ELSEVIER

Aquatic Toxicology 66 (2004) 357–368

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Development and application of a real-time quantitative PCR assay for determining CYP1A transcripts in three genera of salmonids

Christopher B. Rees, Weiming Li*

Department of Fisheries and Wildlife, 13 Natural Resources Building, Michigan State University, East Lansing, MI 48824, USA

Received 1 May 2003; received in revised form 23 September 2003; accepted 19 October 2003

Abstract

The expression of CYP1A (cytochrome P450 1A) can be induced by a large array of aromatic and organic compounds in teleost fishes. We developed a real-time quantitative PCR assay useful for measuring β-naphthoflavone (BNF) induction of liver CYP1A mRNA in four salmonid species. First, to obtain necessary information for the design of a cRNA standard, full-length CYP1A cDNA sequences were determined for two *Salvelinus* species, lake trout (*S. namaycush*) and brook trout (*S. fontinalis*). Each cDNA was found to share the same characteristics with known CYP1A sequences of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*): a start codon, conserved heme-binding region, putative poly-adenylation signal, stop codon, relatively long 3′-untranslated region (UTR; >1 kb), and a protein length of 523 amino acid residues. The brook trout and lake trout CYP1A cDNA's were 2636 and 2672 base pairs (bp) in length and shared greater than 97% coding region sequence identity with Atlantic salmon and rainbow trout CYP1A's. Next, using the generated sequence information, we developed a CYP1A-specific real-time quantitative PCR assay. Primers and a fluorescent-labeled probe were designed from a 68 bp region that was found to be conserved among salmonid CYP1A genes. The assay was designed to allow for simultaneous comparison of CYP1A expression among each experimental group. Finally, groups ($n = 4$–8) of hatchery-raised Atlantic salmon, brook trout, lake trout, and rainbow trout were given an intraperitoneal injection of a corn oil control, 25 mg kg^{-1} BNF, or 50 mg kg^{-1} BNF and sacrificed after 48 h. Liver tissue was collected and CYP1A mRNA levels were estimated. In all species, BNF treated fish showed 1.8–3.0 orders of magnitude higher CYP1A than control fish. The CYP1A induction levels were not different in fish treated with both dosages. Mean base levels of CYP1A expression ranged from 7.24×10^6 (rainbow trout) to 1.05×10^7 (brook trout) transcripts μg^{-1} total RNA. Mean induced levels of CYP1A expression ranged from 1.07×10^8 (lake trout) to 1.05×10^9 (brook trout) trancripts μg^{-1} total RNA.

Keywords: CYP1A; P450; Salmon; Trout; Real-time quantitative PCR; cDNA sequence

1. Introduction

Cytochrome P4501A's (CYP1A) constitute a ubiquitous family of proteins associated with the detoxification of organic compounds such as PCB (polychlorinated biphenyl), PAH (polyaromatic hydrocarbons), and dioxin (Mansuy, 1998; Nelson et al., 1996; Kleinow et al., 1987). These compounds are documented to induce the CYP1A gene in a variety of tissues of many fish species (Levine and Oris, 1999; Hahn et al., 1998; Gooneratne et al., 1997; Nelson et al., 1996; Smolowitz et al., 1992; Miller et al.,

* Corresponding author. Tel.: +1-517-353-9837; fax: +1-517-432-1699.

E-mail address: liweim@msu.edu (W. Li).

0166-445X/$ – see front matter
doi:10.1016/j.aquatox.2003.10.004

1989). Consequently, changes in CYP1A gene expression have been used as a biomarker for contaminant exposure in fish populations (Cousinou et al., 2000; Miller et al., 1999; Campbell and Devlin, 1996; Levine et al., 1994; Stegeman et al., 1992).

A variety of techniques have been applied to estimate CYP1A induction in fish. Protein levels can be measured by determining EROD (ethoxy resorufin-*O*-deethylase) activity (Schlezinger and Stegeman, 2001; Andersson et al., 1985; James and Bend, 1980), immunohistochemistry (Stegeman et al., 2001; Van Veld et al., 1992; Smolowitz et al., 1991), enzyme-linked immunosorbent assay (Sarasquete and Segner, 2000), and Western blotting (Grøsvik et al., 1997). Recently, CYP1A gene expression has been estimated from mRNA levels through Northern blotting (Grøsvik et al., 1997), slot-blotting, or quantitative PCR (Rees et al., 2003; Miller et al., 1999; Campbell and Devlin, 1996). Of all of these methods, quantitative PCR appears to be the most sensitive (Vanden Heuvel et al., 1993). It has been used to assess impact of environmental pollution in marine ecosystems using emerald rockcod (*Trematomus bernacchi*, Miller et al., 1999), guilthead seabream (*Sparus aurata*, Cousinou et al., 2000), and grey mullet (*Liza aurata*, Cousinou et al., 2000). Likewise, numerous quantitative PCR assays have been developed to study CYP1A induction in freshwater teleosts such as Pacific salmon (*Oncorhynchus tshawytscha*, Campbell and Devlin, 1996) and Atlantic salmon (*Salmo salar*, Rees et al., 2003).

The goal of this study was to develop a single real-time quantitative PCR assay and use it to estimate CYP1A levels in at least three genera of salmonids: *Oncorhynchus*, *Salmo*, and *Salvelinus*. This is feasible because orthologous CYP1A genes are often highly conserved. Coding sequences of the CYP1A gene in Atlantic salmon (Rees et al., in press) and rainbow trout (Berndtson and Chen, 1994; Heilmann et al., 1988), for instance, share more than 96% of sequence identity. However, CYP1A sequences from *Salvelinus* species are not available. Therefore, our first objective was to clone CYP1A in two *Salvelinus* species, lake trout (*S. namaycush*) and brook trout (*S. fontinalis*). Our second objective was to develop a quantitative PCR assay useful for all three genera of salmonids. The third objective was to use the anticipated assay to determine and compare the effect of β-naphthoflavone (BNF) treatment on liver CYP1A levels in all species of the three genera.

2. Materials and methods

2.1. Animals, BNF induction, and acquisition of tissues

All species of salmonids used in this study were acquired from nearby fish hatcheries. Juvenile lake trout and brook trout (11 g ± 2 g mean weight, 11 cm ± 2 cm mean length) were acquired from Marquette State Fish Hatchery (Marquette, MI, USA), juvenile Atlantic salmon (20 g ± 3 g mean weight, 13 cm ± 2 cm mean length) were collected from Lake Superior State University Fish Hatchery (Sault Ste. Marie, MI, USA), while juvenile rainbow trout (11 *g* ± 2 g mean weight, 11 *cm* ± 2 cm mean length) were collected from Wolf Lake State Fish Hatchery (Mattawan, MI, USA). All fish were held at Michigan State University where they were acclimated for two weeks at 12 °C in 800-l flow-through tanks (well water, 600 l h^{-1}). A 12-h light-dark cycle was maintained during the acclimation and experiment period. Fish were fed Purina AquaMax© Grower 400 (lot A-5D04; Purina Mills, Inc.; St. Louis, MO) daily at a level of 1.5% body weight. Two days prior to injection, fish were taken off of feed. Individuals were randomly sampled and given an intraperitoneal injection of a corn oil control or β-naphthoflavone (BNF, Sigma Chemical Corp.; St. Louis, MO) dissolved in corn oil at doses of either 25 or 50 mg kg^{-1} body weight. Fish were then placed in a 40-l flow-through aquarium (20 l h^{-1}) for 48 h and then sacrificed using an overdose of MS-222 (Sigma Chemical Corp.). BNF induction of CYP1A reaches maximum in 48 h (Grøsvik et al., 1997). Tissues (gill, liver, and brain) were immediately collected and stored in RNALater© at −80 °C (Ambion; Austin, TX). In addition, gill and liver tissue from one induced lake trout and one induced brook trout were collected and used for cloning of the CYP1A gene in each species.

2.2. RNA isolation

RNALater© preserved tissues were homogenized and extracted for total RNA isolation using Trizol

Reagent (Life Technologies; Carlsbad, CA) according to the manufacturer's protocol. RNA samples were incubated at 37 °C with RNase-free DNase I (Roche Molecular Biochemicals; Mannheim, Germany) then re-suspended in 20–50 μl of diethylpyrocarbonate-treated water (DEPC-H_2O) and quantified (Sambrook et al., 1989) using a GeneQuant *pro* RNA/DNA calculator (Amersham Biosciences; Piscataway, NJ). For long-term storage, RNA samples were supplemented with three volumes of 95% ethanol, 1/10 volume of 3 M sodium acetate, and stored at −80 °C (Sambrook et al., 1989).

2.3. Cloning of full-length cDNA's encoding lake trout and brook trout CYP1A genes

We followed the strategy and procedures of Rees et al. (in press) for cloning these two CYP1A genes. Briefly, RACE was carried out using the Advantage II RACE system (Clontech; Palo Alto, CA) according to the manufacturer's protocol. One μg of total RNA from lake trout gill and brook trout liver was used as a template for synthesis of 3′-RACE Ready cDNA. We conducted a 3′-RACE with a gene specific primer (WML56 5′-CGG CTC ATT TGG CTC ATA ACG GAA GAT-3′) designed from the 5′-UTR sequence of Atlantic salmon CYP1A (Rees et al., in press, GenBank accession number AF361643). This RACE insured that all functional domains including the 5′-UTR and entire 3′-UTR would be included in the cloned cDNA. After cloning, both RACE products were sequenced by the Plant Biology DNA Sequencing Facility, Michigan State University.

2.4. Phylogenetic analysis

The coding region of brook trout, lake trout, and Atlantic salmon CYP1A was aligned to the coding region of a sample of P450 genes using the CLUSTAL W algorithm. Genetic relationships and distances were generated using the Neighbor-joining method. Genes selected for this analysis comprised teleost CYP genes representing families 1–4 (refer to Table 1 for GenBank accession numbers).

2.5. In vitro transcription of cRNA standard

Separate plasmids containing either the full CYP1A cDNA sequence from lake trout, brook trout, and Atlantic salmon (Rees et al., in press) were obtained by standard cloning procedures and sequenced as described previously. To design a cRNA standard, a 491 bp conserved region of the CYP1A gene was amplified from the Atlantic salmon CYP1A clone using the following primers and conditions: forward primer WML 169 5′-TAA TAC GAC TCA CTA TAG GCT GTC TTG GGC TGT TGT GTA CCT TGT G-3′, reverse primer WML 170 5′-TTT TTT TTT TTT TTT TTT-GGA GCA GGA TGG CCA AGA AGA GGT AG-3′, 1 cycle at 94 °C for 4 min, 40 cycles 94 °C for 5 s and 72 °C for 2 min, and 1 cycle at 72 °C for 5 min as added extension. The PCR product contained a

Table 1
CYP genes and accession numbers used in the phylogenetic analysis

Name in analysis (common name)	Reference	GenBank accession number
Anguilla japonica CYP1A (Japanese eel)	Mitsuo et al. (1999) (unpublished)	AB020414
Danio rerio CYP1A (zebrafish)	Yamazaki et al. (2002) (unpublished)	AB078927
Liza saliens CYP1A (mullet)	Sen et al. (1999) (unpublished)	AF072899
Oncorhyncus mykiss CYP1A (rainbow trout)	Berndtson and Chen (1994)	S69278
Oncorhyncus mykiss CYP1A3	Berndtson and Chen (1994)	S69277
Salmo salar CYP1A (Atlantic salmon)	Rees et al. (2003)	AF361643
Salvelinus fontinalis CYP1A (brook trout)	Rees and Li, (this paper)	AF539414
Salvelinus namaycush CYP1A (lake trout)	Rees and Li, (this paper)	AF539415
Stenotomus chrysops CYP1C1 (scup)	Godard et al. (2002) (unpublished)	AF131885
Oncorhyncus mykiss CYP2K4	Yang et al. (1998) (unpublished)	AF043296
Fundulus heteroclitus CYP2P1 (killifish)	Oleksiak et al. (2000) (unpublished)	AF117341
Oryzias latipes CYP3A (Japanese medaka)	Kullman et al. (2000) (unpublished)	AF105018
Oncorhyncus mykiss CYP3A27	Lee et al. (1998)	U96077
Dicentrarchus labrax CYP4DL1 (sea bass)	Sabourault et al. (1998)	AF045468

5′-T7 promoter, 454 bp of CYP1A sequence including the region of the real-time amplicon, and a poly dT tail at the 3′-end. This product was then diluted 1/100 with deionized water, re-amplified and scaled up with the same reaction conditions. Additional handling, amplification, and purification of the cRNA standard were performed as described previously (Rees et al., 2003).

2.6. Quantitative PCR primer and probe design

PCR primers and the fluorescent-labeled probe were designed to conform to several criteria. First, primers should only amplify the CYP1 family of P450 genes and not any other P450 family (i.e. CYP2 or CYP3). In addition, they should anneal to an existing region on the CYP1A gene that was highly conserved (>99%) over all the known salmonid CYP1A sequences. These criteria were met by performing two separate multiple sequence alignments (CLUSTAL W algorithm, DNASTAR; Madison, WI) with known teleost CYP genes. The first alignment was performed with Atlantic salmon CYP1A against six different rainbow trout CYP genes representing families 1–3. Then, a second alignment was performed with all of the known salmonid CYP1A sequences from three different salmonid genera: *Oncorhynchus*, *Salmo*, and *Salvelinus*. We selected a 68 bp region that was conserved among CYP1A genes of *Oncorhynchus*, *Salmo*, and *Salvelinus* and was different from other families of rainbow trout CYP genes.

2.7. RT-PCR

Reverse transcription was performed as described previously (Rees et al., 2003). Each PCR reaction consisted of 25 μl of 2× TaqMan® Universal PCR master mix (Applied Biosystems; Branchburg, NJ), 300 nM of each primer, 100 nM of the TaqMan® probe (5′-6-FAM, 3′-TAMRA quencher), 1 μl of cDNA template, and DI water to a final volume of 50 μl. Reactions were then analyzed on an ABI 7700 real-time PCR thermalcycler (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Amplification plots were generated and CYP1A mRNA levels were estimated against a standard curve.

2.8. Primer and probe optimization

Real-time PCR primers (forward WML158 5′-CCA ACT TAC CTC TGC TGG AAG C-3′ and reverse WML159 5′-GGT GAA CGG CAG GAA GGA-3′) were optimized for quantitative PCR by performing PCR reactions with nine separate concentration combinations in quadruplicate and determining which combination produced the largest ΔR_n. ΔR_n (normalized reporter) represents the signal to noise ratio and indicates the magnitude of the signal generated by a given set of PCR conditions (for more information, consult the Applied Biosystems TaqMan® Universal PCR Master Mix Protocol). Once the primer concentration was chosen, an additional set of reactions was set up to optimize the probe (WML160 5′-TTC ATC CTG GAG ATC TTC CGG CAC TC-3′) concentration for the chosen primer concentration. Five separate probe concentrations were used in quadruplicate and analyzed to see which concentration produced the smallest C_t (threshold cycle). C_t values represent the cycle of amplification at which a PCR reaction reaches a statistically significant increase in ΔR_n (consult the Applied Biosystems TaqMan(r) Universal PCR Master Mix Protocol for more information). The lowest C_t value in these optimization reactions indicates the concentration at which optimal probe binding occurs and point of highest sensitivity for detecting specific template. For all of the above reactions, Atlantic salmon liver cDNA was used as the PCR template.

2.9. Standard curve

A standard curve for each set of samples was generated by performing RT-PCR on a dilution series of the recombinant cRNA standard. The concentration of the standard molecule was estimated in terms of molecules. A 10× dilution series was carried out from 10^3 to 10^{10} molecules. Amplification plots were analyzed on the ABI 7700 and C_t values for each of the reactions in the dilution series were calculated. C_t values were plotted against starting quantity of RNA template to generate the standard curve (refer to Fig. 5 for a representative standard curve). A standard curve was generated for each plate analyzed.

2.10. Statistical analysis

Transcript numbers of CYP1A μg^{-1} total RNA were calculated from the appropriate standard curve and log transformed. Data were analyzed using a two-way analysis of variance (ANOVA; Statistical Analysis Systems v.8; Cary, NC). All pairwise comparisons were tested for significance by using a Tukey–Kramer adjustment (Kramer, 1956).

3. Results

3.1. Brook trout and lake trout CYP1A sequences

3′-Long distance RACE on brook trout cDNA produced a PCR fragment of 2636 bp (Fig. 1). Sequence analysis indicated this product included 33 bp of the 5′-untranslated region (UTR), a 1569 bp coding region, and a 1034 bp 3′-UTR containing three AUUUA sequences. It encodes a protein of 523 amino acid residues. The sequence also possessed all major functional domains and characteristics of previously discovered CYP1A molecules including the heme-binding cysteine (position 463), arginine codon (position 246) integral to enzymatic function, stop codon (position 523), and poly-adenylation signal.

A lake trout CYP1A fragment of 2672 bp long was also cloned and sequenced (Fig. 2). This clone contained 28 bp of the 5′ UTR, a 1569 bp coding region, and a 3′-UTR of 1075 bp in length containing two AUUUA sequences. It also encodes a protein of 523 amino acid residues and contains all of the major functional domains typical of CYP1A's as described above.

```
TGTGCAGAAGCCACTAAAAAAAAACATAATAAAAATGGTTCTCATGATACTACCCATTATCGGCTCTGTCTCTGTGTCTGAGGGGCTGGTTGCCATAGTATCACTATGCCTGGTGTACAT  119
                                  M  V  L  M  I  L  P  I  I  G  S  V  S  V  S  E  G  L  V  A  I  V  S  L  C  L  V  Y  M   29
GCTCATGAAGTACAAGCACACAGAGATCCCAGAGGGACTAAAACGGCTCCCAGGACCAAAGCCCCTGCCCATCATCGGGAATGTGCTGGAGGTGCACAACAACCCTCACCTCAGCCTGA  238
 L  M  K  Y  K  H  T  E  I  P  E  G  L  K  R  L  P  G  P  K  P  L  P  I  I  G  N  V  L  E  V  H  N  N  P  H  L  S  L   68
CTGCCATGAGCGAGCGCTACGGCTCAGTCTTCCAGATCCAAATAGGGATGCGGCCTGTGGTTGTTCTGAGTGGCAGCGAGACAGTCCGCCAGGCTCTTATCAAGCAAGGAGAAGACTTC  357
T  A  M  S  E  R  Y  G  S  V  F  Q  I  Q  I  G  M  R  P  V  V  V  L  S  G  S  E  T  V  R  Q  A  L  I  K  Q  G  E  D  F   108
GCCGGGAGGCCCGATCTATACAGCTTCAAGTTCATCAACGACGGCAAGAGCTTGGCCTTCAGCACCGACAAGGCTGGGGTGTGGCGCGCCCGCCGCAAGCTAGCTATGAGCGCCCTCCG  476
 A  G  R  P  D  L  Y  S  F  K  F  I  N  D  G  K  S  L  A  F  S  T  D  K  A  G  V  W  R  A  R  R  K  L  A  M  S  A  L  R  148
TTCTTTCGCCACCCTGGAGGGATCGACCCCAGAGTACTCCTGTGCCCTGGAGGAGCACGTCTGCAAGGAGGGAGAGTACTTGGTAAAACAGCTGACCTCCGTCATGGATGTCAGTGGCA  595
 S  F  A  T  L  E  G  S  T  P  E  Y  S  C  A  L  E  E  H  V  C  K  E  G  E  Y  L  V  K  Q  L  T  S  V  M  D  V  S  G   187
GCTTTGACCCCTTCCGCCATATTGTCGTATCGGTGGCCAACGTCATCTGTGGAATGTGCTTCGGCCGGCGCTACAGCCATGATGACGAGGAGCTGTTGGGCTTGGTGAACTTGAGTGAT  714
S  F  D  P  F  R  H  I  V  V  S  V  A  N  V  I  C  G  M  C  F  G  R  R  Y  S  H  D  D  E  E  L  L  G  L  V  N  L  S  D   227
GAGTTTGGACAGGTAGTGGGCAGCGGCAACCCTGCAGACTTCATTCCCATCCTTCGTTACCTGCCCAACCGCACCATGAAAAGGTTTATGGATATCAATGACCGTTTCAACACCTTTGT  833
 E  F  G  Q  V  V  G  S  G  N  P  A  D  F  I  P  I  L  R  Y  L  P  N  R  T  M  K  R  F  M  D  I  N  D  R  F  N  T  F  V  267
GCAGAAGATTGTCAGTGAGCACTATGAAAGCTATGACAAGGACAACATCCGTGACATCACTGACTCCCTCATTGACCACTGTGAGGACAGGAAACTAGATGAGAACGCCAACATCCAGG  952
  Q  K  I  V  S  E  H  Y  E  S  Y  D  K  D  N  I  R  D  I  T  D  S  L  I  D  H  C  E  D  R  K  L  D  E  N  A  N  I  Q  306
TTTCTGATGAGAAGATTGTGGGCATTGTCAATGATCTGTTTGGGGCAGGTTTTGACACCATCAGCACAGCTCTGTCATGGGCTGTTGTGTACCTTGTGGCTTACCCAGAGATCCAGGAA 1071
V  S  D  E  K  I  V  G  I  V  N  D  L  F  G  A  G  F  D  T  I  S  T  A  L  S  W  A  V  V  Y  L  V  A  Y  P  E  I  Q  E   346
AGACTGTATCAGGAACTGACGGAAAAGGTGGGAATGAATCGCACTCCCCGTCTCTCAGACAAAACCAACTTACCTCTGCTGGAAGCCTTCATCCTGGAGATCTTCCGGCACTCTTCCTT 1190
 R  L  Y  Q  E  L  T  E  K  V  G  M  N  R  T  P  R  L  S  D  K  T  N  L  P  L  L  E  A  F  I  L  E  I  F  R  H  S  S  F  386
CCTGCCGTTCACCATCCCACACTGCACGATCAAGGATACATCCCTCAATGGCTACTTCATTCCCAAGGACACCTGTGTCTTTATCAACCAGTGGCAGGTCAACCATGACCCGGAGCTGT 1309
 L  P  F  T  I  P  H  C  T  I  K  D  T  S  L  N  G  Y  F  I  P  K  D  T  C  V  F  I  N  Q  W  Q  V  N  H  D  P  E  L   425
GGAAGGAGCCTTCTTTATTCAACCCTGACCGTTTCCTGAGTGCTGATGGCACAGAACTCAACAAGCTGGAGGGGGAGAAGGTGCTCGTATTTGGCATGGGCAAGCGCCGCTGCATTGGT 1428
W  K  E  P  S  L  F  N  P  D  R  F  L  S  A  D  G  T  E  L  N  K  L  E  G  E  K  V  L  V  F  G  M  G  K  R  R  C  I  G   465
GAGGCCATCGGTCGCAACGAGGTCTACCTCTTCTTGGCTATCCTGCTCCAAAGGCTGCGCTTCCAGGAGAAACCTGGGCACCCACTGGACATGACCCCAGAGTACGGCCTCACCATGAA 1547
 E  A  I  G  R  N  E  V  Y  L  F  L  A  I  L  L  Q  R  L  R  F  Q  E  K  P  G  H  P  L  D  M  T  P  E  Y  G  L  T  M  K  505
GCACAAGCGCTGTCAGCTGAAGGCTAGCCTGCGGCCATGGGGGCAGGAGGAGTGAGGGCTATGGTCACATTTATGATTCTCAACATCACAATAACTGATTTATAGTTAGCCCTACATAC 1666
  H  K  R  C  Q  L  K  A  S  L  R  P  W  G  Q  E  E  .                                                                   522
TTGATGGCATGAGATGAGTTCAGATTTAAAAGCCAGAGGGTACATTTTCTCTCCAGGAAAGTCCGGGCTTGACACCTCTCTTGATTCATAGGAGAATTAACAGTGAGGGAAAACTGAGT 1785
CAAATTGTAGACAATTTGTAATTATAAATTATAATTATAATCTGAAATGGTATGTAGGGGGATTCCAAACAAGTGTTGTATTGGATAAAGGACCTTCACAAACTCATAGTTGATTAGTT 1904
TCTATGACCAGCATACCACAGGGCCTACTAGAGTTTGTCAGGTGTTGCTTCAGGAGATATCAGGAGAGACTATAGCTGTTTAGCTGCCTTTTTGTCTACGTCATCGTGTTTTACTGCTC 2023
CTCTTGCCTTGATCACAGCTCATATACTGTATCAGATCGCTTTAAAGAGGATCAATCTATAAAACATACACATAAACCCATGGTGGGGTTTTAAAGGGCATTGTTTTTTAGTTCCATGT 2142
TTGGGTATAATTTTTTGCTACAGAATTTTGAGGCAAAAATGTTACAATCTACATGATAAAGTGCCTGTTCTTGTACTTTTGAGTTATTGTTCAGGTGGTCACAGACAGAGTTGAATGGA 2261
TGTATCCTCCCAATTCTAAGCTAGATATTTTTATTCCACTGAATAAATCTGTGAAATGTGGACATTTGTATCCCACCAACATGTGATTGATTGTTTGTGTACATTTTTAAGCCTAGATG 2380
TATTTTGGAAAGTCATTTTTTGAAATGTGCCTAAAATGTGTATATATTGTGGTGGCTTTGTATGACTATCAGGATACCAAAATACGTATGTATTACTGACTGTGTTATAAATGTCAACA 2499
TTTTATAATGTAACGAAGGACTCTTGTAAACTAAAACCCCAACTGTATACACGATGCATTGTGTTGTTGGTAGCTATCTAGGTAGCTACTGAAATAAAAGCTGAAAACCAAAAAAAAAA 2618
AAAAAAAAAAAAAAAAAA                                                                                                     2636
```

Fig. 1. cDNA and deduced amino acid residue sequence of brook trout CYP1A (GenBank accession number AF539414). The start codon, arginine residue critical to enzymatic function (position 246), heme-binding cysteine codon (position 463), stop codon (positon 523), ATTTA (AUUUA) sequences, and putative poly-adenylation signal are all underlined.

```
TGTGCAGAAGCCACAAAAAAACATACAAATGGTTCTCATGATACTACCCATTATCGGCTCAGTCTCTGTGTCTGAGGGGCTGGTGGCCATGGTAACACTATGCCTGGTGTACATGATCA   119
                            M V L M I L P I I G S V S V S E G L V A M V T L C L V Y M I                                     30
TGAAGTACATGCACACAGAGATCCCAGAGGGACTGAAACGGCTCCCAGGACCAAAGCCCCTGCCCATCATCGGGAATGTGCTGGAGGTGCACAACAACCCTCACCTCAGCCTGACTGCC   238
M K Y M H T E I P E G L K R L P G P K P L P I I G N V L E V H N N P H L S L T A                                                70
ATGAGCGAGCGCTACGGCTCAGTCTTCCAGATCCAGATAGGGATGCGGCCTGTGGTTGTTCTGAGTGGCAGCGAGACAGTCCGCCAGGCTCTTATCAAGCAAGGGGAAGACTTCGCCGG   357
 M S E R Y G S V F Q I Q I G M R P V V V L S G S E T V R Q A L I K Q G E D F A G                                             110
GAGGCCCGATCTATACAGCTTCAAGTTCATCAACGACGGCAAGAGCTTGGCCTTTAGTACCGACAAGGCTGGGGTGTGGCGCGCCCGCCGCAAGCTAGCTATGAGCGCCCTTCGCTCTT   476
  R P D L Y S F K F I N D G K S L A F S T D K A G V W R A R R K L A M S A L R S                                              149
TCGCCACCCTGGAGGGATCGACCCCAGAGTACTCCTGTGCCCTGGAGGAGCACGTCTGCAAGGAGGGAGAGTACCTGGTAAAACAGCTGACTTCCGTCATGGATGTCAGTGGCAGCTTT   595
F A T L E G S T P E Y S C A L E E H V C K E G E Y L V K Q L T S V M D V S G S F                                               189
GACCCCTTCCGCCATATTGTTGTATCGGTGGCCAACGTCATCTGTGGAATGTGCTTCGGCCGGCGCTACAGCCATGATGACCAGGAGCTGTTGGGCTTGGTGAACCTGAGTGATGAATT   714
 D P F R H I V V S V A N V I C G M C F G R R Y S H D D Q E L L G L V N L S D E F                                             229
TGGGCAGGTGGTGGGCAGCGGCAACCCTGCAGACTTCATTCCCATCCTTCGTTACCTACCAAACCGCACCATGAAGAGGTTTATGGATATCAATGACCGTTTCAATACCTTTGTGCAGA   833
  G Q V V G S G N P A D F I P I L R Y L P N R T M K R F M D I N D R F N T F V Q                                              268
AGATTGTCAGTGAGCACTATGAAAGCTATGACAAGGACAACATCCGTGACATCACTGACTCCCTCATTGACCACTGTGAGGACAGGAAACTAGATGAGAACGCCAACATCCAGGTGTCT   952
K I V S E H Y E S Y D K D N I R D I T D S L I D H C E D R K L D E N A N I Q V S                                               308
GATGAGAAGATTGTGGGCATTGTCAATGATCTGTTTGGGGCAGGTTTTGACACCATCAGCACAGCTTTGTCATGGGCTGTTGTGTACCTTGTGGCTTACCCAGAGATCCAGGAAAGACT  1071
 D E K I V G I V N D L F G A G F D T I S T A L S W A V V Y L V A Y P E I Q E R L                                             348
GCATCAGGAACTGAAGGAAAAGGTGGGAATGATTCGCACTCCCCGTCTCTCAGACAAAACCAACTTACCTCTGCTGGAAGCTTTCATCCTGGAGATCTTCCGGCACTCTTCCTTCCTGC  1190
  H Q E L K E K V G M I R T P R L S D K T N L P L L E A F I L E I F R H S S F L                                              387
CGTTCACCATCCCACACTGCACAGTCAAGGATACATCGCTCAATGGCTACTTCATTCCCAAGGACACCTGTGTCTTTATCAACCAGTGGCAGGTCAACCATGACCCGGAGCTGTGGAAG  1309
P F T I P H C T V K D T S L N G Y F I P K D T C V F I N Q W Q V N H D P E L W K                                               427
GAGCCTTCTTCATTCAACCCTGACCGTTTCCTGAGTGCTGATGGCACAGAACTCAACAAACTGGAGGGGGAGAAGGTGCTCGTATTTGGCATGGGCAAGCGCCGCTGCATCGGTGAGGC  1428
 E P S S F N P D R F L S A D G T E L N K L E G E K V L V F G M G K R R C I G E A                                             467
CATTGGACGCAATGAGGTCTACCTCTTTTTGGCCATCCTGCTGCAAAGGCTGTGCTTCAAGGAGAAACCTGGGCACCCGCTGGACATGACCCCAGAGTACGGCCTCACCATGAAGCACA  1547
  I G R N E V Y L F L A I L L Q R L C F K E K P G H P L D M T P E Y G L T M K H                                              506
AGCGCTGTCAGCTGAAGGCTAGCCTGCGGCCATGGGGGCAGGAGGAGTGAGGGCTATGGTCACATTTATGATTCTCAACATCACTATAACTGATTTATAGTTAGCGCTACATACTTGAT  1666
K R C Q L K A S L R P W G Q E E .                                                                                             522
GGCATGAGCTGAGTTCAGATATAAAGCCAGAGGGTACATTTTCTCTCTAGGAAAGACTGGGCTTGACACCTCTCTTGATTCATAGGAAAATTAACAGTGAGGGAAAACTGAGTCAAAT  1785
TGTAGACAATTTGAAATCGATAAAATTATAATCTGAAACACGGTTTGTAGAGGGATTCCAAACAAGTGTTGAATTGGATAAAGGACCTTCACAAACTCATAGAGTTGATTGGTTTCTAT  1904
GACCAGCATACTACAGGCCCTTCTAGAGTTTGTCAGGTGTTTTTTCAGGAGATATCAGGAGAGATTATAGCTGTTTAGCTGCCTTTTTGTCTACATCATTGTGGTTTTACTTCTCCTCT  2023
TGCCTTGATCACAGCTCATATACTGTATCAGATTGCTTTAAAGAGGATGAATCTATAAAACATACACATAACCCCACGGTGGGGTGTTAAAGAGCATTGTTTTTAGTTCCATGTTTGGG  2142
TAAAACATTTTGCTACATATTTTTTTGTGGCAAAAATGTTACTACCTACATGATAAAGTTCTTGTACTTTTGAGTTATTGTTCAGGTGGTCATAGATCACAGACAGAGTTGAATGGATG  2261
TATCCTCCCATTTCTAAGCTAGATATTTTTATTCCACTGAATAAATCAGTCAAACGTGGACACAACACACTGAAGCTATGTTTGTATGCCACCAACATGTGATTGATTGTTTGTGTACA  2380
TATTTAAGCCTAGATGTATTTTGGAAAGTCCTTTTGTGAAATGTGCCTAAAATGTGTATATATTGTGGTGACTTATTATGGTGGCTTTGTATGACTTTATCAGAATACTAAAATACGTA  2499
TGTATTACTGACTGTTATAAATGTCAACATTTTATAATGTAACGAAGGACTCTTGTAAACTAAAGCCCAAACTGTATACACTATGCATTGTGTTGTTGGTAGCTATCTAGGTAGCTACT  2618
GAAATAAAAGCTGAAAATGATAGAAAAACAAAAAAAAAAAAAAAAAAAAAAAAA                                                                        2672
```

Fig. 2. cDNA and deduced amino acid residue sequence of lake trout CYP1A (GenBank accession number AF539415). The start codon, arginine residue critical to enzymatic function (position 246), heme-binding cysteine codon (position 463), stop codon (positon 523), ATTTA (AUUUA) sequences, and putative poly-adenylation signal are all underlined and boldfaced.

3.2. Phylogenetic analysis

For the coding region, the brook trout and lake trout CYP1A genes described here share ∼97% sequence identity with each other. These two genes also share >97% sequence identity with Atlantic salmon and rainbow trout CYP1A genes. In addition, brook trout and lake trout CYP1A genes share between 70–80% nucleotide homology with other teleost CYP1A genes.

Multiple sequence alignment using the CLUSTAL W algorithm followed by construction of a phylogenetic tree using the Neighbor-joining method suggested that all of the salmonid CYP1A genes are highly related (Fig. 3). Minor differences in sequence data grouped brook trout and Atlantic salmon CYP1A genes together. Lake trout CYP1A was genetically closest to rainbow trout CYP1A3. Gene names and GenBank accession numbers used in the phylogenetic analysis can be found in Table 1.

3.3. Primer and probe design and optimization

As discovered from multiple sequence alignments, we determined that the primers and probe chosen would be sufficient to amplify CYP1A from each of the salmonid species listed without cross amplification of alternative teleost CYP genes. The region chosen represents an amplicon of 68 bp long at nucleotides 1103–1170 of the coding region of Atlantic salmon CYP1A. Refer to Fig. 4 for a comparison of this region between salmonid species. The highest mean ΔR_n (2.88) was found with both forward and reverse primers (WML158 and WML159) at 300 nM. Under these primer conditions the probe was found to produce the lowest mean C_t (22.96) at 100 nM.

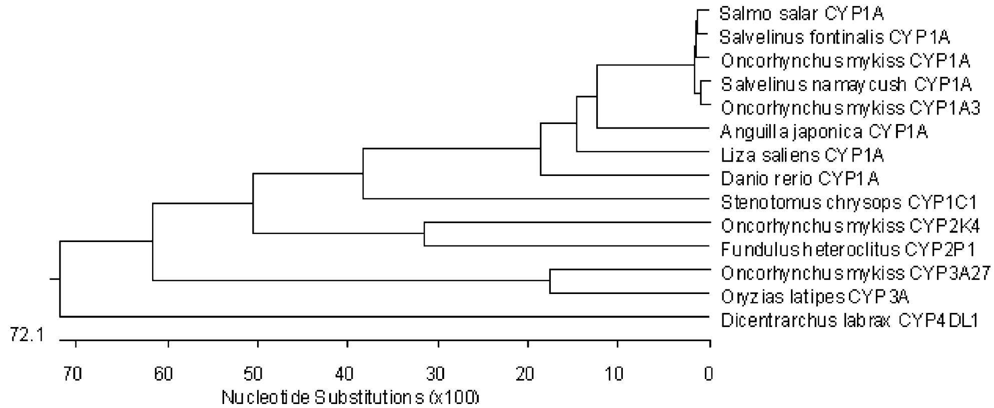

Fig. 3. Phylogenetic analysis of cytochrome P450 genes. Multiple sequence alignment was carried out using the Clustal W algorithm (only coding regions of each respective gene were used). The phylogenetic tree and genetic distances were determined using the Neighbor-joining method.

3.4. Standard curve

The reactions for the standard curve were run on the same plate as all analyzed samples. C_t values were plotted against concentrations of cRNA standard transcripts and analyzed using linear regression. The standard curve had a slope of −3.8 and a coefficient of determination of 0.98 (Fig. 5). All C_t values of RNA extracted from each individual fell within the linear range of the standard curve.

3.5. CYP1A induction in liver of brook trout, lake trout, rainbow trout, and Atlantic salmon

CYP1A was induced approximately 100–1000 fold in all species injected with both dosages (25 mg kg^{-1} body weight and 50 mg kg^{-1} body weight; Fig. 6). A significant interaction was found between species and treatment making main effects irrelevant. Simple effects were determined for each factor by using the SLICE procedure (Statistical Analysis Systems

	C	C	A	A	C	T	T	A	C	C	T	C	T	G	C	T	G	G	A	A	G	C	C	T	T	Majority
										10										20						
1103	C	C	A	A	C	T	T	A	C	C	T	C	T	G	C	T	G	G	A	A	G	C	C	T	T	Salmo salar CYP1A
1103	C	C	A	A	C	T	T	A	C	C	T	C	T	G	C	T	G	G	A	A	G	C	C	T	T	Salvelinus fontinalis CYP1A
1103	C	C	A	A	C	T	T	A	C	C	T	C	T	G	C	T	G	G	A	A	G	C	[T]	T	T	Salvelinus namaycush CYP1A
1103	[T]	C	A	A	C	T	T	A	C	C	T	C	T	G	C	T	G	G	A	A	G	C	C	T	T	Oncorhynchus mykiss CYP1A

	C	A	T	C	C	T	G	G	A	G	A	T	C	T	T	C	C	G	G	C	A	C	T	C	T	Majority
					30										40										50	
1128	C	A	T	C	C	T	G	G	A	G	A	T	C	T	T	C	C	G	G	C	A	C	T	C	T	Salmo salar CYP1A
1128	C	A	T	C	C	T	G	G	A	G	A	T	C	T	T	C	C	G	G	C	A	C	T	C	T	Salvelinus fontinalis CYP1A
1128	C	A	T	C	C	T	G	G	A	G	A	T	C	T	T	C	C	G	G	C	A	C	T	C	T	Salvelinus namaycush CYP1A
1128	C	A	T	C	C	T	G	G	A	G	A	T	C	T	T	C	C	G	G	C	A	C	T	C	T	Oncorhynchus mykiss CYP1A

	T	C	C	T	T	C	C	T	G	C	C	G	T	T	C	A	C	C	Majority
										60									
1153	T	C	C	T	T	C	C	T	G	C	C	G	T	T	C	A	C	C	Salmo salar CYP1A
1153	T	C	C	T	T	C	C	T	G	C	C	G	T	T	C	A	C	C	Salvelinus fontinalis CYP1A
1153	T	C	C	T	T	C	C	T	G	C	C	G	T	T	C	A	C	C	Salvelinus namaycush CYP1A
1153	T	C	C	T	T	C	C	T	G	C	C	G	T	T	C	A	C	C	Oncorhynchus mykiss CYP1A

Fig. 4. Alignment and comparison of the real-time PCR amplicon region (nucleotides 1103–1170) from brook trout, lake trout, rainbow trout, and Atlantic salmon CYP1A genes. Boxed areas show nucleotide differences from the consensus sequence.

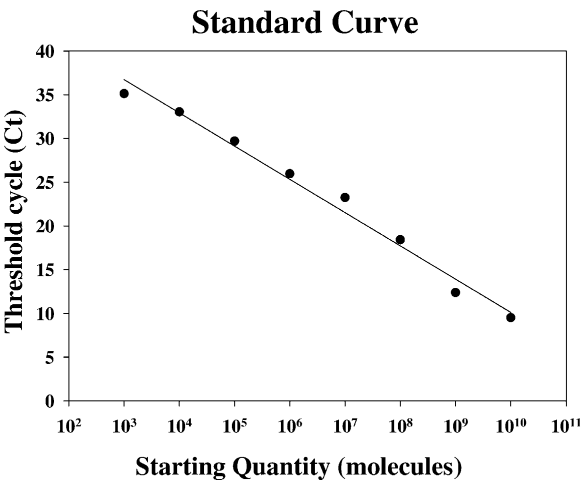

Fig. 5. Standard curve for the real-time CYP1A quantitative PCR assay. A 10-fold dilution series was carried out for the cRNA standard from 10^{10} to 10^3 molecules and amplified for 40 cycles during PCR. C_t (cycle threshold indicating the first detection of CYP1A PCR product) values were plotted against initial concentration followed by standard linear regression ($r^2 = 0.98$).

v.8, Winer, 1971). There was no statistical difference between BNF induction at 25 or 50 mg kg^{-1} body weight in each species ($P > 0.45$). However, ANOVA analysis showed a difference between CYP1A mRNA levels of control and induced groups in each species ($P < 0.0001$). On average, all species had approximately 1×10^6 CYP1A transcripts μg^{-1} total RNA at control levels and 1×10^9 CYP1A transcripts μg^{-1} total RNA under induced conditions. Brook trout demonstrated higher mean basal levels of CYP1A than all other species (ANOVA $P < 0.01$) at 1×10^7 CYP1A transcripts μg^{-1} total RNA. Among BNF induced fish, lake trout had lower levels of CYP1A transcripts at approximately 1×10^8 CYP1A transcripts μg^{-1} total RNA (ANOVA $P < 0.02$) than all other treatment groups except Atlantic salmon treated with 50 mg kg^{-1} BNF.

4. Discussion

It is evident that both of the PCR fragments cloned from lake trout and brook trout represent full-length cDNA clones of CYP1A genes. Each cDNA sequence has characteristics of a full-length teleost CYP1A cDNA: a start codon and a stop codon followed by a poly A tail, a heme-binding domain, an arginine codon integral to enzymatic function, and a rather large 3′-UTR containing two (lake trout) and three (brook trout) AUUUA sequences. The coding region (1569 bp), which encodes a protein of 523 amino acid residues, is the same size as the rainbow trout and Atlantic salmon P450 1A protein. In addition, the brook trout and lake trout CYP1A genes show 97.9 and 97.6% sequence identity to Atlantic salmon CYP1A. The coding regions of CYP1A genes isolated to date in salmonids (Atlantic salmon, rainbow trout, Pacific salmon, brook trout, and lake trout) differ by no more than 3.9%. This high level of sequence identity confirms that the CYP1A gene is highly conserved and thus suitable for developing a real-time quantitative PCR assay to study CYP1A expression dynamics across several genera in response to contaminant exposure.

Real-time PCR analysis indicates that CYP1A induction in liver tissue of lake trout, brook trout, rainbow trout, and Atlantic salmon followed a consistent pattern. In all species, CYP1A expression was induced by BNF injection from approximately 1.8–3.0 orders of magnitude representing a 60–1000-fold difference in CYP1A levels between control and induced levels. This trend of induction was seen in fish injected with 25 mg kg^{-1} and also 50 mg kg^{-1} BNF. Previous quantitative PCR studies have also found a similar level of induction by BNF in Atlantic salmon (Rees et al., 2003) and Pacific salmon (Campbell and Devlin, 1996) in a variety of tissues (liver, kidney, gill, brain, and gonad). Absolute levels of CYP1A expression ranged from a low of approximately 7×10^5 molecules CYP1A μg^{-1} total RNA in rainbow trout liver (control group) to a high of approximately 1×10^9 molecules CYP1A μg^{-1} total RNA in brook trout liver (induced). The CYP1A levels reported here closely resemble CYP1A mRNA expression levels identified in a 28 day BNF induction time course in Pacific salmon liver tissue. Campbell and Devlin (1996) report that at time zero Pacific salmon liver shows similar levels of CYP1A at 5.00×10^5 molecules μg^{-1} total RNA. After 28 days, this expression jumps 160-fold to 8.04×10^7 transcripts μg^{-1} total RNA. However, the results from each of these experiments are 2–3-orders of magnitude lower than those determined using a competitive quantitative PCR (Rees et al., 2003). This

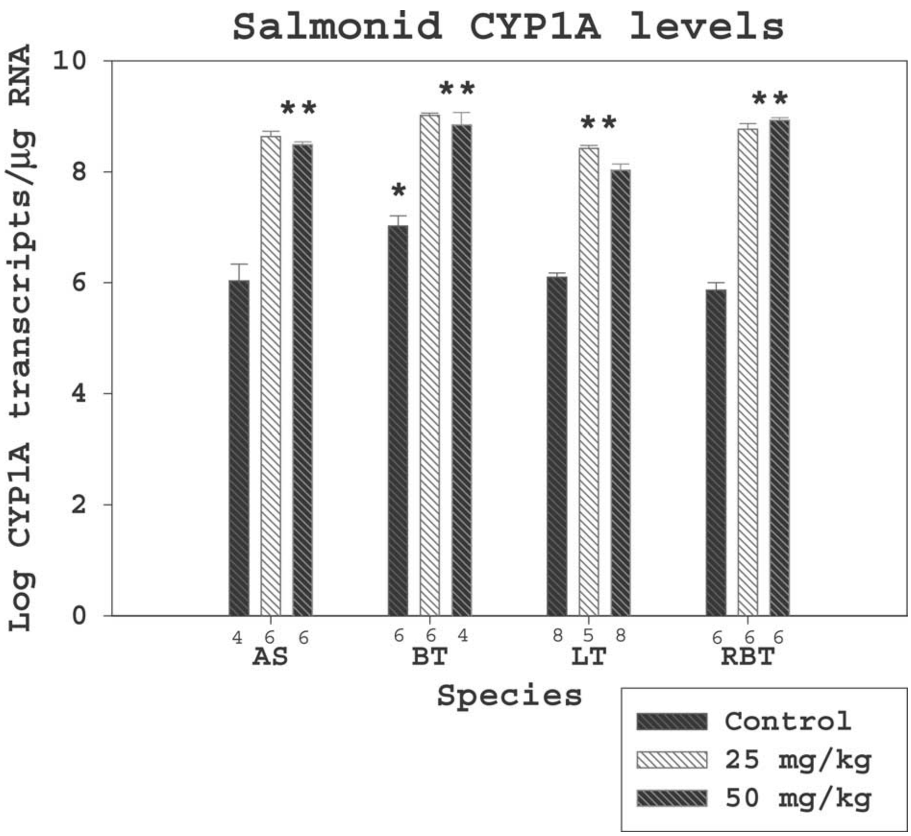

Fig. 6. Real-time PCR analysis of liver CYP1A levels in representative salmonid species (AS: Atlantic salmon, BT: brook trout, LT: lake trout, RBT: rainbow trout). Fish were administered an injection of corn oil only (control), 25 mg kg^{-1} body weight BNF (β-naphthoflavone), or 50 mg kg^{-1} body weight BNF. Total RNA (100 ng) was reverse-transcribed and amplified in real-time from each treatment group ($n = 4$–8; sample size is indicated for each treatment group below each bar) after which CYP1A levels were estimated. Bars represent mean logarithmic values of CYP1A expression μg^{-1} total RNA ± S.E.M. for each treatment group. Comparisons were made between induced and control levels using a Tukey–Kramer adjustment for multiple comparisons. Symbol (✱✱) notes significance of induced groups over each respective control group at $P < 0.0001$. Symbol (✱) notes significantly higher CYP1A levels over other control groups at $P < 0.01$.

discrepancy is likely due to different fish conditions and experimental conditions. Another likely factor is the difference in the standard curves between the two experiments. The real-time PCR standard curve used here covered a larger linear range and had a higher coefficient of determination, thus would be more accurate at estimating CYP1A levels from samples expected to show a large range of difference. Nevertheless, it is evident that each of these assays is useful in showing inducing effects of BNF on CYP1A in fish.

The real-time PCR developed in this study is highly sensitive and versatile. Based on our standard curves, it can measure CYP1A mRNA expression levels down to ∼1000 transcripts of CYP1A μl^{-1} total RNA because the standard curve obtained in this assay covers a large linear range (10^{10}–10^3 molecules) and allows for versatility in measuring CYP1A gene expression through a wide degree of environmental and laboratory conditions. At ∼100 transcripts, a strong signal was observed but the C_t fell out of the linear range of the standard curve. This high level of sensitivity and wide range of applicability will likely enable accurate measurement of CYP1A levels in wild fish from both pristine and highly polluted environments. Because many salmonid species are at threatened status or worse (U.S. Department of Interior, 2000, 2002), this quantitative PCR assay will complement the development of a non-lethal gill biopsy method to monitor contaminant exposure in salmonid populations (Rees et al., in review) without sacrificing individual fish captured in the wild.

Furthermore, this assay makes measuring CYP1A gene expression among various species more accurate, comparable, and quicker because the same primers and probe are used for each species. This allows RNA from various tissues of multiple species to be analyzed on the same plate and compared to the same standard curve. This assay also minimizes the possibility of quantifying false positives such as non-specific PCR products because the probe is single-stranded and only binds to the target sequence. Fluorescence is not emitted unless this binding occurs; therefore, only fluorescence from specific binding is measured (Giuletti et al., 2001). In addition, the time-intensive process during the generation of a "pure" cRNA standard is minimized because the same standard can be used for multiple species. This type of application has been used in the recent past to detect and quantify the same infectious heamatopoietic necrosis virus (IHNV) in multiple salmonid species (Overturf et al., 2001). However, our study documented a use of the real-time PCR assay to measure expression levels of an orthologous gene across several members in a single teleost family.

From an environmental assessment standpoint, it is important to realize the benefits and limits of this quantitative PCR assay. Because the amplification conditions are optimized and primer and probe sequences are known, it will be possible for other laboratories to adopt this method in a matter of days. This approach has the advantage of providing rapid and accurate measures of CYP1A transcripts in various tissues. To develop a comprehensive understanding of CYP1A induction in fish responding to contaminant exposure, this method should be combined with methods that provide better spatial resolution, such as in situ hybridization and immunocytochemistry, and that provide estimated CYP1A protein levels, such as ELISA, Western blotting and EROD analysis.

In conclusion, we have developed a real-time quantitative PCR assay for analysis of CYP1A expression across three salmonid genera, *Salmo*, *Oncorhynchus*, and *Salvelinus*. In development of this assay, we confirmed that CYP1A genes across the salmonid family carry a high degree of sequence homology and is highly induced in liver tissue of BNF-exposed lake trout, brook trout, Atlantic salmon, and rainbow trout after 2 days. We also discovered some species-specific characteristics of CYP1A induction. Brook trout showed higher basal levels and induced levels than all other species. Lake trout showed the lowest induced levels of CYP1A expression. It is notable that brook trout and lake trout, which showed a significant difference in induction, were reared in the same hatchery since embryonic stage. The ecological and physiological implication of this species difference, however, will require further studies to clarify. Finally, the real-time assay has a high degree of specificity for generated CYP1A PCR products as well as a high degree of sensitivity detecting down to 1000 molecules CYP1A μl^{-1} total RNA.

Acknowledgements

John Driver and Jim Aho of the Marquette Fish Hatchery supplied the brook trout and lake trout, Martha Wolmagood and Matthew Hughes of the Wolf Lake State Fish Hatchery provided rainbow trout, and Roger Greil of the Lake Superior State University Fish Hatchery donated Atlantic salmon. Technical discussions on real-time PCR analysis and data interpretation were offered by Jeff Landgraf of the Genomics Technology Support Facility at Michigan State University. Helpful suggestions on statistical analysis were provided by Brad Young. Assistance with fish treatments and injections was given by Yu-Wen Chung-Davidson, Hong Wu, Sang Seon Yun, Brad Young, Jesse Semeyn, Jessica Miller, and Rachel McNinch. This research was funded by National Oceanic Atmospheric Administration and the Great Lakes Fishery Commission.

References

Andersson, T., Pesonen, M., Johansson, C., 1985. Differential induction of cytochrome P-450-dependent monoxygenase, epoxide hydrolase, glutathione transferase, and UDP glucuronyltransferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A. Biochem. Pharmacol. 50 (34), 3309–3314.

Berndtson, A., Chen, T., 1994. Two unique CYP1 genes are expressed in response to 3-methylcholanthrene treatment in rainbow-trout. Arch. Biochem. Biophys. 310, 187–195.

Campbell, P.M., Devlin, R.H., 1996. Expression of CYP1A1 in livers and gonads of Pacific salmon: quantitation of mRNA levels by RT-cPCR. Aquat. Toxicol. 34, 47–69.

Cousinou, M., Nilsen, B., Lopez-Barea, J., Dorado, G., 2000. New methods to use fish cytochrome P4501A to assess marine organic pollutants. Sci. Total Environ. 247, 213–225.

Giuletti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25, 386–401.

Gooneratne, R., Miranda, C.L., Henderson, M.C., Buhler, D.R., 1997. Beta-naphthoflavone induced CYP1A1 and 1A3 proteins in the liver of rainbow trout (*Oncorhynchus mykiss*). Xenobiotica 27, 175–187.

Grøsvik, B.E., Larsen, H.E., Goksøyr, A., 1997. Effects of piperonyl butoxide and B-naphthoflavone on cytochrome P4501A expression and activity in Atlantic salmon (*Salmo salar* L.). Environ. Toxicol. Chem. 16, 415–423.

Hahn, M.E., Woodin, B.R., Stegeman, J.J., Tillitt, D.E., 1998. Aryl hydrocarbon receptor function in early vertebrates: inducibility of cytochrome P450 1A in agnathan and elasmobranch fish. Comparative Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol. 120, 67–75.

Heilmann, L., Sheen, Y., Bigelow, S., Nebert, D., 1988. Trout-P450IA1—cDNA and deduced protein-sequence, expression in liver, and evolutionary significance. DNA 7, 379–387.

James, M.O., Bend, J.R., 1980. PAH induction of cytochrome P4502E1 by nitrogen and sulfur-heterocycles: expression and molecular regulation. Toxicol. Appl. Pharmacol. 120, 257–265.

Kleinow, K.M., Melancon, M.J., Lech, J.J., 1987. Biotransformation and induction—implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environ. Health Perspect. 71, 105–119.

Kramer, C.Y., 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12, 307–310.

Lee, S., Wang-Buhler, J., Cok, I., Yu, T., Yang, Y., Miranda, C., Lech, J., Buhler, D., 1998. Cloning, sequencing, and tissue expression of CYP3A27, a new member of the CYP3A subfamily from embryonic and adult rainbow trout livers. Arch. Biochem. Biophys. 360, 53–61.

Levine, S.L., Oris, J.T., Wissing, T.E., 1994. Comparison of mono-oxygenase induction in gizzard shad (*Dorosoma cepedianum*) following waterborne exposure to benzo[a]pyrene. Comparative Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol. 118, 397–404.

Levine, S.L., Oris, J.T., 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquat. Toxicol. 46, 279–287.

Mansuy, D., 1998. The great diversity of reactions catalyzed by cytochromes P450. Comparative Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol. 121, 5–14.

Miller, M.R., Hinton, D.E., Stegeman, J.J., 1989. Cytochrome-P-450E induction and localization in gill pillar (endothelial) cells of scup and rainbow trout. Aquat. Toxicol. 14, 307–322.

Miller, H.C., Mills, G.N., Bembo, D.G., Macdonald, J.A., Evans, C.W., 1999. Induction of cytochrome P4501A (CYP1A) in *Trematomus bernacchii* as an indicator of environmental pollution in Antarctica: assessment by quantitative RT-PCR. Aquat. Toxicol. 44, 183–193.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6, 1–42.

Overturf, K., Lapatra, S., Powell, M., 2001. Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. J. Fish Dis. 24, 325–333.

Rees, C.B., McCormick, S.D., Li, W., 2003. Quantitative PCR analysis of CYP1A induction in Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 62, 67–78.

Sabourault, C., Berge, J.B., Lafaurie, M., Girard, J.P., Amichot, N., 1998. Molecular cloning of a phthalate-inducible CYP4 gene (CYP4T2) in kidney from the sea bass, *Dicentrarchus labrax*. Biochem. Biophys. Res. Commun. 251, 213–219.

Sambrook, J., Fritsch, E.F., Maniatas, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, New York.

Sarasquete, C., Segner, H., 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. Sci. Total Environ. 247, 313–332.

Schlezinger, J.J., Stegeman, J.J., 2001. Induction and suppression of cytochrome P450 1A by 3,3′,4,4′,5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). Aquat. Toxicol. 52, 101–115.

Smolowitz, R.M., Hahn, M.E., Stegeman, J.J., 1991. Immunohistochemical localization of cytochrome-P-450lA1 induced by 3,3′,4,4′-tetrachlorobiphenyl and by 2,3,7,8-tetrachlorodibenzoafuran in liver and extrahepatic tissues of the teleost *Stenotomus-chrysops* (Scup). Drug Metab. Dispos. 19, 113–123.

Smolowitz, R.M., Schultz, M.E., Stegeman, J.J., 1992. Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis* spp.) by waterborne benzo[a]pyrene. Carcinogenesis 13, 2395–2402.

Stegeman, J.J., Moore, M.N., Hahn, M.E., 1992. Responses of marine organisms to pollutants. 1. Marine Environ. Res. 34 (1–4), R3–R4.

Stegeman, J.J., Schlezinger, J.J., Craddock, K.E., Tillitt, D.E., 2001. Cytochrome P450 1A expression in midwater fishes: potential effects of chemical contaminants in remote oceanic zones. Environ. Sci. Technol. 35, 54–62.

U.S. Department of Interior, 2000. Endangered and Threatened Species; Final Endangered Status for a Distinct Population Segment of Anadromous Atlantic salmon (*Salmo salar*) in the Gulf of Maine. Federal Register 65, 69459–69483.

U.S. Department of Interior, 2002. Endangered and Threatened Species; Take of Anadromous Fish. Federal Register 67, 37392–37393.

Van Veld, P.A., Vogelbein, W.K., Smolowitz, R., Woodin, B.R., Stegeman, J.J., 1992. Cytochrome P4501A1 in hepatic lesions of a teleost fish (*Fundulus heteroclitus*) collected from a polycyclic aromatic hydrocarbon-contaminated site. Carcinogenesis 13, 505–507.

Vanden Heuvel, J.P., Tyson, F.L., Bell, D.A., 1993. Construction of recombinant RNA templates for use as internal standards in quantitative RT-PCR. Biotechniques 14, 395–398.

Winer, B.J., 1971. Statistical Principles in Experimental Design, second ed. McGraw-Hill, New York.