General and Comparative Endocrinology 273 (2019) 152–162

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Research paper

Effects of heavy metals identified in Chascomús shallow lake on the endocrine-reproductive axis of pejerrey fish (*Odontesthes bonariensis*)

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ARTICLE INFO

Keywords:
Atherinopsidae
Gonad alterations
Reproduction
Testis ova
Toxic metals

ABSTRACT

Some heavy metals related to human activities were measured in the water of Chascomús lake. The maximum concentrations were: 0.23 μg/L for Cd, 4.28 μg/L for Cr, 22.09 μg/L for Cu, 2.49 μg/L for Ni, 3.24 μg/L for Pb and 210.76 μg/L for Zn. The values of Cd, Cr, Cr, Pb and Zn were above the Argentine National Guidelines for the Protection of the Aquatic life. The analysis of gonadal condition of pejerrey fish (*Odontesthes bonariensis*) from this lake did not revealed any reproductive damages. However, exposures with environmental concentrations of Cd, Cr, Cu and Zn under laboratory conditions of pejerrey males (14 days), caused a significant increase of the expression of the three variants of *gnrh* in the brain (within Cd exposure) and a decrease in *cyp19a1b* mRNA (within Cu exposure). Furthermore, at pituitary level, a decrease in *fshb* transcript levels was observed in the fish exposed to Cd and Cr and a decrease in the expression of both gonadotropin receptors at gonadal level in Zn exposure. Moreover, the gonads of the fish exposed to all the tested metals suffered structural damages showing shortness of the spermatic lobules, fibrosis, testis ova and the presence of piknotic cells. All these findings alert that heavy metals pollution affects the expression of key reproductive genes and gonadal structure of fish species that represent the predominant group of organisms and are considered sentinel species in the aquatic ecosystems.

1. Introduction

The term "heavy metals" group different metals and metalloids elements directly associated with pollution and with potential toxicological properties (Duffus et al., 2007). Certain heavy metals, such as zinc (Zn), copper (Cu), iron (Fe) and manganese (Mn), are essentials in the regular cellular metabolism but when they are available in high concentrations they could be extremely toxic (Farkas et al., 2000; Handy, 2003). Other nonessentials metals, like arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg) and lead (Pb), caused adverse effects on aquatic organisms even at low concentrations (Cohen et al., 2001; Díaz et al., 2006; Sprocati et al., 2006; Denier et al., 2009; Velma et al., 2009; Jabeen et al., 2012). One peculiarity of heavy metals is their persistence in the environment that let them to bioaccumulate in water, sediments or organic tissues (Linnik and Zubenko, 2000; Uysal et al., 2008). Also, they can become complex solutions that could be even more toxic (Mance, 1987). Due to the characteristics mentioned before, heavy metals are considered one of the most toxic group of pollutants in the aquatic environments such as rivers and lakes (Mance, 1987).

Heavy metals concentrations are increasing in different water bodies around the world in relation with the rise of anthropogenic activities (Beck, 1996; Karadede et al., 2004; Naigaga et al., 2011) like the expansion of agriculture, industries, and the growth of populations and urbanization (Mance, 1987; Echols et al., 2009; Jorgensen, 2012). Metals concentrations reported in aquatic ecosystems were between 1 and 10 μg/L for Cr; 0.20–30 μg/L for Cu; 0.50–10 μg/L for Zn and less than 0.10 μg/L for Cd (Malik et al., 2010; Nawaz et al., 2010; Eroglu et al., 2014; Avigliano et al., 2015). Nevertheless, much higher values were determined in some extreme polluted water bodies in countries like China, India, Pakistan and Poland (Dojlido, 1995; Cheung et al., 2003; Malik et al., 2010; Nawaz et al., 2010).

Chascomús shallow lake (35°38′ S 58°0′ W) is a typical and well-studied water body of the *Pampa* region in Argentina (Diovisalvi et al., 2015). Its specific physicochemical characteristics allow the accumulation of different pollutants including heavy metals (Carriquiriborde and Ronco, 2002; Diovisalvi et al., 2010; Avigliano et al., 2015). *Los Toldos* stream is an important source of heavy metals for the Chascomús lake, since it crosses several industrial and urban areas before flow into the lake (Dangavs et al., 1996). In the last decade, different heavy

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https://doi.org/10.1016/j.ygcen.2018.06.013
Received 26 December 2017; Received in revised form 14 June 2018; Accepted 21 June 2018
Available online 22 June 2018
0016-6480/

metals were measured in the water of Chascomús lake and their concentrations were over the Argentine National Guidelines for the Protection of Aquatic life (National Law N° 24,051; Decree 831/93; Annex II). These values were 3.1 µg/L for Cr, 9.3 µg/L for Cu, 0.28 µg/L for Cd, 27.3 µg/L for Zn, 8.22 µg/L for Pb and 3.22 µg/L for Ni (Schenone et al., 2014). Furthermore, similar levels were also reported for other pampasic lakes (Adela, Barrancas and Chasicó; Avigliano et al., 2015).

Particularly, in Chascomús shallow lake inhabits a large population of pejerrey fish, *Odontesthes bonariensis* (Colautti et al., 2015), an important native fish species from the *Pampa* region of Argentina, due to the sport fishing and its flesh quality (Somoza et al., 2008; Berasain et al., 2015). Moreover, this fish species is considered an excellent model for aquatic ecotoxicology studies due to its sensitivity to different pollutants (Carriquiriborde and Ronco, 2002, 2008; Pérez et al., 2012; Gárriz et al., 2015, 2017; Menéndez-Helman et al., 2015).

Essentially, fishes are the predominant group of vertebrates in the aquatic ecosystems and they are critical for the study of adverse effects that heavy metals can generate in their metabolism and biological functions, therefore fishes act as bio-indicators of pollution (Farkas et al., 2000; Swaibuh Lwanga et al., 2003; Vives et al., 2006; Nawaz et al., 2010; Ambreen et al., 2015). It has been reported by several research groups that heavy metals can alter the behavior, physiology, metabolism, growth and/or reproduction of different fish species (Ali et al., 2003; James et al., 2003; Jezierska et al., 2009). Also, it has been demonstrated that Cu and Zn and their mixtures caused genetic alteration and teratogenesis in rainbow trout (Bagdonas and Vosyliene, 2006). Moreover, fish contaminated with heavy metals could be a potential risk to human health because they are one of the component of human diet (Papagiannis et al., 2004; Agah et al., 2009).

For pejerrey, it was previously reported the LC50 (96 h) for Cd (0.013 mg/L), Cu (0.218 mg/L) and Cr (8.2 mg/L) in early life stage (Carriquiriborde and Ronco, 2002) and it was also demonstrated that these heavy metals generated genotoxic effects in the liver and gill (Gasulla et al., 2016). Also, it was demonstrated that heavy metals can bioaccumulate in pejerrey tissues after exposures under laboratory conditions (Carriquiriborde and Ronco, 2008) and in the wild (Avigliano et al., 2015). It was observed that heavy metals displayed different accumulation patterns in pejerrey tissues. For example, Cd is more likely accumulated in the gill, Cu in the liver and Cr among all tissues. In addition, the bioaccumulation factor was different for each metal, showing maximum accumulation levels for Cd (≈2000), followed by Cu (≈1000), and finally for Cr (≈500).

Nevertheless, there are few studies about the adverse effects of heavy metals as endocrine disruptors on fish reproductive axis (Simmons et al., 2014; Olivares-Rubio et al., 2015) and some of them focus on the early stages of development. For example, rainbow trout *(Oncorhynchus mykiss)* exposed to low concentrations of Cd showed a disruption of steroidogenesis at pituitary and gonadal levels (Tilton et al., 2003). Also, Cd could increase the levels of estradiol (E_2) in juveniles of rainbow trout exposed to concentrations above 0.05 µg/L (Kime, 1984; Lizardo-Daudt and Kennedy, 2008) or decrease both E_2 and testosterone (T) levels in medaka (*Orizias latipes*) exposed to 10 µg/L of Cd (Tilton et al., 2003). Besides, in a recent study, it was demonstrated an increase of FSH, a decrease of LH and alterations of E_2 levels in the brain and gonads of *Girardinichthys viviparus* exposed to relevant environmental concentrations of a mixture of metals (Olivares-Rubio et al., 2017).

In this context, the aims of the current study were: i) to determinate seasonal concentrations of heavy metals in Chascomús lake, ii) to analyze the reproductive health of pejerrey population of this lake and iii) to study the effects of the exposure of environmental concentrations of Cd, Cr, Cu and Zn on key components of the endocrine-reproductive axis in pejerrey males, under laboratory conditions.

2. Materials and methods

2.1. Determination of heavy metals concentrations in Chascomús shallow lake

Water sampling was done during spring (11/9/2011), summer (2/13/2012), autumn (04/24/2012) and winter (7/18/2012) from Chascomús lake in the area where Los Toldos stream discharge its effluents (35°38′ S 58°0′ W; Fig. 1). Analytical method for dissolved Cd, Cr, Cu, Ni, Pb and Zn analysis was conducted according with the standardized protocol described in the Method 200.9 "Determination of Trace Elements by Stabilized Temperature Graphite Furnace Atomic Absorption" (USEPA, 1994). Briefly, water samples were collected in plastic bottles and filtered through a 0.45 µm pore diameter Whatman GF/C filter. Then, they were acidified to pH < 2 adding (1 + 1) nitric acid and refrigerated (4 °C) until processed. One liter of each acid preserved sample was transferred to a 1 L Griffin beaker and 2 mL (1 + 1) nitric acid and 1.0 mL of (1 + 1) hydrochloric acid were added for further digestion and concentration in a sand bath (85 °C, no boil) under a fume hood. Finally, volume was taken to 100 mL with ultrapure water and metals concentrations were measured using an AAnalyst 700 Atomic Absorption Spectrometer with a HGA graphite furnace and an AS-800 auto-sampler (PerkinElmer, MA, USA). All reagents used were ultrapure grade (Suprapur® nitric acid and hydrochloric acid, Merk) and whole process reagent blanks ultrapure water (glass distilled water filtered through aqurium® pro system, Sartorius) were included for quality control. Quantification of each metal was done against a five-point external calibration curve built using certified standards (AccuStandard®) and r values were always above 0.9. The whole method recovery was between 80 and 120% for all assessed metals. Calibration curve regression coefficient, sample list run and metal concentrations were performed using the WinLab32 software (PerkinElmer, MA, USA).

2.2. Pejerrey gonad condition from Chascomús lake

On the same dates of water sampling, pejerrey of both sexes were caught with a trawl net using a rowboat in the same lake area (100 m far from the lagoon coast and approximately 1.2 m in depth). The measure of the net was 50 m and the surface of the lake covered was around 2500 m^2. All fish were immediately taken to the *Instituto de Investigaciones Biotecnológicas/Instituto Tecnológico de Chascomús* (IIB-INTECH) laboratory, euthanized with benzocaine 100 ppm and dissected. Animals were handled and sacrificed in accordance with the UFAW Use and Care Committee Handbook on the Care and Management of Laboratory Animals (http://www.ufaw.org.uk/pubs.htm#Lab) and local regulations.

A portion of the testis and ovaries of these pejerrey adult fish (10 males and 10 females; Weight: 41.17 ± 6.51 g and Standard Length: 14.82 ± 0.56 cm) was fixed in 10% neutral-buffered formalin during 24 h and then preserved in 70% ethanol. These gonads samples were dehydrated and embedded in Paraplast (Leica Biosystems Richmond Inc. USA). Each piece of gonad was cut into 6 µm thick sections. To identify apoptotic cells a TUNEL assay was performed, following the protocol describe by the manufacturer (DeadEndTM Colorimetric TUNEL System, Promega, Madison, USA). In addition, to identify any type of anomalies related with germinal cell proliferation an immunohistochemistry technique was performed using anti-PCNA (Proliferating Cell Nuclear Antigen 1:1000, mouse IgG2a isotype; DAKO, Glostrup, Denmark). In the case of females, the total of apoptotic and PCNA cells were counted in one section per individual. Meanwhile, for the testis, PCNA positive cells were counted in 5 spermatogenic lobules in one section per each fish. Each section was taken from the middle area of each right gonad. In both cases micrographs were taken

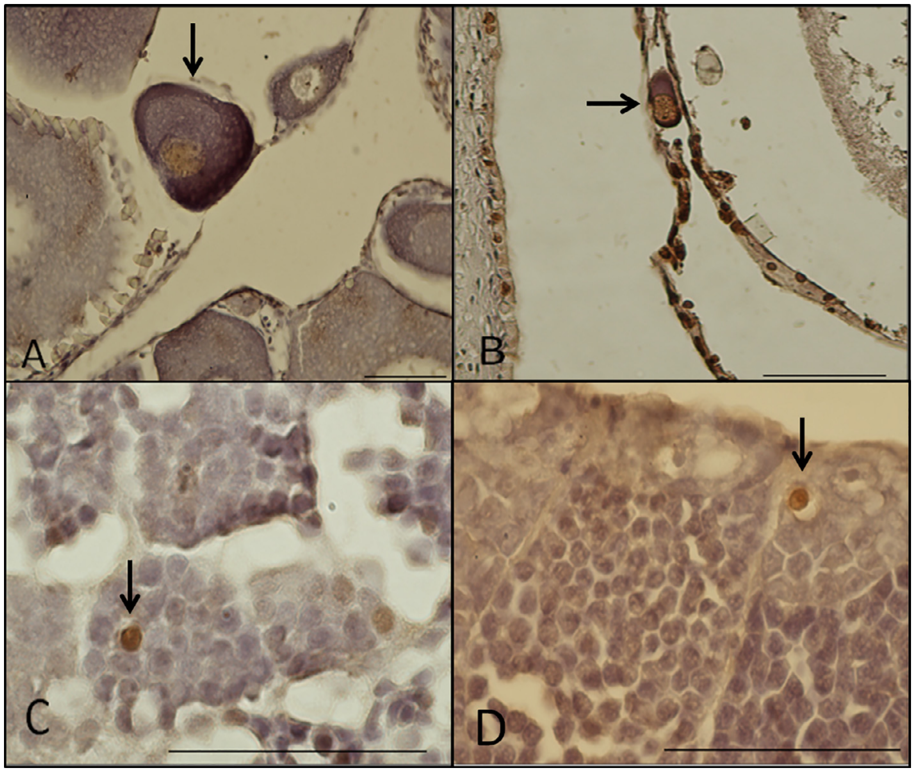

Fig. 1. Apoptotic cells in the gonads of pejerrey from Chascomús lake (pointed with black arrows). A: Apoptotic primary growth oocyte; B: Oogonias; C: Spermatocytes; D: Spermatogonias. A, B, C, D: Bar 5 µm.

with a light microscope Nikon Eclipse E600, attached to a digital photomicrographic system (Nikon Digital Sight DS-U2) and using an Image-Pro Plus software (Rockville, USA). These observations were compared with a control group that consisted of sexual mature pejerrey (10 females and 10 males with similar standard length and weight that wild fish assuming similar age) from the stock of the IIB-INTECH aquaculture facilities that were reared in ground water without any chemicals or pollutants added.

2.3. Heavy metals exposure of pejerrey males

From all the heavy metals measured in Chascomús shallow lake, only Cd, Cu, Cr and Zn were selected for the experimental exposure because their concentrations were above the Argentine guidelines levels. Stock solutions (100 mg/L) for each heavy metal were prepared with distilled water and preserved in the refrigerator (4 °C) for no more than 15 days. The salts used for each metal were: Cadmium chloride ($CdCl_2$; ACS Reagent Sigma-Aldrich, USA), copper sulfate ($CuSO_4$; Mallinckrodt Chemicals, USA), potassium dichromate ($K_2Cr_2O_7$; ACS Reagent Anedra, Argentina), and zinc sulfate 7-hydrated ($ZnSO_4$ 7H2O; Sigma-Aldrich, USA). To reach the environmental concentration for each metal, a dilution of each stock in Chascomús ground water (pH 7.97; Osmolarity 141 mOsm/L; Alcalinity 564 mg CaCO3/L; Hardness 198 mg CaCO3/L; Nitrites 100 µg/L; Nitrates 19 mg/L) was perform. The metal concentrations tested in each treatment were the maximum environmental concentrations of Cd, Cr, Cu and Zn reported in Chascomús shallow lake: 0.25 µg/L Cd, 4 µg/L Cr, 22 µg/L Cu and 211 µg/L Zn.

A total of 60 mature pejerrey males were selected from the stock of the IIB-INTECH aquaculture facilities during spring season (spawning season) and they were kept in a 50 L volume solution in glass aquaria of 60 L total volume. There were maintained in a closed room with controlled air temperature (20 °C) and photoperiod (12 h light: 12 h dark). The aquaria were protected with a polyethylene cover to avoid the adsorption of metals to the glass of the aquariums (Vetillard and Bailhache, 2005). The experiment consisted in 4 heavy metals treatments and a control group with ground water (pH 7,85 ± 0,04; 2740 mg $CaCO_3$/L hardness; Osmolarity 222.7 mOsm/L; Alkalinity 611.3 mg $CaCO_3$/L; Nitrites 100 µg/L; Nitrates 19 mg/L). Each treatment including the control was done by duplicate. A semi-static system was implement for 14 days, with 6 fish per aquarium (5 males and 1 female to keep reproductive activity) provided of gentle aeration. One-half of the volume of each recipient (25 L) was renovated every 48 h and previous this procedure the bottoms of the aquariums were cleaned up to remove feces and remained food. 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). After 14 days, all fish were anesthetized by immersion in benzocaine (100 ppm) and, blood and tissue samples were collected. Brain, pituitary gland and a portion of the testis from each pejerrey were dissected and flash-frozen in liquid nitrogen. All these samples were stored at −80 °C until the moment of analysis. The Gonadosomatic (GSI = gonad weight/body weight × 100) and Hepatosomatic indexes (HSI = liver weight/body weight × 100) were evaluated.

Table 1
Heavy metals concentration in the water of Chascomús lake in different seasons (Spring, Summer, Autumn and Winter) compared with the Argentinian National Guidelines for the aquatic biota protection (*, National Law N° 24051; Decree 831/93; Annex II; ND: Not Detected.

Heavy Metals	Spring (11/9/2011) g/l	Summer (02/13/2012) g/l	Autumn (04/24/2012) g/l	Winter (7/18/2012) g/l	Guidelines Levels* g/l
Cd	0.23	ND	0.11	0.07	0.20
Cr	4.28	3.59	2.64	1.29	2
Cu	22.09	8.04	4.85	5.31	2
Ni	2.49	1.42	0.85	ND	25
Pb	1.64	3.24	ND	0.60	1
Zn	210.76	13.68	38.80	1.36	30
Water Temperature (°C)	18	24.5	13	5.5	
Water Depth (m)	1.88	1.47	1.56	1.64	

2.4. Determination of testosterone (T) plasma levels

Blood samples (300–500 µL) were taken from the caudal vessels using heparinized syringes and collected in 1.5 mL tubes. They were then centrifuged (3000*g* for 15 min at 4 °C) and the plasma was separated and stored at −80° C. Steroids were extracted from plasma with diethyl ether, resuspended in their initial volume with PBS buffer, diluted ½ and the levels of T were measured by an enzyme-linked immunosorbent assay (ELISA) by duplicate using commercial kits and following the manufacturer's protocols (DRG International Inc., USA; EIA-1559 and EIA-2693) previously validated by Chalde et al. (2016). An 8 points standard curve calibration (duplicated) was run for the ELISA plate. The lower limit of detection was 288.99 pg/mL and the optical density was read at 450 nm. The intra-assay coefficients of variance were < 10%.

2.5. Extraction of RNA, cDNA synthesis and mRNA transcript levels 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 and as it was described in Gárriz et al., 2017. The RNA samples were treated with DNase I (Invitrogen, CA, USA), reverse transcribed using SuperScript II RNase H (Invitrogen, CA, USA; except for pituitary gland samples where SuperScript III was used because of 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. The reactions were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) using gene-specific primers for RT-qPCR (Gárriz et al., 2017) with efficiencies 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 Gonadotroping Releasing Hormone (GnRH) variants (*gnrh1, gnrh2, gnrh3*), brain aromatase (*cyp19a1b*), pituitary Gth-β subunits (*fshb, lhb*) and gonadal gonadotropin receptors (*fshr, lhcgr*) genes were determined using real-time RT-PCR with the standard curve method following the procedure published by Applied Biosystems (1997). The mRNA transcript levels data were normalized using two housekeeping genes as reference, *b-actin* and elongation factor 1 alpha (*ef1a*). Because there were not significant differences between both, the graphics using *ef1a* were added as Supplementary Figs. 5 and 6.

2.6. Gonadal histological analysis

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 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 of the 7 columns the total number of abnormal pyknotic nuclei was counted following the criteria of Gárriz et al. (2017). 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 using the Image-Pro Plus software (Rockville, USA).

2.7. Statistical analysis

Data is presented as the mean ± standard error of the mean (SEM). Normal distribution for data was analyzed by the Shapiro–Wilk test, and the Levene test was used to check the homogeneity of variance. As in the experiment with heavy metals no significant differences were observed between replicates, each fish was considered an independent experimental unit. Each treatment (N = 10) for all the assessed parameters. The results were analyzed using one-way analysis of variance (ANOVA, $p < 0.05$) followed by Dunnet's post-hoc test. Logarithmic transformations were used for HSI, GSI, and *gnrh2*, *fshb*, *fshr* because the data lacked the assumptions of the statistical test. Statistical analyses were performed using SPSS Statistics 20.0 (IBM) and GraphPad Prism 5.0 Software.

3. Results

3.1. Heavy metals concentrations in Chascomús shallow lake

The concentrations of Cd, Cr, Cu, Ni, Pb and Zn measured in the water of Chascomús lake were compared with the Argentine Law. Cd, Cr, Cr, Pb and Zn were above of the guidelines levels (Table 1).

3.2. Pejerrey gonad condition from Chascomús lake

Apoptotic oocytes, oogonias (Fig. 1A and B), spermatocytes and spermatogonias (Fig. 1C and D) were identified in pejerrey gonads from Chascomús lake. There were not significant differences in the number of apoptotic cells between wild pejerrey ovaries or testis between

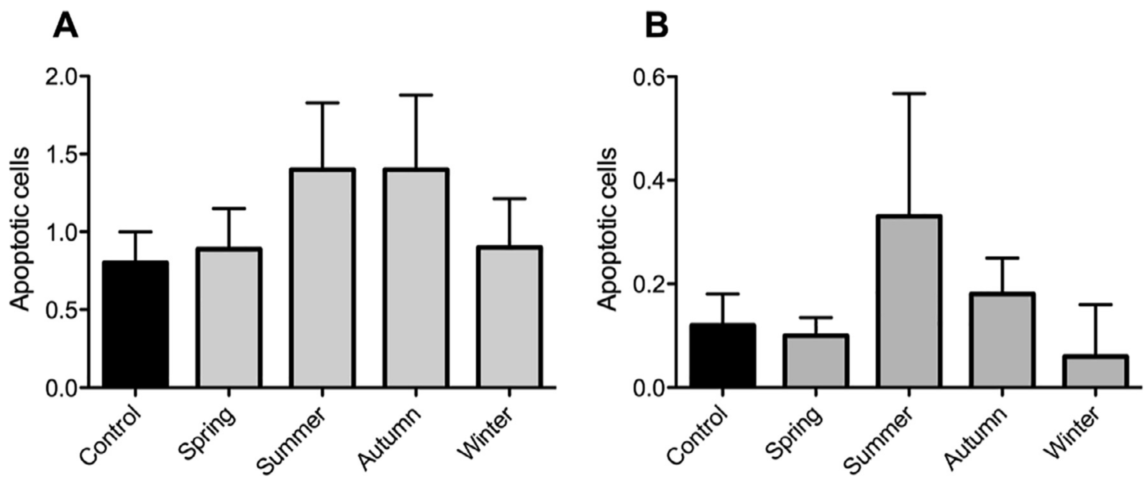

Fig. 2. Number of apoptotic cells in the gonads of pejerrey from Chascomús lake during different seasons. A: Oogonias and B: Spermatogonias (n = 10; $p < 0.05$).

different seasons and compared with the control fish (Fig. 2A and B). PCNA (+) oogonias (Fig. 3A), spermatocytes and spermatogonias (Fig. 3B) were observed in wild pejerrey. There were not statistical differences in the number of PCNA (+) cells in ovaries between wild and control pejerrey fish (Fig. 3C). In the case of testis, there was only a statistically significant increase in the number of PCNA (+) oogonias in spring season compared with other seasons and the control group (Fig. 3D).

3.3. Heavy metal exposure of pejerrey males

There were not dead fish during the heavy metals exposure. Water temperature ranged between 18 and 21 °C, the average for DO was 7,06 ± 0.12 ppm and the pH varied between 7.65 and 8.03 during the experiment. The Hepatosomatic (HSI) and Gonadosomatic (GSI) indexes showed no variation between treated and control fish groups (Fig. 4A and B).

3.4. Testosterone plasma levels

Testosterone plasma levels were not statistically different between exposed and control fish (Fig. 4C).

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

In the brain, a significant increase in the mRNA transcript levels of *gnrh1, 2 and 3* was observed only in fish exposed to 0.25 µg/L of Cd compared with the control group (Fig. 5A–C), meanwhile, *cyp19a1b*

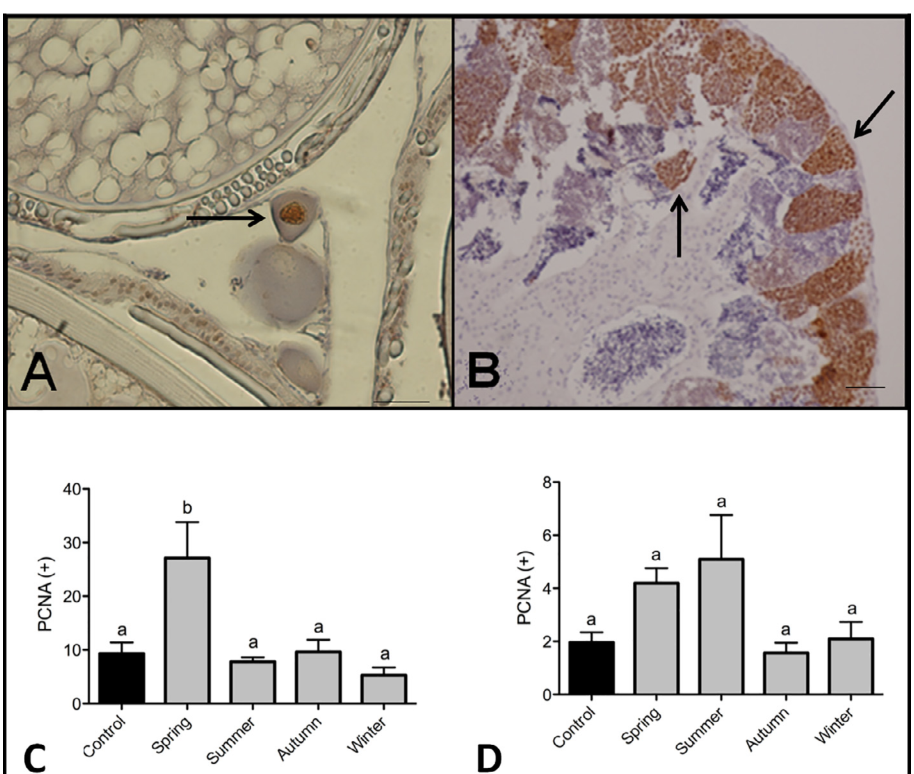

Fig. 3. Proliferating Cell Nuclear Antigen (+) cells in pejerrey gonads from Chascomús. A: Oogonias (Pointed with black arrows; Bar: 5 µm). B: Spermatogonias and spermatocytes (Pointed with black arrows; Bar: 100 µm). C: Number of Proliferating Cell Nuclear Antigen (+) oogonias and D: Number of spermatogonias. Letters above each column indicate significant differences (n = 10; $p < 0.05$).

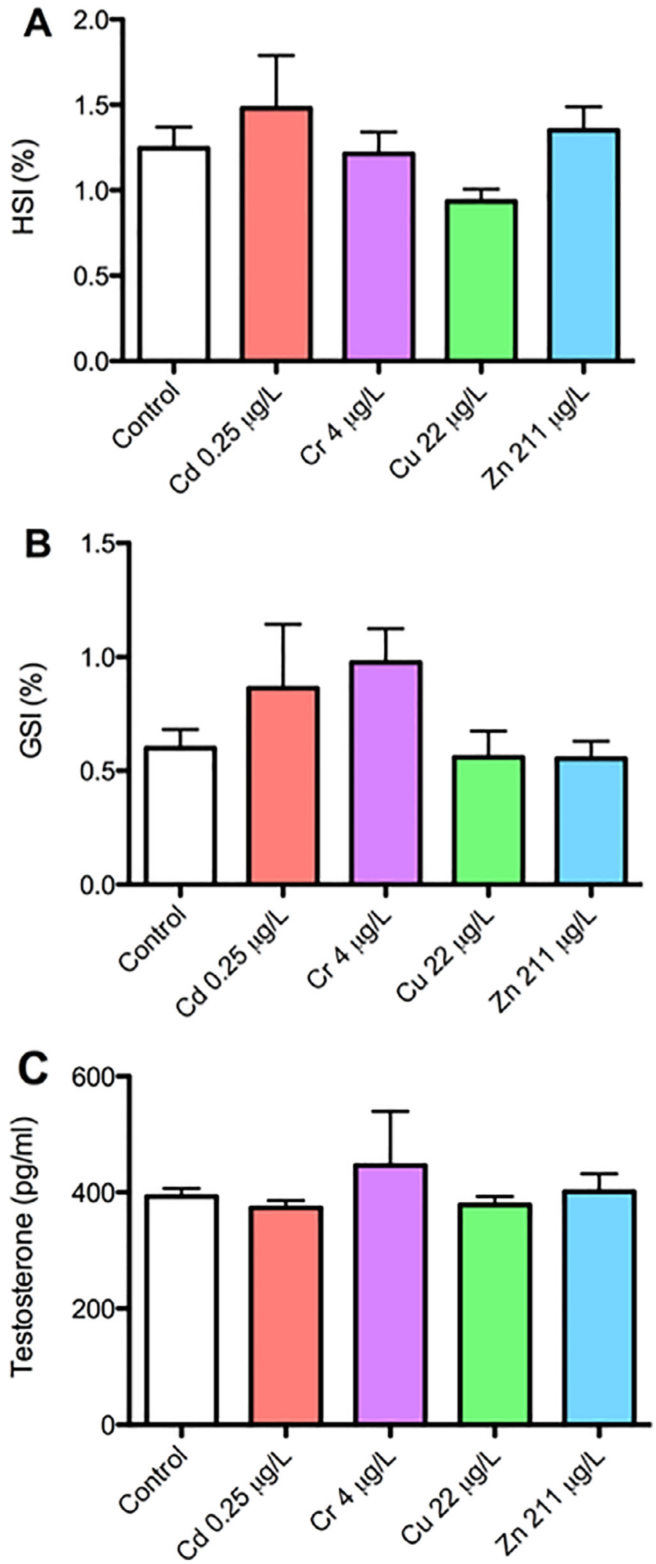

Fig. 4. A: Hepatosomatic Index (HSI); B: Gonadosomatic Index (GSI) and C: Testosterone plasma levels of pejerrey males exposed to heavy metals (Cd, Cr, Cu and Zn; n = 10, $p < 0.05$).

mRNA levels were significantly decreased in fish exposed to Cu (22 µg/L) compared with the control group (Fig. 5D).

At pituitary level, it was observed a statistically significant decrease of *fshb* mRNA levels of pejerrey exposed to Cd (0.25 µg/L) and Cr (4 µg/L), compared with the control fish (Fig. 6A). Otherwise, for *lhb* mRNA levels it was observed a significantly increase in pejerrey exposed to Cu (22 µg/L; Fig. 6B).

In the testis, it was shown a significant decrease of *fshr* mRNA levels in pejerrey exposed to 4 µg/L of Cr, compared with the control (Fig. 6C). Also, a decrease for the same gonadotropin receptor in the Zn treatment was observed but it was not statistically significant (Fig. 6C). In the case of *lhcgr,* mRNA levels were significantly reduced in pejerrey treated with Zn (211 µg/L), compared with the control (Fig. 6D). A similar decrease was observed in the fish exposed to Cr but it was not statistically significant (Fig. 6D).

3.6. Gonadal histology

Different types of gonadal alterations were observed in the testis of pejerrey exposed to heavy metals compared with the control group (Fig. 7A–D). For all treatments, the testis showed fibrosis and shrinkage of the spermatic lobules (Fig. 7B). Moreover, in 2 testes of fish exposed to Cd some oocytes were found (Fig. 7C). Furthermore, it was possible to identify pyknotic cells in the testis of all treated fish (Fig. 7D), where the number of pyknotic cells was statistically higher compared with the control group (Fig. 8A). Besides, the testis of treated fish showed a statistically reduce of the length of the spermatic lobules compared with the control group (Fig. 8B).

4. Discussion

Heavy metals concentrations (Cd, Cr, Cu and Zn) measured in Chascomús lake were in the same range of the ones previously reported by Schenone et al. (2014) after a single sampling in summer, and it is remarkable that in both studies the concentrations were above the Argentine guidelines levels. Such scenario is comparable with that commonly described for waterbodies located nearby industries or urban areas of China (Cheung et al., 2003), India (Malik et al., 2010), Pakistán (Nawaz et al., 2010) and Turkey (Tekin-Özan, 2008) also exceeding the local or the international guidelines (IGWSP-UNU-UNEP, 2016). The source of the measured metals in Chascomús lake need to be determined, but it could be related to the presence of different factories (metallurgy, textile, rail transport and car industries) placed nearby this waterbody.

Despite of the relatively high levels of metals measured in Chascomús lake, the gonadal histological analysis of wild pejerrey did not show damages like to those observed in the exposed fish. Nevertheless, exposures under laboratory conditions to maximum measured metal concentrations of Chascomús lake were able to alter the mRNA transcript levels of key reproductive genes causing different types of damages in the gonad of mature pejerrey males, including the presence of testis ova. Such observed differences between the field and laboratory assessment could be attributed to factors reducing the bioavailability of the metals in surface waters (Luoma, 1983; Väänänen et al., 2018). In addition, high pH, hardness, dissolved organic matter and total suspended matter are characteristic features of Chascomús lake water that could also be involved in this issue (Gómez et al., 2007; Pérez et al., 2010). However, the results obtained in this study are indicating that the metal concentrations measured in this lake represent certain degree of risk for the reproductive success of pejerrey under particular environmental conditions. For all metals, high concentrations were found in the warmer seasons (spring and summer). It is known, that temperature is an important factor increasing water evaporation and concentration of solutes or pollutants (Tekin-Özan, 2008; Malik et al., 2010). In consequence, higher risk would be expected during extreme warm and dry scenarios (Carr and Patiño, 2011).

Within vertebrates, fish are considered excellent bioindicators for

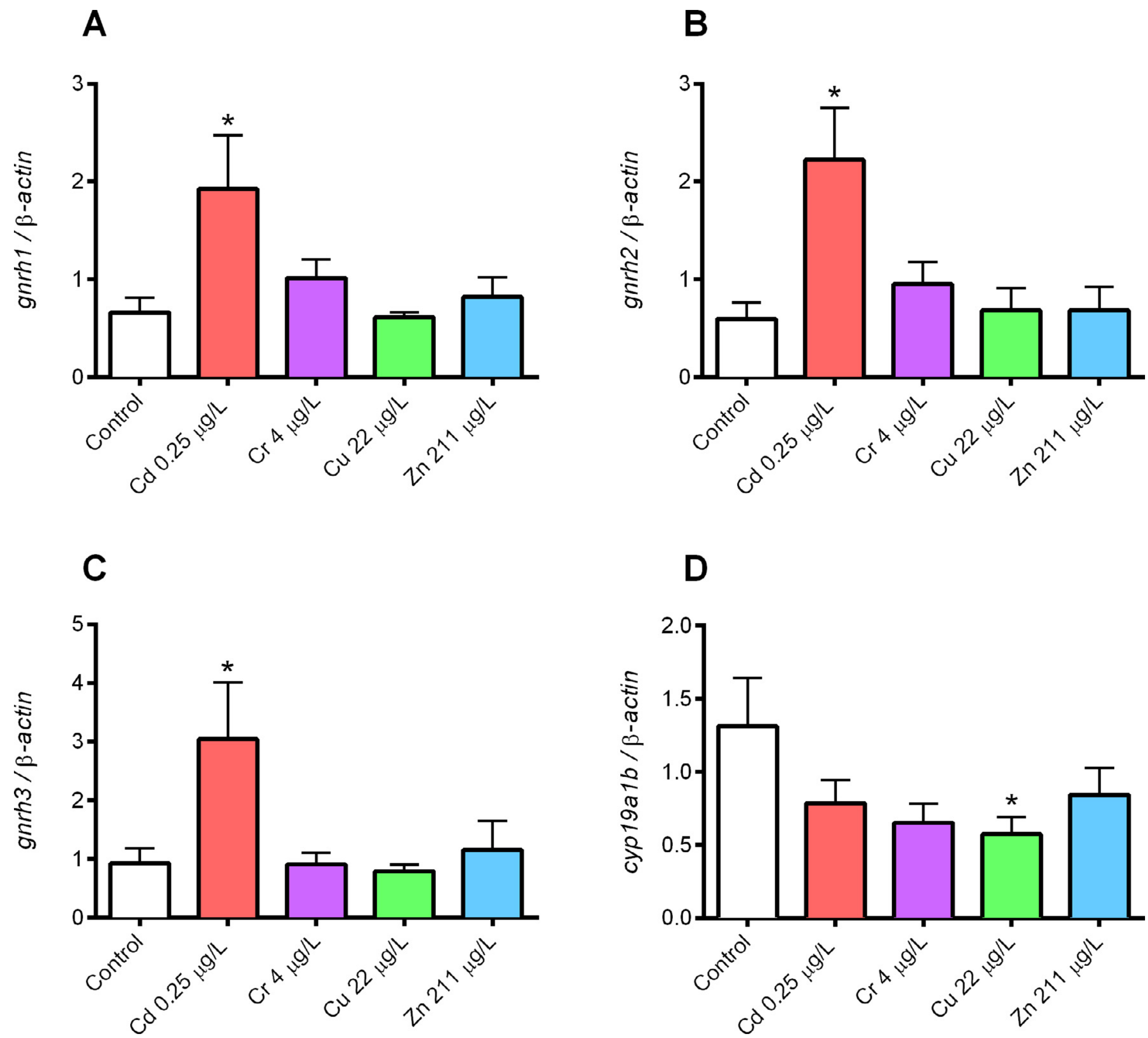

Fig. 5. Transcript levels of A: *gnrh1,* B: *gnrh2,* C: *gnrh3* and D: *cyp19a1b* in the brain of pejerrey males exposed to Cd, Cr, Cu and Zn. ($n = 10$, $p < 0.05$). Black asterisks (*) indicate the statistical differences compared with the control group.

aquatic ecosystem pollution worldwide (Boncompagni et al., 2003; Gale et al., 2004; Vives et al., 2006; Fidan et al., 2008).

Particularly the toxicity of heavy metals at cellular level has been broadly studied in this group of animals and it is known that metals are able to interact mostly with proteins and nucleic acids depending on their bonding tendencies (i.e. HSAB theory). In addition, oxidative stress can be induced by the change in the metals oxidation state (Lemire et al., 2013). However, some adverse effects are not yet fully understood (Almeida et al., 2001). It was reported that some metals can act as endocrine disruptors in early stages of fish development (Hontela, 1998; Lizardo-Daudt and Kennedy, 2008; Luo et al., 2015). For example, it was observed that Cd decreased thyroid hormones levels in rainbow trout disrupting the iodine metabolism (Hontela et al., 1996) and bounds to DNA sequence of estrogen receptor, affecting its transcription levels (Le Guevel et al., 2000). Moreover, Cd was also found to alter calcium homeostasis disrupting hormonal secretion in the pituitary gland and in the gonads (Singhal et al., 1985; Pundir and Saxena, 1992; Piasek and Laskey, 1994; Thomas, 1999). In this sense a recent study with *Girardinichthys viviparus* demonstrated alterations in gonadotropins and E_2 profiles after exposition to environmental concentrations of mixture of metals (Cu, Fe, Mn, Pb, Zn; Olivares-Rubio et al., 2017). Also, Cd could directly inhibit enzymes involved in gonadal steroidogenesis and hepatic steroid metabolism (Singhal et al., 1985). Furthermore, sex mature medaka exposed to 10 µg/L of Cd for 7 weeks showed a drastic decrease of E_2 and T levels (Tilton et al., 2003). On the other hand, there is other study that showed no variation in the plasmatic levels of T for fish exposed to 50 and 100 µg Cd/L (Luo et al., 2015), and agrees with the current study.

Taking in account all the obtained results, this study demonstrated that pejerrey males exposed to Cd exhibited an increase in the expression of the three variants of *gnrh.* Also, in the fishes exposed to Cu a decrease of *cyp19a1b* and a decrease in *fshb* transcript levels was observed. For Cr treatment, a decrease in the expression of *fshb* and *fshr* was found. In the case of the pejerrey exposed to Zn a decrease on *lhcgr* mRNA levels was detected. For our knowledge there are not publication until this one that report effects of heavy metals on the transcript levels of key genes of reproductive axis. Nevertheless, more research must be permorf to establish the mechanism of actions of heavy metals as reproductive endocrine disruptors.

In addition, under histological analyzes in pejerrey testis, it was

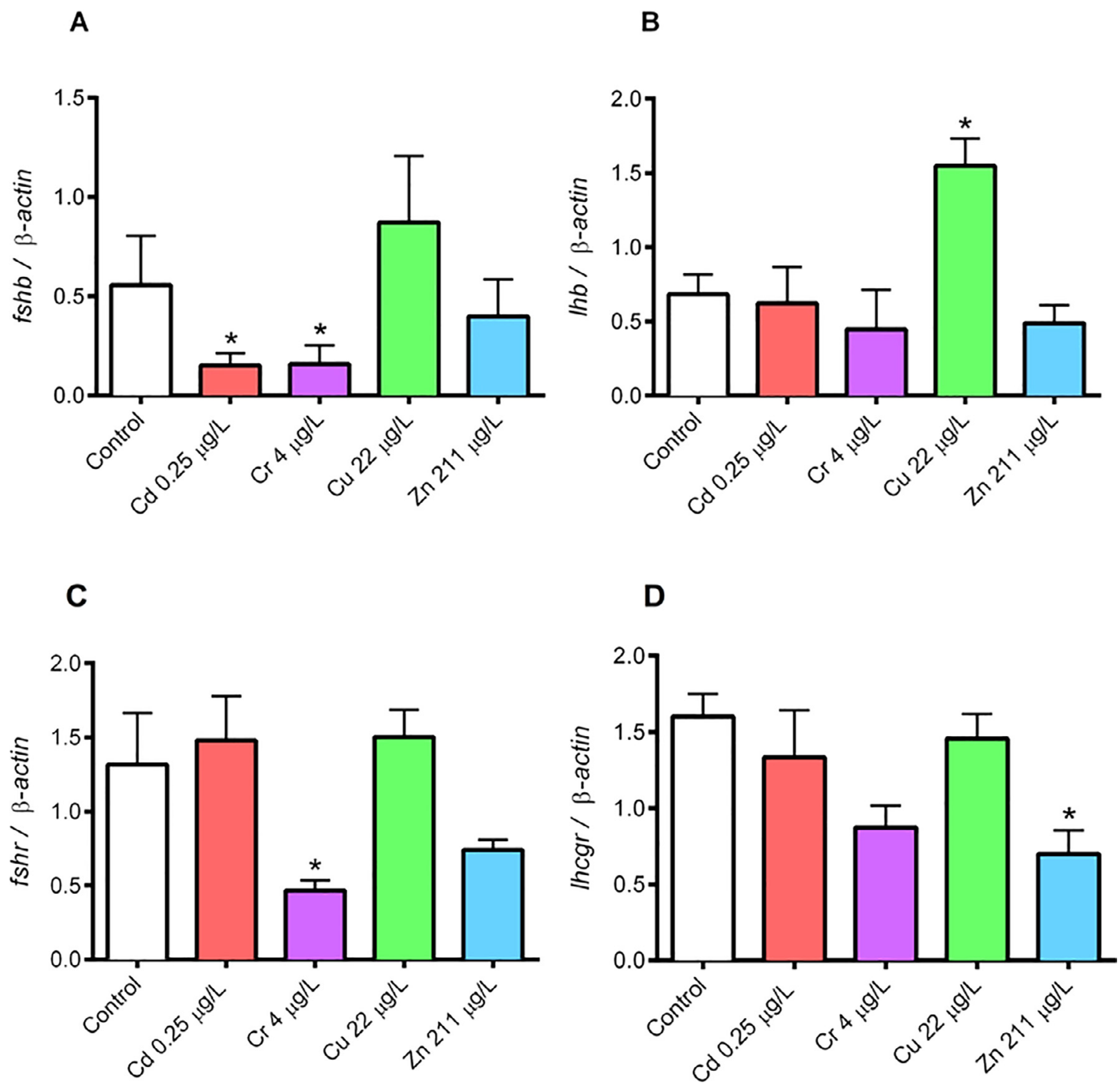

Fig. 6. Transcript levels of A: *fshb, B*: *lhb*, C: *fshr* and D: *lhcgr* pejerrey males exposed to environmental concentrations of Cd, Cr, Cu and Zn (n = 10, $p < 0.05$). Black asterisks (*) indicate the statistical differences compared with the control group.

observed the presence of pyknotic cells in all the heavy metal treatments but not in the control group. Being the highest number of these cells in the pejerrey individuals exposed to Cu. The existence of pyknotic cells among the spermatogonias is a proved sign of a degeneration of germinal cells that can lead to sterility (Ito et al., 2003, 2008; Gárriz et al., 2017). This type of cells were previously observed in pejerrey fish exposed to environmental concentration of estrogens (Gárriz et al., 2017). Several works show evidences that heavy metals generate histological damages in the gonads of different fish species. For example, a reduction of the total amount of spermatocytes and spermatids was reported in fish exposed to Cd (Kumari and Dutt, 1991; Luo et al., 2015). Furthermore, adult males of *Astyanax aff. bimaculatus* exposed to Zn (3 and 20 mg/L) exhibited swollen and broken walls of their spermatic cysts and the presence of pyknotic cells inside them (Marques dos Santos et al., 2015). All these observations support the histological data reported in the current study for *O. bonariensis*. However, this is the first report that show the presence of testis-ova in a fish exposed to Cd. This adverse effect was not previously associated to heavy metals exposure in fish and could be related to the direct action of Cd on steroidogenic tissues such as the gonads (Lizardo-Daudt and Kennedy, 2008).

In conclusion, this study described new seasonal concentrations of Cd, Cu, Cr and Zn in the water of Chascomús lake above the limits allowed by the Argentinean guidelines, and it demonstrated that pejerrey males exposed to environmental concentrations of some of these heavy metals show alterations of the transcript levels of key genes implicated in the endocrine-reproductive axis. Furthermore, the exposed fish showed structural damages of the gonads such as the presence of pyknotic cells, fibrosis and shortness of spermatic lobules and testis ova.

According to the adverse reproductive responses on pejerrey fish to heavy metals pollution, it is plausible that changes or even total loss of the spawning season may occur affecting the future pejerrey populations in the possible worst scenario.

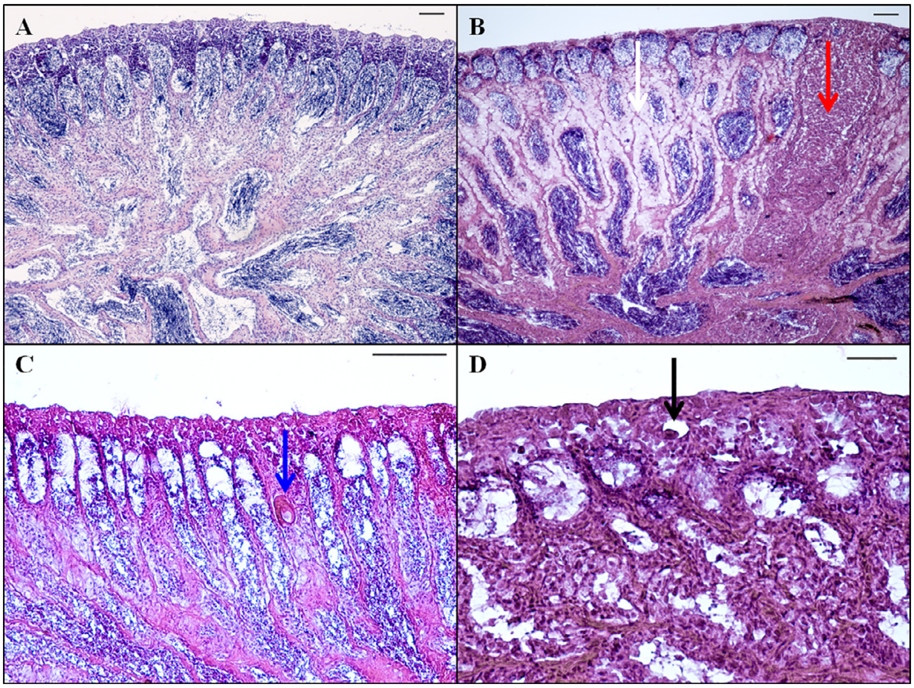

Fig. 7. Testis micrographs of A) Control and pejerrey exposed to heavy metals. Different types of damages were observed in the testis: B) Interstitial fibrosis (red arrow) and shrinkage of spermatic lobules (white arrow) in Cr exposition (4 µg/L). C) Presence of an oocyte (blue arrow) exposed to Cd (0.25 µg/L). D) Pyknotic nuclei in Cu exposition (black arrow). A, C: Bar 100 µm; B, D: Bar 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

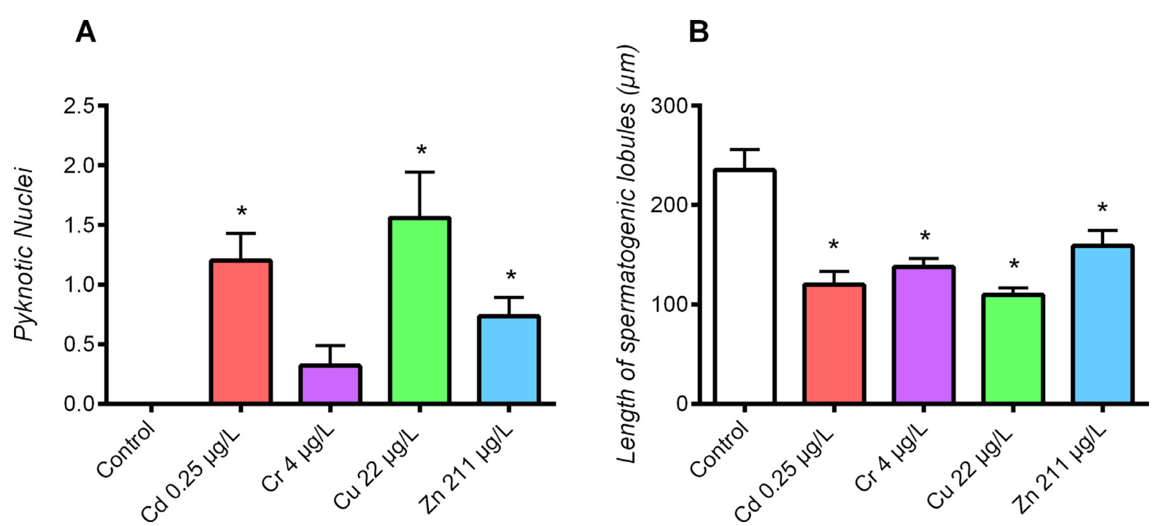

Fig. 8. A) Pyknotic nuclei and B) Spermatic lobules length averages counted in 7 sections of pejerrey testis in the control group and exposed to Cd, Cr, Cu and Zn ($n = 10$, $p < 0.05$). Black asterisks (*) indicate the statistical differences compared with the control group.

Acknowledgements

The authors thank Javier Herdman and Rocio Paladea, for their technical assistance during the experiment and, also acknowledge Emiliano Balsamo for his help in the experimental design. Access to GF-AAS for heavy metals determinations was kindly provided by Dra. Diana Crespo (IMyZA, Inta Castelar, Argentina). This study was supported by grants to L.A.M. (ANPCyT: PICT 2012-1537).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.06.013.

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