CHARACTERIZATION OF AHR SIGNALING AND THE IMPACT OF POLYCHLORINATED BIPHENYLS ON THE ADAPTIVE RESPONSES TO STRESS IN FISH

by

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

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General Abstract

Persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) are widespread in aquatic systems. These toxicants bioaccumulate in the tissues of aquatic organisms, especially fish as they occupy a position near the top of the aquatic food web. Teleost fish respond to stressors, including toxicants, by activating a co-ordinated network of adaptive responses, collectively termed the integrated stress response, which allows animals to regain homeostasis. Depending on the nature of the stressor, this stress response may be a generalised endocrine response that occurs at the organismal level and/or a cellular response involving protein synthesis. The cellular response to PCB insult involves aryl hydrocarbon receptor (AhR) activation and the induction of biotransformation enzymes, including cytochrome P4501A (Cyp1A). However, little is known about the mode of action of PCBs in affecting the adaptive stress response in animals.

The objective of this thesis was to investigate the role played by AhR in mediating PCB impact on the highly conserved physiological responses to secondary stressors in fish. The experimental approach involved whole animal exposure studies with PCBs both in a laboratory setting as well as using feral fish. Also, in vitro mechanistic studies with pharmacological agents [AhR agonist (β-naphthoflavone) and antagonist (resveratrol), Hsp90 inhibitor (geldanamycin), proteasomal inhibitor (MG-132) and transcription (Actinomycin D) and translational inhibitors (cycloheximide D)] were carried out to understand AhR regulation in primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Also, a targeted trout cDNA microarray was developed as a tool to identify stress-responsive genes and signaling networks in fish.

Short-term (3 day) exposure to PCBs, while inducing liver AhR and Cyp1A expression, did not modify the adaptive plasma cortisol response to an acute handling disturbance in rainbow

trout. However, PCBs exposure did modify the metabolic response that is critical for recovery from an acute stressor in rainbow trout. To assess the impact of chronic PCB exposure on cellular stress response, two feral populations of Arctic char (*Salvelinus alpinus*) from Bjørnøya Island, Norway, were utilized. This is because the average PCB load in char liver from Lake Ellasjøen was approximately 25-fold higher than in individuals from Lake Øyangen, providing a natural setting to compare long-term toxicant impact on stress proteins. Liver Cyp1A expression was elevated in the high PCB fish suggesting AhR activation. Changes in mRNA abundance and/or protein expression of glucocorticoid receptor (GR), heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90) in fish from the high PCB lake leads to the proposal that chronic exposures to PCBs is proteotoxic to the fish.

In vitro mechanistic studies with trout hepatocytes revealed for the first time that AhR is autoregulated in response to ligand activation in rainbow trout. Furthermore this AhR regulation as well as AhR signaling involves both the molecular chaperone Hsp90 and the proteasome in hepatocytes. AhR signaling appears to play a role in the cellular response to heat shock in trout hepatocytes. Specifically, AhR signaling appears to be involved in the heat shock-induced Hsp70 and Hsp90 protein expression in trout hepatocytes. This modulation of Hsps by AhR may involve the proteasome. Overall, the results point to a cross-talk between the AhR and Hsps signaling pathways, while the precise mechanism(s) remains to be elucidated.

A targeted rainbow trout cDNA microarray was constructed as a tool to identify stress-responsive genes in trout. This custom cDNA array consisted of 147 rainbow trout genes designed from conserved regions of fish sequences available in GenBank. The targeted genes had established roles in physiological processes, including stress and immune function, growth and metabolism, ion and osmoregulation and reproduction. This targeted array revealed changes

in gene expression suggesting a rapid liver molecular reprogramming as critical for the metabolic adjustments to an acute stressor in fish. Also, transcripts not previously implicated in the stress response process in fish, including genes involved in immune function and protein degradative pathways, were found to be stress-responsive. Many of these transiently elevated stress-responsive transcripts were also shown previously to be glucocorticoid-responsive in fish implicating a key role for genomic cortisol signaling in stress adaptation.

Overall, this thesis demonstrates that PCBs impact the organismal and cellular stress response in fish. AhR autoregulation may be a key aspect of PCBs impact on the cellular stress response pathways. Hsp90 and the proteasome may be involved in AhR regulation and PCB-mediated signaling in fish. The results suggest a cross-talk between AhR and Hsp signaling pathways in fish. Finally, the targeted cDNA microarray will be a useful tool to further expand our knowledge on PCBs impact on the cellular stress signaling pathways in fish.

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Chapter 1

General Introduction

1. Introduction

Persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs) and polychlorinated biphenyls (PCBs) have been detected in almost every environment on earth (Safe, 1992). The manufacturing and use of PCBs in North America was largely banned in late 1970s. However, the highly lipophilic nature of these toxicants means that even today alarmingly high levels of PCBs persist in the environment. As water is often the final destination of environmental pollutants, aquatic organisms face an increased risk of exposure, particularly via their feed. Exposure to PCBs has been linked to a range of conditions, including reproductive toxicity, immunotoxicity, hepatotoxicity, neurotoxicity, apoptosis, necrosis, and endocrine abnormalities (Safe, 1994).

The cellular response to PCB insult is mediated primarily by the aryl hydrocarbon receptor (AhR). The AhR is a ligand activated transcription factor that exists in an inactive conformation with a suite of accessory proteins, including the molecular chaperone Hsp90 (Meyer and Perdew, 1999; Petrulis and Perdew, 2002). Characterisation of AhR regulation and signaling in mammalian systems (Pollenz, 2002) has been fairly extensive. In fact, it is generally accepted that most of the toxic effects resulting from PCB exposure are due to activation of AhR. It is not known if the same hold true for PCB impact on teleost fishes as very little is known about teleost AhR regulation and signaling. As in mammals, the primary characteristic of PCB activation of the AhR is the induction of biotransformation enzymes commonly referred to as cytochrome P450s (Cyp), and the most widely used indicator of PCBs exposure is the Cyp1A1 (Whitlock, 1999; Aluru and Vijayan, 2006). Induction of Cyp1A is thought to be a key adaptive cellular response to offset PCBs impact, but the mechanisms involved are far from clear. This chapter gives an overview of AhR and their role in PCB-mediated cell signaling, the adaptive

response to stress in animals and the impact of PCBs on this highly conserved stress response in teleost fishes.

2. The Aryl Hydrocarbon Receptor (AhR)

The AhR, found in both invertebrates and vertebrates, is an ancient protein, having evolved more than 550 million years ago (Hahn, 2002). AhR is a member of the basic helix-loop-helix Per ARNT-Sim (bHLH-PAS) superfamily of transcription factors. In vertebrates alone, the bHLH-PAS superfamily includes nearly two-dozen PAS-domain containing proteins that act as transcription factors in such processes as development, response to hypoxia, and circadian rhythms (Gu et al., 2000; Denison and Nagy, 2003). AhR was first identified as regulating the induction of cytochrome P450-dependent benzo[*a*]pyrene B[*a*]P hydroxylase (Cyp 1A1) activity (Poland et al, 1976; Whitlock, 1999).

2.1. Structural Diversity of Teleost AhR

An ever increasing number of publications describing teleost AhR structure and function have appeared in the literature over the past decade. From these reports it is obvious that AhR structure and function differs not only between fish and mammalian AhR isoforms, but also across different fish species. The most striking difference between fish and mammalian AhRs is the number of AhRs described in fish. All mammalian species examined to date express only a single AhR gene (AhR1), while the majority of fish species express at least two isoforms of AhR (AhR1 and AhR2), with some species expressing as many as five isoforms (Table 1) (Hahn et al., 2004; Hahn et al., 2006). For example, two isoforms (AhR1 and AhR2) have been identified in the killifish (*Fundulus heteroclitus*) (Karchner et al., 1999), while analysis of the complete genome of the pufferfish (*Fugu rubripes*) revealed a total of five AhR isoforms (two forms of

Table 1. Summary of AhR diversity in teleost fish.

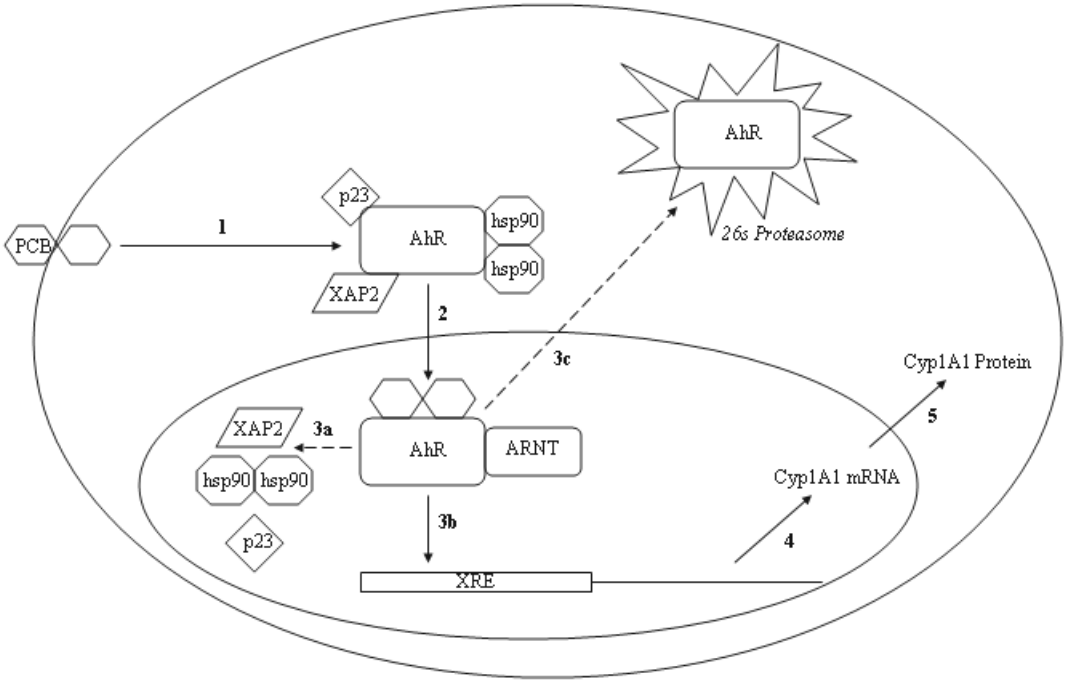
Species	AhR1	AhR2	References
Rainbow trout (*Oncorhynchus mykiss*)	-	3	Pollenz et al., 1996 Abnet et al., 1999a Hannson et al., 2003
Atlantic salmon (*Salmo salar*)	-	4	Hansson et al., 2003 Hannson et al., 2004
Atlantic Killifish (*Fundulus heteroclitus*)	1	1	Hahn et al., 1997 Karchner et al., 1999
Zebrafish (*Danio rerio*)	2	1	Tanguay et al., 1999 Andreasen et al., 2002a Karchner et al., 2005
Atlantic Tomcod (*Microgadus tomcod*)	-	1	Roy and Wirgin, 1997
European Flounder (*Platichthys flesus*)	-	1	Besselink et al., 1998
Top Minnow (*Poeciliopsis lucida*)	-	1	Hahn, 1998
Puffer Fish (*Fugu rubiripes*)	2	3	Karchner and Hahn, 2003
Medaka (*Oryzias latipes*)	2	2	Hahn, 2002

AhR1 and three forms of AhR2) (Karchner and Hahn, 2004). The Salmonids have proven especially rich in AhR diversity. In Atlantic salmon (*Salmo salar*) one isoform of AhR1 and four isoforms of AhR2 have been identified (Hannson et al., 2003). Although an AhR1 gene has not been identified in rainbow trout (*Oncorhynchus mykiss*), at least three AhR2 isoforms have been discovered (Abnet et al., 1999a; Hannson et al., 2003, 2004). Sequence comparisons revealed that rtAhR2α and rtAhR2β are 98% identical at the amino acid level (Abnet et al., 1999a). Analysis of the partial sequence of the third AhR2 isoform revealed that it has greater sequence identity to Atlantic salmon AhR2γ and AhR2δ genes than to the rtAhR2α and rtAhR2β (Hannson et al., 2003, 2004). The presence of a second AhR in teleosts is believed to be the result of a gene duplication that preceded the divergence of the fish and tetraploid lineages (Hahn et al., 1997; Karchner et al., 1999). Consequently, it has been suggested that AhR2 may exist in mammals (Hahn et al., 2006). Despite the multiple isoforms of AhRs in teleost fishes, the functional significance of this receptor diversity is still unclear.

2.2. Mechanism of Xenobiotic Stimulated AhR Signaling

The current model of AhR signaling (Fig.1) has evolved mostly from mammalian studies, although details of the mechanism of teleost AhR signaling are emerging and tend to support the mammalian model (Whitlock et al., 1996; Pollenz, 2002; Song and Pollenz, 2003; Wentworth et al., 2004). Unbound AhR resides either in the cytosol or the nucleus in a complex with a suite of accessory proteins, including the immunophilin XAP2 (X-associated protein 2), the 23 kDa protein p23, and two molecules of the molecular chaperone heat shock protein 90 (Hsp90) (Meyer and Perdew, 1999; Meyer et al., 2000; Petrulis and Perdew, 2002). Chaperone proteins appear to play several roles in the AhR heterocomplex, including masking of the NH-terminal nuclear localization signal, holding the receptor in a ligand receptive conformation, and

Figure 1. Aryl hydrocarbon receptor (AhR)-mediated transcription of xenobiotic metabolizing enzymes. (1) Polychlorinated biphenyls (PCBs) pass through the cell membrane due to their lipophilicity and bind to the cytosolic AhR, which exists as a complex with XAP2, p23, and two molecules of Hsp90. (2) The PCB bound AhR complex translocates to the nucleus and forms a dimer with aryl hydrocarbon nuclear translocator (ARNT) leading to (3a) dissociation of the chaperone proteins. (3b) The PCB-AhR-ARNT complex binds to xenobiotic responsive elements (XRE) in the promoter of target genes, such as the Cyp1A gene modulating (4) transcription and ultimately leading to the (5) expression of Cyp1A1 protein. (3c) Following activation of gene transcription the AhR is degraded via the cytosolic 26S proteasome.


AhR
26s Proteasome
p23
PCB
1
AhR
hsp90
hsp90
XAP2
2
3c
Cyp1A1 Protein
5
XAP2
3a
AhR
ARNT
hsp90
hsp90
Cyp1A1 mRNA
3b
4
p23
XRE

masking ubiquination sites and, thereby, preventing pre-mature proteolysis (Pollenz, 2002). Ligand binding to the AhR stimulates a conformational change in the heterocomplex resulting in nuclear translocation and/or retention of the ligand bound heterocomplex, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), itself a member of the bHLH-PAS superfamily of transcription factors. Subsequently, Hsp90 and other chaperones disassociate from the AhR-ARNT heterodimer and the transcriptionally active complex is free to bind specific regulatory elements, known as xenobiotic response elements (XREs) - also known as dioxin response elements (DRE) or AhR response elements (AhRE) - in the promoter or enhancer of target genes and activate/suppress transcription (Whitlock, 1999). The consensus sequence of the mammalian XRE has been identified as 5'-TNGCGTG-3' (Whitlock, 1999), with the sequence 5'-CGTG-3' absolutely necessary for binding of the AhR-ARNT heterodimer (Chen and Whitlock, 1992; Yao and Denison, 1992). Termination of the AhR signal occurs when the AhR is exported from the nucleus and subjected to 26S proteasome degradation (Davarinos and Pollenz, 1999; Roberts and Whitlaw, 1999; Pollenz, 2002, Song and Pollenz, 2002).

2.3. Regulation of AhR Levels and Signaling

Several mechanisms are thought to regulate AhR mRNA and protein levels and the AhR signaling pathway, including transcriptional and post-transcriptional regulation, the nature of the AhR ligand and regulation of ligand binding, protein-protein interactions, genetic factors, endocrine factors, and the proteasome (Carlson and Perdew, 2002; Harper et al., 2006). The phosphorylation state of AhR and its accessory proteins may play a key role in regulation of the AhR signaling pathway as all of the proteins in the AhR complex are phosphoproteins (Carlson and Perdew, 2002). Berghard et al. (1993) suggested that phosphorylation of one of the AhR

complex proteins, possibly by protein kinase C (PKC), was necessary for AhR binding to the enhancer regions of the Cyp1A1 gene. More recently, it has been demonstrated that phosphorylation of tyrosine residues in the NH-terminal domain of AhR is required for transactivation (Park et al., 2000). The transactivation of genes by AhR is also controlled by cAMP (Oesch-Bartlomoxicz et al., 2005), albeit via a mechanism that is different from xenobiotic stimulation. Activation by cAMP actually impairs dioxin activation of the AhR, possibly because the conformation of the cAMP activated AhR differs from xenobiotic activated AhR, preventing xenobiotics binding or formation the AhR-ARNT heterodimer.

Proteasomal degradation of transcription factors is an established mechanism of regulating signal transduction pathways (Pahl and Baeuerle, 1996). A number of studies in both mammalian systems (Davarinos and Pollenz, 1999; Pollenz, 2002, Song and Pollenz, 2002) and in zebrafish (Wentworth et al., 2004) have demonstrated that 26S proteasomal degradation of AhR plays an important role in regulating AhR stability and signaling. Termination of AhR signaling occurs when the receptor is exported from the nucleus and subjected to proteasomal degradation in the cytoplasm (Pollenz, 2002). Studies have shown that the interaction between the various AhR associated chaperone proteins plays an important role in determining the fate of the AhR. Loss of Hsp90 or changes in the conformation of the heterocomplex upon ligand binding appears to destabilise AhR leading to recognition by the 26S proteasome (Pollenz, 2002). The importance of the Hsp90 association with the AhR heterocomplex is demonstrated by the finding that treatment of cells with geldanamycin (GA), a benzoquinine ansamycin that interacts directly with the ATP/ADP binding site of Hsp90 and disrupts interactions between Hsp90 and associated proteins (Chen et al., 1997; Grenert et al., 1997), promotes proteasomal degradation of ligand-free AhR (Ma and Baldwin, 2000; Song and Pollenz, 2002, 2003; Wentworth et al., 2004). Conclusive evidence for the importance of the 26S proteasome in the

regulation/degradation of both mammalian and zebrafish AhR comes from the finding that the proteasomal inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132) reduces both GA and ligand mediated proteasomal degradation (Ma and Baldwin, 2000, Pollenz, 2002; Wentworth et al., 2004; Pollenz and Buggy, 2006). Proteasomal degradation of AhR protein has been proposed to be important for both removal of misfolded and abnormally localized protein and the removal of protein after it has been recruited to the nucleus and carried out transcriptional activation of target genes (Pollenz et al., 2005).

Xenobiotic stimulated AhR signaling is also regulated by the AhR repressor (AhRR). First identified in mammals (Mimura et al., 1999), the presence of a nuclear AhRR has also been confirmed in teleost fish (Karcher et al., 2002). Due to the presence of a XRE in the promoter region of the AhRR, expression of this gene is controlled by AhR in a ligand-dependent manner. The AhRR competes with the AhR for binding to the ARNT, and ultimately AhRR-ARNT heterodimers competes with AhR-ARNT heterodimers for binding to XREs, thereby acting as a negative regulator of AhR-responsive gene expression (Mimura et al., 1999; Karchner et al., 2002).

2.4. Functional Diversity of AhR Signaling Pathways

Despite the diversity of AhR forms the structure of the protein is fairly well conserved across species. In all AhR proteins characterised to date the highest degree of conservation is found in the bHLH and PAS regions of the N-terminal domain. The diversity in AhR structure is localized to the C-terminal regions of the protein. Fish AhR2s either lack or have a reduced glutamine (Q)-rich transactivation domain that is found in the killifish AhR1 (Karchner et al., 1999) and mammalian AhR1. The AhR2s also have a modified version of the LXCXE motif compared to AhR1. In mammals, this motif mediates interactions of the AhR with the product of

the retinoblastoma tumor suppressor gene. The functional significance of this modification is not yet clear in fishes (Puga et al., 2000).

Functional differences between AhR forms in mammals and teleosts as well as across different teleost species exist both at the level of ligand binding and transcription. For example, AhR-dependent activation of target gene transcription (Abnet et al., 1999b) and toxicity (Walker and Peterson, 1991) in response to mono-ortho substituted PCB exposure is weaker in fish than in mammals. This observation has been attributed to differences in the intrinsic efficiency for activation, not affinity, of the AhR by these ligands (Hestermann et al., 2000). Despite the missing or reduced Q-rich transactivation domain, fish AhR2s retain strong transactivation function (Pollenz et al., 2002). In fact, most full-length fish AhR1 and AhR2 forms are transcriptionally active when expressed in mammalian cells (Abnet et al., 1999b; Pollenz et al., 2002; Karchner et al., 2002). For example, zebrafish AhR1A appears inactive while both AhR1B and AhR2 bind ligands with high affinity and activate transcription in transient transfection assays (Andreasen et al., 2002a, Karchner et al., 2005). The endogenous role for AhR1B remains to be elucidated as morpholino studies have shown this isoform does not play a role in mediating classical responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Mathew et al., 2006). A comparison of rainbow trout AhR2α and AhR2β forms also reveals functional differences. Despite the high degree of identity, AhR2α shows a 10-fold higher enhancer specific transactivation activity when transiently expressed in mammalian cells (Abnet et al., 1999b), a difference that has been linked to amino acid 111 of AhR2α (which corresponds to amino acid 110 of AhR2β) (Andreasen et al., 2002b).

AhR signaling pathways are defined as either adaptive or toxic (Schmidt and Bradfield 1996). Designation of the adaptive response is based on the observation that all XRE containing genes encode enzymes important for the metabolism of xenobiotics. Evolution and maintenance

of the adaptive response is believed to be important in minimizing the deleterious impacts of xenobiotics on important cellular processes. Ironically, designation of a toxic response pathway is based on the observation that many of the metabolites of xenobiotic metabolism impose oxidative stress. An important milestone in the understanding of this toxic response pathway came with the development of AhR knockout mice (Fernandez-Salguero et al., 1996). Based on studies in these mice it is widely accepted that the AhR/ARNT pathway mediates TCDD toxicity as experiments have shown that nearly all signs of TCDD toxicity are missing in AhR-null mice (Fernandez-Salguero et al., 1996; Mimura and Fujii-Kuriyama, 2003). Morpholino studies have shown that knockdown of either zfAHR2 or zfARNT1 blocks dioxin toxicity in zebrafish (Carney et al., 2004; Dong et al., 2004; Antkiewicz et al., 2006; Prasch et al., 2003, 2004, 2006, Mathew et al., 2006).

Expression of a single AhR gene in mammals suggests that the mammalian AhR1 mediates both adaptive responses to PCB exposure, such as the induction of xenobiotic metabolizing enzymes, as well as physiological responses to PCB exposure, such as the regulation of cell growth and differentiation (Schmidt and Bradfield, 1996). However, the evolution of AhR2 in teleosts has led to the speculation that the responses to PCB exposure may be isoform specific. Specifically, it has been suggested that AhR1 regulates physiological processes and AhR2 regulates adaptive processes (Hahn, 2001). However, as AhR1 has not been identified in all fish species, including rainbow trout, the applicability of this designation to all teleost systems is still speculative.

2.5. Cross-Talk Between AhR and Cell Signaling Pathways

The AhR is best understood for its role in mediating the response to PCB exposure, most notably the Cyp1A1 response (Whitlock, 1999; Aluru and Vijayan, 2006). However, AhR

signaling also influences and is influenced by a number of cellular signaling pathways. For example, the AhR interacts with signaling pathways activated by the estrogen receptor (ER), progesterone receptor (PR), hypoxia inducible factor (HIF), nuclear factor kappaB (NFκβ) and retinoblastoma protein (Carlson and Perdew, 2002). The potential for crosstalk between AhR and glucocorticoid receptor (GR) signaling pathways has also been proposed (Vijayan et al., 2005). This crosstalk is largely attributed to similarities in the chaperone proteins, such as Hsp90, and shared dimerization partners, such as ARNT. Direct interactions between AhR and the effector protein of the pathway in question as well as sequence similarity of *cis*-acting elements in enhancer and promoter regions of target genes have also been suggested as potential sites of crosstalk (Carlson and Perdew, 2002; Vijayan et al., 2005; Lee et al., 2006). The example of crosstalk between the AhR and the hypoxia signaling pathway is illustrated below and is given simply to illustrate the multiple levels at which crosstalk between AhR and other signaling pathways may occur.

2.5.1. Cross-Talk Between AhR and Hypoxia Signaling Pathways

The hypoxia responsive signaling pathway is activated by the Hypoxia Inducible Factor 1α (HIF1α). In response to hypoxia, HIF1α forms a heterodimer with ARNT (also know as HIF1β), and this complex modulates the expression of a suite of hypoxia responsive genes. The molecular mechanisms that regulate cross-talk between these two pathways are largely unknown, however, several reports have shown that exposure to AhR agonists, including TCDD, impairs the hypoxia response signaling pathway (Chan et al., 1999; Pollenz et al., 1999; Prasch et al., 2003; Hofer et al., 2004). Interestingly, activation of the AhR signaling pathway does not inhibit the hypoxia-signaling pathway (Prasch et al., 2003; Hofer et al., 2004). The unidirectional nature of this cross-talk may be due to sequestering of ARNT/HIF1β by HIFα during hypoxia,

thereby limiting the pool of free ARNT available for heterodimerization with AhR in response to agonist exposure. However, evidence from several lines of research suggests that ARNT is not a limiting factor and therefore may not act as the lone point of cross-talk between these pathways (Pollenz et al., 1999; Prasch et al., 2003; Lee et al., 2006). Global gene expression analysis and analysis of the regulatory regions of potential targets of AhR/HIF1β cross-talk suggested that co-factors other than ARNT, including response elements and promoter sequences may also play an important role (Lee at al., 2006). Due to the core sequence similarity of the XRE and hypoxic responsive element (HRE), cross-talk between these pathways may occur when AhR and HIF1α compete for the same regulatory sequences (Hogenesch et al., 1997).

2.5.2. The Cyp1A Response

As illustrated in figure 1, upon entry into a cell PCBs bind to and activate the AhR, ultimately modulating transcription of XRE containing target genes. This specific cellular response to PCBs is exemplified by the induction of Cytochrome p450's (Cyp), namely Cyp1A1 (Whitlock et al., 1996, Whitlock, 1999; Pollenz, 2002; Aluru and Vijayan, 2006). Cyp1A1 expression is silent under "resting" conditions but transcription occurs within minutes of exposure to AhR ligands (Whitlock et al. 1996). The induction of Cyp1A1 and XRE containing phase I detoxification enzymes is an adaptive response as these enzymes oxidize PCBs to a more water soluble form. Ironically, the resulting metabolite is often more reactive than the parent compound, increasing the risk of cellular toxicity. Fortunately, the products of phase I metabolism are suitable substrates for phase II enzymes that convert the substrates into readily excreted non-toxic metabolites (Schlenk and Di Giulio, 2002).

Induction of Cyp1A1 is undoubtedly the most studied adaptive response to PCB exposure. In teleost fishes Cyp1A1 is inducible in a variety of tissues, including liver, heart, gill,

intestine, gonad, brain, and spleen, and is detectable at the mRNA, protein, and enzymatic activity level (Bello et al., 2001; Schlenk and Di Guilio, 2002). As Cyp1A1 is silent in non-exposed individuals it is often used as a biomarker of PCB exposure (Schlenk and Di Guilio, 2002; Wirgin and Theodorakis, 2002). However, caution must be taken when utilising Cyp1A1 as a biomarker as number of the factors impact the Cyp1A response. Quantification of Cyp1A1 protein expression and catalytic levels is most commonly employed in biomarker studies. Measurement of mRNA abundance may be advantageous as transcription of Cyp1A1 occurs rapidly with xenobiotic activation of AhR and may provide more accurate dose-related responses than protein and catalytic measurements. In addition, substrate inhibition of Cyp1A1 catalytic activity can be misinterpreted as indicative of low levels of gene expression (Wirgin and Theodorakis, 2002).

Wild fish may be exposed to AhR ligands either alone or in mixtures with other environmental contaminants. Cyp1A1 expression is known to be impacted when animals are exposed to mixtures of individual AhR ligands. Weak AhR agonists may decrease or inhibit stimulation of Cyp1A1 expression by strong AhR agonists, such as B[a]P and TCDD (Hestermann et al., 2000; Wirgin and Theodorakis, 2002). Several studies have shown that a variety of metals can attenuate the Cyp1A1 response (Wirgin and Theodorakis, 2002). Natural factors such as temperature also impact Cyp1A1 expression. In one study the magnitude of the Cyp1A1 response was not impacted by temperature but the half-life of the Cyp1A1 transcript was greater in cold-water acclimated fish than in warm-water acclimated fish (Kloepper-Sams and Stegeman, 1992).

The utility of Cyp1A1 as a biomarker of PCB exposure is also complicated by several endogenous factors (Wirgin and Theodorakis, 2002). The observation that gender and reproductive status may be related to observation that estradiol (E2) E2 may impair AhR

mediated transcription of Cyp1A1 (Williams et al., 1998; Elskus, 2004). Nutritional status may impact Cyp1A1 as increased mobilization of fat reserves in fasted PCB exposed fish ultimately increases hepatic PCB load (Jorgensen et al., 1999). Genetic factors may also impact the Cyp1A1 response. Several populations of different teleost species have acquired resistance to xenobiotic compounds. For example, Cyp1A1 expression is impaired in killifish and Atlantic tomcod from highly polluted waters containing a variety of xenobiotic AhR ligands (Wirgin and Theodorakis, 2002). Primary cultures of hepatocytes from New Bedford Harbour killifish are 14-fold more resistant to TCDD than hepatocytes from a reference population (Bello et al., 2001). Several mechanisms may be responsible for insensitivity of these populations to AhR agonists including point mutations in the AhR receptor or alterations in components of the AhR signaling pathway, including the aryl-hydrocarbon receptor nuclear translocator (ARNT) (Wirgin and Theodorakis, 2002). Hahn et al. (2004) reported that AhR alleles identified in the coding regions of the Atlantic Killifish AhR1 locus differ in frequency between populations from contaminated and non-contaminated waters such that individuals from polluted areas have specific alleles that are underrepresented. It is possible that mutations in the promoter region of Cyp1A1 could play a role in observed insensitivities (Bello et al., 2001). Reduced sensitivity to AhR agonists may also be due to reduced levels of AhR as it has been shown that AhR is down-regulated via the proteasome following exposure to AhR agonists (Davarinos and Pollenz, 1999; Pollenz, 2002; Wentworth et al., 2004). Finally, presence of the recently discovered AhRR (Mimura et al., 1999) in these resistant populations may also play a role in this phenomenon.

Although the induction of Cyp1A1 is a specific adaptive response to PCB exposure there is evidence to suggest that this cellular stress response pathway may be influenced by the generalised stress response (below), mainly via the actions of cortisol. Exposure to glucocorticoids such as cortisol, or synthetic glucocorticoids such as dexamethasone, fails to

stimulate Cyp1A1 expression (Celander et al., 1996). However, in combination with AhR agonists the Cyp1A1 response may be potentiated (Devaux et al., 1992; Celander et al., 1996; Quabius et al., 2005) and this potentiation may be mediated by the glucocorticoid receptor (GR) (Celander et al., 1996). However, in Arctic Char neither cortisol implants nor handling stress potentiated Cyp1A protein expression (Jorgensen et al., 2001).

3. The Stress Response

Hans Selye defined stress as "the non-specific response of the body to any demand made upon it" (Selye, 1973). However, this definition was more accurately defining the stress response - an adaptive response mechanism that allows the fish to recover homeostasis when faced with either real or perceived stressors that threaten homeostasis (Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al., 2006). Simply put, stress can be defined as a state of threatened homeostasis and the stress response is the complex network of biochemical and molecular processes that are vital to coping with the demands placed on the organism by the stressor (either chemical, physical, or perceived) and allow the organism to re-establish homeostasis (Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al., 2006).

Organisms inhabiting the aquatic environment are routinely faced with a plethora of potential stressors – natural or anthropogenic factors that harbour the potential to stimulate a stress response. Teleost fish have evolved numerous behavioural, physiological and molecular strategies to help the organism adapt to and counteract the effect(s) of these stressors. Collectively, these strategies are termed the stress response and can be broadly divided into the cellular stress response and the whole-organism or generalised stress response.

3.1. The Generalized Stress Response

At the organismal level, the stress response can be divided into primary, secondary, and tertiary responses (Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al., 2006). The primary response is invoked when the stressor elicits neuroendocrine stimulation of chromaffin cells and the hypothalamic-pituitary-interrenal (HPI) axis to release catecholamine and corticosteroid hormones, respectively. The secondary stress response is characterized by alterations in respiratory, circulatory and immune function, as well as changes in hydromineral balance and energy metabolism. This stage of the stress response is characterized by an increase in circulating levels of plasma glucose and lactate. These changes are largely dependent upon neuroendocrine involvement in the primary response, and are influenced by the intensity and duration of the stressor (Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al, 2006). The tertiary stress response extends to the level of the whole organism and population, referring to inhibition of growth, reproduction, immune function, and reduced capacity to tolerate subsequent or additional stressors (Wendelaar Bonga, 1997).

Recognition of real or perceived stressors by the central nervous system (CNS) stimulates the release of neuroendocrine factors – catecholamines and corticosteroids - that characterise the primary stress response. Sympathetic nerve fibres which innervate chromaffin cells located in the anterior region of the teleost kidney stimulate the rapid release of catecholamines, primarily epinephrine, resulting in an immediate increase in circulating levels (Randall and Perry, 1992; Reid et al., 1998). The release of catecholamines is important for short-term metabolic adjustments such as the stimulation of glucose production by glycogenolysis. The glucose released from the breakdown of glycogen enters into circulation and is distributed to peripheral tissues where it is used to fuel the increased energy demand associated with stress (Wendelaar Bonga, 1997).

The release of cortisol, the predominant circulating corticosteroid in teleosts, is delayed relative to the release of catecholamines. Secretion of cortisol into circulation involves a series of steps along the HPI axis beginning with the release of corticotrophin-releasing factor (CRF) from the hypothalamus. CRF stimulates the anterior pituitary to release adrencorticotropin hormone (ACTH). ACTH released into circulation stimulates the interrenal cells of the head kidney to synthesise and release corticosteroids, including cortisol, into circulation for distribution to target tissues (Mommsen et al, 1999). Regulation of cortisol levels in circulation is accomplished by a negative feedback control of the hormone at all levels of the HPI axis (Mommsen et al., 1999). The duration of the cortisol response to stress is influenced by the nature and duration of the stressor (Wendelaar Bonga, 1997; Barton et al., 2002). Plasma cortisol levels are transiently increased by acute stressors but remain elevated in response to chronic stressors.

Stress-induced elevation of circulating cortisol levels either directly and/or indirectly affect intermediary metabolism and this response is thought to be critical for stress adaptation (Vijayan et al., 1994; Mommsen et al., 1999). In liver, cortisol stimulates gluconeogenesis by induction of phospho*enol*pyruvate carboxykinase (PEPCK) activity by directly increasing gene transcription (Hanson and Reshef, 1997). Cortisol stimulates gluconeogenesis from amino acid precursors by increasing protein catabolism and increasing aminotransferase activity, namely aspartate aminotransferase (AspAT), alanine aminotransferase (AlaAT) and tyrosine aminotransferase (TAT). Cortisol also plays a role in the regulation of lipid metabolism. Although a clear picture of this effect has yet to emerge the primary effect appears to be stimulation of lipolysis, providing fatty acids as substrates for oxidation and glycerol for gluconeogenesis (Mommsen et al., 1999). These metabolic effects of cortisol highlight the role this stress steroid plays in maintenance of plasma glucose levels and the replenishment of stored glycogen. Cortisol also plays a role in modulating the expression of glycolytic genes as

evidenced by increased transcript abundance of glucokinase (GK) and pyruvate kinase (PK) (Vijayan et al, 1997a; Mommsen et al, 1999; Panserat et al, 2001; Dziewulska-Szwajkowska et al, 2003).

The actions of cortisol are mediated by GR (Ducouret et al., 1995). Much like the AhR, mammalian GR, in the unbound state, resides in the cytosolic heterocomplex with several accessory proteins, including two molecules of Hsp90 (Pratt, 1997). The presence of Hsp90 in this heterocomplex is essential for cortisol binding to the ligand-binding domain of GR (Pratt and Toft, 1997). While the structure of the teleost GR heterocomplex has yet to be elucidated, it is likely that are similar to that seen in mammalian system, including a similar GR-Hsp90 complex also exists in fish (Mommsen et al., 1999; Sathiyaa and Vijayan, 2003; Vijayan et al., 2005). In fact, evidence from ^{3}H-cortisol binding studies have shown that the molecular mass of several teleost GR heterocomplexes is only slightly different from the 330 kDa size of the mammalian GR complex (Mommsen et al., 1999). Additionally, Sathiyaa and Vijayan (2003) have shown that geldanamycin inhibits cortisol-stimulated increases in GR mRNA abundance, suggesting that Hsp 90 is an important component of the GR signaling mechanism in rainbow trout.

Recently, progress has been made in understanding GR regulation during the stress response. Elevation of plasma cortisol levels decrease hepatic GR protein but increase mRNA abundance (Pottinger, 1990; Lee et al., 1992; Vijayan et al., 2003). Sathiyaa and Vijayan (2003) demonstrated that GR protein content is regulated via a negative feedback loop involving GR autoregulation. Essentially, increased cortisol levels decrease GR protein content by proteasomal degradation and this coincides with an increase in GR mRNA abundance. This autoregulation of GR protein content is thought to be critical for maintaining the target tissue responsiveness to cortisol action (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003).

3.1.1. PCB Exposure impacts the Cortisol Response to Stress

In teleost fish the cortisol response to stress is impacted by a variety of environmental pollutants, including PCBs (Quabius et al., 1997; Vijayan et al., 1997b; Hontela et al., 1998; Hontela, 2005). PCBs may act at multiple sites along the HPI axis to weaken the stressor stimulated cortisol response. Proposed mechanisms of this effect include decreased sensitivity of the interrenal cells to ACTH (Hontela, 2005; Wilson et al., 1998; Aluru et al., 2004), abnormal negative feedback regulation of cortisol release (Aluru et al., 2004), altered cortisol clearance (Mommsen et al., 1999), and inhibition of corticosteroidogenesis (Aluru et al., 2005). Activation of AhR signaling by the AhR agonist β-napthoflavone (βNF) impairs ACTH stimulated cortisol synthesis by decreasing mRNA abundance of steroidogenic acute regulatory protein (StAR) and cytochrome P450 cholesterol side chain cleavage (P450scc), key rate-limiting enzymes in the cortisol biosynthesis pathway (Aluru and Vijayan, 2006).

In addition to impacting the cortisol response to stress, PCBs impact protein expression of GR (Vijayan et al., 1997b; Jorgensen et al., 2002; Aluru et al., 2004, Vijayan et al., 2005). For example, in rainbow trout given an injection of 3,3',4,4' tetrachlorobiphenyl (TCBP), a decrease in hepatic GR levels was not seen despite a 3.5-fold increase in plasma cortisol levels (Vijayan et al 1997b), suggesting that PCB exposure modulated GR content to prevent cortisol-mediated GR down-regulation. The environmentally relevant PCB mixture Aroclor 1254 decreases fasting-induced increases in brain GR content in Arctic charr (*Salvelinus alpinus*). The decrease in brain GR protein levels seen in these fish also disrupted the negative feedback inhibition of cortisol secretion (Jorgensen et al., 2002; Aluru et al., 2004). The mechanism(s) by which PCBs exert their effects on GR are not known but possible cross-talk between AhR and GR signaling pathways cannot be ruled out (Vijayan et al., 2005). Overall, the adaptive

responses elicited by animals to stressor exposure is critical for re-establishing homeostasis, while the mode of action of PCBs in modulating this response is still unclear in teleost fishes.

3.2. The Cellular Stress Response

How organisms respond to stress is largely dependent upon the nature of the stressor and the threat it poses. The cellular stress response is a network of transient responses to any stressor that threatens or disrupts cellular homeostasis (Kultz, 2005). Depending on the nature and impact of the stressor the cellular stress response may be non-specific or specific in nature. The most distinguishable feature of the non-specific cellular stress response is the induction of heat shock proteins (Hsps) as a response to any stressor that threatens macromolecule homeostasis, in particular protein homeostasis. The stressor-specific response, termed the cellular homeostasis response, is elicited by activation of stressor specific sensors that monitor changes in environmental variables. A good example of stressor-specific response is the induction of Cyp1A proteins in response to PCB stimulation. This response persists until strain imposing environmental conditions change in order to restore cellular homeostasis (Kultz, 2005).

3.2.1. Heat Shock Proteins

The control and maintenance of protein homeostasis, including protein synthesis, folding and degradation, is vital to cell survival. Each of these processes is dependent upon numerous cellular factors, including Hsps. These evolutionary conserved proteins are represented by several families that when classified by molecular weight (measured in kilodaltons - kDa) are termed Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsps below 30 kDa. Hsps act as ATP-dependent molecular chaperones that bind non-native proteins, aid in protein translocation, inhibit protein aggregation and promote attainment and maintenance of the native state through

interaction with hydrophobic amino acids (Parsell and Lindquist, 1991; Morimoto, 1998; Nollen and Morimoto, 2002). These proteins play important roles during normal physiological processes including cell cycle, embryo development, cell differentiation, and spermatogenesis (Nollen and Morimoto, 2002). Hsps are also critical to cope with proteotoxicity and also to protect cells from subsequent stressors (Hightower, 1991).

Undoubtedly, the Hsp70 family is the most extensively studied. The Hsp70 family includes the constitutive Hsp70, also known as Hsc70, and the stress inducible Hsp70 (Huang et al., 2001; Mayer and Bukau, 2004; Kampinga, 2006). Both Hsp70 and Hsc70 play important housekeeping roles including folding of newly synthesised polypeptides, refolding of denatured, misfolded and aggregated proteins, protein translocation, and controlling the activity of signal transduction pathways such as transcription factors and protein kinases (Mayer and Bukau, 2004). Despite being viewed as functionally interchangeable with one another Hsc70 and Hsp70 are functionally different (Gething and Sambrook, 1992). The most obvious difference occurs at the level of gene expression. Hsc70, as the name implies, is constitutively expressed in both non-stressed and stressed cells, whereas Hsp70 is an inducible protein expressed in stressed cells (Gething and Sambrook, 1992). However, Hsc70 expression is impacted by a variety of conditions, including exposure to cadmium (Hung et al., 1998), ischemia (Kawagoe et al., 1993), hypoxia (Turman et al., 1997), and heat shock (Dressel et al., 1998). In teleost fish increased expression of hsc70 in response to heat shock (Santacruz et al., 1997) and chronic cortisol stimulation (Vijayan et al., 2003) has been reported. However, other studies have reported that hsc70 expression in fish cells does not change in response to heat shock (Zafarullah et al., 1992; Boone and Vijayan, 2002b), metals (Zafarullah et al., 1992), or oxygen limitation (Currie et al., 1999).

Hsp90 is an ATP dependent phospho-protein that plays a vital role in cellular housekeeping (Picard, 2002). In fact, Hsp90 is one of the most abundant proteins in eukaryotic cells, representing 1-2% of the total cellular protein pool. In zebrafish (Krone and Sass, 1994) two isoforms of the Hsp90, designated Hsp90α and Hsp90β and which are 90% identical at the amino acid levels, have been identified. During periods of "non-stress" the expression of Hsp90β is greater than that of Hsp90α; however Hsp90α expression increases in response to heat shock. Levels of Hsp90 may increase up to 10-fold in response to proteotoxic stress. Relatively little is known about the impact of potential stressors on expression of Hsp90 compared to Hsp70. Studies have shown that cortisol administration decreases hsp90 mRNA abundance (Sathiyaa et. al., 2001).

In contrast to Hsp70, Hsp90 is not essential for de novo protein synthesis. However, it can bind to peptides, prevent protein aggregation and function in protein-folding reactions, albeit in a limited capacity (Caplan, 1999). Hsp90 plays a vital role in the maturation, stabilization, and activation of a number of substrate proteins, including protein kinase and transcription factor heterocomplexes (Picard, 2002). Involvement of Hsp90 in the maturation of protein heterocomplexes is highly dependent on interactions with co-chaperones, including Hsp70.

Enhanced expression of Hsps is regulated by a group of transcription factors known as heat shock factors (HSFs). In mammalian systems HSF1 is responsible for stressor induced Hsp expression while HSF4 regulates developmental expression of Hsps (Nollen and Morimoto, 2002). In the unstressed state HSFs reside in the cytoplasm as monomeric units. Disruption of protein homeostasis stimulates formation of HSF1 trimers that bind to heat shock elements (HSE) in the promoter of Hsp genes and stimulate their transcription. Function of HSF is dependent on interactions with Hsp90 and the disruption of Hsp90-HSF interaction by GA treatment, increases expression of Hsps, most notably Hsp70 (Caplan, 1999).

A suite of environmental contaminants, including AhR ligands, impact the expression of Hsps, including Hsp70 and Hsp90 in a number of fish species (Vijayan et al., 2005). Several studies have reported increased Hsp70 protein expression with PCB exposure (Vijayan et al., 1997b; 1998, Janz et al., 1997; Weber and Janz, 2001; Weber et al., 2002). Although little information exists pertaining to the impact of PCBs on Hsp90, Aluru et al. (2004) did show that 5 month fasted char exposed to PCB had decreased expression of brain Hsp90 protein. At this time it is unknown whether the Hsp response to PCB exposure is mediated by AhR activation and/or due to the or is the result of oxidative damage caused by the metabolism of xenobiotics compounds by phase 1 enzymes, such as Cyp1A1. Overall, the highly conserved cellular stress response is modulated by PCBs but the mode of action remains to be determined.

4. Genomic Tools for Identifying Stress-Responsive pathways

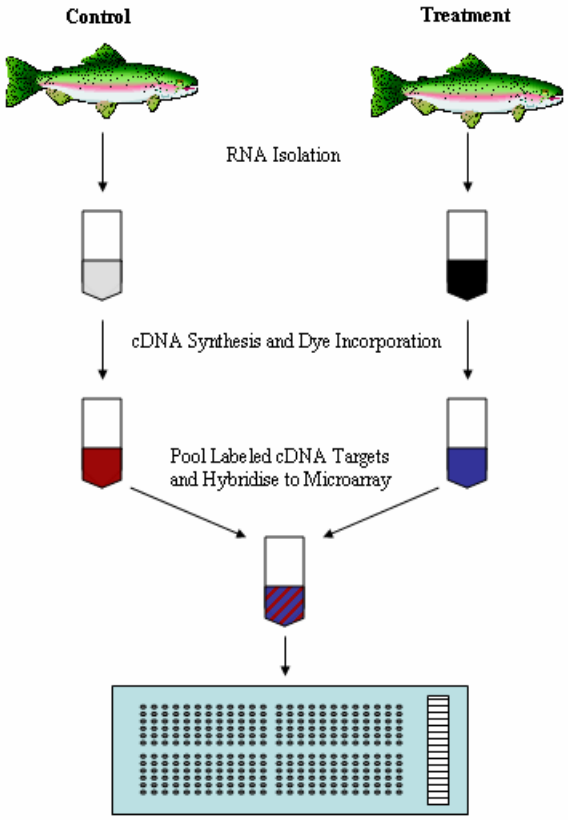
The above sections outlined some of the key players involved in the cellular stress response. However, it is likely that there are other pathways critical for allowing animals to cope with stress but not yet characterized in fish. Until recently, studies of gene regulation in physiology research has been dominated by a hypothesis-driven reductionist approach that attempted to explain physiological responses by understanding functional properties of individual genes (Gracey and Cossins, 2003; Strange, 2007). However, as organisms are much more than the sum of their parts, complex physiological processes cannot be understood by studying how the parts work in isolation. Analysis of the entire transcriptome through large-scale genomic approaches signifies a shift from the conventional hypothesis-driven approach (Brown and Botstein, 1999). These large-scale genomic approaches are often described as discovery-driven or hypothesis generating research (Gracey and Cossins, 2003) and offer views of molecular integration well beyond that achievable via a reductionist approach.

Microarrays are an efficient and powerful tool to monitor changes in expression of a large number of genes simultaneously. In contrast to the northern blot method in which the target mRNA is immobilized onto the solid phase and detected with a labelled liquid phase probe, in the cDNA microarray method (Fig. 2) the cDNA probe is immobilized onto the solid phase, usually a specially coated glass slide and the target molecule is labelled and suspended in the liquid phase. The cDNA probes may be generated by PCR amplification of target genes with either gene specific primers or plasmid specific primers in cases where target genes are cloned into libraries. Alternatively, if sufficient sequence information regarding the genes of interest is available microarrays may be constructed with gene-specific oligonucleotides (Li and Waldbieser, 2006; Santos et al., 2007).

The advent of cDNA microarray technology signifies a major technological advance in the field of genomics and already the impact of this technology on studies of fish physiology is being felt. Researchers have utilised cDNA microarrays to analyse the impact of temperature (Gracey et al., 2004; Podraborsky and Somero, 2004), hypoxia (Gracey et al., 2001; Gracey, 2007), salinity (Kalujnala et al., 2007), disease (Byon et al., 2005), environmental toxicants (Williams et al., 2007), and handling stress (Krasnov et al., 2005; Wiseman et al., 2007). What has become clear from these studies is that the response to any stressor involves a complex network of transcriptional responses involving a battery of genes and molecular pathways. In many instances the role(s) of these genes and pathways in the response to the stressor has been previously uncharacterised.

The microarray technique is most powerfully when applied to model species for which a wealth of sequence information is available from which PCR primers or probe oligonucleotides

Fig. 2. Schematic illustration of the cDNA microarray process. Total RNA is isolated from experimental control and treatment samples. Equal quantities of RNA are reverse transcribed into cDNA and a fluorescent dye, either Cy3 (red) or Cy5 (blue), is incorporated into either the control or treatment sample. The labelled cDNA samples are pooled and hybridized to cDNA that has been immobilized onto a specially coated glass slide.


Control
Treatment
RNA Isolation
cDNA Synthesis and Dye Incorporation
Pool Labeled cDNA Targets
and Hybridise to Microarray

can be synthesised. However, recent work has demonstrated the utility of the cDNA microarray approach in non-model organisms where sequence information may be lacking (Buckley, 2007). Nowhere is this more evident than in the field of teleost genomics. To date cDNA microarrays have been constructed for zebrafish (Ton et al., 2002) a model teleost, and a variety of non-model species including carp (Moen et al., 2006), goldfish (Martyniuk et al., 2006), flounder (Williams et al., 2003), goby (*Gillichthys mirabilis*) (Gracey et al., 2001), Atlantic salmon (Rise et al., 2004), and rainbow trout (Krasnov et al., 2005). Prior to the genomic revolution the lack of sequence information for non-model species' of interest presented a major obstacle to researchers interested in gaining a comprehensive understanding of the genetic regulation of physiological systems. However, it is becoming increasingly clear that adequate sequence identity exists to allow for microarrays developed for one species to be applied to other members of a phylogenetic group. For example, von Schalburg et al., (2005) developed a cDNA microarray consisting of 16000 elements from Atlantic salmon (*Salmo salar*) and rainbow trout, and found that it was applicable for gene expression studies in other closely related species of Salmonids. In addition, RNA isolated from rainbow smelt (*Oncorhynchus mordex*), which is separated from Atlantic salmon by 200 million years of evolution, also hybridised to the array. In the case of cross-species comparisons of gene expression using cDNA microarrays this approach has been termed 'heterologous' hybridization (Renn et al., 2004, Buckley, 2007).

Utilisation of cDNA microarray technology is and will continue to advance our understanding of transcriptional responses to stress. Ultimately, however, the ability to cope with stressor insult results from the action of proteins. Because quantitative changes in transcript amounts may not reflect changes in protein expression the ability to draw physiologically meaningful conclusions based on microarray data is often called into question (Gygi et al., 1999). Using a collection of yeast strains in which each gene is expressed as a GFP-tagged

fusion protein GFP fluorescence was measured to correlate changes in gene expression with changes in the expression of corresponding proteins in response to different environmental stimuli (Newman et al., 2006). Changes in mRNA abundance correlated with changes in protein expression for 87% of those genes in which mRNA abundance changes by greater than twofold. For a minority of the genes, changes in protein abundance were observed without changes in mRNA abundance. Seemingly, mRNA expression profiling is an effective method to identify genes whose protein expression is regulated at the transcriptional level. However, proteomic techniques are required to identify post-translationally regulated genes. In fact, it is suggested that 25% of the changes in protein expression in yeast are due to post-translational effects (Lu et al., 2007). Overall, genomic technology will provide an important means to quickly identify stressors-responsive genes and molecular pathways that are crucial for stress adaptation.

5. Experimental Rationale

When teleost fish are faced with a stressor they will elicit a set of physiological, biochemical and molecular responses, known as the integrated stress response, that are designed to ensure re-establishment of homeostasis. Depending on the nature of the stressor, this stress response may be a generalised response that occurs at the level of the whole organism. Conversely, if cellular homeostasis is threatened, primarily in the form of proteotoxicity, the organism will elicit a cellular stress response. PCBs are a class of anthropogenic compounds that are widely dispersed in the aquatic environment and that pose a threat to fish health. The physiological response to PCB exposure is mediated by AhR activation. The primary response of AhR activation is the induction the Cyp1A1 response, an adaptive response designed to lessen the threat of PCB exposure. Numerous studies have shown that despite activation of the Cyp1A response, exposure to PCBs can disrupt homeostasis. In fact, exposure to PCBs may stimulate or

inhibit the generalised and cellular responses to stress, but very little is known about the role of AhR signaling in modulating the stress response. In order to assess the role of the AhR signaling in regulating the physiological responses to stress, in vivo studies using rainbow trout and in vitro studies using primary cultures of rainbow trout hepatocytes, were performed. In addition, feral populations of Arctic char were utilized to determine impact(s) of chronic PCB exposure on cellular stress response. Consequently, the objective of this thesis was to characterise the rainbow trout AhR and determine the impact of PCB exposure on physiological responses to PCB exposure and stress.

5.1. Research Objectives

The overall objective of this thesis was to determine the impact of PCBs exposure on the adaptive responses to stress in fish and to characterize AhR signaling and its role in modulating the cellular stress response in rainbow trout.

The specific objectives are to:

1. Investigate the impact of short-term PCB exposure on the organismal stress response in rainbow trout.
2. Investigate the impact of long-term PCB exposure on cellular stress response in feral Arctic char.
3. Characterize AhR signaling in trout hepatocytes: examine the role of hsp90 and the proteasome.
4. Examine the role of the AhR in modulating the cellular stress response in trout hepatocytes.
5. Develop a targeted rainbow trout cDNA microarray for identifying stress-responsive genes in fish.

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Chapter 2

Aroclor 1254 affects liver metabolic adjustments associated with recovery from an acute stressor in rainbow trout

Abstract

The objective of this study was to investigate the impact of short-term exposure to polychlorinated biphenyls on the acute stress response in rainbow trout. Fish were fed Aroclor1254 laced food (10mg.kg^{-1} body mass) for three days and then subjected to a 3-min handling disturbance and sampled over a 24 h recovery after the stressor exposure. PCB exposure significantly elevated aryl hydrocarbon receptor (AhR) and cytochrome P4501A1 (Cyp1A1) mRNA abundance and Cyp1A protein expression confirming AhR activation. There was no significant effect of PCB on plasma cortisol and glucose levels, while plasma lactate levels were significantly elevated compared to the sham group. PCB exposure significantly elevated liver glycogen content and hexokinase activity, whereas lactate dehydrogenase activity was depressed. Short-term PCB exposure did not modify the secondary stressor-induced plasma cortisol, glucose and lactate responses. Liver AhR, but not Cyp1A1, transcript levels were significantly reduced during recovery from handling stressor. Also, liver glycogen content dropped significantly after stressor exposure in the PCB group but not in the sham group and this decrease corresponded with a significant elevation in liver LDH activity. This was matched by a significantly higher liver LDH activity and a lower HK activity during recovery in the PCB group suggesting enhanced glycolytic capacity. Collectively, this study demonstrates that short-term PCB exposure impairs the liver metabolic performance critical to cope with the enhanced energy demand associated with a secondary stressor in rainbow trout.

1. Introduction

Exposure to persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), have been linked to reduced health performance, including immunotoxicity, hepatotoxicity, neurotoxicity, and endocrine abnormalities (Safe, 1994). As the aquatic environment is often the final destination for these pollutants, aquatic organisms face an increased risk of exposure to these lipophilic compounds mostly *via* feed. The mode of action of PCBs is thought to be *via* aryl hydrocarbon receptor (AhR) activation and induction of AhR-responsive genes, including cytochrome P4501A1 (Cyp1A1) (Whitlock, 1999; Mimura and Fujii-Kuriyama, 2003; Carney et al., 2004; Ramadoss et al., 2005). While mammals express only a single AhR gene (AhR1), teleost fishes seems to have multiple forms of AhR gene (AhR2), including AhR2 isoforms in rainbow trout (*Oncorhynchus mykiss*) (Abnet et al., 1999; Hannson et al., 2003, 2004; Hahn, 2001; Hahn et al., 2004, 2006). However, the functional significance of AhR isoforms in piscine models is not well understood.

A key adaptive response to stressor exposure, including contaminants, is the acute elevation in plasma cortisol levels (Wendelaar Bonga, 1997; Barton, 2002; Iwama et al., 2006). This generalized whole organism stress response is thought to play an important role in stress adaptation, including metabolic adjustments critical for coping with enhanced energy demand in stressed fish (Wendelaar Bonga, 1997; Mommsen et al., 1999, Barton, 2002; Iwama et al., 2006). Specifically, cortisol via glucocorticoid receptor (GR) is involved in the long-term maintenance of plasma glucose and the replenishment of liver glycogen stores during recovery from the stressor (Mommsen et al., 1999). This energy repartitioning seen with cortisol stimulation involves peripheral proteolysis, amino acid mobilization, target tissue substrate utilization, and higher liver metabolic capacity including gluconeogenesis (see Mommsen et al., 1999 for a review). Consequently, any impact of PCBs on either the cortisol response or intermediary

metabolism will impair stress adaptation. Indeed, in teleost fishes, chronic exposure to PCBs and other AhR agonists impair the adaptive cortisol stress response both in laboratory and field studies (Quabius et al., 1997; Vijayan et al., 1997a; Jørgensen et al., 2002; Aluru and Vijayan, 2004; Aluru et al., 2005; Hontela, 2005). Exposure to PCBs has also been linked to altered expression of the glucocorticoid receptor (GR) (Aluru et al., 2004). As GR mediates the cellular effects of cortisol, it is as an important link between the primary and cellular responses to stress and any effects of PCBs on GR dynamics may ultimately impact cortisol dependent cellular responses to stress.

The cellular stress response is an evolutionary conserved mechanism of cellular defence against damage to macromolecules, including lipid membranes, DNA, and proteins (Kultz, 2005). The cellular response is characterised by stressor non-specific and specific reactions, both of which are directed at re-establishing cellular homeostasis (Kultz, 2005; Iwama et al., 2006). The non-specific response is activated by a diverse set of stressors. An excellent example of this response is gene expression of heat shock protein 70 (hsp70) as a response to proteotoxicity (Hightower, 1991; Iwama et al., 2006). In contrast, stressor specific responses are mediated by stressor specific sensor proteins. For example, a stressor-specific response is the Cyp1A1 expression resulting from PCB-mediated AhR activation (Whitlock, 1999; Aluru and Vijayan, 2006a). Previous studies examined modulation of the organismal and cellular stress response to longer-term exposure to relatively high PCB concentration. However, little is known about the impact of short-term PCB exposure on the stress performance in teleost fishes. The one study that examined PCB impact on the stress response to secondary stressor exposed fish for over 3 months to this contaminant (Jørgensen et al., 2002).

Here our objective was to specifically examine whether a short-term exposure to PCB was sufficient to impair the adaptive organismal and cellular responses in rainbow trout. To this

end, we exposed trout to the environmentally realistic PCB mixture Aroclor 1254 (Muir et al., 1988; Gundersen et al., 2000) for 3 days and examined the organismal and cellular stress responses to a secondary acute handling disturbance. Plasma cortisol, glucose and lactate were measured as indicators of the organismal stress response, while transcript abundance of CYP1A, AhR, hsp70 and GR were measured as indicators of cellular stress response. Liver glycogen content and activities of selected enzymes involved in intermediary metabolism were quantified to assess the liver metabolic capacity.

2. Materials and Methods

2.1. Chemicals

Aroclor 1254 was purchased from LGC Promochem (Boras, Sweden). Protease inhibitor cocktail and Bicinchoninic acid (BCA) reagent were purchased from Sigma-aldrich (St. Louis, MO, USA). All electrophoresis reagents and molecular weight markers were from BioRad (Mississauga, ON, Canada). Cyp1A antibody (mouse anti-cod Cyp1A monoclonal antibody) was from Biosense laboratories (Bergen, Norway). The secondary antibody to Cyp1A was alkaline phosphatase-conjugated to goat anti-mouse IgG (BioRad). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indlyl phosphate salt (BCIP) were obtained from Fisher Scientific (Napean, ON, Canada).

2.2. Animals

Juvenile rainbow trout ((*Oncorhynchus mykiss*, ~200g) were purchased from Rainbow Springs Trout Farm (Thamesford, ON, Canada). Fish were maintained in 200-L tanks with continuous running water at 13 °C and a 12L:12D photoperiod for 1 month prior to the start of

the experiment. Fish were fed once daily to satiety with a 3-point sinking food (Martin Mills Inc., Elmira, ON, Canada) for 5 days a week during the acclimation period.

2.3. Experimental Protocol

Groups of 8-10 fish each were randomly assigned to six aquaria (100-L), three of which were for the sham groups and the other three for the PCB groups. Fish were fed once daily with 2% body weight feed (Martin Mills Inc., Elmira, ON, Canada) for 5 days a week during the acclimation period. Fish were allowed to acclimate for 2 weeks prior to the start of the experiment.

During the exposure period fish were fed 2% body weight feed laced with either ethanol alone (control) or ethanol containing Aroclor 1254 (10mg/kg body mass /day) for 3 days. Briefly, the feed was evenly submerged in 95% ethanol or ethanol containing Aroclor 1254 to ensure adequate coating of the pellets. The ethanol was allowed to evaporate by air-drying, and the feed was stored in a cool and dry place. This method provides an easy means of administering the lipophilic contaminant without stressing the fish (Aluru et al., 2005).

After 3 days of feeding, trout were subjected to an acute stressor consisting of a standardized handling disturbance of netting and chasing for 3 min. Tissue samples were collected either prior to stressor exposure or 1 and 24 h post-stressor exposure. Sampling consisted of quickly netting all fish for each tanks (one sham and one PCB) at the respective time-points and anaesthetizing them with an overdose of 2-phenoxyethanol (1:1,000). Fish were bled by caudal puncture and the blood was collected in heparinised tubes, centrifuged (6,000xg for 5 min at 4 °C), and the plasma was stored frozen at –70 ^{o}C for subsequent cortisol, glucose, and lactate determination. Liver tissues were frozen in liquid nitrogen and stored at –70 ^{o}C for subsequent analyses.

2.4. Plasma Metabolite Determination

Plasma cortisol concentration was determined using a radioimmunoassay (RIA) kit (Medicorp, Montreal, PQ, Canada) according to established protocols (Vijayan et al., 2003). Plasma glucose and lactate concentration were determined colorimetrically using commercially available kits (Sigma).

2.5. Liver Glycogen Concentration and Enzyme Activities

The liver glycogen content was measured after amyloglucosidase hydrolysis according to Keppler and Decker (1974). Optimal enzyme activities for liver hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) were carried out at 22 ^{o}C by continuous spectrophotometry at 340nm on a microplate reader (VersaMax, Molecular Devices Corp., Menlo Park, Califonia, USA) exactly as mentioned before (Vijayan et al., 2006). Enzyme activities were expressed as μmoles of substrate consumed or product liberated per min (U) per gram protein (U.g^{-1} protein).

2.6. RNA Isolation and First Strand cDNA Synthesis

Total RNA isolation, including DNase treatment, was performed using RNeasy mini kits according to the manufacturer's instructions (QIAgen, Mississauga, ON, Canada). The first strand cDNA was synthesized from 1μg of total RNA using a cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada) with an oligo dT primer according to the manufacturer's instructions. A relative standard curve for each gene of interest was constructed using cDNA synthesized using this method. An appropriate volume of cDNA for each gene was determined during quantitative real-time PCR (qPCR) optimization (iCycler, Bio-Rad)

2.7. Primers

Primers (table 1) were designed to amplify either a 100 bp (β-actin, Cyp 1A1, PEPCK, GR, hsp70) or 500 bp (AhR) fragment of the target gene of interest. The Hsp70 primers detect both the inducible (Hsp70) and constitutive (Hsc70) transcripts. Rainbow trout AhR2α and AhR2β (GenBank accession numbers: AF065138 andAF065137 respectively) were aligned and primers were designed from conserved regions of the two genes. As the full-length sequence of the third AhR2 is unavailable this sequence was not included in the primer design.

2.8. Quantitative Real- Time PCR

Relative standard curves for target genes (Cyp1A1, PEPCK, GR, hsp70, AhR) and a housekeeping gene (β-actin) were constructed from a serial dilution of plasmid DNA containing the target gene. Platinum quantitative PCR supermix-UDG (Invitrogen, CA) was used for qPCR and the samples were treated according to the manufacturer's instructions. 0.2 μM forward and reverse primers and 1:100,000 SYBR green I nucleic acid gel stain (Roche, Mississauga, ON, Canada) was used in each PCR reaction. Samples and standards were run in triplicates on 96 well PCR plates (Ultident, Montreal, PQ, Canada) according to the manufacturer's instructions. An optimized volume of cDNA was used for the amplification of each gene. The reaction components were exactly as above and for every single test sample a qPCR for both the target and the housekeeping gene was performed. The following PCR program was used to amplify all genes: 95 °C – 3 min; 40 cycles: 95 °C – 20 sec, Tm (annealing temperature) - 20 sec, 72 °C – 20 sec, followed by 4 °C hold.

2.9. Data Analysis for Quantification of Gene Expression

Calculation of the threshold cycle values (C_T) for every sample was performed using the iCycler iQ™ real time detection software (BioRad). From a standard curve with log input amount and C_T values for each gene, the input amount for each sample was calculated for target gene and β-actin using the appropriate standard curve. The amount of target gene was divided by the amount of β-actin to determine the normalized amount of the target gene. The normalized amount of target gene (a relative unit) was then standardized using an internal calibrator (pre-stress sham samples) and expressed as percent 0 h sham.

2.10. Immunoblot Analysis

Tissue protein concentrations were determined using the bichinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard. Total protein (40 μg) was separated on a 8% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970) for 40 min at 200V using 1X TGS (250mM Tris, 1.92 M glycine, 1% SDS) and transferred onto a 0.45μM nitrocellulose membrane (BioRad) using Trans-blot® SD semi-dry electrophoretic transfer cell (BioRad). A 5% solution of non-fat dry milk in 1X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween 20, pH 7.5) was used as a blocking agent (1 h at room temperature) and for diluting primary and secondary antibodies. The blots were incubated with Cyp1A primary antibody for 1 h at room temperature followed by 1 h incubation with an alkaline phosphatase conjugated secondary antibody (BioRad). The membranes were washed after incubation in either primary (2 x 15 min washes in TTBS (2 mM Tris, 30 mM NaCl, pH 7.5, 0.1% Tween-50) or secondary antibodies (2 x 15 min in TTBS followed by 2 x 15 min in TBS). Proteins were detected using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) / NBT (nitro blue tetrazolium) color substrate (BioRad). The molecular weight was verified by using prestained low range

molecular weight markers (BioRad). Images were captured with Chemi imager™ (Alpha Innotech, San Leandro, CA, USA) and bands were quantified with using the AlphaEase Software (Alpha Innotech).

2.11. Statistical Analyses

All statistical analyses were performed with SPSS version 11.0 (SPSS, Chicago, IL, USA) and data were expressed as mean + S.E.M. Data was log transformed when necessary in order to ensure homogeneity of variance, although non-transformed data are shown in all figures and table. Data were analyzed by 2-way ANOVA with a Bonferonni's post-hoc test to assess the effects of time, treatment and their interaction. When interaction effects between time post-stressor exposure and treatment were observed a 1-way ANOVA was used to compare the effect of time within each treatment group followed by a Bonferonni's post-hoc test, while a two-sample t-test, assuming equal variance, was performed to compare treatment effects at each time point. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma Cortisol, Glucose, and Lactate

Plasma cortisol levels (Fig. 1A) were significantly elevated ($P< 0.05$) at 1h post-stress and returned to pre-stressor levels by 24 h in both the sham and PCB treated groups. Plasma glucose levels were transiently elevated 1 h after stressor exposure and the levels were significantly higher than at 24 h post-stressor exposure but not prior to stress (Fig. 1B). Lactate levels were significantly elevated ($P< 0.05$) at 1 h and 24 h post-stressor exposure compared to the pre-stress groups. Overall, plasma lactate levels were significantly elevated in the PCB

treated groups compared to the sham groups (Fig. 1C). There was no interaction between time and treatment for either plasma cortisol, glucose or lactate levels.

3.2. Liver Glycogen Content

There was a significant interaction between time and Aroclor treatment. In the pre-stress group, PCB treatment significantly elevated liver glycogen content compared to the sham group (Fig. 2). Liver glycogen content was significantly lower at 1 h and 24 h after stressor exposure compared to the pre-stress level in the PCB group, while there was no effect of stressor exposure on liver glycogen content in the sham group (Fig. 2).

3.3. Liver Enzyme Activities

All enzyme activities are summarized in table 2. Neither stress nor PCB exposure had any significant effect on the activities of AspAT and AlaAT (amino acid catabolism) or PK (glycolysis). HK (glycolysis) activity showed a significant interaction between time and PCB treatment. HK activity was significantly higher in the PCB group compared to the sham group prior to stress (Table 2). Also, PCB treatment significantly reduced HK activity at 1 h but not 24 h post-stressor exposure compared to the pre-stress levels, while there was no significant effect of stressor exposure on HK activity in the sham group. There was significant treatment and time effect and interaction for liver LDH (glycolysis) activity. Handling disturbance significantly elevated LDH activity at 1 and 24 h post-stressor exposure compared to the pre-stress levels. PCB treated, but not the sham group showed a significant interaction with LDH activity significantly elevated at 1 h and 24 h post-stressor exposure compared to the pre-stress group. PEPCK (gluconeogenesis) activity showed significant interaction effects between time and PCB

treatment. In the sham group, but not PCB group, PEPCK activity was significantly higher at 1 h post-stressor exposure compared to the pre-stress and 24 h post-stressor exposure groups.

3.4. Liver Transcript Abundance

AhR mRNA abundance showed a significant interaction between PCB treatment and time post-stressor exposure (Fig. 3). AhR mRNA abundance was significantly higher in the PCB group compared to sham group prior to stress (Fig. 3). There was a statistically significant reduction in AhR mRNA abundance in the PCB treated fish at 1 h and 24 h post-stressor exposure compared to the pre-stress group (Fig. 3). AhR mRNA abundance was not statistically different in the sham group between any time points. Cyp1A1 transcript abundance was significantly elevated by Aroclor treatment compared to the sham group (Fig. 4B). However, there was no interaction between time post-stressor exposure and PCB treatment. PEPCK mRNA abundance was not significantly impacted by PCB exposure. PEPCK mRNA abundance was not significantly impacted at either 1 h or 24 h post-stressor exposure relative to the pre-stress group (Fig. 5). PEPCK mRNA abundance was significantly elevated at 24 h post-stressor exposure relative to the 1 h post-stress group. However, there was no interaction between time post-stressor exposure and PCB treatment. Transcript abundance of GR (Fig. 6a) and hsp70 (Fig. 6b) was not significantly impacted by either time post-stressor exposure or by PCB treatment.

3.5. Liver Cyp1A Protein Expression

Cyp1A protein expression (Fig. 4A) was quantified in the pre-stress groups only as previous studies have determined that handling stress does not modulate this response (Vijayan et al., 1997b). Protein expression was significantly elevated in the PCB fed group relative to the sham group.

Table 1: Sequences, annealing temperatures, and corresponding target gene Genbank accession numbers of oligonucleotide primers used in semi-quantitative real-time PCR

Target Gene	Accession #	Primer Sequence (5' – 3')	Annealing Temp (Tm ^{o}C)
β-actin	**AF157514**	Forward: AGA GCT ACG AGC TGC CTG AC Reverse: GCA AGA CTC CAT ACC GAG GA	49
PEPCK	**AF246149**	Forward: TGC TGA GTA CAA AGG CAA GG Reverse: GAA CCA GTT GAC GTG GAA GA	49
Cyp1A1	**U62796**	Forward: GAT GTC AGT GGC AGC TTT GA Reverse: TCC TGG TCA TCA TGG CTG TA	60
GR	**Z54210**	Forward: AGA AGC CTG TTT TTG GCC TGT A Reverse: AGA TGC GCT CGA CAT CCC TGA T	49
AhR	**AF065137** **AF065138**	Forward: CAG CGA AGG GAG CGG TAA Reverse: TGG ACC CGG CCA GTG ATA	60
hsp70	**K02549**	Forward: GAA GGT GTC CAA TGC AGT CA Reverse: GAT CCT CAG CAC ATT CAG C	49

Table 2: Liver hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (AlaAT), and aspartate aminotransferase (AspAT) activities. Rainbow trout were fed either 0 or 10 mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stress or 1 or 24 h post-stress and liver samples were collected for analysis of enzyme activities. Activity is expressed as μmoles of substrate consumed or product liberated per gram protein in the homogenate. Values represent means ± SEM (n = 5 – 6). A Two-way ANOVA was used to assess time and interaction effects. When interaction effects were observed a One-way ANOVA was used to assess the effect of time on enzyme activity within a treatment group. A two-sample t-test was used to assess treatment effects at each time point.

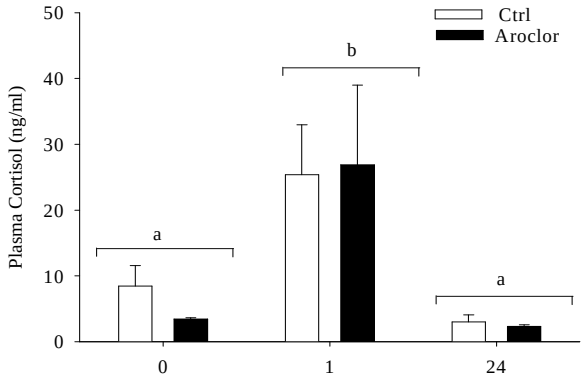
Enzyme	Treatment	0h	1h	24h	Treatment (P < 0.05)	Time (P < 0.05)
HK	**Sham**	0.0110 ± 0.0002 *****	0.0107 ± 0.0003	0.0123 ± 0.0009	NS	NS
	Aroclor	0.0145 ± 0.0001 **A**	0.0111 ± 0.0003 **B**	0.0108 ± 0.0007 **AB**		
PK	**Sham**	0.0527 ± 0.0096	0.0637 ± 0.0126	0.0586 ± 0.0100	NS	NS
	Aroclor	0.0764 ± 0.0100	0.0557 ± 0.0077	0.0545 ± 0.0066		
LDH	**Sham**	0.5640 ± 0.0374 *****	0.4522 ± 0.0326	0.6223 ± 0.0674	S > A	0 > 1, 24
	Aroclor	0.1349 ± 0.0632 **A**	0.5538 ± 0.0003 **B**	0.6238 ± 0.0447 **B**		
PEPCK	**Sham**	0.0030 ± 0.0001 **A**	0.0043 ± 0.0004 **B**	0.0044 ± 0.0004 **A**	NS	NS
	Aroclor	0.0036 ± 0.0005	0.0028 ± 0.0007	0.0070 ± 0.0006		
AlaAT	**Sham**	1.1230 ± 0.1168	0.9304 ± 0.0769	0.9436 ± 0.1209	NS	NS
	Aroclor	0.9557 ± 0.1213	0.8850 ± 0.1368	0.0070 ± 0.0006		
AspAT	**Sham**	0.1260 ± 0.0066	0.1011 ± 0.0083	0.0836 ± 0.0101	NS	NS
	Aroclor	0.0988 ± 0.0340	0.1068 ± 0.0065	0.1157 ± 0.0102		

Different bold uppercase letters indicate significant time effects within a treatment group (P<0.05).

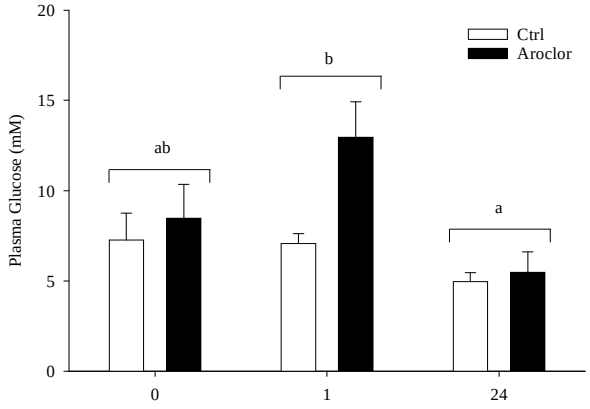
An asterisk (*) indicates a significant treatment effect at a given sampling time point (P<0.05).

Fig. 1. The effect of handling stress on plasma cortisol, glucose and lactate. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stressor exposure and plasma samples were collected for analysis of (A) cortisol, (B) glucose or (C) lactate. Values represent means ± SEM (n = 5 – 6 fish). Different letters denote significant time effects regardless of treatment while the inset shows significant treatment effect (two-way ANOVA; $P < 0.05$).

A


Ctrl
Aroclor
Plasma Cortisol (ng/ml)
50
40
30
20
10
0
a
b
a
0
1
24

B


Ctrl
Aroclor
Plasma Glucose (mM)
20
15
10
5
0
ab
b
a
0
1
24

C

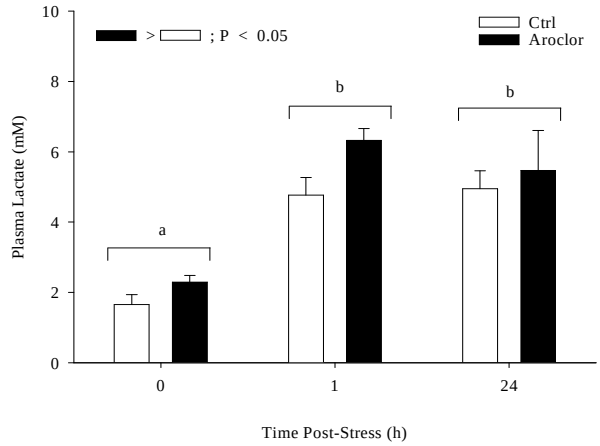

> ; P < 0.05
Ctrl
Aroclor
Plasma Lactate (mM)
10
8
6
4
2
0
a
b
b
0
1
24
Time Post-Stress (h)

Fig. 2. The effect of handling stress on liver glycogen. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stressor exposure and liver samples were collected for analysis of glycogen concentration. Values represent means ± SEM (n = 5 – 6). An asterisk (*) denotes significant treatment effect at a given sampling time (two-sample t-test; $P < 0.05$). Different letters denote significant time effect on liver glycogen in Aroclor fed individuals (one-way ANOVA; $P < 0.05$).

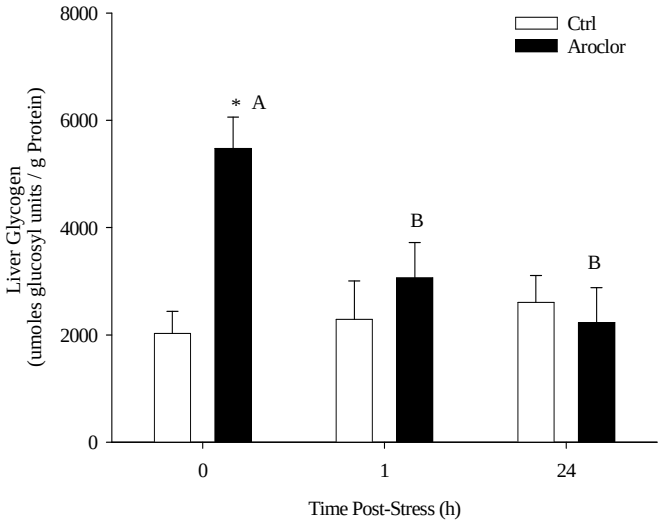

Ctrl
Aroclor
Liver Glycogen
(umoles glucosyl units / g Protein)
8000
6000
4000
2000
0
* A
B
B
0
1
24
Time Post-Stress (h)

Fig. 3. AhR mRNA abundance. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stressor exposure and liver samples were collected for analysis of AhR mRNA abundance. Values represent means ± SEM (n = 5 – 6). An asterisk (*) denotes a significant treatment effect at a given sampling time (two sample t-test; $P < 0.05$). Different letters denote significant time effect on liver AhR mRNA abundance in Aroclor fed individuals (one-way ANOVA; $P < 0.05$).

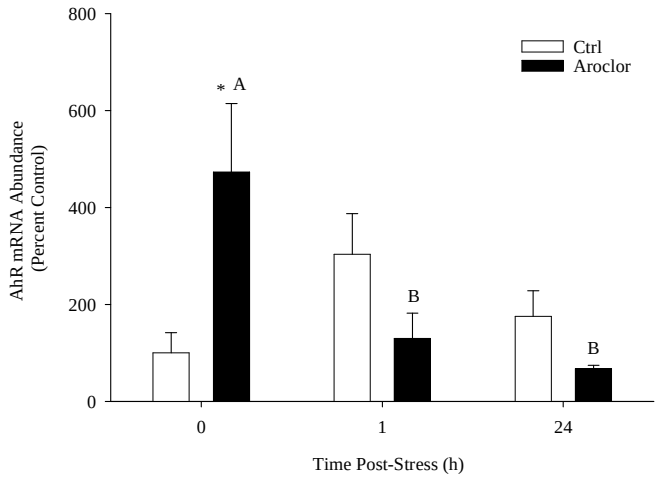
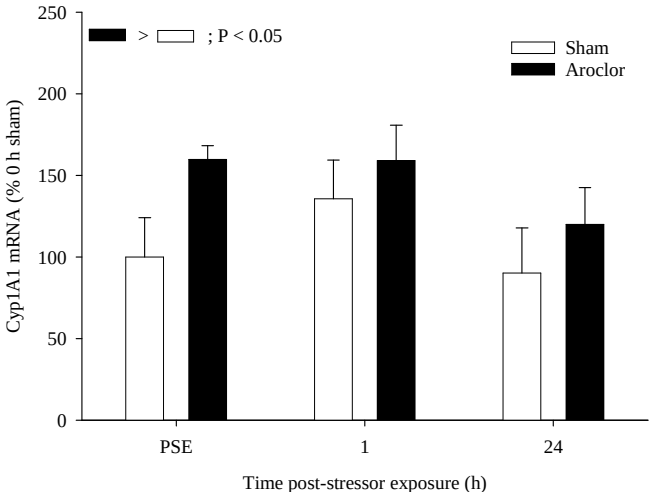

800
600
400
200
0
AhR mRNA Abundance
(Percent Control)
Ctrl
Aroclor
* A
B
B
0
1
24
Time Post-Stress (h)

Fig. 4. Cyp1A(1) protein expression and mRNA abundance. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stressor exposure and liver samples were collected for analysis of (A) Cyp1A1 mRNA abundance over the course of the experiment or (B) Cyp1A protein expression immediately prior to stressor expsoure. Values represent means ± SEM (n = 5 – 6). An asterisk (*) denotes a significant treatment effect (two sample t-test; $P < 0.05$).

A


250
200
150
100
50
0
> ; P < 0.05
Sham
Aroclor
Cyp1A1 mRNA (% 0 h sham)
PSE
1
24
Time post-stressor exposure (h)

B

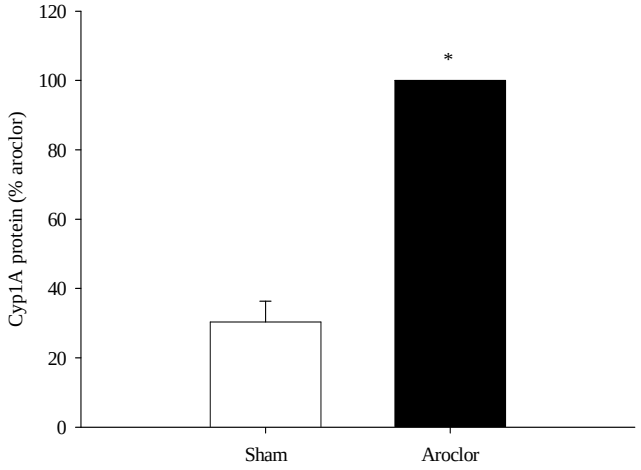

120
100
80
60
40
20
0
*
Cyp1A protein (% aroclor)
Sham
Aroclor

Fig. 5. PEPCK mRNA Abundance. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stress and liver samples were collected for PEPCK mRNA abundance. Values represent means ± SEM (n = 5 – 6). Different letters denote significant time effect on liver PEPCK mRNA abundance (two-way ANOVA; $P < 0.05$).

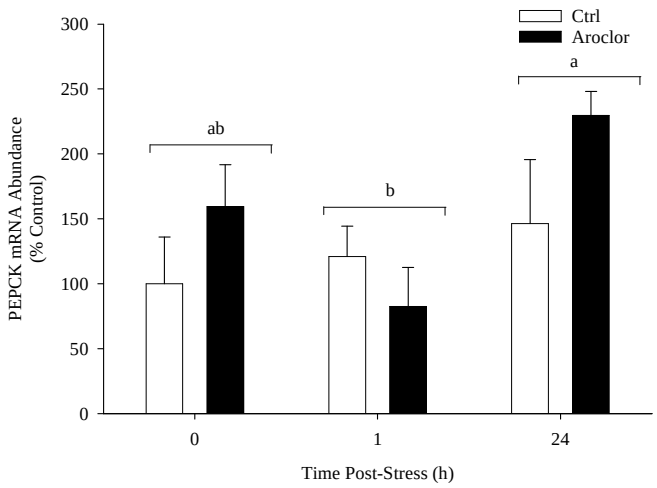
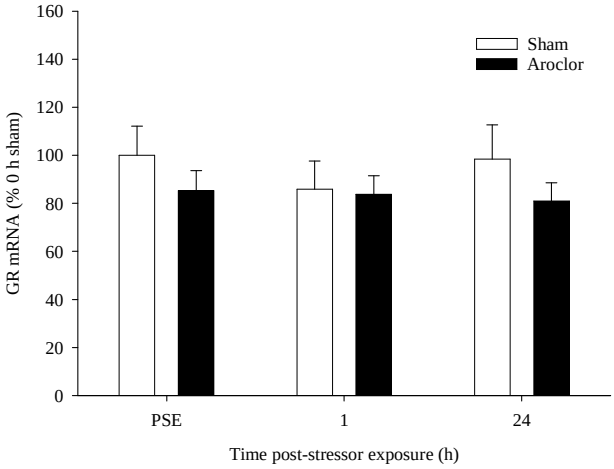
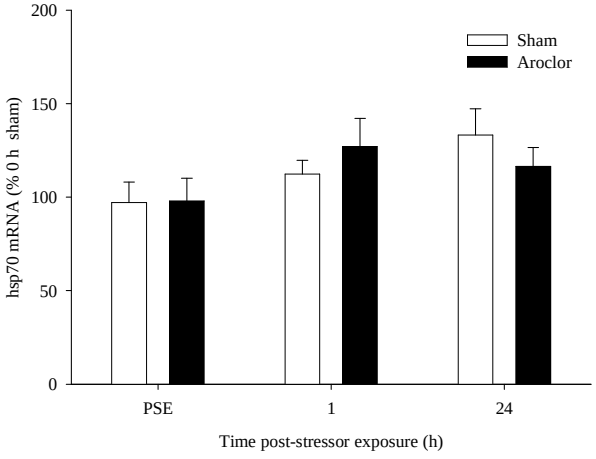

Ctrl
Aroclor
300
250
200
150
100
50
0
PEPCK mRNA Abundance
(% Control)
ab
b
a
0
1
24
Time Post-Stress (h)

Fig. 6. GR and hsp70 mRNA abundance. Rainbow trout were fed either 0 or 10-mg/kg body mass Aroclor 1254 for 3 days prior to being subjected to a standardized 3 minute handling stress. Individuals were sampled either immediately prior to stressor exposure (PSE) or 1 or 24 h post-stressor exposure and liver samples were collected for analysis of (A) GR mRNA abundance and (B) hsp70 mRNA abundance. Values represent means ± SEM (n = 5 – 6).


160
140
120
100
80
60
40
20
0
GR mRNA (% 0 h sham)
Sham
Aroclor
PSE
1
24
Time post-stressor exposure (h)


200
150
100
50
0
hsp70 mRNA (% 0 h sham)
Sham
Aroclor
PSE
1
24
Time post-stressor exposure (h)

4. Discussion

We demonstrate that short-term PCB exposure affects energy substrate repartitioning that is critical for coping with a secondary stressor in rainbow trout. The primary response to acute handling stress is an increase in circulating cortisol levels (reviewed in Mommsen et al., 1999; Barton, 2002, Iwama et al., 2006). Studies have clearly established that exposure to PCBs impairs the adaptive cortisol response to stress (Hontela, 1998; Wilson et al., 1998; Jørgensen et al., 2002; Hontela, 2005) and the mechanism of action may involve AhR-mediated inhibition of corticostroidogenesis (Aluru and Vijayan, 2006b). In the current study, despite activation of AhR signalling, as evidenced by the higher Cyp1A1 mRNA abundance, neither the magnitude nor the duration of the cortisol response to acute handling stress was impacted. In previous studies inhibition of stress induced cortisol secretion was observed at PCB and/or AhR agonist concentrations ranging from 50 mg/kg body mass (Quabius et al., 1997; Wilson et al., 1998; Aluru and Vijayan, 2004; Aluru et al., 2004; Aluru and Vijayan, 2006b) to 100-mg/kg body mass (Aroclor 1254; Jørgensen et al., 2002). The lack of a similar cortisol response at lower PCB concentrations suggests a threshold dose for steroid disruption as seen with Arctic charr (Jørgensen et al., 2002). The target tissue response to cortisol stimulation is mediated by GR activation (Vijayan et al, 2003). GR mRNA abundance was not impacted by PCB treatment in this study. This is in agreement with our recent observation that beta-naphthoflavone (AhR agonist) exposure also did not impact tissue GR protein expression in trout (Aluru and Vijayan, 2004), arguing for a lack of change in target tissue responsiveness to cortisol stimulation in PCB exposed fish. Indeed a reduction in brain GR protein expression with PCB exposure in fasted Arctic charr corresponded with an abnormal cortisol negative feedback regulation in that group suggesting a close link between receptor content and target tissue responsiveness (Aluru et al., 2004).

A key metabolic response to stress is an increase in liver metabolic capacity, including enhanced glucose production to meet the metabolic burden associated with recovery from stress (Wendelaar Bonga, 1997; Mommsen et al., 1999). The immediate plasma glucose response to stress results from catecholamine stimulated breakdown of stored glycogen (Randall and Perry, 1992; Vijayan and Moon, 1994; Vijayan et al., 1994), whereas the longer-term maintenance of plasma glucose levels and glycogen repletion during recovery from stress is primarily mediated by cortisol-induced liver gluconeogenic capacity (Mommsen et al., 1999). The lack of a post-stressor increase in plasma glucose levels in the present study may be related to the stressor and sampling regimes as previous studies saw an increase in plasma glucose only at 3 h post-stressor exposure (Vijayan et al, 1997c). However, there appears to be an increase in plasma glucose concentration at 1 h in the PCB treated group compared to the sham group suggesting an alteration in glucose turnover in this group. A similar disturbance in stressor-induced plasma glucose levels was also seen in fasted Arctic charr chronically treated with PCB (Jørgensen et al., 2002) leading to the proposal that glucose turnover associated with recovery from stressor is disturbed by even shorter-term PCB exposure. The significant drop in liver glycogen content seen only in the PCB group after stressor exposure may have contributed to the elevated glucose response in these stressed animals.

An interesting observation from this study is the higher liver glycogen content seen with PCB exposure. This contrasts previous studies that have reported depressed liver glycogen content in response to PCBs or AhR ligand exposures (Quabius et al., 1997, 1998; Andersen et al., 2003; Vijayan et al., 2006; Tintos et al., 2007). The accumulation of hepatic glycogen is unlikely to have resulted from elevated gluconeogenesis as PEPCK, AspAT, and AlaAT activities were not significantly elevated compared to sham fed trout. While the utilization of circulating glucose for hepatic glycogen is unclear (Moon, 2001), the elevated HK activity

recorded in resting PCB fed trout suggests an enhanced liver capacity for glucose uptake. A similar increase in HK activity has been reported in rainbow trout exposed to the AhR ligand naphthalene (Tintos et al., 2007). This coupled with the significantly lower LDH activity in unstressed PCB treated fish liver further supports increased utilization of free glucose for glycogen synthesis. Taken together, these results suggest that short-term exposure to PCB stimulates liver glycogen synthesis and one mechanism may involve altered capacity for plasma glucose uptake.

PCBs stimulate expression of a suite of proteins, including phase I and phase II enzymes involved in xenobiotic metabolism (Schlenk and Di Giulio, 2002; Shimada, 2006). As protein synthesis is energetically expensive, accounting for nearly 80% of cellular O_2 consumption in isolated rainbow trout hepatocytes (Pannevis and Houlihan, 1992), it follows that PCBs increase energy consumption in exposed organisms. In fact, exposure to pyrene, a weak AhR agonist, increases O_2 consumption in a concentration dependent manner in rainbow trout hepatocytes (Bains and Kennedy, 2004). Consequently, the energetic demand of recovery from acute stress is likely to be greater in the PCB exposed fish. Indeed higher Cyp1A protein expression in the PCB group suggests a higher liver metabolic demand compared to the sham group. Acute handling disturbance resulted in a significant drop in liver glycogen content only in the PCB group. The stressor-induced decrease in liver HK activity coupled with the significant elevation of liver LDH activity in the PCB group clearly supports utilization of this energy store for liver metabolism. Consequently, we propose that glycolysis is a key pathway for coping with the enhanced energy demand in response to PCB exposure. This is further supported by the significantly higher stressor-induced plasma lactate levels in the PCB group. While the mechanism(s) involved in stressor-induced glycolysis is unclear, we cannot rule out the

possibility that PCB exposure alters the cellular response to glycolytic hormone stimulation, including catecholamines (Randall and Perry, 1992; Moon et al., 2002).

Interestingly, handling stress attenuated the Aroclor stimulated increase in AhR transcript abundance. As we have recently demonstrated autoregulation of βNF stimulated rainbow trout AhR (Wiseman and Vijayan, 2007) these results suggest handling stress may disrupt this autoregulation. The impact of handling stress appears to be AhR specific as transcript abundance of Cyp1A1, GR, hsp70 and PEPCK were not impacted by the handling stressor. Interestingly, the decrease in AhR mRNA abundance coincided with the increase in plasma cortisol suggesting a role for this steroid in AhR regulation. This is further supported by the recent observation that cortisol treatment reduced AhR transcript abundance in a GR dependent manner in trout hepatocytes (Aluru and Vijayan, unpublished). Considered together, PCB exposure upregulates AhR mRNA abundance while the handling stressor disrupts the ligand-mediated autoregulation of AhR in rainbow trout. The lack of an associated change in Cyp1A1 transcript abundance argues against lower tissue responsiveness suggesting other factors including perturbations in mRNA stability as a reason for the lower AhR transcript abundance post stressor-exposure in the PCB group. Indeed, studies have shown that cortisol may impact transcript stability (Delany et al., 1995; Rydziel et al., 2004). Given that the AhR mediates many changes in gene expression in response to PCB exposure (Tijet et al., 2006; Aluru and Vijayan, unpublished) attenuation of AhR transcript abundance may represent an adaptive strategy to limit the energy consumed for synthesis of xenobiotic metabolising enzymes. Such a strategy would ultimately conserve energy necessary for metabolic responses to acute handling stressor. Further studies are needed to explore this hypothesis.

5. Conclusions

Stimulation of AhR signaling by the environmentally relevant technical PCB mixture Aroclor 1254 increases transcript abundance of both Cyp1A1 and AhR. Interestingly, exposure to a handling stressor selectively perturbs AhR transcript abundance but not Cyp1A1 gene expression in the PCB group. There was no impact of short-term exposure to PCB on stressor-induced plasma cortisol and glucose levels, but lactate levels were higher suggesting enhanced glycolytic potential. In unstressed trout, PCB exposure elevated liver glycogen content and this coincided with an increased capacity for glucose uptake but not glycolysis. However, PCB exposure enhanced glycogen breakdown in response to a handing stressor and this coincided with an enhanced liver capacity for glycolysis. Taken together, short-term exposure to PCB impairs the energy substrate repartitioning critical for coping with the enhanced energy demand to a secondary stressor in trout. The higher energetic demand associated with PCB exposure may limit the metabolic capacity of fish to recover from a secondary stressor. Consequently, even shorter-term exposure to PCB may decrease the performance of fish to other stressors, including predator avoidance in the environment.

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Chapter 3

Impact of Contaminant Loading in Remote Arctic Lakes in Northern Norway: Biomarker Responses in Arctic Char

Abstract

The island ecosystem of Bjørnøya Island, Norway, offers a unique opportunity to study the impact of lifelong contaminant exposure in wild populations of resident Arctic char. Lake Ellasjøen has persistent organic pollutant (POP) levels that are significantly greater than in nearby Lake Øyangen. Our objective was to examine if this contaminant loading in these remote lakes has biological impact on the resident fish. Specifically, we investigated the impact associated with toxicant exposure on protein and mRNA levels of several cellular stress markers, including cytochrome P4501A (Cyp1A), heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90) and glucocorticoiid receptor (GR) in feral char from the two lakes. The average PCB load in char liver from Lake Ellasjøen was approximately 25-fold higher than in individuals from Lake Øyangen. Liver Cyp1A protein expression and mRNA abundance was significantly higher in individuals from Lake Ellasjøen compared to Lake Øyangen. There was no significant difference in liver Hsp70 or Hsp90 mRNA abundance or Hsp70 protein expression between the two lakes. Brain Hsp70 and Hsp90 mRNA levels were significantly elevated in char from Lake Ellasjøen compared to Lake Øyangen. Liver GR protein expression but not liver and brain GR transcript abundance was significantly higher in the Lake Ellasjøen char compared to Lake Øyangen char. Altered levels of GR, Hsp70 and Hsp90 suggest that these proteins are useful biomarkers of effects associated with chronic PCB exposure in feral char. Additionally, the Hsp response suggests that the brain is more sensitive to PCB exposure than liver in char from these lakes. Altogether, our results for the first time highlight a biological response to contaminant exposure in resident char from remote Arctic lakes.

1. Introduction

High concentrations of persistant organic pollutants (POPs), including polychlorinated biphenyls (PCBs) have been measured in the sediment and biota of remote Arctic lakes (Evenset et al., 2004; 2005; 2007). Specifically, Lake Ellasjøen located in the southern, mountainous part of Bjørnøya Island, Norway, has POPs levels several fold greater than Lake Øyangen, which is located only 6 km north of Ellasjøen on the central plains of the island (Evenset et al., 2004; 2007). The main reason for the high levels of these persistent contaminants in Lake Ellasjøen is due to the transport of contaminants by seabirds from the marine environment through the deposition of guano (Evenset et al., 2005). Indeed some of the highest PCB levels (110 mg/kg lipid weight) in the Arctic ecosystem have been recorded in tissues of Arctic char (*Salvelinus alpinus*) residing in this lake (Evenset et al., 2005; 2007). Interestingly the lake population consists of different morphs of Arctic char and the high PCB levels have been reported in the large, cannibalistic individuals, while lower levels were detected in the dwarfs (Klemetsen et al., 1985; Evenset et al., 2005).

Despite the high contaminant levels reported in tissues of Lake Ellasjøen char, little is known about their biological impact on these animals. Indeed, nearly 4-fold upregulation of hepatic cytochrome P4501A (Cyp1A) enzymes in the liver of Arctic char from Lake Ellasjøen compared to Lake Øyangen suggests exposure to POPs in these animals (Skotvold et al., 1998; Jørgensen and Wolkers, unpublished). The mode of action of PCBs involves arylhydrocarbon receptor (AhR) activation and induction of AhR-responsive genes, including Cyp1A in fishes (Whitlock, 1999; Aluru and Vijayan, 2006). Consequently, Cyp1A gene expression and the activity of the enzyme that this gene codes for are routinely used as biomarkers of PCB exposure in fish (Bucheli and Fent, 1995; Schlenk and Di Giulio, 2002; Hahn, 2002). While the reported high tissue Cyp1A enzyme activity in the Lake Ellasjøen fish suggests clearly that these

individuals are upregulating their phase I biotransformation enzymes in response to longer-term PCBs exposure, the biological impact on target tissues are unknown.

Environmental contaminants, including PCBs, have been shown to impact important components of the cellular stress response in fishes (Vijayan et al., 2006). A key biomarker of cellular effects is the induction of a high conserved family of proteins commonly referred to as the heat shock proteins (Hsps) (Iwama et al., 1998; Vijayan et al., 2006). These proteins are critical for the normal functioning of the cellular protein machinery, as well as defense against proteotoxicity (Hightower, 1991; Parsell and Lindquist, 1993). The most widely studied Hsps in fish are the 70 kilodalton family of proteins and they are present either constitutively in unstressed cells (Hsc70) or induced rapidly (Hsp70) in stressed states (Iwama et al., 1998; Currie et al., 1999; Vijayan et al., 2006). Indeed, Hsp70 expression has been used as a biomarker of effect in response to contaminants, including PCBs in fish (Vijayan et al., 1998; Janz et al., 1997; Weber and Janz, 2001; Vijayan et al., 2006). For instance, sublethal exposure to PCBs corresponded with an elevation in liver Hsp70 levels, whereas lethal concentration of contaminant resulted in a lack of Hsp70 response in trout (Vijayan et al., 1998).

In addition to Hsp70, other proteins thought to be important for coping with toxicants include the 90 kilodalton (Hsp90) member of the Hsps family of proteins and the glucocorticoid receptor (GR) in fish (Vijayan et al., 2006). Hsp90 is a key component of the cellular signaling molecules, including GR. Consequently, any impact on Hsp90 expression may disrupt steroid signaling as was seen with long-term exposure to PCBs in hatchery-reared Arctic char (Aluru et al., 2004). PCB exposure in fasted Arctic char reduced brain GR and Hsp90 protein contents and this corresponded with a disturbance in the plasma cortisol response to stress in this species (Aluru et al., 2004). Taken together, changes in tissue expression of Hsp70, Hsp90 and GR are

indicative of adjustments to cope with stressor insult and may be used as biomarker of cellular effects (Vijayan et al., 2006).

Against this backdrop, the island lake ecosystem of Bjørnøya (Bear Island, Norway, 74°N) offers a unique opportunity to assess the utility of molecular biomarkers as indicators of PCB exposure and effect in feral populations of Arctic char. On the one hand, Lake Ellasjøen is rich in PCBs, dichloro-diphenyl-trichloroethane (DDT) and polybrominated diphenylethers (PBDEs) and various metals, including arsenic (As), cadmium (Cd), cobalt (Co), nickel (Ni), zinc (Zn) and mercury (Hg) (Evenset et al., 2007). On the other hand, Lake Øyangen, located only 6 km north of Lake Ellasjøen on the central plains of the island, muscle PCB concentrations are 10-40 times lower than char from Lake Ellasjøen (Evenset et al., 2004; Blais, 2005). Consequently, these lakes on Bjørnøya provide a natural system for assessing biomarker responses to long-term exposure to different contaminant concentrations in fish with similar environmental conditions. To this end, we investigated the impact of lifelong toxicant exposure and accumulation on protein and mRNA levels of Cyp1A, Hsps and GR in feral char from Lake Ellasjøen and Lake Øyangen. We demonstrate for the first time differences in the mRNA and protein levels of Cyp1A and Hsps in fish from these lakes. Moreover, we show that GR may be a useful indicator of effects associated with chronic PCB exposure in feral populations of char.

2. Materials and Methods

2.1. Chemicals

Protease inhibitor cocktail and Bicinchoninic acid (BCA) reagent were purchased from Sigma-aldrich (St. Louis, MO, USA). All electrophoresis reagents and molecular weight markers were from BioRad (Mississauga, ON, Canada). Cyp1A antibody (mouse anti-cod Cyp1A monoclonal antibody) was from Biosense laboratories (Bergen, Norway). Antibody to

trout glucocorticoid receptor (GR) was developed in our laboratory (Sathiyaa and Vijayan, 2003). Primary antibody to Hsp70 (rabbit anti-rainbow trout Hsp70 polyclonal antibody) has been previously described and detects only the inducible form of Hsp70 and not the constitutive (Hsc70) isoform (Vijayan et al., 1997). Antibody to Hsp90 (rat anti-salmon Hsp90 monoclonal antibody) was from StressGen (Victoria, BC, Canada). This antibody detects both the Hsp90α and Hsp90β isoforms. The secondary antibody to Cyp1A was alkaline phosphatase-conjugated to goat anti-mouse IgG (BioRad). The secondary antibodies to Hsp70 and GR were alkaline phosphatase-conjugated to goat anti-rabbit IgG (BioRad). The seconday antibody to Hsp90 was alkaline phosphatase conjugated togoat anti-rat IgG (BioRad). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indlyl phosphate salt (BCIP) were obtained from Fisher Scientific (Napean, ON, Canada).

2.2. Animals

Arctic char were collected from both Lake Ellasjøen and Lake Øyangen by hook and line capture in September 2002. The small sample size (n=3 independent fish) was due to the fact that only tissues from larger fish was used for this study. This size-pairing was necessary because the lakes had two morphs of char, the dwarfs and the larger cannibalistic variety, and the high contaminant levels were reported only in the larger morphs (Klemetsen et al., 1985; Evenset et al. 2005). The three larger fish sampled from each lake were immature males. Tissues were quickly dissected out and frozen immediately in liquid nitrogen (and stored at –70 ^{o}C) for later analyses of organic contaminants (OCs) levels (only liver) as well as mRNA and protein content (liver and brain) of selected biomarkers.

2.3. PCB Quantification

The chemical analyses were performed at Unilab Analyse AS, Polar Environmental Centre, Tromsø, Norway, using the method described by Brevik (1978). The tissue samples were homogenised prior to extraction. An internal standard (PCB 53) was then added to the homogenised samples (3–5 g) and lipids and OCs extracted with acetone and cyclohexane using an ultrasonic homogenizer (Sonics & Materials Inc, model 501). The extract was washed with 0.5 % NaCl, centrifuged and the volume of the organic fraction was reduced using a rotavapor. The sample was dissolved in dichloromethane and then filtrated before injection on a gel permeation chromatography (GPC) clean-up system (Waters 515 HPLC Pump with Waters Environgel GPC-Clean Up columns) where most of the lipids, sulphur and/or other interfering compounds were separated from the OCs. The elutant from the GPC was concentrated and the dichloromethane solvent was replaced by cyclohexane. Remnants of biological matrix were removed by treatment with sulphuric acid. The volume of the organic phase was then carefully reduced by N_2 –flow to approximately 30-50 µl and the samples were stored on vials at – 20°C before instrumental OC analysis.

Organochlorines were quantified using a gas chromatograph (Hewlett Packard 5890 Series II) equipped with a HP-5 MS 60 m capillary column 60m (0.25 mm i.d., 0.25 µm film thickness) and an electron capture detector. OC identification was based on the retention times for the respective compounds and they were quantified towards the internal standard. The samples were analysed for 10 polychlorinated biphenyl (PCB) congeners (IUPAC Nos., Ballschmiter and Zell, 1980): 28, 31, 52, 101, 105, 118, 138, 153, 180, and 209, hexachlorocyclohexane-isomers (α-HCH and γ-HCH), dichlorodiphenylethanes (*p,p'*-DDT, *p,p'*-DDD, *p,p*'-DDE), hexachlorobenzene (HCB) and pentachlorobenzene (5-CB).

2.4. Quantitative Real-Time PCR (qPCR)

2.4.1. RNA Isolation and First Strand cDNA Synthesis

Total RNA isolation, including DNase treatment, was performed using RNeasy mini kits according to the manufacturer's instructions (Qiagen, Mississauga, ON). The first strand cDNA was synthesized from 1µg of total RNA using a cDNA synthesis kit (MBI Fermentas, Burlington, ON) with an oligo dT primer according to the manufacturer's instructions. A relative standard curve for each gene of interest was constructed using cDNA synthesized using this method. An appropriate volume of cDNA for each gene was determined during qPCR optimization (iCycler, BioRad).

2.4.2. Primers

Primers (Table 1) were designed to amplify either a 100 bp (β-actin, Cyp1A, Hsp70, GR) or 500 bp (Hsp90) fragment of the target genes of interest. As limited sequence information exists for Arctic char, primers were designed to amplify genes of interest in rainbow trout and these were used for Arctic char. Primers for Hsp90 were based on a Chinook salmon Hsp90 cDNA sequence. The identity of all fragments was verified by sequencing to confirm amplification of the target gene in Arctic char.

2.4.3. Relative Standard Curve

Relative standard curves for target genes (Cyp1A, Hsp70, Hsp90, GR) and a housekeeping gene (β-actin) were constructed using plasmid vectors containing the target sequences according to established protocols (Sathiyaa and Vijayan, 2003). The Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, CA) used in qPCR reaction was 2× concentrated. Every 25 µl reaction had 1.5 U Platinum Taq DNA polymerase, 20 mM Tris–HCl (pH 8.4),

50 mM KCl, 3 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dCTP, 400 μM dUTP and 1 U UDG; the reaction also contained 0.2 μM forward and reverse primers, SYBR green I nucleic acid gel stain (1:4000) (Roche, Montreal, QC, Canada) and fluoroscein calibration dye (1:2000) (BioRad). To reduce pipetting errors, master mixes were prepared for triplicate reactions (3 × 25 μl) for each standard and reactions were run in triplicates on 96 well PCR plates (BioRad) according to the manufacturer's instructions (BioRad).

2.4.4. Quantification of Samples

An optimized volume of cDNA was used for the amplification of each gene. The reaction components were exactly the same as the previous section and for every single test sample a qPCR for both the target (Cyp1A, Hsp70, Hsp90, GR) and the housekeeping gene (β-actin) was performed. The following PCR program was used to amplify all genes: 95 °C – 3 min; 40 cycles: 95 °C – 20 sec, Tm (annealing temperature) - 20 sec, 72 °C – 20 sec, followed by 4 °C hold.

2.4.5. Data analysis for Quantification of Gene Expression

Calculation of the threshold cycle values (C_T) for every sample was performed using the iCycler iQ™ real time detection software (BioRad). The transcript amount for each sample was calculated for target gene and β-actin using their respective standard curves with log input amount and C_T values. The amount of target gene was divided by the amount of β-actin to determine the normalized amount of the target gene. The normalized value (a relative unit) was then standardized using an internal calibrator (Lake Øyangen) and expressed as percent of Lake Øyangen levels exactly as described before (Sathiyaa and Vijayan, 2003).

2.5. Immunoblotting

Tissue protein concentrations were determined using the bichinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard. Total protein (40 μg) was separated on a 8% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970) for 40 min at 200V using 1X TGS (250mM Tris, 1.92 M glycine, 1% SDS) and transferred onto a 0.45μM nitrocellulose membrane (BioRad) using Trans-blot® SD semi-dry electrophoretic transfer cell (BioRad). A 5% (w/v) solution of non-fat dry milk in 1X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween 20, pH 7.5) was used as a blocking agent (1 h at room temperature) and for diluting primary and secondary antibodies. All primary antibodies were used at a dilution of 1:3000 in blocking agent. The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse for Cyp1A (1:3000 dilution) or goat anti-rabbit for Hsp70 and GR (1:3000) and goat anti-rat for Hsp90. The blots were incubated with primary antibodies for 1 h at room temperature, followed by 1 h incubation with secondary antibodies. The membranes were washed after incubation in either primary (2 x 15 min washes in TTBS (2 mM Tris, 30 mM NaCl, pH 7.5, 0.1% Tween-50) or secondary antibodies (2 x 15 min in TTBS followed by 2 x 15 min in TBS). Proteins were detected using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) / NBT (nitro blue tetrazolium) chromogenic substrate (BioRad). The molecular weight was verified by using prestained low range molecular weight markers (BioRad). Images were captured with Chemi imager™ (Alpha Innotech, San Leandro, CA, USA) and bands were quantified using Alphaease Software (Alpha Innotech).

2.6. Statistical Analysis

Data was analysed using a two-sample t-test assuming equal variance. Log transformation was carried out whenever necessary to meet the assumptions of homogeneity of

variance, although non-transformed values are shown in the figures. A probability of $P < 0.05$ was considered statistically significant. All data are shown as mean ± SEM (n = 3 independent fish).

3. Results

3.1. Fish Size and Liver OC load

There were no significant differences in either body mass or length between fish caught from the two lakes (Table 1). The average PCB and DDT load in char liver from Lake Øyangen was 11.30 ng/g wet weight (ww) and 0.93 ng/g ww, respectively, whereas in the Lake Ellasjøen char it was 281.33 ng/g ww and 39.57 ng/g ww, respectively. Mean PCBs load in the liver was approximately 25-fold higher in the liver of char from Lake Ellasjøen compared to individuals from Lake Øyangen (Fig. 1). Of the DDT group, *p,p'*-DDE constituted more than 75% in all fish tissues, while concentrations of 5-CB, HCB and HCHs were all below 2 ng/g wet weight (data not shown).

3.2. Cyp1A mRNA and Protein

Liver Cyp1A mRNA levels were significantly elevated (5-fold) in char from Lake Ellasjøen compared to those from Lake Øyangen (Fig. 2A). Liver Cyp1A protein expression was also significantly higher (approximately 50-fold) in char from Lake Ellasojen compared to Lake Øyangen (Fig. 2B). The Cyp1A mRNA abundance in the brain was lower than that observed in the liver, but there was no significant difference in the brain Cyp1A transcript abundance between individuals from the two lakes (Fig. 2C).

3.3. Hsp70 mRNA and Protein

There was no significant difference in liver Hsp70 mRNA abundance or protein content in char between the two lakes (Fig. 3A and Fig. 3B). The brain Hsp70 mRNA abundance was significantly elevated in char from Lake Ellasjøen compared to Lake Øyangen (Fig. 3C).

3.4. Hsp90 mRNA

There was no statistical significant difference in liver Hsp90 transcript levels between char from Lake Ellasojen and Lake Øyangen (Fig. 4A). However, brain Hsp90 mRNA abundance was significantly higher in char from Lake Ellasjøen compared to Lake Øyangen (Fig.4B).

3.5. GR Protein and mRNA

There was no significant difference in liver GR transcript levels in char between the two lakes (Fig. 5A). Liver GR protein expression was significantly higher in the Lake Ellasojen char compared to Lake Øyangen char (Fig. 5B). The brain GR mRNA content was significantly higher in the Lake Ellasojen char compared to Lake Øyangen char (Fig. 5C).

Table 1: Primer sequence, annealing temperatures, and corresponding target gene Genbank accession numbers of oligonucleotide used in quantitative real-time PCR.

Target Gene	Accession #	Primer Sequence (5' – 3')	Annealing Temp (Tm ^{o}C)
β-actin	**AF157514**	Forward: AGA GCT ACG AGC TGC CTG AC Reverse: GCA AGA CTC CAT ACC GAG GA	49
Cyp1A	**U62796**	Forward: GAT GTC AGT GGC AGC TTT GA Reverse: TCC TGG TCA TCA TGG CTG TA	60
Hsp70	**K02549**	Forward: GAAGGTGTCCAATGCAGTCA Reverse: GATCCTCAGCA CATTCAGC	49
Hsp90	**U89945**	Forward: AAG CTG GGA ATC CAT GAA GA Reverse: CAG GGA GAC CAT TTC GTC AG	49
GR	**Z54210**	Forward: AGA AGC CTG TTT TTG GCC TGT A Reverse: AGA TGC GCT CGA CAT CCC TGA T	49

Table 2. Individual body mass and length and the mean ± S.E.M. (n=3) of Arctic char sampled from Lake Øyangen and Lake Ellasjøen, Bjørnøya Island, Norway.

	Lake Øyangen		Lake Ellasjøen	
	Body mass (g)	**Length (mm)**	**Body mass (g)**	**Length (mm)**
Fish 1	1321	484	**950**	**470**
Fish 2	1193	457	**1260**	**505**
Fish 3	979	430	**1125**	**462**
Average ± SEM	1164 ± 99.8	457 ± 16	**1111 ± 89.7**	**479 ± 13**

Figure 1. Liver PCB levels in char from Lake Ellasjøen and Lake Øyangen. Values represent mean ± SEM (n = 3) of the 10 PCB congeners analysed; *statistically significant (unpaired Student's t-test, $P < 0.05$).

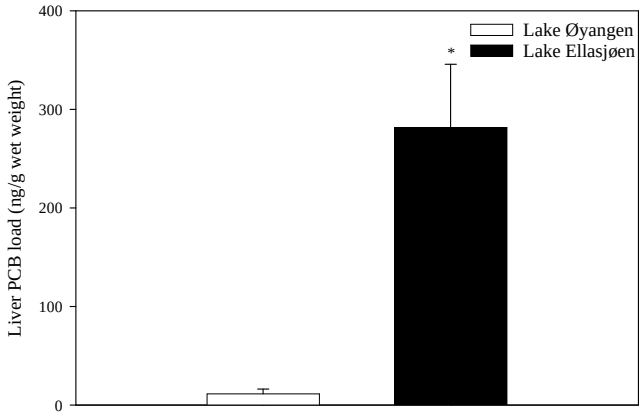
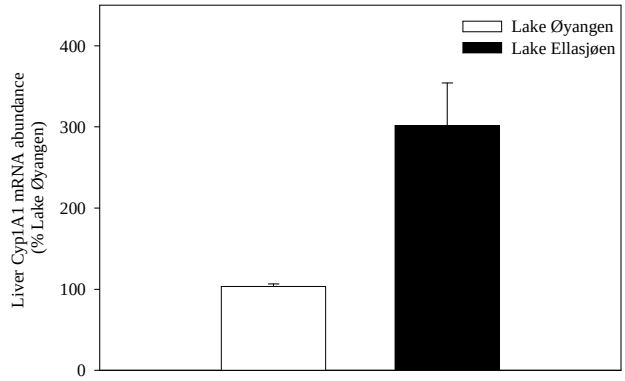
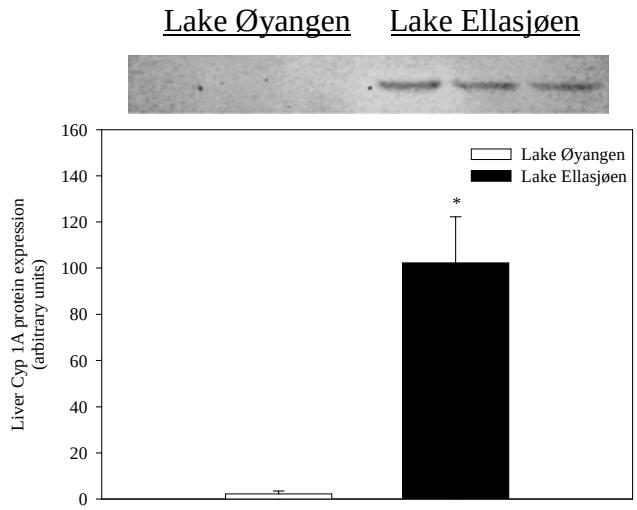

Liver PCB load (ng/g wet weight)
400
300
200
100
0
Lake Øyangen
Lake Ellasjøen
*

Figure 2. Cyp1A mRNA abundance and protein expression. Transcript abundance of (A) liver and (B) brain Cyp1A was quantified in individuals from Lake Ellasjøen and Lake Øyangen. Protein expression of (C) Cyp1A was quantified in liver of individuals from Lake Ellasjøen and Lake Øyangen. An immunoblot of Cyp1A protein expression is shown above the histogram. Each of the three lanes represents a different fish sampled from Lake Ellasjøen and Lake Øyangen. Values represent mean ± SEM (n = 3); *statistically significant (unpaired Student's t-test, $P < 0.05$).

A


Lake Øyangen
Lake Ellasjøen
Liver Cyp1A1 mRNA abundance
(% Lake Øyangen)
400
300
200
100
0

B


Lake Øyangen
Lake Ellasjøen
Lake Øyangen
Lake Ellasjøen
Liver Cyp 1A protein expression
(arbitrary units)
*
160
140
120
100
80
60
40
20
0

C

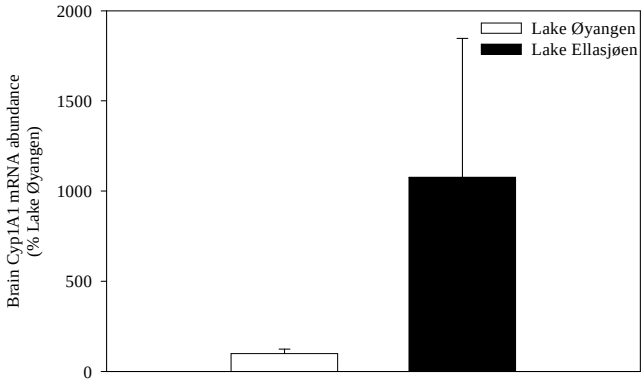
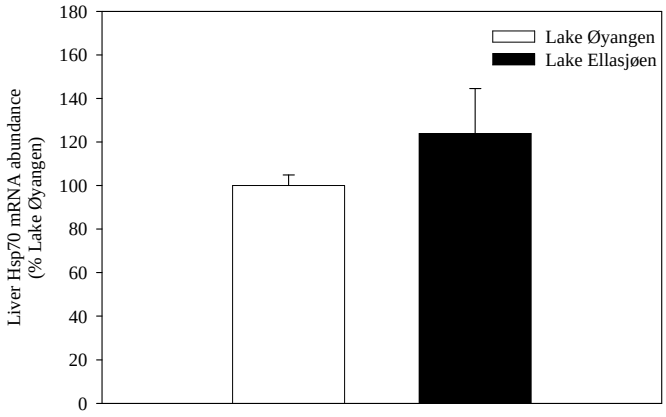
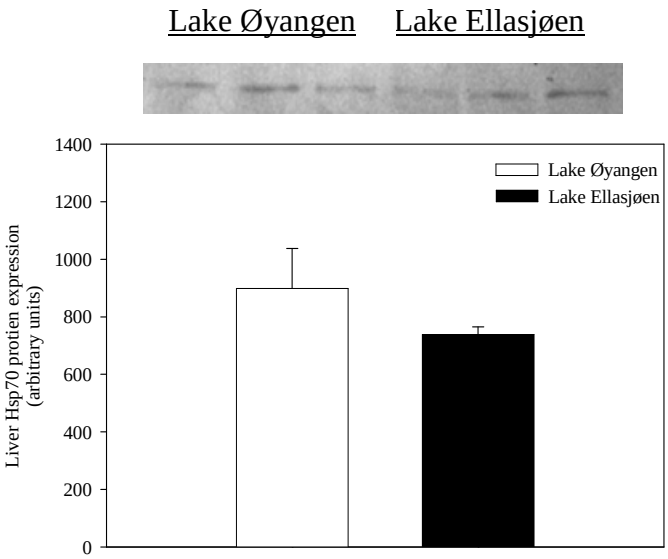

Lake Øyangen
Lake Ellasjøen
Brain Cyp1A1 mRNA abundance
(% Lake Øyangen)
2000
1500
1000
500
0

Figure 3. Hsp70 mRNA abundance and protein expression. Transcript abundance of (A) liver and (B) brain Hsp70 was quantified in individuals from Lake Ellasjøen and Lake Øyangen. Protein expression of (C) Hsp70 was quantified in liver of individuals from Lake Ellasjøen and Lake Øyangen. An immunoblot of Hsp70 protein expression is shown above the histogram. Each of the three lanes represents a different fish sampled from Lake Ellasjøen and Lake Øyangen. Values represent mean ± SEM (n = 3); *statistically significant (unpaired Student's t-test, $P < 0.05$).

A


Lake Øyangen
Lake Ellasjøen
Liver Hsp70 mRNA abundance
(% Lake Øyangen)
180
160
140
120
100
80
60
40
20
0

B


Lake Øyangen
Lake Ellasjøen
Lake Øyangen
Lake Ellasjøen
Liver Hsp70 protien expression
(arbitrary units)
1400
1200
1000
800
600
400
200
0

C

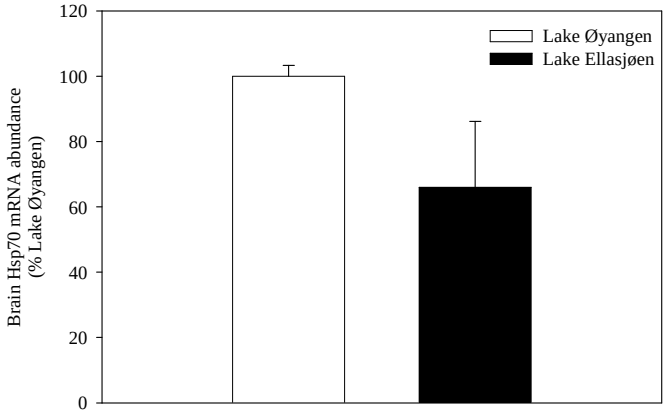
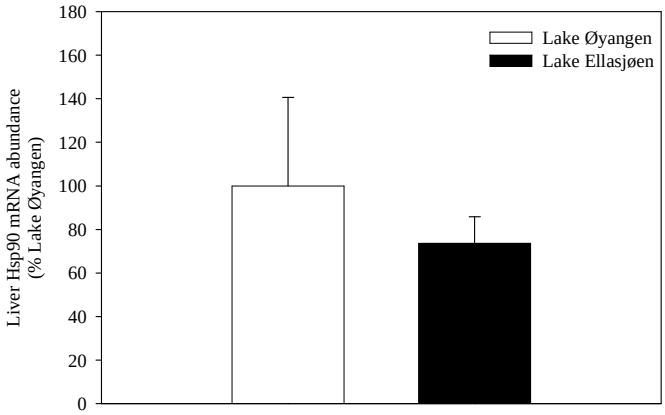

Lake Øyangen
Lake Ellasjøen
Brain Hsp70 mRNA abundance
(% Lake Øyangen)
120
100
80
60
40
20
0

Figure 4. Hsp90 mRNA abundance. Transcript abundance of (A) liver and (B) brain Hsp90 was quantified in individuals from Lake Ellasjøen and Lake Øyangen. Values represent mean ± SEM (n = 3); *statistically significant (unpaired Student's t-test, $P < 0.05$).

A


Liver Hsp90 mRNA abundance
(% Lake Øyangen)
180
160
140
120
100
80
60
40
20
0
Lake Øyangen
Lake Ellasjøen

B

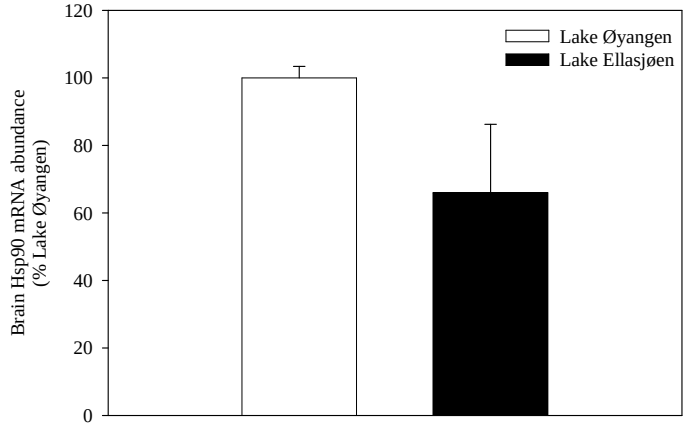
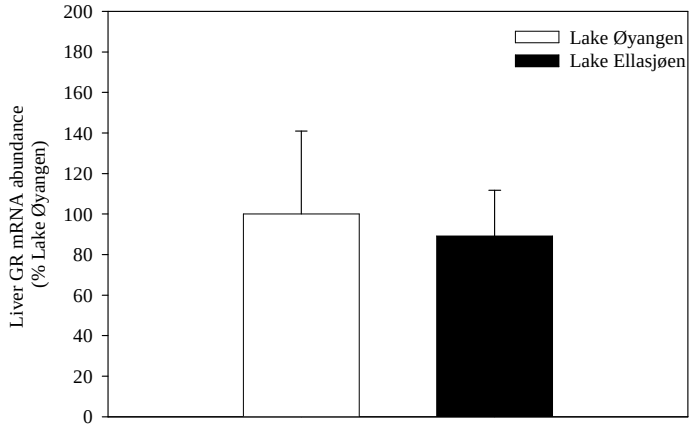

Brain Hsp90 mRNA abundance
(% Lake Øyangen)
120
100
80
60
40
20
0
Lake Øyangen
Lake Ellasjøen

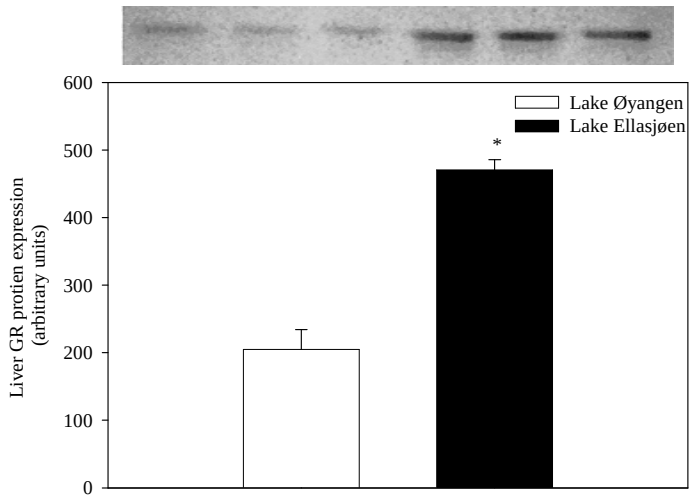
Figure 5. GR mRNA abundance and protein expression. Transcript abundance of (A) liver and (B) brain GR was quantified in individuals from Lake Ellasjøen and Lake Øyangen. Protein expression of (C) GR was quantified in liver of individuals from Lake Ellasjøen and Lake Øyangen. An immunoblot of GR protein expression is shown above the histogram. Each of the three lanes represents a different fish sampled from Lake Ellasjøen and Lake Øyangen. Values represent mean ± SEM (n = 3); *statistically significant (unpaired Student's t-test, $P < 0.05$).

A

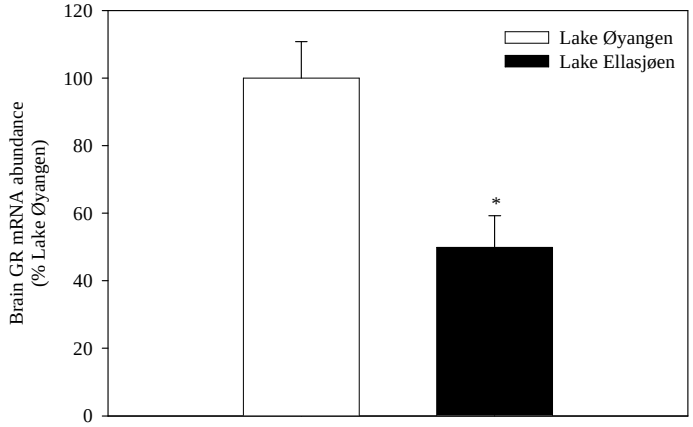


Lake Øyangen Lake Ellasjøen

B



C



4. Discussion

Our study for the first time highlights a clear tissue effect associated with long-term contaminant exposure in char residing in remote Arctic lakes. The liver PCB concentration confirms previous studies showing that the muscle burden of polychlorinated biphenyls (PCB) was higher in Lake Ellasjøen char compared to fish inhabiting Lake Øyangen (Evenset et al., 2004). However, based on the 10 PCB congeners analysed, the magnitude of difference (25-fold) in liver PCB load between the two lakes is approximately 2-fold greater than the PCB load seen in muscle (Evenset et al., 2004) suggesting increased lipophilic contaminants in the liver of fish. This is not surprising given the fact that liver is a major organ accumulating lipophilic contaminants, including PCBs in fish (Jørgensen et al., 2006). Indeed, the significant elevation of liver Cyp1A mRNA abundance and protein expression in the Lake Ellasjøen char clearly support activation of AhR signaling in response to higher PCB exposure. This is in accordance with elevated levels of EROD activity reported in Lake Ellasjøen char (Skotvold et al., 1998). Increased abundance of both Cyp1A protein and mRNA levels is a well established response to PCB exposure (Hahn and Stageman, 1994; Whitlock, 1999; Hahn, 2002; Aluru and Vijayan, 2006) and has been used as a biomarker of exposure to this lipophilic contaminant (Bucheli and Fent, 1995; Schlenk and Di Giulio, 2002).

While char from Lake Ellasjøen showed elevated levels of Cyp1A, other studies have demonstrated reduced sensitivity to AhR agonists in teleosts inhabiting PCB contaminated sites, including killifish (*Fundulus heteroclitus*; Bello et al., 2001; Van Veld and Westbrook, 1995, Hahn et al., 2004), carp (*Cyprinus carpio;* Fisher et al., 2006), Atlantic tomcod (*Microgadus tomcod*; Roy and Wirgin, 1997) and Atlantic salmon (*Salmo salar*; Hannson et al., 2006). A variety of mechanisms may be responsible for insensitivity of these populations to AhR agonists, including point mutations in the AhR receptor (Poland et al., 1994; Sun et al., 1997; Pohjanvirta

et al., 1998) and alterations in post-receptor signaling pathway (Pollenz et al., 1996; Wilson et al., 1997). Hahn et al. (2004) reported that AhR alleles identified in the coding regions of the Atlantic killifish AhR 1 locus differ in frequency between populations from contaminated and non-contaminated waters. Specifically, individuals from polluted areas have specific alleles that are underrepresented compared to non-resistant fish from uncontaminated sites. It is possible that mutations/alterations in the promoter region of Cyp1A also could play a role in observed insensitivities (Bello et al., 2001). Reduced sensitivity to AhR agonists in populations experiencing prolonged exposure to AhR agonists may also be due to reduced AhR protein expression as it has been shown that AhR is down-regulated via the proteasome following exposure to AhR agonists (Davarinos and Pollenz, 1999; Pollenz, 2002; Wentworth et al., 2004). Finally, the presence of the recently discovered AhR repressor (AhRR; Mimura et al., 1999) in these resistant populations may also play a role in this phenomenon. All the above factors either singly or in combination may be involved in the insensitivity to Cyp1A response observed with generational PCB exposures in feral fish.

However, the higher Cyp1A expression in char from Lake Ellasjøen suggests that these fish have not acquired resistance to chronic exposure to AhR agonists. While the mechanism is unknown, we hypothesize that the unique lifestyle of Arctic char may be responsible for the observed effects. For instance, Arctic char in these lakes are seasonal feeders, fattening during the summer months (2 months) and undergoing extended fasting during the winter months (10 months) (Jørgensen et al., 1997; Jobling et al., 1998). During their summer fattening period, char accumulate PCBs in lipid depots. The lipids are mobilized during winter emaciation resulting in the redistribution of PCBs to peripheral tissues, including liver and brain (Jørgensen et al., 2006). Eventually, given the extended duration of the fasting period, the whole body burden of PCBs may decrease due to the activation of phase I biotransformation enzymes as

evidenced by the higher Cyp1A protein levels and EROD activity (Fig. 2; Jorgensen et al., 2006). According to this model, char would endure an annual cycle of PCB loading and unloading and this may be a reason for the observed response to chronic PCBs exposure not seen in other species (see above). As the fish in this study were sampled in September, the PCB levels seen are those representing a well fed fish. We hypothesize that the tissue levels of PCBs will be lower in fish sampled in the spring. However, we cannot rule out the possibility that genetic differences between char and other species, that have developed insensitivity to PCB exposure, may be playing a role, but this remains to be determined.

Our results clearly suggest that exposure to higher contaminant load in fish from Lake Ellasjøen is eliciting a cellular stress response. Hsp70 expression is commonly used as an indicator of cellular stress in animals as this protein is critical for defending cells against proteotoxicity (Hightower, 1991; Iwama et al., 1998). The higher Hsp70 expression in the brain, but not liver, of char from Lake Ellasjøen suggests an adaptive response to defend against proteotoxicity in this tissue (Sanders, 1993; Hightower, 1991; Ryan and Hightower, 1996). The absence of any effect on Hsp70 transcript level leads us to propose that contaminant exposure alters the brain Hsp70 protein turnover in these animals. We did not measure OC levels in the brain, but the higher Cyp1A mRNA abundance in this tissue in Lake Ellasjøen fish points to a higher exposure to these lipophilic contaminants. These results suggest the brain is more sensitive to PCB insults relative to the liver in these fish resulting in the heat shock response. Recently we showed that chronic exposure to PCB in fasted char leads to reduced brain hsp70 and hsp90 levels in Arctic char and this coincided with a reduced brain function (Aluru et al., 2004). The difference in response seen in this study may be related to the feeding status of the animal. As these were well fed animals, it raises the possibility that this adaptive response may be compromised during the winter months when the animals undergo extended fasting.

Consequently, brain Hsp70 levels may be a good biomarker of brain function with chronic PCB exposure, but this response may be seasonal in feral Arctic char.

Similar to Hsp70, the Hsp90 transcript levels were higher in the brain but not in the liver in response to PCB exposure. Hsp90 acts as a molecular chaperone and is crucial for AhR signaling (Song and Pollenz 2003; Cox and Miller, 2004; Wentworth et al., 2004; Pollenz and Buggy, 2006; Wiseman and Vijayan, 2007). When AhR is activated two molecules of Hsp90 dissociate from the receptor which then translocates to the nucleus and is eventually degraded via the proteasome (Davarinos and Pollenz, 1999; Pollenz, 2002; Wentworth et al., 2004). As Hsp90 is a key signaling molecule for transcription factor activation, including AhR and GR signaling, the higher brain Hsp90 transcript abundance suggests a key adaptive response to facilitate brain function. Although little information exists pertaining to the impact of PCBs on Hsp90, Aluru et al. (2004) did show that fasted char exposed to PCBs for four months had decreased expression of brain Hsp90 protein. As seen with hsp70 response, the likely explanation for the lower hsp90 in the fasted char relative to the fish in this study may be related to the nutritional status of the animal. Together, based on the Hsp response, it appears that the brain has a lower threshold for PCB-mediated proteotoxicity relative to the liver in Arctic char. Consequently, higher levels of Hsp70 and Hsp90 may serve an important neuroprotective role in Lake Ellasjøen char, preventing neuronal loss and helping to maintain neuroendocrine function (Aluru et al., 2004). However, it appears likely that this adaptive heat shock response may be compromised during the annual fasting cycle during the winter months in these animals.

A novel finding from this study was the significant liver GR protein expression in the Lake Ellasjøen char compared to the Lake Øyangen char. GR is a key transcription factor mediating cortisol action in fish (Vijayan et al., 2005). As cortisol is involved in all aspects animal function, including energy metabolism, osmo- and ionic-regulation, immune function and

stress adaptation, the changes in tissue GR content reflect target tissue responsiveness to this steroid stimulation (Mommsen et al., 1999; Vijayan et al., 2005). We were unable to obtain plasma samples from these fish due to logistical problems and, therefore, unable to obtain cortisol levels in these fish. It is known that plasma cortisol levels modulate liver GR content (Vijayan et al., 2003) and, therefore, we hypothesize that circulating cortisol levels are lower in char from contaminated lakes leading to a compensatory increase in target tissue receptor content. In support of this argument studies have shown reduced circulating steroid levels and decreased capacity for stress-induced cortisol production in feral fish collected from PCBs contaminated sites (Hontela, 2005). Interestingly GR transcript abundance was not significantly different in the liver (Fig. 2b) but was significantly lower in brain tissue (Fig. 3b) from Lake Ellasjøen char. Disruption of GR homeostasis has been shown to impact the cortisol response to stress by disrupting negative feedback regulation of cortisol levels (Aluru et al., 2004). It remains to be seen whether PCB exposure in Lake Ellasjøen char compromises the ability of these individuals to regulate a cortisol response to secondary stressor exposure.

5. Conclusion

Unlike other populations of teleost species that endure lifelong, even multigenerational exposure to PCBs, Lake Ellasjøen char do respond to PCB exposure by inducing Cyp1A expression. We hypothesize that this lack of insensitivity to chronic PCB exposure may be related to the seasonal feeding and fasting life style of these fish. Our results suggest that the brain is more sensitive to PCB exposure and elicits a heat shock protein response to cope with proteotoxicity in char from the contaminated lake. Changes in liver GR content suggests alteration in target tissue responsiveness to stimulation by the stress adapting hormone cortisol in feral char from the contaminated lake. Taken together, contaminant effect studies on wild

animal populations suffer from many factors related to differences between individuals and populations caused by natural variations in different ambient factors, making causative conclusions difficult. The natural setting on Bjørnøya, with two lakes in close proximity but with substantially differing contaminant loads provides a unique opportunity for elucidating possible effects of contaminants, and also identifying biomarkers, in fish sharing many similarities at the genotypic, phenotypic and ecological levels. Our results for the first time demonstrate that Arctic char living in these remote lakes are impacted by contaminant exposure. Future studies on animal performance, including stress responsiveness and immune performance of these char populations will help gain insight into the reliability of these biomarker responses as indicators of effects associated with longer-term PCB exposure in feral populations of fish.

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Chapter 4

Aryl hydrocarbon receptor signaling in rainbow trout hepatocytes: role of hsp90 and the proteasome

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Abstract

The objective of this study was to investigate the role of heat shock protein 90 (hsp90) and the proteasome in regulating arylhydrocarbon receptor (AhR) activation and cytochrome P450 1A (Cyp1A) protein expression in rainbow trout (*Oncoryhynchus mykiss*). We exposed trout hepatocytes in primary culture to the AhR agonist β-napthoflavone (βNF; 10^{-6} M) and examined AhR and Cyp1A expression. βNF induced a significant temporal accumulation of AhR and Cyp1A1 mRNA abundance in trout hepatocytes. This transcript response was followed by a significantly higher AhR and Cyp1A protein expression. Exposure to geldanamycin (GA; 1000 $ng.ml^{-1}$), a benzoquinone ansamycin antibiotic used to inhibit hsp90 function, significantly reduced (~70%) βNF-induced Cyp1A protein expression in trout hepatocytes. Also, exposure to the proteasomal inhibitor MG-132 (50 μM) completely abolished βNF-induced Cyp1A protein expression in trout hepatocytes. In addition, MG-132 treatment further enhanced the GA-mediated suppression of the Cyp1A response in trout hepatocytes. The effect of MG-132 on Cyp1A response corresponded with a significant inhibition of BNF-mediated AhR mRNA abundance, but not protein content. Altogether our results suggest a βNF-mediated autoregulation of AhR content in trout hepatocytes. We propose a key role for hsp90 and the proteasome in this ligand-mediated AhR regulation and Cyp1A response in trout hepatocytes.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix Per ARNT-Sim (bHLH-PAS) superfamily of transcription factors. In mammals only a single AhR gene (AhR1) has been identified, whereas teleosts have multiple isoforms, including AhR2 (reviewed in Hahn, 2000; Hahn et al., 2005). While teleost species, including Atlantic killifish (*Fundulus heteroclitus*) and zebrafish (*Danio rerio*) express both AhR1 and AhR2 (Hahn et al., 1997; Tanguay et al., 1999; Andreasen et al., 2002; Hahn et al., 2005), so far AhR1 gene has not been identified in rainbow trout (*Oncorhynchus mykiss*). However, three AhR2 isoforms have been identified in trout and sequence comparisons revealed that rtAhR2α and rtAhR2β are 98% identical at the amino acid level (Abnet et al., 1999a). Analysis of the partial sequence of the third AhR2 isoform in trout revealed that it has greater sequence identity to Atlantic salmon AhR2γ and AhR2δ genes than to rtAhR2α and rtAhR2β (Hannson et al., 2003, 2004). Although, multiple forms of AhR exist in fish, the functional significance in relation to isoform-specific cellular signaling is unclear.

In the unliganded state, AhR exists as a heterocomplex with other proteins, including two molecules of heat shock protein 90 (hsp90) (Meyer and Perdew, 1999; Petruilis and Perdew, 2002). According to the model of AhR action, the binding of ligands to the receptor promotes the dissociation of chaperone proteins and the formation of nuclear AhR-ARNT (AhR nuclear translocator) heterodimers that bind to xenobiotic response elements (XRE) in the promoter of target genes (Whitlock, 1999; Swanson, 2002). Indeed, AhR activation by ligand regulates the expression of a variety of genes, notably those encoding the cytochrome P450 (Cyp) family of enzymes (Whitlock, 1999; Hanokakoski and Negishi, 2000; Ma, 2001). A number of studies have demonstrated that the association between AhR and hsp90 is crucial for AhR signaling, while disruption of this interaction leads to proteasomal degradation of this receptor (Chen et al.,

1997; Meyer et al., 2000; Song and Pollenz, 2002; Wentworth et al., 2004). In addition, ligand binding also leads to AhR degradation, which is mediated *via* the proteasomal pathway (reviewed in Pollenz, 2002; Wentworth et al., 2004).

While the mechanism of AhR activation is mostly based on mammalian studies, little is known about factors regulating AhR signaling in piscine models. Only one study with zebrafish showed that, as in mammals, zfAhR2 is also rapidly degraded by the proteasome in response to ligand exposure (reviewed in Pollenz, 2002; Wentworth et al., 2004). However, a similar ligand-induced AhR reduction was not seen in rainbow trout. Specifically, trout liver AhR protein content was elevated in response to β-naphthoflavone (βNF) exposure *in vivo* as well as *in vitro* using hepatocytes in primary culture (Aluru et al., 2005; Aluru and Vijayan, 2006) suggesting species-specific differences in AhR regulation. In order to address this distinct AhR response in trout, we tested the hypothesis that hsp90 and the proteasome are critical for AhR signaling in trout hepatocytes. To this end, geldanamycin (GA), a benzoquinone ansamycin antibiotic and a well established inhibitor of hsp90 function (Grenert et al., 1997), was used as a tool to tease out the role of this chaperone in AhR signaling. We also used MG-132, a well established proteasomal inhibitor (Lee and Goldberg, 1998), to evaluate the role of the proteasome in AhR signaling and Cyp1A response.

2. Materials and Methods

2.1. Chemicals

Collagenase, βNF, L-15 medium, protease inhibitor cocktail, antibiotic and antimycotic solution, and bicinchoninic acid (BCA) reagent were purchased from Sigma-aldrich (St. Louis, MO, USA). Geldenamycin was purchased from Bioshop (Burlington, ON, Canada) and MG-132 (carbobenzoxyl-leucinyl-leucinyl-leucinal) was purchased from Calbiochem (San Diego, CA,

USA). Multiwell (6-well) tissue culture plates were obtained from Sarstedt (Piscataway, NJ, USA). All electrophoresis reagents and molecular weight markers were from BioRad (Mississauga, ON, Canada). Cyp1A antibody (mouse anti-cod Cyp1A monoclonal antibody) was from Biosense laboratories (Bergen, Norway). Hsp90 antibody (rat anti-hsp90 monoclonal antibody) was from Abcam (Cambridge, MA, USA). Polyclonal antibody to trout glucocorticoid receptor (GR) was developed in our laboratory (Sathiyaa and Vijayan, 2003). The antibody to salmon AhR was custom made (Invitrogen, Sunnydale, CA, USA) based on a conserved region of rainbow trout AhR2α and AhRβ peptide (Abnet et al., 1999) and has been used to immunodetect AhR in Baltic salmon (*Salmo salar*; Vuori et al., 2004) and rainbow trout (Aluru et al., 2005, Aluru and Vijayan, 2006). The secondary antibodies to Cyp1A, hsp90, or GR were alkaline phosphatase-conjugated to either goat anti-mouse IgG for Cyp1A (BioRad), goat anti-rat IgG for hsp90 (Stressgen, Victoria, BC, Canada) or goat anti-rabbit IgG for GR (BioRad). The secondary antibody for AhR was horseradish peroxidase-conjugated to rabbit anti-rat IgG (BioRad). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indlyl phosphate salt (BCIP) were obtained from Fisher Scientific (Nepean, ON, Canada). ECL plus substrate was purchased from Amersham Biosciences (Baie d'Urfe, QC, Canada).

2.2. Primary Cell Culture

Rainbow trout were obtained from Humber Springs Trout farm (Mono Mills, ON) and kept at the University of Waterloo Aquatic Facility at 12 ± 1 °C on a 12-h light/dark cycle and fed once daily to satiety (3 pt sinking food; Martin Mills Inc., Elmira, ON). The fish were acclimated for at least 2 weeks prior to the start of experiments and all the protocols were approved by the animal care committee. Hepatocytes were isolated using collagenase perfusion according to established protocols (Sathiyaa et al, 2001) and plated in 6-well plates in L15

medium at a density of $0.75x10^6$ cells/ml ($1.5x10^6$ cells/well). Hepatocytes were maintained at 13°C for the duration of the experiments. βNF and GA were dissolved in ethanol, while MG132 was dissolved in DMSO. The final concentration of either ethanol or DMSO in the incubation medium did not exceed 0.01%. The control cells received the same amount of ethanol or DMSO as the treatment groups. Cells were harvested, centrifuged (13,000 x g for 1 min), the supernatants removed and the cell pellets flash frozen on dry ice and stored at - 70 °C until required for analysis.

2.3. Experimental Treatments

Temporal studies were conducted to investigate the impact of βNF stimulation on Cyp1A1 and AhR mRNA abundance and protein expression. The experimental protocol consisted of replacing the media with fresh media containing βNF (final in well concentration of 10^{-6} M). Media (for lactate dehydrogenase (LDH) leakage) and cells were collected either immediately prior to βNF addition or 1, 2, 4, 8 and 24 h post-βNF treatment for analysis of either mRNA abundance or protein expression.

The impact of GA exposure on Cyp1A protein expression was conducted to determine whether hsp90 was important in AhR signaling. The experimental protocol consisted of replacing the media with fresh media containing either control (0.01% ethanol), βNF (10^{-6} M), GA (1000 ng ml^{-1}) or a combination of βNF (10^{-6} M) and GA (1000 ng ml^{-1}), where GA was added 30 min prior to addition of βNF. The GA concentration used was previously shown to block hsp90 binding to GR in trout hepatocytes (Sathiyaa and Vijayan, 2003). Cells were harvested at 1, 2, 4 or 24 h post- βNF addition and analysed for Cyp1A protein expression. Hsp90 protein expression was used as a positive control to confirm GA effect as reported previously in trout hepatocytes (Sathiyaa and Vijayan, 2003).

The proteasomal regulation of AhR signaling consisted of replacing the media with fresh media containing control (0.01% ethanol), MG-132 (50 μM), βNF (10^{-6} M), or a combination of MG-132 (50 μM) and βNF (10^{-6} M), where MG-132 was added 24 h prior to BNF. Cells were harvested either 4 h post βNF addition for quantification of AhR mRNA abundance or 24 h post-BNF addition to determine AhR, Cyp1A, and GR protein expression. GR expression was used as a positive control as previously we showed that MG-132 treatment enhanced this steroid receptor expression in trout hepatocytes (Sathiyaa and Vijayan, 2003).

For all studies the cell viability was determined by measuring the release of LDH into the medium (leakage), according to Boone and Vijayan (2002). LDH activity was low and not statistically different among the different treatment groups (data not shown).

2.4. Quantitative Real-Time PCR (qPCR)

2.4.1 RNA Isolation and First Strand cDNA Synthesis

Total RNA isolation, including DNase treatment, was performed using RNeasy mini kits according to the manufacturer's instructions (Qiagen, Mississauga, ON). The first strand cDNA was synthesized from 1μg of total RNA using a cDNA synthesis kit (MBI Fermentas, Burlington, ON) with an oligo dT primer according to the manufacturer's instructions. A relative standard curve for each gene of interest was constructed using cDNA synthesized using this method. An appropriate volume of cDNA for each gene was determined during qPCR optimization (iCycler, BioRad).

2.4.2. Primers

Primers (Table 1) were designed to amplify either a 100 bp (β-actin; GenBank accession number **AF157514** and Cyp 1A1; GenBank accession number **U62976**) or 500 bp (rainbow trout AhR) fragment of the target gene of interest. Rainbow trout AhR primer design consisted of aligning rtAhR2α and rtAhR2β (GenBank accession numbers: **AF065138** and **AF065137** respectively) and primers were designed from conserved regions of the two genes. As the full-length sequence of the third rtAhR2 is unavailable this sequence was not included in the primer design.

2.4.3. Relative Standard Curve

Relative standard curves for target genes (AhR and Cyp1A1) and a housekeeping gene (β-actin) were constructed using plasmid vectors containing the target sequences according to established protocols (Sathiyaa and Vijayan, 2003). The Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, CA) used in qPCR reaction was 2× concentrated. Every 25 µl reaction had 1.5 U Platinum Taq DNA polymerase, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dGTP, 200 µM dATP, 200 µM dCTP, 400 µM dUTP and 1 U UDG; the reaction also contained 0.2 µM forward and reverse primers, SYBR green I nucleic acid gel stain (1:4000) (Roche, Montreal, QC, Canada) and fluoroscein calibration dye (1:2000) (BioRad). To reduce pipetting errors, master mixes were prepared for triplicate reactions (3× 25 µl) for each standard and reactions were run in triplicates on 96 well PCR plates (BioRad) according to the manufacturer's instructions (BioRad).

2.4.4. Quantification of Samples

An optimized volume of cDNA was used for the amplification of each gene. The reaction components were exactly the same as the previous section and for every single test sample a qPCR for both the target (AhR and Cyp1A1) and the housekeeping gene (β-actin) was performed. The following PCR program was used to amplify β-actin: 95 °C – 3 min; 40 cycles: 95 °C – 20 sec, 49 °C – 20 sec, 72 °C – 20 sec, followed by 4 °C hold. Cyp1A1 and AhR were amplified using the following protocol: 95 °C – 3 min; 40 cycles: 95 °C – 20 sec, 49 °C – 20 sec, 72 °C – 20 sec, followed by 4 °C hold.

2.4.5. Data analysis for Quantification of Gene Expression

Calculation of the threshold cycle values (C_T) for every sample was performed using the iCycler iQ™ real time detection software (BioRad). The transcript amount for each sample was calculated for target gene and β-actin using their respective standard curves with log input amount and C_T values. The amount of target gene was divided by the amount of β-actin to determine the normalized amount of the target gene. The normalized value (a relative unit) was then standardized using an internal calibrator (control samples of each experiment) and expressed as percent control exactly as described before (Sathiyaa and Vijayan, 2003).

2.5. Immunoblot Analysis

Total protein (40 μg; unless otherwise specified) was separated on a 8% SDS-PAGE set at 200 V for 40 min using 1X TGS (250mM Tris, 1.92 M glycine, 1% SDS) and transferred onto a 0.45μM nitrocellulose membrane (BioRad) using Trans-blot® SD semi-dry electrophoretic transfer cell (BioRad). A 5% solution of non-fat dry milk in 1X TTBS (2 mM Tris, 30 mM

NaCl, 0.01% Tween 20, pH 7.5) was used as a blocking agent (1 h at room temperature) and for diluting antibodies. The blots were incubated with primary antibodies for either 1 h (Cyp1A, GR, hsp90) or 2 h (AhR; 80 ug protein loaded) at room temperature followed by 1 h incubation with the appropriate secondary antibody. Membranes were washed after incubation in either primary (2 x 15 min washes in TTBS) or secondary antibodies (2 x 15 min in TTBS followed by 1 x 5 min in TBS, 2 mM Tris, 30 mM NaCl, pH 7.5). Band detection was carried out with either BCIP-NBT substrate for Cyp1A, GR and hsp90 or with ECL plus for AhR. Images were captured with either Chemi imager™ (Alpha Innotech, San Leandro, CA, USA) for Cyp1A, GR and hsp90 or a Typhoon 9400 Variable Mode Imager (Amersham Biosciences) for AhR. All protein bands were quantified using Chemi imager™ software (Alpha Innotech).

2.6. Statistical Analyses

All statistical analyses were performed with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). A logarithmic transformation was used wherever necessary to ensure homogeneity of variance, but non-transformed values are shown in the figures. Data was analysed with either paired Student's t-test or one-way ANOVA to assess the changes in both mRNA and protein levels. A probability level of $p \leq 0.05$ was considered significant. All data are shown as mean ± standard error of mean (S.E.M.)

3. Results

3.1. AhR Expression

There was a temporal change in AhR mRNA accumulation in response to βNF exposure (10^{-6} M) in trout hepatocytes (Fig. 1A). Relative to the 0 h time point, AhR mRNA abundance was significantly higher at 4 h post-βNF addition and remained significantly elevated for the

duration of the exposure ($P < 0.05$; Fig. 1A). Peak mRNA abundance was achieved at 8 h post-βNF addition, and levels declined thereafter, although this decrease was not statistically significant. The AhR protein content was significantly higher in the βNF group by approximately 75% compared to the controls (Fig. 1B).

3.2. Cyp1A1 Expression

Cyp1A1 transcript levels showed a temporal increase after βNF addition and were significantly elevated above control (0 h) levels at 2 h and thereafter remained significantly elevated for the duration of the exposure ($P < 0.05$; Fig. 2A). Cyp1A protein expression was significantly higher at 24 h after βNF exposure compared to the control group ($P < 0.05$; Fig. 2B).

3.3. Effect of GA

There was significantly higher hsp90 protein content in cells exposed to GA compared to control cells (Fig. 3A). Addition of GA alone did not significantly impact Cyp1A protein expression compared to the control group (Fig. 3B). However, GA treatment for 24 h significantly depressed (~60%) βNF-induced Cyp1A protein expression in trout hepatocytes compared to the βNF group ($P < 0.05$; Fig. 3B). Exposure to GA even for 1, 2, 4 or 8 h prior to βNF addition caused a significant inhibition (~40%) of Cyp1A protein expression relative to the βNF positive control ($P < 0.05$; Fig. 3C).

3.4. Effect of MG-132

There was significantly higher GR protein content in cells exposed to MG-132 compared to control cells (Fig. 4A). Exposure to MG-132 did not alter AhR mRNA levels in trout

hepatocytes (Fig. 4B). However, βNF-induced AhR mRNA abundance was significantly depressed (approximately 70 %) by MG-132 ($P < 0.05$; Fig. 4B). MG-132 either alone or in combination with BNF did not significantly affect AhR protein expression compared to control cells in trout hepatocytes (Fig. 4C).

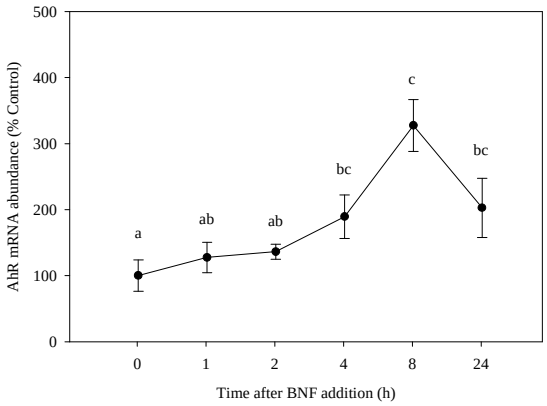
Exposure to MG-132 alone had no significant impact on Cyp1A protein expression compared to control cells (Fig. 5A). However, MG-132 significantly suppressed βNF-induced Cyp1A protein expression in trout hepatocytes compared to cells treated with βNF alone (Fig. 5A). Also, MG-132 completely abolished the Cyp1A protein response seen with βNF exposure either alone or in combination with GA (Fig. 5B).

Table 1: Sequences of oligonucleotide primers used in q PCR

Gene of Interest	Primer Sequence
β-actin	Forward: 5’ - AGA GCT ACG AGC TGC CTG AC – 3’ Reverse: 5’ - GCA AGA CTC CAT ACC GAG GA – 3’
Cyp1A1	Forward: 5’ – GAT GTC AGT GGC AGC TTT GA – 3’ Reverse: 5’ – TCC TGG TCA TCA TGG CTG TA – 3’
AhR	Forward: 5’ – CAG CGA AGG GAG CGG TAA – 3’ Reverse: 5’ – TGG ACC CGG CCA GTG ATA – 3’

Fig. 1. Effect of βNF exposure (10^{-6}M) on AhR mRNA abundance (A) and protein content (B) in trout hepatocytes in primary culture. AhR protein was immunodetected with a rabbit polyclonal anti-salmon antibody; a representative immunoblot is shown above Fig. 1B and the bands correspond to the x-axis labels; data represent mean ± SEM (n = 3 for mRNA; n = 8 for protein); time points (Fig. 1A) or bars (Fig. 1B) with different letters are significantly different (paired t-test; $P \leq 0.05$). Specific experimental details are given in the materials and methods.

A


AhR mRNA abundance (% Control)
500
400
300
200
100
0
a
ab
ab
bc
c
bc
0
1
2
4
8
24
Time after BNF addition (h)

B

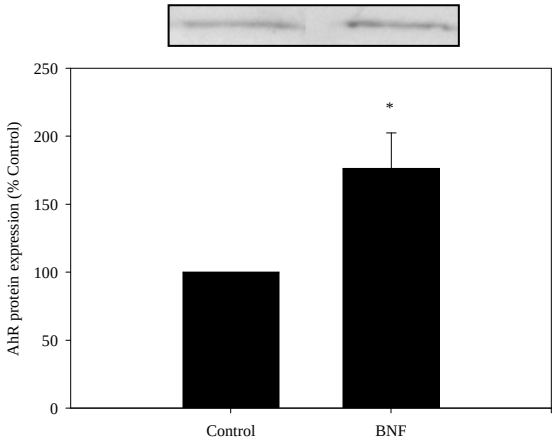
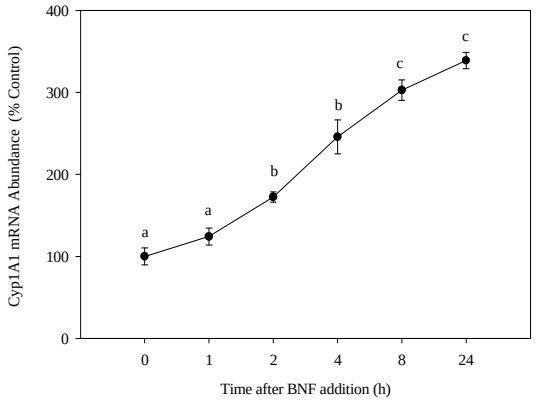

AhR protein expression (% Control)
250
200
150
100
50
0
*
Control
BNF

Fig. 2. Effect of βNF exposure (10^{-6}M) on Cyp1A1 mRNA abundance (A) and Cyp1A protein content (B) in trout hepatocytes in primary culture. Cyp1A protein was immunodetected with a monoclonal mouse anti-cod antibody; a representative immunoblot is shown above Fig. 1B and the bands correspond to the x-axis labels; data represent mean ± SEM (n = 4 for mRNA; n = 22 for protein); time points (Fig. 1A) or bars (Fig. 1B) with different letters are significantly different (paired t-test; $P \leq 0.05$). Specific experimental details are given in the materials and methods.

A


Cyp1A1 mRNA Abundance (% Control)
400
300
200
100
0
a
a
b
b
c
c
0
1
2
4
8
24
Time after BNF addition (h)

B

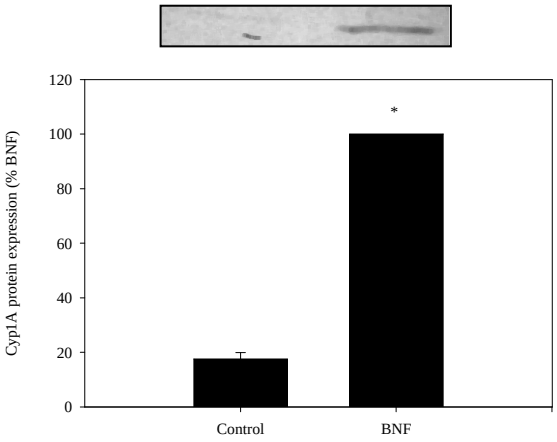
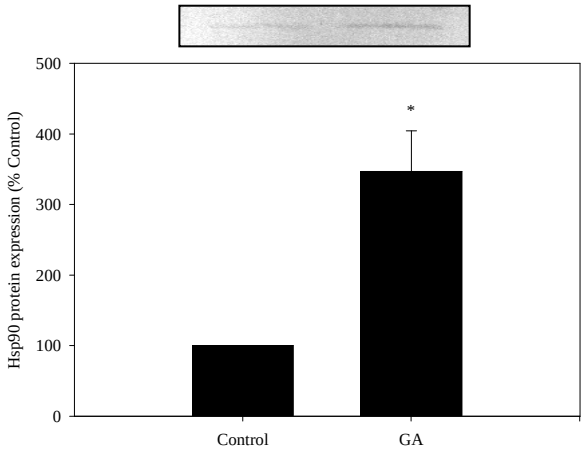
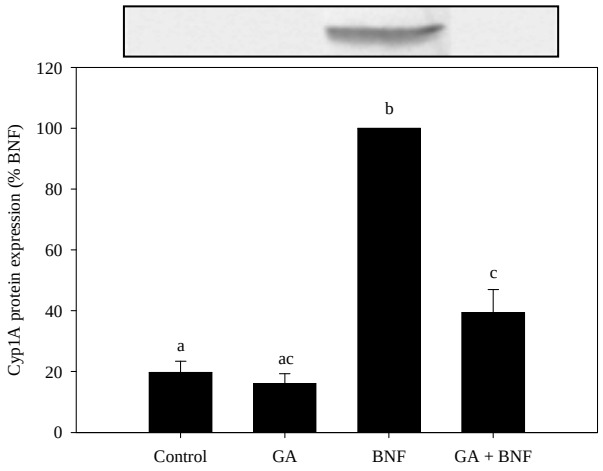

Cyp1A protein expression (% BNF)
120
100
80
60
40
20
0
*
Control
BNF

Fig. 3. Impact of geldanamycin (GA) on hsp90 (A) and Cyp1A protein expression, after either 24 h (B) or 1-8 h exposure to the drug (C), in trout hepatocytes. Hsp90 was immunodetected with a monoclonal rat anti-hsp90 antibody, while a monoclonal anti-cod antibody was used for Cyp1A; protein content was expressed as % control for hsp90 and % βNF for Cyp1A; a representative immunoblot is shown at the top of each histogram and the bands on the blot correspond to the respective x-axis labels; values represent mean ± SEM (n = 5 (A); n=10 (B); n=6 (C)); * significantly different (paired students t-test; $P \leq 0.05$); treatments with different letters are significantly different (paired t-test; $P \leq 0.05$). Specific experimental details are given in the materials and methods.

A


Hsp90 protein expression (% Control)
500
400
300
200
100
0
*
Control
GA

B


Cyp1A protein expression (% BNF)
120
100
80
60
40
20
0
a
ac
b
c
Control
GA
BNF
GA + BNF

C

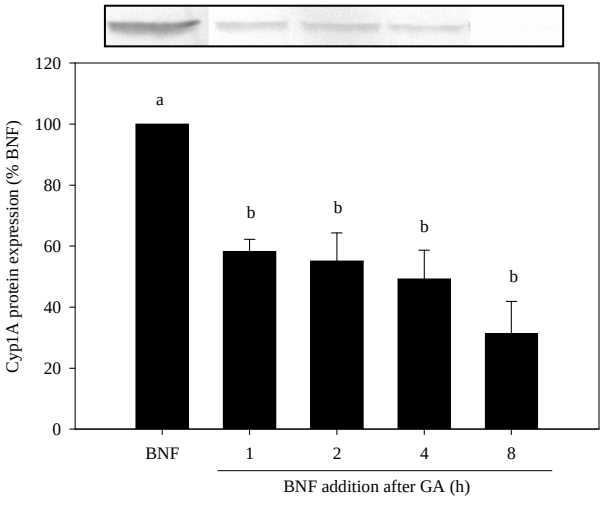
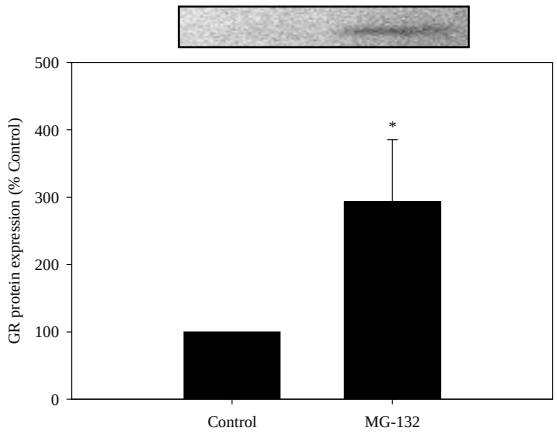
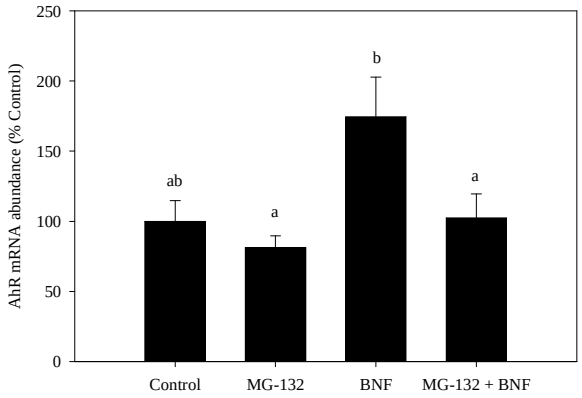

Cyp1A protein expression (% BNF)
120
100
80
60
40
20
0
a
b
b
b
b
BNF
1
2
4
8
BNF addition after GA (h)

Fig. 4. Impact of MG-132 on glucocorticoid receptor protein content (GR; A), AhR mRNA abundance (B) and AhR protein content (C) in trout hepatocytes. GR was immunodetected with a rabbit polyclonal anti-trout antibody, while a rabbit polyclonal anti-salmon antibody was used for AhR; a representative immunoblot is shown at the top of each protein histogram and the bands on the blot correspond to the respective x-axis labels; values represent mean ± SEM (n = 4 (A); n=3-7 (B); n=5-8 (C)); * significantly different (paired students t-test; $P \leq 0.05$); treatments with different letters are significantly different (paired t-test; $P \leq 0.05$). Specific experimental details are given in the materials and methods.

A


GR protein expression (% Control)
500
400
300
200
100
0
*
Control
MG-132

B


AhR mRNA abundance (% Control)
250
200
150
100
50
0
ab
a
b
a
Control
MG-132
BNF
MG-132 + BNF

C

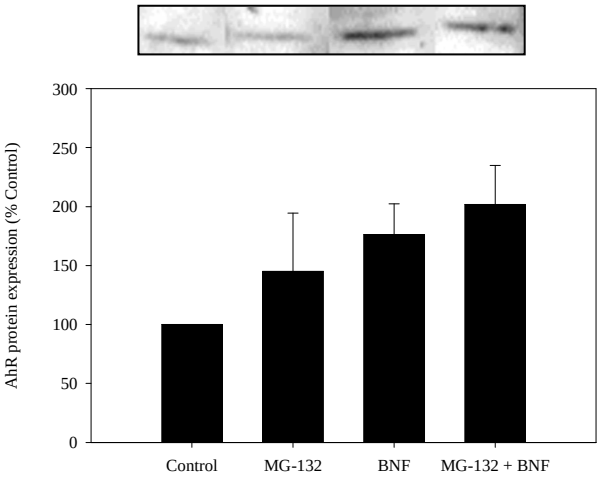
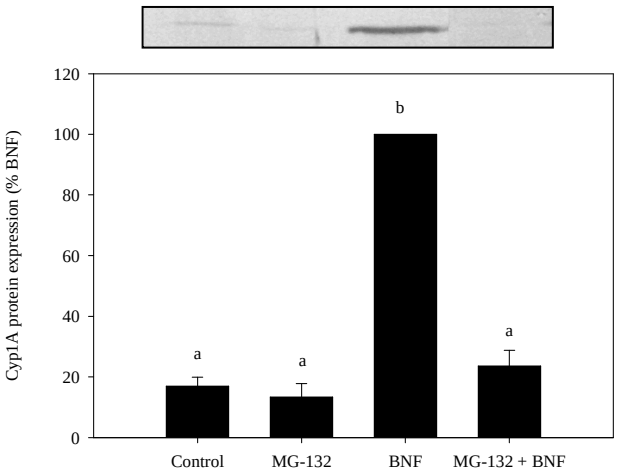
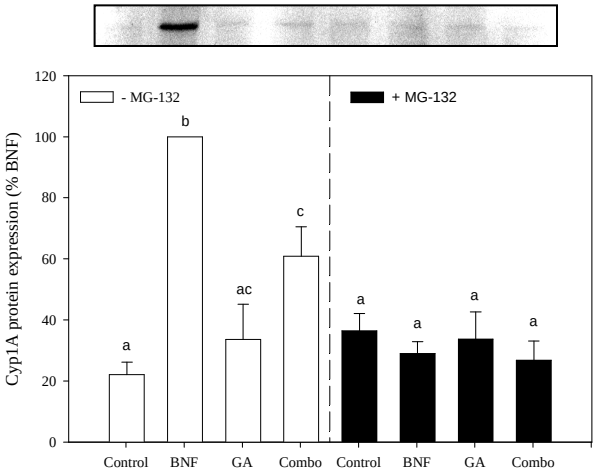

AhR protein expression (% Control)
300
250
200
150
100
50
0
Control
MG-132
BNF
MG-132 + BNF

Fig. 5. Impact of MG-132 on Cyp1A protein expression either in the absence (A) or presence of GA (B). Cyp1A protein was immunodetected with a monoclonal mouse anti-cod antibody; a representative immunoblot is shown above each histogram and the bands correspond to the respective x-axis labels; data is shown as percent BNF; values represent mean ± SEM (n = 8); treatments with different letters are significantly different (paired t-test; $P \leq 0.05$).

A


Cyp1A protein expression (% BNF)
120
100
80
60
40
20
0
a
a
b
a
Control
MG-132
BNF
MG-132 + BNF

B


- MG-132
+ MG-132
Cyp1A protein expression (% BNF)
120
100
80
60
40
20
0
a
b
ac
c
a
a
a
a
Control
BNF
GA
Combo
Control
BNF
GA
Combo

4. Discussion

We demonstrate for the first time that hsp90 and the proteasome are critical for AhR activation and βNF-mediated Cyp1A response in trout hepatocytes. Ligand binding leads to a rapid decrease in mammalian AhR1 (reviewed in Pollenz, 2002; Davarinos and Pollenz, 1999; Ma and Baldwin, 2000) and zfAhR2 protein content (Wentworth et al., 2004) and this ligand-mediated receptor degradation involves the proteasome (reviewed in Pollenz, 2002; Wentworth et al., 2004). Interestingly in trout hepatocytes AhR agonist stimulation resulted in higher AhR mRNA and protein content. This agrees with previous studies demonstrating higher AhR protein expression in response to βNF exposure in trout liver *in vivo* as well as hepatocytes in primary culture *in vitro* (Aluru et al., 2005; Aluru and Vijayan, 2006). Also, our results support the TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)-induced rtAhR2α and rtAhR2β mRNA abundance seen in trout (Abnet et al., 1999a). Although analysis of human, mouse and tomcod (*Microgadus tomcod*) AhR promoter regions failed to identify a XRE (Schmidt et al., 1993; Roy and Wirgin, 1997; Mimura et al., 1999), our results suggest that a functional XRE may exist in the promoter region of trout AhR leading to autoregulation of this receptor by ligand binding.

AhR forms a heterocomplex with several chaperone proteins, including two molecules of hsp90, in mammalian cells and this association is critical for the receptor stability, ligand binding and receptor activation (Chen et al., 1997; Meyer and Perdew, 1999; Meyer et al., 2000). Ligand binding promotes the dissociation of chaperone proteins and the formation of nuclear AhR-ARNT heterodimers that binds to XRE in the promoter of target genes, including Cyp1A1 (Berndtson and Chen, 1994; Whitlock, 1999; Swanson, 2002). Although hsp90 protein content is elevated with GA treatment as seen in other studies (Kim et al., 1999; Sittler et al., 2001; Sathiyaa and Vijayan, 2003), the chaperone protein is not functional. This is because the drug interacts directly with the ATP/ADP binding site of hsp90 and disrupts interactions between this

chaperone and associated proteins (Grenert et al., 1997). Indeed, mammalian studies demonstrated enhanced AhR degradation *via* the 26S proteasomal pathway as a mechanism for GA-mediated reduction in receptor content (Chen et al., 1997; Song and Pollenz, 2002; Meyer et al., 2000; Song and Pollenz, 2003). Also, this AhR protein degradation correlated with a 60% reduction in ligand-induced Cyp1A1 protein expression (Song and Pollenz, 2002), clearly establishing a key role for hsp90 in modulating AhR signaling. Consequently, the inhibition of βNF-induced Cyp1A expression by GA in this study leads us to propose that hsp90 is critical for AhR signaling in trout hepatocytes.

One hypothesis we tested was that the proteasome is involved in the hsp90 modulation of Cyp1A expression in trout hepatocytes. Indeed the 26S proteasome has been implicated in the TCDD-stimulated mammalian AhR1 and zfAhR2 degradation (Roberts and Whitelaw, 1999; Ma and Baldwin, 2000; Song and Pollenz, 2003; Wentworth et al., 2004; Pollenz and Buggy, 2006). Also, mammalian studies demonstrated that inhibition of the proteasome by MG-132 abolished GA-mediated reductions in AhR protein content pointing to a key role for the proteasome in hsp90-mediated AhR regulation (Santiago-Josefat et al., 2001; Song and Pollenz, 2002; Song and Pollenz, 2003). Also, in mammalian models proteasomal inhibition resulted in a greater TCDD-mediated Cyp1A1 transcript levels as well as TCDD driven luciferase activity in mouse Cyp1A1 promoter-based reporter assays, suggesting that the proteasome is involved in ligand-mediated degradation of AhR (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000). This may be due to the accumulation of nuclear AhR-ARNT heterodimers that are capable of binding DNA and activating gene transcription (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Ma et al., 2000). However, in our study MG-132 was unable to recover the GA-mediated inhibition of AhR signaling as evidenced by the suppression of the Cyp1A response even in the presence of the proteasomal inhibitor in trout hepatocytes (Fig. 5). Also, MG-132 completely

abolished the βNF -induced Cyp1A response, whereas GA reduced it by only 60% suggesting a direct role for the proteasome in regulating AhR signaling in trout hepatocytes. The observation that MG-132 suppressed AhR transcript levels, without changes in AhR protein levels lead us to hypothesize that the proteasome may be involved in the transcriptional regulation of AhR in trout hepatocytes. Taken together, these results point to an important role for the proteasome in βNF-mediated autoregulation of AhR in trout hepatocytes, while the mechanism(s) involved remains to be determined.

Attenuation of βNF-induced AhR signaling by proteasomal inhibition is a novel finding. Studies to date have reported a lack of agonist-mediated AhR degradation with proteasomal inhibitors (Santiago-Josefat et al., 2001; Song and Pollenz, 2002; Song and Pollenz, 2003, Wentworth et al., 2004) and this corresponded with a greater Cyp1A1 response (Davarinos and Pollenz, 2000; Ma and Baldwin, 2000). With the exception of a single study in zebrafish (Wentworth et al., 2004) these studies have been performed in mammalian systems where only one form of AhR (AhR1) is found. Analysis of mammalian and teleost AhR sequences revealed that the teleost AhR2 shares structural similarity with the mammalian N-terminal region of the AhR1 protein (Tanguay et al., 1999; Andreasen et al., 2002). However, major differences in the C-terminal ligand binding and transactivation domains of AhR1 compared to AhR2 do exist (Tanguay et al., 1999; Andreasen et al., 2002; Abnet et al., 1999a). Consequently, the regulation of rtAhR2α and rtAhR2β may differ from that of mammalian AhR1. In addition, studies have further shown differences in the ligand binding potential and enhancer specificity of rainbow trout AhR2α and AhR2β (Pollenz et al., 2002; Abnet et al., 1999b). As the primers and antibody used in this study do not differentiate between the rtAhR2α and rtAhR2β isoforms, we are unable to decipher whether the responses we see are attributed to both isoforms or due to changes in only one of the isoforms. Nonetheless, βNF-induced autoregulation of AhR appears distinct in

trout hepatocytes relative to the mammalian model leading us to hypothesize a key role for the proteasome in xenobiotic-mediated AhR activation and Cyp1A upregulation in trout.

5. Conclusion

In conclusion, hsp90 and the proteasome are playing a key role in modulating AhR signaling and Cyp1A response in trout hepatocytes. The mode of action of the proteasome in modulating AhR signaling in trout hepatocytes appears distinct from that seen in other animal models. βNF stimulated an increase in AhR mRNA abundance and a subsequent increase in AhR protein expression leading us to propose that βNF stimulates AhR autoregulation in trout hepatocytes. We hypothesize that this AhR autoregulation may represent a key adaptation in maintaining tissue responsiveness even after long-term exposures to AhR ligands, including polychlorinated biphenyls in rainbow trout (Celander and Förlin, 1995).

Acknowledgement

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Chapter 5

Aryl hydrocarbon receptor signaling is involved in the heat shock proteins response in rainbow trout hepatocytes

Abstract

The objective of this study was to determine whether functional cross-talk between the AhR signaling pathway and the cellular heat shock protein response occurs in rainbow trout (*Oncorhynchus mykiss*). We exposed trout hepatocytes in primary culture to the AhR antagonist resveratrol (RVT, 10^{-5} M), with or without the AhR agonist β-napthoflavone (βNF; 10^{-6} M) and cells were subjected to a heat shock to stimulate Hsp70 and Hsp90 protein expression. βNF treatment for 24 or 48 h induced a significant increase in Cyp1A protein expression and this response was attenuated by RVT. Activation of AhR signaling by βNF did not impact Hsp70 or Hsp90 protein expression in either non-heat shocked or heat shocked hepatocytes at 24 h, except for a significant elevation in unstimulated Hsp70 protein expression at 48 h in the BNF groups. Inhibition of AhR signaling by RVT reduced the heat shock-induced Hsp70 and Hsp90 protein expression in trout hepatocytes. Exposure to actinomycin-D (10^{-5} M) or cyclohexamide (10^{-5} M), inhibitors of transcription and translation respectively, confirmed that the majority of Hsp70 and 90 response to heat shock involved protein synthesis, but this was not impacted by RVT. Exposure of heat shocked cells to the proteasomal inhibitor MG-132 (50 μM) abolished RVT-mediated effects on Hsp70 protein but not Hsp90 protein expression. We propose that AhR regulation of heat shock proteins response involves either transcriptional and/or post-translational modifications, while the ligand stimulating this receptor activation with heat shock remains to be explored. Overall, our results suggest a role for AhR signaling in the heat shock-induced Hsp70 and Hsp90 response in trout hepatocytes.

1. Introduction

The cellular stress response ensures survival of healthy cells and the controlled death of unhealthy cells during stressful environmental conditions (Reviewed in Kultz, 2005). One of the best known indicators of cellular stress response is the non-specific induction of a highly conserved family of heat shock proteins (Hsps), including Hsp70 and Hsp90, in response to stressor-induced damage to the protein machinery (reviewed in Parsell and Lindquist, 1993; Iwama et al, 1998). The Hsps induction is a key aspect of the cellular adaptive response to defend against proteotoxicity (Hightower, 1991).

In addition to the non-specific cellular response, stressor-specific protein responses are also important part of the stress coping mechanisms (Reviewed in KKultz, 2005). For instance, cellular responses to polychlorinated biphenyls (PCBs) exposure involves induction of phase I enzymes, including cytochrome P4501A (Cyp1A) that facilitates biotransformation of these lipophilic contaminants to more water soluble products for excretion (Rushmore and Kong, 2002; Xu et al., 2005). This contaminant-specific response to PCB stimulation is mediated by binding of the ligand to aryl hydrocarbon receptor (AhR) and activating the signaling pathways, including AhR-responsive genes (Hahn and Stagemann, 1994; Whitlock, 1999; Nebert et al., 2004; Ma, 2001).

The AhR is a ligand mediated transcription factor that exists in a heterocomplex with a suite of accessory proteins, including two molecules of Hsp90 (Meyer and Perdew, 1999; Petruilis and Perdew, 2002). The association between AhR and Hsp90 is crucial for both AhR stability and signaling, as disruption of this interaction leads to proteasomal degradation of AhR and inhibition of AhR-responsive gene induction (Chen et al., 1997; Meyer et al., 2000; Song and Pollenz, 2002; Wentworth et al., 2004; Wiseman and Vijayan, 2007). While Hsps play a role in PCBs-mediated signaling, several studies also showed that this lipophilic contaminant

exposure impact Hsp70 and Hsp90 protein expression in piscine models (Vijayan et al., 1997, 1998; Janz et al., 1997; Weber and Janz, 2001; Weber et al., 2002; Aluru et al., 2004). While these results suggest a link between AhR activation and the Hsp response in fish cells, the mode of action is unknown. AhR interacts with a number of signaling pathways, including those associated with hypoxia (Nie et al., 2001; Prasch et al., 2004; Lee et al., 2006), estrogen receptor (Wormke et al., 2000; Safe et al., 2000; Matthews and Gustafsson, 2006), and glucocorticoid receptor (Celander et al., 1997; Vijayan et al., 2005) signaling pathways. Several mechanisms have either been identified or hypothesized as mediating crosstalk between AhR and these other signaling pathways (Carlson and Perdew, 2002; Vijayan et al., 2005). Most notably Hsp90 plays an important chaperone role in many of these signaling pathways and, therefore, thought to be a key regulator of AhR signaling (Nollen and Morimoto, 2002; Vijayan et al., 2005). However, to our knowledge no study has addressed the possibility that AhR-activation may be involved in the heat shock response as part of the adaptive defense mechanism against stressor insult. Taken together, as AhR function is dependent on its association with Hsp90 and PCBs induce Hsp70 and Hsp90 protein expression, we hypothesized that crosstalk between AhR signaling pathway and Hsp response occurs in fish cells. To address this, we used the AhR antagonist resveratrol (RVT) as a tool to block AhR signaling (Aluru and Vijayan, 2006a,b) and determined the impact of AhR on heat shock stimulated Hsp70 and Hsp90 protein expression in primary cultures of rainbow trout hepatocytes. The AhR agonist β-naphthoflavone (BNF)-stimulated Cyp1A protein expression was used as a positive control to confirm AhR activation.

2. Materials and Methods

2.1. Chemicals

Collagenase, βNF, RVT, actinomycin D (ACT D), cyclohexamide (CHX), L-15 medium, protease inhibitor cocktail, antibiotic and antimycotic solution, and bicinchoninic acid (BCA) reagent were purchased from Sigma-aldrich (St. Louis, MO, USA). MG-132 (carbobenzoxyl-leucinyl-leucinyl-leucinal) was purchased from Calbiochem (San Diego, CA, USA). Multiwell (6-well) tissue culture plates were obtained from Sarstedt (Piscataway, NJ, USA). All electrophoresis reagents and molecular weight markers were from BioRad (Mississauga, ON, Canada). Cyp1A antibody (mouse anti-cod Cyp1A monoclonal antibody) was from Biosense laboratories (Bergen, Norway). Hsp90 antibody (rat anti-Hsp90 monoclonal antibody) was from Abcam (Cambridge, MA, USA) and detects both the Hsp90α and Hsp90β isoforms. Primary antibody to Hsp70 (rabbit anti-rainbow trout Hsp70 polyclonal antibody) has been described previously. This antibody detects the inducible Hsp70 isoform but does not detect the constitutive (Hsc70) isoform (Vijayan et al., 1997). The secondary antibodies to Cyp1A, Hsp90, or Hsp70 were alkaline phosphatase-conjugated to either goat anti-mouse IgG for Cyp1A (BioRad), goat anti-rat IgG for Hsp90 (Stressgen, Victoria, BC, Canada) and goat anti-rabbit IgG for Hsp70 (BioRad). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indlyl phosphate salt (BCIP) were obtained from Fisher Scientific (Nepean, ON, Canada).

2.2 Primary Cell Culture

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Humber springs trout farm (Mono-Mills, ON, Canada). Trout were maintained in running well water at the University of Waterloo aquatic facility in 500 L aquaria and fed once daily to satiety (3 pt sinking food; Martin Mills Inc., Elmira, ON, Canada). The water temperature was maintained at 12±1 °C under a 12 h

light/dark photoperiod and fish were acclimated for at least 2 weeks before experimentation. All fish were maintained in accordance with the Canadian Council of Animal Care guidelines.

For all experiments hepatocytes were isolated using collagenase perfusion according to established protocols (Sathiyaa et al, 2001) and plated in 6-well plates in L15 medium at a density of $0.75x10^{-6}$ cells/ml ($1.5x10^{-6}$ cells/well). Unless otherwise stated all cells were allowed to recover for at least 24 h prior to any treatments and media was replaced with fresh media. Control hepatocytes were maintained at 13 °C for the duration of the experiments, while heat shock consisted of exposing cells to 28 ^{o}C (+15 ^{o}C) for 1 h and returning the plates to 13 °C.

2.3. Experimental Details

A series of studies were performed in order to investigate whether the AhR plays a role in the Hsp70 and Hsp90 response to heat shock. All experiments were conducted with hepatocytes isolated from at least 4 different fish. In each experiment, hepatocyte viability was determined by measuring lactate degydrogenase (LDH) leakage into the medium following standard protocols (Boone and Vijayan, 2002). LDH leakage was very low and was not statistically different from control cells (data not shown).

2.3.1. RVT Impact on Cyp1A, Hsp70, and Hsp90 Protein Expression

Approximately 24 h after cells were plated, the media was replaced with fresh media and cells were exposed to RVT (10^{-5} M), βNF (10^{-6} M), or a combination of both. In all experiments RVT was added 30 min prior to addition of βNF. Thirty minutes after the addition of BNF one set of plates were subjected to heat shock (+15 ^{o}C for 1 h) and then returned to 13^{o}C, while the other set served as the unstimulated control. Cells and media from all treatment groups were collected 24 h after heat shocking and stored at –80^{o}C until needed.

2.3.2 Longer-Term Impact of RVT on Hsp70 and Hsp90 Protein Expression

Approximately 24 h after cells were plated, the media was replaced with fresh media and all cells were exposed to RVT (10^{-5} M), βNF (10^{-6} M), or a combination of both exactly as mentioned above. However, here the cells were exposed to the drugs for 24 h prior to heat shocking and the samples were collected 24 h after heat shocking (or 48 h after drugs exposure) exactly as above. Cells and media from all treatment groups were collected and stored at –80 ^{o}C until needed.

2.3.3. Effect of RVT on Transcription and Translation of Hsp70 and Hsp90 Protein Expression

Cells were exposed to RVT (10^{-5} M), CHX, ACT D or a combination of CHX (10^{-5} M) and RVT (10^{-5} M) or ACTD (10^{-5} M) and RVT (10^{-5} M). Thirty minutes after the addition of inhibitors, one set of plates were subjected to heat shock (+15 ^{o}C for 1 h) and then returned to 13^{o}C. Cells and media from all treatment groups were collected 24 h post heat shock and stored at –80 ^{o}C until needed.

2.3.4. Effect of the Proteasome on RVT-Mediated Attenuation of Hsp70 and Hsp90 Protein Expression

Hepatocytes were exposed to MG-132 (50 μM), a proteasomal inhibitor, and then exposed to RVT (10^{-5} M), βNF (10^{-6} M), or a combination of both exactly as mentioned before. Thirty minutes later the cells were subjected to a heat shock (+15 ^{o}C for 1 h) and then returned to 13 ^{o}C as explained before. Cells and media from all treatment groups were collected 24 h post heat shock and stored at –80 ^{o}C until needed.

2.4. Immunoblotting

Protein concentration of hepatocytes was determined using the bichinchoninic acid (BCA) method using bovine serum albumin (BSA) as the standard. Total protein (40 μg) was separated on 8% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Proteins were separated at 200 V for 50 min using 1X TGS (250mM Tris, 1.92 M glycine, 1% SDS) and transferred onto a 0.45μM nitrocellulose membrane (BioRad) using Trans-blot® SD semi-dry electrophoretic transfer cell (BioRad). A 5% solution of non-fat dry milk in 1X TTBS (2 mM Tris, 30 mM NaCl, 0.01% Tween 20, pH 7.5) was used as a blocking agent (1 h at room temperature) and for diluting antibodies. The blots were incubated with primary antibodies for either 1 h (Cyp1A1 and Hsp70) or 2 h (Hsp90) at room temperature, followed by 1 h incubation with the appropriate an alkaline phosphatase conjugated secondary antibody. The membranes were washed after incubation in primary (2 x 15 min washes in TTBS (2 mM Tris, 30 mM NaCl, pH 7.5, 0.1% Tween-50) and secondary antibodies (2 x 15 min in TTBS followed by 2 x 15 min in TBS). Proteins of interest were detected using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) / NBT (nitro blue tetrazolium) color substrate (BioRad). The protein bands were quantified using Chemi imager™ and Alphaease softeware (Alpha Innotech, CA).

2.5. Statistics

All statistical analyses were performed with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). A logarithmic transformation was used wherever necessary to ensure homogeneity of variance, but non-transformed values are shown in the figures. Data was analysed with either paired Student's t-test or one-way ANOVA to assess the changes in protein levels. A probability level of $p \leq 0.05$ was considered significant. All data are shown as mean ± standard error of mean (S.E.M.).

3. Results

3.1. Impact of RVT on Cyp1A, Hsp70, and Hsp90 Protein Expression.

Regardless of heat shock, βNF exposure for 24 h significantly increased Cyp1A protein expression in trout hepatocytes, while RVT by itself did not affect Cyp1A expression (Fig. 1A). The βNF -induced Cyp1A response was attenuated in the presence of RVT (combination group). We were interested in determining whether inhibition of AhR signaling impacted Hsp70 and Hsp90 protein expression. In non-heat shocked cells, Hsp70 and Hsp90 protein expressions were not significantly impacted by βNF, RVT, or the combination of βNF and RVT. Similarly, exposure to βNF did not significantly affect Hsp70 or Hsp90 protein expression in heat shocked cells. However, RVT exposure either alone or in combination with βNF significantly reduced heat shock-induced Hsp70 protein expression relative to the control cells but not the BNF-treated cells (Fig. 1B). Heat shock-induced Hsp90 protein expression was also significantly lower in the RVT group compared to the control but not the βNF group, while the Hsp90 expression in the combination group was significantly lower than both the control and βNF groups (Fig. 1C).

3.2. Impact of Longer-Term RVT Exposure on Hsp70 and Hsp90 Protein Expression

In the absence of heat shock, exposure to βNF for 48 h either alone or in combination with RVT significantly increased Hsp70 protein levels compared to control cells (Fig. 2A). Longer-term exposure to BNF either alone or in combination with RVT did not significantly affect Hsp90 protein expression, while RVT alone significantly reduced Hsp90 levels compared to the control group (Fig. 2B).

Heat shocking significantly elevated Hsp70 and Hsp90 protein expression compared to unstimulated cells, while RVT either alone or in combination with BNF significantly reduced the

heat shock-induced Hsp70 and Hsp90 protein expression compared to the control cells (Fig. 2A and B).

3.3. Effect of Actinomycin D (ACTD) and Cycloheximide (CHX) on Hsp70 and Hsp90 Protein Expression

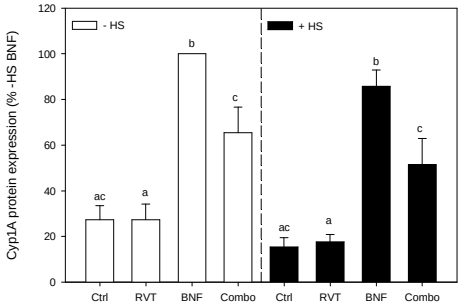
Exposure to CHX or ACTD alone significantly decreased heat shock-induced Hsp70 and Hsp90 protein expression (Fig. 3 and B). Co-exposure with RVT did not significantly affect protein expression compared to CHX or ACTD alone.

3.4. Effect of the Proteasome Inhibitor MG-132 on Hsp70 and Hsp90 Protein Expression

Exposure to MG-132 completely abolished the significant reduction in heat shock-mediated Hsp70 expression seen in the RVT treated cells either alone or in combination with BNF compared to the control cells (Fig. 1B and Fig. 4A). However, the heat shock-mediated significant reduction in Hsp90 protein expression in the RVT and combination groups compared to the control group (Fig. 1C) was not significantly affected by MG-132 treatment (Fig. 4B).

Fig. 1. Impact of resveratrol (RVT) on Cyp1A (A), Hsp70 (B), and Hsp90 (C) protein expression. Hepatocytes were treated with control (0.01% ethanol), RVT, BNF, or a combination of RVT and BNF in which case RVT was added 30 min prior to BNF. Half of the cells were subjected to a 1 h heat shock at + 15 ^{o}C and then returned to a 13 ^{o}C incubator. All cells were collected 24 h later and protein was isolated for immunoblotting. All values represent mean ± S.E.M . ($N \geq 4$) and bars with different letters are statistically different (paired t-test; $P < 0.05$).

A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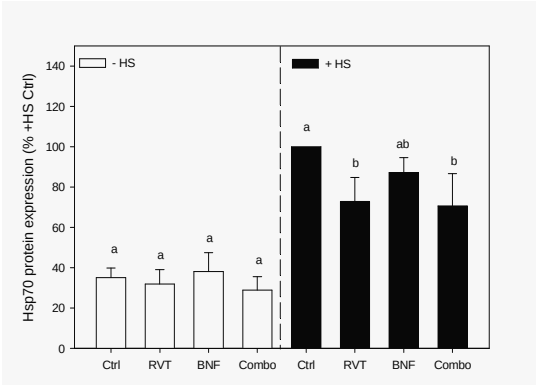
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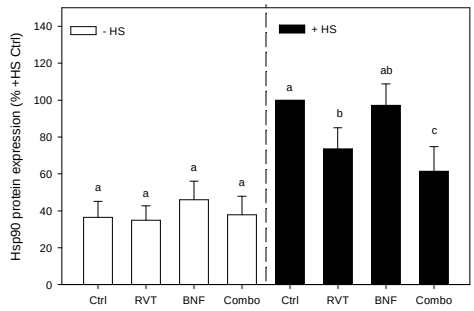
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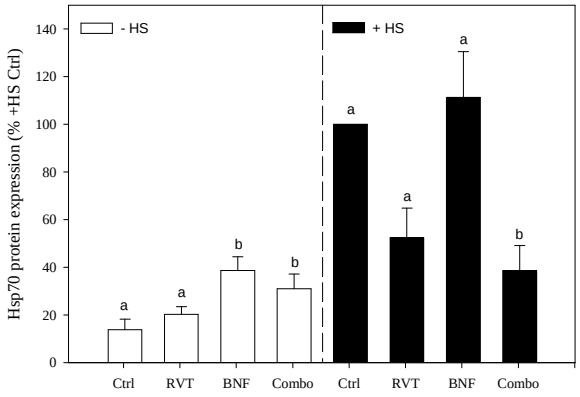
Fig.2. Impact of elevated AhR protein expression on Hsp70 (A) and Hsp90 (B) protein expression. Cells were exposed to RVT (10^{-5} M), βNF (10^{-6} M), or a combination of both in which case cells were exposed to RVT 30 min prior to addition of BNF. All cells were then incubated at 13 ^{o}C for 24 h. At the end of the 24 h incubation period a media change was performed and cells were exposed to the same treatment regime described above. Thirty minutes after addition of βNF half of the cells were subjected to heat shock (+15 ^{o}C for 1 h) and then returned to 13 ^{o}C. Cells and media from all treatment groups were collected 24 h post heat shock and stored at –80^{o}C until needed. All cells were collected 24 h later and protein was isolated for immunoblotting. All values represent mean ± S.E.M . ($N \geq 4$) and bars with different letters are statistically different (paired t-test; $P < 0.05$).

A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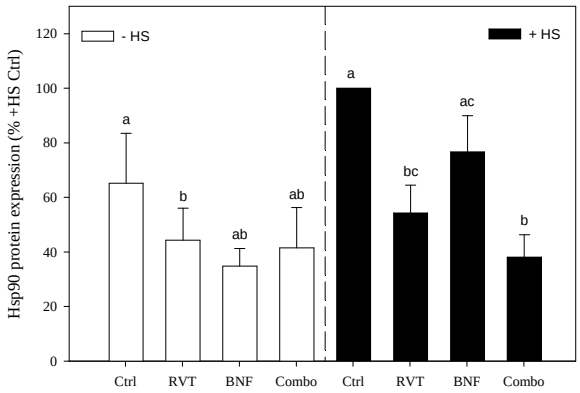
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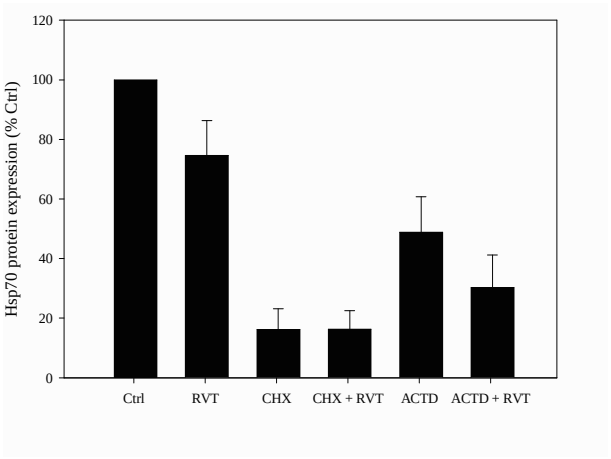
Fig. 3. Impact of transcription and translation inhibition on Hsp70 (A) and Hsp90 (B) protein expression. Hepatocytes were treated with control (0.01% ethanol), RVT, ACTD, CHX, or a combination of ACTD and RVT or CHX and RVT. In the combination groups RVT was added 30 min prior to ActD or CHX. Heptocytes were subjected to a 1 h heat shock at + 15 ^{o}C and then returned to a 13 ^{o}C incubator. All cells were collected 24 h later and protein was isolated for immunoblotting. All values represent mean ± S.E.M . ($N \geq 4$) and bars with different letters are statistically different (paired t-test; $P < 0.05$).

A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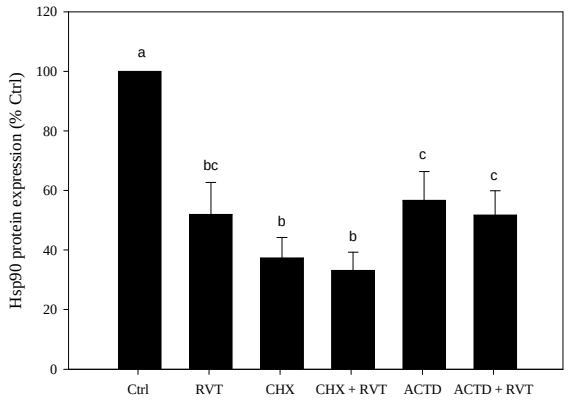
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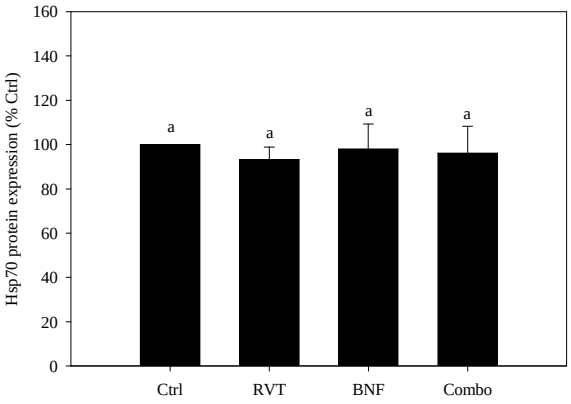
ACTD + RVT

Fig. 4. Impact of proteasomal inhibition and heat shock on Hsp70 (A) and Hsp90 (B) protein expression. Cells were treated with the proteasomal inhibitor MG-132 30 min prior to treatment with either control (0.01% ethanol), RVT, BNF, or a combination of BNF and RVT in which case BNF was added 30 min prior to RVT. Cells were subjected to a 1 h heat shock at + 15 ^{o}C and then returned to a 13 ^{o}C incubator. All cells were collected 24 h later and protein was isolated for immunoblotting. All values represent mean ± S.E.M . ($N \geq 4$) and bars with different letters are statistically different (paired t-test; $P < 0.05$).

A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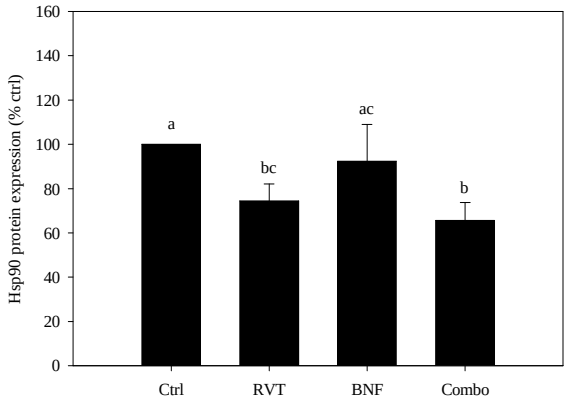
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4. Discussion

We demonstrate for the first time that inhibition of AhR signaling attenuates the heat shock-induced Hsp70 and Hsp90 protein expression in trout hepatocytes. Although the mechanism of action is unclear, our results suggest a cross-talk between AhR signaling and the proteasomal pathways in modulating the Hsp70 response in rainbow trout.

Recent studies have established the role of RVT as an AhR antagonist in animal models, including piscine cells (Aluru and Vijayan, 2006a, b). Several mechanisms have been proposed for RVT inhibition of AHR signaling in mammalian models, including competition with agonists for binding sites (Casper et al., 1999; Chen et al. 2004). Also, RVT binding stimulates the nuclear translocation of AhR and binding to response elements, but the transactivation of AhR-responsive genes, including Cyp1A1, is inhibited (Casper et al., 1999). Our results are in agreement with other studies clearly showing an attenuation of the AhR agonist-induced Cyp1A expression with RVT (Ciolino et al., 1998, Casper et al., 1999; Chen et al., 2004; Aluru and Vijayan, 2006a,b) confirming inhibition of AhR signaling in trout hepatocytes.

Induction of Hsps is a non-specific response to any acute or chronic stressor that threatens or damages cellular macromolecules, in particular protein homeostasis (Lindquist and Craig, 1988; Morimoto, 1998). The protective role of Hsps is largely attributed to their role as molecular chaperones involved in protein folding, maintenance of native protein conformation, and repair or promotion of the degradation of misfolded proteins (Lindquist and Craig, 1988; Hightower, 1991; Morimoto, 1998). The actions of Hsps allow cells to re-establish protein homeostasis in response to toxic insults. The goal of this study was to explore whether AhR signaling is involved in this highly conserved heat shock response to defend cells against proteotoxicity. Clearly βNF stimulated Hsp70 but not Hsp90 protein expression in trout hepatocytes, but this was evident only after longer-term exposure (48 h) suggesting activation of

the cellular stress response to chronic exposure of this drug. We have shown before that βNF exposure in vivo stimulates liver Hsp70 expression in rainbow trout (Vijayan et al., 1997). However, the Hsp70 response to βNF is of a lower magnitude compared to heat shocked cells, a well established proteotoxic stressor. Also, RVT failed to inhibit this Hsp70 response leading to the proposal that AhR signaling is not involved in the unstimulated Hsp70 response to this AhR ligand. We propose that the βNF -mediated Hsp70 response involves AhR- independent pathways, but the precise mechanism remains to be determined.

Indeed, the reduction of heat shock-induced Hsp70 and Hsp90 protein expression with RVT for the first time establishes a role for AhR signaling in the heat shock response. Also, the greater magnitude of this response with longer RVT exposures further confirms the involvement of AhR receptor in this response. However, to our knowledge no study has reported a role for AhR or RVT in the cellular response to heat shock. As this Hsp response to RVT was not seen in the absence of heat shock, it appears likely that AhR signaling is modulating the induction of heat shock proteins. This is supported by the observation that both transcriptional and translational inhibitors suppressed the heat shock-induced Hsps in trout hepatocytes, while this response was not modified by RVT treatment. Consequently, we hypothesize that cross-talk between the AhR signaling and the heat shock signaling pathway may be involved in the heat shock response in trout hepatocytes. However, the ligand mediating this AhR activation in response to heat shock remains to be elucidated. The absence of Hsps response with βNF in the heat shocked cells leads us to propose that heat shock response may involve AhR signaling pathways independent of βNF activation. It remains to be seen if this response is specific to RVT stimulation and independent of AhR activation in trout hepatocytes. Other studies with RVT showed both increased (Wang et al., 2006; Bezstarosti et al., 2006) and decreased (Bezstarosti et al., 2006) Hsp27 expression. Using 2D gel electrophoresis Bezstarosti et al. (2006) identified two

molecular forms of Hsp27 that were differentially impacted by RVT. The authors suggest that RVT may impact Hsp27 expression through its stimulation of MAP kinases. Resveratrol increased the expression of the phosphorylated for of Hsp27.

As it is well established that the proteasome plays an important role in regulating cellular protein levels (Ciechanover, 2005) we determined whether inhibition of AhR signaling by RVT attenuates Hsp70 and Hsp90 protein expression via a proteasome-mediated mechanism. Indeed, inhibition of the proteasome abolished the Hsp70 response to RVT exposure leading to the suggestion that this AhR-mediated signaling prevents Hsp70 degradation. However, the Hsp90 response to RVT was not altered by the proteasomal inhibitor leading to the proposal that distinct mechanisms of action may be involved in AhR-mediated regulation of Hsp70 and Hsp90 protein expression in trout hepatocytes. Taken together, AhR signaling may be involved in maintaining the Hsps response to heat shock stimulation, but the mode of action appears distinct.

5. Conclusion

In conclusion, this study further demonstrates the utility of RVT as a tool to elucidate the dynamic role of AhR in cellular response pathways. This is the first study to demonstrate that the AhR plays a role in the Hsp70 and Hsp90 response to heat shock. Although the mechanism of this action is unclear it appears that the proteasome plays a role in the degradation of Hsp70 but not Hsp90. Future studies are needed to elucidate AhR involvement in the cellular response to heat shock, in particular to understand the mechanism of action of AhR in modulating the Hsps response. Although the physiological implications of these findings are unclear, these results demonstrate a cross-talk between the AhR and the heat shock signaling pathway in trout hepatocytes

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Chapter 6

Transient gene expression in the liver during recovery from an acute stressor in rainbow trout

Abstract

The physiological response to stressors, including hormonal profiles and associated tissue responsiveness, has been extensively studied with salmonid fish, but less is known about the genetic basis of this adaptive response. As liver is the major target organ for metabolic adjustments, we exploited a selective transcriptomics approach to address molecular response in this tissue during acute stress adaptation in rainbow trout. The stressor consisted of a standardized 3 min handling disturbance of trout, and plasma and liver samples were collected either prior to or 1 and 24 h after stressor exposure.

We developed a low density custom cDNA array consisting of 147 rainbow trout genes designed from conserved regions of fish sequences available in GenBank. All amplicons were similar in size (~450-550bp) and the targeted genes had established roles in physiological processes, including stress and immune function, growth and metabolism, ion and osmoregulation and reproduction. The acute stress response and recovery was confirmed by the transient elevation in plasma cortisol concentration at 1 h, which returned to pre-stress levels over a 24 h period. This was accompanied by significant upregulation of 40 genes at 1 h, and 15 genes at 24 h after stressor exposure in trout liver. Many of these genes were involved in energy metabolism, implicating a rapid liver molecular reprogramming as critical for the metabolic adjustments to an acute stressor. Several other transcripts not previously implicated in the stress response process in fish, including genes involved in immune function and protein degradative pathways, were found to be stress-responsive in trout. A large number of these transiently elevated stress-responsive transcripts were also shown previously to be glucocorticoid-responsive in fish.

Transient upregulation of metabolic genes may play a key role in regaining homeostasis after an acute stressor. Our results suggest a role for stressor-mediated genomic cortisol signaling

in the liver molecular programming associated with stress in fish. Overall, the study demonstrates the complex nature of the adaptive stress response at the genetic level and underscores the utility of functional arrays for identifying stress coping mechanisms.

1. Introduction

The cortisol response to stressor exposure has been studied in a number of teleostean fishes. The magnitude and duration of this stress steroid response in large part is determined by the type, duration and the intensity of the stressor and is also species-specific (Bonga, 1997; Barton et al, 2002; Iwama et al, 2006). This conserved stress response has adaptive value and is thought to allow animals to regain homeostasis after a stressor insult (Bonga, 1997; Mommsen et al, 1999; Barton et al, 2002; Iwama et al, 2006). While most studies have examined plasma cortisol response to stressors, very little is known about the genetic basis of this adaptive response.

The biochemical response that accompanies the stress response does play a role in the metabolic adjustments critical for meeting the increased energy demand (Mommsen et al, 1999). Most studies so far have focused on the glucose response as this metabolite is a key fuel to meet the enhanced energy demand to regain homeostasis. The glucose response to stressors is mediated in part by the stress hormones catecholamines and glucocorticoid and involves enhanced liver metabolic capacity, including glycogenolysis, glycolysis and gluconeogenesis. While catecholamine impact is thought to be immediate and involves glucose production by glycogenolysis, the cortisol response allows for longer-term glucose maintenance and glycogen repletion during stress recovery (Mommsen et al, 1999). The enhanced liver gluconeogenic and amino acid catabolic capacity, along with peripheral proteolysis and elevated plasma amino acid levels in cortisol treated fish supports this contention. Taken together, stress-mediated cortisol response may be critical in the liver metabolic programming to regain homeostasis.

While most studies focused on tissue and plasma metabolite levels to stressors, very few studies have examined molecular responses to stressors in fish. Recent development of microarrays for non-model species, including teleostean fishes (Gracey et al, 2001; Ton et al,

2002; Larkin et al, 2003; Krasnov et al, 2005; Moens et al, 2006; Li and Waldbieser, 2006) will rapidly improve this situation (Gracey and Cossins, 2003). While a genomics approach was utilized to examine the impact of repeated handling stress on brain and kidney gene expression patterns in rainbow trout (Krasnov et al, 2005), no study has addressed the impact of acute stressor exposure and recovery on liver transcriptomics. As liver metabolism is crucial for stress adaptation, the associated transcriptional response is particularly important in order to identify the genetic basis of homeostatic adjustments.

To this end, we assessed gene expression pattern in the liver during recovery from a standardized acute handling disturbance in rainbow trout. Previous studies have established that such an acute, short handling stressor, followed by recovery, elevates plasma cortisol level rapidly, but the levels return to basal, unstressed levels, within 24 h of recovery confirming homeostasis of this steroid in circulation (Bonga, 1997; Barton et al, 2002; Vijayan et al, 2005). As liver is the main tissue in the metabolic adjustments to stress, we investigated transcriptional responses of some of the well established proteins involved in intermediary metabolism and endocrine signaling of liver function (Gracey et al., 2001). To this end, we developed a low density custom array consisting of rainbow trout genes with established roles in physiological processes, including stress and immune function, growth and metabolism, ion and osmoregulation and reproduction, to examine their role during stress adaptation in trout. Although larger high-density arrays allow for simultaneous analysis of thousands of genes, our custom array, with its high percentage of metabolic and endocrine related genes is ideally suited to the study of the stress response as it has been well established that recovery from stressor exposure is energetically demanding, and involves hormone signaling (Wendelaar Bonga, 1997; Gracey et al., 2001).

2. Materials and Methods

2.1 Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*, ~200g) were purchased from Rainbow Springs Trout Farm (Thamesford, ON, Canada). Groups of 6-7 fish each were maintained in three 100-L tanks with continuous running water at 13 °C and 12L:12D photoperiod for 1 month prior to the start of the experiment. Fish were fed once daily to satiety with 3-point sinking food (Martin Mills Inc., Elmira, ON, Canada) for 5 days a week. Food was withheld 24 h prior to the stress experiment.

2.2 Experimental Protocol

Trout were stressed using a standardized handling disturbance consisting of 3 minutes of netting and chasing, as previously described by Vijayan et al. (1997b). Tissue samples were collected at 0, 1 and 24 h after stressor exposure. All animal handling and tissue collection was done quickly. Sampling consisted of netting the fish at the respective times and anaesthetizing them with an overdose of 2-phenoxyethanol (1:1,000). Fish were bled by caudal puncture and the blood was collected in heparinized tubes, centrifuged (6,000g for 5 min at 4 °C), and the isolated plasma was frozen for subsequent cortisol and glucose determination. Liver tissues were frozen in liquid nitrogen and stored at -70^{o}C for microarray analysis. Plasma cortisol concentration was determined using a commercially available radioimmunoassay (RIA) kit (ICN Biomedicals, CA, USA) according to established protocols (Vijayan et al., 2003). Plasma glucose concentration was measured colorimetrically using the Trinder method (Sigma-Aldrich, St. Louis, MO, USA).

2.3 Isolation and Quantification of Total RNA

Total RNA was isolated from 30 mg tissue from each fish separately using either the RNeasy RNA isolation system (Qiagen, Valencia, CA, USA; acute stress study) or the Trizol method (Gibco-BRL, Burlington, ON, Canada; generation of cDNA clones). Isolated RNA was digested with DNase1 and all steps were performed according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically by the A260/A280 method, the RNA integrity confirmed by formaldehyde agarose gel electrophoresis, and stored at -70^{o}C until further use.

2.4 cDNA Clones

A total of 147 unique cDNA fragments were amplified by RT-PCR from total RNA isolated from rainbow trout liver, intestine, ovary, brain or kidney tissue. Primers (17-21 nucleotides) were generally designed to amplify fragments between 450-550 base pairs in length using either the internet primer design program Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA). The primer pairs were designed from the most conserved region of the cDNA sequence for each gene, based on sequence alignment with all available gene sequences, including other species of fish. Primer pairs that consistently amplified only a single product, confirmed by separating on a 1% agarose gel stained with ethidium bromide, were used in the array construction. Primers were obtained from Alpha DNA (Montreal, QC, Canada).

PCR products were cloned into the Invitrogen pCR-Topo II vector (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol, except all reaction volumes were halved. Ligation of the PCR products into the vector was verified by PCR with the vector specific M-13 forward and reverse primers. Plasmids containing the desired PCR fragments

were isolated using a Qiaprep Spin miniprep plasmid isolation kit (QIAgen). All plasmids were sequenced, for correct insert confirmation, at the Center for Environmental Health, at the University of Victoria (Victoria, B.C. Canada).

2.5 Probe Preparation

Each gene fragment was amplified in ten PCR reactions (100 µl each or 1 ml total) in a thermal cycler (MJ Research, Watertown, MA, USA) using gene specific primers in the following PCR reaction: 1X Taq Polymerase buffer containing magnesium chloride, each dNTP at a concentration of 300 µM, 3.75 U of Taq (Promega, Madison, WI, USA), 3 ng of plasmid, and 0.6 µM of each specific primer. The following conditions applied: 35 cycles of 94°C for 30 s, appropriate annealing temperature (45 to 55^{o}C depending on gene specific primers) for 60 s, and 72 °C for 2 min followed by a final 12 min extension at 72°C. PCR products were visualized on a 1.2% agarose gel stained with 1 µg of ethidium bromide/ml to confirm correct size of amplicon as well as minimal primer dimer. The ten PCR reactions for each gene fragment were pooled and purified using the Qiagen QIAquick PCR Purification kit. Concentration of each gene fragment was determined via spectrophotometry, the samples were speed vacuum dried, resuspended in 3X SSC (0.45 M sodium chloride, 0.045 M sodium citrate, pH 7.0) to a final concentration of 0.5 µg/ml for each gene fragment. The gene fragment was confirmed with a 1.2% agarose gel.

2.6 Array Printing

Prior to printing, cDNA clones were diluted to 50 ng/µL in 3x SSC and placed in a 384 well plate. The cDNA fragments were printed onto poly-L lysine coated glass slides (Telechem, Sunnyvale, CA, USA) using a BioRad ChipWriter Pro robotic System (BioRad, Mississauga,

ON, Canada) equipped with 6 Stealth 2 pins (Telechem). Each cDNA fragment was printed side by side in triplicate. In addition, a 500 bp fragment from the Lambda Q bacterial gene was printed onto each slide in order to control for hybridization inefficiencies and for dye normalization. After printing, all slides were UV cross linked at 70 mJ for 2 min. Slides were then washed in 0.2% SDS for 2 min., followed by 3 washes in milli-Q water for 2 min each, and finally a 5 min. wash in 95^0C milli-Q water to denature the cDNA probes.

2.7 Preparation of Fluorescently Labeled cDNA Targets

The procedure used to label RNA was adapted from protocols for *Arabidopsis thaliana* arrays described in detail at the Institute for Genomic Research site (TIGR; http: atarrays.tigr.org/arabprotocols.html). As there were 6-7 fish per tank, three independent RNA samples were generated at each time point (0,1 and 24 h) after stressor exposure by pooling equal quantities of total RNA from 2 fish livers. For each of the 6 microarray slides used in the study 2 separate reverse transcription reactions were performed (RNA from control and stressed trout) for a total of 3 slides each for the 1 and 24 h samplesafter stressor . The same control RNA was used to hybridize the 1 h and 24 h sample post-stressor exposure. For a single reaction, 60 μg total RNA (from either control or stressed fish) was mixed with 0.75 μg oligo$(dT)_{12\text{-}18}$ primer (Invitrogen) and spiked with 5 ng lambda Q bacterial mRNA which had been *in vitro* transcribed from the lambda Q cDNA fragment used in array printing. The reaction was mixed, heat denatured at 70^oC for 10 min and then snap cooled on ice. The remaining components of the reverse transcription reaction were added as follows: 6 μL 5x Superscript II reverse transcription buffer (Invitrogen), 3 μL 0.1M DTT, 400 U Superscript II reverse Transcriptase (Invitrogen), 0.6 μl of 50x aminoallyl dNTP mix (containing 25mM each of dATP, dCTP, dGTP, 15mM dTTP, and 10mM aminoallyl-dUTP; Sigma-Aldrich, Oakville, ON, Canada), and 40 U ribonuclease

inhibitor (MBI fermentas, Burlington, ON, Canada). Aminoallyl cDNA was synthesized at 42^{o}C for 3 h. The reaction was stopped by heating at 70^{o}C for 15 min along with the addition of 10 µL 1M NaOH and 10 µL 0.5 M EDTA to hydrolyse the RNA, and 10 µL 1M HCl to neutralize the reaction. After drying in a speed vac centriguge, the purified aminoallyl cDNA was mixed with 40 nmol of either Cy3 (control samples) or Cy5 (post-stress samples) dye (Amersham) suspended in 9 µl 0.1 M sodium bicarbonate. The dye coupling reaction was incubated in the dark for 90 min. The coupling reaction was terminated by the addition of 7.5 µl of 4M hydroxylamine and the reaction was allowed to proceed for 15 min. in the dark. A QIAquick PCR purification kit (Qiagen) was used to purify the aminoallyl labeled cDNA and the target was concentrated in a speed vac centrifuge.

2.8 Array Hybridization and Washing

Hybridization buffer containing the Cy3 and Cy5 labeled targets were spread onto the microarray surface and covered with a 22mm x 22mm hybrislip (Sigma). Hybridizations were carried out in Corning hybridization chambers submerged in a 50^{o}C water bath for 16 h. After hybridization, the slides were rinsed in 0.2x SSC in order to remove the hybrislips. The slides were washed three times with agitation at 37^{o}C in 0.1% SDS with 0.1 x SSC, followed by three washes with agitation at room temperature in 0.1 x SSC, and dried by centrifugation at 2000 x g for 2 min.

2.9 Image analysis

Microarray slides were scanned at 80% laser power and 800 sensitivity using Versarray chipreader software Version 2.0 (BioRad, Canada). The excitation setting was at 635 nm (Cy5) and 532 nm (Cy3) and the images were captured in TIFF format. Spot finding and quantification

was carried out with ImaGene 3.0 microarray image analysis software (BioDiscovery; Los Angeles, CA). Briefly, a grid was created taking into account the number of spots, the printing pattern, and the size of the spots. Spot finding was performed using a semiautomatic method in which each spot was checked for proper alignment within the grid manually. Finally, spot quantification was performed to obtain the expression level of each gene on the array.

2.10 Data analysis

The data analysis was carried out using microarray data analysis system (MIDAS) (Saeed et al, 2003). Poor or negative control spots were flagged as unreliable and were excluded from the analysis. For each spot, signal/noise (SN) threshold was calculated using the following formula: *[I(A or B) + Bkg (A or B)]/ Bkg(A or B)*, where I(A) and I (B) denotes background corrected signal intensity for channel A and B; Bkg(A) and Bkg(B) denotes background intensity for channel A and B respectively. The SN ratios for each channel was compared to the set threshold value of 2.0 and any spots which were less than or equal to 2.0 were marked as bad and excluded from downstream analysis. The data were then LOWESS normalized before calculating the gene expression ratios. The data was calculated as a ratio of normalized gene intensity for samples taken after stressor exposure to that of the 0 h time unstressed samples. The raw dataset has been deposited into the Arrayexpress repository under accession number E-MEXP-622. Six genes from the array were randomly selected for gene quantification using quantitative real-time PCR to check for consistency in gene expression patterns observed with the microarray.

2.11 Semi-Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was performed on cDNA generated from 1μg of RNA obtained from the same pooled RNA samples used in the microarray study. Primers were designed using rainbow trout GR, PEPCK, GLUT II, AhR, prostaglandin D synthase, cathepsin D and β-actin cDNA to amplify a short fragment (~100-500 bp) for qPCR (Table 1). Relative standard curves for the target genes and housekeeping gene (β-actin) were constructed from a serial dilution of their plasmid DNA according to Sathiyaa and Vijayan (2003). Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, CA) used was 2X concentrated and every 25 μl reaction had 1.5 U Platinum Taq DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM dGTP, 200 μM dATP, 200 μM dCTP, 400 μM dUTP, and 1 U UDG; the reaction also contained 0.2 μM forward and reverse primers, fluoroscein calibration dye (1:2000; BioRad) and SYBR green I nucleic acid gel stain (1:100,000; Roche, Mississauga, ON). Samples and standards were run in triplicates on a 96 well PCR plate (Ultident, Montreal, PQ) and an optimized volume of cDNA was used for the amplification of each gene. The reaction components were exactly as above and for every single test sample a qPCR for both the target and the housekeeping gene was performed. The following PCR program was used for gene amplification: 95°C – 3 min; 40 cycles: 95°C – 20 s, Tm (annealing temperature) – 20 s, 72°C – 20 s, followed by 4 °C hold.

2.12 Data analysis for quantification of gene expression

Calculation of the threshold cycle values (C_T) for every sample was performed using the iCycler iQ™ real time detection software (BioRad). Serial dilutions of plasmids containing inserts of genes of interest were used for standard curves. The C_T values for β-actin (house

keeping gene) were consistent between treatments. The input amount for each sample was calculated for target gene and β-actin using the appropriate standard curves. The amount of target gene was divided by the amount of β-actin to determine the normalized amount of the target gene. The target gene was then standardized using an internal calibrator (0 h time samples) and the mRNA abundance post-stressor exposure expressed as percent 0 h unstressed levels.

2.13 Statistical analysis

The data are shown as mean + S.E.M. Statistical differences in data sets between the post-stressor exposure time points (1 and 24 h) from the unstressed control group (0 h) was compared using Student's t-test (n = 3 independent samples) as mentioned before for gene expression profiling (34). For statistics, where necessary data was log-transformed for homogeneity of variance, but non-transformed values are shown in the figures and tables. Significance level was set at $P \leq 0.05$.

3. Results

3.1 Array construction

A low density (147 gene) array was constructed from cDNA fragments amplified from multiple rainbow trout tissues, including liver, intestine, ovary, brain and kidney (Table 2). Most fragments were between 450-550 base pairs in length and amplified from the most conserved regions of the gene. The genes chosen for the array were broadly categorized into functional groups including, metabolism, transport, signaling, chaperones, structural, oncogenes, endocrine, immune and developmental-related genes (Table 2), while the biological function of significant genes were grouped based on gene ontology (Tables 3 and 4). Hybridization with fluorescently labeled trout liver cDNA consistently revealed about half of the 147 genes on the array.

3.2 Plasma cortisol and glucose response

When we subjected rainbow trout to a standardized handling disturbance and recovery, plasma cortisol concentration showed the expected transient elevation that was significant at 1 h after the handling disturbance and dropped to unstressed levels at 24 h post-handling stressor (Fig. 1A). Plasma glucose levels were not affected by the acute handling stressor and remained stable over the 24 h experimental period (Fig. 1B).

3.3 Transcriptional response

The transcript levels of several genes showed transient changes in the liver of trout in response to handling stressor (Tables 3 and 4). Specifically, 40 transcripts were significantly higher at 1 h (Table 3), while only 15 transcripts were significantly higher at 24 h (Table 4) after stressor exposure compared to the 0 h unstressed group. Of the 15 genes elevated at 24 h, six were significantly higher even at 1 h (Tables 3 and 4). Prostaglandin D2 synthase (PGDS) was the only gene that was significantly lower in response to acute stressor in trout liver (Table 4). This decrease was statistically significant from the 0 h control at 24 h but not at 1 h (even though the levels were ~ 200% lower) after stressor exposure. However, qPCR clearly revealed a significantly lower PGDS mRNA abundance at 1 and 24 h after stressor exposure (Fig. 2D). Most of the transcripts upregulated by stress belonged to genes encoding proteins important in metabolism, immune function and cell signaling.

The metabolic genes upregulated at 1 h post-stressor exposure include glucokinase (GK), glucose transporter II (GLUT II), pyruvate kinase (PK), glutamine synthetase 3 (GS-3), arginase, cystatin (proteolytic), ubiquitin (proteolytic), cathepsin D (proteolytic), vitelline envelope proteins alpha (VEPα) and gamma (VEPγ) (structural), lipoprotein lipase, nitric oxide synthase (NOS-2), cytochrome P450 2M1, retinal binding protein (RBP) and ATPase B (Table 3). Also,

transcript levels of hormones and receptors involved in metabolism were upregulated at 1 h after stressor exposure including glucagon, insulin-like growth factor 1 (IGF-1), androgen receptor (AR), arylhydrocarbon receptor (AhR), glucocorticoid receptor (GR), insulin receptors b (IRb) and c (IRc) (Table 3). A number of immune-related genes, including cyclooxygenase 2 (COX-2), major histocompatibility complex 2 (MHC-2) and tumor necrosis factor alpha (TNF α), were also significantly upregulated at 1 h after stress in trout liver (Table 3). Most of these genes were not significantly different from the unstressed levels at 24 h post-stressor exposure (Table 4). A few metabolic genes stayed elevated over the 24 h period, including Cyp450, AhR, COX-2, cathepsin D, lipoprotein lipase and GLUT2 (Tabel 4). In addition there was significant upregulation of other metabolic genes including 20β-hydroxysteroid dehydrogenase (HSD), IGF-2 and GS-4 (Table 4). We checked the reliability of changes in mRNA abundance observed with the array by quantifying the gene expression pattern of six randomly chosen genes using qPCR. Indeed the transcript changes seen with qPCR post-stressor exposure for GR, AhR, cathepsin D and PGDS, but not GLUT II and PEPCK, seem to agree with the microarray pattern, although the magnitude of change appeared to be different (Figs. 2A-F).

Table 1: Sequences, annealing temperatures, amplicon size, and corresponding target gene Genbank accession numbers of oligonucleotide primers used in quantitative real-time PCR.

Target Gene	Accession #	Primer Sequence (5' – 3')	Amplicon Size (bp)	Annealing Temp (Tm ^{o}C)
β-actin	**AF157514**	F: AGA GCT ACG AGC TGC CTG AC R: GCA AGA CTC CAT ACC GAG GA	100	49
PEPCK	**AF246149**	F: TGC TGA GTA CAA AGG CAA GG R: GAA CCA GTT GAC GTG GAA GA	500	49
GLUT II	**AF321816**	F: CTG TCC TCA GGG CTA GTT CC R: AAT GTT CCC AGA GAG GAT TC	500	50
AhR	**AF065138**	F: CAG CGA AGG GAG CGG TAA R: TGG ACC CGG CCA GTG ATA	500	60
Prostaglandin D2 Synthase	**AF281353**	F: ATG CCT CAG AAA GAC TTC AA R: AAT GTT CCC AGA GAG GAT TC	500	50
Cathepsin D	**U90321**	F: TGC TCC TTC ACA GAC ATC G R: CCT CAC AGC CTC CCT TAC A	500	55
GR	**Z54210**	F: AGA AGC CTG TTT TTG GCC TGT A R: AGA TGC GCT CGA CAT CCC TGA T	500	49

Table 2. List of genes contained on the low-density targeted rainbow trout cDNA array. Gene names (abbreviation), class and their GenBank accession numbers are shown.

Gene	Gene Class	Accession #
Apolipoprotein A-I-2 (Apo A-1-2)	Binding/Metabolism	**AF042219**
Apolipoprotein E (Apo E)	Binding/Metabolism	**AJ132620**
Metallothionein (MT)	Cell Stress	**M18103**
Heat shock protein 70 induced (Hsp70)	Cell Stress / Molecular Chaperone	**K02549**
Heat shock protein 90 (Hsp90)	Cell Stress / Molecular Chaperone	**N/A**
Heat shock protein 70 constitutive (Hsc70)	Cell Stress / Molecular Chaperone	**S85730**
β-Actin	Cell Structure	**AJ438158**
Matrix metalloproteinase-2 (MMP-2)	Cell Structure	**AB021698**
Matrix metalloproteinase-1 (MMP-1)	Cell Structure	**AB043536**
α-Tubulin	Cell Structure	**M36623**
α-21Collagen	Cell Structure	**AB075699**
KeratineS5	Cell Structure	**Y14289**
Nuclearpore complex glycoprotein p62	Cell Structure	**AB008798**
Otolith matrix protein-1 (omp-1)	Cell Structure	**AB030389**
α –actin	Cell Structure	**AF503211**
Myosin heavy chain fast	Cell Structure	**AF231706**
Myosin heavy chain slow	Cell Structure	**AF231708**
Myostatin	Cell Structure	**AF503209**
Vitelline envelope protein- α (VEP-α)	Developmental Processes	**AF231707**
Vitelline envelope protein-β (VEP-β)	Developmental Processes	**Z48794**
Vitelline envelope protein-γ (VEP- γ)	Developmental Processes	**AY009125**
Vasa	Developmental Processes	**AF479825**
Inhibitor of DNA binding/differentiation-1 (Id-1)	Developmental Processes	**Y08368**
Inhibitor of DNA binding/differentiation-2 (Id-2)	Developmental Processes	**Y08369**
Cholecystokinin (CCK-Asn-Thr)	Endocrine	**AJ011846**
Chicken GnRH (cGnRH)	Endocrine	**AF125973**
Growth hormone-1 (GH-1)	Endocrine	**M24683**
Glucagon	Endocrine	**U19914**
Insulin-like growth factor-1 (IGF-1)	Endocrine	**M95183**
Insulin-like growth factor-2 (IGF2)	Endocrine	**M95184**
Melanin concentrating hormone (MCH)	Endocrine	**X73837**
Proopiomelanocortine A (POMC-A)	Endocrine	**X69808**
Proopiomelanocortine B (POMC-B)	Endocrine	**X69809**
Prolactin	Endocrine	**M24738**
Somatostatin-2 (SST-2)	Endocrine	**U32471**
Thyrotropin-b	Endocrine	**D14692**
Urotensin-1	Endocrine	**AJ005264**
Vig-1	Endocrine	**AF076620**
Gonadotropin releasing hormone 1(GnRH-1)	Endocrine	**AF232212**
Gonadotropin releasing hormone 2 (GnRH-2)	Endocrine	**AF232213**
Bcp pre-cerebellin-like protein (BpCLP)	Immune	**AF192969**
β -2 microglobulin (β -2-M)	Immune	**L63537**
β-Globin-2	Immune	**AB015451**
β –Globin	Immune	**D82926**
Chemokine CK-1 (CK-1)	Immune	**AF093814**
Chemokine CXCR (CXCR)	Immune	**AJ001039**
Chemokine R (CKR)	Immune	**AJ003159**
Complement Bf1	Immune	**AF089861**
Low molecular mass protein 2d (Lmp2d)	Immune	**AF115540**
Major histocompatability complex-1 (MHCI)	Immune	**AF296359**
Major histocompatability complex-2 (MHCII)	Immune	**AF296390**
Mx-1	Immune	**U30253**

Mx-2	Immune	**U47945**
Natural resistance associated macrophage-α (Nramp- α)	Immune	**AF054808**
Natural resistance associated macrophage β (Nramp- β)	Immune	**AF048761**
Interleukin 1 β	Immune	**AJ223954**
Low molecular mass protein-2 (LMP-2)	Immune	**AF115541**
Tumor necrosis factor (TNF)	Immune	**AJ277604**
Arylalkylamine-n-acetyltransferase (AANAT)	Metabolism	**AB007294**
ATPase b	Metabolism	**AF140022**
Carbamoyl-phosphate synthetase-3 (CPS-3)	Metabolism	**U65893**
Carbamoyl-phosphate synthetase-2 (CPS-2)	Metabolism	**AF014386**
Carbonyl reductase/20- β HDA (CR-20- β HDA)	Metabolism	**AF100933**
Creatine kinase (CK)	Metabolism	**X53859**
Cystatin	Metabolism	**U33555**
Glucose-6-phosphatase (G-6-Pase)	Metabolism	**AF120150**
Lipoprotein lipase	Metabolism	**AF358669**
Monoamineoxidase (MAO)	Metabolism	**L37878**
NADH-dehydrogenase subunit-6 (NADH-6)	Metabolism	**AF125047**
Nitric oxide synthase-2 (NOS-2)	Metabolism	**AJ300555**
Terminal deoxynucleotidyl transferase (TDT)	Metabolism	**U53366**
Tmyogenin	Metabolism	**Z46912**
Vitellogenin	Metabolism	**AJ011691**
Arginase	Metabolism	**AY056477**
Glutamine synthetase-1 (GSase-1)	Metabolism	**AF390022**
Gluatmine Synthase-2 (GSase-2)	Metabolism	**AF390021**
Hexokinase IV (glucokinase)	Metabolism	**AF053331**
Phosphoenolpyruvatecarboxykinase (PEPCK)	Metabolism	**AF246149**
Glutamine Synthase-3 (GSase-3)	Metabolism	**AF390023**
Pyruvate kinase (PK)	Metabolism	**AY113695**
Glutamate dehydrogenase-3 (GDH-3)	Metabolism	**AF427344**
11- β -Hydroxylase (11-β-hyd)	Metabolism	**AF179894**
17-α-Monooygenase (17-α-mono)	Metabolism	**X65800**
3- β -Hydroxysteroid dehydrogenase (3-β-HSD)	Metabolism	**S72665**
Cholesterol sidechain cleavage (p450scc)	Metabolism	**S57305**
Prostaglandin D2 synthase (PGDS)	Metabolism	**AF281353**
StAR	Metabolism	**AB047032**
GAD65	Metabolism	**AF503210**
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Metabolism	**AF027130**
RtSox-23	Metabolism	**AB007906**
Sox-24	Metabolism	**AB010741**
Sox-9	Metabolism	**AB006448**
SoxLZ	Metabolism	**D61688**
SoxP1	Metabolism	**D83256**
Cyclooxygenase-2 (COX-2)	Metabolism	**AJ238307**
Cytp450 1A3 (Cyp1A3)	Metabolism	**U62797**
Cytp450 monooxygenase 2K1V2 (Cyp 2K1V2)	Metabolism	**L11528**
Cytp450 2M1 (Cyp 2M1)	Metabolism	**U16657**
Cytochrome c oxidase subunit VIA (Cyt CCO)	Metabolism	**U83980**
Cytp450 2K5 (Cyp 2K5)	Metabolism	**AF151524**
Glutathione peroxidase (GP)	Metabolism	**AF281338**
18S rRNA	Nucleic Acid Processing	**AF243428**
Ras	Oncogene	**U45968**
Retinoblastoma	Oncogene	**AF102861**
Myc	Oncogene/Regulatory	**S79770**

Ubiquitin	Protein Catabolism	**AB036060**
Cathepsin D	Protein Catabolism	**U90321**
Small ubiquitin-related modifier (SUMO-1)	Protein Catabolism	**AB036430**
TFZR1	Receptor	**AB006153**
Androgen receptor α + β (AR α + β)	Receptor/Endocrine	**AB012096**
Glucocorticoid receptor (GR)	Receptor/Endocrine	**Z54210**
Mineralocorticoid receptor (MR)	Receptor/Endocrine	**AF209873**
Aryl hydrocarbon receptor (AhR)	Receptor/Transcription factor	**AF065138**
Insulin-like growth factor 1a Receptor (IGF1a-R)	Receptor/Endocrine	**AF062499**
Insulin receptor-a (IR-a)	Receptor/Endocrine	**AF062496**
Insulin receptor-b (IR-b)	Receptor/Endocrine	**AF062497**
Insulin receptor-c (IR-c)	Receptor/Endocrine	**AF062498**
Melatonin receptor (MlR)	Receptor/Endocrine	**AF156262**
Estrogen receptor-α (ER-α)	Receptor/Endocrine	**AJ242741**
Thyroid hormone receptor-α (THR-α)	Receptor/Endocrine	**AF132752**
Thyroid hormone receptor-β (THR-β)	Receptor/Endocrine	**AF302246**
GnRH receptor	Receptor/Endocrine	**AJ272116**
MyoD	Regulatory	**X75798**
Recombinant activating protein (rag-1)	Regulatory	**U15663**
Secreted protein, acidic, rich in cysteine (SPARC)	Regulatory	**U25721**
Transforming growth factor-β (TGF-β)	Signaling	**X99303**
Lipoprotein receptor (LR)	Signaling	**AJ417877**
Fibroblast growth factor-6 (FGF-6)	Signaling	**Y16850**
Forcystein	Signaling	**Y17313**
P53	Signaling	**AF223794**
RbtStat-1	Signaling	**U60331**
Vitellogenin receptor (VR)	Signaling	**AJ003118**
AhR nuclear translocator (ARNT)	Signaling	**U73841**
Pit-1	Transport	**D16513**
Cardiac sodium-calcium exchanger (C-NCX)	Transport	**AF175313**
Transferrin	Transport	**D89083**
α-Globin	Transport	**D88113**
Hemopexin	Transport	**Z68112**
Fatty acid binding protein (H-FABP)	Transport	**U95296**
Retinol binding protein (RBP)	Transport	**AF503212**
Glucose transporter-1 (GLUT-1)	Transport	**AF247728**
Kidney Na/Pi cotransporter (K-NPC)	Transport	**AF297186**
Intestine Na/Pi cotransporter (I-NPC)	Transport	**AF297184**
Glucose transporter-2 (GLUT-2)	Transport	**AF321816**
Galectin	Transport	**AB027452**
Anion exchanger	Transport	**Z50848**
Tap2	Transport	**AF002180**

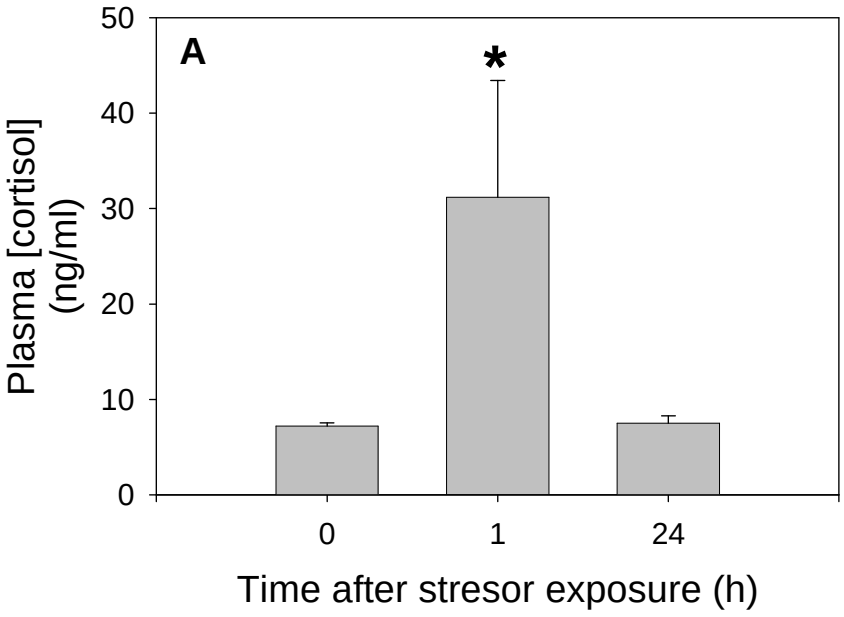
Table 3. Genes significantly different at 1 h after stressor exposure (Student's t-test; $P<0.05$). Data shown as percent change from the 0 h unstressed group (% change from 0 h); values represent mean ± SEM. (n = 3 independent samples); GO number: genes assigned a gene ontology (GO) biological process number.

Gene Name	Gene Symbol	GO Biological Process	GO Number	% Change from 0 h
Glucokinase	GK	Carbohydrate metabolism	0005975	96±18
Pyruvate kinase	PK	Glucose metabolism	0006006	51±19
Androgen receptor	AR	Steroid hormone receptor signaling pathway	0030518	28±7
Arginase, type II	Arginase	Amino acid metabolism	0009063	51±15
Cystatin C	Cystatin	Cellular protein metabolism	0044267	43±16
Ubiquitin	Ubiquitin	Cellular protein metabolism	0044267	96±13
Cathepsin D	Cathepsin D	Cellular protein metabolism	0044267	50±8
Vitelline envelope protein gamma	VEPγ	Binding of Sperm to Zona Pellucida	0007339	82±14
Aryl hydrocarbon receptor	AhR	Xenobiotic metabolism	0006805	42±5
ATPase B	ATPase B	ATP synthesis coupled proton transport	0042776	37±7
Cyclooxygenase-2	COX-2	Prostaglandin metabolism	0006693	71±19
Cytochrome p450	CYP p450	Electron transport	0006118	31±8
TFZR-1	TFZR-1	Regulation of Cell Cycle	0051726	29±6
Galectin	Galectin	Signal transduction	0007165	36±7
Glucocorticoid receptor	GR	Steroid hormone receptor metabolism	0030518	19±7
Glucagon	Glucagon	Glucose homeostasis	0042593	36±2
Hemopexin	Hemopexin	Iron homeostasis	0006879	32±12
Inhibitor of DNA binding/differentiation-2	ID-2	Morphogenesis	0009653	21±7

Table 4. List of genes significantly different at 24 h post-stressor exposure (Student's t-test; $P<0.05$). Data are shown as percent change from the 0 h unstressed group (% change from 0 h); values represent mean ± SEM. (n = 3 independent samples); GO number: genes assigned a gene ontology (GO) biological process number.

Gene Name	Gene Symbol	GO Biological Process	GO Number	% change from 0 h
Aryl hydrocarbon receptor	AhR	Xenobiotic metabolism	0006805	60±20
Solute carrier family 2, member 1 (facilitated glucose transporter)	GLUT 2	Transport	0051179	40±14
Glutamate-ammonia ligase (glutamine synthase)	GS-4	Amino acid metabolism	0006520	31±6
20-B-hydroxysteroid dehydrogenase	20ß-HSD	Steroid metabolism	0008202	42±9
Cytp450 monooxygenase 2k5	CYP 2k5	Electron transport	0006118	60±12
Cathepsin D	Cathepsin D	Cellular protein metabolism	0044267	64±25
VASA	VASA	Cellular differentiation	0030154	28±6
Sox-9	Sox-9	Regulation of transcription	0045449	56±21
Pit-1	Pit-1	Regulation of transcription	0045449	34±13
Cyclooxygenase-2	COX-2	Prostaglandin metabolism	0006693	50±6
Insulin-like growth factor-2	IGF-2	Insulin like growth factor receptor signaling pathway	0048009	53±8
α-21 Collagen	α-21 Collagen	Skeletal development	0001501	60±22
Lipoprotein lipase	Lipoprotein Lipase	Fatty acid metabolism	0006631	62±20
Bcp pre-cerebellin-like protein	BcpCLP	Nervous system development	0007399	19±7
Prostaglandin D2 synthase	PGDS	Prostaglandin metabolism	0006693	- 425±48

Figure 1. Effect of handling disturbance on plasma cortisol (A) and glucose (B) levels in rainbow trout sampled either prior to or 1 and 24 h after stressor exposure. *Significantly different from the 0 and 24 h samples (1-Way ANOVA, $P<0.05$). All values represent mean + S.E.M. ($n = 4 –6$ fish).


A
*
Plasma [cortisol] (ng/ml)
50
40
30
20
10
0
0
1
24
Time after stresor exposure (h)

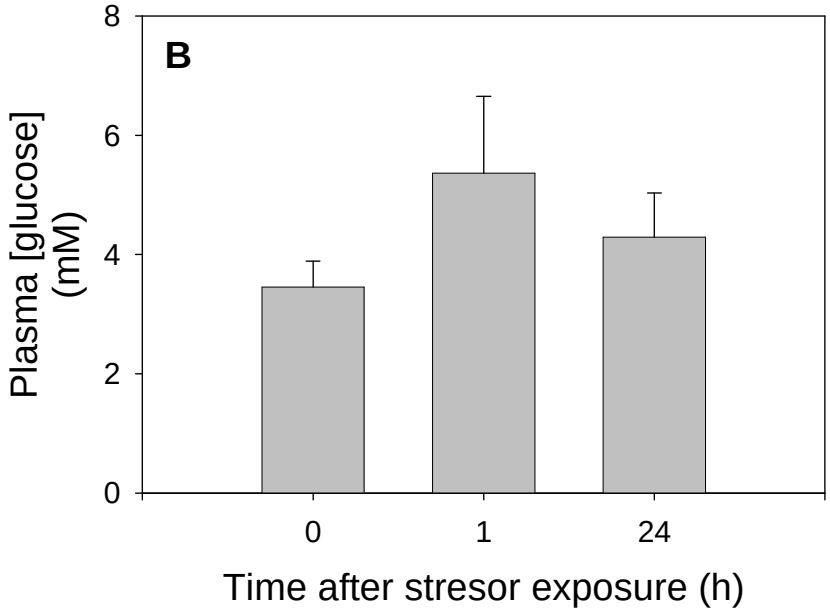
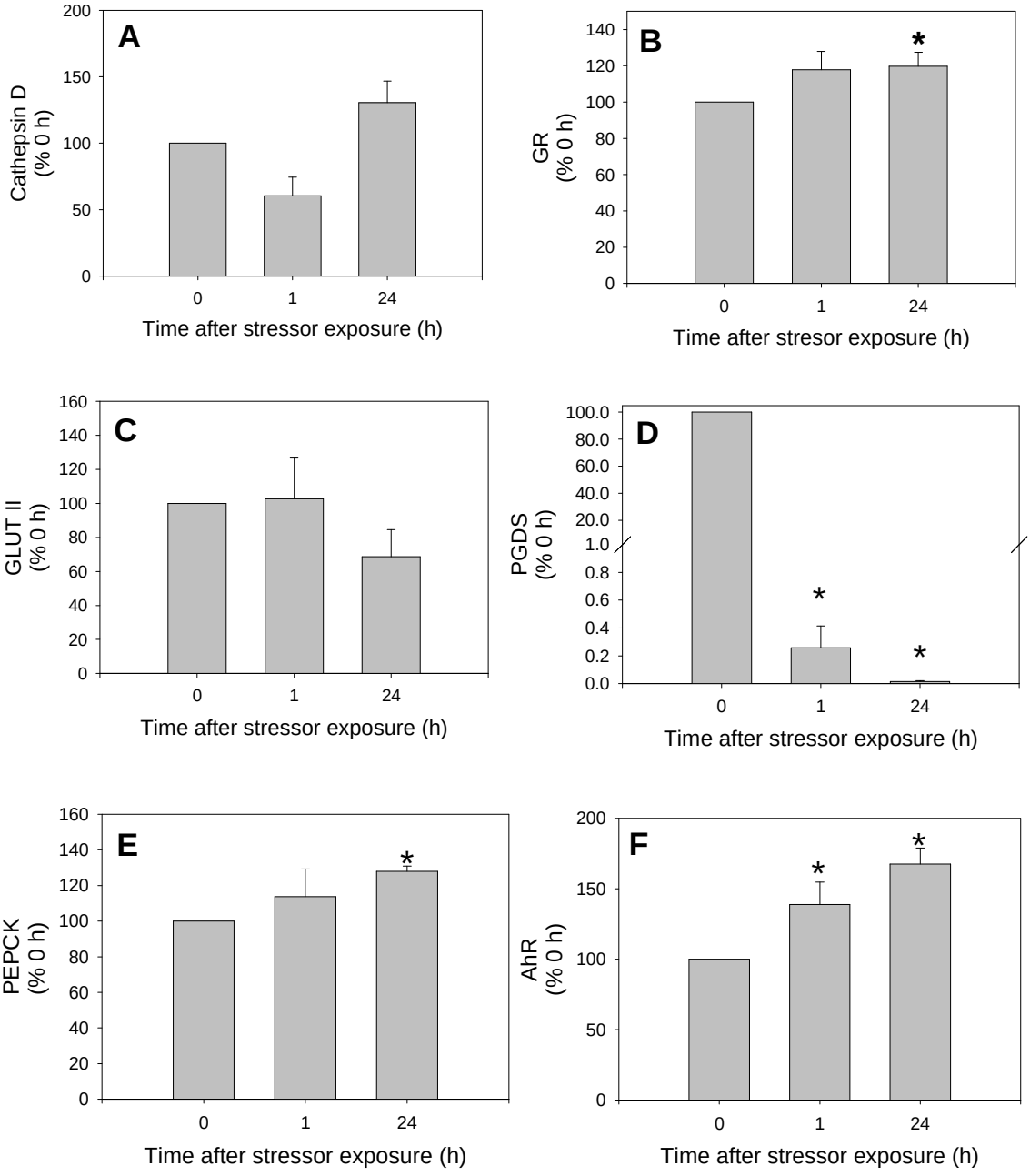

B
Plasma [glucose] (mM)
8
6
4
2
0
0
1
24
Time after stresor exposure (h)

Figure 2. Quantitative real-time PCR analysis of mRNA abundance of cathepsin D (A), glucocorticoid receptor (GR; B), glucose transporter II (GLUT II; C), prostaglandin D2 synthase (PGDS; D), phosph*enol*pyruvate carboxykinase (PEPCK; E) and arylhydrocarbon receptor (AhR; F). Bars represent mean + S.E.M. (n = 3 independent samples); values shown as percent 0 h unstressed group (% 0 h); *significantly different from the 0 h unstressed group (1-Way ANOVA, $P<0.05$).


A
Cathepsin D
(% 0 h)
200
150
100
50
0
0
1
24
Time after stressor exposure (h)
B
GR
(% 0 h)
140
120
100
80
60
40
20
0
*
0
1
24
Time after stresor exposure (h)
C
GLUT II
(% 0 h)
160
140
120
100
80
60
40
20
0
0
1
24
Time after stressor exposure (h)
D
PGDS
(% 0 h)
100.0
80.0
60.0
40.0
20.0
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0
1
24
Time after stressor exposure (h)
E
PEPCK
(% 0 h)
160
140
120
100
80
60
40
20
0
*
0
1
24
Time after stressor exposure (h)
F
AhR
(% 0 h)
200
150
100
50
0
*
*
0
1
24
Time after stressor exposure (h)

4. Discussion

We demonstrate for the first time the transient changes in liver gene expression pattern associated with recovery from an acute stressor in rainbow trout. While homeostatic adjustments of plasma stress hormones and metabolites in response to acute stress have been well documented, little was known about the underlying genetic components. Indeed our stress protocol showed a transient elevation in plasma cortisol concentration, while homeostasis of this steroid in circulation was attained within 24 h (Fig. 1A). Previous studies have clearly established association of this transient elevation in plasma cortisol concentration with acute stress, unlike chronic stress which maintains elevated plasma cortisol levels over a longer period, depending on the intensity and duration of the stressor (Bonga, 1997; Barton et al, 2002).

Many of the genes upregulated in the liver in response to stress encoded proteins involved in energy metabolism. This pattern of expression is consistent with previous findings that stress increases liver metabolic capacity in teleostean fishes (Mommsen et al, 1999). One of the key metabolic responses to stress involves enhanced glucose production to meet the increased energy demand associated with stress adaptation. Indeed stress increases the metabolic rate in salmonids and this is coincident with elevated plasma glucose levels, an important fuel for metabolism (Mommsen et al, 1999). While the rapid output of liver glucose in response to stress is thought to involve adrenergic signaling of the glycogenolytic pathway, the longer-term maintenance of plasma glucose levels, as well as the replenishment of depleted liver glycogen content, involves upregulation of the gluconeogenic pathway (Mommsen et al, 1999). This is further corroborated by the significantly higher PEPCK transcript levels seen in stressed trout (Fig. 2E) underscoring the enhanced liver capacity for gluconeogenesis as an adaptive response to cope with stress (Vijayan et al, 1996; Vijayan et al, 1997a; Mommsen et al, 1999). The absence of an increase in plasma glucose levels at 1 h post-stressor exposure in this study is not

surprising given the fact that rapid glucose response is dependent upon the intensity and duration of the stressor. Furthermore, a stress regimen similar to this study elicited a significant glucose response only at 3 h, but not at 1 h after a handling disturbance (Vijayan et al, 1997b).

In addition to activation of the gluconeogenic pathway, our results also highlight the rapid activation of the glycolytic genes post-stressor exposure in trout. Specifically, transcript levels of glucokinase, (hexokinase IV) -a high Km hexokinase - were upregulated at 1 h post-stressor along with pyruvate kinase, the rate-limiting step in glycolysis. While glucokinase transcript abundance and activity is tightly regulated by circulating glucose levels in trout (Panserat et al, 2001), the rapid elevation of this transcript level at 1 h, even in the absence of any concurrent increases in plasma glucose levels, suggests other signaling pathways regulating the transcript abundance seen with stress. The fact that liver PEPCK and PK activities increased in response to cortisol treatment in fish (Vijayan et al, 1997a; Mommsen et al, 1999; Panserat et al, 2001; Dziewulska-Szwajkowska et al, 2003) coupled with our recent observation that cortisol upregulates PEPCK mRNA abundance in trout liver (Vijayan et al, 2003) leads us to propose that cortisol signaling plays a pivotal role in the molecular regulation of the hyperglycemic response to stress in fish. The substrates for gluconeogenesis are predominantly amino acids in trout (Mommsen et al, 1999) and clearly stress did elevate some of the genes involved in protein metabolism, including GS, cathepsin D and arginase (Tables 3 and 4). Also, GS and arginase have been shown to be glucocorticoid-responsive in fish liver (Mommsen et al, 1992; Hopkins et al, 1995; Vijayan et al, 1996; Mommsen et al, 2003) further highlighting a key role for cortisol in the metabolic adjustments to stress (Mommsen et al, 1999, Vijayan et al, 2003). Whether this transcriptional response is related to either direct cortisol signaling or indirect changes in overall metabolism remains to be elucidated. Nonetheless, changes in transcript levels of both the

glucose utilization (PK and glucokinase) and gluconeogenic pathways (PEPCK) post-stressor exposure imply a fine-tuning of liver metabolism that may be hormonally regulated.

The tissue response to glucocorticoid stimulation is mediated via glucocorticoid receptor (GR) and studies have shown a downregulation of GR affinity and capacity after stress in fish (see Vijayan et al, 2003 for references). However, recent studies points to an autoregulation of GR mRNA abundance in response to cortisol stimulation (Vijayan et al, 2003, Sathiyaa and Vijayan, 2003). The elevated GR transcript level at 1 h post-stressor along with higher plasma cortisol level at that time compared to the unstressed group support a role for cortisol in regulating GR expression in fish. The recent observation that GR mRNA levels were lowered in sea bass (*Dicentrarchus labrax*) liver in response to chronic crowding stress (Terova et al, 2005) suggests that the stress effect on GR message may be either species-specific and/or dependent on the type, intensity and duration of the stressor. However, the adaptive significance of GR dynamics associated with stress awaits further study, but our recent observation that GR turnover is autoregulated by cortisol (Sathiyaa and Vijayan, 2003; Vijayan et al, 2003) suggests that GR response may be an important part of the adaptive stress response that is regulated by plasma cortisol level in trout.

While cortisol is a key hormone associated with the stress response, our results also implicate a role for insulin-like growth factors, testosterone and insulin in the stress response process. Specifically, the transcript levels of receptors for testosterone and insulin showed transient elevation after a handling disturbance (Table 3) signifying a complex interplay of the endocrine signals in the adaptive stress response. As most receptor changes were evident at 1 h post-stressor, it raises the possibility that cortisol signaling may be involved in this molecular regulation. In support of this argument, glucocorticoid have been implicated in the stress-mediated suppression of plasma testosterone levels in fish (Goos and Consten, 2002), while the

higher AR and 20β-HSD mRNA abundance suggests a cortisol –mediated effect on target tissue androgen response (Table 3) and metabolism (Table 4), respectively. As these hormones and receptors play a role in energy metabolism, we hypothesize that genomic cortisol signaling either directly and/or indirectly with other hormones are involved in liver metabolic reorganization essential for homeostatic adjustments to stress in fish. Together, these results underscore a complex interplay of corticosteroids along with other endocrine signals in the adaptive stress response process.

A novel observation from this study is the increased abundance of transcripts encoding proteins involved in protein degradation. For instance, cystatin, cathepsin D and ubiquitin mRNA levels increased after handling stressor. Although very little is known about the protein degradative pathway activation with stress, several lines of evidence suggest a role for proteolysis in the process. Cathepsin D plays a key role in fish muscle proteolysis, attacking native proteins such a myosin heavy chain, actin and myosin (Nielsen and Nielsen, 2001; Mommsen, 2004). Analysis of mammalian cathepsin D gene structure identified a corticosteroid-responsive element (Dardevet et al, 1995). In fact, mammalian muscle cathepsin D transcript levels are upregulated by the synthetic corticosteroid dexamethasone via a glucocorticoid receptor mediated pathway (Dardevet et al, 1995). Additionally, it has been demonstrated that decreased levels of hepatic GR in response to stress and cortisol exposure is due to proteasomal degradation (Sathiyaa and Vijayan, 2003). Ubiquitin, a major player in the proteasomal pathway of protein degradation (Ciechanover, 1994; Martin et al, 2002), is stress-activated in fish (Ryan et al, 1995). Collectively, these studies, as well as our results, argue for an increased proteolytic potential in fish liver that may be mediated by glucocorticoid signaling. Secondarily, this would point towards the possibility that susceptible hepatic proteins may serve as transient sources of amino acids for hepatic metabolism, including gluconeogenesis. Also, the

recent finding that the proteasome may be involved in GR autoregulation (Sathiyaa and Vijayan, 2003) is a clear indication that the relationship between the protein breakdown pathways and GR signaling is not simple and may be an important control point for homeostatic adjustments to stress.

Our study is also the first to show a transient change in immune-related gene expression patterns in response to an acute stressor in fish liver. The physiological significance of MHC-2, COX-2 and TNF, important in the antigen presentation and inflammatory response, upregulation in the liver in response to stressor is unknown. However, it remains to be seen whether the enhanced potential for liver proteolytic capacity with stress may also be activating the immune response pathways, as part of the adaptive mechanism aiding cellular homeostasis. Of particular interest is the significant downregulation of liver PGDS, an enzyme converting prostaglandin (PG)H2 to PGD2, an important mediator of inflammation (Urade and Hayaishi, 2000). However, the physiological or immunological role for this molecule in fish remains relatively unknown. Recently, PGDS transcripts were shown to be downregulated in the skin of carp, but not in the liver in response to ectoparasitic infection (Gonzalez et al, 2006). The pronounced suppression of PGDS transcripts in trout liver in response to acute stressor exposure may suggest a role for this tissue in the inflammatory response, but this remains to be tested. Overall, cell and tissue-specific adjustments of immune response pathways may be part of the adaptive mechanism aiding cellular homeostasis post-acute stressor exposure in trout.

5. Conclusions

Using a custom-made low density rainbow trout cDNA array, we identified transient upregulation of genes involved in energy metabolism in trout liver post-stressor exposure. The majority of these metabolic genes were glucocorticoid-responsive in trout. Also, several

endocrine genes involved in energy substrate metabolism were transiently upregulated by handling disturbance and recovered over the 24 h period leading to the proposal that the stress-mediated metabolic reprogramming may be mediated by cortisol either directly and/or indirectly with other hormones. In addition, our study also identified several genes involved in protein breakdown and immune response pathways, as stress-responsive in trout. As most of these genes were also glucocorticoid-responsive, our results highlight a key role for genomic cortisol signaling in allowing fish to regain homeostasis after an acute stressor exposure. Collectively, the study demonstrates the complex nature of the adaptive stress response process at the genetic level and underscores the utility of functional arrays for identifying pathways, which could be studied in greater detail to provide insights regarding the transcriptional regulation of the stress response.

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Chapter 7

General Conclusion

Experiments described in this thesis were designed to characterize aryl hydrocarbon receptor (AhR) signaling in rainbow trout (*Oncorhynchus mykiss*) and investigate the impact(s) of polychlorinated biphenyls (PCBs) exposure on the adaptive responses to stressors in fish. To this end a series of in vivo and in vitro studies were performed and the following conclusions were derived.

1. Short-term exposure to PCBs (Aroclor 1254) impacts liver metabolic capacity that is critical for coping with the increased energy demand associated with acute stressor exposure in fish. **(Chapter 2)**

2. In contrast to populations of teleost species that show reduced responsiveness to lifelong, even multigenerational, exposure to PCBs, feral Arctic char (*Salvelinus alpinus*) maintain the ability to elicit a cytochrome P4501A (Cyp1A) response. Tissue-specific glucocorticoid receptor (GR), heat shock protein 70 (Hsp70) and Hsp90 expressions appears to be a useful indicator of effects associated with chronic contaminant exposure in these feral fish. **(Chapter 3)**

3. The AhR agonist β-napthoflavone (βNF) stimulates autoregulation of AhR content in trout hepatocytes. In addition, Hsp90 and the proteasome are important in βNF-mediated AhR autoregulation and Cyp1A response in trout hepatocytes. **(Chapter 4)**

4. AhR singaling appears to play a role in the adaptive cellular response to heat shock as protein expression of Hsp70 and Hsp90 were impaired by the AhR antagonist resveratrol (RVT). Although further research is needed to completely understand the crosstalk between AhR and Hsp signaling, attenuated Hsp70 response to heat shock in BNF treated cells involves the proteasome. **(Chapter 5)**

5. Microarrays offer a powerful approach to identify genes that are involved in the adaptive response to stressors in fish. A targeted trout cDNA microarray was developed and

using this array we identified pathways that were stress-responsive in fish liver. Interestingly, a number of genes that were stress-responsive were also previously shown to be cortisol-responsive, providing a framework to expand on the role of this steroid in adapting animals to stress. Also, this microarray can be utilized to identify other stressor-responsive pathways, including xenobiotics impact in fish. **(Chapter 6)**

Persistent organic pollutants (POPs), including PCBs, accumulate in the aquatic environment, both in sediment and in tissues of aquatic organisms. As teleost fish sit atop the aquatic food web, they are at an increased risk of bioaccumulation from lipophilic contaminants. Depending on the aquatic environment in question, fish may be exposed to varying levels of PCBs and for varying periods of time. In some cases, fish may be forced to endure lifelong or even generational exposure to high levels of PCBs. The results from this thesis suggest that short-term exposure to relatively low doses of PCBs has the potential to impact liver metabolic capacity, but not the expression of stress-related proteins, including hsp70 and GR. However, longer-term exposure to PCBs alters hsp70, hsp90 and GR mRNA and protein levels in the liver and brain of Arctic char. Consequently, fish residing in polluted may have different adaptive strategies to cope with pollutants and this may be dependent upon various factors, including contaminant levels, duration of exposure and species-specific effects.

An interesting finding from the Arctic char study was that despite lifelong exposure to PCB, the ability to mount a Cyp1A response is retained. Other teleost species that endure similar exposure conditions have lost the ability to mount a Cyp1A response, in some cases due to the loss of AhR protein expression. The ability to mount this response may be related to the feeding and fasting lifestyle of these fish. According to this model, durng feeding PCBs accumulate in lipd deposits and these PCBs are redistributed to peripheral tissues during periods of fasting when lipid deposits are mobilised as energy reserves. This results in intermittent PCBs exposure

to critical tissues only during periods of fast and may be a reason for the lack of tissue unresponsiveness to PCBs despite longer-term exposures. Alternatively, one cannot discount the possibility that the differences in CYP1A response may be simply related to a species effect associated with different AhR isoforms, but this remains to be tested.

The results presented in this thesis reveal interesting details regarding AhR mechanism of action and further highlight AhR as a dynamic player in the cellular response to stress. In rainbow trout, as in all species studied to date, PCBs activate AhR signaling, leading to expression of phase I detoxification proteins typified by Cyp1A proteins. In accordance with other studies, both Hsp90 and the proteasome are required for AhR function. However, it is demonstrated for the first time that rainbow trout AhR proten expression is autoregulated in response to agonist exposure. This AhR autoregulation by its ligand may have be a mechanism to maintain tissue responsiveness in the face of persistant PCBs exposure. This model of AhR regulation contrasts that seen in mammalian systems and in zebrafish, where AhR protein levels decrease in response to ligand stimulation. This unique aspect of rainbow trout AhR regulation may be related to the diversity of this receptor isoforms found in trout. However, this remains to be characterized, but would shed light on the functional significance of AhR signalling in altering the stress response.

While ligand stimulated autoregulation of AhR may have eveolved as a mechanism to maintain tissue responsiveness to persistant PCB exposure it appears that this autoregulation may be compromised by secondary stressors that elicit an organismal stress response. Specifically, the observation that handling stress stimulates a decrease in AhR mRNA abundance in PCB exposed trout suggests that cortisol may disrupt AhR autoregulation by decreasing AhR protein expression. Although the precise mechanism of this action is unknown, studies have demonstrated that cortisol may stimulate proteasomal degradation of critical proteins, including

glucocorticoid receptor. Disruption of ligand stimulated AhR autoregulation may negatively impact organismal health by decreasing the capacity for detoxification reactions required for the clearance of lipophilic ligands, including PCBs.

An interesting observation from this work is the possible involvement of AhR signalling in the cellular response to heat shock. Indeed, other studies have suggested that AhR may participate in cross-talk with other cell signaling pathways, including the response to hypoxia stress. That the AhR may play a role in multiple cell stress responsive pathways exemplifies the importance of maintaining minimal functional levels of AhR. Again, it is interesting to speculate that the presence of multiple AhR isoforms in trout may be reflective of the multiple roles played by AhR, but this remains to be determined. Moreover, ligand stimulated AhR autoregulation may be an important adaptation in maintaining the cellular AhR pool in order to ensure that these multiple cellular pathways, dependent on AhR function, are maintained in the face of PCBs exposure.

The thesis highlights the notion that AhR is a dynamic player in cellular stress response pathways. As multiple AhR forms exist in rainbow trout it will be important to determine the role of individual AhR isoforms in regulating AhR signaling and their involvement in disrupting homeostasis. Utilizing gene silencing technology, including small interfering RNA (siRNA) and morpholino's will be a valuable tool in this regard. Harnessing the power of emerging genomic technology, such as cDNA microarrays, and combining this with traditional physiological and molecular biology approaches will provide additional insight into AhR function as well as other adaptive stress response pathways impacted by PCBs and AhR signaling.