Aquatic Toxicology 156 (2014) 30–40

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Nonmonotonic response of vitellogenin and estrogen receptor α gene expression after octylphenol exposure of *Cichlasoma dimerus* (Perciformes, Cichlidae)

G. Genovese[a,b,*], M. Regueira[a], R.H. Da Cuña[a,b], M.F. Ferreira[a], M.L. Varela[a], F.L. Lo Nostro[a,b]

[a] *Laboratorio de Ecotoxicología Acuática, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428EGA, Argentina*
[b] *IBBEA, CONICET-UBA, Argentina*

ARTICLE INFO

Article history:
Received 15 May 2014
Received in revised form 20 July 2014
Accepted 24 July 2014
Available online 1 August 2014

Keywords:
Cichlid fish
Vitellogenin gene expression
Estrogen receptors
Octylphenol
Nonmonotonic dose–response

ABSTRACT

In oviparous vertebrates, vitellogenin (VTG) is mainly produced by the liver in response to estrogen (E_2) and its synthesis is traditionally coupled to estrogen receptor alpha induction. Even though VTG is a female-specific protein, chemicals that mimic natural estrogens, known as xenoestrogens, can activate its expression in males causing endocrine disruption to wildlife and humans. Alkylphenols such as nonylphenol (NP) and octylphenol (OP) are industrial additives used in the manufacture of a wide variety of plastics and detergents, and can disrupt endocrine functions in exposed animals. For more than a decade, the freshwater cichlid fish *Cichlasoma dimerus* has been used for ecotoxicological studies in our laboratory. We recently found an up-regulation of VTG gene expression in livers of male fish exposed to OP, from a silent state to values similar to those of E_2-induced fish. To better understand the underlying mechanisms behind the action of xenoestrogens, the aim of this study was to analyze the dose–response relationship of *C. dimerus* VTG and estrogen receptors (ERs) gene expression after waterborne exposure to 0.15, 1.5, 15, and 150 μg/L OP for up to 1 month (0, 1, 3, 7, 14, 21, and 28 days). At the end of the experiment, histological features of exposed fish included active hepatocytes with basophilic cytoplasm and high eosinophilic content in their vascular system due to augmented expression of VTG. In testis, high preponderance of sperm was found in fish exposed to 150 μg/L OP. A classic dose–response down-regulation of the expression of Na^+/K^+-ATPase, a "non-gender specific gene" used for comparison, was found with increasing OP concentrations. No VTG and very low levels of ERα were detected in control male livers, but an up-regulation of both genes was found in males exposed to 0.15 or 150 μg/L OP. Moreover, VTG transcripts were significant as early as day 3 or day 1 of exposure to these OP concentrations, respectively. Nearly no response was detected in 1.5 and 15 μg/L OP exposed-fish. Data was curve-fitted evidencing a nonmonotonic dose–response curve. Interestingly, ERβ2 mRNA expression was augmented above baseline levels only when males were exposed to the lowest OP concentration. We speculate that genomic control of vitellogenesis is under control of multiple steroid receptors with different affinities for ligands. ERβ isoform, only up-regulated with very low concentrations of ligand, would act as a sensors of OP (or E_2) to induce ERα and VTG. With high OP concentrations, the expression of ERα isoform is promptly augmented, with the concomitant VTG transactivation.

Abbreviations: APE, alkylphenol polyethoxylates; Ctrl, control; E_2, 17β-estradiol; OP, 4-tert-octylphenol; VTG, vitellogenin; NP, nonylphenol; AP, alkylphenols.

* Corresponding author at: Laboratorio de Ecotoxicología Acuática, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428EGA, Argentina. Tel.: +54 11 4576 3348; fax: +54 11 4576 3384.
E-mail addresses: genovese@bg.fcen.uba.ar, grigenovese@hotmail.com (G. Genovese).

http://dx.doi.org/10.1016/j.aquatox.2014.07.019
0166-445X/

1. Introduction

In oviparous vertebrates the yolk precursor vitellogenin (VTG), a complex phospholipoglycoprotein, is produced mainly by the liver in response to estrogens. It reaches the ovaries through the bloodstream where it is incorporated into growing oocytes *via* receptor-mediated endocytosis. Vitellogenin is cleaved into yolk proteins, which participate in the physiology of the ovulated eggs and in the nutrition of the developing embryos (Hiramatsu et al., 2006; Babin et al., 2007). Production of VTG has been tightly coupled to the induction of estrogen receptors (ERs) at transcriptional and post-transcriptional levels (Flouriot et al., 1996). Nuclear ERs belong to the steroid/thyroid/retinoic acid superfamily of ligand-activated transcription factors with a modular structure that consists of a *trans*-activation domain (A/B), a highly conserved DNA binding domain (C), a hinge region (D), a well-conserved ligand binding domain (E), and a C-terminal region (F) (Andreassen et al., 2003; Menuet et al., 2004). In the absence of ligand, ERs are mostly found in the cytoplasm as complexes with inhibitory heat shock proteins. In the presence of ligand, the heat shock protein complex is lost. The receptors form homodimers or heterodimers between ERα and/or ERβ, relocating to the nucleus to bind to specific regions on the DNA (termed estrogen response elements or ERE) where they interact with nuclear coactivators or corepressors modulating the transcription of target genes (Nelson and Habibi, 2013). It has been proposed that ER forms characterized in teleost fish would have distinct functions since they are differentially expressed in a development, gender, and organ-fashion and they respond differently to E_2 or specific estrogen receptor modulators (SERM) (Davis et al., 2008; Chandrasekar et al., 2010; Nagler et al., 2012).

Even though VTG is a female-specific protein, as it is not synthesized by juveniles or male fish, its expression can be transactivated by exogenous estrogens and chemicals that mimic natural estrogens known as xenoestrogens (Yadetie et al., 1999; Bowman et al., 2000; Arukwe et al., 2001; Genovese et al., 2011, 2012; Chandra et al., 2012). These xenoestrogens are wild life and human health hazards capable of causing endocrine disruption. Among known xenoestrogens are alkylphenols (AP), such as 4-tert-octylphenol (OP) and nonylphenol (NP), non-ionic surfactants used in the manufacture of detergents, plastics, paints, pesticides and cosmetics, and can disrupt endocrine functions in fish (Trudeau and Tyler, 2007).

The freshwater cichlid fish *Cichlasoma dimerus* belongs to one of the largest Perciform families (Nelson, 2006), the Cichlidae, and inhabits inland waters of Argentina and Brazil. This species displays an elaborate social system and organized biparental breeding activities and can be easily adapted to laboratory conditions (Pandolfi et al., 2009). *C. dimerus* has been extensively used in our laboratory for ecotoxicological studies (Moncaut et al., 2003; Rey Vázquez et al., 2009; Rey Vázquez and Lo Nostro, 2014; Da Cuña et al., 2011, 2013; Genovese et al., 2011, 2012; Piazza et al., 2011). As a biomarker of early detection of pollution, we have already shown VTG as a good estrogen-responsive gene; for which we previously obtained a partial sequence of liver VTGAb mRNA for this species, highly homologous to other teleosts' VTG (Genovese et al., 2011, 2012). We were also able to detect an up-regulation of VTG gene expression in liver of male fish exposed to 150 μg/L OP for 28 days, from a silent state typical of male fish and reaching the same level of induction caused by estradiol (Genovese et al., 2012). Furthermore, OP exerted induction of plasma vitellogenin, and histological liver and testis damage; the latter possibly causing impairment of spermatogenesis (Rey Vázquez et al., 2009; Genovese et al., 2011, 2012). After transference of OP-treated fish to OP-free water, histological damage could not be restored; however molecular biomarkers such as plasma protein induction and mRNA expression of estrogen-responsive genes were completely recovered after adequate time of depuration (28 days) (Genovese et al., 2012).

Some dose–response studies of the effects caused by xenoestrogens or natural estrogens in fish report a linear dose-dependent induction of estrogen-responsive genes such as VTG, estrogen receptor (ER), *zona pellucida* protein (ZP) and/or corresponding proteins (Yadetie et al., 1999; Andersson et al., 2007; Calabrese, 2004). However, recent studies (Villeneuve et al., 2012; Beausoleil et al., 2013) report nonmonotonic concentration-responses after endocrine disruption. To our knowledge, no such studies have been done in estrogen-responsive genes in cichlid fish.

As we were particularly interested in whether the response to OP agreed with a threshold, non-threshold linear, or biphasic model, the aim of this work was to study the dose–response relationship of the *C. dimerus* VTG gene expression to environmental relevant concentrations of OP, as a valuable tool for risk management of environmental estrogens since effects at higher doses not always readily predict the effect at low-doses.

2. Materials and methods

2.1. Animals

Adult *Cichlasoma dimerus* (Perciformes, Cichlidae) of both sexes, caught in Esteros del Riachuelo, Corrientes Province, Argentina (27°35′ S 58°45′ W), were housed in 100 L glass aquaria with filtered tap water (pH 7.3, alkalinity 36.5 mg/L, hardness 55 mg/L, conductivity 219 μS/cm, dissolved oxygen 8 mg/L, sodium 20 mg/L) under conditions that mimic their natural habitat (Casciotta et al., 2005) for at least one month before the start of the experiments. 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 daily with cichlid pellets (Tetra®). All experiments were performed in accordance with international standards on animal welfare (World Medical Association Statement on Animal Use in Biomedical Research, 2006), and local regulations.

2.2. Experimental design

Adult male fish (45.6 ± 1.6 g body weight; n = 4–6 for each treatment) were transferred to 50 L glass aquaria and exposed during 0, 1, 3, 7, 14, 21, and 28 days to 0.15; 1.5; 15 and 150 μg/L (0.73, 7.3, 73, 730 nM respectively) 4-tert-octylphenol (OP, Sigma–Aldrich, USA), previously dissolved in ethanol 96° (the final concentration of alcohol in each aquarium was 0.001%). Control groups were exposed to ethanol alone under the same conditions. Concentrations of OP were selected to range from environmentally relevant in surface waters (reported levels usually below 1 μg/L), occasionally occurring higher values (as high as 22 μg/L), to levels above those detected (Céspedes et al., 2005; Wang et al., 2012; Wu et al., 2013; Babay et al., 2014). In order to confirm that initial nominal and actual OP levels were in good agreement, water samples were analyzed by reverse phase HPLC coupled to fluorescence detection according to Rey Vázquez et al. (2009). Briefly, water samples were treated by solid phase extraction on a C18 column, eluted by methanol and injected into the HPLC. Water renewal including OP was performed twice a week when OP values decreased by 80% from the initial concentration (data not shown). Octylphenol was not detected in OP-free water at any time point.

Female fish were i.p. injected with 17β-estradiol (10 μg/g bw) (E_2; Sigma–Aldrich, USA) to strongly induce VTG expression. The obtained mucus, plasma, and liver samples were used as positive controls in Western blots and gene expression assays.

No mortality was registered in E_2-injected females or in OP exposed males.

2.3. Sample collection

After the exposure period, fish were sedated (Jungle Hypno, Fish Calmer, USA), and total body weight (g) was determined. Blood was drawn by caudal puncture with a heparin-coated syringe, coupled with a 27 gauge × 1/2 in needle. Mucus was gently scraped from the body surface with a metal spatula. All samples were collected in plastic tubes and processed according to Genovese et al. (2011) 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 sacrificed by decapitation and quickly dissected. The distal section of each liver was divided in two pieces; one portion was fixed in Bouin's solution for 18 h for histological processing, and the other was immersed in cold RNAlater (Ambion, USA) for 24 h and frozen at −20 °C for gene expression analysis. Testes were fixed in Bouin's solution for 18 h for histological processing.

2.4. SDS-PAGE, Western blot

Samples with equal amounts of protein (40 μg for plasma; 50 μg for mucus) were mixed with loading buffer, boiled for 5 min and briefly spun down before loading them into polyacrylamide gel wells. A molecular weight standard was loaded in a separate well (SeeBlue Plus2 Pre-Stained Standard, Invitrogen Corporation, USA). A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970) and modified by Genovese et al. (2011), was performed at a constant 100 V using 4% stacking and 8% separating gels (Mini-Protean III, Bio-Rad, USA). Transference to nitrocellulose membranes (ECL Amersham Biosciences, UK) was achieved at 100 V for 90 min.

VTG was immunodetected according to Genovese et al. (2011) using rabbit anti-perch VTG antiserum 1:5000 (*Perca fluviatilis*; donated by Dr. B. Allner, Germany) for 90 min at RT. The specificity and cross-reactivity of the primary antibody has been previously tested in *C. dimerus* (Moncaut et al., 2003). After three 5-min washes with Tris buffered saline with Tween® 20 (TTBS), membranes were incubated with biotinylated anti-rabbit secondary antibody 1:1000 for 1 h at RT, streptavidin–HRP (Dako, USA) 1:3000 for 1 h, and 0.1% 3,3′-diaminobenzidine in Tris–HCl buffer (Dako, USA) for 5 min, for amplification and detection of the signal.

2.5. Gene expression

Liver RNA was extracted and purified following the phenol–chloroform–isoamyl alcohol protocol (RNAgent total RNA isolation system, Promega Corporation, USA). Each RNA extract was analyzed for quantity and quality by microfluidic electrophoresis with RNA 6000 Nano Chip Kit and Agilent 2100 Bioanalyzer (Waldbronn, Germany). High quality of RNA and normalization to total RNA are necessary to produce biologically relevant and reliable data using real-time PCR (Bustin et al., 2005). A complete absence of degradation products or genomic DNA was found. Two micrograms of each total RNA sample were reverse transcribed to single-stranded cDNA with SuperScript III First-Strand Synthesis System for real time-PCR (Invitrogen, USA) using oligo(dT) as primers.

Specific primers for the VTG sequence previously described for *C. dimerus* (Genovese et al., 2012) (forward primer 5′-CGGCGTGGTCAGTAGAGTG-3′ and reverse primer 5′-GGCTACCAGGTGATTCATAGTG-3′) were used for real time quantitative PCR. cDNA was amplified in the presence of SYBR-Green dye using Qiagen Quantitect chemistry and the Stratagene MX4000 Multiplex Quantitative PCR System (Stratagene, USA). The thermal profile for real-time PCR consisted of an activation step at 95 °C for 10 min and 40 cycles of denaturing at 95 °C for 30 s, annealing at 45 °C for 60 s and elongation at 72 °C for 30 s. After the last amplification cycle, the temperature was increased to 95 °C for 1 min and then decreased to 45 °C to run 82 cycles, increasing by 0.5 °C per cycle, to obtain melting curves, which confirmed the absence of non-specific PCR products or primer dimers. As done previously (Jayasundara et al., 2007; Beale et al., 2008; Genovese et al., 2011, 2012), mRNA expression levels were normalized to total RNA content, a preferred method of normalization for gene expression studies in systems lacking a validated housekeeping gene (Bustin et al., 2005), by using triplicate 1 μl aliquots of each 20 μl cDNA reaction mixture that was produced with 2 μg total RNA. cDNA in each qPCR incubation was thus derived from 0.1 μg total RNA. Adult females were used as positive controls after a single i.p injection of 10 μg/g 17β-estradiol according to Genovese et al. (2011) (body weight 28 ± 8 g; GSI 2.9 ± 0.8; HSI 2.1 ± 0.3; $N = 4$). This protocol guarantees VTG production by the liver in an efficiently manner as high levels of VTG transcripts are measured after 3 days of the single dose. Reference liver preparations from these estrogenized females served as the basis for a standard dilution series, demonstrating a linear relationship between threshold cycle (Ct) and $\log_{10}$ of template availability. They were also used for calculating relative abundance values in the remaining samples.

The same protocol but at an annealing temperature of 55 °C was used for Na^+/K^+-ATPase (NAK) and estrogen receptors (ERα and β2) gene expression. For ERα, a partial sequence of 443 bp was used to design specific primers (GenBank EU158258; forward primer 5′-CACCACTGGCTGCTACTCGG-3′ and reverse primer 5′-CCTTACGCATACCTCCTTTCATC-3′) and for ERβ2 the partial sequence of 434 bp (GenBank EU158259) was used (forward primer 5′-GTCTGCATCCCCTCTCCGTA-3′ and reverse primer 5′GCTTTTACGTCGGTTCTTGTCTAT3′). No ERβ1 could be sequenced in *C. dimerus* so far.

The quantitative expression of NAK as an estrogen-responsive gene and a "non-gender specific gene" was also analyzed for comparison, using forward primer 5′-ACTCTGCAACCGTGCCGTCTTT-3′ and reverse primer 5′-AGCGTCCTTCATCTCATCATCC-3′.

2.6. Histological analysis

Liver and testes samples were dehydrated in alcohol and embedded in Paraplast® (Oxford, USA). Seven micrometers thick sections were stained with hematoxylin–eosin. Photomicrographs were taken with a Microphot FX (Nikon) microscope coupled with a Coolpix 5400 digital camera (Nikon, Japan).

2.7. Statistical analysis

For gene expression, data was compared using 1 way ANOVA followed by Tukey's multiple comparison test. When assumptions where not met, data was log-transformed. Means were considered statistically different if $p < 0.05$. Nonlinear curve fitting to expression levels of VTG, ERs and NAK at 28 days was performed to define the model representing each relationship. A free version of Graph Pad Prism software was used for all analyses (http://www.graphpad.com).

3. Results

3.1. Immunodetection of vitellogenin in plasma and mucus samples of control fish

In order to compare male VTG induction caused by OP, we used vitellogenic and estrogenized *C. dimerus* adult females as positive controls. SDS-PAGE followed by Western blot of female plasma samples is depicted in Fig. 1A. Plasma of vitellogenic females

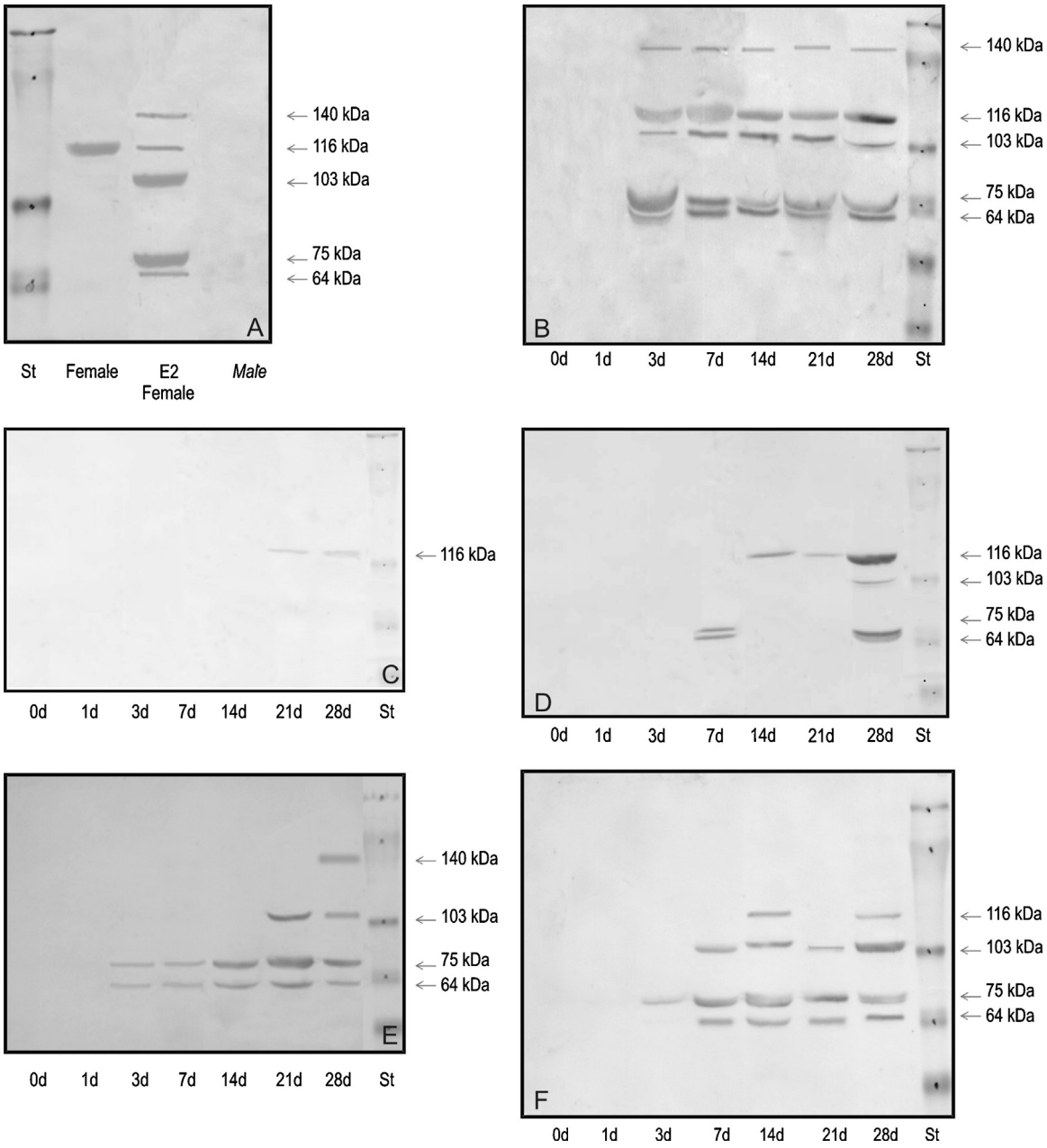

Fig. 1. (A) Western blot of plasma samples from vitellogenic and E_2-induced VTG of *Cichlasoma dimerus*. St: molecular weight standards; female: vitellogenic female; E_2 female: E_2-challenged female; male: unexposed male. (B–E) Plasma samples of *C. dimerus* males exposed to different concentrations of OP. (B) 0.15 μg/L OP; (C) 1.5 μg/L OP; (D) 15 μg/L OP; (E) 150 μg/L OP. (F) VTG was only detected in mucus samples of males exposed to 150 μg/L OP. Days of exposure are indicated below each lane.

showed a single immunoreactive (ir) band of 116 kDa (lane 2), while females i.p. injected with 17β-estradiol (E_2) showed several VTG ir-bands: 140, 116, 103, 75 and 64 kDa (lane 3). Unexposed males did not exhibit VTG bands (lane 4).

3.2. Immunodetection of vitellogenin in plasma and mucus samples of fish after waterborne exposure to octylphenol

No VTG induction was detected in plasma of control male fish or in plasma of males exposed to the lowest OP concentration (0.15 μg/L) on day 1. From day 3 onward, five VTG bands of 140, 116, 103, 75 and 64 kDa could be observed (Fig. 1B). Mucus revealed no VTG bands at any given day post-exposure (data not shown).

In 1.5 μg/L OP exposure, plasma of male fish became VTG immunoreactive after 21 days of OP exposure; only one VTG band, similar to that of mature vitellogenic females, was detected (116 kDa) (Fig. 1C). In mucus samples no VTG bands were seen (data not shown).

Exposure to 15 μg/L OP had a slight effect on male plasma samples after day 7, with VTG ir-bands detected at 75 and 64 kDa. By day 28 of OP exposure additional bands were also detected (116, 103, 75 and 64 kDa) (Fig. 1D). Mucus samples showed no VTG ir-bands (data not shown).

When males were exposed to 150 μg/L OP, plasma VTG could be immunodetected after the third day of exposure. Between days 3 and 14, two VTG bands of 75 and 64 kDa were observed. At day 21 heavier VTG bands were apparent (140, 103, 75 and 64 kDa) (Fig. 1E). In mucus samples, one VTG ir-band was detected after day 3 of exposure, and additional bands were evident from day 7 onward (116, 103, 75 and 64 kDa) (Fig. 1F).

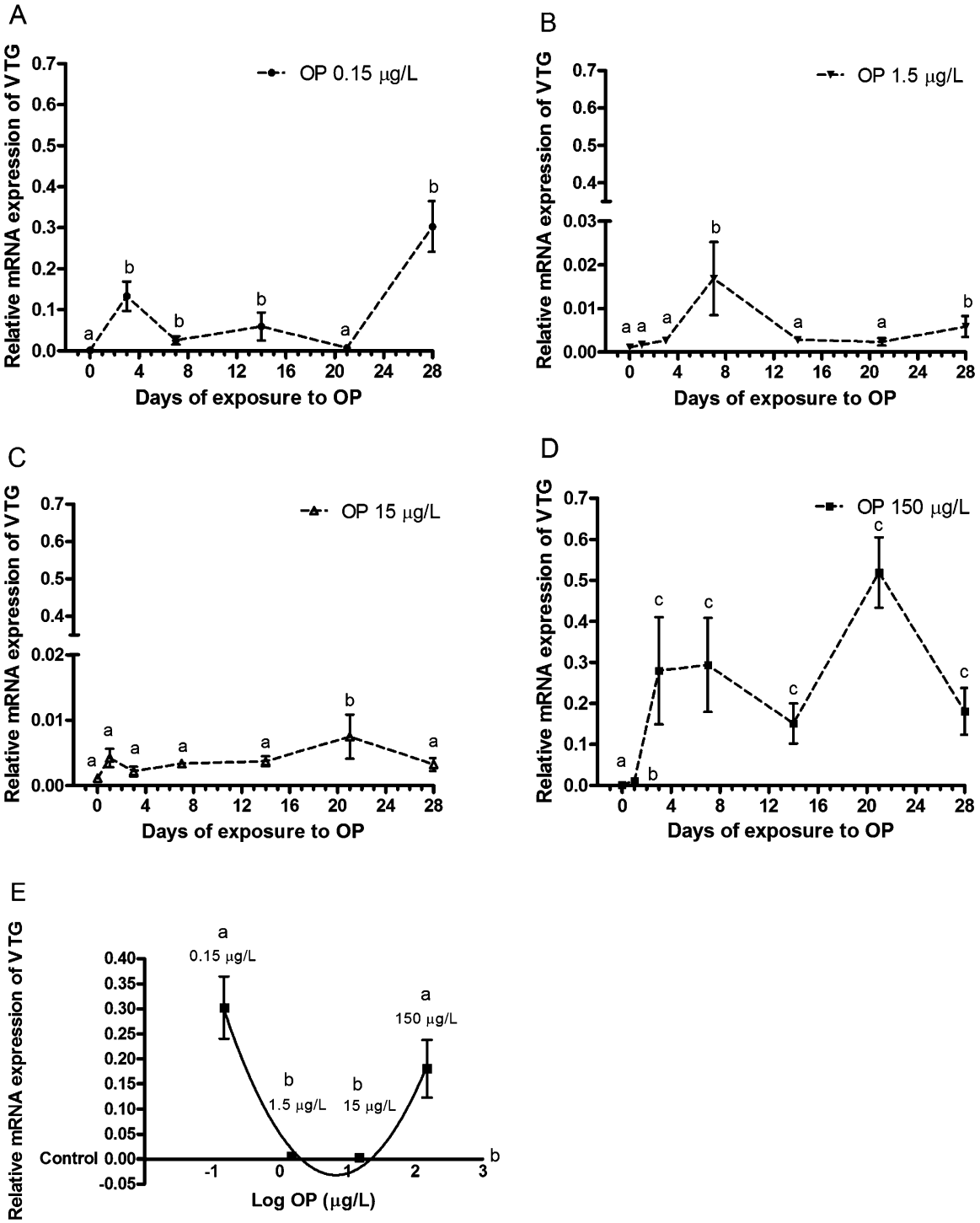

Fig. 2. Time course of liver vitellogenin (VTGAb) mRNA up-regulation of *Cichlasoma dimerus* males exposed to different concentrations of octylphenol (OP). Mean ± SE are represented. Different letters denote significant differences with $p < 0.05$ after Tukey's comparisons. After high (D) and low (A) OP exposition, a significant up-regulation of VTG was detected on day 1 and 3, respectively. This up-regulation continued and increased until the end of the experiment. Note the two-segment scale only for 1.5 (B) and 15 µg/L OP (C) due to low mRNA expression. Males exposed to solvent (control) did not express VTG at any time point. (E) Best fit after 28 days of OP showing nonmonotonic dose–response curve (nonlinear regression, second order polynomial) (R^2: 0.68).

3.3. *Quantitative gene expression of VTG, ERα, ERβ2, and Na^+/K^+-ATPase in liver of C. dimerus after waterborne exposure to OP*

Time course of VTG mRNA expression was measured in liver of male *C. dimerus* exposed to different concentrations of OP. Fig. 2A depicts an up-regulation of VTG mRNA as early as day 3 of exposure to the lowest OP concentration (0.15 µg/L), from a silent state typical of male fish to values significantly different from those at the beginning of the experiment. This increase in VTG expression was particularly noticeable after 28 days of OP exposure. Fig. 2B and C shows a slight VTG mRNA up-regulation at a few time points (notice the two-segment scale due to low signal of VTG expression when males were exposed to 1.5 or 15 µg/L OP). On the contrary 150 µg/L

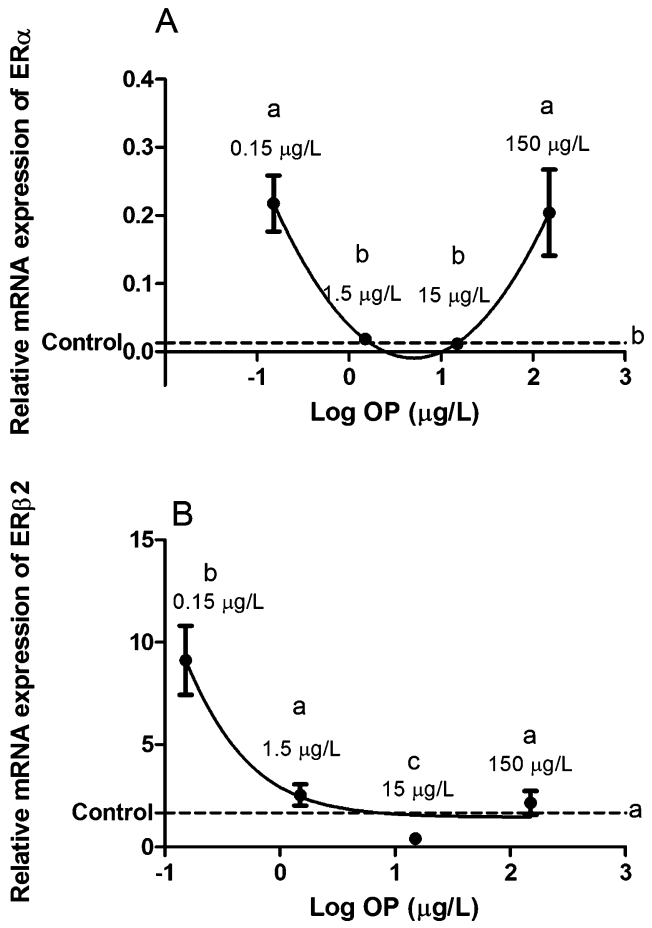

Fig. 3. Liver estrogen receptor alpha (ERα) and beta (ERβ2) mRNA expression of *Cichlasoma dimerus* males exposed for 28 days to different concentrations of octylphenol (OP). Mean ± SE are represented. Different letters denote significant differences with $p < 0.05$ after Tukey's comparisons. Dashed-lines show the gene expression measured in control fish. (A) Best fit of ERα showing nonmonotonic dose–response curve (non linear regression, second order polynomial) (R^2:0.63). (B) Best fit of ERβ2 showing nonlinear regression dose response (R^2: 0.71).

OP caused a significant up-regulation of VTG gene expression as early as day 1 of the experiment. Values continued to increase until they reached similar levels to those of E_2-females. Plotting VTG expression *vs.* OP concentration at day 28 revealed a U-shaped dose–response curve and the best fit for these data was a quadratic curve explaining 68% of variance (*R*-squared 0.68). Fig. 2E shows high values of mRNA expression both at low and high OP concentrations and very low expression at intermediate concentrations, which were not significantly different from those of control male fish. The same type of response was found after measuring the mRNA expression of ERα in liver of males exposed to OP, with a significant up-regulation only for 0.15 and 150 µg/L OP (best fit was a quadratic curve with *R*-squared 0.63). Basal transcripts of ERα expression in control male fish were minimal and similar to those found for 1.5 and 15 µg/L OP (Fig. 3A). Fig. 3B shows an up-regulation of the expression of ERβ2 when fish were exposed to 0.15 µg/L OP (best fit was a non linear regression dose response with *R*-squared 0.71). For the remaining OP concentrations, values were similar to those of control male fish, except for 15 µg/L OP, where a down-regulation was detected.

Liver Na^+/K^+-ATPase (NAK) gene expression showed a down-regulation after OP exposure as soon as day 1 and thereafter for all concentrations except 0.15 µg/L OP. The best fit after 28 days of OP showed a monotonic sigmoidal dose–response curve explaining 76% of variance (*R*-squared 0.76) (Fig. 4).

3.4. Effects on liver and gonad histology

The liver of males exposed to 0.15, 1.5 or 15 µg/L OP showed no histological damage, and were similar in appearance to that of control males (Fig. 5A). On the contrary, the liver of males exposed to 150 µg/L OP during 28 days showed general tissue disarrangement, eccentric and euchromatic nuclei with conspicuous nucleoli, and intense perinucleolar basophilia (Fig. 5B and inset, arrows), as reported by previous work in the same species (Genovese et al., 2012). A reduction of vacuolization of the cytoplasm was also evident. Several eosinophilic granule cells were seen mostly around main blood vessels and pancreatic tissue. Within blood vessels, the homogeneous content was intensely stained with eosin (Fig. 5B). No testicular damage could be observed at any concentration/time similar to control males (unrestricted lobular testis type) (Fig. 5C); though sperm predominated over other germinal stages in fish exposed to 150 µg/L OP (Fig. 5D).

4. Discussion

In oviparous vertebrates, vitellogenin (VTG) synthesis was traditionally coupled to estradiol-dependant up-regulation of the estrogen receptor alpha (ERα) expression in female liver (Bowman et al., 2002; Lange et al., 2003; Davis et al., 2008). However, recent evidences support the participation of liver ERβ2 isoform during vitellogenesis (Nagler et al., 2012; Nelson and Habibi, 2013). To date, four ER isoforms have been described which appeared after an additional genome duplication event in fishes (Nagler et al., 2007; Zhu et al., 2008). The expression of these receptors can be induced by xenoestrogens such as nonylphenol (NP) and octylphenol (OP) and this induction is followed by that of genes regulated by ER, like *zona pellucida* proteins (ZP) and VTG (Yadetie et al., 1999; Andreassen et al., 2005; Palermo et al., 2012). The estrogenic effect of these chemicals is apparently only mediated by specific binding to estrogen receptors (White et al., 1994) and not due to elevation of endogenous estradiol since Yadetie et al. (1999) reported that plasma levels of E_2 remained constant in fish exposed to NP. In fact, plasma E_2 and testosterone levels in *C. dimerus* males exposed to 150 µg/L OP for 1 month was lower than those of control male fish which could block spermatogenesis after failure of Sertoli cells function (Rey Vázquez, 2012). Although, Rey Vázquez (2012) found changes in Sertoli cell morphology after fish were exposed for two months to 300 µg/L OP, no such alterations were observed in that study using lower concentrations of OP.

In the present study we demonstrated that adult male *C. dimerus*, which normally do not synthesize female-specific proteins, possess the ability to synthesize VTG after only 1 day of exposure to a nominal concentration of 150 µg/L OP. VTG transcription is followed by mRNA translation into protein which explains VTG detection in Western blots on day 3. Further induction was seen as the exposure continued. Nuclear hypertrophy, conspicuous nucleoli and prevalence of euchromatin in hepatocytes are distinctive of active gene transcription. The consequence of protein synthesis and accumulation in liver tissue is evidenced by the intense cytoplasmic basophilia within hepatocytes due to proliferation of rough endoplasmic reticulum for mRNA translation of estrogen-inducible proteins such as ZP and VTG (Yadetie et al., 1999; Arukwe et al., 2002; Bowman et al., 2002; Tollefsen et al., 2002; Woods et al., 2009). Without a target organ for egg proteins in males (lack of ovaries), VTG accumulates within the liver and in the bloodstream (Moncaut et al., 2003). Consequently, VTG can be easily detected in plasma. At the end of the experiment (28 days) numerous VTG immunoreactive bands were observed as previously reported for OP-exposed males of the same species (Genovese et al., 2012). This five VTG bands pattern was identical to the one found in E_2-females, indicating the similarities of both chemicals on inducing

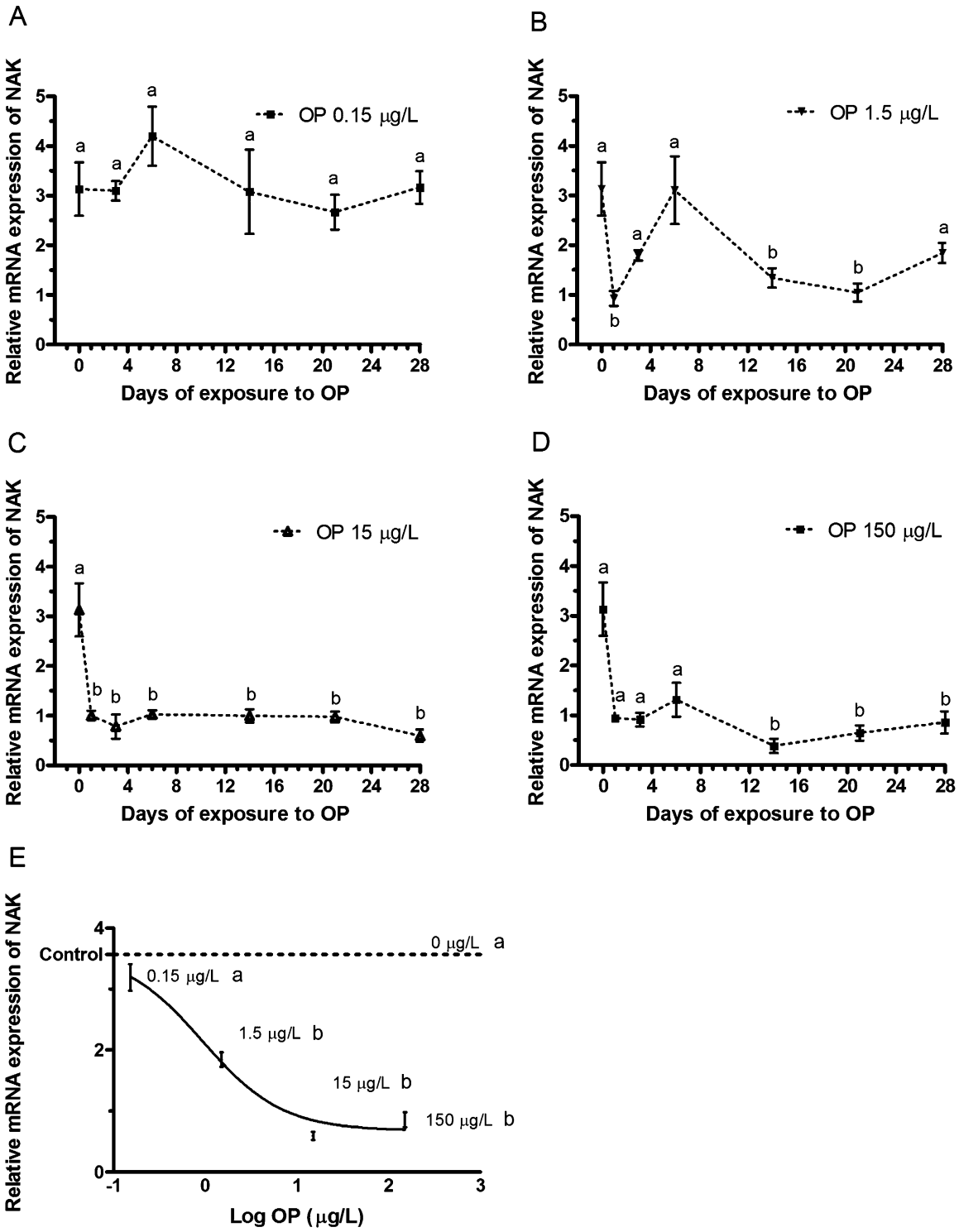

Fig. 4. Time course of mRNA down-regulation of liver Na^+/K^+-ATPase (NAK) of *Cichlasoma dimerus* males exposed to different concentrations of octylphenol (OP). Different letters denote significant differences with $p < 0.05$ after Tukey's comparisons. Low OP concentration did not affect NAK expression (A), while increasing the concentration of OP (B–D) caused a reduction on the expression of NAK. Control males exposed to solvent were not affected. (E) Best fit after 28 days of OP showing a monotonic dose–response curve (non linear regression, sigmoidal model) (R^2: 0.76).

estrogen-responsive genes. Simultaneously, in male mucus samples, detection of only one VTG ir-band was found on day 3, and additional bands were detected from day 7 onward. The presence of VTG in skin of estrogenized fish might be due to elimination of excess plasma proteins and/or *in situ* synthesis of VTG (Moncaut et al., 2003; Meucci and Arukwe, 2005; Arukwe and Røe, 2008; Jin et al., 2008; Genovese et al., 2011, 2012). Although control males of this species exhibit no VTG in mucus samples, it is well known that fry of some cichlids get nutritional components (vitellogenin) by biting mucus from both parents during the first weeks of parental care (Buckley et al., 2010). *C. dimerus* does not exhibit this nipping behavior but the presence of VTG in mucus could be evolutionarily significant for nutritional purposes. In this context, the immunodetection of VTG in body surface mucus is a sensitive, easy, and

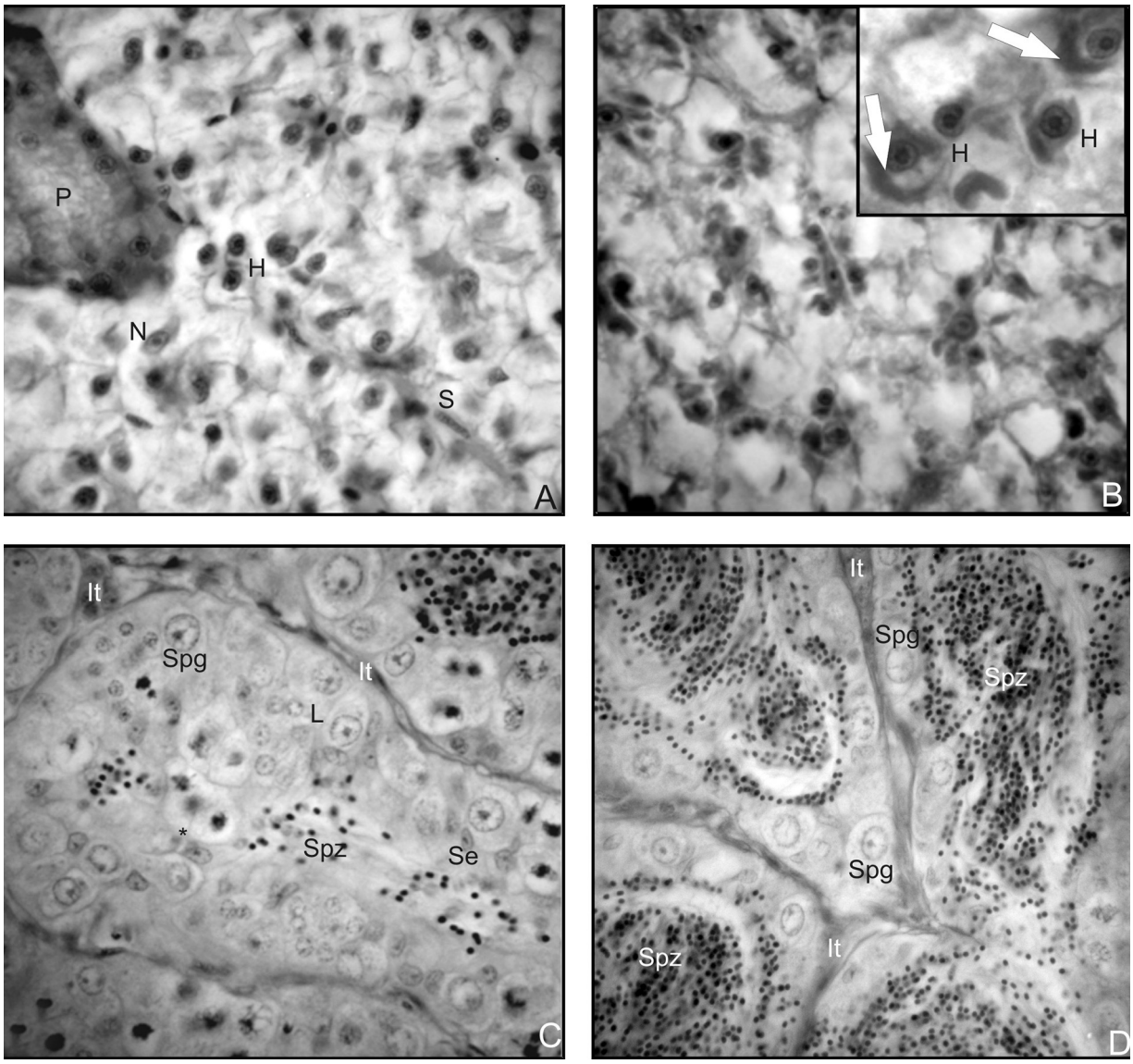

Fig. 5. Liver and testis sections from control males of *Cichlasoma dimerus* (A and C), and from males exposed during 28 days to 150 μg/L OP (B and D) (hematoxilin–eosin; 600×). H: hepatocyte, L: Testis lobule containing spermatocysts with different stages of spermatogensis and surrounded by interstitial tissue (It), N: nuclei, P: pancreas, S: sinusoid, white arrows: intense perinucleolar basophilia, Se: Sertoli cell, Spg: spermatogonia, Spz: sperm, *: mitotic germ cells.

noninvasive technique to address endocrine disruption and thus it can be used after short exposures to xenoestrogens (Jin et al., 2008; Genovese et al., 2011, 2012).

In spite of the valuable results cited above, high concentrations of OP are far from being found in nature but were necessary to obtain a maximum induction of the aforementioned biomarkers. For this reason, we additionally exposed male *C. dimerus* to lower OP concentrations (0.15, 1.5, and 15 μg/L) much more likely to be present in water bodies since reported environmental concentrations in surface water of OP and NP are usually below 1 μg/L, with occasional values as high as 22 μg/L (Céspedes et al., 2005; Wang et al., 2012; Wu et al., 2013; Babay et al., 2014). The experimental design was done in an ecologically relevant pulse-exposure fashion, since fish are not usually exposed to constant levels of pollutants in the environment. We aimed to analyze the time course effect as well as to find the lowest observed effect concentration (LOEC). The lowest concentration of OP did not affect the normal expression of NAK in liver. The lack of effect of 0.15 μg/L OP on NAK expression in contrast to the up-regulation of VTG confirms a different behavior of these two estrogen-responsive genes probably due to recruitment of different coactivator/corepressor proteins (Martyniuk et al., 2007; Nelson and Habibi, 2013).

In contrast, a down-regulation of NAK expression was found for concentrations ≥1.5 μg/L very early during the exposure period. In fish it was reported that estrogens can disrupt the sodium pump in different tissues through estrogen receptors. Particularly in zebrafish, the liver NAK mRNA was down-regulated after exposure to ethinylestradiol, a widely used contraceptive (Martyniuk et al., 2007). NAK is a key enzyme that provides the driving forces for the control of the ionic environment in the cytosol, cell volume, intracellular pH, and transport of nutrients, and metabolites (Suzuki-Yagawa et al., 1992). In the present study, fish continued to feed normally and no mortality was registered. It seems that the levels of NAK transcripts in *C. dimerus* liver after OP insult were sufficient to maintain the homeostasis in hepatocytes. However, it would be interesting to analyze the effects in liver metabolic functions through the study of hepatic enzymes and or energy stores.

Surprisingly, the dose–response for VTG or ERα expression was non-linear lacking a threshold (no LOEC could be found), as it was

previously reported by Yadetie et al. (1999) for estrogen responsive genes in juvenile salmon. These models of dose–response are the twin pillars of toxicology from which researchers and regulators have derived the majority of guidelines for risk assessment, but they do not necessarily reflect one of the most fundamental toxicological models, which is the nonmonotonic dose–response model. This later model is defined as a non-linear relationship between dose and effect, where the slope of the curve changes sign somewhere within the range of doses studied (Kohn and Melnick, 2002). They usually have U- or inverted U-shapes, often referred to as biphasic dose–response curves because responses show ascending and descending phases in relation to dose. Southam and Ehrlich (1943) first described this at the time unusual biphasic dose–response curve while studying the effects of red cedar extracts on fungi. The nonmonotonic curve of environmental toxicants should be distinguished from "hormesis", a specific type of nonmonotonic dose–response in which "the various points along the dose–response curve can be interpreted as beneficial or detrimental, depending on the biological or ecological context in which they occur" (Cook and Calabrese, 2006). Calabrese (2008) explained that hormesis represents overcompensation to a disruption in homeostasis. However, the view that hormesis is an adaptive mechanism where overcompensation takes place as a repairing process has no relevance for estrogenic stimulatory responses initiated by low doses of manmade xenoestrogens within a physiological range of estrogenic activity (Weltje et al., 2005; Azzam, 2011).

Low-dose effects and non-monotonicity are remarkably common in studies of natural hormones and EDCs (Vandenberg et al., 2012; Beausoleil et al., 2013). Biphasic effects of xenoestrogens have been reported by vom Saal et al. (1997), Murono et al. (1999) and Calabrese and Baldwin (2001). Biphasic dose–response relationships acting *via* receptor-based mechanisms can be explained by the presence of two receptor subtypes with different ligand affinities (Calabrese, 2004). Studies performed in human transfected cells and teleost ER constructs expressed into competent cells, demonstrated a higher binding affinity of OP for ERβ than for ERα (Routledge et al., 2000; Hawkins and Thomas, 2004). Moreover, in fish ERβ2 has a 1.8 fold higher affinity for E_2 than other ER forms and the percentages of identity between these receptors suggest the existence of distinct genes (Menuet et al., 2002). An overlapping distribution of ER forms within the brain, pituitary, liver, and gonads in zebrafish suggests that each ER subtype could regulate different genes implicated in different physiological processes (Menuet et al., 2002). In oviparous vertebrates ERα induces its own expression (Teitsma et al., 1998; Yadetie et al., 1999; Menuet et al., 2004); in fish ERβ2 was also shown to induce ERα promoter activity, suggesting that ERβ2 could be involved in the maintenance of ERα gene expression when E_2 levels are low during the reproductive cycle (Menuet et al., 2004; Nagler et al., 2012; Nelson and Habibi, 2013). In *C. dimerus*, two estrogen receptor subtypes (ERα and ERβ2, GenBank accession number EU158258 and EU158259, respectively) have been partially sequenced. Liver of unexposed cichlid male fish such as *C. dimerus* and *Oreochromis mossambicus* have very low levels of ERα transcripts, similar to those found in muscle of the same species (Davis et al., 2008; Genovese et al., 2008). However, in *C. dimerus* males the expression of liver ERα can be strongly up-regulated by very low or very high OP concentrations (0.15 or 150 μg/L), in a U shaped dose–response curve similar to VTG (Fig. 2E). On the contrary, baseline levels of ERβ2 in male *C. dimerus* livers are high and can only be induced by very low concentrations of OP. Therefore, we speculate that at very low concentrations of OP (0.15 μg/L) *C. dimerus* ERβ2 would directly influence (up-regulate) ERα gene expression and the latter would induce VTG expression. For concentrations between 1.5 and 15 μg/L ERβ2 would be silent and no significant ERα up-regulation would occur. On the contrary, high concentrations of OP (150 μg/L) would up-regulate ERα gene expression directly. The differential induction of ER by xenoestrogens both *in vitro* and *in vivo* suggests that ERα and ERβ play a differential modulation of gene regulation (Paech et al., 1997; Pennie et al., 1998; Menuet et al., 2004). The physiological significance of liver ERs in teleosts has been addressed by Nelson and Habibi (2013). As it is known that teleost ERβ forms have higher affinity for E_2 than ERα (Hawkins and Thomas, 2004), Nelson and Habibi (2013) hypothesized that the ERβ forms would act as a sensor for E_2 at the beginning of ovary recrudescence. Thus, as E_2 levels rise, they act *via* the ERβ forms to increase both vitellogeneisis and importantly ERα expression (Menuet et al., 2004). As ERα levels rise, E_2 can increase its signal through ERα to induce vitellogenesis.

5. Conclusions

We conclude that the two liver ER isoforms involved in *C. dimerus* vitellogenesis are differentially regulated by ligand concentration, ERα expression is augmented both at high and very low OP concentrations following a nonmonotonic dose–response curve, but ERβ2 is only up-regulated at very low OP concentration, VTG expression pattern is similar to that of ERα. Nonmonotonic responses demand toxicologists to focus on low doses of pollutants that generally reflect a more realistic scenario since the effects of low doses of OP on estrogen-responsive genes cannot be predicted by those observed at high doses. Toxicity data cannot be interpreted in a qualitative, binary mode to inform risk assessment, but a chemical specific mode of action should be highlighted in order to identify key events, responsible for responses and thresholds.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Acknowledgments

This manuscript is dedicated to my mentor Dra. María Cristina Maggese to whom I am deeply grateful. The gene expression studies were carried out at the Mount Desert Island Biological Laboratory (Salisbury Cove, Maine, USA) with a new investigator award and a Fulbright fellowship to G. Genovese. We are eternally indebted to Dr. David W. Towle who shared knowledge, friendship, and expertise with great generosity. He will live forever in our memories. We appreciate the valuable comments from both reviewers which improved the significance of this manuscript. The present work was supported by Universidad de Buenos Aires (UBACyT X620, Genovese) and CONICET (PIP 2302, Lo Nostro).

References

Andersson, C., Katsiadaki, I., Lundstedt-Enkel, K., Örberg, J., 2007. Effects of 17alpha-ethynylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). Aquat. Toxicol. 83, 33–42.

Andreassen, T.K., Skjoedt, K., Anglade, I., Kah, O., Korsgaard, B., 2003. Molecular cloning, characterisation, and tissue distribution of oestrogen receptor alpha in eelpout (*Zoarces viviparus*). Gen. Comp. Endocrinol. 132, 356–368.

Andreassen, T.K., Skjoedt, K., Korsgaard, B., 2005. Upregulation of estrogen receptor a and vitellogenin in eelpout (*Zoarces viviparus*) by waterborne exposure to 4-tert-octylphenol and 17beta-estradiol. Comp. Biochem. Physiol. C 140, 340–346.

Arukwe, A., Kullman, S.W., Hinton, D.E., 2001. Differential biomarker gene and protein expressions in nonylphenol and estradiol-17beta treated juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C 129, 1–10.

Arukwe, A., Kullman, S.W., Berg, K., Goksøyr, A., Hinton, D.E., 2002. Molecular cloning of rainbow trout (*Oncorhynchus mykiss*) eggshell zona radiata protein complementary DNA: mRNA expression in17beta-estradiol- and nonylphenol-treated fish. Comp. Biochem. Physiol. B 132, 315–326.

Arukwe, A., Røe, K., 2008. Molecular and cellular detection of expression of vitellogenin and zona radiata protein in liver and skin of juvenile salmon (*Salmo salar*) exposed to nonylphenol. Cell Tissue Res. 331, 701–712.

Azzam, E.I., 2011. Exposure to low level environmental agents: the induction of hormesis. Mutat. Res. 726, 89–90.

Babay, P.A., Itria, R.F., Romero Ale, E.E., Becquart, E.T., Gautier, E.A., 2014. Ubiquity of endocrine disruptors nonylphenol and its mono- and di-ethoxylates in freshwater, sediments, and biosolids associated with high and low density populations of Buenos Aires, Argentina. CLEAN – Soil Air Water 42 (6), 731–737.

Babin, P.J., Carnevali, O., Lubzens, E., Schneider, W.J., 2007. Molecular aspects of oocyte vitellogenesis in fish. In: Babin, P.J., Cerdà, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, New York, pp. 39–76.

Beale, K.M., Towle, D.W., Jayasundara, N., Smith, C.M., Shields, J.D., Small, H.J., Greenwood, S.J., 2008. Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: molecular characterization and transcriptional response to *Vibrio fluvialis* challenge. Comp. Biochem. Physiol. D 3, 263–269.

Beausoleil, C., Ormsby, J.N., Gies, A., Hass, U., Heindel, J.J., Holmer, M.L., Nielsen, P.J., Munn, S., Schoenfelder, G., 2013. Low dose effects and non-monotonic dose responses for endocrine active chemicals: science to practice workshop: workshop summary. Chemosphere 93, 847–856.

Bowman, C.J., Kroll, K.J., Hemmer, M.J., Folmar, L.C., Denslow, N.D., 2000. Estrogen induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). Gen. Comp. Endocrinol. 120, 300–313.

Bowman, C.J., Kroll, K.J., Gross, T.G., Denslow, N.D., 2002. Estradiol-induced gene expression in largemouth bass (*Micropterus salmoides*). Mol. Cell. Endocrinol. 196, 67–77.

Buckley, J., Maunder, R.J., Foey, A., Pearce, J., Val, A.L., Sloman, K.A., 2010. Biparental mucus feeding: a unique example of parental care in an *Amazonian cichlid*. J. Exp. Biol. 213, 3787–3795.

Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W., 2005. Quantitative real-time RT-PCR – a perspective. J. Mol. Endocrinol. 34, 597–601.

Calabrese, E.J., 2004. Hormesis: from marginalization to mainstream. A case for hormesis as the default dose–response model in risk assessment. Toxicol. Appl. Pharmacol. 197, 125–136.

Calabrese, E.J., 2008. Converging concepts: adaptive response, preconditioning, and the Yerkes–Dodson Law are manifestations of hormesis. Ageing Res. Rev. 7, 8–20.

Calabrese, E.J., Baldwin, L.A., 2001. Hormesis: U-shaped dose responses and their centrality in toxicology. Trends Pharmacol. Sci. 22, 285–291.

Casciotta, J.R., Almirón, A.E., Bechara, J., 2005. Peces del Iberá. Hábitat y Diversidad. Grafikar, La Plata, Argentina; UNDP, Fundación Ecos, UNLP–UNNE, 244 pp.

Céspedes, R., Lacorte, S., Raldúa, D., Ginebreda, A., Barceló, D., Piña, B., 2005. Distribution of endocrine disruptors in the Llobregat River basin (Catalonia, NE Spain). Chemosphere 61, 1710–1719.

Chandra, K., Bosker, T., Hogan, N., Lister, A., MacLatchy, D., Currie, S., 2012. Sustained high temperature increases the vitellogenin response to 17α-ethynylestradiol in mummichog (*Fundulus heteroclitus*). Aquat. Toxicol. 118–119, 130–140.

Chandrasekar, G., Archer, A., Gustafsson, J.A., Andersson Lendahl, M., 2010. Levels of 17b-estradiol receptors expressed in embryonic and adult zebrafish following in vivo treatment of natural or synthetic ligands. PLoS ONE 5 (3), e9678.

Cook, R., Calabrese, E.J., 2006. The importance of hormesis to public health. Environ. Health Perspect. 114, 1631–1635.

Da Cuña, R., Rey Vázquez, G., Piol, N., Verrengia Guerrero, N., Maggese, M.C., LoNostro, F., 2011. Assessment of the acute toxicity of the organochlorine pesticide endosulfan in *Cichlasoma dimerus* (Teleostei, Perciformes). Ecotoxicol. Environ. Saf. 74, 1065–1073.

Da Cuña, R.H., Pandolfi, M., Genovese, G., Piazza, Y., Ansaldo, M., Lo Nostro, F.L., 2013. Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquat. Toxicol. 126, 299–305.

Davis, L.K., Pierce, A.L., Hiramatsu, N., Sullivan, C.V., Hirano, T., Grau, E.G., 2008. Gender-specific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of 17 beta-estradiol in the male tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. 156, 544–551.

Flouriot, G., Pakdel, F., Valotaire, Y., 1996. Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. Mol. Cell. Endocrinol. 124, 173–183.

Genovese, G., Maggese, C., Towle, D., 2008. Differential induction of estrogen receptors in *Cichlasoma dimerus* (Teleostei, Perciformes) exposed to octylphenol. Bull. MDI Biol. Lab. 47, 56–57.

Genovese, G., Da Cuña, R., Towle, D.W., Maggese, M.C., Lo Nostro, F., 2011. Early expression of zona pellucida proteins under octylphenol exposure in *Cichlasoma dimerus* (Perciformes, Cichlidae). Aquat. Toxicol. 101, 175–185.

Genovese, G., Regueira, M., Piazza, Y., Towle, D.W., Maggese, M.C., Lo Nostro, F., 2012. Time–course recovery of estrogen-responsive genes of a cichlid fish exposed to waterborne octylphenol. Aquat. Toxicol. 114–115, 1–13.

Hawkins, M.B., Thomas, P., 2004. The unusual binding properties of the third distinct Teleost estrogen receptor subtype ERβa are accompanied by highly conserved amino acid changes in the ligand binding domain. Endocrinology 145 (6), 2968–2977.

Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C.V., Hara, A., 2006. Multiple piscine vitellogenins: biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Mar. Biol. 149, 35–47.

Jayasundara, N., Towle, D.W., Weihrauch, D., Spanings-Pierrot, C., 2007. Gill-specific transcriptional regulation of Na^+/K^+-ATPase alpha-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: sequence variants and promoter structure. J. Exp. Biol. 210, 2070–2081.

Jin, Y., Wang, W., Sheng, G.D., Liu, W., Fu, Z., 2008. Hepatic and extrahepatic expression of estrogen-responsive genes in male adult zebrafish (*Danio rerio*) as biomarkers of short-term exposure to 17beta-estradiol. Environ. Monit. Assess. 146, 105–111.

Kohn, M.C., Melnick, R.L., 2002. Biochemical origins of the nonmonotonic receptor-mediated dose–response. J. Mol. Endocrinol. 29, 113–123.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lange, I.G., Hartel, A., Meyer, H.H., 2003. Evolution of oestrogen functions in vertebrates. J. Steroid Biochem. Mol. Biol. 83, 219–226.

Lowry, O.M., Rosenbrough, N.J., Farr, L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Martyniuk, C.J., Gerrie, E.R., Popesku, J.T., Ekker, M., Trudeau, V.L., 2007. Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. Aquat. Toxicol. 84, 38–49.

Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881–1892.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERα, ERβ1 and ERβ2. J. Mol. Endocrinol. 32, 975–986.

Meucci, V., Arukwe, A., 2005. Detection of vitellogenin and zona radiate protein expressions in surface mucus of immature juvenile Atlantic salmon (*Salmo salar*) exposed to waterborne nonylphenol. Aquat. Toxicol. 73, 1–10.

Moncaut, N., Lo Nostro, F.L., Maggese, M.C., 2003. Vitellogenin detection in surface mucus of the South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) induced by estradiol-17beta. Effects on liver and gonads. Aquat. Toxicol. 63, 127–137.

Murono, E.P., Derk, R.C., de León, J.H., 1999. Biphasic effects of octylphenol on testosterone biosynthesis by cultured Leydig cells from neonatal rats. Reprod. Toxicol. 13, 451–462.

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad III, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERα2 and both ERβ isoforms. Gene 392 (1–2), 164–173.

Nagler, J.J., Cavileer, T.D., Verducci, J.S., Schultz, I.R., Hook, S.E., Hayton, W.L., 2012. Estrogen receptor mRNA expression patterns in the liver and ovary of female rainbow trout over a complete reproductive cycle. Gen. Comp. Endocrinol. 178, 556–561.

Nelson, J.S., 2006. Fishes of the World, 4th ed. Wiley & Sons, Inc., NJ, USA, pp. 622.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24.

Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J., Kushner, P.J., Scanlan, T.S., 1997. Differential ligand activation of estrogen receptors ERalpha and ERbeta at [1AP] sites. Science 277, 1508–1510.

Palermo, F.A., Cocci, P., Angeletti, M., Polzonetti-Magni, A., Mosconi, G., 2012. PCR–ELISA detection of estrogen receptor β mRNA expression and plasma vitellogenin induction in juvenile sole (*Solea solea*) exposed to waterborne 4-nonylphenol. Chemosphere 86, 919–925.

Pandolfi, M., Cánepa, M.M., Meijide, F.J., Alonso, F., Vázquez, G.R., Maggese, M.C., Vissio, P.G., 2009. Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Percifomes, Cichlidae). Biocell 33, 1–18.

Pennie, W.D., Aldridge, T.C., Brooks, A.N., 1998. Differential activation by xenoestrogens of ERα and ERβ when linked to different response elements. J. Endocrinol. 158, 11–14.

Piazza, Y., Pandolfi, M., Lo Nostro, F., 2011. Effect of the organochlorine pesticide endosulfan on GnRH and gonadotrope cell populations in fish larvae. Arch. Environ. Contam. Toxicol. 61, 300–310.

Rey Vázquez, G., Meijide, F.J., Da Cuña, R.H., Lo Nostro, F.L., Piazza, Y.G., Babay, P.A., Trudeau, V.L., Maggese, M.C., Guerrero, G.A., 2009. Exposure to waterborne 4-tert-octylphenol induces vitellogenin synthesis and disrupts testis morphology in the South American freshwater fish *Cichlasoma dimerus* (Teleostei, Perciformes). Comp. Biochem. Physiol. C 150, 298–306.

Rey Vázquez, G., (Doctoral Thesis) 2012. Espermatogénesis, ciclo reproductivo anual y parámetros hematológicos de *Cichlasoma dimerus* (Teleostei, Perciformes). Efecto de la exposición a 4-tert-octilfenol. Universidad de Buenos Aires, pp. 145.

Rey Vázquez, G., Lo Nostro, F., 2014. Changes in hematological parameters of *Cichlasoma dimerus* (Teleostei, Perciformes) exposed to sublethal concentrations of 4-tert-octylphenol. Arch. Environ. Contam. Toxicol. 66, 463–469.

Routledge, E.J., White, R., Parker, M.G., Sumpter, J.P., 2000. Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) α and ERβ. J. Biol. Chem. 275 (46), 35986–35993.

Southam, C.M., Ehrlich, J., 1943. Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology 33, 517–524.

Suzuki-Yagawa, Y., Kawakami, K., Nagano, K., 1992. Housekeeping Na, K-ATPase α1 subunit gene promoter is composed of multiple cis elements to which common and cell type-specific factors bind. Mol. Cell. Biol. 12, 4046–4055.

Teitsma, C., Lethimonier, C., Tujague, M., Anglade, I., Saligaut, D., Bailhache, T., Pakdel, F., Kah, O., Ducouret, B., 1998. Identification of potential sites of cortisol actions on the reproductive axis in rainbow trout. Comp. Biochem. Physiol. C 119, 243–249.

Tollefsen, K.E., Mathisen, R., Stenersen, J., 2002. Estrogen mimics bind with similar affinity and specificity to the hepatic estrogen receptor in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 126, 14–22.

Trudeau, V., Tyler, C., 2007. Endocrine disruption. Gen. Comp. Endocrinol. 153, 13–14.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.

Villeneuve, D.L., Garcia-Reyero, N., Escalon, B.L., Jensen, K.M., Cavallin, J.E., Makynen, E.A., Durhan, E.J., Kahl, M.D., Thomas, L.M., Perkins, E.J., Ankley, G.T., 2012. Ecotoxicogenomics to support ecological risk assessment: a case study with bisphenol A in fish. Environ. Sci. Technol. 46, 51–59.

vom Saal, F.S., Timms, B.G., Montano, M.M., Palanza, P., Thayer, K.A., Nagel, S.C., Dhar, M.D., Ganjam, V.K., Parmigiani, S., Welshons, W.D., 1997. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc. Natl. Acad. Sci. U. S. A. 94, 2056–2061.

Wang, L., Ying, G.G., Chen, F., Zhang, L.J., Zhao, J.L., Lai, H.J., Chen, Z.F., Tao, R., 2012. Monitoring of selected estrogenic compounds and estrogenic activity in surface water and sediment of the Yellow River in China using combined chemical and biological tools. Environ. Pollut. 165, 241–249.

Weltje, L., vom Saal, F.S., Oehlmann, J., 2005. Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. Hum. Exp. Toxicol. 24, 431–437.

White, R., Jobling, S., Hoare, S.A., Sumpter, J.P., Parker, M.G., 1994. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135, 175–182.

2006. World Medical Association Statement on Animal Use in Biomedical Research, http://www.wma.net/en/30publications/10policies/a18/

Woods, M., Kumar, A., Barton, M., Woods, A., Kookana, R., 2009. Localisation of estrogen responsive genes in the liver and testis of Murray rainbowfish *Melanotaenia fluviatilis* exposed to 17beta-estradiol. Mol. Cell. Endocrinol. 303, 57–66.

Wu, M., Wang, L., Xu, G., Liu, N., Tang, L., Zheng, J., Bu, T., Lei, B., 2013. Seasonal and spatial distribution of 4-tert-octylphenol, 4-nonylphenol and bisphenol A in the Huangpu River and its tributaries, Shanghai, China. Environ. Monit. Assess. 185, 3149–3161.

Yadetie, F., Arukwe, A., Goksøyr, A., Male, R., 1999. Induction of hepatic estrogen receptor in juvenil Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. Sci. Total Environ. 233, 201–210.

Zhu, P., Zhang, Y., Zhuo, Q., Lu, D., Huang, J., Liu, X., Lin, H., 2008. Discovery of four estrogen receptors and their expression profiles during testis recrudescence in male *Spinibarbus denticulatus*. Gen. Comp. Endocrinol. 156, 265–276.