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Drospirenone intake alters plasmatic steroid levels and *cyp17a1* expression in gonads of juvenile sea bass☆

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ABSTRACT

Drospirenone (DRO) is one of the most widely used progestins in contraceptive treatments and hormone replacement therapies. The pharmacokinetics and potential toxicological effects of DRO were investigated in juvenile sea bass (*Dicentrarchus labrax*) exposed through the diet (0.01–10 μg DRO/g) for up to 31 days. DRO was detected in the blood (4–27 ng/mL) of fish exposed to the highest concentration, with no significant bioaccumulation over time and no alteration of hepatic metabolizing enzymes, namely, CYP1A and CYP3A-catalysed activities and UDP-glucuronyltransferase (UGT). Pregnenolone (P5), progesterone (P4), 17α-hydroxyprogesterone (17P4), 17α-hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T) were determined in plasma and gene expression of *cyp17a1*, *cyp19a1a* and *cyp11β* analysed by qRT-PCR in gonads. The significant increase in plasmatic levels of 17P5, 17P4 and AD detected after 31 days exposure to 10 ng DRO/g together with the increased expression of *cyp17a1* in females evidence the ability of DRO to alter steroid synthesis at low intake concentrations (7 ng DRO/day). However, the potential consequences of this steroid shift for female reproduction remain to be investigated.

1. Introduction

Drospirenone (DRO) (6β,7β,15β,16β-dimethylene-3-oxo-17a-pregn-4-ene-21,17-carbolactone) is a new generation progestin and a derivative of the synthetic mineralocorticoid 17α-spironolactone, with a pharmacologic profile similar to progesterone, and with anti-mineralocorticoid, anti-aldosterone and slight antiandrogenic activity (Krattenmacher, 2000; Rapkin and Winer, 2007). It is prescribed in contraceptive treatments and hormone replacement therapies in combination with estradiol or ethynilestradiol at doses up to 150-fold higher than estrogens, and it is one of the most widely used synthetic progestins in Europe (Fent, 2015). Thus, environmental concentrations of DRO are expected to be in the same range or higher than other synthetic estroges and progestins, which are detected at the low ng/L range in effluents (Besse and Garric, 2009). However, DRO and some other new generation progestins have not yet been extensively monitored in aquatic systems (Fent, 2015).

In teleost fish, natural progestins play an important role in the stimulation of oocyte growth and maturation as well as in spermatogenesis and sperm maturation, and they also act as sex pheromones (Nagahama and Yamashita, 2008; Scott et al., 2010). Moreover, fish possess similar drug targets as humans, and consequently, synthetic progestins can interact with those conserved targets and adversely affect reproduction (Runnalls et al., 2013). Thus, levonorgestrel or gestodene at concentrations of 100 ng/L stopped spawning almost completely in the fathead minnow; gestodene concentrations as low as 1 ng/L had significant effects on reproduction over 21 days, whereas desogestrel was less potent, but still reduced egg production at concentrations of or above 1 μg/L. Zeilinger et al. (2009) reported reduced fertility of fathead minnow at concentrations of levonorgestrel as low as

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0.8 ng/L and DRO of 6.5 μg/L, while reproduction was not affected at lower concentrations of DRO (100 ng/L) (Runnalls et al., 2013). Additionally, norethindrone decreased fecundity in fathead minnow and medaka, and levonorgestrel disrupted the seasonal breeding cycle in male sticklebacks, both at concentrations in the low ng/L range (Paulos et al., 2010; Svensson et al., 2014).

Despite of these evidences, the mechanisms which mediate endocrine disruption by synthetic progestins are poorly understood. Modulated gonadotropin expression in the pituitary and changes of plasma sex steroid levels underlie many of the reproductive effects, as reduced fecundity or disturbed gonad development. However, the concentrations needed to induce changes in steroid levels are usually higher than those to reduce fecundity (Kumar et al., 2015). *In vitro* exposure of fathead minnow ovaries to progesterone resulted in increased synthesis of pregnenolone, 17α-hydroxyprogesterone, 17α,20β-dihydroxypregnenone and testosterone, while norethindrone had no significant effect, despite a non-significant decrease of testosterone production (Petersen et al., 2015). Interestingly, DRO inhibited CYP17 activity (metabolism of 17α-hydroxyprogesterone to androstenedione) in carp testis mitochondrial fractions *in vitro* (IC50: 3.8 μM). DRO was a stronger inhibitor than cyproterone acetate (IC50: 183 μM), while other synthetic progestins (levonorgestrel and norethindrone) did not affect CYP17 activity (Fernandes et al., 2014).

Progestins are shown to alter the expression of steroidogenic enzymes in zebrafish and fathead minnows (Overturf et al., 2014; Fent, 2015). Transcriptional changes were generally more sensitive than changes on steroid levels and revealed a number of affected pathways, including steroid hormone receptor activities and steroid hormone mediated signaling pathways, cellular response to steroid hormone stimulus and thyroid hormone receptor activity (Zucchi et al., 2014). More recently, Zhao et al. (2015) reported significant and dose-dependent alterations of the circadian rhythm network in the brain of zebra fish exposed to progesterone and DRO.

Within this context, the present study was designed to investigate the effect of DRO exposure in the hepatic metabolism (CYP1A and CYP3A-catalysed activities and UDP-glucuronyltransferase (UGT)), circulating steroid levels and expression of key steroidogenic enzymes (*cyp17a1*, *cyp19a1a*, *cyp11β*) in gonads of juveniles of European sea bass (*Dicentrarchus labrax*), with the aim of gathering information on the dynamics and the mode of action of this synthetic progestin in juvenile fish, in a period particularly sensitive to the effect of exogenous steroids (Piferrer et al., 2005).

2. Material and methods

2.1. Chemicals

Drospirenone, *p*-nitrophenol (pNP), uridine 5′-diphosphoglucuronic acid (UDPGA), NADPH, 7-ethoxyresorufin (7-ER), bovine serum albumin (BSA; fatty acid free, ≥ 99% purity), methyl tert-butyl ether (MTBE) and hydroxylamine hydrochloride were purchased from Sigma-Aldrich (Steinheim, Germany). D8-17-hydroxyprogesterone (d8-17P4) was obtained from C/D/N Isotopes (Quebec, Canada); d9-progesterone (d9-P4), d4-pregnenolone (d4-P5), d3-testosterone (d3-T), pregnenolone (P5), progesterone (P4), 17-hydroxyprogesterone (17P4), 17- hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T) were obtained from Sigma-Aldrich (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin (7-BFC) was from Cypex (Dundee, Scotland, UK). Dulbecco's Phosphate Buffered Saline (DPBS) was obtained from Gibco (Life technologies). Primers of selected genes and SuperScript III Reverse Transcriptase were obtained from Invitrogen. All solvents were from Merck (Darmstadt, Germany).

2.2. Experimental design

Juvenile European sea bass – 243 days post-hatching (dph) (100–185 mm length) reared at the experimental animal facility of the Institute of Marine Science (Barcelona, Spain), were randomly distributed into six 50 L tanks (30 individuals per tank) for acclimatization at a flow rate of 1.2 L water/min with 80% oxygen saturation, natural temperature (14.5–15 °C) and photoperiod (light:dark, 9:15). After the acclimatization period (4 weeks), fish were fed with commercial pellets spiked with DRO at a concentration of 0.01, 0.1, 1 and 10 μg/g, with a daily average intake of 0.7 g of pellet feed per fish. The experimental diets were prepared following the alcohol evaporation method adapted for the sea bass (Blázquez et al., 1995). Briefly, food pellets were sprayed with the different concentrations of DRO dissolved in ethanol, being the solvent completely evaporated afterwards. A solvent control (SC) group–pellets only sprayed with ethanol–and a control group––untreated pellets-were also included in the study. The highest concentrations (1 & 10 μg/g of DRO) corresponded to typical doses of estrogens or androgens used in experiments of sex reversal in this species (Blázquez et al., 1998, 2001), while concentrations of 0.01 and 0.1 μg/g are close to the human therapeutical dose (0.05 μg/g of DRO).

Fish were sampled after 2, 4, 8, 16 and 31 days of exposure. They were anesthetized with 0.2% phenoxyethanol and the individual weights and lengths measured. Blood (approx. 1 mL) was taken from the caudal vein, transferred into heparinised tubes, centrifuged (1000 × *g*; 15 min), and the plasma separated and stored at −80 °C. Immediately after, fish were sacrificed by quickly severing the spinal cord and the liver and the gonads were dissected. A fragment of the central part of the left gonad from each fish was separated and fixed in 4% PAF (buffered paraformaldehyde) for further histological analysis. The rest of the gonads and the liver were immediately frozen in liquid nitrogen and stored at −80 °C. All fish were treated in accordance with the Spanish regulations (Royal Decree Act 53/2013) and the European legislation (2010/63 EU) concerning the protection of vertebrates used for experimental and other scientific purposes. All the steps were taken to reduce suffering of the animals.

2.3. Analysis of drospirenone in plasma

Circulating levels of DRO in plasma were determined after 2, 4, 8, 16 and 31 days of exposure to the highest concentration (10 μg/g). Acetonitrile (400 μL) was added to 100 μL of plasma, centrifuged at 4000 × *g* for 10 min and the resulting supernatant (10 μL) injected in an UPLC-MS/MS system (Ultra Acquity LC System, TQ Detector, Waters, USA). To determine extraction efficiency a known concentration of DRO was added to plasma of non-exposed individuals and extracted as mentioned above. DRO was detected under positive electrospray ionization (ES+) and multiple reaction mode (MRM) measuring the transition of precursor ion fragmentation (367 *m*/*z*) to product ions (97/91 *m*/*z*) under a collision energy of 41/75 Ev and with a capillary voltage of 3.50 Kv and cone voltage of 40 V. The analysis was performed using a Zorbax Eclipse Plus C-18 column (2.1 mm × 50 mm, 1.8 μm) (Agilent, Loveland, U.S) connected to a pre-column Zorbax Eclipse Plus C-18 (2.1 mm × 5 mm, 1.8 μm) (Agilent, Loveland, U.S) with a mobile phase composed of acetonitrile (A) and Milli-Q water containing 0.1% (v/v) of formic acid (B). The run consisted of 0.5 min at 25% A, a 8 min linear gradient from 25% A to 90% A, 2 min at 90% A and back over to initial state at 1 min, allowing 1 min for column re-equilibration. The total run-time was 12 min at a flow rate of 0.3 mL/min. The column was

maintained at 50 °C. The retention time of DRO, under our assay conditions, was of 4 min and the limit of detection was <1 μg/L. The efficiency of the extraction procedure was of 98 ± 0.5% (n = 6).

2.4. Plasma steroid analysis

Plasmatic steroid levels (i.e. pregnenolone (P5), progesterone (P4), 17-hydroxyprogesterone (17P4), 17- hydroxypregnenolone (17P5), androstenedione (AD) and testosterone (T)) were analysed after 4 and 31 days of exposure. Plasma samples were extracted according to the procedure described by Keski-Rahkonen et al. (2011). 400 μL of plasma together with 10 μL of internal standard (IS; mix solution of deuterated steroids) at 10 ppb was placed into glass screw top tubes, vigorously mixed and extracted twice with 1 mL of MTBE after centrifugation at 2000 rpm for 10 min. The resulting organic layer was transferred into and HPLC vial, evaporated under nitrogen current and reconstituted in 100 μL of hydroxylamine solution (100 mM in 50% methanol (v/v)) and heated at 60 °C for 1 h before being injected (10 μL) into the UPLC-MS/MS system.

Calibration curve was prepared by substituting the plasma with 4% of BSA solution (2 g of BSA in 50 mL of DPBS). Thus, working solutions of mix steroids (P5, d4-P5, P4, d9-P4, 17P4, d8-17P4, 17P5, AD, T, d3-T) dissolved in methanol were added to the BSA solution, before being extracted twice with 1 mL of MTBE, evaporated under nitrogen and reconstituted in hydroxylamine solution (heated at 60 °C for 1 h). The calibration curve consisted of ten concentration levels: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 ppb, a zero sample (only IS added, to calculate extraction efficiency), and a blank (no standards added).

Samples were analysed by an UPLC-MS/MS system (Ultra Acquity LC System, TQ Detector, Waters, USA), with a Zorbax Eclipse Plus C-18 column (2.1 mm × 50 mm, 1.8 μm) (Agilent, Loveland, U.S) connected to a pre-column Zorbax Eclipse Plus C-18 (2.1 mm × 5 mm, 1.8 μm) (Agilent, Loveland, U.S). Steroids (T, d3-T, AD, P4, d9-P4, P5, d4-P5, 17P4, d8-17P4 and 17P5) were detected under positive electrospray ionization (ES+) and multiple reaction mode (MRM), measuring the transition of precursor ion fragmentation to product ions under a capillary voltage of 3.00 Kv and cone voltage of 34 V (see Table 1). The mobile phase consisted of methanol (A) and Milli-Q water (B) both containing 0.1% (v/v) of formic acid. The run was as follows: 0.1 min at 10% A, a 2 min linear gradient from 10% A to 70% A, a 3 min linear gradient from 70% A to 90% A, 2 min at 90% A and back to initial conditions at 1 min, allowing 2 min for column re-equilibration. The total run-time was 10 min at a flow rate of 0.2 mL/min. The column was maintained at 48 °C. The limit of detection was <50 ng/L for all steroids, with the exception of P5 < 10 ng/L. Deuterated steroids added to the samples in order to compensate for matrix effects and to evaluate the efficiency of the extraction procedure, showed recoveries of 79 ± 9% for d3-T, 68 ± 14% for d4-P5, 97 ± 11% for d8-17P4 and 84 ± 22% for d9-P4 (n = 20).

2.5. Histological analysis

Histological analyses were performed in order to unequivocally assess the sex of the fish used in the study since visual classification was not possible due to the low degree of sexual development of the gonads. With this purpose, portions of gonadal tissues were fixed in 4% PAF at 4 °C overnight. Fixed gonads were dehydrated using a graded series of ethanol concentrations (70–100%) and embedded in paraplast (Leica, Germany). Sections for histological examination were cut at 5 μm on a retracting microtome and stained with haematoxylin and eosin.

2.6. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA from gonads of control and DRO-exposed groups after 2 and 31 days of treatment was isolated with TRIZOL (Invitrogen, Paisley, UK) following the manufacturer's instructions. The concentration and quality of the RNA was assessed by spectrophotometry and gel electrophoresis. Total RNA (2 μg) was reverse transcribed to cDNA with Superscript III (200 units; Invitrogen) and random hexamer primer (50 mM) following the manufacturer's instructions. The resulting cDNAs were used as a template to amplify and quantify the expression levels of *cyp17a1*, *cyp19a* and *cyp11β* genes. Previously, A TBLASTN search (Altschul et al., 1997) was conducted to identify the *cyp17a1* sequence in the European sea bass genome database (Tine et al., 2014), using as queries various known vertebrate sequences. Once identified, specific primers were designed for its complete amplification. Full CDS sequence was cloned and sequenced in order to verify its identity when compared with the sea bass genome. The verified and cloned sequence was finally deposited in the GenBank database under the accession number KT932710. Full sequences for sea bass *cyp19a1a* and *cyp11beta* were available in the GenBank under accession numbers AJ318516 and AF449173, respectively. Specific primers for detecting housekeeping and target genes were designed using primer 3 software (http://www.bioinformatics.nl//cgi-bin/primer3plus/primer3plus.cgi) and examined for their specificity and amplification efficiency using serial dilutions of template (Table 2). The melting curve analysis (60 °C for 15 s and 95 °C for 15 s) after the amplification phase displayed a single peak for every gene, ensuring primers specificity. Moreover, PCR efficiencies (E) of all primers ranged between 90% and 110%. All qRT-PCR reactions were analysed on 7300 Real Time PCR System (Applied Biosystems) using SYBR Green (Select Master Mix, Applied Biosystems). Samples were run in triplicate in 96-well plates in a 15 μL reaction, containing 7.5 μL of SYBR Green, 0.75 μL of each of forward and reverse primer, 1 μL of cDNA sample and 5 μL nuclease-free water. The qRT-

Table 1
Masses of precursor and product ions of steroids detected in plasma by UPLC-MS/MS, obtained under positive electrospray ionization (ES+) subsequent to collision induced dissociation with multiple reaction monitoring (MRM).

	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Fragmentor voltage (V)	Collision energy (eV)
Androstenedione	317	112; 124	40	30; 30
Testosterone	304	112; 124	50	30; 30
d3-Testosterone	307	112; 124	40	30; 30
Progesterone	345	112; 124	40	30; 30
d9-Progesterone	354	115; 128	50	30; 30
Pregnenolone	332	86; 60	40	25; 25
d4-Pregnenolone	336	90	40	30
17α-Hydroxyprogesterone	361	112; 124	40	30; 30
d8-17α-Hydroxyprogesterone	369	115; 128	50	35; 35
17α-Hydroxypregnenolone	348	330; 312	10	10; 20

Table 2
Primer sequences and variables used for the amplification and calculation of the efficiency (E) of the real-time qPCR reactions.

Gene	Primer sequence (5′→3′)	Slope	E	R^2	Amplicon size (bp)
cyp17a1	Sense: TTGCAGGAAGACCCAGAACT Antisense: TCTCCAAACATGCACAGAGC	−2.92	2.20	0.97	133
cyp19a1a	Sense: AGACAGCAGCCCAGGAGTTG Antisense: TGCAGTGAAGTTGATGTCCAGTT	−3.33	1.99	0.99	106
cyp11β	Sense: CTGGAAGCCAGTTGCCATGT Antisense: TCCTCCACTGCCCAAATAA	−3.33	1.99	0.99	98
18S	Sense: GCATGCCGGAGTCTCGTT Antisense: TGCATGGCCGTTCTTAGTTG	−3.33	1.99	0.99	70

PCR amplification was carried out using the following program: an activation step for 10 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and a 1 min annealing/extension step at 60 °C. Finally, a dissociation step of 15 s at 95 °C followed by 15 s at 60 °C was added. The 18s rRNA was selected as reference gene for normalization due to its stable expression among the solvent control and treated groups. Data were collected and compiled using SDS 2.3 software (Applied Biosystems) that was used to calculate gene expression levels. For each gene, a non-template control was included to confirm the absence of DNA contamination. In addition, the same control sample was used in all runs to calculate the intra- and inter-assay variations. Control values were adjusted for differences in E of each primer set using Q-gene. Values were normalized (normalized expression; NE) to the reference gene following the equation $NE = (Eref)^{Ctref}/(Etarget)^{Cttarget}$. Replicates were averaged and shown as mean normalized expression (MNE) ± SEM.

2.7. Biochemical determinations

Hepatic microsomal fractions were prepared as described in Fernandes et al. (2013). Livers (0.5 g) were flushed with ice-cold 1.15% KCl and homogenized in 1:4 w/v cold homogenization buffer containing 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 × *g* for 15 min, and the obtained supernatant centrifuged at 12,000 × *g* for 45 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 60 min to obtain the microsomal fraction. Microsomal pellets were resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA. Proteins were measured according to the method of Bradford (1976), using BSA as standard.

EROD activity was determined in the microsomal fraction of the liver and was assayed by incubating 0.1 mg of protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 (final volume of 250 μL) at 30 °C for 10 min. The reaction was stopped by adding 400 μL of ice-cold acetonitrile and after centrifugation (2000 × *g*; 10 min) an aliquot of the supernatant (200 μL) was transferred into a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelength pairs of 537/583 using a Varioskan microplate reader (Thermo Electron Corporation). Quantification was performed using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute.

BFCOD activity was analysed according to the procedure described by Thibaut et al. (2006). The assay consisted in incubating 25 μg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume of 250 μL) at 30 °C for 10 min. The reaction was stopped by addition of 75 μL of acetonitrile (20:80, v/v), the fluorescence was read in a 200 μL aliquot transferred into a 96-multiwell plate at the excitation/emission wavelength pairs of 409 and 530 nm, using a Varioskan microplate reader (Thermo Electron Corporation), and the activity calculated as the amount of 7-hydroxy-4-(trifluoromethyl)-coumarin (pmol) generated per milligram of protein per minute.

UDP-glucuronosyltransferase activity (UGT) was determined in hepatic microsomal fraction and was assayed by incubating 0.25 mg of proteins (pre-treated for 15 min with 0.2% Triton X-100 on ice) with 3.0 mM of UDPGA in 50 mM Tris-HCl buffer pH 7.4, containing 10 mM $MgCl_2$ (final volume of 260 μL). The reaction was initiated by the addition of 81 μM *p*-nitrophenol (*p*NP) and run for 30 min at 30 °C in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid (TCA), centrifuged (1500 × *g*; 15 min), alkalinized with 0.1 mL of 10 N KOH and the remaining *p*NP was measured spectrophotometrically at 405 nm. Activity was calculated as the amount of pNP (nmol) consumed per milligram of protein per minute.

2.8. Statistical analysis

Plasma steroid levels were determined in pooled samples of two individuals. Enzymatic activities were determined in duplicate in six individual fish per tank. Comparisons between treatment groups were made using one-way ANOVA followed by multiple independent group comparison (Dunnett and Tukey's test). DRO levels in plasma were analysed by non-parametric Kruskal-Wallis test. RNA gene expression was determined in six individual fish per treatment and results were expressed as mean normalised expression (MNE) ± SEM and analysed by non-parametric Mann-Whitney test comparing results between control and exposed groups.

In all instances transformations of the data were performed when the assumption of normality of residuals was not met. All statistical analyses were performed with the software package SPSS 15.0 (SPSS Inc., Chicago, IL) and p values lower than 0.05 were considered statistically significant.

3. Results

3.1. Biological parameters and histological examination of the gonads

The morphometric parameters of the fish are presented in Table 3. Individuals were rather similar in body weight, length and condition factor (CF) and no significant differences were observed among the different groups. In addition, histological examination of the gonads revealed no effects of DRO in treated fish at any concentration and exposure length.

Table 3
Biological parameters of sea bass (*Dicentrarchus labrax*). Values are mean ± SEM (n = 5–9). Condition factor (CF) was calculated as (weight/length^3) × 100.

Exposure time	Concentration	Length (cm)	Weight (g)	Condition factor (g/cm^3)
2 days	Control (C)	13.7 ± 1.1	35.8 ± 3.6	1.5 ± 0.5
	Solvent control (SC)	13.4 ± 0.5	32.0 ± 5.2	1.4 ± 0.7
	0.01 μg/g	12.5 ± 0.6	36.3 ± 7.8	1.7 ± 0.2
	0.1 μg/g	12.4 ± 0.7	32.0 ± 5.2	1.6 ± 0.1
	1 μg/g	12.0 ± 0.3	32.7 ± 2.5	1.9 ± 0.2
	10 μg/g	10.8 ± 1	30.5 ± 3.6	1.8 ± 0.3
4 days	Control (C)	12.9 ± 0.4	34.5 ± 2.4	1.6 ± 0.04
	Solvent control (SC)	12.8 ± 0.5	34.4 ± 4.4	1.6 ± 0.05
	0.01 μg/g	12.9 ± 0.4	38.3 ± 4.0	1.7 ± 0.03
	0.1 μg/g	12.7 ± 0.3	35.4 ± 2.2	1.7 ± 0.04
	1 μg/g	12.0 ± 0.4	29.3 ± 2.9	1.7 ± 0.08
	10 μg/g	12.6 ± 0.6	36.4 ± 5.0	1.8 ± 0.03
8 days	Control (C)	12.2 ± 0.2	29.1 ± 1.9	1.6 ± 0.04
	Solvent control (SC)	12.7 ± 0.4	34.0 ± 3.7	1.6 ± 0.05
	0.01 μg/g	12.5 ± 0.5	36.6 ± 3.6	1.9 ± 0.09
	0.1 μg/g	13.0 ± 0.2	38.4 ± 1.4	1.8 ± 0.06
	1 μg/g	12.9 ± 0.2	36.5 ± 1.6	1.7 ± 0.02
	10 μg/g	12.9 ± 0.4	36.4 ± 3.6	1.7 ± 0.04
16 days	Control (C)	12.5 ± 0.6	34.9 ± 5.3	1.7 ± 0.02
	Solvent control (SC)	12.4 ± 0.4	32.4 ± 3.7	1.7 ± 0.05
	0.01 μg/g	15.6 ± 0.5	33.0 ± 3.4	1.6 ± 0.04
	0.1 μg/g	12.0 ± 0.6	30.9 ± 4.2	1.8 ± 0.04
	1 μg/g	12.8 ± 0.5	38.4 ± 5.1	1.8 ± 0.07
	10 μg/g	13.8 ± 0.6	45.5 ± 6.2	1.7 ± 0.07
31 days	Control (C)	12.4 ± 0.5	31.6 ± 3.7	1.6 ± 0.15
	Solvent control (SC)	12.1 ± 0.5	31.2 ± 5.2	1.7 ± 0.2
	0.01 μg/g	13.3 ± 0.3	38.7 ± 2.7	1.6 ± 0.03
	0.1 μg/g	13.3 ± 0.6	40.4 ± 4.7	1.7 ± 0.08
	1 μg/g	13.0 ± 0.5	35.4 ± 3.9	1.6 ± 0.1
	10 μg/g	13.4 ± 0.5	40.8 ± 5.1	1.6 ± 0.15

3.2. Drospirenone in plasma

DRO, in the range of 4–27 ng/mL, was detected circulating in plasma of individuals exposed to 10 μg DRO/g food (Fig. 1). The highest concentration was detected after four days of exposure and the lowest at 31 days. Levels of DRO in plasma of individuals exposed to 0.01–1 μg DRO/g were below the detection limit of the method (1 ng/mL).

3.3. Steroids in plasma

Steroids were analysed in the plasma of individuals exposed to DRO for 4 and 31 days. No significant differences in plasma steroid levels were observed between exposed and control groups after 4 days of treatment (Fig. 2). However, levels of 17P5 (up to 7-fold), 17P4 (up to 3-fold) and AD (up to 2-fold) were significantly increased in individuals exposed to the lowest concentration of DRO (0.01 μg DRO/g food) for 31 days, while no changes were observed for P5, P4 and T (Fig. 2). Exposure to higher concentrations of DRO (0.1, 1 and 10 μg DRO/g food) did not significantly alter circulating steroid levels in juvenile sea bass.

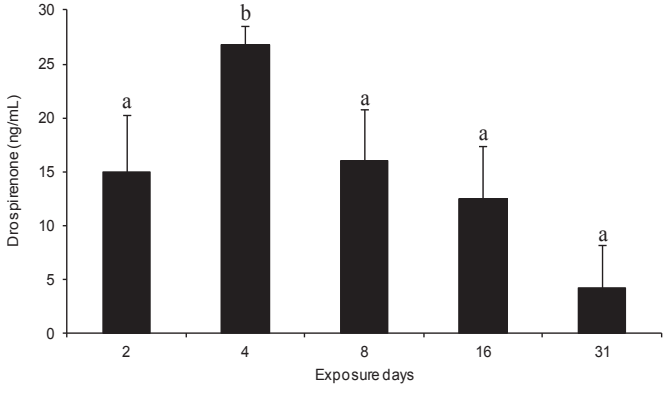

Fig. 1. Drospirenone concentrations in plasma of sea bass (*Dicentrarchus labrax*) after exposure to pellet feed contaminated with 10 μg/g of DRO. Values are mean ± SEM (n = 5–6). Distinct letters indicate significant differences between exposure days.

3.4. RNA gene expression

Data from control and exposed fish, regardless of exposure concentration, were pooled for the analysis in order to obtain a statistically robust sample size that could take into consideration males and females as separate variables. This is particularly important considering that the expression levels of the genes *cyp19a1a* and *cyp11beta* exhibit sex-related differences (Blázquez et al., 2009). After 2 days of DRO administration, a significant 2-fold induction of *cyp17a1* was detected in the ovaries of exposed females (Fig. 3). Expression of *cyp17a1* was one order of magnitude higher in male gonads, and it was not altered by exposure. Neither *cyp19a1a* nor *cyp11β* expression were altered by DRO treatment. After 31 days of dietary exposure to DRO, no differences in the expression of *cyp17a1*, *cyp19a1a* or *cyp11β* were observed between control and treated fish (Fig. 3).

3.5. Hepatic metabolism

DRO had no significant effect in the activity of EROD, BFCOD or UGT after 2 or 31 days of treatment (Fig. 4). Nonetheless, a tendency towards lower EROD and UGT activities was detected after 2 days of exposure to 0.1 μg DRO/g food. EROD activity ranged from 32 to 59 pmol/min/mg protein at 2 days of exposure, while after 31 days of exposure the variability among tanks was smaller (31–43 pmol/min/mg protein). A similar tendency was observed for UGT.

EROD, BFCOD and UGT activities were also determined after 8 days of exposure, and no statistically significant differences were detected among treatments (data no shown).

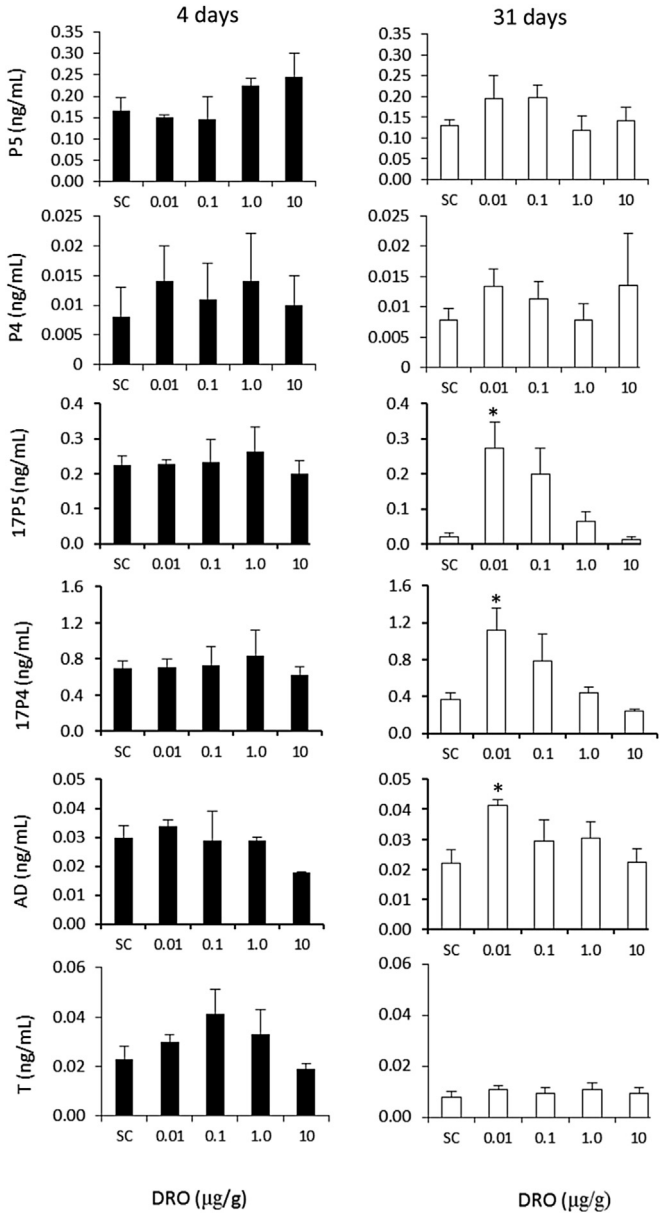

Fig. 2. Plasma steroid levels after 4 and 31 days of exposure. Values are mean ± SEM (n = pools of 2 individuals). P5: pregnenolone; 17P5: 17α-hydroxypregnenolone; P4: progesterone; 17P4: 17α-hydroxyprogesterone; AD: androstenedione; T: testosterone. Results of control samples are shown as mean of control and solvent control. *Significant differences relative to the control ($P < 0.05$).

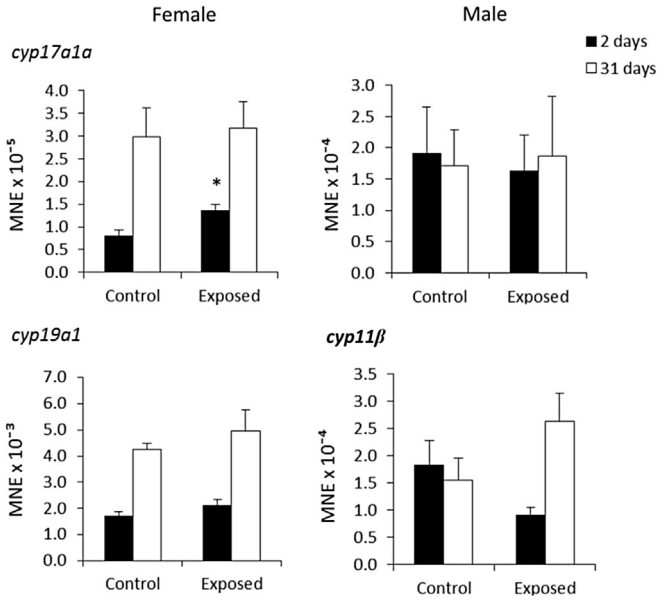

Fig. 3. Mean normalised expression (MNE ± SEM) of *cyp17a1*, *cyp19a1a* and *cyp11β* genes in gonads of sea bass (*Dicentrarchus labrax*) exposed for 2 and 31 days to DRO. *Significant differences relative to the control ($P < 0.05$). Data from the different exposure concentrations (n = 13 males, 7 females) and from control groups (n = 4 males, 6 females) were pooled.

4. Discussion

The detection of DRO in plasma of sea bass after 2, 4, 8, 16 and 31 days of exposure confirms the uptake of the compound and its distribution in the body. Considering a daily intake of 0.7 g food pellets/fish, the concentration of DRO detected in plasma represents only 0.06–0.4% of the amount theoretically ingested daily. If we assume that, as in humans, the absolute bioavailability of DRO after oral administration is of 76% and that approximately 95–97% of the compound binds to serum proteins (Krattenmacher, 2000), a maximum of 160 ng DRO/mL would be expected in the plasma of sea bass exposed to 10 µg/g. However, the maximum plasmatic concentration detected was 27 ng/mL after four days of exposure, and decreased to 5 ng/mL after 31 days. Certainly, some desorption of DRO from food might occur while in water, prior to being ingested, although food was eaten within 5 min. Altogether, the results indicate no significant bioconcentration of DRO in plasma over time and suggest that the compound is metabolized by juvenile sea bass. Similarly, DRO was reported to have a relatively low bioconcentration factor (BCF: 36) in mussels after 2 weeks of exposure to 100 mg DRO/L; concentrations were below detection limit for those mussels exposed to 0.01 mg/L (Gilroy et al., 2014). However, despite the evidence of a metabolism of DRO in juvenile sea bass, CYP1A, CYP3A and UGT activities determined in liver microsomal fractions were not significantly altered by exposure, suggesting that the metabolism of DRO was probably CYP-independent. In humans, DRO is mainly metabolized in the liver to 4,5-dihydrodrospirenone-3-sulfate and to the acid form that is generated by opening of the lactone ring, both metabolites are reported not to be pharmacologically active and are CYP-independent (Krattenmacher, 2000). Nonetheless, it has been reported that DRO is metabolized to a minor extent (4–7%) by CYP3A4 in human liver microsomes, and an inhibition of CYP3A4 can moderately increase DRO exposure in individuals treated with DRO, suggesting a potential involvement of the enzyme in DRO metabolism (FDA, 2006; Wiesinger et al., 2015).

No significant changes in length, weight and condition factor were observed in sea bass regardless of the concentration or exposure length. Similarly, Zucchi et al. (2014) reported no significant morphometric changes in zebrafish (*Danio rerio*) exposed up to 5 µg/L DRO for 14 days, despite a significant decrease in the gonadosomatic index (GSI), strong inhibition of VTG mRNA and altered transcription of *cyp19a1a*. In the present work, the expression of *cyp19a1a* and *cyp11β* in gonads of juvenile sea bass was not altered by exposure, but a 2-fold up-regulation of *cyp17a1* was detected in ovaries of exposed females. *Cyp17a1* encodes for a protein that has both 17α-hydroxylase and 17,20-lyase activities and catalyzes the conversion of pregnenolone and progesterone to their 17α-hydroxylated products, and subsequently to dehydroepiandrosterone (DHEA) and androstenedione. Although our findings should be interpreted with caution, as females from all exposure concentrations were pooled, it is interesting to notice that

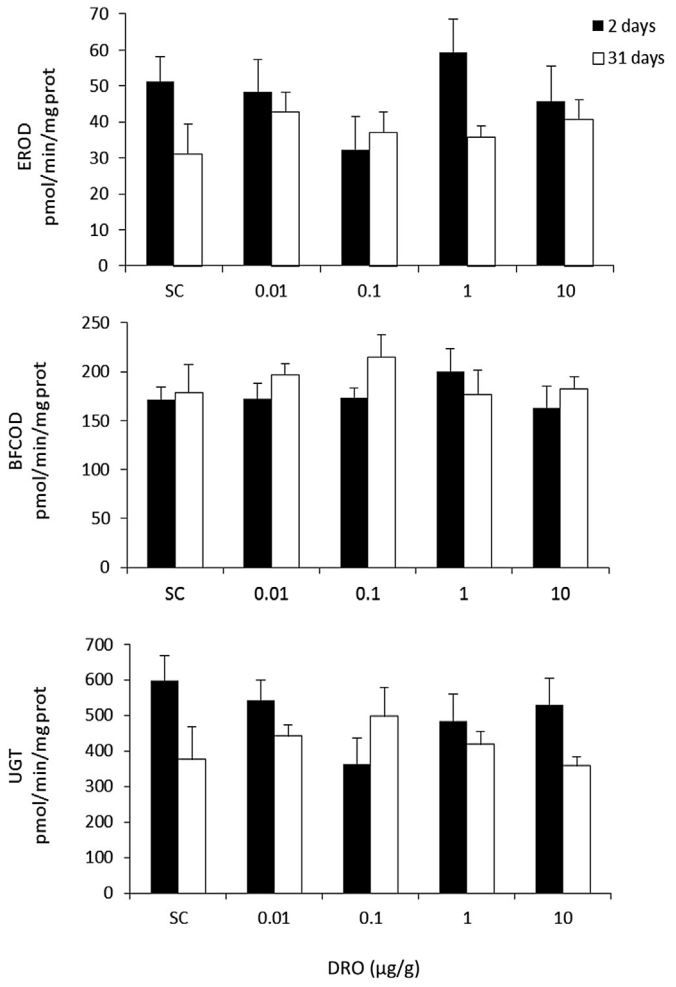

Fig. 4. EROD (CYP1A), BFCOD (CYP3A) and UDP-glucuronosyltransferase (UGT) activities determined in liver microsomal fractions of juvenile sea bass exposed for 2 and 31 days to different concentrations of DRO (0.01, 0.1, 1 and 10 µg/g of pellet feed). Values are mean ± SEM (n = 6).

in agreement with an activation of *cyp17a1*, significantly increased plasmatic levels of 17P4, 17P5 and AD were detected in sea bass exposed to 0.01 µg DRO/g food for 31 days, while T levels were not altered (Fig. 2). Similarly, Runnalls et al. (2013) reported no effect of DRO in plasmatic concentrations of T and 11-ketotestosterone after 31 days of exposure in males of fathead minnow. Neither Zeilinger et al. (2009) observed masculinization of female fathead minnow exposed to DRO (70 µg/L). Altogether, these findings are in agreement with the fact that DRO is not an agonist of fish androgen receptors (ARα and ARβ), but a weak agonist of the progesterone receptor (Ellestad et al., 2014; Bain et al., 2015), and as such, it might have progesterone-like action at relatively low concentrations.

The analysis of pharmaceuticals in fish plasma can be an excellent tool to assess the risk for pharmacological effects, as plasma concentrations can be easily compared with human therapeutic levels (Fick et al., 2010). Thus, concentrations of levonorgestrel of 2.4 ng/L were enough to bioaccumulate in plasma of rainbow trout at levels exceeding up to 4 times the human therapeutic dose. Nevertheless, in the present study, only sea bass from the high exposure group had plasmatic concentrations of DRO (5–27 ng/mL) relatively close to human therapeutic concentrations (20–25 ng/mL; maximum 60–87 ng/mL). Nonetheless, fish from the low exposure group, with plasmatic concentrations well below human therapeutic doses, showed increased plasmatic levels of 17P4, 17P5 and AD after 31 days exposure. These results, together with the increased expression of *cyp17a1* in females, evidence the ability of DRO to alter steroid synthesis in juvenile sea bass with a mean uptake of 7 ng DRO/day (10 ng/g × 0.7 g food/day). However, the consequences in terms of impaired gonad development and/or reproduction in adult sea bass remain unknown.

Overall, the work contributes to the better understanding of the impact of DRO in fish. The question on whether environmental concentrations of DRO are likely to have an endocrine effect in fish still remain open, as so far, concentrations of DRO in water are unknown and it is uncertain whether the uptake of DRO through the water will have the same endocrine disruptive effect as through the food. However, this work (a) evidences the ability of DRO to increase plasmatic levels of 17P5, 17P4 and AD in juvenile sea bass and to increase the expression of *cyp17a1* in ovaries after a daily intake of 7 ng DRO for up to 31 days, and (b) stresses the need of performing further exposure experiments at the low ng/L concentration range to ascertain whether the same endocrine disruptive effect is likely to occur at environmentally relevant concentrations of DRO.

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