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Time-course recovery of estrogen-responsive genes of a cichlid fish exposed to waterborne octylphenol

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ABSTRACT

The aim of this study was to describe the time-course of estrogen-induced gene expression, corresponding plasma protein detection and histological alterations after cessation of octylphenol (OP) exposure of *Cichlasoma dimerus*, to test differential responses of biomarkers suitable for environmental monitoring. Male fish were exposed to a nominal concentration of 150 μg/L OP for 28 days, and later transferred to OP-free water aquaria for 1, 3, 7, 14, 21 or 28 days. Blood and mucus samples were obtained in order to analyze vitellogenin (VTG) and zona pellucida (ZP) proteins by Western blot; liver samples were used for gene expression and to assess tissue damage and further recovery of all the analyzed endpoints. Partial sequences of *C. dimerus* VTG and Na^+/K^+-ATPase were obtained. Comparison with VTGs of several teleosts supports that the partial sequence obtained for *C. dimerus* belongs to VTGAb type. ZP and VTG expression was highly up-regulated by OP. Immunoreactive (ir-) bands of 62, 52 and 50 kDa for ZP and 140, 103, 75 and 64 kDa for VTG, were detected after 28 days of OP exposure in plasma and mucus samples. After transfer of treated fish to clean water, ZP ir-bands in plasma disappeared rapidly (day 3), while VTG ir-bands decreased gradually; no ir-bands were detected on day 28 of recovery. Similarly, ZPB transcripts abruptly returned to background levels (day 3), earlier than for ZPC (day 7) or VTG (day 14). Liver from OP treated fish showed tissue disarrangement, eccentric and euchromatic hepatocytes nuclei and intense perinuclear basophilia. After the recovery period, these changes were still evident though less pronounced, accounting for irreversibility of tissue damage or the requirement for a longer period of depuration. The present results confirm that for biochemical and molecular biomarkers, such as induction of female proteins in male fish exposed to OP, complete recovery is achieved after adequate time of depuration (28 days). Male ZPB expression reflects a recent exposure to estrogenic contaminants, while VTG may reveal past exposures. The combination of biomarkers with different temporal responses such as *C. dimerus* ZP and VTG provides a more comprehensive interpretation of pollution status.

1. Introduction

Alkylphenol poly-ethoxylates (APE) are used as non-ionic surfactants in the manufacture of detergents, plastics, paints, pesticides, and cosmetics. The two most common APE degradation products are nonylphenol (NP) and octylphenol (OP). The presence of these xenoestrogens in the environment may alter embryogenesis, steroidogenesis, socio-sexual behavior, reproduction, viability of offspring, and natural endocrine development. A decreasing trend in fertility of wildlife and human populations has been reported lately, causing profound concern to be raised over the mimicking or antagonizing effects of APE regarding natural estrogens (Gronen et al., 1999; Fox, 2001; Iguchi et al., 2001; Arukwe and Goksøyr, 2003; Robinson et al., 2004; Bangsgaard et al., 2006; Saradha and Mathur, 2006; Mendiola et al., 2009).

Abbreviations: APE, alkylphenol poly-ethoxylates; Ctrl, Control; E_2, 17β-estradiol; EE_2, ethinylestradiol; LvH, lipovitellin heavy chain; LvL, lipovitellin light chain; OP, 4-tert-octylphenol; Pv, phosvitin; VTG, vitellogenin; ZP, zona pellucida proteins.

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Several field studies performed in Europe and USA reported water concentrations of APE – enough to explain the estrogenicity of these samples – in the range of 15–76 μg/L, and maximum levels of NP and OP of 644 and 42 μg/L, respectively (Rudel et al., 1998; Blackburn et al., 1999; Céspedes et al., 2005). In South America, water measurements of OP and NP levels in the environment are scarce. Fiedler et al. (2007) noticed that OP was the dominant contaminant in sediments of rural areas of Brazil. In Argentina, ecotoxicological studies in freshwater male fish exposed to waterborne OP within the range of 30–300 μg/L, produced estrogenic effects (Rey Vázquez et al., 2009). Although the estrogenic potency of OP is 10–20 fold that of NP, it has been seldom considered in ecological studies due to its lower industrial usage (White et al., 1994).

Among the estrogen-responsive genes that can be activated by xenoestrogens in juveniles or male fish that do not normally synthesize them are zona pellucida proteins (ZP) and vitelogenin (VTG) (Yadetie et al., 1999; Bowman et al., 2000; Arukwe et al., 2001; Genovese et al., 2011). In vertebrates, ZP form the egg coat that mediates sperm-oocyte binding, induction of acrosome reaction, sperm penetration, eggshell hardening and prevents polyspermy (Spargo and Hope, 2003; Modig et al., 2007). In many teleosts, ZP are synthesized by the liver of mature females under estrogenic control (Arukwe and Goksøyr, 2003). Similarly, VTG is produced in oviparous vertebrates in response to estrogens and transferred to the ovaries through the bloodstream. Within oocytes, VTG is cleaved into yolk proteins, which participate both in the physiology of the ovulated eggs and the nutrition of developing embryos (reviewed by Hiramatsu et al., 2006 and Babin et al., 2007). In teleosts, recent findings suggest that the liver is not the only site for VTG synthesis and that several other tissues may be involved in this process (Tingaud-Sequeira et al., 2011).

We previously confirmed that the liver of mature *Cichlasoma dimerus* females is the only site of ZP and VTG synthesis for this species (Genovese et al., 2006 and unpublished results), and that i.p. injections of OP exert a prompt and strong effect causing early mRNA expression of ZP, induction of plasma and mucus VTG and ZP, as well as histological damage in liver and testis of adult fish (Genovese et al., 2011). Therefore, ZP and VTG are suitable biomarkers for endocrine disruption in males of *C. dimerus*. Since detection of VTG and ZP in skin mucus can be assessed without killing the fish (Genovese et al., 2011), it would be interesting to test if exposure to environmentally relevant concentrations of OP can cause mucus VTG and ZP induction. Moreover, only two previous studies analyzed recovery of the effects caused by exposure to OP (Robinson et al., 2004; Bangsgaard et al., 2006) but to our knowledge no time-course experiment has been performed thus far.

C. dimerus belongs to the cichlid family, one of the largest perciform families (Nelson, 2006), and inhabits inland waters of Argentina and Brazil. This species shows biparental care and a highly organized breeding activity (Alonso et al., 2011). It has been used in ecotoxicological studies in our laboratory (Moncaut et al., 2003; Rey Vázquez et al., 2009; Da Cuña et al., 2011; Genovese et al., 2011; Piazza et al., 2011), and it is considered an appropriate native species for xenobiotic toxicity assays by the Argentinean Institute of Standardization and Certification (IRAM, 2008).

The objective of this study was to describe the time-course recovery of estrogen-responsive genes, corresponding plasma proteins and histological damage after cessation of exposure of *C. dimerus* males to octylphenol, to test differential responses and reversibility of biomarkers suitable for environmental monitoring. It is imperative to understand the temporal changes of biomarkers before applying them in environmental monitoring and risk evaluation. A thorough understanding of the kinetic profile encompassing hepatic mRNA regulation of estrogen-stimulated genes and elimination of induced plasma proteins is required for the effective field application of adequate endpoints as biomarkers of estrogenic exposure (Hemmer et al., 2002).

2. Materials and methods

2.1. Animals

Adult *C. dimerus* fish were caught in Esteros del Riachuelo, Corrientes Province, Argentina (27°35′S 58°45′W). Prior to experimentation, fish (42 ± 2 g body weight) were housed in 100 L glass aquaria under conditions that mimic their natural habitat (Casciotta et al., 2005) for at least one month. Aquaria were kept at 26 ± 1 °C, 14: 10 light: dark cycle with full spectrum illumination, external filtration, constant aeration and regulated pH 7.3. Fish were fed ad libitum daily with cichlid pellets (Tetra®). All experiments were conducted in accordance to international standards on animal welfare (NIH, 2011).

2.2. Experimental design

Male fish were transferred to 50 L glass aquaria containing 4-tert-octylphenol (OP) (Sigma–Aldrich, USA) previously dissolved in ethanol 96% (final ethanol concentration in each aquaria was 0.001%). Fish were exposed to the nominal concentration of 150 μg/L OP for 28 days, according to Rey Vázquez et al. (2009). Control groups were exposed to ethanol under the same conditions.

Water renewal including OP was performed twice a week. In order to confirm that initial nominal and actual OP levels were in good agreement, water samples were analyzed according to Rey Vázquez et al. (2009); detection limit was 1 μg/L.

After 28 days of OP exposure, fish were transferred to 50 L OP-free water aquaria during 0, 1, 3, 7, 14, 21 or 28 days ($N = 6$ for each recovery time).

Six female fish were i.p. injected with 17β-estradiol (10 μg/g bw) (E_2; Sigma–Aldrich, USA) to produce estrogen-induced proteins. The obtained samples were used as positive controls in Western blots and gene expression assays.

2.3. Sample collection

Male fish from each recovery time, as well as controls, were sedated (Jungle Hypno, Fish Calmer, USA) and total weight (g) was determined. Blood was drawn by caudal puncture with an heparin-coated syringe, 27 gauge × 1/2 in. needle. Mucus samples were scraped with a metal spatula from the body surface. All samples were collected in plastic tubes with 10 μL of protease inhibitor cocktail (Sigma–Aldrich, USA). PBST (phosphate buffer saline, 0.1 M, pH 7.4, 0.5% Tween 20) was also added to mucus samples. After centrifugation at 3000 rpm for 15 min at 4 °C, plasma and mucus samples – free of debris and scales – were stored at −20 °C until SDS-PAGE and Western blot assays were performed. Protein concentrations were measured by Lowry's method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951).

Fish were quickly dissected and livers were weighed for the calculation of the hepatosomatic index (HSI; liver weight/(body weight − liver weight) × 100). The distal section of each liver was divided in two pieces; one portion was fixed in Bouin's solution for 18 h for later histological processing, and the other was immersed in 2 mL cold RNAlater (Ambion, USA) for 24 h and frozen at −20 °C for gene expression studies.

2.4. SDS-PAGE and Western blot

Samples with equal amounts of protein (40 μg for plasma; 50 μg for mucus) were mixed with loading buffer (120 mM Tris–HCl,