Modulation of Extracellular Heat Shock Protein 70 Levels in Rainbow Trout

by

Leslie Erin Faught

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2013

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.

I understand that my thesis may be made electronically available to the public.

Abstract

At the cellular level, the stress response involves the synthesis of a highly conserved family of heat shock proteins (Hsps). These proteins are essential for maintenance of cellular homeostasis, both in times of stress and in normal cell functioning. Some of the most abundant forms of Hsps in the cell are members of the 70 kDa family. Intracellular heat shock protein 70 (Hsp70) expression in response to proteotoxicity is a highly conserved cellular stress response, but little is known about the role of extracellular Hsp70 (eHsp70) in fish.

In order to begin characterizing eHsp70 in fish, the hypothesis that an acute stressor will elevate plasma Hsp70 levels in rainbow trout (*Oncorhynchus mykiss*) was tested. Subsequent *in vitro* studies examined whether eHsp70 level was modulated by cortisol and if this involved the action of the glucocorticoid receptor (GR), a ligand-activated transcription factor. The effect of cortisol on the eHsp70 response is important to consider because this steroid is elevated as a result of stressor exposure to allow for short-term allocation of energy stores to cope with stress. Cortisol is the primary corticosteroid in fish and exerts its main effects by binding to either GR or mineralocorticoid receptors (MR). Furthermore, eHsp70 has been previously implicated as having important immunoregulatory roles in mammalian models, but nothing has yet been reported in fish. To this end, a hypothesis tested here was that eHsp70 levels will increase after exposure to the bacterial endotoxin lipopolysaccharide (LPS), and that this response is modulated by cortisol. Finally, research on the effects of exogenous Hsp70 has not been reported in lower vertebrates; however, the relevance of this protein in intercellular signaling, especially in regards to immune regulation, is gaining increasing importance in mammalian models. Therefore, an experiment to determine whether Hsp70 would elicit upregulation of key immunoregulatory cytokines was also conducted.

To accurately measure the low levels of Hsp70 in the plasma, a competitive antibody-capture enzyme-linked immunosorbent assay (ELISA) was developed. In the *in vivo* study, fish exposed to an acute heat shock (1h at 10°C above ambient temperature) exhibited a significant elevation in red blood cell Hsp70 levels over a 24 h period. There was also a significant increase in plasma Hsp70 levels at 4 h, but not at 24 h post-heat shock. To more specifically determine how cortisol affected the release of Hsp70, *in vitro* studies using primary cultures of hepatocytes demonstrated that cortisol significantly decreased eHsp70 levels in the medium at 24 h when compared with untreated controls, and this response was abolished in the presence of a GR antagonist, mifepristone (RU486). This result

for the first time established a link between cortisol signaling and eHsp70 release in any animal model.

When hepatocytes were exposed to LPS *in vitro*, eHsp70 levels were significantly lower in the LPS (30 µg/ml) group; however, heat shock abolished this effect at 24 h. Though eHsp70 levels in the heat shocked hepatocytes treated with low-dose LPS (10 µg/ml) was similar to untreated control levels, high-dose LPS treated hepatocytes showed significant elevation of eHsp70 levels above the low dose group. The ability of LPS to modulate eHsp70 release was not observed to be further regulated by cortisol. While this work suggests the modulation of eHsp70 by LPS, the physiological role remains to be elucidated. Finally when hepatocytes were exposed to exogenous Hsp70, there was no effect on key immunoregulatory genes (IL-1β and IL-8) transcript levels; however, the effect of this protein remains to be tested using other cell systems, including immune cells in fish.

Overall, eHsp70 concentration was measured in trout plasma using a competitive ELISA and demonstrates for the first time that stressor exposure affects plasma eHsp70 levels in fish. Furthermore, cortisol, the primary corticosteroid in teleosts, modulates eHsp70 release in trout hepatocytes and this is action is mediated by GR signaling. Also, while trout hepatocytes secrete eHsp70 in response to endotoxin shock, a role for eHsp70 in eliciting an immune response is not clear in lower vertebrates. Taken together the results from this study suggest a role for eHsp70 in acute stress adaptation in fish, but the target tissues involved and the physiological responses remain to be elucidated. Further work on the effects of eHsp70 on target tissues effects, and the mechanisms involved, may have important implications in our understanding of the role of this stress protein in cell signaling and stress adaptation in fish.

Acknowledgements

I would first like to thank Dr. Matt Vijayan for the opportunity to work in his lab. His invaluable guidance, encouragement, and patience over the last three years have taught me so much.
I would also like to thank my committee members Dr. Zoya Leonenko, who first gave me the opportunity to work in her lab, as well as Dr. Brenden McConkey for their constructive input.
Thank you to all the members of the Vijayan lab - past, present, official and honorary - for all the help you have given me. A special thanks to Nita and Oana for their unending patience with all my questions, to Carol, Laura and Dinu for discussions on life and science, and to Liz and Navdeep for their perpetual optimism. Lastly, a special thanks to my sisters and James, for their unending support and encouragement.

.

Table of Contents

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

List of Tables

Chapter 4:

Appendix:

Chapter 1
General Introduction

1.1 Introduction

1.1.1 The Heat Shock Response and Heat Shock Proteins

The stress response is an important adaptation in all living organisms as it allows for the maintenance of homeostasis when challenged with one or more stressors (Iwama et al., 2006). An animal's physiological response to stress can be broadly categorized into primary, secondary, and tertiary responses (Wendelaar Bonga, 1997). The primary response involves the rapid release of stress hormones, catecholamines and cortisol, into circulation (Iwama et al., 1999). The secondary response, in part, is mediated by these stress hormones, and involves the biochemical and physiological adjustments associated with stress. For example, stress hormones will activate a number of metabolic pathways, such as glycolysis, that result in various alterations in blood chemistry (Iwama et al., 1999). Effects of stress on the whole animal and population level changes can be categorized under the tertiary response. For example, in circumstances of stress when an animal is unable to effectively adapt, there may be subsequent decreases in reproduction and growth (Iwama et al., 1999).

At the cellular level, the stress response involves the upregulation of several classes of heat shock proteins (Hsps), generally categorized as the heat shock response (Richter et al., 2010). Stressors, such as heat, have several deleterious effects at the cellular level such as reorganization or aggregation of cytoskeleton components. This leads to the loss of correct organelle localization, a decrease in transcription and translation as well as an increase in membrane permeability causing changes in intracellular pH and ion concentrations (Richter et al., 2010). The induction of Hsps are adaptive and they provide cytoprotection to offset the proteotoxicity in response to stressors (Hightower, 1989). Hsps can be broadly classified into seven categories based on function, including the traditional molecular chaperones, components of the proteolytic system, RNA – and DNA-modifying enzymes, metabolic enzymes, transcription factors and kinases, as well as transport, detoxifying and membrane-modulating proteins. The heat shock response is conserved amongst species; however, the composition and rate of up-regulation of each class of proteins will vary (Richter et al., 2010). The class of proteins whose primary role is to act as molecular chaperones are divided into five families based on molecular mass, including Hsp 27, 60, 70, 90, and 110, (Kregel, 2002; Calderwood et al., 2007; Richter et al., 2010). These stress proteins comprise 5-10% of total

protein in an unstressed cell, but can be induced to encompass up to 15% of total protein during times of stress (Calderwood et al., 2007; Pockley et al., 2008).

The Hsps are primarily known for their intracellular roles in cell signaling and molecular chaperoning (Parsell and Lindquist, 1993). Hsp70 (68 -73 kDa) is one of the most extensively studied, and most highly conserved of the Hsps (Pockley et al., 2008). Within this family, there are two main isoforms, Hsc70 which is constitutively expressed in all cells and the inducible Hsp70 that is synthesized rapidly in response to proteotoxicity. Hsc70 is involved in proper protein folding, intracellular localization and secretion (Feder and Hoffman, 1999) while the intracellular role of Hsp70 includes molecular chaperoning, folding nascent polypeptide chains, mediating the repair or degradation of denatured proteins, and protein transport (Kregel et al. 2002). Higher molecular mass Hsps, including Hsp90 (85 -90 kDa), are important in intracellular cell signaling, particular as a chaperone for steroid hormone receptors. Low molecular mass Hsps (16 – 40 kDa) are expressed only during stress and have more diverse, species-specific roles within the cell (Kregel et al., 2002). Although HSPs were first observed in response to heat shock, we now know that any stressor affecting protein stability will stimulate the synthesis of these proteins in various species. Briefly, these stimuli include hyperthermia (Burdon et al., 1982; Flanagan et al., 1995; Kregel et al., 1995; Kregel and Moseley, 1996; Parsell and Lindquist, 1993; Skidmore et al., 1995), bacterial pathogens (Ackerman, et al., 2001,Forsyth et al., 1997), heavy metals (Boone and Vijayan, 2002a), oxidative stress (Parsell and Lindquist, 1993), xenobiotics (Vijayan et al., 1998; Vijayan et al., 2005), amino acid analogues (Thomas and Mathews, 1984), energy depletion (Sciandra and Subjeck, 1983) and acidosis (Weitzel., 1985) (Fig 1.1).

The regulation of the heat shock response and synthesis of heat shock proteins occurs through the activation of heat shock factors (HSF) which will bind to the promoter region of heat shock genes during stress, initiating transcription (Fig 1.1). There are four possible heat shock factors. HSF1 is the principal transcription factor in mammals and lower vertebrates (Sarge et al., 1993; Deane and Woo, 2011). HSF4 has been found to be constitutively expressed (Nakai, 1999) and HSF2 expression has been observed at different stages of development (Pirkkala et al., 2001). Within avian species, HSF3 is the main transcription factor induced by stress (Pirkkala et al., 2001). In the absence of stress, HSF1 is found within the cytoplasm in a latent monomeric state bound to HSP70 (Kregel, 2002). Upon detection of a stressor, the Hsp70 or ubiquitin is removed from HSF1 leading to homotrimerization and subsequent transport to the nucleus where it is hyperphosphorylated by several

kinases (Richter et al., 2010). The trimer can then bind to the heat shock element (HSE), located in the promoter region of the HSP70 gene, inducing transcription. Regulation of HSF1 binding to the HSE is mediated by heat shock binding protein 1 which can be subject to negative feedback by Hsp70 thereby repressing synthesis (Shi et al., 1998).

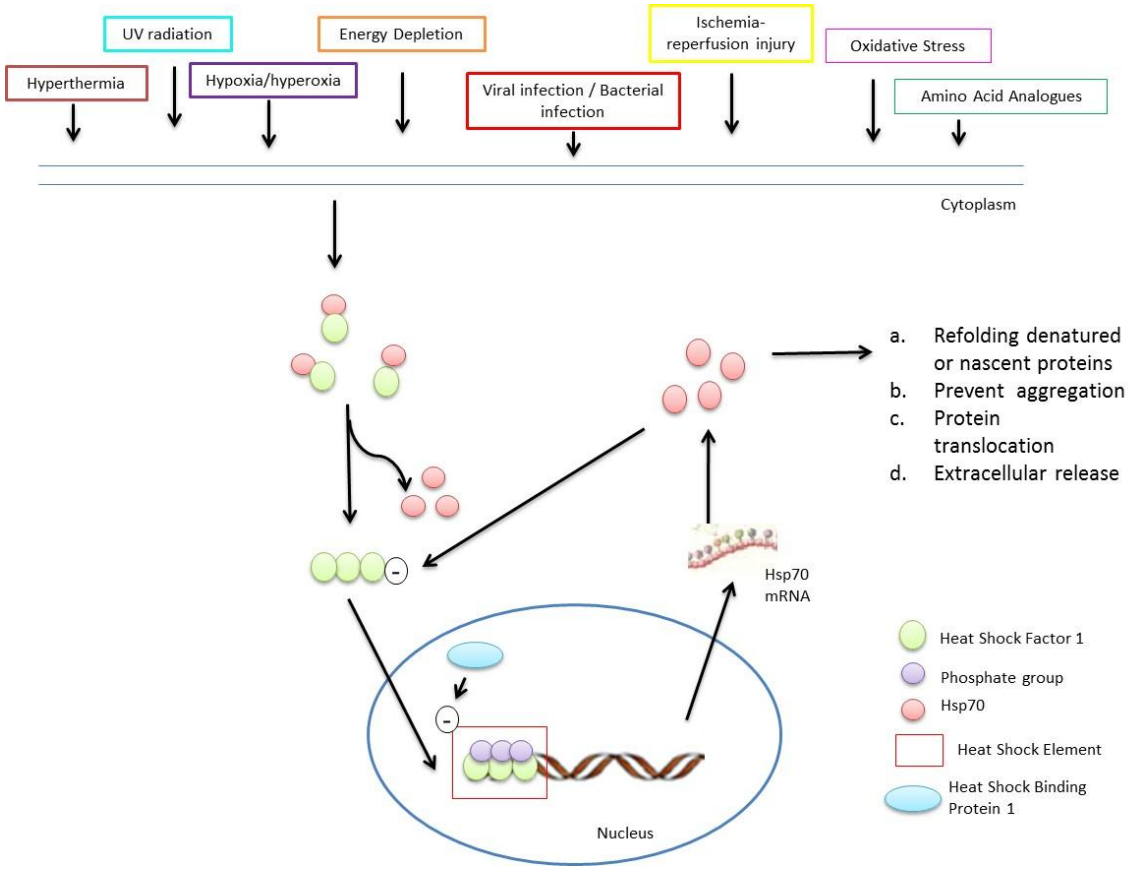

Figure 1.1: Hsp70 Synthesis

Synthesis by a variety of stressors activates Heat shock factor 1(HSF1), which is found in the cytoplasm in a monomeric state bound to Hsp70. Hsp70 is removed upon stressor detection leading to homotrimerizaton and translocation to the nucleus where it is phosphorylated. This complex will then bind to the heat shock element and initiate transcription (Adapted from Richter, 2010).

1.1.2 Hsp70 Expression of Fish

The intracellular chaperoning roles of heat shock proteins in fish have been well characterized (see reviews by Iwama et al., 1999; Deane and Woo, 2011). In the aquatic environment fish are exposed to a variety of stressors which can either be natural, such as diurnal or seasonal changes in temperature, or anthropogenic, such as chemical pollutants and nitrogenous waste from industry (Deane and Woo, 2011). Fish are therefore an excellent model organism, as they are directly exposed to these stressors in the aquatic environment. Physiological changes, such as elevations in Hsps, may be instrumental in determining the stress level and health of an organism and subsequently the quality of its environment. Hsps have been studied in a variety of fish, utilizing various tissues, experimental designs and endpoints (Hsp family, mRNA transcripts or protein expression). This thesis focuses on the regulation of Hsp70 and therefore only this family will be described further in this section. In regards to teleosts and hsp70, upregulation of mRNA transcripts have been demonstrated in response to acute heat shock in silver sea bream (*Sparus sarba*) liver (Deane and Woo, 2005), black sea bream (*Mylio macrocephalus*) fibroblasts (Dean and Woo 2006); gill of common killifish (*Fundulus heteroclitus*; Fangue et al., 2006), rainbow trout gonadal (RTG-2) cell lines (Ojima et al., 2005a,b), primary cultures of goldfish (*Carassius auratus*) caudal fin cells (Kondo et al., 2004); liver and muscle of common carp (*Cyrprinus carpio*; Ali et al., 2003), and embryo cells of Japanese flounder (*Paralichthys olivaceus*; Yokoyama et al., 1998). Protein expression has also been shown to be upregulated under a variety of conditions and species, including changes to salinity (Long jawed mudsucker [*Gillichthys mirabilis*: Kultz, 1996] and common carp ([De Wachter et al., 1998]), exposure to pathogens (Forsyth et al., 1997; Ackerman and Iwama, 2001; Deane et al., 2004) and rainbow trout hepatocytes (Philip et al., 2012). Increased expression of Hsp70 has also been demonstrated in cells due to hypoxia (Mestril et al., 1994; Kobayashi and Welsh, 1995) and chemical toxicants (Boone and Vijayan, 2002a). In contrast to the inducible form, Hsc70 is relatively constant as there was no change detected in cadmium-treated or heat-shocked rainbow trout hepatocytes (Boone & Vijayan, 2002a), top minnow hepatocytes (White et al., 1994; Norris et al., 1995), or in response to heavy metal exposure or hypoxia in rainbow trout RBCs (Currie et al., 1999). Some contrasting results remain, as an increase in Hsc70 mRNA was observed in zebrafish (*Danio rerio*) embryos and medaka (*Oryzias latipes*) cell lines in response to heat shock (Santacruz et al., 1997). Physical stressors did not affect the expression of liver Hsp70, despite the transient elevation in cortisol and glucose levels after stressor exposure (Vijayan et al., 1997; Washburn et al., 2002). Overall the expression of Hsp70 is consistently upregulated in various tissues of multiple species in

response to a variety of stressors, though the degree of induction is dependent on species, tissue, and experimental design. The majority of studies in regards to fish and Hsp70 expression show causal evidence for Hsp70 regulation by stressors; however, there is a lack of mechanistic research which would fully elucidate its regulation.

1.1.3 Extracellular Hsp70 (eHsp70)

There are various stress proteins released into the extracellular milieu to help maintain homeostasis. eHsp70 was first observed being released into the extracellular space in the squid giant axon and subsequently taken up by neurons when exposed to a heat stressor (Tytell et al., 1986). It was next observed in mammals when Hightower and Guidon (1989) observed the release of 71, 73 and 110 kD Hsps in cultured rat embryo cells, in the absence of necrosis. Recent studies have recently confirmed the release of Hsps from a variety of mammalian models. Whole animal studies have established release into plasma in rat [Fisher 344] (Johnson et al., 2005; Campisi and Fleshner, 2003) and release from various primary cell cultures in vitro, including human cell lines (Lancaster and Febbraio, 2005), rat peripheral blood mononuclear cells (Hunter-Lavin et al., 2004), natural killer cells (Gastpar et al., 2005), human tumor cells (Mambula and Calderwood, 2006), and human β cells (Clayton et al., 2005). eHsp70 release has also detected from established cell lines such as human liver carcinoma (HepG2; Vega et al., 2010), human T98G glioma cells and differentiated LA-N-5 neuroblastoma cells (Guzhova et al., 2001).

To date, no research has been published regarding the mechanism of release of ehsp70 or the effect of eHsp70 in lower vertebrates. In fish, recently eHsp70 was detected in the culture medium of peripheral blood lymphocytes (PBL) of grass carp (*Ctenopharyngodon idella*) in vitro (Zhang et al., 2011), but no study has looked at the mechanism of release from cells in lower vertebrates.

1.1.3.1 Mechanism of Hsp70 Release in Mammalian Models

To traverse the semipermeable plasma membrane, proteins have adapted to be actively transported across this barrier. Briefly, secreted proteins encode an N-terminal hydrophobic leader sequence which can be inserted into the ER membrane allowing them to translocate across this membrane where the leader sequence is then cleaved. The protein is then passed along cellular organelles until these structures fuse with the plasma membrane (Tytell, 2005). Recently, non-classical secretion has been implicated in several proteins which lack a leader sequence despite having primarily extracellular roles, such as interleukin-1β (Andrei et al., 1999). There are currently several hypotheses

for leaderless release of Hsp70 from the cell; however, whether the release is dependent on species or is tissue specific still remains to be determined (Mambula and Calderwood, 2006).

The release of Hsp70 by cell necrosis, in part, contributes to circulating or medium levels of eHsp70 (Calderwood et al., 2005; Pockley and Multhoff, 2010). However, assays for other intracellular enzymes show no correlation with the rate of Hsp70 release, suggesting that this protein is actively released from the cells (Broquet et al., 2003, Hunter-Lavin et al., 2004; Lancaster and Febbraio, 2005). The exact mechanism of release has yet to be elucidated in any organism; however, several have been speculated. The classical secretion pathway was eliminated as a possibility through the inability of Brefeldin A, an antagonist of transport through the ER-Golgi system, to modulate eHsp70 levels (Lancaster and Febbraio, 2005; Broquet et al., 2003). However, monensin, an antagonist of transport through the Golgi did inhibit release (Hunter-Lavin et al., 2004), suggesting that the Golgi may play a role. Golgi-mediated Hsp70 release has been linked to its localization on the plasma membrane of lysosomes (Hunter-Lavin et al., 2004). These secretory lysosomes have been proposed as a possible method of Hsp70 release as the rate of Hsp70 secretion is highly correlated to the lysosomal marker LAMP1 on the cell surface (Mambula and Calderwood, 2006). These secretary lysosomes will migrate to the cell surface and release their contents into the extracellular space. This pathway has been observed in macrophages stimulated with LPS and ATP (Andrei et al., 1999; Andrei et al., 2004). Cells that use secretory lysosomes are usually derived from the haematopoietic lineages (Blott and Griffths, 2002), indicating a cell-specific method of release as eHsp70 has been observed from various cell types. Secretion by this method is also regulated by ATP-binding cassette (ABC) transport proteins at the plasma membrane. Interestingly, this mechanism of release appears to be only active post heat shock, as basal levels were not affected by the non-specific ABC transport inhibitor glibenclamide in prostate carcinoma cell lines (Mambula and Calderwood, 2006). This lends increasing complexity to the release of this protein. Another possible mechanism is the secretion of exosomes which are small membrane vesicles secreted from all cell types. In this method, once the exosome enters the extracellular environment it will either lyse releasing its contents (De Maio, 2011) or, alternatively, the entire exosome will communicate with a target cell (Mathivanan et al., 2010). Hsp70 has been detected in exosomes derived from human peripheral blood lymphocytes (PMBC's; Lancaster and Febbrio et al., 2005), human tumor cells (Gastpar et al, 2005) and rat hepatocytes (Conde-Vancells et al., 2008). While exosomal protein composition varies depending in the cell type of origin, conserved sets of proteins have been identified in exosomes regardless of origin including, Hsp70, metabolic enzymes, and annexin (membrane trafficking and fusion) (Mathivanan et al., 2010).

Though Hsp70 is present in the plasma membrane (Arispe and De Maio, 2000), particularly in association with phosphatidylserine (PS; Arispe et al., 2004; Schilling et al., 2009) and other sphinglolipids (Gehrmann et al., 2008; Sugawara et al., 2009), treatment with the raft disrupting drug methyl-β-cyclodextrin, had no effect on Hsp70 release (Lancaster and Febbraio, 2005; Broquet et al., 2003). The release of Hsp70 may differ based on cell type, both in regards to the amount of Hsp70 released and the pathways involved. This is especially the case in heat shocked cell, as the stressor activates alternative pathways of release (Mambula and Calderwood, 2006). The mechanism behind leaderless release of various cytokines, and heat shock proteins has interesting applications in our knowledge of the dynamics of intracellular signaling. This is especially important during times of stress, as the heat shock response is essential to cellular survival. Very little is known about the modulation of eHsp70 release in fish during times of stress. Elucidation of this regulation has important implications if eHsp70 were to be used as a biomarker of stress in the aquatic environment.

1.1.3.2 Immunoregulatory Effects of eHsp70

One of the major roles for eHsp70 is thought to involve modulation of the innate immune system; however, the effects of circulating eHsp70 and the physiological implications are far from clear (Pockley and Multhoff, 2010). The immunoregulatory effects of Hsp70 have not been studied in fish and this is an important area as pathogens are found ubiquitously in environment, and Hsps may be essential in mounting an effective immune response (Deane and Woo, 2011).

In mammalian models, Hsp70 appears to interact with various receptors on the plasma membrane of target cells. Depending on the cellular origin of Hsp70, there have been completely contrasting results, as eHsp70 has been demonstrated to have both pro-inflammatory and anti-inflammatory properties (Pockley et al., 2008). In mammals, exogenous applications of Hsp70 have demonstrated primarily pro-inflammatory properties, while endogenous application has been shown to be anti-inflammatory (Pockely et al., 2008). The mechanism behind this phenomenon has yet to be elucidated. It was first demonstrated that Hsp70 could bind to CD14 (Asea et al, 2002) a covalently anchored receptor to the cellular membrane via glycosylphosphatidylinositol. This receptor lacks intrinsic signaling capabilities (Chen et al., 2004) and, therefore, acts in conjunction with transmembrane receptors such as toll-like receptors (TLRs). TLRs will subsequently elicit the nuclear factor – κB (NF-κB) pathway. Activation of TLRs by heat shock has been shown in a series of Hsps, including 60, 70 and gp96 (Asea et al, 2002; Vabulas et al., 2001; Vabulas et al., 2002; Vabulas and Wagner, 2005). Members of the NF-κB family are nuclear transcription factors essential for

regulation of the immune stress responses, apoptosis and cellular differentiation. These signaling pathways are tightly regulated and closely coordinated with other signaling pathways (Oeckinghaus et al., 2011). The upregulation of pro-inflammatory cytokines through this pathway upon treatment with exogenous Hsp70 implicated eHsp70 as an important modulator of the innate immune system. However, further work demonstrated that activation through this pathway was due, in part, to contamination with bacterial endotoxins such as lipopolysaccharide (LPS; Gao and Tsan, 2003a), as most recombinant Hsp70 is produced in Escherichia coli. Both Hsp60 and Hsp70 are known to associate with and increase the immune-stimulating effects of LPS (Habich et al., 2005; Osterloch et al., 2008 and Tsan and Gao, 2009). With this taken into account, highly-purified Hsp70 from murine liver was reported to have no cytokine effects at concentrations as high as 200-300 µg/ml; however, LPS-contaminated Hsp70 at concentrations as low as 0.05-0.1 µg/ml were able to induce cytokines (Wallin et al., 2002). Further studies confirmed that other Hsps such as gp96 were also incapable of upregulating cytokines through the NF-κB pathway (Reed et al., 2003) and Hsp70 had no effect on 112 cytokine genes in lymphocytes (Tsan and Gao, 2009). Because Hsp60, gp96 and Hsp70 can bind and enhance the immune-stimulating effects of LPS this indicates that Hsps are important in the in vivo recognition of Gram-negative bacterial infection and that it will modulate the response via TLR4. Regardless of previous discrepancies, the immunoregulatory effects of Hsp70 are still viable (Henderson, et al., 2010). For instance, while Hsp72 did not up-regulate expression of IL-6 and TNFα2 in murine hepatocytes, this protein at concentrations of 100 ng/ml and 1000 ng/ml upregulated the expression of macrophage inflammatory protein 2 (MIP-2/CCL4), suggesting regulation of pro-inflammatory chemokines (Galloway et al., 2008). The concentration of Hsp70 is important as higher concentrations (20-50 µg) have been demonstrated to have cytotoxic effects (rabbit arterial smooth muscle cells; Johnson et al., 1995)

Hsp70 has also been demonstrated to bind to the LDL-binding proteins CD91 and the tumor necrosis family member CD40 (Tytell, 2005). CD40 plays a major role in the maturation of antigen presenting cells (APC) through binding to its counter receptor on activated T cells (Tytell, 2005). Mycobacterial Hsp70 can bind to CD40 which may indicate the protein's ability to activate APCs and release cytokines (CCL3, CCL4, and CCL5; Wang et al., 2001). Furthermore, Hsp 60, 70, and 90 can all bind to lectin-type oxidized LDL receptor 1 (LOX-1) (Theriault et al., 2005, Delneste et al., 2002). It has also been proposed that receptor recognition will vary within members of the same HSP family (Theriault et al., 2005). Receptors such as TLR, CD40, and CCR5 may be adapted for transmembrane signaling, while CD91 and SR may play a more important role in internalization of HSP (Theriault et

al., 2005). The application of exogenous Hsp70, for any purpose, has yet to be investigated in lower vertebrates yet the interaction of Hsp70 with target tissues suggest novel roles for this protein in autocrine, paracrine and even endocrine signaling. This not only has important implications in possible novel signaling pathways, essential in our understanding of the stress response, but it also has interesting applications in the health field, as the role of Hsp70 as a signaling molecule is already being investigated for cancer therapies (Schmitt et al, 2007; Murshid et al. 2011).

1.1.3.3 Hsp70 and Lipid Membranes

In addition to receptors, it has also been demonstrated that Hsp70 will interact directly with lipid membranes which is important in not only understanding both the mechanism of release but also characterizing the effects of Hsp70 on target cells. Interaction of Hsp70 with membranes is important, particularly during times of stress, as permeability of membranes will change in response to stressors and may have further physiological implications. Both Hsp70 and Hsp90 are known to associate with these micro-domains which are important in both assembly and sorting platforms for signal transduction complexes, and intercellular interactions. Most studies regarding the interaction of Hsp70 and lipids have been carried out using either artificial membranes or membranes isolated from mammals (De Maio, 2011). Leakage of fluorescent dye was observed from lysosomes (lipid composition: lecithin, cephalin, and phosphatidylinositol) when they were exposed to human Hsp70 or mycobacterial Hsp65 (Alder et al., 1990). When Hsp70 (5-22.5 µg/ml) was added to planar phospholipid bilayers composed of either the former composition or simply diphytanoylphosphatidylcholine (DPPC), stepwise increases in electrical conductivity resembling the formation of ion-conducting channels were observed (Alder et al., 1990). Interestingly, the addition of Hsp70 to these membranes also promotes the insertion of other proteins into the membranes (Adler et al., 1990). Hsp70 has also been shown to form ATP-dependent, cation-selective channels in artificial lipid bilayers (lipid composition: phosphatidylserine and phosphatidylthanolamine) (Arispe et al., 2002). At concentrations of 15 ng/ml both isoforms of Hsp70 will promote the fusion of phosphatidylserine liposomes. Hsp70 can also bind to sulphogalactolipids which are found in the outer leaflet of the plasma membrane primarily in neurons. This is due to the highly conserved feature of the ATPase domain of HSP70 and binding can inhibit the ATPase activity of HSP70 (Arispe et al., 2002). Furthermore, potassium channels in human promonocyte cells are activated when exposed to 30-100 µg of a 2:3 mixture of Hsc70 and Hsp70 (Negulyaev et al., 1996). In rabbit aortic smooth muscle cells concentrations of 20 – 50 µg/ml were reported to be cytotoxic (Johnson et al., 1995).

There is a lack of information, beyond speculation, regarding the role of eHsp and the interaction that it has with the lipid bilayer. The interaction between Hsp70 and the plasma membrane become important when assessing the extracellular role of this protein.

The idea that treating damaged cells and tissues with exogenous Hsp70 to increase survival of the targeted cells has been explored in cultured arterial smooth muscle cells (Johnson et al., 1990; Johnson et al., 1993; Johnson et al., 1995), and prevented axotomy-induced cell death of sensory neurons but not motor neurons in neonatal mouse model of sciatic nerve transections (Houenou et al., 1996; Li et al., 1994; Lo et al., 1995). When a 1:1 mixture of Hsp70/Hsc70 was injected into the eye of the albino rat after light exposure, significantly less photoreceptor degradation was observed when compared with the controls (Barbe et al., 1988; Tytell et al., 1994; Yu et al., 2001). Further cytoprotective effects were seen in a variety of in differentiated neuroblastoma cells and primary cultures of embryonic chick motor neurons (Guzhava et al., 2001). These studies suggest that the interaction of Hsp70 with lipid membranes may be beneficial for an organism in the stress response. However, little is known about such interactions of Hsp70 with membranes in non-mammalian models. For the first time this study will reveal changes in eHsp release in the plasma of erythrocytes and the medium of hepatocytes. The release of Hsp70 will be monitored in the presence of stress hormones and pharmaceuticals to further elucidate the conditions under which eHsp is released from the cell.

1.1.4 Effects of Stress Hormones (Glucocorticoids and Catecholamines) on eHsp70

The primary effect, in response to stress, is the release of catecholamines (epinephrine and nonepinephrine) which are released by the chromaffin cells near the head kidney in teleosts (Wendelaar Bonga, 1997). These hormones have low resting levels (1nM), but are rapidly elevated post stressor (20 nM); however, this increase is transient (Wendelaar Bonga, 1997). In mammals, Johnson et al. (2005) suggested that the release of extracellular Hsp72 during exposure to stress is mediated in part by catecholamines via stimulation of the α1-adrenergic receptor–mediated pathway. Adrenergic receptors are a class of G-protein-coupled receptors that are the primary targets of catecholamines. Interestingly, rats treated with either labetolol (alpha/beta adrenergic antagonist) or prozosin (alpha-adrenergic blocker) completely blocked the stress induced increase in extracellular HSPs (Johnson et al., 2005). It was concluded that activation of α1- adrenoceptors elevates circulating HSP72, whereas activation of β-adrenoreceptors inhibits circulating Hsp72 in tail shock stress (Johnson et al., 2005). This indicates a role in stress hormonal regulation of the cellular heat shock

response by catecholamines as this elevation was not seen with glucocorticoids (Johnson et al., 2005). Another notable class of stress hormones is glucocorticoids, which in contrast to catecholamines remain in circulation for a longer period, mediating the effects of stress. The primary glucocorticoid in teleosts is cortisol (Mommsen et al., 1999). This hormone is secreted by the adrenal gland in mammals, or interrenal cells (analogous to the adrenal cortex) of the head kidney in teleosts. Plasma cortisol levels are elevated in response to stress in fish and involve the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Mommsen et al., 1999) (Fig 1.2). Briefly, a stressor will stimulate the release the hypothalamic neuropeptide corticotropin releasing factor (CRF) which acts on the pituitary gland to stimulate the release of adrenocorticoptropic hormone (ACTH). ACTH will subsequently stimulate the interrenal cells to release cortisol. Cortisol will subsequently exert its effects on target tissues by binding to either the glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) (Iwama et al., 2006). In teleosts, cortisol will work to restore homeostasis during stress by regulating osmotic and metabolic adjustments in target tissues. It is important to examine the interaction involved in the release of stress hormones and their effect on the expression and subsequent release of stress proteins such as Hsp70. In fish the effects of cortisol on heat shock proteins are varied. Cortisol has been shown to attenuate the HSP response because of its effect on HSF, which regulates heat shock genes (Iwama et al., 2006). Interestingly, GR will modulate levels of intracellular Hsp70 in macrophages and fibroblasts in sea bream which was correlated with increased cell survival (Deane et al., 2006). In rainbow trout hepatocytes, it was demonstrated that while cortisol will not affect the rate of protein degradation, it will reduce *de novo* synthesis post stressor (Boone et al., 2002b). Comparatively the constitutive form, Hsc70, is up-regulated by cortisol in rainbow trout liver (Iwama et al., 2006), which may lead to an increase of the stress threshold in animals. Cortisol and epinephrine attenuated the heat shock-mediated elevation in extracellular Hsp70 levels in RBCs at 24 hours post heat shock exposure (Henrickson, 2010). Though preliminary studies have been completed, the effects of stress hormones on Hsp70 release and the importance of eHsp70 in circulation is still far from clear.

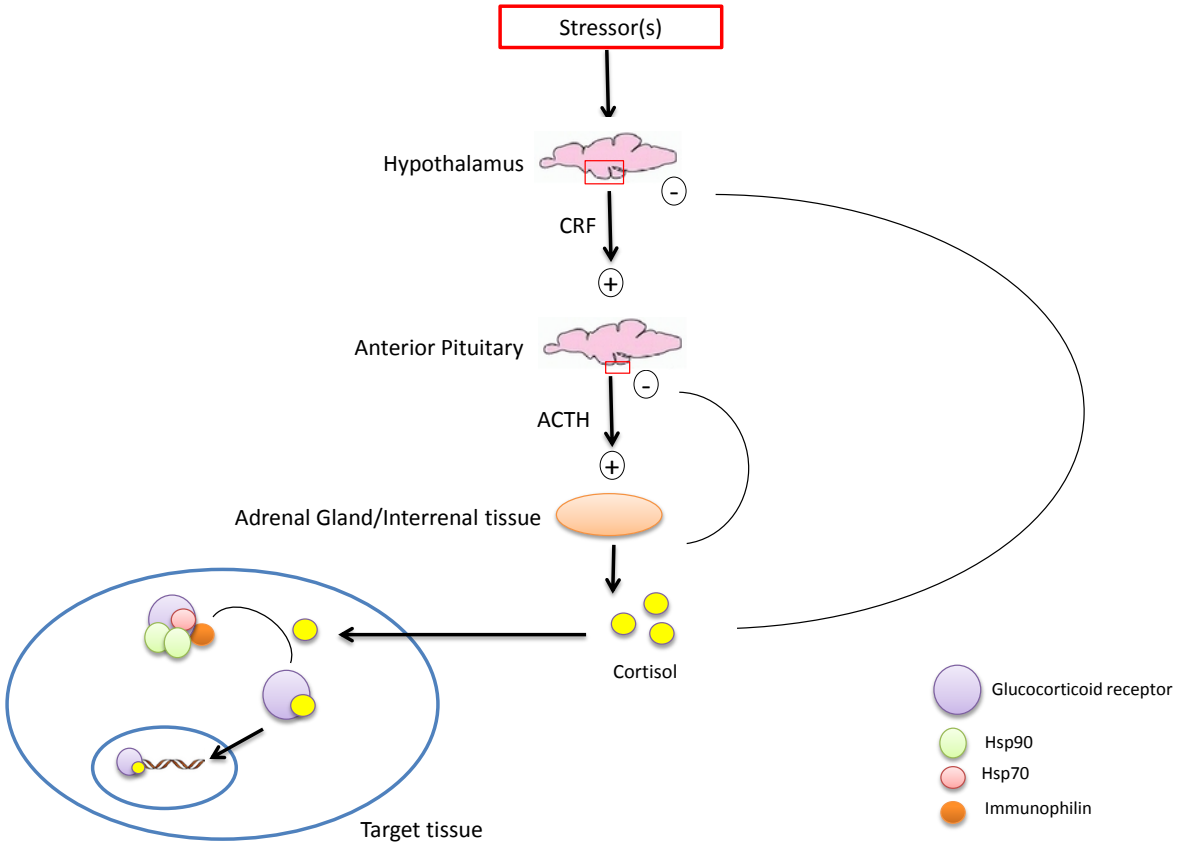

Figure 1.2: The Hypothalamus-Pituitary-Interrenal (HPI) axis

In teleosts, the stress axis activation involves the release of corticotropin releasing factor (CRF) from the hypothalamus, which in turn stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH). ACTH stimulates the interrenal tissue of the teleost head kidney (analogous to the adrenal cortex in mammals), leading to the stimulation of corticosteroid biosynthesis. Cortisol, the primary corticosteroid in teleosts, primarily exerts its effects by binding to either the glucocorticoid receptors (GR) or mineralocorticoid receptors (MR).

1.2 Experimental Rationale

All research regarding the release of Hsp70 and its possible roles in immunoregulatory function and intercellular signaling have been carried out in mammalian models. There have been no studies of eHsp70 in fish, beyond preliminary detection in the medium in response to LPS exposure (Zhang et al., 2011) or stress hormones (Henrickson, 2010). It has been proposed that eHsp70 during stress may be essential for the maintenance of homeostasis and may have important physiological implications on the health of the organism, but this is yet to be tested. Given this, measurement of eHsp70 in fish has practical applications for studying the impact of stress in fish, using minimally invasive measures. Therefore this project will, for the first time, try and characterize the release of eHsp70 in rainbow trout (*Oncorhynchus mykiss*) both *in vivo* and *in vitro* and examine how its release is modulated by various stressors and the stress hormone cortisol. The hypotheses tested are: i) Hsp70 will be released into the plasma and its release is modulated in response to stressor exposure, ii) stressor–induced cortisol elevation will modulate extracellular release of Hsp70 in rainbow trout hepatocytes, and iii) eHsp70 will elicit an immune response in trout hepatocytes.

1.3 Research Objectives

1. Determine stressor-induced changes in plasma Hsp70 levels in rainbow trout *in vivo* (Chapter 2)
2. Establish stressor-induced release of Hsp70 from trout hepatocytes *in vitro* (Chapter 3)
3. Determine the role of cortisol in regulating Hsp70 release from trout hepatocytes *in vitro* (Chapter 3)
4. Determine the effect of immune-stimulant on eHsp70 release, and its modulation by cortisol, in trout hepatocytes (Chapter 4)
5. Determine the effect of exogenous Hsp70 exposure on immune response in trout hepatocytes (Chapter 4)

Chapter 2
Modulation of Plasma Hsp70 Levels in Rainbow Trout

2.1 Introduction

An organism's response to stress can be generally categorized into primary, secondary and tertiary responses. The primary response is characterised by the rapid release of catecholamines, epinephrine and norepinephrine, in addition to the release of corticosteroids (Wendelaar Bonga, 1997). Cortisol, the primary corticosteroid in teleosts, primarily exerts its effects by binding to either the glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) (Iwama et al., 2006). The secondary response is characterized by an increase in metabolic rate and a subsequent increase in gluconeogenesis and plasma glucose. The prolonged effects of stress are classified under the tertiary response in which the aforementioned physiological changes will lead to reduction in growth and reproduction (Wendelaar Bonga, 1997).

One aspect of the primary stress response involves changes at the cellular level to re-establish homeostasis. This is characterized by the heat shock response, which is essential in increasing the survival of a cell in response to a variety of stressors (Pardue et al., 1992). In the presence of a stressor, such as heat, changes occur in membrane permeability leading to a drop in cytosolic pH and changes in ion homeostasis as well as a decrease in the rate of transcription and translation effectively halting cell growth and proliferation (Ritcher et al., 2010). Degradation of the cytoskeleton and localization of internal organs are also affected leading to a breakdown in intracellular transport processes. The subsequent heat shock response is characterized primarily by the induction of heat shock proteins (HSPs) which can be broadly categorized into seven classes, based on intracellular function (Richter et al., 2010). The class of heat shock proteins which act as molecular chaperones are comprised of five families of highly conserved ubiquitous proteins. In general, these HSPs offer cytoprotection during stressful periods through folding and refolding of denatured proteins, maintaining protein structure, protein translocation across membranes, preventing protein aggregation, and degradation of unstable proteins (see reviews, Parsell and Lindquist, 1993 and Kregel, 2002). They are classified into families based on their molecular mass ranging from the large Hsps (>110 kiloDalton; kDa) to small Hsps (>40 kDa). Hsps are not only highly conserved among species but they also comprise 5-10% of the total proteins found in the cell during unstressed periods (Pockley, 2003). One of the most abundant and highly conserved of these proteins is the 70 kDa

family, comprising the inducible HSP70 and the constitutive HSC70. The intracellular expression of these two isoforms in response to stressors has been well characterized in animals, including fish (see review Iwama et al., 1999).

The interaction between the primary endocrine stress response and the cellular stress response is still far from clear. While cortisol and Hsps has been previously documented in fish (Vijayan et al., 2005) the results are variable. This suggests that the effects of cortisol on Hsps are dependent on experimental design, species, and even tissue type. Cortisol attenuates the heat shock response, in gill and liver of rainbow trout (*Oncorhynchus mykiss*; Basu et al., 2001; Boone et al., 2002a) and common carp exposed to copper (*Cyprinus carpio*; De Boeck et al., 2003); however, exogenous cortisol exposure did not affect hepatic levels of Hsp70 or *hsp70* mRNA levels in silver sea bream (*Sparus sarba) in vitro* or in cutthroat trout (*Oncorhynchus clarkii*) *in vivo* (Ackerman et al., 2000). While cortisol also decreased *hsp90* mRNA abundance in primary cultures of trout hepatocytes (Sathiyaa et al., 2001), protein expression of Hsp90 was elevated *in vivo* in the trout liver (Vijayan et al., 2003). These variations highlight the need for establishing a baseline of how the organismal stress response *in vivo* will affect the cellular stress response, as this is far from clear (Ritcher et al., 2010).

Although the role of intracellular Hsp70 has been well characterized, the extracellular release of Hsp70 (eHsp70)(Tytell et al., 1986; Hightower and Guidonin, 1989) has been observed and several novel roles for this protein have been speculated (Calderwood et al., 2007). However, the mechanism of release from the cell and the possible functions this protein may have once in the extracellular milieu has yet to be established. Preliminary research in mammals suggests that the release of extracellular Hsp70 (eHsp70) is stimulated by a variety of pathological and environmental stressors and may signal danger to the organism (Calderwood, 2007). It is known that Hsp70 lacks the leader sequence which allows proteins to be secreted through the endoplasmic reticulum (ER) and the Golgi apparatus (Calderwood et al., 2007). Consequently, several methods of release have been hypothesized in mammalian models, including: i) release through cell necrosis (Calderwood et al., 2007), ii) active transport across the plasma membrane by ATP binding cassette (ABC) protein transporters (Mambula and Calderwood., 2006), iii) exosomal secretion (Hunter-Lavin et al., 2004; Lancaster and Febbraio., 2005) and iv) direct association with membranes (Alder et al., 1990; Gehrmann et al., 2008). However, no conclusive results on the mode of secretion of HSP70 have been reported. Also, a functional role for eHSP70 is unclear, but recent studies have hypothesized an immunoregulatory role for this protein in mammalian models (Calderwood, 2007)

While most studies on eHsps have been carried out in mammalian models, very little is known about eHsp dynamics in response to stress in lower vertebrates. While further work on the physiological implications of plasma Hsp70 are required, the characterization of plasma Hsp70 in fish has important implications in monitoring the stressed state of the organism, and the quality of their environment. Therefore, the objectives of this study were to i) to further refine already developed methods for immunodetection of Hsp70 (Henrickson, 2010) in order to quantify plasma Hsp70 levels, and ii) to determine if stressor exposure (heat shock) will alter plasma Hsp70 levels in rainbow trout *in vivo*.

2.2 Materials and Methods

2.2.1 Experimental Fish

Immature rainbow trout (110 ± 20 g body mass) were obtained from Alma Research Station (Alma, Ontario) and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1°C on a 12-h light: 12-h dark cycle. The fish were fed once daily to satiety with commercial trout pellet (Martin Mill, Elmira, Ontario). The fish were acclimated for 2 weeks before the experiments.

2.2.2 Experimental Design

2.2.2.1 Short-term Heat Shock Response

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 post-heat shock. Fish were quickly netted and euthanized with an overdose of 2-phenoxy ethanol (1 ml/1L well water; Sigma, St Louis, MO, USA). Blood was collected in tubes containing 500 µl physiological saline (1x Hanks medium, 5mM $NaHCO_3$, 1.5 mM $CaCl_2$ + 5 mM Glucose) with 2mM EDTA per fish and centrifuged for five minutes at 3000 x g to separate the plasma which was stored separately then blood at -80°C for later analysis.

2.2.2.2 Long-term Heat Shock Response

Two groups of 6 fish each were divided into two 100 L tanks in a flow through system using well water maintained at 13°C exactly as above. They were acclimated for a minimum of two weeks. One group of fish was transferred into a tank maintained at 23°C (heat shock group: +10°C heat shock for one hour), while the other group was transferred to another tank maintained at 13°C (control group). Fish were then transferred back to their original tanks at the end of the one hour heat shock exposure. Repeated plasma samples were collected at 1, 4, and 7 days for measuring eHSP70 concentration later. Fish were anaesthetized with 2- phenoxy ethanol (non-lethal dose of 1:10,000; Sigma) and 300 µl of blood was taken from caudal vein with a syringe (22 ½ G). They were also tagged on the first day with fin clippings for identification on subsequent sampling days. Fish were then returned to the tank where they recovered within 10 minutes. On day 7, fish were lethally anaesthetized with an overdose of 2-phenoxy ethanol (1 ml/1L well water; Sigma). Each fish were bled by caudal puncture into a sterilized centrifuge tube containing 500 µl physiological saline (1x Hanks medium, 5mM $NaHCO_3$, 1.5 mM $CaCl_2$ + 5 mM Glucose) with 2mM EDTA. Blood was centrifuged for 5 min at 5000 x g, and plasma was collected and stored separately for Hsp70 detection later.

2.2.3 Sample Analysis

2.2.3.1 Plasma Cortisol and Glucose

Plasma cortisol levels were analyzed using ^3H cortisol radioimmunoassay following established protocols (Ings and Van der Kraak, 2006). Plasma glucose was measured calorimetrically as described previously (Bergmeyer, 1983)

2.2.3.2 Hsp70 ELISA

Plasma Hsp70 was measured using a competitive sandwich ELISA according to Specker and Anderson (1994). Briefly, Chinook salmon Hsp70 recombinant protein (Stressgen) was used to coat wells of a medium binding 96-well plate with 50 mM carbonate buffer, pH 9.6. After washing with Tris-buffered saline with Tween 20 (TTBS) and blocking with 1% BSA, previously incubated samples or standards was added (1:1 ratio of homogenate to polyclonal rabbit anti-salmon Hsp70 primary antibody; 1:100,000, StressMarq). After overnight incubation, wells were washed and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:5000; BioRad) diluted in 5% skim milk was added to each well. Following a1 hour incubation the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 h the reaction was stopped with 8.5M

acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

2.2.3.3 Enzyme Activity: Lactate Dehydrogenase

Lactate dehydrogenase (LDH: EC 1.1.127) activity was measured in the plasma to assess putative cellular rupture and cytosolic contamination. LDH were measured in 50 mM imidazole-buffered enzyme reaction (0.12 mM NADH initiated with 1 mM pyruvic acid; pH 7.4; Sigma) at 22°C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax) as described previously in Vijayan et al. (2006).

2.2.4 Statistical Analysis

The experimental design utilized a two-way ANOVA for statistical comparison between treatment (control and heat shock) and time as independent factors. 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 was log transformed, wherever necessary, 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 Hsp70 Validation

Validation of an immunoassay is characterized by precision, sensitivity, and accuracy (Specker and Anderson, 1994). The primary antibody (Polyclonal rabbit anti-salmon Hsp70) was previously shown to cross react with the inducible Hsp70 protein in rainbow trout (Henrickson, 2010). The refinement of the ELISA to detect low levels of the plasma Hsp70 included validation of the immunoassay. Precision is expressed as the reproducibility of a result indicated by intra-assay and inter-assay coefficient of variation (%CV= [SD/mean]*100), and should general be <20% (Specker and Anderson, 1994). The overall all intra-assay precision was calculated to be 3.46% and the inter-assay 25.55% (See Appendix A). Sensitivity can be defined as the least detectable sample concentration that is significantly different from the zero standards, which was determined by running a series of low standards to determine the lowest standard different from the zero (Specker and Anderson, 1994). For this ELISA the lowest detectable limit was determined to be 5 ng/ml. Accuracy can be evaluated by measuring parallelism and recovery. A serial dilution of heat shocked RBCs (Fig

2.1B) shows step-wise decreases with increasing dilution, mirroring the standard curve, ensuring that samples can be run different dilutions. The standard curve (shown as % B/Bo) ranges from 100% to approximately 30% (Fig 2.1 A). Taken together the ELISA was able to reliably detect levels of plasma and tissue Hsp70.

2.3.2 Short-term Heat Shock Response

2.3.2.1 Plasma Hsp70 levels

Over the 24 h sampling period there was no statistically significant treatment (control vs. heat shock) or time effects (1, 4 and 24 h) in plasma Hsp70 levels (Fig: 2.2 A).

2.3.2.2 Enzyme Activity: Lactate dehydrogenase (LDH)

There was no temporal differences or treatment effect in plasma LDH levels between the control and heat-shocked trout over a 24h period (Fig. 2.2 B).

2.3.3 Long-term Heat Shock Response

2.3.3.1 Plasma Cortisol and Glucose levels

When exposed to a +10°C heat shock (23°C) plasma cortisol levels were significantly decreased (2.7-fold) at 7 d when compared with 1d. However, there was no statistical difference between 4d and 1d or 7d post-heat shock. In the absence of heat shock, at each sampling time point (1d, 4d and 7d) there was no statistical difference in plasma cortisol levels between control and heat shocked fish (Fig 2.4 B).

At 1d plasma glucose levels were 1.2 fold higher in controls (4.7± 0.2 mM), compared to the heat shocked group (3.9 ± 0.2 mM). At both 7d and 4 d there was no statistical difference in plasma glucose levels between control and heat shocked fish (Fig 2.4 A).

2.3.3.2 RBC Hsp70 levels

Exposure to a +10 ºC heat shock above ambient temperature caused a 1.7-fold increase in heat-shocked RBC Hsp70 levels (431 ± 91.1 ng/ml) at 1 d post heat shock when compared to control levels (256 ± 47.9 ng/ml). By 4 d and 7 d there was no statistical difference in RBC Hsp70 levels between control and heat-shocked fish RBC Hsp70 levels. There was a significant treatment effect with heat-shocked fish having significantly higher Hsp70 levels compared to the control group (Fig 2.3 A).

2.3.3.3 Plasma Hsp70 levels

There was a temporal difference in plasma Hsp70 levels in response to stress in the present study. Two-way ANOVA indicated a significant sampling time effect (1 d, 4 d and 7d); plasma Hsp70 levels were significantly higher at each sampling day compared to the previous sampling day (Fig. 2.3B). Also, there was significant interaction between treatment (heat shock vs control) and sampling time in the present study. In the absence of heat shock, there was a 1.6-fold increase in plasma Hsp70 levels in the control fish at day 7 compared to day 1, while there was no statistical difference between day 4 and day 1 control fish. In the heat shocked group, plasma hsp70 levels were significantly higher at day 4 and day7 compared to day 1, while there was no statistical difference in hsp70 levels between day 4 and day7 (Fig. 2.3 B). In the heat shock group, there was a significant decrease in plasma Hsp70 levels at day 1 (1.6-fold) and day 7 (1.3-fold) compared to the control group. There was no difference in plasma Hsp70 levels between the control and heat shock group at day 7.

2.3.3.4 Plasma Lactate Dehydrogenase levels

There was no temporal difference in LDH levels nor was there a difference between levels in control or heat-shocked fish at each sampling time point

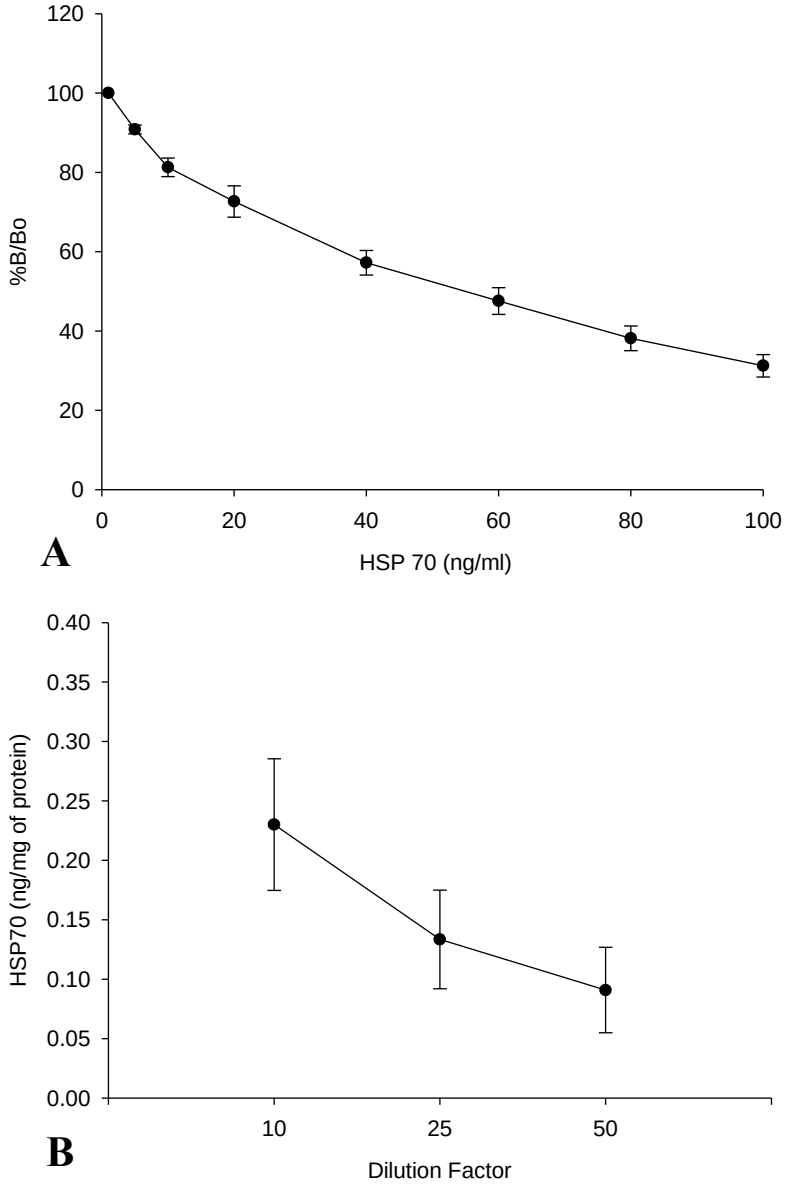

Figure 2.1: Standard Curve and Serial Dilution

Hsp70 standard curve for the ELISA (A) from 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. A serial dilution of heat shocked RBCs (+10ºC above ambient temperature) using the ELISA (B). The dilution factor is shown along the x axis and corresponding Hsp70 values along the y-axis.

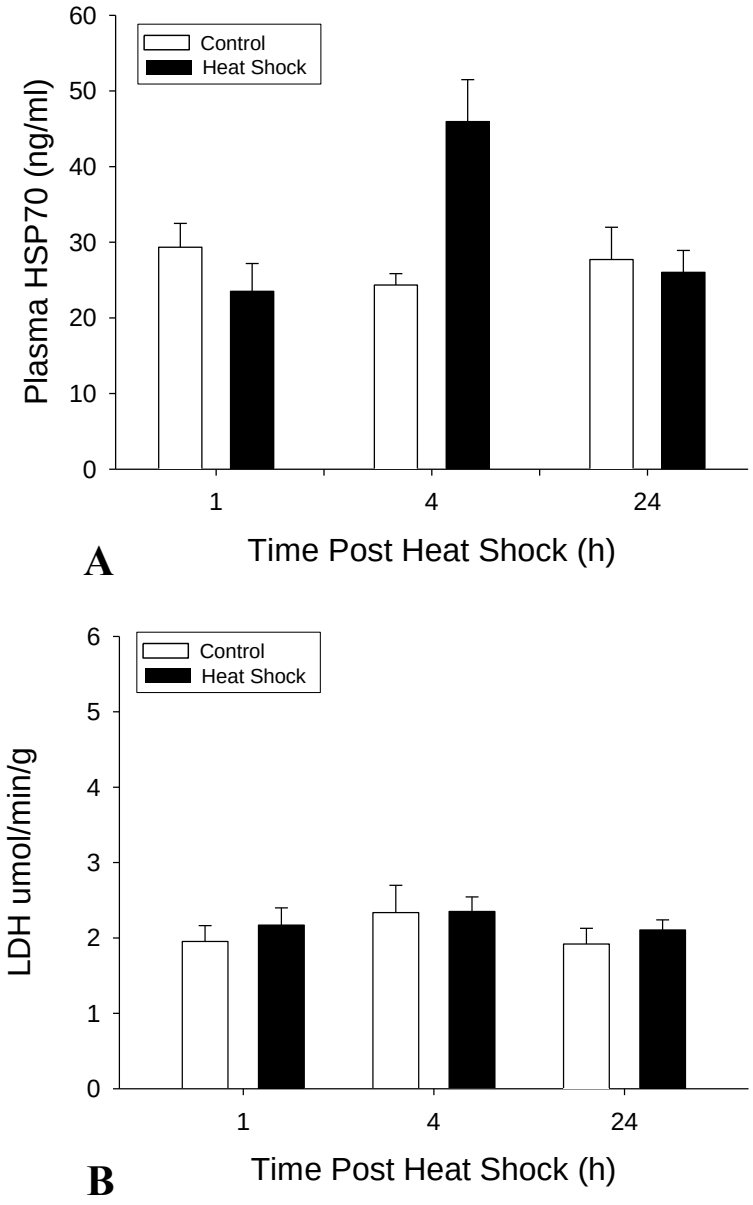

Figure 2.2: Short Term Heat Shock Response:

Plasma Hsp70 (A) and lactate dehydrogenase (LDH) levels (B) in rainbow trout exposed to heat shock. Plasma samples were collected from trout exposed to either no heat shock (control) or one hour heat shock (+12°C above ambient temperature) and then allowed to recover at ambient temperature (13°C). Samples were collected immediately at 1, 4, and 24 hours post heat shock. Inset shows significant treatment effect ($p < 0.05$; two way ANOVA); values represent means ± SEM (n = 5-6). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

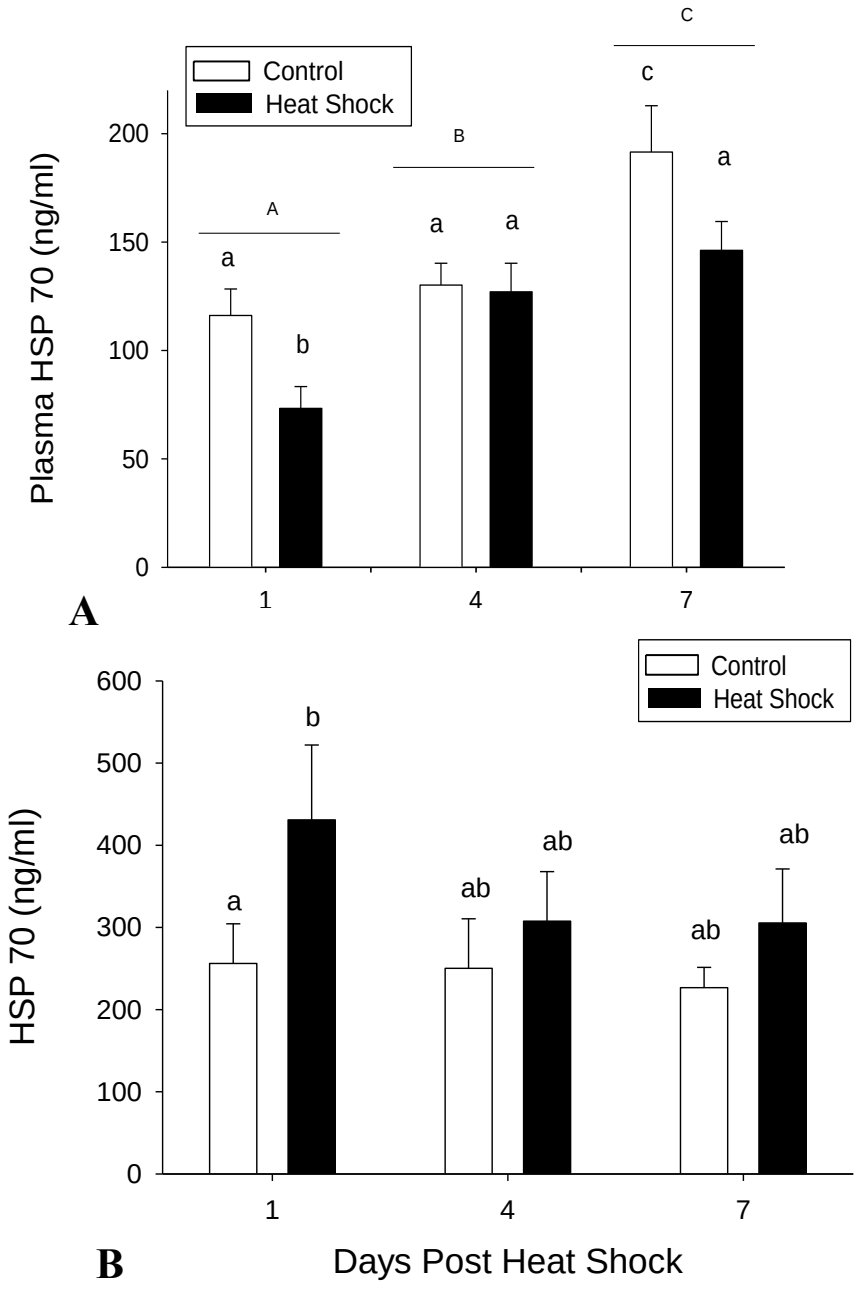

Figure 2.3: Long Term Heat Shock Response:

Plasma Hsp70 (A) and intracellular Hsp70 (B) levels in rainbow trout exposed to heat shock. Plasma samples were collected from trout exposed to either no heat shock (control) or one hour heat shock (+10°C above ambient temperature) and then allowed to recover at ambient temperature (13°C). Samples were collected immediately at 1, 4, and 7 days post heat shock. Inset shows significant treatment effect ($p < 0.05$; two way ANOVA); values represent means ± SEM ($n = 5$-6). Bars with different capital letters (A,B,C) indicate significant time effect, while bars with different lower case letters (a, b, c) denote significant treatment effect ($p<0.05$, two way repeated measures ANOVA).

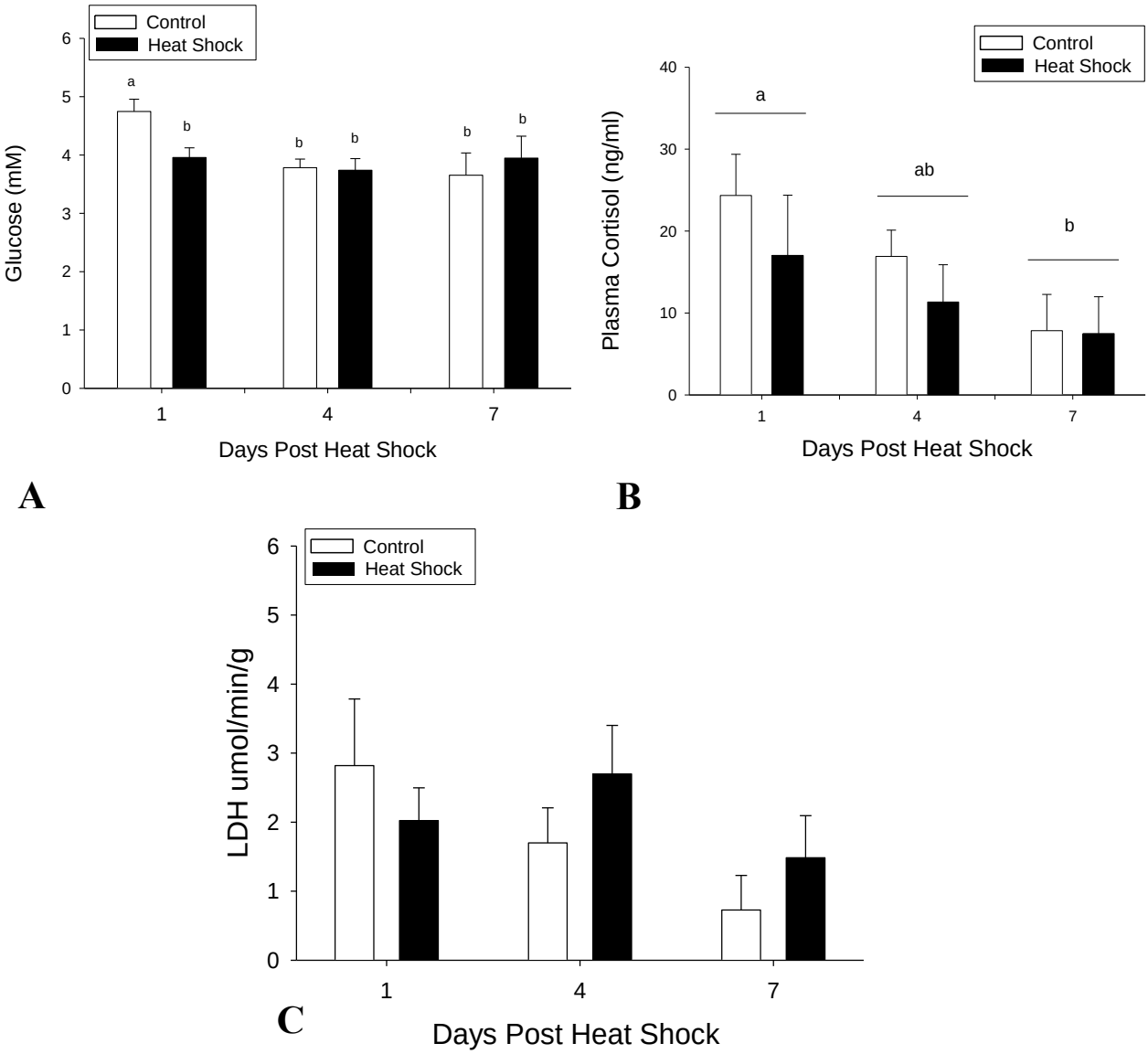

Figure 2.4: Long Term Stress Response

Plasma glucose (A), cortisol (B) and lactate dehydrogenase (LDH) levels (C) in rainbow trout exposed to heat shock. Plasma samples were collected from trout exposed to either no heat shock (control) or 1 h heat shock (+10°C above ambient temperature) and then allowed to recover at ambient temperature (13°C). Samples were collected at 1, 4, and 7 d post-heat shock. Values represent means ± SEM (n = 5-6). Bars with different letters are significantly different ($p<0.05$, two way repeated measures ANOVA).

2.4 Discussion:

A competitive antibody-capture ELISA that specifically detects inducible Hsp70 concentration in rainbow trout plasma and tissue was developed. Quantification of these proteins has been carried out using a variety of methods, such as western blotting which only gives a semi-quantitative representation of changes in Hsp expression. Comparatively, ELISAs are a sensitive method, especially for quantification of proteins that are in low abundance in cells. ELISAs have been used previously in mammalian studies (Skidmore et al., 1995), and semi-quantitative ELISAs have also been developed to increase the specificity of Hsp70 detection in fish (Vijayan et al., 1997; De Boeck et al., 2003; Parmini & Rani, 2008). However, there is no commercially available ELISA kit available for fish. In the present study, the use of a salmonid Hsp70 antibody and standards made of recombinant salmon Hsp70 protein ensured specificity to the species of interest and this was confirmed through western blotting (Henrickson, 2010).

For the first time we show that Hsp70 is released into the plasma in rainbow trout. We further demonstrate that this release is modulated over the long-term, by both heat and handling stress. The intracellular RBCs levels of Hsp70 were elevated in both studies as expected by 24 h post heat shock (Henrickson, 2010 and Fig: 2.2A). However, when examining plasma Hsp70 level over the short-term sampling period, no significant difference between control and heat shocked groups were observed (Fig: 2.2A). Interestingly when fish were sampled over 7d we again observed release of Hsp70 into the plasma; however, this release was attenuated by heat shock (Fig: 2.3A).The lack of correlation between RBC Hsp70 levels and plasma levels suggests that either extracellular Hsp70 levels may not be entirely dependent on intracellular levels, or more likely RBCs are not the primary source of Hsp70 in the plasma. The cellular source of plasma Hsp70 is indeterminable and may be a result of the release by multiple tissues. While not directly comparable, these results are in contrast to mammalian models which have shown Hsp70 elevation *in vitro* in a variety of cells in response to heat shock (Guzhova et al., 2001; Hunter-Lavin et al., 2004; Lancaster and Febbraio, 2005; Calderwood and Mambula 2006) as well as exercise (Walsh et al., 2001). Over the long term sampling, lower plasma Hsp70 levels in heat-shocked fish compared with the controls at both 4d and 7d could be attributed to protein degradation; however, there are also alternative explanations based on mammalian models. Firstly, lower plasma Hsp70 suggests an increased demand intracellularly which may restrict the release into the extracellular environment, through yet unidentified mechanisms. This heat induced restriction in Hsp70 was observed to only occur over a certain heat threshold in human prostate cancer (PC-3) and human prostate adrenocarcinoma (LNCaP) cell lines

(Mambula and Calderwood, 2006). Secondly, the decreased levels in the plasma, despite intracellular elevation may indicate an increase in cellular uptake of Hsp70. It has been hypothesized that Hsp70 is released in order to aid cells deficient in this protein (Calderwood et al., 2007). Receptor mediated endocytosis has been previously demonstrated *in vitro* in human antigen presenting cells (APCs; Arnold-Schild et al., 1999) and in human neuroblastoma cells (Guzhova et al., 2001).

Another novel finding of this study was the modulation of plasma Hsp70 by handling stress. While it has been previously established that handling stress does not affect intracellular Hsp70 levels in rainbow trout (Vijayan et al., 1997), and was further confirmed in this study, the rise in plasma Hsp70 over the 7d period in non-heat shocked fish suggests a longer-term modulation of this protein in circulation by a handling stressor (Fig: 2.3 A). The lack of correlation between intracellular and extracellular Hsp70 indicates that the release may not be entirely dependent on intracellular levels; however, the physiological implications remain to be determined.

Enhanced energy demand due to temperature elevation will cause metabolic adjustments to maintain homeostasis (Iwama et al., 2006). The effect of heat shock on cortisol, glucose and tissue Hsp70 levels have been discussed previously (Henrickson, 2010). When fish were sampled over a longer time period (7d) heat shock elicited both an organismal and cellular stress response in rainbow trout. The organismal stress response was evidenced by elevated plasma cortisol and glucose levels, which are key indicators of stress (Iwama et al., 2006). While plasma cortisol was not elevated in the heat shock group as observed in the first study (Fig: 2.4B) this is expected as cortisol returned to resting levels by 24h, and the second study had sampling over the course of 7d. Furthermore, it has been previously established that cortisol will increase gluconeogenesis in the liver (Aluru et al., 2007; Sathiyaa et al., 2009) and given that plasma cortisol levels were not elevated in response to heat shock the corresponding rise in glucose levels was not observed (Fig:2.4A).

The ability to measure the amount of Hsp70 in the blood has particular importance as it allows for repeated sampling of blood from the same animal, eliminating the need to sacrifice the whole animal to collect other tissues. Heat shock in red blood cells has been well documented (Currie et al., 1997; Currie et al., 1999) and Hsp70 levels have been shown to increase significantly with exposure to heat shock, which was confirmed in this study. 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, 2011).

In conclusion, we developed a sensitive ELISA to quantify both intracellular and plasma Hsp70 levels in rainbow trout. We further demonstrate that plasma Hsp70 is modulated by both heat shock and

handling stress over a 7d period. While this suggests the importance of this protein in both the cellular and generalized stress response, the physiological implications of eHsp70 remains to be determined.

Chapter 3
Modulation of Hsp70 Release in Rainbow Trout Hepatocytes

3.1 Introduction

The stress response can be broadly categorized into organismal and cellular responses. The organismal stress response is characterized by the release of catecholamines and glucocorticoids. Cortisol, the primary glucocorticoid in teleost fish, is responsible for various physiological processes such as immunoregulation, growth, and reproduction (Mommsen et al., 1999). Cortisol is synthesized in the head kidney interrenal tissue in teleosts, analogous to the adrenal cortex of mammals. Activation of the hypothalamus-pituitary-interrenal axis in fish is necessary for the synthesis and secretion of cortisol. Briefly, corticotropin releasing factor (CRF) produced in the hypothalamic preoptic area stimulates the pituitary corticotropes to secrete adrenocorticotropic hormone (ACTH). ACTH stimulates the biosynthesis and secretion of cortisol from the interrenal tissue (Iwama et al., 2006). The effects of cortisol on target tissues are mediated by either the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR). Most teleost species express a single MR and two GRs (GR1 and GR2), with the exception of zebrafish where only one GR has been detected in the genome (Alsop and Vijayan, 2008, 2009). The glucocorticoid receptor is known to associate with several heat shock proteins, specifically Hsp56, Hsp70 and Hsp90 (Basu et al., 2003).

The cellular stress response is characterized by the expression of heat shock proteins (Hsps) which are a large family of highly conserved proteins essential for establishing cellular homeostasis (see reviews by Parsell and Lindquist, 1993; Kregel, 2002). Hsps are categorized by molecular weight with the most extensively studied group being Hsp70 (heat shock proteins ~ 70 kDa). Of the two isoforms, one is constitutively expressed (Hsc70), and the other is induced during times of stress (Hsp70). The primary role of intracellular Hsp70 is cytoprotection and it is responsible for reducing protein aggregation and refolding denatured proteins, thus improving cell survival when exposed to a stressor (Parsell and Lindquist, 1993; Kregel, 2002). While the intracellular role of Hsp70 has been well characterized in fish (see reviews Iwama et al., 1999; Deane and Woo, 2011), little is known about Hsps released into the extracellular space (eHsps) in both mammals and lower vertebrates. There is increasing evidence from mammalian studies that Hsp70 is released into the extracellular environment (eHsp70) from different tissues, including brown fat (Campisi and Fleshener, 2003), neuronal cells (Tytell et al., 1986), glial cells (Guzhova et al., 2001), β cells (Clayton et al., 2005), tumor cells (Gastpar et al., 2005), and human peripheral blood mononuclear cells (Lancaster and

Febbraio, 2005). Furthermore, the release of this protein is independent of cell death, suggesting active secretion from the cells (Broquet et al., 2003; Hunter-Lavin et al., 2004; Hightower et al., 1989). Recently, studies in fish have shown that Hsp70 is released into the circulation *in vivo* (Chapter 2) as well as from cells *in vitro*, including peripheral blood lymphocytes (Zhang et al., 2011) and red blood cells (Henrickson, 2010), but the mechanisms involved have yet to be studied in fish. The stress-steroid cortisol has been shown to regulate the intracellular expression of Hsps in fish. For instance, elevated cortisol levels attenuate the intracellular heat shock response in several species of fish, including cutthroat trout (*Oncorhynchus clarkii*; Ackerman et al., 2000), rainbow trout (*Oncorhynchus mykiss*; Basu et al., 2001: Boone et al., 2002) and the Mossambique tilapia (*Oreochromics mossambicus*; Basu et al., 2001). Cortisol also attenuated the gene expression of *hsp90* in primary cultures of trout hepatocytes (Sathiyaa et al., 2001). In sea bass head kidney cortisol caused an increase in Hsp70 and Hsp90 levels at both 3 and 7 d post treatment (Celi et al., 2012) and Hsp90 and Hsc70 were both increased above control levels in the liver when exposed to elevated levels of cortisol (via intraperitoneal injection) in rainbow trout (Vijayan et al., 2003). However, cortisol injections had no effect on Hsp70 levels in sea bream (Deane et al., 2000). The mode of action of cortisol in modulating the Hsp70 expression in fish tissues is far from clear. While GR signaling is critical for cortisol action, the role of receptor activation in Hsp70 modulation has not been assessed. This may be an important factor as elevated levels of cortisol, similar to that seen in stressed fish, down regulate GR expression in fish (Vijayan et al., 2003).

Taking these findings into account, the present study tested the hypothesis that stressed levels of cortisol seen in trout will enhance the release of Hsp70 from fish cells and this response is mediated by GR activation. A well-established GR antagonist, mifepristone (RU486), was used to pharmacologically block the activation of GR as described previously (Aluru and Vijayan, 2007). Trout hepatocytes in primary culture were used as a model cell system to test Hsp70 regulation because liver is a key target for cortisol action (Mommsen et al., 1999). Trout hepatocytes have been used previously to assess stress hormone modulation of the heat shock response (Boone et al., 2002b). While several studies have examined the effects of steroid hormones such as cortisol on intracellular Hsp70 levels, this is the first study to examine the modulatory effects of cortisol on the release of Hsp70 in fish.

3.2 Materials and Methods

3.2.1 Experimental Fish

Immature rainbow trout (150± 20 g mean body mass) were obtained from Alma Research Station (Alma, Ontario) and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1°C on a 12: 12-h light dark cycle. The fish were fed once daily to satiety with commercial trout pellet (Martian Mill, Elmira, Ontario). The fish were acclimated for 2 weeks before the experiments.

3.2.2 Primary culture of trout hepatocytes

Trout hepatocytes were isolated by *in situ* perfusion of liver with collagenase (Sigma, St Louis, MO, USA) exactly as described before (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). Trypan blue dye exclusion method was used to confirm hepatocyte viability (> 95%). Cells were plated in six well tissue culture pates (Sarstedt Inc., NC, USA) at a density of 1.5 million cells/well (0.75 million cells/ml) in L-15 medium and were maintained at 13°C for 24 h. For all experiments, samples were collected and the cells and media separated by centrifugation (13,000 x g, 1 min). The cell pellet and media were stored at -80 for later analysis.

3.2.3 Experimental Design

To test whether Hsp70 was being released from hepatocytes and whether this was being modulated by cortisol, hepatocytes from five independent fish were exposed to control media (L-15) or media containing cortisol (100 ng/ml; Sigma), mifepristone (1000 ng/ml Sigma), or a combination of mifepristone and cortisol. In the combination group, cells were incubated with mifepristone 0.5 h before the addition of cortisol. The cells were then subjected to a 1 h heat shock (+15°C above ambient). At the end of the experimental period (4 or 24 h post heat shock), the medium was collected, and the cells were centrifuged (13,000g for 1 min), supernatants removed, and the cell pellets were flash frozen on dry ice and stored at -80 °C.

3.2.4 Sample Analysis

3.2.4.1 Intracellular Hsp70 levels

Hsp70 expression was measured using a direct trout Hsp70 enzyme-linked immunosorbent assay (ELISA) following the protocol of Specker and Anderson (1994) with slight modifications. Briefly, all samples were diluted to 2 µg/ml in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6) and

100 µl of each sample was added to a medium binding 96 well plate (Corning, MA, USA) in duplicate. Chinook salmon Hsp70 recombinant protein (Stressgen, NY, USA) was used to create a standard curve (0 ng/ml – 100 ng/ml) in coating buffer and 100 ul of each were added to the wells in duplicate. After overnight incubation at 4°C the plate was washed with Tris-buffered saline with 0.05% Tween 20 (TTBS) and blocking with 1% bovine serum albumin (BSA; Sigma). After a two hour incubation at room temperature with primary antibody (polyclonal rabbit anti-salmon HSP 70 (1:100,000; StressMarq Biosciences, BC, Canada) wells were washed and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000; BioRad, CA, USA) diluted in 5% skim milk was added to each well. Following a1 hour incubation the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 hour the reaction was stopped with 8.5M acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

3.2.4.2 Hsp70 Release

Hsp70 in the medium was measured using a competitive sandwich ELISA according to Specker and Anderson (1994). Briefly, chinook salmon (*Oncorhynchus tshawytscha*; Hsp70 recombinant protein (Stressgen) was used to coat wells of a medium binding 96-well plate with 50 mM carbonate buffer, pH 9.6. After washing with Tris-buffered saline with Tween 20 (TTBS) and blocking with 1% BSA, previously incubated samples or standards was added (1:1 ratio of homogenate to polyclonal rabbit anti-salmon Hsp70 primary antibody; 1:100,000, StressMarq). After overnight incubation, wells were washed and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:5000; BioRad) diluted in 5% skim milk was added to each well. Following a1 hour incubation the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 h the reaction was stopped with 8.5M acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

3.2.4.3 Enzyme Activity: Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH: EC 1.1.127) activity was measured in the media to assess putative cell rupture and cytosolic contamination. LDH activity was measured in 50 mM imidazole-buffered enzyme reaction (0.12 mM NADH; 1 mM pyruvic acid; pH 7.4) at 22°C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax: Molecular Devices Corp) exactly as described previously (Vijayan et al., 2006). The blank and sample were run in duplicate.

3.2.5 Statistical Analysis

All statistical analysis were performed using Sigmaplot 11 (Systat Software Inc, Chicago, USA) and data is shown as mean ± standard error of mean (SEM). Statistical comparisons used two way repeated measures analysis of variance (ANOVA). The data was log transformed, wherever necessary, to meet the assumptions of homogeneity of variance although non-transformed values are shown in the figures. Significant differences between treatment groups were compared using the Holm-Sidak post hoc test. A probability level of $p<0.05$ was considered significant.

3.3 Results

3.3.1 Intracellular Hsp70 Levels

There was a 4.2-fold increase in intracellular Hsp70 levels at 4 h post heat shock, which was seen to a greater extent at 24 hours where there was an 8.1-fold increase in Hsp70 above levels at 4 h. Furthermore, while Hsp70 levels in the 4h control (5.8 ± 1.22 µg/mg of protein) and 4h heat shock groups (24.5± 7.0 µg/ mg of protein) were not statistically different (Fig.3.1 A) at 24 h the heat-shocked hepatocytes had 19-fold higher Hsp70 levels (199.5 ± 80 µg/ mg of protein) when compared to controls (10.0 ± 3.1µg/ mg of protein; Fig.3.1A). In both the absence and presence of heat shock, there was no statistical difference in hepatic Hsp70 levels in any of the treatments (cortisol, mifepristone, or a combination of cortisol and mifepristone; Fig 3.1 B); however, at 4h, heat-shocked hepatocytes had 2.7-fold higher levels of Hsp70 when compared to controls and at 24 h heat-shocked hepatocytes had 14-fold higher levels of Hsp70 when compared to non-heat shocked controls.

3.3.2 Extracellular Hsp70 Levels

At both 4 and 24 h post-heat shock, Hsp70 levels in the control and heat shock groups were not statistically different (Fig.3.2A). While levels of Hsp70 in the medium were 1.4-fold less in heat shocked cells at 24 h compared to 4 h, there was no significant difference in Hsp70 levels in the medium of the control group at either 4 or 24 h (Fig 3.2 A).

In the absence of heat shock, 4 h post treatment (Fig 3.2 B) there was no statistical difference in medium Hsp70 levels between control (60.4± 9.07µg/ mg of protein) and cortisol-treated hepatocytes (66.5± 38.1 µg/ mg of protein); however, there was a 2.0-fold decrease in Hsp70 levels in medium of mifepristone treated hepatocytes (30.0 ± 2.3 µg/ mg of protein) and a 1.9-fold decrease in medium Hsp70 of hepatocytes treated with a combination of cortisol and mifepristone (31.7± 2.8 µg/ml; Fig 3.2 B), respectively. At 24 h post-heat shock (Fig 3.2 C) however there was a 3.1-fold decrease in the

amount of medium Hsp70 detected in cortisol treated hepatocytes (14.0 ± 5.6 µg/ mg of protein) when compared to Hsp70 levels in medium of untreated controls (43.1 ± 4.0µg/ mg of protein). Addition of mifepristone (35.8 ± 6.1 µg/ mg of protein) and a combination of mifepristone and cortisol (36.0 ± 4.4µg/ mg of protein) abrogated this decrease.

At 4 h post-heat shock exposure medium Hsp70 levels were statistically lower in cortisol (4.2-fold), mifepristone (3.2-fold) and the combination of mifepristone and cortisol-treated (8.2 - fold) hepatocytes when compared to the medium of untreated controls. Medium Hsp70 levels were statistically lower in cortisol (2.8 fold), mifepristone (2.6 fold) and the combination of mifepristone and cortisol-treated (6.5 fold) hepatocytes when compared to the medium of control hepatocytes (37.4 ± 5.7 µg/ mg of protein).

3.3.3 Cell Viability: Lactate Dehydrogenase (LDH)

Cell viability was unaffected as there was no statistical difference in levels of LDH, an intracellular enzyme, detected in the medium as either temporally or a result of treatments (Fig 3.5 A and B)

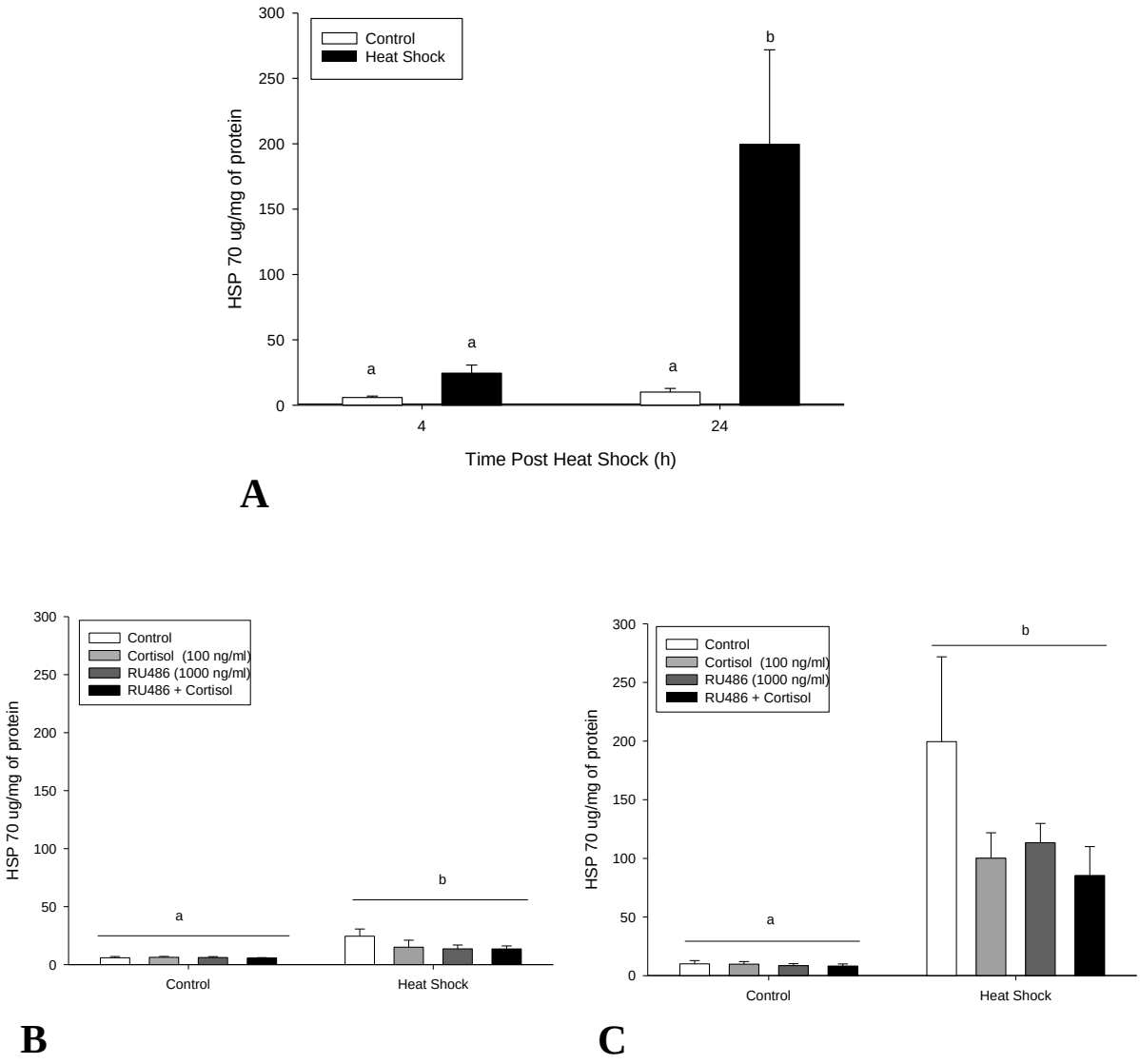

Figure 3.1 Intracellular HSP70 levels

Hsp70 levels of rainbow trout hepatocytes subjected to either heat shock *in vitro* at 4 h and 24 h (A) or treated with cortisol and its receptor antagonist at 4 h (B) and 24 h (C) after a heat shock. Hepatocytes were exposed to either control media, cortisol (100 ng/ml), RU486 (mifepristone; 1,000 ng/ml), or a combination of RU486 + cortisol (in the combination group, RU486 was added to the hepatocytes 30 min before cortisol addition), and subjected to a heat shock (+15°C above ambient temperature [13°C] for one hour before allowing recovery at ambient temperature. Samples were collected at 4, and 24 hours post heat shock. Hsp70 was detected via a direct ELISA using a polyclonal rabbit anti-salmonid antibody (1:10,000 dilution). Values represent means ± SEM (n = 5). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

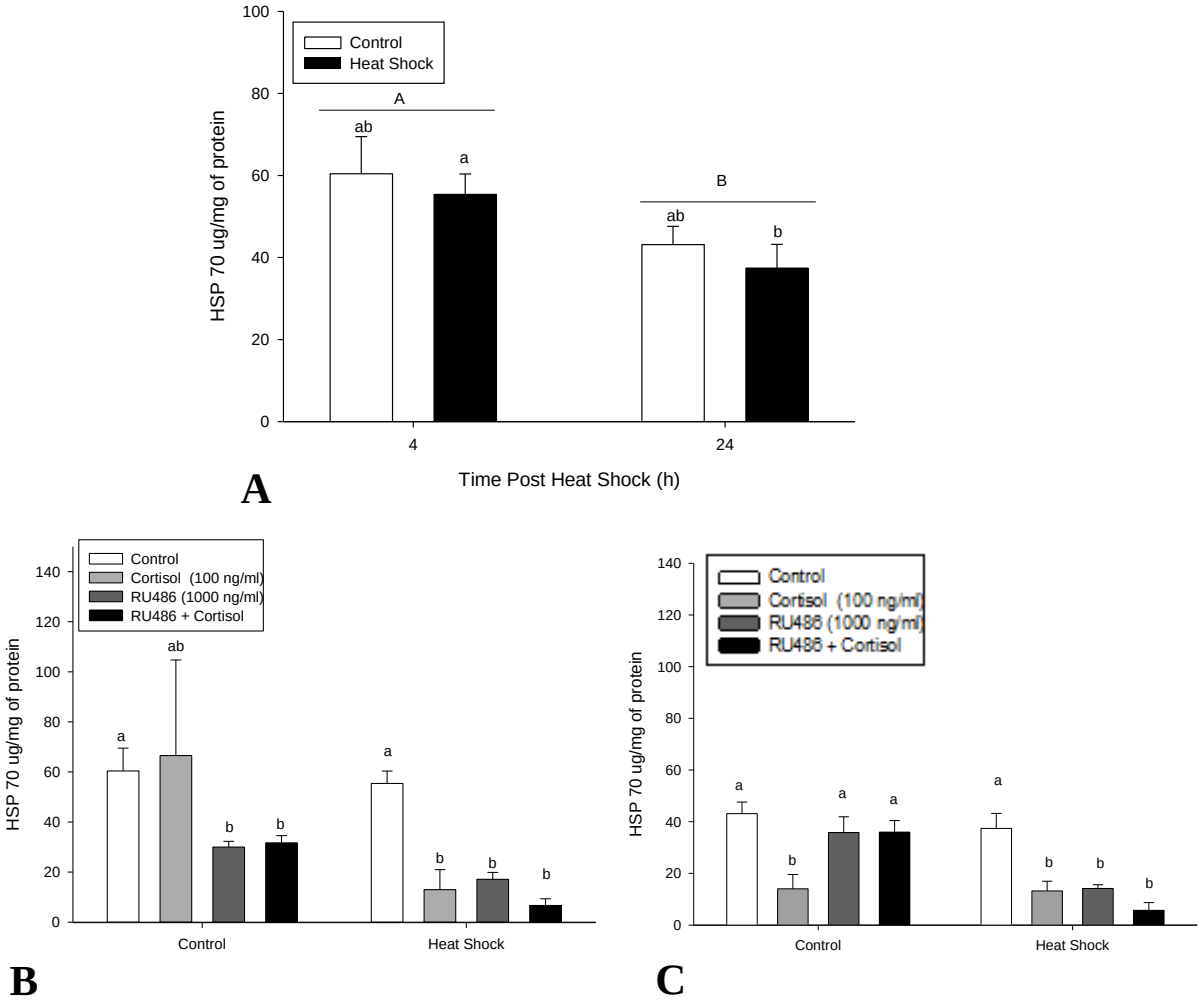

Figure 3.2 Extracellular Hsp70 levels

Hsp70 levels in medium collected from rainbow trout hepatocytes subjected to either heat shock *in vitro* (A) or exposed to cortisol and its receptor antagonist at 4 h (B) and 24 h (C) after a heat shock. Hepatocytes were exposed to either control media, cortisol (100 ng/ml), RU486 (1,000 ng/ml), or a combination of RU486 + cortisol (in the combination group, RU486 was added to the hepatocytes 30 min before cortisol addition), and subjected to a heat shock (+15°C above ambient temperature [13°C] for one hour before allowing recovery at ambient temperature. Medium samples were collected at 4, and 24 hours post heat shock. Hsp70 was detected via a competitive ELISA using a polyclonal rabbit anti-salmonid antibody (1:100,000 dilution). Values represent means ± SEM (n = 5). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

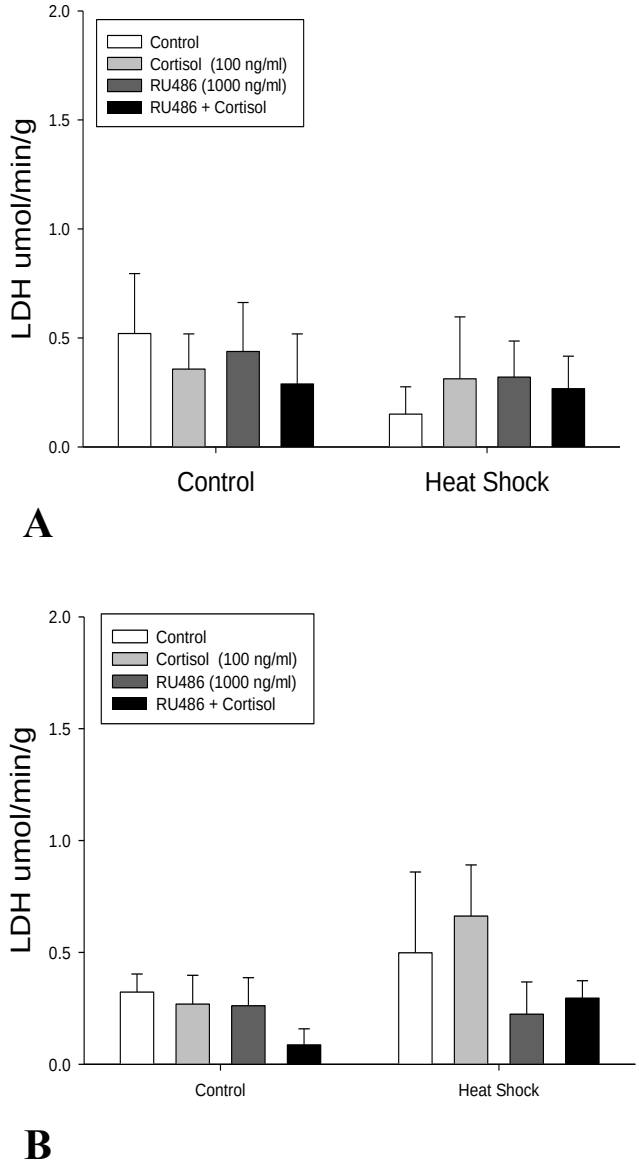

Figure 3.3 Lactate Dehydrogenase (LDH) levels

LDH levels in medium collected from rainbow trout hepatocytes subjected to heat shock *in vitro* at 4 (A) and 24 hours (B) post heat shock. Hepatocytes were exposed to either control media, cortisol (100 ng/mL), RU486 (mifepristone; 1,000 ng/ml), or a combination of RU486 + cortisol (in the combination group, RU486 was added to the hepatocytes 30 min before cortisol addition), and subjected to a heat shock (+15°C above ambient temperature (13°C) for one hour before allowing recovery at ambient temperature. Medium samples were collected at 4, and 24 hours post heat shock. Values represent mean ± SEM (n = 5). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

3.4 Discussion

The results of this study demonstrate for the first time that Hsp70 is actively released from rainbow trout hepatocytes into the extracellular medium and that this release is modulated by physiologically relevant levels of cortisol. This observation provides a framework for elucidating how stress hormones can modulate the cellular stress response, and whether they mediate extracellular stress signaling. The cortisol-mediated decrease in medium Hsp70 levels in the absence of heat shock implicates a novel role for GR in cell stress signaling.

In order to measure Hsp70 in the medium we developed a competitive sandwich ELISA to quantify levels of eHSP (see Chapter 2), as detection via western blotting was not possible due to low levels of medium Hsp70. Comparatively, intracellular Hsp70 was quantified using a direct ELISA, which differs from more traditional methods previously used to report these levels, such as western blotting, because the ELISA measures absolute concentration. The increase of Hsp70 in the liver post heat shock has been previously documented at both the tissue level (Koban et al., 1991; Forsyth et al., 1997) and the cellular level (Koban et al., 1987; Vijayan et al., 1997). Previous work has shown that cortisol attenuates the expression of intracellular Hsp70 levels in fish (Basu et al., 200; De Boeck et al., 2003; Boone and Vijayan, 2002 a,b; Henrickson, 2010). However, in the present study, cortisol did not affect intracellular Hsp70 levels in trout hepatocytes (Fig 3.1 A and B). While the reason is not known, a similar absence of cortisol-mediated effects on Hsp70 expression was observed *in vitro* in head kidney preparations (*Sparus aurata*: Celi et al., 2012) and hepatocytes (*Sparus sarba*: Deane et al., 1999; rainbow trout: Philip et al., 2012) and *in vivo* cutthroat trout liver (*Oncorhynchus clarkii*: Ackerman et al., 2000). Differences in the effect of cortisol on Hsp expression suggests that results may depend on several factors, including tissue type, fish species or experimental conditions.

This study demonstrates for the first time that 24 h after the addition of cortisol there is a significant decrease in medium Hsp70 levels and that this effect can be abrogated with the addition of the GR antagonist, mifepristone, suggesting that the release of Hsp70 may be GR-mediated (Fig: 3.2 B). The regulation of Hsp70 by GR has been shown before in sea bream fibroblasts and macrophages (Deane et al., 2006). Intracellular levels were not modulated by cortisol in the present study; however, whether intracellular levels are indicative of extracellular release remains to be determined. The observed decrease in medium Hsp70 levels after the addition of cortisol at 24 h may be due to several possibilities. Firstly, cortisol may act intracellularly to attenuate the release of Hsp70 through an unknown mechanism or Hsp70 may be subject to re-uptake. Though there was no change in intracellular Hsp70 levels after the addition of cortisol it has previously been established that cortisol

will lower nascent Hsp70 synthesis (Boone et al., 2002b) suggesting that eHsp70 release may also be dependent, in part, on *de novo* synthesis. However, our data supports the possibility that Hsp70 may be subject to re-uptake by hepatocytes as there was an observable decrease in medium Hsp70 levels of cortisol treated hepatocytes at 4 hours compared to 24 hours. While this decrease could be attributed to protein degradation, it has been previously reported that cortisol does not affect the rate of protein degradation intracellularly in hepatocytes (Boone et al., 2002b). Furthermore, uptake of Hsp70 has been established previously in mammalian models including human macrophages and dendritic cells expressing the cell surface protein CD40 (Becker et al., 2002), human neuroblastoma cells (Guzhova et al., 2001), human peripheral blood lymphocytes (Fujihara and Nadler, 1999) and antigen presenting cells (Castellino et al., 2000).

The novel finding of GR-mediated release of Hsp70 highlights the importance of the generalized stress response on the cellular stress response. Briefly, the effect of cortisol on target tissues is mediated intracellularly by the glucocorticoid receptor (GR) and in the absence of hormonal activation, GR exists as a heterodimer of Hsp90, Hsp70 and several other co-chaperones (Pratt and Toft, 2008) . The amount of Hsp70 in the final heterocomplex is variable and the reasons for this are unknown but may be tissue-specific, lending more complexity to GR regulation of Hsp70. Mifepristone is a well-established antagonist which has been previously found to pharmacologically inhibit the transcription factor activity of GR (Aluru and Vijayan, 2008). Whether GR is modulating the release of Hsp70 by altering intracellular levels or regulating this protein through association with membrane associated GR isoforms (Lowenberg et al., 2007) has yet to be determined. Interestingly, in the presence of heat the effect of cortisol no longer appears dependent on GR, and mifepristone independently suppresses the release of eHsp70. This suggests that heat may up-regulate an alternative pathway of release which is more highly regulated by cortisol and sensitive to mifepristone, in order to encourage the cell to retain Hsps to maintain homeostasis during times of stress. The differential regulation of Hsp70 release has been demonstrated before, as Hsp70 release was only regulated through ABC transporters in the presence of heat (Mambula and Calderwood, 2006). We propose that in the absence of heat shock, stress hormones will encourage the cell to retain heat shock proteins through modulation of cytosolic hormone receptors, however in the presence of heat shock it will act more rapidly through modulation of yet unidentified mechanisms to maintain homeostasis by limiting extracellular release of Hsp70. Given that an alternative pathway is sensitive to mifepristone regulation, this suggests that Hsp70 release could be regulated through non-genomic intracellular signaling as it has been shown to induce phosphorylation of the extracellular signal-

regulated kinase (ERK/MAPK) signaling pathway (Chen et al., 2012) or through modulation of the plasma membrane as mifepristone has also been shown to affect membrane order in rainbow trout hepatocytes (Dindia et al., 2012). Further work needs to be completed to fully understand the regulation behind stress signaling and eHsp70 release. Finally the observation that Hsp70 is released in the absence of cell death (as measured by lactate dehydrogenase) indicates a role for active secretion of this protein, however, further work is required to elucidate possible mechanisms of release and the biological significance of this release remains to be investigated in fish.

In conclusion, the novel role of GR signaling in regulating eHsp70 levels during stress has implications in our understanding of how the stress induced endocrine response affects the cellular heat shock response. Furthermore, because heat shock was able to differentially modulate the effects of cortisol on eHsp70 levels this suggests that there may be greater physiological implications in regards to the stress signaling abilities of this molecule.

Chapter 4
Modulation of Extracellular Hsp70 in Rainbow Trout

4.1 Introduction

The cellular stress response is characterized by an induction of heat shock proteins (Hsps), which are a family of highly conserved ubiquitous proteins essential for maintaining cellular homeostasis (Kregel et al., 2002). These proteins have been characterized from bacteria to mammals and are classified by molecular weight, ranging from the large >110 kDa family to the small heat shock protein family that are < 40 kDa (Kregel at al., 2002). The Hsp70 family is the most extensively studied, and they have a variety of intracellular roles based on isoform. Mammalian cells contain several different isoforms, including the constitutively expressed Hsc70, stress inducible Hsp70, mitochondrial Hsp75, and Grp78 localized in the endoplasmic reticulum (Schmitt et al., 2007). Hsp70 has been implicated in various essential intracellular roles, specifically acting as intracellular chaperones, folding nascent or denatured proteins, assisting in the assembly of multi-protein complexes and transporting proteins across cellular membranes (Parsell and Lindquist, 1993 and Kregel, 2002; Schmitt et al., 2007). While most work has been completed in mammals, Hsps have also been well characterized in fish, both in whole animal studies and primary cell cultures (see review by Iwama et al., 1999, Chapter 3, Chapter 4), though the effects of the generalized stress response on the cellular stress response have yet to be fully elucidated.

New roles for Hsp70 are continuously being discovered and the detection of this protein in the extracellular milieu has led to speculation of a role in immune regulation (De Maio, 2011). Extracellular Hsp70 (eHsp70) was first observed to be released from glial cells in the giant squid axon (Tytell et al, 1986), but has since been observed to be released from various mammalian cell types (see review by De Maio, 2011). In teleosts eHsps have been detected in grass carp (*Ctenopharyngodon idella*) from peripheral blood lymphocytes (Zhang et al., 2011), plasma of rainbow trout (*Oncorhynchus mykiss*) (Chapter 2) and extracellular media of trout red blood cells in culture (Henrickson, 2010).

The mechanism by which Hsp70 is released from the cell and what role it plays once released has yet to be fully characterized in mammals or lower vertebrates (De Maio, 2011). Once released eHsp70 is expected to have immunoregulatory targets and roles (Calderwood, 2007). eHsp70 specifically binds to Toll like receptors (TLR) on antigen presenting cells (APC; Asea et al., 2002) and exogenous application of recombinant Hsp70 has been shown to modulate cytokine protein levels in rat

hepatocytes (macrophage inflammatory protein-2; Galloway et al., 2008) and human macrophages (Asea et al., 2003), however the results vary based on both species and tissue type. The mechanism of extracellular release and the role of Hsp70 on target cells have yet to be elucidated in fish, underscoring the novelty of this study.

Given that Hsp70 has been implicated as having immunoregulatory effects, the modulation of these proteins in response to an immune stimulant is important to establish its roles in both the adaptive and innate immune responses. Lipopolysaccharide (LPS), a major component of the outer membrane of gram negative bacteria, will up-regulate cytokines through activation of the CD14 receptor (Wright et al., 1990). Upon stimulation with LPS, an association of Hsp70/Hsp90 with chemokine receptors, and the TLR4/CD14 receptor complex in lipid rafts was initiated, indicating a role for lipid bound Hsp70 in bacterial recognition by the innate immune system (Schmitt et al., 2007). In human PMBCs, treatment with LPS significantly inhibited the expression of Hsp70 (Schroeder et al., 1999). Hsp70 has been shown to associate with high affinity to LPS (Byrd et al., 1999) and will suppress LPS-induced production of pro-inflammatory cytokines through modulation of nuclear factor-kappa B (NF-κB), a key transcription factor that mediates important functions including survival, apoptosis, proliferation, and inflammation (Oeckinghaus et al., 2011) as well as augmenting the production of anti-inflammatory cytokines (Stolte et al., 2009).

In fish LPS has been previously shown to regulate immune responsive genes (Engelsma, 2002; MacKenzie et al., 2006) and increase the cellular stress response and metabolic capacity of trout hepatocytes (Philip et al., 2012). Elevated cortisol levels have recently been shown to have immunosuppressive effects in fish (Tort, 2011), and cortisol is known to modulate immune function by inhibiting LPS-induced cytokine expression in fish immune cells (Zou et al., 2000; Engelsma et al.,2003; MacKenzie et al., 2006). Preliminary effects of endotoxin shock on heat shock proteins in fish show that intracellular levels will significantly increase in rainbow trout hepatocytes following LPS treatment (Philip et al., 2012). The extracellular release of Hsp70 in response to LPS exposure has been preliminarily reported in common carp (Zhang et al., 2011), however its physiological implications have yet to be elucidated. No research has yet been reported on the effects of hsps on immune function in fish. Therefore, this study tested the hypotheses that i) endotoxin shock (LPS treatment) will increase the release of eHsp70 in trout hepatocytes and this is modulated by cortisol, and ii) salmon Hsp70 stimulates the innate immune response in rainbow trout hepatocytes.

4.2 Materials and Methods

4.2.1 Experimental fish

Immature rainbow trout (150± 20 g mean body mass) were obtained from Alma Research Station (Alma, Ontario) and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1°C on a 12: 12-h light dark cycle. The fish were fed once daily to satiety with commercial trout pellet (Martian Mill, Elmira, Ontario). The fish were acclimated for 2 weeks before the experiments.

4.2.2 Primary culture of trout hepatocytes

Trout hepatocytes were isolated by *in situ* perfusion of liver with collagenase (Sigma, St Louis, MO, USA) exactly as described before (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). Trypan blue dye exclusion method was used to confirm hepatocyte viability (> 95%). Cells were plated in six well tissue culture pates (Sarstedt Inc., NC, USA) at a density of 1.5 million cells/well (0.75 million cells/ml) in L-15 medium and were maintained at 13 C for 24 h.

4.2.3 Experimental Design

4.2.3.1 Modulation of Hsp70 release from immune-stimulated cells

The temporal profile of basal Hsp70 release and its modulation by either cortisol (100 ng/ml) or LPS (30 µg/ml) was examined by treating trout hepatocytes over a 24 h period. Freshly isolated trout hepatocytes were plated and the cells allowed to recover for 2 h before replacing the medium with the respective treatments. 20 µl of media was collected at 0, 1, 2, 4, 6, 18 and 24 h and stored at -30 °C for Hsp70 analysis later. This was repeated with hepatocytes isolated from 4 independent fish.
To test whether Hsp70 is released from LPS treated (immune -stimulated) cells, trout hepatocytes in primary culture were exposed to either control media or media containing 10 µg/ml or 30 µg/ml LPS concentrations for 24 h. LPS was used in this experiment to mimic the effects of endotoxin and the concentration used has been shown before to elicit an immune response in trout hepatocytes (Philip et al., 2012). To examine if the release was modulated by the stress steroid cortisol, the above treatments were also carried out either with or without cortisol (100 ng/ml). A subset of these treatment cells were also subjected to a 1 h heat shock (+15°C above ambient) to 28°C and allowed to recover at ambient temperature for 23 h. At the end of the experimental period, the media and cells (obtained by centrifuging at 13,000 x g for 1 min) were flash frozen on dry ice and stored at -80 °C. The experiment was repeated with hepatocytes isolated from five independent fish.

4.2.3.2 Hepatocyte response to Hsp70 exposure

To assess the effect of exogenous Hsp70 on cellular response, hepatocytes from six independent fish were exposed to either control media or media containing recombinant salmon Hsp70 (Stressgen, NY, USA; 40 or 400 ng/ml) with and without LPS (30 µg/ml) over a 24 h period. At the end of the experimental period, the media and cells (obtained by centrifuging at 13,000 x g for 1 min) were flash frozen on dry ice and stored at -80 °C. The experiment was repeated with hepatocytes isolated from five independent fish.

4.2.4 Sample Analysis

4.2.4.1 Intracellular Hsp70 levels

Hsp70 expression was measured using a direct trout Hsp70 enzyme-linked immunosorbent assay (ELISA) following the protocol of Specker and Anderson (1994) with slight modifications. Briefly, all samples were diluted to 2 µg/ml in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6) and 100 µl of each sample was added to a medium binding 96 well plate (Corning, MA, USA) in duplicate. Chinook salmon Hsp70 recombinant protein was used to create a standard curve (0 ng/ml – 100 ng/ml) in coating buffer and 100 ul of each were added to the wells in duplicate. After overnight incubation at 4°C the plate was washed with Tris-buffered saline with 0.05% Tween 20 (TTBS) and blocking with 1% bovine serum albumin (BSA; Sigma). After a two hour incubation at room temperature with primary antibody (polyclonal rabbit anti-salmon Hsp70 (1:100,000; StressMarq Biosciences, BC, Canada) wells were washed and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3000; BioRad, CA, USA) diluted in 5% skim milk was added to each well. Following a1 h incubation, the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 h the reaction was stopped with 8.5M acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

4.2.4.2 Hsp70 Release

Hsp70 in the medium was measured using a competitive sandwich ELISA according to Specker and Anderson (1994). Briefly, chinook salmon Hsp70 recombinant protein (Stressgen) was used to coat wells of a medium binding 96-well plate with 50 mM carbonate buffer, pH 9.6. After washing with Tris-buffered saline with Tween 20 (TTBS) and blocking with 1% BSA, previously incubated samples or standards was added (1:1 ratio of homogenate to polyclonal rabbit anti-salmon Hsp70

primary antibody; 1:100,000, StressMarq). After overnight incubation, wells were washed and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:5000; BioRad) diluted in 5% skim milk was added to each well. Following a1 h incubation the detection reagent was added (41 mM TMB in 200 mM potassium citrate, pH 4). After 1 h the reaction was stopped with 8.5M acetic acid in 0.5 M sulfuric acid. Wells were read at 450 nm using a microplate reader (VersaMax, Molecular Devices, CA, USA).

4.2.4.3 Lactate dehydrogenase activity

Lactate dehydrogenase (LDH: EC 1.1.127) activity was measured in the media to assess putative cell rupture and cytosolic contamination. LDH activity was measured in 50 mM imidazole-buffered enzyme reagent (0.12 mM NADH; 1 mM pyruvic acid; pH 7.4) at 22°C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax: Molecular Devices Corp) exactly as described previously (Vijayan et al., 2006). The blank and samples were run in duplicate.

4.2.4.4 Quantitative real time PCR (qPCR)

Total RNA was isolated from hepatocytes using Trizol reagent according to the manufacturer's instructions (Invitrogen, CA, USA) and the concentration determined at 260/280 nm using a Nanodrop spectrophotometer (Thermo Scientific, IL, USA). The RNA samples were DNase-treated (MBI Fermentas, ON, Canada) to avoid genomic contamination. cDNA was synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The mRNA abundance of target genes were measured using gene-specific primers (see Table 1) exactly as described before (Aluru and Vijayan, 2007). PCR products were subjected to melt curve analysis to confirm the presence of a single amplicon. Control reactions were conducted with no cDNA template and with RNA to determine the level of background or genomic contamination. Standard curves and gene quantification were carried out exactly as previously described (Aluru and Vijayan, 2007). EF1α threshold cycle (CT) values were similar across all experimental treatment and hence used for the normalization of transcript abundance. The mRNA abundance with treatment was expressed as percentage of control.

4.2.5 Statistical Analysis

All statistical analysis were performed using Sigmaplot 11 (Systat Software Inc, Chicago, USA) and data is shown as mean ± standard error of mean (SEM). Statistical comparisons used two way repeated measures analysis of variance (ANOVA). The data was log transformed, wherever

necessary, to meet the assumptions of homogeneity of variance although non-transformed values are shown in the figures. Significant differences between treatment groups were compared using the Holm-Sidak post hoc test. A probability level of $p<0.05$ was considered significant.

4.3 Results

4.3.1 Hsp70 Release from immune-stimulated hepatocytes

4.3.1.1 Intracellular Hsp70 Levels

Heat shock exposure elevated intracellular Hsp70 levels by 41-fold compared to the control group at 24 h post-heat shock (Fig.4.1A). In both the control hepatocytes and those subjected to heat shock, there was no statistical difference in hepatic Hsp70 levels in any of the treatments (low dose LPS, high dose LPS; a combination of cortisol and low LPS or a combination of cortisol and high LPS) when compared to untreated controls.

4.3.1.2 Extracellular Hsp70 Levels

When we treated hepatocytes with LPS there was a 17-fold increase in medium Hsp70 at 24 h when compared with untreated controls (Fig 4.2B). There was no statistical difference temporally in eHsp70 release with any of the treatments over the 24 h period. However there was a significant 1.6-fold increase in medium Hsp70 levels of LPS-treated hepatocytes when compared to Hsp70 levels in the medium of both cortisol treated and untreated controls.

In order to determine if the above response was modulated by heat shock, cells were subject to a 1h acute heat shock (+15 °C above ambient temperature). We observed that when subjected to this heat shock (Fig 4.1 B), there was a significant increase in Hsp70 levels of high dose LPS treated hepatocytes (7.7-fold; 9.06 ± 3.0 µg/ml) when compared to low-dose LPS treated hepatocytes (1.7 ± 0.8 µg/ml). There was no statistical difference between Hsp70 levels in hepatocytes treated with cortisol either in combination with low or high dose LPS when compared to untreated controls.

In the absence of heat shock, there was a significant decrease (8-fold) in the amount of eHsp70 detected in cortisol treated hepatocytes (1.2 ± 0.5 µg/ml) when compared to medium of untreated controls (9.8 ± 2.2 µg/ml; data not shown). Interestingly, when treated with high-dose LPS there was a significant decrease (2-fold) in Hsp70 levels (4.6 ± 1.3 µg/ml) when compared to the medium of untreated controls. There was no statistical difference between any of the other treatments (low-dose LPS, a combination of cortisol and low-dose LPS, or a combination of cortisol and high-dose LPS).

4.3.2 Hepatocyte response to Hsp70 exposure

4.3.2.1 IL-1β and IL-8

In order to test whether exogenous Hsp70 would upregulate immunoregulatory genes we treated the cells with both a high and low dose of Hsp70, in combination with LPS. As a result, when cells were treated with LPS we observed an expected increase (20-fold) in IL-1β (Fig 4.4A) and a 2.6 fold increase in IL-8 (Fig 4.4B) compared to untreated controls. Treatment with Hsp70 did not modify this response.

4.3.3 Cell Viability

Cell viability was unaffected as there was no statistical difference in levels of LDH, an intracellular enzyme, detected in the medium as either temporally or a result of treatments (Fig 4.2)

Gene ID	GenBank Accession #	Primer sequences (5'-3') and reference	Temp (C)	Amplicon size (bp)
IL-1B	AJ223954	F: GGAGAGGTTAAAGGGTGGCGA R: TGCCGACTCCAACTCCAACA Gioacchini et al., 2008	60	121
IL - 8	AJ279069	F: CACTGAGATCATTGCCACTCTGA R: ATGACCCTCTTGACCCACGG Gioacchini et al., 2008	60	81
EF 1α	AF498320.1	F: CATTGACAAGAGAACCATTGA R: CCTTCAGCTTGTCCAGCAC Aluru et al., 2010	56	95

Table 4.1: Gene specific primers [forward (F) and reverse (R)] for quantitative real time PCR for Interleukin 1β (IL-1β), interleukin 8 (IL-8) and elongation factor 1α (EF-1α), along with their appropriate references, GenBank Accession numbers, melting temperature and amplicon size.

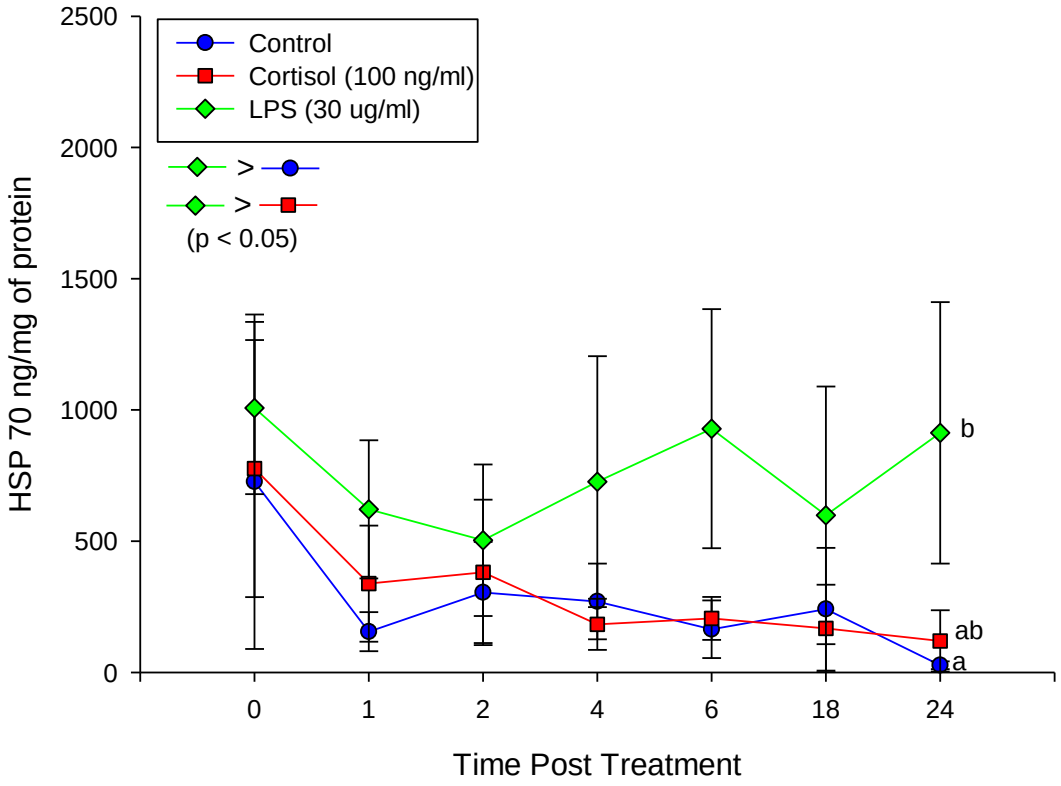

Figure 4.1 Hsp70 levels in medium collected from rainbow trout hepatocytes over a 24 h period

Hepatocytes were exposed to either control media, cortisol (100 ng/ml), or LPS (30 ug/ml). Medium samples were collected at 1, 2, 4, 6, 18 and 24 hours post heat shock. Hsp70 was detected via a competitive ELISA using a polyclonal rabbit anti-salmonid antibody (1:100,000 dilution). Inset shows significant treatment effect ($p < 0.05$; two way ANOVA); values represent mean ± SEM (n = 4). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

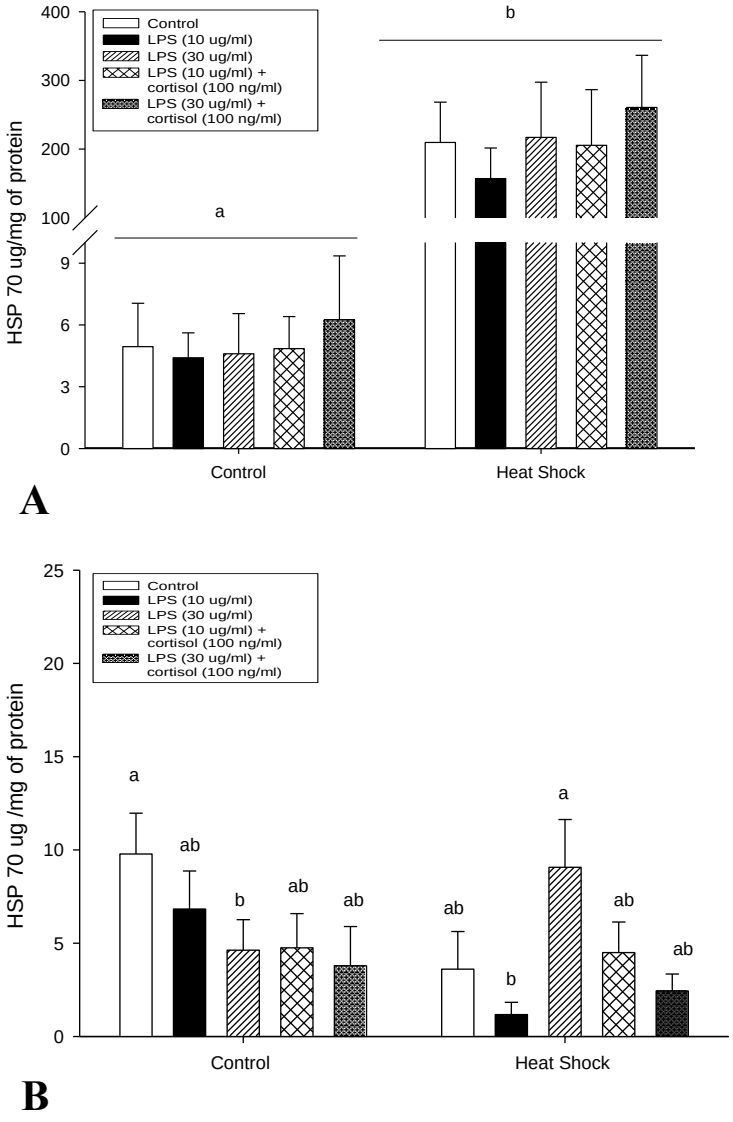

Figure 4.2: Hsp70 levels in rainbow trout hepatocytes (A) and Hsp70 levels in medium (B) collected from rainbow trout hepatocytes subjected to heat shock *in vitro*

Hepatocytes were exposed to either control media, cortisol (100 ng/ml), high (30 µg/ml) or low (10 µg/ml) of LPS, or a combination of high or low LPS + cortisol. Cells were then subjected to a heat shock (+15°C above ambient temperature [13°C] for one hour before allowing recovery at ambient temperature. Medium samples were collected 24 hours post heat shock. Hsp70 levels in hepatocytes were detected via a direct ELISA using a polyclonal rabbit anti-salmonid antibody (1:10,000 dilution), and Hsp70 levels in the medium were detected via a competitive ELISA (1:100,000 dilution). Values represent means ± SEM (n = 5). Bars with different letters (a, b) are significantly different ($p<0.05$, two way repeated measures ANOVA).

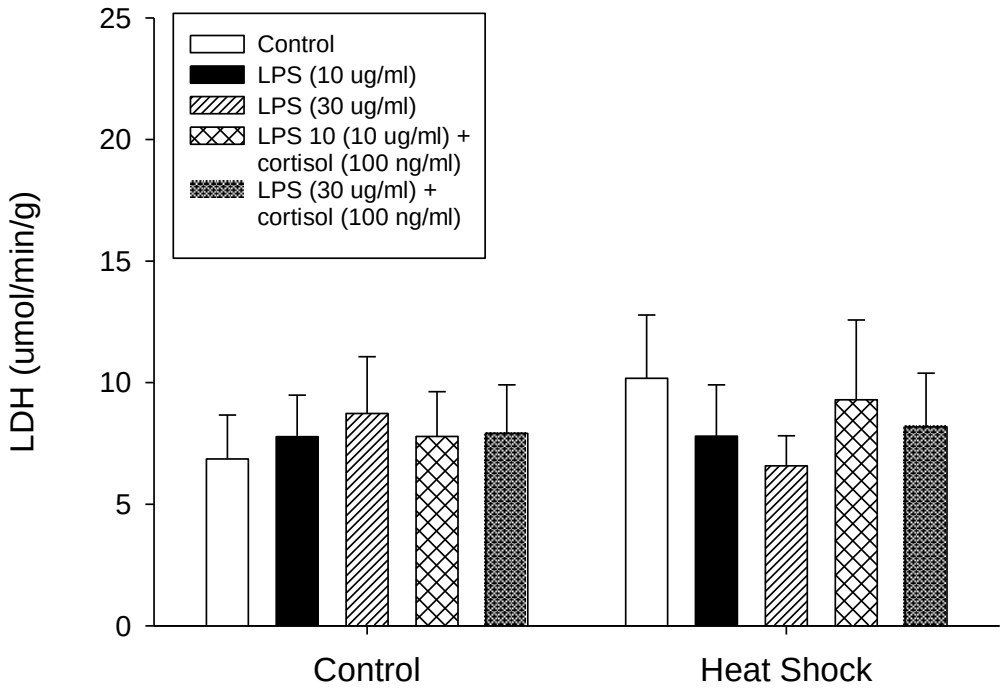

Figure 4.3: Lactate Dehydrogenase (LDH) levels

LDH levels in medium collected from rainbow trout hepatocytes subjected to a 1 h heat shock *in vitro* at 24 hours post heat shock. Hepatocytes were exposed to either control media, cortisol (100 ng/ml), high (30 µg/ml) or low (10 µg/ml) of LPS, or a combination of high or low LPS + cortisol. Cells were then subjected to a heat shock (+15°C above ambient temperature [13°C] for one hour before allowing recovery at ambient temperature. Medium samples were collected 24 hours post heat shock. Values represent means ± SEM (n = 5).

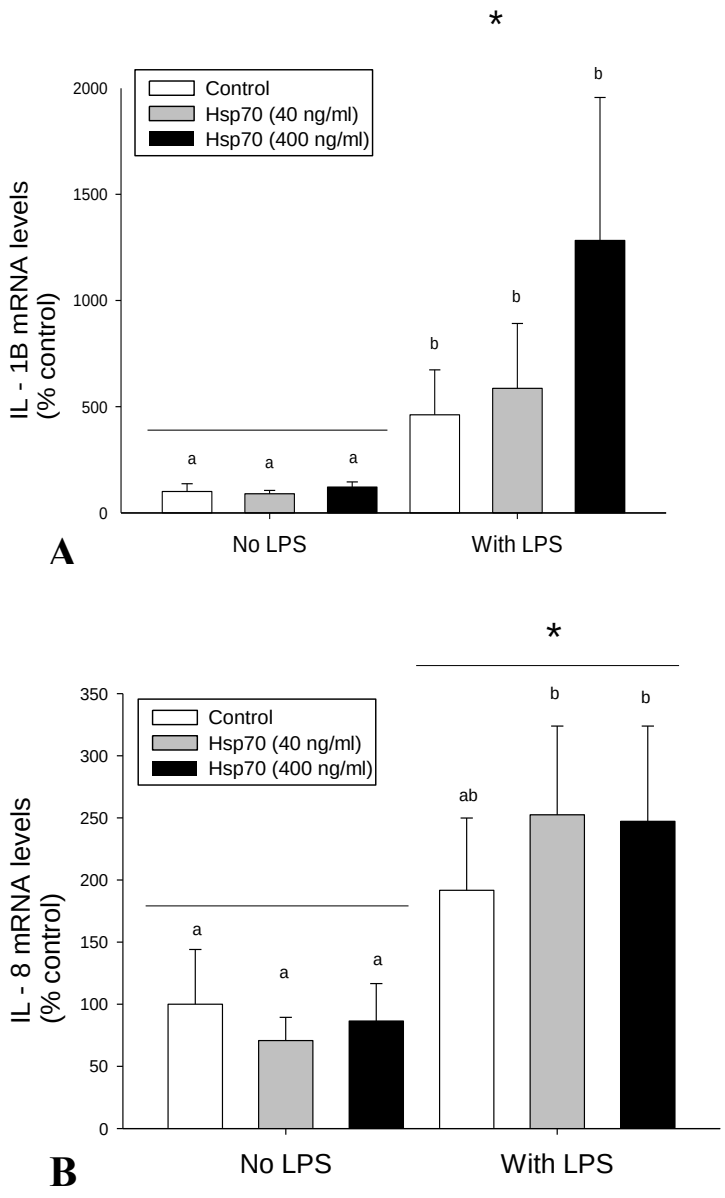

Figure 4.4 Effect of HSP70 treatment on expression of immune-responsive genes

(A) IL-1b (B) IL-8 in trout hepatocytes either in the presence (30 µg/ml) or absence of LPS. Hepatocytes were exposed to either control, low levels (40 ng/ml) or high levels (400 ng/ml) of Hsp70. All values represent mean ± SEM (n = 4 independent fish); bars with different letters are significantly different within each No LPS or with LPS group; LPS group significantly different from the No LPS group (two-way repeated measures ANOVA; $p<0.05$).

4.4 Discussion

In this study we demonstrate for the first time that extracellular Hsp70 is modulated by exposure to an immune stimulant in rainbow trout hepatocytes. Interestingly, modulation by both LPS and cortisol appear to be dependent on the presence or absence of an acute heat shock, suggesting modulation of heat activated intracellular signaling cascades in modulating extracellular release. We further demonstrate that exogenous Hsp70 is not able to elicit an immune response in rainbow trout hepatocytes.

4.4.1 Effect of Endotoxin Shock on Hsp70 Release

A novel finding of this study was that eHsp70 release is modulated by endotoxin stimulation in rainbow trout hepatocytes, emphasizing the importance of eHsp70 during an immune challenge (Fig 4.1 and 4.2). The release of heat shock proteins in response to bacterial endotoxins have yet to be characterized in fish; however, preliminary studies in mammals have shown modulation via endotoxin exposure of small Hsps in human intestinal epithelial cells (Kojima et al., 2004) and an increase in exosomal Hsp70 from murine macrophages in response to mycobacteria (Anand et al., 2010). The modulation of Hsps was in part due to activation of the MAP kinase signaling pathways (Kojima et al., 2004) through which LPS has been shown to regulate the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and high mobility group box 1 (HMGB1) from murine macrophages (Chen et al., 2004) implicating a possible role for these secondary signaling cascades in modulating the release of Hsp70. HMGB1, is a primarily cytosolic protein which has been previously suggested to be secreted in a similar fashion to Hsp70 through ATP binding cassette (ABC) transporters on the plasma membrane (Mambula and Calderwood, 2006). The induction of Hsps after LPS-stimulation showed significant elevation by 24 h, suggesting that eHsp70 levels may be regulated through intracellular mechanisms in a stressor-dependent manner as cortisol did not modulate eHsp70 during this time period. This was in contrast to our previous work (Chapter 3) in which cortisol decreased medium Hsp70 levels at 24 h. Reasons for this discrepancy could be attributed to divergent cortisol responses in trout which have been shown to modulate the intracellular heat shock response (LeBlanc et al., 2012), and may be reflected in the extracellular release. Though this is unlikely to be the sole reason it highlights the importance of cortisol in modulating eHsp70, particularly in combination with additional stressors such as heat or endotoxin exposure. While it has been established that cortisol treatment will abolish LPS-induced intracellular Hsp70 expression in rainbow trout hepatocytes (Vijayan et al., 2010; Philip et al., 2012), it did not regulate intracellular

levels of Hsp70 in common carp head kidney phagocytes in response to LPS-mediated increases in Hsps and GR expression (Stolte et al., 2009), nor was it observed in the current study (Fig 4.2A). Previous work in sea bass exposed to elevated levels of cortisol (via intraperitoneal injection) had increased intracellular Hsp70 levels in head kidney at 3d and 7d post treatment but not before 24h (Celi et al., 2012). eHsp70 levels appeared to be modulated, in part, by intracellular Hsp70 levels and therefore over a longer time scale modulation in response to cortisol may have been seen in the medium. Further work *in vivo* would be required to confirm this. Given this information, we tested the hypothesis that endotoxin-mediated Hsp70 release would be modulated by cortisol. As 24 h was the only time point that showed significant elevation in LPS-treated hepatocytes above untreated controls, this was used as the sampling time in our subsequent experiments.

Interestingly, release of Hsp70 was differentially regulated by LPS in the presence of heat shock. In contrast to the results discussed above, LPS significantly reduced the amount of medium Hsp70 at 24h (Fig 4.1 B). However, when hepatocytes were subjected to heat shock and stimulated with LPS we again observed a significant rise in medium Hsp70. This differential regulation in the presence of heat shock may be due to the modulatory effects of elevated intracellular Hsp70 levels, or other heat induced proteins, on heat activation of signaling cascades. This indicates that regardless of stressor the release of eHsp70 is highly regulated by levels of intracellular Hsp70. p38 mitogen activated protein (MAP) kinase and extracellular signal-regulated kinase (ERK) signaling cascades have been previously shown to modulate the release of cytokines (IL-8 and IL-6) from human bronchial epithelial cells (Laan et al., 2009). LPS is known to stimulate p38 MAP kinase pathways (Herleer et al., 1999) and heat has been shown to induce signaling pathways such c-Jun NH2- terminal kinases (JNK; apoptotic-signaling pathway) and ERK (survival pathway; Ma et al., 2001; Woessmann et al., 1999; Gabai et al., 2002). Therefore, the increase in Hsp70 after an acute heat shock may be due, in part, to the up-regulation of intracellular signaling cascades by both heat and endotoxin exposure. Alternatively, in the absence of heat shock LPS appears to act in a suppressive fashion, attenuating the release of eHsp70 through an unidentified pathway. Further work using pharmacological agents to modulate intracellular signaling cascades could further clarify how LPS is able to differentially modulate the release of Hsp70. However, regardless of mechanism, the effect of endotoxin shock on the cellular stress response is important to maintain cellular homeostasis by retaining Hsp70 to better increase cellular survival, in a similar way to cortisol (Chapter 3). However, after an acute heat shock, LPS will encourage the release of these proteins which may assist neighboring cells deficient in Hsp70, to increase survival in response to an immune challenge, suggesting a role for this protein

in paracrine signaling (Mambula and Calderwood, 2006). In the presence of heat shock, eHsp70 levels were unaffected by cortisol administration in combination with LPS; however, if treated individually LPS will stimulate the release of Hsp70 and cortisol will attenuate the release, this may be an adaptive cellular response that elevates eHsp70 regardless of other stressors when subject to an immune challenge. The exact mechanism, as well as the biological significance of eHsp70 release remains to be determined in future studies

4.4.2 Immunoregulatory effects of Hsp70 on rainbow trout hepatocytes

To the best of our knowledge studies on the effects of exogenous Hsp70 in teleosts have not yet been examined. However, given that Hsp70 has been implicated as having immunoregulatory roles, elevation may be an indicator of overall health of an organism. In mammalian models exogenous Hsp70 has been shown to activate potassium channels in promonocytes (Negulyaev et al., 1996) and stimulate the production of IL-1β, IL – 6, IL-12, and TNF –α in human monocytes (Asea et al., 2000). However, Basu et al.(2000) did not observe stimulation of cytokines or maturation of dendritic cells after exposure to exogenous Hsp70. Follow-up research into these discrepancies concluded that much of the presumed immunoregulatory role of Hsp70 was due to LPS contamination of recombinant Hsp70 (Tsan and Gao, 2009). Therefore while the immunoregulatory role of eHsp70 does not induce cytokines to the degree that was once believed (Tsan, 2011), treatment with recombinant Hsp70 was observed to stimulate the release of macrophage inflammatory protein 2 (MIP-2) from rat hepatocytes (Galloway et al., 2008), suggesting that Hsp70 may be an inducer of chemokines in the liver. Therefore we examined, for the first time, the extent to which exogenous Hsp70 was able to regulate both key cytokine (IL-1β) and chemokine (IL-8) genes in rainbow trout hepatocytes. The recombinant Hsp70 used in this study was not further purified post receipt from the manufacturer though preliminary work did not induce a response in either IL-8 and IL-1β (Fig: 4.4 A and B) after treatment with either high (400 ng/ml) or low doses of Hsp70 (40 ng/ml). The concentrations of Hsp70 were chosen to be physiologically relevant, as observed previously in rainbow trout plasma (chapter 2). LPS significantly elevated the expression of these genes which has been previously reported in rainbow trout (Philip et al., 2012). Overall, while Hsp70 did not regulate immune responsive genes further experiments are required to fully elucidate the physiological role of eHsp70. Specifically, the effect of eHsp70 may be tissue-specific, especially if the primary extracellular role is immunoregulatory then it can be hypothesized that target tissue may not be the liver. Given that hepatocytes elicit a strong heat shock response, Hsp70 release, and subsequent

modulation by both cortisol and LPS further suggests that hepatic derived eHsp70 may have alternative target tissues.

In conclusion we show for the first time that eHSP70 is actively released from rainbow trout hepatocytes and that this is modulated by exposure to both cortisol (discussed previously in chapter 3) and LPS treatment in rainbow trout hepatocytes. Interestingly, modulation by both LPS and cortisol appear to be dependent on the presence or absence of an acute heat shock suggesting the regulation of eHsp70 by the intracellular heat shock response. Furthermore while treatment of exogenous Hsp70 has been examined in various mammalian cell types, this was the first time it was examined in fish. Overall, further work needs to be completed on both the mechanism of release and the role of Hsp70 once it is released to fully understand how exposure to endotoxins and stress hormones modulate extracellular levels. Regardless of how stress hormones and endotoxin exposure modulate the release of eHsp70 from the cell it is clear that its release and continued presence in the extracellular environment is essential in times of stress, which may have broader implications in the overall health of the organism.

Chapter 5
General Conclusion

Heat shock proteins are essential for the survival of cells during periods of stress. As such, their intracellular role has been well characterized; however, their possible functions in the extracellular *milieu* have yet to be conclusively proven. While the release of Hsp70 and the immunoregulatory functions of eHsp70 have been studied in mammals, there have only been a few studies in fish addressing the release of Hsp70 into the extracellular environment (Henrickson, 2010; Zhang et al., 2011). This thesis presents, for the first time, the release of Hsp70 into the circulation in response to stress in fish.

The first hypothesis tested was that eHsp70 was released into the plasma in response to a heat shock (+10-12°C above ambient temperature) in rainbow trout *in vivo* (Chapter 2). Furthermore, circulating levels of Hsp70 were quantified in the plasma using an in-house developed competitive ELISA. Interestingly, while there was a significant increase in Hsp70 at 4 h post heat shock when compared to 1h and 24 h, over a long period the plasma heat shock response was lower at 4 d and 7d post-heat shock compared to 1d. Therefore, while Hsp70 may be elevated in the plasma at 4 h, it is either subject to degradation, or uptake by target tissues. Plasma Hsp70 levels appear to be due to secretion, as opposed to cell lysis, because there were no differences in plasma LDH levels, a cytoplasmic marker indicative of cell lysis. Further studies are required to understand the mode of release and the modulation of plasma Hsp70 levels in response to stress. Because circulating Hsp70 may be indicative of chronic stress in fish this could have interesting applications as a non-lethal biomarker.

To further examine how eHsp70 levels were modulated by stress, the hypothesis that cortisol would regulate Hsp70 levels was tested using rainbow trout hepatocytes as a model (Chapter 3). While *in vivo* results may vary, this gave us a controlled environment to examine the release of Hsp70 in response to the stress hormone cortisol. Interestingly, 24 h after cortisol treatment there was a significant decrease in eHsp70 levels compared to control. This attenuation was abolished after the addition of the GR antagonist, mifepristone, suggesting a novel role for GR in regulating the release of eHsp70 in trout hepatocytes. Interestingly, the GR mediated release of Hsp70 was abrogated by heat shock suggesting that cellular content of Hsp70 may modulate GR signaling. Similarly, changes in cell signaling event in response to cellular Hsp70 content has been observed before with ABC transporters (Mambula and Calderwood, 2006). However, further work is warranted to understand the mechanism(s) leading to eHsp70 release in trout hepatocytes. Another interesting result from this

chapter was the significant decrease in eHsp70 levels in medium of hepatocytes from 4 h to 24 h after a heat shock. This suggests that eHsp70 is either subject to protein degradation or protein uptake, further supporting a role for Hsp70 in intercellular signaling (Fig 5.1). In this regard, further studies can be carried out using labeled Hsp70 to determine if Hsp70 is localized intracellularly.

In Chapter 4, the modulation of Hsp70 by bacterial endotoxins (LPS) was examined, as well as whether eHsp70 levels were further regulated by heat shock and stress hormones. LPS-mediated release of Hsp70 was differentially regulated by heat shock. In the absence of heat shock, LPS-treated hepatocytes (30 µg/ml) showed a significant decrease in eHsp70 when compared to controls. However, in the presence of heat shock, there was no change in Hsp70 release from basal levels. In heat-shocked cells, high-dose LPS treatment elicited significantly more eHsp70 release when compared to hepatocytes treated with a low dose of LPS (10 µg/ml). Interestingly, when we examined the rate of release over 24 h, there was significantly more eHSP70 in LPS treated hepatocytes compared to controls at 24 h. These contrasting results require further study.

Lastly, the ability of Hsp70 to elicit an immune response in rainbow trout hepatocytes was studied. Though we saw no regulation of some cytokine genes with exogenous Hsp70 treatment, the results are preliminary at best and will have to be fully characterized in the future. These may include examining upregulation of other key cytokines, which have been previously shown to be up-regulated in rat hepatocytes (Galloway et al., 2008). Examining the effects of eHsp70 on intracellular signaling (MAP kinases and NF-κB) may also assist in determining the effects of this protein on target cells. Furthermore, whether the effects of Hsp70 are tissue-specific also remains to be determined by examining the effects of eHsp70 on other cell types.

In conclusion, this study demonstrates for the first time the release of Hsp70 in trout plasma *in vivo* and the modulation of this response by heat and handling stress. Subsequent *in vitro* experiments further established modulation of eHsp70 release by both cortisol and endotoxin shock (LPS) from trout hepatocytes. In contrast to our original hypothesis, all stressors that we used to modulate eHsp70 failed to increase levels above basal, with the exception of LPS, and in many instances significantly decreased the amount of Hsp70 released into the plasma or medium. Further research involving mechanisms behind this regulation may have important implications in our understanding of cell stress signaling, and may have broader consequences on the health of the organism, and also in stress detection for environmental monitoring.

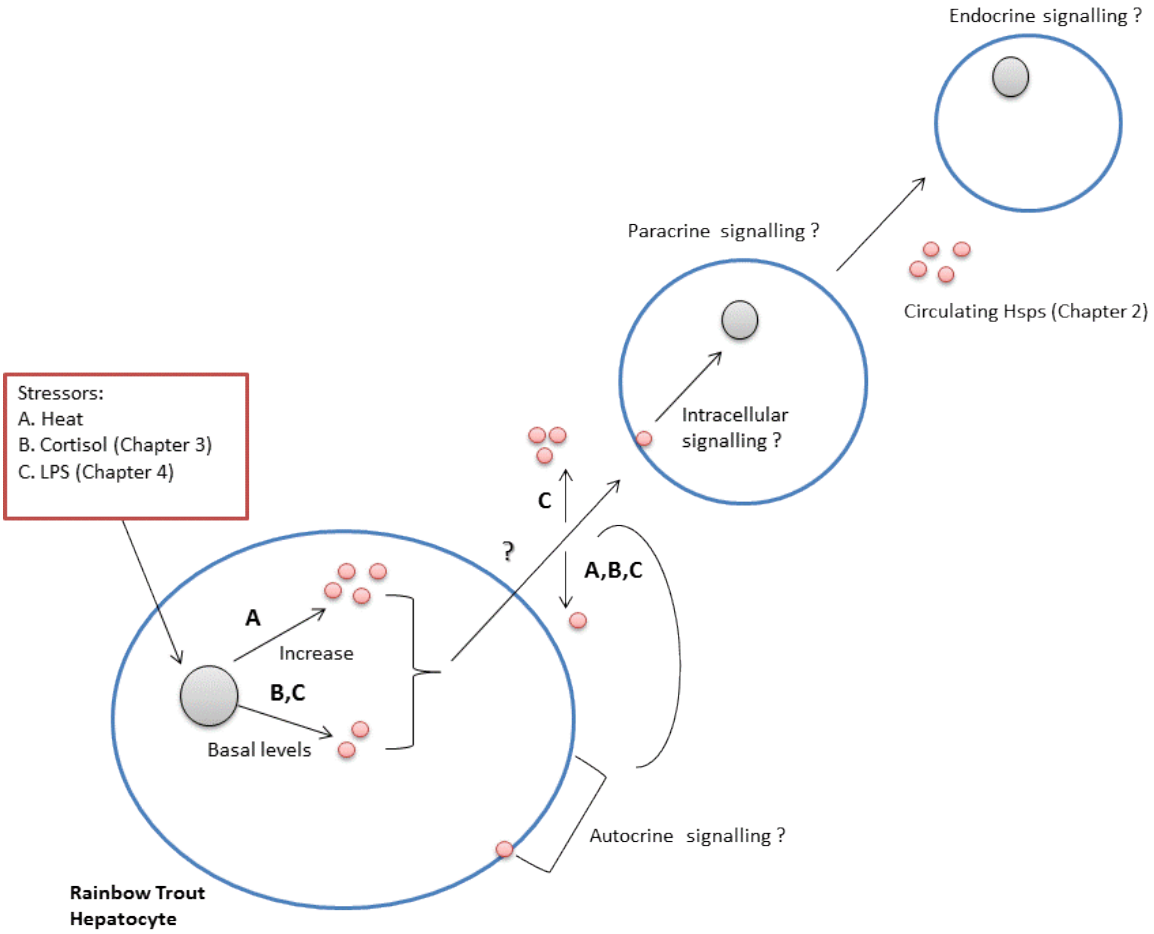

Figure 5.1: Modulation of eHsp70 Levels:

A simplified schematic of possible role for eHsp70 during stress. Exposure to heat shock (A) causes a rise in intracellular levels in rainbow trout hepatocytes, but levels of eHsp70were significantly decreased in the medium. Treatment with the stress hormone cortisol (B) and the bacterial endotoxin, LPS (C) did not change basal intracellular Hsp70 levels, but caused a significant decrease in eHsp70 levels when compared to basal levels. LPS was also observed to increase eHsp70 levels over a 24 h period. Modulation by stressors in combination is also discussed further in each specified chapter. Possible role for eHsp70 in autocrine and paracrine signaling, and in modulating immunoregulatory functions, warrants further research. Presence of plasma Hsp70 levels may suggest a role in endocrine signaling, but this remains to be tested (Adapted from Henderson, 2010).

Appendix A

ELISA Protocols and Validation Tables

5.1 Antibody-Capture Competitive Trout Hsp70 Enzyme-Linked Immunosorbent Assay Detailed Protocol

Day 1:

1) A medium binding 96 well plate (Costar) was coated with 200 µl chinook salmon Hsp70 recombinant protein (60 ng/mL; Stressgen) in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6).

2) A standard curve Hsp70 recombinant protein was serial diluted in dilution buffer (0.1 M phosphate buffered saline, pH 7.4, 0.05% Tween 20). Standards ranged from 0 ng/ml to 100 ng/ml with the total volume of each standard being 0.4 ml. To tubes containing these standards, 0.4 ml of polyclonal rabbit anti-salmon Hsp70 primary antibody (1:100,000; StressMarq) was added (1:1 ratio). The tubes were then incubated with constant rocking overnight at 4°C.

3) To prepare the samples dilute either homogenized RBCs (1:1 ration of RBCs to Tris +Protease Inhibitor [PI]) or plasma (5x) to a final volume of 0.25 ml in dilution buffer (0.1 M PBS; pH 7.4; 0.05% Tween 20). To these tubes add 0.25 ml of polyclonal rabbit anti-salmon Hsp70 primary antibody (1:100,000; StressMarq). The tubes were then incubated with constant rocking overnight at 4°C.

Day 2:

4) Using a plate washer (BioRad) the plate was washed with at least 250 ul of washing buffer (TTBS (20 mM Tris, pH 7.4, 300 mM NaCl, 0.05% Tween 20)) 4 times.

5) The plate was blocked with a 200 ul/well of blocking solution (1% bovine serum albumin (Fisher Scientific) in 1x TTBS) for one hour at room temperature with constant shaking. Wash plate as described in step 4.

6) 200 ul of either the Ab:standard or the Ab:sample was distributed from the incubated tubes to each well (200 ul/well). The standards were added in triplicate and the samples in duplicate. The plate was then incubated overnight at 4°C, with constant rocking.

Day 3:

7) Plate contents were washed as described in step 4 and reacted with enzyme bound secondary antibody. Add 200 µl/well of horseradish peroxidase conjugated goat anti-rabbit secondary antibody

(1:5,000; BioRad) diluted in 5% skim milk. Incubated for 2 hours at room temperature, with constant shaking.

8) Wash plate as described in step 4. Add the enzyme substrate (200 ul/well), 3,3',5,5'-tetramethylbenzidine (TMB) (41 mM). Incubate at room temperature for 30 minutes. The reaction was stopped with 8.5M acetic acid in 0.5 M sulfuric acid

9) The plate was read immediately at 405 nm using a microplate reader (VersaMax).

Table A.1: Competitive ELISA Intra-assay variation

CV% was calculated for each set of replicates (n=3) within the standard curve of each ELISA run. These values were averaged, and standard deviation (SD) was calculated to determine average CV% (σ/SD*100). % Bound over unbound (B/Bo) was calculated to determine the sensitivity of the assay.

	Mean absorbance (405 nm)						
ng Hsp70/ml	1	2	3	Mean	SD	%CV	%B/Bo
100	0.294	0.289	0.289	0.291	0.003	1	100
80	0.356	0.343	0.327	0.342	0.015	4.3	87.6
60	0.413	0.381	0.374	0.389	0.021	5.3	74.5
40	0.512	0.493	0.497	0.501	0.01	2	62.5
20	0.675	0.649	0.715	0.680	0.033	4.9	46.0
10	0.832	0.787	0.815	0.811	0.022	2.8	35.7
5	0.942	1.002	0.914	0.953	0.045	4.7	31.4
0	1.078	1.123	1.064	1.088	0.031	2.8	26.7
Average						**3.5**	

Table A.2: Competitive ELISA Inter-assay variation. CV% was calculated for each set of replicates (n=4) , which represent 4 ELISA standard curves run on different days within the standard curve of each ELISA run. These values were averaged, and standard deviation (SD) was calculated to determine average CV% (σ/SD*100).

	Mean absorbance (405 nm)						
ng Hsp70/ml	1	2	3	4	Mean	SD	%CV
100	1.088	1.083	1.25	1.206	1.156	0.073	6.3
80	0.953	1.005	1.121	0.131	0.803	0.392	48.9
60	0.811	0.927	1.03	1.007	0.944	0.086	9.0
40	0.680	0.833	0.933	0.959	0.85	0.109	12
20	0.501	0.678	0.853	0.071	0.525	0.291	55.2
10	0.389	0.527	0.671	0.675	0.566	0.118	20.9
5	0.342	0.417	0.605	0.6	0.491	0.115	23.3
0	0.291	0.304	0.558	0.51	0.415	0.120	28.8
Average							**25.6**

5.2 Direct Trout Hsp70 Enzyme-Linked Immunosorbent Assay Detailed Protocol

Day 1:

1) A medium binding 96 well plate (Costar) was coated with 100 µl of either standards (ranging from 0-200 ng/ml) of 200 µl chinook salmon Hsp70 recombinant protein (60 ng/ml; Stressgen) or samples diluted to 2 µg/ml in coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6). Both samples and standards were done in duplicate

Note: The coating buffer was made fresh each day and was cold (4°C) when diluting samples and the recombinant protein.

2) Each plate was incubated with constant rocking overnight at 4°C.

Day 2:

3) Using a Plate Washer (BioRad) the plate was washed with at least 250 ul of washing buffer (TTBS (20 mM Tris, pH 7.4, 300 mM NaCl, 0.05% Tween 20)) 4 times.

4) Block each plate with a 150 µl/well of blocking solution (1% bovine serum albumin (Fisher Scientific) in 1x TTBS) for one hour at room temperature with constant shaking. Wash plate as described in step 3.

5) 100 µl of polyclonal rabbit anti-salmon Hsp70 primary antibody (1:10,000; StressMarq) was added. Allow plates to incubate for 2 h at room temperature with constant shaking. Wash plate as described in step 3

6) Add enzyme bound secondary antibody (100 µl/well of horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:3,000; BioRad) diluted in 1% skim milk)). Incubate for 1 h at room temperature, with constant shaking. Wash plate as described in step 3

7) Add the enzyme substrate (200 ul/well), 3,3',5,5'-tetramethylbenzidine (TMB) (41 mM). Incubate at room temperature for 1 h. Stop the reaction with 8.5M acetic acid in 0.5 M sulfuric acid

8) Read immediately at 405 nm using a microplate reader (VersaMax).

Table A.3: Direct ELISA Intra-assay variation. CV% was calculated from samples run in duplicate (n=2) within the standard curve of each ELISA run. These values were averaged, and standard deviation (SD) was calculated to determine average CV% (σ/SD*100). % Bound over unbound (B/Bo) was calculated to determine the sensitivity of the assay.

	Mean absorbance (405 nm)				
ng Hsp70/ml	1	2	Mean	SD	%CV
200	0.234	0.225	0.229	0.007	3
150	0.207	0.202	0.205	0.004	1.8
100	0.102	0.144	0.123	0.029	23.9
50	0.057	0.048	0.052	0.006	11.6
25	0.037	0.032	0.034	0.004	11.3
10	0.021	0.02	0.02	0.001	3.9
5	0.012	0.011	0.011	0	4.4
0	0.004	0.003	0.004	0.001	16.5
Average					**9.55**

Table A.4: Direct ELISA Inter-assay variation. CV% was calculated for each set of replicates (n=5) , which represent 4 ELISA standard curves run on different days within the standard curve of each ELISA run. These values were averaged, and standard deviation (SD) was calculated to determine average CV% (σ/SD*100).

	Mean absorbance (405 nm)							
ng Hsp70/ml	1	2	3	4	5	Mean	SD	%CV
200	0.436	0.139	0.165	0.229	0.223	0.2384	0.104	43.8
150	0.285	0.118	0.124	0.205	0.164	0.179	0.061	34.2
100	0.138	0.072	0.099	0.123	0.125	0.111	0.023	21.0
50	0.101	0.046	0.041	0.052	0.057	0.059	0.022	36.8
25	0.04	0.024	0.024	0.034	0.027	0.030	0.006	21.1
10	0.027	0.014	0.013	0.02	0.016	0.018	0.0051	28.3
5	0.013	0.008	0.008	0.011	0.007	0.0094	0.002	23.9
0	0.009	0.006	0.006	0.004	0.001	0.006	0.003	51.6
Average								**32.4**

Bibliography

Ackerman, P. A. & Iwama, G. K. (2001). Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. J. Aquat. Anim. Health., 13: 173–180.

Ackerman, P. A., Forsyth, R. B., Mazur, C. F. & Iwama, G. K. (2000). Stress hormones and the cellular stress response in salmonids. Fish. Physiol. Biochem., 23, 327–336.

Alder G.M., Austen B.M., Bashford C.L., Mehlert A., Pasternak C.A.. (1990). Heat shock proteins induce pores in membranes. Biosci Rep, 10:509–518.

Ali K.S., Dorgai L., Gazdag A, Abraham M, Hemesz E. (2003). Identification and induction of hsp70 gene by heat shock and cadmium exposure in carp. Acta. Biol. Hung. 54:323– 334

Alsop, D., Vijayan, M. M., (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol Regul Integr Comp Physiol. 294**,** R711-719.

Alsop, D., Vijayan, M. M., (2009). Molecular programming of the corticosteroid stress axis during zebrafish development. Comp Biochem Physiol A Mol Integr Physiol. 153**,** 49-54.

Aluru, N., Vijayan, M.M., (2007). Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. Physiol. Genomics, 31, 483–491.

Anand, P.K., Anand, E., Bleck, C.K.E., Anes, E., Griffiths, G. 2010. Exosomal Hsp70 Induces a Pro-Inflammatory Response to Foreign Particles Including Mycobacteria. PLoS ONE 5(4): e10136. doi:10.1371/journal.pone.0010136

Andrei C, Dazzi C, Lotti L, Torrisi MR, Chimini G, Rubartelli A (1999). The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. Mol. Biol. Cell., 10:1463–1475

Andrei,C., Margiocco, P., Poggi, A., Lotti, L.V. Torrisi, M.R. and Rubartelli, A. (2004). Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. Proc. Natl. Acad. Sci., 101: 9745–9750.

Asea, A., Kraeft, S. K., Kurt-Jones, E. A., Stevenson, M. A., Chen, L. B., Finberg, R. W., et al. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med., 6: 435-442.

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare O., Auron, P. Stevenson M. A., and Calderwood S.K. (2002). Novel Signal Transduction Pathway Utilied by Extracellular HSP70: Role of Toll-Like receptor (TLR) 2 and TLR4. J. Biol. Chem., 277 (17): 15028-15034.

Asea, A., (2003). Chaperone-Induced Signal Transduction Pathways. Exerc. Immunol. Rev. 9:25-33.

Arispe N., and De Maio A. (2000) ATP and ADP modulate a cation channel formed by Hsc70 in acidic phospholipid membranes. J. Bio. Chem. 275:30839–30843.

Arispe N, Doh M, De Maio A. (2002). Lipid interaction differentiates the constitutive and stress-induced heat shock proteins Hsc70 and Hsp70. Cell Stress Chaperon., 7:330–338.

Barbe M.F., Tytell M., Gower D.J., and Welch W.J. (1988). Hyperthermia protects against light damage in the rat retina. Science, 241:1817–1820.

Basu, N., Nakano, T., Grau, E. G. & Iwama, G. K. (2001). The effects of cortisol on heat shock protein 70 in two fish species. Gen. Comp. Endocrinol., 124: 97-105.

Basu N., Kennedy C.J., and Iwama G.K. (2003). The effects of stress son the association between hsp70 and the glucocorticoid receptor in rainbow trout. Comp. Biochem. Physiol: A Mol. and Integr. Biol.134 (3): 655 -663

Basu, S., Binder R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000). Necrotic by not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. Int. Immunol. 12 (11): 1539-1546.

Bausinger, H., Lipsker, D., Ziylan, U., Manie, S., Briand, J. P., Cazenave, J. P., Muller, S., Haeuw, J. F., Ravanat, C., de la Salle, H., Hanau, D. (2002) Endotoxin-free heat shock protein 70 fails to induce APC activation. Eur. J. Immunol. 32: 3708–3713.

Becker, T., Ulrich Hartl, F., Wieland, F. (2002). CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J. Cell. Viol. 158 (7): 1277 - 1285

Bergmeyer HU 1983. Methods of Enzymatic Analysis, Academic Press, New York

Blott, E. J., & Griffiths, G. M. (2002). Secretory lysosomes. Nat. Rev. Mol. Cell Bio., 3(2), 122– 31.

Boone, A. N. & Vijayan, M. M. (2002a). Constitutive heat shock protein 70 expression in rainbow trout hepatocytes: effect of heat shock and heavy metal exposure. Comp Biochem. Physiol. C Toxiocol. Pharmacol., 132(2), 223-233.

Boone A. N. & Vijayan, M. M. (2002b). Glucocorticoid-mediated attenuation of hsp70 response in trout hepatocytes involves the proteasome. Am. J. Physiol. Regul. Integr. Comp. Physiol., 283, (R): 680-687.

Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G., and Bachelet M. (2003). Expression of Molecular Chaperone Hsp70 in Detergent-resistant Microdomains correlates with its membrane Delivery and Release. J. Biol. Chem., 278(24): 21601-21606

Burdon, R. H., Slater, A., McMahon, M., & Cato, A. C. (1982). Hyperthermia and the heat-shock proteins of HeLa cells. Br J Cancer, 45(6), 953-963.

Byrd, C. A., Bornmann, W., Erdjument-Bromage, H., Tempst, P., Pavletich, N., Rosen, N., & Ding, A. (1999). Heat shock protein 90 mediates macrophage activation by Taxol and bacterial lipopolysaccharide. Proc. Natl. Acad. Sci., 96(10): 645-5650.

Calderwood, S. K., Mambula, S. S., Gray, P. J. & Theriault, J. R. (2007). Extracellular heat shock proteins in cell signaling. FEBS Letters, 581(19): 3689-3694.

Calderwood, S. K. (2005). Evolving connections between molecular chaperones and neuronal function. Int. J. Hyperther, 21(5), 375-378.

Campisi, J. & Fleshner, M. (2003). Role of extracellular HSP72 in acute stress-induced potentiation of innate immunity in active rats. J. Appl. Physiol., 94(1): 43-52.

Castellino, F., Boucher, P.E., Eichelberg, K., Mayhew, M., Rohman, J.E., Houghton, A.N., and Germain R. N. (2000). Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med., 191 (5): 1958-64.

Celi, M., Vazzana, M., Sanfratello, M. A., & Parrinello, N. (2012). Elevated cortisol modulates Hsp70 and Hsp90 gene expression and protein in sea bass head kidney and isolated leukocytes. Gen. Comp. Endocr., 175(3): 424–31.

Chen, G., Li, J., Ochani, M., Rendon-Mitchell, B., Qiang, X., Susarla, S., Ulloa, L., Yang, H., Fan, S., Goyert, S. M., Wang, P., Tracey, K. J., Sama, A. E., Wang, H. (2004) Bacterial endotoxin stimulates macrophages to release HMGB1 partly through CD14- and TNF-dependent mechanisms. J. Leukoc. Biol. 76: 994–1001.

Clayton, A., Turkes, A., Navabi, H., Mason, M. D. & Tabi, Z. (2005). Induction of heat shock proteins in B-cell exosomes. J. Cell. Sci., 118(16): 3631-3638.

Conde-vancells, J., Rodriguez-suarez, E., Embade, N., Gil, D., Matthiesen, R., Valle, M., Elortza, F., et al. (2008). Characterization and Comprehensive Proteome Profiling of Exosomes Secreted by Hepatocytes research articles. J. Proteome Res., 7: 5157–5166.

Currie, S. Tufts, B. L., & Moyes, C. D. (1999). Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. Am J Physiol Regul Integr Comp Physiol, 276, 990-996.

De Boeck, G., De Wachter, B., Vlaeminck, A., & Blust, R. (2003). Effect of cortisol treatment and/or sublethal copper exposure on copper uptake and heat shock protein levels in common carp, Cyprinus carpio. Enviro. Toxicol. Chem., 22(5): 1122-1126.

De Maio, A. (2011). Extracellular heat shock proteins, cellular export vesicles and the Stress Observation System: a form of communication during injury, infection and cell damage. Cell Stress Chaperon., 16:235-249

De Wachter B, Scholliers A, Blust B (1998) Semiquantitative immunoblot detection of 70 kDa stress proteins in the carp Cyprinus carpio. Bull Environ Contam. Toxicol. 60:37–44

Deane E.E., and Woo, N.Y.S. (2011). Advances and perspectives on the regulation and expression of piscine heat shock proteins. Rev. Fish. Biol. Fish., 21: 153-185.

Deane, E. E. & Woo, N. Y. S. (2006). Impact of heavy metals and organochlorines on hsp70 and hsc70 gene expression in black sea bream fibroblasts. Aquat. Toxicol., 79: 9–15.

Deane E.E., Woo N.Y.S. (2005) Cloning and characterization of the hsp70 multigene family from silver sea bream: modulated gene expression between warm and cold temperature acclimation. Biochem. Biophys. Res. Commun. 330:776–783

Deane E.E., Woo N.Y.S. (2004). Differential gene expression associated with euryhalinity in sea bream (*Sparus sarba*). Am. J. Physiol. 287:R1054–R1063

Deane E.E., Kelly S.P., Chow I.N.K, Woo N.Y.S.(2000) Effect of a prolactin pharmacological stimulant (sulpiride) and sup- pressant (bromocriptine) on heat shock protein 70 expression in Sparus sarba. Fish. Physiol Biochem 22:125–133

Deane, E. E., Kelly, S. P., Lo, C. K. M. & Woo, N. Y. S. (1999). Effects of GH, prolactin and cortisol on hepatic heat shock protein 70 expression in a marine teleost Sparus sarba. J Endocrinol, 161, 413-421.

Delneste,Y. et al. (2002). Involvement ofLOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17: 353–362.

Dindia, L., Murray, J., Faught, E., Davis, T.L., Leonenko, Z., Vijayan, M.M. (2012) Novel Nongenomic Signalling by Glucocorticoid May Involve Changes to Liver Membrane Order in Rainbow Trout. PLoS ONE: IN PRESS

Engelsma, M.Y., Stet, R.J.M., Saeij, J.P., Verburg-van Kemenade, B.M.L., 2003. Differential expression and haplotypic variation of two interleukin-1beta genes in the common carp (Cyprinus carpio L.). Cytokine 22: 21–32.

Fangue NA, Hofmeister M, Schulte PM (2006) Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus.J. Exp. Biol. 209:2859–2872

Flanagan, S. W., Ryan, A. J., Gisolfi, C. V., & Moseley, P. L. (1995). Tissue-specific HSP70 response in animals undergoing heat stress. Am J Physiol Regul Integr Comp Physiol, 268(1): 28-32.

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Ann. Rev. Physiol. 61: 243–82.

Forsyth, R. B., Candido, E. P. M., Babich, S. L. & Iwama, G. K. (1997). Stress protein expression in coho salmon with bacterial kidney disease. J. Aquat. Anim. Health., 9:18–25.

Fujihara, M.S. and Nadler, S.G. (1999). Intranuclear targeted delivery of functional NF-κB by 70 kDa heat shock protein. EMBO J.,18 (2): 411-419.

Gabai, V.L., and Sherman M.Y. 2002.Invited Review: Interplay between molecular chaperones and signalling pathways n survival of heat shock. J. Appl. Physiol., 92: 1743-1748

Galloway, E., Shin, T., Huber, N., Eismann, T., Kuboki, S., Schuster, R., Blanchard, J., Wong, H.R., and Lentsch, A.B. (2008). Activation of hepatocytes by extracellular heat shock protein 72. Am. J. Physiol. Cell. Physiol., 295: C514-C520.

Gao, B., Tsan, M-F. (2003a) Endotoxin contamination in recombinant human Hsp70 preparation is responsible for the induction of TNF release by murine macrophages. J. Biol. Chem. 278:174–179.

Gao, B., Tsan, M-F. (2003b) Recombinant human heat shock protein 60 does not induce the release of tumor necrosis factor from murine macrophages. J. Biol. Chem. 278, 22523–22529.

Gastpar, R., Gehrmann, M., Bausero, M. A., Asea, A., Gross, C., Schroeder, J. A., et al. (2005). Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res., 65(12): 5238-5247.

Gehrmann, M., Liebisch, G., Schmitz, G., Anderson, R., Steinem, C., De Maio, A., Pockley, G., and Multhoff, G. (2008). Tumor-Specific Hsp70 Plasma Membrane Localization is enabled by the Glycosphingolipid Gb3. PLoS ONE 3(4): e1925 doi:10.1371/journal.pone.0001925

Guzhova, I., Kislyakova, K., Moskaliova, O., Fridlanskaya, I., Tytell, M., Cheetham, M., et al. (2001). In vitro studies show that HSP70 can be released by glia and that exogenous HSP70 can enhance neuronal stress tolerance. Brain Res, 914(1-2), 66-73.

Habich, C., Kempe, K., van Der Zee, R., Rumenapf, R., Akiyama, H., Kolb, H., Burkart, V. (2005) Heat shock protein 60: specific binding of lipopolysaccharide. J. Immunol. 174: 1298–1305.

Henderson, B., Calderwood, S.K., Coates, A.R.M. Cohen, I., van Eden W., Lehner T., and Pockley, A.G. (2010). Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants. Cell Stress Chaperon., 15:123-141.

Henderson, B. (2010). Integrating the cell stress response : a new view of molecular chaperones as immunological and physiological homeostatic regulators. *Cell Biochem.Funct.*, 28: 1–14.

Henrickson, L., (2010). Extracellular Heat Shock Protein 70 Regulation in Rainbow Trout. MSc Dissertation. University of Waterloo, Waterloo, ON, Canada.

Hightower, L.E., (1991). Heat shock, stress proteins, chaperones and proteotoxicity. Cell. 66, 191–197.

Hightower, L. E. & Guidon, P. T., Jr. (1989). Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. J. Cell. Physiol., 138(2), 257-266.

Houenou L.J., Li L., Lei M., Kent C.R., and Tytell M. (1996). Exogenous heat shock cognate protein Hsc70 prevents axotomy-induced death of spinal sensory neurons. Cell Stress & Chaperones, 1:161–166.

Hunter-Lavin, C., Davies, E. L., Bacelar, M. M., Marshall, M. J., Andrew, S. M. & Williams, J. H. H. (2004). HSP70 release from peripheral blood mononuclear cells. Biochem. Biophys. Res. Commun., 324(2): 511-517.

Ings J. S. & Van der Kraak, G. J. (2006). Characterization of the mRNA expression of StAR and steroidogenic enzymes in zebrafish ovarian follicles. Mol. Reprod. Dev, 73(8), 943-54.

Iwama, G. K., Afonso, L. O. B. & Vijayan, M. M. (2006). Stress in fishes. The Physiology of Fishes, 3rd Edition. Taylor & Francis, p. 319-342.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B. & Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. Amer. Zool., 39: 901-909.

Johnson, J. D., Campisi, J., Sharkey, C. M., Kennedy, S. L., Nickerson, M., & Fleshner, M. (2005). Adrenergic receptors mediate stress-induced elevations in extracellular Hsp72. J. Appl. Physiol., 99(5): 1789–95.

Johnson, D.A., Berberian, P.A., Tytell, M., and Bond, G.M. (1995).Differential Distrubution of 70kD Heat Shock Protein in Aherosclerosis: Its Potential role in Arterial SMC survival. Arterioscl. Thromb. Vas., 15:27-36.

Johnson A.D., Berberian P.A., Bond M.G. (1990). Effect of heat shock proteins on survival of isolated aortic cells from normal and atherosclerotic cynomolgus macaques. Atheroscl, 84:111–119.

Johnson A.D., and Tytell M. (1993). Exogenous hsp70 becomes cell associated, but not internalized, by stressed arterial smooth muscle cells. In Vitro Cell Dev Biol, 29A:807–812.

Koban, M., Graham, G. & Prosser, C.L. (1987). Induction of heat-shock protein synthesis in teleost hepatocytes: effects of acclimation temperature. Physiol. Zool., 60: 290-296.

Koban, M., Yup, A. A., Agellon, L. B. & Powers, D. A. (1991). Molecular adaptation to environmental temperature: heat-shock response of the eurythermal teleost Fundulus eteroclitus. Mol. Mar. Biol. Biotech, 1: 1-17.

Kondo H, Harano R, Nakaya M, Watabe S (2004) Characterization of goldfish heat shock protein-30 induced upon severe heat shock in cultured cells. Cell Stress Chaperon. 9:350–358

Kobayashi S, Welsh FA (1995). Regional alterations of ATP and heat shock protein 72 mRNA following hypoxia- ischemia in neonatal rat brain. J .Cereb. Blood. Flow. Metab. 15:1047–1056

Kojima, K., Musch, M. W., Ropeleski, M. J., Boone, D. L., Ma, A., & Chang, E. B. (2004). Escherichia coli LPS induces heat shock protein 25 in intestinal epithelial cells through MAP kinase activation. Am. J. Physiol. Gastrointest. Liver Physiol., 286(4), G645-G652.

Kregel, K. C. (2002). Invited review: Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol., 92(5): 2177-2186.

Kregel, K. C., & Moseley, P. L. (1996). Differential effects of exercise and heat stress on liver HSP70 accumulation with aging. J. Appl. Physiol., 80(2): 547-551.

Kregel, K. C., Moseley, P. L., Skidmore, R., Gutierrez, J. A., & Guerriero, V. (1995). HSP70 accumulation in tissues of heat-stressed rats is blunted with advancing age. J. Appl. Physiol, 79(5): 1673-1678.

Kultz D, Madhany S, Burg MB (2002) Hyperosmolality causes growth arrest of murine kidney cells: induction of GADD45 and GADD153 by osmosensing via stress- activated protein kinase 2. J. Biol. Chem. 273:13645–13651

Laan, M., Lotvall, J., Chung, K.F., Linden A. 2001. IL-17 induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen activated protein (MAP) kinases. Br. J. of Pharmacol., 133 (1): 200-206.

Lancaster, G. I., & Febbraio, M. A. (2005). Exosome-dependent trafficking of HSP70: A novel secretory pathway for cellular stress proteins. J. Biol. Chem., 280(24): 23349-23355.

Li L., Oppenheim R.W., Lei M., and Houenou L.J.. (1994). Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J of Neuro, 25:759–766

LeBlanc, S., Hoglund, E., Gilmour, K.M. and Currie, S. (2012). Hormonal modulation of the heat shock response: insights from fish with divergent cortisol stress responses. Am. J. Physiol. Regul. Integr. Comp. Physiol. 302 (R):184-192

Lo A.C., Houenou L.J., and Oppenheim R.W. (1995). Apoptosis in the nervous system: Morphological features, methods, pathology, and prevention. Archives of Histology and Cytology, 58:139–149.

Lowenberg, M., Verhaar A. P., van den Brink, G. R., and Hommes D. W. (2007) Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. Trends Mol.Med., 13 (4): 158-163

Ma N, Jin J, Lu F, Woodgett J, and Liu FF. The role of protein kinase B (PKB) in modulating heat sensitivity in a human breast cancer cell line. Int J Radiat Oncol Biol Phys 50: 1041–1050, 2001.

MacKenzie, S., Iliev, D., Liarte, C., Koskinen, H., Planas, J.V., Goetz, F.W., Mölsä, H., Krasnov, A., Tort, L., 2006. Transcriptional analysis of LPS-stimulated activation of trout (Oncorhynchus mykiss) monocyte/macrophage cells in primary culture treated with cortisol. Mol. Immunol. 43: 1340–1348.

Mathivanan, S., Ji, H., & Simpson, R. J. (2010). Exosomes: Extracellular Organelles Important in Intercellular Communication. J. proteomics, 73(10): 1907–20.

Mestril R, Chi SH, Sayen R, Dillmann WH (1994) Isolation of a novel inducible rat heat shock protein (hsp70) gene and its expression during ischemia/hypoxia and heat shock. Biochem J 298:561–569

Mambula, S. S., & Calderwood, S. K. (2006). Heat Shock Protein 70 Is Secreated from Tumor Cells by a Nonclassical Pathway Involving Lysosomal Endosomes. J. Immunol., 177: 7849–7857.

Mommsen, T. P., Vijayan, M. M. & Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish. Biol. Fish., 9, 211-268.

Murshid, A., Gong, J., Stevenson, M. A., & Calderwood, S. K. (2011). Heat shock proteins and cancer vaccines: developments in the past decade and chaperoning in the decade to come. Expert Rev. Vaccines, 10(11): 1553-1568.

Nakai, A. (1999). New aspects in the vertebrate heat shock factor system: Hsf3 and Hsf4. Cell Stress Chap, 4(2), 86-93.

Negulyaev, Y. A., Vedernikova, E. A., Kinev, A. V., and Voronin, A.P. (1996), Exogenous heat shock protein hsp70 activates potassium channels in U937 cells. Biochim. Biophys. Acta. 1282 (273): 156 -279.

Norris, C. E., di Iorio, P. J., Schultz, R. J. & Hightower, L. E. (1995). Variation in heat shock proteins within tropical and desert species of poeciliid fishes. Mol. Biol. Evol., 12, 1048–1062.

Oeckinghaus, A., Hayden, M. S., & Ghosh, S. (2011). Crosstalk in NF-κB signaling pathways. Nat. Immunol., 12(8): 695–708.

Ojima N, Yamashita M, Watabe S (2005a) Quantitative mRNA expression profiling of heat-shock protein families in rainbow trout cells. Biochem. Biophys. Res. Commun. 329:51–57

Ojima N, Yamashita M, Watabe S (2005b) Comparative expression analysis of two paralogous Hsp70s in rainbow trout cells exposed to heat stress. Biochim. Biophys. Acta. 1681:99–106

Osterloh, A. and Breloer,M. 2008. Heat Shock proteins: linking danger and pathogen recognition. Med. Microbiol. Immunol., 197:1-8

Parmini, E. & Usha Rani, M. (2008). Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 151(3): 278-285.

Pardue, M. L., Ballinger, D. G., & Hogan, N. C. (1992). The heat shock response. Cells coping with transient stress. Ann. N. Y. Acad. Sci., 663: 125–38.

Parsell, D. A. & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet., 27(1): 437-496.

Philip, A. M., Kim, D.S., Vijayan M.M. (2012). Cortisol modulates the expression of cytokines and suppressors of cytokine signaling (SOCS) in rainbow trout hepatocytes. Dev. and Comp. Immunol. 38 (2) 360-367.

Pirkkala, L., Nykanen, P. & Sistonen, L. E. A. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J, 15(7): 1118-1131.

Pockley, A. G., & Multhoff, G. (2010). Circulating Hsp70 as an endogenous Cytoprotector. Heat Shock and Whole Body Physiology. (Asea & B. K. Pedersen, Eds.): Springer Science + Media: 317–326.

Pockley, A. G., Muthana M., and Calderwood S.K..(2008). The dual immunoregulatory roles of stress proteins. Trends Biochem. Sci., 33 (2): 71-79.

Pratt, W.B. and Toft D. O., 2008. Regulation of Signalling Protein Function and Trafficking by hsp90/hsp70 – based Chaperone Machinery. Exp. Biol. and Med., 228(2): 111-133.

Reed, R. C., Berwin, B., Baker, J. P., Nicchitta, C. V. (2003) GRP94/gp96 elicits ERK activation in murine macrophages: a role for endotoxin contamination in NF_B activation and nitric oxide production. J. Biol. Chem., 278: 31853–31860.

Ritcher, K., Haslbeck., M., and Buchner, J. (2010) The Heat Shock Response: Life on the Verge of Death. Mol. Cell., 40 (2): 253-266.

Sciandra, J. J. & Subjeck, J. R. (1983). The effects of glucose on protein synthesis and thermosensitivity in chinese hamster ovary cells. J. Biol. Chem., 258(20): 12091-12093.

Skidmore, R., Gutierrez, J. A., Guerriero, V. & Kregel, K. C. (1995). HSP70 induction during exercise and heat stress in rats: Role of internal temperature. Am J Physiol Regul Integr Comp Physiol, 268(1), 92-97.

Santacruz, H., Vriz, S. & Angelier, N. (1997). Molecular characterization of a heat shock cognate cDNA of zebrafish, hsc70, and developmental expression of the corresponding transcripts. Dev Genet, 21, 223–233.

Sathiyaa, R., Campbell, T. & Vijayan, M. M. (2001). Cortisol modulates HSP90 mRNA expression in primary cultures of trout hepatocytes. Comp. Biochem. Physiol. B Comp. Biochem., 129: 679-685.

Sarge, K. D., Murphy, S. P. & Morimoto, R. I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol. Cell. Biol., 13(3): 1392-1407.

Schroeder, S., Bischoff, J., Lehmann, L.E., Hering, R., von Spiegel, T., Putensen, C., Hoeft A., and Stuber, F., (1999). Endotoxin inhibits heat shock protein 70 (Hsp70) expression in peripheral blood mononuclear cells of patients with severe sepsis. Intensive Care Medicine 25 (1): 52-52

Schmitt, E., Gehrmann, M. Brunet, M., Multhoff, G., Garrido, C. (2007). Intracellular and extracellular functions of heat shock proteins repercussions in cancer therapy. J. Leukoctye Biol., 81: 15-27

Shi, Y., Mosser, D. D. & Morimoto, R. I. (1998). Molecularchaperones as HSF1-specific transcriptional repressors. Genes Develop, 12(5), 654-666.

Specker, J. L. & Anderson, T. R. (1994). Developing an ELISA for a model protein – vitellogenin. Biochemistry and Molecular Biology of Fishes Volume 3: Analytical Techniques. Elsevier, p 567-578.

Stolte, E.H., Chadzinska, M., Przybylska, D., Filk, G, Savelkoul, H. F. J., Verburg-van Kemenade, L. B. M. (2009). The immune response differentially regulates Hsp70 and glucocorticoid receptor expression in vitro and in vivo in common carp (Cyprinus carpio L.) Fish. Shellfish. Immun.., 27 (1): 9-16

Theriault, J.R. et al. (2005). Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. FEBS Letters, 579: 1951–1960.

Thomas GP, Mathews MB (1984) Alterations of transcription and translation in HeLa cells exposed to amino acid analogs. Mol. Cell. Biol., 4: 1063-1072

Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol., 35: 1366–1375

Tsan, M-F.,and Gao, B. (2009). Heat shock proteins and the immune system. J Leukocyte Biol., 85: 905-910

Tsan, M.-F. (2011). Heat shock proteins and high mobility group box 1 protein lack cytokine function. J. leukocyte. Biol., 89(6): 847–53.

Tytell, M. (2005). Release of heat shock proteins (hsps) and the effects of extracellular hsps on neural cells and tissues. Int. J. Hyperthermia, 21(5): 445-455.

Tytell M., Barbe M.F., and Brown I.R. (1994). Induction of heat shock (stress) protein 70 and its mRNA in the normal and light damaged rat retina after whole body hyperthermia. J. of Neuro. Res., 38:19–31

Tytell, M., Greenberg, S. G., & Lasek, R. J. (1986). Heat shock-like protein is transferred from glia to axon. Brain Res, 363(1): 161-164.

Vabulas, R.M. et al. (2001). Endocytosed Hsp60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J. of Bio. Chem. 276: 31332–31339.

Vabulas, R.M. et al. (2002). Hsp70 as endogenous stimulus of the Toll/interleukin- 1 receptor signal pathway. J. of Bio. Chem., 277: 15107–15112.

Vabulas, R.M., and Wagner, H. (2005). Toll-Like Receptor-Dependent Activation of Antigen-Presenting Cells by Hsp60, Gp96 and Hsp70. Molecular Chaperones and Signalling. Cambridge University Press, p113-132

Vega, V.L., Charles, W. and De Maio, A. (2010). A new feature of the stress response: increase in endocytosis mediate by Hsp70. Cell stress Chaperon., 15: 517-527.

Vijayan, M.M., Aluru, N., Leatherland, J.F., (2010). Stress Response and the Role of Cortisol, second ed., in: Fish Disease. Noninfectious Disorders. vol. 2. C.A.B. International, Oxon, pp. 182–201.

Vijayan, M. M., Aluru, N., Maule, A. G., & Jørgensen, E. H. (2006). Fasting augments PCB impact on liver metabolism in anadromous arctic char.Toxic. Sci, 91(2): 431-439.

Vijayan, M. M., Pereira, C., Forsyth, R. B., Kennedy, C. J. & Iwama, G. K. (1997). Handling stress does not affect the expression of heat shock protein 70 and conjugation enzymes in rainbow trout treated with b-naphthoflavone. Life Sci, 61, 117–127.

Vijayan, M. M., Pereira, C., Kruzynski, G. & Iwama, G. K. (1998). Sublethal concentrations of contaminant induce the expression of hepatic heat shock protein 70 in two salmonids. Aquat. Toxicol, 40: 101–108.

Vijayan, M. M., Prunet, P. & Boone, A. N. (2005). Xenobiotic impact on corticosteroid signaling. *In*: Biochemistry and Molecular Biology of Fishes, Volume 6, Environmental Toxicology. Edited by T.W. Moon and T.P. Mommsen. Elsevier, Amsterdam., pp. 365-394.

Vijayan, M. M., Raptis, S. & Sathiyaa, R. (2003). Cortisol treatment affects glucocorticoid receptor and glucocorticoid responsive genes in the liver of rainbow trout. Gen. Comp. Endocrinol, 132: 256–263.

Wallin, R. P., Lundqvist, A., More, S. H., von Bonin, A., Kiessling, R., Ljunggren, H. G. (2002) Heat-shock proteins as activators of the innate immune system. Trends Immunol. 23, 130–135.

Wang, Y., Kelly, C. G., Karttunen, J. T., Whittall, T., Lehner, P. J., Duncan, L., et al. (2001). CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity, 15(6): 971-983.

Washburn B.S., Moreland J.J., Slaughter A. M. et al. (2002). Effect of Handling on heat shock protein expression in rainbow trout (oncorhynchus mykiss). Environ. Toxicol. Chem., 21: 557 -560.

Wendelaar Bonga, S. E. (1997). The stress response in fish. Physiol. Rev, 77(3): 591-625.

Weitzel, G., Pilatus, U. & Rensing, L. (1985). Similar dose response of heat shock protein synthesis and intracellular pH change in yeast. Exp. Cell Res., 159(1): 252-256

White, C. N., Hightower, L. E. & Schultz, R. J. (1994). Variation in heat-shock proteins among species of desert fishes (Poeciliidae, Poeciliopsis). Mol. Bio. Evol, 11: 106–119.

Woessmann W, Meng YH, and Mivechi NF. (1999). An essential role for mitogen-activated protein kinases, ERKs, in preventing heatinduced cell death. J. Cell. Biochem. 74: 648– 662.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., & Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 249(4975), 1431.

Yokoyama Y, Hashimoto H, Kubota S, Kinoshita M, Toyohara H, Sakaguchi M, Tanaka M, Seikai T, KanamoriM(1998). cDNA cloning of heat inducible HSP70, a 70 kDa heat shock protein in Japanese flounder, Paralichthys oliva-ceus. Fish Sci 64:964–968

Yu Q., Kent C.R., and Tytell M. (2001). Retinal uptake of intravitreally injected Hsc/Hsp70 and its effect on susceptibility to light damage. Mol. Vision, 7:48–56.

Zhang, A., Zhou, X., Wang, X., Zhou, H., 2011. Characterization of two heat shock proteins (Hsp70/Hsc70) from grass carp (Ctenopharyngodon idella): evidence for their differential gene expression, protein synthesis and secretion in LPS-challenged peripheral blood lymphocytes. Comparative Biochemistry and Physiology B Biochemical and Molecular Biology 159, 109–114.

Zou, J., Holland, J., Pleguezuelos, O., Cunningham, C., Secombes, C.J., 2000. Factors influencing the expression of interleukin-1b in cultured rainbow trout (Oncorhynchus mykiss) leucocytes. Dev. Com. Immunol. 24: 575–582