STRESS PHYSIOLOGY AND IMMUNE RESPONSES OF ATLANTIC COD (*Gadus morhua*) SUBJECTED TO VARIOUS CHALLENGES

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Stress Physiology and Immune Responses of Atlantic Cod (*Gadus morhua*) Subjected to Various Challenges

by

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A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy

Department of Biology/School of Graduate Studies/Faculty of Science

Memorial University of Newfoundland

2012

Abstract

The Atlantic cod is an economically important species in Canada, as well as in several European countries including Norway, Iceland and Scotland. However, collapse of Atlantic cod stocks has threatened the global Atlantic cod fishing industry. The aquaculture of Atlantic cod could be an alternative source of fish for these markets, but its establishment has been slow, due to the lack of knowledge with regards to certain aspects of this species' biology (e.g health/disease, optimal diet formulation and early maturation). Therefore, the objectives of this thesis were to develop tools to study the stress physiology and immune function of cod, and to apply these to aquaculture relevant questions. In Chapter 2, I: 1) demonstrated that cortisol responsiveness was negatively correlated with growth parameters; and 2) identified differences in the magnitude of the cortisol stress response to handling and potential quantitative trait loci (QTL) associated with this phenotype. In Chapter 3, I investigated the molecular mechanisms that mediate differences in cortisol responsiveness by measuring the mRNA expression of genes involved in cortisol synthesis and tissue responsiveness in Atlantic cod with different magnitudes of cortisol response. In Chapter 4, I report on a stress relevant gene discovery effort conducted as part of the Atlantic Cod Genomics and Broodstock Development Project (CGP), that involved the sequencing and characterization of over 5000 ESTs. Several of the genes identified in this work were validated using quantitative real-time reverse transcription-polymerase chain reaction (QPCR) as heat shock responsive genes, and were subsequently incorporated into the CGP 20K Atlantic cod microarray. Finally, in Chapter 5, I used this microarray platform to study the effect of a gradual temperature increase (from 10 to 16°C over several weeks) on immune-relevant gene transcription,

and demonstrated that these increases in temperature dysregulated the anti-viral transcriptomic response of Atlantic cod; fish held at 16°C responding earlier than Atlantic cod held at 10°C. This research has contributed significantly to the resources available for investigating aquaculture relevant questions in Atlantic cod, and generated novel information that can be used to develop molecular markers for use in Atlantic cod broodstock selection programs.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my parents, because a lot of what I am is what they believed I could be, much more than even I thought possible.

I also would like to thank my supervisors, Dr. Kurt Gamperl and Dr. Matthew Rise. This was an intense rollercoaster ride (fish died, factories burnt to the ground, partners walked out from research programs), but both of you ensured that I made to the end of it largely unharmed and somewhat sane. To Dr. Luis Afonso I owe this opportunity, and for that I will always be grateful.

Thanks also goes to the other CGP partners, collaborators, researchers and technicians that helped me so much throughout my program. In particular, I would like to acknowledge: Dr. Stewart Johnson, Dr. Sharen Bowman, Dr. Sophie Hubert, Dr. Laura Brown, Dr. Amber Garber, Dr. Andrew Robinson, Dr. Mark Fast, Jennifer Hall, Charles Feng, Danny Boyce, Jane Osborne, Sandra Sperker, Catherine Kozera, Cynthia Stone, Jennifer Kimball, Gord Nash, Jennifer Monk, Francine Fortune, Tasha Harrold and Lori Thorne. A special thanks goes to Dr. Marlies Rise, our project manager supreme and all-around good friend.

To the friends I left and to the ones I made anew in Canada, whatever "life" I had during the last years is thanks to you. In a thesis that is already more than 200 pages long I can't name all of you, but some names I feel compelled to spell out. Isabel Costa, Johanne Lewis and I are bound by an address and a friendship that I am grateful for. Thanks also to JPNR and Fabio Peixoto, who came to explore Newfoundland with an open heart and I think also fell in love with it. Finally, I would like thank Dr. Marije

Booman for understanding, liking and bringing out the geekiest part of me (and also for teaching me R and mySQL and providing me with tons of eBooks).

A large part of this thesis would not have been possible without the efforts of hundreds of unnamed hackers (and some famous ones), who spend their time developing Linux, perl, mySQL, R, python, apache and PHP, when they should really be doing "work". Thanks go out the Open Source movement, the Free Software Foundation, the GNU project, the Bioconductor project, the R project and all their collaborators.

I would like to thank all the staff at the OSC for doing a little of everything whenever anything was needed, specially Winnie Sparkes, Delores Wheeler, Joanne Green, Danny Au, Connie Short and Wally, you guys rock!

I would also like to thank the Institute for Marine Biosciences of the National Research Council, Newfoundland is one of the best things that ever happened to me and if not for IMB, I wouldn't live here.

Last but not least, I would like to thank my wife Kim. Truth to be told, I am not always a nice person, but she persevered through the worse of my mood swings and always saw the best in me (sometimes with the help of a scope).

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3βHSD – 3 Beta-hydroxysteroid-dehydrogenase
ACS – After the cessation of stress
ACTH – Adrenocorticotropic hormone
ANOVA – Analysis of variance
AS – After stress
ATF – Activating transcription factor
ATPS - ATP synthase H^+ transporting, mitochondrial Fo complex, subunit F2
aa – Amino acids
BHS – Before heat shock
BLAST – Basic local alignment search tool
BP – Biological process
BI – Before injection
BS – Before stress
bp – Base pair
°C – Degree centigrade
C – Control
CBI – Control before injection
CBTI – Control before temperature increase
CC – Cellular component
CCL – Chemokine (C-C) ligand
CCT – T-complex containing chaperonin
CD – Compact disc
CF – Condition factor
CGP – Atlantic cod genomics and broodstock development project
CRF – Corticotropin releasing factor
CT – Control transferred
C_T – Threshold cycle
CS – Cessation of stress
CTM – Critical thermal maximum
cAMP – Cyclic adenosine monophosphate
cDNA – Complementary DNA
cM – Centimorgan
DBD – DNA binding domain
DHX – DEAH (Asp-Glu-Ala-His) box polypeptide
DFO – Department of Fisheries and Oceans
DNA – Deoxyribonucleic acid
DO – Dissolved oxygen
E – Efficiency
ELISA – Enzyme linked immunosorbent assay
ER – Endoplasmic reticulum
EST – Expressed sequence tag
EtBr –Ethidium bromide
F - Family

FDR – False discovery rate
Fig – Figure
G – Gauge
GaP – Genomics and proteomics facility
GEO – Gene expression omnibus
GH – Growth hormone
GO – Gene ontology
GOI – Gene of interest
Gp96 – Glucose regulated protein 94
GR – Glucocorticoid receptor
GRE – Glucocorticod responsive elements
GRP – Glucose regulated protein
GSP – Gene specific primer
GST – Glutathione-S-transferase
g – Grams
g – Gravitational force
HBI – Heat exposed before injection
HBTI – Heat exposed before temperature increase
HK – Head kidney
HPI – Hours post-injection
HR – High responder
HS – Heat shocked
HSC – Heat shock cognate
HSP – Heat shock protein
h^2 – Heritability
hr – Hour
ID – Identity
IFN – Interferon
IFNAR – Interferon alpha/beta receptor
IGF – Insulin-like Growth Factor
IKK – Inhibitor of NF-κB kinase
IL – Interleukin
IP – Intraperitonial
IRAK – Interleukin-1 receptor-associated kinase
IRF –Interferon regulatory factor
ISG – Interferon stimulated gene
Ig – Immunoglobulin
JNK – c-jun terminal kinase
K – Potassium
kDa – Kilodalton
LB – Luria-Bertani medium
LBD – Ligand binding domain
LPS – Lipopolysaccharide
LR – Low responder
L – Liver

l – Litre
ln – Natural logarithm
M – Skeletal muscle
MAS – Marker assisted selection
MDA – Melanoma differetiation-associated gene
MHC – Major histocompatibility complex
Min – Minimum
Max – Maximum
MF – Molecular function
MMLV – Moloney murine leukemia virus
MR – Mineralocorticoid receptor
MUN – Memorial University of Newfoundland
m – Metre
mg – Milligram
min – Minute
ml – Millilitre
mRNA – Messenger RNA
Na – Sodium
NB – New Brunswick
NCBI – National center for biotechnology information
NF-κB – Nuclear factor kappa B
NR – Nuclear receptor
NRC-IMB – National research council institute for marine biosciences
NTC – Non-template control
NUPR – Nuclear protein
ng – Nanogram
nM – Nanomolar
nr – Non-redundant
OSC – Ocean Sciences Centre
P450c17a2 – Cytochrome P450 - 17-alpha-2-hydroxylase activity
P450c21 – Cytochrome P450 – 21-hydroxylase activity
P450scc – Cytochrome P450 - 20,22 R-hydroxylase cholesterol side chain cleavage activity
PAMP – Pathogen associated molecular pattern
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PEPCK – Phosphoenolpyruvate carboxykinase
PERK – Eukaryotic translation factor initiation factor 2-alpha kinase 3
PDI – Protein disulfide isomerase
PFK – Phosphofructokinase
PGM – Phosphoglucomutase
PIT – Passive integrated transponder
PKR – Double stranded RNA activated protein kinase
PMT – Photomultiplier tube
PPIase – Prolyl-peptidyl-isomerase

PRR – Pattern recognition receptor
PTA – Paracel transcript assembler
pIC – Polyriboinosinic polyribocytidylic acid
p – p-value
QC – Quality check
QPCR – Quantitative real-time reverse transcription PCR
QTL – Quantitative trait locus/loci
RACE – Rapid amplification of cDNA ends
RIG – Retinol induced gene
RLR – RIG-I like receptors
RNA – Ribonucleic acid
RQ – Relative quantity
RSAD2 – Radical S-adenosyl methionine domain containing 2 (alias Viperin)
R&D – Research and development
SACS – Sacsin (alias spastic-ataxia of Charlevoix-Saguenay)
SAM – Significance analysis of microarrays
SCYA – Small inducible cytokine
S.E. – Standard error of the mean
SGR – Specific growth rate
SNP – Single nucleotide polymorphism
SOD – Superoxide dismutase
SSH – Suppression subtractive hybridization
STAT – Signal transducer and activator of transcription
StAR – Steroidogenic acute regulatory protein
S – Svedberg
sec – Seconds
TAGC – The atlantic genome centre
Taldo – Transaldolase
TANK – TRAF family member-associated NF-κB activactor
TBK – TANK-Binding kinase
TCP – T-complex
TCTP – Translationally controlled tumor protein
TLR – Toll-like receptor
TMS – Tricaine methane sulphonate
TNFAIP – Tumor necrosis factor alpha-interacting protein
U - Units
UTR – Untranslated region
VNN – Viral nervous necrosis
YC – Year-class
ZNFX1 – Zinc finger, NFX1-type containing 1
Z_t – Total Z-score
µl – Microlitre

Co-authorship statement

In this thesis, I was primarily responsible for experimental design, implementation of the experiments, data analysis and the preparation of manuscripts. Exceptions are: a) the experimental design used in Chapter 2 was developed in collaboration with Dr. Luis Afonso; b) the QTL analysis in Chapter 2 was done by Dr. Andrew Robinson and his research group at the University of Guelph, ON; and c) cDNA sequencing and EST analysis (i.e. trimming, assembly and annotation) for the libraries reported in Chapter 4 were performed at the Atlantic Genome Centre in Halifax, NS.

Dr. Andrew Robinson, **Dr. Gordon Vander-Voort** and **Christina Hastings** developed the statistical model used for QTL analysis in Chapter 2 and performed the QTL analysis reported therein.

Dr. Sharen Bowman, **Dr. Sophie Hubert** and **Jennifer Kimball** were responsible for the sequencing and analysis of the ESTs reported in Chapter 4 and also for the development and implementation of the EST database used in Chapter 4.

Chapter 1: Introduction

1.1 Introduction

Marine fisheries are important to the economic and cultural fabric of many countries, including Canada, Japan, the United States, Iceland and Norway. Among the species relevant to this industry on the east coast of Canada, the Atlantic cod (*Gadus morhua*) has played an extremely important role, both historically and economically (Rose, 2007). However, several of the wild stocks of Atlantic cod in Canadian waters have collapsed due largely to overfishing (Myers et al., 1997), and the Atlantic cod fishing industry in Canada has been impacted greatly by the decline in this species' populations and the subsequent Atlantic cod moratorium that was imposed by the Canadian government (Department of Fisheries and Oceans) in 1992.

The global decline in Atlantic cod landings (from 3.1 millions of tons in 1970 to 890,000 tons in 2002 – www.fao.org), combined with the considerable success achieved by the salmonid aquaculture industry, has led to an increased interest in rearing Atlantic cod in captivity to supply the global market (Roselund and Skretting, 2006). Even though it is generally agreed that there is great economic potential in an Atlantic cod aquaculture industry, there is also a strong consensus that its success will depend heavily on research and development (R&D) (Kjesbu et al., 2006; Roselund and Skretting 2006; Genome Atlantic, 2010). One of the obstacles faced by those trying to establish the Atlantic cod as a commercially viable aquaculture species is the incomplete understanding of many aspects of its' biology [causes of larval mortality, slow growth, disease, responses to improper fish husbandry or changes in the environment, early maturation, and the use of non-optimal feeds (Bricknell et al., 2006; Kjesbu et al., 2006; Roselund and Skretting,

2006; Pérez-Casanova et al., 2008a and 2008b; Pérez-Casanova, 2009; Solsletten, 2009; Genome Atlantic, 2010)]. For example, high mortalities that are likely associated with increasing temperatures have been reported during the summer months in sea-cages in Newfoundland (Pérez-Casanova, 2009) and disease (including those caused by viruses belonging to the Nodaviridae family) have been identified as one of the major factors limiting the aquaculture of marine fish, including gadoid species (Samuelsen et al., 2006; Lang et al., 2008).

To start to address a number of these challenges, the Genome Canada funded Atlantic Cod Genomics and Broodstock Development Project (CGP, http://codgene.ca) generated genomic tools and knowledge of cod biology that will hopefully contribute to the future development of a highly successful Atlantic cod aquaculture industry. For example, this program generated information on production-relevant traits such as the Atlantic cod's growth, stress response and disease resistance that can be used to identify elite broodstock, and to improved husbandry practices. This type of information, coupled with high-throughput genomic approaches to identify molecular markers for use in marker-assisted selection (MAS), has been fundamental to improving the livestock performance of many commercial species such as the domesticated pig (*Sus scrofa*) and cattle (e.g. *Bos taurus*) (Lande and Thompson, 1990; Gjedrem, 2000; Hu et al., 2009; Togashi and Lin, 2010; Verbyla et al., 2010; Paux et al., 2011).

1.2 Fish stress physiology

There is a large body of literature on the response of fish to acute stressors (Vijayan and Moon, 1994; Fabri et al., 1998; Tort, 1998; Mommsen et al., 1999; Barton, 2002;

Aslop and Vijayan, 2008; Aluru and Vijayan, 2009). The responses to acute stress are usually adaptive and help the fish to survive alterations in environmental conditions or interactions with conspecifics and/or predators. However, the stress response can become maladaptive when the animal is continuously exposed to the stressor, i.e. the animal becomes chronically stressed (Barton, 2002). Chronic stress can lead to losses in aquaculture production mainly due to its negative impacts on fish immunity, appetite, growth and reproductive performance (Mazeaud et al., 1977; Pickering et al., 1989; Tort, 1998; Morgan et al., 1999; Barton, 2002). Several factors can lead to chronic stress in commercial scale aquaculture, including elevated or rapid changes in temperature, high stocking densities, low oxygen levels and the accumulation of nitrogenous compounds (e.g. ammonia or nitrite) (reviewed in Iwama et al., 1997). Therefore, a better knowledge of the response of Atlantic cod to stress, and of the effects of long-term stress on Atlantic cod physiology, may have a positive outcome on this species' management and aquaculture production.

Corticosteroids (a class of steroid hormones) and catecholamines are the principal hormones released into the blood stream of vertebrates in response to stress. In teleost fish, cortisol is the main corticosteroid produced. This hormone is synthesized in the interrenal cells of the head kidney (analogous to the adrenal cortex of mammals) following the release of the corticotropin releasing factor (CRF) and adrenocorticotropic hormone (ACTH) from the hypothalamus and pituitary, respectively. Once it is released into the bloodstream, cortisol can circulate as a free molecule or bound to proteins, but only the former is able to affect target tissues (Mommsen et al., 1999). Once it reaches its target tissues (e.g. liver), cortisol is known to have a wide range of physiological effects.

These are mediated by its interaction with intracellular receptors, that once bound to this hormone act as transcription factors, and potentially through non-genomic effects mediated by membrane receptors (e.g. changes in protein phosphorylation status) (Mommsen et al., 1999; Barton, 2002; Aluru and Vijayan, 2009). These cortisol-induced physiological effects include changes in: 1) energy mobilization [e.g. release of glucose from the liver (Boone et al., 2002), increased gluconeogenesis and lipid oxidation (Mommsen et al, 1999; Aluru and Vijayan, 2007)]; 2) osmoregulation [e.g. increased branchial Na^+/K^+-ATPase activity (McCormick, 1995; Madsen et al., 1995) and changes in paracellular (i.e. between epithelial cells) permeability (Kelly and Chasiotis, 2011)]; and 3) fish behaviour [e.g. increased chirp production in the electric eel (*Apteronotus leptorhynchus*) (c.f. Dunlap et al., 2011) and changes in amphibious behaviour in the mudskipper (*Periophthalmus modestus*) (c.f. Sakamoto et al., 2011)].

1.3 Divergence in the stress response

The magnitude of the response to stress can vary between individuals of the same population, and is defined as a divergent response. Divergent stress responses, based on plasma cortisol levels, have been demonstrated in many fish species including the rainbow trout (*Oncorhynchus mykiss*) (c.f. Pottinger et al., 1992), Atlantic salmon (*Salmo salar*) (c.f. Fevolden et al., 1991), striped bass (*Morone saxatilus*) (c.f. Wang et al., 2004) and gilthead sea bream (*Sparus aurata*) (c.f. Tort et al., 2001). In fish that demonstrate a divergent stress response, a proportion of the population presents a high cortisol response when subjected to stress, and are therefore termed high responders (HR). Other fish that have a low or non-consistent cortisol response to stress can be categorized as low

responders (LR) and non-consistent responders, respectively. The magnitude of the cortisol response in fish appears to be, at least in part, genetically determined as several groups have been able to produce stress resistant (LR) strains of Atlantic salmon and/or rainbow trout (e.g. see Fevolden et al., 1991; Fevolden et al., 1993; Fevolden and Røed., 1993; Pottinger and Carrick, 1999a; Pottinger and Carrick, 1999b; Fevolden et al., 2002). Moreover, Fevolden et al. (2002) reported a realised heritability (h^2) of 0.50 for the cortisol response in rainbow trout, while Vallejo et al. (2009) reported h^2 values ranging from 0.22 to 0.39. These results suggest that the selection of LR phenotypes could lead to improved growth. However, whether LR and HR phenotypes exist in the Atlantic cod, and if enhanced production traits are associated with HR vs. LR phenotypes in this species, has never been investigated. Further, there is no information on molecular markers that could be used to select favourable genotypes (e.g. SNPs) that correlate with stress responsiveness.

1.4 Regulation of cortisol synthesis

Hormonal actions and tissue responsiveness are regulated by a wide range of mechanisms and processes. These include hormone synthesis, receptor levels and affinity, feedforward and feedback mechanisms, transport mechanisms and metabolism (Baxter et al., 2004). Although the mechanisms that regulate the actions of hormones and tissue responses to catecholamines and glucocorticoids are well understood in mammals (Mommsen et al., 1999), there is limited information on these mechanisms for lower vertebrates such as fish. With respect to Atlantic cod, the mechanisms that are important in the regulation of the cortisol response are largely unknown.

Cortisol production and secretion are primarily regulated by the hypothalamic-pituitary-interrenal axis. In this hormone pathway, CRF released from the hypothalamus induces the release of ACTH from the anterior pituitary gland, and ACTH acts via a cyclic adenosine monophosphate (cAMP) cascade, leading to the subsequent activation of protein kinases A and C that activate cortisol production in the interrenal cells (Mommsen et al., 1999; Baxter et al., 2004). These two protein kinases in turn regulate the activity of proteins [cholesterol esterase and steroidogenic acute regulatory protein (StAR)] that control the availability of cholesterol for steroid synthesis (Lacroix and Hontela, 2001; Aluru et al., 2005; Aluru and Vijayan, 2006). The synthesis of corticosteroids in all vertebrates is accomplished through the activity of cytochrome P450 dependent and hydroxysteroid dehydrogenase enzymes present in the membranes of the endoplasmic reticulum and mitochondria (Nunez, 1996). The rate limiting step in steroid (e.g. cortisol) synthesis is generally thought to be the delivery of cholesterol to the mitochondrial cytochrome CYP11A1 (20, 22 R-hydroxylase cholesterol side-chain cleavage activity - P450scc) by StAR (Mommsen et al., 1999; Le Roy et al., 2000; Lingappa and Mellon, 2004; Aluru et al., 2005; Aluru and Vijayan, 2006). However, recent studies in fish have shown an increase in steroidogenic enzyme mRNA expression and activity in response to stress, and question whether StAR is important for regulating cortisol synthesis under all circumstances. For example, Gelsin and Auperin (2004) reported an increase in mRNA expression of P450scc in rainbow trout submitted to stress. Nunez and Trant (1999) showed increased activity of 3β hydroxysteroid dehydrogenase (3βHSD), but not in StAR protein levels, after stress in the Atlantic stingray (*Dasyatis sabina*). Finally, other experiments on rainbow trout have demonstrated that increases in transcript levels for

these mediators of cortisol synthesis are dependent on the magnitude of stress response (Kusakabe et al., 2002; Gelsin and Auperin, 2004; Hagen et al., 2006). Thus, these data suggest that any or all of these steps could play an important role in determining whether a particular individual is a high, average or low cortisol responder.

1.5 Effects of temperature on fish physiology and gene transcription

Fluctuations in temperature are known to cause many physiological changes in fish, including: increases in the levels of circulating corticosteroids (Varsamos et al., 2006; Pérez-Casanova et al., 2008a and 2008b; Hayashi et al., 2010; Methling et al., 2010; Cook et al., 2011; LeBlanc et al., 2012) and the transcription and/or translation of genes belonging to the heat shock protein family (Currie and Tufts, 1996; Iwama et al., 1999; Hori et al., 2010 – Chapter 4; LeBlanc et al., 2012); reductions in food consumption, growth and feed conversion efficiency (Pérez-Casanova et al., 2010); alterations in cardiac function (Joaquim et al., 2004; Gollock et al., 2006; Mendonça and Gamperl, 2009); changes in oxygen consumption (Clark et al., 2008); and disturbances in immune function (Bowden, 2008; Pérez-Casanova et al., 2008b).

Atlantic cod in their natural environment usually remain at preferred temperatures (~ 5 - ~ 15°C; Swain and Kramer, 1995; Despatie et al., 2001; Petersen and Steffensen, 2003) by changing their position in the water-colunm when temperatures become too cold or too warm (Claireaux et al., 1995). However, Atlantic cod confined to sea-cages cannot avoid acute or seasonal temperature changes, and significant mortalities have occurred in Atlantic Canada when summer water temperatures approach 20°C (Gollock et al., 2006). Interestingly, these mortalities do not appear to be due to the direct effects of temperature

as both the acute and chronic critical thermal maximum (CTM) values for Atlantic cod juveniles and adults are in excess of 21°C (Gamperl et al., unpublished data; Kelly et al., unpublished data). Changes in temperature also cause several changes in immune-related gene transcription and/or immune function in fish [rainbow trout (*Oncorhychus mykiss* - Alishahi and Buchmann, 2006; Raida and Buchmann, 2007; Raida and Buchmann, 2008; Bettge et al., 2009; Lewis et al., 2010), European sea bass (*Dicentrarchus labrax* - Varsamos et al., 2006), orange spotted grouper (*Epinephelus coioides* - Cheng et al., 2009), Atlantic salmon (*Salmo salar* - Eggset et al., 1997)], including increased susceptibility of European sea bass to *Nodavirus* (the causative agent of viral nervous necrosis) (Varsamos et al., 2006). Therefore, it is possible that the mortalities observed at Atlantic cod cage sites during the summer months may be the result of interactions between elevated temperature and the immune system. This is an interesting hypothesis that is supported by a few studies that have reported modulation of immune parameters or immune-relevant mRNA expression in Atlantic cod exposed to changes in temperature (e.g. Magnadottir et al., 1999; Pérez-Casanova et al., 2008b). Nonetheless, very little is known about the effects of temperature on gene transcription in this species, or how elevated (high) temperatures affect the capacity of Atlantic cod to mount an effective immune response if they encounter a pathogen or other immune stimuli [e.g. pathogen associated molecular patterns (PAMPs) or pathogen mimics].

This research was part of the Genome Canada/Genome Atlantic funded Atlantic Cod Genomics and Broodstock Development Project (http://codgene.ca.) and represents a portion of a much larger effort to improve our understanding of cod biology and genomics, and to enhance cod aquaculture in Canada.

1.6 Overall thesis objectives

Given the potential for Atlantic cod aquaculture in Newfoundland and elsewhere in Atlantic Canada, the overall objectives of this thesis were to: 1) investigate whether inter-individual and -family differences in cortisol responsiveness exist in Atlantic cod, the mechanisms involved, and the correlation of cortisol responsiveness on Atlantic cod production traits (e.g. growth rate); 2) study the effect of acute heat stress on Atlantic cod gene transcription and develop genomic tools for stress-related studies in this species; and 3) use these functional genomic tools to investigate how elevated temperature affects constitutive and viral mimic-induced immune gene transcription in the Atlantic cod. To accomplish these objectives, in Chapters 2 and 3, I quantified the magnitude of cortisol response to handling stress in 10 families of Atlantic cod, examined the relationship between HR and LR status and growth-related parameters, and determined how cortisol responsiveness influenced the mRNA expression of genes that regulate cortisol synthesis and target tissue responsiveness. In Chapter 4, I used suppression subtractive hybridization (SSH) cDNA library construction and characterization to identify transcripts that were dysregulated by heat shock in liver, skeletal muscle and head kidney of Atlantic cod. Finally, in Chapter 5, I exposed Atlantic cod to an incremental increase in temperature from 10 to 16°C and used the CGP 20K microarray (which contained many of the temperature dysregulated genes identified in Chapter 4), followed by quantitative real-time reverse transcription – polymerase chain reaction (QPCR), to investigate how elevated temperature affects the Atlantic cod spleen transcriptome response to the intraperitoneal injection of a viral mimic (polyriboinosinic polyribocytidylic acid, pIC).

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Chapter 2: Inter-Individual and -Family Differences in the Cortisol Responsiveness of Atlantic Cod (*Gadus morhua*)

This chapter has been published in the journal Aquaculture as: Hori, T.S., Gamperl, A.K., Hastings, C.E., Vander-Voort, G.E., Robinson, J.A.B., Johnson, S.C., Afonso, L.O.B., 2012. Inter-individual and -family differences in the cortisol responsiveness of Atlantic cod (*Gadus morhua*). Aquaculture 324-325, 165-173.

1.1 Abstract

Cortisol is released into the bloodstream of fish in response to several exogenous stimuli referred to as stressors, and long-term or repetitive exposure to stressors can lead to poor growth and disease susceptibility in aquaculture-reared fish. Thus, as has been shown for other fish species (e.g. rainbow trout), the identification of fish with an attenuated cortisol response to stress may be beneficial to the emerging Atlantic cod aquaculture industry. In this study, I: 1) examined if differences in the magnitude of the cortisol response to a standardized stressor (30 sec. handling) could be consistently detected within a multi-family population of Atlantic cod; 2) determined if these differences were related to growth, and independent of stressor type; and 3) assessed whether single nucleotide polymorphisms (SNPs) identified by the Atlantic Cod Genomics and Broodstock Development Project (CGP) were correlated significantly with the magnitude of cortisol response. In this study, I was able to consistently separate fish with a high cortisol response (HR) from those with a low cortisol response (LR) to handling and heat stress. I showed that the percentage of HR and LR fish differed dramatically amongst the 10 Atlantic cod families (with some families having no LR or HR individuals), and that significant negative correlations existed between cortisol responsiveness (total Z score) and wet mass, specific growth rate (SGR) and condition factor (CF); LR fish were approximately 55% heavier at the end of the year long study. Finally, in collaboration with Dr. Andrew Robinson's research group at the University of Guelph, I identified four polymorphic SNPs (QTL) that were significantly associated with the magnitude of cortisol response to handling stress. This research suggests that there is a genetic component to this phenotype (HR vs. LR) in Atlantic cod, and that

responsiveness to stress at the juvenile stage may be an important trait to consider in broodstock selection.

2.2 Introduction

The corticosteroid cortisol is released into the blood of fish in response to many stressors (e.g. Mommsen et al., 1999; Iwama et al., 2005; Hosoya et al., 2007; Hori et al., 2010 - see Chapter 4), and affects a multitude of physiological processes including appetite and feeding (e.g. Pickering, 1993; Bernier et al., 2004), osmoregulation (e.g. Shrimpton and McCormick, 1999), intermediary metabolism (e.g. Montero et al., 1999) and immune function (e.g. Fevolden et al., 1992; MacKenzie et al., 2006, Fast et al., 2008). Stressors such as grading, vaccination, changes in water temperature and crowding cannot be completely avoided within an aquaculture setting. Therefore, several studies have assessed whether fish can be separated into high and low cortisol responders (HR and LR, respectively), and how cortisol responsiveness relates to production relevant traits. Indeed, the cortisol response to a standardized stressor (e.g. handling) has been demonstrated to vary in magnitude between individual fish and fish populations, and to be consistently higher or lower in certain groups of fish when compared to the population average (Pottinger and Carrick 1999a; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Wang et al., 2004; Weber et al., 2008). However, the nature of the relationship between cortisol responsiveness and aquaculture relevant traits is highly variable. For example, while research by Fevolden et al. (2002), Trenzado et al. (2003) and Pottinger (2006) showed that LR rainbow trout (*Oncorhynchus mykiss*) grow significantly faster, other works (Pottinger and Carrick, 1999b; Weber and Silverstein, 2007; Weber et al., 2008) have reported that the rainbow trout in their studies showed a positive relationship between cortisol responsiveness and growth. Weil et al. (2001) found a negative correlation between growth and the stress response of rainbow trout, but suggested that

growth was negatively correlated with the levels of cortisol at 3 hours post-stress, and thus, to the rate of cortisol clearance. Finally, Tort et al. (2001) reported improved growth in HR sea bream (*Sparus auratus*) when compared to LR fish, but only with respect to males.

Although the relationship between cortisol responsiveness and growth differs between studies and species, there is strong evidence that cortisol responsiveness is a heritable trait. Several authors have been able to establish groups of high and low responding rainbow trout using selective breeding, and calculated heritabilities of cortisol response magnitude in this species range from 0.26 to 0.56 (e.g. Pottinger and Carrick, 1999b, Fevolden et al., 2002; Weber et al., 2008; Vallejo et al., 2009). Thus, it may be possible to select low cortisol responding, and potentially fast-growing fish that are better suited for production under intensive rearing conditions. The Atlantic cod is a marine cold-water fish that has significant aquaculture potential and is under commercial or pre-commercial production in the Nordic countries, Scotland, Canada and the United States (Roselund and Skretting, 2006). However, Atlantic cod aquaculture currently faces several challenges, including the supply and price of quality juveniles, production costs, disease, early maturation and less than optimal growth rates (Kjesbu et al., 2006).

The objectives of this work were to: 1) determine whether there are differences in the magnitude of cortisol response (with respect to total and free plasma concentrations) to handling stress within and between 10 different families of Atlantic cod (*Gadus morhua*); 2) test if fish that are consistently high and low responders retain their magnitude of response after being removed from their initial family tanks, and when a different stressor is applied (i.e. heat stress); and 3) examine if cortisol responsiveness is correlated with

growth parameters [i.e. body mass, specific growth rate (SGR) and condition factor (CF)] and any of the ~ 1298 Atlantic cod single nucleotide polymorphisms (SNPs) in the Atlantic Cod Genomics and Broodstock Development Project (CGP) Illumina Golden Gate genotyping platform (Hubert et al., 2010).

2.3 Materials and methods

2.3.1 Fish husbandry

All experiments were carried out following the guidelines of the Canadian Council on Animal Care and approved by the animal care committee of the National Research Council's Institute for Marine Biosciences (NRC-IMB).

Thirty fish each from ten different families (F3, F4, F6, F8, F11, F12, F31, F33, F34 and F73) (~ 30 g) raised at the Huntsman Marine Science Centre (St. Andrews, New Brunswick) in 2005 as part of CGP, were passive integrated transponder (PIT)-tagged (AVID, Norco, CA) and their mass and length were recorded. These fish were then transported to the NRC-IMB's aquaculture facilities (Ketch Harbour, Nova Scotia). Fish were initially held together for a month in two 500 l tanks supplied with flow-through seawater at 10°C and with dissolved oxygen (DO) levels maintained above 90%. Following this initial 4-week acclimation period, the fish had their PIT-tag scanned, their standard length and mass recorded (sampling point S0), and they were separated according to family into 150 l tanks (henceforth referred to as "family tanks") receiving the same water supply. In order to investigate different questions regarding the magnitude of the cortisol response to stress I performed 3 experiments.

2.3.2 Experiment #1: Monthly plasma cortisol levels in response to a standardized handling stress, and the identification of high (HR) and low (LR) cortisol responders

One month prior to the first application of the standardized stressor, the resting cortisol level of all fish (one family tank at a time) was measured. To do this, fish were quickly netted from their tanks in groups of 3, and lightly anesthetized in a bath containing 100 mg l^{-1} of tricaine methane sulphonate (Syndel Laboratories, Qualicum Beach, BC) (TMS). Then, a small sample of blood (~ 300 µl) was obtained within 2 min. of the initiation of anesthesia from the caudal vein using heparinized (80 units ml^{-1}) (Sigma Co., St. Louis, MO) syringes with 28G 1/2" needles, and fish had their PIT-tags read and body mass and standard length recorded. Blood was transferred to 1.5 ml microcentrifuge tubes, centrifuged (3000 *g* for 15 min. at 4°C), and the plasma immediately aliquoted into 0.6 ml microcentrifuge tubes, and frozen at -80°C. After sampling, fish were placed in a recovery tank with the same water supply as the family tanks. When all fish from one family had been sampled, and all fish resumed normal swimming behavior, they were moved back to their family tanks. This sampling point was designated as S1.

Subsequently, to determine cortisol levels in response to a standardized stressor, fish in each family were carefully netted from their tanks in groups of ~ 6, held in the net for 30 sec. and placed in a different tank with the same dimensions (150 l) and water supply as the original family tanks. Each group of 6 fish was netted 10 min. after the previous group to ensure that every fish had as close as possible to one hour to elicit the cortisol response before being sampled. When the first set of fish had reached one-hour post-stress, they were sampled as described for S1 except that the amount of blood sampled

was 500 µl. This procedure was repeated until all fish from one family were sampled (see Figure 2.1 for a graphical representation). Three to four families were sampled per day, with the entire sampling regime lasting 3 days. This regime was repeated 5 times (S2-S6) with at least a one month interval between sampling points. For each sampling, the order in which the families were sampled was determined randomly. One hour post-stress was selected based on a preliminary experiment (data not shown), which showed that cortisol levels were maximum at this time point (sampling points investigated were: 10 min., 30 min., 1 hr., 3 hrs. and 6 hrs. post-stress).

Since the absolute magnitude of post-stress plasma cortisol levels varied between sampling points, I calculated a total Z-score for each fish, and used this value as the variable for the categorization of individuals. This method standardizes values for an individual based on the ratio between its deviation from the overall cortisol mean and the overall standard deviation (Weil et al., 2001). Z-scores were calculated for each fish at all 5 sampling points (i.e. S2-S6) as:

$$Z = (\text{cortisol}_{ind} - \text{avg. cortisol}_{allind})/SD_{allind}$$

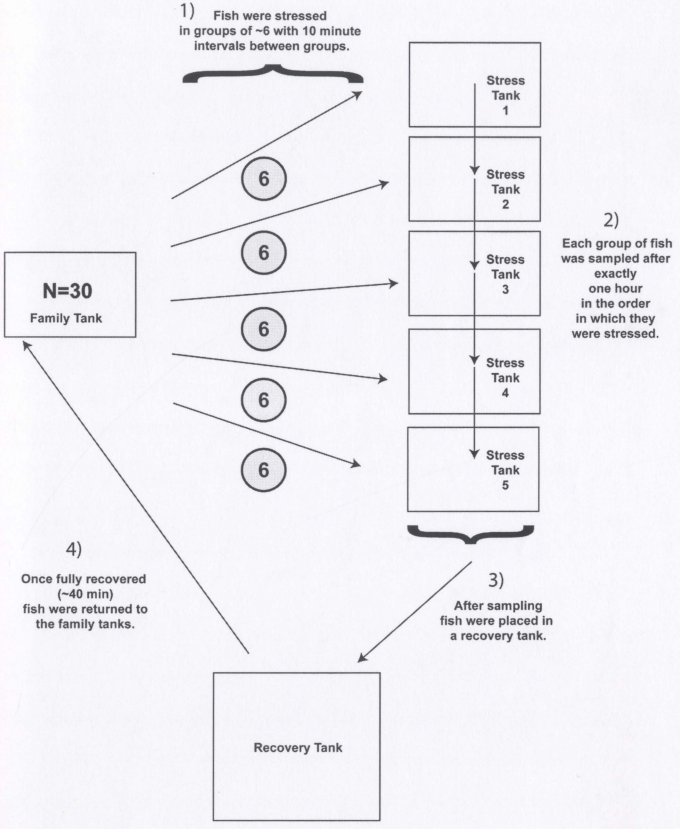
where cortisol_{ind} is the value for a given individual, avg. cortisol_{allind} is the average cortisol value for all individuals at a given sampling point (e.g. S2), and SD_{allind} is the standard deviation based on all the fish sampled at a particular sampling point. This resulted in 5 Z-scores for each fish, each representing its cortisol level at one sampling point. To categorize the individuals as high or low cortisol responders, the Z-scores for all sampling points were added, and the resulting value was referred to as Z_t (total Z-score). Fish

belonging to the first quartile of the Z_t distribution (i.e. with the lowest Z_t) were categorized as low cortisol responders (LR), whereas fish belonging to the fourth quartile of the Z_t distribution (i.e. with the highest Z_t) were categorized as high cortisol responders (HR). All remaining fish were considered non-consistent responders and were not used in Experiments #2 and #3 (see below).

2.3.3 Experiments #2 and #3: Are Atlantic cod consistently high or low responders?

A second experiment was performed to investigate whether fish categorized as LR or HR in Experiment #1 would maintain the magnitude of their cortisol response to the same stressor (handling stress) after being removed from their family tanks and mixed with other fish from the same category (e.g. HR and LR). In this experiment, 20 fish categorized as consistent low cortisol responders (i.e. belonging to the first quartile of the Z_t distribution) and 20 fish categorized as high cortisol responders (i.e. belonging to the fourth quartile of the Z_t distribution) that remained after the ones with highest and lowest

Figure 2.1 Schematic diagram of the sampling procedure used to investigate whether fish from a multi-family population of Atlantic cod could be classified as high- or low-cortisol responders.


1) Fish were stressed in groups of ~6 with 10 minute intervals between groups.
Stress Tank 1
Stress Tank 2
Stress Tank 3
Stress Tank 4
Stress Tank 5
6
6
6
6
6
N=30
Family Tank
2) Each group of fish was sampled after exactly one hour in the order in which they were stressed.
3) After sampling fish were placed in a recovery tank.
4) Once fully recovered (~40 min) fish were returned to the family tanks.
Recovery Tank

Z_t scores were sampled for measurements of stress-related gene expression [Hori et al., 2012– see Chapter 3], were divided equally into four 250 l tanks, 2 tanks for each category. One week later, 8 fish from each category (four from each tank) were subjected to the same stressor and sampling procedures as described above.

In the final experiment (Experiment #3), the 20 fish categorized as HR and 20 fish categorized as LR in Experiment #2 were again used (see above). Eight fish from each category were sampled directly from their tanks following euthanasia in 400 mg l^{-1} of TMS; these fish were used as before stress (i.e. heat shock) (BS) controls. After the control fish had been sampled, all the remaining fish from each category were transferred to a different tank with water temperature set at 18°C. Six fish from each category were then sampled at the cessation of the 3 hours heat shock (CS) and 3 hours after they were transferred back to their 10°C holding tanks. This point called 3 hours after the cessation of heat shock (3ACS). Blood was sampled and stored as described previously.

2.3.4 Separation of the free cortisol fraction and cortisol measurement

In a subsample of fish (10 per family) from time point S6 in Experiment #1, and for all fish in Experiments #2 and #3, the amount of free cortisol in the plasma was measured in addition to total plasma cortisol. The free plasma cortisol fraction was separated from that bound to proteins in the blood using the method described in Hosoya et al. (2007). Briefly, 300 µl of plasma were loaded onto ultracentrifugation columns (Centrifree® - MPS micropartition devices, Millipore Corp., Billerica, MA) and centrifuged at 2000 *g* for 30 minutes at 10°C. The resulting filtrate was saved and used for the measurement of free cortisol. Levels of free and total plasma cortisol were determined (in ng ml^{-1}) using

an ELISA (enzyme linked immunosorbent assay) as described in Hori et al. (2010 – Chapter 4). The percentage of free cortisol was calculated as:

([free cortisol] / [total cortisol]) x 100

2.3.5 Growth parameters

The specific growth rate (SGR) of each fish were calculated as:

((ln final body mass – ln initial body mass) / days) x 100

For each sampling point SGR was calculated using the body mass of the fish at S0 as the initial mass. Condition factor (CF) was calculated as:

(body mass x 100) / length^3

2.3.6 Quantitative trait loci (QTL) identification

DNA extraction and genotyping to identify single nucleotide polymorphisms (SNPs) followed the same procedures as described in Hubert et al. (2010). The Atlantic cod is a diploid species with 46 chromosomes (Johansen et al, 2009). A total of 1298 SNPs were tested for association with the magnitude of the cortisol response.

In this analysis, the difference (DCortisol) between the resting cortisol level at sampling point S1 (RCortisol) and the average of the 5 post-handling cortisol levels (S2 –

S6) (MCortisol) was fitted using straight association analysis (SAS 9.1, mixed procedure). Relationships between wild-caught parent stocks were not known, limiting pedigree information to a single generation of parent and offspring, so Sire and Dam were simply fitted as random classification effects. A chromosome-wise adjustment for error rate was used assuming each chromosome was independent; False Discovery Rate (FDR, Benjamini and Hochberg, 1995).

The model for DCortisol = (MCortisol – Rcortisol) was:

$$DCortisol_i = \mu + Sire + Dam + Zt_i + mass_i + e_i$$

where $DCortisol_i$ was the difference in cortisol level for the i-th fish, Zt_i was the average Z score for the i-th fish over the 5 sampling periods (S2 –S6), $mass_i$ was the average wet mass over the 5 month sampling period for the i-th fish, and e_i was the residual error associated with the i-th fish.

2.3.7 Statistical analyses

For Experiment #1 I used: 1) one-way ANOVAs, followed by Tukey's post-hoc tests for multiple comparisons, to detect differences between families in SGR and total cortisol (see Fig. 2.3); 2) repeated measures two-way ANOVAs [with category (LR vs. HR) and sampling point as main factors], followed by Tukey's post-hoc tests ($p<0.05$) for multiple comparisons, to test for between category differences and for between sampling point

differences (within category) in plasma cortisol, body mass, SGR, and condition factor (see Fig. 2.5); and 3) two-tailed t-tests to test for differences in free cortisol and % free cortisol between LR and HR Atlantic cod (see Fig. 2.6). For Experiment #2 I used two-tailed t-tests to compare the cortisol response (total, free and % free) of LR and HR fish to handling stress (see Table 2.1). For Experiment #3, I used a two-way ANOVA (with category and sampling point as the main factors), followed by a t-test to compare HR and LR fish at each sampling point and a one-way repeated measures ANOVA to compare each category (e.g. HR) between sampling points (see Table 2.1). Finally, Pearson's correlation analysis was performed to examine the relationships between: 1) final body mass and the average post-stress cortisol value from S2 to S6; 2) SGR and the average post-stress cortisol value from S2 to S6; and 3) Z_t and the growth parameters of final mass, SGR and condition factor. The data for % free cortisol were arcsine transformed prior to statistical analysis. Statistical analyzes were analyzed using MiniTab v14 (MiniTab Inc., PA) with $p<0.05$ set as the level of statistical significance. All data presented in the text, tables and figures are means ± standard error (S.E.), with exception on Table 2.2.

2.4 Results

2.4.1 Experiment #1

There was significant variation in the final body mass of the families. For example, fish in families 4 and 6 had the smallest average masses (146.2 ± 10.9 g and 214.1 ± 14.9 g, respectively) at the end of the experiment, when the mean mass of the Atlantic cod in families 12 and 73 was over 350 g (Figs. 2.2, 2.3A). In contrast, only the SGR of Family

73 (F73) was significantly different (by approximately 20% to 35%) as compared with all of the other families (Fig. 2.3B). When the families were ordered from highest to lowest with regards to mean cortisol levels from S2 – S6, there appeared to be a weak negative relationship between this parameter and both final body mass and overall SGR (Figs. 2.3A and B). This was also seen when the data for individual fish was analyzed, although the relationship was stronger between average post-stress cortisol level from S2-S6 and final body mass. The relationship between individual final body mass and average post-stress cortisol had a Pearson's correlation coefficient of of -0.15 ($p = 0.001$), whereas that for individual SGR values vs. average post-stress cortisol (S2- S6) was -0.04 ($p = 0.001$).

When the Atlantic cod were categorized according to Z_t, it was apparent that there was considerable variability in the proportion of LR and HR fish that comprised the families (Fig. 2.4). For example, while F3 and F73 had both LR and HR fish, some families had no LR (e.g. F31) or HR (e.g. F8 and F11) fish. Families with a high number of LR fish (e.g. F8 and F11) were substantially larger by the end of Experiment #1 as compared with families that had a high proportion of HR fish (e.g. F6, F31 and F34) (see Figs. 2.3A and 2.4). This relationship between LR vs. HR status (i.e. Z_t score) and growth parameters was confirmed when data for the LR and HR fish (54 fish in each category) was analyzed separately (Fig. 2.5). Low responder fish (LR) had post-stress cortisol levels that were on average only 29% of those measured in HR fish (i.e. 37.6 ± 6.9 ng ml^{-1} vs. 128.9 ± 30.7 ng ml^{-1}) (see Fig. 2.5A), and these fish grew 14% faster overall and were 55% larger at the final sampling point (S6) (Fig. 2.5B and C). Finally, a strong negative relationship was found between the Z_t for LR and HR fish and final mass (Pearson's correlation coefficient = -0.385 and $p < 0.0001$).

Differences in cortisol responsiveness were also found when the free plasma cortisol concentration of LR and HR fish from sampling point S6 was compared (Fig. 2.6). Consistent with the data for total plasma cortisol, HR fish had a free cortisol concentration approximately 4-fold higher than measured in LR fish (48.7 ± 4.4 ng ml^{-1} vs. 204.1 ± 24.7 ng ml^{-1}, respectively). However, there was no difference in the percentage of free cortisol, this value was ~ 55% for both groups.

2.4.2 Experiments #2 and #3

When the LR and HR fish were stocked into tanks containing fish of the same category, low and high responders had mean post-stress cortisol levels of 29.6 ng ml^{-1} and 74.3 ng ml^{-1} respectively (30 sec. of handling) ($p < 0.05$) (Table 2.1). These cortisol levels were lower than observed for these LR and HR fish in Experiment #1 (~ 74 ng ml^{-1} and ~ 127 ng ml^{-1}, respectively). However, the fold difference (i.e. mean cortisol levels in HR fish / mean cortisol levels in LR fish) in cortisol responsiveness (2.5) was comparable to the range of values reported in the first experiment (2.9 to 4.6 between S2 and S6).

Resting cortisol levels in HR fish from Experiment #3 were approx. 4.5 fold higher than measured in LR fish (48.8 vs.11.4 ng ml^{-1}, respectively), but this difference was not significant (Table 2.1). This result is very similar to that seen in Experiment #1 (see Fig. 2.5A). At the cessation of the 3 hour heat shock (sampling point CS) the average plasma cortisol value for LR fish (109.5 ± 12.3 ng ml^{-1}) was significantly lower than that measured in HR fish (193.6 ± 51.5 ng ml^{-1}) ($p<0.05$). However, both values were significantly higher than measured when the fish were exposed to a 30 sec. handling stress (i.e. Experiment #2) (Table 2.1). The difference in cortisol levels between LR and

Table 2.1 Total, free and percent free plasma cortisol levels (in ng ml^{-1}, ng ml^{-1} and %, respectively) in LR and HR fish subjected to handling (S2 – S6 Experiment #1; Experiment #2) and heat shock (Experiment #3).

	Sampling Point	LR Total Cortisol	LR Free Cortisol[1]	HR Total Cortisol	HR Free Cortisol[1]
Experiment 1	Average Data S2-S6	73.8 ± 4.7	_	126.5 ± 11.0*	_
Experiment 2	1ACS	29.6 ± 3.0	15.2 ± 5.9 (51.9%)	74.3 ± 19.2*	39.2 ± 3.9 (58.1%)
Experiment 3	BS	11.1 ± 4.4[a]	5.0 ± 0.9[a] (44.4%)	48.8 ± 23.0[a]	22.9 ± 6.1[a] (41.5%)
	CS	109.5 ± 12.3[b]	36.7 ± 2.0[b] (37.6%)	193.6 ± 51.5[b]*	79.5 ± 6.1[b]* (41.6%)
	3ACS	116.2 ± 17.6[b]	52.2 ± 4.4[b] (43.6%)	108.4 ± 29.9[c]	49.2 ± 6.0[b] (42.1%)

Asterisks (*) represents statistically significant ($p<0.05$) difference between LR and HR fish at a particular sampling point. Different letters represent statistically significant differences ($p<0.05$) within categories (e.g LR fish). 1ACS = 1 hour after the cessation of the handling stress; BS = Before heat shock, CS = at the cessation of heat shock, 3ACS = 3 hours after the cessation of heat shock.
[1]Percent free cortisol was calculated as the average of the (ratio between free and total cortisol x 100) for each individual in a given experimental group.

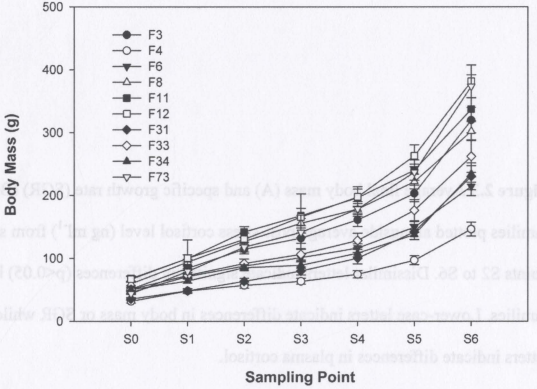
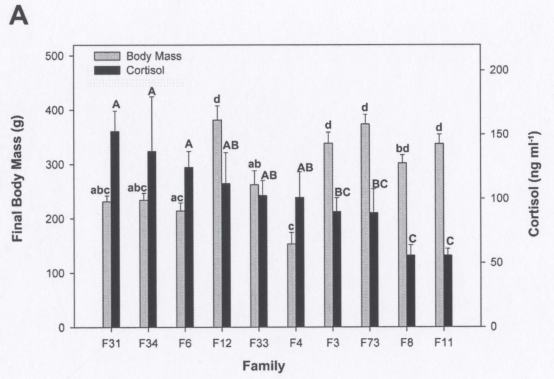
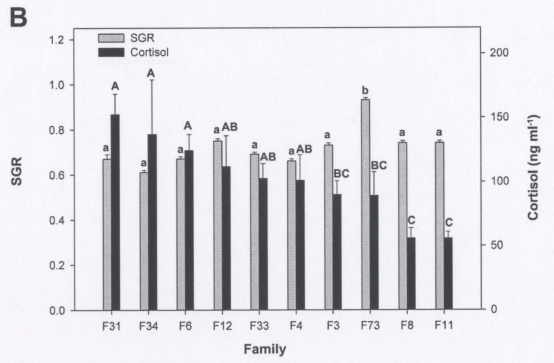


Figure 2.2 Average (± S.E.) body mass of all families from sampling points S0-S6. S0 shows the body mass of fish when they were sorted into their family tanks. S1 shows the body mass of the fish when they were sampled for resting cortisol. S2-S6 show the body mass of fish at the sampling points for post-stress plasma cortisol.

Figure 2.3 Average final body mass (A) and specific growth rate (SGR) (B) of all families plotted alongside average post-stress cortisol level (ng ml^{-1}) from sampling points S2 to S6. Dissimilar letters indicate significant differences ($p<0.05$) between families. Lower-case letters indicate differences in body mass or SGR while lower-case letters indicate differences in plasma cortisol.


A
Body Mass
Cortisol
Final Body Mass (g)
Cortisol (ng ml-1)
abc
abc
ac
d
AB
ab
AB
c
AB
d
BC
d
BC
bd
C
d
C
A
A
A
F31
F34
F6
F12
F33
F4
F3
F73
F8
F11
Family


B
SGR
Cortisol
SGR
Cortisol (ng ml-1)
a
a
a
a AB
a
a AB
a
b
a
a
BC
BC
C
C
A
A
A
AB
AB
F31
F34
F6
F12
F33
F4
F3
F73
F8
F11
Family

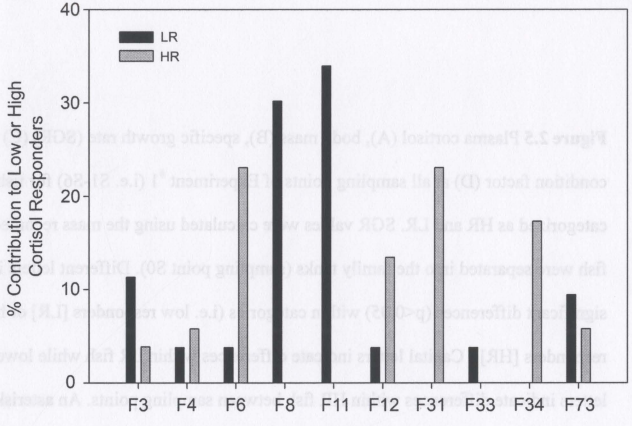
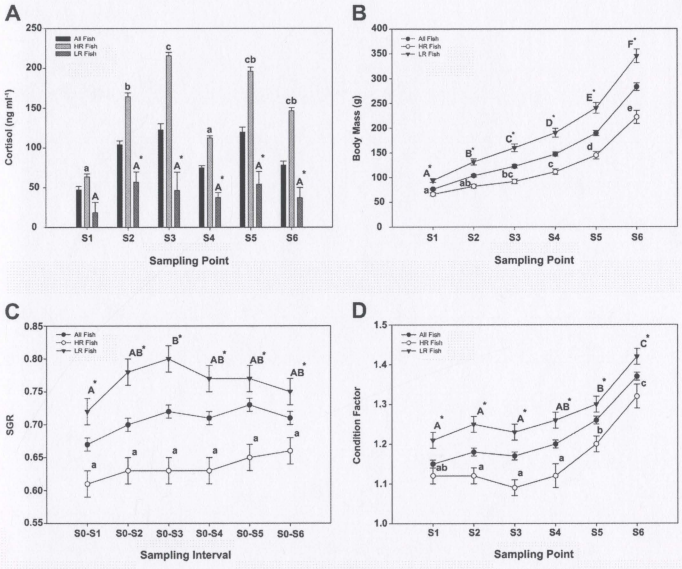


Figure 2.4 Contribution of each family (%) to the total number of fish classified as high responders (HR) or low responders (LR).

Figure 2.5 Plasma cortisol (A), body mass (B), specific growth rate (SGR) (C) and condition factor (D) at all sampling points of Experiment #1 (i.e. S1-S6) for fish categorized as HR and LR. SGR values were calculated using the mass recorded when the fish were separated into the family tanks (sampling point S0). Different letters indicate significant differences ($p<0.05$) within categories (i.e. low responders [LR] or high responders [HR]). Capital letters indicate differences within LR fish while lower-case letters indicate differences within HR fish between sampling points. An asterisk (*) indicates a significant difference ($p<0.05$) between LR and HR fish at a sampling point. The middle trace (filled-circles) is the average of all fish sampled.


A
All Fish
HR Fish
LR Fish
Cortisol (ng ml⁻¹)
Sampling Point
S1
S2
S3
S4
S5
S6
B
Body Mass (g)
Sampling Point
C
SGR
Sampling Interval
S0-S1
S0-S2
S0-S3
S0-S4
S0-S5
S0-S6
D
Condition Factor
Sampling Point

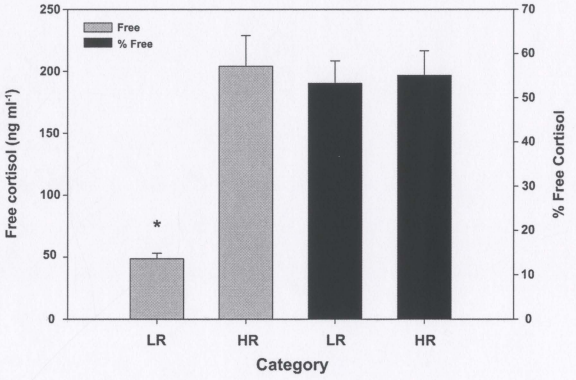


Figure 2.6 Free (grey - in ng ml^{-1}) and percent free (black) cortisol in a subset of fish categorized as HR or LR at sampling point S6 (N = 10 per family). An asterisk (*) indicates a significant difference ($p < 0.05$) between HR and LR fish.

HR fish was not evident at 3 hours after the heat shock (at 3ACS) where both groups had circulating cortisol values of approximately 120 ng ml^{-1}. The results were similar when the data for free plasma cortisol were examined, where the percentage of free cortisol ranged from 38 to 44% (Table 2.1).

2.4.4 QTL analysis

Four polymorphic SNPs were found to be significantly associated with the magnitude of cortisol response (Dcortisol). The CGP SNP identification (Hubert et al., 2010) of these markers is given in Table 2.2. Three of these had a False Discovery Rate (FDR) p-value ≥ 0.017, whereas the p-value for the SNP cgpGmo-S487 was 0.00002. The SNPs found to be significantly correlated with Dcortisol mapped to linkage groups 6, 13, 18 and 20 (Hubert et al., 2010).

2.5 Discussion

2.5.1 Stress response of juvenile Atlantic cod

In Experiment #1, mean cortisol levels 1 hour following a 30 sec. handling stress ranged from ~ 50 ng ml^{-1} to 100 ng ml^{-1} between S2 and S6. These values are comparable to those reported by King and Berlinsky (2006) and King et al. (2006) for Atlantic cod (100 and 80 ng ml^{-1}, respectively), and measured by Hosoya et al. (2007) and Afonso et al. (2008) in the haddock (*Melanogrammus aeglefinus*) (40–50 ng ml^{-1}). Further, the variability in cortisol levels that was observed across sampling periods was not unexpected. Similar results have been reported in several experiments with other fish

Table 2.2 Summary statistics for total Z-Score (Zt), post-stress cortisol levels (ng ml^{-1}), resting cortisol levels (ng ml^{-1}) and body mass (g), and a list of single nucleotide polymorphisms (SNPs) associated with the magnitude of cortisol response. The preferred genotype is the one associated with low cortisol responsiveness.

Trait	Mean	Min	Max	SD
Zt	0.11	-1.05	1.70	0.73
Post-Stress Cortisol	108.6	18.1	264.9	58.7
Resting Cortisol	42.9	0.1	426.5	69.5
Body Mass	155.4	38.4	362.4	64.6

Linkage Group	Preferred Genotype	fdr_p[1]	Position (cM)	Locus Name
6	TT	0.0174	3.382	cgpGmo-S848
13	GG	0.00002	42.546	cgpGmo-S487
18	TT	0.0287	48.440	cgpGmo-S975a
20	AA	0.0369	41.030	cgpGmo-S525

[1]False discovery rate p-value. SD = Standard Deviation

species that involved repeated sampling (see Weil et al. 2001; Fevolden et al. 2002; Castranova et al., 2005; Weber and Silverstein, 2007). For HR fish, the handling stress imposed during Experiment #2 resulted in an average post-stress cortisol level of 74.3 ng ml^{-1}, whereas plasma cortisol levels of 193.6 ng ml^{-1} were measured when these same fish were exposed to an 8°C heat shock (Experiment #3). This trend was also seen for LR fish, and suggests that high temperatures are extremely stressful for Atlantic cod on the basis of plasma cortisol. This finding is consistent with the data of Pérez-Casonova et al. (2008a) who exposed Atlantic cod to an incremental increase in water temperature from 10 – 24°C, and reported that cortisol levels were elevated by 16°C and reached peak levels in 10 and 50 g fish of approximately 200 and 450 ng ml^{-1}, respectively, by 22°C.

In Experiments #1 and #3, the percentage of free cortisol ranged from approximately 38 to 58%, and was not influenced by the type of stressor or the magnitude of the stress response (Table 2.1, Fig. 2.6). The percentage of free cortisol measured in these Atlantic cod is similar to what Hosoya et al. (2007) reported for stressed haddock (i.e. subjected to an acute handling stress) (40% - 60%), and collectively, these data suggest that at least in gadoids total cortisol is a suitable proxy for biologically available free cortisol.

2.5.2 High and low cortisol responsiveness in Atlantic cod

In this study, I used a multi-family population of Atlantic cod juveniles to calculate total Z scores (Z_t) for post-stress cortisol values, and classified low responder fish (LR) as those within the first quartile of Z_t values and high responders (HR) as those within the fourth quartile. While it is important to recognize that the limits used to classify the fish were arbitrary, and that LR and HR fish probably represent the extremes of a continuous

phenotype, my results clearly demonstrate that Z_t scores can be used to separate Atlantic cod with regards to the magnitude of their cortisol response. For example, the fold difference in total post-stress cortisol between HR and LR fish in response to a 30 sec. handling stress ranged from 2.9 to 4.6 over the 5 different sampling points (Fig. 2.5A). A difference in the magnitude of total cortisol response was still evident when a select group of HR and LR fish were reared separately (i.e. not within their respective families), and exposed to a different stressor (i.e. heat shock; fold difference 1.8 between LR and HR fish – Experiment #3) (Table 2.1). Finally, the percentage of cortisol present as free cortisol was similar in HR and LR fish (approx. 55%), as was the fold difference in post-stress cortisol levels between HR and LR at sampling point S6 when calculated based on free or total cortisol values (approximately 4) (Figure 2.6). This latter point is important as free cortisol is thought be the biologically available, and therefore, the active form of this steroid hormone (Breuner and Orchinik, 2002).

This difference in cortisol responsiveness between LR and HR Atlantic cod could be related to alterations in the hypothalamic-pituitary-interrenal axis, in interrenal biosynthetic capacity, and/or in aspects of cortisol clearance. Indeed, there is evidence for all three of these possibilities. The similar values for plasma cortisol at 3 hr post-stress in Experiment #3, despite a 1.8 fold difference at the cessation of stress, suggests that the two phenotypes differ in their ability to clear cortisol from the circulation. In Chapter 3, I demonstrate that the constitutive expression of transcripts that encode rate limiting proteins in cortisol synthesis (steroidogenic acute regulatory protein, P450 side-chain cleavage enzyme, and 3-beta-hydroxysteroid dehydrogenase) are significantly elevated (by ~ 2.5 fold) in HR Atlantic cod from these experiments [Hori et al., 2012 (Chapter 3)].

Finally, ACTH (adrenocorticotropin) injection results in a 2-fold higher plasma cortisol levels in HR vs. LR rainbow trout (Pottinger and Carrick, 2001). In contrast, it is unlikely that the difference in cortisol responsiveness in this study was due to differences at the level of the hypothalamus or pituitary. Pottinger and Carrick (2001) reported that plasma ACTH levels were not different between HR and LR rainbow trout.

2.5.3 Relationship between cortisol responsiveness and growth

Differences in the magnitude of the cortisol response to stress have been previously found in commercially important fish species such as the rainbow trout, striped bass and sea bream (Fevolden et al., 1991; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Wang et al., 2004; Castranova et al., 2005). However, there are contrasting data regarding how cortisol responsiveness relates to growth. For example, growth rate has been shown to be both negatively (Fevolden et al. 2002; Trenzado et al. 2003; Pottinger, 2006) and positively (Pottinger and Carrick, 1999b; Weber and Silverstein, 2007; Weber et al., 2008) correlated with cortisol responsiveness in rainbow trout. Further, Tort et al. (2001) showed that male, but not female, HR sea bream had greater values for SGR. In the present study, I report a significant, but weak, negative correlation between average cortisol level (from S2-S6) and either final mass or SGR. However, I also showed that Atlantic cod classified as LR based on Z_t values were significantly heavier than HR fish (by 55%) by the end of Experiment #1, that LR fish had significantly higher values for CF and SGR when compared to HR fish (Fig. 2.5), and that the relationship between Z_t and final mass had a r^2 value of -0.385. These latter data suggest that selection of Atlantic cod

based on cortisol responsiveness will lead to increased growth rates, and thus, increased production and a shorter time to market.

I did not perform any analyses to identify the factors that mediate the difference in growth performance between HR and LR fish. However, there is considerable data suggesting that the enhanced growth (e.g. final mass, SGR) exhibited by LR Atlantic cod was primarily due to differences in behavioral attributes between the two phenotypes. First, selection for cortisol responsiveness in rainbow trout is associated with a range of behavioral differences, with LR and HR fish usually (see Schjolden et al., 2005 for an exception) exhibiting behaviors characteristic of proactive and reactive coping styles, respectively (reviewed by Koolhaas et al., 1999). Specifically, LR rainbow trout are more aggressive and normally win fights for dominance in LR/HR pairings, tend to resume feeding more rapidly when placed in a novel environment, and display a reduced locomotor response in a territorial intrusion test (Pottinger and Carrick, 1999a; Overli et al., 2002; Ruiz-Gomez et al., 2008). These differences in behaviour could translate into enhanced growth through more favourable positioning of LR fish within the tank or a faster response to the presence of food (Pottinger, 2006). Indeed, Overli et al. (2006) showed that LR rainbow trout grew 24% faster than HR rainbow trout in the 2 weeks following a transport stress, and that this enhanced growth was associated with less food wastage and a greater feed conversion efficiency. Second, Pottinger (2006) showed that differences in growth between LR and HR rainbow trout were context dependent. When strains of LR and HR fish were reared separately, there were no detectable differences in growth. On the other hand, when the LR and HR fish were reared together, like in the present study, significant differences in body mass were evident. These data strongly

suggest that growth differences in LR and HR rainbow trout are not related to physiological differences. This is supported by Lankford and Weber (2006) who reported that, although significant differences in cortisol responsiveness to a 3 hour confinement stress were identified amongst families, there was no correlation between post-stressor cortisol levels and thermal growth coefficient or resting plasma insulin-like growth factor 1 and growth hormone levels.

2.5.4 Is cortisol responsiveness in Atlantic cod genetically determined?

I could not calculate heritability values for cortisol responsiveness of the studied Atlantic cod due to the limited number of families used (10) and the lack of known pedigree among the wild-caught parent stocks. Nonetheless, I found that there were large differences in the contribution of the various families to fish categorized as LR and HR (Fig. 2.4), with some families (e.g. 8 and 11) contributing large numbers of LR fish but no fish classified as HR, whereas others (e.g. 6, 31 and 34) had large numbers of HR fish. Further, in collaboration with Dr. Andrew Robinson's team at the University of Guelph, I was able to identify 4 polymorphic SNPs that were associated with cortisol responsiveness as determined using total Z scores (Z_t; Table 2.2). These data strongly suggest that a selective breeding program for low stress responsiveness should be possible, and is in agreement with the work that has been conducted on rainbow trout. For this species, calculated heritabilities for cortisol response magnitude range from 0.26 to 0.56 (e.g. Pottinger and Carrick, 1999b, Fevolden et al., 2002; Weber et al., 2008; Vallejo et al., 2009), and strains of LR and HR fish have been successfully developed and appear to be stable over several generations (Pottinger and Carrick ,1999b; Ruiz-Gomez et al.,

2008). However, the mechanisms by which these associated genomic regions (i.e. QTL) have an effect on cortisol response in Atlantic cod are not known, and thus, marker-assisted selection should be undertaken with care as it could have deleterious effects on other traits.

With regards to the identified QTL, 3 had associations with cortisol responsiveness that were marginally significant (FDR p-value>0.017), while one (locus cgpGmo-S487) had a FDR p-value of 0.0002. These results suggest that an interplay between several genomic regions underlies the variation in cortisol response in these families of Atlantic cod, but that the influence of cgpGmo-S487 in determining whether a fish has a LR or HR phenotype may be considerable. Although additional work must be performed to confirm this, Bayesian methods of complex segregation analysis for cortisol responsiveness to confinement stress in rainbow trout suggest that the stress (cortisol) response in this species is determined by the interplay between one or more major cortisol-decreasing genes and a large number of minute-effect additive polygenes; these major genes explaining as much as 63% of the total stress response variance (Vallejo et al., 2009). Clearly, fine scale mapping of the regions of the genome containing cgpGmo-S487 and the other QTL should be performed to identify these genes, and to investigate their role in determining cortisol responsiveness in Atlantic cod. To my knowledge, these are the first QTL for stress (cortisol) responsiveness identified for any species of fish.

2.5.5 Summary

In this study, I: 1) used 10 families of Atlantic cod to examine whether the cortisol response of this species is repeatable and to determine whether HR and LR phenotypes

can be indentified using Z scores; 2) established that a negative relationship exists between cortisol responsiveness (Z_t) and growth parameters; and 3) identified 4 QTL that correlate with stress responsiveness in this species. These results strongly suggest that there is a genetic component to the LR vs. HR phenotype in Atlantic cod, and that selected lines for low cortisol responsiveness can be developed by integrating such markers into a selective breeding program for this species (see Bowman et al., 2010).

However, it is presently unclear to what extent a breeding program based on the selection of LR fish would benefit the emerging Atlantic cod aquaculture industry in Canada, or potentially worldwide. First, when LR and HR rainbow trout were reared separately, as opposed to communally, Pottinger (2006) was unable to demonstrate differences in growth between the two phenotypes. Second, the relationship between cortisol responsiveness and growth can be positive or negative depending on the population of rainbow trout (Pottinger and Carrick, 1999b; Fevolden et al. 2002; Trenzado et al. 2003; Pottinger, 2006; Weber and Silverstein, 2007; Weber et al., 2008) and in this study I used a single population of Atlantic cod produced from broodstock collected at Cape Sable (NAFO Division 4X). Finally, although the chronic elevation of plasma cortisol results in a dose-dependent increase in mortality to common fungal and bacterial diseases in salmonids (Pickering and Pottinger, 1989), the limited number of studies on the relationship between cortisol responsiveness (HR vs. LR) and disease resistance are equivocal. Weber et al. (2008) were not able to correlate breeding and phenotypic values for cortisol responsiveness with survival following *Yersinia ruckeri* (the causative agent of enteric red mouth disease) exposure. Fevolden et al. (1992) showed that mortality of the HR phenotype was higher following exposure to *Aeromonas*

salmonicida (the causative agent of furunculosis), while the opposite was true following a *Vibrio angullarum* challenge (the causative agent of vibriosis).

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Chapter 3: Cortisol Axis Related Gene Transcript Expression in Atlantic Cod (*Gadus morhua*) Categorized as High or Low Responders

This chapter was published in the journal General and Comparative Endocrinology as: Hori, T.S., Rise, M.L., Johnson, S.C., Afonso, L.O.B, Gamperl, A.K., 2012. The mRNA expression of cortisol-axis related genes differs in Atlantic cod (*Gadus morhua*) categorized as high or low responders. Gen. Comp. Endocrinol. 175(2), 311-320.

3.1 Abstract

Cortisol is a major stress hormone in fish and is known, under normal or stressful conditions, to affect several physiological processes including growth and immunity. Thus, efforts have been made for several cultured finfish species, including the Atlantic cod, to determine whether fish with a high or low cortisol response to stress can be identified and selected. However, we have a limited understanding of the mechanisms that determine these two phenotypes. Thus, in this Chapter, I measured total and free plasma cortisol levels in high and low responding Atlantic cod when subjected to a 30 sec. handling stress, and the mRNA expression of 4 key genes in the glucocorticoid (i.e. cortisol) stress axis both pre- and post-stress. The cortisol data is consistent with the findings for Atlantic cod presented in Chapter 2, with high responding (HR) fish having ~ 3 fold higher total and free plasma cortisol levels when compared to low responder (LR) fish. Three of the transcripts studied encode key proteins involved in steroidogenesis (StAR, P450scc and 3βHSD), and the constitutive expression of all three genes was significantly higher (~ 2 fold) in the head kidney of HR fish when compared to LR Atlantic cod. The other gene of interest was the glucocorticoid receptor (GR). I partly cloned and characterized a cDNA from Atlantic cod likely to be this fish's ortholog of the teleost GR1, and showed that while there was no difference in hepatic constitutive GR mRNA expression between groups, HR fish had liver GR mRNA levels that were significantly (1.8 fold) higher at 3 hours post-stress as compared to LR fish. My results suggest that the different magnitude of cortisol response between LR and HR fish is at least partially determined by the capacity of the interrenal tissue to produce steroids.

3.2 Introduction

Cortisol is a key hormone in the fish stress response, and is known to regulate several important metabolic processes (e.g. carbohydrate metabolism) (Mommsen et al., 1999) and to have negative consequences if chronically elevated (Barton, 2002; Fast et al., 2008). Consequently many studies have investigated the response of this steroid hormone to a diverse array of stimuli (i.e. stressors) (for reviews see Mommsen et al., 1999; Iwama et al., 2005). Several studies in finfish [e.g. European sea bass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), sea bream (*Sparus aurata*)] have shown that the magnitude of the cortisol response to stress varies greatly between individuals of a given population, and that high and low cortisol responders (HR and LR, respectively) can be identified and selected (Fevolden et al., 1991; Pottinger and Carrick 1999; Tort et al., 2001; Weil et al., 2001; Fevolden et al., 2002; Fevolden et al., 2003; Trenzado et al., 2003; Wang et al., 2004). In Chapter 2, I showed that differences in the magnitude of cortisol response are also observed in Atlantic cod (*Gadus morhua*), that LR vs. HR status is independent of the type of stressor, and that cortisol responsiveness in this species was associated with 4 quantitative trait loci (QTL). However, the mechanisms underlying the different responsiveness of fish to the same stressor remain poorly understood, and must be elucidated before the benefits/consequences of selecting fish for stress responsiveness can be fully evaluated.

In teleost fish, cortisol is synthesized in the interrenal cells dispersed in the head kidney (Mommsen et al., 1999), and like other steroid hormones, its biosynthesis begins with the precursor molecule cholesterol and occurs primarily within the mitochondria (Hu et al., 2001). The first step in steroidogenesis (i.e. the synthesis of steroids) is the active

transport of cholesterol across the outer mitochondrial membrane. This is accomplished by active transport facilitated by steroidogenic acute regulatory (StAR) protein and the peripheral-type benzodiazepine receptor (PBR), a process that is thought to be the main rate-limiting step in steroidogenesis (Manna et al., 2009; Hu et al., 2010). Subsequently, several enzymatic reactions occur within the mitochondria before the final steroid hormone (e.g. cortisol) is released. These include the conversion of cholesterol into pregnenolone [catalyzed by cytochrome P450 side chain clevage (scc) – CYP11A1] and its subsequent oxidative conversion to progesterone (catalyzed by 3β hydroxysteroid dehydrogenase - 3βHSD). These three steps are conserved among most steroid biosynthetic pathways, and are thought to determine the amounts of steroid hormone produced in response to a given stimuli (Hu et al., 2001; Manna et al., 2009).

The glucocorticoid receptor (GR) is another key element of the stress response. It is a member of the nuclear receptor family and acts as a transcription factor when bound to cortisol, regulating the expression of genes containing the glucocorticoid response elements (GREs) in their promoter regions (Mommsen et al., 1999). In several fish species, the presence of two GR paralogs has been reported (Prunet et al., 2006), and these have been shown to have different binding characteristics in rainbow trout (Stolte et al., 2006). On the other hand, at least one fish species [i.e. zebrafish (*Danio rerio*)] appears to have only one copy of the GR (Aslop and Vijayan, 2009). Given that the effects of cortisol on fish physiology are primarily mediated through the GR (Mommsen et al., 1999), levels of its gene expression (or the expression of different paralogs) may determine the effects of this hormone. Several studies have examined GR mRNA expression in teleost fish in response to both acute and chronic stress (Sathiyaa and

Vijayan, 2003; Vijayan et al., 2003; Terova et al., 2005, Stolte et al., 2009). However, very little is known about the GR in Atlantic cod or GR regulation in fish categorized as high and low cortisol responders, with the exception of the recent work by Johansen et al. (2011).

Given the above, the objectives of this study were to: 1) characterize the GR in Atlantic cod; and 2) measure the mRNA expression of three key steroidogenic genes (StAR, P450scc and 3βHSD) in the head kidney, and of the GR in the liver, of Atlantic cod categorized as LR and HR.

3.3 Material and methods

3.3.1 Fish husbandry and categorization of HR and LR fish

All experiments were carried out with approval of the institutional animal care committees of Memorial University of Newfoundland and the National Research Council of Canada, and followed the guidelines of the Canadian Council on Animal Care.

Fish husbandry, and the experiments conducted to categorize fish as high (HR) and low (LR) cortisol responders, have been described in detail elsewhere (Hori et al., 2012 – see Chapter 2). Briefly, I subjected a multi-family population of Atlantic cod (300 fish in total) to 5 handling stress events (30 sec. air exposure) separated by approximately 1 month, and measured plasma cortisol for every individual at each sampling point. Based on the cortisol data, Z-scores were then calculated, and total Z scores (Z_t; Weil et al, 2001; Hori et al., 2012 – Chapter 2) were used to classify fish according to their cortisol responsiveness. Fish with Z_t scores in the first quartile of the Z_t distribution were

classified as LR, while those having Z_t scores in the fourth quartile of the distribution were designated as HR.

For this experiment, I selected the fish (mean mass ± S.E.: 377.7 ± 137.0 g) with the 30 lowest and 30 highest Z_t values [identified by their unique passive integrated transponder (PIT) tags], and placed fish in each category (LR and HR) into two separate 250 l tanks supplied with 10°C seawater (dissolved oxygen >90%) and lined with a net. Fish were then allowed to acclimate to their new tanks for 2 weeks.

3.3.2 Experimental design and sampling

After the 2-week acclimation period, 8 fish from each tank (i.e. 8 HR and 8 LR fish) were netted directly from their respective tanks, and euthanized by placing them in a bucket filled with seawater from the tanks that contained a lethal dose (400 mg l^{-1}) of tricaine methane sulphonate (TMS) (Syndel Laboratories, Qualicum Beach, BC). Aliquots of blood were quickly (i.e. within 2 minutes) obtained from the caudal vein using heparizined syringes with 28G 1/2" needles, and centrifuged at 3000 *g* (15 min. at 4°C) to separate the plasma. The head kidney (i.e. the part of the kidney anterior to the pectoral fins) and liver were sampled with dissecting instruments pre-cleaned with RNAseAway (Molecular Bioproducts, San Diego, CA) and placed in certified RNAse and DNAse free tubes (Fisher Scientific, City, USA). Both the plasma and the tissue samples were quickly frozen in liquid nitrogen, and stored at -80°C until cortisol analysis and RNA extraction, respectively, were performed. These fish served as undisturbed control fish, i.e. before stress (BS) samples.

The remaining fish in each tank were subjected to a 30 sec. handling stress by lifting the nets that lined the tanks out of the water. Following application of the stressor, 8 fish from each tank were sampled at 3 (3h), 12 (12h) and 24 (24h) hours after the handling stress (AS). At these sampling points, plasma and tissue samples were obtained using the methods described above.

3.3.3 Total and free cortisol separation and quantification

Total and free cortisol were separated and quantified as described in Hosoya et al. (2007) and Hori et al. (2012 – see Chapter 2). Briefly, 300 µl of plasma were pipetted onto ultracentrifugation columns (Centrifree - MPS micropartition devices, Millipore Corp., Billerica, MA) and centrifuged at 2000 *g* for 30 min. at 10°C. The filtrate, which is a protein-free extract, was then used for the measurement of free cortisol. Plasma values of free and total plasma cortisol were measured (in ng ml^{-1}) using an ELISA (enzyme linked immunosorbent assay; Neogen, Lexington, KY). The percentage of free cortisol was calculated as:

% free cortisol = [(free cortisol) / (total cortisol)] x 100

3.3.4 RNA extraction and column purification

RNA extractions, DNAse-I treatment and column purification were performed using the TRIzol reagent (Invitrogen, Carlsbad, CA), RNAse-free DNAse Set (Qiagen, Valencia, CA) and RNeasy MinElute Clean-up Kits (Qiagen), respectively. All

procedures were done according to the manufacturers' instructions with minor changes as described in Hori et al. (2010 – Chapter 4).

3.3.5 Cloning and sequence analysis of the Atlantic cod glucocorticoid receptor (GR) partial cDNA

Primers (see Table 3.1) were designed to amplify a conserved region of the fathead minnow (*Pimephales promelas*) glucocorticoid receptor (GR) described in Filby and Tyler (2007). One μg of DNAse-I treated, column cleaned, Atlantic cod liver RNA was reverse transcribed using MMLV reverse transcriptase (Invitrogen) as described in Rise et al. (2008). PCR amplification was then carried out using the TopTaq polymerase kit (Qiagen) following the manufacturer's instructions, and 14 cycles of touchdown PCR [94°C for 30 sec., 60°C (with a 0.5°C decrease per cycle) for 30 sec. and 70°C for 30 sec.], followed by 26 cycles of (94°C for 30 sec., 53°C for 30 sec. and 70°C for 30 sec.). PCR products were electrophoretically separated on a 1.5% agarose gel, and stained with ethidium bromide (EtBr) alongside an 100 bp ladder (Invitrogen). A ~ 300 bp (expected size based on Filby and Tyler, 2007) amplicon was extracted from the gel using the QIAQuick gel purification kit (Qiagen) following the manufacturer's instructions. This extracted PCR product was then cloned into the pGEM-T Easy TA cloning vector (Promega, Madison, WI). Plasmids were transformed into chemically competent DH5α *E.coli* and cells were plated onto Luria-Bertani (LB)-agar plates containing 100 μg ml^{-1} of ampicillin and X-Gal (50 μg ml^{-1}) (Invitrogen). Four individual white colonies were picked, grown overnight in liquid LB containing 100 μg ml^{-1} of ampicillin, and plasmids from these cultures were isolated using the QIAprep kit (Qiagen) following the

manufacturer's instructions. Recombinant plasmids were then sequenced as many times as required to ensure a 6 fold coverage of the insert at the Genomics and Proteomics (GaP) facility of Memorial University of Newfoundland using the BigDye terminator v3.1 chemistry and the ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Resulting sequences were assembled using the SeqMan function of Lasergene v7.2 software package (DNASTAR, Madison, WI) and generated a 307 bp consensus sequence that was confirmed by BLASTx to represent an Atlantic cod GR-like transcript. Based on this partial cDNA sequence, I designed gene specific primers (GSPs) to be used in 3' and 5' rapid amplification of cDNA ends (RACE) (Table 3.1), and used the 3' and 5' RACE components of the SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA) to extend the partial cDNA sequences. In this step, all PCR amplifications were performed using the high-fidelity Advantage 2 polymerase kit (Clontech). One μg of DNAse-I treated and column cleaned liver RNA was used for the RACE specific reverse transcription reaction using the poly (T) primers provided with the kit. All other steps were carried out following the manufacturer's instructions. RACE PCR products were cloned and sequenced and assembled as before.

3.3.6 GR amino acid sequence analysis

Since my attempts at characterizing the 5' end of the Atlantic cod GR cDNA failed, and a draft assembly of the Atlantic cod genome has recently become available (Star et al., 2011), I used the 1149 bp 3'RACE product to search the collection of putative transcripts based on this assembly (Star et al., 2011; http://codgenome.no/) using the

BLASTn server provided by the Atlantic cod genome project. I identified 2 full-length sequences (ENSGAUG00000005668 and ENSGAUG00000017783) representing GR-like transcripts and one representing a mineralocorticoid receptor (MR)-like (ENSGAUG00000001897) transcript of Atlantic cod. These sequences were translated using SeqBuilder (Lasergene software package v7.2) and hypothetical amino acid sequences were aligned with GRs of other vertebrates using the Japanese medaka (*Oryzias latipes*) (BAH59525) and Atlantic cod MRs as out-groups. Multiple alignment of sequences was performed using the ClustalW algorithm in the MegAlign software (Lasergene v7.2, DNASTAR), and a phylogenetic tree was constructed based on the alignment results using the Neighbour-Joining method and the bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in units of "number of amino acid substitutions per site". Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

3.3.7 QPCR of cortisol axis related transcripts

Primers for QPCR were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3) based on sequences available in GenBank [StAR (AY291434), P450scc (AY706102) and 3βHSD (EF464646)] or on the Atlantic cod GR-like sequence (i.e. *gmGR*) identified in this work. Every primer set was quality checked

before use as described in Hori et al. (2010 – Chapter 4). Briefly, a 5 point 1:5 fold dilution series was analyzed to calculate PCR amplification efficiencies according to Pfaffl (2001). Dissociation curves were also analyzed to make sure that there was only one sharp peak and no indication of primer dimers. Lastly, PCR products were electrophoretically separated on a 1.5% agarose gel alongside a 100 bp ladder (Invitrogen), and stained with EtBr, to verify amplicon size. Primer sequences, amplification efficiencies and amplicon sizes are shown in Table 3.1.

Complementary DNA (cDNA) synthesis for QPCR was performed with 1 μg of DNAse-I treated column purified head kidney (StAR, P450scc and 3βHSD) or liver (GR) total RNA using the MMLV reverse transcriptase (Invitrogen) as described in Rise et al. (2008) and diluted with 180 μl of nuclease-free water (Invitrogen). QPCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) in 13 μl reactions using 2 μl of cDNA (10 ng of input total RNA), 50 nM each of forward and reverse primer and 1X Power SYBR Green PCR Master Mix (Applied Biosystems). Transcript expression levels of the genes of interest (GOI) were normalized to 18S ribosomal RNA. PCR cycling parameters were as in Hori et al. (2010 – Chapter 4). On each 96-well plate, GOI and the normalizer gene were amplified in duplicate (Nolan et al., 2006). The 7500 Software Relative Quantification Study Application (Version 2.0) (Applied Biosystems) was used to determine threshold cycle (C_T) values. The relative starting quantity (RQ) of each transcript was determined using the delta-delta C_T method for relative quantification (Livak and Schmittgen, 2001), using the individual with the lowest gene of interest expression (i.e. lowest normalized expression) within a study as a

Table 3.1 Primers used in the polymerase chain reaction (PCR) and quantitative real-time reverse transcription - PCR (QPCR) experiments

Primer	Sequence (5' to 3')	Amplicon Size (bp)	r^2	Amplification efficiency (%)	Application
GR_cl_pp_F	GAGTACCTGTGCATGAAGGTCC				
GR_cl_pp_R	TTTCGGTAATTGGTTGCTGATGAT	~ 300	-	-	PCR
gmGR_3'RACE	GGCAGGAACGACTGCATCATCGACA				
gmGR_3'RACE_Nested	GCCCGAGGTGGTCTACTCCGGCTAC	-	-	-	3'RACE
STAR_QPCR_F	GGAACAGATGGGAGACTGGA				
STAR_QPCR_R	CACAGCGGACACTGACAAAG	150	0.969	98.5	QPCR
P450scc_QPCR_F	GATGCAGCTCTTCCTCATCC				
P450scc_QPCR_R	GCAGAGGCTTGATGGTCAAT	118	0.997	92.8	QPCR
3βHSD_QPCR_F	GGAGGTGCTGAGGTTCTGTC				
3βHSD_QPCR_R	TGAACGGCGTGTTGAGCAT	113	0.996	86.3	QPCR
GR_QPCR_F	GCTGCCCTTTATGAATGACC				
GR_QPCR_R	ATCCTTTGGTACGGTGCTGA	130	0.999	90.6	QPCR
18S_QPCR_F	ATGGCCGTTCTTAGTTGGTG				
18S_QPCR_R	GGACATTTAAGGGCGTCTCA	180	0.999	109.0	QPCR

calibrator sample. Overall fold-changes were always calculated as (mean HR RQ) / (mean LR RQ).

3.3.8 Statistical analyses

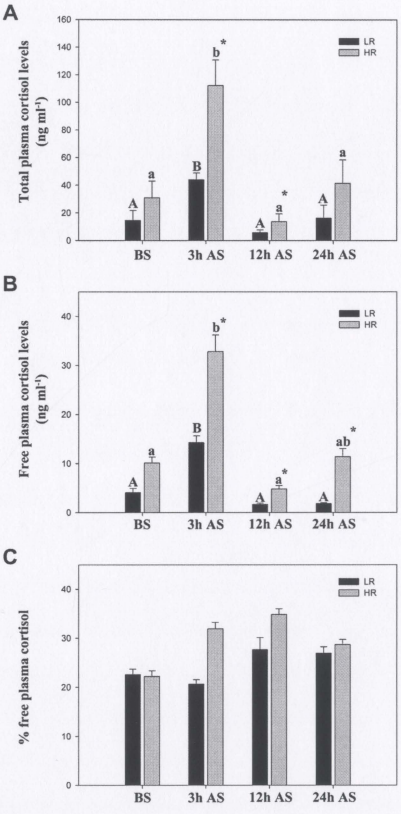
All statistical analyses were performed using Prism software v5 (GraphPad, La Jolla, CA). The percent free cortisol data were arcsine transformed using Prism before any statistical analysis was performed. The plasma cortisol (i.e. total, free and percent free) and QPCR RQ data were tested for normality using the Kolmogorov-Smirnov test. For both data-sets, I first conducted a two-way analysis of variance (ANOVA) with sampling point and category (i.e. LR or HR) as main effects. When the main effects had a significant impact on the studied variable, subsequent analyses were performed: 1) a t-test to compare LR and HR fish within each time point; and 2) one-way ANOVAs followed by Tukey's post-hoc tests within each category (e.g. LR) across time points.

3.4 Results

3.4.1 Plasma cortisol levels in LR and HR fish

Plasma total and free cortisol levels were 30.9 ± 12.8 and 10.1 ± 3.4 ng ml^{-1} (mean ± S.E.), respectively, in HR fish before application of the stressor. Both of these values were approximately 2-fold higher than those measured in LR fish, but neither of these differences were significant (p=0.269 and p=0.169, respectively) (Figs. 3.1A and B). This fold-difference in cortisol levels was maintained at 3 hrs. post-stress where total plasma cortisol values reached 112.3 ± 19.9 in the HR fish but only 43.9 ± 5.7 in LR Atlantic cod

Figure 3.1 Plasma total (A) (ng ml^{-1}) and free (B) cortisol levels (ng ml^{-1}), and the percentage of free cortisol (C), in Atlantic cod categorized as high (HR) and low (LR) cortisol responders. Different letters indicate significant differences within categories (i.e. low responders [LR] or high responders [HR]). Capital letters indicate differences within LR fish, while lower-case letters indicate differences within HR fish between sampling points. An asterisk (*) indicates a significant difference ($p<0.05$) between LR and HR fish at a sampling point.

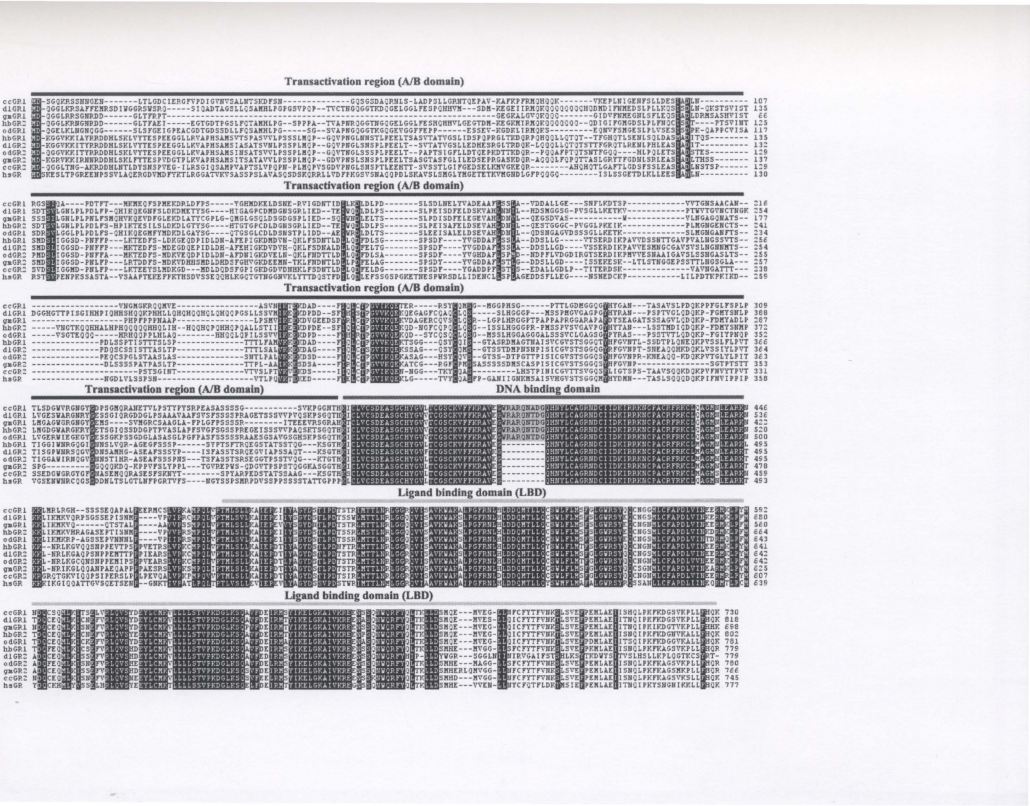

A
Total plasma cortisol levels (ng ml-1)
LR
HR
BS
3h AS
12h AS
24h AS
B
Free plasma cortisol levels (ng ml-1)
C
% free plasma cortisol

($p<0.05$); values for both total and free cortisol were also significantly higher than pre-stress levels within categories (e.g. LR) (Fig. 3.1A and B). Plasma cortisol levels (free and total) returned to pre-stress values by 12 hours AS (Fig. 3.1A and B). However, free cortisol levels were again significantly higher at both 12 and 24AS in HR fish, and total plasma cortisol was significantly higher in this group at 12h AS. The percentage of free cortisol ranged from 22% to 35%, but was not significantly different between the groups at any sampling point (Fig. 3.1C).

3.4.2 Cloning and sequence analysis of a Atlantic cod GR-like transcript

I initially identified a 307 bp Atlantic cod GR cDNA fragment using primers from Filby and Tyler (2007). BLASTx alignment of this fragment against the non-reduntant (nr) protein database of GenBank indicated that it represented an Atlantic cod GR-like transcript. Its best BLASTx hit was "Atlantic salmon (*Salmo salar*) glucocorticoid receptor (GenBank accession number ACS91455)", and this hit had an E-value of $3e^{-49}$ with 100 identities over 102 aligned putative amino acids (i.e. 98% identity). BLASTp analysis showed that these 102 amino acids were likely to represent the highly conserved part of the Nuclear Receptor Ligand Binding Domain (NR_LBD) (Fig. 3.2). I performed 3' RACE to further characterize this cDNA and obtained a 1149 bp sequence that contained the original 307 bp fragment. This cDNA sequence, which will be referred to throughout the paper as *gmGR*, was submitted to GenBank and is available under accession number JF896315.

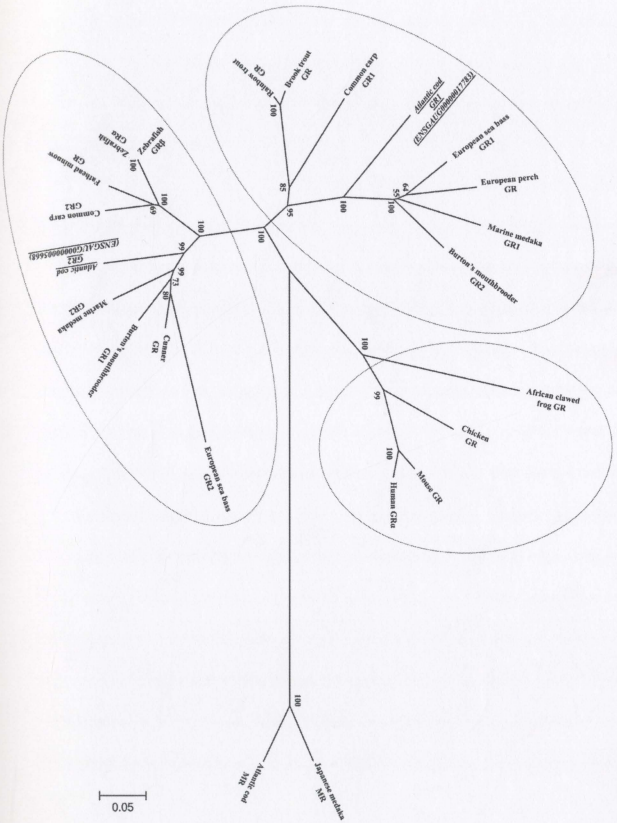
Figure 3.2 Amino acid multiple alignment of several vertebrate glucocorticoid receptor (GR) sequences showing the transactivation region (A/B domain) (black bar), the DNA binding domain (DBD) (dark grey bar) and the ligand binding domain (LBD) (light gray bar). Conserved residues or residues presenting conservative substitutions are shaded in black. The nine amino acid insert between the 2 zinc-fingers characteristic of the teleost GR1 DBD is shaded in grey. ClustalW within Lasergene was used to generate the alignment of *Cyprinus carpio* GR1 (ccGR1) (AJ879149) and GR2 (ccGR2) (AM183668); *Dicentrarchus labrax* GR1 (dlGR) (AY549305) and GR2 (AY619996); *Gadus morhua* GR1 (ENSGAUG00000017783) and GR2 (ENSGAUG00000005668); *Haplochromis burtoni* GR1 (hbGR1) (AF263738) and GR2 (hbGR2) (AF263740); *Oryzias dancena* GR1 (odGR1) (HM598068) and GR2 (odGR2) (HM598069); and *Homo sapiens* GRα (hsGR) (P04150).


Transactivation region (A/B domain)
Transactivation region (A/B domain)
Transactivation region (A/B domain)
Transactivation region (A/B domain)
DNA binding domain
Ligand binding domain (LBD)
Ligand binding domain (LBD)

This fragment encodes 237 putative amino acids, and its best BLASTx hit was "nuclear receptor subfamily 3 group C member 1" (glucocorticoid receptor) of the Japanese flounder (*Paralichthys olivaceus*) with an E-value of $2e^{-127}$ and 223 identities over 237 aligned amino acids (i.e. 94% identity). BLASTp analysis of the conceptual translated sequence indicated that it contains the Nuclear Receptor Ligand Binding Domain Glucocorticoid Receptor-like (NR_LBD_GR_like). I identified a stop codon (TAG) at position 713-715 bp, and thus, the remaining 434 nucleotides represent the 3' untranslated region (UTR) of this cDNA [including the poly(A) tail]. When I compared the coding region of this cDNA to the Norwegian collection of predicted transcripts (Star et al., 2011), based on their genomic scaffolds, I identified 2 sequences that were similar to the *gmGR*. The first one (ENSGAUG00000017783) was 99% (709/714 aligned nucleotides) identical to the cDNA I identified and the second one (ENSGAUG00000005668) showed only 87% (480/547 aligned nucleotides) similarity to *gmGR*.

Multiple sequence analysis showed that the predicted Atlantic cod transcript (ENSGAUG00000017783) that is 99% identical to *gmGR* (cloned in this work) has high similarity with the orthologous sequences of other teleost fish (Fig. 3.2). The phylogenetic analysis based on the putative translation of the full-coding sequences for several fish GRs shows two distinct clusters within the teleost GRs (Fig. 3.3), similar to that reported by Kim et al. (2011). It is also worth noting that the GRs for the Burton's mouthbrooder (*Haplochromis burtoni*) clustered in inverted groups (*H. burtoni* GR1 clustering with the other teleost GR2s, and *H. burtoni* GR2 clustering with the other teleosts GR1s – Fig. 3.3).

Figure 3.3 Phylogenetic tree showing the evolutionary relationships between the GRs of several teleosts and tetrapods. The tree was constructed in MEGA5 using the neighbour-joining method after multiple alignments were performed using ClustalW within Lasergene. The Japanese medaka (*Oryzias latipes*) (BAH59525) and Atlantic cod MRs were used as outgroups and tree reliability was verified by bootstrapping using 1000 replicates. Any branches that were present in less than 50% of bootstrap replicates were collapsed. Common carp (*Cyprinus carpio*) GR1 (AJ879149) and GR2 (AM183668); Marine medaka (*Oryzias dancena*) GR1 (HM598068) and GR2 (odGR2) (HM598069); Rainbow trout (*Oncorhynchus mykiss*) GR1 (P49843); Brook trout (*Salmo trutta*) GR (AY863149); Atlantic cod (*Gadus morhua*) GR1 (ENSGAUG00000017783) and GR2 (ENSGAUG00000005668); Burton's mouthbrooder (*Haplochromis burtoni*) GR1 (AF263738) and GR2 (AF263740); Zebrafish (*Danio rerio*) GRα (EF436284) and GRβ (EF436284); European sea bass (*Dicentrarchus labrax*) GR1 (AY549305) and GR2 (AY619996); Fathead minnow (*Pimephales promelas*) GR (AY533141); European perch (*Perca fluviatilis*) GR (EU861040); Cunner (*Tautogolabrus adspersus*) (GU596482); African clawed frog (*Xenopus laevis*) GR (P49844); Chicken (*Gallus gallus*) GR (DQ227738); Mouse (*Mus musculus*) GR (P06537); Human (*Homo sapiens*) GRα (P04150).


Rainbow trout GR
Brook trout GR
Common carp GR1
Atlantic cod GR1 (ENSGALG00000015681)
European sea bass GR1
European perch GR
Marine medaka GR1
Burton's mouthbreeder GR2
African clawed frog GR
Chicken GR
Mouse GR
Human GRα
Japanese medaka NIR
Atlantic cod NIR
0.05
European sea bass GR2
Cichlid GR
Burton's mouthbreeder GR1
Blue-fin medaka GR2
Atlantic cod GR2 (ENSGALG00000015681)
Common carp GR2
Fathead minnow GR
Zebrafish GRβ
Zebrafish GRβ

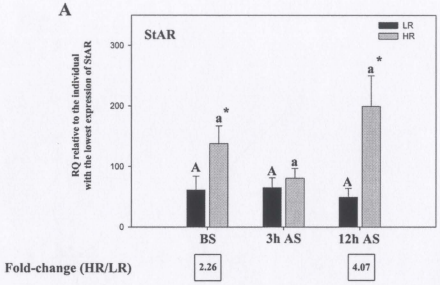
This is probably due to the way the authors (Greenwood et al., 2003) who identified them named these paralogs (Kim et al., 2011). Based on the high similarity between the sequence presented herein and one of the GR-like transcripts (ENSGAUG00000017783) used in the multiple alignment and its position on the phylogenetic tree, the cDNA identified in this work (i.e. *gmGR*) for the Atlantic cod is a putative ortholog of the teleost GR1 (Fig. 3.3).

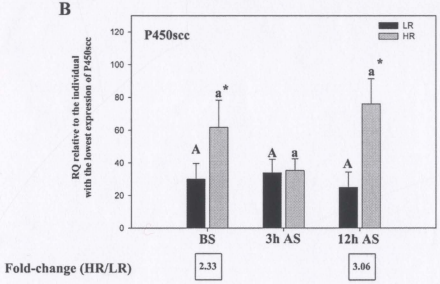
3.4.3 StAR, P450scc, 3βHSD, and GR transcript in LR and HR Atlantic cod

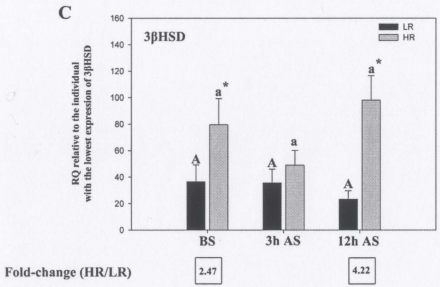
Transcript expression levels were only determined on samples collected up to 12 hours AS because cortisol levels had already returned to basal levels by this time post-stress. There were no significant differences in StAR, P450scc and 3βHSD mRNA expression in either category (HR or LR) over time. However, StAR, P450scc and 3βHSD mRNA expression levels were significantly ($p < 0.05$) higher in HR as compared to LR fish both before the stressor was applied and at 12h AS (Fig. 3.4A-C).

Glucocorticoid receptor (GR) mRNA (*gmGR*) expression in the Atlantic cod liver was similar for the LR and HR groups before the stressor (Fig. 3.5). However, the effect of the 30 sec. handling stress on GR mRNA expression was different for the two groups. GR mRNA levels in the LR fish were not elevated at either time-point post-stress as compared to LR fish at BS. In contrast, HR fish had significantly ($p <0.05$) higher GR mRNA levels at 3 hours post-stress when compared to the BS time-point or LR fish at 3h AS; overall fold-change values were approximately 2.4 and 1.8 fold, respectively (Fig. 3.5).

Figure 3.4 QPCR analysis of transcript levels of genes involved in steroidogenesis: (A) StAR, (B) P450scc, and (C) 3βHSD. Data is presented as mean ± S.E. RQs (relative quantities) were normalized to the expression of 18S rRNA and calibrated to the individual with the lowest expression of a given GOI. Different letters indicate significant differences ($p<0.05$) within categories (i.e. low responders [LR] or high responders [HR]). Capital letters indicate differences within LR fish, while lower-case letters indicate differences within HR fish between sampling points. An asterisk (*) indicates a significant difference ($p<0.05$) between LR and HR fish at a sampling point. Fold-changes presented in the white boxes were always calculated as (mean RQ of HR fish/mean RQ of LR fish).


A
StAR
LR
HR
RQ relative to the individual with the lowest expression of StAR
300
200
100
0
A
a
*
BS
3h AS
12h AS
Fold-change (HR/LR)
2.26
4.07


B
P450scc
LR
HR
RQ relative to the individual with the lowest expression of P450scc
120
100
80
60
40
20
0
A
a
*
BS
3h AS
12h AS
Fold-change (HR/LR)
2.33
3.06


C
3βHSD
LR
HR
RQ relative to the individual with the lowest expression of 3βHSD
160
140
120
100
80
60
40
20
0
A
a
*
BS
3h AS
12h AS
Fold-change (HR/LR)
2.47
4.22

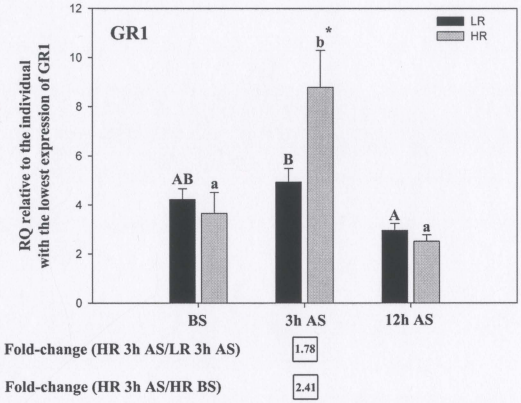


Figure 3.5 QPCR analysis of Atlantic cod GR-like (*gmGR*) transcript levels. Data is presented as mean ± S.E. RQs (relative quantities) were normalized to the expression of 18S rRNA and calibrated to the individual with the lowest normalized GR mRNA expression. Different letters indicate significant differences ($p<0.05$) within categories (i.e. low responders [LR] or high responders [HR]). Capital letters indicate differences within LR fish, while lower-case letters indicate differences within HR fish between sampling points. An asterisk (*) indicates a significant difference ($p<0.05$) between LR and HR fish at a sampling point.

3.5 Discussion

In this study, I report that the elevated cortisol responsiveness of HR vs. LR Atlantic cod is associated with increases in mRNA expression levels for StAR, P450scc, 3βHSD and GR, but that the 2.5 fold higher levels of cortisol are cleared from the blood of HR fish in the same period as for LR fish. Further, I partially cloned and sequenced a GR-like transcript in Atlantic cod (*gmGR*). These findings provide further insights into how HR vs. LR cortisol status in fish is regulated and its potential physiological impacts, and contributed to the development of new tools for the study of stress physiology in Atlantic cod.

3.5.1 Plasma cortisol in HR vs. LR fish

In Hori et al. (2012 – Chapter 2) I reported pre- and post-stress plasma cortisol levels for 10 Atlantic cod families, and demonstrated that it was possible to categorize fish as high or low cortisol responders based on plasma cortisol values at 1 hour post-handling. In this study, I took these HR and LR fish from their family units, grouped them separately for 2 weeks, and subsequently subjected both groups to handling stress. Based on the 2.5 fold difference in plasma cortisol levels between HR and LR fish at 3 hours post-stress, the present study confirms the categorization of fish as HR and LR as in Hori et al. (2012 – Chapter 2). Thus, HR and LR fish maintained their cortisol response to a stressor even when taken out of their family structure. This suggests that cortisol responsiveness (LR vs. HR) is not influenced by behavioral (social interactions) or morphological (size) differences, and provides further evidence that this trait is heritable in Atlantic cod. This finding is consistent with what is known for rainbow trout. In this

species, the magnitude of the cortisol response is heritable, and fish selected as HR and LR display consistent cortisol responsiveness over time/generations (Pottinger and Carrick, 1999; Fevolden et al., 2002; Weber et al., 2008; Vallejo et al., 2009).

3.5.2 Differences in gene expression associated with cortisol responsiveness

The higher cortisol levels in HR fish compared to LR fish at 3h AS were not associated with an up-regulation of mRNA expression levels for StAR, P450scc or 3βHSD at this time point, but with significantly higher transcript levels for all three of these genes prior to the stressor (Figs. 3.1A and 3.1B; Fig. 3.4). Interpretation of the lack of change in mRNA expression for these 3 proteins involved in cortisol synthesis is difficult as previous studies show considerable variability in the response of these transcripts to stress. For example, while Gelsin and Auperin (2004) showed that neither a 5 min. chase or ACTH injection affected the mRNA expression levels of StAR, P450scc and 3βHSD in rainbow trout, these authors reported that confinement stress (2 min.) followed by anesthesia (3 min.) resulted in increased mRNA expression levels of StAR and P450scc. Further, Nematollahi et al. (2009) showed that the mRNA expression of 3βHSD and P450c17a2, but not StAR or P450c21, changed in common carp (*Cyprinus carpio*) during and following confinement stress, and that the time-course for the changes in 3βHSD and P450c17a2 mRNA expression were very different; transcript levels of 3βHSD peaked at 20 min. of confinement, while P450c17a2 mRNA expression levels peaked at 4 hours after the confinement stress.

To my knowledge, no previous studies have measured the constitutive expression of transcripts encoding proteins involved in interrenal steroidogensis in HR and LR fish.

Nonetheless, these results suggest that differences in constitutive levels of StAR, P450scc and 3βHSD are associated with the magnitude of cortisol response to an acute stress in these two Atlantic cod phenotypes. This is an interesting hypothesis and it is consistent with Pottinger and Carrick's (2001) conclusion that cortisol responsiveness in HR vs. LR rainbow trout is not mediated by differences in the hypothalamus-pituitary axis, and with Rotllant et al. (2003) who showed that HR sea bream (*Sparus aurata*) interrenal cells have higher *in vitro* cortisol biosynthetic activity as compared with LR individuals. However, I cannot exclude the possibility that the elevated cortisol levels in HR Atlantic cod were related to differences in translation or post-translational modifications of steroidogenic proteins (as suggested by Gelsin and Auperin, 2004), or sensitivity of the interrenal cells to ACTH. Although their results are contradictory with regard to the influence of HR vs. LR status, both Rotllant et al. (2003) and Pottinger and Carrick (2001) show that ACTH-induced cortisol production is different in these two groups. The higher StAR, P450scc and 3βHSD transcript levels in HR Atlantic cod could be due to an increase in the number of interrenal cells, and/or the levels of expression per cell. I did not quantify the number of interrenal cells or measure their size, but there is information in the literature suggesting that both mechanisms could be involved in the increase in mRNA expression of steroidogenic genes reported here. Touma et al. (2008) showed that HR mice have enlarged adrenal glands as compared to LR individuals, and Ruane et al. (2005) showed that common carp with a disease state that affects cortisol production had associated interrenal hyperplasia (by 212%) and increased interrenal cell size (by 28%).

In addition to differences in cortisol biosynthesis, it appears HR Atlantic cod can clear higher amounts of cortisol than LR in the same time interval. In this study, HR

Atlantic cod had cortisol levels at 3h AS of 112.3 ng ml^{-1} vs. 43.9 ng ml^{-1} for LR fish, but at 12h AS levels were 13.6 and 5.8 ng ml^{-1}, respectively; this represents a drop of ~ 100 ng ml^{-1} for HR fish but only of ~ 38 ng ml^{-1} in LR fish. This result is consistent with Hori et al. (2012 – Chapter 2) who exposed the two phenotypes to a 3h heat shock and monitored cortisol levels at the end of the heat shock and post-stress, and suggested that a more efficient drop in post-stress cortisol levels may limit the impact of the stressor on the physiology of HR Atlantic cod. However, I only had a limited number of sampling points in this study, and cannot exclude the possibility that LR Atlantic cod had a more sustained cortisol elevation. Although no studies have examined whether cortisol clearance is different between fish categorized as HR and LR, Tuoma et al. (2008) showed that the fecal accumulation of cortisol between ACTH injection and 6 hours post-injection was 2 – 3 fold higher in HR mice.

One of the mechanisms controlling the levels of cortisol following a stressor is the negative feedback effects of this hormone on both corticotropin releasing factor (CRF) and ACTH via the glucocorticoid receptor in the brain (Aluru and Vijayan, 2007). However, it is unlikely that differences in feedback are responsible for the enhanced decline in plasma cortisol levels in HR Atlantic cod. Pottinger and Carrick (2001) showed that ACTH levels post-stress were very similar in LR and HR rainbow trout, and Johansen et al. (2011) showed that mRNA expression of GR1 and GR2 did not differ in the hypothalamus of HR vs. LR rainbow trout. These latter findings suggest that HR Atlantic cod may be able to clear more cortisol from the blood in a given interval when compared to LR fish.

3.5.3 Cloning and sequence analysis of the Atlantic cod GR-like transcript (*gmGR*)

At the time this experiment was initially conducted there were no publicly available sequences for the Atlantic cod GR. Therefore, I used a combination of PCR cloning and 3' RACE to obtain a cDNA representing a fragment of an Atlantic cod GR-like transcript (*gmGR*). The conceptual translation of this cDNA yielded a 237 amino acid (aa) partial protein, which is likely to represent the ligand binding domain (LBD) of the Atlantic cod GR as indicated by its BLASTp alignment results. Since then, the cod genome project (Star et al., 2011; http://www.codgenome.no) has made its draft assemblies and a collection of predicted transcripts publicly available. Using my cloned sequence (i.e. *gmGR*), I found two predicted transcripts (based on genomic scaffolds) representing the whole coding sequence for two putative Atlantic cod GR paralogs using BLASTn (http://www.codgenome.no). Multiple alignments of the putative translation of these sequences with those of other teleost GRs (Fig. 3.3), showed a high level of similarity between the LBD of these sequences and the LBD of the putative Atlantic cod GRs (85% - 95%). This is evidence that these putative transcripts represent GR-like genes in Atlantic cod.

For several species of teleost [e.g. the common carp, the European sea bass, the rainbow trout, the fugu (*Takifugu rubipres*) and the Japanese medaka] two copies of GR have been reported. In others like the Japanese flounder, the gilthead sea bream, brook trout (*Salmo trutta*) and zebrafish (*Danio rerio*) only one copy has been found. However, the zebrafish is the only teleost species in which the whole genome has been sequenced and there is no evidence for the existence of a second GR loci (Alsop and Vijayan, 2009). Further, as suggested by Kim et al. (2011) it is likely that there are two GR loci in many

fish species due to the teleost specific whole genome duplication (Prunet et al., 2006). Thus, it is probable that the two GR paralogs of Atlantic cod identified based on transcripts predicted from genomic scaffolds are orthologous with the teleost GR1 and GR2, respectively (Fig. 3.3). Kim et al. (2011) and Stolte et al. (2006) point out that the Burton's mouthbrooder GR1 and GR2 are orthologs of the teleost GR2 and GR1, respectively. That is also evidenced by my results, as the Burton's mouthbrooder GR1 clustered with the GR2 of other teleosts while its GR2 clustered in the GR1 branch of my tree (Fig. 3.3).

3.5.4 mRNA GR1 expression levels in LR and HR fish

I showed that *gmGR* (representing the Atlantic cod GR1) pre-stress mRNA levels were not different between HR and LR Atlantic cod, but that levels increased significantly in the former group at 3h AS. Once cortisol binds to GR, the receptor heterocomplex translocates into the nucleus where it binds to the glucocorticoid response elements (GREs) and regulates the transcription of several genes that are key in energy metabolism, osmoregulation and other important physiological processes (Mommsen et al., 1999). Therefore, higher levels of GR mRNA could lead to even greater effect of cortisol on these processes in HR fish. This hypothesis is supported by mammalian studies that show a positive correlation between GR mRNA and protein expression (Vedeckis et al., 1989; Breslin et al., 2001), but not by studies on fish. Numerous studies have reported lower glucocorticoid receptor protein levels in fish concomitant with elevated plasma cortisol (Maule and Schreck, 1991; Pottinger et al., 1994; Shrimpton and Randall, 1994; Mommsen et al., 1999; Shrimpton and McCormick, 1999; Sathiyaa and

Vijayan, 2003; Vijayan et al., 2003). Trezado et al. (2003) reported that HR trout exposed to chronic confinement showed a greater reduction in GR binding sites as compared to LR fish. Finally, Sathiyaa and Vijayan (2003) demonstrated that cortisol treatment results in increased levels of GR mRNA, but a decrease in GR protein expression and Aluru and Vijayan (2007) suggested that increased GR mRNA expression following stress was indicative of the negative feedback regulation of cortisol on GR protein content. In humans, it has been demonstrated that there is a GRE in one of the promoter regions of the GR gene (Breslin et al., 2001), and thus, this provides a potential mechanism through which GR mRNA levels might be regulated by cortisol exposure.

A decrease in hepatocyte GR protein levels would be consistent with the apparent more efficient clearance of plasma cortisol in HR fish, and thus, the suggestion that there are mechanisms that limit the physiological effects of the elevated cortisol levels on HR fish. Clearly, more studies must be conducted to resolve this issue, and future studies should include simultaneous measurements of GR and GR mRNA levels in HR and LR Atlantic cod, and measurements of cortisol mediated cellular responses. Hepatocytes would be a suitable model for such studies as they are an important target tissue for corticosteroids, there is considerable information on GR and GR mRNA regulation in fish hepatocytes/liver (Mommsen et al., 1999; Boone et al., 2002; Aluru and Vijayan, 2007), and glucose levels could be easily measured as an index of stress-responsiveness.

3.6 References

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Chapter 4: Heat Shock-Responsive Genes Identified and Validated in Atlantic Cod (*Gadus morhua*) Liver, Head Kidney and Skeletal Muscle Using Genomic Techniques

This chapter has been published in the journal BMC Genomics as: Hori, T.S., Gamperl, A.K., Afonso, L.O.B., Johnson, S.C., Hubert, S., Kimball, J., Bowman, S., Rise, M.L. 2010. Heat shock-responsive genes identified and validated in Atlantic cod (*Gadus morhua*) liver, head kidney and skeletal muscle using genomic techniques, BMC Genomics 11:72.

4.1 Abstract

Daily and seasonal changes in temperature are challenges that fish within aquaculture settings cannot completely avoid, and are known to elicit complex organismal and cellular stress responses and can lead to increased mortality. In this chapter, I used suppression subtractive hybridization (SSH) cDNA library construction and characterization, and quantitative real-time reverse transcription – polymerase chain reaction (QPCR) to identify and validate transcripts that were responsive to by heat shock (i.e. exposure for 3 hours to an 8°C elevation in temperature) in liver, skeletal muscle and head kidney of Atlantic cod. A total of 5980 ESTs were sequenced and characterized, 4394 (1524 from liver, 1451 from head kidney and 1419 from skeletal muscle) from three "forward subtracted" libraries (enriched for genes up-regulated by heat shock) and 1586 from a liver "reverse subtracted" library (enriched for genes down-regulated by heat shock). Several cDNAs encoding putative chaperones belonging to the heat shock protein (HSP) family were found in these libraries, and "protein folding" was among the gene ontology (GO) terms highly represented in the libraries. QPCR analysis of several known chaperones (e.g. HSP90α, HSP70-1, HSP47, GRP78 and GRP94-like transcripts) showed that these transcripts were significantly upregulated in all 3 tissues following heat shock (some by up to 100 – fold). Chaperones are an important part of the cellular response to stress, and genes identified in this work may play important roles in resistance to thermal-stress. In contrast, Toll-like receptor 22 (TLR22), found in the liver reverse SSH library, was shown by QPCR to be significantly down-regulated in the head kidney after heat shock. This transcript is a key immune response gene, and its down-regulation may be a component of heat-induced immunosuppression. These genes are good candidates for the

development of molecular markers to be used in the identification of elite Atlantic cod broodstock for the aquaculture industry.

4.2 Introduction

Temperatures are known to vary considerably at aquaculture cage-sites (Gollock et al., 2006) and can approach levels that are lethal for Atlantic cod (*Gadus morhua*). These changes can occur both rapidly [e.g. increase of ~ 8°C in less than 12 hours during thermocline inversions (Gollock et al., 2006)] and seasonally. Fish confined to cages cannot completely avoid these temperatures, and therefore, are likely to be exposed to stressful conditions (Gollock et al., 2006). The stress response consists of numerous modifications to an organism's physiology and behavior that are necessary to regain and maintain homeostasis once it has been challenged by changes in the environment, e.g. changes in temperature (Barton, 2002). The cellular response to stress is the coordinated reaction to a threat of macromolecular damage and protects the cell against the potentially hazardous consequences of such events (Kultz, 2005). Cortisol is often regarded as a suitable indicator of stress, and one of the key hormones regulating the stress response (Barton and Iwama, 1991; Barton, 1997; Mommsen et al., 1999). Most actions of cortisol are thought to be mediated by the glucocorticoid receptor (GR), which upon binding to the hormone, moves into the nucleus and acts as a transcription factor that interacts with specific promoter regions known as glucocorticoid response elements (GREs) (Mommsen et al., 1999). Stress can therefore have a significant impact on the transcription of specific genes. Thermal stress is also known to alter the transcription of a variety of genes including those encoding proteins that are involved in the response to oxidative stress, apoptosis, protein folding, energy metabolism, protein synthesis, membrane fluidity and immune function (Yiangou et al., 1998; Basu et al., 2002; Podrabsky and Somero, 2004; Buckley et al., 2006; Kassahn et al., 2007; Pérez-Casanova et al., 2008b). The proteins

encoded by these transcripts include some of the elements that comprise and/or regulate both the organismal and cellular stress responses, and may help to protect the animal against the deleterious effects of stress. Among these are chaperones (e.g. members of the heat shock protein gene family), anti-oxidative enzymes [e.g. catalase, superoxide dismutases (SODs), glutathione-S-transferases (GSTs)] and enzymes of carbohydrate metabolism (e.g. glycogen phosphorylase and phosphofructokinase) (Aluru and Vijayan, 2009).

Transcriptomic studies have been used to investigate the impacts of environmental stress on several organisms including fish (Gracey and Cossins, 2003; Meisterzheim et al., 2007; Wang et al., 2007; Lelandais et al., 2008; Garcia-Reyero et al., 2009; Perrigault et al., 2009; Romanuik et al., 2009). Specifically, suppression subtractive hybridization (SSH) libraries have been used to identify fish genes that are responsive to diverse stimuli such as polyriboinosinic polyribocytidylic acid (pIC, a viral mimic) injection (Rise et al., 2008), formalin-killed atypical *Aeromonas salmonicida* injection (Feng et al., 2009), osmotic stress (Kultz et al., 2007), cadmium (Reynders et al., 2006), and pesticide exposure (Marchand et al., 2006). In order to better characterize the genes and molecular pathways involved in the Atlantic cod response to heat shock, I, in collaboration with The Atlantic Genome Centre (TAGC), constructed, sequenced, and characterized reciprocal SSH libraries enriched for transcripts up-regulated or down-regulated by heat shock. Candidate heat shock-responsive Atlantic cod cDNAs identified in the libraries were further investigated using real-time quantitative reverse transcription – polymerase chain reaction (QPCR). The work described in this chapter is the first to use high-throughput genomic techniques [SSH library construction, sequencing of expressed sequence tags

(ESTs), cDNA sequence assembly, identification and functional annotation of assembled sequences in an EST database, and QPCR] to characterize the transcriptomic response of Atlantic cod to thermal stress. This work was part of the Genome Canada funded Atlantic Cod Genomics and Broodstock Development Project (CGP, http://codgene.ca), which developed many tools to study stress physiology in this species. A better understanding of changes in Atlantic cod gene transcription in response to heat shock will potentially lead to new tools and techniques [e.g. molecular biomarkers, QPCR assays, and single nucleotide polymorphism (SNP) genotyping assays] for studying the impacts of environmental stress on Atlantic cod, and for selecting individuals (broodstock) that can tolerate higher water temperatures.

4.3 Material and methods

4.3.1 Heat shock and sampling

One hundred and fifty juvenile Atlantic cod (~ 35 g) from a single CGP family (06NL04) were divided equally into 3 x 250 l saltwater flow-through tanks (10°C, dissolved oxygen >90% of air saturation). The tanks were then randomly assigned as control (C), control + handling stress (CT) and heat shocked (HS), and fish were allowed to acclimate to their new environment for one week. In addition, another two tanks with the same water conditions were set-up, these tanks adjacent to the tanks where the fish were stocked. During the one week acclimation period, the Atlantic cod were fed 1.5% of average body mass once daily. After the acclimation period one of the additional two tanks had the water flow interrupted and was heated to 18°C using a 1500 watt bayonet style immersion heater (Process Technologies, Tampa, FL), while the other tanks were

left as 10°C flow-through tanks. Fish from the CT group were quickly (~ 5 min) netted and transferred to the 10°C additional tank while fish belonging to the HS group were quickly netted and transferred to the 18°C additional tank. During the heat exposure period, oxygen levels were constantly monitored in both tanks using a dissolved oxygen (DO) meter and probe (Oxyguard, HandiPolaris, Denmark), and pure oxygen was gently bubbled into the 18°C tank to maintain the DO levels above 90% of air saturation. The heat shock lasted for 3 hours; after this period the immersion heater was turned-off and water in the HS tank was quickly (within 10 minutes) brought back to 10°C by re-establishing the flow of 10°C seawater. Eight fish from each tank were sampled before the heat shock (BHS; i.e. while still in their acclimation tank), at the cessation of the 3 hour heat shock (i.e. after cold water flow was re-established) (CS), and at 3, 12, and 24 hours after the cessation of heat shock (ACS). For lethal sampling, fish were quickly netted from their tanks and placed in a bath containing an overdose of anaesthetic [400 mg l^{-1} of tricaine methane sulphonate (TMS) (Syndel Laboratories, Qualicum Beach, BC)]. Head kidney, liver, and skeletal muscle samples were rapidly removed by team dissection, flash-frozen in liquid nitrogen and stored at -80°C until RNA extractions were performed. All sampling instruments were cleaned with RNAse Away® (Molecular BioProducts, San Diego, CA) between individuals. An aliquot of blood was centrifuged at 5000 *g* for 10 min. at 10°C to obtain plasma for cortisol determination.

4.3.2 Plasma cortisol

Plasma cortisol levels were determined in duplicate using an enzyme linked immunosorbent assay (ELISA) kit that has been previously validated for fish (Neogen

Corp. Lexington, KY), and parallelism to the standard curve was confirmed using serially-diluted plasma samples (as described by Hosoya et al., 2007). Intra- and inter-assay variation was determined and never exceeded 10%. The cortisol data were analyzed statistically using MiniTab v14 (MiniTab Inc., PA). Data were tested for normality using the Anderson-Darling normality test. Treatment and time point were used in a general linear model with 2 crossed factors, considering all possible interactions between factors (A B A*B model). When the effects of each factor on a given variable were found to be significant ($p < 0.05$), two separate analyses were performed: 1) a one-way ANOVA within each group (e.g. CT) across sampling points was used to determine if groups were different from the respective before heat shock (BHS) group ($p < 0.05$); and 2) an ANOVA within each sampling point was used to determine if the CT and HS groups were different from the undisturbed control (C) ($p < 0.05$). When groups were identified as significantly different through ANOVA, Tukey's multi-comparison pairwise post-hoc test was used to test the hypothesis that means were significantly different between groups.

4.3.3 RNA extractions

RNA was extracted from flash-frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with modifications. Samples (~ 50 mg of tissue) were disrupted in 1.5 ml RNAse-free microcentrifuge tubes using RNAse-free disposable pestles and a handheld homogenizer (Kimble Chase, Vineland, NJ) and further homogenized using QIAshredder spin columns (QIAGEN, Mississauga, ON) following the manufacturer's instructions. The remainder of the protocol was carried

out following the manufacturer's instructions. RNA samples were treated with RNAse free DNAse-I (QIAGEN) to degrade any residual genomic DNA contamination and then purified from salts, proteins and nucleotides using RNeasy MinElute (QIAGEN) spin columns following the manufacturer's instructions. RNA quantity and quality were assessed using NanoDrop spectrophotometry and 1% agarose gel electrophoresis, respectively. Only high quality total RNA samples (260/280 ratio >1.8, with tight 18S/28S ribosomal RNA bands) were used for library construction and QPCR.

4.3.4 mRNA isolation

Poly $(A)^+$ RNA (mRNA) was isolated from total RNA pools using the MicroPurist mRNA isolation kit (Ambion, Austin, TX) following the manufacturer's instructions. For each one of the tissues used for library construction [head kidney (HK), liver (L) and skeletal muscle (M)], column-cleaned, DNAse-I treated total RNA samples from the CT and HS groups taken at sampling times CS, 3ACS, 12ACS, and 24ACS were pooled for mRNA isolation (N = 32 for each group within each tissue). Each one of the 32 individuals contributed an equal quantity of cleaned total RNA (HK = 10 μg; L = 8 μg; M = 10 μg) to each tissue/treatment group-specific pool. Purified mRNA quantity and quality were assessed by NanoDrop spectrophotometry and 1.5% agarose gel electrophoresis, respectively. Poly $(A)^+$ RNA yield ranged from 1.5 - 4% of total RNA.

4.3.5 SSH library construction

Suppression subtractive hybridization (SSH) was performed using the PCR-Select cDNA Subtraction kit (Clontech, Mountain View, CA) and the manufacturer's

instructions. Pooled HS mRNA samples were used as testers in the forward subtractions and as drivers in the reverse subtractions. Pooled CT samples were used as drivers in the forward subtractions and as testers in the reverse subtractions. "Forward subtracted" libraries were designed to be enriched for genes that were up-regulated by heat shock, and "reverse subtracted" libraries were designed to be enriched for genes that were down-regulated by heat shock. Two μg of mRNA were used for each first-strand cDNA synthesis. After second strand synthesis, cDNA samples were *RsaI* digested for 1.5 hours at 37°C. For SSH enrichment two rounds of hybridization (6h for the first hybridization and 16h for the second hybridization) were performed in a hybridization oven at 68°C. All other procedures were performed according to the manufacturer's instructions.

The resulting SSH cDNA libraries were cloned into pGEM-T Easy (Promega, Madison, WI) vectors following the manufacturer's instructions. The ligation reactions were then transformed into chemically competent Max Efficiency DH5α cells (Invitrogen) using standard molecular biology techniques. Prior to sequencing, library insert size and complexity were evaluated as described in Rise et al. (2008).

4.3.6 DNA sequencing, sequence assembly and annotation

DNA sequencing, sequence assembly and annotation were performed by the TAGC team and are described in detail in Rise et al. (2008). Briefly, individual bacterial clones were inoculated into LB/glycerol with 100 μg ml^{-1} ampicillin in 384-well format and incubated overnight at 37°C. Sequencing reactions were carried out using the ET terminator chemistry (GE Healthcare, Piscataway, NJ) and after removal of excess fluorescent terminators, samples were loaded onto MegaBACE (GE Healthcare) capillary

sequencers. The resulting ESTs were analyzed for quality, trimmed and assembled as described by Rise et al. (2008) using Phred (Ewing and Green, 1998; Ewing et al., 1998) and Paracel Transcript Assembler (PTA). Each EST set resulting from the 4 different libraries (e.g. liver forward) was assembled separately. All ESTs were annotated by an automated pipeline using AutoFACT (Koski et al., 2005) and have been deposited in the GenBank dbEST under the accession numbers presented in Additional Files 4.1 and 4.2 (Tables S1 and S2 – **provided with the thesis on a CD**). One hundred and seventy nine ESTs were not submitted to GenBank due to low quality. Gene ontology (GO) annotation was obtained using AutoFACT (Koski et al., 2005) and GOblet (Groth et al., 2004). Annotations presented within the text were obtained using BLASTx manually, and reflect a more updated state of the NCBI's non-redundant (nr) protein database. AutoFACT summary results are stored in the CGP EST database (http://codgene.ca).

4.3.7 cDNA synthesis and quantitative real-time reverse transcription – polymerase chain reaction (QPCR)

Complementary DNA (cDNA) was synthesized from 1 μg of high quality, DNAse-I treated, column-purified total RNA (the same individual samples that were pooled for SSH library construction) using the High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Resulting cDNAs were diluted to a final concentration of 5 ng of input total RNA μl^{-1} with 180 μl of nuclease free water (Invitrogen).

Candidate stress-responsive transcript levels were quantified by QPCR using Power SYBR Green I chemistry and the 7500 Fast Real-Time PCR System (Applied

Biosystems). For QPCR studies, I used 6 individuals per treatment per time-point out of the 8 individuals that were used for SSH library construction. Individuals for QPCR were selected based on RNA concentration and quality.

The sequences of the primers used in mRNA expression analyses are presented in Table 4.1. Each primer set was tested for quality before use. QPCR primer quality control included running 5 serial 1:5 dilutions (with the exception of TLR22 for which a 1:2 dilution was used) for both CT and HS samples using cDNA (a pool of 6 individuals sampled at CS) at a starting concentration of 10 ng of input total RNA in order to calculate amplification efficiency ($E=10^{[-1/\text{slope}]}$) (Pfaffl, 2001). I also performed melt-curves (+1% increases every 30 seconds from 60°C to 95°C) to verify that the primers amplified a single product and that there were no primer dimers or amplification in the no-template controls (NTC). Furthermore, random samples of each QPCR amplicon were subjected to 1.5% agarose gel electrophoresis with ethidium bromide staining and compared with a DNA size marker (100 bp ladder, Invitrogen) to confirm that the amplicons were of the expected sizes.

PCR amplification was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems) in 13 µl reactions using 2 µl of cDNA (10 ng of input total RNA), 50 nM each of forward and reverse primer and 1X Power SYBR Green PCR Master Mix (Applied Biosystems). Expression levels of the genes of interest (GOI) were normalized to 18S ribosomal RNA. The suitability of 18S as a normalizer was confirmed by calculating the standard deviation (SD) of all 18S fluorescence threshold cycle (C_T) values for a given tissue. The highest SD found was 0.36 in the skeletal muscle, with

values of 0.28 and 0.30 in the head kidney and liver, respectively. The average 18S C_T values were 25.66, 25.90, and 27.99 for muscle, head kidney and liver, respectively. Moreover, the average 18S C_T for each group (e.g. HS) was calculated and shown not to differ by more than 0.3 cycles from the average of any other group. The QPCR cycling parameters consisted of 1 cycle of 50°C for 5 minutes to activate AmpErase Uracil N-glycosylase (UNG), 1 cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 15 sec. and 60°C for 1 min. On a given 96-well plate, target and normalizer genes were run in duplicate (Nolan et al., 2006). The C_T values were determined using the 7500 Software Relative Quantification Study Application (v2.0) (Applied Biosystems) with automated threshold determination and walking baseline. Each data set from a tissue was analyzed as a multi-plate study. The relative quantity (RQ) of each transcript was determined using the comparative C_T method for relative quantification (Livak and Schmittgen, 2001), using the individual with the lowest GOI expression (i.e. lowest normalized expression) within a given tissue as calibrator. Calculated amplification efficiencies (Table 4.1) were used to calculate RQs. Overall fold up-regulation for each group (e.g. HS at CS) was calculated as (average RQ)/(average RQ of the appropriate BHS group). Overall fold down-regulation (if applicable) was calculated as the inverse of overall fold up-regulation.

The RQs obtained from the software were statistically analyzed using MiniTab (v14). Data were tested for normality using the Anderson-Darling normality test. Treatment and time point were then used in a general linear model with 2 crossed factors, considering all possible interactions between factors (A B A*B model). When the effects of each factor

on a given variable were found to be significant ($p<0.05$) two separate analyses were performed. A one-way ANOVA in each group was used across sampling points to determine if groups (e.g. CT) were different from their respective BHS group ($p<0.05$); when groups were significantly different, Tukey's multi-comparison pairwise post-hoc test was used. A t-test within each sampling point was used to determine if the HS group was different from the CT group ($p<0.05$).

4.4 Results

4.4.1 Plasma cortisol levels

Plasma cortisol did not change significantly in the control (C) group over the course of the experiment, averaging 30.4 - 41.2 ng ml^{-1} (Fig. 4.1). In contrast, plasma cortisol levels were significantly ($p<0.05$) increased in the "transferred but not heat shocked control" (CT) and heat shocked (HS) groups at the cessation of the 3 hour heat shock (CS) when compared to their respective before heat shock groups (BHS) (Fig. 4.1). At this time point (CS), the average plasma cortisol level in the heat shocked group (HS: 132.4 ng ml^{-1}) was 2.3-fold higher than for the CT group (57.8 ng ml^{-1}) and 5.7-fold higher than for the HS "before heat shock" (BHS) group (23.4 ng ml^{-1}) (Fig. 4.1). Plasma cortisol in fish that were only transferred to a new tank at 10°C (CT) returned to basal levels (29.9 ng ml^{-1}) at 3 hours after the cessation of heat shock (3ACS), while cortisol in the heat shocked fish reached its highest level at this time-point (164.8 ng ml^{-1}), and remained significantly elevated at 12 (12ACS) and 24 (24ACS) hours after the cessation of heat shock; values at these time points were 3.5-fold and 2.8-fold higher than those measured in BHS fish, respectively.

Table 4.1 Primers used in quantitative real-time reverse transcription - polymerase chain reaction (QPCR)

Primer Name	Sequence	Gene name of the best named BLASTx hit	Efficiency	Amplicon Size (bp)
HSP90α F	5'- GAA CAA GAC CAA GCC CCT TT -3'	Heat shock protein 90 alpha	107%	133
HSP90α R	5'- CTG ACC CTC CAC CGA GAA GT -3'			
GRP94 F	5'- AGT GTT TCT CTC GAC ACG TTC A -3'	Heat shock protein 90kDa beta, member 1 (synonym: GRP94)	91%	97
GRP94 R	5'- CAG ACG ACT TCC ATG ACA TGA T -3'			
HSP70-1 F	5'- GAG AAC AAG ATC ACC ATC ACG A -3'	HSP70-1 (synonym: *HSPA1A*)	97%	123
HSP70-1 R	5'- GGC TGT TAC TTT CTC TCC CTG A -3'			
GRP78 F	5'- CTC CTT CAT TTT GGT CAG AAC C -3'	78 kDa glucose-regulated protein (synonym: GRP78)	90%	132
GRP78 R	5'- CTC AAG TTC CTC CCA TTC AAA G -3'			
HSP47 F	5'- ATG GAA GTC AGC CAC AAC CT -3'	Heat Shock Protein 47	93%	100
HSP47 R	5'- TCT TGC CCG TGA TGT TAG AC -3'			
CCT1 F	5'- GCA GGC GTT TGG GAT AAC TA -3'	T-complex-1	95%	127
CCT1 R	5'- GCG CTT AAC CCT TCA GAG AA -3'			
CCT5 F	5'- CCA GGC GAG GTT GAA GAA TA -3'	Chaperonin containing TCP1, subunit 5 (epsilon)	92%	91
CCT5 R	5'- TAG AAC AGG GGA GTG GTG GT -3'			
TCTP F	5'- ACC AAG CCA GAG AGA GTG GA -3'	Translationally-controlled tumor protein	101%	147
TCTP R	5'- ATC CTC ACG GAA GTC AAG CA -3'			
Enolase F	5'- GGA CGG CAC TGA AAA CAA AT -3'	Enolase 3	93%	115
Enolase R	5'- ACA GAG GAA CCC CCT TCT CC -3'			
PFK F	5'- TGT TTG CCA ACT CCC CAG AGA -3'	Phosphofructokinase, muscle a	96%	121
PFK R	5'- TCC GGT GCT TGA AGT CTG TCA -3'			
Aldolase F	5'- TGA CAT TGC TCA GAG GAT GG -3'	Aldolase B	91%	143
Aldolase R	5'- TAG CGA CGG TTC TCC TCA CT -3'			
Bikunin F	5'- GCC ACT GAG TTC ACA GAC G -3'	Alpha-1-microglobulin/bikunin precursor	90%	107
Bikunin R	5'- CAG CTC ATG GAG GAG GAG T -3'			
IgM F	5'- GAG CAT CCA CTG GCT CTT TA -3'	Immunoglobulin heavy chain, secretory form	93%	97
IgM R	5'- GCA GCA AGC TAT ATC CAG GT -3'			
Nupr1 F	5'- CTT TCT TCT CGC TGT TCT GC -3'	Nuclear protein 1	88%	101
Nupr1 R	5'- GGA AGG ACC AAG AAG GAG TC -3'			
IL-8 F	5'- CTT CAG CAT CCA GAC AGA CC -3'	Interleukin-8	100%	133
IL-8 R	5'- CAG ACA GAG AGC CGT CAG AT -3'			
TLR22 F	5'- TGC AGG TAA TCA CGA CTG AC -3'	TLR22	85%	93
TLR22 R	5'- GAG ACT TCC AGC CAG ACC TA -3'			
18S F	5'- ATG GCC GTT CTT AGT TGG TG -3'	18S ribosomal RNA (normalizer gene)	109%	180
18S R	5'- GGA CAT TTA AGG GCG TCT CA -3'			

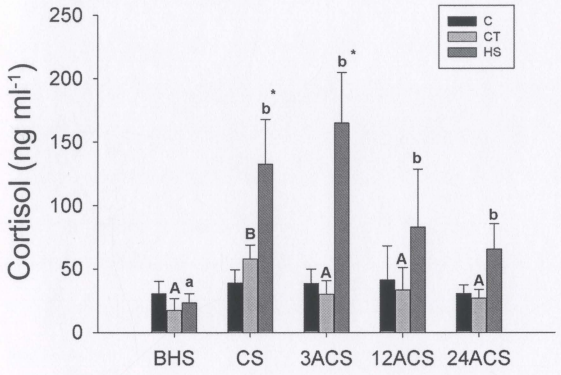


Figure 4.1 Average (±S.E.) plasma cortisol (ng ml^{-1}) levels in Atlantic cod before and following a 3 hour heat shock (transfer from 10°C to 18°C). Plasma cortisol levels in undisturbed control (C), control transferred (CT) and heat shocked (HS) groups are shown before heat shock (BHS), at the cessation of heat shock (CS) and 3h after the cessation of heat shock (3ACS), 12h after the cessation of heat shock (12ACS) and 24h after the cessation of heat shock (24ACS). Different letters indicate significant differences between sampling points within the same group (upper case for CT and lower case for HS) ($p<0.05$). An asterisk (*) indicates a significant difference between groups within a sampling point ($p<0.05$). Cortisol levels in the C group did not change significantly during the experiment.

4.4.2 Characterization of SSH libraries and identification of candidate heat shock responsive transcripts

I constructed and characterized 3 forward SSH libraries (liver, head kidney and skeletal muscle) and 1 reverse SSH library (liver). The SSH library method requires 2 μg of poly $(A)^+$RNA (mRNA) (Diatchenko et al., 1996), and mRNA usually only represents 1 - 5% of total RNA. In order to obtain adequate quantities of mRNA for SSH library construction, I isolated mRNA from pooled total RNA samples (see Methods). For SSH library construction, mRNA from heat shocked (HS) (i.e. netted and transferred to a new tank at 18°C) fish were subtracted against mRNA from tissues of control transferred (CT) fish that were subjected to a handling stress (i.e. netted and transferred to a new tank, identical to the HS tank but at the same temperature) but without heat shock. The mRNA used for SSH library construction consisted of samples taken at the cessation of the stressor and throughout the recovery period (up to 24 hours after the cessation of heat shock). The resulting SSH libraries were enriched for transcripts that were responsive to the combined handling and heat shock, but not those transcripts that were responsive to handling stress alone. I used three tissues to facilitate the identification of Atlantic cod transcripts that were stress responsive across tissues, and therefore likely to be linked to the organism's overall sensitivity or resistance to thermal stress. I built reciprocal SSH libraries to identify transcripts that were up-regulated by heat shock (i.e. "forward subtracted" libraries) as well as transcripts that were down-regulated by heat shock (i.e. "reverse subtracted" libraries). A total of 5980 expressed sequence tags (ESTs) were sequenced by TAGC, 1524 from the liver forward library, 1451 from the head kidney forward library, 1419 from the skeletal muscle forward library, and 1586 from the liver

reverse library (Table 4.2). Although reverse SSH libraries were also made for the head kidney and skeletal muscle, initial complexity evaluation and/or sequencing showed that they were of low complexity (i.e. dominated by a few highly abundant transcripts), and they were therefore not subjected to deeper sequencing (Table 4.2). The sequences were assembled into contiguous sequences (contigs) using Paracel Transcript Assembler (PTA) and annotated using AutoFACT (Koski et al., 2005). Selected defense (i.e. stress and immune) relevant sequences are presented in Tables 4.3 (forward) and 4.4 (reverse). In these tables, I do not include contigs and singletons with BLASTx hit E-values above 10^{-5}, as well as many sequences that are unclassified (i.e. no BLAST hit), redundant, or with BLAST hits that have non-stress related functional annotations. A complete list of assembled sequences (i.e. contigs and singletons), ESTs contributing to contigs, EST accession numbers, associated AutoFACT hit descriptions (including BLASTx statistics) and functional annotations, can be found in Additional File 4.1 (Table S1) and Additional File 4.2 (Table S2). Haemoglobin subunit alpha was the largest contig (17 contributing sequences) in the head kidney forward library (Additional File 4.1, Table S1A), and haemoglobin subunit beta was the largest contig (25 contributing sequences) in the liver forward library (Additional File 4.1, Table S1B). In the skeletal muscle forward library the largest contig was parvalbumin (66 contributing sequences) (Additional File 4.1, Table S1C). In order to identify and validate transcripts that were up-regulated in response to heat shock, I looked for Atlantic cod cDNAs that: a) had significant BLASTx hits (i.e. E-values lower than 10^{-5}) against proteins with stress-relevant functional annotations; b) were in contigs in more than one library; and/or c) had a relatively large number of contributing sequences (more than 5).

A list of selected contigs from the forward libraries, with detailed BLASTx statistics and functional annotations, is presented in Table 4.3. These include several Atlantic cod cDNAs with BLASTx hits against putative chaperone or chaperone-related proteins, including the following: the translationally-regulated tumor protein (TCTP) (found in skeletal muscle, head kidney and liver); heat shock protein 47 (HSP47) (skeletal muscle); several putative members of the T-complex (TCP) containing chaperonin system (CCT) including chaperonin containing TCP1, subunit 5 (synonym: CCT5) (skeletal muscle and liver), T complex protein 1 subunit beta (synonym: CCT2) (liver), T complex 1 (synonym: CCT1) (liver, skeletal muscle and head kidney) and TCP1 theta (synonym: CCT8) (head kidney); heat shock protein 90 alpha (HSP90α) (head kidney and skeletal muscle); heat shock protein 90 kDa beta, member 1 (synonym : glucose regulated protein 94, GRP94) (liver and head kidney); HSP70-1 (synonym: *HSPA1A*) (skeletal muscle); and the 78 kDa glucose-regulated protein (synonym: GRP78) (liver).

I also identified several Atlantic cod transcripts with significant BLASTx hits against genes and proteins involved in carbohydrate metabolism (Table 4.3 and Additional File 4.1, Table S1). These include enolase 3 (skeletal muscle), phosphofructokinase, muscle a (PFK) (skeletal muscle), transaldolase 1 (taldo 1) (head kidney and skeletal muscle), aldolase B (liver), phosphoglucomutase 1 (PGM1) (liver and skeletal muscle), glycogen phosphorylase (head kidney) and phosphoenolpyruvate carboxykinase (PEPCK) (liver).

In the liver reverse SSH library (i.e. enriched for transcripts down-regulated by heat shock) the 3 largest contigs were also haemoglobins (subunits alpha-1, beta-1 and beta-2) (Additional File 4.2, Table S2B). Some of the defense-relevant transcripts identified in this library were: tetraspanin-6, nuclear factor interleukin-3 regulated protein, alpha 1-

microglobulin/bikunin (bikunin), lectin, IgM heavy chain, hepcidin, interleukin-8 (IL-8), Toll-like receptor 22 (TLR22) and TNFAIP3-interacting protein 1. I also identified the cell cycle regulator nuclear protein 1 (NUPR1, synonym: p8).

Stress has been shown to influence fish immune function (Varsamos et al., 2006), and therefore for QPCR studies I chose primarily Atlantic cod cDNAs representing acute phase and immune-relevant genes as heat shock-responsive candidate transcripts from the liver reverse SSH library. Detailed information on these selected Atlantic cod transcripts is presented in Table 4.4. For a more comprehensive list (i.e. containing all contigs and ESTs identified in the reverse libraries along with AutoFACT hit descriptions, BLASTx statistics and functional annotations), refer to Additional File 4.2, Table S2.

4.4.3 Functional (Gene Ontology) annotation of assembled ESTs from SSH libraries

The ESTs from the 3 forward SSH libraries represented 958 (head kidney: 212 contigs and 746 singletons), 812 (liver: 200 contigs and 612 singletons) and 642 (skeletal muscle: 159 contigs and 483 singletons) putative transcripts, and ESTs from the liver reverse library represented 846 putative transcripts (178 contigs and 668 singletons) (Table 4.2). Using AutoFACT (Koski et al., 2005) and GOblet (Groth et al., 2004), these assembled sequences were assigned to 143, 157, 165 and 125 gene ontology (GO) terms (belonging to all 3 GO categories), respectively. A comprehensive list of assembled ESTs, and detailed BLAST statistics and functional annotations (e.g. associated GO terms) can be found at the CGP's website (http://codgene.ca/) and in Additional files 4.1-4.3 (Tables S1-S3). Protein biosynthesis and transport were among the GO terms with the highest proportion of associated assembled ESTs in all 4 libraries (Figs. 4.2 and 4.3). I

Table 4.2 Statistics for expressed sequence tags (ESTs) generated for all suppression subtractive hybridization (SSH) libraries[1]

Library Name	HK_F	HK_R[6]	SM_F	L_F	L_R
Tissue	Head Kidney	Head Kidney	Skeletal Muscle	Liver	Liver
Direction[2]	forward	reverse	forward	forward	reverse
CGP ID[3]	sb_gmnlkfta	sb_gmnlkrta	sb_gmnlmfta	sb_gmnllfta	sb_gmnllrta
# of ESTs	1451	93	1419	1524	1586
Average EST length[4]	297 bp	400 bp	340 bp	241 bp	227 bp
# of contigs[5]	212	8	159	200	178
# of singletons	746	35	483	612	668
# of non-redundant ESTs[7]	958	43	642	812	846
% redundancy[8]	33.9%	53.7%	54.7%	46.7%	46.6%

[1]Agarose gel electrophoresis of the resulting muscle reverse library revealed the presence of 5 clear bands, which indicated a low-complexity library and therefore sequencing was not attempted.
[2]The forward SSH libraries were constructed to be enriched for genes that were up-regulated by heat shock, and the reverse SSH libraries were constructed to be enriched for genes down-regulated by heat shock.
[3]The identifiers (ID) or names of the SSH libraries in the CGP EST database: http://codgene.ca.
[4]The ESTs were trimmed with Phred (Ewing and Green, 1998; Ewing et al., 1998) with the trim_alt and trim_cutoff fixed at 0.06, followed by the removal of known contaminant sequences and short sequences (<75 bp), and the average EST length was calculated based on edited sequences.
[5]Sequences generated were then clustered using Paracel Transcript Assembler (PTA), with the cluster threshold set at 100 for relatively stringent clustering.
[6]The 4 largest contigs in this library (Additional file 4.2, Table S2A) were annotated as haemoglobins and contained a total of 45 sequences, which represented almost 50% of the good sequences obtained for it. Thus, this library was deemed low-complexity and not sequenced further.
[7]The number of non-redundant ESTs is the sum of the number of contigs plus the number of singletons.
[8]Percent redundancy is the proportion of redundant ESTs in each library, calculated as [1 – (Number of non-redundant ESTs/total number of ESTs)] multiplied by 100.

Table 4.3 Selected cDNAs[1] from all 3 forward (enriched for genes up-regulated by heat shock) SSH libraries representing stress response related genes

Contig ID (Accession Number)	Tissue (Library)	QPCR	# ESTs	BLASTx identification[4] of selected contigs			Gene Ontology or function of putative ortholog[7]
				Gene Name [Species of best named BLASTx Hit]	%ID (aa length of align.)	E-value	
16.C1 (ES784003)	Muscle (gmnlmfta)	Fig.4.5I[2]	13	Enolase 3 (beta muscle) [*Danio rerio*]	91% (159/174)	2e-81	Glycolysis (BP), Cytoplasm (CC).
76.C1 (ES784315)	Muscle (gmnlmfta)	Fig.4.5H[2]	8	Translationally-controlled tumor protein[5] (TCTP) [*Cyprinus carpio*]	68% (116/170)	9e-62	Calcium Binding and apoptosis regulation (Bommer and Thiele, 2004).
45.C1 (ES783752)	Muscle (gmnlmfta)	Fig.4.4E[2]	7	Heat Shock Protein 47 (HSP47) [*Onchorhynchus mykiss*]	75% (132/174)	2e-76	Serine-type endopeptidase inhibitor activity (MF)
31.C1 (ES783410)	Muscle (gmnlmfta)	Fig.4.5G[2]	7	Chaperonin containing TCP1, subunit 5 (epsilon) (synonym: CCT 5) [*Danio rerio*]	93% (149/159)	2e-98	Protein folding (BP)*
27.C1 (ES783916)	Muscle (gmnlmfta)	Not sig.[3]	5	Phosphofructokinase, muscle a [*Danio rerio*]	88% (173/195)	4e-98	Glycolysis (BP)*
79.C1 (ES781823)	Liver (gmnllfta)	Not done	5	Cyclophilin A[6] [*Argopecten irradians*]	80% (132/164)	2e-75	Protein folding (BP)*
37.C1 (ES780395)	H. Kidney (gmnlkfta)	Not done	5	Taldo1 protein[5, 6] (synonym: transaldolase) [*Danio rerio*]	82% (120/145)	6e-63	Pentose-phosphate shunt (BP)*
65.C1 (EY973473)	H. Kidney (gmnlkfta)	Fig.4.4A[2]	4	Heat shock protein 90 alpha (HSP90α) [*Paralichthys olivaceus*]	93% (93/99)	5e-47	Protein folding (BP)*, Response to stress (BP)*
62.C1 (ES781078)	Liver (gmnllfta)	Not done	4	Glutathione S-transferase pi[5] [*Carassius auratus*]	69% (46/66)	9e-14	Metabolic process (BP)*
24.C1 (ES781741)	Liver (gmnllfta)	Not done	4	T-complex protein 1 subunit beta[6] [*Salmo salar*]	88% (174/196)	2e-77	Protein folding (BP)*
41.C1 (EY973602)	H. Kidney (gmnlkfta)	Not done	3	Copper/zinc superoxide dismutase [*Epinephelus coioides*]	82% (125/152)	2e-71	Superoxide metabolic process (BP), Superoxide dismutase activity (MF)[8]
113.C1 (ES781350)	Liver (gmnllfta)	Fig.4.4B[2]	3	Heat shock protein 90kDa beta, member 1[5, 6] (synonym: GRP94) [*Danio rerio*]	91% (122/134)	4e-54	Protein folding (BP), Response to stress (BP)*
146.C1 (ES781990)	Liver (gmnllfta)	Fig.4.5F[2]	2	T-complex 1[6] (synonym: CCT 1) [*Pan troglodytes*]	98% (59/60)	3e-28	Assists folding of tubulin and other cytoskeleton proteins (Lopez-Fanarraga et al., 2001)
128.C1 (ES783784)	Muscle (gmnlmfta)	Fig.4.4C[2]	2	HSP70-1 protein[6] (synonym: *HSPA1A*) [*Oryzias latipes*]	92% (170/183)	7e-76	Response to stress (BP)*, ATP Binding (MF)
5.C1 (EX190083)	H. Kidney (gmnlkfta)	Not done	2	TCP1-theta[6] [*Notothenia coriiceps*]	90% (56/62)	2e-25	Protein folding (BP), Protein Binding (MF)[9]
10.C1 (ES781650)	Liver (gmnllfta)	Fig.4.4D[2]	2	78 kDa glucose-regulated protein[6] (synonym: GRP78) [*Salmo salar*]	98% (60/61)	1e-28	ATP binding (MF), Ig chain folding (Haas and Wabl, 1983)[10]
27.C1	Liver	Not sig.[3]	2	Aldolase B[5] [*Poecilia*	91%	8e-20	Glycolysis (BP)[11]

Table 4.3 cont'd

(ES780987)	(gmnllfta)			*reticulata*]	(45/49)		
84.C1 (ES781112)	Liver (gmnllfta)	Not done	2	Pdia4 protein[6] [*Danio rerio*]	71/92 (77%)	5e-36	Cell redox homeostasis (BP)*
172.C1 (ES781772)	Liver (gmnllfta)	Not done	2	Phosphoglucomutase 1 [*Danio rerio*]	(96%) (38/41)	1e-14	Carbohydrate metabolic process (BP)*
174.C1 (EX190172)	H. Kidney (gmnlkfta)	Not done	2	Glycogen phosphorylase [*Oreochromis mossambicus*]	(84%) (69/82)	4e-34	Carbohydrate metabolic process (BP)*

[1]ESTs from each individual forward library (e.g. liver) were assembled separately. Contigs identified in the forward library that were also found in the liver reverse library were included in this table if present as one contig of 2 or more ESTs in at least 2 forward libraries or if validated by QPCR. For genes represented by multiple contigs in a given library or across tissues (i.e. in different forward libraries), the contig with the highest number of contributing sequences is shown. Possible reasons for the presence of multiple same named contigs within the same library are given in the discussion. All SSH contigs and singletons for the 3 libraries with all BLASTx statistics and GenBank accession numbers are presented in the Additional file 4.1, Table S1. The GenBank accession number of one representative EST from each contig is given in this table. The name of each SSH library on the CGP EST database are gmnllfta (liver), gmnlkfta (head kidney), gmnlmfta (muscle).

[2]These genes showed at least one statistically significant difference between 2 groups and/or time points (e.g. CT at BHS versus CT at CS, or CT at CS versus HS at CS) in at least 1 tissue.

[3]These 2 genes were analyzed with QPCR at the individual level, but the data is not presented because there were no statistically significant differences between groups and/or time-points.

[4]The BLASTx hit with the lowest *E*-value and a gene name (e.g. not predicted or hypothetical) is shown. BLAST statistics were collected on the 4th of December, 2008 and reflect the entries on the nr protein database up to that date. aa = amino acids.

[5]These cDNAs were represented by a contig or a singleton in the liver reverse library.

[6]Synonyms for protein names were retrieved from the Swiss-Prot Protein Knowledgebase Database (http://ca.expasy.org/sprot/):
Cyclophilin A: Peptidyl-prolyl-isomerase A, PPIase.
Taldo1 protein: Transaldolase 1.
T-complex protein 1 subunit beta: CCT2.
Heat shock protein 90kDa beta, member 1: HSPB1, Chaperone protein GP96, Tumor rejection antigen (gp96) 1.
T-complex 1: Chaperonin-containing T-complex polypeptide alpha subunit.
HSP70-1: HSP70, Heat Shock Protein 70 kDa 1, HSP72.
TCP1-theta: T-complex protein 1 subunit theta; CCT8.
Glucose-regulated protein 78kDa: Heat Shock Protein 70 kDa protein 5.
Pdia4 protein: Protein disulfide isomerase-associated 4.

[7]Functional annotation associated with the Atlantic cod cDNA's best BLAST hit or an annotated putative ortholog from *Homo sapiens* or *Mus musculus* (* or reference in []). Gene ontology (GO) categories: Biological Process (BP), Molecular Function (MF) and Cellular Component (CC).

[8]Other functional annotations associated with this cod EST are: Endoplasmic Reticulum (CC) and Nucleotide Binding (MF).

[9]Other functional annotations associated with this cod EST are: Oxidoreductase activity (MF), Zinc Ion Binding (MF) and Copper Ion Binding (MF).

[10]Other functional annotations associated with this cod EST are: Cellular Protein (CC), Cytoplasm (CC), Metabolism (BP), ATP Binding (MF), Nucleotide Binding (MF).

[11]Other functional annotations associated with this cod EST are: Catalytic Activity (MF), Fructose Bisphosphate Aldolase Activity (MF) and Metabolic Process (BP).

Table 4.4 Selected cDNAs[1] from the liver reverse (enriched for genes down-regulated by heat-shock) SSH library representing immune/stress related genes

Contig or Sequence ID (Accession Number)	QPCR	# ESTs	BLASTx identification[4] of selected contigs			Gene Ontology or function of putative ortholog[7]
			Gene Name [Species of best BLASTx Hit]	%ID (aa length of align.)	E-value	
154.C1 (ES783183)	Not done	3	Map4k4[5,6] [*Mus musculus*]	92% (24/26)	4e-05	Protein amino acid phosphorylation (BP)*
111.C1 (FL634330)	Not done	2	Glutathione peroxidase [*Xenopus tropicalis*]	55% (30/54)	1e-11	Response to oxidative stress (BP)*
121.C1 (ES782223)	Not done	2	Tetraspanin-6 [*Salmo salar*]	96% (28/29)	9e-09	G-protein coupled receptor protein signaling pathway (BP)*
129.C1 (ES782343)	Not done	2	Nuclear factor interleukin-3 regulated [*Danio rerio*]	73% (31/42)	6e-08	Immune response (BP)*
143.C1 (ES782977)	Not done	2	Chaperonin containing TCP1- subunit 3[4,6] [*Salmo salar*]	94% (127/134)	7e-67	Protein folding (BP)*
128.C1 (ES782638)	Fig.4.6B[2]	2	Nuclear protein 1[5,6] (NUPR1) [*Salmo salar*]	50% (35/70)	4e-11	Cell Growth (BP)*, Acute Inflammatory Response (BP)*
168.C1 (ES782218)	Fig.4.6C[2]	2	Alpha-1-microglobulin/bikunin precursor [*Oncorhynchus mykiss*]	55% (21/38)	1e-04	Serine-type endopeptidase inhibitor activity (MF), Endopepitidase (MF), Transporter Activity (MF)
69.C1 (ES783083)	Fig.4.6D[2]	2	Immunoglobulin heavy chain, secretory form[5] (IgM) [*Gadus morhua*]	97% (137/140)	1e-70	Immune response (BP)*
91.C1 (ES782456)	Not done	2	Lectin [*Oncorhynchus mykiss*]	48% (25/52)	2e-13	Sugar binding (MF)*
1e08 (ES782565)	Not done	1	Hepcidin precursor[5] [*Gadus morhua*]	98% (78/79)	1e-26	Innate immune response (BP)*
2i08 (ES782607)	Fig.4.6A[2]	1	TLR22[4,6] [*Takifugu rubripes*]	88% (39/44)	3e-29	Immune response (BP), Inflammatory Response (BP)[8]
7m20 (FL634530)	Not sig.[3]	1	Interleukin-8 [*Melanogrammus aeglefinus*]	82% (63/76)	4e-36	Immune response (BP), Cytokine Activity (MF)[9]
7p13 (FL634573)	Not done	1	TNFAIP3 interacting protein 1 [*Danio rerio*]	86% (74/86)	1e-34	Negative regulation of viral response genome duplication (BP)

[1]ESTs from the liver reverse library were assembled separately. These cDNAs were not represented by any contig of 2 or more ESTs in any of the 3 forward libraries. For genes represented by multiple contigs in this library, the one with the highest number of contributing sequences is shown. Possible reasons for the presence of multiple same named contigs within the same library are given in the discussion. All SSH contigs and singletons for the liver reverse library with all BLASTx statistics and GenBank accession numbers are presented in the Additional file 4.2, Table S2. The GenBank accession number of one representative EST from each contig is given in this table. The name of this SSH library on the CGP EST database is gmnllrta (liver). Sequence names reflect the plate number and the well number (e.g. 7p13 – plate 7, well p13).

[2]These genes showed at least one statistically significant difference between 2 groups and/or time points (e.g. CT at BHS versus CT at CS, or CT at CS versus HS at CS) in at least 1 tissue.

[3]This gene was analyzed with QPCR at the individual level, but the data is not presented because there were no statistically significant differences between groups and/or time-points.

[4]Refer to Table 4.3.

[5]These cDNAs were each represented by one singleton in the liver forward library.

[6]Synonyms for protein names were retrieved from the Swiss-Prot Protein Knowledgebase Database (http://ca.expasy.org/sprot/):

Table 4.4 cont'd

Map4k4: Mitogen-activated protein kinase kinase kinase kinase 4.
Chaperonin containing TCP1- subunit 3: CCT3.
Nuclear protein 1: Protein p8, Candidate of metastasis 1.
TLR22: Toll-like receptor 22.
[7]Refer to Table 4.3.
[8]Other functional annotations associated with this cod EST are: Innate Immune Response (BP), Receptor Activity (MF), Integral Part to Membrane (CC).
[9]Other functional annotations associated with this cod EST are: Extracellular Region (CC), Extracellular Space (CC).

identified many sequences that were assigned to GO terms relevant to stress or immune responses (e.g. metabolism, protein folding, immune response, proteolysis, glycolysis, response to stress and signal transduction). The proportions of assembled ESTs associated with GO annotations belonging to specific biological process categories relative to the total number of assembled ESTs with biological process GO annotations within each SSH library are shown in Figs. 4.2 and 4.3.

4.4.4 Expression of candidate heat shock-responsive transcripts

I used QPCR to validate and further study the effects of heat shock on 16 SSH-identified Atlantic cod transcripts. While SSH libraries were constructed using pooled mRNA samples, QPCR was conducted using individual RNA templates to assess biological variability. Eight Atlantic cod cDNA sequences selected for QPCR had significant BLASTx hits against proteins with chaperone functions (TCTP, HSP47, CCT-5, HSP90α, GRP94, CCT-1, HSP70-1 and GRP78), 3 had significant BLASTx hits against proteins involved in carbohydrate metabolism (enolase, PFK and aldolase), 3 were identified as immune-relevant transcripts (IgM, TLR22 and IL-8), 1 was most similar at the predicted amino acid level to an acute phase protein (bikunin) and 1 cDNA was identified as the transcript for a gene involved in regulation of the cell cycle (NUPR1). The number of contributing sequences and libraries where these transcripts were found are detailed in Tables 4.3 and 4.4.

Of the 11 cDNAs arising from the forward libraries and analyzed with QPCR (Table 4.3), all of those encoding proteins with putative chaperone function were shown to be

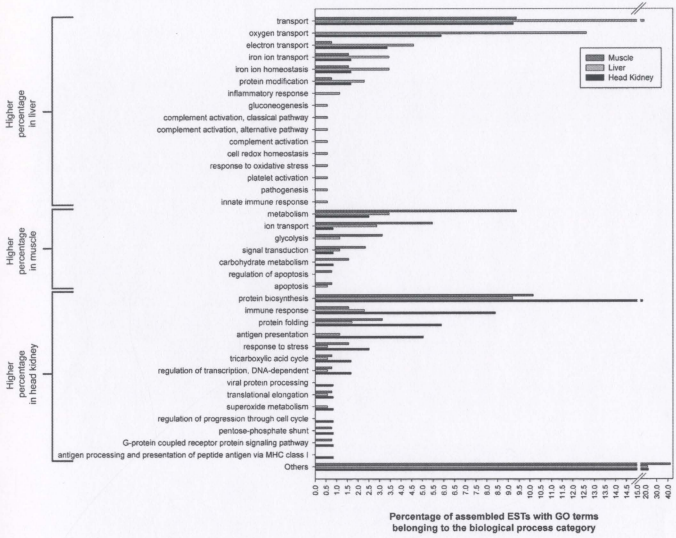


Figure 4.2 Summary of Gene Ontology (GO) functional annotation of assembled ESTs identified in the forward SSH libraries. GO annotations were obtained using AutoFACT (Koski et al., 2005) and GOblet (Groth et al., 2004) analysis of clusters. Numbers represent the percentage of ESTs with a particular GO annotation relative to the total number of sequences with GO annotation (all belonging to the Biological Process GO category). Dark grey: skeletal muscle. Light grey: liver. Black: head kidney.

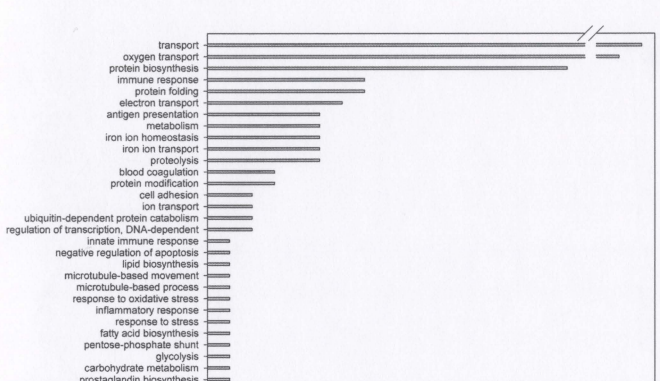


Figure 4.3 Summary of GO functional annotation of assembled ESTs identified in the liver reverse SSH library. GO annotations were obtained using AutoFACT (Koski et al., 2005) and GOblet (Groth et al., 2004) analysis of clusters. Numbers represent the percentage of ESTs with a particular GO annotation relative to the total number of sequences with GO annotation (all belonging to the Biological Process GO category).

significantly up-regulated at the mRNA level by heat shock in at least one tissue and at least one time point post heat shock (Fig. 4.4 A-E and 4.5 A-D) with the exception of TCTP. TCTP mRNA levels were responsive to handling alone (Fig. 4.5C) in the head kidney, with an average fold down-regulation of 3.0 and 1.6 at CS and at 3ACS, respectively. I also found that HSP70-1 (Fig. 4.4C) and GRP78 (Fig. 4.4D) transcripts responded significantly to handling alone in the liver. HSP70-1 mRNA was significantly up-regulated by an average of 47.1-fold at 12ACS in the CT group when compared to the before heat shock (BHS) group, while GRP78 mRNA was significantly down-regulated by an average of ~ 3.0-fold at all time points in the same group. These two transcripts showed no response to handling alone in the other tissues studied. Of the cDNAs with significant BLASTx hits against proteins involved in carbohydrate metabolism, enolase mRNA expression was significantly responsive to handling alone in only a single tissue (i.e. average down-regulation of 1.9-fold in the head kidney of CT group at the CS time point, Fig. 4.5D), but was not significantly responsive to heat shock.

The genes with the highest significant mRNA up-regulation in response to heat shock were HSP90α [Fig. 4.4A – average fold-changes of 168.7 (liver - CS) and 61.3 (skeletal muscle - 3ACS)]; HSP70-1 [Fig. 4.4C – average fold-changes of 100.2 (liver - 12ACS) and 17.8 (head kidney - 12ACS)], GRP94 [Fig. 4.4B – average fold-changes of 25.1 (head kidney - 3ACS) and 17.5 (liver - 3ACS)]; and HSP47 [Fig. 4.4E – average fold-changes of 20.4 (liver - 3ACS) and 16.9 (skeletal muscle - 12ACS)]. Of the 7 transcripts significantly up-regulated by heat shock that were identified in the forward libraries (i.e. HSP90α, GRP94, HSP70-1, GRP78, HSP47, CCT1 and CCT5 – Figs. 4.4 and 4.5 A-B), only two transcripts (CCT-1 and CCT-5, Fig. 4.5A and B) were not significantly up-

regulated (i.e. in at least one heat shock time point relative to BHS groups) in all 3 tissues tested. Within the tissues, HSP90α mRNA presented the greatest fold up-regulation in liver (average 168.7-fold up-regulation at CS) and skeletal muscle (average 61.3-fold up-regulation at 3ACS), while GRP94 mRNA presented the highest fold up-regulation in the head kidney (average 25.1-fold up-regulation at 3ACS). The transcripts with significant up-regulation in response to heat shock also showed differences in timing of expression in the three tissues studied. As previously stated, HSP90α mRNA expression in the liver peaked relative to the BHS group at CS (Fig. 4.4A). However, many stress-relevant transcripts had significant maximum fold up-regulation at 3ACS relative the BHS group. These were: HSP90α in the skeletal muscle (Fig. 4.4A, 61.3 fold-change); GRP94 in all tissues [Fig. 4.4B – average fold-changes of 17.5 (liver), 25.1 (head kidney), and 11.9 (skeletal muscle)]; GRP78 in all tissues [Fig. 4.4D – average fold-changes of 5.2 (liver), 4.4 (head kidney), and 3.8 (skeletal muscle)]; HSP47 in the liver (Fig. 4.4E – average fold-change of 20.4); and CCT1 (Fig. 4.5A – average fold-change of 9.3) and CCT5 (Fig. 4.5B – average fold-change of 10.8) both in the liver. Transcripts for HSP90α in the head kidney (Fig. 4.4A – average fold-change of 6.3), HSP47 in the skeletal muscle and head kidney [Fig. 4.4E – average fold-changes of 16.9 (skeletal muscle) and 1.6 (head kidney)], and HSP70-1 in all tissues [Fig. 4.4C, 100.2 (liver), 17.8 (head kidney) and 10.1 (skeletal muscle)] had maximum significant mRNA fold up-regulation at 12ACS relative to the BHS group. The timing and magnitude of the changes in expression of transcripts for HSP90α (Fig. 4.4A), HSP47 (Fig. 4.4E), and GRP78 (Fig. 4.4D) also varied between different tissues. For example, HSP90α transcript was maximally up-regulated at CS relative to their BHS groups in the liver but at 3ACS in the skeletal muscle.

From the 5 cDNAs selected for QPCR studies from the reverse liver library (Table 4.4), NUPR1 and TLR22 were the only Atlantic cod transcripts that responded significantly to heat shock. The transcript levels for TLR22 were down-regulated by an average of 2.2- and 2.6-fold in the HS group in the head kidney at CS and 3ACS, respectively, when compared to the levels in the BHS group (Fig. 4.6A). Interestingly, NUPR1 transcripts were significantly up-regulated by heat shock at CS in both liver (average 2.7-fold change) and head kidney (average 3.0-fold change), and at 3ACS the HS group still showed significantly higher levels of NUPR1 transcript than in the CT group in both tissues (Fig. 4.6B). Bikunin and IgM mRNAs displayed similar responses in CT and HS groups (Fig. 4.6C and D) and IL-8 mRNA showed no significant changes (data not shown). Bikunin transcripts in the liver and head kidney were reduced by both stressors. They were significantly lower in the CT group in the liver at 3ACS when compared to levels in the BHS group, and in the head kidney from HS fish when compared to those from the CT group at the CS time-point (Fig. 4.6C).

4.5 Discussion

Thermal stress can pose a significant challenge to Atlantic cod in aquaculture cage sites as seasonal temperatures may approach the upper critical thermal limit for this species and/or change rapidly during the day (due to thermocline inversions, i.e. when bays "turn-over") (Gollock et al., 2006). Heat stress is known to cause many physiological changes in Atlantic cod including the release of stress hormones

Figure 4.4 QPCR of selected transcripts with stress relevant functional annotations identified in the forward SSH libraries (designed to be enriched for transcripts up-regulated by heat shock). Part A. The RQs (relative quantities), normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest expression of each gene of interest (see methods section), are presented as averages ± S.E. The levels of gene expression of heat shock protein 90 alpha (HSP90α: **A**), heat shock protein 90kDa beta, member 1 (GRP94: **B**), HSP70-1 protein (HSP70-1: **C**), 78 kDa glucose regulated protein (GRP78: **D**), heat shock protein 47 (HSP47: **E**) are shown for the control transferred (CT) and heat shocked (HS) groups before heat shock (BHS), at the cessation of heat shock (CS), 3h after the cessation of heat shock (3ACS), and 12h after the cessation of heat shock (12ACS). Different letters indicate significant differences between sampling points within the same treatment ($p<0.05$). An asterisk (*) indicates significant differences between CT and HS groups at a given sampling point ($p<0.05$). Numbers in the boxes represent overall fold-changes. For each treatment, overall fold up-regulation was calculated relative to the appropriate before heat shock (BHS) value as (average RQ of time point)/(average RQ of appropriate group at the BHS time point), and overall fold down-regulation was calculated when necessary (i.e. if overall fold up-regulation was <1) as the inverse of overall fold up-regulation. Each panel shows the expression for a given gene of interest in all 3 tissues studied. L: liver. HK: head kidney. M: skeletal muscle.

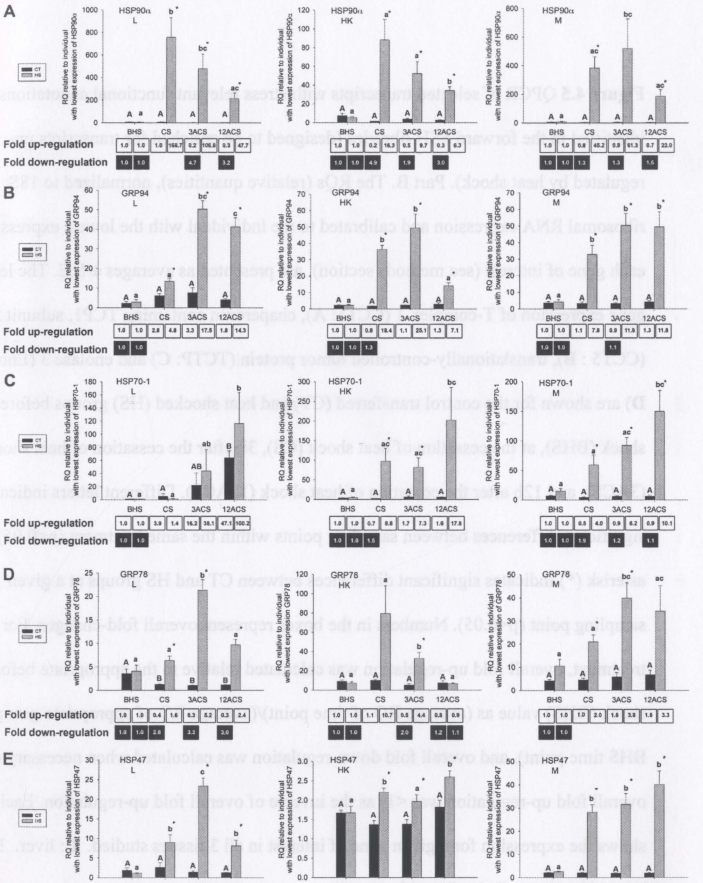

A
HSP90α
L
HK
M
B
GRP94
C
HSP70-1
D
GRP78
E
HSP47
CT
HS
RQ relative to individual with lowest expression of HSP90α
RQ relative to individual with lowest expression of GRP94
RQ relative to individual with lowest expression of HSP70-1
RQ relative to individual with lowest expression GRP78
RQ relative to individual with lowest expression of HSP47
BHS
CS
3ACS
12ACS
Fold up-regulation
Fold down-regulation

Figure 4.5 QPCR of selected transcripts with stress relevant functional annotations identified in the forward SSH libraries (designed to be enriched for transcripts up-regulated by heat shock). Part B. The RQs (relative quantities), normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest expression of each gene of interest (see methods section), are presented as averages ± S.E. The levels of gene expression of T-complex 1 (CCT1: **A**), chaperonin containing TCP1, subunit 5 (CCT5 : **B**), translationally-controlled tumor protein (TCTP: **C**) and enolase 3 (Enolase: **D**) are shown for the control transferred (CT) and heat shocked (HS) groups before heat shock (BHS), at the cessation of heat shock (CS), 3h after the cessation of heat shock (3ACS), and 12h after the cessation of heat shock (12ACS). Different letters indicate significant differences between sampling points within the same treatment ($p<0.05$). An asterisk (*) indicates significant differences between CT and HS groups at a given sampling point ($p<0.05$). Numbers in the boxes represent overall fold-changes. For each treatment, overall fold up-regulation was calculated relative to the appropriate before heat shock (BHS) value as (average RQ of time point)/(average RQ of appropriate group at the BHS time point), and overall fold down-regulation was calculated when necessary (i.e. if overall fold up-regulation was <1) as the inverse of overall fold up-regulation. Each panel shows the expression for a given gene of interest in all 3 tissues studied. L: liver. HK: head kidney. M: skeletal muscle.

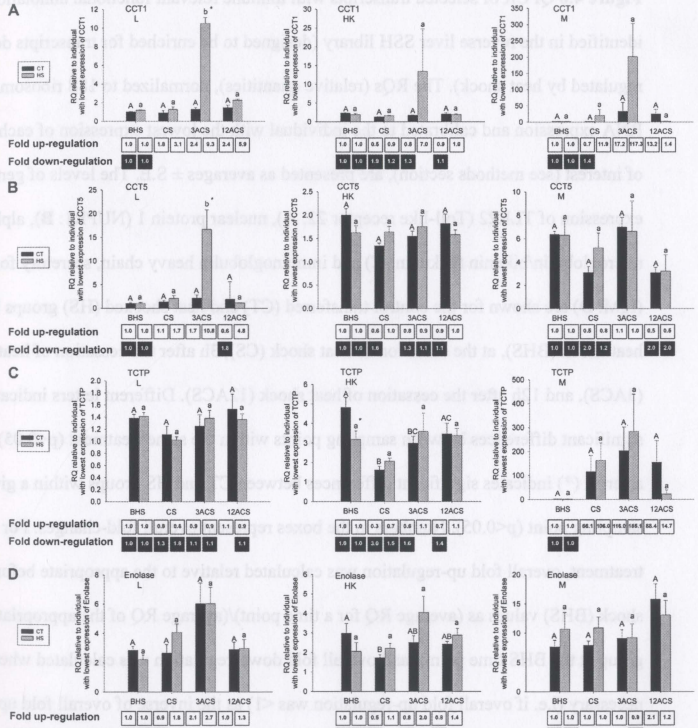
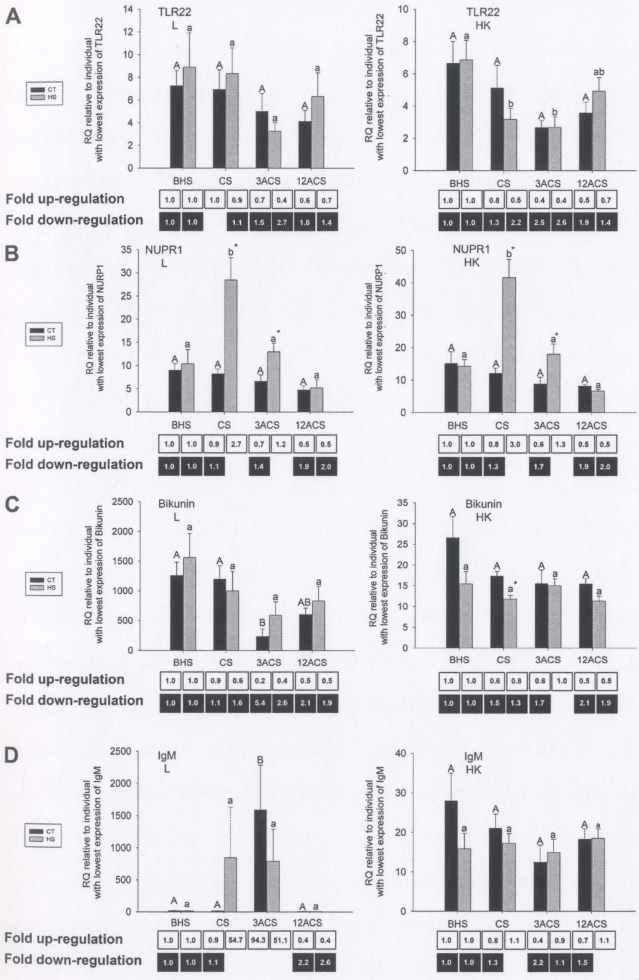

A
CCT1
L
CCT1
HK
CCT1
M
RQ relative to individual with lowest expression of CCT1
CT
HS
BHS
CS
3ACS
12ACS
Fold up-regulation
Fold down-regulation
B
CCT5
L
CCT5
HK
CCT5
M
RQ relative to individual with lowest expression of CCT5
C
TCTP
L
TCTP
HK
TCTP
M
RQ relative to individual with lowest expression of TCTP
D
Enolase
L
Enolase
HK
Enolase
M
RQ relative to individual with lowest expression of Enolase

Figure 4.6 QPCR of selected transcripts with immune relevant functional annotations identified in the reverse liver SSH library (designed to be enriched for transcripts down-regulated by heat shock). The RQs (relative quantities), normalized to 18S ribosomal RNA expression and calibrated to the individual with the lowest expression of each gene of interest (see methods section), are presented as averages ± S.E. The levels of gene expression of TLR22 (Toll-like receptor 22: **A**), nuclear protein 1 (NUPR1: **B**), alpha 1-microglobulin/bikunin (Bikunin: **C**) and immunoglobulin heavy chain, secretory form (IgM: **D**) are shown for the control transferred (CT) and heat shocked (HS) groups before heat shock (BHS), at the cessation of heat shock (CS), 3h after the cessation of heat shock (3ACS), and 12h after the cessation of heat shock (12ACS). Different letters indicate significant differences between sampling points within the same treatment ($p<0.05$). An asterisk (*) indicates significant differences between CT and HS groups within a given sampling point ($p<0.05$). Numbers in the boxes represent overall fold-changes. For each treatment, overall fold up-regulation was calculated relative to the appropriate before heat shock (BHS) values as (average RQ for a time point)/(average RQ of the appropriate group at the BHS time point), and overall fold down-regulation was calculated when necessary (i.e. if overall fold up-regulation was <1) as the inverse of overall fold up-regulation. Each panel shows the expression for a given gene of interest in both tissues studied. L: liver. HK: head kidney.


A
TLR22
L
TLR22
HK
RQ relative to individual with lowest expression of TLR22
CT
HS
BHS
CS
3ACS
12ACS
Fold up-regulation
Fold down-regulation
1.0 1.0 1.0 0.9 0.7 0.4 0.6 0.7
1.0 1.0 1.1 1.5 2.7 1.8 1.4
1.0 1.0 0.8 0.5 0.4 0.4 0.5 0.7
1.0 1.0 1.3 2.2 2.5 2.6 1.9 1.4
B
NUPR1
L
NUPR1
HK
RQ relative to individual with lowest expression of NURP1
1.0 1.0 0.9 2.7 0.7 1.2 0.5 0.5
1.0 1.0 1.1 1.4 1.9 2.0
1.0 1.0 0.8 3.0 0.6 1.3 0.5 0.5
1.0 1.0 1.3 1.7 1.9 2.0
C
Bikunin
L
Bikunin
HK
RQ relative to individual with lowest expression of Bikunin
1.0 1.0 0.9 0.6 0.2 0.4 0.5 0.5
1.0 1.0 1.1 1.6 5.4 2.6 2.1 1.9
1.0 1.0 0.6 0.8 0.6 1.0 0.5 0.5
1.0 1.0 1.5 1.3 1.7 2.1 1.9
D
IgM
L
IgM
HK
RQ relative to individual with lowest expression of IgM
1.0 1.0 0.9 54.7 94.3 51.1 0.4 0.4
1.0 1.0 1.1 2.2 2.6
1.0 1.0 0.8 1.1 0.4 0.9 0.7 1.1
1.0 1.0 1.3 2.2 1.1 1.5

(e.g. cortisol), changes in the expression of immune relevant transcripts, alterations in oxygen consumption and heart rate, and increased mortality (Pérez-Casanova et al., 2008a; Pérez-Casanova et al., 2008b; Gollock et al., 2006). Therefore, a better understanding of the mechanisms mediating the response to thermal stress should provide insights into how to mitigate and/or avoid the deleterious effect of such environmental challenges. I observed a significant elevation in average plasma cortisol in both control transferred (CT) and heat shocked (HS) Atlantic cod at CS, but the increase in HS fish was 2.3-fold higher at this time point when compared to CT Atlantic cod and 5.5-fold higher in HS Atlantic cod when compared to the CT group at 3ACS. Further, cortisol levels in HS fish remained elevated during the recovery period. In contrast, average plasma cortisol levels in the CT group had returned to basal levels (i.e. were not significantly different from the CT group at BHS) by 3ACS. These results confirm that 3 hours of exposure to 18°C was a severe stressor for these juvenile Atlantic cod.

Some of the terms more frequently represented amongst the GO annotated ESTs derived from the forward libraries were protein folding, signal transduction, immune response, and response to stress (Fig. 4.2). The genes associated with these GO terms may be important in the strategies involved with coping with stress, and variability in their sequences (e.g. exonic, intronic, or regulatory region SNPs) and/or timing and magnitude of mRNA expression (i.e. expression profiles) could reveal markers for increased resistance to thermal and other stressors. This is the first study to use high-throughput genomic techniques to investigate the response to heat shock in Atlantic cod, and to provide expression profiles of a wide range of transcripts encoding putative chaperone genes in tissues of fish that have distinct physiological roles. Many of the transcripts

validated at the individual level using QPCR in this study had BLASTx hits that were associated with the GO terms mentioned above and exhibited differences in expression profiles between tissues. These findings indicate that the cellular response to heat shock in Atlantic cod is complex, involves many genes, and may also be controlled by different cues and/or different transcription regulation mechanisms in different tissues, as has been observed in human cells (Watowich and Morimoto, 1988).

I have shown in all three tissues studied, an increase in transcript levels of a putative member of the HSP70 family [HSP70-1, a putative ortholog of the human *HSPA1A* gene as per the nomenclature proposed by Kampinga et al. (2009)]. Previous reports on Atlantic cod and haddock (*Melanogrammus aeglefinus*) (Zakhartsev et al., 2005; Afonso et al., 2008; Pérez-Casanova et al., 2008a) did not detect an increase in HSC71/HSP70-1 protein expression in the gills and liver of Atlantic cod or haddock in response to thermal stress. It could be that HSC71/HSP70-1 protein elevation was not detected in Atlantic cod or haddock in the aforementioned studies due to the fact that the antibodies [i.e. polyclonal anti-rainbow trout (*Oncorhynchus mykiss*) HSP70 (Agrisera, Sweden) and monoclonal anti-mouse (*Mus musculus*) HSP70/HSC71 (Sigma Co., St. Louis)] used were not generated against Atlantic cod or haddock HSP70-1 and may not have recognized the HSP70-1 protein in these species. However, Methling and collegues (2010) were able to show differences in HSP70-1 protein expression in the gills but not livers of Atlantic cod acclimated to different temperatures using the anti-mouse HSP70/HSC71 antibody. Clearly, Atlantic cod-specific antibodies are necessary to further elucidate the response of HSP70 to different temperatures in this species.

Several of the cDNAs identified in this study were represented by more than one contig in a single library. There are several possible reasons for the presence of more than one contig with the same annotation in a given library. Multiple, same-named contigs may represent: a) different paralogs; b) different alleles at a given locus; or c) non-contiguous segments of a given cDNA. (The last-mentioned potential cause of multiple same-named contigs may be the most likely since SSH library construction includes a restriction digest with *RsaI*, potentially resulting in more than one contig from a given full mRNA.) For example, I report two contigs that were annotated as HSP90α in the head kidney forward library (Additional File 4.1, Table S1A). Further analysis of these contigs using nucleotide alignments against a full-length sequence obtained from Chinook salmon (*Oncorhynchus tshawytscha*) [GenBank: U89945] (Palmisano et al., 1999) suggests that these are likely to represent non-contiguous regions of the same Atlantic cod cDNA (data not shown). However, the HSP family provides important examples of differential expression (i.e. constitutive and induced expression profiles) between distinct paralogs (e.g., HSC71/HSP70-1) (Voellmy and Boellmann, 2007) and further studies addressing this question will be needed to determine the roles of different Atlantic cod chaperone paralogs in thermal tolerance.

Molecular chaperones play important roles in cell physiology in both unstressed and stressed situations. These proteins assist with the folding of nascent peptides and the *de-novo* folding of denatured proteins, the transport of unfolded proteins across membranes, quality control and conformational changes that affect function (Genevaux et al., 2007). Sudden or chronic increases in temperature are known to induce both mRNA and protein

expression of several chaperones, such as those belonging to the HSP family (Voellmy and Boellmann, 2007). Among the clients that these proteins bind to are physiologically relevant proteins such as the glucocorticoid (Mommsen et al., 1999) and aryl hydrocarbon (Bell and Poland, 2000) receptors (clients of HSP90), heat shock factor 1 (Prahlad and Morimoto, 2009) (client of HSP70), Immunoglobulin (Ig) heavy chain (Haas and Wabl, 1983) (client of GRP78) and the Toll-like receptors (Yang et al., 2007) (clients of GRP94). Transcripts encoding putative orthologs of all of these chaperones were identified in my libraries, and all of them were confirmed to be heat shock-responsive mRNAs. HSP90α mRNA expression was up-regulated in the liver more than 150-fold at CS relative to the before heat shock (BHS) group (Fig. 4.4A). The up-regulation of HSP90s in response to heat shock has been demonstrated at both mRNA and protein levels in different species of fish (Palmisano et al., 2000; Cara et al., 2005). For example, Cara et al. (2001) detected a ~ 6000% increase in HSP90 proteins and a ~ 600% increase in HSP70 proteins in fasted +10°C heat shocked rainbow trout larvae. In the present study, there was a higher fold-induction of HSP90α transcripts compared to HSP70-1 transcripts in both liver and muscle following heat shock. I also observed a significant increase in HSP70-1 mRNA expression in the liver of CT fish at 12ACS, which may have been a result of fasting. Cara et al. (2001) observed increased HSP70 protein expression in fasted non heat shocked rainbow trout larvae. Among the many clients of these chaperones are heat shock factor 1 (HSP70) and the glucocorticoid receptor (HSP90). Therefore, the increase in the levels of mRNAs encoding these chaperones may indicate

that their products are essential in maintaining signal transduction during stress, and that they are likely to be proteins involved in heat stress tolerance.

Stress also has an impact on the fish's immune system, and temperature stress has been shown to decrease plasma immunoglobulin (Ig) content and increase the susceptibility of sea bass (*Dicentrarchus labrax*) to nodavirus (Varsamos et al., 2006). Nodaviruses belong to the family *Nodaviridae*, and are the causative agents of viral nervous necrosis (VNN). These viral pathogens also infect Atlantic cod, and can cause high levels of morbidity and mortality (Johnson et al., 2002). GRP78 is essential for the appropriate folding and secretion of immunoglobulin (Ig) light and heavy chains from the endoplasmic reticulum (ER) (Haas and Wabl, 1983; Schroder and Kaufman, 2005). IgM heavy chain transcripts were significantly up-regulated by handling stress but not by heat shock in my study (Fig. 4.6D), and thus, GRP78 may be important for the proper folding of this immune-relevant protein following exposures to only some types of stressors. GRP78 mRNA, which encodes the ER-resident member of the HSP70 family, was up-regulated by heat shock in all tissues studied. GRP94 (synonym: Gp96), the ER-resident member of the HSP90 family, is the major chaperone for the Toll-like receptors (TLRs) (Yang et al., 2007). Yang et al. (2007) demonstrated that Gp96 null mice were also macrophage-TLR null and highly susceptible to *Listeria* infections. In my study, GRP94 transcripts were up-regulated in all tissues after heat shock, with the head kidney presenting a 25.1-fold up-regulation at 3ACS (Fig. 4.4B) relative to GRP94 mRNA levels before heat shock. TLRs may play an important role in the defense against viral infections and have been shown to be up-regulated by the viral mimic pIC in fugu (*Takifugu rubripes*) (Bell and Poland, 2000). Therefore, divergent forms of the encoding sequences,

or different expression profiles of the GRP genes between families and/or populations, could be important in preventing temperature-related immunosuppression. Given that TLR22 mRNA was significantly down-regulated in the head kidney of heat shocked Atlantic cod (Fig. 4.6A) when compared to its levels before heat shock, and that this receptor in fish recognizes double-stranded RNA and induces genes of the interferon pathway (Matsuo et al., 2008), it is possible that its down-regulation following thermal stress may result in reduced protein levels and be linked to decreased resistance to viruses in stressed fish (Varsamos et al., 2006). However, stress does not always correlate negatively with disease resistance. Weber et al. (2006) have shown that a single 3 hour crowding event does not affect rainbow trout survival to a challenge with *Yersinia ruckeri* (the causative agent of enteric redmouth disease). On the other hand, the work by Fevolden et al. (1992) indicates that the impact of stress on immune competence may be pathogen-specific. These authors have shown that rainbow trout strains selected for high cortisol response had lower survival rate when challenged with *A. salmonicida* (the causative agent of furunculosis), but higher survival rates when challenged with *Vibrio anguillarum* (the causative agent of vibriosis), when compared to strains selected for low cortisol response. Clearly, the relationships between stress and immune responses in fish are complex and require further investigation.

Other Atlantic cod transcripts encoding molecular chaperone-like proteins were identified in this work, including several putative members of the T-complex-containing chaperones (CCT), prolyl-peptidyl-isomerase (PPIase, synonym: cyclophilin A), protein disulfide isomerase (PDI) (the two latter being classified as foldases, enzymes that catalyze reactions which accelerate protein folding and are an important part of the ER

chaperone machinery) (Schroder and Kaufman, 2005), and an ER-resident chaperone (HSP47) that is essential for the normal synthesis of procollagen and its stabilization during stress (Nagata and Hosokawa, 1996). Collagen is an essential and ubiquitous component of the extracellular matrix and a potential target for denaturation and aggregation. I found HSP47 mRNA fold-induction by heat shock to be 16.9 in skeletal muscle tissue at 12ACS and 20.4-fold in liver tissue at 3ACS (Fig. 4.4E) when compared to its levels before heat shock.

In mammalian cells, apoptosis induced by the denaturation and aggregation of proteins is one of the causes of death following heat shock. Over-expression of the HSP70-1 protein (synonym: *HSPA1A*) plays an important role in protecting cells from apoptosis, presumably by preventing protein aggregation and inactivating the c-jun terminal kinase (JNK) pro-apoptotic pathway (Gabai and Sherman, 2002). In my study, the mRNA encoding the putative Atlantic cod ortholog of this particular chaperone was one of the most highly induced transcripts in the liver, with an 100.2-fold up-regulation (at 12ACS – Fig. 4.4C) relative to the before heat shock (BHS) group. Although HSP70s have been shown to be anti-apoptotic in sea bream (*Sparus auratus*) primary macrophage cultures (Deane et al., 2006), previous studies have reported that HSP70-1 protein is not responsive to heat stress in Atlantic cod (Zakhartsev et al., 2005; Pérez-Casanova et al., 2008a). However, these studies relied on anti-mouse HSC71/HSP70 or anti-rainbow trout HSP70 protein commercial antibodies, which may not efficiently cross-react with orthologous HSP70 protein in Atlantic cod. I have demonstrated that, at least at the transcriptional level, there is a significant up-regulation of a HSP70-1-like gene in response to heat shock. Likewise, Methling et al. (2010) were able to show increased

HSP70-1 protein levels in the gills of Atlantic cod acclimated at 5°C when compared to fish held at 15°C. The maximum-fold up-regulation of HSP70-1 mRNA at 12ACS in all tissues (Fig. 4.4C), is consistent with its reported role in acquired thermal tolerance during the recovery of mildly heat shocked mammalian cells (Kampinga, 1993).

GRP78 is also known to protect cells against apoptosis, since it interacts with the key players in the ER stress signalling system (e.g. ATF6 and PERK) in non-stressed cells, preventing pro-apoptotic signalling (Lee, 2005). Misfolded proteins in the ER interact with GRP78, which causes the activation of the pro-apoptotic ER stress signalling cascades (Lee, 2005). Thus, up-regulation of the GRP78 transcripts may lead to elevated levels of this protein that would still be able to silence the pro-apoptotic ER stress signalling pathway. The up-regulation of NUPR1 (synonym: p8) mRNA may also lead to increased expression of this protein, and be an indication of increased levels of anti-apoptotic factors in both liver and head kidney. This protein has been correlated with reduced apoptosis in pancreatic cancer cells (Su et al., 2001). Protein aggregation is known to trigger apoptosis (Kultz, 2005), and therefore, cell viability under thermal stress may depend on the ability to elicit a significant anti-apoptotic response through the expression of transcripts such as those encoding HSP70-1 and NUPR1. However, up-regulation of NUPR1 has also been linked to the acute phase response to pancreatitis in mammals (Mallo et al., 1997). Bikunin transcript, which also encodes an acute phase protein, was down-regulated by handling stress in the liver and by heat shock in the head kidney. Thus, heat shock may also affect the inflammatory response. In the spleen of Atlantic cod, bikunin transcript levels were not affected by saline control injection (which includes general handling stress), but were significantly suppressed by viral mimic (pIC)

injection at 2 and 6 h post-injection, and significantly induced by the viral mimic at 24 h post-injection, relative to saline injected controls at these time points (Rise et al., 2008).

Finally, it is worth noting that while NUPR1 was identified as a contig of 2 sequences in the reverse liver library (enriched for genes down-regulated by heat shock), QPCR showed that this transcript was up-regulated by heat shock in the liver and head kidney of Atlantic cod. This was not surprising however as, in at least one of the previous works by the Rise research group (Rise et al., 2008), the SSH technique was less effective at enriching for genes that are down-regulated by a stressor (i.e. in reverse subtractions) than at enriching for genes that are up-regulated by a stressor (i.e. in forward subtractions). As evidence of this, three out of four transcripts identified in a reverse spleen SSH library designed to be enriched for Atlantic cod transcripts that were down-regulated by exposure to a stressor (viral mimic) could not be confirmed by QPCR as significantly down-regulated by the stressor (i.e. no statistically significant differences were detected) (Rise et al., 2008). Therefore, the presence of NUPR1 as a contig of 2 sequences in the reverse liver library in the current study could be an artifact of the SSH technique.

The timing of up-regulation of some transcripts encoding putative chaperone proteins suggests that HSP90α may be a first line of defense against heat stress, while HSP70-1 may be more important during recovery. Moreover, given that most of the studied chaperone genes peaked in mRNA expression either at CS or 3ACS, I hypothesize that early time points may be crucial in the process of recovery and repair of damaged proteins. Two other transcripts, CCT 1 and CCT 5, putative members of the TCP1 complex, were up-regulated by thermal stress in the liver (at 3ACS – Fig. 4.5A and B). Of

the 8 known mammalian members of this complex, I identified cDNAs for 6 putative orthologs in cod: CCT 1, 2, 3, 5, 6 and 8 [Tables 4.3 and 4.4, and Additional Files 4.1 (Table S1) and 4.2 (Table S2)]. These chaperonins are known to form heterologous polymers that assist in the folding of actins and tubulins (Lopez-Fanarraga et al., 2001), important components of the cytoskeleton. Structural proteins seem to be among the most heat-labile proteins and their misfolding and/or denaturation contributes greatly to protein aggregation (Lee and Lai, 1995).

Although I only saw a small, albeit significant, down-regulation (3.0 fold; CS-Fig. 4.4C) in head kidney TCTP mRNA expression in the CT group relative to its appropriate BHS group, this transcript may still represent an important component of the molecular mechanism involved in thermal resistance. The product of the TCTP gene, a ubiquitously expressed protein in most mammalian cells, is known to bind to calcium and tubulin and to be responsive to stressors such as starvation and heat stress (Bommer and Thiele, 2004). In addition, Bonnet et al. (2000) have shown that in yeast cells exposed to heat shock there is a down-regulation of TCTP mRNA, and in rat (*Rattus norvegicus*) C6.9 glioma cells TCTP is up-regulated in response to induced programmed cell-death (Baudet et al., 1998). The down-regulation of TCTP in response to heat shock in yeast may be part of the mechanisms that prevents heat-induced apoptosis, and it is possible that this down-regulation, which was detected in the present experiment (e.g. 1.5 fold down-regulation in HS head kidney at CS), was not significant due to high variance between biological replicates (Fig. 4.5C). Down-regulation of TCTP may also play a role in preventing apoptosis triggered by other stressors (i.e. handling) since I found it to be significantly down-regulated by 3.0 fold at CS in the head kidney.

I saw little change in the mRNA expression of genes with carbohydrate metabolism related functional annotations (enolase, aldolase, PFK) (Fig. 4.5D, Table 4.3) with heat shock at 18°C. However, this finding does not preclude the possibility that carbohydrate metabolism is increased when Atlantic cod are acutely exposed to elevated temperatures. This is because glycolysis had a relatively high prevalence (3.13%) amongst the biological process GO terms in the muscle forward library (Fig. 4.2). PFK and glycogen phosphorylase (identified in the head kidney forward library) are the rate limiting enzymes of glycolysis, and allosteric regulation of these enzymes is likely to be the main mechanism through which carbohydrate metabolism is re-organized during acute stress. Finally, the results of Pérez-Casanova et al. (2008b) suggest that carbohydrate metabolism (based on measurements of plasma glucose) is not up-regulated significantly in Atlantic cod until temperature reaches at least 20°C during acute thermal stress.

Interestingly, transcript levels of enolase were significantly down-regulated by 1.9-fold in the head kidney in the control transferred (CT) group at the CS time point relative to the CT BHS group, and GRP78 mRNA was significantly down-regulated in the liver in the CT group at all time points relative to the CT BHS group. These results suggest that, even though there is a conserved general stress response, some responses at the transcriptome level are stressor specific (i.e. responsive to either heat shock or handling stress).

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Chapter 5: A Moderate Increase in Ambient Temperature Alters the Atlantic Cod (*Gadus morhua*) Spleen Transcriptome Response to Intraperitoneal Viral Mimic Injection

5.1 Abstract

Atlantic cod reared in sea-cages can experience large variations in temperature, and these have been shown to affect their immune function. I used the new 20K Atlantic cod microarray to investigate how a water temperature change which simulates that seen in sea-cages in Newfoundland during the spring-summer (i.e. from 10 to 16°C, 1°C increase every 5 days) impacted the Atlantic cod spleen transcriptome response to the intra-peritoneal injection of a viral mimic (polyriboinosinic polyribocytidylic acid, pIC). The temperature regime alone did not cause any significant increases in plasma cortisol levels and only minor changes in spleen gene transcription. However, it had a considerable impact on the fish spleen transcriptome response to pIC [i.e. 290 and 339 significantly differentialy expressed genes between 16°C and 10°C at 6 and 24 hours post-injection (HPI), respectively]. Seventeen microarray-identified transcripts were selected for QPCR validation based on immune-relevant functional annotations. Fifteen of these transcripts (88%), including TLR9, DHX58, STAT1, IRF7, ISG15 and RSAD2, were shown by QPCR to be significantly induced by pIC. Furthermore, the temperature increase appeared to accelerate the spleen immune transcriptome response to pIC. Functional analysis of the genes selected for QPCR indicates that changes in NF-κB and type I interferon signal transduction pathways may play key roles in controlling temperature-regulated changes in the spleen's global transcript expression response to pIC. Moreover, interferon effector genes such as ISG15 and RSAD2 were differentially expressed between fish injected with pIC at the different temperatures (10°C or 16°C) at 6 hours post-injection. These findings: generate new hypotheses, suggest further experiments aimed at better understanding the impacts of temperature fluctuations on Atlantic cod

immune responses and disease resistance; and may lead to the development of molecular markers (e.g. SNPs or mRNA expression profiles that correlate with desired traits) for use in the development of elite broodstock (e.g. with robust anti-viral immune responses at various rearing temperatures) for the Atlantic cod aquaculture industry.

5.2 Introduction

The Atlantic cod (*Gadus morhua*) is an important commercial species in several countries including Canada, the USA and Norway, whose supply has been threatened by declining wild stocks (Myers et al., 1997; Kjesbu et al., 2006; Rose, 2007). In recent years, the aquaculture of Atlantic cod has emerged as a potential alternative source of fish for these markets (Brown et al., 2003; Roselund and Halldórsson, 2007). Unfortunately, however, the development of Atlantic cod aquaculture still faces many challenges, including our incomplete understanding of how changes in the environment (e.g. seawater temperature) impact gadoid culture (Roselund and Skretting, 2006; Kjesbu et al., 2006; Pérez-Casanova et al., 2008a, 2008b).

Atlantic cod reared in sea-cages are confined within a limited space, and therefore, are more likely than wild fish to be subjected to seasonal fluctuations in temperature. For example, high levels of mortality in farmed Atlantic cod have been observed when temperatures increase during the summer months (Gollock et al., 2006; Pérez-Casanova et al., 2008b). The temperatures to which farmed Atlantic cod are often exposed in the summer months (e.g. 16 - 20°C; Gollock et al., 2006) in themselves, however, are unlikely to be lethal for this species. In fact, previous work from the Gamperl research group indicates that Atlantic cod juveniles can survive an incremental temperature increase of 1°C every 5 days until the temperature reaches ~ 22°C (Gamperl et al., unpublished data).

Elevated temperatures have been shown to modulate the immune response of several commercially important fish species such as the rainbow trout (*Oncorhynchus mykiss*) (Alishahi and Buchmann, 2006; Raida and Buchmann, 2007; Raida and Buchmann, 2008;

Bettge et al., 2009), European sea bass (*Dicentrarchus labrax*) (Varsamos et al., 2006), orange-spotted grouper (*Epinephelus coioides*) (Cheng et al., 2009), Atlantic salmon (*Salmo salar*) (Eggset et al., 1997) and Atlantic cod (Magnadottir et al., 1999; Pérez-Casanova et al., 2008b). However, the influence of temperature on fish immune function is variable. For example, while constant sub-lethal elevated temperatures can enhance the immune response of salmonids (e.g. improve the protection conferred by vaccines; Raida and Buchmann, 2008), variable temperatures (i.e. daily fluctuations in temperature) can be immune suppressive in European sea bass (Varsamos et al., 2006). Since the thermal regime to which Atlantic cod are exposed during the spring-summer months in Newfoundland is characterized by a gradual increase in temperature (i.e. a variable thermal environment) and temperatures do not tend to reach the Atlantic cod's critical thermal maximum (CTM) (Pérez-Casanova et al., 2008b; Gamperl et al., unpublished data), I hypothesized that a temperature-dependent modulation of the immune system, which could lead to increased susceptibility to pathogens, may be associated with the losses sometimes observed in Atlantic cod sea-cages.

Microarrays have been widely used to study the immune responses of fish to pathogens and pathogen-associated molecular patterns (PAMPs) (e.g. Rise et al., 2004; Milev-Milovanovic et al., 2009; Workenhe et al., 2009; Booman et al., 2011). In this study, I used the 20,000 element (20K) oligonucleotide microarray platform (GEO accession #GPL10532; Booman et al., 2011) which includes sequences from both suppression subtractive hybridization (SSH) and normalized libraries enriched for immune and heat stress responsive transcripts (Rise et al., 2008; Feng et al., 2009; Hori et al., 2010 – Chapter 4; Rise et al., 2010; Bowman et al., 2011), and reverse transcription –

quantitative polymerase chain reaction (QPCR), to investigate the effects of an increasing temperature regime (gradually from 10 to 16°C) on the Atlantic cod spleen response to the intraperitoneal (IP) injection of the viral mimic polyriboinosinic polyribocytidylic acid (pIC). A better understanding of how moderately increased ambient temperature affects the genes and pathways involved in the Atlantic cod's anti-viral response may shed light on the potential mechanisms of temperature induced immune suppression (Steinhagen et al., 1998; Magnadottir et al., 1999; Hamel, 2005; Varsamos et al., 2006; Verma et al., 2007). This knowledge will not only be important for the emerging Atlantic cod aquaculture industry, but may also be valuable to our understanding of how accelerated global climate change (Hansen et al., 2006) may impact cold-water marine finfish species.

5.3 Materials and methods

All experimental procedures were conducted with approval of Memorial University's Institutional Animal Care Committee, and followed the guidelines of the Canadian Council on Animal Care.

5.3.1 Experimental animals

Passive integrated transponder (PIT)-tagged Atlantic cod belonging to 10 different families (~ 30 fish per family) from the Atlantic Cod Genomics and Broodstock Development Project (CGP) year-class 3 (YC3) (mean ± S.E.: 52.6 ± 2.5 g) were obtained from the Huntsman Marine Science Centre in St. Andrew's, New Brunswick. The fish were transported to the Ocean Sciences Centre of Memorial University of

Newfoundland in October of 2008, and placed in a 3000 l tank supplied with flow-through seawater (at 10°C and >90% oxygen saturation). After one month of acclimation, the fish were distributed amongst eight 500 l tanks (~ 36 fish per tank) (Fig. 5.1A), with approximately equal numbers of fish from each of the ten families in each tank. Fish were fed to apparent satiation throughout the acclimation period.

5.3.2 Experimental design

Figure 5.1 provides an overview of the complete experimental design. Four tanks out of the 8 were randomly assigned as temperature control tanks (i.e. kept at 10°C for the entire duration of the experiment), while the remaining 4 tanks were exposed to a gradual increase to 16°C (Fig. 5.1A). After the fish were allowed to acclimate to the 500 l tanks for 2 weeks (Fig. 5.1B), 3 fish from each tank (N = 12 per temperature) had their blood sampled for the determination of plasma cortisol levels. These groups were designated "control before temperature increase" (CBTI) and "heat exposed before temperature increase" (HBTI). Thereafter, water temperature in the heat exposed tanks was increased by 1°C every 5 days. These tanks reached 16°C after approximately 1 month (Fig. 5.1C), and were held for another ~ 1 week at this temperature (Fig. 5.1C); fish were fed to apparent satiation during this ~ 5 week period. At this point, 8 fish per tank were sampled for plasma cortisol analysis, 2 fish per tank had their spleens removed for subsequent microarray and QPCR analyses, and the remaining fish were given an IP injection of either polyriboinosinic polyribocytidylic acid (pIC) (Sigma Co, St. Louis, MO) [2 μg of pIC g^{-1} wet mass, 0.5 μg μl^{-1} in ice-cold 0.2 μm filtered phosphate buffered saline (PBS) (Rise et al., 2008)] or an equivalent volume of ice-cold 0.2 μm filtered PBS after

recording their PIT tag ID (Fig. 1D). The fish sampled prior to pIC injection were designated as "control before injection" (CBI) and "heat exposed before injection" (HBI). Finally, at both temperatures (10 and 16°C), 5 fish from each of two tanks (N=10 per group/temperature) were sampled at 6 hours post-injection (6HPI) and at 24HPI (Fig. 5.1E) to examine the impact of elevated rearing temperature on the spleen's transcriptome response to the viral mimic pIC.

5.3.3 Immune stimulation

After being lightly anesthetized using a non-lethal dose (100 mg l^{-1}) of tricaine-methane-sulphonate (TMS) (Syndel Laboratories, Qualicum Beach, BC), equal numbers of fish were injected with pIC or PBS (Fig. 5.1). Thereafter, they were allowed to recover (i.e. re-establish normal swimming behavior) in a "recovery-tank" filled with seawater from the same system and at the same temperature as before injection, and then fish were returned to their original tanks. Oxygen levels in the recovery tanks were constantly monitored and maintained above 90% of air saturation.

5.3.4 Tissue and blood sampling

At all sampling points, the fish were starved for at least 24 hours, then quickly netted from their tanks and placed in an anaesthetic bath containing a lethal dose of TMS (400 mg l^{-1}). For cortisol analysis, whole blood was quickly obtained from the caudal vein using heparinized (100 U of sodium heparin ml^{-1}) 1 ml syringes with 28G 1/2" needles (BD Medical Supplies, Franklin Lakes, NJ), centrifuged at 5000 *g* for 10 minutes (4°C), and the resulting plasma was placed in separate 1.5 ml microcentrifuge tubes and frozen

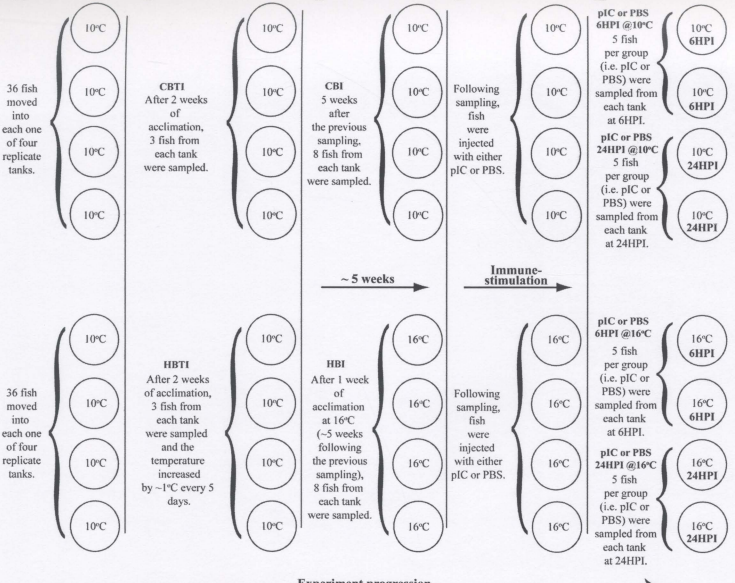
in liquid nitrogen. Spleen samples were collected immediately after blood samples were obtained using standard aseptic molecular biology techniques, placed in certified RNAse free 1.5 ml microcentrifuge tubes and flash-frozen in liquid nitrogen. All spleen samples were stored at -80°C until RNA isolations were performed.

Twelve fish (three per tank) were sampled for plasma cortisol before the temperature increase (BTI), and compared with values before injection (BI), to allow me to determine the degree of stress induced by the increasing temperature regimen experienced by the fish and being held at 16°C for one week. Thirty-two fish were sampled prior to the pIC and PBS injections (BI) to allow for correlation analysis of the relationship between temperature-induced plasma cortisol levels and constitutive (pre-injection) spleen immune-related gene mRNA expression using QPCR. However, because holding the fish at 10 vs. 16°C only resulted in 6 genes being significantly differentially expressed (see results), this analysis was not performed.

5.3.5 Plasma cortisol analyses

An enzyme linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY) was used to measure plasma cortisol levels, and the methods are described in detail elsewhere (Hori et al., 2010 – Chapter 4). The cortisol data was subjected to a Kolmogorov-Smirnov normality test, and a two-way analysis of variance (ANOVA) (with temperature and time point as main effects). However, neither of these factors had a significant impact on cortisol data, and thus, no further statistical analyses were carried out.

Figure 5.1. Overview of the experimental design used to expose fish to increases in temperature simulating those observed in Newfoundland sea-cages in the spring-summer, and to investigate the impact of this temperature increase on the Atlantic cod's response to the viral mimic polyriboinosinic polyribocytidylic acid (pIC). CBTI = Control group with no temperature increase; HBTI = Heat-exposed before temperature increase. HPI = hours post-injection.


A
B
C
D
E
36 fish moved into each one of four replicate tanks.
10ºC
CBTI
After 2 weeks of acclimation, 3 fish from each tank were sampled.
CBI
5 weeks after the previous sampling, 8 fish from each tank were sampled.
Following sampling, fish were injected with either pIC or PBS.
pIC or PBS 6HPI @10ºC
5 fish per group (i.e. pIC or PBS) were sampled from each tank at 6HPI.
6HPI
pIC or PBS 24HPI @10ºC
5 fish per group (i.e. pIC or PBS) were sampled from each tank at 24HPI.
24HPI
~ 5 weeks
Immune-stimulation
36 fish moved into each one of four replicate tanks.
16ºC
HBTI
After 2 weeks of acclimation, 3 fish from each tank were sampled and the temperature increased by ~1ºC every 5 days.
HBI
After 1 week of acclimation at 16ºC (~5 weeks following the previous sampling), 8 fish from each tank were sampled.
Following sampling, fish were injected with either pIC or PBS.
pIC or PBS 6HPI @16ºC
5 fish per group (i.e. pIC or PBS) were sampled from each tank at 6HPI.
6HPI
pIC or PBS 24HPI @16ºC
5 fish per group (i.e. pIC or PBS) were sampled from each tank at 24HPI.
24HPI
Experiment progression

5.3.6 RNA extractions, DNAse-I treatment and column purification

RNA extraction, DNAse-I treatment and column purification were performed using the TRIzol reagent (Invitrogen, Carlsbad, CA), RNAse-free DNAse-I set (Qiagen, Valencia, CA) and RNeasy MiniElute Clean-up kit (Qiagen), respectively. All procedures were done according to the manufacturers' instructions with minor changes as described in Hori et al. (2010 – Chapter 4).

5.3.7 Microarray hybridizations

A schematic of the microarray experimental design is presented in Figure 5.2. In summary, 6 individuals from each of the following groups were used for microarray analysis: CBI; HBI; pIC 6HPI@10°C; PBS 6HPI@10°C; pIC 6HPI@16°C; PBS 6HPI@16°C; pIC 24HPI@10°C; PBS 24HPI@10°C; pIC 24HPI@16°C; PBS 24HPI@16°C. In these abbreviations, the "@" followed by a number indicates the temperature at which fish were held, pIC refers to fish immune-stimulated with the viral mimic pIC, PBS refers to sham-injected (i.e. saline-injected) fish and HPI stands for "hours post-injection". These abbreviations will be used throughout this chapter to refer to these groups.

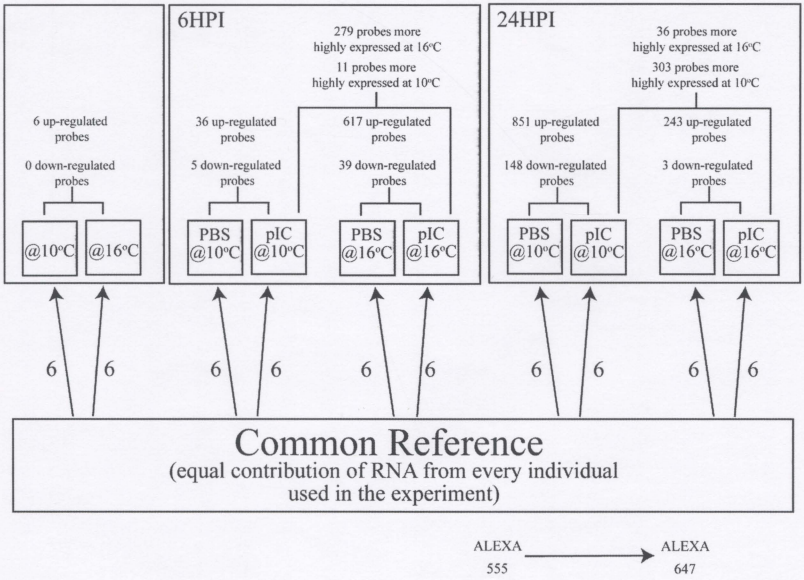
For all of the above groups, 3 individuals from each of 2 different replicate tanks (N = 6) were selected based on RNA quantity and quality for microarray analysis. Seven μg of spleen DNAse I-treated and column-purified total RNA from each individual used in the microarray experiment was pooled to generate a "common reference" sample. After pooling, the common reference quantity was measured using NanoDrop spectrophotometry and its quality was checked by 1% agarose gel electrophoresis. For

each experimental sample (e.g. pIC 6HPI@10°C, individual 1) or the common reference, 5 μg of DNAse I-treated, column-purified RNA was used for target synthesis. Complementary DNA (cDNA) synthesis and labeling were performed using Invitrogen's SuperScript Direct Labeling kit following the manufacturer's instructions. The experimental sample was always labeled with ALEXA 647 fluorophore and the common reference was always labeled with ALEXA 555 fluorophore (Fig. 5.2). Labeled cDNA from one experimental sample and the common reference were pooled together and hybridizations were performed at 42°C for ~ 16 hours as described in Booman et al. (2011). Detailed protocols on all hybridizations and washing are available in Booman et al. (2011).

5.3.8 Microarray data acquisition and pre-processing

Microarrays were scanned using a ScanArray Gx Plus scanner and ScanExpress software v4.0 (Perkin Elmer, Woodbridge, ON) with photomultiplier tube (PMT) settings adjusted for each channel of each array and laser power set at 90%. For each channel (i.e. ALEXA 647 or 555) on a given array, the average signal was measured in a circle of ~ diameter 200 in pixels. PMT settings were adjusted in subsequent scans until the average signal was between 600 and 1000 signal units and there was no more than 200 signal units difference between channels. Raw microarray data was saved as TIFF images and probe intensities were extracted using Imagene v7.5 (BioDiscovery, El Segundo, CA). In R, flagged and control spots were removed and the mArray package of Bioconductor was used to $\log_2$ transform and Loess normalize the data by sub-grid (i.e. print-tip Loess).

Figure 5.2. Overview of the microarray experimental design and results. Brackets connecting 2 boxes represent a direct comparision between these groups using significance analysis of microarrays (SAM) (Tusher et al., 2001 - see methods section for details on statistical analyses). Numbers beside the arrows represent the number of biological replicates (i.e. individual fish on individual microarrays). The base of the arrow is on the sample labeled with ALEXA 555, while the arrowhead points to the sample labeled with ALEXA 647. All genes reported were found to be significantly differentially expressed at FDR = 1%. In the abbreviations used in this figure, the "@" followed by a number indicates the temperature at which fish were held, pIC refers to fish immune-stimulated with the viral mimic pIC, PBS refers to sham-injected (i.e. phosphate buffered saline-injected) fish, BI stands for "before injection" and HPI stands for "hours post-injection". For detailed gene lists refer to Additional File 5.1.


6HPI
24HPI
279 probes more highly expressed at 16°C
11 probes more highly expressed at 10°C
36 probes more highly expressed at 16°C
303 probes more highly expressed at 10°C
6 up-regulated probes
0 down-regulated probes
36 up-regulated probes
5 down-regulated probes
617 up-regulated probes
39 down-regulated probes
851 up-regulated probes
148 down-regulated probes
243 up-regulated probes
3 down-regulated probes
@10°C
@16°C
PBS @10°C
pIC @10°C
PBS @16°C
pIC @16°C
6
Common Reference
(equal contribution of RNA from every individual used in the experiment)
ALEXA 555
ALEXA 647

Thresholding and averaging of the probes were performed as described in Booman et al. (2011). The R scripts are described in full in the supplemental materials of Booman et al. (2011). This microarray dataset is described in the Gene Expression Omnibus (GEO) seriesGSE27299, and individual samples are available under GEO accession numbers GSM675013-GSM675072 (including both pre- and post-normalization data).

5.3.9 Microarray data analysis

The Significance Analysis of Microarrays (SAM) algorithm (Tusher et al., 2001) was used to perform two-class comparisons between groups (e.g. pIC 6HPI@10°C vs. pIC 6HPI@16°C) in order to identify differentially (FDR = 1%) expressed genes. Prior to SAM analysis, all probes that were absent in more than 25% of the arrays in the entire experiment (i.e. 15 or more out of 60 arrays) were removed from the dataset. This procedure generated a final dataset containing 12,154 probes. Any remaining missing values were imputed using the EM_array method from the LSimpute algorithm (Bø et al., 2004; Celton et al., 2010). I used the Bioconductor implementation of SAM available in the siggenes package (Schwender et al., 2006) and set my False Discovery Rate (FDR) threshold to 1% to generate gene lists. Detailed R scripts are available in the supplemental materials of Booman et al. (2011). Resulting data (i.e. gene lists) were then clustered in Genesis (Sturn et al., 2002) using Pearson uncentered correlation and complete linkage hierarchical clustering.

Resulting gene lists were re-annotated using the expressed sequence tags (ESTs) or contiguous sequences (contigs) from which 50 mer oligonucleotide probes on the array were designed. For details on probe design please refer to Booman et al. (2011).

Automated BLASTx alignment of these sequences against the NCBI nr protein database was performed using the Blast2GO tool (Conesa et al., 2005) with thresholds set as follows: *E*-value $<10^{-5}$, maximum number of hits = 100 and high-scoring segment pairs (HSP) >33. BLASTx results for each EST or contig representing a probe were mapped to Gene Ontology (GO) terms using Blast2GO. For detailed information on how BLASTx results are mapped to GO terms, refer to the Blast2GO manual and Conesa et al. (2005). Biological process GO mapping results for the genes differentially expressed between fish injected with pIC at the different temperatures (i.e. 10 vs. 16°C) were normalized to GO term hierarchy level 2 and plotted as pie charts (Fig. 5.3 A-D). GO term enrichment analysis was performed to compare the Atlantic cod spleen response to pIC at different temperatures using Fisher's exact test as implemented by GOSSIP in the Blast2GO software with a p-value cutoff of <0.01. I compared the distribution of GO terms between lists of genes responded to pIC compared to PBS at the different temperatures [e.g. (genes up-regulated in pIC injected fish @10°C vs. time-matched PBS injected controls @10°C) compared to (genes up-regulated in pIC injected fish @16°C vs. time-matched PBS-injected controls @16°C)].

5.3.10 QPCR analyses

From the informative gene lists that were generated using SAM analysis, the following 17 genes of interest (GOI) were selected for QPCR analysis based on their known transcript expression responses in fish exposed to viruses or viral mimics (Rise et al., 2008; Workenhe et al., 2009; Rise et al., 2010): Deltex3, DHX58 (DExD/H box RNA helicase; aliases LGP2, RIG-I-c terminal domain containing protein), IκBα (NF-κB

inhibitor alpha), IL-8 (interleukin-8 variant 5), IRAK4 (interleukin-1 receptor-associated kinase 4), IRF1 (interferon regulatory factor 1), IRF7 (interferon regulatory factor 7), IRF10 (interferon regulatory factor 10), ISG15 (interferon stimulated gene 15, paralogs 1 and 3), PKR (double stranded RNA activated protein kinase), RSAD2 (radical S-adenosyl methionine domain containing protein 2; alias Viperin), SACS (sacsin; alias spastic ataxia of Charlevoix-Saguenay), SCYA123 [small inducible cytokine 123; this follows the nomenclature for Atlantic cod CC chemokines from Borza et al. (2010)], STAT1 (signal transducer and activator of transcription 1), TLR3 (Toll-like receptor 3), TLR9 (Toll-like receptor 9) and ZNFX1 (zinc finger, NFX1-type containing 1). Nine of these (DHX58, IL-8, IRAK4, IRF1, ISG15, PKR, RSAD2, SCYA123 and ZNFX1) were amongst the 96 overlapping probes between the list of 279 genes more highly expressed in the pIC 6HPI@16°C vs. pIC 6HPI@10°C comparison and the list of 303 genes more highly expressed in the pIC 24HPI@10°C vs. pIC 24HPI@16°C comparison (Figs. 5.2 and 5.4). Primers were either chosen from previous publications from the Rise lab (Rise et al., 2008, 2010) or designed based on sequences representing informative probes using Primer3 (http://frodo.wi.mit.edu). Every primer set was quality-checked (QC) using reference cDNA templates generated by reverse transcription of the microarray experiment reference RNA. The QC process was carried out as described in Booman et al. (2011). Briefly, a 5-fold dilution (starting with 10 ng of input total RNA) standard curve consisting of 5 points was used to calculate amplification efficiencies (Pfaffl, 2001), and melt-curve analysis was carried out to ensure primers sets amplified a single product and there was no detectable primer dimers. The primer sequences, amplification efficiencies and amplicon sizes are shown in Table 5.1.

Gene expression was normalized to ATPS [ATP synthase H^+ transporting, mitochondrial Fo complex, subunit F2; CGP microarray probe identifier (ID) #36304] mRNA levels. Several steps were taken to ensure that this gene was a suitable normalizer for this QPCR study. Initially, several candidate probes that had overall normalized ratios (i.e. $\log_2$ transformed ALEXA 647 / ALEXA 555) close to 0 across all 60 microarrays in this study were selected as potential normalizers. For these, the probe IDs for the ones with the lowest standard deviation were searched in all generated gene lists of differentially expressed genes (with FDR = 1%) (Additional File 5.1 - Supplemental Tables S1-S8), and the ones that were not present in any gene lists were tested with QPCR in a subset of individuals (3 from each condition and time point). The assay for ATPS had the lowest threshold cycle (C_T) range (1.2). Lastly, in every multi-plate experiment C_T values for ATPS were always checked; if their range was >1.5 cycles, the samples more distant from the overall C_T mean were removed from the study. For any given GOI, no more than 2 individuals had to be removed from the study.

All cDNAs were reverse transcribed from 1 μg of DNAse I-treated, column purified RNA using the RETROScript kit (Ambion, Austin, TX) following the manufacturer's instructions for the protocol that includes template denaturation. Six individuals from each group were analyzed in triplicate reactions. To QPCR validate the microarray experiment-identified GOI, I used 3 individuals that had been used in the microarray and 3 individuals that were not used for microarray hybridizations. Fish included in the QPCR experiment were also selected based on RNA quality and quantity. PCR amplification and cycling were performed as in Booman et al. (2011). In all plates a linker sample consisting of cDNA made from the reference RNA was included for every target

(including the normalizer). Prior to analysis, all linker C_T values were checked; inter-plate variation was never >0.5 cycles. All thresholds were set automatically, and relative quantities (RQs) were calculated with the linker as a calibrator using the Applied Biosystems (Foster City, CA) 7500 Software Relative Quantification Study Application (v2.0) (Livak and Schmittgen, 2001) using calculated amplification efficiencies.

Resulting RQs were $\log_2$ transformed in Excel (Microsoft Co., Seattle, WA) and analyzed statistically using Prism (v5.0, GraphPad Software Inc., La Jolla. CA). All groups were initially subjected to a Kolmogorov-Smirnov normality test. For each time point (e.g. 6HPI) a two-way ANOVA with injection (i.e. pIC or PBS) and temperature (10°C or 16°C) as factors was then carried out. When the effects of a given factor were statistically significant ($p<0.05$), t-tests were carried out to compare pIC-injected groups to their temperature-matched controls (i.e. PBS injected) at a particular time point, PBS-injected fish held at different temperatures, or pIC-injected fish held at different temperatures. Overall fold-change values were calculated as 2^{A-B} as suggested by Cui and Churchill (2003), where A is the average $\log_2$ transformed RQ from an experimental group (pIC always considered experimental relative to PBS, and 16°C always considered experimental relative to 10°C), and B is the average $\log_2$ transformed RQ from the time- or temperature-matched control.

5.4 Results

5.4.1 Plasma cortisol

Plasma cortisol averaged 15.3 ± 6.4 and 18.2 ± 6.3 ng ml^{-1} in the CBTI and HBTI groups, respectively. These values were approximately two-fold higher in both the CBI

Table 5.1 Quantitative real-time reverse transcription – polymerase chain reaction (QPCR) Primers

Gene		Primer sequence 5' to 3'	Amplification Efficiency (%)	r^2	Amplicon Size (bp)
Deltex3[b]	Forward	TCCACCACAAGACCAGCATCA	98.5	0.99	110
	Reverse	ACTTCACTCGATGCCTTTCGC			
DHX58	Forward	ACAGAAGCCATCGCAGAAAT	92.9	0.99	105
	Reverse	TTTTGCAGCACGAATCAAAC			
IκBα	Forward	GCCAGCAACTGATAAAGCATC	92.1	0.99	132
	Reverse	GGTCACAGAGGGAGACAGAAAA			
IL-8	Forward	GTGTTTCCAGCAGATCACTCG	94.9	0.99	118
	Reverse	TGTTCCACTTGGTGAGGAGTC			
IRAK4	Forward	CGTGGATTACAAGATGGATAAGC	95.8	0.99	102
	Reverse	TCGTCGGGGTCTAAAAAGTC			
IRF1[a]	Forward	AGAAGGACGCCAGTCTGTTCAA	88.5	0.99	100
	Reverse	GCGGAAGTTGGCTTTCCATT			
IRF7[a]	Forward	GGTCGTCGGAGTTCTTGGAGTT	95.1	0.99	102
	Reverse	CCAAACGACAAGGCCAAATG			
IRF10[a]	Forward	CGAGGCGGTAGACCTTGTAG	104.6	0.94	161
	Reverse	GGGCAGGTACAAAGGGAAAT			
ISG15	Forward	AGGACCAACAAAGGCTGATG	94.7	0.99	110
	Reverse	CAGCCGTCCGTTAAGGTAGA			
PKR	Forward	ATTGCATCAGGTTCCCAATC	94.4	0.99	174
	Reverse	GCCGTTACCAGACCAAAATC			
RSAD2	Forward	TGTTTCCACACAGCGAAGAC	95.8	0.99	108
	Reverse	TCCGCCAGAGAAGTTGATCT			
SACS[b]	Forward	CTCCCACTGCCAATGTCATTC	88.5	0.99	102
	Reverse	TCAAGAAAACGTCCCAAGGC			
SCYA123	Forward	GCTCTGGGTCGTGTACCTCT	94.1	1.00	189
	Reverse	TCTCTCTGGACGAACAAGCA			
STAT1[b]	Forward	GCCAATGCCATGTGTTTATG	97.5	0.99	100
	Reverse	ACCTGGAGCAGTTCGTCAGT			
TLR3	Forward	CCTGAAACGCAACTCTATCTCC	90.6	0.99	121
	Reverse	GCCATCAAACATACCCTTCTTT			
TLR9	Forward	TTGCTCGCCAAAACACTATG	99.5	0.99	150
	Reverse	GGAATCCAGTCCCTCTCCTC			
ZNFX1[b]	Forward	ATGCCACTATCGGTGGACAGA	87.3	0.99	108
	Reverse	TCAACAGATTATTGCCCTCGG			
ATPS	Forward	ACATGGATAAATGGCTTTTTGC	99.6	0.98	155
	Reverse	TTGAAGAAGTAGTGTGGCTGGA			

[a]These primers were previously designed and used in Rise et al. (2008) but were still quality checked for the present experiment using the reference cDNA as template.
[b]These primers were previously designed and used in Rise et al. (2010) but were still quality checked for the present experiment using the reference cDNA as template.

fish (33.1 ± 6.8 ng ml^{-1}) and the HBI fish (37.7 ± 7.5 ng ml^{-1}). However, there were no significant differences in cortisol levels between the groups at either time point, or within the groups between time points.

5.4.2 Impact of a non-lethal chronic elevation in temperature on the spleen transcriptome

The first analysis carried out using SAM assessed the impact of the temperature increase alone on the Atlantic cod spleen transcriptome. I compared 6 individuals from the CBI group to 6 individuals from the HBI group using a modified t-test assuming unequal variances in the SAM algorithm (Tusher et al., 2001) as implemented in the Bioconductor package siggenes. The gradual 6°C increase in temperature over ~ 30 days had a very minor impact on spleen mRNA expression. Only 6 probes (all up-regulated) were identified as differentially expressed (at FDR = 1%) between the CBI and the HBI groups (Fig. 5.2 and Additional File 5.1 - Supplemental Table S1 – **All Additional Files have been supplied in a CD that accompanies this thesis**).

5.4.3 Impact of pIC injection on the spleen transcriptome

pIC injected fish were also compared to their time- and temperature-matched PBS controls using SAM. This allowed for the following four comparisons: pIC 6HPI vs. PBS 6HPI (at 10 and 16°C) and pIC 24HPI vs. PBS 24HPI (at 10 and 16°C). I identified 41 (36 up-regulated and 5 down-regulated) differentially expressed probes (at FDR = 1%) between the pIC 6HPI vs. PBS 6HPI at 10°C (Fig. 5.2, Table 5.2, Additional file 5.1 - Table S2), 656 probes (617 up-regulated and 39 down-regulated) between the pIC 6HPI

vs. PBS 6HPI at 16°C (Table 5.2, Additional File 5.1 - Table S3), 999 probes (851 up-regulated and 148 down-regulated) between pIC 24HPI vs. PBS 24HPI at 10°C (Fig. 5.2, Table 5.3, Additional File 5.1 - Table S4), and 246 probes (243 up-regulated and 3 down-regulated) between pIC 24HPI vs. PBS 24HPI at 16°C (Fig. 5.2, Table 5.3, Additional File 5.1 - Table S5). Selected probes having immune-related functional annotations are presented alongside overall fold-change values [i.e. experimental ratio of (ALEXA 647 / ALEXA 555) / control ratio of (ALEXA 647 / ALEXA 555)] in Tables 5.2 and 5.3. For complete information on microarray-identified informative probes, including raw p-values, d-values and standard deviations, please refer to Additional File 5.1 - Tables S2-S5.

I also compared the PBS injected fish to the temperature-matched pre-injected fish (i.e. CBI and HBI) to ensure that they were suitable controls. In this analysis, I found 29 probes differentially expressed (with FDR = 1%) between the PBS 6HPI@10°C and CBI groups, 40 differentially expressed probes between the PBS 6HPI@16°C and HBI groups, 18 differentially expressed probes between the PBS 24HPI@10°C and the CBI group and 97 differentially expressed probes between the PBS 24HPI@16°C and the HBI group (for tables containing information on the above lists see Additional File 5.1 -Table S6). Given that only a small overlap (less than 5%) was observed between the probes identified in these comparisons and the pIC vs. PBS comparisons (Additional File 5.1 - Tables S2 – S5), I concluded that the PBS groups were a suitable control for injection stress.

5.4.4 Impact of increased temperature on pIC induced mRNA expression

In order to study the effect of the gradual temperature increase on the Atlantic cod spleen transcript expression response to pIC, I performed two direct comparisons of the 20K microarray data: pIC 6HPI@16°C vs. pIC 6HPI@10°C and pIC 24HPI@16°C vs. pIC 24HPI@10°C. This approach detected 290 significantly differentially expressed probes (with FDR = 1%) (279 more highly expressed at 16°C and 11 more highly expressed at 10°C) between pIC 6HPI@16°C and pIC 6HPI@10°C (Fig. 5.2 and Table 5.2, Additional File 5.1. - Table S7) and 339 differentially expressed probes (36 more highly expressed at 16°C and 303 more highly expressed at 10°C) between pIC 24HPI@16°C and pIC 24HPI@10°C (Fig. 5.2 and Table 5.2, Additional File 5.1 - Table S8). Selected probes having immune-related functional annotations are presented with fold-change values in Tables 5.2 and 5.3. For complete information on these probes, including raw p-values, d-values, standard deviations and sequence ID of the ESTs from which informative probes were designed, please refer to Additional File 5.1 - Tables S7 and S8.

These genes were further analyzed using gene ontology (GO) and hierarchical clustering analyses. Two distinct analyses were used to look at GO terms mapped to genes differentially expressed between fish injected with pIC at 10 and 16°C. One analysis consisted of plotting level 2 GO terms (belonging to the biological process branch) mapped to the genes differentially expressed between fish injected with pIC at 10 and 16°C (Fig. 5.3). The second analysis consisted of using GOSSIP as implemented in Blast2GO to perform Fisher's exact test to check if any GO terms (belonging to any branch) were significantly ($p<0.01$) enriched in the genes responding to pIC (compared to

Table 5.2 Selected probes representing immune-relevant genes that were differentially expressed between fish injected with PBS or pIC and sampled at 6 hours post-injection (6HPI) at 10 and 16°C, or injected with pIC but held at 10 vs. 16°C and sampled at 6HPI.

Probes identified as differentially expressed (FDR=1%) between PBS 6HPI and pIC 6HPI@10°C, and between PBS 6HPI and pIC 6HPI@16°C[1]				
Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @16°C /pIC @10°C)[3]
38613	Interferon stimulated gene 15 [*Gadus morhua*]	55.97	5.29	8.45
38285	DExD/H box RNA helicase [*Paralichthys olivaceus*] (Aliases: DHX58, LGP2)	5.13	2.44	2.41
45882	Retinoic acid receptor responder protein 3 [*Osmerus mordax*] (Alias: HRAS-suppressor like 3)	12.17	5.51	-
38260	Phosphoinositide phospholipase C-eta-2 [*Homo sapiens*]	5.08	2.19	2.28
44546	Interferon regulatory factor 1 [*Gadus mohua*] (Alias: IRF1)	4.74	1.88	2.48
36426	Cell division cycle 42 [*Sus scrofa*]	4.00	2.16	1.85
38042	Deoxyribonuclease gamma precursor [*Oncorhynchus mykiss*]	3.41	1.45	2.18
41397	Prostaglandin E synthase 3 [*Osmerus mordax*]	2.86	1.87	-
44530	NF-kappa-B inhibitor alpha [*Gadus morhua*] (Alias: IκBα)	2.15	3.12	-
40721	Nostrin protein [*Danio rerio*]	2.12	2.04	-
39371	Novel immune-type receptor 4 [*Oncorhynchus mykiss*]	2.03	1.52	-
41181	Ubiquitin carboxyl-terminal hydrolase 5 [*Salmo salar*]	1.88	2.04	-
36482	CXC chemokine [*Psetta*	1.74	1.71	-

Table 5.2 cont'd

maxima]

Probes identified as significantly differentially expressed (FDR=1%) between PBS 6HPI@10°C and pIC 6HPI@10°C only[1]				
Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @ 16°C /pIC @10°C)[3]
46031	Synaptic vesicle glycoprotein 2B [*Harpegnathos saltator*]	-	3.35	-
44581	NF-kappa-B inhibitor alpha [*Gadus morhua*] (Alias: IκBα)	-	1.46	-
37564	Heat shock protein 90 alpha [*Paralichthys olivaceus*]	-	1.50	-
49256	Unknown	-	10.59	-

Probes identified as significantly differentially expressed (FDR=1%) between PBS 6HPI@16°C and pIC 6HPI@16°C only[1]				
Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @16°C /pIC @10°C)[3]
44448	Small inducible cytokine SCYA104 [*Paralabidochromis chilotes*] (Alias: gmSCYA123[a])	24.34	-	7.29
38356	Sacsin [*Homo sapiens*] (Alias: SACS)	12.54	-	11.14
48390	Endonuclease domain-containing 1 protein precursor [*Salmo salar*]	9.94	-	-
39328	Zinc finger, NFX1-type containing 1 [*Homo sapiens*] (Alias: ZNFX1)	9.05	-	2.67
43196	VHSV-induced protein [*Oncorhynchus mykiss*]	8.03	-	3.35
38639	Interleukin-8 variant 5 [*Ictalurus punctatus*] (Alias: IL-8)	7.60	-	5.88
43201	Viperin [*Niphon spinosus*] (Aliases: Radical S-adenosyl methionine domain containing	6.89	-	5.43

Table 5.2 cont'd

	protein 2, RSAD2)			
50762	Unknown	6.70	-	-
37299	GADD45 alpha [*Anoplopoma fimbria*]	2.27	-	3.19
38617	Interferon-inducible GTPase a [*Salmo salar*]	6.53	-	2.80
47384	Unnamed protein product [*Tetraodon nigroviridis*]	6.50	-	7.71
36121	Anti-apoptotic protein NR-13 [*Gadus morhua*]	4.44	-	-
37609	Hepcidin precursor [*Gadus morhua*]	4.30	-	-
36384	CC chemokine type 3 [*Gadus morhua*]	3.55	-	-
44371	Probable E3 ubiquitin-protein ligase RNF144A-A [*Esox lucius*]	3.43	-	-
36190	Bloodthirsty [*Gadus morhua*]	3.38	-	2.05
37904	Type 2 double stranded RNA activated protein kinase [*Gadus morhua*] (Alias: PKR)	2.98	-	2.52
44579	Mitogen-activated protein kinase kinase 4 [*Gadus morhua*]	2.76	-	1.90
38599	Interferon regulatory factor 10 [*Paralichthys olivaceus*] (Alias: IRF10)	2.63	-	-
38655	Interferon regulatory factor 7 [*Psetta maxima*] (Alias: IRF7)	2.49	-	2.15
38788	Mannose-specific lectin precursor [*Esox lucius*]	2.39	-	-
39119	Ras homolog gene family, member T1a [*Danio rerio*]	2.25	-	1.75
38625	Interleukin 12 receptor beta 2.b [*Danio rerio*]	2.12	-	-
36407	CD9 antigen [*Salmo salar*]	2.05	-	1.44
44615	Toll-like receptor 9 [*Gadus morhua*]	2.03	-	-

Table 5.2 cont'd

	(Alias: TLR9)			
37854	Interferon-inducible protein Gig2 [*Siniperca chuatsi*]	2.01	-	-
35877	Alpha-2-macroglobulin [*Epinephelus coioides*]	1.94	-	-
41703	Ras-related protein Rab-10 [*Salmo salar*]	1.90	-	-
36380	CC chemokine type 2 [*Gadus morhua*]	1.89	-	-
36142	Beta-2-microglobulin [*Gadus morhua*]	1.73	-	-
38629	Interleukin-1 receptor-associated kinase 4 [*Gadus morhua*] (Alias: IRAK4)	1.67	-	-
38090	GTPase IMAP family member 7 [*Salmo salar*]	1.64	-	-
36328	FLICE-like inhibitory protein [*Oryzias latipes*]	1.58	-	1.40
45132	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10-like [*Bos taurus*]	1.55	-	-
44434	Type 1 death domain-containing protein [*Gadus morhua*]	1.48	-	-
41613	Dhx33 protein [*Xenopus laevis*]	1.47	-	-
37498	GTP-binding nuclear protein Ran [*Osmerus mordax*]	1.43	-	1.36
36122	Novel protein similar to BCL2-related ovarian killer [*Danio rerio*]	1.43	-	-
44431	Caspase 10 [*Gadus morhua*]	1.33	-	1.34
37561	Stress-70 protein, mitochondrial precursor [*Salmo salar*] (Synonym: Mortalin)	1.32	-	-
44606	Toll-like receptor 3 [*Gadus morhua*] (Alias: TLR3)	1.32	-	-

[1]Fold-change in probe expression is also shown for pIC @16°C vs. pIC @10°C when significant differential expression was found in the PBS vs. pIC comparisons.
[2]Informative genes were re-annotated using the contig or EST from which the probe printed in the array was designed by comparing the referred sequence to the NCBI's nr

database using the BLASTx algorithm as implemented by Blast2GO (Conesa et al., 2005). The best BLASTx hit (*E*-value cutoff of 10^{-5}) with an informative name (e.g. not predicted, probable or unnamed protein), if available, is presented in this table. Synonyms were obtained from SWISS-PROT, GENE CARDS and other BLASTx hits as long as they had an *E*-value < 10^{-5}, or from the literature ([a]Borza et al., 2010). Some genes without a BLASTx hit (e.g. unknowns) were included because they presented a pIC induced fold-change higher than 5, and therefore, could represent novel immune-relevant genes. For same named probes within a given comparison, the one with the highest significant fold-change is presented in this table.

[3]Fold-changes are only reported when probes were significantly differentially expressed (FDR=1%) in a given comparison (e.g. pIC@10°C vs. pIC@16°C) and are shown as outputted by siggenes; otherwise a dash (-) is presented in place of the fold-change. Fold-changes presented in white font with black background represent overall fold down-regulation for a particular comparison. For standard deviation, p- and d-values, please refer to Additional file 5.1 - Tables S2-S5 and S7-S8.

Table 5.3 Selected probes representing immune-relevant genes that were differentially expressed between fish injected with PBS or pIC at 24 hour post-injection (24HPI) at 10 and 16°C, or injected with pIC but held at 10 vs. 16°C and sampled at 24HPI.

Probes identified as significantly differentially expressed (FDR=1%) between PBS 24HPI and pIC 24HPI@10°C, and PBS 24HPI and pIC 24HPI@16°C[1]				
Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @16°C /pIC @10°C)[3]
38604	Interferon stimulated gene 15 [*Gadus morhua*] (Alias: ISG15)	38.35	90.24	3.05
45882	Retinoic acid receptor responder protein 3 [*Osmerus mordax*] (Alias: HRAS-suppressor like 3)	31.10	27.01	-
44590	RIG-I C-terminal domain-containing protein 1 [*Gadus morhua*] (Aliases: DHX58, LGP2)	12.13	30.48	-
48390	Endonuclease domain-containing 1 protein precursor [*Salmo salar*]	11.53	14.24	-
38356	Sacsin [*Homo sapiens*] (Alias: SACS)	9.88	17.37	-
44448	Small inducible cytokine SCYA104 [*Paralabidochromis chilotes*] (Alias: gmSCYA123[a])	7.28	66.64	2.34
38638	Interleukin-8 variant 5 [*Ictalurus punctatus*] (Alias: IL-8)	6.92	25.33	5.66
35718	A disintegrin and metalloproteinase domain 8a [*Danio rerio*]	6.12	3.02	-
47329	Unnamed protein product [*Tetraodon nigroviridis*]	5.81	5.52	-
41269	Regulator of nonsense transcripts 1-like [*Bos taurus*] (Alias: ZNFX1)	5.07	13.93	2.16
44859	Novel protein [*Danio rerio*]	4.98	11.50	2.34
37854	Interferon-inducible protein Gig2	3.41	4.14	-

Table 5.3 cont'd

	[*Siniperca chuatsi*]			
44324	Aminopeptidase N [*Camponotus floridanus*]	3.04	3.45	-
44472	CC chemokine type 3 [*Gadus morhua*]	2.75	1.75	-
55231	Unknown	2.65	5.17	-
47410	Probable E3 ubiquitin-protein ligase RNF144A-A [*Salmo salar*]	2.64	7.10	2.83
47613	Unnamed protein product [*Tetraodon nigroviridis*]	2.48	5.22	2.72
37542	10 kDa heat shock protein, mitochondrial [*Esox lucius*]	2.45	1.61	-
44919	Novel protein similar to vertebrate IGSF3 [*Danio rerio*]	2.43	3.12	-
44371	Probable E3 ubiquitin-protein ligase RNF144A-A [*Esox lucius*]	2.35	4.56	1.92
37299	GADD45 alpha [*Anoplopoma fimbria*]	2.25	4.52	-
44598	STAT1 [*Gadus morhua*]	2.12	1.98	-
36190	Bloodthirsty [*Gadus morhua*]	2.11	3.43	1.78
36612	Complement component C3 [*Paralichthys olivaceus*]	2.07	2.47	-
43196	VHSV-induced protein [*Oncorhynchus mykiss*]	2.02	4.04	-
44546	Interferon regulatory factor 1 [*Gadus mohua*] (Alias: IRF1)	1.86	3.35	1.84
37566	Heat shock protein HSP 90-alpha [*Salmo salar*]	1.80	1.34	1.55
36407	CD9 antigen [*Salmo salar*]	1.78	1.93	-
36426	Cell division cycle 42 [*Sus scrofa*]	1.66	1.93	-
35957	Apolipoprotein A-IV precursor [*Salmo salar*]	1.60	2.58	1.53
38545	IgD heavy chain constant region variant b [*Gadus morhua*]	1.60	1.43	-
44865	Hect domain and RLD 5	1.52	1.94	1.40

Table 5.3 cont'd

	[*Bos Taurus*]			
36923	DnaJ-like subfamily A member 4 [*Paralichthys olivaceus*]	1.51	1.43	-

Probes identified as significantly differentially expressed (FDR=1%) between PBS 24HPI@10°C and pIC 24HPI@10°C only[1]

Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @16°C /pIC @10°C)[3]
38617	Interferon-inducible GTPase a [*Salmo salar*]	-	8.88	-
49247	Unknown	-	5.74	2.86
43201	Viperin [*Niphon spinosus*] (Aliases: Radical S-adenosyl methionine domain containing protein 2, RSAD2)	-	4.68	2.45
38260	Phospholipase C-eta-2 [*Homo sapiens*]	-	4.11	-
39368	Novel immune type receptor protein [*Danio rerio*]	-	3.23	
40397	Deltex-3-like [*Salmo salar*]	-	2.76	2.18
39119	Ras homolog gene family, member T1a [*Danio rerio*]	-	2.66	-
44579	Mitogen-activated protein kinase kinase 4 [*Gadus morhua*]	-	2.61	1.66
40174	60 kDa heat shock protein, mitochondrial precursor [*Salmo salar*]	-	2.46	-
37609	Hepcidin precursor [*Gadus morhua*]	-	2.44	-
36339	Caspase-1 [*Dicentrarchus labrax*]	-	2.42	1.78
38788	Mannose-specific lectin precursor [*Esox lucius*]	-	2.30	1.80
36364	Cathepsin L precursor [*Anoplopoma fimbria*]	-	2.11	-
38599	Interferon regulatory factor 10		2.05	-

Table 5.3 cont'd

	[*Paralichthys olivaceus*] (Alias: IRF10)			
44503	Type 2 double stranded RNA activated protein kinase [*Gadus morhua*] (Alias: PKR)	-	1.93	-
45146	DNA (cytosine-5-)-methyltransferase 7 [*Danio rerio*]		1.89	1.79
44434	Type 1 death domain-containing protein [*Gadus morhua*]	-	1.89	-
36797	Cytotoxic and regulatory T cell protein [*Oncorhynchus mykiss*]	-	1.87	1.81
40721	Nostrin protein [*Danio rerio*]	-	1.86	-
44839	Disulfide-isomerase A3 precursor [*Salmo salar*]	-	1.79	-
44518	Fas [*Gadus morhua*]	-	1.74	1.77
36121	Anti-apoptotic protein NR-13 [*Gadus morhua*]	-	1.67	-
37498	GTP-binding nuclear protein Ran [*Osmerus mordax*]	-	1.64	1.54
44550	Interleukin-1 receptor-associated kinase 4 [*Gadus morhua*] (Alias: IRAK4)	-	1.63	1.37
36328	FLICE-like inhibitory protein [*Oryzias latipes*]	-	1.59	-
38619	Interferon-inducible protein Gig1 [*Psetta maxima*]	-	1.55	-
44615	Toll-like receptor 9 [*Gadus morhua*] (Alias: TLR9)	-	1.54	-
42836	Tumor necrosis factor receptor-2 [*Paralichthys olivaceus*]	-	1.54	-
42266	Strawberry notch homologue 1 [*Danio rerio*]	-	1.53	-
35877	Alpha-2-macroglobulin [*Epinephelus coioides*]	-	1.52	-
42501	T-complex protein 1	-	1.46	-

Table 5.3 cont'd

	subunit delta [*Takifugu rubripes*]			
41397	Prostaglandin E synthase 3 [*Osmerus mordax*]	-	1.45	1.49
36967	E3 SUMO-protein ligase RanBP2 [*Homo sapiens*]	-	1.42	-
38108	Inhibitor of nuclear factor kappa-B kinase subunit alpha [*Danio rerio*] (Alias: IKKα)	-	1.41	-
38631	Interleukin-11a [*Takifugu rubripes*]	-	1.40	-
40138	78 kDa glucose-regulated protein precursor [*Salmo salar*]	-	1.39	-
37118	Novel protein similar to vertebrate EIF4E [*Danio rerio*]	-	1.35	1.31
41690	RAS guanyl-releasing protein 1 [*Xenopus tropicalis*]	-	1.33	-
37568	Glucose-regulated protein 94 [*Paralichthys olivaceus*]	-	1.33	-
39371	Novel immune-type receptor 4 [*Oncorhynchus mykiss*]	-	1.31	1.33
39063	MHC class Ia antigen [*Gadus morhua*]	-	1.28	-
41711	Rab8b protein [*Xenopus tropicalis*]	-	1.27	-
35876	Alpha-2-macroglobulin receptor-associated protein [*Homo sapiens*]	-	1.26	-
41613	Dhx33 protein [*Xenopus laevis*]	-	1.24	-
45113	Methyltransferase-like protein 2 [*Salmo salar*]	-	1.48	1.44
38713	KH domain-containing, RNA-binding, signal transduction-associated protein 1 [*Salmo salar*]	-	1.54	-
41688	Novel protein similar to vertebrate IQGAP2 [*Danio rerio*]	-	1.65	-

Table 5.3 cont'd

37695	G1/S-specific cyclin-D1 [*Salmo salar*]	-	1.68	-
35934	Annexin max3 [*Oryzias latipes*]	-	1.83	-
38596	Interferon induced protein 2 [*Ictalurus punctatus*]	-	2.38	-

Probes identified as significantly differentially expressed (FDR=1%) between pIC 24HPI@16°C and PBS 24HPI@16°C only[1]

Probe ID	Best BLASTx hit[2]	Fold-change (pIC @16°C /PBS @16°C)[3]	Fold-change (pIC @10°C /PBS @10°C)[3]	Fold-change (pIC @16°C /pIC @10°C)[3]
44465	CC chemokine type 2 [*Gadus morhua*]	3.76	-	-
37456	Goose-type lysozyme 2 [*Gadus morhua*]	3.36	-	-
36381	CC chemokine type 2 [*Gadus morhua*]	2.98	-	-
42545	Thioredoxin [*Oncorhynchus mykiss*]	2.35	-	2.17
36388	CEBPA protein [*Bos taurus*]	1.86	-	-
36468	CCT epsilon subunit [*Carassius auratus*]	1.66	-	1.49
38014	Ctssa protein [*Danio rerio*]	1.66	-	-
35923	Annexin A4 [*Ctenopharyngodon idella*]	1.62	-	-
42502	Tcp1 protein [*Danio rerio*]	1.56	-	-
36813	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23 [*Xenopus tropicalis*]	1.55	-	-
40508	Glutathione S-transferase Mu 3 [*Anoplopoma fimbria*]	1.53	-	1.61
45010	Heat shock protein 90 alpha [*Paralichthys olivaceus*]	1.52	-	1.66
43222	Warm temperature acclimation protein 65-1 [*Dicentrarchus labrax*]	1.42	-	-

Table 5.3 cont'd

37574	Heat shock protein 90 beta [*Paralichthys olivaceus*]	1.37	-	1.52
39904	Peptidylprolyl isomerase A (cyclophilin A) [*Danio rerio*]	1.33	-	-

Probes identified as significantly differentially expressed (FDR = 1%) between pIC 24HPI@10°C and pIC 24HPI@16°C only				
Probe ID	**Best BLASTx hit[2]**	**Fold-change (pIC @16°C /PBS @16°C)[3]**	**Fold-change (pIC @10°C /PBS @10°C)[3]**	**Fold-change (pIC @16°C /pIC @10°C)[3]**
37555	Heat shock protein 47 [*Oncorhynchus mykiss*]	-	-	1.77
40546	Heat shock protein HSP 90-alpha [*Harpegnathos saltator*]	-	-	1.64
36470	Chaperonin containing TCP1 subunit 6A [*Paralichthys olivaceus*]	-	-	1.54
44902	HSP90 multi-domain protein (pfam 00183)	-	-	1.47
37544	Heat shock cognate protein 70 [*Pelodiscus sinensis*]	-	-	1.35
42467	TANK-binding kinase 1 [*Gadus morhua*]	-	-	1.37
37203	Ferritin heavy subunit [*Epinephelus awoara*]	-	-	1.47
41479	Protein kinase C, delta [*Danio rerio*]	-	-	1.47

[1]Fold-change in probe expression is also shown for pIC @16°C vs. pIC @10°C when significant differential expression was found in the PBS vs. pIC comparisons.

[2]Informative genes were re-annotated using the contig or EST from which the probe printed in the array was designed by comparing the referred sequence to the NCBI's nr database using the BLASTx algorithm as implemented by Blast2GO (Conesa et al., 2005). The best BLASTx hit (E-value cutoff of 10^{-5}) with an informative name (i.e. not predicted, probable or unnamed protein), if available, is presented in this table. Synonyms were obtained from SWISS-PROT, GENE CARDS other BLASTx hits as long as they had an E-value < 10^{-5}, or from the literature ([a]Borza et al., 2010). Some genes without a BLASTx hit (e.g. unknowns) were included because they presented a pIC induced fold-change higher than 5, and therefore, could represent novel immune-relevant genes. For same named probes within a given comparison, the one with the highest significant fold-change is presented in this table.

[3]Fold-changes are only reported when probes were significantly differentially expressed (FDR=1%) in a given comparison (e.g. pIC @10°C vs. pIC @16°C) and are shown as

Table 5.3 cont'd

outputted by siggenes; otherwise a dash (-) is presented in place of the fold-change. Fold-changes presented in white font with black background represent overall fold down-regulation for a particular comparison. For standard deviation, p- and d-values, please refer to supplemental Additional File 5.1 - Tables S2-S5 and S7-S8.

PBS) at 10 and 16°C for both time points. Figure 5.3 shows four pie charts depicting the proportions of level 2 biological process GO terms of: A) the 279 genes (Fig. 5.2) more highly expressed in fish injected with pIC and sampled six hours post-injection at 16°C in comparison to the time-matched pIC injected fish at 10°C; B) the 11 genes (Fig. 5.2) more highly expressed in fish injected with pIC at 10°C and sampled six hours post-injection in comparison to the time-matched pIC injected fish at 16°C; C) the 36 genes (Fig. 5.2) more highly expressed in fish injected with pIC at 16°C and sampled twenty-four hours post-injection in comparison to the time-matched pIC injected fish at 10°C; and D) the 303 genes (Fig. 5.2) more highly expressed in fish injected with pIC at 10°C and sampled twenty-four hours post-injection in comparison with the time-matched pIC injected fish at 16°C.

Several biological processes were highly represented (i.e. by more than 10 sequences) in the 629 genes differentially expressed between fish stimulated with pIC at the different temperatures (i.e. identified in the direct comparisons between fish injected with pIC at 10 and 16°C and sampled at 6HPI and 24HPI). Among these were: "cellular component organization", "cellular process, developmental process", "signaling, biological regulation", "response to stimulus", "immune system process", and "death". Only 4 biological process level 2 GO terms were mapped to the 11 genes more highly expressed in fish stimulated at 10°C and sampled at 6HPI compared to those stimulated with pIC at 16°C and sampled at the same time point (Fig. 5.3). This is likely a reflection of the low number of probes in this particular gene list. Eighteen of the level 2 biological process GO terms that were mapped to the genes more highly expressed in fish stimulated with pIC at 16°C and sampled at 6HPI (Fig 5.3A) were also mapped to the genes that

were more highly expressed in fish stimulated with pIC at 10°C and sampled at 24HPI (Fig. 5.3D) (each compared to its time- and temperature-matched pIC injected counterpart). Ninety-six probes were present in both gene lists (Fig. 5.4 – Additional File 5.1 – Table S9). However, many of the GO terms that were in common between these lists had more sequences mapped to them in the list of probes more highly expressed in fish stimulated with pIC at 10°C and sampled at 24HPI (Fig. 5.3D) compared to fish stimulated with pIC at 16°C and sampled at 6HPI (Fig 5.3A). Examples are "signalling" (20 for pIC 6HPI@16°C and 26 for pIC 24HPI@10°C), "immune system process" (15 for pIC 6HPI@16°C and 19 for pIC 24HPI@10°C), "death" (8 for pIC 6HPI@16°C and 15 for pIC 24HPI@10°C), "biological regulation" (44 for pIC 6HPI@16°C and 64 for pIC 24HPI@10°C), "response to stimulus" (18 for pIC 6HPI@16°C and 41 for pIC 24HPI@ 10°C) and "cellular process" (68 for pIC 6HPI@16°C and 96 for pIC 24HPI@10°C) (Fig. 5.3 A and D).

The GO enrichment analysis also identified several GO terms with significantly different over- or under-representation between the genes up-regulated by pIC (relative to PBS) at 24HPI at the two temperatures. Most GO terms (55 out of 57) identified were over-represented in the genes up-regulated by pIC at 16°C compared with those up-regulated by pIC injection at 10°C (all relative to PBS). Examples of these over-represented GO terms in the 24HPI list of pIC responsive genes at 16°C are: "protein folding" (GO:0006457), "negative regulation of cellular process" (GO:0048523), "negative regulation of protein metabolic process" (GO:0051248), "regulation of interferon-gamma-mediated-signaling" (GO:0060334), "regulation of signaling process"

(GO:0023051), "regulation of signal transduction" (GO:0009966), "regulation of cytokine-mediated signaling pathway" (GO:0001959), "unfolded protein binding" (GO:0051082) and "enzyme regulatory activity" (GO:0030234). Two GO terms were under-represented in the 24HPI list of pIC responsive genes at 16°C: "macromolecule modification" (GO:0043412) and "protein modification process" (GO:0006464).

Two separate clustering results are displayed as heat-maps (Figs. 5.5, 5.6). In these heat-maps, all individuals (60) were clustered based on their expression for the genes identified as differentially expressed between the pIC@16°C and pIC@10°C groups at 6HPI (Fig. 5.5) or at 24HPI (Fig. 5.6). In both figures, panel A shows the entire heat-map, while panel B shows a sub-tree (marked as a blue bar on the right of panel A) dominated by genes belonging to the interferon (IFN) pathway. Both clustering results show a clear distinction (with a few outliers) between the groups of fish injected with pIC at the different temperatures and time points. Based on the expression of the genes identified in the above-mentioned comparisons, the pIC 6HPI@10°C group had an expression profile more similar to that of the non-injected fish, while the pIC 6HPI@16°C group's expression profile was more similar to that of the pIC 24HPI@10°C group. For the PBS-injected and non-injected fish the clusters were less distinctive.

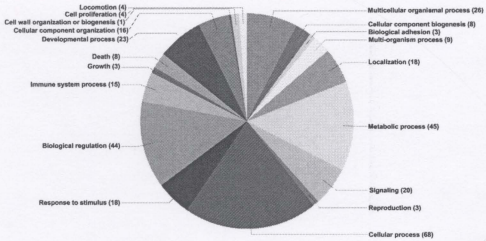
5.4.5 QPCR validation of selected immune-relevant genes

Genes of interest (GOI) were selected from the 20K microarray-identified pIC-responsive transcripts for QPCR analysis based on their putative roles in pathogen detection, signal transduction/transcription control and as immune effectors; this QPCR

Figure 5.3 Summary of gene ontology (GO) terms belonging to the biological process branch, represented as pie charts, for genes differentially expressed between fish injected with pIC at 10 and 16°C. Genes found to be significantly differentially expressed between pIC@10°C and pIC@16°C at both 6HPI and 24 HPI time points were normalized to GO term hierarchy level 2. GO terms that mapped to the 279 genes more highly expressed in pIC 6HPI@16°C compared to pIC 6HPI@10°C are shown in A. The terms that mapped to the 11 genes more highly expressed in pIC @10°C compared to pIC @16°C at 6HPI are shown in B. C (36 genes) and D (303 genes) represent the same analyses, respectively, for the 24HPI time point.

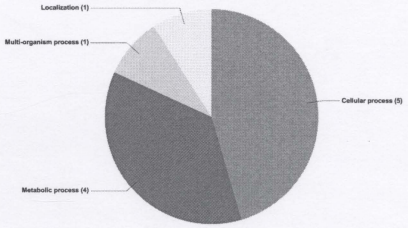
A

279 genes more highly expressed in fish injected with pIC at 16°C and sampled at 6HPI



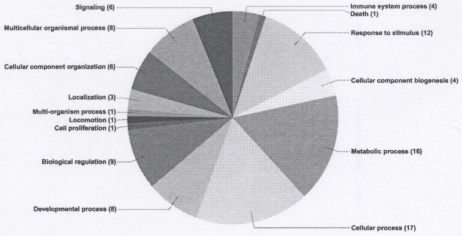
B

11 genes more highly expressed in fish injected with pIC at 10°C and sampled at 6HPI



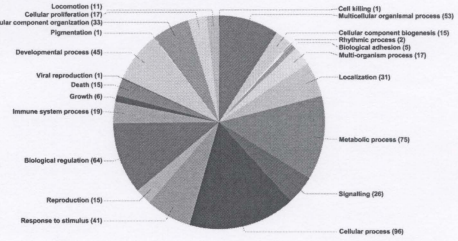
C

36 genes more highly expressed in fish injected with pIC at 16°C and sampled at 24HPI



D

303 genes more highly expressed in fish injected with pIC at 10°C and sampled at 24HPI



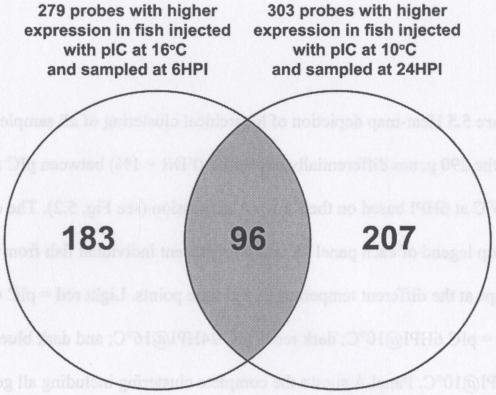


Figure 5.4 Venn diagram showing the overlap between the 279 probes with significantly higher mRNA expression in pIC 6HPI@16°C compared to pIC 6HPI@10°C and the 303 probes with significantly higher mRNA expression in pIC 24 HPI@10°C compared to pIC 24HPI@16°C (Fig. 5.2).

Figure 5.5 Heat-map depiction of hiearchical clustering of all samples (i.e. microarrays) and the 290 genes differentially expressed (FDR = 1%) between pIC @10°C and pIC @16°C at 6HPI based on their mRNA expression (see Fig. 5.2). The colored boxes below the top legend of each panel (A and B) represent individual fish from the pIC injected groups at the different temperatures and time points. Light red = pIC 6HPI@16°C; light blue = pIC 6HPI@10°C; dark red = pIC 24HPI@16°C; and dark blue = pIC 24HPI@10°C. Panel A shows the complete clustering including all genes. Panel B shows only the cluster marked with a blue bar on A, which is enriched for genes belonging to the interferon pathway. For a detailed high resolution image refer to Additional File 5.2, Figure S1).

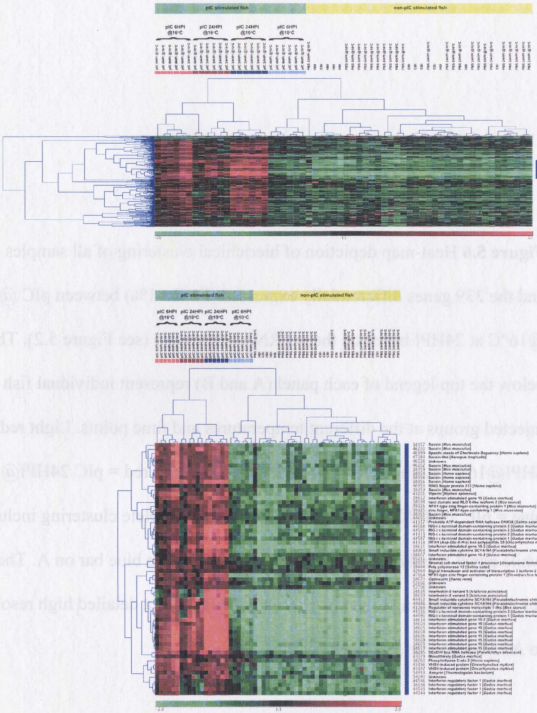
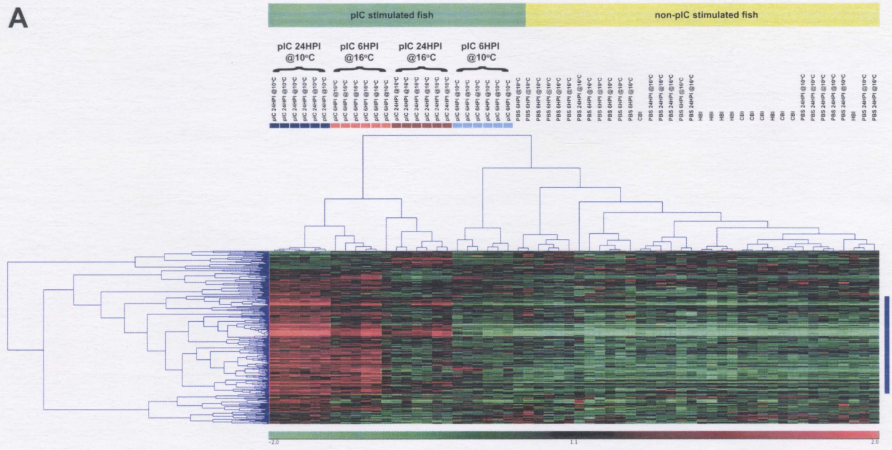
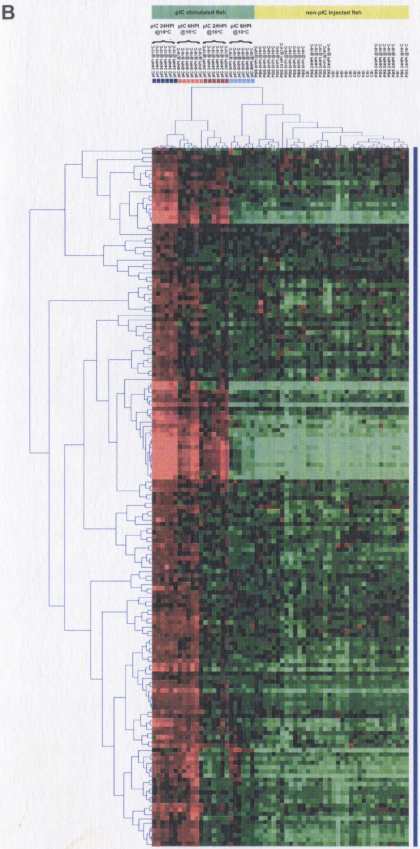

A
pIC stimulated fish
non-pIC stimulated fish
pIC 6HPI @16°C
pIC 24HPI @16°C
pIC 24HPI @16°C
pIC 6HPI @16°C
B
pIC stimulated fish
non-pIC stimulated fish
pIC 6HPI @16°C
pIC 24HPI @16°C
pIC 24HPI @16°C
pIC 6HPI @16°C

Figure 5.6 Heat-map depiction of hierichical clustering of all samples (i.e. microarrays) and the 339 genes differentially expressed (FDR =1%) between pIC @10°C and pIC @16°C at 24HPI based on their mRNA expression (see Figure 5.2). The colored boxes below the top legend of each panel (A and B) represent individual fish from the pIC injected groups at the different temperatures and time points. Light red = pIC 6HPI@16°C, Light Blue = pIC 6HPI@10°C; Dark red = pIC 24HPI@16°C and Dark blue = pIC 24HPI@10°C. Panel A shows the complete clustering including all genes, while panel B only shows the cluster marked with a blue bar on A. The latter is enriched for genes belonging to the interferon pathway. For a detailed high resolution image refer to Additional File 5.2, Figure S2).


A
pIC stimulated fish
non-pIC stimulated fish
pIC 24HPI @10°C
pIC 6HPI @16°C
pIC 24HPI @16°C
pIC 6HPI @10°C
-2.0
1.1
2.0


B
pIC stimulated fish
non-pIC injected fish
pIC 24HPI @18°C
pIC 6HPI @18°C
pIC 24HPI @18°C
pIC 6HPI @18°C
-2.0
1.1
2.0

data is presented in Figures 5.7, 5.8, and 5.9, respectively, as average $\log_2$ transformed RQs ± S.E. Fifteen of the immune-relevant genes (88%; all except TLR3 and IRAK4) were validated as being differentially ($p < 0.05$) expressed in at least one comparison (e.g. pIC 6HPI@10°C vs. PBS 6HPI@10°C).

Of the 4 genes with putative pathogen detection roles that were subjected to QPCR, 3 (TLR9; PKR; DHX58) were validated (i.e. showed a significant difference in at least one of the group comparisons corresponding to the microarray comparison identifying the genes as differentially expressed) (Fig. 5.7B-D). In the QPCR experiment, DHX58 showed the highest fold up-regulation in response to pIC (13.00-fold at 6HPI@16°C and 34.86-fold at 24HPI@10°C) and the highest fold differential expression between fish injected with pIC at different temperatures (at 6HPI, DHX58 was 13.64-fold more highly expressed in fish injected with pIC at 16°C and at 24HPI it was 4.45-fold more highly expressed in fish injected with pIC at 10°C) (Fig. 5.7D). PKR showed the same trend but with lower, and yet significant, fold-changes (at 6HPI, 1.56-fold higher in pIC @16°C vs. PBS @16°C and 1.86-fold higher in pIC @16°C vs. pIC@10°C; at 24HPI, 2.73-fold higher in pIC @10°C vs. PBS @10°C, 1.65-fold higher in pIC @16°C vs. PBS @16°C and 1.19-fold higher in pIC @10°C vs. pIC@16°C) (Fig. 5.7C).

Of the 8 microarray-identified genes with putative roles in signal transduction/transcription control analyzed with QPCR, 7 (IκBα, IRF1, IRF7, IRF10, STAT1, Deltex3, ZNFX1) were validated (Fig. 5.8A-E and G-H). QPCR analysis showed that IκBα mRNA was only differentially expressed between groups at the 6HPI time point (Fig. 5.8A - 3.82-fold higher in pIC @10°C vs PBS @10°C, 1.94-fold higher in pIC@16°C vs. PBS@16°C and 1.69-fold higher in pIC@10°C vs. pIC@16°C). All three

studied IRFs (1, 7, and 10, Fig. 5.8B-D, respectively) showed very similar expression profiles at the mRNA level. These genes responded significantly to pIC at both temperatures and time points, although the magnitude of the fold up-regulation was variable. Of all studied IRFs, IRF7 at the 6HPI time point showed the highest response to pIC (13.26-fold at 16°C) and the highest fold difference between fish injected with pIC at 16 vs. 10°C. (4.66-fold). At the 24HPI time point, IRF10 showed the highest response to pIC (9.07-fold at 10°C) and the highest fold difference between fish injected with pIC at 10 vs. 16°C (4.47-fold). Of all the genes with putative roles in signal transduction/transcription control, ZNFX1 showed the highest response to pIC at 10°C (Fig. 8H - 15.30 fold up-regulation at 24HPI). The microarray results for IRAK4 (Tables 5.2 and 5.3) were not validated by QPCR analysis (Fig. 5.7F).

All 5 of the putative immune-effector genes studied with QPCR showed significant differential expression at 6HPI and 24HPI between fish injected with pIC vs. PBS at 10 and/or 16°C (Fig. 5.9A-E). Further, they showed the highest levels of change of all the studied transcripts. At 6HPI, ISG15 showed the highest response to pIC at both temperatures (20.77-fold at 10°C and 134.8-fold at 16°C), and RSAD2 (Fig. 5.10E) (alias viperin) showed the highest differential mRNA expression between fish injected with pIC at the two temperatures (13.47-fold higher in 16°C vs.10°C). At 24HPI, ISG15 also showed the highest response to pIC at both temperatures (155.2-fold at 10°C and 82.40-fold at 16°C), while IL-8 presented the highest fold difference between fish stimulated with pIC at 10 vs. 16°C (11.14-fold higher at 10°C). ISG15 was the only microarray identified, QPCR studied, putative immune-effector transcript for which not all differences detected on the microarray were validated by QPCR. While ISG15 was

Figure 5.7 QPCR results for transcripts with putative roles in pathogen detection. Data is presented as mean $\log_2$ transformed RQs ± S.E. Different letters represent significant differences between fish injected with pIC at different temperatures within each time point. An asterisk (*) represents a difference between a given pIC injected group and the time- and temperature-matched PBS injected group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Fold-changes in black font in white-filled boxes (shown beneath each panel) are always pIC/PBS or 16°C/10°C. Only differences that were significant are shown. Values in white font in black-filled boxes (shown beneath each panel) were calculated as 1/fold-change for comparisons that yielded fold-change values less than one (for more details on fold-change calculations, refer to the methods section). A) TLR3; B) TLR9; C) PKR; D) DHX58.

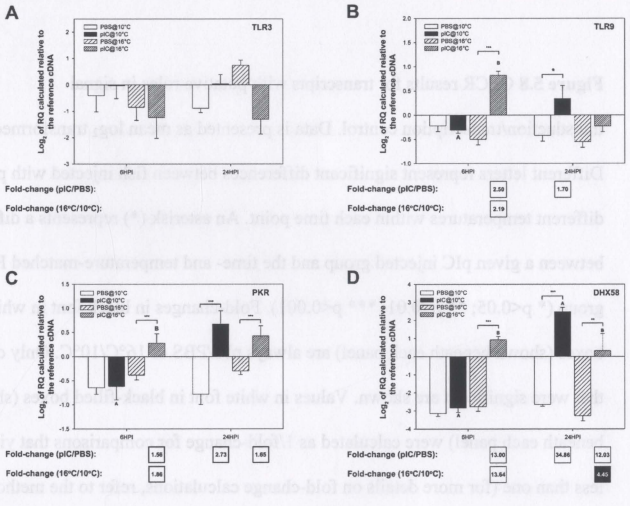

A
TLR3
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS):
Fold-change (16°C/10°C):
B
TLR9
2.58
1.70
2.19
C
PKR
1.56
2.73
1.65
1.86
D
DHX58
13.00
34.86
12.03
13.64
4.43

Figure 5.8 QPCR results for transcripts with putative roles in signal transduction/transcription control. Data is presented as mean $\log_2$ transformed RQs ± S.E. Different letters represent significant differences between fish injected with pIC at different temperatures within each time point. An asterisk (*) represents a difference between a given pIC injected group and the time- and temperature-matched PBS injected group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Fold-changes in black font in white-filled boxes (shown beneath each panel) are always pIC/PBS or 16°C/10°C. Only differences that were significant are shown. Values in white font in black-filled boxes (shown beneath each panel) were calculated as 1/fold-change for comparisons that yielded values less than one (for more details on fold-change calculations, refer to the methods section). A) IκBα; B) IRF1; C) IRF7; D) IRF10; E) STAT1; F) IRAK4; G) Deltex3; H) ZNFX1.

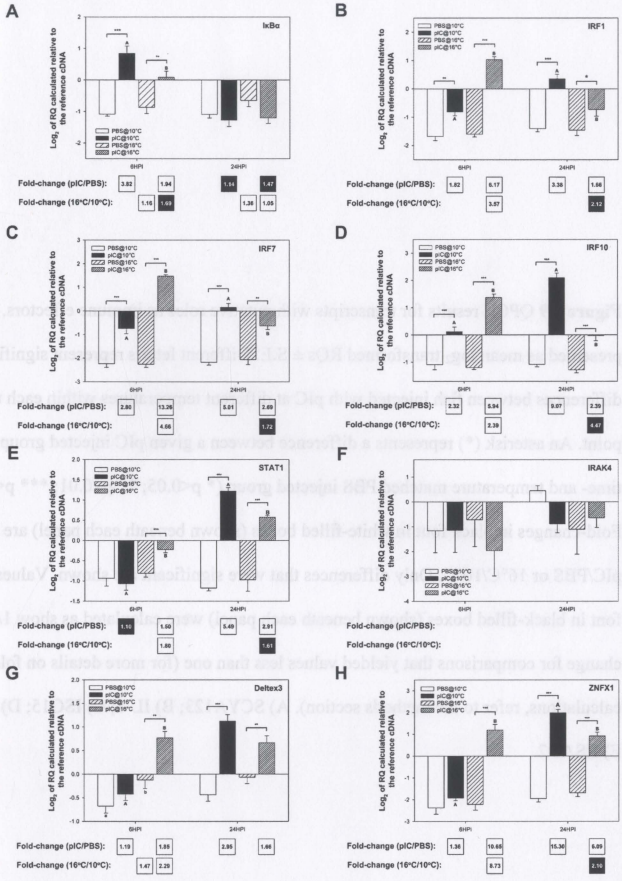
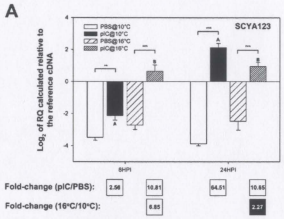
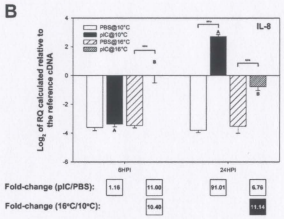
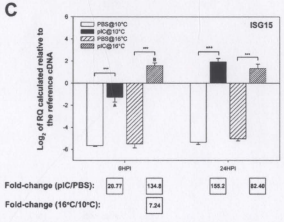
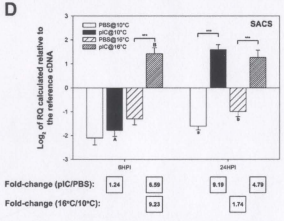

A
IκBα
B
IRF1
C
IRF7
D
IRF10
E
STAT1
F
IRAK4
G
Deltex3
H
ZNFX1
Log2 of RQ calculated relative to the reference cDNA
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
6HPI
24HPI
Fold-change (pIC/PBS):
Fold-change (16°C/10°C):

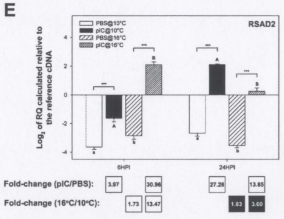
Figure 5.9 QPCR results for transcripts with putative roles as immune effectors. Data is presented as mean $\log_2$ transformed RQs ± S.E. Different letters represent significant differences between fish injected with pIC at different temperatures within each time point. An asterisk (*) represents a difference between a given pIC injected group and the time- and temperature matched PBS injected group (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Fold-changes in black font in white-filled boxes (shown beneath each panel) are always pIC/PBS or 16°C/10°C. Only differences that were significant are shown. Values in white font in black-filled boxes (shown beneath each panel) were calculated as show 1/fold-change for comparisons that yielded values less than one (for more details on fold-change calculations, refer to the methods section). A) SCYA123; B) IL-8; C) ISG15; D) SACS; E) RSAD2.


A
SCYA123
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS): 2.56 10.81 64.01 10.65
Fold-change (16°C/10°C): 6.65 2.27


B
IL-8
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS): 1.16 11.00 91.01 6.76
Fold-change (16°C/10°C): 10.48 11.14


C
ISG15
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS): 20.77 134.8 155.2 82.46
Fold-change (16°C/10°C): 7.24


D
SACS
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS): 1.24 6.59 8.19 4.79
Fold-change (16°C/10°C): 9.23 1.74


E
RSAD2
PBS@10°C
pIC@10°C
PBS@16°C
pIC@16°C
Log2 of RQ calculated relative to the reference cDNA
6HPI
24HPI
Fold-change (pIC/PBS): 3.07 30.96 27.26 13.65
Fold-change (16°C/10°C): 1.73 13.47 1.83 3.03

identified as being differently expressed (FDR = 1%) between fish injected with pIC at 10 vs. 16°C at 24HPI in the microarray experiment, only a non-significant change in the same direction was observed in the QPCR at 24HPI.

5.5 Discussion

Variations in temperature are an inevitable element of sea-cage based aquaculture (Gollock et al., 2006; Johansson et al., 2007; Oppedal et al, 2007). I used a regimen of incremental changes in seawater temperature that simulated the environmental conditions to which the Atlantic cod is often exposed during the spring-summer transition in sea-cages in Newfoundland, and microarrays and QPCR, to study the impact of changes in rearing temperature on the Atlantic cod spleen transcriptome response to pIC. This is the first study to use global gene expression analysis to investigate the impact of elevated water temperature on the immune response of this species. I was able to demonstrate that, at the mRNA level, while a non-lethal increase in water temperature to 16°C only caused minor changes in the spleen's transcriptome, it had a major influence on the Atlantic cod spleen's gene expression response to IP injection with the viral mimic pIC.

5.5.1 Influence of elevated temperature on plasma cortisol and the spleen transcriptome

In the present study, plasma cortisol levels did not increase significantly when Atlantic cod were exposed to the chronic increasing temperature regimen. This finding suggests that the juvenile Atlantic cod in the present study were not stressed under these conditions, but is in contrast to the results of Pérez-Casanova et al. (2008b) who reported

plasma cortisol values of ~ 52 ng ml^{-1} in similar sized Atlantic cod that had been warmed from 10 to 16°C using a similar protocol. This discrepancy, however, it is likely due to the fact that Pérez-Casanova et al. (2008b) sampled their fish as temperature reached 16°C, whereas I sampled the Atlantic cod after they were given a week to acclimate to this temperature. It is probable that the extra week to which my fish were exposed at 16°C allowed their cortisol (stress) level to return towards basal values. This hypothesis is also supported by Pérez-Casanova et al. (2008b), as these authors reported that Atlantic cod plasma cortisol levels decreased on day 40 of their experiment (temperature ~ 17°C) and that values remained at pre-stress levels as temperature was increased further.

Gradually increasing water temperature to 16°C had a very small impact on the Atlantic cod spleen transcriptome (only 6 up-regulated probes were detected at FDR = 1%, Fig. 5.2). Further, using microarrays, I was unable to detect any differences in expression of β2-microglobulin, MHC-I or IgM heavy chain due to increases in water temperature alone. This result is again different from Pérez-Casanova et al. (2008b) who showed, using QPCR with blood cDNA templates, that these genes were differentially expressed between Atlantic cod at 16°C and at 10°C. Although I cannot rule out tissue-specific differences (spleen vs. blood) in the response of these genes to the temperature increase, it likely that the slightly different sampling regimes (see above) largely explain these disparate findings. When this data is combined with that for plasma cortisol levels, it suggests that prolonged exposure of Atlantic cod juveniles to sea-cage temperatures of 16°C is unlikely to have negative consequences with regards to their stress state or basal immune transcript expression.

5.4.2 Temperature effects on the Atlantic cod spleen anti-viral transcriptome

Compared to the time- and temperature-matched PBS controls, pIC altered the expression of 41 (36 up-regulated and 5 down-regulated) and 999 (851 up-regulated and 148 down-regulated) transcripts in 10°C fish at 6HPI and 24HPI, respectively, and 656 (617 up-regulated and 39 down-regulated) and 246 (243 up-regulated and 3 down-regulated) transcripts in 16°C fish at 6HPI and 24HPI, respectively (Fig. 5.2). Figure 5.3 shows a summary of the GO terms that were associated with the genes that were differentially expressed between fish injected with pIC at the different temperatures and sampled at 6HPI (Fig. 5.3A-B) and 24HPI (Fig. 5.3C-D). Not surprisingly, "immune system process", "signaling", and "response to stimulus" were found in 3 out of the 4 pie charts in relatively high-proportions (Fig. 5.3A, C and D). These results indicate, as previous studies have shown (Raida and Buchmann, 2007; Pérez-Casanova et al., 2008b), that elevated temperatures can induce changes in the fish's immune response to an antigen. Furthermore, I suggest that the genes associated with these GO terms (e.g. "biological regulation", "death", "growth", "metabolic process"; see Fig. 5.3) may be good candidates for the development of molecular markers (e.g. exonic, intronic or regulatory region single nucleotide polymorphisms) for use in marker assisted selection (MAS) programs aimed at developing elite (e.g. immune-robust, heat stress resistant) broodstock for the Atlantic cod aquaculture industry.

The sample clustering based on genes differentially expressed between the groups injected with pIC and sampled at 6HPI shows that the expression profile of pIC injected fish held at 10°C and sampled at 6HPI was more similar to that of the non-immune stimulated (i.e. non-injected or injected with PBS) fish than to the profile of any other

group injected with pIC (Fig. 5.5A). In addition, clustering based on the 290 (Fig. 5.5) or 339 (Fig. 5.6) genes differentially expressed between fish injected with pIC at the different temperatures and sampled at 6HPI or 24HPI, respectively, indicates that the mRNA expression profile of pIC-injected fish held at 16°C and sampled 6HPI was most similar to that of fish injected with pIC at 10°C and sampled at 24HPI. Collectively, these data suggest that the moderate increase in water temperature caused a time shift in the transcriptomic response of the Atlantic cod spleen to pIC, with fish held at elevated temperature (16°C) having an earlier maximum response (i.e. at 6HPI) and fish held at optimal temperature (10°C) having a later maximum response (i.e. at 24HPI). Raida and Buchmann (2007) report similar data for mRNA expression of immune-relevant genes in rainbow trout injected with a *Yersinia ruckeri* bacterin. These authors found that peak transcript expression of IL-1β, IL-10 and IFN-γ occured earlier in fish stimulated at 15°C or 25°C when compared to fish stimulated at 5°C. Furthermore, it is clear from my heat-maps that, overall, the magnitude of the response to pIC for these genes was greatest in the pIC stimulated fish at 10°C and sampled at 24HPI. This result, however, must be interpreted with caution. While it is possible that holding Atlantic cod at 16°C results in a faster, but weaker, immune response in this species, this hypothesis must be confirmed by sampling pIC-injected Atlantic cod exposed to similar temperature regimes at more frequent intervals post-injection.

Using the clustering of genes based on their expression profiles in each sample, I was able to identify many different clusters, including one from each time point that was highly enriched for putative members of the interferon (IFN) pathway. These clusters are marked with a blue bar on Figures 5.5A and 5.6A and shown in detail in Figures 5.5B and

5.6B. The IFN pathway is a key part of the fish innate response to viruses (Robertsen, 2006; Robertsen, 2007; Rise et al., 2008; Workenhe et al., 2009; Rise et al., 2010), and my results indicate that elevated temperature had a considerable impact on the Atlantic cod early innate immune response to a viral mimic.

5.5.3 Impacts of the gradual temperature increase on transcript expression

5.5.3.1 Genes with putative viral detection roles

One of the key steps in mounting an anti-viral innate immune response (e.g. expression of type I interferons and proinflammatory cytokines) is the detection of an invading pathogen. This recognition often occurs via the detection of a set of pathogen associated molecular patterns (PAMPs). PAMPs bind specifically to germ-line pattern recognition receptors (PRRs), which in turn activate signaling pathways that induce the innate immune response (Phelan et al., 2005; Galligan et al., 2006; Rebl et al., 2010). The immune-stimulant (pIC) used in this experiment is a double-stranded RNA (dsRNA) that mimics the genome and/or RNA intermediates of several viruses and is recognized by PRRs, including the Toll-like receptor 3 (TLR3). While TLR3-like transcript was shown to be slightly up-regulated by pIC in 16°C fish at 6HPI on the microarray (1.32 fold – Table 5.2), the QPCR analysis did not confirm this result (Fig. 5.7A). In fact, I did not detect any significant changes in TLR3 due to pIC injection using QPCR. This is not surprising as Rise et al. (2008) obtained similar QPCR results for TLR3 (using the same primer pair) in the spleen of Atlantic cod stimulated with pIC, and Rodriguez et al. (2005) did not find any significant induction of TLR3 mRNA in spleen or head kidney of rainbow trout following injection of this viral mimic. However, it suggests that the

differences in response to pIC between fish held at 10 vs. 16°C were not likely to be caused by an enhanced dsRNA-induced over-expression of TLR3 in the spleen of fish held at 16°C and injected with pIC. Interestingly, Rodriguez et al. (2005) and Chiou et al. (2007) have shown up to ~ 30-fold induction of rainbow trout TLR3 transcripts by pIC in isolated anterior kidney leukocytes/macrophages, and this is similar to what has been observed in mammalian macrophages (Matsumoto and Seya, 2008). These data suggest that the effects of pIC stimulation on TLR3 mRNA expression may be tissue/cell type-specific.

In this chapter, TLR9 transcripts were found by both the microarray and QPCR analyses to be up-regulated by pIC at 6HPI in 16°C fish and at 24HPI for fish held at 10°C, and the QPCR analysis showed that mRNA levels of TLR9 were significantly different between 10 and 16°C pIC injected fish sampled at 6HPI (Fig. 5.7B). As in mammals, the main ligand of TLR9 in fish is thought to be viral/bacterial unmethylated CpG DNA (Palti, 2011; Rebl et al., 2010), not dsRNA. Thus, it is unclear what the roles of TLR9 during the host response to viral dsRNA may be. While it is apparent that temperature can impact the responses of fish to other pathogens such as bacteria by modulating TLR9 mRNA expression (Rebl et al., 2010), further research is warranted to elucidate how antigens and/or changes in the environment affect the dynamics of TLR expression.

Other important sensors of viruses are cytosolic PRRs, such as the retinol induced gene I (RIG-I)-like receptors (RLRs) and the dsRNA activated protein kinase (PKR). DHX58 (alias LGP2) is a RLR that was found to be highly induced by pIC compared to PBS at both 10°C and 16°C, and also differentially expressed between fish injected with

pIC at 10 and 16°C at both time points in the QPCR and microarray experiments (Tables 5.2 and 5.3, Fig. 5.7D). Previous studies on Atlantic cod have reported up-regulation of DHX58 mRNA by pIC in both spleen and brain (Rise et al, 2008, 2010). In addition, DHX58 mRNA was found to be significantly up-regulated in the brain (Rise et al. 2010), but not the spleen (Rise et al. 2008), of asymptomatic high nodavirus carrier Atlantic cod. As in Rise et al. (2008, 2010), I detected maximum up-regulation of DHX58 mRNA at 24HPI for fish injected with pIC at 10°C, whereas, in the spleen of fish injected with pIC at 16°C, the fold up-regulation (compared to the PBS control) of DHX58 transcript was similar at 6HPI and 24HPI (13-fold and 12-fold, respectively) (Fig. 5.7D). The roles of DHX58 in the anti-viral response are still unclear (Kawai and Akira, 2008; Rise et al., 2008; Wilkins and Gale, 2010). However, there is some experimental evidence that it may induce or repress RIG-I/MDA5 dependent signal transduction in a virus-dependent manner (Kawai and Akira, 2008). I observed similar differences in the expression of PKR mRNA (Fig. 5.7C). Like TLR9, DHX58, STAT1, Deltex3, ZNF1, IL-8 and SACS, PKR did not respond to pIC at 6HPI in fish held at 10°C, but it was significantly up-regulated in the spleens of fish held at 16°C and injected with pIC. In mammals, the main anti-viral function of PKR was originally thought to be the phosphorylation of eIF2α, leading to reduced protein synthesis (Gil et al., 2000). More recently, it has been demonstrated that PKR has roles in modulating apoptosis via NF-κB and the growth-inhibitory activity of IRF1 (Garcia et al., 2007). These results suggest that the observed temperature-time interaction on PKR mRNA expression could impact the fish's ability to fight a viral infection.

5.5.3.2 Genes with putative signal transduction and transcription regulation roles

In mammals, downstream signaling of TLRs and RLRs involves activation of NF-κB transcription factors (Kawai and Akira, 2006; Kawai and Akira, 2008). There is some evidence of a similar mechanism in fish, since expression constructs containing a constitutively-active form of the zebrafish (*Danio rerio*) TLR3 transfected into ZFL cells induced NF-κB mediated luciferase fluorescence (Phelan et al., 2005). An important step in this process is the activation of the inhibitor of NF-κB kinases (IKKs), which in turn phosphorylate the inhibitor of NF-κB proteins (IκBs). Phosphorylation targets IκBs for degradation, and in their absence, the NF-κB proteins accumulate in the nucleus and regulate mRNA transcription (Li and Verma, 2002; Bonizzi and Karin, 2004). In mammals, IκBα is thought to regulate transient activation of NF-κB. As such, it is rapidly degraded in response to stressful stimuli, but then quickly re-synthesized due to the presence of a NF-κB response element in its promoter region (reviewed by Li and Verma, 2002). At 6HPI, both the microarray and the QPCR studies showed an up-regulation of IκBα mRNA by pIC at both temperatures (Table 5.2 and Fig. 5.8A). Such a response would enable the re-synthesis IκBα protein in the spleen cells following pIC stimulation. However: 1) the QPCR-detected up-regulation of IκBα mRNA by pIC at 6HPI was higher in fish held at 10°C than in fish held at 16°C (3.82-fold vs.1.94-fold, respectively); and 2) the difference in mRNA expression of IκBα between 10 and 16°C fish injected with pIC at 6HPI was also significant (both in the QPCR and the microarray studies). This suggest that the early response of IFN related genes observed in the fish held at 16°C (see Fig. 5.7) was in part due to a reduced re-synthesis of IκBα, and that this lead to a more pronounced NF-κB-mediated induction of transcription. At 24HPI, I detected no

significant differences in the mRNA expression of IκBα in either the microarray (Table 5.3) or QPCR (Fig. 5.8A) studies; although the microarray analysis indicated that IKKα was significantly up-regulated by pIC at this time point only in the spleens of fish held at 10°C (Table 5.3). Since IKKs are also essential to NF-κB activation, this difference may have contributed to the differences in immune-related gene expression observed at 24HPI (e.g. as shown by the hierarchical clustering; see Figs. 5.5 and 5.6). Overall, these results suggest that, as in higher vertebrates (Li and Verma, 2002), the timing and intensity of Atlantic cod immune responses are partly regulated by NF-κB transcription factors.

The interferon regulatory factor (IRF) genes are also key to the activation of the IFN pathway (Tamura et al., 2008). In this work all 3 IRFs (i.e. IRF1, IRF7 and IRF10) that had been previously identified as pIC-responsive in Atlantic cod (Rise et al., 2008) were shown to be up-regulated at the mRNA level in spleen by this viral mimic (Table 5.2 and 5.3; Fig. 5.8B, C and D). Moreover, all studied IRFs presented the same overall expression profile as most IFN pathway-related genes identified in this study. This profile is characterized by a higher expression at 6HPI in fish injected with pIC and held at 16°C vs. 10°C, but greater expression in this latter group at 24HPI. In higher vertebrates both IRF1 and IRF7 are thought to be positive modulators of the type I IFN response to viruses, with IRF7 being essential for interferon-related gene expression (Tamura et al., 2008). In the current microarray experiment, TANK-binding kinase 1 (TBK1), which activates IRF7 by phosphorylation, was also more highly expressed in the spleens of fish injected with pIC at 10°C and sampled at 24HPI (1.37 fold – Table 5.3) as compared to spleens of 16°C pIC-injected fish at this time point. Thus, it is likely that temperature effects on the expression of IRFs and TBK1 in fish stimulated with pIC contributed to the

global expression profiles (i.e. with several IFN-related genes peaking at different times in fish injected with pIC at 10°C as compared to those injected with pIC at 16°C) depicted in Figures 5.5 and 5.6.

Once type I interferons (e.g. IFN-α/β) are expressed they interact with specific cell surface receptors (Interferon α/β receptors [IFNAR]), and trigger the expression of interferon stimulated genes (ISGs) (Noppert et al., 2007). This signaling cascade occurs partly through the heterologous dimerization of the signal transducer and activator of transcription (STAT) 1 and 2 proteins, and their interaction with IRF9. The STAT1/STAT2/IRF9 complex then further induces STAT1 expression (Noppert et al., 2007). In Atlantic cod, STAT1 mRNA has been shown to be more highly expressed in the brains of fish with high nodavirus carrier status compared to fish with no/low nodavirus status, and to be significantly up-regulated in the brain 24 and 72 h after IP injection with pIC (Rise et al., 2010). In the current study I found that: 1) at 6HPI STAT1 was only up-regulated in fish injected with pIC at 16°C; 2) as with the IRFs at 24HPI, both pIC-injected groups showed STAT1 mRNA up-regulation; and 3) the STAT1 QPCR-detected up-regulation was more pronounced in the 10°C pIC-injected fish sampled at 24HPI (5.49-fold at 10°C vs. 2.91-fold at 16°C) (Fig. 5.8E). These results suggest that modulation of the expression of IRFs at the mRNA level could have affected the productions of type I IFN, and together with the observed STAT1 transcript expression profiles, may also have contributed to the global gene expression profiles depicted in Figures 5.5 and 5.6.

5.5.3.3 Genes with putative functions as immune effectors

The shift in the timing of the transcriptome response to pIC between fish stimulated at 10 vs. 16°C (Figs. 5.5 and 5.6) was also observed for several immune effector genes (Fig. 5.9A-E). Of the genes studied with QPCR, IL-8 transcript expression is known to be under the control of NF-κB in mammals as evidenced by marked reductions in its expression in $IKK\varepsilon^{-/-}$ mouse cells (Bao et al., 2010). In the present work IL-8 transcript was ~ 11 fold more highly expressed in the spleens of pIC injected fish held at 16°C and sampled at 6HPI as compared to 16°C PBS controls. Further, IL-8 mRNA was 91-fold up-regulated in the spleens of pIC-injected fish held at 10°C at 24HPI as compared with the time- and temperature-matched PBS controls, but only ~ 7-fold up-regulated in the spleens of pIC-injected fish held at 16°C at 24HPI as compared to time- and temperature-matched PBS controls. Two of the genes identified in the microarray study and subjected to QPCR validation are known ISGs: ISG15 and RSAD2 (alias viperin) (Liu et al, 2011). These genes were previously reported to be pIC-responsive in the Atlantic cod spleen (Rise et al., 2008) and significantly up-regulated in the brains of Atlantic cod by asymptomatic high nodavirus carrier state and IP pIC treatment (Rise et al., 2010). Thus, it was not surprising that these genes presented the same mRNA expression profiles as the IRFs (Fig. 5.9C and E). For example, ISG15 was ~ 7 fold more highly expressed in 16°C pIC injected fish compared to 10°C pIC injected fish at 6HPI and 1.51-fold more highly expressed in 10°C pIC-injected fish compared to 16°C injected fish sampled at 24HPI [Note: this latter difference was only significant in the microarray and the disagreement between the microarray and the QPCR data may be due to the fact that my primers only amplified two of the 3 known ISG15 paralogs (Furnes et al., 2009)]. RSAD2 mRNA was

~ 13 fold more highly expressed in 16°C pIC-injected fish at 6HPI as compared to 10°C pIC-injected Atlantic cod at 6HPI, and ~ 4 fold more highly expressed in 10°C pIC-injected fish at 24HPI as compared to 16°C fish at the same time point. However, unlike what was observed for IL-8, the peak fold-change in ISG15 and RSAD2 mRNA expression in pIC-stimulated fish was similar for 16°C fish at 6HPI as compared with 10°C Atlantic cod at 24 HPI.

SCYA123 (Rise et al., 2008) and SACS (Rise et al., 2010) have been previously identified as responsive at the mRNA level to pIC in Atlantic cod, and the expression of both of these genes was significantly up-regulated in the spleen of Atlantic cod injected with pIC at 10 and 16°C (Figure 5.9 A and D). However, little is known about the roles of these proteins in the anti-viral response. SCYA123 is a small inducible cytokine, and phylogenetic clustering indicates that it is most related to the CCL19 group of higher vertebrates (Borza et al., 2010). In mammals, the CCL19 chemokines are constitutively expressed and thought to only have a minor role in immunity (Borza et al., 2010). The very large sacsin protein (4579 residues) encoded by SACS possesses heat shock protein (HSP)-like ATPase domains and HSP-like activity (Anderson et al., 2010), and brain levels of SACS mRNA are up-regulated by asymptomatic high nodavirus carrier state and IP pIC treatment in Atlantic cod (Rise et al., 2010) and by ISA (infectious salmon anemia) virus infection in salmonid TO cells (Workenhe et al., 2009). Nonetheless, it is clear that these chemokine-like transcripts are responsive to pIC and other immune stimuli (e.g. formalin-killed atypical *Aeromonas salmonicida*) in the spleen of Atlantic cod (Rise et al., 2008; Feng et al, 2009; Borza et al., 2010 Booman et al., 2011), and therefore, at least in this species, that they have some role in immunity. However, further

studies are warranted to elucidate which are the anti-viral mechanisms of action of this protein.

5.5.3.4 Cellular response to stress and innate immunity

The mild increase in temperature alone did not cause up-regulation of the genes considered to be part of the conserved cellular stress response (e.g. HSP40, HSP70 and peptidyl-prolyl-isomerase; Kultz, 2005). In contrast, several chaperone-like genes were more highly expressed at 24HPI in cod injected with pIC at 16°C as compared to fish injected with pIC at 10°C. In fact, at this time point, 2 GO terms related to protein folding ("protein folding" and "unfolded protein binding") were significantly enriched in the list of genes up-regulated by pIC at 16°C when compared to those up-regulated by pIC at 10°C. In the microarray experiment, chaperone-like genes such as HSP90-alpha, HSP90-beta, HSC71, HSP47, peptidyl-prolyl-isomerase A, CCT1 and CCT6 (Table 5.3) were identified as either being induced at 24HPI by pIC in fish injected at 16°C but not in fish injected at 10°C or differentially expressed between the pIC @16°C and pIC @10°C fish at 24HPI (Table 5.3). Three of these genes (i.e. HSP90-alpha, HSP47 and CCT1) were previously identified in suppression subtractive hybridization (SSH) cDNA libraries, and QPCR-validated, as heat shock-responsive in the Atlantic cod liver (Hori et al., 2010 – Chapter 4). This suggests that even though heat alone did not elicit a robust cellular stress response, the combination of elevated temperature and pIC induced the expression of molecular biomarkers of cellular stress.

There is evidence in the mammalian literature that HSPs play a key role in modulating the innate immune response (Salminen et al., 2008). Interestingly at 24HPI,

the GO terms "regulation of interferon-gamma-mediated-signaling", "regulation of signaling process", "regulation of signal transduction", and "regulation of cytokine-mediated signaling pathway" were significantly over-represented in the list of genes up-regulated by pIC at 16°C compared to those up-regulated by pIC at 10°C. These GO terms were largely associated with probes representing HSP90s or HSP70. It is known that, at least in higher vertebrates, the cellular response to stress prevents innate immune signaling (Salminen et al., 2008), and that these effects are to some extent mediated by HSPs. Thus, it is possible that the reduced pIC induction observed for some immune-relevant genes (e.g. DHX58, PKR, STAT1, SCYA123, IL-8) in Atlantic cod held at 16°C and sampled at 24HPI may have been a result of the activation of the cellular stress response prior to this time point. However, I cannot exclude the possibility that the mRNA expression of immune relevant genes in the spleen of fish held at 16°C and injected with pIC had peaked at an early time point (i.e. between 6 and 24HPI).

5.5.4 Conclusions

In this study I used microarrays, followed by QPCR, to investigate the interaction between a moderate temperature increase and the early innate anti-pIC immune response of Atlantic cod. This is the first time that global gene expression analysis has been used to investigate the interaction between changes in environmental conditions and the antiviral immune response of this commercially important species. I was able to demonstrate that exposure to elevated temperatures, similar to those experienced by Atlantic cod in sea-cages in Newfoundland during the summer, caused a massive shift in the timing and magnitude of the early spleen transcriptome response to a viral mimic. Furthermore, I

present evidence to suggest that these changes may be, in part, regulated by changes in signal transduction (particularly in the NF-κB and IFN pathways) and by interactions between the cellular stress response and the innate immune response. Collectively, this new information enhances our understanding of the interactions between temperature and the immune system, and will be valuable in global efforts to improve cod aquaculture practices and broodstock.

5.6 References

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Chapter 6: Summary

6.1 Summary of findings

The first goal of my thesis was to gain a better understanding of Atlantic cod stress physiology. The results reported in Chapter 2 (Hori et al., 2012a) show that, like in other fish species (Fevolven et al., 1992; Tort et al., 2001; Wang et al., 2004), there are Atlantic cod with consistently different magnitudes of cortisol response [i.e. high responders (HR) and low responders (LR)] to handling stress, and that these differences are repeatable over time and when fish are subjected to heat stress. The data also indicate that there is a relatively strong inverse correlation between the magnitude of cortisol response and growth. However, it remains unclear whether cortisol responsiveness has a direct impact on growth or if this variable is related to other traits that are co-inherited with LR responsiveness. Finally, in collaboration with Dr. Andrew Robinson's research team at the University of Guelph, I was able to identify four QTL that correlate significantly with the magnitude of cortisol response; the first QTL identified for cortisol responsiveness in any fish species.

In Chapter 3 (Hori et al, 2012b), I focused on the mRNA expression of genes related to the cortisol stress axis in an effort to understand which molecular mechanisms underlie differences in Atlantic cod cortisol responsiveness to stress (i.e. LR vs. HR status). The transcript expression data showed that all three of the key genes involved in steroidogenesis that I studied (i.e. StAR, P450scc and 3βHSD) had higher constitutive expression in fish with a higher cortisol response to handling. This data strongly suggests that steroid synthesis in the interrenal tissues of Atlantic cod is a primary determinant of whether a fish is LR or HR. I also measured the mRNA expression of a glucocorticoid

receptor (GR) in these fish, since it is the main effector of cortisol induced changes in the cell (Mommsen et al., 1999). Unfortunately, there were no available sequences for any Atlantic cod GRs in public databases at the start of my study. Thus, I had to clone and further characterize a cDNA representing a cod GR-like gene before I could begin my expression work. My attempts to characterize the 5' end of this cDNA failed after several attempts. Nonetheless, I was still able to clone and sequence a 1149 bp fragment encoding a putative 237 aa fragment of an Atlantic cod GR, and phylogenetic analysis showed that this cDNA is most likely to be the ortholog of the teleost GR1. The subsequent GR expression studies showed that HR fish had higher mRNA expression of GR at three hours AS when compared to LR fish. However, whether this results in greater post-stress physiological responses in HR fish is still unclear. Research on other fish species shows that GR receptor protein levels are usually lower in individuals that have higher plasma cortisol levels (Sathiyaa and Vijayan, 2003; Aluru and Vijayan, 2007). Sathiyaa and Vijayan (2003) suggested that elevated GR mRNA levels are indicative of the negative feedback of cortisol on GR receptor density. Finally, my data suggest HR Atlantic cod maybe be able to clear more cortisol than LR fish in the same period of time, and this may limit the impact of the elevated circulating levels of this hormone.

The second goal of my thesis was to generate genomic tools to study stress physiology in Atlantic cod, and to apply these to aquaculture relevant questions. In Chapter 4 (Hori et al, 2010), as part of the Atlantic Cod Genomics and Broodstock Development Project (CGP), I exposed Atlantic cod to a three hour (8°C) heat shock, and constructed and characterized four SSH libraries from head kidney, liver and skeletal muscle [3 forward (all tissues) – enriched for transcripts up-regulated by heat shock, and

1 reverse (only liver) – enriched for transcripts down-regulated by heat shock). From these SSH libraries more than 5500 ESTs were generated, and several transcripts identified in these were subsequently validated as heat shock-responsive using QPCR. Among the most responsive genes were chaperones, such as HSP90α, HSP70, GRP78 and GRP94. These genes are good candidates for the development of molecular markers to be used in the production of elite broodstock for the Atlantic cod aquaculture industry. Moreover, these ESTs were part of the collection used to design the Atlantic cod 20,000 (20K) oligonucleotide microarray (Bowman et al., 2011; Booman et al., 2011), and helped to ensure that this platform would be valuable for investigating aspects of stress physiology and temperature stress in this species.

Finally, in Chapter 5, I used the 20K cod microarray to investigate how an increasing temperature regimen (from 10 – 16°C) affected the constitutive expression of immune-related genes, and gene expression following intraperitonial (IP) injection of the viral mimic pIC. This is an important question for the Atlantic cod aquaculture industry, given that large mortalities can be observed in the summer months on cage-sites in Newfoundland (Gollock et al., 2006), and that viral pathogens are of considerable concern to the health of both aquaculture-reared and wild fishes (Samuelsen et al., 2006; Lang et al., 2009). My global gene expression results show that: 1) although exposure to the chronic temperature increase (to 16°C) had very little effect on constitutive gene expression, it caused a massive dysregulation of the anti-viral transcriptome with fish held at 16°C exhibiting a much earlier immune response to pIC; and 2) genes with putative roles in the perception of a virus (e.g. PKR), in signal transduction following virus detection (e.g. STAT1) and as effectors of the immune response (e.g. ISG15), were

significantly differentially expressed (as measured using QPCR) at the two temperatures. These results indicate that many important immune pathways/processes were affected by the temperature increase. It remains unknown, however, how these different mRNA expression profiles correlate with survival following a viral challenge/exposure, and this is the next logical step to understanding the interaction between stress, temperature, and the disease resistance of Atlantic cod.

The research presented herein highlights the complexity of the physiological stress response, and that its impacts on fish biology and welfare are not straightforward; i.e. they may be stressor-, population-, environment- and phenotype-dependent. Furthermore, it shows that we are far from completely understanding how cortisol levels (stress) and temperature impact the health, growth and survival of the Atlantic cod. This is partially because it is not clear how changes in mRNA expression relate to those in protein expression and physiology, and that functional analyses carried out to interpret transcriptomic data in fish are largely based on the functional annotation of higher vertebrate genes. The latter is an issue as one must assume that orthology can be accurately assigned between genes from different species (taxa), and that these orthologs retained their function(s) throughout evolution.

6.2 Perspectives and future research

In spite of the extensive literature on the effects of cortisol on physiological and immunological variables in fish (Fevolden et al., 1992; Shrimpton and McCormick, 1999; Barton, 2002; Lankford and Weber, 2006; Fast et al., 2008; Hori et al., 2010; Hori et al., 2012a; Hori et al., 2012b), the consequences of acute, repeated and chronic stress are not

yet clear, and there are contrasting results with regards to the impact of cortisol responsiveness (HR vs. LR) on disease resistance (e.g. Fevolden et al., 1992; Lankford and Weber, 2006). With regards to cortisol responsiveness, there is growing evidence that: 1) as in other vertebrates, there are proactive and reactive phenotypes in fish (Koolhaas et al., 1999; Øverli et al., 2002; Øverli et al., 2006); and 2) that this may influence variability in the stress response between individuals and its impact on physiology and survival (McEwen, 1998; Korte et al., 2005).

In Chapter 2 of this thesis, I showed that fish with a higher cortisol response (HR) to handling had a lower final mass and condition factor as compared to their LR counterparts. A difference in the magnitude of the adrenal (i.e. interrenal and chromaffin) tissue response to stress is one of the defining characteristics of proactive vs. reactive phenotypes (Koolhaas et al., 1999), and thus, these findings suggest that it may be important to take into account stress coping phenotype when trying to elucidate how stress will impact an individual and/or selecting fish for aquaculture production. Further, it is unknown how the QTL markers of stress responsiveness identified in my work relate to reactive phenotype, or whether cortisol responsiveness is directly or indirectly related to the growth characteristics of Atlantic cod. Such studies should be conducted before these molecular markers are used for broodstock selection for the Atlantic cod aquaculture industry.

In Chapter 3 (Hori et al, 2012b), I showed that fish with a higher magnitude of cortisol response, had higher constitutive mRNA expression for genes involved in steroidogenesis (StAR, P450ssc and 3βHSD), but lower GR mRNA levels at 3 hours after the cessation of stress (AS). These findings suggest that the HR phenotype may be largely

due to enhanced cortisol synthesis capacity, but question the physiological impact of the elevated cortisol levels. Clearly, specific antibodies for these key proteins involved in the stress response should be developed as these would allow for protein quantification and intracellular localization studies using western blots and immunohistochemistry, respectively. Such studies could provide a more definitive answer as to how HR vs. LR status influences fish physiology and immunology. In this thesis, I contributed a partial sequence of an Atlantic cod glucocorticoid receptor-like cDNA, the full-length cDNA for StAR has been previously characterized (Goetz et al., 2004) and the recent draft of the cod genome (Star et al., 2011) has made over 20,000 putative full length transcripts available (including two putative GR-like sequences). Thus, the resources required to design new Atlantic cod antibodies for future protein expression are available. Finally, fine mapping of the QTL identified in Chapter 2 (Hori et al., 2012a) should be performed. This would allow us to determine whether they are linked to any of the steroidogenic genes studied in Chapter 3, and/or other genes directly related to the cortisol axis.

In Chapters 4 (Hori et al., 2010) and 5, I used a heat-stress experiment to construct four SSH libraries, validated several of the identified genes as heat-stress responsive using QPCR, and used the 20K cod microarray (which contains many of the genes identified in the SSH libraries) to study how elevated temperature affects the capacity of Atlantic cod to mount an immune response to a viral mimic. The data from this latter experiment indicate that even a mild, non-lethal increase in temperature (i.e. from 10 to 16°C) can shift the timing of the immune response to the viral mimic pIC at the transcriptome level. However, the results from this experiment were equivocal with regards to whether the overall magnitude of the response was also affected. Although the

overall magnitude of the response to pIC was lower for most genes in fish held at 16°C, the fish were not sampled frequently enough post-stress to ensure that I measured peak gene (mRNA) expression in the two groups. Clearly, a QPCR-based study of the immune relevant genes identified in Chapter 5 should be performed, where fish are sampled every 2 – 4 hours until 24 hours after pIC injection. Such an experiment would provide a much more comprehensive picture of how elevated temperatures affect the anti-viral transcriptome of the Atlantic cod.

As stated previously, changes in mRNA expression do not always translate into alterations at the protein level, and this highlights the importance of developing antibodies for some of the key genes identified as differentially expressed between pIC injected fish at 10 vs. 16°C; these include IκBα, STAT1, IRF1, ISG15 and IL-8. Finally, although the pIC study is very informative, the effects of temperature on immune function may be different when Atlantic cod are exposed to live pathogens (e.g. viral or bacterial). There are several viral (e.g. nodavirus; infectious pancreatic necrosis virus; viral haemorrhagic septicaemia virus) and bacterial pathogens (e.g. *Vibrio* sp.; typical and atypical *Aeromonas* sp.) that have been associated with significant losses of Atlantic cod in sea-cage sites (Samuelsen et al., 2006), and it will be essential to perform similar experiments with some of these live pathogens. Such studies have not been possible at the Ocean Sciences Centre (OSC) to date. However, a Level 3 biosafety (containment) facility is currently being constructed at the OSC, and this facility and its infrastructure/equipment, will allow for such studies to be performed.

6.3 Relevance of current findings to the Atlantic cod aquaculture industry

The worldwide decline of Atlantic cod fisheries, due to the collapse of wild stocks (Myers et al., 1997), stimulated interest in developing an aquaculture industry for this species in several countries including Norway, Iceland, Canada, Scotland and the United States (Roselund and Skretting, 2006). However, obstacles to this industry's success remain (Kjesbu et al., 2006; Roselund and Skretting, 2006), including less than optimal feed formulations, early maturation, losses associated with disease and environmental stressors, and market conditions (Bricknell et al.,, 2006; Kjesbu et al. 2006; Solsletten, 2009; Genome Atlantic, 2010).

One of the main objectives of the CGP was to improve our knowledge of how disease and stress impact the Atlantic cod. As part of this large project, I helped to identify Atlantic cod with different magnitudes of cortisol response, characterized SNPs that could be used for marked assisted selection (MAS) of stress resistance, and provided novel information on how the cortisol stress response is regulated. The CGP also made significant contributions to the genomic toolbox for Atlantic cod, including a collection of ~ 150,000 ESTs (Rise et al., 2008; Feng et al., 2009; Hori et al., 2010; Rise et al., 2010; Bowman et al. 2011), a collection of informative SNPs (Hubert et al., 2010) and a 20K oligonucleotide microarray platform (Booman et al., 2011). The work presented in this thesis contributed to all of these elements. For example, I contributed ~ 5000 ESTs enriched for heat shock responsive genes that were used both for SNP discovery and to build the 20K microarray, and I identified and validated several heat shock and viral mimic responsive genes. The latter are candidate molecular markers for resistance to changes in temperature and viral infection, and could be valuable to the Atlantic cod

aquaculture industry. Significant mortalities are associated with high temperatures at Atlantic cod sea-cages in Newfoundland (Gollock et al., 2006; Pérez-Casanova et al., 2009), and data from the Gamperl lab (Gamperl et al., unpubl.) indicates that these mortalities are not related to temperature alone.

My thesis also generated several hypotheses, and ideas for experiments, to further investigate the interactions between the Atlantic cod transcriptome, changes in the cage culture environment, and Atlantic cod stress physiology and immunology. A better understanding of these interactions, and their impact on the overall health and survival of this species is paramount to the success and profitability of the Atlantic cod aquaculture industry which relies almost exclusively on marine sea-cages for growout. Finally, the microarray data from Chapter 5 builds on previous research conducted by the CGP (e.g. see Rise et al., 2008; Rise et al., 2010), and significantly expands the catalogue of Atlantic cod anti-viral genes available for study, or as potential targets for SNP discovery and use as molecular markers of disease resistance. Such markers would be very valuable to the industry since viruses such as those belonging to the Nodaviridae family can cause great losses to cod farmers (Samuelsen et al., 2006; Lang et al., 2009).

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