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Effects of salinity acclimation on the pesticide-metabolizing enzyme flavin-containing monooxygenase (FMO) in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Thioether-containing pesticides are more toxic in certain anadromous and catadromous fish species that have undergone acclimation to hypersaline environments. Enhanced toxicity has been shown to be mediated through the bioactivation of these xenobiotics by one or more flavin-containing monooxygenases (FMOs), which are induced by hyperosmotic conditions. To better understand the number of FMO genes that may be regulated by hyperosmotic conditions, rainbow trout (*Oncorhynchus mykiss*) were maintained and acclimated to freshwater (<0.5 g/L salinity) and to 18 g/L salinity. The expression of 3 different FMO transcripts (A, B and C) and associated enzymatic activities methyl *p*-tolyl sulfoxidation (MTSO) and benzydamine *N*-oxygenation (BZNO) were measured in four tissues. In freshwater-acclimated organisms FMO catalytic activities were as follows: liver>kidney>gills>olfactory tissues; in hypersaline-acclimated animals activities were higher in liver>gills>olfactory tissues>kidney. Acclimation to 18 g/L caused a significant induction in the stereoselective formation of *R*-MTSO in gill. In olfactory tissues, stereoselective (100%) formation of *S*-MTSO was observed and was unaltered by acclimation to hypersaline water. When specific transcripts were evaluated, salinity-acclimation increased FMO A in liver (up to 2-fold) and kidney (up to 3-fold) but not in olfactory tissues and gills. FMO B mRNA was significantly down-regulated in all tissues, and FMO C was unchanged by hypersaline acclimation. FMO B and C failed to correlate with any FMO catalytic activity, but FMO A mRNA expression linearly correlated to both FMO catalytic activities (MTSO and BZNO) in liver ($r^2=0.92$ and $r^2=0.88$) and kidney microsomes ($r^2=0.93$ and $r^2=0.90$). FMO A only correlated with MTSO activity in gills ($r^2=0.93$). These results indicate unique tissue specific expression of FMO genes in salmonids and are consistent with salinity-mediated enhancement of thioether-containing pesticide bioactivation by FMO which may occur in liver or kidney after salinity acclimation.

Keywords

Flavin-containing monooxygenases; biotransformation; salmonids; extrahepatic metabolism

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1. Introduction

Flavin-containing monooxygenases (FMOs) are a polymorphic family of enzymes found in the smooth endoplasmic reticulum of multiple tissues that generally catalyze the oxidation of soft nucleophilic heteroatom substances to their respective oxides (Cashman, 2008; Krueger and Williams, 2005; Ziegler, 2002). FMO substrates include alkaloids, pesticides, and pharmaceutical compounds (Cashman and Zhang, 2006; Rettie and Fisher, 1999). Five FMO isoforms have been identified in mammals and differ in tissue distribution, regulation, and substrate specificity (Cashman and Zhang, 2006; Krueger and Williams, 2005; Luo and Hines, 2001). Only 5 of 11 genes express functional proteins in mammals (Lawton et al., 1994). FMO genes are located in two clusters on the long arm of chromosome 1 with 5 pseudogenes on one of the clusters (Hernandez et al., 2004). FMOs have been shown to be regulated in response to changes in diet (Katchamart et al., 2000), exposure to sex hormones (Lattard et al., 2002; Lee et al., 1993; Ripp et al., 1999), and pregnancy (Williams et al., 1985). In other organisms, hypoxia (Shen et al., 2005), cortisol (El-Alfy et al., 2002), urea, diminished temperature (Larsen and Schlenk, 2002), and hyperosmotic conditions (Rodriguez-Fuentes et al., 2009).

Several Salmonid species of fish routinely move between fresh and saltwater using olfaction to locate 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). Hypersaline conditions increased mortality resulting from fenthion exposure in rainbow trout and striped bass (Bawardi et al., 2007). Enhanced formation of the more potent metabolite fenoxon from fenthion and fenoxon sulfoxide from fenoxon, and reduced fenthion ester cleavage was observed in liver microsomes from rainbow trout after undergoing acclimation to hypersaline conditions and may explain the increased toxicity (Lavado et al., 2009). Similar results were observed with other thio-ether anticholinesterase pesticides, aldicarb and phorate, which were converted to the more potent metabolite, aldicarb sulfoxide and phorate-oxon, under hypersaline conditions in salmonids and other euryhaline fish (El-Alfy and Schlenk, 1998; El-Alfy et al., 2001; Lavado et al., 2011).

In fish and mammals, biotransformation enzymes are primarily found in the liver, although extra-hepatic tissues are also involved in the biotransformation of xenobiotics (e.g. gut, kidneys, gills and the olfactory system) (Buhler and Wang-Buhler, 1998; Matsuo et al., 2008; Monod et al., 1994; Stegeman, 1989). Despite extensive investigation on the role of Phase I enzymes in liver and gills, little is known about the activity of these enzymes in the olfactory systems of fish which is of particular interest given the fundamental importance of olfaction in migratory salmonids (Tierney et al., 2008). In addition, because the kidney can significantly contribute to extra-hepatic biotransformation and bioactivation of nephrotoxic xenobiotics such as haloalkane thioethers (Barshteyn and Elfarra, 2007; Elfarra, 1995), it is also critical to understand the impact of physiological alterations, which influence expression of FMO within kidney as well. The hypothesis that osmotic pressure plays a direct role on FMO expression and activity was evaluated by analyzing the expression of 3 novel FMO genes in rainbow trout (FMO A, B and C) and the correlation with FMO-associated enzymatic activities in different tissues (liver, gills, olfactory tissues and kidney) from juvenile rainbow trout acclimated to freshwater (<0.5 g/L salinity) and hypersaline conditions (18 g/L salinity).

2. Material and Methods

2.1. Standards reagents

Methyl-*p*-tolyl sulfide (MTS), (*R*)- and (*S*)-methyl-*p*-tolyl sulfoxides ((*R*)- and (*S*)-MTSO), benzydamine (BZN) and benzydamine *N*-oxide (BZNO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, ethanol and acetonitrile were of analytical grade (Fisher, Pittsburg, PA). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Organisms

Juvenile rainbow trout (*Oncorhynchus mykiss*) (age approximately 5 months, 16 ± 3 cm), without discernible gonadal morphology indicative of gender, were obtained from Jess Ranch Fish Hatchery (Apple Valley, CA, USA). Organisms were maintained in a flow-through living-stream system with dechlorinated carbon-filtered municipal water at 11–13 °C and acclimatized for 2 months before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT, USA).

2.3. Hypersalinity 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, 12 and 18 g/L saline concentration in 24 L aquaria. At 18 g/L salinity, they were left for two weeks. 8 animals and 3 replicates (total 24 animals) were used per experimental group. After acclimation, animals were measured and blood samples were collected from the dorsal aorta using a latex-free 1 syringe and a 27-gauge needle (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The samples were immediately centrifuged at 5,000 *g* for 5 min, plasma was collected before and after the last 2 weeks and its osmolality was measured using a vapor pressure osmometer 5520 VAPRO™ (Wescor Inc, Mission Viejo, CA, USA), as it is an indicator of hypersaline acclimation in euryhaline fish (Bjerknes et al., 1992; Ferraris et al., 1988). 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, olfactory tissues and kidney were dissected and frozen in liquid nitrogen and stored at –80 °C. A small portion of each tissue (approximately 20 mg) was stored in RNeasy Lysis Buffer (Applied Biosystems, Carlsbad, CA, USA) for qPCR determinations. All animals were euthanized according to IACUC guidelines (#20080017) approved by the University of California Riverside-IACUC.

2.4. FMO mRNA expression

Total mRNA was extracted from cells 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 (Table 1) for specific FMO genes: FMO A (E1-2F/E-3R), FMO B (5qL1/3qL1) and FMO C (5qL3/3qL3). Primers for FMO A were designed from previous sequences (bp 131–341) obtained from Rodriguez-Fuentes et al. (2008), and the cDNAs of FMO B (bp 393–623) and FMO C (bp 604–747). β -Actin was used as housekeeping gene in all cases and as sense primer 5'-GTCCTTCATGATTCTCTGCTGA-3' and antisense primer 5'-ACTCGGGTTCATTTGCATAAACA-3'. Each primer (200 nMoles; FMO or β -actin) was added to 25 μ L PCR reactions containing SYBR® Green RT-PCR Reaction Mix (Bio-Rad), 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).

2.5. Subcellular fractionation

Aliquots for mRNA expression and catalytic activity were obtained from the same individual tissue sample. Olfactory tissue samples were pooled from 6–8 different individuals, but gills, liver and kidneys were analyzed from individual organisms. Livers, gills, olfactory tissues and kidney were selected due to their physiological roles in biotransformation (liver), osmoregulation (gill and kidney), and behavior (olfactory tissues). Subcellular fractionation was performed according to Lavado et al. (2011). Briefly, after weighing each tissue or tissue pool, it was rinsed in ice-cold 1.15% KCl and homogenized in 1:5 w/v of cold 100 mM KH₂PO₄/K₂HPO₄ 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. 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 KH₂PO₄/K₂HPO₄ 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 Coomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL, USA) and using bovine serum albumin as a standard.

2.6. FMO-associated activities

FMO-associated activities were measured using methyl-*p*-tolyl sulfide (MTS) and benzydamine (BZN) as substrates. Stereoselective methyl-*p*-tolyl sulfoxidation was measured as described in Lavado et al. (2012), 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 MgCl₂, 1 mM MTS in a 50 mM glycine buffer pH 9.0 were incubated at 25 °C for 1 h. 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 started with the addition of the substrate after pre-incubation of buffer, NADPH and microsomal proteins for 5 min. 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 were incubated at 25 °C for 1 h. This was in the linear range for catalytic activity. As for MTS oxidation, the reaction was started with the addition of the substrate after pre-incubation of buffer, NADPH and microsomal proteins for 5 min. 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 membrane and 40 µL were injected into the HPLC system. To minimize effects of cytochrome P450, co-incubation with 1 mM ketoconazole (Sigma-Aldrich) were carried out as this compound is a cytochrome P450 inhibitor in rainbow trout (Miranda et al., 1998).

2.7. HPLC methods

Separation of methyl-*p*-tolyl sulfide (MTS) and its sulfoxides was performed on a SCL-10AVP Shimadzu 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 with the same instrument and an Agilent Hypersil ODS (4.0 mm × 250 mm) column and using an isocratic system with the mobile phase composed of methanol-acetonitrile-water-NH₄OH (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 $\lambda_{\text{ex}}=307$ and $\lambda_{\text{em}}=377$, quantified by integrating the area under the peaks and identified with co-elution of authentic standards. The detection limit of each metabolite was 0.2 pmol/min/mg protein.

2.8. Statistical procedures

Statistical differences between two groups were assessed using Student's *t* test, and to evaluate differences between more than two groups (tissues), one-way ANOVA was applied, with the use of GraphPad Prism v5.0 for Mac OS 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 multiple range tests were performed. Samples showing levels below the detection limits were considered as having 50% of the minimal values detectable only for statistical comparisons. All data was analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests. Correlation between variables was calculated using the Spearman rank order method by using the same software package described before.

3. Results

3.1. FMO comparisons

Nucleotide-derived amino acid sequences showed a high degree of similarity between the 3 genes (Supplemental Figure 1). FMO A and FMO B were the most similar with 68% identity, while FMO A and FMO C shared 65% identity. FMO B and FMO C had the least similarity (58%). FMO B possessed the classic FAD- and NADPH-binding sites found in all FMO proteins, but did not have the FATGY motif. FMO C had the NADPH binding site, but did not have the FATGY nor the FAD-binding sites.

3.2 FMO mRNA expression and salinity effects

In freshwater-acclimated animals, FMO A mRNA was up to 10-fold higher in liver ($p<0.05$) compared to the rest of the tissues (Table 2). FMO B transcripts were approximately 2-fold higher in the liver relative to gills, olfactory tissues and kidney. All 3 FMO mRNAs were detected in kidney (B>C>A). The magnitude of expression of FMO A and B were as follows: liver>gills=olfactory tissues=kidney. For FMO C, gills=olfactory tissues>liver>kidney. Salinity acclimation (at 18 g/L) significantly increased FMO A mRNA expression in all tissues ($p<0.05$). FMO A mRNA was increased up to 2.3-, 8.8-, 2.8- and 2.8-fold in liver, gills, olfactory tissues and kidney, respectively. In contrast, salinity acclimation significantly reduced the expression of FMO B mRNA in all tissues

($p < 0.05$), with reductions ranging 3.2- to 8.5-fold in the different tissues. FMO C expression was not significantly altered by salinity acclimation in any of the tissues.

3.2. Methyl-p-tolyl S-oxidase (MTS) and benzydamine N-oxidase (BZN) activities

In freshwater-acclimated animals, MTS oxidase activity was higher in liver, olfactory tissues and kidney than gills (Figure 1A). Salinity acclimation significantly increased MTS biotransformation in liver, gills and kidney (up to 2.1-, 1.8- and 3.4-fold, respectively) ($p < 0.05$). No effect was observed in olfactory tissues. Co-incubations with 1 mM ketoconazole did not significantly alter MTS oxidation.

Stereoselective MTSO formation was observed in microsomes from liver and gill of freshwater and hypersaline fish. Stereoselective sulfoxidation was observed in microsomes from olfactory tissues. Stereoselective transformation of MTS was not observed in kidney microsomes although a slight trend toward formation of the *S*-oxide was observed in hypersaline fish. In gill microsomes, a significant increase of the *R*-oxide was observed in hypersaline animals.

The highest BZN oxidase activity was observed in kidney relative to liver and gills but was not observed in olfactory tissues (Figure 1B). Salinity acclimation significantly increased BZN biotransformation in liver and kidney. Benzydamine *N*-oxide formation in the saline acclimated-group increased up to 2.8-fold in liver and up to 3.5-fold in kidney when compared to freshwater groups ($p < 0.05$). Co-incubations with 1 mM ketoconazole did not significantly alter BZN oxidation ($p < 0.05$).

3.3. Correlations between FMO expression and associated enzymatic activities

In liver, FMO A expression strongly correlated with MTS oxidation (Pearson's: 0.96, $p < 0.01$) and BZN oxidation (Pearson's: 0.94; $p < 0.01$) (Figure 2A). In gills, FMO A expression only correlated with MTS oxidation (Pearson's: 0.96; $p < 0.01$), but not with BZN biotransformation (Figure 2B). In kidney, both activities also strongly correlated with FMO A (Pearson's: 0.96 for MTS oxidation and Pearson's: 0.95 for BZN oxidation; both of them at $p < 0.01$) (Figure 2C).

4. Discussion

Previous studies in euryhaline fish and isolated hepatocytes have indicated up-regulation of FMO expression and catalytic activity after acclimation to hypersaline conditions (Schlenk and Lavado, 2011). However, the substrate specificity of the enzyme encoded by that specific FMO gene and the role of other FMO genes in osmoregulation has not been explored. Three cDNAs from 3 novel FMO genes have been cloned and sequenced from rainbow trout with only FMO A (previously reported as hFMO), encoding a full-length protein (Rodriguez-Fuentes et al., 2008). The high degree of similarity of the fish FMO transcripts (58–68%) with other mammalian FMO transcripts (52–55%) of functional proteins (FMO1–5) and the lack of a clear nomenclature for non-mammalian forms have precluded the numerical identification of novel genes and have relegated their identities to alphabetical or parallel numerical categories. In this particular study, the transcripts of three FMO genes from rainbow trout (A, B, and C) were evaluated in rainbow trout in freshwater and after acclimation to saltwater.

In freshwater organisms, FMO A and B transcripts were highest in liver with relatively similar expression in gill, olfactory tissues, and kidney. In contrast, FMO C mRNA was highest in gills and olfactory tissues relative to liver, and just above detection in kidney. These results contrast previous studies where FMO protein expression in microsomes of different tissues using anti-mammalian FMO1 antibodies, and thiourea oxidase as an FMO-

specific catalytic activity did not show significant differences between liver, kidney, gill or gut (Larsen and Schlenk, 2001). However, comparable rates of thiourea oxidase in these tissues were also observed in the current study where MTSO activities in liver, olfactory tissues and kidney were not significantly different. In contrast with Larsen and Schlenk (2001), gill activities were significantly lower than liver activities. Catalytic activities have been shown to vary significantly between studies. For example, in studies that preceded those of Larsen and Schlenk (2001), thiourea oxidase was 10 times lower in gill microsomes compared to liver microsomes of rainbow trout (Schlenk, 1998). In contrast, thiourea oxidase in the genetically similar coho salmon were significantly higher in gill relative to liver in freshwater animals (Matsuo et al., 2008). The lack of correspondence between MTSO and previous thiourea oxidase in gill may reflect this variability.

MTSO activity has been previously characterized in liver microsomes of rainbow trout where unique stereoselective properties were observed relative to mammalian isoforms of FMO (Schlenk et al., 2004). For example, FMO1 catalyzes stereoselective oxidation of MTS to the *R*-oxide (>99%), whereas trout liver microsomes selectively catalyze formation of the *S*-oxide (67–74%). Similar stereoselectivity was observed in the current study with gill, and to a lesser extent in kidney (52–55%). In contrast, olfactory tissues catalyzed the stereoselective oxidation of MTS to the *S*-oxide. Acclimation to salinity only significantly altered stereochemical oxidation in gill tissues where formation of the *R*-oxide was enhanced. Transformation of the organophosphate insecticide fenthion from *S*- to *R*-sulfoxides was also observed in gill microsomes of rainbow trout that were acclimated to hypersaline conditions (Lavado et al., 2009). The transformation of fenthion is significant because the *R*-sulfoxide oxon metabolite of fenthion is a more potent inhibitor of acetylcholinesterase (Gadepalli et al., 2007) and may help explain the greater toxicity observed in rainbow trout maintained in hypersaline conditions (Bawardi et al., 2007; Lavado et al., 2009). Whilst BZNO was not observed in olfactory tissues, BZNO activities in the other three tissues were not significantly different from each other. FMO-catalyzed *N,N*-dimethylamine-*N*-oxidation has been previously observed in microsomes of gill and liver from several fish (Schlenk, 1998). DMA oxidase activities were significantly higher in gill of Atlantic flounder *Platichthys flesus* (Schlenk et al., 1996), but were approximately 10 times lower in gill of rainbow trout relative to liver (Schlenk, 1998). The higher expression in osmoregulatory organs such as the gill indicated that FMO may play a role in osmoregulation perhaps through the formation of “compatible solutes” such as trimethylamine oxide which is formed by FMO to counter the enhanced formation of urea which occurs in fish undergoing saltwater acclimation (Larsen and Schlenk, 2002). In the current study, N-oxygenation did not correlate with expression of FMOA in the gill. Explanations may be related to unique factors within gill microsomes relative to liver microsomes that impair BZNO, but not DMA oxidase, or the occurrence of splice variants of FMOA that have unique substrate specificities (see discussion below). Whether FMO has an osmoregulatory function that is related to tissue-specific expression is unclear, but the induction and down-regulation of specific genes and their correlation with some catalytic activities suggest an association.

In saltwater acclimated animals, expression of the FMOA transcript increased in all tissues with a greater increase in gill (nearly 10 fold) relative to other tissues (approximately 3 fold). The greater response in the gill is consistent with earlier studies showing induction of FMO catalytic activities and expression (Larsen and Schlenk, 2001). MTSO formation was likewise increased after hypersaline acclimation in all tissues except olfactory tissues and BZNO formation was enhanced in liver and kidney. Overall catalytic activity strongly correlated with FMOA mRNA content and suggests MTSO and BZNO activities may be likely substrates. Previous studies in our laboratory attempted to express FMO A in several heterologous expression systems. While saturating NADPH oxidation was observed in the

expressed protein for several substrates including TMA and MTS, formation of oxygenated product was not observed (Schlenk, unpublished). Consequently, the significant correlation observed in tissues of fish after hypersaline acclimation suggests FMOA may be involved in osmoregulation. Whether this enzyme is responsible for TMAO formation to counter osmotic stress is unclear, but future studies are underway to explore this possibility.

Salinity acclimation down-regulated expression of FMO B in all four tissues. While the deduced amino acid sequence of FMO B possessed the classic FAD- and NADPH-binding sites found in all FMO proteins, it lacked the FATGY motif. Similarly, FMO C had the NADPH binding site, but did not have the FATGY, nor the FAD-binding sites. These data suggest the transcripts would not be able to encode a functional protein and may be derived from pseudogenes or could be splice variants of one of the other FMO genes. The limited difference in sequence identities between the 3 transcripts is consistent with this hypothesis. Earlier studies suggested at least 7 genes or variants are present in rainbow trout (Rodriguez-Fuentes et al., 2008). Splice variants of FMO genes have been identified in humans and shown to have unique catalytic properties (Hines et al., 2002; Lattard et al., 2004). Without catalytic function, it is unclear why expression of FMO B would be regulated by hypersaline conditions. Inverted tissue specific expression between FMO A and B with hyperosmotic conditions suggests some functional role. A similar phenomenon occurs in fetal livers of humans, where FMO1 is the predominant form and then undergoes down-regulation at birth with concurrent up-regulation of FMO3 (Dolphin et al., 1996; Yeung et al., 2000). Given the presence of multiple FMO genes in this species, FMO B may be a variant of another uncharacterized FMO gene that encodes a full-length protein that has a unique catalytic activity or cellular function.

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A.

B.

Figure 1.

FMO-associated activities in microsomal fractions isolated from rainbow trout acclimated to different salinities (<0.5 g/L and 18 g/L). Methyl-*p*-tolyl sulfoxidation (A) and benzydamine *N*-oxidation (B) are shown as mean $\pm$ standard deviation ($n=8-10$). Significant differences between salinity groups are indicated as * ($p < 0.05$) and ** ($p < 0.01$) (Student's *t*-test). Different letter indicated significant differences between tissues ($p < 0.05$; One-way ANOVA). **BDL**: Below detection limit (<0.2 pmol/min/mg protein).

Figure 2.
Correlations between methyl-*p*-tolyl sulfoxidation (MTS-oxidase) and benzydamine *N*-oxidation (BZD *N*-oxidase) activities and FMO A expression in liver (A), gills (B) and kidney (C) in rainbow trout acclimated to different salinities.

Table 1

Primers used for quantitative-PCR.

Name	Nucleotide Sequence (5'-3')
FMO A	
E1-2F	gga agt tca agg aag ttt ctg agc cc
E2-3R	ccg cgc act ggt ctg gaa gt
FMO B	
5qL1	tat tcc tca gta cca ggc gtg g
3qL1	gac tgg gcc atc ttt aac acc ctt
FMO C	
5qL3	ggg tgt tag ctc gta tgt cca gta g
3qL3	ggt ggt cat atc ggt gat taa ggg

Table 2

Expression of FMO mRNA in different tissues from rainbow trout (*O. mykiss*) acclimated to freshwater (<0.5 g/L salinity) and hypersaline water (18 g/L salinity). Data are presented as pg/μg total RNA and as mean ± standard deviation (n=8–10).

Salinity	FMO A		FMO B		FMO C	
	<0.5 g/L	18 g/L	<0.5 g/L	18 g/L	<0.5 g/L	18 g/L
Liver	1.27 ± 0.42 ^a	3.02 ± 0.75 ^{a*}	3.24 ± 1.16 ^a	0.38 ± 0.19 ^{a*}	3.12 ± 1.50 ^a	3.65 ± 1.44 ^a
Gills	0.12 ± 0.05 ^b	1.06 ± 0.26 ^{b*}	1.06 ± 0.55 ^b	0.24 ± 0.12 ^{a*}	9.37 ± 4.29 ^{ab}	14.52 ± 2.87 ^b
Olfactory tissues	0.12 ± 0.01 ^b	0.34 ± 0.09 ^{c*}	1.83 ± 0.69 ^{ab}	0.57 ± 0.04 ^{a*}	9.60 ± 2.62 ^b	5.47 ± 2.80 ^a
Kidney	0.16 ± 0.08 ^b	0.46 ± 0.16 ^{bc*}	1.52 ± 0.55 ^b	0.30 ± 0.21 ^{a*}	0.74 ± 0.39 ^c	0.22 ± 0.13 ^c

Significant differences between salinity groups are indicated as * ($p < 0.05$; Student's *t*-test).

Different letter indicated significant differences between tissues ($p < 0.05$; One-way ANOVA).

Table 3

S-oxidation of methyl-*p*-tolyl in microsomal fractions isolated from rainbow trout acclimated to different salinities (<0.5 g/L and 18 g/L). Data are presented as % of total metabolites and as mean $\pm$ standard deviation (n=8–10).

	<i>R</i> -MTSO		<i>S</i> -MTSO	
	<0.5 g/L	18 g/L	<0.5 g/L	18 g/L
Liver	32 $\pm$ 4 ^a	36 $\pm$ 4 ^a	67 $\pm$ 6 ^a	63 $\pm$ 6 ^a
Gills	33 $\pm$ 4 ^a	41 $\pm$ 6 ^{ac}	66 $\pm$ 6 ^a	58 $\pm$ 4 ^a
Olfactory tissues	<i>bdl</i> ^b	<i>bdl</i> ^b	100 $\pm$ 0 ^b	100 $\pm$ 0 ^b
Kidney	47 $\pm$ 7 ^c	45 $\pm$ 5 ^c	52 $\pm$ 3 ^c	55 $\pm$ 5 ^a

Different letter indicated significant differences between tissues ($p < 0.05$; One-way ANOVA).

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

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

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