Impact of non-steroidal anti-inflammatory drugs on the adaptive responses to stress in rainbow trout

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

Pharmaceutical drugs are used extensively by humans and domestic animals. The detection of compounds such as non-steroidal anti-inflammatory drugs (NSAIDs) in effluents of sewage treatment plants and surface waters has raised concerns about whether these drugs have the potential to impact aquatic organisms. However, little is known about either the mechanism of action of NSAIDs or their impact on aquatic organisms. A key indicator of animal stress performance is the elevation in plasma cortisol levels, the primary circulating corticosteroid in teleosts, and the associated metabolic changes in response to stressor exposure. The secretion of cortisol is under the control of the hypothalamus-pituitary-interrenal (HPI) axis with the terminal step involving the activation of interrenal steroidogenesis by adrenocorticotropic hormone (ACTH) from the pituitary gland. Cortisol, predominantly *via* glucocorticoid receptor (GR) activation, is involved in a wide array of animal functions, including growth and metabolism, osmo- and iono-regulation, stress and immune function and reproduction, all of which play a role in regaining homeostasis after stressor insult.

The overall objective of this thesis was to investigate the role of NSAIDs in impacting the evolutionarily-conserved adaptive stress response in a model teleost fish, the rainbow trout (*Oncorhynchus mykiss*). Specifically, the impact of NSAIDs on stress coping mechanisms was investigated by examining: i) interrenal steroid biosynthetic capacity and cortisol production, ii) target tissue GR function, iii) cellular heat shock protein response, iv) tissue-specific metabolic response to stressors, and iv) ionoregulatory performance in seawater. The experimental approach involved a series of whole animal *in vivo* and *in vitro* studies, using rainbow trout interrenal cell preparations, with two NSAIDs, salicylate and ibuprofen, commonly detected in our surface waters. Fish were subjected to stressors of varying intensity and duration, including

handling disturbance, heat shock and salinity exposures, to identify targets impacted by NSAIDs in fish.

NSAIDs did not affect resting plasma cortisol levels but disrupted the acute ACTH-stimulated corticosteroidogenesis *in vitro* and stressor-induced plasma cortisol response *in vivo*. The mode of action of NSAIDs in disrupting cortisol production involves inhibition of the key rate-limiting step, the steroidogenic acute regulatory protein (StAR), in steroidogenesis. Also, tissue (brain, liver and gill) GR protein content is a target for endocrine disruption by NSAIDs leading to abnormal negative feedback regulation of plasma cortisol levels and reduced target tissue responsiveness to cortisol after stressor exposure. The drugs also clearly affected the cellular stress response in rainbow trout by perturbing the expression of heat shock protein 70 (hsp70), a highly conserved stress coping mechanism. This impaired heat shock response with NSAIDs corresponded with an altered tissue metabolic capacity suggesting disturbances in biochemical adjustments to stressor. Specifically, the dynamics of glucose, the primary fuel to cope with the enhanced tissue metabolic demand, was disrupted in a drug-specific manner in rainbow trout. Exposure to NSAIDs also disrupted the ionoregulatory mechanisms critical for seawater acclimation in rainbow trout. The targets for ionoregulatory disturbance in seawater by NSAIDs include the major ion transporter gill Na^+/K^+-ATPase as well as gill GR, a key signaling protein for Na^+/K^+-ATPase upregulation in fish.

Altogether, NSAIDs disrupt the adaptive endocrine and metabolic stress coping mechanisms in rainbow trout. The targets for endocrine disruption by NSAIDs include multiple sites along the HPI axis as well as target tissue response to cortisol action in fish. Specifically, the mode of action of NSAIDs involves disruption of StAR and GR, two key proteins critical for cortisol production and target tissue responsiveness to this steroid, respectively. While the work

presented here identified the mechanism(s) of action of NSAIDs, the environmental relevance of this finding, specifically the impact of concentrations of NSAIDs present in our waterways on fish stress performance, remains to be explored.

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CHAPTER 1

General Introduction

1. Introduction

Pharmaceutical drugs and personal care products have been detected in the aquatic environment. However, few studies have addressed the impact of these drugs on aquatic organisms. The intended therapeutic use of non-steroidal anti-inflammatory drugs (NSAIDs) involves modulation of immune and endocrine functions in targeted species. Consequently, adverse effects, including endocrine disruption by NSAIDs may likely occur in aquatic organisms chronically exposed to pharmaceutical drugs in the environment. However, little is known about either the impact or the mechanism(s) of action of NSAIDs in impacting animal performance. This thesis investigates the impact of NSAIDs on the endocrine and metabolic pathways critical for coping with stress in a teleost fish, the rainbow trout (*Oncorhynchus mykiss*). Specifically, the work attempts to identify the mechanism(s) of action of NSAIDs in modulating the highly conserved adaptive stress response in a piscine model.

2. Pharmaceuticals in the environment

2.1 Detection and abundance

Prescription and non-prescription drugs are used in large quantities (hundreds to thousands of tons) to treat human diseases and in animal husbandry (Halling-Sorensen et al., 1998; Ternes, 1998). Nearly 30 to 90% of the dose originally administered is excreted in urine and feces in an active form (Halling-Sorensen et al., 1998). These compounds eventually pass through wastewater treatment processes, as treatment plants often are not designed to remove pharmaceutical products from sewage (Jorgensen and Halling-Sorensen, 2000). They can also be landfilled onto agricultural lands, which could lead to runoff into surface water or ground water. Consequently, contamination of the aquatic environment by pharmaceuticals is a major concern.

Until recently, most of the data on distribution and concentrations of pharmaceuticals in the aquatic environment came from Europe (for review Halling-Sorensen et al., 1998). However, recent studies clearly highlight the presence of these bioactive compounds in ng/L to µg/L concentrations, especially near discharges from sewage treatment plants (STPs), in American and Canadian waters (Metcalfe et al., 2003a,b). A large variety of pharmaceutical drugs were identified in surface waters, including several analgesic/anti-inflammatory drugs, a variety of lipid regulating agents, beta-blockers, $beta_2$-sympathomimetics, antibiotics and synthetic estrogens (Ternes 1998; Halling-Sorensen et al., 1998; Buser et al., 1999; Daughton and Ternes 1999; Ternes et al., 1999 and 2001; Heberer, 2002). Sewage effluents collected after treatment from STPs from different municipalities across Canada often showed detectable levels of non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, naproxen and salicylic acid at µg/L concentrations (Metcalfe et al., 2003a). Although there is now substantial evidence that numerous pollutants discharged into the environment from anthropogenic activities have the capacity to interfere with endocrine functions, very little is known about the effects of NSAIDs on endocrine axes.

2.2 Impact of NSAIDs in fish

Information concerning possible sublethal effects of NSAIDs on aquatic organisms is scarce. In most cases, studies have been restricted to short-term acute lethality tests in algae, invertebrates and fish (Webb, 2001). However, chronic exposure to diclofenac in rainbow trout revealed histopathological alterations and cytological effects in liver, kidney, gills and intestine that were correlated with a dose-related tissue accumulation of the drug (Schwaiger et al., 2004; Triebskorn et al., 2004). These studies are consistent with the well known gastrointestinal toxicity by NSAIDs in mammals (Wolfe et al., 1999). Concurrent histological analysis in fish

revealed an increase in granulocyte numbers in primary gill filaments, as well as granulocyte accumulation and enhanced major histocompatibility complex (MHC) II expression in kidney, suggesting an inflammatory response in these organs (Hoeger et al., 2005). In addition, diclofenac, at sublethal but environmentally relevant concentrations, inhibited the EROD activity in primary cultures of trout hepatocytes (Laville et al., 2004), suggesting potential impact on biotransformation of xenobiotics in fish.

NSAIDs are widely used for their analgesic and anti-inflammatory properties. The therapeutic effects are based on the fact that their amphiphilic acid structure binds to the lipid-water interphase of cell membrane proteins such as the prostaglandin-G/H synthase and inhibit their function. This enzyme exhibits two catalytic activities, hydroperoxidase and cyclooxygenase, which together catalyze the transformation of arachidonic acid to prostaglandins (PGs) and thromboxanes (Smith, 1989). Most NSAIDs are known inhibitors of cyclooxygenase activity. For instance, ibuprofen, indomethacin, diclofenac and acetylsalicylic acid (ASA) are commonly used in mammalian studies as well as in fish (Wade and Van der Kraak, 1993) as inhibitors of cyclooxygenase-2 (COX-2) activity. Consequently, NSAIDs will inhibit the synthesis and release of PGs, which are involved in the inflammatory response and also in modulating the functioning of the pituitary-adrenal axis in humans (Cavagnini et al., 1979; Hockings et al., 1993) and fish (Gupta et al., 1985; Wales, 1988). To this end, a recent study demonstrated that ASA administered *in vivo* to Mozambique tilapia (*Oreochromis mossambicus*) disturbed the endocrine axes, including attenuated plasma cortisol response to a stressor, leading to the proposal that PGs are involved in the stress response process in fish (van Anholt et al., 2003).

3. The General Stress Response

Stress is a physical condition in which the homeostasis of the animal is disturbed as a result of the actions of stimuli called stressors. These stressors will elicit a range of behavioral and physiological compensatory changes enabling the animal to overcome the threat. These adaptive changes characterize the stress response (Wendelaar Bonga, 1997). The general stress response can be broadly categorized into the primary, secondary and tertiary response (Mazeaud et al., 1977; Wedemeyer et al., 1990). The primary response represents the initial perception of stress and the initiation of a neuroendocrine/endocrine response characterized by the rapid release of stress hormones, the catecholamines and cortisol into the circulation (Wendelaar Bonga, 1997). Elevation in circulating levels of cortisol is the most common indicator of stress in fish (Mazeaud and Mazeaud, 1981; Donaldson, 1981). The secondary response comprises the various biochemical and physiological effects associated with stress, resulting mostly from the actions of the stress hormones like cortisol. Change in plasma glucose concentration is a good secondary indicator of stress in fish (Wedemeyer et al., 1990). The tertiary response represents changes at the whole-animal or at the population level, such as reduced growth and/or reproduction. These changes may be as a consequence of energy repartitioning to cope with the enhanced energy demand associated with stress (Wendelaar Bonga, 1997).

Cortisol is produced in response to a variety of biotic and abiotic stressors (Wendelaar Bonga, 1997), including pollutants (Hontela, 1997). Cortisol secretion plays an essential role in regaining homeostasis through effects on growth and metabolism, iono- and osmo-regulation and immune function (Wendelaar Bonga, 1997; Mommsen et al., 1999). Consequently, any adverse effects on cortisol production would compromise the ability of fish to respond to additional stressors (Hontela, 1998; Barton et al., 2002).

4. Hypothalamo-Pituitary-Interrenal (HPI) axis

4.1 Cortisol stress axis

The circulating level of cortisol is tightly regulated by the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). For instance, stress stimulates the release of corticotrophin-releasing factor (CRF) from the hypothalamus, which in turn stimulates the release of adrenocorticotropic hormone (ACTH), the main secretogogue for cortisol biosynthesis, from the pituitary. ACTH binds to the interrenal cells (steroidogenic cells), located predominantly in the anterior part of the kidney (head kidney), to activate the steroidogenic pathway leading to the release of cortisol (Wendelaar Bonga, 1997; Mommsen et al., 1999; Hontela, 2005). The rate of cortisol production is under the control of ACTH and one key step involves the transport of cholesterol from sites of storage to the inner mitochondrial membrane. Very little is known about the intracellular signaling pathway for cortisol in fish, but the importance of cAMP as a second messenger has been demonstrated (Ilan and Yaron, 1980; Patiño et al., 1986; Leblond et al., 2001; Lacroix and Hontela, 2001).

4.2 Cortisol Regulation

ACTH hormonal stimulation of the adrenal can be divided into two phases: acute and chronic regulation. In the acute response, ACTH stimulates the cAMP-protein kinase-A signaling cascade that leads to mobilization of cholesterol from cellular stores to CYPscc in the inner mitochondrial membrane at an accelerated rate, producing a rise in cortisol output within minutes of stimulation (Mokuda et al., 1997; Thomson, 1998). The chronic regulation is a secondary and longer lasting response to ACTH occurring after approximately an hour, and involves the upregulation of genes encoding the enzymes involved in the steroidogenic process, thereby maintaining optimal steroidogenic capacity (Hum and Miller, 1993; Orme-Johnson,

1990; Parker and Schimmer, 1995). Circulating levels of cortisol are tightly regulated by a negative feedback loop involving glucocorticoid receptor (GR) signaling in the brain. This results in reduced CRF and ACTH secretion from the hypothalamus and pituitary, respectively, in response to elevated stressor-induced plasma cortisol levels (Hontela 2005; Mommsen et al 1999; Wendelaar Bonga, 1997; Balm et al., 1994).

4.3 Cortisol biosynthesis

In mammals, the transport of cholesterol between the inner and the outer mitochondrial membrane is thought to be the rate limiting step in steroidogenesis, and this process require at least two key regulatory proteins: the steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor (PBR).

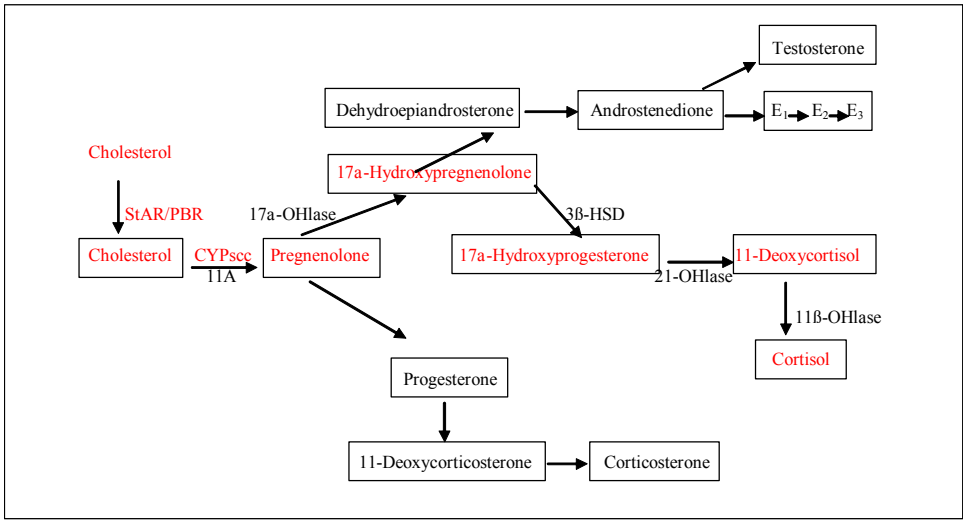
4.3.1. StAR: The acute regulation of steroid hormone synthesis is mediated by *de novo* protein synthesis of StAR (Stocco, 2005). This acute stimulation in response to peptide hormones/cAMP-mediated signaling is directly correlated with rapid increases in StAR mRNA levels. It is still unclear how cAMP activates StAR transcription during the acute steroidogenic response, but phosphorylation of StAR may be playing a role in this process (Jones et al., 2000). Four distinct models of StAR action have been proposed to explain cholesterol transport from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Miller, 2007), including that StAR: i) serves as a contact site between OMM and IMM, ii) desorbs cholesterol from the OMM into the mitochondrial intramembranous space (IMS) under the form of micro-droplets from the OMM before being taken up by the IMM, iii) must act as a cholesterol shuttle in the IMS (but this model was rejected because of the inconsistency with StAR being active only at the OMM), and iv) undergoes a molten globule conformational change at pH 3.5 aided by protonated phospholipids. Indeed, the most recent research provides evidence

for a pH-dependant molten globule model. However, in order for StAR to deliver up to 400 molecules of cholesterol to the IMM, while remaining on the OMM imply that StAR is part of a larger molecular mechanism for cholesterol transport (Miller, 2007). Pharmacological studies suggest that PBR is another key molecule required for mitochondrial cholesterol import (Papadopoulos, 1993).

4.3.2. PBR: This protein is primarily localized on the outer mitochondrial membrane and is extremely abundant in steroidogenic cells (Anholt et al., 1986). PBR is a high-affinity drug ligand and cholesterol binding protein (Li et al., 2001; Lacapère et al., 2001) involved in various cell functions, including steroid biosynthesis, mitochondrial respiration, cell proliferation and apoptosis (Papadopoulos, 1993; Gavish et al., 1999; Casellas et al., 2002). PBR ligands stimulate steroidogenesis and induce cholesterol movement from the OMM to the IMM. PBR is a multifunctional transmembrane protein, which is associated among others with the voltage-dependant anion channel (VDAC) located in the outer mitochondrial membrane and with the adenine nucleotide carrier (ANC) in the inner mitochondrial membrane. While a physical interaction between PBR and STAR has not been established, there is strong evidence for a functional interaction and a role for the cholesterol recognition amino acid consensus (CRAC) domain of PBR (Hauet et al., 2005). The predominant view used to be that StAR delivers cholesterol to PBR, which once activated by StAR, could serve as a regulated cholesterol channel to expose cholesterol to the active site of P450scc. However, inconsistencies with other studies have lead to a most recent model suggesting that StAR removes cholesterol from the cholesterol binding domain (CRAC) of PBR and delivers it to the IMM (Miller, 2007). It seems likely that other proteins would participate in this action, probably proteins in association with PBR (Miller, 2007). Although the mechanism of action is still unclear, these studies underscore

the importance of PBR and StAR in mammalian steroidogenesis (Miller, 2007), but very little is known in fish.

4.3.3. Steroidogenesis: (Fig. 1) Once cholesterol has reached the inner mitochondrial membrane of steroidogenic cells, the first and common step in the steroid biosynthesis pathway is the conversion of cholesterol to pregnenolone (Jefcoate et al; 1986, Stocco and Clark 1996) catalysed by cytochrome P450 side-chain cleavage enzyme (CYPscc) localized on the matrix side of the inner mitochondrial membrane (Hall, 1985; Stocco, 2001). The specific steroids, including cortisol, are synthesized through the enzymatic cascade, in which the conversion of 11-deoxycortisol by 11ß-hydroxylase in the mitochondria is the final step in cortisol synthesis (Payne and Hanes, 2004).



1. **Figure 1.** Cortisol biosynthesis pathway in teleost fish.

StAR: steroidogenic acute regulatory protein; PBR: peripheral-type benzodiazepine receptor; CYPscc: cytochrome P450 side chain cleavage (CYP 11A); 17α-OHlase: 17α-hydroxylase; 3β-HSD: 3β-hydroxy-steroid dehydrogenase; 21-OHlase: 21-hydroxylase (CYP21A2); 11β-OHlase: 11β-hydroxylase (CYP 11B1).

4.4 Adrenal/interrenal steroidogenesis as a target for toxicity

It is well known that the adrenal gland is a target organ for endocrine toxicity (Ribelin, 1984; Colby, 1996; Harvey, 1996; Hinson and Raven, 1999; Rossol et al., 2001; Harvey and Everett, 2003). Among others, some of the factors that predispose the adrenal to direct toxic assault include its disproportionately large blood supply per unit mass, its lipophilicity and its high concentration of cytochrome P-450 (CYP) and other enzymes normally involved in steroidogenesis, but that can also bioactivate toxicants (Hinson and Raven, 1999). Toxic effects on steroidogenesis in mammals have been reported in several studies presenting multiple molecular targets, ranging from general effects on all steroidogenic tissues to specific targets affecting only the adrenal function. These potential targets include a wide variety of cytochrome P-450s, dehydrogenases and the StAR protein (reviewed in Harvey and Everett, 2003). Recent studies have shown that the antiandrogenic effects of some peroxisome proliferators in rat MA-10 Leydig cells inhibited PBR expression by a direct transcriptional inhibition (Gazouli et al., 2002) or by accelerating PBR mRNA decay (Boujrad et al., 2000). Some compounds have more than one molecular target, indicating the vulnerability of the process of steroidogenesis to endocrine-disrupting chemicals (Harvey and Everett, 2003).

Endocrine disruption is not exclusive to mammals. In fact, the first reports on endocrine modulators originated in wildlife because of their constant exposure to the contaminated environment. Impairment of adrenal function has been reported in different species of wildlife, including fish exposed to a variety of environmental contaminants. Brown trout living in metal contaminated rivers (Norris et al., 1999) and yellow perch from metal contaminated lakes (Brodeur et al., 1998; Girard et al., 1998; Lévesque, 2003) exhibited disruption of HPI axis function. Also, different species of fish sampled at sites contaminated by polychlorinated

biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Hontela et al., 1992; 1995), and also from downstream of a point source of bleached kraft mill effluent (BKME) (Hontela et al., 1997), showed impaired interrenal function. The altered internal response to ACTH stimulation in these animals suggests that the normal stress-response may be disrupted and thus the ability of those impaired fish to cope with environmental stressors may be significantly reduced. In the laboratory, impaired cortisol response to stress have been demonstrated in fish exposed to PCB126 (Quabius et al., 1997), PCB agonist BNF (Wilson et al., 1998; Aluru and Vijayan, 2006) and Aroclor 1254 (Jorgensen et al., 2002). *In vitro* studies using interrenal cell suspension of rainbow trout have shown that ACTH- and cAMP-stimulated cortisol secretion was inhibited by pesticides (Bisson and Hontela, 2002), heavy metal (cadmium: Leblond and Hontela, 1999) and PCB agonists (Aluru and Vijayan, 2004, 2006). The impaired cortisol secretion induced by cadmium was reversed by a treatment with pregnenolone, indicating that Cd inhibited interrenal steroidogenesis at a step downstream from cAMP formation and prior to pregnenolone synthesis (Lacroix and Hontela, 2004). Also, a recent study showed that β-phytosterol, a plant sterol identified as one potential source of the reproductive malfunction in fish living downstream from pulp mills, impairs cholesterol mobilization across the mitochondrial membrane of goldfish testes *in vitro* (Leush and MacLatchy, 2003). Taken together, it appears likely that contaminants can impact steroidogenic pathways at multiple levels and laboratory studies are required to understand the possible mechanisms of action of environmental contaminants on this important endocrine axis in fish. In addition to direct effects, toxicants may indirectly impact steroidogenesis, including changes in the heat shock protein status of steroidogenic cells (Khanna et al., 1994; 1995).

5. The heat shock response

5.1 Heat shock proteins

While plasma cortisol levels are indicative of organismal stress response, heat shock proteins (hsps) levels are commonly used as an indicator of cellular stress response. Hsps are a family of intracellular proteins that share a high degree of similarity in their amino acid sequences among organisms and hsp70 (the 70 kilodalton family of hsps) is one of the most highly conserved among the groups (Iwama et al., 1998). Hsps are molecular chaperones constitutively expressed in cells to maintain a number of critical cellular processes. They are known to be involved in protein assembly, folding and translocation and also involved in regulating interactions between hormones and their receptors (Welch, 1993). These proteins are also induced rapidly in cells in response to a variety of stressors affecting the protein machinery to enhance cell survival. Heat shock is the most common stressor used in the characterization of the heat shock protein responses (Hightower, 1991). Stressors would act by damaging native proteins inside the cell. As a result, the misfolded or damaged proteins activate binding of heat shock factor-I (HSF-I) to heat shock elements (HSE) on the promoter regions of heat shock protein genes and activate transcription. The resultant protein synthesis will aid in either damaged protein repair or destruction (Morimoto et al., 1996). Stress proteins such as hsp70 are therefore used as indicator of cellular stress in fish. The lack of induction of these proteins in response to a stressor results in cell death (Hightower, 1991).

5.2 Toxicants and the Hsp response

Studies have shown that contaminants can induce the expression of hsp70 in fish (Iwama et al., 1998; Vijayan et al., 2005). While studies on HS response induction by xenobiotics have

focused on organic contaminants and heavy metals, very little is known about the impact of other toxicants, including pharmaceutical drugs in the aquatic environment. Several NSAIDs regulate different COX- independent pathways including the heat shock response (Lee et al., 1995; Ishihara et al., 2003; Lagunas et al., 2004). The induction of hsp70 and activation of the HSF-1 DNA-binding complex with a variety of NSAIDs have been reported in mammalian systems (Jurivich et al., 1992; 1995; Lee et al., 1995). Also, some NSAIDs can lower the cellular stress threshold by reducing the temperature and time threshold for induction of hsp70 in some mammalian cell lines (Lee et al., 1995; Batulan et al., 2005). While NSAIDs increase hsp70 transcript levels in mammalian cells, their role in the cellular stress response in lower vertebrates is largely unknown. As protein synthesis is energy demanding, the induction of stressor-induced heat shock protein synthesis will affect cellular energy status of cells. Consequently, impact of toxicants on tissue metabolic capacity may impair heat shock protein response to subsequent stressors in fish (Feng et al., 2003; Vijayan et al., 2005).

6. Energetics of ionregulation

6.1 Metabolic cost of ionregulation

There is an increased metabolic cost associated with seawater exposure in teleost fish. This increased energy demand is thought to include gill biogenesis as well as upregulation of gill ion transporters to facilitate ion excretion in seawater (Shepherd et al., 2005; Singer et al., 2007). Gills are the primary site for ion transport in teleost fish, taking up salts in fresh water and secreting them in seawater (Evans et al., 1999). The fish gill epithelium is characterized by the presence of three main cell types: pavement cells (PVCs), which represent most of the gill epithelium and lamellar surface, mitochondria-rich cells (MRCs) also called chloride cells, and accessory cells (ACS) (Evans, 2002). Both chloride cells and PVCs are involved in sodium and

chloride uptake in fresh water (McCormick, 2001), while in seawater, MRCs are the site of secretion of excess salt (Foskett and Scheffey, 1982b). The ability of fish to move between FW and SW requires remodeling of the gill, including changes in chloride cells phenotypes according to their external environment (Hiroi et al., 1999; Wong and Chan, 1999). Thus the FW and SW gill phenotypes can be distinguished based on their morphology, but also on the expression of their main ion transporters: Na^+/K^+-ATPase, Na^+/K^+-$2Cl^-$ cotransporter (NKCC) and a Cl^- channel named cystic fibrosis transmembrane conductance regulator (CFTR). Indeed, Na^+/K^+-ATPase, NKCC and CFTR mRNA abundance increase upon transfer from FW to SW (Singer et al., 1998; Pelis and McCormick, 2001; Marshall et al., 2002). This change in gill phenotype with salinity and the osmoregulatory adaptations are energy demanding and highly influenced by the cortisol axis, which act as a link between environmental changes and physiological responses.

6.2 Role of cortisol in ion regulation

Cortisol plays a key role not only in energy substrate mobilization to provide fuel to cope with increased tissue energy demand, but also increases chloride cell biogenesis and ion transporter activities in teleost fishes (Mommsen et al., 1999; McCormick, 2001). In seawater, cortisol upregulates gill ion transporters. Specifically, studies have shown that cortisol elevates gill Na^+/K^+-ATPase activity and CFTR expression in teleost fishes (McCormick, 1995; 2001; Singer et al., 1998; 2007). The number of gill cortisol receptors is strongly correlated with the capacity of cortisol to stimulate Na^+/K^+-ATPase activity *in vivo* and *in vitro* (McCormick et al., 1991a; Shrimpton et al., 1994; Shrimpton and McCormick, 1999). Also, gill GR transcript abundance increases upon exposure of trout to higher salinities (Singer et al., 2007), supporting a key role for gill GR signaling in modulating ion transporters during seawater acclimation. In addition, cortisol along with other hormones, including growth hormone and insulin-like growth

factor-I, upregulate ion transporters leading to improved salinity tolerance (Madsen, 1990b; Madsen and Korsgaard, 1991; McCormick, 1996; Mancera and McCormick, 1999).

6.3 Energy demand: role of cortisol

Stressed animals have higher metabolic rate and this increased energy demand is associated with metabolic adjustments to regain homeostasis. The cortisol response associated with stress is thought to play a key role in the energy substrate mobilization critical for coping with the increased energy demand (Barton et al., 2002; Iwama et al., 2006; Mommsen et al., 1999; Wendelaar Bonga, 1997). Glucose is an important fuel for metabolism and certain tissues, including brain and gills in fish, rely primarily on this metabolite for energy production (Mommsen et al., 1999). One of the effects of cortisol is the production of glucose during stress in fish. This is accomplished by cortisol-induced enhancement of liver metabolic capacity, including gluconeogenesis, glycogenolysis, proteolysis and lipolysis, all of which are crucial for the metabolic adjustments to stress in fish (Mommsen et al., 1999).

In seawater, cortisol is involved in the energy substrate re-partitioning to cope with the increased energy demand. Specifically, cortisol is thought to play a role in enhancing gluconeogenesis to provide glucose for gill metabolism. This is supported by the higher plasma glucose levels and liver gluconeogenic capacity, along with elevated plasma cortisol levels upon seawater exposure in fish (Mommsen et al., 1999; Laiz-Carrion et al., 2002; Shepherd et al., 2005; Singer et al., 2007). Also, the gill glycolytic capacity is enhanced in seawater suggesting an enhanced utilization of glucose for tissue metabolism (Vijayan et al., 2001; Laiz-Carrion et al., 2002). Considered together, proper functioning of the cortisol axis is critical for stress tolerance, including seawater acclimation in rainbow trout.

6.4 Impact of toxicants on ion regulation

Several studies have investigated the potential effects of environmental contaminants on gill osmoregulatory capacity of fish. Inhibition of gill Na^+/K^+-ATPase activity was reported in fish exposed to metals (Morgan et al., 1997; Lionetto et al., 1998) and organic pollutants, including pesticides and PCBs (Davis et al., 1972; Koch et al., 1972; Lerner et al., 2007). Also, xenoandrogens and xenoestrogens have been shown to impact salinity tolerance in salmon, including inhibition of ion transporter function and impaired the adaptive hormonal response to salinity (Berglung et al., 1994; Lundqvist et al., 1989; McCormick et al., 2005). The impact on ion regulation may at least in part be mediated by a decreased gill and liver metabolic capacity as seen in tilapia exposed to estrogens (Vijayan et al., 2001). Together, these results highlight a role for pollutants in disrupting the conserved adaptive response to salinity challenge in teleosts either by affecting directly the ion transporters or indirectly through perturbation in intermediary metabolism. However, whether NSAIDs have a role in impacting the seawater acclimation process remains to be determined.

7. Hypothesis and Objectives

Elevated plasma cortisol levels seen after a stressor exposure plays an important role in the stress adaptation process in fish. This steroid not only has specific effects on stress coping mechanisms, including upregulation of ion transporters, but also exerts a generalized effect by modulating the energy substrate re-partitioning, which is essential for coping with the higher demand for energy seen in stressed animals. Consequently, any impact on the cortisol stress axis and/or the metabolic status of the cells will impair the adaptive responses critical for coping with stress. The impact of aquatic pollutants on the stress axis in fish has focused mainly on PAHs

and metals, while little is known about the role of pharmaceutical drugs. The hypothesis tested in this thesis is that: non-steroidal anti-inflammatory drugs (NSAIDs) impair the adaptive stress response by disrupting the cortisol stress axis in rainbow trout. To test this hypothesis, the specific objectives are to determine whether NSAIDs

- disrupt corticosteroidogenesis in trout
- impair the adaptive cortisol and metabolic response to stressors
- alter the highly conserved heat shock protein response
- impair tissue-specific metabolic and ionoregulatory capacities in seawater stressors

References

Aluru N, Vijayan MM. 2004. beta-Naphthoflavone disrupts cortisol production and liver glucocorticoid responsiveness in rainbow trout. Aquat Toxicol. 67(3):273-85.

Aluru N, Vijayan, M.M. 2006. Ah receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis. Endocrinol. 147(4):1895-1903.

Anholt RR, Pedersen PL, De Souza EB, Snyder SH. 1986. The peripheral-type benzodiazepine receptor. Localization to the mitochondrial outer membrane. J.Biol.Chem. 261(2):576-583.

Balm PH, Pepels P, Helfrich S, Hovens ML, Bonga SE. 1994. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol. 96(3):347-60.

Barton BA, Morgan JD, Vijayan MM. 2002. Physiological and condition-related indicators of environmental stress in fish. In: Adams, S.M. (Ed.), Biological Indicators of Stress in Fish, Second Edition. American Fisheries Society, Bethesda, Maryland, pp 111-148.

Batulan Z, Nalbantoglu, J., Durham, H.D. 2005. Nonsteroidal anti-inflammatory drugs differentially affect the heat shock response in cultured spinal cord cells. Cell Stress. Chaper.10(3):185-196.

Bisson M, Hontela A. 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed *in vitro*. Toxicol Appl Pharmacol. 180(2):110-7.

Boone AN, Vijayan MM. 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. Amer. J. Physiol. Regul, Integr. Comp. Physiol. 283, R680-R687.

Boujrad N, Vidic B, Gazouli M, Culty M, Papadopoulos V. 2000. The peroxisome proliferator perfluorodecanoic acid inhibits the peripheral-type benzodiazepine receptor (PBR) expression and hormone-stimulated mitochondrial cholesterol transport and steroid formation in Leydig cells. Endocrinology. 141(9):3137-48.

Brodeur JC, Sherwood G, Rasmussen JB, Hontela A. 1998. Impaired cortisol secretion in yellow perch (*Perca flavescens*) from lakes contaminated mwith heavy metals: in vivo and in vitro assessment. Can.J.Fish.Aquat.Sci. 54: 2752-2758.

Burglund I, Lundqvist H, and Fangstam, H. 1994. Downstream migration of immature salmon (*Salmo salar*) smolts blocked by implantation of the androgen 11-ketoandrostenedione, Aquaculture 121: 269–276.

Buser HR, Muller, MD, Theobald N. 1998a. Occurrence of pharmaceutical drud clofibric acid and the herbicide mecorpop in various Swiss lakes and in the North Sea. Environ Sci Technol 32: 188-192.

Buser HR, Poiger T, Muller MD. 1998b. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. Environ Sci Technol 32: 3449-3456.

Buser HR, Poiger T, Muller MD.1999. Occurrence and environmental behaviour of the chiral pharmaceutical drug ibuprofen in surface waters and wastewater. Environ Sci Technol 33: 2529-2535.

Casellas P, Galiegue S, Basile AS. 2002. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int. 40(6):475-86.

Cavagnini F, Di Landro A, Maraschini C, Invitti C, Pinto ML. 1979. Effect of two prostaglandin synthesis inhibitors, indomethacin and acetylsalicylic acid, on plasma ACTH and cortisol levels in man. Acta Endocrinol (Copenh). 91(4):666-73.

Colby HD. 1986. The adrenal cortex as a toxicological target organ. In The adrenal in toxicology: Target organ and modulator of toxicity, Harvey PW (ed.). Taylor and Francis: London; 131-163.

Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ Health Perspect. 107 Suppl 6:907-38.

Davis PW, Friedhoff JM, Wedemeyer GA. 1972. Organochlorine insecticide, herbicide and polychlorinated biphenyl (PCB) inhibition of NaK-ATPase in rainbow trout. Bull Environ Contam Toxicol. 8(2):69-72.

Donaldson EM. 1981. The pituitary-interrenal axis as an indicator of stress in fish. In Stress and fish. pickering AD. (Ed.). Academic Press, New York. pp.11-47.

Evans DH. 2002. Cell signaling and ion transport across the fish gill epithelium. J.Exp.Zool. 293: 336-347.

Evans DH, Piermarini PM, Potts WW. 1999. Ionic transport in the fish gill epithelium. Review. J. Exper. Zool. 283:641–652.

Evans DH, Roeser JM, Stidham JD. 2002. Natriuretic peptide hormones do not inhibit NaCl transport across the opercular skin of the killifish, Fundulus heteroclitus. Bull Mt Desert Isl Biol Lab 41:47.

Feng Q, Boone AN, Vijayan MM. 2003. Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135C: 345-355.

Fortner NA, Pickford GE. 1982. The effects of hypophysectomy and replacement therapy with prolactin, cortisone, or their combination on the blood of the black bullhead *Icatalurus melas*. Gen. Comp. Endocrinol. 47:111–119.

Foskett JK, Scheffey C. 1982. The chloride cell: Definitive identification as the salt-secretory cell in teleosts. Science. 215:164–166.

Gagne F, Blaise C, Fournier M, Hansen PD. 2006. Effects of selected pharmaceutical products on phagocytic activity in Elliptio complanata mussels. Comp Biochem Physiol C Toxicol Pharmacol. 143, 179-186.

Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, Weizman A. 1999. Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev.51(4): 629-50.

Gazouli M, Yao ZX, Boujrad N, Corton JC, Culty M, Papadopoulos V. 2002. Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor alpha. Endocrinol. 143(7):2571-83.

Girard C, Brodeur JC, Hontela A. 1998. Responsiveness of the interregnal tissue of yeloow perch (*Perca flavescens*) from contaminated sites to an ACTH challenge test in vivo. Can.J. Fish. Aquat. Sci. 55: 438-450.

Gupta OP, Lahlou B, Botella J, Porthe-Nibelle J. 1985. *In vivo* and *in vitro* studies on the release of cortisol from interrenal tissue in trout. I. Effects of ACTH and prostaglandins. *Exp. Biol.* 43: 201-212.

Hall PF. 1985. Role of cytochromes P-450 in the biosynthesis of steroid hormones. Vitam. Horm. 42: 315-68.

Halling-Sorensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lutzhoft HC, Jorgensen SE. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment—a review. Chemosphere. 36(2):357-393.

Harvey PW. 1996. The adrenal in toxicology: Target organ and modulator of toxicity. Taylor and Francis: London.

Harvey PW, Everett DJ. 2003. The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: critical omissions from regulatory endocrine disrupter screening strategies for human health? J Appl Toxicol. 23(2):81-7.

Hauet T, Yao ZX, Bose HS, Wall CT, Han Z, Li W, Hales DB, Miller WL, Culty M, Papadopoulos V. 2005. Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. Mol. Endocrinol. 19: 540-554.

Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett. 131(1-2):5-17.

Herndon, TM., McCormick, SD., Bern, HA. 1991. Effects of prolactin on chloride cells in opercular membrane of seawater-adapted tilapia. Gen.Comp.Endocrinol. 83: 283–289.

Hightower LE. 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66: 191-197.

Hinson JP, Raven PW. 1999. Adrenal toxicology. In. Endocrine and Hormonal Toxicology, Harvey PW, Rush KC, Cockburn A (eds). John Wiley: Chichester; 67-89.

Hiroi J, Kaneko T, Tanaka M. 1999. *In vivo* sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. J. Exp. Biol. 202:3485–3495.

Hockings GI, Grice JE, Crosbie GV, Walters MM, Jackson AJ, Jackson RV. 1993. Aspirin increases the human hypothalamic-pituitary-adrenal axis response to naloxone stimulation. J.Clin.Endocrinol.Metab.77(2):404-408.

Hoeger B, Kollner B, Dietrich DR, Hitzfeld B. 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). Aquat Toxicol. 75(1):53-64.

Hontela A. 1997. Endocrine and physiological responses of fish to xenobiotics: role of glucocorticoid hormones. Rev Toxicol. 1: 1-46.

Hontela A. 1998. Interrenal dysfunction in fish from contaminated sites: in vivo and in vitro assessment. Annual Review Environ. Toxicol. Chem. 17: 44-48.

Hontela A. 2005. Stress and the hypothalamo-pituitary-interrenal axis: adrenal toxicology-Effects of environmental pollutants on the structure and function of the HPI axis. In: T.W.Moon and T.P.Mommsen (Eds.), Biochemical and molecular biology of fishes, Vol.6 Environmental Toxicology. Elsevier, Amsterdam, p 331-363.

Hontela A, Dumont P, Duclos D, Fortin R. 1995. Endocrine and metabolic dysfunction in yellow perch, Perca flavescens, exposed to organic contaminants and heavy metals in the St-Lawrence River. Environ.Toxicol.Chem. 14: 725-731.

Hontela A, Rasmussen JB, Audet C, Chevalier G. 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch Environ Contam Toxicol. 22(3):278-83.

Hum DW, Miller WL. 1993. Transcriptional regulation of human genes for steroidogenic enzymes. Clin Chem. 39(2):333-40.

Ilan Z, Yaron Z. 1980. Stimulation of cortisol secretion in vitro from the interrenal tissue of the cichlid fish, Sarotherodon aureus, by adrenocorticotrophin or cyclic AMP. J Endocrinol. 86(2):269-77.

Ishihara K, Horiguchi K, Yamagishi N, Hatayama T. 2003. Identification of sodium salicylate as an hsp inducer using a simple screening system for stress response modulators in mammalian cells. Eur. J. Biochem. 270: 3461-3468.

Iwama GK, Alfonso LOB, Vijayan MM. 2006. Stress in Fish. In: Evans, D.H., Clairborne, J.B. (Eds.), The Physiology of Fishes, Third Edition. CRC Press, Boca Raton, pp 319-342.

Iwama GK, Thomas PT, Forsyth RB, Vijayan MM. 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fisher. 8: 35-56.

Jefcoate CR, McNamara BC, and DiBartolomeis, MS. 1986. Control of steroid synthesis in adrenal fasciculata. Endocr. Res. 12: 315–350.

Jones PM, Sayed SB, Persaud SJ, Burns CJ, Gyles S, Whitehouse BJ. 2000. Cyclic AMP-induced expression of steroidogenic acute regulatory protein is dependent upon phosphoprotein phosphatase activities. J Mol Endocrinol. 24(2):233-239.

Jorgensen EH, Vijayan MM, Aluru N, Maule AG. 2002. Fasting modifies Aroclor 1254 impact on plasma cortisol, glucose and lactate responses to a handling disturbance in Arctic charr. Comp Biochem Physiol C Toxicol Pharmacol.132(2) :235-45.

Jorgensen SE, Halling-Sorensen B. 2000. Drugs in the environment. Chemosphere 40: 691-699.

Jurivich DA, Pachetti C, Qiu L, Welk JF. 1995. Salicylate triggers heat shock factor differently than heat. J. Biol .Chem. 270: 24489-24495.

Jurivich DA, Sistonen, L, Kroes RA, Morimoto RI. 1992. Effect of sodium salicylate on the human heat shock response. Science 255: 1243-1245.

Kelly SP, Chow INK, Woo NYS. 1999. Effects of prolactin and growth hormone on strategies of hypoosmotic adaptation in a marine teleost, *Sparus sarba*. Gen.Comp. Endocrinol.113: 9–22.

Khanna A, Aten RF, Behrman HR. 1994. Heat shock protein induction blocks hormone-sensitive steroidogenesis in rat luteal cells. Steroids. 59(1):4-9.

Khanna A, Aten RF, Behrman HR. 1995. Physiological and pharmacological inhibitors of luteinizing hormone-dependent steroidogenesis induce heat shock protein 70 in rat luteal cells. Endocrinol. 136: 1775-1781.

Kirschner LB. 1993. The energetics of osmotic regulation in ureotelic and hypoosmotic fishes. J. Exp. Zool. 267: 19–26.

Koch RB, Desaiah D, Yap HH, Cutkomp LK. 1972. Polychlorinated biphenyls: effect of long-term exposure on ATPase activity in fish, Pimephales promelas. Bull Environ Contam Toxicol. 7(2):87-92.

Lacapere JJ, Delavoie F, Li H, Peranzi G, Maccario J, Papadopoulos V, Vidic B. 2001. Structural and functional study of reconstituted peripheral benzodiazepine receptor. Biochem Biophys Res Commun. 284(2):536-41.

Lacroix A, Hontela A. 2004. A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, Oncorhynchus mykiss, and yellow perch, Perca flavescens. Aquat Toxicol. 67(1):13-21.

Lacroix M, Hontela, A. 2001. Regulation of acute cortisol synthesis by cAMP-dependent protein kinase and protein kinase C in a teleost species, the rainbow trout (Oncorhynchus mykiss). J. Endocrinol. 169(1):71-8.

Lagunas L, Bradbury MC, Laszlo A, Hunt CR, Gius D. 2004. Indomethacin and ibuprofen induce Hsc70 nuclear localization and activation of the heat shock response in HeLa cells. Biochem .Biophys. Res .Commun. 313: 863-870.

Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Míguez JM, Soengas JL, Mancera JM. 2002. Energy Metabolism in Fish Tissues Related to Osmoregulation and Cortisol Action. Fish Physiol.Biochem. 27: 179-188.

Laurent P, Dunelerb S, Chevalier C, Lignon J. 1994. Gill epithelial-cells kinetics in a freshwater teleost, Oncorhynchus mykiss during adaptation to ion-poor water and hormonal treatments. Fish Physiol.Biochem. 13: 353-370.

Laurent P, Perry SF. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tissue Res. 259: 429–442.

Laville N, Ait-Aissa S, Gomez E, Casellas C, Porcher JM. 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. Toxicology 196(1-2):41-55.

Leblond VS, Bisson M, Hontela A. 2001. Inhibition of cortisol secretion in dispersed head kidney cells of rainbow trout (Oncorhynchus mykiss) by endosulfan, an organochlorine pesticide. Gen Comp Endocrinol. 121(1):48-56.

Leblond VS, Hontela A. 1999. Effects of in vitro exposures to cadmium, mercury, zinc, and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane on steroidogenesis by dispersed interrenal cells of rainbow trout (Oncorhynchus mykiss). Toxicol Appl Pharmacol. 157(1):16-22.

Lee BS, Chen J, Angelidis C, Jurivich DA, Morimoto RI. 1995. Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage. Proc. Natl. Acad. Sci .U.S.A. 92: 7207-7211.

Lerner DT, Bjornsson BT, McCormick SD. 2007. Effects of aqueous exposure to polychlorinated biphenyls (Aroclor 1254) on physiology and behavior of smolt development of Atlantic salmon. Aquat. Tox. 81 (3): 329-336.

Leusch FD, MacLatchy DL. 2003. *In vivo* implants of ß-sitosterol cause reductions of reactive cholesterol pools in mitochondria isolated from gonads of male goldfish (*Carassius auratus*). Gen. Comp. Endocrinol. 134, 255-263.

Levesque HM, Dorval J, Hontela A, Van Der Kraak GJ, Campbell PG. 2003. Hormonal, morphological, and physiological responses of yellow perch (Perca flavescens) to chronic environmental metal exposures. J Toxicol Environ Health A. 66(7):657-76.

Li H, Yao Z, Degenhardt B, Teper G, Papadopoulos V. 2001. Cholesterol binding at the cholesterol recognition/ interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. Proc Natl Acad Sci U S A. 98(3):1267-72.

Lin H, Randall DJ. 1993. Proton-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol.180:163–174.

Lionetto MG, Maffia M, Cappello MS, Giordano ME, Storelli C, Schettino T. 1998. Effect of cadmium on carbonic anhydrase and Na+-K+-ATPase in eel, Anguilla anguilla, intestine and gills. Comp.Biochem.Physiol. A. Mol. Integ. Physiol. 120 (1): 89-91.

Lundqvist H, Borg B, and Berglund I. 1989.Androgens impair seawater adaptability in smolting Baltic salmon (*Salmo salar*). Can. J. Zool. 67:1733–1736.

Madsen SS. 1990. The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). Gen. Comp. Endocrinol. 79:1–11.

Madsen SS, Korsgaard B. 1991. Opposite effects of 17B-estradiol and combined growth hormone cortisol treatment on hypo-osmoregulatory performance in sea trout presmolts, *Salmo trutta* Gen. Comp. Endocrinol. 83: 276–282.

Mancera J. M., McCormick, S. D. 1999. Influence of cortisol, growth hormone, insulin-like growth factor I and 3,3′,5-triiodo-L-thyronine on hypoosmoregulatory ability in the euryhaline teleost Fundulus heteroclitus. Fish. Physiol. Biochem. 21: 25–33.

Marshall WS, Lynch EM, Cozzi RRF. 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish Fundulus heteroclitus to seawater. J Exp Biol 205:1265–1273.

Marshall WS. 2002. Na+, Cl-, Ca2+ and Zn2+ transport by fish gills: retrospective review and prospective synthesis. J.Exp.Zool. 293: 264-283.

Mazeaud MM, Mazeaud F, Donaldson EM. 1977. Primary and secondary effects of stress in fish. Some new data with a general review. Trans.Am.Fish.Soc. 106: 201-212.

Mazeaud MM, Mazeaud F. 1981. Adrenergic responses to stress in fish. In Stress and Fish. AD Pickering (Ed.). Academic Press, New York. pp 49-75.

McCormick SD. 1996. Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+,K^+-ATPase in Atlantic salmon (*Salmo salar*): Interactions with cortisol. Gen. Comp. Endocrinol. 101:3–11.

McCormick SD. 2001. Endocrine control of osmoregulation in teleost fish. Amer.Zool. 41: 781-794.

McCormick S D, Dickhoff WW, Duston J, Nishioka, RS, Bern HA. 1991. Developmental differences in the responsiveness of gill Na^+,K^+-ATPase to cortisol in salmonids. Gen. Comp. Endocrinol. 84:308–317.

Metcalfe CD, Koenig BG, Bennie DT, Servos M, Ternes TA, Hirsch R. 2003a. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ Toxicol Chem. 22(12):2872-80.

Metcalfe CD, Miao XS, Koenig BG, Struger J. 2003b. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem. 22(12):2881-9.

McCormick SD, O'Dea MF, Moeckel A, Lerner, DT and Björnsson BT. 2005. Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17beta-estradiol.Gen Comp Endocrinol.142(3):280-288.

Miller MR, Wentz E, Ong S. 1999. Acetaminophen alters estrogenic responses in vitro: inhibition of estrogen-dependent vitellogenin production in trout liver cells. Toxicol Sci. 48(1):30-7.

Miller WL. 2007. Mechanism of StAR's regulation of mitochondrial cholesterol import. Mol Cell Endocrinol. 265-266, 46-50.

Miller WL, Strauss JF. 3rd. 1999 Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. J Steroid Biochem Mol Biol. 69, 131-141.

Miller WL. 1988. Molecular biology of steroid hormone synthesis. Endocr Rev. 9(3):295-318.

Mokuda O, Ubukata E, Sakamoto Y. 1997. Glucocorticoids potentiate effect of ACTH on cortisol secretion in isolated perfused guinea pig adrenal glands. Exp Clin Endocrinol Diabetes. 105(2):119-21.

Mommsen TP. 1984. Metabolism of the fish gill. In *Fish Physiology*, eds. W.S. Hoar and D.J. Randall, pp 203-238, Academic, New York.

Mommsen TP. 1997. Growth and metabolism. In *The Physiology of Fishes, Second Edition*, ed. D.H. Evans, pp 65-97, CRC Press, Boca Raton, Florida.

Mommsen TP, Vijayan MM, Moon TW. 1999. Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. Reviews Fish Biol. Fish. 9: 211-268.

Morgan IJ, Henry RP, Wood CM. 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na+ and Cl- transport. Aquat. Tox. 38 (1-3): 145-163.

Morimoto RI, Kroeger PE, Cotto JJ.1996. The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions.EXS. 77:139-63. Review.

Nakano K, Tagawa M, Takemura A, Hirano T. 1997. Effects of ambient salinities on carbohydrate metabolism in two species of Tilapia: *Oreochromis mossambicus* and *O. niloticus*. Fish Sci 63:338–343.

Norris DO, Donahue S, Dores RM, Lee JK, Maldonado TA, Ruth T, Woodling JD. 1999. Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. Gen Comp Endocrinol. 113(1):1-8.

Olson KR. 2002. Gill circulation: regulation of perfusiondistribution and metabolism of regulatory molecules. J.Exp.Zool. 293: 320–335.

Orme-Johnson NR. 1990. Distinctive properties of adrenal cortex mitochondria. Biochim Biophys Acta. 1020(3):213-31.

Pannevis MC, Houlihan DF. 1992. The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. [B] 162: 393-400.

Papadopoulos V. 1993. Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. Endocr Rev. 14(2):222-40.

Parwez I, Goswami SV. 1985. Effects of prolactin, adrenocorticotrophin, neurohypophysial peptides, cortisol and androgens on some osmoregulatory parameters of the hypophysectomized catfish, *Heteropneustes fossilis* (Block). Gen. Comp. Endocrinol. 58: 51–68.

Parker KL, Schimmer BP. 1995. Transcriptional regulation of the genes encoding the cytochrome P-450 steroid hydroxylases. Vitam Horm. 51:339-70. Review.

Patino R, Bradford CS, Schreck CB. 1986. Adenylate cyclase activators and inhibitors, cyclic nucleotide analogs, and phosphatidylinositol: effects on interrenal function of coho salmon(Oncorhynchus kisutch) in vitro. Gen Comp Endocrinol. 63(2):230-5.

Payne AH, Hales DB. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25, 947–970.

Pelis RM, McCormick SD. 2001. Effects of growth hormone and cortisol on Naþ,Kþ,2Cl_ cotransporter localization and abundance in the gills of Atlantic salmon. Gen Comp Endocrinol 124:134–143.

Perry SF, Goss GG, Laurent P.1992. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. Can. J. Zool. 70:1775–1786.

Pisam M, Auperin B, Prunet P, Rentier-Delrue F, Martial J, Rambourg A. 1993. Effects of prolactin on a and b chloride cells in the gill epithelium of the saltwater-adapted tilapia *Oreochromis niloticus*. Anat.Rec. 235: 275–284.

Quabius ES, Balm PH, Wendelaar Bonga SE. 1997. Interrenal stress responsiveness of tilapia (*Oreochromis mossambicus*) is impaired by dietary exposure to PCB 126. Gen Comp Endocrinol. 108(3):472-82.

Ribelin WE. 1984. The effects of drugs and chemicals upon the structure of the adrenal gland. Fundam Appl Toxicol. 4(1):105-19.

Rossol TJ, Yarrington JT, Latendresse J, Capen CC. 2001. Adrenal gland: structure, function and mechanisms of toxicity. Toxicol. Pathol. 29: 41-48.

Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele RD. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac; Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol. 68(2):141-50.

Shepherd BS, Drennon K, Johnson J, Nichols JW, Playle RC, Singer TD, Vijayan, MM. 2005. Salinity acclimation affects the somatotropic axis in rainbow trout. Am. J. Physiol. Reg.Integ.Comp.Physiol. 288: R1385-R1395.

Shrimpton JM, Devlin RH, Mclean E, Byatt JC, Donaldson EM, Randall DJ. 1995. Increases in gill corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) treated with growth hormone and placental lactogen. Gen. Comp. Endocrinol. 98:1–15.

Shrimpton JM, Bernier NJ, Randall DJ. 1994. Changes in cortisol dynamics in wild and hatchery-reared juvenile coho salmon (*Oncorhynchus kisutch*) during smoltification. Can. J. Fish. Aquat. Sci. 51:2179–2187.

Shrimpton J.M. and McCormick, S.D., 1998. Seasonal differences in plasma cortisol and gill corticosteroid receptors in upper and lower mode juvenile Atlantic salmon. Aquaculture 168: 205–219.

Shrimpton JM, McCormick SD. 1999. Responsiveness of gill Na+/K+-ATPase to cortisol is related to gill corticosteroid receptor concentration in juvenile rainbow trout. J. Exp. Biol. 202:987–995.

Singer TD, Raptis S, Sathiyaa R, Nichols JW, Playle, RC and Vijayan MM. 2007 Tissue-specific modulation of glucocorticoid receptor expression in response to salinity acclimation in rainbow trout. Comp Biochem Physiol B Biochem Mol Biol. 146(2):271-278.

Singer TD, Tucker SJ, Marshall WS, and Higgins CF. 1998. A divergent CFTR homolog: highly regulated salt transport in the euryhaline teleost *F. heteroclitus.* Am J Physiol Cell Physiol 274: C715–C723.

Smith WL. 1989. The eicosanoids and their biochemical mechanisms of action. Biochem. J. 259(2):315-24.

Stocco DM. 2001. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol. 63:193-213.

Stocco DM, Wang XJ, Jo Y, Manna PR. 2005. Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought. Mol. Endocrinol. 19, 2647-2659.

Stocco DM, Clark BJ. 1996. Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev. 17(3):221-44.

Tegeder I, Pfeilschifter J, Geisslinger G. 2001. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J.15: 2057-2072.

Ternes TA. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res 12: 3245-3260.

Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants--I. Investigations in Germany, Canada and Brazil. Sci Total Environ. 225(1-2):81-90.

Ternes T, Bonerz M, Schmidt T. 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr A. 938(1-2):175-85.

Thomson M. 1998. Does the CRH binding protein shield the anterior pituitary from placental CRH? Endocrine. 9(3):221-6.

Triebskorn R, Casper H, Heyd A, Eikemper R, Kohler HR, Schwaiger J. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac; Part II.Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol. 68(2):151-66.

van Anholt RD, Spanings T, Koven W, Wendelaar Bonga SE. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (Oreochromis mossambicus). Am J Physiol Regul Integr Comp Physiol. 285(5):R1098-106.

Van der Boon J, Van den Thillart GEEJM, Addink ADF. 1991. The effects of cortisol administration on intermediaty metabolism in teleost fish. Comp Biochem Physiol 100A:47–53.

Vijayan MM, Ballantyne JS, Leatherland JF. 1990. High stocking density alters the enrgy-metabolism of brook charr, Salvelinus fontinalis. Aquacult. 88, 371-381.

Vijayan MM, Ballantyne JS, Leatherland JF. 1991. Cortisol-induced changes in some aspects of the intermediary metabolism of Salvelinus fontinalis. Gen.Comp.Endocrinol. 82: 476-486.

Vijayan MM, Foster GD, Moon TW. 1993. Efects of cortisol on hepatic carbohydrate-metabolism and responsiveness to hormones in the sea raven *Hemitripterus americanus*. Fish Physiol.Biochem. 12, 327-335.

Vijayan MM, Moon TW. 1992. Acute handling stress alters hepatic glycogen metabolism in food deprived rainbow trout *Oncorhynchus mykiss*. Can.J.Fish.Aquat.Sci. 49 (11): 2260-2266.

Vijayan MM, Pereira C, Moon TW. 1994. Hormonal-stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. Comp.Biochem.Physiol.C.-Pharmacol.

Toxicol.Endocrinol. 108: 321-329.

Vijayan MM, Prunet P, Boone AN. 2005. Xenobiotic impact on corticosteroid signaling. In *Biochemistry and molecular biology of fishes,* vol. 6, eds. T. P. Mommsen, and T. W. Moon, pp. 365–394, Elsevier, Amsterdam.

Vijayan MM, Takemura A, Mommsen TP, 2001. Estradiol impairs hyposmoregulatory capacity in the euryhaline tilapia, *Oreochromis mossambicus*. Am. J. Physiol. 281, R1161–R1168.

Wade MG, Van der Kraak G. 1993. Arachidonic acid and prostaglandin E2 stimulate testosterone production by goldfish testis in vitro. Gen Comp Endocrinol. 90(1):109-18.

Wales NM. 1988. Hormones studies in *Myxine glutinosa*: effects of the eicosanoids arachidonic acid, prostaglandin E_1, E_2, A_2, F_{2a}, thromboxane B_2 and of indomethacin on plasma cortisol, blood pressure, urine flow and electrolyte balance. J. Comp. Endocrinol. B. 158: 621-626.

Webb SF. 2001. A data based perspective on the environmental risk assessment of human pharmaceuticals II –aquatic risk characterization. In. Kummerer, K. (Ed.),Pharmaceuticals in the environment. Sources, fate, effects and risks. Springer-Verlag Berlin, Heidelberg, New York, pp.203-219.

Wedemeyer GA, Barton BA, McLeay DJ. 1990. Stress and acclimation. In: Methods for Fish Biology, edited by C. B. Schreck and P. B. Moyle. Bethesda, MD: Am. Fish. Sot.270: 451-489.

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

Wilson ND, Shear NM, Paustenbach DJ, Price PS. 1998. The effect of cooking practices on the concentration of DDT and PCB compounds in the edible tissue of fish. J.Expo.Anal.Environ. Epidemiol. 8(3):423-40.

Wolfe MM, Lichtenstein DR, Singh G. 1999. Gastrointestinal Toxicity of Nonsteroidal Antiinflammatory Drugs.N.Eng.J.Med. 340:1888-1899.

Wong CKC, Chan,DKO. 1999. Chloride cell subtypes in the gill epithelium of Japanese eel Anguilla japonica. American Journal of Physiology—Regulatory Integr. Comp. Physiol. 46:517–R522 R.

Yamauchi K, Nishioka RS, Young G, Ogasawara T, Hirano T, Bern HA. 1991. Osmoregulation and circulating growth hormone and prolactin in hypophysectomized coho salmon (*Oncorhynchus kisutch*) after transfer to freshwater and seawater. Aquaculture 952: 33-42.

CHAPTER 2

Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout

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v1.1

Abstract

Varying levels of pharmaceuticals, including salicylate, ibuprofen and acetaminophen, have been reported in the aquatic environment, but few studies have actually addressed the impact of these drugs on aquatic organisms. We tested the hypothesis that these pharmaceuticals are endocrine disruptors in fish by examining their impact on interrenal corticosteroidogenesis in rainbow trout. Indeed, acute adrenocorticotrophic hormone (ACTH)-mediated cortisol production in trout interrenal cells *in vitro* was significantly depressed (20-40%) by these pharmaceutical drugs. Furthermore, we investigated whether this interrenal dysfunction involved inhibition of the steroidogenic capacity in rainbow trout. To this end, we fed trout salicylate-laced feed (100 mg/kg body wt) for three days and assessed the transcript levels of key proteins involved in corticosteroidogenesis, including steroidogenic acute regulatory protein (StAR), peripheral-type benzodiazepine receptor (PBR), cytochrome P450 cholesterol side chain cleavage (P450scc) and 11β-hydroxylase. Salicylate treatment did not affect the resting plasma cortisol or glucose levels, whereas the acute ACTH-stimulated cortisol production was significantly depressed in the interrenal tissue. This disruption of steroidogenesis by salicylate corresponded with a significant drop in the gene expression of StAR and PBR, but not P450scc or 11β-hydroxylase, compared to the sham treated fish. Also, brain glucocorticoid receptor (GR) protein content, and not GR mRNA level, was significantly reduced by salicylate. Taken together, salicylate is a corticosteroid disruptor in trout and the targets include the key rate-limiting step in interrenal steroidogenesis and brain glucocorticoid signaling.

1. Introduction

Cortisol, the main corticosteroid released in response to stress in teleostean fishes, is thought to play a key role in enabling animals to regain homeostasis after stressor exposure (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton et al., 2002). The circulating level of this steroid is tightly regulated by the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). For instance, stress stimulates the release of corticotropin releasing factor (CRF) from the hypothalamus, which in turn stimulates the release of adrenocorticotropic hormone (ACTH), the main secretagogue for cortisol biosynthesis, from the pituitary. ACTH binds to the interrenal cells (steroidogenic cells), located predominantly in the anterior part of the kidney (head kidney) in fish, to activate the steroidogenic pathway leading to the release of cortisol (Wendelaar Bonga, 1997; Mommsen et al., 1999; Hontela, 2005). However, the circulating cortisol levels are also tightly regulated by a negative feedback loop, including glucocorticoid receptor (GR) signaling in the brain, inhibiting the release of trophic hormones (CRF and/or ACTH) in response to elevated steroid levels (Wendelaar Bonga, 1997; Mommsen et al., 1999). Consequently, any adverse effect on the functioning of the HPI axis would compromise the ability of the animal to respond to additional stressors (Barton et al., 2002; Hontela, 2005). Indeed, xenobiotics disrupt cortisol response to stress by targeting multiple sites along the HPI axis, including impaired steroidogenesis and brain glucocorticoid signaling (Aluru et al., 2004; Hontela, 2005; Vijayan et al., 2005; Aluru and Vijayan, 2006).

A key rate-limiting step in steroid biosynthesis involves the transport of cholesterol from intracellular sites of storage to the inner mitochondrial membrane. The cholesterol transport process is thought to require at least two key regulatory proteins: the steroidogenic acute regulatory protein (StAR) (Stocco, 2001; Stocco et al., 2005) and the peripheral-type

benzodiazepine receptor (PBR) (Hauet et al., 2002; Delavoie et al., 2003; Papadopoulos, 2004). Together, StAR and PBR facilitate the transfer of cholesterol to the active site on cytochrome P450 side chain cleavage (P450scc), thereby initiating the steroid biosynthetic cascade (Culty et al., 1999; Papadopoulos, 2004). The ultimate step for cortisol biosynthesis is catalysed by 11β-hydroxylase, which was shown not to be a limiting factor in the production of cortisol (Aluru and Vijayan, 2006). Most studies on cholesterol transport proteins in steroidogenic cells involved mammalian cell systems and very little is known about the regulation of StAR and/or PBR in lower vertebrates. Recently, StAR was cloned and sequenced from trout (Kusakabe et al., 2002) and this protein showed stress-induced elevation similar to that seen in mammalian models (Kusakabe et al., 2002; Geslin and Auperin, 2004; Aluru and Vijayan, 2006; Hagen et al., 2006). Also, aryl hydrocarbon receptor (AhR) signaling was shown to disrupt corticosteroidogenesis by targeting StAR transcript levels (Aluru et al., 2005; Aluru and Vijayan, 2006). Together, these studies highlight StAR as a key target for steroidogenic disruption by toxicants, however very little is known about the role of PBR expression in fish.

While studies on corticosteroid disruption by xenobiotics have focused on organic contaminants and heavy metals (Hontela, 2005; Vijayan et al., 2005), very little is known about the impact of other toxicants, including pharmaceutical drugs in the aquatic environment. Indeed, human and veterinary prescription and nonprescription drugs have become a matter of concern in aquatic toxicology due to the fact that many of these compounds have been detected in the aquatic environment (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Heberer, 2002; Metcalfe et al., 2003a and b; Trudeau et al., 2005). However, most studies on the impact of pharmaceutical drugs in aquatic animals have been restricted to short-term acute toxicity bioassays (Webb, 2001; Cleuvers, 2004) and very little is known about either the sublethal

effects of these drugs or their mechanism(s) of action. A recent study demonstrated that acetylsalicylic acid (ASA) altered plasma thyroid hormone levels and also attenuated the stress-induced cortisol response to stress in tilapia (*Oreochromis mossambicus*) (van Anholt et al., 2003). However, the mechanism(s) involved in this disruption of cortisol response to stress was not ascertained.

The objective of this study was to investigate whether the mode of action of pharmaceutical drugs involved steroidogenic disruption in fish. For this, we initially screened three drugs, two non-steroidal anti-inflammatory drugs (NSAIDS: salicylate and ibuprofen) and one analgesic/antipyretic agent (acetaminophen), for their impact on acute ACTH-mediated cortisol production in rainbow trout (*Oncorhynchus mykiss*) interrenal cells *in vitro*. Furthermore, we tested the hypothesis that key proteins in the corticosteroid biosynthetic pathway are targets for impact by pharmaceutical drugs. To this end, we fed trout salicylate *in vivo*, according to the protocol followed by van Anholt et al. (2003), and examined the interrenal capacity for cortisol production, including mRNA abundance of StAR, PBR, P450scc and 11β-hydroxylase in the interrenal tissue. In addition, brain glucocorticoid receptor (GR) expression was assessed, especially since this protein is crucial for the negative feedback regulation of cortisol, to determine if salicylate targets multiple sites along the corticosteroid stress axis.

2. Material and Methods

2.1 Chemicals

Leibovitz's (L-15) culture medium, porcine ACTH (1-39), acetaminophen, ibuprofen, salicylate (salicylic acid, sodium salt), protease inhibitor cocktail, bicinchoninic acid (BCA) reagent and 2-phenoxyethanol were purchased from Sigma (St Louis, MO) and collagenase/dispase was from Roche (Montreal, QC). Multiwell (24-well plate) tissue culture

plates were from Falcon (Becton Dickinson Labware, NJ). The electrophoresis reagents and molecular weight markers were from BioRad (Hercules, CA). Polyclonal rabbit anti-trout GR antibody was developed in our laboratory (Sathiyaa and Vijayan, 2003). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (StressGen, Victoria, BC). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2 Experimental animals

Juvenile rainbow trout (~150 g body mass) were obtained from Humber Springs Hatchery, Mono Mills, Ontario. Fish were maintained for at least a month in aerated 200L tanks with constant water flow at 13˚C with a 12 hL: 12hD photoperiod prior to their use for either the *in vitro* or the *in vivo* study.

2.3 In vitro study – interrenal cells

2.3.1 Interrenal cell preparation: Cortisol production *in vitro* was determined using interrenal cell suspension following the protocol described by Leblond and Hontela, (1999) with slight modification. Briefly, fish were anaesthetized with 2-phenoxyethanol and the caudal peduncle dissected to expose the caudal vein. An initial blood sample was collected, after which the caudal vein was perfused with a syringe containing saline solution (0.7% NaCl) to clear blood from the kidney. The interrenal tissue was dissected and placed in fresh L-15 medium supplemented with antimycotic/antibiotic, $NaHCO_3$ and 5mM glucose, with pH adjusted to 7.6. The tissue was washed and resuspended in fresh L-15 medium supplemented with 2mg/ml collagenase/dispase and incubated for 60 min at 13˚ C with gentle agitation in a 15ml conical centrifuge tube. Following enzymatic digestion, the solution was filtered through a 40 μm mesh and the filtrate

was centrifuged at 250 x g for 10 min. The supernatant was removed and the pellet resuspended in fresh L-15 medium and the cell density adjusted to 75 x 10^6 cells/ml.

2.3.2 Exposure to drugs: Cells were plated in a 24-well culture plate with 500 µl of the cell suspension per well. Protocol for exposure and ACTH-stimulation was similar to that followed by Leblond and Hontela (1999), with slight modification. All incubations were performed at 13˚ C with slow agitation and the plated cells were allowed a 2 h settling period. The cells were then centrifuged for 3min at 200 x g, supernatant removed, and the cells exposed to either L-15 alone or L-15 containing acetaminophen, ibuprofen or salicylate (1nM to 1000 µM) for 22 h. Exposure to $CdCl_2$ (160 µM) was used as a positive control to confirm the effectiveness of this system, as previous studies clearly showed that this concentration of cadmium inhibits cortisol production without affecting cell survival (Leblond and Hontela, 1999; Lacroix and Hontela, 2004). After the incubation, ACTH was added to each well (0.5 IU/mL final concentration) and medium collected 2 h later for cortisol analysis. This concentration of ACTH gave maximal cortisol response with trout interrenal preparations (Aluru and Vijayan, 2004). There was no significant effect of drug treatments on cell viability as determined by measuring lactate dehydrogenase leakage (data not shown).

2.4 In vivo study - salicylate exposure

Groups of five trout each were separated into two aquaria (60L capacity) and maintained exactly as above for 2 weeks. The fish were fed to satiety with trout chow (five-point sinking food, Martin Mills Inc., Elmira, ON) once daily 5 days a week. After two weeks of acclimation, one group of fish were fed control diet (trout chow at 2% body mass), while the other group was fed the same amount of feed laced with salicylate (100 mg/kg). The drug incorporation into the feed and the feeding protocol was exactly as described before for rainbow trout (Aluru et al.,

2005). Briefly, salicylate was dissolved in 100% ethanol and sprayed on the trout chow and the ethanol allowed to evaporate overnight. A control feed was also prepared exactly as the test feed, but without the drug. The salicylate concentration chosen for this study, as well as the administration route, was based on the methodology followed by van Anholt et al., (2003).

The fish were fed the treatment diet for three days and sampled on the fourth day. Sampling consisted of quickly netting all fish from each tank and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). Fish were bled by caudal puncture into heparinized tubes and plasma collected after centrifugation (10 min at 6000 x g) was kept frozen at -70˚C for cortisol and glucose analyses. Pieces of brain and head kidney tissues were collected and immediately frozen on dry ice and stored at -70˚C for mRNA and protein determination later. Also, pieces of fresh head kidney tissue, immediately after sampling, were placed in L-15 media and subjected to an *in vitro* ACTH challenge test (see below).

2.4.1 Cortisol production: The *in vitro* incubation and ACTH challenge were conducted exactly as described before by Aluru and Vijayan (2004). Briefly, head kidney pieces from each fish were finely minced (~1mm^3 size) and rinsed with cold L-15 media and then distributed into two wells (10 fish x 2 wells each = 20 wells) of a 24-well Falcon tissue culture plate. The tissue slices were allowed to recover for 2 h at 13˚C with gentle shaking. Media was collected after the recovery period for determination of basal cortisol production, after which the tissue slices (one set each) were exposed to either fresh L-15 media alone (control wells) or fresh L-15 media containing 0.5 IU/ml ACTH. The incubation lasted for 2 h and the media was collected and stored frozen at -70°C for later determination of cortisol concentration. The tissue wet weight was measured in order to normalize the cortisol production rates.

2.5 Plasma cortisol and glucose analysis

Plasma cortisol concentration was measured using a commercially available ImmuChem™ ^{125}I RIA kit (MP Biomedicals, CA) that was validated for fish plasma (Aluru and Vijayan, 2004). Plasma glucose level was determined by a colorimetric assay using a commercial kit (Trinder method; Sigma).

2.6 Immunodetection of GR

Tissue homogenization and sample preparation for immunodetection was described in detail previously (Boone et al., 2002). Briefly, samples in Laemmli's buffer were loaded (40µg protein/sample) onto 8% SDS-PAGE and proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semi-dry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris pH7.5, 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02% sodium azide) buffer, probed for 1 h at room temperature with polyclonal rabbit anti-trout GR (1:1000) in the blocking solution, washed with TTBS and incubated for 1 h with goat-anti-rabbit IgG conjugated with alkaline phosphatase (1:3000 dilution). The protein bands were detected with NBT and BCIP and the intensity of the scanned bands were quantified using the AlphaEase software (AlphaEase Innotech, CA).

2.7 Transcript levels – quantitative real-time PCR (qPCR)

2.7.1 RNA isolation and cDNA synthesis: Total RNA (DNAse treated) was isolated from tissues using the RNeasy Mini Kit protocol (Qiagen) and the RNA was quantified by spectrophotometry at 260nm. The first strand cDNA was synthesized from 1µg total RNA using First Strand cDNA Synthesis kit (MBI Fermentas). Briefly, total RNA was heat denatured (70°C) and cooled on ice and the sample was used in a 20 µl reverse transcriptase reaction using 0.5 µg oligo d(T) primers

and 1mM each dNTP, 20 U ribonuclease inhibitors and 40 U M-MuLV reverse transcriptase. The reaction was started by incubating at 37°C for 1 h and stopped by heating at 70°C for 10 min.

2.7.2 Primers: The primers (Table1) were designed using rainbow trout GR, StAR, P450scc, 11β-hydroxylase, PBR and β-actin cDNA sequences (GenBank accession nos. Z54210, AB047032, S57305, AF179894, AY029216 and AF157514, respectively). The primer pairs amplified either a ~100 bp fragment for GR and β-actin or a ~500 bp fragment for all the other genes used in qPCR (iCycler, BioRad).

1. **Table 1.** Oligonucleotide primers used in quantitative real-time PCR and their annealing temperatures.

Gene	Primers	Annealing temp. (°C)
GR	Forward: 5'-AGAAGCCTGTTTTTGGCCTGTA-3' Reverse: 5'-AGATGAGCTCGACATCCCTGAT-3'	49
StAR	Forward: 5'-CGCTGGCATCTCCTACA-3' Reverse: 5'-GGGACTTCGTTAGTGTTCG-3'	58
P450scc	Forward: 5'-GAGGAGGGTAGGAGCCA-3' Reverse: 5'-CCTTGTGGGACTCTGGT-3'	60
11ß-hydroxylase	Forward: 5'-ACCTCTTCCGCTTCGC-3' Reverse: 5'-GGGTAGCGTGGGCAAGA-3'	58
PBR	Forward: 5'GGCCACCATTTCATTTGTTCATTTCA-3' Reverse: 5'-GCCATGCAAGGTAGGGTGTCAGGA-3'	60
ß-actin	Forward: 5'-AGAGCTACGAGCTGCCTGAC-3' Reverse: 5'-GCAAGACTCCATACCGAGGA-3'	49

2.7.3 Data analysis

Quantification of transcript levels were performed as previously described (Sathiyaa and Vijayan, 2003) by qPCR using Platinum® Quantitative PCR SuperMix-UDG (In vitrogen, CA). Briefly, cDNA samples were used as template for amplification of the housekeeping gene (β-actin) and target genes (GR, StAR, P450scc, 11β-hydroxylase and PBR) using appropriate primers and their respective annealing temperatures (Table 1). A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95°C- 3min; 40 cycles: denature 95°C- 20s, anneal-20s, and extend 72°C-20 s; then cool 4°C-hold. The iCycler IQTM real time detection software (Biorad) was used to determine the threshold values (CT) for every sample. A standard curve with log input amount and CT were established for each gene and used to calculate the transcript levels in samples. The target gene transcript levels were normalized by taking a ratio of target gene to housekeeping gene. This normalized value was standardized using an internal calibrator (sham group) and transcript levels expressed as percent sham.

2.8 Statistical analyses

The difference in cortisol production *in vitro* in response to different concentrations of drugs were compared statistically using one-way analysis of variance (ANOVA) followed by Tukey's test. For the *in vivo* study with salicylate, significant differences among the sham and treated groups were ascertained using an unpaired Student's t-test. Data were log transformed, wherever necessary, for homogeneity of variance, while non-transformed data are shown in the tables and figures. A significance level of $\alpha=0.05$ was considered statistically significant.

3. Results

3.1 In vitro study

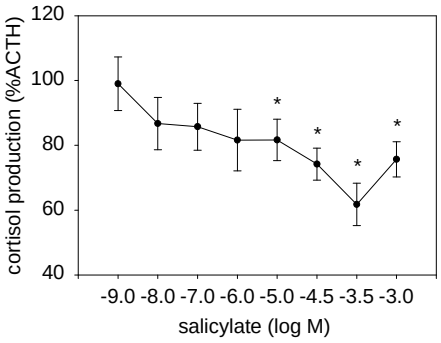
Salicylate, ibuprofen and acetaminophen, at the concentrations tested, were not cytotoxic to interrenal cells as determined by lactate dehydrogenase leakage (0.5 units/ ml/ 75 x 10^6 cells). Salicylate and acetaminophen at concentration of 10^{-5}M and above significantly depressed cortisol production compared to the control group (Figs. 1A and 1B). Ibuprofen exposure significantly decreased cortisol production at concentrations of 10^{-7}M and above, except for a lack of significant difference at 10^{-6} and 10^{-5}M concentrations compared to the control group (Fig. 1C). The maximal suppression of cortisol production with salicylate ($10^{-3.5}$M) and ibuprofen ($10^{-4.5}$M and $10^{-3.5}$M) was around 40% and acetaminophen (10^{-5}M to 10^{-3}M) was 20% (Figs. 1A-C).

2. **Figure 1**. The effect of salicylate (A), acetaminophen (B) and ibuprofen (C) on ACTH-mediated cortisol production by trout interrenal cells in vitro.

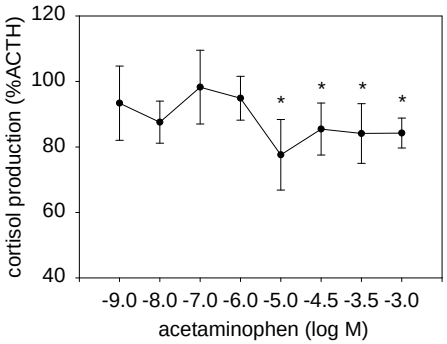
The cortisol production in the presence of drugs is shown as % ACTH stimulation (maximal cortisol production = ~30 ng/ml in the control groups); values represent means ± SEM (n = 4-7 fish); *statistically significant from the ACTH group (One-way ANOVA; $P<0.05$).

Fig.1

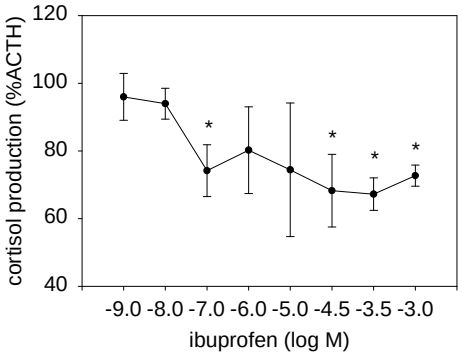
A


cortisol production (%ACTH)
120
100
80
60
40
-9.0 -8.0 -7.0 -6.0 -5.0 -4.5 -3.5 -3.0
salicylate (log M)

B


cortisol production (%ACTH)
120
100
80
60
40
-9.0 -8.0 -7.0 -6.0 -5.0 -4.5 -3.5 -3.0
acetaminophen (log M)

C


cortisol production (%ACTH)
120
100
80
60
40
-9.0 -8.0 -7.0 -6.0 -5.0 -4.5 -3.5 -3.0
ibuprofen (log M)

3.2 In vivo study

3.2.1 Cortisol and glucose concentration

Salicylate treatment did not significantly affect either plasma cortisol or glucose concentrations in rainbow trout (Table 2). However, salicylate significantly inhibited (~50%) the acute ACTH-mediated cortisol production compared to the sham group (Fig. 2). There was no effect of salicylate treatment on unstimulated cortisol production by trout head kidney slices.

3.2.2 StAR, PBR, P450scc and 11β-hydroxylase transcript abundance

Transcript abundance of genes encoding proteins involved at different stages of the cortisol biosynthetic pathway was measured in the interrenal tissue (Fig. 3). Transcript levels of StAR and PBR, two key proteins involved in cholesterol transport to the inner mitochondrial membrane, were significantly depressed in salicylate-treated fish compared to the sham fish (Figs. 3A and B). There was no significant difference in 11β-hydroxylase and P450scc transcript levels between the salicylate-treated and sham groups (Figs. 3C and D).

3.2.3 Glucocorticoid receptor (GR)

GR mRNA abundance in the brain of salicylate group was not significantly different from the sham group in the present study (Fig. 4A). However, GR protein content in the brain was significantly reduced (~50%) with salicylate treatment compared to the sham fish (Fig. 4B).

2. **Table 2.** Plasma cortisol and glucose concentrations in trout fed salicylate for four days

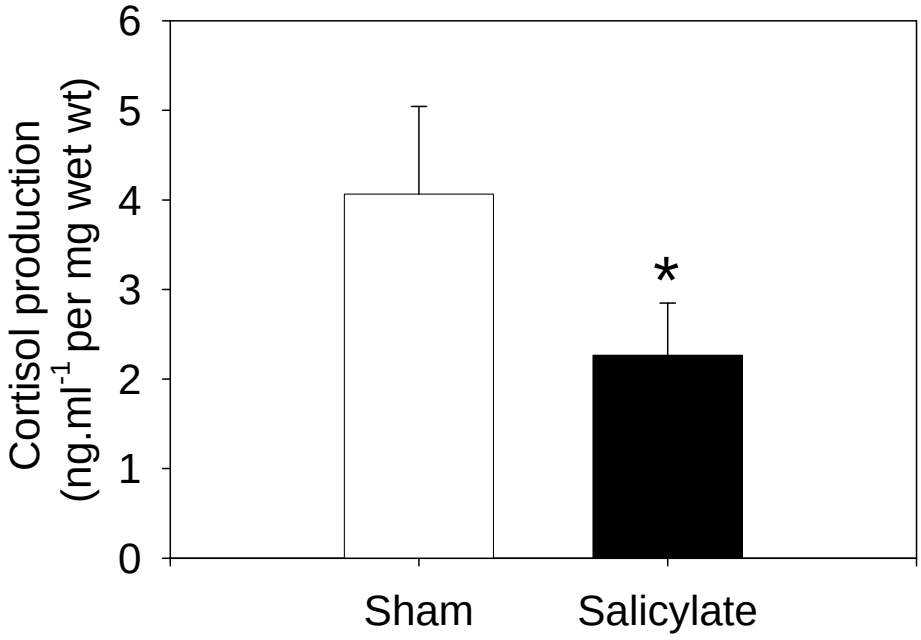
	Cortisol (ng/ml)	Glucose (mM)
Sham	2.38 ± 0.12	7.23 ± 0.37
Salicylate	2.75 ± 0.50	7.35 ± 0.75

Values represent mean ± SEM (n = 5 fish).

3. **Figure 2.** Acute ACTH-mediated cortisol production by head kidney slices obtained from trout fed salicylate for four days.

Data shown as magnitude of cortisol production (unstimulated levels subtracted from the ACTH-stimulated cortisol production) in both groups; values represent mean ± SEM (n = 5 fish); *statistically significant from the sham group (Student's *t* test; $P<0.05$).

Fig. 2

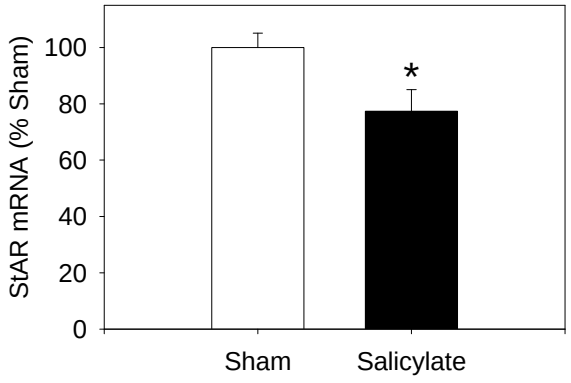

Cortisol production
(ng.ml-1 per mg wet wt)
6
5
4
3
2
1
0
*
Sham
Salicylate

4. **Figure 3.** Steroidogenic acute regulatory protein (StAR; A), peripheral-type benozodiazepine receptor (PBR; B), cytochrome P450 cholesterol side chain cleavage (scc; C) and 11ß-hydroxylase (D) transcript levels in the head kidney slices obtained from transcript levels in the head kidney slices obtained from trout fed salicylate for four days.

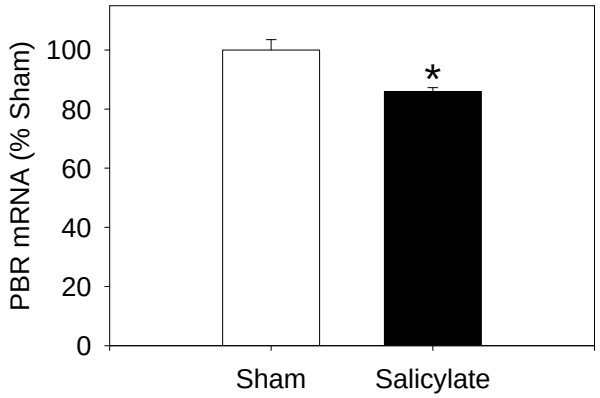
Values represent mean ± SEM (n = 5 fish); *statistically significant from the sham group (Student's *t* test; $P<0.05$).

Fig. 3

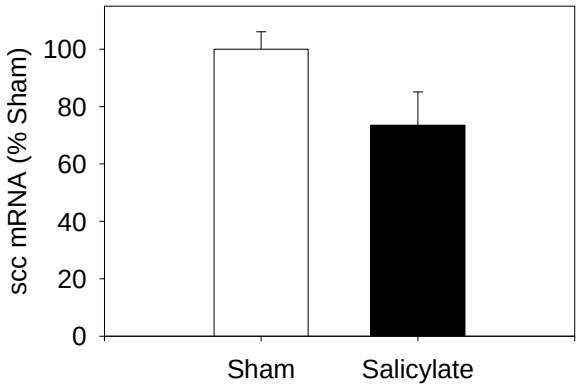
A


StAR mRNA (% Sham)
100
80
60
40
20
0
*
Sham
Salicylate

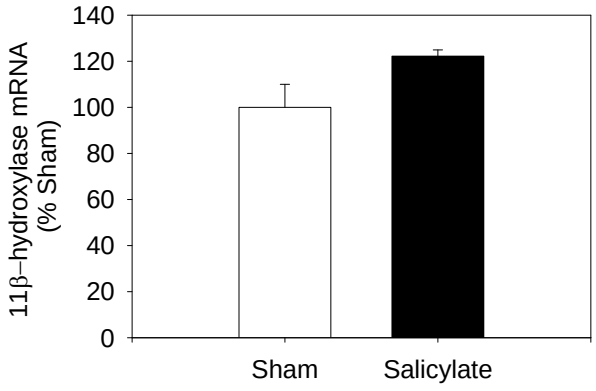
B


PBR mRNA (% Sham)
100
80
60
40
20
0
*
Sham
Salicylate

C


scc mRNA (% Sham)
100
80
60
40
20
0
Sham
Salicylate

D

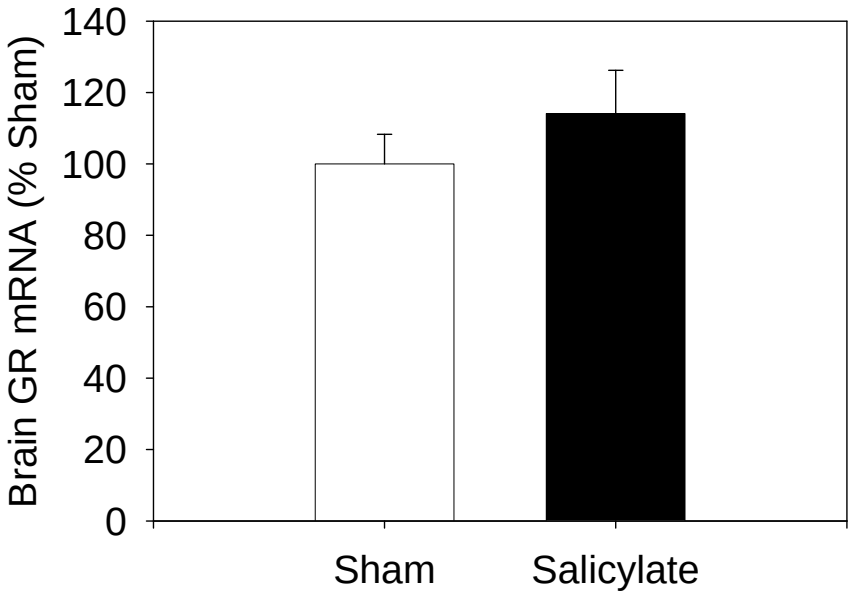

11β–hydroxylase mRNA (% Sham)
140
120
100
80
60
40
20
0
Sham
Salicylate

5. **Figure 4.** Effect of salicylate treatment in vivo on glucocorticoid receptor mRNA abundance (A) and protein content (B) in the brain of rainbow trout.

The image above figure 4B shows a representative western blot of GR protein expression. Values represent mean ± SEM (n = 5 fish). *statistically significant from the sham group (Student's t test; $P<0.05$).

Fig. 4

A


Brain GR mRNA (% Sham)
140
120
100
80
60
40
20
0
Sham
Salicylate

B


Sham
Salicylate
GR (90 kDa)
Brain GR protein (% Sham)
140
120
100
80
60
40
20
0
*
Sham
Salicylate

4. Discussion

We demonstrate that pharmacological levels of salicylate, ibuprofen and acetaminophen disrupt interrenal capacity for cortisol production in rainbow trout. The interrenal cell preparation used in this study has been extensively used to test the impact of xenobiotics on cortisol biosynthetic capacity in teleostean fishes (Hontela, 2005). Those studies have identified several organic contaminants and heavy metals as adrenotoxicants in fish (Hontela, 2005), but this is the first report of pharmaceutical drugs impacting steroidogenesis in fish.

Recently, ASA administration *in vivo* was shown to attenuate the stressor-induced cortisol response in Mozambique tilapia, while the mode of action of the drug was not ascertained (van Anholt et al., 2003). The impaired acute ACTH-mediated cortisol response with drugs, including salicylate, in the present study (Fig. 1) leads us to propose steroidogenic disruption as a possible mechanism for the muted cortisol response to stress in tilapia. In support of this notion, salicylate exposure *in vivo* depressed acute ACTH-mediated interrenal corticosteroidogenesis by 50% supporting interrenal steroidogenic pathway as a target for salicylate impact in fish. The absence of any significant changes in resting plasma cortisol or glucose levels with either salicylate (Table 2) or ASA (van Anholt et al., 2003) suggests that the drug does not elicit a stress response, but instead the mode of action appears to be the inhibition of acute stressor(s)-mediated upregulation of corticosteroidogenesis in fish.

A key rate-limiting step in steroidogenesis involves the transport of cholesterol from the outer to the inner mitochondrial membrane and this is mediated by two critical proteins, the StAR and PBR (Stocco et al., 2005; Papadopoulos, 2004). While StAR transports cholesterol across the mitochondrial membrane, the multifunctional protein PBR is thought to serve as a regulated channel to direct cholesterol to the active site on P450scc (Culty et al., 1999;

Papadopoulos, 2004). In addition, recent studies suggest that cholesterol uptake in the mitochondria may be mediated in part by PBR functioning as a cholesterol exchanger against steroids produced by P450scc enzymatic action (Lacapere and Papadopoulos, 2003; Hauet et al; 2005). Although the mechanism of action of cholesterol delivery is still unclear, a key role for StAR and PBR in mammalian steroidogenesis is unequivocal, while little is known about their regulation in piscine models. Upregulation of StAR transcript levels has been implicated in the stressor/ACTH-induced cortisol biosynthesis (Kusakabe et al., 2002; Geslin and Auperin, 2004; Aluru and Vijayan, 2006; Hagen et al., 2006), while the role of PBR in the stress response process in fish is unknown.

Our results for the first time highlight StAR and PBR as targets for salicylate-mediated disruption of steroidogenesis in trout. StAR protein was shown before to be a target for xenobiotics-mediated endocrine disruption in animals (reviewed in Harvey and Everett, 2003; Mutoh et al., 2006), including fish (Aluru et al., 2005; Aluru and Vijayan, 2006), but few studies have investigated the toxic impact on PBR levels (Boujrad et al., 2000; Gazouli et al., 2002). The depressed StAR and PBR transcript levels along with a reduced steroid output from interrenal cells points to an impairment of the cholesterol transport process. This notion finds support from a recent study that showed impaired mitochondrial cholesterol transport as a limiting factor for the steroidogenic disruption by β-sitosterol in male goldfish (*Carassius auratus*) gonads (Leusch and MacLatchy, 2003). Also, the lack of a significant change in P450scc and 11β-hydroxylase transcript levels argues against pathways downstream of cholesterol transport to be a limiting factor in the inhibition of cortisol production by salicylate.

While our study showed only a marginal reduction in StAR and PBR transcript levels by salicylate (~15-20%), the interrenal tissue capacity for acute cortisol production was

substantially lower (~50%). This is in agreement with other studies clearly showing that despite marginal, but significant, transcript level changes in StAR and P450scc with either stressor- or ACTH-stimulation, the inhibition of cortisol output was of a much greater magnitude relative to transcript changes (Geslin and Auperin, 2004; Aluru et al., 2005; Aluru and Vijayan, 2006; Hagen et al., 2006). Indeed, we showed that abolishment of this minimal transcript response completely reverted the cortisol production capacity of interrenal tissue reflecting the important role for the rate-limiting step in modulating steroidogenesis in fish (Aluru and Vijayan, 2006). The reason for the minor changes in the transcript levels of key steroidogenic proteins in teleostean studies compared to mammalian studies (Boujrad et al., 2000; Walsh et al., 2000a and c; Gazouli et al., 2002) is unclear. Nonetheless, it appears likely that salicylate-mediated disruption of steroidogenesis contributes to the impaired cortisol response to stressor seen in fish (van Anholt et al., 2003), and the mode of action involves reduction in StAR and PBR mRNA levels in fish.

Although the mechanism(s) involved in steroidogenic disruption by salicylate is not clear, a few likely scenarios emerge. For instance, salicylate inhibits cyclooxygenases (Brooks and Day, 1991; Borne, 1995) and, therefore, will reduce the formation of arachidonic acid metabolites, including prostaglandins (PG). It is well known that PGs modulate the functioning of the hypothalamus-pituitary-adrenal axis in mammals (Cavagnini et al., 1979; Hockings et al., 1993; Nye et al., 1997) and even in fish (Gupta et al., 1985; Wales, 1988). Also, administration of ASA to tilapia lowered plasma PGE_2 concentration (van Anholt et al., 2003) and this coincided with a lowered cortisol response to stress. Together, these results point to a role for PG as a link for salicylate impact on cortisol response in fish. However, recent studies also point to a PG independent action of arachidonic acid signaling in modulating the cortisol response to stress

in fish (van Anholt et al., 2004a and b). It has also been demonstrated that several NSAIDs, including salicylate, exhibit anti-inflammatory effects independent of cyclooxygenase activity (Tegeder et al., 2001; Amann and Peskar, 2002). This raises the possibility that other pathways, in addition to PG, may be involved in salicylate-mediated disruption of cortisol biosynthesis in fish. Specifically, the findings that NSAIDS can also affect the activation of transcription factors, including the peroxisome proliferator-activated receptors (PPARs), a key regulator of steroidogenesis (Komar, 2005), suggests a molecular mechanism(s) for salicylate-mediated inhibition of the rate limiting step in steroidogenesis. Indeed, this argument finds support from the fact that PPAR alpha stimulation depressed PBR transcript levels in a mammalian cell model (Gazouli et al., 2002) leading to our hypothesis that PPARs activation by salicylate may be a mechanism for inhibition of steroidogenesis in fish.

Although elevated plasma cortisol levels post-stressor is associated with enhanced interrenal steroidogenesis, a key player in the cortisol response also involves activation of the hypothalamus-pituitary axis. For instance, elevation of cortisol levels is tightly regulated in part by a negative feedback loop, whereby cortisol levels, via GR activation, reduces CRH and ACTH release from the hypothalamus and pituitary, respectively (Wendelaar Bonga, 1997; Mommsen et al., 1999; Bernier and Peter, 2001; Huising et al., 2004). Indeed reduction in brain GR protein content with PCBs was accompanied by a disturbed plasma cortisol profile in response to stressors in Arctic charr (*Salvelinus alpinus*: Aluru et al., 2004) suggesting impaired feedback regulation. While we did not assess the cortisol response to acute stressors in the present study, the reduced GR protein content (~50%) in the salicylate treated fish leads us to hypothesize that this drug also disrupts the negative feedback regulation of cortisol in rainbow trout. However, this lower GR protein content was not accompanied by changes in GR mRNA

content in the salicylate group, suggesting increased GR protein breakdown. Considered together, the disruption of the cortisol response to stress by pharmaceutical drugs, including NSAIDs, may involve multiple targets along the corticosteroid stress axis in fish.

In conclusion, salicylate, acetaminophen and ibuprofen are endocrine disruptors in fish and have the potential to impair the adaptive cortisol response to stressors. We further demonstrate that the inhibition of the acute cortisol production capacity seen with salicylate involves depressed StAR and PBR transcript levels, two proteins crucial for cholesterol transport to the inner mitochondria membrane for its utilization by steroidogenic enzymes. In addition, salicylate also reduced brain GR protein content in rainbow trout. Altogether, StAR, PBR and GR, key proteins involved in the activation of corticosteroid stress axis are targets for salicylate-mediated impairment of the adaptive cortisol response to stress in fish.

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References

Aluru, N., Jorgensen, E.H., Maule, A.G., and Vijayan, M.M. (2004). PCB disruption of the hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor downregulation in anadromous Arctic charr. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287,** R787-R793.

Aluru, N., Renaud, R., Leatherland, J.F., and Vijayan, M.M. (2005). Ah receptor-mediated impairment of interrenal steroidogenesis involves StAR protein and P450scc gene attenuation in rainbow trout *Toxicol. Sci*. **84,** 260-269.

Aluru, N., and Vijayan, M.M. (2004). ß-naphthoflavone disrupts cortisol production and liver glucocorticoid responsiveness in rainbow trout. *Aquat. Toxicol*. **67,** 273-285.

Aluru, N., and Vijayan, M.M. (2006). Ah receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis. *Endocrinol.* 10.1210/en.2005-1143.

Amann, R., and Peskar, B.A. (2002). Anti-inflammatory effects of aspirin and sodium salicylate. *Eur. J. Pharmacol*. **28,** 1-9.

Barton, B.A., Morgan, J.D., and Vijayan, M.M. (2002). Physiological and condition-related indicators of environmental stress in fish. *In*: Adams SM (Ed.), Biological indicators of aquatic ecosystem health. American Fisheries Society, pp.111-148.

Bernier, N.J., and Peter, R.E. (2001). The hypothalamic-pituitary-interrenal axis and the control of food intake in teleost fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129,** 639-644.

Boone, A.N., Ducouret, B., and Vijayan, M.M. (2002). Glucocorticoid-induced glucose release is abolished in trout hepatocytes with elevated hsp70 content. *J. Endocrinol*. **172,** R1-R5.

Borne, R.F. (1995). Nonsteroidal anti-inflammatory drugs. In: WO Foye, TL Lemke and DA Williams, (Eds), *Principles of medicinal chemistry*, Williams & Wilkins, Media, pp. 535–580.

Boujrad, N., Vidic, B., Gazouli, M., Culty, M., and Papadopoulos, V. (2000). The peroxisome proliferator perfluorodecanoic acid inhibits the peripheral-type benzodiazepine receptor (PBR) expression and hormone-stimulated mitochondrial cholesterol transport and steroid formation in Leydig cells. *Endocrinol*. **141,** 3137-3148.

Brooks, P.M., and Day, R.O. (1991). Nonsteroidal anti-inflammatory drugs-differences and similarities. *N. Engl. J. Med.* **324,** 1716–1725.

Cavagnini, F., Di Landro, A., Maraschini, C., Invitti, C., and Pinto, M.L. (1979). Effect of two prostaglandin synthesis inhibitors, indomethacin and acetylsalicylic acid, on plasma ACTH and cortisol levels in man. *Acta. Endocrinol.* (Copenh). **91,** 666-673.

Cleuvers, M. (2004). Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotox. Environ. Saf*. **59,** 309-315.

Culty, M., Li, H., Boujrad, N., Amri, H., Vidic, B., Bernassau, J.M., Reversat, J.L., and Papadopoulos, V. (1999*). In vitro* studies on the role of the peripheral-type benzodiazepine receptor in steroidogenesis. *J. Steroid. Biochem. Mol. Biol*. **69,** 123-130.

Daughton, C.G., and Ternes, T.A. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ. Health. Perspect*. **107,** 907-938.

Delavoie, F., Li, H., Hardwick, M., Robert, J.C., Giatzakis, C., Peranzi, G., Yao, Z.X., Maccario, J., Lacapere, J.J., and Papadopoulos, V. (2003*). In vivo* and *in vitro* peripheral-type benzodiazepine receptor polymerization: functional significance in drug ligand and cholesterol binding. *Biochem*. **42,** 4506-4519.

Gazouli, M., Yao, Z.X., Boujrad, N., Corton, J.C., Culty, M., and Papadopoulos, V. (2002). Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene

expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor alpha. *Endocrinol*. **143,** 2571-2583.

Geslin, M., and Auperin, B. (2004). Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*. **135,** 70-80.

Gupta, O.P., Lahlou, B., Botella, J., Porthe-Nibelle, J. (1985). *In vivo* and *in vitro* studies on the release of cortisol from interrenal tissue in trout. I. Effects of ACTH and prostaglandins. *Exp. Biol.* **43,** 201-212.

Hagen, J., Kusakabe, M., and Young, G. (2006). Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11ß-hydroxylase messenger RNAs in rainbow trout interrenal cells: Relationship with *in vitro* cortisol production. *Gen. Comp. Endocrinol***. 145,** 254-262.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., and Jorgensen, S.E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment—a review. *Chemosphere*. **36,** 357-393.

Harvey, P.W., and Everett, D.J. (2003). The adrenal cortex and steroidogenesis as cellular and molecular targets for toxicity: critical omissions from regulatory endocrine disrupter screening strategies for human health? *J. Appl. Toxicol*. **23,** 81-87.

Hauet, T., Liu, J., Li, H., Gazouli, M., Culty, M., and Papadopoulos, V. (2002). PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. *Endocr. Res*. **28,** 395-401.

Hauet, T., Yao, Z.X., Bose, H.S., Wall, C.T., Han, Z., Li, W., Hales, D.B., Miller, W.L., Culty, M., and Papadopoulos, V. (2005). Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. *Mol. Endocrinol*. **19,** 540-554.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol. Lett*. **131,** 5-17.

Hockings, G.I., Grice, J.E., Crosbie, G.V., Walters, M.M., Jackson, A.J., and Jackson, R.V. (1993). Aspirin increases the human hypothalamic-pituitary-adrenal axis response to naloxone stimulation. *J. Clin. Endocrinol. Metab*. **77,** 404-408.

Hontela, A. (2005). Stress and the hypothalamo-pituitary-interrenal axis: adrenal toxicology-Effects of environmental pollutants on the structure and function of the HPI axis. In: T.W.Moon and T.P.Mommsen (Eds.) *Biochemical and molecular biology of fishes*, **Vol.6** *Environmental Toxicology*. Elsevier, Amsterdam p 331-363.

Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermsen, T., Verburg-van Kemenade, B.M., and Flik, G. (2004). Structural characterisation of a cyprinid (*Cyprinus carpio*

L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. *J Mol. Endocrinol.* **32,** 627-648.

Komar, C.M. (2005). Peroxisome proliferator-activated receptors (PPARs) and ovarian function--implications for regulating steroidogenesis, differentiation, and tissue remodeling. *Reprod. Biol. Endocrinol.* **3,** 41-55.

Kusakabe, M., Todo, T., McQuillan, J.H., Goetz, F.W., and Young, G. (2002). Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. *Endocrinol*. **143,** 2062-2070.

Lacapere, J.J. and Papadopoulos, V. (2003). Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids*. **68,** 569-585.

Lacroix, A, and Hontela, A. (2004). A comparative assessment of the adrenotoxic effects of cadmium in two teleost species, rainbow trout, *Oncorhynchus mykiss*, and yellow perch, *Perca flavescens*. *Aquat. Toxicol*. **67,** 13-21.

Leusch, F.D., and MacLatchy, D.L. (2003). *In vivo* implants of ß-sitosterol cause reductions of reactive cholesterol pools in mitochondria isolated from gonads of male goldfish (*Carassius auratus*). *Gen. Comp. Endocrinol*. **134,** 255-263.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Leblond, V.S., and Hontela, A. (1999). Effects of *in vitro* exposures to cadmium, mercury, zinc, and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane on steroidogenesis by dispersed interrenal cells of rainbow trout (*Oncorhynchus mykiss*). *Toxicol. Appl. Pharmacol*. **157,** 16-22.

Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., and Hirsch, R. (2003a). Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environ. Toxicol. Chem*. **22,** 2872-2880.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., and Struger, J. (2003b). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ. Toxicol. Chem*. **22,** 2881-2889.

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

Mutoh, J., Taketoh, J., Okamura, K., Kagawa, T., Ishida, T., Ishii, Y., and Yamada, H. (2006). Fetal Pituitary Gonadotropin as an Initial Target of Dioxin in Its Impairment of Cholesterol Transportation and Steroidogenesis in Rats. *Endocrinol.* **147,** 927-936.

Nye, E.J., Hockings, G.I., Grice, J.E., Torpy, D.J., Walters, M.M., Crosbie, G.V., Wagenaar, M., Cooper, M., and Jackson, R.V. (1997). Aspirin inhibits vasopressin-induced hypothalamic-pituitary-adrenal activity in normal humans. *J. Clin. Endocrinol. Metab*. **82,** 812-817.

Papadopoulos, V. (2004). In search of the function of the peripheral-type benzodiazepine receptor. *Endocr. Res*. **30,** 677-684.

Sathiyaa, R., and Vijayan, M.M. (2003). Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. *Am. J. Physiol. Cell Physiol.* **284,** C1508-C1515.

Stocco, D.M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annu. Rev. Physiol.* **63,** 193-213.

Stocco**,** D.M., Wang, X.J., Jo, Y., and Manna, P.R. (2005). Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought. *Mol. Endocrinol*. **19,** 2647 - 2659.

Tegeder, I., Pfeilschifter, J., and Geisslinger, G. (2001). Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* **15,** 2057-2072.

Trudeau, V.L., Metcalfe, C.D., Mimeault, C. and Moon, T.W. (2005). Pharmaceuticals in the environment: Drugged fish? In: Mommsen T.P. and T.W. Moon (Eds.) *Biochemistry and Molecular Biology of Fishes,* **Vol. 6,** Elsevier, Amsterdam, pp 87-101.

van Anholt, R.D., Spanings, F.A., Koven, W.M., and Bonga, S.E. (2004a). Dietary supplementation with arachidonic acid in tilapia (*Oreochromis mossambicus*) reveals physiological effects not mediated by prostaglandins. *Gen. Comp. Endocrinol*. **139,** 215-226.

van Anholt, R.D., Spanings, F.A., Koven, W.M., Nixon, O., and Bonga, S.E. (2004b). Arachidonic acid reduces the stress response of gilthead seabream *Sparus aurata* L. *J. Exp. Biol*. **207,** 3419-3430.

van Anholt, R.D., Spanings, F.A., Koven, W.M., and Bonga, S.E. (2003). Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **285,** R1098-R1106.

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

Wales, N.M. (1988). Hormones studies in *Myxine glutinosa*: effects of the eicosanoids arachidonic acid, prostaglandin E_1, E_2, A_2, F_{2a}, thromboxane B_2 and of indomethacin on plasma cortisol, blood pressure, urine flow and electrolyte balance. *J. Comp. Endocrinol. B*. **158,** 621-626.

Webb, S.F. (2001). A data based perspective on the environmental risk assessment of human pharmaceuticals II –aquatic risk characterization. In. Kummerer, K. (Ed.),*Pharmaceuticals in the environment. Sources, fate, effects and risks.* Springer-Verlag, New York, pp.203-219.

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

CHAPTER 3

Salicylate impacts the physiological responses to an acute handling disturbance in rainbow trout

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Abstract

While salicylates (non-steroidal anti-inflammatory drugs) have been detected in the aquatic environment, few studies have focused on the mechanism of action of these pharmaceuticals on aquatic organisms. We reported previously that salicylate disrupted the acute trophic hormone-stimulated corticosteroidogenesis in rainbow trout (*Oncorhynchus mykiss*) interrenal tissue *in vitro*. Here we tested the hypothesis that this drug will inhibit the adaptive plasma cortisol response and the associated metabolic response to an acute stressor in trout. Fish were fed salicylate-laced feed (100mg/kg body weight) for 3 days, subjected to an acute (3 min) handling disturbance and sampled 1, 4 and 24 h after the stressor exposure. Salicylate treatment attenuated the stressor-induced plasma cortisol but not glucose or lactate elevations. The disruption of cortisol response corresponded with a significant reduction in transcript levels of the steroidogenic acute regulatory protein (StAR), but not peripheral-type benzodiazepine receptor, cytochrome P450 side chain cleavage or 11β-hydroxylase. Salicylate did not modify the stressor-induced elevation of brain glucocorticoid receptor (GR) protein expression, while liver GR protein content was reduced. Salicylate impact on liver metabolic capacity involved depressed liver glycogen content, whereas no significant changes in liver hexokinase, glucokinase, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate carboxykinase, aspartate aminotransferase and alanine aminotransferase activities were observed. Taken together, salicylate impairs the stressor-mediated plasma cortisol response and the associated liver metabolic capacity in trout. The mode of action of salicylate involves disruption of StAR and liver GR, two key proteins critical for cortisol production and target tissue responsiveness to this steroid, respectively.

1. Introduction

A key indicator of fish stress performance is the activation of the hypothalamus-pituitary –interrenal (HPI) axis and the associated metabolic changes (Mommsen et al., 1999; Iwama et al., 2006). Cortisol, the main corticosteroid in teleosts, is released in response to stressor-mediated stimulation of interrenal tissue by ACTH (Wendelaar Bonga, 1997; Mommsen et al., 1999). The rate-limiting step in corticosteroid biosynthesis is thought to be the transport of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR; Stocco et al., 2005; Miller, 2007) and the peripheral-type benzodiazepine receptor (PBR; Papadopoulos, 2004). In addition, cytochrome P450 side-chain cleavage (P450scc) and 11β-hydroxylase are also two enzymes critical for cortisol biosynthesis (Payne and Hanes, 2004).

The stressor-induced acute cortisol response is a key signal orchestrating the metabolic adjustments critical to cope with the enhanced energy demand to stress (Mommsen et al., 1999; Iwama et al., 2006). For instance, cortisol enhances liver metabolic capacity, including higher amino acid catabolism, gluconeogenesis and glycogenolysis, leading to increased glucose production (Wendelaar Bonga, 1997; Mommsen et al., 1999; Iwama et al., 2006). The rise in plasma cortisol levels after an acute stressor is quickly regulated by a negative feedback loop, which involves glucocorticoid receptor (GR) signaling in the brain (Mommsen et al., 1999; Aluru et al., 2004; Vijayan et al., 2005). Also, modulation of target tissue responsiveness to cortisol during stress may involve ligand-mediated GR autoregulation in trout (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Collectively, the functioning of the HPI axis, including GR dynamics, is a key aspect of the highly conserved adaptive stress response to regain homeostasis.

Recent studies suggest that the highly conserved adaptive cortisol response in fish is impacted by environmental pollutants (Hontela, 2005; Aluru and Vijayan, 2006; Gravel and Vijayan, 2006). While most of those studies focused on arylhydrocarbon receptor (AhR) agonists and metals, very little is known about the impact of pharmaceutical drugs in modulating the stress axis in fish. Indeed the detection of pharmaceutical drugs in the aquatic environment in μg/L concentrations has raised concerns about the potential adverse effects of these drugs on aquatic organisms. Non-steroidal anti-inflammatory drugs (NSAIDs), due to their high consumption, are among the most important group detected in surface waters (Heberer, 2002; Metcalfe et al., 2003a,b). While toxicity studies with pharmaceutical drugs have been restricted to acute toxicity studies (Webb, 2001; Cleuvers, 2004; Trudeau et al., 2005), little is known about either the mechanism of action of these drugs or their impact on fish stress performance. A recent study showed that acetylsalicylic acid treatment attenuated the stressor-mediated cortisol response in Mozambique tilapia, *Oreochromis mossambicus* (van Anholt et al., 2003). Also, we demonstrated that StAR and PBR are targets for salicylate impact in rainbow trout (*Oncorhynchus mykiss*) interrenal tissue leading to the proposal that this NSAID is a corticosteroid disruptor (Gravel and Vijayan, 2006).

To further explore the role of NSAIDs as endocrine disruptors in fish, we tested the hypothesis that salicylate disrupts stressor-induced plasma cortisol level and the associated metabolic response in rainbow trout. To this end, we fed trout salicylate for three days exactly as described before (Gravel and Vijayan, 2006) and subjected the fish to a standardized handling disturbance (Aluru and Vijayan, 2006). Plasma cortisol, glucose and lactate levels were measured as indicators of organismal stress response, while liver glycogen and glucose content and activities of several enzymes involved in intermediary metabolism were determined to assess

the liver metabolic capacity. We also evaluated the cortisol biosynthetic capacity of trout interrenal tissue during recovery from an acute stressor by quantifying the mRNA abundance of StAR, PBR, P450scc and 11ß-hydroxylase, while brain and liver GR content was determined to investigate the impact of salicylate on cortisol feedback regulation and target tissue signaling, respectively.

2. Material and Methods

2.1 Chemicals

Sodium salicylate, protease inhibitor cocktail, bicinchoninic acid (BCA) reagent, and 2-phenoxyethanol were purchased from Sigma (St Louis, MO). Costar 96-well EIA/RIA flat bottom microplates were from Corning Inc. (Corning, NY), while the electrophoresis reagents, molecular weight markers and alkaline phosphatase-conjugated goat anti-rabbit IgG were from BioRad (Hercules, CA). Polyclonal rabbit anti-trout GR antibody to trout was designed in our laboratory (Sathiyaa and Vijayan, 2003). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (StressGen, Victoria, BC). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2 Animals

Juvenile rainbow trout (~ 40 g body mass) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario. Groups of 14 trout each were maintained for a month in four aerated tanks (40L capacity) with constant water flow at 13˚C with a 12 hL: 12hD photoperiod prior to the experiment. The fish were fed to satiety with trout chow (five-point sinking food, Martin Mills Inc., Elmira, ON) once daily for 5 days a week.

2.3 Experimental design

After acclimation, fish in two aquaria were fed control diet (trout chow at 2% body mass), while the other two aquaria were fed the same amount of feed as sham group but laced with salicylate (100mg/kg sodium salicylate) exactly as described before (Gravel and Vijayan, 2006). Briefly, sodium salicylate was dissolved in 100% ethanol and sprayed on the trout chow, while control diet received only ethanol. The fish were fed the treatment diet for three days and sampled on the fourth day. Sampling consisted of quickly netting seven fish each from the sham or salicylate-fed tanks and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). These fish were the unstressed fish, after which fish in all four tanks were subjected to a 3 min handling disturbance according to Aluru and Vijayan (2006) and sampled at 1, 4 and 24 h after stressor exposure. Fish were bled by caudal puncture into heparinized tubes and plasma was collected after centrifugation (10 min at 6000 x g) and kept frozen at -70˚C for cortisol, glucose and lactate analyses. Pieces of liver, brain and head kidney tissues were collected and immediately frozen on dry ice and stored at -70˚C for glycogen, enzyme activity, mRNA and protein determination later. The experimental protocol was in accordance with the Canadian Council for Animal Care guidelines and approved by the animal care committee at the University of Waterloo.

2.3.1 Plasma analyses

Plasma cortisol concentration was measured using a commercially available ImmuChem™ ^{125}I radioimmunoassay kit (MP Biomedicals, CA). Plasma glucose (modified Trinder method; Rainchem, San Diego, CA) and lactate (Trinity Biotech, St-Louis, MO) levels were measured using commercial kits.

2.3.2 Liver glycogen, glucose and enzyme activities

Livers were homogenized (Ultra Turrax; IKA Works, Wilmongton, NC), followed by sonication (Microson, Farmingdale, NY), in a homogenization buffer (50% glycerol, 21mM Na_2HPO_4, 0.5mM EDTA-Na_2, 0.2% BSA, 5mM β-mercaptoethanol and protease inhibitors, pH adjusted to 7.5) exactly as described before (Vijayan et al., 2006). Liver glycogen content was analyzed by measuring glucose content before and after amyloglucosidase hydrolysis according to Vijayan et al. (2006). The glycogen content is shown as micromoles glucosyl units per gram protein in the homogenate. The enzyme activities were measured in 50mM imidazole-buffered enzyme reagent (pH 7.4) at 22^{o}C by continuous spectrophotometry at 340nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA). The following assay conditions were used to obtain optimal activities:

Hexokinase (HK: E.C. 2.7.1.1): 1 mM glucose, 5mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 1 mM ATP, 5 U/ml lactate dehydrogenase (LDH) and 2.5 U/ml pyruvate kinase.

Glucokinase (GK: E.C. 2.7.1.2): 15 mM glucose, 5mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 1 mM ATP, 5 U/ml lactate dehydrogenase (LDH) and 2.5 U/ml pyruvate kinase.

Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and 25mM pyruvic acid.

Phosphoenolpyruvate carboxykinase (PEPCK: E.C. 4.1.1.32): 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, 5 mM deoxyguanosine diphosphate, and 0.12 mM NADH.

Alanine aminotransferase (AlaAT: E.C. 2.6.1.2): 0.12 mM NADH, 200 mM L-alanine, 0.025 mM pyridoxal 5-phosphate, 262.5 mM a-ketoglutarate, and 12 U/ml LDH.

Aspartate aminitransferase (AspAT: E.C. 2.6.1.1): 1000 mM aspartatic acid, 7 mM a-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/ml malate dehydrogenase.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein in the homogenate.

2.4 Transcript analysis

Total RNA (DNase treated) was isolated from interrenal tissue using the RNeasy Mini Kit (Qiagen, Mississauga, ON), and the RNA was quantified by spectrophotometry at 260nm. The first strand cDNA was synthesized from 1μg total RNA using First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON). Quantification of transcript levels were performed exactly as previously described (Sathiyaa and Vijayan, 2003) using Platinum® Quantitative PCR SuperMix-UDG (In Vitrogen, Carlsbad, CA). Briefly, cDNA were used as template for amplification of the housekeeping gene (β-actin) and target genes (StAR, PBR, P450scc and 11ß-hydroxylase) using appropriate primers and annealing temperatures (Table 1). The primers (Table1) were designed using rainbow trout StAR, P450scc, 11β-hydroxylase, PBR, and β-actin cDNA sequences (GenBank accession nos. AB047032, S57305, AF179894, AY029216, and AF157514, respectively). The primer pairs amplified either a ~100 bp fragment for β-actin or a ~500 bp fragment for all target genes used in quantitative real-time PCR (qPCR; iCycler, Biorad).

A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95°C- 3min; 40 cycles: denature 95°C- 20s, anneal-20s, and extend 72°C-20 s; then cool 4°C-hold. The iCycler IQ^{TM} real time detection software (BioRad)

was used to determine the threshold values (C_T) for every sample. A standard curve with log input amount and C_T was established for β-actin and the genes of interest and used to calculate the transcript levels in samples. The target gene transcript levels were normalized by taking a ratio of target gene to housekeeping gene. This normalized value was standardized using an internal calibrator (Sham unstressed group) and the transcript levels were expressed as percent of internal calibrator according to established protocols (Gravel and Vijayan, 2006).

3. **Table 1.** Oligonucleotide primers used in quantitative real-time PCR and their annealing temperatures.

Gene	Primers	Annnealing temp. (°C)
StAR	Forward: 5'-CGCTGGCATCTCCTACA-3' Reverse: 5'-GGGACTTCGTTAGTGTTCG-3'	58
P450scc	Forward: 5'-GAGGAGGGTAGGAGCCA-3' Reverse: 5'-CCTTGTGGGACTCTGGT-3'	60
11ß-hydroxylase	Forward: 5'-ACCTCTTCCGCTTCGC-3' Reverse: 5'-GGGTAGCGTGGGCAAGA-3'	58
PBR	Forward: 5'GGCCACCATTTCATTTGTTCATTTCA-3' Reverse: 5'-GCCATGCAAGGTAGGGTGTCAGGA-3'	60
ß-actin	Forward: 5'-AGAGCTACGAGCTGCCTGAC-3' Reverse: 5'-GCAAGACTCCATACCGAGGA-3'	49

2.5 GR Protein quantification

Tissue homogenization and sample preparation for GR immunodetection was described in detail previously (Boone and Vijayan, 2002). Briefly, samples in Laemmli's buffer were loaded (40 μg protein/sample) onto 8% SDS-PAGE and the proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semi-dry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris (pH 7.5), 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02% sodium azide), probed for 1 h at room temperature with primary antibody (anti-trout GR; 1:1000 dilution) in the blocking solution, washed with TTBS (3 times for 5 min each) and incubated for 1 h with secondary antibody (goat-anti-rabbit IgG conjugated with alkaline phosphatase; 1:3000 dilution). The membranes were washed with TTBS (2 x 5 min) followed by one wash with TBS and the protein bands were detected with NBT and BCIP. The intensity of the scanned bands was quantified using the AlphaEase software (AlphaEase Innotech, CA) and the protein values expressed as percent control.

2.6 Statistical analysis

The effect of salicylate on acute stress response was compared using a two-way ANOVA, with treatment (sham, salicylate) and time (pre-stress, 1, 4 and 24 h) as independent factors, followed by LSD (post hoc) test. In case of significant interaction between treatment and time, the data were analyzed within each time-point using one-way ANOVA followed by LSD test. Data were log transformed, wherever necessary, to meet the assumptions of homogeneity of variance, while non-transformed data are shown in the figures and tables. A significance level of α=0.05 was considered statistically significant.

3. Results

3.1 Plasma cortisol, glucose and lactate levels

Plasma cortisol levels were significantly elevated at 1 h after stressor exposure in both the sham and salicylate-fed groups compared to other time-points (Fig. 1A). The levels returned to pre-stress levels in both groups at 4 h and was maintained over the 24 h period (Fig. 1A). Salicylate treatment significantly attenuated the stressor-induced plasma cortisol response compared to the sham group (Fig. 1A). Plasma glucose concentration was significantly higher at 4 h, but not at 1 or 24 h after stressor exposure compared to the pre-stress group in both the sham and salicylate-fed fish (Fig. 1B). There was no significant effect of salicylate on stressor-induced plasma glucose response in the present study (Fig. 1B). Plasma lactate concentrations were significantly higher at 1 h following handling disturbance in both the sham and salicylate-fed groups compared to the pre-stress levels (Fig. 1C). The plasma lactate levels dropped to pre-stress levels by 4 h and this level was maintained over the 24 h period after stressor exposure (Fig. 1C). Salicylate treatment did not significantly affect plasma lactate levels compared to the sham group (Fig. 1C).

6. **Figure 1.** Effect of salicylate on plasma cortisol (A), glucose (B) and lactate (C) concentrations either prior to handling stress (pre-stress) or at 1, 4 and 24 h after stress in rainbow trout.

Values represent mean ± SEM (n = 5-7 fish); different upper case letters denote significant time effect regardless of treatment (two-way ANOVA; $P<0.05$); * significantly different from the sham group within each time point ($P<0.05$).

Fig. 1A

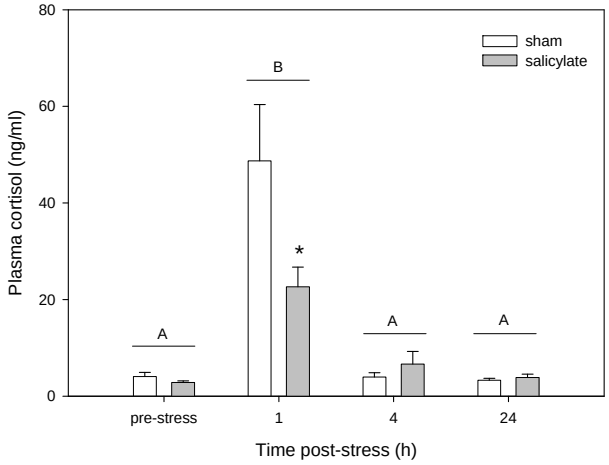


Fig. 1B

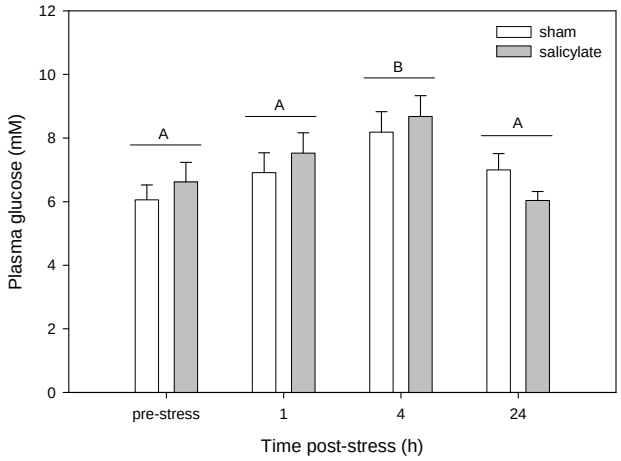
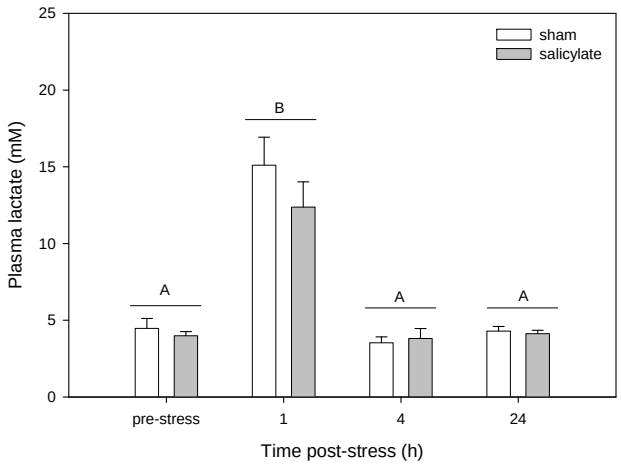


Fig. 1C



3.2 Interrenal transcript levels

Acute handling disturbance did not significantly affect StAR mRNA abundance compared to the pre-stress group (Fig, 2A). However, salicylate treatment significantly reduced stressor-mediated StAR transcript levels compared to the sham group (Fig. 2A; inset showing treatment effect). Transcript levels of PBR (Fig. 2B), P450scc (Fig. 2C) and 11ß-hydroxylase (Fig. 2D) showed a transient elevation after stressor exposure with levels significantly higher at 1 h and 4 h compared to pre-stress and 24 h groups. The temporal response in transcript abundance to stressor exposure was not significantly modified by salicylate treatment (Figs. 2B, C and D).

7. **Figure 2.** Effect of salicylate on steroidogenic acute regulatory protein (StAR; A), peripheral-type benzodiazepine receptor (PBR; B), cytochrome P450 side chain cleavage (cypSCC; C) and 11ß-hydroxylase mRNA abundance either prior to handling stress (pre-stress) or at 1, 4 and 24 h after stress in rainbow trout.

Values represent mean ± SEM (n = 6 fish); different upper case letters denote significant time effect regardless of treatment, while inset shows significant treatment effect (two-way ANOVA; $P<0.05$).

Fig. 2A

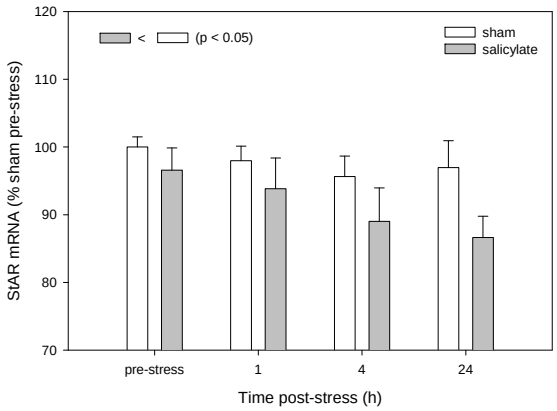


Fig. 2C

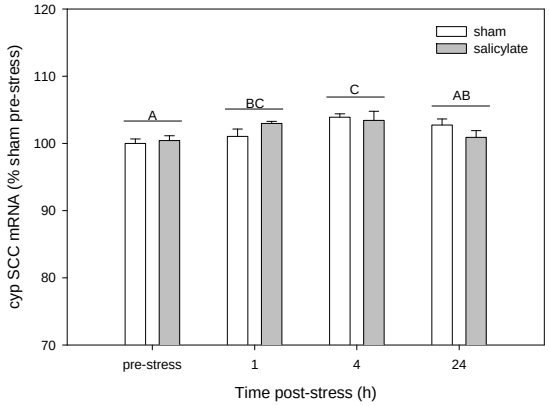


Fig. 2B

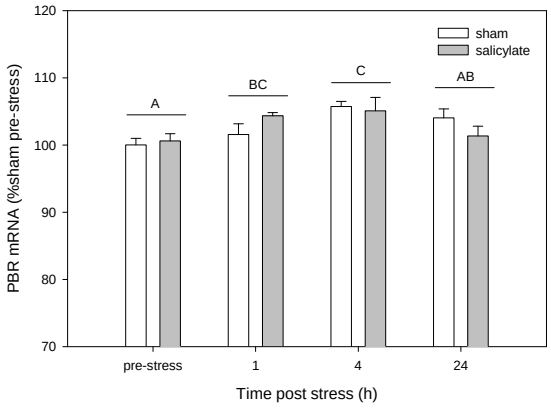
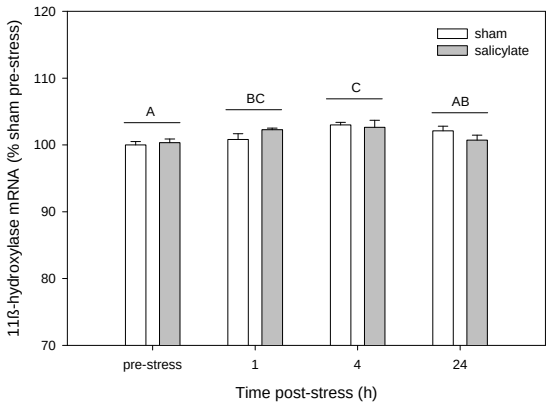


Fig. 2D



3.3 Liver glycogen content

There was a significant treatment effect as well as interaction (time x treatment) on liver glycogen content in the present study. Glycogen content was significantly lower in the salicylate-fed fish compared to the sham fish (Fig. 3). Specifically, the glycogen content in the salicylate group was significantly lower in the pre-stress and 1 h after stressor groups compared to their respective sham groups. In the sham fish, stressor exposure significantly reduced liver glycogen content at 24 h compared to the other time-points. In the salicylate group, there was no significant temporal change in liver glycogen content after stressor exposure compared to the pre-stress group (Fig. 3).

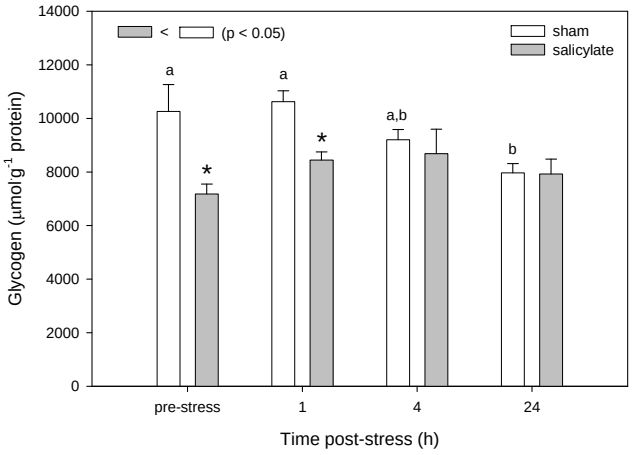
3.4 Liver glucose and enzyme activities

There was a significant elevation in liver glucose concentration at 4 h after stressor exposure compared to all other time points in the sham group (Table 2). There was no stressor-mediated change in glucose levels in the salicylate-fed fish. However, glucose levels at 4 h after stressor exposure in the salicylate group was significantly lower compared to the respective sham group (Table 2). Activities of liver enzymes involved in glycolysis (HK and LDH), gluconeogenesis (PEPCK) and amino acid catabolism (AlaAT and AspAT) were significantly higher at 4 and 24 h, but not at 1 h after stress exposure compared to the pre-stress group (Table 2). The liver GK activity, a glycolytic enzyme, was significantly higher at 1, 4 and 24 h compared to the pre-stress groups (Table 2). Salicylate treatment had no significant effect on liver enzyme activities measured in this study compared to the sham group (Table 2).

8. **Figure 3.** Effect of salicylate on liver glycogen content either prior to handling stress (pre-stress) or at 1, 4 and 24 h after stress in rainbow trout.

Values represent mean ± SEM (n = 6-7 fish); *inset* shows significant treatment effect (two-way ANOVA; $P<0.05$); different lower case letters show significant interaction between time points within a treatment group; *significantly different from the sham group within each time point ($P<0.05$).

Fig. 3


14000
12000
10000
8000
6000
4000
2000
0
Glycogen (µmol g-1 protein)
< (p < 0.05)
sham
salicylate
a
a
a,b
b
*
*
pre-stress
1
4
24
Time post-stress (h)

4. **Table 2.** Effect of salicylate on liver glucose levels (μmol/g protein) and enzyme activities (U/g protein) either prior to handling stress (pre-stress) or at 1, 4 and 24 h after stress in rainbow trout.

Liver	Time Post-stress	Sham	Salicylate	Time effect
Glucose	Pre-stress	5.69 ± 1.04 [a]	6.92 ± 0.68	none
	1h	6.00 ± 0.43 [a]	6.08 ± 0.49	
	4h	9.03 ± 0.75 [b]	6.26 ± 0.57 *	
	24h	5.35 ± 0.31 [a]	6.90 ± 1.02	
HK	Pre-stress	6.89 ± 0.54	7.82 ± 0.50	A
	1h	8.29 ± 0.40	8.05 ± 0.68	AB
	4h	8.86 ± 0.32	9.07 ± 0.54	BC
	24h	9.19 ± 0.53	9.49 ± 0.57	C
GK	Pre-stress	7.08 ± 0.60	7.80 ± 0.23	A
	1h	8.98 ± 0.45	8.34 ± 0.59	B
	4h	9.83 ± 0.41	9.96 ± 0.91	C
	24h	9.42 ± 0.26	9.73 ± 0.56	BC
LDH	Pre-stress	436.01 ± 43.93	566.92 ± 34.11	A
	1h	507.82 ± 38.79	513.55 ± 52.97	A
	4h	713.75 ± 39.06	741.18 ± 37.40	B
	24h	669.34 ± 68.32	655.45 ± 52.06	B
PEPCK	Pre-stress	3.32 ± 0.23	3.87 ± 0.21	A
	1h	3.89 ± 0.21	4.18 ± 0.34	A
	4h	5.21 ± 0.20	4.45 ± 0.24	B
	24h	4.41 ± 0.27	4.94 ± 0.44	B
Ala AT	Pre-stress	42.69 ± 4.08	60.20 ± 6.78	A
	1h	58.89 ± 4.52	52.56 ± 4.58	AB
	4h	62.48 ± 5.08	78.38 ± 9.40	C
	24h	70.14 ± 11.80	66.77 ± 8.02	BC
Asp AT	Pre-stress	87.33 ± 4.88	93.52 ± 4.42	A
	1h	99.49 ± 9.25	92.43 ± 6.68	A
	4h	108.66 ± 7.83	136.52 ± 15.50	B
	24h	129.32 ± 13.05	136.27 ± 9.94	B

Note: Values represent mean ± SEM (n = 6 fish); HK = hexokinase; GK = glucokinase; LDH = lactate dehydrogenase; PEPCK = phosphoenolpyruvate carboxykinase; AlaAT = alanine aminotransferase; AspAT = aspartate aminotransferase; different upper case letters denote significant time effect regardless of treatment (two-way ANOVA; $P<0.05$); different lower case letters show significant interaction between time points within a treatment group; * significantly different from the sham within each time point (two-way ANOVA; $P<0.05$).

3.5 Glucocorticoid receptor signaling

Brain GR content showed a significant transient elevation at 1 h after stressor exposure compared to the pre-stress level (Fig. 4A). The GR levels dropped after an hour but the levels were significantly higher at 4 h, but not at 24 h, compared to the pre-stress group (Fig. 4A). Salicylate treatment did not significantly affect brain GR content compared to the sham group (Fig. 4A). There was no significant effect of either time after stressor exposure or interaction (time x treatment) on liver GR expression (Fig. 4B). However, salicylate treatment significantly reduced liver GR protein content compared to the sham group (Fig. 4B inset).

9. **Figure 4.** Effect of salicylate on brain (A) and liver (B) glucocorticoid receptor (GR) protein expression prior to handling stress (pre-stress) or at 1, 4 and 24 h after stress in rainbow trout. A representative Western blot image of GR protein expression is shown above each figure. Values represent mean ± SEM (n = 6-7 fish); different upper case letters denote significant time effect regardless of treatment, while *inset* shows significant treatment effect (two-way ANOVA; $P<0.05$).

Fig. 4A

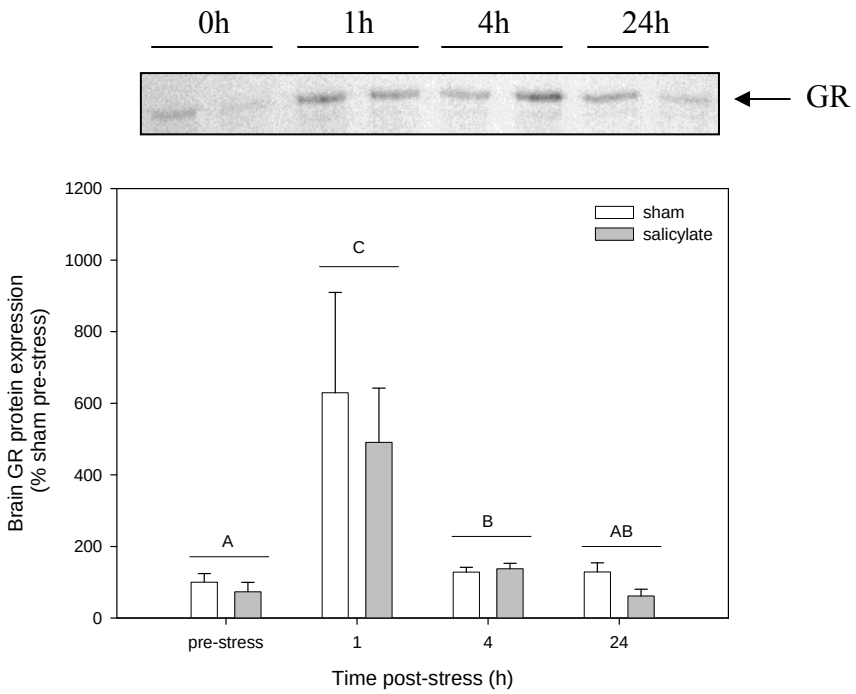
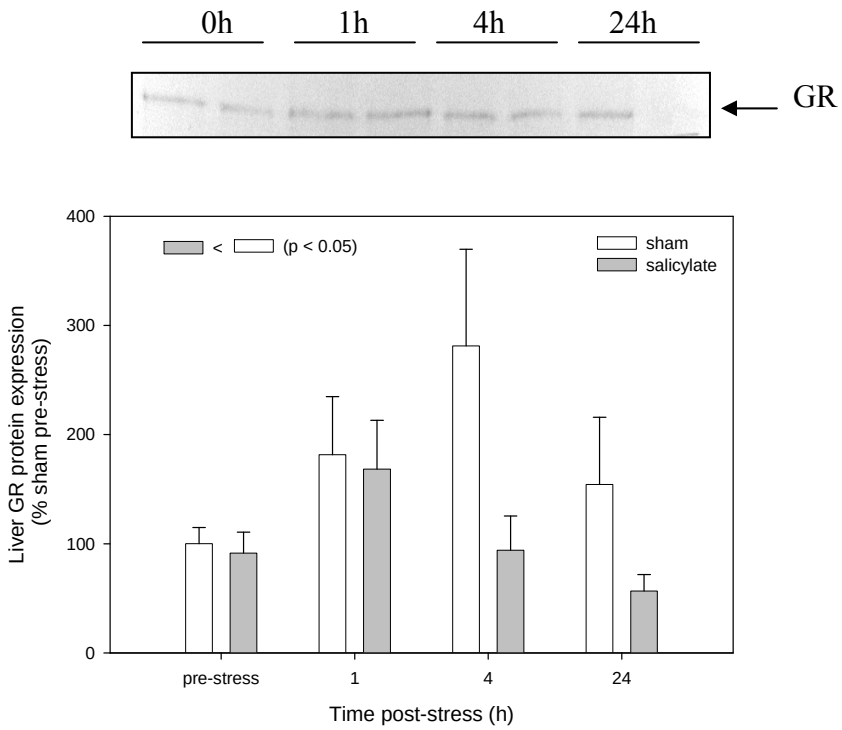

0h
1h
4h
24h
GR
1200
1000
800
600
400
200
0
Brain GR protein expression
(% sham pre-stress)
sham
salicylate
A
C
B
AB
pre-stress
1
4
24
Time post-stress (h)

Fig. 4B


0h
1h
4h
24h
GR
400
300
200
100
0
Liver GR protein expression
(% sham pre-stress)
< (p < 0.05)
sham
salicylate
pre-stress
1
4
24
Time post-stress (h)

4. Discussion

We demonstrate for the first time that salicylate disrupts the adaptive cortisol response to stress in rainbow trout. While the concentration of salicylate used in this study is not environmentally relevant, our result does highlight the mode of action of this drug in affecting fish stress performance. Specifically, key proteins involved in cortisol biosynthesis as well as target tissue responsiveness to cortisol stimulation are targets for salicylate-mediated endocrine disruption in fish.

4.1 Acute stress response in trout

The acute stress response has been well characterized in teleost fish and involves the elevation of plasma cortisol levels upon stressor challenge (Wendelaar Bonga, 1997). However, the changes in transcript levels of genes encoding key proteins and enzymes involved in steroidogenesis, as well as the adaptive metabolic response have been less well studied. Our results are in agreement with others showing that elevation in plasma cortisol, lactate and glucose in response to an acute stressor is transient in rainbow trout (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton et al., 2002; Iwama et al., 2006). The timing, duration and magnitude of these plasma indicators of stress vary with the animal model and also the type and intensity of the stressors (Barton et al., 2002; Iwama et al., 2006). As seen here, the plasma steroid levels during recovery from an acute handling disturbance regain homeostasis quickly and this is due in part to a negative feedback regulation involving GR signaling in the brain. Indeed, a positive correlation between plasma cortisol levels and brain GR protein content clearly implicate a key role for this receptor dynamics in regulating plasma cortisol levels (Aluru et al., 2004).

The transient changes in plasma cortisol levels in response to stress corresponded with a significant, albeit minimal, increase in PBR, P450scc and 11β-hydroxylase, but not StAR mRNA content in trout interrenal tissue. In mammals, under acute ACTH stimulation, transcription of StAR is rapidly (within 30min) upregulated (Fleury et al., 1998; Lehoux et al., 1998; Ivell et al., 2000), whereas increased transcription of steroidogenic enzymes, especially P450scc, has been reported only with chronic ACTH stimulation (Di Blasio et al., 1987; Lehoux et al., 1998). In fish, StAR mRNA levels were also upregulated in response to an acute stressor (Kusakabe et al., 2002; Geslin and Auperin, 2004; Aluru and Vijayan, 2006), but this transcript response appears to be related to the magnitude of cortisol response. For instance, only high intensity (30-fold increase in cortisol levels), but not low intensity stressor (5-fold change in cortisol), resulted in elevated StAR and P450scc mRNA abundance in the interrenal tissue of trout (Geslin and Auperin, 2004). Consequently, the lack of change in StAR mRNA levels in our study may be related to the stressor regimen, which was similar to the low intensity stressor of Geslin and Auperin (2004).

The elevated cortisol response to stressor did not affect liver GR protein content in trout liver. Although liver GR changes were not significant, liver GR content tended to show a transient increase with stress and coincided with enhanced liver metabolic capacity after stressor exposure. Indeed, the enhanced liver metabolic capacity along with plasma glucose elevation after stressor exposure suggests modulation of tissue responsiveness to cortisol stimulation with stress. Further support for this is provided by the enhanced liver glycolytic and gluconeogenic enzymes and transaminase activities after stress, as these pathways are GR-responsive in fish (Vijayan et al., 1994, Mommsen et al., 1999; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003; Wiseman et al., 2007). Thus target tissue GR regulation may be a key process in the signaling of

glucocorticoid-responsive genes and an essential player in the enhanced liver metabolic capacity to cope with stress (Vijayan et al., 2003; Wiseman et al., 2007).

Our results suggest a greater tissue capacity for glucose utilization associated with the acute stress response in rainbow trout. Glucose has been shown to be a primary substrate to fuel metabolic processes in fishes, especially to cope with enhanced energy demand associated with stress (Mommsen et al., 1999). Indeed, the enhanced activities of glycolytic and gluconeogenic enzymes after stressor exposure coincided with a reduction in liver glycogen content and an increase in liver glucose levels suggesting glycogen mobilization to fuel liver metabolism as well as providing glucose for extra-hepatic metabolism. Liver glycogen stores represent an important energy reserve and previous studies have shown increased glycogen depletion in stressed fish (Barton et al., 2002; Iwama et al., 2006) and enhanced liver glycolytic capacity with cortisol treatment (Mommsen et al., 1999). The concomitant increase in transaminases and PEPCK supports a higher liver capacity for amino acid catabolism and channeling of C3 substrates for gluconeogenesis (Mommsen et al., 1999). Considered together, the acute stress response in trout involves the synthesis and release of cortisol into the circulation, which may modulate target tissue responsiveness to this steroid. This stressor-induced cortisol response is essential for the metabolic adjustments to cope with stress in fish. Consequently, any impact on either cortisol biosynthesis and/or GR signaling will impair the adaptive stress response.

4.2 Role of salicylate

Our results demonstrate for the first time that salicylate exposure disrupts the adaptive cortisol and metabolic response to stress in trout. The attenuation of cortisol response to stressor exposure with salicylate agrees with a recent study showing a similar steroid response in tilapia fed acetylsalicylic acid and subjected to a confinement stressor (van Anholt et al., 2003). The

corresponding suppression of interrenal StAR transcript levels in the present study establishes a mechanistic link for the impaired cortisol response by NSAIDs in fish. The results confirm our earlier observation that NSAIDs attenuate the acute ACTH-stimulated StAR and PBR transcript abundance in trout interrenal tissue, leading to impaired cortisol production in trout (Gravel and Vijayan, 2006). We also reported previously that salicylate exposure affected brain GR content suggesting disturbances in negative feedback regulation of cortisol (Gravel and Vijayan, 2006). However, the lack of changes in brain GR protein content with salicylate in the present study argues against negative feedback dysregulation as a possible reason for the attenuated cortisol response to handling disturbance. Taken together, our results implicate a role for salicylate in disrupting steroidogenesis leading to a reduced stress performance to subsequent stressor insults.

As cortisol is important in the metabolic adjustment to stress (Mommsen et al., 1999), the lower cortisol response to stress with salicylate may also lead to altered energy repartitioning. To this end, salicylate reduced liver GR protein content in rainbow trout, which would lead to a lower liver responsiveness to cortisol stimulation. As this effect was apparent during recovery from stressor (Fig. 4B), it appears likely that salicylate impacts stressor-induced GR modulation in trout. The elevation in brain and liver GR content after stressor exposure in the sham group, which coincides with elevated steroid levels, supports the notion that the reduced cortisol levels with salicylate may be playing a role in the depressed liver GR protein content. This also agrees with a recent study that showed a positive correlation between plasma cortisol and brain GR content in Arctic charr (*Salvelinus alpinus*; Aluru et al., 2005). Despite the potential for reduced tissue responsiveness to cortisol, we did not observe major changes in the liver metabolic capacity with salicylate. However, salicylate reduced liver glycogen content and also eliminated the stressor-induced glycogen mobilization seen in the sham fish. This was further supported by

the absence of a stressor-induced liver glucose peak, seen in the sham group, with salicylate. Taken together, salicylate affects liver glycogen metabolism, but the lack of a change in stressor-induced plasma glucose levels leads us to propose a disruption in glucose turnover by salicylate in trout. Indeed this observation is supported by our recent finding that waterborne salicylate exposure inhibited the stressor-mediated drop in whole body glucose levels in trout, suggesting disturbances in the clearance mechanism (Gravel and Vijayan, 2007).

While StAR is a target for salicylate impact on steroidogenesis, the mechanism(s) of action is unclear. For instance, as a COX inhibitor, salicylate prevents prostaglandins (PGs) production, which is a key modulator of HPA axis functioning in humans (Cavagnini et al., 1979; Hockings et al., 1993) and also in fish (van Anholt, 2003). Furthermore, NSAIDs are known to activate a variety of transcription factors, including peroxisome proliferators (PPARs), involved in the regulation of steroidogenesis (Komar, 2005). Disruption of steroidogenesis and cholesterol transfer was shown to be PPARα-dependant in mammalian cell lines (Gazouli et al., 2002), raising the possibility that this transcription factors may be involved in salicylate-mediated endocrine disruption. Considered together, while the disruption of cortisol response to stress by NSAIDs may involve multiple modes of action, clearly the key proteins involved in cortocosteroidogenesis are targets for endocrine disruption by salicylates in trout.

In conclusion, we demonstrate that salicylate impaired the adaptive cortisol response to an acute stressor in rainbow trout. Although fish in the aquatic ecosystems are unlikely to be exposed to such high levels of NSAIDs, we subjected the fish to a high concentration in order to understand the mechanism of action of salicylate. The attenuation of cortisol response with salicylate involved reduction in StAR mRNA levels, a key protein involved in the acute regulation of steroidogenesis. Salicylate did not affect brain GR content arguing against

disturbed feedback regulation as a reason for the attenuated cortisol response to stressor. However, salicylate depressed liver GR protein content and also modified the stressor-mediated liver metabolic capacity in trout suggesting a role for this drug in reducing target tissue responsiveness to cortisol. Altogether, salicylate impaired the adaptive cortisol response and the associated metabolic adjustments critical for coping with stress in fish.

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References

Aluru, N., Vijayan, M.M. 2006. Ah receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis. Endocrinol. 147, 1895-1903.

Aluru, A., Jorgensen, E.H., Maule, A.G., Vijayan, M.M. 2004. PCB disruption of the hypothalamus-pituitary-interrenal axis involves brain glucocorticoid receptor downregulation in anadromous Arctic charr. Am. J Physiol. Regul. Integr. Comp. Physiol. 287, R787-793.

Barton, B.A., Morgan, J.D., Vijayan, M.M. 2002. Physiological and condition-related indicators of environmental stress in fish. In: Adams, S.M. (Ed.), Biological Indicators of Stress in Fish, Second Edition. American Fisheries Society, Bethesda, Maryland, pp 111-148.

Boone, A.N., Vijayan. M.M. 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. Amer. J. Physiol. Regul, Integr. Comp. Physiol. 283, R680-R687.

Cavagnini, F., Di Landro, A., Maraschini. C., Invitti, C., Pinto, M.L. 1979. Effect of two prostaglandin synthesis inhibitors, indomethacin and acetylsalicylic acid, on plasma ACTH and cortisol levels in man. Acta Endocrinol (Copenh). 91, 666-73.

Cleuvers, M. 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotox. Environ. Saf. 59, 309-315.

Di Blasio, A.M., Voutilainen, R., Jaffe, R.B., Miller, W.L. 1987 Hormonal regulation of messenger ribonucleic acids for P450scc (cholesterol side-chain cleavage enzyme) and P450c17 (17 alpha-hydroxylase/17,20-lyase) in cultured human fetal adrenal cells. J Clin Endocrinol Metab. 65, 170-175.

Fleury, A., Ducharme, L., Hales, D.B., Stocco, D.M., LeHoux, J.G. 1998. Acute in vivo effects of ACTH on the expression of steroidogenic acute regulatory protein in rat adrenal. Endocr Res. 24, 571-574.

Gazouli, M., Yao, Z.X., Boujrad, N., Corton, J.C., Culty, M., Papadopoulos, V. 2002. Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor alpha. Endocrinol. 143, 2571-2583.

Geslin, M., Auperin, B. 2004. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 135, 70-80.

Gravel, A., Vijayan, M.M. 2006. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. Toxicol. Sci. 93, 41-49.

Gravel, A., Vijayan, M.M. 2007. Non-Steroidal Anti-Inflammatory Drugs Disrupt the Heat Shock Response in Rainbow Trout. Aquat.Toxicol. 81, 197-206.

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett. 131, 5-17.

Hockings, G.I., Grice, J.E., Crosbie, G.V., Walters, M.M., Jackson, A.J., Jackson, R.V. 1993. Aspirin increases the human hypothalamic-pituitary-adrenal axis response to naloxone stimulation. J Clin Endocrinol Metab.77, 404-408.

Hontela, A. 2005. Stress and the hypothalamo-pituitary-interrenal axis: adrenal toxicology-Effects of environmental pollutants on the structure and function of the HPI axis. In: T.W.Moon and T.P.Mommsen (Eds.), Biochemical and molecular biology of fishes, Vol.6 Environmental Toxicology. Elsevier, Amsterdam, p 331-363.

Ivell, R., Tillmann, G., Wang, H., Nicol, M., Stewart, P.M., Bartlick, B., Walther, N., Mason, J.I., Morley, S.D. 2000. Acute regulation of the bovine gene for the steroidogenic acute regulatory protein in ovarian theca and adrenocortical cells. J Mol Endocrinol. 24,109-118.

Iwama, G.K., Alfonso, L.O.B., Vijayan, M.M. 2006. Stress in Fish. In: Evans, D.H., Clairborne, J.B. (Eds.), The Physiology of Fishes, Third Edition. CRC Press, Boca Raton, pp 319-342.

Komar, C.M. 2005. Peroxisome proliferator-activated receptors (PPARs) and ovarian function--implications for regulating steroidogenesis, differentiation, and tissue remodeling. Reprod. Biol. Endocrinol. 3, 41-55.

Kusakabe, M., Todo, T., McQuillan, J.H., Goetz, F.W., Young, G. 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinol. 143, 2062-2070.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lehoux, J.G., Fleury, A., Ducharme, L. 1998. The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. Endocrinol. 139, 3913-3922.

Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., Hirsch, R. 2003a. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxicol. Chem. 22, 2872-2880.

Metcalfe, C.D, Miao, X.S., Koenig, B.G., Struger, J. 2003b. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem. 22, 2881-2889.

Miller, W.L. 2007. Mechanism of StAR's regulation of mitochondrial cholesterol import. Mol Cell Endocrinol. 265-266, 46-50.

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

Payne, A.H., Hales, D.B. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25, 947–970.

Papadopoulos, V. 2004. In search of the function of the peripheral-type benzodiazepine receptor. Endocr. Res. 30, 677-684.

Sathiyaa, R., Vijayan, M.M. 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Amer. J. Physiol. Cell Physiol. 284, C1508-C1515.

Stocco, D.M., Wang, X.J., Jo, Y., Manna, P.R. 2005. Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought. Mol. Endocrinol. 19, 2647-2659.

Trudeau, V.L., Metcalfe, C.D., Mimeault, C., Moon, T.W. 2005. Pharmaceuticals in the environment: Drugged fish? In: Mommsen, T.P., Moon, T.W. (Eds), Biochemistry and Molecular Biology of Fishes, Vol. 6. Elsevier, Amsterdam, pp 87-101.

van Anholt, R.D., Spanings, T., Koven, W., Wendelaar Bonga, S.E. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R1098-R1106.

Vijayan, M.M., Reddy, P.K., Leatherland, J.F., Moon, T.W. 1994. The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. Gen. Comp. Endocrinol. 96, 75-84.

Vijayan, M.M., Pereira, C., Grau, E.G., Iwama, G,K. 1997. Metabolic responses associated with confinement stress in tilapia: The role of cortisol. Comp.Biochem.Physiol. C-Pharmacol.Toxicol. 116, 89-95.

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

Vijayan, M.M., Prunet, P., Boone, A.N. 2005. Xenobiotic impact on corticosteroid signaling. In: Mommsen, T.P., Moon, T.W. (Eds). Biochemistry and Molecular Biology of Fishes, Vol. 6. Elsevier, Amsterdam, pp 87-101.

Vijayan, M.M., Aluru, N., Maule, A.G., Jorgensen, E.H. 2006. Fasting augments PCB impact on liver metabolism in anadromous arctic char. Toxicol. Sci. 91, 431-439.

Webb, S.F. 2001. A data based perspective on the environmental risk assessment of human pharmaceuticals II –aquatic risk characterization. In. Kummerer, K. (Ed.), Pharmaceuticals in the environment. Sources, fate, effects and risks. Springer-Verlag Berlin, Heidelberg, New York, pp.203-219.

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

Wiseman, S., Osachoff, H., Bassett, E., Malhotra, J., Bruno, J., VanAggelen, G., Mommsen, T.P., Vijayan, M.M. 2007. Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. Comp. Biochem. Physiol. Part D: Genomics and Proteomics, doi: 10.1016/j.cbd.2007.04.005.

CHAPTER 4

Physiological responses to waterborne exposure to salicylate and ibuprofen in rainbow trout fry: a concentration-response study.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as salicylic acid and ibuprofen are detected in effluents from sewage treatment plants and surface waters at concentrations of 1µg/L (Heberer, 2002; Metcalfe et al., 2003a,b). However, very little is known about the physiological effects associated with water-borne exposure to NSAIDs in fish. Acute toxicity tests performed according to ISO laboratory assays showed that ibuprofen is almost non toxic to bluegill sunfish *Lepomis machrochirus* (96h-LC_{50}=173mg/L) and sheepshead minnow *Cyprinodon variegates* (96h-NEL >300mg/L) (Knoll, BASF, 1995). Lethality concentrations (LC_{50}) values for salicylic acid and ibuprofen in fish either *in vitro* with fish cell lines or *in vivo* are in the low mM (~10^6 µg/L) range (Caminada et al., 2006). Lethal and sublethal effects of NSAIDs reported in aquatic organisms are seen at higher concentrations (Halling-Sorensen et al., 1998; Cleuvers, 2004; Caminada et al., 2006) that are not found in the environment.

The objective of this study was to establish a concentration-related response of salicylate and ibuprofen on some of the physiological end-points routinely used for steroid production, stress response and metabolism in fish. To this end, a waterborne exposure (0, 0.1, 1, 10, 100 and 1000 µg/L) to salicylate and ibuprofen was performed on rainbow trout (*Oncorhynchus mykiss*) fry for 96h exactly as mentioned before (Gravel and Vijayan, 2007). The physiological endpoints were selected in terms of their relevance to other studies in the thesis. Liver hsp70 expression was measured as an indicator of cellular stress response (Iwama et al., 1998). Because plasma analysis was not possible in these very small fish, cortisol was not measured. However, transcript levels of enzymes and proteins involved in cortisol biosynthesis were measured in the interrenal tissue as an indicator of steroidogenic activity (Gravel and Vijayan, 2006). Gill Na^+/K^+-ATPase activity was selected as an indicator of ion regulation capacity, while liver

glycogen and glucose content and lactate dehydrogenase (glycolytic enzyme) activity were determined to indicate liver metabolic capacity.

2. Material and Methods

2.1 Material

Ibuprofen (sodium salt), salicylate (salicylic acid sodium salt), protease inhibitor cocktail, bicinchoninic acid (BCA) reagent, and 2-phenoxyethanol were purchased from Sigma (St Louis, MO). Costar 96-well EIA/RIA flat bottom microplates were from Corning Inc. (Corning, NY), while the electrophoresis reagents, molecular weight markers and alkaline phosphatase-conjugated goat anti-rabbit IgG were from BioRad (Hercules, CA). Polyclonal rabbit anti-trout total hsp70 antibody was a generous gift from Dr E. Peter M. Candido (Biochemistry Department, UBC). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (StressGen, Victoria, BC). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2 Fish

Fry rainbow trout (~ 1.5 g body mass; ~ 5 cm length) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario. During two weeks acclimation period, fish were maintained in aerated tanks with constant water flow at 13˚C with a 12 hL: 12hD photoperiod and fed to satiety twice daily (ground commercial trout chow, Martin Mills Inc., Elmira, ON).

2.3 Experimental design

Groups of 8 fry each were transferred to a static system of eleven aerated aquaria (2.5 L capacity) partially immersed in flowing water in order to keep water temperature constant to13˚

C and maintained under photoperiod and feeding regimen described above. Aquaria were cleaned by siphoning left over food and feces and replacing 2/3rd water twice daily. After one week of acclimation to the static system, fry were exposed to the drugs for 4 days, to be consistent with most lethality bioassays conducted on a 96h time period. Ibuprofen or salicylate was dissolved in water to reach a final concentration of 0.1, 1, 10, 100 or 1000 μg/L, while an additional aquarium with no drugs served as the control group. Lower concentrations were representative of levels detected in the environment (0.1 and 1μg/L) (Heberer, 2002; Metcalfe et al., 2003a and b). Food was withheld during the exposure period. Sampling consisted of quickly netting fish from each aquaria and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). There were no mortalities associated with any of the treatments. Gills, liver and interrenal tissues were immediately frozen on dry ice and stored at -70˚C for later biochemical analysis as described below.

2.4 Gill Na^+K^+-ATPase activity

Gill was homogenized in SEI buffer (0.3M sucrose, 0.02M EDTA, 0.1M imidazole buffer pH 7.3) containing 0.1% sodium deoxycholate and briefly centrifuged. Na^+K^+-ATPase activity was determined from fresh homogenate by measuring ouabain-sensitive ATPase activity (NADH-mediated ATP hydrolysis) in a microplate according to protocol by McCormick (1993). Activity was calculated relative to protein quantification in homogenate by BCA method.

2.5 Liver glycogen and glucose analysis

Livers and gills were homogenized (Ultra Turrax; IKA Works, Wilmongton, NC), followed by sonication (Microson, Farmingdale, NY) in a homogenization buffer (50% glycerol, 21mM Na_2HPO_4, 0.5mM EDTA-Na_2, 0.2% BSA, 5mM β-mercaptoethanol and protease

inhibitors, pH adjusted to 7.5) exactly as described before (Vijayan et al., 2006). For determination of liver glucose and glycogen, an aliquot of the liver homogenate was treated with perchloric acid and liver glucose was determined by a colorimetric assay using a commercial kit (modified Trinder method; Rainchem, San Diego, CA). Liver glycogen content was analyzed by measuring glucose content before and after amyloglucosidase hydrolysis (Keppler and Decker, 1974). The glycogen activity is represented as micromoles per gram protein in the homogenate.

2.6 Hepatic enzyme activity

The lactate dehydrogenase (LDH) enzyme activity was measured in 50mM imidazole-buffered enzyme reagent (pH 7.4) at 22^{o}C by continuous spectrophotometry at 340nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA). The following assay conditions were shown to provide optimal activities:

Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and 25mM pyruvic acid.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein in the homogenate.

2.7 Transcript analysis

Total RNA (DNase treated) was isolated from interrenal tissue using the RNeasy Mini Kit protocol (Qiagen, Mississauga, ON), and the RNA was quantified by spectrophotometry at 260nm. The first strand cDNA was synthesized from 1μg total RNA using First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON). Quantification of transcript levels were performed exactly as previously described (Sathiyaa and Vijayan, 2003) using Platinum® Quantitative PCR SuperMix-UDG (In Vitrogen, Carlsbad, CA). Briefly, cDNA were used as template for amplification of the housekeeping gene (β-actin) and target genes (StAR, PBR, and CYPscc) with appropriate primers (Table1) designed using rainbow trout StAR, P450scc, PBR,

and β-actin cDNA sequences (GenBank accession nos. AB047032, S57305, AY029216, and AF157514, respectively). The primer pairs amplified either a ~100 bp fragment for β-actin or a ~500 bp fragment for all target genes used in quantitative real-time PCR (qPCR; iCycler, Biorad).

A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95°C- 3min; 40 cycles: denature 95°C- 20s, anneal-20s, and extend 72°C-20 s; then cool 4°C-hold. The iCycler IQ^{TM} real time detection software (BioRad) was used to determine the threshold values (C_T) for every sample. A standard curve with log input amount and C_T were established for β-actin and each target genes to calculate the transcript levels in samples. The target gene transcript levels were normalized by taking a ratio of target gene to housekeeping gene. This normalized value was standardized using an internal calibrator (control group) and transcript levels expressed as percent of internal calibrator according to established protocols (Gravel and Vijayan, 2006).

5. **Table1:** Oligonucleotide primers used in quantitative real-time PCR and their annealing temperatures.

Gene	Primers	Annnealing temp. (°C)
StAR	Forward: 5'-CGCTGGCATCTCCTACA-3' Reverse: 5'-GGGACTTCGTTAGTGTTCG-3'	58
P450scc	Forward: 5'-GAGGAGGGTAGGAGCCA-3' Reverse: 5'-CCTTGTGGGACTCTGGT-3'	60
PBR	Forward: 5'GGCCACCATTTCATTTGTTCATTTCA-3' Reverse: 5'-GCCATGCAAGGTAGGGTGTCAGGA-3'	60
ß-actin	Forward: 5'-AGAGCTACGAGCTGCCTGAC-3' Reverse: 5'-GCAAGACTCCATACCGAGGA-3'	49

2.8 Protein quantification

Tissue homogenization and sample preparation for hsp70 immunodetection was described in detail previously (Boone and Vijayan, 2002). Briefly, samples in Laemmli's buffer were loaded (40 μg protein/sample) onto 8% SDS-PAGE and proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semi-dry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris (pH 7.5), 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02% sodium azide), probed for 1 h at room temperature with primary antibody (anti-trout hsp70; 1:3000 dilution) in the blocking solution, washed with TTBS (3 times for 5 min each) and incubated for 1 h with secondary antibody (goat-anti-rabbit IgG conjugated with alkaline phosphatase; 1:3000 dilution). Our anti-trout hsp70 antibody recognizes both the constitutive and the inducible isoform of hsp70 (Boone and Vijayan, 2002). The protein bands were detected with NBT and BCIP. The intensity of the scanned bands were quantified using the AlphaEase software (AlphaEase Innotech, CA) and the protein values, expressed as percent control, were shown as figures.

2.9 Statistical analysis

The concentration-related effects of NSAIDs were analysed using one-way analysis of variance (ANOVA), followed by LSD (post hoc) test. A t-test was used to compare differences between control and each individual concentration separately. Data were log transformed, wherever necessary, for normal distribution and homogeneity of variance, while non-transformed data are shown in the figures. A significance level of $\alpha=0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Steroidogenesis

The rate-limiting step in steroidogenesis is the delivery of cholesterol, the precursor of all steroid hormones to the active site of cytochrome P450 side chain cleavage (CYPscc), which will initiate the enzymatic cascade of steroidogenesis in mammals and fish (Payne and Hanes, 2004). The steroidogenic acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBR) are two key proteins involved in cholesterol transfer across the double mitochondrial membrane (Papadopoulos, 2004; Stocco et al., 2005; Miller, 2007). Therefore, transcript levels of StAR, PBR and CYPscc were measured as indicators of steroidogenesis. Although the steroidogenic acute regulatory protein (StAR) mRNA abundance tended to decrease with salicylate concentration, the regression did not show a significant concentration-response relationship and none of the salicylate concentrations used had a significant effect on StAR transcript levels (Fig. 1A). Ibuprofen exposure had no significant effect on StAR mRNA in trout fry (Fig. 1B). Ibuprofen significantly inhibited PBR transcripts levels at 0.1,10 and 100ug/L (Fig. 2A) but none of the two NSAIDs tested followed a significant linear concentration-response relationship on PBR mRNA abundance (appendix Fig. 2A and B). These results are at odds with our earlier study (Gravel and Vijayan, 2006) showing reduced StAR and PBR mRNA abundance in the interrenal tissue of rainbow trout after three days of feeding 100mg/kg body weight salicylate (Gravel and Vijayan, 2006). However, this may be due to the fact that the previous study examined acute ACTH-stimulated cortisol production *in vitro*, whereas in the present study only the constitutive levels of the transcripts were measured. This supports the contention that NSAIDs impact on StAR and PBR are not seen in unstimulated tissues and evident only after an acute stimulation by either stressors or ACTH administration (Gravel and Vijayan, 2006). .

Interrenal transcript levels of cytochrome P-450 side chain cleavage (CYPscc) were not significantly affected by 4-d exposure to a range of concentration of either salicylate (Fig. 3A) or ibuprofen (Fig. 3B) in rainbow trout fry and agrees with our previous study that this steroidogenic enzyme was also not impacted by NSAIDs in trout (Gravel and Vijayan, 2006). Overall, our results suggest that constitutive transcript levels of key steroidogenic proteins and enzyme in the interrenal tissue are not a major target of water-borne NSAIDs in rainbow trout fry, at least at the concentrations and under the conditions tested.

10.**Figure 1.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on StAR mRNA levels in the interrenal tissue of rainbow trout fry.

Values represent mean ± SEM (n= 4-8 fish); values are expressed % control (0 µg/L); * means significantly different from the control (Student T-test $P \leq 0.05$). Regression lines are presented in appendix.

Fig. 1A

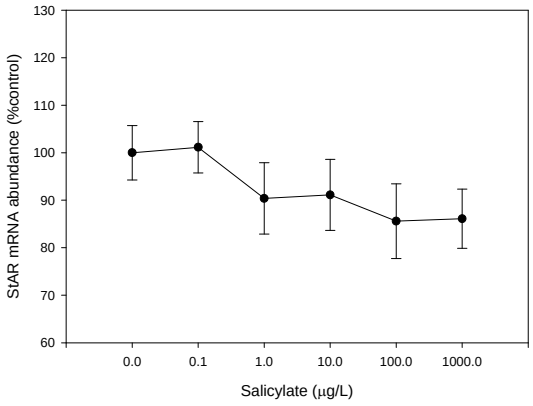
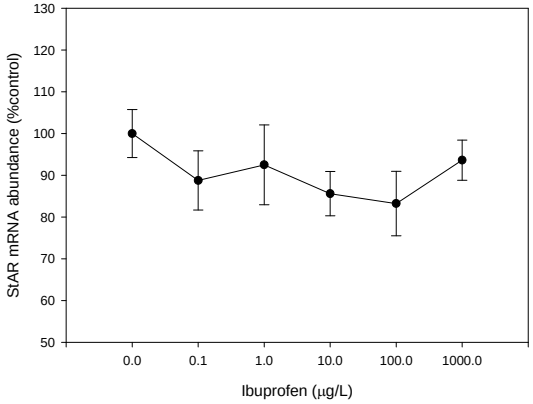

StAR mRNA abundance (%control)
130
120
110
100
90
80
70
60
0.0
0.1
1.0
10.0
100.0
1000.0
Salicylate (μg/L)

Fig. 1B


StAR mRNA abundance (%control)
130
120
110
100
90
80
70
60
50
0.0
0.1
1.0
10.0
100.0
1000.0
Ibuprofen (μg/L)

11. **Figure 2.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on PBR mRNA levels in the interrenal tissue of rainbow trout fry.

Values represent mean ± SEM (n= 4-8 fish); values are expressed % control (0 µg/L); different letters represent significant differences between treatment (One-way ANOVA); * means significantly different from the control (Student T-test $P \leq 0.05$). Regression lines are presented in appendix.

Fig. 2A

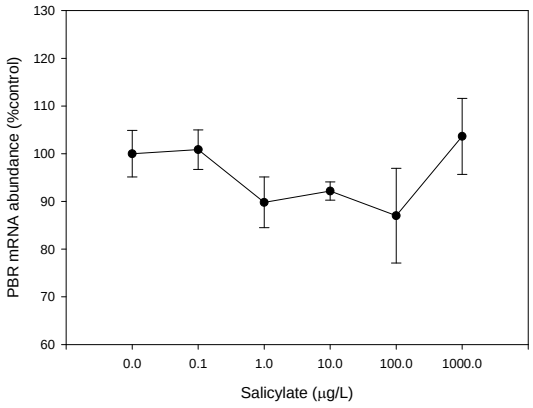
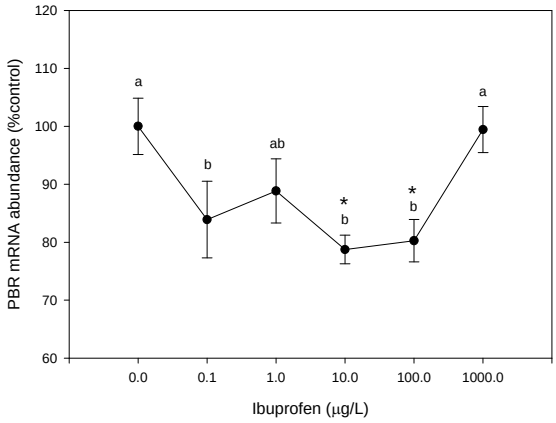

PBR mRNA abundance (%control)
130
120
110
100
90
80
70
60
0.0
0.1
1.0
10.0
100.0
1000.0
Salicylate (μg/L)

Fig. 2B


PBR mRNA abundance (%control)
120
110
100
90
80
70
60
a
b
ab
*
b
*
b
a
0.0
0.1
1.0
10.0
100.0
1000.0
Ibuprofen (μg/L)

12. **Figure 3.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on CYPscc mRNA levels in the interrenal tissue of rainbow trout fry.

Values represent mean ± SEM (n= 4-6 fish); values are expressed % control (0 µg/L); * means significantly different from the control (Student T-test $P \leq 0.05$). Values at 0.1 µg/L not available. Regression lines are presented in appendix.

Fig. 3A

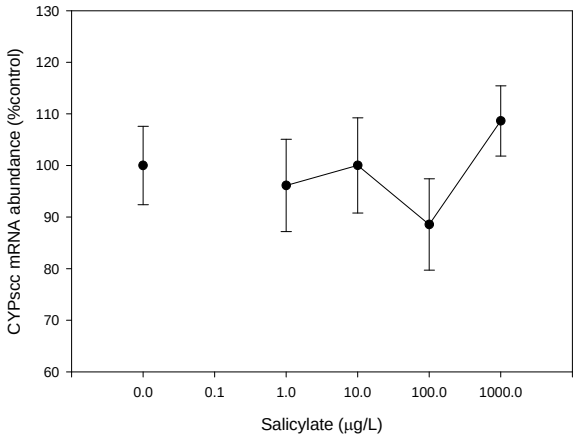
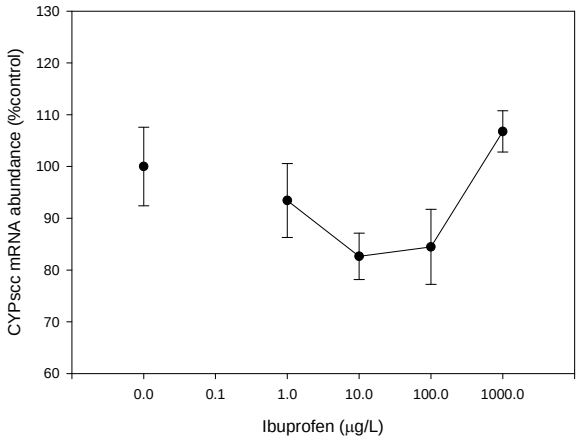

CYPscc mRNA abundance (%control)
130
120
110
100
90
80
70
60
0.0
0.1
1.0
10.0
100.0
1000.0
Salicylate (μg/L)

Fig. 3B


CYPscc mRNA abundance (%control)
130
120
110
100
90
80
70
60
0.0
0.1
1.0
10.0
100.0
1000.0
Ibuprofen (μg/L)

3.2 Gill Na^+/K^+-ATPase activity

A major transporter involved in maintaining constant osmotic pressure in fluids of fish is the gill Na^+/K^+-ATPase. The activity level of the sodium pump is commonly used as an indicator of ion regulation (Marshall and Brysson, 1998; McCormick, 2001; Evans, 2002). Exposure to a range of concentrations of salicylate and ibuprofen showed no significant linear concentration-dependant response on gill Na^+/K^+-ATPase activity (appendix Fig. 4A and B). However, salicylate exposure significantly increased gill Na^+/K^+-ATPase activity compared to the control at 100 μg/L, but not at any other concentrations (Fig. 4A). Increase in gill Na^+/K^+-ATPase activity was significant only at the highest concentration of ibuprofen exposure (Fig. 4B). In our study, the activity of the sodium pump was moderately affected only at high concentrations of NSAIDs in trout maintained in a fresh water environment but the function of this ion transporter gains importance at higher salinity level. Increase in gill Na^+/K^+-ATPase plays an essential role in fish survival in hyperosmotic environment by mediating excess salt excretion during seawater adaptation (Marshall and Brysson, 1998; McCormick, 2001; Evans, 2002). Therefore, effects of NSAID exposure in combination with a change in salinity environment could potentially affect the ion regulation capacity in rainbow trout and impair the adaptive response to salinity.

13. **Figure 4.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on gill Na^+/K^+-ATPase activity in rainbow trout fry.

Values represent mean ± SEM (n= 6 fish); values are expressed % control (0 µg/L); * means significantly different from the control (Student T-test $P \leq 0.05$). Regression lines are presented in appendix.

Fig. 4A

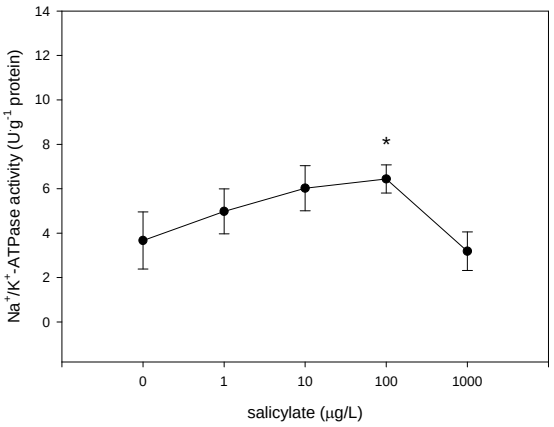
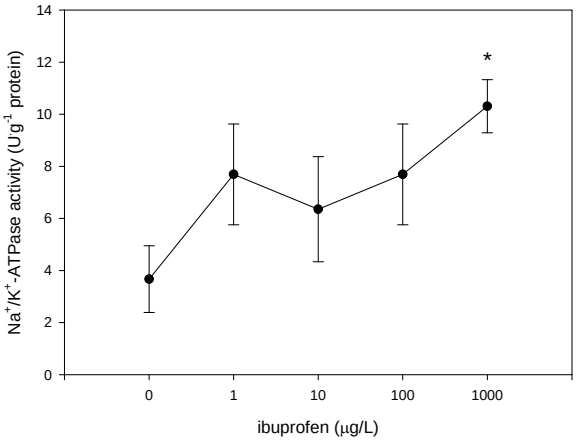

14
12
10
8
6
4
2
0
Na+/K+-ATPase activity (U·g-1 protein)
*
0
1
10
100
1000
salicylate (µg/L)

Fig. 4B


14
12
10
8
6
4
2
0
Na+/K+-ATPase activity (U·g-1 protein)
*
0
1
10
100
1000
ibuprofen (µg/L)

3.3 Liver metabolic capacity

Glucose is an important fuel for metabolism and certain tissues, including brain and gills in fish, rely primarily on this metabolite for energy production (Mommsen et al., 1999). Liver glycogen stores represent an important energy reserve enabling enhanced glucose production through glycogenolysis to cope with increased energy demand (Mommsen et al., 1999). Since liver is a major site of metabolic activity, glycogen content and the activity of a glycolytic enzyme were measured in liver of trout exposed to NSAIDs. Liver glycogen was not significantly affected by salicylate exposure (Fig. 5A). Ibuprofen significantly increased glycogen content only at 10µg/L but not at other concentrations (Fig. 5B) (appendix Fig. 5B). Liver glucose levels did not change significantly with either salicylate (Fig. 6A) or ibuprofen (Fig. 6B) exposure. Hepatic lactate dehydrogenase activity remained unchanged with both NSAIDs throughout the concentration range tested (Fig. 7A and B). Overall, liver metabolic capacity was not affected with a waterborne exposure to salicylate or ibuprofen at concentrations ranging from 0 to 1000 µg/L in rainbow trout fry. However, exposure to stressors including toxicants initiate alteration in glycogen and/or glucose metabolism, as part of the stress response, which enables the fish to cope with increased energy demand (Mazeaud and Mazeaud, 1981; Vijayan et al., 1991). Therefore, we hypothesize that NSAID exposure coupled with an additional stressor will impact the energy substrate re-partitioning and consequently compromise the ability of fish to cope with stress.

14. **Figure 5.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on liver glycogen content in rainbow trout fry.

Values represent mean ± SEM (n= 6 fish); values are expressed % control (0 µg/L); * means significantly different from the control (Student t-test $P \leq 0.05$). Regression lines are presented in appendix.

Fig. 5A

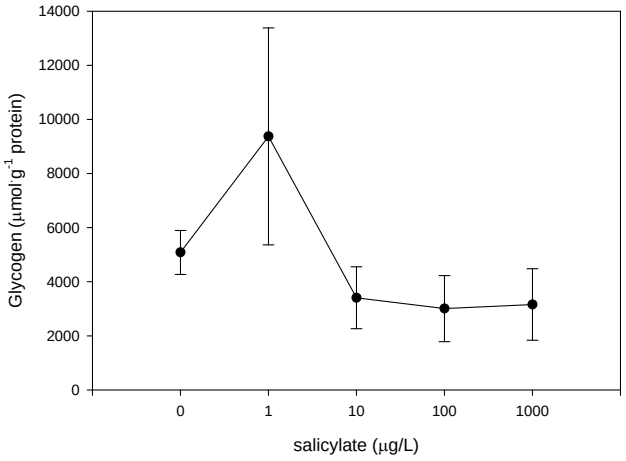
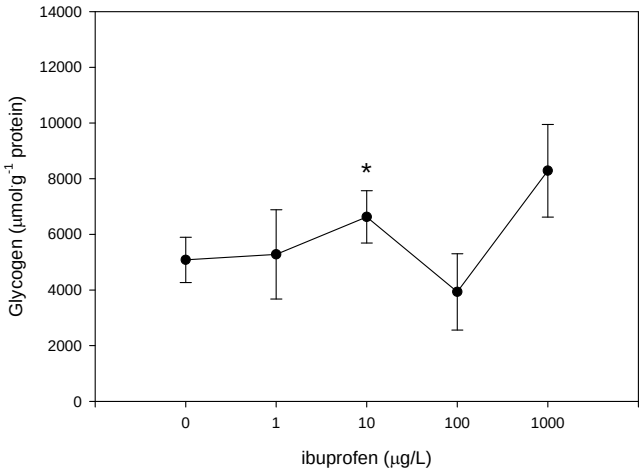

14000
12000
10000
8000
6000
4000
2000
0
Glycogen (µmol·g-1 protein)
0
1
10
100
1000
salicylate (µg/L)

Fig. 5B


14000
12000
10000
8000
6000
4000
2000
0
Glycogen (µmol·g-1 protein)
*
0
1
10
100
1000
ibuprofen (µg/L)

15. **Figure 6.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on liver glucose content in rainbow trout fry.

Values represent mean ± SEM (n= 6 fish); values are expressed % control (0 µg/L). Regression lines are presented in appendix.

Fig. 6A

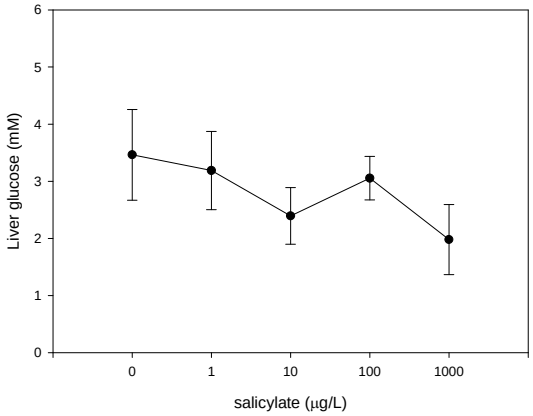
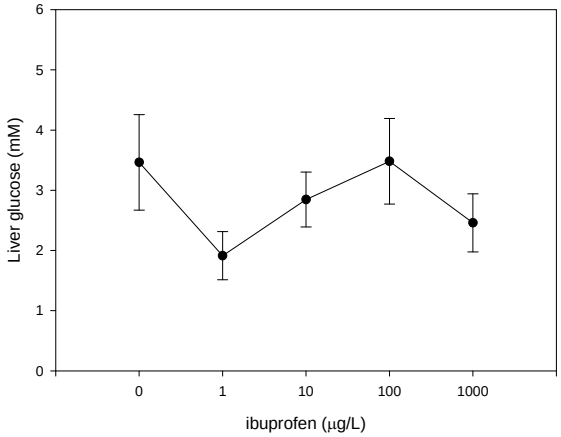

Liver glucose (mM)
0
1
2
3
4
5
6
0
1
10
100
1000
salicylate (μg/L)

Fig. 6B


Liver glucose (mM)
0
1
2
3
4
5
6
0
1
10
100
1000
ibuprofen (μg/L)

16. **Figure 7.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on hepatic lactate dehydrogenase activity content in rainbow trout fry.

Values represent mean ± SEM (n= 5-6 fish); values are expressed % control (0 µg/L).

Regression lines are presented in appendix.

Fig. 7A

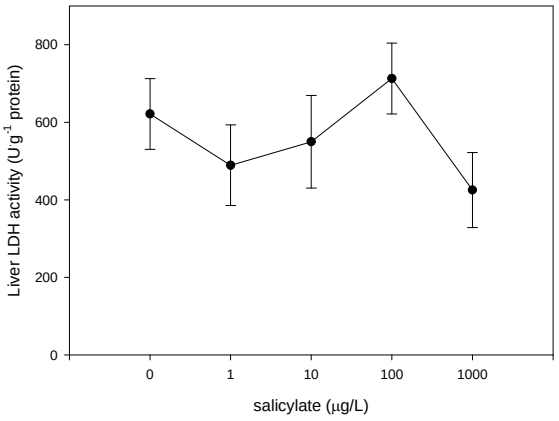
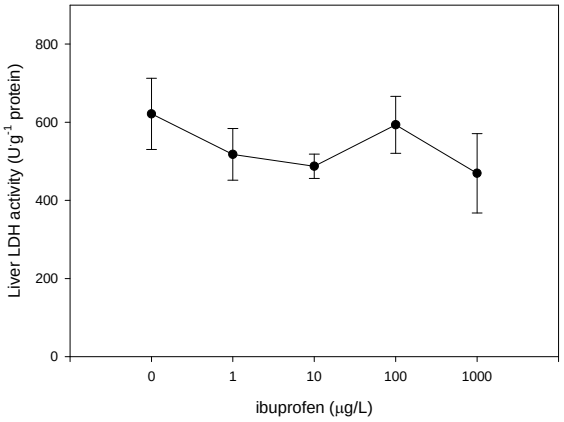

Liver LDH activity (U g-1 protein)
800
600
400
200
0
0
1
10
100
1000
salicylate (μg/L)

Fig. 7B


Liver LDH activity (U g-1 protein)
800
600
400
200
0
0
1
10
100
1000
ibuprofen (μg/L)

4. Conclusion

In conclusion, the physiological endpoints measured in this study in response to a waterborne exposure to salicylate or ibuprofen had little or no concentration-related impact. Most often the biochemical changes were detected at concentrations that do not reflect levels detected in the aquatic environment. This is consistent with acute toxicity tests showing that sublethal effects on aquatic organisms occur at elevated concentrations of these pharmaceutical drugs (Halling-Sorensen et al., 1998; Cleuvers, 2004; Caminada et al., 2006). Overall, these results suggest that NSAIDs do not impact the unstimulated hormonal and metabolic end-points routinely used as indicators of stress in fish. However, further studies are necessary to investigate whether these drugs at environmentally relevant concentrations could impact the animal's performance to secondary stressors in fish.

5. Acknowledgements

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References

Boone, A.N., Vijayan. M.M. 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. Amer. J. Physiol. Regul, Integr. Comp. Physiol. 283, R680-R687.

Caminada, D., Escher, C., and Fent, K. 2006. Cytotoxicity of pharmaceuticals found in aquatic systems: Comparison of PLHC-1 and RTG-2 fish cell lines. Aquat.Tox. 79: 114-123.

Cleuvers, M. 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotox. Environ. Saf. 59, 309-315.

Evans, D.H. 2002. Cell signaling and ion transport across the fish gill epithelium. J.Exp.Zool. 293: 336-347.

Gravel, A., Vijayan, M.M. 2006. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. Toxicol. Sci. 93, 41-49.
Gravel, A., Vijayan, M.M. 2007. Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout. Aquat.Toxicol. 81, 197-206.
Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett. 131, 5-17.
Halling-Sorensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten, Lutzhoft., and HC, Jorgensen SE. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment—a review. Chemosphere. 36(2):357-93.
Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M. 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fisher. 8: 35-56.
Keppler D. and Decker, K. 1974. Glycogen: determination with amyloglucosidase. In Methods of enzymatic Analysis (ed. H.-U. Bergmeyer), pp.1127-1131. Weinheim: Verlag Chemie.
Knoll, BASF. 1995. Pharma safety data sheet Ibuprofen. Issue/revision date 6/4/95. Knoll Pharmaceuticals, Notthingham, NG2 3AA, England.
Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
Marshall, WS., Bryson, SE.1998. Transport mechanisms of seawater teleost chloride cells—an inclusive model of a multifunctional cell. Comp. Biochem. Physiol. A—Molecular and Integrative Physiology. 119:97–106.
Mazeaud, MM., and Mazeaud, F. 1981. Adrenergic responses to stress in fish. In Stress and Fish. AD Pickering (Ed.). Academic Press, New York. pp 49-75.
McCormick, SD**.** 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can J Fish Aquat Sci 50: 656–658.
McCormick, S.D. 2001. Endocrine control of osmoregulation in teleost fish. Amer.Zool. 41: 781-794.
Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., Hirsch, R. 2003a. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxicol. Chem. 22, 2872-2880.
Metcalfe, C.D, Miao, X.S., Koenig, B.G., Struger, J. 2003b. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem. 22, 2881-2889.
Miller, W.L. 2007. Mechanism of StAR's regulation of mitochondrial cholesterol import. Mol Cell Endocrinol. 265-266, 46-50.
Mommsen, T.P., Vijayan, M.M., Moon, T.W. 1999. Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. Reviews Fish Biol. Fish. 9, 211-268.
Payne, A.H., Hales, D.B. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25, 947–970.
Papadopoulos, V. 2004. In search of the function of the peripheral-type benzodiazepine receptor. Endocr. Res. 30, 677-684.
Sathiyaa, R., Vijayan, M.M. 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Amer. J. Physiol. Cell Physiol. 284, C1508-C1515.
Stocco, D.M., Wang, X.J., Jo, Y., Manna, P.R. 2005. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol. Endocrinol. 19, 2647-2659.

Vijayan, M.M., Aluru, N., Maule, A.G., Jorgensen, E.H. 2006. Fasting augments PCB impact on liver metabolism in anadromous arctic char. Toxicol. Sci. 91, 431-439.

Vijayan, MM., Ballantyne, JS., Leatherland, JF. 1991. Cortisol-induced changes in some aspects of the intermediary metabolism in *Salvelinus fontinalis*. Gen.Comp.Endocrinol. 82: 476-486.

APPENDIX

Fig. 1A

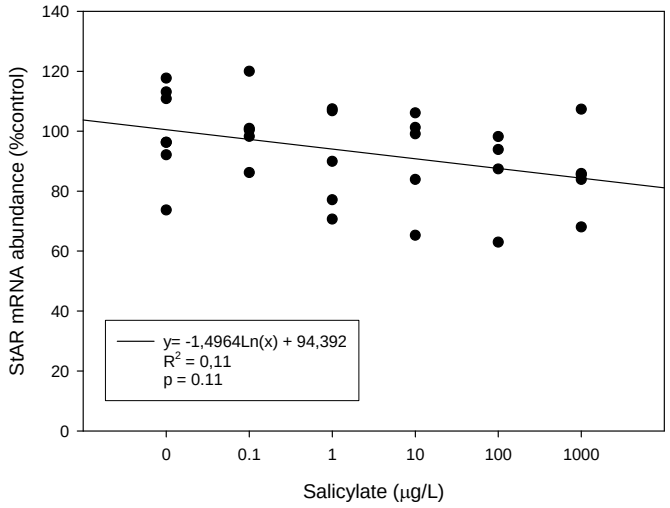


Fig. 1B

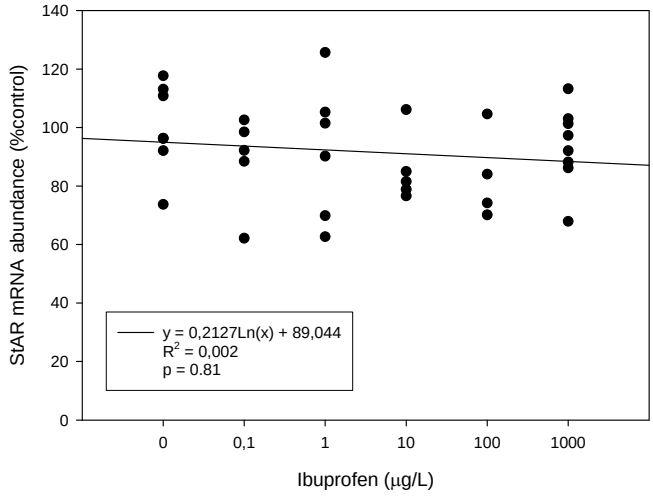


Fig. 2A

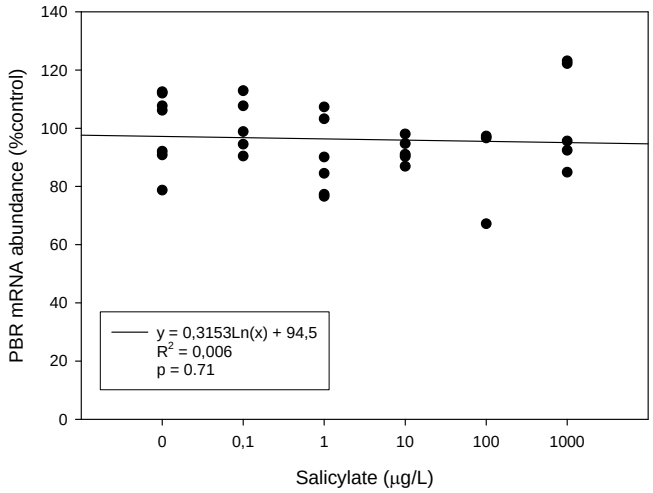

PBR mRNA abundance (%control)
140
120
100
80
60
40
20
0
y = 0,3153Ln(x) + 94,5
R2 = 0,006
p = 0.71
0
0,1
1
10
100
1000
Salicylate (μg/L)

Fig. 2B

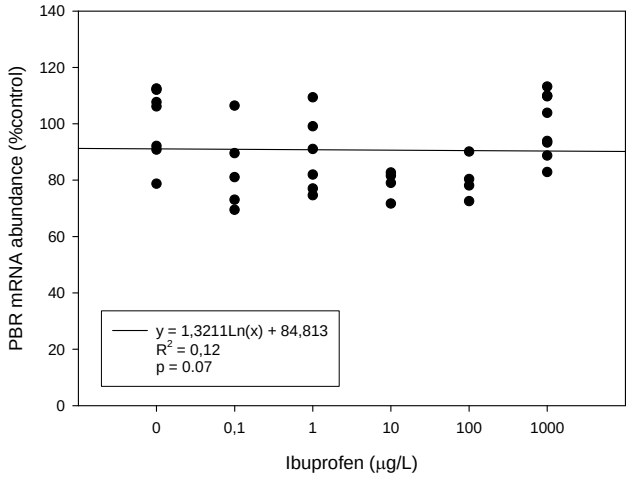

PBR mRNA abundance (%control)
140
120
100
80
60
40
20
0
y = 1,3211Ln(x) + 84,813
R2 = 0,12
p = 0.07
0
0,1
1
10
100
1000
Ibuprofen (μg/L)

Fig. 3A

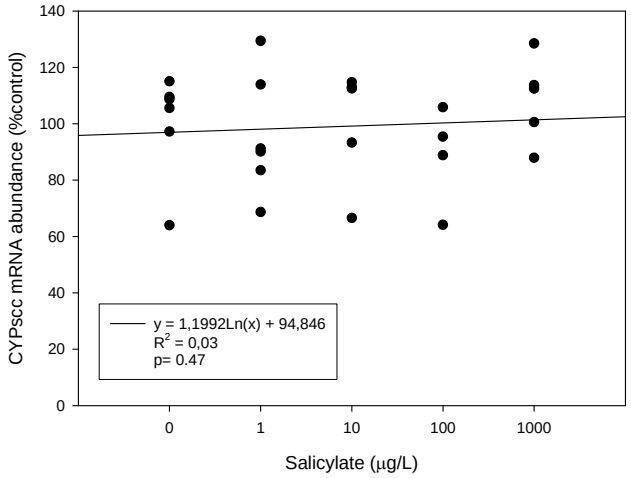

CYPscc mRNA abundance (%control)
140
120
100
80
60
40
20
0
y = 1,1992Ln(x) + 94,846
R2 = 0,03
p= 0.47
0
1
10
100
1000
Salicylate (μg/L)

Fig. 3B

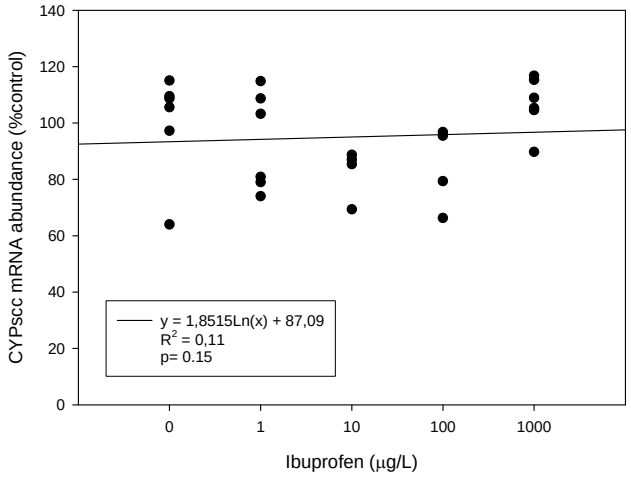

CYPscc mRNA abundance (%control)
140
120
100
80
60
40
20
0
y = 1,8515Ln(x) + 87,09
R2 = 0,11
p= 0.15
0
1
10
100
1000
Ibuprofen (μg/L)

Fig. 4A

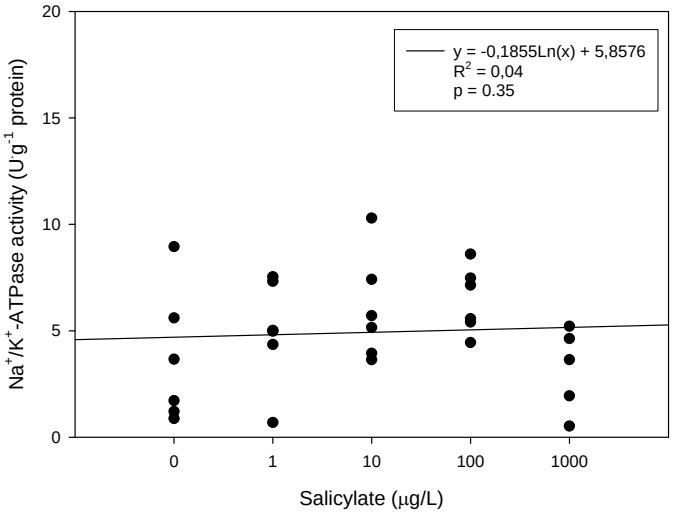

y = -0,1855Ln(x) + 5,8576
R2 = 0,04
p = 0.35
Na+/K+-ATPase activity (U·g-1 protein)
Salicylate (μg/L)
0
5
10
15
20
0
1
10
100
1000

Fig. 4B

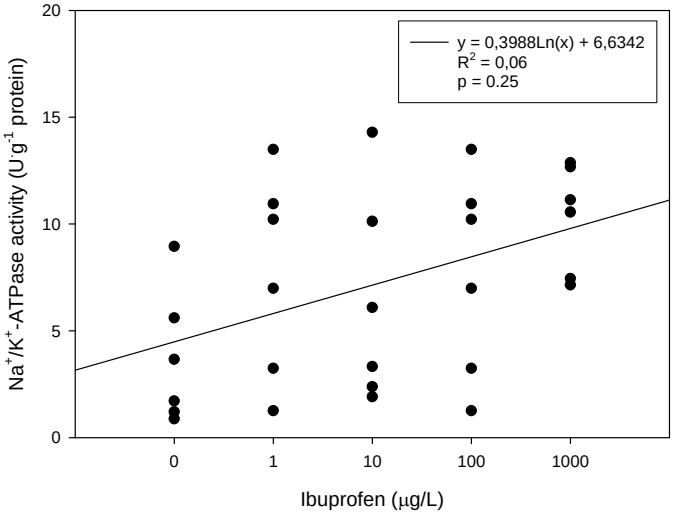

y = 0,3988Ln(x) + 6,6342
R2 = 0,06
p = 0.25
Na+/K+-ATPase activity (U·g-1 protein)
Ibuprofen (μg/L)
0
5
10
15
20
0
1
10
100
1000

Fig. 5A

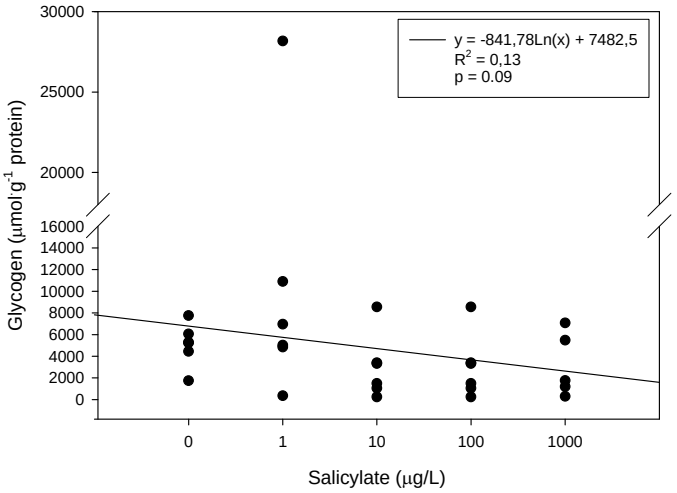

y = -841,78Ln(x) + 7482,5
R2 = 0,13
p = 0.09
Glycogen (µmol g-1 protein)
Salicylate (µg/L)
0
1
10
100
1000

Fig. 5B

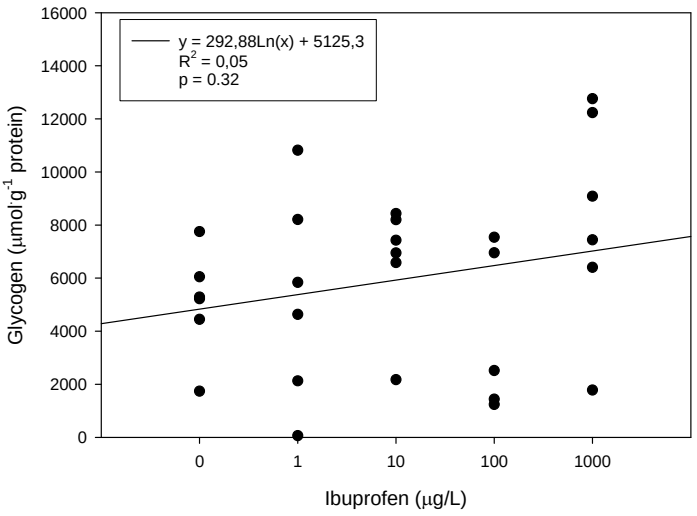

y = 292,88Ln(x) + 5125,3
R2 = 0,05
p = 0.32
Glycogen (µmol g-1 protein)
Ibuprofen (µg/L)
0
1
10
100
1000

Fig. 6A

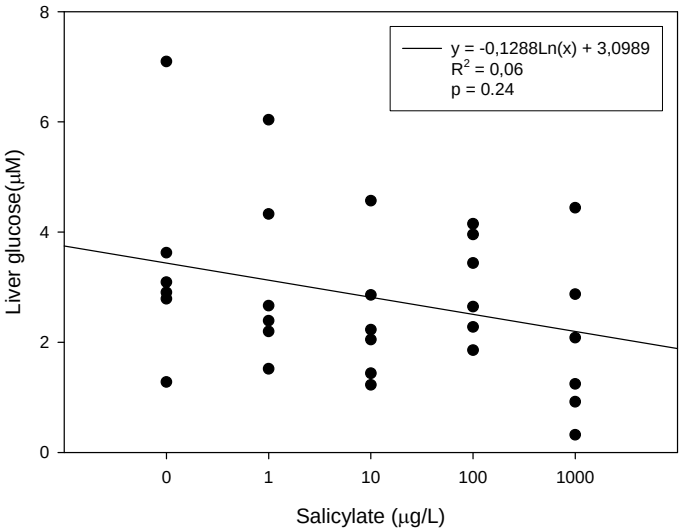

y = -0,1288Ln(x) + 3,0989
R2 = 0,06
p = 0.24
Liver glucose(μM)
Salicylate (μg/L)
0
2
4
6
8
0
1
10
100
1000

Fig. 6B

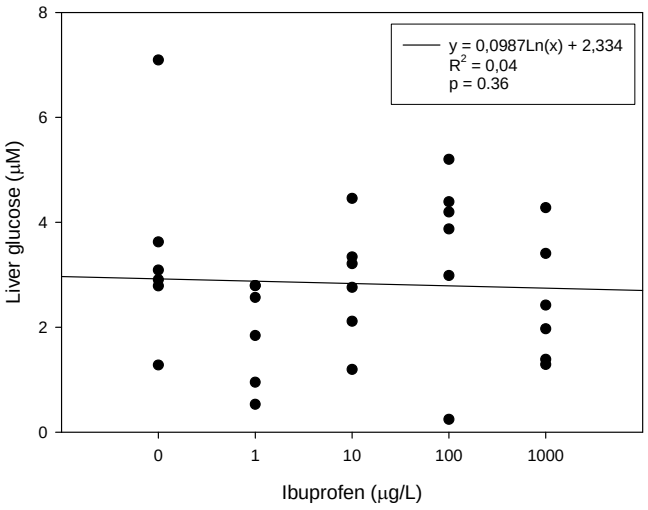

y = 0,0987Ln(x) + 2,334
R2 = 0,04
p = 0.36
Liver glucose (μM)
Ibuprofen (μg/L)
0
2
4
6
8
0
1
10
100
1000

Fig. 7A

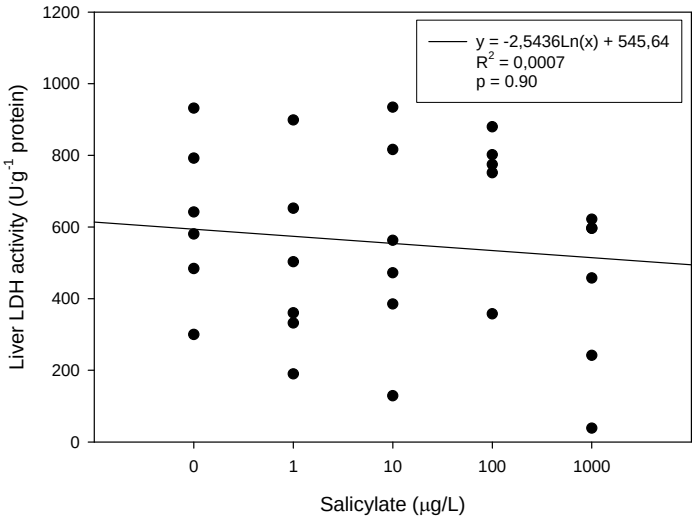

y = -2,5436Ln(x) + 545,64
R2 = 0,0007
p = 0.90
Liver LDH activity (U·g-1 protein)
1200
1000
800
600
400
200
0
0
1
10
100
1000
Salicylate (μg/L)

Fig. 7B

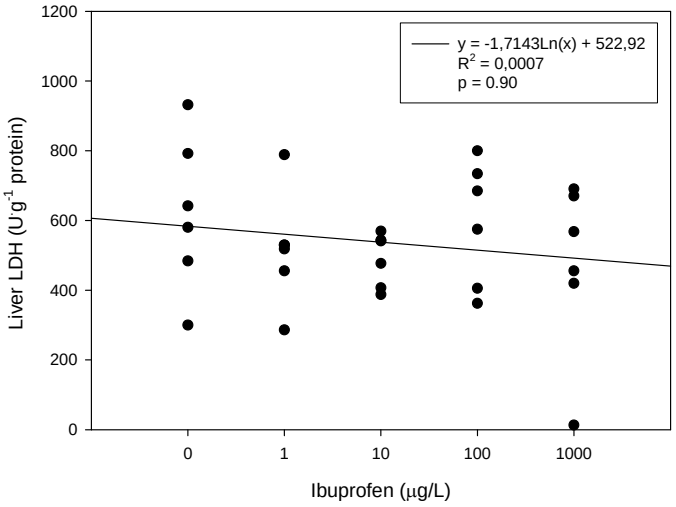

y = -1,7143Ln(x) + 522,92
R2 = 0,0007
p = 0.90
Liver LDH (U·g-1 protein)
1200
1000
800
600
400
200
0
0
1
10
100
1000
Ibuprofen (μg/L)

Figure 8. (appendix only). Regression line of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on liver hsp70 protein expression in rainbow trout fry. (n= 6 fish); values are expressed as % control (0 µg/L).

Fig. 8A

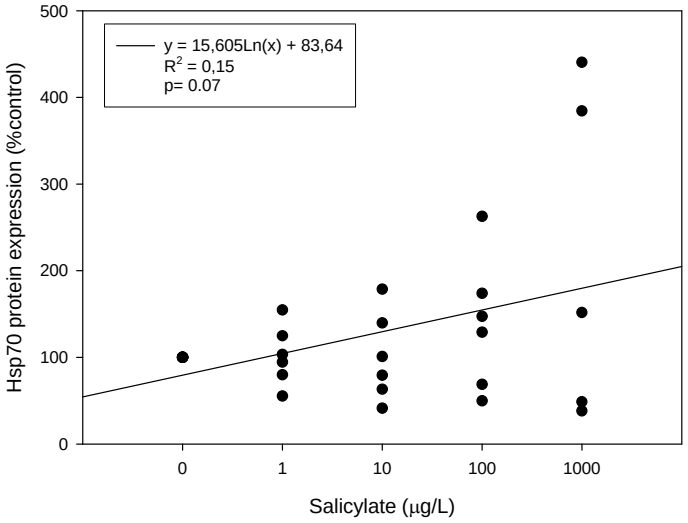
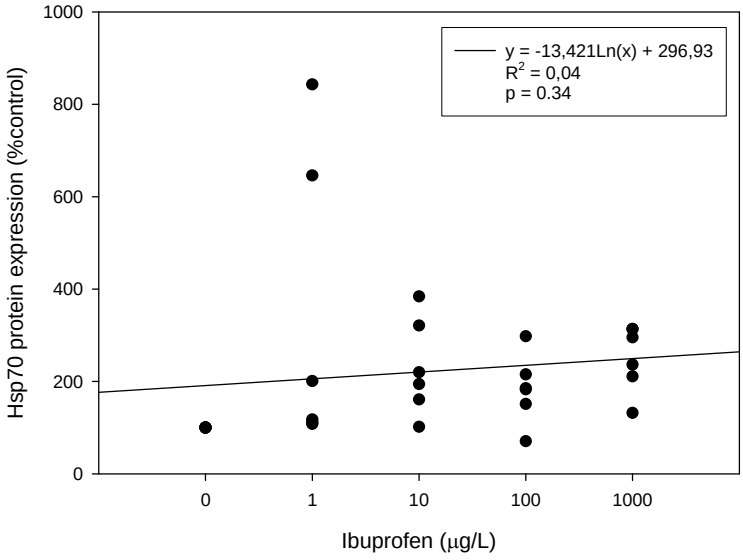


Fig. 8B



CHAPTER 5

Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDS) have been detected in the aquatic environment, but little is known about either their impact or mode of action in aquatic organisms. We tested the hypothesis that NSAIDs disrupt the evolutionarily conserved heat shock response, critical for defence against stressor-mediated proteotoxicity, in rainbow trout (*Oncorhynchus mykiss*). Trout fry were exposed by immersion to a range of salicylate or ibuprofen concentrations (1,10,100 or 1000 ug/L) for 4 d. Ibuprofen, but not salicylate, at all concentrations induced heat shock protein 70 (hsp70) in trout liver. We used the highest concentration of the drugs to investigate their mode of action on the heat shock response. Fry were subjected to a standardized heat shock, 10ºC above ambient (13^{o}C) for 1 h, and the temporal changes in liver hsp70 mRNA and protein content as well as glucose dynamics during recovery from the heat stressor assessed. Ibuprofen exposure did not modify hsp70 mRNA abundance, but significantly depressed the heat shock-induced hsp70 protein expression in the liver and gill of trout. Salicylate exposure elevated hsp70 mRNA abundance and delayed the hsp70 expression after a heat shock. Liver glucose levels and the activities of hexokinase, pyruvate kinase and lactate dehydrogenase, were elevated by NSAIDs suggesting enhanced tissue glycolytic capacity. Heat shock-mediated whole body glucose dynamics were either absent with ibuprofen or completely modified by salicylate. Overall, NSAIDs disrupt the heat shock response in rainbow trout, while the mode of action of salicylate and ibuprofen in impacting the cellular stress response appears distinct.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmacological agents that are cyclooxygenase (COX) inhibitors and commonly used for the treatment of fever, pain and inflammation (Smith, 1989; Vane, 1971; Vane and Blotting, 2003). Recent reports of the presence of pharmaceutical drugs, including NSAIDs at μg/L concentrations, in the aquatic environment has raised concerns about their impact on biota (Metcalfe et al., 2003a, 2003b; Trudeau et al., 2005). However, there is a paucity of information on either the impact or mode of action of these drugs in non-mammalian vertebrates. Recently we showed that NSAIDs are endocrine disruptors and impair corticosteroidogenesis in teleostean fishes (Gravel and Vijayan, 2005; van Anholt et al., 2003). While elevated cortisol production to stressors is a key adaptive response at the organismal level to regain homeostasis (Barton et al., 2002; Iwama et al., 2006; Mommsen et al., 1999; Wendelaar Bonga, 1997), our study did not test the impact of these pharmaceutical agents on the adaptive stress response that is crucial for coping with stressor insults.

In this connection, the heat shock response is a key evolutionarily conserved defense mechanism against stressors and involves the synthesis of a suite of proteins belonging to the heat shock protein family (Fink, 1999; Parsell and Lindquist 1993). These proteins can be broadly categorized as those belonging to the low molecular mass (<30kDa), 60 kDa, 70 kDa, 90 kDa and the high molecular mass (>100 kDa) family of hsps. The role of these proteins in protecting cells from the harmful effect of stressors, including heat shock, as well as providing cellular stress tolerance have been extensively studied (Fink, 1999; Parsell and Lindquist 1993). While a variety of stressors have been shown to induce hsps, heat shock is the most common stressor used in the characterization of the heat shock protein responses (Hightower, 1991). The

timing and duration of hsps induction vary with the animal model and the type of stressors, but the lack of induction of these proteins in response to a stressor results in cell death. Consequently, the induction of hsps is one of the fundamental responses of the cells to defend against stressor-mediated proteotoxicity (Hightower, 1991).

In teleosts, several studies examined hsp expression in response to stressors, including heat shock and contaminants, both *in vivo* as well as *in vitro* using a variety of cell systems (Iwama et al., 1998; Vijayan et al., 2005). The majority of those studies focused on stressor-impact on hsps belonging to the 70 kDa (hsp70) family in juvenile fishes (Iwama et al., 1998; Vijayan et al., 2005). The induction of hsps is energy demanding as *de novo* protein synthesis constitutes the major metabolic demand of a cell (Mommsen, 1997; Pannevis and Houlihan, 1992). Glucose is an important fuel for metabolism and certain tissues, including brain and gills in fish, rely primarily on this metabolite for energy production (Mommsen, 1984). Indeed, we showed that the glucose production capacity of hepatocytes is diminished in cells with elevated hsp70 leading to the proposal that high cost of protein synthesis in heat shocked cells compromised other energy demanding pathways, including gluconeogenesis (Boone et al., 2002). This metabolic hypothesis may also in part explain the attenuated heat shock response seen in trout hepatocytes with elevated hsp70 content (Feng et al., 2003). Together, these results imply that heat shock response is energy demanding and compromise other critical pathways that are essential for coping with stress. In spite of this, very few studies have examined the tissue energy substrate utilization associated with a heat shock response in animal models.

Against this backdrop, we tested the hypothesis that NSAIDs disrupt the heat shock response in trout fry. Specifically, we investigated whether salicylate and ibuprofen, two NSAIDs present in the aquatic environment (Metcalfe et al., 2003a, b), effect on the hsp70

response to a heat shock in rainbow trout (*Oncorhynchus mykiss*) involved a common mode of action. To this end, we examined the role of the two drugs in impacting the energy substrate repartitioning, critical for mounting the hsp 70 response to stressor insult. As early life stages of fish show a very pronounced heat shock response, and also are sensitive to contaminants relative to juvenile fishes (Cara et al., 2005; Deane and Woo, 2003; Krone et al., 2005), we used fry trout in our study. Characterization of the heat shock response involved determining temporal changes in liver hsp70 mRNA and protein content over a 24 h period after heat shocking (Cara et al., 2005). The metabolic response associated with this heat shock response was determined by measuring whole body and liver glucose levels and the activities of key enzymes involved in the glycolytic pathway (hexokinase, pyruvate kinase and lactate dehydrogenase) in the gill and liver of fry trout.

2. Material and Methods

2.1 Chemicals

Ibuprofen, sodium salicylate, protease inhibitor cocktail, bicinchoninic acid (BCA) reagent and 2-phenoxyethanol were purchased from Sigma (St Louis, MO). Costar 96-well EIA/RIA flat bottom microplates were from Corning Inc. (Corning, NY), while the electrophoresis reagents, molecular weight markers and alkaline phosphatase-conjugated goat anti-rabbit IgG were from BioRad (Hercules, CA). Polyclonal rabbit anti-trout total hsp70 antibody was a generous gift from Dr E. Peter M. Candido (Biochemistry Department, UBC). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2 Animals

Rainbow trout fry (~ 1.5 g body mass; ~ 5 cm length) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario. Fish were maintained for two weeks in aerated tank (60L capacity) with constant water flow at 13˚C with a 12 hL: 12hD photoperiod and fed to satiety twice daily (ground commercial trout chow, Martin Mills Inc., Elmira, ON).

2.3 Experimental design

2.3.1 Dose-response study

Groups of 8 fry each were transferred to a static system of nine aerated aquaria (2.5 L capacity) partially immersed in flowing water in order to keep water temperature constant at 13˚ C and maintained under identical photoperiod and feeding regimen described above. Aquaria were cleaned by siphoning left over food and feces and replacing 2/3rd water twice daily. After one week of acclimation to the static system, fry were exposed to the drugs for 4 days, consistent with the 96 h LC_{50} bioassay protocol followed for rainbow trout. Ibuprofen or salicylate was dissolved in water to reach a final concentration of 1, 10, 100 or 1000 μg/L, while the ninth aquarium with no drugs served as the control group. The lowest concentration (1 μg/L) was representative of environment levels, while a pharmacological concentration (1000 μg/L) was used to study the mechanism of action of NSAIDs. Food was withheld during the exposure period. Sampling consisted of quickly netting fish from each aquaria and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). There were no mortalities associated with any of the treatments. Liver was immediately frozen on dry ice and stored at -70˚C for hsp 70 protein detection as described below.

2.4 Heat shock response study

Based on the results from the above study, the highest concentration (1000 μg/L) of the drugs were chosen for characterizing the heat shock response in trout fry. Groups of 64 fry each were transferred to three aerated aquaria (15L capacity) and maintained in a static system as described in the dose-response study. Either ibuprofen or salicylate was dissolved in water to reach a final concentration of 1000 μg/L, while the third aquarium with no drugs served as the sham group. The fry were exposed to the drugs for 4 d and sampling consisted of netting 16 fry from each of the three treatment aquaria and anaesthetizing with an overdose of 2-phenoxyethanol (1:1000). This batch represents the prior to heat shock (PHS) group. The remaining fish in the aquaria were subjected to a standardized heat shock regimen (Cara et al., 2005). There were no mortalities associated with either drug exposure or heat shocking in the present study. One dead fry in the ibuprofen group was seen 24 h after heat shocking.

Briefly, heat shocking consisted of transferring fry to aquaria set at 23°C (+10^{o}C heat shock) for 1 h following which the fish were allowed to recover at ambient temperature (13^{o}C), in the absence of drugs, for 24 h. Sampling consisted of quickly netting 16 fry from each group at 1, 4 and 24 h after heat shocking. Gills and liver were collected from 8 fry and were immediately frozen on dry ice and stored at -70˚C for later determination of mRNA, protein and enzyme activities. The remaining 8 fry from each group were frozen whole at -20˚C for later whole body glucose measurement.

2.5 Whole body glucose analysis

Fry (without the liver) were homogenized in 3 volumes (ml) of phosphate buffer exactly as described in Gravel et al. (2005). The homogenate was centrifuged (13000 x g) three times for 10 min each and the supernatant was further diluted (1:1 with buffer) and then sonicated

(Microson, NY). An aliquot of the homogenate was used for protein quantification using BCA method with bovine serum albumin (BSA) as the standard. Another aliquot was treated with perchloric acid (2% final) to precipitate proteins before glucose analysis. Whole body glucose concentration was determined by a colorimetric assay using a commercially available kit (Trinder method, Sigma) and expressed as unit protein.

2.5.1 Liver glucose analysis

Liver glucose content was measured from samples homogenized in a lysis buffer (RLT buffer, RNeasy Mini Kit from Qiagen, Mississauga, ON) for total RNA extraction (see below). An aliquot of the homogenate was treated with perchloric acid and glucose analyzed exactly as described above. Liver glucose levels were expressed as unit total RNA content (see below).

2.6 Enzyme activities

Liver and gills were homogenized (Ultra Turrax; IKA Works, Wilmongton, NC), followed by sonication (only livers) (Microson, Farmingdale, NY) in a homogenization buffer (50% glycerol, 21mM Na_2HPO_4, 0.5mM EDTA-Na_2, 0.2% BSA, 5mM β-mercaptoethanol and protease inhibitors, pH adjusted to 7.5) exactly as described before (Vijayan et al., 2006). The enzyme activities were measured in 50mM imidazole-buffered enzyme reagent (pH 7.4) at 22oC by continuous spectrophotometry at 340nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA). The following assay conditions were shown to provide optimal activities:

Hexokinase (HK: E.C. 2.7.1.1): 1 mM glucose, 5mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 1 mM ATP, 5 U/ml lactate dehydrogenase (LDH) and 2.5 U/ml pyruvate kinase.

Pyruvate kinase (PK: E.C. 2.7.1.40): 30 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 2.5 mM PEP and 5U/ml LDH.

Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and 1mM pyruvate.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein in the homogenate.

2.7 Transcript analysis

Total RNA (DNase treated) was isolated from tissues using the RNeasy Mini Kit protocol (Quiagen, Mississauga, ON), and the RNA was quantified by spectrophotometry at 260nm. The first strand cDNA was synthesized from 1μg total RNA using First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON). Quantification of transcript levels were performed exactly as previously described (Sathiyaa and Vijayan, 2003) using Platinum® Quantitative PCR SuperMix-UDG (In Vitrogen, Carlsbad, CA). Briefly, cDNA were used as template for amplification of the housekeeping gene (β-actin) and target genes (hsp70) using appropriate primers and an annealing temperature of 49°C. The primers were designed using rainbow trout β-actin (Forward: 5'-AGAGCTACGAGCTGCCTGAC-3'; Reverse: 5'-CAAGACTCCATACCGAGGA-3') and hsp70 (Forward: 5'-GAAGGTGTCCAATGCAGTCA-3'; Reverse: 5'-GATCCTCAGCA CATTCAGC-3') cDNA sequences (GenBank accession nos. AF157514 and K02549, respectively). The primer pairs amplified a ~100 bp fragment that was used in quantitative real-time PCR (qPCR; iCycler, BioRad).

A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95°C- 3min; 40 cycles: denature 95°C- 20s, anneal-20s, and extend 72°C-20 s; then cool 4°C-hold. The iCycler IQ™ real time detection software (BioRad)

was used to determine the threshold values (C_T) for every sample. A standard curve with log input amount and C_T were established for β-actin and hsp70 and used to calculate the transcript levels in samples. The target gene transcript levels were normalized by taking a ratio of target gene to housekeeping gene. This normalized value was standardized using an internal calibrator (Sham PHS group) and transcript levels expressed as percent of internal calibrator according to established protocols (Gravel and Vijayan, 2006).

2.8 Protein quantification

Tissue homogenization, sample preparation and sample loading for hsp70 immunodetection was described in detail previously (Boone and Vijayan, 2002; Cara et al., 2005). Briefly, samples in Laemmli's buffer were loaded (60μg protein) onto 8% SDS-PAGE and proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semi-dry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris (pH 7.5), 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02% sodium azide), probed for 1 h at room temperature with primary antibody (anti-trout hsp70; 1:3000 dilution) in the blocking solution, washed with TTBS (3 times for 5 min each) and incubated for 1 h with secondary antibody (goat-anti-rabbit IgG conjugated with alkaline phosphatase; 1:3000 dilution). Our anti-trout hsp70 antibody recognizes both the constitutive and the inducible isoform of hsp70 (Boone and Vijayan, 2002). The protein bands were detected with NBT and BCIP and with this antibody only a single protein band at 70 kDa is detected as shown previously (Boone et al., 2002; Boone and Vijayan, 2002; Feng et al., 2003; Cara et al., 2005). The intensity of the scanned bands were quantified using the AlphaEase software (AlphaEase Innotech, CA) and the protein values, expressed as percent control, were shown as histograms.

2.9 Statistical analysis

The dose-related effect of NSAIDs on heat shock protein expression was compared statistically using one-way analysis of variance (ANOVA). The heat shock response was compared using two-way ANOVA, with treatment (sham, salicylate and ibuprofen) and time (PHS, 1, 4 and 24 h) as independent factors, followed by LSD (post hoc) test. In case of significant interaction between treatment and time, the data were analyzed within each time-point using one-way ANOVA followed by Tukey-Kramer's test. Data were log transformed, wherever necessary, for homogeneity of variance, while non-transformed data are shown in the figures. A significance level of $\alpha=0.05$ was considered statistically significant.

3. Results

3.1 Liver hsp70 expression

Exposure to various concentrations of salicylate did not significantly affect hsp70 expression in the present study (Fig. 1A). Ibuprofen exposure for 4 d significantly elevated hsp70 expression in all groups compared to the controls (Fig. 1B).

Heat shocking of trout fry transiently elevated liver hsp70 mRNA accumulation and the levels were significantly higher at 1 h and 4 h compared to PHS and 24 h after heat shock groups (Fig. 2A). The mRNA abundance at 1 h after heat shocking was significantly higher than all other time-points. Exposure to either salicylate or ibuprofen for 4 d did not significantly affect hsp70 mRNA abundance in the present study (Fig. 2A; PHS). However, salicylate, but not ibuprofen, exposure significantly elevated the heat shock-stimulated hsp70 transcript levels compared to the sham group (Fig. 2A *inset*).

The liver hsp70 protein expression showed a gradual increase after heat shock in the sham group and the levels were significantly higher at 4 and 24 h compared to the PHS group

(Fig. 2B). Ibuprofen exposure, but not salicylate, for 4 d significantly elevated hsp70 expression several-fold in trout fry (Fig. 2B; PHS). However, both salicylate and ibuprofen exposures impacted the temporal hsp70 response to a heat shock. The liver hsp70 content was significantly higher in the ibuprofen group compared to the sham and salicylate group at 1 h after heat shock. At 4 h after heat shock, there was no significant difference in hsp70 levels between the ibuprofen and sham group, but the salicylate group had significantly lower levels compared to the other two groups. The hsp70 response was highest at 24 h after heat shock, but the ibuprofen group had significantly lower levels compared to the sham and salicylate groups (Fig. 2B). Overall, regardless of heat shocking, ibuprofen treatment significantly elevated hsp70 protein content, whereas salicylate exposure significantly suppressed hsp70 content compared to the sham group (Fig. 2B; *inset*).

3.2 Gill hsp70 expression

The hsp70 protein expression in the gills was below detectable limit prior to heat shock. Heat shocking significantly elevated hsp70 protein expression at 24 h in the sham group compared to the PHS group (Fig. 3). Ibuprofen exposure, but not salicylate, significantly attenuated this heat shock-induced hsp70 expression in fry trout gills (Fig. 3).

17. **Figure 1.** Effect of exposure to increasing concentrations of salicylate (A) and ibuprofen (B) on liver heat shock protein 70 (hsp70) expression in rainbow trout fry.

A representative western blot is shown above each histogram. Values represent mean ± SEM (n = 6 fish); Values are expressed as % control (0 ug/L group); *significantly different from the control (one-way ANOVA; $P \leq 0.05$).

Fig. 1A

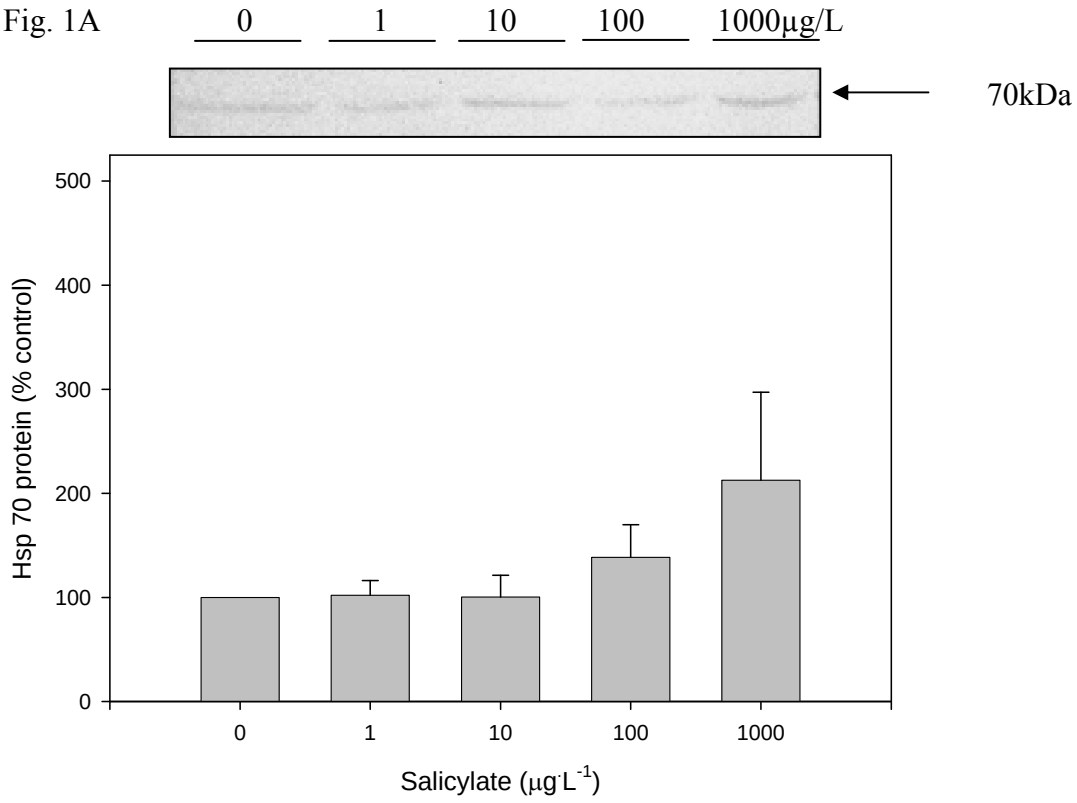
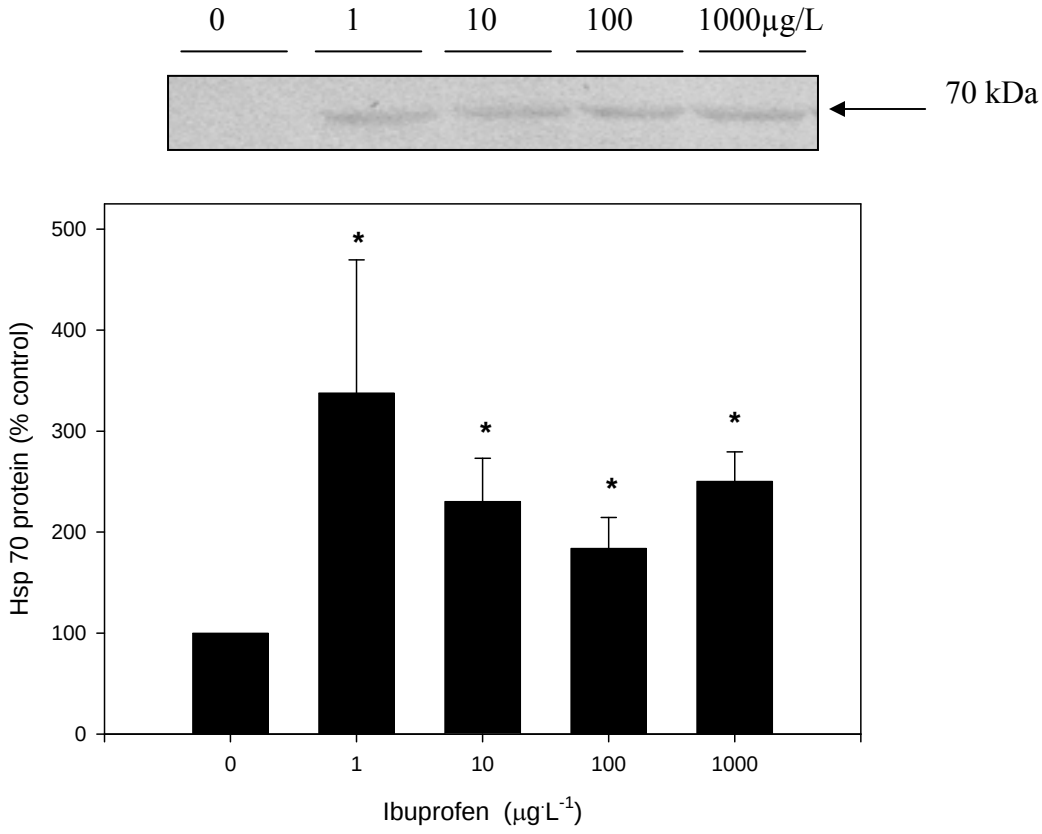

0
1
10
100
1000µg/L
70kDa
Hsp 70 protein (% control)
500
400
300
200
100
0
0
1
10
100
1000
Salicylate (µg·L-1)

Fig. 1B


0
1
10
100
1000µg/L
70 kDa
Hsp 70 protein (% control)
500
400
300
200
100
0
*
*
*
*
0
1
10
100
1000
Ibuprofen (µg·L-1)

18. **Figure 2.** Effect of salicylate and ibuprofen exposure on liver heat shock protein (hsp) 70 mRNA abundance (A) and protein expression (B) either prior to heat shock (PHS) or at 1, 4 and 24 h after a heat shock in rainbow trout fry.

Values represent mean ± SEM (n = 6 fish); Values are expressed as % control (sham group PHS); different upper case letters denotes significant time effect regardless of treatment, while *inset* shows significant treatment effect (two-way ANOVA; $P<0.05$); different lower case letters shows significant interaction between treatments for each time point (two-way ANOVA; $P<0.05$).

Fig. 2A

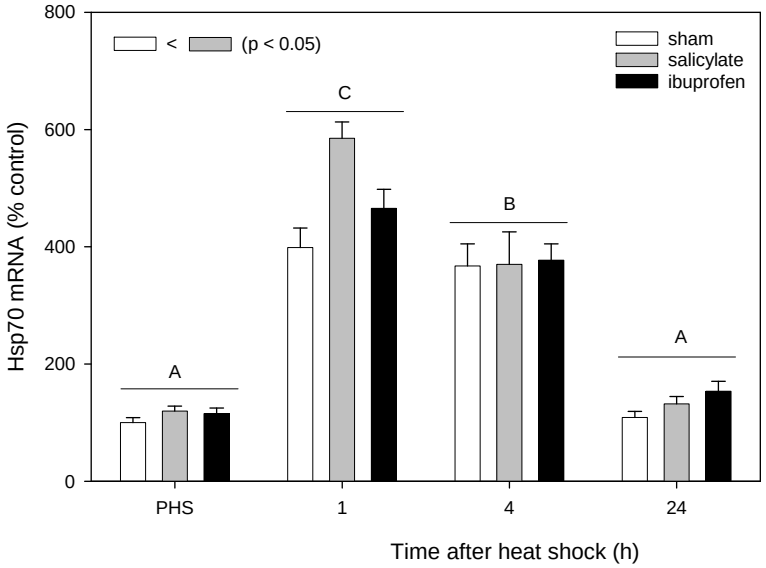
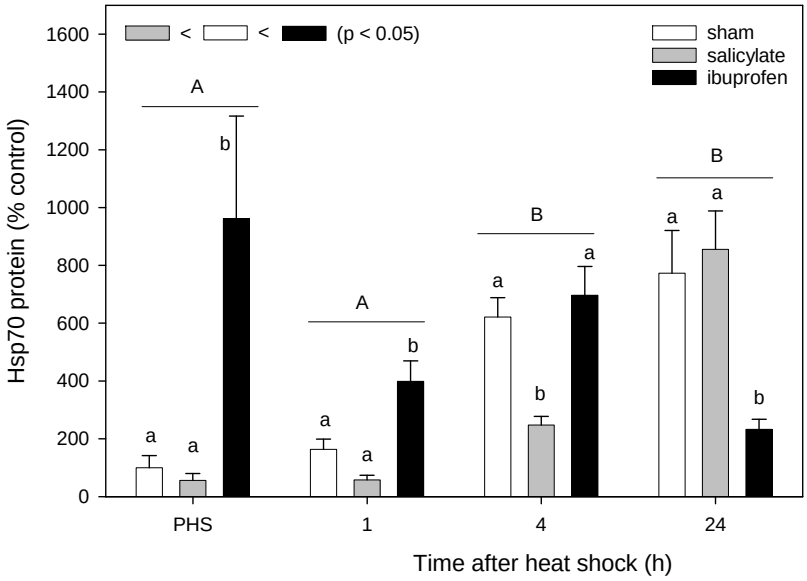

800
600
400
200
0
Hsp70 mRNA (% control)
< (p < 0.05)
sham
salicylate
ibuprofen
A
C
B
A
PHS
1
4
24
Time after heat shock (h)

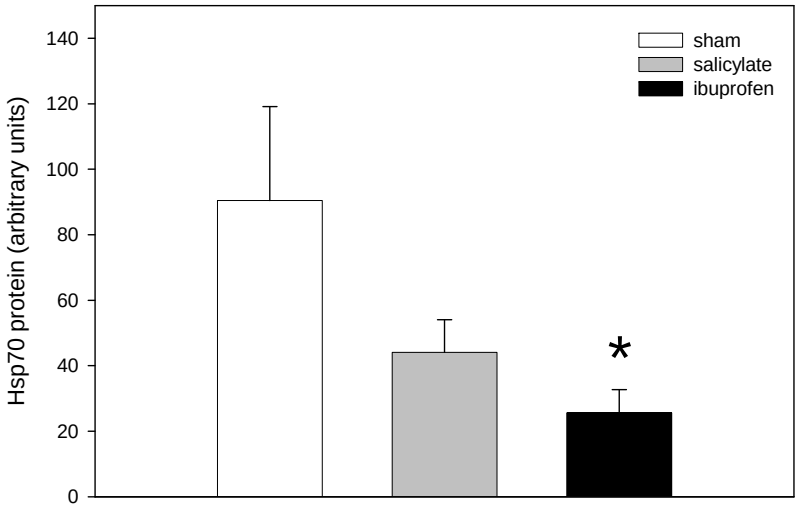
Fig. 2B


1600
1400
1200
1000
800
600
400
200
0
Hsp70 protein (% control)
< < (p < 0.05)
sham
salicylate
ibuprofen
A
B
A
B
a
a
b
a
a
b
a
b
a
a
b
PHS
1
4
24
Time after heat shock (h)

19. **Figure 3.** Effect of salicylate and ibuprofen exposure on heat shock protein (hsp) 70 expression in rainbow trout fry gills at 24 h after a heat shock.

Values represent mean ± SEM (n = 6 fish); *statistically significant from the sham group ($P<0.05$).

Fig. 3


Hsp70 protein (arbitrary units)
0
20
40
60
80
100
120
140
sham
salicylate
ibuprofen
*

3.3 Whole body glucose content

Exposure to NSAIDs for 4 d did not significantly affect glucose levels in trout fry (Fig. 4A; PHS). In response to heat shock, the sham group showed a transient reduction in whole body glucose content at 4 and 24 h after heat shock compared to the 1 h and PHS groups (Fig. 4A). This drop in body glucose content with heat shock in the sham group was greater than 70% in trout fry. NSAIDs did modify the heat-induced whole body glucose response. Exposure to ibuprofen did not show any significant change in the temporal glucose levels after a heat shock (Fig. 4A). Salicylate exposure significantly elevated whole body glucose content at 1, 4 and 24 h after heat shocking compared to the PHS group. Overall, salicylate exposure resulted in significantly higher body glucose content in response to heat shock compared to the sham and ibuprofen groups (Fig. 4A).

3.3.1 Liver glucose content

Liver glucose content was significantly higher after 4 d exposure to NSAIDs compared to the sham group. Even within NSAIDs, the ibuprofen group had significantly higher glucose levels compared to the salicylate group (Fig. 4B; PHS). Heat shock resulted in a transient elevation in liver glucose levels at 1, 4 and 24 h compared to the PHS group. Overall, ibuprofen exposure significantly elevated liver glucose content compared to the other two groups, while salicylate exposure also significantly elevated glucose levels compared to the sham group (Fig. 4B; *inset*). Significant interaction (treatment and time) was observed in liver glucose content with salicylate group having lower liver glucose levels at 4 h compared to the sham group, while at 24 h there were no significant differences in liver glucose content between the three groups (Fig. 4B).

20. **Figure 4.** Effect of salicylate and ibuprofen exposure on whole body (A) and liver (B) glucose content either prior to heat shock (PHS) or at 1, 4 and 24 h after a heat shock in rainbow trout fry.

Values represent mean ± SEM (n = 6 fish; except 4 h ibuprofen group in fig. 4B which has an n of 2); *inset* shows significant treatment effect, while different upper case letters denote significant time effect regardless of treatment (Two-way ANOVA; $P<0.05$); different lower case letters denote significant treatment differences for each time-point, while numbers represent significant differences between time-points for each treatment (significant interaction; two-way ANOVA; $P<0.05$).

Fig. 4A

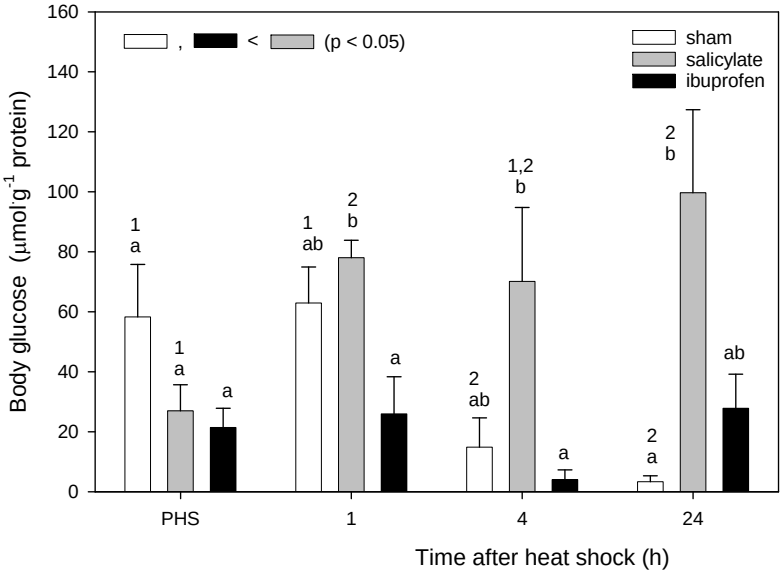
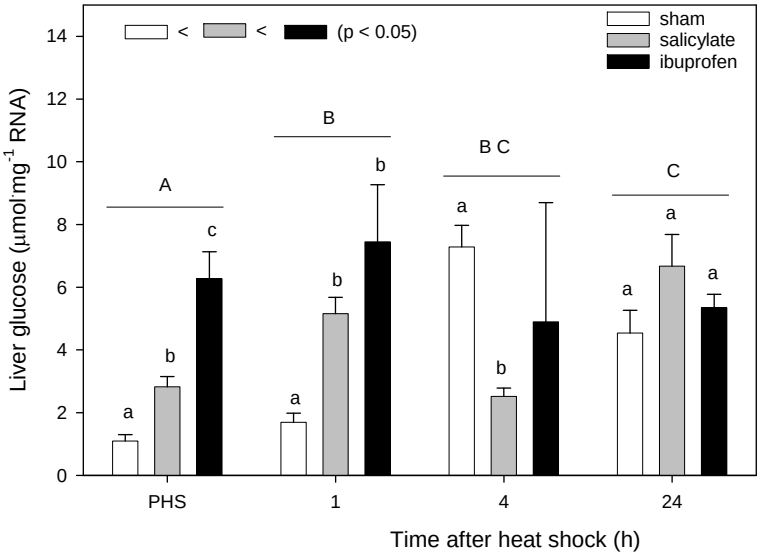

, < (p < 0.05)
sham
salicylate
ibuprofen
Body glucose (µmol·g-1 protein)
Time after heat shock (h)
PHS
1
4
24
1 a
1 a
a
1 ab
2 b
a
2 ab
1,2 b
a
2 a
2 b
ab

Fig. 4B


< < (p < 0.05)
sham
salicylate
ibuprofen
Liver glucose (µmol·mg-1 RNA)
Time after heat shock (h)
PHS
1
4
24
A
B
B C
C
a
b
c
a
b
b
a
b
a
a
b
a
a
a

3.4 Liver glycolytic capacity

Liver HK activity was significantly higher at 24 h compared to PHS, 1 or 4 h after heat shocking (Fig. 5A). Both salicylate and ibuprofen exposure significantly elevated liver HK activity compared to the sham group (Fig. 5A; *inset*). Liver PK activity showed no significant changes temporally in response to heat shock. However, salicylate exposure resulted in significantly higher PK activity compared to the sham and ibuprofen exposed fry (Fig. 5B; *inset*). Liver LDH activity was also significantly higher at 24 h compared to PHS, 1 or 4 h after heat shocking. Also, both ibuprofen and salicylate exposure significantly elevated liver LDH activity compared to the sham group (Fig. 5C; *inset*).

3.5 Gill glycolytic capacity

All three glycolytic enzymes, HK, PK and LDH activities were significantly higher in the gills at 24 h after heat shock compared to the PHS group (Figs. 6 A-C). Ibuprofen exposure for 4 d significantly elevated gill HK activity (Fig. 6; PHS), but did not affect PK or LDH activities (Fig. 6 B and C). Salicylate exposure for 4 d significantly lowered gill PK activity, but did not significantly affect HK or LDH activities (Fig. 6). Overall, salicylate exposure resulted in a significantly lower gill PK activity compared to the sham and ibuprofen groups in trout fry (Figs. 6 B; *inset*).

21. **Figure 5.** Effect of salicylate and ibuprofen exposure on hepatic hexokinase (A), pyruvate kinase (B) and lactate dehydrogenase (C) activities either prior to heat shock (PHS) or at 1, 4 and 24 h after a heat shock in rainbow trout fry.

Values represent mean ± SEM (n = 6 fish); *inset* shows significant treatment effect, while different upper case letters denote significant time effects (Two-way ANOVA; $P<0.05$); different treatment effects for each time point is shown by different lower case letters (significant interaction; two-way ANOVA; $P<0.05$).

Fig. 5A

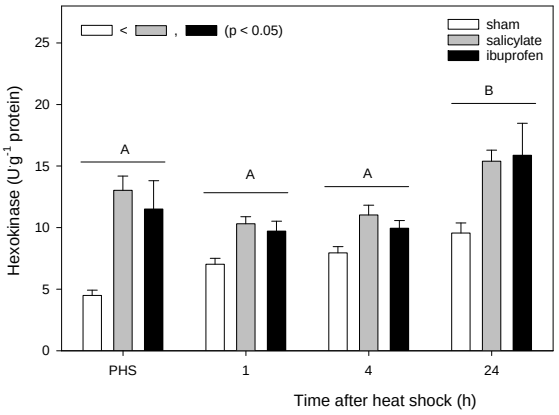


Fig. 5B

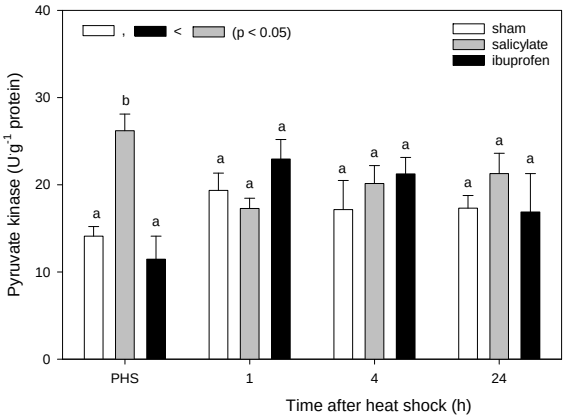
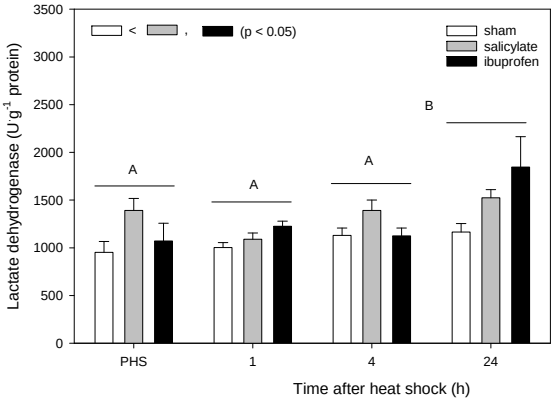


Fig. 5C



22. **Figure 6.** Effect of salicylate and ibuprofen exposure on gill hexokinase (A), pyruvate kinase (B) and lactate dehydrogenase (C) activities either prior to heat shocking (PHS) or 24 h after a heat shock (24 h HS) in rainbow trout fry.

Values represent mean ± SEM (n = 6 fish); *inset* shows significant treatment effect, while different upper case letters denote significant time effect (Two-way ANOVA; $P<0.05$); different lower case letters denote significant treatment effect for each time point (significant interaction; two-way ANOVA; $P<0.05$).

Fig. 6A

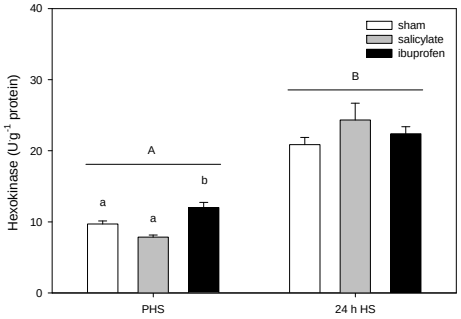


Fig. 6B

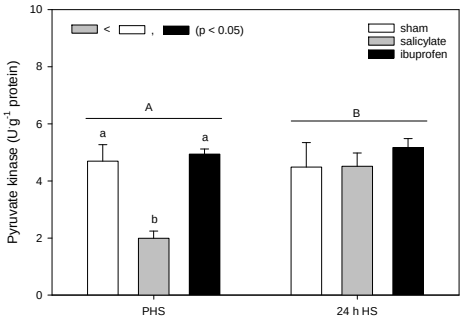
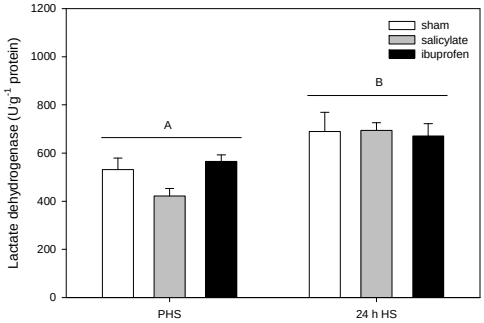


Fig. 6C



4. Discussion

We demonstrate for the first time that NSAIDs disrupt the cellular stress response in rainbow trout. The hsps synthesis to heat shock is an evolutionarily conserved phenomenon protecting cells from stressors, especially those affecting the protein machinery (Hightower, 1991; Iwama et al., 1998). The two NSAIDs used in this study, salicylate and ibuprofen, have been detected in the aquatic environment, but their impact on the heat shock response in aquatic organisms is unknown. Here we show that environmentally realistic concentration (1 μg/L) of ibuprofen, but not salicylate, is an hsp70 inducer in trout fry. However, both NSAIDs disrupt the heat shock-induced hsp70 expression. While the concentration of drugs (1000 μg/L) used to characterize the heat shock response is not environmentally relevant, it does provide valuable insight into the mechanism(s) of action of these two NSAIDs in trout. This is important given the fact that mixtures of pharmaceuticals, including other NSAIDs, are usually present in the aquatic environment (Metcalfe et al., 2003a, 2003b). Specifically, salicylate exposure elevated hsp70 transcript levels and delayed the hsp70 expression that was observed in heat shocked trout fry. On the other hand, ibuprofen inhibited the heat shock-induced hsp70 protein expression. Also, both NSAIDs perturbed the heat shock-mediated glucose dynamics in trout fry. Together, our results demonstrate that NSAIDs disrupts the heat shock response in trout fry. However, differences in hsp70 expression and the energy substrate re-partitioning associated with salicylate and ibuprofen exposures argues against a common mode of action for these pharmaceutical agents in impairing the cellular stress response in rainbow trout.

4.1 Heat shock response in trout:

The heat shock response has been well characterized in model systems and involves the synthesis of a suite of proteins belonging to the heat shock protein family (Parsell and Lindquist,

1993). However, the adaptive heat shock response in teleostean fishes is less well studied. Our results in trout fry are in agreement with other animal models showing that the hsp70 gene expression in response to heat shock is transient (Parsell and Lindquist, 1993). The return to unstimulated levels of the message within 24 h of recovery in trout from heat shock was also seen recently in the silver sea bream (Deane and Woo, 2005). The timing, duration and magnitude of hsps induction vary with the animal model and also the type and intensity of the stressors (Cara et al., 2005; Deane and Woo, 2003). However, the induction of hsps is one of the fundamental responses of the cells to defend against stressor-mediated proteotoxicity, while the lack of hsp synthesis to proteotoxic stressors is associated with cell death (Hightower, 1991).

The drop in hsp70 mRNA levels at 4 h in trout coincides with the significant elevation of hsp70 protein expression supporting the activation of the transcriptional and translational machinery in response to heat shock (Parsell and Lindquist, 1993). The timing of the hsp70 protein expression to heat shock is delayed and also the expression lasts longer during recovery in trout compared to mammalian and drosophila models (Ashburner and Booner, 1979; DiDomenico et al., 1982; Morimoto, 1991; Parsell and Lindquist, 1993). Our results are in agreement with other studies clearly showing an accumulation of hsp70 protein content over a 24 h period in trout in response to heat shock both *in vivo* and in cell systems *in vitro* (Basu et al., 2003; Boone and Vijayan, 2002; Cara et al., 2005). To this end, we showed previously that the half-life of hsp70 protein in trout hepatocytes was greater than 24 h, unlike only a few hours for mammalian models (Boone and Vijayan, 2002). The reason may be attributed to the lower ambient temperature of trout (13ºC) compared to mammals (37ºC) resulting in lower metabolic rates and the associated overall delay in protein turnover (Boone and Vijayan, 2002). Despite changes in the timing and the magnitude of hsp70 induction with heat shock, it is clear that the

hsp70 response to heat shock is conserved in animal models and supports the well established role for this protein in protecting cells against proteotoxicity (Hightower, 1991).

The synthesis of hsps is energy demanding, especially since greater than 50% of the oxygen demand in a cell is accounted for by protein synthesis (Mommsen, 1997; Pannevis and Houlihan, 1992). Our results for the first time suggest a greater tissue capacity for glucose untilization associated with the heat shock response in rainbow trout. Glucose has been shown to be a primary substrate to fuel metabolic processes in fishes, especially to cope with enhanced energy demand associated with stress (Mommsen et al., 1999). Indeed, the elevation in liver and gill hsp70 protein expression in trout fry in response to heat shock coincided with a reduction in whole body glucose content and elevation in liver glucose levels. This coupled with the enhanced gill and liver glycolytic capacity at 24 h after a heat shock leads us to hypothesize that glucose is a key energy substrate to fuel the heat shock response in fish. The increased tissue metabolic capacity seen even at 24 h after heat shock is not surprising given the fact that trout tissues synthesize hsp70 even at 24 h after recovery from heat shock (Boone and Vijayan, 2002). Considered together, the heat shock response in trout fry involves the synthesis of hsp70 protein, while the energy demand associated with this process is met at least in part by glucose utilization and enhanced tissue glycolytic capacity. Consequently, any impact on either the hsp70 synthesis and/or glucose dynamics will impair the adaptive heat shock response.

4.2 Role of NSAIDs in the heat shock response:

Our results demonstrate clearly that NSAIDs modify the heat shock response in rainbow trout. In trout, 4 d exposure to ibuprofen elevated liver hsp70 expression in a dose-independent manner, but that was not the case for salicylate. While mammalian studies did demonstrate that NSAIDs stimulate hsp70 expression (Ishihara et al., 2003; 2004; Lagunas et al., 2004;

Yamagishi et al., 2006), the role of salicylate in this heat shock response is equivocal. For instance, salicylate was shown to activate heat shock factor (HSF), but this did not result in hsp gene induction (Jurivich et al., 1992; 1995; Winegarden et al., 1996). While the reason for this is unknown, it may be related to the difference in the hyperphosphorylation of HSF by salicylate compared to heat shock (Jurivich et al., 1995). However, recent studies showed induction of hsp70 expression, but only with >20 mM concentrations of salicylate (Ishihara et al., 2003; 2004; Yamagishi et al., 2006). While the lack of hsp70 response in trout may be due to the lower salicylate exposure concentration (6.3 μM) in the present study, our result underscores the difference in the mode of action even within NSAIDs in stimulating hsp70 expression in trout.

The differential effects of salicylate and ibuprofen exposure on hsp70 expression in unstimulated fry were also evident in heat shocked fish. For instance, salicylate exposure modified the heat shock-mediated hsp70 transcript levels and also delayed the hsp70 protein expression (seen only at 24 h with salicylate as opposed to 4 and 24 h in the sham group), whereas ibuprofen group showed no further increase in hsp70 expression after heat shocking. The higher hsp70 mRNA abundance in the salicylate exposed fry, without a corresponding increase in protein expression, suggests changes in mRNA turnover. Whether this involves increased transcription and/or decreased translation remains to be determined. The observation that salicylate does not stimulate hsp70 transcription in mammalian cells (Jurivich et al., 1992; 1995; Winegarden et al., 1996) leads us to propose that this NSAID affects hsp70 mRNA stability in heat shocked rainbow trout.

Previous studies suggested a synergistic effect of NSAIDs in lowering the heat threshold for a heat shock response in a mammalian cell system (Lee et al., 1995). However, that study only examined the HSF1 activation and did not examine the hsp70 transcriptional and/or

translational responses. We did not observe any greater induction of the hsp70 response to heat shock in NSAIDs-exposed fry. In fact, while salicylate showed no change, the hsp70 expression in the gill and liver at 24 h after heat shock was reduced with ibuprofen. One possibility for the attenuated response to ibuprofen may be due to the already elevated hsp70 content in these fish and the associated tissue energy demand, which may curtail the heat shock response. This metabolic hypothesis finds support from the elevated liver glucose content, as well as enhanced tissue HK activity with ibuprofen exposure in trout fry. A similar decrease in the heat shock response as a result of elevated hsp70 content was also seen before in trout hepatocytes and included a reduction in hepatocyte glucose production (Borgs et al., 1996; Feng et al., 2003). This suggest that the impairment of the heat shock response to secondary stressors, seen with hsp inducers, including ibuprofen, may involve a combination of enhanced tissue demand for glucose coupled with a decrease in glucose production. To this end, higher glucose levels and glycolytic enzyme activities in the liver with NSAIDs supports enhanced energy demand in this tissue and may interfere with other energy demanding pathways, including hsp70 synthesis (Feng et al., 2003).

The reduction in whole body glucose content after a heat shock seen in the sham fish was either completely abolished or absent with NSAIDs, suggesting perturbations also in glucose dynamics. While the ibuprofen exposed fish showed no change in whole body glucose content after a heat shock, the levels in the salicylate exposed fish actually was elevated. The low whole body glucose content along with elevated liver glucose levels with ibuprofen imply an increased glucose utilization by this tissue and supports the increased energy demand associated with hsp70 synthesis (see above). Salicylate exposure had higher whole body glucose levels in spite of elevated liver glucose content compared to the sham group. The higher whole body glucose

content despite an enhanced capacity for glucose utilization and glycolysis in the salicylate group argues for an impaired glucose turnover in response to heat shock. Whether this elevated glucose response involves glucose production and/or glucose utilization remains to be explored. However, studies have shown that salicylate attenuate the acute plasma glucose response to stress in a teleost fish (van Anholt et al., 2003). As glycogen breakdown is the main contributor for the immediate glucose elevation after stress (Mommsen et al., 1999), it is likely that salicylate is inhibiting glycogenolysis. This is further supported by the observation that NSAIDs inhibited nitric oxide-induced glucose production and this involved inactivation of phosphorylase (Borgs et al., 1996). Together, these results would suggest that the elevated whole body glucose seen in the salicylate group is due to gluconeogenesis, but this hypothesis remains to be tested.

In conclusion, an environmentally relevant concentration of ibuprofen, but not salicylate, is an hsp70 inducer in trout fry. However, the heat shock-stimulated hsp70 expression was inhibited by high concentration of this drug in the gill and liver of trout. Salicylate exposure modified hsp70 mRNA and protein response to a heat shock differently from that of ibuprofen. The metabolic response, critical for hsp70 synthesis, was also perturbed by NSAIDs. Specifically, both NSAIDs (ibuprofen greater than salicylate) increased liver glycolytic capacity and this coincided with elevated liver glucose content. However, the whole body glucose dynamics in response to heat shock was different between the two drugs. While ibuprofen did not result in the heat-shock induced transient glucose reduction, salicylate exposure significantly elevated whole body glucose content after a heat shock. Considered together, salicylate and ibuprofen, two NSAIDs routinely detected in the aquatic environment, disrupt the evolutionarily conserved adaptive heat shock response in trout fry. However, the differences in the hsp70 synthetic machinery, as well as the energy substrate repartitioning, critical for mounting the heat

shock response, between the two NSAIDs argues against a common mechanism of action for these pharmaceutical agents in rainbow trout.

5. Acknowledgements

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References

Ashburner, M., Bonner, J.J. 1979. The induction of gene activity in Drosophila by heat shock. Cell 17: 241-254.

Barton, B.A., Morgan, J.D., Vijayan, M.M. 2002. Physiological and condition-related indicators of environmental stress in fish. In *Biological Indicators of Stress in Fish, Second Edition*, ed. S.M. Adams, pp 111-148, Bethesda, Maryland: American Fisheries Society.

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

Boone, A.N., Ducouret, B., Vijayan, M.M. 2002. Glucocorticoid-induced glucose release is abolished in trout hepatocytes with elevated hsp70 content. J. Endocrinol. 172: R1-R5.

Boone, A.N., Vijayan. M,M. 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. Amer. J. Physiol. Regul, Integr. Comp. Physiol. 283: R680-R687.

Borgs, M., Bollen, M., Keppen,s S., Yap, S.H., Stalmans, W., Vanstapel, F. 1996. Modulation of basal hepatic glycogenolysis by nitric oxide. Hepatol. 23: 1564-1571.

Cara, J.B., Aluru, N., Moyano, F.J., Vijayan, M.M. 2005. Food-deprivation induces HSP70 and HSP90 protein expression in fry gilthead sea bream and rainbow trout. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 142: 426-431.

Deane, E.E., Woo, N.Y. 2003. Ontogeny of thyroid hormones, cortisol, hsp70 and hsp90 during silver sea bream fry development. Life Sci. 72: 805-818.

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

DiDomenico, B.J., Bugaisky, G.E., Lindquist, S. 1982. The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell 31: 593-603.

Feng, Q., Boone, A.N., Vijayan, M.M. 2003. Copper impact on heat shock protein 70 expression and apoptosis in rainbow trout hepatocytes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135C: 345-355.

Fink, A.L. 1999. Chaperone-mediated protein folding. Physiol. Rev. 79: 425-449.

Gravel, A., Vijayan, M.M. 2006. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. Toxicol. Sci. 93: 41-49.

Gravel, A., Campbell, P.G.C., Hontela. A. 2005. Disruption of the hypothalamo-pituitary-interrenal axis in 1+ yellow perch (*Perca flavescens*) chronically exposed to metals in the environment. Can. J. Fish. Aquat. Sci. 62: 982-990.

Hightower, L.E. 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66: 191-197.

Ishihara, K., Horiguchi, K., Yamagishi, N., Hatayama, T. 2003. Identification of sodium salicylate as an hsp inducer using a simple screening system for stress response modulators in mammalian cells. Eur. J. Biochem. 270: 3461-3468.

Ishihara, K., Yamagishi, N., Hatayama, T. 2004. Suppression of heat- and polyglutamine-induced cytotoxicity by nonsteroidal anti-inflammatory drugs. Eur. J.Biochem. 271: 4552-4558.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M. 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fisher. 8: 35-56.

Iwama, G.K., Alfonso L.O.B., Vijayan, M.M. 2006. Stress in Fish. In *The Physiology of Fishes, Third Edition*, eds. D.H. Evans and J.B. Claiborne, pp 319-342, CRC Press, Boca Raton, Florida.

Jurivich, D.A., Pachetti, C., Qiu, L., Welk, J.F. 1995. Salicylate triggers heat shock factor differently than heat. J. Biol .Chem. 270: 24489-24495.

Jurivich, D.A., Sistonen, L., Kroes, R.A., Morimoto, R.I. 1992. Effect of sodium salicylate on the human heat shock response. Science 255: 1243-1245.

Krone, P.H., Blechinger, S.R., Evans, T.G., Ryan, J.A., Noonan, E.J., Hightower, L.E. 2005. Use of fish liver PLHC-1 cells and zebrafish embryos in cytotoxicity assays. Methods 35: 176-187.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lagunas, L., Bradbury, M.C., Laszlo, A., Hunt, C.R., Gius, D. 2004. Indomethacin and ibuprofen induce Hsc70 nuclear localization and activation of the heat shock response in HeLa cells. Biochem .Biophys. Res .Commun. 313: 863-870.

Lee, B.S., Chen, J., Angelidis, C., Jurivich, D.A., Morimoto, R.I. 1995. Pharmacological modulation of heat shock factor 1 by antiinflammatory drugs results in protection against stress-induced cellular damage. Proc. Natl. Acad. Sci .U.S.A. 92: 7207-7211.

Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servo,s M., Ternes, T.A., Hirsch. R. 2003a. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxico .Chem. 22: 2872-2880.

Metcalfe, C.D., Miao, X.S., Koenig, B.G., Struger, J. 2003b. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol. Chem. 22: 2881-2889.

Mommsen, T.P. 1984. Metabolism of the fish gill. In *Fish Physiology*, eds. W.S. Hoar and D.J. Randall, pp 203-238, Academic, New York.

Mommsen, T.P. 1997. Growth and metabolism. In *The Physiology of Fishes, Second Edition*, ed. D.H. Evans, pp 65-97, CRC Press, Boca Raton, Florida.

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

Morimoto, R.I. 1991. Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. Cancer Cells 3: 295-301.

Pannevis, M.C., Houlihan, D.F. 1992. The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. [B] 162: 393-400.

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

Sathiyaa, R., Vijayan, M.M. 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Amer. J. Physiol. Cell Physiol. 284: C1508-C1515.

Smith, W.L. 1989. The eicosanoids and their biochemical mechanisms of action. Biochem. J. 259: 315-324.

Trudeau, V.L., Metcalfe, C.D., Mimeault, C., Moon, T.W. 2005. Pharmaceuticals in the environment: Drugged fish? In *Biochemistry and Molecular Biology of Fishes,* Vol. 6, eds. T.P. Mommsen and T.W. Moon, pp 87-101, Elsevier, Amsterdam.

van Anholt, R.D., Spanings, F.A., Koven, W.M., Wendelaar Bonga, S.E. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). Amer.J. Physiol. Regul. Integr. Comp. Physiol. 285**:** R1098-R1106.

Vane, J.R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat. New Biol. 23: 232-235.

Vane, J.R., Blotting, R.M. 2003. The mechanism of action of aspirin. Thromb. Res. 110: 255-258.

Vijayan, M.M., Aluru, N., Maule, A.G., Jorgensen, E.H. 2006. Fasting augments PCB impact on liver metabolism in anadromous arctic char. Toxicol. Sci. 91: 431-439.

Vijayan, M. M., Prunet, P., Boone, A. N. 2005. Xenobiotic impact on corticosteroid signaling. In *Biochemistry and molecular biology of fishes,* vol. 6, eds. T. P. Mommsen, and T. W. Moon, pp. 365–394, Elsevier, Amsterdam.

Wang, Q., Mosser, D.D., and Bag. J. 2005. Induction of HSP70 expression and recruitment of HSC70 and HSP70 in the nucleus reduce aggregation of a polyalanine expansion mutant of PABPN1 in HeLa cells. Hum. Mol. Genet. 14: 3673-3684.

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

Winegarden N.A., Wong, K.S., Sopta, M., Westwood, J.T. 1996. Sodium salicylate decreases intracellular ATP, induces both heat shock factor binding and chromosomal puffing, but does not induce hsp 70 gene transcription in *Drosophila*. J .Biol. Chem. 271: 26971-26980.

Yamagishi, N., Tokunaga, S., Ishihara, K., Saito, Y., Hatayama, T. 2006. The phenylic hydroxyl group is essential for the induction of stress response by sodium salicylate. Biochem. Biophys. Res. Commun. 350: 131-137.

CHAPTER 6

Non-steroidal anti-inflammatory drugs affect seawater acclimation in rainbow trout

This chapter will be submitted to American Journal of Physiology, Gravel, A., Wilson, JM., Pedro, D., Vijayan, MM. Non-steroidal anti-inflammatory drugs affect seawater acclimation in rainbow trout.

Abstract

Recently we demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) disrupt cortisol production in rainbow trout. As cortisol is important for seawater (SW) adaptation, we hypothesized that NSAIDs will disrupt the adaptive responses critical for SW acclimation in fish. To this end, rainbow trout (*Oncorhynchus mykiss*) were exposed to waterborne salicylate or ibuprofen for three days and then exposed sequentially to 50% SW for two days followed by 100% SW for an additional two days. Elevated plasma osmolality and concentrations of Na^+, Cl^- and K^+ with NSAIDs compared to the sham group in SW suggests disturbances in ion regulation. Gill Na^+/K^+-ATPase activity but not protein content was elevated in 100%SW, while this response was inhibited in the presence of ibuprofen but not salicylate. NSAIDs also enhanced gill glycolytic capacity and reduced liver glycogen content in SW. Exposure to 100% SW significantly elevated plasma cortisol levels in trout and this response was further enhanced in the presence of the drugs. Salicylate exposure resulted in a greater plasma cortisol response compared to the other groups at both 50% and 100%SW. This cortisol response did not accompany changes in the transcript levels of key proteins involved in steroidogenesis, while the reduced brain GR content in the salicylate group suggests disturbed cortisol dynamics. Taken together, NSAIDs impaired the adaptive responses to seawater acclimation in rainbow trout. Specifically, salicylate and ibuprofen disturbed ion regulation and disrupted the endocrine and metabolic responses critical for salinity adaptation in fish, while the mode of action of these drugs appears to be distinct.

1. Introduction

As teleost fishes move from fresh water to salt water, excess ions are secreted across the gills to maintain plasma osmotic and ionic homeostasis. Although several proteins in the gills are involved in this salt excretion process, the key players include the Na^+/K^+-ATPase and the Na^+/K^+-$2Cl^-$ cotransporter (NKCC) in fish (Evans et al., 2005). Indeed, the activity and/or expression of these transport proteins in the gills are elevated upon exposure of fishes, including rainbow trout, to seawater (Evans et al., 2005). Recent studies also suggest that there are isoform-specific changes in Na^+/K^+-ATPase transcript levels during seawater acclimation, but little is known about the physiological consequence of this isoform switching in teleosts (Richards et al., 2003).

Several hormones have been implicated in the seawater acclimation process in fish, including cortisol, growth hormone, insulin-like growth factors (IGFs) and prolactins (McCormick, 1995; Shepherd et al., 2005). However, the role of cortisol in adapting fish to seawater has received the most attention. Indeed plasma cortisol levels are transiently elevated upon transfer of fish from fresh water to higher salinities (Wendelaar Bonga, 1997; McCormick, 1995). This increase may involve either enhanced cortisol production capacity and/or altered clearance of this steroid hormone in seawater (Wendelaar Bonga, 1997; Mommsen et al., 1999; Shepherd et al., 2005). However, while most studies examined plasma cortisol response to salinity or seawater exposure, few studies have actually focused on the dynamics (production, regulation and clearance) of this steroid hormone in circulation. The role of this hormone in regulating plasma ion concentrations in seawater is well established in teleosts. For instance, cortisol promotes the development and proliferation of gill chloride cells (McCormick, 1990), stimulates Na^+/K^+-ATPase activity and enhances mRNA abundance of the Na^+/K^+-ATPase α-

subunit (McCormick, 1995; Madsen et al., 1995). Also, cortisol has a modulatory role in NKCC abundance (Pelis and McCormick, 2001), as well as CFTR expression (Singer et al., 1998). In addition to the role of this steroid in modulating ion transporters, cortisol also plays a key role in energy substrate mobilization, which is critical for coping with the increased energy demand associated with seawater adaptation (Vijayan et al., 1996; Morgan and Iwama, 1998, 1999; Mommsen et al., 1999; Laiz-Carrión et al., 2002).

The action of cortisol is mediated by the glucocorticoid receptor (GR), a ligand-bound transcription factor, and recently studies have identified multiple isoforms of this steroid receptor in fish (Prunet et al., 2006; Vijayan et al., 2005). Also, the discovery of a mineralocorticoid receptor (MR) in fish suggests a role for this receptor in cortisol signaling, however a MR-specific ligand, as in mammals, that is functionally relevant has yet to be identified in fish (Prunet et al., 2006; Vijayan et al., 2005). The changes in ion transporters with cortisol treatment in seawater and also the decreased seawater adaptability with GR antagonist (RU486) clearly implicate a role of cortisol signaling in the salinity acclimation process (McCormick, 1995; Shaw et al., 2007). Indeed, GR transcript abundance, protein expression and receptor capacity are modulated in response to salinity exposure in teleosts (McCormick, 2001; Shrimpton et al., 1995; Dean et al., 2003; Singer et al., 2007). Also, the number of gill GR receptors is strongly correlated with the capacity of cortisol to stimulate gill Na^+/K^+-ATPase activity *in vitro* and *in vivo* (McCormick et al., 1991a; Shrimton et al., 1994; Shrimpton and McCormick, 1999). Consequently, any impact on either plasma cortisol dynamics and/or target tissue responsiveness to cortisol stimulation will impact seawater adaptability in teleost fishes. To this end, we recently demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) disrupt acute adrenocorticotropic hormone (ACTH)-mediated cortisol production by targeting key proteins,

the steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor (PBR), in the steroid biosynthetic pathway in trout (Gravel and Vijayan, 2006). Also, NSAIDs attenuated the stressor-induced plasma cortisol response in fish (van Anholt et al., 2003; Gravel and Vijayan, in review) as well as reducing the target tissue GR protein (Gravel and Vijayan, 2006) and modifying the stressor-mediated metabolic responses in trout (Gravel and Vijayan, 2007).

As NSAIDs are present in μg/L concentrations in sewage effluents and surface water (Heberer, 2002; Metcalfe et al., 2003a, b), and little is know about the physiological consequences to animals, we tested the hypothesis that endocrine disruption of the cortisol stress axis by NSAIDs will lead to impaired seawater acclimation in rainbow trout. The fish were exposed to the drugs by immersion and the salinity acclimation regimen followed included exposing trout to 50% seawater for 2 days after which the salinity was increased to 100% seawater. Fish were sampled after 2 days each in 50% and 100% seawater. We examined: i) the functioning of the hypothalamus-pituitary-interrenal (HPI) axis by measuring plasma cortisol concentration, target tissue GR content and the transcript levels of key proteins involved in corticosteroidogenesis in the interrenal tissue, ii) indicators of ion regulation, including measuring plasma ion levels, osmolality, gill Na/K-ATPase activity and protein content, as well as the distribution of Na/K-ATPase and Na/K/2Cl-cotransporter immunoreactivity in gill ionocytes, and iii) tissue metabolic capacity by measuring liver glycogen and glucose content as well as the activities of select gluconeogenic (liver) and glycolytic enzymes (gill and liver) in rainbow trout.

2. Material and Methods

2.1 Material

Ibuprofen (sodium salt), sodium salicylate, protease inhibitor cocktail, bicinchoninic acid (BCA) reagent, and 2-phenoxyethanol were purchased from Sigma (St Louis, MO). Heparinized micro-hematocrit capillary tubes were from VWR (Mississauga, ON). Costar 96-well EIA/RIA flat bottom microplates were from Corning Inc. (Corning, NY), while the electrophoresis reagents, molecular weight markers and alkaline phosphatase-conjugated goat anti-rabbit IgG were from BioRad (Hercules, CA). Polyclonal rabbit anti-trout GR antibody was custom made in our laboratory (Sathiyaa and Vijayan, 2003). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (StressGen, Victoria, BC), while nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were purchased from Fisher Scientific (Ottawa, Ontario).

2.2 Fish

Juvenile rainbow trout (~ 170 g body mass) were obtained from Rainbow Springs Hatchery, Thamesford, Ontario. Groups of 9 fish were maintained in nine separate aerated tanks (40L capacity; 3 treatments x 3 sampling times) with constant water flow at 13˚C with a 12 hL: 12hD photoperiod and fed to satiety twice daily (ground commercial trout chow, Martin Mills Inc., Elmira, ON). The fish were maintained at the Aqualab, University of Guelph, and the protocol was approved by the animal care committee. The fish were acclimated for two weeks prior to the commencement of the experiment.

2.3 Experimental design

The concentrations of the drugs chosen for the study (1000 ug/L) were based on the effects seen with our earlier concentration-response studies in fresh water (Gravel and Vijayan,

2006, 2007). At the start of the exposure, the flow-through tanks were converted to static systems. Food was withheld during the exposure period. For each salinity group, either ibuprofen or salicylate was dissolved in water to reach a final concentration of 1000 μg/L, while a third tank with no drugs served as the sham group. One half of the water in all tanks was replenished on day 2. One set of fish (three tanks) was sampled on day 4 after exposure to drugs and this batch represents the fresh water (FW) group. In the remaining tanks artificial seawater was added to reach (within an hour) a salinity of 50% seawater (an hour after addition of seawater) and a second set of three tanks were sampled 2 days later (50%SW). Finally, the last three aquaria were filled with 100% seawater and sampled 2 days later (100%SW). The concentration was chosen based on our preliminary study (0, 1, 10, 100 and 1000 ug/L of either salicylate or ibuprofen), which showed a significant drug effect on gill Na^+/K^+-ATPase activity only at the high concentration (data not shown).

The sampling consisted of netting all the fish from each aquarium and anaesthetizing them quickly with an overdose of 2-phenoxyethanol (1:1000). Fish were bled by caudal puncture, hematocrit determined, and the plasma was collected after centrifugation (10 min at 6000 x g) and kept frozen at -70˚C for analyses later. Pieces of gill, liver, brain and head kidney (interrenal tissues) were collected and immediately frozen on dry ice and stored at -70˚C for later enzyme activity, mRNA and protein determination later. The second gill arch from each fish was immediately fixed in 4% paraformaldehyde / phosphate buffered saline (PBS, pH 7.4) at 4°C for 24 h for histology and immunostaining.

There were no mortalities in the FW groups, while there was one mortality in the 50% SW group (ibuprofen only) and three in the 100% SW groups (one with salicylate and 2 with ibuprofen) after two days of exposure.

2.4 Blood and plasma analyses

Plasma cortisol concentration was measured using a commercially available ImmuChem™ ^{125}I RIA kit (MP Biomedicals, CA), while plasma glucose (modified Trinder method; Rainchem, San Diego, CA) and lactate (Trinity Biotech, St-Louis, MO) levels were determined by a colorimetric assay using a commercially available kit. Plasma sodium and potassium ion levels were measured using a flame photometer while plasma chloride ions were measured using a chloridometer.

2.5 Gill Na^+K^+-ATPase activity

Gill was homogenized in SEI buffer (0.3M sucrose, 0.02M EDTA, 0.1M imidazole buffer pH 7.3) containing 0.1% sodium deoxycholate and briefly centrifuged. Na^+K^+-ATPase activity was determined from fresh homogenate by measuring ouabain-sensitive ATPase activity (NADH-mediated ATP hydrolysis) in a microplate exactly as described by McCormick (1993). The protein concentration in the homogenate was determined by BCA method using bovine serum albumin as the standard, and the enzyme activity was expressed as U/g protein.

2.6 Liver glycogen and glucose levels and metabolic enzyme activity

Liver and gill were homogenized (Ultra Turrax; IKA Works, Wilmongton, NC), followed by sonication (Microson, Farmingdale, NY) in a homogenization buffer (50% glycerol, 21mM Na_2HPO_4, 0.5mM EDTA-Na_2, 0.2% BSA, 5mM β-mercaptoethanol and protease inhibitors, pH adjusted to 7.5) exactly as described before (Vijayan et al., 2006). For determination of liver glucose and glycogen, an aliquot of the liver homogenate was treated with perchloric acid and liver glycogen content analyzed by measuring glucose content before and after amyloglucosidase hydrolysis (Vijayan et al., 2006). The glycogen content is shown as micromoles glucosyl units per gram protein in the homogenate. The enzyme activities were measured in 50mM imidazole-

buffered enzyme reagents (pH 7.4) at 22°C by continuous spectrophotometry at 340nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA). The following assay conditions were shown to provide optimal activities:

Hexokinase (HK: E.C. 2.7.1.1): 1 mM glucose, 5mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 1 mM ATP, 5 U/ml lactate dehydrogenase (LDH) and 2.5 U/ml pyruvate kinase.

Glucokinase (GK: E.C. 2.7.1.2): 15 mM glucose, 5mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 1 mM ATP, 5 U/ml lactate dehydrogenase (LDH) and 2.5 U/ml pyruvate kinase.

Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and 25mM pyruvic acid.

Phosphoenolpyruvate carboxykinase (PEPCK: E.C. 4.1.1.32): 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 0.5 mM phosphoenolpyruvate, 5 mM deoxyguanosine diphosphate, and 0.12 mM NADH.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein in the homogenate.

2.7 Immunocytochemistry

Gill tissue was fixed in 4% paraformaldehyde/PBS solution at pH 7.4 for 24h at 4˚C. The tissue was then decalcified (24h in 35% Formic Acid, 13% Trisodium Citrate, pH 2.3) and processed for paraffin embedding. Sections (5 μm) were collected onto APS coated slides, air dried overnight and dewaxed and rehydrated. Sections were then blocked with 5% normal goat serum in 0.05% tween-20/ 1% bovine serum albumin/ PBS (pH 7.3) and incubated with primary antibodies (a combination of an affinity purified rabbit anti-peptide polyclonal specific for Na^+/K^+-ATPase α1 subunit (αRbNKA 1:500;) and a mouse anti-NKCC monoclonal antibody (T4 1:200; Lytle et al., 1995) overnight at room temperature. Slides were then rinsed with TPBS and

incubated with secondary antibodies (goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568) for 1h at 37°C. Slides were rinsed and nuclei counter stained with DAPI and cover slips mounted with 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 M Tris (pH 8.5). Sections were viewed on a Leica DM6000 B wide field epifluorescence microscope and images captured with a digital camera (DFC340FX, Leica). Optimal exposure settings were predetermined and all images captured under these settings. For quantitative analysis pictures were taken of filament sagital sections toward the afferent side within the interlamellar region. This area typically has the highest Na^+/K^+-ATPase IR cell densities (Wilson and Laurent, 2002).

The frequency of Na^+/K^+-ATPase and NKCC immunopositive cells was quantified in lamellar and filament epithelia. Cell Na^+/K^+-ATPase immunofluorescence labelling was quantified in digital images (average intensity and area of labelled cells) and the cell's shape factor calculated ($4\ \pi$ cell area $\cdot$ perimeter^{-2}) using commercial image analysis software (SigmaScanPro 5.0, SPSS). The data are presented as average intensity of cell labelling (arbitrary units), area of the labelled cell (μm^2), and the total fluorescence per cell (cell fluorescence = Area $\cdot$ Intensity). On average > 60 cells were analyzed per fish.

2.8 Transcript analysis

Total RNA (DNase treated) was isolated from interrenal tissue using the RNeasy Mini Kit protocol (Quiagen, Mississauga, ON), and the RNA was quantified by spectrophotometry at 260nm. The first strand cDNA was synthesized from 1μg total RNA using First Strand cDNA Synthesis kit (MBI Fermentas, Burlington, ON). Quantification of transcript levels were performed exactly as previously described (Sathiyaa and Vijayan, 2003) using Platinum® Quantitative PCR SuperMix-UDG (In Vitrogen, Carlsbad, CA). Briefly, cDNA were used as template for amplification of the housekeeping gene (β-actin) and target genes (StAR, PBR, and

P450scc –expand this first time) using appropriate primers and an annealing temperature (Table 1). The primers were designed using rainbow trout StAR, P450scc, PBR, and β-actin cDNA sequences (GenBank accession nos. AB047032, S57305, AY029216, and AF157514, respectively). The primer pairs amplified a ~100 bp fragment of β-actin and ~500 bp fragments for the target genes used in quantitative real-time PCR (qPCR; iCycler, Biorad).

A relative standard curve for each gene was constructed using either serial dilutions of cDNA or plasmid vector with inserted target sequences and subjected to the same qPCR cycles and conditions as test samples: 95°C- 3min; 40 cycles: denature 95°C- 20s, anneal-20s, and extend 72°C-20 s; then cool 4°C-hold. The iCycler IQ^{TM} real time detection software (BioRad) was used to determine the threshold values (C_T) and a standard curve with log input amount and C_T were established for β-actin and each target genes. Using these standard curves, a ratio of target gene to housekeeping gene is obtained and this normalized value is then standardized using an internal calibrator (FW sham). The transcript levels for the gene of interest are shown as percent internal calibrator exactly as described before (Gravel and Vijayan, 2006).

6. **Table1:** Oligonucleotide primers used in quantitative real-time PCR and their annealing temperatures.

Gene	Primers	Annnealing temp. (°C)
StAR	Forward: 5'-CGCTGGCATCTCCTACA-3' Reverse: 5'-GGGACTTCGTTAGTGTTCG-3'	58
P450scc	Forward: 5'-GAGGAGGGTAGGAGCCA-3' Reverse: 5'-CCTTGTGGGACTCTGGT-3'	60
PBR	Forward: 5'GGCCACCATTTCATTTGTTCATTTCA-3' Reverse: 5'-GCCATGCAAGGTAGGGTGTCAGGA-3'	60
ß-actin	Forward: 5'-AGAGCTACGAGCTGCCTGAC-3' Reverse: 5'-GCAAGACTCCATACCGAGGA-3'	49

2.9 Protein quantification

Tissue homogenization and sample preparation for GR immunodetection was described in detail previously (Boone and Vijayan, 2002), while gill Na^+/K^+-ATPase (NKA) detection followed the protocol of Wilson et al. (2004). Briefly, samples in Laemmli's buffer were loaded (40 μg protein/sample) onto 8% (GR) or 10% (NKA) SDS-PAGE and proteins separated using a discontinuous buffer (Laemmli, 1970). The proteins were transferred onto nitrocellulose membranes using a semi-dry transfer unit (BioRad). The membranes were blocked for 1 h with 5% skim milk in TTBS (20mM Tris (pH 7.5), 300mM NaCl and 0.1% (v/v) Tween 20 with 0.02% sodium azide) and probed for 1 h at room temperature with either anti-trout GR(1:1000 dilution) or mouse α5 monoclonal anti-α1-subunit of chicken Na^+/K^+-ATPase (1:400) in the blocking solution. The membranes were washed with TTBS (3 times for 5 min each) and incubated for 1 h with either goat-anti-rabbit IgG (GR) or goat anti-mouse IgG (NKA) conjugated with alkaline phosphatase (1:3000 dilution). The protein bands were detected with NBT and BCIP. The intensity of the scanned bands was quantified using the AlphaEase software (AlphaEase Innotech, CA) and the protein values are shown as percent FW sham.

2.10 Statistical analyses

Statistical analysis was carried out using two-way analysis of variance (ANOVA) with treatment (sham, salicylate, ibuprofen) and salinity (FW, 50%, SW) as independent factors, followed by LSD (post hoc) test. In case of significant interaction between treatment and salinity, the data were analyzed using one-way ANOVA followed by LSD test. Data were log transformed, whenever necessary, for normal distribution and homogeneity of variance, while non-transformed data are shown in the figures. A significance level of $\alpha=0.05$ was considered statistically significant.

3. Results

3.1 Plasma cortisol, glucose, lactate and protein levels

Plasma cortisol concentration increased proportionally with salinity in all groups (Fig. 1A upper case letters). Overall salicylate exposure increased the response to cortisol regardless of salinity (Fig. 1A inset). In FW or 50% SW there were no significant drug effects on plasma cortisol concentration, whereas in 100% SW both drugs significantly elevated cortisol levels compared to the sham group. The salicylate effect on plasma cortisol levels in 100% SW was significantly greater than ibuprofen (Fig. 1A). Plasma glucose concentrations were significantly higher in 100% SW compared to either FW or 50% SW groups (Fig. 1B upper case letters). In general, ibuprofen exposure significantly reduced plasma glucose concentration compared to the other two treatment groups regardless of environmental salinity (Fig. 1B inset). In 50% SW, both NSAIDs significantly reduced plasma glucose concentration compared to the sham group, while in 100% SW, salicylate significantly elevated glucose levels compared to the other two groups (Fig. 1B). There was no significant effect of either salinity or treatment with NSAIDs on plasma lactate levels. However, plasma lactate levels were significantly higher in the 100% SW group compared to the 50% SW but not the FW groups (Fig. 1C inset). Plasma protein levels were significantly higher in 100% SW compared to 50% SW and FW groups regardless of the treatment (Table 2). There was no drug effect on plasma protein levels in the present study (Table 2).

23. **Figure 1.** Effect of salicylate and ibuprofen on plasma cortisol (A), glucose (B) and lactate (C) either in fresh water (FW), in 50% seawater (50%SW) or in full strength seawater (SW) in rainbow trout.

Values represent mean ± SEM (n = 6-9 fish); different upper case letters denote significant salinity effect regardless of treatment (two-way ANOVA; $P<0.05$); numbers show significant interaction between salinity within a treatment group, while different small case letters mean a significant interaction effect between treatments within this salinity (one-way ANOVA; $P<0.05$).

Fig. 1A

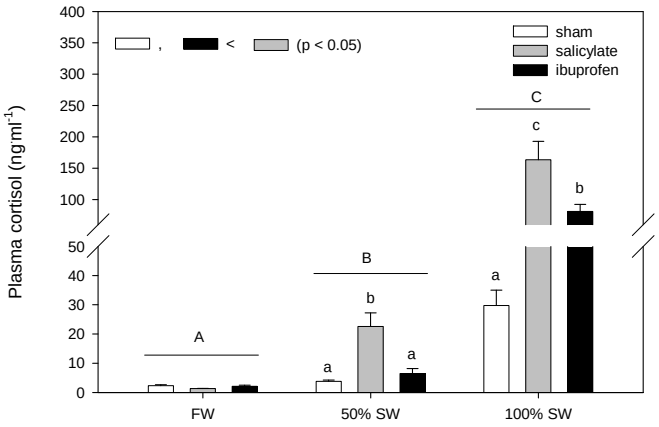


Fig. 1B

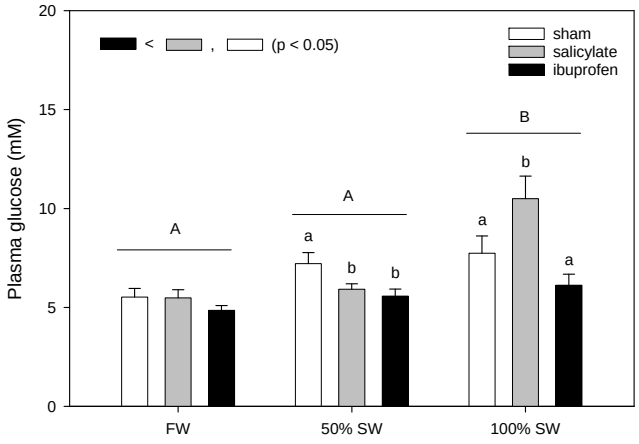
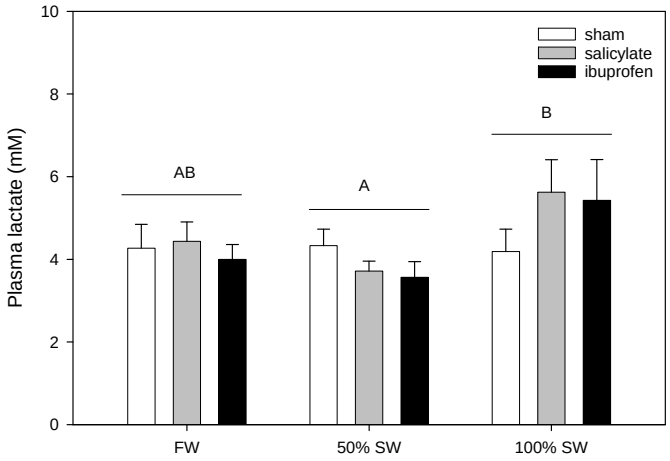


Fig. 1C



7. **Table 2.** Effect of salicylate and ibuprofen exposure on blood and plasma components of rainbow trout in response to salinity.

	Salinity	Sham	Salicylate exposure	Ibuprofen exposure	Treatment effect	Salinity effect
% Blood Hct.	FW	41.93 ± 0.78	40.62 ± 1.62	40.76 ± 1.88		AB
	50% SW	39.55 ± 0.50	39.96 ± 2.03	38.22 ± 1.47	none	A
	100% SW	40.39 ± 1.44	40.82 ± 1.69	40.89 ± 2.37		B
Plasma protein ($mg \cdot ml^{-1}$)	FW	26.69 ± 1.16	30.59 ± 1.23	30.24 ± 1.10		A
	50% SW	29.30 ± 1.60	28.61 ± 2.43	30.36 ± 1.63	none	A
	100% SW	39.89 ± 2.31	34.91 ± 1.24	38.63 ± 1.62		B

Note: Values represent mean ± SEM (n= 6-9 fish). Two-way ANOVA: Different capital letters represent significant salinity effect regardless of treatment ($P<0.05$). Hct: Hematocrit.

3.2 Hematocrit and plasma osmolality, sodium, chloride and potassium levels

Hematocrit was not significantly affected by either salinity or drug treatment (Table 2). There was slight but significant elevation of hematocrit in the 100% SW group compared to the 50% SW group but not the FE group (Table 2). Plasma osmolality was significantly higher in 100% SW compared to 50% SW and FW groups regardless of the treatment (Fig. 2A capital letters). Also, salicylate but not ibuprofen exposure significantly elevated plasma osmolality compared to the sham group (Fig. 2A inset). Plasma sodium ion concentration increased proportionally with salinity (Fig. 2B capital letters). A significant interaction effect between treatment and salinity revealed that in 50% SW group sodium levels were significantly higher with ibuprofen compared to the other two treatments (Fig. 2B lower case letters). As with sodium, plasma chloride concentration also increased proportionally with salinity regardless of treatment groups (Fig. 2C capital letters). In 100% SW group, salicylate, but not ibuprofen, treatment significantly elevated plasma chloride levels compared to the sham group (Fig. 2C

lower case letters). Plasma potassium concentration also increased proportionally with salinity regardless of the treatment (Fig. 2D capital letters). Both drugs significantly elevated plasma potassium levels compared to the sham group, with ibuprofen showing significantly greater potassium levels even compared to the salicylate treatment (Fig. 2D inset).

24. **Figure 2.** Effect of salicylate and ibuprofen on plasma osmolality (A), sodium ions (B), chloride ions (C) and potassium ions (D) either in fresh water (FW), in 50% seawater (50%SW) or in full strength seawater (SW) in rainbow trout.

Values represent mean ± SEM (n = 6-9 fish); different upper case letters denote significant salinity effect regardless of treatment (two-way ANOVA; $P<0.05$); numbers show significant interaction between salinity within a treatment group, while different small case letters mean a significant interaction effect between treatments within this salinity (one-way ANOVA; $P<0.05$).

Fig.2A

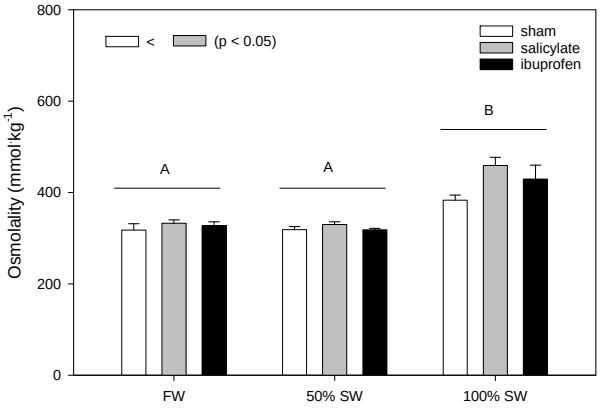


Fig. 2C

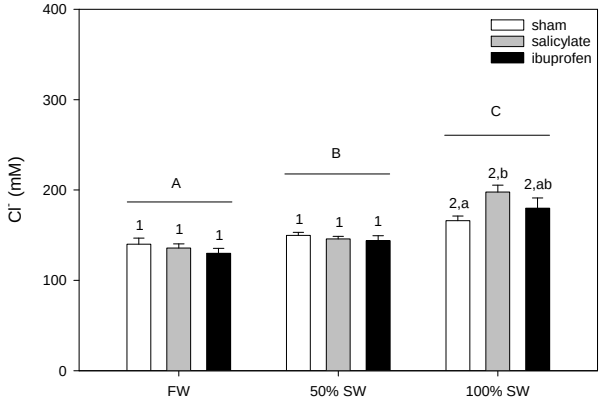


Fig. 2B

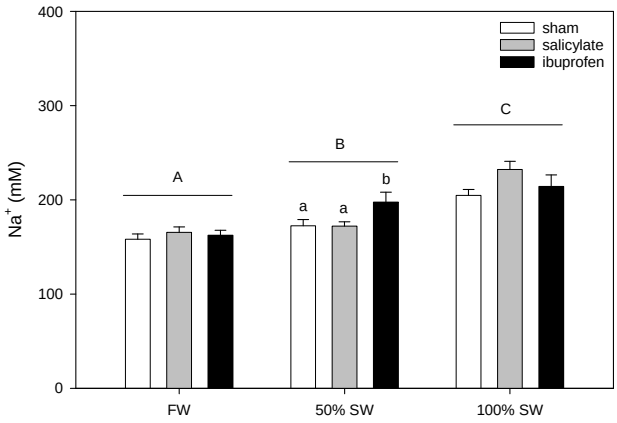
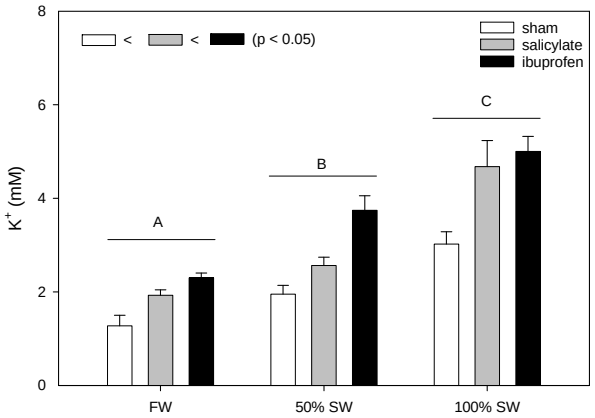


Fig. 2D



3.3 Gill ion transporters

Overall gill Na^+/K^+-ATPase activity was significantly higher in 100% SW compared to 50% SW and FW groups (Fig. 3A capital letters). A significant interaction between treatment and salinity revealed that while both sham and salicylate groups significantly elevated Na^+/K^+-ATPase activity, ibuprofen exposure failed to increase the ionic pump activity in 100% SW (Fig. 3A numbers). Western blotting revealed that gill Na^+/K^+-ATPase protein expression was significantly reduced in 100% SW compared to the FW group (Fig. 3B). There was no significant effect of the drugs on gill Na^+/K^+-ATPase protein expression. We were unable to measure NKCC protein expression by Western blotting in rainbow trout gills.

Na^+/K^+-ATPase and NKCC immunoreactive (IR) cells were identified in both the filament and lamellar epithelia of both freshwater and seawater transferred fish (Fig 4). NKCC was found co-localized with a subpopulation of Na^+/K^+-ATPase IR cells. Two days after transfer to seawater, the abundance of Na^+/K^+-ATPase-IR cells was significantly less compared to fresh water. (c.15% filament and c.60% lamellae) (Table 3). Following seawater transfer the filament Na^+/K^+-ATPase IR cells were also smaller and the lamellar Na^+/K^+-ATPase IR cells were less round. There were no treatment related effects on Na^+/K^+-ATPase IR cell numbers or morphology. The number of NKCC IR cells did not change with salinity or treatment (Table 3).

25. **Figure 3.** Effect of salicylate and ibuprofen on gill Na^+/K^+-ATPase activity (A), Na^+/K^+-ATPase protein expresion (B) either in fresh water (FW), in 50% seawater (50%SW) or in full strength seawater (SW) in rainbow trout.

A representative Western blot image of Na^+/K^+-ATPase protein expression is shown above figure B. Values represent mean ± SEM (n = 5-9 fish); different upper case letters denote significant salinity effect regardless of treatment (two-way ANOVA; $P<0.05$); numbers show significant interaction between salinity within a treatment group, one-way ANOVA; $P<0.05$).

Fig. 3A

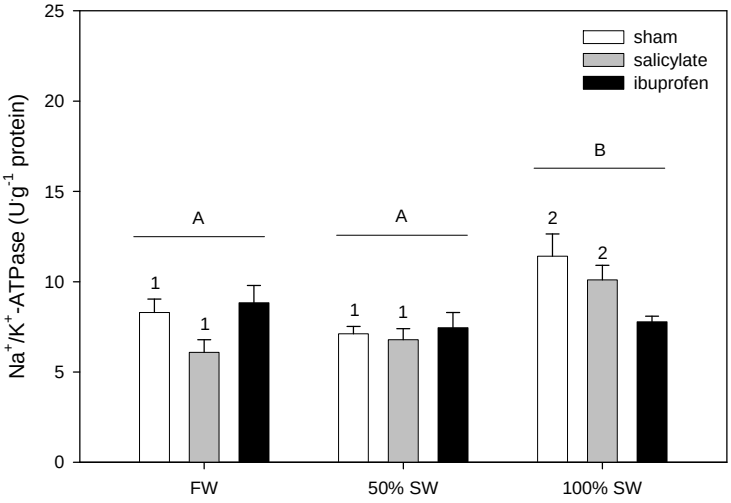
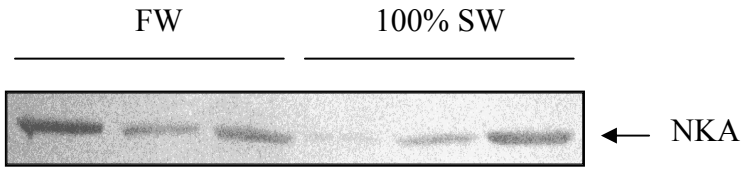
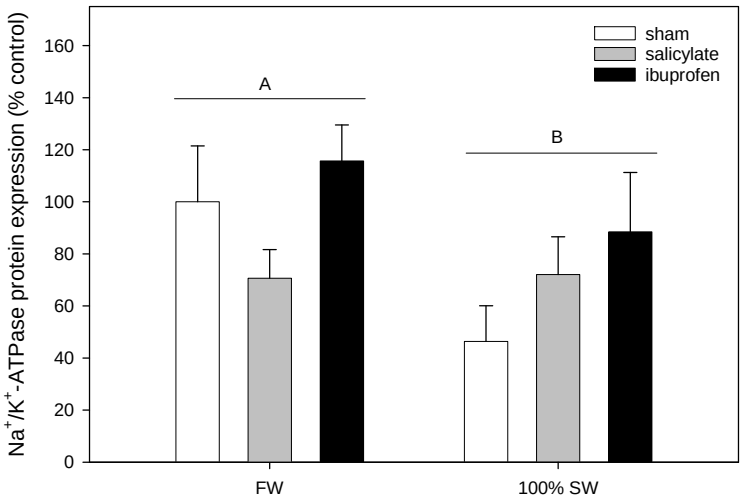

sham
salicylate
ibuprofen
Na+/K+-ATPase (U·g-1 protein)
25
20
15
10
5
0
A
A
B
1
1
1
1
2
2
FW
50% SW
100% SW

Fig.3B

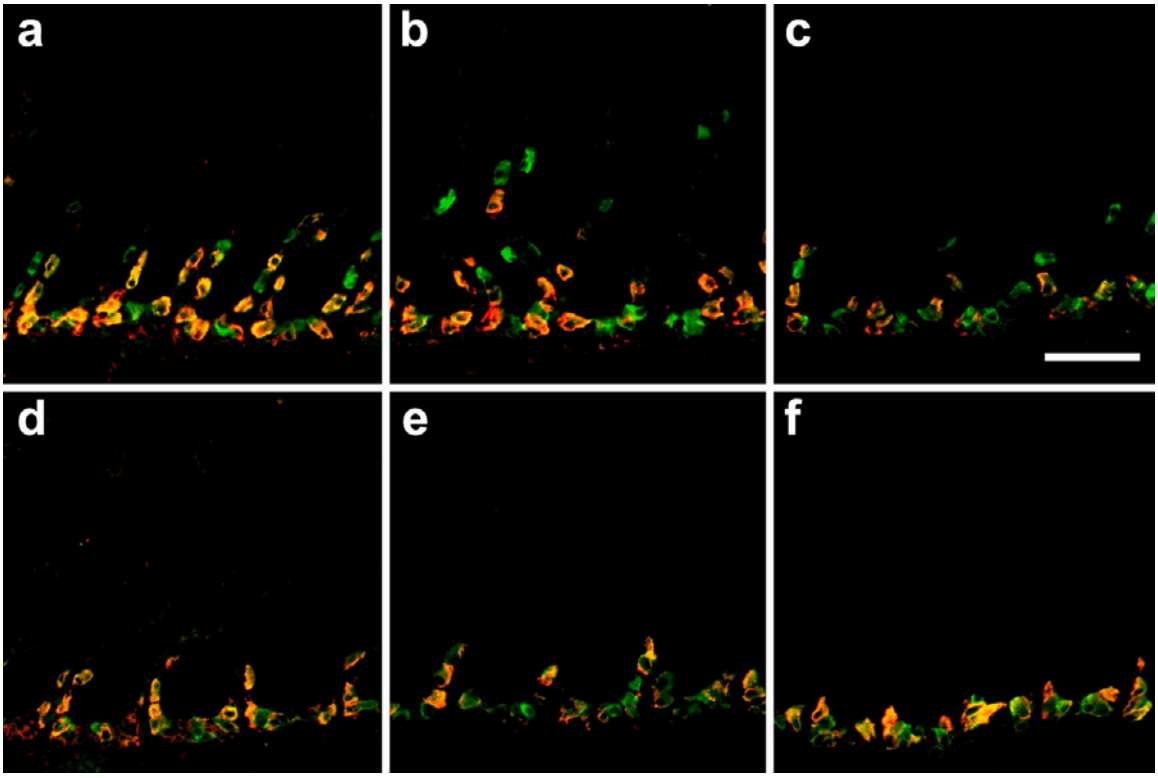

FW
100% SW
NKA


sham
salicylate
ibuprofen
Na+/K+-ATPase protein expression (% control)
160
140
120
100
80
60
40
20
0
A
B
FW
100% SW

26. **Figure 4.** Double immunofluorescence labeling of sagital sections of the gills of rainbow trout using antibodies against Na^+/K^+-ATPase and NKCC.

Trout were exposed to (a) sham, (b) ibuprofen, or (c) salicylate in fresh water and were transferred to 50% seawater followed by 100% seawater (d, e, and f, respectively). Scale bar = 50 μm.

Fig.4



8. **Table 3.** Effect of salicylate and ibuprofen exposure on quantification of branchial Na^+/K^+-ATPase and NKCC immunoreactive (IR) cell number and morphometrics of Na^+/K^+-ATPase IR cells in rainbow trout in response to salinity.

	Salinity	Sham	Salicylate exposure	Ibuprofen exposure	Treatment effect	Salinity effect
Filament Na^+/K^+-ATPase IR cells						
NKA IR cell #	FW	6.08 ± 0.70	5.43 ± 0.58	5.74± 0.58	none	A
	100% SW	2.44 ± 0.36	2.60 ± 0.67	1.67 ± 0.31		B
NKCC IR cell #	FW	2.80 ± 0.73	2.71 ± 0.44	2.67 ± 0.53	none	none
	100% SW	2.34 ± 0.36	2.36 ± 0.70	1.44 ± 0.28		
Cell area (μm^2)	FW	7.94 ± 0.50	7.23 ± 0.21	8.31 ± 0.86	none	none
	100% SW	8.26 ± 0.65	7.88 ± 0.52	8.11 ± 0.48		
Avg. Fluor. Int.	FW	48.95 ± 2.81	44.98 ± 0.93	49.16 ± 2.41	none	none
	100% SW	46.20 ± 2.24	44.25 ± 1.28	44.84 ± 1.23		
Cell Immunofluor.	FW	394.5 ± 43.69	325.1 ± 12.03	415.4 ± 55.89	none	none
	100% SW	386.4 ± 47.36	350.3 ± 32.59	365.4 ± 28.63		
Cell Shape factor	FW	0.30 ± 0.03	0.32 ± 0.02	0.35 ± 0.04	none	A
	100% SW	0.34 ± 0.03	0.42 ± 0.04	0.35 ± 0.02		B
Lamellar Na^+/K^+-ATPase IR cells						
NKA IR cell #	FW	5.71 ± 0.28	5.40 ± 0.34	5.58 ± 0.57	none	A
	100% SW	4.93 ± 0.26	4.59 ± 0.33	4.50 ± 0.28		B
NKCC IR cell #	FW	2.97 ± 0.32	2.68 ± 0.21	2.96 ± 0.43	none	none
	100% SW	3.51 ± 0.22	2.64 ± 0.36	3.02 ± 0.31		
Cell area (μm^2)	FW	6.99 ± 0.33	5.65 ± 0.21	7.33 ± 0.47	SA < Ibu	none
	100% SW	6.40 ± 0.41	5.95 ± 0.40	6.63 ± 0.79		
Avg. Fluor. Int.	FW	50.37 ± 2.29	43.85 ± 1.74	49.46 ± 2.23	none	none
	100% SW	45.10 ± 1.93	43.74 ± 2.23	42.53 ± 1.62		
Cell Immunofluor.	FW	355.2 ± 30.70	248.7 ± 16.08	366.5 ± 36.32	none	none
	100% SW	289.7 ± 26.46	262.9 ± 31.69	283.8 ± 37.95		
Cell Shape factor	FW	0.47 ± 0.02	0.41 ± 0.02	0.46 ± 0.02	none	none
	100% SW	0.43 ± 0.01	0.44 ± 0.04	0.43 ± 0.02		

Note: Values represent mean ± SEM (n= 6 fish). NKA IR cell #; Na^+/K^+-ATPase immunoreactive (IR) cell numbers and NKCC IR cell #; $NK2Cl^-$ cotransporter immunoreactive cell numbers per interlamellar space; Avg. Fluor. Int.: Average fluorescence intensity; Cell Immunofluor: Cell Immunofluorescence. Two-way ANOVA: different upper case letters denote significant salinity effect regardless of treatment, while SA < Ibu represent significant treatment effect between salicylate (SA) and ibuprofen (Ibu) ($P<0.05$).

3.4 StAR, PBR and P450scc transcript levels

Neither salinity nor NSAIDs had any significant effect on either StAR or P450scc mRNA abundance in trout interrenal tissue (Table 4). However, PBR mRNA abundance was inversely proportional to salinity. This significant drop in PBR transcript levels with salinity was seen only with salicylate and ibuprofen but not in the sham group (Table 4).

9. **Table 4.** Effect of salicylate and ibuprofen exposure on steroidogenic transcript levels in interrenal tissue of rainbow trout in response to salinity.

	Salinity	Sham	Salicylate exposure	Ibuprofen exposure	Treatment effect	Salinity effect
StAR	FW	100.00 ± 4.43	99.03 ± 3.60	109.70 ± 8.12		
	50% SW	112.51 ± 6.61	107.00 ± 4.65	106.35 ± 4.11	none	none
	100% SW	105.01 ± 10.00	103.38 ± 6.04	109.73 ± 7.33		
PBR	FW	100.00 ± 6.29	110.57 ± 3.74 [1]	117.24 ± 3.90 [1]		A
	50% SW	109.78 ± 4.13	97.47 ± 4.36 [2]	97.94 ± 2.23 [2]	none	B
	100% SW	100.53 ± 5.00	92.65 ± 1.94 [2]	92.32 ± 1.96 [2]		C
CYPscc	FW	100.00 ± 5.04	97.44 ± 2.88	106.47 ± 8.38		
	50% SW	106.64 ± 6.16	109.12 ± 5.39	102.15 ± 4.69	none	none
	100% SW	99.94 ± 9.14	94.27 ± 6.14	103.79 ± 7.22		

Note: Values represent mean ± SEM (% sham FW; n= 6 fish). Two-way ANOVA: Different capital letters represent significant salinity effect regardless of treatment. Interaction effect between time and treatment are represented by different numbers, meaning statistically significant between salinity groups within the same treatment (One-way ANOVA $P<0.05$). StAR: Steroidogenic acute regulatory protein; PBR: Peripheral-type benzodiazepine receptor; CYPscc: Cytochrome P-450 side chain cleavage.

3.5 Liver glycogen, glucose and enzyme activities

Liver glycogen and glucose concentrations were significantly reduced in 100% SW compared to FW in all treatment groups (Figs. 5A and 5B). Both drugs significantly depressed liver glycogen and glucose content compared to the sham group in 100% SW but in the FW group (Figs. 5A and 5B). There was no significant effect of either salinity or drugs on liver glycolytic (HK, GK, PK, LDH) and gluconeogenic enzyme (PEPCK) activities in this study (Table 5).

27. **Figure 5.** Effect of salicylate and ibuprofen on liver glycogen (A) and liver glucose (B), either in fresh water (FW) or in full strength seawater (SW) in rainbow trout.

Values represent mean ± SEM (n = 6 fish); different upper case letters denote significant salinity effect regardless of treatment (two-way ANOVA; $P<0.05$); *, meaning significant interaction between FW and SW within a treatment group (Student T-test; $P<0.05$), while different small case letters mean a significant interaction effect between treatments within this salinity (one-way ANOVA; $P<0.05$).

Fig. 5A

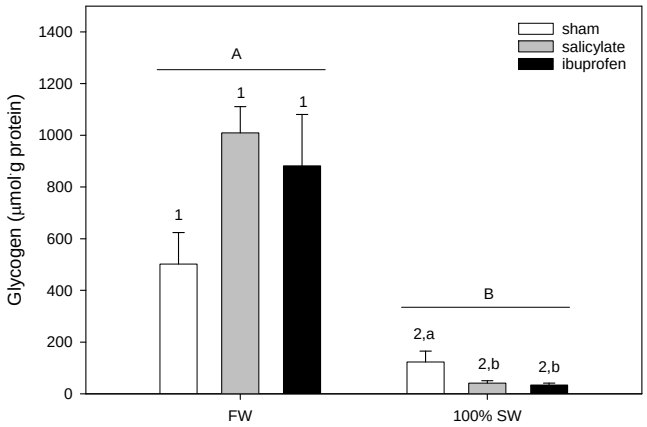
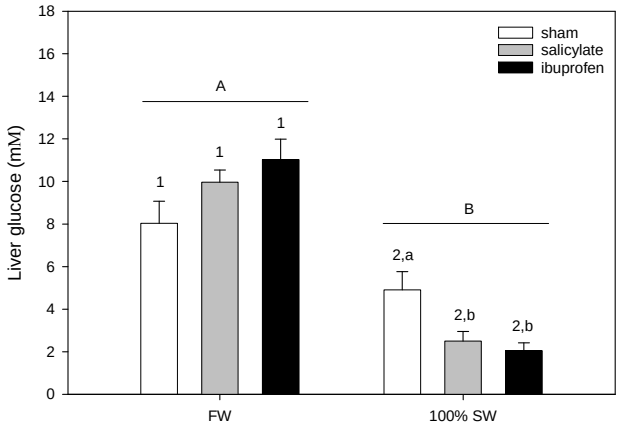

sham
salicylate
ibuprofen
Glycogen (µmol g protein)
1400
1200
1000
800
600
400
200
0
A
B
1
1
1
2,a
2,b
2,b
FW
100% SW

Fig. 5B


sham
salicylate
ibuprofen
Liver glucose (mM)
18
16
14
12
10
8
6
4
2
0
A
B
1
1
1
2,a
2,b
2,b
FW
100% SW

10. **Table 5.** Effect of salicylate and ibuprofen exposure on hepatic enzyme activity of rainbow trout in response to salinity.

	Salinity	Sham	Salicylate exposure	Ibuprofen exposure	Treatment effect	Salinity effect
HK	FW	9.50 ± 0.61	7.83 ± 1.56	8.96 ± 0.33	none	none
	100% SW	7.11 ± 1.07	7.59 ± 0.33	7.74 ± 0.34		
GK	FW	8.29 ± 0.66	7.00 ± 1.50	8.03 ± 0.27	none	none
	100% SW	6.55 ± 0.86	6.79 ± 0.29	6.93 ± 0.33		
PK	FW	7.69 ± 0.83	8.96 ± 2.05	8.07 ± 0.91	none	none
	100% SW	6.12 ± 1.43	9.87 ± 2.73	5.09 ± 0.94		
LDH	FW	653.07 ± 48.81	646.84 ± 88.86	586.97 ± 43.60	none	none
	100% SW	556.63 ± 44.91	593.49 ± 33.97	640.24 ± 24.80		
PEPCK	FW	3.15 ± 0.26	2.82 ± 0.62	2.23 ± 0.38	none	none
	100% SW	1.98 ± 0.34	2.00 ± 0.31	2.66 ± 0.40		

Note: Values represent mean ± SEM ($mU \cdot g^{-1}$ protein; n= 6 fish). Two-way ANOVA showed no change. HK: Hexokinase; GK: Glucokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase; PEPCK: Phosphoenol pyrruvate.

3.6 Gill glycolytic enzyme activities

Gill HK and LDH activities were significantly elevated in 100% SW compared to the FW groups (Table 6). This increase was mostly due to a significant increase in gill activities of HK and LDH in the salicylate group but not in the other two treatment groups (Table 6). Both salicylate and ibuprofen also significantly reduced HK activity compared to the sham group in FW but not in 100% SW, while LDH activity was significantly elevated by the two drugs in 100% SW and not FW. Gill PK activity was not significantly affected by either salinity or the two drugs in the present study (Table 6).

11. **Table 6.** Effect of salicylate and ibuprofen exposure on enzymatic activity in gills of rainbow trout in response to salinity.

	Salinity	Sham	Salicylate exposure	Ibuprofen exposure	Treatment effect	Salinity effect
HK	FW	11.02 ± 0.74 a	7.10 ± 0.43 b	9.31 ± 0.73 a	none	A
	100% SW	10.21 ± 0.50	11.92 ± 0.75 *	9.60 ± 0.80		B
PK	FW	19.01 ± 2.94	14.07 ± 1.85	21.23 ± 2.43	none	none
	100% SW	22.33 ± 2.58	25.03 ± 3.98	21.88 ± 4.16		
LDH	FW	74.82 ± 4.18	64.83 ± 2.15	70.09 ± 2.20	none	A
	100% SW	72.37± 3.43 a	87.56 ± 4.19 *,b	87.96 ± 2.76 b		B

Note: Values represent mean ± SEM ($mU \cdot g^{-1}$ protein; n= 6 fish). Two-way ANOVA: Different capital letters represent significant salinity effect regardless of treatment. Interaction effect between time and treatment are represented by lower case letters meaning statistical difference between treatment at this salinity (One-way ANOVA $P<0.05$). *****, meaning statistically significant from the FW group (Student's *t* test; $P<0.05$). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

3.7 GR protein expression

There was no significant effect of either drug treatments or salinity exposure on GR protein content in the gill and brain on rainbow trout in the present study (Figs. 6 A and B). However, there was a significant interaction between salinity and drugs revealing a significant drop in gill and brain GR protein expression only in the salicylate group in 100% SW compared to the FW group (Figs. 6A and B).

28. **Figure 6.** Effect of salicylate and ibuprofen on gill GR (A) and brain GR (B) protein expression either in fresh water (FW) or in full strength seawater (SW) in rainbow trout.

A representative Western blot image of GR protein expression is shown above each figure. Values represent mean ± SEM (n = 5-6 fish). *, meaning significant interaction (two-way ANOVA; $P<0.05$) between FW and SW within a treatment group (Student T-test; $P<0.05$).

Fig. 6A

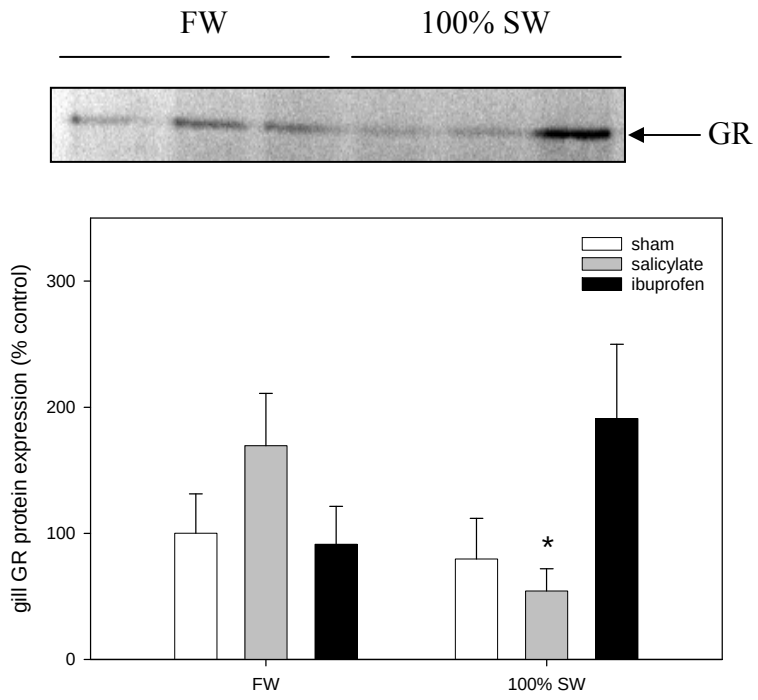
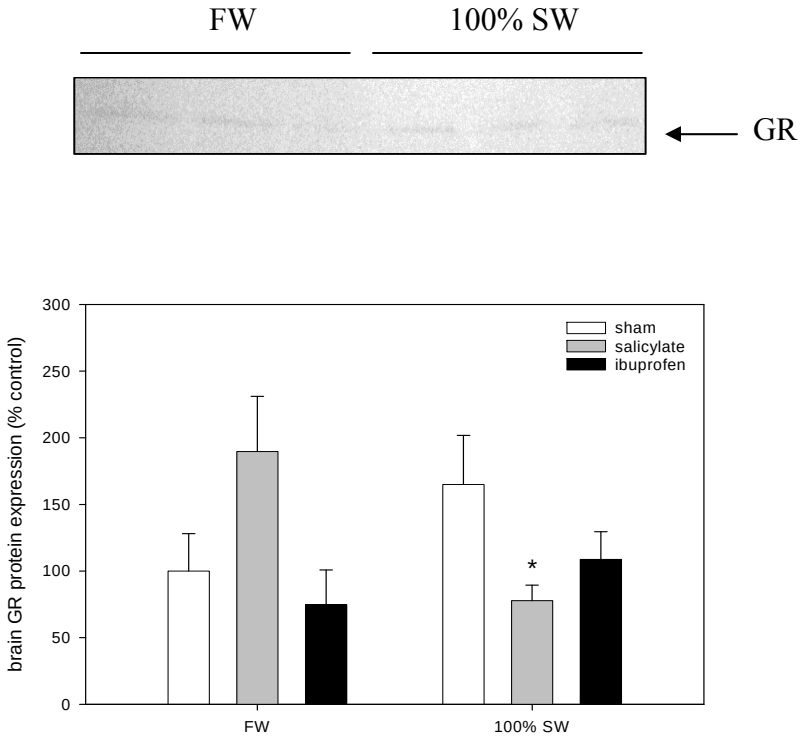

FW
100% SW
GR
sham
salicylate
ibuprofen
gill GR protein expression (% control)
300
200
100
0
*
FW
100% SW

Fig. 6B


FW
100% SW
GR
sham
salicylate
ibuprofen
brain GR protein expression (% control)
300
250
200
150
100
50
0
*
FW
100% SW

4. Discussion

We demonstrate for the first time that NSAIDs alter the adaptive responses associated with seawater acclimation in rainbow trout. Specifically, plasma cortisol dynamics and target tissue responsiveness to cortisol stimulation were targets for NSAIDs impact on seawater adaptation in trout. Although the concentration of drugs used in this study is not environmentally relevant, our results highlight the distinct mode of action of salicylate and ibuprofen in affecting the adaptive responses to salinity exposure in fish.

4.1 Seawater acclimation

As expected 100% SW exposure elevated plasma osmolality and this corresponded with higher sodium and chloride concentrations in rainbow trout (Bystriansky et al., 2006). These ionic changes were minimal in 50% SW supporting efficient regulation of ions at lower salinity as demonstrated previously in trout (Shepherd et al., 2005). Salinity exposure increased plasma protein concentration (by 30%) in 100% SW, while blood hematocrit remained unchanged demonstrating a small change in plasma volume with high salinity. The elevated osmolality, sodium and chloride values in 100%SW is similar to values observed in rainbow trout following direct transfer to 67-100%SW (Prunet et al., 1985; Bolton et al., 1987; Sakamoto and Hirano, 1991; 1993) and supports ionoregulatory disturbances that usually takes a few days to recover (Sakamoto and Hirano, 1991; 1993). These results suggest that complete ion regulation was attained within 2 days at 50%SW, whereas longer time-frame is required for acclimation to higher salinities in trout. This notion is further supported by a lack of changes in Na^+/K^+-ATPase activity in 50%SW, whereas at 100%SW the sodium pump activity was significantly higher compared to FW.

While a number of ion transporters are involved in ion extrusion in SW, the Na^+/K^+-ATPase activity is a key player for establishing the ionic gradient in SW, and also received the most attention (McCormick, 2001). Interestingly, a decrease in gill Na^+/K^+-ATPase protein expression and Na^+/K^+-ATPase immunofluorescent cell size and numbers was observed in rainbow trout exposed to SW for 2 days despite the increase in this enzyme activity. While the reason for this is unknown a recent study in trout also showed that sodium pump protein expression remained unchanged after 2 d of SW exposure (Bystriansky et al., 2006), suggesting either enhanced chloride cell and/or sodium pump turnover upon SW exposure in trout. However, the longer-term (30 d) upregulation of sodium pump protein expression is thought to be due to the longer SW acclimation period required for chloride cell biogenesis in rainbow trout, which are not pre-adapted for SW osmoregulation (Bystriansky et al., 2006).

The higher sodium pump activity in 100% SW corresponds nicely with an elevation in plasma cortisol levels supporting a role for this steroid in stimulating Na/K-ATPase activity in teleosts (McCormick, 1995). Indeed elevation in plasma cortisol levels seen here in 100% SW is a key hormonal response to salinity exposure and is in agreement with other studies in teleosts (McCormick, 2001; Evans, 2005). The lack of a cortisol response in 50% SW supports our earlier finding in trout (Shepherd et al., 2005). The absence of a handling stress associated with gradual salinity increment, unlike abrupt transfer of fish to lower salinities (Sakamoto and Hirano, 1991, 1993), may be the reason for the lack of a cortisol response at lower salinities (Shepherd et al., 2005). However, the pronounced increase in plasma cortisol levels at 100% SW leads to the proposal that a higher salinity threshold is required for activation of the HPI axis in rainbow trout.

We show for the first time that this enhanced cortisol response in SW was not accompanied by changes in the mRNA abundance of key proteins involved in corticosteroidogenesis. While StAR transcript levels were elevated in response to acute stress and/or acute ACTH stimulation of cortisol production in trout (Kusakabe et al., 2002; Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Gravel and Vijayan, 2006), the absence of any change in this study may be due to the longer residence (2 d) in SW. It remains to be seen if the StAR protein expression, rather than transcript level, is a better indicator of interrenal steroid capacity in trout. While we cannot rule out the possibility that the interrenal capacity for cortisol production is enhanced in SW, the clearance of the hormone may also be affected leading to elevated cortisol levels in SW (Redding et al., 1984). The lack of change in brain GR in SW argues against disturbance in the negative feedback regulation of cortisol as a reason for the elevated plasma concentration of this steroid leading to the proposal that higher salinity (>50% SW) stimulates the HPI axis functioning in rainbow trout.

The elevated cortisol response in SW is thought to play a role in the energy substrate mobilization to cope with the higher metabolic cost associated with ion regulation in fish (Kirschner, 1993; Mommsen et al., 1999). Glucose is the preferred fuel for gill metabolism, including upregulation of ion transporters in SW (Mommsen, 1984; Mommsen et al., 1999). Indeed, there was an increase in plasma glucose levels in 100%SW, while the liver metabolic capacity was not affected by this SW residence in the present study. This coupled with the significant decrease in liver glycogen content implicates a role for glycogenolysis in the observed glucose response in SW. Liver glycogen stores represent an important energy reserve and previous studies have clearly shown an enhanced capacity for glycogen depletion with stress (Mommsen et al., 1999), including SW acclimation process (Singer et al., 2007). The enhanced

glycolytic capacity of trout gills in 100%SW clearly establishes enhanced glycogen mobilization as a key SW acclimation response critical for providing fuel for gill function. Considered together, SW acclimation in trout involves activation of the HPI axis leading to elevated plasma cortisol levels in trout. SW acclimation involved enhanced gill sodium pump activity, while the enhanced energy demand associated with this gill function was reflected in the higher glycolytic capacity of this tissue. Breakdown of liver glycogen stores appear to be a key source of glucose to fuel gill function in SW. We hypothesize that the cortisol response seen in 100%SW assist with the upregulation of ion extrusion mechanisms, including sodium pump activity, as well as alter target tissue metabolism to cope with the higher energy demand in SW.

4.2 Impact of NSAIDs on SW acclimation

Our results clearly demonstrate that NSAIDs disturb ion regulation and modify the physiological response to salinity exposure in rainbow trout. While 100%SW exposure resulted in elevated plasma osmolality and ion concentrations, salicylate, more than ibuprofen, further disturbed ion regulation in rainbow trout. Particularly, the regulation of plasma sodium, chloride and potassium ion levels with NSAIDs was disturbed after salinity exposure. However, the salicylate-induced ionoregulatory disturbance was not correlated with any changes in the activity or protein expression (and immunoreactive cell numbers and size) of Na^+/K^+-ATPase, a key branchial enzyme involved in NaCl excretion. However, this is consistent with results of ASA exposure in fresh water-adapted tilapia (van Anholt et al, 2003). Salicylate-induced ionic impairment is therefore independent of branchial sodium pump activity and maybe targeting drinking, renal or other aspects of branchial ion regulation.

In contrast, ibuprofen treatment clearly failed to activate the sodium pumps when fish were transferred to SW, despite no further accumulation of sodium or chloride ions in plasma.

Our results suggest that ibuprofen inhibits the activation of Na^+/K^+-ATPase in SW, but it did not result in significantly greater ion impairment. A possible mode of action of ibuprofen-induced inhibition of the sodium pump is via PGs. NSAIDs like ibuprofen are COX inhibitors preventing formation of PGs. PGs are known to be involved in the regulation of Na^+/K^+-ATPase activity in kidney of mammals (Scherzer et al., 1992; Kreydiyyeh and Markossian, 2006; Borsick et al., 2006) and there are growing evidence of their involvement in osmoregulation in fish gills (Evans et al., 2002a). Indeed, in killifish COX2 has been identified and localized to branchial chloride cells (Choe et al., 2006). Constitutive expression is higher in SW versus FW-acclimated animals although hyper- or hypo-osmotic shock causes a transient increase (Choe et al., 2006). PGs can also modulate the functioning of the HPI axis in fish (Gupta et al., 1985; Wales, 1988). Therefore, impaired ion regulation in ibuprofen treated fish could be indirectly mediated through inhibition of the PG pathway acting on the cortisol response and/or the sodium pump activation. Since salicylate is also a COX inhibitor, the disturbed ion regulation with ibuprofen relative to salicylate suggests distinct mode of action of these drugs in impairing seawater acclimation in trout.

The pronounced cortisol response at higher salinities with NSAIDs is novel and contrasts the suppression of ACTH- or stressor-stimulated cortisol production *in vitro* and *in vivo* observed before in rainbow trout (Gravel and Vijayan, 2006; 2007). Specifically, salicylate showed the greatest cortisol response, while the higher levels compared to the sham and ibuprofen groups even in 50%SW suggest a distinct mode of action of this drug in SW cortisol dynamics. The changes in plasma cortisol levels were not reflected in the transcript abundance of key proteins involved in corticosteroidogenesis. In fact, the lack of changes in StAR and a lower PBR mRNA abundance with salinity exposure argues against enhanced cortisol biosynthesis with the drugs.

This is supported by the observation that lower StAR and PBR mRNA abundance with NSAIDs corresponded with lower cortisol production in trout interrenal tissue (Gravel and Vijayan, 2006). This raises the possibility that cortisol clearance mechanism may be disturbed by NSAIDs. The suppression of brain GR protein expression with salicylate in SW supports a disturbed negative feedback regulation of cortisol leading to elevated plasma cortisol levels in trout. However, the lack of a similar GR response with ibuprofen, despite higher cortisol levels in 100%SW, suggests additional mechanisms responsible for the elevated cortisol response but this remains to be determined.

The metabolic response, essential for SW acclimation, was also perturbed by NSAIDs. Specifically, the depressed liver glycogen and glucose content with NSAIDs, coupled with the absence of any changes in activities of enzymes involved in intermediary metabolism, points to enhanced capacity for glycogenolysis in SW. However, this was reflected in a higher plasma glucose concentration only with salicylate and not ibuprofen suggesting disturbances in glucose clearance mechanisms. This agrees with our recent report that these two drugs have different modes of action on stressor-mediated glucose metabolism in trout (Gravel and Vijayan, 2007). Indeed, the higher gill glycolytic capacity with NSAIDs suggests a higher metabolic demand for gill function in SW. Specifically, the higher gill HK and LDH, key glycolytic enzymes, activities with salicylate in SW compared to FW support a higher tissue capacity for glucose utilization and metabolism, critical for fueling ion transporters. Consequently, the depressed glycogen stores seen here with NSAIDs may limit the adaptive glucose response critical to cope with the increased energy demand for gill function in SW. Furthermore, the pronounced plasma cortisol levels in the salicylate group along with a reduced gill GR protein content suggests a lower tissue responsiveness in this group that may be playing a role in the disturbed ion regulation.

Considered together, salicylate and ibuprofen disturbed ion regulation that is associated with SW acclimation in rainbow trout. This response involved impaired cortisol response and energy substrate re-partitioning critical for coping with the enhanced energy demand associated with SW acclimation. However, given the differences between the two NSAIDS in perturbing ion regulation and the associated adaptive responses to SW exposure, we hypothesize a distinct mode of action for the two NSAIDs in disrupting the SW acclimation process in rainbow trout.

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References

Aluru, N., and Vijayan, M.M. 2006. Ah receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate limiting steps in steroidogenesis. *Endocrinol.* 147(4):1895-1903.

Bolton J. P., N. L. Collie, H. Kawauchi, T. Hirano. 1987. Osmoregulatory actions of growth hormone in rainbow trout (*Salmo gairdneri*). J. Endocrinol. 112:63–68.

Boone, A.N., Vijayan. M,M. 2002. Glucocorticoid-mediated attenuation of the hsp70 response in trout hepatocytes involves the proteasome. Amer. J. Physiol. Regul, Integr. Comp. Physiol. 283: R680-R687.

Borsick M, Rajkhowa T, and Taub M. 2006 Evidence for post-transcriptional regulation of Na,K-ATPase by prostaglandin E1.Biochem Biophys Res Commun. 345(2):739-745.

Bystriansky JS, Richards JG, Schulte PM, Ballantyne JS. 2006. Reciprocal expression of gill Na^+/K^+-ATPase alpha-subunit isoforms alpha1a and alpha1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. J Exp Biol. 209(Pt 10):1848-1858.

Choe KP, Havird J, Rose R, Hyndman K, Piermarini P, Evans DH. 2006. COX2 in a euryhaline teleost, Fundulus heteroclitus: primary sequence, distribution, localization, and potential function in gills during salinity acclimation. J. Exp. Biol. 209(Pt 9):1696-1708.

Dean DB, Whitlow, ZW and Borski, RJ. 2003 Glucocorticoid receptor upregulation during seawater adaptation in a euryhaline teleost, the tilapia (*Oreochromis mossambicus*).Gen Comp Endocrinol. 132(1):112-118.

Evans DH, Roeser JM, Stidham JD. 2002a. Natriuretic peptide hormones do not inhibit NaCl transport across the opercular skin of the killifish, Fundulus heteroclitus. Bull Mt Desert Isl Biol Lab 41:47.

Evans, D.H. Piermarini, PM., Choe, KP. 2005. The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol.Rev. 85 (1): 97-177.

Geslin, M., and Auperin, B. 2004. Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 135, 70-80.

Gravel, A., and Vijayan, MM. 2007. Non-steroidal anti-inflammatory drugs disrupt the heat shock response in rainbow trout. Aquat.Toxicol. 81, 197-206.

Gravel, A., Vijayan, MM. Salicylate impacts the physiological responses to an acute handling disturbance in rainbow trout. *In review.*

Gravel, A., Vijayan, M.M. 2006. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. Toxicol. Sci. 93: 41-49.

Gupta, O.P., Lahlou, B., Botella, J., Porthe-Nibelle, J. 1985. *In vivo* and *in vitro* studies on the release of cortisol from interrenal tissue in trout. I. Effects of ACTH and prostaglandins. Exp. Biol. 43: 201-212.

Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol Lett. 131(1-2):5-17.

Kirschner, LB. 1993. The energetics of osmotic regulation in ureotelic and hypoosmotic fishes. J. exp. Zool. 267: 19–26.

Kreydiyyeh SI and Markossian, S. 2006; Tumor necrosis factor alpha down-regulates the Na^+-K^+ ATPase and the Na^+-K^+2Cl^- cotransporter in the kidney cortex and medulla. Cytokine. 33(3):138-44.

Kusakabe, M., Todo, T., McQuillan, J.H., Goetz, F.W., and Young, G. 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinol. 143: 2062-2070.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laiz-Carrión, R., Sangiao-Alvarellos, S. Guzmán, JM.,Martín del Río, MP., Míguez, JM.' Soengas, JL., Mancera, JM. 2002. Energy metabolism in fish tissues related to osmoregulation and cortisol action. Fish Physiol.Biochem. 27: 179-188.

Lytle C, Xu JC, Biemesderfer D, Forbush B 3rd. 1995. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. Am J Physiol.269(6 Pt 1):C1496-1505.

Madsen S. S., M. K. Jensen, J. Nohr, K. Kristiansen. 1995. Expression of Na+-K+-ATPase in the brown trout, Salmo trutta: In vivo modulation by hormones and seawater. Amer. J. Physiol.— Regul. Integr. C. 38:1339–R1345 R.

McCormick S. D. 1995. Hormonal control of gill Na+,K+-ATPase and chloride cell function. *In* C. M. Wood and T. J. Shuttleworth (eds.), *Fish physiology*, Vol. XIV, *Ionoregulation: Cellular and molecular approaches,* pp. 285–315. Academic Press, New York.

McCormick S. D., W. W. Dickhoff, J. Duston, R. S. Nishioka, H. A. Bern. 1991.. Developmental differences in the responsiveness of gill Na^+K^+-ATPase to cortisol in salmonids. Gen. Comp. Endocrinol. 84:308–317.

McCormick SD. 1990. Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. Am J Physiol 259:R857–R863.

McCormick SD**.** 1993. Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Can J Fish Aquat Sci 50: 656–658.

McCormick, S.D. 2001. Endocrine control of osmoregulation in teleost fish. Amer.Zool. 41: 781-794.

Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., and Hirsch, R. 2003a. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxicol. Chem. 22: 2872-2880.

Metcalfe CD, Miao XS, Koenig BG, Struger J. 2003b. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. Environ Toxicol Chem. 22(12):2881-9.

Mommsen TP, Vijayan MM, Moon TW. 1999. Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. Reviews Fish Biol. Fish. 9: 211-268.

Mommsen, T.P. 1984. Metabolism of the fish gill. In *Fish Physiology*, eds. W.S. Hoar and D.J. Randall, pp 203-238, Academic, New York.

Morgan, JD, Iwama, GK. 1998. Salinity effects on oxygen consumption, gill Na^+,K^+-ATPase and ion regulation in juvenile coho salmon. J.Fish Biol. 53 (5): 1110-1119.

Morgan, JD, Iwama, GK. 1998. Energy cost of NaCl transport in isolated gills of cutthroat trout. Am.J.Physiol.Reg.Int.Comp.Physiol. 277 (3): R631-R639.

Pelis RM, McCormick SD. 2001. Effects of growth hormone and cortisol on Naþ,Kþ,2Cl_ cotransporter localization and abundance in the gills of Atlantic salmon. Gen Comp Endocrinol 124:134–143.

Prunet P, Bouef G, and Houdebine LM**.** 1985. Plasma and pituitary prolactin levels in rainbow trout during adaptation to different salinities. J Exp Zool 235: 187–196.

Prunet P, Sturm, A., Milla, S. 2006. Multiple corticosteroid receptors in fish: from old ideas to new concepts. Gen Comp Endocrinol. 147(1):17-23

Redding, JM., Patino, R., Schreck, CB. 1984. Clearance of corticosteroids in yearling coho salmon, *Oncorhynchus kisutch*, in fresh water and seawater and after stress.Gen.Comp. Endocrinol. 54(3):433-443.

Richards, JG., Semple, JW., Bystriansky, JS.,Schulte, PM. 2003. Na^+/K^+-ATPase (alpha-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J.Exp.Biol. 206 (24): 4475-4486.

Sakamoto T., T. Hirano. 1991. Growth hormone receptors in the liver and osmoregulatory organs of rainbow trout: Characterization and dynamics during adaptation to seawater. J. Endocrinol. 130:425–433.

Sakamoto T., T. Hirano. 1993. Expression of insulin-like growth factor-I gene in osmoregulatory organs during seawater adaptation of the salmonid fish: Possible mode of osmoregulatory action of growth hormone. Proc. Natl. Acad. Sci. U.S.A. 90:1912–1916.

Sathiyaa, R., Vijayan, M.M. 2003. Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. Amer. J. Physiol. Cell Physiol. 284: C1508-C1515.

Scherzer P, Wald H, Rubinger D, and Popovtzer, MM. 1992. Indomethacin and sodium retention in the rat: role of inhibition of prostaglandin E2 synthesis, Clin. Sci. 83: 307–311.

Shaw JR, Gabor K, Hand E, Lankowski, A., Durant, L., Thibodeau, R., Stanton, CR., Barnaby, R., Coutermarsh, B., Karlson, KH., Sato, JD., Hamilton, JW., Stanton, BA. 2007. Role of glucocorticoid receptor in acclimation of killifish (*Fundulus heteroclitus*) to seawater and effects of arsenic. Am.J.Physiol.-Reg.Int.Comp.Physiol. 292 (2): R1052-R1060.

Shepherd BS, Drennon K, Johnson J, Nichols, JW, Singer TD and Vijayan, MM. 2005. Salinity acclimation affects the somatotropic axis in rainbow trout. Am J Physiol Regul Integr Comp Physiol. 288(5):R1385-95.

Shrimpton J. M., N. J. Bernier, D. J. Randall. 1994. Changes in cortisol dynamics in wild and hatchery-reared juvenile coho salmon (*Oncorhynchus kisutch*) during smoltification. Can. J. Fish. Aquat. Sci. 51:2179–2187.

Shrimpton J. M., R. H. Devlin, E. Mclean, J. C. Byatt, E. M. Donaldson, D. J. Randall. 1995. Increases in gill corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) treated with growth hormone and placental lactogen. Gen. Comp. Endocrinol. 98:1–15.

Shrimpton J. M., S. D. McCormick. 1999. Responsiveness of gill Na^+/K^+-ATPase to cortisol is related to gill corticosteroid receptor concentration in juvenile rainbow trout. J. Exp. Biol. 202:987–995.

Singer TD, Raptis S, Sathiyaa R, Nichols JW, Playle, RC and Vijayan MM. 2007 Tissue-specific modulation of glucocorticoid receptor expression in response to salinity acclimation in rainbow trout. Comp Biochem Physiol B Biochem Mol Biol. 146(2):271-278.

Singer TD, Tucker SJ, Marshall WS, and Higgins CF. 1998. A divergent CFTR homolog: highly regulated salt transport in the euryhaline teleost *F. heteroclitus.* Am J Physiol Cell Physiol 274: C715–C723.

van Anholt RD, Spanings T, Koven W, Wendelaar Bonga SE. 2003. Effects of acetylsalicylic acid treatment on thyroid hormones, prolactins, and the stress response of tilapia (*Oreochromis mossambicus*). Am J Physiol Regul Integr Comp Physiol. 285(5):R1098-106.

Vijayan, M.M., Aluru, N., Maule, A.G., Jorgensen, E.H. 2006. Fasting augments PCB impact on liver metabolism in anadromous arctic char. Toxicol. Sci. 91: 431-439.

Vijayan, MM, Morgan JD, Sakamoto T, Grau EG, and Iwama GK. 1996. Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. J Exp Biol 199: 2467-2475.

Vijayan, M.M., Prunet, P., Boone, A.N. 2005. Xenobiotic impact on corticosteroid signaling. In: Mommsen, T.P., Moon, T.W. (Eds). Biochemistry and Molecular Biology of Fishes, Vol. 6. Elsevier, Amsterdam, pp 87-101.

Wales, N.M. 1988. Hormones studies in *Myxine glutinosa*: effects of the eicosanoids arachidonic acid, prostaglandin E_1, E_2, A_2, F_{2a}, thromboxane B_2 and of indomethacin on plasma cortisol, blood pressure, urine flow and electrolyte balance. J. Comp. Endocrinol. B. 158**:** 621-626.

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

Wilson, JM., Antunes, JC., Bouca, PD., Coimbra, J. 2004. Osmoregulatory plasticity of the glass eel of *Anguilla anguilla*: freshwater entry and changes in branchial ion-transport protein expression. Can.J.Fish.Aquat.Sci. 61 (3): 432-442.

Wilson, JM., Laurent, P. 2002. Fish gill morphology: inside out. J. Exp. Zool. 293(3):192-213.

CHAPTER 7

General Conclusions

12.**Table 1:** Summary of principle effects of NSAIDs on the adaptive responses to stressors in rainbow trout.

Steroidogenesis and the HPI axis (Gravel and Vijayan, 2006)
In vitro exposure Inhibition of ACTH-stimulated cortisol production at $\geq 10^{-5}$ M salicylate; $\geq 10^{-5}$ M acetaminophen*; $\geq 10^{-4.5}$ M ibuprofen.
In vivo salicylate exposure (100mg/kg body wt.) 50% inhibition of in vitro ACTH-stimulated cortisol production. Reduction in StAR mRNA and PBR mRNA levels. 50% reduction brain GR protein expression.
Acute (handling) stress response: *In vivo* salicylate exposure (100mg/kg body wt.)
50% inhibition of cortisol response to handling stress. Reduction in StAR mRNA levels. Reduction in liver glycogen levels. Reduction in liver GR protein expression

Concentration-responses to waterborne exposure to 0-1000 µg/L NSAIDs	
Salicylate:	Ibuprofen:
No effect measured at environmentally relevant concentrations	Induction of hsp70 protein expression at ≥ 1 µg/L.

Heat shock (HS) response: waterborne exposure to 1000 µg/L NSAIDs (Gravel and Vijayan, 2007)	
Salicylate:	Ibuprofen:
Elevated liver hsp70 mRNA levels. Delayed HS-induced liver hsp70 protein. Higher whole body glucose levels post-HS. Increased liver glycolytic enzyme activities	Hsp70 protein inducer in liver Reduction of hsp70 protein expression at 24h post HS in gills and liver. Absence of a HS-mediated decrease in whole body glucose levels. Increased liver glycolytic enzyme activities

Seawater (SW) acclimation: waterborne exposure to 1000 µg/L NSAIDs	
Salicylate:	Ibuprofen:
Elevated plasma cortisol levels in 50% and 100% SW. Increased plasma osmolality and K^+ levels in SW Enhanced glycogen breakdown in SW. Higher LDH activity in SW.Reduction in liver and gill GR protein content.	Elevated plasma cortisol levels in 100% SW. Increased plasma K^+ levels in SW. Absence of a SW-mediated elevation in gill Na^+K^+-ATPase activity. Enhanced glycogen breakdown in SW. Higher LDH activity in SW.

Note: * acetaminophen is an analgesic/antipyretic but not NSAID.

The significant finding from this thesis, about the effect of salicylate and ibuprofen on the adaptive responses to stressors in trout, is outlined in Table 1. Based on the results, the specific conclusions are: i) salicylate and ibuprofen did not affect basal corticosteroidogenesis (chapters 2, 3, and 6), but perturbed acute hormone- (chapter 2) and stressor-stimulated (chapters 3 and 6) cortisol production; ii) the disruption of steroidogenesis by salicylate corresponded with a reduced gene expression of key proteins involved in the rate-limiting step in steroidogenesis (chapters 2 and 3); iii) the abnormal stressor-mediated plasma cortisol levels with salicylate corresponded with a reduced GR expression in the brain suggesting disturbance in the negative feedback regulation (chapters 2 and 6); iv) salicylate and ibuprofen affected intermediary metabolism and perturbed the plasma glucose dynamics (chapters 3, 5 and 6); v) salicylate effect on tissue metabolic capacity to stressors corresponded with depressed GR levels reflecting disturbances in target tissue cortisol responsiveness (Chapters 3 and 6); vi) both NSAIDs also impaired ion regulation capacity (chapter 6) and cellular stress response (chapter 5); and viii) adverse effects of NSAID were observed at high concentrations of the drugs, which are not environmentally relevant (chapters 2 and 4), with the exception of the induction of hepatic hsp70 protein expression with ibuprofen (1μg/L) (chapter 5).

Taken together, salicylate and ibuprofen impair the organismal and cellular stress responses in rainbow trout. Specifically, salicylate disrupted corticosteroidogenesis and target tissue responsiveness to cortisol stimulation and the key targets are the steroidogenic acute regulatory protein (StAR) and glucocorticoid receptor (GR), respectively. At the cellular levels, these NSAIDs altered the highly conserved heat shock protein response 70 (hsp70) that is critical for defense against proteotoxicity. However, ibuprofen only, but not salicylate, is an hsp70 inducer in trout fry. This suggests that ibuprofen mode of action could involve targeting the

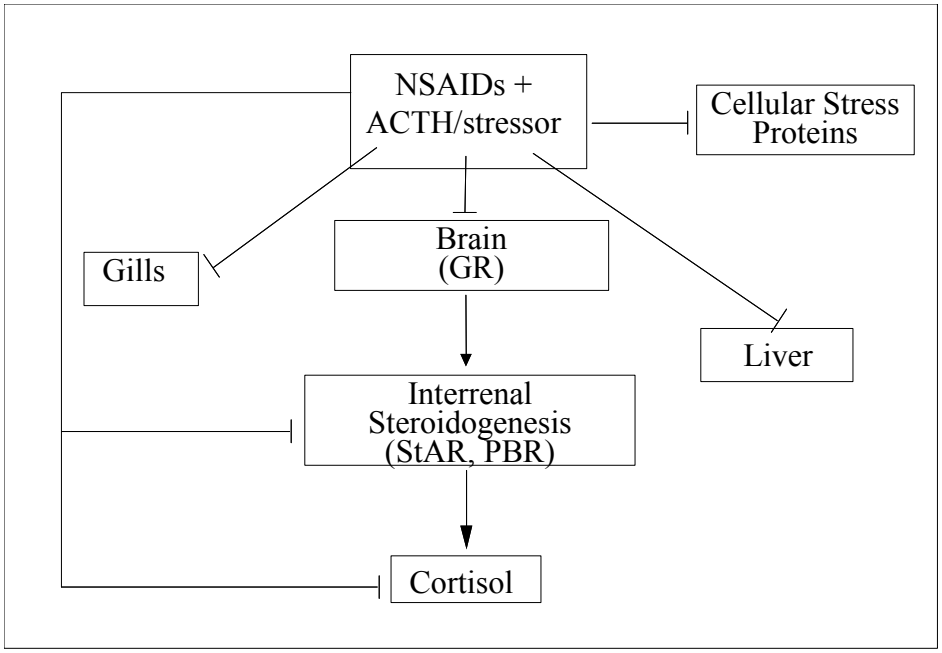
protein machinery. Also, the glucose response, the primary metabolic fuel for stress coping mechanisms, was impaired leading to disturbances in metabolic adjustments to stressors. Altogether, salicylate and ibuprofen are corticosteroid disruptors in trout and impair the adaptive responses to secondary stressors in this species.

Aquatic organisms are challenged with increasing amounts of contaminants, including pharmaceutical drugs that can affect the endocrine system of animals. The impact of NSAIDs on the functioning of the HPI axis may have adverse consequences at the population level. This stems from the fact that cortisol is involved in all aspects of animal function, including growth, metabolism, immune function, osmoregulation and reproduction. Also, since the rate-limiting step in steroidogenesis is common between corticosteroids and sex steroids, the impact of salicylate on StAR may reflect not only impaired cortisol response but also reproductive function. While the mode of action of ibuprofen and salicylate in impacting the adaptive response appears distinct, clearly both compounds are homeostatic disruptors in fish. However, future studies should be designed to understand the mode of action of these two compounds as the results suggest that their presence in the aquatic environment may have distinct physiological consequences to the animal. This information may be particularly important for risk assessment because most often these chemicals are present as complex mixtures leading to multiple sites of impact on the organism.

Although our results showed endocrine disruption with NSAIDs at high concentrations, further studies are required to investigate whether NSAIDs can have adverse effects at environmentally relevant concentrations. Also, studies using specific agonists and antagonists for hormone action, including corticosteroid receptors, would shed light on the cause and effect relationship of NSAIDs on cortisol signaling in fish. Indeed, the induction of a cellular stress

response by exposure of rainbow trout fry to ibuprofen at environmental concentrations (chapter 5) suggests that some aspects of animal stress performance may be targeted at low concentrations of the drugs. Also, most adverse effects of NSAIDs were observed when drug exposure was coupled to a secondary stressor, such as following ACTH or handling stimulation, a heat shock or a transfer from fresh water to seawater, as opposed to when fish were under basal or unstimulated condition.

Therefore, future studies should investigate the impact of low concentrations of NSAIDs either alone or in combination with other chemicals, including other NSAIDs, in impacting the stress performance of fish to secondary stressors. As this study focused on the mechanistic aspect of NSAID`s impact on fish performances, only short term (3, 4 or 7days) acute responses to high concentrations were documented. Longer term exposures to environmentally realistic concentrations of the drugs are required to understand their toxic potential on aquatic organisms. However, it is worth mentioning that some of the NSAIDs, especially salicylates, are broken down very rapidly in the aquatic environment and that may be a factor influencing the physiological responses in chronic exposure studies with these chemicals. Finally, since contaminants are usually found as a complex mixture in the environment, studies should focus on the interaction of pharmaceutical drugs with other pollutants in mediating the toxic effects in fish. Overall, based on the results of this thesis, NSAIDs impact the adaptive stress response by targeting multiple pathways in rainbow trout (Fig. 1).



29. **Figure 1.** NSAIDs impact multiple aspects of the adaptive responses to stressors in rainbow trout.