



ORIGINAL ARTICLE

## Changes of cytochrome P4501A mRNA expression and physiology responses in the olive flounder, *Paralichthys olivaceus*, exposed to benzo[a]pyrene

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### Abstract

Benzo[a]pyrene (BaP) is generated by the incomplete combustion of organic substances such as oil and coal, and is a widespread organic environmental contaminant in terrestrial and aquatic ecosystems. To determine the effects of BaP on organisms, we investigated its time- and dose-related effects on the levels of cytochrome P4501A (P4501A) mRNA in the liver and gills of the olive flounder (*Paralichthys olivaceus*) using quantitative polymerase chain reaction (QPCR) and measured the plasma glucose, cortisol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. The full-length olive flounder P4501A cDNA consists of 1566 nucleotides and encodes a 521-amino-acid protein. In the liver and gills, the expression of P4501A mRNA was highest 6 h after exposure to both 10 and 30  $\mu\text{g l}^{-1}$  BaP, and then decreased. In addition, the plasma parameters increased with exposure. These results suggest that P4501A plays an important role in the detoxification of BaP, which stressed the olive flounder. Therefore, these physiological parameters may be indicators of BaP-induced stress responses.

**Key words:** ALT, AST, benzo[a]pyrene, cytochrome P450, olive flounder, stress response

### Introduction

Benzo[a]pyrene (BaP), a widespread organic environmental contaminant in terrestrial and aquatic ecosystems, is a representative polycyclic aromatic hydrocarbon (PAH) generated by the incomplete combustion of organic substances such as oil and coal (Mcgroddy & Farrington 1995). When absorbed by marine organisms, it accumulates in their tissues (Boleas et al. 1998) and passes through cell plasma membranes. This may cause lipoperoxidation (Livingstone 1993), resulting in carcinogenic, mutagenic, cytotoxic, teratogenic, and other adverse effects (Gelboin 1980; Borhoumi et al. 2002).

Cytochrome P450s (P450s) are the principal enzymes catalysing the oxidative metabolism of toxicants, including important environmental chemicals, and they catalyse the oxidative steps in the biosynthesis and degradation of endogenous regulatory molecules such as steroids and eicosanoids

(Nebert & Russell 2002). The P450 superfamily has many members with multiple functions, but the induction of P450s by xenobiotics is the most important characteristic of the P450 enzyme system (Andersson & Förlin 1992). Cytochrome P4501A (P4501A) metabolizes xenobiotics such as PAHs, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyl congeners (PCBs) (Van der Weiden et al. 1994; Hahn 2002). Since P4501A has a vital role as a biomarker of environmental contamination and is an enzyme that metabolizes xenobiotics (Stegeman et al. 1992; Levine & Oris 1997), it is the most studied member of the P450 superfamily.

P4501A cDNA has been cloned in many species of fish, including killifish (*Rivulus marmoratus*) (Lee et al. 2005), European sea bass (*Dicentrarchus labrax*) (Stien et al. 1998), European plaice (*Pleuronectes platessa*) (Leaver et al. 1993), and striped seabream

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(*Lithognathus mormyrus*) (Tom et al. 2002). In addition, P4501A expression has been studied in fish exposed to xenobiotics, including zebrafish (*Danio rerio*) (Jönsson et al. 2007), rainbow trout (*Oncorhynchus mykiss*) (Levine et al. 1999), and gizzard shad (*Dorosoma cepedianum*) (Levine & Oris 1997). BaP is a typical PAH that induces P4501A for its detoxification and metabolism (Van der Weiden et al. 1994). The expression of P4501A following exposure to BaP has been studied in European sea bass (Stien et al. 1998) and rainbow trout (Jönsson et al. 2006). Therefore, P4501A can be used as a bioindicator for organic chemicals (Stegeman et al. 1992; Levine & Oris 1997).

In fish, xenobiotics such as BaP are absorbed by the gills, metabolized there and in the liver and kidneys, and then excreted (Varanasi 1989). The gills are the metabolic tissue in direct contact with chemicals dissolved in water (Kolok et al. 1996), while the liver plays the main role in detoxifying organic chemicals and metabolizing hydrocarbon (Stegeman 1981; Livingstone 1993).

The olive flounder *Paralichthys olivaceus* Temminck and Schlegel, 1846, is a bottom dweller that is exposed to accumulated contaminants (Winzer et al. 2002). In this study, we isolated the full-length P4501A cDNA from the liver of olive flounder, and investigated BaP metabolism in the liver and gills by measuring P4501A expression after exposure to BaP. In addition, we measured the changes in the plasma glucose, cortisol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels to examine the physiological stress induced by BaP.

## Material and methods

### Experimental fish

Olive flounders ( $n = 50$ , length  $14.2 \pm 0.8$  cm, weight  $25.6 \pm 5.6$  g) were obtained from a commercial fish farm located in Gijang (Hwa-nam fisheries, Busan, Korea). The fish were reared for 4 weeks in 300 l circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at  $20 \pm 1^\circ\text{C}$  and a 12L/12D cycle, respectively.

### BaP exposure

The experimental fish were exposed to BaP (Supelco, Bellefonte, PA, USA) dissolved in acetone in four 50 l tanks (control, acetone control, and 10 and  $30 \mu\text{g l}^{-1}$  BaP groups) for 0, 6, 12, and 24 h. No food was supplied during the experiments.

### Sampling

Five fish from each treatment were selected randomly for blood and tissue sampling. The fish were anesthetized with  $200 \text{ mg l}^{-1}$  tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) before collecting blood from the caudal vein using a 3 ml heparin-coated syringe. The plasma samples were separated by centrifugation ( $4^\circ\text{C}$ ,  $10,000 \times g$ , 5 min) and stored at  $-80^\circ\text{C}$  until analysis. To collect liver and gill tissues, the fish were killed by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until total RNA was extracted for analysis.

### Identification of P4501A cDNA

Primers for P4501A were designed using highly conserved regions of European plaice P4501A (GenBank accession no. X73631) and gilthead seabream (*Sparus aurata*) P4501A (AF011223): P4501A forward primer (5'-TTC CAG ATC CAG ATC GGC AT-3') and P4501A reverse primer (5'-CTG CCA CTG ATT GAT GAA GAC-3'). Total RNA was extracted from the liver using a TRIzol kit (Gibco/BRL, Grand Island, NY, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using  $2 \times$  Taq Premix I (Solgent, Seoul, Korea). PCR consisted of an initial 2 min at  $95^\circ\text{C}$ , followed by 40 cycles at  $95^\circ\text{C}$  for 20 s,  $54^\circ\text{C}$  for 40 s, and  $72^\circ\text{C}$  for 1 min, with a final 7 min extension at  $72^\circ\text{C}$ . The amplified PCR product was electrophoresed in 1% agarose gels. The PCR product was purified and ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA). The transformed colonies were cultured in DH5 $\alpha$  (RBC Life Sciences, Seoul, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Seoul, Korea) and *EcoRI* (Fermentas, Hanover, MD, USA). Using the plasmid DNA, the P4501A cDNA was sequenced using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

### Rapid amplification of P4501A cDNA 3' and 5' ends (3' and 5' RACE)

For the P4501A RACE reaction, total RNA was extracted from the liver using a TRIzol kit (Gibco/BRL). Using  $3 \mu\text{g}$  of total RNA as a template, 3' RACE cDNA and 5' RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Seoul, Korea). First-strand cDNA was

synthesized using an oligo (dT) anchor primer (5'-CTG TGA ATG CTG CGA CTA CGA T(T)<sub>18</sub>-3') and a CapFishing™ adaptor (Seegene).

P4501A-specific primers were designed for the PCR product. For 3' RACE, the 50 µl PCR reaction mixture contained 5 µl of 3' RACE cDNA, 1 µl of 10 mM 3' RACE target primer (5'-CTG TGA ATG CTG CGA CTA CGA T-3'), 1 µl of 10 mM 3' RACE gene-specific primer (5'-CAG TGG CAG ATC AAC CAT GAT CCT GAG C-3'), and 25 µl of SeeAmp Taq Plus Master Mix. PCR consisted of an initial 94°C for 5 min, followed by 40 cycles at 94°C for 40 s, 62°C for 40 s, and 72°C for 1 min, with a final 5 min at 72°C.

For 5' RACE, the 50 µl PCR reaction mixture contained 5 µl of 5' RACE cDNA, 1 µl of 10 mM 5' RACE target primer (5'-GTC TAC CAG GCA TTC GCT TCA T-3'), 1 µl of 10 mM 5' RACE gene-specific primer (5'-TTG ATG AAG CGG AAG CTG TAC AGG TCA G-3'), and 25 µl of SeeAmp Taq Plus Master Mix. PCR consisted of an initial cycle at 94°C for 5 min, 35 cycles at 94°C for 40 s, 62°C for 40 s, and 72°C for 1 min, followed by 5 min at 72°C. The amplified PCR product was electrophoresed in 1% agarose gels. Transformation and sequencing were performed as described above.

#### Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of P4501A mRNA in the total RNA extracted from the BaP-exposed liver and gills. Primers for QPCR were designed from the known sequences of olive flounder and were P4501A forward primer (5'-ATG TCA GAC GAG AAG ATT GTA GG-3'), P4501A reverse primer (5'-ATT GGG TTT ATC AGA GAG AAG AGG-3'), β-actin forward primer (5'-GGA CCT GTA TGC CAA CAC TG-3'), and β-actin reverse primer (5'-TGA TCT CCT TCT GCA TCC TG-3'). PCR amplification was conducted using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR consisted of an initial cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 20 s and 55°C for 20 s. As an internal control, the experiments were duplicated with β-actin, and all data were expressed as the change relative to the calculated β-actin threshold cycle (CT) levels.

#### Plasma parameters analysis

Plasma glucose, AST, and ALT were determined using a biochemistry autoanalyser (model 7180;

Hitachi, Tokyo, Japan). Plasma cortisol was determined using a radioimmunoassay (RIA) with an RIA kit (Diagnostic System Laboratories, Webster, TX, USA).

#### Statistical analysis

All data were analysed using the SPSS statistical package (version 10.0; SPSS Inc., Cary, NC, USA). One-way ANOVA followed by Dunnett's post hoc test was used to compare the differences with control values ( $p < 0.05$ ).

## Results

#### Identification of P4501A full-length cDNA

Using total RNA, RT-PCR was used to clone a fragment of P4501A cDNA. A single PCR product of the expected size [1020 base pairs (bp)] was obtained. A PCR-based cloning strategy (PCR followed by 3' and 5' RACE) was used to clone the full-length cDNA encoding P4501A. The full-length P4501A cDNA contained 1566 nucleotides, including an open reading frame (ORF) that was predicted to encode a 521-amino-acid protein (GenBank accession no. EF451958) (Figure 1). The amino acid sequence of olive flounder P4501A was compared to those deduced from the cDNA of other teleosts (Figure 1). The amino acid similarities were as follows: 90% with European plaice P4501A (X73631), 90% with marbled flounder P4501A (AB120566), 90% with European sea bass P4501A (U78316) and 86% with gilthead seabream P4501A (AF011223) (Figure 1).

#### QPCR for P4501A mRNA expression

Using QPCR, we measured the expression of P4501A mRNA in the liver and gills. In the liver, the P4501A mRNA expression at 6 h in the 10 and 30 µg l<sup>-1</sup> BaP groups was about 6.7 and 131 times higher than in the control group, respectively, and then the levels decreased (Figure 2A). Similarly, in the gills, the expression of P4501A mRNA at 6 h in 10 and 30 µg l<sup>-1</sup> BaP groups was 7.5, and 28 times higher than in the control group, respectively, and then subsequently decreased (Figure 2B).

#### Plasma parameters

Figure 3 shows the plasma glucose, cortisol, AST, and ALT levels following exposure to BaP. The plasma glucose peaked at 0.012 ± 0.002 mM l<sup>-1</sup> (with 10 µg l<sup>-1</sup> BaP) and 0.015 ± 0.002 mM l<sup>-1</sup> (with 30 µg l<sup>-1</sup>) at 6 h, and then decreased. Cortisol peaked at 6.7 ± 0.5 µg l<sup>-1</sup> (10 µg l<sup>-1</sup>)

ofP450	1: MVLMLLPFIGSVSVSESLVAMTTVCLVYLILKFFHTDIPKGLRRLPGPKPLPIIGNVLEV	60
epP450	1: MMLMMLPFIGSVSVSESLVAMTTMCLVYLILKFFQTEIPEGLRRLPGPKPLPIIGNVLGL	60
mfP450	1: MMLMMLPFIGSVSVSESLVAMTTMCLVYLILKYLQTEIPEGLRRLPGPKPLPIIGNVLEL	60
esP450	1: MVLMLLPFIGSVSVSESLVALTTVCLVYLILKFFRTEIPEGLHRLPGPKPLPIIGNVLEV	60
gsP450	1: MVLMLLPFVGPVSVSESLVAIITMCLVYMLKFFRTEIPEGLCQLPGPKPLPIIGNVLEV	60
ofP450	61: GSKPYLSLTAMSKRYGHVFQIQIGMRPVVLSGSETVRQALIKQGEFFSGRPDLYSFRFI	120
epP450	61: GSKPYLSLTAMSKRYGHVFQIQIGMRPVVLSGSETVRQALIKQGEFFAGRPDLYSFRFI	120
mfP450	61: GSKPYLSLTAMSKRYGHVFQIQIGMRPVVLSGSETVRQALIKQGEFFAGRPDLYSFRFI	120
esP450	61: GNKPYLSLTAMSKRYGDVFQIQIGMRPVVLSGSETVRQALIKQGEFFAGRPDLYSFRFI	120
gsP450	61: GRNPYLSLTAMSKRYGDVFQIQIGMRPVVLSGSETVRQALIKQGEFFAGRPDLYSFRFI	120
ofP450	121: SDGKSLAFSTDQAGVWRARRKLAAYSALRSFSTLEGTTPEYSCVLEEHCKEGEYLKIQLN	180
epP450	121: NAGKSLAFSTDQAGVWRARRKLAAYSALRSFSTLEGTTPEYSCVLEEHCKEGEYLKIQLN	180
mfP450	121: NEGKSLAFSTDQAGVWRARRKLAAYSALRSFSLGTTPEYSCVLEEHCKEGEYLKIKRLN	180
esP450	121: NDGKSLAFSTDQAGVWRARRKLAAYSALRSFSSLGTTPEYSCVLEEHCKEGEYLKIQLN	180
gsP450	121: NDGKSLAFSTDQAGVWRARRKLAAYSALRSFSTLEGTTPEYSCALEEHVSKAEAYLVKQLN	180
ofP450	181: TVMKADGSFDPFRHIVVSVANVICGMCFGRRYDHDQELVSLVNLSDVFGQVVGSGNPAD	240
epP450	181: TVMKADGSFDPFRHIVVSVANVICGMCFGRRYDHDQELVSLVLSDEFGRVVGSGNPAD	240
mfP450	181: TVMKADGSFDPFRHIVVSVANVICGMCFGRRYDHDQELVGLVLSDEFGRVVGSGNPAD	240
esP450	181: TVMKADGSFDPFRHIVVSVANVICGMCFGRRYDHDQELLSLVNLSDVFGQVVGSGNPAD	240
gsP450	181: TVMETDGSFDPFRHIVVSVANVICGMCFGRRYDHNQELLSLVNLSDVFGQVVGSGNPAD	240
ofP450	241: FIPILQFLPSKSMKNFMSINERFISFVQKIVTEHYATFDKDNIRDITDSLIDHCEDRKL	300
epP450	241: FIPILQYLPSEAMKNFLRINEHFEFVQKIVTEHYTTFNKNIRDITDSLIDHCEDRKL	300
mfP450	241: FIPILQYLPSEAMKNFVRINARETTFVQKIVTEHYTTFNKNIRDITDSLIDHCEDRKL	300
esP450	241: FIPVLQFLPSTTMKKEFMDINARFNKVFQKIVSEHYTTFYDKDNIRDITDSLIDHCEDRKL	300
gsP450	241: FIPILQYLPSTSMKKEFVSINDRENAFVQKIVSEHYTTFDKDNIRDITDSLIDHCEDRKL	300
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ofP450	301: ENSNIQMSDEKIVGIVNDLFGAGFDITSTALSWSVMYLVAYPEIQERLYEEIKEKVGGLDR	360
epP450	301: ENSNVQMSDEKIVGIVNDLFGAGFDITVSTALSWSVMYLVAHPEIQERLYQEIEDKVGGLDR	360
mfP450	301: ENSNVQMSDEKIVGIVNDLFGAGFDITVSTAMSWSVMYLVAHPEIQERLYQEIEDKVGGLDR	360
esP450	301: ENSNVQMSDEKIVGIVNDLFGAGFDITSTALSWSVMYLVAYPEIEERLYQELKENVGGLDR	360
gsP450	301: ENSNVQMSDEKVVGIVNDLFGAGFDITSTALSWSVMYLVAYPEIQERLYQEMKESVGLDR	360
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ofP450	361: TPLLSDKPNLLFLEAFILEIFRHSSFLPFTIPHCTSKDTSLNGYFIPKDTCVFINQWQIN	420
epP450	361: MPLLSDKPNLPFLEAFILEILRHSSFLPFTIPHCTTKDTSLNGYFIPKDTCVFINQWQIN	420
mfP450	361: MPLLSDKPNLPFLEAFILEVFRHSSFLPFTIPHCTSKDTSLNGYFIPKDTCVFINQWQIN	420
esP450	361: TPLLCDRPNLPFLEAFILEIFRHSSFLPFTIPHCTSKDTSLNGYFIPKDTCVFINQWQIN	420
gsP450	361: TPCLSDKPKLPFLEAFILEIFRHSSFLPFTIPHCSKDTSLNGYFIPKDTCVFINQWQIN	420
ofP450	421: HDEELWKDPSSFNDRFLSADGSGIKKLEGEKVMVFGMKRRCIGEVIAARNEVYLFLLAI	480
epP450	421: HDPELWKDPSSFNDRFLSADGSEVNKLDGEKVMVFGMKRRCIGEVIAARNEVYLFLLAI	480
mfP450	421: HDPELWKDPSSFNDRFLSADGSEVNKLDGEKVMVFGMKRRCIGEVIAARNEVYLFLLAI	480
esP450	421: HDPELWKDPSSFNDRFLSDGTGTELNKLEGEKVMVFGKRRRCIGEVIAARNEVYLFLLAI	480
gsP450	421: HDPELWKDPSSFNDRFLNTDGTGTELNKLEGEKVMVFGKRRRCIGEVIAARNEVYLFLLAI	480
ofP450	481: IQKLHFHTMPGELLDMTPEYGLTMKHKRCHLKATMRARNEH	521
epP450	481: IQKLHFLLPIPGEKLDMTPEYGLTMKHKRCHLKATMRARNEH	521
mfP450	481: IQKLHFLLPIPGEKLDMTPEYGLTMKHKRCHLKATMRARNQH	521
esP450	481: VQKLHFKTLPGPEPLDMTPEYGLTMKHKRCHLRATMRA-SE-	519
gsP450	481: VQNLRFHAKPGEPLDMTPEYGLTMKHKRCHLRAAMSRNEE	521
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Figure 1. Comparison of amino acid sequence of olive flounder, *Paralichthys olivaceus* P4501A, European plaice, *Pleuronectes platessa* P4501A, marbled flounder, *Pseudopleuronectes yokohamae* P4501A, European sea bass, *Dicentrarchus labrax* P4501A and gilthead seabream, *Sparus aurata* P4501A optimally aligned to match identical residues, indicated by shaded box. The sequences were taken from the GenBank/EMBL/DBJ sequence databases. The P4501A sequences used for alignment are olive flounder P4501A (ofP4501A, EF451958), European plaice P4501A (epP4501A, X73631), marbled flounder P4501A (mfP4501A, AB120566), European sea bass P4501A (esP4501A, U78316), and gilthead seabream P4501A (gsP4501A, AF026800). An asterisk indicates the most conserved regions among the sequences.

and  $12.2 \pm 1 \mu\text{g l}^{-1}$  ( $30 \mu\text{g l}^{-1}$ ) at 6 h, and then decreased. AST began to increase at 12 h and peaked at  $551 \pm 100 \text{ IU l}^{-1}$  ( $10 \mu\text{g l}^{-1}$ ) at 24 h, while it peaked at  $994 \pm 10.4 \text{ IU l}^{-1}$  at 6 h with  $30 \mu\text{g l}^{-1}$  BaP, and then decreased. ALT peaked at  $12.0 \pm 3 \text{ IU l}^{-1}$  ( $10 \mu\text{g l}^{-1}$ ) at 12 h and at  $22.7 \pm 1.5 \text{ IU l}^{-1}$  ( $30 \mu\text{g l}^{-1}$ ) at 6 h, and then decreased, similar to AST (Figure 3).

## Discussion

Fish express cytochrome P4501A as a physiological response to toxic stress. This study determined the

full-length cDNA sequence of P4501A from the liver of the olive flounder and investigated BaP metabolism in fish through the increased P4501A expression in the liver and gills after exposure to BaP. In addition, we measured the changes in the plasma glucose, cortisol, AST, and ALT levels to compare the physiological stress induced by BaP with the level of P4501A expression.

The olive flounder P4501A cDNA consists of a 1566-nucleotide ORF that encodes 521 amino acids (GenBank accession no. EF451958). Vertebrate P4501A typically has two highly conserved regions, LDNSN and MTPEYG, which are at residues 299

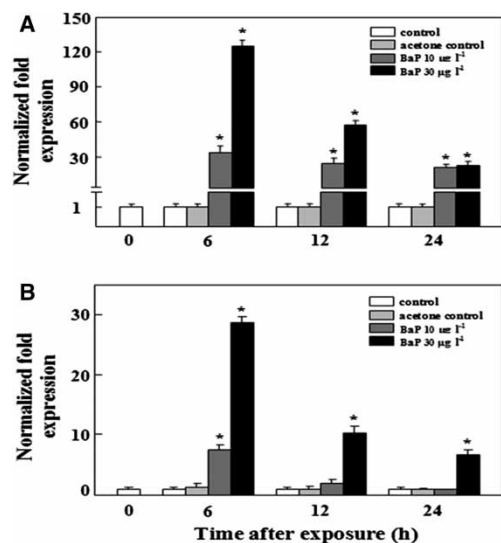


Figure 2. Dose- and time-related effects of BaP on P4501A transcript levels in the olive flounder liver and gill. One microgram of total RNA prepared from the liver (A) and gill (B) were reverse transcribed and amplified using gene-specific primers by quantitative PCR. Results are expressed as normalized fold expression with respect to  $\beta$ -actin levels for the same sample. Values with an asterisk indicates significantly different from control in the same time of sampling ( $p < 0.05$ ). Values are means  $\pm$  SD ( $n = 5$ ).

and 496, respectively, in olive flounder P4501A (Figure 1).

Using QPCR, P4501A mRNA expression in the flounder liver was highest at 6 h with both the 10 and 30  $\mu\text{g l}^{-1}$  BaP treatments (Figure 2A). This concurs with studies that observed high P4501A expression in the liver of European sea bass injected with BaP at 20  $\text{mg kg}^{-1}$  (Stien et al. 1998), and gizzard shad

exposed to waterborne BaP at 1  $\mu\text{g l}^{-1}$  (Levine & Oris 1997). Xenobiotics entering the body undergo phase I and II metabolic reactions. P4501A, which takes part in the phase I reaction, metabolizes xenobiotics by adding an oxygen molecule to lipophilic molecules to make them water-soluble (Van der Weiden et al. 1994; Andersson & Förlin 1992). Therefore, the high P4501A mRNA expression was induced in the liver following BaP treatment for BaP detoxification. Organic chemicals, such as PAH and chlorinated hydrocarbons, induce P4501A in the liver (Stegeman et al. 1992) and can be used as a biomarker of these xenobiotics.

The expression of P4501A mRNA in the flounder gills matched that in the liver, and peaked at 6 h with both the 10 and 30  $\mu\text{g l}^{-1}$  BaP treatments (Figure 2B). Similarly, P4501A expression increased rapidly in the gills of rainbow trout exposed to 1  $\mu\text{g l}^{-1}$  BaP for 6 h (Levine & Oris 1999) and the epithelium and pillar cells of the gills of top minnows (*Poeciliopsis lucida*) treated with 1  $\text{mg l}^{-1}$  BaP (Smolowitz et al. 1992). Similarly, ethoxyresorufin O-deethylase (EROD) was activated in the gills of rainbow trout exposed to 25  $\mu\text{g l}^{-1}$  BaP (Jönsson et al. 2006), while the gills reduced the internal accumulation of di-2-ethylhexyl phthalate (DEP) (Barron et al. 1988). The gills of fish are exposed to xenobiotics directly; before entering the blood, xenobiotics must pass through the epithelial cells, where they are metabolized (Levine & Oris 1999). Since the gills are exposed to aquatic xenobiotics and are influenced by them directly, the P450 enzyme system of the gills

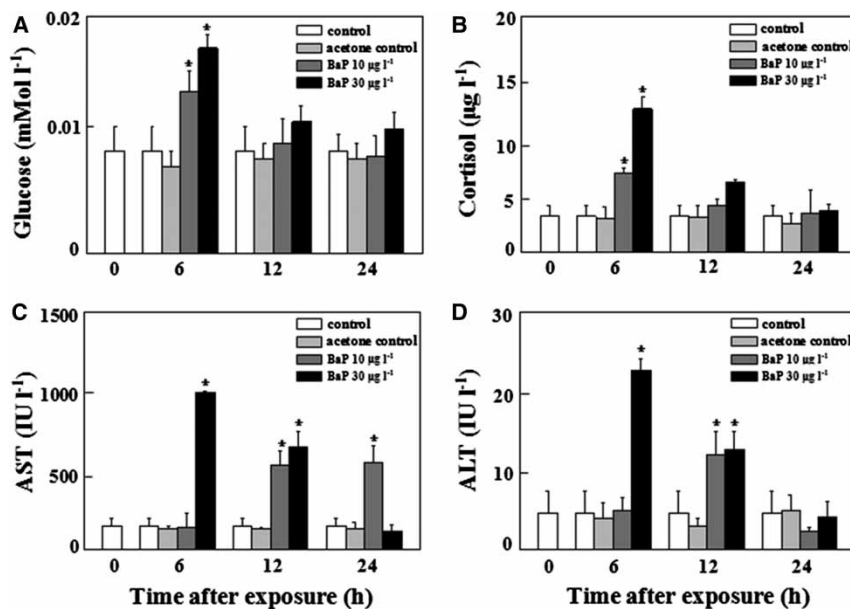


Figure 3. Level of plasma glucose, cortisol, AST, and ALT in olive flounder after BaP exposure. Values with an asterisk indicates significantly different from control in the same time of sampling ( $p < 0.05$ ). Values are means  $\pm$  SD ( $n = 5$ ).

plays an important role in detoxification (Levine & Oris 1999).

Since P4501A expression was high in the liver, the major tissue in which P4501A is activated to metabolize xenobiotics (Binder et al. 1984), and in the gills, then P4501A also likely metabolizes xenobiotics in the gills. A previous study also found that BaP was metabolized rapidly in both the liver and gills (Ueng et al. 1992; Kennedy & Walsh 1994). The gills have an oxidative-conjugating enzyme system that takes part in the metabolism of xenobiotics, although the enzyme system of the gills is relatively less active than that in the liver (Lindstrom-Seppa et al. 1981; Ueng et al. 1992). This supports the greater expression we observed in the liver.

In fish, blood parameters are commonly used as indicators of the physiological stress response to endogenous or exogenous changes. We also analysed the changes in the plasma glucose, cortisol, AST, and ALT on exposure to BaP. Xenobiotics such as BaP stress fish (Hontela 2005) and the primary stress responses induce secondary responses that affect energy requirements, such as increases in plasma glucose (Carmichael et al. 1984), AST, and ALT (Almeida et al. 2002), and changes in electrolyte homeostasis in the blood and tissues (Carmichael et al. 1984).

The plasma cortisol levels were highest 6 h after exposure to both 10 and 30  $\mu\text{g l}^{-1}$  BaP. Therefore, BaP was absorbed by the tissues and identified as a stressor, activating the hypothalamus–pituitary–interrenal axis (Perry & Reid 1993). Subsequently, the cortisol normalized.

Therefore, the stress is reduced by BaP is metabolized to nontoxic products. Since cortisol is involved in metabolism (Mommsen et al. 1999), the increased cortisol levels may reflect an increase in energy metabolism in response to BaP, which may in turn lead to increased glucose levels (Carmichael et al. 1984). It is thought that P450 detoxified the toxicity of BaP and cortisol was increased by a general toxic-stress response (Aluru & Vijayan 2004; Oliveira et al. 2007). Like cortisol, the plasma glucose levels were highest 6 h after exposure to both 10 and 30  $\mu\text{g l}^{-1}$  BaP, suggesting that stress-induced increases in cortisol promote gluconeogenesis in the liver. Hyperglycemia via this mechanism supplements the increased energy requirements due to stress (Vijayan et al. 1997).

The amino group transferases AST and ALT are general indices of liver function in vertebrates. In fish, these are widely used to evaluate stress responses to changes in water temperature, hypoxia, pH, ammonia, and heavy metals (Pan et al. 2003). In our study, the plasma AST and ALT increased

with BaP exposure, implying that liver cell damage led to decreased liver function. Since no mortality occurred during exposure and the AST and ALT levels normalized with time, we deduce that the BaP toxicity was counteracted by P4501A, thereby gradually reducing cell damage.

In summary, P4501A was expressed in the liver and gills of the flounder to metabolize and detoxify BaP. The increased plasma glucose, cortisol, AST, and ALT levels all indicated that BaP stresses flounders. Since the levels normalized with prolonged exposure to BaP, the toxicity of BaP was counteracted by P4501A. We anticipate that P4501A will be used as a bio-indicator for aquatic environmental pollution during exposure to BaP. Further studies of diverse environmental conditions are still needed to evaluate their impact on the P4501A enzyme system.

## References

- Almeida JA, Diniz YS, Marques SFG, Faine LA, Ribas BO, Burneiko RC. 2002. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. *Environment International* 27:673–9.
- Aluru N, Vijayan MM. 2004.  $\beta$ -Naphthoflavone disrupts cortisol production and liver glucocorticoid responsiveness in rainbow trout. *Aquatic Toxicology* 67:273–85.
- Andersson T, Förlin L. 1992. Regulation of the cytochrome P450 enzyme system in fish. *Aquatic Toxicology* 24:1–20.
- Barron MG, Schultz IR, Hayton WL. 1988. Presystemic branchial metabolism limits di-2-ethylhexyl phthalate accumulation in fish. *Toxicology and Applied Pharmacology* 98:49–57.
- Binder JR, Melancon MJ, Lech JJ. 1984. Factors influencing the persistence and metabolism of chemicals in fish. *Drug Metabolism Reviews* 15:697–724.
- Boleas S, Fernandez C, Beyer J, Tarazona JV, Goksøyr A. 1998. Accumulation and effects of benzo[a]pyrene on cytochrome P450 1A in waterborne exposed and intraperitoneal injected juvenile turbot (*Scophthalmus maximus*). *Marine Environmental Research* 46:17–20.
- Borhoumi R, Mouneimne Y, Awooda I, Safe SH, Donnelly KC, Burghardt RC. 2002. Characterisation of calcium oscillations in normal and benzo[a]pyrene-treated clone 9 cells. *Toxicological Sciences* 68:444–50.
- Carmichael GJ, Tomasso JR, Simco BA, Davis KB. 1984. Characterization and alleviation of stress associated with hauling largemouth bass. *Transactions of the American Fisheries Society* 113:778–85.
- Gelboin HV. 1980. Benzo[a]pyrene metabolism, activation and carcinogenesis: role of regulation of mixed-function oxidases and related enzymes. *Physiological Review* 60:1107–66.
- Hahn ME. 2002. Aryl hydrocarbons receptor: diversity and evolution. *Chemico-Biological Interactions* 141:131–60.
- Hontela A. 2005. Adrenal toxicology: environmental pollutants and the HPI axis. In: Mommsen TP, Moon TW, editors. *Biochemistry and Molecular Biology of Fishes*. Environmental Toxicology, Vol. 6. Amsterdam: Elsevier. p 331–63.
- Jönsson EM, Abrahamson A, Brunström B, Brandt I. 2006. Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo[a]pyrene and

- 3,3',4,4',5-pentachlorobiphenyl. *Aquatic Toxicology* 79: 226–32.
- Jönsson ME, Orrego R, Woodin BR, Goldstone JV, Stegeman JJ. 2007. Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. *Toxicology and Applied Pharmacology* 221:29–41.
- Kennedy CJ, Walsh PJ. 1994. The effects of temperature on the uptake and metabolism of benzo[a]pyrene in isolated gill cells of the gulf toadfish (*Opsanus beta*). *Fish Physiology and Biochemistry* 13:93–103.
- Kolok AS, Huckins JN, Petty JD, Oris JT. 1996. The role of water ventilation and sediment ingestion in the uptake of benzo[a]pyrene in gizzard shad (*Dorosoma cepedianum*). *Environmental Toxicology and Chemistry* 15:1752–9.
- Leaver MJ, Pirrit L, George SG. 1993. Cytochrome P450 1A1 cDNA from plaice (*Pleuronectes platessa*) and induction of P450 1A1 mRNA in various tissues by 3-methylcholanthrene and isosafrole. *Molecular Marine Biology and Biotechnology* 2:338–45.
- Lee YM, Williams TD, Jung SO, Lee JS. 2005. cDNA cloning and expression of cytochrome P450 1A (CYP1A) gene from the hermaphroditic fish *Rivulus marmoratus*. *Marine Pollution Bulletin* 51:769–75.
- Levine SL, Oris JT. 1997. Induction of CYP1A mRNA and Catalytic activity in gizzard Shad (*Dorosoma cepedianum*) after waterborne exposure to benzo[a]pyrene. *Comparative Biochemistry and Physiology Part C* 118:397–404.
- Levine SL, Oris JT. 1999. CYP1A expression in liver and gill of rainbow trout following waterborne exposure: implication for biomarker determination. *Aquatic Toxicology* 46:279–87.
- Lindstrom-Seppa P, Koivuasaari U, Hanninen O. 1981. Extra-hepatic xenobiotic metabolism in North-European freshwater fish. *Comparative Biochemistry and Physiology Part C* 69: 259–63.
- Livingstone DR. 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology & Biotechnology* 57:195–211.
- Mcgroddy SE, Farrington JW. 1995. Sediment porewater partitioning of polycyclic aromatic hydrocarbon in three cores from Boston harbor, Massachusetts. *Environmental Science & Technology* 29:1542–50.
- Mommsen TP, Vijayan MM, Moon TW. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* 9:211–68.
- Nebert DW, Russell DW. 2002. Clinical importance of the cytochromes P450. *Lancet* 360:1155–62.
- Oliveira M, Pacheco M, Santos MA. 2007. Cytochrome P4501A, genotoxic and stress responses in golden grey mullet (*Liza aurata*) following short-term exposure to phenanthrene. *Chemosphere* 66:1284–91.
- Pan CH, Chien YH, Hunter B. 2003. The resistance to ammonia stress of *Penaues monodon* Fabricius juvenile fed diets supplemented with astaxanthin. *Journal of Experimental Marine Biology and Ecology* 297:107–18.
- Perry SF, Reid SD. 1993. b-Adrenergic signal transduction in fish: interactive effects of catecholamines and cortisol. *Fish Physiology and Biochemistry* 11:195–203.
- Smolowitz RM, Schultz ME, Stegeman JJ. 1992. Cytochrome P-4501A induction in tissues, including olfactory epithelium, of top minnows (*Poeciliopsis* spp.) by waterborne benzo[a]pyrene. *Carcinogenesis* 13:2395–402.
- Stegeman JJ. 1981. Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. In: Ts'o POP, editor. *Polycyclic Hydrocarbons and Cancer*. New York: Academic Press. p 1–60.
- Stegeman JJ, Brouwer M, Di Giulio RT, Forlin L, Fowler BA, Sanders BM. 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Hugget RJ, Kimerle RA, Mehrle PM, Bergman HL, editors. *Biomarkers: Biochemical, Physiological, and Histopathological Markers of Anthropogenic Stress*. Chelsea, MI: Lewis publishers. p 235–51.
- Stien X, Amichot M, Bergé JB, Lafaurie M. 1998. Molecular cloning of a CYP1A cDNA from the teleost fish *Dicentrarchus labrax*. *Comparative Biochemistry and Physiology Part C* 121:241–8.
- Tom M, Myers CR, Waterman MR. 2002. Evaluating molar CYP1A level in fish hepatic microsomes by competitive ELISA using recombinant membrane-free CYP1A standard protein. *Aquatic Toxicology* 59:101–14.
- Ueng TH, Ueng YF, Park SS. 1992. Comparative induction of cytochrome P-450-dependent monooxygenases in the livers and gills of tilapia and carp. *Aquatic Toxicology* 23:49–64.
- Van der Weiden MEJ, Hanegraaf FHM, Eggens ML, Celandier M, Seinen W, Van der Berg M. 1994. Temporal induction of cytochrome P4501A in the mirror carp (*Cyprinus carpio*) after administration of several polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry* 13:797–802.
- Varanasi U. 1989. *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*. Boca Raton, FL: CRC Press.
- Vijayan MM, Pereira CE, Grau G, Iwama GK. 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comparative Biochemistry and Physiology Part C* 116:89–95.
- Winzer K, Van Noorden CJF, Köhler A. 2002. Sex-specific biotransformation and detoxification after xenobiotic exposure of primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). *Aquatic Toxicology* 59:17–33.

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