Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 92 (2004) 485–494
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish

Rémi Thibaut*, Cinta Porte

Environmental Chemistry Department IIQAB-CSIC. C/Jordi Girona, 18, 08034 Barcelona, Spain

Received 19 March 2004; accepted 15 October 2004

Abstract

The interactions of estrogenic (nonylphenol, dicofol, atrazine), androgenic (organotins, phthalates, fenarimol) and anti-androgenic compounds (vinclozolin, diuron, *p,p′*-DDE) with key enzymatic activities involved in both synthesis and metabolism of sex hormones was investigated. Carp testicular microsomes incubated in the presence of androstenedione and different xenobiotics evidenced higher sensitivity of 5α-reductase activity than 17β-hydroxysteroid dehydrogenase activity towards those chemicals. Dicofol, organotins and phthalates were among the most effective inhibitors. In contrast, ovarian synthesis of maturation-inducing hormones (20α- and 20β-hydroxysteroid dehydrogenase activities) were enhanced by nonylphenol, dicofol, fenarimol and *p,p′*-DDE. Metabolic clearance pathways of hormones were also affected. Fenarimol, nonylphenol and triphenyltin inhibited the glucuronidation of testosterone and estradiol at concentrations as low as 10, 50 and 100 μM, respectively. Triphenyltin, tributyltin and nonylphenol were also inhibitors of estradiol sulfation with IC_{50} values of 17, 18 and 41 μM. Overall, the data indicates the interaction of selected chemicals with key enzymatic pathways involved in both synthesis and metabolism of sex hormones. This interference might be one of the underlying mechanisms for the reported hormonal disrupting properties of the tested compounds, and might finally affect physiological processes such as gamete growth and maturation.

Keywords: Endocrine disrupters; 17β-HSD; 5α-Reductase; 20α/β-HSD; UGT; Estradiol-SULT; Fish

1. Introduction

There is now clear evidence that numerous xenobiotic compounds act as endocrine disrupters (EDs) in wildlife affecting reproductive functions. The effects of EDs have been extensively reported in fish; they include inhibition of gonad growth, reduction in the number and quality of germ cells, feminisation and masculinisation [1]. However, the mechanisms of action of those compounds are not well understood.

Sex steroids have two classical functions in fish development: they act as morphogenic factors during sex differentiation, and as activational factors during sexual maturation. Sex differentiation and gamete growth are regulated by androgens and estrogens [2], while maturation of both oocyte and spermatozoa depends on maturation-inducing hormones (MIH) [3,4]. Both, sex steroids and MIH, act through specific nuclear or membrane receptors in target cells, and many studies have reported the interaction of EDs with those receptors [5–8]. The interference of xenobiotics with the synthesis and clearance of key sex hormones may also alter bioavailable amounts of active hormones within the organism, and be a potential mechanism of endocrine disruption viz. polycyclic aromatic hydrocarbons (PAHs) had an inhibitory effect on CYP17, 17β-hydroxysteroid dehydrogenase (17β-HSD), and P450 aromatase in vitellogenic ovarian tissue of the flounder (*Platichthys flesus* L.) [9]. Other studies have shown inhibition of P450 aromatase activity by fungicides [10,11]. Since a number of cytochrome P450 subfamilies (CYP2K and CYP3A) participate in both steroid and xenobiotic metabolism in fish [12,13], interactions between foreign chemicals (e.g. alkylphenols, prochloraz, several imidazole compounds) and different P450 isoforms involved in steroid metabolism have often been reported [14–16].

* Corresponding author. Tel.: +34 93 400 61 00; fax: +34 93 204 59 04.
E-mail address: cpvqam@cid.csic.es (R. Thibaut).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.10.008

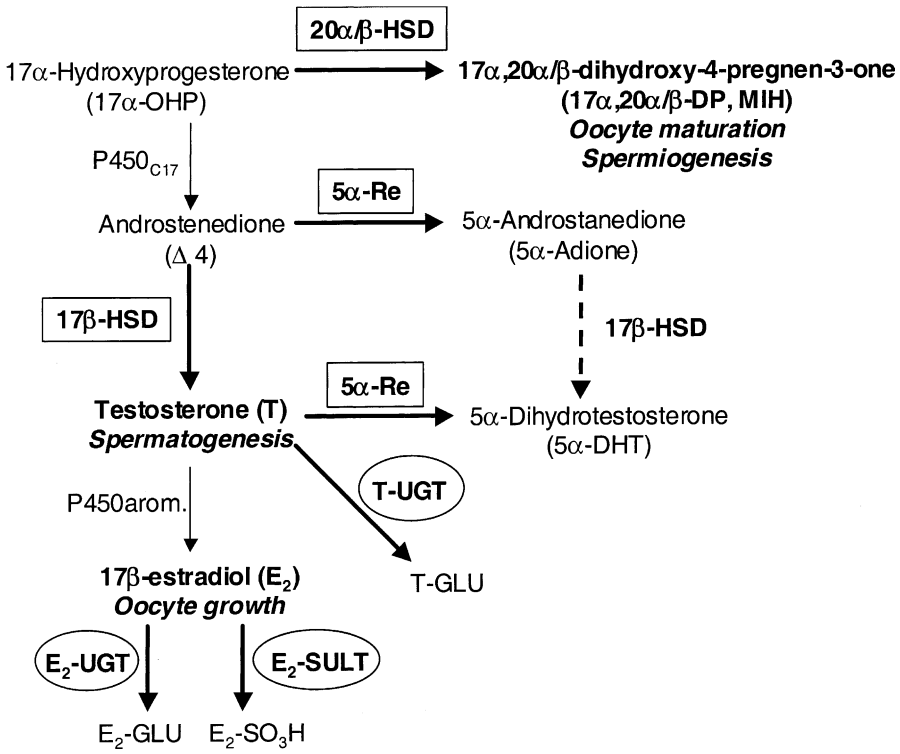

Fig. 1. Schematic representation of the steroid synthesis and metabolism pathways studied in carp. HSD, hydroxysteroid dehydrogenase; 5α-Re, 5α-reductase; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; -GLU, glucuronide; -SO_3H, sulphate; MIH, maturation-inducing hormone.

However, less attention has been paid to the interaction of xenobiotics with steroid phase II metabolism. Glucuronidation and sulfation are important routes of clearance of active hormones, generating a pool of hormone-conjugates that may be excreted or re-converted to the active form in peripheral tissues. Thus, alteration of those metabolic pathways may greatly affect levels of active hormones. Moreover, alkylphenols have been shown to reduce *p*-nitrophenol and testosterone glucuronidation in salmonoids [15,17] whereas polychlorinated biphenyls (PCBs) increased estradiol glucuronidation [18,19]. Estrogen-sulfotransferase activities have been recently described in fish [20], and the interference of straight chain 4-*n*-alkylphenols with estrone sulfation reported [21].

Thus, this study was designed to further assess the in vitro interactions of EDs with key enzymatic activities involved in both synthesis and metabolism of sex hormones in fish by focussing on those pathways where few or no data is available, namely: (a) testicular metabolism of androstenedione, the classical androgen precursor; (b) ovarian synthesis of MIH; and (c) sulfation and glucuronidation of testosterone and estradiol. Any interference of xenobiotics with those enzymatic pathways would affect key physiological processes, such as gamete growth and maturation (Fig. 1). To this end, widely used xenobiotic compounds referenced in the literature as estrogenic (4-nonylphenol, dicofol, atrazine), androgenic (triphenyltin —TPT-, tributyltin —TBT-, dibutyltin —DBT-, di-*n*-butyl phthalate —DBP-, di-(2-ethylhexyl) phthalate —DEHP-, fenarimol), and anti-androgenic compounds (vinclozolin, diuron, *p,p′*-DDE) were selected and incubated in vitro with subcellular fractions isolated from carps (*Cyprinus carpio*).

2. Materials and methods

2.1. Chemicals

[1β-^{3}H(N)]-androstenedione was purchased from PerkinElmer Life Sciences (Boston, MA), [6,7-^{3}H]-estradiol and [4-^{14}C]-testosterone were from Amersham (Buckinghamshire, England). The radiochemical purity of labelled androstenedione, estradiol and testosterone was analysed by Radio-HPLC and found to be >97%. Testosterone (T), estradiol (E_2), androstenedione (Δ4), 17α-hydroxyprogesterone (17α-OHP), 5α-dihydrotestosterone (5α-DHT), UDPGA and NADPH were obtained from Sigma (Steinheim, Germany) and PAPs (adenosine 3′-phosphate 5′-phosphosulfate, tetralithium salt) were from Calbiochem (Darmstadt, Germany). 5α-Androstene-3,17-dione (5α-Adione), 17α,20α-dihydroxy-4-pregnen-3-one (17α,20α-DP) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) were purchased from Steraloids (Wilton, NH). Acetonitrile used for HPLC was of analytical grade (Merck, Darmstadt, Germany).

2.2. Microsomes and cytosol preparation

Male and female carps (*C. carpio*) were collected by electrofishing from the Ebro River (Spain). Fish were sacrificed and the liver and gonads immediately dissected, frozen in liquid nitrogen, and stored at −80 °C until preparation of subcellular fractions. Gonad and livers were flushed with ice cold 1.15% KCl, and homogenised in 1:5 (w/v) of cold 100 mM potassium-phosphate buffer pH 7.4, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenantroline and 0.1 mg/mL trypsine inhibitor. Homogenates were centrifuged at 1500 × *g* for 15 min, the fatty layer removed and the obtained supernatant centrifuged at 12,000 × *g* for 20 min. The 12,000 *g* supernatant was further centrifuged at 100,000 × *g* for 60 min to obtain the cytosolic and microsomal fractions. Gonad microsomal pellets were washed in homogenised buffer and centrifuged again at 100,000 × *g* for 30 min. Microsomes were resuspended in a small volume of 100 mM potassium-phosphate buffer pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenantroline, 0.1 mg/mL trypsine inhibitor and 20% (w/v) glycerol. Proteins were measured according to Lowry et al. [22], using bovine serum albumin as standard.

2.3. Androstenedione testicular metabolism

According to histology of gonads determined in a previous study [23], male carps showing a pronounced spermatogenic activity were used to investigate the effects of chemicals on androgen synthesis. Assays were carried out by preincubating 0.1 mg of testicular microsomal protein in Tris–HCl buffer (50 mM), $MgCl_2$ (10 mM), pH 7.4 with 100 μM of xenobiotic for 10 min at 25 °C. Assays were started with NADPH (300 μM) after addition of ^{3}H-androstenedione (0.1 μM as final concentration) and additional incubation for 15 min in a shaking water bath maintained at 25 °C (final volume 250 μL). Incubations were stopped by adding 250 μL of acetonitrile and after centrifugation (1500 × *g*, 10 min), 200 μL of supernatant were injected onto the RP-HPLC column.

2.4. Ovarian synthesis of MIH

According to histology of gonads [23], female carps being in a maturing stage, with oocytes near maximum size and containing numerous densely-packed vitelline granules, were used to investigate the effects of chemicals on MIH synthesis. Assays were carried out by preincubating 1 mg of ovarian microsomal protein in Tris–HCl buffer (50 mM), $MgCl_2$ (10 mM), pH 7.4 with 1 mM of xenobiotics for 10 min at 25 °C. Assays were started with NADPH (2 mM) after addition of 17α-hydroxyprogesterone (200 μM as final concentration) and additional incubation for 2 hours in a shaking water bath maintained at 25 °C (final volume 0.5 mL). Incubations were stopped by adding 1 mL of ethyl acetate, and extraction of metabolites and unchanged 17α-hydroxyprogesterone (17α-OHP), was completed by 2 × 1 mL ethyl acetate. The ethyl acetate fraction was evaporated to dryness under nitrogen stream, redissolved in acetonitrile-water (1:1, v/v), and injected onto the reverse phase HPLC column.

2.5. Testosterone and estradiol UDP-glucuronosyltransferase activities

Testosterone and estradiol UDP-glucuronosyltransferase (T-UGT and E_2-UGT) activities were assayed by preincubating 0.4 mg of liver microsomal protein in Tris–HCl buffer (50 mM), $MgCl_2$ (10 mM), pH 7.4 with xenobiotic for 20 min at 25 °C. Assays were started with UDPGA (3 mM) after addition of ^{14}C-testosterone or ^{3}H-estradiol (100 μM as final concentration and final volume 0.25 mL). Then, an additional incubation of 30 min was carried out in a shaking water bath maintained at 25 °C. The reaction was stopped by adding 2 mL of ethyl acetate, and the extraction of unmetabolized steroids was completed by 2 mL × 2 mL of ethyl acetate. An aliquot (50 μL) of the remaining aqueous phase containing glucuronides was quantified by liquid scintillation counting. Effects of chemicals on T- and E_2-UGT activities were firstly investigated at 1 mM, and when a striking effect was detected, assays were repeated at 500, 100, 50, 10, 1, 0.1 and 0.01 μM in order to calculate concentrations resulting in 50% inhibition (IC_{50}).

2.6. Estradiol-sulfotransferase activity

The first step of the study consisted on the determination of the kinetic parameters (K_m, V_{max}) of estradiol-sulfotransferase (E_2-SULT) activity and the protein concentration range in which the activity was linear, in order to characterise this enzymatic activity and to find the best incubation conditions to investigate xenobiotics effects. E_2-SULT kinetic parameters (K_m and V_{max}) were determined using ^{3}H-E_2 concentrations ranging from 0.001 to 10 μM. Assays were carried out by incubating 0.025–1 mg of liver cytosolic protein with the substrate and 10 μM PAPS in Tris–HCl buffer (50 mM), $MgCl_2$ (10 mM), pH 7.4 and Na_2SO_3 (5 mM) as sulfatase inhibitor (final volume 170 μL). Samples were incubated for 30 min in a shaking water bath maintained at 25 °C. The reaction was stopped with 3 mL of dichloromethane after addition of 200 μL of Tris–HCl buffer (50 mM) pH 8.2 in order to avoid sulphate hydrolysis. The extraction of unmetabolized E_2 was completed by 3 mL of dichloromethane and an aliquot (200 μL) of the remaining aqueous phase containing E_2-sulfate was quantified by liquid scintillation counting. Effects of chemicals on E_2-SULT activity were assessed by preincubating 25 μg of liver cytosolic protein with xenobiotics in Tris–HCl buffer (50 mM), $MgCl_2$ (10 mM), pH 7.4 and Na_2SO_3 (5 mM) (final volume 170 μL). The reaction was started with PAPS (10 μM) after addition of ^{3}H-estradiol (0.01 μM as final concentration). Samples were incubated for 30 min in a shaking water bath maintained at 25 °C. Reaction

was stopped and the samples were treated as described above. Effects of chemicals on E_2-SULT were firstly investigated at 100 μM and when a striking effect was detected, assays were repeated at 50, 25, 10, 5, 1, 0.1, 0.01 and 0.001 μM in order to calculate concentrations resulting in 50% inhibition (IC_{50}).

2.7. Analysis of metabolites

HPLC separations were performed on a Perkin–Elmer Binary LC pump 250 system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 μm) reversed-phase column (Merck, Darmstadt Germany) protected by a guard column LiChrospher 100 RP-18 (5 μm). Separation of ^{3}H-androstenedione metabolites was performed at 1 mL/min with a mobile phase composed of: (A) 75% water and 25% acetonitrile; and (B) 25% water and 75% acetonitrile. The run was set as follows: 0–30 min, linear gradient from 100% A to 100% B, 30–35 min 100% B. For separation of 17α-OHP metabolites, the HPLC gradient system elution was pumped at 1 mL/min with a mobile phase composed of: (A) 71% water and 29% acetonitrile; and (B) 100% acetonitrile. The run consisted on a 30 min linear gradient from 100% A to 60% B, 30–35 min linear gradient from 60% B to 100% B, and 34–40 min 100% B. Radioactive metabolite peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks.

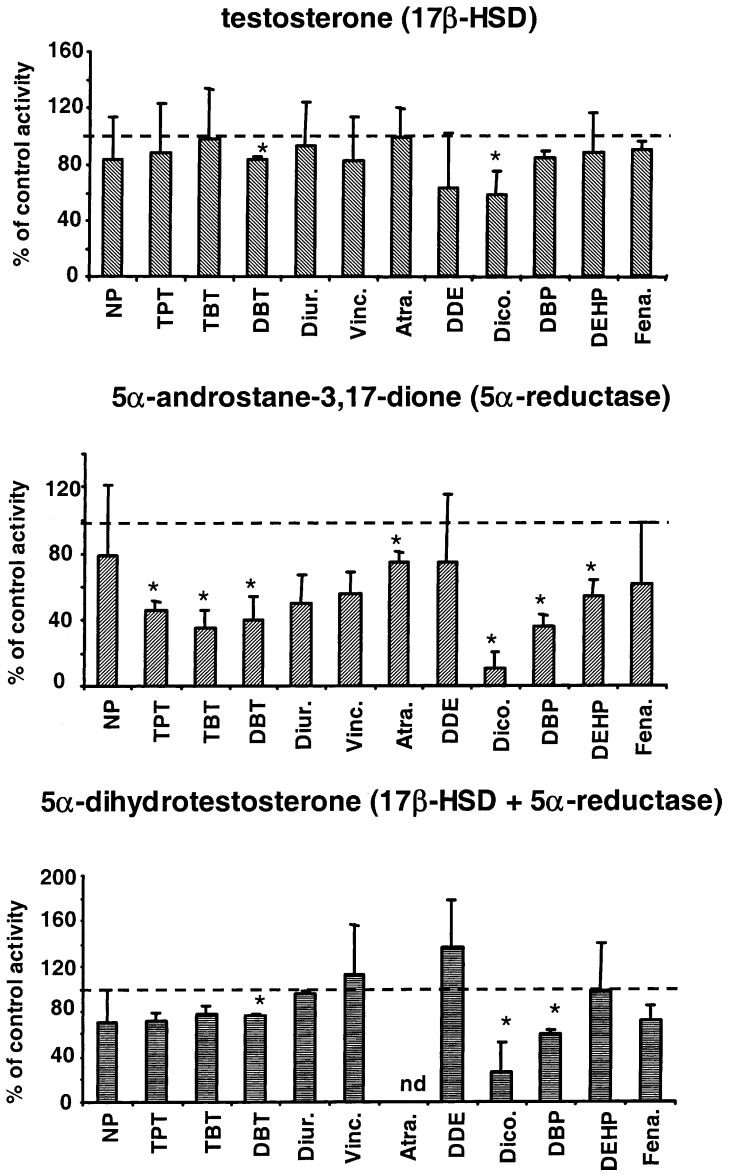

Fig. 2. Effect of chemicals on Δ4 metabolism by carp testicular microsomes. Assays containing 0.1 μM Δ4 and 100 μM xenobiotic were carried out in triplicate. Data presented as mean ± S.D. *Significantly different from control. nd = not determined.

The elution of non-radioactive metabolites was monitored at 254 nm using an UV-detector (Knauer LC-photometer). Metabolites were quantified by integrating the area under the peaks and using the calibration curve of authentic standards.

The metabolites were analysed by GC–MS (EI+) as trimethylsilyl (TMS) derivatives and the chemical structures were identified by comparison of the retention times and the mass spectra with authentic standards. Silylation was achieved using 100 μL of BMSTFA and allowed to stand for 1 h at 70 °C. After the reaction, the samples were evaporated to dryness under a nitrogen stream, and redissolved in iso-octane before injection. Mass spectra were obtained on a Fisons GC 8000 Series chromatograph coupled to a Fisons MD800 mass spectrometer fitted with a HP-5MS (30 mm × 0.25 mm i.d., crosslinked 5% PH ME siloxane) column (Hewlett-Packard). The carrier gas was helium at 1 mL/min. The oven temperature was programmed as follows: 90 to 130 at 12 °C/min and from 130 to 320 at 6 °C/min. The injector temperature was 250 °C and the ion source and the analyser were maintained at 200 °C and 250 °C, respectively.

2.8. Data analyses

The Michaelis–Menten parameters (K_m and V_{max}) were estimated by analysing Lineweaver–Burk plots and using Eadie–Hofstee transformation. Statistical analyses were performed using Student's *t*-test with the level of significance set at $p \leq 0.05$ unless otherwise indicated.

3. Results

3.1. Effect of chemicals on androstenedione testicular metabolism

In testicular microsomes, the radio-HPLC profile of ^{3}H-androstenedione (R_T = 22 min) exhibited three metabolites; testosterone (T) at 20 min, 5α-dihydrotestosterone (5α-DHT) at 24 min, and 5α-androstenedione (5α-Adione) at 26 min. Dicofol and dibutyltin at a concentration of 100 μM significantly inhibited the synthesis of T (42% and 16%, respectively) (Fig. 2). An inhibitory effect was observed for *p,p′*-DDE but due to high variability, the effect was not significant. In contrast, seven out of the twelve chemicals tested led to a significant inhibition of 5α-Adione production (Fig. 2). The strongest inhibitor was dicofol, at a concentration of 100 μM led to 90% inhibition. Organotin compounds (TPT, TBT, and DBT) and phthalates (DBP, DEHP) inhibited the formation of 5α-Adione in the range of 45–65%. A weak inhibitory effect (25%) was observed for atrazine. Concerning the synthesis of 5α-DHT, dicofol was the strongest inhibitor (74%), followed by DBP (41%) and DBT (24%) (Fig. 2). The effect of atrazine on 5α-DHT synthesis is not presented because of the great discrepancy observed between samples.

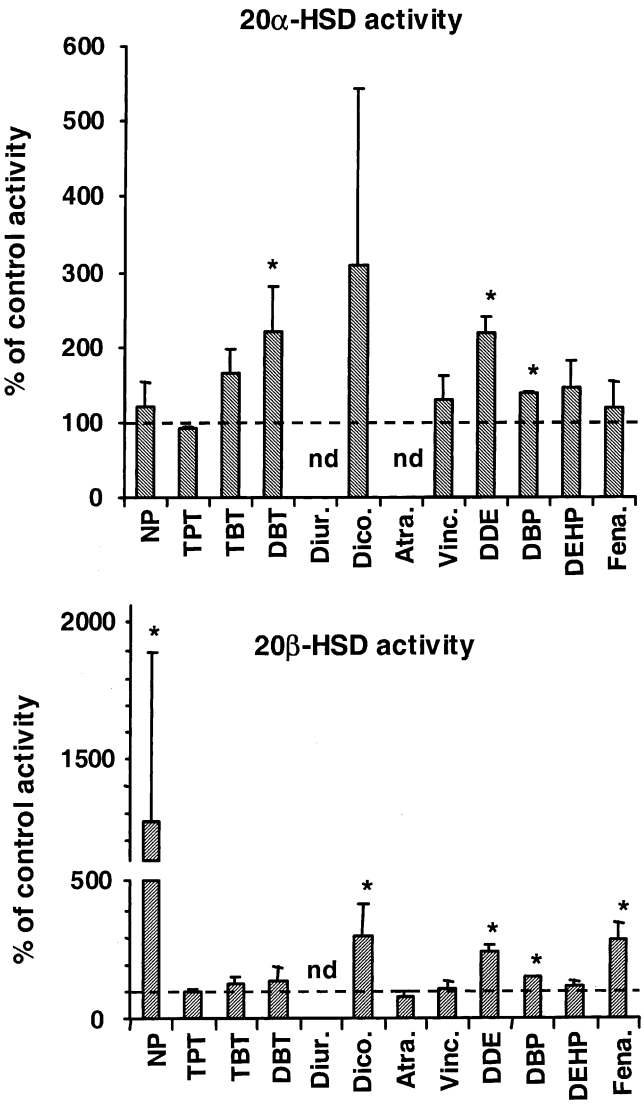

Fig. 3. Effect of chemicals on MIH synthesis by carp ovarian microsomes. Assays containing 200 μM 17α-OHP and 1 mM xenobiotic were carried out in triplicate. Data presented as mean ± S.D. *Significantly different from control. nd = not determined.

3.2. Effect of chemicals on ovarian MIH synthesis

The HPLC profile of 17α-OHP (R_T = 21.1 min) incubated with ovarian microsomes exhibited two metabolites; 17α,20α-dihydroxy-4-pregnen-3-one (17α,20α-DP) at 16.8 min, and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) at 18.4 min. Control microsomes formed 326 ± 104 pmoles/mg/h of 17α,20β-DP, and 23 ± 6 pmoles/mg/h of 17α,20α-DP. Interestingly, DBT, *p,p′*-DDE and DBP at a concentration of 1 mM increased the synthesis of 17α,20α-DP in the range of 138 to 220% (Fig. 3). Dicofol increased the synthesis of 17α,20α-DP to 310%, but due to high variability between samples, this increase was not statistically significant. The synthesis of 17α,20β-DP was strongly enhanced by dicofol (307%), but also by fenarimol (284%), *p,p′*-DDE (241%), and DBP

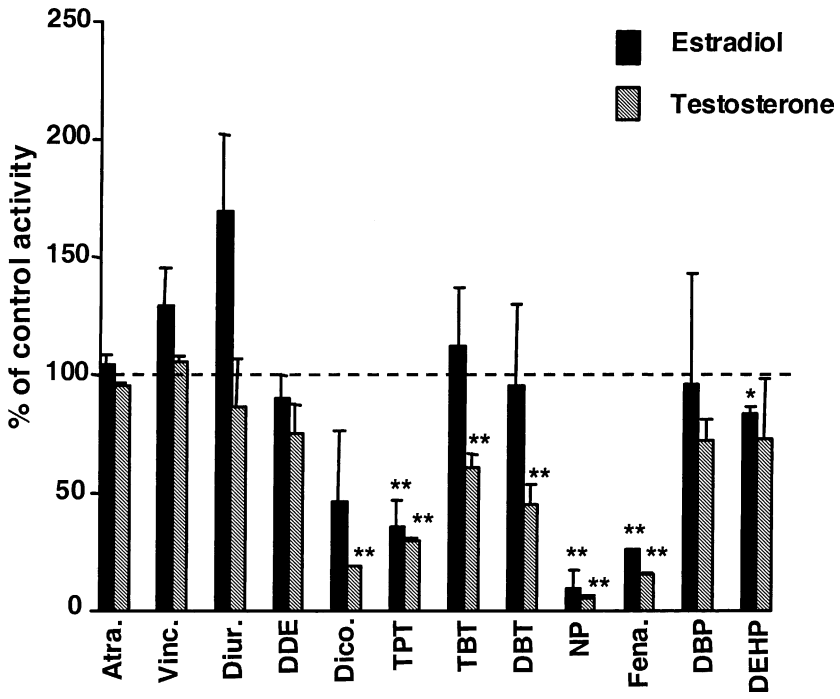

Fig. 4. Effect of chemicals on T- and E_2-UGT activities in carp liver microsomes. Assays containing 100 μM T or E_2 and 1 mM xenobiotic were carried out in triplicate. Data presented as mean ± S.D. Significantly different from control $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

(156%). The most striking effect was detected for NP, which enhanced 8 – 17-fold the synthesis of this metabolite (Fig. 3). The effect of atrazine and diuron was not assessed because of co-elution with metabolites in the HPLC system.

3.3. Effect of chemicals on T-UGT and E_2-UGT activities

Fig. 4 shows the effect of chemicals on T- and E_2-UGT activities when tested at a concentration of 1 mM. Kinetics of inhibition for NP, dicofol, TPT and fenarimol are presented in Fig. 5. E_2-UGT activity was strongly reduced by TPT, fenarimol and NP, the percentage of inhibition being 64%, 74%, and 90%, respectively. Inhibitions were dose-dependent, and IC_{50} values were estimated at 692 ± 134, 92 ± 63, and 224 ± 25 μM, respectively. TPT, fenarimol and NP were even stronger inhibitors of T glucuronidation. When tested at a concentration of 1 mM, 70%, 85%, and 95% inhibition was measured. Inhibition was dose-dependent, and IC_{50} values were of 623 ± 192, 139 ± 8 and 71 ± 20 μM, respectively. Interestingly, while TPT, fenarimol and NP strongly affected both UGT activities, dicofol significantly inhibited T glucuronidation (81% inhibition; IC_{50} 293 ± 11 μM) with no significant effect on E_2-UGT. Similarly, TBT and DBT were only inhibitors of T glucuronidation.

3.4. Effect of chemicals on E_2-SULT activity

E_2-SULT activity did not follow a strict Michaelis–Menten kinetic. A break in the Eadie-Hofstee transformation of the kinetic data suggested the presence in the liver cytosol of two SULT activities able to conjugate E_2 (data not shown). The activity with the greatest affinity for E_2 showed a K_m of ≈ 17 nM while the other activity exhibited a K_m of ≈ 3.2 μM. Inhibition experiments were conducted using 0.01 μM E_2, and thus with the sulfotransferase activity showing the greatest affinity for E_2. Only three compounds, NP, TPT and TBT were found to inhibit significantly E_2-SULT activity. Inhibitions were dose-dependent and NP, TPT and TBT showed IC_{50} values of 41 ± 6, 17 ± 3 and 18 ± 13 μM, respectively (Fig. 6).

4. Discussion

In carp gonad microsomes, the metabolism of Δ4 led to the production of T, 5α-Adione and 5α-DHT. T is synthesised by the reduction of Δ4, a pathway involving the reductive 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, whereas 5α-Adione and 5α-DHT come from 5α-reduction of Δ4 and T, respectively (Fig. 1). Among tested compounds, dicofol and DBT were the only inhibitors of 17β-HSD, and the effect of DBT was rather weak (Fig. 2). Thus, the inefficiency of most of the tested compounds to inhibit T synthesis may indicate that 17β-HSD are not likely targets for EDs. In fact, several vertebrate studies support this hypothesis, viz. Mc Vey and Cooke [24] examined the effects in rat testis of a range of organotin compounds (mono-, di-, tri-butyltin, mono-, di-, tri-octyltin) on the activities 3β-HSD and 17β-HSD, and reported that 17β-HSD was

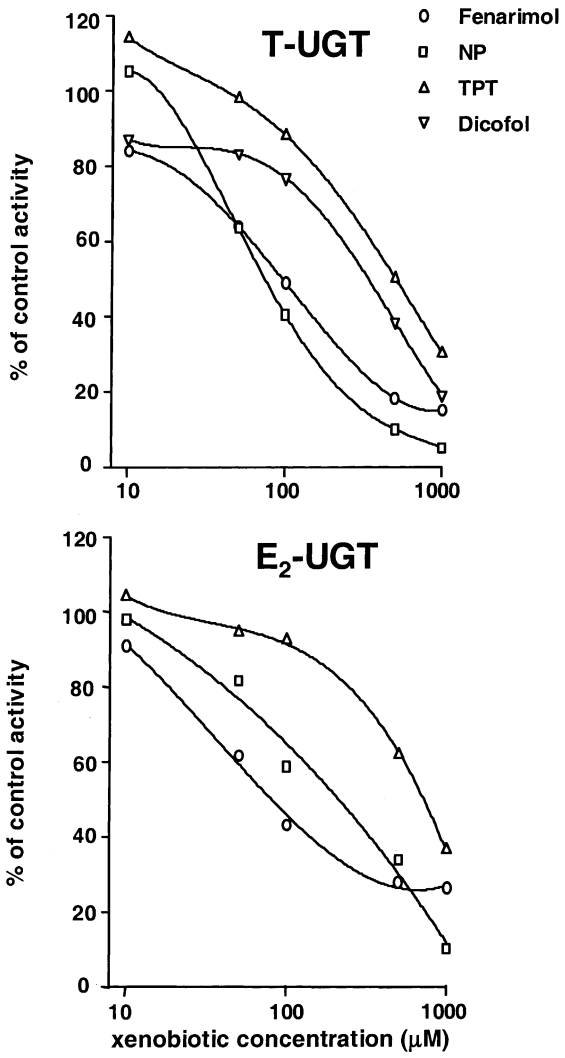

Fig. 5. Dose-dependent effects of EDs on T- and E_2-UGT activities in carp liver microsomes. Assays were carried out in triplicate, and presented as mean values.

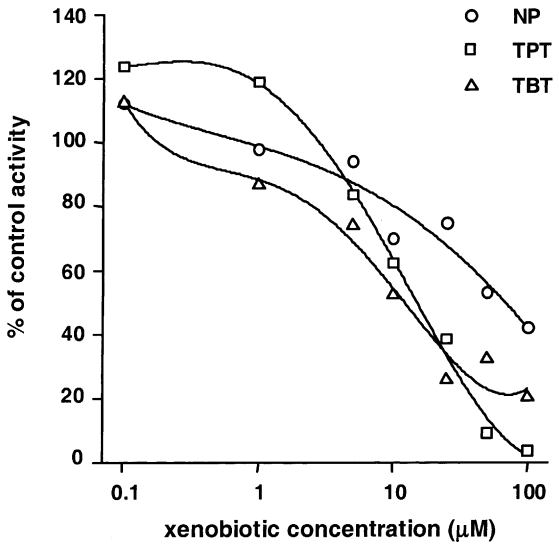

Fig. 6. Dose-dependent effects of EDs on E_2-SULT activity in carp liver cytosol. Assays were carried out in triplicate, and presented as mean values.

unaffected while 3β-HSD was only inhibited by mono-octyltin and TBT. Similarly, Doering et al. [25] assessed the effects of butyltins on human androgen metabolism, and reported no effect on brain 17β-HSD activity working with 0.1 μM Δ4 as substrate and concentrations up to 200 μM of butyltins. Also, octylphenol has been shown not to affect 17β-HSD activity in cultured rat Leydig cells [26]. The only positive result described so far is the inhibition by TPT of human 17β-HSD cytosolic (type 1) and microsomal (type 3) activities [27]. IC_{50} values were estimated at 10.5 μM for 17β-HSD 1 and 4.2 μM for 17β-HSD 3.

Δ4 and T are both substrates of 5α-reductase and they are reduced to 5α-Adione and 5α-DHT, respectively (Fig. 1). Whether the same or distinct 5α-reductase isoforms are involved in the reduction of Δ4 and T remains to be determined, but the effect of xenobiotics on 5α-reductase activity were more marked when assessed by quantification of 5α-Adione synthesis than 5α-DHT production (Fig. 3). The formation of 5α-Adione was inhibited by organotin compounds (TPT, TBT and DBT), phthalates (DBP, DEHP), and dicofol; while the formation of DHT was only affected by dicofol, DBP and DBT. Few investigations have been conducted on the effects of environmental contaminants on 5α-reductases, but in agreement with our data, TPT and TBT have been shown to inhibit human brain 5α-reductase (type 1) and prostate 5α-reductase (type 2) activities [25,27]; and DBT is reported to inhibit human brain 5α-reductase [25]. In contrast, vinclozolin did not affect the conversion of T to 5α-DHT in rats [28], nor in carps (this study).

Incubation of ovarian microsomes with 17α-OHP lead to the formation of 17α,20α-DP and 17α,20β-DP, the 20β-epimer being the major metabolite. In salmonoid fish, 17α,20β-DP is known to play a major role in the final gamete maturation in both male and females [29,3]. Additionally, free and sulfated forms of 17α,20β-DP were among the most important pheromonal steroids released by female goldfish, indicative of spawning readiness [30]. To our knowledge, this is the first report on the effects of chemicals on 20α- and 20β-hydroxysteroid dehydrogenase activities (20α-HSD and 20β-HSD), the key enzymes involved in the formation of 17α,20α-DP and 17α,20β-DP, respectively. The obtained data shows a significant effect of NP that increases the synthesis of the 20β-epimer, but also dicofol, *p,p′*-DDE, DBP and fenarimol; *p,p′*-DDE, DBP, and DBT increased the synthesis of the 20α-epimer. This effect was observed at concentrations of xenobiotic five-fold higher than substrate. Nonetheless, the mechanism behind the activation remains unclear. It has been reported that 20α- and 20β-HSD are members of aldo-keto reductase superfamily [31]. These enzymes can metabolise xenobiotics, e.g. 20α-HSD oxidised trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (B[a]P trans-dihydrodiol) to benzo[a]pyrene-7,8-dione (B[a]P o-quinone) [32]. Moreover, 20β-HSD was shown to exhibit a carbonyl reductase-like

structure and activity in rainbow trout ovary, catalysing the NADPH-dependent reduction of a variety of endogenous and foreign carbonyl compounds (4-nitrobenzaldehyde and menadione) to the corresponding alcohols [33]. The simultaneous binding of two substrate molecules, 17α-OHP and a xenobiotic in the active site of the enzyme, is likely to occur and could be one of the mechanisms accounting for the observed 20α-HSD and 20β-HSD activations as previously indicated for some P450 isoenzymes [34]. Among the tested compounds, DBP contains two carbonyl groups and it is, therefore, a potential substrate for carbonyl reductases. NP, dicofol and fenarimol possessing an alcohol function are also likely to be substrates of aldo-keto reductases and thus possible ones for 20α/β-HSD. Alternatively, a specific inhibition of those compounds on 17α-hydroxylase/C17,20 lyase (P450c17), that converts 17α-OHP to Δ4 (Fig. 1), would lead to an increased amount of substrate available for 20α/β-HSD in our in vitro system, and consequently increased formation of 17α,20α/β-DP. In fact, the ability of NP to directly inhibit P450c17 activity in vitro has been demonstrated in rat testis [40]. In this case, the effect of NP and other active xenobiotics would be comparable to the shift from Δ4 to 17α,20α/β-DP production observed when the transition from gamete growth to gamete maturation occurs.

Finally, xenobiotic disruption of metabolic clearance pathways of hormones was investigated by measuring effects on glucuronidation and sulfation. The in vitro data evidenced that fenarimol, NP and TPT inhibited the glucuronidation of estradiol at a concentration as low as 20, 50 and 100 μM, respectively. The glucuronidation of testosterone was also significantly inhibited at 10 (fenarimol), 50 (NP and dicofol), and 100 μM (TPT). Other compounds, such as tributyltin, have been shown to selectively inhibit the glucuronidation of testosterone at a dose as low as 5 μM, but not that of estradiol [35]. Sturm et al. [16] also indicated that 15 μM Igepal (a mixture of nonylphenol mono- and di-ethoxylates) significantly inhibited testosterone glucuronidation in trout hepatocytes. Overall, the data available supports the hypothesis that some compounds (pesticides, alkylphenols) may alter the glucuronidation of testosterone and estradiol, and this interference may account for significant in vivo alterations. In vivo studies are rather limited, but NP was shown to decrease testosterone UDP-glucuronosyltransferase activities in salmon and trout [15,17].

Among the 12 tested xenobiotics, NP, TPT and TBT were the only three compounds inhibiting E_2 sulfation. Octyl- and nonylphenol were potent inhibitors of the sulfation of E_2 in human platelets [36], and straight chain 4-*n*-alkylphenols inhibit the sulfation of estrone in chub liver, being longer chain compounds the most potent inhibitors [21]. The authors estimated an IC_{50} value for octylphenol and 4-n-nonylphenol below 1 μM. In this study, the IC_{50} for NP is 40 μM, but we were using commercial NP that is a mixture of isomeric compounds differing in the structure of the alkyl chain. TPT and TBT were even stronger inhibitors of E_2 sulfation with IC_{50} values at 17 μM. Lineweaver-Burk plots analyses of inhibition kinetics showed competitive and non-competitive inhibition types for NP and TPT, respectively (data not shown).

Table 1 summarises the effects of selected EDs on the enzymatic activities. Among the biosynthesis pathways, 5α-reductases were more sensitive to xenobiotics modulation than 17β-HSD. Hence, dicofol, atrazine, organotins and phthalates had an inhibitory effect on 5α-reductase. This led to a decrease in the synthesis of 5α-Adione and 5α-DHT. In mammals, 5α-DHT is a potent androgen that binds to the androgen receptor but neither 5α-DHT nor 5α-Adione functions have been elucidated in fish. However, a novel fish androgen receptor was shown to have stronger affinity for DHT than T or 11-ketotestosterone (the classical active androgens in fish) suggesting that DHT may also have a physiological function in fish [37,38]. As shown in Fig. 1, 5α-Adione is, like T, a potential precursor of 5α-DHT. Moreover, 5α-reductase activi-

Table 1
Summary of the in vitro effects of selected EDs on Δ4 testicular metabolism, MIH ovarian synthesis, T and E_2 hepatic glucuronidation and sulfation

Endocrine disrupters	17β-HSD	5α-Re	20α-HSD	20β-HSD	T-UGT	E_2-UGT	E_2-SULT
Estrogenic							
NP	—	—	—	↑	↓	↓	↓
Dicofol	↓	↓	—	↑	↓	—	—
Atrazine	—	↓	nd	—	—	—	—
Androgenic							
TPT	—	↓	—	—	↓	↓	↓
TBT	—	↓	—	—	↓	—	↓
DBT	↓	↓	↑	—	↓	—	—
DBP	—	↓	↑	↑	—	—	—
DEHP	—	↓	—	—	—	↓	—
Fenarimol	—	—	—	↑	↓	↓	—
Anti-androgenic							
Vinclozolin	—	—	—	—	—	—	—
Diuron	—	—	nd	nd	—	—	—
pp′-DDE	—	—	↑	↑	—	—	—

HSD, hydroxysteroid dehydrogenase; 5α-Re, 5α-reductase; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase. ↓ = Inhibition, ↑ = activation, — = no significant effect, nd = not determined.

ties are involved in hepatic androgen metabolism [39]. Consequently, the interaction of EDs with 5α-reductases may alter both androgen synthesis and metabolism and male sexual maturation and development. A number of compounds also interfere with 20α/β-HSD activities, enhancing the formation of 17α,20α-DP and 17α,20β-DP metabolites that are known to act as maturation-inducing hormones [3] and pheromones [30]. Thus, activation of those pathways at an improper period of the reproductive cycle may alter gamete quality and quantity and disrupt the synchronization of spawning and mating behaviour. Among the compounds that exert an effect on 20α/β-HSD it is interesting to mention *p,p′*-DDE, a metabolite of *p,p′*-DDT, still very abundant in some areas [23], that did not affect any of the other pathways tested. About phase II activities, UGTs were more sensitive to xenobiotic disruption than SULT, and NP and organotin compounds had an inhibitory effect on both pathways. Overall, estrogenic (NP and dicofol) and androgenic (organotins and fenarimol) compounds were the most active chemicals towards the metabolic pathways investigated, while the antiandrogenic chemicals tested were not so efficient. All these compounds may exert their effects in vivo, by virtue of their ability to bioaccumulate in fish, and particularly in those organs with a high percentage of lipids (gonads and liver) where target enzymes are located. Additionally, the high affinity of these compounds for steroid receptors, and the ability to induce the subsequent biological responses should be considered together with these findings.

Acknowledgements

This study was supported by the Spanish National Plan for Research under Project Ref. REN2002-01709/HID. Dr. Rémi Thibaut acknowledges a postdoctoral fellowship from the Spanish government. We acknowledge Ramón Lavado for his valuable help and comments on parts of this work.

References

[1] A. Arukwe, Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction, Mar. Pollut. Bull. 42 (8) (2001) 643–655.
[2] F. Piferrer, Endocrine sex control strategies for the feminization of teleost fish, Aquaculture 197 (2001) 229–281.
[3] Y. Nagahama, 17α,20β-Dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action, Steroids 62 (1997) 190–196.
[4] R.H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, Aquaculture 208 (2002) 191–364.
[5] A.K. Loomis, P. Thomas, Binding characteristics of estrogen receptor ER in Atlantic croaker *Micropogonias undulatus* testis: different affinity for estrogens and xenobiotics from that of hepatic ER, Biol. Reprod. 61 (1999) 51–60.
[6] A.K. Loomis, P. Thomas, Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testis in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor, Biol. Reprod. 62 (2000) 995–1004.
[7] W.R. Kelce, L.E. Gray, E.M. Wilson, Antiandrogens as environmental endocrine disruptors, Reprod. Fertil. Dev. 10 (1998) 105–111.
[8] S. Das, P. Thomas, Pesticides interfere with the nongenomic action of progestogen on meiotic maturation by binding to its plasma membrane receptor on fish oocytes, Endocrinology 140 (4) (1999) 1953–1956.
[9] P.R. Rocha Monteiro, M.A. Reis-Henriques, J. Coimbra, Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.), Aquat. Toxicol. 48 (2000) 549–559.
[10] G. Monod, A. De Mones, A. Fostier, Inhibition of ovarian microsomal aromatase and follicular estradiol secretion by imidazole fungicides in rainbow trout, Mar. Environ Res. 35 (1993) 153–157.
[11] E. Noaksson, M. Linderoth, A.T.C. Bosveld, L. Balk, Altered steroid metabolism in several teleost species exposed to endocrine disrupting substances in refuse dump leachate, Gen. Comp. Endocrinol. 134 (2003) 273–284.
[12] D.R. Buhler, J.-L. Wang-Buhler, Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring, Comp. Biochem. Physiol. 121 (C) (1998) 107–137.
[13] R. Thibaut, L. Debrauwer, E. Perdu, A. Goksøyr, J.-P. Cravedi, A. Arukwe, Regio-specific hydroxylation and the involvement of CYP2K- and CYP2M-like iso-enzymes in Atlantic salmon (*Salmo salar*), Aquat. Toxicol. 56 (2002) 177–190.
[14] C.L. Miranda, M.C. Henderson, D.R. Buhler, Evaluation of chemicals as inhibitors of trout cytochrome P450s, Toxicol. Appl. Pharmacol. 148 (1998) 237–244.
[15] A. Arukwe, L. Förlin, A. Goksøyr, Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound: 4-nonylphenol, Environ. Toxicol. Chem. 16 (1997) 2576–2583.
[16] A. Sturm, J.-P. Cravedi, E. Perdu, M. Baradat, H. Segner, Effects of Prochloraz and nonylphenol diethoxylate on hepatic biotransformation enzymes in trout: a comparative in vitro/in vivo-assessment using cultured hepatocytes, Aquat. Toxicol. 53 (2001) 229–245.
[17] R. Thibaut, E. Perdu, J.-P. Cravedi, Testosterone metabolism as a possible biomarker of exposure of fish to nonylphenol, Rev. Méd. Vét. 153 (8–9) (2001) 589–590.
[18] T. Andersson, M. Pesonen, C. Johansson, Differential induction of cytochrome P450–dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyltransferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A50, Biochem. Pharmacol. 34 (1985) 3309–3314.
[19] L. Förlin, C. Haux, Increased excretion in the bile of 17β-[^{3}H]-estradiol derived radioactivity in the rainbow trout treated with β-naphthoflavone, Aquat. Toxicol. 6 (1985) 197–208.
[20] Z. Tong, M.O. James, Purification and characterization of hepatic and intestinal phenol sulfotransferase with high affinity for benzo[a]pyrene phenols from channel catfish, *Ictalurus punctatus*, Arch. Biochem. Biophys. 376 (2) (2000) 409–419.
[21] C.J. Kirk, L. Bottomley, N. Minican, H. Carpenter, S. Shaw, N. Kohli, M. Winter, E.W. Taylor, R.H. Waring, F. Michelangeli, R.M. Harris, Environmental endocrine disrupters dysregulate estrogen metabolism and Ca^{2+} homeostasis in fish and mammals via receptor-independent mechanisms, Comp. Biochem. Physiol. 135 (A) (2003) 1–8.
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[23] R. Lavado, R. Thibaut, D. Raldúa, R. Martín, C. Porte, First evidence of endocrine disruption in feral carp from the Ebro River, Toxicol. Appl. Pharmacol. (2004).
[24] M.J. McVey, G.M. Cooke, Inhibition of rat testis microsomal 3β-hydroxysteroid dehydrogenase activity by tributyltin, J. Steroid Biochem. Mol. Biol. 86 (2003) 99–105.

[25] D.D. Doering, S. Steckelbroeck, T. Doering, D. Klingmüller, Effects of butyltins on human 5α-reductase type 1 and type 2 activity, Steroids 67 (2002) 859–867.
[26] E.P. Murono, R.C. Derk, J.H. de León, Differential effects of octylphenol, 17β-estradiol, endosulfan, or bisphenol A on the steroidogenic competence of cutured adult rat Leydig cells, Reprod. Toxicol. 15 (2001) 551–560.
[27] S. Lo, A. Alléra, P. Albers, J. Hembrecht, E. Jantzen, D. Klingmüller, S. Steckelbroeck, Dithioerythritol (DTE) prevents inhibitory effects of triphenyltin (TPT) on the key enzymes of the human sex steroid hormone metabolism, J. Steroid Biochem. Mol. Biol. 84 (2003) 569–576.
[28] W.R. Kelce, E. Monosson, M.P. Gamcsik, S.C. Law, L.E. Gray, Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites, Toxicol Appl. Pharmacol. 126 (1994) 276–285.
[29] D.E. Kime, Classical" and "non-classical" reproductive steroids in fish, Rev. Fish. Biol. Fisheries 3 (1993) 160–180.
[30] A.P. Scott, P.W. Sorensen, Time course of release of pheromonally active gonadal steroids and their conjugates by ovulatory goldfish, Gen. Comp. Endocrinol. 96 (1994) 309–323.
[31] E. Maser, Xenobiotic carbonyl reduction and physiological steroid oxidoreduction, Biochem. Pharmacol. 49 (1995) 421–440.
[32] M.E. Burczynski, R.G. Harvey, T.M. Penning, Expression and characterization of four recombinant human dihydrodiol dehydrogenase isoforms: oxidation of trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to the activated o-quinone metabolite benzo[a]pyrene-7,8-dione, Biochemistry 37 (1998) 6781–6790.
[33] G. Guan, M. Tanaka, T. Todo, G. Young, M. Yoshikuni, Y. Nagahama, Cloning and expression of two carbonyl reductase-like 20β-hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*), Biochem. Biophys. Res. Commun. 255 (1999) 123–128.
[34] K.R. Korzekwa, N. Krishnamachary, M. Shou, A. Ogai, R.A. Parise, A.E. Rettie, F.J. Gonzalez, T.S. Tracy, Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrate can simultaneously bind to cytochrome P450 active site, Biochemistry 37 (1998) 4137–4147.
[35] Y. Morcillo, G. Janer, S.C.M. O'Hara, D.R. Livingstone, C. Porte, Interaction of tributyltin with hepatic cytochrome P450 and uridine diphosphate-glucuronosyl transferase systems of fish: in vitro studies, Environ, Toxicol Chem. 23 (4) (2004) 164–170.
[36] R.M. Harris, R.H. Waring, C.J. Kirk, P.J. Hughes, Sulfation of "estrogenic" alkylphenols and 17β-estradiol by human platelet phenol sulfotranferases, J. Biol. Chem. 275 (1) (2000) 159–166.
[37] T.S. Sperry, P. Thomas, Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities, Endocrinology 140 (4) (1999) 1602–1611.
[38] T.S. Sperry, P. Thomas, Identification of two nuclear androgen receptors in Kelp bass (*Paralabrax clathratus*) and their binding affinities for xenobiotics: comparison with Atlantic croaker (*Micropogonias undulatus*) androgen receptors, Biol. Reprod. 61 (1999) 1152–1161.
[39] B. Truscott, Steroid metabolism in fish: II. Testosterone metabolites in the bile of the marine winter flounder *Pseudopleuronectes americanus* and the freshwater Atlantic salmon *Salmo salar*, Gen. Comp. Endocrinol. 51 (1983) 460–470.
[40] E.M. Laurenzana, G. Balasubramanian, C. Weis, B. Blaydes, R.R. Newbold, K.B. Delclos, Effect of nonylphenol on serum testosterone levels and testicular steroidogenic enzyme activity in neonatal, pubertad, and adult rats, Chem Biol. Interact. 139 (2002) 23–41.