Comparative Biochemistry and Physiology, Part C 150 (2009) 298–306

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

# Exposure to waterborne 4-*tert*-octylphenol induces vitellogenin synthesis and disrupts testis morphology in the South American freshwater fish *Cichlasoma dimerus* (Teleostei, Perciformes)

G. Rey Vázquez [a,*], F.J. Meijide [a,b], R.H. Da Cuña [a,b], F.L. Lo Nostro [a,b], Y.G. Piazza [a,b], P.A. Babay [c], V.L. Trudeau [d], M.C. Maggese [a,b], G.A. Guerrero [a]

[a] Lab. de Embriología Animal, Dpto. de Biodiversidad y Biología Experimental, FCEyN-UBA, Ciudad Universitaria, Pabellón II, 4° piso (C1428EHA), Buenos Aires, Argentina
[b] CONICET, Rivadavia 1917 (C1033AAJ), Buenos Aires, Argentina
[c] Gerencia de Química, Centro Atómico Constituyentes, CNEA. Av. Gral. Paz 1499 (1650), Pcia. de Buenos Aires, Argentina
[d] Centre for Advanced Research in Environmental Genomics, Dept. of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

ARTICLE INFO

*Article history:*
Received 29 January 2009
Received in revised form 15 May 2009
Accepted 19 May 2009
Available online 27 May 2009

*Keywords:*
*Cichlasoma dimerus*
Cichlids
Liver and testis pathology
Octylphenol
Surface mucus
Teleosts
Vitellogenin
Xenoestrogens

ABSTRACT

Exposure to environmental pollutants may disrupt endocrine functions and cause reproductive effects in human and wildlife populations. Various groups of chemicals have estrogen-like effects, including degradation products of alkylphenol polyethoxylates, such as 4-*tert*-octylphenol (OP). Laboratory studies have shown that exposure of male fish to xenoestrogens results in induction of circulating vitellogenin (Vtg), inhibition of testicular growth, testis abnormalities and formation of intersex gonads. In this study, the impact of the exposure to waterborne OP on reproductive aspects in the South American cichlid fish *Cichlasoma dimerus* was evaluated using qualitative changes in the levels of Vtg in plasma and surface mucus and histological alterations in the liver and gonads as endpoints. Adult males and females were exposed to OP via immersion during 60 days in aquaria under semi-static conditions, water changes being made every 84 h. Treatment groups were: control (ethanol 0.005%), OP 30, 150 and 300 μg/L. Using Western and Dot blot analysis, Vtg was detected in plasma and mucus of control and treated females and treated males, while no Vtg was observed in samples from control males. Morphological changes in the hepatocytes due to the accumulation of Vtg were observed in OP-exposed males. Impairment of testicular structure became apparent in males treated with the highest OP concentrations. The most salient pathological change was the alteration of lobular organization with increased testicular fibrosis and progressive disruption of spermatogenesis. No major changes were observed in ovarian architecture. Our results indicate that detection of Vtg in surface mucus may be a sensitive and non-invasive biomarker of the endocrine disrupting effects of environmental estrogens, resulting in a useful method for field monitoring.



## 1. Introduction

A wide range of chemicals introduced into the environment by human activities may be producing adverse health effects in human and wildlife populations. The term endocrine disrupting chemicals (EDCs) is applied to a wide range of compounds that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, that regulate homeostasis, reproduction, development and behavior (Nishi et al., 2002). Among EDCs, various groups of chemicals have estrogen-like effects, being referred to as xenoestrogens. Alkylphenol polyethoxylates (APEs) are one of the classes of nonionic surfactants most widely used in the manufacturing of cleaning agents, plastics, pulp and paper, textiles, agrochemicals, cosmetics and food products (Routledge and Sumpter, 1997; Ying et al., 2002). Primary degradation of APEs in wastewater treatment plants or in the environment generates more persistent shorter-chain APEs and alkylphenols (APs) such as 4-*tert*-octylphenol (OP), one of the most biologically active products. It has been suggested that the levels of APE metabolites present in the aquatic environment may be well above the threshold necessary to induce endocrine disruption in wildlife. These findings have raised public concern over their environmental and human health effects (Ying et al., 2002). Laboratory and field studies have shown that exposure of male fish to alkylphenolic compounds results in induction of circulating vitellogenin (Vtg), inhibition of testicular growth, testis abnormalities and formation of intersex gonads, among other signs of reproductive impairment (White et al., 1994; Sumpter and Jobling, 1995; Jobling et al., 1996; Gray and Metcalfe, 1997; Christiansen et al., 1998; Gronen et al., 1999; Kinnberg et al., 2000; Folmar et al., 2001; Metcalfe et al., 2001; Van den Belt et al., 2001; Knörr and Braunbeck,

* Corresponding author. Tel.: +54 11 45763348; fax: +54 11 45763384.
*E-mail address:* grarey@bg.fcen.uba.ar (G. Rey Vázquez).

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doi:10.1016/j.cbpc.2009.05.012

2002; Kang et al., 2003; Rasmussen and Korsgaard, 2004; Rasmussen et al., 2005).

Vitellogenin is a complex phospholipoglycoprotein normally produced in the liver of mature female fish in response to increasing circulating estrogen levels leading up to spawning (Arukwe et al., 2000). Circulating Vtg is taken up by developing oocytes and is a constituent of yolk which serves as a source of nutrients for the developing embryo. Adult male fish do not usually produce Vtg, but they do possess the hepatic estrogen receptor and the *Vtg* gene, which is normally silent. Therefore, Vtg synthesis in males can be induced by treatment with xenoestrogens (Flouriot et al., 1997; Gronen et al., 1999; Ackerman et al., 2002; Park et al., 2003; Van den Belt et al., 2003; Knoebl et al., 2004; Meucci and Arukwe, 2005; Arukwe and Røe, 2008). Various field investigations have reported Vtg detection in male fish exposed to industrial and domestic effluents (Sumpter and Jobling, 1995; Harries et al., 1997; Nichols et al., 1999; Wahli et al., 1998; Hashimoto et al., 2000; Folmar et al., 2001). These effluents are known to carry a wide range of structurally diverse chemicals with the capability of exerting estrogenic actions.

Detection of Vtg in the surface mucus has been suggested as a mean of determining sex in fish lacking sexual dimorphism or as a screening method of maturity state (Kishida et al., 1992; Kishida and Specker, 1994, 2000; Takemura and Oka, 1998). In addition, it has been proposed that the possibility of detecting Vtg in the surface mucus could result in a useful tool for monitoring the presence of estrogenic pollutants in the environment (Moncaut et al., 2003; Meucci and Arukwe, 2005; Van Veld et al., 2005; Maltais and Roy, 2007; Arukwe and Røe, 2008). On the other hand, the teleost liver is one of the most sensitive organs to show alterations in biochemistry, physiology and structure following exposure to various types of environmental pollutants (Hinton and Couch, 1998; Andersson et al., 2007; Perez Carrera et al., 2007).

The South American cichlid fish *Cichlasoma dimerus*, a perciform teleost, is common in quiet shallow waters of the Paraguay River and most of the Paraná River basins (Kullander, 1983). This species is representative of teleosts in the La Plata River Basin and results relevant to the Argentinean riverine ecosystems. In addition, *C. dimerus* adapts easily to captivity and shows notable reproductive features such as a complex social and breeding behavior, which includes parental care and, most important, a high spawning rate (about every 25 days during 8 months) (Meijide and Guerrero, 2000), providing an amenable model for laboratory studies, including ecotoxicological testing.

Due to the lack of studies dealing with the effects of environmental estrogens in South American perciform freshwater fish, the aim of this work was to evaluate the effects of waterborne exposure to OP at sublethal concentrations in *C. dimerus* using qualitative changes in the levels of Vtg in plasma and surface mucus, and histological alterations in the liver and gonads as endpoints.

## 2. Materials and methods

### 2.1. Fish

The adult specimens of *C. dimerus* used in this study were captured from the natural environment in Esteros del Riachuelo, Corrientes, Argentina (27° 25′ S, 58° 15′ W). They were allowed to acclimate to captivity conditions for a month prior to the start of experimentation. A total of 32 fish (24 males and 8 females) were weighed and total length (TL) measured (females: 27.14 ± 8.95 g and 10.50 ± 1.17 cm; males: 50.15 ± 11.50 g and 12.78 ± 0.98 cm) and kept in 100 L aquaria at 26.5 ± 1 °C, pH 7.3, with 12:12 h photoperiod and an average density of 6.4 g/L. Laboratory aquaria were well aerated and provided with external filtration and a layer of gravel on the bottom. Fish were normally fed once a day with pelleted commercial food (Tetra food sticks).

### 2.2. Treatment and sampling

The test substance, 4-*tert*-octylphenol (OP) (>97% pure) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Concentrations of OP for the exposures were selected from preliminary studies and a 96 h acute toxicity test (LC 50–96 h = 780 µg/L).

Previous to the onset of exposure, three males and one female fish were transferred to each 50 L glass tank under the same physical conditions and alimentary ratio, except that the layer of gravel on the bottom was removed. Animals were allowed to acclimate to the new experimental conditions for a week before the experiment was started. Fish were exposed to nominal concentrations of 0 (solvent control; ethanol 0.005%), 30, 150 and 300 µg/L OP in duplicate aquaria. Exposure to OP was performed during 60 days under semi-static conditions, water changes being made twice a week. Stock solutions were prepared once a week by dissolving OP in 100% ethanol and stored in darkness at 4 °C. During each water renewal, small aliquots of the stock solution were added to filtered tap water in order to obtain the desired concentrations.

At the end of the experiment, mucus and blood samples were carefully and quickly collected after fish were softly narcotized with fish calmer (dose: 6 drops/2 L; active ingredients: acetone, dimethylketone alpha methyl quinoline; Jungle Hypno, USA). Mucus was collected by scraping the body surface with a stainless steel spatula into microcentrifuge tubes containing the same volume of 0.1 M phosphate-buffered saline, pH 7.4 with Tween 20 and 2.5 µL of 1 mM PMSF (phenylmethylsulfonyl fluoride, protease inhibitor). After centrifugation at 2500 *g* for 15 min at 4 °C, the supernatant was separated and stored at −20 °C. Peripheral blood was collected by puncture of the caudal vein with a heparin-coated 25 gauge × 1/2 in. needle, attached to a 1 mL syringe. Samples with the addition of 2.5 µL of 1 mM PMSF were centrifuged at 2500 *g* for 15 min at 4 °C to obtain plasma which was stored at −20 °C. Immediately following, the fish were sacrificed by decapitation under anesthesia and liver and gonads were quickly removed. The samples were fixed for subsequent histological and immunohistochemical examination.

### 2.3. Immunoblot

Plasma and surface mucus samples were analyzed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) using a 5% stacking gel and an 8% resolving gel followed by Western blot. Samples were diluted in sample buffer containing 0.3 M Tris/HCl, pH 6.8, 10% SDS, 12% glycerol, 11% β-mercaptoethanol and 0.2% bromophenol blue. Equal amounts of protein (20 µg), as measured by Lowry et al. (1951), were loaded into each lane. Molecular mass was estimated using pre-stained molecular mass standards (Invitrogen). Following separation by electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia) for 90 min at 4 °C and 100 V in transfer buffer (25 mM Tris, 187 mM glycine, 20% (v/v) methanol). Non-specific binding of membranes was blocked with 5% non-fat powdered milk in TBST (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5) overnight at 4 °C. Membranes were then incubated with the primary antiserum, rabbit anti-perch Vtg (donated by Dr. B. Allner, Hessisches Landesamt für Umwelt und Geologie, Wiesbaden, Germany; see Hennies et al., 2003) 1:3000, for 90 min at room temperature. This antiserum has already proven to be effective for Vtg detection in *C. dimerus* (Moncaut et al., 2003). After three 5 min washes in TBST, membranes were incubated with a biotinylated anti-rabbit IgG antibody (Vector Lab., Burlingame, CA, USA) diluted 1:1000 for 1 h and washed again, followed by incubation with horseradish peroxidase-conjugated streptavidin (Dako, Carpenteria, CA, USA) diluted 1:3000 for 1 h in the dark. Immunoreactivity was developed with 0.1% 3, 3′ diaminobenzidine in 0.1 M Tris buffer pH 7.6 (DAB) and 0.02% water peroxide ($H_2O_2$). Preadsorption with pure