Ecotoxicology and Environmental Safety 124 (2016) 82–90

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of waterborne exposure to 17β-estradiol and 4-*tert*-octylphenol on early life stages of the South American cichlid fish *Cichlasoma dimerus*

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form
24 September 2015
Accepted 5 October 2015

Keywords:
Teleosts
Environmental estrogens
Gonad development
Sex differentiation
Testicular oocytes
Acute toxicity

ABSTRACT

Estrogenic chemicals are often detected in the aquatic environment and can negatively affect animal development and reproduction. In teleost fishes, the hormonal regulation during a critical period of larval development has a strong influence on gonadal sex differentiation; thus this process may be affected by the exposure to environmental estrogens. In this study, we first assessed the lethal acute toxicity of the natural estrogen 17β-estradiol (E_2) and the weaker estrogen mimics 4-*tert*-octylphenol (OP) and 4-nonylphenol (NP) on larval stages of the South American cichlid fish *Cichlasoma dimerus*. In a further experiment, we analyzed the effects of chronic waterborne exposure to E_2 and OP on gonad development and sex differentiation. Exposure to high concentrations of E_2 had a pronounced feminizing effect directing sex differentiation towards ovarian development, while testis development was inhibited at a lower, environmentally relevant concentration. Among OP-exposed fish, 15–38.5% of the males exhibited testicular oocytes (TOs), a commonly reported biomarker of estrogenic exposure. However, since TOs were also recorded in control males and the proportion of males with TOs was not significantly higher in OP treatments, their occurrence could not be attributed to OP exposure. In addition, TOs did not seem to impair male gonad development and functionality since normal spermatogenesis was observed in testes of OP-treated fish. These results indicate that E_2 occurring in the South American aquatic environment may affect male reproductive development and pose a risk for wild *C. dimerus*, especially under prolonged exposure, while the effects of weaker xenoestrogens such as OP would be negligible for gonad development in this species. As illustrated by this study, the natural occurrence of TOs indicates that conclusions concerning the causes of this phenomenon must be drawn with care.

1. Introduction

Chemicals capable of affecting the endocrine function of aquatic organisms are often detected in waters that receive effluents from municipal and industrial wastewater treatment plants or untreated wastewaters. One class of such endocrine disruptors is represented by estrogenic chemicals such as the natural estrogen 17β-estradiol (E_2), the synthetic estrogen 17α-ethynylestradiol (EE_2) used in birth-control pills, or weaker estrogen mimics such as nonylphenol (NP) and octylphenol (OP) (see White et al., 1994; Desbrow et al., 1998; Giesy and Snyder, 1998; Sumpter and Jobling, 2013). Like EE_2, E_2 reaches the aquatic environment via human excretion or through improper disposal of unused pharmaceuticals containing it as active ingredient. Also effluents from feedlots are a significant source of E_2 input into the aquatic systems in rural areas with intensive cattle breeding (Ying et al., 2002b; Soto et al., 2004). Alkylphenol ethoxylates (APEs) are nonionic surfactants used as detergents, emulsifiers, wetting and dispersing agents in products for agricultural, industrial, commercial and domestic applications. Although the use of APEs has been restricted in the European Union (Soares et al., 2008; Sumpter and Jobling, 2013), they are still widely employed in other regions, including South America, where no action has been taken to reduce or eliminate their usage. As two of the main breakdown products of APEs, both NP and OP are ubiquitous in the aquatic

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http://dx.doi.org/10.1016/j.ecoenv.2015.10.004
0147-6513/

environment. NP is the preponderant alkylphenol (AP), constituting 80% of APs found in surface waters and sediments (Ying et al., 2002c). In comparison, OP accounts for about 15% of the commercial AP input (Bennett and Metcalfe, 1998). Due to its lower usage, OP has been less analyzed than NP in ecotoxicological studies. The reports about the relative estrogenic potency of both APs are inconclusive; while OP probed to be more estrogenic than NP in rainbow trout vitellogenin production and human breast cancer cell growth assays (Jobling and Sumpter, 1993; White et al., 1994), NP presented a higher estrogenicity factor in a recombinant yeast estrogen receptor (ER) assay (Céspedes et al., 2004). In addition, while OP probed to be more potent than NP when competing with E_2 for binding to the trout hepatic ER (White et al., 1994), both APs had a similar affinity for the ER when tested for competitive binding with E_2 to the rat uterine ER (Laws et al., 2000).

Frequently, the concentrations of estrogens recorded in the aquatic environment (ng/L to μg/L range) are above the thresholds for which adverse reproductive effects have been reported for teleost fishes, raising concern about possible negative impacts on wild populations. Laboratory studies have shown that exposure to environmentally relevant concentrations of these chemicals can cause reproductive disorders of various types, including abnormal gonad development, altered secondary sex characteristics, changes in reproductive behavior, altered hormone levels and reduced reproductive success (Jobling et al., 1996; Gray and Metcalfe, 1997; Panter et al., 1998; Gray et al., 1999a; Kinnberg et al., 2000; Harris et al., 2001; Knörr and Braunbeck, 2002; Kang et al., 2002, 2003; Seki et al., 2002, 2003; Balch et al., 2004; Nash et al., 2004; Fenske et al., 2005; Balch and Metcalfe, 2006; Salierno and Kane, 2009; Guyón et al., 2012; Maltais and Roy, 2014; Roggio et al., 2014). Field assessments have demonstrated that estrogenic exposure can lead to reduction of the reproductive performance (Harris et al., 2011) and even to collapse (Kidd et al., 2007) of wild fish populations. In South America, records on environmental levels of estrogenic chemicals are scarce. However, the generally defective treatment of effluents and the lack of control of effluent discharges suggest that the concentration of these compounds in the aquatic systems might exceed the levels causing endocrine disruption in fishes. In addition, although the reproductive effects of environmental estrogen exposure have been analyzed in a number of teleosts, little is known about the sensitivity of native South American species to this class of pollutants.

The process of sex differentiation in teleosts is highly dependent on the hormonal regulation during a critical period of larval development. The administration of exogenous sex steroids can strongly influence the course of sex differentiation, suggesting that they play a critical role in this process (Devlin and Nagahama, 2002). The lability of sex-determination systems in this group of fishes makes them particularly sensitive to the influence of environmental estrogens. Few studies have addressed the effects of waterborne exposure to these chemicals on gonadal morphology and histology during the period of sex differentiation. A commonly reported sign of feminization is the presence of previtellogenic oocytes within the testicular tissue of exposed males. However, the natural occurrence of testicular oocytes is largely unknown for most fish species, and therefore conclusions concerning the causes of this phenomenon must be drawn with care. This concern is especially valid regarding the ambisexual nature of teleosts that results in a sexual plasticity that is not yet completely understood (Hecker et al., 2006).

The acará, *Cichlasoma dimerus*, is a neotropical cichlid quite common in shallow waters of the Paraguay river and most of the Paraná river basins (Kullander, 1983). This species is representative of the teleost fish fauna in the La Plata River Basin and results relevant to the riverine ecosystems of Argentina. It adapts easily to laboratory conditions and displays an elaborate social and reproductive behavior, which includes biparental breeding activities (Pandolfi et al., 2009). In addition, *C. dimerus* has been extensively used in our laboratory for ecotoxicological research (Moncaut et al., 2003; Rey Vázquez et al., 2009; Da Cuña et al., 2011, 2013; Genovese et al., 2011, 2012, 2014; Piazza et al., 2011; Rey Vázquez and Lo Nostro, 2014), and it is considered an appropriate native species for xenobiotic toxicity assays by the Argentinean Institute of Standardization and Certification (IRAM 29112, 2008). In a previous study, we showed that sublethal OP exposure induced vitellogenin synthesis and disrupted testis morphology in adult *C. dimerus* (Rey Vázquez et al., 2009). The aims of this study were to assess the lethal acute toxicity of E_2, OP and NP on larval stages of *C. dimerus* and to evaluate the effects of sublethal concentrations of E_2 and OP on gonad development and sex differentiation. As far as we are aware, this is the first study reporting the effects of waterborne exposure to environmental estrogens on early life stages of a South American freshwater fish.

2. Materials and methods

2.1. Animals

Adult specimens of *C. dimerus* were captured by local fishermen from the natural environment in Esteros del Riachuelo, Corrientes, Argentina (27° 25′ S, 58° 15′ W), an area with minimal human influence. Fish were held in 100 L aquaria where they were allowed to acclimate to laboratory conditions for two months prior to the onset of experimentation. Pairs formed in community tanks were isolated in 45 L aquaria and maintained at 26 ± 2 °C and a 12:12 h photoperiod. Aquaria were well aerated and provided with a layer of gravel and smooth stones for egg deposition on the bottom. Fish were fed once a day with pelleted commercial food (Tetra food sticks). Larvae used in the experiments were obtained from natural spawns of 6 pairs. On the 10th day posthatch (PH), each lot of offspring was isolated in a bare 20 L aquarium and reared until they reached the larval stage used in each experiment. Fry were initially fed with freshly hatched nauplii of *Artemia sp.* and later fed finely ground, dried flake food. Guidelines on the care and use of fish in research and testing from the Canadian Council on Animal Care (2005) were followed.

2.2. Tests conditions and chemicals

Experiments were performed at 26 ± 1 °C and a 12:12 h photoperiod, using filtered tap water (pH 7.8, conductivity 250 μS/cm, total alkalinity 44.1 mg/L, O_2 8 mg/L). The test substances, 17β-estradiol (E_2), 4-*tert*-octylphenol (OP) and 4-nonylphenol (NP) (> 97% pure) were obtained from Sigma (St. Louis, MO). Stock solutions of each chemical were prepared every week by dissolving them in 100% ethanol and stored in the darkness at 4 °C. During each water renewal, the necessary volume of stock solution was added to the aquarium water in order to achieve the desired final concentrations (solvent = 0.01% *per* treatment).

2.3. Lethal acute toxicity

Since NP is the preponderant alkylphenol in the aquatic environment (Ying et al., 2002c), the lethal toxicity of this compound was assessed in addition to that of OP and E_2. Toxicity of the three substances was assessed in larvae at 10, 18 and 26 days PH. For each larval stage, a 96 h exposure was conducted under semi-static conditions with daily renewal of water and test chemical solutions. Exposure concentrations were determined by a preliminary range-finding test for each chemical. In the definitive

toxicity tests, larvae were exposed to nominal concentrations ranging 250–2000 μg/L E_2, 100–500 μg/L OP and 50–400 μg/L NP. Treatments were performed in 500 mL glass beakers in which larvae were allowed to acclimate for 48 h before the initiation of the experiment. Each concentration was tested by triplicate with seven individuals *per* test group. The solvent control treatment contained ethanol 0.01%. Mortality was recorded daily and dead larvae were removed upon detection. In order to avoid mortality due to cannibalism, larvae were fed nauplii of *Artemia sp.* 60 min before each solution renewal. Data were discarded and the test was repeated when mortality in the control group was higher than 10%.

2.4. Effects on gonadal development and sex differentiation

Larvae obtained from two simultaneous spawns were pooled and randomly transferred to bare 4 L aquaria, where they were allowed to acclimate to tests conditions for 48 h before the experiment was started. Afterwards, larvae were exposed to 0.1, 1 and 10 μg/L E_2 and 1, 10 and 100 μg/L OP from day 24 to day 115 PH, covering the period of gonadal sex differentiation (Meijide et al., 2005). The nominal concentrations of the test substances included environmentally realistic levels of estrogens and alkylphenols for the Argentinean aquatic environment, where concentrations $>$ 100 ng/L E_2 and $>$ 10 μg/L NP (toxic equivalents) were recorded in streams with low dilution capacity (see Babay et al., 2014; Valdés et al., 2015). Treatments including control (water) and solvent control (ethanol 0.01%) groups were done by triplicates with 9 individuals *per* test group. Exposure was conducted under semi-static conditions, water and the test chemical solutions being renewed three times a week. Aquaria were provided with artificial aeration. Fish were fed daily, initially with nauplii of *Artemia sp.* and later with dried flake food. Mortality was recorded daily and dead fish were removed upon detection. At the end of the experiment, fish were sacrificed by immersion in a concentrated benzocaine solution. The standard length of each specimen was measured with a caliper to the nearest mm. Afterwards, the body cavity was dissected and the sex was recorded by gross inspection of the gonads under a stereomicroscope. For histological analysis, gonads were fixed in Bouin's solution for 24 h at room temperature and then preserved in 70% ethanol. Samples were gradually dehydrated in an ethanol series, embedded in glycol methacrylate (Leica Historesin) and transversally sectioned at 5 μm (Leica RM 2155 rotary microtome, Germany). Sections taken from the anterior, mid and posterior region of the gonads were stained with Toluidine blue. The slides were examined and photographed with a Nikon Microphot FX microscope coupled with a Coolpix 5400 digital camera (Nikon, Japan). Gonad anomalies resulting from chemical exposures were described in accordance with Hecker et al. (2006).

2.5. Measurement of actual E_2 and OP concentrations

In order to evaluate the decrease of test chemicals in the aquarium water, actual concentrations of E_2 and OP were measured by reverse-phase HPLC coupled to fluorescence detection following the approaches by Ahel et al. (1985), Ying et al. (2002a) and Yoon et al. (2003). The column employed was a PRP-1 of 250×4.1 mm^2, 10 μm particle size and 100 Å pore diameter (Hamilton, USA), and elution was performed with a 80:20 methanol/water mixture, at a flow rate of 0.8 mL/min. Detector was set at excitation and emission wavelengths of 280 and 310 nm for E_2, and 230 and 300 nm for OP. Duplicate water samples were taken from the 10 μg/L E_2, 10 μg/L OP and solvent control aquaria upon addition of the chemicals (time 0), after three hours, and then every 24 h during the last week of the experiment, and treated by solid phase extraction (SPE) on C18 with methanol elution before injection in the HPLC. Data were acquired and analyzed with the Konikrom 5.2 software (Konik Instruments, Spain). For quantifications, calibration curves were constructed for peak areas, from injection of standard solutions daily prepared by adding known amounts of E_2 and OP to control water and processed in the same manner as the samples. For each set of replicate samples, mean and standard deviations were calculated after interpolation of E_2/OP chromatographic peak area in the calibration curve ($R^2 = 0.99$).

2.6. Statistical analysis

96 h lethal concentrations (LC_{10} and LC_{50}) of the test chemicals were statistically estimated for each larval stage by using the USEPA Probit program (USEPA, 1988). Comparisons of the LC_{10} and LC_{50} values were made considering the difference statistically significant when the higher LC/lower LC ratio exceeded the critical value (95% confidence interval) established by APHA, AWWA, WEF (2005). One way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons, was used to establish differences between treatments in the standard length of fish at the end of the chronic experiment. Differences in mortality, sex ratio and the proportion of males exhibiting testicular oocytes at each treatment were assessed by means of the Fisher's exact test (Statistica 7.0, StatSoft, Inc., 2004). The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. Lethal acute toxicity

The results of the acute toxicity tests for NP, OP and E_2 on larvae of *C. dimerus* aged 10, 18 and 26 days posthatch (PH) are presented in Table 1. Assessment of the lethal toxicity by means of LC_{50}

Table 1
96 h lethal concentrations (LC_{10}, LC_{50}) of NP, OP and E_2 (μg/L) for larval stages of *C. dimerus* at three ages posthatch (PH).

	Age of larvae (days PH)	LC (Confidence interval) (μg/L)		
		NP	OP	E_2
LC_{10}	10	139.7 (122.0–150.1)^{a}	177.8 (158.0–190.9)^{bc}	495.3 (375.3–565.2)^{e}
	18	149.2 (117.2–165.3)^{ab}	197.6 (152.1–220.9)^{bc}	388.8 (257.7–497.0)^{de}
	26	158.2 (96.8–185.6)^{ab}	283.7 (240.8–308.7)^{d}	559.5 (400.5–674.3)^{e}
LC_{50}	10	164.9 (154.6–174.8)^{a}	219.3 (207.1–231.0)^{c}	709.4 (636.0–806.8)^{e}
	18	181.3 (162.9–195.3)^{ab}	251.3 (226.7–271.3)^{c}	772.6 (632.1–919.8)^{e}
	26	209.1 (172.9–234.3)^{bc}	345.2 (319.7–369.6)^{d}	867.0 (732.3–995.3)^{e}

For each parameter, different letters denote significant differences ($p < 0.05$).

values revealed that NP resulted slightly more toxic than OP at all larval stages, while both APs showed higher toxicity than E_2, rendering an order of decreasing toxicity NP > OP > E_2. In addition, younger larvae showed higher susceptibility to NP and OP, while there was no significant difference in the toxicity of E_2 for the three larval stages assessed. A similar pattern of relative toxicity between chemicals and relative sensitivity of larval stages was observed when using the LC_{10} estimates.

3.2. Effects on gonadal development and sex differentiation

Similar values of all the analyzed parameters were observed in replicates of the same treatment and are then presented jointly. 2 of 27 fish (7.4%) died in the control and solvent control groups during the exposure period. Mortality in the E_2 and OP treatment groups ranged from 1 (3.7%) to 4 (14.8%) and was not significantly different from that of the control groups. Consequently, the mortality observed was assumed not to be related to chemical exposure. The standard length of fish at the end of the experiment ranged from 15.7 ± 1.2 mm (control group) to 16.6 ± 1.6 mm (0.1 μg/L E_2 group) and did not vary significantly between treatments. Fish from all treatments exhibited no abnormalities, neither in their swimming behavior nor in their external morphology.

Sex ratios in the OP treatments did not differ significantly from those of the control groups, nor from the expected 50:50 proportion (Fig. 1). Upon dissection, gonads of OP-treated fish appeared as developed as those of control fish (not shown). Histologically, testes of control males showed a normal lobular architecture and contained spermatogonia as well as cysts with germ cells at all stages of spermatogenesis, including spermatozoa (Fig. 3A). Testes of OP-exposed males displayed a similar progression of spermatogenesis to those of control males. Up to 38.5% of OP-treated males (5 out of 13 males from the 10 μg/L group, Fig. 1) exhibited oocyte-like cells in their testes, interspersed in an otherwise normal looking testicular tissue. These testicular oocytes (TOs) had a similar appearance to oocytes at the perinucleolar step of primary growth present in ovaries of control females and were characterized by having a basophilic cytoplasm and peripheral nucleoli within the nucleoplasm (Fig. 3C and D). TOs were also detected in 2 out of 24 males from the control treatments (Figs. 1 and 3B). The percentage of males exhibiting

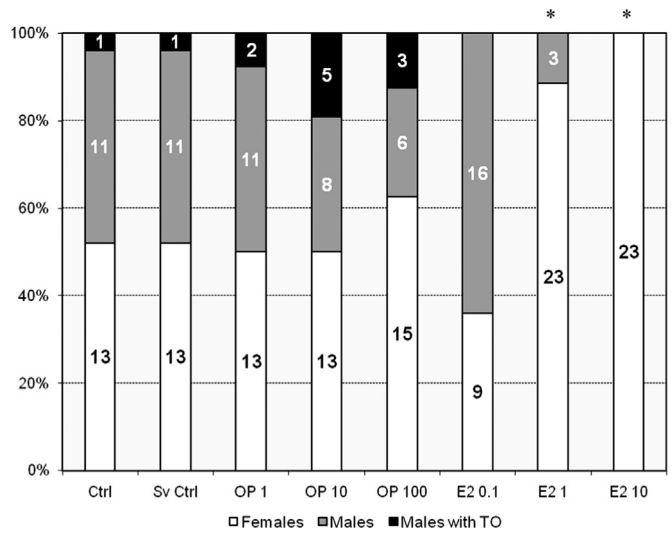

Fig. 1. Percentages of females, males and males with testicular oocytes (TO) recorded in each treatment at the end of the chronic-exposure experiment. The number of fish is indicated on the bars. Differences in the number of males with TOs were not statistically significant. The proportion of females was significantly higher in the 1 and 10 μg/L E_2 treatments with respect to control groups ($^*p < 0.05$).

TOs did not differ significantly between the different OP concentrations tested, nor from the control groups (Fig. 1). The number of TOs *per* testis section was usually one or two (Fig. 3C), but occasionally rose to up to nine *per* section (Fig. 3D).

Exposure to 1 and 10 μg/L E_2 had a marked feminizing effect with 88.5% and 100% of fish differentiating as females at each treatment, respectively (Fig. 1). In the 0.1 μg/L E_2 treatment, the sex ratio was not significantly different from that of the control groups (Fig. 1). Males from the 0.1 and 1 μg/L E_2 treatments exhibited underdeveloped testes as compared to those of control males (Fig. 2A and B). Histologically, a delay of spermatogenesis was evidenced in E_2-treated males, as their testes exhibited just spermatogonia and few spermatocytes that had already entered meiosis (Fig. 3E). These features were characteristic of recently differentiated testes and contrasted to the more advanced development of control testes in which all stages of spermatogenesis, including spermatozoa, were observed (Fig. 3A).

Ovaries of OP and E_2-treated females did not differ neither in size nor histologically from ovaries of control females (Figs. 2C, D and 3F, G). They presented numerous follicles containing oocytes at the perinucleolar step of primary growth, as well as early meiotic oocytes and oogonia (Fig. 3F and G).

3.3. Concentration of E_2 and OP in the water

The measured concentrations of E_2 and OP in replicate water samples taken at 0 h, 3 h and every 24 h from the 10 μg/L treatment group are indicated in Table 2. The initial nominal and actual levels of both chemicals were in good agreement. A marked decline of the actual concentration was observed over the period of solution renewal, measured levels of E_2 and OP at 72 h decreasing to approximately 25% of the initial concentration. These chemicals were not detected in samples from the solvent control treatment.

4. Discussion

4.1. Occurrence of estrogens in the aquatic environment

Even though research on environmental estrogens has been conducted during the latest 20 years, the issue of estrogens in the aquatic environment is still a matter of debate (Sumpter and Jobling, 2013), and assessment of their effects on teleost fishes is still relevant, especially regarding endangered species (Maltais and Roy, 2014) or native species inhabiting poorly investigated regions such as South America. The concentrations of E_2 and OP used in our chronic-exposure experiment span from environmentally realistic concentrations to high, sublethal concentrations. E_2 has been generally reported in the low ng/L, although concentrations ranging up to 27 ng/L and 160 ng/L have been measured in Japan and US waterbodies, respectively (see Ying et al., 2002b; Kolpin et al., 2002). APs are generally found in concentrations < 10 μg/L (see Blackburn and Waldock, 1995; Bennie, 1999; Soares et al., 2008) but levels of up to 53 μg/L and 644 μg/L have been reported in rivers from UK and Spain, respectively (see Ying et al., 2002c). For South America, information about the occurrence and concentrations of estrogens in the aquatic environment is very limited. In Brazil, concentrations of up to 63 ng/L E_2 and EE_2, and 2 μg/L NP were reported for surface waters (Kuster et al., 2009; Moreira et al., 2009, 2011). In Argentina, Babay et al. (2014) determined concentrations of NP-toxic equivalents of up to 12.2 μg/L in surface waters of streams running across densely urbanized and industrialized zones. More recently, E_2 and EE_2 were detected at concentrations of 369 and 43 ng/L, respectively, in streams receiving municipal sewage effluents (Valdés et al., 2015). Both studies indicated a potential ecotoxicological risk of the reported

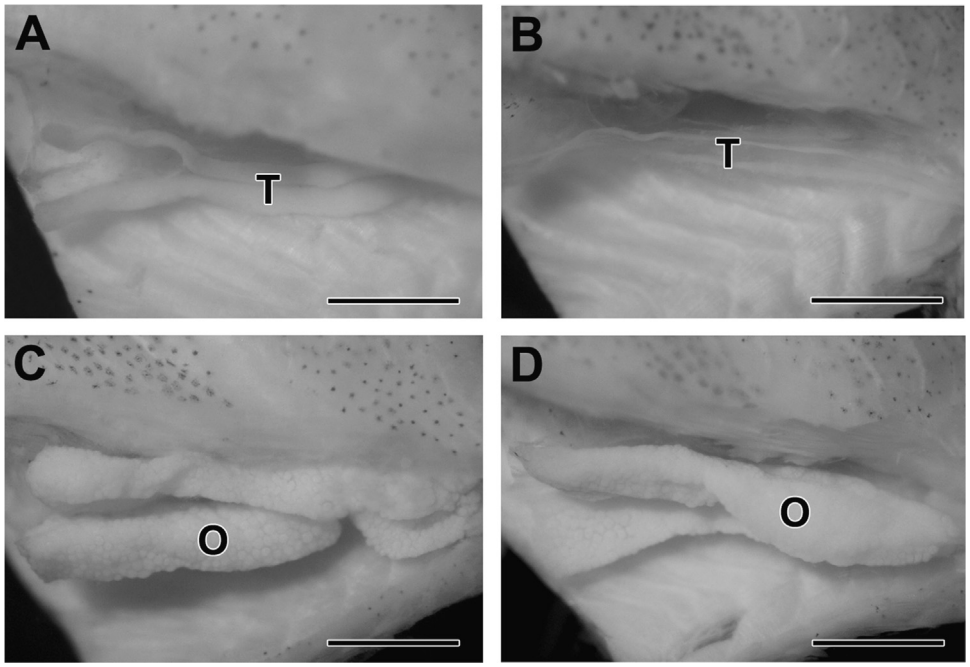

Fig. 2. Gonads of juvenile *C. dimerus* as observed upon fixation at the end of the chronic-exposure experiment. Stereomicroscope photographs. A, C: solvent control; B, D: 0.1 μg/L E_2. Testes of E_2-treated males (B) were underdeveloped as compared to those of control males (A). Ovaries of control (C) and E_2-treated (D) females exhibited a similar degree of development, oocytes becoming visible within them. O, ovaries; T, testes. Scale bars = 1 mm.

estrogens to the local aquatic biota.

4.2. Acute toxicity

Before this study, data on the acute toxicity of environmental estrogens on fish species native to South America were non-existent. Here, we determined the lethal toxicity of E_2, OP and NP in larval stages since they usually represent the most sensitive period of development (Mohammed, 2013). According to USEPA ecotoxicity categories for aquatic organisms, these chemicals resulted highly toxic (100 μg/L < LC_{50} < 1000 μg/L) for *C. dimerus* larvae. NP resulted the most toxic compound, with an LC_{50} slightly but significantly lower than that of OP in the three larval stages assessed (see Table 1). In addition, the susceptibility to APs declined as larval development progressed, probably due to an increased ability to metabolize the compounds and operate detoxification mechanisms at later stages of development. The 96 h CL_{50} estimates of NP and OP for *C. dimerus* larvae are comparable to those reported for other teleosts at different life stages, with ranges of 128–310 μg/L for NP and 280–495 μg/L for OP (see McLeese et al., 1981; Ferrara et al., 2001; Toft and Baatrup, 2003; Brooke et al., 2005). The data available indicate that OP is less toxic than NP, as observed in our study. In contrast to NP and OP, the E_2 LC_{50} did not differ between larval stages, i.e. E_2 was similarly toxic for all of them. The LC_{50} of E_2 for *C. dimerus* larvae are about half of the value reported for EE_2 in adult zebrafish, *Danio rerio* (1.7 mg/L, Versonnen et al., 2003). Regulatory thresholds are often applied for effects characterization based on the LC_{50} for compounds. However, the LC_{50} might not be fully representative of the entire concentration–response curve, and a lower effects benchmark, as the LC_{10}, might be a more appropriate safety factor for a level of concern due to the slope of the toxicity curve. In addition, the use of LC_{10}s has been advocated for deriving protective concentrations in lieu of no-effect concentrations (NOECs) estimated using hypothesis-testing techniques, which are usually driven by experimental design issues (Carriger et al., 2011). In our study, the closeness of the LC_{50} and LC_{10} values of the test chemicals are indicative of a steep slope of the concentration–effect curves. In addition, both LC_{50} and LC_{10} estimates are above any of the levels likely to be recorded in the surface waters. Then, the presence of these chemicals in the aquatic environment would not represent a threat in terms of direct effects on fish survival. However, the sublethal effects produced by environmental estrogens, including endocrine disruption and impairment of sexual development, might be detrimental for wild fish populations.

4.3. Effects on gonadal development and sex differentiation

With the exception of a previous study on adult *C. dimerus* (Rey Vázquez et al., 2009) and the studies by Guyón et al. (2012) and Roggio et al. (2012, 2014) on adult males of the one-sided livebearing fish, *Jenynsia multidentata*, there is no other research on the reproductive effects of waterborne exposure to estrogenic chemicals on South American fishes. Teleost gonads are extremely sensitive to environmental factors, including xenobiotics, around the time of sex differentiation. Endocrine disruptors such as estrogens and estrogen mimics can act during critical periods inducing temporary or permanent morphological changes. The results of various studies performed in teleost species suggest that E_2 synthesized in female gonads mediates female sexual differentiation by stimulating development of undifferentiated XX gonads through ERs. Expression of ERs in XY gonads early during differentiation may explain the susceptibility of males to feminization by exogenous E_2 (Paul-Prasanth et al., 2011). In this study, chronic exposure to sublethal concentrations of E_2 during early development of *C. dimerus* resulted in an impairment of gonadogenesis and sex differentiation. In this species, the usual 1:1 sex ratio recorded in progenies under natural breeding conditions indicates that the sex is genetically determined (Meijide, unpublished results). Exposure to 1 and 10 μg/L E_2 was capable of overriding genetic sex determination, most or all the individuals differentiating as phenotypic females. In contrast, exposure to 0.1 μg/L, an environmentally relevant concentration of E_2, did not result in a female-skewed sex ratio; rather it had an inhibitory effect on male gonadogenesis. In *C. dimerus*, gonad development is dependent on somatic growth (Meijide et al., 2005), so that larger

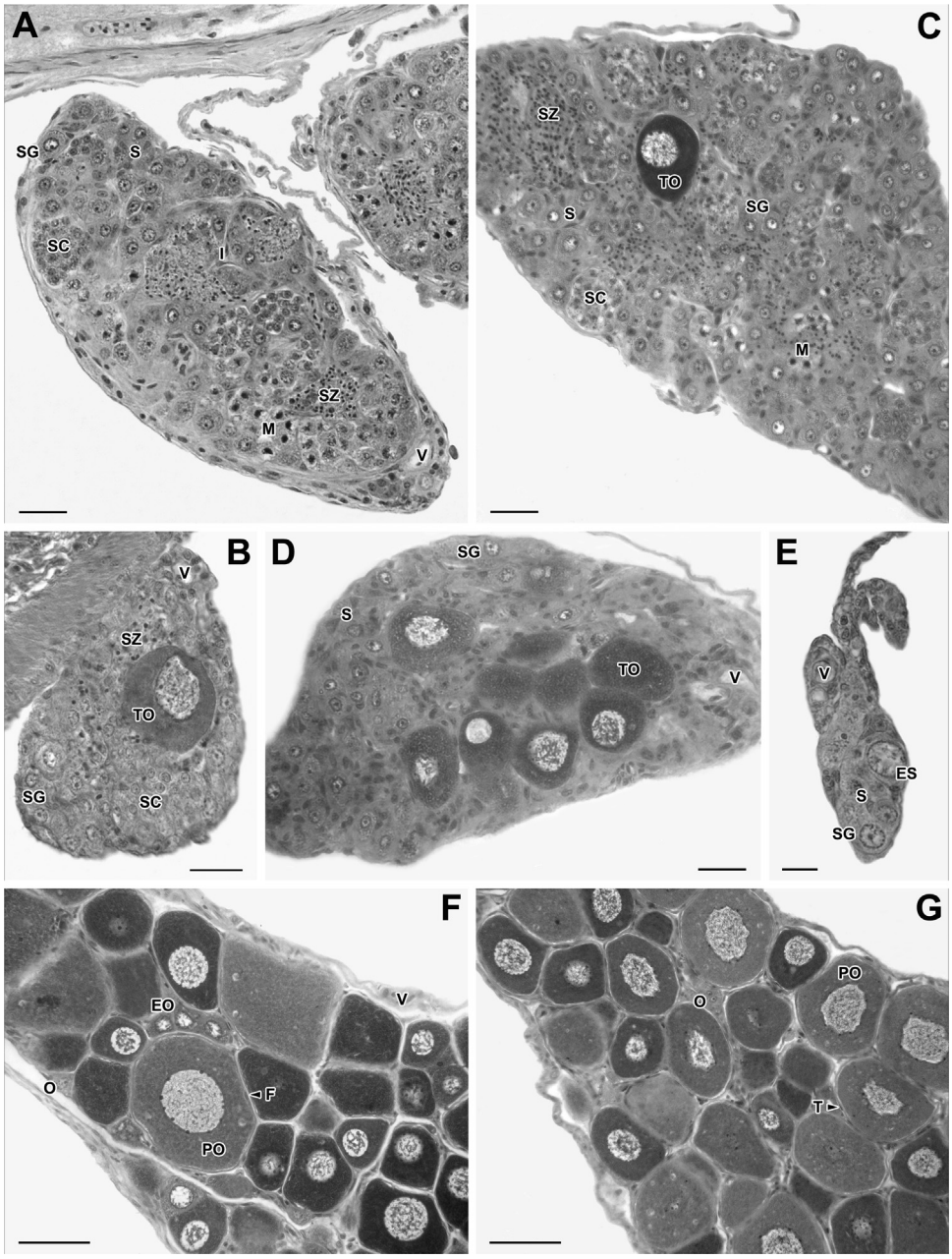

Fig. 3. Cross sections of gonads from juvenile *C. dimerus* processed at the end of the chronic-exposure experiment. Light-microscope photographs. (A) Testis of a male from the solvent control treatment; (B) Testis of a control male showing a testicular oocyte (TO); (C) Testis of a male exposed to 10 μg/L OP showing a single TO; (D) Testis of a male exposed to 100 μg/L OP showing multiple TOs; (E) Underdeveloped testis of a male exposed to 0.1 μg/L E_2; (F) Ovary of a control female; (G) Ovary of a female exposed to 10 μg/L OP. Spermatogenesis seemed not to be impaired by the presence of TOs (B–D). Delayed spermatogenesis was evidenced in E_2-treated males (E). EO, early meiotic oocyte; ES, early meiotic spermatocyte; F, follicle cell; I, interstitial tissue; M, mitotic figures; O, oogonia; PO, perinucleolar oocyte; S, Sertoli cell; SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa; T, thecal cell; TO, testicular oocyte; V, blood vessel. Scale bars = 10 μm (E); 20 μm (A–D); 50 μm (F, G).

male fish exhibit more developed testes and more advanced spermatogenesis. Males from the 0.1 μg/L E_2 treatment presented smaller testes than control males, even though their body sizes were similar. Histologically, testes of E_2-exposed males contained only spermatogonia and early meiotic spermatocytes, which contrasted with the more advanced stages of spermatogenesis observed in control males. This finding indicates that the reproductive potential of the males might be disturbed by long-term exposure to E_2, raising concern about possible population-level impacts for wild fishes. Coincidently with our results, an arrest of male gonad development was observed in zebrafish exposed to EE_2 during full life cycle (Nash et al., 2004; Fenske et al., 2005). Inhibited development of testicular tissue and delayed spermatogenesis were also reported in male fathead minnow, *Pimephales promelas*, and pearl dace, *Margariscus margarita*, exposed to EE_2 in lake experiments (Palace et al., 2002, 2006). The inhibitory effect of exogenous E_2 or EE_2 may be explained by a negative feedback action at the hypothalamus-pituitary-gonadal axis resulting in reduced levels of androgens, which are known to promote spermatogenesis. This is supported by the findings that EE_2 exposure reduced T and 11 KT in fish plasma (Salierno and Kane, 2009; Flores-Valverde et al., 2010; Maltais and Roy, 2014). In medaka, *Oryzias latipes*, exposed to E_2 from 1 day to 100 days posthatch under a static-renewal design comparable to that of our study, 98–100% feminization was reported at 1 μg/L (Metcalfe et al., 2001; Balch and Metcalfe, 2006), coincidently with our

Table 2
Actual concentrations of E_2 and OP in the aquarium water during the last week of the chronic experiment. Measurements were made 0, 3, 24, 48 and 72 h after addition of the chemicals. Values are means ± standard deviations of the concentrations recorded in two samples from the 10 μg/L treatment group. % are values for the measured concentrations expressed as percentage of the nominal concentration.

Nominal concentration			10 μg/L	
Time (h)	E_2		OP	
	Actual concentration (μg/L)	%	Actual concentration (μg/L)	%
0	9.74 ± 0.14	97.4 ± 1.4	10.07 ± 0.26	100.7 ± 2.6
3	9.14 ± 0.45	91.4 ± 4.5	9.34 ± 0.60	93.4 ± 6.0
24	5.09 ± 0.55	50.9 ± 5.5	5.85 ± 0.52	58.5 ± 5.2
48	3.90 ± 0.38	39.0 ± 3.8	3.37 ± 0.53	33.7 ± 5.3
72	2.45 ± 0.54	24.5 ± 5.4	2.64 ± 0.57	26.4 ± 5.7

finding of a high proportion of females at this concentration. Differently from our results, treatment with 0.1 μg/L E_2 resulted in a still, though less significantly female-biased sex ratio and the induction of an "intersex condition" in males (Metcalfe et al., 2001), which ranged from the presence of dispersed oocytes (testicular oocytes, TOs) to the occurrence of organized ovarian tissue within the testis (ovotestis) (*sensu* Hecker et al., 2006). However, flow-through exposure to 0.1 μg/L E_2 during the entire life cycle in medaka and the guppy, *Poecilia reticulata*, did not have a significant feminizing effect (Knörr and Braunbeck, 2002; Toft and Baatrup, 2003). Together, these results point at 1 μg/L E_2 as a minimum concentration capable of inducing feminization in teleosts.

APs are estrogenic chemicals since like E_2, they can bind to ERs and cause the expression of estrogen-inducible genes (White et al., 1994; Metcalfe et al., 2001). However, the modes of action of xenoestrogens also include ER-independent mechanisms (Gillesby and Zacharewski, 1998). TOs are usually reported as a common sign of estrogenic exposure. Whether the induction of TOs is mediated by an ER-dependent mechanism remains unknown. In *C. dimerus*, exposure to OP during the sex differentiation period did not affect neither sex ratios nor the development of gonads. TOs were evidenced in a small proportion of OP-exposed and control males. Since TOs were recorded in control males and the percentage of males with TOs was not significantly higher in OP treatments, their occurrence cannot be attributed to OP exposure. Given the plasticity in teleost sexual differentiation, which sometimes includes development of the testis from earlier all-female stages or via developmental hermaphroditic stages, residual occurrences of single or low numbers of oocytes appear to be common for some species (Hecker et al., 2006). In fact, the observation of intersex conditions in several fish species in areas with low levels of contamination indicate that a certain degree of intersexuality in gonochoristic fish could be natural (Scholz and Klüver, 2009). For gonad histology to be used as an endpoint in endocrine disruption testing, it is important to know the normal pattern of sex differentiation of the test species. *C. dimerus* is a differentiated gonochorist, in which the undifferentiated gonad develops directly into an ovary or testis (Meijide et al., 2005). Under normal conditions, TOs are detected within the developing testis on rare occasions (Meijide, unpublished observations). As argued by Hecker et al. (2006), it is possible that the occurrence of individual organisms with TOs as observed in studies with relatively small sample sizes is a function of natural variability of this phenomenon rather than a direct chemical effect; thus TOs should be carefully used as an endpoint in judging the estrogenicity of chemicals. In experiments with medaka and zebrafish exposed to similar concentrations of OP or NP for long terms (including the sex differentiation period), the occurrence of TOs in males was attributed to chemical treatments. Variable results were reported in these studies, ranging from no clear concentration-dependent effects and less than 50% of males exhibiting TOs in all treatments (Knörr and Braunbeck, 2002; Hill and Janz, 2003; Seki et al., 2003) to a significant concentration-dependent pattern, with over 80% of males showing TOs at the higher concentration (100 μg/L) (Gray and Metcalfe, 1997; Balch and Metcalfe, 2006). This variability in the responses to AP treatments might be explained by differences in the exposure time windows used in each study and the differential sensitivity of each test species to the action of APs. In addition, while AP exposure at the adult stage induced TOs in male medaka (Gronen et al., 1999; Kang et al., 2003), TOs were not observed in adult males of *C. dimerus* after treatment with OP (Rey Vázquez et al., 2009). In the present study, TOs usually appeared as isolated cells within a normal looking testicular tissue, in which spermatogenesis seemed not to be affected. Then, it can be hypothesized that the presence of TOs would not directly impact the reproductive capability of male *C. dimerus* in case they are still present upon gonad maturation. This is in agreement with the finding that male medaka with TOs had the same ability to fertilize eggs as males without intersex gonads (Balch et al., 2004). Although the presence of TOs has been correlated with poor reproductive success in feral fish populations (Harris et al., 2011), and even linked to population collapse in case of short-lived species (Kidd et al., 2007), these effects cannot be explained by the intersex condition alone. Other factors such as hormonal alterations, impairment of reproductive behavior and altered secondary sex characteristics may be implicated in the reproductive failures reported in wild fishes.

4.4. Considerations about the exposure design

Whenever a static-renewal system is employed to perform chemical exposure experiments, the difference between measured and nominal concentrations tends to be noticeable. Drop of actual concentrations may be explained by glass adsorption, uptake by fish, microbial degradation and photodegradation (Ekelund et al., 1993; Ahel et al., 1994; Lewis and Lech, 1996). In this study, actual E_2 and OP concentrations decreased to 34–39% of the nominal concentrations over a 48 h period and resulted only about a 25% after 72 h. This tendency of decline was determined from measurements done only in the 10 μg/L treatments; however comparable results are expected with the other concentrations employed. A similar trend was recorded in various studies using static-renewal systems, in which measured concentrations of estrogenic chemicals, including E_2, EE_2, OP and NP, were reported to drop to 25–50% of the nominal concentrations over a 48–72 h period (Gray and Metcalfe, 1997; Gray et al., 1999a, 1999b; Kinnberg et al., 2000; Metcalfe et al., 2001; Balch and Metcalfe, 2006). Therefore, it can be argued that the effects registered under these conditions are being produced by exposure to concentrations considerably lower than the nominal values. Semi-static experiments as the one used in our study do not result in a permanent exposure of fish to the chemicals at the nominal concentrations; however they emulate what happens when environmental contamination is caused by pollutants arising mainly as a result of discharges from point sources to surface waters. Exposure by regular pulses results ecologically relevant, since fish are not usually exposed to constant water concentrations of xenobiotics in the environment.

5. Conclusions

The results of our study indicate that long-term exposure to E_2

at concentrations reported in South American water bodies could be capable of impairing sexual development and future reproductive success in male *C. dimerus*, posing a potential risk for wild populations, while environmental levels of the weaker xenoestrogen OP would not represent a major threat for this species. It should be noted that although the concentrations of single estrogenic chemicals may be below the thresholds for which adverse reproductive effects are reported, different compounds may coexist in the aquatic environment and additive responses may induce reproductive impairment in fish chronically exposed to this class of pollutants. Such evidences suggest that further assessment of the effects of environmental estrogens, including exposure to estrogen mixtures and field studies is required for South American teleosts. The natural occurrence of TOs, as shown in this study, indicates that conclusions concerning the causes of this phenomenon must be drawn with care, since TOs may represent a function of natural variability rather than a direct effect of chemical exposure.

Acknowledgments

This study was performed with financial support from the University of Buenos Aires (EX 303) and CONICET (PIP 2302). We deeply appreciate the valuable comments and the support of our mentor Dr. Graciela A. Guerrero.

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