



Expression of a novel cytochrome P450 4T gene in rare minnow (*Gobiocypris rarus*) following perfluorooctanoic acid exposure

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ABSTRACT

Cytochrome P450s play an important role in the biotransformation of endogenous substrates and xenobiotics; however, little is known about the function of the CYP4T subfamily in the transformation of environmental pollutants in fish. We isolated a full-length cDNA sequence (designated as CYP4T11) from rare minnow (*Gobiocypris rarus*) liver by rapid amplification of cDNA ends. The open reading frame encoded a 467-residue protein that exhibited 87% and 71% identity with zebrafish CYP4T8 and European sea bass CYP4T2, respectively. CYP4T11 was predominantly expressed in liver and intestine with lower expression in the gill and brain. To further examine the function of CYP4T11 in pollutant metabolism, the effects of perfluorooctanoic acid (PFOA) exposure on the transcriptional expression of CYP4T11 and two possible upstream regulators, peroxisome proliferator-activated receptor alpha (PPAR α) and peroxisome proliferator-activated receptor gamma (PPAR γ), were determined in rare minnow gills and livers. PFOA induced a consistent significant upregulation of both PPAR α and PPAR γ and a nonsignificant increase of CYP4T11 in the gill. In the liver, induced expression of PPAR γ was observed, although no obvious changes in PPAR α expression were observed. Induction of CYP4T11 was only observed in males at the highest concentration of PFOA. These results suggest that the PPAR-CYP4T11 signaling pathway may be involved in PFOA-induced gill toxicity. Since the induced expression of CYP4T11 in liver was not consistent with the PPAR regulators, complex tissue-specific transcriptional regulation of CYP4T11 following PFOA exposure likely occurs.

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1. Introduction

Cytochrome P450s (CYPs or P450s) are a large superfamily of heme-binding enzymes, which play an important role in the synthesis and metabolism of endogenous substrates and in the biotransformation of xenobiotics (Guengerich 1991; Mansuy 1998). The potential of P450s to contribute to the detoxification of xenobiotics, which can also be oxidized to reactive intermediates, has led to considerable interest in understanding their function. The large superfamily of CYPs is divided into families and subfamilies (Nelson et al., 1993), and each subfamily has broad but distinct substrate specificities. Among the P450 families, the CYP4 family has received considerable attention for its important role in fatty acid metabolism and lipid signal transduction.

The CYP4 family often catalyzes the ω -hydroxylation of fatty acids as well as arachidonic acid and derivatives such as leukotrienes and prostanoids (Hsu et al., 2007). This family of P450 enzymes evolved approximately 1.25 billion years ago, following the divergence of steroid-synthesizing CYP genes (Simpson 1997). Since then, this gene family has diversified to eighteen subfamilies in animals, and seven subfamilies (CYP4A, CYP4B, CYP4E, CYP4T, CYP4V, CYP4X, and CYP4Z)

have been identified in vertebrate animals (Nelson 2003). Recent research on the CYP4 family has been remarkable in the past few years, especially on the members of CYP4A, which are, by far, the best understood acid hydroxylases with regard to their induction by peroxisome proliferators and their ability to prevent lipid toxicity (Hardwick 2008). The induction of CYP4A in response to specific xenobiotics, principally peroxisome proliferators, is mediated by peroxisome proliferator-activated receptor (PPAR)-activated pathways (Waxman 1999; Palut et al., 2002). In response to ligand activation, all PPAR isoforms bind to peroxisome proliferator response elements (PPREs) in the gene promoter area. PPARs bind as heterodimers with retinoid X receptor (RXR) to modulate the expression of the target genes (Shearer and Hoekstra, 2003). Several studies reported the induction of CYP4 by activation of PPAR α or PPAR γ (Muerhoff et al., 1992; Green 1995).

Perfluorooctanoic acid (PFOA) is a perfluorinated compound (PFC), which consists of a carbon backbone and a hydrogen replaced by fluorine. The stable C–F bonds result in remarkable chemical stability, and these compounds therefore do not undergo photolysis, hydrolysis, or biodegradation. The wide commercial application of perfluorinated compounds over the past several decades has resulted in a broad distribution of stable precursors/metabolites in wildlife in terrestrial and aquatic environments (Key et al., 1997; Giesy and Kannan, 2001). In teleosts, PFOA affects the circulating sex steroid levels, increases

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hepatic fatty acyl-CoA oxidase activity, and increases oxidative damage (Oakes et al., 2004; Ankley et al., 2005). Studies in rodents have demonstrated that PFOA exerts reproductive and developmental toxicities and, in particular, causes hepatotoxicity, including hepatomegaly and the induction of hepatic peroxisomes (Biegel et al., 2001; Lau et al., 2007). This PFOA-induced hepatotoxicity in rodents is directly related to PPAR pathways.

Aquatic ecosystems serve as the ultimate sink for many environmental pollutants that accumulate in fish species. Research on environmental pollutant-induced toxicity in fish is often P450-related (Tseng et al., 2005; Oh et al., 2008). Given the structural similarity of PFOA to fatty acids and the fact that the CYP4A subfamily may be derived from the CYP4T subfamily (Fujita et al., 2004), we hypothesized that PFOA-induced toxicity in fish may be associated with CYP4T. Despite the rapid expansion of P450 studies, little is known about the CYP4T subfamily, which is exclusively found in fish and amphibians. A total of ten CYP4Ts have been reported (<http://drnelson.utm.edu/CytochromeP450.html>) since the identification of the first member of CYP4T subfamily in 1997 (Falckh et al., 1997). Among these CYP4Ts, six were from frog species, and only two full-length CYP4T sequences have been reported in fish (Falckh et al., 1997; Sabourault et al., 1998).

We identified a CYP4-like sequence fragment from our previously generated rare minnow (*Gobiocypris rarus*) liver cDNA library, suggesting the presence of CYP4-like protein in this species. In order to clarify the molecular and physiological functions of this enzyme, we isolated the full length cDNA sequence and examined its transcriptional expression patterns. Furthermore, we investigated the toxic effects and possible mechanisms of PFOA exposure on CYP4T signaling pathways in the gill and liver of both female and male rare minnows by quantitative real-time polymerase chain reaction (qPCR).

2. Materials and methods

2.1. Fish and exposure experiments

PFOA (98%) was purchased from Acros Organics (Geel, Belgium). Rare minnows (*Gobiocypris rarus*, Cyprinidae, Cypriniformes) (about 9 months old), with a body mass of 1.4 ± 0.4 g and total length of 47.7 ± 3.6 mm, were obtained from a laboratory hatchery. Exposure experiments were performed as follows. Briefly, male and female rare minnows were randomly allocated into four treatment groups (0, 3, 10, or 30 mg/L PFOA). These concentrations were selected based on previous reports (Oakes et al., 2004). Six males and six females were included in each treatment group. After a 28-day exposure in dechlorinated tap water under flow-through conditions at 25 ± 2 °C with a 16 h L:8 h D photoperiod, fish were anesthetized on ice and then sampled. The gills and livers from fish in the PFOA treatment groups and the muscle, livers, brains, gonads, gills, and intestines from fish in the control group were removed, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

2.2. Total RNA isolation and cloning of full-length CYP4T from rare minnow

Total RNA was isolated from gill and liver using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was treated with RNase-free DNase I (Qiagen, Hilden, Germany) to remove any remaining genomic DNA. The integrality of total RNA was assessed by denaturing agarose gel electrophoresis, and the concentration was determined by the absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Kyoto, Japan). Approximately 1 µg of total RNA was reverse transcribed using an oligo (dT) 15 primer and M-MuLV reverse transcriptase as described by the manufacturer (Promega, Madison, WI, USA).

To obtain a full-length CYP4T cDNA, 5'- and 3'-rapid amplification of cDNA ends (RACE) analyses were performed according to the protocol of the BD SMART™ RACE cDNA Amplification Kit (Clontech

Table 1
Primer sequences used for PCR amplification.

Primers used for rapid amplification of cDNA ends (5' to 3')		
GSP1	AGCAAGCCATCACCTAACCAAGGGATG	
GSP2	CACTGGCTCTTCGGACACGTTAAAGAG	
Primers used for amplification of PPARα and PPARγ fragments		
Target gene	Primer sequences (5' to 3')	Product length (bp)
PPARα	F: AACCGAAACAAGTGCCAATAC	743
	R: GGGACGATCTCCACAGCAG	
PPARγ	F: GCCGCAACAAGTGCCAAT	221
	R: GGCTTTGGTCAGAGGGAAGT	
	R: GGCTTTGGTCAGAGGGAAGT	
Primers used for quantitative real-time PCR		
Target gene	Primer sequences (5' to 3')	Product length (bp)
PPARα	F: CTGTGTGGGAGAGAGAAGTGG	141
	R: GCAGACTCACCTGGTCGTTG	
PPARγ	F: GCCGCAACAAGTGCCAAT	221
	R: GGCTTTGGTCAGAGGGAAGT	
CYP4	F: TTGAATATCCACCACCCATC	110
GAPDH	R: ACAAGCAAGCCATCACCTAA	138
	F: CGTGTCTGTCCAGTCCAA	
	R: GCCGCTTCTGCCTTAACCT	

Laboratories, USA). The gene-specific primers (GSP1 and GSP2) were designed according to the partial sequence from our cDNA library (GenBank accession no. EE399163; Table 1). Amplification of cDNA ends was performed for 32 cycles with the following conditions: 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. After purification by agarose gel electrophoresis and recycling, the amplified products were cloned into a pGEM-T vector and sequenced.

2.3. Sequence analysis

The obtained nucleotide sequence was compared to sequences in GenBank using the BLAST function in order to test the degree of sequence homology with other CYP4 sequences. The deduced amino acid sequence was analyzed using online SMART software (<http://smart.embl-heidelberg.de/>). To examine the sequence conservation of rare minnow CYP4T, orthologs from other species were retrieved, and their amino acid sequences were aligned using ClustalW multiple alignment software in EBI. The BoxShade software (http://www.ch.embnet.org/software/BOX_form.html) was used to display the alignment results.

2.4. Semi-quantitative PCR

To assess the transcriptional expression of CYP4T in different tissues, total RNA was prepared from tissue from various organs, including skeletal muscle, liver, brain, gonad, gill, and intestine, from three female and three male fish of unexposed individuals. Reverse transcription was performed using 1 µg of RNA and an oligo-d(T) primer. The resultant cDNA was used as a template for subsequent experiments. Expression was analyzed by semi-quantitative PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Primers were designed according to the partial sequences in the rare minnow cDNA library (GenBank accession no. EE397198), and the primer sequences are listed in Table 1. For quantitation of expression, different cycles of semi-quantitative PCR assays were optimized. A cycle number within the exponential phase of the amplification curve was chosen. The PCR products were separated in a 2% (w/v) agarose gel, and the band densities were measured using Bandleader Software (Magnitec Ltd., Israel).

2.5. Amplification of rare minnow PPARα and PPARγ fragments

As there was no sequence information about rare minnow PPARα and PPARγ, oligonucleotides for PCR were derived from the corresponding genes of other teleosts, such as zebrafish (*Danio rerio*), red seabream

(*Pagrus major*), Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*), and European hake (*Merluccius merluccius*). Conserved regions were identified, and primers for rare minnow PPAR α and PPAR γ were subsequently designed (Table 1). The resultant PCR products were cloned into a pGEM-T vector and sequenced. The primers used in the subsequent quantitative real-time PCR for PPAR α and PPAR γ were designed based on this acquired sequence information.

2.6. Quantitative real-time PCR

Quantitative PCR (qPCR) was performed to evaluate the expression of CYP4T, PPAR α , and PPAR γ in the gills and livers from PFOA-exposed rare minnows. The qPCR reactions were performed on a Stratagene Mx3000p qPCR system (Stratagene, La Jolla, CA, USA). The reaction

(25 μ L) contained 12.5 μ L of 2 \times SYBR Premix Ex Taq (Takara, Dalian, China), target-specific primer (0.1 μ M each; Table 1), 0.5 μ L of ROX reference Dye II, nuclease-free water, and 1 μ L of cDNA template. Each sample was quantified in triplicate. Thermal cycling conditions were as follows: 95 $^{\circ}$ C for 10 s followed by 40 cycles of 95 $^{\circ}$ C for 5 s, 55 $^{\circ}$ C for 15 s, and 72 $^{\circ}$ C for 10 s. A melting curve analysis was also performed to demonstrate the specificity of the PCR product as displayed by a single peak (data not shown). After adjusting the amplifying efficiencies of CYP4T, PPAR α , PPAR γ and GAPDH to render them approximately equivalent, differences in target gene mRNA transcription were calculated with the $2^{-\Delta\Delta C_t}$ method, where C_t represents the cycle in which the fluorescence signal is first significantly different from background and $\Delta\Delta C_t$ is $(C_{t, target} - C_{t, GAPDH})_{treatment} - (C_{t, target} - C_{t, GAPDH})_{control}$ (Livak and Schmittgen, 2001).

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1  acgcggggagactgacagcacagctcagctcatgcc
37  atgcagctttacgagattttcccattgaatttgaagcactctccataaacatgcctttccaactgcattgtctgtg
   M Q L Y E I F P L N L E A L S I N H A F P L A L L V
115 tgtttggtgatagtgatagatataatgactatgaagaggaggggatgcgaactatggagccttttctggacctcct
   C L V I V D R L M T M K R R G M R T M E P F P G P P
193 gcacactggctcttcggacacgttaagagtttaccaggatggacacgatttggaaaagattatcatgtggtgtgaa
   A H W L F G H V K E F H Q D G H D L E K I I M W C E
271 cagtatccatttgccttttccactgtggttggcccgtcattgtccattttgaaatccaccaccatcatatgtgaag
   Q Y P F A F P L W F G P S L S I L N I H H P S Y V K
349 accattctcaccacaacagaacccaagatgactatgcatataagtttttcatcccttggtttaggtgatggcttgcct
   T I L T T T E P K D D Y A Y K F F I P W L G D G L L
427 gtgtctgctggacagaaatggtttcggcatagacgactcctcactcctggtttccattatgatgtttgaagccttat
   V S A G Q K W F R H R R L L T P G F H Y D V L K P Y
505 gtgaaacttatttctgattcaaccaagtcattgataaagggaattgtatgccaaatcagaagagtcatttggag
   V K L I S D S T K V M L D K W E L Y A K S E E S F E
583 ctgtttaagcatgtaagtctcatgaccctggacagcatcatgaaatgtcattcagctgtaacagcaactgtcagaca
   L F K H V S L M T L D S I M K C A F S C N S N C Q T
661 gacagtggaaccaaccatacatccaggcagtggtttgacctctgccacctgggaacctcagggttcagagtttccca
   D S G T N P Y I Q A V F D L C H L V N L R F R V F P
739 taccacagcaaggccatatttcatctcagtcacatggatacaaatcaggaaggcagccggcatggcacacaatcat
   Y H S K A I F H L S P H G Y K F R K A A G M A H N H
817 acagcggaagtattcagaaaaaggaaggaagtccttgaagatggagaaggaacagggcattgttaaaaaaacgatat
   T A E V I R K R K E V L K M E K E Q G I V K N K R Y
895 ttgactttttggatattcttctgtctgcccagacgagaatcagcagggtttgtcagatgaggatatacagggtgaa
   L D F L D I L L S A R D E N Q Q G L S D E D I R A E
973 gtggacacatcatgtttgaagccatgataccacagccagtgaataatcttggatcttctacaatctggcgtgtaac
   V D T F M F E G H D T T A S G I S W I F Y N L A C N
1051 cctgagcaccaggagaatgcagagaggagatcttacgagctctggatggaaaagacacactggagtggaagacctc
   P E H Q E K C R E E I L R A L D G K D T L E W E D L
1129 aataaaatccttataccacaatgtgtatataaagaatctctgcggtttgcccctctgtgccaggaatatacagaaag
   N K I P Y T T M C I K E S L R L C P P V P G I S R K
1207 ttgactaaaccttgacctttttgatggacggactgtacctgaaggtgtaccattggagtagcatttatgggtagc
   L T K P L T F F D G R T V P E G C T I G V S I Y G I
1285 cacttgaatgccaccgtgtgggagaatccatattgatccactgagatttctgcctgagaacactgctaagagg
   H L N A T V W E N P Y V F D P L R F L P E N T A K R
1363 tcaccacatgcttttgccttctctgctggaccagaaaagaaaattcttcaaggagctcttctctgtacagtgaa
   S P H A F V P F S A G P R K G K F F K E L F P V Q *
1441 cagttcatagtaactatagatgtcaatttacaattctgtgttaaacgaactatcaatcaatcatttaacttcaacttc
1519 ttaaatctgaagcaacggggcgtactaagcgatctcaagccaccaaacactgttgagatgcaagtgaatggccagaa
1597 gttgaaaatggtgccgtgtgagtggtcccaagtaataacggaaataaatcaatcaaaaacttactttagctgtaca
1675 ataacttttctctagagctgtgtacagccaaaacaatcaacaagatgagatataaaaaaacaataattttaaacat
1753 aagaccttttcatatgacaaaaa
    
```

Fig. 1. The nucleotide and deduced amino acid sequences of the CYP4T11 transcript. The numbers at the head of each line indicate the nucleotide positions. The start codon (ATG), stop codon (TAA), and consensus polyadenylation site (AATAAA) are underlined. The amino acid sequence, which is characteristic of the CYP4 family, is indicated in a hatched box.

2.7. Statistical analyses

All results are expressed as mean \pm SE from one representative experiment, and all experiments were performed three times to confirm the results. Values were analyzed for significant differences ($p < 0.05$) using a one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test. All analyses were carried out using SPSS software (Version 13.0).

3. Results and discussion

The full length sequence of a CYP4 gene was obtained by the method of 5'- and 3'-rapid amplification of cDNA ends (RACE) (Fig. 1).

We obtained a 1783 nt cDNA sequence, including a 36-nt 5'-untranslated region (UTR) and a 343-nt 3'-UTR. A polyadenylation signal, AATAAAA, was found upstream of the poly(A) tract. The open reading frame (1404 nt) encoded a 467 amino acid protein with a calculated molecular mass of 54,192 Da and a theoretical pI of 7.95. The assignment of CYP450 sequences into families and subfamilies requires a sequence homology of $\geq 59\%$ for CYPs being placed within the same gene family, whereas CYPs within the same subfamily should be $\approx 70\%$ similar (Lewis et al., 1998). The sequence, which was forwarded to Dr. D. Nelson (University of Tennessee) from the P450 Gene Superfamily Nomenclature Committee, was assigned the name CYP4T11 (GenBank accession no. EU593896) on the basis of its sequence identity. Sequence comparisons between trout CYP4T11 and

G. rarus	1	---	MQLYEIFPLNLEALS	-	INHAFPLALVLC	LVIVDR	LMTMKRR	-	GMRTMEP	FPGPPA	AHWLFG	HVKEFH	QDGH	DLEKII												
D. rerio	1	---	MLLYGISPF	--	VLS-VNHVFAL	IFLACL	LLTVVKLL	IVRRK	-	GVKTMER	FPGPPA	AHWLFG	HVKEFR	QDGH	LEKIV											
D. labrax	1	---	MELTEAFLTL	HWGLPR	DHLLAL	LCIVAV	VVYK	LATL	LAKRRD	VFRSYED	FPGPP	THWLF	HVLEFK	QDGT	DFDTLM											
X. tropicalis	1	M	ASTLWKAL	SSPWL	SVNII	YIQGF	VALL	CVLL	LLLLKAY	LHSRGR	RFAAAL	APFP	GPPA	AHWL	VGHV	NQFR	RDGK	LDLRLM								
M. musculus	1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---								
P. cinereus	1	---	MNSPLS	FAWF	QGNV	SGLL	QVSF	LLGL	GLLV	LKVA	QYVRR	QRL	LD	FQNF	FP	PPS	SHWLV	GNTE	FFPVE	-	ELPKMA					
H. sapiens	1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---				
consensus	1																									
G. rarus	75	M	WCEQY	PFAP	PLWFG	PSLSI	LN	IHHPS	YV	KTIL	TTTE	PKDD	YAYK	FFI	PWL	GD	GLLV	SAG	G	KWFR	HRR	LL	TPG	FHY	DVLK	
D. rerio	72	K	WMLYQ	FA	PLWFG	PSLAV	LN	IHHPS	YAKT	IL	TTTE	PKDD	YAYK	FFI	PWL	GD	GLLV	ST	G	KWFR	HRR	LL	TPG	FHY	DVLK	
D. labrax	77	A	WTQY	PVAF	PLWFG	PF	CV	LN	IHHDP	VYK	T	L	AST	EP	KDD	VSYR	F	L	S	W	I	G	E	G	L	V
X. tropicalis	81	V	VV	NKYP	NA	F	L	W	I	G	K	F	F	G	T	L	I	T	D	D	Y	A	K	V	V	F
M. musculus	72	T	W	TEQ	FP	Y	A	H	P	L	W	L	G	Q	F	V	F	L	N	I	E	P	D	Y	A	K
P. cinereus	76	S	L	A	E	K	F	P	C	A	F	R	W	L	T	G	S	W	N	L	Q	V	D	P	E	Y
H. sapiens	72	S	W	A	H	Q	F	P	Y	A	H	P	L	W	G	Q	F	I	G	L	N	I	E	P	D	Y
consensus	81																									
G. rarus	155	P	V	V	K	L	I	S	D	S	T	K	V	M	L	D	K	W	E	L	Y	A	K	S	E	S
D. rerio	152	P	V	V	K	L	I	S	D	S	T	K	V	M	L	D	K	W	E	V	H	S	R	S	E	S
D. labrax	157	P	V	V	L	M	S	D	S	T	K	T	M	L	D	K	W	G	S	Y	A	N	S	N	E	
X. tropicalis	161	P	V	V	S	L	I	S	D	S	T	K	L	M	L	D	E	L	D	V	S	N	K	D	E	
M. musculus	152	P	V	V	A	I	F	A	E	S	T	R	V	M	L	D	K	W	E	K	K	S	E	N	K	
P. cinereus	156	P	V	V	G	L	M	A	S	R	V	M	L	D	K	W	E	K	L	S	Y	Q	P	L	S	
H. sapiens	152	P	V	V	A	F	T	E	S	T	R	V	M	L	D	K	W	E	K	A	R	E	G	K		
consensus	161																									
G. rarus	235	Y	H	S	K	A	I	F	H	L	S	P	H	G	Y	K	F	R	K	A	A	G	M	A	N	
D. rerio	232	Y	H	S	K	A	I	F	H	L	S	P	H	G	Y	R	F	R	K	A	A	S	I	A	N	
D. labrax	237	Y	H	S	D	L	I	F	Y	L	S	P	H	G	Y	R	Y	R	K	A	T	K	V	A		
X. tropicalis	240	Y	H	S	N	L	I	F	Y	L	S	P	H	G	Y	R	F	R	K	A	C	R	I	A		
M. musculus	231	Y	H	N	D	F	I	Y	W	L	T	P	H	G	R	R	F	L	R	A	C	I	A			
P. cinereus	236	Y	H	N	D	V	L	Y	W	F	S	S	A	G	R	R	T	R	E	A	A	Q	R			
H. sapiens	231	Y	H	N	D	F	I	Y	W	L	T	P	H	G	R	R	F	L	R	A	C	I				
consensus	241																									
G. rarus	315	T	F	M	F	E	G	H	D	T	T	A	S	G	I	S	W	I	F	Y	N	L	A	C		
D. rerio	312	T	F	M	F	E	G	H	D	T	T	A	S	G	I	S	W	I	F	Y	N	L	A			
D. labrax	317	T	F	M	F	E	G	H	D	T	T	A	S	G	I	S	F	I	L	Y	A	L	A			
X. tropicalis	320	T	F	M	F	E	G	H	D	T	T	S	G	I	S	W	I	L	Y	C	M	A				
M. musculus	311	T	F	M	F	E	G	H	D	T	T	S	G	I	S	W	F	L	Y	C	M					
P. cinereus	316	T	F	M	F	E	G	H	D	T	T	A	S	G	I	S	W	L	Y	A						
H. sapiens	311	T	F	M	F	E	G	H	D	T	T	S	G	I	S	W	F	L	Y							
consensus	321																									
G. rarus	395	L	T	F	D	G	R	T	V	P	E	G	C	T	I	G	V	S	I	Y	G	I	H			
D. rerio	392	L	T	F	D	G	R	T	V	P	E	G	C	T	I	G	V	S	I	Y	G	I				
D. labrax	397	I	T	F	C	D	G	R	T	L	P	E	G	S	I	G	T	S	V	F	G					
X. tropicalis	400	I	T	F	H	D	G	R	S	L	P	A	G	M	L	V	S	L	Q	I	Y					
M. musculus	391	V	T	F	D	G	R	S	L	P	A	G	S	L	I	S	L	H	Y	A						
P. cinereus	396	I	T	F	D	G	R	I	L	R	A	G	N	S	V	S	L	H	Y							
H. sapiens	391	V	T	F	D	G	R	S	L	P	A	G	S	L	I	S	M	H								
consensus	401																									
G. rarus	459	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---			
D. rerio	453	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---			
D. labrax	477	L	K	K	Y	L	I	E	D	P	N	W	K	P	K	I	I	P	R	L	V					
X. tropicalis	480	L	K	R	F	E	L	S	P	D	L	S	K	P	L	K	Q	P	L	V						
M. musculus	471	L	L	R	F	E	L	S	P	D	P	S	K	I	P	K	V	P	L							
P. cinereus	476	L	L	R	F	E	L	K	P	D	M	K	P	L	P	I	S	L								
H. sapiens	471	L	L	R	F	E	L	S	L	D	P	S	R	L	P	I	K									
consensus	481																									

Fig. 2. Alignment of the deduced amino acid sequence of CYP4T11 with other full-length CYP4T sequences and some mammalian CYP4 sequences. Residues conserved in all sequences are enclosed in dark boxes. The "*" and "." indicate constitute and semi-constitute amino acids. Dashes represent gaps introduced to optimize alignment.

rare minnow CYP4T11 showed 73% identity over the length of the partial CYP4T1 cDNA sequence. While the full length sequence of CYP4T11 shared 87% and 71% identity with zebrafish CYP4T8 and European sea bass CYP4T2, respectively. The deduced amino acid sequence contained four residues that are invariant in all cytochrome P450 sequences. The N-terminal region of CYP4T11 was highly hydrophobic similar to other membrane-bound CYPs, suggesting a function as membrane-anchor signals. The C-terminus contained a cysteine-rich region that corresponds to the CYP heme-binding area (Werck-Reichhart and Feyereisen, 2000).

CYP4T11 was aligned with other full length CYP4Ts and several representative mammalian sequences obtained via a BLAST search. The alignment results clearly indicated an extremely conserved thirteen-residue sequence around position 315 (I helix) throughout these species (Fig. 2). This conserved peptide sequence defines the CYP4 family and suggests that these enzymes act on closely related substrates, even though several sequence variations were reported in trout CYP4T1 and the CYP4F and CYP4D subfamilies (Falckh et al., 1997; Simpson 1997). A phylogenetic tree was generated using Clustal X and Tree View (Fig. 3). This tree showed that the CYP4T had a

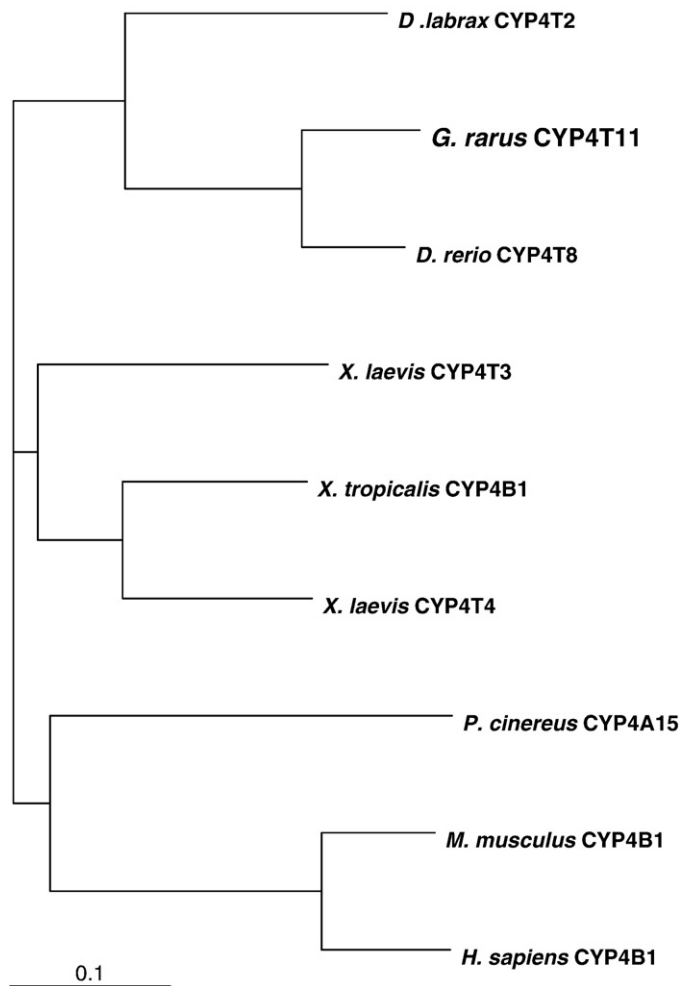


Fig. 3. Phylogenetic tree of the full-length CYP4T sequences and selected mammalian CYP4 sequences. The phylogenetic tree was constructed using Clustal X followed by Tree View (Version 1.6.6) for graphic. The scale bar represents 0.1 amino acid replacements per amino acid site. All the CYP sequences were obtained from the GenBank databases. GenBank accession numbers: rare minnow (*G. rarus*) ACH53593; zebrafish (*D. rerio*) NP_954686; sea bass (*D. labrax*) AAC98961; western clawed frog (*X. tropicalis*) AAH98079; African clawed frog (*X. laevis*) BAA85387 and NP_001079027; house mouse (*M. musculus*) NP_031849; human (*H. sapiens*) AAA35712; koala (*P. cinereus*) AAO15579.

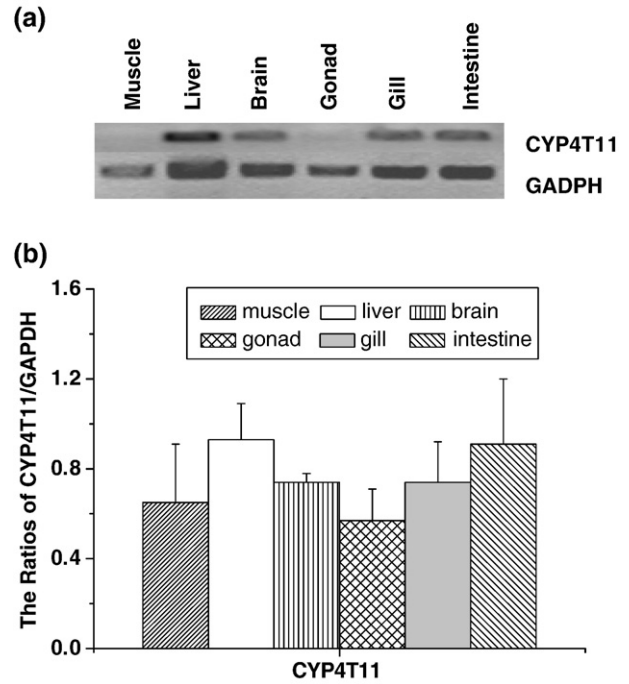


Fig. 4. Expression of rare minnow CYP4T in various tissues. (a) Semi-quantitative PCR products from the indicated tissues were separated on a 2% gel. The expression of GAPDH was used as an internal control. (b) The density of the electrophoretically separated PCR products was determined using Bandler Software and normalized for GAPDH levels. Each value represents the mean \pm SE of three replicates from one representative experiment.

distinct divergence from mammalian CYP4. In addition, the fish CYP4T sequences were assembled in a branch that was quite different from *xenopus* CYP4T. Compared with sea bass CYP4T2, zebrafish CYP4T8 had a closer relationship with the rare minnow CYP4T11. Only three full-length *xenopus* sequences were employed in the phylogenetic tree, while there were still four partial-length *xenopus* sequences, which were also assigned to CYP4T subfamilies in the CYP database. In *Xenopus laevis*, four CYP4T isoforms (including two full-length CYP4T3 and 4T4, partial-length 4T6 and 4T7) have been identified. For the teleosts, however, only one isoform of CYP4T has been identified for one species.

To gain insight into the functional role of CYP4T11, the tissue distribution of the expression of this gene was examined by semi-quantitative PCR using primer pairs specific for each gene (Fig. 4). The cDNA prepared from muscle, livers, brains, gonads (including both testis and ovaries), gills, and intestines from untreated rare minnows were amplified with CYP4T11-specific primers and compared to GAPDH levels. Being the main detoxifying organ in mammals and in fish, liver has been reported to be the major site of expression of many CYP isoforms. In this analysis, CYP4T11 was also predominately expressed in liver and intestine, and to a lesser extent in gill and brain. Furthermore, expression of CYP4T11 was at trace levels in muscle and was undetectable in gonad. The expression distribution of CYP4T11, however, was quite distinct from that of sea bass CYP4T2, which was most abundantly expressed in kidney with no expression in gill or muscle (Sabourault et al., 1998).

The toxic effects of the widespread persistent environmental pollutant PFOA have been reported in several studies, and induction of P450 expression following PFOA exposure has also been demonstrated in rodents (Permadi et al., 1992; Guruge et al., 2006). In humans, rats, and mice, the toxic effects of PFOA were connected with its potential role as a peroxisome proliferator and the induction of CYP4 mRNA following PFOA exposure was found to be mediated by the PPAR pathway (Abbott 2008). Recently, PFOA induction of the PPAR α -

CYP4A signaling pathway was also reported in a waterborne mammal, the Baikal seal (*Pusa sibirica*) (Ishibashi et al., 2008). In teleosts, however, the involvement of CYP4 in PFOA-induced toxicity has not yet been studied. Moreover, the CYP4 subfamilies in fish are quite different from those in mammals as only the CYP4F, CYP4T, and CYP4V subfamilies have been found in fish (Mark, 2007). Thus, in the present study, we investigated whether CYP4T11 acts as the corresponding mammalian CYP4A in fish and functions following PFOA exposure in rare minnows. Rare minnows were used for the toxicity experiments due to their sensitivity to chemicals, small size, wide temperature range, ease of laboratory culture, and short embryonic development period (Qun-Fang et al., 2002; Zhong et al., 2005). We focused on the toxic effect of PFOA on the gill and liver, mainly because gills are the primary tissue exposed directly to aquatic xenobiotic compounds and the liver is the major site for detoxication of xenobiotics. Moreover, from our previous observations, PFOA induces obvious histopathological changes in rare minnow gill and liver, such as capillary lumen dilation, blood congestion and disintegrated pillar cell systems in the gill, hepatocellular hypertrophy, vacuolar degeneration, and necrosis and cytolysis of hepatocytes in the liver (Liu et al., 2008; Wei et al., 2008). Following PFOA exposure, the expression of both PPAR α and PPAR γ was induced in the gills (Fig. 5a and b). PPAR α upregulation was statistically significant in the 10 mg PFOA/L treated females and in the 30 mg PFOA/L treated males ($p < 0.05$). PPAR γ expression was significantly elevated in the 10 and 30 mg PFOA/L-treated females and in the 30 mg PFOA/L-treated males ($p < 0.05$). In addition, the expression of CYP4T11 in gills was increased as well following PFOA exposure although the changes were not significant (Fig. 5c). For each sex, the alteration trends of PPAR α , PPAR γ , and CYP4T11 was almost consistent in PFOA treatment group. As for liver, PFOA exposure failed to cause significant alterations of PPAR α expression in males and females (Fig. 6a). The expression of PPAR γ was significantly upregulated in females exposed to 10 mg PFOA/L ($p < 0.05$), while the upregulation was significant in both 3 and 10 mg PFOA/L-treated males ($p < 0.05$). The expression of CYP4T11 showed a significant 1.8-fold induction in males exposed to 30 mg PFOA/L; however, the induction was not significant for females in any of the PFOA treatment groups. In each sex, trends of altered expression for PPAR α , PPAR γ , and CYP4T11 were quite different.

Since PFOA is a fatty acid mimic, PFOA-induced hepatotoxicity in rodents has been linked with interference of fatty acid metabolism and lipid homeostasis (Kudo et al., 1999; Kennedy et al., 2004). Activation of PPAR by PFOA regulates the expression of a series of target genes, including CYP4 members. In the present study, the consistency of PFOA-induced expression alterations for PPAR α , PPAR γ , and CYP4T11 in gills indicated the involvement of the PPAR pathway in the regulation of CYP4T11. In the liver, however, the transcription of PPAR α was not statistically changed in both sexes although the expression of the putative downstream gene, CYP4T11 was significantly induced in males. Moreover, the induction of PPAR γ in male rare minnow livers following PFOA exposure was not concomitant with the upregulation of CYP4T11. The lack of upregulation of PPAR α in the liver following PFOA exposure in fish despite an increase in rodents may be attributed to species difference. Other reports have indicated that rainbow trout also failed to respond to exposure with peroxisome proliferative effects, and this difference may be related to low concentrations of PPAR in the animal or to the defective nature of the PPAR present in this species (Baldwin et al., 1993; Scarano et al., 1994; Whitlock and Denison, 1995). In addition, as the precise physiological role of CYP4T11 remains to be fully elucidated, the observed inconsistencies in the transcription of rare minnow CYP4T and the regulators following exposure to PFOA may stem from complex interactions and cross-talk between different signaling pathways. Since activation of CYP450 may be accomplished through complex regulation of any of its many upstream nuclear receptors, and there may exist competitive inhibition or enhancing

effects of coactivators for many upstream nuclear receptors (Shaban et al., 2004). For example, coexposure to an AHR agonist and a PPAR ligand could lead to an additive effect on CYP1A1 inducibility in Caco-2 cells (Fallone et al., 2005). Thus, taken together, our results suggest that the PPAR-CYP4T11 signaling pathway may be involved in PFOA-

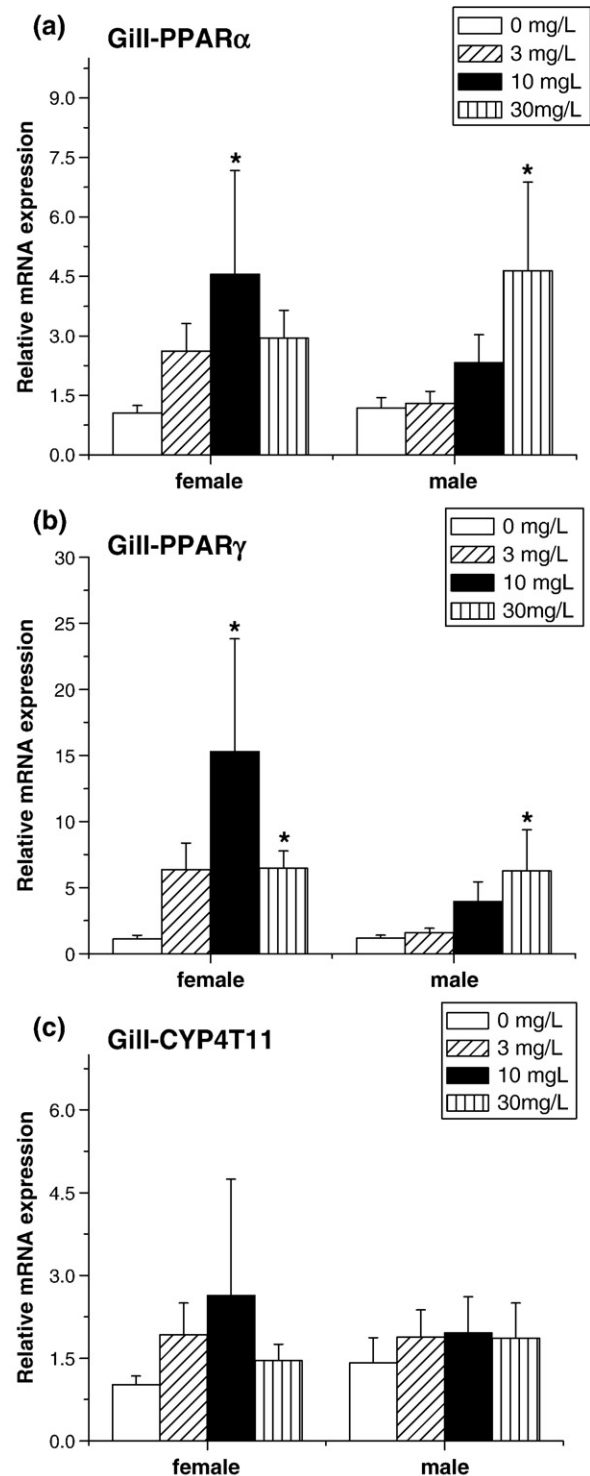


Fig. 5. Quantitative real-time PCR analysis of mRNA levels of PPAR α (a), PPAR γ (b), and CYP4T11 (c) in rare minnow gills. Gene expression levels represent the relative mRNA expression compared to the control. Values are presented as the mean \pm SE of six individual fish per group. Statistical significance is indicated by * for $p < 0.05$ compared to the control.

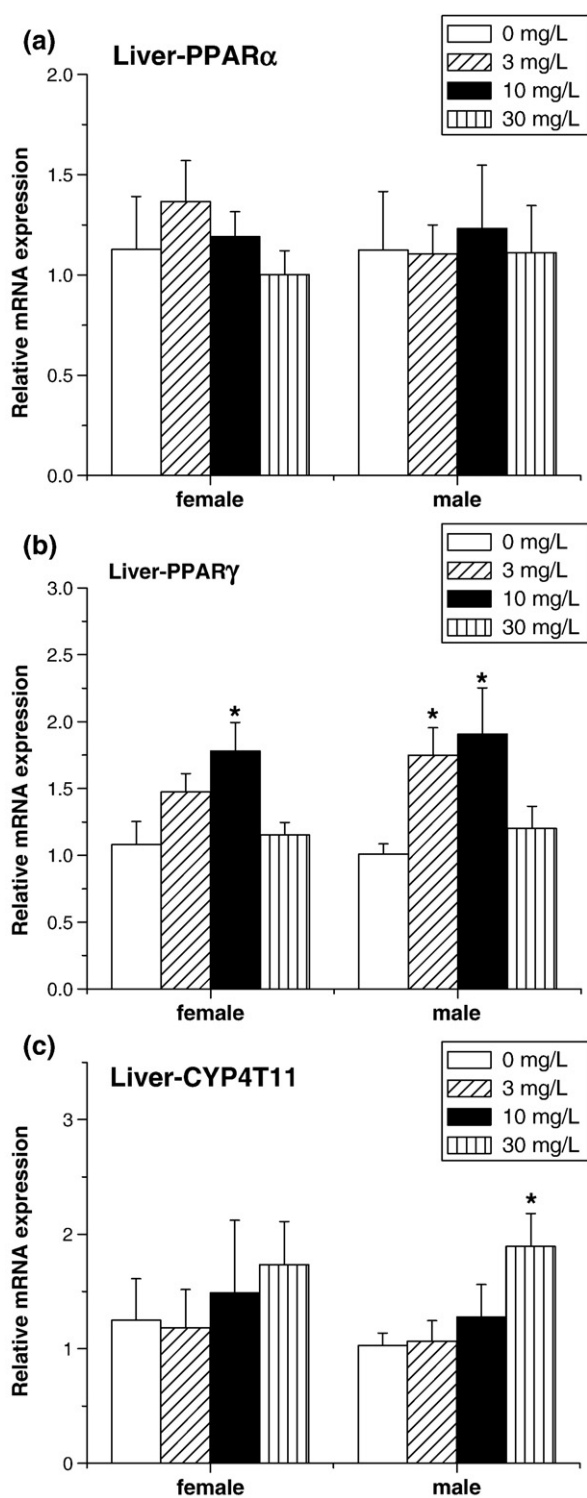


Fig. 6. Quantitative real-time PCR analysis of mRNA levels of PPAR α (a), PPAR γ (b), and CYP4T11 (c) in rare minnow livers. Gene expression levels represent the relative mRNA expression compared to the control. Values are presented as the mean \pm SE of six individual fish per group. Statistical significance is indicated by * for $p < 0.05$ compared to the control.

induced gill toxicity. However, since the induced expression of CYP4T11 in liver was not consistent with the PPAR regulators, complex tissue-specific transcriptional regulation of CYP4T11 following PFOA exposure likely occurs. Future work will be performed to more thoroughly investigate the complex interactions of CYP4T11 and

PPARs and the cross-talk between different signaling pathways in the response to PFOA exposure.

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