Brain CYP1A in seabream, *Sparus aurata* exposed to Benzo(a)pyrene

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Summary. This study compares basal and induced expression of cytochrome P4501A-CYP1A in the brain of gilthead seabream, *Sparus aurata*. Larval or adult seabream were exposed to benzo(a)pyrene -B(a)P- and the CYP1A response was assessed by analyzing CYP1A mRNA (RT-PCR), CYP1A protein (expression levels: ELISA, western blotting; cellular localization: immunohistochemistry), and CYP1A catalytic activity (7-ethoxyresorufin-O-deethylase-EROD).

In the brain of adult *S. aurata*, CYP1A immunostaining was generally detected in the vasculature. It was present in the neuronal fibers and glial cells of the olfactory bulbs and the ventral telencephalon. ELISA and RT-PCR analyses confirmed CYP1A expression in the brains of non-exposed seabream. B(a)P exposure led to increased CYP1A staining mainly in neuronal fibers and glial cells of the olfactory bulbs, but also in the vascular endothelia. EROD activity, however, could not be detected in the brain of adult seabream, neither in control nor in exposed fish.

In the developing brain of *S. aurata* larvae, immunohistochemical staining detected CYP1A protein exclusively in endothelia of the olfactory placode and in retina. Staining intensity of CYP1A slightly increases with larval development, especially in vascular brain endothelia. Exposing the larvae to 0.3 or 0.5 µg B(a)P/L from hatching until 15 days post hatching (dph) did not result in enhanced CYP1A immunostaining in the brain. In samples of whole seabream larvae, both from controls and BaP treatments, neither CYP1A mRNA, protein nor catalytic activity were detectable.

The results demonstrate that CYP1A is expressed already and inducible in the larval brain, but that the regional and cellular expression differs partly between larval and adult brain. This may have implications for the toxicity of CYP1A-inducing xenobiotics on early and mature life stages of seabream.

Key words: *Sparus aurata*, Larvae, Adult, Brain, CYP1A, EROD, B(a)P

Introduction

Cytochrome P-450 monooxygenases are a multigene family of constitutive and inducible haeme- proteins containing enzymes, which are involved in the oxidative metabolism of a broad range of substrates, with physiological functions in inter-and intracellular signalling, such as fatty acids, prostaglandins, steroids, neurohormones, and for metabolizing lipophilic xenobiotics and drugs (Nebert and González, 1987; Nebert et al., 1991, 1996; Parkinson, 1995; Mansuy, 1998; Sarasquete and Segner, 2000; Hoffman and Oris, 2006). CYP enzymes may have homeostatic functions since have been shown, in the mammalian brain, to participate in the synthesis of neuroactive steroids and also in cerebral blood vessel tone. Moreover, CYP isoforms have been localized in discrete cerebral areas and may alter the local action or concentration of neuroactive drugs (Majewska et al., 1986; Walther et al., 1986, 1987).

The most intensively studied P450 gene in fish is CYP1A (Nelson et al., 1996), which are inducible by a wide variety of lipophilic contaminants, particularly polychlorodibenzo dioxins (PCDDs), polycyclic aromatic hydrocarbons (PAHs), polyhalogenated

aromatic hydrocarbons (PHAHs) and polychloro-biphenyls (PCBs) (Stegeman and Hahn, 1994; Parkinson, 1995). These xenobiotics activate CYP1A gene expression via ligand binding to the arylhydrocarbon receptor (AhR) pathway (Nebert and Gonzalez, 1987). As a consequence of receptor activation, levels of CYP1A mRNA and newly synthesized CYP1A proteins are increased and proteins subsequently undergone 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 CYP1A induction (Goksoyr and Förlin, 1992; Sarasquete and Segner, 2000).

Fish can be exposed to lipophilic xenobiotics via water, sediment and/or food. Once taken up by the fish, the contaminants may be metabolized and excreted and/or they may produce physiological dysfunctions and pathological alterations. The brain may be particularly vulnerable to damage by toxic compounds due to the limited regenerative capability of the neurons. The blood-brain barrier which renders some protection to the brain by preventing the entry of many types of circulating toxic molecules is well developed in mammals but might be not fully developed in fish species (Andreas, 1994). Lipophilic compounds such as the AhR-activating HAHs and PAHs may diffuse through the endothelial cells of the brain vessels, and in fact, high accumulation factors of these compounds in fish brain have been reported (Varanasi et al., 1989). CYP1A-mediated *in situ* metabolism and cellular toxicity of xenobiotics in the brain may have far-reaching consequences, causing disruption of neuronal and neuroendocrine functions (Andersson et al., 1993; Andersson and Goksoyr, 1994; Huang et al., 2000). Adverse consequences of xenobiotic accumulation and activation of the AhR pathway may differ between larval and adult fishes, as the larval brain is still under differentiation, however, to date it is not known whether at all larval brain expresses an inducible AhR pathway.

The present study aims to compare CYP1A expression and potential benzo(a)pyrene –B(a)P-induction in the brains of developing as well as adult seabream, *Sparus aurata*. For this purpose, a battery of molecular, cellular and biochemical approaches was used to localize CYP1A cellular and regional distribution in the brain, as well as to quantify CYP1A mRNA (by means of RT-PCR) and protein levels (Western Blot, ELISA), as well as CYP1A catalytic activities (7-ethoxyresorufin-O-deethylase –EROD-activity) in the brain. For the larvae, both the endotrophic and exotrophic nutrition phases were investigated. The gilthead seabream, *Sparus aurata*, is a protandric species which develops as male during the first year of life and later undergoes sex conversion into females. The use of all-male fish has the advantage to exclude a possible sex influence on the CYP1A response. *Sparus aurata* was selected as an experimental species because of its economical importance, and

because the morphological and neuroanatomical description of its central nervous system, CYP1A immunodistribution and cerebral larvae developing was already studied and described in detail for *Sparus aurata* and *Solea senegalensis* (Muñoz-Cueto et al., 2001; Sarasquete et al., 1999, 2001; Ortiz-Delgado et al., 2002; Piñuela et al., 2004). This anatomical knowledge provides an indispensable tool for the interpretation of the findings on basal CYP1A distribution and xenobiotic induction.

Material and Methods

Adult fish

Xenobiotic exposure

Immature male specimens of gilthead seabream, *Sparus aurata* (1 year old, average weight: 250-300 gr) were obtained from CUPIMAR S.A (San Fernando, Cádiz, Spain). Until the xenobiotics exposure, specimens were kept in tanks during 2 weeks for acclimatisation. The tanks were supplied with continuously flowing seawater at constant temperature ($19\pm1°C$).

After the acclimatisation, fish were randomly distributed to the experimental tanks for 20 days and submitted to the following treatments: (a) only vehicle added, toluene, as control, and (b) exposure to 100 μg/L B(a)P (SIGMA, Aldrich). Treatments were applied in triplicate, with 12 fish per each 120-L experimental tank. A stock solution of B(a)P was prepared in toluene and added to the water in suitable quantities to give nominal concentrations of B(a)P. The maximum solvent concentration in the water was 0.05 μl/L toluene.

The fish were exposed to the toxicant under semi-static conditions. The water was renewed every 24 hours, followed by the addition of suitable quantities of B(a)P. Before the experiments were initiated, tanks were filled with water and B(a)P solutions were added and maintained during 24 h in order to guarantee complete adsorption of the compounds on the walls. Fish samples were taken after 5, 10 15 and 20 days of the experimental period (3 fish per each sampling point and treatment group). At sampling, fish were then anaesthetised by immersion in 2-phenoxyethanol (3 ml/L) and disected for immnuohistochemical and biochemical purposes. Water temperature ($18.8\pm0.2°C$), pH (7.5 ± 0.2), NO_2^- (<0.1 mg.L^{-1}), NO_3^- (<8mg.L^{-1}), NH_4^+ (<3 mg.L^{-1}), dissolved oxygen (8.3 ± 0.3 mg.L^{-1}) and salinity (32‰) were measured daily during the experimental period. No mortalities were recorded during experimental exposure treatment.

Samples of brain from control and exposed fish were taken at the end of the experimental period. Tissue samples corresponding to whole brain/B; olfactory bulbs/OB, cerebral hemispheres/Part I, diencephalon/mesencephalon/Part II, and cerebellum/medulla oblongata/Part III, were taken for CYP1A mRNA, CYP1A proteins/ELISA immunohistochemical analysis and EROD activities

Larvae

Fertilized seabream *Sparus aurata* eggs from a commercial fish farm (CUPIMAR S.A, San Fernando, Cadiz, Spain), were maintained under controlled laboratory conditions until hatching. Physico-chemical parameters, such as salinity (32‰), pH (7.5±0.2), and dissolved oxygen (8.3±0.3 mg/L) were maintained at appropriate values. From hatching and during 15 days post-hatching/dph, larvae were transferred to 1-L glass vessels (30 larvae per each vessel) and exposed to the following treatments: (a) control (only toluene added), (b) exposure to 0.3 μg/L B(a)P, (c) exposure to 0.5 μg/L B(a)P during 24 h for toxicological studies. The experimentas were performed in a termostatic chamber at constant temperature (22±1°C)and photoperiod (12 h light/ 12 h darkness). Groups of larvae from each one of the 15 age classes (neonate, 2-15 dph) were exposed during 24 h to B(a)P and inmediately euthanized with 200 mg/L and preserved for immunohistochemical and biochemical purposes. The experiments for each treatment groups (control and B(a)P exposure) were carried out in triplicate. No mortalities were recorded during the experimental period.

Experimental procedures and B(a)P concentrations used in this work were previously described and justified by Ortiz-Delgado and Sarasquete (2004) and Ortiz Delgado et al. (2008).

CYP1A mRNA expression

For RNA extraction, 50-100 mg of whole brain sample or of the various brain regions were mixed with 1 ml TRIZOL and homogenized. Two-hundred microliters of chloroform were added, and the solution was centrifuged at 10000xg for 15 min. The RNA-containing supernatant was precipited by adding 500 μl of isopropanol and was centrifuged at 10000xg for 30 min. After centrifugation, the pellet was washed with 1 ml cold ethanol (70%), air dried and re-dissolved in DEPC-H20 (diethyl pyrocarbonate, 0.1%). The RNA content was quantified using UV spectrophotometer.

Fish-strand cDNA was produced following a protocol of the SuperScript II RT kit (Boehringer Mannheim, Germany) using 1 μg of total RNA. Two microliters of the resulting cDNA were amplified in the PCR reaction. PCR cycling conditions were as follow: 94°C for 45 s, 60°C for 30 s and 72°C for 35 s. The CYP1A reaction was run in 35 cycles in order to be in the linear range of product increase. The primer sequences used for RT-PCR on seabream CYP1A mRNA were taken from Cousinou et al. (2000).

The PCR products were electrophoresed on 1.6% agarose gel and visualized with ethidium bromide staining. Their identity was confirmed by sequencing

CYP1A protein analysis

Protein concentrations in homogenates and microsomes were determined by using the BIO-RAD Dc (detergent compatible) protein kit which is based on a modified Lowry method.

Western Blot

Western blotting analyses were performed in order to verify the specific cross-reactivity of the C-10-7 monoclonal antibody prepared against peptide 277-294 of rainbow trout (*Oncorhynchus mykiss*) CYP1A (Biosense AS, Bergen, Norway) with the seabream CYP1A protein. Microsomal preparations were separated in 9% sodium dodecylsulfate polyacrylamine gel electrophoresis (SDS-PAGE) (10 μg of protein/lane). Transfer of the protein to nitrocellulose sheets was performed in a Mini-Protean II blotting cell (Bio-rad) according to the manufacturer's procedures. The blots were then probed overnight with a 1:200 diluted C10-7 antibody at room temperature and a peroxidase-conjugated secondary anti-mouse IgG antibody from goat. The immunoreaction was visualized using 4-chloronaphtol as substrate for peroxidase. Molecular weights of stained proteins on the Western blotting were determined by comparing the relative mobilities with molecular weight standards.

ELISA

An indirect ELISA method was performed according to Goksøyr et al. (1991) and Scholz et al. (1997) in whole brain and different cerebral parts after 20 days to B(a)P. The microsomal samples were adjusted to a protein content of 10 ng microsomal protein/ml. The primary antibody used for the ELISA was the C10-7 CYP1A monoclonal antibody directed against peptide 277-294 of rainbow trout (*Oncorhynchus mykiss*) CYP1A (Biosense AS, Bergen, Norway) diluted 1:500. As previously pointed out by Ortiz-Delgado et al., (2005), the C10-7 antibody presented cross reactivity with the CYP1A from *S. aurata*. The primary reaction was followed by a horseradish-peroxidase-conjugated goat IgG anti-mouse (Dako) as secondary antibody. Staining was performed using 3,3'-diaminobenzidine as substrate. The peroxidase reaction product was measured as optical density in a spectrophotometer (Pharmacia, Freiburg, Germany) at 405 nm; controls (non-specific binding, blank) were included.

Immunohistochemistry of CYP1A

Sections of 5 μm thickness of adult seabream, *Sparus aurata* brain and whole larvae were prepared from paraffin embedded samples. The sections after blocking of unspecific binding sites were incubated overnight in a humid chamber at room temperature with the primary monoclonal mouse C10-7 CYP1A antisera at dilution of 1:250. For further staining, the ABC kit (Vectastain, USA) including a biotinylated anti-mouse IgG secondary antibody was applied. Sections incubated with normal fish serum instead of the primary antibody, were used as negative controls.

EROD analysis

EROD activity was measured as described by Scholz et al. (1997). Tissue samples corresponding to whole brain/B, olfactory bulbs/OB, cerebral hemispheres/Part I, diencephalon/mesencephalon/Part II, cerebellum/medulla oblongata/Part III, were homogenized in Tris-based homogenisation buffer and microsomes were prepared by means of ultracentrifugation (Scholz et al., 1997). The microsomes were suspended in 200 μl homogenisation buffer and EROD activities were determined in a microplate format assay using a fluorometer plate reader (Fluostar, SLT-Tecan). The assay was run with 47 μM NADPH and 0.4 μM ethoxyresorufin in phosphate-buffered saline. The concentrations of the reagents in the assay were optimised in preliminary experiments. The rate of resorufin formation in the assay was measured at an excitation wavelength of 344 nm and at an emission wavelength of 590 nm. A resorufin standard curve was used to convert the fluorescent readings into the amount of resorufin formed. Volume activity of EROD was normalized to microsomal protein.

Handling of the fish was always done according to the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other scientific purposes. All experimental procedures were performed according to the Spanish Bioethical Commission (CSIC and MEC).

Statistics

Results are presented as mean ± standard deviation (S.D.). Significant differences were evaluated by means
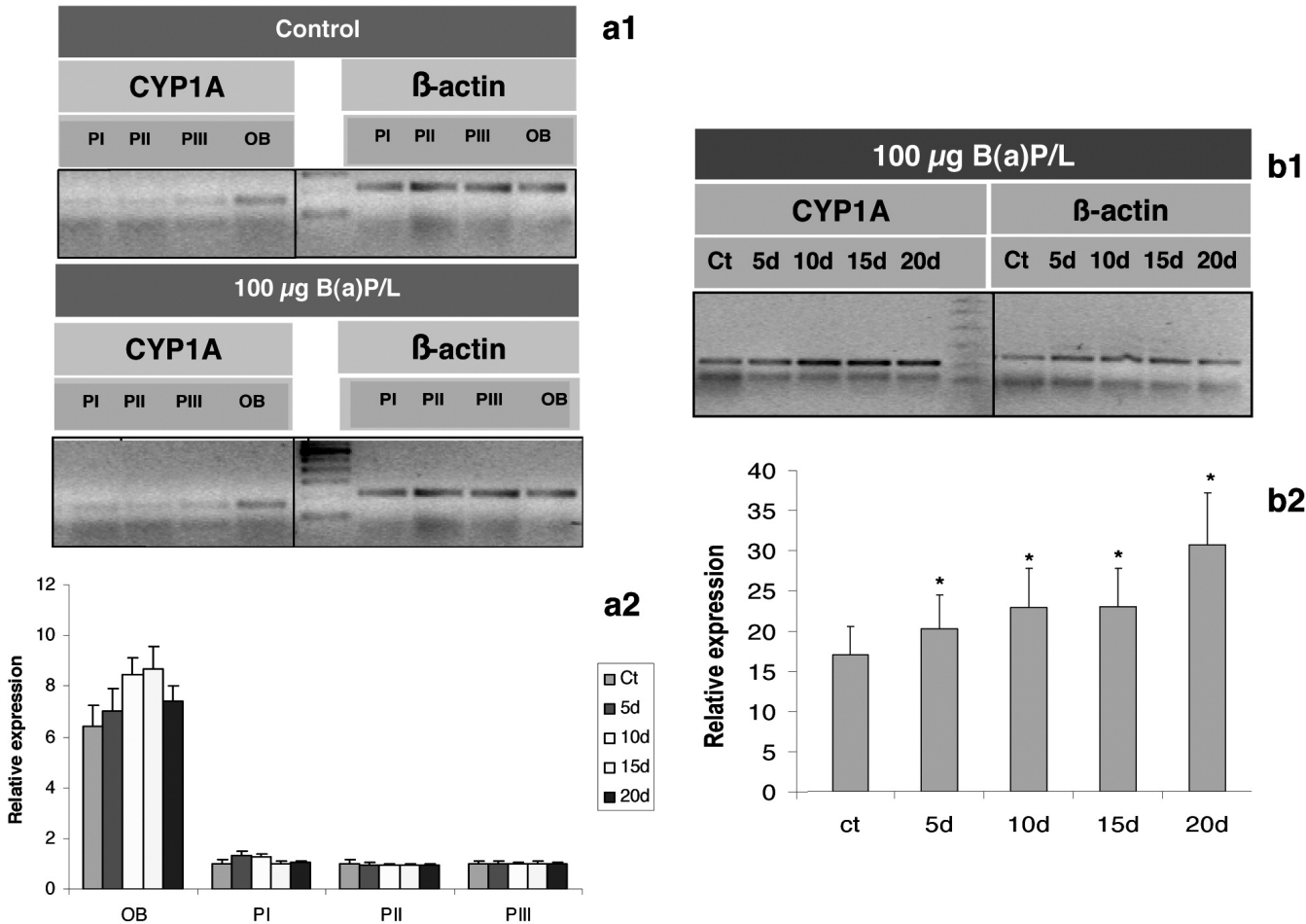
Fig. 1. Changes of CYP1A mRNA protein levels in liver and brain from *S. aurata* control and exposed individuals (100 μg B(a)P/L, during 20 days). Changes of CYP1A levels in different brain regions of control and treated specimens at 5, 10, 15 and 20 days of exposure, were assessed by means of RT-PCR **(a1)** and products of mRNA for CYP1A1 and ß-Actin (housekeeping gene) were analyzed by means of a densitometer (gel doc-BIORAD) **(a2)**. Hepatic changes of mRNA for CYP1A as a positive control assessed by means of RT-PCR **(b1)** and products of mRNA for CYP1A1 and ß-actin (housekeeping gene) in liver samples analyzed by means of a densitometer (gel doc-BIORAD) **(b2)**. Results are expressed as means ± S.D. (n= 9 fish per each sampling point). Asterisks denote significant differences (p<0.05) with respect to the control group. OB: olfactory bulb; PI: part I; PII: part II; PIII: part III.

of a non-parametric ANOVA followed by the Tukey-Kramer test. The significance level adopted was p<0.05.

Results

The expression and synthesis of CYP1A and catalytic EROD activity show differences between the brains of larval and adult seabream, *Sparus aurata*, which may be related to ontogenetic changes in the CYP1A expression patterns. A preferential site of CYP1A expression is the olfactory bulbs. Throughout the brain, vascular endothelia are immunoreactive to CYP1A staining (Figs. 1-5).

Adult fish

CYP1A mRNA

In the brain of both control and B(a)P exposed adult *Sparus aurata* specimens, the expression of CYP1A mRNA was detectable only in telencephalon and here specifically in the olfactory bulbs (OB). Other brain regions (cerebral hemispheres, diencephalon-mesencephalon and cerebellum-medulla oblongata) were CYP1A-negative (Fig. 1a1,a2). Also, no expression was detected in whole brain samples (data not shown). Exposure to B(a)P (100 μg/L) did not induce significant changes of CYP1A mRNA levels (Fig. 1a1,a2), whereas hepatic CYP1A mRNA expression (used as positive control) was markedly elevated (Fig. 1b1,b2).

CYP1A proteins

Western blotting was used in order to verify a cross-reactivity of the C10-7 commercial mouse antibody with the CYP1A protein of *S. aurata*, using liver microsomes from seabream and rainbow trout (positive control). In the liver and brain of B(a)P exposed seabream fish, the antibody detects specifically one single band of approximately 60 kDa molecular weight (Fig. 2a), indicating that C10-7 is specifically cross-reactive to CYP1A of seabream.

For a detailed analysis of the regional distribution of CYP1A in the brain, an immunoenzymatic assay/ELISA was used. In both control and B(a)P exposed fish, CYP1A was only found in olfactory bulb microsomes of seabream, with the levels of control and B(a)P-exposed fish being similar (Fig. 2b).

Immunohistochemistry of CYP1A

In adult *Sparus aurata* brain, CYP1A immuno-staining was detected in neuronal fibers of the glomerular layer of the olfactory bulbs. In the cerebral hemispheres, the ventralis telencephali pars ventrali area (vv) also contained CYP1A positive fibers and cell bodies. The vascular endothelia of all brain regions were strongly immunostained for Cyp1A (Fig. 3a,b). In B(a)P-exposed seabream (100 μg/L B(a)P, 20 days of exposure), an increase of CYP1A staining intensity was

detected in the neuronal fibers of the olfactory bulbs (endothelium, fibers and glial cells), and particularly in the vascular endothelia (Fig. 3c).

Catalytic activity

EROD activity was analyzed in microsomes from homogenates of whole brain *S. aurata* specimens, as well as of specific brain regions as described in the Material and Methods section. Liver homogenates taken from the same individuals used for brain analysis were used as positive controls (Fig. 4). In the brains of *Sparus aurata* exposed to 100μg/L B(a)P for 20 days, no cerebral EROD activity could be detected, neither in the whole brain nor in specific brain regions (OB, Part I, Part II and Part III). However, control liver samples showed well detectable EROD levels (10 pmol resorufin min^{-1} mg protein-1), as well as hepatic EROD activities considerably increased after exposure to 100μg/L B(a)P for 20 days (Fig. 4). These findings confirm the previous observations of Ortiz-Delgado et al. (2008).
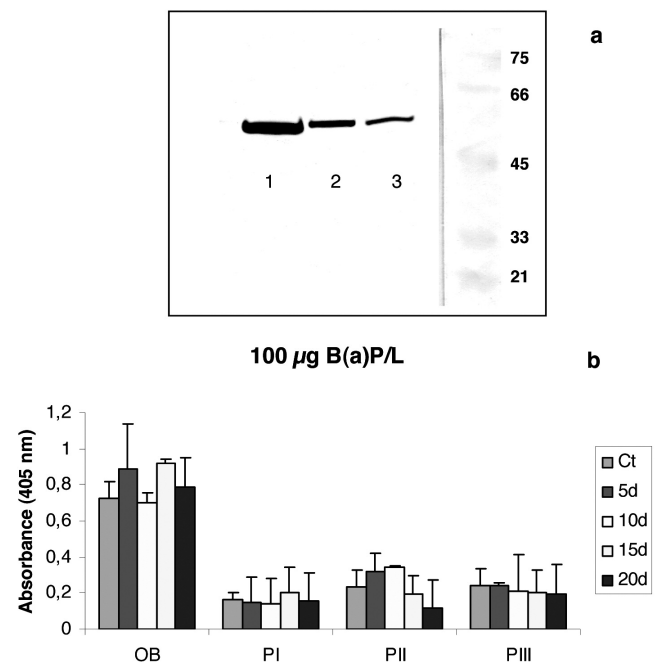
Fig. 2. a. Western blot of CYP1A protein in brain and liver from control and exposed specimens (20 days of treatment). The amount of protein applied to each lane was 20 μg. Liver of rainbow trout (*O. mykiss*) was used as a positive control. (Lane 1: hepatic microsomes from 100 μg B(a)P/L *S. aurata* exposed organisms; Lane 2: liver from rainbow trout; Lane 3: microsomes from whole brain homogenates of B(a)P exposed fish; Right lane: molecular weight marker). The antibody used was the monoclonal antibody C10-7 (Biosense, Bergen, Norway) directed against CYP1A of rainbow trout. **b.** Changes of CYP1A protein levels assessed by means of Elisa technique in S. aurata exposed to 100 μg/L during 20 days. Results are presented in means ± S.D. OB: olfactory bulb; PI: part I; PII: part II; PIII: part III.
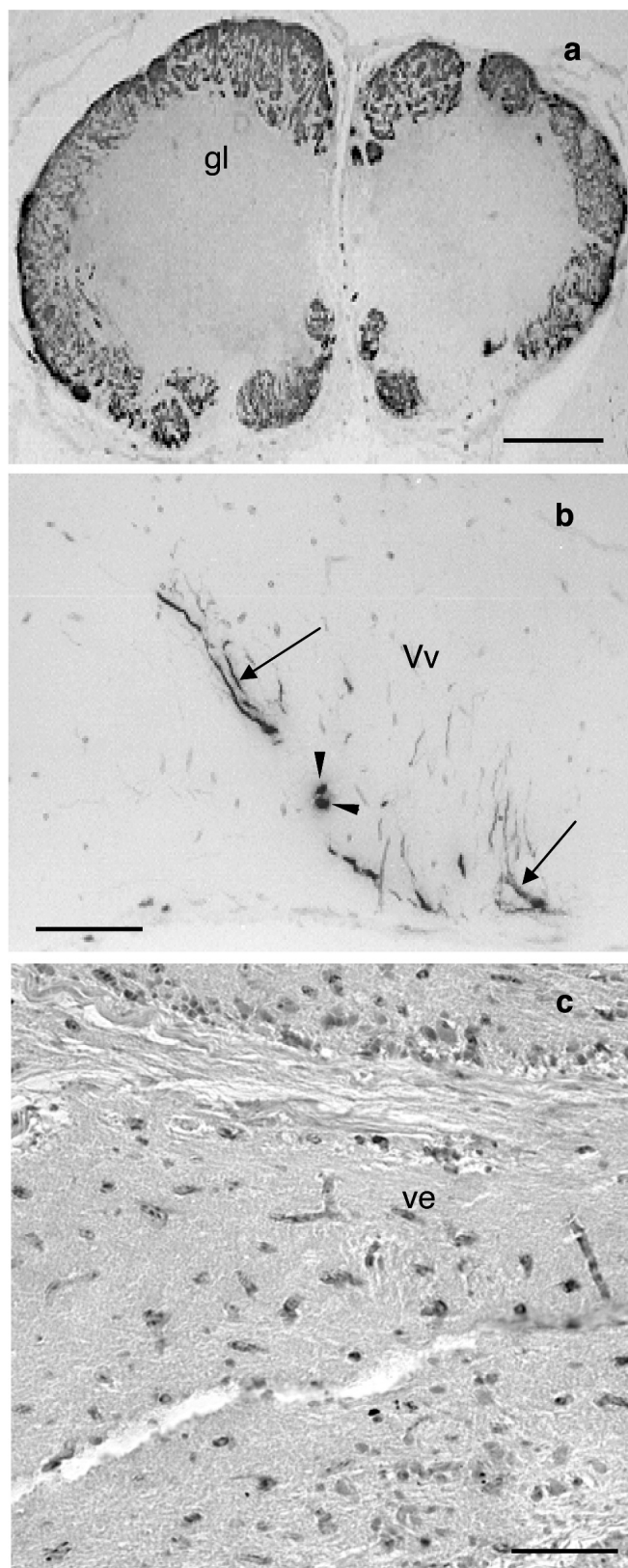
Larvae

By means of ELISA, RT-PCR and EROD, no CYP1A expression could be detected in samples prepared from whole seabream larvae, from hatching until 15 dph.

In *Sparus aurata* larvae from hatching until 15 dph, vascular brain endothelium, olfactory organ and folds of the retina sensory cells showed basal CYP1A immunoreactivity. The staining intensity increased weakly during larval development, especially the immunostaining of the brain endothelia (Fig. 5a-c). In B(a)P-exposed larvae (0.3 and 0.5μg/L for 24 h), a distribution of CYPIA similar to controls was observed in vascular endothelium, retina and in olfactory organ (ciliated olfactory sensory neurons, olfactory receptor neurons and olfactory bulbus) (Fig. 5d-f).

Discussion

Induction of CYP1A can enhance metabolism of lipophilic xenobiotics. Basal expression of CYP1A as well as xenobiotic induction shows inter- and intra-species differences among teleosts, related to developmental patterns, to the respective target organ
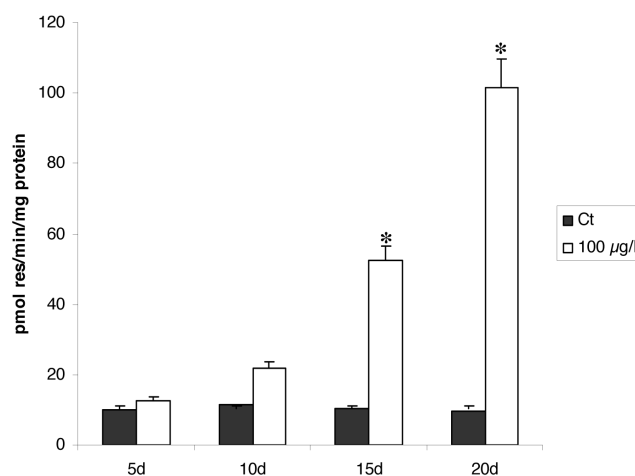
Fig. 4. EROD induction in the liver of B(a)P (100μg/L) exposed *Sparus aurata*. Values represent means ± S.D. Asterisks denote significant differences ($p<0.05$) with respect to the control group.

Fig. 3. Photomicrographs of transverse sections through the brain of *S. aurata*, showing CYP1A immunoreactive cell bodies and neuronal fibers. **a.** Strong CYP1A immunoreactivity in the glomerular layer (gl) of the olfactory bulbs of control *S. aurata* brain. **b.** Ventralis telencephali pars ventrali area (Vv) from control *S. aurata* brain showing CYP1A immunoreactive fibers (arrows) and cell bodies (arrowheads). **c.** CYP1A induction in cerebellum from B(a)P exposed fish (100 μg/L) restricted to the vascular endothelia (ve). Scale bars: 100 μm.
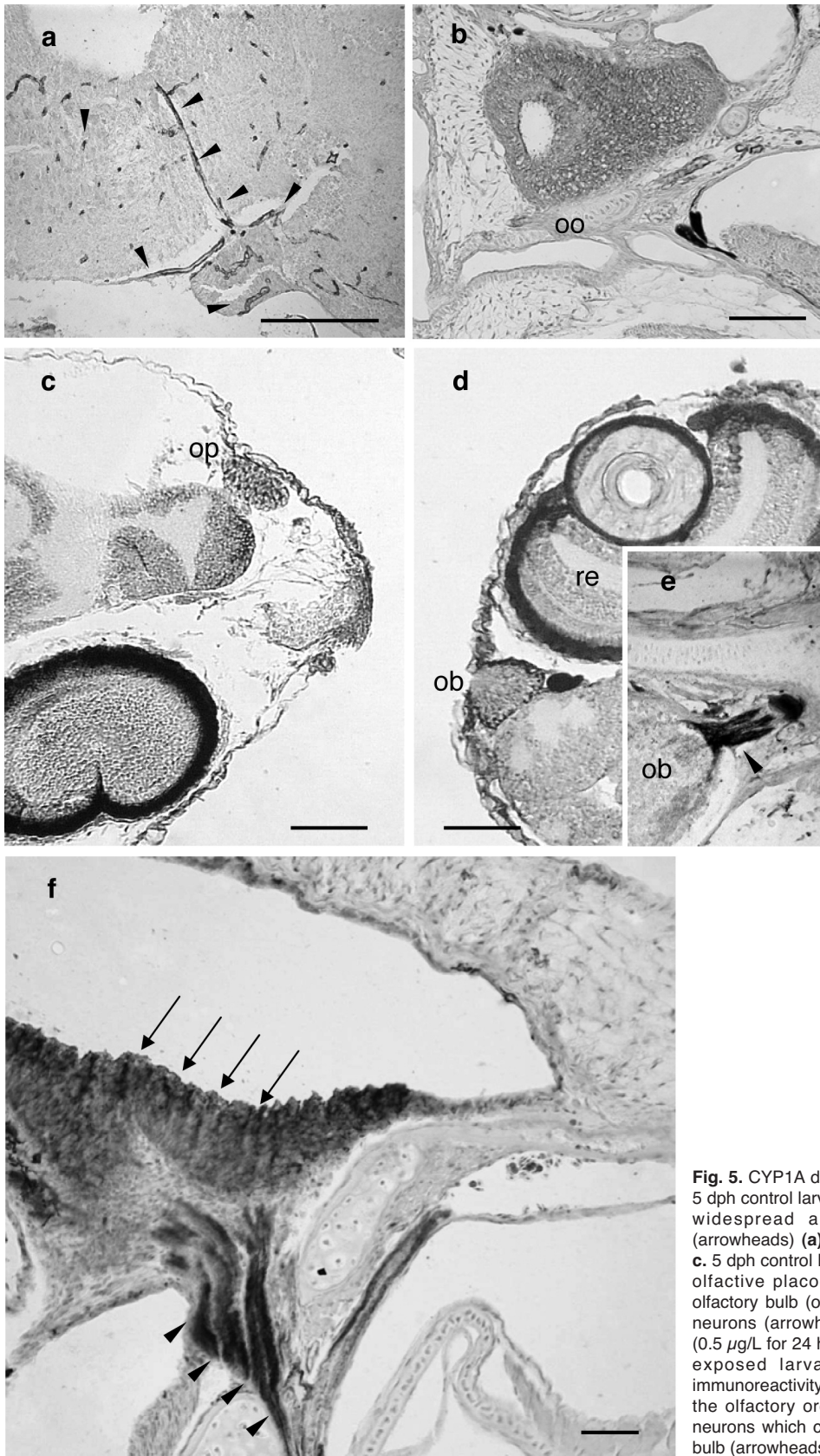
Fig. 5. CYP1A distribution in brain from *Sparus aurata* larvae. 5 dph control larvae showing a strong CYP1A immunostaining widespread along the endothelium of vasculature (arrowheads) **(a)** and located in the olfactory organ (oo) **(b). c.** 5 dph control larvae showing CYP1A immunostaining in the olfactive placoda (op). CYP1A immunoreactivity in the olfactory bulb (ob), the retina (re) **(d)** and in olfactory reptor neurons (arrowhead) **(e)** from 5 dph B(a)P exposed larvae (0.5 µg/L for 24 h). **f.** Detail of the olfactory organ from 15 dph exposed larvae (0.5 µg/L) showing strong CYP1A immunoreactivity in the ciliated olfactory sensory neurons of the olfactory organ (arrows) and in the olfactory receptor neurons which connect the olfactory organ with the olfactory bulb (arrowheads). Scale bars: 100 µm.

and cells, to sex, to routes of incorporation (food, water, sediment) and to concentration and type of contaminant, i.e. metabolizable or persistent (Smolowitz et al., 1991, 1992; Stegeman et al., 1991; Goksøyr and Förlin, 1992; Andersson and Goksøyr, 1994; Monod et al., 1994; Stegeman and Hahn, 1994; Guiney et al., 1997; Sarasquete and Segner, 2000; Arellano et al., 2001; Ortiz-Delgado et al., 2002, 2005, 2008; Ortiz-Delgado and Sarasquete, 2004; Hornung et al., 2007, among others).

Adult Seabream brain

In brain of *Sparus aurata* adults, CYP1A was constitutively expressed in both glial and neuronal cells from mainly olfactory region, and it was expressed throughout the brain in the vascular endothelia. Similar regional and cellular distribution of CYP1A has been reprorted for higher vertebrates (Walther et al., 1985), and for other fish species (Smolowitz et al., 1992; Monod et al., 1994; Gu et al., 2000; Ortiz-Delgado et al., 2002). Because of the presence of CYPs in the synaptic mitochondria of the nerve fibres, it was suggested that xenobiotic oxidation and detoxification can occur inside nerve endings. The strong induction of CYP1A in cerebral glial cells is of significance, because these cells are components of the blood-brain-barrier and also can have a potential role in the toxication-detoxication mechanisms (Walther et al., 1986, 1987).

In fish species, due to the direct and continuous contact of the olfactory organ to the external environment, the cells of the olfactory system may be potential targets of toxicant uptake, metabolism and action, thereby contributing to neurotoxicity effects (Smolowitz et al., 1992; Monod et al., 1994). The presence of highly inducible CYP1A proteins in the olfactory bulb and cerebellum is an interesting observation, since both regions are readily accessible by xenobiotics. The olfactory bulb serves the first protection line in the brain because the olfactory receptors neurons (*via* their apical dendrites) are in direct and continuous contact with the external environment (Chung-Davidson et al., 2004). Moreover, olfactory receptor neurons innervating the olfactory bulbs provide direct access to the central nervous system for certain toxicants (Hastings and Evans, 1991).

In B(a)P exposed seabream, a CYP1A induction in the brain was detectable only by immunohistochemical techniques. This observation may be explained by a high dilution of CYP1A-positive cells in the whole brain homogenates, i.e. a specific increase in the CYP1A-positive cells may turn undetectable in a whole brain homogenate containing mainly CYP1A-negative cells. In contrast to our findings on sea bream, in *Fundulus heteroclitus* and *Brachydanio rerio* exposed to B(a)P, the highest relative induction of CYP1A and mRNA expression were detected in head, brain and testis, but not in the liver (Hoffman and Oris, 2006; Wang et al., 2006).

No CYP1A catalytic activity was detected neither in the whole brain nor in specific brain regions (olfactory bulbs-cerebral hemispheres/part I, mesencephalon-diencephalon/part II and cerebellum-medulla oblongata/part IIII) of both control and B(a)P exposed *Sparus aurata* specimens. Similar negative results were indicated for TCDD exposed fish (Ortiz-Delgado et al., 2002). EROD enzymatic levels in *Sparus aurata* brain were too low to give an unequivocal signal in the catalytic assays on brain homogenates. Interestingly, CYP1A proteins are constitutively expressed and synthesized in seabream brain, and especially in the olfactory bulbs (endothelial and glial cells and nerve fibers). A CYP1A induction by B(a)P was detected by immunohistochemical analysis exclusively in seabream brain. However, detectable levels of ß-naphtoflavone-inducible EROD activity and CYP1A proteins were observed in brain of both control and exposed rainbow trout (Andersson and Goksoyr, 1994). In ß-naphtoflavone exposed trout, a higher EROD induction in olfactory organ than in liver was observed (Goksoyr and Forlin, 1992).

In brain of *Sparus aurata* adults from the control group, as previously pointed out (Ortiz-Delgado et al., 2002), CYP1A immunoreactive neuronal fibers were present in the glomerular layer of the olfactory bulbs. In the cerebral hemispheres, the ventralis telencephali pars ventrali area also contained CYP1A-positive fibers and cell bodies. In addition to neuronal tissue, the endothelia of the cerebral vascular system showed especially strong CYP1A immunostaining. In B(a)P-exposed seabream , a strong induction of CYP1A was detected in olfactory bulbs (glia, nerve fibres) and in the endothelia of the brain vascular system. CYP1A induction after xenobiotic exposure was also detected in chemosensory structures (olfactory and taste bud epithelia) of topminnows, *Poeciliopsis* sp exposed to B(a)P (Smolowitz et al., 1992). A constitutive CYP1A immunoreactivity was detectable only in the non-sensory epithelium of the olfactory organ, whereas in ß-naphtoflavone-exposed fish, CYP1A localization extends to the sensory epithelium (Saucier et al., 1999). CYP1A was clearly induced by ß-naphtoflavone in the olfactory bulbs (endothelial and glial cells and the nerve fibers) and the valvula of the cerebellum. In contrast, in the juvenile lake trout, *Salvelinus namaycush*, both control and exposed ß-naphtoflavone juvenile lake trout, showed similar CYP1A immunoreactivities in endothelia, glial cells and neurons, in the tectum mesencephali, corpus cerebelli and in the accessory cerebelloid structure (Chung-Davidson et al., 2004). In the brain of TCDD-exposed seabream, a strong induction of CYP1A mRNA and protein, but not of EROD activity was detected (Ortiz Delgado et al., 2002). Interestingly, TCDD-exposed fish showed CYP1A-immunoreactive fibres and cell bodies in the telencephalon (terminal nerve ganglion cells, cerebral hemispheres), mesencephalon (neuronal cells and fibers on the optic tectum and tegmentum, diencephalon (preoptic area), mesencephalon,

rombencephalon (area octavolateralis, 3trd ventricule), cerebellum (valvula cerebeli, corpus cerebeli and lobus vestibulolateralis) and adenohypophysis (Ortiz-Delgado et al., 2002).

Larval brain

At hatching and during larval ontogeny of seabream, CYP1A was already expressed in the developing brain. The early appearance of basal and inducible CYP1A is important for the ability of fish embryos and larvae to metabolize, eliminate and eventually detoxify endogenous substances and lipophilic xenobiotics (Sarasquete and Segner, 2000). In medaka, *Oryzias latipes*, when the larval gastrointestinal tract became functional a few days after hatching, the B(a)P metabolites were rapidly eliminated (Hornung et al., 2007). These findings indicate that some of the earliest embryonic tissues are metabolically competent and that redistribution of benzo(a)pyrene and its metabolic products occurs throughout development. Rapid metabolism of B(a)P substantially reduces the body burden of the parent chemical in the developing embryo, potentially reducing toxicity (Hornung et al., 2007).

The cellular staining for CYP1A was often weak in tissues of young fish, except for the vascular endothelia, which showed a comparatively strong immunoreactivity (Reinecke and Segner, 1998; Sarasquete et al., 1999, 2001). Strong inducibility of CYP1A by lipophilic xenobiotics (dioxins, ß-naphthoflavone), in the vascular endothelia of fish has been reported by several authors (Guiney et al., 1997; Chung-Davidson et al., 2004; Ortiz-Delgado and Sarasquete, 2004).

During larval growth of *Sparus aurata*, CYP1A immunostaining was detected from hatching onwards in olfactory placode, endothelia of the vascular system and in retina sensory cells. Staining intensity was increasing progressively during larval development. A similar tissue distribution and intensity of CYP1A immunostaining as in control seabream larvae was detected in the brains of B(a)P-exposed larvae. Sea bream larvae exposed to TCDD, which is one of the most potent inducers of CYP1A, also showed an induction of CYP1A in neuronal cells in the proximity of the olfactory bulbs (Ortiz-Delgado and Sarasquete, 2004). No CYP1A induction, in somatic or cerebral areas, was detected in seabream larvae exposed to sublethal concentrations of linear alkylbenzene suphonate (Hampel et al., 2004). Recently, Brzuzan et al. (2007) observed elevated levels of CYP1A mRNA in the brain of juvenile trout after exposure to B(a)P. Overall, it appears that accumulation of AhR-binding xenobiotics by fish can result in an induction of AhR-regulated genes such as CYP1A in the fish brain, with possible adverse consequences on brain development and function.

In fish brain, basal CYP1A immunoreactivities in endothelia, glial cells and neurons have been observed in larvae, juvenile and adult fish species (Smolowitz et al., 1991; Stegeman et al., 1991; Reinecke and Segner, 1998; Sarasquete et al., 1999, 2001; Sarasquete and Segner, 2000; Arellano et al., 2001; Ortiz-Delgado et al., 2002; Ortiz-Delgado and Sarasquete, 2004). According to Chung-Davidson et al. (2004), in *Salvelinus namaycush* exposed to ß-naphtoflavone, most of glial cells that showed CYP1A immunoreactivities were attached to the blood vessels. It is likely that these glial cells can absorb xenobiotic chemicals from the blood vessels and they constitutively produce CYP1A proteins to serve a protective function by eliminating exogenous and metabolic substances from the central nervous system. Interestingly, as previously pointed out (Walther et al., 1986, 1987), the xenobiotic oxidation and detoxification can occur inside nerve endings.

In different fish species, including *Sparus aurata*, the endothelia of the vascular system of different cerebral and somatic organs and tissues showed basal levels of CYP1A proteins and a strong xenobiotic CYP1A induction (Sarasquete et al., 1999, 2001; Sarasquete and Segner, 2000; Arellano et al., 2001; Ortiz-Delgado et al., 2002, 2005, 2007; Ortiz-Delgado and Sarasquete, 2004). Endothelial cells are the first site of interaction with blood-borne toxicants, and it has been suggested that the vascular endothelium with its wide range of physiological functions (i.e vasoactive regulators, such as arachidonic acid metabolites, prostaglandins, steroids, etc) may be the primary target for the toxic action of CYP1A-inducing substances (Stegeman and Hahn, 1994). At the same time, endothelial cells are involved in the transfer of chemicals from the blood to the underlying cells and tissues (Guiney et al., 1997; Sarasquete and Segner, 2000). Potentially, CYP1A induction and "first pass" metabolism in vascular endothelial cells may protect the underlying cells from toxic pollutants. Additionally, activated intermediate metabolites in the endothelial cells may affect both the cells and the underlying parenchymal cells. The toxicity of AhR ligands appears to correlate with CYP1A induction in endothelia, and activation of toxicants could contribute to endothelial damage and vascular pathologies (Cantrell et al., 1996; Guiney et al., 1997; Henry et al., 1997; Goksoyr and Husoy, 1998; Chung-Davidson et al., 2004).

In conclusion, from hatching onwards *Sparus aurata* expresses immunoreactive CYP1A in the brain, with preferential localization in the cerebral endothelia and in sensory organs (olfactory placode and retina) although no measurable CYP1A induction was detected in B(a)P exposed larvae. Expression levels increase during ontogeny (somatic and brain tissues). In adult male seabream, CYP1A proteins appear to be expressed constitutively at very low levels in the brain parenchyma, and the expression of CYP1A in the olfactory bulbs and endothelia can be weakly induced by B(a)P exposure. Thus, B(a)P reaching the brain *via* blood could be metabolized at least partly metabolized already in the cerebral endothelial system.

From a technical point of view, the use of

immunohistochemical methods is particularly advisable for heterogeneously composed tissues such as the brain containing only a small fraction of CYP1A-positive cells.

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