

GREAT LAKES FISHERY COMMISSION

2003 Project Completion Report¹

Effects of Low Level Aquatic Contaminants on Lake Trout Reproduction: Implication in Lake Trout Rehabilitation

by:

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April 2003

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COMPLETION REPORT FOR GLFC RESEARCH PROJECT

PROJECT TITLE

**Effects of Low Level Aquatic Contaminants on Lake Trout Reproduction:
Implication in Lake Trout Rehabilitation**

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PROJECT DURATION

January 2002 – April 2003

30 April 2003

Abstract

We have completed this project and anticipate submitting in total of three manuscripts. Briefly, we developed several novel methods to further understand and characterize the induction and function of this gene in lake trout and other salmonids species. First, we developed the measurement of gill CYP1A using quantitative PCR with the sampling of non-lethal gill biopsies in both wild and cultured Atlantic salmon. This study demonstrated that gill biopsies coupled with quantitative PCR analysis was a potentially valuable tool in environmental assessment of wild fish populations. Second, advances in the quantitative PCR method and instruments led us to develop a real-time quantitative PCR assay useful for measuring CYP1A mRNA in four salmonid species; lake trout, brook trout, rainbow trout, and Atlantic salmon. In order to obtain necessary information for the design of a cRNA standard, full-length CYP1A cDNA sequences were determined for two *Salvelinus* species, lake trout (*Salvelinus namaycush*) and brook trout (*Salvelinus fontinalis*). Each cDNA was found to share the same characteristics with known CYP1A sequences of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*, and shared greater than 97% coding region sequence. The developed a CYP1A-specific real-time quantitative PCR assay indicated that BNF treated fish showed 1.8 to 3.0 orders of magnitude higher CYP1A than control fish in all four species studied. Finally, we examined the effects BNF exposure had on juvenile lake trout brain tissue using a multidisciplinary approach. Over a 32 day time-course, CYP1A mRNA induction in response to BNF exposure occurs rapidly and continued to rise in the BNF-treated lake trout after 4 hours, 8 hours, and 24 hours with a peak in CYP1A mRNA expression after 2 days. At each of these time periods, significantly higher levels of CYP1A expression were found in each induced group over their respective control groups (Tukey-Kramer, $p < 0.0001$). *In situ* hybridization study supports the Q-RT-PCR results in that CYP1A mRNA level was universally induced in the brain of BNF-exposed fish, and that CYP1A mRNA were mainly expressed in the endothelia and occasionally in the neurons or glial cells. CYP1A immunoreactivity was induced in the olfactory bulb and valvula cerebelli of BNF-treated fish. Notably, some BNF-treated fish contained multifocal hemorrhages in the brain tissue. These fish had overall depressed CYP1A immunoreactivity in the brain. These results show the relationship between transcriptional and translational effects of contaminant exposure in the brain of juvenile lake trout and provide knowledge to the potential physiological effects sublethal levels of contaminants have on fish from the population level.

Introduction and Summary of Results

This project was proposed to conduct a preliminary investigation on the toxicological and physiological consequences of chronic exposure to sub-lethal levels of toxicants that contribute to reproduction impairment in Great Lakes lake trout (*Salvelinus namaycush*). In particular, we attempted to examine lake trout Cytochrome P450 1A, enzymes inducible by a wide variety of persistent toxicants found in the Great Lakes, to establish a biological and molecular indicator for these responses. Our specific objectives were: (1) clone a CYP1A gene from lake trout; (2) develop a sensitive biomarker for lake trout; and (3) use this marker to assess effects of chronic exposure to contaminants.

We have completed this project and anticipate submitting in total of three manuscripts. The first chapter, A non-lethal method to estimate CYP1A expression in laboratory and wild Atlantic salmon (*Salmo salar*), which is a manuscript submitted to the *Journal of Fish Biology*, describes a sensitive method to estimate the CYP1A expression in salmonids without sacrificing the fish. The second chapter, Development and application of a real-time quantitative PCR assay for determining CYP1A transcripts in three genera of salmonids, which is a manuscript submitted to the *Aquatic Toxicology*, describes the cDNA encoding a Lake trout CYP1A. In addition, this chapter describes a cDNA encoding a CYP1A in closely related species, brook trout (*S. fontinalis*). It also illustrates a real time quantitative PCR based assay for expression levels of CYP1A in lake trout and brook trout. Finally, the third chapter, Quantitative PCR Time Course study and Brain Localization for β -naphthoflavone induced CYP1A in juvenile lake trout (*Salvelinus namaycush*), is a manuscript under development. It describes a multi-disciplinary study that integrates several cutting edge technologies, such as real time quantitative PCR, in situ hybridization and immunocytochemistry to examine the expression of CYP1A in the brain of lake trout. This study also identifies for the first time in teleost fish the expression patterns of CYP1A in neurons of different parts of the brain. Here is a summary of our results. The detailed description can be found in chapter 1 through 3.

The expression of CYP1A (cytochrome P450 1A) can be induced by a large array of aromatic and organic compounds in teleost fishes and has been used as a biomarker for possible exposure to contaminants such as PCB's and dioxins. We developed several novel methods to further understand and characterize the induction and function of this gene in lake trout and other salmonids species.

First, we incorporated the measurement of gill CYP1A using quantitative PCR with the sampling of non-lethal gill biopsies in both wild and cultured Atlantic salmon. Groups of ten Atlantic salmon juveniles (48-76 g) received an intraperitoneal injection of $50 \mu\text{g g}^{-1}$ β -naphthoflavone (BNF) or vehicle. Their gill tissues were repeatedly sampled by non-lethal biopsies on day 0, 1, 2 and 7. Control fish showed static levels of CYP1A over the course of sampling. BNF treated salmon demonstrated similar levels of CYP1A to control fish at day 0 and higher levels over the course of each additional sampling point. Gill biopsies from wild salmon sampled from Millers River (South Royalston, Worcester County, MA, USA), known to contain PCBs, showed significantly higher CYP1A levels over a pristine stream, Fourmile Brook (Northfield, Franklin County, MA, USA). This

study demonstrated that gill biopsies coupled with Q-RT-PCR analysis can be a potentially valuable tool in environmental assessment of wild Atlantic salmon populations and many other populations of fish as well.

Second, advances in the quantitative PCR method led us to the next development, a real-time quantitative PCR assay useful for measuring β -Naphthoflavone (BNF) induction of liver CYP1A mRNA in four salmonid species; lake trout, brook trout, rainbow trout, and Atlantic salmon. First, to obtain necessary information for the design of a cRNA standard, full-length CYP1A cDNA sequences were determined for two *Salvelinus* species, lake trout (*Salvelinus namaycush*) and brook trout (*Salvelinus fontinalis*). Each cDNA was found to share the same characteristics with known CYP1A sequences of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*): a start codon, conserved heme-binding region, putative poly-adenylation signal, stop codon, relatively long 3' untranslated region (UTR; >1 kb), and a protein length of 523 amino acid residues. The brook trout and lake trout CYP1A cDNA's were 2636 and 2672 base pairs (bp) in length and shared greater than 97% coding region sequence identity with Atlantic salmon and rainbow trout CYP1A's. Next, using the generated sequence information, we developed a CYP1A-specific real-time quantitative PCR assay. Primers and a fluorescent-labeled probe were designed from a 68 bp region that was found to be conserved among salmonid CYP1A genes. The assay was designed to allow for simultaneous comparison of CYP1A expression among each experimental group. Finally, groups ($n = 4-8$) of hatchery-raised Atlantic salmon, brook trout, lake trout, and rainbow trout were given an intraperitoneal injection of a corn oil control, 25 mg kg⁻¹ BNF, or 50 mg kg⁻¹ BNF and sacrificed after 48 h. Liver tissue was collected and CYP1A mRNA levels were estimated. In all species, BNF treated fish showed 1.8 to 3.0 orders of magnitude higher CYP1A than control fish. The CYP1A induction levels were not different in fish treated with both dosages. Mean base levels of CYP1A expression ranged from 7.24 x 10⁶ (rainbow trout) to 1.05 x 10⁷ (brook trout) transcripts μg^{-1} total RNA. Mean induced levels of CYP1A expression ranged from 1.07 x 10⁸ (lake trout) to 1.05 x 10⁹ (brook trout) transcripts μg^{-1} total RNA. This real-time CYP1A specific quantitative PCR assay was a stepping stone to our next series of investigations.

Finally, we examined the effects BNF exposure had on juvenile lake trout brain tissue. Many factors are hypothesized to contribute to the decline in successful naturally-reproducing populations of lake trout (*Salvelinus namaycush*) in the Great Lakes such as changes in food-web dynamics, the introduction of the sea lamprey, or the sublethal effects of pollutants on various stages of lake trout development. Some of these pollutants, such as polychlorinated biphenyls and dioxins, are known to induce expression of the cytochrome P450 (CYP) 1A subfamily of genes. We applied a multidisciplinary approach to assess the molecular effects of sublethal contaminant exposure in lake trout brain CYP1A using quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), *in situ* hybridization, western blot and immunocytochemistry.

Over a 32 day time-course, CYP1A mRNA induction in response to BNF exposure occurs rapidly and continued to rise in the BNF-treated lake trout after 4 hours, 8 hours, and 24 hours with a peak in CYP1A mRNA expression after 2 days. At each of these

time periods, significantly higher levels of CYP1A expression were found in each induced group over their respective control groups (Tukey-Kramer, $p < 0.0001$). Induction fell after 4 days and this trend continued after 16 days of exposure. For in situ hybridization, western blot and immunocytochemistry experiments, 4-day BNF exposure was chosen for maximum induction of CYP1A. *In situ* hybridization study supports the Q-RT-PCR results in that CYP1A mRNA level was universally induced in the brain of BNF-exposed fish, and that CYP1A mRNA were mainly expressed in the endothelia and occasionally in the neurons or glial cells. Western blot showed no detectable CYP1A in the brain extracts of fish treated with or without BNF. However, a 65 kDa protein was induced in the liver extract of BNF-exposed fish. CYP1A immunoreactivity was induced in the olfactory bulb and valvula cerebelli of BNF-treated fish. Other brain areas showed constitutive CYP1A immunoreactivity in both control and BNF-treated fish. Some BNF-treated fish contained multifocal hemorrhages in the brain tissue, and these fish had overall depressed CYP1A immunoreactivity in the brain. These results show the relationship between transcriptional and translational effects of contaminant exposure in the brain of juvenile lake trout and provide knowledge to the potential physiological effects sublethal levels of contaminants have on fish from the population level.

Chapter 1

A non-lethal method to estimate CYP1A expression in laboratory and wild Atlantic salmon (*Salmo salar*)

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Short title: Non-lethal Detection of CYP1A Expression

Expression of cytochrome P450 1A (CYP1A) has been used as a biomarker for possible exposure to contaminants such as PCB's and dioxins in teleost fish. Using a quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) and a non-lethal gill biopsy, we estimated levels of CYP1A mRNA expression in Atlantic salmon. Groups of ten Atlantic salmon juveniles (48-76 g) received an intraperitoneal injection of 50 $\mu\text{g g}^{-1}$ β -naphthoflavone (BNF) or vehicle. Their gill tissues were repeatedly sampled by non-lethal biopsies on day 0, 1, 2 and 7. Control fish showed static levels of CYP1A over the course of sampling. BNF treated salmon demonstrated similar levels of CYP1A to control fish at day 0 and higher levels over the course of each additional sampling point. Gill biopsies from wild salmon sampled from Millers River (South Royalston, Worcester County, MA, USA), known to contain PCBs, showed significantly higher CYP1A levels over a pristine stream, Fourmile Brook (Northfield, Franklin County, MA, USA). We conclude that gill biopsies coupled with Q-RT-PCR analysis is a valuable tool in environmental assessment of wild Atlantic salmon populations and many other populations of fish as well.

Key words: Atlantic salmon, gill biopsies, CYP1A, quantitative PCR, *Salmo salar*

INTRODUCTION

For endangered fish species, it has become increasingly important to assess the health and stress levels in remaining wild stocks. However, direct assessment through conventional lethal sampling procedures is difficult, if not impossible, to justify. For instance, the wild Atlantic salmon (*Salmo salar*), once an abundant and prized sport fish, is in danger of becoming extinct from the United States and some areas of Northeastern Canada. Habitat destruction, over fishing, damming of rivers (MacCrimmon and Gots 1979), disease (Bakke and Harris 1998), competition from hatchery-stocked salmon (Youngson and Verspoor 1998), and contaminants (Elson 1967, Fairchild et al. 1999) have contributed to Atlantic salmon declines, especially in the southern reaches of their native range (Parrish et al. 1998). This has resulted in the listing of Atlantic salmon on the US Endangered Species List as of November 2000 in the state of Maine (U.S. Department of Interior 2000). They are also considered endangered in the Inner Bay of Fundy in New Brunswick and Nova Scotia of Eastern Canada (Committee on the Status of Endangered Wildlife in Canada 2002). To conserve this species, extensive physiological and toxicological studies are needed to confirm or eliminate potential causes for the decline of wild Atlantic salmon.

In fact, intensive studies have been carried out on Atlantic salmon populations derived from stocked hatchery fish. A series of investigations have been carried out recently focusing on the sub-lethal mechanisms associated with contaminant exposure and reproductive impairment in fish populations (Jones et al. 1998, Moore and Waring 2001; topic in review McMaster 2001). Some have used correlational studies to link the locational dwindling of salmon stocks to historical application of pesticides in those same areas (Fairchild et al. 1999). Others have studied the effects of pesticides on reproductive development in related salmonid species (Fitzsimmons 1995). Determination of contaminant body burdens or organismal response to exposure usually requires destructive (lethal) sampling. As a result, direct assessment of this response in endangered wild stocks, which would directly implicate their current physiological conditions and potential stress by environmental factors, is rare.

In this study, we attempted to develop a non-lethal method to assess levels of gene expression rapidly and accurately. Expression of particular genes is often associated with specific physiological function or response to environmental changes, and is a good indicator for fish health. Our primary objective is to illustrate a concept that can be adapted to study the expression of virtually any gene of fish in tissues suitable for biopsies. Specifically, we selected CYP1A mRNA of Atlantic salmon as our model system to develop this approach for several reasons. First, this enzyme is involved with the detoxification of polychlorinated biphenyls (PCB's), polyaromatic hydrocarbons (PAH's), and dioxins (Goksøyr and Husøy 1998). Historically, CYP1A induction by these compounds has been used as an indicator to monitor chemical contamination and health of aquatic ecosystems (Bucheli and Font 1995, Flammarión et al. 2001). Its expression has also been found to influence vitellogenesis, zonagenesis, and oocyte maturation (Arukwe et al. 2001, Navas and Segner 2001). Therefore, such an assay could be used directly to study responses to a variety of toxicants in fish. More importantly, we have established a quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) assay and used it to measure levels of CYP1A mRNA in Atlantic salmon tissues induced by xenobiotics (Rees et al. 2003). This is important because one of our primary

objectives is to compare the anticipated data from the new gill biopsy tissues directly to the previous set of data on CYP1A expression, collected from tissues through destructive sampling (Rees et al. 2003). Such a comparison provides further evaluation of the reliability of a gill biopsy for estimating levels of CYP1A gene expression. Here we describe development of this new approach, report the first time course of CYP1A induction established in a single group of fish, and show utility of this new method in assessing gene expression of fish from the wild.

MATERIALS AND METHODS

GILL BIOPSIES

Non-lethal gill biopsies were collected according to McCormick (1993). Briefly, Atlantic salmon were anaesthetised by immersion in buffered 100 ng l⁻¹ MS-222 (pH 7.0). The distal half of six gill filaments were excised from the first gill arch (refer to Figure 1 for a picture of the area of sampling), stored immediately in 300 µl of RNALater[®] (Ambion; Austin, TX), and packed on dry ice. Samples were stored at -80 °C for further analysis.

TOTAL RNA EXTRACTIONS

For quantitative PCR analysis, it is essential that the extracted RNA samples be highly pure with no genomic DNA. To achieve the desired results, we used a RNA filtration based technique (Absolutely RNA[™] Nanoprep Kit; Stratagene; La Jolla, CA). Total RNA was resuspended in 10 µL of diethylpyrocarbonate-treated water (DEPC-H₂O) and quantified (Sambrook et al. 1989) using a Beckman DU 7400 spectrophotometer (Fullerton, CA).

RNA QUALITY

Quality of RNA was verified by use of a spectrophotometer and by analysing samples on an agarose gel. Subsets of extracted RNA samples were size-fractionated on a 1 % agarose gel containing 0.1 µg ml⁻¹ ethidium bromide (Sambrook et al. 1989) and analysed for genomic DNA contamination. Based on spectrophotometer and agarose gel analysis, we determined that RNA samples with an A260/A280 ratio of = 1.0 or an A230 of < 0.1500 were considered adequate for the Q-RT-PCR assay. Samples which did not meet these criteria (10% of the total) were discarded from the statistical analysis.

TIME-COURSE INDUCTION STUDY

Atlantic salmon juveniles (15-19 cm, 48-76 g) were held at the Conti Anadromous Fish Research Center in Turners Falls, MA, USA in 1 m diameter tanks with flow-through Connecticut River water (4 l min⁻¹) maintained at 10 – 11 °C under natural daylight conditions. Salmon were fed by hand to satiation twice per day (Ziegler's; Gardners, PA.). Two days prior to injections, salmon were taken off of feed. Gill biopsies were collected from each individual immediately before injection and stored in RNALater. After biopsies were taken at time 0, one group of salmon was given an intraperitoneal injection of corn oil only (n = 10) while the other group (n = 10) received an injection of corn oil with β-naphthoflavone (BNF, 50 µg g⁻¹), a known inducer of the

CYP1A gene in Atlantic salmon (Rees et al. 2003). After injections, non-lethal gill biopsies were taken at 1, 2, and 7 days. Salmon were sacrificed on the 7th day of the experiment by an overdose of MS-222. At that time, liver was also sampled for use as a reference tissue.

SALMON FROM THE WILD

To show the utility of the Q-RT-PCR assay in fish gill biopsies from the wild, we sampled juvenile Atlantic salmon (9-68 g) by electro-shocking from two rivers in Massachusetts, Fourmile Brook and Millers River (25.8 km apart from each other), on November 16th, 2001 (6.3 – 7.3 °C). Millers River had been found in the past to contain contaminated waters, sediment, and fish with high levels of PCB contamination (Colman 2001). Fourmile Brook was considered a ‘pristine’ site. Fish in these streams were planted as fry as part of the Connecticut River Restoration Program. Ten fish were collected from each site after which gill biopsies were immediately taken and stored in 300 µl of RNALater. The juveniles were returned to the stream.

QUANTITATIVE RT-PCR

Construction of the internal standard, reaction conditions and primers in RT-PCR, and other aspects of the quantitative PCR assay used in this study were described previously (Rees et al. 2003).

STATISTICAL ANALYSIS

All analyses were carried out using Statistical Analyses System (SAS Institute; Cary, NC). Q-RT-PCR data was transformed logarithmically to increase the homogeneity of variance. Time and treatment effects for the gill biopsy-lab induction study were analysed using a repeated measures mixed effect ANOVA (PROC MIXED). A Tukey-Kramer adjustment was used to determine differences between biopsy samples from experimental and control groups. Student *t*-tests were used to compare means between Millers River and Fourmile Brook CYP1A levels and also between control and induced liver samples.

RESULTS

TIME-COURSE LAB INDUCTION EXPERIMENT

BNF treatment significantly increased the levels of CYP1A mRNA in gills (ANOVA, $P < 0.0001$; Figure 2a). Gill biopsies from each group of salmon demonstrated no difference in CYP1A levels prior to injection (Tukey-Kramer, $P > 0.10$). In contrast, after injection, gill biopsies from salmon injected with BNF showed a 12x induction of CYP1A mRNA over control salmon after 1 day (Tukey-Kramer, $P < 0.0001$). Maximal induction of 85x over control levels occurred after 2 days and remained at this level after a seven-day exposure (Tukey-Kramer, $P < 0.0001$). A higher level of CYP1A mRNA was found in liver tissues of salmon treated with BNF after seven days of exposure compared to control salmon (Student *t*-test, $P < 0.0001$; Figure 2b).

GILL BIOPSIES FROM SALMON IN THE WILD

Gill CYP1A mRNA levels in Atlantic salmon from the Millers River were 66x higher than salmon from Fourmile Brook (Student *t*-test, $P < 0.0001$; Figure 3). In addition, levels of gill CYP1A mRNA from Fourmile Brook salmon corresponded closely to levels found in control fish of the lab induction study. Likewise, Atlantic salmon CYP1A levels from Millers River were approximately equal to levels of CYP1A from BNF-induced salmon in the lab.

DISCUSSION

Using non-lethal gill biopsies from Atlantic salmon we have shown that CYP1A is highly inducible and that the results are consistent with those collected from destructive sampling. In particular, after one day of exposure induced salmon demonstrated ~1 order magnitude induction of CYP1A mRNA over control levels. The trend of induction continued after two days of exposure where BNF-induced salmon showed ~2 orders of magnitude induction of CYP1A over control individuals. Maximal induction was reached at this point in the gill samples. This level of induction was maintained over the remainder of the seven-day time-course experiment. These results are consistent with previous findings. In Atlantic salmon liver tissue, previous research has demonstrated using Northern blotting techniques that CYP1A mRNA maximal induction is reached after two days (Grosvik et al. 1997). In addition, ELISA analysis of CYP1A protein demonstrated maximal levels after 96 h of exposure (Grosvik et al. 1997). As a reference tissue in the laboratory induction time-course, liver samples after seven days of exposure showed 370x as much CYP1A mRNA in induced fish than control fish. This is similar to the induction levels observed in liver tissue after two days of exposure in Rees et al. (2003). Likewise, maximum induction of CYP1A estimated from gill tissues taken by destructive sampling from Atlantic salmon under virtually identical conditions (Rees et al. 2003) was very similar to the induction levels in the present study. This high level of similarity further confirms the accuracy of non-lethal sampling approaches to measure CYP1A levels.

Our analysis of CYP1A from salmon of Millers River and Fourmile Brook demonstrated that the gill biopsy-Q-RT-PCR approach is useful in measuring CYP1A activity in salmon from the wild. The elevated level of CYP1A in Millers River fish is similar to those seen previously using large quantities of gill tissue from destructive sampling (Rees et al. 2003). These elevated levels of CYP1A mRNA are likely due to induction by PCB's, known to be higher in Millers River (Colman 2001) than in Fourmile Brook. Although temperature and other factors may affect CYP1A activity (Kloepper-Sams and Stegeman 1992; Grosvik et al. 1997, Rees et al. 2003), temperature profiles in these streams were similar and sampling on the two rivers occurred within a short period of time during which the temperature varied less than 1 °C.

This study extends the use of gill biopsies to direct assessment of gene expression, in addition to studies of proteins and enzymatic activities. In the past, gill biopsies have been used for many applications in monitoring fish health, particularly in the aquaculture industry (Montgomery-Brock et al. 2001). The effect of gill biopsies (2 x 3 mm) has also been tested on age-0 juvenile Pacific salmon (*Oncorhynchus tshawytscha*.) that had been implanted with radio tags showing that salmon with both a radio tag and gill biopsy have no adverse health or survival effects compared to salmon with only radio tags (Martinelli-Liedke et al. 1999). The effects gill biopsies have on health of Atlantic salmon has been

studied as well. Specifically, it has been shown that juvenile Atlantic salmon receiving a gill biopsy showed no differences in mortality, growth rate, and the ability to regulate plasma sodium when exposed to saltwater as salmon without biopsies (McCormick 1993). Likewise, Siegler et al. (1996) observed no differences in mortality or growth rate (length and weight) between the biopsy group and the control group of smolting salmon. In this study, we extended the application of this non-lethal technique to measuring changes of gene expression induced by environmental contaminants. Generally, all of the studies above took larger amounts of gill tissue for biopsies compared to this study and did not engage in repeated sampling of the same individual fish over time. This study sampled the same fish four times repeatedly over the course of seven days, used smaller amounts of gill tissue than any of the aforementioned experiments, and fish that were the same size or smaller than any of the previous studies. Therefore, the methods described in this study expand the utility of using gill biopsies to study genetic, physiological, and toxicological effects on fish populations.

Previous studies of CYP1A induction in teleosts relied on EROD (ethoxy resorufin-*O*-deethylase) activity or ELISA to measure effects on CYP1A protein and Northern blotting or slot blotting to examine the kinetics of induction on CYP1A mRNA levels (Goksøyr and Husøy 1998). The size of gill biopsies described here would be too small to carry out induction studies with any of the techniques described above due to the sensitivities associated with each of the methods. 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 (Vanden Heuvel 1994). This level of sensitivity offered by Q-RT-PCR enabled us to study gene expression in smaller amounts of biopsy tissues.

Gill biopsies have never been used to assay levels of gene expression in fish populations. We have determined in this study that non-lethal gill biopsies of Atlantic salmon coupled with Q-RT-PCR offers a conservation-based approach to studying gene expression in fish populations. Our specific assay may be useful to study CYP1A levels in most salmonid species due to the high level of conservation of CYP1A genes in many fish species. In theory, quantitative PCR has the adaptability to study virtually any gene in any tissue of any fish species. With modifications in primer sequences and internal standards, the levels of any known gene can be analysed. It will be even more useful in a few years when the genomic data for several teleost species become readily available due to the many genome projects that are currently under way. The methods described in this paper will allow for repeated sampling of gill tissue from individual fish to study gene expression over time. This characteristic alone will lower the number of samples needed for studying gene expression in fish as well as offering an accurate and sensitive method in related time-course experiments. As more and more species are added to the Endangered Species list, this technique gives researchers and managers the needed flexibility to study physiological and genetic changes in threatened and possibly endangered fish populations without lowering an already small population.

Appreciation is extended to Amy Moeckel, Mike O'Dea, Darren Lerner and Junya Hiroi who helped in collecting Atlantic salmon gill biopsy samples, and to Hong Wu for assisting in the extraction of RNA. Dr. Kim Scribner offered the use of gel imaging and electrophoresis equipment. Dr. Bradley Young offered advice and

suggestions for statistical analysis. This research was funded by the National Oceanic Atmospheric Administration and by the Great Lakes Fishery Commission.

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Figure 1.

The location of sampling for gill biopsies. All biopsies were taken from centrally located filaments only from the distal end. For non-lethal sampling, 5 - 7 filaments (< 0.5 cm in length) are adequate for Q-RT-PCR analysis.

Figure 2.

- a. Log mean number of CYP1A transcripts/ μg total RNA over time. Gill biopsies were taken (non-lethally) from both control (?) and experimental (?) groups ($n = 10$ for each) immediately before intraperitoneal injection and 1, 2, and 7 days after injection. Asterisks indicate significance of difference (Tukey-Kramer, $P < 0.0001$) between CYP1A mRNA levels from gill biopsies of BNF-injected (50 mg kg^{-1} BNF) and corn oil-injected salmon for each day respectively. The vertical bars indicate the standard error (STE).
- b. Log mean number of CYP1A transcripts/ μg total RNA in liver tissue of Atlantic salmon seven days post-injection. The liver samples came from the same animals used for taking gill biopsies during 0, 1, 2, and 7 days in the time-course experiment. Asterisks indicate significance of difference (Students t -test, $P < 0.0001$) between CYP1A mRNA levels from liver tissue of BNF-injected and corn oil-injected salmon. The vertical bars indicate the standard error (STE).

Figure 3.

a. Representative gel pictures for gill biopsies taken from feral Atlantic salmon of Millers River and Fourmile Brook. RNA (100 ng) was co-transcribed and co-amplified with a RNA internal standard of a known concentration (IS). Each lane represents one individual (n = 10). 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. 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.

b. Log mean number of CYP1A transcripts/ μ g total RNA from gill biopsies of feral Atlantic salmon from two Massachusetts streams, Millers River (PCB contaminated) and Fourmile Brook (pristine site). Asterisks indicate significance of difference (Students *t*-test, $P < 0.0001$) between CYP1A levels from gill biopsies of salmon sampled from Fourmile Brook and Millers River.

Vertical bars indicate the standard error (STE).

Figure 1.

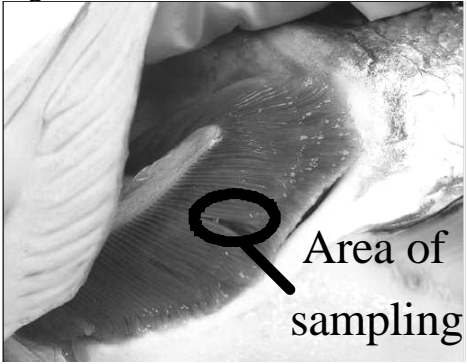
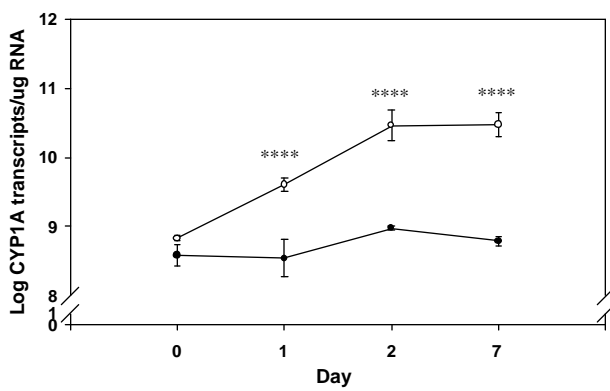


Figure 2.

a.



b.

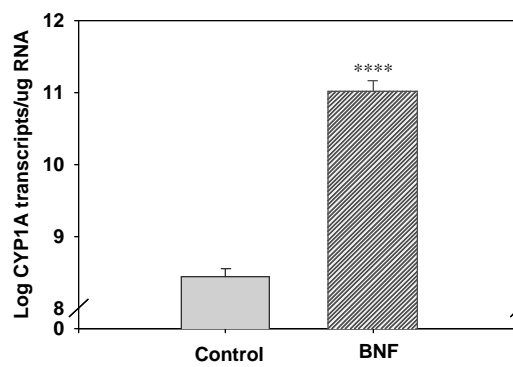
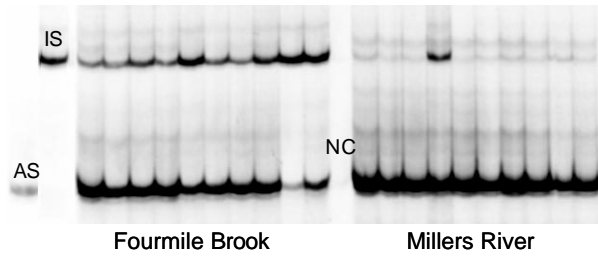
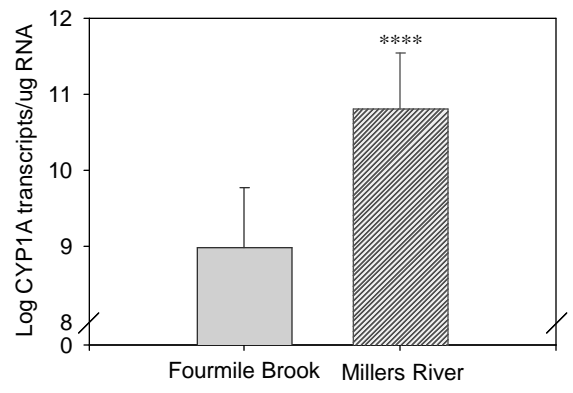


Figure 3.
a.



b.



Chapter 2

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

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Abstract

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

Key Words: CYP1A, P450, salmon, trout, real-time quantitative PCR, cDNA sequence

Introduction

Cytochrome P4501A's (CYP1A) constitute a ubiquitous family of proteins associated with the detoxification of organic compounds such as PCB (polychlorinated biphenyl), PAH (polyaromatic hydrocarbons), and dioxin (Buhler and Wang-Buhler 1998; Mansuy 1998; Nelson et al. 1996). These compounds are documented to induce CYP1A in a variety of tissues of many teleost species (Levine and Oris 1999; Hahn et al. 1998; Gooneratne et al. 1997). Consequently, changes in CYP1A gene expression have been used as a biomarker for contaminant exposure in fish populations (Cousinou et al. 2000; Miller et al. 1999; Campbell and Devlin 1997).

A variety of techniques have been applied to estimate CYP1A induction in fish. Protein levels can be measured by determining EROD (ethoxy resorufin-O-deethylase) activity (Schlezingner and Stegeman 2001), immunohistology (Stegeman et al. 2001), enzyme-linked immunosorbent assay (Sarasquette and Segner 2000), and Western blotting (Grøsvik et al. 1997). Recently, CYP1A gene expression has been estimated from mRNA levels through Northern blotting (Grøsvik et al. 1997), slot-blotting, or quantitative PCR (Rees et al. 2003; Miller et al. 1999; Campbell and Devlin 1996). Of all of these methods, quantitative PCR appears to be the most sensitive (Vanden Heuvel 1994). It has been used to assess impact of environmental pollution in marine ecosystems using emerald rockcod (Trematomus bernacchi, Miller et al. 1999), guilthead seabream (Sparus aurata, Cousinou et al. 2000), and grey mullet (Liza aurata, Cousinou et al. 2000). Likewise, numerous quantitative PCR assays have been developed to study CYP1A induction in freshwater teleosts such as Pacific salmon (Oncorhynchus tshawytscha, Campbell and Devlin 1996) and Atlantic salmon (Salmo salar, Rees et al. 2003).

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

Materials and Methods

Animals, BNF induction, and acquisition of tissues

All species of salmonids used in this study were acquired from nearby fish hatcheries. Juvenile lake trout and brook trout ($11 \text{ g} \pm 2 \text{ g}$ mean weight, $11 \text{ cm} \pm 2 \text{ cm}$

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

RNA isolation

RNALater® preserved tissues were homogenized and extracted for total RNA isolation using Trizol Reagent (Life Technologies; Carlsbad, CA.) according to the manufacturer's protocol. RNA samples were incubated at 37 °C with RNase-free DNase I (Roche Molecular Biochemicals; Mannheim, Germany) then re-suspended in 20 – 50 μ L of diethylpyrocarbonate-treated water (DEPC-H₂O) and quantified (Sambrook et al. 1989) using a GeneQuant *pro* RNA/DNA calculator (Amersham Biosciences; Piscataway, NJ). For long-term storage, RNA samples were supplemented with 3 volumes of 95% ethanol, 1/10 volume of 3 M sodium acetate, and stored at -80 °C (Sambrook et al. 1989).

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

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

Phylogenetic analysis

The coding region of brook trout, lake trout, and Atlantic salmon CYP1A was aligned to the coding region of a sample of P450 genes using the CLUSTAL W algorithm.

Genetic relationships and distances were generated using the Neighbor-joining method. Genes selected for this analysis comprised teleost CYP genes representing families 1 through 4 (Refer to Table 1 for GenBank accession numbers).

In vitro transcription of cRNA standard

Separate plasmids containing either the full CYP1A cDNA sequence from lake trout, brook trout, and Atlantic salmon (Rees et al. in press) were obtained by standard cloning procedures and sequenced as described previously. To design a cRNA standard, a 491 bp conserved region of the CYP1A gene was amplified from the Atlantic salmon CYP1A clone using the following primers and conditions: forward primer WML 169 5' TAA TAC GAC TCA CTA TAG GCT GTC TTG GGC TGT TGT GTA CCT TGT G 3', reverse primer WML 170 5' TTT TTT TTT TTT TTT TTT GGA GCA GGA TGG CCA AGA AGA GGT AG 3', 1 cycle at 94 °C for 4 min, 40 cycles 94 °C for 5 sec and 72 °C for 2 min, and 1 cycle at 72 °C for 5 min as added extension. The PCR product contained a 5' T7 promoter, 454 bp of CYP1A sequence including the region of the real-time amplicon, and a poly dT tail at the 3' end. This product was then diluted 1/100 with deionized water, re-amplified and scaled up with the same reaction conditions. The concentrated PCR product was cleaned using the QIAquick® PCR Purification Kit (Qiagen; Valencia, CA) and transcribed using the Riboprobe In Vitro Transcription System (Promega Corp.; Madison, WI) according to standard protocol. The cRNA 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 centrifuged for 10 min at 12,000 g, re-suspended in 20 µL DEPC-H₂O, and filtered through a NucAway™ Spin Column (Ambion; Austin, TX). The size and quality of the cRNA standard was verified by analysis on an agarose gel and quantified at 260 nm using a spectrophotometer. This RNA standard was then used to generate standard curves useful for the real-time quantitative PCR analysis of CYP1A.

Quantitative PCR Primer and Probe Design

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

RT-PCR

Reverse transcription (all reagents were from Invitrogen Life Technologies) was performed on all samples in a final volume of 20 μ L containing a 1x concentration of First Strand Buffer, 0.01 M dithiothreitol, 1 mM of each deoxynucleotide triphosphate, 2.5 μ M oligo(dT), 5 units of MMLV reverse transcriptase, 1 unit rRNasin (Promega Corp.), 100 ng of total RNA, and varying amounts of cRNA standard predetermined from initial range-finding experiments. The reaction mixture was incubated at 37°C for 50 min and inactivated at 70°C for 15 min. Then, 2 μ L of the cDNA sample was spiked into a PCR master mix. Each PCR reaction consisted of 25 μ L of 2x TaqMan[®] Universal PCR master mix (Applied Biosystems; Branchburg, NJ), 300 nM of each primer, 100 nM of the TaqMan[®] probe (5' 6-FAM, 3' TAMRA quencher), 100 ng of cDNA template, and DI water to a final volume of 50 μ L. Reactions were then analyzed on an ABI 7700 real-time PCR thermalcycler (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min. Amplification plots were generated and CYP1A mRNA levels were estimated against a standard curve.

Primer and Probe Optimization

Real-time PCR primers (forward WML158 5' CCA ACT TAC CTC TGC TGG AAG C 3' and reverse WML159 5' GGT GAA CGG CAG GAA GGA 3') were optimized for quantitative PCR by performing PCR reactions with 9 separate concentration combinations in quadruplicate and determining which combination produced the largest ΔR_n . ΔR_n (normalized reporter) represents the signal to noise ratio and indicates the magnitude of the signal generated by a given set of PCR conditions (for more information, consult the Applied Biosystems TaqMan[®] Universal PCR Master Mix Protocol). Once the primer concentration was chosen, an additional set of reactions was set up to optimize the probe (WML160 5' TTC ATC CTG GAG ATC TTC CGG CAC TC 3') concentration for the chosen primer concentration. Five separate probe concentrations were used in quadruplicate and analyzed to see which concentration produced the smallest C_t (threshold cycle). C_t values represent the cycle of amplification at which a PCR reaction reaches a statistically significant increase in ΔR_n (consult the Applied Biosystems TaqMan[®] Universal PCR Master Mix Protocol for more information). The lowest C_t value in these optimization reactions indicates the concentration at which optimal probe binding occurs and point of highest sensitivity for detecting specific template. For all of the above reactions, Atlantic salmon liver cDNA was used as the PCR template.

Standard curve

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

Statistical analysis

Transcript numbers of CYP1A μg^{-1} total RNA were calculated from the appropriate standard curve and log transformed. Data were analyzed using a 2-way analysis of variance (ANOVA; Statistical Analysis Systems v.8; Cary, NC.). All pairwise comparisons were tested for significance by using a Tukey-Kramer adjustment (Kramer 1956).

Results

Brook trout and lake trout CYP1A sequences

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

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

Phylogenetic analysis

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

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

Primer and Probe Design and optimization

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

Standard curve

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

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

CYP1A was induced approximately 100-1000 fold in all species injected with both dosages (25 mg kg⁻¹ body weight and 50 mg kg⁻¹ body weight; Figure 6). A significant interaction was found between species and treatment making main effects irrelevant. Simple effects were determined for each factor by using the SLICE procedure (Statistical Analysis Systems v.8, Winer 1971). There was no statistical difference between BNF induction at 25 or 50 mg kg⁻¹ body weight in each species ($p > 0.45-0.99$). However, ANOVA analysis showed a difference between CYP1A mRNA levels of control and induced groups in each species ($p < 0.0001$). On average, all species had approximately 1×10^6 CYP1A transcripts μg^{-1} total RNA at control levels and 1×10^9 CYP1A transcripts μg^{-1} total RNA under induced conditions. Brook trout demonstrated higher mean basal levels of CYP1A than all other species (ANOVA $p < 0.01$) at 1×10^7 CYP1A transcripts μg^{-1} total RNA. Among BNF induced fish, lake trout had lower levels of CYP1A transcripts at approximately 1×10^8 CYP1A transcripts μg^{-1} total RNA (ANOVA $p < 0.02$) than all other treatment groups except Atlantic salmon treated with 50 mg kg⁻¹ BNF.

Discussion

It is evident that both of the PCR fragments cloned from lake trout and brook trout represent full-length cDNA clones of CYP1A genes. Each cDNA sequence has characteristics of a full-length teleost CYP1A cDNA: a start codon and a stop codon followed by a poly A tail, a heme-binding domain, an arginine codon integral to enzymatic function, and a rather large 3' UTR containing 2 (lake trout) and 3 (brook trout) AUUUA sequences. The coding region (1569bp), which encodes a protein of 523 amino acid residues, is the same size as the rainbow trout and Atlantic salmon P450 1A protein. In addition, the brook trout and lake trout CYP1A genes show 97.9% and 97.6% sequence identity to Atlantic salmon CYP1A. The coding regions of CYP1A genes isolated to date in salmonids (Atlantic salmon, rainbow trout, Pacific salmon, brook trout, and lake trout) differ by no more than 3.9%. This high level of sequence identity confirms that the CYP1A gene is highly conserved and thus suitable for developing a real-time quantitative PCR assay to study CYP1A expression dynamics across several genera in response to contaminant exposure.

Real-time PCR analysis indicates that CYP1A induction in liver tissue of lake trout, brook trout, rainbow trout, and Atlantic salmon followed a consistent pattern. In all species, CYP1A expression was induced by BNF injection from approximately 1.8 – 3.0 orders of magnitude representing a 60 – 1000 fold difference in CYP1A levels between control and induced levels. This trend of induction was seen in fish injected with 25 mg kg⁻¹ and also 50 mg kg⁻¹ BNF. Previous quantitative PCR studies have also found a

similar level of induction by BNF in Atlantic salmon (Rees et al. 2003) and Pacific salmon (Campbell and Devlin 1996) in a variety of tissues (liver, kidney, gill, brain, and gonad). Absolute levels of CYP1A expression ranged from a low of approximately 7×10^5 molecules CYP1A μg^{-1} total RNA in rainbow trout liver (control group) to a high of approximately 1×10^9 molecules CYP1A μg^{-1} total RNA in brook trout liver (induced). The CYP1A levels reported here closely resemble CYP1A mRNA expression levels identified in a 28 day BNF induction time course in Pacific salmon liver tissue. Campbell and Devlin (1992) report that at time zero Pacific salmon liver shows similar levels of CYP1A at 5.00×10^5 molecules μg^{-1} total RNA. After 28 days, this expression jumps 160-fold to 8.04×10^7 transcripts μg^{-1} total RNA. However, the results from each of these experiments are 2-3 orders of magnitude lower than those determined using a competitive quantitative PCR (Rees et al. 2003). This discrepancy is likely due to different fish conditions and experimental conditions. Another likely factor is the difference in the standard curves between the two experiments. The real-time PCR standard curve used here covered a larger linear range and had a higher coefficient of determination, thus would be more accurate at estimating CYP1A levels from samples expected to show a large range of difference. Nevertheless, it is evident that each of these assays is useful in showing inducing effects of BNF on CYP1A in fish.

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

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

In conclusion, we have developed a real-time quantitative PCR assay for analysis of CYP1A expression across three salmonid genera, Salmo, Oncorhynchus, and Salvelinus. In development of this assay, we confirmed that CYP1A genes across the salmonid family carry a high degree of sequence homology and is highly induced in liver tissue of BNF-exposed lake trout, brook trout, Atlantic salmon, and rainbow trout after 2 days. We also discovered some species-specific characteristics of CYP1A induction. Brook trout showed higher basal levels and induced levels than all other species. Lake trout showed the lowest induced levels of CYP1A expression. This assay has a high degree of specificity for generated CYP1A PCR products as well as a high degree of sensitivity detecting down to 1000 molecules CYP1A μL^{-1} total RNA.

Acknowledgments

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

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

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

Figure Captions

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

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

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

Figure 4.

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

Figure 5.

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

Figure 6.

Real-time PCR analysis of liver CYP1A levels in representative salmonid species (AS = Atlantic salmon, BT = brook trout, LT = lake trout, RBT = rainbow trout). Fish were administered an injection of corn oil only (control), 25 mg kg⁻¹ body weight BNF (β-Naphthoflavone), or 50 mg kg⁻¹ body weight BNF. Total RNA (100 ng) was reverse-transcribed and amplified in real-time from each treatment group (n = 4-8; sample size is indicated for each treatment group below each bar) after which CYP1A levels were estimated. Bars represent mean logarithmic values of CYP1A expression μg⁻¹ total RNA ± S.E.M. for each treatment group. Comparisons were made between induced and control levels using a Tukey-Kramer adjustment for multiple comparisons.

** notes significance of induced groups over each respective control group at $p < 0.0001$.

* notes significantly higher CYP1A levels over other control groups at $p < 0.01$.

Figure 2.

TGTGCAGAAGCCACAAAAAATACAAA**TGG**TTCTCATGATACTACCCATTATCGGCTCAGTCTCTGTGTCTGAGGGCTGGTGCCATGGTAACACTATGCCTGGTGATACATGATCA 119
M V L M I L P I I G S V S V S E G L V A M V T L C L V Y M I 30
TGAAGTACATGCACACAGAGATCCCAGAGGGACTGAAAACGGCTCCAGGACCAAAGCCCTGCCCATCATCGGGAATGTGCTGGAGGTGCACACAAACCTCACCTCAGCTGACTGCC 238
M K Y M H T E I P E G L K R L P G P K P L P I I G N V L E V H N N P H L S L T A 70
ATGAGCGAGCGCTACGGCTCAGTCTTCCAGATCCAGATAGGGATGCGGCTGTGGTTGTTCTGAGTGGCAGGACAGTCCGCCAGGCTTTATCAAGCAAGGGGAAGACTTCGCCGG 357
M S E R Y G S V F Q I Q I G M R P V V V L S G S E T V R Q A L I K Q G E D F A G 110
GAGGCCGATCTATACAGCTTCAAGTTTCATCAACGACGGCAAGAGCTTGGCCTTTAGTACCACAAAGCTGGGGTGTGGCGCGCCCGCAAGTAGCTATGAGCGCCCTTCGCTCTT 476
R P D L Y S F K F I N D G K S L A F S T D K A G V W R A R R K L A M S A L R S 149
TCGCCACCTGGAGGGATCGACCCAGAGTACTCCTGTGCCCTGGAGGACAGCTGTCAAGGAGGAGAGTACCTGGTAAACAGCTGACTTCCGCTCATGGATGTCAGTGGCAGCTTT 595
F A T L E G S T P E Y S C A L E E H V C K E G E Y L V K Q L T S V M D V S G S F 189
GACCCCTCCGCCATATTGTTGATCGGTGGCCAACTCATCTGGAATGTCTCGGCCGGCGCTACAGCCATGATGACCAGGAGCTGTGGCTTGGTGAACCTGAGTGAAT 714
D P F R H I V V S V A N V I C G M C F G R R Y S H D D Q E L L G L V N L S D E F 229
TGGCAGGTGTGGGCGAGCCAAACCTGCAGACTTCATCCCATCTT**CGT**TACCTACCAAACCGCACCATGAAGAGTTTATGGATATCAATGACCGTTTCAATACCTTTTGCAGA 833
G Q V V G S G N P A D F I P I L R Y L P N R T M K R F M D I N D R F N T F V Q 268
AGATTGTGAGTACTATGAAAGCTATGACAAAGGACAACTCCGTGACATCAGTACTCCCTCATTGACCAGTGTGAGGACAGGAACTAGATGAGAACGCCAACATCCAGGTGTCT 952
K I V S E H Y E S Y D K D N I R D I T D S L I D H C E D R K L D E N A N I Q V S 308
GATGAGAAGATTGTGGGATGTCAATGATCTGTTGGGGCAGGTTTGGACACCATCAGCACAGCTTGTGATGGGCTGTTGTACCTTGTGGCTTACCCAGAGATCCAGGAAAAGACT 1071
D E K I V G I V N D L F G A G F D T I S T A L S W A V V Y L V A Y P E I Q E R L 348
GCATCAGAACTGAAGAAAAGTGGGAATGATTCGCACTCCCGTCTCTCAGACAAAACCAACTTACCTCTGCTGGAAGCTTTCATCCTGGAGATCTCCCGCACTTTCCTTCTCTGC 1190
H Q E L K E K V G M I R T P R L S D K T N L P L L E A F I L E I F R H S S F L 387
CGTTCACCATCCACACTGCACAGTCAAGGATACATCGCTCAATGGCTACTTCAATCCCAAGGACACCTGTGTCTTTATCAACCACTGGCAGGTCAACATGACCCGGAGCTGTGGAAG 1309
P F T I P H C T V K D T S L N G Y F I P K D T C V F I N Q W Q V N H D P E L W K 427
GAGCCTTCTTCAACCTGACCGTTTCTGAGTCTGATGGCAGAGAACTCAACAACTGGAGGGGAGAGGTGCTCGTATTGGCATGGCAAGCGCCGCT**GC**ATCGGTGAGGC 1428
E P S S F N P D R F L S A D G T E L N K L E G E K V L V F G M G K R R C I G E A 467
CATTGGAGCAATGAGGTCTACCTTTTTGGCCATCCTGCTGCAAGGCTGTGCTTCAAGGAGAACTGGGCAACCGCTGGACATGACCCAGAGTACGGCTCACCATGAAGCACA 1547
I G R N E V Y L F L A I L L Q R L C F K E K P G H P L D M T P E Y G L T M K H 506
AGCGCTGCAGCTGAAGGCTAGCTTGGGCCATGGGGCAGGAGG**TGAGGG**CTATGGTCA**ATTAT**GATTCTCAACATCACTATAACT**GATTTA**TAGTTAGCGCTACATACTTGGAT 1666
K R C Q L K A S L R P W G Q E E . 522
GGCATGAGCTGAGTTCAGATATAAAAGCCAGAGGTACATTTCTCTCTAGGAAAGACTGGGCTTGACACCTCTCTTGATTATAGGAAAATTAACAGTGAAGGAAAAGTGAAGTCAAAT 1785
TGATAGCAATTTGAAATCGATAAAATATAATCTGAAACCGTGGTTGTAGAGGGATTCCAAACAGTGTGAATGGATAAAGACCTTCACAACTCATAGATTGATTGGTTTCTAT 1904
GACCAGCATACTACAGGCCCTCTAGAGTTTGTGAGGTGTTTTTCAGGAGATACAGGAGAGATTATAGCTGTTTAGCTGCTTTTTGTCTACATCATTGGTGTTCCTCTCTCT 2023
TGCCTTGATCAGCTCATATACTGTATCAGATTGCTTTAAAGAGGATGAATCTATAAAACATACACATAACCCACGGTGGGTTAAAGAGCATTGTTTTAGTCCATGTTGGG 2142
TAAACATTTTGTACATATTTTTTGTGGCAAAAATGTTACTACCTACATGATAAAGTCTTGTACTTTTGAGTTATGTTTCAGTGGTTCATAGATCACAGACAGAGTGAATGGATG 2261
TATCTCCCAATTTAAGCTAGATATTTTTATTCCACTGAATAAATCAGTCAACAGTGGACACAACTGAAAGCTATGTTTGTATGCCACCAACATGATTGATTGTTTGTGTACA 2380
TATTTAAGCTAGATGATTTTGGAAAGTCTTTTGTGAAATGTGCTAAAATGTGTATATATTTGGTACTTATTATGGTGGCTTTGTATGACTTTATCAGAATACTAAAATACGTA 2499
TGATTTACTGACTGTTATAAATGTCAACATTTTATAATGTAACGAAGGACTCTGTAACCTAAAGCCAACTGTATACACTATGCATTGTGTTGGTGGTAGCTATCTAGGTAGCTACT 2618
GAAATAAAAGCTGAAAATGATAGAAAACAAAAAAAAAAAAAAAAAAAAAAAAAA 2672

Figure 3.

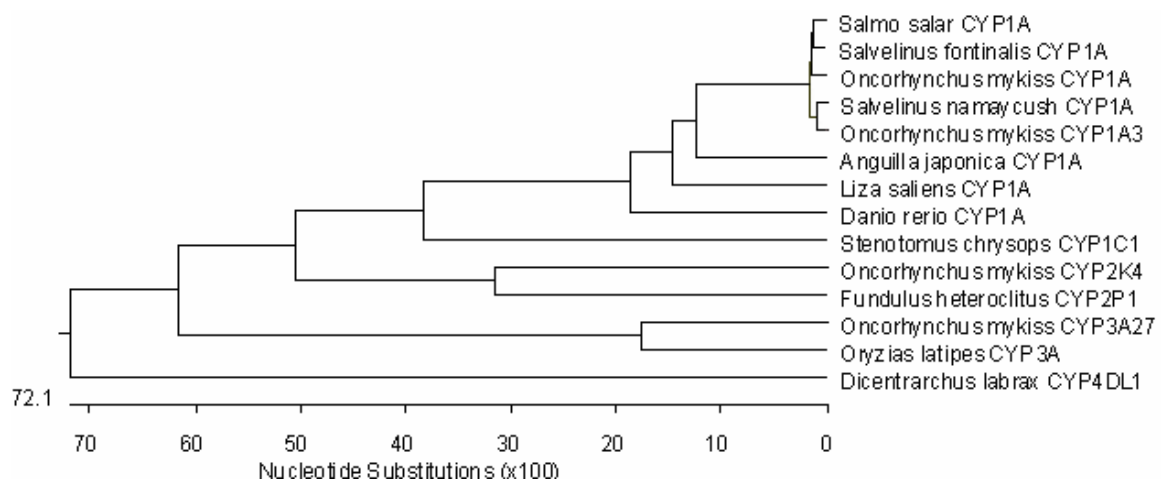


Figure 5.

Standard Curve

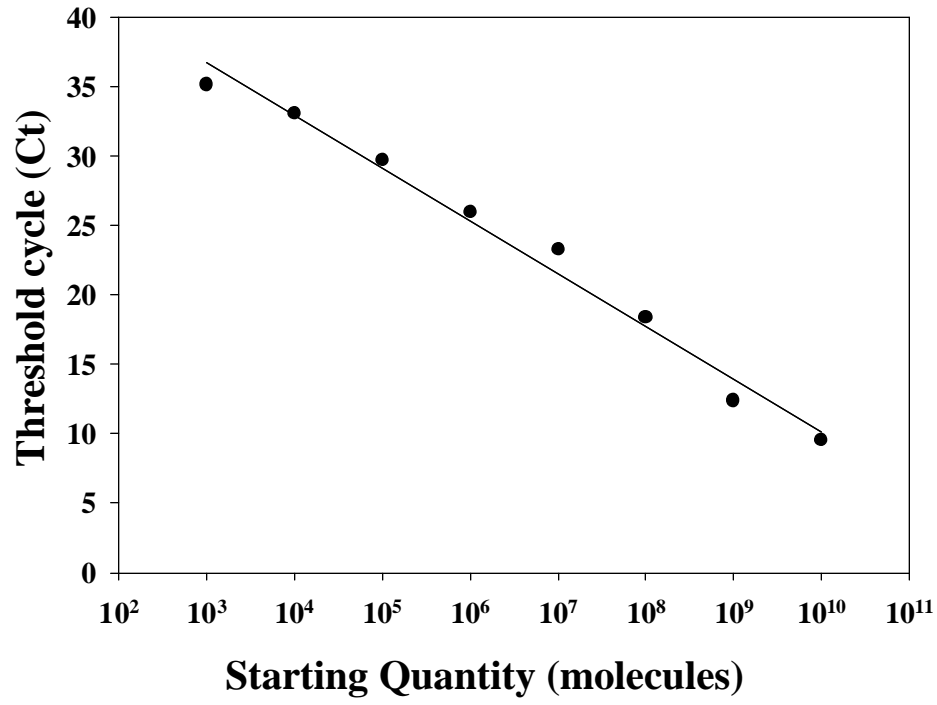
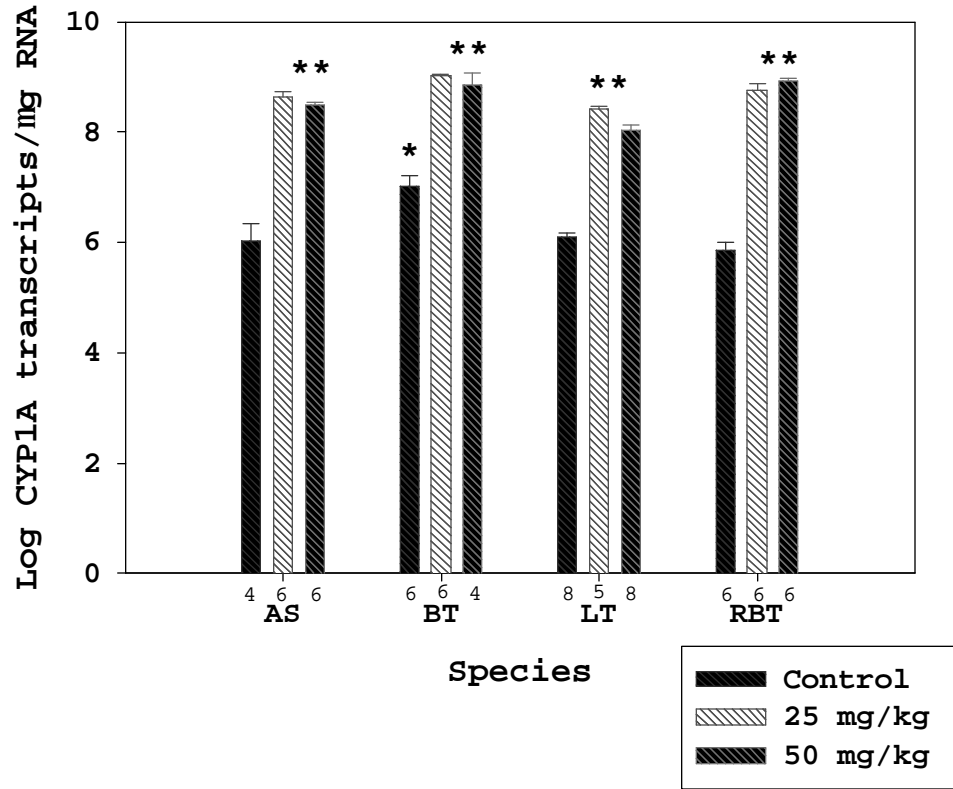


Figure 6.

Salmonid CYP1A levels



Chapter 3

Quantitative PCR Time Course study and Brain Localization for β -naphthoflavone induced CYP1A in juvenile lake trout (*Salvelinus namaycush*)

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Running Head: CYP1A induction in juvenile lake trout

Abstract

Many factors are hypothesized to contribute to the decline in successful naturally-reproducing populations of lake trout (*Salvelinus namaycush*) in the Great Lakes such as changes in food-web dynamics, the introduction of the sea lamprey, or the sublethal effects of pollutants on various stages of lake trout development. Some of these pollutants, such as polychlorinated biphenyls and dioxins, are known to induce expression of the cytochrome P450 (CYP) 1A subfamily of genes. We applied a multidisciplinary approach to assess the molecular effects of sublethal contaminant exposure in lake trout brain CYP1A using quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), *in situ* hybridization, western blot and immunocytochemistry. β -naphthoflavone (BNF) was chosen as the contaminant of interest since it was a well known CYP1A inducer.

Over a 32 day time-course, CYP1A mRNA induction in response to BNF exposure occurs rapidly and continued to rise in the BNF-treated lake trout after 4 hours, 8 hours, and 24 hours with a peak in CYP1A mRNA expression after 2 days. At each of these time periods, significantly higher levels of CYP1A expression were found in each induced group over their respective control groups (Tukey-Kramer, $p < 0.0001$). Induction fell after 4 days and this trend continued after 16 days of exposure.

For *in situ* hybridization, western blot and immunocytochemistry experiments, 4-day BNF exposure was chosen for maximum induction of CYP1A. *In situ* hybridization study supports the Q-RT-PCR results in that CYP1A mRNA level was universally induced in the brain of BNF-exposed fish, and that CYP1A mRNA were mainly expressed in the endothelia and occasionally in the neurons or glial cells. Western blot showed no detectable CYP1A in the brain extracts of fish treated with or without BNF. However, a 65 kDa protein was induced in the liver extract of BNF-exposed fish. CYP1A immunoreactivity was induced in the olfactory bulb and valvula cerebelli of BNF-treated fish. Other brain areas showed constitutive CYP1A immunoreactivity in both control and BNF-treated fish. Some BNF-treated fish contained multifocal hemorrhages in the brain tissue, and these fish had overall depressed CYP1A immunoreactivity in the brain. These results show the relationship between transcriptional and translational effects of contaminant exposure in the brain of juvenile lake trout and provide knowledge to the potential physiological effects sublethal levels of contaminants have on fish from the population level.

Introduction

The cytochrome P-450 (CYP) is a multi-gene family (Nelson et al., 1996) of constitutive and inducible heme-containing enzymes. They function in the metabolism of a wide spectrum of xenobiotics, and of numerous endogenous substances with physiological functions in inter- and intra-cellular signaling, such as steroid hormones, prostaglandins and fatty acids (Stegeman & Hahn, 1994, Parkinson, 1995). These enzymes are primarily found in the liver and adrenal glands; however, they are also distributed throughout the body in diverse areas, such as the brain, heart, intestine, kidney, lung, and skin (Norris et al., 1996; Sarasquete & Segner, 2000). CYP enzymes in the mammalian brain are approximately 0.5 to 3% of the content in the liver. These enzymes have been shown to be highly localized in discrete areas within the brain and may thus alter the local action or concentration of neuroactive drugs (Majewska et al., 1986). In addition, cytochrome P450 in the mammalian brain may have homeostatic functions since CYP isoforms have been shown to participate in cerebral blood vessel tone and also in the synthesis of neuroactive steroids (Walther et al., 1987; Warner et al., 1994; Harder et al., 1997). Current evidence shows that several CYP1A isoforms exist in fish (Teramitsu et al., 2000) and they are distributed in most tissues such as liver, gut, kidney, gill and heart (Sarasquete & Segner, 2000). Very few CYP1A studies in fish are focused on the brain tissue. Available data in fish show that constitutive CYP1A immunoreactivity has been found in neurons and brain endothelia, and some inducible CYP1A has also been found in the pituitary cells and brain endothelia (Sarasquete & Segner, 2000).

Many chemically different compounds induce *de novo* synthesis of CYP1A protein (Nebert & Gonzalez, 1987; Nebert et al., 1989; Parkinson, 1995; Stegeman & Hahn, 1994). The inductive response in this subfamily is known to occur via the high affinity binding of aromatic hydrocarbons to an intracellular receptor complex (the *Ah* receptor), involving the 90-kD heat shock protein (Hsp 90) and a nuclear translocation factor. Translocation of the inducer-receptor complex to the nucleus results in the transcriptional activation of the genes in the *Ah* battery (Nebert & Jones, 1989; Nebert et al., 1989; Hoffman et al., 1991); therefore, levels of CYP1A messenger RNA and newly synthesized CYP1A proteins are increased and the CYP1A proteins subsequently undergo processing, heme insertion and folding to yield the catalytically active enzymes. Each of these steps, i.e., mRNA, protein, and catalytic activity, can be analyzed with a suitable probe to detect induction (Goksøyr & Förlin, 1992).

The major site of induced CYP1A expression in teleost fish is the liver; however, the enzyme is also present in other tissues (Stegeman & Hahn, 1994, Goksøyr & Husoy, 1998, Sarasquete & Sagner, 2000). The CYP1A-mediated *in situ* metabolism and cellular toxicity of xenobiotics in the brain may have far-reaching consequences by causing disruption of neuronal and neuroendocrine function (Andersson et al., 1993, Morse et al., 1998, Huang et al., 2000). In this report, we applied a multidisciplinary approach to assess the molecular effects of sublethal contaminant exposure in lake trout brain using quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), *in situ* hybridization, western blot and immunocytochemistry. β -naphthoflavone (BNF) was chosen as the contaminant since it was a well known Ah receptor agonist and CYP1A inducer (Smeets et al., 1999).

Materials and methods

Animals

Juvenile lake trout (11.5 cm ± 4 cm, 12.5 g ± 3 g) were gathered from Marquette Fish Hatchery (Marquette, MI) and maintained at the Michigan State University Lower River Laboratory (East Lansing, MI). Trout were given time to acclimate for two weeks at 12°C in an 800-L flow-through tank (600 L/hr). During the acclimation period, lake trout were fed twice daily to satiation a diet of Purina AquaMax® Grower 400 (Purina Mills, Inc.; St. Louis, MO). A photoperiod of 10 h light and 14 h dark was kept during the duration of the experiment.

Experiment 1. Time-course Quantitative PCR for BNF-Induced CYP1A mRNA

Chemical Exposure

Two days prior to injections, trout were taken off of feed. Individuals were randomly sampled and anesthetized by immersion in buffered 100 ng/l MS-222 (pH 7.0). Anesthetized fish were given an intraperitoneal injection of either β-Naphthoflavone (BNF, Sigma Chemical Corp.; St. Louis, MO; 50 mg/kg body weight) dissolved in corn oil (10 mg/ml) or corn oil alone (corn oil was autoclaved and sonicated prior to administration). Lake trout were divided into ten gallon experimental aquaria where flow rate (0.75 R) and temperature (12 °C) were kept constant. Each aquaria received either a group (n = 8) of BNF-induced individuals or control individuals. One control and one experimental group of lake trout was sampled prior to injection (time zero), and after each exposure period of 2 h, 4 h, 8 h, 24 h, 2 d, 4 d, 8 d, 16 d, and 32 d.

Tissue collection and storage

After the appropriate exposure, lake trout were given an overdose of tricaine methanesulfonate (Sigma Chemical Corp.), whole brain tissue was excised with a pair of small forceps, submerged in 0.5 mL RNALater® (Ambion; Austin, TX), and immediately placed on ice. Samples were then frozen at -80 °C for long term storage.

RNA isolation and storage

For isolation of total RNA, brain tissue was removed from RNALater® with sterile forceps, homogenized, and placed in 1 mL Trizol Reagent (Life Technologies; Carlsbad, CA.). RNA pellets were reconstituted in varying amounts of diethylpyrocarbonate-treated water (DEPC-H₂O) dependent upon pellet size. Genomic DNA was digested by incubation at 37 °C with 1 μL RNase-free DNase I (Roche Molecular Biochemicals; Mannheim, Germany) and 0.3 μL rRNasin (Promega Corp.; Madison, WI) per 100 μL total RNA. DNase was inactivated by heating samples to 70 °C for 10 minutes. Total RNA was quantified (Sambrook et al. 1989) using a GeneQuant *pro* RNA/DNA calculator (Amersham Biosciences; Piscataway, NJ). For long-term storage, RNA samples were supplemented with 3 volumes of 95% ethanol, 1/10 volume of 3 M sodium acetate, and placed at -80 °C (Sambrook et al. 1989).

Reverse transcription – polymerase chain reaction (RT-PCR)

Reverse transcription (all reagents were from Invitrogen 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, 1 mM of each deoxynucleotide triphosphate,

2.5 mM oligo(dT), 5 units of MMLV reverse transcriptase, 1 unit rRNasin (Promega Corp.), and 100 ng of total RNA. The reaction mixture was incubated at 37°C for 50 min and inactivated at 70°C for 15 min. Then, 1 µL of the cDNA sample was spiked into a PCR master mix. Each PCR reaction consisted of 12.5 µL of 2x TaqMan® Universal PCR master mix (Applied Biosystems; Branchburg, NJ), 300 nM of each primer (forward WML158 5' CCA ACT TAC CTC TGC TGG AAG C 3' and reverse WML159 5' GGT GAA CGG CAG GAA GGA 3'), 100 nM of the TaqMan® probe (WML160 5' TTC ATC CTG GAG ATC TTC CGG CAC TC 3') that contained a 3' TAMRA quencher and a 6-FAM fluorescent label at the 5' end, 100 ng of cDNA template, and DI water to a final volume of 25 µL. Reactions were then analyzed on an ABI 7700 real-time PCR thermalcycler (Applied Biosystems) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min. Amplification plots were generated and CYP1A mRNA levels were estimated against a standard curve.

Recombinant RNA standard and generation of standard curves

A CYP1A recombinant RNA standard was used to generate standard curves in each set of reactions. This recombinant RNA was synthesized as follows. A 491 bp conserved region of the CYP1A gene was amplified from the Atlantic salmon CYP1A clone using the following primers and conditions: forward primer WML 169 5' TAA TAC GAC TCA CTA TAG GCT GTC TTG GGC TGT TGT GTA CCT TGT G 3', reverse primer WML 170 5' TTT TTT TTT TTT TTT TTT GGA GCA GGA TGG CCA AGA AGA GGT AG 3', conditions 1 cycle at 94 °C for 4 min, 40 cycles 94 °C for 5 sec and 72 °C for 2 min, and 1 cycle at 72 °C for 5 min as added extension. The PCR product generated contained a 5' T7 promoter, 454 bp of CYP1A sequence including the region of the real-time amplicon, and a poly dT tail at the 3' end. This product was then diluted 1/100 with deionized water, re-amplified and up-scaled with the same reaction conditions. The concentrated PCR product was cleaned using the QIAquick® PCR Purification Kit (Qiagen; Valencia, CA) and transcribed using the Riboprobe In Vitro Transcription System (Promega Corp.; Madison, WI) according to standard protocol. The cRNA 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 for 10 min at 12,000 g, resuspended in 20 µL DEPC-H₂O, and filtered through a NucAway™ Spin Column (Ambion; Austin, TX). The size and quality of the cRNA standard was verified by analysis on an agarose gel and quantified at 260 nm using a spectrophotometer. This RNA standard was then used to generate standard curves useful for the real-time quantitative PCR analysis of CYP1A.

In order to quantify CYP1A levels, each plate of samples was normalized against a set of standard curve reactions. To generate standard curves, RT-PCR was carried out on a dilution series (10¹⁰ – 10³ molecules) of the CYP1A real-time cRNA molecule. Amplification plots were analyzed on the ABI 7700 and Ct values for each of the reactions in the dilution series were calculated. Ct values were plotted against starting quantity of RNA template to generate the standard curve. Additional control reactions were also run on each plate including a no template (water) negative control, a no

amplification (RNA) control, a negative reverse-transcription (water added) control, and a CYP1A positive control by adding a 1 μ L of a plasmid containing a full-length CYP1A cDNA sequenced from lake trout (GenBank accession number AF539415) to the PCR reaction mixture.

Statistical analysis

All data were log-transformed to fulfill normality requirements and analyzed using a two-way analysis of variance (Statistical Analysis Systems v.8; Cary, NC.). A significant interaction was found between time and treatment making main effects irrelevant. Simple effects were determined for each factor by using the SLICE procedure (Statistical Analysis Systems v.8, Winer 1971). All pairwise comparisons were tested for significance by using a Tukey-Kramer adjustment (Kramer 1956).

Experiment 2. In situ hybridization for BNF-Induced CYP1A mRNA in the Brain

After four days BNF induction (as described in Experiment 1), 12 (6 control and 6 induced) juvenile lake trout (*Salvelinus namaycush*) were anesthetized with 0.05% MS222 (tricaine methanesulfonate, Sigma) and perfused with saline for five min and decapitated to excise their brains. The tissues were fixed in 4 % paraformaldehyde, 0.1M PBS solution for 3h. Following cryoprotection in 25 % sucrose, 4 % paraformaldehyde, 0.1M PBS buffer overnight at room temperature, the tissues were embedded in Tissue Tek O.C.T compound (Sakura Finetek, Torrance, CA, USA) and rapidly frozen in -80 °C. The brain was sectioned into 20- μ m slices using a Leica 1850 cryostat, adhered to Superfrost plus (Fisher, Orangeburg, NY, USA) microslides and stored at -80 °C.

Synthesis of Digoxigenin labeled cRNA probes

The Digoxigenin labeled antisense RNA Probe (500bp) was generated from the lake trout CYP1A full-length cDNA clone using the Riboprobe In vitro Transcription Systems (Promega, Madison, WI, USA). In brief, 2 μ g linearized vector were transcribed in the presence of 700 nmol digoxigenin-11-UTP. The cRNA was collected by ethanol precipitation and resuspended in DEPC water. The sense RNA was prepared in the same way and used as the negative control.

Hybridization

Tissue sections were brought to room temperature, treated with proteinase K (20 μ g/ml in PBS) for 5 min and post fixed for 15 min in 4% paraformaldehyde, 0.1M PBS solution. Sections were rinsed three times for 10 min each in PBS before a 2h incubation in prehybridization solution [50% deionized formamide, 1 \times Denhart's solution, 750 mM sodium chloride, 25mM ethylenediaminetetraacetic acid (EDTA), 25 mM piperazine-N,N'-bis (2-ethanesulfonic acid)(PIPES), 0.25 mg/ml calf thymus DNA, 0.25 mg/ml Poly A acid and 0.2 % sodium dodecyl sulfate (SDS)]. Sections were then hybridized with antisense or sense RNA probe in hybridization solution (prehybridization solution containing 5 % dextran sulfate) at 60 °C for 16-20 h. After hybridization, sections were washed three times for 10 min each in 2 \times SSC -0.3 % polyoxyethylenesorbitan monolaurate (Tween-20) followed by three washes in 0.2 \times SSC -0.3 % Tween-20 at 65 °C.

Immunovisualisation of Digoxigenin

For detection of digoxigenin-labeled probes, the sections were blocked for 1 h in 4% dry milk, 2 % bovine albumin and 0.3 % triton. The sections were incubated for 3h with alkaline phosphatase-conjugated sheep anti digoxigenin Fab fragments (1:1000 in blocking solution (Boehringer Mannheim, Indianapolis, IN, U.S.A.). The color was developed with incubation in nitroblue tetrazolium chloride and 5-bromo-4-chloro-3 indolyl phosphate substrate (NBT/BCIP, Boehringer Mannheim) about 20-30 min. Subsequently, the “Nuclear Fast Red” (Vector Laboratories, Burlingame, CA, U.S.A) was used to stain the nuclei. The sections were mounted in DPX Mountant and examined under a Nikon Microscope.

Experiment 3. Western Blot for BNF-Induced CYP1A Proteins

The brain and liver tissues of non-treated and treated lake trout with BNF were homogenized separately in 200 µl ice-cold 10 mM Tris buffer, pH 7.4 containing 25 µg/ml leupeptin, 5 µg/ml aprotinin, 40 µg/ml phenylmethylsulfonyl fluoride, 50 µg/ml benzamidine, and 0.5 µg/ml pepstatin. at 0°C. Protein concentration was determined using a DCA protein analysis kit (Pierce). 25 µg of protein were then applied to 10% acrylamide/N,N'-methylenebisacrylamide (29:1 mix, Bio-Rad Laboratories) SDS-PAGE at 150 V for 45 min (Laemmli, 1970). Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Immobion-P) by electroblotting. The PVDF membranes were then blocked with 5% (w/v) nonfat dry milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.04% Tween 20) overnight. The PVDF membranes were incubated in the primary antibodies (1:200, Cayman, Ann Arbor, USA) in 5% nonfat dry milk for 1h. After washing 3 times with Tris buffered saline/0.05% tween 20 (TBST), the PVDF membranes were then reacted with goat anti-rabbit antibody conjugated with horseradish peroxidase (Pierce) at a 1:10,000 dilution for 1h. Protein signal was detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent kit (Pierce).

Experiment 4. Immunocytochemistry for BNF-Induced CYP1A Protein in the Brain

Twelve juvenile lake trout (*Salvelinus namaycush*) received an intraperitoneal injection of 50 µg/g BNF or vehicle (corn oil). The fish were anesthetized with 0.05% MS222 (tricaine methanesulfonate, Sigma) and perfused with saline. The brain was exposed and fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) overnight at 4°C and then transferred to 4% paraformaldehyde with 20% sucrose for storage. The brain was horizontally sectioned into 20-µm slices using a Leica1850 cryostat, subsequently mounted onto L-glycine coated slides (Sigma) and immunostained for CYP1A.

The general procedure for immunocytochemistry was as follows. The sections were washed in 50 mM Tris buffer saline (TBS) three times (5 min each) in between each step. All the procedures followed the manufacturer's instruction. Unless otherwise specified, all the reactions were performed at room temperature. Sections were first reacted with 0.01% H₂O₂ (DAB substrate kit, Vector) for 10 min to eliminate the endogenous peroxidase activity. Sections were then reacted with avidin- and then biotin-blocking solutions 10 min each (Avidin-Biotin Blocking Kit, Vector) to eliminate endogenous biotin. Sections were incubated at 4°C overnight in the primary antibody solution (1: 200 rabbit-anti-rainbow trout CYP1A, CP-226, Cayman Chemical) in 50 mM

TBS with 0.05% Triton X-100 and normal goat serum (Vectastain ABC kit for rabbit IgG, Vector). Sections were reacted with the biotinylated secondary antibody (goat-anti-rabbit, Vectastain ABC kit, Vector) for 2 hours, incubated in ABC solution (Vectastain ABC kit, Vector) for 2 hours, reacted with 3,3'-diaminobenzidine and NiCl_2 (DAB substrate kit, Vector) for 15 min, counter stained with hematoxylin (Sigma) for 5 min, dehydrated through an ethanol series (70%, 95%, 100%, 2 min each), clarified twice by xylene (5 min and 10 min), and covered with glass using DPX mounting media (Sigma). Stained sections were visualized using a Nikon light microscope and images were captured by a digital camera.

Results

Experiment 1:

Standard curves

There were two plates run to analyze the entire 160 sample data set. Both standard curves exhibited a correlation coefficient (r^2) of at least 0.995. An example of one of these standard curves is shown in Fig.1.

Lake trout CYP1A induction time-course in brain

Over a 32 day time-course, data revealed that CYP1A mRNA induction in response to BNF exposure occurs rapidly. Both control and induced lake trout groups at time zero showed nearly identical mean CYP1A levels at 3.98×10^4 and 4.44×10^4 transcripts CYP1A μg^{-1} total RNA respectively. After two hours exposure, both control and induced groups showed significantly higher CYP1A levels (Tukey-Kramer, $p < 0.05$) however, this difference may be due to stress-induced increases of CYP1A expression. CYP1A levels continued to rise in the BNF-treated lake trout after 4 hours, 8 hours, and 24 hours with a peak in CYP1A mRNA expression after 2 days at 2.90×10^7 transcripts CYP1A μg^{-1} total RNA. At each of these time periods, significantly higher levels of CYP1A expression were found in each induced group over their respective control groups (Tukey-Kramer, $p < 0.0001$). Induction fell after 4 days and 8 days of exposure slightly. This trend continued after 16 days of exposure where 5 x less CYP1A expression was found compared to maximal levels. Control levels during this time were statistically the same in all time groups. We also found that after 32 days, a large degree of variation in CYP1A expression was found between individuals. Some trout returned to basal levels at that time period while others remained in an induced state. To make sure this was not due to experimental error, total RNA samples were run on agarose gels and checked for densitometry comparisons and ribosomal band integrity. All RNA samples were found to be similar in RNA integrity and band density (data not shown). The results of the brain CYP1A time-course are shown in Fig.2.

Fig.1.

Standard Curve

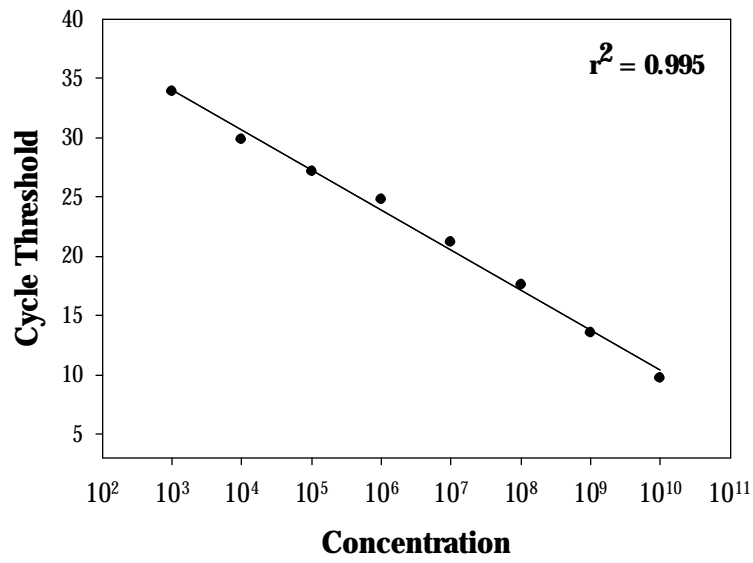
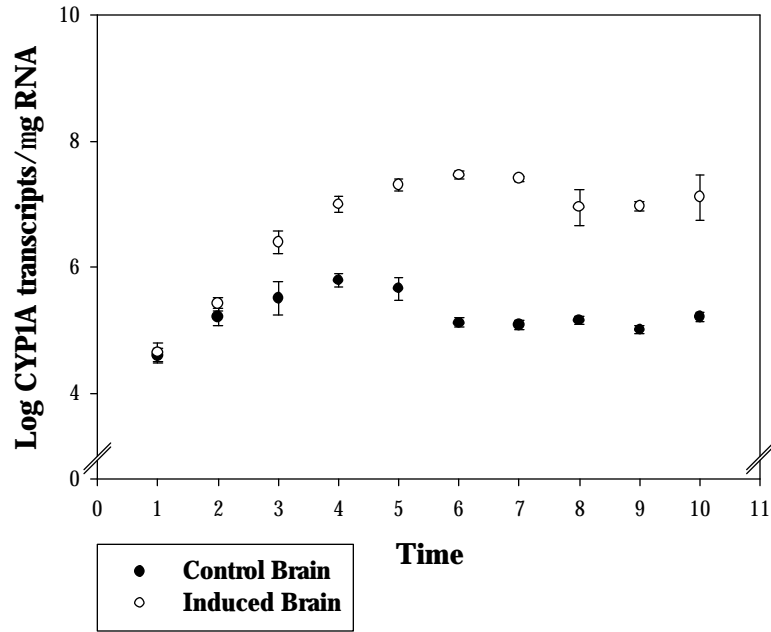


Fig.2.

Brain CYP1A levels



Experiment 2:

Four-day BNF exposure induced CYP1A mRNA expression in lake trout brain. As shown in Fig.3A no CYP1A mRNA was detectable in control lake trout brain. After 4-day exposure to BNF, CYP1A mRNA was highly induced (Fig.3B) and universally expressed in the brain, mainly in the endothelium cells, glial cells (Fig.3C), and in some neurons (Fig.3D).

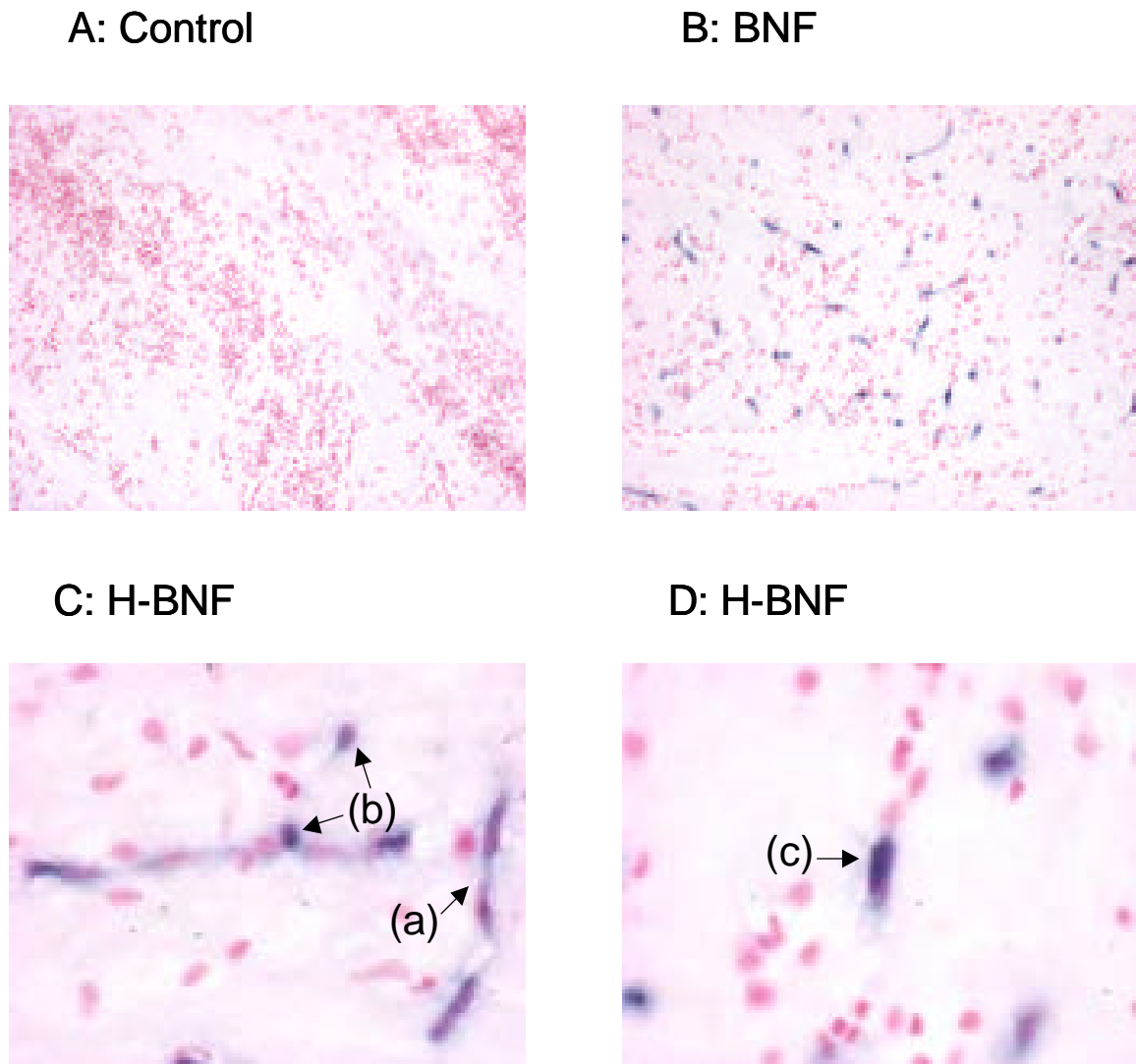


Fig.3. Expression patterns of CYP1A mRNA in juvenile lake trout brains. *In situ* hybridization on the brain section of juvenile lake trout treated with β -naphthoflavone (BNF: B,C,D) or corn oil (Control: A) for four days. Arrows in C, D show the staining cells (a): endothelia, (b): glial cells, (c) neuron.

Experiment 3:

Western blot analysis using an antiserum against CYP1A protein identified a clear protein band at 65 kDa and revealed a strong induction of the protein in the BNF exposed liver tissues (Fig.4). No significant expression of CYP1A protein was observed in the brain tissues exposed to BNF.



Fig.4. Western blot analysis of lake trout liver tissues exposed to BNF. Lane 1: liver tissues injected with corn oil as control. Lane 2: liver tissues injected with BNF. Note that strong expression of CYP1A is observed at the sample exposed to BNF.

Experiment 4:

BNF induced CYP1A immunoreactivity in specific brain areas. Control juvenile lake trout showed constitutive expression of CYP1A in neurons, endothelial and glial cells in the brain. Some of the glial cells coursed along and directly contacted the blood vessels (Fig.5). BNF appeared to induce more CYP1A expression in the olfactory bulb (Fig.6A, B). In some fish, BNF also induced more CYP1A expression in the valvula cerebelli (Fig.6C, D). However, CYP1A induction in this brain region showed individual difference. There is no difference in the induction of CYP1A immunoreactivity in other brain areas examined (Fig.7). Some BNF-treated juvenile lake trout showed depressed CYP1A immunoreactivity in the brain compared to the control fish (Fig.8). Upon careful examination, in the fish that showed CYP1A depression, sporadic hemorrhage sites were discovered (Fig.9).

Fig.5. CYP1A-immunoreactive (black) glial cells in the corpus cerebelli (A) and the tectum mesencephali (B) of the control juvenile lake trout. Note that the glial cell courses along the blood vessels and the end feet (black arrows in A) and dendritic spines (black arrows in B) are visible. Horizontal section (20- μ m thick) is counterstained with hematoxylin nuclear stain (purple).

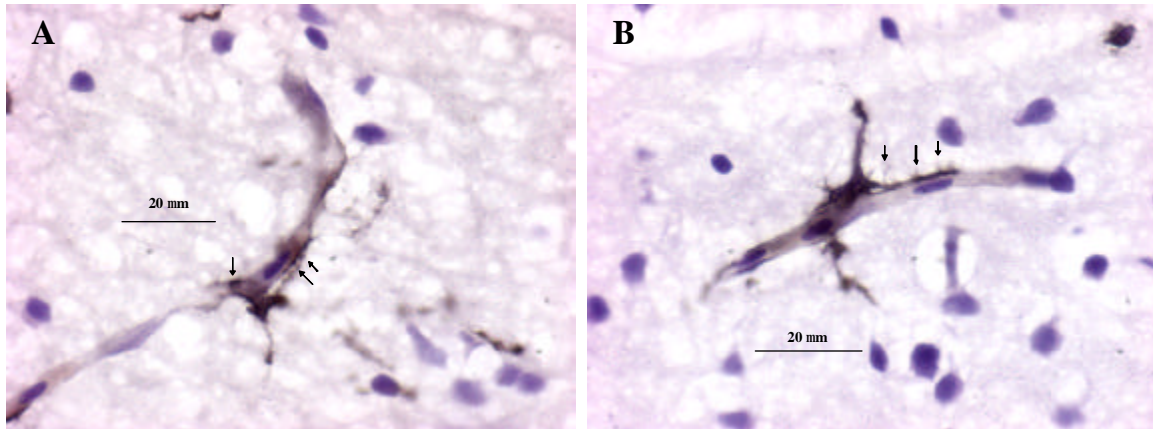


Fig.6. CYP1A immunoreactivity (brown/black) in the olfactory bulbs (A, B) and valvula cerebelli (C, D) of the control (A, C) and β -naphthoflavone (BNF)-treated (B, D) juvenile lake trout. Horizontal sections (20- μ m thick) are counterstained with hematoxylin nuclear stain (purple).

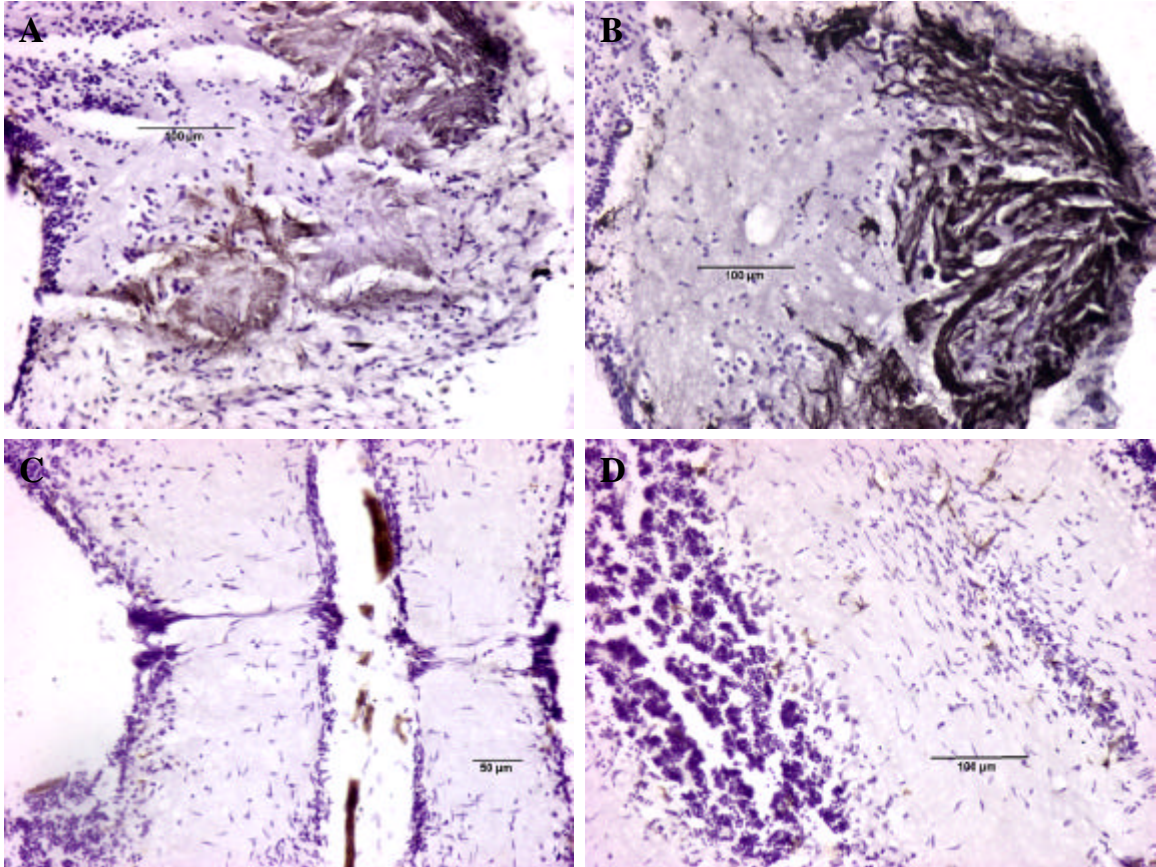


Fig.7. CYP1A immunoreactivity (brown/black) in the habenula (A, B), cerebellum (C, D), and tectum mesencephali (E, F), and the of the control (A, C, E) and β -naphthoflavone (BNF)-treated (B, D, F) juvenile lake trout. Horizontal sections (20- μ m thick) are counterstained with hematoxylin nuclear stain (purple).

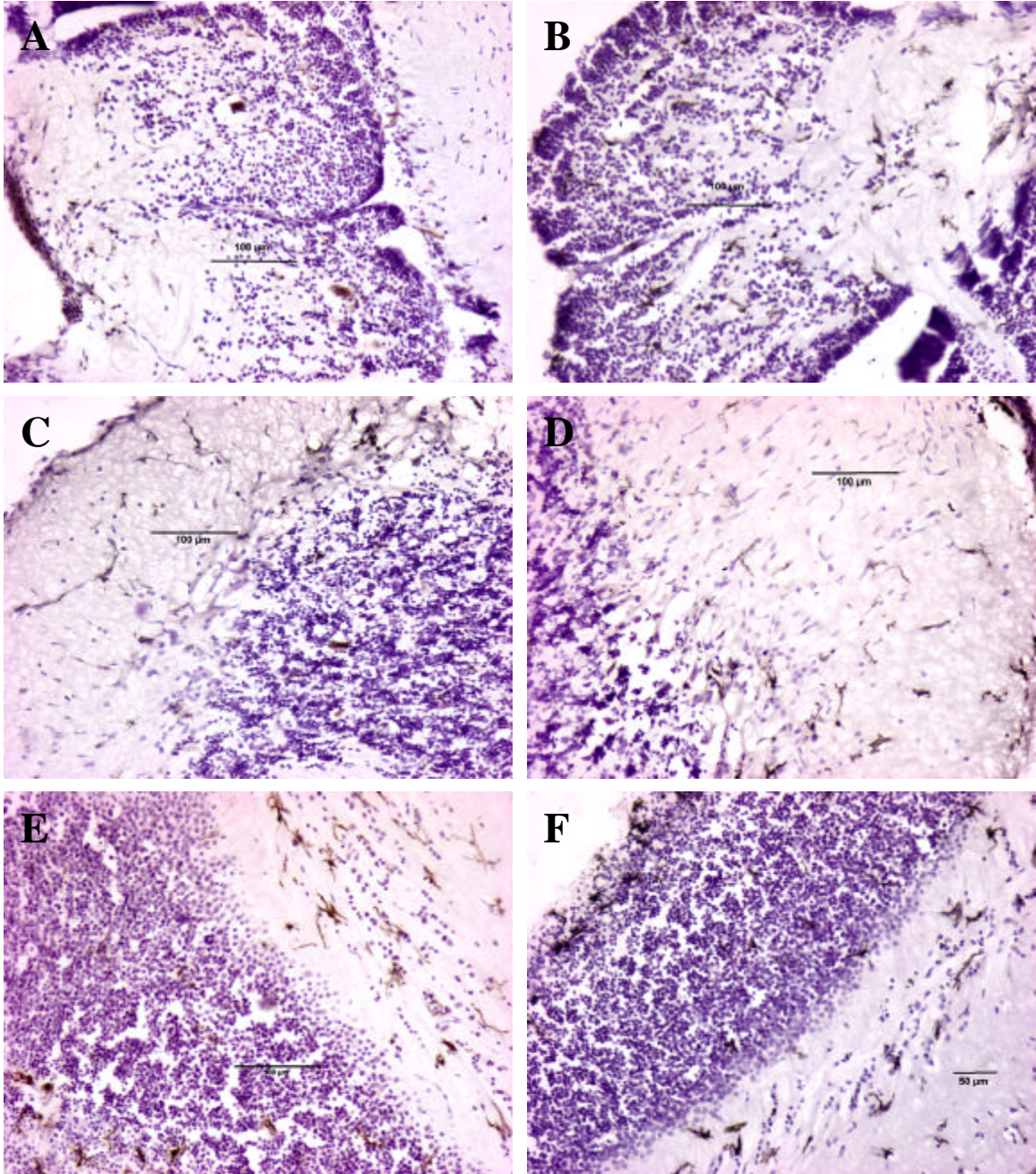
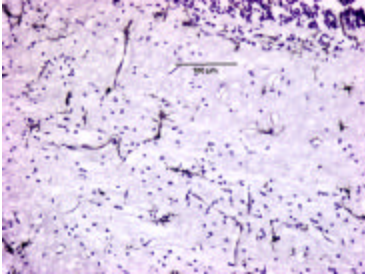
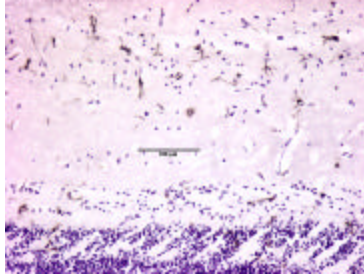


Fig.9. CYP1A immunoreactivity (brown/black) in the tectum mesencephali of the control and β -naphthoflavone (BNF)-treated lake trout. Hemorrhage depressed the immunoreactivity of CYP1A in BNF-treated juvenile lake trout (BNF-H). Horizontal sections (20- μ m thick) are counterstained with hematoxylin nuclear stain (purple).

(A) Control



(B) BNF



(C) BNF-H

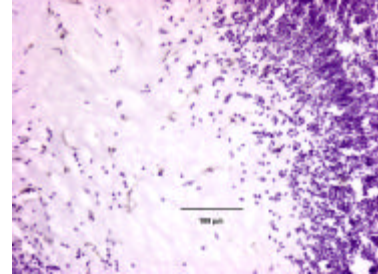
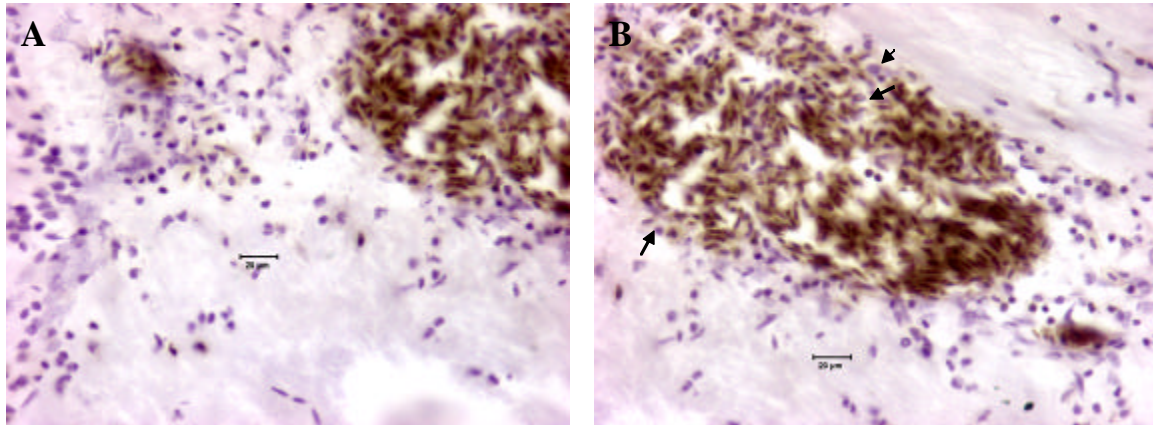


Fig.10. Multifocal hemorrhage (brown) in the midbrain of the β -naphthoflavone-treated juvenile lake trout. Note that some blood cells have infiltrated into tissues. Black arrows indicated multinucleated white blood cells. Horizontal sections (20- μ m thick) are counterstained with hematoxylin nuclear stain (purple).



Discussion

Juvenile lake trout respond to BNF exposure by induction of CYP1A expression. Increased CYP1A mRNA in the brain of BNF-exposed fish was confirmed by Q-RT-PCR and *in situ* hybridization. The response is rapid (increased within hours) and distributed universally in the brain for days. On the other hand, the induction of CYP1A protein in the brain is area-specific (in olfactory bulb and valvula cerebelli) and not as robust as the induction of CYP1A mRNA.

Constitutive expression of CYP1A in neurons, endothelial and glial cells was observed in the brain of control juvenile lake trout, which was consistent with the immunohistochemical studies by Smolowitz et al. (1991) and Stegeman et al. (1991) in that CYP1A was localized at vascular endothelia of fish brain. Other studies also provided the evidence that cerebral CYP1A was not restricted to the endothelia but was also localized in neuronal tissue (Reinecke & Segner, 1998, Sarasquete et al., 1999). It is interesting that most of the glial cells that showed CYP1A immunoreactivity were attached to the blood vessels. It is likely that these glial cells can absorb xenobiotic chemicals from the blood vessels and they constitutively expressed CYP1A protein to serve a protective function by eliminating xenobiotics from the central nervous system.

CYP1A immunoreactivity in specific brain areas such as the olfactory bulb is induced by BNF. The peripheral olfactory system was readily exposed to a wide variety of xenobiotic compounds since the olfactory receptor neurons, via their apical dendrites, were directly and continuously in contact with the external environment. In addition, these neurons whose axons terminated in the olfactory bulb, provided a direct access to the central nervous system for certain toxicants (Gottofrey & Tjalve, 1991, Hastings & Evans, 1991). The presence of constitutive CYP1A1 in the trout olfactory system has been confirmed (Andersson & Goksøyr, 1994; Monod et al., 1994, 1995; Ortiz-Delgado et al., 2002). CYP1A1 immunoreactivity has also been detected in the olfactory organ of topminnows (*Poeciliopsis sp.*) exposed to waterborne benzo[a]pyrene (Smolowitz et al., 1992). Ethoxyresorufin *O*-deethylation (EROD) induction level, an indicator of CYP1A activity, is higher in the trout olfactory organ than in the liver after a 4-day exposure to waterborne BNF (Goksøyr & Förlin, 1992). BNF induced more CYP1A immunoreactivity in the olfactory bulb of juvenile lake trout and was consistent with those previous reports.

It is surprising that some BNF-treated juvenile lake trout showed depressed CYP1A immunoreactivity in the brain compared to the control fish. In these fish we found sporadic hemorrhage sites. It is known that BNF mimics the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in altering local brain circulation (Dong et al., 2002). TCDD has been shown to cause zebrafish and lake trout embryo multifocal hemorrhages (Andreasen et al., 2002; Spitsbergen et al., 1991; Toomy et al., 2001). It is likely that 4-day BNF treatment caused multifocal hemorrhages in some juvenile lake trout. However, these pathological effects varied among individuals.

Hemorrhage may depress BNF-induced CYP1A expression. Local hemorrhages in the brain may induce the inflammatory response (Perry et al., 1993, Rothwell et al., 1996) in which glial cells, specifically astrocytes and microglia, become activated and stimulate the acute phase response. This process is characterized, in part, by the release of immune mediators including cytokines, prostanoid products, and nitric oxide (Rivest et al., 2000), which can produce a large number of responses involving the autonomic,

endocrine, and behavioral systems (Rothwell et al., 1996). The activation of host defense mechanisms down-regulates microsomal CYP expression in cell culture, humans and animals (Nicholson & Renton, 2001; Paton & Renton, 1998; Renton et al., 1999; Renton & Nicholson, 2000; Shimamoto et al., 1998; Stanley et al., 1991). Most cytokines that are found to decrease basal CYP expression can counteract *Ah* receptor-mediated induction of CYP1A protein and its associated EROD activity. In this report, CYP1A protein immunocytochemistry was depressed in the BNF-treated fish which developed hemorrhages. It is likely that CYP1A depression in these fish is due to hemorrhage-induced immunodepression.

The mechanism to depress CYP1A protein apparently requires protein synthesis since treatment with cycloheximide in combination with *Ah*-receptor agonist led to superinduction of CYP1A mRNA (Abdel-Razzak et al., 1994). In this report the staining for CYP1A mRNA in situ hybridization was increased while CYP1A protein immunocytochemistry was depressed in BNF-treated fish showing hemorrhage in the brain. This supports the notion that immunodepression of CYP1A protein requires protein synthesis.

To summarize, BNF induced CYP1A mRNA expression in the brain of juvenile lake trout. The induction of CYP1A protein by BNF in the brain is less robust and area specific since constitutive expression of CYP1A protein has been observed in juvenile lake trout. BNF may induce hemorrhage in some individuals and may cause the immunodepression of CYP1A protein in the brain.

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