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Vitellogenesis and other physiological responses induced by 17-β-estradiol in males of freshwater fish *Rhamdia quelen*

D.D. Moura Costa [a], F. Filipak Neto [a], M.D.M. Costa [b], R.N. Morais [c], J.R.E. Garcia [d], B.M. Esquivel [d], C.A. Oliveira Ribeiro [a,*]

[a] Departamento de Biologia Celular, Universidade Federal do Paraná, Curitiba, PR, Brazil
[b] Departamento de Patologia Básica, Universidade Federal do Paraná, Curitiba, PR, Brazil
[c] Departamento de Fisiologia, Universidade Federal do Paraná, Curitiba, PR, Brazil
[d] Estação de Piscicultura Panamá, Paulo Lopes, SC, Brazil

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ABSTRACT

This study investigated the effects of different doses of 17-β-estradiol (E_2) in *Rhamdia quelen*. Groups of males exposed to different doses of E_2 (0.1 mg kg^{-1}, 1 mg kg^{-1} and 10 mg kg^{-1}) were compared with non-exposed male and female fish groups. Among the considered biomarkers, no significant differences were observed for micronuclei test, reduced glutathione concentration and lipid peroxidation. All E_2-treated individuals had decreased glutathione *S*-transferase activity. Increased catalase and superoxide dismutase activities, increased vitellogenin expression and decreased metallothionein concentration were observed in males treated with the highest dose. Liver of all test groups showed necrotic areas, but cytoplasm vacuolization was again found only in the individuals exposed to highest dose. E_2 causes deleterious hepatic effects to *R. quelen*, and vitellogenin expression, catalase and superoxide dismutase activity and metallothionein concentration represent appropriate biomarkers for studying E_2 effects. Additionally, the response of some biomarkers was similar in males exposed to E_2 and unexposed females, and therefore exposure to endocrine disruptors may cause consequences for fish populations.

1. Introduction

Nowadays, the potential hazardous effects that hormone-like chemicals have on wildlife and human populations still constitute a challenge to environmental studies. According to Colborn (1998) these chemicals mimic endogenous hormones or inhibit their activity, modulating the endocrine system. As such, many organic compounds introduced into the environment by human activity can disrupt the endocrine system of animals, including fish (Lyle et al., 1997), reptiles (Guilette, 2000), birds (Berg et al., 1998) and mammals (Brouwer et al., 1989).

Estrogens are a group of steroid hormones that can act as endocrine disruptors when present in non-physiological sex-dependent concentrations, and data about the effects of these compounds on vertebrates are still controversy. Laboratorial experiments support that some endocrine disruptors are able to cause breast and testicular cancer, cryotochidism and decreased sperm production (Witorsch, 2002). However, a study case released by National Research Council and American Council of Safety and Health concluded that a consistent link between environmental pollutants and endocrine disruption in vertebrates, particularly humans, is not supported by the epidemiologic data. Therefore one need of ecotoxicology is to investigate this issue, since endocrine disruptors can impair the reproduction and other physiological parameters of natural populations of organisms.

The 17-β-estradiol (E_2) reaches aquatic environments through domestic effluents (Gagné et al., 2005), livestock waste (Ying et al., 2002) and agriculture runoff (Céspedes et al., 2004), and it has been increasingly reported as a potent environmental contaminant (Ying et al., 2002; Imai et al., 2005; Dorabawila and Gupta, 2005). The most important source of estrogens in aquatic ecosystems is domestic effluents because this kind of drain has higher concentrations of E_2 coming from human and veterinary medicines. Estrogens like E_2 are highly potent even at low concentrations. However, endocrine changes may be particularly important at sublethal doses as they constitute early responses to disturbances, preceding the onset of pathology and mortality.

The increasing number of studies suggesting adverse effects of endocrine disruptors for wildlife species (Kavlock et al., 1996; Campbell and Hutchinson, 1998) urged for studies of screening and detecting disturbances related to exposure (EMWAT, 1997; EDSTAC, 2000). A biomarker is a cellular, molecular, genetic or physiologic response altered in an organism or population in response to a

* Corresponding author. Universidade Federal do Paraná - Setor de Ciências Biológicas - Centro Politécnico - Curitiba - PR, - PO Box 19031 - 81531990, Brasil. Fax: +55 41 3361 1756.
E-mail address: dani_coxa@hotmail.com (M.D.M. Costa).

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chemical stressor. The most obvious biomarkers for endocrine disruption involve assessment of reproductive processes. However, the endocrine system is complex, involving processes that operate at several levels of biological organization and this complexity is frequently underappreciated and oversimplified by the evaluation of only reproductive and developmental abnormalities, leading to skewed conclusions about the cause of certain adverse effects. Because of this, several biomarkers must be used for a more realistic evaluation of the effects of endocrine disruptors.

Depending on the exposure dose, estrogens potentially alter hepatic-somatic index in males, reduce egg production in females, induce vitellogenesis in males and juveniles, decrease overall fish fertility (Mills et al., 2001; Kang et al., 2002), and disrupt non-reproductive endocrine events. For instance, genotoxic (Liehr, 2000; Joosten et al., 2004) and morphological effects in target organs such as liver (Warner, 1994) as well as disturbance on the cellular redox milieu (Maria et al., 2007) were reported for E_2. These disturbances results, in some part, from increased production of reactive oxygen species (ROS), impairment of antioxidant defense systems or both. Antioxidant defense systems usually respond to increases of ROS, protecting cells against insults to macromolecules. Then, the activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are important biomarkers for investigating the cellular redox milieu. Similarly to these antioxidant enzymes, glutathione *S*-transferase (GST) usually helps protecting cells against chemical insults and GST participates in phase II detoxication reactions, conjugating glutathione (GSH) to chemicals. Not least important, metallothioneins can protect from metal and nonmetal-induced oxidative stress caused by pro-oxidants chemicals (Boelsterli, 2007). However, if the aforementioned defense systems and many others are not able to degrade or scavenge chemical stressors, damage to biomolecules may ensue. Particularly, the integrity of the membrane lipids and DNA can be affected, impairing essential processes for the functioning and survival of cells, then being implicated with several vertebrate diseases.

Fishes are rich in unsaturated membrane lipids, the most susceptible lipids to oxidative damage, and directly exposed to estrogenic compounds such E_2 derived mainly from sewage effluents. *Rhamdia quelen* (jundiá) is a freshwater fish that was chosen as biological model to investigate the E_2 effects because of its omnivorous diet feeding on sediment where lipophilic compounds such as E_2 adsorb. In addition, the species is widely spread in South America from Argentina to Mexico, and is intensively cultured in fish farms for human feeding (Gomes et al., 2000). The sexual maturity is reached within one year and mature fishes are sexually dimorphic.

The aim of the current study was to induce vitellogenin expression by 17-β-estradiol in male *R. quelen* and verify its dose-dependent physiological effects using biomarkers that cover the cellular redox milieu (CAT, SOD, GST and GPx enzymes, GSH and metallothionein content), DNA (nuclear abnormalities) and lipid (lipid peroxidation) damages and morphological hepatic alterations (liver histology and hepatic-somatic index).

2. Material and methods

2.1. Chemicals

17-β-estradiol, Paraplast, Bradford reagent, trichloroacetic acid, xylenol orange, chloroform, HCl, bovine serum albumin, tris–HCl and hydrogen peroxide 30% were purchase from Sigma-Aldrich (MO, USA). Canola oil was from Cargill S.A., Brazil. β-Mercaptoethanol and phenylmethylsulphonylfluoride (PSMF) were from Fluka (MO, USA). Ethanol, formol and acetic acid were purchased from Merck (North American, S.A.). Xylocaine was obtained from Cristalia Laboratories (SP, BR). Hematoxylin, eosin, giemsa and ethylenediamine tetraacetic acid (EDTA) were purchased from VETEC (Duque Caxias, RJ, BR). Glutathione-S-transferase (GST), reduced glutathione (GSH), chlorodinitro benzene (CDNB), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR), nitroblue tetrazolium (NBT), butylated hydroxytoluene (BHT), and ammonium ferrous sulfate were from Acros Organics (Geel, Belgium). Coomassie brilliant blue, nitrocellulose membrane, non-fat dry milk were obtained from Bio-Rad Laboratories (CA, USA). Polyclonal rabbit anti-sea bream vitellogenin was from Biosense Laboratories (Bergen, Norway) and the antibody peroxidase labeled goat anti-rabbit IgG was from Gaithersburg (MD, USA). Pierce chemiluminescent kit was purchased from Pierce (IL, USA).

2.2. Experimental design

Forty mature males (29.5 cm ± 2.2, 411.28 g ± 9.6) and ten mature females (25.6 cm ± 3.1, 333.8 g ± 12.6) of *R. quelen,* all one year old, were obtained from a fish farm (Piscicultura Panama, Santa Catarina State - Brazil). The tested doses (0.1, 1 and 10 mg E_2 kg^{-1}) have been reported to have experimental physiological effects in other studies using fishes on estrogenic effects after intraperitoneal injection of E_2 (Carrera et al., 2007; Pait and Nelson, 2003; Zaroogian et al., 2001). For exposure, 17-β-estradiol was dissolved in canola oil and five experimental groups (10 fish each) were established: female (FC) were not injected with 17-β-estradiol or canola oil; male control group (MC) were injected only with canola oil; and the remaining three groups were intraperitoneally injected with the estrogen at doses of 0.1, 1, or 10 mg E_2 kg^{-1}. Sampling was performed 17 days after injection, because this period had been previously reported as being necessary for vitellogenin induction (Carrera et al., 2007; Pait and Nelson, 2003). After 17 days, fishes were anesthetized with xylocaine (2%) and blood was collected from animals still alive as described by Silversand et al. (1993). Blood was mixed with the anti-proteolytic PMSF (phenyl-methyl-sulfonyl fluoride −10 mM) diluted in saline solution (NaCl at 0.9%), and centrifuged at 3000 *g* for 30 min and 4 °C to obtain the plasma, which was stored at −75 ºC. Blood smears on glass slides were used for posterior analyses of nuclear alterations. After blood sampling, fish were killed by surgical incision between the first vertebra and skull, and liver samples were collected and frozen (−75 ºC, for biochemical analyses) or fixed in Alfac solution (for histological analysis).

2.3. Biometry

Intact fishes and livers were weighted for determining the hepatic-somatic index (HSI) through the equation: HSI = liver mass × 100 × (body mass)$^{-1}$.

2.4. Liver histological aspects

For light microscopy, samples of liver tissue were preserved in ALFAC-fixative solution (ethanol, formaldehyde and glacial acetic acid) for 16 h, dehydrated in graded series of ethanol and xylene, and embedded in Paraplast resin (Sigma®). Sections of 5 μm thick were obtained, stained with hematoxylin/eosin and observed under the Leica® DME light microscope. A qualitative analysis was performed, considering the presence or absence of alterations.

2.5. Micronucleus test

Micronucleus test (MNT) was performed through the visualization and counting of two thousand peripheral erythrocytes stained with Giemsa per fish. The identification of nuclear morphological alterations and the statistical analysis was performed according to Carrasco et al. (1990).

2.6. Biochemical assays

For GST (EC 2.5.1.18), CAT (EC 1.11.1.6), GPx (EC 1.11.1.9), GSH and lipid peroxidation analyses, 0.4 g of liver were homogenized in 1.0 mL of phosphate buffer saline (PBS, pH 7.4) and centrifuged at 9000*g* for 30 min at 4 °C. Then, the supernatant (S9 fraction) was transferred to 2 mL tubes and frozen at −75 °C; prior to the assays samples were thawed on ice and supernatants were diluted by addition of ice-cold PBS in a volume that varied according to the assay's requirement (see dilution ratio on specific assays). For metallothionein, 0.2 g of liver were homogenized in 1.0 mL of 20 mM Tris–HCl, 500 mM sucrose, pH 8.6, centrifuged at 15,000 *g* for 30 min, 4 °C and the supernatant after centrifugation was immediately utilized for the assay.

2.6.1. Protein content

Total protein content was quantified following Bradford (1976). A volume of 10 μL of supernatant (diluted 1:20–50) and 250 μL of Bradford reagent (Sigma®) were placed in a 96-well microplate and absorbance was measured at 620 nm. Protein content was calculated through comparison with a standard curve of bovine serum albumin.

2.6.2. Superoxide dismutase (SOD) activity

SOD (EC 1.15.1.1) activity was measured by inhibition of the reduction of nitrobluetetrazolium (NBT) caused by $O_2^{-\bullet}$ generated by hydroxylamine in alkaline solution (Crouch et al., 1981). Thawed supernatant was diluted (1:10), mixed with pure ethanol (4:1) and centrifuged at 12,000 *g* for 20 min and 4 °C. Then, 20 μL of the resulting new supernatant and 70 μL of solution containing 286 μM NBT chloride and 0.05 mM EDTA were mixed in a 96-well microplate. The reaction was initiated through addition of 110 μL of 67 mM hydroxylamine chloride in 182 mM sodium carbonate (pH 10.2). The absorbance was measured immediately after hydroxylamine addition and after 1 h at 560 nm. The "blank" was determined by replacing sample supernatants with PBS and ethanol (ethanol at 25%). One unit of SOD was defined as the enzymatic activity able to inhibit the reduction of NBT to 50% of the blank rate.

2.6.3. Catalase (CAT) activity

CAT activity was assayed spectrophotometrically in S9 fraction measuring H_2O_2 consumption (Aebi, 1984). Supernatant (20 μL, diluted 1:10) was mixed with reaction medium (980 μL of 20 mM H_2O_2, 50 mM Tris–base, 0.25 mM EDTA, pH 8.0, at 25 °C) in a quartz cuvette and H_2O_2 absorbance decrease was immediately measured at 240 nm for 40 s in intervals of 1 s; blanks consisted of reaction medium and PBS. The first interval of 30 s (r^2>0.99) was used to calculate the enzyme activity, using the molar extinction coefficient for H_2O_2 of 40 M^{-1} cm^{-1}.

2.6.4. Glutathione S-transferase (GST) activity

GST activity was measured using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (Keen et al., 1976). Supernatant (20 μL, diluted 1:10) was placed in 96-well microplate, immediately followed by reaction medium (180 μL, 1.5 mM GSH, 2.0 mM CDNB, 0.1 M potassium phosphate buffer, pH 6.5). Absorbance increase was immediately measured at 340 nm for 2 min in intervals of 12 s. Blanks consisted of reaction medium and PBS. GST activity was determined at the first interval of 1 min (r^2>0.99) with the molar extinction coefficient for CDNB of 9.6 mM^{-1} cm^{-1}.

2.6.5. Reduced glutathione (GSH) measurement

GSH was measured on supernatant after protein precipitation by 10% trichloroacetic acid and centrifugation at 1000*g* for 15 min and 4 °C. Supernatant (50 μL, undiluted) and 230 μL of Tris (0.4 M, pH 8.9) were placed in a 96-well microplate, followed by addition of 20 μL of 2.5 mM DTNB in 25% methanol]. Absorbance was determined at 415 nm and GSH concentration was calculated by comparison with the standard curve for GSH (Sedlak and Lindsay, 1968). Blanks consisted of PBS instead of samples.

2.6.6. Glutathione peroxidase (GPx) measurement

GPx activity was measured based on the decrease of absorbance of NADPH at 340 nm, encouraged by the reduction of GSSG, catalyzed by GR, in the presence of NAPH (Sies et al., 1979). A volume of 10 μL of supernatant (diluted 1:10) and 130 μL reaction medium (0.1 M sodium phosphate buffer, pH 7.0, 3,08 mM sodium azide, 0.308 mM NADPH, 3,08 GSH and 1.54 U mL^{-1} Glutathione reductase) were placed in 96-well microplate. After 2 min, 60 μL of hydrogen peroxide solution (5 mM hydrogen peroxide in 0.1 M sodium phosphate buffer, pH 7.0) were added. The absorbance decrease was immediately measured for 2 min, at intervals of 10 s. Samples were replaced by PBS in the blanks. GPx activity was determined at the first interval of 1 min (r^2>0.99) using the molar extinction coefficient for NAPH 6.22 mM^{-1} cm^{-1}.

2.6.7. Lipid peroxidation (LPO) measurement

LPO was measured by ferrous oxidation-xylenol (FOX) assay (Jiang et al., 1992). A volume of 200 μL of supernatant (not diluted) and 1400 μL of reaction solution (0.1 mM xylenol orange, 25 mM H_2SO_4, 4.0 mM butylated hydroxytoluene (BHT) and 0.25 mM ammonium ferrous sulfate ($FeSO_4.NH_4$) in pure methanol) were mixed in 2 mL tubes. The tube's content was mixed once every 3 min by inversion during 20 min. Then, tubes were centrifuged at 9000*g* for 10 min, and 300 μL of supernatant was carefully added to a 96-well microplate. Blank was prepared by replacing the supernatant with PBS. Absorbance was measured at 570 nm and hydroperoxides concentration was determined using the apparent molar extinction coefficient for H_2O_2 and cumene hydroperoxide of 4.3×10^4 M^{-1} cm^{-1}.

2.6.8. Metallothionein measurement

Metallothionein (MT) was quantified according to Viarengo et al. (1997). After homogenization and centrifugation (see Section 2.6), 50 μL of supernatant was stored (for protein quantification) and 300 μL of it was transferred to another tube. To the tubes containing 300 μL of supernatant, 342 μL of solution 1 (318 μL ethanol and 24 μL chloroform, at −20 °C) were added and the tube contents were mixed. After centrifugation (6000 *g*, 10 min, 0 °C), 490 μL of this new supernatant and 1502 μL of solution 2 (32 μL HCl and 1470 μL ethanol, at −20 °C) was added and mixed in a second new tube; the tubes were kept in the freezer (−20 °C) for 1 h. Then, tubes were centrifuged one more time (6000 *g*, 10 min, 0 °C) and supernatant was carefully removed and discarded. Precipitated proteins were suspended and mixed with 1000 μL of solution 3 (870 μL ethanol, 10 μL chloroform and 120 μL of 20 mM Tris–HCl buffer (pH 8.6)), and tubes were centrifuged (6000*g*, 10 min, 0 °C). Supernatant was discarded and precipitated proteins were suspended in 50 μL of 250 mM NaCl; after mixing, 50 μL of the solution containing 4 mM EDTA and 1 M HCl was added (blank was prepared in a separate tube where the solution of NaCl and EDTA-HCl were both added). Finally, 1000 μL of Ellman's reagent (0.43 mM DTNB and 2 M NaCl in 0.2 M sodium phosphate buffer, pH 8.0) was added to all tubes and mixed; tubes were centrifuged for the last time (3000*g*, 5 min, 0 °C) and 300 μL from these final supernatants were added to a 96-well microplate. Absorbances were measured at 412 nm and a standard curve of GSH mixed with Ellman's reagent was used as reference to determine thiol groups (SH) content. We used the metallothionein thiol content of mussel (30%) to estimate the metallothionein content of *R. quelen* (Viarengo et al., 1997).

2.7. Measurement of plasma estradiol

For 17-ß-estradiol measurements individual or within group pooled plasma samples (when individual sample volume was bellow 400 μL) were extracted in diethyl ether (1:4; v:v). The ether fraction was poured

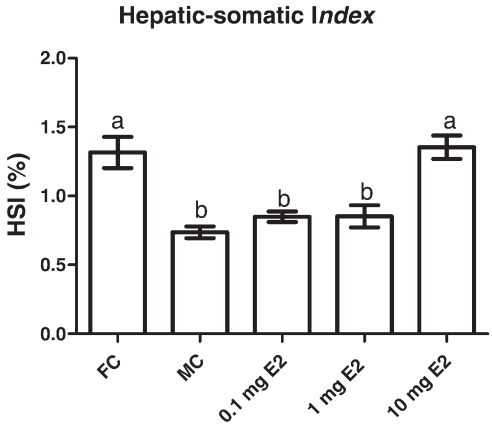


Fig. 1. Hepatic-somatic index. FC: non-exposed females; MC: non-exposed male; 0.1 mg E2: male exposed to 0.1 mg E_2 kg^{-1}; 1.0 mg E2: male exposed to 1.0 mg E_2 kg^{-1}; 10 mg E2: male exposed to 10 mg E_2 kg^{-1}. Different letters (i.e. a, b) indicate differences ($P<0.05$) among groups.

off into clean glass tubes and evaporated in a fume hood. Extracts were reconstituted in 200 μL assay buffer (NaH_2PO_4; Na_2HPO_4; NaCl; BSA; pH 7.0) and analyzed by enzyme immunoassay using a polyclonal anti-17-ß-estradiol antibody (R 0008/1:12.000 dilution) and HRP-conjugate (1:200.000) obtained from Coralie Munro at the University of California (Davis, CA, USA). Serial dilutions of pooled plasma extracts produced displacement curve parallel to those of standards ($r^2 = 0.96$; $F = 0.000253838$. DFn = 1 DFd = 13, $p = 0.9875$). Samples were measured in a single run with an intra-assay coefficient of variation of 3.73% and results are expressed in ng/mL.

2.8. SDS PAGE and Western blot analysis

A volume of 100 μg of total plasma protein was resolved by 8% SDS-PAGE (Mini Protean II, BioRad®). Proteins from two identical gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose membrane in order to perform the Western blot. The Western blot was initiated by rehydrating the membrane in the blocking solution (5% of non-fat dry milk in Tris-buffered saline (TBS) with 0.5% Tween-20 (TTBS)). The membrane was then probed for Vg with commercial rabbit anti-sea bream vitellogenin polyclonal antibodies (1:500, Biosense, Norway) in blocking solution. Bound antibodies were detected with goat anti-rabbit antibodies coupled to HRP (Horseradish peroxidase 1:4000, Sigma). The reaction was developed using the Pierce chemiluminescent kit (Chemiluminescent super signal, Pierce).

2.9. Statistical analysis

For the biochemical assays, statistical analyses were performed using Prism software and differences among controls and exposed groups were tested by one-way ANOVA followed by Tukey test when appropriate. For the evaluation of the frequency of nuclear morphological abnormalities, the comparisons among the groups were performed through Kruskal–Wallis test (Carrasco et al., 1990). Results with $p<0.05$ or least were considered statistically significant.

3. Results

3.1. Hepatic-somatic index

According to the hepatic-somatic index, male and female control groups were different, as well as male control and 10 mg E_2 kg^{-1} male exposed group. In both, an increase of hepatic-somatic index occurred in comparison with male control group (Fig. 1). No differences among male control group and both 0.1 and 1 mg E_2 kg^{-1} exposed groups (Fig. 1) were observed, while the 10 mg E_2 kg^{-1} exposed group presented an increased hepatic-somatic index in comparison to the other two exposed groups (0.1 and 1 mg E_2 kg^{-1}).

3.2. Liver histology

General liver structure differed between the controls groups: the hepatocytes of female present an intense cytoplasm “vacuolization” (Fig. 2 A), that was not observed in male control group (Fig. 2 B). The

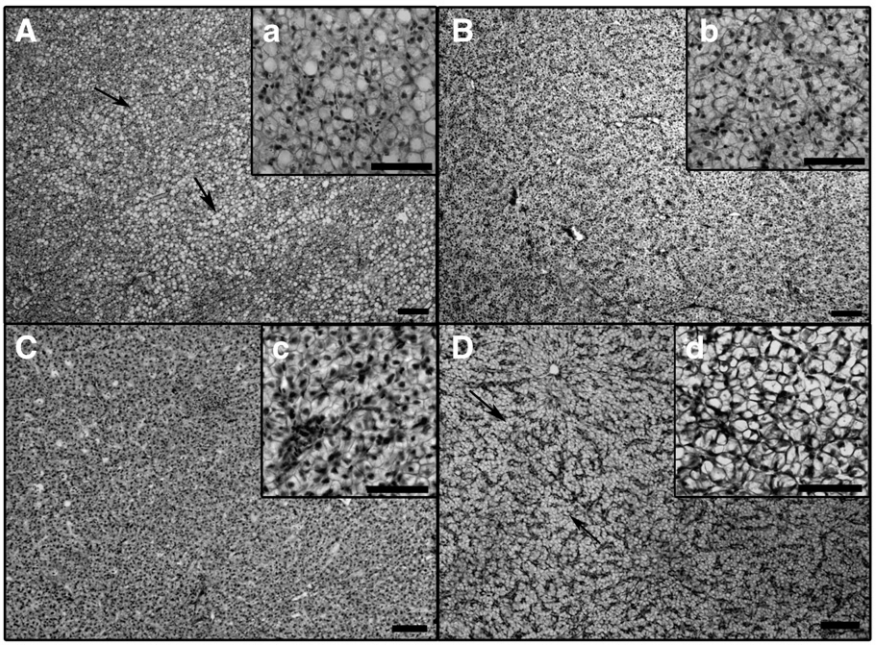


Fig. 2. Liver histology. (A) Female liver structure. Vacuolated cells (arrows). Bar scale = 100 μm. (a) Details of vacuolated cells. Bar scale = 50 μm. (B) – Male non-exposed liver structure. Bar scale = 100 μm. (b) Details of male non exposed liver structure. Bar scale = 50 μm. (C) Liver structure from male exposed to 1 mg E_2 kg^{-1}. There no differences in liver structure between male exposed to 0.1 and 1.0 mg E_2 kg^{-1}. Bar scale = 100 μm. (c) Details of liver structure from male exposed to 1 mg E_2 kg^{-1}. Bar scale = 50 μm. (D) – Liver structure from male exposed to 10 mg E_2 kg^{-1}. Vacuolated cells (arrows). Bar scale = 100 μm. (d) – Details of vacuolated cells. Bar scale = 50 μm.

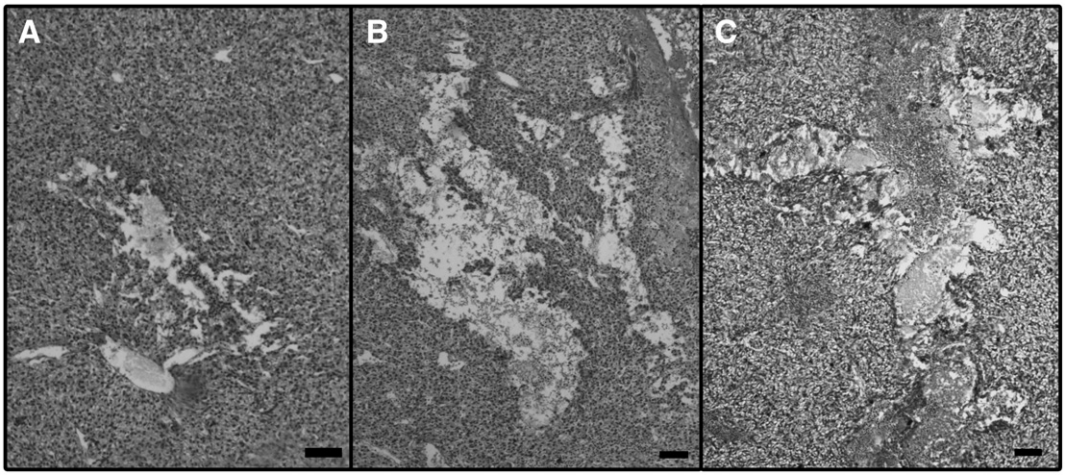


Fig. 3. Necrosis. (A) Necrosis area in males exposed to 0.1 mg E_2 kg^{-1}. Bar scale = 50 μm. (B) Necrosis area in males exposed to 1.0 mg E_2 kg^{-1}. Bar scale = 50 μm. (C) Necrosis area in males exposed to 10 mg E_2 kg^{-1}. Bar scale = 50 μm.

same alteration was observed in males exposed to 10 mg E_2 kg^{-1} (Fig. 2 D), but not in males exposed to 0.1 and 1 mg E_2 kg^{-1} (Fig. 2 C). The male and female control groups did not have necrotic lesions, which were observed in individuals from all exposed groups (Fig. 3). The presence and intensity of the necrosis areas is similar among the exposed groups. Leukocyte infiltration, that occurs during inflammatory responses, was observed similarly in all groups, except for the 10 mg E_2 kg^{-1} exposed group (Fig. 4).

3.3. Nuclear morphological abnormalities

Nuclear abnormalities were present in all tested groups, but no significant differences occurred among the groups. Table 1 presents the mean and standard deviation of nuclear abnormalities per group.

3.4. Biochemical assays

SOD and CAT activities increased in the 10 mg E_2 kg^{-1} male exposed group in comparison with male and female controls, but were similar among the male exposed groups. Also, these enzymatic activities were similar in the female control, male control and the males injected with the two lowest doses (Fig. 5 A and B). GST activity decreased at similar magnitude in all male exposed groups comparatively to the male and female controls, which presented similar activities (Fig. 5 C). GSH concentration (Fig. 5 D), GPx activity (Fig. 5 E) and LPO (Fig. 5 F) had similar values among all the experimental groups. The metallothionein (Mt) concentration decreased in the female and 10 mg E_2 kg^{-1} male exposed groups in comparison with the others experimental groups. No differences among the male

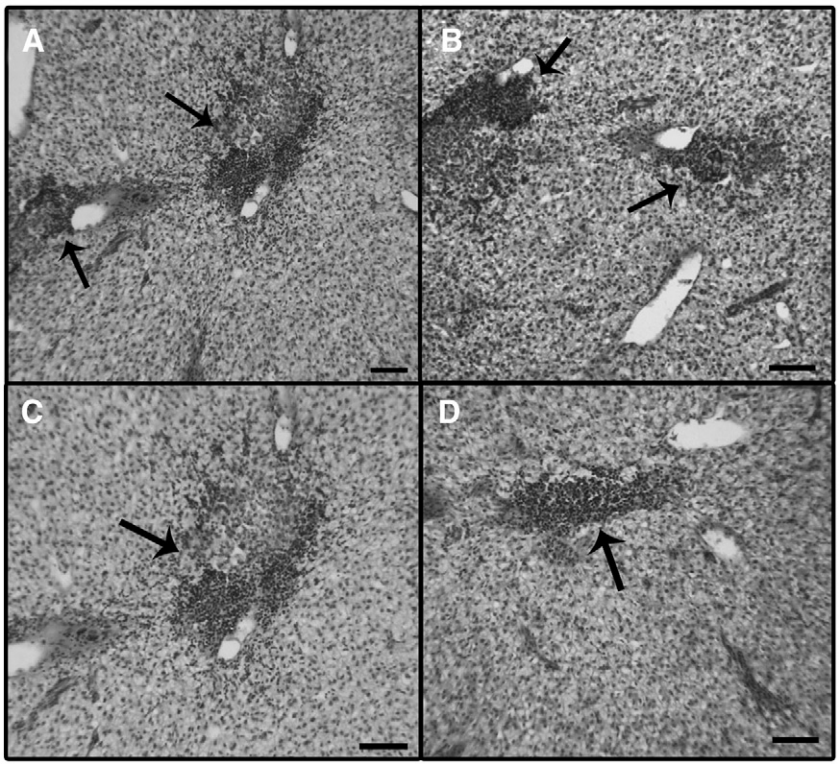


Fig. 4. Inflammatory response. (A) Leukocyte infiltration (arrows) in female control group. (B) Leukocyte infiltration (arrows) in males non exposed. (C) Leukocyte infiltration (arrows) in males exposed to 0.1 mg E_2 kg^{-1}. (D) Leukocyte infiltration (arrows) in males exposed to 1.0 mg E_2 kg^{-1}. Bar scale = 50 μm.

Table 1
Mean of nuclear abnormalities per group (NMA).

Group	FC	MC	0.1 mg E_2	1.0 mg E_2	10 mg E_2
NMA	3.12 ± 2.66	2.15 ± 1.45	3.55 ± 2.55	3.07 ± 2.77	6.12 ± 3.27

FC: female non-exposed; MC: male non-exposed; 0.1 mg E_2 kg^{-1}: male exposed to 0.1 mg E_2 kg^{-1}; 1.0 mg E_2: male exposed to 1 mg E_2 kg^{-1}; 10 mg E_2: male exposed to 10 mg E_2 kg^{-1}.
Mean ± Standard deviation. No significant differences among all groups.

control and males exposed to 0.1 or 1 mg E_2 kg^{-1} were observed (Fig. 5 G).

3.5. Plasma estradiol

Plasma 17-β-estradiol concentration was similar between females and males exposed to 10 mg E_2 kg^{-1} and both had higher E_2 concentrations than all other male groups. Males at the control group and males exposed to 0.1 or 1 mg E_2 kg^{-1} had similar concentrations of plasma estradiol (Fig. 5 H).

3.6. Vitellogenin expression

Vitellogenin (Vg) expression was observed in females and in both 1 and 10 mg E_2 kg^{-1} male exposed groups. Males injected with highest dose of E_2 over-expressed Vg, while males injected with 1 mg E_2 kg^{-1} slightly-expressed the protein and so, Vg expression can be considered dose-dependent in *R. quelen*. Vitellogenin was not detected in the male control group and in the 0.1 mg E_2 kg^{-1} male exposed group (Fig. 6 A and B).

4. Discussion

Occurrence of steroid hormones in natural aquatic environments strongly increased in the last decades due to medicine application and livestock farming release, becoming a matter of concern for ecotoxicologists. Although intraperitoneal injections are not the most environmentally relevant mode of exposure, they do provide a means to determining whether a species responds to compounds of interest and the kinds of responses that are relevant for that species. Different intraperitoneal doses of E_2, with significant physiological effects have been reported by others authors (Flynn and Benfey, 2007; Maria et al., 2007; Zarrogian et al., 2000). Some of the doses tested in this study were different from those used by Flynn and Benfey (2007) and Maria et al. (2007), but were similar to those tested by Zaroogian et al. (2001). We chose the doses based on the literature and on a previous study in which lethality was observed in *R. quelen* for 20 mg E_2 kg^{-1} and 50 mg E_2 kg^{-1} (unpublished data), even though the value of 20 mg E_2 kg^{-1} was reported as not lethal to *Paralichthys dentatus* (Mills et al., 2001). Then, different species of fish have different sensibility to estrogens.

Estrogenic compounds accumulate in the aquatic environment at concentrations of few micrograms per liter, whereas sewage sludge effluents contain up to some hundreds of micrograms per liter (Ahel et al., 1994; Naylor et al., 1992).

The estradiol concentration on plasma of females of *R. quelen* was similar to the concentration found elsewhere (Barcellos et al., 2001; Soso et al., 2007), and greater than the concentration observed for mammals possibly due to the differences in reproductive strategies of fishes and mammals. Females in the reproductive period and males exposed to 10 mg E_2 kg^{-1} present similar concentrations of E_2, demonstrating that the tested dose have physiological significance. However, not exposed males and males exposed to 0.1 and 1.0 mg of E_2 had similar concentrations of the hormone, indicating that this hormone was possibly metabolized and excreted by the organism during the seventeen days that followed the intraperitoneal injection. Literature reports that 17-β-estradiol is converted in the liver to estrone and subsequently metabolized into 2-, 4- or 16-a-hydroxyestrone. The 2-Hydroxyestrone (2-OHE) is rapidly excreted by the organism, whereas the 16-a-hydroxyestrone (16a-OHE1) is not, being a potent estrogen that exhibits toxic characteristics (Greenspan and Gordon, 1997). Due to this biotransformation and subsequent excretion of estrogens some of their effects can be reversible after discontinuing the exposure.

The increased HSI in individuals exposed to the highest dose can be interpreted as a metabolic disturb related to the increase on Vg production and the vacuolization of hepatocytes. These results were also reported for *Paralichthys dentatus* after 2 and 20 mg E_2 kg^{-1} exposure (Mills et al., 2001).

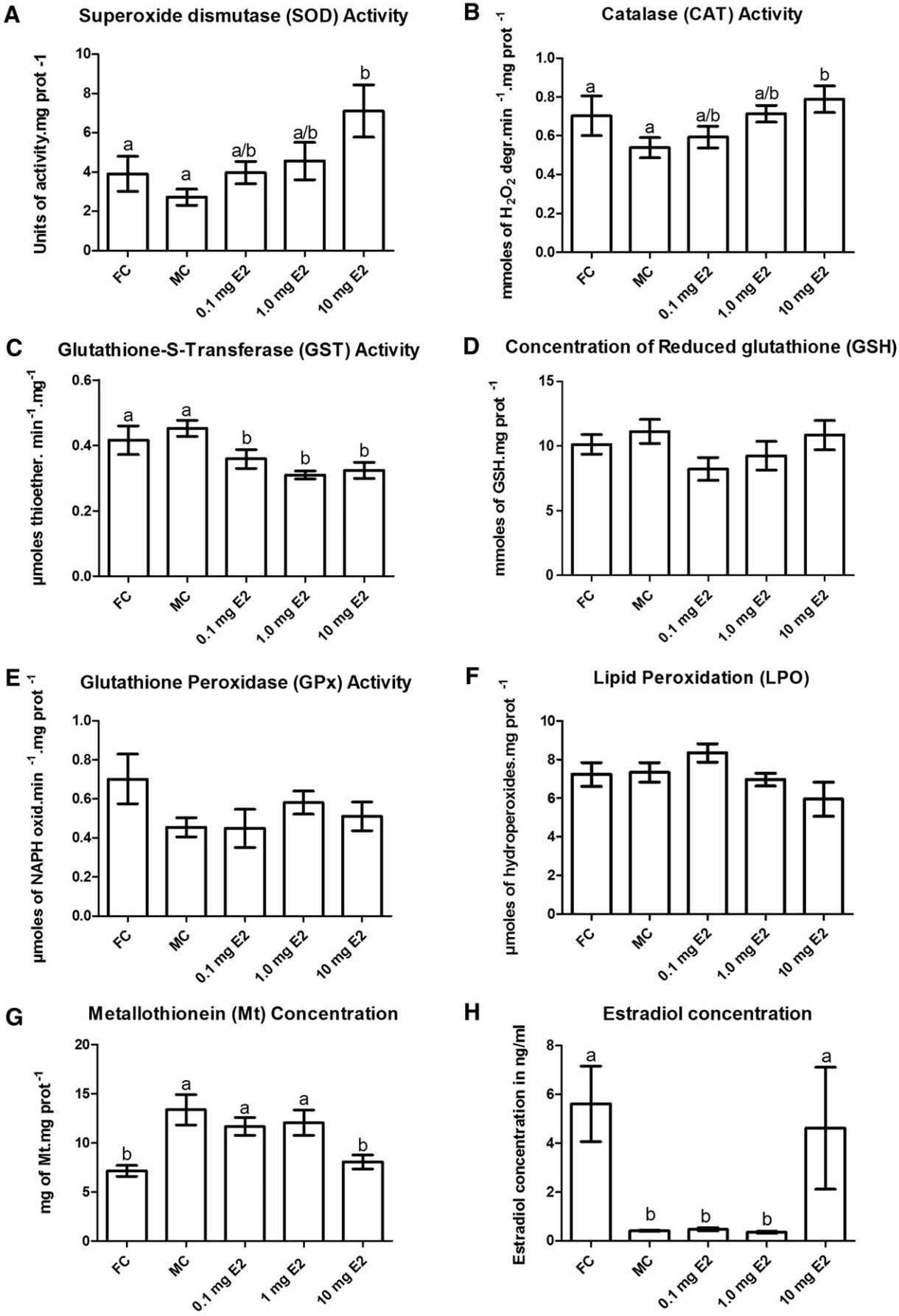
Histological damages have been poorly used as biomarkers to study the toxic effects of E_2 in fishes. The presence of necrosis in all E_2 exposed groups suggests a cause-effect relationship. Excessive cell death through necrosis may lead to immediate or long-term tissue and organ failure. Hepatic disarrays in *Cichlasoma dimerus* (Moncaut et al., 2002) and necrosis in the liver of *P. dentatus* (Zaroogian et al., 2001) were also reported after E_2 injection, demonstrating that high doses of E_2 are hepatotoxic and corroborating the findings for *R. quelen*. Necrosis can occur as a consequence of oxidative stress (Lu et al. 2004), however the occurrence of oxidative stress was not confirmed in *R. quelen* after seventeen days after the injection with E_2. One possibility is that the cell damages such as necrosis were a consequence of the acute exposure to E_2 and redox unbalance not evaluated in the beginning of the experiment, meaning that the absence of oxidative stress after seventeen days is due to a modulation after long-term exposure (cell response) as described by Hughes and Gallagher (2004) in *Micropterus salmoides*. According to the same authors the oxidative stress occurred after 48 h of estrogen exposure, but after 7 and 14 days of exposure the normal conditions were reestablished, corroborating our hypothesis for *R. quelen*.

In both groups, female control and males injected with the highest dose of E_2, cytoplasm vacuolization were observed in the hepatocytes. This morphological finding could be a lipid accumulation for egg yolk production, since a high Vg synthesis was observed in females group and males exposed to 10 mg E_2 kg^{-1} group; in females it is a normal physiological situation, but in males it can be considered an alteration since they do not produce eggs.

The effects of estrogens in immune system of fishes are still unclear, but these effects on mammal immune systems are widely studied (Josefsson et al., 1992). In *Cyprinus carpio* and some mammals, the phagocytic activity of leukocytes was suppressed by estradiol (Magnusson and Fossum, 1992; Yamaguchi et al., 2001), and in mammals estradiol inhibits leukocyte production in bone marrow and affects the distribution of polymorphonuclear cells in peripheral blood (Josefsson, et al., 1992). The action of E_2 on important physiological activities such as leukocyte production and activity may lead to inflammatory suppression in *R. quelen* male fishes exposed to the highest dose of E_2, and thus to impairment of immunity and tissue repair.

According to Liehr (2000) and Joosten et al. (2004) high concentration of estrogens are genotoxic to mammals and E_2 was classified as a class I carcinogen (IARC, 1999). No morphological nuclear alterations indicating genotoxic effects on erythrocytes after short-term exposure were observed in the current and on other works, for *Dicentrarchus labrax* (Teles et al., 2004) and for *Spaurus aurata* (Teles et al., 2005). Despite of that, other authors have observed DNA breakage and DNA adduct formation in cells and tissues after E_2 long-term exposure, meaning that 17 days are probably not enough to cause DNA damages in *R. quelen* observable by microscopic analysis even injecting high doses.

Metallothioneins are highly conserved proteins responsible for metal sequestration and detoxication (Smirnov et al., 2005), having its induction affected by metals and other chemicals stressors (Smirnov


A
Superoxide dismutase (SOD) Activity
Units of activity.mg prot -1
B
Catalase (CAT) Activity
mmoles of H2O2 degr.min -1.mg prot -1
C
Glutathione-S-Transferase (GST) Activity
µmoles thioether. min-1.mg-1
D
Concentration of Reduced glutathione (GSH)
mmoles of GSH.mg prot -1
E
Glutathione Peroxidase (GPx) Activity
µmoles of NAPH oxid.min -1.mg prot -1
F
Lipid Peroxidation (LPO)
µmoles of hydroperoxides.mg prot -1
G
Metallothionein (Mt) Concentration
mg of Mt.mg prot-1
H
Estradiol concentration
Estradiol concentration in ng/ml
FC
MC
0.1 mg E2
1.0 mg E2
1 mg E2
10 mg E2

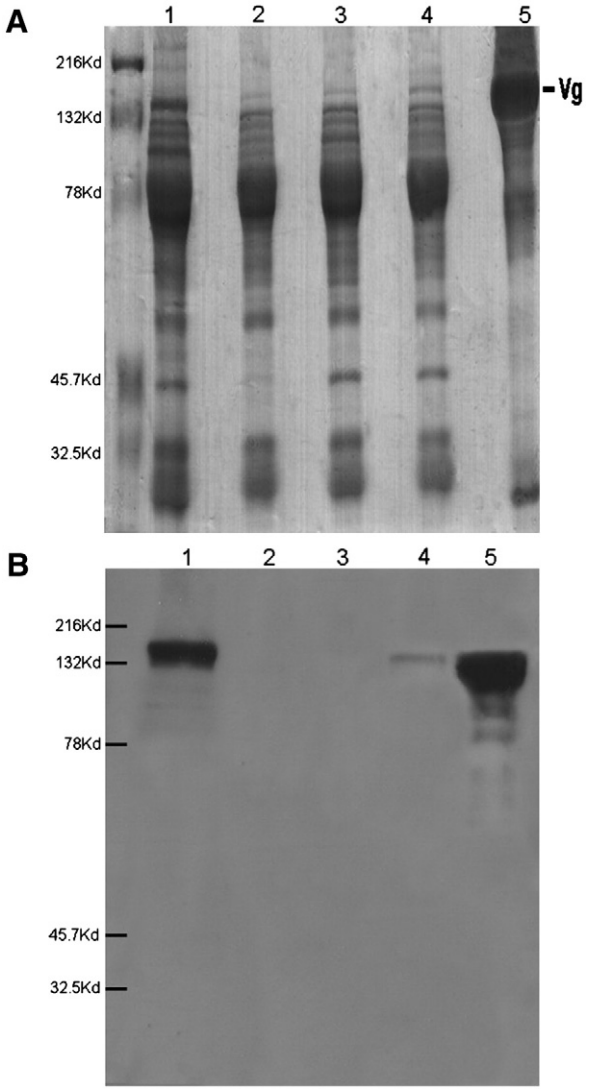


Fig. 6. Differential expression of vitellogenin. (A) Equal amounts of protein were resolved by SDS-PAGE, gel was stained with Coomassie brilliant blue. (B) Same samples were analyzed by western blot, using the rabbit anti-sea bream vitellogenin polyclonal antibody MM: molecular mass – Kd = kDa; Lane 1: plasma from female; Lane 2: plasma from male non exposed; Lane 3: plasma from male exposed to 0.1 mg E_2 kg^{-1}; Lane 4: plasma from male exposed to 1.0 mg E_2 kg^{-1}; Lane 5: plasma from male exposed to 10 mg E_2 kg^{-1}.

et al., 2005). Because elevated MT levels have frequently been associated with metal exposure, MT measurement has been used as a biomarker of metal pollution (Amiard et al., 2006; Costa et al., 2007). However, there are several factors that influence MT expression (Smirnov et al., 2005), such as age, sex and tissue type, both in the control and exposed fishes (Rhee et al., 2009). The levels of metallothionein decreased in the liver of *R. quelen* in males exposed to the highest E_2 dose, corroborating the findings described for *Salvelinus alpinus* (Gerpe et al., 2000) and for *Salvelinus namaycush* (Werner et al., 2003). Metallothionein levels in the liver of *R. quelen* were influenced by sex, indicating that the tested E_2 levels can modulate the metallothionein expression in this tissue down to the levels found in females. This study highlights that endocrine disruptors can modulate MT expression and vitellogenin together with MT are suitable for biomonitoring areas impacted by endocrine disruptor compounds.

Since E_2 is normally metabolized by hepatic enzymes the decrease in GST activity observed was not expected, and can compromise the metabolism of endobiotic molecules and xenobiotics, sensitizing the organism to the effects of pollutants that require GST conjugating activities for detoxication. In the current study, GST activity was inhibited in all E_2-treated groups and the same results were observed in *D. labrax* (Vaccaro et al., 2005). These findings may have an important impact on hepatic cell survival since GST expression is necessary for normal cell metabolism of many endobiotic molecules and protection against many xenobiotics.

The CAT and SOD increases, but not the GPx activity, represent an efficient modulation of the cellular defense mechanism against oxidative stress and cellular damage in the fishes exposed to the highest dose of E_2, meaning an initial direct or indirect disturbance to redox milieu caused by E_2 that was successfully neutralized by the increased expression of both enzymes, so that GSH, DNA structure and lipid peroxidation levels remained unaltered. The same biochemical responses were also observed in *S. auratus* (Vaccaro et al., 2005; Carrera et al., 2007), but not in *Cyprinus carpio* (Solé et al., 2000), which had a slight decrease of selenium-dependent GPx.

Vg is a large phospholipoglycoprotein precursor of egg yolk found on oviparous vertebrates, including fish (Hiramatsu et al., 2002; Pantino and Sullivan, 2002). It is a female-specific protein (Hara et al., 1980) synthesized in response to endogenous estrogens (Stifani et al., 1990) involved in both, defensive reactions and yolk proteins synthesis (Shi et al., 2006). Here and in other studies (Funkenstein et al., 2000; Moncaut et al., 2002; Pait and Nelson, 2003), there are strong indicatives that Vg production in males are dose and time-dependent. In natural environment fishes are "not pierced by needles" containing estrogens, however their chronic exposure to low levels of such compounds may induce Vg expression as well, as reported by Folmar et al. (2000).

In the present study, we observed that exposure to E_2 caused important damage to liver (necrosis) and can increase the Vg levels, the hepatocyte cytoplasm vacuolization, the HSI and the metallothionein concentration in *R. quelen* males, particularly on those fishes exposed to the highest E_2 dose. Foremost, E_2 injection resulted in levels of these biomarkers that are similar in females and exposed males. Then, E_2 can be considered harmful to *R. quelen* species and could potentially be hazardous for other tropical fishes on natural environment, because of the impairment on the capacity of fishes to maintain male-characteristic levels of those biomarkers, which are normally different from those of females.

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Fig. 5. Biochemical response on liver and estradiol on plasma. (A) Super oxide dismutase (SOD) activity in unities of activity per milligram of protein; (B) Catalase (CAT) activity in millimoles of H_2O_2 degraded per min per mg of protein; (C) Glutathione-S-transferase (GST) activity in micromoles of thioether produced per min per mg of protein; (D) Reduced glutathione (GSH) concentration in micromoles of cellular reduced glutathione per mg of protein; (E) Glutathione peroxidase (GPx) activity in micromoles of NAPH oxidized per min per mg of protein; (F) Lipid peroxidation (LPO) in micromoles of hydroperoxides per mg of protein; (G) Metallothionein concentration in mg of metallothionein per mg of protein. (H) 17-β-estradiol in ng of estradiol per mL of plasma. FC: female non-exposed; MC: male non-exposed; 0.1 mg E2: male exposed to 0.1 mg E_2 kg^{-1}; 1.0 mg E2: male exposed to 1.0 mg E_2 kg^{-1}; 10 mg E2: male exposed to 10 mg E_2 kg^{-1}. Different letters (i.e. a, b) mean statistical differences ($P<0.05$) among groups.

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