growth temperatures, low levels of HSP70 can be seen in the cytoplasm; however, with heat shock there is a rapid synthesis of large amounts of HSP70 within the nucleus and nucleolus (Ulrich Hartl, 1996; Welch & Feramisco, 1984). Heat shock cognate 70 (HSC70) is present during normal physiological states and is not affected by heat shock (Lowe & Moran, 1984).

HSP70 is involved in the repair of heat damaged nucleoli by binding hydrophobic surfaces and aiding in refolding of proteins (Pelham, 1984). As well, it resolubilizes

hydrophobic precipitates formed by abnormal or denatured proteins through repeated binding and release (Lewis & Pelham, 1985). In general, HSP70 improves cell survival following a stressor exposure by reducing protein aggregation, refolding of denatured proteins, and other chaperoning functions (Ulrich Hartl, 1996). It has also been shown to have slow proteolytic action (Burdon, 1986). Transcription of the HSP70 gene suppresses inflammatory cytokine gene transcription, thereby limiting the amount of inflammatory cytokines (Cahill et al., 1997) and preventing extraneous cell death from inflammation (Zugel & Kaufmann, 1999). It is also thought to stimulate anti-cancer immunity (Srivastava & Heike, 1991), inhibit chronic inflammation (van Eden et al., 2005) and contribute to autoimmune disease (Kaufmann, 1990a; Kaufmann, 1990b). This latter function is due to recognition by natural killer and cytotoxic T cells without the activation of the major histocompatibility complex class I (MHC I) pathway when HSP70 is located on the cell surface (Johnson & Fleshner, 2006). It has also been shown to downregulate heat shock factor 1 (HSF1) activity through ATP binding ATPase activity (Pockley, 2001).

HSC70 is involved in trafficking proteins to lysosomal compartments, mediating lysosomal proteolysis of intracellular proteins and autophagy (Cuervo & Dice, 1997). It prevents premature folding by associating with integral mitochondrial matrix proteins prior to translocation from the cytoplasm, allowing the entry of these proteins into the mitochondrial matrix (Neupert & Brunner, 2002). Other members of this family, including Hsp78/Glucose-related protein 78 (Grp78) or binding immunoglobulin protein (BiP) (located in the endoplasmic reticulum) and mtHSP70 or Grp75 (located in mitochondria),

bind to extended polypeptides to prevent aggregation of unfolded peptides (Pockley, 2001) and are also antiapoptotic (Garrido et al., 2001; Kregel, 2002).

1.1.2 Heat Shock Protein Synthesis

Synthesis of HSPs involves the activation of heat shock factors (HSFs) which bind to the promoter region of the heat shock gene during stress. Four HSFs can be found within different organisms: HSF1, HSF2, HSF3, and HSF4. HSF1 is highly conserved and ubiquitous in vertebrates and is induced by stressors (Nakai, 1999). This is the main HSF found in mammalian systems (Sarge et al., 1993). HSF2 is also highly conserved within vertebrates (Nakai & Morimoto, 1993) but the expression of this protein is selective and induced only during early development and differentiation (Pirkkala et al., 2001). Within avian species, HSF3 functions as the main heat shock factor and is induced by stressors (Nakai, 1999). HSF4 has been found to be constitutively expressed in mammals (Nakai, 1999), and had been found to be involved with lens development and the formation of cataracts (Fujimoto et al., 2004; Shi et al., 2008).

During unstressed states, HSF1 is found in the cytoplasm in a latent monomeric state bound to HSP70 (Kregel, 2002) or ubiquitin (Burdon, 1986) and is unable to bind DNA in this state. Stress activates HSF1 by directly removing the HSP or by ubiquitin removal via ubiquitination of aberrant or damaged proteins (Burdon, 1986). A protein kinase then phosphorylates HSF1, which subsequently trimerizes and translocates to the nucleus (Morimoto, 1998). The trimer binds to the heat shock element (HSE) located in the promoter region of the gene leading to the synthesis of new HSPs (Kregel, 2002). Because of the lack of introns in the inducible isoform of HSP70, this synthesis occurs very rapidly (Deane &

Woo, 2010). This process is highly regulated in order to prevent pathological gene function (Eustace & Jay, 2004). Regulation occurs via protein binding. For instance, HSPs will bind to the transactivation domain of HSF1 to repress synthesis (Shi et al., 1998), while heat shock binding protein 1 (HSBP1) inhibits that ability of HSF1 to bind DNA (Satyal et al., 1998).

1.1.3 Extracellular HSPs

Extracellular HSP70 (eHSP70) was first shown to be released from glia cells and taken up by neighbouring neurons (Tytell et al., 1986; Hightower & Guidonin, 1989). Neurons show a deficit in HSP expression in the cytoplasm of the synapse due to the long axial length that must be traversed in order to reach the synapse from the neuronal body, and also due to decreased *de novo* synthesis (Tonkiss & Calderwood, 2005). To overcome this deficit, one theory is that the HSPs synthesized in neighbouring glial cells are released into the extracellular environment and taken up by neuronal receptors (Chen & Brown, 2007; Guzhova et al., 2001). Although the function of eHSP70 is unknown, it is thought to play a chaperoning role and protect the neurons against apoptosis (Calderwood, 2005).

In general, release of eHSPs is not dependent on basal levels or *de novo* expression and is thought to be a result of active secretion in response to cellular insults (Mambula & Calderwood, 2006b). A variety of stimuli may trigger the release of these proteins, including physical, behavioural and immunological stressors (Calderwood et al., 2007). The release may signal danger to the organism (Mambula et al., 2007). For instance, eHSP70 levels are elevated following trauma or bacterial infection (Hunter-Lavin et al., 2004), and these proteins are dominant antigens eliciting an immune response (Burnie et al., 2006).

Release of eHSP70 has been seen from a variety of tissues and cells, including brown fat following behavioural stress (Campisi & Fleshner, 2003), neuronal cells (Tytell et al., 1986), glial cells (Guzhova et al., 2001), β cells (Clayton et al., 2005), tumour cells (Gastpar et al., 2005) and human peripheral blood mononuclear cells (Lancaster & Febbraio, 2005). A mild elevation in body temperature has been shown to lead to HSP70 release, with more severe temperatures inducing release via cell lysis through necrotic death (Mambula & Calderwood, 2006a).A number of diseases and afflictions lead to chronically elevated eHSP70 levels in circulation. These include renal disease (Wright et al., 2000), hypertension (Pockley, 2002) and atherosclerosis (Pockley et al., 2003). eHSP70 may also exacerbate inflammatory disease like atherosclerosis, Alzheimer's and inflammatory bowel disease (Pockley, 2002). As well, there is an increase in eHSP70 concentration in the blood following exposure to normal physiological states, including acute physical and/or psychological stress (Johnson & Fleshner, 2006).

Recent studies suggest an immunological role for eHSP70 in animals. For instance, *in vitro* studies have shown the eHSP70 stimulates the production of nitric oxide synthase (Panjwani et al., 2002), nitric oxide, tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β) and IL-6 (Asea et al., 2000; Campisi & Fleshner, 2003) in macrophages and neutrophils. It also induces the complement cascade via C1q activation without antibody presence (Prohászka et al., 2002). Immunological differences in the termini of HSP70 have also been noted. The C terminus has been shown to stimulate cytokine production, induce T helper cell 1 (T_H1) polarization, and stimulate dendritic cell formation and maturation (Lehner et al., 2004). As well, in microbes and humans the C terminus will bind to CD14, CD14, TLR4,

and CD40 on antigen presenting cells (APCs) which may lead to stimulation of the innate immune system through APC activation leading to T cell responses (Wang et al., 2001).

1.1.4 Mechanisms of Release

There are a number of proposed and confirmed mechanisms of release for eHSPs. These proteins do not contain a leader sequence so they cannot be secreted through classical secretory pathways (Calderwood et al., 2007). There is most likely a posttranslational modification to trigger the protein for secretion into the extracellular environment (Mambula & Calderwood, 2006b). Release may occur via many mechanisms, including cell lysis, secretory vesicles, ATP-binding cassette (ABC) transport proteins and apocrine secretion (Hamon et al., 2000 Wewers, 2004; Clayton et al., 2005; Tytell, 2005; Mambula & Calderwood 2006a, b). Another mechanism for eHSP release is thought to involve the stressor-induced activation of the sympathetic nervous system, leading to the release of norepinephrine (NE). NE activates α1-adrenergic receptor (ADR) on the surface of the cell leading to an increase in intracellular Ca^{++} (Guarino et al., 1996) and the subsequent release of exosomes containing HSP70 (Savina et al., 2003; Clayton et al., 2005). The activation of α1-ADR by NE also elevates intracellular HSP70 levels (Matz et al., 1996), which would also lead to an increase in eHSP70 release. HSP70 release from exosomes can be increased in two ways, either by the increase in concentration of HSP70 within the exosome itself or by increasing the number of exosomes containing HSP70 (Johnson & Fleshner, 2006).

1.1.5 HSPs in Fish

The majority of studies on the mechanisms of HSP induction, and their mode of action in maintaining cellular protein homeostasis have been carried out with mammalian models. In fish, studies related to HSPs expression have focussed on the tissue expression of these proteins as markers of cellular stress in response to stressor exposures, including heat shock (Vijayan et al., 2005; Deane & Woo, 2010). Due to their aquatic environment, fish are at an increased risk of contact with a variety of stressors, including changes in salinity, temperature and oxygen as well as the presence of pathogens and chemicals, all of which have been shown to elicit a cellular HSP response (Deane & Woo, 2010). The most widely studied HSP in fish is the expression of the inducible 70 kDa protein (HSP70; Vijayan et al., 2005; Deane & Woo, 2010).

Changes in environmental variables may lead to a tissue-specific heat shock response. For instance, a change in salinity, whether it is transient or chronic leads to osmotic and/or ionic imbalance in fish. This can lead to the formation of aberrant proteins resulting in the induction of the heat shock response (Deane et al., 2002). The most important organ in coping with changes in surrounding ion concentrations is the gill, and this tissue elevates HSP70 expression in response to different salinities (Deane & Woo, 2010). Interestingly, a mild heat shock prior to a salinity challenge was shown to increase tolerance to osmotic stress in Atlantic salmon (*Salmo salar*; Du Beau et al., 1998). This is thought to be due to the synthesis of HSPs, which confers cellular protection from damage due to subsequent stressor challenges (Deane & Woo, 2010). However, no increase in osmotolerance was seen in tidepool sculpin (*Oligocottus maculosus;* Todgham et al., 2005) and only a minor effect was

seen in rainbow trout (*Oncorhynchus mykiss*; Niu et al., 2008) when they were first treated with heat shock prior to an osmotic stressor. Similarly, a tissue-specific expression of HSP70 is also seen with other environmental variables including hypoxia and elevated temperatures, but the mechanisms leading to this protein induction and the long term consequences on animal acclimation to these environmental stressors are poorly understood in fish (Methling et al., 2010; Deane & Woo, 2010).

Pathogens also induce the synthesis of HSPs, which have been found to play an integral role in the immune response (Deane et al., 2010). Infection with disease causing bacteria elicits an HSP response in fish. For instance, bacterial kidney disease increases both hepatic and renal HSP70 levels in coho salmon (*Oncorhynchus kisutch*; Forsyth et al., 1997). This was also the case with silver sea bream (*Sparus sarba*) infected artificially with *Vibrio alginolyticus*, showing elevated hepatic HSP70 expression (Deane et al., 2004). Acute bacterial infection with *Vibrio anguillarum* also showed an increase in HSP70 in liver and head kidney of rainbow trout (Ackerman & Iwama, 2001). Overall, the tissue HSP70 expression is elevated in response to pathogen exposure, but the mechanism leading to induction of this protein in unclear in fish.

HSP70 expression in response to chemical exposure has been extensively studied in fish (see reviews by Vijayan et al., 2005; Deane & Woo, 2010). Most of these studies revealed a tissue-specific enhanced expression of HSP70 gene and protein in response to contaminants, including polychlorinated biphenyls and metals, supporting the utility of using this protein expression as a biomarker of cellular effect in fish (see reviews by Vijayan et al., 2005; Deane & Woo, 2010). The observation that acute handling stress and the associated

elevation in plasma cortisol levels did not modulate the HSP70 expression further confirmed the use of HSP70 as a biomarker of cellular effects for aquatic environmental monitoring (Vijayan et al., 1997). Also, exposure of trout to various concentration of PCBs resulted in elevated hepatic HSP70 expression that correlated with animal survival (Vijayan et al., 1998). The lack of hepatic HSP70 expression at higher concentrations of PCB corresponded with increased mortality clearly supporting a role for this protein expression in protecting fish from PCB toxicity (Vijayan et al., 1998). Taken together, HSP70 expression is a reliable marker of cellular effects in fish, while the mechanisms leading to this protein induction, as well as their role in protecting cells against stressor insults needs further work in lower vertebrates.

1.1.6 HSP70 in Fish Red Blood Cells

Unlike mammalian red blood cells (RBCs), the RBCs of teleost fish are nucleated. The presence of this nucleus renders these cells capable of protein synthesis (Speckner et al., 1989). Because of this ability and its ease of collection and manipulation, RBCs of fish are an excellent candidate for use as a non-lethal biomarker of stress. Indeed, studies have demonstrated that fish RBCs synthesize HSP70 and their expression is modulated by stressors, including heat shock and metals (Currie & Tufts, 1997; Currie et al., 1999; Lund et al., 2003; Fulladosa et al., 2006). Expression of HSP70 in RBCs was found to increase with heat shock both *in vitro* and *in vivo*. When isolated RBCs were subjected to a heat shock there were significant increases in HSP70 levels (Currie & Tufts, 1997; Currie et al., 1999). However, neither hypoxia nor energy depletion elicited a heat shock response in trout RBCs in vitro (Currie et al., 1999). Brook trout (*Salvelinus fontinalis*) exposed to temperatures *in*

vivo ranging from +2 – +7ºC above ambient temperature (18ºC) showed significant elevation in RBC HSP70 expression from 22ºC onwards (Lund et al., 2003). The only study so far examining RBC HSPs expression in response to contaminant exposure showed significant increases in HSP70 levels in RBCs of sea bream (*Sparus auratus*) in response to cadmium exposure *in vitro* (Fulladoa et al., 2006). This study demonstrated that RBCs were sensitive to cadmium toxicity with HSP70 expression seen at cadmium concentration as low as 0.1 µM. This study also showed elevated HSP70 expression with lead and chromium, but the heat shock response was attenuated at higher concentrations of these metals (Fulldosa et al., 2006). Together the results suggest the potential benefits of using HSP70 expression in RBCs as an indicator of cellular stress in fish. However, no study has yet characterized the reliability and specificity of using this protein expression in RBCs as a biomarker of cellular stress effect for environmental monitoring.

1.1.7 Linking the Organismal Stress Response to the Cellular Stress Response

The organismal response allows animals to cope with stress and to re-establish homeostasis. There are three levels of the stress response: primary, secondary and tertiary. The primary level of response involves the release of catecholamines (CAs), including epinephrine and norepinephrine, and corticosteroids. The secondary level of response involves the tissue changes associated with the stimulation of these primary hormones. This comprises of physiological responses, including increased cardiac output, oxygen uptake, mobilization of energy substrates and disturbances in hydromineral balance. The last level of response, the tertiary level, can be seen at the level of the whole animal and population,

reflecting growth inhibition, reproductive and immune dysfunction, and a reduction in the ability to cope with additional stressors (Wendelaar Bonga, 1997).

The primary effect seen in response to stressor exposure is the release of epinephrine and norepinephrine, which are involved in the "fight or flight" reflex (Wendelaar Bonga, 1997). These CAs are released by chromaffin cells, near the head kidney in fish, and function in the enhancement of blood oxygen transport and energy mobilization. Resting levels of CAs are generally less than 1 nM, but within minutes of exposure to an acute stressor these levels can increase to over 20 nM (Wendelaar Bonga, 1997). Due to their short circulating half-life of less than ten minutes, CA levels undergo a rapid decrease following their initial peak (Mommsen et al., 1999). These hormones work to increase ventilation rate, blood flow and oxygen transportation capacity of blood. As well, epinephrine is involved in mobilizing energy stores via glycogenolysis (Mommsen et al., 1988; Wright et al., 1989). Chronic stress may lead to the desensitization of target cells through downregulation of adrenergic receptors (ADR), the catecholamine receptors (Gilmour et al., 1994).

The other notable stress hormone is the corticosteroid, cortisol in teleosts, which is elevated in response to stressor exposure and involves the activation of the hypothalamus-pituitary-interrenal (HPI) axis (see Fig. 1.1). Briefly, a stressor stimulates the hypothalamus to secrete corticotropin releasing factor (CRF), a hypothalamic neuropeptide, which acts on the pituitary gland and stimulates the release of adrenocorticotropic hormone (ACTH). ACTH, in turn, stimulates the interrenal cells to release cortisol (Mommsen et al., 1999). Cortisol works at the level of the tissue, particularly gill, liver and intestine, to bring about metabolic and ionic and osmotic regulation that will allow the organism to restore

homeostasis during stress. The release of cortisol is regulated via a negative feedback control at each level of the HPI axis (Vijayan et al., 2005). This multifaceted hormone is important for physiological adjustment to stress, including regulation of hydromineral balance, energy metabolism and immune functions (Wendelaar Bonga, 1997; Mommsen et al., 1999; Vijayan et al., 2005; Iwama et al., 2006).

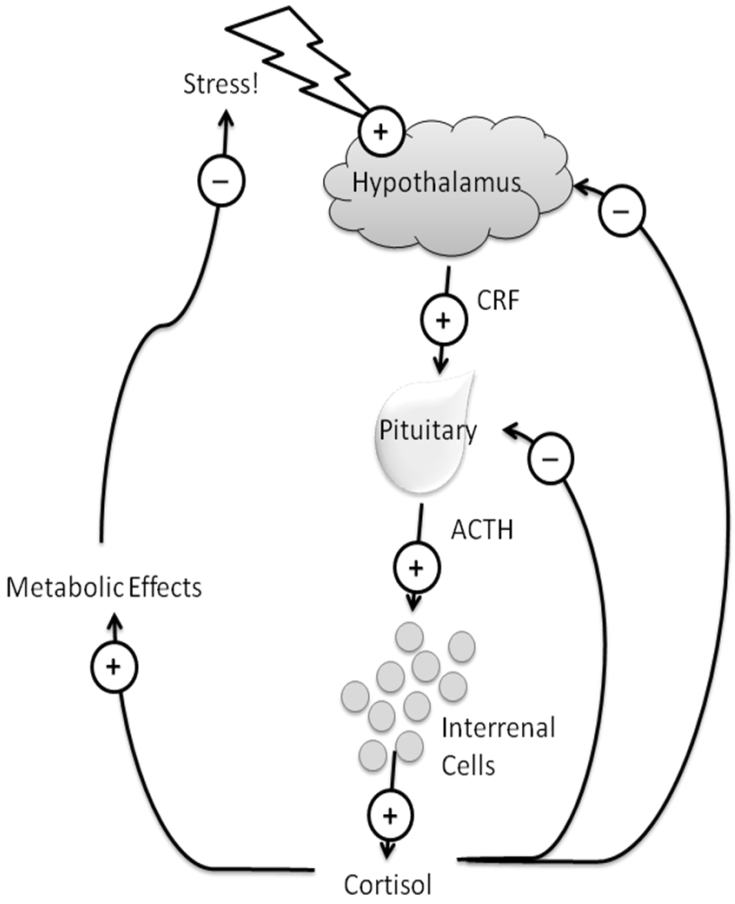


Figure 1.1: The Hypothalamus-Pituitary-Interrenal (HPI) axis. Exposure to a stressor stimulates the hypothalamus to release corticotrophin releasing factor (CRF) which acts on the pituitary stimulating the release of adrenocorticotropic hormone (ACTH). ACTH will cause the release of cortisol from interrenal cells, which acts on tissues such as liver to bring about metabolic effects in order to return to homeostasis. Cortisol regulates itself via negative feedback at each level of the axis.

The interactions of cortisol and the heat shock response have been documented in fish (Vijayan et al., 2005). It has been repeatedly shown that increases in cortisol will cause a decrease in the heat shock response. Increased cortisol has been shown to decrease HSP70 expression in the liver of rainbow trout by 30% and in gill by 66% (Basu et al., 2001). When treated with cortisol prior to exposure to copper, common carp showed a decrease in HSP70 levels (De Boeck et al., 2003). In rainbow trout hepatocytes, HSP70 accumulation decreased over a 24 h period when treated with cortisol prior to heat shock (Boone & Vijayan, 2002a). No change was seen in gill HSP70 levels of cutthroat trout (*Oncorhynchus clarkii*) administered cortisol prior to heat shock (Ackerman et al., 2000). HSP70 levels were also unchanged in the liver of silver sea bream injected daily with cortisol (Deane et al., 1999). However, silver sea bream fibroblasts HSP70 levels were shown to increase after cortisol administration *in vitro* (Deane et al., 2006).

Other HSPs are also affected by increased cortisol levels. Cortisol at 1000 ng/mL decreased heat shock-induced HSP90 mRNA abundance in rainbow trout hepatocytes in primary culture (Sathiyaa et al., 2001). However, cortisol administration *in vivo* elevated liver HSP90 protein expression in rainbow trout (Vijayan et al., 2003). A decrease in gill HSP30 levels was seen in cutthroat trout with cortisol administration followed by heat shock (Ackerman et al., 2000). Taken together, these results support a link between the organismal stress response and the cellular stress response in fish. Consequently, the organismal stress response may modulate the HSP70 response in fish, but this remains to be tested under different conditions. While the liver HSP70 expression in response to beta-naphthflavone was not modulated by the acute stress of handling (a scenario that involved transiently

elevated plasma cortisol levels) over a 24 h period (Vijayan et al., 1997), little is known about the effect of chronic stress and sustained cortisol elevation on HSP70 response in fish. Also, nothing is known about the role of stress hormones in regulating HSP70 expression in fish RBCs.

1.2 Experimental Rationale

While knowledge of intracellular regulation of HSPs in fish has been increasing over recent years, there is still a lack of information regarding HSP70 expression in RBCs and its modulation by stress hormones. HSP70 levels in RBCs of fish has the potential to be a reliable marker of cellular stress effects, particularly because, unlike other tissues, they can be collected non-lethally by repeated sampling of fish. Also, nothing is known about extracellular heat shock proteins in fish. The hypotheses tested are: *i*) RBC HSP70 concentration is a marker of acute and chronic cellular stress effects in rainbow trout, and *ii*) extracellular HSP70 levels are elevated in response to acute stress in rainbow trout.

1.3 Research Objectives

The specific objectives are:

1. Determine if HSP70 expression follow the same pattern in RBCs as they do in other tissues in rainbow trout.
2. Develop an enzyme-linked immunosorbent assay (ELISA) for measuring HSP70 concentration in trout RBCs.
3. Determine if acute and chronic stressor exposure affects RBC HSP70 concentration in rainbow trout.
4. Determine if cortisol and epinephrine modulates HSP70 level in trout RBCs.
5. Examine whether RBCs HSP70 is released into the extracellular medium, and if this secretion is modulated by stress hormones in trout.

Chapter 2
Tissue-Specific Heat Shock Proteins Expression in Rainbow Trout

2.1 Introduction

In response to stressor exposure fish elicits an organismal stress response, including elevation in plasma catecholamine, cortisol, glucose and lactate levels (Wendelaar Bonga, 1997; Iwama et al., 2006). The primary hormonal response, including epinephrine and cortisol, plays a role in the secondary metabolite response seen during stress in fish (Iwama et al., 2006). While epinephrine is rapidly elevated (within seconds to minutes) in response to stress, the cortisol release into the circulation is delayed by several minutes after a stressor exposure. Therefore, it is possible to obtain resting level of cortisol with sampling unlike epinephrine. Consequently, cortisol is the hormone of choice as a marker of acute stress in fish (Iwama et al., 2006). The organismal stress response, and the associated hormone release, plays a key role in allowing animals to cope with stress, including reestablishment of homeostasis (Mommsen et al., 1999; Iwama et al., 2006).

At the cellular level, the stress response involves the synthesis of a suite of proteins, termed the HSPs (see Iwama et al., 2006 for a review). HSPs are a family of highly conserved and ubiquitous proteins that are essential to cope with cellular stress, as well as for normal cell functioning (Kregel, 2002). These proteins are classified into different families based on their molecular mass, including small HSPs (sHSP), HSP40, HSP60, HSP70, HSP90 and HSP110. They make up 5-10% of the total cellular proteins pool even in unstressed cells (Welch et al., 1983; Pockley, 2003).). In general, HSPs offer cytoprotection

during stressful periods through folding and refolding of denatured proteins, maintaining proteins structure, protein translocation across membranes, preventing protein aggregation and degradation of unstable proteins (Kregel, 2002). Some of the most abundant forms of HSPs in the cell include members of the 70 kDa family, the inducible HSP70 and the constitutive HSC70.

In fish, several studies have examined the expression of HSP70 and HSC70 in response to a variety of stressors (Iwama et al., 2006; Deane and Woo, 2010). At normal growth temperatures, HSP70 is found at low levels in a cell; however, exposure to heat shock or other stressors affecting the protein machninery causes a rapid synthesis of this protein (Iwama et al., 2006; Deane & Woo, 2010). In general, HSP70 improves cell survival in response to stress by re-establishing protein homeostasis, including reducing protein aggregation, refolding denatured proteins and degrading damaged proteins (Ulrich Hartl, 1996). HSC70 is constitutively expressed in the cell and is involved in trafficking damaged proteins to lysosomal compartments for proteolysis (Cuervo & Dice, 1997). It also prevents premature folding of integral mitochondrial matrix proteins (Neupert & Brunner, 2002). In fish, HSC70 expression is maintained at the constitutive levels and not modulated by stressors, including heat shock and metals (Zarfullah et al., 1992; White et al., 1994; Norris et al., 1995; Boone & Vijayan, 2002a).

While most studies have examined HSP70 and HSC70 expression in the liver, very few studies have measured these protein expressions in the red blood cells (RBCs) of fish. Indeed trout (*Oncorhynchus mykiss*) RBCs showed a robust HSP70 gene and protein expressions in response to heat shock (Currie & Tufts, 1997). However, HSC70 expression

was unaffected by stressors, including heat shock, sodium azide, hypoxia and zinc exposures in rainbow trout RBCs (Currie et al., 1999). Few studies have compared the stressor-induced HSP70 response in RBCs in relation to that seen in other tissues, including liver, gill and brain in fish. This tissue-specific comparison would provide important information on the feasibility of using RBC HSP70 expression as a marker of cellular stress response in trout. Also, since fish can be bled repeatedly without sacrificing the animal, the HSP70 expression in response to stressors in RBCs will provide an excellent non-lethal marker of cellular stress in fish. Hence, the objective of this study was to examine the tissue-specific expression of HSP70 and HSC70 in response to a standardized heat shock regimen (1 h at +12ºC above ambient temperature) in rainbow trout. HSP70 and HSC70 protein expression were measured in several tissues, including brain, liver, gill and RBCs. Plasma cortisol, glucose and lactate levels were also measured as indicators of organismal stress response in rainbow trout (Iwama et al., 2006).

2.2 Materials and Methods

2.2.1 Animals

Juvenile rainbow trout (average body mass of 125 g) used for experiments conducted at the University of Waterloo (Waterloo, Ontario) were obtained from Alma Aquaculture Research Station (Alma, Ontario) and were acclimated for at least two weeks prior to any experimentation in 200 L aerated tanks with constant water flow at 13°C. Fish were maintained on a 12 h light/dark photoperiod and fed once daily to satiety (3 part sinking food; Martin Mills Inc., Elmira, Ontario). All experiments conducted were approved by the

Animal Care and Use committee of the University of Waterloo and is in accordance with guidelines established by the Canadian Council for Animal Care.

2.2.2 Experimental Design

Groups of 12 fish each were separated into four 100 L tanks in a static system containing well water. Water in two tanks was heated to a temperature of approximately 25°C (+12ºC above ambient), while two tanks were maintained at ambient temperature (13°C). Fish were subjected to the +12°C heat shock for one hour after which they were brought back to 13°C within 15 minutes. Six fish from each of the heat shocked and control (no heat shock) groups were sampled immediately following heat shock and at 4 and 24 h after the heat shock. Fish were quickly netted and euthanized with an overdose of 2-phenoxy ethanol (1 mL/1L well water; Sigma). Liver, brain, and gill filaments from the first and second arches and blood samples were collected. Blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (5 mg in Hank's medium; Fischer Scientific) and centrifuged for five minutes at 3000 x g to separate the RBCs from plasma. All samples were stored at -80°C for analyses later.

2.2.3 Sample Analysis

2.2.3.1 Plasma Cortisol, Glucose and Lactate

Plasma cortisol levels were analyzed using 3H cortisol radioimmunoassay following established protocol (Ings & Van der Kraak, 2006). Plasma glucose (Rainchem, San Diego, CA, USA) and lactate levels (Trinity Biotech, St-Louis, MO, USA) were measured with an end-point colourimetric assay using commercially available kits.

2.2.3.2 HSP70 and HSC70 expression

2.2.3.2.1 SDS-PAGE and Western Blotting

SDS-Page and western blotting were used to determine expression of HSP70 and HSC70 in liver, brain, gill and RBC samples following established protocols (Boone & Vijayan, 2002b). The protein concentration in the cell lysate was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) using bovine serum albumin as the standard. Sample proteins (40 ug) were separated on 8% polyacrylamide gels using discontinuous buffer system of Laemmli (Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane (BioRad, 0.45 mm pore size) (20 V for 25 minutes) with semidry transfer unit (BioRad) in 10% transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 10% (v/v) methanol). Membranes were blocked with 5% skim milk in TTBS (20 mM Tris pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20 with 0.05% sodium azide) for one hour and subsequently probed with primary antibody accordingly for one hour at room temperature. For HSP70, a polyclonal rabbit anti-salmonid HSP70 primary antibody (1:5000; StressMarq) was used while HSC70 was detected using a polyclonal rabbit anti-trout HSC70 antibody (1:3000; Boone & Vijayan, 2002a). Following primary antibody probing, membranes were washed 3x10 min with TTBS and probed with horseradish peroxidase conjugated goat anti-rabbit (1:3000; BioRad) secondary antibody for one hour at room temperature. Membranes were washed 3x10 min with TTBS and washed 1x10 min with TBS. Bands were revealed with ECL plus Western Blotting Detection System (GE Healthcare, Amersham) and scanned using a Typhoon scanner. The molecular mass of target proteins was confirmed using

Precision Plus Protein Standards (BioRad). Band intensities were quantified with AlphaEase software (Alpha Innotech, CA).

2.2.4 Statistical Analysis

The experimental design utilized a two-way ANOVA for statistical comparison with treatment (control and heat shock) and time as independent factors. However, the HSPs expression in the control group (non-heat shocked group) at the different time points were not statistically different (one-way ANOVA; $p<0.05$). Therefore, for HSPs analysis, the control groups were pooled together as one group and compared with the heat shocked groups at 1, 4 and 24 h post-heat shock using a one-way ANOVA. Significant differences were compared using the Holm-Sidak post hoc test with the level of significance for all tests set at $p<0.05$. The data were transformed to meet the assumptions of homogeneity of variance although non-transformed data are shown in the figures. Results are presented as mean ± standard error of mean (SEM) for all groups.

2.3 Results

2.3.1 Plasma Cortisol levels

While there were no significant temporal differences in plasma cortisol levels,the heat shocked group had significantly higher plasma cortisol levels compared to the control group and this was clearly evident at 4 and 24 h post-heat shock treatment (Fig. 2.1A).

2.3.2 Plasma lactate levels

Plasma lactate levels were significantly higher at 1 h in both the control and heat shocked fish compared to 4 and 24 h samples (Fig. 2.1B). Plasma lactate levels in the heat shocked groups were significantly higher than the control group only at 1 h but not at 4 and 24 h after heat shock treatment (Fig. 2.1B).

2.3.3 Plasma glucose levels

Plasma glucose concentration showed a significant time effect with levels significantly lower at 24 h compared to the 1 and 4 h time points after heat shock treatment (Fig. 2.1C). There was also a significant treatment effect with heat shocked fish having significantly higher plasma glucose levels compared to the control group (Fig. 2.1C).

2.3.4 HSP Expression: Brain

Exposure to a one hour heat shock of 12ºC above ambient temperature caused a significant increase in HSP70 expression in the brain of rainbow trout at 4 and 24 h, but not at 1 h post-heat shock exposure (Fig. 2.2A). Expression levels of HSC70 in the brain tissues did not change significantly from the control in response to heat shock (Fig. 2.2B).

2.3.5 HSP Expression: Liver

As with the brain, the expression of HSP70 in the liver of heat shocked fish was significantly higher at 4 and 24 h but not at 1 h after heat shock exposure compared to the control group (Fig. 2.3A). There was no significant difference in HSC70 expression in the heat shocked group compared to the control group (Fig. 2.3B).

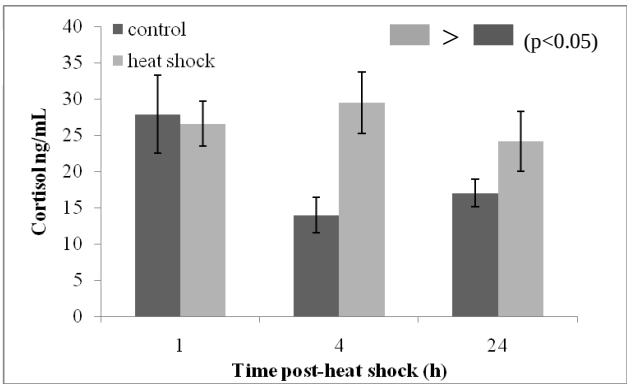
2.3.6 HSP Expression: Gill

The expression of HSP70 in gill filaments was significantly increased even at 1 h post-heat shock. This protein expression stayed significantly higher at 4 and 24 h post-heat shock compared to the control group (Fig. 2.4A). There was no significant effect of heat shock in constitutive HSC70 expression in the gill of rainbow trout (Fig. 2.4B).

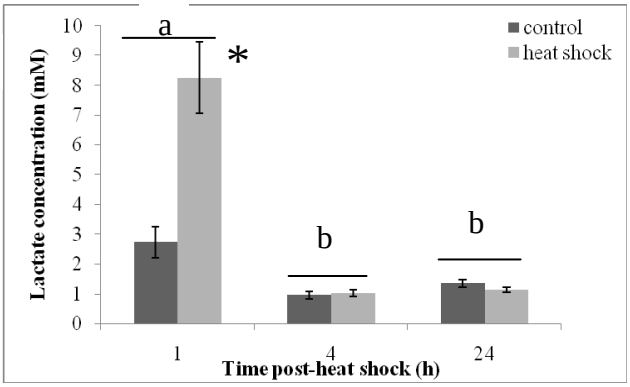
2.3.7 HSP Expression: RBCs

HSP70 expression in RBCs was significantly higher at 1 h compared to the control group. The expression of this protein increased further and was significantly higher at 4 and 24 h compared to the control and 1 h post-heat shock groups (Fig. 2.5A). There was no significant effect of heat shock on HSC70 expression in the RBCs compared to the control group (Fig. 2.5B).

A.



B.



C.

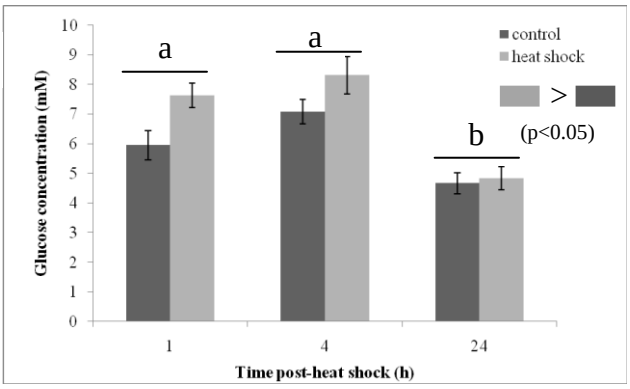


Figure 2.1: Plasma cortisol (A), lactate (B) and glucose (C) levels in rainbow trout exposed to heat shock. Plasma samples were collected from trout exposed either to no heat shock (control) or one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. Values represent means ± SEM (n = 6). Inset shows significant treatment effect (P<0.05; two-way ANOVA); bars with different letters (a, b) are significantly different (p<0.05, two-way ANOVA); asterisks denote a significant difference from their respective control (p<0.05, two-way ANOVA).

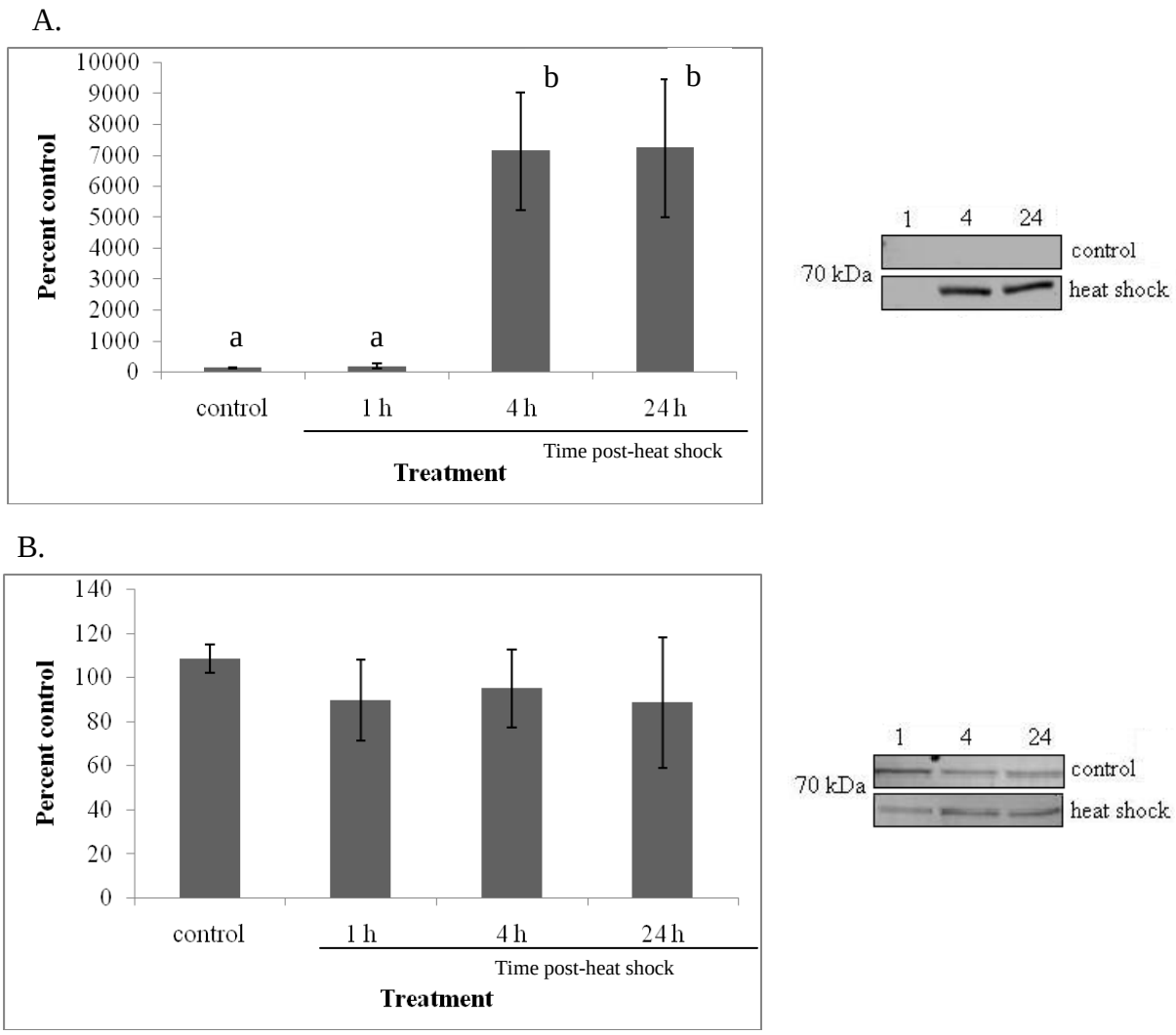


Figure 2.2: Brain HSP70 (A) and HSC70 (B) protein expressions in rainbow trout in response to heat shock. Brain samples were collected from trout exposed to a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. Values represent means ± SEM (n = 6). Bars with different letters (a, b) are significantly different ($p<0.05$, one-way ANOVA). A representative western blot image for HSP70 and HSC70 is shown beside their respective histograms; the blots was probed with either polyclonal rabbit anti-salmonid HSP70 (1:5000 dilution) or polyclonal rabbit anti-trout HSC70 (1:3000 dilution).

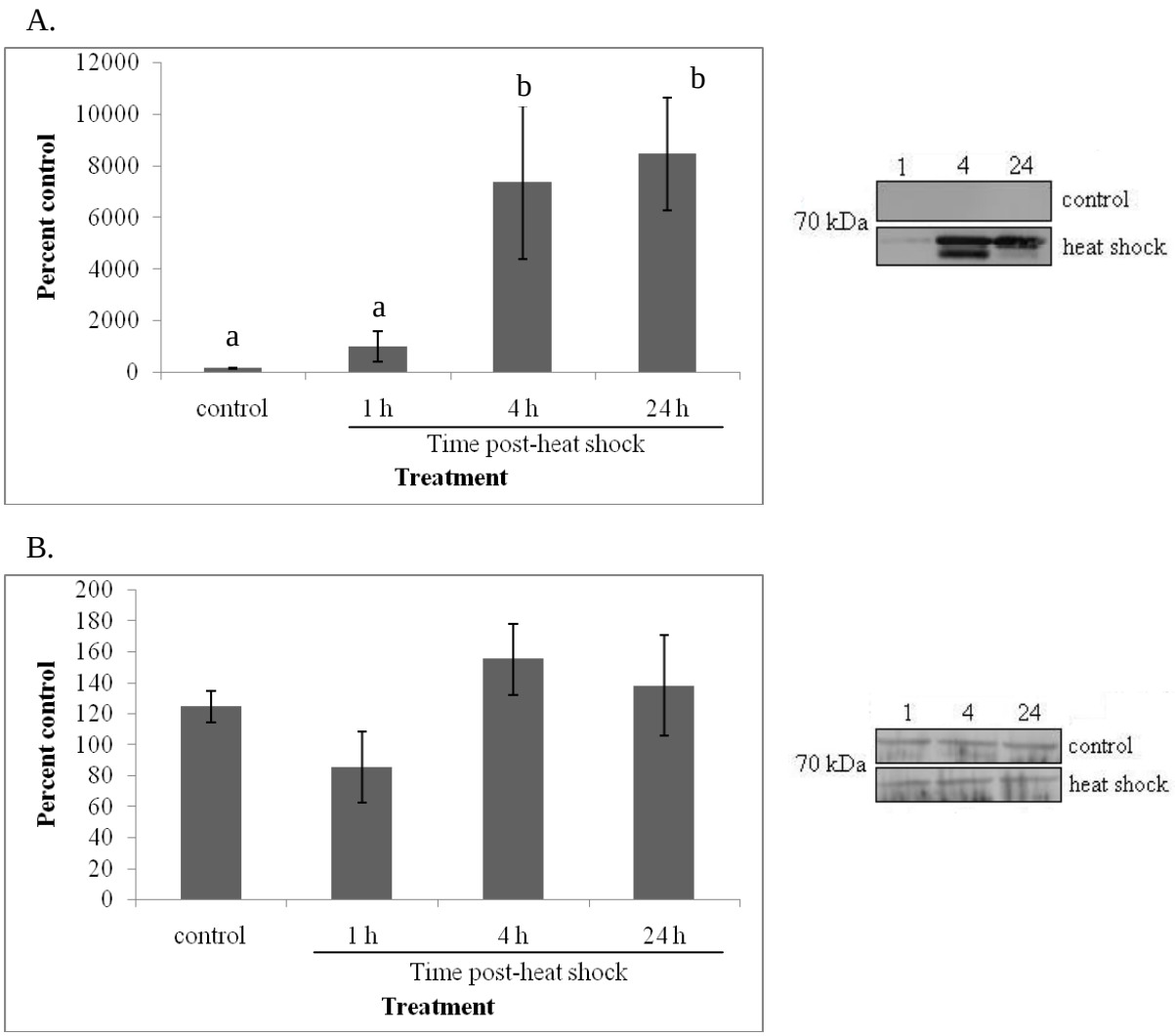


Figure 2.3: Liver HSP70 (A) and HSC70 (B) protein expressions in rainbow trout in response to heat shock. Brain samples were collected from trout exposed to a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. Values represent means ± SEM (n = 6). Bars with different letters (a, b) are significantly different ($p<0.05$, one-way ANOVA). A representative western blot image for HSP70 and HSC70 is shown beside their respective histograms; the blots was probed with either polyclonal rabbit anti-salmonid HSP70 (1:5000 dilution) or polyclonal rabbit anti-trout HSC70 (1:3000 dilution).

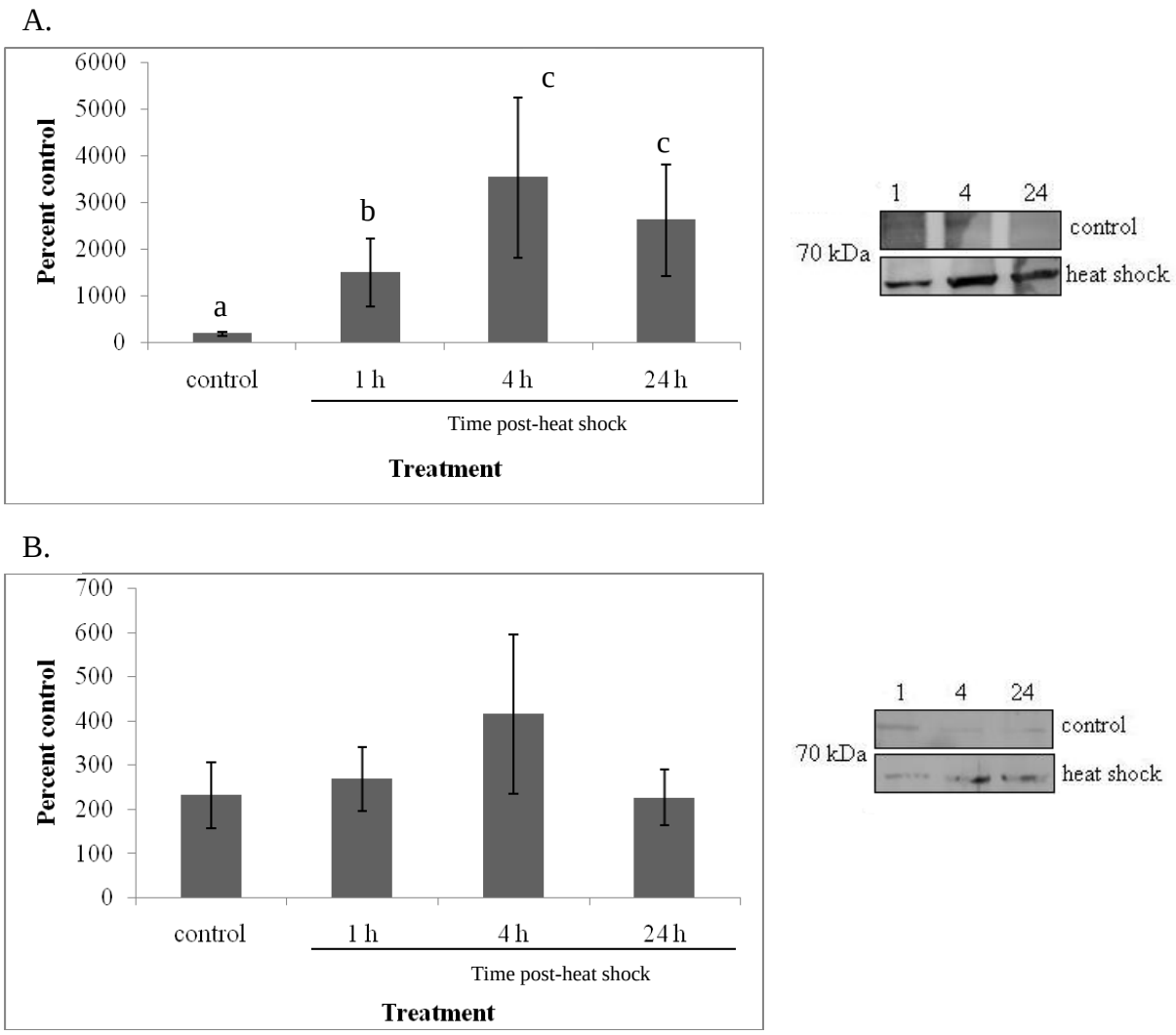
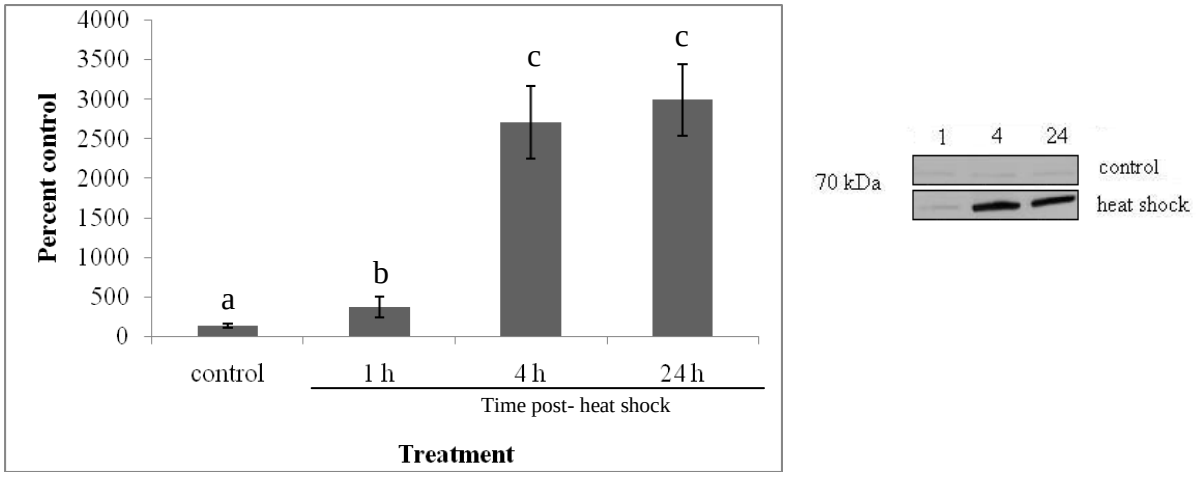


Figure 2.4: Gill HSP70 (A) and HSC70 (B) protein expressions in rainbow trout in response to heat shock. Brain samples were collected from trout exposed to a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. Values represent means ± SEM (n = 6). Bars with different letters (a, b) are significantly different ($p<0.05$, one-way ANOVA). A representative western blot image for HSP70 and HSC70 is shown beside their respective histograms; the blots was probed with either polyclonal rabbit anti-salmonid HSP70 (1:5000 dilution) or polyclonal rabbit anti-trout HSC70 (1:3000 dilution).

A.



B.

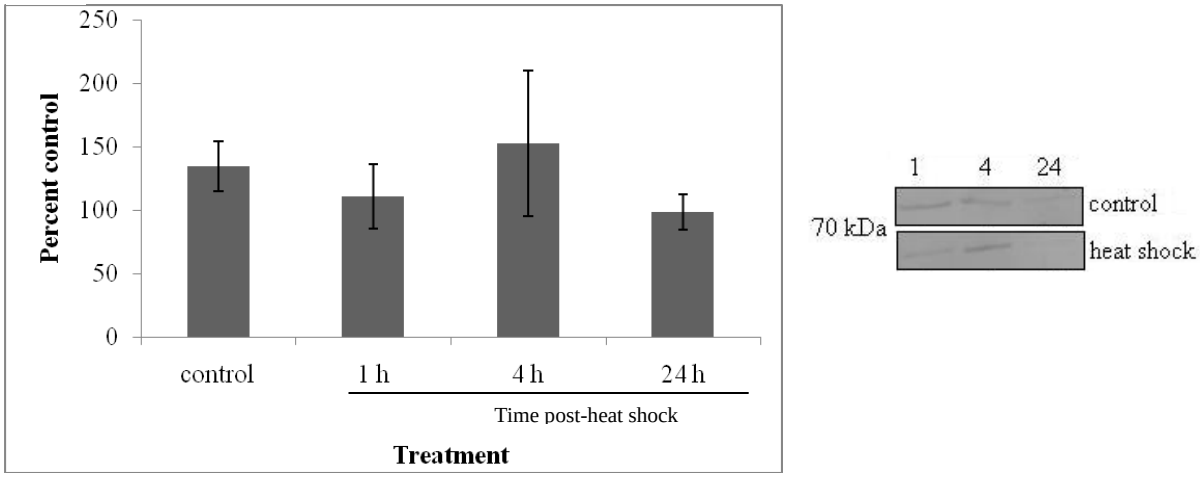


Figure 2.5: Red blood cell HSP70 (A) and HSC70 (B) protein expressions in rainbow trout in response to heat shock. Brain samples were collected from trout exposed to a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. Values represent means ± SEM (n = 6). Bars with different letters (a, b) are significantly different ($p<0.05$, one-way ANOVA). A representative western blot image for HSP70 and HSC70 is shown beside their respective histograms; the blots was probed with either polyclonal rabbit anti-salmonid HSP70 (1:5000 dilution) or polyclonal rabbit anti-trout HSC70 (1:3000 dilution).

2.4 Discussion

Heat shock elicited an organismal and cellular stress response in rainbow trout. The organismal stress response was evident by the elevated plasma cortisol, lactate and glucose levels, key players in allowing an animal to metabolically cope with stress (Iwama et al., 2006). The elevated plasma cortisol levels also seen in the non-heat shocked fish at 1 h were due to the stress associated with transfer and confinement of fish (Iwama et al., 2006). However, the plasma cortisol levels dropped to resting levels in the non-heat shocked controls over a 24 h period but not in the heat shocked group. The maintenance of elevated plasma cortisol levels in the heat shock group would account for the elevated plasma glucose levels seen in the heat shocked fish. This is because it is well established that cortisol is a key stimulator of gluconeogenesis in fish (Mommsen et al., 1999). The organismal stress response elicited by the fish may be a playing a role in metabolically adjusting the animals to the enhanced energy demand associated with temperature elevation in fish (Iwama et al., 2006).

This study demonstrated that HSP70 expression in RBCs reflects the cellular heat shock response seen in other key tissues in rainbow trout. All tissues showed a significant increase in HSP70 expressions with heat shock. Brain and liver showed no increase at 1 h but was seventy fold higher at 4 h post-heat shock. This HSP70 expression was maintained over 24 h post-heat shock. Gill and RBCs showed a rapid induction in HSP70 expression at 1 h and this continued to increase and was significantly higher at 4 and 24 h after heat shock. The rapid induction seen in the gills and RBCs in response to heat shock may reflect their close

contact with the external environment, allowing them to sense the changes prior to internal tissues, including liver and brain.

Dyer and associates (1991) found that HSP70 levels in brain increased with increasing temperatures when heat shocked fathead minnow (*Pimephales promelas*) were examined. It was found however, that brain does synthesize much less protein than other tissue but produces more HSP70 than any other HSP when exposed to elevated temperatures (Dyer et al., 1991).The increase of HSP70 in heat shocked liver has been well documented in a number of different species at the tissue level (Koban et al., 1991; Forsyth et al., 1997) and the cellular level (Koban et al., 1987; Vijayan et al., 1997). Dyer (1991) found that gill synthesized the largest amount of HSPs in response to heat shock when compared to other tissues and found HSP70 was increased up until 34ºC. The rapid increase in gill HSP70 levels following heat shock may be due to the direct exposure to heat, unlike the interior organs which will be affected more slowly. Currie in two separate studies (1997, 1999) showed that HSP70 levels in rainbow trout RBC increase significantly with exposure to heat shock. The increase in HSP70 shortly after exposure to heat shock may occur for reason similar to that of gill induction, as blood may be one of the first tissues to be affected because of its close proximity to the external environment as it traverses the gills.

HSC70 levels were not significantly altered in any of the studied tissues. As this is the constitutive isoform of the HSP70, this result is reasonable. As well, this has been confirmed in a number of fish studies. Rainbow trout hepatocytes exposed to both cadmium or heat shocks showed no change in HSC70 levels (Boone & Vijayan, 2002a). Heat shocked top minnow hepatocytes HSC70 levels did not change either (White et al., 1994; Norris et al.,

1995). Cell lines treated with heat shock (rainbow trout gonadal (RTG) cells) or heavy metals (Chinook salmon embryo (CHSE) cells) also did not have altered levels of HSC70 (Zarafullah et al., 1992). Currie et al. (1999) exposed rainbow trout RBCs to azide, hypoxia and zinc and did not find any change in HSC70 expression. A number of studies have shown that HSC70 levels will increase in response to stressor exposure. However, the majority of these studies have been conducted using mammalian tissues. Mammalian brain samples have shown to increase HSC70 in response to cadmium (rat, Hung et al., 1998), electric shock (mouse, Kaneko et al., 1993) and ischemia (gerbil, Kawagoe et al., 1993). Rat kidney cells have also shown an elevation in HSC70 levels in response to heat shock (Sakakibra et al., 1992). A few studies on fish, however, have revealed increases in HSC70 mRNA in response to heat shock (zebrafish (*Danio rerio*) embryo, Santacruz et al., 1997; medaka (*Oryzias latipes*) cell lines, Aria et al., 1995). One study using a human cell line, IB3-1, treated with sodium 4-phenylbutyrate showed a decrease in HSC70 (Rubenstein & Zeitlin, 2000). Overall, unlike HSC70 expression, HSP70 expression in tissues is sensitive to stressor exposure in fish (Deanne & Woo, 2010). RBCs and gill show an early temporal response to the elevated temperature by the induction of HSP70 in comparison to other tissues, including brain and liver. This temporal difference may reflect their access to the external environment, as RBCs and gill are in close contact with the aquatic environment relative to the brain and liver.

2.5 Conclusion

The heat shocked fish showed an organismal stress response, including elevated plasma cortisol, glucose and lactate levels. The HSP70 expression was higher in all tissues tested over a 24 h period after heat shock, supporting the activation of the cellular stress response. The expression of HSP70 in response to heat shock showed temporal differences among tissues. The tissues with close contact to the external environment, including gill and RBCs, showed an early heat shock response relative to the liver and brain. Overall, the rapidity in HSP70 response and the sustained elevation over a 24 h period demonstrate the usefulness of RBC HSP70 expression as a non-lethal biomarker of cellular stress effect in fish.

Chapter 3
Development of an Enzyme Linked Immunosorbent Assay (ELISA) for HSP70 Measurement in Rainbow Trout Red Blood Cells

3.1 Introduction

Heat shock proteins (HSPs) are a collection of highly conserved, ubiquitous proteins involved in all aspects of cell function and essential for defending against cellular stress (Kregel, 2002). These proteins are induced by a variety of stressors, including hyperthermia (Burdon et al., 1982; Parsell & Lindquist, 1993; Flanagan et al., 1995; Kregel et al., 1995; Skidmore et al., 1995; Kregel & Moseley, 1996), and chemicals (Parsell & Lindquist, 1993). HSPs are categorized into families depending on their molecular mass and range from 110 kDa (HSP110) down to proteins less than 20 kDa, known as the small HSP family (sHSPs). The HSP70 family is the most widely studied as they are sensitive to thermal shock and also the most highly conserved of the HSPs (Kregel, 2002). The two main members of this family are the inducible HSP70 or HSP72 and the constitutively expressed HSC70 or HSP73 (Pockley, 2001). While inducible HSP70 can be found at low levels in cytoplasm at normal growth temperature, the exposure to a heat shock causes a rapid increase within the nucleus and nucleolus (Ulrich Hartl, 1996; Welch & Feramisco, 1984). HSP70 functions during periods of stress on the organism, increasing cell survival by reducing protein aggregation, refolding of denatured proteins, and chaperoning of proteins (Ulrich Hartl, 1996).

Unlike mammals, fish possess nucleated red blood cells (RBCs), allowing high levels of aerobic metabolism (Boutilier & Ferguson, 1989), which in turn, allows for more cellular

processes to occur, including protein synthesis (Speckner et al., 1989). In the previous chapter, it was shown that rainbow trout (*Oncorhynchus mykiss*) RBCs increase the expression of heat shock protein when subjected to elevated temperatures, with significant increases at 4 and 24 h post heat-shock. This supports the finding that RBCs are capable of synthesizing HSP70 protein when faced with a heat shock of 11 – 15 ºC above ambient temperature (Currie & Tufts, 1997). To date, the effects of stress exposure on HSP70 expression in RBCs has focused only on exposure to acute stressors (Currie & Tufts, 1997; Currie et al., 1999). Very little is known about HSP70 expression in RBCs in response to chronic stressors.

Studies suggest that HSP70 response to acute and chronic stressor exposures may differ. For instance, acute cadmium exposure induced significant increases in HSP70 expression in rainbow trout hepatocytes and in cell lines (Boone & Vijayan, 2002a; Misra et al, 1989), while there was no significant effect associated with chronic cadmium exposure (1.5 µg/L for 21 days) in trout liver (Ait-Aissa et al., 2003).

Very seldom are chemicals found in isolation in the environment. Municipal waste water effluent (MWWE), widely distributed in the aquatic environment, contains an assortment of contaminants that may vary from region to region depending on the treatment process. MWWEs may contain human, industrial, commercial and institutional wastes, which in turn can contain a variety of components, including pharmaceuticals, pathogens, household cleaners, metals, fertilizers and other chemicals (CCME, 2006). However, no study has documented the impact of chronic exposure to MWWEs on HSPs expression in fish.

Because of the ability to take repeated blood samples without sacrificing the animal, HSP70 expression in RBCs have the potential to be used as a non-lethal marker of cellular stress in fish. However, to be used as a reliable marker of cellular stress response, it is important to accurately measure HSP70 concentration in RBCs. The majority of studies in fish have used SDS PAGE and western blotting as a method for analyzing HSP70 expression. While western blotting is a well established technique for the detection of specific protein expression in tissues, it is at best semi-quantitative. An enzyme linked immunosorbent assay (ELISA) is a sensitive technique for measuring specific protein concentration by immunodetection. There are no commercially available ELISAs for the measurement of HSP70 levels in fish. Therefore, the primary objective was to develop an ELISA for quantifying HSP70 concentration in trout RBCs. The second objective was to assess whether RBC HSP70 levels were elevated in response to acute and chronic stressors. Recovery from heat shock (see chapter 2) over a 24 h period was the acute stressor, while MWWE and cadmium exposures over a 14 or 28 d period, respectively, were used as a tool to assess HSP70 expressions in response to chronic stressors (long term exposures).

3.2 Materials and Methods

3.2.1 Animals

Juvenile rainbow trout (average body mass of 125 g), used for the heat shock study conducted at the University of Waterloo (Waterloo, Ontario), were obtained from Alma Aquaculture Research Station (Alma, Ontario) and were acclimated for at least two weeks prior to any experimentation in 200 L aerated tanks with constant water flow at 13°C. Fish

were maintained on a 12 h light/dark photoperiod and fed once daily to satiety (3 point sinking food; Martin Mills Inc., Elmira, Ontario).

The cadmium exposure study was carried out at the Wilfrid Laurier University (Waterloo, Ontario) and juvenile trout (approximate body mass of 20 g) were purchased from Rainbow Springs Trout Farm (Thamesford, Ontario). Fish were acclimated and maintained in well water at 11°C in two 200 L polyethylene tanks with constant flow (700 mL/min) and aeration under flow-through conditions with a 12 h light/dark photoperiod. Fish were fed Nutra ST 1.0 salmonid feed (Skettring Fish Food, Vancouver, BC) once daily at 2% body weight.

Juvenile rainbow trout (average body mass of 23 g) were purchased from Silvercreek Aquaculture Ltd (Erin, Ontario) for the MWWEs study at the University of Guelph (Guelph, Ontario) and acclimated for one week prior to exposure. Fish were maintained at 13ºC in 34 L aquaria, with tank water changed with fresh well water every 48 h on a 12 h light/dark photoperiod. Fish were not fed for the duration of the exposure.

All experiments conducted were approved by the Animal Care and Use committee of the respective universities and is in accordance with guidelines established by the Canadian Council for Animal Care.

3.2.2 Experimental Design

3.2.2.1 Heat shock study:

Groups of 12 fish each were separated into four 100 L tanks in a static system using well water. Water in two tanks was heated to a temperature of approximately 25°C, while two tanks were maintained at ambient temperature (13°C). Fish were subjected to the +12°C

heat shock above ambient temperature for one hour. Following the heat shock, the immersion heaters were removed and the temperature brought back to 13°C within 15 minutes. Six fish from the heat shocked and non-heat shocked tanks were sampled immediately following heat shock and at 4 and 24 h after the onset of heat shock. Fish were quickly netted and euthanized with an overdose of 2-phenoxy ethanol (1 mL/L well water; Sigma). Blood was collected into tubes, containing ethylenediaminetetraacetic acid (EDTA; 5 mg in Hank's medium; Fischer Scientific). Blood samples were spun for five minutes at 3000 X g and separated into RBCs and plasma. Samples were stored at -80°C for further analysis.

3.2.2.2 Cadmium Exposure:

This exposure was performed at Wilfrid Laurier University. Six 200 L polyethylene tanks with flow through systems (700 mL/min) and continuous aeration from common 60 L polyethylene mixed head tank were set up. Fish (420 total) were equally distributed into tanks and subjected to one of three experimental conditions (in duplicate): hard water control (pH 7.2; hardness of 140mg $CaCO_3$/L; 11°C); low cadmium dose (0.75 µg/L) and high cadmium dose (2.0 µg/L). Cadmium (acidified stock solution in $CdCl_2$ form) was delivered via three smaller 11.2 L polypropylene split head tanks via Q-pumps. Split head tanks received water from the common mixed head tank. Actual cadmium concentrations in the water samples were determined by graphite furnace atomic absorption spectrometry (GFAAS) (Milne & McGeer, unpublished data, 2010). The cadmium level in the control group was below detection, while it was 0.85 ± 0.23 µg Cd/L and 2.08 ± 0.55 µg Cd/L in the low and high cadmium groups, respectively.

Fish were acclimated for two week and fed Nutra ST 1.0 salmonid feed once daily at 2% body weight. Food was withheld 24 h prior to sampling which occurred at 1, 7 and 28 d post–cadmium exposure. Four fish per tank (eight fish per treatment per time point) were quickly netted and euthanized with an overdose of tricain methanesulfonate (MS222; 1:3 with $NaHCO_3$; Synde Laboratories Ltd). Blood was collected with heparin and spun for five minutes at 3000 X g. Samples were subsequently separated into RBCs and plasma and stored frozen at -80°C for further analysis.

3.2.2.3 Municipal Waste Water Effluent Exposure:

Experiment was carried out at the University of Guelph Aqualab Facility (Guelph, Ontario). Fish were maintained in 34 L aquaria in 0% (control), 20% (low dose) or 90% (high dose) municipal waste water effluent (MWWE) collected from Guelph sewage treatment plant. Tanks contained a mixture of MWWE and well water maintained at 13°C. Water was changed every 48 h with fresh effluent collected from Guelph sewage treatment plant. In order to minimize unnecessary stress on the fish, duplicate tanks were set up. Fish were transferred to the second tank every 48 h which contained a fresh water supply. Three tanks per dose (18 tanks overall, including duplicates) were set up, containing six fish per tank. Fish were not fed throughout the duration of the exposure and were sampled at 2, 8 and 14 d post-MWWE exposure. Two fish per tank (6 in total per treatment) were collected and given a lethal dose of MS222 (0.6 g/L; Sigma). Blood was collected via caudal puncture with heparin-coated (10 mg/L; Sigma) syringe and needle and spun for 5 minutes at 3000 X g. Samples were separated into plasma and RBCs, which were frozen on dry ice and stored at -80°C at the University of Waterloo for further analysis.

3.3 Sample Analysis

3.3.1 Antibody-Capture Competitive HSP70 ELISA

ELISA protocol was adapted from established protocols according to Specker and Anderson (1994). An EIA/RIA medium binding 96 well plate (Costar) was coated with 200 uL chinook salmon HSP70 recombinant protein (100 ng/mL; Stressgen) in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6). Standards consisted of a serial dilution of the same HSP70 recombinant protein in dilution buffer (0.1 M phosphate buffered saline, pH 7.4, 0.05% Tween 20). Polyclonal rabbit anti-salmon HSP70 primary antibody (1:20000; StressMarq) was added in 1:1 ratio to each standard. Samples were also prepared to analyze non-specific binding and background absorbances. RBC samples were diluted (10x) in dilution buffer (0.1 M PBS; pH 7.4; 0.05% Tween 20) and primary antibody was added at a 1:1 ratio. Coated ELISA plate, standards and samples were incubated overnight at 4°C with constant shaking. Following incubation, the ELISA plate was washed 4 X with TTBS (20 mM Tris, pH 7.4, 300 mM NaCl, 0.05% Tween 20). A blocking solution (1% bovine serum albumin in dilution buffer; Fisher Scientific) was added to each well (200 uL/well) and plate was incubated one hour at room temperature with constant shaking. Blocking solution was then discarded and unknown samples and standards were added to the ELISA plate at 200 uL/well and incubated for 2 h at room temperature with constant shaking. After incubation, the contents were discarded and the plate was washed 4 X with TTBS. Horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000; BioRad) diluted in 5% skim milk was added to each well at 200 uL/well. The plate was incubated for 2 h at room temperature, with constant shaking, after which the contents were discarded and the plate

was washed 4 times with TTBS. Enzyme substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (41 mM), was added to wells at 200 uL/well and after 10 min the plate was read at 655 nm using a microplate reader (VersaMax, Molecular Devices, CA). Intra- and interassay comparisons were made by calculating the coefficient of variance (CV) of standard curves and comparing between plates and within plates. Within a plate, CVs were calculated between standard replicates the mean was taken to determine intra-assay variation. For interassay variation, mean CVs from each plate run were compared.

3.3.2 SDS-PAGE and Western Blotting (for ELISA validation)

SDS-Page and western blotting was used to determine the expression of inducible HPS70 in RBC samples following established protocols (Boone & Vijayan, 2002b). The protein concentration in the cell lysate was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) using bovine serum albumin as the standard. Sample proteins (40 ug) were separated on 8% polyacrylamide gels using discontinuous buffer system of Laemmli (Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane (BioRad, 0.45 mm pore size) (20 V for 25 minutes) with semidry transfer unit (BioRad) in 10% transfer buffer (25 mM Tris pH 8.3, 192 mM glycine and 10% (v/v) methanol). Membranes were blocked with 5% skim milk in TTBS (20 mM Tris pH 7.5, 300 mM NaCl, 0.1% (v/v) Tween 20 with 0.05% sodium azide) for one hour and subsequently probed with primary antibody, a polyclonal rabbit anti-salmonid HSP70 (1:5000; StressMarq), for one hour at room temperature. Following primary antibody probing, membranes were washed 3x10 min with TTBS and probed with horseradish peroxidase conjugated goat anti-rabbit (1:3000; BioRad) secondary antibody for one hour at room temperature. Membranes were washed

3x10 min with TTBS and washed 1x10 min with TBS. Bands were revealed with ECL plus Western Blotting Detection System (GE Healthcare, Amersham) and scanned using a Typhoon scanner. The molecular mass of target proteins was confirmed using Precision Plus Protein markers (BioRad). Band intensities were quantified with AlphaEase software (Alpha Innotech, CA). HSP70 expression validation was based on data shown in Fig. 2.5A in chapter 2.

3.3.3 Statistical Analysis

Data was log transformed and statistically compared using two-way analysis of variance (ANOVA). Significant differences were compared using the Holm-Sidak method with the level of significance for all tests set at $p<0.05$. Results are presented as mean ± standard error of mean (SEM) for all groups.

3.4 Results

3.4.1 ELISA validation

With increasing concentration of HSP70 in the standard samples, a decrease in percent bound was seen (Fig. 3.1). When concentrations were log transformed and plotted on a semi-log graph, a linear curve was generated (Fig. 3.1 inset). The validity of the ELISA was determined by comparing inter-assay and intra-assay coefficients of variance (CV; see appendix). The inter-assay CV was determined to be 26 ± 4% (SEM), while the intra-assay CV was 11 ± 2% (SEM).

The ELISA antibody specificity was confirmed by western blotting. The antibody detected a single 70 kDa protein that was clearly over expressed in the heat shocked cells

compared to the control RBCs (Fig. 3.2). The HSP70 expression in the control cells were noticeably lower compared to the heat shocked groups and did not change over the 24 h period (Fig. 3.2). The HSP70 expressions in the heat shocked cells were higher at 4 and 24 h compared to 1 h post-heat shock exposure.

3.4.2 Heat shock exposure:

An acute heat shock exposure significantly elevated HSP70 concentration in trout RBCs at 4 and 24 h after heat shock compared to the non-heat shocked control groups (Fig. 3.3). There was no time effect on RBC HSP70 levels post heat shock exposure (Fig. 3.3). This data obtained with ELISA is in agreement with the RBC HSP70 expression data in response to heat shock obtained with western blotting (see Chapter 2, Fig. 2.5A).

3.4.3 Chronic Sublethal Cadmium Exposure:

Chronic exposure to sublethal water-borne cadmium exposure (low dose 0.75 µg/L; high dose 2.0 µg/L) had no significant effect on HSP70 concentration in trout RBCs over a 28 d period (Fig. 3.4). Neither cadmium concentration nor duration of exposure (1, 7 and 28 d) significantly impacted trout RBC HSP70 levels (Fig. 3.4).

3.4.4 Chronic MWWE Exposure:

Chronic exposure to varying concentration of MWWE (low – 20% MWWE; high – 90% MWWE) over a 14 d period did not significantly affect RBC HSP70 concentration (Fig. 3.5). There was neither a treatment (MWWE concentrations) nor a time (2, 8 and 14 d) effect on HSP70 levels in trout RBCs (Fig. 3.5).

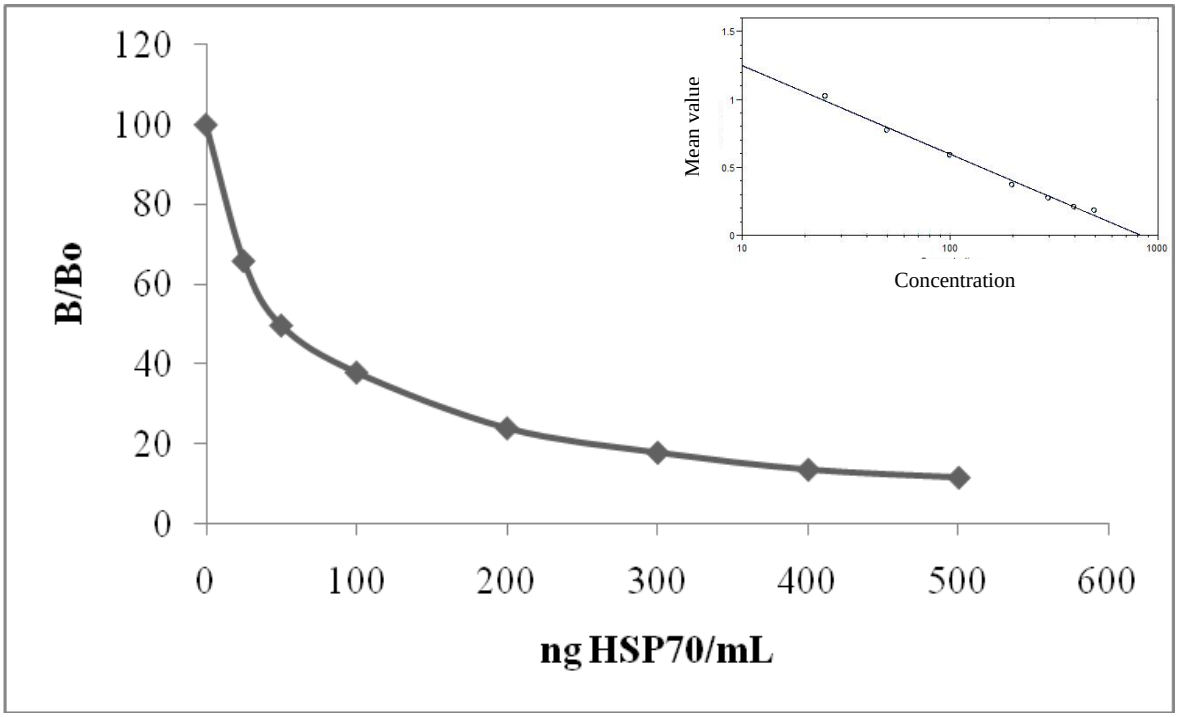


Figure 3.1: HSP70 standard curve using the ELISA. A serial dilution of recombinant chinook salmon HSP70 protein. The B/Bo (percent bound) values are plotted along the y-axis along with their corresponding salmon HSP70 concentration (ng/ml) on the x-axis. Inset shows a semi-log plot with absorbance on the y-axis and HSP70 (ng/ml) on the x-axis.

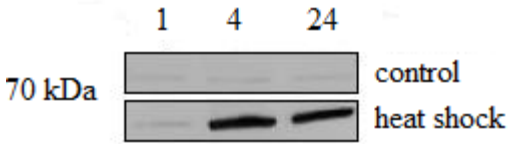


Figure 3.2: Representative western blots of red blood cells HSP70 expression in rainbow trout exposed to heat shock. RBCs were collected from trout exposed to a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 1, 4, and 24 h post-heat shock. The blot was probed with an antibody specific for inducible HSP70 (polyclonal rabbit anti-trout HSP70; 1:5000 dilution).

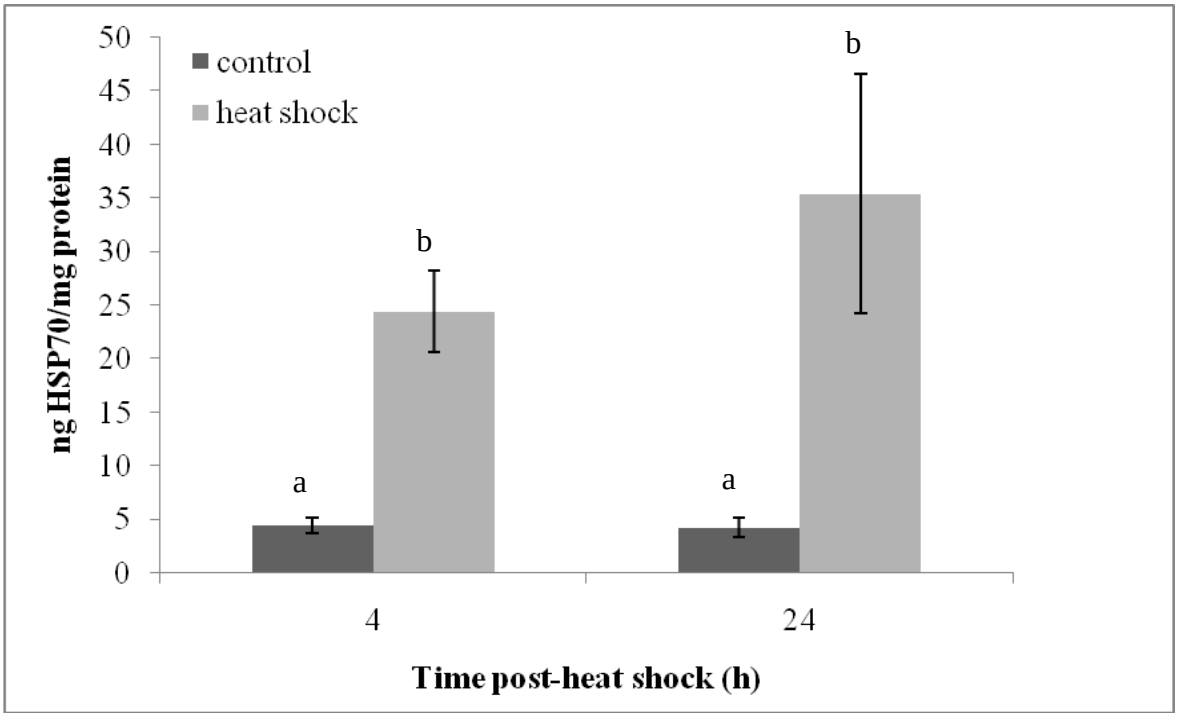


Figure 3.3: HSP70 concentrations in rainbow trout red blood cells exposed to heat shock. RBCs were collected from trout exposed to either no heat shock (control) or a one hour heat shock (+12ºC above ambient temperature) and then allowed to recover at ambient temperature (13ºC). Samples were collected at 4, and 24 h post-heat shock. HSP70 concentration in the samples was analyzed using a capture ELISA. Values represent means ± SEM (n = 6). Bars with different letters are significantly different ($p<0.05$, two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 (1:20000 dilution) was used.

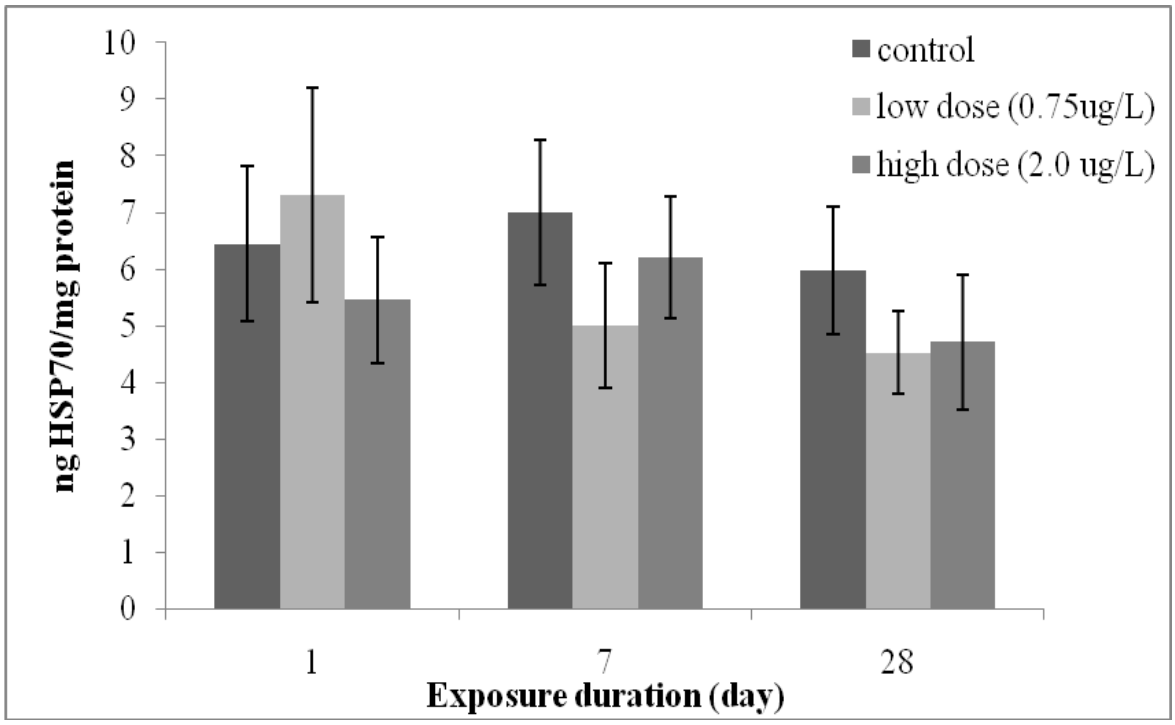


Figure 3.4: HSP70 concentrations in rainbow trout red blood cells exposed to chronic sublethal concentrations of cadmium. Trout were exposed to a control, low dose (0.75 µg/L) and high dose (2.0 µg/L) of cadmium in a flow-through system for a total of 28 days. Fish were sampled at 1, 7 and 28 d post-exposure. RBC samples were analyzed using HSP70 capture ELISA. Values represent mean ± SEM (n = 6; two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

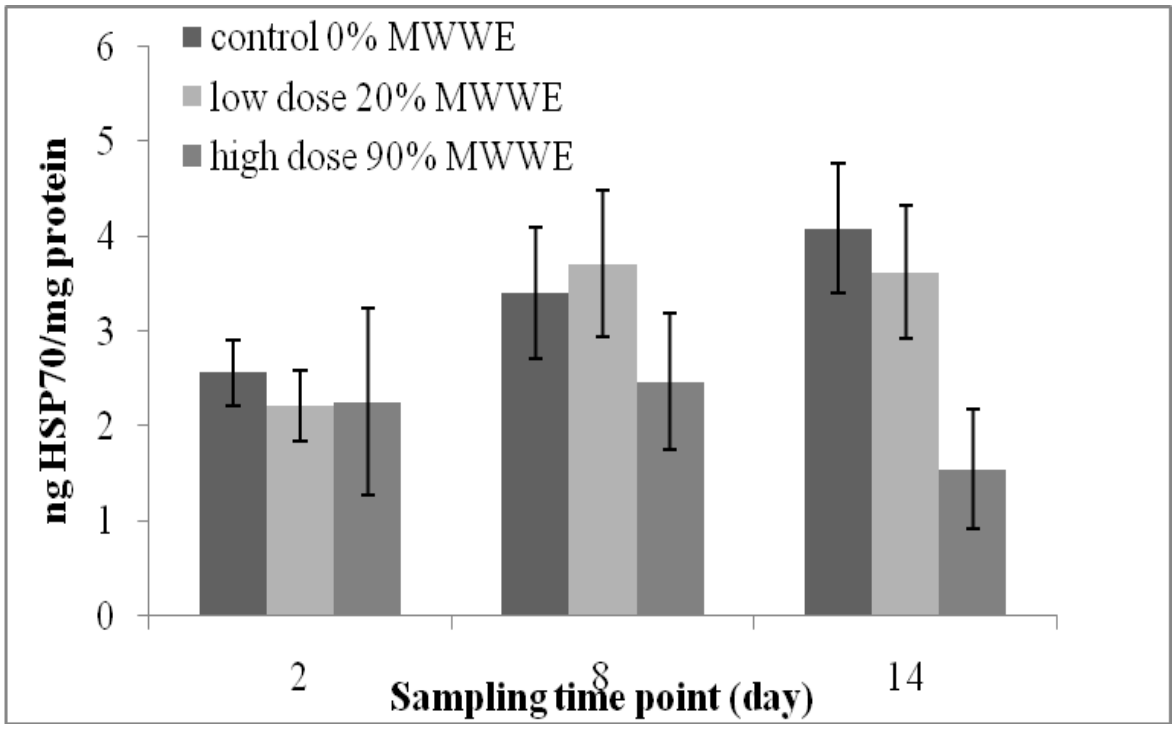


Figure 3.5: HSP70 concentrations in rainbow trout red blood cells exposed to chronic sublethal concentrations of municipal wastewater effluent. Trout were exposed to a control, low dose (20%) and high dose (90%) of municipal wastewater effluent (MWWE) in a static system for a total of 14 days. Fish were sampled at 2, 8 and 14 d post-exposure onset. RBC samples were analyzed using HSP70 capture ELISA. Values represent mean ± SEM (n = 5; two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

3.5 Discussion

A competitive antibody-capture ELISA that specifically detects inducible HSP70 concentration in trout RBCs was developed. ELISAs have been used previously in mammalian studies (Skidmore et al., 1995), but no commercial kit is available for use in fish studies. However, a few researchers have reported the development of semi-quantitative ELISAs in order to increase the specificity of HSP70 detection (Vijayan et al., 1997; De Boeck et al., 2003; Parmini & Rani, 2008). Previously, quantification of these proteins has been carried out using a variety of methods including, western blotting, dot blotting, radioimmunoassay (RIA) and ELISA. Western blotting has been the most widely used methodology for the majority of studies involving mammalian and fish species (Burdon et al., 1982; Welch & Feramisco, 1984; Flanagan et al., 1995; Kregel et al., 1995; Polla et al., 1995; Kregel & Moseley, 1996; Currie & Tufts, 1997; Currie et al., 1999; Schroder et al., 1999; Boone & Vijayan, 2002a). While the wide spread use of western blotting lends to its usefulness in measuring stress protein levels, it is at best semi-quantitative and can give only a general representation of the HSPs changes that are occurring in the organism or tissue in response to stressor exposure. ELISA is a sensitive method, especially for quantification of proteins that are in low abundance in cells.

In the present study, the use of a salmonid HSP70 antibody and a standard made of recombinant salmon HSP70 protein ensured specificity to the species of interest. Antibody specificity for the inducible form was also confirmed by western blotting. The ELISA results showing a significant increase in RBC HSP70 concentrations at 4 and 24 h post-heat shock

clearly supports similar results obtained earlier with western blotting (see Chapter 2, Fig. 2.5A).

This is in agreement with other studies showing an elevation in HSP70 expression and protein synthesis in rainbow trout RBCs following a heat shock (Currie & Tufts, 1997; Currie et al., 1999). The increase in HSP70 levels may have a protective role in defending the cells against proteotoxicity associated with heat shock (Hightower, 1991; Iwama et al., 2006; Deanne & Woo, 2010). Consequently, tissue HSP70 level is a sensitive indicator of cellular effects associated with stressor exposure, including contaminant exposure in fish (Iwama et al., 2006; Deanne & Woo, 2010). The finding that RBC HSP70 expression reflects this protein expression in other tissues, including liver, gill and brain, underscores the utility of using blood samples as a non-lethal means of detecting the cellular stress in fish (see Chapter 2). This has particular importance as it allows for repeated sampling of blood from the same animal as well as eliminating the need to sacrifice the animal to collect other tissues to determine the cellular stress response.

While the RBC HSP70 levels were modulated by an acute stressor, this was not the case in response to long term exposures to cadmium and MWWEs. Exposure to sublethal cadmium concentrations have been shown to induce HSP70 expression in fish. This has been shown in primary cultures of hepatocytes of salmon (*Salmo salar L*;1-100 µM for 24 h; Grosvik & Goksoyr, 1996), emerald rock cod (*Trematomus bernacchii*; 100 µM for 24 h; Buckley et al., 2004) and rainbow trout (hsp70 transcripts; 0.1 µM for 1 h; Boone & Vijayan, 2002a), PLHC-1 and renal cell lines (10 µg/mL for 24 h and 6 µg/mL for 1 h, respectively; Ryan & Hightower, 1994), black sea bream fibrobasts (*Spondyliosoma cantharus*; 0.01 µM

for 4 h; Deane & Woo, 2006) and silver sea bream (*Sparus sarba*) RBCs (0.1 µM for 1 h; Fulladosa et al., 2006). Despite the evidence that cadmium exposure will induce an increase in HSP70 expression in fish, no change was seen in the current study. This could be for a variety of reasons. With the exception of one study (Deane & Woo, 2006), all previous studies have used concentrations of cadmium far greater than those used in this study. It may be that cadmium will not elicit a heat shock response at low concentrations. Tissue-specific responses to a stressor have been previously demonstrated (chapter 2). While previous studies have shown increases in HSP70 expression in response to cadmium, these levels were examined in cell lines and liver tissue. Fulladosa et al. (2006) did shown that RBC HSP70 expression does increase with cadmium exposure, but concentrations used in their study were at least tenfold greater than those used in the current study. Another explanation for lack of change in expression levels may be due to a transient increase in HSP70 expression within the first 24 h. Sampling occurred after 1 d of exposure, it may be possible that the low concentrations of cadmium elicited a response that occurred and ended prior to sampling.

Exposure to MWWE induced a number of physiological changes in the fish (Ings, Servos & Vijayan, unpublished data), but no changes were seen in levels of HSP70 expression. Wastewater effluents contain a number of contaminants including industrial and residential wastes. A portion of contaminants found in wastewater are pharmaceuticals. Non-steroidal anti-inflammatory drugs (NSAIDS) have been shown to modulate the heat shock response. Ibuprofen has been shown to decrease HSP70 expression in liver and gill of rainbow trout exposed first to the drug and then subjected to a one hour +10ºC above ambient temperature heat shock, while salicylate caused a delay in the heat shock response, with

significant increases in HSP70 expression occurring only after 4 h post-heat shock (Gravel & Vijayan, 2007). However, this study used high concentrations of pharmaceuticals relative to the concentration detected in MWWEs. A recent study showed no significant change in liver HSP70 expression in trout exposed to MWWE over a 14 d period (Ings, Servos & Vijayan, unpublished data). It remains to be seen whether chronic exposure to MWWE will impact the heat shock-induced HSP70 levels in fish as seen with exposure to ibuprofen and salicylate (Gravel & Vijayan, 2007)., suggesting disruption in the cellular heat shock performance in fish.

3.6 Conclusion

An ELISA for measuring HSP70 levels in salmonid RBCs was developed. The RBCs HSP70 levels appear to be a sensitive indicator of acute heat stress, but not chronic stress due to either cadmium or MWWEs exposures in trout. The HSP70 levels in RBCs have the potential as a non-lethal marker of acute cellular stress effect in trout.

Chapter 4
Modulation of HSP70 Levels in Rainbow Trout Red Blood Cells by Stress Hormones

4.1 Introduction

When an organism encounters a stressor, a number of physiological processes are initiated to help re-establish homeostasis. In general, these processes are termed the stress response (Wendelaar Bonga, 1997). The organismal response involves the release of catecholamines and glucocorticoids, while the cellular response involves not only the effect of these hormones on cells, but also the synthesis of stress proteins, commonly known as the HSPs (Deane & Woo, 2010). The most highly conserved family of HSPs is the HSP70 family (Kregel, 2002), with its two main members, HSP70, the inducible isoform, and HSC70, the constitutive isoform (Pockley, 2001). When an organism encounters a protein denaturing stressor, a rapid synthesis of HSP70 is seen (Ulrich Hartl, 1996; Welch & Feramisco, 1984). HSP70 functions to improve cell survival by reducing protein aggregation, refolding denatured proteins, and chaperoning proteins (Ulrich Hartl, 1996). The red blood cells (RBCs) of teleost fish are nucleated rendering the cell capable of protein synthesis (Speckner et al., 1989). While there is little information available on the regulation of HSP70 in RBCs, studies have shown an elevated HSP70 expression in response to heat stress (Currie & Tufts, 1997; chapters two, three). However, nothing is known about extracellular release of HSP70 in fish.

In mammals, the extracellular release of HSP70 (eHSP70) was first reported from glia cells and this was taken up by neighbouring neurons (Tytell et al., 1986; Tonkiss & Calderwood, 2005; Chen & Brown, 2007; Guzhova et al., 2001). However, the function of these HSPs is relatively unknown, but thought to be utilized by cells for chaperoning and protection against apoptosis (Calderwood, 2005). Recently, a number of mammalian studies have reported eHSP70 from a variety of tissues and cells, including brown fat following behavioural stress (Campisi & Fleshner, 2003), neuronal cells (Tytell et al., 1986), glial cells (Guzhova et al., 2001), β cells (Clayton et al., 2005), tumour cells (Gastpar et al., 2005) and human peripheral blood mononuclear cells (Lancaster & Febbraio, 2005). An increase in eHSP70 has been seen in blood following exposure to normal physiological states, including acute physical and/or psychological stress (Johnson & Fleshner, 2006). Also, mild heat shock in the fever range has been shown to induce the release of HSP70 into the extracellular environment (Mambula & Calderwood, 2006a). In addition, other pathological conditions, including renal disease (Wright et al., 2000), hypertension (Pockley, 2002) and atherosclerosis (Pockley et al., 2003) leads to chronically elevated eHSP70 levels in circulation.

The organismal stress response, a highly conserved adaptive response, involves the release of epinephrine and cortisol, two key stress hormones in animals. Catecholamines, including epinephrine and norepinephrine, are released rapidly into circulation in response to stressor exposure from chromaffin cells, located in the head kidney in fish (Wendelaar Bonga, 1997). Epinephrine and norepinephrine are key players in "fight or flight" response (Wendelaar Bonga, 1997), enhancing the blood oxygen transport and energy mobilization.

The elevated corticosteroid response to stressors involves the activation of the hypothalamus-pituitary-interrenal (HPI) axis. A stressor, acting on the hypothalamus, causes an increase in corticotrophin releasing factor (CRF), which in turn increases levels of adrenocorticotropic hormone (ACTH) in the pituitary. ACTH acts on the interrenal cells inducing an increase in cortisol biosynthesis (Mommsen et al., 1999). Cortisol signalling action occurs at the tissue level, particularly gill, liver and intestine, to bring about metabolic effects such as stimulation of gluconeogenesis and increasing liver glycogen content, causing proteolysis and modulating enzymes involved in glutamate processing (Mommsen et al., 1999) to restore homeostasis during stress. Cortisol release is regulated via a negative feedback at each level of the axis (Bradford et al., 1992; Mommsen et al., 1999).

Both the organismal response and the cellular response are vital to an organism's survival during periods of stress. While there has been much research into the effects of cortisol on HSP70 expression in various tissues and species (Barr & Dokas, 1999; Basu et al., 2001; Sathiyaa et al., 2001; Boone & Vijayan, 2003; De Boeck et al., 2003), very little is known about the effect of these stress hormones on the nucleated RBCs of fish. The objective of this study was to determine whether: *i*) rainbow trout (*Oncorhynchus mykiss*) RBC will release HSP70 into the extracellular environment, *ii*) stress hormones, cortisol and epinephrine regulate RBC HSP70 levels in trout, and *iii*) cortisol and epinephrine modify eHSP70 levels in trout.

4.2 Materials and Methods

4.2.1 Animals

Juvenile rainbow trout (body mass ranging between 300 – 600 g) used for experiments conducted at the University of Waterloo (Waterloo, Ontario) were obtained from Alma Aquaculture Research Station (Alma, Ontario) and were acclimated for at least two weeks prior to any experimentation in 200 L aerated tanks with constant water flow at 13°C. Fish were maintained on a 12 h light/dark photoperiod and fed once daily (3 part sinking food; Martin Mills Inc., Elmira, Ontario). All experiments conducted were approved by the University of Waterloo Animal Care and Use committee and are in accordance with guidelines established by the Canadian Council for Animal Care.

4.2.2 *In Vitro* RBC Heat Shock Experiment:

RBCs were obtained following protocol adapted from established methods (Currie & Tufts, 1997). Two fish per sample were netted and euthanized with an overdose of 2-phenoxyethanol (1 mL/1L well water; Sigma). Fish were bled by caudal severance into a sterile centrifuge tube containing 1 mL of physiological saline (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPOP_4, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM Hepes; pH 7.63 at room temperature; 1.5 mM $CaCl_2$, 5 mM glucose) and EDTA (10 mg/mL). After five minutes on ice, blood was diluted to approximately 20% hematocrit with physiological saline containing EDTA. Cells were plated at 500 uL/well into a 24 well culture plate (Grenier Bio-One, Germany) and were subjected to one of four treatments: control; 10 ng/mL cortisol; 100 ng/mL cortisol; or 10 nM epinephrine (all hormone treatments diluted in physiological

saline). One set of these treatments were subjected to a one hour heat shock (+15ºC above ambient temperature), while the other was maintained at 9ºC (non-heat shocked group). Both groups were gently shaken throughout the incubation period. The samples were collected immediately following heat shock (1 h) and at 4 and 24 h post-heat shock. Samples were spun for five minutes at 5000 x g and RBCs and supernatant were stored frozen at -70ºC for HSP70 analysis later.

4.2.3 Sample Analysis

4.2.3.1 Antibody-Capture Competitive HSP70 ELISA

The protein concentration in the cell lysate was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) using bovine serum albumin as the standard. ELISA protocol was adapted from established protocols according to Specker and Anderson (1994). An EIA/RIA medium binding 96 well plate (Costar) was coated with 200 uL Chinook salmon HSP70 recombinant protein (100 ng/mL; Stressgen) in a coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6). Standards consisted of a serial dilution of the same HSP70 recombinant protein in dilution buffer (0.1 M phosphate buffered saline, pH 7.4, 0.05% Tween 20). Polyclonal rabbit anti-salmon HSP70 primary antibody (1:20000; StressMarq) was added in 1:1 ratio to each standard. Samples were also prepared to analyze non-specific binding (no primary antibody) and background absorbance (blanks). RBC homogenate was diluted (10x) in dilution buffer (0.1 M PBS; pH 7.4; 0.05% Tween 20) and primary antibody was added at a 1:1 ratio. Coated ELISA plate, standards and samples were incubated overnight at 4°C with constant shaking. Following incubation, the ELISA plate

was washed 4 X with TTBS (20 mM Tris, pH 7.4, 300 mM NaCl, 0.05% Tween 20). A blocking solution (1% bovine serum albumin in dilution buffer; Fisher Scientific) was added to each well (200 uL/well) and plate was incubated for one hour at room temperature with constant shaking. Blocking solution was then discarded and unknown samples and standards were added to the ELISA plate at 200 uL/well and incubated for 2 h at room temperature with constant shaking. After incubation, the contents were discarded and the plate was washed 4 X with TTBS. Horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000; BioRad) diluted in 5% skim milk was added to each well at 200 uL/well. The plate was incubated for 2 h at room temperature, with constant shaking, after which the contents were discarded and the plate was washed 4 times with TTBS. Enzyme substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (41 mM), was added to wells at 200 uL/well and after 10 min the plate was read at 655 nm using a microplate reader (VersaMax, Molecular Devices, CA).

4.2.3.2 Lactate Dehydrogenase (LDH) Leakage

LDH leakage was determined by measuring the LDH content in the RBCs and medium. The assay consisted of an enzyme buffer [0.12 mM NADH in 50 mM immidazole buffer (pH 7.5)] and 25 mM pyruvate in 50 mM imidazole to start the reaction. Enzyme activity was measured, in duplicate, kinetically at 340 nm over a five minute period at room temperature using a microplate reader (VersaMax, Molecular Devices, CA). LDH leakage was calculated as percent LDH in the medium relative to total LDH activity (cells + medium).

4.2.4 Statistical Analysis

Data was log transformed and statistically compared using two-way analysis of variance (ANOVA). Significant differences were compared using the Holm-Sidak method with the level of significance for all tests set at $p<0.05$. Results are presented as mean ± standard error of mean (SEM) for all groups.

4.3 Results

4.3.1 HSP70 levels in heat shocked RBCs *in vitro*

RBCs subjected to a one hour heat shock showed a significant increase in HSP70 levels compared to the control cells at 24 h post-heat shock, but not at 1 or 4 h post-heat shock (Fig. 4.1). Levels of HSP70 were approximately 5 ng/mg protein in both control and heat shocked samples at 1 and 4 h post-heat shock. The heat shocked RBCs had ~11 ng/mg at 24 h compared to ~5 ng/mg in the control cells (Fig. 4.1). Western blot analysis of control and heat shocked samples from the 24 h group confirmed the ELISA results; there was higher expression of HSP70 in the heat shocked cells, while control cells showed very low expression (see representative blot).

4.3.2 HSP70 levels in hormone-treated and heat-shocked RBCs *in vitro*

RBCs were treated with stress hormones and then subjected to a heat shock. After 4 h post-heat shock levels of HSP70 ranged between 2.6 – 5.6 ng/mg protein, but there were no treatment effects on RBC HSP70 concentration (Fig. 4.2). After 24 h post-heat shock, a significant increase in HSP70 concentration was seen in the heat-shocked cells compared to

the control cells (Fig. 4.3). Neither cortisol nor epinephrine significantly altered the basal or heat shock-induced HSP70 levels in trout RBCs.

4.3.3 HSP70 levels in medium of heat-shocked RBCs *in vitro*

Levels of HSP70 released into the medium were on average 20% of that seen in cells. The medium HSP70 concentration was not significantly different between the control and heat-shocked cells at either 1 or 4 h post-heat shock exposure (Fig. 4.4). There was s significantly higher HSP70 concentration in the medium collected from heat-shocked cells relative to the control cells at 24 h post-heat shock exposure (Fig. 4.4).

4.3.4 HSP70 levels in medium of hormone-treated and heat-shocked RBCs *in vitro*

There was not effect of hormones on either basal or heat shock-mediated extracellular HSP70 levels in trout RBCs at 4 h post-heat shock exposure (Fig. 4.5). Hormone treatment also had no significant effect on unstimulated extracellular HSP70 levels at 24 h post-heat shock (Fig. 4.6). However, both cortisol and epinephrine attenuated the heat shock-mediated elevation in extracellular HSP70 levels in RBCs at 24 h post-heat shock exposure (Fig. 4.6).

4.3.5 Cell viability

Release of LDH into the medium was normalized to percent control. Levels of LDH release into the medium showed very little change over the 24 h sampling period in the control and heat-shocked groups (Fig. 4.7). Hormone treatment did not significantly modify the % LDH release into the medium in either the control or the heat-shocked cells (Fig. 4.8).

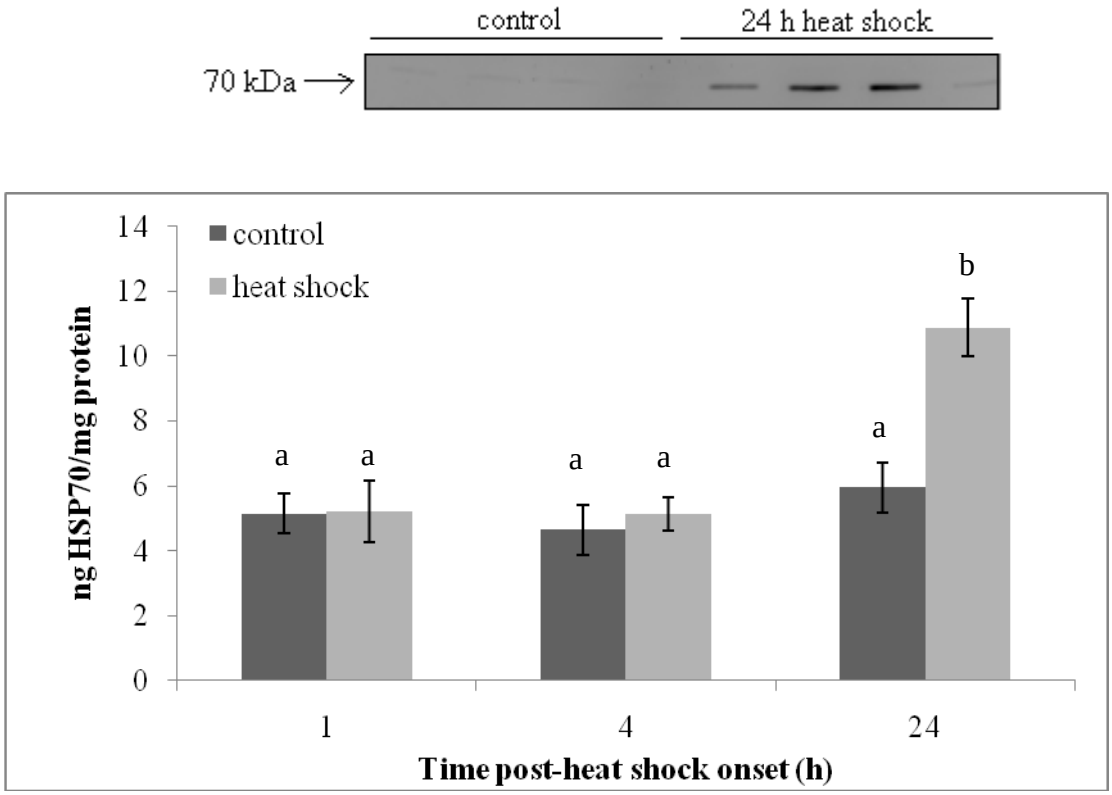


Figure 4.1: HSP70 levels of rainbow trout red blood cells subjected to heat shock *in vitro*. RBCs were plated and subjected to a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 1, 4 and 24 h post-heat shock. Values represent mean ± SEM (n = 6). Bars with different letters are significantly different ($p<0.05$, two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used. A representative western blot image of HSP70 expression in heat-shocked and control RBCs at 24 h is shown above the histogram; the blot was probed with polyclonal rabbit anti-salmonid HSP70 (1:5000 dilution).

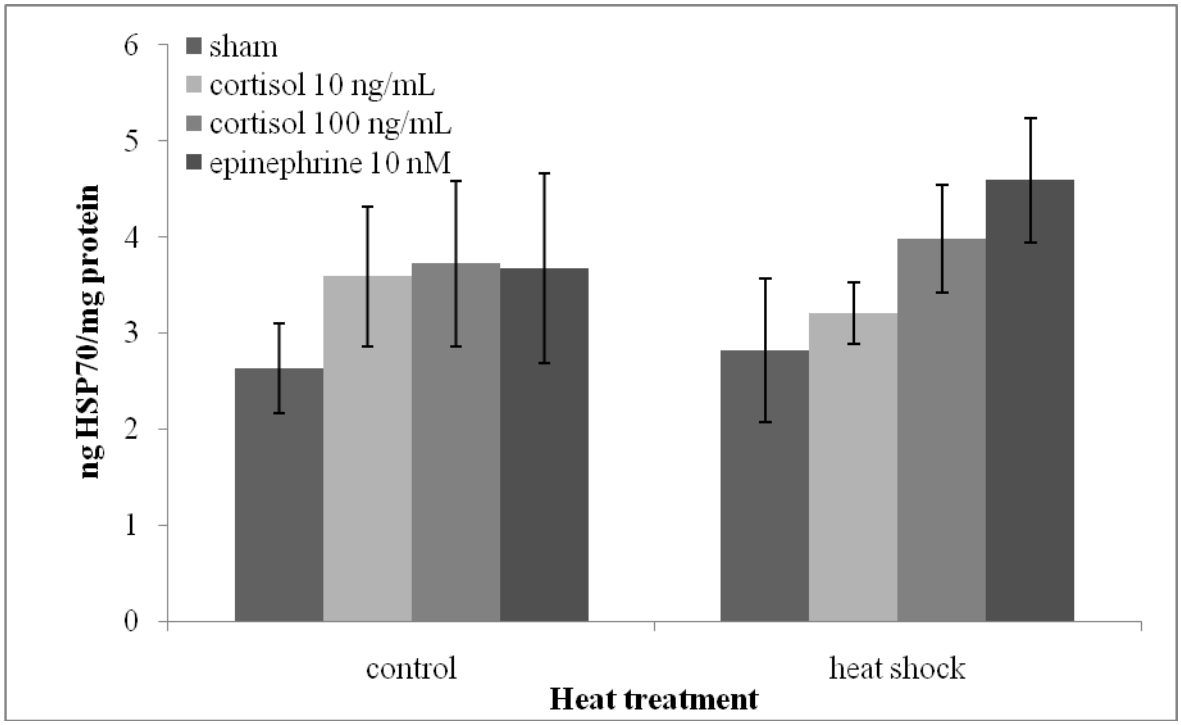


Figure 4.2:HSP70 levels of hormone treated red blood cells at 4 h post-heat shock. RBCs were isolated, plated and treated with one of four hormone treatments: no hormone (sham), low cortisol (10 ng/mL), high cortisol (100 ng/mL) or epinephrine (10 nM). Samples were given a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 4 h post-heat shock. Values represent mean ± SEM (n = 6; two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

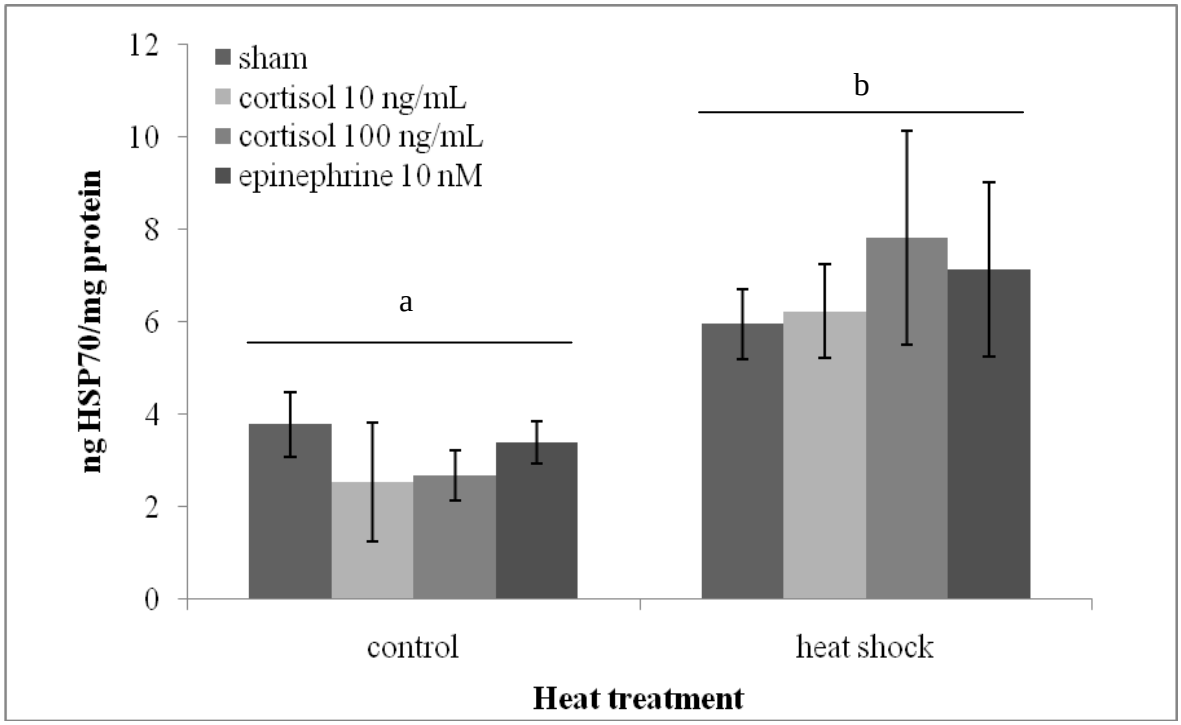


Figure 4.3: HSP70 levels of hormone treated red blood cells at 24 h post-heat shock. RBCs were isolated, plated and treated with one of four hormone treatments: no hormone (sham), low cortisol (10 ng/mL), high cortisol (100 ng/mL) or epinephrine (10 nM). Samples were given a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 24 h post-heat shock. Values represent mean ± SEM (n = 6). Bars with different letters are significantly different ($p<0.05$, two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

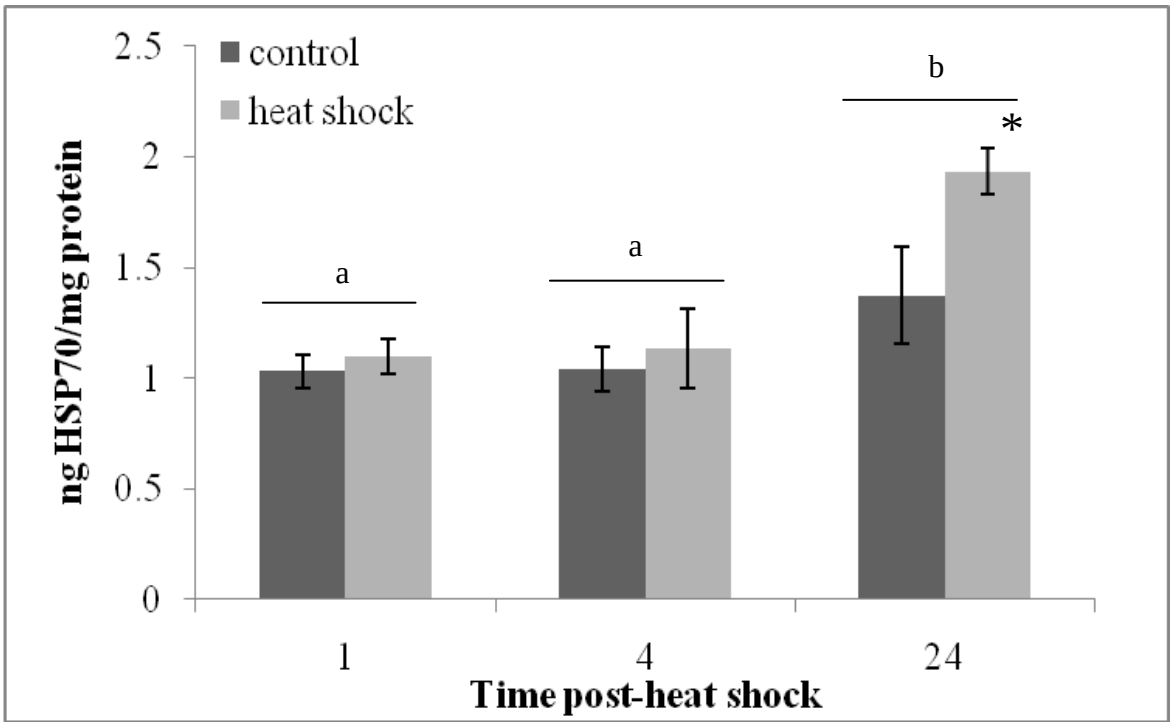


Figure 4.4: HSP70 levels of in medium collected from rainbow trout red blood cell subjected to heat shock *in vitro*. RBCs were isolated, plated and subjected to a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Medium samples were collected at 1, 4 and 24 h post-heat shock. Values represent mean ± SEM (n = 5). Bars with different letters are significantly different, while the asterisk denotes significant difference from the respective control ($p<0.05$, two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

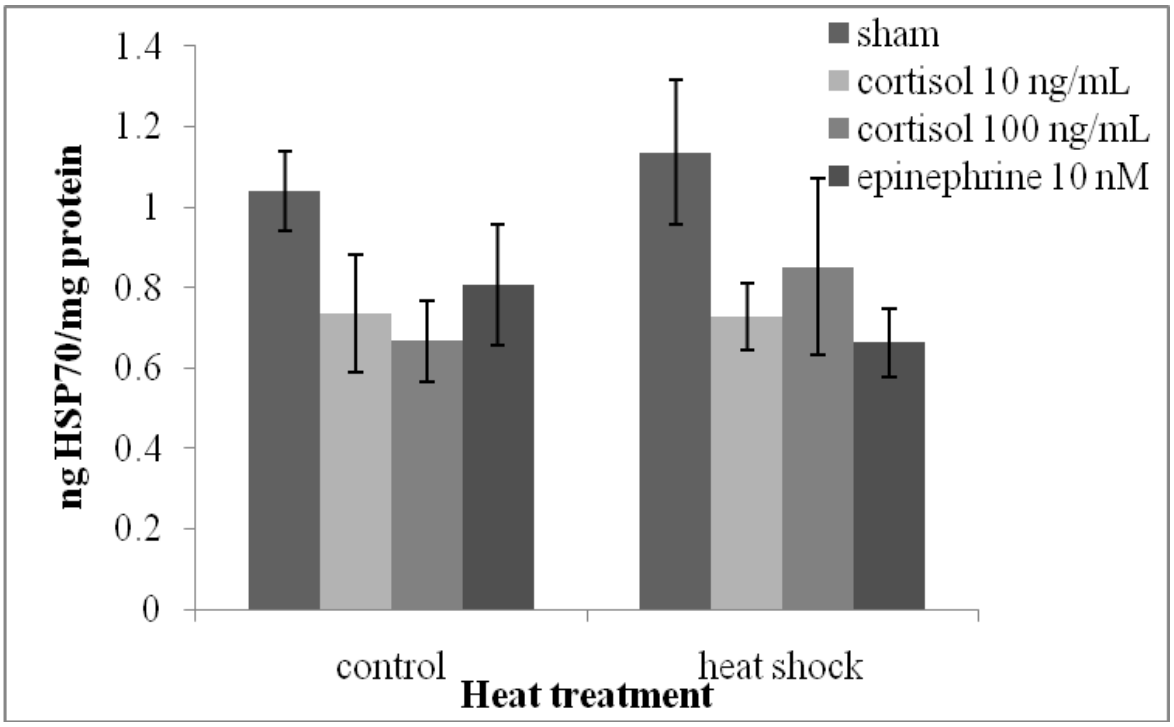


Figure 4.5: HSP70 levels in medium collected from hormone treated red blood cells at 4 h post-heat shock. RBCs were isolated, plated and treated with one of four hormone treatments: no hormone (sham), low cortisol (10 ng/mL), high cortisol (100 ng/mL) or epinephrine (10 nM). Samples were given a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 4 h post-heat shock. Values represent mean ± SEM (n = 6; two-way ANOVA repeated measure). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

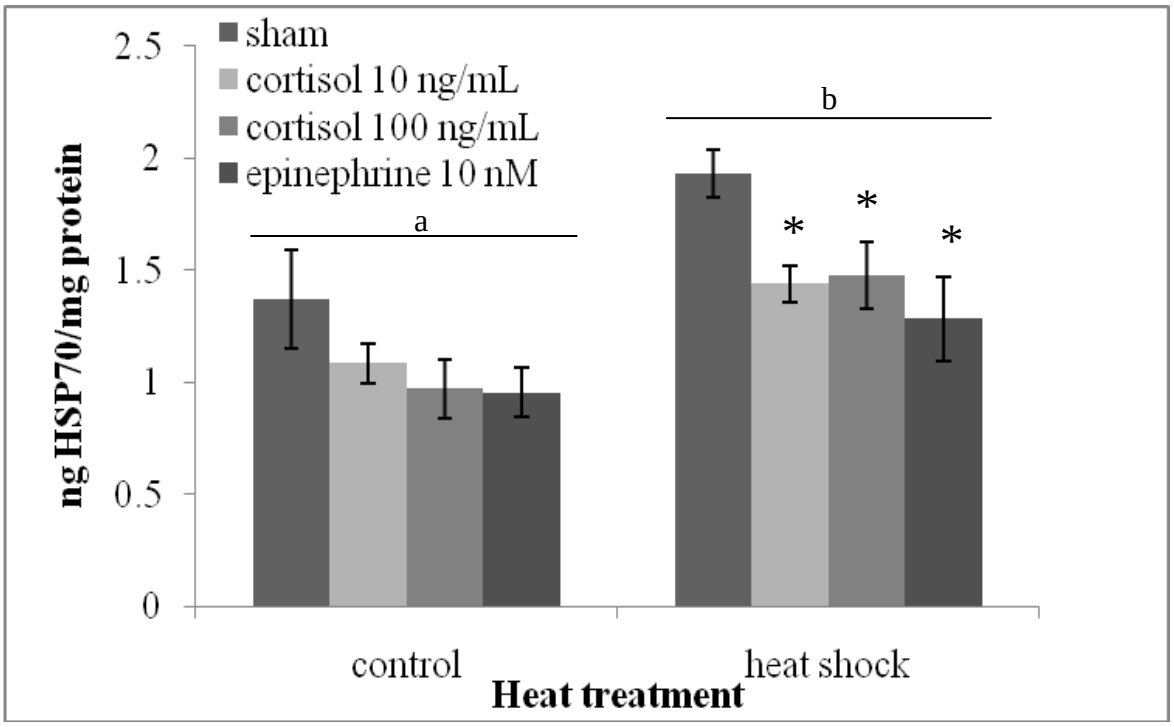


Figure 4.6: HSP70 levels in medium of hormone treated red blood cells at 24 h post-heat shock. RBCs were isolated, plated and treated with one of four hormone treatments: no hormone (sham), low cortisol (10 ng/mL), high cortisol (100 ng/mL) or epinephrine (10 nM). Samples were given a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 24 h post-heat shock. Values represent mean ± SEM (n = 6). Bars with different letters are significantly different, while the asterisk denotes significance from their respective sham group ($p<0.05$, two-way ANOVA). A polyclonal rabbit anti-salmonid HSP70 antibody (1:20000 dilution) was used.

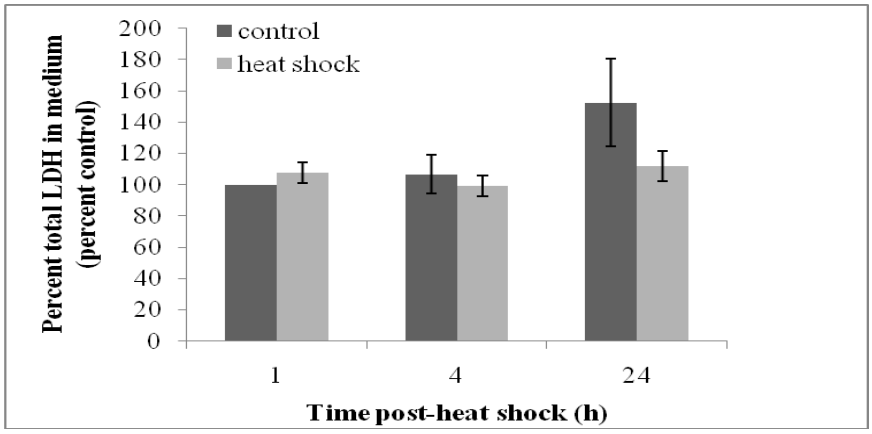


Figure 4.7: Percent total LDH in medium collected from heat shocked rainbow trout RBCs *in vitro*. RBCs were isolated, plated and subjected to a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Samples were collected at 1, 4 and 24 h post-heat shock. Percent total LDH was calculated by determining medium concentrations and dividing my total LDH concentrations (medium + cell). Values represent mean ± SEM (n = 5; two-way ANOVA).

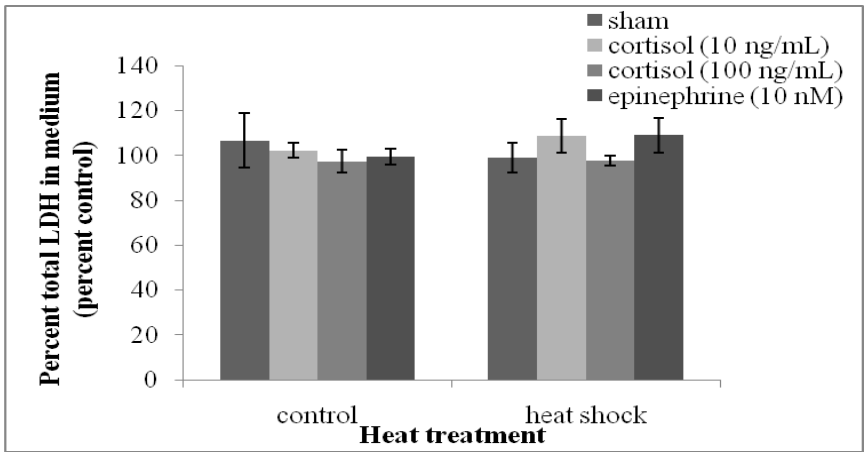


Figure 4.8: Percent total LDH in medium collected from heat shocked and hormone treated rainbow trout RBCs at 4 h post-heat shock. RBCs were isolated, plated and treated with one of four hormone treatments: no hormone (sham), low cortisol (10 ng/mL), high cortisol (100 ng/mL) or epinephrine (10 nM). Samples were given a heat shock of 15ºC above ambient temperature for one hour and then returned to ambient temperature to recover. Values represent mean ± SEM (n = 6; two-way ANOVA).

4.4 Discussion

The results from this study demonstrate for the first time that HSP70 is released extracellularly from RBCs of rainbow trout and that this release is modulated by stress hormones. While mammalian studies have shown the release of eHSP70, there has been a lack of study on their release in teleost fish. To date, data collected on eHSPs have shown that these proteins have a stimulatory effect on the immune response (see reviews by Pockley, 2003), but this remains to be established in fish.

Cell viability was not affected by heat shock, suggesting that the elevated HSP70 in medium was not associated with cell necrosis. It has also been shown that HSP70 levels in trout RBCs are not altered by either anoxia or energy deprivation (Currie et al., 1999) providing further evidence that the HSP70 levels were not due to a lack of oxygen or nutrients to the cell during the experimental period. Release of HSP70 into the extracellular environment is a recent discovery, first demonstrated being released from glia cells for uptake by nearby neurons (Tytell et al., 1986). Since then, it has been found to be released from a number of cells, many of which play vital roles in the immune response [glial cells (Guzhova et al., 2001); β cells (Clayton et al., 2005); tumour cells (Gastpar et al., 2005); human peripheral blood mononuclear cells (Lancaster & Febbraio, 2005); brown fat (Campisi & Fleshner, 2006)]. The presence of HSP70 in the blood has been associated with a number of diseases (Pockley, 2002) and has been found in blood following acute physical and/or psychological stresses (Johnston & Fleshner, 2006).

Previous studies have shown that cortisol has a pronounced effect on HSP70 levels. Basu et al. (2001) showed that a combination of heat shock and exogenous cortisol

administration (50 µg/g body weight) suppressed expression of HSP70 in liver of rainbow trout by over 30% and 66% in gill. Cortisol has also been shown to suppress heat shock induced HSP70 levels in rat brain slices (Barr & Dokas, 1999) and in copper treated fish (De Boeck et al., 2003). Boone and Vijayan (2002b) showed a decrease in HSP70 accumulation over a 24 h period in rainbow trout hepatocytes treated with cortisol. Vijayan et al., (1997) also showed that a handling stress will induce cortisol production but not HSP70 production, which may indicate a suppression of HSP70 expression by the rapid cortisol response as it has been shown that psychological stress will increase the HSP70 concentration in blood of mammals (Johnson & Fleshner, 2006). High levels of cortisol (1000 ng/mL) have been shown to decrease production of other essential HSPs. Sathiyaa et al. (2001) showed a decrease in HSP90 mRNA in fish subjected to heat shock after been treated with a high dose of cortisol. HSP30 expression is also attenuated after cortisol treatment and subsequent heat shock in gill of cutthroat trout (*Oncorhynchus clarkii*; Ackerman et al., 2000). In this study, however, they found no change in gill HSP70 levels. Another study consisting of daily injections of cortisol (4 µg/g) into silver sea bream (*Sparus sarba*) did not show changes in hepatic HSP70 levels (Deane et al., 1999). While the cortisol impact of HSP70 response is equivocal, the reason for the lack of response in RBCs is unclear. It is likely that various factors, including the experimental design, type and intensity of stressor, duration of exposure, species and tissue type, all play a role in the hormonal action on HSP70 response. Also, little is known about cortisol signalling in RBCs and it remains to be seen if that may play a role in the observed response.

While data is very limited on the effect of epinephrine on HSP70 levels, all current studies show that increased epinephrine will cause an increase in HSP70 expression. Epinephrine was shown to be a potent inducer of HSP72 in human fibroblasts (Hakoto et al., 1996). Studies carried out in fish using physiologically relevant concentrations of epinephrine also showed an increase in HSP70 expression. Cutthroat trout subjected to either 10^{-7} M or 10^{-5} M epinephrine showed increased HSP70 levels in gill (Ackerman et al., 2000), while beta-adrenergic stimulation by an agonist, isoproternol, caused an increase in the heat shock proteins (Currie et al., 2008). It remains to be seen if the lack of hormone response on HSP70 levels is tissue-specific, as indicated by the lack of response in RBCs, or due to other factors, including the concentration of the hormone and the duration of exposures. While the mechanism leading to reduced eHSP70 with epinephrine in the present study is unclear, one hypothesis is that this hormone may be inhibiting the release of intracellular HSP70. This is supported by the reduced eHSP70 levels in heat shocked RBCs treated with epinephrine and cortisol.

4.5 Conclusion

We have shown for the first time, the release of HSP70 into the extracellular environment by heat shocked RBCs. The increase in HSP70 levels in medium samples corresponded with the increase in HSP70 levels seen within the cell. Hormone treatments to cells prior to a heat shock exposure had no effect on HSP70 levels within the cell. However, suppression in protein concentration released into the medium was seen in response to hormone administration in heat shocked RBCs. This suggests for the first time a hormonal regulation of eHSP70 in fish, while the mechanisms involved remains to be elucidated. Overall, the results further support the use of HSP70 expression in RBCs as a reliable marker of acute cellular stress effect as neither cortisol nor epinephrine interferes with the heat shock response in RBCs.

Chapter 5
General Conclusions

The expression of HSP70 is a very reliable marker of cellular stress in organisms. This protein is expressed in response to a wide variety of stressors, including physical, chemical and psychological, and in several species. The elevated HSP70 expression is thought to be important in maintaining the protein homeostasis in response to stressor insults. In teleosts, studies have examined stressor-induced HSP70 expression in a number of tissues, including liver, gill and heart. While fish red blood cells (RBCs) induce HSP70 expression in response to heat shock, few studies have been carried out to examine the effect of different stressors and/or hormones on HSP expression in RBCs. RBCs in fish have a functional nucleus and molecular machinery that is geared for protein synthesis. However, few studies have characterized protein biomarkers expression in fish RBCs as a tool for non-lethal detection of stress status. In the present study, the hypothesis tested was that exposure to acute and chronic stressors modulate HSP70 levels in RBCs in order to assess the feasibility of using this protein level as a marker of cellular stress effect in fish. To test this, we first determined the tissue-specific expression of HSPs in response to an acute stressor. Secondly, we developed a method of quantification of HSP70 levels in rainbow trout (*Oncorhynchus mykiss*) RBCs. Next, the effects on HSP70 levels in RBCs chronically exposed to contaminants were examined. Finally, the effects of stress hormones on levels of intracellular and extracellular HSP70 in RBCs were analyzed.

In chapter 2, studies were carried out using a standardized heat shock protocol (+12ºC heat shock of 1 h, then recovery at ambient temperature with sampling occurring at 1, 4, and 24 h post-heat shock) as a model to examine the tissue-specific expression of HSP70 in rainbow trout. The HSP70 expression was analyzed using immunodetection by western blotting with anti-trout HSP70 antibodies. The results clearly displayed a heat shock-induced expression of HSP70 in all tissues tested, including RBCs. This confirmed our prediction that HSP70 expression in RBCs is a potentially non-lethal method for determining the cellular stress response in fish. Although, western blotting is widely used for protein expression analysis, the method is semi-quantitative at best. Therefore, an enzyme-linked immunosorbent assay (ELISA) that is specific for measuring trout HSP70 level was developed (Chapter 3). This competitive antibody-capture ELISA was developed using a commercially available rabbit polyclonal anti-salmon HSP70 and a recombinant Chinook salmon HSP70 protein. The specificity and reliability of the ELISA was confirmed with RBCs collected from heat shocked (see chapter 2) rainbow trout. An acute heat shock (1 h at +15ºC above ambient) significantly elevated RBC HSP70 levels at 1, 4 and 24 h post-exposure. However, chronic exposure to cadmium (0.75 µg/L or 2.0 µg/L) over a 28 d period did not alter the RBC HSP70 levels in rainbow trout. A similar lack of HSP70 response in RBCs was also seen in trout exposed to municipal waste water effluent (MWWE: 0, 20 or 90% of effluent) over a 14 d period.

A key aspect of the organismal stress response involves the release of stress hormones, including catecholamines and corticosteroids, into the circulation in response to stressors. The stressor-induced elevation in epinephrine (a catecholamine) and cortisol (the

principal corticosteroid in teleosts) allows the animal to re-establish homeostasis. While studies have shown linkages between the organismal stress response and the cellular HSP70 response in fish, the role of cortisol and epinephrine in regulating RBC HSP70 expression is far from clear. In chapter 4, *in vitro* studies with trout RBCs showed that neither epinephrine (10 nM) nor cortisol (10 or 100 ng/ml) modified either the basal or heat shock-induced HSP70 levels. This finding is significant because it suggests that the stressor-induced elevation in circulating stress hormone levels will not modulate the existing HSP70 level in RBCs. This further underscores the utility of using RBC HSP70 levels as a reliable marker of cellular stress in rainbow trout.

A novel finding from this study was that RBC HSP70 is released into the extracellular fluid in response to an acute heat shock in rainbow trout. The HSP70 levels in the medium increased significantly post-heat shock in a manner similar to the elevation seen in RBC levels. This increase in the medium was not associated with cell death as the percent lactate dehydrogenase release (a marker of cell viability) was not affected by heat shock. Interestingly, the heat shock-induced extracellular release of HSP70 from RBCs was attenuated in the presence of epinephrine and cortisol. Although the significance is unclear, the results lead to the proposal that the stress hormones may have a protective role by limiting the release of intracellular HSP70 in RBCs. The mechanism leading to the release of HSP70 extracellularly as well as a physiological role for this protein in the extracellular environment warrants further attention.

The results suggest that the HSP70 level in RBCs is a reliable marker of cellular effects in response to acute stressors. The lack of a cortisol or epinephrine effect on RBCs

HSP70 expression in response to heat stress makes this tissue a highly useful tool in stress research. This is because the results suggest that repeated sampling of fish for bleeding, and the associated stress hormone responses, may not modulate the prior HSP70 expression of RBCs in fish. In the future, more research is needed with respect to the effects of chronic stressor exposure on HSP70 levels in RBCs. The demonstration of extracellular release of HSP70 for the first time in isolated fish cells underscores the utility of HSP70 levels in blood (plasma and RBCs) as a biomarker of cellular effects in fish. The mechanisms leading to the extracellular HSP70 release and their implications on adaptations to stress remains to be elucidated.

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Appendix A
ELISA Intra-assay Variation Calculations

ELISA standards were run in triplicate. The coefficient of variation (%CV) was calculated for each set of replicates. The mean %CV was determined by averaging values for each ELISA run. The overall average %CV was calculated to be 11 ± 2 (SEM) for intra-assay variation.

Table 1: Coefficient of variance (%CV) for intra-assay ELISA. %CV was calculated for each set of replicates within the standard curve of each ELISA run. These values were averaged to determine average %CV per run. The mean of these values was determined to obtain an average %CV for intra-assay variation of 11 ± 2% (SEM).

	%CV Individual ELISAs							
ng HSP70/mL	1	2	3	4	5	6	7	8
0	7.8	0.8	8.5	12.4	3	12.6	1.1	2.4
25	17.9	0.1	2	24.6	2.6	11.5	0.1	5.4
50	18	2.8	8.8	23.5	3.2	0.2	6.5	0.2
100	13.3	26.5	2.9	23.9	24.8	0.3	0.3	12.1
200	33.9	9.4	5.2	24.9	22.8	7.3	17.4	9.8
300	18	10.7	12.2	14.8	23.3	12.7	0.2	16.2
400	13.6	0.7	17.6	27.4	10.3	4.4	2.3	8.4
500	20.4	14.1	3	12.7	12.8	19.4	8.6	22.9
Mean %CV	17.9	8.1	7.5	20.5	12.9	8.6	4.6	9.7
							Intra-assay % CV = 11	

Appendix B

ELISA Inter-assay Variation Calculations

Mean results for each standard curve replicate were calculated. %CV was calculated for each standard concentration from the replicate mean of each ELISA run. The mean of these values was determined to obtain the inter-assay variation of 26 ± 4 (SEM).

Table 2: Coefficient of variance (%CV) for inter-assay ELISA. Mean absorbance for each standard was calculated. %CV was calculated for each standard from means obtained from all ELISA runs. The average %CV was calculated from these values to obtain a overall inter-assay variation of 26 ± 4 (SEM).

	ELISA Run Number								
ng HSP70/mL	1	2	3	4	5	6	7	8	%CV
0	1.448	1.426	1.551	1.387	1.5	1.587	1.469	1.339	5.6
25	1.181	1.409	1.021	0.869	1.035	1.158	0.897	1.052	16.0
50	0.996	1.22	0.77	0.674	0.839	0.849	0.642	0.977	21.8
100	0.662	1.034	0.587	0.498	0.613	0.565	0.408	0.658	29.4
200	0.414	0.574	0.37	0.371	0.418	0.399	0.267	0.451	21.2
300	0.405	0.605	0.275	0.171	0.28	0.29	0.177	0.379	43.7
400	0.154	0.284	0.209	0.137	0.208	0.196	0.14	0.353	35.7
500	0.166	0.315	0.178	0.125	0.192	0.191	0.138	0.205	30.7
									Inter-assay %CV = 26