Sparus aurata L. liver EROD and GST activities, plasma cortisol, lactate, glucose and erythrocytic nuclear anomalies following short-term exposure either to 17h-estradiol (E_2) or E_2 combined with 4-nonylphenol

M. Teles, M. Pacheco, M.A. Santos *

Biology Department, Campus de Santiago, Aveiro University, 3810-193 Aveiro, Portugal

Abstract

Immature Sparus aurata L. (gilthead seabream) were exposed to 17h-estradiol (E_2) 4000 ng/l and to the same E_2 concentration mixed with 50,000 ng/l 4-nonylphenol (E_2 + NP) during 4, 8, 12 and 16 h. E_2 availability and E_2 plasma level variations were assessed. Liver biotransformation capacity was measured as ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) activities. Plasma cortisol, lactate and glucose were also determined. Genotoxicity was assessed through erythrocytic nuclear anomalies (ENA) frequency. Liver EROD activity significantly decreased during the whole experiment for both treatments, with the exception of 16 h exposure to E_2. Liver GST activity was significantly increased after 8 and 12 h of exposure either to E_2 or E_2 + NP. An endocrine disruption expressed as plasma cortisol decrease was observed after 16 h exposure under both tested conditions, concomitantly with a plasma lactate increase. No genotoxic responses, measured as ENA frequency, were detected. Analyzing the E_2 water concentration in aquaria without fish it was demonstrated an intense and fast E_2 loss, considerably reducing its availability to fish. In the presence of fish, E_2 water levels were drastically reduced after 4 h exposure, being this reduction more pronounced in E_2 aquarium when compared to E_2 + NP aquarium. In addition, it was demonstrated a rapid E_2 uptake from the water since the highest E_2 plasma concentrations were observed after 4 h exposure, followed by a continuous decrease, which became more pronounced between 8 and 12 h of exposure. Furthermore, during the first 8 h exposure to E_2 and E_2 + NP, seabream plasma E_2 concentrations were higher than the initial water exposure concentration. Comparing the E_2 plasma levels in both seabream-exposed groups, it was clear that its concentration is always higher in E_2 + NP-treated fish. Despite the previous results, no significant differences were found in the measured responses between E_2 and E_2 + NP.

Keywords: Seabream; Biotransformation; Genotoxicity; Endocrine disruption

* Corresponding author. Tel.: +351-234370965; fax: +351-234426408.

E-mail addresses: mteles@bio.ua.pt (M. Teles), mpacheco@bio.ua.pt (M. Pacheco), monteiro@bio.ua.pt (M.A. Santos).

1. Introduction

The aquatic environment contamination by xenoestrogens—substances that modulate or mimic the action of sex steroid hormones, mainly 17h-estradiol (E_2)—

has received considerable scientific, government and public attention (White et al., 1994; Brighty, 1996). However, natural estrogens such as E_2 and estrone can also be regarded as aquatic contaminants. The discovery of significant E_2 concentrations in municipal sewage treatment plants effluents (Brighty, 1996) became a matter of concern, being the assessment of its effects on fish at different levels, strongly recommended.

The degradation products of nonylphenol polyethoxylates, such as 4-nonylphenol (NP), are able to activate fish estrogen-dependent gene expression (Ackermann et al., 2002), revealing estrogenic properties either in vitro (White et al., 1994) or in vivo (Jobling et al., 1996). The polymer industry is the most important source of NP environment contamination, being the major part (95%) released to water. The majority of fish studies carried out on this subject concerned the effects on reproductive aspects. Either E_2 or NP elevate plasma vitellogenin and zona radiata proteins in both males and females (Ackermann et al., 2002), induce intersexuality (Jobling et al., 1996), inhibit spermatogenesis and alter gonadosomatic index (Sepúlveda et al., 2003). Besides the known interference on reproduction, it is also important to study the interaction of synthetic and natural steroids with other biological responses. In this perspective, a substantial lack of information concerning the effects of this type of compounds on nonreproductive endocrine responses is evident. Considering the important role of cortisol on fish physiological responses to stressors through intermediary metabolism regulation, immune function and hydromineral homeostasis (Hontela, 1997), its alterations after exposure to xeno/estrogens must be investigated.

Different authors (Pajor et al., 1990; Arukwe et al., 1997) proposed a cross-talk mechanism between P450 activities and steroid synthetic pathways. Thus, cytochrome P450-based mixed-function oxygenase (MFO) system is involved in xenobiotics and endogenous steroids metabolism. Despite the lack of information concerning CYP1A regulation by endogenous compounds, it was demonstrated that both EROD activity and CYP1A respond to estrogens (Solé et al., 2000). A significant reduction in EROD activity and CYP1A protein was reported in several fish species, namely Pseudopleuronectes americanus, Stenotomus chrysops (Gray et al., 1991) and Salmo salar (Arukwe and Goksøyr, 1997) after E_2 exposure. The same pattern of response was also observed in S. salar (Arukwe et al., 1997) after NP exposure. Metabolites resulting from biotransformation processes (phase I) may be conjugated with endogenous molecules (phase II) making them more readily excretable (GoksØyr and Förlin, 1992). Gallagher et al. (2001) found that conjugase activities are also modulated by endogenous steroids. Additionally, effects of synthetic or natural steroids include genotoxicity (Liehr, 2000) as it was proposed that they could induce genetic damage and gene mutations in mammals (Roy and Liehr, 1999; Yared et al., 2002). However, to our knowledge only a few studies are available concerning genotoxicity of these compounds in non-mammal species. NP was reported as genomic DNA damaging agent to crustacean larvae (Atienzar et al., 2002), and genotoxic to sea bass, measured as erythrocytic nuclear abnormalities (ENA) (Teles et al., 2004a).

Gilthead seabream, Sparus aurata L., seawater teleost widespread in Atlantic and Mediterranean coastal waters, is one of the most commercially important species, and as protandrous hermaphrodite is particularly appropriate as a biological model to endocrine studies.

Environmental waters are frequently contaminated simultaneously by different natural and synthetic steroids, being therefore relevant to study xeno/estrogen mixtures effects, as well as their eventual interference on single compounds effects, in fish.

The aims of the present research work, using juvenile seabream, were to assess the waterborne E_2 or E_2 mixed with NP (E_2 + NP) effects on biotransformation, measured as total P450 content, ethoxyresorufin-O-deethylase (EROD) activity (phase I) and glutathione-S-transferase (GST) activity (phase II). The variation on E_2 concentrations in aquaria water and in seabream plasma was determined during the whole experiment. Liver somatic index (LSI) and alanine transaminase (ALT) activity were measured as general hepatic condition indicators. Additionally, the stress responses were evaluated as plasma cortisol, glucose and lactate, and genotoxicity was investigated using the ENA assay.

2. Material and methods

2.1. Chemicals

NP and E_2 were purchased from Sigma-Aldrich (Germany). Marine salt was supplied by Sera Premium (Germany). All the other chemicals were of analytical

grade obtained from Sigma-Aldrich, Roche (Germany) and E. Merck-Darmstadt (Germany).

2.2. Test animals

The experiment was carried out with juvenile S. aurata specimens obtained from a local fish farm, Materaqua- Ílhavo, Portugal. Seabream weighing 34 F 0.3 g were transported in aerated saltwater and acclimated to laboratory conditions for 1 week prior to experimentation. During acclimatization and experimental periods, fish were kept in 80-l aquaria, at 20 jC in aerated (dissolved oxygen: 7.4 F 0.2 mg/l) and filtered artificial seawater (34 g/l salinity), with a pH of 8.4 F 0.2.

2.3. Experimental design

Fish were divided in three lots: one as control and the others were exposed either to E_2 or E_2 mixed with NP (E_2 + NP), during 4, 8, 12 and 16 h. The appropriate amount of each chemical was previously dissolved in 1 ml of DMSO and added to the experimental aquaria in order to prepare the following nominal concentrations: 14.68 nM (4000 ng/l) of E_2 and 183.5 nM (50,000 ng/l) of NP. The same volume of DMSO was added to the control aquarium, since this concentration has no damaging effects on fish (Pacheco and Santos, 1998). Experiments were carried out using test groups of five seabream (n = 5). E_2 or E_2 + NP (in the previous nominal concentrations) were added to two additional aquaria (E_2nf and $E_2 + NP_{nf}$), kept without fish, in order to determine the E_2 availability to fish after the eventual adsorption and degradation occurring during the same exposure period.

Fish were killed at each sampling point and their blood and liver were collected. Liver was immediately frozen in liquid nitrogen, stored at − 80 jC until homogenization. Blood smears were prepared. Blood plasma was isolated using an Eppendorf centrifuge (14,000 rpm).

2.4. Biochemical analysis

2.4.1. Liver cytochrome P450 content and EROD activity

Cytochrome P450 content was determined using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm, as previously described by Hermens et al. (1990).

The liver EROD activity was measured in microsome suspension as described by Burke and Mayer (1974) as adapted by Pacheco and Santos (1998). The reaction was carried out, at 25 jC, in the fluorometer cuvette containing 1 ml 0.5 AM ethoxyresorufin (in 0.1 M Tris–HCl pH 7.4, containing 0.15 M KCl and 20% glycerol) and 100 Al of microsomal suspension. The reaction was initiated by adding 10 Al of NADPH (10 mM) and the progressive increase in fluorescence, resulting from the resorufin formation, was measured for 3 min (excitation wavelength 530 nm, emission wavelength 585 nm). EROD-activity was expressed as picomoles per minute per milligram of microsomal protein.

2.4.2. Liver GST activity

GST activity was determined in the cytosolic fraction as described by Lemaire et al. (1996), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). The assay was carried out in a quartz cuvette with a 2-ml mixture of 0.2 M phosphate buffer (pH 7.4), 0.2 mM CDNB and 0.2 mM GSH. The reaction was initiated by the addition of 10 Al sample at 25 jC. The increase in absorbance was recorded at 340 nm wavelength during 3 min.

2.4.3. Liver ALT activity

ALT activity was measured, in the supernatant resulting from microsomal isolation, according to a colorimetric method based on the measurement of the pyruvate produced by the transamination reaction (Reitman and Frankel, 1957).

2.4.4. Protein measurement

Microsomal and cytosolic protein concentrations were determined according to the Biuret method (Gornal et al., 1949) using bovine serum albumin (E. Merck-Darmstadt) as a standard.

2.4.5. Cortisol, glucose and lactate measurement

The determination of cortisol was performed in plasma using a diagnostic ELISA direct immunoenzymatic kit (Diametra, Italy). The cortisol in the sample competes with horseradish peroxidase-cortisol for binding onto the limited number of anti-cortisol sites in the microplate. Cortisol concentration in the sample is calculated based on a series of standards and the

color intensity is inversely proportional to the cortisol concentration in the sample. The method allows the determination of cortisol from 10 to 500 ng/ml.

Plasma glucose was measured spectrophotometrically (340 nm) according to the method modified from Banauch et al. (1975) based on the quantification of NADH after a glucose oxidation catalysed by the glucose-dehydrogenase. The quantity of NADH formed is proportional to the glucose concentration.

Plasma lactate levels were determined spectrophotometrically (340 nm) according to the method modified from Noll (1974) using lactate-dehydrogenase (LDH), ALT and NAD, measuring the NADH appearance.

2.4.6. 17b-Estradiol measurement

The E_2 determination was performed using a diagnostic ELISA direct immunoenzymatic kit (Diametra). E_2 in the sample competes with horseradish-peroxidase E_2 for binding onto the limited number of anti E_2 sites on the microplates. E_2 concentration in the sample is calculated based on a series of standard; the color intensity is inversely proportional to the E_2 concentration in the sample. The method allows the determination of E_2 from 20 to 4000 pg/ml.

Plasmatic and water E_2 measurements were carried out during the entire experiment. The E_2 quantification on water was performed in the experimental aquaria (E_2, $E_2 + NP$) containing seabream, as well as in two additional aquaria kept without fish (E_2nf, $E_2 + NP_{nf}$).

2.5. Liver somatic index

LSI results were presented as a frequency (%) resulting from the following expression: [liver mass (g)/body mass (g)] × 100%.

2.6. ENA assay

The blood smears were fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. In order to evaluate genotoxicity the erythrocytic nuclear abnormalities were scored in 1000 mature erythrocytes sample per fish, according to the criteria of Schmid (1976), Carrasco et al. (1990) and Smith (1990), adapted by Pacheco and Santos (1996). According to these authors, nuclear lesions were scored into one of the following categories: micronuclei, lobed nuclei, dumbbell shaped or segmented nuclei and kidney shaped nuclei. The final result was expressed as the mean value ($\bar{x}$) of the sum for all the individual lesions observed.

2.7. Statistical analysis

Statistica software (StatSoft, Tulsa, OK) was used for statistical analyses. All the data were first tested for normality and homogeneity of variance to meet statistical demands. ANOVA analysis was used to compare results between fish groups, followed by LSD test (Zar, 1996). Differences between means were considered significant when $P < 0.05$.

3. Results

3.1. Water and plasma 17b-estradiol quantification (Table 1)

A time-related reduction in 17h-estradiol concentrations was observed either in aquaria with fish (E_2 and $E_2 + NP$) or without fish (E_2nf and $E_2 + NP_{nf}$),

Table 1
17h-Estradiol (E_2) measurement in the aquaria water 5 min, 4, 8, 12 and 16 h and in the S. aurata plasma 4, 8, 12 and 16 h after the adition of E_2 (4000 ng/l) or E_2 mixed with 4-nonylphenol (NP)—$E_2 + NP$—4000 and 50,000 ng/l, respectively

Water estradiol (ng/l)						Plasma estradiol (ng/l)		
Time	Control	E_2	$E_2 + NP$	E_{2nf}	$E_2 + NP_{nf}$	Control	E_2	$E_2 + NP$
5 min	33.05	666.01	1618.18	3426.21	3879.99	–	–	–
4 h	29.09	31.46	258.86	1605.71	2039.32	59.14	5997.43	14329.58
8 h	17.38	24.16	102.08	1238.95	1481.23	145.55	5111.98	10040.00
12 h	29.75	17.62	32.05	958.90	1203.81	101.31	882.13	4051.00
16 h	32.98	10.18	19.99	630.71	686.84	51.41	206.10	1355.04

The water quantification was performed in aquaria with fish (E_2 and $E_2 + NP$) or without fish (E_2nf and $E_2 + NP_{nf}$).

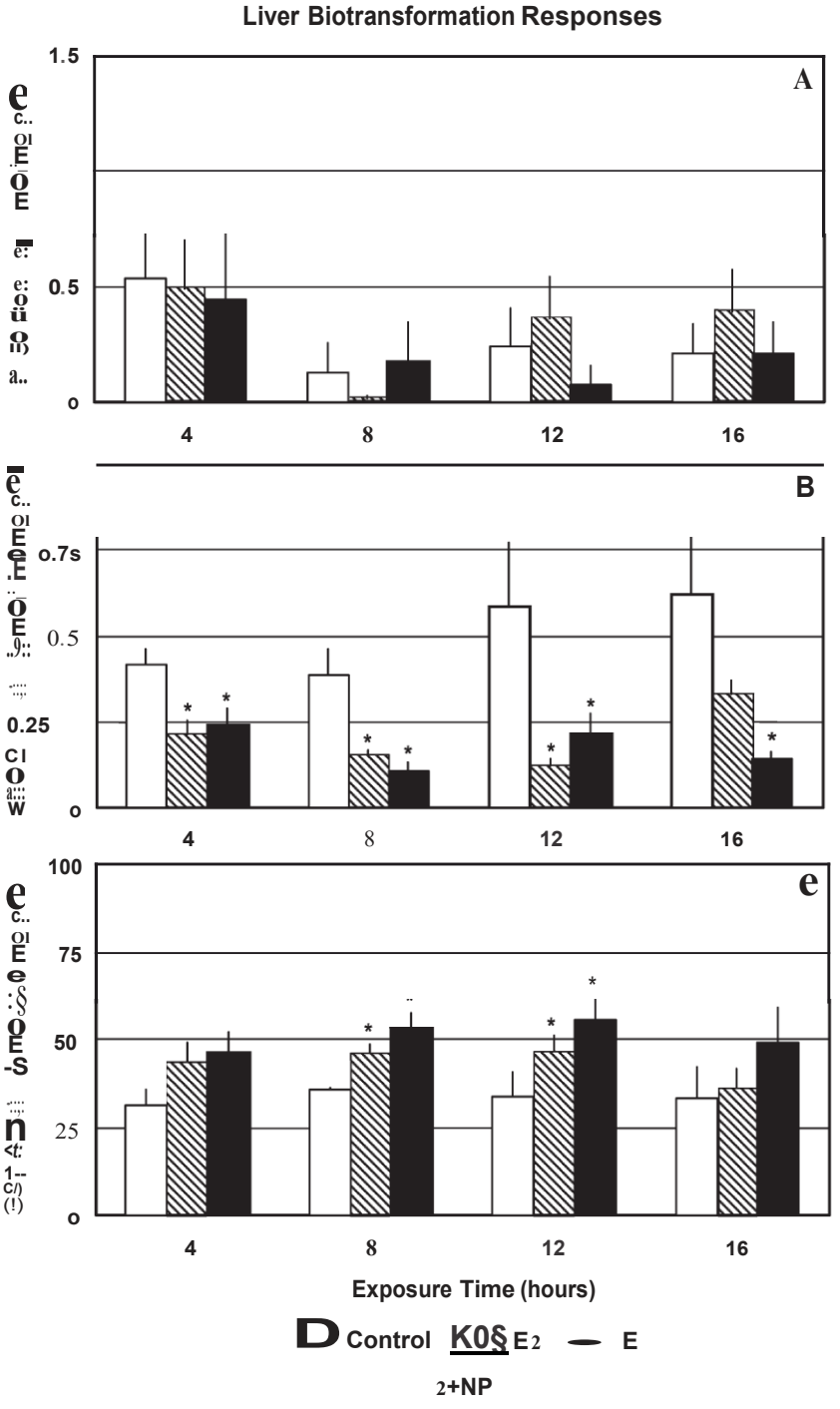

Fig. 1. S. *aurata* liver P450 content (A), liver EROD activity (B) and liver GST activity (C) after 4, 8, 12 and 16 h exposure to (4000 ng/l) of 17f>-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP)--E_2 + NP-4000 and 50,000 ng/l, respectively. Values represent the means and S.E. (n = 5/treatment). Differences from control: *P< 0.05.

during the entire experiment. However, this E_2 reduction was more pronounced in aquaria containing fish. On the other hand, the $E_2 + NP$ aquaria always presented clearly higher E_2 concentrations than the E_2 aquaria, either in the fish presence or absence. After 4 h exposure E_2 levels in the aquaria with fish were drastically reduced, i.e., a 99% reduction in E_2 aquarium and a 93.5% reduction in $E_2 + NP$ aquarium. Furthermore, the control level was reached after 4 h in E_2 aquarium, whereas in $E_2 + NP$ aquarium this reduction occurred after 12 h of exposure. The maximum E_2 plasma levels were observed after 4 h exposure in both treated groups, presenting thereafter a time-related decrease. Seabream exposed to $E_2 + NP$ exhibited E_2 plasma concentrations higher, at least two-fold, than E_2-treated group. In the period between 4 and 8 h, it was observed, for treatments, the lowest E_2 plasma reduction, 15% and 30% reduction for E_2- and $E_2 + NP$-treated fish, respectively. The highest plasma E_2 reduction was observed after 12 h in E_2 (83% reduction) and at 16 h in $E_2 + NP$-treated fish (67% reduction) Table 1.

Taking into account the E_2 levels detected in the E_2nf and $E_2 + NP_{nf}$ aquaria, indicating the E_2 availability, it is noticeable that its uptake from the water in

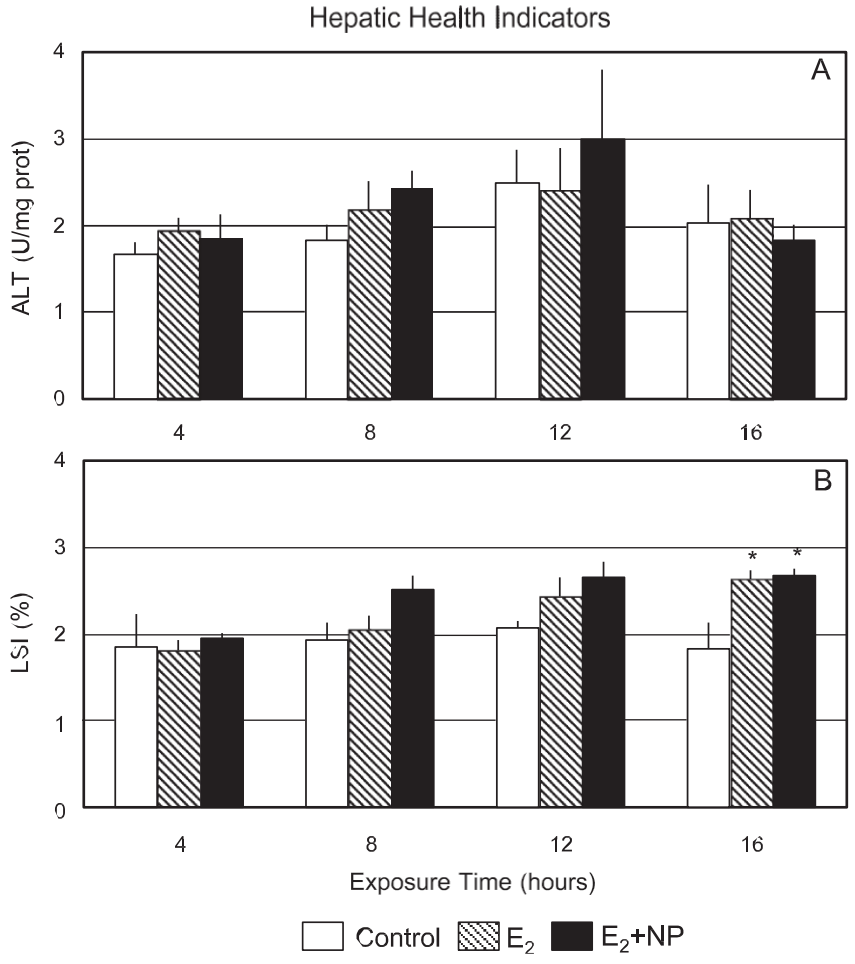

Fig. 2. S. aurata liver ALT activity (A) and LSI (B) after 4, 8, 12 and 16 h exposure to (4000 ng/l) of 17h-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP)—$E_2 + NP$—4000 and 50,000 ng/l, respectively. Values represent the means and S.E. (n = 5/treatment). Differences from control: *P < 0.05.

E_2 group was slightly lower than in E_2 + NP group during the first 4 h.

3.2. Liver biotransformation responses (Fig. 1)

P450 content did not exhibit any statistically significant alteration (Fig. 1A). However, a significant EROD activity decrease was observed in seabream after 4, 8 and 12 h exposure to E_2 and E_2 + NP compared to the control (Fig. 1B). Furthermore, EROD activity inhibition significantly persisted at 16 h of exposure to E_2 + NP. Seabream exposed either to E_2 or to E_2 + NP did not demonstrate any significant differences between them. A significant increase in GST activity was observed in seabream at 8 and 12 h exposure to E_2 or E_2 + NP (Fig. 1C).

3.3. Hepatic health indicators (Fig. 2)

Liver ALT activity did not present any significant alteration (Fig. 2A). However, LSI significantly rises after 16 h exposure to E_2 and E_2 + NP (Fig. 2B).

3.4. Genotoxic response (Fig. 3)

The ENA frequency in treated fish was similar to that displayed by the control (Fig. 3).

3.5. Stress responses (Fig. 4)

Seabream plasma cortisol levels significantly decreased after 16 h exposure to E_2 or E_2 + NP despite its decreased tendency since the initial exposure (Fig. 4A). The plasma glucose concentration did not exhibit any significant alteration (Fig. 4B). Plasma lactate levels displayed a significant increase after 16 h exposure either to E_2 or E_2 + NP, though this increasing tendency was noticed from 8 h exposure onwards (Fig. 4C).

4. Discussion

4.1. 17b-Estradiol measurements

E_2 has been detected in effluent sewage treatment plants in different countries such as USA, Japan, Germany, Italy and the Netherlands, at concentrations up to 64 ng/l (Spengler et al., 2001). Considering the previous environmental data, a spiking concentration was adopted in the current study, to ensure a substantial uptake in a short period. Thus, this fact should be kept in mind in eventual extrapolations of the current findings to environmental situations.

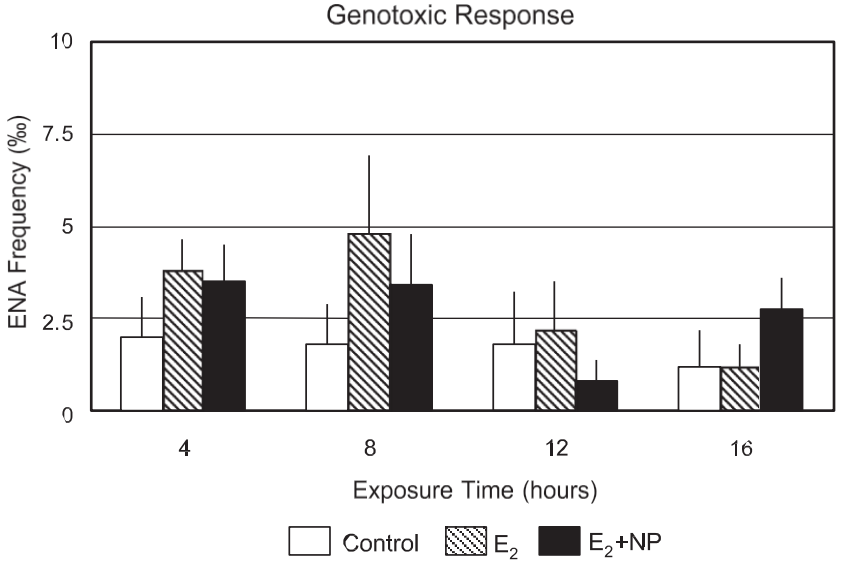

Fig. 3. S. aurata ENA frequency after 4, 8, 12 and 16 h exposure to (4000 ng/l) of 17h-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP)—E_2 + NP—4000 and 50,000 ng/l, respectively. Values represent the means and S.E. (n = 5/treatment). Differences from control: *P < 0.05.

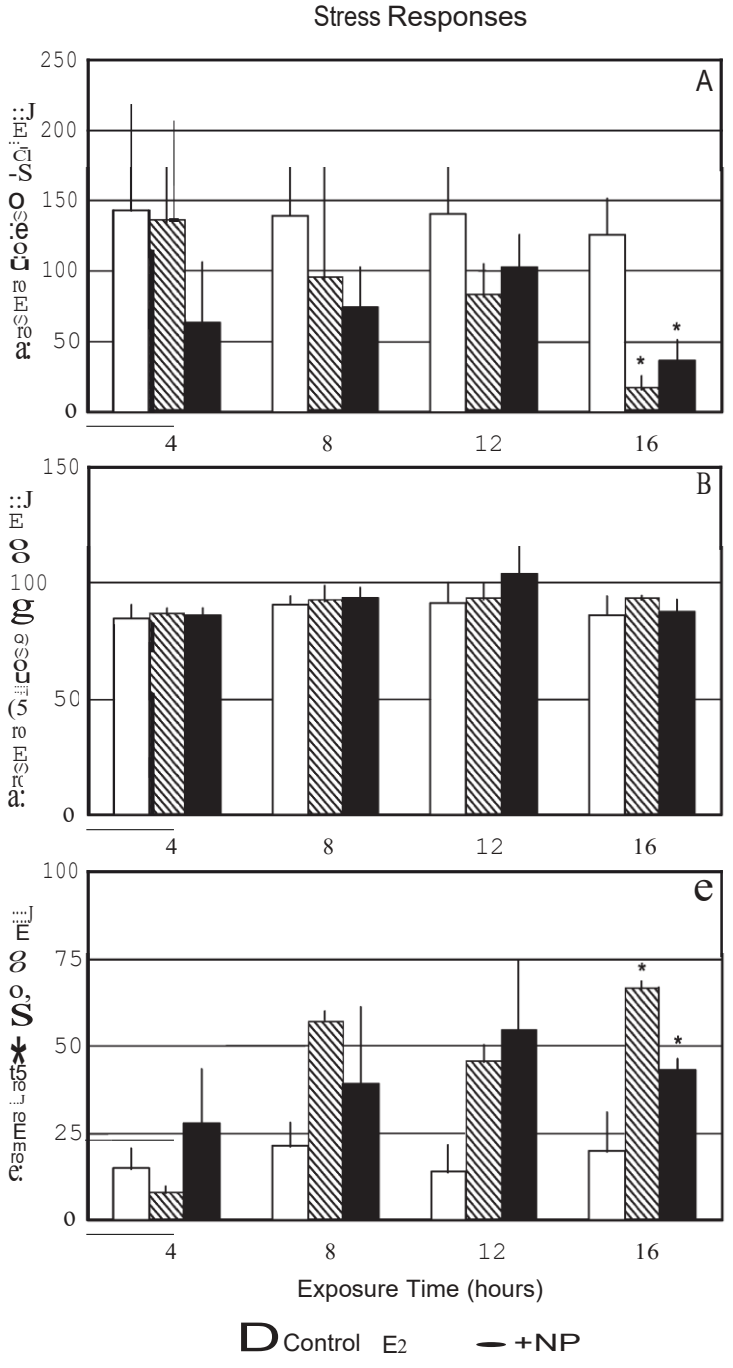

Fig. 4. S. *aurata* plasma cortisollevels (A) plasma glucose levels (B) and plasma lactate levels (C) after 4, 8, 12 and 16 h exposure to (4000 ng/1) of l7f>-estradiol (E_2), and to a mixture of E_2 and 4-nonylphenol (NP)— E_2 + NP--4000 and 50,000 ng/1, respectively. Values represen!the means and S.E. (n=5/treatment). Differences from control: *$P<0.05$.

The NP concentrations detected in environment ranged from near or below limits of detection up to 180 Ag/l (Nielsen et al., 2000).

The measurement of E_2 in aquaria without fish demonstrated a time-related reduction in E_2 concentration, which is less pronounced in the presence of NP. Some studies were previously carried out on E_2 sorption and degradation, which can help to explain the current results. A study testing an E_2 concentration range which includes the current E_2 concentration revealed that microorganisms were capable of transforming E_2 with half-lives of 0.2–9 days (Jurgen et al., 2002). According to the same authors, E_2 was susceptible to photodegradation with half-lives in order of 10 days. Additionally, Holthaus et al. (2002) observed that 80–90% of E_2 bound to bed sediments within 1 day, whereas Ying et al. (2003) stated that E_2 had a modest sorption capability to sediment.

The E_2 decrease in water was more pronounced in aquaria with than without seabream, suggesting its rapid uptake from the water, which is corroborated by the plasma E_2 increase. Thus, E_2 on water was drastically reduced during the first 4 h of exposure, and concomitantly the highest E_2 plasmatic concentrations were detected after 4 h, followed by its continuous decrease, which became more pronounced between 8 and 12 h exposure. Moreover, plasma E_2 concentrations were higher than the initial water exposure concentration (4000 ng/l), during the first 8 and 12 h in fish, respectively exposed to E_2 and E_2 + NP. The present results agree with Specker and Chandlee (2003) who found that E_2 uptake by Paralichthys dentatus reached an initial plateau within 30 min, being accumulated in the whole-body above the exposure concentrations. Comparing the E_2 plasma levels in seabream exposed either to E_2 or E_2 + NP, it is clear that its concentration was always higher in the last group. This fact may reflect alterations on E_2 water/plasma/tissue uptake, and excretion, as a consequence of the simultaneous NP exposure.

Despite the different E_2 plasma levels between seabream exposed to E_2 and E_2 + NP, in general the measured responses were not different, as discussed below.

4.2. Biotransformation responses

The CYP1A induction measured either by immunodetection or through its catalytic activity is probably the best-studied biomarker (Bucheli and Font, 1995). Hence, EROD activity has been widely used as a biomarker for fish exposure to substances that bind to the aryl hydrocarbon (Ah) receptor (Lemaire et al., 1996; Pacheco and Santos, 1998, 2001).

In the present research work P450 content did not demonstrate any alteration after E_2 or E_2 + NP exposures, which is in agreement with other authors who observed an unaltered total P450 content in Cyprinus carpio treated with the synthetic steroid 17a-ethynylestradiol (Solé et al., 2000) and in D. labrax exposed to E_2 (Teles et al., 2004a).

It is known that steroid hormones can regulate CYP1A expression. A decrease in the catalytic CYP1A activities was observed in sexually mature females with elevated levels of E_2, as well as in E_2-treated fish, namely Oncorhynchus mykiss (Andersson and Rafter, 1990), P. americanus and S. chrysops (Gray et al., 1991). Thus, the current EROD activity decrease regularly found during the whole experiment in both exposed groups is not surprising. Though, the interaction mechanism between E_2 and CYP1A is not well understood and different possibilities were presented. It was suggested that the possible binding of E_2 to the estrogen receptor (ER) exhibits an anti-CYP1A influence on the CYP1A gene, indicating a probable receptor and/or gene cross-talk between CYP1A and estrogen-responsive genes (Arukwe et al., 1997; Navas and Segner, 2000). However, the ability of steroids, including E_2 binding to the CYP1A molecule, acting as a competitive inhibitor of its enzymatic activities, e.g. EROD, was established in fish (Chan and Hollebone, 1995).

Despite NP estrogen-like properties (White et al., 1994) its interference with CYP1A function was demonstrated as an EROD activity reduction in juvenile S. salar (Arukwe et al., 1997). Furthermore, BNF potential to induce EROD was also reduced either by NP or E_2 D. labrax exposure (Teles et al., 2004a). Considering the previous findings a higher EROD activity reduction should be expected in E_2 + NP than in E_2 seabream exposed groups; however, current results did not confirm this hypothesis, since there was no NP additional effect under co-exposure.

The less pronounced decrease in EROD activity was observed at 16 h exposure to E_2 coinciding with the lowest E_2 plasma concentration and thus suggesting an E_2 metabolic turnover in the hepatocytes.

Furthermore, the E_2 inhibitory action was kept up to the end of the experiment in E_2 + NP exposed fish which coincided with a substantially higher E_2 blood concentration than that observed in E_2 exposed fish.

P450 enzymes are constitutively expressed being also modulated (induced/inhibited) by chemicals. Since no potential CYP1A inducers were used in the present study, it seems that E_2 affects the constitutive liver CYP1A expression, as previously demonstrated by Navas and Segner (2000) in monolayer cell cultures of O. mykiss.

The physiological implications of P450 MFO activities inhibition have not been fully established (Arukwe et al., 1997). Nevertheless, this MFO inhibition by environmental estrogens may reduce fish ability to metabolize and excrete xenobiotics as well as endogenous estrogens, causing alterations at different biological levels and not only on reproduction. These metabolic changes may have important ecological consequences, as fish are frequently exposed in the environment to different classes of chemicals. Furthermore, the mechanisms involved in these interaction processes should be studied.

To our knowledge, no previous data concerning E_2 effects on fish GST activity are available. Concerning synthetic steroids, 17a-ethynylestradiol induced no alterations on C. carpio GST activity (Solé et al., 2000). On the other hand, a mammalian study demonstrated that bisphenol A increased GST activity (Nieminen et al., 2002). The GST activity increase observed in the current study is suggestive of an increased hepatic steroid catabolism in fish. CYP induction and conjugation activities may occur simultaneously, depending on the enzyme involved and its regulation. Thus, the inhibition detected on CYP1A activity was not extended to other CYP families. Taking into account that steroid metabolism and elimination may be catalyzed by CYP3A and that previous investigations on synthetic steroids effects demonstrated a CYP1A down-regulation and a CYP3A up-regulation, further laboratory studies on E_2 biotransformation, namely CYP3A induction should be carried out (Lee et al., 1996a,b).

4.3. Hepatic condition indicators

The LSI increase observed in both treatments at 16 h exposure indicates metabolic alterations. The ALT kept constant, indicating that no cell damage was induced under the experimental conditions. Concerning LSI and ALT no differences were detected between E2 single exposure and the NP co-exposure.

4.4. Genotoxic response

Recent works on genotoxicity of aquatic environment contaminants have demonstrated the suitability of the ENA assay, based on micronuclei and other nuclear anomalies detection in mature erythrocytes. This test, successfully adopted in different fish species (Pacheco and Santos, 1996, 1998, 2001, 2002; Ayllón and Garcia-Vazquez, 2001; Gravato and Santos, 2002), is expanding in application due to its simplicity, rapidity, sensitivity, and low cost. However, its utilization may present some limitations in the presence of intense contamination since it can be masked by an increased splenic erythrocytic catabolism and/or erythropoiesis rate reduction (Pacheco and Santos, 2002).

In the present study, E_2 or E_2 + NP exposure did not induce any significant ENA increase. However, an ENA increasing trend was observed at 4 and 8 h that returned to control levels after 12 h exposure to E_2 or E_2 + NP. Comparing the ENA frequency and E_2 plasmatic levels the explanation previously presented for EROD and GST activities can be adopted, i.e., the ENA highest values were found concomitantly to the highest plasma E_2 concentrations. Thus, the occurrence of an ENA expression limitation in the presence of intense contamination, as previously suggested by Pacheco and Santos (2002), does not apply to this particular situation.

E_2 was classified as nonmutagenic and nongenotoxic, based on the failure to induce gene mutations in classical bacterial and mammalian mutation assays; however, this matter seems to be highly controversial (Roy and Liehr, 1999). In a recent review work on estrogens DNA damage and mutations Roy and Liehr (1999) stated that this classification was probably related to the lack of a sensitive method for detection of estrogen covalent binding to DNA bases, and because the classical mutation assays performed were designed to uncover mutations only at one specific locus and could not have detected other types of mutations or changes in other genes. According to

International Agency for Research on Cancer (IARC) (1999) E_2 genotoxicity was not demonstrated in vivo, neither in humans, nor in mice; nevertheless, E_2 was classified as a carcinogen, class I (IARC, 1999).

In the present study, the E_2 genotoxic potential was not demonstrated. However, the importance of further fish studies on estrogen genotoxicity is increased by the lack of data in nonmammal organisms, being recommended the adoption of longer exposures in realistic conditions.

4.5. Stress responses

The studied stress responses and the respective methodologies proved their suitability and reproducibility in previous studies with different fish species and different classes of contaminants (Teles et al., 2003a,b, 2004a,b), encouraging their utilization on toxicological endocrine research works.

The plasma cortisol decrease observed for both exposure conditions is an indication of endocrine impairment. Despite the lack of fish studies concerning cortisol alterations after E_2 exposure, plasma cortisol decrease was also found in various fish studies after short-term exposure to different contaminants (Pacheco and Santos, 2001; Teles et al., 2003a). The mechanism involved in this response is not completely clarified. Santos and Pacheco (1996) found that the interrenal cortisol release into the blood is prevented by the contaminant (i.e. endocrine disruptor) since an interrenal cortisol accumulation was observed in parallel with a reduced plasma cortisol concentration. The observed cortisol secretion dysfunction may reduce fish physiological competence, growth, and survivorship since this hormone is required for a wide range of important homeostatic mechanisms, including fuel reserves mobilization.

The E_2 potential to induce a plasma glucose increase was previously demonstrated in D. labrax (Teles et al., 2004a); however, this effect was not confirmed for the current S. aurata study.

The plasma lactate increase, observed for E_2 and E_2 + NP exposures can be regarded as an indication of stress. This response seems to be expectable since it was demonstrated for a wide range of xenobiotics (Santos and Pacheco, 1996; Pacheco and Santos, 2001; Teles et al., 2003a), despite the lack of any publications concerning specifically the current compounds.

Cortisol effect includes the gluconeogenic activation, mobilizing substrates such as lipids, amino acids and lactate as fuel energy, besides carbohydrates. Analyzing simultaneously the plasma cortisol and lactate responses it is evident an opposite trend of variation; thus, it can be suggested that the observed plasma lactate increase could have been potentiated by a reduction of the cortisol induced gluconeogenesis.

5. Conclusions

The current data concerning seabream exposure either to E_2 or to its mixture with NP revealed:

- a liver EROD activity depression after both treatments, while GST activity was elevated;
- an endocrine disruption, expressed as plasma cortisol decrease after exposure either to E_2 or E_2 + NP. Furthermore, a plasma lactate increase was measured for both conditions;
- that despite the higher E_2 levels measured in plasma of E_2 + NP comparing to E_2-treated fish, no significant differences were detected on the biological responses due to NP co-exposure.

It was also demonstrated a high E_2 spontaneous loss in the aquaria, and a rapid E_2 uptake by fish (in the first 4 h).

Acknowledgements

The authors express their appreciation for the financial support provided by the Aveiro University Research Institute (CESAM) and by the ‘‘Fundação Ciência e Tecnologia’’ (FCT-Grant no. SFRH/BD/6607/2001).

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