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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,

pH 6.8, 3% sodium dodecyl sulfate, 10% glycerol, 2% bromophenol blue and 1% β-mercaptoethanol), boiled for 5 min and briefly spun down before loading them into polyacrylamide gel wells. Molecular weight standard was loaded in a separate well (SeeBlue Plus2 Pre-Stained Standard, Invitrogen, USA). A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970), was performed at constant 100 V using 4% stacking and 9% separating gel (Mini-Protean III, Bio-Rad, USA), and 124 mM Tris–HCl, pH 8.8 running buffer. Transference to nitrocellulose membranes (ECL Amersham Biosciences, UK) was achieved at 100 V for 90 min, in 25 mM Tris, 187 mM glycine and 20% methanol.

Subsequently, membranes were soaked with TTBS (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5), and endogenous peroxidases were blocked with 2% 30 vol H_2O_2 in TTBS for 5 min. Afterwards, non-specific binding sites were blocked with 3% skimmed milk and 3% BSA in TTBS overnight at 4 °C.

According to methods described previously for this species by Genovese et al. (2011), the immunodetection of *zona pellucida* proteins (ZP) was performed using mouse anti-salmon ZP monoclonal antibody (*Salmo salar*; MN-8C4, Biosense Laboratories, Norway) 1:500 overnight at 4 °C. For vitellogenin (VTG) immunodetection, membranes were incubated with rabbit anti-perch VTG antiserum 1:2000 for 90 min at RT (*Perca fluviatilis*; donated by Dr. B. Allner, Germany).

After three 5 min washes with TTBS, membranes were incubated with HRP anti-mouse secondary antibody for ZP and enzyme-linked enhanced chemiluminescence (Amersham Biosciences, UK) was performed. For VTG, biotinylated anti-rabbit secondary antibody 1:1000 for 1 h at RT; then ABC kit (Dako, USA) 1:3000 for 1 h, and 0.1% 3,3′-diaminobenzidine in Tris–HCl buffer (Dako, USA) for 5 min were used. In both cases, omission of the primary antibody was also performed and no ir-bands were detected in any sample (data not shown). Membranes were scanned and molecular weights were estimated using SigmaGel software (Jandel Scientific software 1.0, USA).

2.5. RNA extraction, sequencing, and quantitative mRNA 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). The results showed complete absence of degradation products or genomic DNA. 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 primer.

Degenerated primers were designed based on full-length VTG sequences published in the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov), accession numbers AF017250.1 for *Oreochromis aureus*, and AB074891.1 for *Oryzias latipes*. Conventional PCR was performed at an annealing temperature of 45 °C using RedTaq polymerase (Sigma REDTaq® ReadyMixTM PCR Reaction Mix), and forward and reverse primers (5′-ATHAARTTYGARTAYWSNAAYGGNGTNGT-3′, and 5′-TTRTCDATRTTNGCRAANGCNATYTCYTG-3′, respectively). Amplification products were isolated electrophoretically on 0.8% agarose gels (Promega, USA) and, following gel extraction with a MiniElute Gel Extraction Kit (Qia-gen, USA), they were sequenced and analyzed with Chromas 2.33 (http://www.technelysium.com.au). PCR products were subcloned using TA cloning (Invitrogen, USA) and inserts in individual plasmid preparations were then sequenced. The partial VTG sequence of 715 bp obtained was submitted to GenBank (accession number EU081907.1).

Primers specific to the VTG sequence of *C. dimerus*, designed using Primer Premier software, (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 and primer dimers. As done previously by several authors (Jayasundara et al., 2007; Beale et al., 2008; Genovese et al., 2011), 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. Reference liver preparations from estrogenized females, shown to exhibit high expression levels, served as the basis for a standard dilution series, demonstrating a linear relationship between threshold cycle (Ct) and $\log_{10}$ of template availability, used for calculating relative abundance values in the remaining samples.

Quantitative mRNA expression of two forms of ZP (ZPA and ZPB) was performed following the protocol detailed in Genovese et al. (2011). For comparison, the expression of a "non-gender specific gene", Na^+/K^+-ATPase, was also analyzed. In order to obtain the Na^+/K^+-ATPase sequence of *C. dimerus*, we used degenerated primers previously designed at Mount Desert Island Biological Laboratory for sequencing gill α-subunit of Na^+/K^+-ATPase of *Pachygrapsus marmoratus* crab (Crustacea, Decapoda), according to Jayasundara et al. (2007). These primers proved to work in other metazoans (Towle DW, personal communication). As a positive control for conventional PCR, cDNA of posterior gill 7 of the same crab was used (Jayasundara et al., 2007). The forward (NAK10F) and reverse (NAK16R) degenerated primers used were 5′-ATGACIGTIGCICAYATGTGG-3′ and 5′-GGRTGRTCICCIGTIACCAT-3′, respectively. After confirming with BLAST analysis, the partial Na^+/K^+-ATPase sequence of 656 bp was submitted to GenBank (accession number JN993160). For quantitative gene expression, *C. dimerus* specific primers were designed: forward primer 5′-ACTCTGCAACCGTGCCGTCTTT-3′ and reverse primer 5′-AGCGTCCTTCATCTCATCATCC-3′, following the above protocol but at an annealing temperature of 55 °C.

Alignment of sequences between different species and a cladogram showing estimated phylogenetic relationship of VTG forms was performed with ClustalW2 available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

2.6. Histological analysis

Liver samples were dehydrated in alcohol and embedded in Paraplast® (Oxford, USA). 7 μm 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

Non-parametric (Kruskal-Wallis) test followed by Dunn's multiple comparisons was performed for gene expression analysis. A free version of Graph Pad Prism 4 software was used (http://www.graphpad.com).

3. Results

3.1. OP concentrations and mortality

In this study, the nominal (150 μg/L) and the initial actual (152 ± 1 μg/L) OP levels were comparable. Actual OP concentration decreased with time, reaching values of 60 ± 2 μg/L at 24 h and 50 ± 0.5 μg/L at 48 h. At 72 h actual OP values decreased 80% from the initial concentration (32 ± 2 μg/L); at that time water and OP solution was renewed. OP was neither detected in water samples from the control treatment nor in OP-free water at any time.

The dose of E_2 injected to females and the OP concentration assayed in waterborne exposed males, were not lethal under the conditions tested since no mortality was registered.

3.2. Partial sequencing and gene expression

A broad and clear band of approximately 700 bp was obtained after gel electrophoresis of PCR products using degenerated primers previously designed for Na^+/K^+-ATPase of the marbled crab *Pachygrapsus marmoratus* and liver cDNA of female of *C. dimerus* induced with 17β-estradiol (data not shown). A positive control of PCR was run in parallel using the same primers and posterior gill cDNA of *P. marmoratus*. Purification of the band and sequencing analysis yielded a partial sequence of *C. dimerus* Na^+/K^+-ATPase of 656 bp. Fig. 1A shows a 63–74% homology between the partial sequence of gill Na^+/K^+-ATPase of the crab *P. marmoratus* with species of other animal groups. Alignment of liver Na^+/K^+-ATPase obtained for *C. dimerus* was compared with available sequences of different vertebrate species (Fig. 1A). Amino-acid sequence similarities evidenced 95 and 92% homology between Na^+/K^+-ATPase of *C. dimerus* and other cichlid fish such as blackchin tilapia *Sarotherodon melanotherodon* and Mozambique tilapia *Oreochromis mossambicus*, respectively, and 87% homology with zebrafish *Danio rerio*. When compared to tetrapods (*Xenopus laevis*, *Gallus gallus* and *Mus musculus*) the homology was 80–82%.

Comparison of *C. dimerus* vitellogenin partial sequence to VTGAb of other teleosts showed 91% homology with chameleon cichlid fish *Australoheros facetus*, 79% homology with blue tilapia *O. aureus*, and 72% homology with white perch *Morone americana* and red seabream *Pagrus major*, all perciform species (Fig. 1B). A cladogram that shows estimated phylogenetic relationships of different VTG forms between fish species is depicted in Fig. 1C. VTGAb of species belonging to other orders such as rainbow trout *Oncorhynchus mykiss*, common carp *Cyprinus carpio*, Japanese medaka *Oryzias latipes*, flathead mullet *Mugil cephalus*, and Western mosquitofish *Gambusia affinis* showed 62–69% homology with *C. dimerus.* Comparison of *C. dimerus* VTG with VTGAa of other species showed 49–59% homology. Vitellogenin C showed 25–31% homology to the *C. dimerus* VTG partial sequence.

After exposure to a nominal concentration of 150 μg/L OP for 28 days, male ZP and VTG expression was markedly up-regulated. More than a 7000-fold increase was observed for ZPB and 300-fold for ZPC and VTG when compared with control male baseline levels (Fig. 2, 28 days of OP vs. Ctrl). Values obtained after OP exposure were in the same order as those obtained for estrogenized females.

Fig. 2 depicts the time course recovery of estrogen-responsive genes after transfer of exposed male fish to OP-free water for 28 days. ZPB transcripts decreased immediately after transfer of fish to clean water and from day 3 onwards values were similar to those of control males, with the exception of day 14. The ZPB expression obtained at the end of the recovery period (28 days of OP-free water) was 0.5% of maximum ZPB expression levels obtained after 28 days of OP exposure (Fig. 2A). ZPC expression decreased in a time-dependent manner, but was still up-regulated until day 3; from day 7 onwards values rapidly reached control levels (Fig. 2B; 1.7% of OP-induced expression). VTG remained highly induced till day 7, but gradually returned to baseline levels from day 14 to day 28 of recovery. By the end of the experiment, 1.8% of the maximum VTG expression due to OP was found (Fig. 2C).

Liver Na^+/K^+-ATPase gene expression was strongly down-regulated in OP-treated *C. dimerus* males (Fig. 2D). This inhibition was 61% of control values after 28 days of OP exposure and 48% of control values on day 1 following transfer to clean water. Na^+/K^+-ATPase transcripts reached baseline levels on day 3 following transfer to clean water and remained similar to control fish values till the end of the recovery period. A significant up-regulation of Na^+/K^+-ATPase was detected on day 21 (Fig. 2D).

3.3. Immunodetection of vitellogenin and zona pellucida proteins in plasma and mucus samples

SDS-PAGE followed by Western blot of plasma and mucus samples of estrogenized *C. dimerus* females, used as positive controls, revealed three ir-ZP bands: 62, 52 and 50 kDa (Fig. 3A, E_2 lane), and two ir-VTG bands: 75 and 64 kDa (Fig. 3, E_2 lane). Control males did not exhibit any ZP or VTG ir-bands (data not shown).

After 28 days of exposure to a nominal concentration of 150 μg/L OP, plasma of male fish became ZP- and VTG-immunoreactive (Fig. 3A and C, lane 0d + OP), showing a similar ZP pattern to that found in induced females and the addition of two heavier bands for VTG (140 and 103 kDa). A similar ZP band pattern was detected in mucus samples of males exposed to OP for 28 days and that of estrogenized females (Fig. 3B). However, for mucus, the band pattern of VTG in males exposed to OP for 28 days showed only two ir-bands (116 and 103 kD bands: Fig. 3D).

On day 1 of transfer of exposed fish to aquaria free of OP, plasma ZP was still immunodetected. After 3 days bands were no longer evidenced. Mucus samples were free of ZP ir-bands from day 1 of recovery till the end of the experiment (Fig. 3A and B). Four VTG ir-bands (140, 103, 75 and 64 kDa) were evident in plasma samples from day 1 till day 7 following transfer to clean water but only three bands (103, 75 and 64 kDa) could be detected later on, until day 28, when no bands were detected (Fig. 3C). In mucus samples two ir-VTG bands (116 and 103 kDa) were detected from days 1 through 7 of recovery and no VTG signal was found from day 14 till the end of the experiment (Fig. 3D).

3.4. Effects on liver

Hepatosomatic index (HSI) in control male fish was 1.5 ± 0.2%. A significant increase in HSI was found in fish treated with OP for 28 days (3.05 ± 0.5%; Table 1). Throughout the recovery period, HSI never recovered to control levels, though values were not statistically different from those of control fish, with the exception of HSI on day 14.

As it was previously reported by Genovese et al. (2011), polyhedral hepatocytes in normal male liver of *C. dimerus* were arranged in rows around blood sinusoids; these cells had central nucleus with one nucleolus and weakly eosinophilic vacuolated cytoplasm in hematoxylin-eosin stained sections (Fig. 4A). Interdispersed pancreatic tissue could also be seen (data not shown). On the contrary, the liver of males exposed to a nominal concentration of 150 μg/L OP showed general tissue disarrangement,

A

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Cichlasoma        ----------------------------------------TENQSGTSFDRSSPTWA 17
Sarotherodon      NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDRSSPTWA 419
Oreochromis       NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGTSFDRSSATWA 419
Danio             NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDSQIHEADTTENQSGTTFDRSSPTWS 420
Gallus            NLEAVGTLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGASFDKSSATWL 417
Mus               NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGVSFDKTSATWF 419
Xenopus           NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNQIHEADTTENQSGASFDKSSPTWT 419
Pachygrapsus      NLEAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDNTIIEADTSEDQSGCQYDKSSEGWK 324
                                                          :*:***  :*::*  *

Cichlasoma        ALSRIAGLCNRAVFLADQANIPILKRDVAGDASEAALLKCIELCCGSVSGMREKYPKIAE 77
Sarotherodon      NLSRIAGLCNRAVFLADQSNIPILKRDVAGDASEAALLKCIELCCGSVSGMREKYPKIAE 479
Oreochromis       NLSRIAGLCNRAVFLADQSNIPILKRDVAGDASEAALLKCIELCCGSVNEMREKYPKIAE 479
Danio             ALARVAGLCNRAVFLADQRNVPILKRDTAGDASESALLKCIELCCGSVNEMREKYTKIAE 480
Gallus            ALSRIAGLCNRAVFQANQENVPILKRAVAGDASESALLKCIELCCGSVKEMRERYPKVVE 477
Mus               ALSRIAGLCNRAVFQANQENLPILKRAVAGDASESALLKCIEVCCGSVMEMREKYSKIVE 479
Xenopus           ALSRVAGLCNRAVFQAGQENTPILKRDVAGDASESALLKCIELCCGSVRDMREKNHKVAE 479
Pachygrapsus      TLSRIAALCNRAEFKTGQEDVPILKREVNGDASEAALLKCVELAVGDVRGWRSRNKKVCE 384
                   *:*:*.***** * :.* : ***** . *****:*****:*:. *.*   *.:  *: *

Cichlasoma        IPFNSTNKYQLSIHKNTTPGESKHLLVMKGAPERILDRCSTIVIQGKEQPLDDEMKDAFQ 137
Sarotherodon      IPFNSTNKYQLSIHKNTTPGETKHLLVMKGAPERILDRCSSIMLQGKEQPLDDEMKDAFQ 539
Oreochromis       IPFNSTNKYQLSIHKNTTPGETKHLLVMKGAPERILDRCNSIVLQGKVQALDDEMKDAFQ 539
Danio             IPFNSTNKYQLSIHKNPNSSEPKHLLVMKGAPERILDRCSTILIQGKQQPLDEEMKDAFQ 540
Gallus            IPFNSTNKYQLSIHKNANAGESRHLLVMKGAPERILDRCDSILIHGKVQPLDEEIKDAFQ 537
Mus               IPFNSTNKYQLSIHKNPNASEPKHLLVMKGAPERILDRCSSILLHGKEQPLDEELKDAFQ 539
Xenopus           IPFNSTNKYQLSVHKNANPSESRYILVMKGAPERILDRCTSIILQGKEQPLDEELKDAFQ 539
Pachygrapsus      IPFNSTNKYQVSIHETQDKNDPRYLVVMKGAPERILERCSTIFMNGEEKPLDEEMKESFN 444
                  **********:*:*:.   .:.::::***********:** :*.::*: :.**:*:*::*:

Cichlasoma        NAYVELGGLGERVLGFCQFHLPDDQFPQGFAFDTDEVNFPTENLCFIGLMAMIDPPRAAV 197
Sarotherodon      NAYVELGGLGERVLGFCHFHLPDDQFPEGFAFDTDEVNFPTENLCFVGLMAMIDPPRAAV 599
Oreochromis       NAYVELGGLGERVLGFCHYHLPDDEFPEGFAFDTDEVNFPTENLCFVGLMAMIDPPRAAV 599
Danio             NAYVELGGLGERVLGFCHFCLPDDQFPEGFAFDTEEVNFPTENLCFVGLMSMIDPPRAAV 600
Gallus            NAYLELGGLGERVLGFCHLALPDDQFPEGFQFDTDEVNFPVEKLCFVGLMSMIDPPRAAV 597
Mus               NAYLELGGLGERVLGFCHLLLPDEQFPEGFQFDTDDVNFPVDNLCFVGLISMIDPPRAAV 599
Xenopus           NAYLELGGLGERVLGFCHLALPDDQFPDGFQFDTEEVNFPTENLCFVGLISMIDPPRAAV 599
Pachygrapsus      NAYLELGGLGERVLGFCDYMLPSDKYPLGYPFDADSVNFPVHGLRFVGLMSMIDPPRAAV 504
                  ***:**************.  **.:::* *: **::.****.. * *:**::********* 

Cichlasoma        PDAVG---------------------------------------------------------- 202
Sarotherodon      PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPVSEVNPRD 659
Oreochromis       PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNVPVSEVNPRD 659
Danio             PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPVGEVNPRD 660
Gallus            PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISDGNETVEDIAARLNIPVSQVNPRD 657
Mus               PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPVNQVNPRD 659
Xenopus           PDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIISEGNETVEDIAARLNIPVNQVNPRD 659
Pachygrapsus      PDAVAKCRSAGIKV---------------------------------------------- 518
                  ****.
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Fig. 1. (A) Alignment of the partial sequence of Na^+/K^+-ATPase alpha-subunit of *Cichlasoma dimerus* and other species. Tilapia fish species: *Sarotherodon melanotherodon* (ADB03120.1), *Oreochromis mossambicus* (AAD11455.2) and zebrafish *Danio rerio* (AAH54630.1), african clawed frog *Xenopus laevis* (AAC59759.1), chicken *Gallus gallus* (NP_990852.1), house mouse *Mus musculus* (AAH33435.1) and marbled crab *Pachygrapsus marmoratus* (ABA02166.1). (B) Alignment of partial sequence of vitellogenin of *Cichlasoma dimerus* and other teleosts: chameleon cichlid *Australoheros facetus* VTGA partial sequence (ABC86694.1), blue tilapia *Oreochromis aureus* VTGAb (AAD01615.1), common carp *Cyprinus carpio* VTGAb (BAF73406.1), white perch *Morone americana* VtgAa (AAZ17415.1), VtgAb (AAZ17416.1), VtgC (AAZ17417.1), red seabream *Pagrus major* VtgAa (BAE43870), VtgAb (BAE43871.1), VtgC (BAE43872.1), flathead mullet *Mugil cephalus* VTGAa (BAF64835.1), VTGAb (BAF64836.1) VTGC (BAF64837.1), Japanese medaka *Oryzias latipes* VTGAb (BAB79591.1), western mosquitofish *Gambusia affinis* VTGAa (BAD93697.1), VTGAb (BAD93698.1), VTGC (BAD93699.1), Japanese sillago *Sillago japonica* (VtgAa BAC20186.1), orange-spotted grouper *Epinephelus coioides* VtgAa (AAW29031.1), rainbow trout *Oncorhynchus mykiss* VTGA (CAA63421.1), Mozambique tilapia *Oreochromis mossambicus* VTGC (ABQ81855.1), *Acanthogobius flavimanus* VtgAa (BAC06190.1), VtgC (BAC06191.1). The lysine (**K**) residue of the short motif HLT**K**SKDL is presumed to be a key functional residue in the putative receptor-binding domain of vitellogenin. The motif RGILN is widely conserved between vertebrates. Blanks (-) are inserted to optimize alignment of the sequences. (*) means that amino-acids in that column are identical in all sequences in the alignment. (:) means that conserved substitutions have been observed, according to amino-acid type. (.) means semi-conserved substitutions. (C) Cladogram showing estimated phylogenetic relationship of VTG forms between species used in (B).

hepatocytes with eccentric and euchromatic nuclei and conspicuous nucleoli, and intense perinuclear basophilia (Fig. 4B). A decrease in the vacuolization of the cytoplasm was also evident. Several eosinophilic granule cells were mostly seen around main blood vessels and pancreatic tissue. Inside blood vessels, homogeneous content was intensely stained with eosin surrounding oval erythrocytes (Fig. 4B). Liver damage was clearly evident at the beginning of the recovery period (Fig. 4C), but at the end of the experiment signs of exposure were less pronounced: rows of swollen hepatocytes were identified, eccentric nuclei and cytoplasmic vacuolization with eosinophilic content was seen (Fig. 4D).

B

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CichlasomaVtg                ------ALIKFEYSNGVVSRVSAPAGVSTTVLNIYRGILNILQLNVKKTQ 44
AustraloherosVtgpartial      LAAQLSTPIKFEYANGVVSRVSAPAGVSTPVLNIYRGILNILQLNVKKTQ 70
OreochromisVtgAb             LATQLSTPIKFEYTNGVVGRLAAPPGVSTTVLNIYRGIINLLQLNVKKTQ 148
MoroneVtgAb                  LAAQLLTPIKFEYTNGVVGRVFAPAGVSVTVLNIYRGILNIFQLNIKKTQ 148
PagrusVtgAb                  LAAQLLTPIKFEYANGVVGKVFAPAGVSATVLNIYRGILNILQLNIKKTQ 148
MugilVtgAb                   LAAQLLTPIKFEYANGVVGKVFAPAGVSATVLNIYRGILNILQLNIKKTQ 148
OryziasVtgAb                 LAAQLLTPIKFEYTNGVVGKVFAPPGISTSVLNIYRGLLNILQLNIKKTQ 148
GambusiaVtgAb                LSAQLLTPIKFEYANGVIGKVYAPAGVSTAVLNIFRGILNIFQINIKKTM 148
CyprinusVtgAb                LAAQLQIPIKFEYANGVVGKVFAPAGVSPTVLNLHRGILNILQLNLKKTQ 148
PagrusVtgAa                  LAAQLMTPIKFEYTNGVVGKMFAPEGVSTMVLNVYRGILNVLQLNIKKTH 148
SillagoVtgAa                 LAAQLMTPIKFEYVNGVVGKMFAPEGISTMVLNVYRGILNVLQLNIKKTQ 148
MoroneVtgAa                  LAAQLMIPIKFEYANGVVGKMFAPEGISTMVLNVHRGILNVLQLNIKKTQ 148
EpinephelusVtgAa             LAAQLLTPIKFEYANGVVGKVFAPEGISTMVLNVYRGILNVLQLNIKKTQ 113
MugilVtgAa                   LAAQLVTPIKFEYVSGVVGKIFAPEGISTMVLNIYRGILNVLQLNIKKTQ 150
GambusiaVtgAa                LAPQFAIPIKFEYTNSVVGKIFAPEGVSPVVLNIHRGIVNVLQLNIKKTH 148
OncorhynchusVtgA             LAAQFSIPIKFEYAKGVVGKVLAPTAVSETVLNVHRGILNILQLNIKKTQ 148
AcanthogobiusVtgAa           LEAHLKTPIKFRYNNGTVREILAPESVPTMVINMYRGILNVLQLNIKKTQ 147
MoroneVtgC                   IAAQLIKPFMFNYTCGHVSDIHASAEISDTVVNIVRGILGFFQVTVKTTQ 148
PagrusVtgC                   IAAQIVKPFMFDYTSGHVGDIRAPAEVSDTVVNIVRGILGFLQVTVKTTQ 148
MugilVtgC                    IAAQLAKPFMFEYASGHVGDIRAAAEISDTVVNIVRGILGFFQFTVKTTQ 148
OreochromisVtgC              LAAQLAKPFKFEHSNGHVRQIQAAPDVSETTVNIVRGILSFFHATVKTSP 102
GambusiaVtgC                 ITSELAQPFLFEYDNGHIGDISGHSKVSTTVVNIIRGILSFFQVTVKNTQ 148
AcanthogobiusVtgC            IAAQLEKPIGFTYTAGNVGEIKASPEVSDTVVNIVRGILGFFQVTVKSNQ 149
                                   : * :  . :  : .   :.  .:*: **::..:: .:*..

CichlasomaVtg                NVYELQESGVHGVCKTQYVIREDAKAERIHLTKSKDLNHCQERIVKDIGL 94
AustraloherosVtgpartial      NVYELQESGVHGVCKTQYVIREDAKAERIYLTKSKELNHCQERIVKDIGL 120
OreochromisVtgAb             NVYEMQESGAHGVCKTNYVIREDARAERIHLTKTKDLNHCQEKIMKAIGL 198
MoroneVtgAb                  NVYELQEPGAQGVCKTHYVISEDAKADRILLTKTKDMNQCQERIIKDIGL 198
PagrusVtgAb                  NVYELQEPGAQGVCKTHYVISEDAKADRILLTKTKDLNHCQERIVKDIGL 198
MugilVtgAb                   NVYELQEPGAQGVCKTHYAISEDAKADRIVLTKTKDLNHCQERIIKDIGM 198
OryziasVtgAb                 NVYELQEPGTQGVCKTHYVISEDSKADRIHLSKTKDLNHCQERIYKDVGL 198
GambusiaVtgAb                NVYDLQETGVKGVCKTSYVLKEDPKADRLHLTKTTDLNLCTEKINMDVGM 198
CyprinusVtgAb                NIYELQEAGAQGVCRTHYVISEDPKANHITVTKSKDLSHCQERIMKDVGL 198
PagrusVtgAa                  NVYELQEAGAQGVCKTLYAIAEDEKAERILLTKTRDLNHCQEKIMKDLGL 198
SillagoVtgAa                 NIYELQEGGAQGVCKTLYAITEDEKADRILLTKTRDLNHCQEKIIKDIGL 198
MoroneVtgAa                  NVYELQEAGAQGVCKTLYAITEDDKAERILLTKTRDLNHCQEKIIKDLGL 198
EpinephelusVtgAa             NVYEMQEAGAQGVCKTLYAISEDEKAERILLTKTRDLNHCQEKVIKDMGL 163
MugilVtgAa                   NVYELQEAGTQGVCKTLYAISEDEKAERILLTKTRDMNQCQEKIIKDMGL 200
GambusiaVtgAa                KVFDLQEVGTQGVCKTLYSISEDARNENILLTKTRDLNNCQERLIKDMGL 198
OncorhynchusVtgA             NVYELQEAGAQGVCKTHYVIREDAKAERIHLTKSKDLNNCQQRIMKDFGL 198
AcanthogobiusVtgAa           NIYDLQEEGVQGVCRTQYAITENEKTERILLTKSRNLNQCQEKVMKDIGL 197
MoroneVtgC                   RIYELEEVGIHGKCQSNYATEENMETKDMTITQVVDVSGCREKAAIYRGM 198
PagrusVtgC                   TVYELEEVGIHGKCQSNYATEENTETKDMTITQVVDVGACKEKAAIYRGM 198
MugilVtgC                    RIYELEEIGIHGKCQSNYATEENTETQDMTITQVVDVSNCREKAAIYRGM 198
OreochromisVtgC              TVYELEEAGIHGVCQSNYATEINKQTNNMNITQVVDIDNCRMKVAMQNGM 152
GambusiaVtgC                 TIYELRETGIHGNCHNDYAIEEKEGTKDWIVTQVVDVTNCWERAAMYSGM 198
AcanthogobiusVtgC            DIYELEEIGIHGKCLSSYATKINEQEKVMDLTQVVDVTNCREKAMFQTGM 199
                              ::::.* * :* * . *    .   .   :::  ::  *  :     *:

CichlasomaVtg                D-FLEKCHDCEARGKALEGTASYNYIMKPTPSGSLIMEAVATEVIQFSPF 143
AustraloherosVtgpartial      E-FQEKCHECKARGKALEGTASYNYIMKPTPSGSLIMEAVATEVIQFSPF 169
OreochromisVtgAb             E-HVEKCHDCEARGKSLKGTASYNYIMKPAPSGSLIMEAVAREVIEFSPF 247
MoroneVtgAb                  A-YTEKCVECEARGKILKGAAAFNYIMKPTATGALLLEATATELIQFSPF 247
PagrusVtgAb                  A-YTERCVECEARGTTLKGTAAFNYVMKPTATGALLLEATATELIQFSPF 247
MugilVtgAb                   A-YTQTCFECDARGKTLKGAAAFNYIMKPAPTGALILEASATELIQFSPF 247
OryziasVtgAb                 AGYTESCTECEASGKTMKGAAAINYVMKPSTTGSLILEATATELIQYSPI 248
GambusiaVtgAb                AGYTNKCEECVRRGKVFSGAMSLSYVMKPSASGALILEATATELLQYSPF 248
CyprinusVtgAb                A-YTERCAECTERIKSLIETATYNYIMKPASAGVLITEATVEEVHQFSPF 247
PagrusVtgAa                  A-YTEKCAKCQQDSKNLRGATAYNYILKQAPSGIVILEAAVNELIQFSPF 247
SillagoVtgAa                 A-YIEKCPKCQQESKNLRGATAYNYILKPVASGILILEAAVNELIQFSPF 247
MoroneVtgAa                  A-YTEKCAKCQQDSKNLRGATAYNYILKPVASGILILEAAVNELIQFSPF 247
EpinephelusVtgAa             A-YTEKCAKCQQDSKNLRGATSYNYVLKPVGHGILILEATVNELIQFSPF 212
MugilVtgAa                   A-YTEKCARCQQDSKNLRGATSYNYILKPVASGIMILEVAVNEVIQFSPF 249
GambusiaVtgAa                A-YTQKCERCQEETKNLRGTTTFSYTLKPVANAIMILKVDVNELIQFLPF 247
OncorhynchusVtgA             A-YTEKCVECRQRGEALMGAATYNYLMKPADNGALILEATVTELHQFTPF 247
AcanthogobiusVtgAa           A-YTKTSHKYQQDSKSLRGTTGYTYKLKPVPGGTIIEEATGNEIIQFTPF 246
MoroneVtgC                   A-TAVLDKVSKQRGESVISTVRYVYTVKATEEGGLITRAHGLERQHFSSF 247
PagrusVtgC                   A-TAVLDQVSKQRGESVISTVRYVYTVKPTAEGGLITRAHGLERQHFSPF 247
MugilVtgC                    A-TAVLDKISKQRGESVISTVRCVYTVKPTAEGGLITRAHGLERQHFSPF 247
OreochromisVtgC              A-SAVLDKDSKQRGESVISTVQYLYTIQPTEEGGIVKSAYGLEQQHFSPF 201
GambusiaVtgC                 A-TAEWDRLAKERGENVISSMKYNYTIKPTGDGGLITRAQGFERQHFSAF 247
AcanthogobiusVtgC            A-TAVEDKVSRQRGESVFSTVKYTYNIKATEEAGLITKAQALELQFFTPF 248
                                       .  :    * ::    . ::  .   *   : .:

CichlasomaVtg                NILNGAAQMQSRQNLTFVNMENTPVEPARNDYVQHGSLQYEYGREVLQTP 193
AustraloherosVtgpartial      NILNGAAQMQSRQNLTFLNMENSPVAHARNDYVQHGSLQYEYGREVLQMP 219
OreochromisVtgAb             NILNGAAQMESKQILTFLDIENTPVDHARYTYVHRGSLQYEHGSEILQTP 297
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Fig. 1. (*Continued*)

4. Discussion

Whenever a static renewal system is employed to perform chemical compounds exposure experiments, the difference between measured and nominal concentrations in exposure aquaria tends to be especially noticeable due to glass adsorption, uptake by fish, microbial degradation, and photolysis (Lewis and Lech, 1996). We found a marked decrease in OP levels with time, reaching less than 20% of the nominal concentration by the time of water renewal, ensuring that fish were exposed to concentrations comparable to those reported in field studies (Céspedes et al., 2005). Since OP has a half-life of 1.7 days in liver of fish but does not accumulate in that organ (Pedersen and Hill, 2002; Pedersen et al., 2003), a repeated exposures design guaranteed high levels of this contaminant for longer periods, which could cause endocrine disruption in exposed *C. dimerus*, as it was previously demonstrated

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MoroneVtgAb                NILNGAAQMEAKQILTFLEIEKTPVVPIRADYLHRGSLQYEFGSELLQTP 297
PagrusVtgAb                NILNGAAQMEAKQSLTFLEIQNTPVEPIRAEYLHRGSLQYEFGSELLQTP 297
MugilVtgAb                 NILNGAAQMEAKQILTFLEIKRAPLEPIRAEYLHRGSLQYEFGSELLQTP 297
OryziasVtgAb               NILNGAAQMEAKQTLTFLEIKKIPVEPISADYLPRGSLKYEFGSELLQTP 298
GambusiaVtgAb              NIVNGAAQMEARQNITFVDVKKTPLVPIKADYVPRGTLKYELSTEVLQTP 298
CyprinusVtgAb              NEIHGAAQMEAKQTLAFVEIEKTLVVPIKADYLARGSLQYEFATEILQTP 297
PagrusVtgAa                TEMNGAAQMQTKQSLVFLEIQKAPIVPITAQYLHRGSLKYEFSTELLQTP 297
SillagoVtgAa               NQMNGATQMQTKQSLVFLEIQRAPIVPIEAQYLHRGSLKYEFSTELLQTP 297
MoroneVtgAa                TELNGAAQMQTKQSLVFLEIQRATILPNEAQYLHRGSLKYEFSTELLQTP 297
EpinephelusVtgAa           AEMNGAAQMETKQSLVFLEIQRAPIVPIQAEYLHRGSLKYEFSTELLQTP 262
MugilVtgAa                 SELNGAAQMETRQSLVFLEIQGAPIVPIEAQYIDRGSLKYQFSSELLQTP 299
GambusiaVtgAa              SEDNGATQMRTKQSFQFLEIQKEPITPINAVYRHRGSLKYEFANELLQAP 297
OncorhynchusVtgA           NEMSGAAQMEAKQMLTFVEIKKDPIIVPDNNYVHRGSIRYEFATEILQMP 297
AcanthogobiusVtgAa         NELNGVASMQTLQHLVFIQVENAPIVPISAEYRHRGSLKYEFSTEIQQSP 296
MoroneVtgC                 NVKGGSFKMQAMKEIVLLGVSDTARAVIFGPMENKGNLVYKFVNAEANVP 297
PagrusVtgC                 NVKGGSFKMQAMKEMVLLCVSDTARAFTYGPMESKGNLVYKFVNAEANVP 297
MugilVtgC                  NVKGGSFKMQAMKEMVLLGVSDTARAITFGPMESKGNLVYKFVNAEANIP 297
OreochromisVtgC            NVKRGSFRMEAKKEMVLIRVSEGGKPANVGQLQNRGDLVHKLVNSDANIP 251
GambusiaVtgC               NVKGGSFKMEATKDLVLLSMNRTARGRTYGPLEKKGKIIYSFEDVDINIP 297
AcanthogobiusVtgC          NLKGGTFKMEAMKELVLTTVKDKTQDTPNRQMESRGNIVYKVVKNWANVP 298
                                *   *.: : : :  :.             :* : :.      : *
CichlasomaVtg              IHLLKVTNAEEQIVNTMNHLVASNVDRVHEDAPLKFVELIQLLRV----- 238
AustraloherosVtgpartial    IHLLRVTNAEEQIVSTVNHLVASNMDKVHEDAPLKFVELIQLLRVARFET 269
OreochromisVtgAb           IHLLRVTHAEAQIVSTLNHLVASNVAKVHEDAPLKFVELIQVMRVARFET 347
MoroneVtgAb                IQLLKISNAEAQIVEVLNHLVTFNAAKVHEDAPLKFIELIQLLRVARYES 347
PagrusVtgAb                IQLLRISNAEAQMVEILNHLVANNVAKVHEDAPLKFIELIQLLRVARFEN 347
MugilVtgAb                 IQLLRISNAEAQIIEILNHLVTFNVAMVHEDAPLKFIELVQLLRVARFES 347
OryziasVtgAb               VELLRISNAEAQIVETLNKLVTLNMGKAHEDSPLKFIELIQLLRVARYES 348
GambusiaVtgAb              IQLLRITNAEAQIVETLNNLVSLNLGKAHEDSPLKFIELIQLLRVARYES 348
CyprinusVtgAb              IHLMKISDAPAQIIEVLKHLVANNVAMVHEDAPLKFVQLIQLLRVSTLEN 347
PagrusVtgAa                IQLIKVNNVQTQIVEILNHLVTHNMQTVHEDAPLKFLELIQLLRSARFED 347
SillagoVtgAa               IQLVKVNNVQAQIVEVLNHLVSHNVERVHEDAPLKFLEFVQLLRAARLED 347
MoroneVtgAa                IQLIKINNVQAQIVEILNHLVTNNVERVHEDAPLKFLELIQLLRAARFED 347
EpinephelusVtgAa           IQLIKITNVQAQIVEVLNHLVTRNVEKVHEDAPLKFLELIQLLRVAKFED 312
MugilVtgAa                 IQLIKIKNAQAQIAEVLNHLVIHNVEKVHEDAPLKFLELVQLFRETRLED 349
GambusiaVtgAa              IKLVKISNAKAQTAEVMNKLATINVENLHENAPMKFLELVQLLRLARYED 347
OncorhynchusVtgA           IQLLKISNARAQAVKILNHLVTYNTAPVHEDAPLKFLQFIQLLRMASSET 347
AcanthogobiusVtgAa         IRIFKMKEVQEQIPEVLNHLVVNNRDKVHEDAPLKFLELIQLLRASDITQ 346
MoroneVtgC                 IMMQNLEDPLPKAVELIKQLAEANKYQIDSATTEDTIKLYQLLRVMPYEE 347
PagrusVtgC                 IMMQNLDDPVPKAIELIKQLAEANKYEVDSATTEVTIQLYQLLRVIPYEG 347
MugilVtgC                  IVMQNLDNPVPKAVELFKQLAQANRYQIDKATTEDTIKLYQLLRVMPYEG 347
OreochromisVtgC            LVMQDLAEPKAKAVRMIKQLAEDHKNQINRETTEDTLKVYQLLRMLQDRD 301
GambusiaVtgC               TMMQKLDNPGPKATELIKRLSEANSGTINSATTEDSIKLYHLLRVTPYEE 347
AcanthogobiusVtgC          IMMQKLDDPVPKATELIKRLVQANTNQLDSTTTEDAIKLYQLLRVIPLEK 348
                             :  : : .   :    .::.*   :    .  :.   ::. :::*
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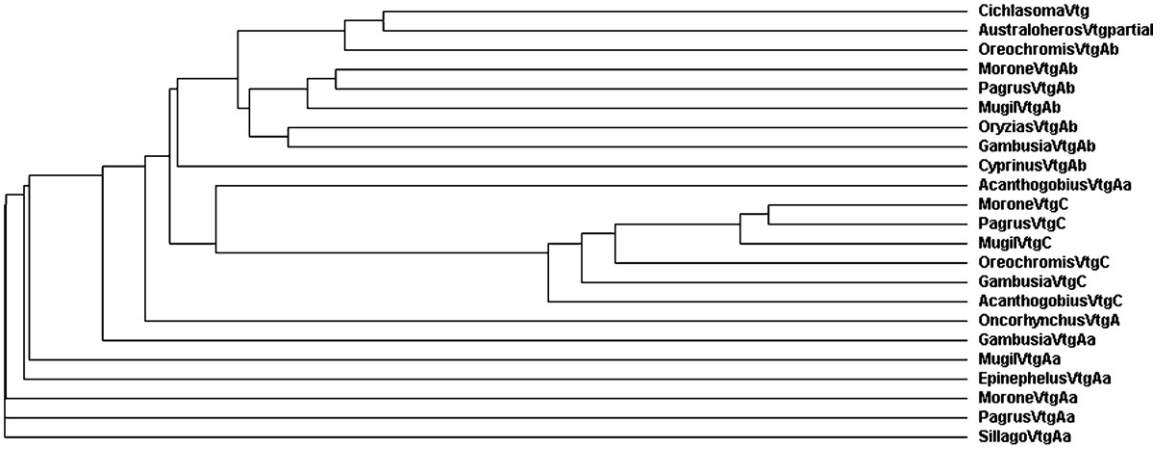

Fig. 1. (*Continued*)

Table 1
Mean hepatosomatic index (HSI) with standard error (S.E.) for *Cichlasoma dimerus* adult males exposed to a nominal concentration of 150 μg/L octylphenol (OP) for 28 days (day 0 of recovery) and further transferred to OP-free water for 1–28 days. Mean HSI value of males exposed to vehicle is also included (Control). Significant values ($p < 0.05$), after comparison with control values, are indicated by asterisks.

Treatment	Mean	S.E.
Control	1.5	0.2
Day 0 of recovery (28 days of OP)	3.0*	0.5
Day 1 of recovery	2.8	0.4
Day 3 of recovery	2.6	0.3
Day 7 of recovery	2.3	0.2
Day 14 of recovery	3.2*	0.4
Day 21 of recovery	2.9	0.3
Day 28 of recovery	2.8	0.2

for this species (Rey Vázquez et al., 2009). Moreover, pulse exposure, as was the case in the present work, is ecologically relevant since fish are not usually exposed to constant levels of pollutants in the environment. In this experimental design, we were able to detect marked changes in estrogen-responsive genes and plasma proteins, and also in liver histology of *C. dimerus* males after exposure to150 μg/L OP during 28 days.

Among these estrogen-responsive genes, we choose Na^+/K^+-ATPase since it is a ubiquitous enzyme involved in homeostasis in metazoan cells (Suzuki-Yagawa et al., 1992). The Na^+/K^+-ATPase provides the driving forces for the control of the ionic environment in the cytosol, cell volume, intracellular pH, and transport of nutrients and metabolites. Its amino-acid sequence is widely

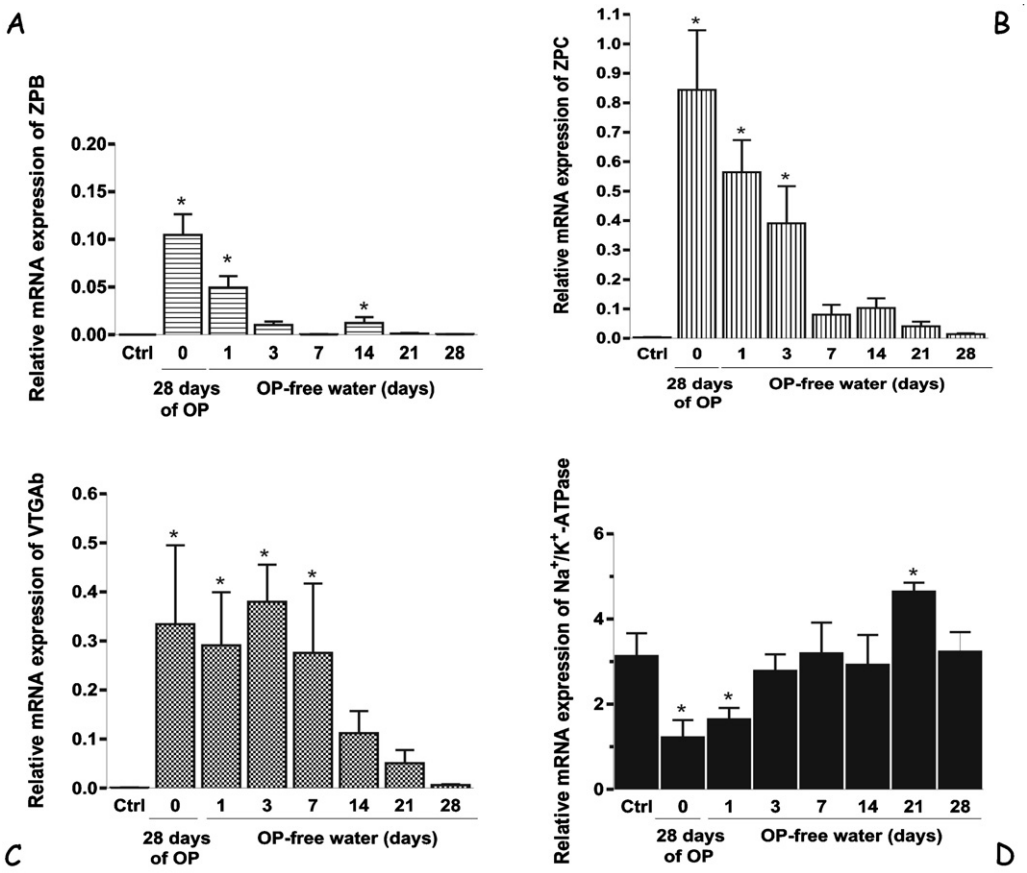

Fig. 2. Time course recovery of gene expression of zona pellucida proteins (ZPB and ZPC), vitellogenin (VTGAb) and Na^+/K^+-ATPase in liver of *Cichlasoma dimerus* adult males exposed to a nominal concentration of 150 μg/L octylphenol (OP) for 28 days and further transferred to OP-free water for 1–28 days. Values were first normalized to total RNA content, and then expressed relative to the estrogenized female value which shown to exhibit high expression levels. Error bars represent standard error of the mean. Significant values ($p < 0.05$), after comparison with control values (Ctrl), are indicated by asterisks.

conserved among opisthokonts (Pressley, 1992; de Souza and Gomes, 1998; Feng et al., 2002) which allowed the use of degenerated primers already designed for an invertebrate species (crab), and liver cDNA of a vertebrate species (*C. dimerus* fish) to amplify the partial sequence of *C. dimerus* Na^+/K^+-ATPase. This partial sequence of 656 bp corresponds to the alpha subunit of Na^+/K^+-ATPase. Not surprisingly, the greatest homology (≥92%) was found with species belonging to the cichlid family, such as tilapia, whereas an 80% coincidence was found with other vertebrate groups. In both fish and mammals it was reported that estrogens are capable of disrupting the sodium pump in different tissues through estrogen receptors. In zebrafish, alpha and beta subunits of liver Na^+/K^+-ATPase mRNA were down-regulated after exposure to an environmentally relevant concentration of ethinylestradiol (EE_2) (Martyniuk et al., 2007). Xenoestrogens, including OP, not only affect mRNA expression and enzyme activity of Na^+/K^+-ATPase but also membrane lipid composition, all of which may alter sodium pump function (Davis et al., 1978; Lee et al., 2001; Bangsgaard et al., 2006). In the present study, baseline levels of liver Na^+/K^+-ATPase transcripts in *C. dimerus* control males were 3.13 ± 0.5. After 28 days of OP exposure, down-regulation of Na^+/K^+-ATPase expression was found, reaching relative values of 1.22 ± 0.4 (Fig. 2D day 0). Similar values were obtained for E_2-females, which were used to calculate relative abundance values, supporting similar effects of OP and E_2 on estrogen-responsive genes. The down-regulation of Na^+/K^+-ATPase was rapidly reversed, reaching control values after 3 days following transfer to OP-free water, accounting for a rapid recovery of its normal function. An increase in Na^+/K^+-ATPase gene expression was detected on day 21 of recovery probably due to a modest overcompensation after a toxic insult (Calabrese and Baldwin, 2001). It is interesting to note that the expression profile of Na^+/K^+-ATPase due to OP, was completely different from that of female proteins such as VTG or ZP, so estrogen-responsive genes can behave differentially when exposed to the same contaminant.

Vitellogenins belong to an ancient family of genes conserved from protostomes to deuterostomes (Baker, 1988; Finn and Kristoffersen, 2007). It is speculated that they have emerged from an ancestral gene designed to ensure a pivotal event in cellular transfer of liposoluble substances (Babin et al., 1999). While attempting the sequencing of *C. dimerus* VTG with conventional PCR, we found overlaid products, because this technique does not differentiate highly conserved genes such as VTG forms (VTGAa, VTGAb, VTGC) and related ones. Therefore, we used TA cloning technique, which isolates individual PCR products from heterogeneous amplifications, to obtain a single VTG partial sequence. VTG is a highly lipidated protein consisting of two polypeptides, known as lipovitellins. In vertebrates, the VTG coding sequence is arranged in a linear fashion from N-termini as follows: lipovitellin heavy chain (LvH)-phosvitin (Pv)-lipovitellin light chain (LvL) and C-terminal coding sequence (Romano et al., 2004). The Pv domain possesses calcium-binding properties. In teleosts, downstream from the LvL, a small yolk protein containing neither lipid nor phosphorous was discovered and named β′-component (Hiramatsu et al., 2002; Sawaguchi et al., 2005). The sequence region obtained for *C. dimerus* codifies for LvH, highly homologous to LvH of other cichlids. The new nomenclature, based on a recent whole genome duplication

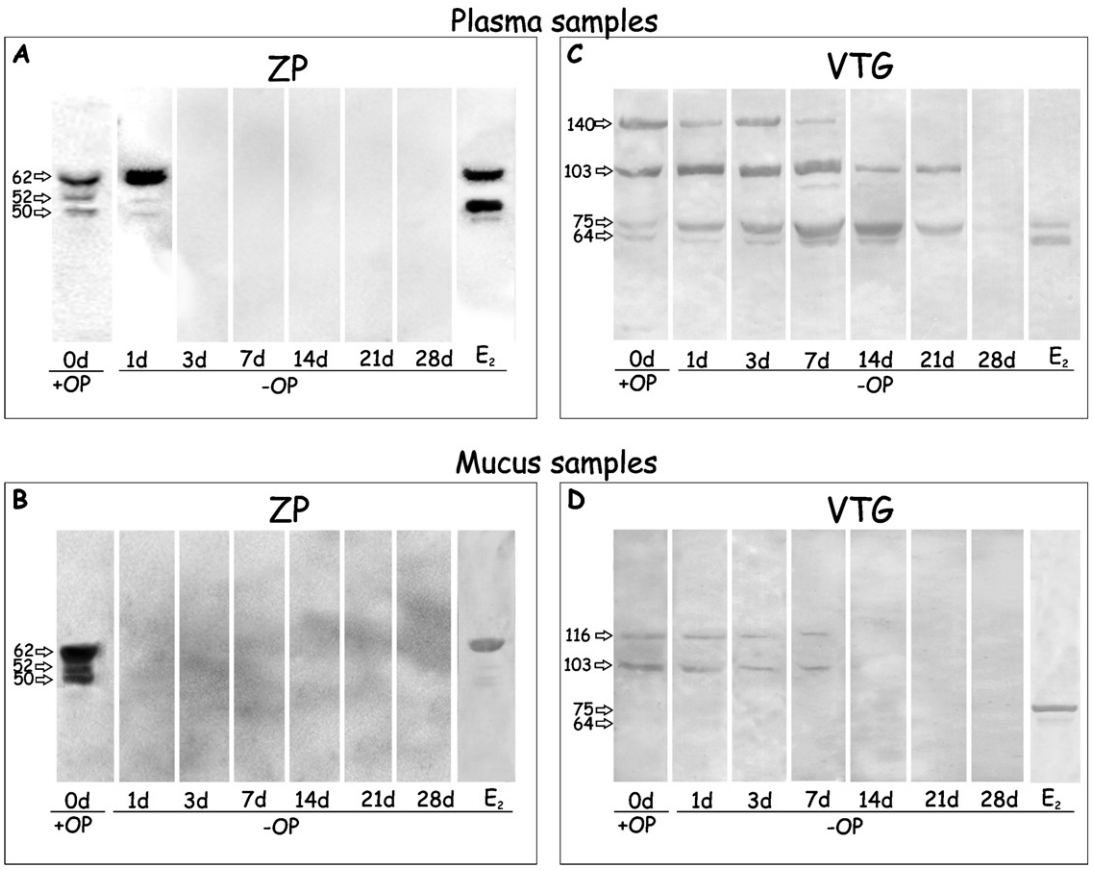

Fig. 3. Western blot of (A and B) zona pellucida proteins (ZP) and (C and D) vitellogenin (VTG) from plasma and mucus samples of *Cichlasoma dimerus* males exposed to a nominal concentration of 150 μg/L octylphenol during 28 days (+OP) and further transferred to OP-free water for 28 days (-OP). Plasma and mucus ZP and VTG patterns of females induced with 17-β estradiol are also shown (E_2). Numbers on the left of each figure represent molecular weight in kilodaltons (kDa). Numbers at the bottom of each column of (-OP) indicate the days following transfer to clean water. Control males did not exhibit ir-bands for ZP and VTG, neither in plasma samples nor in mucus samples, (data not shown).

event (Finn and Kristoffersen, 2007), categorizes the two longest cDNAs of VTG as VTGAa and VTGAb, while the shorter cDNA, encoding a phosvitinless VTG, was categorized as VTGC. In acanthomorph teleosts, VTGAa is completely cleaved into free amino-acids which function as osmotic effectors during oocyte hydration. In contrast the lipovitelin derived from VTGAb and VTGC remains mostly intact and may be used by late stages during embryogenesis (Matsubara et al., 1999; Finn et al., 2009; Reading et al., 2009). Comparison with VTGs of several teleosts supports that the partial sequence obtained for *C. dimerus* belongs to VTGAb type (Mouchel et al., 1996; Li et al., 2003; Sawaguchi et al., 2005). Even though the characterization of VTG is complex and full-length CDS have been obtained for only few teleosts so far (Sawaguchi et al., 2005; Hiramatsu et al., 2006; Reading et al., 2009), VTG has been widely used as a biomarker to assess estrogenic contamination in fish (Moncaut et al., 2003; Arukwe and Goksøyr, 2003).

We recently concluded that the expression of ZP, which is induced by xenoestrogens in *C. dimerus*, is a suitable biomarker for endocrine disruption because males do not normally express female proteins (Genovese et al., 2011). In our laboratory, ZPB and ZPC (but not ZPA) were partially sequenced to quantify their mRNA expression. We found that ZPB is more sensitive as a biomarker than ZPA. In the present study, VTG and ZP were markedly expressed in males exposed to a nominal concentration of 150 μg/L OP during 28 days, reaching the same level of induction caused by a single dose of 10 μg/g E_2; hence OP and E_2 generate strong estrogenic responses (Moncaut et al., 2003; Genovese et al., 2011; present work). At least 1–3 days of exposure to the nominal concentration of 150 μg/L OP is necessary to significantly induce mRNA abundance of VTG and ZP in *C. dimerus* (Genovese et al., 2007). The prolonged expression of female proteins indicates that the liver is capable of maintaining active the genomic machinery needed to promote the process of vitellogenesis and zonagenesis for a relatively extended period (Flouriot et al., 1996; Martyniuk et al., 2007).

Three immunoreactive ZP bands (62, 52, 50 kDa) were detected in plasma and mucus samples of males exposed to OP, though we previously reported four bands in OP-injected fish (Genovese et al., 2011). The 62 kDa band was the broader one probably containing two irresolvable bands. The fact that different proteins appear in different positions could be partially attribute to different glycoforms and thus have a different migration behavior in SDS-PAGE (Izquierdo-Rico et al., 2009). Only two ZP isoforms were partially sequenced in *C. dimerus* so other subfamilies, besides ZPB and ZPC, may also be present in this species. Hiramatsu et al. (2002) identified a 180 kDa band as the primary polypeptide subunit of perch vitellogenin and bands migrating at 110 kDa and 74 kDa as lipovitellin and β′-component derived from vitellogenin. A similar VTG pattern was obtained for plasma samples of exposed *C. dimerus* males. In addition to the VTG bands observed in estrogenized females, two extra bands of higher molecular weights were detected in plasma of OP-males probably due to a differential induction of E_2 and OP, related to the estrogenic potency of the compounds (Rey Vázquez et al., 2009; Genovese et al., 2011).

Immunodetection of VTG and ZP in skin mucus ceased before plasma detection (day 14 for mucus VTG vs. day 28 for plasma VTG; day 1 for mucus ZP vs. day 3 for plasma ZP). We previously

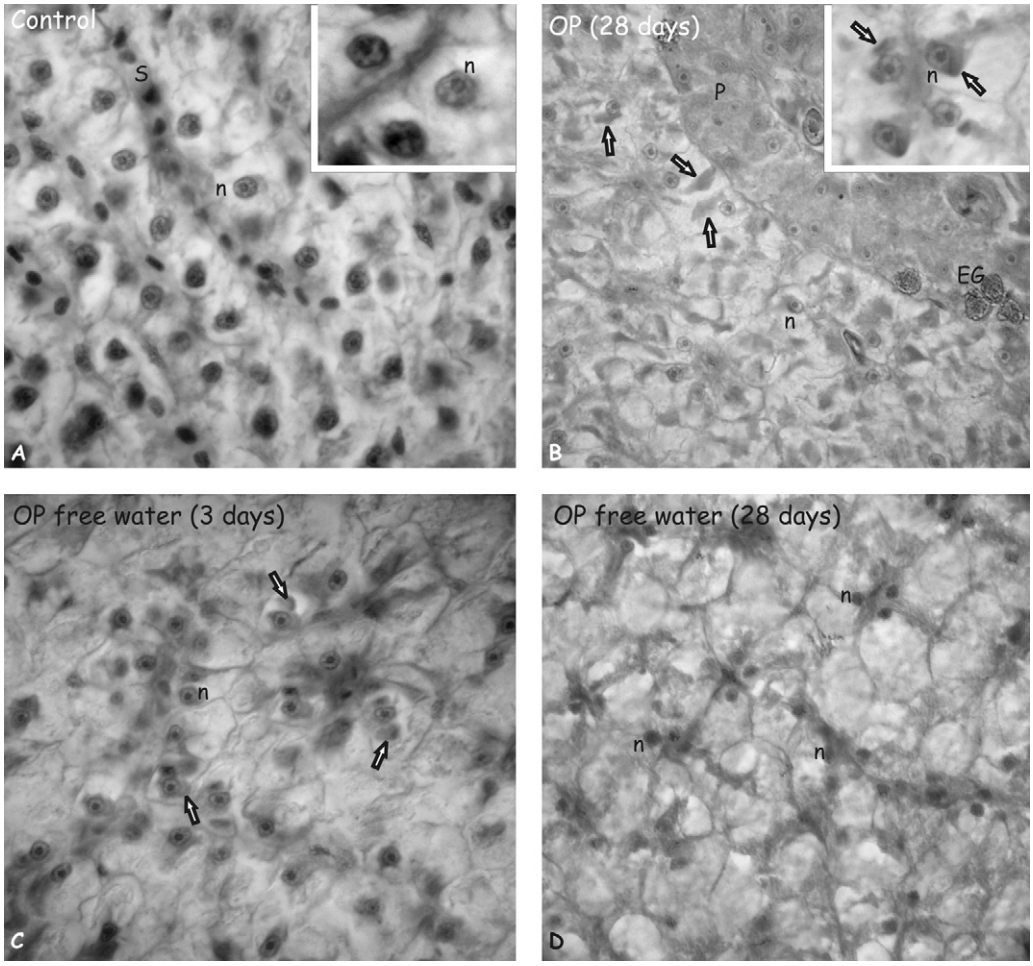

Fig. 4. Liver cross sections of *Cichlasoma dimerus* males. (A) Control fish showing the normal structure of liver, 600X, inset 1000X. (B) Liver of fish exposed to a nominal concentration of 150 μg/L OP for 28 days displays cellular disarrangement, euchromatic nuclei, and intense cytoplasmic basophilia (indicated by arrows), 400X, inset 1000X. Similar alterations were seen during the whole recovery period. (C) and (D) 3 and 28 days of recovery, respectively, 400X. Hematoxylin-eosin. EG, eosinophilic granule cell; n, nucleus of hepatocyte; P, pancreas; S, sinusoid.

suggested that OP is less potent eliciting an estrogenic response in mucus than in plasma, since ZP and VTG were detected initially in plasma and later on in mucus of OP injected fish (Genovese et al., 2011). It was also proposed that the skin of estrogenized fish could serve as an excretory pathway for surplus plasma proteins, however we cannot rule out the possibility that the fish skin could be a site for synthesis and secretion of VTG and ZP (Moncaut et al., 2003; Meucci and Arukwe, 2005; Arukwe and Røe, 2008; Jin et al., 2008; Genovese et al., 2011). With the present results, we validate immunodetection of VTG and ZP in skin mucus as an easy and non-invasive technique to address endocrine disruption caused by APE derivates (Arukwe and Røe, 2008; Genovese et al., 2011). The fact that these parameters can be assessed without killing the fish is especially useful for threatened species or fishes where sampling plasma may be too stressful or not practicable (Maltais and Roy, 2009).

The present results confirm that the induction of female proteins in *C. dimerus* males exposed to OP is reversible after cessation of exposure. Previous results indicate a reduction in the mRNA abundance of estrogen receptors (ERα) when fish are returned to clean water (Genovese et al., 2008). ZPB transcripts returned abruptly to background levels sooner than ZPC or VTG, decreasing to half the level achieved after 28 days of OP in only 1 day following transfer to clean water. On day 3 of recovery significant ZPC transcripts were detected but no ZP protein could be detected in plasma probably due to low affinity of the antibody to ZPC and/or high mRNA stability of ZPC. In addition, gene expression has greater sensitivity than Western blot (Genovese et al., 2011). Plasma VTG protein and liver VTG mRNA levels both decreased gradually upon transfer of males to OP-free water. For all biochemical and molecular biomarkers used in the present study, the recovery was complete after an adequate time of depuration (28 days). Reversibility of endocrine disruption caused mainly by E_2 and EE_2 has been evaluated before in fish species including *Oryzias latipes*, *Cyprinodon variegates*, *Danio rerio* and *Salmo salar*, among others (Gronen et al., 1999; Bowman et al., 2000; Hemmer et al., 2002; Van den Belt et al., 2002; Andersen et al., 2003; Seki et al., 2003; Craft et al., 2004; Robinson et al., 2004; Bangsgaard et al., 2006). To our knowledge, partial or complete recovery of liver and gonad histology, loss of sexual characters and baseline return of VTG induction was reported only by Bowman et al. (2000), Van den Belt et al. (2002) and Craft et al. (2004). Irreversible effects such as abnormal development of embryos, gonad alterations, presence of ovo-testis and complete feminization of exposed males were reported by the other mentioned authors.

In the present work normal histology of *C. dimerus* liver could not be restored after waterborne OP exposure and transfer to clean water. As previously reported, fish exposed to xenoestrogens show active hepatocytes, nuclear hypertrophy and intense cytoplasmic basophilia (Van den Belt et al., 2002; Rey Vázquez et al., 2009;

Genovese et al., 2011). This basophilia is probably a result of an important proliferation of rough endoplasmic reticulum due to increased mRNA translation of estrogen-inducible genes (Islinger et al., 2003; Zha et al., 2008; Woods et al., 2009), typical of mature vitellogenic females (Ribeiro et al., 2006). Moreover, an increase in the number of eosinophilic granule cells (or mast cells) found in the liver of OP-treated *C. dimerus* is typical of chronically inflamed tissues (Reite and Evensen, 2006). The liver due to its multiple functions in protein, lipid and carbohydrate metabolism, detoxification of foreign substances and biotransformation, is the major target organ for xenobiotics, and thus one of the most affected organs (Au, 2004). In OP treated *C. dimerus*, liver tissue disarrangements and the accumulation of ZP and VTG might explain the increased HSI (Rey Vázquez et al., 2009; Da Cuña et al., 2011). A future challenge would be to verify if the reported liver damage still present after the recovery period could affect physiological parameters and reproduction of *C. dimerus*.

VTG and ZP induction apparently provide the same information regarding exposure to contaminants but they can have different sensitivities to xenoestrogens (Westerlund et al., 2001; Genovese et al., 2007). Wu et al. (2005) classified six types of biomarkers according to the initial time of induction and recovery period. As they suggested, monitoring programs based on biomarkers of a single type are not desirable. They also argued that the time required for initial induction, maximum induction, adaptation and recovery of stress responses must first be fully understood before using them in environmental monitoring, or else erroneous conclusions may be drawn when interpreting results. According to Wu's classification ZP expression would correspond to a type 5 biomarker (fast induction-fast recovery), and VTG gene expression to a type 6 biomarker (fast induction-slow recovery) (Genovese et al., 2007 and present study). The use of biomarkers with different temporal responses (highly sensitive and long term recovery) such as ZP and VTG as was shown for *C. dimerus*, provides a more trustworthy interpretation of pollution status.

5. Conclusions

In the present work we reported that the histological damage found in *C. dimerus* liver after OP treatment and further transference to OP-free water was not restored. On the other hand, biochemical and molecular biomarkers such as plasma protein induction and mRNA expression of ZP or VTG, up-regulated by OP, were completely recovered after adequate time of depuration (28 days). We conclude that ZP gene expression (particularly ZPB) in males of *C. dimerus* reflects recent exposure to xenoestrogens, whereas, VTG transcripts, which are prolonged in time after OP exposures, may reveal past exposures up to a few weeks. Therefore, combining both ZP/VTG expression profiles should differentiate between recent and past exposures of fish sampled from contaminated environments.

Conflict of interest

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

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