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Effects of salinity acclimation on the expression and activity of Phase I enzymes (CYP450 and FMOs) in coho salmon (*Oncorhynchus kisutch*)

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Abstract

Phase I biotransformation enzymes are critically important in the disposition of xenobiotics within biota and are regulated by multiple environmental cues, particularly in anadromous fish species. Given the importance of these enzyme systems in xenobiotic/endogenous chemical bioactivation and detoxification, the current study was designed to better characterize the expression of Phase I biotransformation enzymes in coho salmon (*Oncorhynchus kisutch*) and the effects of salinity acclimation on those enzymes. Livers, gills and olfactory tissues were collected from coho salmon (*Oncorhynchus kisutch*) after they had undergone acclimation from freshwater to various salinity regimes of seawater (8, 16 and 32 g/L). Using immunoblot techniques coupled with testosterone hydroxylase catalytic activities, 4 orthologs of cytochrome P450 (CYP1A, CYP2K1, CYP2M1 and CYP3A27) were measured in each tissue. Also the expression of 2 transcripts of flavin-containing monooxygenases (FMO A and B) and associated activities were measured. With the exception of CYP1A, which was down-regulated in liver, protein expression of the other 3 enzymes was induced at higher salinity, with the greatest increase observed in CYP2M1 from olfactory tissues. In liver and gills, 6 - and 16 -hydroxylation of testosterone was also significantly increased after hypersaline acclimation. Similarly, FMO A was up-regulated in all 3 tissues in a salinity-dependent pattern, whereas FMO B mRNA was down-regulated. FMO-catalyzed benzydamine *N*-oxygenase and methyl *p*-tolyl sulfoxidation were significantly induced in liver and gills by hypersalinity, but was either unchanged or not detected in olfactory tissues. These data demonstrate thatenvironmental conditions may significantly alter the toxicity of environmental chemicals in salmon during freshwater/saltwater acclimation.

Keywords

Salmonids; salinity acclimation; pesticide biotransformation; CYP450; FMO

Introduction

Pacific salmon populations have declined markedly in the Western United States due to a multitude of factors including loss of habitat, over-fishing, dam construction/operation, predation, diseases, parasites, climatic changes, oceanic shifts, and water pollution (Lackey 2003). The widespread contamination of surface waters and sediments, in particular, appears to be a limiting factor for the recovery of wild salmon stocks. Water quality monitoring conducted by the United States Geological Survey has indicated that many Pacific Northwest surface waters contain pesticide residues in river beds used by salmon for

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spawning and early life stages (Gilliom et al. 2006; Scholz et al. 2006). Pollutants in water may affect the physiology of fish olfaction, disrupting biologically relevant signals essential in their behavior that ultimately affect species survival (Moore and Lower 2001; Scott et al. 2003). In addition, several salmonid species of fish routinely move between fresh and saltwater to locate a critical habitat for spawning. While hypersaline conditions can provide a certain degree of protection to aquatic organisms from some xenobiotics such as metals, interactions with pesticides which often occur in urban and agriculturally impacted estuaries are less clear (Hall and Anderson 1995).

One of the most important processes in the disposition of xenobiotic and endogenous chemical fate within organisms is biotransformation. Biotransformation occurs through biochemical processes in which hydrophobic compounds are converted to more hydrophilic derivatives to promote or facilitate their elimination from the organism. Such processes are often categorized into Phase I and Phase II reactions. Phase I reactions carry out hydrolysis, oxidation, and reduction reactions, typically by adding or exposing functional groups (e.g., hydroxyl, sulfhydryl, carboxyl, amino). Phase II reactions tend to conjugate the chemical (often a Phase I metabolite) with endogenous biomolecules such as glucuronides or sulfates to further increase the water solubility of the compound (Kleinow et al. 2008).

The cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) enzymes are two well-studied oxidative enzymes of Phase I metabolism. CYP and FMO have been identified in fish and catalyze the sulfoxidation and bioactivation of several organophosphate (Lavado et al. 2009) and carbamate insecticides (Wang et al. 2001). A unique feature of FMO and CYP3A regulation within the salmonid, rainbow trout, is the up-regulation of expression and catalytic activity under hypersaline conditions (Lavado et al. 2009; Rodriguez-Fuentes et al. 2008; Rodriguez-Fuentes et al. 2009). Organophosphate (OP) and thioether carbamate insecticides, have significantly greater acute toxicity in several euryhaline fish species, including salmonids, following acclimation to hypersaline conditions (El-Alfy and Schlenk 1998; El-Alfy et al. 2001; Lavado et al. 2009). In each case where enhanced toxicity was observed, the pesticides were activated to more toxic oxon and/or sulfoxide intermediates following saltwater acclimation. Aldicarb sulfoxide was more than 100-fold more toxic than aldicarb in rainbow trout and Japanese medaka (El-Alfy and Schlenk 1998; Perkins and Schlenk 2000). Stereoselective formation of the most potent oxon-sulfoxide metabolite of fenthion was enhanced by acclimation to hypersaline conditions as was its precursor, the more potent fenoxon; and the less potent fenthion sulfoxide (Lavado et al. 2009). Oxidation of OPs and carbamates to more potent cholinesterase inhibitors occurs through orthologs of CYP and FMO, induced by hypersaline conditions in euryhaline fish (Larsen and Schlenk 2001; Lavado et al. 2011).

While previous studies showing impacts of hypersaline conditions on biotransformation have been reported in rainbow trout, few have examined other salmonids. The current study was designed to better characterize the expression and activity of Phase I biotransformation enzymes in coho salmon (*Oncorhynchus kisutch*) and the effects of salinity acclimation on those enzymes. Using real-time quantitative polymerase chain reaction (qPCR) and Western blotting, we characterized the expression pattern of different CYP isoforms (CYP1A, CYP2K1, CYP2M1, and CYP3A27) and different FMOs in fish acclimated to 4 different salinities: freshwater (<0.5 g/L), 8, 16 and 32 g/L. In addition, catalytic activities of CYP2-dependent and CYP3A27-dependent testosterone hydroxylase, and FMO-mediated methyl *p*-tolyl sulfoxidase and benzydamine *N*-oxidase activities were determined in microsomal fractions isolated from liver, gills and olfactory tissues. Salinities were chosen to mimic environmental conditions in the Pacific Northwest areas used by salmon for spawning (freshwater, <0.5 g/L) and early life stages (8 and 16 g/L). The highest salinity (32g/L) was chosen to mimic estuarine salinity (Cohen 2000; Knowles and Cayan 2002).

Material and Methods

Animals

Juvenile coho salmon (*Oncorhynchus kisutch*) were obtained from Nimbus Hatchery (Gold River, CA, USA). Organisms were maintained in a flow-through living-stream system with dechlorinated carbon-filtered municipal water at 13-15°C and acclimated for 2 months as minimum before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT, USA).

Salinity acclimation

After tank acclimation, fish were transferred and sequentially acclimated for two days to hypersaline water at concentrations of 4 g/L, followed by 8, 16, and 32 g/L saline concentration in 24 L aquaria. At selected salinities, blood samples were collected from the dorsal aorta using a latex-free 1 mL syringe and a 27-gauge needle (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to measure osmolality and they were left for one week. 5 animals and 2 replicates (total 10 animals) were used per experimental group. After acclimation, animals were weighted, measured and blood samples were collected again. The blood samples were immediately centrifuged at 5,000*g* for 5 min, plasma was collected and its osmolality was measured using a vapor pressure osmometer 5520 VAPRO™ (Wescor Inc, Mission Viejo, CA, USA), as it is a well-established indicator of hypersaline acclimation in euryhaline fish (Bjerknes et al. 1992; Ferraris et al. 1988). After that, fish were anesthetized using tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA, USA), killed by a blow to the head and the liver, gills and olfactory tissue were dissected and frozen in liquid nitrogen and stored at −80°C. All animals were euthanized according to IACUC guidelines (#20080017) approved by the University of California Riverside-IACUC.

FMO mRNA expression

Total mRNA was extracted from tissues using SV Total RNA Isolation System kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. FMO mRNA was quantified by qPCR by using iScript™ One-step RT-PCR kit with SYBR® Green from Bio-Rad (Hercules, CA, USA) and using different primers for specific FMO genes: FMO A (GenBank accession number EF063736) (sense primer: 5′-GGAAGTTCAAGGAAGTTTCTGAGCCC-3′; antisense primer: 5′-CCGCGCACTGGTCTGGAAGT-3′; amplification efficiency 96.3%), and FMO B (GenBank accession number EF063739) (sense primer: 5′-TATTCCTCAGTACCAGGCGTGC-3′; antisense primer: 5′-GACTGGGCCATCTTTAACCTT-3′; amplification efficiency 93.1%). Primer design was based on previous studies in rainbow trout (Lavado et al. 2013). Transcript size and sequence were identical between rainbow trout and coho salmon (data not shown). An absolute quantification (Pfaffl and Hageleit 2001) was performed by using standard calibration of FMO A and B cDNAs obtained from Lavado et al. (2013). 200 nM of each primer was added to 25 μL PCR reactions containing SYBR® Green RT-PCR Reaction Mix (Bio-Rad, Hercules, CA), 100 ng mRNA sample and iScript Reverse Transcriptase for One-Step RT-PCR from Bio-Rad. Thermocycling parameters were as follows: 10 min at 50°C (cDNA synthesis); 5 min at 95°C (iScript Reverse transcriptase inactivation); 40 cycles of 10 s at 95°C and 30 s at 59.5°C. Fluorescence data were collected at the end of each cycle. Following the amplification reaction, a melting curve analysis was carried out between 55°C and 95°C, fluorescence data were collected at 0.1°C intervals. The C(t) was selected to be in the linear phase of amplification. All real-time reactions were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad) and data analysis was done using IQ5 (Bio-Rad).

Subcellular fractionation

Livers, gills and olfactory tissues were selected due to their physiological roles in biotransformation (liver), osmoregulation (gills), and behavior (olfactory tissues). The subcellular fractionation was performed according to Lavado et al. (2011). Briefly, after weighing each tissue, it was rinsed in ice-cold 1.15% KCl and homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM 1,10-phenanthroline. Olfactory tissues were pooled from 3 different individuals, but gills and liver were analyzed from individual organisms. Homogenates were centrifuged at 500*g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000*g* for 20 min. The 12,000*g* supernatant was further centrifuged at 100,000*g* for 60 min to obtain microsomal fractions. Microsomal pellets were resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 100 mM KCl, 20% (w/v) glycerol, 1 mM EDTA, 0.1 mM PMSF and 0.1 mM 1,10-phenanthroline. Protein concentrations were determined by the Coomasie Blue method using a commercial kit (Pierce Inc., Rockford, IL, USA) using bovine serum albumin as a standard.

CYP1A1, CYP2M1, CYP2K1 and CYP3A27 immunoblot evaluations

CYP1A, CYP2K1, CYP2M1 and CYP3A27 protein levels were determined by Western immunoblot as described in Lavado et al. (2009) with minor modifications. Briefly, microsomal fractions were boiled for 5 min in SDS-PAGE buffer (Laemmli 1970), and 40 μg of proteins were loaded into 10% polyacrylamide gels. Proteins were separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. They were probed using a 1:1000 mouse anti-rainbow trout monoclonal CYP1A antibodies from Biosense (Bergen, Norway), 1:500 rabbit anti-rainbow trout polyclonal CYP2M1, 1:500 rabbit anti-rainbow trout polyclonal CYP2K1 provided by Dr. D. R. Buhler (Oregon State University, USA), and 1:1000 rabbit anti-rainbow trout polyclonal CYP3A27 antibodies provided by Dr. Malin Celander (University of Goteborg, Sweden). Blots were incubated at room temperature overnight and rinsed three times with Tris-buffered saline containing 0.2% Tween 20 (v/v) and 0.5% gelatin (w/v). The membranes were incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG, the excess of secondary antibody was removed, and immunoreactive bands were visualized by incubation with the substrates *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate from a commercial alkaline phosphatase conjugation kit (Bio-Rad, Hercules, CA, USA). Semi-quantification by densitometry was carried out using Quantity One (Bio-Rad) in a Molecular Imager Gel Doc XR System (Bio-Rad) image analyzer and presented as optical density units (ODU)/mg total protein.

[^{14}C]Testosterone hydroxylase activity

Testosterone hydroxylation activities were measured as described in Lavado et al (2009). Briefly, 0.4 mg hepatic microsomal protein was incubated with 12 μM [^{14}C]testosterone (150 μCi/μmol; 97.6% purity) and 600 μM NADPH in a final volume of 0.25 mL of 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4. Samples were incubated for 1 h at 25°C. Incubations were stopped by adding 250 μL of acetonitrile and after centrifugation (10,000*g*; 10 min), 350 μL of supernatant was injected into a reverse-phase HPLC column.

FMO-associated activities

FMO-associated activities were measured using methyl *p*-tolyl sulfide (MTS) and benzydamine (BZN) as substrates. Methyl *p*-tolyl sulfoxidation was measured as described in Lavado et al. (Lavado et al. 2013), which was a modification of Rettie et al. (1994). A 0.25 mL reaction volume containing 400 μg of microsomal protein, 1 mM NADPH, 3.3 mM

$MgCl_2$, 1 mM MTS in a 50 mM glycine buffer pH 9.0 was incubated at 25°C for 1 hour. This was in the linear range for catalytic activity. Blanks consisted in the addition of boiled proteins or the omission of NADPH. The reaction was stopped by the addition of 75 μL acetonitrile and centrifuged at 13,000*g* for 5 min. Supernatant was filtered with Millipore Durapore (Bedford, MA, USA) membrane and 40 μL were injected into the HPLC system. Benzydamine *N*-oxidation was measured as described in Schlenk et al. (2002) with minor modifications. A 0.25 mL reaction volume containing 200 μg of microsomal protein, 1 mM NADPH and 300 μM BZN in a 50 mM glycine buffer pH 9.0 was incubated at 25°C for 1 hour. This was in the linear range for catalytic activity. Blanks consisted in the addition of boiled proteins or the omission of NADPH. The reaction was stopped by the addition of 250 μL acetonitrile and centrifuged at 13,000*g* for 5 min. Supernatant was filtered with Millipore Durapore (Bedford, MA, USA) membrane and 40 μL were injected into the HPLC system. To minimize effects of cytochrome P450, co-incubations with 1mM ketoconazole (Sigma-Aldrich, St. Louis, MO, USA) were realized as this compound is a known cytochrome P450 inhibitor in rainbow trout (Miranda et al. 1998).

HPLC methods

For testosterone hydroxylated metabolites, HPLC analyses were performed on a SCL-10AVP Shimadzu HPLC system equipped with a 250 × 4.6 mm ODS Hypersil C18 (5 μm) reverse-phase column (Thermo Scientific, Waltham, MA, USA). Separation of testosterone metabolites employed an HPLC gradient system elution at a flow rate of 1 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile. The run consisted of a 40 min linear gradient from 100% A to 100% B and 40-45 min 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a radio-flow detector β-ram Model 3 (IN/US Systems Inc., Tampa, FL, USA) using In-Flow 2:1 (IN/US Systems Inc.) as scintillation cocktail. Metabolites (6β-, 16α- and 16β-hydroxytestosterone) were identified by co-chromatography with authentic standard compounds and quantified by integrating the area under the radioactive peaks (recovery was from 95.9% to 97.6% for each metabolite and the detection limit was 0.2 pmol/min/mg protein). Separation of methyl *p*-tolyl sulfide (MTS) and its sulfoxides was performed on the same HPLC system equipped with a Regis Technologies (*R*,*R*) Whelk-01 10/100 Chromasil chiral column. Samples were eluted with methanol 46% (v/v) (0-7 min) that was slowly increased to 100% (7-20 min). Purified (*R*)- and (*S*)-MTSO were used to establish a standard curve. Peaks were monitored with a UV-detector SPD-10AVP Shimadzu at 237 nm, quantified by integrating the area under the peaks and identified with co-elution of authentic standards. (*R*)- and (*S*)-enantiomers were eluted with retention times of 13 min and 15 min, respectively and the detection limit of each metabolite was 0.3 pmol/min/mg protein. Benzydamine *N*-oxide was analyzed in the same HPLC equipment but with an Agilent Hypersil ODS (4.0 mm × 250 mm) C18 (5 μm) reverse-phase column and using an isocratic system with the mobile phase composed of methanol-acetonitrile-water-NH_4OH (46:36:17.5:0.5) at flow rate of 1 mL/min. Purified benzydamine *N*-oxide was used to establish a standard curve. Peaks were monitored with a fluorescent detector RF-10AXL Shimadzu, using λ_{ex}=307 and λ_{em}=377, quantified by integrating the area under the peaks and identified with coelution of authentic standards. The detection limit of each metabolite was 0.2 pmol/min/mg protein.

Statistical procedures

Statistical significance was assessed using a Student's *t* test and one-way ANOVA to evaluate differences between groups, with the use of GraphPad Prism version 5.00 for MacOS X (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's and Bonferroni's multiple range tests were performed. Samples showing

levels below the detection limits were considered as having 50% of the minimal values detectable for statistical comparisons. All data was analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests.

Results

Morphometric parameters

The impacts of acclimation to hypersaline conditions are provided in Table 1. Acclimation did not alter the body weight and length, or condition factor (CF), calculated as weight/lentgh^3. During the acclimation process, plasma osmolality increased in a dose-dependent manner, but after acclimation (one week at selected salinity), plasma osmolality recovered to freshwater baseline levels (Sower and Schreck 1982; Wertheimer 1984).

Immunoblotting and CYP catalytic activities

Western blots of coho salmon microsomes confirmed the presence of CYP1A, CYP2K1, CYP2M1, and CYP3A27 proteins (Table 2). Salinity acclimation significantly reduced ($p<0.05$) the expression of hepatic CYP1A below detection levels (<0.10 ODU/mg protein). CYP1A was not detected in gills nor olfactory tissues. CYP2K1 was observed in liver and olfactory tissues at similar levels but was not detected in gills. Salinity acclimation significantly increased CYP2K1 protein levels in liver in the highest salinity group (32 g/L). CYP2M1 protein was detected in each of the three tissues, but was 5.6-fold higher in liver and olfactory tissues compared to gills. Salinity acclimation significantly increased ($p<0.05$) the expression in the 3 tissues, with increases of up to 1.5-fold observed in liver, 1.4-fold in gills and 2.8-fold in olfactory tissues compared to freshwater (<0.5 g/L) animals. CYP3A27 protein was also detected in the three tissues with the highest levels observed in liver. Salinity acclimation also significantly increased ($p<0.05$) expression in the 3 tissues, with increases in the highest salinity group of up to 1.6-fold in liver, 1.8-fold in gills and 3.9-fold in olfactory tissues when compared to freshwater (<0.5 g/L) acclimated animals.

Testosterone 16 -hydroxylation was detected only in liver and it was not significantly altered by salinity acclimation ($p<0.05$) (Table 3). CYP2K1 and CYP3A27-dependent activities of testosterone 6 - and 16 -hydroxylation, respectively, were observed only in liver and gills, but not in olfactory tissues. Both enzymatic activities were higher (up to 3.8-fold) in liver compared to gills ($p<0.05$). Salinity acclimation significantly increased both activities in a dose-dependent manner in both tissues (Table 3).

FMO expression and catalytic activities

The results of the qPCR analysis of FMO gene expression in coho tissues are presented in Figure 1. Both FMO mRNAs were detected in liver, gills and olfactory tissues. The magnitude of expression of FMO A was as follows: liver=gills>olfactory tissues; and for FMO B, liver=olfactory tissues>gills. Salinity acclimation significantly increased FMO A mRNA expression in all tissues in a dose-dependent manner, being significant only in the 32 g/L salinity acclimation group ($p<0.05$). A 5.8-fold increase of FMO A mRNA was observed in liver following saltwater acclimation. Respective 8.9- and 5.3-fold increases were observed in gills and olfactory tissues from animals acclimated to 32 g/L. In contrast to FMO A, salinity acclimation significantly reduced the expression of FMO B mRNA in all tissues ($p<0.05$), with reductions ranging from 1.2- to 5.1-fold in the different tissues.

FMO-associated activities, methyl *p*-tolyl *S*-oxidase (MTS) and benzydamine *N*-oxidase (BZN) are shown in Figure 2. MTS oxidase activity was higher in liver than in gills and olfactory tissues. Salinity acclimation significantly increased MTS biotransformation in liver and gills in the groups acclimated to 16 and 32 g/L ($p<0.05$). In olfactory tissues, salinity

acclimation did not alter MTSO formation (Figure 2A). Stereoselective formation of *S*-MTSO was observed in liver and gill microsomes with approximately 65% formation of *S*-MTSO (Table 4). In olfactory tissues, 100% stereoselectivity for *S*-MTSO was observed. Acclimation to salinity had no significant change on *S*-oxidation stereoselectivity. BZN oxidase activity was also higher in liver than in gills and olfactory tissues, being below detection levels (<0.2 pmol/min/mg protein) in that tissue. BZN oxide formation in saline acclimated-animals increased significantly ($p<0.05$) up to 5.8-fold in liver and up to 5.2-fold in gills of fish acclimated to the highest salinity regime compared to freshwater (<0.5 g/L) acclimated fish. Co-incubations with 1 mM ketoconazole did not significantly alter MTS and BZN oxidation ($p<0.05$) (data not shown) showing that CYP did not contribute to these oxidation reactions.

Discussion

Salmonids undergo significant endocrine and physiological changes moving from freshwater and saltwater environments. Studies in rainbow trout have reported that enzymes involved in the bioactivation and detoxification of xenobiotics or steroids can be significantly altered and that the toxicity of compounds can be enhanced or diminished depending upon the role the enzymes play in activation or detoxifying the compound (Kennedy 2011; Lavado and Schlenk 2011). Although studies have demonstrated the linkage between hypersaline acclimation, chemical biotransformation and toxicity in rainbow trout, few have done so in other salmonids. The purpose of this study was to better understand this linkage in coho salmon (*O. kisutch*).

CYP1A is one of the most studied xenobiotic enzymes in aquatic organisms because of its use as a biomarker of exposure to planar aromatic hydrocarbons such as PAHs and PCBs (Stegeman and Hahn 1994). Consistent with earlier studies with rainbow trout (Lavado et al. 2009), acclimation to hypersaline conditions in coho salmon diminished CYP1A protein expression. In human hepatocytes, hyperosmotic conditions enhanced expression of CYP1A1 mRNA, but diminished expression of CYP1A2 (Ito et al. 2007). The authors suggested that hypersaline conditions could negatively alter the stability of the CYP1A2 transcript. However, given the structural similarities between the CYP1A1 and CYP1A2 transcripts it is unclear why the stability of only one would be affected and not the other. Other studies in salmonids showed that reduction of CYP1A may be mediated through enhanced glucocorticoid (e.g. cortisol) production, which occurs in salmonids undergoing acute stress (Schreck 1981). Hepatic CYP1A mRNA and protein expression were reduced following treatment with the glucocorticoid, dexamethasone in rainbow trout (Haasch et al. 1994). Stress was also shown to impair CYP1A catalytic activity in the Artic charr (*Salvelinus alpinus*) (Jorgensen et al. 2001). Given the importance of CYP1A in PAH and PCB bioactivation, reduction of their toxicity would be expected in fish after saltwater acclimation. It is also important to mention that toxicity of other pollutants, normally detoxified by the CYP1A, may increase and due to reduced CYP1A some pollutants might be more bioaccumulated, which could pose serious risk related to human consumption. It is unclear whether movement into freshwater upon spawning would induce CYP1A in salmonids leading to enhanced toxicity, but the possibility of this phenomena suggests additional studies are needed given the co-occurrence of substrates activated by CYP1A (e.g. PAHs, PCBs) in estuarine environments.

In contrast to CYP1A, CYP2M1, CYP2K1 and CYP3A27 proteins were all increased in liver microsomes after salinity acclimation. CYP2M1 was increased about 30 and 50% in gills and liver, respectively. However, it was increased nearly 300% in olfactory tissues. While purified CYP2M1 from rainbow trout liver had minimal steroid hydroxylase activities, its predominant catalytic activity was −6 hydroxylation of lauric acid (Miranda et

al. 1990). Similarly CYP2K1 also possessed significant fatty acid hydroxylase activities (Buhler and Wang-Buhler 1998; Miranda et al. 1989). Other CYP2 family proteins have been shown to catalyze oxygenation of cellular fatty acids including arachidonic acid (AA) in fish and mammals (Arnold et al. 2010; Oleksiak et al. 2000). Oxidation of AA may play a significant role with regard to cellular membrane stability, osmolyte efflux and osmoregulation (Furlong et al. 1991). Although both CYP isoforms catalyze lauric acid hydroxylase, it is unclear whether CYP2M1 or CYP2K1 catalyzes the oxidation of AA in salmonids and what impact this may have in cellular osmoregulation or in olfactory function. The significant expression of CYP2M1 and 2K1 in olfactory tissues and the inducibility of CYP2M1 by saline acclimation is novel and warrants further characterization.

The differential regulation and expression of CYP2K1 and CYP2M1 in salmon tissues suggests unique functional roles for each isoform. CYP2K1 is the major constitutive form in rainbow trout liver and catalyzes the oxidation of several endogenous substrates including fatty acids, the 2-hydroxylation of E2, the 16 -hydroxylation of testosterone and the 16 -hydroxylation of progesterone (Buhler and Wang-Buhler 1998). The induction of 16 -testosterone hydroxylase with hypersaline acclimation in coho salmon liver is consistent with expression of CYP2K1 in the liver. However, the nearly 3-fold induction of activity in gills did not correspond to expression of CYP2K1 protein suggesting that other CYP450 isoforms may contribute to 16 - testosterone hydroxylation in this tissue. Although 6 -hydroxylase activity was high for purified CYP3A27 in rainbow trout, significant 16 -hydroxylase activity was also noted for CYP3A27(Miranda et al. 1989). The corresponding increase of both testosterone hydroxylase activities with CYP3A27 protein confirms the induction of both CYP2K1 and CYP3A27 in the liver by hypersaline acclimation. This is the first reported observation of CYP2K1 induction. Since the gill is the primary osmoregulatory organ of fish, the lack of CYP2K1 expression in the gill indicates it may not play a significant role in the key function of gills and olfactory tissue where its expression was unaltered by hypersaline acclimation.

In contrast to CYP2K1 and CYP2M1, CYP3A27 is the predominant steroid hydroxylase of the salmonid cytochrome P450s (Miranda et al. 1989) and, contrary to CYP1A, is induced (not as significantly as mammals) by glucocorticoid receptor agonists (see Buhler and Wang-Buhler (1998) for review). The reduction of CYP1A and induction of CYP3A by hypersaline conditions is consistent with the concomitant production of cortisol during saltwater acclimation (Schreck 1981). Previous studies in rainbow trout also reported significant induction of hepatic CYP3A27 protein and 6 - and 16 -hydroxylase activities in fish after acclimation to 17 ppth (g/L) seawater (Lavado et al. 2009).

Expression of FMO A mRNA strongly correlated with two catalytic activities in liver and gills, and each were enhanced by hypersaline acclimation in a concentration-dependent manner. Although similar results were recently observed in livers of rainbow trout after saltwater acclimation (Lavado et al. 2013), one notable exception was the concurrent increase of BZNO and MTSO formation in gill of coho salmon. In rainbow trout, only MTSO formation correlated with FMOA mRNA (Lavado et al. 2013). One possible explanation may be that coho salmon are more tolerant to saline water with exposures occurring without mortality up to full strength seawater (32 g/L). Rainbow trout could only tolerate 18 g/L using the same acclimation protocol (Lavado et al. 2013; Lavado et al. 2009). FMO may play a role in the osmoregulation through the formation of “compatible solutes” such as trimethylamine oxide which is formed to counter the enhanced formation of urea which occurs in fish undergoing saltwater acclimation (Larsen and Schlenk 2002). The higher tolerance of salmon may be due to the expression of an FMO orthologous with greater catalytic efficiency in the gill, or a splice variant that carries out both *S*- and *N*-oxygenation. FMO catalytic efficiency is relatively sensitive to pH and temperature

(Cashman and Zhang 2006; Ziegler 2002). Alternatively, splice variants of FMO have been observed in mammalian studies and have significant differences in substrate specificity and turnover (Hines et al. 2002; Lattard et al. 2004). Since nucleotide sequences of the FMO A transcript were identical between rainbow trout and coho salmon, post-translational modification or splice variant formation appears to be more likely.

As with rainbow trout, FMO B mRNA expression in coho salmon was significantly reduced in all tissues following hypersaline acclimation. The corresponding down-regulation of FMO B and CYP1A is consistent with recent studies in mammals showing the potential for co-regulation of each enzyme potentially through downstream aryl-hydrocarbon receptor or P53 signaling (Celius et al. 2010). In mouse liver and immortal hepatocytes treated with AhR agonists, expression of the transcripts of various FMOs did not correspond to the magnitude of induction to corresponding protein or catalytic activities (Celius et al. 2010). Likewise, FMO B transcripts did not correspond to either catalytic activity in all tissues evaluated. Whether FMO B has different substrate specificity than FMO A requires additional study. And the salinity-dependent diminishment of FMO B in the same tissues of two salmonid species following acclimation likewise warrants additional investigation.

In conclusion, acclimation of coho salmon to various salinity regimes led to salinity-dependent increases and reductions of critical xenobiotic biotransformation enzymes or their transcripts. Given the importance of these enzymes in the bioactivation and/or detoxication of numerous environmental chemicals that have previously been observed to co-occur in areas where fish undergo acclimation, and have been shown to be more toxic, additional studies exploring the regulatory mechanisms in salmonids are necessary to better understand the combined stress of chemical insult with physiological acclimation in catadromous and anadromous species of fish.

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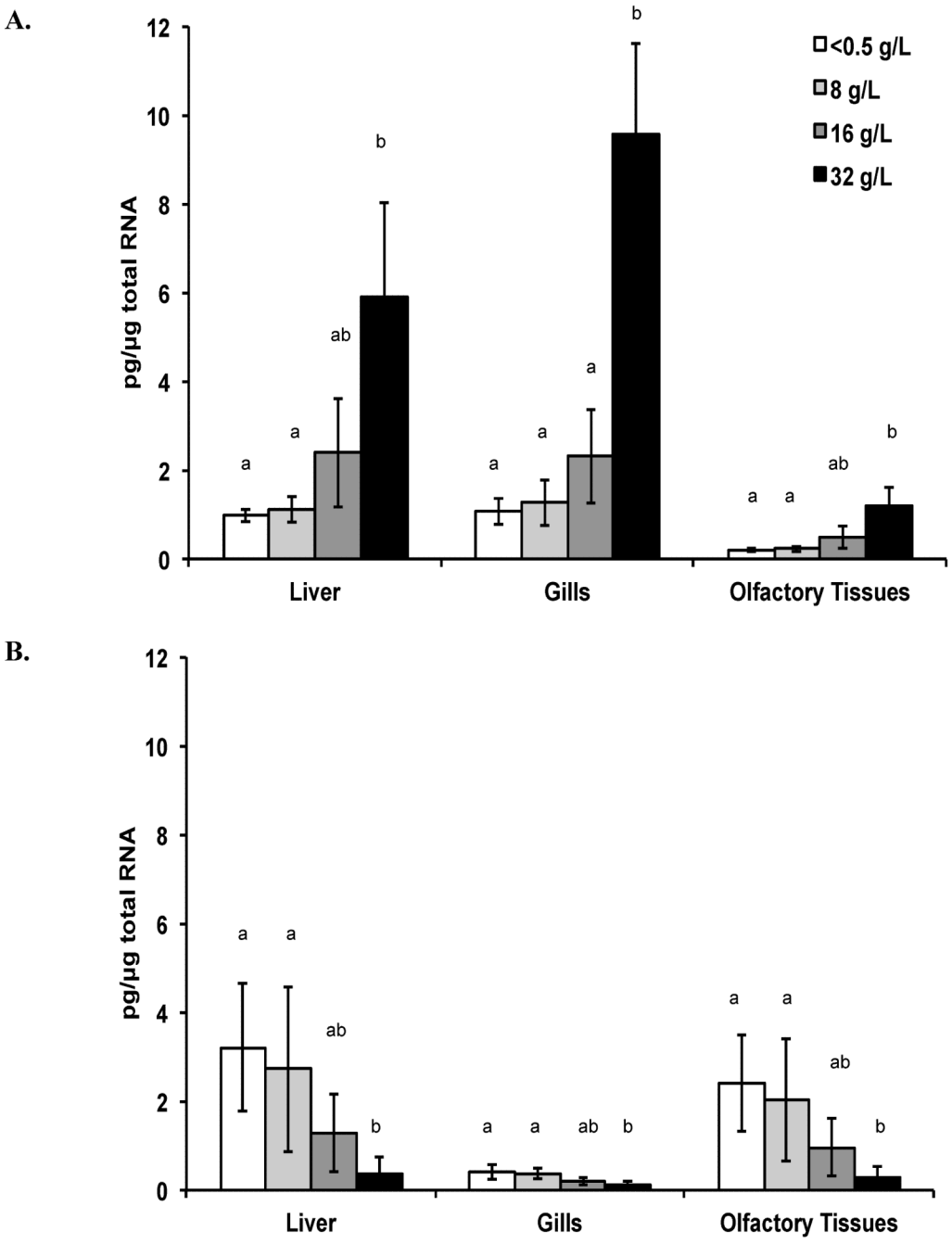

Fig. 1.

Effects of different saline acclimations on the expression of different FMO mRNAs: A) FMO A and B) FMO B in liver, gills and olfactory tissues from coho salmon (*Oncorhynchus kisutch*). Data are expressed as pg/μg of total mRNA and as mean ± SD (n=8-10). Different letters indicate significant differences ($p<0.05$; One-way ANOVA, Tukey's test).

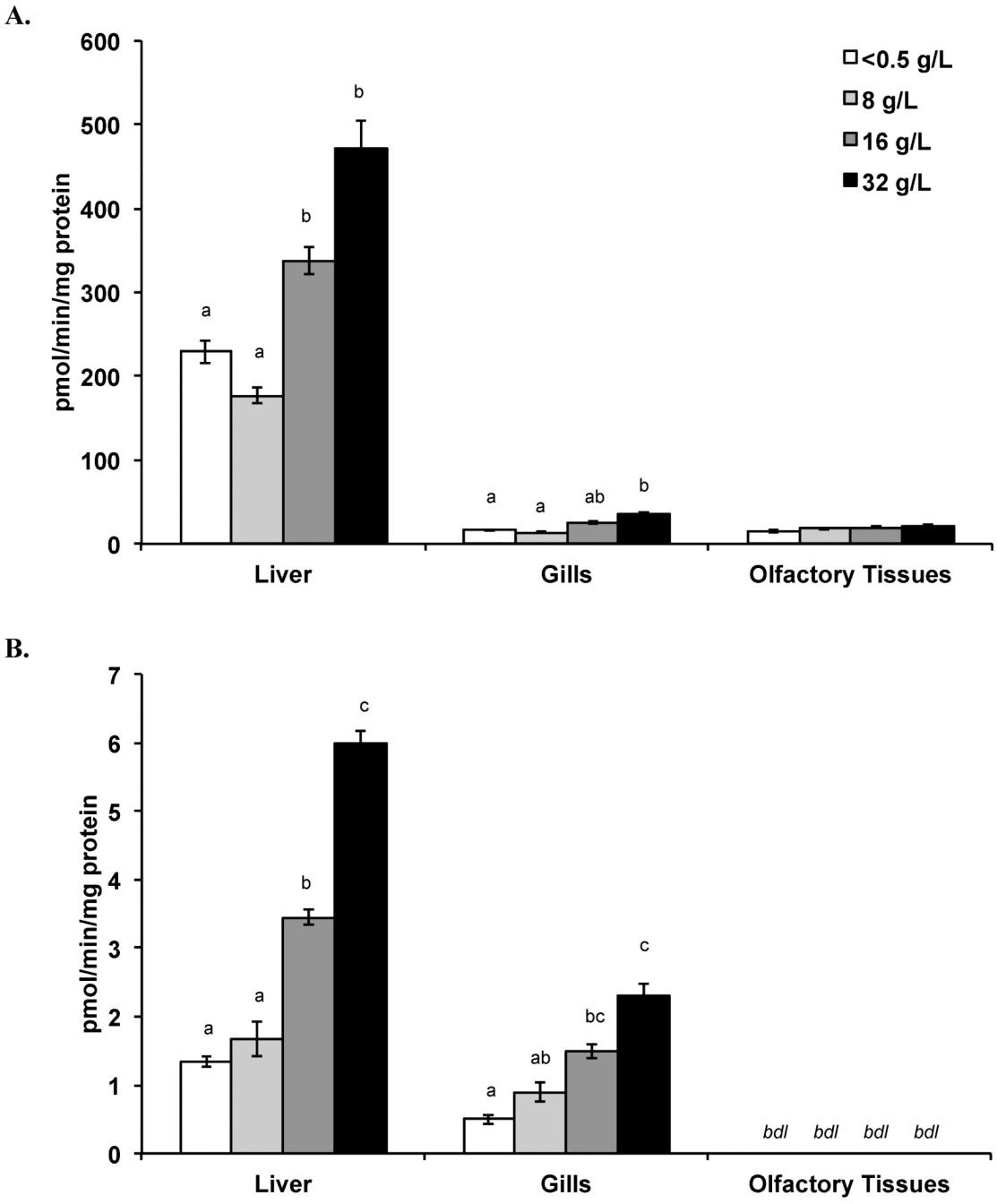

Fig. 2.

FMO-associated activities in microsomal fractions isolated from liver, gills and olfactory tissues from coho salmon (*Oncorhynchus kisutch*) acclimated to different salinities (<0.5, 8, 16 and 32 g/L). Methyl *p*-tolyl sulfoxidation (A) and benzydamine *N*-oxidation (B) are shown as mean ± SD (n=8-10 for liver and gills; n=3 pools of 3 animals each for olfactory tissues). Different letters indicate significant differences ($p<0.05$; One-way ANOVA, Tukey's test). *bdl*: below detection level (<0.2 pmol/min/mg protein).

Table 1

Morphometric and plasma osmolality of coho salmon acclimated to different salinities. Values are presented as mean ± SD (n=8-10). CF: condition factor calculated as (weight/length^3) × 100. Lowercase letters are used for statistical comparisons; different letters indicate significant differences between salinity groups ($p<0.05$; One-way ANOVA, Tukey's test).

	Salinity			
	<0.5 g/L	**8 g/L**	**16 g/L**	**32 g/L**
Total length (cm)	21.3 ± 2.2	22.3 ± 1.5	21.8 ± 1.7	21.3 ± 1.4
Body weight (g)	93.7 ± 32.4	112.6 ± 29.9	101.9 ± 25.5	94.4 ± 26.8
CF	0.94 ± 0.10	1.00 ± 0.07	0.97 ± 0.07	0.96 ± 0.19
Plasma osmolarity at T=0 of acclimation (mmol/kg)	270.6 ± 7.1^a	289.4 ± 4.4^a	306.2 ± 5.6^b	346.3 ± 1.6^c
Plasma osmolarity one week after acclimation (mmol/kg)	274.6 ± 2.4	269.3 ± 10.2	276.8 ± 3.4	280.7 ± 11.1

Table 2

Effects of different saline acclimations on the expression of different cytochromes P450 (CYP1A1, CYP2K1, CYP2M1 and CYP3A27) in microsomal fractions isolated from liver, gills and olfactory tissues from coho salmon (*Oncorhynchus kisutch*). Data are expressed as Optical Density Units (ODU)/mg total protein and as mean ± SD (n=8-10 for liver and gills; n=3 pools of 3 animals each for olfactory tissues). Different letters indicate significant differences ($p<0.05$; One-way ANOVA, Tukey's test).

CYP	Salinity			
Tissue	**<0.5 g/L**	**8 g/L**	**16 g/L**	**32 g/L**
CYP1A1				
Liver	2.19 ± 0.20[a]	0.71 ± 0.29[b]	*bdl* [c]	*bdl* [c]
Gills	*bdl*	*bdl*	*bdl*	*bdl*
Olfactory Tissues	*bdl*	*bdl*	*bdl*	*bdl*
CYP2K1				
Liver	15.10 ± 1.15[a]	14.60 ± 2.35[a]	17.68 ± 5.86[ab]	21.43 ± 2.03[b]
Gills	*bdl*	*bdl*	*bdl*	*bdl*
Olfactory Tissues	11.86 ± 2.16	10.35 ± 1.25	12.78 ± 0.64	10.56 ± 2.50
CYP2M1				
Liver	10.25 ± 1.01[a]	10.39 ± 1.44[a]	13.97 ± 1.63[b]	15.32 ± 1.28[b]
Gills	1.82 ± 0.36[a]	1.39 ± 0.28[a]	2.85 ± 0.65[b]	2.42 ± 0.60[b]
Olfactory Tissues	13.55 ± 1.25[a]	23.41 ± 3.68[b]	37.09 ± 3.92[c]	37.35 ± 1.85[c]
CYP3A27				
Liver	16.26 ± 2.06[a]	20.07 ± 2.48[a]	21.16 ± 4.27[ab]	26.12 ± 2.58[b]
Gills	4.11 ± 1.04[a]	5.57 ± 1.62[ab]	6.08 ± 1.98[ab]	7.50 ± 1.33[b]
Olfactory Tissues	1.90 ± 0.59[a]	4.32 ± 1.79[ab]	7.16 ± 1.42[b]	7.52 ± 0.82[b]

bdl: below detection limit (<0.10 ODU/mg protein)

Table 3

Effects of different saline acclimations on the *in vitro* biotransformation of testosterone in microsomal fractions isolated from liver, gills and olfactory tissues from coho salmon (*Oncorhynchus kisutch*). Data are expressed as pmol/min/mg protein and as mean ± SD (n=8-10 for liver and gills; n=3 pools of 3 animals each for olfactory tissues). Different letters indicate significant differences ($p<0.05$; One-way ANOVA, Tukey's test).

Metabolite	**Salinity**			
Tissue	**<0.5 g/L**	**8 g/L**	**16 g/L**	**32 g/L**
16α-Hydroxytestosterone				
Liver	3.12 ± 0.63	3.40 ± 1.15	3.39 ± 1.68	4.14 ± 1.01
Gills	*bdl*	*bdl*	*bdl*	*bdl*
Olfactory Tissues	*bdl*	*bdl*	*bdl*	*bdl*
6β-Hydroxytestosterone				
Liver	13.16 ± 3.30^{a}	10.42 ± 2.84^{a}	16.82 ± 4.98^{ab}	20.44 ± 2.51^{b}
Gills	2.31 ± 0.18^{a}	2.94 ± 1.42^{ab}	2.17 ± 1.97^{ab}	3.78 ± 0.34^{b}
Olfactory Tissues	*bdl*	*bdl*	*bdl*	*bdl*
16β-Hydroxytestosterone				
Liver	20.38 ± 4.87^{a}	20.40 ± 4.14^{a}	26.39 ± 5.08^{ab}	35.58 ± 4.91^{b}
Gills	5.43 ± 3.17^{a}	6.49 ± 3.03^{a}	11.19 ± 3.85^{ab}	15.02 ± 5.70^{b}
Olfactory Tissues	*bdl*	*bdl*	*bdl*	*bdl*

bdl: below detection limit (<0.024 pmol/min/mg protein)

Table 4

S-oxidation of methyl *p*-tolyl in microsomal fractions isolated from coho salmon (*O. kisutch*) acclimated to different salinities (<0.5 g/L and 18 g/L). Data are presented as % of total metabolites and as mean ± standard deviation (n=8-10 for liver and gills; n=3 pools of 3 animals each for olfactory tissues). Different letter indicated significant differences between tissues ($p<0.05$; One-way ANOVA).

	***R*-MTSO**				***S*-MTSO**			
	<0.5 g/L	**8 g/L**	**16 g/L**	**32 g/L**	**<0.5 g/L**	**8 g/L**	**16 g/L**	**32 g/L**
Liver	31 ± 2[a]	34 ± 6[a]	29 ± 5[a]	37 ± 6[a]	68 ± 8[a]	65 ± 4[a]	70 ± 5[a]	62 ± 4[a]
Gills	30 ± 4[a]	34 ± 2[a]	34 ± 4[a]	38 ± 7[a]	69 ± 6[a]	65 ± 8[a]	65 ± 6[a]	61 ± 3[a]
Olfactory tissues	*bdl* [b]	*bdl* [b]	*bdl* [b]	*bdl* [b]	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]

R-MTSO: *R*-Methyl *p*-tolyl sulfoxide.

S-MTSO: *S*-Methyl *p*-tolyl sulfoxide.

bdl: below detection limit (<0.3 pmol/min/mg protein).