Stress-Immune-Growth Interactions in Fish: Mechanisms and Nutritional Modulation

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2014

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Chapter 2

AMP and MMV designed the experiments. AMP and SDK performed the experiments. SDK conducted the Western and enzyme activity analysis. AMP conducted all the remaining analysis including gene expressions, data processing and interpretation. AMP and MMV wrote the manuscript.

Chapter 3

EHJ and AGM were involved in the maintenance of fish and sampling. AMP carried out all the analyses, data processing and interpretation. AMP and MMV wrote the manuscript.

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Chapter 6

AMP and MMV designed the experiments. AMP carried out all the experiments, analyses, data processing and interpretation. AMP and MMV wrote the paper.

Abstract

The primary adaptive organismal response to stress involves the activation of the hypothalamic-sympathetic-chromaffin cell (HSC) axis leading to rapid secretion of catecholamines, predominantly epinephrine. The hypothalamus-pituitary-interrenal (HPI) axis activation follows, leading to the secretion of cortisol in teleosts. Both these hormones play key roles in fueling the increased energy demand associated with stress. On prolonged exposure to periods of chronic stress, the stress response shifts from adaptive to maladaptive, eventually resulting in decreased disease resistance, reduced growth and an overall decline in fitness. This reduction of performance can be viewed as a consequence of the animals altered energy budget, with an increase in the metabolic requirements to cope with stress, leading to a reduction in body defense and growth potential. However, the mechanisms linking stress effects on growth and immune performance are far from clear. The suppressors of cytokine signalling (SOCS) are gaining increasing attention in mammalian models and particularly in human medicine for their ability to regulate diverse physiological functions like immunity, growth and development. Even though homologues of the SOCS genes have been identified in fishes, their functional roles are unknown. This paucity of information on the role of SOCS, combined with the knowledge that they are key regulators of energy demanding pathways in mammals, led to the hypothesis that the SOCS genes may be playing a critical role during stress to divert energy away from immune and growth processes in fishes.

To test this, liver was used as a model because this tissue plays an important role in stress adaptation, immune response and growth. The ability of cortisol to modulate immune responses in the liver was investigated by exposing rainbow trout (*Oncorhynchus mykiss*) hepatocytes to lipopolysaccharide (LPS), a potent immunostimulant, along with cortisol and mifepristone- a glucocorticoid receptor (GR) antagonist, to tease out the role of cortisol signalling on immune function. The results showed that LPS stimulation increases the cellular stress response and metabolic capacity and induces the expression of innate response mediators in trout hepatocytes. Cortisol modulates these responses and this involves GR signalling. The results demonstrated for the first time that cortisol upregulates SOCS-1 and

SOCS-2 mRNA abundance in trout liver, leading to the proposal that these proteins may be involved in stress/cortisol-mediated immune suppression.

The SOCS are also potentially involved in energy–reallocations associated with nutritional restriction. To test this, immune responses and SOCS regulation in response to LPS challenge were investigated between two salmonids exhibiting different life-strategies, the anadromous Arctic charr (*Salvelinus alpinus*), which undergo natural long-term fasting, and the rainbow trout, that do not naturally fast. Arctic charr and rainbow trout were fed or fasted for 85 and 118 d, respectively, and injected with LPS to examine their ability to evoke an immune response despite their negative energy balance. While fasting did not alter stress parameters like plasma cortisol and glucose levels in the Arctic charr, nutrient restriction modified plasma glucose and lactate levels and liver glycogen content in rainbow trout. Additionally the fasted charr showed lower cytokine responses to LPS than the fed charr, while there was no difference in the degree of cytokine responses between the fed and fasted rainbow trout. Fasting also upregulated SOCS isoforms in the Arctic charr, but reduced SOCS-1 expression in rainbow trout. LPS upregulated SOCS-3 in the Arctic charr, but downregulated SOCS-2 levels in rainbow trout. Together, these results suggest differences in the stress, cytokine and SOCS responses to fasting and LPS stimulation between these two salmonid species. Specifically, SOCS upregulation by fasting in charr may be adaptive to restrict energy demanding pathways, including inflammatory response and growth, to cope with the negative energy balance during overwintering. To assess if SOCS are acutely regulated by stress and if this response was modulated by fasting and LPS stimulation, trout were fed or fasted for 118 d and then injected with LPS, and 72 h later subjected to a handling disturbance. Prior fasting and LPS stimulation altered the acute stressor-mediated changes in plasma cortisol, glucose and lactate levels and liver glycogen content and GR expression in trout. Acute stress also modulated liver SOCS-2 and SOCS-3 mRNA levels in rainbow trout. Overall the results suggest that liver SOCS-2 upregulation by acute stress may be playing a role in the metabolic adjustments essential to cope with stress in fishes.

Finally, a series of *in vitro* studies to identify possible mechanisms involved in SOCS mediated immune and growth suppression were carried out. The objective was to examine whether upregulation of SOCS genes, a key negative regulator of JAK/STAT signalling by cortisol, is a key molecular link in the suppression of growth and immune responses during stress in fish. Cortisol exposure suppressed growth hormone (GH)-stimulated insulin like growth factor (IGF-1) expression and this involved reduced STAT5 phosphorylation/ activation and decreased total JAK2 protein levels. Cortisol also suppressed LPS-induced IL-6 transcript levels. While LPS reduced GH signalling, this was mediated by the downregulation of GH receptors and not due to upregulation of SOCS genes. These results highlight a novel molecular mechanism, involving SOCS upregulation by cortisol, linking stress effects on growth and immune suppression in rainbow trout. Altogether, the results for the first time highlight novel functional roles for the SOCS genes as regulators and integrators of stress-immune-growth processes, and the mode of action involves their regulation by cortisol signalling in fishes.

Acknowledgements

This is perhaps the easiest and hardest chapter that I have to write. It will be simple to name all the people that helped with this work, but it will be hard to thank them enough. I will nonetheless try…

My head bows before God Almighty for His innumerable blessings and the knowledge, wisdom and understanding that enabled me to complete this thesis.

Much gratitude is due to my Supervisor Dr. Matt Vijayan for giving me the opportunity to work in his lab. Throughout my Ph.D., he provided sound mentoring, encouragement, good teaching, good company and lots of good ideas! I would have been lost without him. I would also like to thank my committee members, Dr. Brendan McConkey and Dr. Brian Dixon for taking the time out of their busy schedules to review my work and provide helpful feedback.

This acknowledgement will never be complete without the special mention of my lab seniors Ms. Nita Modi and Dr. Maryam Kamkar, for all their support and motivation during my initial days as a Ph.D. student and for the lifelong friendship that it turned into. I also want to extend my heartfelt thanks to all the members of the Vijayan Lab- past and present for their co-operation, coffee-breaks and everything else that made this long journey more enjoyable and memorable.

Finally I thank my wonderful parents Philip and Annie and my little brother Ajit, who have raised me to be the person I am today. You have been with me every step of the way, through good times and bad. Thank you for all the unconditional love, guidance and support that you have always given me, helping me to move ahead and instilling in me the confidence that I am capable of doing anything I put my mind to.

Lastly, but most importantly, I thank my husband Aby and our son Alex. Aby has been my most powerful cheerleader, by best friend, my amazing husband, a wonderful father to Alex and not to mention a great cook! His willingness to work around my lab schedule and be Alex's primary caregiver when I wasn't around, helped me to complete this thesis right on track in 4 years. He has seen me through the ups and downs of this Ph.D. process and has shared this amazing journey with me. As for Alex, it is this two year old's bright smile, hugs and naughtiness that helped me get over the "bad science days" and put it all into perspective. He reminds me daily of the wonderful times ahead and all that is yet to be.

Thank You All!

Dedication

To my family

who taught me the richness of learning

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Chapter 1
General Introduction

1.1 Introduction

Even though an organism's response to stress has adaptive value, exposure to chronic or severe stress, which is not uncommon in mammals as well as lower vertebrates like teleosts, can have adverse effects on growth and immunity (Tort and Teles, 2011). This reduction in immune and growth performance can be viewed as a consequence of the animal's altered energy budget, with an increase in the metabolic requirements to cope with stress, leading to a reduction of immune and growth potential. Consequently, sustained activation or suppression of the stress axis may not only wreak havoc in an individual but can potentially damage population dynamics (Fefferman and Romero, 2013). Several studies have looked at the bi-directional communication between stress and immune responses as well as stress and growth processes. However, there is a paucity of information on the molecular mechanisms linking stress effects on growth and the suppression of immune responses in mammalian as well as teleost models.

In fishes, feed restriction is a natural occurrence in wild populations and occurs frequently in cultured fishes as well (Liu et al., 2013). This can also have negative effects on growth and immunity due to alterations in the animal's energy budget (Martin et al., 2010). Salmonids, like the anadromous Arctic charr (*Salvelinus alpinus*) undergo voluntary long-term fasting as part of a life-history strategy as opposed to other species like the rainbow trout (*Oncorhynchus mykiss*) (Jørgensen et al., 1997). But the mechanisms involved in cellular energy re-partitioning during long-term fasting in fishes are poorly understood, and it is not known if species that naturally resort to extended fasting have unique molecular adaptations that arose as a consequence of their life-style.

The suppressors of cytokine signalling (SOCS) are a class of proteins gaining increasing recognition for their role in mammalian development and disease, by acting as regulators of diverse physiological functions (Kile and Alexander, 2001; Trengove and Ward, 2013). In mammals, the SOCS act as negative regulators of cytokine and growth hormone (GH) signalling. They negatively regulate the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, a signalling pathway common to GH, immunostimulants like lipopolysaccharide (LPS) and cytokines (Croker et al., 2008). Yet, the

effects of stress and stress hormones on SOCS expression have not been explored. Homologues of all the SOCS family members have been identified in teleosts (Wang et al. 2011), but their functional roles in fishes are far from clear. The purpose of this thesis was to investigate the effects of stress as well as altered nutritional status on SOCS expression in fishes, and to see if the SOCS can act as a molecular link in integrating and regulating stress-immune-growth interactions in fishes.

1.2 Stress in Fishes

Life exists by maintaining a complex dynamic equilibrium or homeostasis, which is constantly challenged by internal or external adverse forces, the stressors (Chrousos et al., 1996). Thus stress is defined as a state in which homeostasis is actually threatened or perceived to be so (Chrousos et al., 1996). Homeostasis is re-established by a complex array of molecular, biochemical, physiological and behavioral adaptive responses of the organism, collectively referred to as the stress response (Mommsen et al., 1999). The physiological response to stress is often characterized temporally by three phases, the primary, secondary and tertiary responses (Mommsen et al., 1999; Barton, 2002) (Figure 1). The primary response is rapid and involves neuroendocrine activation, leading to the release of catecholamines and corticosteroids in circulation (Barton, 2002). The catecholamines (epinephrine and norepinephrine) in circulation are released from the chromaffin cells distributed in the head kidney region of fishes, in response to the stressor-induced activation of the sympathetic nervous system (hypothalamic-sympathetic-chromaffin cell (HSC) axis) (Reid et al., 1998; Vijayan et al., 2010).

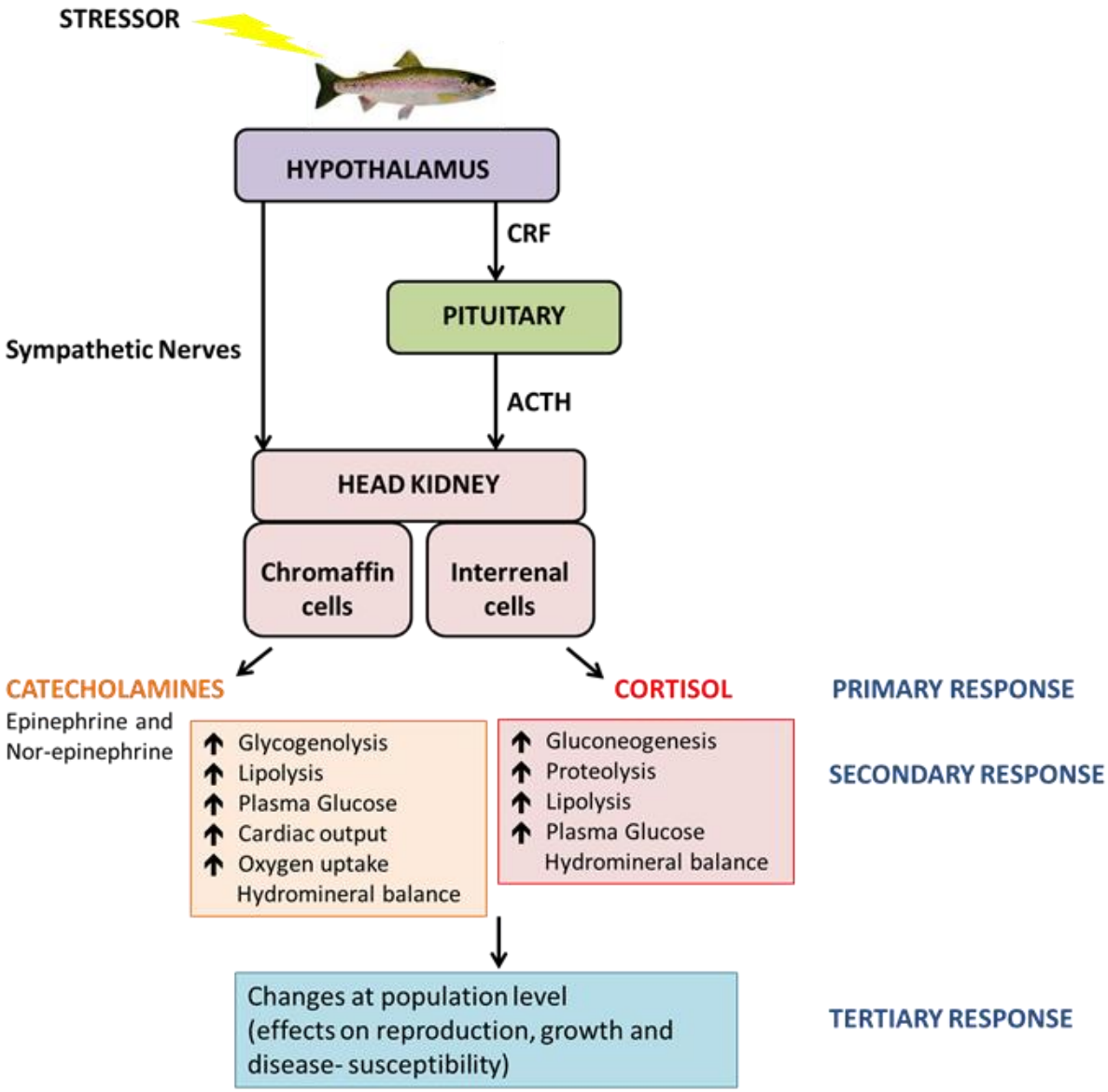
Cortisol, the principal corticosteroid in teleosts, is released from the interrenal tissue located in the head kidney region in response to the stressor-induced activation of the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997; Mommsen et al., 1999; Vijayan et al., 2010). Briefly, activation of the HPI axis is initiated by perception of the stressor, leading to the release of corticotrophin releasing factor (CRF) from the hypothalamus. This neuropeptide then acts on the anterior pituitary and stimulates the release of adrenocorticotropic hormone (ACTH) into the general circulation. This pituitary peptide is the primary secretagogue for *de novo* synthesis and secretion of cortisol and involves the

activation of melanocortin 2 receptors (MC2R) on the steroidogenic cells located in the head kidney (Aluru and Vijayan, 2008). Cortisol biosynthesis is accomplished through a series of reactions starting with the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR; Stocco et al., 2005) and peripheral-type benzodiazepine receptor (PBR; Lacapère and Papadopoulos, 2003), often referred to as the rate limiting step in steroid hormone biosynthesis. Additionally, cytochrome P450 side chain cleavage (P450scc) or cyp11A1 and 11β-hydroxylase are two key enzymes important for cortisol biosynthesis (Mommsen et al., 1999; Payne and Hales, 2004).

The primary hormonal stress response is important in the regulation of many physiological and cellular processes, including energy substrate mobilization and reallocation, increased cardiac output and blood flow and enhanced oxygen uptake and transport at the gills, all essential for acute stress adaptation (Vijayan et al., 2010). Together, these physiological and biochemical changes constitute the secondary stress response (Barton, 2002). In the short-term, stress responses are geared towards metabolic adjustments and fuel delivery to tissues that have a higher energy demand, while in the long-run this may lead to negative consequences, including impaired immune function, growth and reproduction, due to diversion of energy resources from these growth and health processes in order to re-establish homeostasis. This in turn can result in adverse changes at the population level, including decreased reproductive potential, increased disease susceptibility and mortality, constituting the tertiary stress response (Barton, 2002).

Figure 1: The teleost stress axis

Following stressor perception, the hypothalamic-sympathetic-chromaffin cell (HSC) and the hypothalamic-pituitary-interrenal cell (HPI) axes are activated, stimulating the release of catecholamines and cortisol respectively, and together constitute the primary stress response. Elevation of these stress hormones mediate important physiological adjustments required for stress adaptation, collectively referred to as the secondary stress response. Prolonged or chronic elevation of stress hormones leads to negative consequences or maladaptation, bringing about adverse changes at the population level and constitute the tertiary stress response.


STRESSOR
HYPOTHALAMUS
CRF
PITUITARY
Sympathetic Nerves
ACTH
HEAD KIDNEY
Chromaffin cells
Interrenal cells
CATECHOLAMINES
Epinephrine and Nor-epinephrine
CORTISOL
PRIMARY RESPONSE
Glycogenolysis
Lipolysis
Plasma Glucose
Cardiac output
Oxygen uptake
Hydromineral balance
Gluconeogenesis
Proteolysis
Lipolysis
Plasma Glucose
Hydromineral balance
SECONDARY RESPONSE
Changes at population level (effects on reproduction, growth and disease- susceptibility)
TERTIARY RESPONSE

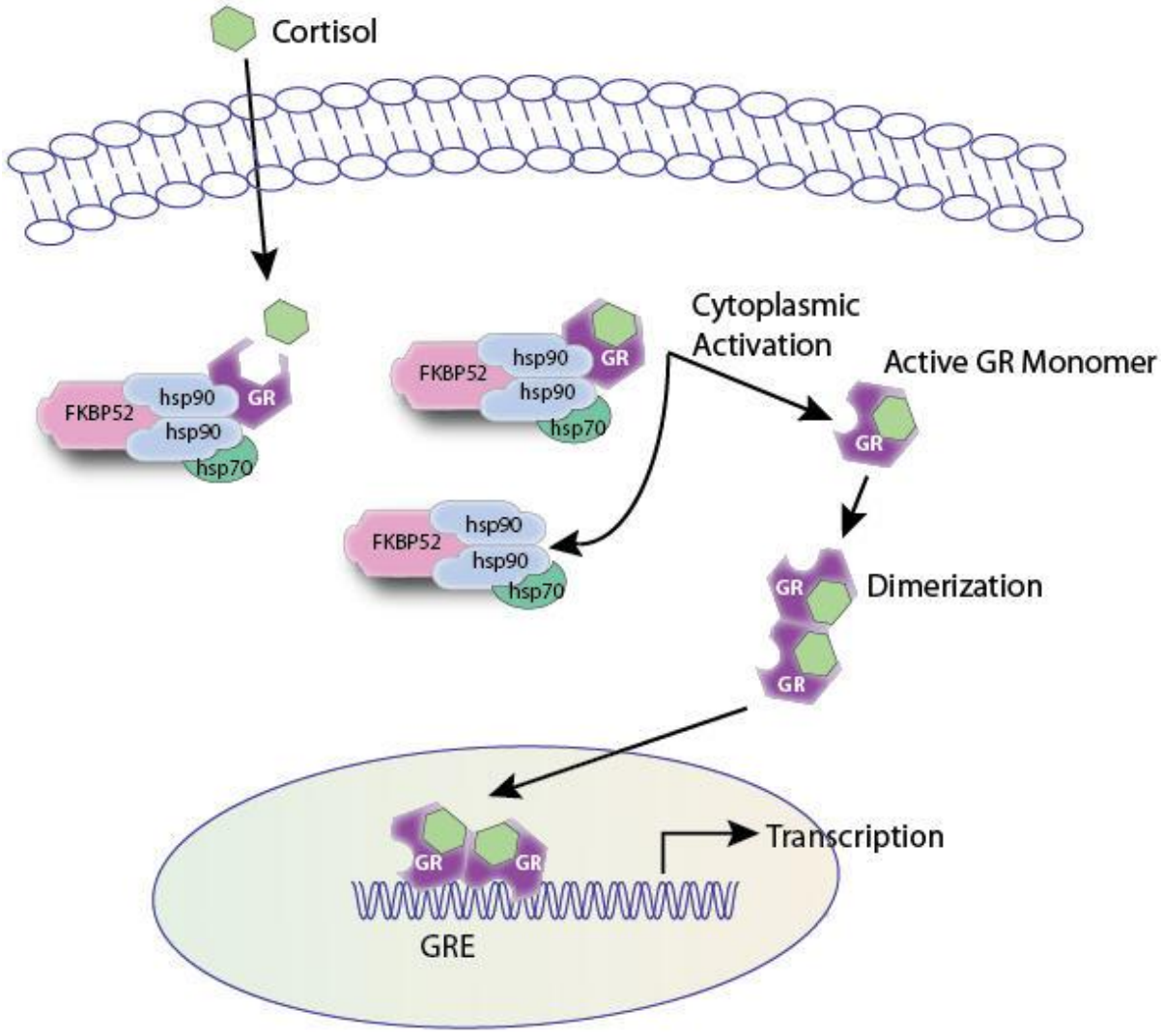
1.3 Glucocorticoid Signalling

Cortisol action is mediated predominantly by binding to intracellular corticosteroid receptors (Funder, 1997), namely the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Mommsen et al., 1999; Bury and Sturm, 2007; Prunet et al., 2006; Alsop and Vijayan, 2009; Nesan and Vijayan, 2013). Unlike other vertebrates, most teleosts exhibit multiple GR genes as a result of genome-wide duplication events, which occurred 25-100 million years ago (Meyer and Van de Peer, 2005; Koop and Davidson, 2008). Most teleosts possess two genes encoding GR namely GR1 and GR2 (Bury and Sturm, 2007), the only exception being zebrafish (*Danio rerio*) with one GR (Alsop and Vijayan 2009). GR1 and GR2 have different activation properties. GR2 is considered to be more sensitive than GR1 and is subsequently activated at lower cortisol concentrations while GR1 is thought to be activated at high, stress-induced levels of cortisol (Prunet et al., 2006; Sturm et al., 2011). Teleost also express an analogous MR receptor (Colombe et al., 2000) but it is controversial whether fishes possess a distinct endogenous MR ligand other than cortisol. Though 11-deoxycorticosterone has been suggested as a putative ligand for MR in fishes (Sturm et al., 2005), their physiological actions are not very clear.

Classical cortisol signalling starts with cortisol binding to and activating GR. This results in receptor dimerization and translocation into the nucleus, where the activated glucocorticoid-receptor complex modulates target gene expression by binding to glucocorticoid response elements (GREs) upstream of target genes (Mommsen et al., 1999) (Figure 2). In the absence of corticosteroid binding, GR forms a multi-protein complex with chaperone proteins namely heat shock proteins and immunophilins (Nicolaides et al., 2010), although this has not been confirmed in teleosts. In addition to the classical GR signalling pathway, cortisol can also stimulate rapid non-genomic effects, which are independent of gene transcription (Borski, 2000; Dindia et al., 2013). Yet, these effects are less explored compared to the classical genomic effects.

Figure 2: Schematic diagram of classical glucocorticoid receptor activation and signalling

Cortisol released into circulation from the interrenal tissue, crosses the plasma membrane of target cells and binds to the cytosolic glucocorticoid receptor (GR). Cortisol binding causes the dissociation of GR from its chaperone proteins and the dimerization of the activated receptor, which then translocates into the nucleus to act as a transcription factor and modulate target gene expression.



(Adapted from www.affymetrix.com)

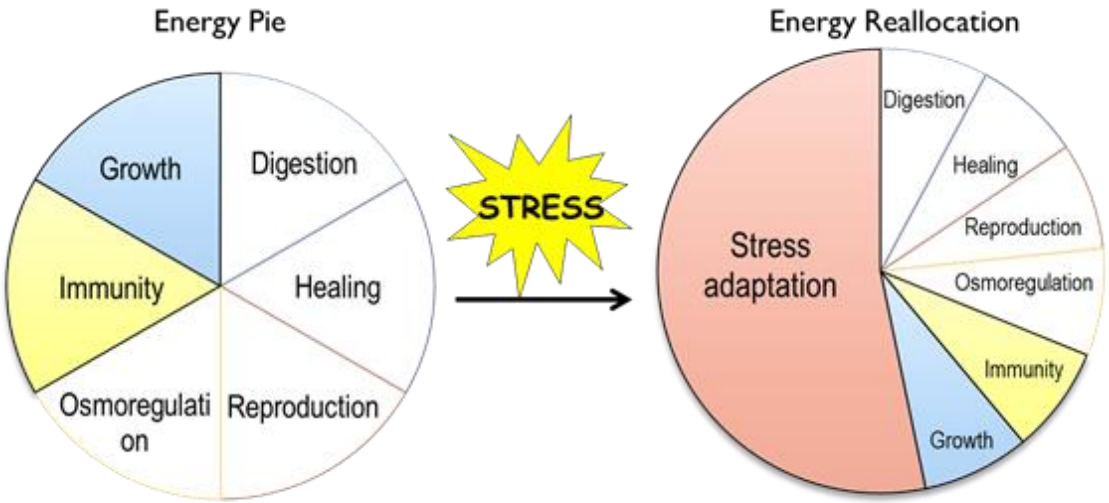
1.4 Metabolic effects of stress and changes in energy budget

In fishes, high plasma cortisol levels associated with stress have a wide range of metabolic effects including, the modulation of carbohydrate metabolism through gluconeogenesis, increases in protein turnover and increased lipolysis (reviewed in Mommsen et al., 1999). Plasma metabolite levels such as glucose and lactate are routinely used as indices for stressor and hormone effects on metabolism (Vijayan et al., 1994; 2003; 2010). A clear link has been made between increased cortisol and increased plasma glucose levels (Mommsen et al., 1999), as the primary role of cortisol is to mobilize energy substrates in order to cope with the increased energy demand associated with stress. The initial stressor-induced release of glucose is mediated by catecholamine-induced glycogenolysis, while the maintenance of plasma glucose levels in response to stressor exposure is through cortisol-induced hepatic gluconeogenesis (Mommsen et al., 1999). Previous studies have shown that stress and cortisol elevation increase the activity as well as the transcript abundance of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (the rate-limiting step in gluconeogenesis that converts oxaloacetate into phosphoenolpyruvate and carbon dioxide) (Wiseman et al., 2007; Sathiyaa and Vijayan 2003; Vijayan et al., 2003). An increase in the activity of hepatic and extra-hepatic glycolytic enzymes, including hexokinase, pyruvate kinase and lactate dehydrogenase, have also been associated with acute stress and cortisol exposure, likely as a means to provide metabolic fuel required to cope with the increased energy demand associated with stress adaptation (Mommsen et al., 1999; Ings et al., 2011).

Thus any response or adaptation to stress requires the expenditure of energy that would otherwise be utilized for maintaining normal body functions, including immune function and growth (Figure 3). If the total energy budget in an organism is considered as a pie with only so many pieces, stress adaptation consumes a large portion of this energy (Barton, 2002). However, the mechanisms involved in stress-related cellular energy re-partitioning are not clear.

Figure 3: Schematic representation of the effect of stress on the animal's energy budget

Animals are thought to have a limited amount of energy, which it uses to support normal physiological functions and maintain physiological equilibrium. Stress alter an animal's energy budget by consuming a large amount of energy to deal with the increased metabolic demand associated with stress, which in turn means less energy is available for normal body functions like immune function and growth.



1.5 Fish Immune Response and the role of Cytokines

The basic mechanisms governing an immune response follow certain patterns that are conserved throughout evolution. These include macrophage activity, cytokine or complement factor signalling as well as specific targeting of pathogens (Janeway and Walport, 2001). Yet, fishes rely to a large extent on a highly diversified innate immune response controlled by cytokines, due to limitations in their adaptive immune system, including limited array of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007).

In fishes, the innate immune response is commonly divided into three compartments, the epithelial/mucosal barrier of the skin, gills and alimentary tract, the humoral parameters and the cellular components (Uribe et al., 2011). The innate immune system recognizes conserved molecular structures common to pathogenic microorganisms, including LPS, polysaccharides, peptidoglycans, bacterial DNA, and double-strand viral RNA, through their interaction with specific receptors like toll receptors (TLRs) (Reyes-Cerpa et al., 2012). This receptor-mediated pathogen recognition may lead directly to successful removal of pathogens, or may trigger additional protective responses through activation of the adaptive immune pathways. As in mammals, phagocytosis and inflammation are two non-specific innate immune responses that appear to be important in fishes (Corbel, 1975). The immediate innate immune response to the presence of any "foreign" agent or pathogen includes inflammatory cytokines, acute phase proteins (APP) and antimicrobial peptides (Ellis, 2001; Nakao et al., 2011). Firstly, non-specific humoral factors, including lysozyme, acute phase proteins [e.g. C-reactive protein (CRP), serum amyloid protein A (SAA), transferrin, lectin], complement and interferons (IFN) hamper the spread and multiplication of the pathogen and activate the cellular part of the immune system (Nakao and Yano, 1998; Bayne and Gerwick, 2001). Most of these proteins are normally present in the serum and often induced upon infection. Macrophages and neutrophils are the principal cell types associated with carrying out innate immune reactions in fishes (Uribe et al., 2011). Cells of the innate immune system have a wide array of functions. Some cells are phagocytic and will engulf and degrade pathogenic particles. Phagocytosis is an important and ancient defense mechanism which is

most important in poikilothermic animals because it is a process that is least influenced by temperature (Uribe et al., 2011). Both macrophages and neutrophils exert phagocytic functions in fishes (Secombes and Fletcher, 1992). These cells release a number of reactive oxygen species and nitric oxide (NO) to kill intracellular or extracellular pathogens (Uribe et al., 2011). Other cells produce and secrete cytokines and chemokines to help guide the migration of cells, and further direct the immune response (Secombes et al., 2001).

The adaptive immune response is initiated when antigenic particles are taken-up and processed by specialized antigen presenting cells (APC) and subsequently presented on major histocompatibility complex (MHC) class II molecules (Dixon et al., 1995). Additionally, MHC class I molecules present an array of self antigens whose altered expression is indicative of virus infected cells (Stet et al., 1998). These presented antigens activate cells of the adaptive immune system namely T lymphocytes and B lymphocytes, to eliminate the pathogen involved (Uribe et al., 2011). When B lymphocytes are activated, they are capable of differentiating into plasma cells that can secrete antibodies (Janeway and Walport, 2001). T lymphocytes upon activation differentiate into either helper T cells or cytotoxic T cells (Janeway and Walport, 2001). Helper T cells are capable of activating other cells of the adaptive immune response such as B lymphocytes and macrophages, while cytotoxic T cells upon activiation are able to kill cells that have been infected (Janeway and Walport, 2001). Together, they build-up long lasting immunological memory and faster response when exposed to the same pathogen thereafter (Uribe et al., 2011). Initiation of an effective immune response requires close interactions between the innate and adaptive immune responses with cytokines playing a predominant role in this interaction.

1.5.1 Cytokines

Cytokines are secreted proteins which act as regulators of the immune system and control cell-to-cell communication. Most cytokines are pleiotropic, meaning they have multiple sources, targets and also multiple functions (Reyes-Cerpa et al., 2012) and are classified into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines (Savan and Sakai, 2006). Like in mammals, the innate immune response in fishes is initiated by cytokines, including IL-1β, tumor necrosis factor α

(TNFα), IL-2, IL-6 and interferon γ (IFNγ) (Uribe et al., 2011). These are often referred to as pro-inflammatory cytokines due to their role in initiating and facilitating the inflammatory response. While IL-1β has also been shown to be involved in the anti-viral response (Haugland et al., 2005), the immune response against virus mainly relies on the IFNs which in turn induce the production of anti-viral proteins (Uribe et al. 2011). Activation of Type I IFNs leads to the production of anti-viral protein Mx while activation of Type II IFN (IFNγ) leads to production of anti-viral protein γ inducible protein (γIP also called IP10) (Sun et al., 2011; Zou and Secombes, 2011). IL-10 and transforming growth factor β (TGFβ) are anti-inflammatory cytokines involved in the innate immune response and function to modulate and prevent over activity of the inflammatory responses (Bogdan et al., 1992). Macrophages secrete IL-1, IL-6, IL-12, TNFα, and chemokines such as IL-8 and monocyte chemo-attractant protein-1 (MCP-1), which activate and recruit macrophages, neutrophils and lymphocytes to the infected tissues (Svanborg et al., 1999). Meanwhile, cytokines released by phagocytes in tissues can also induce the synthesis of acute phase proteins, including mannose-binding lectin (MBL), CRP and SAA (DeVries et al. 1999; Talbot et al. 2009). Furthermore, cytokines involved in leukocyte differentiation, including granulocyte colony stimulating factor (CSF), macrophage-CSF and IL-7 have all been identified in fishes (Uribe et al., 2011).

Several cytokines are also involved in modulating the adaptive immune response. As in mammals, it is believed that cytokines will drive the activation and differentiation of T helper (Th) cell subsets to release different cytokine arrays (Secombes, 2008). IFNγ, among others, initiates the activation of the adaptive immune response. This cytokine together with IL-2 are expressed by Th1 cells and promotes their proliferation, whereas IL-4, IL-5 and IL-10 are expressed by Th2 cells and further promote their proliferation (Uribe et al., 2011). Even though the functionality of a Th1/Th2 response and the existence of other Th cell subsets, as defined in mammals have not been verified in fishes, the presence of cell markers and cytokine patterns suggests that these functions may indeed exist in fishes (Reyes-Cerpa et al., 2012).

Some of the cytokines commonly studied in fishes- IL-1β, TNFα, IL-8 and IL-6, along with their signalling pathways, are reviewed in detail below.

IL-1β

IL-1β is produced and released by most cell types in the body and is commonly used as a a marker of overall immune activation (Uribe et al., 2011). IL-1β was the first interleukin to be characterized in fishes and has since been identified in several fish species and is involved in the regulation of immunity through the stimulation of T cells (Uribe et al., 2011). It is an 'early' cytokine in the inflammatory response and mediates most of its effects by up- or downregulating other cytokines. Biological activity of IL-1β requires processing into a mature form through cleavage by interleukin converting enzyme (ICE) (Secombes et al., 2001). There are two types of IL-1 receptors namely IL-1R type I and IL-1R type II, but only binding of IL-1β to the type I receptor evokes signal transduction. Upon binding of IL-1β, the cytokine-receptor complex forms a heterodimer with the IL-1 receptor accessory protein (IL-lRAcP). This leads to signal transduction and activation of the nuclear factor (NF)- κB pathway. NF-κB resides in the cytosol in an inactive form complexed to the chaperone protein inhibitor IκB. Upon phosphorylation, IκB dissociates from the complex and NF-κB enters the nucleus to bind NF-κB response elements in the promotor regions of target genes (Martin and Falk, 1997; May and Ghosh, 1998). As in mammals, teleost IL-1β is regulated by various stimuli, including LPS and poly I:C (Reyes-Cerpa et al., 2012).

TNFα

TNFα is another pro-inflammatory cytokine that plays important roles in cell proliferation, differentiation, necrosis, apoptosis, and the induction of other cytokines (Reyes-Cerpa et al., 2012). TNFα and IL-1β act synergistically to activate the inflammatory response (Stahl et al., 2003). TNFα has been identified, cloned, and characterized in several fish species including Japanese flounder, rainbow trout, gilthead seabream, carp, catfish, tilapia, turbot and goldfish (Reyes-Cerpa et al., 2012). It is mainly produced by macrophages and is often used as a marker for inflammation in medicine. Studies in several fish species have shown that TNF causes the activation of macrophages, leading to increased respiratory

activity, phagocytosis and nitric oxide production (Uribe et al., 2011). Two receptors TNF-R1 and TNF-R2 have been identified (Orlinick and Chao, 1998). TNFα can induce either NF-κB mediated survival or apoptosis, depending on the cellular context (Rahman and McFadden, 2006). Both receptors bind TNFα but only signalling via TNF-R1 leads to the induction of apoptosis (Scaffidi et al., 1999). The intracellular pathway leading to activation of NF-κB is similar to that in the IL-1R signalling pathway.

IL-8

IL-8 is an important chemokine involved in the pro-inflammatory process and it's activity is coordinated by binding to G-protein-linked receptors having seven transmembrane domains (Secombes et al., 2001). IL-8 has been characterized in several fish species like flounder, trout, catfish, and lamprey (Reyes-Cerpa et al., 2012). IL-8 attracts neutrophils, T lymphocytes and basophils *in vitro,* but not macrophages or monocytes (Mukaida et al., 1998). Many cell-types, including macrophages, produce IL-8 in response to a variety of stimuli including LPS, cytokines like IL-1β and TNFα, and viruses (Reyes-Cerpa et al., 2012). The biological effects of IL-8 on neutrophils include increased cytosolic calcium levels, respiratory burst, changes in neutrophil shape and chemotaxis (Laing et al., 2002).

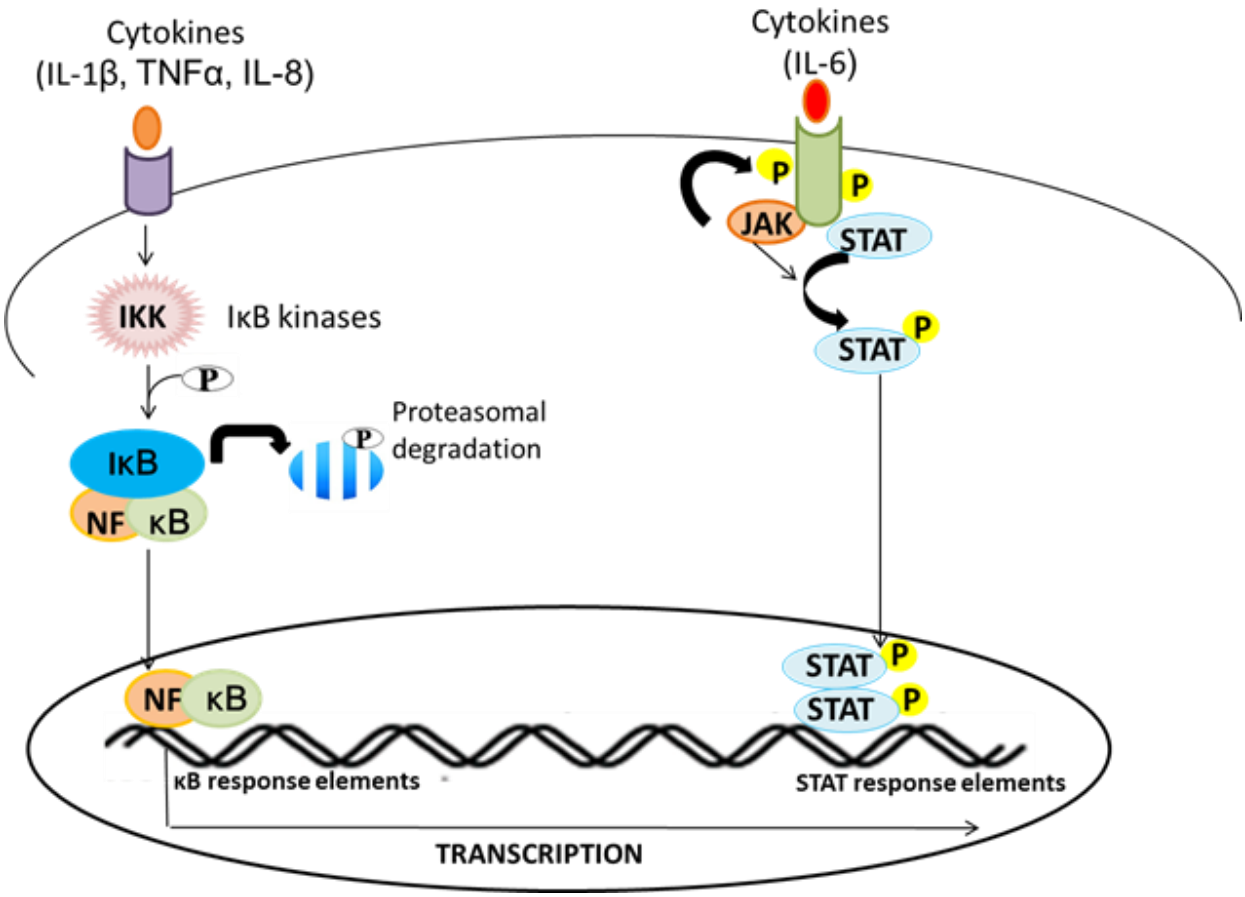
IL-6

IL-6 is another inflammatory response cytokine exhibiting pro- as well as anti-inflammatory properties. The expression of IL-6 is up-regulated by IL-1β as well as TNFα and through inhibitory effects on IL-1β and TNFα, IL-6 can also function as an anti-inflammatory mediator (Tilg et al., 1994). IL-6 is also important as the major mediator of the acute phase reactions (Gruys et al., 2005). IL-6 binds the IL-6Rα receptor and induces formation of a homodimer of the cell surface protein gp l30. The gp l30 signal-tranduction system involves triggering of the JAK/STAT signalling pathway (Croker et al., 2008). Cytokine binding induces receptor dimerization, activating the associated JAKs, which phosphorylate themselves and the receptor. The phosphorylated sites on the receptor and JAKs serve as docking sites for the STAT proteins which in turn get phosphorylated by the JAKs. Phosphorylated STATs dimerize and translocate into the nucleus to regulate target

gene transcription (Croker et al., 2008). IL-6 has been identified in several fish species but little is known about the function and signalling pathways of IL-6 in fishes. LPS, poly I:C and rIL-1β induce IL-6 expression in rainbow trout macrophages and the monocyte cell line RTS-11 (Costa et al., 2011) Moreover, IL-6 induces its own expression, suggesting that it can act in an autocrine and paracrine fashion with the potential to both amplify and limit the inflammatory response (Reyes-Cerpa et al., 2012).

Figure 4: Simplified diagram showing IL-1β, TNFα, IL-8 and IL-6 signalling pathways

The cytokines IL-1β, TNFα and IL-8 signal through the NF-κB pathway. The binding of cytokine to its respective receptor leads to the recruitment and activation of the I kappa B kinase complex (IKK) which in turn phosphorylates IκB, an inhibitory protein that is bound to NF-κB. The phosphorylation of IκB induces its release from its complex with NF-κB, after ubiquitination and degradation by proteasomes. The now active NF-κB then translocates to the nucleus to activate target genes regulated by κB sites. The cytokine IL-6 signals through the JAK/STAT pathway. Binding of IL-6 to its receptor initiates cellular events leading to the activation of JAK proteins. Activated JAKs phosphorylate themselves and the receptor and also phosphorylate and activate STAT transcription factors. Phosphorylated STATs dimerize and translocate into the nucleus to activate transcription of genes containing STAT response elements.



1.6 Stress-Immune interactions in Fishes

As described in the previous sections, there are high energetic costs associated with the stress response. This can adversely affect normal body functions like mounting a successful immune response to pathogens. Table 1 summarizes the effects of stress on immune function in fishes.

Table 1: Stimulatory effects of acute stress and suppressive effects of chronic stress on immune parameters

Acute Stress	**Chronic stress**
↑ lysozyme levels (Demers and Bayne, 1997)	↓ lysozyme levels (Sunyer and Tort, 1995)
↑ C3 levels (Sunyer and Tort, 1995)	↓complement activity (Sunyer and Tort, 1995)
↑ leukocyte numbers (Tort, 2011)	↓ leukocyte distribution and differentiation (Tort, 2011)
↑ glucocorticoid receptor sites in head kidney leukocytes (Maule and Schreck, 1991)	↓ agglutination activity (Sunyer and Tort, 1995)
↑ activated macrophages in skin (Dhabhar, 2002)	↓ antibody titres (Sunyer and Tort, 1995)
↑ T-cell activation (Dhabhar, 2002)	↓ circulating B lymphocytes (Verburg-VanKemenade et al., 2009)
↑ recruitment of surveillance T cells in the skin (Dhabhar, 2002)	↓ IgM levels and ↑ susceptibility to viral and parasitic infections (Varsamos et al., 2006; Saej et al., 2003)
↑ Th1 response (Tort, 2011)	↑ Th2 response (Tort, 2011)
	↓ rates of cytotoxicity (Vazzana et al., 1992)
	Induces apoptosis of immune cells (Franco et al., 2009)

The outcome of a stress response depends largely on the intensity of the stressor and its duration. Acute stress is often considered to be adaptive (stress resulting in potential advantages), leading to immunoactivation or immunoenhancing processes (Tort, 2011). Acute stress is characterised by a rapid physiological stress response which rapidly shuts down after cessation of the stressor. In contrast, chronic stress is considered to be maladaptive leading to a dysregulation or suppression of overall immune function (Tort, 2011). Chronic stress is characterised by a physiological stress response which persists long after the stressor has ceased or is activated repeatedly resulting in overall and sustained increases in stress hormone levels (Dhabhar, 2008).

1.6.1 Effects of Cortisol on Immune Regulation in Fishes

The suppressive effects of stress on immune responses have been mostly associated to the activity of cortisol (Tort, 2011). Lymphocyte proliferation, phagocytic activity as well as antibody and complement responses are suppressed by cortisol (Engelsma et al., 2002; Espelid et al., 1996, Ortuño et al. 2001). High levels of cortisol correlate with the severe immune suppression observed with cold-shock and the reduced levels of complement observed in response to high density stress in sea bream (Tort et al., 1998; Montero, 1999). In carp, cortisol triggers B cells to undergo apoptosis while it rescues neutrophilic granulocytes from apoptosis (Weyts et al., 1998b; Weyts et al., 1998a). Consequently, cortisol injection increases circulating blood granulocytes and decreases circulating lymphocyte numbers in carp (Wojtaszek et al., 2002). Cortisol pre-incubation of head kidney cells, resembling a chronic stress paradigm, negatively affects the immune responsiveness to contaminant challenge (Quabius et al., 2005). Cortisol also inhibits inflammatory cytokine expression *in vitro* in immune cells (Saeij et al., 2003). It suppresses the expression of cytokines (TNF, TGFβ, IL-6) in isolated macrophages (Castillo et al., 2009). It is also known to down-regulate LPS induced cytokine expression *in vitro* (Castillo et al., 2009). It modulates immune function in rainbow trout leukocytes and carp phagocytic cells by inhibiting LPS induced IL-1β expression (Zou et al., 2000; Engelsma et al., 2001). It also significantly inhibits LPS induced TNF α2 expression in trout macrophage cultures (MacKenzie et al., 2006).

Even though the immunosuppressive effects of cortisol are well documented and accepted, the molecular mechanisms involved in this interaction are far from clear especially in teleost models. In mammals, cortisol has been shown to control cytokine expression in several ways. In addition to the traditional GR signalling pathway, the activated GR complex can downregulate transcription factors, including activator protein-1 (AP-1), cAMP responsive element binding protein (CREB) and NF-κB by protein–protein interactions (Barnes, 1998; McKay and Cidlowski, 1999). Pathogenic stimuli induce the expression of most cytokines via the NF-κB pathway. Cortisol can counter this by interacting with NF-κB, preventing its binding to κB responsive elements, or by upregulating IκB transcript levels (McKay and Cidlowski, 1999). However, it is not known whether cortisol modulates cytokine expression in fishes through similar mechanisms.

1.6.2 Immune Regulation of the HPI Axis

Clear evidence exists for cytokine mediated hypothalamus-pituitary-adrenal (HPA) axis activation in mammals, including cytokine mediated upregulation of corticotrophin releasing hormone (CRH) and ACTH expressions (Kemppainen and Behrend, 1998; Katahira et al., 1998; Turnbull and Rivier, 1999). However, information on the immune regulation of the HPI axis in fishes is limited. Recombinant IL-1β injection has been shown to activate the HPI axis in rainbow trout and raise cortisol levels (Holland et al., 2002). Also, LPS injection elevates cortisol levels in fishes, and GR is expressed in most tissues 6-72 h post injection (Swain et al., 2008; Acerete et al., 2007). Additionally, in fishes, a number of immune cells have been shown to produce small amounts of stress hormones like proopiomelanocortin (POMC), CRF and ACTH (Ottaviani et al., 1995; Ottaviani et al., 1998; Arnold and Rice, 2000). The very low production rates of these hormones would favour a paracrine or autocrine action over a classical endocrine one, as contribution to plasma levels of these hormones will be very small. Thus the immune regulation of the HPI axis may be mediated in part by cytokines and also by the inherent ability of immune cells to produce neuroendocrine mediators.

1.7 Growth in Fishes and GH signalling

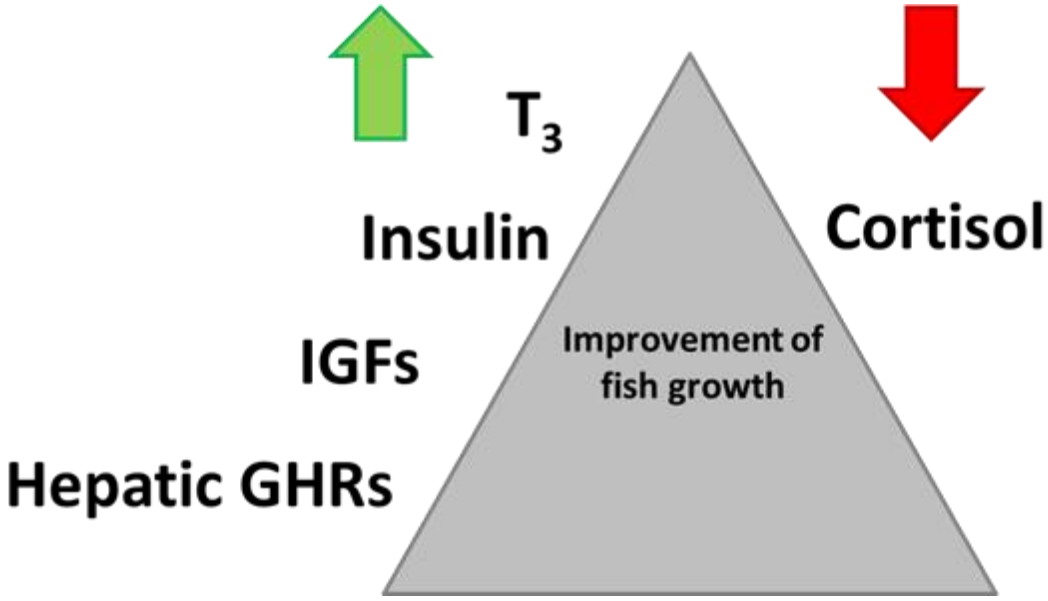
Fish growth is a complex function mostly regulated by the growth hormone (GH)/ insulin-like growth factor (IGF) system (Gabillard et al., 2006). GH is the pre-dominant regulator of growth in fishes, and has complex metabolic functions. GH is also considered to be an osmoregulatory 'seawater adapting' hormone for its role in salmon smoltification (Björnsson et al., 2004) and is known to have roles in reproduction (Gomez et al., 1999; Le Gac et al., 1993) and immune function (Perez-sanchez, 2000). The release of GH from the anterior pituitary is stimulated by growth hormone-releasing hormone (GH-RH) and suppressed by somatostatin from the hypothalamus (Reinecke, 2010). GH released from the anterior pituitary acts mainly on the liver via the growth hormone receptor (GHR) and leads to the synthesis of IGF-I and its release into the circulation (Reinecke, 2010). IGF-I reaches its target cells in numerous organs *via* the blood stream, where it interacts with the type 1 IGF receptor (IGF-1R) and exerts its effects. IGF-I, in turn, specifically inhibits GH gene transcription and secretion *via* a negative feedback mechanism. Furthermore, IGF-I is also expressed in several other organs, including the gonads, gills, kidney, cartilage, pancreas and muscle, where it is thought to act in an autocrine/paracrine manner (Reinecke, 2010).

It has been observed that best growth performance is achieved by increasing growth hormone sensitivity and growth hormone interactions with other hormones (Perez-sanchez and Bail, 1999) (Figure 5). Insulin and thyroid hormones have been shown to be required for adequate hepatic GHR expression and GH signal transduction (Perez-sanchez and Bail, 1999). Insulin augments the *in vitro* stimulatory action of GH on IGF-I transcription (Duan et al., 1992). Moreover in fasted and stunted coho salmon exhibiting low insulin levels, the GH signal is not transferred to the hepatic IGF-I synthesizing machinery in spite of high circulating levels of GH (Plisetskaya et al, 1994). Similarly, in sea bream and rainbow trout, decreased growth rates induced by fasting and dietary protein restriction is linked to the increase of circulating GH levels, and to a loss of hepatic GHRs suggesting a state of GH resistance (Perez-sanchez and Bail, 1999). Furthermore, a strong positive correlation between growth rates and circulating levels of the thyroid hormone T_3 have been shown in fishes (Perez-sanchez and Bail, 1999). T_3 increases the pituitary levels of GH mRNA in

rainbow trout (Moav and McKeown, 1992). Moreover, thyroid hormone (TH) responsive elements are found on the GH gene in both Atlantic salmon and rainbow trout (Yang et al., 1997; Farchi-Pisanty et al., 1997). However, maintaining high levels of cortisol either experimentally or by stressors, inhibits the GH axis and growth and this is discussed in more detail in the following sections.

Figure 5: Simplified representation of factors influencing growth and growth performance in fishes

Growth performance in fishes is influenced by factors contributing to GH sensitivity as well as interactions of GH with other hormones. Low plasma cortisol levels, in combination with a high concentration of hepatic GHRs and high circulating levels of IGFs, insulin and T_3 yield better growth and growth performance.



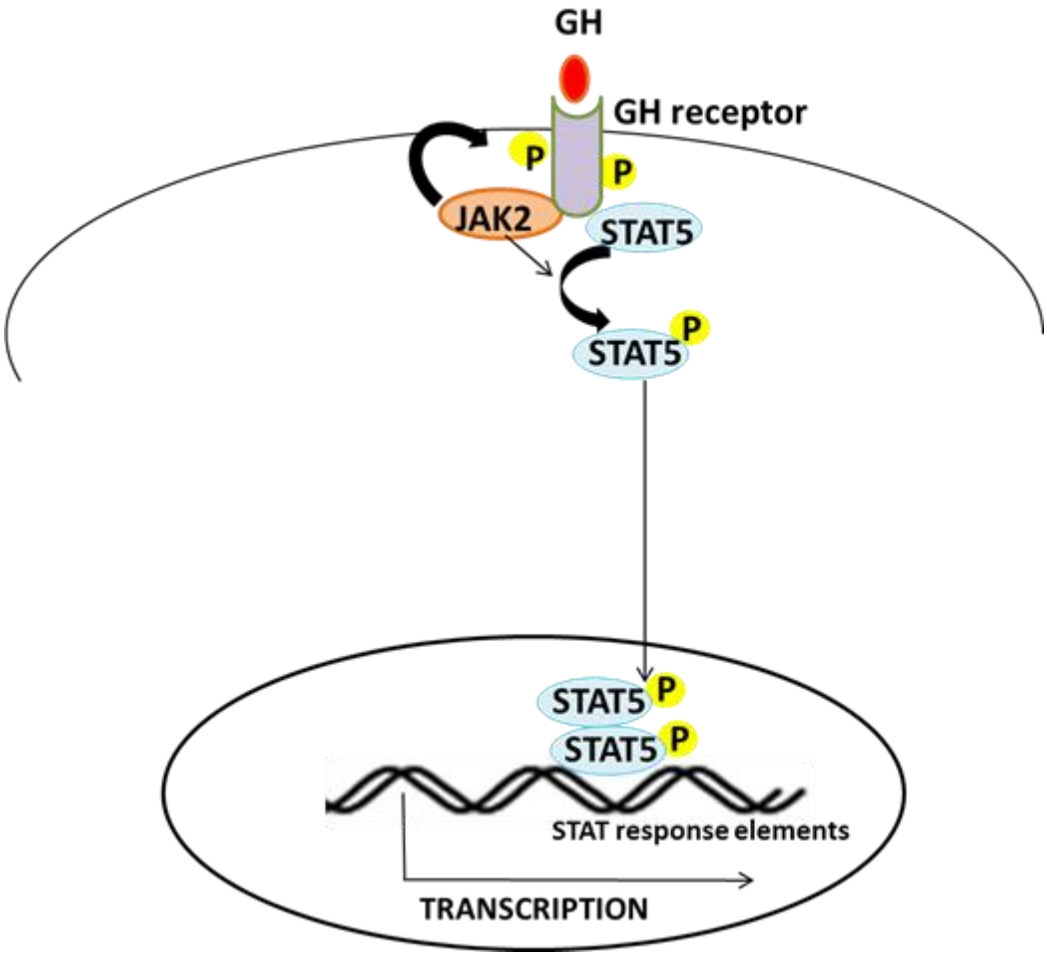
(Adapted from Perez-sanchez and Bail, 1999)

1.7.1 GH signalling

The diverse effects of GH on growth and metabolism in fishes are mediated by signalling via at least three major pathways, including STATs, ERK1/2, and phosphatidylinositol 3-kinase/Akt (Reindl et al., 2011). The JAK/STAT pathway is the principal signalling mechanism for a wide array of cytokines and growth factors (Rawlings et al., 2004). It was recently shown that GH signalling in fishes is also pre-dominantly mediated through the JAK/STAT pathway (Figure 6; Reindl et al., 2011). The JAK proteins are seen associated with the cytoplasmic domains of GHRs. The interaction of GH with GHR causes receptor multimerization promoting the association of the cellular tyrosine kinase JAK2 with the GHR and induces JAK2 activation. The activated JAK2s subsequently phosphorylate both the receptors and the major substrates, STAT5s. STAT5s are latent transcription factors that reside in the cytoplasm until activated. Once activated, they enter the nucleus where the dimerized STAT5s bind specific regulatory sequences to activate (IGF-1) or repress transcription of target genes (Rawlings et al., 2004).

Figure 6: Simplified diagram showing GH induced JAK/STAT signalling in fishes

Binding of GH to the GHR causes the receptor to dimerize, activating the JAK2 protein kinase. The activity of JAK2 mediates many of the downstream responses to growth hormone through phosphorylation of STAT5 transcription factors. Once activated, the phosphorylated STAT5s enter the nucleus where they activate or repress the transcription of genes containing STAT response elements



1.8 Stress-Growth interactions in Fishes

Stress generally shows depressive effects on energetics and growth and is known to inhibit feeding behaviour in all vertebrates (Tort and Teles, 2011; Pickering, 1990; Bernier et al., 2004). The effect of CRH (CRF in fishes) to inhibit feeding is observed in mammals, birds and fishes (Tort and Teles, 2007; Bernier et al., 2004). Stressors, including crowding and handling impact growth in several salmonid species (Wedemeyer 1976; Fagerlund et al., 1981; Pickering and Stewart, 1984; McCormick et al., 1998). It has been shown that handling and confinement stress elicits a reduction in plasma GH levels concomitant with increased HPI activity in rainbow trout (Pickering et al., 1991). IGF-1 transcript levels have also been shown to be lowered in response to crowding stress in Atlantic salmon (Solberg et al., 2012). Additionally, administration of cortisol, the major stress hormone in teleosts has been found to reduce growth in both rainbow trout and channel catfish (Davis et al., 1985; Barton et al., 1986), providing a direct link between this hormone and stress-related changes in growth. Yet the exact mechanism for this interaction is not clear.

Even though the negative effects of stress on immune responses and growth processes has been extensively studied and reviewed in the previous sections, very few studies have looked in greater depth into the molecular mechanisms underlying stress-immune-growth interactions in fishes. The presence of common signalling pathways namely the JAK/STAT pathway that is shared by cytokines, LPS and GH (Kimura et al., 2005; Croker et al., 2008) suggest a possibility for pathway cross-talk, leading us to look in more detail at the negative regulators of this pathway and their modulation.

1.9 SOCS as regulators of immune and growth processes

The SOCS proteins are a family of intracellular proteins that are centrally involved in vertebrate growth, development and immunity via their effects as negative feedback regulators of cytokine (and hormone) signalling (Kile and Alexander, 2001). In mammals, SOCS molecules comprise eight proteins, SOCS-1 to 7 and cytokine inducible SRC homology 2 (SH2) – containing protein (CISH) (Dalpke et al., 2008). The role played by SOCS genes in the negative control of cytokine signalling has been well documented in

mammals (Kile and Alexander, 2001). They regulate the JAK/STAT pathway, which is an intracellular signalling pathway shared by a variety of cytokines as well as leptin, GH, and prolactin (PRL). Hence they are also referred to as JAK- binding protein (JAB) or STAT-induced STAT inhibitors (SSIs) (Kile and Alexander, 2001). All SOCS proteins share a number of common structural regions including the N-terminal Src homology 2 (SH2) and kinase inhibitory region (KIR) domains and the C-terminal SOCS-box, which enable these proteins to negatively regulate the JAK/STAT signalling pathway in various ways (e.g., inhibiting JAK docking, STAT transphosphorylation and increased signalling protein degradation) (Croker et al., 2008). SOCS-1 and SOCS-3 are sensitive to induction by a wide variety of cytokines, and in turn act in a negative feedback loop to inhibit signalling by these cytokines (Krebs and Hilton, 2000). Cytokines, including IL-11, IL-6, IL-1β as well as bacterial endotoxin, have been shown to stimulate SOCS-3 expression (Mao et al., 1999; Krebs and Hilton, 2000). In mice macrophages, SOCS-1 has been shown to participate in the negative regulation of LPS-induced inflammatory responses by inhibiting LPS induced STAT and NF κB activation (Nakagawa et al., 2002). SOCS-1 knock out mice fail to thrive beyond three weeks of age suggesting a role for SOCS genes in development (Kile and Alexander, 2001). SOCS-2 has been shown to associate with GH and IGF-1 receptors and SOCS-2 deficient mice exhibit accelerated growth, implicating a role for SOCS genes in the negative control of GH signalling (Metcalf et al., 2000). Mice lacking SOCS-3 have been reported to die at mid gestation with delayed development and excessive erythropoiesis (Marine et al., 1999). SOCS-3 also inhibits leukemia inhibitory factor (LIF) induced POMC gene expression and ACTH secretion, thus providing an intracellular negative feedback on cytokine-induced activation of the HPA axis (Auernhammer et al., 1998). Additionally, SOCS-3 has also been shown to act as a negative regulator of leptin signalling in mice (Bjorbak, 1999).

Homologues of all the eight mammalian SOCS family members have been discovered in fishes, with many of them having multiple copies in fishes (Wang et al., 2011). The functional roles of fish SOCS genes have just begun to emerge. Recent studies have documented the effects of immune stimulation on SOCS gene expression and their regulation

by immune cytokines in teleosts (Wang and Secombes, 2008; Wang et al., 2010) . Fish SOCS gene expression is under strict control, and different members follow different kinetics in response to the same stimulants. For example, in response to IL-6 stimulation, trout SOCS-3 expression peaks within half an hour, while SOCS-1 and SOCS-2 peak later, and no difference is seen after 6 h of stimulation (Wang et al., 2011). LPS and cytokines, including IL-1β and IFN γ have been shown to up regulate SOCS 1-3 in rainbow trout cell lines, while bacterial infection (*Yersinia ruckeri*) upregulates CISH and SOCS-3 expression in trout splenocyte cultures (Wang and Secombes, 2008; Wang et al., 2010; Wang et al., 2011). With regards to growth in fishes, it has been observed that homozygote GH-transgenic zebrafish who express double the amount of GH compared with hemizygote individuals, also express higher levels of SOCS-1 and SOCS-3 and display slower growth rates (Studzinski et al., 2009) suggesting that the heightened expression of SOCS-1 and 3 in homozygotes downregulates GH signalling. Yet the mechanism for this is not clear in fishes. Additionally, even though a couple of studies have shown conditions of handling and injection stress to modulate SOCS expression in fishes (Shepherd et al., 2012; Wang et al., 2010), the mechanisms for this are far from clear and no direct impact of cortisol on SOCS expression has been demonstrated.

Thus this thesis investigates the possibility of the SOCS genes being a key link in integrating stress-immune-growth processes in fishes; more specifically the effects of cortisol on SOCS expression and the subsequent effects on immune and growth processes are explored.

1.10 Nutritional modulation of stress-immune-growth processes

Fishes show major physiological changes following conditions of fasting or malnutrition. Fasting is characterized by a sequential utilization of glycogen, lipid and protein reserves (Collins and Anderson, 1995) and an overall reduction in metabolic capacity which in turn might have profound effects on stress, immune and growth functions. The effects of dietary restriction on stress parameters are variable between fish species. Plasma cortisol and glucose levels have been variously reported to be unaffected by fasting, reduced

by fasting or increased by fasting in different fish species (Pottinger et al., 2003). Additionally, low protein intake has been associated with reduced gluconeogenic enzyme expression in rainbow trout which might in turn affect their stress performance (Kirchner et al., 2003). Starvation decreases immune gene transcription in response to infection and non-specific immune parameters like haemagglutinating activity and respiratory burst activity (Caruso et al., 2011). Starvation in blackspot seabream reduces lysozyme content in the mucus and plasma and haemolytic activity (Caruso, et al., 2011b). In salmonids, prolonged starvation leads to growth retardation and reduction in the condition factor (Reinecke, 2010). Nutritional status is considered to be the principal regulator of circulating GH and IGF-I levels (Reinecke, 2010). Protein restriction or fasting reduces circulating IGF-I and liver IGF-I levels in several fish species including coho salmon, chinook salmon, seabream, tilapia and the Arctic charr (Fox et al., 2006; Jørgensen et al., 2013). Yet, normal or elevated levels of GH have been observed in fasted salmonids, suggesting reduced negative feedback by hepatic IGF-I or a state of GH resistance (Fox et al., 2006). Moreover fasting reduces GHR levels in both black seabream and catfish (Deng et al., 2004; Small et al., 2006).

Food withdrawal occurs naturally during the life cycle of many salmonids like the Arctic charr (Jørgensen et al., 2013). Yet, the mechanisms involved in cellular energy re-partitioning during nutritional restriction are not known. Whether such species differentially modulate their stress, immune and growth responses in comparison with other salmonids like the rainbow trout that don't undergo voluntary fasting remain to be determined.

1.11 Role of the liver in stress-immune-growth processes

Stress, immune and growth responses as well as nutritional status have major consequences to whole animal physiology with alterations occurring in many cellular processes across numerous tissues (Martin et al., 2010). However, most of this thesis focuses on the liver as a target tissue for studying stress-immune-growth interactions, since it is a metabolically active tissue known to play important roles in the stress response, immune response and growth and is also substantially affected by changes to nutritional status. It is a prime target for the action of cortisol and cortisol signalling plays a key role in the molecular

regulation of liver metabolism which is essential to cope with stress (Vijayan et al., 2010). During an immune response, the liver is also the primary site of acute phase response protein synthesis in response to cytokines, including IL-6 and TNFα (Bayne and Gerwick, 2001). Also, mammalian liver contains specific macrophages, the Kupffer cells (Janeway and Walport, 2001). Yet, the role of liver as an actual immune tissue and whether it directly responds to immunostimulants like LPS in fishes is unknown. The liver is also involved in fish growth. It responds to circulating GH by synthesizing and secreting IGF-1 which in turn, stimulates cell growth and differentiation in a variety of target tissues via distinct IGF-1 receptors (Reindl et al., 2011).

The liver also plays an important role in intermediary metabolism by storing reserve compounds such as lipids (triacylglycerol) and glycogen. Consequently, mounting a successful stress response, immune response and growth response may all be related to the availability of sufficient energy reserves and free amino acids together with protein synthesis costs for the *de novo* synthesis of metabolic enzymes to cope with stress, acute phase proteins involved in the immune response and growth mediators like IGF-1. All this makes the liver a good target for studies looking at the interactions between stress-immune-growth processes and the nutritional modulation of these processes.

1.12 Thesis objectives

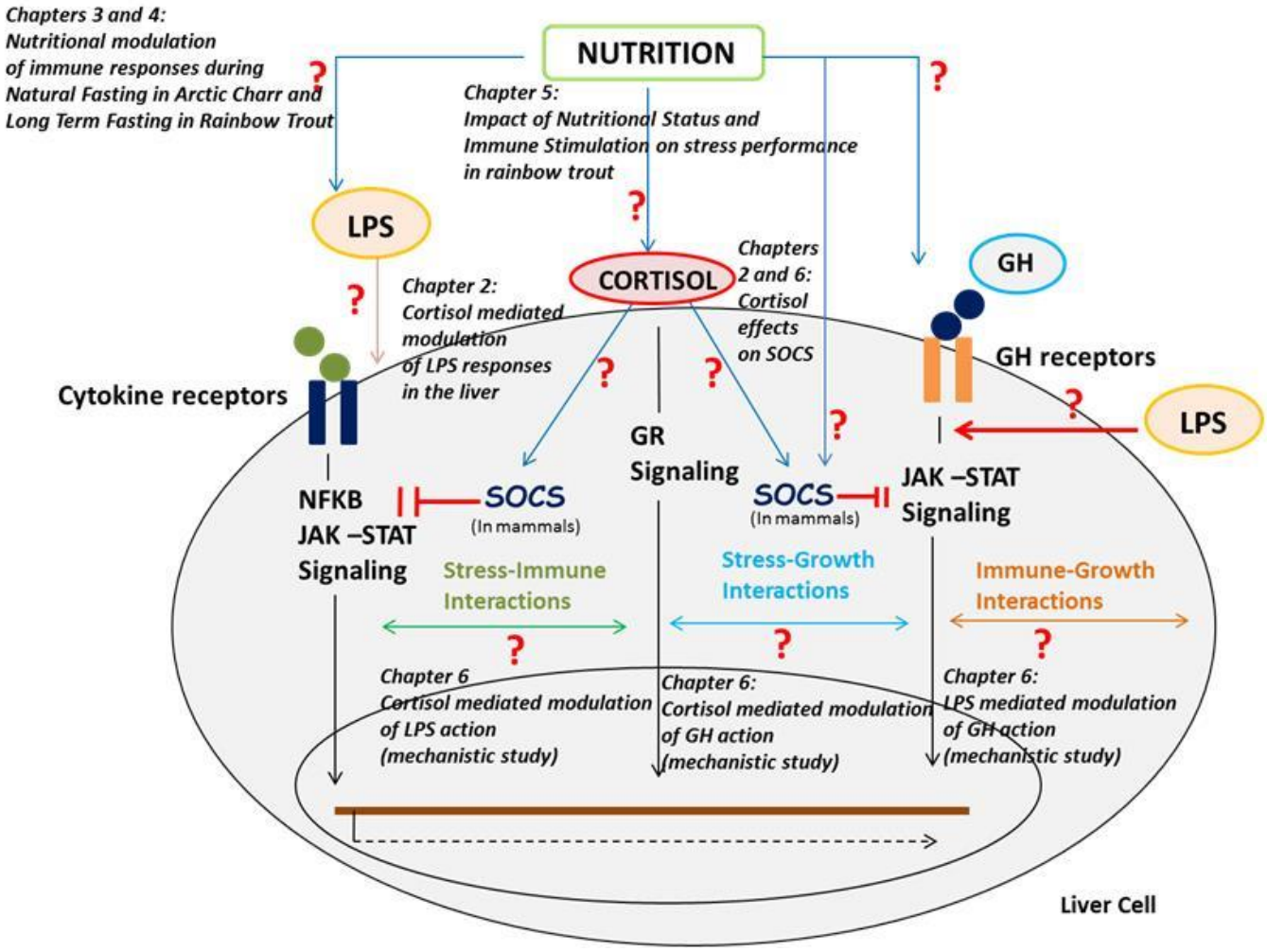
The overall objective of this thesis was to understand the molecular basis of stress-immune-growth interactions and to determine if there are any common molecular mechanisms linking stress effects on immune function and growth. The overall hypothesis was that conditions of stress as well as altered nutritional status modulate SOCS expression in fishes, which in turn will negatively regulate immune and growth processes. We tested this hypothesis through a series of whole animal studies, as well as *in vitro* experiments (see Figure 7). Specific objectives were to see whether:

1. Liver is an immune tissue in fish and cortisol modulates immune responses in the liver (Chapter 2)
2. Cortisol regulates SOCS expression (Chapters 2, 6)

3. Nutritional restriction modulates SOCS expression and this influences immune response and growth parameters in fish (Chapters 3, 4)
4. Acute stress modulates SOCS expression and this response is modulated my nutritional status and immune stimulation (Chapter 5)
5. Cortisol modulates GH and LPS signalling (Chapter 6)

Figure 7: Layout of data chapters

Stress-Immune-Growth interactions in fish: the "known" and the "unknown"



Chapter 2

Cortisol modulates the expression of cytokines and suppressors of cytokine signalling (SOCS) in rainbow trout hepatocytes

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2.1 Overview

Although liver is a key target for corticosteroid action, its role in immune function is largely unknown. We tested the hypothesis that stress levels of cortisol down regulate immune-relevant genes in rainbow trout (*Oncorhynchus mykiss*) liver. Hepatocytes were treated with lipopolysaccharide (LPS) for 24 h either in the presence or absence of cortisol. LPS stimulated heat shock protein 70 expression, enhanced glycolytic capacity, and reduced glucose output. LPS stimulated mRNA abundance of cytokines and serum amyloid protein A (SAA), while suppressors of cytokine signalling (SOCS)-3 was reduced. Cortisol increased mRNA abundances of IL-1β, SOCS-1 and SOCS-2, while inhibiting either basal or LPS-stimulated IL-8, TNF α2 and SAA. These cortisol-mediated effects were rescued by mifepristone, a glucocorticoid receptor antagonist. Altogether, cortisol modulates the molecular immune response in trout hepatocytes. The upregulation of SOCS-1 and SOCS-2 by cortisol may be playing a key role in suppressing cytokine signalling and the associated inflammatory response.

2.2 Introduction

Cortisol is the major corticosteroid that is released in response to stress in teleosts, and this hormone is important for re-establishing homeostasis (Wendelaar Bonga, 1997; Mommsen et al., 1999; Aluru and Vijayan, 2009). Stressor-induced elevation of plasma cortisol involves the activation of the hypothalamus-pituitary-interrenal (HPI) axis, including the release of corticotropin-releasing factor from the hypothalamus, the adrenocorticotrophic hormone from the pituitary, and the stimulation of steroid biosynthesis in the interrenal tissues (analogous to the adrenal gland in mammals) (Wendelaar Bonga, 1997; Mommsen et al., 1999). This stress steroid has wide ranging effects and the actions are mediated by the corticosteroid receptors in target tissues. There are multiple glucocorticoid receptors (GRs) in teleosts due to whole genome duplication events, with the only exception so far being the zebrafish that has only a single GR in the genome (Prunet et al., 2006; Bury and Sturm, 2007; Alsop and Vijayan, 2009). Teleosts also express a mineralocorticoid receptor (MR), however the function(s) of activating this receptor remain unclear (Sturm et al., 2005).

Cortisol is a key modulator of physiological processes, including stress response, growth, metabolism and immune response (Mommsen et al., 1999; Vijayan et al., 2010; Tort, 2011). A key response to stress that is cortisol mediated is the enhancement of liver metabolic capacity, including gluconeogenesis and increasing glucose output from this tissue (Mommsen et al., 1999; Aluru and Vijayan, 2007). This metabolic adjustment is essential for restoring homeostasis, as glucose is the preferred fuel for tissues to meet the increased energy demand that is essential to cope with stress (Mommsen et al., 1999; Aluru and Vijayan, 2007). While several studies have examined the role of stressor-induced cortisol in modulating liver metabolic capacity (Aluru and Vijayan, 2009), little is known about liver function in response to stressors eliciting an immune response in fishes.

Stress, and the associated plasma cortisol elevation, has immunosuppressive effects in fishes (Tort, 2011). For instance, cortisol modulates immune function by inhibiting lipopolysaccharide (LPS)-induced cytokine expression in fish immune cells (Zou et al., 2000, Engelsma et al., 2003 and MacKenzie et al., 2006). However, to our knowledge no study has examined the effect of either stress or cortisol on the expression of suppressors of cytokine signalling (SOCS) in fishes. Three isoforms of SOCS (SOCS-1, SOCS-2 and SOCS-3) were recently cloned and sequenced in rainbow trout, and they showed differential tissue expression (Wang and Secombes, 2008; Wang et al., 2010). While the functions of these proteins in fishes are unclear, SOCS are known to integrate several physiological processes, including immune function, growth and embryonic development in mammals (Kile and Alexander, 2001).

Against this backdrop we tested the hypothesis that cortisol will suppress expression of immune responsive genes in rainbow trout hepatocytes. Trout hepatocytes are a model metabolic system to study cortisol effects (Aluru and Vijayan, 2007), but few studies have examined immune response with this cell system. LPS was used as an immune-stimulant in this study because it is a well-established tool to regulate immune responsive genes, including cytokines and acute phase proteins in fishes (Engelsma, 2002; MacKenzie et al., 2006; Martin et al., 2010). As a marker of immune response, we measured the expression of interleukin1β (IL-1β), IL-8 and tumor necrosis factor α2 (TNF α2), serum amyloid protein A

(SAA) and the three isoforms of SOCS using real-time quantitative PCR in trout hepatocytes. The cellular stress response was ascertained by determining the expressions of heat shock protein 70 and glucocorticoid receptor, key markers of disturbance to protein homeostasis (Deane and Woo, 2010) and metabolic response (Aluru and Vijayan, 2007), respectively. A GR antagonist (mifepristone) was also used to tease out the direct role of cortisol signalling involving GR activation on immune and metabolic responses to an immunostimulant in hepatocytes. The metabolic capacity was ascertained by measuring the activities of glycolytic (hexokinase, glucokinase, pyruvate kinase), citric acid cycle (malate dehydrogenase, isocitrate dehydrogenase) and gluconeogenic (phosphoenolpyruvate carboxykinase) enzymes along with glucose release (Aluru and Vijayan, 2007).

2.3 Materials and Methods

2.3.1 Experimental Fish

Immature rainbow trout (*Oncorhynchus mykiss;* 150 ±10g body mass) were obtained from Alma Research Station (Alma, ON, CAN), and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1^{o}C on a 12:12-h light/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.

2.3.2 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 et al., 2003; Aluru and Vijayan, 2007). Trypan blue dye exclusion method was used to confirm hepatocyte viability and >95% cells were viable. Cells were plated in six-well tissue culture plates (Sarstedt Inc., NC, USA) at a density of 1.5 million cells/well (0.75 million cells/ml) in L-15 media and were maintained at 13^{o}C for 24 h. After 24 h, the L-15 media was replaced with fresh L-15 and cells were incubated at 13^{o}C for 2 h prior to experimental treatments.

2.3.3 Experimental treatments

The experimental protocol for this study consisted of treating hepatocytes from each fish with either 0 or 30 μg/mL LPS (*Escherichia coli*, 055:B5; Sigma) along with either control media (0.01% ethanol used as vehicle) 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 30 min before the addition of cortisol. The cells were then maintained at 13^{o}C for 24 h before sampling. The LPS concentration used was shown previously to elicit pro-inflammatory cytokine expression in fish cells (Stolte et al., 2008). The cortisol concentration used represents a typical stress level for trout, while the Mifepristone concentration used was shown previously to block cortisol-mediated metabolic effects in trout hepatocytes (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). At the end of the experimental period, the medium was collected, and the cells were centrifuged (13,000*g* for 1 min), supernatants were removed, and the cell pellets were flash frozen on dry ice and stored at -70°C. There were six wells in total for each of the eight treatments. Two were pooled for glucose and enzyme analysis, two for protein expression analysis (immunodetection) and the remaining two for quantitative real-time PCR (qPCR). The experiment was repeated with hepatocytes isolated from seven independent fish.

2.3.4 Glucose release and cell viability

Media glucose was determined colorimetrically using a commercially available kit (Raichem, Cliniqa Corporation, CA, USA). The cell viability in culture was determined by measuring the leakage of lactate dehydrogenase (LDH) into the medium (Aluru and Vijayan, 2007). The LDH activity was determined as described below.

2.3.5 Enzyme activity

The enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at 22°C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA) exactly as described before (Vijayan et al., 2006). The following assay conditions were used:

***Hexokinase (HK: EC 2.7.1.1)*:** 1 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/mL lactate dehydrogenase (LDH) and 5U/mL pyruvate kinase; reaction started with 4 mM ATP.

***Glucokinase (GK:EC 2.7.1.2)*:** 20 mM glucose, 5 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate (PEP), 20 U/mL lactate dehydrogenase (LDH) and 5U/mL pyruvate kinase; reaction started with 4 mM ATP.

***Pyruvate kinase (PK: EC 2.7.1.40)*:** 30 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 20 U/mL LDH; reaction started with 2.5 mM PEP.

***Lactate dehydrogenase (LDH: EC 1.1.1.27)*:** 0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.

***Malate dehydrogenase (MDH: EC 1.1.1.37)*:** 0.12mM NADH; reaction started with 0.5mM oxaloacetate.

***Isocitrate dehydrogenase (IDH: EC 1.1.1.42)*:** 4mM $MgCl_2$, 0.4mM NADP; reaction started with 0.6mM isocitrate.

***Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32)*:** 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 0.2 mM deoxyguanosine diphosphate

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute per gram protein.

2.3.6 SDS-PAGE and immunodetection

Sample protein concentrations were measured with bicinchoninic acid (BCA) reagents using BSA as the standard. SDS–PAGE and immunodetection were carried out exactly as described before (Aluru and Vijayan, 2007). Briefly, sample protein (40 μg protein per lane) was separated on 10% polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes. The membranes were probed with either rabbit polyclonal anti-trout GR (1:1000; Sathiya and Vijayan, 2003) or anti-salmon hsp 70 (1: 5000; StressMarq, Victoria, BC, CAN). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; BioRad, Hercules, CA, USA). Protein bands were detected with ECL plus (Amersham Biosciences, Piscataway, NJ, USA) and the

molecular mass confirmed with precision plus molecular weight markers (BioRad). The bands were scanned with a Typhoon 9400 (Amersham) and the band intensity quantified by AlphaEase software (Alpha Innotech, CA, USA). The band intensity was normalized to ß-actin and shown as percent No LPS control.

2.3.7 Quantitative real-time PCR (qPCR)

Total RNA was isolated from hepatocytes using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and the concentration determined at 260/280 nm using a Nanodrop. The RNA samples were DNase-treated (MBI Fermentas, ON, CAN) to avoid genomic contamination. The first strand cDNA was synthesized from 1µg 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). EF1alpha threshold cycle (C_T) values were similar across all experimental treatments and used for the normalization of transcript abundance. The mRNA abundance with treatment was expressed as percentage of No LPS control.

Table 1: **Gene-specific primers for quantitative real time PCR.**

The table provides the list of genes (Gene ID), their GenBank accession numbers, forward and reverse primer sequences, annealing temperature, amplicon size and the appropriate references. IL-1β – interleukin-1 beta; IL-8: interleukin-8; TNF α2: Tumor necrosis factor α2; SAA: serum amyloid protein A; SOCS: suppressors of cytokine signalling; EF1α: elongation factor 1α.

Gene ID	GenBank Accession #	Primer sequences (5'-3') and reference	Temp (^{o}C)	Amplicon size (bp)
IL-1β	AJ223954	Fwd: GGAGAGGTTAAAGGGTGGCGA Rev: TGCCGACTCCAACTCCAACA Gioacchini et al., 2008	60	121
IL-8	AJ279069	Fwd: CACTGAGATCATTGCCACTCTGA Rev: ATGACCCTCTTGACCCACGG Gioacchini et al., 2008	60	81
TNF α2	AJ401377	Fwd: GGAGGCTGTGTGGCGTTCT Rev: TGCTGACACCAGGCAAAGAG Gioacchini et al., 2008	60	73
SAA	X99387	Fwd: TTGTTCTGACCCTCGTTGTAGGAGC Rev: CATGTCGCCATATGCACGCC Talbot et al., 2009	60	101
SOCS-1	AM748721	Fwd: GATTAATACCGCTGGGATTCTGTG Rev: CTCTCCCATCGCTACACAGTTCC Wang and Secombes, 2008	63.3	136
SOCS-2	AM748722	Fwd: TCGGATGACTTTTGGCCTAC Rev: CCGTTCTTCTCTCGTTTTCG	60	102
SOCS-3	AM748723	Fwd: TAGCCCTGAGCCTGGAAGTA Rev: GGTTGCTAGGCAGTTTCCTG	60	113
EF 1 α	AF498320.1	Fwd: CATTGACAAGAGAACCATTGA Rev: CCTTCAGCTTGTCCAGCAC Aluru et al., 2010	56	95

2.3.8 Statistical analysis

Data are shown as mean ± standard error of mean (SEM). Outliers, value that was two standard deviations above or below the mean, were omitted from the analyses. Statistical comparisons used two-way repeated measures analysis of variance (ANOVA) followed by Tukey's *post-hoc* test to determine significance between groups (SigmaStat, Systat Software Inc., San Jose, CA, USA). The data were log transformed, wherever necessary, for homogeneity of variance, but non-transformed values are shown in the figures and table. A probability level of $p < 0.05$ was considered significant.

2.4 Results

2.4.1 Metabolic response

LPS treatment did not affect cell viability. LDH leakage was minimal (<7% of total LDH) in all groups, and there were no significant difference among treatments (data not shown). Hepatocyte metabolic capacity was ascertained by measuring glucose release into the medium as well as the activities of key glycolytic, citric acid cycle and gluconeogenic enzymes. LPS exposure significantly reduced medium glucose release over a 24 h period in trout hepatocytes. Additionally, LPS exposure significantly increased hepatic PK and MDH activities. As expected, cortisol treatment increased medium glucose levels, but LPS did not modify this response (Fig. 1). Neither LPS nor cortisol had any significant effect on hepatocyte HK, GK, LDH, IDH and PEPCK activities. Cortisol and Mifepristone treatments enhanced PK activity in trout hepatocytes in the absence of LPS, but none of the other enzymes were significantly affected either in the presence or absence of LPS (Table 2).

Figure 1: **Glucose production by trout hepatocytes.**

Hepatocytes were exposed to either control (vehicle; 0.01% ethanol), cortisol (100 ng/ml), Mifepristone (MP; 1,000 ng/ml), or a combination of MP + cortisol, in the presence (30μg/ml) or absence of LPS. In the combination group, MP was added to the hepatocytes 30 min before cortisol addition. Media and hepatocytes were sampled 24 h after treatment, and media glucose release was measured. All values represent means ± SEM (n=5 independent fish); *LPS group significantly different from the No LPS group; the inset shows significant treatment effect (two-way repeated measures ANOVA; $P < 0.05$).

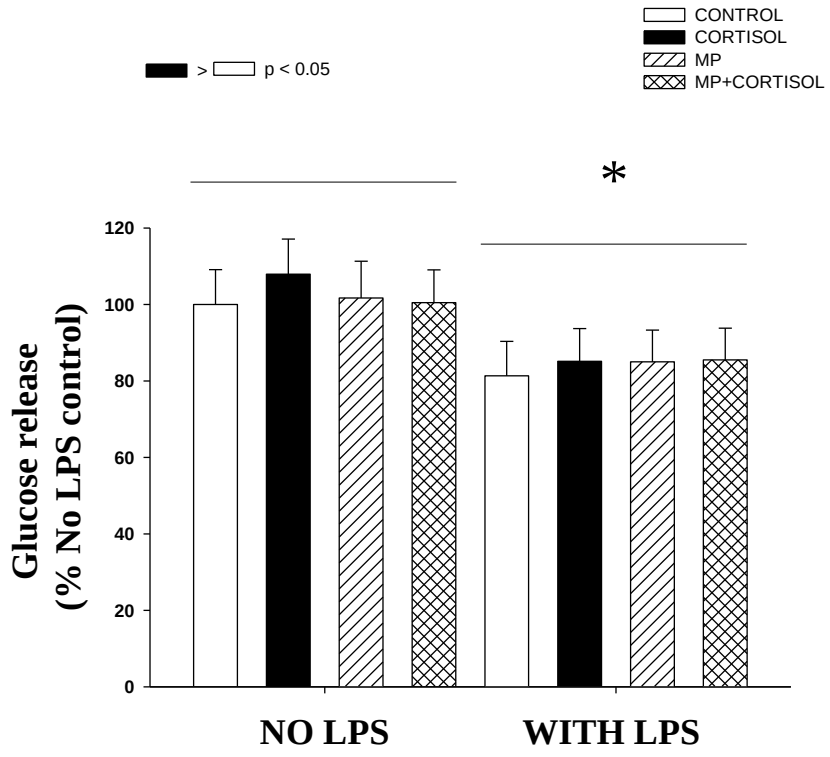


Table 2: **Effect of cortisol and LPS exposures on enzyme activities in trout hepatocytes.** Cells were exposed to control (vehicle; 0.01% ethanol), cortisol (100 ng/ml), Mifepristone (MP; 1,000 ng/ml) or a combination of MP + cortisol, in the presence (30μg/ml) or absence of LPS. In the combination group, MP was added to the hepatocytes 30 min before cortisol addition. All values represent means ± SEM (n=5 independent fish); different letters indicate significant difference within a group; * significantly different between groups (No LPS and With LPS) (two-way repeated measures ANOVA; $P < 0.05$); enzyme activity is shown as μmol/min per g protein.

Enzyme	Treatments	No LPS	With LPS	
Hexokinase	Control	4.1 ± 0.5	4.5 ± 0.3	
	Cortisol	4.6 ± 0.3	4.5 ± 0.2	
	MP	4.4 ± 0.3	4.3 ± 0.1	
	MP + Cortisol	4.3 ± 0.3	4.1 ± 0.3	
Glucokinase	Control	4.6 ± 0.4	4.4 ± 0.4	
	Cortisol	4.7 ± 0.4	4.7 ± 0.3	
	MP	4.4 ± 0.3	4.7 ± 0.4	
	MP + Cortisol	4.4 ± 0.3	4.3 ± 0.2	
PK	Control	5.1 ± 1.0 [a]	10.0 ± 2.2	
	Cortisol	8.4 ± 1.6[b]	11.2 ± 2.4	*
	MP	7.6 ± 1.9[b]	9.4 ± 2.0	
	MP + Cortisol	7.2 ± 1.5[ab]	9.4 ± 2.2	
LDH	Control	47.6 ± 5.4	48.7 ± 5.4	
	Cortisol	50.2 ± 5.8	49.9 ± 5.3	
	MP	49.4 ± 5.3	48.9 ± 4.8	
	MP + Cortisol	48.7 ± 5.6	47.2 ± 4.4	
MDH	Control	3.2 ± 0.4	3.5 ± 0.4	
	Cortisol	3.5 ± 0.4	3.4 ± 0.4	*
	MP	3.3 ± 0.4	3.6 ± 0.5	
	MP + Cortisol	3.3 ± 0.4	3.5 ± 0.4	
IDH	Control	10.8 ±1.6	11.0 ± 1.5	
	Cortisol	11.2 ± 1.6	10.9 ± 1.6	
	MP	10.9 ± 1.7	10.7 ± 1.5	
	MP+Cort	10.3 ± 1.4	10.1 ± 1.4	
PEPCK	Control	1.4 ± 0.04	1.5 ± 0.06	
	Cortisol	1.5 ± 0.05	1.4 ± 0.06	
	MP	1.4 ± 0.13	1.3 ± 0.09	
	MP + Cortisol	1.3 ± 0.05	1.2 ± 0.07	

2.4.2 GR and HSP70 protein expression

The cellular stress response was ascertained by determining GR protein and heat shock protein 70 expressions. LPS stimulation had no significant effect on GR protein expression in trout hepatocytes, while cortisol treatment significantly decreased GR protein expression and this was not modified by LPS (Fig. 2). LPS exposure significantly increased total hsp70 protein expression in trout hepatocytes. Cortisol by itself did not affect hsp70 expression, whereas LPS-induced hsp70 expression was blocked by treating the cells with this steroid (Fig. 3).

Figure 2: Effect of cortisol and LPS treatment on GR protein expression.

Hepatocytes were exposed to either control (vehicle; 0.01% ethanol) or cortisol (100 ng/ml) in the presence (30μg/ml) or absence of LPS. Representative blots with immunodetected GR and β-actin are shown above the histogram; GR was immunodetected using polyclonal rabbit anti-trout antibody (see material and methods for detail); The GR band intensities were quantified and normalized to β-actin (as a loading control). Values represent means ± SEM (n=5 independent fish); bars with different letters are significantly different within each No LPS or with LPS group (two-way repeated measures ANOVA; $P < 0.05$).

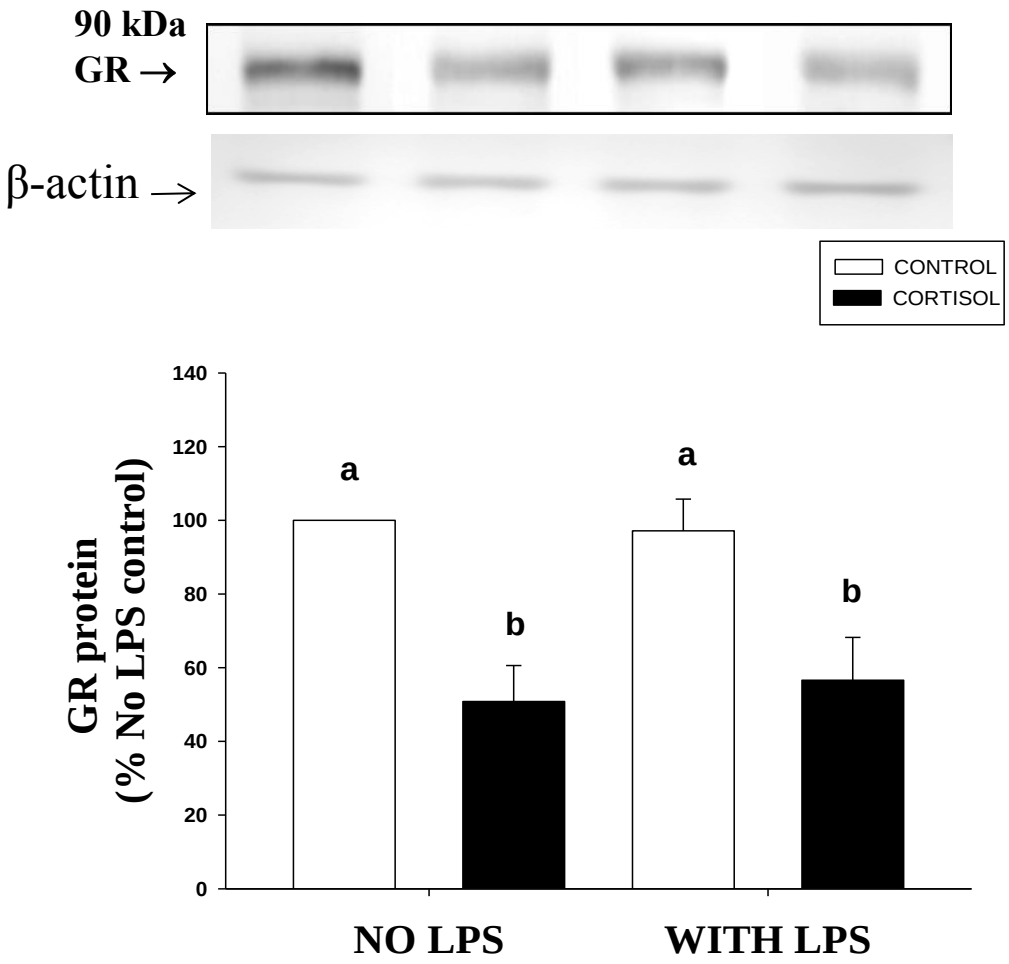
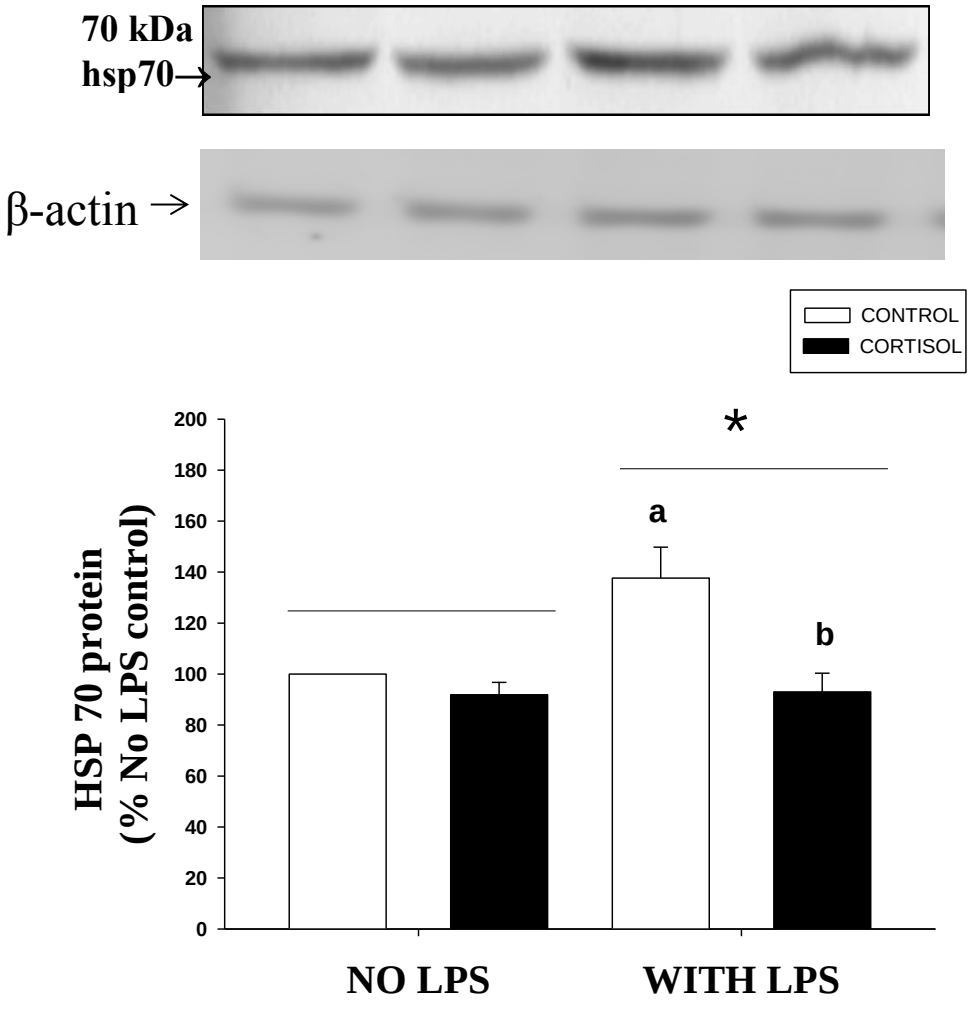


Figure 3: Effect of cortisol and LPS treatment on hsp70 protein expression.

Hepatocytes were exposed to either control (vehicle; 0.01% ethanol) or cortisol (100 ng/ml) in the presence (30μg/ml) or absence of LPS. Representative blots with immunodetected hsp70 and β- actin are shown above the histogram; hsp70 was immunodetected using polyclonal rabbit anti-salmon total hsp70 antibody (see material and methods for detail). The hsp70 band intensities were quantified and normalized to β-actin (as a loading control). Values represent means ± SEM (n=5 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; $P < 0.05$).



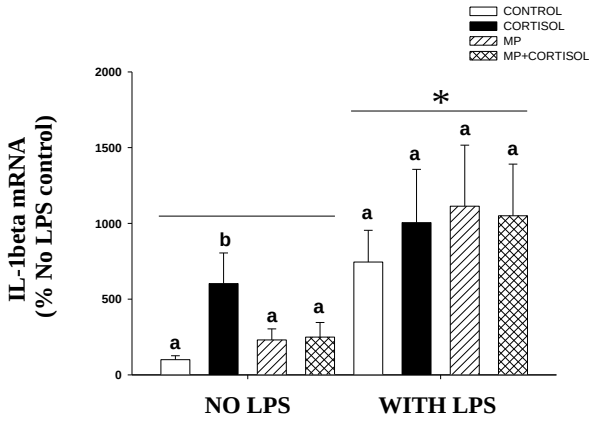
2.4.3 Immune-related genes

Using qPCR we determined changes in immune-related mRNA transcript levels in response to LPS and cortisol treatments in trout hepatocytes. LPS treatment significantly elevated IL-1β (3.3-fold), IL-8 (2.4-fold), TNF α2 (1.2-fold) and SAA (1.2-fold) mRNA transcript levels in trout hepatocytes (Fig 4A-D). Cortisol treatment by itself significantly elevated IL-1β (6.0-fold), whereas IL-8, TNF α2 and SAA, mRNA levels were significantly reduced in trout hepatocytes (Figs 4A-D). Cortisol treatment also significantly suppressed the LPS-induced up-regulation of IL-8, TNF α2 and SAA but not IL-1β in trout hepatocytes (Fig 4A-D). Mifepristone by itself did not modify gene expressions, but blocked the cortisol-induced regulation of IL-1β and TNF α2 genes (Figs 4A and 4C). Also, Mifepristone blocked the cortisol effect on LPS-mediated TNF α2 and IL-8 (only partially) gene expressions (Fig 4B and 4C).

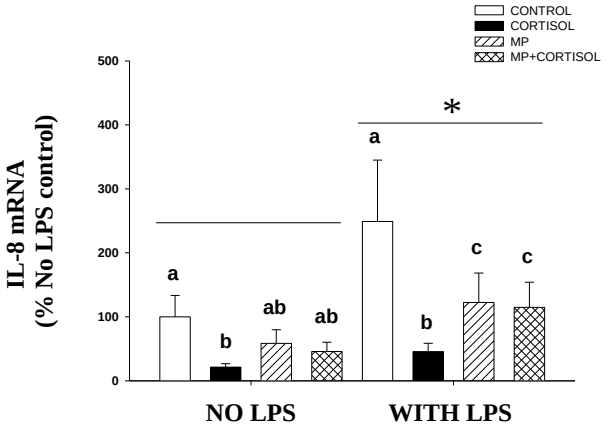
Figure 4: **Effect of cortisol and LPS treatment on expression of immune response genes.**

Effect of cortisol and mifepristone (MP) on (A) IL-1β (B) IL-8, (C) TNFα2 and (D) SAA mRNA abundances in trout hepatocytes either in the presence (30μg/ml) or absence of LPS. Hepatocytes were exposed either to control (vehicle; 0.01% ethanol), cortisol (100 ng/ml), MP (1,000 ng/ml), or a combination of MP + cortisol, in the presence (30μg/ml) or absence of LPS. In the combination group, MP was added to the hepatocytes 30 min before cortisol addition. All values represent means ± SEM (n=6-7 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; $P < 0.05$).

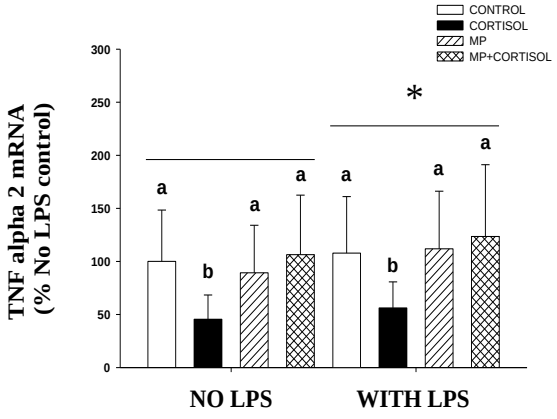
A


CONTROL
CORTISOL
MP
MP+CORTISOL
IL-1beta mRNA
(% No LPS control)
2000
1500
1000
500
0
*
a
b
a
a
a
a
a
a
NO LPS
WITH LPS

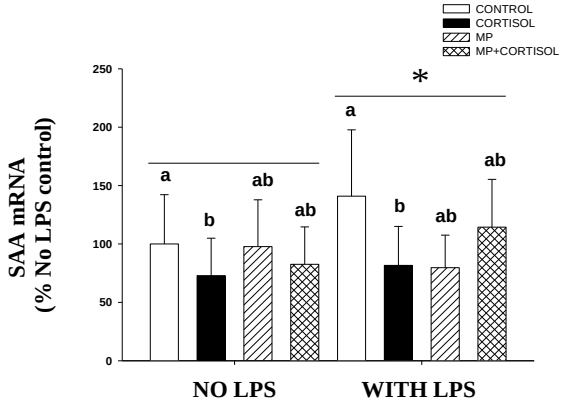
B


CONTROL
CORTISOL
MP
MP+CORTISOL
IL-8 mRNA
(% No LPS control)
500
400
300
200
100
0
*
a
b
ab
ab
a
b
c
c
NO LPS
WITH LPS

C


CONTROL
CORTISOL
MP
MP+CORTISOL
TNF alpha 2 mRNA
(% No LPS control)
300
250
200
150
100
50
0
*
a
b
a
a
a
b
a
a
NO LPS
WITH LPS

D

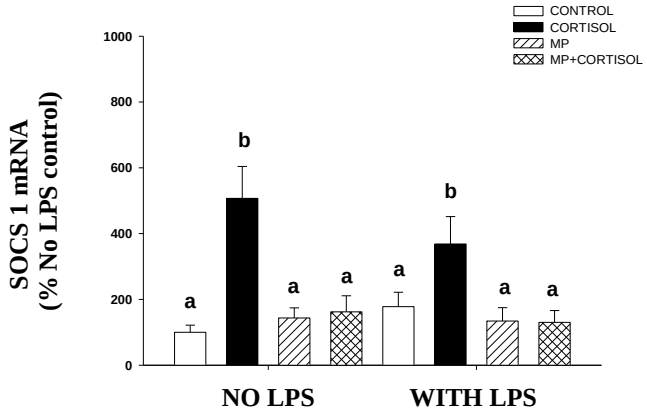

CONTROL
CORTISOL
MP
MP+CORTISOL
SAA mRNA
(% No LPS control)
250
200
150
100
50
0
*
a
b
ab
ab
a
b
ab
ab
NO LPS
WITH LPS

2.4.4 Suppressors of cytokine signalling (SOCS)

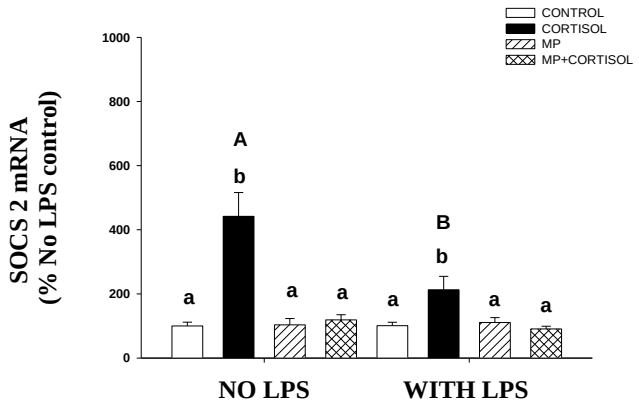
Exposure to LPS did not significantly affect SOCS-1 or SOCS-2 mRNA levels, but this immune-stimulant significantly reduced SOCS-3 mRNA abundance (1.4-fold) in trout hepatocytes (Fig. 5 A-C). Cortisol treatment significantly elevated SOCS-1 (5.1-fold) and SOCS-2 (4.4-fold), but not SOCS-3 mRNA transcript levels in trout hepatocytes (Figs. 5A-C). Cortisol-induced up-regulation of SOCS-1 and SOCS-2 was completely abolished by Mifepristone treatment in trout hepatocytes (Fig. 5A and 5B). There was a significant reduction in cortisol-induced SOCS-2 mRNA abundance in the presence of LPS in trout hepatocytes (Fig. 5B).

Figure 5: Effect of cortisol and LPS treatment on mRNA abundance of SOCS genes. Effect of cortisol and mifepristone (MP) on (A) SOCS-1 (B) SOCS-2 and (C) SOCS-3 gene expression in trout hepatocytes either in the presence (30μg/ml) or absence of LPS. Hepatocytes were exposed either to control (vehicle; 0.01% ethanol), cortisol (100 ng/ml), MP (1,000 ng/ml), or a combination of MP + cortisol, in the presence (30μg/ml) or absence of LPS. In the combination group, MP was added to the hepatocytes 30 min before cortisol addition. All values represent means ± SEM (n=6-7 independent fish); bars with different letters are significantly different within each No LPS or with LPS group; different upper case letters indicate cortisol effect between the No LPS and LPS groups; *LPS group significantly different from the No LPS group (two-way repeated measures ANOVA; $P < 0.05$).

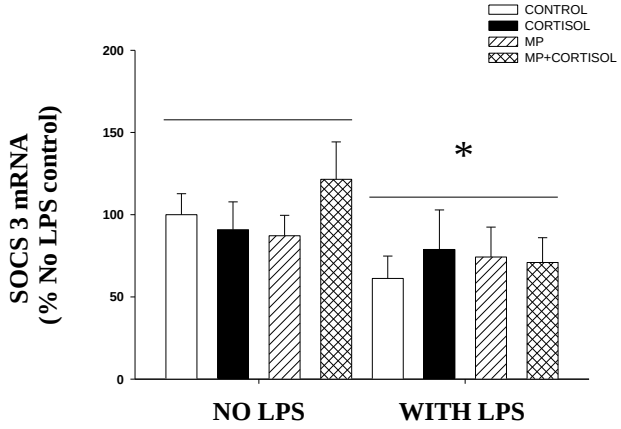
A


CONTROL
CORTISOL
MP
MP+CORTISOL
SOCS 1 mRNA
(% No LPS control)
1000
800
600
400
200
0
a
b
a
a
a
b
a
a
NO LPS
WITH LPS

B


CONTROL
CORTISOL
MP
MP+CORTISOL
SOCS 2 mRNA
(% No LPS control)
1000
800
600
400
200
0
a
A
b
a
a
a
B
b
a
a
NO LPS
WITH LPS

C


CONTROL
CORTISOL
MP
MP+CORTISOL
SOCS 3 mRNA
(% No LPS control)
200
150
100
50
0
*
NO LPS
WITH LPS

2.5 Discussion

We demonstrated that the stress hormone cortisol modulates mRNA transcript levels of genes encoding key mediators of innate immune response in rainbow trout hepatocytes. Our results reveal for the first time the regulation of SOCS-1 and SOCS-2 mRNA levels by cortisol in trout hepatocytes. We propose that up-regulation of SOCS genes by cortisol may be an adaptive response that restricts activation of cytokine signalling. This restriction in immune signalling would divert energy substrates away from endogenous (within the liver) immune-related pathways to those essential for coping with stress, including gluconeogenesis.

2.5.1 Metabolic response to LPS challenge

While liver is an important organ for the metabolic adjustment to stress (Mommsen et al., 1999), the response of this tissue to LPS challenge is far from clear. It is well known that inflammatory and immune responses, also associated with elevated plasma cortisol levels (Tort, 2011), are energy demanding and lead to pronounced shifts in target tissue metabolism (Kominsky et al., 2010). Here we show that LPS stimulation increases the cellular stress response and metabolic capacity in trout hepatocytes, supporting the general increase in energy demand during inflammation in fishes (Tacchi et al., 2010). The higher expression of hsp70, an inducible isoform of the highly conserved family of 70 kDa heat shock proteins, supports disruption in protein homeostasis and the associated adaptive response (Deane and Woo, 2010; Vijayan et al., 2010) to LPS-mediated cellular damage in trout hepatocytes. The elevated hsp70 protein levels in response to immune-stimulants appears to be a general phenomenon, as studies have shown a similar upregulation of hsp70 expression in fish immune cells (Stolte et al., 2009; Zhang et al., 2011). Although the mechanism by which LPS causes cellular stress is unclear, it may involve the generation of oxygen-free radicals and the associated proteotoxicity (Ghezzi et al., 1986; Boltana et al., 2009; Deane and Woo, 2010; Vijayan et al., 2010).

Cellular protein synthesis is energy demanding and accounts for ~80% of total oxygen consumption in trout hepatocytes (Pannevis and Houlihan, 1992). Consequently, the stress protein synthesis in response to LPS challenge may occur at the expense of other

energy demanding pathways, including anabolic processes. In support of this, trout hepatocytes with up-regulated hsp70 expression showed reduced responsiveness to cortisol stimulation of glucose production, a key adaptive metabolic response to stress (Mommsen et al., 1999; Boone et al., 2002). The suppression of glucose release from hepatocytes in the present study supports the notion that hsp70 expression in response to LPS may compromise other energy demanding pathways, including gluconeogenesis (Boone et al., 2002). This reduction was also seen in the presence of cortisol pointing to an overall reduced capacity for stress-mediated metabolic performance, as glucose is a key fuel to cope with the enhanced energy demand (Mommsen et al., 1999). Further support for increased energy demand due to LPS stimulation is seen from the higher PK and MDH activity in the LPS group suggesting enhanced glycolytic potential for endogenous energy use by the liver to cope with the immune challenge (Tacchi et al., 2010). Taken together, LPS treatment elicited a cellular stress response and enhanced the metabolic capacity of trout hepatocytes leading to a reduction in other energy demanding pathways, including gluconeogenesis.

Interestingly, cortisol treatment abolished the LPS-induced hsp70 expression suggesting a protective effect of this stress steroid on LPS-induced proteotoxicity. Similar inhibition of stressor-induced hsp70 expression by cortisol in fish tissues, both *in vivo* and *in vitro*, has been shown before (Vijayan et al., 2010). This cortisol effect did not involve any changes in GR protein expression in LPS-treated hepatocytes. The suppression of GR by cortisol is consistent with the response seen previously in trout hepatocytes *in vitro* and fish liver *in vivo* (Vijayan et al., 2003; Sathiyaa and Vijayan, 2003; Alderman et al., 2012), while LPS treatment did not seem to alter GR expression. Previous studies showed a rapid modulation of GR mRNA levels upon LPS treatment *in vitro* in head kidney phagocytes of common carp (*Cyprinus carpio* L.), suggesting that GR responses may be tissue-specific (Stolte et al., 2008, 2009). However, it is unclear whether the transcript changes mimic GR protein expression in this species. Overall our results underscore an enhanced energy demand in hepatocytes to elicit an inflammatory response to LPS, which may compromise the highly conserved glucose response to cortisol stimulation in trout hepatocytes.

2.5.2 Liver immune response

While stress, including endotoxin shock, and the associated elevated cortisol response is a key modulator of immune response in fishes (Tort, 2011), the role of liver in this process is far from clear. A well established acute stress response is the rapid elevation of circulating corticosteroid levels and our results demonstrate that stressed levels of this steroid modulate immune response in trout hepatocytes. Activation of innate immunity is a first line of defense against infection and involves the transcriptional up-regulation of pro-inflammatory cytokines, including IL-1β, TNF α2, IL-8 and acute phase proteins in fishes (Tort, 2011). cDNA libraries of liver, kidney and spleen obtained from fishes stimulated by LPS have proven to be an excellent source of genetic information concerning immune function in fishes (Xia and Yue, 2010). Indeed, fish immune cells, including splenocytes, peripheral blood leucocytes, lymphocytes, and head kidney macrophages express cytokines and acute phase proteins in response to immune-stimulants in trout (Zou et al., 1999, 2000; MacKenzie et al., 2003; Stolte et al., 2008; MacKenzie et al., 2006; Castro et al., 2011). Also, a recent study demonstrated that erythrocytes (a non-immune cell) elicit an immune response to immune stimulant challenge in trout (Morera et al., 2011). Our results add hepatocytes to the list of immune-responsive cells in fishes.

The immunosuppressive effect of cortisol reported for fish immune cells (Tort, 2011) was also evident in trout hepatocytes. The suppression of hepatocyte TNF α2, IL-8 and SAA mRNA abundances by cortisol is in agreement with previous studies demonstrating that stress-induced glucocorticoid levels down-regulate cytokine mRNA levels in gilthead seabream head kidney cells (Castillo et al., 2009) and trout macrophage cell line (Castro et al., 2011). However, cortisol elevated IL-1β mRNA levels in trout hepatocytes, unlike seabream head kidney cells (down-regulation; Castillo et al., 2009) and trout macrophage cell line (no change; Castro et al., 2011), pointing to a cell-specific difference in this cytokine regulation during stress in fishes. Also, repeated handling stress and elevating plasma cortisol levels increased the constitutive expression of IL-1β in head kidney macrophages in Atlantic salmon (Fast et al., 2008), suggesting a tissue- and species-specific differences in IL-1β expression.

In addition to cortisol impact on the constitutive levels of immune related gene expression, this stress steroid also modulated the LPS stimulated gene expression in trout hepatocytes. Specifically, the suppression of LPS-induced TNF α2, IL-8 and SAA expression by cortisol supports an immunosuppressive effect of this steroid on fish immune cells (Stolte et al., 2008; MacKenzie et al., 2006; Castro et al., 2011). For instance, cortisol administered at stress levels to carp head kidney phagocytes as well as rainbow trout monocyte/macrophage cells *in vitro* inhibited the LPS-induced TNF α2 mRNA abundances (Stolte et al., 2008; MacKenzie et al., 2006). The finding that the cortisol effects on unstimulated and LPS-stimulated immune-related gene expressions, including IL-1β and TNFα2 were abolished by Mifepristone, a well established GR antagonist in trout (Aluru and Vijayan, 2007), underscores a direct role for glucocorticoid receptor signalling in regulating liver immune response during stress. The failure of Mifepristone to completely block cortisol effect on LPS-stimulated IL-8 gene expression suggests other non-GR mediated signalling in trout (Aluru and Vijayan, 2007).

2.5.3 Liver SOCS regulation

A key regulator of the inflammatory response to cytokine stimulation is the suppressor of cytokine signalling (SOCS) proteins (Kile and Alexander, 2001). Multiple isoforms of SOCS genes, including SOCS-1, SOCS-2 and SOCS-3, have been cloned and sequenced in trout (Wang et al., 2008; Wang et al., 2010), but their functional significance is far from clear in fishes. Our results reveal for the first time that stress level of cortisol up-regulates SOCS-1 and SOCS-2 but not SOCS-3 in trout hepatocytes, implicating liver as a target for stress-immune interactions in fish. Bioinformatic analysis revealed putative glucocorticoid response elements on the promoter of SOCS-1 and SOCS-2 genes in zebrafish (*Danio rerio*; see Appendix A) supporting GR regulation of these genes. Furthermore, cortisol regulation of SOCS-1 and SOCS-2 were abolished by Mifepristone, a GR antagonist (Aluru and Vijayan, 2007), supporting a direct role for GR signalling in regulating SOCS expression during stress in fish. However, it remains to be established whether SOCS function in fish are similar to that described in mammals (Kile and Alexander, 2001). As endotoxin challenge elevates plasma cortisol levels (Acerete et al., 2007), we propose that

up-regulation of SOCS gene expression by this steroid may be an adaptive mechanism to suppress target tissue cytokine signalling, thereby limiting infection-induced inflammatory responses and the associated metabolic costs.

Upregulation of SOCS (CIS, SOCS 1-3) have been shown to inhibit JAK/STAT signalling in rat models (Croker et al., 2008), which play an important role in suppressing cytokine signalling and modulating other cellular energy demanding pathways. For instance, growth hormone (GH), which is a key regulator of postnatal somatic growth, signals primarily through the JAK2–STAT5b pathway. It has been shown that SOCS proteins interact with GH receptor (GHR) signalling, and over-expression of SOCS-2 interferes with the JAK2–STAT5b pathway (Croker et al., 2008). Also, phenotypically SOCS-2-deficient mice resemble GH-transgenic mice, displaying increased body weight resulting from enhanced bone size and an enlargement of most organs, supporting a negative regulation of GH actions by SOCS genes (Croker et al., 2008). Taken together, the glucocorticoid responsive SOCS genes in the liver of trout may be playing a key role in diverting energy resources away from growth promoting action of GH during stress in fish. This may be a key adaptive response that not only suppresses cytokine signalling, but also favours energy substrate repartitioning away from growth to metabolically cope with the enhanced energy demand associated with stress.

As in mammals, LPS challenge stimulates SOCS-1, SOCS-2 and SOCS-3 expression in fish immune cells (Jin et al., 2008; Jin et al., 2007a; Jin et al., 2007b), including rainbow trout leukocyte cell line, RTS-11 and freshly prepared trout splenocytes over a 24 h period (Wang et al., 2010). However, we were unable to detect a stimulatory effect of LPS on SOCS-1 and SOCS-2 mRNA abundances in trout hepatocytes, while expression of SOCS-3 was reduced. It is possible that the expression may be transient in liver tissue as recently seen in yellow perch liver where SOCS-1 and SOCS-3 expression peaked between 6 -12 h and was back to the pre-LPS exposure level at 24 h (Shepherd et al., 2012). Together, these results suggest that LPS stimulates transient expression of SOCS genes in a tissue-, cell- and isoform-specific manner in fishes, but this remains to be established in trout hepatocytes.

In conclusion, we demonstrate that trout hepatocytes respond to LPS challenge by up-regulating key mediators of the innate immune response. The molecular response in hepatocytes is modulated by stress levels of cortisol and this involves GR signalling. We identified SOCS-1 and SOCS-2 to be glucocorticoid responsive, while SOCS-3 was modulated by LPS in trout hepatocytes. We propose that the regulation of SOCS-1 and SOCS-2 by cortisol may be a key adaptive strategy to reallocate energy substrates away from growth and inflammatory response to those essential to cope with stress, including glucose production.

2.6 Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council of Canada discovery grant and Discovery Accelerator Supplement to MMV.

Chapter 3

Tissue-specific molecular immune response to lipopolysaccharide challenge in emaciated anadromous Arctic charr

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3.1 Overview

Anadromous Arctic charr (*Salvelinus alpinus*) undergo voluntary winter fasting for months in the Arctic. We tested the hypothesis that extended fasting will compromise the ability of this species to evoke an immune response. Charr were either fed or fasted for 85 days and challenged with lipopolysaccharide (LPS), and the molecular immune response in the liver and spleen assessed at 8 and 96 h post-injection. LPS increased IL-1β, IL-8, and serum amyloid protein A (SAA) mRNA levels in both groups, but the liver IL-1β and IL-8, and spleen IL-8 responses were reduced in the fasted group. Fasting upregulated SOCS-1 and SOCS-2 mRNA abundance, while LPS stimulated SOCS-3 mRNA abundance and this response was higher in the fasted liver. Collectively, extended fasting and emaciation does not curtail the capacity of charr to evoke an immune response, whereas upregulation of SOCS may be a key adaptation to conserve energy by restricting the inflammatory response.

3.2 Introduction

It is well established that pro-inflammatory cytokines, including IL-1β and IL-8, are expressed in salmonids during the early phases of an infection, thereby initiating a strong innate inflammatory response (Sigh et al., 2004; Engelsma, 2002). While most studies in fishes have examined the inflammatory response using immune cells, recent studies also alluded to immunostimulation of extra immune-related tissues, including red blood cells and hepatocytes, (Morera et al., 2011; Philip et al., 2012). The liver plays an important role in intermediary metabolism and, therefore, may be particularly sensitive to changes in nutritional status of the animal. The liver is also a major site of acute phase proteins (APPs) synthesis, which is a key component of the innate immune response (SaranyaRevathy et al., 2012; Talbot et al., 2009). Serum amyloid protein A (SAA) is a major APP and an effector of innate immunity in all vertebrates, including fishes (Steel and Whitehead, 1994; Talbot et al., 2009). Recently, we demonstrated that trout hepatocytes can mount an innate immune response to lipopolysaccharide (LPS) stimulation, suggesting a role for this tissue in the inflammatory response, including upregulation of IL-1β, IL-8 and SAA (Philip et al., 2012).

Suppressors of cytokine signalling (SOCS) proteins are thought to play a key role in the modulation of cytokine signalling (Kile and Alexander, 2001).The negative regulation of cytokine signalling by SOCS involves activation of the JAK2-STAT5 pathway (O'Sullivan et al., 2011). Homologues of all the eight mammalian SOCS family members have been discovered in fish (Wang et al., 2011), while SOCS-1, SOCS-2 and SOCS-3 have been characterized in salmonids (Wang and Secombes, 2008). Although the functional significance of SOCS transcript abundance is unclear, the mRNA abundance of SOCS-1-3 have been shown to be modulated by immunostimulants (Philip et al., 2012; Shepherd et al., 2012; Wang et al., 2011), but their expression with fasting is less clear.

Fasting leads to a negative energy balance and can restrict energy demanding pathways, including mounting an immune response (Berczi 1986; Houston et al., 2007; Martin et al., 2010). Most studies that have examined the direct effects of nutritional status on the immune response have focused on mammalian models and showed a decreased capacity to defend against infection (Sun et al., 2001; Walrand et al., 2001). For instance, in mice and rat models, starvation reduces the number of T cells, suppresses the development of T-cell-mediated immunity, and induces apoptosis of lymphocytes, thereby impairing immune function (Wing et al., 1988; Pires et al., 2007). However, very little is known about the effect of dietary restriction on the immune response in fishes. A recent study showed that in the Atlantic salmon (*Salmo salar*), starvation for 28 days led to a reduction in mRNA abundance of immune-related genes in the liver (Martin et al., 2010). Also, starvation for 31 days decreased the non-specific immune parameters, including haemagglutinating activity and respiratory burst activity in the sea bass (*Dicentrarchus labrax*) and blackspot sea bream (*Pagellus bogaraveo*) (Caruso et al., 2011). These studies suggest that fasting also compromises the molecular immune responses and function in teleosts as seen in mammalian models.

While reduced capacity for immune response activation in response to negative energy balance appears to be the norm in most animals (Martin et al., 2010; Berczi, 1986), those studies utilized animal models that do not naturally experience extended fasting. We asked the question whether animals exhibiting extreme adaptations, including extended

fasting as part of a life-history strategy, show the same degree of immune response to immunostimulants as fed animals. To test this, we utilized the anadromous Arctic charr (*Salvelinus alpinus*), the northernmost freshwater fish and considered as the most cold-adapted species in the salmonid family (Siikavuopio et al., 2009). This species makes an annual seaward migration each spring where they exhibit concentrated bouts of heavy feeding before returning to freshwater every fall to overwinter. Overwintering is characterized by anorexia and energy substrate depletion leading to a continuous negative energy balance until they resume appetite again in May (Jørgensen et al., 1997). These seasonal patterns of appetite and growth are even exhibited by captive offspring of anadromous Arctic charr in spite of providing food in excess (Tveiten et al., 1996; Frøiland et al., 2010), making them an excellent model for mechanistic studies on the influence of nutritional status on immune function.

In the present study we tested the hypothesis that extended fasting will compromise the immune competence in a fish species which undergoes voluntary seasonal emaciation. This was carried out by comparing the molecular immune response in the liver, a metabolically active tissue, and spleen, a well established tissue model for immune studies (Stolte, 2008), in response to LPS (a well-established immunostimulant; Engelsma, 2002; MacKenzie et al., 2006; Fast et al., 2007) stimulation between fed and fasted anadromous Arctic charr. As markers of the inflammatory response, we measured the expression of pro-inflammatory cytokines IL-1β and IL-8 as well as the three isoforms of SOCS, SOCS-1, SOCS-2 and SOCS-3, while SAA was used as an indicator of the acute phase response.

3.3 Materials and Methods

3.3.1 Fish and experimental conditions

The study was carried out at Tromsø Aquaculture Research Station (69^{o} N), Norway. Fish used were 3-year old, hatchery-reared offspring of wild, anadromous Arctic charr. The original broodstock was captured in Lake Vårflusjøen, Svalbard (79^{o} N) in 1990. Eggs hatched in January 2008 and were held in fresh water at 6^{o} C under continuous light until July 2008. The fry were then transferred to a circular, 3000-l tank and held at ambient water

temperature and natural photoperiod (transparent roof) until March 2010. Until then all fish were fed to satiety with commercial dry pellet (Skretting, Stavanger, Norway) using automatic disc feeders.

On March 9, a total of 68 fish were sorted out from the stock tank, anaesthetised in benzocaine (50 ppm) and individually tagged (Floy FTF-69 fingerling tags, MGF, Seattle, USA). They were then randomly distributed among four 300-l tanks with ambient fresh water (0.5^{o} C) and held at a simulated, natural light regime (69^{o} N) and continuous feeding (Nutra Parr 2,0 mm; Skretting) until March 25. After the acclimation period, water temperature was elevated to 5^{o} C and the fish in two tanks were deprived of food and those in the two other tanks were fed as before. The temperature was raised to 5^{o} C to achieve body mass measures comparable to emaciated wild charr in three months. This regimen was maintained until June 17, 2010, when the energy status of the fasted and fed fish (as evidenced by their condition factor) were comparable to that of emaciated, wild charr in late winter and well-fed, wild charr in mid-summer, respectively (Jorgensen et al., 1997).

3.3.2 Experimental design

This experimental protocol was approved by the Norwegian Committee on Ethics in Animal Experimentation. An initial sampling on June 17 (0 time) consisted of netting 10 fed and 10 fasted fish (n = 5 from each replicate tank) and euthanizing them with an overdose of benzocaine (150 ppm). Body mass (BM; g) and fork length (FL; cm) were recorded and blood was collected from the caudal vein using heparinised vacutainers. The blood samples were held on ice until plasma was separated by centrifugation (6000*g*, for 10 min) and stored at -20º C for glucose analysis later. The liver from each fish was excised and weighed before a small piece was removed and stored at -20^{o} C for glycogen analyses. Finally the rest of the carcass was frozen at -20^{o} C for later total fat analyses. BM, FL and liver weight (LW) were used to calculate the condition factor (CF; BM/FL^{3} x 100) and hepatosomatic index (HSI; LW/BM x 100). Specific growth rate (SGR) was calculated by the formula [(ln BM_T – ln BM_t/T-t) x 100] where BM_T and BM_t are the weights of the fish in June and March, respectively, and T-t the number of days between weight measurements.

On June 18, the remaining fish (12 per tank) were injected (injection volume 2ml/kg fish wt) with either LPS (Sigma L2630; 2.5mg/Kg fish wt in PBS) in one tank each for fed and fasted groups] or the vehicle (PBS; Sigma P5493). The fish were euthanized at 8 and 96 h post-injection (n = 6 for each time-point) as described above, after which pieces of the liver and spleen were excised and stored in RNAlater, first at 4^{o}C for 24 h, and then at – 80^{o} C until measuring transcript levels later.

3.3.3 Analyses

Plasma glucose was determined by a Randox GL 1611glucose kit (RANDOX Laboratories Ltd., Crumlin, UK). The same kit was used to determine liver glycogen content by analysing liver glucose content before and after amyloglucosidase hydrolysis (Keppler and Decker, 1974). Liver protein content was analysed using bicinchoninic acid Protein Assay Reagent Kit (Pierce # 23227; Pierce Chemical Company, Rockford, Illinois) using bovine serum albumin as the standard. Total body fat was extracted by petroleum benzine (Merck, Darmstadt, Germany) in a Behrotest extraction system (Behr Labor-technik, Düsseldorf, Germany) according to a protocol described before (Frøiland et al., 2010). Plasma cortisol levels were analysed in diethyl ether extracted plasma by radioimmunoassay and validated for Arctic charr as described previously (Tveiten et al., 2010).

3.3.4 Quantitative real-time PCR (qPCR)

RNA extraction and cDNA synthesis: Total RNA was extracted from liver and spleen tissue using the RNeasyextraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The concentration of total RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Napean, ON, CAN). First-strand cDNA was synthesized from 1µg of total RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

Quantification: Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The genes of interest were IL-1β, IL-8, SAA, SOCS-1, SOCS-2 and SOCS-3, while elongation factor 1

alpha (EF1α) was the housekeeping gene. Primer pairs (Table 1) for these genes were designed from rainbow trout cDNA sequences using Primer 3 version 0.4.0. software. Threshold cycle values for each sample were calculated using iCycler iQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 2.5 μL cDNA, 2.5 μL of primer pair (10 mM each of the respective forward and reverse primers), 40 μL of SYBR green mix and 35 μL of nuclease-free water was prepared for each sample, after which 25 μL was added to each of three wells. Each sample was assayed for the genes of interest and the housekeeping gene. The following PCR conditions were used for amplification: 95 °C for 3 min; 40 cycles of 95 °C for 20 s and annealing temperature (see Table 1) for 20 s; 95 °C for 1 min; 55 °C for 1 min followed by melt curve analysis to confirm the presence of a single amplicon, starting at 55 °C and increasing in 0.5 °C increments to 95 °C every 10 s. Copy number of transcripts for each gene was determined with the threshold cycles (CT) using plasmid standard curves exactly as described before (Aluru et. al., 2010). EF1α was used as the housekeeping gene for normalization as this transcript level remained unchanged between treatment groups. Briefly, standard curves were generated using a serial dilution of plasmid vector stock (pGEM-Teasy cloning vector; Promega,Valencia, CA, USA) with inserted target sequences to attain varying copy number of insert sequences ($3x10^{10} – 3x\ 10^{1}$ copies). PCRs were performed as described above with 2.5 μL of the different plasmid dilutions added as template. Background subtracted CT values were plotted against log of standard copy numbers to obtain standard curves.

Table 1: **Gene-specific primers for quantitative real-time PCR.**

List of genes (Gene ID), forward and reverse primer sequences, annealing temperature and amplicon size. IL-1β: interleukin-1 beta; IL-8: interleukin-8; SAA: serum amyloid protein A; SOCS: suppressors of cytokine signalling; EF1α: elongation factor 1α.

Gene ID	Primer sequences (5'-3')	Temp (^{o}C)	Amplicon size (bp)
IL-1β	Fwd: GGAGAGGTTAAAGGGTGGCGA Rev: TGCCGACTCCAACTCCAACA	60	121
IL-8	Fwd: CACTGAGATCATTGCCACTCTGA Rev: ATGACCCTCTTGACCCACGG	60	81
SAA	Fwd: TTGTTCTGACCCTCGTTGTAGGAGC Rev: CATGTCGCCATATGCACGCC	60	101
SOCS-1	Fwd: TCAGCGTACGCATCGTCTAT Rev: CGGTCAGGCTTTTCTTAGAGG	55.7	120
SOCS-2	Fwd: TCGGATGACTTTTGGCCTAC Rev: CCGTTCTTCTCTCGTTTTCG	60	102
SOCS-3	Fwd: GAACAACACAAGATATCAAGCTCAAGG Rev: GAAGGTCTTGTAACGGTGAGGCAG	65.7	351
EF1α	Fwd: CATTGACAAGAGAACCATTGA Rev: CCTTCAGCTTGTCCAGCAC	56	95

3.3.5 Statistical analysis

Data are shown as mean ± standard error of mean (SEM). Outliers, values that were two standard deviations above or below the mean, were omitted from the analyses. Three-way ANOVA followed by Holm Sidak's *post-hoc* was used to compare the effect of different treatments on mRNA levels. The units for mRNA levels for each gene are copy numbers for that particular gene obtained from the standard curve and then normalised to EF1alpha. These numbers were used for statistical analysis although percentages are shown in the Figures. Student *t*-test was used to compare the effect of different treatments on body mass, condition factor, SGR, HSI, body fat, liver glycogen and plasma glucose and cortisol levels. The data were log transformed, wherever necessary, for homogeneity of variance, but non-transformed values are shown in the figures. A probability level of $p<0.05$ was considered significant. Statistical analyses were performed with SigmaStat (Systat Software Inc., San Jose, CA, USA).

3.4 Results

3.4.1 Body mass, specific growth rate, condition factor and fat content

As evidenced by the fish sampled at 0 time, long-term fasting significantly reduced body mass, specific growth rate, condition factor and body fat content in anadromous Arctic charr. The mean body mass of fasted charr was reduced by ~60%, this was also reflected in significant differences in SGR (Table 2). The body fat content was ~88% lower in the fasted charr, and the condition factor was also reduced in the fasted charr by ~38% compared to the fed charr (Table 2). The mean body mass and condition factor of the fish used for the immune challenge were similar to those of the fish sampled at 0 time, being 327 ± 24.5 g and 1.17 ± 0.03, respectively, in fed fish and 128 ± 5.3 g and 0.75 ± 0.01, respectively, in fasted fish.

3.4.2 HSI, liver glycogen content and plasma glucose and cortisol levels

The HSI (~48%) and liver glycogen content (~88%) were significantly reduced by fasting compared to the fed charr (Table 2). There were no significant differences in either plasma glucose or cortisol levels between the fed and fasted charr (Table 2).

Table 2: Biometrical measurements and metabolic and stress parameters.

The table provides the body mass (BM), condition factor (CF), specific growth rate (SGR), body fat content, hepatosomatic index (HSI), liver glycogen content and plasma glucose and cortisol levels in the fed and fasted charr sampled at time 0 prior to lipopolysaccharide treatment. Different letters denote significant difference. All values represent means ± SEM (n denotes the number of fish) (t test; $P<0.05$).

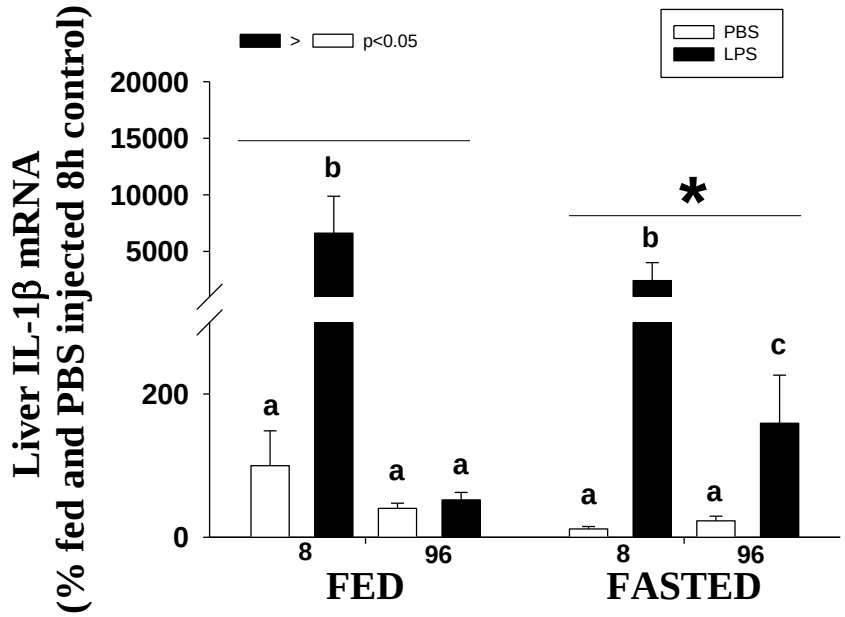
Biometrical measurements	**FED (n=7)**	**FASTED (n=10)**
BM (g)	339 ± 52^{a}	138 ± 7.5^{b}
CF	1.20 ± 0.06^{a}	0.75 ± 0.01^{b}
SGR	0.79 ± 0.08^{a}	-0.17 ± 0.01^{b}
Body fat (%)	10.4 ± 1.5^{a}	1.25 ± 0.38^{b}
HSI	1.96 ± 0.12^{a}	1.03 ± 0.04^{b}
Metabolic and stress parameters		
Liver glycogen(µmol/g protein)	1408 ± 182^{a}	168 ± 42^{b}
Plasma glucose (mM)	4.27 ± 0.17^{a}	4.25 ± 0.23^{a}
Plasma cortisol (ng/ml)	16.5 ± 4.8^{a}	16.8 ± 5.4^{a}

3.4.3 Gene expression

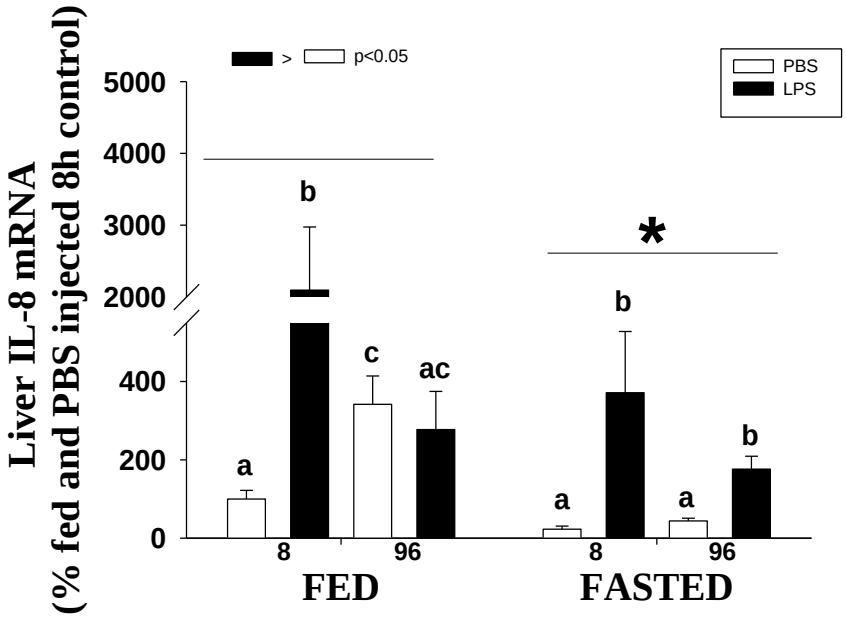
Cytokine expression: LPS stimulation increased IL-1β and IL-8 mRNA abundances in the liver and spleen of both fed and fasted Arctic charr. In fed charr, IL-1β (66-fold increase in the liver; 68-fold increase in the spleen) and IL-8 (21-fold increase in the liver; 35-fold increase in the spleen) expression levels peaked at 8 h post-LPS injection and returned to control levels by 96 h in both tissues examined (Fig. 1A-D). However, tissue-specific temporal differences in cytokine mRNA levels were observed in the fasted charr; IL-1β and IL-8 expression levels, which peaked at 8 h post-LPS injection, failed to return to control levels by 96 h in the liver, but did so in the spleen of fasted charr (Fig. 1A-D). Moreover, there was a temporal increase in liver IL-8 levels in the control fed group at 96 h post-injection. Overall, fasted charr showed significantly lower mRNA levels of IL-8 in both tissues, compared to fed charr. IL-1β was significantly reduced in the liver but not the spleen of fasted charr compared to fed charr.

Figure 1: Tissue-specific mRNA abundance of IL-1β (A and C) and IL-8 (B and D) Arctic charr that were either fed or fasted for 85 days were injected with LPS (2.5mg/Kg wt) or PBS. Liver (A and B) and spleen (C and D) were sampled 8 and 96 h post-injection. All values represent means ± SEM (n=5-6 independent fish) relative to the value obtained for the fed fish injected with PBS and sampled at 8 h post-injection (100%); bars with different letters are significantly different within the fed and fasted groups; * denotes fasted group significantly different from the fed group; the inset shows overall significant treatment (LPS vs PBS) effects (three-way ANOVA; $P < 0.05$).

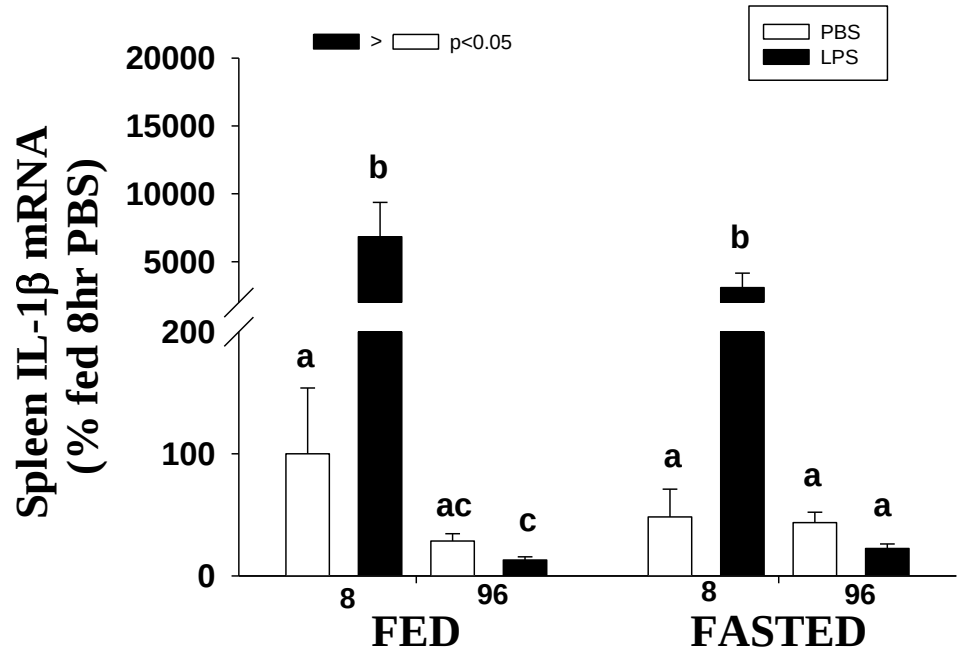
A


Liver IL-1β mRNA
(% fed and PBS injected 8h control)
> p<0.05
PBS
LPS
20000
15000
10000
5000
200
0
b
a
a
a
*
b
c
a
a
8
96
8
96
FED
FASTED

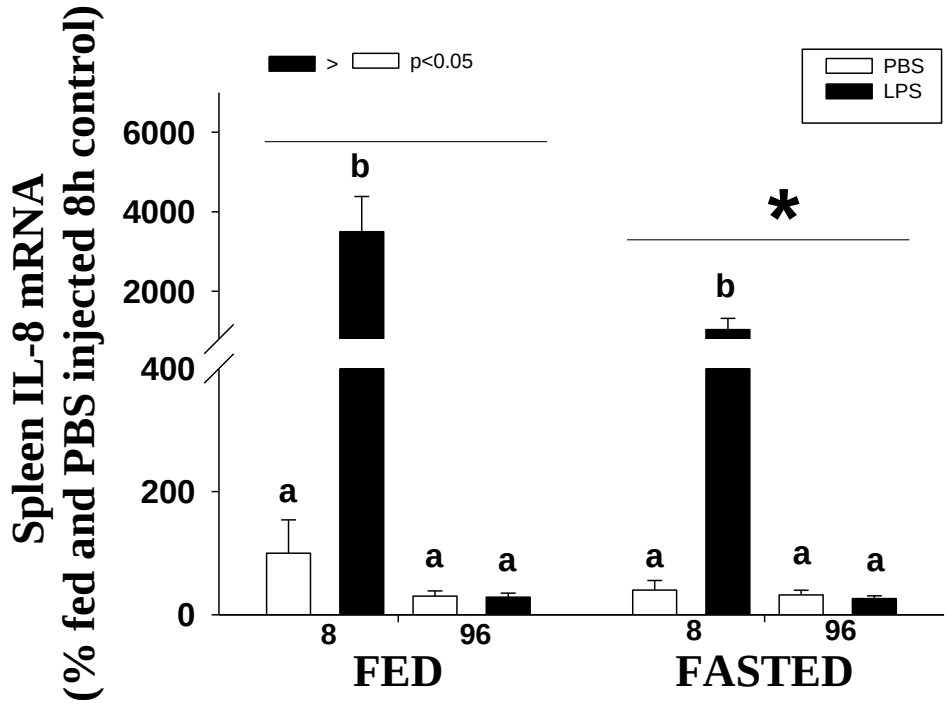
B


Liver IL-8 mRNA
(% fed and PBS injected 8h control)
> p<0.05
PBS
LPS
5000
4000
3000
2000
400
200
0
b
a
c
ac
*
b
a
a
b
8
96
8
96
FED
FASTED

C


> p<0.05
PBS
LPS
Spleen IL-1β mRNA
(% fed 8hr PBS)
20000
15000
10000
5000
200
100
0
a
b
ac
c
a
b
a
a
8
96
8
96
FED
FASTED

D

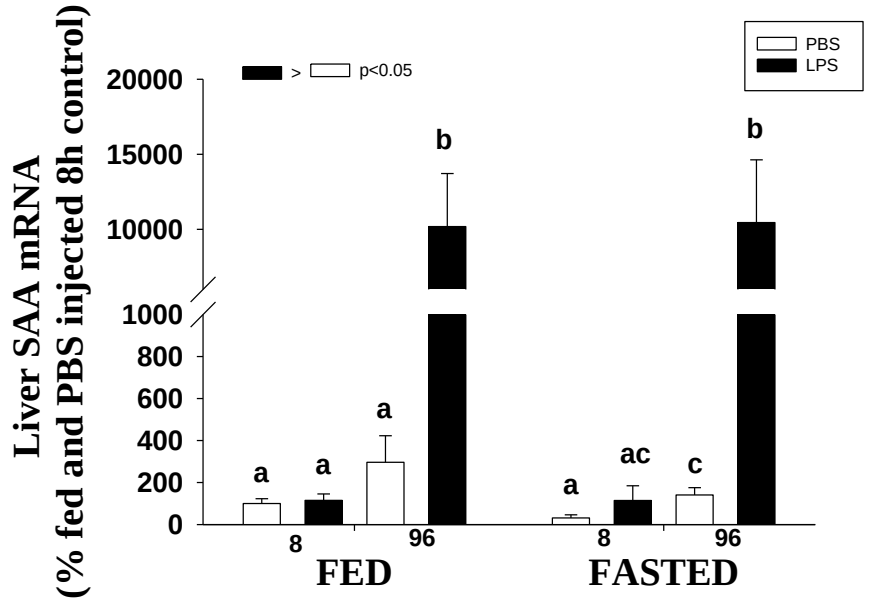

> p<0.05
PBS
LPS
Spleen IL-8 mRNA
(% fed and PBS injected 8h control)
6000
4000
2000
400
200
0
a
b
a
a
a
b
a
a
*
8
96
8
96
FED
FASTED

SAA expression: LPS injection increased SAA transcript levels in the liver and spleen of both fed and fasted Arctic charr. In the liver of both fed and fasted charr, SAA mRNA levels peaked at 96 h post-LPS injection (34-fold increase for fed charr; 74-fold increase for fasted charr) with no significant change at 8 h post- injection compared to the controls (Fig. 2A). Fasted charr showed the same overall level of SAA mRNA abundance as fed charr. There was a temporal increase in liver SAA levels in the control fasted fish at 96 h post-injection. An interaction between nutritional status and time post-LPS injection was observed in the spleen. In the spleen of fed charr, SAA levels peaked at 96 h post-LPS injection (134-fold) with no significant change at 8 h, while in the spleen of fasted charr, SAA expression was significantly higher at both 8 h (24-fold) and 96 h (71-fold) post-LPS injection compared to the control groups (Fig. 2B).

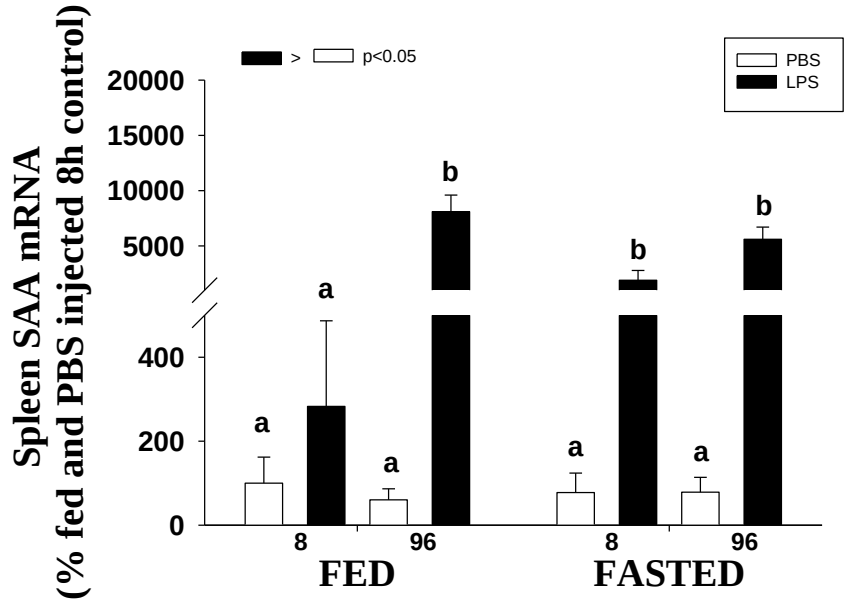
Figure 2: Tissue-specific mRNA abundance of serum amyloid protein A (SAA)

Arctic charr that were either fed or fasted for 85 days were injected with LPS (2.5mg/Kg wt) or PBS. Liver (A) and spleen (B) were sampled 8 and 96 h post-injection. All values represent means ± SEM (n=5-6 independent fish) relative to the value obtained for the fed fish injected with PBS and sampled at 8 h post-injection (100%); bars with different letters are significantly different within the fed and fasted groups; the inset shows overall significant treatment (LPS vs PBS) effects (three-way ANOVA; $P < 0.05$).

A


Liver SAA mRNA
(% fed and PBS injected 8h control)
PBS
LPS
> p<0.05
20000
15000
10000
1000
800
600
400
200
0
a
a
a
b
a
ac
c
b
8
96
8
96
FED
FASTED

B


Spleen SAA mRNA
(% fed and PBS injected 8h control)
PBS
LPS
> p<0.05
20000
15000
10000
5000
400
200
0
a
a
a
b
a
b
a
b
8
96
8
96
FED
FASTED

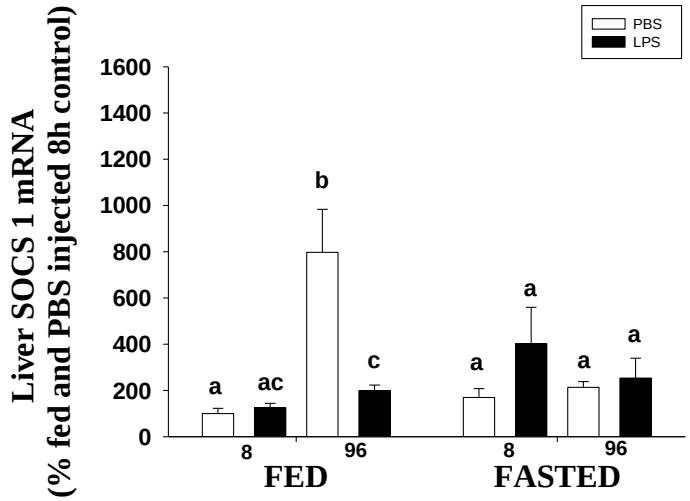
***SOCS expression**:* In general, fasted charr, irrespective of LPS/PBS injection upregulated the overall mRNA abundances of the three SOCS isoforms in a tissue-specific manner (Figs. 3-5). SOCS-2 was upregulated by fasting (9-fold increase in the liver; 3-fold increase in the spleen) regardless of LPS/PBS injection in both tissues (Fig. 4A and 4B), whereas SOCS-1 and SOCS-3 were significantly higher with fasting compared to the fed charr only in the spleen (1.4- fold increase; Fig. 3B) and liver (4-fold increase; Fig. 5A), respectively.

The fed and fasted charr differentially expressed their SOCS isoforms in response to LPS both temporally and in a tissue specific manner. There was a temporal increase in liver SOCS-1 in the control fed group at 96 h post-injection and this was not seen in the fasted group (Fig. 3A). In the spleen, fed charr showed elevated SOCS-1 levels in response to LPS at 96 h (Fig. 3B), while this change was not observed in the fasted charr (Fig. 3B). There was no interaction between LPS and fasting on liver SOCS-2 mRNA levels (Fig. 4A), whereas fasted charr showed lower spleen SOCS-2 mRNA levels in response to LPS at 96 h post-injection (Fig. 4B). LPS-injection significantly increased SOCS-3 mRNA levels in the liver and spleen of fed and fasted charr (see inset; Fig. 5A and B). There was a significant temporal reduction in spleen SOCS-3 mRNA levels in the control group at 96 h compared to 8 h post-injection in both the fed and fasted charr (Fig. 5B).

Figure 3: Tissue-specific mRNA abundance of SOCS-1

Arctic charr that were either fed or fasted for 85 days were injected with LPS (2.5mg/Kg wt) or PBS. Liver (A) and spleen (B) were sampled 8 and 96 h post-injection. All values represent means ± SEM (n=5-6 independent fish) relative to the value obtained for the fed fish injected with PBS and sampled at 8 h post-injection (100%); bars with different letters are significantly different within the fed and fasted groups; * denotes fasted group significantly different from the fed group (three-way ANOVA; P <0.05).

A



B

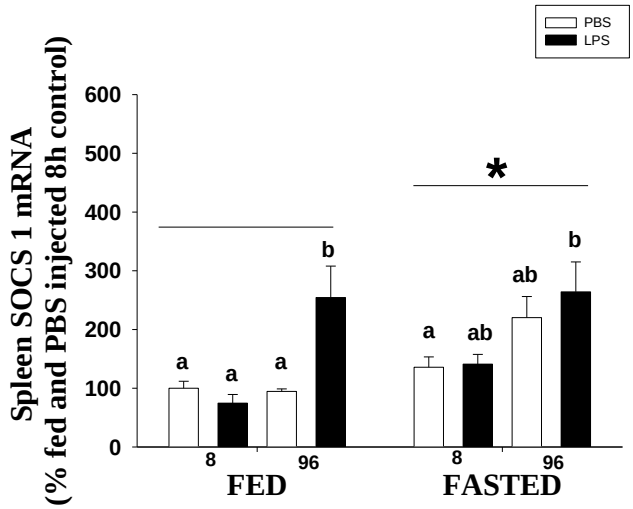
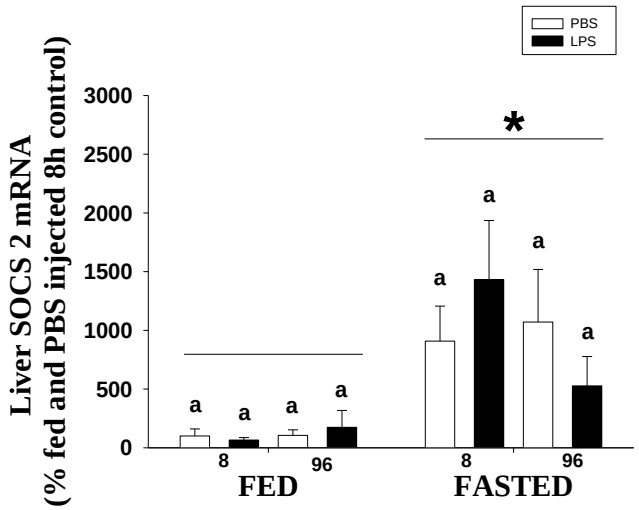


Figure 4: Tissue-specific mRNA abundance of SOCS-2

Arctic charr that were either fed or fasted for 85 days were injected with LPS (2.5mg/Kg wt) or PBS. Liver (A) and spleen (B) were sampled 8 and 96 h post-injection. All values represent means ± SEM (n=5-6 independent fish) relative to the value obtained for the fed fish injected with PBS and sampled at 8 h post-injection (100%); bars with different letters are significantly different within the fed and fasted groups; * denotes fasted group significantly different from the fed group (three-way ANOVA; P <0.05).

A



B

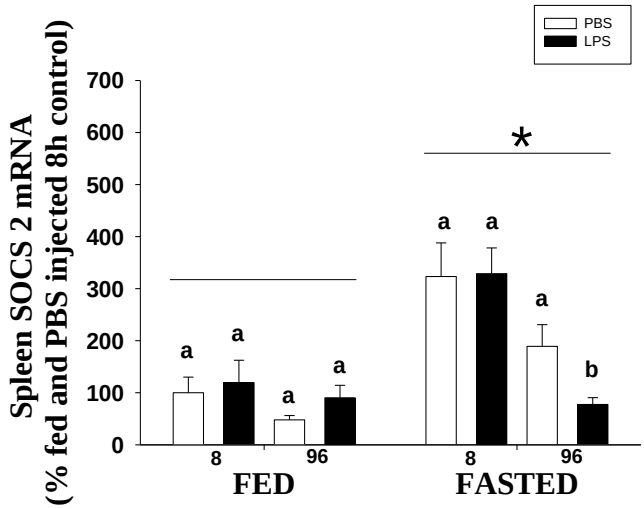
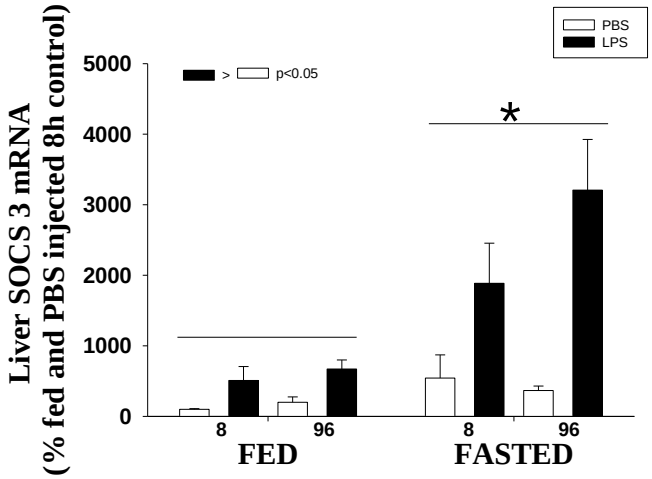


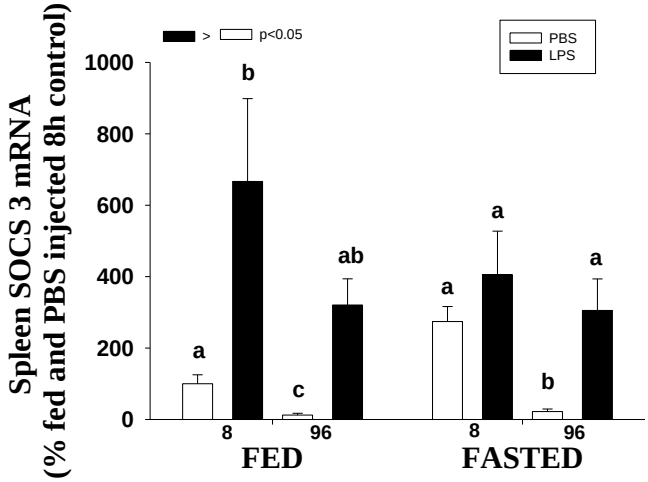
Figure 5: Tissue-specific mRNA abundance of SOCS-3

Arctic charr that were either fed or fasted for 85 days were injected with LPS (2.5mg/Kg wt) or PBS. Liver (A) and spleen (B) were sampled 8 and 96 h post-injection. All values represent means ± SEM (n=4-6 independent fish) relative to the value obtained for the fed fish injected with PBS and sampled at 8 h post-injection (100%); bars with different letters are significantly different within the fed and fasted groups; * denotes fasted group significantly different from the fed group; the inset shows overall significant treatment (LPS vs PBS) effects (three-way ANOVA; P <0.05).

A



B



3.5 Discussion

High-latitude fish species have developed adaptive mechanisms that enable them to cope with the strong seasonality of the Arctic environment, including extended fasting and emaciation during the overwintering phase and intense feeding and energy deposition during the brief summer phase. We demonstrate that an emaciation comparable to that experienced by wild, anadromous Arctic charr during overwintering does not compromise their ability to evoke an immune response to LPS stimulation. This indicates that their immune competence is not compromised by long-term fasting and emaciation associated with this aspect of their life-history. Maintaining the immune competence may be essential for this species to fight infection, especially when they migrate back into the ocean in the summer. Our results show that extended fasting in this species upregulates SOCS-1, SOCS-2 and SOCS-3 mRNA levels in a tissue-specific manner. We hypothesize that upregulation of SOCS may be a key adaptive strategy to curtail energy demanding pathways, including growth hormone and cytokine signalling, during overwintering to metabolically cope with the extended fasting in this species.

3.5.1 Innate immune modulation

Cytokines and acute phase proteins are key mediators of the innate immune response and their expression is a key marker of immune function (Engelsma, 2002). A key finding from this study was that LPS-mediated upregulation of cytokines, including IL-1β and IL-8 were observed in both the fed and fasted Arctic charr. This is unlike other animal models where even short term starvation reduces the capacity for immune response activation (Martin et al., 2010). IL-1β is important in initiating inflammatory responses while IL-8 is a potent neutrophil chemotactic factor (Fast et al., 2007) and, together, they act as key mediators of innate immunity. However, the steady-state mRNA levels in fasted charr were lower than in the fed group and may be related to their negative energy balance. This is supported by the lower liver metabolic capacities associated with winter fasting (this study; Jørgensen et al., 2013) suggesting an overall metabolic suppression as an adaptation to extended fasting in these animals. Additionally, the absence of any difference in plasma cortisol and glucose levels between the fed and fasted charr supports the notion that these

animals have unique metabolic adaptations to cope with the extreme seasonality without eliciting a stress response (Jørgensen et al., 2002) and attendant immune suppression.

There were tissue-specific differences in the temporal expression profiles of these cytokines due to extended fasting. Although liver is a non-classical immune tissue, unlike the spleen, we recently demonstrated that trout heaptocytes respond to LPS challenge by upregulating cytokine transcript levels (Philip et al., 2012). Our results confirm that liver also responds to immune challenges in Arctic charr. The transcript levels of LPS-stimulated IL-1β and IL-8 were back to the unstimulated steady-state levels by 96 h post-injection in the spleen but not the liver of fasted fish suggesting a tissue-specific difference in transcript dynamics. It remains to be seen if these changes were associated with enhanced mRNA stability in the liver or due to transcriptional activation. The temporal increase in liver IL-8 levels in the control fed group at 96 h post-injection may be due to the stress of handling and injection (Fast et al., 2007).

During an inflammatory response the liver synthesizes high levels of APPs that assists with the neutralization of invading pathogens (Martin et al., 2010). SAA is a positive APP, whose levels have been shown to increase during inflammation (Uhlar and Whitehead, 1999). Furthermore, the upregulationof SAA mRNA levels has been demonstrated in Arctic charr following infection with *A. salmonicida* (Jensen et al., 1997). Our results reveal that fasted charr also upregulate SAA transcript abundance in response to LPS challenge. In the liver of both fed and fasted charr, SAA expression peaked at 96 h post-LPS injection with no significant change at 8 h post-injection. Given that cytokine expression occurred at 8 h after LPS injection compared to 96 h for SAA expression, and also because cytokines are the key stimulators of APP expression in the liver (Jensen and Whitehead, 1998), the delayed SAA response may be due to the kinetics of the response and the lag time associated with cytokine stimulation. The increased liver SAA expression seen in the control fasted fish at 96 h post-injection supports previous studies showing that fasted fish show a heightened acute phase response (Martin et al., 2010). Although acute phase proteins are synthesized predominantly in the liver (Talbot et al., 2009), our results show SAA expression profile to be similar in the spleen of fed charr in response to LPS stimulation. This is not surprising since recent studies

have provided evidence of extrahepatic synthesis and cellular expression of APPs (Colten, 1992; Goetz et al., 2004; SaranyaRevathy et al., 2012).However, the faster SAA response to LPS stimulation in the spleen of fasted charr along with the cytokine response leads us to propose a paracrine and/or autocrine role for cytokines in APP response in this tissue. Although cytokines were upregulated by 8 h post-LPS injection in the fed group, the lack of a SAA response similar to that seen in the fasted fish suggests a potential interaction of nutritional status on APP response in charr.

3.5.2 SOCS modulation

While cytokines and APPs play an indispensable role in mediating immune and inflammatory responses in complex organisms, excessive cytokine signalling can be energy demanding and lead to chronic inflammation and disease (Shepherd et al., 2012). The SOCS genes act as key negative-regulators of cytokine signalling. Homologues of all the mammalian SOCS family members including SOCS-1, SOCS-2 and SOCS-3, have been identified in fishes (Wang et al., 2011), but a functional role has not been thoroughly described yet. Our results reveal for the first time that fasted Arctic charr increase the expression of SOCS genes in a tissue specific manner. The results confirm the recent finding that emaciation *per se* increases the expression of liver SOCS-2 and SOCS-3 in Arctic charr (Jørgensen et al., 2013). Also, fasting in this species increases the mRNA abundance of SOCS-1 and SOCS-2 in the spleen. This corresponds with the lower levels of cytokine expression that we observed in the fasted fish. As SOCS genes are involved in attenuating the inflammatory response to cytokines in animals, including fish (Martin et al., 2010; Zhu et al., 2012), we propose that during conditions of extended fasting, this might be a key adaptation to protect against energy demanding processes, including excessive cytokine signalling.

Our results indicate that SOCS expression in response to LPS stimulation is also tissue-specific. While LPS upregulated liver and spleen SOCS-3 transcript levels, it did not alter SOCS-1 and SOCS-2 mRNA levels in the liver. In the spleen of fed charr, it was seen that LPS increasd SOCS-1 expression over time while in the spleen of fasted charr, LPS decreased SOCS-2 expression over time suggesting a potential interaction of nutritional status on LPS-mediated SOCS responses in charr. Together, this supports previous studies

suggesting transient expression of SOCS genes in a species-, tissue- and isoform-specific manner in fishes in response to LPS stimulation (Philip et al., 2012; Shepherd et al., 2012). The reason for the temporal increase in liver SOCS-1 in the control fed group at 96 h post-injection or the temporal reduction in spleen SOCS-3 levels in the control fed and fasted groups at 96 h post-injection is not known. We cannot rule out the possibility that the stress of handling and injection may be modulating SOCS expression (Shepherd at al., 2012; Wang et al., 2010), but this warrants further investigation.

Although most SOCS proteins are induced by cytokines, they have also been shown to be induced by various other stimuli, such as pathogen associated molecular patterns (PAMPs), and bacterial, viral, and parasitic infection (Akhtar and Benveniste, 2011).We recently showed the SOCS genes to be responsive to cortisol, the major stress hormone in fishes, which highlights the role played by the SOCS genes in integrating different physiological responses (Philip et al., 2012). Additionally, it has also been shown that SOCS proteins interact with GH receptor (GHR) signalling (Wang et al., 2011). Fish growth is a complex function mostly regulated by the growth hormone (GH)/insulin-like growth factor (IGF) system (Gabillard et al., 2006). Over-expression of SOCS-2 has been shown to interfere with the JAK2–STAT5b pathway and inhibit growth hormone signalling (Croker et al., 2008). Moreover homozygote GH-transgenic zebrafish who express double the amount of GH compared with hemizygote individuals, also express higher levels of SOCS-1 and SOCS-3 and display slower growth rates (Studzinski et al., 2009). The increased SOCS expression that we observed with fasting, especially SOCS-2 which was increased both in the liver and spleen, correlates well with the reduced body weight of the fasted charr and the reduced hepatic IGF-1 levels, marker of GH signalling, suggesting inhibition of GH signalling (Jørgensen et al., 2013). Consequently, the heightened expression of SOCS genes with extended fasting in Arctic charr may be an adaptative strategy restricting energy demanding processes, including growth and immune responses, to offset the reduced substrate availability during overwintering.

In summary, extended fasting and emaciation did not deter the capacity to elicit an innate immune response to LPS challenge in the anadromous Arctic charr. This supports our

previous finding that resistence toward furunculosis was not compromised by extended fasting in Arctic charr (Maule et al., 2005). This is a unique adaptation as even short-term fasting in animals, including mammals and fishes, reduced the capacity for immune response activation (Wing et al., 1988; Pires et al., 2007; Caruso et al., 2011; Martin et al., 2010). We propose that maintenance of immune competence in Arctic charr despite extended fasting as an adaptive trait that arose along with their anadromous life-strategy, preparing the emaciated animals to defend against pathogenic challenges when they migrate from fresh water to seawater. A key finding was that extended fasting upregulated SOCS transcript levels in Arctic charr. As negative regulators of cytokine and growth hormone signalling, the upregulation of SOCS by fasting may be a key strategy during overwintering to conserve or limit energy substrate utilization by suppressing energy demanding pathways, including growth hormone and cytokine signalling. While the factor(s) involved in SOCS upregulation with fasting is unclear and warrants further investigation, we propose a key role for the SOCS in the energy substrate repartitoning to cope with the extended fasting in Arctic charr.

3.6 Acknowledgements

This study was supported by the Tromsø Research Foundation, University of Tromsø and the Natural Sciences and Engineering Research Council of Canada Discovery Grant.

Chapter 4

Extended fasting differentially regulates SOCS but not cytokine responses to lipopolysaccharide stimulation in rainbow trout

4.1 Overview

Nutritional restriction alters the energy budget and results in metabolic suppression in fishes. We tested the hypothesis that fasting will evoke a stress response in rainbow trout and modify their immune response to lipopolysaccharide (LPS) challenge. Rainbow trout were either fed or fasted for 118 d and challenged with LPS, and the plasma stress response and molecular immune response in the liver assessed at 8, 72 and 98 h post-injection. Fasting did not alter plasma cortisol levels, but LPS injection in the fasted trout resulted in higher plasma cortisol levels compared to the fasted and saline injected trout. Fasting and LPS injection reduced plasma glucose and lactate levels in trout. Fasting also suppressed liver glycogen content. Moreover, fasting and LPS injection modified suppressors of cytokine signalling (SOCS) mRNA abundances in trout. Fasting downregulated SOCS-1, upregulated SOCS-2 and had no effect on SOCS-3 mRNA abundance. LPS downregulated SOCS-2 but had no effect on SOCS-1 and SOCS-3 expression. Fasted trout showed the same level of cytokine and acute phase protein responses to LPS stimulation as fed trout. Collectively, extended fasting differentially modulates SOCS expression in rainbow trout and does not reduce their ability to evoke an immune response to LPS stimulation.

4.2 Introduction

Prolonged fasting in fishes result in metabolic suppression and is associated with reduced plasma glucose, liver glycogen and liver metabolic capacity (Foster and Moon, 1991; Navarro and Gutiérrez, 1995). The liver plays an important role in intermediary metabolism and is a key energy substrate depot for lipids (triacylglycerol) and glycogen. We recently demonstrated the ability of this tissue to express immune response mediators in response to endotoxin shock, suggesting a role for this tissue in the inflammatory response (Philip et al., 2012). Even though conditions of fasting are well tolerated by most fish species, dietary restriction may be deleterious with respect to immune function (Martin et al., 2010).

Fish innate immune responses involve the synthesis of pro-inflammatory cytokines, including IL-1β and IL-8, and acute phase proteins (APP) like serum amyloid protein A

(SAA) (Uribe et al., 2011). Pro-inflammatory cytokines are expressed in salmonids during the early phases of an infection, while APPs are synthesized in the liver in response to pro-inflammatory cytokines to aid in pathogen elimination (Saranya et al., 2012). Mounting an immune response requires energy for protein synthesis, leading to increase in metabolic activity (Martin et al., 2010). Dietary restrictions lead to reduced growth and heightened stress response which in turn impact the effectiveness of a robust immune response (Liu et al., 2013; Martin et al., 2010). In teleosts, the impact of these changes has been shown to differ depending on duration of feed restriction, pathogen dynamics as well as species-specific evolutionary adaptations to seasonal changes in food availability (Liu et al., 2013).

Feed deprivation for a week leads to decreased mortality rates to *Edwardsiella ictaluri* and higher mortality to *Flavobacterium columnare* in channel catfish (Shoemaker et al., 2003; Wise et al., 2008). Starvation for 28 days leads to a reduction in mRNA abundance of immune-related genes in the liver of Atlantic salmon (*Salmo salar*) while starvation for 31 days decreases non-specific immune parameters, including haemagglutinating activity and respiratory burst activity in the sea bass (*Dicentrarchus labrax*) and blackspot sea bream (*Pagellus bogaraveo*) (Martin et al., 2010; Caruso et al., 2011). Fasting in anadromous Arctic charr (*Salvelinus alpinus*), a salmonid who resorts to long term fasting as a life history strategy, leads to a reduction in cytokine response to LPS, which is likely an adaptation to conserve energy resources (Philip et al., 2014). The cortisol or stress response to food deprivation has been equivocal and this may be due to various factors, including species differences, duration of fasting and the life history of the animal (Pottinger et al., 2003; Vijayan et al., 2010). Though it is clear that nutritional perturbations can modulate immune responses in teleosts, the molecular mechanisms for this are largely unknown.

The suppressors of cytokine signalling (SOCS) are key modulators of the immune response and are affected by fasting (Philip et al., 2014). Even though they traditionally function as negative regulators of cytokine signalling, they are also known to regulate diverse physiological functions, including growth and development in mammals (Croker et al., 2008; Kile and Alexander, 2001). Homologues of all the eight mammalian SOCS family members have been discovered, with many of them having multiple copies in fish (Wang et al., 2011).

SOCS-1-3 have been characterized in salmonids (Wang and Secombes, 2008), but their functional roles are just beginning to emerge. It has been shown that SOCS affect cytokine and growth hormone (GH) signalling by acting as negative regulators of the JAK/STAT pathway (Croker et al., 2008). Stressed levels of cortisol upregulate SOCS-1 and SOCS-2 expression in rainbow trout liver, suggesting a role for the SOCS in regulating stress-immune interactions in fish (Philip et al., 2012). An adaptive role for the SOCS during natural fasting in Anadromous arctic charr was also recently shown by us (Philip et al., 2014). We proposed that the upregulation of SOCS expression with fasting may be a key adaptive strategy to conserve energy reserves by curtailing energy demanding pathways like growth hormone and cytokine signalling (Philip et al., 2014). While the Arctic charr resorts to voluntary long-term fasting as a life-strategy, other salomonids, including rainbow trout do not exhibit natural fasting as a life-history strategy. It remains to be seen if fasting has similar effects on SOCS expression and cytokine responses to LPS in the rainbow trout.

Here we examine further the broader molecular effects of long term food deprivation on the stress response, SOCS expression and the molecular response to LPS challenge in rainbow trout. We tested the hypothesis that fasting will evoke a stress response in rainbow trout and modify their SOCS expression and innate immune response to LPS challenge. Rainbow trout were fed or fasted for 118 d and injected with LPS, a well-established immunostimulant (Engelsma, 2002; MacKenzie et al., 2006) and sampled at 8, 72 and 98 h post-injection. We examined plasma cortisol, glucose and lactate levels and liver glycogen content and expressions of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) protein as markers of the stress response. To assess inflammatory response, we measured the expression of pro-inflammatory cytokines, IL-1β and IL-8 as well as the three isoforms of SOCS, SOCS-1, SOCS-2 and SOCS-3, while SAA was used as an indicator of the acute phase response.

4.3 Materials and Methods

4.3.1 Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*; 55± 10 g body mass) were obtained from Washington Hatchery Klickitat River, WA, USA and maintained at the Columbia River Research Laboratory, USGS, WA, USA, at 6-7^{o}C on a 12:12-h light/dark cycle. Fish were fed daily to satiety with commercial dry pellet till the start of the experiment. Fish were acclimated for 2 weeks prior to use in the following experiments.

4.3.2 Experimental design

On 10th January 2012, rainbow trout were weighed, fork length measured and tagged and distributed randomly into 4 tanks (n=20 per tank).Rainbow trout in two of the tanks were fed as stated before, while the rainbow trout in the other two tanks were fasted till 7th May 2012 (118 days). On 7th May, the fish were reweighed and fork length measured again. Body mass (BM) and fork length (FL) were used to calculate the condition factor (CF; BM/FL^3 x 100). Specific growth rate (SGR) was calculated by the formula [(ln BM_T – ln BM_t/T-t) x 100] where BM_T and BM_t are the weights of the fish in May and January, respectively, and T-t the number of days between weight measurements. On the same day, half the fish in each tank were injected with LPS [(*Escherichia coli*, 0111:B4; Sigma; 2.5mg/Kg wt in saline) and the other half with the saline vehicle and redistributed into 4 tanks (fed and LPS injected, n=20; fed and saline injected, n=20; fasted and LPS injected, n=20; fasted and saline injected, n=20).The fish were then sampled (n=6-8) at 8, 72 and 98 h post-injection. Fish were euthanized with an overdose of neutralized MS222 and blood samples were immediately centrifuged at 5000x g for 5 min. Plasma was separated and stored at -80 °C to measure cortisol, glucose and lactate levels later. Liver tissues were stored at -80 °C for glycogen, transcript and protein expression analyses later.

4.3.3 Plasma cortisol, glucose, lactate levels and liver glycogen content

Plasma cortisol levels were measured using a [3H]-labeled cortisol radioimmunoassay as described previously (Alsop et al., 2009). Plasma glucose levels were

measured by monitoring NAD reduction by the hexokinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) assay method in Tris buffer (120 mM Tris-base, 80 mM Tris-HCl, 5 mM NAD, 2 mM MgS04, 5 mM ATP). The reaction was started with G6PDH (0.4 U/ml) and HK (0.5 U/ml). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA). The same protocol was used to determine liver glycogen content by analysing liver glucose content before and after amyloglucosidase hydrolysis. Plasma lactate levels were measured by monitoring NAD reduction by Lactate dehydrogenase (LDH) assay method in Hydrazine buffer (0.2 M; pH 9.5). The reaction was started with LDH (10U/well). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA).

4.3.4 SDS-PAGE and immunodetection

Sample protein concentrations were measured with bicinchoninic acid (BCA) reagents using BSA as the standard. SDS–PAGE and immunodetection were carried out exactly as described before (Aluru and Vijayan, 2007). Briefly, sample protein (40 μg protein per lane) was separated on 10% polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes. The membranes were probed with either rabbit polyclonal anti-trout GR (1:1000; Sathiya and Vijayan, 2003) or rabbit polyclonal anti-trout MR (1: 1000; Jeffrey et al., 2012). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; BioRad, Hercules, CA, USA). Protein bands were detected with ECL plus (Amersham Biosciences, Piscataway, NJ, USA) and the molecular mass confirmed with precision plus molecular weight markers (BioRad). The bands were scanned with a Typhoon 9400 (Amersham) and the band intensity quantified by AlphaEase software (Alpha Innotech, CA, USA). Equal loading was confirmed by incubation of membranes with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, 1:1000) for 1 h at room temperature.

4.3.5 Quantitative real-time PCR (qPCR)

***RNA extraction and cDNA synthesis*:** Total RNA was extracted from liver tissue using the RNeasyextraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The concentration of total RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Napean, ON, CAN). First-strand cDNA was synthesized from 1μg of total RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

***Quantification*:** Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The genes of interest were IL-1β, IL-8, SAA, SOCS-1, SOCS-2 and SOCS-3, while elongation factor 1 alpha (EF1α) was the housekeeping gene. Primer pairs (Table 1) for these genes were designed from rainbow trout cDNA sequences using Primer 3 version 0.4.0. software. Threshold cycle values for each sample were calculated using iCycleriQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 2.5 μL cDNA, 2.5 μL of primer pair (10 mM each of the respective forward and reverse primers), 40 μL of SYBR green mix and 35 μL of nuclease-free water was prepared for each sample, after which 25 μL was added to each of three wells. Each sample was assayed for the genes of interest and the housekeeping gene. The following PCR conditions were used for amplification: 95 °C for 3 min; 40 cycles of 95 °C for 20 s and annealing temperature (see Table 1) for 20 s; 95 °C for 1 min; 55 °C for 1 min followed by melt curve analysis to confirm the presence of a single amplicon, starting at 55 °C and increasing in 0.5 °C increments to 95 °C every 10 s. Copy number of transcripts for each gene was determined with the threshold cycles (CT) using plasmid standard curves exactly as described before (Aluru et. al., 2010). EF1α was used as the housekeeping gene for normalization as this transcript level remained unchanged between treatment groups.

Table 1: Gene-specific primers for quantitative real-time PCR.

The table provides the list of genes (Gene ID), forward and reverse primer sequences, annealing temperature and amplicon size. IL-1β: interleukin-1 beta; IL-8: interleukin-8; SAA: serum amyloid protein A; SOCS: suppressors of cytokine signalling; EF1α: elongation factor 1α.

Gene ID	Primer sequences (5'-3')	Temp (^{o}C)	Amplicon size (bp)
IL-1β	Fwd: GGAGAGGTTAAAGGGTGGCGA Rev: TGCCGACTCCAACTCCAACA	60	121
IL-8	Fwd: CACTGAGATCATTGCCACTCTGA Rev: ATGACCCTCTTGACCCACGG	60	81
SAA	Fwd: TTGTTCTGACCCTCGTTGTAGGAGC Rev: CATGTCGCCATATGCACGCC	60	101
SOCS-1	Fwd: GATTAATACCGCTGGGATTCTGTG Rev: CTCTCCCATCGCTACACAGTTCC	63.3	136
SOCS-2	Fwd: TCGGATGACTTTTGGCCTAC Rev: CCGTTCTTCTCTCGTTTTCG	60	102
SOCS-3	Fwd: TAGCCCTGAGCCTGGAAGTA Rev: GGTTGCTAGGCAGTTTCCTG	60	113
EF1α	Fwd: CATTGACAAGAGAACCATTGA Rev: CCTTCAGCTTGTCCAGCAC	56	95

4.3.6 Statistical analysis

Data are shown as mean ± standard error of mean (SEM). Outliers, values that were two standard deviations above or below the mean, were omitted from the analyses. Two-way ANOVA followed by Holm Sidak's post-hoc was used to compare the effect of different treatments on stress, metabolic and immune parameters. Student *t*-test was used to compare the effect of fasting on BM, CF and SGR. The data were log transformed, wherever necessary, for homogeneity of variance, but non-transformed values are shown in the figures. A probability level of $p<0.05$ was considered significant. Statistical analyses were performed with SigmaStat (Systat Software Inc., San Jose, CA, USA).

4.4 Results

4.4.1 Body mass, condition factor and specific growth rate

Long-term fasting significantly reduced body mass, condition factor and specific growth rate in rainbow trout. The mean body mass of fasted trout was reduced by ~68%, this was also reflected in significant differences in SGR (Table 2). The condition factor was also reduced in the fasted trout by ~26% compared to the fed trout (Table 2).

Table 2: Biometrical measurements.

The table provides the body mass (BM), condition factor (CF) and specific growth rate (SGR) of the fed and fasted rainbow trout used in this study. Different letters denote significant difference. All values represent means ± SEM (n denotes the number of fish) (t test; $P<0.05$).

Biometrical measurements	**FED** (n=16)	**FASTED** (n=16)
BM (g)	145 ± 21^{a}	46 ± 7.0^{b}
CF	1.32 ± 0.08^{a}	0.98 ± 0.07^{b}
SGR	0.82 ± 0.09^{a}	-0.18 ± 0.02^{b}

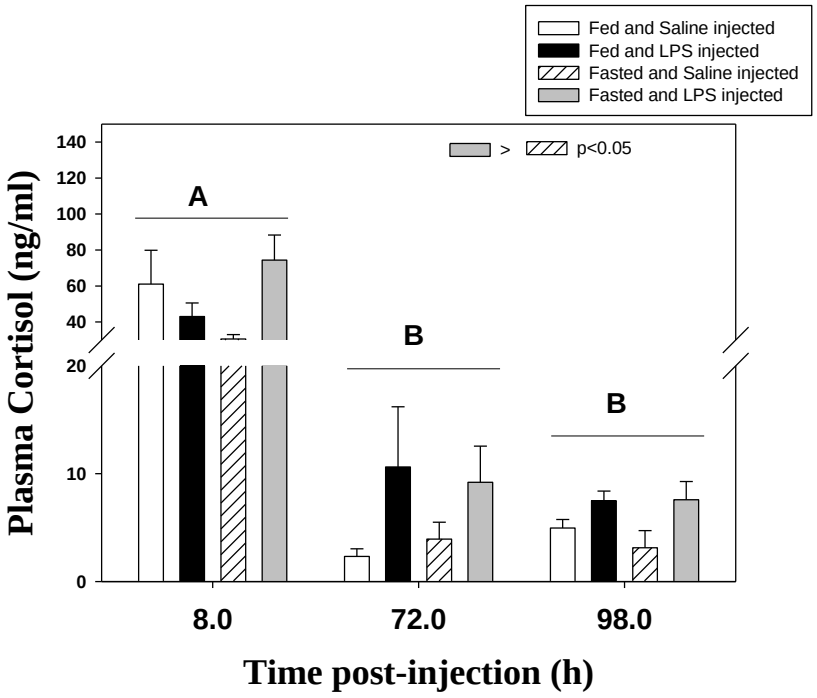
4.4.2 Plasma analysis and liver glycogen content

All the four treatment groups showed elevated cortisol levels (stress levels of cortisol) at 8 h post injection, which returned to non-stress levels ($\leq$ 10ng/ml) by 72 and 98 h post-injection (Fig. 1A). Fasting did not modify plasma cortisol levels. LPS injection in fasted fish resulted in higher cortisol levels compared to fasted and saline injected trout. Similarly, all the four treatment groups showed elevated plasma glucose levels at 8 h post-injection when compared to 72 and 98 h post-injection. Fasting and LPS injection reduced overall plasma glucose levels compared to the fed and saline injected control trout (Fig. 1B). Fasting reduced plasma lactate levels in trout (Fig. 1C). LPS injection reduced plasma lactate levels at 98 h post-injection compared to other timepoints. While lactate levels in fed and saline injected trout were higher at 98 h compared to 8 h post-injection, lactate levels in fasted and LPS injected trout were higher at 8 h and declined by 72 and 98 h post-injection. Fasting reduced liver glycogen content in trout (Fig. 2). Liver glycogen content was also higher in all the treatment groups at 8 h post-injection compared to 72 and 98 h post-injection.

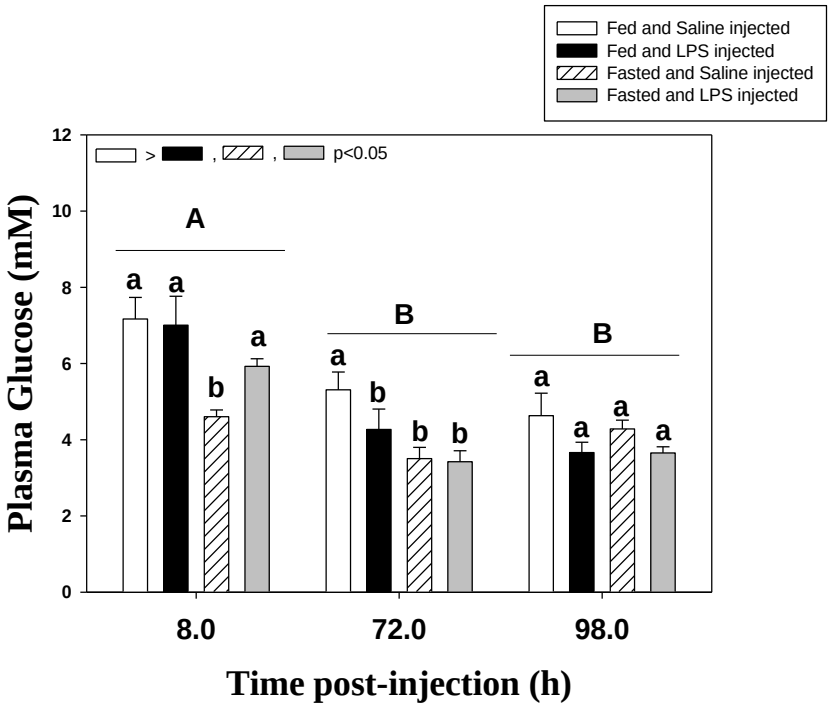
Figure 1: Effect of nutritional status (Fed vs Fasted) and LPS injection on plasma cortisol (A), plasma glucose (B) and plasma lactate (C) levels in rainbow trout.

Data represents mean ± S.E.M (n = 6-8). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each timepoint and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).

A


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
> p<0.05
Plasma Cortisol (ng/ml)
140
120
100
80
60
40
20
10
0
A
B
B
8.0
72.0
98.0
Time post-injection (h)

B


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
> , , p<0.05
Plasma Glucose (mM)
12
10
8
6
4
2
0
A
B
B
a a b a
a b b b
a a a a
8.0
72.0
98.0
Time post-injection (h)

C

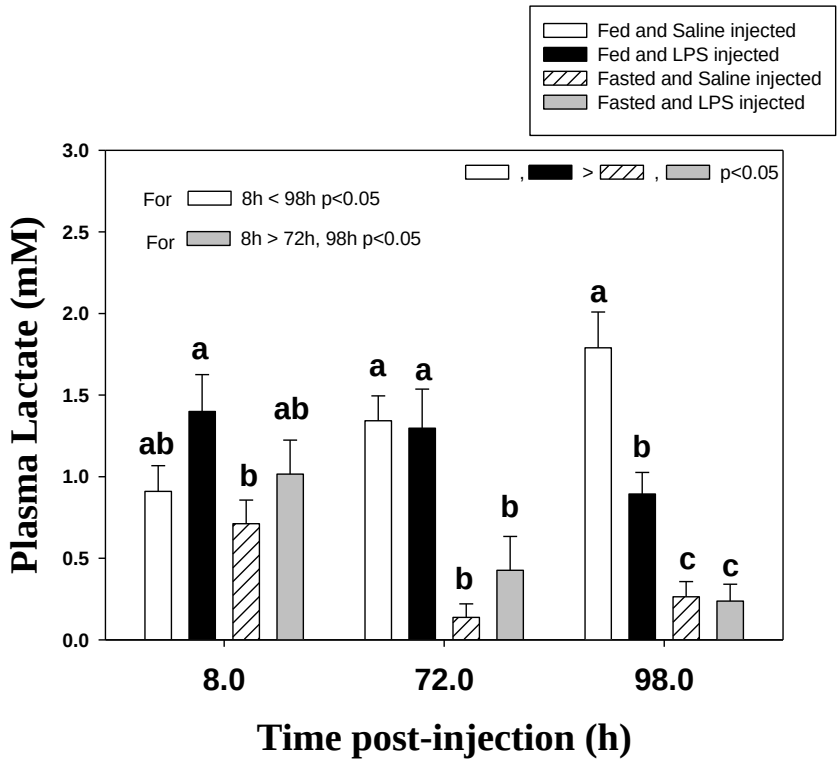
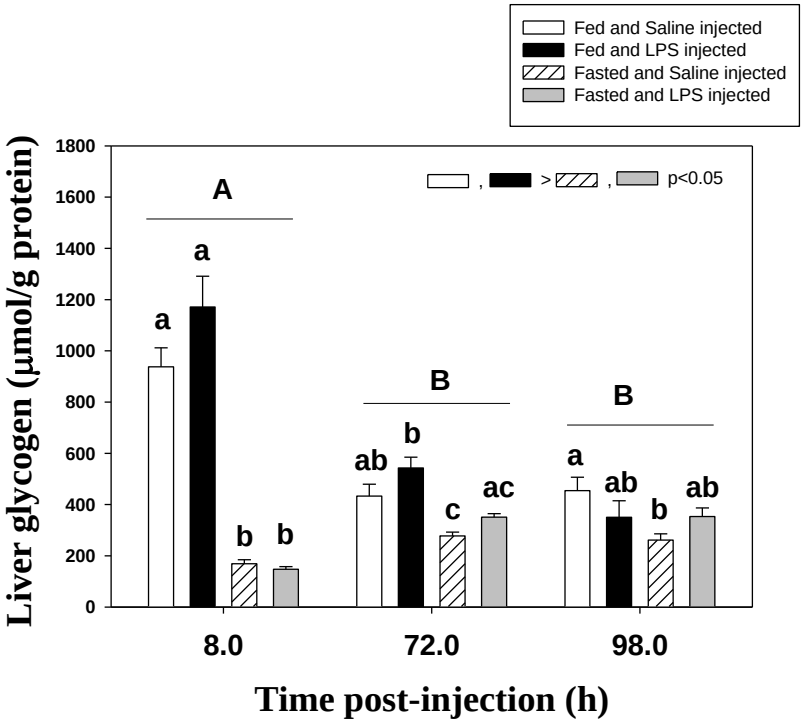

Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
, > , p<0.05
For 8h < 98h p<0.05
For 8h > 72h, 98h p<0.05
Plasma Lactate (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ab
a
b
ab
a
a
b
b
a
b
c
c
8.0
72.0
98.0
Time post-injection (h)

Figure 2: Effect of nutritional status (Fed vs Fasted) and LPS injection on liver glycogen content in rainbow trout.

Data represents mean ± S.E.M (n = 6-8). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each timepoint and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).



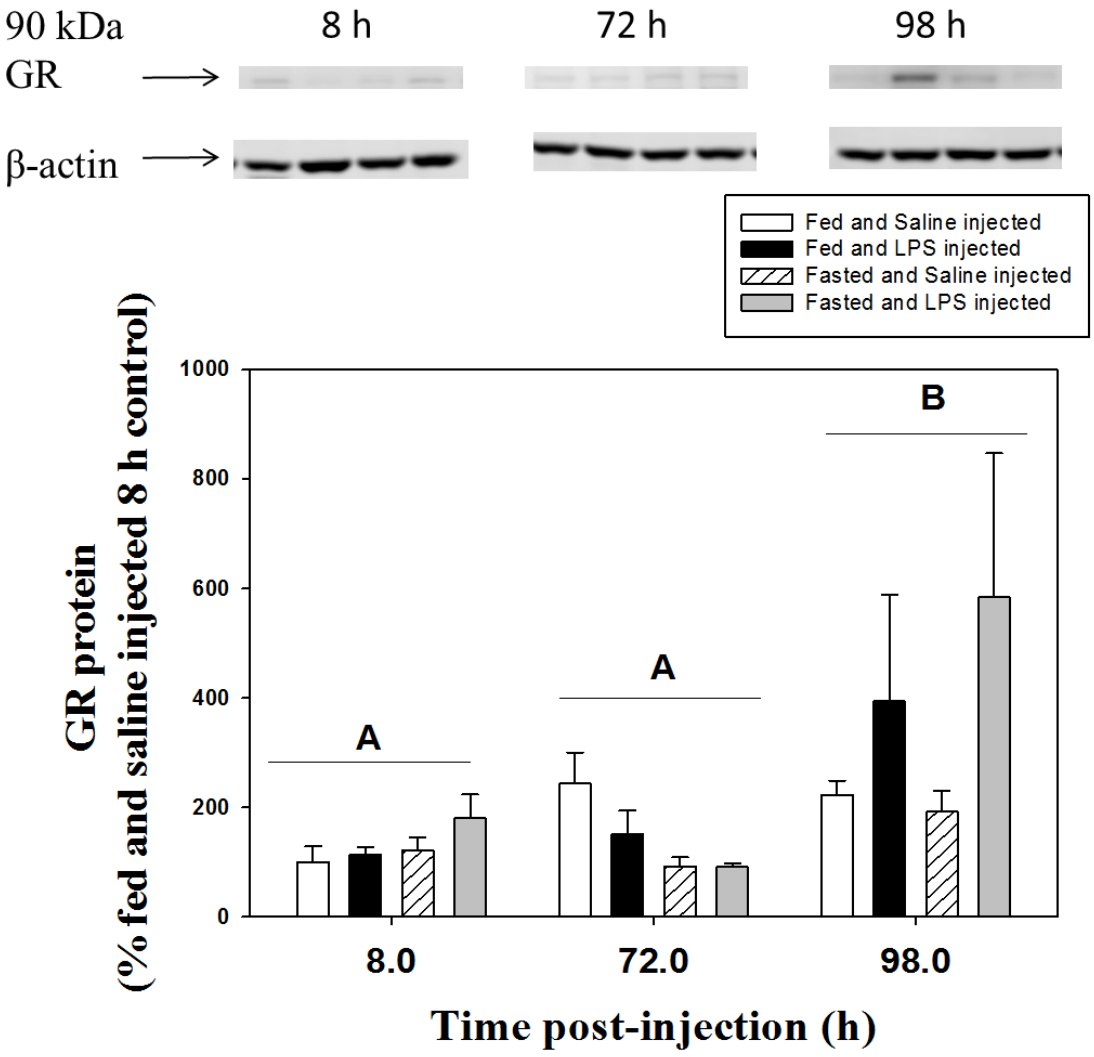
4.4.3 Glucocorticoid Receptor and Mineralocorticoid Receptor Protein Expression

Neither fasting nor LPS injection had any effect on GR (Fig. 3A) or MR (Fig. 3B) protein expression at any of the time points. Overall, GR expression was higher at 98 h compared to 8 and 72 h post-injection, while MR expression was higher at 72 and 98 h compared to 8 h post-injection.

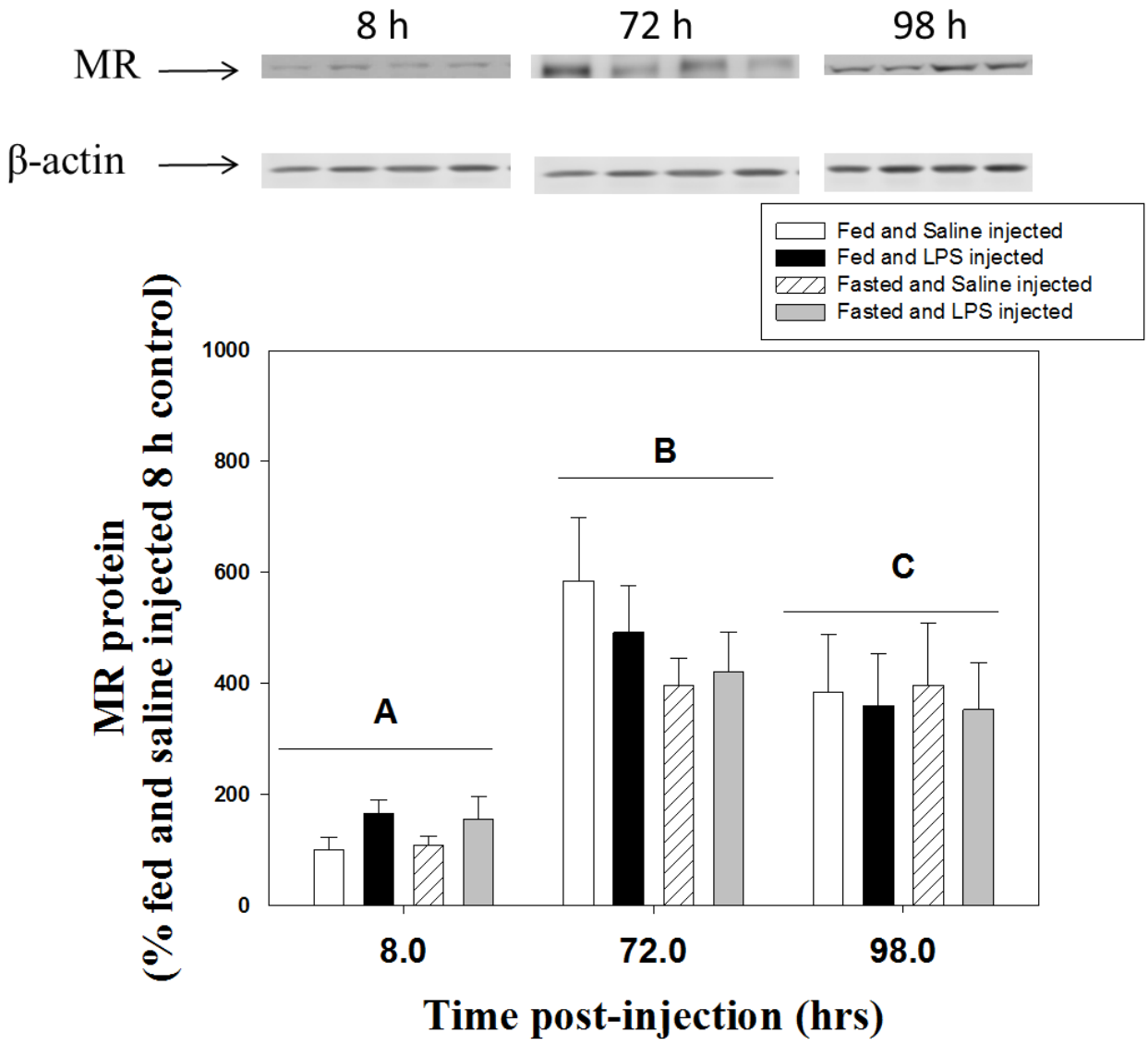
Figure 3: Effect of nutritional status (Fed vs Fasted) and LPS injection on GR and MR protein levels in rainbow trout liver

Densitometric values for GR (A) and MR (B) are plotted as % fed and saline injected 8 h control and show mean ± S.E.M (n = 5-6 independent fish). Different upper case letters indicate significant time effects (two way ANOVA, $p < 0.05$). Protein loading was corrected by normalizing to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Representative blots are shown above the histogram.

A



B


8 h
72 h
98 h
MR
β-actin
Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
MR protein
(% fed and saline injected 8 h control)
1000
800
600
400
200
0
A
B
C
8.0
72.0
98.0
Time post-injection (hrs)

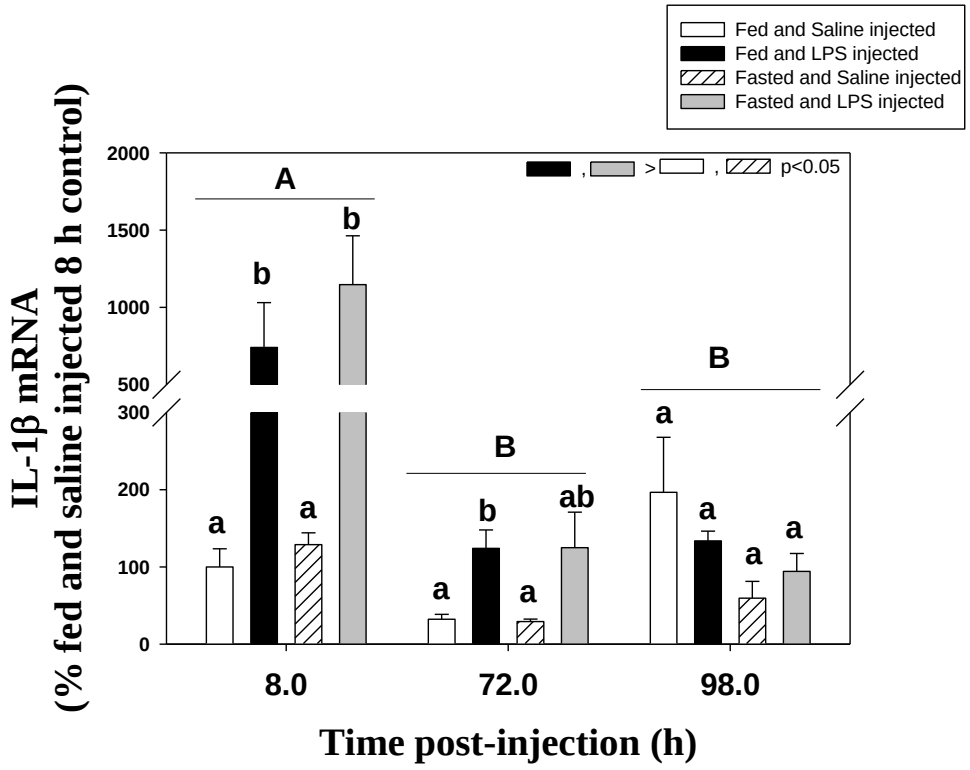
4.4.4 Cytokine and acute phase protein transcript abundance

LPS injection increased IL-1β (Fig. 4A) and IL-8 (Fig. 4B) transcript levels in both fed and fasted trout. Fasting did not reduce or increase the cytokine response to LPS. Cytokine levels were higher at 8 h compared to 72 and 98 h post-injection in both the fed and fasted fish. While fed and LPS injected trout still showed elevated IL-1β and IL-8 levels compared to the controls at 72 h post-injection, both cytokines reached control levels in the fasted and LPS injected trout by 72 h. LPS injection increased SAA levels in both fed and fasted trout at 72 and 98 h post-injection (Fig. 4C). Fasting did not modulate the SAA response to LPS.

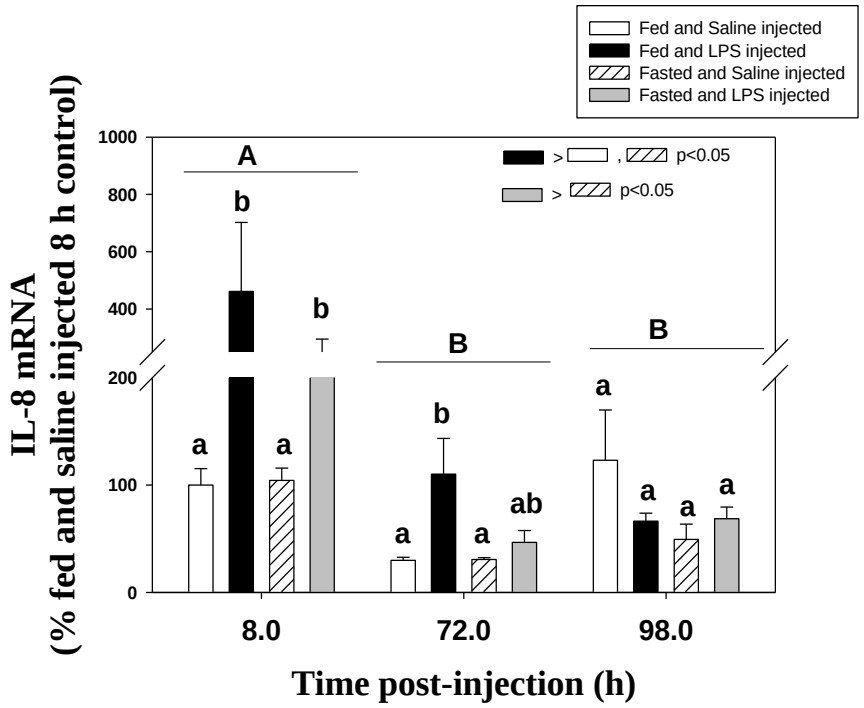
Figure 4: Effect of nutritional status (Fed vs Fasted) and LPS injection on IL-1β (A), IL-8 (B) and SAA (C) transcript levels in rainbow trout liver.

Data are plotted as % fed and saline injected 8 h control and show mean ± S.E.M (n = 5-6 independent fish). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each timepoint and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).

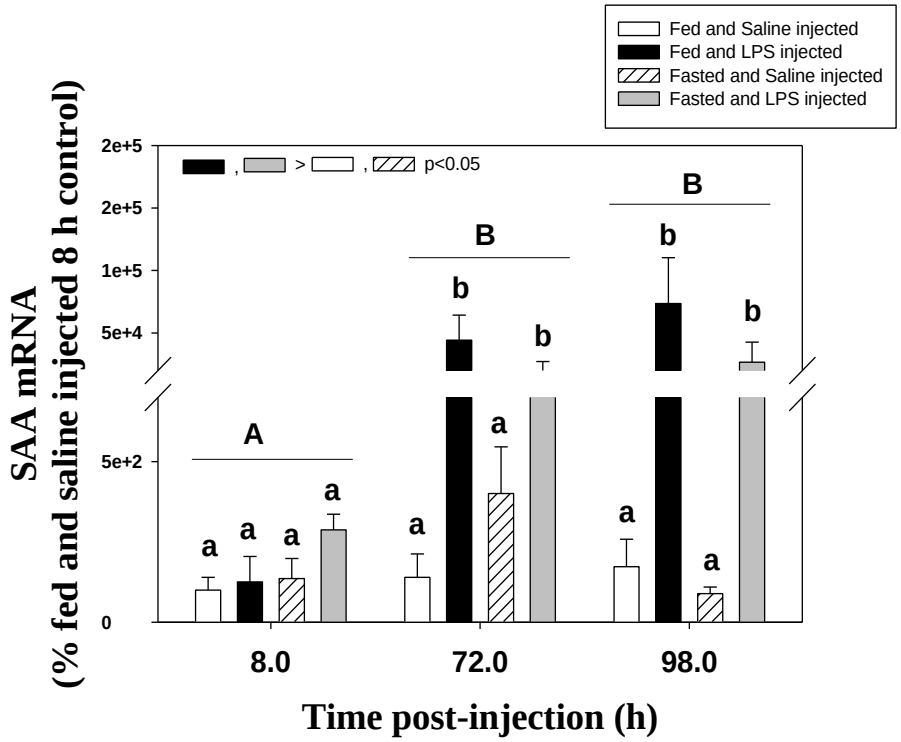
A



B


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
IL-8 mRNA
(% fed and saline injected 8 h control)
1000
800
600
400
200
100
0
A
B
B
> , p<0.05
> p<0.05
a
b
a
b
a
b
a
ab
a
a
a
a
8.0
72.0
98.0
Time post-injection (h)

C


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SAA mRNA
(% fed and saline injected 8 h control)
2e+5
2e+5
1e+5
5e+4
5e+2
0
, > , p<0.05
A
B
B
a
a
a
a
a
b
a
b
a
b
b
8.0
72.0
98.0
Time post-injection (h)

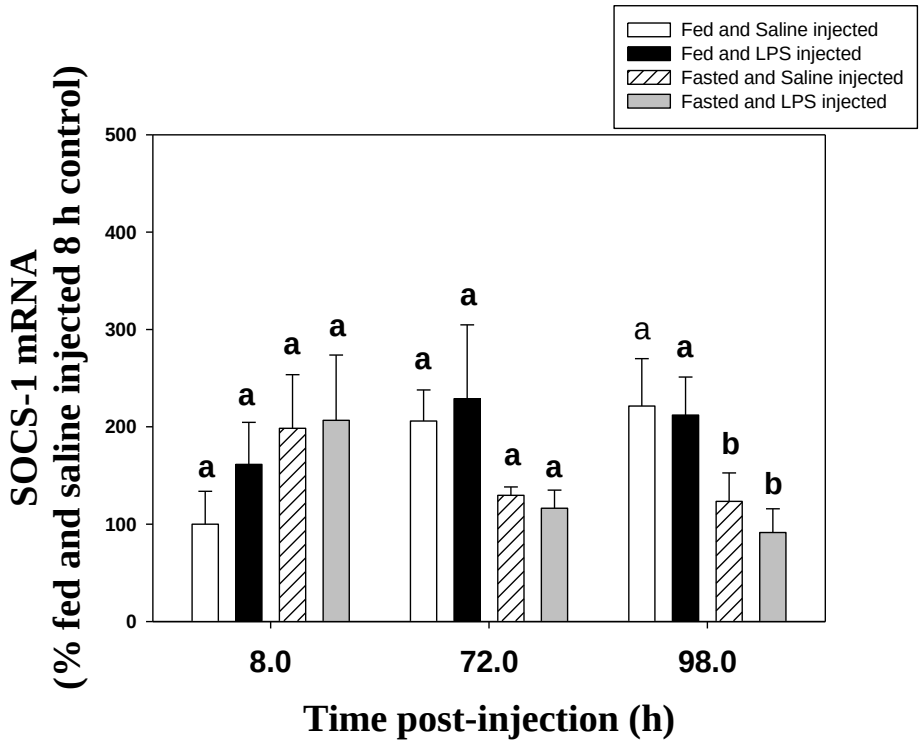
4.4.5 SOCS transcript abundance

Fasting reduced SOCS-1 expression at 98 h post-injection compared to fed trout (Fig. 5A). However, fasted trout showed overall higher SOCS-2 expression compared to fed trout (Fig. 5B). Moreover, while LPS injection reduced SOCS-2 expression in fed trout at 72 and 98 h post-injection, LPS injection reduced SOCS-2 expression in fasted trout at 8, 72 and 98 h post-injection. Overall, SOCS-2 and SOCS-3 expression were lower at 72 h compared to 8 h post-injection. Neither fasting nor LPS had any effect on SOCS-3 expression (Fig. 5C).

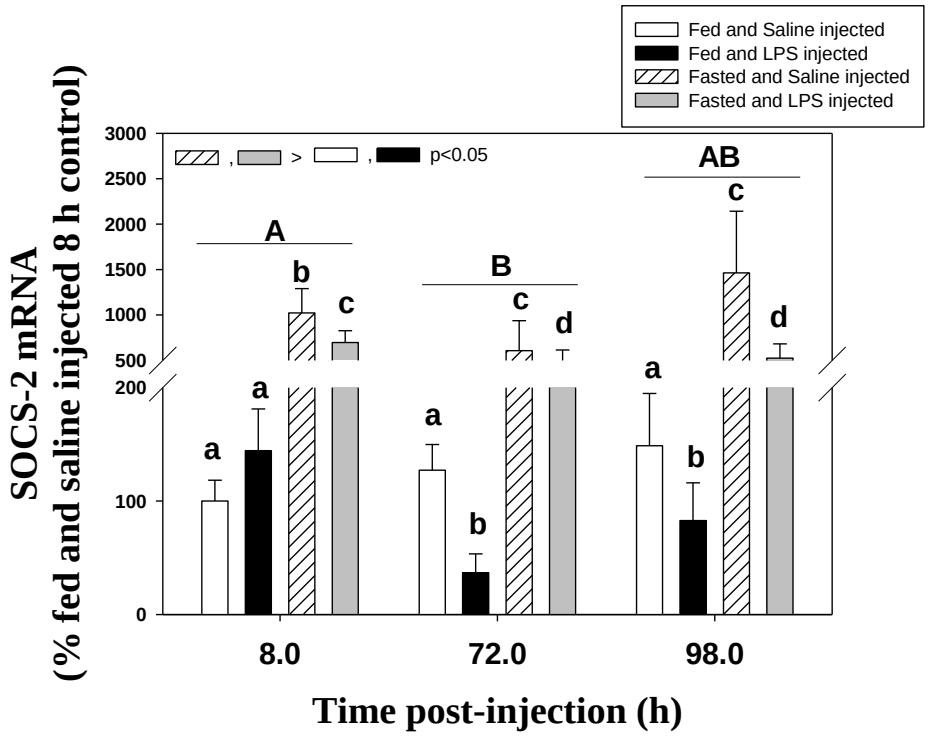
Figure 5: Effect of nutritional status (Fed vs Fasted) and LPS injection on SOCS-1 (A) SOCS-2 (B) and SOCS-3 (C) transcript levels in rainbow trout liver.

Data are plotted as % fed and saline injected 8 h control and show mean ± S.E.M (n = 5-6 independent fish). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each timepoint and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).

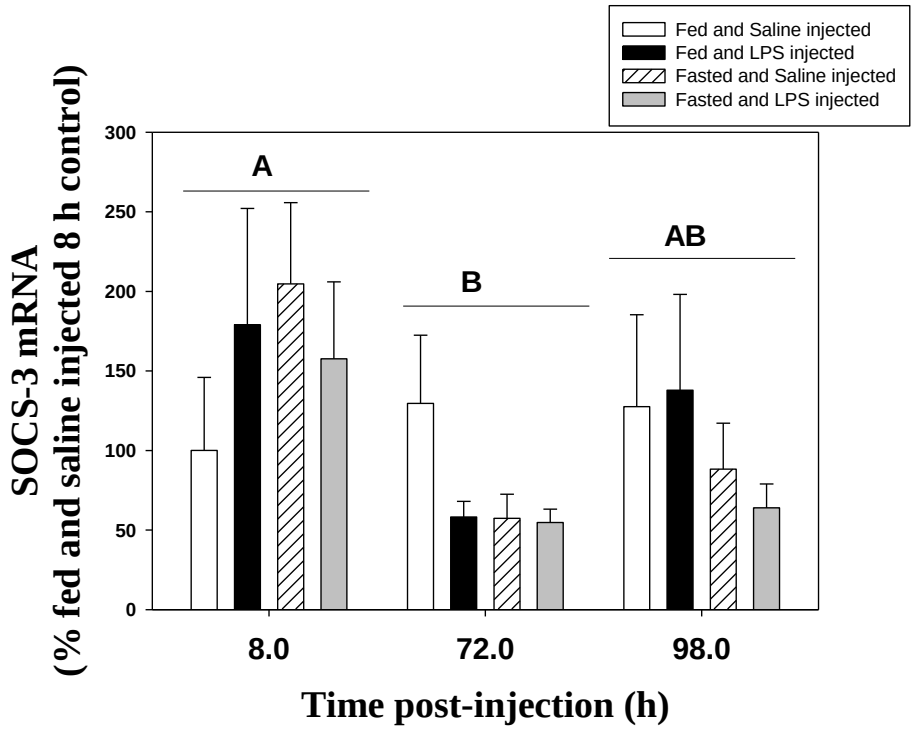
A


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SOCS-1 mRNA
(% fed and saline injected 8 h control)
500
400
300
200
100
0
a
a
a
a
a
a
a
a
a
a
a
b
b
8.0
72.0
98.0
Time post-injection (h)

B


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SOCS-2 mRNA
(% fed and saline injected 8 h control)
3000
2500
2000
1500
1000
500
200
100
0
, > , p<0.05
A
B
AB
a
a
b
c
a
b
c
d
a
b
c
d
8.0
72.0
98.0
Time post-injection (h)

C


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SOCS-3 mRNA
(% fed and saline injected 8 h control)
300
250
200
150
100
50
0
A
B
AB
8.0
72.0
98.0
Time post-injection (h)

4.5 Discussion

We demonstrate for the first time that long term fasting in rainbow trout does not compromise their ability to evoke an immune response to LPS stimulation. Fasted rainbow trout showed the same initial degree of cytokine response to LPS as the fed trout, which might be due to the fact that there is no overriding effect of fasting or LPS to increase SOCS expression, a potent negative regulator of cytokine responses. However the ability of fasted trout to sustain and prolong a cytokine response seems to be compromised which might be due to the reduced glucose and glycogen levels observed in these fish, leading to a quicker depletion of energy stores, essential to mounting a successful immune response.

4.5.1 Effects on stress indicators

Fishes can tolerate conditions of feed restriction better than mammals (Martin et al., 2010). While plasma corticosteroid levels (role involves mobilizing energy substrates in order to cope with the increased energy demand associated with stress) are consistently elevated in response to fasting or malnutrition in homeotherms, including rodents and humans (Pottinger et al., 2003), response in fishes are equivocal. Studies in rainbow trout have reported levels of plasma cortisol to be unaffected, reduced or increased by fasting (Sumpter et al., 1991; Farbridge and Leatherland, 1992; Blom et al., 2000; Vijayan and Moon, 1992). In the present study, fasting did not appear to have any effect on plasma cortisol levels supporting previous observations in trout (Pottinger et al., 2003). This lack of response may be adaptive with extended fasting to spare proteins, as elevated cortisol levels will lead to enhanced muscle proteolysis (Mommsen et al., 1999). The lack of an increase in cortisol levels with LPS injection in the fed fish is in contrast to what is reported previously in rainbow trout, where LPS injection increased cortisol levels to support the general increase in energy demand during inflammation (Holland et al., 2002). In the present study, all the fish groups, including the fed controls (saline injected) had elevated plasma cortisol levels at 8 h post-LPS injection and this is due to the stress of handling and injection. This is further supported by the observation that plasma cortisol levels dropped to unstressed levels (reported previously for trout; Barton et al., 2002) by 72 and 98 h post-LPS injection. This suggests that the injection procedure itself was eliciting a stress response, which may have

masked the effects of fasting and/or LPS on plasma cortisol levels. However, LPS injection elicited a high cortisol response in the fasted fish compared to saline control suggesting that nutritional status modulates LPS-mediated plasma cortisol levels in trout.

In addition to cortisol, plasma glucose concentration is another widely used index of stress in fish (Pottinger and Carrick, 1999). All the fish groups, including the controls (fed and saline injected) showed elevated plasma glucose levels at 8 h post-injection compared to 72 and 98 h, which is likely due to cortisol-mediated gluconeogenesis (Mommsen et al., 1999). The higher glycogen levels observed at 8 h along with stress-levels of cortisol support a key role for gluconeogenesis in maintaining elevated plasma glucose levels associated with the injection and handling stressor. The drop in liver glycogen and plasma glucose at 72 and 98 h post-injection compared to 8 h suggests utilization of this substrate for endogenous use by the liver. This is all the more evident in the fed group as the liver glycogen content was significantly higher than the fasted fish. The LPS-injected fed fish showed reduced overall plasma glucose levels similar to that seen in fasted fish, despite the lack of changes in liver glycogen content, pointing to increased glucose utilization, but this remains to be tested. A similar LPS-induced hypoglycemia was also observed in mice and humans resulting from increased glucose utilisation to cover energy costs associated with inflammation (Raetzsch et. al., 2009). Plasma lactate levels, which reflect the muscular activity of the animal, was also measured as an indicator of the organismal stress response (Wendelaar Bonga, 1997; Iwama et al., 2006). Overall lactate levels were lower in the fasted fish compared to the fed fish, suggesting limited muscle activity (Iwama et al., 2006). The lower lactate levels seen in the fed and LPS injected fish at 98 h post-injection compared to the fed controls may be related to lower muscular activity. Whether this is a direct effect of the endotoxin remains to be determined. Neither fasting nor LPS injection had any effect on GR and MR protein expression. Overall, GR and MR protein expressions were lower at 8 h compared to the other timepoints, which correlated well with the elevated cortisol levels observed at 8 h post-injection. This supports previous studies showing suppression in GR protein expression with elevated plasma cortisol levels in fish (Vijayan et al., 2003; Sathiyaa and Vijayan, 2003).

4.5.2 Innate immune modulation

A key finding from this study was that the LPS-mediated upregulation of cytokines, including IL-1β and IL-8 were observed in both the fed and fasted trout, with the fasted trout showing the same degree of cytokine response to LPS as the fed fish. Cytokines and acute phase proteins are critical to a successful innate immune response. While IL-1β is one of the early response pro-inflammatory cytokines that induces a cascade of effects leading to inflammation, IL-8 is a chemokine involved in controlling the movement of immune cells (Gioacchini et al., 2008; Walz et al., 1987). This result from this study contrasts the response observed in anadromous Arctic charr where IL-1β and IL-8 expression was lower in the fasted fish compared to the fed charr (Philip et al., 2014). Fasting in both these species result in a negative energy balance (Philip et al., 2014; BM, CF and SGR values in this study) and so the significance of this difference is not clear. It has been suggested that the Arctic charr undergo extended periods of fasting naturally and, therefore, may employ an overall metabolic suppression as an adaptation to conserve energy resources (Philip et al. 2014). Consequently, the high initial cytokine response in fasted rainbow trout despite their negative energy status, may affect their ability to prolong and sustain a successful immune response due to lack of sufficient energy fuels like glucose (Martin et al., 2010). Moreover, in both fed and fasted trout, cytokine expression in response to LPS peaked at 8 h post injection. In the fed and LPS injected trout, cytokine expression was still elevated at 72 h post-injection compared to the controls, while cytokine expression in the fasted and LPS injected fish reached control levels by 72 h, supporting our theory that the fasted trout may be unable to sustain a cytokine response to LPS.

Acute phase proteins (APP) are made in response to cytokine stimulation and are also critical to a successful immune response (Talbot et al., 2009) SAA is the major APP in all vertebrates including teleosts (Talbot et al., 2009). The induction of SAA in the liver and several extra-hepatic tissues has been reported in rainbow trout after the induction of the acute phase response either by natural infections or by challenge with pathogen-associated molecular patterns (PAMPs) including LPS (Talbot et al., 2009; Philip et al., 2012). Our results reveal that fasted rainbow trout also upregulate SAA transcript abundance in response

to LPS challenge. In the liver of both fed and fasted trout, SAA expression peaked at 72 and 98 h post-LPS injection with no significant change at 8 h post-injection. Given that cytokine expression occurred at 8 h after LPS injection compared to 72 and 98 h for SAA expression, and also because they are key stimulators of APP expression in the liver (Jensen and Whitehead, 1998), the delayed SAA response may be due to the kinetics of the reaction and the lag-time associated with cytokine stimulation (Philip et al., 2014).

4.5.3 SOCS modulation

Although SOCS were discovered as inhibitors of cytokine signalling, they are now known to act as negative regulators of not only cytokines, but also leptin and growth hormone signalling in mammals (Croker et al., 2008). In the present study, fasting and LPS stimulation did not have an overriding effect on upregulating SOCS expression in rainbow trout. Moreover, fasting and LPS lowered liver SOCS-1 and SOCS-2 expression, respectively. This is contrary to the SOCS responses observed in the anadromous Arctic charr, where fasting and LPS upregulated SOCS expression, possibly as a key strategy during overwintering to conserve or limit energy substrate utilization by suppressing energy demanding pathways, including growth hormone and cytokine signalling (Philip et al., 2014). Consequently, this study highlights fundamental differences in the SOCS responses to fasting and LPS challenge between two salmonids. We propose that these species-specific differences in SOCS modulation with fasting may have an evolutionary significance in adapting fish species like the Arctic charr to their unique life-style of voluntary long-term fasting (Philip et al., 2014). The fasting- and LPS-induced reduction in SOCS transcripts correlate with the fact that fasted trout injected with LPS showed the same initial degree of cytokine responses as their fed counterparts with no attempt to reduce cytokine responses in view of their negative energy state. However, fasted trout irrespective of saline or LPS injection showed higher SOCS-2 expression than fed trout injected with saline or LPS, similar to that observed in the Arctic charr (Philip et al., 2014). Over-expression of SOCS-2 has been shown to interfere with the JAK/STAT pathway and is thought to be the predominant SOCS isoform inhibiting growth hormone signalling (Croker et al., 2008). This heightened expression of SOCS-2 with fasting correlates well with the reduced body weight

and specific growth rate of the fasted trout observed in this study. Overall SOCS-2 and SOCS-3 levels were also higher at 8 h post-injection compared to 72 h. This might be an effect of the elevated cortisol levels observed at 8 h post injection, since the SOCS genes have been shown to be cortisol responsive and upregulated by stress levels of cortisol (Philip et al., 2012).

In summary, extended fasting and LPS stimulation altered plasma cortisol, glucose and lactate levels in rainbow trout. However, fasting did not deter the capacity to elicit an innate immune response to LPS challenge. At the same time the capacity to prolong or sustain a cytokine response to LPS may be compromised in the fasted trout. The findings from this study leads us to propose a fundamental species-specific differences in the cytokine and SOCS responses to fasting and LPS challenge between rainbow trout and the anadromous Arctic charr. The differences observed may partly be due to species-specific evolutionary adaptations to seasonal changes in food availability.

4.6 Acknowledgements

This study as supported by funding from the Natural Sciences and Engineering Research Council of Canada, Discovery Grant to MMV.

Chapter 5

Handling stressor affects liver SOCS mRNA levels in rainbow trout: modulation by long-term fast and lipopolysaccharide challenge

5.1 Overview

The objective of this study was to determine how nutritional status and LPS stimulation affect SOCS expression during acute stress in rainbow trout (*Oncorhynchus mykiss*). Fish were fed or fasted for 118 d, after which a subset of fish were injected with either lipopolysaccharide (LPS) or saline and allowed to recover. All fish were subjected to a handling disturbance 72 h after LPS injection. Handling stressor elevated plasma cortisol, glucose and lactate levels and these changes were modulated by long-term fast and prior LPS injection. Handling disturbance did not affect liver glycogen content and glucocorticoid receptor (GR) protein expression, but fasting and LPS injection did modulate their response to stressor exposure. Mineralocorticoid receptor (MR) protein expression in the liver was significantly reduced by handling disturbance and this was not altered by fasting or LPS injections. Our results demonstrate for the first time that a handling stressor will alter the liver mRNA abundances of SOCS-2 and SOCS-3 but not SOCS-1 in rainbow trout. Fasting has a significant effect on SOCS-1 (reduced) and SOCS-2 (increased) mRNA, while LPS significantly reduced SOCS-2 mRNA levels only in the fed and not fasted trout. There was no effect of fasting or LPS injection on SOCS-3 mRNA levels in rainbow trout. Our results suggest that stress acutely upregulates SOCS-2 mRNA levels in rainbow trout and this response is modulated by fasting and LPS injection in trout. We hypothesize that SOCS-2 regualtion may be playing a role in the metabolic adjustments essential to cope with a handling stressor in fish.

5.2 Introduction

Organisms have evolved a series of physiological responses, collectively referred to as the integrated stress response, to help them regain homeostasis when exposed to stressors (Barton, 2002). A key aspect of this integrated stress response is the mobilization and reallocation of energy substrates, including glucose to cope with the increased energy demand associated with stress (Mommsen et al., 1999). In teleosts, this metabolic adjustment to stress involves the activation of two neuroendocrine pathways, the hypothalamic-sympathetic-chromaffin cell (HSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis,

culminating in the release of catecholamines and corticosteroids, respectively (Wendelaar Bonga, 1997). Cortisol is the major corticosteroid in teleosts and a key player in metabolic adjustments to stress. It is synthesised in the interrenal tissues of the head kidney in response to adrenocorticotropic hormone (ACTH) activation of melanocortin 2 receptors (MC2R) on steroidogenic cells (Aluru and Vijayan, 2008). Key rate limiting steps in cortisol biosynthesis involve the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR; Stocco et al., 2005) and the conversion of the cholesterol to pregnenolone by the cytochrome P450 side chain cleavage (P450scc) enzyme (Payne and Hales, 2004). Cortisol action is mediated by corticosteroid receptors in target tissues, primarily the glucocorticoid receptor (GR; Vijayan et al., 2010). Stressor-induced elevation of plasma cortisol and its activation of GR are essential in maintaining elevated plasma glucose levels to fuel energy demanding pathways including protein synthesis (Mommsen et al., 1999; Aluru and Vijayan, 2007; Vijayan et al., 2010). This is mediated by cortisol stimulation of hepatic gluconeogenesis (Mommsen et al., 1999). Fishes also express a mineralocorticoid receptor (MR); however, the function (s) associated with this receptor activation remain unclear (Sturm et al., 2005).

Studies have shown that the functioning of the cortisol stress axis is affected by the nutritional status of fish, but there is a lot of variation in the responses observed. Plasma levels of cortisol have been shown to be unaffected, reduced or increased by fasting in fish species (Pottinger et al., 2003). Moreover, fasting also impacts stressor-induced hyperglycemia which in turn can affect overall stress performance, since glucose is a key energy substrate channeled to pathways needed to re-establish homeostasis (Barton et al, 1988; Mommsen et al., 1999). Studies have also shown the functioning of the cortisol stress axis to be modulated by immune stimulation. Exposure to immunostimulants, including lipopolysaccharide (LPS) leads to elevated cortisol levels in several fish species (Swain et al., 2008). Conversely, glucocorticoids are known to limit inflammatory cascades initiated by LPS (Zou et al. 2000; Engelsma et al. 2003; MacKenzie et al. 2006). Previous studies have also shown rapid elevation in heat shock protein (HSP) and GR expression in response to

LPS, both significant players in the adaptive response to stress (Stolte et al., 2009; Philip et al., 2012).

The suppressors of cytokine signalling (SOCS) proteins are a family of intracellular proteins that are centrally involved with vertebrate growth, development and immunity via their effects as negative feedback regulators of cytokine (and hormone) signalling (Kile and Alexander, 2001). We recently showed SOCS expression was upregulated by cortisol in trout, as well as by fasting in anadromous Arctic charr (Philip et al., 2012; Philip et al., 2014). Also, SOCS upregulation by cortisol exposure for 24 h downregulate growth hormone and immune signalling in fish (Chapter 6). Given these results, we proposed a role for the SOCS in energy substrate re-partitioning in response to sustained elevated cortisol levels that is usually associated with longer-term (chronic) stress in fish. However it is not known if SOCS modulation plays a role in the metabolic adjustments that is associated with acute stressor exposure in fish.

We tested the hypothesis that an acute stressor and the attendant elevation in plasma cortisol levels will modulate SOCS levels in rainbow trout. This hypothesis was tested under two scenarios known to modulate SOCS levels, feed restriction (Philip et al., 2014) and LPS stimulation (Philip et al., 2012; 2014). Rainbow trout that were fed or fasted for 118 d, so that they are in a negative energy balance, were injected with LPS and subjected to an acute handling stressor 72 h post-LPS injection. Plasma cortisol, glucose, lactate and liver glycogen content as well as GR and MR protein expression were assessed as indicators of metabolic stress response, along with the transcript abundances of liver SOCS-1, SOCS-2 and SOCS-3 in rainbow trout.

5.3 Materials and Methods

5.3.1 Fish

Juvenile rainbow trout (*Oncorhynchus mykiss*; 55± 10 g body mass) were obtained from Washington Hatchery Klickitat River, WA, USA and maintained at the Columbia River Research Laboratory, USGS, WA, USA, at 6-7^{o}C on a 12:12-h light/dark cycle. Fish were

fed daily to satiety with commercial dry pellet till the start of the experiment. Fish were acclimated for 2 weeks prior to use in the following experiment.

5.3.2 Experimental design

On 10th January 2012, rainbow trout were weighed, fork length measured and tagged and distributed randomly into 4 tanks (n=18 per tank). Rainbow trout in two of the tanks were fed as stated before while the rainbow trout in the other two tanks were fasted till 7th May 2012 (118 days). On 7th May, the fish were reweighed and fork length measured again. Body mass (BM) and fork length (FL) were used to calculate the condition factor (CF; BM/FL^3 x 100). Specific growth rate (SGR) was calculated by the formula [(ln BM_T – ln BM_t/T-t) x 100] where BM_T and BM_t are the weights of the fish in May and January, respectively, and T-t the number of days between weight measurements. On the same day, half the fish in each tank were injected with LPS (*Escherichia coli*, 0111:B4; Sigma; 2.5mg/Kg wt in saline) and the other half with the vehicle (saline) and redistributed into 4 tanks (fed and LPS injected, n=18; fed and saline injected, n=18; fasted and LPS injected, n=18; fasted and saline injected, n=18). After 72 h on 10th May, 2012, blood and liver samples were collected from 6 fish from each treatment (0 h time point). Following the 0 h sampling, 12 fish from each treatment were subjected to an handling disturbance (a standardised handling disturbance in which trout were netted and held out of the water for 1 min; Wiseman et al., 2011) after which they were allowed to recover. Blood and liver samples were taken at 1and 4 h post-stressor exposure (n=6 at each time point for each of the four treatment groups) to assess the acute stress response (Ings et al., 2011). Fish were euthanized with an overdose of neutralized MS222 and blood samples were immediately centrifuged at 5000x g for 5 min. Plasma was separated and stored at -80 °C to measure cortisol, glucose and lactate levels later. Liver tissues were stored at -80 °C for glycogen, transcript and protein expression analyses later.

5.3.3 Plasma cortisol, glucose, lactate levels and liver glycogen content

Plasma cortisol levels were measured using a [3H]-labeled cortisol radioimmunoassay as described previously (Alsop et al., 2009). Plasma glucose levels were

measured by monitoring NAD reduction by the hexokinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) assay method in Tris buffer (120 mM Tris-base, 80 mM Tris-HCl, 5 mM NAD, 2 mM MgS04, 5 mM ATP). The reaction was started with G6PDH (0.4 U/ml) and HK (0.5 U/ml). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA). The same protocol was used to determine liver glycogen content by analysing liver glucose content before and after amyloglucosidase hydrolysis (Birceanu et al., 2013). Plasma lactate levels were measured by monitoring NAD reduction by Lactate dehydrogenase (LDH) assay method in Hydrazine buffer (0.2 M; pH 9.5). The reaction was started with LDH (10U/well). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA.

5.3.4 SDS-PAGE and immunodetection

Sample protein concentrations were measured with bicinchoninic acid (BCA) reagents using BSA as the standard. SDS–PAGE and immunodetection were carried out exactly as described before (Aluru and Vijayan, 2007). Briefly, sample protein (40 μg protein per lane) was separated on 10% polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes. The membranes were probed with either rabbit polyclonal anti-trout GR (1:1000; Sathiya and Vijayan, 2003) or rabbit polyclonal anti-trout MR (1: 1000; Jeffrey et al., 2012). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; BioRad, Hercules, CA, USA). Protein bands were detected with ECL plus (Amersham Biosciences, Piscataway, NJ, USA) and the molecular mass confirmed with precision plus molecular weight markers (BioRad). The bands were scanned with a Typhoon 9400 (Amersham) and the band intensity quantified by AlphaEase software (Alpha Innotech, CA, USA). Equal loading was confirmed by incubation of membranes with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, 1:1000) for 1 h at room temperature.

5.3.5 Quantitative real-time PCR (qPCR)

***RNA extraction and cDNA synthesis*:** Total RNA was extracted from liver tissue using the RNeasyextraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The concentration of total RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Napean, ON, CAN).First-strand cDNA was synthesized from 1μg of total RNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions.

***Quantification*:** Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The genes of interest were SOCS-1, SOCS-2 and SOCS-3 while elongation factor 1 alpha (EF1α) was the housekeeping gene. Primer pairs (Table 1) for these genes were designed from rainbow trout cDNA sequences using Primer 3 version 0.4.0. software. Threshold cycle values for each sample were calculated using iCycleriQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 2.5 μLcDNA, 2.5 μL of 10 mM primer pair, 40 μL of SYBR green mix and 35 μL of nuclease-free water was prepared for each sample, after which 25 μL was added to each of three wells. Each sample was assayed for the genes of interest and the housekeeping gene. The following PCR conditions were used for amplification: 95 °C for 3 min; 40 cycles of 95 °C for 20 s and annealing temperature (see Table 1) for 20 s; 95 °C for 1 min; 55 °C for 1 min followed by melt curve analysis starting at 55 °C and increasing in 0.5 °C increments to 95 °C every 10 s. Copy number of transcripts for each gene was determined with the threshold cycles (CT) using plasmid standard curves and was normalized to EF1α as described previously (Aluru et al., 2010). The abundance of EF1α was unchanged between treatment groups and, therefore, used as a housekeeping gene in this experiment.

Table 1: Gene-specific primers for quantitative real-time PCR.

The table provides the list of genes (Gene ID), forward and reverse primer sequences, annealing temperature and amplicon size. SOCS: suppressors of cytokine signalling; EF1α: elongation factor 1α.

Gene ID	Primer sequences (5'-3')	Temp (^{o}C)	Amplicon size (bp)
SOCS-1	Fwd: GATTAATACCGCTGGGATTCTGTG Rev: CTCTCCCATCGCTACACAGTTCC	63.3	136
SOCS-2	Fwd: TCGGATGACTTTTGGCCTAC Rev: CCGTTCTTCTCTCGTTTTCG	60	102
SOCS-3	Fwd: TAGCCCTGAGCCTGGAAGTA Rev: GGTTGCTAGGCAGTTTCCTG	60	113
EF1α	Fwd: CATTGACAAGAGAACCATTGA Rev: CCTTCAGCTTGTCCAGCAC	56	95

5.3.6 Statistical analysis

Data are shown as mean ± standard error of mean (SEM). Outliers, values that were two standard deviations above or below the mean, were omitted from the analyses. Two-way ANOVA followed by Holm Sidak's post-hoc was used to compare the effect of different treatments and time and their interactions. Student *t*-test was used to compare the effect of fasting on BM, CF and SGR. The data were log transformed, wherever necessary, for homogeneity of variance, but non-transformed values are shown in the figures. A probability level of $p<0.05$ was considered significant. Statistical analyses were performed with SigmaStat (Systat Software Inc., San Jose, CA, USA).

5.4 Results

5.4.1 Body mass, condition factor and specific growth rate

Long-term fasting significantly reduced body mass, condition factor and specific growth rate in rainbow trout. The mean body mass of fasted trout was reduced by ~68%, this was also reflected in significant differences in SGR (Table 2; chapter 4). The condition factor was also reduced in the fasted trout by ~26% compared to the fed trout (Table 2; chapter 4).

5.4.2 Plasma analysis and liver glycogen content

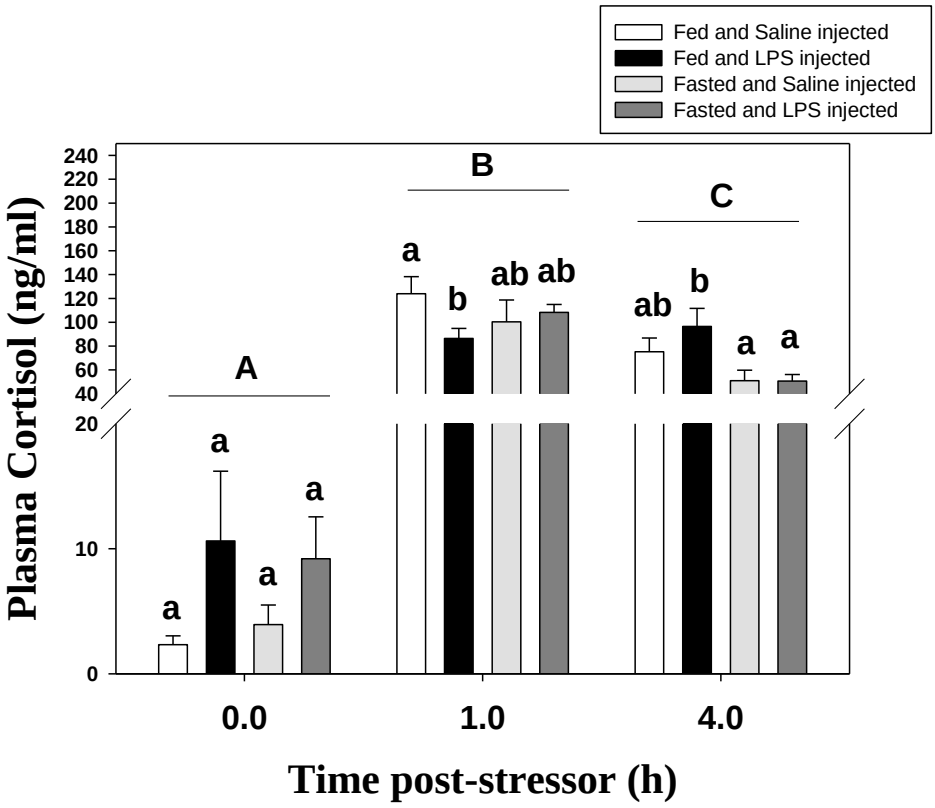
Following the acute stressor, plasma cortisol levels increased in all the treatment groups and was significantly different from the 0 time at 1 and 4 h post-stressor exposure. The stressor-induced cortisol response was reduced by LPS treatment at 1 h only in the fed fish but not the fasted fish (Fig. 1A). None of the other treatment groups were significantly different at 1 h post-stressor exposure. At 4 h the LPS treated fed fish were significantly higher than the fasted groups but not from the fed saline group (Fig. 1A). Similarly, following the acute stressor, plasma glucose levels were also elevated in all the treatment groups. However prior fasting and LPS stimulation attenuated the stressor-induced plasma glucose response in trout (Fig. 1B). Plasma lactate levels also increased in all the treatment groups following the acute stressor. However, prior fasting, regardless of LPS treatment, attenuated the stressor-induced plasma lactate response compared to the fed control fish (Fig.

1C). Overall, fasted trout had significantly lower liver glycogen content compared to the fed trout (Fig. 2). There was significant interaction between treatment and time post-stressor exposure on liver glycogen content. At 4 h post-stressor, the fasted and LPS injected trout showed higher liver glycogen content than the fasted and saline injected trout. Also, the fed and LPS injected trout showed higher liver glycogen levels at 4 h post-stressor compared to 1 h post-stressor (Fig. 2).

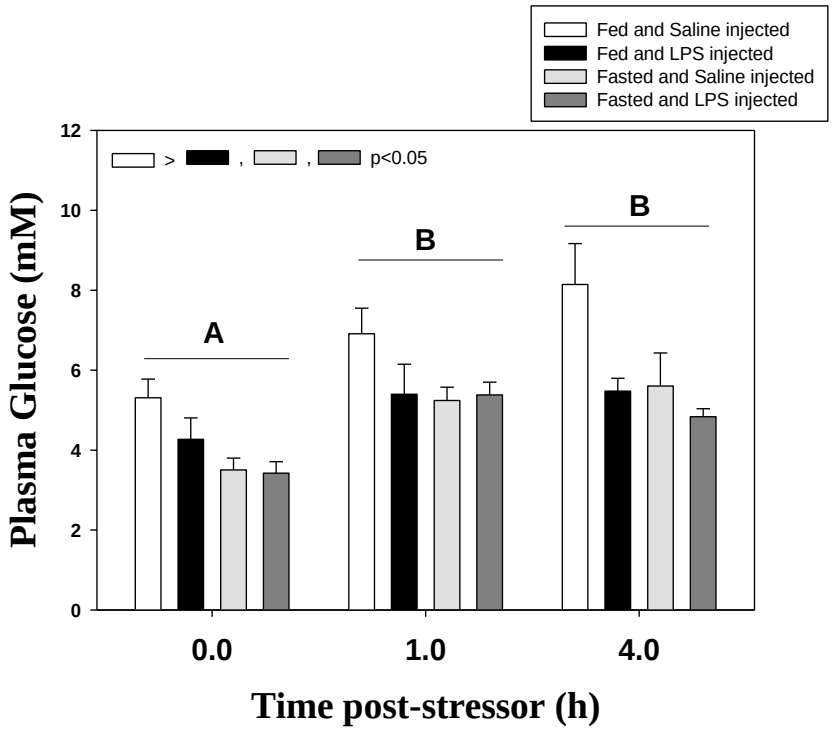
Figure 1: Effect of nutritional status (Fed vs Fasted) and LPS injection on plasma cortisol (A) plasma glucose (B) and plasma lactate (C) levels following an acute stressor

Data represents mean ± S.E.M (n = 6). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each time-point and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).

A



B


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
> , , p<0.05
Plasma Glucose (mM)
0
2
4
6
8
10
12
A
B
B
0.0
1.0
4.0
Time post-stressor (h)

C

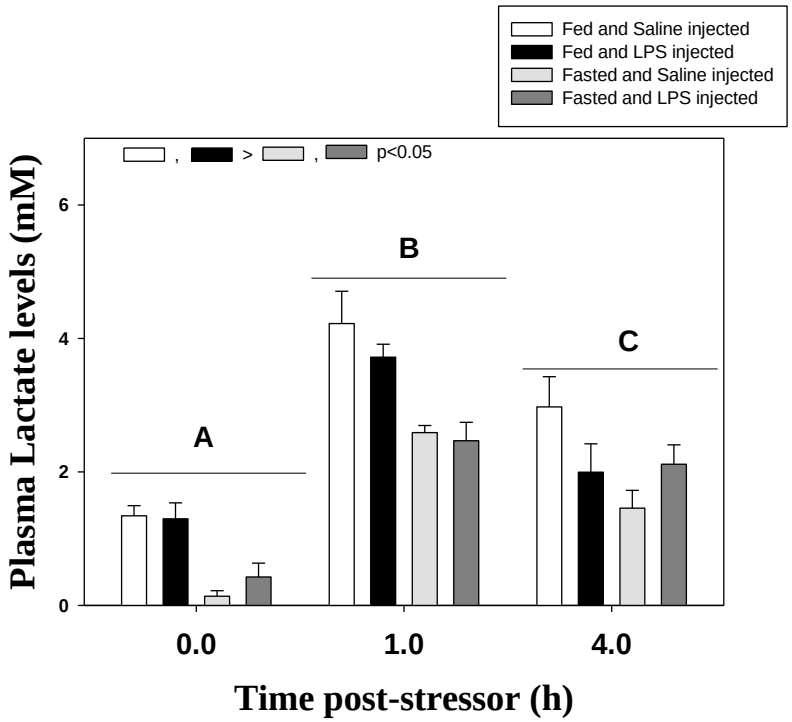
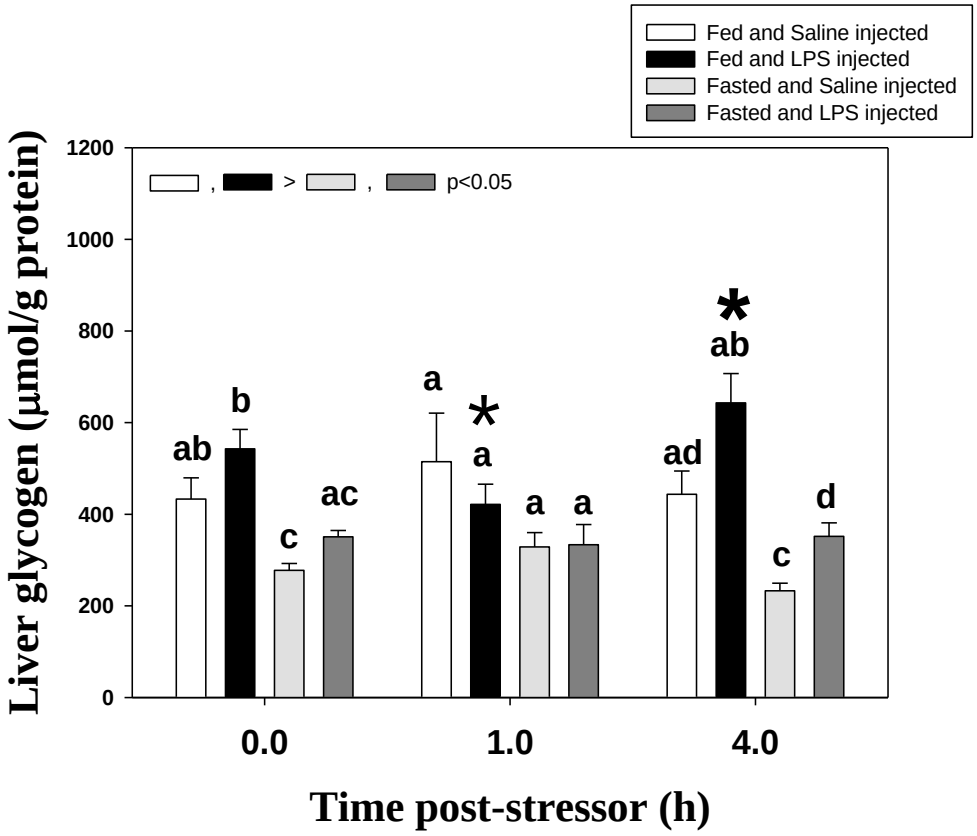

Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
, > , p<0.05
Plasma Lactate levels (mM)
0
2
4
6
A
B
C
0.0
1.0
4.0
Time post-stressor (h)

Figure 2: Effect of nutritional status (Fed vs Fasted) and LPS injection on liver glycogen content following an acute stressor

Data represents mean ± S.E.M (n = 6). Different lower case letters indicate significant treatment effects within each time-point; * denotes treatment effects between the different time points and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).



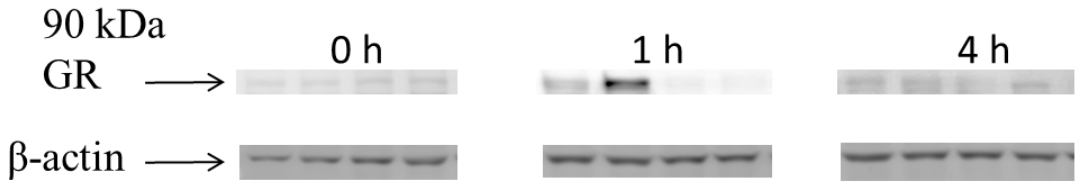
5.4.3 Glucocorticoid Receptor and Mineralocorticoid Receptor Protein Expression

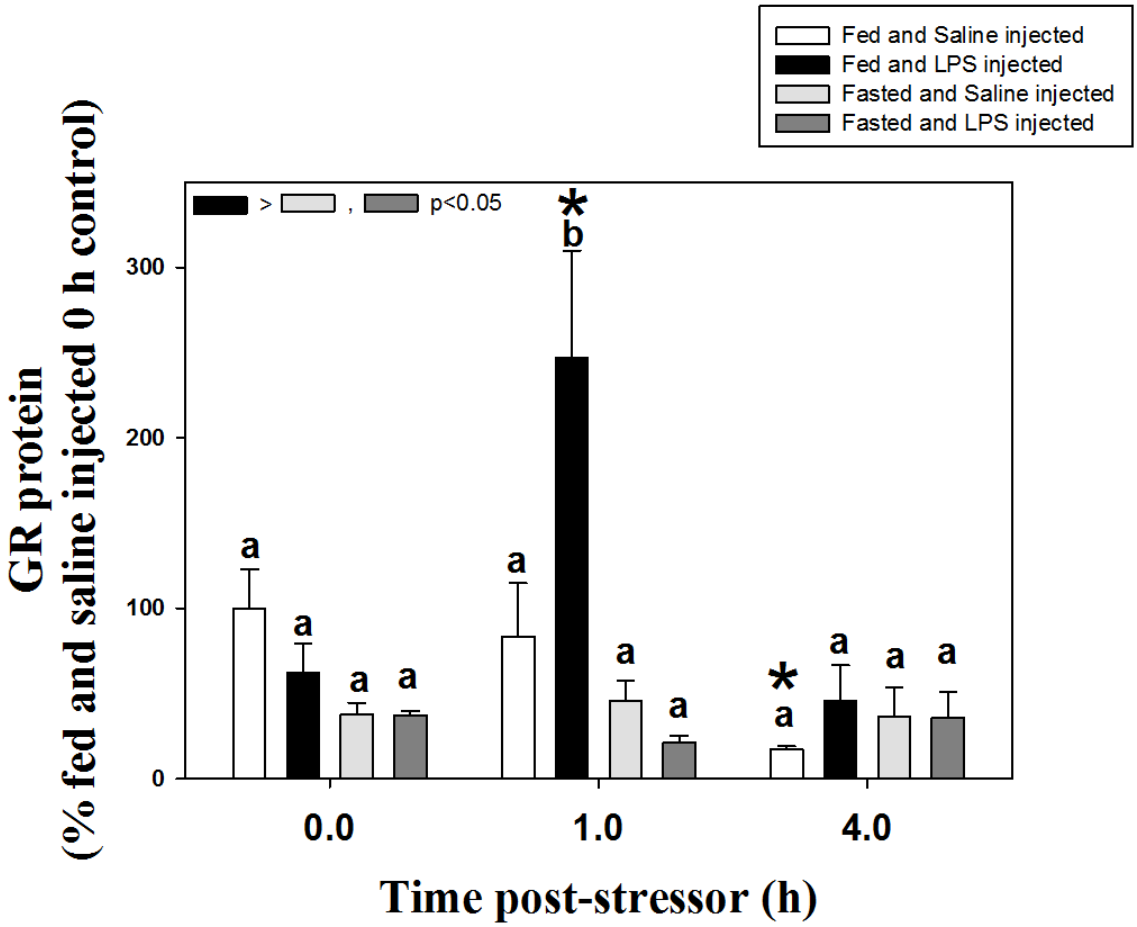
There was a significant LPS effect and interaction between treatment and time after stressor exposure. LPS injection increased liver GR expression post-stressor exposure in the fed compared to the fasted trout (Fig. 3A). The fed and LPS injected trout showed higher GR protein levels at 1 h post-stressor compared to all the other three treatment groups. The fed and LPS injected trout also showed higher GR levels at 1 h compared to the 0 and 4 h post-stressor time-points, while the GR expression in the fed and saline injected trout at 4 h was significantly lower compared to 0 and 1 h post-stressor exposure (Fig. 3A). Neither fasting nor LPS injection had any effect on MR protein expression. Acute stressor exposure, regardless of treatments, significantly affected liver MR expression. Overall, liver MR expression was significantly lower at 1 and 4 h post-stressor exposure compared to 0 h time-point (Fig. 3B). The MR expression at 4 h was significantly higher than 1 h post-stressor exposure (Fig. 3B).

Figure 3: Effect of nutritional status (Fed vs Fasted) and LPS injection on GR and MR protein expression in rainbow trout liver following an acute stressor

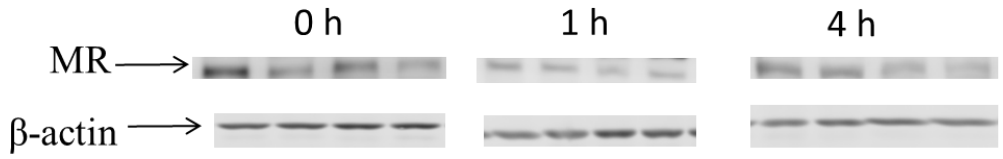
Densitometric values for GR (A) and MR (B) are plotted as % fed and saline injected 0 h control and show mean ± S.E.M (n = 6 independent fish). Different upper case letters indicate significant time effects; different lower case letters indicate significant treatment effects within each time-point; * denotes treatment effects between the different time points and inset indicates overall significant treatment effects (two way ANOVA, $p < 0.05$). Protein loading was normalized to β-actin (monoclonal mouse antibody; Sigma, St. Louis, MO). Representative blots are shown above the histogram.

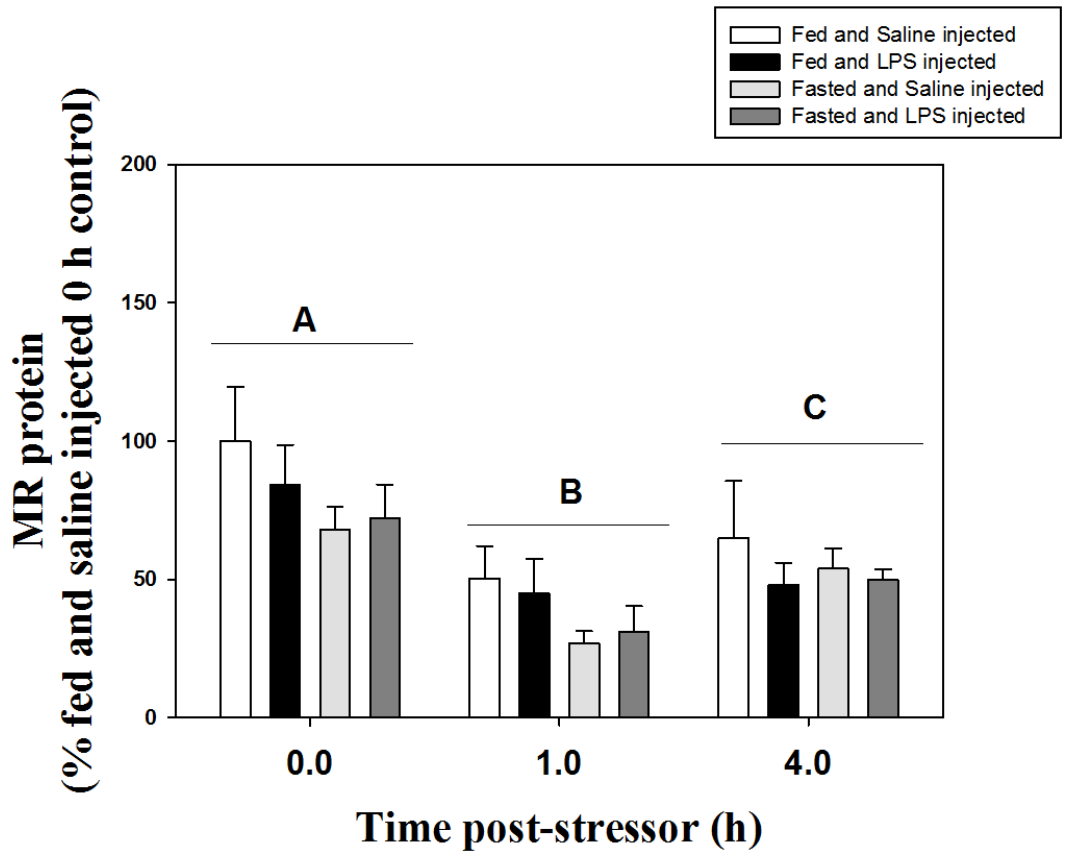
A


90 kDa
GR
β-actin
0 h
1 h
4 h


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
> , p<0.05
GR protein
(% fed and saline injected 0 h control)
300
200
100
0
a
a
a
a
a
*
b
a
a
*
a
a
a
a
0.0
1.0
4.0
Time post-stressor (h)

B


0 h
1 h
4 h
MR
β-actin


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
MR protein
(% fed and saline injected 0 h control)
200
150
100
50
0
A
B
C
0.0
1.0
4.0
Time post-stressor (h)

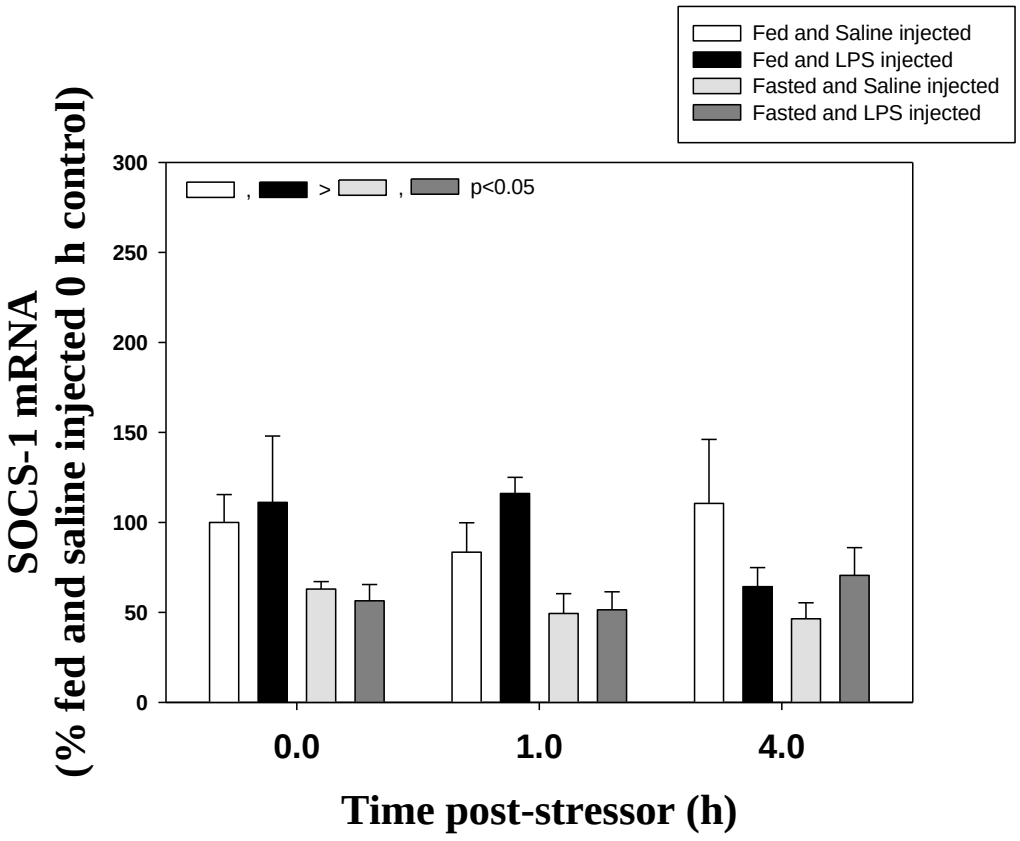
5.4.4 SOCS transcript abundance

Acute stress had no significant effect on liver SOCS-1 mRNA abundance (Fig. 4A). Fasting, but not LPS injection, significantly reduced SOCS-1 mRNA abundance. Liver SOCS-2 mRNA levels were significantly higher at 4 h post-stressor exposure compared to levels prior to stress but not 1 h post-stressor exposure (Fig. 4B). There was also a significant treatment effect with SOCS-2 mRNA levels significantly higher in the fasted fish compared to the fed fish (Fig. 4B). Within the fed group LPS injection significantly reduced SOCS-2 mRNA levels compared to the saline control group, while no such differences were seen in the fasted fish (Fig. 4B). Liver SOCS-3 mRNA levels were significantly lower at 1 h post-stressor exposure compared to the pre-stress values, while the SOCS-3 mRNA levels at 4 h was not significantly different from the other time-points (Fig. 4C). Neither prior fasting nor LPS injection had any effect on SOCS-3 expression (Fig.4C).

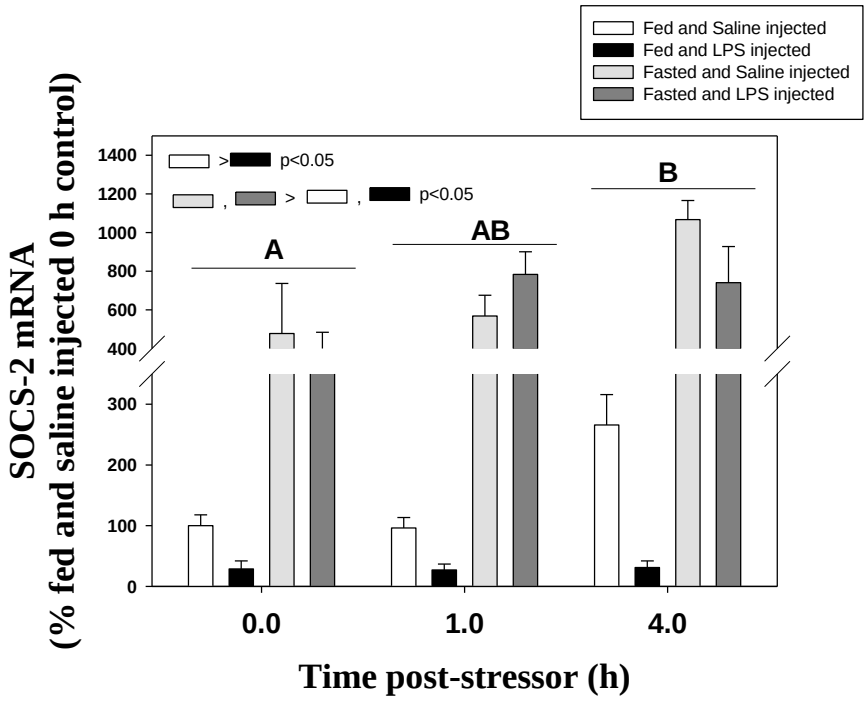
Figure 4: Effect of nutritional status (Fed vs Fasted) and LPS injection on SOCS-1 (A) SOCS-2 (B) and SOCS-3 (C) transcript levels in rainbow trout liver following an acute stressor

Data are plotted as % fed and saline injected 0 h control and show mean ± S.E.M (n = 6 independent fish). Different upper case letters indicate significant time effects and inset indicates overall significant treatment effects (two-way ANOVA, $p < 0.05$).

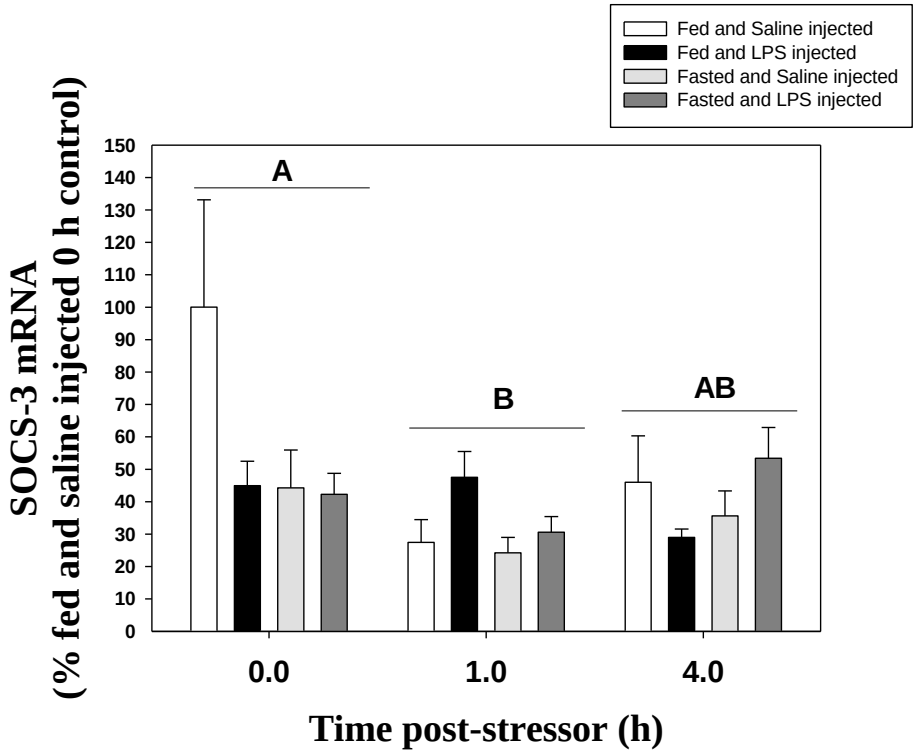
A



B


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SOCS-2 mRNA
(% fed and saline injected 0 h control)
> p<0.05
, > , p<0.05
A
AB
B
1400
1200
1000
800
600
400
300
200
100
0
0.0
1.0
4.0
Time post-stressor (h)

C


Fed and Saline injected
Fed and LPS injected
Fasted and Saline injected
Fasted and LPS injected
SOCS-3 mRNA
(% fed and saline injected 0 h control)
A
B
AB
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0.0
1.0
4.0
Time post-stressor (h)

5.5 Discussion

The results of this study demonstrate for the first time that an acute handling disturbance regulates SOCS mRNA levels in rainbow trout. While SOCS-3 was downregulated, SOCS-2 was significantly elevated in response to stressor exposure leading us to propose a key role for this protein in the metabolic adjustments to cope with an acute stress in fish

5.5.1 Stress performance

Stressor-induced elevation in plasma cortisol levels is a highly conserved adaptive response that is important for the metabolic adjustments necessary to cope with stress in fishes (Vijayan et al., 2010). Even though all the treatment groups showed a typical cortisol response to acute stress, with cortisol levels peaking at 1 h and coming down by 4 h post-stressor (Mommsen et al. 1999; Ings et al. 2011), the LPS injected fish showed lower plasma cortisol levels than the saline injected fish in the fed group suggesting an attenuation of the cortisol response to the handling stressor. It should be noted that the handling stressor was applied 72 h post LPS injection, which in itself was a stressor leading to elevated cortisol levels that returned to basal unstressed levels prior to the secondary handling stressor (see chapter 4). The prior hyperactivity of the interrenal tissue by LPS may be a reason for the attenuated response seen here to the handling disturbance in this group. For instance, studies have shown interrenal exhaustion to chronic stress leading to a reduced cortisol response to subsequent stressors (Barton et al., 2002; Hontela and Vijayan, 2008). Moreover, temporally separated effects of stressors on responses to subsequent stressors can have "carry over" effects and can modulate a fish's response to subsequent stressors despite physiological recovery from the initial stressor (McConnachie et al., 2012; Harrison et al., 2011). The attenuated cortisol response in the LPS injected fish may happen at multiple levels including the hypothalamus, pituitary and/or at the interrenal tissue of trout, but this remains to be determined. LPS injection in the fasted fish did not attenuate their cortisol response to acute stress, suggesting better stress performance which may be because during fasting, a number of physiological changes occur which allow the fish to use stored energy depots for metabolic maintenance which in turn appears to increase the tolerance of the fish to

subsequent stressors including immune challenges and handling (Davis and Gaylord, 2011). Moreover, fasted trout showed the same level of cortisol response to acute stress as fed trout underscoring this theory.

Energy, mainly in the form of glucose, is required to meet the metabolic needs associated with stress recovery (Mommsen et al., 1999; Barton, 2002). The fed and LPS injected as well as the fasted trout (both saline and LPS injected) showed attenuated stressor-induced plasma glucose levels compared to the fed and saline injected trout. The immediate stressor stimulated increase in plasma glucose levels results from catecholamine stimulated glycogenolysis, whereas the maintenance of this response and repletion of liver glycogen stores involves cortisol-induced hepatic gluconeogenesis (Mommsen et al., 1999; Vijayan et al., 2010) . Thus increases in plasma glucose during a glucocorticoid stress response are mainly the result of mobilization of carbohydrate energy stores. However, these resources are finite (Mommsen et al., 1999; Barton 2002). Fasted fish had lower glycogen stores compared to their fed counterparts, and therefore, displayed reduced circulating glucose levels, in response to the handling stressor. LPS challenge induces hypoglycemia in fish similar to that observed in mice and humans (Raetzsch et. al., 2009). This response in the present study may relate to the attenuated cortisol response seen with LPS in the fed fish leading to the proposal that this steroid may be playing a key role in maintaining plasma glucose levels during stress in fish (Aluru and Vijayan, 2009). The attenuated stressor-induced plasma lactate response seen in the fasted fish suggest decreased muscle activity. This may be due to the decreased muscle glycogen content as previous studies have shown that fasting depletes muscle glycogen in fish (Lim and Ip, 1989). This is further supported by the reduced liver glycogen content seen in the fasted trout in the present study.

The physiological effects of cortisol on target tissues is mediated by GR and MR in teleosts (Aluru and Vijayan, 2009). It is known that plasma cortisol levels modulate liver GR content (Vijayan et al., 2003). Consequently, the lower cortisol levels in the fed and LPS injected trout at 1 h post-stressor may be playing a role in the compensatory increase in target tissue GR content (Sathiyaa and Vijayan, 2003). This upregulation of target tissue GR expression will lead to increased cortisol responsiveness and may favour glucocorticoid-

mediated suppression of endotoxicity (Salkowski and Vogel, 1992). While GR expression was not affected by acute stress, liver MR expression was reduced post-stressor exposure in trout. The role of MR in stress adaptation is unknown, as most studies have examined the role of GR in stress adaptation (Aluru and Vijayan, 2009). Our results suggest that MR signalling may be affected by acute stress, but whether the changes are mediated by stressor-induced elevated plasma cortisol levels remains to be determined. Overall, long term fast and the associated negative energy balance compromise the capacity of the animal to evoke a physiological stress response. Our results suggest that LPS treatment, may also compromise the ability of the animals to evoke a stress response similar to that seen in fasted trout.

5.5.2 SOCS modulation

The SOCS proteins are negative regulators of cytokine signalling and play a critical role in protecting against the pathological effects of excessive cytokine signalling. They regulate the janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway which is an intracellular signalling pathway shared by a variety of cytokines, as well as leptin, growth hormone (GH), and prolactin (PRL) (Croker et al., 2008). Their roles in diverse functions, including immunity, growth and development have been demonstrated highlighting their potential in integrating different physiological processes. Homologues of all the mammalian SOCS family members including SOCS-1, SOCS-2 and SOCS-3, have been identified in fish (Wang and Secombes 2008; Wang et al., 2011), but their functional roles are just emerging. We recently showed that SOCS-1 and SOCS-2 in rainbow trout are cortisol responsive and bioinformatics analysis suggested putative glucocorticoid response elements (GREs) in their promoters (Philip et al., 2012). We also showed that long-term fasting in the anadromous Arctic charr upregulates SOCS isoforms in a tissue specific manner possibly as an adaptive strategy to restrict energy demanding pathways like GH and cytokine signalling in an attempt to conserve energy resources (Philip et al., 2014). We followed this up with a study that showed that upregulation of SOCS genes by cortisol suppresses GH and LPS signalling in fish liver slices *in vitro* (Chapter 6). Recent studies also showed that handling and injection stress modulates SOCS expression in fishes (Shepherd et al., 2012; Wang et al., 2010).

Our results show that acute stress differentially modulates SOCS mRNA levels in trout. While it had no effect on SOCS-1 mRNA levels, it upregulated SOCS-2 and downregulated SOCS-3 levels with different temporal dynamics, highlighting that SOCS expression is transient and tissue, cell and isoform-specific (Wang et al., 2011; Philip et al., 2012). The elevated cortisol levels associated with handling disturbance may be responsible for the upregulation of SOCS-2 (Philip et al., 2012). This may serve to restrict energy demanding pathways and re-direct energy resources for stress adaptation. Genomic effects of cortisol culminate in gene transcription and are slower than non-genomic effects that involves phosphorylation/dephosphorylation events (Dindia et al., 2013). The genomic effect of cortisol on SOCS regulation is further supported by the presence of putative glucocorticoid response elements (GREs) in the SOCS-2 promoter of fish (Appendix A). This may explain the absence of SOCS-2 modulation during the initial hour after stressor exposure. The decrease in SOCS-2 expression in the fed and LPS injected fish may be related to the lower cortisol levels seen in these fish, underscoring the effects of this steroid on SOCS-2 expression. However, it is not clear if cortisol is also responsible for the downregulation of SOCS-3 levels seen at 1 h post stressor. The downregulation of SOCS-3, a potent negative regulator of immune signalling, may facilitate the immune-stimulatory effects of acute stress (Tort, 2011). Yet, the mechanisms involved in SOCS-3 downregulation are not clear and warrants further investigation. The effects of fasting on SOCS-1 and SOCS-2 expression in response to a handling disturbance suggests a carry over effect since the same effects were observed even at 0 time (prior to stressor exposure), but whether this has any adaptive value needs to be further investigated.

Overall, our results suggest that acute handling disturbance regulates SOCS mRNA levels in trout. Specifically, stressor exposure upregulated SOCS-2 mRNA levels in trout. As this gene regulation is under the control of cortisol, we propose that upregulation of SOCS-2 mRNA levels by this steroid during acute stressor exposure may be playing a key role in the metabolic adjustments essential to cope with stress in fish. We demonstrate that fasting and LPS challenge modify the physiological stress response, including alterations in plasma cortisol, glucose and lactate levels and liver glycogen content and GR protein expression,

suggesting the importance of looking at the carry-over, cumulative and long-term effects of different types of stressors while looking at stress performance in fish.

5.6 Acknowledgements

This study as supported by funding from the Natural Sciences and Engineering Research Council of Canada, Discovery Grant to MMV.

Chapter 6
Stress-Immune-Growth Interactions in Fish: A Role for Suppressors of Cytokine Signalling

6.1 Overview

Chronic stress is thought to be a major factor in the poor growth and immune performances of salmonids in aquaculture. However, the molecular mechanisms linking stress to growth and immune suppression in fishes are unknown. We tested the hypothesis that upregulation of suppressors of cytokine signalling (SOCS), a key negative regulator of JAK/STAT signalling, by cortisol is a key molecular link in the suppression of growth and immune responses. Exposure of trout liver slices to stressed levels of cortisol upregulated SOCS-1 and SOCS-2 and this was abolished by mifepristone. Cortisol exposure suppressed growth hormone (GH)-stimulated IGF-1 expression and this involved reduced STAT5 phosphorylation and decreased total JAK2 protein expression. Cortisol also suppressed lipopolysaccharide (LPS)-induced IL-6 but not IL-8 transcript levels. LPS also reduced GH signalling and this was mediated by the downregulation of GH receptors and reduced STAT5 phosphorylation. Altogether, our results underscore SOCS-1 and SOCS-2 upregulation by cortisol as a novel mechanism linking stress effects on growth and immune suppression in trout.

6.2 Introduction

Plasma corticosteroid elevation is a highly conserved response to stressor exposure in vertebrates and is essential to adapt animals to stress (Tort and Teles, 2011). Coping with stress is energy demanding and elevated corticosteroid levels increase the metabolic rate and energy substrate mobilization in animals (Charmandari et al., 2005). Animals have a limited amount of energy, which it uses to support normal functions and maintain physiological equilibrium. Thus, stress alters animal energy budget by consuming a large amount of energy, at the expense of other energy demanding activities, including growth and immunity (Charmandari et al., 2005; Tort and Teles, 2011) . However, the mechanisms involved in stress-related cellular energy re-partitioning are far from clear.

In fishes, exposure to stressors, including handling for grading, crowding and transportation are part and parcel of aquaculture operations (Segner et al., 2011). These hatchery practices lead to reduced growth and increased disease susceptibility, resulting in an

overall decrease in fish production (Ashley, 2006). However, the link between the stressor exposure and effects on growth and immune alterations are far from clear. Stressor-induced elevation in circulating cortisol, the primary circulating corticosteroid in teleosts, has been implicated in affecting growth (Bernier et al., 2004; Vijayan et al., 2010; Barton, 2002) and immune suppression (Tort, 2011). As in other animals, the growth hormone (GH)/insulin-like growth factor (IGF) axis is the key driver of the growth process in fishes (Reinecke, 2010; Reinecke et al., 2005). The JAK/STAT (Janus Kinase/Signal transducers and activators of transcription) pathway is the principal GH signalling mechanism (Reindl et al., 2011), leading to transactivation and/or repression of target genes, including IGF-1 (Rawlings et al., 2004). Consequently, IGF-1 hormone levels and/or the mRNA abundance of this gene is used as a marker of GH signalling in fishes (Reindl et al., 2011).

Similar to growth, immune response is another energy demanding pathway that is curtailed during stress in fishes (Tort, 2011). Cortisol has been shown to suppress the expression of cytokines, including IL-1β, IL-8, IL-6 and TNFα2, in a variety of cell types in response to immunostimulants in fishes (MacKenzie et al., 2003; Castillo et al., 2009; Philip et al., 2012). Lipopolysaccharide (LPS) is the most common form of immunostimulant used and this endotoxin also elicits a cortisol response in fishes (Swain et al., 2008). In mammals, LPS is recognized by TLR4 and down-stream signalling mediated through the NF-κB and JAK/STAT pathways (Kimura et al., 2005). Most fish species lack a TLR4 and the molecular mechanisms involved in LPS signalling in teleost models are less clear (Sepulcre et al., 2009).

Although stress and/or cortisol exposure reduces growth (Reinecke, 2010; Pierce et al., 2011) and suppresses immune response in fishes (Tort, 2011) , the molecular mechanism(s) mediating these changes during stress is far from clear. We recently showed that suppressors of cytokine signalling (SOCS) transcript levels in the liver are modulated by stress and/or cortisol treatment in rainbow trout (Philip et al., 2012).The SOCS genes act as negative regulators of both cytokine and GH signalling in mammals by targeting the JAK/STAT pathway (Croker et al., 2008). Homologues of all eight mammalian SOCS family members have been discovered in fishes, while SOCS-1, SOCS-2 and SOCS-3 have been characterized

in salmonids (Wang and Secombes, 2008). Although the functional significance of SOCS transcript abundance is unclear in fishes, the mRNA abundance of SOCS1-3 have been shown to be modulated by cytokines, immunostimulants, nutritional status and cortisol exposure in fishes (Shepherd et al., 2012; Wang et al., 2011; Philip et al., 2012, 2014). Consequently, SOCS upregulation may limit GH, cytokine and LPS signalling, given they all share the JAK/STAT signalling pathway (Croker et al., 2008; Kimura et al., 2005).

Against this backdrop we tested the hypothesis that stress levels of cortisol upregulate SOCS mRNA abundance and this will negatively regulate growth and immune response by inhibiting the JAK2-STAT5 signalling pathway in rainbow trout. We used a series of *in vitro* experiments using liver as a model to understand the mechanisms underlying the effect of cortisol on growth and immune response in fishes. The liver was used in this study because it is a key tissue for energy substrate reallocation during stress, and is also a key target for cortisol action, including the expression of glucocorticoid receptor (GR) (Mommsen et al., 1999; Aluru and Vijayan, 2009; Vijayan et al., 2010). Also, liver plays important roles during immune responses and growth in fishes (Bayne and Gerwick, 2001; Reindl et al., 2011). Our results indicate that cortisol upregulation of SOCS-1 and SOCS-2 may be a key mechanism reducing GH signalling and suppressing cytokine production during stress in fishes. This may have adaptive value by reallocating energy substrates away from growth and immune function in order to meet the increased metabolic demands essential to cope with stress.

6.3 Materials and Methods

6.3.1 Experimental fish

Immature rainbow trout (*Oncorhynchus mykiss*; 150 ± 10 g body mass) were obtained from Alma Research Station (Alma, ON, CAN), and maintained at the University of Waterloo Aquatic Facility, at 12 ± 1^{o}C on a 12:12-h light/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. Experiments were approved by the University of Waterloo Animal Care Protocol review committee and adhere to guidelines

established by the Canadian Council on Animal Care for the use of animals in teaching and research.

6.3.2 Liver slices

Liver slices were prepared by quickly excising and washing the liver in ice cold modified Hank's buffer (110 mM NaCl, 3 mM KCl, 1.25 mM K_2HPO_4, 5 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 1 mM $MgCl_2$ and 10 mM Hepes; 1.5 mM $CaCl_2$, 5 mM glucose; pH 7.63 at room temperature). Livers were then sliced into 8-10 mm pieces (500 μm maximum width) using a MD-1100 tissue slicer (Munford, USA), washed three times with modified Hank's buffer and were placed in 24-well tissue culture plates (approximately 50 mg of tissue/well) with 2 mL of L15 media/well. Liver slices were maintained at 13°C with constant rocking for 2 h after which media was changed and replaced with the treatments. We used cortisol-mediated glucose release (see Fig.S1) as a positive control in each experiment to confirm liver viability (Aluru and Vijayan, 2007).

6.3.3 Cortisol effects on SOCS expression

The aim of this study was to see if SOCS genes are temporally regulated in response to cortisol stimulation. We used stressed levels of cortisol reported for rainbow trout (100 ng/ml; Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007) for our exposures. Liver slices were incubated with either control media or media containing cortisol (100ng/ml; Sigma) for 1, 4, 6, 8 and 24 h for SOCS-1 and SOCS-2 transcript analysis, since these two SOCS isoforms have been shown to be cortisol responsive (Philip et al., 2012). The liver slices were washed twice in ice cold modified Hank's buffer and immediately frozen at -80°C. To address if cortisol effects are mediated through GR signalling, liver slices were incubated with control media or media containing cortisol (100 ng/ml), mifepristone (a GR antagonist; 1000 ng/mL; Sigma) or a combination of mifepristone and cortisol. The Mifepristone concentration used was shown previously to block GR-mediated metabolic effects in trout (Sathiyaa and Vijayan 2003; Aluru and Vijayan, 2007). In the combination group, liver slices were incubated with Mifepristone 30 min before the addition of cortisol. Liver slices

were collected at 24 h, washed twice in ice cold modified Hank's buffer, and immediately frozen at -80°C for transcript analysis later.

6.3.4 Cortisol effects on GH signalling

The aim of this study was to see whether cortisol mediated upregulation of SOCS expression downregulates GH signalling. Liver slices were pre-incubated with control media or media containing cortisol (100ng/ml) for 24 h to upregulate SOCS expression based on our previous study (Philip et al., 2012). After 24 h, the media was changed and the liver slices were incubated with control media or media containing bovine GH (bGH; 100 ng/ml or 1000 ng/ml). These GH concentrations were shown previously to upregulate IGF-1 expression in trout hepatocytes (Reindl et al., 2011). Following GH addition, liver slices were collected either at 10 min to confirm JAK/STAT activation or at 6 h for IGF-1, SOCS-1 and SOCS-2 transcript analysis. Total JAK-2 protein levels and rapid changes in phosphorylation status of STAT5 substrate proteins were used to confirm modulation of GH signalling pathways in response to cortisol treatment. The liver slices were washed twice in ice cold modified Hank's buffer and immediately frozen at -80°C.

6.3.5 Cortisol effects on LPS signalling

The aim of this study was to examine whether cortisol-mediated upregulation of SOCS expression affects LPS signalling. Liver slices were pre-incubated with control media or media containing cortisol (100ng/ml) for 24 h. After priming the tissue with cortisol, media was replenished and the control and cortisol groups were either exposed to control media or media containing LPS (30µg/ml; *Escherichia coli*, 055:B5; Sigma) for 6 h. LPS is a well-established immunostimulant and the concentration used here upregulates cytokines in trout hepatocytes (Philip et al., 2012). Liver slices were collected as described above for IL-6, IL-8, and SOCS transcript analysis.

6.3.6 Cortisol and LPS effects on GH signalling

The aim of this study was to identify the mechanisms by which cortisol and LPS modulate GH signalling. Liver slices were pre-incubated with control media or media

containing cortisol (100ng/ml; Sigma), LPS (30μg/ml) or a combination of cortisol and LPS for 24 h (0 time) after which they were incubated with control media or media containing bGH (500ng/ml). Following GH addition, liver slices were collected either at 10 min to confirm JAK/STAT activation or at 6 h for IGF-1 transcript analysis. SOCS-1, SOCS-2, GHR-1 and GHR-2 transcript abundance were also measured at 0 time.

6.3.7 Glucose analysis

Media glucose levels were measured by monitoring NAD reduction by the hexokinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) assay method in Tris buffer (120 mM Tris-base, 80 mM Tris-HCl, 5 mM NAD, 2 mM MgS04, 5 mM ATP). The reaction was started with G6PDH (0.4 U/ml) and HK (0.5 U/ml). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA).

6.3.8 SDS-PAGE and Immunodetection

Liver slices were sonicated in lysis buffer (50mM Tris buffer; pH 7.5) containing phosphatase inhibitors [Na_3VO_4 (2 mM) and NaF (5mM)] and protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche). Protein concentration was measured using the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as the standard. All samples were diluted in Laemmli's sample buffer (1 M tris-HCl, pH 6.8, 60 mM, glycerol 25%, SDS 2%, β-mercaptoethanol 14.4 mM, bromophenol blue 0.1%). Total protein (50 μg) was separated on a 7.5% SDS-PAGE and transferred to nitrocellulose membrane and blocked with 5% solution of BSA in 1 X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween, pH 7.5) for 1 h at room temperature. This was followed by an overnight incubation (1:1000 dilution) with either total-JAK2, total-STAT5 or phospho-STAT5 (Tyr 694) monoclonal rabbit antibodies (Cell Signaling Technology, Beverly, MA). Blots were then incubated for 1 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibody (Bio-rad; 1:3000 dilution in 5% BSA). Protein bands were detected with Luminata Crescendo Western HRP substrate (EMD Millipore) and imaged using Pharos FX Molecular Imager (Bio-rad). Protein band intensity was quantified using AlphaImager HP™ (Alpha

Innotech, CA). Equal loading was confirmed by incubation of membranes with Cy3™ conjugated monoclonal mouse β-actin antibody (Sigma, 1:1000) for 1 h at room temperature.

6.3.9 Quantitative real-time PCR (qPCR)

Total RNA was isolated from liver slices using RiboZol reagent according to the manufacturer's instructions (Amresco, OH, USA) and the concentration determined at 260/280 nm using a Nanodrop. The RNA samples were DNase-treated (MBI Fermentas, ON, CAN) to avoid genomic contamination. The first strand 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 treatments and used for the normalization of transcript abundance.

6.3.10 Statistical analysis

Data are shown as mean ± standard error of mean (SEM). Statistical comparisons used analysis of variance (ANOVA) followed by Holm-Sidak's post hoc test to determine significance between groups (SigmaStat, Systat Software Inc., San Jose, CA, USA). Statistics were performed either on raw or log transformed data, when necessary to meet normality and equal variance assumptions. A probability level of $p < 0.05$ was considered significant. All statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA).

Table 1: **Gene-specific primers for quantitative real-time PCR.**

List of genes (Gene ID), forward and reverse primer sequences, annealing temperature and amplicon size. IL-1β: interleukin-1 beta; IL-8: interleukin-8; IL-6: interleukin-6; SOCS: suppressors of cytokine signalling; IGF-1: insulin like growth factor-1; GHR-1: growth hormone receptor-1; GHR-2: growth hormone receptor-2; EF1α: elongation factor 1α.

Gene ID	Primer sequences (5'-3')	Temp (^{o}C)	Amplicon size (bp)
IL-8	Fwd: CACTGAGATCATTGCCACTCTGA Rev: ATGACCCTCTTGACCCACGG	60	81
IL-6	Fwd: CTTCATCATCAGTCAGGAG Rev: CCCCTTAACTAACACCAC	59	118
SOCS-1	Fwd: GATTAATACCGCTGGGATTCTGTG Rev: CTCTCCCATCGCTACACAGTTCC	63.3	136
SOCS-2	Fwd: TCGGATGACTTTTGGCCTAC Rev: CCGTTCTTCTCTCGTTTTCG	60	102
IGF-1	Fwd: TGGACACGCTGCAGTTTGTGTGT Rev: CACTCGTCCACAATACCACGGTT	68	120
GHR-1	Fwd: TGAACGTTTTTGGTTGTGGTCTA Rev: CGCTCGTCTCGGCTGAAG	60	61
GHR-2	Fwd: CATGGCAACTTCCCACATTCT Rev: GCTCCTGCGACACAACTGTTAG	60	65
EF1α	Fwd: CATTGACAAGAGAACCATTGA Rev: CCTTCAGCTTGTCCAGCAC	56	95

6.4 Results

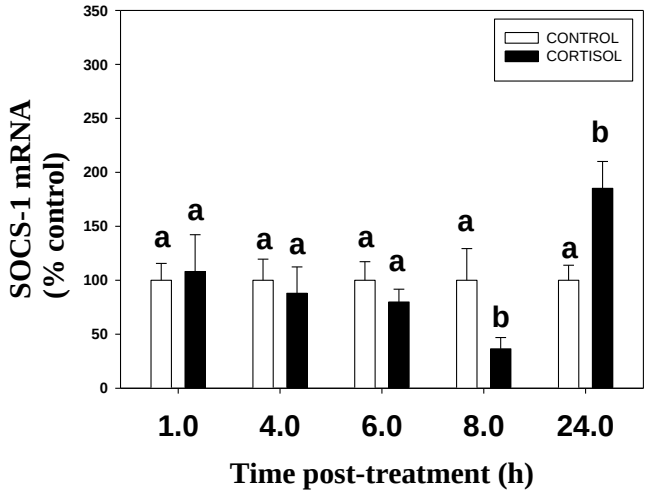
6.4.1 Cortisol upregulates SOCS expression

Exposing liver slices to stress levels of cortisol (100 ng/ml) upregulated liver SOCS-1 and SOCS-2 mRNA levels transiently (Figs. 1A and 1B). The cortisol effect was significant at 24 h, but not at earlier time-points, up to 8 h after-exposure. There was a significant drop in SOCS-1 mRNA level in the cortisol group at 8 h compared to the control, but not at any other time points (Fig. 1A). Cortisol had no significant effect on SOCS-1 or SOCS-2 mRNA abundances at 1, 4 and 6 h after hormone addition. The significant increase in SOCS-1 and SOCS-2 mRNA abundance seen with cortisol at 24 h was completely abolished by Mifepristone, a GR antagonist (Figs. 2A and 2B).

Figure 1: Cortisol upregulates SOCS expression

The effect of cortisol on the temporal profiles of SOCS-1 (A) and SOCS-2 (B) mRNA abundance in rainbow trout liver. Liver slices were incubated with either control media or media containing cortisol (100ng/ml) for 1, 4, 6, 8 and 24 h. Values are plotted as % control and show mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects within each timepoint; (two way ANOVA, $p < 0.05$).

A



B

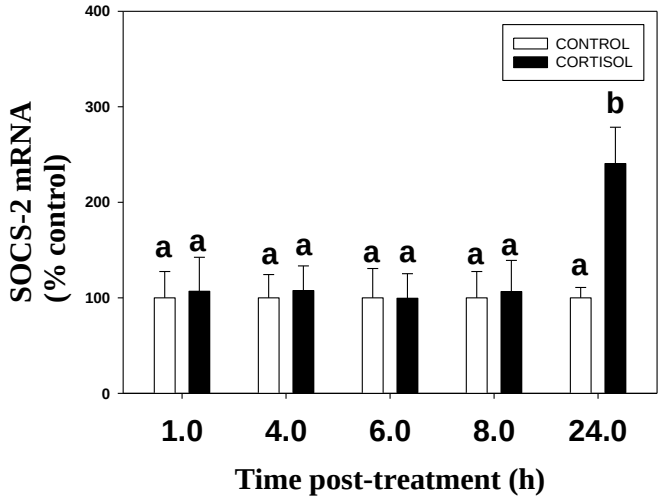
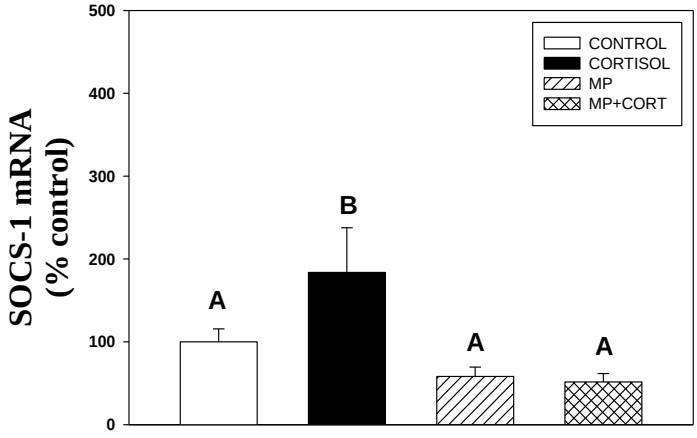


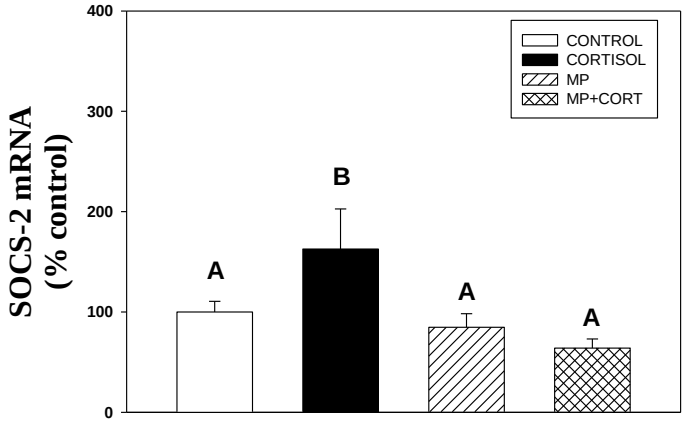
Figure 2: Glucocorticoid receptor signalling is involved in SOCS upregulation

The effect of cortisol and mifepristone either alone or in combination on SOCS-1(A) and SOCS-2 (B) mRNA abundance in rainbow trout liver. Liver slices were incubated with control media or media containing cortisol (100 ng/ml), mifepristone (1000 ng/mL; Sigma) or a combination of mifepristone and cortisol for 24 h. Values are plotted as % control and show mean ± S.E.M (n = 6 independent fish); different upper case letters denote significant treatment effects (One way repeated measures ANOVA, $p < 0.05$).

A



B



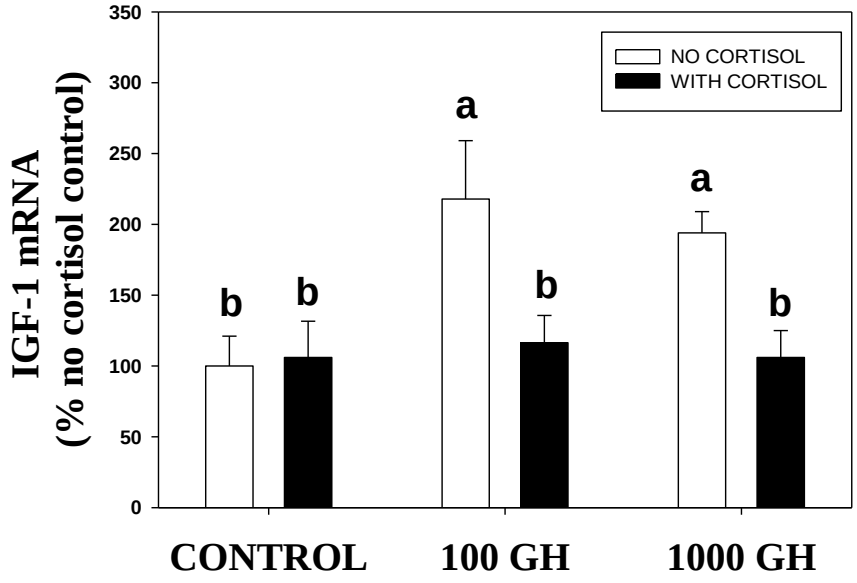
6.4.2 Cortisol suppresses GH signalling

As shown before in trout hepatocytes (Reindl et al., 2011), GH increased liver IGF-1 mRNA abundance at 6 h after hormone addition (Fig. 3A). This corresponded with increased STAT5 activation (ratio of phospho STAT5 to total STAT5) by GH, especially at the highest concentration (Fig. 3B). GH treatment did not significantly affect total JAK2 expression compared to the controls (Fig. 3C). Cortisol exposure for 24 h did not significantly affect IGF-1 transcript levels (Fig. 3A), but the steroid incubation significantly reduced GH-induced IGF-1 mRNA abundance in trout liver (Fig. 3A). Cortisol incubation also significantly reduced GH-induced STAT5 activation (Fig. 3B). Moreover, cortisol by itself downregulated total JAK2 levels, which is a key player in STAT5 activation (Fig. 3C).

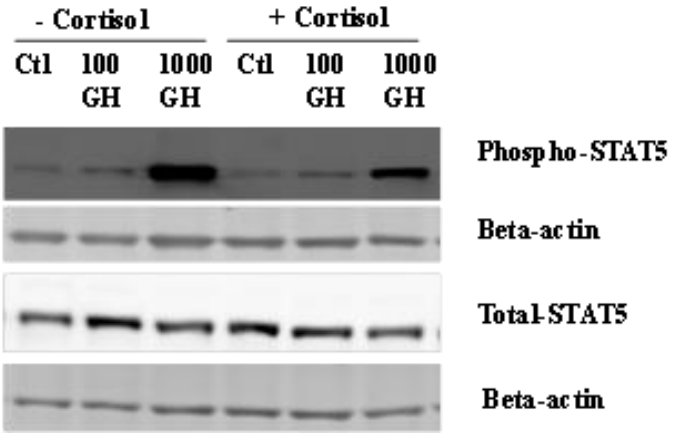
Figure 3: Cortisol suppresses GH signalling

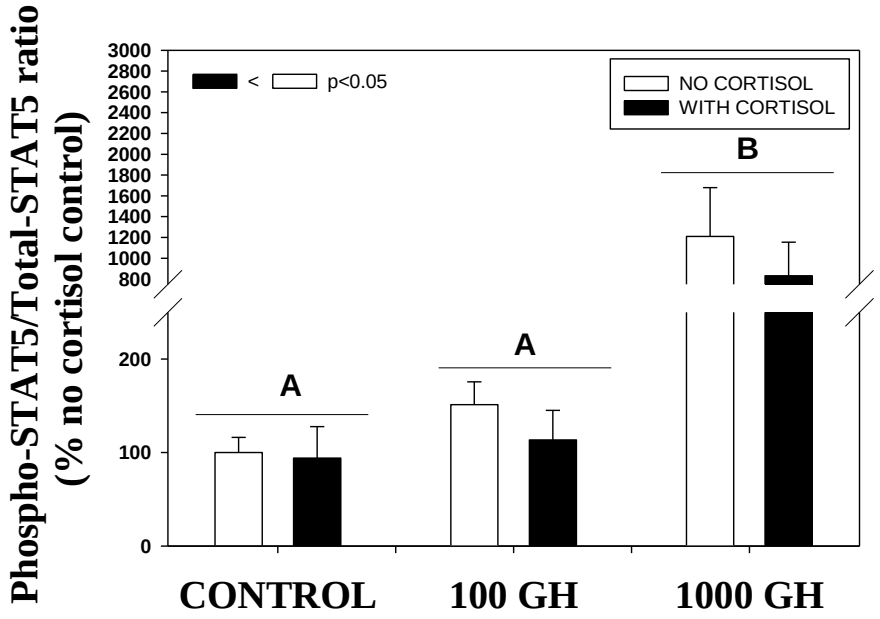
The effect of cortisol in combination with GH on IGF-1 mRNA abundance (A), STAT5 phosphorylation (B) and total JAK2 protein expression (C) in rainbow trout liver. Liver slices were pre-incubated with cortisol (100ng/ml; Sigma) or control media for 24 h and then stimulated with GH (100ng/ml or 1000ng/ml) for either 10 min (JAK/STAT) or 6 h (IGF-1). Values are plotted as % no cortisol control and shown as mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects; different upper case letters denote overall treatment effects between the control, 100 GH and 1000 GH groups; the inset shows overall cortisol effects (two way repeated measures ANOVA, $p < 0.05$). Representative western blots are shown above the histogram in figures 3B and 3C.

A

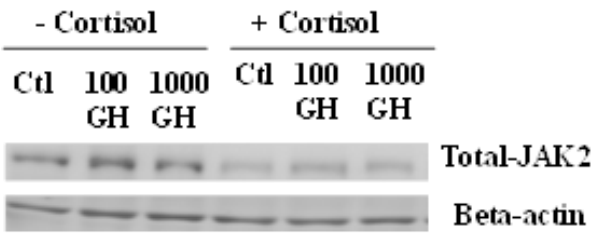


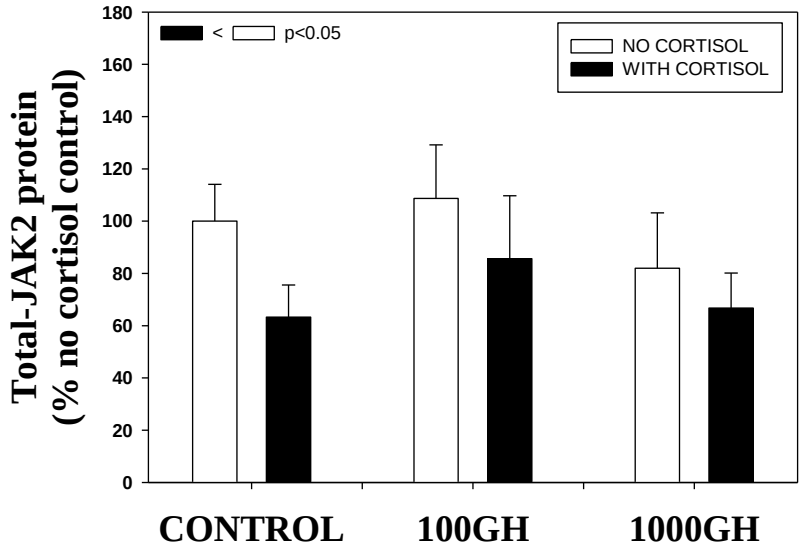
B


- Cortisol
+ Cortisol
Ctl
100 GH
1000 GH
Ctl
100 GH
1000 GH
Phospho-STAT5
Beta-actin
Total-STAT5
Beta-actin


Phospho-STAT5/Total-STAT5 ratio
(% no cortisol control)
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
200
100
0
< p<0.05
NO CORTISOL
WITH CORTISOL
A
A
B
CONTROL
100 GH
1000 GH

C


- Cortisol
+ Cortisol
Ctl
100 GH
1000 GH
Ctl
100 GH
1000 GH
Total-JAK2
Beta-actin


Total-JAK2 protein (% no cortisol control)
180
160
140
120
100
80
60
40
20
0
< p<0.05
NO CORTISOL
WITH CORTISOL
CONTROL
100GH
1000GH

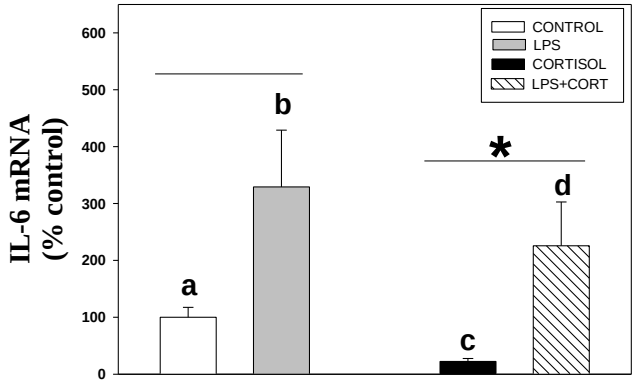
6.4.3 Cortisol suppresses LPS signalling

Exposure of liver slices to LPS significantly increased liver IL-6 and IL-8 mRNA abundances (Figs. 4A and 4B) confirming LPS stimulation. The LPS-induced IL-8 transcript levels were not altered by co-incubation with cortisol (Fig. 4B). However, cortisol incubation significantly reduced the LPS-induced increase in IL-6 transcript levels in trout liver slices (Fig. 4A). This corresponded with an overall increase in cortisol-induced SOCS-2 mRNA abundance in trout liver slices (Fig. 4C).

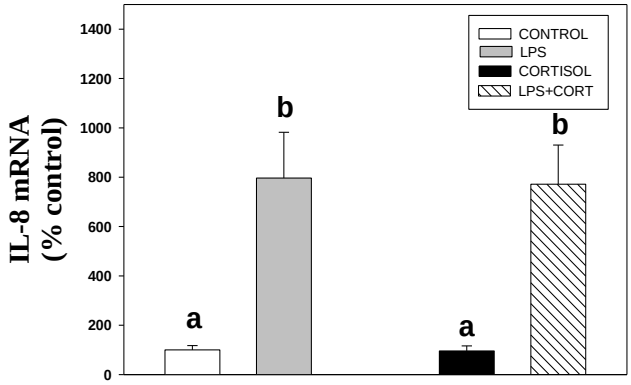
Figure 4: Cortisol suppresses LPS signalling

Cortisol modulates LPS-induced IL-6 (A) but not IL-8 (B) mRNA abundance in rainbow trout liver. This is paralleled by an increase in SOCS-2 expression with cortisol exposure (C). Liver slices were pre-incubated with control media or media containing cortisol (100ng/ml) for 24 h after which they were incubated with control media or media containing LPS (30μg/ml) for 6 h. Values are plotted as % control and shown as mean ± S.E.M (n = 7 independent fish); different lower case letters denote significant treatment effects; * denotes overall cortisol effects (two way repeated measures ANOVA, $p < 0.05$).

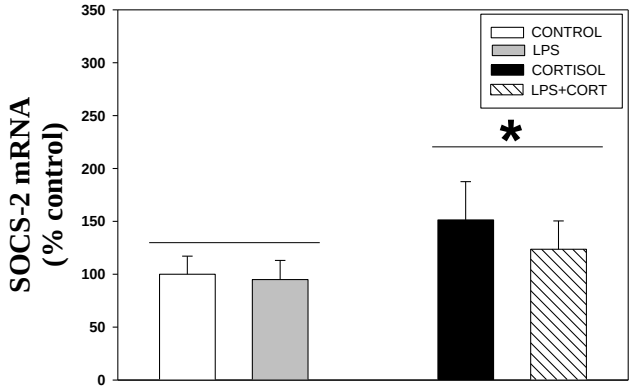
A


IL-6 mRNA
(% control)
CONTROL
LPS
CORTISOL
LPS+CORT
600
500
400
300
200
100
0
a
b
c
d
*

B


IL-8 mRNA
(% control)
CONTROL
LPS
CORTISOL
LPS+CORT
1400
1200
1000
800
600
400
200
0
a
b
a
b

C


SOCS-2 mRNA
(% control)
CONTROL
LPS
CORTISOL
LPS+CORT
350
300
250
200
150
100
50
0
*

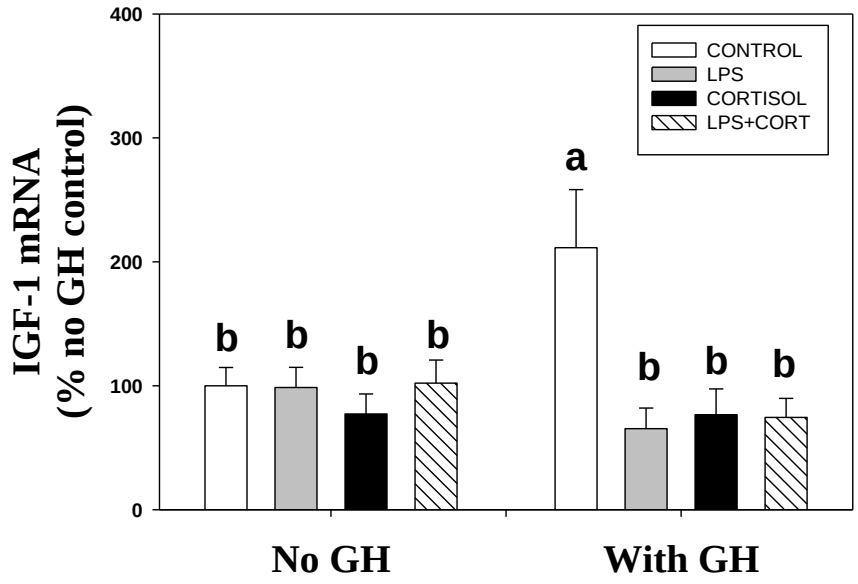
6.4.4 LPS suppresses GH signalling

LPS incubation for 24 h did not have any significant effect on IGF-1 transcript levels (Fig. 5A), but LPS alone or in combination with cortisol significantly reduced GH-induced IGF-1 mRNA abundance in liver slices (Fig. 5A). LPS incubation either alone or in combination with cortisol significantly reduced GH-induced STAT5 activation (Fig. 5B). This reduction seen by co-incubation of cortisol and LPS was greater than in the presence of cortisol alone (Fig. 5B). LPS either alone or in combination with cortisol did not have any significant effect on total JAK2 protein expression, whereas cortisol by itself significantly reduced total JAK2 protein expression (Fig. 5C). LPS treatment significantly reduced GHR-1 (Fig. 6A) and GHR-2 (Fig. 6B) mRNA abundance in trout liver slices. While cortisol incubation either alone or in combination with LPS significantly increased GHR-1 (Fig. 6A), there was no significant effect of either cortisol alone or in combination with LPS on GHR-2 mRNA abundance (Fig. 6B).

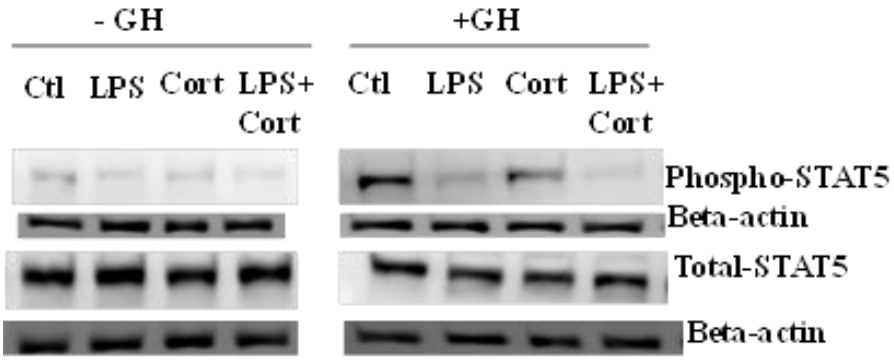
Figure 5: Interaction of cortisol and LPS on GH signalling

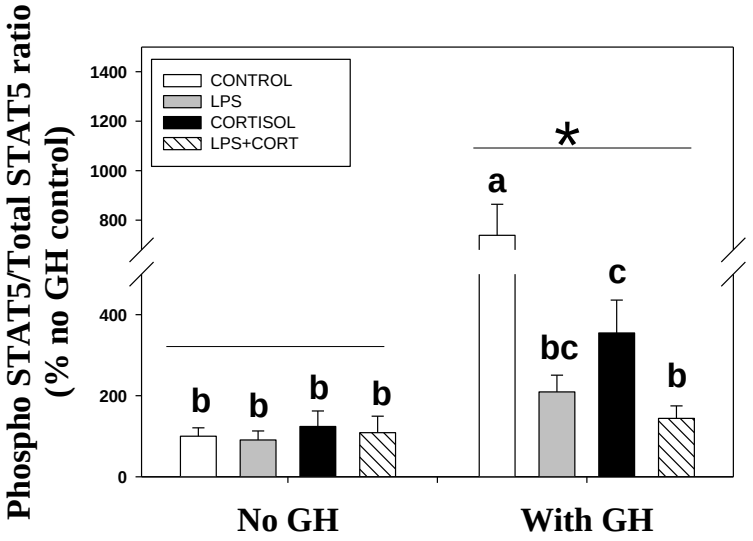
The graphs show the effects of cortisol and LPS either singly or in combination in modulating GH stimulation of IGF-1 mRNA abundance (A), STAT5 phosphorylation (B) and total JAK2 protein expression (C) in rainbow trout liver. Rainbow trout liver slices were pre-incubated with control media or media containing cortisol (100ng/ml; Sigma), LPS (30μg/ml) or a combination of cortisol and LPS for 24 h, after which they were incubated with or without GH (500ng/ml) for either 10 min (JAK/STAT) or 6 h (IGF-1). Values are plotted as % no GH control and show mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects; * denotes overall GH effects; the inset shows overall cortisol effects (two way repeated measures ANOVA, $p < 0.05$). Representative western blots are shown above the histogram in figures 5B and 5C.

A

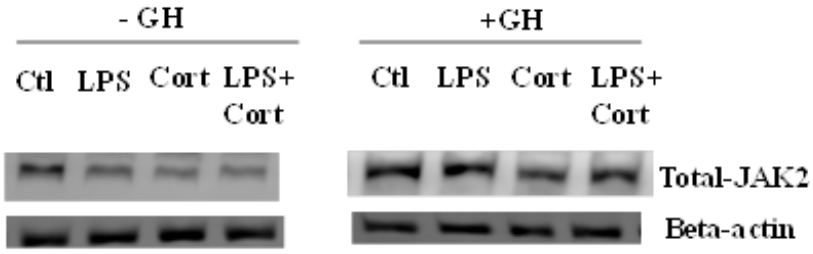


B


- GH
+GH
Ctl
LPS
Cort
LPS+ Cort
Ctl
LPS
Cort
LPS+ Cort
Phospho-STAT5
Beta-actin
Total-STAT5
Beta-actin


Phospho STAT5/Total STAT5 ratio (% no GH control)
CONTROL
LPS
CORTISOL
LPS+CORT
1400
1200
1000
800
400
200
0
*
a
bc
c
b
b
b
b
b
No GH
With GH

C


- GH
+GH
Ctl
LPS
Cort
LPS+ Cort
Ctl
LPS
Cort
LPS+ Cort
Total-JAK2
Beta-actin

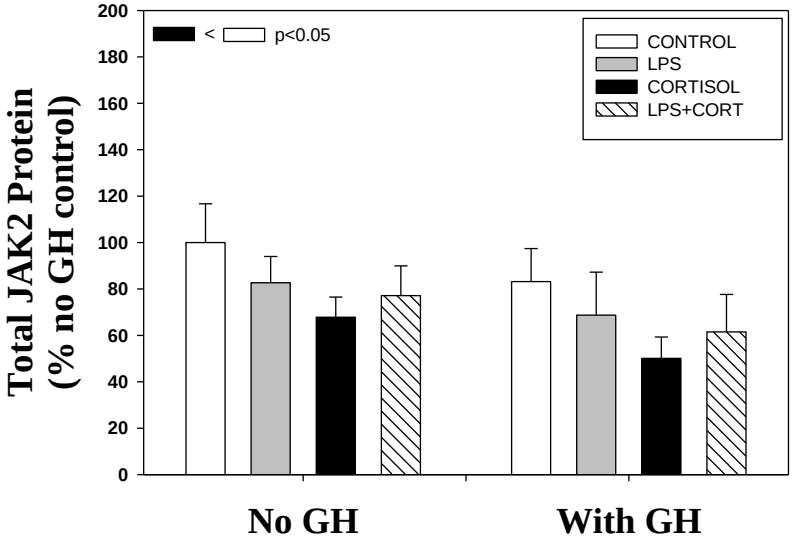
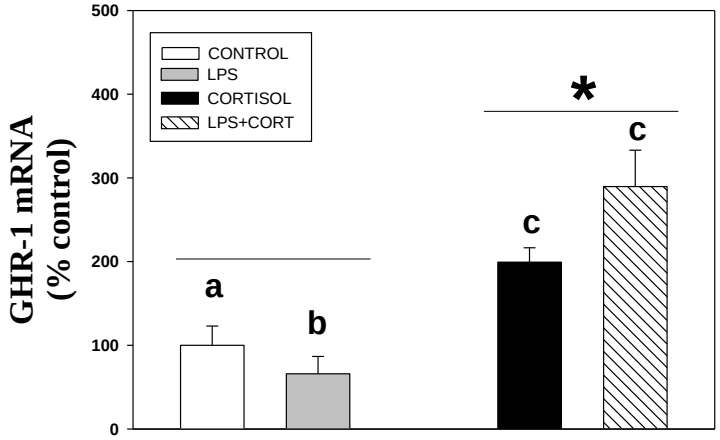

Total JAK2 Protein (% no GH control)
200
180
160
140
120
100
80
60
40
20
0
< p<0.05
CONTROL
LPS
CORTISOL
LPS+CORT
No GH
With GH

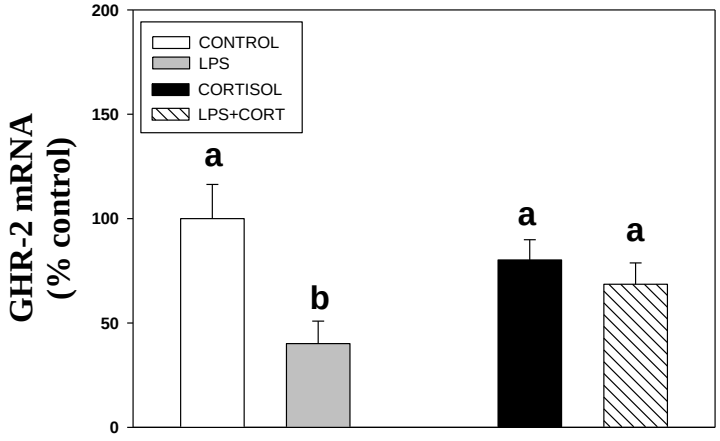
Figure 6: Effect of cortisol and LPS on GH receptors.

The effect of cortisol and LPS on GHR-1 (A) and GHR-2 (B) mRNA abundance in rainbow trout liver. Rainbow trout liver slices were pre-incubated with control media or media containing cortisol (100ng/ml; Sigma), LPS (30μg/ml) or a combination of cortisol and LPS for 24 h. Values are plotted as % control and show mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects; * denotes overall cortisol effects (two way repeated measures ANOVA, $p < 0.05$).

A



B



6.5 Discussion

We demonstrate a novel mechanism by which cortisol signalling curtails energy demanding growth and inflammatory responses during stress in fishes. This cross-talk between stress-immune-growth processes in fishes involves upregulation of SOCS-1 and SOCS-2 by cortisol, and the attendant reduction in JAK/STAT signalling.

The SOCS genes are traditionally known for their role as negative regulators of cytokine signalling. They regulate the JAK/STAT pathway which is an intracellular signalling pathway shared by a variety of cytokines, as well as leptin, GH, and prolactin (Croker et al., 2008). Consequently, SOCS may integrate and regulate diverse physiological functions involving energy substrate re-partitioning (Philip et al., 2012, 2014). In addition, we recently showed that the SOCS genes are cortisol responsive, suggesting a role for this protein in energy re-allocation during stress in fishes (Philip et al., 2012; this study). Stressed levels of cortisol upregulates SOCS-1 and SOCS-2 and this is mediated by GR signalling. This response is not rapid but is longer term and may be playing a role in the delayed immune and growth suppression observed with stress (Tort, 2011; Vijayan et al., 2010). A key role for cortisol during the stress response is to mobilize and reallocate energy substrates to cope with the energy demands associated with stress adaptation. Consequently, prolonged exposure to chronic stress will increase energy mobilization resulting in decreased disease resistance, reduced growth and an overall decline in fitness (Tort and Teles, 2011). This reduction of performance can be viewed as a consequence of the animals altered energy budget, with an increase in the metabolic requirements to cope with stress, leading to a reduction of immune and growth potential. We propose cortisol induced upregulation of SOCS may be playing a role in this energy substrate reallocation during stress in fishes.

Our results indicate that stressed levels of cortisol inhibit GH action by downregulating JAK/STAT signalling. GH signalling in fishes involve activation of the JAK2-STAT5 pathway. IGF-1 is a primary mediator of the effects of GH and the major endocrine promoter of growth in salmonids. The liver IGF-1 transcript abundance is consistently elevated in response to GH stimulation and used as a marker of GH signalling (Reindl et al., 2011). This was also the case in the present study, and the higher IGF-1

mRNA levels with GH corresponded with an upregulation in STAT5 phosphorylation in trout liver and supports previous studies in fishes (Pierce et al., 2011). However, cortisol levels mimicking chronic stress reduced GH signalling and the corresponding IGF-1 expression in trout liver. The cortisol mediated suppression of IGF-1 gene expression is in agreement with previous studies in mammals and teleosts (Unterman et al., 1993; Rodgers et al., 1995; Pierce et al., 2011; Kajimura et al., 2003), but the mode of action was unclear. Here we show for the first time that cortisol inhibition of GH signalling in the liver involves inhibition of STAT5 phosphorylation/activation in trout. Additionally, cortisol also downregulated total JAK2 levels, which are key players in STAT5 activation. These effects correlate with the cortisol mediated upregulation of SOCS-1 and SOCS-2, which are important negative regulators of GH action (Kile and Alexander, 2001). The SOCS proteins are known to down-regulate mammalian GH signalling by multiple complementary mechanisms. They either directly inhibit JAK activity by acting as pseudosubstrates, prevent STAT phosphorylation by competing with STAT proteins for specific receptor phosphotyrosine residues or ubiquitinate putative targets such as JAK2 , directing their subsequent degradation through the proteasome (Croker et al., 2008). The cortisol-induced upregulation of SOCS, together with the downregulation of total JAK2 levels and STAT5 phosphorylation by this steroid, leads us to propose SOCS as a novel mechanism linking stress to growth inhibition in teleosts.

SOCS proteins also function in a negative feedback loop to restrain inflammatory responses, and their involvement in glucocorticoid-mediated immunosuppression in teleost was recently proposed (Philip et al., 2012). Here we provide evidence to support this proposal. Cytokines are key mediators of the innate immune response and their expression is a key marker of immune function (Engelsma et al., 2002). We saw a strong activation of typical pro-inflammatory cytokines IL-6 and IL-8 in response to LPS, similar to that observed in previous studies (Castro et al., 2011). Chronic stress suppresses immune responses in fishes (Tort, 2011), and this is supported by the cortisol-mediated reduction in LPS-induced IL-6 expression. But cortisol had no effect on LPS induced IL-8 expression in the liver. While IL-6 is a cytokine signalling through the JAK/STAT pathway, IL-8 is a

chemokine mediating it's effects through G-protein-linked receptors (Secombes et al., 2001). Furthermore, the SOCS genes have been shown to inhibit LPS-induced IL-6 production by regulating JAK/STAT signalling in mammals (Kimura et al., 2005). Taken together, these results further confirm that the cortisol effect on immune suppression involve SOCS upregulation. We propose that SOCS upregulation by cortisol inhibits LPS-induced IL-6 production and downstream IL-6 signalling in fishes by regulating JAK/STAT signalling. It should be noted that cortisol mediated suppression of LPS induced IL-6 expression was observed at 6 h post LPS addition. This does not exclude the possibility of cortisol down-regulating other cytokines through inhibitory interactions with pro-inflammatory transcription factors, such as NF-κB and AP-1 (Castro et al., 2011) at other time points.

Mounting an immune response can itself be energy-demanding. This can result in energy trade-offs and the mobilisation of energy stores and redirection of energy towards immune function at the expense of normal body processes like growth (Rauw, 2012). LPS challenge and a combination of LPS challenge and cortisol also reduced GH signalling and the corresponding IGF-1 expression in rainbow trout liver by preventing STAT5 phosphorylation/activation. LPS has been previously shown to downregulate growth hormone signalling in mammalian models by impairing STAT5 activation and JAK/STAT signal transduction (Chen et al., 2007; Wang et al., 2008). Our results also reveal for the first time that LPS suppresses GH signalling in fish. Since LPS reduced SOCS-2 expression, the LPS mediated reduction in STAT5 phosphorylation seems to be SOCS independent. This is contrary to mammalian studies where LPS-mediated downregulation of GH signalling involved an increase in SOCS isoforms (Chen et al., 2007). In trout, our results indicate that LPS effect on GH action occurs upstream of STAT5 modulation. Recent studies have described the co-existence of two clades of putative receptors for GH (GHR1 and GHR2) in fishes, both of which are highly expressed in the liver (Jiao et al., 2006; Pierce et al., 2012). However, functional differences between GHR1 and GHR2 are not clear. We show that LPS decreases the expression of both GHR1 and GHR2 and this may be playing a role in the attenuated GH signalling in trout liver. This is in agreement with mammalian studies showing that LPS directly suppresses GHR expression, thereby contributing to GH resistance

(Dejkhamron et al., 2008; Wang et al., 2008). The down-regulation of the GHR is achieved through complex mechanisms that involve rapid ubiquitin-dependent endocytosis of the receptor, the action of tyrosine phosphatases, and the degradation by the proteasome (Flores-Morales et al., 2006) . However, the mechanisms involved in the downregulation of GHR in fish by LPS remains to be determined. In contrast, cortisol stimulation increased GHR1 expression in rainbow trout liver similar to that seen in the seabream (*Pagellus bogaraveo* species) (Jiao et al., 2006). Cortisol being the main mediator of metabolic adjustments to stress in the liver (Vijayan et al., 2010) taken together with the fact that GHR1 levels appear to be regulated by cellular metabolic status and may be involved in liver metabolism (Pierce et al., 2012) suggest a functional significance to this observation warranting further investigation. The LPS+cortisol combination group showed lower levels of STAT5 phosphorylation than the cortisol treated group which might be explained as an additive effect of LPS mediated downregulation of GHR receptors and cortisol mediated up-regulation of negative regulators of GH signalling, namely the SOCS.

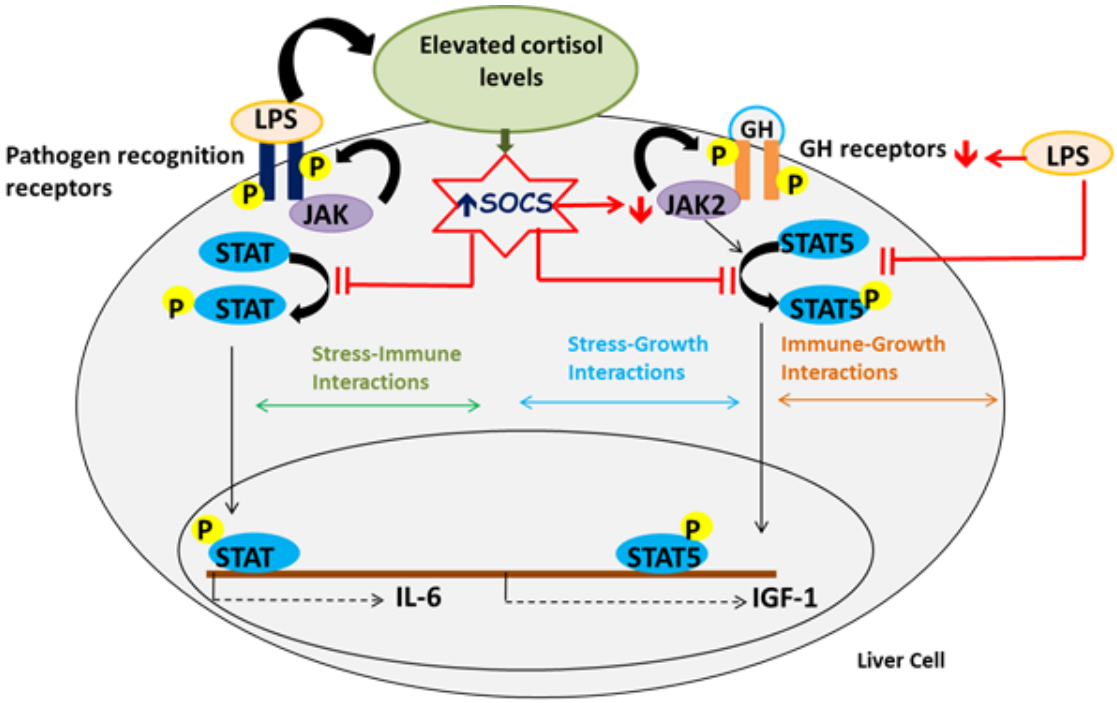
In summary, the study reveals novel mechanisms involved in the cross-talk between stress-immune-growth processes in rainbow trout (Figure 7). Our results demonstrate that cortisol upregulates SOCS expression during stress, which in turn inhibits GH and LPS responses by targeting JAK/STAT signalling. In contrast, immune challenge with LPS inhibits GH responses by downregulating growth hormone receptors and not by the modulation of SOCS expression. Stress-immune, stress-growth and immune-growth interactions so far had been investigated completely independently from each other. Our findings provide a novel molecular link namely the cortisol mediated upregulation of SOCS as a mechanism linking stress effects on growth and the suppression of immune responses in fishes.

6.6 Acknowledgements

This study was supported by funding from the Natural Sciences and Engineering Research Council of Canada, Discovery Grant to MMV.

Figure 7: Mechanism of cortisol mediated inhibition of growth and immune signalling.

Cortisol conditions mimicking chronic stress increase SOCS expression and reduce GH signalling and the corresponding IGF-1 expression in rainbow trout liver by preventing STAT5 phosphorylation and also by decreasing total JAK2 protein levels. The cortisol-induced upregulation of SOCS may be playing a role in the suppression of LPS-induced IL-6 expression (a cytokine signalling through the JAK/STAT pathway). Immune challenge with LPS may indirectly inhibit GH signalling by elevating plasma cortisol levels or directly inhibit GH signalling and the corresponding IGF-1 expression by downregulating growth hormone receptors 1 and 2 and by preventing STAT5 phosphorylation.



Chapter 7
General Conclusions

7.1 Summary of findings

The overall goal of this thesis was to understand the relationships and interactions between stress-immune-growth processes in fishes, with special focus on identifying molecular mechanisms, key to this interaction, and the impact of nutritional restriction on these processes. To this end, a series of *in vivo* and *in vitro* experiments were performed, attempts were made to correlate *in vivo* findings to *in vitro* data, and the following conclusions were drawn:

1. Though a non-classical immune tissue, the liver is still capable of directly responding to an immune stimulation by upregulating innate immune response mediators, including cytokines and acute phase response proteins (APP). Additionally, stressed levels of cortisol modulate these responses and most of these cortisol effects were mediated through glucocorticoid receptor (GR) signalling. This was also the first study to demonstrate that cortisol upregulates suppressors of cytokine signalling (SOCS) 1 and SOCS-2 mRNA levels in fishes, and this involves GR signalling. Overall, this study underscores a novel role for the liver in integrating stress-immune responses in trout with the possibility that the upregulation of SOCS genes by cortisol may be playing a key role in suppressing cytokine signalling and the associated inflammatory response during stress in fishes (chapter 2)
2. Fundamental differences occur in the cytokine and SOCS responses to fasting and LPS stimulation between two salmonids, namely the anadromous Arctic charr (*Salvelinus alpinus*) that undergo natural long term fasting as a life strategy (chapter 3), and the rainbow trout (*Oncorhynchus mykiss*) that do not naturally fast (chapter 4). In the rainbow trout there was no change in the degree of cytokine response to LPS stimulation between the fed and fasted groups, while fasted charr showed a lesser degree of cytokine response to LPS compared to the fed charr. This correlated with the fact that in the rainbow trout, fasting and LPS stimulation downregulated some of the SOCS isoforms, while in the Arctic charr, fasting and LPS stimulation upregulated the SOCS isoforms, possibly an adaptive trait that arose along with their anadromous life-strategy to conserve energy resources by restricting energy

demanding pathways, including growth hormone and cytokine signalling. Overall, these studies underscore a novel role for the SOCS genes in integrating stress-immune-growth responses in fishes *in vivo* and a potential role for the SOCS genes in energy re-partitioning.

3. Carry-over effects of prior fasting and LPS stimulation are evident and reflected on the metabolic and molecular responses to handling stress in rainbow trout, even after apparent recovery from these previous stressors. It is also evident from this study that handling stress modulates SOCS expression and this response is influenced by fasting and LPS injection. The upregulation of SOCS-2 during handling stress may be playing a key role in the metabolic adjustments associated with acute stress adaptation in fishes (chapter 5).
4. Cortisol mediated upregulation of SOCS mRNA levels is seen primarily during prolonged exposure to cortisol. This suppresses GH and LPS induced JAK/STAT signalling, demonstrating a novel role for SOCS in linking stress effects on immune function and growth. Specifically, cortisol reduced STAT5 phosphorylation/activation and decreased total JAK2 protein levels. LPS also suppressed GH induced JAK/STAT signalling but this involved the downregulation of growth hormone receptors (GHRs). Overall these studies underscore a role for the SOCS genes in integrating stress-immune-growth responses in fishes and provide a novel molecular mechanism, namely cortisol-mediated upregulation of SOCS, linking stress effects on growth and the suppression of immune responses in fishes (chapter 6).

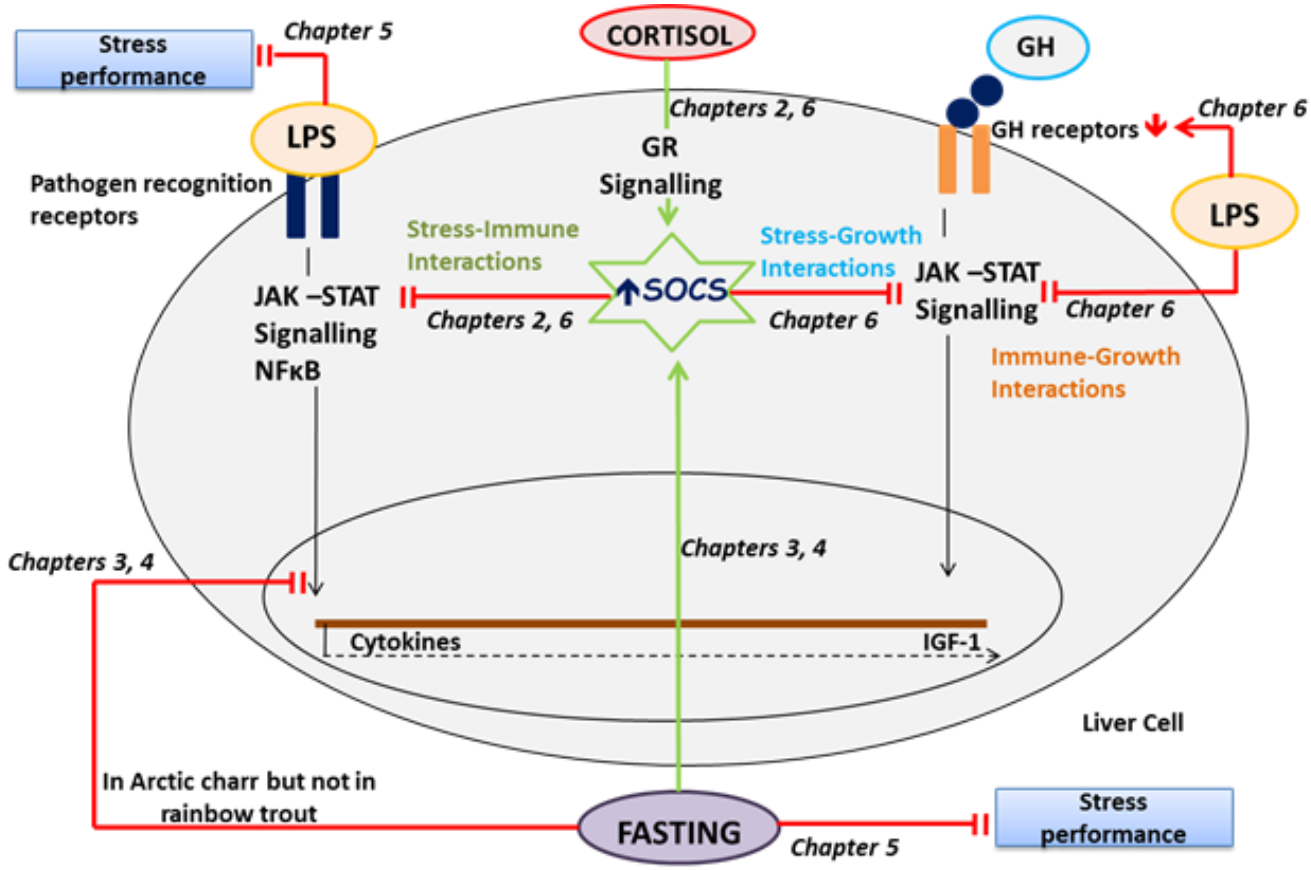
7.2 Scientific relevance and future perspectives

Taken together, the work presented in this thesis adds a great deal to the relative paucity of information on the molecular basis of stress-immune-growth interactions in fishes (see Figure 1 for summary). Stress-immune, stress-growth and immune-growth interactions so far, had been investigated independent from each other. The results from this thesis for the first time suggest novel functional roles for the SOCS genes as integrators of stress-immune-

growth processes in fishes and their involvement in energy re-partitioning during stress and natural life-history events.

Figure 1: Stress-Immune-Growth interactions in fishes

Conceptual diagram visualizing the major contributions of this thesis to the better understanding of stress-immune-growth interactions in fish



LPS was used as the immunostimulant throughout this thesis. Though LPS elicited a robust immune response by upregulating cytokines and APP expression in tissues, including the liver and spleen, the exact mechanism by which LPS exerts its endotoxic effects in fishes are not clear. High concentrations of LPS (μg/ml) are needed to elicit an immune response in fishes as opposed to mammals (ng/ml) (Sepulcre et al., 2009). Moreover, the lack of a TLR4 ortholog in some fish species and the lack of the essential costimulatory molecules for LPS activation via TLR4 (i.e., myeloid differentiation protein 2 (MD-2) and CD14) in the fish genome suggests that LPS recognition in fish may be different from that of mammals (Sepulcre et al., 2009). Though it has been suggested that beta-2 integrins, may play a primary role in the activation of fish immune cells by LPS (Iliev et al., 2005), the exact mechanism for LPS endotoxicity in fishes warrants further investigation.

The stress/cortisol responsiveness of the SOCS genes and its impact on other physiological processes like growth and immune function is another avenue that warrants further research. Genome duplication events have led to multiple copies of SOCS fanily members like the three trout SOCS-2s in fish (Wang et al., 2011). Yet, the functional significance of having multiple copies of these SOCS family members are not clear. Nevertheless, initial functional studies show that fish SOCS affect cytokine and GH signalling via the JAK2-STAT5 pathway like in mammals suggesting conserved functional roles for the SOCS family members across species (Wang et al., 2011; Reindl et al., 2011). However, fully elucidating the function of fish SOCS proteins will be complicated, given the multiple paralogues of SOCS members and cytokines/receptors, but is essential and should be explored.

The role of the SOCS genes in integrating different physiological processes may also have ramifications beyond our observed findings and should be further explored. Using the zebrafish as a model organism for these studies would allow the use of gene knockdown techniques to look at the impact of SOCS knockdown on stress, immunity, growth, reproduction and development. Moreover, the SOCS genes are now well recognized for their role in human disease, particularly their tumor suppressor and anti-inflammatory functions, and have been implicated in different human cancers, type 2 diabetes, tuberculosis and

metabolic syndromes (Trengove and Ward, 2013). Extensive anatomical, physiological, and genomic homologies between zebrafish and mammals will permit the translation of insights gained in zebrafish into advances in human medicine (Lieschke and Currie, 2007). Another aspect that should be further explored is the mechanisms responsible for SOCS upregulation during long-term fasting in the anadromous Arctic charr. The answer to this question might have implications in human medicine since fasting-induced GH resistance brought about by impairment of JAK/STAT signalling is a common observation in mammalian models (Beauloye et al., 2002). Moreover GH resistance is associated with conditions including sepsis, trauma, burns, AIDS, cancer, and renal or liver failure in humans (Jenkins and Ross, 1998). The carry-over effects of prior fasting and LPS stimulation on stress performance (chapter 5) is another avenue that should be investigated further since the cumulative and long-term effects of different types of stressors may impair stress performance in fishes over a life time. It is important for future studies to determine whether such cumulative effects have observable implications in wild fish, leading to a failure to grow and survive.

7.3 Conclusions

Wild fish populations and cultured fish species alike are increasingly important to Canada's economy. Stress from physical and chemical factors in different shapes and forms affect wild and cultured populations of fishes. While wild fishes are more prone to stress from temperature fluctuations, salinity changes and pollution, cultured fishes are exposed to routine hatchery stressors, including handling and crowding. Stress alters an animal's energy budget by consuming a large amount of energy, which in turn means less energy is available for normal body functions, including growth, immunity and reproduction. This can have long-term consequences on overall-health, fitness and eventually population dynamics. Better understanding of molecular mechanisms involved in stress-immune-growth interactions can indeed benefit stress management in aquaculture practices, and policy making for conservation of our wild fish populations. The results from this thesis suggest a novel role for SOCS in energy susbtrate partitioning during stress and natural life-history events in fishes.

Appendix A
Supporting Information for Chapter 2

Analysis of zebrafish SOCS-1 promoter for putative GREs

Promoter Prediction

The bioinformatics tool Promoter 2.0 was used for promoter prediction. The Promoter 2.0 prediction server can be found at http://www.cbs.dtu.dk/services/Promoter/. Promoter 2.0 which is used for the prediction of eukaryotic PolII promoters from DNA sequences, takes advantage of a combination of elements similar to neural networks and genetic algorithms to recognize a set of discrete sub patterns with variable separation as one pattern: a promoter. The neural networks use as input a small window of DNA sequence, as well as the output of other neural networks. Through the use of genetic algorithms, the weights in the neural networks are optimized to discriminate maximally between promoters and non-promoters. Typically, neural network- substrate interactions preset to reflect transcription factor binding to known sites such as the TATA box, cap site, CCAAT box and GC box are used to distinguish promoters from non promoter regions (Knudsen, 1999).

The zebrafish SOCS-1 genomic sequence was obtained from ENSEMBL and extended to include a 5' 3700 bp flanking region. This 5' 3700 bp flanking sequence was used as input for the promoter 2.0 program. Promoter 2.0 gave a highly likely prediction with a score of 1.143 at position 3300. This means that it is highly likely (95% confidence) that the transcription start site occurs within 100 base pairs upstream from position 3300.

Figure 1: Promoter 2.0 prediction results for zebrafish SOCS-1

Promoter 2.0 Prediction Results

INPUT SEQUENCE:

```
>Sequence
CATGATAAGAATTGTTTCATATAAATAAACAAGGGTAGATGGATTTCTAAACACTGGCTG        60
CTATTCAAGGAAACCACTCGAGTCTTTTTCTATTTGTGGCTTGTGGGGTTTAGTTCACGT      120
GACCTCGAACTGAAAGTGACCGGTCAGAATTACTTTAATGATAAGTGACAGGATGATAAA      180
TGTGAGACTAAATGTATAGTTAAGTATATCTTTGCAGGCTTGCAGCAAATAATATAATAC      240
ATTTAAATAGGCTACTCCCCATTTTTCAGAAAAACGCATGAAAACGTAAGGTAATTTAGA      300
GAAAAAAACATTAGAGAATTACAACGCCATCTCCAGGTAGCTTTGTATTAAAGCAGTTCA      360
CTAGCTTTCGCTTTCGTTTTCCTGTAAAGCAGTGGGATACGCAGAAAGAAACAGACTGAA      420
TTGTTTGTATATGCAAACATATTGCTATTGTACCATAAAGGAACAAGGTAAGGATTATAT      480
TCATATATTTATGACTATACAACTGCATATGGCTACAGACTGTATATATGTTGCAATAAT      540
TTTTCAGATTTCACCTCGAGTCTCTGCATGAGAGAGGGGATTCAGTCAGATAGCTTATTA      600
TAAATCTGAAGCACTGCATTGTGAATGACACCGATTTACACGGACATGCAAATTAATTAT      660
GTTAGTCTACAGTCTGTGAGATGCACTTGGAGGATATGTGCATGCATATACAGTTCTCAG      720
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TATATATTGGTGCATTTGAACAAAACAGATTTATTAAACAGATATATTTATTGCAATAAT      840
AAACTGTTCGTTACAGAACATCTCTGGAAATTAAAAGATACATTTAAATTCATGCAAAAT      900
ATAGCAAAAACATTTTATATAATATGTAATATTTATTATAATAATATTTTCCCTAATATA      960
TAAATTTTGGTTACTAATTTTTGAACCAATGTTGTAAGTTATTTTGTTAGATTGGCTCCA     1020
GATTTAGCTTCAGTGCTGACTAATCTAATGTATCTGCACAAATATAATATTGTAAAGCAA     1080
TCATATAGAACAGTGGTTCTCAAACTTTTTTCATCAAGTACCACCTCAGGAAAAAAATTG     1140
TCTCTCCAAGTACCACCAAAATGAGCAGTATTGAAATATACAGTAGCATAGTACGCCCAG     1200
TTAAGCAGCTGCAACTCTGCACAGTTAAAAAACGTGGCATATTAGCCCAGAAATATAGGC     1260
TATATGTCATATATGGCATATGATATGCATAATTTTTGATAAATTTTGAAAAGTGTGTAG     1320
CATATGCATGACATATTTAATTCCTCCGCGTACCACTAGAAGGAAGCCTGCGTACCAGTA     1380
GAAGTACATGTACCACAGTTTGAGAACCAATGATATAGAAAATAGTAATTTAAATGAGAT     1440
ATTTGTGAGGGGTGTACACATATATGCTGAGGACTGTATGACTGTTAAATTAAGACCTCA     1500
AACCTATCATCACGTAAAGTAGACACGCAATATATGGTCTTTCTGCCAAGATATCTAGAT     1560
TAAGCTAAAACTCGAATGATTACACTGGCGTCATGGTCTCAAAATGTCACTGCATAAAAA     1620
TGTGCTAAACGCGTTCAAGCAGTTCCTGCATCTGATAAAGTGGCTTGTTTCACGGAAGAT     1680
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ACTCTCCTCTAGTGGTGCTTCAACTAAATATCGTCTTTCTGAGAAATACTAGCGAAACCA     1800
AAAGTATAGGAGCCAGTAATTCTACTCCAATCCAAACAAGTCGTTTGAAAGGCAACCCTA     1860
ATCTGCATGATTGGAATTTCAGTTTCGCAATGATATCATTTAAATGAAACACAGGGGCAG     1920
GAGAGTTCATACTCAAAATGTTCCAAAGGTGTGCTAATAATAATGATGTCATTCATCTTG     1980
TTGACTTGGCTATAGGAATACAATTAGTTTGCAACTAAAGAGTACAAGCTCATGCAGATA     2040
TTGGATAAACACACACACACACACACACACACACACACACACACACACACACACACACAC     2100
ACACACAAAACAAATTAATCAAAAACGCATGCAAACCTACACTTAAGAAAAAGTTTCAAG     2160
AATGATTCCTTGGAATCATTATTCATTATCACTATTTTTTTAAAGTTAAGTGGTTGTAAA     2220
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GGTGGTTTAAATTTAACCTATTTAAGCCTAATCAAGAGAATAAAGATGTTTGCATAAGTG     2340
TGTTTAATAAACAGGTGGCTTTTGGTGCAGTTGTGCAGTGTTTACCACATCTGAGTGTAT     2400
ACAGACATCTCAGTCTATCTCTATTTGTAAGAAATGAAACTCATGGATAAATGTTCGCCG     2460
GGTGCGCAGGTGTGGAGTTGCAGGCCAAAATTGAGTTTCATTTATGGCTAAAACTGTACG     2520
CAGTGGACATCAGCTTACTTTCCAACTAACAGTCAAAAAAAAAAAAAAAACAGGTCAACTG     2580
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CAGATATGCATCAATAATATGTATGTTTATGTGCAATGCATGACATGGATAATGTGCAAA     2640
CCCAGGACGCATGAGTTTTTATTTCATAAAAAGAGATAACTCGAAAAAGTGCATATGTTT     2700

ATATTCAGGATTGTGAAATTTCGTTTTACATCTGTGCAGTGCAATAGAAGATAGATGTAC     2760
ATTTTCAAATACTTATATACTATGGTAACATGTTAAATACATGCATACATGTGCTTCTCC     2820
GCTTTCCAACAGTCAAAAATATTCAATAAAATTCATCTTCATTTCTATAGTGATTTTACA     2880
ATGTAGATTGTGTCAAAGCAGCTTAACATATAATATCAAAACAAATGCAGATGGGCATTG     2940
TCAATATTGACTTTGTTTATTCACAATGTACAGTGAGACACATAATGTGCAAACCCTGTA     3000

GTATGCTTATTTTCAGGATTGTGATATTAAGTTTTACATCTGGACATTGCAATAGAAGTT     3060
AGATGTACATATTTTAAATAATGATATGATAAAATGTTGTTAATTCTGTCCAGACAGACA     3120
CACACACATACATACATGAAATGTACAAAATTGTATTATAGTATTGAAAATGTACTTATA     3180
TCTTCTATTGAAATGCACAGATGTAAAACAAACTAAGTCCTGAAAATAAGCATATGCACT     3240
TTTCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATC     3300
TATCTGTCTATCTGTCTATCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTC     3360
TGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTAGCCTGCTTCTACTGAT     3420
TAACTGAGCTTTTTTACAAACACATGACAGTTTACAAACACATGACAGCATGATTATAGA     3480
TGTCATTTTCTTCCTCTTTGAAAGTTTATCATAAATGCCGCTTTTGTTTGGAAAGGCGTG     3540
TGTTTAAAAGAGAAGTGAAAAAATAAAGGGCTACTGCTTTCAATTCAGAATGGACTAATT     3600
GTTCTGATTTGCTGTTTCCAAGAAATGGGCGGGGCTGGCTGTAAGCTCAAAGTATATCTG     3660
AATGCGCCTCTCGAGCATTTCATTCTGACATTACAAAGAG                         3720
```

PREDICTED TRANSCRIPTION

START SITES:

Sequence, 3700 nucleotides

Position	Score	Likelihood
3300	1.143	Highly likely prediction

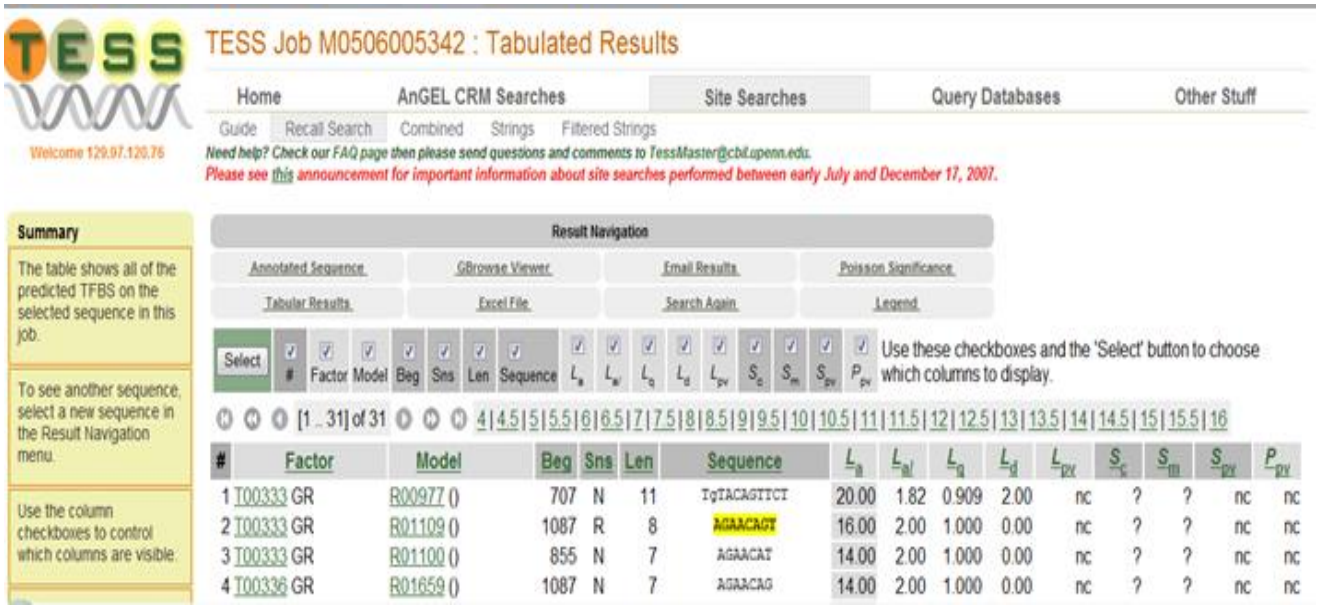
Transcription Factor Search Tools

These tools are used to predict transcription factor binding sites in DNA sequences. Identification of a good binding site in the promoter of a gene suggests the possibility that the corresponding factor may play a role in the regulation of that gene (Schug, 2003). Transcription Element Search System (TESS) and Transcription Factor Search (TF SEARCH) are two tools used in this regard. TESS uses two different kinds of models of sites, strings and positional weight matrices, and offers facilities for browsing and querying data from databases like TRANSFAC, IMD, CBIL-GibbsMat and JASPAR (Schug, 2003). TF SEARCH employs simple correlation calculation with binding site profile matrices. TF SEARCH searches highly correlated sequence fragments versus TFMATRIX transcription factor binding site profile database in TRANSFAC (Akiyama, 2010; Heinemeyer et al., 1998). It is recommended to use more than one independent search tool to see if the results correlate and hence both these tools were used in this study.

Based on the results obtained from the promoter 2.0 prediction server, the 1-3300 bp region of the 5' 3700 bp flanking sequence was used as input for these two searches. The factor filter option in TESS was set to GR. The search matrix in TF SEARCH was set to vertebrates and the threshold score was set to 75.

TESS identified 58 putative GREs. However, based on the log -likelihood ratio based scores [L_a (Log likelihood score)- higher the better, $L_{a/}$ (Density) - higher the better; maximum is 2, L_d (Deficit) – 0 is best; higher is worse, and L_q (Quotient)– higher the better; best is 1], the putative GRE at position 1087 was identified as the best scoring hit. TF SEARCH also identified a putative GRE at position 1087 with a score of 79.2. Based on these results, a putative GRE is present in the promoter of zebrafish SOCS-1.

Figure 2: A high scoring putative GRE identified at position 1087 in the zebrafish SOCS-1 promoter using TESS



TESS Job M0506005342 : Tabulated Results

Home | AnGEL CRM Searches | Site Searches | Query Databases | Other Stuff

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Summary

The table shows all of the predicted TFBS on the selected sequence in this job.

To see another sequence, select a new sequence in the Result Navigation menu.

Use the column checkboxes to control which columns are visible.

Result Navigation

Annotated Sequence | GBrowse Viewer | Email Results | Poisson Significance

Tabular Results | Excel File | Search Again | Legend

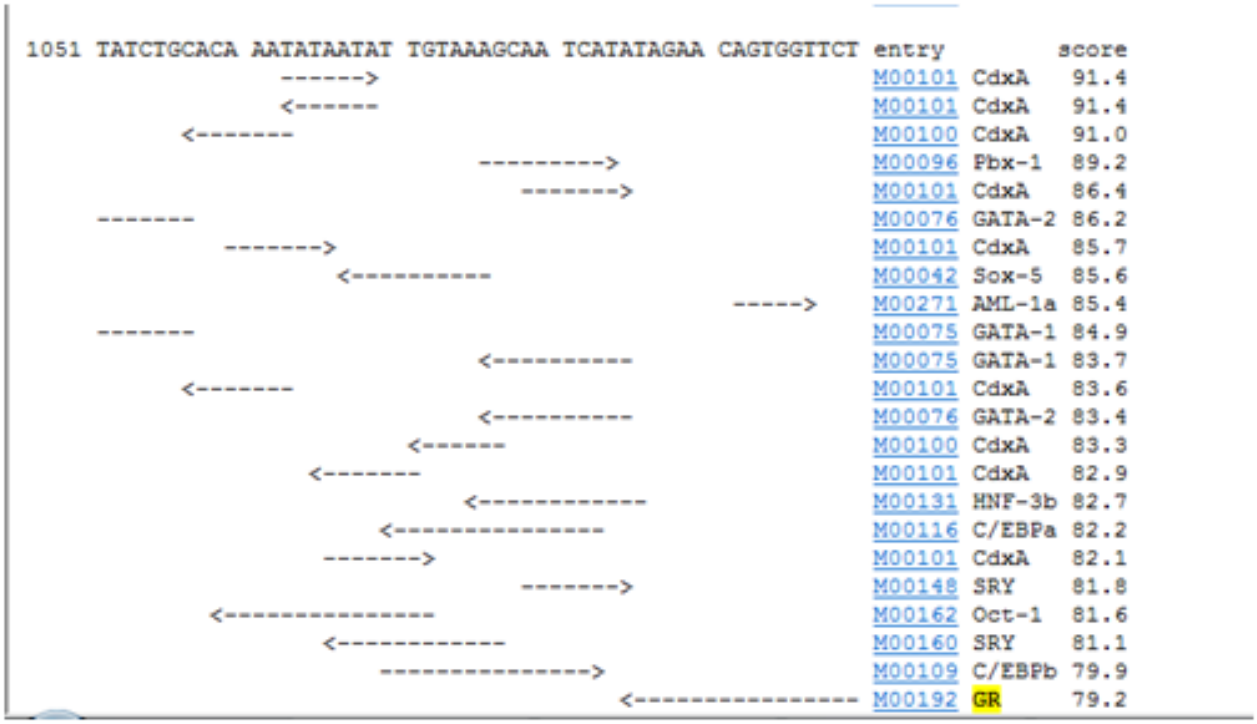
Select | # | Factor | Model | Beg | Sns | Len | Sequence | L_a | $L_{a'}$ | L_q | L_d | L_{pv} | S_c | S_m | S_{pv} | P_{pv}

Use these checkboxes and the 'Select' button to choose which columns to display.

[1 .. 31] of 31 | 4 | 4.5 | 5 | 5.5 | 6 | 6.5 | 7 | 7.5 | 8 | 8.5 | 9 | 9.5 | 10 | 10.5 | 11 | 11.5 | 12 | 12.5 | 13 | 13.5 | 14 | 14.5 | 15 | 15.5 | 16

#	Factor	Model	Beg	Sns	Len	Sequence	L_a	$L_{a'}$	L_q	L_d	L_{pv}	S_c	S_m	S_{pv}	P_{pv}
1	T00333 GR	R00977 ()	707	N	11	TgTACAGTTCT	20.00	1.82	0.909	2.00	nc	?	?	nc	nc
2	T00333 GR	R01109 ()	1087	R	8	AGAACAGT	16.00	2.00	1.000	0.00	nc	?	?	nc	nc
3	T00333 GR	R01100 ()	855	N	7	AGAACAT	14.00	2.00	1.000	0.00	nc	?	?	nc	nc
4	T00336 GR	R01659 ()	1087	N	7	AGAACAG	14.00	2.00	1.000	0.00	nc	?	?	nc	nc

Figure 3: A high scoring putative GRE (score of 79.2) identified at position 1087 in the zebrafish SOCS-1 promoter using TF SEARCH



Upon literature review, a putative STAT binding site has been previously described in the zebrafish SOCS-1 promoter (Jin et al., 2007). In this study, TESS and TF SEARCH also identified a STAT binding site at position 1777 in the zebrafish SOCS-1 promoter. This is further confirmation of the reliability of these two tools.

Analysis of zebrafish SOCS-2 promoter for putative GREs

Promoter Prediction:

The zebrafish SOCS-2 genomic sequence was obtained from ENSEMBL and extended to include a 5' 2000 bp flanking region. This 5' 2000 bp flanking sequence was used as input for the promoter 2.0 program. Promoter 2.0 gave a highly likely prediction with a score of 1.251 at position 1700. This means that it is highly likely (95% confidence) that the transcription start site occurs within 100 base pairs upstream from position 1700.

Figure 4: Promoter 2.0 prediction results for zebrafish SOCS-2

Promoter 2.0 Prediction Results

INPUT SEQUENCE:

```
>Sequence
AGTGGCATCGATGGTTCCTCAAAAAATATTTAACAATCATAAATCTTTGAGTTTATTTAT 60
AGTGCAAATAATTTTTAGTATTAAATGTTCTTCACACTAAGAGAAAACATGTCATTTTAA 120
GCACTCTGAATGTTCTTCTATATTGCATTGCTGCAAAAACACTAATATTTATTTAATTAT 180
TTGTTTTCGGGGGCGTGCAGCCACGTTACGCATTCTGTCGTCTGAAACACATTTCTTATT 240
TATTTATTTATTGTTTTGTTTTTTCAGATCGGCTCAGCCAAATAAAATCCACGGACGCTG 300
TGGAAGACTTCTCACATGTTTTGGTTGATTGTAAGAGTCAGTCGTCTCTGAGGGATAATT 360
ATAGACATTGACTGGATGCAGAACGCGGTTCTCCAATGACCTGTCACTCATCCGACTCCA 420
CGGAAAGCATCGAGAATGAAAGGAGATCGCAATCCGAAACCCAAGTCGCCGACACCGAGC 480
AGAGTCGCATTGCCACTGCCATGAGAGACCTTAAAAACACTGGTAAACGTTTACTGCTCT 540
TTTTTTACTTCCACATTCTCTGCATTTGTTGCAGTAGGCCTATATTACTCTCTGAGTTCA 600
TGTTGTGTAGTCGACACGCAGCCTCGGGGCAGAGCTTCATGTAAGGAATAATAATTTTAA 660
ATGCATCAAATTTGATGTTGGTAGTAAAAAAAAAACCTGTTTAAGCGATTATGTGGAATT 720
TTAATAAAACGTTTAAAGGTCGTCTTTAGTGTCATTTAAACTGGCAGCAGGTCTGCATTA 780
AAACGTGTTCATGGCATGAGTTTGTTAATATATTTAAAGCAGGTTTGTTTTGGTATTTGG 840
TTGAACTTGTTGTTTCAGGACTGCTGTTAATGTATTATTACACATTATTACACTTTTGCT 900
TTTTTGTTTGAGCAGAAGATAATCATGAAAATATAAAAAGGGGCCATTTGAACCAATTTG 960
GGTTTCTGGAAAAAAAAATGCATGTTCTTGGTGAGAGAGTTTTTCACTCTGTTACATGAG 1020
TAGTTGAACTTAGTGAATTCACTGATCCAAATAATCTGTGTTTATTATATATGAAGATTA 1080
AAAATTATTCAAATGCTATTGCTAAGTAATACTAAGTATTTACAATGTTCGCCCCCACAA 1140
ACTGAATTCCAGGGTCATTTAGTAACTTTTACGTGGCATTTTTCCCATAAGCCCCCTTTT 1200
TGACCCTGAGATGGAGTAAATGAATGCAAGTAGGAGCCATGTGAATTTCCTGACAAAGCT 1260
TTTACTGTTTACATTTATTGTTTTGCTATTTTGCAACAAAAGAGTGACATTATCATGTCT 1320
ATAAAGCCATTGTTATGTTTCCTGTCCTGGCTGGAACATTTGTCCTCACATCTCTACCGC 1380
ATGCCTTTCTGTTTTTAATTTATTCCATTGCACATTATATTAAGAGAAAAAAACAACATA 1440
CAGTGATAATGATTCTACAAAATGTCCACTTTATGTTTGTTTTCTTGTGGAGCAAAACAT 1500
TCTGAGGCGTTTTGGGAACATCTGCCAAACATCTGCCTTTAAAAAATTTCATAAACTCCC 1560
AAGACGAAACAGTCAGATGGTCTAGTTCAGTATTTCAGGAAAGATACGTCATCTTTGTTG 1620
ACATTTTTCGCAGCGTTGTTTAGTCTTGCTGTGCCACATTTGCCATAATGTTTTCAATTA 1680
ACGTTTATTTAACAGCTCATTTCGGAAGTGCTTGCTTTTTGCTGCATTATCAGATGCGAT 1740
TTAGCCCTGTGCATCAGGGGAAACAGAGCTGCGAGGTTGAACGTGACGTGCTGAGACTTC 1800
TTATTCATCTATTCAGAAAATGCCTGACAAAAAAAAACACAGAAGAGCCAGTTTATGACT 1860
ATGTAGCTGTGTCGGTTTTTGTGTGCGTGTCCTTAGAGGAACGAGTTATGTGTCTCATGT 1920
TACACAAGCAAACGACTGAAACAGATACAGTGGAAGCGGATATGCATGACCATCTCTGTG 1980
TCTTCTTTTCTTGGAAGCAG                                          2040
```

PREDICTED TRANSCRIPTION START SITES:
Sequence, 2000 nucleotides

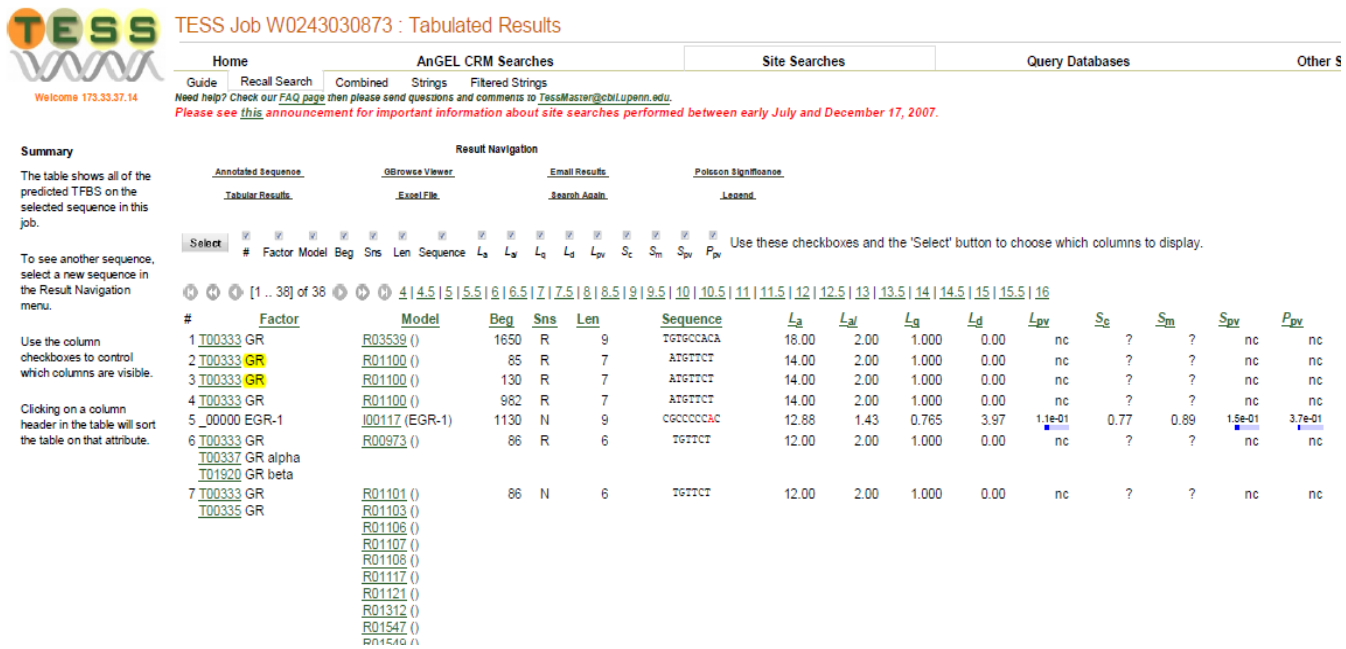
```
Position Score Likelihood

    1700 1.251 Highly likely prediction
```

Transcription Factor Search Tools

Based on the results obtained from the promoter 2.0 prediction server, the 1-1700 bp region of the 5’ 2000 bp flanking sequence was used as input for these two searches. The factor filter option in TESS was set to GR. The search matrix in TF SEARCH was set to vertebrates and the threshold score was set to 75.

Based on the combined results from TESS and TF SEARCH, GREs at positions 85 (TF search score of 81.1) and 130 (TF search score of 76.9) were identified as the best scoring hits and designated as putative GREs.

Figure 5: High scoring putative GRE identified in the zebrafish SOCS-2 promoter using TESS



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Summary

The table shows all of the predicted TFBS on the selected sequence in this job.

To see another sequence, select a new sequence in the Result Navigation menu.

Use the column checkboxes to control which columns are visible.

Clicking on a column header in the table will sort the table on that attribute.

Result Navigation

Annotated Sequence | GBrowse Viewer | Email Results | Poisson Significance

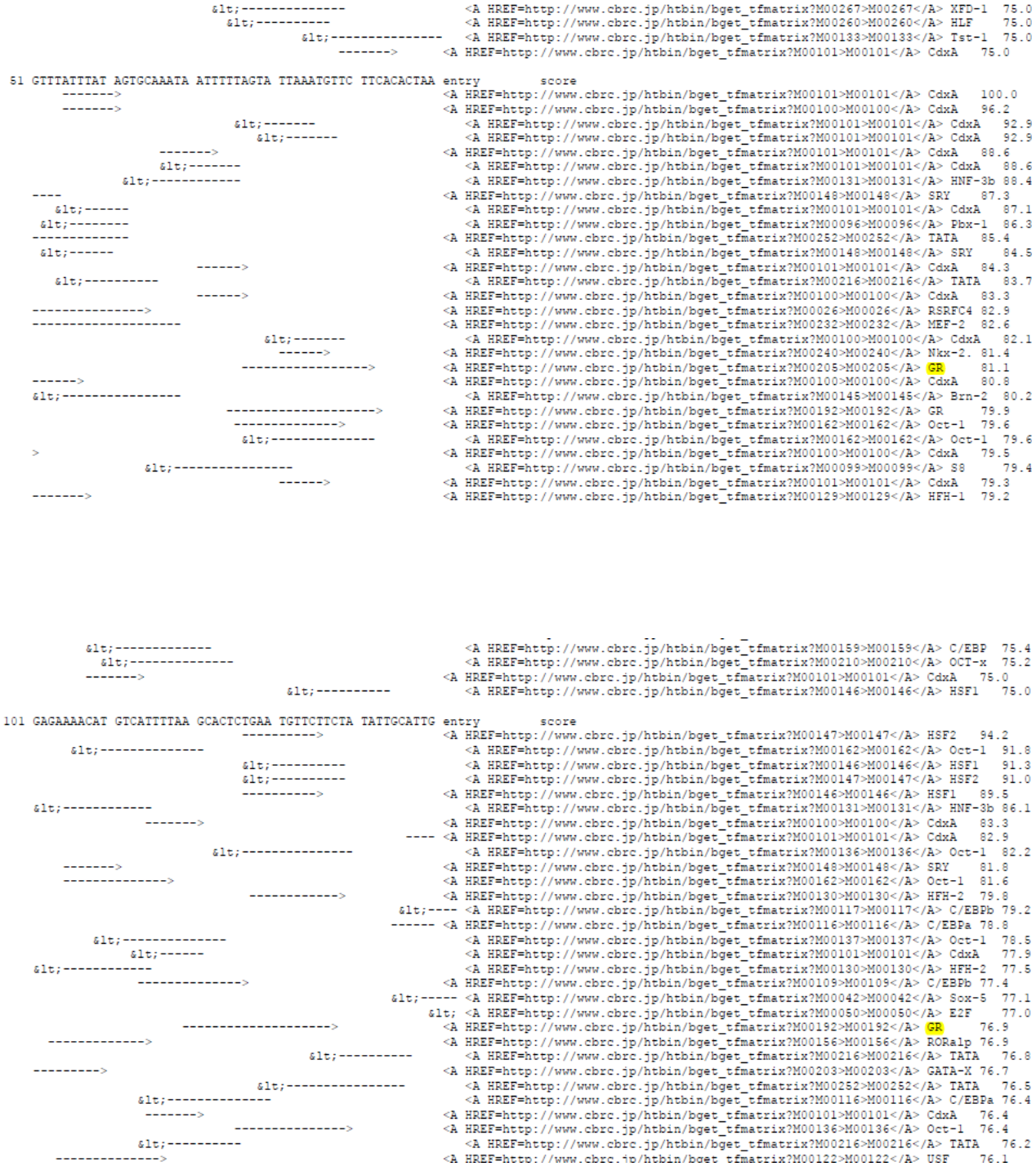
Tabular Results | Excel File | Search Again | Legend

Select # Factor Model Beg Sns Len Sequence L_a L_{al} L_q L_d L_{pv} S_c S_m S_{pv} P_{pv} Use these checkboxes and the 'Select' button to choose which columns to display.

[1 .. 38] of 38 4 | 4.5 | 5 | 5.5 | 6 | 6.5 | 7 | 7.5 | 8 | 8.5 | 9 | 9.5 | 10 | 10.5 | 11 | 11.5 | 12 | 12.5 | 13 | 13.5 | 14 | 14.5 | 15 | 15.5 | 16

#	Factor	Model	Beg	Sns	Len	Sequence	L_a	L_{al}	L_q	L_d	L_{pv}	S_c	S_m	S_{pv}	P_{pv}
1	T00333 GR	R03539 ()	1650	R	9	TGTGCCACA	18.00	2.00	1.000	0.00	nc	?	?	nc	nc
2	T00333 GR	R01100 ()	85	R	7	ATGTTCT	14.00	2.00	1.000	0.00	nc	?	?	nc	nc
3	T00333 GR	R01100 ()	130	R	7	ATGTTCT	14.00	2.00	1.000	0.00	nc	?	?	nc	nc
4	T00333 GR	R01100 ()	982	R	7	ATGTTCT	14.00	2.00	1.000	0.00	nc	?	?	nc	nc
5	_00000 EGR-1	I00117 (EGR-1)	1130	N	9	CGCCCCCAC	12.88	1.43	0.765	3.97	1.1e-01	0.77	0.89	1.5e-01	3.7e-01
6	T00333 GR T00337 GR alpha T01920 GR beta	R00973 ()	86	R	6	TGTTCT	12.00	2.00	1.000	0.00	nc	?	?	nc	nc
7	T00333 GR T00335 GR	R01101 () R01103 () R01106 () R01107 () R01108 () R01117 () R01121 () R01312 () R01547 () R01549 ()	86	N	6	TGTTCT	12.00	2.00	1.000	0.00	nc	?	?	nc	nc

Figure 6: High scoring putative GREs identified at position 85 and 130 in the zebrafish SOCS-2 promoter using TF SEARCH



Appendix B

Supporting Information for Chapter 6

Supplementary figures

Figure S1: Cortisol effects on glucose release from the liver

The graph shows a representative plot of the cortisol induced glucose release seen in liver slices incubated with 100ng/ml cortisol for 24 hours.

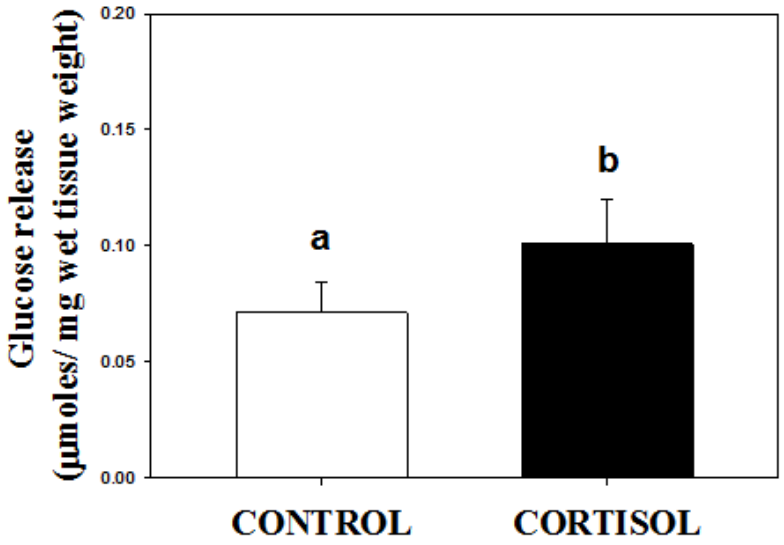
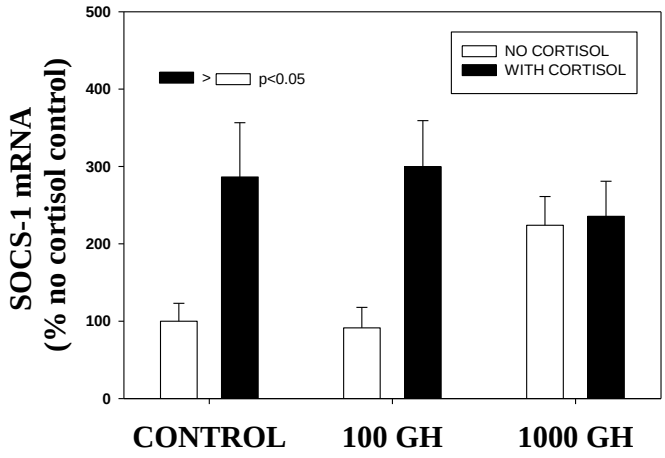


Figure S2: Effect of cortisol and GH on SOCS-1 (A) and SOCS-2 (B) expression

The graphs show the effect of cortisol in combination with GH on SOCS-1 (A) and SOCS-2 (B) mRNA abundance in rainbow trout liver. Liver slices were pre-incubated with cortisol (100ng/ml; Sigma) or control media for 24 h and then stimulated with GH (100ng/ml or 1000ng/ml) for 6 h. Values are plotted as % no cortisol control and show mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects the inset shows significant cortisol effects (two way repeated measures ANOVA, $p < 0.05$).

A



B

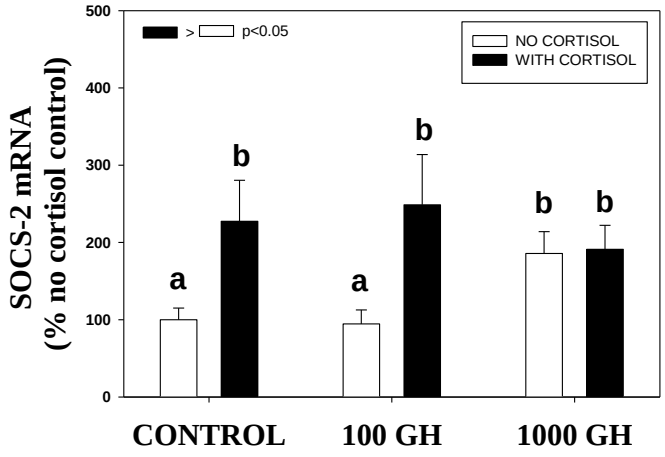
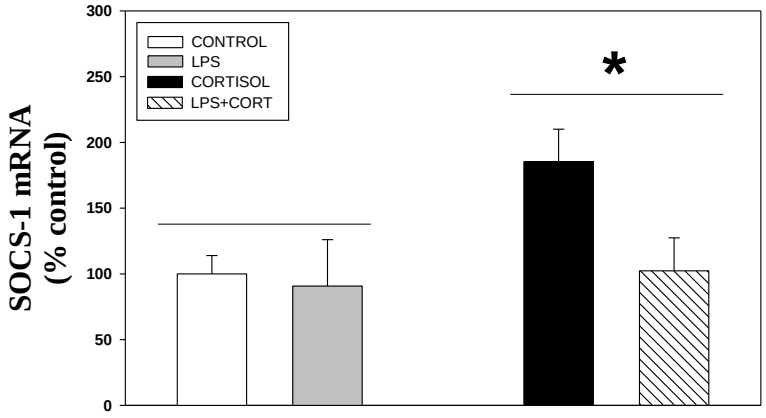


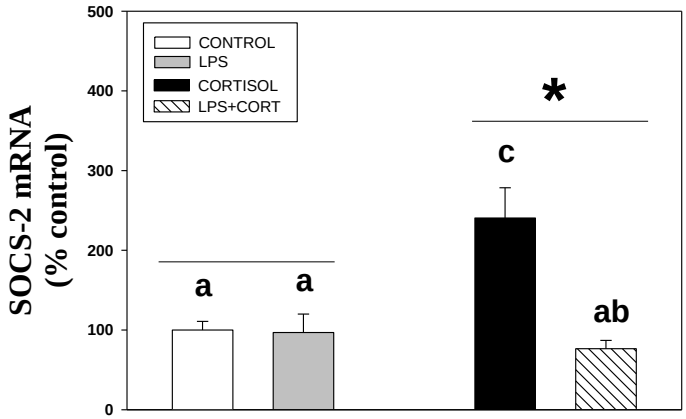
Figure S3: Effect of cortisol and LPS on SOCS-1 (A) and SOCS-2 (B) expression at 24 h post-incubation

The graphs show the effects of cortisol and LPS on SOCS-1 (A) and SOCS-2 (B) mRNA abundance in rainbow trout liver. Rainbow trout liver slices were pre-incubated with control media or media containing cortisol (100ng/ml; Sigma), LPS (30µg/ml) or a combination of cortisol and LPS for 24 h. Values are plotted as % control and show mean ± S.E.M (n = 6 independent fish); different lower case letters denote significant treatment effects; * denotes overall significant cortisol effects (two way repeated measures ANOVA, $p < 0.05$).

A



B



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Publication: Developmental & Comparative Immunology

Publisher: Elsevier

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