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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