Ecotoxicology and Environmental Safety 144 (2017) 45–53

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Exposure to E_2 and EE_2 environmental concentrations affect different components of the Brain-Pituitary-Gonadal axis in pejerrey fish (*Odontesthes bonariensis*)

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ARTICLE INFO

Keywords:
Endocrine disruptors
Estradiol
Ethinylestradiol
Reproductive axis
Pejerrey

ABSTRACT

The present study focuses on the effects of E_2 and EE_2 environmental concentrations on different components of the reproductive axis of pejerrey (*Odontesthes bonariensis*), a native fish species from *Pampas* lakes of *Argentina*. The results obtained demonstrated that E_2 and EE_2 separate or mixed, could disrupt key pathways of the pejerrey Brain-Pituitary-Gonadal axis. First, it was observed that at the brain level, *gnrh-III* and *cyp19a1b* mRNA expression increased significantly in the exposed fish. Secondly, in the pituitary *fshb* and *lhb* mRNA expression levels, the study did not show any differences between treated and control groups. Thirdly, *fshr* and *lhcgr* transcript levels showed a significant decrease at testicular level. Nevertheless, testosterone plasmatic levels remained unchanged in exposed fish. In addition, in a histological analysis, it was possible to find pyknotic nuclei in estrogen only on treated fish testis linked to a reduction in the GSI index and a decrease in the length of spermatogenic lobules. All these findings highlighted the fact that environmental concentrations of E_2, EE_2 and their mixture disrupted the endocrine-reproductive axis of pejerrey, being the testis the main direct target.

1. Introduction

Estrogenic substances such as 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) are pollutants that contribute to the estrogenic activity in waters of the world (Desbrow et al., 1998). In vertebrate ovaries, E_2 is produced during oocyte growth (Zohar et al., 2010), while EE_2 is a synthetic hormone used in contraceptive pills (Kolpin et al., 2002; Laurenson et al., 2014). Both estrogens are excreted through the urine in the aquatic environment (Guengerich, 1990) making it possible to measure concentrations from less than 1 up to 300 ng/L for EE_2 (Kolpin et al., 2002; Laurenson et al., 2014; Caspillo et al., 2014), and 1 up to 631 ng/l for E_2 (Desbrow et al., 1998; Valdés et al., 2015).

Chascomús shallow lake (35°38′S 58°0′W) is a typical water body of Argentina's *Pampa* region and due to its particular physicochemical characteristics (Diovisalvi et al., 2010) it is possible to accumulate different pollutants as estrogens (Valdés et al., 2015). In this sense, E_2 and EE_2 (369 and 45 ng/L respectively) were measured in the waters of the "*Girado*" stream which is connected to *Chascomús* shallow lake, and where the effluents of the City of Chascomús are discharged. The reported concentrations are higher compared to other lakes around the world This may be due to a poor service of the local sewage plant of Chascomús city. In all *Pampa's* lakes, and specially in Chascomús, a population of an important native fish species, pejerrey (*Odontesthes bonariensis*), lives (Somoza et al., 2008; Colautti et al., 2015). Moreover, pejerrey is an excellent model to study environmental aquatic toxicology due to its sensitivity to pollutants (Pérez et al., 2012; Gárriz et al., 2015).

Although, estrogens levels found in dischargeable effluents are relatively low, E_2 concentrations recorded on the surface of water are usually higher than EE_2 (Desbrow et al., 1998; Kolpin et al., 2002; Laurenson et al., 2014; Valdés et al., 2015). And these two pollutants can act as sexual endocrine disruptors (ED) in different fish species (Brion et al., 2004; Cheshenko et al., 2008; Caspillo et al., 2014).

The reproductive cycle of teleost fish is regulated by different hormones and enzymes of the Brain–Pituitary–Gonadal axis in order to stimulate gametogenesis and spawning (Zohar et al., 2010). The gonadotropin releasing hormone (GnRH) is the neuromodulator that coordinates the synthesis and the release of pituitary gonadotropins (Gths) follicle, stimulating hormone (FSH) and luteinizing hormone (LH). These hormones play a central role in the regulation of gametogenesis and the production of sexual steroids, through their receptors at gonadal level (Levavi-Sivan et al., 2010). Sexual steroids exert negative

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http://dx.doi.org/10.1016/j.ecoenv.2017.06.002
Received 19 January 2017; Received in revised form 29 May 2017; Accepted 2 June 2017

and positive feed-back effects at the pituitary and hypothalamus levels to modulate the synthesis and release of GnRH and Gths (Zohar et al., 2010). The whole reproductive cycle regulation represents a complex process in fish, including the reception of environmental cues that can be disrupted by environmental stressors (Miranda et al., 2013). There is also a wealth of data that proves that environmental estrogens can influence or disrupt the normal endocrine regulation of the reproductive cycle (Johns et al., 2011). These kinds of reproductive alterations include the induction of different genes and proteins, changes in the gonadal structure and in the expression of secondary sex characteristics, and/or in the sexual behavior (Johns et al., 2011; Guyón et al., 2012; Caspillo et al., 2014; Roggio et al., 2014). Exposures to estrogens cause also a decrease in the gonadosomatic index (GSI), morphological changes in the gonads (Van den Belt et al., 2002; Versonnen and Janssen, 2004), an increase in Vitellogenin (Vtg; Tyler et al., 1996) and a rise of its expression in the liver of zebrafish (*Danio rerio*) males (Henry et al., 2009). Even more, EE_2 was able to modify zebrafish sex differentiation process producing sex reversal in males (Örn et al., 2003). But a peculiar characteristic of this pollutant is that the effects on adult fish could be reversible depending on timing, duration and concentration of the exposure (Van den Belt et al., 2002; Maack and Segner, 2004; Schäfers et al., 2007).

Looking for new and better estrogens biomarkers, Caspillo et al. (2014) demonstrated that the exposures of adult zebrafish males to 0.5 or 25 ng/L of EE_2 for 14 days produced both, a significant down-regulation of male-predominant genes such as *amh* (*anti*-mullerian hormone) and *dmrt1* (double sex and mab-3 related gene 1), and a significant induction of *vtg* transcription in the liver, suggesting a demasculinization process of the testis. In addition, fathead minnows (*Pimephales promelas*) males exposed to relevant concentrations of E_2 (32, 100 and 320 ng/L) during 14 days, showed a dose-dependent induction of plasma Vtg and an inhibition of testis growth (Halm et al., 2002).

Since the gonadal steroidogenesis pathway, the enzyme P450arom plays an important role in the sexual differentiation and gonadal development in all vertebrates, because it catalyzes the conversion of testosterone (T) to E_2 during steroidogenesis (Simpson et al., 2002). Two different aromatase isoforms exist in teleost fish: *cyp19a1a* (P450aromA) expressed in the gonads and *cyp19a1b* (P450aromB) expressed in the brain (Kishida and Callard, 2001). It has been reported that estrogens exposures cause an upregulation of *cyp19a1b* gene (Halm et al., 2002; Kishida and Callard, 2001; Kazeto et al., 2004) and an increase of the aromatase activity in males of fathead minnow (Halm et al., 2002), medaka (*Oryzias latipes;* Contractor et al., 2004), and cunner (*Tautogolabrus adspersus*; Mills et al., 2014). Also, *Jennynsia multidentata* adult males exposed to E_2 (75 and 150 ng/L) and EE_2 (50, 100 and 250 ng/L) showed an increase of *cyp19a1b* gene expression (Guyón et al., 2012; Roggio et al., 2014). All the data mentioned above indicates that aromatase expression is a good estrogens exposure's biomarker and a key gene that could be related to abnormalities in the reproductive axis and in sex behavior (Guyón et al., 2012).

Steroids plasmatic levels could vary in different ways under estrogen exposure. For example, elevated 11-ketosterone (11-KT) plasma levels were reported in rainbow trout males (*Oncorhynchus mykiss*) exposed to 10 ng/L of EE_2 but at the same time, a reduction of 11-KT plasma levels was observed when these fish were exposed to higher EE_2 concentration (100 ng/L; Schultz et al., 2003). Also, 11-KT levels in estrogen treated goldfish (*Carassius auratus*) were reduced and, as expected, the E_2 levels were increased (Golshan et al., 2015).

There are only two works about the effects of estrogens in *O. bonariensis,* in which larvae were fed with estrogens through the diet. One of the studies demonstrated that E_2 administration (20 and 50 mg/kg) produced 100% females (Strüssmann et al., 1996), and the other one reported that EE_2 administration led to a feminization process in the pejerrey larvae during the first weeks after hatching, increasing the expression of *cyp19a1a* (Pérez et al., 2012). Furthermore, a recent study has demonstrated that E_2 and EE_2 environmental concentrations negatively affected sperm motility, fecundity, and embryo and larval survival (Gárriz et al., 2015).

There are many studies that describe adverse effects of environmental estrogens in different fish species. However, these works usually focus more on the effects in a particular developmental stage, sex or only one component of the reproductive system. Within this context, the aim of the present study was to evaluate in-depth the effects of the exposition of environmental concentrations of E_2 and EE_2 and their mixture in the pejerrey endocrine-reproductive axis, analyzing the expression of several genes at brain, pituitary and gonadal levels. Additionally, in order to find a possible damage in the testis, a detailed gonadal histology analysis was performed.

2. Materials and methods

2.1. Fish exposure and sampling

Pejerrey mature males (Weight: 42.31 ± 3.43 g; Standard Length: 16.21 ± 0.36 cm) were selected from the stock of IIB-INTECH aquaculture facilities during the spawning season (spring) and kept in 60 L glass aquariums containing 50 L of volume solution in a closed room at air temperature (20 °C) and photoperiod (12 h light: 12 h dark). A static exposure system was implemented with 6 fishes per aquarium (5 males and 1 female to keep reproductive activity) and artificial aeration. For this study it was used water with the estrogens environmental concentrations in the surface water of *Girado* stream as reported by Valdés et al. (2015): 350 ng/L (1.28 nM) of E_2, 45 ng/L (0.15 nM) of EE_2 and the mixture of both estrogens at these same concentrations. First, a 100 mg/l stock solution for each estrogen was diluted in ethanol. Both stock solutions were diluted for a second time in ground water, 1/100 times for EE_2 and 1/10 times for E_2, and from these stocks the doses for each treatment were prepared. The quantity of ethanol added to the aquariums was less than 0.07% of the total volume. Since there is a previous study performed in the same fish species where there were no differences between solvent and control groups (Gárriz et al., 2015), and also to avoid the unnecessary death of fish, the solvent control was not performed in this experiment. The control group consisted only with ground water (pH 7.97; Osmolarity 222.7 mOsm/L; Alkalinity 611.3 mg $CaCO_3$/L; Hardness 202.7 mg $CaCO_3$/L; Nitrites 100 µg/L; Nitrates 19 mg/L). The time of exposure was 14 days and the estrogen compounds were added every 48 h, taking into consideration their half-life (24–48 h) reported for E_2 (Guyón et al., 2012; Meijide et al., 2016) and for EE_2 (Hashimoto et al., 2008; Roggio et al., 2014). Before pouring the estrogens compounds, the bottoms of the aquariums were cleaned up to remove feces and remained food and then, the volume of water was restored up to 50 litters. The physicochemical parameters such as dissolved oxygen (DO), ammonia levels, pH and water temperature were recorded every day. All the fish were fed once a day (1% body weight) with commercial food 3 mm pellet (Protein: 42.9%; Lipids: 1.5%; Carbohydrates: 43.8% Phosphorus: 2.9%; Shullet, Bs. As., Argentina). Each estrogen treatment and control were tested by duplicate.

All fish were euthanized with benzocaine (100 ppm) at the end of the treatment (day 14), and sperm, blood and tissue samples were collected. The brain, pituitary gland and testis from each pejerrey were carefully collected and immediately flash-frozen in liquid nitrogen. The brain was dissected into three sections: rostral (including the olfactory bulbs and telencephalon), medium (from the optic tectum to the hypothalamus) and the posterior section (containing the cerebellar body, vagal lobe and medulla oblongata) that was discarded. All these samples were stored at −80 °C until the moment of analysis. The gonadosomatic (GSI = gonad weight/body weight × 100) and hepatosomatic index (HSI = liver weight/body weight × 100) were also assessed.

2.2. Determination of sex steroids

Fish were anesthetized by immersion in a 100 ppm benzocaine, and then blood samples (300–500 μL) were taken from the caudal peduncle and collected in 1.5 mL tubes by heparinized syringes. They were then centrifuged (3000*g* for 15 min at 4 °C) and the plasma was separated and stored at −80° C. Prior to use, serum samples were extracted with diethyl-ether and suspended in their initial volume of PBS buffer as it was described in Elisio et al. (2012). E_2 and T plasma levels were measured by an enzyme-linked immunosorbent assay (ELISA) based on the principle of competitive binding, using commercial kits and following the manufacturer's protocols (DRG International Inc., Mountainside, NJ, USA; E_2: EIA-2693 and T: EIA-1559) previously validated by Chalde et al. (2016). A standard curve was run for each ELISA plate. The lower limits of detection were 288.99 pg/mL for T and 32.41 pg/mL for E_2. The optical density was read at 450 nm, for both T and E_2. The intra-assay coefficients of variance were < 10%.

2.3. Extraction of RNA, cDNA synthesis and gene expression measurements by quantitative real-time qPCR

Total RNA was extracted from the brain, pituitary gland and testis using Trizol (Invitrogen, CA, USA) following the manufacturer's instructions. Briefly, 50 mg of tissue were homogenized in 0.5 mL of Trizol and chloroform (100 μL) were added. After mixing, samples were centrifuged (12,000 × *g* for 15 min) and supernatants were transferred into new tubes containing an equal volume of isopropanol. The mixture was centrifuged (12,000*g* for 10 min) and the precipitated RNA pellet was washed once using 0.5 mL of ethanol 75%. Total RNA concentration was estimated using a Sinergy H1 spectrophotometer (BioTek Instruments Inc, Winooski, Vermont, USA) and its purity was verified by A260 nm/A280 nm ratio. The RNA samples were treated with DNase I (Invitrogen) and reverse transcribed using SuperScript II RNase H (Invitrogen, except for pituitary gland samples where SuperScript III was used because its greater efficiency) and oligo(dT) to obtain cDNA samples. The cDNA template quality was checked before the RT-qPCR analysis using *β*-actin as control. Gene-specific primers for RT-qPCR analysis were designed to generate amplicons no longer than 155 bp using the Primer Express software (Applied Biosystems, Foster City, CA, USA; see Table 1). The PCR mix consisted of 2 μL of diluted cDNA (ca. 100 ng), 1 pmol of each primer and 5 μL of FastStart Universal SYBR Green Master (ROX, Roche Applied Science, Mannheim, Germany) in a final volume of 10 μL. The reactions were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The efficiencies of RT-qPCR ranged between 87% and 100%. Dissociation-curves analyses were run after each real-time experiment to ensure that there was only one product. A reverse-transcriptase negative control was run for each template and primer pair. The relative transcript levels of brain GnRH variants (*gnrh-I, gnrh-II* and *gnrh-III*), brain aromatase (*cyp19a1b*), pituitary Gth-*β* subunits (*fshb, lhb*) and gonadal Gth receptors (*fshr, lhcgr*) genes were determined in each individual using real-time RT-PCR with the standard curve method following the procedure published by Applied Biosystems (1997). The gene expression data were normalized using two housekeeping genes: *β*-actin and elongation factor 1-α (EF1α) as reference. Because there were no significant differences between them, the graphics only show *β*-actin results.

2.4. Histological analysis

For histological procedures a portion of each testicle was fixed in 10% neutral-buffered formalin, dehydrated, cleared and embedded in Paraplast (Leica Biosystems Richmond Inc. USA). Each tissue block was sectioned in 7 serial columns containing 7 sections of 6 μm thick. There was a 100 μm section space between each column so as to cover a representative testicle area. The samples were stained with hematoxylin and eosin for observation under a light microscope. In one of the section of each 7 columns the total number of abnormal pyknotic nuclei was counted following the criteria of Patiño and Takashima (1995). In addition, the length of 5 spermatogenic lobules was measured in the same sections, by micrographs taken with a light microscope Nikon Eclipse E600, attached to a digital photomicrographic system (Nikon Digital Sight DS-U2), and Image-Pro Plus software (Rockville, USA).

Table 1
Oligonucleotid primers used for real time RT-PCR.

Primers	(5′ - 3′)	Source sequence
gnrh-I (103):		
Forward	TGCACCTTGCCTGTTGTGG	AY744689
Reverse	GCGTCCATTTTCCCTGTCGGT	
gnrh -II (104):		
Forward	CTACTTGAGACCCCAGAGGCAGAA	AY744687
Reverse	AGCAGCGAAAGATGGAAAGCAGTC	
gnrh -III (154):		
Forward	GAGGCAAGCAGCAGAGTTATGGTG	AY744688
Reverse	CTCCTCCTGTGCCCATCATCCT	
cyp19a1b (71):		
Forward	CCATCTTGATTACTCTGTTGTCTCGTT	AY380061
Reverse	CTTGATGCTGTTGAGGTTGCA	
fshb (103):		
Forward	GGCTGCCACCTCGACTGTTAT	AY319832
Reverse	TGAAGCACAGTCCTTCACATATGG	
lhb (96):		
Forward	CATCCAGTGGAAGCAACCATCT	AY319833
Reverse	CGTGCACACACTTTGGTACATGT	
fshr (83):		
Forward	TGGCAAAACTAACGTACCCTTCA	GQ258853
Reverse	GTCGCCACAAAACAAGTTCCA	
lhcgr (98):		
Forward	GCCATGCCAACACTGACTTCTATAG	GQ258852
Reverse	GGGTTTCTGTTGGCCACTTGT	
b-actin (83):		
Forward	CTCTGGTCGTACCACTGGTATCG	EF044319
Reverse	GCAGAGCGTAGCCTTCATAGATG	

2.5. Statistical analysis

As no great differences were found in all the parameters analyzed between males of duplicate aquariums for the statistics analysis, they were grouped giving a n = 10 for each estrogen treatment. Data is presented as the mean ± standard error of the mean (SEM). The differences for all the parameters were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The results were considered statistically significant at $p < 0.05$. Logarithmic transformations were used for *cyp19a1b*, *lhcgr* and *fshr* because the data lacked the assumptions of the statistical test. Statistical analyses were performed using GraphPad Prism 5.0 Software and SPSS Statistics 20.0 (IBM).

3. Results

3.1. Water quality, fish survival and body indexes (HSI and GSI)

Mean water temperature during the experiment was 22.14 ± 0.11 °C. There were not significant differences between treatments and between days of exposures in DO and pH measures, being the total average 7.17 ± 0.47 mg/L and 8.17 ± 0.02, respectively. The levels of ammonia were lower than 20 ppm, being bellow pejerrey tolerance limits (Gómez et al., 2007). Fish did not show signs of stress during the experiment and only two fish died in the mix treatment. GSI decreased in all the treatments compared with the control group, but it was significantly different with the mix group ($p < 0.05$; Fig. 1A).

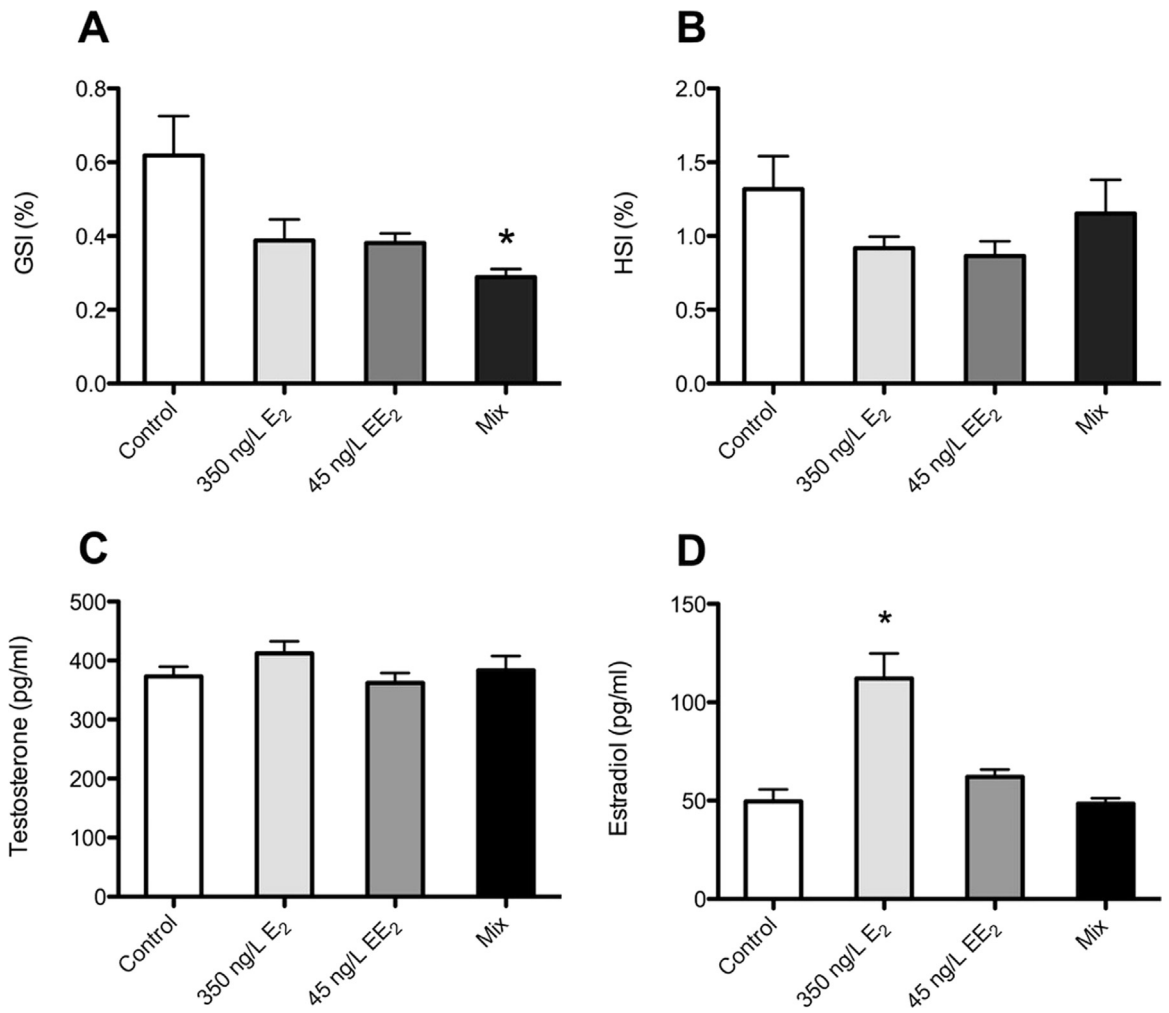

Fig. 1. Gonadosomatic Index (GSI; A), Hepatosomatic Index (HSI; B), and plasma levels of T (C) and E_2 (D) measured in *O. bonariensis* fish exposed to E_2 (350 ng/L), EE_2 (45 ng/L) and the mix of both estrogens (n = 10; * differences between control and treatments $p < 0.05$).

There were not differences in HSI among the control and the estrogens treatments ($p < 0.05$; Fig. 1B).

3.2. Sperm release and plasma sex steroids levels

It was not possible to obtain sperm samples from treated fish meanwhile a low quantity of sperm was collected from the control fish. For this reason, the quality of the sperm was not assessed. After 14 days of exposure, there were not differences in T levels between treated fish and the control group (Fig. 1C). However, E_2 levels were significantly higher only in E_2 treated group compared with the control (Fig. 1D).

3.3. mRNA transcript levels of Brain-Pituitary-Gonadal axis genes

It was observed that *gnrh-I* and *gnrh-III* expression tended to increase in the rostral brain section of treated fish. However, it was only significantly different in *gnrh-III* expression between EE_2 treated fish and the control group (Fig. 2). Otherwise, all the analyzed genes in the medium brain section (*gnrh-I*, *gnrh-II* and *cyp19a1b)* showed an expression increase in all the estrogenic treatments. Only the *cyp19a1b* expression was significantly higher in the mix group compared with the control group ($p < 0.05$, Fig. 3).

At pituitary level, there were not clear differences for *fshb* and *lhb* transcript levels between treated and control fish (Fig. 4A, B).

In the testis, *fshr* and *lhcgr* transcript levels decreased in all the exposed fish compared to the control group. The differences were statistically significant between EE_2 and the mix group compared with the control group for *fshr* (Fig. 4C); and between EE_2 treatment and the mix group for *lhcgr* (Fig. 4D).

3.4. Gonadal histology

Severe testicular histological alterations were observed in all treated fish compared to control fish. It was highly remarkable that exposed fish presented testis with pyknotic nuclei (probably degenerating spermatogonia), a shrinkage of the spermatic lobules, the loss of the cyst structure with severe interstitial fibrosis, and containing markedly fewer or even lacking spermatocytes and Leydig cells (Fig. 5). The number of pyknotic nuclei was increased (Fig. 6A) in the mix treatment compared with the control group, and the length of spermatic lobules was significantly reduced in all the estrogen treatment compared with the control group (Fig. 6B).

4. Discussion

The present study demonstrated that relevant environmental concentrations of E_2 and EE_2 had the ability to disrupt the Brain-Pituitary-Gonadal axis of pejerrey fish, mainly at gonadal level.

Emphasizing the particulars effects on the endocrine-reproductive axis, the results of the present study showed that environmental concentrations of E_2 and EE_2 altered the expression pattern of key genes that control the whole axis, upregulating *gnrh-III, cyp19a1b,* and down-

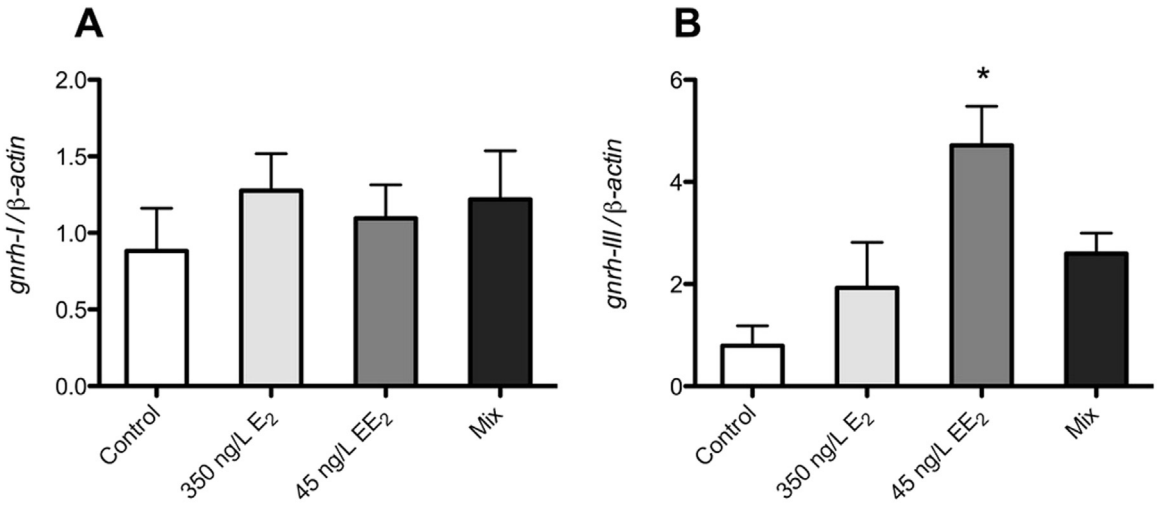

Fig. 2. Gene transcript levels of *gnrh-I* (A) and *gnrh-III* (B) in the rostral region of the brain of *O. bonariensis* males exposed to E_2 (350 ng/L), EE_2 (45 ng/L) and the mix of both estrogens (n= 10; * differences between control and treatments $p < 0.05$).

regulating *fshr* and *lhcgr*. Precisely, *cyp19a1b* expression, is strongly regulated by E_2 through an estrogen-responsive element (ERE) localized in the promoter of the gene, even at very low doses of estrogenic compounds (Kazeto et al., 2004; Menuet et al., 2005). This experiment also showed, that in all treated pejerrey the brain aromatase transcripts levels increased after the exposure but they were significantly higher only in the mix. Other experiments could be done in order to test whether under a longer time of exposure to each estrogen separately major changes occur. The alteration of *cyp19a1b* gene expression levels was associated with a fluctuation in the activity of this enzyme in the

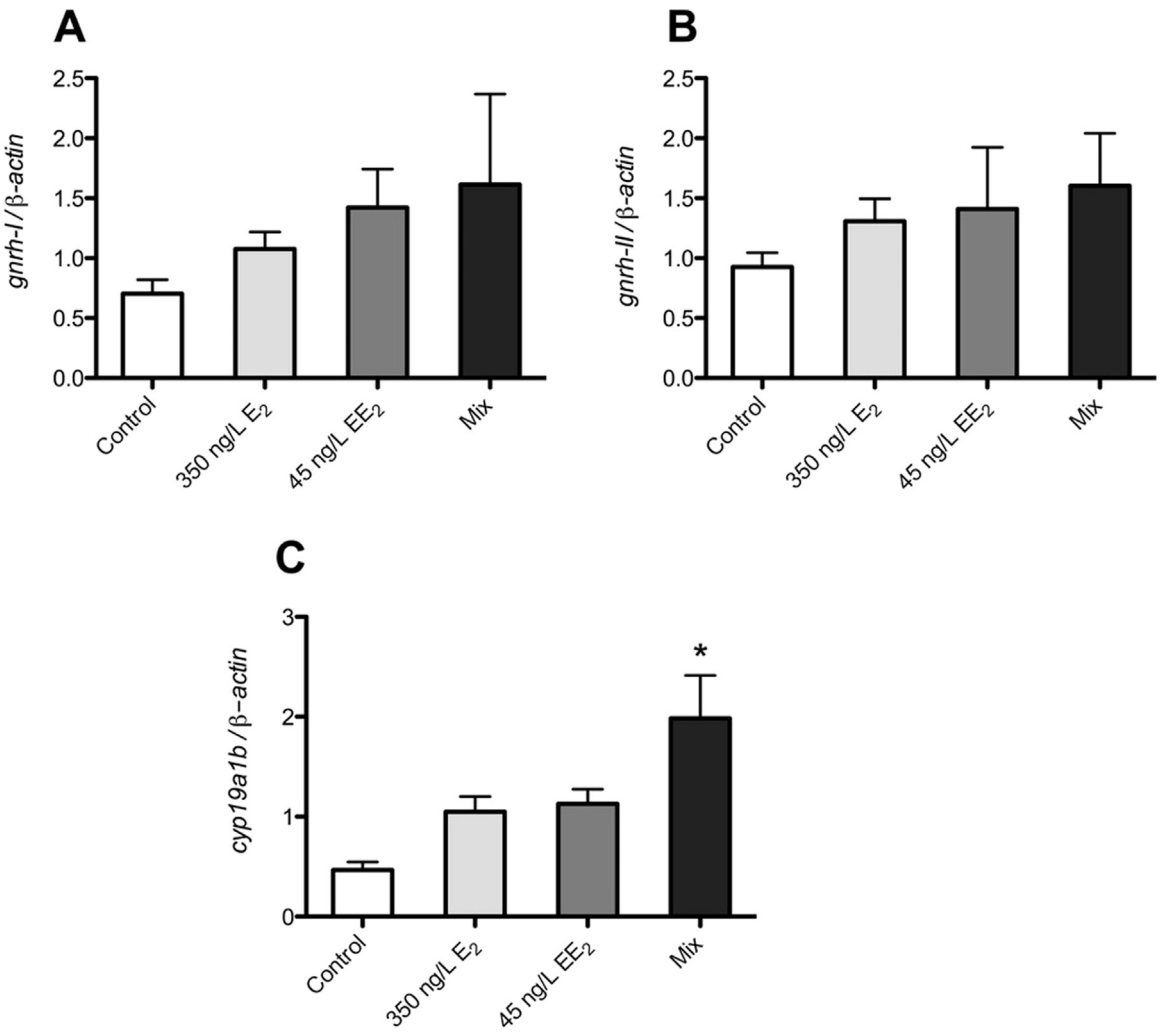

Fig. 3. Gene transcript levels of *gnrh-I* (A), *gnrh-II* (B) and *cyp1a1b* (C) in the medium brain region of *O. bonariensis* fish exposed to E_2 (350 ng/L), EE_2 (45 ng/L) and the mix of both estrogens (n =10; * differences between control and treatments $p < 0.05$).

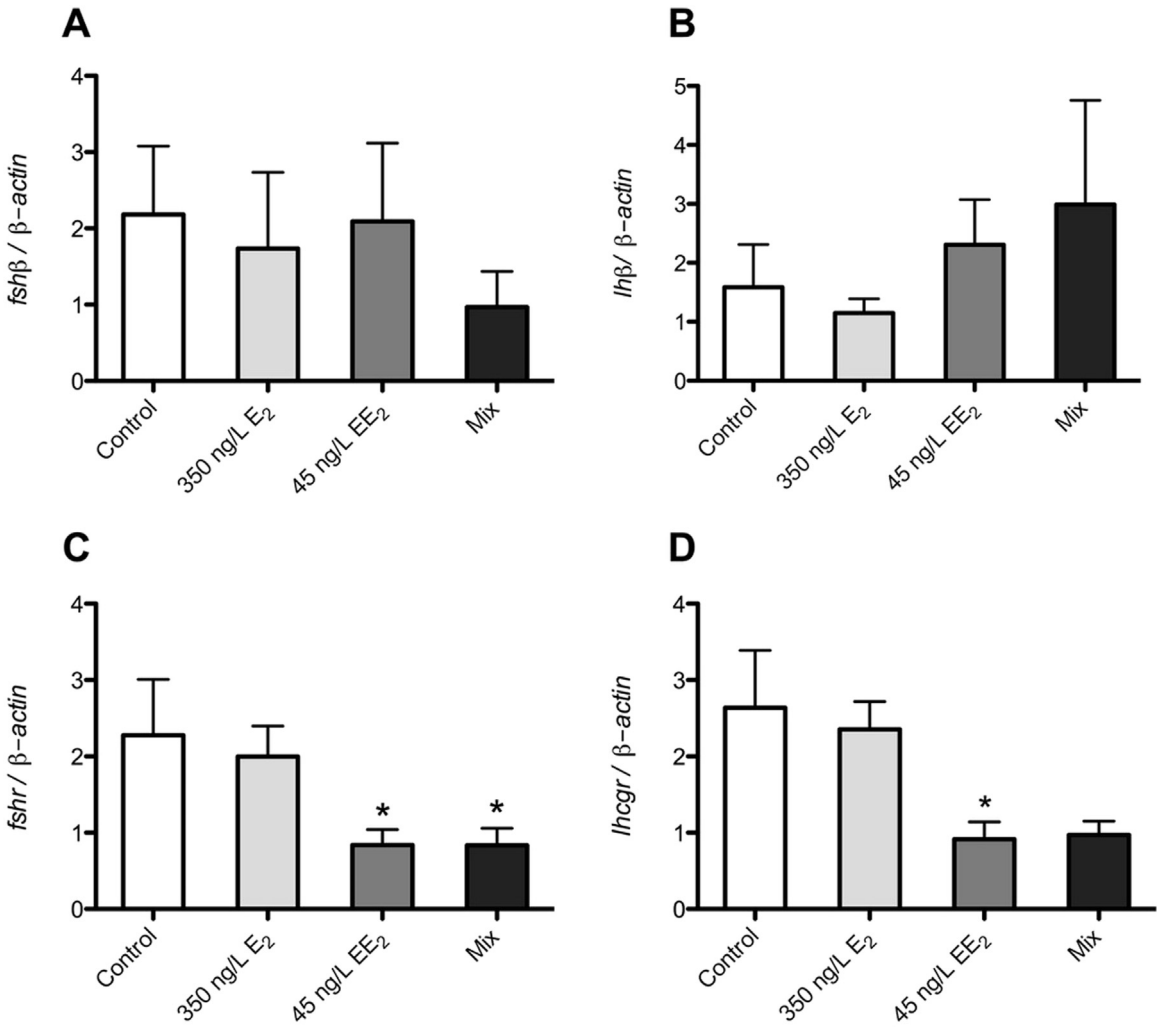

Fig. 4. Gene transcript levels of *fshb* (A) and *lhb* (B) in the pituitary gland and *fshr* (C) and *lhcgr* (D) in the testis of *O. bonariensis* fish exposed to E_2 (350 ng/L), EE_2 (45 ng/L) and the mix of both estrogens (n= 10; * differences between control and treatments $p < 0.05$).

brain and gonads (Cheshenko et al., 2008). The *cyp19a1b* expression changes after an estrogens exposition depending on the different stages of fish development, sex, exposure times and fish species, although normally the result is an upregulation (Guyón et al., 2012; Roggio et al., 2014). However, it has been recently observed that *cyp19a1b* mRNA expression showed no clear response in zebrafish after discontinued exposure to EE_2 (Baumann et al., 2014). Otherwise, the effects on gonadal aromatase expression (*cyp19a1a*) are much more inconsistent giving ambiguous conclusions (Scholz and Gutzeit, 2000; Cheshenko et al., 2008; Pérez et al., 2012).

Although there are many estrogen exposures studies, only a few of them focus on the analysis of *gnrh* expression disruption. In the present study, *gnrh-III* expression increased in all the treatments, being only statistically significant in EE_2 treated fish, reflecting a positive feedback mechanism. However, in goldfish treated with 5 μg/L E_2 (Golshan et al., 2015) and in *Clarias garjepinus* exposed to 1 μg/L of EE_2 (Swapna and Senthilkumaran, 2009) a decrease in *gnrh-III* and in *gnrh-I* expression was observed, respectively. Also, Parhar et al. (2000) have shown an increase of POA-H GnRH neuronal number but no alteration in the midbrain *gnrh-II* mRNA levels in *Oreochromis niloticus* sexually immature males treated with 5 μg/g of E_2. These contradictory results, according to the authors may be due to the differences in fish reproductive stages, the species, and experimental conditions.

There is practically no-data on the variations of Gths gene expression under estrogens exposure. Goldfish treated with E_2 showed that circulatory LH levels decreased after 30 days of exposure (Golshan et al., 2015), but there is no information available about *lhb* and *fshb* gene expression. The study carried out showed a high variation of both Gths gene expression in treated fish but not with a clear pattern, perhaps it should have required longer times of exposure to find significant differences. However, the effect of estrogens was evident in both Gths receptors showing a significant decrease of *lhcgr* and *fshr* transcript levels in EE_2 and mix treatments, possibly due to a negative feedback mechanism. Other explanation, could be the absence of Leydig cells where gonadotropin receptors had been identified (Levavi-Sivan et al., 2010). Similar results were reported in pejerrey after the exposure to abnormal high temperatures, where a decrease of *fshr* expression jointly with an alteration of gonads structure were demonstrated (Ito et al., 2008; Elisio et al., 2012).

The reduction of the GSI and the fact that it was not possible to obtain releasable sperm of the exposed fish may be related to the severe histological damage observed in the testis of treated fish. Nevertheless, only a significantly decline in the GSI was found in the fish group treated with the mix of E_2 and EE_2 where high number of pyknotics cells and a marked shortening of the spermatogenic lobules were observed, possibly due, to a synergistic effect provoked by the action of both estrogens at the same time. It was previously reported that a decrease in the GSI and a clear disorganization of the testis structure, with the lack of germinal cells and severe interstitial fibrosis, is a common adverse effect of estrogens exposures in different fish species (Jobling et al., 1996; Weber et al., 2003; Swapna and Senthilkumaran, 2009; Velasco-Santamaría et al., 2010, 2013; Oropesa et al., 2013). These

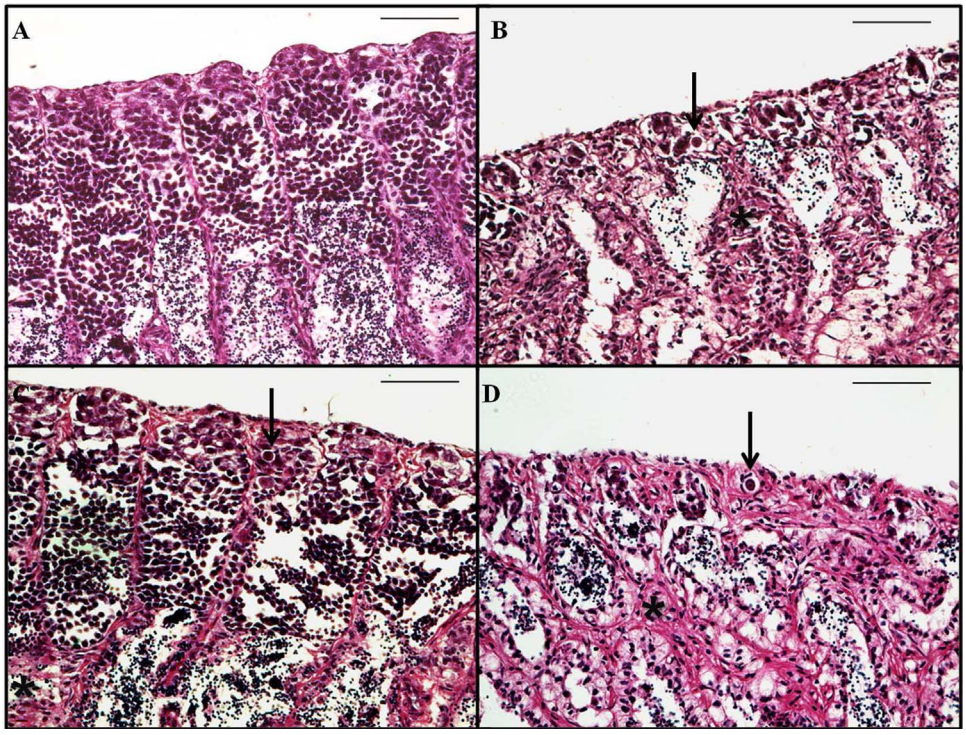

Fig. 5. Testis micrographs of *O. bonariensis* fish. A) Control, B) E_2 exposed fish (350 ng/L), C) EE_2 exposed fish (45 ng/L), D) Mix estrogens treated fish. Black arrows: Pyknotic cells; Black asterisks: Interstitial fibrosis (Bar: 50 μm).

outcomes were also associated with alterations in other aspects of fish reproduction, such as a decrease in egg or milt production, or abnormalities in the testicular development (Bjerselius et al., 2001; Guyón et al., 2012; Roggio et al., 2014; Golshan et al., 2015).

It is known that sexual steroids play a key role in the regulation of the reproductive endocrine axis through feedback mechanism between brain, pituitary and gonads (Zohar et al., 2010). In the current study, a significant increase in E_2 plasmatic levels were only observed in the E_2 treated fish, perhaps reflecting the incorporation of this steroid inside the fish body. The T plasma levels were unchanged in the treated fish, demonstrating the requirement of higher doses of estrogens or longer time of exposition to see an alteration at this level. In this sense, it was reported that E_2 exposure increased E_2 and decreased 11-KT plasma levels in goldfish males (Golshan et al., 2015), meanwhile EE_2 exposure decreased E_2, T and 11-KT levels in goldfish and in fathead minnows males (Salierno and Kane, 2009). However, the deviation of sex steroids levels might differ based on the concentration of the pollutant, time of exposure and the reproductive phase of the exposed fish.

In the current study the significant increase in *cyp19a1b* ARNm expression was observed in the mix treatment, added to a significant reduce in the GSI and the length of the spermatic tubules, and also a greater number of pyknotic cells. These results together could suggest a synergetic effect of E_2 and EE_2 on the reproductive axis of pejerrey, as it was demonstrated in other fish species such as zebrafish (Silva et al., 2012; Chen et al., 2015).

All these findings highlighted the idea that the gonads represented the main target of estrogens compounds in pejerrey fish. The presence of spermatogonia degeneration and the absence of spermatocytes are signs of a gonadal regression process that could lead to permanent sterility, by a complete eradication of the germ cells involving apoptosis (Ito et al., 2008). Apparently, spermatocytes and spermatids are the most heat-sensitive cells in the mammalian testis (Chowdhury and Steinberger, 1970) and the same could be happening for fish exposed to estrogens.

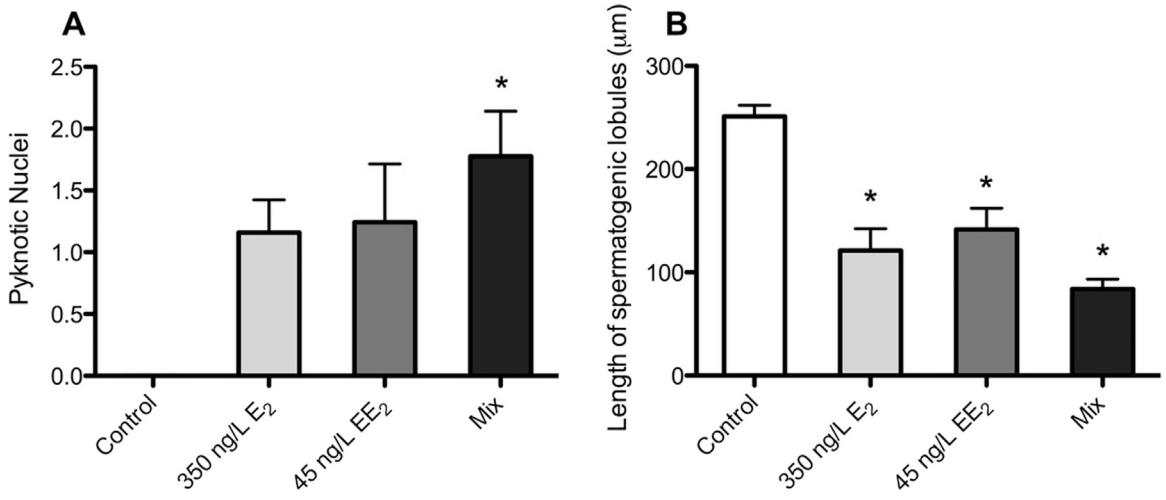

Fig. 6. Pyknotic nuclei (A) and spermatic lobules length (B) averages counted in 7 sections of *O. bonariensis* testis in the control group, exposed to E_2 (350 ng/L), EE_2 (45 ng/L) and the mix of both estrogens (n = 10; * differences between control and treatments $p < 0.05$).

For many years, the reproductive organs have been considered to be the main target of estrogenic endocrine disruptors compounds, causing many types of alterations (Sumpter and Jobling, 2013; Caspillo et al., 2014). But it should be taking in account that the effects might differ based on the fish species, its reproductive phase and the analyzed pollutant concentration. Even so, more research and experiments should be needed to deeply understand how environmental estrogens or endocrine disruptors in general affect the molecular and genetic mechanisms in fish reproductive system.

5. Conclusion

This study demonstrated that environmental concentrations of estrogens altered key genes of pejerrey endocrine-reproductive axis. At brain level caused the upregulation of *gnrh-III* and *cyp19a1b* but the effects were more evident at gonadal level, causing down-regulation of both gonadotropin receptors (*fshr* and *lhcgr*), the appearance of pyknotic nuclei, the absence of spermatocytes and the shrinkage of the germinal epithelium in the testis that consequentially could lead to sterility. The presence of this kind of pollutants in the *Pampa*'s water bodies represent a real threat to wild fish species such as pejerrey, an emblematic fish species of this region.

Acknowledgement

The authors thank Javier Herdmann, Renata Menendez-Hellman and Oswaldo Tovar for their technical assistance during the experiment and the English professor Monica Reggini for her help in the revision of the English version of this manuscript. This study was supported by grants to L.A.M. (ANPCyT: PICT 2012-1537, CONICET: PIP 1673/12).

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