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Vitellogenin, zona radiata protein, cathepsin D and heat shock protein 70 as biomarkers of response to xenobiotics

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Abstract
The antagonistic and/or synergistic effects of different chemical compounds were examined in the marine teleost, *Gobius niger*, by testing a series of biomarkers involved in fish reproduction. Among the biomarkers analysed, vitellogenin (VTG) and zona radiata proteins (ZRP) are key molecules involved in reproduction, widely used to detect the presence of pollutants in the marine environment, while heat shock protein 70 (HSP70) and cathepsin D (CATD) have recently been introduced as bioindicators of endocrine disruption. The detection of VTG and ZRP in the plasma of wild male specimens is universally accepted as an early warning signal of environmental pollution. The evaluation of VTG, ZRP and CATD expression demonstrated the oestrogenic effect of nonylphenol on both male and female fish; on the contrary beta-naphthoflavone behaves mainly as an anti-oestrogen although, when co-injected with compounds with oestrogenic activity, it enhances ZRP gene expression. Regarding the chaperone, all treatments stressed the fish, inducing an increase in HSP70 gene transcription. The results obtained underlined the importance of testing the effects of compound mixtures: fish in the wild are subjected to a blend of chemicals and the effects observed derive from the synergic or antagonistic interactions of these compounds.

Keywords: *Gobius niger, nonylphenol, oestradiol, beta-naphthoflavone, endocrine disruptors, molecular biology*

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Introduction

The release of pollutants into the environment is a source of increasing concern, mainly because evidence demonstrates that there is a link between pollution and human health. For this reason, in particular, many studies have been carried out to predict the effects of the wide spectrum of chemicals released into the surroundings. The results of these studies provide a fast and effective starting point for all biomonitoring approaches. The identification of biomarkers is an important and fast tool for investigating the health of organisms and this, in turn, reflects the quality of the environment.

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The aim of this study was to investigate the changes induced by some toxic compounds on fish physiology. Attention was addressed in particular to the effects of nonylphenol (NP), 17β-oestradiol (E2), beta-naphthoflavone (bNF) and a mixture of these compounds (NP+bNF and E2+bNF) on male and female *Gobius niger* caught during the reproductive season.

Vitellogenin (VTG) and zona radiata proteins (ZRP) were examined because these two proteins have a pivotal role in oocyte maturation. VTG represents the precursor of yolk proteins; it is synthesised in the liver under oestrogenic control, released in the blood stream and transported to the ovary until the final receptor-mediated endocytosis into developing oocytes occurs (Tyler & Sumpter 1996). Xenoestrogens can bind with high affinity to the oestradiol receptor (ER) and initiate the cell processes typical of natural oestrogens, thus triggering VTG gene transcription even in male fish (Sumpter 1998, Tyler et al. 1998, Cardinali et al. 2004, Maradonna et al. 2004). ZRP consist of two to four protein monomers (Begovac & Wallace 1989, Hyllner et al. 1994) which build the egg envelope and have an important role for species-specific sperm binding and, in the fish egg, for the prevention of polyspermy. After fertilisation, the eggshell undergoes a Ca^{2+}-dependent hardening (Lønning et al. 1984) that ensures protection of the developing embryo. This shell is shed when hatching is induced by developmental and environmental signals (Yamagami et al. 1992). Synthesis of ZRP and VTG in teleosts is interrelated because both processes are controlled by E2 (Wallace 1985, Oppen-Berntsen et al. 1994). To date, the presence of an oestrogen-responsive element (ERE) in the promoter of the VTG gene has been well documented both in fish and amphibians (Teo et al. 1998). In contrast, an ERE site for ZRP genes has not been identified in fish, although ZRP response to E2 is very similar to that of VTG (Arukwe & Goksøyr 2003). For this reason, both molecules have been widely used as biomarkers of oestrogenic endocrine disrupters released in the environment (Sumpter & Jobling 1995, Oppen-Bernsten et al. 1999, Arukwe et al. 1997, 2000). The presence of a documented ERE even in the promoter region of the cathepsin D (CATD) gene (Augereau et al. 1994) led us to focus on its regulation by oestrogenic compounds (Carnevali & Maradonna 2003). CATD is a member of the aspartic protease family present in a large number of organisms and it has been shown to be involved in various physiological pathways, including intracellular proteolysis (Barret 1977). In oviparous vertebrates, it has been demonstrated that CATD has a key role in reproduction, being involved in the ovary, in yolk formation (Retzek et al. 1992, Sire et al. 1994, Yoshizaki & Yonezawa 1994, Carnevali et al. 1999a,b, 2006) and in yolk mobilisation during early embryogenesis (Brooks et al. 1997, Carnevali et al. 2001). As a novel biomarker, the expression of heat shock protein 70 (HSP70) was also investigated. HSP70 has a pivotal role in protein metabolism under normal and stress conditions, including *de novo* protein folding, membrane translocation, degradation of misfolded proteins and other regulatory processes (Morimoto 1998). Numerous studies have clearly demonstrated that thermal shock (Mosser & Bols 1988), hypoxia (Airaksinen et al. 1998) and heavy metals (Misra et al. 1989, La Porte 2005) induce HSP70 expression in fish. The aim of this study was to provide an overview of the effects of different pollutants on fish physiology. In particular, the expressions of three biomarkers involved in fish reproduction (VTG, ZRP, CATD) were examined using molecular biological and biochemical tools; HSP70 expression was analysed to provide a fast signal of fish stress. Reproductive *G. niger* were injected with different doses and mixtures of

compounds to evaluate how they modulate the selected biomarkers. In particular, NP, a breakdown product of alkylphenol polyethoxylates (APES), was chosen for its well-documented oestrogenic activity (Ahel et al. 1994a,b, Jobling et al. 1996, Tyler et al. 1998, Cionna et al. 2006). E2 was used as positive control. bNF was used from the large group of polycyclic aromatic hydrocarbons (PAHs); a cocktail of either NP+bNF or E2+bNF was used to detect any synergic or antagonistic effects of these chemicals.

Materials and methods

Fish sampling and treatments

Male and female black goby, *G. niger*, weighing 10–15 g, were captured in the Adriatic Sea during the reproductive period (May) and maintained in tanks in a closed system with a salinity of 35 parts per thousand with a natural photoperiod and at a constant temperature of 18°C. After a recovery period of 3 weeks, the fish were divided in different groups of 20 (10 males and 10 females) and treated. Doses and time of exposure were determined on the basis of the highest biological response obtained as described in Maradonna et al. (2004). Toxicants were dissolved in cocoa butter and injected intraperitoneally (i.p.) as follows:

- NP1: 50 μg kg^{-1} NP
- NP2: 500 μg kg^{-1} NP
- bNF: 60 mg kg^{-1} bNF
- E2: 3.5 μg kg^{-1} E2
- NP+bNF: 500 μg kg^{-1} + 60 mg kg^{-1} NP and bNF, respectively
- E2+bNF: 3.5 μg kg^{-1} + 60 mg kg^{-1} E2 and bNF, respectively
- C (control): injected i.p. with cocoa butter

After 72 h, all specimens were anaesthetized with MS222 (Sigma-Aldrich, St. Louis, MO, USA) and immediately sacrificed. Liver samples were taken, frozen in liquid nitrogen and kept at −80°C until assayed. Plasma samples were prepared by centrifugation at 3000 rpm for 15 min at 4°C and stored at −80°C until use.

NP was obtained from Fluka (Buchs, Switzerland) as a mixture of isomers with differently branched nonyl side chains and containing approximately 85% *p*-isomers. The major impurities were 2-nonylphenol (*o*-isomers), dodecylphenol and dinonylphenol, which together comprise approximately 10% of the 4-NP mixture. E2 and bNF were purchased from Sigma (St. Louis, MO, USA).

Vitellogenin and zona radiata protein analysis

Plasma samples were electrophoresed as previously described in Maradonna et al. (2004). Briefly, 20 μg of each plasma protein sample were separated using 4% stacking and 15% separating sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), and electroblotted onto a Bio-Rad filter using a Bio-Rad mini-trans-blot electrophoretic transfer cell. The transfer was carried out at 7 V cm^{-1} overnight at 4°C using a 25 mM Tris base, 192 mM glycine, and 20% methanol as an electrode solution. The nitrocellulose membrane was soaked

in 5% Nonidet-P40 for 1 h to remove SDS and incubated with 2% bovine serum albumin (BSA; Sigma) in PBS buffer. For the VTG assay, the primary antibody (anti-VTG *Sparus aurata*) diluted 1:1000 in a solution containing 2% BSA, 0.01% NaN_3 in PBS, was incubated for 2 h at room temperature (about 20°C) and rinsed three times with PBS plus 0.05% Tween 20. For the ZRP assay, the primary polyclonal antibody (rabbit anti-salmon ZRP) was purchased from Biosense Laboratories AS, Bergen, Norway and diluted 1:1000 in a solution containing 2% BSA, 0.01% NaN_3 in PBS, and incubated for 2 h at room temperature (about 20°C) and rinsed three times with PBS plus 0.05% Tween 20. For both assays, the second antibody solution (HRP-conjugated anti-rabbit IgG; BioRad) diluted 1:1000 in 2% BSA in PBS buffer was incubated for 1 h. The filter was rinsed again with PBS without Tween 20. The blot was developed using as substrate ECL+Plus Western Blotting Detection System (Amersham Biosciences, UK).

RNA extraction and RT-PCR

Total RNA was extracted from 30 mg of liver following the RNeasy® Mini Kit protocol (Quiagen, Milan, Italy); 5 μg of RNA were used for cDNA synthesis, employing 1 ml oligo d(T)+adapter primer, 5′-GACTGCAGTCGACATCGA TTTTTTTTTTTTTTTTTT-3′, in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of dNTP and 200 units of Superscript II RT (Invitrogen, Life Technologies, Milan, Italy), with incubation at 42°C for 50 min. VTG, ZRP, CATD and HSP70 primers were designed on the basis of the homology among the sequences available in GenBank (VTG for 5′-GGACCA AACACAGGAACAGAAC-3′ VTG rev 5′-AGCCGTTCGTTGGGTGTG3′; ZRP for 5′GG(CT)TCCATCAC(AC)AG(AG)GACAG3′ ZRP rev 5′CCA(AG)(AG) (AGT)GA(GT)GG(AC)(AT)GGCACAC3′, CATD for 5′GCTCCTCCAACC TGTGGGT3′ CATD rev CTGGTGAGGTAGAAAGAGAAAA3′; HSP70 for 5′CAATGACTC(ACT)CAGGG(ACT)CA3′ HSP70 rev 5′GGTGATGGAGGTG TAGAAGT3′). cDNA was amplified with 5 units of Taq DNA polymerase (Dynazyme) in 20 μl of master mix containing 1 ×PCR buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 100 pmol of each set of primers. Acidic ribosomal posphoprotein P0 (ARP) liver expression was used to standardise the expression of each biomarker. (ARP for 5′-GAAAATCATCCAATTGCTGGATG'-3′, ARP rev: 5′-CTTCCCACGCAAGGACAGA -3′.)

Cloning and sequencing

PCR products amplified with the above-mentioned specific primers were cloned as already described in Cionna et al. (2006). Briefly, the single PCR product was purified using the PCR purification kit (Qiagen) and then cloned into the p-GEM T easy vector (Promega) following the manufacturer's protocol. The plasmid was transformed into DH5α cells by the TransformAid™ kit (MBI Fermentas). Several positive clones were analysed by PCR and restriction cutting in order to verify the presence of the insert and then sequenced using an ABI model 310 DNA sequencer (Perkin-Elmer, Oak Brook, IL, USA).

Southern blot

The PCR product was run onto a 1.5% TAE agarose gel, the blotting was made under a rapid protocol, using a light alkaline buffer, on a nylon membrane (Nytran Super charge, Schleicher & Schuel, Dassel, Germany). DIG-labelled DNA probe was made using the same homologous fragment as the template. The labelling was performed using the DIG DNA labelling kit (Roche, Basel, Switzerland), based on random primed reaction, following the manufacturer's instructions. Briefly, the membrane was hybridised ON with the homologous DNA probe at 60°C in a standard hybridisation buffer (5X SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 2% blocking reagent, 50% formamide). After the hybridisation, the blot was first washed two times in a solution containing 0.1% SDS and 2X SSC for 5 min at room temperature, then twice in 0.1% SDS and 0.1X SSC at 65°C for 15 min. After these washings, the blot was incubated with an antibody against digoxigenin alkaline phosphatase conjugated and a chemiluminescent detection procedure was performed with the DIG luminescent detection kit (Roche, Basel, Switzerland), according to the manufacturer's protocol. The signal was then observed by autoradiography using the Kodak BioMax Light-1 film (Sigma). The films were scanned by a laser scanner (Sharp Electronics, Milan, Italy) and then subjected to densitometric analysis by ImageQuant software v. 1.2 (Molecular Dynamics, Amersham Biosciences, Sunnyvale, USA).

Gene expression quantification

The variations in VTG, ZRP, CATD and HSP70 mRNA expressions were evaluated by semiquantitative PCR using ARP as an internal standard. After the chemiluminescent detection, films were scanned using a laser scanner (Sharp Electronics) and then subjected to densitometric analysis by ImageQuant software v. 1.2 (Molecular Dynamics).

Enzymatic activity

The assay used to test the enzymatic activity of CATD was performed as previously described by Takahashi & Tang (1981) using haemoglobin as substrate. To test the enzymatic assay specificity, pepstatin an inhibitor of CATD was added in the test tube. In addition, to test the substrate specificity of the enzymatic preparation, the activity was assayed using both benzoyl-L-argininamide and glycil-L-phenylalaninamide specific substrate for B and L, respectively (Mycek 1970).

Data analysis

Data presented in this paper are in the form mean $\pm$ SD of means. Results were examined by one-way ANOVA followed by Student–Newman–Keul's test or the Student's *t*-test as appropriate, using a statistical software package, Stat View 512+ TM (Brain Power Inc., Calabasas, CA, USA). A p value of 0.05 was used as the limit of statistical significance.

Results

PCR optimisation

The parallel amplification efficiency was obtained at 27, 22, 30, 28 and 20 cycles for VTG, ZRP, CATD, HSP70 and ARP, respectively.

VTG gene and protein expression

A single fragment of 455 bp was obtained with RT-PCR, using a specific VTG set of primers as described in the Materials and methods section. Its sequence, submitted to GenBank (A# DQ073804) provided an 81% match with the *Acanthogobius flavimanus* Vg-530 mRNA for vitellogenin (A #AB088473).

In males, the densitometric analysis of the Southern blot hybridised with homologous VTG probe showed considerable hepatic variation ($p < 0.05$) among the different experimental groups. Specifically, the results obtained highlighted NP oestrogenic potency; a markedly higher induction was found in fish treated with the highest dose of contaminant. The highest VTG induction was found in E2-treated males, while in fish treated with the cocktail of either NP+bNF or E2+bNF, the antagonistic effects of bNF on the oestrogenic effect exerted by NP or E2 was recorded. In fact, in fish treated with the mixture of compounds, the induction of VTG was lower than that observed after the single injection of NP or E2 (Figure 1A).

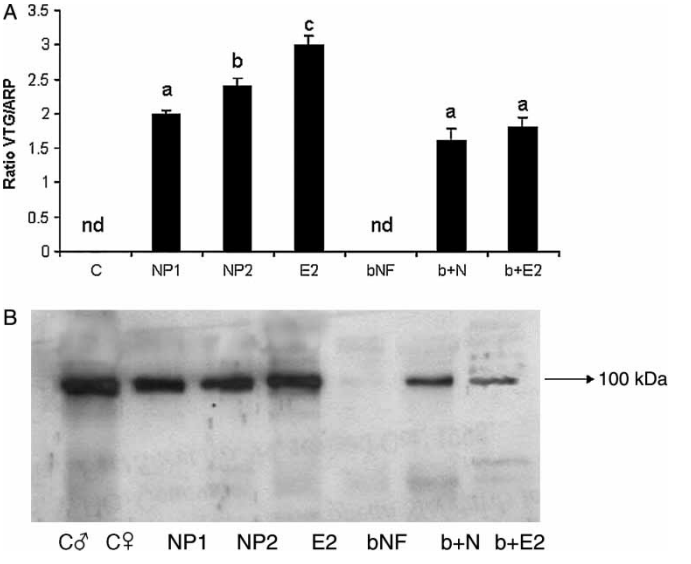

Figure 1. (A) Southern blot analysis of vitellogenin (VTG) mRNA expression in the male. Data, normalised using ARP, are the means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control male; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+ nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Western blot analysis of vitellogenin in male plasma tissue probed with anti-*Sparus* VTG (diluted 1:1000). C♂, control male; C♀, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

These results on gene expression were confirmed at protein level by Western blot analysis (Figure 1B). A single band of 100 kDa was detected in plasma samples of all treated groups, except for bNF group.

In females, VTG gene expression was significantly induced only in fish treated with E2 or bNF +E2 ($p < 0.05$). No significant variations compared with control levels were recorded for the other experimental groups. The contrasting effect of bNF on the oestrogenic induction of VTG found in the males was not evident in reproductive females. Western blot analysis revealed a different VTG pattern for the different groups: except for control and bNF-injected fish, where a single 100 kDa band was detected, in all the other treatments two more VTG bands were evident at the apparent molecular mass of 85 and 75 kDa (Figure 2A,B), indicating multiple forms of this protein.

ZRP expression

Using RT-PCR, a single fragment of 611 bp for ZRP was obtained. Its sequence, submitted to GenBank (A# DQ073801) showed an 80% match with *Danio rerio* ZP2 (A# AF331968). The densitometric analysis of the Southern blot hybridised with the homologous probe showed considerable variation ($p < 0.05$) in hepatic gene expression among the different experimental groups. In males, ZPR was slightly induced by the lowest dose of NP (NP1), while in NP2- and E2-treated fish the induction was markedly higher. BNF co-injected with NP or E2 strengthened the oestrogenic potency of these compounds ($p < 0.05$) (Figure 3A). In females, significant variations

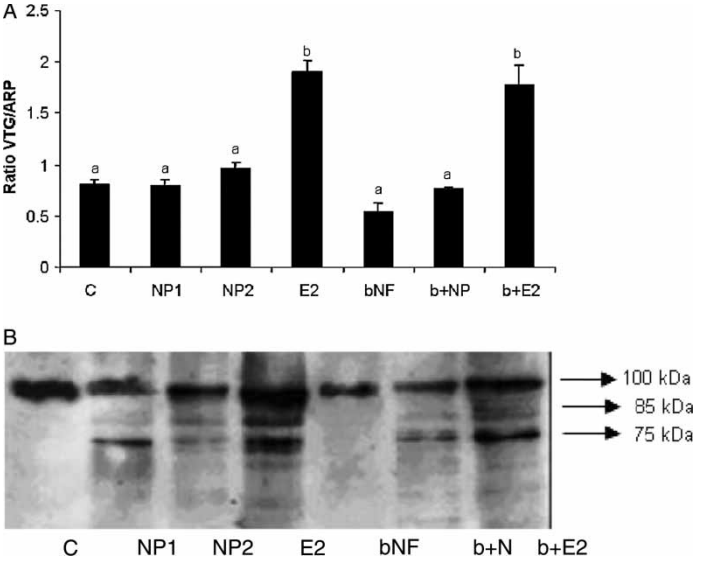

Figure 2. (A) Southern blot analysis of vitellogenin (VTG) mRNA expression in the female. Data, normalised using ARP, are the means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+NP, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Western blot analysis of VTG in female plasma tissue probed with anti-*Sparus* VTG (diluted 1:1000). C, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

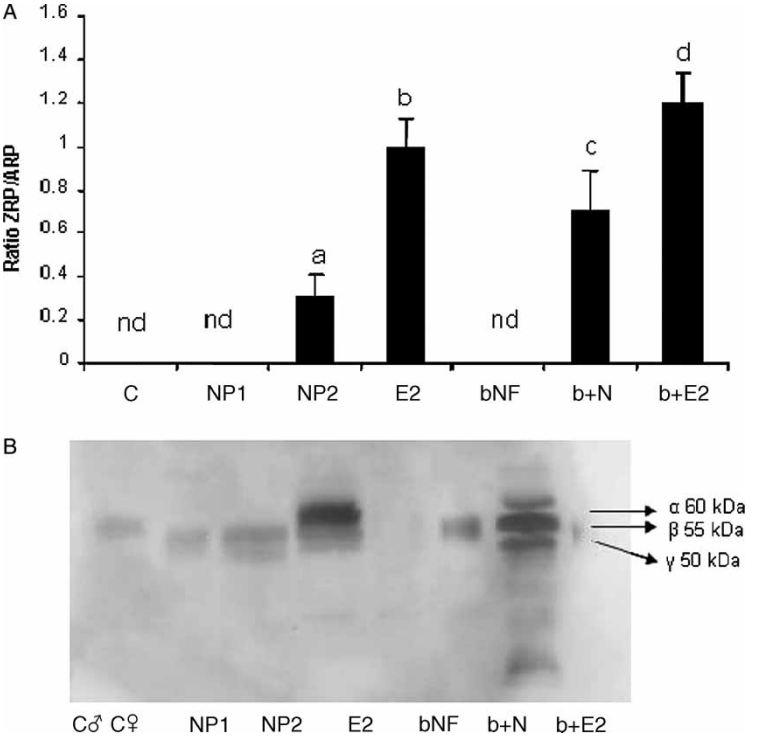

Figure 3. (A) Southern blot analysis of zona radiata proteins (ZRP) mRNA expression in the male. Data, normalised using ARP, are the means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control male; NP1, nonylphenol 50 μg/ kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Western blot analysis of ZRP in male plasma tissue probed with rabbit anti-salmon ZRP (diluted 1:1000). C♂, control male; C♀, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+NP, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

of ZRP gene expression were observed only with the highest NP dose or in the presence of E2 (Figure 4A).

At the proteic level, the hybridisation of plasma protein with the polyclonal antibody anti-salmon ZRP, highlighted the different sensitivity of fish to chemicals. As expected, no band was detected in control and in bNF male plasma. ZRP-β was induced in all male groups, whilst ZRP-γ was mainly induced in NP2-, E2- and bNF+E2-treated fish. Treatments with E2 (both E2 and E2+bNF) also induced the α-form (Figure 3B). The ZRP-α form was detectable only in the controls and NP1 females, and was not evident in the other treatments where the appearance of the β form was recorded (Figure 4B).

CATD expression

A single band of 370 bp was obtained using CATD specific primers, as previously published by Carnevali & Maradonna (2003). The densitometric analysis of the

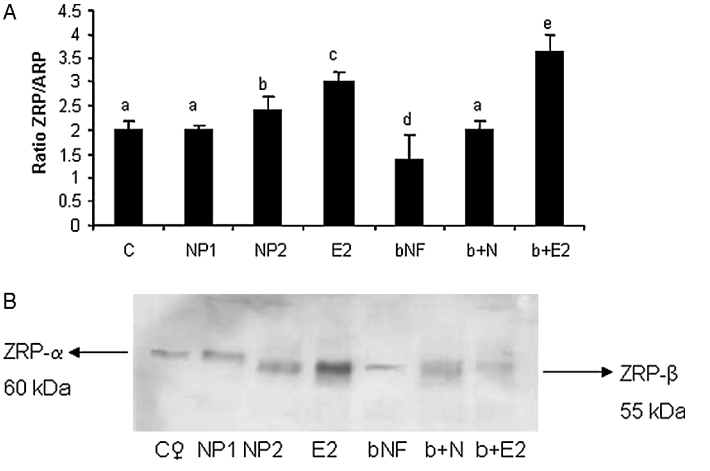

Figure 4. (A) Southern blot analysis of zona radiata proteins (ZRP) mRNA expression in the female. Data, normalised using ARP, are the means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Western blot analysis of ZRP in female plasma tissue probed with rabbit anti-salmon ZRP (diluted 1:1000). C♀, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

Southern blot hybridised with homologous CATD probe showed that the lowest dose of NP showed no variation in the hepatic expression of this messenger, either in male or in female fish. On the contrary, at the highest dose injected, NP proved to be the most oestrogenic agent. bNF determined a significant reduction in CATD expression in both males and females.

Regarding the treatments with NP+bNF and E2+bNF, although the similarity of the oestrogenic potency of NP to E2 was confirmed, it is worth noting that their effect was inhibited by bNF. CATD gene expression in the co-injected groups was lower compared with the groups who had the single injection of NP or E2 (Figure 5A,B).

CATD enzymatic activity

In males, basal levels of CATD changed significantly compared with the control only in NP2- and E2-treated fish. bNF alone had no effect. Regarding the co-injections, individual bNF did not exert any biological effects on NP, although when injected with E2 it produced a significant reduction in gene expression compared with E2-treated fish (Figure 6A). In females, all treatments generally increased CATD activity except for bNF alone which did not exert any action. However, when bNF was co-injected with NP or E2 cathepsin levels decreased, indicating its anti-oestrogenic role (Figure 6B).

HSP70 expression

The densitometric analysis of the Southern blot showed that different treatments determined a variation in the expression of HSP70 both in male and female fish.

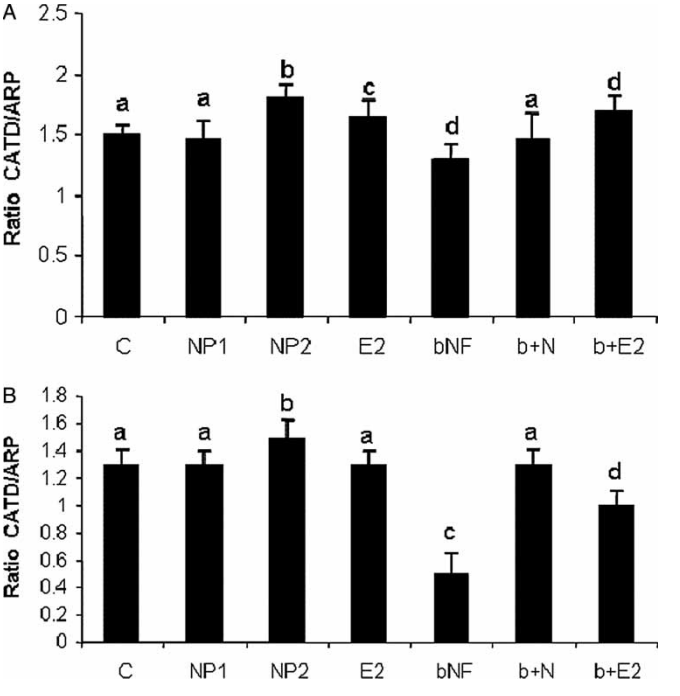

Figure 5. (A) Southern blot analysis of cathepsin D (CATD) mRNA expression in the male. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control male; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Southern blot analysis of CATD mRNA expression in the female. Data, normalised using ARP, are means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

Males also seemed to be more responsive to the different doses of stressors (Figure 7A); in fact, in females the NP effect was not related to the dose in the same way as observed in males (Figure 7B). In both males and females, bNF determined a significant increase in HSP70 gene expression and the cocktails of bNF+NP and bNF+E2 induced a synergistic effect (Figure 7A,B).

Discussion

In this study, fish were treated with different kinds of chemicals: NP and bNF, the effects of which have already been documented in this species (Carnevali & Maradonna 2003, Maradonna et al. 2004) in freshwater fish (Aubry et al. 2005) and in *in vitro* studies (Smeets et al. 1999, Navas & Segner 2000). As in the wild, species are subjected to mixtures of xenobiotics that may behave differently when acting in isolation or in mixtures and the biological effect observed may raise questions about additive, synergistic or antagonistic interactions, fish were also co-injected with cocktails of the above-mentioned compounds.

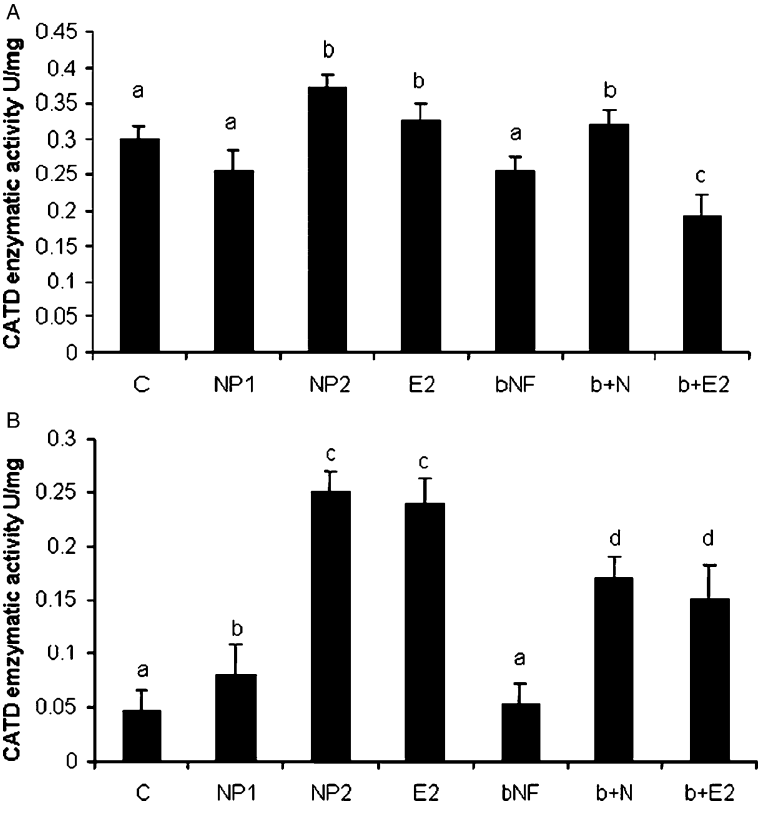

Figure 6. (A) Cathepsin D (CATD) activity in male fish liver. C, control male; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) CATD activity in female fish liver. C, control female; NP1, nonylphenol 50 μg/kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. All points are averages of determinations of ten individual animals; different letters indicate significant differences ($p < 0.05$).The activities are expressed as U mg^{-1}; a unit (U) of enzymatic activity is defined as the amount of enzyme that produces at 280 nm an absorbance value of 1.0 after 20 min of incubation at 37°C in the presence of a haemoglobin saturated solution as substrate (Carnevali et al. 1999a). To test the substrate specificity of the enzymatic preparation, the activity was assayed using both benzoyl-L-argininamide and glycyl-L-phenylalaninamide as substrates (Mycek 1970).

In general terms, the effects exerted by pollutants were easily detected in males while in females the peak of endogenous E2, usually recorded at reproductive time, might have determined a levelling of the results obtained. In the plasma of oestrogen-treated males, a single VTG band was detected, indicating this form as the inducible one. In contrast, in females two more VTG bands at lower molecular mass were observed. These data are supported by studies performed in the plasma of both *Marone americana* and *Gambusia affinis*; in these species three forms of VTG, named Vg-A type, Vg-B type and a smaller sub-unit PV-less type, were detected (Matsubara et al. 2003) and each form seemed to have a physiologically diverse function. In barfin flounder, Vg-A and Vg-B have distinct roles in modulating egg viability via selective

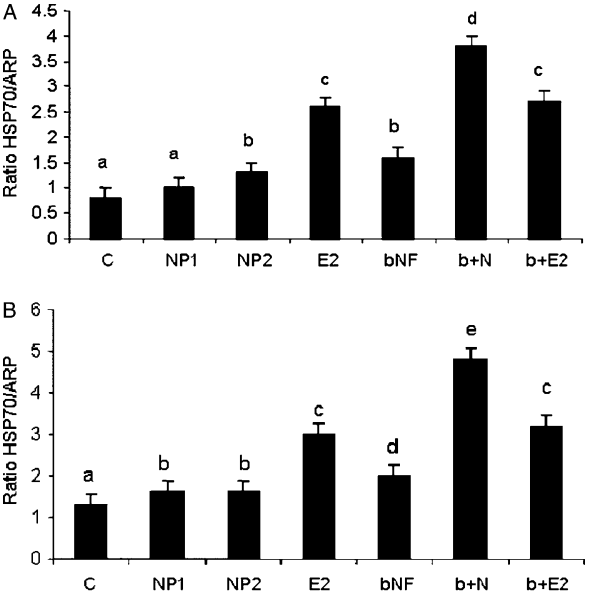

Figure 7. (A) Southern blot analysis of heat shock protein 70 (HSP70) mRNA expression in the male. Data, normalised using ARP, are the means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control male; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}. (B) Southern blot analysis of HSP70 mRNA expression in the female. Data, normalised using ARP, are means of ten individual fish. Data are expressed as mean (SD); different letters indicate statistical significant values ($p < 0.05$). C, control female; NP1, nonylphenol 50 μg kg^{-1}; NP2, nonylphenol 500 μg kg^{-1}; E2, 17β-estradiol 3.5 μg kg^{-1}; bNF, b-naphthoflavone 60 mg kg^{-1}; b+N, b-naphthoflavone 60 mg kg^{-1}+nonylphenol 500 μg kg^{-1}; b+E2, b-naphthoflavone 60 mg kg^{-1}+17β-estradiol 3.5 μg kg^{-1}.

proteolysis of their lipovitellin products during oocyte maturation (Matsubara et al. 1999).

The different responsiveness of ZRP to chemicals was shown using Western blot analysis. In males, NP and bNF+NP mainly induced the β form as confirmed by results obtained in *Salmo salar* (Arukwe et al. 1997). Only treatments with E2 induced the three different forms of ZRP (ZRPα,β,γ), while in females the constitutive form, ZRPα-like, was replaced by the lower molecular weight form, the ZRPβ-like. These data provide clear evidence that *G. niger* eggshell ZRP originate in extra-ovarian tissue, the liver, and are transported in the blood for deposition in the target organ, the ovary.

The synergistic effect recorded in males after the co-injections of bNF with NP or E2 is very interesting. This effect is also evident in females treated with bNF+E2. Since the existence of an ERE-like region in ZRP gene has not yet been documented, we may not only speculate that xenoestrogens/biotics act by binding the ER, leading to the final transactivation of the gene, but also that the existence of additional pathways might be considered.

Regarding CATD, the lack of correlation between gene expression and enzyme activity observed for some groups can be explained considering the very complex structure of the gene promoter: oestrogen induction of CATD is mediated by

interaction of ER with a non-consensus ERE that requires synergy with other elements located upstream and/or downstream of this central ERE (Augereau et al. 1994). The other issue which should be taken into consideration is the initial appearance of CATD as a pre-pro-enzyme undergoing several proteolytic cleavages to produce the mature form (Richo & Conner 1994). Following the initial co-translational removal of the signal peptide to yield pro-CATD, sugars are attached at two *N*-linked glycosylation sites and targeted to lysosomes where cysteine proteases yield an active intermediate single-chain molecule (Samarel et al. 1989).

The last biomarker analysed was HSP70. All treatments induced a rapid and significant increase in its constitutive levels. HSP70 can be considered an early signal of the presence of all types of stressors. This is due to the simple activation mechanism of this gene which results from the binding of an activated heat shock transcription factor (HSF) to heat shock elements (HSEs) upstream of the gene (Morimoto et al., 1992). Since most inducible HSPs do not contain introns, the mRNA is rapidly translated into nascent protein within minutes of exposure to a stressor (Mayer et al. 2000). For this reason HSP70 is a non-discriminating marker although its role as chaperone against heat shock has been largely documented (Chen et al. 1988, Koban et al. 1991, Airaksinen et al. 1998) and in recent years it has also been used as a biomarker of pollutants (Carnevali & Maradonna 2003, Weber 2004, Migliarini et al. 2005).

It is well documented that the release of pollutants in the environment interferes with the preservation of biodiversity although to our knowledge, few studies have been conducted on fish to assess the genotropic potential of xenoestrogens and hydrocarbons (Schlenk et al. 1994, Teles et al. 2004, Aubry et al. 2005). This type of study is, however, ecotoxicologically relevant to determine the effects of pollutants at the level of DNA transcription. The data obtained in this study demonstrate that while NP behaves as an oestrogen, the role of bNF is worth noting as both an anti-oestrogenic and an oestrogenic chemical. This leads to the consideration that in a monitoring study including the analysis of oestrogen – modulated proteins, such as VTG, ZRP and CATD, as biomarkers of xenoestrogens should also evaluate any negative or positive interference of compounds with different properties, such as many aryl hydrocarbon receptor agonists. For this reason great attention must be focused not only on the effect exerted by the single compounds but also on the interaction set up between pollutants.

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