MOLECULAR RESPONSE OF ATLANTIC COD'S (*Gadus morhua L.*) CYP1A, PROLACTIN AND ZONA RADIATA GENES UPON EXPOSURE TO POLYCHLORINATED BIPHENYLS

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ABSTRACT
A molecular response study was conducted to determine whether Polychlorinated Biphenyl or PCB (Clophen A40) had an effect on the induction of the zona radiata, prolactin and cytochrome P4501A (CYP1A) mRNAs in matured Atlantic cod (Gadus morhua). A total of 127 fishes were maintained under natural photoperiod in tanks and fed with wet pellets containing Clophen A40. RNA samples were taken from the gonads, pituitary and the liver from day 0, 48 and 105 after sacrificing the fish and stored at -20^{O}C prior to analysis. Analysis of gene induction by Northern hybridization assay showed the induction of prolactin and CYP1A in the pituitary and the liver tissues respectively after PCB treatment. The induction was found to be sex, age and seasonal specific. Males had greater gene expression than the females. There was however, no observed differences between the controlled and exposed fishes in terms of zona radiata gene expression. Although the picture is far from complete, the findings demonstrate the potential of using gene induction as a biomarker of aquatic pollution.

Keywords: *Cytochrome P4501A, Biomarker, Gene expression*

INTRODUCTION

As industrial and human waste increase with the growth of new factories and population, the aquatic environment is increasingly being used as 'humanity's wastebin'. In recent years, some fish species have been the focus of a number of biomonitoring studies assessing the impact of environmentally borne xenobiotics (Courtenay *et al*., 1993; Courtenay *et al*., 1994). In many of these studies, induction of CYP1A mRNA was the indicator of exposure to contaminants. The level of polyaromatic hydrocarbons (PAHs) in the sediments has correlated well with increased prevalence of several categories of toxicopathic hepatic lesions (Myers *et al*., 1994).

Biotransformation or metabolism of PCBs and other related compounds are considered to be a detoxification process and the enzyme systems involved regarded as detoxifying enzymes. One of these enzymes is the cytochrome P450 mono-

oxygenase system. Nebert *et al.*, (1989) reported cytochrome P450 induction in fish exposed to low levels of persistent environmental pollutants. The biochemistry and molecular biology of these enzymes have attracted the attention of many investigators, who seek to understand and control the processes of xenobiotic transformation and its effects. The application of biochemical and molecular biological methods and probes is changing our understanding of mechanisms in aquatic toxicology (Stegeman and Hahn, 1994).

In studying the effects of environmental pollution, the most relevant subfamily of cytochrome P450 enzymes appear to be that of CYP1A genes, which is a monooxygenase induced by specific types of foreign chemicals like planar PCBs, planar PAHs, chlorinated dioxins and furans. Induction of CYP1A is therefore widely used as a biomarker for such type of pollution in fish (Stegeman and Hann, 1994; Goksøyr,1995). Induction of CYP1A gene and other genes like prolactin and zona radiata are generally measured at one of three levels: by catalytic activity of the enzyme, using primarily ethoxyresolufin-O-deethylase assays (Upshall, *et al*., 1993; Martel, *et al.*, 1994), cytochrome P4501A (CYP1A) protein concentration (Collier *et al*.,1992; Goksøyr and Husøy, 1992), or by mRNA that encodes for the protein.

The use of CYP1A induction as an environmental indicator requires characterization of several factors which can increase variability. Variations in CYP1A expression with temperature, age, sex and season have been observed in fish (Goksøyr and Forlin, 1992). Other factors like species differences, diet, photoperiod, pressure, salinity may also affect variation in expression but studies on these factors are virtually nonexistent, and therefore make their impact on background variabilities difficult to evaluate.

Interpretation of field data collected in biomonitoring studies should include laboratory experimentation with pure chemicals to calibrate the system and better interprete environmental responses. Laboratory experimentation is necessary to determine which contaminants induce gene expression, to be able to determine the dose-response relationship of inducing contaminants, and also to characterize the dynamics of the response with respect to time. The study detected and assessed the molecular response of CYP1A, prolactin and the zona radiata genes to PCB treatment and to possibly use the result as a biomarker of aquatic pollution.

MATERIALS AND METHODS

Animals and Tissues Preparation

A total of 127 juvenile Atlantic cod (*Gadus morhua* L.), approximately 1+ years old and body weight of 100- 200g were purchased from Sævareid Smolt Producer A/S in Fusa County (near Bergen, Norway). They were brought to the Industrial Laboratory (ILAB) at the High Technology Center in Bergen (HIB). They were held in tanks and maintained under natural photoperiod (Bergen, 60^{o}N) with continuously running sea water (salinity 35‰) at a constant temperature of 10^{o}C and fed with wet pellets of wheatbran containing Clophen A40 which is a technical PCB mixture.

Total RNA Isolation

Depending on sex and the day of administration of PCB, RNA samples were extracted from the gonad, pituitary gland and the liver on day 0, 48 and 105 and homogenized in TRIzol. This was thawn at room temperature and the supernatant (containing the RNA) collected after 15 min of centrifugation (15000g/min) at 4^{o}C. It was then precipitated with isopropanol (0.5 ml of isopropanol per 1 ml of TRIzol), washed with 70% and 96% ethanol and dissolved in 0.4 ml of 0.5% SDS and then stored at -20^{o}C. The quantification of the RNA was done by measuring the OD (A260nm/A280nm) of diluted aliquot (1:40) of RNA with GeneQuant II (Pharmacia Biotech).

Plasmid construction and Polymerase Chain Reaction

The cDNA used for the PCR amplification and

as probes for the CYP1A were kindly supplied by Dr. D. Nebert and Dr. J.E. Jones (LPD/ NICHD, NIH, Bethesda, Maryland, USA). This oligonucleotide contains most of the CYP1A1 gene sequence from rainbow trout and was inserted into multiple cloning site of pUC 18. PCR was used to amplify a CYP1A1 fragment from base pairs 661 to 1433 using primers P450 I (5'-GGCTTGGTGAACATGAGT-3') and P450 II (5'-GCCAAGAAGAGGAAGACC-3'). The amplified fragment was performed on 10ng cDNA, plasmids products in a 100-µl volume containing 1X Taq buffer (10 mM Tris/HCl, pH 8.3, at 20°C, 1.5 mM MgCl2, 50mM KCl, 0.1% Triton X-100), 50µM each dNTP, 0.2 µM primer, 2.5U *Taq* polymerase and run for 25-30 cycles (94°C, 55°C and 72°C each for 1 min) using QIAquick purification kit (Qiagen). The Atlantic salmon prolactin cDNA fragment (isolated from a pituitary cDNA library) of about 700 base pairs was obtained from S.A.M. Martin (Belfast, Northern Ireland) and was cloned into pBluescript between EcoR I and BamH I sites. The zona radiata cDNA was obtained from Professor R. Male (MBI, UiB, Bergen, Norway) and was cloned into a pGEM7zf(+) (Promega) vector between the Bst XI and Xba I sites. Digestion by these two restriction enzymes yielded two fragments of about 800 bp and 700 bp. Comparing these two fragments with the nucleotide sequence (Data bank; Accession number AJ000665), the 700 bp band was shown to contained the 3' end of the zona radiata transcript (designated as DOOB insert) and was therefore used as the probe.

RNA analysis and Northern blotting

Total RNA from the tissues were resolved on a 1% denaturation agarose ethidium bromide-stained gels containing 2% formaldehyde and transferred to nylon membranes (MSI) by capillary blots overnight in 20X NaCl/P_i (Sambrook *et al*., 1989). The RNA were fixed to the membranes by baking at 80°C under a UV illuminator (TF-35M/312nm-8000 mW; Vilber Lourmat) for 1 min and stored at 4°C in a non-electrostatic plastic foil. The sizes were estimated from the migration of an RNA marker (Boehringer Mannheim).

Labelling of probes and hybridization

The membrane was prehybridized overnight in a Hybaid rolling oven at 42°C in a prehybridization solution containing 2 X SSPE (1 X SSPE is 180 mmol NaCl/l, 10 mmol sodium phosphate/l and 1 mmol EDTA/l, pH 7.7), 5 X Denhardt's solution, 0.5% (w/v) SDS and calf thymus DNA (20 mg/l). Hybridization was carried out 42°C overnight in a Hybaid rolling oven overnight by adding the probe, to the prehybridization solution. The oligonucleotides were labeled at the 5' end using 10µCi/µl (3000 Ci/mmol) of [α-^{32}P] dCTP (Amersham International plc.) and 0.5µl (= 2.5u) of Klenow fragment (Promega). All radiolabeled probes were purified by chromatography using a Sephadex G-25 column (Pharmacia). The blots were washed twice for 15 min in 2 X SSPE/0.1% SDS and then 0.5 X 0.1% SDS 42°C and 55°C respectively. The membranes were then exposed to Kodak X-ray film (2 sides useable).

RESULTS AND DISCUSSION

Isolation of probe (insert) from plasmids

Three different cDNAs were used as probes in this study. Digestion of the zona radiata protein plasmid with Bst XI and Xba I yielded a 700bp (Fig.1) and a 800bp (results not shown).

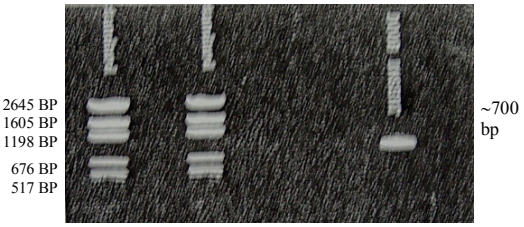


Fig. 1: Agarose gel electrophoresis of the purified fragment of the zona radiata cDNA, represented as DOOB insert with pGEM DNA marker as a standard

Nucleotide sequences comparison (Data bank Accession number AJ000665) of the two bands revealed that the 700bp contained the 3' end of the zona radiata transcript designated as DOOB insert with pGEM DNA marker as a standard. The prolactin cDNA fragment of about 700bp was cloned into pBluescript between EcoR I and BamH I and endonuclease restriction cutting using these two enzymes yielded two bands of 2150 bp and 700 bp. Polymerase Chain Reaction was used to amplify a CYP1A1 fragment cloned into an unknown site of pUC18 from base pair 661 to 1433 using primers P450 I and P450 II as described in the methods. This yielded a 772 bp fragment which represents the 5' end of the trout CYP1A1 and contained a conserved part of the CYP sequence (Fig. 2).

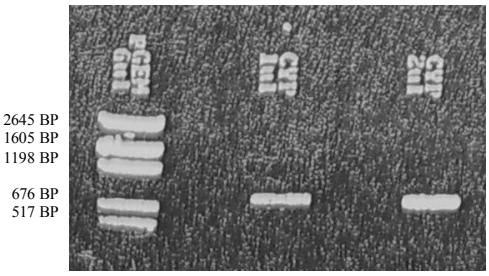


Fig. 2: Agarose gel electrophoresis of the PCR amplified CYP1A cDNA probe using P450I and P450II as primers with pGEM DNA marker as a standard

RNA samples

The quality of the RNAs were assessed by examining the gel under UV-light (Table 1and Fig. 3) and evaluated from the appearance of the ribosomal 28S and 18S RNA bands. A third lower band beneath the 18S RNA band was characteristic of all the female gonadal RNA samples. The intensity of the lower bands decreased with the time of collection.

Recent reports suggest that teleostean eggshell protein are of extra-ovarian origin (Oppen-Bernsten *et al*., 1992). The zona radiata (Zrp) cDNA probe was used to Northern hybridize the RNA samples from the gonads, pituitary and the liver. Of the over 80 RNA samples (both males and females and PCB treated and untreated) subjected to Northern hybridization assay, the probe could not detect any zona radiata protein transcript in any of the samples.

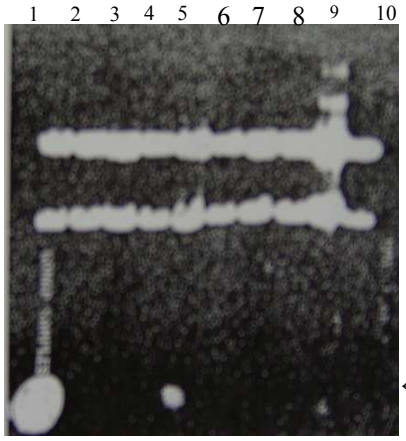
The prolactin (PRL) cDNA probe was able to detect and recognize the prolactin transcript in some pituitary (both males and females and PCB treated and untreated) RNA samples (Table 2).

There was however, no expression or recognition of prolactin transcript in either the liver or the gonads RNA samples. The rainbow trout CYP1A cDNA was also able to detect the cod CYP1A transcript in 20 liver samples (both males and

Table 1: Quantitative results of the intensity of the third gonadal band observed on the gel

Times/day	**0**	**48**		**105**	
PCB	Untreated	Untreated	Treated	Untreated	Treated
Presence of third band	Yes	Yes	Yes	Yes	Yes
Intensity of third band	******	****	***	**	*

****** very intense and very strong third band **** strong third band
*** weak third band ** very weak third band * very faint third band



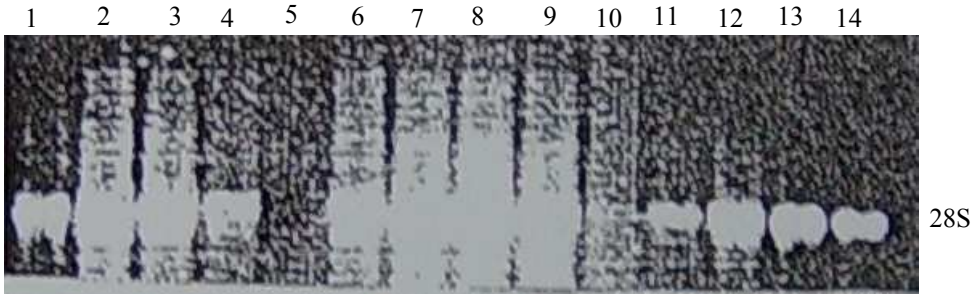
Approximately 20µg of total RNA were electrophoresed in each lane of a 1% agarose/formaldehyde gel.
Lanes 1 and 2 are female and male control fish collected on day 0
Lanes 3 and 4 are female and male untreated fish collected on day 48
Lanes 5 and 6 are female and male treated fish collected on day 48
Lanes 7 and 8 are female and male untreated fish collected on day 105
Lanes 9 and 10 are female and male treated fish collected on day 105

Fig. 3: Electrophoresis of gonad RNA samples from PCB treated and untreated cods.

females and PCB treated and untreated) (Table 3). Using this same probe to Northern hybrididize the gonads and the pituitary RNA samples, there was no detectable signal with the probe even after 10 days of film incubation.

The Northern blotting assay in this study has shown that PCB apparently influenced the expression of CYP1A and prolactin genes in the liver and the pituitary respectively. There were no observed expression of the zona radiata gene in any of the selected organs. Since PCBs are complex mixtures, their biological and molecular effects might be expected to represent an integrated response to individual components of these mixtures (Wolff and Toniolo, 1995). In their function, some PCBs can exhibit estrogenic and antiestrogenic activity for congeners with specific chlorine substitution pattern (Krishnan and Safe, 1993).

In the electrophoretical separation of the gonadal RNA samples, an unusual third lower band (PCB treated and untreated) beneath the 18S ribosomal



Approximately 20µg of total RNA were loaded per well of a 1% agarose/formaldehyde gel.
Lanes 1 and 2 are male and female control fish collected on day 0
Lanes 3 and 4 are female (F_1 and F_2) untreated fish collected on day 48
Lanes 5 and 6 are male (M_1 and M_2) untreated fish collected on day 48
Lanes 7 and 8 are female (F_3 and F_4) treated fish collected on day 48
Lanes 9 and 10 are male (M_3 and M_4) treated fish also collected on day 48
Lanes 11 and 12 are female (F_5) and male (M_5) untreated fish collected on day 105
Lanes 13 and 14 are female (F_6) and male (M_6) treated fish also collected on day 105

Fig. 4: Pituitary RNA electrophoresis from PCB treated and untreated fish.

Table 2: Response of Treated and Untreated Fish with the Prolactin probe using the Northern Blot Assay on pituitary samples.

Day	Sex	PCB Untreated Samples	PCB Treated Samples
0	F (control)	x	x
	M (control)	x	x
48	F_1	x	-
	F_2	x	-
	M_1	x	-
	M_2	x	-
	F_3	-	+
	F_4	-	+
	M_3	-	+
	M_4	-	++
105	F_5	x	-
	M_5	+	-
	F_6	-	++
	M_6	-	++++

x no expression; + very weak expression; ++ weak expression; +++ strong expression; +++++ very strong expression

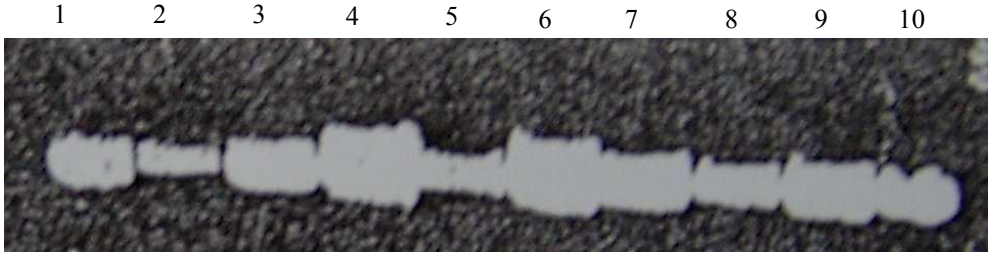


Fig. 5: Liver RNA electrophoresis from PCB treated and untreated fish

were observed in all the females (Fig. 3 and Table 1). The intensity of these bands decreased with the times of sample collection. The control sample produced a very strong and intense band. There was however, a gradual decrease in the band intensity in the samples collected on day 48 and 105. This decreased in band intensity was probably not from the PCB induction but perhaps from an unknown endogenous substance which when acted in combination with the PCB resulted in the decrease (Addo, 1998). Another possible explanation is that, the band may be a processed or unprocessed 5S ribosomal RNA in the gonads.

Although the samples were taken during gonadal maturation and Zrp was expected to be produced, there was no transcript recognition in any of the samples. This may be due in part to the fact that, the Zrp expression was very weak to the extend that detection by this assay was impossible. Similar study undertaken by Arukwe (1998) using Western blot assay on some of these samples

Table 3. Response of Treated and Untreated Fish with the CYP1A specific probe using the Northern blot assay on liver samples

Day	Sex	PCB Untreated Samples	PCB Treated Samples
0	F (control)	x	x
	M (control)	x	x
48	F_1	++	-
	M_1	++	-
	F_2	-	+++
	M_2	-	++++++
105	F_3	+	-
	M_3	++	-
	F_4	-	+++
	M_4	-	++++

x no expression; + very weak expression; ++ weak expression;
+++ strong expression; +++++ very strong expression

indicated a very weak Zrp expression in the liver. Immunochemical studies also support the fact that teleostan Zrp has three major proteins α, β and χ (Oppen-Berntsen *et al*., 1990) and therefore the salmon eggshell protein can probably cross-react with the cod Zrp to compare their similarities if the latter is expressed. The Clophen A40 may have some induction effects on some parameters other than the gonads, the pituitary and the liver.

In general, males had relatively stronger prolactin (PRL) gene expression than the females (Table 2). In all cases, the untreated samples showed no response or very weak gene expression. The PCB treated fish showed varying degree of expression. Both the males and females on day 48 indicated very weak band. Male samples collected on day 105 (Table 2, M_6) showed very strong expression due to possible varying amount of RNA on the gel. The PCB treated female sample on day 105 also showed some gene expression (Table 2, F_6). The reason for this pattern of expression in the treated samples is not known but it may be due to the fact that, the application of the chemical might have inhibited the expression in the females whilst enhancing that in the males. It is also not possible to make any definite conclusion with this number of samples, even though the treated male fish apparently seemed more sensitive than their female counterparts to PCB exposure as they mature from day 0 to 105.

In the CYP1A hybridization, the untreated samples collected on day 48 indicated a weak CYP1A gene expression whilst those collected on day 105 gave a contrasting CYP1A expression with the males being relatively strong. The male treated liver samples collected on day 48 showed a greater CYP1A mRNA levels than the female samples (Table 3, F_2 and M_2). This increase from day 0 to 48 was probably due to the accumulation of CYP1A mRNA following the addition of PCB. There was however, a decline in the CYP1A mRNA levels in the PCB treated samples collected on day 105 (Table 3, F_4 and M_4). This may be due to the fact that, in addition to transcriptional activation, post-transcriptional mechanisms such as stabilization of mRNA can regulate the expression of P450.

The study revealed that, fish administered with PCB had stronger CYP1A mRNA levels than those that were not treated with PCB, and males had higher levels than females. Williams *et al*.,

(1997) injected tomcods (*Microgadus tomcod*) with PCB and in all cases there were higher CYP1A mRNA expression in the males than in the females. Other studies also showed that seasonal changes and sexual factors affect the level of CYP1A systems under natural conditions (Larsen *et al*., 1992; Andersson *et al*., 1990). Perhaps, the main contributing factor for this observation was the timing of the experiment which coincided with the onset of spawning in the cods (October – December). Similar differences between males and females tested during the spawning period have been demonstrated many times with other species, including Atlantic salmon (Goksøyr and Larsen, 1991). The mechanism for the difference is not clear, but the hormone estradiol appears to inhibit induction in the females (Elskus *et al*., 1992). The CYP1A probe could not recognize any transcript in both the gonad and the pituitary for both the treated and untreated fish. Immunological studies have shown that, apart from the liver, CYP1A is also located in vascular endothelial as well as epithelial cells of gills and kidneys.

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