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Beta-naphthoflavone inhibits the induction of hepatic oestrogen-dependent proteins by 17alpha-ethynylestradiol in mosquitofish (*Gambusia holbrooki*)

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Abstract
The interactive effects of an aryl hydrocarbon receptor (AhR) agonist and of a xenoestrogen on biomarker responses were studied in the liver of male mosquitofish (*Gambusia holbrooki*). Hepatic 7-ethoxyresorufin *O*-deethylase (EROD) enzymatic activity was measured as a biomarker of exposure to the model AhR agonist beta-naphthoflavone (bNF). Hepatic proteins indicating the exposure of males to the synthetic oestrogen 17alpha-ethynylestradiol (EE2) were monitored by Western blot analysis using immunoserum prepared for this study. After a semi-static exposure only to waterborne EE2, Western blot analysis of liver homogenate revealed the induction of two protein bands (a double band at 205 kDa and a single band at 125 kDa). The interaction between bNF and EE2 was investigated by analysing, on the one hand, EROD activity and, on the other hand, immunoreactivity corresponding to the two oestrogen-dependent protein bands in the liver of fish exposed to different concentrations of bNF for 2 days, then to the same concentrations of bNF plus 0.1 $\mu g\,l^{-1}$ EE2 for 5 days. EE2 changed neither the basal activity of EROD nor its rate of induction with 1.0 and 4.0 $\mu g\,l^{-1}$ bNF. On the other hand, the induction of oestrogen-dependent proteins with 0.1 $\mu g\,l^{-1}$ EE2 was inhibited by exposure to 4.0 $\mu g\,l^{-1}$ bNF. These results together with literature data suggest that field monitoring of xenoestrogen contamination through the analysis of oestrogen-dependent protein in male fish as a biomarker should take into account the possible negative interference of AhR agonists.

Keywords: *Fish, hepatic 7-ethoxyresorufin O-deethylase (EROD), oestrogen-dependent protein, beta-naphthoflavone, 17alpha-ethynylestradiol, interaction*

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Introduction

Aquatic ecosystems act as a receptacle for the great majority of anthropogenic chemicals to which aquatic organisms are subsequently exposed. Fish living in polluted ecosystems can incorporate micropollutants in their body by direct uptake

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from the water filtered through their gills or by ingestion of contaminated prey. Fish can be used as an indicator of the chemical contamination of the ecosystem where they live through the chemical analysis of bioaccumulated micropollutants in their tissues. A complementary approach is to measure the response of fish biological parameters (biomarkers) to chemical pollution (for a review, see Van der Oost et al. 2003).

Induction of cytochrome P4501A (CYP1A) in fish has been associated with exposure to major micropollutants, such as dioxins, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Stegeman & Hahn 1994). Induction of CYP1A can be monitored through the measurement of CYP1A-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity. For the last 25 years, numerous studies have dealt with EROD induction in fish exposed to chemicals in the laboratory and in the field (for a review, see Whyte et al. 2000). The mechanism underlying CYP1A induction has been extensively studied in mammals. It relies on the binding of an inducer to the cytosolic aryl hydrocarbon (Ah) receptor (Poland et al. 1976, Nebert et al. 1993, Swanson & Bradfield 1993, Hankinson 1995). The Ah receptor (AhR) is a ligand-activated transcription factor that forms a heterodimer with the AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to specific regions of DNA designated as xenobiotic-responsive elements (XREs). This binding initiates the transcription of several genes of biotransformation enzymes (Hankinson 1995) and the subsequent synthesis of proteins including apoprotein of CYP1A (Whitlock 1999). Fish differ from mammals in having not one, but at least two AhR genes (Hahn 1998, 2002). Nevertheless, induction of CYP1A in fish is thought to occur through a similar mechanism to mammals (Stegeman & Hahn 1994, Pollenz et al. 2002).

Over the last decade, the induction of circulating oestrogen-dependent proteins, especially vitellogenin (Vtg) in male fish has been used as a biomarker of exposure to chemicals exhibiting oestrogenic activity (xenoestrogens) in many studies (Matthiessen & Sumpter 1998, Denslow et al. 1999). In females, Vtg is synthesized in the liver after stimulation by oestradiol (E2), then transported by the blood to the ovary and incorporated into growing oocytes where it constitutes the main egg trophic reserve. Males normally do not synthesize Vtg, but upon exposure to oestrogen or oestrogen mimicking compounds, their liver will be induced to synthesize Vtg. Thus, finding Vtg in males is a bioindication of exposure to an oestrogenic chemical (Sumpter & Jobling 1995). Eggshell proteins are also hepatic oestrogen-dependent proteins specific to female fish which are induced in males exposed to oestrogens. Their induction in male fish has also been proposed as a biomarker of exposure to xenoestrogens (Arukwe et al. 1997c). Induction of oestrogen-dependent proteins in fish is based on transcriptional activation of the corresponding genes by E2 (Pakdel et al. 1997). The mechanism of the action of E2 is supposed to be the same as that which exists in mammals. Briefly, after entering the cell, E2 binds to its receptor (ERalpha), then the E2–ERalpha complex binds to the oestrogen-responsive elements (ERE). Binding of the oestrogen-receptor complex to the ERE results in production of mRNA coding for a number of oestrogen-responsive proteins, such as Vtg and eggshell proteins. In fish, *in vitro* as well as *in vivo* studies show an increase in the transcription rate of oestrogen-dependent genes after exposure to xenoestrogens (Flouriot et al. 1995, Petit et al. 1997, Bowman & Denslow 1999, Arukwe et al. 2001a, Islinger et al. 2002, Thomas-Jones et al. 2003).

In the field, chemical contamination is generally constituted by a great variety of compounds that originate from numerous sources of pollution. Thus, fish are actually

exposed to chemical mixtures rather than to single chemicals, and one can therefore wonder if the response of a biomarker could not be modulated by chemicals other than those that normally induce this response. In this case, interpretation of biomarker-based monitoring of chemical pollution should be complicated. In fish, a repressive effect of oestrogens on CYP1A was suggested *in vivo* (Förlin & Hansson 1982, Vodicnik & Lech 1983) as well as *in vitro* (Navas & Segner 2001). On the other hand, several *in vitro* studies conducted in fish liver cells show that AhR agonists can diminish E2-induced Vtg synthesis (Anderson et al. 1996a, Smeets et al. 1999, Navas & Segner 2000, Bemanian et al. 2004). However, conflicting results are reported in *in vivo* studies (Anderson et al. 1996b, Arukwe et al. 2001b).

The aim of the present study was to study the *in vivo* effect of waterborne beta-naphthoflavone (bNF), a model AhR agonist, on the induction of the synthesis of hepatic oestrogen-dependent proteins by the synthetic oestrogen 17alpha-ethynylestradiol (EE2) in male mosquitofish (*Gambusia holbrooki*). For that purpose, we decided to expose fish concomitantly to EE2 and bNF after pre-exposure to bNF.

Materials and methods

Chemicals

7-Ethoxyresorufin, resorufin, AEBSF, ethylene diamino tetraacetate (EDTA), beta-naphthoflavone (5,6-benzoflavone, bNF) and alpha-naphthoflavone (7,8-benzoflavone, aNF) were purchased from Sigma-Aldrich (Saint-Quentin, Fallavier, France). Dithiothreitol (DTT) and NADPH were purchased from Roche (Meylan, France). Acetone Uvasol® was purchased from Merck (Foutenay-Sousbois, France).

Fish

Mosquitofish (*Gambusia holbrooki*) were reared at 25°C under a 14-h light and 10-h dark photoperiod at the Unité Expérimentale d'Écologie et d'Écotoxicologie Aquatique (INRA, Rennes, France). For all experiments, male individuals were approximately 6 months old and had a standard length of 19–22 mm and a body weight of 120–160 mg.

Treatment

Exposure to chemicals was performed following a semi-static procedure in stainless steel tanks filled with 10 litres dechlorinated tap water at 25°C under a 14-h light and 10-h dark photoperiod. Fish were fed with Tetramin® flakes twice a day. Three times a week, with intervals of 2 or 3 days, water and chemicals were renewed. A nominal concentration of chemicals was achieved by adding chemicals dissolved in acetone (0.1% final volume). The actual concentration of chemicals was not measured. Therefore, only nominal concentrations are indicated in the following text.

Preparation and analysis of samples

After sacrifice of fish, the liver was immediately excised. Entire liver was used for the preparation of subcellular fractions. Briefly, liver was homogenized in 50 mM ice-cold phosphate buffer (pH 7.6), containing 0.15 M KCl, 1 mM EDTA, 1 mM DTT,

0.1 mM AEBSF and 20% glycerol (v/v), and homogenized using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2000*g* for 15 min at 4°C. The supernatant was stored at −80°C. Glycerol was shown to prevent the decline of EROD activity during storage of subcellular fractions of fish liver at low temperature (Monod & Vindimian 1991).

EROD assay was carried out in a 1-ml final volume containing 0.1 mM phosphate buffer, 0.1 mM NADPH, supernatant and substrate in DMSO (1% final). The reaction was stopped with 2 ml acetone. After centrifugation, the resorufin fluorescence was measured at 583 nm with an excitation wavelength of 537 nm. Fluorescence was measured using a Photon Technology International spectrofluorimeter.

The protein content in the supernatant was determined using a Coomassie protein assay reagent (Interchim, Mountluçon, France).

A rabbit polyclonal antibody against mosquitofish oestrogen-dependent hepatic proteins was prepared. Male mosquitofish were maintained at 23°C and exposed for 4 days to 1 $\mu g\,l^{-1}$ EE2. Fish were then sacrificed and livers briefly minced with a cutter and incubated in M199 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine and placed in an incubator under O_2 atmosphere conditions at 23°C and cultured for 3 days. The culture medium was then collected, centrifuged and dialysed (centilutor C10; Millipore, Bedford, MA, USA). Samples containing 200 μg proteins were mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich). One rabbit was immunized subcutaneously and boosted with the same quantity of proteins mixed with Freund's incomplete adjuvant on days 17, 38, 59 and 83 after the primary immunization. The rabbit was bled and serum prepared. The specificity of the serum was checked by Western blotting.

For Western blot analysis, 10 μg total protein from liver tissues were run on a 7% SDS-PAGE (Laemmli 1970). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France) for 80 min at 90 V using a mini trans-blotting system (Bio-Rad). The filters were soaked for 120 min in 5% non-fat dried milk in TBS-T (20 mM Tris, pH 7.5, 137 mM NaCl, 1% Tween 20) and further incubated overnight with the rabbit antibody raised against the oestrogenic-dependent proteins (1/10 000) diluted in TBS-T-1% BSA. The membranes were rinsed three times for 15 min in TBS-T and incubated for 1 h in an anti-rabbit phosphatase alkaline conjugated secondary antibody (Sigma) at 1/30 000. The membranes were then washed three times in TBS-T and once in TBS. Proteins were visualized using 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) phosphatase alkaline substrate (Sigma-Aldrich).

The signal intensity of immunoreactive bands was determined using ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA, USA). The mean intensity of oestrogen-dependent band signal from the liver of the treated fish was expressed as the per cent of the mean intensity of oestrogen-dependent band signal from a pool of livers of male mosquitofish exposed to 0.1 $\mu g\,l^{-1}$ EE2 for 3 days and submitted to the same Western blot analysis (internal standard).

Statistical analysis

Statistical analyses were performed by the non-parametric Mann–Whitney *U*-test using StatView (SAS Institute, Inc., Cary, NC, USA).

Results

Induction of hepatic EROD activity by bNF

Preliminary investigations were carried out in male mosquitofish in order to determine optimal incubation conditions for EROD activity measurement (data not shown). Reaction was shown to be linear with time (up to 20 min) and with protein concentration. Maximum velocity was obtained with 2.5 μM ethoxyresorufin, optimum pH was 7.2 and optimum temperature was 35°C. In the present study, EROD activity was measured according to these conditions, except for temperature (25°C). Furthermore, EROD activity was strongly inhibited by aNF, an inhibitor of CYP1A (50% inhibition at 10^{-9} M aNF).

The EROD activity in the liver of male mosquitofish exposed for 3 days to waterborne bNF is shown in Figure 1. EROD activity was not modified by exposure to the solvent vehicle acetone, whereas it was induced for all the bNF concentrations tested. EROD induction increased from threefold at 3.84 $\mu g\,l^{-1}$ bNF to sevenfold at 150 $\mu g\,l^{-1}$ bNF. Statistical analysis shows that the induction rate did not differ from 9.6 to 150 $\mu g\,l^{-1}$ bNF.

The time trend of the induction of EROD activity in the liver of male mosquitofish exposed to 9.6 $\mu g\,l^{-1}$ bNF is shown in Figure 2. The induction rate increased from 3 to 5 days of exposure then levelled off up to day 7.

Induction of hepatic oestrogen-dependent proteins by EE2

Western blot analyses of oestrogen-dependent proteins in mosquitofish tissues are shown in Figure 3. A double band at 205 kDa and a single band at 125 kDa were detected in the liver of control females undergoing vitellogenesis as well as in the liver of males exposed to 0.4 $\mu g\,l^{-1}$ EE2 for 7 days. These bands were absent from the

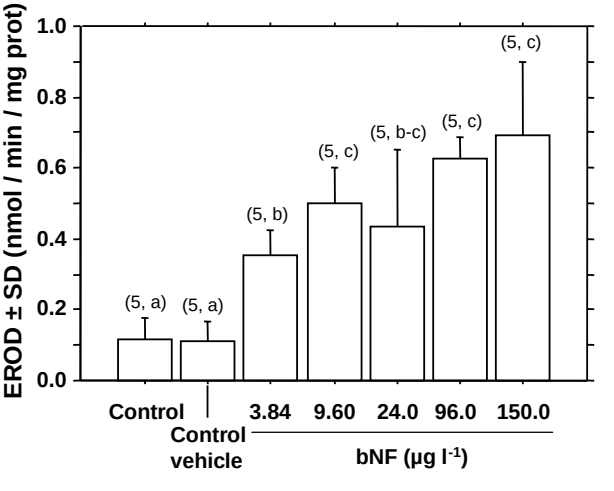

Figure 1. Hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in male mosquitofish exposed to different concentrations of beta-naphthoflavone (bNF) for 3 days. Fish were exposed statically to the indicated nominal bNF concentrations from days 0 to 3. Data are the mean ± standard deviation. The number of analysed fish and the statistical ranking of each treatment group are indicated between parentheses. Two groups of data labelled with different letters have significantly different EROD activities ($p < 0.05$, Mann–Whitney *U*-test).

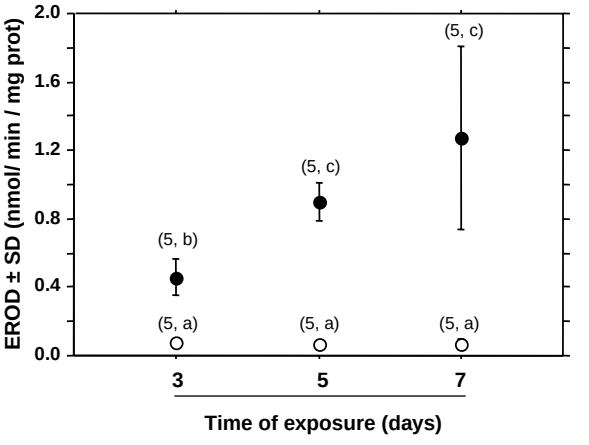

Figure 2. Temporal induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity in male mosquitofish exposed to 9.6 $\mu g\,l^{-1}$ beta-naphthoflavone (bNF) nominal concentration (●) or to solvent vehicle (○). Fish were exposed to bNF on day 0, and water contaminated by nominal concentration of bNF was renewed on days 3 and 5. Data are the mean ± standard deviation. The number of fish analysed and the statistical ranking of each treatment group are indicated between parentheses. Two groups of data labelled with different letters have significantly different EROD activities ($p < 0.05$, Mann–Whitney *U*-test).

muscle of EE2-exposed males as well as from the liver and the muscle of untreated males.

In additional experiments (data not shown), 205- and 125-kDa protein bands were absent in the liver of males exposed to 0.001 $\mu g\,l^{-1}$ EE2 nominal concentration, but a dose-dependent increase of immunoreactivity was observed for EE2 nominal concentrations ranging from 0.01 to 1.0 $\mu g\,l^{-1}$ for 7 days. Furthermore,

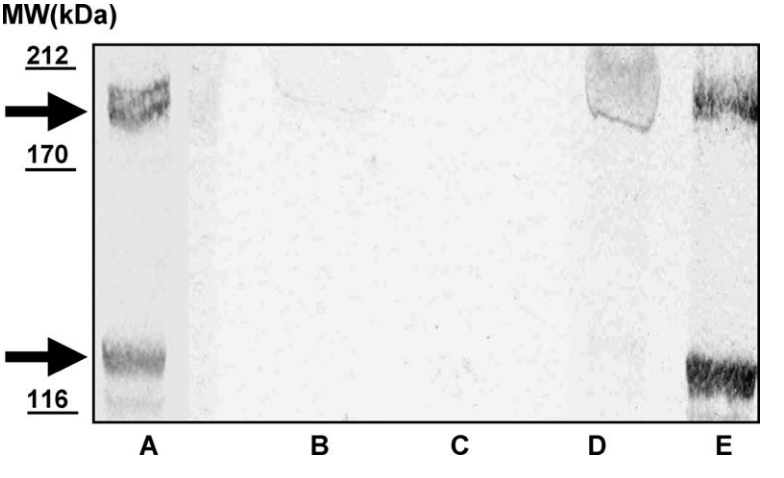

Figure 3. Western blotting analysis performed with a polyclonal antibody raised against oestrogen-dependent proteins from mosquitofish (see the Materials and methods for polyclonal antibody preparation and for Western blot analysis). Analysed tissues: liver (A) and muscle (B) of female undergoing vitellogenesis; liver of untreated male (C), muscle (D) and liver (E) of male after exposure to 0.4 $\mu g\,l^{-1}$ EE2 for 7 days. Each lane (10 μg proteins) corresponds to one fish. Arrows show protein bands of 205 and 125 kDa, respectively.

oestrogen-dependent proteins were detected at a significant level from 3 days of exposure.

Interaction between bNF and EE2

The effect of bNF on the induction of oestrogen-dependent proteins by EE2 in the liver of male mosquitofish was studied following the protocol shown in Figure 4.

The effects of the different treatments on the hepatic EROD activity are shown in Figure 5. The EROD activities in the liver of fish exposed to 0.1 $\mu g\,l^{-1}$ EE2, 0.25 $\mu g\,l^{-1}$ bNF or 0.1 $\mu g\,l^{-1}$ EE2 plus 0.25 $\mu g\,l^{-1}$ bNF are not statistically different from that in control fish. The EROD activities in the liver of fish exposed to 1.0 or 4.0 $\mu g\,l^{-1}$ only or in combination with 0.1 $\mu g\,l^{-1}$ EE2 are significantly higher than those measured in control fish, with the EROD activity in the liver of the fish exposed to 4.0 $\mu g\,l^{-1}$ bNF being significantly higher than that recorded in the fish exposed to 1.0 $\mu g\,l^{-1}$. The induction rates of the EROD activity by 1.0 or 4.0 $\mu g\,l^{-1}$ bNF are not statistically modified by co-exposure to 0.1 $\mu g\,l^{-1}$ EE2.

The effect of the different treatments on the synthesis of hepatic oestrogen-dependent protein is shown in Figure 6. The results show that the pattern of response is the same for the 205-kDa as for the 125-kDa protein bands. No immunoreactivity was detected in control fish nor in fish exposed to bNF only, whereas the liver of fish exposed to 0.1 $\mu g\,l^{-1}$ EE2 exhibited marked induction of 205- and 125-kDa protein

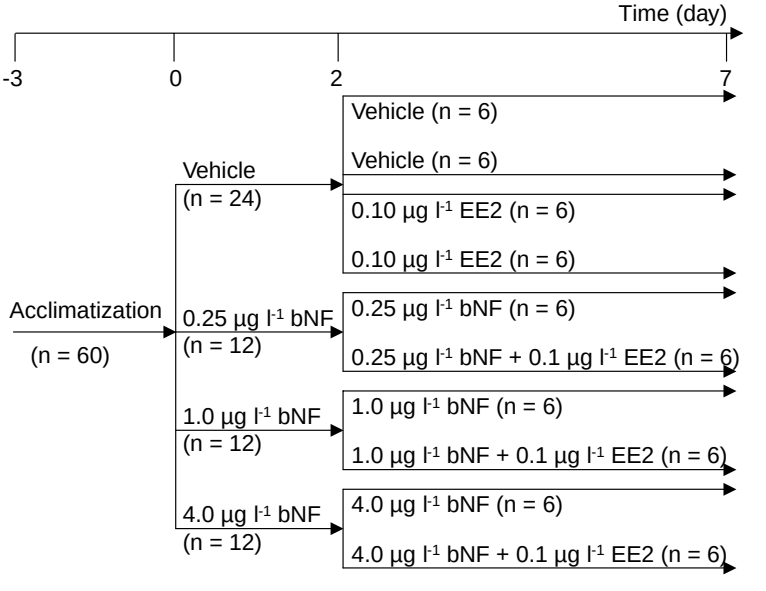

Figure 4. Schematic representation of the experimental protocol carried out to study the interaction between beta-naphthoflavone (bNF) and 17alpha-ethynylestradiol (EE2). Sixty male mosquitofish were divided in ten groups of six fish. After 3 days of acclimatization, water was renewed: four groups were exposed to the solvent, two groups were exposed to 0.25 $\mu g\,l^{-1}$ bNF, two groups were exposed to 1.0 $\mu g\,l^{-1}$ bNF, and two groups were exposed to 4.0 $\mu g\,l^{-1}$ bNF. After 2 days, the water was renewed and the different groups were exposed to the chemicals as shown. The water and chemicals were renewed again on day 5. Fish were sacrificed on day 7. Indicated concentrations are nominal concentrations. The number of fish used for solvent vehicle and for EE2-only treatments was higher than that of other treatment groups in order to improve the statistical weight of the experiment.

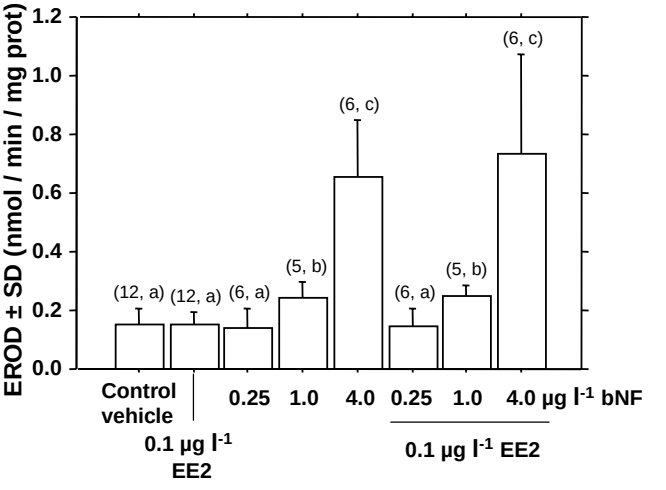

Figure 5. Hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in male mosquitofish exposed to beta-naphthoflavone (bNF) only or in combination with 17alpha-ethynylestradiol (EE2). For the protocol of exposure to bNF and EE2, see Figure 4. Data are the mean ± standard deviation. The number of fish analysed and the statistical ranking of each treatment group are indicated between parentheses. Two groups of data labelled with different letters have significantly different EROD activities ($p < 0.05$, Mann–Whitney *U*-test).

bands (Figure 6A). This induction was not modified by co-exposure to 0.25 or 1.0 $\mu g\, l^{-1}$ bNF, but a significant reduction was observed after co-exposure to 4.0 $\mu g\, l^{-1}$ bNF (Figure 6B). Following the same protocol, an additional experiment showed that no immunoreactivity was detected in the liver of male mosquitofish exposed to 0.1 $\mu g\, l^{-1}$ EE2 plus 20.0 $\mu g\, l^{-1}$ bNF (data not shown).

Discussion

Our study showed that the induction of EROD activity, a biomarker of exposure to the AhR agonists, and the induction of oestrogen-dependent proteins, a biomarker of exposure to xenoestrogens, can be monitored in the liver of male mosquitofish. Furthermore, the results showed that in this species, the *in vivo* induction of hepatic oestrogen-dependent proteins by the xenoestrogen EE2 can be inhibited by co-exposure to the EROD inducer bNF.

Liver as target tissue

When studying the effect of (xeno)estrogens in fish, analysis of oestrogen-dependent proteins was usually performed on blood samples (Tyler et al. 1996, Arukwe et al. 1997b, Denslow et al. 1999, Parks et al. 1999). Alternatively, analysis on whole-body homogenate was shown to be convenient in small fish (Holbech et al. 2001). In the present study, we decided to carry out analysis on liver homogenate. Liver is the tissue where Vtg and eggshell proteins are synthesized after oestrogenic stimulation, and it is also the main tissue for CYP1A induction. Therefore, we thought that liver was a relevant target tissue for studying the interaction between ER- and AhR-signalling pathways.

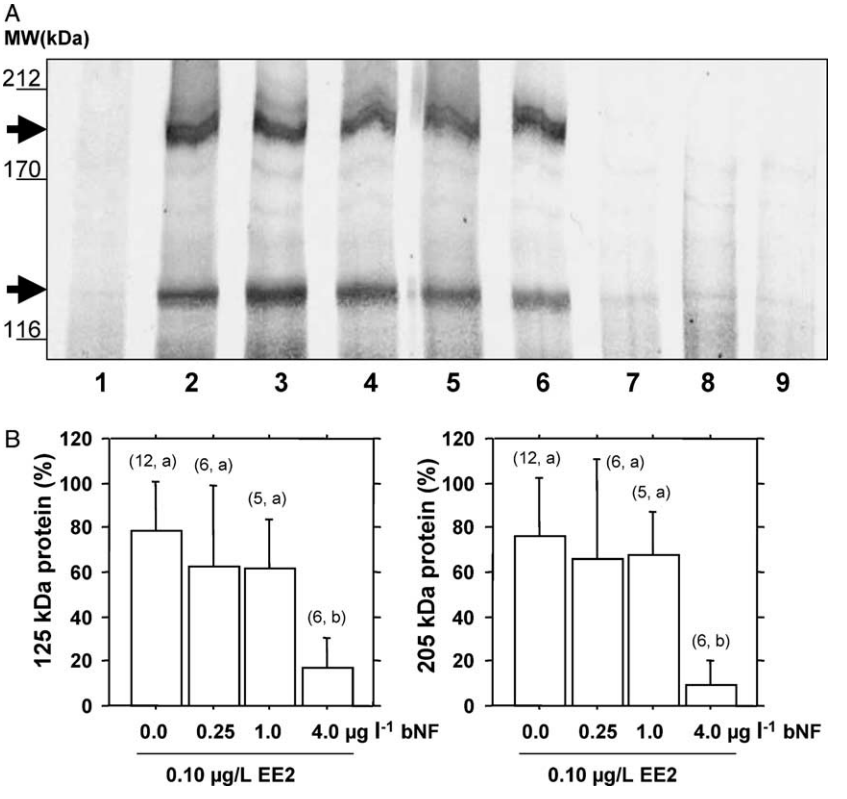

Figure 6. Effect of beta-naphthoflavone (bNF) on the induction of hepatic oestrogen-dependent proteins by 17alpha-ethynylestradiol (EE2) in male mosquitofish. For the protocol of exposure, see Figure 4. (A) Typical Western blot analysis using polyclonal antibody raised against oestrogen-dependent proteins (see the Materials and methods for polyclonal antibody preparation). Lanes 1, 2 and 4–9 correspond to one fish; lanes 1 and 8, fish exposed to solvent vehicle for 5 days; 2 and 4, fish exposed to 0.1 $\mu g\,l^{-1}$ EE2 only for 5 days; 3, pool of livers from males exposed to 0.1 $\mu g\,l^{-1}$ EE2 only for 5 days (internal standard); 5, fish exposed for 2 days to 0.25 $\mu g\,l^{-1}$ bNF and then to 0.25 $\mu g\,l^{-1}$ bNF plus 0.1 $\mu g\,l^{-1}$ EE2 for 5 days; 6, fish exposed to 1.0 $\mu g\,l^{-1}$ bNF for 2 days and then to 1.0 $\mu g\,l^{-1}$ bNF plus 0.1$\mu g\,l^{-1}$ EE2 for 5 days; 7, fish exposed to 4.0 $\mu g\,l^{-1}$ bNF for 2 days and then 4.0 $\mu g\,l^{-1}$ bNF plus 0.1 $\mu g\,l^{-1}$ EE2 for 5 days; and 9, fish exposed to 4.0 $\mu g\,l^{-1}$ bNF only for 7 days. Arrows show protein bands of 205 and 125 kDa, respectively. (B) Immunoreactive signal intensity of 205- and 125-kDa bands in male mosquitofish exposed to EE2 only or in combination with bNF. The signal intensity of immunoreactive bands was determined using ImageQuant image analysis software (Molecular Dynamics, Sunnyvale, CA, USA). The mean intensity of the oestrogen-dependent band signal from the liver of the treated fish was expressed as the per cent of the mean intensity of the oestrogen-dependent band signal from a pool of livers of male mosquitofish exposed to 0.1 $\mu g\,l^{-1}$ EE2 for 3 days and submitted to the same Western blot analysis (internal standard). Data are the mean $\pm$standard deviation. The number of fish analysed and the statistical ranking of each treatment group are indicated between parentheses. Two groups of data labelled with different letters have significantly different immunoreactive signal intensity ($p < 0.05$, Mann–Whitney U-test).

EROD

In the present study, a dose-dependent induction of EROD activity in the liver of male mosquitofish exposed to waterborne bNF was observed. In fish exposed to bNF, an induction level of hepatic EROD activity ranging from 1 to 283 was reported depending on species and conditions of exposure (Whyte et al. 2000). Relatively few

data have been published about EROD induction in *Poeciliidae* and particularly mosquitofish. Goddard et al. (1987) reported a two–six-fold increase in EROD activity in the liver of killifish *Poeciliopsis lucida* and *P. monacha* exposed to waterborne PAHs. Pulp mill effluents were shown to induce a four–eight-fold increase in EROD activity in mosquitofish and guppy (*Poecilia reticulata*), respectively (Larsson et al. 2002, Pacheco et al. 2002). From our data, the induction rate of hepatic EROD activity in mosquitofish after exposure to waterborne bNF was comparable with that reported in other *Poeciliid* species. Furthermore, the inhibition of hepatic EROD activity in mosquitofish by a low concentration of aNF, an inhibitor of CYP1A (Koley et al. 1997), strongly suggests that in this species EROD activity is CYP1A-dependent. CYP1A induction has been shown to be associated to AhR activation in several fish species, and partial sequencing of AhR was realized in a cell line from *Poeciliopsis lucida* (Hahn 1998). Accordingly, the induction of EROD activity in mosquitofish is likely to follow from the activation of AhR-signalling pathway, and can then be used as a biomarker of exposure to the AhR agonists.

Oestrogen-dependent proteins

Using the anti-serum prepared in our laboratory, Western blot analysis makes possible the detection of 205 kDa (double band) and 125 kDa protein bands in the liver of control female mosquitofish as well as in the liver of males exposed to EE2. From all the data recorded in the experiment reported in Figure 6, a close positive correlation is observed between the intensity of the 205 kDa immunoreactive band and the intensity of the 125 kDa immunoreactive band ($p < 0.001$, data not shown). Moreover, Western blot analysis performed in the liver of females sacrificed at different times after their parturition (data not shown) showed that those proteins were mainly detected during the 10 days following the parturition, which is the period corresponding to active vitellogenesis in mosquitofish (Koya et al. 2003). Tolar et al. (2001) reported the induction of a 429 kDa protein (non-denaturing conditions) in the blood of male mosquitofish fed with EE2-supplemented food and of control female. They suggested that this protein is Vtg. More recently, three distinct forms of Vtg were described in the plasma of E2-treated female mosquitofish (Sawaguchi et al. 2003). Further studies are needed to characterize precisely the identity of the oestrogen-dependent proteins detected in mosquitofish by our immunoserum, but nevertheless we could expect Vtg to be one of them.

Influence of EE2 on EROD activity

In the present study, the exposure of male mosquitofish to 0.1 $\mu g\, l^{-1}$ EE2 for 5 days altered neither the basal level nor the rate of induction of hepatic EROD activity by bNF. In fish, during sexual maturation hepatic CYP1A-dependent enzyme activities were shown to be lower in females than in males (Lindström-Seppa 1985, Förlin & Haux 1990, Masfaraud et al. 1990, Arukwe et al. 1997b). *In vivo* studies suggested a repressive effect of oestrogens or xenoestrogens on these enzyme activities (Förlin & Hansson 1982, Vodicnik & Lech 1983, Arukwe et al. 1997a). In cultured rainbow trout hepatocytes, E2 was shown to decrease basal EROD activity as well as basal CYP1A mRNA level (Navas & Segner 2001). In the same study, the authors showed that the presence of the ER antagonist tamoxifen abolished the inhibitory action of E2 on EROD activity, suggesting that the ER is involved in the suppressive action of E2

on CYP1A. However, in rainbow trout hepatocytes E2 did not overcome induction of CYP1A by bNF (Navas & Segner 2001) or TCDD (Bemanian et al. 2004). In mammalian Hepa-1 cells, E2 treatment blocked dioxin-induced accumulation of CYP1A1, and AhR-mediated activation of the CYP1A1 promoter (Kharat & Saatcioglu 1996). However, conflicting results were published when using other mammalian systems (Sarkar et al. 2000, Son et al. 2002). Further studies are needed, particularly by testing a range of concentrations of EE2, before concluding on the absence of the EE2 effect on EROD activity in mosquitofish.

Influence of bNF on oestrogen-dependent protein induction by EE2

Our data showed that EE2-induced production of oestrogen-dependent proteins in the liver of male mosquitofish can be inhibited by exposure to the AhR agonist bNF. In fish, the repressive control of AhR-signalling pathway towards the ER-signalling pathway has been suggested in some other studies. For example, *in vitro* studies using cultured liver cells of rainbow trout and carp demonstrated a repressive effect of AhR agonists on E2-induced vitellogenesis (Anderson et al. 1996a, Smeets et al. 1999, Navas & Segner 2000, Bemanian et al. 2004). However, *in vivo* studies reported conflicting results. Data in favour of *in vivo* anti-oestrogenic effects of AhR agonists include reduced level of circulating Vtg induced by E2 in juvenile rainbow trout fed with PCB-contaminated diet (Chen et al. 1986), impairment of ovarian recrudescence paralleled by a decrease in plasma E2 concentration in Atlantic croaker (*Micropogonias undulatus*) fed with a PCB-contaminated diet (Thomas 1989), and impairment of the development of pre-vitellogenic to vitellogenic oocytes in female zebrafish (*Brachydanio rerio*) exposed to dioxin-contaminated food (Wannemacher et al. 1992). In contrast, intraperitoneal injection of female striped bass (*Morone saxatilis*) by dioxin-like 3,3′,4,4′-tetrachlorobiphenyl (TCB) during gonadal recrudescence did not disrupt circulating E2, testosterone and Vtg levels (Monosson et al. 1996). Juvenile rainbow trout injected intraperitoneally with 3,3′,4,4′,5,5′-pentachlorobiphenyl, then with E2, did not show a decrease in circulating Vtg levels when compared with fish exposed to E2 only. Other studies suggested that the *in vivo* response could be dependent on conditions of exposure. For example, treatment of juvenile rainbow trout with 0.5 mg kg^{-1} E2 plus either 25 or 50 mg kg^{-1} bNF showed that bNF depressed E2-induced Vtg synthesis by the liver. Alternatively, juvenile trout treated with 5 mg kg^{-1} E2 plus either 25 or 50 mg kg^{-1} bNF showed a potentiation of Vtg synthesis relative to E2-only injected fish (Anderson et al. 1996b). Arguments supporting the dependence of *in vivo* response to the conditions of interaction between AhR agonists and xenoestrogens were also given by Arukwe et al. (2001b). In a set of experiments, these authors treated juvenile Atlantic salmon (*Salmo salar*) with the xenoestrogen nonylphenol (NP) only or in combination with TCB. Data showed a stimulatory effect by TCB on NP-induced plasma Zrp and Vtg levels and their respective liver mRNA species during some of the experimental conditions, whereas the effects were not detected, or an anti-oestrogenic effect was observed during other conditions. No absolutely clear explanation could be deduced from the results of Arukwe et al. (2001b), but the responses appear to be dependent on the ratio of NP and TCB doses, and the temporal sequence of exposure.

Two main mechanisms have been suggested to explain the anti-oestrogenic activity of AhR agonists (Safe 1995, Gillesby & Zacharewski 1998). The latter could trigger

enhancement of the catabolism of oestrogenic compounds through AhR-mediated induction of biotransformation enzymes. Alternatively, AhR activation could also impair transcription of oestrogen-responsive genes.

In vertebrates, the biotransformation enzymes whose gene expressions are regulated by an AhR-signalling pathway including CYPs and UDP glucuronyl transferases are involved in steroid catabolism. Hydroxylation of oestrogens by CYPs has been demonstrated in fish as well as in mammals (Hansson & Rafter 1983, Snowberger & Stegeman 1987, Martucci & Fishman 1993, Lee et al. 2003). Therefore, enhancement of oestrogen catabolism as a consequence of induction of biotransformation enzymes by AhR agonists has been suggested. For example, in rainbow trout injected with radiolabelled E2 after pretreatment by bNF, the induction of biotransformation enzymes was paralleled by an increase of the biliary excretion of E2 derivatives (Förlin & Haux 1985). In human breast cancer cell line MCF-7, data reported an increase of E2 metabolism after exposure to the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Spink et al. 1990), and PAHs (Arcaro et al. 1999). However, in studies conducted in cultured rainbow trout hepatocytes, the authors failed to observe a decreased E2 level when CYP1A was induced (Anderson et al. 1996a, Navas & Segner 2000), and several studies conducted in mammals show that AhR-mediated anti-oestrogenicity can be observed in the absence of oestrogen metabolism (DeVito et al. 1992, Safe 1995, Rogers & Denison 2002). In the experiment reported in the present study, the exposure of male mosquitofish to the highest concentration of bNF could have increased the metabolic capabilities of fish towards EE2 through induction of biotransformation enzymes and thus could have decreased the oestrogenic signal. However, EE2 is a more stable and a more potent oestrogen than E2 (Arcand-Hoy et al. 1998), and in the present study the tested EE2 concentration was relatively high. Furthermore, continuous and long-lasting exposure to oestrogen is not necessary to induce oestrogenic effects. For example, in the human breast cancer cell line MCF-7, a pulse of 1 nM E2 as short as 1 min was sufficient to induce oestrogenic responses (Otto 1995). Similarly, in fathead minnows (*Pimephales promelas*) exposed continuously or intermittently to 0.12 $\mu g\, l^{-1}$ E2, intermittent exposure resulted in plasma Vtg concentrations approximately equal to those in response to continuous exposure and significantly higher than continuous exposure to the equivalent time-weighted average concentration (Panter et al. 2000). From all these data, we could suggest that in the present study EE2 catabolism was unlikely to be sufficient to explain fully the inhibition of the oestrogen-dependent protein synthesis by bNF in the liver of male mosquitofish.

The AhR-mediated disruption of ER signalling pathway is another mechanism of action that has been proposed to explain the anti-oestrogenic activity of AhR agonists (Safe et al. 2000). Studies conducted in mammals provide evidence of inhibition of E2-induced genes by AhR-ligand complex through inhibition of the transactivation capacity of ERalpha (Kharat & Saatcioglu 1996, Klinge et al. 1999, Safe et al. 2000). This general mechanism should also govern AhR-mediated down-regulation of ERalpha since this receptor is autoregulated (Tian et al. 1998, Rogers & Denison 2002). Recently, Ohtake et al. (2003) demonstrated that the agonist-activated AhR/ARNT heterodimer directly associates with ER-alpha and -beta, and that the function of liganded ER is subsequently attenuated. Interestingly, the authors show that in the absence of E2, the association between agonist-activated AhR/ARNT and ER can lead to oestrogenic effects.

In fish, direct involvement of activated-AhR in the disruption of ER signalling pathway was strongly suggested in liver cell cultures incubated with various CYP1A inducers including bNF (Anderson et al. 1996a, Smeets et al. 1999, Navas & Segner 2000). A recent study in cultured Atlantic salmon hepatocytes shows that activation of AhR by TCDD results in a marked reduction of ERalpha and Vtg mRNA levels (Bemanian et al. 2004). Vtg gene expression was also negatively affected by bNF but to a lesser extent than by TCDD. This study also suggested that activation of the AhR-signalling pathway caused a marked decrease in the number of the nuclear ERalpha or that activated AhR blocked the ability of ERalpha to bind to its target DNA sequence in a similar way as suggested in mammals.

In the present study, fish were pre-exposed to bNF for 2 days before being exposed to EE2 plus bNF for an additional 5 days. The experiment (Figure 2) shows that EROD induction was highly significant after 3 days of exposure to bNF. One can therefore suppose that the 2 days of pre-exposure to bNF should be enough for bNF to activate AhR. Our data show that EROD activity was dramatically induced and the synthesis of oestrogen-dependent proteins was significantly inhibited in fish treated by EE2 plus 4.0 $\mu g\,l^{-1}$ bNF when compared with fish exposed to EE2 only. The synthesis of the oestrogen-dependent proteins was not different in fish treated by EE2 plus 1.0 $\mu g\,l^{-1}$ bNF when compared with fish exposed to EE2 only, whereas EROD activity in fish treated by EE2 plus 1.0 $\mu g\,l^{-1}$ bNF was only slightly induced when compared with control vehicle fish. In fish treated by EE2 plus 0.25 $\mu g\,l^{-1}$ bNF neither the synthesis of oestrogen-dependent proteins nor EROD activity level were altered when compared with fish treated by EE2 only and by control vehicle, respectively. Taken altogether, our data suggest that pre-exposure then co-exposure to 4.0 $\mu g\,l^{-1}$ bNF could disrupt the ER signalling pathway activation by 0.1 $\mu g\,l^{-1}$ EE2 in the liver of mosquitofish. In these conditions, it might be hypothesized that AhR was activated by bNF and that AhR activation was at least in part involved in this disruption. On the other hand, 1.0 $\mu g\,l^{-1}$ bNF might lead to AhR activation, which is insufficient to overcome the activation of the ER-signalling pathway by 0.1 $\mu g\,l^{-1}$ EE2. Similarly, in carp hepatocytes concomitantly exposed to E2 and TCDD, the decrease in Vtg production did not parallel induction of EROD, and it started when EROD was maximally induced (Smeets et al. 1999). Further studies will be necessary to characterize the exact mechanism underlying the repressive effect of bNF on the induction of oestrogen-dependent protein by EE2 in mosquitofish.

Prospect for field monitoring

Our study shows experimentally that the induction of hepatic oestrogen-dependent proteins by the xenoestrogen EE2 can be inhibited by concomitant exposure to the AhR agonist bNF in male mosquitofish. Due to their widespread use by human activities, oestrogenic compounds and AhR agonists are likely to contaminate simultaneously the great majority of aquatic ecosystems with great variation of their respective proportions (Zacharewski et al. 1995, Michallet-Ferrier et al. 2004). In fish, the inhibitory effect of AhR agonists on the induction of oestrogen-dependent protein synthesis could be confusing for the interpretation of biomarker-based monitoring of field contamination by xenoestrogens. To an extreme degree, a location might be classified xenoestrogen-free while, in fact, it is actually contaminated by xenoestrogen and also by inhibiting concentrations of AhR agonists. To a lesser degree, locations

differentially contaminated by xenoestrogens might be detected through the measurement of oestrogen-dependent proteins in male fish, but they might be incorrectly ranked on the scale of oestrogenic contamination level due to differential interferring effects of AhR agonists also present in the same locations. Accordingly, in field studies dealing with the monitoring of xenoestrogen contamination through the analysis of oestrogen-dependent proteins in male fish, it would be recommended to measure EROD or CYP1A in parallel.

In conclusion, the present study shows that the induction of EROD activity, a biomarker of exposure to the AhR agonists, and the induction of oestrogen-dependent proteins, a biomarker of exposure to xenoestrogens, can be monitored in the liver of male mosquitofish. Furthermore, in this species the *in vivo* induction of hepatic oestrogen-dependent proteins by the xenoestrogen EE2 can be inhibited by co-exposure to the EROD inducer bNF. This last observation together with literature data suggest that field monitoring of xenoestrogen contamination through the analysis of oestrogen-dependent protein in male fish as a biomarker should take into account the possible negative interference of AhR agonists.

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