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Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads—A stereological study using the zebrafish (*Danio rerio*) as model

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ABSTRACT

In natural environments fish populations are exposed to many potential xenoestrogens, whereby understanding the impacts of mixtures continue to be of great interest. The main objective of this study was, therefore, to understand whether and how an environmentally relevant mixture of xenoestrogens found in the Douro River estuary can disrupt the normal gametogenesis in fish. For this purpose, adult zebrafish of both sexes were exposed for 21 days to an environmental mixture (MIX) of 11 xenoestrogens from diverse sources. A 100 ng/L ethinylestradiol (EE2) positive control was added. A quantitative (stereological) analysis with systematic sampling was made in the gonads, and using light microscopy both the relative and the absolute volumes of the gametogenic stages were estimated. Data point that the EE2 stimulus induced changes in structural compartments; with decreasing trends for the advanced maturation stages both in males and females. There was also a trend for a greater amount of interstitial tissue in males. Along with an interstitial fibrosis increase detected, the presence of a proteinaceous fluid was observed in both sexes and experimental groups (EE2 and MIX). Other histopathologic alterations were observed in the EE2 female group, such as the presence of foci of granulomatous inflammation and follicular mineralization in the germinal parenchyma and luminal areas. The most interesting finding of this study was that the exposure to the MIX caused a decrease of the relative volume of spermatozoa in zebrafish. This kind of estrogenic effect has not earlier been structurally quantified in such a fine detail with unbiased stereology in fish gonads. Despite the ultimate consequences of such disruptions being unknown, it could be logically argued that reduction or slowing-down of the appearance of the most mature cohorts and/or eventual interstitial fibrosis and other pathologic changes can adversely affect breeding. The findings add further explanatory bases for understanding the negative impacts of xenoestrogens.

1. Introduction

Over the past twenty years, considerable and growing attention has been given to the fact that chemical compounds can be biologically active by disrupting the normal functions of the endocrine systems of animals. Those substances, called endocrine disrupting chemicals (EDCs), are widespread throughout the aquatic ecosystems and their effects and modes of action have been documented (Depledge and Billinghurst, 1999; Porte et al., 2006; Segner et al., 2003). Aquatic species are exposed to EDCs through a variety of sources, but wastewater is the main source of EDCs such as those that mimic the effects of natural estrogens (xenoestrogens) (Falconer et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2001; Ying et al., 2002).

Under laboratory conditions, xenoestrogens have been reported to cause a variety of effects in fish reproduction such as changes in plasma hormone concentrations (Khan and Thomas, 1998), gonadal size (Ashfield et al., 1998; Gray and Metcalfe, 1997; Jobling et al., 1996), development of intersex gonads (ovotestis) (Gray and Metcalfe, 1997) and induction of the female yolk precursor protein, vitellogenin (VTG) (Jobling et al., 1996). Although there has been an increased effort to understand the mechanisms by which

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xenoestrogens exert final effects, fundamental doubts still remain, such as the impact on the kinetics of gametogenesis. Several studies suggest that exposure to xenoestrogens may cause suppression of gametogenesis in both male and female fish (Blázquez et al., 1998; Christiansen et al., 1998; Drèze et al., 2000; Gimeno et al., 1998; Papoulias et al., 2000; Piferrer and Donaldson, 1992; Tanaka and Grizzle, 2002; Van Den Belt et al., 2002; Weber et al., 2003). All those histological investigations involved a tissue microscopic analysis, which resulted in visual qualitative descriptions and/or semi-quantitative data or in quantifications by two-dimensional measurements, which are to be avoided as they are well-known now to suffer from uncontrolled biases (Howard and Reed, 2005). The application of stereology to such issues, however, can result in unbiased quantitative data sets, and thus could be a far more adequate approach for investigating the impact of xenoestrogen exposure on gametogenesis kinetics. Most research on effects of xenoestrogens on fish, done under laboratory conditions, has focused primarily on exposure to single chemicals, despite the fact that the aquatic environment receives influxes of different chemicals. This wide range of xenoestrogens in the aquatic environment highlights the importance of improving our understanding of the combinatory effects of the chemicals, at least in model organisms. The existence of interactive effects implies that the estrogenic effect of a mixture may somehow deviate from what would be expected of each single agent acting on its own.

In view of the above, we wanted to study whether and how one environmentally relevant mixture of xenoestrogens as found in the Douro River (Rocha et al., 2011) could disrupt the normal cellular kinetics of both spermatogenesis and oogenesis in fish. As a positive control we exposed fish to 17α-ethinylestradiol (EE2) at a high concentration (100 ng/L) to promote the appearance of more marked effects that could serve as reference. Another study with the same experimental design, and testing both the same MIX and EE2, disclosed changes in sex steroidogenesis at the molecular level and a different behavior of the MIX and EE2 on specific transcription factors in the zebrafish brain (Urbatzka et al., 2012). Herein, we specifically tested the hypothesis that such exposures could evoke volume changes in different germ cells, detectable only with stereological tools. The zebrafish (*Danio rerio*, Hamilton) was chosen because it has been recommended for assessing effects of toxicants on development and reproduction (Andersen et al., 2000; Segner et al., 2003).

2. Material and methods

2.1. Test compounds and their nominal and measured concentrations

Stock solutions of EE2 (Sigma–Aldrich) and the Douro's environmental mixture (MIX) were prepared in ethanol at the beginning of the exposure. The xenoestrogens that composed the mixture and their chosen nominal concentrations were based on what was found by chemical screening in the Douro River estuary (Rocha et al., 2011) and are shown on Table 1. This also provides the measured concentrations in the aquaria (analytical details below). Measured concentrations were similar to the nominal ones, and variations were minimal. The estrogenic potency of the MIX was estimated to be 20.73 ng/L EE2-equivalents (Urbatzka et al., 2012). As the focus herein was the MIX, the estrogenic potency of each chemical was not evaluated. Also, it is well known that they differ in potencies (Laws et al., 2000; Gutendorf and Westendorf, 2001; Preuss et al., 2006).

During the trials, the stock solutions were diluted, daily, in water, and used for renewal of the test solutions in the aquaria. Ethanol (*p.a.* grade) was used as vehicle, and its final concentration in the aquaria, in both treatment and controls, was ≈0.001%. This was the minimum amount necessary to dissolve the chemicals of the mixture. The nominal used concentration was 10 times lower than the maximum solvent concentration recommended by OECD (100 μl/L). Additionally, the concentration used was half the conservative safe-guard limit of 20 μl/L proposed by Hutchinson et al. (2006), when reviewing acute and chronic effects of carrier solvents in aquatic animals.

Table 1
Xenoestrogens in the Douro's environmental mixture (MIX) used in the exposure assay. Measured concentration is a pooled value (±standard deviation) between times 0 h (just after water renewal) and 24 h (just before the next water renewal).

Xenoestrogen	Nominal Concentration (ng/L)	Measured Concentration (ng/L)
Estrone (E1)	2.8	2.8 ± 0.0
17β-Estradiol (E2)	20.0	20.0 ± 0.0
17α-Ethinylestradiol (EE2)	4.3	4.2 ± 0.2
4-t-Octylphenol (4-t-OP)	17.9	16.9 ± 1.5
4-n-Octylphenol (4-n-OP)	6.0	6.0 ± 0.0
Bisphenol A (BPA)	25.7	25.7 ± 0.0
4-Octylphenol monoethoxylate (OP1EO)	10.5	10,0 ± 0.7
4-Octylphenol diethoxylate (OP2EO)	16.0	16.0 ± 0.0
4-Nonylphenol monoethoxylate (NP1EO)	21.9	21.9 ± 0.0
4-Nonylphenol diethoxylate (NP2EO)	1608.0	1608.0 ± 0.0
4-n-Nonylphenol (4-n-NP)	3.5	3.1 ± 0.6

Water samples (1 L) were weekly collected after renewing the test solutions and just before the next renewal from each of the experimental aquaria (see below Section 2.3). These samples were then added with internal deuterated surrogates (E2-d_2 and BPA-d_{16}) and submitted to solid phase extraction (SPE) (Rocha et al., 2010). Briefly, the water samples were loaded onto SPE Oasis HLB™ (hydrophilic–lipophilic balance) cartridges at a constant flow rate of 5 mL/min followed by a washing step of 10 mL of ultrapure water and methanol (9:1). Cartridges were dried under vacuum for 60 min and then eluted with 10 mL of ethyl acetate, at 1 mL/min. The resulting extracts were evaporated to dryness in a heating block at 40 °C under a gentle stream of nitrogen and reconstituted in 500 μL of anhydrous methanol. Due to the low volatility of the majority of the present compounds, 50 μL of each extracted fraction were transferred into GC vials and evaporated to dryness under a gentle nitrogen stream. Fifty μL of pyridine were added to the dry residues which were derivatized by the addition of 50 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) added with 1% trimethylchlorosilane (1% TMCS). The vials were mixed using a vortex system and heated, in a heating block, for 30 min at 65 °C. Then each sample was analyzed in a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation) coupled with an ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC–MS^n), an autosampler (Thermo Scientific TriPlus™) and a TR5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium carrier gas (99.999% purity) was maintained at a constant flow rate of 1.0 mL/min and the column oven temperatures were programmed according to Rocha et al. (2010). Finally, the quantitative analysis was made in a selected ion monitoring mode (SIM) using external calibration. After renewing the test solutions the measured concentrations matched the nominal ones. Immediately before the next renewal the actual concentrations averaged 95% of the nominal ones, with no detectable variations in most compounds (Table 1).

2.2. Experimental animals

Adult zebrafish were obtained from a local specialized dealer. Fish were received at the Interdisciplinary Centre for Marine and Environmental Research (Porto) bioterium and were kept for 4 weeks, separated by sex, in a 600 L tank with dechlorinated water.