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CLONING AND EXPRESSION ANALYSIS OF ATLANTIC SALMON (*SALMO SALAR*) CYP1A

By

Christopher Benjamin Rees

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ABSTRACT

CLONING AND EXPRESSION ANALYSIS OF
ATLANTIC SALMON (*SALMO SALAR*) CYP1A

By

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Environmental pollution, such as polyaromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), and dioxins, is suspected of causing recent declines in Atlantic salmon (*Salmo salar*) populations. I hypothesize that cytochrome P4501A (CYP1A) is inducible by β-Naphthoflavone (BNF), a PAH, in Atlantic salmon. To characterize the function and expression of this gene, I first determined the cDNA sequence of Atlantic salmon CYP1A through molecular cloning, the first CYP1A gene cloned for any salmon species. In addition, phylogenetic analysis of the CYP1A coding region as well as the deduced amino acid sequence placed this gene most closely related to rainbow trout CYP1A genes. I then demonstrated the inducibility of this gene by treating salmon with β-Naphthoflavone and visualizing through Northern blotting procedures. Furthermore, based on the Atlantic salmon CYP1A sequence, a highly specific and sensitive competitive reverse transcription-polymerase chain reaction was developed and used to determine the levels of CYP1A expression. This method was utilized to quantify the levels of inducibility of CYP1A in liver, gill, kidney and brain tissue as well as to demonstrate induction of CYP1A in wild Atlantic salmon from streams known to contain PCB's, PAH's, and dioxins. These studies provide a foundation for studying CYP1A in salmon as well as other species, for quantitatively assessing biomarker responses in Atlantic salmon, and for providing valuable tools to manage dwindling salmon stocks.

DEDICATION

To my family and friends for all of their love and support.

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CHAPTER ONE: Cloning and induction of CYP1A in Atlantic salmon (*Salmo salar*)

ABSTRACT

Environmental contaminants are implicated for recent declines in Atlantic salmon stocks across the eastern coast of the United States and Canada (Fairchild et al. 1999). To understand the function of cytochrome P450 enzymes in the metabolism of these toxicants, I have cloned a CYP1A gene in Atlantic salmon using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The cloned cDNA possesses all characteristic motifs of teleost CYP1A genes including the start codon, heme-binding region, stop codon, and putative poly-adenylation signal. This gene is also characterized by a long 3' untranslated region (UTR; 1025bp) containing three AUUUA sequences, which are a target for its rapid degradation. The Atlantic salmon CYP1A shares 97.4% and 96.7% sequence identity with the corresponding rainbow trout CYP1A1 and CYP1A3 genes, respectively. In addition, the deduced amino acid sequence of salmon CYP1A shows 96.9% and 96.2% identity with rainbow trout P4501A1 and P4501A3 enzymes, respectively. Phylogenetic analysis of a sample of P450 genes established that this Atlantic salmon CYP1A gene is closely related to rainbow trout CYP1A genes as well. Northern analysis showed that CYP1A mRNA is significantly higher in the liver of Atlantic salmon injected intraperitoneally with β-Naphthoflavone (BNF) suggesting that the cloned gene is inducible (Rees 2001; this paper). The sequence of the CYP1A cDNA is useful in development of biomarkers for exposure of Atlantic salmon to a broad range of xenobiotics.

INTRODUCTION

Cytochrome P450 constitutes a ubiquitous enzyme system noted for its inducibility and diversity (Andersson and Forlin 1992). These enzymes have been found in all species studied to date including plants, animals, bacteria, and other organisms (Nelson et al. 1996). P450's are involved with the detoxification of organic compounds such as xenobiotics (Mehmood et al. 1996, Goksoyr and Husoy 1998), inhaled odorants (Dahl and Hadley 1991, Nef et al. 1989, Lazard et al. 1991), steroids (Brittebo and Rafter 1984, Ding and Coon 1994), drugs (Nef et al. 1989, Schlenk et al. 1993), and carcinogens (Hrelia et al. 1996, Kawajiri and Fujii-Kuriyama 1991). Historically, mammals have been the primary targets of P450 examinations. More recently, fish species have become models of P450 research.

In fish, the most intensively studied P450 genes are CYP1A's (Nelson et al. 1996). Their sequences have been determined in rainbow trout (Heilmann et al. 1988, Berndtson and Chen 1994), plaice (Leaver et al. 1993), Atlantic tomcod (Roy et al. 1995), toadfish and scup (Morrison et al. 1995), killifish (Morrison et al. 1998), red sea bream (Mizukami et al. 1994), and sea bass (Stien et al. 1998). These experiments have provided tools to understand the structure and function of cytochrome P450 proteins in general, and the role of CYP1A in metabolism of a variety of toxicants in particular.

I attempted to clone a CYP1A gene from Atlantic salmon to determine its structure and evolutionary relationship to other teleost CYP1A cDNA's. This information will be useful to understand the steep decline of Atlantic salmon populations in response to environmental pollutants across the eastern seaboard, including both the United States and Canada (Tufts 2000). These pollutants have long been suspected of

negatively impacting Atlantic salmon populations (Waldichuk 1979). Recent research has focused on the possibility that pesticides may be affecting the return rates of Atlantic salmon in Canada (Fairchild et al. 1999). The problems associated with salmon exposure to environmental pollution are two fold. Firstly, one of the most sensitive stages of stream life, smoltification (Moyle and Cech 2000), coincides seasonally with the peak agricultural application of pesticides (Albanis et al. 1998). Secondly, many studies suggest that pesticides may have profound effects (decreased reproductive success; lower recruitment, etc.) on teleosts at sublethal concentrations (Little et al. 1990, Jones et al. 1998, Gagne et al. 1999), which over time, may lead to population decline. Therefore, the acute effects pesticides have on Atlantic salmon development may be subtle, emphasizing the need for molecular markers. In order to understand these effects at the molecular level, I examined the cDNA sequence, deduced amino acid sequence, and inducibility of CYP1A in Atlantic salmon liver.

MATERIALS AND METHODS

Fish

Thirty Atlantic salmon (*Salmo salar*) approximately 20cm in length and 50 grams in weight were acquired from a fish hatchery (Lake Superior State University, Sault Ste. Marie, Michigan) and acclimated for 2 weeks at the Michigan State University Lower River Laboratory in an 800 l flow through tank (1 fish 20 l^{-1}) at 11.5°C. A 12 hour light-dark cycle was maintained during the acclimation period. Salmon were fed Purina AquaMax© Grower 400 (lot A-5D04) daily at a level of 3.0% body weight. Two days prior to tissue collection, salmon were taken off of feed.

Tissue collection

Fish were given an overdose of MS-222. The liver was removed, sectioned 3 times, immediately stored in at least a 10x volume of RNALater© (Ambion; Austin, Texas), and subsequently stored at -80°C.

RNA extraction

Total RNA was extracted using TRIzol® Reagent according to the manufacturer's protocol (Life Technologies; Rockville, MD), resuspended in 50μl diethylpyrocarbonate-treated water, and quantified on a UV spectrophotometer (Beckman DU® 7400; Fullerton, CA). RNA solution was mixed with 3 volumes of 70% ethanol and 3 M sodium acetate to a final concentration of 0.3 M and stored at -80°C until further analysis. Storage of RNA in this condition is stable (Sambrook et al. 1989).

First strand cDNA synthesis

Total RNA (1-5 μg) was mixed with 10pM μl^{-1} Oligo $d(T)^{18}$ and diluted with deionized water to a final volume of 12μl. This mixture was heated at 70°C for 10 minutes and quick chilled on ice. Next, 4μl 5x first strand buffer, 2μl 0.1 M dithiothreitol, and 1μl 10mM dNTP were also added. After incubation of this mixture at 42°C for 2 minutes, 200 units of MMLV-RT (Life Technologies) were added making the final reaction volume 20μl. The reaction mixture was incubated at 42°C for 50 minutes and inactivated at 70°C for 15 minutes. Finally, 10 units of RNAseH (Life Technologies) were added, incubated at 37°C for 20 minutes, and inactivated at 94°C for 5 minutes. The synthesized cDNA was stored at -20°C until used for PCR.

Polymerase chain reaction (PCR)

To isolate a CYP1A gene in Atlantic salmon liver, I first aligned three rainbow trout CYP1A sequences (Heilmann et al. 1988; Berndtson and Chen 1994) using the CLUSTAL W algorithm. Then, using a region spanning the heme-binding domain and the helical region, I selected degenerate primers that flanked a region of approximately 580bp in known teleost genes (Table 1). Degenerate primers were synthesized (Macromolecular Structure, Synthesis, and Sequencing Facility, Michigan State University).

PCR master mix consisted of the following constituents (final reaction concentrations in parentheses): 2μl of cDNA, dNTP's (200μM), 5x PCR Buffer (1x), $MgCl_2$ (4mM), WML38 sense primer (500nM), and WML39 antisense primer (500nM). The final 50μl reaction was amplified in a PTC-200 MJ Research Thermocycler (1 cycle

94°C 4 min; 30 cycles 94°C 1 min, 56°C 45s, 72°C 1 min; 1 cycle 72°C 5 min; 1 cycle 4°C forever). The product was subsequently cloned and sequenced as described in the Materials and Methods section.

Rapid amplification of cDNA ends (RACE)

Gene specific primers (GSP) for 5' and 3' RACE were designed by using the sequence of the previous PCR product. RACE was carried out utilizing the Advantage II RACE system (Clontech; Palo Alto, CA) according to the manufacturer's protocol. One μg of total RNA from Atlantic salmon liver was used as template for synthesis of 5' and 3' RACE Ready cDNA. GSP's were used individually with the universal primer mix supplied in the kit for both 3' and 5' RACE. Nested GSP's were used to enhance the efficiency of RACE amplification (Table 1).

To clone a full length CYP1A cDNA, a two-step long distance RACE-PCR was utilized. Briefly, a 3' RACE GSP (WML51) was designed to extend the cDNA to the 3' end (poly A tail) while a 5' RACE GSP (WML52) was used to extend to the 5' end. The two RACE products produced by this method were expected to have a region of overlap of ~200bp. Both RACE products were cloned and sequenced as previously described and analyzed using BLAST on the world-wide-web. To eliminate the possibility of producing a hybrid sequence of a full length CYP1A gene, I conducted a second RACE with a GSP designed upstream from the start codon. The expected product from this RACE would include all functional domains of a single, full-length CYP1A gene.

Molecular cloning

All PCR products were size-fractionated on a 1% agarose gel containing 0.1μg/ml ethidium bromide (Sambrook et al. 1989). PCR products were cleaned using the Wizard DNA clean-up system (Promega Corporation; Madison, WI), ligated into a pGEM – T Easy vector (Promega Corporation), and subsequently electroporated into DH5α competent cells (Clontech). Plasmid DNA minipreps were extracted by Wizard DNA Miniprep Kit (Promega Corporation).

Sequencing

Preliminary sequencing reactions were performed using the Sequitherm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies; Madison, WI). Hex-labeled T7 (forward sequencing primer) and SP6 (reverse sequencing primer) primers were purchased from Integrated DNA Technologies (Coralville, IA). Once CYP1A positive inserts were identified using dideoxy sequencing and BLAST, extended sequencing was carried out by the Plant Biology DNA Sequencing Facility, Michigan State University. Primer walking was used for shorter inserts, between 1-1.5kb. In vitro transposon insertion was used (Genome Priming System, New England Biolabs) for sequencing of templates greater than 1.7kb. DNA sequence comparison and analysis was performed either with BLAST (http://www.ncbi.nlm.nih.gov/BLAST), Sequencher 3.1 (Gene Codes Corporation; Ann Arbor, MI), MacVector 6 (Oxford Molecular Group PLC; Huntsville, MD), or DNASTAR (DNASTAR, Inc.; Madison, WI).

Phylogenetic analysis

The coding region of 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. This same analysis was also performed on CYP1A amino acid sequences as well. Genes selected for this analysis comprised mostly teleost CYP1A genes, several mammalian CYP1A genes, and two rainbow trout P450 genes of other subfamilies (CYP2K1 and CYP3A27). Initially, teleost genes were aligned after which subsequent mammalian genes were aligned. Finally, rainbow trout CYP2K1 and CYP3A27 were added to the alignment.

Induction

To demonstrate the inducibility of the CYP1A gene in Atlantic salmon, randomly selected individuals (n=4) were sampled and given either an intraperitoneal injection of β-Naphthoflavone (BNF, a potent inducer of CYP1A; 50mg kg^{-1} body weight) dissolved in corn oil (10mg BNF ml^{-1} corn oil) or corn oil alone. Individual salmon were then placed for 48 hrs in a 40 l flow-through aquarium (20 l h^{-1}; 0.5R), sacrificed with an overdose of MS-222, and dissected for liver tissues which were immediately stored in RNALater© (Ambion).

Northern blotting

Total RNA (7μg) from control and induced samples were separated on 1% agarose-formaldehyde gels and transferred to positively charged nylon membranes (Ambion). P450 full sequence clones were used as probes and were labeled using the

DIG Random Primed DNA Kit (Roche Diagnostics Corp.; Indianapolis, IN). Blots were hybridized according to the manufacturer's protocol and washed (68°C, 0.1x SSC, 0.1% SDS) followed by color detection reactions (Roche Diagnostics Corp.). Probes for actin mRNA were produced by the same labeling technique as above from an Atlantic salmon β-actin cDNA (Rogers et al. 1999, GenBank Accession AF012125) fragment confirmed by sequencing.

RESULTS

Atlantic salmon CYP1A

Initial PCR amplification of CYP1A from Atlantic salmon liver cDNA produced a fragment approximately 580bp long (Figure 1A). Molecular cloning and sequencing of this PCR product established that it was homologous to rainbow trout CYP1A and exactly 576bp long. GSP's obtained from this preliminary sequence extended to the 3' and 5' end by RACE reactions. The resulting CYP1A fragments were 1,669bp and 1,394bp respectively with a region of overlap of 205bp (Figure 1B). Both 3' and 5' RACE fragments were roughly 95% homologous to both rainbow trout CYP1A1 and CYP1A3 cDNA molecules.

To verify that the two overlapping products encode a single CYP1A gene, a long distance 3' RACE fragment of 2626bp was obtained (Figure 1C). Sequence analysis indicated this product included 32bp of the 5' untranslated region (UTR), a 1569bp coding region, and a 1025bp 3' UTR containing 3 AUUUA sequences. It encodes a protein of 523 amino acid residues. The obtained sequence also possesses 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 putative polyadenylation signal (Figure 2).

Phylogenetic analysis

This gene shares 97.4% sequence identity with the corresponding rainbow trout CYP1A1 gene and 96.7% sequence identity with rainbow trout CYP1A3. In addition,

this gene shares between 70-76% nucleotide homology with other teleost CYP1A genes. The deduced amino acid sequence shows 96.9% amino acid identity with rainbow trout p4501A1 and 96.2% amino acid similarity with rainbow trout P4501A3. Likewise, the salmon CYP1A protein demonstrates between 68-83% amino acid homology with other teleost CYP1A proteins. The heme-binding region encompassing the amino acid sequence FGMGKRRCIG (positions 456-465) is highly conserved over all teleosts including Atlantic salmon (Figure 3).

Multiple sequence alignment using the CLUSTAL W algorithm followed by construction of phylogenetic tree using the Neighbor-joining method suggested that *Salmo salar* CYP1A is most highly related to *Oncorhyncus mykiss* CYP1A genes of the genes compared (Table 2). The phylogenetic tree also showed that Atlantic salmon CYP1A is related to representative teleost CYP1A genes, followed by mammalian CYP1A cDNA's, and finally members of *Oncorhyncus mykiss* CYP subfamilies 2 and 3. The same analysis based on predicted amino acid sequences demonstrated the same relationship (Table 3). The phylogenetic methods carried out here showed that Atlantic salmon CYP1A is a close relative of rainbow trout CYP1A genes.

Induction of CYP1A

In the Northern analysis, the CYP1A probe hybridized with mRNA approximately 2.7kb in length from salmon treated with β-Naphthoflavone (Figure 4). Total RNA extracted from control salmon did not show a visible hybridization band. In addition, a probe designed from an amplified PCR product of the Atlantic salmon β-actin gene hybridized with mRNA of approximately 1.2kb from both control and induced samples

with both bands showing roughly the same density. This suggests the difference in CYP1A mRNA levels between control and induced salmon is not due to different loading of total RNA (data not shown).

DISCUSSION

There are several lines of evidence that suggest the cDNA I have cloned is a full-length CYP1A molecule from Atlantic salmon. It contains all of the positional characteristics of a full-length coding cDNA including the start codon and a stop codon followed by the poly A tail. This cDNA also carries qualities characteristic of many teleost CYP1A genes such as the heme-binding domain, arginine codon integral to enzymatic function, and a rather large 3' UTR (1025bp). The coding region (1569bp), which encodes a protein of 523 amino acid residues, is the same size as the rainbow trout P4501A protein. This gene shows 97.4% and 96.7% sequence identity with rainbow trout CYP1A1 and CYP1A3, respectively. The deduced amino acid sequence of this salmon gene is highly homologous to rainbow trout CYP1A1 and CYP1A3 proteins (96.9% and 96.2% respectively). Furthermore, this gene was demonstrated to be inducible by BNF, a strong characteristic of CYP1A genes. This is the first sequenced cytochrome P450 gene in Atlantic salmon.

It is especially interesting that this gene has a 3' UTR as long as 1025bp. The RNA sequence AUUUA recurs frequently in the 3' UTR of many CYP1A genes. In mammalian species, where CYP1A1 genes generally have 1 or 2 AUUUA (Sagami et al. 1991), this sequence has been postulated to be involved with RNA degradation (Fukuhara et al. 1989; Shaw and Kamen 1986; Binder et al. 1989). This property of 3' UTR's in teleost CYP1A molecules has not been reported or discussed previously. Atlantic salmon CYP1A has 3 AUUUA sequences in the 3' UTR suggesting this RNA is likely to be targeted for rapid removal in cells.

Whether Atlantic salmon has two CYP1A genes needs to be explored. It has been demonstrated that rainbow trout have two separately functioning CYP1A genes, CYP1A1 and CYP1A3 (Berndtson and Chen 1994). *Salmo*, which is the sister genus of *Oncorhyncus* (Allendorf and Waples 1996), is likely to possess two CYP1A molecules in all species of the genus. This raises two issues. The first issue dealt with isolation of one single CYP1A gene. During initial PCR amplification, the primer sequences utilized in these experiments showed 100% homology to both rainbow trout CYP1A genes. In addition, the product amplified also showed less than a 1% difference in sequence identity between both CYP1A genes in rainbow trout. Therefore, the RACE primers initially utilized also were "non-distinguishing" between the possibility of two CYP1A genes in Atlantic salmon liver. The first CYP1A gene published in rainbow trout was named CYP1A1 (Heilmann et al. 1988). Recently, it has been reported that this gene actually was a chimeric sequence incorporating both CYP1A1 and CYP1A3 character (Rabergh et al. 2000). For that reason, even though overlapping 5' and 3' RACE CYP1A products were produced, I was not sure whether this was a chimeric sequence. The CYP1A genes in rainbow trout differ mostly in their 5' UTR. Therefore, I designed another RACE primer in a nonhomologous 5' region upstream from the start codon. The long distance RACE product obtained using this GSP contained 32bp of the 5' UTR as well as the entire coding region and 3' UTR. Therefore, it is likely this cDNA is a single CYP1A gene from Atlantic salmon liver.

The second issue dealt with the naming of this gene. As discussed in previous teleost CYP1A cloning papers (Morrison et al. 1998; Nelson et al. 1996), careful considerations are essential to correctly assign nomenclature to each new CYP1A gene

published. In recent years, functional properties as well as extended sequence analyses have become necessities in distinguishing families and subfamilies of CYP1A genes. One of the major ways to distinguish between CYP1A1 and CYP1A3 in rainbow trout is by looking for presence or absence of xenobiotic regulatory elements (XRE) in the 5' flanking region of each gene. CYP1A1 contains no XRE's while CYP1A3 contains two XRE's (Berndtson and Chen 1994). Unfortunately, the 5' UTR of this salmon gene isn't large enough to verify whether or not it contains XRE's. Another possible way to assign a name to the CYP1A gene from Atlantic salmon is by comparison to rainbow trout CYP1A1and CYP1A3. However, rainbow trout CYP1A genes show 96% homology suggesting a more recent gene duplication event, and thus making comparisons in this manner problematic. Conservatively, I have assigned this Atlantic salmon P450 gene as CYP1A. However, based on strict sequence comparison, it is likely this cDNA is a CYP1A1 molecule.

The nucleotide sequence of CYP1A is useful as a tool for continued research on Atlantic salmon. Highly accurate and expedient techniques now exist which use sequence information to quantitatively assess expression levels of particular genes. One of these techniques, quantitative PCR, has been used previously to study expression of CYP1A genes (Vanden Heuvel et al. 1994; Miller et al. 1999). Using CYP1A as a biomarker in this manner will open a new avenue of pesticide research and hopefully will shed light on some of the causes of Atlantic salmon decline over the last few decades. For instance, this biomarker would make it possible to characterize the impact of environmental exposure across many salmon streams or even across entire regions, thus making it easier for scientists to focus their efforts for salmon restoration.

Further studies are needed to characterize the family of cytochrome P450 genes in Atlantic salmon and to understand their complex diversity and evolutionary relationships to the same genes of other teleosts and mammals. It is apparent that P450 genes have been genetically modified in many ways to account for and metabolize the many compounds with which fish interact throughout their life history. P450 genes from subfamily 2 and 3 have been identified in rainbow trout. Based on the genetic similarity of rainbow trout and Atlantic salmon, these gene subfamilies would be expected to exist in Atlantic salmon as well. Likewise, sequence and evolutionary comparisons are needed from the Pacific salmon species to grasp knowledge of how salmonid species in general have adapted to their changing environments.

In conclusion, I have cloned a single, full-length CYP1A cDNA from Atlantic salmon. This cDNA contains all functional domains of teleost CYP1A genes, is most closely related to rainbow trout CYP1A1 through sequence homology comparisons and phylogenetic analysis, and is BNF inducible. I also believe this gene is targeted for rapid degradation due to the presence of AUUUA sequences in its 3' UTR.

CHAPTER TWO: Quantitative PCR analysis of CYP1A induction in Atlantic salmon (*Salmo salar*)

ABSTRACT

CYP1A genes are highly inducible by a broad range of environmental pollutants (Goksoyr and Husoy 1998, Croce et al. 1995) and are suspected of causing declines in populations of Atlantic salmon (*Salmo salar*; Fairchild et al. 1999). I developed a highly sensitive technique, quantitative reverse transcription-polymerase chain reaction (RT-PCR), for measuring the levels of induction of salmon CYP1A gene expression. Two groups of 100 salmon maintained at 11°C and 17°C received an intraperitoneal injection (50mg kg^{-1}) of either β-Naphthoflavone (BNF) in corn oil (10mg BNF ml^{-1} corn oil) or corn oil alone. After a 48 h exposure salmon gill, kidney, liver, and brain were collected for RNA isolation and analysis. The highest base levels of CYP1A expression (2.56 $X10^9$ molecules/100ng RNA) were found in gill tissue. In addition, all tissues show induction of CYP1A by BNF. Kidney had the highest mean induction at 5 orders of magnitude while gill tissue showed the lowest mean induction at 2 orders of magnitude. This technique was also applied to salmon sampled from two Massachusetts streams. Salmon liver and gill tissue sampled from Miller's River (South Royalston, Worcester County), known to contain polychlorinated biphenyls (PCB), showed on average a 2 order of magnitude induction over those collected from a stream with limited contamination, Fourmile Brook (Northfield, Franklin County). These results suggest that quantitative PCR analysis of CYP1A expression is advantageous in studying ecotoxicity on populations of wild Atlantic salmon.

INTRODUCTION

Atlantic salmon populations across Eastern Canada and the United States have been suffering a steady decline for the past 30 years (Anderson et al. 2000). This decline has resulted in the listing of Atlantic salmon as an endangered species in the state of Maine as of November, 2000 (U.S. Department of Interior 2000). Fairchild et al. (1999) suggested that endocrine disrupting chemicals inhibit salmon development during stream life, home stream imprinting, or possibly smoltification. Other compounds such as polyaromatic hydrocarbons, polychlorinated biphenyls, dioxins, and furans can also have physiological and pathological effects on fish populations at sublethal concentrations (Goksoyr and Husoy 1998). These compounds and other pesticides are known to stimulate expression of various members of the cytochrome P450 family of genes (Beyer et al. 1996; Hodgson et al. 1995).

The cytochrome P450 detoxification system is involved in the metabolism of compounds such as steroids, prostaglandins, eicosanoids, drugs, and xenobiotics (Nelson et al. 1996; Larsen et al. 1992). It is an extensively studied enzyme system and has been found in bacteria, plants, and animals. Cytochrome P450 genes are highly diverse (Buhler et al. 1998; Sarasquete and Segner 2000). Approximately 120 different subfamilies of cytochrome P450 (CYP) genes have been identified (Nelson et al. 1996) and characterized by a wide range of xenobiotic-metabolizing functions.

The most intensively studied cytochrome P450 molecule is arguably P4501A1. Its gene (CYP1A1) is highly inducible by polyaromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), furans, and dioxins. The mechanism of this induction has been examined closely in rainbow trout (Buhler et al 1998; Cao et al. 2000; Porter

and Coon 1991). There are many factors other than environmental pollutants that also affect the expression of this gene such as stage of development, age, temperature, season, species, and genetic strain differences (Grosvik et al. 1997; Vignier et al. 1996; Goksoyr and Larsen 1991). Because this gene is inducible by a wide variety of compounds, it has been utilized as a biomarker for detecting environmental contamination and stress in fish populations (Anderson et al. 1995).

Studies of CYP1A inducibility and expression in fish have previously relied on techniques such as Northern blotting, Western blotting, ELISA, or 7-ethoxyresorufin O-deethlyase (EROD) enzyme kinetics (Anderson and Goksoyr 1994; Croce et al. 1995; Schlezinger and Stegeman 2001; Grosvik et al. 1997). These methods, although informative, require much fish tissue, time, and are often qualitative rather than quantitative in nature. In the last 10 years, a new and innovative technique for measuring gene expression, quantitative reverse transcription-polymerase chain reaction (RT-PCR), has been developed (Cousinou et al. 2000; Miller et al. 1999). As reported by Vanden Heuvel et al. (1993), RT-PCR is at least 10 fold more sensitive in detecting CYP1A induction over EROD activity and radioimmunoassay and at least 100 fold more sensitive than Northern or slot blotting in measuring CYP1A RNA. Also, because PCR is an amplification process, only a very small amount of tissue is required for analysis. A requirement in studying P450 levels with quantitative PCR is one must know the nucleotide sequence of the CYP1A cDNA in order to design highly specific primers for use in the procedure. The sequence of CYP1A in Atlantic salmon has been determined (see Chapter 1, this thesis).

The immediate goal of this study is to develop a sensitive and expedient assay to study toxicity responses of Atlantic salmon to persistent organic contaminants known to induce the CYP1A gene. This paper will show that quantitative PCR is a highly advantageous technique in studying P450 expression in Atlantic salmon in both lab-based experiments and specifically biomarker response studies with wild populations. There were two parts in developing this study. The first part attempted to develop the quantitative PCR method to study CYP1A induction using Atlantic salmon as a subject in the laboratory. Secondly, I applied the PCR method to assess toxic responses of wild Atlantic salmon. The ultimate goal of my study is to develop a highly sensitive biomarker to study the toxicity effects of PCB's, dioxins, and polyaromatic hydrocarbons on wild Atlantic salmon populations.

MATERIALS AND METHODS

Fish handling and sampling

For the laboratory induction study, 200 juvenile Atlantic salmon weighing 35g ± 7g and 15cm ± 2cm in length were acquired from Adirondack Fish Hatchery, Saranac Lake, NY and transported to Michigan State University where they were acclimated for 2 weeks at 11°C. The fish were then divided into two equal groups for another two-week acclimation period, one group at 11°C, the other at 17°C. Randomly selected individuals were sampled through unbiased netting procedures and given either an intraperitoneal injection of β-Naphthoflavone (BNF, Sigma Chemical Corp.; St. Louis, MO; 50mg kg^{-1} body weight) dissolved in corn oil (10mg ml^{-1}) or corn oil alone. Individual salmon were then placed for 48 h in an appropriate temperature 40 l flow-through aquarium (20 l h^{-1}). A 48 h exposure results in the maximum expression of CYP1A (Grosvik et al. 1997). Injected salmon were then sacrificed with an overdose of MS-222 (Sigma Chemical Corp.) and tissues (gill, liver, brain, and kidney) were collected and immediately stored in RNALater© at -20°C (Ambion; Austin, TX).

To sample wild salmon, 10 juvenile Atlantic salmon were collected by electro-shocking from two Massachusetts streams 25.8km apart, Miller's River (South Royalston, Worcester County) and Fourmile Brook (Northfield, Franklin County). Fourmile Brook samples were collected on October 17th (2000) and Miller's River was sampled on November 8th (2000). Miller's River, known to contain fish with tissue concentrations of PCB's between 0.8 – 5.5 μg/g (US Army Corps of Engineers 1995), was expected to have salmon with higher levels of CYP1A expression. All tissues

collected were immediately stored in RNALater© and shipped to Michigan State University for further analysis.

Total RNA isolation and storage

Tissue samples that had been stored in RNALater© (1 sample of liver, gill, brain, and kidney from each injected salmon) were homogenized and total RNA was extracted using Trizol Reagent (Life Technologies; Rockville, MD) according to the manufacturer's protocol. Total RNA was resuspended in 50 μl of diethylpyrocarbonate-treated water (DEPC-H_2O) and quantified (Sambrook et al. 1989) using a Beckman DU 7400 spectrophotometer (Fullerton, CA). Long-term storage of RNA samples was carried out by adding 3 volumes of 95% ethanol, 1/10 volume of 3 M sodium acetate, and placing at -80°C. Storage of RNA in this manner maintains stability of RNA for greater than 6 months (Sambrook et al. 1989).

Internal standard synthesis

An internal standard (IS) that contained a T7 promoter, both CYP1A forward and reverse primer sequences, and a poly-deoxythymidilic acid tail was synthesized by the method of Vanden Heuvel et al. (1993) and is outlined in Figure 5. Briefly, using human genomic DNA as a template for PCR, the WML53 5'-TAA TAC GAC TCA CTA TAG GCT GTC TTG GGC CGT TGT GTA CCT TGT GCA ACT TCA TCC ACG TTC ACC-3' and WML54 5'-TTT TTT TTT TTT TTT TTT TAT CCT TGA TCG TGC AGT GTG GGA TGG GAA GAG CCA AGG ACA GGT AC-3' internal standard primers (Macromolecular Structure, Sequencing, and Synthesis Facility, Michigan State

University) amplified a β-globin product of approximately 360bp under the following conditions: 3 mM $MgCl_2$, 0.4 mM dNTP's, 0.6nM forward internal standard primer, 0.6 nM reverse internal standard primer, a 1x concentration of PCR buffer, and 2.5 units of Taq DNA Polymerase (all reagents were from Life Technologies). This reaction was performed with 1 cycle at 94°C for 4 min, 30 cycles at 94°C for 20 sec, 59°C for 30 sec, and 72°C for 30 sec, and 1 cycle at 72°C for 5 min. The size of the product was verified on a 1% TAE agarose gel with a 100bp DNA ladder loaded (Life Technologies). I then diluted this product 1/100 with DI H_2O and reamplified the IS PCR product with the same reaction conditions. Unincorporated primers and primer-dimers were cleaned from the concentrated PCR product using the Wizard DNA Clean-Up System (Promega Corp.; Madison, WI). Once a purified PCR product was obtained, in vitro transcription was carried out using the Riboprobe In Vitro Transcription System (Promega Corp.) according to standard protocol. The rcRNA was then treated with RNase-free Dnase (Promega Corp.) to remove excess DNA template and subsequently extracted with water-saturated (pH 4.9) phenol/chloroform (24:1). The aqueous phase was isolated and extracted with chloroform/isoamyl alcohol (24:1) followed by an overnight ethanol precipitation at -20°C. To remove free nucleotides, the precipitated sample was spun down for 10min at 12,000g, resuspended in 20 μl DEPC-H_2O, and filtered through a G-50 Sephadex column (Amersham Pharmacia Biotech; Piscataway, NJ) pre-equilibrated in 0.1% SDS. The filtered sampled was precipitated overnight. After spinning the sample for 10min at 12, 000g the rcRNA pellet was washed with 70% ethanol, resuspended in DEPC-H_2O, and quantified with a UV spectrophotometer.

Competitive RT-PCR

It is necessary to gain an estimate of the levels of P450 mRNA in each tissue prior to spiking each RNA sample with internal standard. A standard curve was generated for each tissue and treatment to be analyzed (data not shown for all tissues) by co-reverse transcription and co-amplification of a constant amount of total RNA (100ng) against a dilution series of internal standard (10^{10} molecules – 10^3 molecules). These "range finding" experiments allow one to determine the relative levels of a particular gene of interest between several tissues and/or treatments (Vanden Heuvel 1998). These preliminary reactions help to determine the exact amount of internal standard to spike into each sample. Once initial range-finding experiments were concluded for each tissue, it was determined that it would be possible to use only one of the standard curves generated, thus reducing errors introduced through the use of several curves. Next, reverse transcription (all reagents were from Life Technologies) was performed on all samples in a final volume of 20 μl containing a 1x concentration of First Strand Buffer, 0.01 M dithiothreitol, 1mM of each deoxynucleotide triphosphate, 2.5μM oligo$(dT)_{18}$, 5 units of MMLV reverse transcriptase, 1 unit RNAsin (Promega Corp.), 100ng of total RNA, and varying amounts of internal standard predetermined from initial range-finding experiments. The reaction mix was incubated at 42°C for 50 min and inactivated at 70°C for 15 min. Immediately, 1 unit of RNase H (Life Technologies) was added and then each reaction was incubated at 37°C for 20 min, inactivated at 94°C for 5 min, after which 2 μl were taken and spiked into a PCR master mix. The 50 μl PCR mix contained 3 mM $MgCl_2$, 2.5 units Taq Polymerase, 30 pmol of each hex-labeled (Integrated DNA Technologies; Coralville, IA) forward and reverse primer (WML51 5'- CTG TCT TGG

GCC GTT GTG TAC CTT GTG-3' and WML52 5'- TAT CCT TGA TCG TGC AGT GTG GGA TGG-3'), and 0.4 mM dNTP's. A "hot start" was utilized where each reaction was heated to 94°C for 2 min after which Taq was added. Then, the reactions were heated to 94°C for 4 min, followed by 30 cycles of a 94°C denaturation for 20 sec, a 70°C annealing step for 30 sec, and a 72°C extension step for 30 sec. An additional 5 min extension step was included at the conclusion of the 30 cycle main reaction.

Because the efficiency of reverse transcription and PCR varies from tube to tube (Vanden Heuvel et al. 1994), four controls were used in my reactions. First, the internal standard controls for variability of reverse transcription and PCR amplification. The IS is roughly the same size as the target gene product and contains the same primer recognition sequence, thus it should amplify at the same efficiency as the target gene. Secondly, to assure IS was spiked at the expected concentration, a blank IS RT-PCR reaction was also included and visualized. Thirdly, the laser scanner used to visualize PCR products was calibrated across all gels by loading an absorbance standard (AS), which was simply a $1/10^{th}$ dilution of a single P450 PCR product. Finally, to assure the total RNA was loaded for each RT-PCR reaction at expected levels, I adapted the standard procedure used in many quantitative PCR studies (Vanden Heuvel et al. 1994; Loitsch et al. 1999). I sampled several cDNA samples that corresponded to lower or higher levels of P450 compared to other samples in a group of Atlantic salmon. Using hex-labeled primers ACTIN1 and ACTIN2 designed from an Atlantic salmon β-actin cDNA (Rogers et al. 1999, GenBank Accession AF012125) fragment confirmed by sequencing, I amplified a fragment of the actin gene using 25 cycles of the same conditions as was used for P450. If actin fragment amplification was equal across all samples I then concluded that any

difference in P450 levels in the corresponding samples was due to individual variation and not experimental error introduced by RNA loading.

PCR fragment visualization and data generation

PCR products were electrophoresed on a 4% non-denaturing polyacrylamide (BioRad; Hercules, CA) gel at 20 V cm^{-1}. The size of the products was verified using a hex-labeled MAPMARKER™ molecular size standard (Bioventures Inc.; Murfreesboro, TN). Densitometric readings were calculated using an FMBIO II Laser Scanner (Hitachi Genetic Systems; Alameda, CA) and software (ReadImage version 1.5, Analysis v8.0). Target RNA was computed as described by Vanden Heuvel (1998).

Statistical analysis

The data (estimated copies of RNA) were transformed logarithmically to bring data to normality. The main effects and possible interactions of treatments in laboratory induction experiments were analyzed with a 2-way ANOVA. For samples collected from streams, student *t*-tests were utilized for detecting differences between streams. All analyses were carried out using Statistical Analyses System (SAS Institute; Cary, NC).

RESULTS

Internal standard and standard curves

A PCR reaction using internal standard primers WML53 and WML54 amplified a fragment from human genomic DNA approximately 360bp long. Transcription of this PCR product yielded the rcRNA molecule of approximately 360bp. Reverse transcription of this rcRNA molecule and subsequent PCR amplification using primers WML51 and WML52 resulted in a cDNA of approximately 320bp. The observations of these IS products are in accordance with expected results.

Standard curves for all tissues and treatment groups were estimated and showed a correlation coefficient (r) of 0.85 or higher. The standard curve used for this study obtained from liver tissue is shown in Figure 6. The point at which log [mRNA/IS] = 0 tells the amount of internal standard to spike into the RT reactions. It was determined that a range of internal standard concentrations ($1x10^7$-$1x10^9$ molecules per reaction) could be used for subsequent analysis. After these initial range-finding experiments, final computation of P450 RNA in all tissues was estimated through the use of one representative standard curve (Figure 6).

Induction of CYP1A in brain, gill, liver, and kidney in hatchery-raised salmon

Mean levels of CYP1A mRNA are reported for all treatment groups in Table 5. Representative gel pictures of these results are given in Figures 7 and 8. In a controlled laboratory setting, CYP1A mRNA was affected by treatment with BNF in all four tissues (gill, liver, kidney, and brain; ANOVA, $p < 0.05$). Gill tissue demonstrated the highest overall base level of P450 expression at 2.56×10^9 molecules per 100ng total RNA. The

lowest base level, 6.52 x 10^5 molecules per 100ng total RNA of P450 expression, was seen in brain tissue. Kidney tissue showed the greatest induction potential from base levels with a mean induction of ~4 orders of magnitude. The lowest mean induction for the tissues studied was in gill at approximately 2 orders of magnitude. In all cases, base levels of CYP1A mRNA were lower in salmon maintained at 17°C than salmon maintained at 11°C (ANOVA $p < 0.05$). Overall, ANOVA indicated that no interactions existed between BNF treatment and temperature ($p > 0.05$).

In cases with highly variable results, total RNA samples were analyzed with actin amplification to make sure initial RNA concentrations were accurately quantified and diluted. A representative gel picture for actin visualization is given in Figure 9. These results showed that initial total RNA dilutions were accurate.

CYP1A expression in wild salmon

The quantitative PCR analysis showed that Miller's River salmon CYP1A levels were on average approximately 100 times greater in both gill and liver tissue than salmon sampled from Fourmile Brook. Representative gel pictures of these results are given in Figure 10 and a summary of these results are given in Table 6.

DISCUSSION

I have demonstrated that CYP1A is highly inducible in Atlantic salmon gill, liver, kidney, and brain tissues. Each tissue showed at least 2 orders of magnitude induction over control levels. Interestingly, gill tissue samples had the highest base levels of P450 expression. Generally, it has been reported that liver tissue demonstrates the highest levels of CYP1A (Goksoyr and Husoy 1998). It is likely that high concentrations of CYP1A do exist in the gill lamellae for the following reasons. The gill constitutes less than 1% of the body weight of a fish but more than 90% of the surface area. It is a primary route of exposure for water borne contaminants, and an important secondary route for ingested compounds since it directly receives all of the blood flow from the heart. In addition, it was discovered that an induction limit exists across all tissues. All inductive responses seen in the laboratory study crested at approximately $1x10^{11}$ molecules per 100ng total RNA 1 order of magnitude except for brain tissue. This result could possibly be due to the presence of AUUUA sequences, which is believed to be involved with degradation of mRNA molecules, in the 3' untranslated region of CYP1A (in Chapter 1, this thesis). Even in kidney tissue, which showed the second lowest (brain tissue had the lowest) base levels of CYP1A expression, induced samples had a mean mRNA level of ~ $1x10^{11}$ molecules per 100ng total RNA. Therefore, I conclude that kidney tissue has the highest induction potential of all tissues. Brain mRNA levels appeared to have a delayed response to BNF induction, perhaps simply due to the fact that liver, kidney, and gill CYP1A metabolized the majority of the injected sample before it reached the brain. Brain CYP1A levels also could have been less

pronounced due to a blood-brain-barrier although this possibility has not been investigated.

I also discovered that there was an overall mean decrease in CYP1A expression in all tissues during acclimation at higher temperatures (ANOVA $p < 0.05$). Previous studies on P450 levels have also found significant effects of temperature (Grosvik et al. 1997; Andersson and Forlin 1992). There are several possibilities that may explain this finding. The first is the solubility of BNF increases with increasing temperatures thus making it easier physiologically for salmon to rid the body of the contaminant. However, this seems unlikely since we are only dealing with an increase in temperature of 6°C. Another possibility is increased temperatures suppress the Ah-receptor complex which mediates production of CYP1A mRNA transcripts (Goksoyr and Husoy 1998). Thirdly, there may be a decreased activation of heat shock protein complexes that are involved with stimulation of the Ah-receptor complex (Goksoyr and Husoy 1998). This last possibility could also be related to suppression of the Ah-receptor complex.

I developed a standard curve-based quantitative PCR method to assess expression levels of CYP1A in Atlantic salmon gill, kidney, liver, and brain tissue. This method was utilized in both a controlled lab induction study as well as on samples from natural streams. I believe developing quantitative PCR assays such as this will be useful in the following years in monitoring the current status of Atlantic salmon populations, helping to discover cause and effect relationships for the reasons behind Atlantic salmon decline, and providing solutions to establish sound management plans for restoration of wild salmon.

Recently, a quantitative PCR study was carried out using standard curves with the Antarctic fish *Trematomus bernacchi* and the effects environmental pollution has on P450 levels in this species (Miller et al. 1999). The current study and the Miller paper contradict traditional quantitative PCR experiments where single samples are quantified using a dilution series of internal standard for each sample (Vanden Heuvel et al. 1993). Using standard curves in quantitative PCR studies allow for more samples to be analyzed in a shorter amount of time while still producing results that correspond to results seen in traditional quantitative PCR experiments (Tsai and Wiltbank 1996). In fact, the time between sampling to production of results can be reduced to just three days. Overall, the quantitative PCR experiment becomes more economical without reducing accuracy and sensitivity. Finally, quantitative PCR is both quantitative and qualitative in nature, a contradiction to traditional RNA-based biomarker analysis where qualitative data is the result. This characteristic can enhance understanding of biomarker responses seen in Atlantic salmon and other species.

Finally, further research is needed in studying the overall effects PCB's and similar compounds are having on the health of Atlantic salmon. I have demonstrated that it is possible to assess CYP1A levels in wild Atlantic salmon using quantitative PCR. Using this method, it would be possible to quantify CYP1A levels in Atlantic salmon through gill biopsy sampling, a definite advantage from the viewpoint of restoration and conservation. This type of sampling could be performed on either adult or juvenile fish. However, knowing how CYP1A is altered in response to PCB's and other compounds is not enough. There is a need to acquire better physiological knowledge of how increased levels of CYP1A correlate with levels of sex steroids or hormones, activity of Na^+,K^+-

ATPase, or other metabolic processes. In addition, further research is needed to establish a distribution of PCB related contamination in New England rivers and streams. Using quantitative PCR as a tool, it would be possible to quantify the CYP1A levels of different Atlantic salmon runs across the east coast, thus making it easier to focus research projects and restoration efforts. Moreover, the possibility exists to adapt quantitative PCR to study other gene expression in fish, such as olfactory receptor expression during different life stages. Some pesticides, namely carbofuran at a concentration of 1-6ppb, can abolish olfactory sensitivity in fish (Waring and Moore 1997). Olfactory function is essential for juvenile imprinting and the ability for adult salmon to find their natal stream for spawning. Thus olfaction is integral for survival of this species. It is still unknown why Atlantic salmon stocks are decreasing across the Eastern portion of North America. Is it a case of endocrine disruption? What is causing increased precocity in salmon populations? Could this effect be due to abolishment of olfactory sensitivity during exposure to low levels of pesticides? I feel with the usefulness of a biomarker such as CYP1A expression as well as the analytical power of quantitative PCR these questions can be answered.

Table 1: Primers used in PCR and RACE amplifications of CYP1A cDNA.

Application	Nucleotide sequence	Primer Name
PCR	5'-CCT TGK KRA TGA ART AGC CMT TGA GG-3'	WML38
	5'-CCA ACR TMA TCT SWG GMA TGT GC-3'	WML39
RACE	5'-CTG TCT TGG GCC GTT GTG TAC CTT GTG-3'	WML51
	5'-TAT CCT TGA TCG TGC AGT GTG GGA TGG-3'	WML52
	5'-GCT CAG TTC CTG ATG CAG TCT TTC CTG-3'	WML55
	5'-CGG CTC ATT TGG CTC ATA ACG GAA GAT-3'	WML56

Table 2: Phylogenetic analysis of cytochrome P450 genes.

Gene accession numbers and references for the sequences used in this analysis are given in table 4. Multiple sequence alignment was carried out using the Clustal W algorithm (1515 nucleotides of the coding region). The phylogenetic tree and genetic distances were determined using the Neighbor-Joining Method. Cytochrome P450 nomenclature followed that of Morrison et al. 1998.

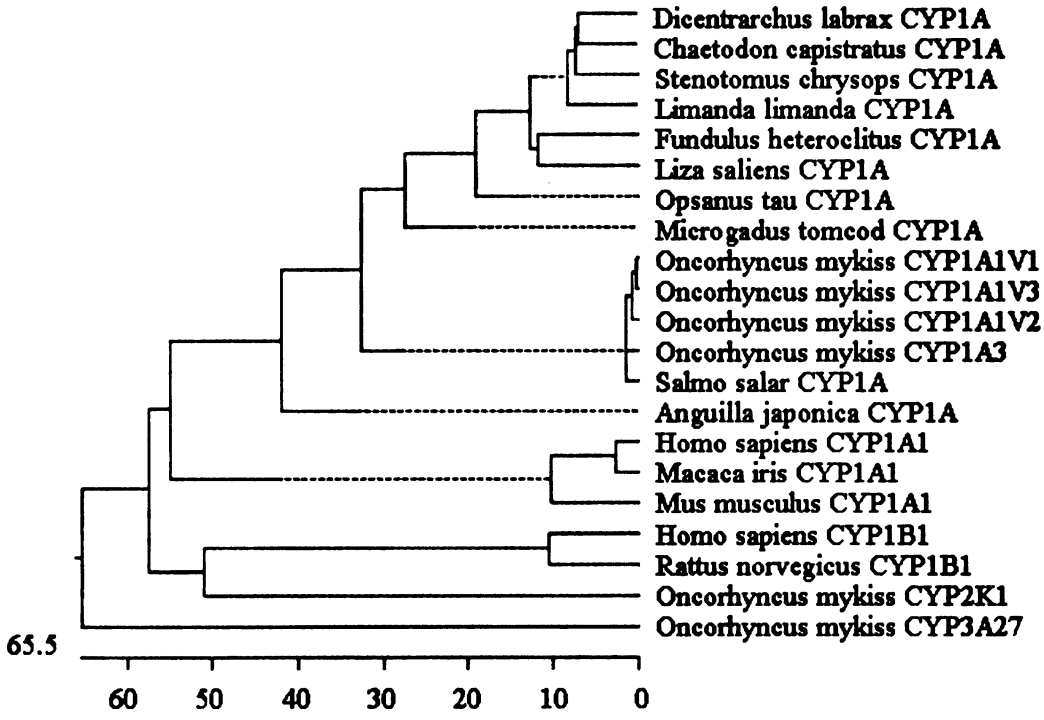


Table 3: Phylogenetic analysis of cytochrome P450 proteins.

Analysis was carried out as described in Table 2 utilizing 505 amino acid residues of each enzyme. Accession numbers for the genes encoding these enzymes are given in table 4.

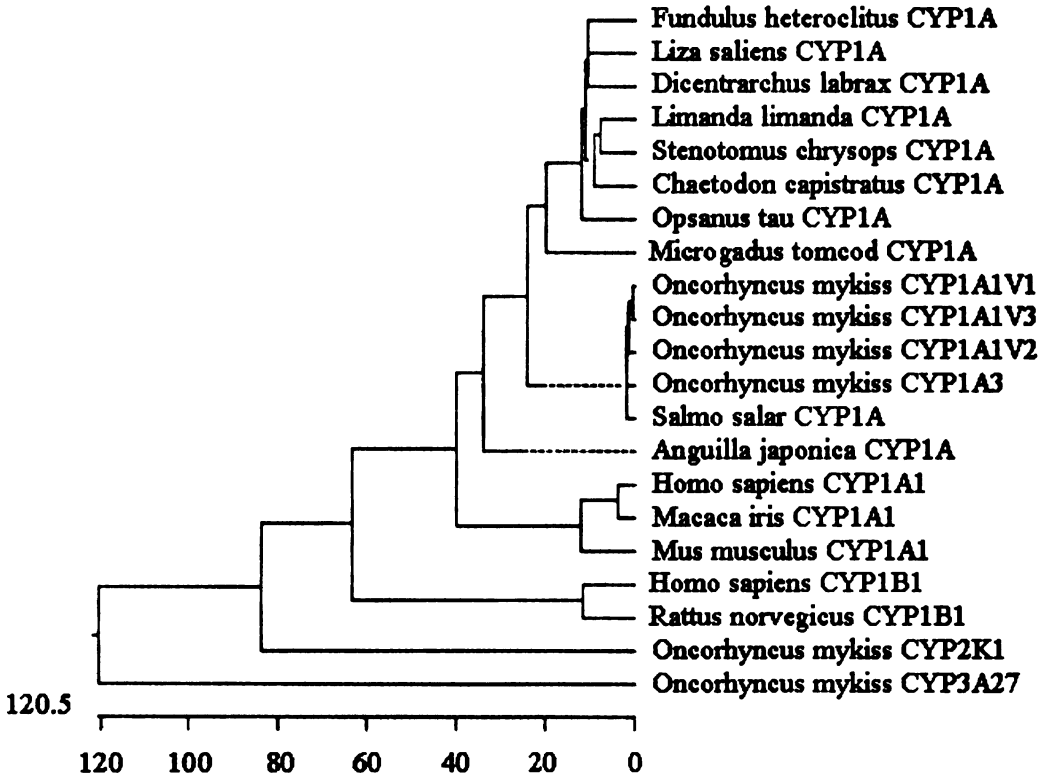


Table 4: CYP genes and accession numbers used in the phylogenetic analysis.

Name in analysis (General Name)	Reference	GenBank Accession Number
Fundulus heteroclitus CYP1A (Killifish)	Morrison et al., 1998	AF026800
Liza saliens CYP1A (Mullet)	Sen et al., 1999	AF072899
Dicentrarchus labrax CYP1A (Seabass)	Stien et al., 1998	U78316
Limanda limanda CYP1A (Sand dab)	Robertson, 1997	AJ001724
Stenotomus chrysops CYP1A (Scup)	Morrison et al., 1995	U14162
Chaetodon capistratus CYP1A (Butterflyfish)	Vrolijk et al., 1995	U19855
Opsanus tau CYP1A (Toadfish)	Morrison et al., 1995	U14161
Microgadus tomcod CYP1A (Tomcod)	Roy et al., 1995	L41886
Oncorhyncus mykiss CYP1A1V1 (Rainbow trout)	Berndtson and Chen, 1994	S69278
Oncorhyncus mykiss CYP1A1V3	Bailey et al., 1997	U62797
Oncorhyncus mykiss CYP1A1V2	Bailey et al., 1997	U62796
Oncorhyncus mykiss CYP1A3	Berndtson and Chen, 1994	S69277
Salmo salar CYP1A (Atlantic salmon)	Rees 2001 (this paper)	AF361643
Anguilla japonica CYP1A (Japanese eel)	Mitsuo et al., 1999	AB020414
Homo sapiens CYP1A1 (Human)	Jaiswal et al., 1985	K03191
Macaca iris CYP1A1 (Crab eating monkey)	Ohmachi et al., 1993	D17575
Mus musculus CYP1A1 (Mouse)	Kimura et al., 1984	Y00071
Homo sapiens CYP1B1	Sutter et al., 1994	U03688
Rattus norvegicus CYP1B1 (Rat)	Battacharyya et al., 1995	X83867
Oncorhyncus mykiss CYP2K1	Buhler et al., 1994	L11528
Oncorhyncus mykiss CYP3A27	Lee et al., 1998	U96077

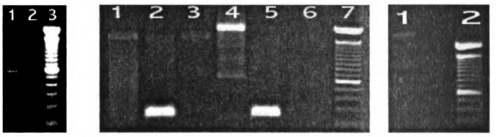
Table 5: Quantitative RT-PCR analysis of CYP1A levels in tissues of hatchery salmon. Each value represents the mean number of CYP1A mRNA transcripts per 100ng total RNA for each treatment group (n = 6-9). Induced animals were treated with 50mg kg^{-1} BNF (see Methods section). In all four tissues, the control P450 mRNA level was significantly lower than the induced P450 mRNA level ($p < 0.05$). In addition, for each tissue, there was a significant decrease in P450 mRNA during increased temperatures ($p < 0.05$). No interaction was seen between temperature and BNF treatment ($p > 0.05$).

Low Temp = 11°C; High Temp = 17°C

	Control		Induced	
Tissue	High Temp	Low Temp	High Temp	Low Temp
Gill	**2.56E+09**	**2.03E+11**	**3.94E+11**	**2.29E+12**
Liver	**1.25E+07**	**9.00E+08**	**1.07E+11**	**5.76E+11**
Kidney	**1.21E+06**	**2.79E+06**	**1.00E+10**	**2.49E+11**
Brain	**6.52E+05**	**2.86E+06**	**1.39E+09**	**3.17E+09**

Table 6: Quantitative RT-PCR analysis of CYP1A expression in tissues of wild salmon. Each value represents the mean number of CYP1A mRNA transcripts per 100ng total RNA for each tissue sampled (n = 7). Miller's River P450 mRNA levels were significantly higher than Fourmile Brook in both liver and gill tissue samples ($p < 0.05$). See Methods section for description of rivers.

	mRNA Levels	
Tissue	Fourmile Brook	Miller's River
Gill	**6.39E+07**	**1.43E+10**
Liver	**1.29E+09**	**1.97E+11**



A. B. C.

Figure 1: Electrophoretic analysis of amplified CYP1A cDNA's. All of these 1% agarose gels were stained with ethidium bromide (0.1μg/ml).

A) RT-PCR products using primers WML38 and WML39. Representative lanes are: (1) CYP1A PCR product using *Salmo salar* liver cDNA as the template; (2) negative control with no template loaded; (3) 100bp DNA ladder (Gibco BRL).

B) 5' and 3' RACE-PCR products.

Gene specific primers (GSP) were WML51 for 3' RACE and WML52 for 5' RACE. Representative lanes are: (1) 5' RACE; (2) GSP1 + GSP2 positive control; (3) GSP1 negative control; (4) 3' RACE; (5) GSP1 + GSP2 positive control; (6) GSP2 negative control (7) 100bp DNA ladder.

C) 3' long distance RACE product using GSP's WML52 and WML56. Representative lanes are: (1) 3' RACE showing 2.7kb RACE product and (2) 100bp DNA ladder.

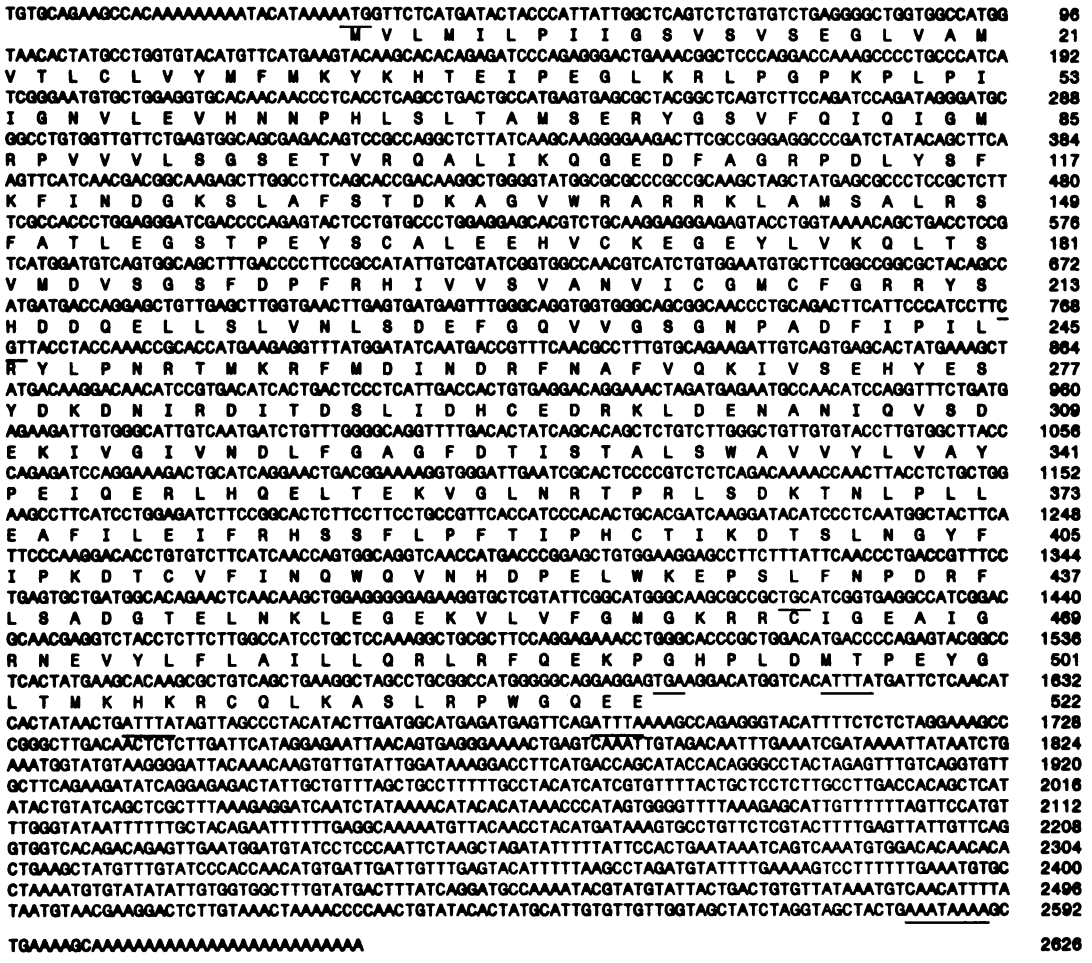


Figure 2: cDNA and deduced amino acid residue sequence of Atlantic salmon CYP1A. The start codon, arginine residue critical to enzymatic function (position 246), heme-binding cysteine codon (position 463), stop codon (positon 523), ATTTA (AUUUA) sequences, and putative poly-adenylation signal are all underlined.

F G M G K R R C I G	Butterflyfish 1A	Vrolijk et al. 1995
F G L G R R R C I G	Killifish 1A	Morrison et al. 1998
F G M G K R R C I G	Mullet 1A	Sen et al. 1999
F G M G K R R C I G	Salmon 1A	Rees (this study)
F G M G K R R C I G	Sand dab 1A	Robertson 1997
F G M G K R R C I G	Scup 1A	Morrison et al. 1995
F G L G K R R C I G	Seabass 1A	Stien et al. 1998
F G L G K R R C I G	Toadfish 1A	Morrison et al. 1995
F G M G K R R C I G	Tomcod 1A	Roy et al. 1995
F G M D K R R C I G	Trout 1A1V1	Berndtson and Chen 1994
F G M G K R R C I G	Trout 1A1V2	Bailey et al. 1997
F G M G K R R C I G	Trout 1A1V3	Bailey et al. 1997
F G M G K R R C I G	Trout 1A3	Berndtson and Chen 1994

Figure 3: Alignment of heme-binding region (amino acid residues 456-465). Comparison of heme-binding regions from representative teleost P4501A enzymes. Boxed areas show amino acid residue differences from salmon P4501A.

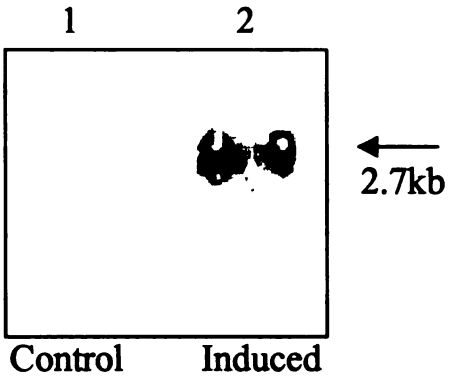


Figure 4: Northern blot analysis of CYP1A total RNA from Atlantic salmon liver. Lane 1: untreated salmon liver; Lane 2: salmon liver treated with β-Naphthoflavone. The 2.7kb CYP1A mRNA was hybridized with a digoxigenin-labeled CYP1A full-length cDNA probe.

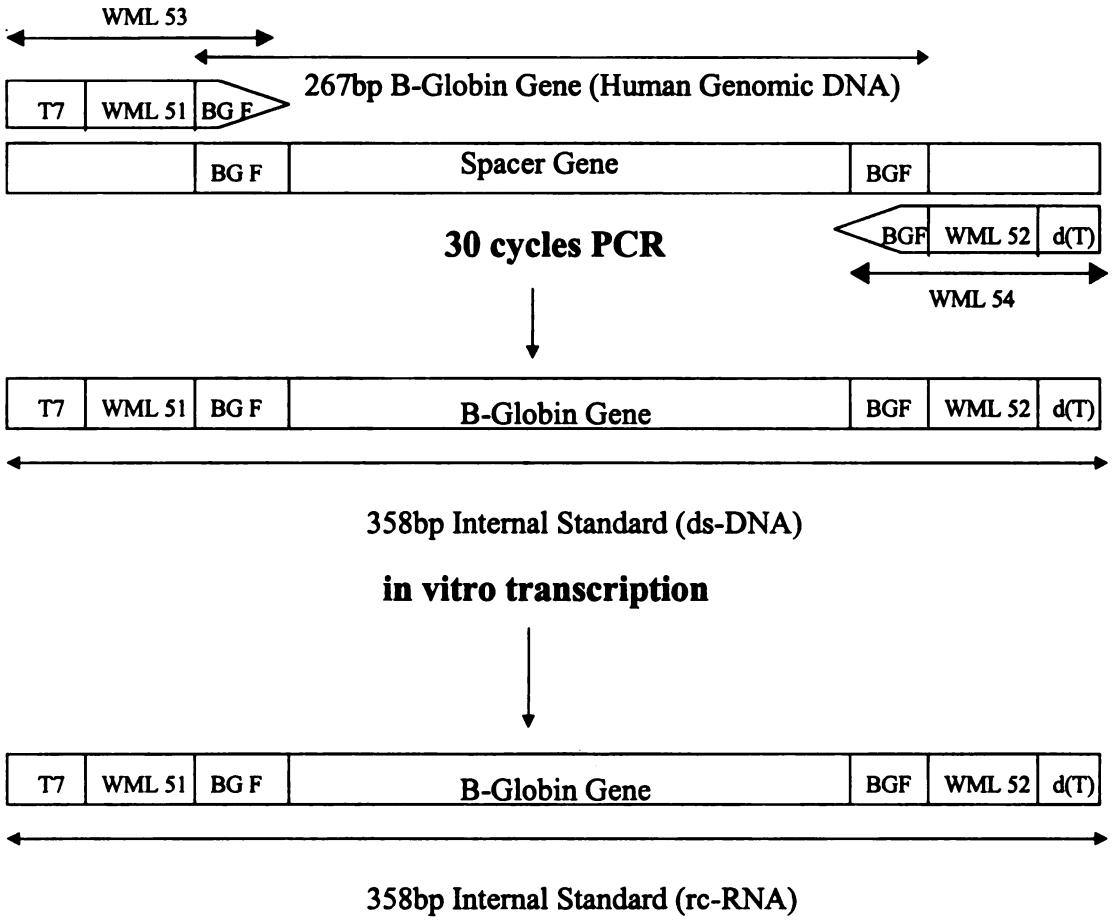


Figure 5: Construction of internal standard.

A schematic flow diagram showing the steps for synthesis of the rcRNA internal standard used in this quantitative PCR study (modified from Vanden Heuvel et al., 1993).

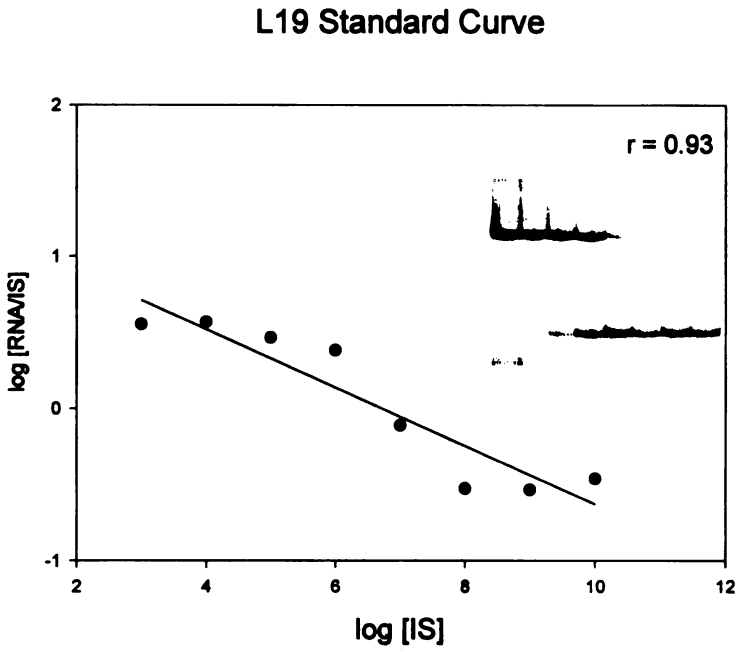


Figure 6: Generation of standard curve.

The standard curve used for quantifying the number of CYP1A transcripts in 100ng of total RNA. A constant amount of total RNA (100ng) was co-amplified against a dilution series of internal standard (10^{10}-10^{3} molecules IS). Data points used in the curve are generated by taking log absorbance [RNA/IS]/log absorbance [IS].

Abbreviations: IS, internal standard; CYP1A, cytochrome P4501A

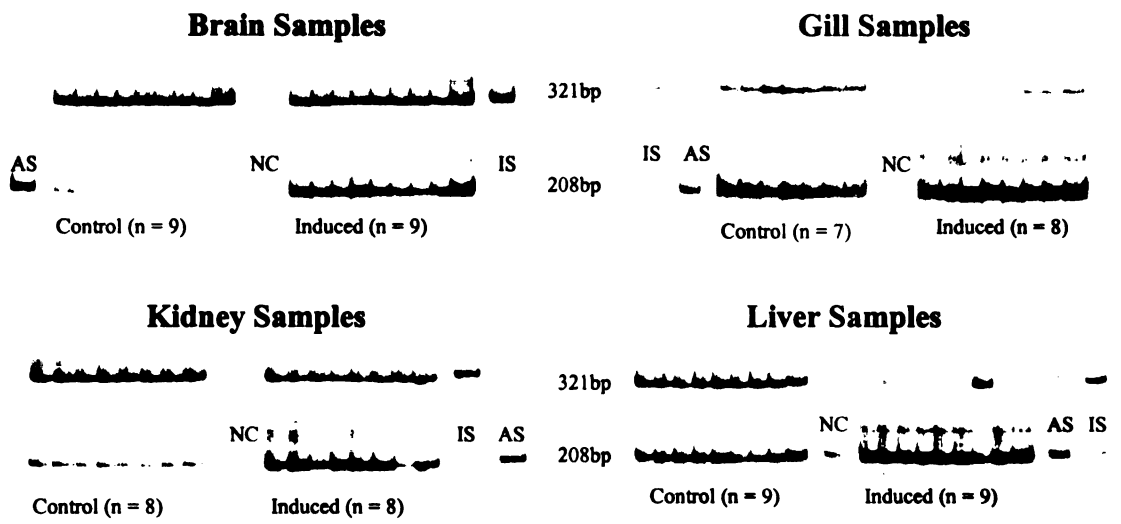


Figure 7: Representative gel pictures for gill, liver, kidney, and liver samples acclimated at 17°C. RNA (100ng) was co-amplified against a known concentration of internal standard (IS). Induced salmon received an intraperitoneal injection of β-Naphthoflavone (50mg kg^{-1} body weight) while control salmon received an injection of corn oil alone. Sample size is indicated in parentheses for each treatment group. The bands near the top of each gel are the 321bp IS. The bands at the bottom of each gel represent the 208bp CYP1A fragment. CYP1A mRNA levels are determined by taking the density ratio of IS/CYP1A.

Abbreviations: NC, negative control (water control); AS, absorbance standard; IS, internal standard positive control.

Samples Acclimated to 11 ° C

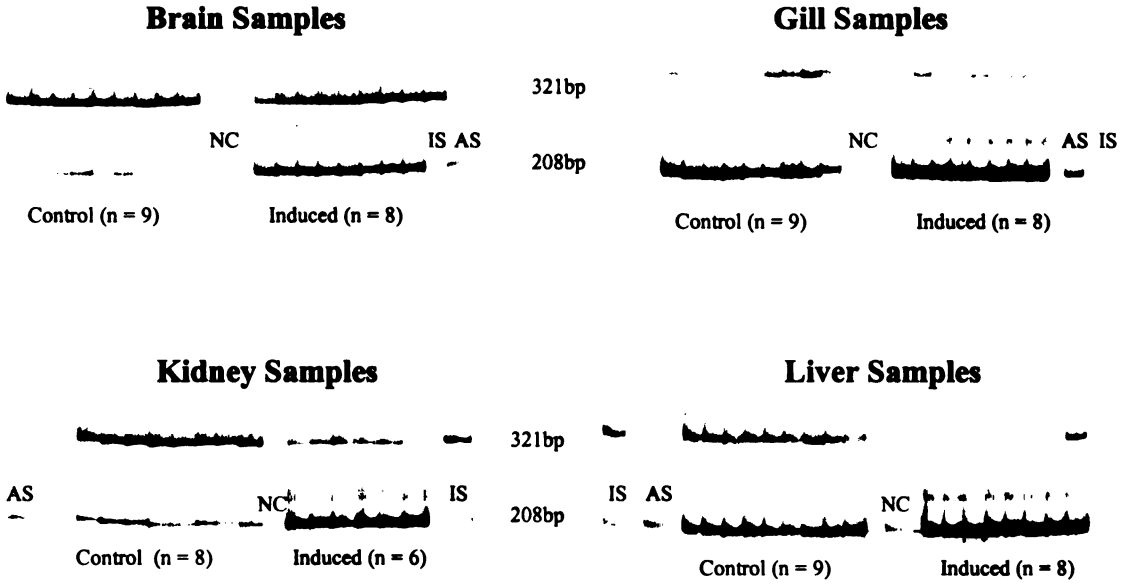


Figure 8: Representative gel pictures for gill, liver, kidney, and liver samples acclimated at 11°C. RNA (100ng) was co-amplified against a known concentration of internal standard (IS). Sample size is indicated in parentheses for each treatment group. Induced salmon received an intraperitoneal injection of β-Naphthoflavone (50mg kg^{-1} body weight) while control salmon received an injection of corn oil alone. The bands near the top of each gel are the 321bp IS. The bands at the bottom of each gel represent the 208bp CYP1A fragment. CYP1A mRNA levels are determined by taking the density ratio of IS/CYP1A.

Abbreviations: NC, negative control (water control); AS, absorbance standard; IS, internal standard positive control.

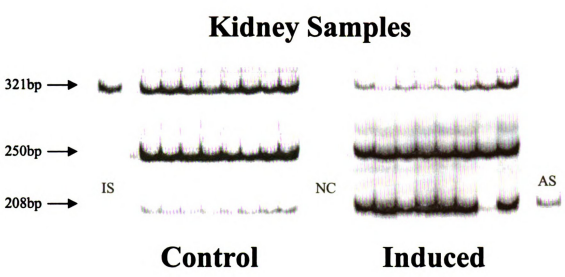


Figure 9: Representative gel picture of actin normalization. RNA (100ng) was co-amplified against a known concentration of internal standard (IS). Induced salmon received an intraperitoneal injection of β-Naphthoflavone (50mg kg^{-1} body weight) while control salmon received an injection of corn oil alone. The bands near the top of the gel are the 321bp IS. The bands at the bottom of the gel represent the 208bp CYP1A fragment. The bands in the middle are the 250bp actin fragments.

Abbreviations: NC, negative control (water control); AS, absorbance standard; IS, internal standard positive control.

n = 8 for both control and induced groups.

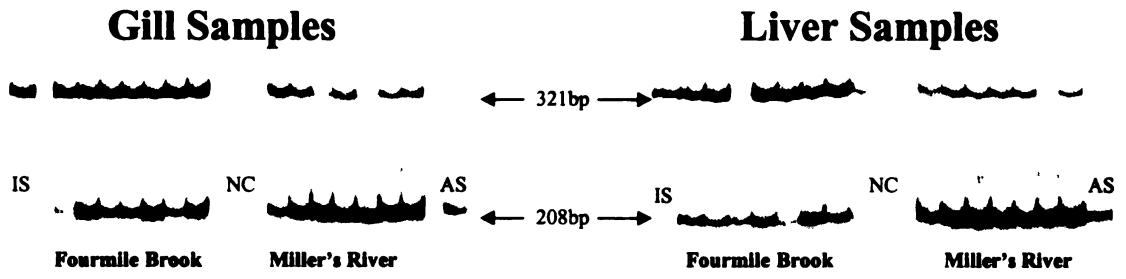


Figure 10: Quantitative PCR of gill and liver tissue CYP1A for samples collected from wild salmon from two Massachusetts rivers. RNA (100ng) was co-amplified against a known concentration of internal standard (IS). The bands near the top of each gel are the 321bp IS. The bands at the bottom of each gel represent the 208bp CYP1A fragment. mRNA quantities are determined by taking the density ratio of IS/CYP1A.

Abbreviations: NC, negative control (water control); AS, absorbance standard; IS, internal standard positive control.

n = 7 for all groups.

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