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Effects of 17β-estradiol and 4-nonylphenol on osmoregulation and hepatic enzymes in gilthead sea bream (*Sparus auratus*)

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Abstract

Sexually immature *Sparus auratus* were injected intraperitoneally with coconut oil either alone (control) or containing 17β-estradiol (E_2, 10 μg/g body mass) or 4-nonyphenol (4-NP, 100 and 200 μg/g body mass) and sampled 10 days later. Gill and kidney Na^+,K^+-ATPase activities, plasma levels of E_2 and cortisol, plasma osmolites (osmolality, sodium and chloride) and metabolites (glucose, lactate, proteins and triglycerides) were examined. Livers were used for measuring hepatosomatic index (HSI) and determinations of the activities of antioxidant defences catalase (CAT) and total glutatione peroxidase (t-GPX), the CYP1A-dependent, 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione *S*-transferase (GST). HSI and plasma levels of E_2 were significantly increased in E_2-treated fish. E_2 treatment enhanced plasma osmolality, glucose, triglycerides and proteins, but had no effect on plasma cortisol, and gill and kidney Na^+,K^+-ATPase activities. Hepatic activities of EROD, GST and CAT were significantly decreased after E_2 administration, whereas t-GPX remained unaffected. Treatment with 200 μg/g 4-NP caused a slight increase in plasma E_2 relative to the control group. Plasma glucose and protein levels were not affected by 4-NP, while triglycerides were increased. Fish treated with the higher dose of 4-NP displayed a clear reduction in kidney Na^+,K^+-ATPase activity, together with increases in plasma osmolality, relative to the control group. High 4-NP also caused a significant decrease in EROD and an increase in GST activity. Our results confirm the regulation of the natural estrogen E_2 and the weak xenoestrogen 4-NP on osmoregulation and biotransformation enzymes in a partially similar manner. The actions of xenoestrogens on critical physiological processes may have an ecological significance as it can reduce adaptability and capacity to metabolise xenobiotics under stressful conditions.

Keywords: 17β-Estradiol; 4-Nonylphenol; Gilthead sea bream; Osmoregulation; Metabolising enzymes

1. Introduction

Alkylphenol polyethoxylates (APEs), and one of their degradation products, 4-nonylphenol (4-NP), are compounds of significant environmental concern due to their estrogenic effects (Nimrod and Benson, 1996; Cravedi and Zalko, 2005). Many studies have provided evidence of the estrogenicity of 4-NP in fish, although to a lesser extent than that caused by the natural estradiol (E_2), either in vitro (White et al., 1994; Folmar et al., 2002) or in vivo (Jobling et al., 1996; Yadetie et al., 1999; Casini et al., 2002). This evidence is mainly based on the presence of vitellogenin in male or immature fish, a broadly accepted biomarker of estrogenicity (Sumpter and Joblin, 1995). Although many reproductive disorders associated with 4-NP exposure have been reported in fish (Jobling et al., 1996; Christensen et al., 1999; Ackermann et al., 2002), less attention has been paid to the actions of 4-NP on other crucial physiological processes such as osmoregulation (Madsen et al., 1997, 2004; McCormick et al., 2005), the xenobiotic metabolising system (Arukwe et al., 1997, 2001; Thibaut et al., 2002; Hughes and Gallagher, 2004; Thibaut and Porte, 2004) or both processes together (Teles et al., 2004, 2005).

Several studies have reported a negative effect of steroids (17α methyltestosterone and 17β-estradiol (E_2)) on the

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hypoosmoregulatory capacity of fish (see McCormick, 1995). E_2 treatment reduces gill chloride cell density and gill Na^+,K^+-ATPase activity in different salmonid species (Madsen and Korsgaard, 1989, 1991; Madsen et al., 1997, 2004). This fact could explain the negative effect of sexual maturity on seawater adaptability in salmonids. In non-salmonid species such as *Oreochromis mossambicus* and *Fundulus heteroclitus*, E_2 treatment reduced branchial Na^+,K^+-ATPase activity (Vijayan et al., 2001; Mancera et al., 2004). However, in the euryhaline teleost *Sparus auratus* maintained in seawater, E_2 treatment significantly increased gill Na^+,K^+-ATPase activity (Guzmán et al., 2004). The influence of 4-NP on osmoregulatory system has also been studied in salmonids (Madsen et al., 1997, 2004; McCormick et al., 2005) showing a negative effect on the smoltification process.

The cytochrome P450-dependent monooxygense system plays a key role in the oxidative metabolism of endogenous compounds (e.g. steroids), as well as a wide range of xenobiotics. In fish, this phase I system also responds to estrogenic compounds in a very selective manner (Arukwe et al., 1997). As a component of this system, the isozyme CYP1A or, in particular, its associated catalytic activity, 7-ethoxyresorufin *O*-deethylase (EROD) responds to estrogens and 4-NP (Arukwe et al., 1997; Solé et al., 2000; Teles et al., 2004, 2005). The actions of estrogen-like compounds, and 4-NP, over the reproductive system are well understood either by direct mimicking of natural E_2 (Yadetie et al., 1999; Arukwe et al., 2001) or acting over the E_2 feedback system of the pituitary (Jobling et al., 1996). However, the actions of 4-NP on the xenobiotic metabolising system are not so fully understood. Several hypotheses have been suggested, from cross-talk between estrogen receptors and aryl hydrocarbon receptors and actions over selective CYP isoforms (Navas and Segner, 2000; Thibaut et al., 2002), or over conjugating phase II enzymes (Arukwe et al., 2001; Thibaut and Porte, 2004). Both hypotheses bring in sound arguments on actions over the two enzymatic systems as both seem to be regulated by 4-NP.

As a consequence of endogenous/xenobiotic metabolism, reactive oxygen species (ROS) can be generated as byproducts of this transformation process (Livingstone, 2001). To face that, aerobic organisms, including fish, have evolved defense systems against oxidative damage such as antioxidant scavengers and specific antioxidant enzymes, namely catalase (EC 1.11.1.6) and glutathione peroxidase (GPX; EC 1.11.1.9).

The gilthead sea bream (*S. auratus*) is a proterandric, euryhaline teleost fish with high economic interest and a broad geographic distribution, capable of adapting to different environmental salinities. In this species, our group has studied several aspects of osmoregulatory and metabolic changes induced by E_2 (Guzmán et al., 2004; Sangiao-Alvarellos et al., 2004). Nevertheless, to our knowledge there is only one study that reports the effects of xenoestrogens, and in particular 4-NP, on this species (Teles et al., 2005). The reported study differs from ours in the route of exposure (waterborne versus injection) and the way of administrating the compounds (coexposure versus independent administration). Therefore, the aim of the present study was to observe the influence of the weak xenoestrogen 4-NP in respect to E_2 as a steroid model, on the osmoregulatory system of this species. Effects on isoenzymes of the cytochrome P450 system, phase II GST and selected, antioxidant enzymes were measured, as both are highly significant physiological processes in determining fish homeostasis. For this purpose, immature fish were selected, as sexual maturation itself has a great influence on the selected parameters. The information obtained will help to understand the modulation of E_2 and 4-NP over physiological processes, and their significance as factors that may compromise fish adaptation under stressful environmental situations either caused by salinity changes or chemical insults. This is highly significant in our area of study (Cadiz, SW Spain) where this species naturally breads in marsh areas subjected to strong salinity fluctuations and where high levels of 4-NP have been reported (Petrovic et al., 2002).

2. Materials and methods

2.1. Fish

Immature male gilthead sea bream (*S. auratus* L., 50–70 g body mass) were provided by Planta de Cultivos Marinos (C.A. S.E.M., University of Cádiz, Puerto Real, Cádiz, Spain) and transferred to the wet laboratories at Faculty of Marine Science (Puerto Real, Cádiz). Fish were acclimated to seawater (SW, 38 ppt salinity, 1000 mosm kg^{-1} H_2O) in 300 L aquaria for 2 weeks in an open system under natural photoperiod (July 2003), and constant temperature (18 °C). Fish were fed daily with 1% body mass commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). They were fasted for 24 h before injection and throughout the experiment.

2.2. Experimental protocol

Five different groups (10 individuals per group) of SW-acclimated fish were used. Fish were anaesthetized with 2-phenoxyethanol (Sigma) (0.5 mL/L water), weighed, injected intraperitoneally with 5 μL/g body mass of coconut oil (Sigma) alone (control) or containing 17β-estradiol (E_2, Sigma, 10 μg/g body mass) or 4-nonyphenol (4-NP, Aldrich 29085-8, 100 and 200 μg/g body mass), returned to SW and sampled after 10 days. One group, not injected served as control. No mortality was observed during the experiments. The dose of E_2 was based on observations from previous studies shown to have osmoregulatory and metabolic effects in *S. auratus* (Guzmán et al., 2004; Sangiao-Alvarellos et al., 2004). Doses of 4-NP were based on fish studies that reported disrupting effects after i.p. injection of this compound (Arukwe et al., 1997; Christensen et al., 1999; Yadetie et al., 1999; Casini et al., 2002).

2.3. Sampling

Fish were anaesthetized with 2-phenoxyethanol (1 mL/L water) and weighed. Mixed venous and arterial blood was sampled from the caudal peduncle using ammonia-heparinized

syringes. Plasma was separated from cells by centrifugation for 20 min at 900 g and stored at −80 °C. A biopsy of gill and kidney tissue was placed in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C. Livers were dissected, weighed and frozen at −80 °C. Hepatosomatic index (HSI) was calculated as [liver mass (g)/body mass (g)]×100.

2.4. Plasma measurements

Plasma levels of free E_2 were quantified by enzyme-linked immunosorbent assay (ELISA) following the method described by Rodríguez et al. (2002). Free steroids were extracted from 3.5–8 μL plasma in methanol (Panreac). E_2 standard was purchased from Sigma-Aldrich (Madrid, Spain). Mouse antirabbit IgG monoclonal antibody and specific antisteroid antibody (E_2-Ab) and enzymatic tracer (steroid acetylcholinesterase conjugate: E_2) were obtained from the Cayman Chemical Company (Michigan, USA). Microtiter plates (MaxiSorp™) were purchased from Nunc (Roskilde, Denmark). A standard curve from 6.1×10^{-4} to 2.5 ng mL^{-1} was established in all assays. Standards and extracted plasma samples were run in duplicate. The lower limits of detection (90% of binding, ED90) was 1.69 pg mL^{-1}. The inter-assay coefficients of variation at 50% of binding was 3.38%. The intra-assay coefficients of variation (calculated from the samples duplicates) was 4.65±0.75%. Details on cross-reactivity for antibody was given by the supplier.

Plasma cortisol levels were measured by indirect enzyme immunoassay (ELISA) validated for gilthead sea bream (Tintos et al., 2006). Briefly, Covalink microplates (Nunc) pre-treated with disuccinimidyl suberate were coated with a given amount of a conjugate of bovine serum albumin with the active ester of 3-carboxymethyl oxime prepared with cortisol. After incubation and blocking with BSA, competition was started by the addition of samples and anticortisol antibody raised in rabbit. Goat antirabbit IgG-conjugated peroxidase was added as second antibody and then incubated with OPD as substrate. Reaction was stopped with 0.1 M HCl and absorbance was read at 450 nm in an automatic plate reader. The standard curve was linear (logit/log) from the lower limit of sensitivity of the assay (0.3 ng mL^{-1}) to approximately 3000 ng mL^{-1}. Dose–response inhibition curves using serially diluted plasma samples consistently showed parallelism with the standard curve using cortisol. The ELISA satisfied the criteria of specificity (testing cross-reactivity with other steroids), reproducibility (inter-assay coefficient of variation <6%), precision (intra-assay coefficient of variation <4%), and accuracy (average recovery >98%).

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mosm kg^{-1}. Plasma Cl^- was measured using commercial microplate kits from Spinreact (Spain) and plasma Na^+ was measured using an atomic absorption spectrophotometer.

Plasma glucose and lactate were measured using commercial microplate kits from Spinreact (Spain) (Stein, 1963; Iwama et al., 1989). Plasma protein was measured using the bicinchoninic acid method (Smith et al., 1985) with the BCA protein kit (Pierce, Rockford, USA) for microplates using bovine serum albumin as standard. Plasma triglycerides were determined enzymatically with a commercial kit (Sigma #334-UV; Bucolo and David, 1973) in microplates. These assays were run on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics, Inc. NJ, USA).

2.5. Gill and kidney Na^+,K^+-ATPase activities

Na^+,K^+-ATPase activity was determined using the micro-assay method of McCormick (1993). Gill tissue was homogenized in 125 μL of sucrose–EDTA–imidazole (SEI) buffer with 0.1% deoxycholate, then centrifuged at 2000 g for 30 s. Duplicate 10 μL homogenate samples were added to 200 μL assay mixture with and without 0.5 mM ouabain in 96-well microplates at 25 °C and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as μmol ADP mg $protein^{-1}$ h^{-1}. Protein homogenate was determined using Pierce BCA Protein kit. Both assays were run on a microplate reader using DeltaSoft3 software for Macintosh.

2.6. Liver measurements

Cytosolic and microsomal fractions were prepared essentially as described in more detail in Solé et al. (2000). Briefly, livers were flushed with ice-cold 1.15% KCl and homogenized in 4 vol. of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM phenyl-methylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 g×10 min, the fat layer was removed and the resulting supernatant further centrifuged at 10,000 g×20 min; the obtained supernatant at 100,000 g for 60 min originated the cytosolic and microsomal fractions. Microsomal pellets were resuspended in a small volume of 100 mM Tris–HCl (pH 7.4) containing 0.15 M KCl, 20% w/v glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. Cytosolic and microsomal protein contents were measured by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Antioxidant enzymes and GST were measured in the cytosolic fraction as described in Solé et al. (2000). Catalase activity was measured by the decrease in absorbance at 240 nm (ext. coeff. 40 M^{-1} cm^{-1}) using 50 mM H_2O_2 as substrate. GPX activity was measured by the NADPH consumption at 340 nm (ext. coeff. 6.2 mM^{-1} cm^{-1}) during the formation of reduced glutathione by commercial glutathione reductase using 3 mM cumene hydroperoxide (sum of Se-dependent and Se-independent activities and referred as total-GPX) as substrate. Glutathione *S*-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the final reaction mixture containing 1 mM CDNB and 1 mM reduced glutathione.

In the microsomal fraction, 7-ethoxyresorufin *O*-deethylase activity (EROD) was determined at 30 °C as described in Burke and Mayer (1974); 10 μL of microsomes of about 10 mg mL^{-1}

Table 1
Effect of an implant of coconut oil alone (control), containing E_2 (10 μg/g body mass) or 4-NP (100 or 200 μg/g body mass) on hepatosomatic index (HSI) and plasma parameters of *S. auratus* after 10 days of treatment

	Non-injected	Control	E_2	4-NP (100)	4-NP (200)
HSI (%)	1.55±0.13[a]	1.62±0.31[a]	3.76±0.49[b]	1.72±0.18[a]	1.49±0.16[a]
E_2 (ng mL^{-1})	0.20±0.03[a]	0.22±0.03[a]	6.34±0.44[c]	0.58±0.10[a,b]	0.70±0.10[b]
Cortisol (ng mL^{-1})	6.62±0.98[a]	7.07±1.53[a]	7.84±1.10[a]	8.67±3.16[a]	10.71±1.38[a]
Osmolality (mosm kg^{-1})	379±4[a]	377±5[a]	409±18[b]	394±5[b]	404±11[b]
Na^+ (mM)	161±3[a]	164±4[a]	159 ±5[a]	163±4[a]	160±5[a]
Cl^- (mM)	180±7[a]	184±3[a]	176±6[a]	168±4[a]	167±4[a]
Glucose (mM)	2.6±0.2[a]	2.7±0.2[a]	6.2±0.3[b]	2.6±0.2[a]	3.4±0.2[c]
Lactate (mM)	1.8±0.1[a,b]	1.8±0.2[a,b]	3.1±0.3[c]	2.3±0.2[b,c]	2.9±0.1[c]
Protein (mg mL^{-1})	44.2±1.3[a]	43.4±1.6[a]	142.6±11.5[b]	39.5±0.9[a]	44.2±1.7[a]
Triglycerides (mM)	1.5±0.1[a]	1.6±0.1[a]	2.4±0.2[c]	2.0±0.1[a,b]	2.2±0.1[b,c]

Values are the mean±SEM (n=9–10). Same letters indicate no differences among groups (P<0.05, one-way ANOVA, SNK multiple comparison test).

protein content were incubated for 10 min in a final volume of 1 mL containing 90 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.22 mM NADPH and 3.70 μM 7-ethoxyresorufin. The reaction was stopped by adding 2.0 mL of ice-cold acetone, samples were centrifuged at low speed, and 7-hydroxyresorufin fluorescence determined in a Perkin-Elmer LS-5 spectrofluorimeter at 537/583 nm excitation/emission wavelengths.

2.7. Statistics

A unique replica of each control and experimental group (10 fish per group) was used in the experiment. Significant differences among groups were tested by one-way ANOVA. Logarithmic transformations of the data were made when necessary to fulfil the conditions of the analysis of variance but the data are shown in their decimal values for clarity. Post-hoc comparisons were made using a Student–Newman–Keuls multiple comparison test (SNK), with the differences considered to be statistically significant at P<0.05.

3. Results

3.1. Biological parameters

No mortality occurred in any animal groups during the treatment period. Body mass remained similar to initial experimental values (50–70 g body mass) with no differences among groups (data not shown). Liver weight and HSI were significantly enhanced only in the E_2-treated group (Table 1).

3.2. Plasmatic and osmoregulatory parameters

E_2 treatment significantly increased plasma levels of E_2. In addition, treatment with 4-NP enhanced plasma levels of E_2 with respect to non-injected fish and the control group only at the highest dose (200 μg/g body mass) (Table 1). Plasma levels of cortisol were neither affected by E_2 nor 4-NP.

Treatment with E_2 enhanced plasma osmolality and other metabolic parameters (glucose, lactate, triglycerides and proteins), but did not affect plasma Na^+ or Cl^- levels. In contrast, increases of plasma glucose, lactate and triglycerides levels were observed only in fish treated with the highest dose of 4-NP. However, plasma Na^+, Cl^- and protein levels were not affected by treatment with any doses of 4-NP.

Gill Na^+,K^+-ATPase activity was not affected by E_2 or 4-NP treatment (Fig. 1) while fish treated with the highest dose of 4-NP displayed a clear reduction in kidney Na^+,K^+-ATPase activity (Fig. 1).

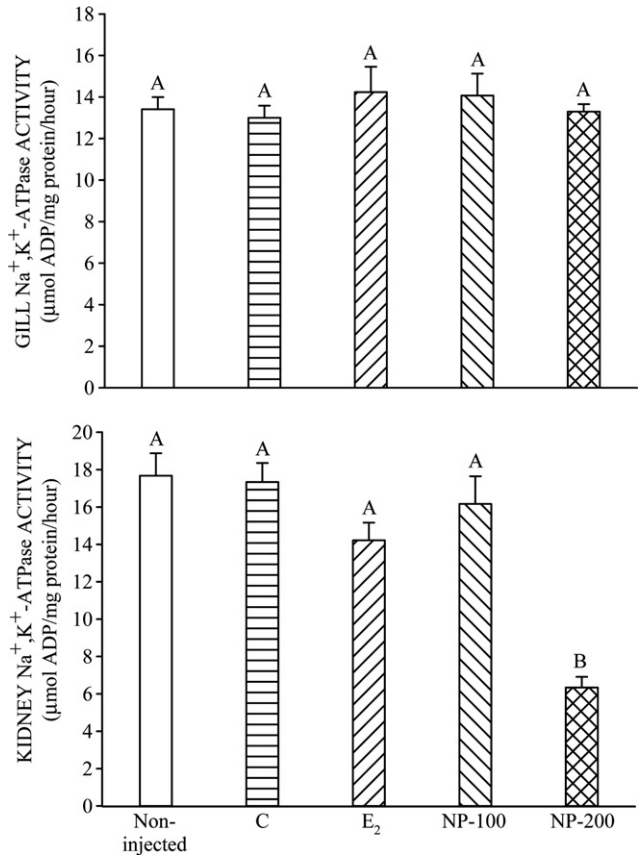

Fig. 1. Effect of an implant of coconut oil alone (control) or containing E_2 (10 μg/g body mass) or 4-NP (100 or 200 μg/g body mass) on gill (top) and kidney (bottom) Na^+,K^+-ATPase activities of *S. auratus* after 10 days of treatment. Values are the mean±SEM (n=7–8). Different letters indicate significant differences (P<0.05, one-way ANOVA, SNK multiple comparison test) among groups.

Table 2
Effect of an implant of coconut oil alone (control), containing E_2 (10 μg/g body mass) or 4-NP (100 or 200 μg/g body mass) on several hepatic enzymes of *S. auratus* after 10 days of treatment

	Non-injected	Control	E_2	4-NP (100)	4-NP (200)
Catalase (μmol min^{-1} mg $prot^{-1}$)	320±50[a]	407±53[a]	92±8.5[b]	420±76[a]	512±89[a]
t-GPX (nmol min^{-1} mg $prot^{-1}$)	21.0±3.0[a]	25.1±2.9[a]	28.2±5.8[a]	43.1±9.4[a]	48.3±9.7[a]
GST (nmol min^{-1} mg $prot^{-1}$)	383±81[a]	536±88[a]	185±21[b]	448±93[a]	973±191[c]
EROD (pmol min^{-1} mg $prot^{-1}$)	308±67[a]	207±90[a,b]	4.2±2.9[c]	116±37[a,b]	68±28[b]

Values are the mean±SEM ($n=6$). Same letters indicate no differences among groups ($P<0.05$, one-way ANOVA, SNK multiple comparison test).

3.3. Hepatic biomarkers

CAT activity was lower in E_2-treated fish, while t-GPX showed no changes due to either E_2 or 4-NP (Table 2). GST activity was clearly inhibited after E_2 treatment, while it was enhanced in 4-NP-treated fish at the highest dose (200 μg/g body mass). EROD activity decreased in E_2-treated fish, to the extent that, in some individuals, it was unmeasurable. Treatment with 4-NP also caused an inhibitory effect of 78% at the high dose (200 μg/g body mass) (Table 2).

4. Discussion

The dose and way of administering E_2 have been shown effective in increasing the levels of plasma E_2 in *S. auratus* in several studies, including ours (Mosconi et al., 1998; Guerreiro et al., 2002; Cavaco et al., 2003). In addition, hepatosomatic index and plasma protein levels were significantly higher in E_2-treated fish compared with controls, which is a fair indicator of the effect of the E_2 treatment.

It has been demonstrated that the modulation of the estrogenic response caused by E_2 and other estrogen-like compounds, such as 4-NP is mediated through their binding to the estrogen receptor or to an E_2 feedback at the pituitary level (White et al., 1994; Jobling et al., 1996; Tollefsen et al., 2002). In addition, the differential effects of 4-NP and E_2 treatment on the expression of estrogen receptor α gene in smolting sockeye salmon could explain the different physiological effects observed between these treatments (Luo et al., 2005).

It is interesting to remark that 4-NP treatment induced a small increase in E_2 plasma values at the highest dose (200 μg/g body mass). In juvenile *S. auratus*, coexposure of waterborne E_2 and E_2 plus 4-NP resulted in a two-fold increase of plasma E_2 under coexposure (Teles et al., 2005). Similarly, a study conducted with fathead minnows (*Pimephales promelas*) linked 4-NP exposure to plasma E_2 elevation (Giesy et al., 2000). Conversely, in *Salmo salar*, Arukwe et al. (1997) did not show an elevation in plasma E_2 after injection of a 4-NP dose of 125 μg/g (intermediate to ours) but a decrease in this hormone was shown at lower 4-NP doses (1 and 5 μg/g). Further studies are needed to demonstrate the mechanisms by which 4-NP stimulates release of E_2. Factors such as species, dose, time and method of administration are factors to be considered.

The osmoregulatory system of teleosts is affected by E_2. Treatment with this hormone reduces gill Na^+,K^+-ATPase activity in salmonids (Madsen and Korsgaard, 1989, 1991; Madsen et al., 1997) as well as in non-salmonid species (*O. mossambicus*: Vijayan et al., 2001; *F. heteroclitus*: Mancera et al., 2004). In a previous experiment, our group showed that *S. auratus* treated with similar doses of E_2 for 5 days clearly increased gill Na^+,K^+-ATPase activity, but showed a decrease in this activity after 9 days post-implant (Guzmán et al., 2004). In the present study we sampled fish after 10 days of E_2 treatment and gill Na^+,K^+-ATPase activity did not increase. Similar to Guzmán et al. (2004), we propose either a loss in sensitivity of gill chloride cells to exogenous E_2 or an exhaustion of gill chloride cells as a consequence of high E_2 levels as the possible cause for the lack of effect on gill Na^+,K^+-ATPase activity. However, recently Luo et al. (2005) have demonstrated that gill E_2 receptors are up-regulated by E_2 treatment in gills of sockeye salmon and these data did not support the previous hypothesis of loss in sensitivity of gill chloride cells with a decrease in affinity or number of E_2 by excess of exogenous E_2. Further studies are necessary to test the possibility of exhaustion of gill chloride cells.

In *S. salar*, treatment with 4-NP for 30 days impaired smolting with a reduction in gill Na^+,K^+-ATPase activity, relative α-subunit Na^+,K^+-ATPase mRNA expression and gill chloride cells density as well as salinity tolerance (Madsen et al., 1997; 2004). In addition, treatment with 4-NP for 14 days (0.5–150 μg/g body mass) induced several endocrine disfunctions of parr–smolt transformation and decreased salinity tolerance but not gill Na^+,K^+-ATPase activity in the same species (McCormick et al., 2005). Treatment with 4-NP at the two doses assessed in our study (100 and 200 μg/g body mass) did not influence gill Na^+,K^+-ATPase activity in *S. aurata*. These negative results could be due to a lower sensitivity of *S. auratus* to this compound or to the necessity of longer exposures to 4-NP in this species to achieve any changes in gill Na^+,K^+-ATPase activity (see Madsen et al., 2004).

In contrast, kidney Na^+,K^+-ATPase activity showed a clear reduction in fish treated with the high dose of 4-NP (200 μg/g body mass), while no change was observed after E_2 treatment. To our knowledge, this is the first report of an inhibitory action of this compound on kidney Na^+,K^+-ATPase activity in fish. The kidney plays an important role in marine fish and Na^+,K^+-ATPase activity drives the extrusion of excess of divalent ions (Beyenbach, 1995). In *S. auratus*, a linear relationship between kidney Na^+,K^+-ATPase activity and environmental salinity has been demonstrated, with higher values in marine fish compared to brackish water adapted fish (Sangiao-Alvarellos et al., 2003). In this way, a reduction in kidney Na^+,K^+-ATPase activity by treatment with 4-NP could indicate its negative effect on the capacity of *S. auratus* in adapting to hyperosmotic environments. However, plasma Na^+ and Cl^- levels did not change

after 4-NP treatment. Clearly, further research is necessary to clarify the suggested negative influence of 4-NP on osmoregulatory capacity of *S. auratus*.

E_2 treatment increased plasma glucose, lactate and triglycerides. These results agree with those previously reported in *S. auratus* under the same hormonal treatment (Sangiao-Alvarellos et al., 2004; Guzmán et al., 2004). These parameters were also increased by 4-NP treatment, suggesting the existence of cross-reactivity between E_2 receptors and 4-NP as has been suggested previously (White et al., 1994; Jobling et al., 1996; Tollefsen et al., 2002).

In *S. auratus* treatment with E_2 and 4-NP had no effect on plasma cortisol levels (present results), in agreement with that previously reported for *O. mossambicus* (Vijayan et al., 2001) and *S. salar* (McCormick et al., 2005). However, Teles et al. (2005) showed a reduction in plasma cortisol levels in *S. auratus* using a short-term exposure to either E_2 or E_2+4-NP. Our differing results using the same species could be ascribed to the different route or time of exposure used. It has been previously demonstrated that cortisol administration enhances glucose, lactate and triglyceride levels in plasma of *S. auratus* (Laiz-Carrión et al., 2003). Our experimental fish also increased plasma glucose and lactate, but only with the higher doses of 4-NP, suggesting the existence of a stress situation, without significant increases of plasma cortisol levels (see Wendelaar Bonga, 1997).

With respect to hepatic metabolising enzymes, several studies have shown a significant reduction in EROD activity and CYP1A protein levels in different fish species in relation to E_2 (Arukwe et al., 1997), ethynylestradiol (EE_2) (Solé et al., 2000), E_2 and 4-NP alone (Arukwe et al., 2001; Thibaut et al., 2002; Vaccaro et al., 2005) or E_2 and 4-NP coexposures including *S. aurata* (Teles et al., 2004, 2005). Our data agree with previous studies, as a strong depletion in EROD activity was observed in E_2-treated animals. The mechanism responsible for CYP1A inhibition by estrogens or estrogen-like compounds is yet undefined. Of all the mechanisms proposed (see Introduction) a selective action over the CYP isoforms (CYP1A or EROD down-regulation) has been commonly observed for E_2 and 4-NP in our study and others (Thibaut et al., 2002). In any case, EROD inhibition was not so pronounced in 4-NP treatment as it was in E_2 injected fish and the response to 4-NP was dose-dependent, as also shown in juvenile salmon (Arukwe et al., 1997) or *D. labrax* (Vaccaro et al., 2005).

Modulation of estrogenic compounds over phase II enzymes has also been suggested (Arukwe et al., 1997) and demonstrated (Hughes and Gallagher, 2004; Thibaut and Porte, 2004; Teles et al., 2004; Vaccaro et al., 2005). In our study, GST activity was strongly inhibited in E_2-treated fish, but was enhanced in fish treated with the high 4-NP dose. An increase in GST was seen in *O. mykiss* after waterborne exposure to 4-NP for 1 week (Uguz et al., 2003) or in *S. aurata* exposed to E_2 or E_2 plus 4-NP (Teles et al., 2005) but not in *D. labrax* (Teles et al., 2004). In the same species, *D. labrax*, GST was inhibited in a time and dose-dependent manner by injected E_2 but not 4-NP (Vaccaro et al., 2005). This coincides with our finding with E_2 injected *S. auratus*. As for 4-NP, the lack of effect over GST in *D. labrax* can be due to the doses administered (5 and 50 μg/g), which are lower than ours (200 μg/g).The different behaviour of phase II GST after either E_2 or 4-NP administration can be partially explained by alternative ways of metabolising steroids or xenobiotics. The doses and exposure route (i.p. injection and waterborne exposure) are also factors to consider as modulators of response even in the same species. In *S. salar*, Arukwe et al. (1997) reported a certain decrease, although not significant, in another phase II enzyme (UDPGT) after administration of E_2 or increasing doses of 4-NP (1–125 μg/g body mass). Similarly, UDPGT was reduced while GST was unaffected in *C. carpio* after EE_2 injection (Solé et al., 2000).

Response of the antioxidant enzymes seems to parallel actions on the redox cycling processes. Strong EROD inhibition caused by an estrogen-mimicking compound, could reduce the amount of oxyradical species originated as byproducts of this process and therefore reduce the response in the antioxidant defences. In this sense, CAT was lowered in E_2-treated fish. The particular response on antioxidant defences after E_2 and 4-NP administration supports the previous responses in GST. These observations reinforce the feasibility of, not only quantitatively but also qualitatively, different routes of metabolism for either a natural steroid or a xenobiotic with estrogenic capacity. Other antioxidant enzymes, such as selenium-dependent GPX, catalase and superoxide dismutase (SOD) slightly decreased in EE_2 injected carp (Solé et al., 2000), however no effect was seen on DT-diaphorase activity after E_2 or 4-NP injection in *D. labrax* (Vaccaro et al., 2005).

In conclusion, osmoregulatory results obtained after E_2 treatment agreed with those previously described for this species under similar hormonal treatment (Guzmán et al., 2004), while results with 4-NP indicate a reduction in kidney Na^+,K^+-ATPase activity which suggested a negative effect of this compound on the hypoosmoregulatory capacity of *S. auratus*. In addition, concomitant depletion of EROD, GST and CAT activities after E_2 administration indicates a decrease in the xenobiotic transformation metabolism in *S. auratus*. In contrast, 4-NP displayed some estrogenic capacity, measured as plasma E_2, and caused EROD depletion, especially at the higher dose. Nevertheless, it also showed particular routes of biotransformation, more in line with those observed for organic xenobiotics (increased GST and t-GPX). A consistent indication of CYP1A down-regulation was seen after E_2 and 4-NP administration.

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