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EXPRESSION OF CYP1A IN THE HEPATOPANCREAS OF *MERLUCCIUS MERLUCCIUS*, *TRIGLA LUCERNA*, AND *LIZA RAMADA* (PISCES) IN THE WIDER VICINITY OF BAR HARBOR MONTENEGRO

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Abstract - The expression of CYP1A, a biomarker for the presence of xenobiotic compounds, was examined in three fish species from the wider vicinity of Bar harbor in winter and spring. Induction of CYP1A was observed in winter and increased further in spring. Several PCBs were found in seawater in winter. They decreased below the limit of detection in spring, when the PAH fluorene was detected. It is concluded that the constant presence of CYP1A expression is probably due to pollutants in the environment, whereas increased expression of CYP1A in spring results from exposure of the fish to fluorene.

Key words: CYP1A, PAHs, PCBs, fish, Montenegro

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INTRODUCTION

Cytochrome P450 isozymes are responsible for the biotransformation of xenobiotic compounds such as PAHs and PCBs. In fish, they are represented by the CYP1A subfamily of proteins (G o s k ø y r and F ö r l i n, 1992; S t e g e m a n and H a h n, 1994). The presence of CYP1A is a well-established *in vivo* biomarker of exposure of fish to xenobiotic compounds. Induction of CYP1A occurs through ligand binding of xenobiotic compounds to a cytoplasmic arylhydrocarbon receptor (AhR) (V a n d e r O o s t *et al.*, 2003). Ligand binding activates the receptor, which leads to dissociation of heat-shock and immunophilin-related proteins. Upon heterodimerization with the arylhydrocarbon nuclear translocator, nuclear translocation of the complex proceeds. This is followed by its specific binding to the xenobiotic-response element on the DNA upstream from the CYP1A gene promoter and upregulation of gene transcription, which leads to elevated mRNA and protein levels and increased CYP1A catalytic activity (Fig. 1).

Several approaches exist for the determination of CYP1A induction: measurement of CYP1A mRNA by Northern analysis, evaluation of increased protein levels by Western blotting, ELISA, the use of histochemical techniques, and catalytic measurements of either ethoxyresorufin-O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH) activities (B u c h e i l and F e n t, 1995). Generally, there is good correlation between CYP1A mRNA, protein levels, and EROD activity.

In this work, relative changes of CYP1A concentrations in the hepatopancreas of common hake (*Merluccius merluccius*), tub gurnard (*Trigla lucerna*) and thin-lipped gray mullet (*Liza ramada*) caught near Bar (Montenegro) were examined by immunoblot analysis. This locality is characterized by moderate anthropogenic influence and industrial activity, and all of the examined fish species are of considerable commercial importance. Our aim was to characterize the possible ecological impact of chemical contaminants in the light of their toxicological potential. The examination of CYP1A expression described here is the first of its kind to be carried out in Serbia and Montenegro.

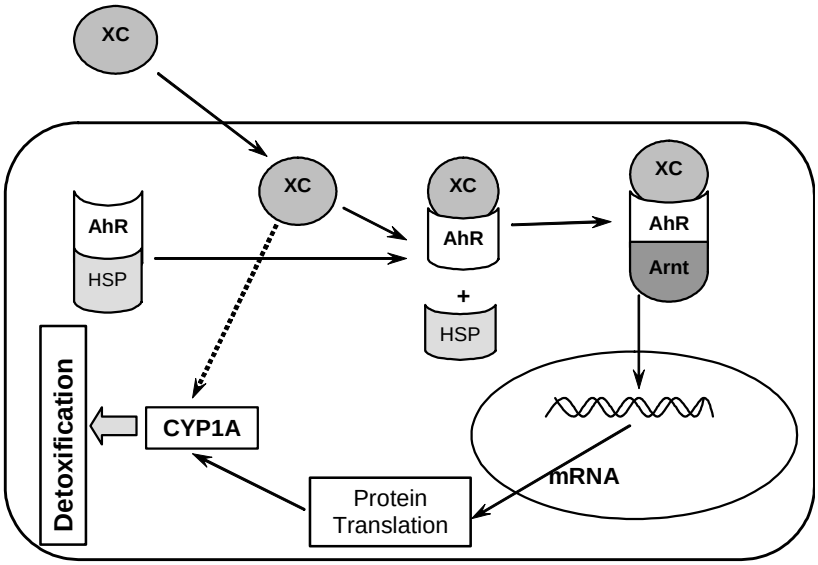

Fig. 1. Schematic illustration of CYP1A induction.
XC-xenobiotic compounds, AhR-aryl hydrocarbon receptor, Arnt-aryl hydrocarbon nuclear translocator, HSP-heat shock protein.

MATERIALS AND METHODS

Animals

Specimens of *Merluccius merluccius, Trigla lucerna* and *Liza ramada* were collected by trawling in the wider vicinity of Bar harbor (UTM CM 3.6) as shown in Fig. 2. The investigations took place in winter (25th February) and spring (25th May). At least seven (and up to nine) individual fish of each species during one season were pooled. The fish were killed immediately by spinosectomy according to standard animal care regulations. The hepatopancreas was quickly removed, washed in ice-cold 0.15 M NaCl and frozen in liquid nitrogen. Individuals of the same size were selected to ensure uniformity of samples.

Isolation of the microsomal fraction

The microsomal fraction of the hepatopancreas was prepared following the procedure of K r a u s s *et al.* (1983). Tissues were excised and homogenized (1g liver/1ml) in STM buffer: 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 1 mM PMSF) and pelleted at 10,000 g and 4°C for 25 min. The post-mitochondrial supernatant was then centrifuged at 150,000 g and 4°C for 60 min. The obtained microsomal pellets were resuspended in STM buffer.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) microsomal proteins (20 μg) were loaded onto 4% stacking/12% separating slab gels as described by L a e m m l i (1970). The gels were stained using Coomassie Brilliant Blue R-250. Proteins separated by SDS-PAGE were electroblotted onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). Immunoblot analysis was performed according to T o w b i n *et al.* (1979) using a polyclonal antibody to fish CYP1A (CP226, Biosense Laboratories, Norway). Immunoreactive bands were identified with the aid of an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. Antigen-antibody complexes were analyzed with Total Lab (Phoretix) electrophoresis software and changes of the relative concentrations of CYP1A in different samples were compared. Protein concentrations were determined according to L o w r y *et al.* (1951).

Determination of PCBs and PAHs in water

Concentrations of PCBs were determined by gas chromatography (GC) with an ECD detector and linear programmable temperature vaporizer (PTV) injector. Those of PAHs concentrations were determined by gas

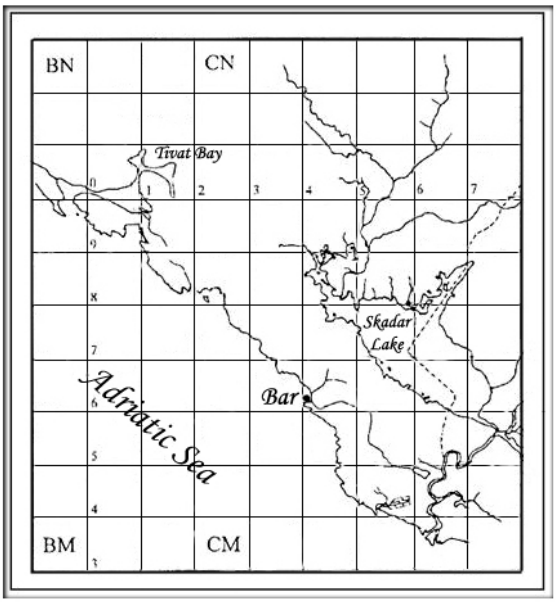

Fig. 2. Map of the Adriatic coast of Montenegro. The location in the wider vicinity of Bar harbor (UTM CM 3.6) where the sea water samples were collected and the fish caught is indicated.

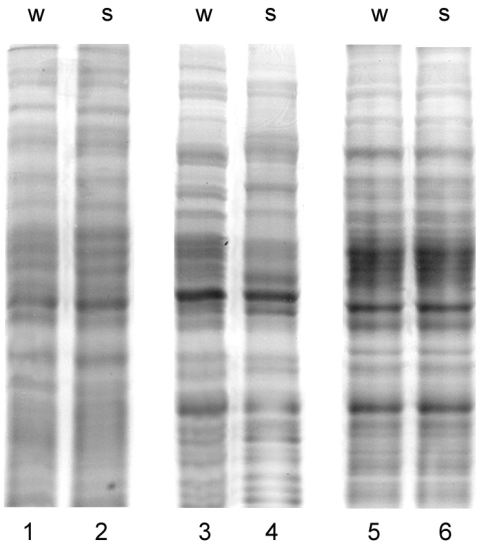

Fig. 3. Electrophoretic profiles of microsomal fraction proteins prepared from the hepatopancreas of *Merluccius merluccius* (lanes 1 and 2), *Trigla lucerna* (lanes 3 and 4), and *Liza ramada* (lanes 5 and 6); w-winter, s-spring. Proteins (20 μg) were subjected to 12% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250.

chromatography (GC) with a FID detector and a linear programmable temperature vaporizer (PTV) injector. The absence of individual peaks was reported not as zero, but as less than the detection limit. Seawater was always sampled from the greatest possible depth (60 m).

RESULTS AND DISCUSSION

Whereas heavy pollution causes the sudden death of large numbers of fish, exposure to sublethal levels of pollutants has to be estimated by measurements of specific biochemical, physiological, or histological responses of fish (M o n d o n *et al*., 2001). Although chemical analyses of the environment and tissues provides information about the presence of specific xenobiotic compounds, they are not particularly indicative of the concentrations to which the animals were exposed and cannot serve as bioaccumulation markers for exposure assessment. For example, in field studies the highest PAH tissue levels are frequently observed in fish from sites with the lowest

Merluccius merlucius		*Trigla lucerna* L.		*Liza ramada*	
winter	spring	winter	spring	winter	spring
29.524	**33.297**	**20.612**	**22.224**	**66.791**	**97.889**

Fig. 4. Immunoblot analysis with anti-CYP1A antibody (A) and quantification of antigen-antibody complexes (B). Lanes 1 and 2 – *Merluccius merluccius*; 3 and 4 – *Trigla lucerna*; 5 and 6 – *Liza ramada*; w – winter; s – spring. Proteins (20 μg) were subjected to 12% SDS-PAGE and electroblotted to membranes. Immunoblotting was performed with a polyclonal antibody for CYP1A. Antigen-antibody complex formation was detected using an ECL detection system. Antigen-antibody complexes (changes of the relative concentrations of CYP1A), were analyzed by densitometry using Total Lab (Phoretix) electrophoresis software.

Table 1. Concentrations of PAHs (A) and PCBs (B) in seawater collected from a location in the wider vicinity of Bar harbor (UTM CM 3.6).

A

PAHs \ seasons	winter (ng/l)	spring (ng/l)
Acenaphthylene	<10	<10
Fluorene	<10	**554**
Phenantrene	<10	<10
Anthracene	<10	<10
Pyrene	<10	<10
Benz(A)anthracene	<10	<10
Chrysene	<10	<10
Benzo(B)fluoranthene	<10	<10
Benzo(K)fluoranthene	<10	<10
Benzo(A)pyrene	<10	<10
Benzoperylene	<10	<10
Indeno(1.2.3.cd)pyrene	<10	<10
Dibenzo(A)anthracene	<10	<10

B

PCBs \ seasons	winter (ng/l)	spring (ng/l)
2,4,4'-trichlorobiphenyl (pcb28)	**20**	<10
2,2',5,5'-tetrachlorobiphenyl (pcb52)	**15**	<10
2,2',4,5,5'-pentachlorobiphenyl (pcb101)	<10	<10
2,2',3,4,4',5'-heksachlorobiphenyl (pcb138)	<10	<10
2,2',4,4',5,5'-heksachlorobiphenyl (pcb153)	**15**	<10
2,2',3,4,4',5,5'-heptachlorobiphenyl (pcb180)	<10	<10

PAH sediment levels, probably because low levels of induction in fish from sites with low contamination limit PAH metabolism, whereas increased metabolic clearance occurs in fish from polluted sites due to greater induction of enzymatic activity (Van der Oost *et al*., 1997). A highly sensitive *in vivo* biomarker of exposure to PAHs and PCBs in a wide variety of different fish species is represented by the inducible hepatic enzyme CYP1A. The induction of CYP1A is an early-warning signal that reflects adverse biological responses to environmental toxins (Buchelli and Fent, 1995). The given enzyme is an important sensitive biomarker because observable response-inducing concentrations of xenobiotics are usually too low to be detected by other methods (Machala *et al*., 2000). In this work, we compared changes of CYP1A levels with seasonal fluctuations of xenobiotic concentrations in the environment.

Proteins prepared from the hepatopancreatic microsomal fraction were separated by SDS-PAGE and stained with Coomassie Blue. Whereas interspecies differences in protein profiles were established (Fig. 3, lanes 1 and 2, 3 and 4, and 5 and 6), no qualitative or quantitative intraspecies or seasonal variations in protein profiles were observed (Fig. 3). Following Western analysis with a polyclonal antibody to CYP1A (Fig. 4A) and quantification of antigen-antibody complexes (Fig. 4B), species-specific levels of CYP1A expression were observed in all three species in winter. In spring, the relative concentrations of CYP1A increased in a species-specific manner. In *Merluccius merluccius*, *Trigla lucerna*, and *Liza ramada*, the relative CYP1A concentration increased by about 13%, 8%, and 47%, respectively (Fig. 4A, lanes 2, 4, and 6).

Chemical analyses of PAH and PCB content in seawater are shown in Table 1. The presence of PAHs was observed only in spring, when the concentration of fluorene was 0.554 μg/l (Table 1A). Compared to the values reported in the criteria for PAH concentration in marine water for Canada, this concentration was below the recommended maximum concentration (12 μg/I) (Nagpal, 1993). In winter, the concentrations of PAHs were below the limit of detection, as established using a GC column (<0.01 μg/l), whereas the concentrations of several PCBs were increased. The concentrations of pcb28, pcb52 and pcb153 were 20, 15, and 15 ng/l, respectively (Table 1B). In light of the recommended maximal total PCB concentration of 0.1 ng/l (Nagpal, 1992), the concentrations of PCBs in the wider vicinity of Bar harbor could be taken as significant contamination of the seawater. However, compared to the minimal concentration of PCBs of 42 ng/L reported to exert a negative impact on marine organisms as stated in the Environmental Quality Standards for the Mediterranean Sea in Israel (2002), these concentrations remained at an acceptable level.

The apparently elevated constitutive levels of CYP1A that were observed in fish caught in winter in the wider vicinity of Bar harbor probably resulted from the presence of PCBs. The observed induction of CYP1A in spring primarily resulted from the exposure of fish to an elevated concentration of the PAH fluorene. The observed change at the molecular level represents the first signal demonstrating that pollutants have entered the organisms, been distributed among their tissues, and elicited toxic effects on critical targets. However, our findings do not necessarily imply deleterious effects, as links between the levels of exposure, the degree of tissue contamination, and early adverse effects on fish need to be established further.

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ЕКСПРЕСИЈА CYP1A У ХЕПАТОПАНКРЕАСУ *MERLUCCIUS MERLUCCIUS, TRIGLA LUCERNA* И *LIZA RAMADA* (PISCES) У ШИРЕМ РЕГИОНУ ЛУКЕ БАР, ЦРНА ГОРА

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Експресија CYP1A, биомаркера на присуство ксенобиотика, посматрана је у три врсте риба уловљених са ширег локалитета барске луке током зиме и пролећа. Индукција CYP1A која је уочена у зимском периоду, додатно је увећана у пролећном. Неколико PCB који су пронађени у морској води током зиме, није детектовано у пролеће када је откривен PAH флуорен. Закључено је да је стално присуство експресије CYP1A највероватније последица присуства полутаната у животној средини; додатно увећање експресије CYP1A у пролеће последица је излагања риба флуорену.