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Effects of 17β-estradiol, 4-nonylphenol and PCB 126 on the estrogenic activity and phase 1 and 2 biotransformation enzymes in male sea bass (*Dicentrarchus labrax*)

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Abstract

The endocrine system of wildlife is exposed to a wide variety of natural and man-made chemicals which may lead to damage to the reproductive system and other adverse effects, including alteration of drug-metabolizing enzymes. In the present study, the effects of in vivo exposure to a natural (17β-estradiol: E2) or a xenoestrogen (4-nonylphenol: NP) estrogen or an anti-estrogen (3,3′,4,4′,5-pentachlorobiphenyl: PCB 126) upon vitellogenin (VTG) synthesis and hepatic phase 1 and 2 enzymes have been investigated in adult male sea bass. By means of ELISA analysis with the use of polyclonal antibodies prepared against VTG purified from E2-treated sea bass, we assessed the time course and sensitivity of VTG induction in the plasma of sea bass treated with E2 at 0.1, 0.5, 2.5 and 5.0 mg/kg doses or NP at 5.0 or 50 mg/kg doses, respectively. Sea bass sensitivity to this induction was found to be similar to that of other fish species, but with a delay in maximal response. E2 treatment also caused a selective time- and dose-dependent inhibition of hepatic CYP1A-linked EROD and phase 2 glutathione *S*-transferase (GST) activities, without affecting the activity of CYP3A-linked 6β-testosterone hydroxylase, (ω)- and (ω-1)-lauric acid hydroxylases or phase 2 DT-diaphorase. A similar selective inhibition on CYP1A was also observed in fish treated with 50 mg/kg NP. The results regarding CYP1A and CYP3A were also confirmed by Western blot analysis. When the sea bass were treated with either 10 or 100 μg/kg PCB 126, an AhR ligand not yet tested in vivo in fish to assess its anti-estrogenicity, a modest and selective induction of EROD and DT-diaphorase activities was observed. Interestingly, both these activities were recovered to their control levels in sea bass co-treated with 0.5 mg/kg E2 and 10 or 100 μg/kg PCB 126, probably through a cross-talk mechanism between the estrogen receptor and AhR or other transcription factors that regulate the expression of these enzymes. Furthermore, it was demonstrated that PCB 126 possesses a potent anti-estrogenic activity in the sea bass in vivo as it inhibited the E2-induced VTG synthesis with an IC50 of 28 μg/kg. The results of this study suggest that the exposure of fish to xenoestrogens or anti-estrogens

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may alter, in addition to various physiological processes, the expression of specific CYPs and phase 2 enzymes, thereby reducing the capability of their detoxication system.

Keywords: Sea bass; *Dicentrarchus labrax*; Vitellogenin; 17β-Estradiol; 4-Nonylphenol; PCB 126; CYP1A; CYP3A; Glutathione *S*-transferase; DT-diaphorase

1. Introduction

There is increasing evidence that man-made chemical compounds can interact with the endocrine systems of fish and wildlife, inducing reversible and irreversible alterations to the reproductive and developmental hormone-related processes (Vos et al., 2000; Fairbrother et al., 1999). Some endocrine-disrupting compounds, the so-called xenoestrogens, which mimic the endogenous estrogens, can bind to the estrogen (ER) or androgen (AR) receptors and stimulate abnormal hormonal responses. Alternatively, certain endocrine disrupters may act as anti-estrogenic agents which antagonize the normal hormone receptor pathway, inhibiting the expression of target genes, including the gene coding vitellogenin (VTG), the egg yolk protein precursor (Guillette et al., 1994; Knudsen et al., 1997).

Many studies have focused on reproductive and developmental disturbances in fish, caused by chemicals with an estrogenic or anti-estrogenic activity, but less attention has been given to their potential to alter the xenobiotic-metabolizing system (phase 1 and 2), and therefore the detoxification capability of these organisms (Segner et al., 2003).

In regard to oxidative enzymes, a number of reports have indicated that the suppression of the hepatic CYP1A and associated EROD activity occurs in various male or female fish after experimental treatment with 17β-estradiol (E2) or other xenoestrogens (Forlin and Haux, 1990; Buhler et al., 1997; Arukwe and Goksøyr, 1997), although investigations into the effects of this hormone on other CYP isoforms in fish species are scarce.

Buhler et al. (2000) showed that in juvenile trout, the administration of E2 depresses the hepatic CYP content, the expression of CYP2K1- and CYP2M1-linked lauric acid hydroxylase and CYP3A-dependent 6β-progesterone hydroxylase in the female, but only 6β-progesterone hydroxylase in the male. Arukwe et al. (1997) described the inhibition by E2 of 6β-progesterone in the juvenile Atlantic salmon, but Hasselberg et al. (2004) did not observe this inhibition in the liver of male adult cod.

In regard to the phase 2 enzymes, previous studies in largemouth bass have demonstrated that the expression of glutathione *S*-transferase (GST) and DT-diaphorase may be altered by in vivo exposure to E2 and alkylphenols in a dose- and time-dependent manner (Pham et al., 2004; Hughes and Gallagher, 2004).

Regarding the anti-estrogenicity of xenobiotics, which is generally assessed by the suppression of the estrogen-dependent synthesis of vitellogenin, in vitro and in vivo studies in various fish species have demonstrated that many polycyclic aromatic compounds, including the polychlorinated derivatives, possess this activity (Safe and Krishnan, 1995; Anderson et al., 1996; Santodonato, 1997; Navas et al., 2004). Furthermore, it has been observed that the anti-estrogenicity of these compounds is linked to their ability to bind to the aryl hydrocarbon receptor (AhR) which regulates the activation of CYP1A and, at least in mammals, other genes that codify phase 1 and 2 enzymes (Nebert et al., 1990). In mammalian cells and in fish, it has also been ascertained that inhibition of estrogen-induced responses by AhR ligands is due to a complex molecular interaction (cross-talk) between AhR and ER (Brunnberg et al., 2003; Bemanian et al., 2004; Pascussi et al., 2004).

The present work was undertaken to ascertain the in vivo susceptibility of the estrogen receptor and oxidative and non-oxidative drug-metabolizing enzymes in adult male sea bass (*Dicentrarchus labrax*) to respond in a time- and concentration-dependent manner to the challenge of estrogenic and anti-estrogenic pollutants which may be present in specific aquatic environments.

The sea bass was selected since this fish species is widely cultured in brackish-marine water of the

Mediterranean basin. This study was performed only in male fish, as the specific conditions, including warm temperature and feeding of many Italian intensive aquacultures of sea bass, select nearly exclusively (75–100%) this sex (Piferrer et al., 2005).

The estrogenic sensitivity of sea bass by using E2 and the xenoestrogen 4-nonylphenol (NP), which is resistant to biodegradation and bioaccumulates in aquatic organisms (Ahel et al., 1994), was assessed.

The anti-estrogenic sensitivity of sea bass was assessed by the co-administration of E2 and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), a strong AhR agonist. PCB 126 is a very lipophilic planar PCB which can promote hepatocarcinoma, developmental interference and immunotoxicity, and is ubiquitous in the environment (Safe, 1994). This PCB congener has been selected for its potency to induce the liver detoxification system through its high-intrinsic efficacy in binding to AhR (Hestermann et al., 2000). Although PCB 126 has been found to be the most toxic congener in fish, with a toxic equivalent factor (TEF) of 0.1 (Van den Berg et al., 1998; Newsted et al., 1995), its anti-estrogenic activity has not yet investigated in vivo in any piscine species. In addition, the effects of various treatments with E2, NP and PCB 126 on the biotransformation of some CYP isoforms, GST and DT-diaphorase enzymes were evaluated in sea bass liver.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2), 4-nonylphenol (NP), horseradish peroxidase-conjugated goat anti-rabbit antibodies (HRP), phenylmethylsulphonylfluoride (PSMF), 1,2-phenylenediamine dihydrochloride (OPD) were purchased from Sigma (St. Louis, MO, USA); 3,3′,4,4′,5-pentachlobiphenyl (PCB126) was from Dr. Ehrenstorfer (GmbH, Germany); 6B-Sepharose and DEAE-Sepharose were from Amersham Biosciences (UK). Rabbit polyclonal antibodies against rat CYP1A1 were obtained in our lab, as previously described by Novi et al. (1998), while the anti-trout CYP3A27 was kindly supplied by Dr. M. Celander (Goteborg, Sweden). Anti-seabream/anti-turbot/anti-salmon/anti-carp VTG antibodies were from commercial source (Biosense Laboratories AS, Bergen, Norway). All other chemicals and solvents were of analytical grade and were obtained from common commercial sources.

2.2. Experimental animals

Adult male, sexually mature, European sea bass (*D. labrax*, L., 1758) (520 ± 130 g) were obtained from commercial fish farms and maintained in 500 l tanks containing 35‰ salinity marine water maintained at a temperature of 18 °C. Experiments began after an acclimatization period of at least 2 weeks.

Different groups of fish were injected i.p. (in corn oil as vehicle) with:

1. E2 0.1, 0.5, 2.5 and 5 mg/kg for 3, 7, 14 and 28 days.
2. NP 5 and 50 mg/kg for 3, 7, 14 and 28 days.
3. PCB126 10 and 100 μg/kg for 3, 7, 14 and 28 days.
4. PCB126 10 and 100 μg/kg + E2 0.5 mg/kg for 3, 7, 14 and 28 days.

For NP and PCB126 experimental doses we referred to Arukwe et al. (2001) and to Schlezinger and Stegeman (2001), respectively.

Ten fish, acting as control, received only the vehicle; fish were sacrificed at day 3 ($n = 4$), day 7 ($n = 3$) and day 28 ($n = 3$) post the vehicle treatment.From each fish, blood samples were collected from the caudal vein in heparinized syringes, and were centrifuged immediately (1500 × g for 20 min). Fish were then sacrificed, livers excised and microsomes immediately prepared in accordance with the method described by Novi et al. (1998). Procedures for the care and management of animals were performed in accordance with the provisions of the EC 91 Council Directive 86/609 EEC, recognized and adopted by the Italian Government (DL 27.01.1992, no. 116).

2.3. Hormonal induction of VTG synthesis and blood sampling

Two adult male sea bass were injected i.p. with 2 mg/kg body weight of E2 dissolved in corn oil. The E2-treated males received four injections, spaced 2 days apart. Fifteen days after the last injection, blood was collected from the caudal veins, using 10 ml lithium-heparinized vacuum tubes. Plasma was stored in the presence of PMSF (1 mM) in order to reduce proteolysis and degradation of VTG prior to isolation. Plasma was then separated by centrifugation at

1500 × *g* for 20 min (4 °C) and frozen (−80 °C) until further analysis was undertaken. After the blood was collected, the fish were killed by a blow to the head, dissected, and sex and maturational status were determined by visual inspection of gonads.

2.4. Purification of VTG and antibody production

VTG of sea bass was isolated in accordance with the method described by Mañanós et al. (1994). Briefly, 1 ml plasma from E2-treated fish was applied to a 6B-Sepharose column and fractions containing VTG (identified by native and SDS-PAGE gel electrophoresis) were applied to a DEAE-Sepharose column. The whole procedure was performed at 4 °C. Freshly purified VTG was used for polyclonal antibody production, coating of plate wells and as the standard in the ELISA assay (stored at −80 °C).

Polyclonal antibodies to purified sea bass VTG were raised in rabbits using standard immunological methods. The specific antibodies against VTG were purified from rabbit serum by affinity chromatography in accordance with the method described by Korsgaard and Pedersen (1998).

2.5. Electrophoresis and Western blot

Serum samples and purified VTG were analysed by native and SDS-PAGE electrophoresis performed in accordance with the method described by Laemmli (1970), using a 4.5% stacking gel and a 9% resolving gel of acrylamide. Gels from electrophoresis were stained with Coomassie Brilliant Blue R-250 or were used for Western blot analysis (Towbin et al., 1979), using both heterologous and homologous anti-seabream/anti-turbot/anti-salmon/anti-carpVTG antibodies. The immunodetection for the CYPs in the control and treated microsomes was performed using polyclonal antibodies raised against rat P450 1A1 and trout hepatic P450 3A27, as previously described (Novi et al., 1998). The quantification of Western blots was performed using Scion Image Software (Scion Corporation, Frederick, MD, USA).

2.6. ELISA

Using a procedure modified from Specker and Anderson (1994), an antibody capture ELISA was developed for measuring VTG in plasma. In this assay, antigens bound to the wells of 96-well microtiter plates (Nunc-Immuno Plates, 96-well plate) and unbound sample antigens compete for the anti-VTG antibody-binding sites. Absorbance values were measured at 490 nm using a Biorad, microplate-plate reader (Model 550). In the ELISA analysis, the range of the standard curve was between 4 and 1000 ng/ml, with 50% of binding around 64 ng/ml. Interassay variation determined for 30, 50 and 80% of binding were 7.3 ± 1.9, 7.2 ± 2.4 and 4.8 ± 2.9%, respectively, calculated for 20 standard curves. The intra-assay variation within one assay conducted on different plates, calculated at 50%, binding was 4.3 ± 1.2% ($n = 8$, VTG standard replicates). Serial dilutions of plasma samples from adult female and estrogenized male sea bass showed a good parallelism with the standard VTG curve.

2.7. Enzyme assays

Erythromycin *N*-demethylase (ErD) activity was determined by measuring the formation of formaldehyde (Tu and Yang, 1983); ethoxyresorufin-*O*-deethylase (EROD) activity was determined by measuring the resorufin formation in a Perkin-Elmer spectrofluorimeter (Krijgsheld and Gram, 1984) and protein concentrations were determined in accordance with the method described by Lowry et al. (1951) using bovine serum albumin as the standard. Testosterone hydroxylase was assayed in accordance with an HPLC method described by Platt et al. (1989), with slight modifications (Longo et al., 1991). The testosterone metabolites were resolved in a C18 RP Supelco column (250 mm × 4.6 mm) using an isocratic elution with methanol plus tetrahydrofuran (THF) 7.5% and H_2O + THF 7.5% (27:73, v/v) for 8 min, followed by a linear gradient reaching a 42:58 (v/v) ratio for 12 min and a final period of isocratic elution for a further 10 min. (ω)- and (ω-1)-lauric acid hydroxylase were determined by HPLC (Zanelli et al., 1996). Glutathione *S*-transferase was determined in the cytosolic fraction, using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (Habig et al., 1974). DT-diaphorase activity was determined at 600 nm following the reduction of dichlorophenolindolphenol (an electron acceptor) to an uncoloured metabolite (Benson et al., 1980).

2.8. Statistical analysis

Results are reported as means ± S.D. Significant differences between means of various fish treatment groups were determined by analysis of variance (ANOVA) and means were contrasted using Dunnett's *t*-test. Some comparisons were performed by Student's *t*-test. All statistical analyses were carried out using Prism, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Preparation of vitellogenin and antibodies

VTG was purified from plasma of E2-treated adult male fish as described in Section 2. VTG was purified as a major band of about 180 kDa with four minor bands corresponding to breakdown products, as previously reported (Mañanós et al., 1994).

Polyclonal antibodies against this purified VTG were prepared, and their specificity was assayed by Western blotting of plasma from control and E2-treated male sea bass. These antibodies recognized in the blood serum of E2-treated male fish a strong protein band, which had the same M_r (180 kDa) as purified VTG, and other minor bands with a lower M_r, possibly breakdown products, while no signal was detected in untreated male sea bass.

For a further characterization of specificity, the immunoreactivity of commercial available heterologous anti-VTG antibodies was tested against the purified sea bass VTG by Western blot and ELISA. In Western blot analysis anti-seabream and anti-turbot VTG cross-reacted very weakly with the purified sea bass VTG, whereas anti-salmon and anti-carp VTG completely failed to detect the protein (not shown). In ELISA the high specificity of the anti-sea bass VTG compared to heterologous antibodies and the absence of reactivity of the heterologous antibodies was demonstrated (Fig. 1).

3.2. VTG induction by E2 and NP

E2 treatment induced an increase in plasma levels of VTG showing a dose- and time-dependent effect (Fig. 2). All the E2 doses caused a rapid formation of VTG in plasma, with a peak on day 14. The pattern of VTG induction appeared to be qualitatively similar for all E2 doses. The highest levels of VTG synthesis (5231 ± 1360 μg/ml of plasma) were found at the dose of 0.5 mg/kg on day 14, with an increase of about 1300-fold with respect to the control value.

Whereas the 5 mg/kg NP-treated fish had a time course profile of VTG levels not significantly different from that of controls, the 50 mg/kg NP-treated fish showed an induction pattern of VTG synthesis (not shown) quite similar to that of 0.5 mg/kg E2-treated fish, with a maximum of synthesis at the day 14 (4922 ± 922 μg/ml plasma).

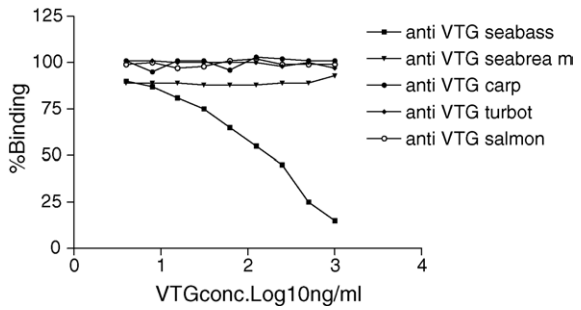

Fig. 1. Dilution curves and % of binding of homologous and heterologous antibodies against purified sea bass VTG.

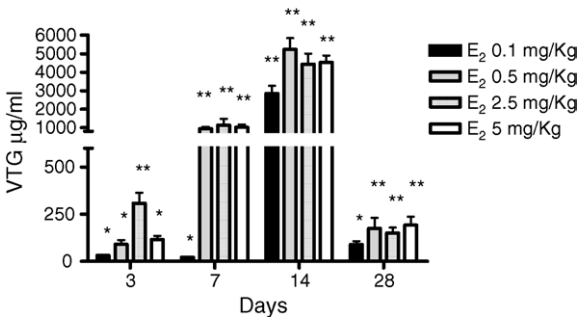

Fig. 2. Time course and dose-dependent VTG induction in plasma of E2-treated (i.p., single injection) adult male sea bass at the dose of 0.1, 0.5, 2.5 and 5.0 mg/kg and sampled at day 3, 7, 14 and 28, respectively. Values are expressed as μg/ml of plasma. VTG level in control fish was 4.03 ± 2.32 μg/ml. Control fish received only the vehicle (corn oil). Data are means ± S.D. (bars) of five fish. **Significantly different from control values for $p < 0.01$, $^{*}p < 0.05$ (ANOVA).

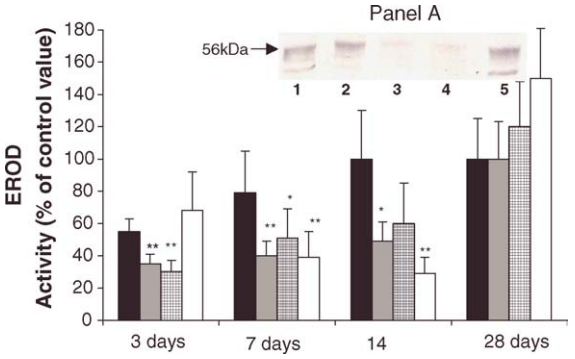

Fig. 3. Time course and dose-dependent inhibition of EROD activity in microsomes from liver of fish treated with a single injection of 0.1 mg/kg (■), 0.5 mg/kg (▩), 2.5 mg/kg (▦) and 5 mg/kg (□) E2. Values are expressed as percentage of the hepatic microsomal EROD activity (81 ± 32 pmol/min/mg protein) of control fish set at 100%. The control EROD value was the mean ± S.D. of the very similar activities obtained from three groups of fish sacrificed at day 3 ($n = 4$), 7 ($n = 3$) and 28 ($n = 3$) post vehicle-injection. Data are the means ± S.D. of five experiments. *Significantly different from control at $p < 0.05$ or **$p < 0.01$ (ANOVA). (Panel A) Western blots of hepatic microsomes from control and 5 mg/kg E2-treated fish—lane 1: control microsomes; lane 2: microsomes from E2-treated fish at day 3; lane 3: microsomes from E2-treated fish at day 7; lane 4: microsomes from E2-treated fish at day 14; lane 5: microsomes from E2-treated fish at day 28. Microsomes (30 μg of protein) were subjected to electrophoresis, blotted in nitrocellulose sheets and probed with polyclonal antibody anti-CYP1A1.

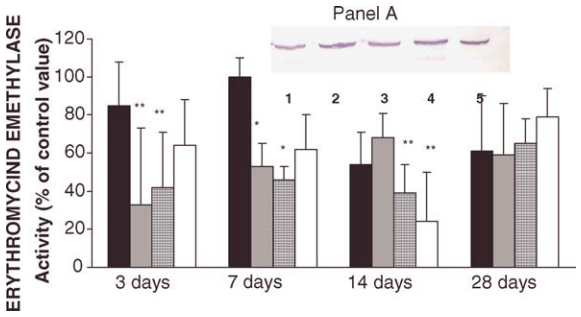

Fig. 4. Time course and dose-dependent inhibition of erythromycin demethylase activity in microsomes from liver of fish treated with a single injection of 0.1 mg/kg (■), 0.5 mg/kg (▩), 2.5 mg/kg (▦) and 5 mg/kg (□) E2. Values are expressed as percentage of the hepatic microsomal erythromycin demethylase activity (848 ± 399 pmol/min/mg protein) of control fish (determined as in Fig. 3) set at 100%. Data are the means ± S.D. of five experiments. *Significantly different from control at $p < 0.05$; **$p < 0.01$(ANOVA). (Panel A) Western blots of hepatic microsomes from control and 5 mg/kg E2-treated fish—lane 1: control microsomes; lane 2: microsomes from E2-treated fish at day 3; lane 3: microsomes from E2-treated fish at day 7; lane 4: microsomes from E2-treated fish at day 14; lane 5: microsomes from E2-treated fish at day 28. Microsomes (30 μg of protein) were subjected to electrophoresis, blotted in nitrocellulose sheets and probed with polyclonal antibody anti-CYP3A27.

3.3. Effects of E2 on CYP activities

Sea bass treatment with E2 at 0.1, 0.5, 2.5 and 5 mg/kg caused a time- and concentration-dependent decrease of the activities of hepatic EROD (Fig. 3) as a marker of CYP1A (Stegeman and Hahn, 1994) and erythromycin demethylase (Fig. 4), as a marker for various CYP isoforms (Stegeman and Hahn, 1994), in comparison to control values.

At 0.1 mg/kg, E2 had no significant effect on these activities at any of the times, while higher E2 concentrations, 3 and 7 days post treatment, showed a consistent reduction, although not always significant, of both these monooxygenases, whereas on day 14 only a 2.5 mg/kg or higher E2 concentration caused a decrease in these activities. After any E2 treatment, the activities of both EROD and erythromycin demethylase had recovered the control levels on day 28.

When 6β-testosterone hydroxylase, a known marker of CYP3A (Buhler et al., 2000; Hasselberg et al., 2004), was assayed, no significant reduction of its activity was observed in the hepatic microsomes of treated fish compared with the control value (86 ± 18 pmol/min/mg protein). Also (ω)- and (ω-1)-lauric acid hydroxylases, which displayed values of 51 ± 16 and 41 ± 24 nmol/min/mg protein, respectively, in control fish, and are probably associated to CYP2K and CYP2M in sea bass, as in trout (Buhler et al., 2000), were not significantly affected by any E2 treatment.

The activity data related to CYP1A and CYP3A were confirmed by Western blotting analysis. As shown in panel A of Fig. 3, polyclonal anti-CYP1A1 antibodies recognized a major protein band in control liver microsomes which was found to be drastically reduced to about 10% of the control value in microsomes from 5 mg/kg E2-treated fish on day 7 and day 14, but was recovered to the control level on day 28. A similar pattern was observed in the immunoblots (not shown) of microsomes from fish treated with 0.5 or 2.5 mg/kg E2. The minor protein band with a lower M_r recognized by

these antibodies may be a cross-reaction or a fragment of the CYP1A breakdown. The anti-trout CYP3A27 recognized a single band in control microsomes, which was not altered in any microsomes from the 5 mg/kg E2-treated fish (panel A of Fig. 4), consistently with the activity data obtained for 6β-testosterone hydroxylase.

3.4. Effects of NP on CYP activities

Compared with the corresponding control values sea bass treatment with 50 mg/kg NP significantly reduced both the EROD and erythromycin demethylase activities only on day 14 but at day 28 these activities had recovered from NP-induced decrease (Fig. 5). The treatment with a lower concentration of NP (5 mg/kg) failed to decrease these monooxygenases compared with control values and, as observed with E2 neither of the NP treatments influence 6β-testosterone hydroxylase at any time (data not shown). Also in this case, immunoblot analysis confirmed the activity results. As shown in Fig. 5 (panel A), anti-rat CYP1A1 recognized a major protein band in the liver microsomes from 50 mg/kg NP treated fish, which decreased with time, reaching the minimum value (35% of control) 14 days post treatment; only on day 28 was the intensity of this band partially restored to the control value. The minor band, an immunoreactive contamination or a fragment of CYP1A breakdown, was less sensitive to reduction by NP treatment. In agreement with the 6β-testosterone hydroxylase activity, the intensity of the protein band recognized by anti-trout CYP3A27 was not influenced by NP treatment at any time.

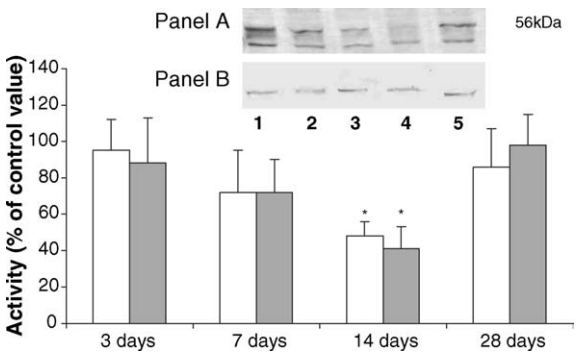

Fig. 5. Time course inhibition (3, 7, 14 and 28 days) of EROD (□) and erythromycin demethylase (■) activity in microsomes from liver of fish treated with a single injection of 50 mg/kg NP. Values are expressed as percentage of the hepatic microsomal EROD activity (81 ± 32 pmol/min/mg protein) and erythromycin demethylase activity (848 ± 399 pmol/min/mg protein) of control fish (determined as in Fig. 3) set at 100%. Data are the means ± S.D. of five experiments. *Significantly different from control at $p < 0.05$ (ANOVA). (Insert) Western blots of hepatic microsomes from control and 50 mg/kg NP-treated fish—lane 1: control microsomes; lane 2: microsomes from NP-treated fish at day 3; lane 3: microsomes from NP-treated fish at day 7; lane 4: microsomes from NP-treated fish at day 14; lane 5 microsomes from NP-treated at day 28. Microsomes (30 μg of protein) were subjected to electrophoresis, blotted in nitrocellulose sheets and probed with polyclonal antibody anti-CYP1A1 (panel A), anti-CYP3A27 (panel B).

3.5. Effects of E2 and NP on GST and DT-diaphorase activities

As shown in Fig. 6, the GST activity was significantly inhibited in a dose- and time-dependent manner by E2 treatment. This activity returned to the control level on day 7 of 0.1 mg/kg E2 exposure, whereas following 0.5 and 2.5 mg/kg E2 exposure, the activity was restored only on day 14 and day 28, respectively. In the case of 5 mg/kg treatment, the GST activity remained significantly inhibited at 28 days. Unlike GST, none of the E2 treatments altered the control level (1.6 ± 0.45 nmol/min/mg protein) of DT-diaphorase activity at any time. In the case of 50 mg/kg NP-treated fish, there were no significant effects on the activities of either hepatic GST or DT-diaphorase (data not shown).

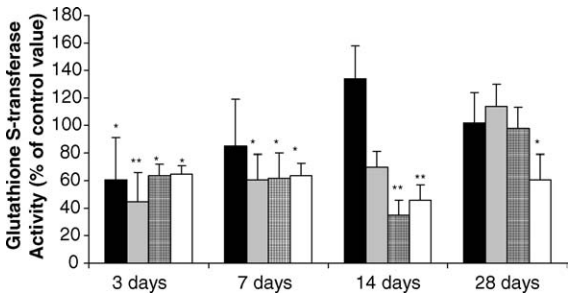

Fig. 6. Time course and dose-dependent inhibition of glutathione *S*-transferase activity in cytosol from liver of fish treated with a single injection of 0.1 mg/kg (■), 0.5 mg/kg (▩), 2.5 mg/kg (▦) and 5 mg/kg (□) E2. Values are expressed as percentage of the hepatic cytosol GST activity (61.8 ± 12.3 pmol/min/mg protein) of control fish (determined as in Fig. 3) set at 100%. Data are the means ± S.D. of four experiments. *Significantly different from control at $p < 0.05$; **$p < 0.01$ (ANOVA).

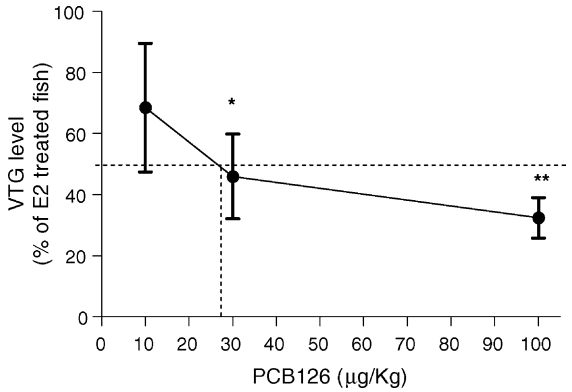

Fig. 7. The anti-estrogenicity of PCB 126 in sea bass. Fish were co-treated with 0.5 mg/kg E2 and PCB 126 at doses of 10, 30 or 100 μg/kg and sampled 14 days post-treatment. VTG levels were expressed as percentage of 0.5 mg/kg E2-treated sea bass VTG value (5231 ± 1360 μg/ml of plasma). Data are the means ± S.D. (bars) of five fish. **Significantly different from E2-treated sea bass VTG value for $p < 0.01$, *$p < 0.05$ (ANOVA).

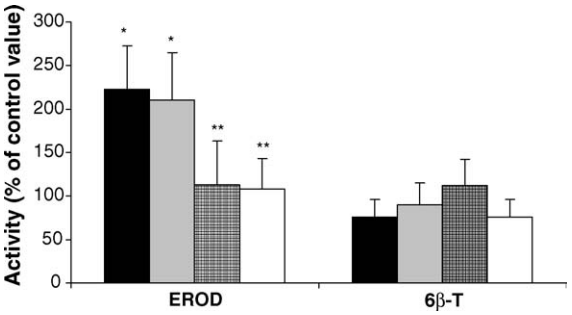

Fig. 8. Effects on the hepatic microsomal EROD and 6β-testosterone hydroxylase activities by a single injection of 10 μg/kg (■) or 100 μg/kg (▩) PCB alone or PCB 10 μg/kg + E2 0.5 mg/kg (▤) or PCB100 μg/kg + E2 0.5 mg/kg (□) in fish 7 days post treatment. Values are expressed as percentage of the hepatic microsomal EROD (81 ± 32 pmol/min/mg protein) and 6β testosterone hydroxylase activity (86 ± 18 pmol/min/mg protein) of control fish set at 100%. Data are the means ± S.D. of five experiments. *Significantly different from control value by Student's *t*-test at $p < 0.05$. **Significantly different from microsomal activities of 10 or 100 μg/kg PCB treated fish, respectively, by Student's *t*-test at $p < 0.05$.

3.6. Anti-estrogenic activity of PCB 126 on E2-induced VTG synthesis

In order to assess the reduction of VTG secretion by PCB 126, fish were co-administered 0.5 mg/kg E2 and three doses (10, 30 and 100 μg/kg) of the poly chlorinated compound, and were then sacrificed after 14 days. A dose-dependent reduction of plasmatic VTG caused by PCB 126 was clearly observed (Fig. 7). The PCB 126 concentration that corresponded to a 50% reduction (IC50) of the E2-induced VTG synthesis was about 28 μg/kg. The administration of 100 μg/kg PCB 126 alone was without any effect on the VTG synthesis.

3.7. Effects of PCB 126 and PCB 126/E2 co-treatment on CYP1A and CYP3A

The administration of 10 or 100 μg/kg PCB 126 in the sea bass similarly increased hepatic EROD in a time-dependent manner; this CYP1A marker activity was about 2.5-fold above the control level 7 days after both PCB treatments (Fig. 8) and stayed as a plateau for at least 14 days, while after 28 days it nearly returned to the control value (not shown). By contrast, 6β-testosterone hydroxylase was not influenced by these treatments (Fig. 8). The co-administration of 0.5 mg/kg E2 and PCB 126 at either 10 or 100 μg/kg led to a reduction of EROD activity to the control level, whereas no effect was observed in 6β-testosterone hydroxylase activity.

Confirmation of the PCB 126 effects on these activities was provided by the immunoblots (Fig. 9). As previously shown in Fig. 3 panel A, anti-CYP1A1 recognized two protein bands in the microsomal samples. However, only the intensity of the band with the higher M_r, corresponding to CYP1A, was significantly increased (to about 330–360% of control) by both PCB

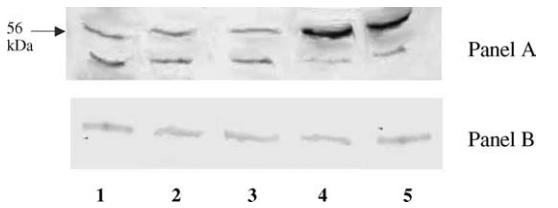

Fig. 9. Western blots of hepatic microsomes from control and treated fish with 10 or 100 μg/kg PCB alone or treated with PCB 10 or 100 μg/kg + E2 0.5 mg/kg at day 7—lane 1: control microsomes; lane 2: microsomes from treated fish with PCB 10 μg/kg + E2; lane 3: microsomes from treated fish with PCB 100 μg/kg + E2; lane 4: microsomes from treated fish with PCB 10 μg/kg; lane 5: microsomes from treated fish with PCB 100 μg/kg. Microsomes (10 μg of protein) were subjected to electrophoresis, blotted in nitrocellulose sheets and probed with polyclonal antibody anti-CYP1A1 (panel A), anti-CYP3A27 (panel B).

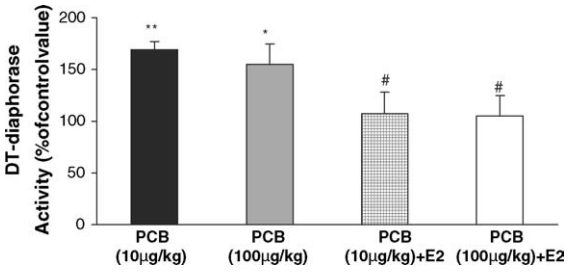

Fig. 10. DT-diaphorase activity in hepatic cytosol from fish 7 days post a treatment with a single dose of only PCB at 10 μg/kg (■) and 100 μg/kg (▩) or PCB 10 μg/kg (▤) and 100 μg/kg (□) in combination with 0.5 mg/kg E2. Enzymatic activity is expressed as % of control value (1.60 ± 0.45 nmol/min/mg protein), set at 100%. Data are the means ± S.D. (bars) of four experiments. *Significantly different from the control at $p < 0.05$ or **$p < 0.01$ (ANOVA). #Significantly different from cytosolic activities of 10 and 100 μg/kg PCB treated fish, respectively, at $p < 0.05$ (ANOVA).

treatments, whereas the co-treatment of E2 with the two doses of PCB 126 reduced the intensity of the CYP1A bands to the control level. On the other hand, no difference was seen in the anti-CYP3A27 immunoreactive proteins among microsomes from control and treated fish.

3.8. Effects of PCB 126 and PCB 126/E2 co-treatment on DT-diaphorase and GST activities

Treatment with PCB 126 at either 10 or 100 μg/kg showed a similar increase of the DT-diaphorase activity on day 7, which was found to be significantly higher (about 1.6-fold) than the control value (Fig. 10). Co-treatment of 0.5 mg/kg E2 with PCB 126 at 10 or 100 μg/kg reduced this activity to the control level. On the other hand, the GST activity was not influenced by the treatments of either of the PCB 126 concentrations.

4. Discussion

The present study preliminarily determined the estrogenic sensitivity of sea bass in terms of VTG induction by E2 and NP using an ELISA method with specific antibodies. The available commercial heterologous antibodies raised against VTG from other fish species were scarcely able or even unable, to detect plasma VTG in the sea bass, in keeping with previous findings on the VTG of other fish (Watts et al., 2003). The sensitivity of VTG detection in sea bass by ELISA was about 4 ng/ml, similar to the sensitivity found for *Pleuronectes vetulus* (Lomax et al., 1998). However, on day 14, after E2 doses of 0.5 mg/kg or higher ones, we observed a saturation of the VTG response which appeared to be delayed compared with those (of about 5–8 days) found in rainbow and brown trout (Sherry et al., 1999; Schultz et al., 2001) or zebrafish (Rose et al., 2002).

A similar delay in the VTG maximum induction, unlike in juvenile Atlantic salmon (Arukwe et al., 2001), was also noted in the plasma of fish treated with the NP (50 mg/kg), further suggesting a specificity in the time course of the ER response to natural or xenoestrogens in sea bass.

An important aim of the present investigation was to assess the effects of E2 and NP on some phase 1 and 2 drug-metabolizing enzymes in the adult male sea bass. Consistently with previous reports (Forlin et al., 1984; Buhler et al., 1997; Arukwe and Goksøyr, 1997), the present study, shows that E2 treatments in sea bass decreased, in an apparently dose- and time-dependent manner, the hepatic microsomal activity of EROD and ErD, the latter catalysed by various CYP isoforms (Stegeman and Hahn, 1994). Further, E2 treatment did not alter the (ω)- or (ω-1)-lauric acid hydroxylase activities, in agreement with the data found for the juvenile male trout (Buhler et al., 2000). However in sea bass, unlike in juvenile male trout (Buhler et al., 2000) and juvenile salmon (Arukwe et al., 1997), the microsomal 6β-testosterone hydroxylase activity [a marker of CYP3A isoforms (Buhler et al., 2000; Hasselberg et al., 2004)], was not affected by any E2 treatment.

Confirmation of this pattern was also offered by Western blot analysis. Also the administration of 50 mg/kg NP, known to mimic the action of the endogenous estrogens, caused a significant decrease of EROD and ErD activities, but not a decrease of 6β-testosterone hydroxylase activity. Taken together, the results concerning the effects of estrogens on CYPs suggest that the expression and activity of CYPs in the adult male sea bass are less susceptible to respond to xeno- or natural estrogens than the juvenile male and/or female trout and salmon, where a reduction by estrogens of CYP activities linked to the isoforms 2K1, 2M1 an 3A, in addition to 1A, was observed (Buhler et al., 2000). Consistent with the present findings, Hasselberg et al. (2004) demonstrated that in the adult male cod, only

CYP1A but not CYP3A was decreased by E2 or 4-alkylphenol treatment.

Thus, a down-regulation by estrogens of CYP3A-associated activities observed in juvenile male trout and salmon, but not in adult sea bass and cod, may reflect a difference in the hormonal regulation of CYP3A isoforms during the development and adulthood of teleostes, as found in mammals (Lund et al., 1991), rather than being a feature of certain fish species.

On the other hand, the down-regulation of CYP1A by estrogens found in adult sea bass confirms that this is a general phenomenon in fish. Thus, the adult male sea bass from aquaculture exposed to xenoestrogens are less efficient in the CYP1A-dependent detoxification of PAH, as ubiquitous contaminants, and consequently they may be more prone to undergo toxicity and cancer.

This aspect may be further confirmed by considering that E2 treatment, although not with the less estrogenic NP, reduced in a dose- and time-dependent manner the activity of GST, a very important family of enzymes to cope with the risk of cellular damage provoked by electrophilic xenobiotics. At variance with our results, inhibition by E2 and/or NP of GST activity, as measured by the general substrate CDNB, was not clearly observed in the juvenile sea bass (Teles et al., 2004) and in the juvenile largemouth bass, although in the latter the estrogens were administered in a similar manner and doses as in the present study (Hughes and Gallagher, 2004).

The other aim of this study was to investigate the anti-estrogenic sensitivity of adult male sea bass towards PCB 126 in terms of inhibition of VTG synthesis and of phase 1 and 2 drug-metabolizing enzymes. The results showed that PCB 126 has a strong anti-estrogenic activity as it inhibited E2-induced VTG synthesis with an IC50 of about 28 μg/kg. The single administration of PCB 126 at 10 μg/kg induced about 2.5-fold the CYP1A-dependent EROD activity. However, PCB 126 at 100 μg/kg did not further induce EROD, as it would be expected for a classic CYP inducer, whereas both the PCB 126 treatments increased the immunoreactive CYP1A apoprotein by about 3.8-fold. This discrepancy between apoprotein and activity levels may be due, as previously observed in scup by Schlezinger and Stegeman (2001), to a specific inactivation of CYP1A enzyme in sea bass by reactive oxygen species produced by uncoupling during the in vivo PCB 126 metabolism.

The co-administration of PCB 126 and E2 reduced the EROD activity to approximately the control level without affecting the CYP3A-linked 6β-testosterone hydroxylase activity. Western blot analysis confirmed this selective inhibition of CYP1A, but not of the CYP3A, probably through a mechanism of ER and AhR cross-talk, as previously reported (Brunnberg et al., 2003; Bemanian et al., 2004; Pascussi et al., 2004).

Previous reports have demonstrated that co-treatment of E2 and β-naphthoflavone (a typical AhR ligand) down-regulates the CYP1A expression and EROD activity in cultured trout hepatocytes (Navas and Segner, 2000, 2001) and in vivo experiments in juvenile sea bass (Teles et al., 2004). As previously seen in mammals, PCB 126 at both 10 and 100 μg/kg doses induced cytosolic DT-diaphorase activity (about 1.6-fold of control), indicating that this enzyme, at least partially, might be regulated also in fish by AhR (Nebert et al., 1990). This result was unexpected as a strong ligand like β-naphthoflavone failed to induce DT-diaphorase in adult sea bass (Novi et al., 1998). However, interestingly, the co-administration of PCB 126 and E2 reduced DT-diaphorase activity to the control value, as found for the EROD activity, suggesting that the cross-talk between AhR and ER might be influenced by the PCB 126-induced oxidation stress and thereby modulate, in addition to CYP1A, also the expression of DT-diaphorase.

The anti-estrogenic activity of PCB 126, when present as a pollutant in the aquatic environments and sea bass aquacultures, might be of toxicological significance on the reproductive system. Indeed, it has been demonstrated that sea bass fed with a diet including β-naphthoflavone is associated with a significant reduction of plasmatic VTG levels and possibly with a disturbed reproduction (Navas et al., 2004).

In conclusion, the present study indicates that: (i) male adult sea bass are sensitive to natural and xenoestrogens; (ii) heterologous commercial anti-VTG antibodies cannot be used to replace species-specific anti-VTG for monitoring VTG as a marker of sea bass exposure to endocrine-disrupting chemicals; (iii) PCB 126 is a potent anti-estrogenic compound in adult male sea bass; (iv) the natural or xenoestrogens depress selectively the activity associated with CYP1A and GST and thereby may compromise the detoxification capability of sea bass aquacultures exposed to

environmental pollutants such as polycyclic aromatic hydrocarbons.

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