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

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Abstract

Environmental pollutants are hypothesized to be one of the causes of recent declines in wild populations of Atlantic salmon (*Salmo salar*) across Eastern Canada and the United States. Some of these pollutants, such as polychlorinated biphenyls and dioxins, are known to induce expression of the CYP1A subfamily of genes. We applied a highly sensitive technique, quantitative reverse transcription-polymerase chain reaction (RT-PCR), for measuring the levels of CYP1A induction in Atlantic salmon. This assay was used to detect patterns of CYP1A mRNA levels, a direct measure of CYP1A expression, in Atlantic salmon exposed to pollutants under both laboratory and field conditions. Two groups of salmon were acclimated to 11 and 17 °C, respectively. Each subject then received an intraperitoneal injection (50 mg kg^{-1}) of either β-naphthoflavone (BNF) in corn oil (10 mg BNF ml^{-1} corn oil) or corn oil alone. After 48 h, salmon gill, kidney, liver, and brain were collected for RNA isolation and analysis. All tissues showed induction of CYP1A by BNF. The highest base level of CYP1A expression (2.56×10^{10} molecules/μg RNA) was found in gill tissue. Kidney had the highest mean induction at five orders of magnitude while gill tissue showed the lowest mean induction at two orders of magnitude. The quantitative RT-PCR was also applied to salmon sampled from two streams in Massachusetts, USA. Salmon liver and gill tissue sampled from Millers River (South Royalston, Worcester County), known to contain polychlorinated biphenyls (PCBs), showed on average a two orders of magnitude induction over those collected from a stream with no known contamination (Fourmile Brook, Northfield, Franklin County). Overall, the data show CYP1A exists and is inducible in Atlantic salmon gill, brain, kidney, and liver tissue. In addition, the results obtained demonstrate that quantitative PCR analysis of CYP1A expression is useful in studying ecotoxicity in populations of Atlantic salmon in the wild.

Keywords: Atlantic salmon; P450; CYP1A; Induction; Quantitative PCR

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1. Introduction

Atlantic salmon populations across Eastern Canada and the United States have suffered 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 (US Department of Interior, 2000). In some cases, this decline has been attributed to the sublethal effects of pesticides. Fairchild et al. (1999) suggested that endocrine disrupting chemicals have caused declines in salmon populations by altering or inhibiting the parr-smolt transformation. Other compounds such as polyaromatic hydrocarbons, polychlorinated biphenyls, dioxins, and furans can also have physiological and pathological effects on fish populations at sublethal concentrations (Goksøyr and Husøy, 1998). These compounds are known to stimulate expression of various members of the cytochrome P450 family of genes, particularly those of the CYP1 family (Hahn and Stegeman, 1994; Hahn et al., 1998; Stegeman et al., 2001).

The cytochrome P450 detoxification system is an extensively studied enzyme system and has been found in bacteria, plants, and animals. It is involved in the metabolism of compounds such as steroids, prostaglandins, eicosanoids, drugs, and xenobiotics (Larsen et al., 1992; Nelson et al., 1996). Cytochrome P450 genes are highly diverse, 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 (Mansuy, 1998).

The most intensively studied cytochrome P450 protein is arguably cytochrome P4501A (CYP1A). 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 (Porter and Coon, 1991; Buhler and Wang-Buhler, 1998; Cao et al., 2000) and many other teleosts. As this gene is inducible by a wide variety of compounds, its induction has been used as a biomarker for detecting environmental contamination in fish populations (Bucheli and Font, 1995).

Previous studies of CYP1A inducibility and expression in fish have largely relied on techniques such as Northern blotting, Western blotting, ELISA (enzyme-linked immunosorbent assay), or 7-ethoxyresorufin O-deethlyase (EROD) enzyme kinetics (Andersson and Goksøyr, 1994; Croce et al., 1995; Grosvik et al., 1997; Sarasquete and Segner, 2000; Schlezinger and Stegeman, 2001). Although these methods are informative, they require substantial fish tissue, time, and some are qualitative rather than quantitative. 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 (Campbell and Devlin, 1996; Miller et al., 1999; Cousinou et al., 2000). As reported by Vanden Heuvel et al., 1994), 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. As PCR is an amplification process, only a very small amount of tissue is required for analysis. In order to design highly specific primers for development of a quantitative PCR assay, one must know the nucleotide sequence of the target gene. We have determined the sequence of CYP1A in Atlantic salmon through RT-PCR and RACE techniques (Rees et al., GenBank accession number AF361643).

The goal of this study was to develop a sensitive and time efficient assay to study toxicity responses of Atlantic salmon to persistent organic contaminants known to induce the CYP1A subfamily of genes. Our first objective was to develop a quantitative PCR method to estimate CYP1A mRNA levels. The second objective was to apply the PCR method to assess toxic responses of laboratory and feral Atlantic salmon. Our results clearly demonstrate that quantitative PCR is a sensitive technique in measuring P450 expression of Atlantic salmon in both lab-based induction experiments and responses to contaminant exposure in the wild.

2. Materials and methods

2.1. Fish handling and sampling

For the laboratory induction study, 200 juvenile Atlantic salmon with an average body mass of 35 g and total length of 15 cm 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 in an 800 l flow-through tank (600 l h^{-1}). The fish were then divided into two equal groups for another 2-week acclimation period, one group at 11 °C, the other at 17 °C (flow-rate and size of tank remained the same). A 12 h light-dark cycle was maintained during the acclimation period. Salmon were fed Purina AquaMax© Grower 400 (lot A-5D04; Purina Mills, Inc.; St. Louis, MO) daily at a level of 3.0% body weight. Two days prior to injection, salmon were taken off of feed. Individuals were randomly sampled and given an intraperitoneal injection of either β-naphthoflavone (BNF, Sigma Chemical Corp.; St. Louis, MO; 50 mg kg^{-1} body weight) dissolved in corn oil (10 mg 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 using 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, ten juvenile Atlantic salmon were collected by electro-shocking from two Massachusetts streams located 25.8 km apart from one another, Millers River (South Royalston, Worcester County) and Fourmile Brook (Northfield, Franklin County). Fourmile Brook was sampled on October 17th, 2000 (10.4 °C) while Millers River was sampled on November 8th, 2000 (6.2 °C). Millers River, known to contain fish with tissue concentrations of PCB's between 0.8 and 5.5 μg g^{-1} (Colman, 2001), 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.

2.2. Total RNA isolation and storage

Tissue samples that had been stored in RNALater© (one 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). For long-term storage, RNA samples were supplemented with 3 volumes of 95% ethanol, 1/10 volume of 3 M sodium acetate, and stored at −80 °C (Sambrook et al., 1989).

2.3. Internal standard synthesis

The cDNA template for an internal standard (IS) 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 Fig. 1. 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 TAT CCT TGA TCG TGC AGT GTG GGA TGG GAA GAG CCA AGG ACA GGT AC-3′ IS primers (Macromolecular Structure, Sequencing, and Synthesis Facility, Michigan State University) amplified a β-globin product of approximately 360 bp under the following conditions: 3 mM $MgCl_2$, 0.4 mM dNTP's, 0.6 μM forward IS primer, 0.6 μM reverse IS 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 s, 59 °C for 30 s, and 72 °C for 30 s, and 1 cycle at 72 °C for 5 min. The size of the product was verified on a 1% TAE agarose gel with a 100 bp molecular size standard (Life

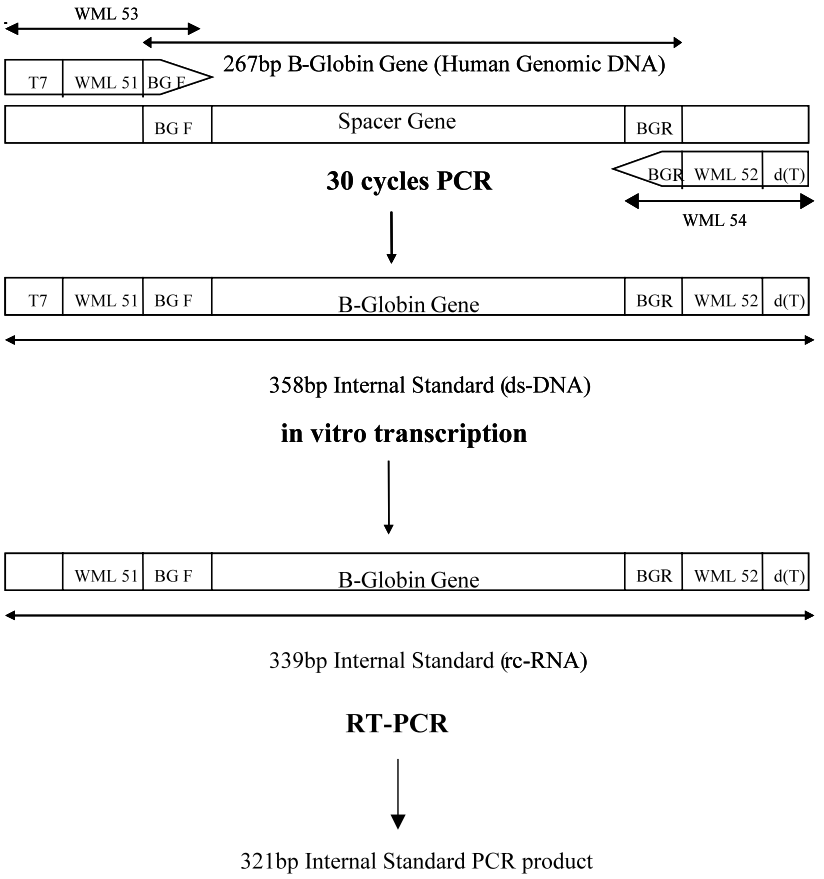

Fig. 1. Construction of IS. A schematic flow diagram showing the steps for synthesis of the rcRNA IS used in this quantitative PCR study (modified from Vanden Heuvel et al., 1993).

Technologies). This product was then diluted 1/100 with deionized water and amplified with the same reaction conditions. The concentrated PCR product was cleaned using the Wizard DNA Clean-Up System (Promega Corp.; Madison, WI) and transcribed 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 10 min at 12 000 g, resuspended in 20 μl DEPC-H_2O, and filtered through a G-50 Sephadex column (Amersham Pharmacia Biotech; Piscataway, NJ) pre-equilibrated in 10 mM Tris-HCl (pH 7.5) and 0.1% SDS (sodium dodecyl sulfate). The filtered sample was precipitated overnight. After spinning the sample for 10 min at 12 000 g, the rcRNA pellet was washed with 70% ethanol, resuspended in DEPC-H_2O, quantified with a UV spectrophotometer, and used as the IS.

2.4. Competitive RT-PCR

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 (100 ng) against a dilution series of the IS (10^9–10^4

molecules). These 'range finding' experiments allow for relative determination of the levels of a particular gene of interest between several tissues and treatments (Vanden Heuvel, 1998), and thus the exact amount of IS to spike into each sample. Our initial range-finding experiments showed 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 1 × concentration of First Strand Buffer, 0.01 M dithiothreitol, 1 mM of each deoxynucleotide triphosphate, 2.5 μM oligo$(dT)_{18}$, five units of MMLV reverse transcriptase, 1 unit rRNasin (Promega Corp.), 100 ng of total RNA, and varying amounts of IS predetermined from initial range-finding experiments. The reaction mixture 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 used where each reaction was heated to 94 °C for 2 min after which Taq was added. Then, the reaction mix was heated to 94 °C for 4 min, followed by 30 cycles of a 94 °C denaturation for 20 s, a 70 °C annealing step for 30 s, and a 72 °C extension step for 30 s. An additional 5 min extension step was included at the conclusion of the 30-cycle main reaction. Preliminary experimentation demonstrated that 30 cycles of PCR would keep CYP1A product formation in log-phase rather than plateau.

As the efficiency of reverse transcription and PCR varies from tube to tube (Vanden Heuvel et al., 1994), four controls were used in each of the reactions. First, the IS 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 approximately the same efficiency as the target gene. Secondly, a blank IS RT-PCR reaction was also included to act as an additional size marker and to verify that the IS was amplified as a single product. Third, the laser scanner used to visualize PCR products was calibrated across all gels by loading an absorbance standard (AS), which was simply a 1/10th dilution of a single CYP1A PCR product. Finally, to assure the total RNA was loaded for each RT-PCR reaction at expected levels, we adapted the standard procedure used in many quantitative PCR studies (Vanden Heuvel et al., 1994; Loitsch et al., 1999). We sampled several cDNA samples that corresponded to lower or higher levels of CYP1A compared with other samples in a group of Atlantic salmon. Using hex-labeled primers ACTIN1 (5′ GAG CGT AAC CCT CGT AGA TGG GTA CTG TGT 3′) and ACTIN2 (5′ ATC ACA CCT TCT ACA ACG AGC TGA GAG TGG 3′) designed from an Atlantic salmon β-actin cDNA (Rogers et al., 1999, unpublished data, GenBank Accession AF012125) fragment confirmed by sequencing, we amplified a fragment of the actin gene using 25 cycles of the same conditions as were used for CYP1A. In this case, due to the higher copy number of actin mRNA, only 25 cycles of PCR were required to produce log-phase amplification. If actin fragment amplification was equal across all samples we 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.

2.5. 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 MAP-MARKER^{TM} 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).

2.6. Statistical analysis

The data (estimated copies of RNA) were transformed logarithmically to increase the homogeneity of variance. The main effects and possible interactions of treatments in laboratory induction experiments were analyzed using 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).

3. Results

3.1. Internal standard and standard curves

A PCR reaction using IS primers WML53 and WML54 amplified a fragment from human genomic DNA approximately 360 bp long. Transcription of this PCR product yielded a single rcRNA molecule of approximately 340 bp. Reverse transcription of this rcRNA molecule and subsequent PCR amplification using primers WML51 and WML52 resulted in a cDNA of approximately 320 bp. These observed IS products were in accordance with expected sizes.

Standard curves for all tissues and treatment groups were estimated and showed a correlation coefficient (r^2) of 0.85 or higher. The standard curve used for this study was obtained from liver tissue and is shown in Fig. 2. The point at which log [mRNA/IS] = 0 tells the amount of IS to spike into the RT reactions. It was determined that a range of IS concentrations (1×10^7–1×10^9 molecules per reaction) could be used for subsequent analysis. After these initial range-finding experiments, final computation of CYP1A RNA in all tissues was estimated through the use of one standard curve (Fig. 2).

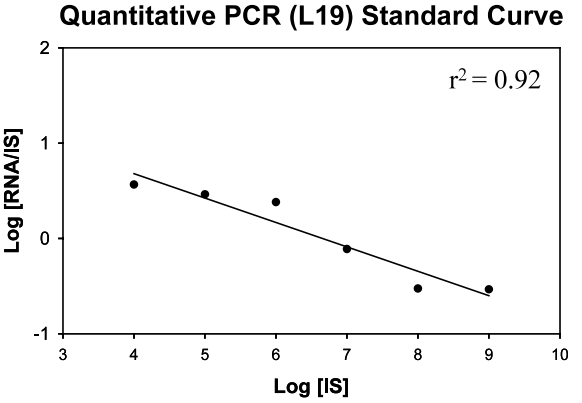

Fig. 2. Representative standard curve. The standard curve used for estimating the number of CYP1A transcripts in 100 ng of total RNA. A constant amount of total RNA (100 ng) was co-amplified against a dilution series of IS (10^{10}–10^3 molecules IS). Data points used in the curve were generated by taking log absorbance [RNA/IS]/log absorbance [IS].

3.2. Induction of CYP1A in brain, gill, liver, and kidney

In a controlled laboratory setting, CYP1A mRNA was affected by treatment with BNF in all four tissues sampled: gill, liver, kidney, and brain (ANOVA, $P < 0.05$). Gill tissue demonstrated the highest overall base level of P450 expression at 2.56×10^{10} molecules/μg total RNA. The lowest base level, 6.52×10^6 molecules/μg 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 about five orders of magnitude. The lowest mean induction for the tissues studied was in gill at approximately two 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, $P < 0.05$). Specifically, salmon maintained at 11 °C demonstrated anywhere from 2 to 80 times more CYP1A mRNA than salmon acclimated to 17 °C in each respective tissue. Overall, the ANOVA indicated that no interactions existed between BNF treatment and temperature ($P > 0.05$). Mean levels of CYP1A mRNA are reported for all treatment groups in Table 1.

Table 1
Quantitative RT-PCR analysis of CYP1A levels in tissues of Atlantic salmon

	Control		BNF Induced	
Tissue	17 °C	11 °C	17 °C	11 °C
Gill	10.20±0.19	11.31±0.39	12.23±0.26	12.55±0.31
Liver	7.84±0.20	9.09±0.25	11.47±0.42	12.38±0.34
Kidney	6.86±0.17	7.25±0.16	10.38±0.50	11.96±0.26
Brain	6.65±0.12	7.09±0.20	9.84±0.20	10.30±0.15

Each number represents the logarithmic mean value of CYP1A mRNA transcripts/μg total RNA±standard error of the mean (S.E.M.) for each treatment group ($n = 6–9$). Induced animals were treated with 50 mg kg^{-1} BNF. In all four tissues, the control P450 mRNA level was significantly lower than the induced CYP1A mRNA level ($P < 0.05$). In addition, for each tissue, there was a significant decrease in CYP1A mRNA during increased temperatures ($P < 0.05$). No interaction was seen between temperature and BNF treatment ($P > 0.05$).

In cases where outliers were apparent, 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 Fig. 3. These results showed that initial total RNA dilutions and loading were accurate. Thus all the samples estimated for CYP1A were included for the ANOVA and subsequent analyses.

3.3. CYP1A expression of salmon in the wild

The quantitative PCR analysis showed CYP1A levels were greater in both gill and liver tissue in Millers River salmon than salmon sampled from Fourmile Brook. In particular, Millers River salmon had approximately 220 times more CYP1A mRNA in their liver tissue than salmon from Fourmile Brook ($n = 7$, $P < 0.05$, student t-test). Likewise, individuals sampled from Millers River had approximately 150 times more CYP1A mRNA in their gill tissue than salmon from Fourmile Brook ($n = 7$, $P < 0.05$, student t-test). Representative gel pictures of these results are given in Fig. 4 and a summary of these results is given in Table 2.

4. Discussion

We have demonstrated that CYP1A is highly inducible by BNF in Atlantic salmon gill, liver, kidney, and brain tissues. Each tissue showed at least two orders of magnitude induction over

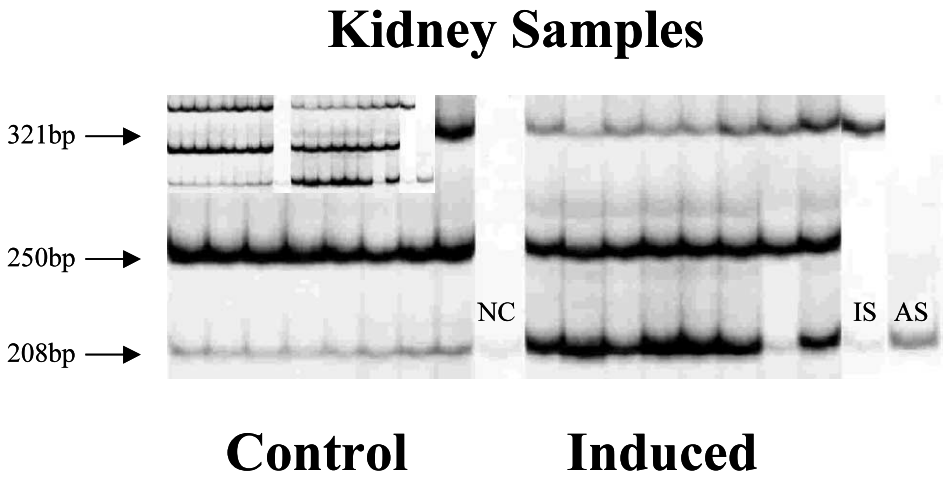

Fig. 3. Representative gel picture of actin normalization. RNA (100 ng) was co-amplified against a known concentration of IS. Induced salmon received an intraperitoneal injection of β-Naphthoflavone (50 mg kg^{-1} body weight) while control salmon received an injection of corn oil alone. The bands near the top of the gel are the 321 bp IS. The bands at the bottom of the gel represent the 208 bp CYP1A fragment. The bands in the middle are the 250 bp actin fragments.Abbreviation: NC, negative control (water control). $n = 8$ for both control and induced groups.

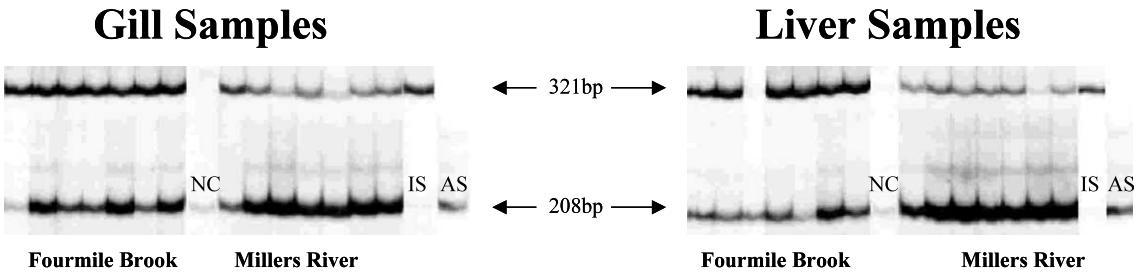

Fig. 4. Quantitative PCR of gill and liver tissue CYP1A for samples collected from salmon in the wild in two Massachusetts rivers. RNA (100 ng) was co-amplified against a known concentration of IS. The bands near the top of each gel are the 321 bp IS. The bands at the bottom of each gel represent the 208 bp CYP1A fragment. mRNA quantities are determined by taking the density ratio of IS/CYP1A. $n = 7$ for all groups.

Table 2
Quantitative RT-PCR analysis of CYP1A expression in tissues of salmon from the wild

mRNA Levels		
Tissue	Fourmile brook	Millers river
Gill	8.81 ± 0.76	11.15 ± 0.62
Liver	10.11 ± 1.03	12.29 ± 0.84

Each number represents the logarithmic mean value of CYP1A mRNA transcripts/μg total RNA ± S.E.M. for each tissue sampled ($n = 7$). Millers river (south Royalston, Worcester county, Massachusetts, USA) CYP1A mRNA levels were significantly higher than Fourmile Brook (Northfield, Franklin county, Massachusetts, USA) in both liver and gill tissue samples ($P < 0.05$). See Section 2 for description of rivers.

control levels. This level of induction is similar to that reported in Chinook salmon (*Oncorhynchus tshawytscha*) which showed a 160-fold induction of CYP1A in liver during a period of 4 weeks (Campbell and Devlin, 1996). Our results also indicate that gill tissue on average had the highest base levels of CYP1A mRNA, yet, it has been reported previously that liver tissue demonstrates the highest levels of CYP1A mRNA in Atlantic salmon (Goksøyr and Husøy, 1998). Other studies have also demonstrated the expression of CYP1A in gill tissues of fishes (Miller et al., 1989; Smolowitz et al., 1991, 1992). The higher basal levels of steady state CYP1A mRNA found in gill tissue may reflect the function of the gills as a primary route of exposure for water borne contaminants and as an important secondary route for ingested compounds. In fish, the gill constitutes less than 1% of the body weight but more than 90% of the surface area, and directly receives all of the blood flow from the heart. Thus, it is likely that gills do express very high levels of CYP1A during times of acute exposure to organic contaminants, perhaps even more than the liver (Van Veld et al., 1997; Levine and Oris, 1999). In addition, previous comparative time-course experiments with waterborne benzo(a)pyrene CYP1A induction in rainbow trout (*Oncorhynchus mykiss*) have shown that a rapid rise in CYP1A mRNA occurs during the first 24 h of exposure in both liver and gill tissue (Levine and Oris, 1999). More importantly, gill tissue demonstrates a maximal induction after only 6 h of exposure, and this level of induction is sustained for up to 120 h. However, liver tissue CYP1A expression is maximally induced after 24 h, and returns back to basal levels after 72 h of exposure (Levine and Oris, 1999). Although the dynamics of induction demonstrated in various tissues of rainbow trout may not be the same in Atlantic salmon tissues, it is likely that similar differences in induction dynamics contributed to the differences in induction levels of various tissues found in this study. Regardless, it is likely that gill tissue plays an important role in both acute and prolonged biotransformation of xenobiotics.

Intensive investigations have been carried out in measuring the transcriptional (mRNA production) as well as translational (protein production) properties of CYP1A activity (Kloepper-Sams and Stegeman, 1992; Grosvik et al., 1997; Levine and Oris, 1999). In *Fundulus heteroclitus* liver, CYP1A

mRNA levels remain in an induced state for only 24 h after exposure (Kloepper-Sams and Stegeman, 1992), whereas CYP1A protein remains active (measuered by ethoxyresorufin-O-deethylase activity) for 4 days (Kloepper-Sams and Stegeman, 1994). We found that an expression limit existed across all tissues. All inductive responses seen in the laboratory seemed to reach a threshold after which no additional CYP1A mRNA could be transcribed. There are several possible explanations that may account for this observance. One possibility is this level represents the kinetic limit on the production of CYP1A mRNA transcripts. However, this crest could also be explained by the presence of three AUUUA sequences found in the 3′ untranslated region of CYP1A mRNA of Atlantic salmon (Rees et al., unpublished data). AUUUA sequences are believed to be involved with rapid degradation of mRNA molecules, (Shaw and Kamen, 1986; Binder et al., 1989; Fukuhara et al., 1989) and serve as a post-transcription mechanism for gene regulation. Based on these observances as well as earlier studies (Kloepper-Sams and Stegeman, 1992; Levine and Oris, 1999), it appears post-transcriptional factors play an important regulatory role in CYP1A expression. Brain mRNA levels appeared to have a lower response to BNF induction. In scup (*Stenotomus chrysops*), it has been shown that induction of CYP1A in brain occurs in the endothelium, or, the blood brain barrier (Smolowitz et al., 1991). Perhaps the measured low induction in brain is due to a dilution of endothelial CYP1A mRNA among total RNA extracted from all cell types of the brain. Our results have shown that there was an overall mean reduction of CYP1A expression in all tissues during acclimation to higher temperatures (ANOVA, $P < 0.05$). Previous studies on P450 levels have also found that temperature has compensatory effects on CYP- related activities (Stegeman, 1979; Andersson and Förlin, 1992; Kloepper-Sams and Stegeman, 1992; Grosvik et al., 1997). There are several possibilities that may explain the effect temperature has on CYP1A expression. Temperature compensation may be due to changes in membrane lipid composition, because membranes with more unsaturated fatty acids have greater fluidity than membranes with higher levels of saturated fatty acids (Grosvik et al., 1997). Fish acclimated to lower temperatures have increased levels of unsaturated fatty acids in the liver (Carpenter et al., 1995; Hazel, 1995; Grosvik et al., 1997), and probably in other tissues. The greater fluidity of membranes may allow for increased CYP1A-associated metabolic activity due to an overall increase in enzymatic activity. Alternatively, the temperature compensation can be explained by the Q10 effect. That is, a lower level of enzyme is needed to metabolize the same level of toxicants at a higher temperature. Another possibility is increased temperatures suppress the Ah-receptor complex (Hahn, 1998; Hahn et al., 1998) that mediates production of CYP1A mRNA transcripts. These results as well as findings from prior investigations demonstrate that temperature can have a significant effect on the transcriptional-control mechanisms of CYP1A mRNA production. Temperature adaptation of CYP1A transcription as well as translation requires further experimentation.

Quantitative PCR analysis of tissues from Atlantic salmon in Fourmile Brook and Millers River showed that animals from Millers River demonstrated 150 times more CYP1A mRNA in gill tissue and 220 times more CYP1A mRNA in liver tissue than salmon from Fourmile Brook. This result was expected due to the higher level of PCB's in Millers River (Colman, 2001). Another contributing factor to the higher levels of observed CYP1A mRNA in Millers River animals could be differences in temperature. During sampling, the ambient water temperature of Millers River was 6.2 °C while Fourmile Brook was 10.4 °C. Therefore, temperature compensation would also be expected to result in higher levels of CYP1A mRNA in Millers River salmon. In laboratory induction experiments, although lower temperatures resulted in a higher number of CYP1A mRNA, differences between high temperature (17 °C) and low temperature (11 °C) samples did not differ by more than 25 times. Since the temperature difference in the two streams was even smaller than the 6 °C used in these laboratory studies, it is likely that most of the observed differences in levels of CYP1A mRNA in Millers

River and Fourmile Brook was due to induction by elevated PCB's found in Millers River.

These experiments resulted in a standard curve-based quantitative PCR method to assess expression levels of CYP1A in Atlantic salmon gill, kidney, liver, and brain tissue. This study used a semi-heterologous recombinant RNA standard designed from a portion of the human β-globin gene with a size discrepancy of 116 bp from the native CYP1A amplified fragment. This type of IS has been used frequently (Vanden Heuvel, 1998), and is particularly useful when limited information about the endogenous gene is known (Vanden Heuvel et al., 1993, 1994; Vanden Heuvel, 1998). Potentially, inaccuracies may result from differences in reverse transcription efficiency between native RNA molecules and RNA competitors due to different secondary structures or other factors (Hayward et al., 1998). To reduce potential discrepancy in reverse transcription and PCR amplification, we designed our IS with the same primer recognition sequences as the native CYP1A fragment as well as a poly A tail. In addition, we used a calibration curve, a sensitive labeling technique, and a laser scanner to increase the calculation accuracy of the assay.

This method provides a direct measure of CYP1A mRNA copy numbers, and thus a direct assessment of salmon response to pollutants. This was confirmed in both a controlled lab induction study as well as on samples from natural streams. 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 populations in the North Atlantic. This assay thus will be useful in monitoring the toxicological stress of Atlantic salmon populations, helping to discover the reasons behind Atlantic salmon declines, and potentially providing solutions to establish sound management plans for restoration of wild salmon. Further research is needed in studying the overall effects PCB's and similar compounds are having on the health of Atlantic salmon. We have demonstrated that it is possible to assess CYP1A levels in Atlantic salmon in the wild using quantitative PCR. Further, an assay measuring tissues that are in direct contact with the ambient environment, specifically gill or intestine, is preferred when looking at toxic responses of fish to waterborne contaminants (Levine and Oris, 1999). Using quantitative PCR, it would be possible to quantify CYP1A levels in Atlantic salmon through non-lethal 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 (McCormick, 1993). However, knowing how CYP1A is altered in response to PCB's and other compounds is not enough. Quantitative PCR can be adapted to study the effects pesticides in general have on expression of many other genes not only in Atlantic salmon but other fish species as well.

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). Our method and the method developed by Miller et al. (1999) are an improvement to traditional quantitative PCR experiments where single samples were quantified using a dilution series of IS for each sample (Vanden Heuvel et al., 1993). Using standard curves in quantitative PCR studies allows 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 3 days. Further, with the development of real-time PCR thermal cyclers, this time can be shortened even more. Overall, the quantitative PCR experiment has become more economical without reducing accuracy and sensitivity. These characteristics allow direct estimation of mRNA levels, enabling rapid quantification of CYP induction dynamics at the transcription level. Clearly, quantitative PCR complements the existing quantitative methods for estimating P450 protein levels. Future experimentation is needed to compare the relative merits and accuracies associated with quantitative PCR, and its utility in environmental assessment.

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