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The SSRI fluoxetine exhibits mild effects on the reproductive axis in the cichlid fish *Cichlasoma dimerus* (Teleostei, Cichliformes)

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HIGHLIGHTS

- The purpose of this study was to evaluate the endocrine disrupting potential of fluoxetine (FLX) over the reproductive axis in a cichlid fish.
- Fluoxetine injection caused an increase on LH levels in females of *C. dimerus.*
- Testis abnormalities were observed in FLX-exposed fish males.
- FLX is acting as a mild endocrine disrupting compound in adults of *C. dimerus.*

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ABSTRACT

Among the wide variety of pharmaceuticals released into the environment, Fluoxetine (FLX), a selective serotonin reuptake inhibitor, is one of the most prescribed for the treatment of major depression. It inhibits serotonin (5-HT) reuptake at the presinaptic membrane, increasing serotonergic activity. In vertebrates, including fish, the serotonergic system is closely related to the Hypothalamic Pituitary Gonadal (HPG) axis which regulates reproduction. As FLX can act as an endocrine disrupting compound (EDC) by affecting several reproductive parameters in fish, the aim of this study was to provide an integral assessment of the potential effect of FLX on the reproductive axis of the Neotropical freshwater fish *Cichlasoma dimerus*. Adult fish were intraperitoneally injected with 2 μg g^{-1} FLX or saline every third day for 15 days. No significant differences were found on serotonergic turnover (5-HIAA/5-HT ratio). Pituitary βLH content in FLX injected females was significantly higher than control females; no significant differences were seen for βFSH content. Sex steroids remained unaltered, both in males and females fish, after FLX treatment. No plasma vitellogenin was induced in treated males. Some alterations were seen in testes of FLX injected males, such as the presence of foam cells and an acidophilic PAS positive, Alcian-Blue negative secretion in the lobular lumen. Although there is no clear consensus about the effect of this drug on reproductive physiology, these results indicate that FLX is acting as a mild EDC in adults of *C. dimerus*.

1. Introduction

Thousands of pharmaceutically active compounds are commercially produced nowadays, among which a wide variety of substances designed to improve animal and human health can be found (Rehman et al., 2015; Wen et al., 2014). In addition, widespread usage of these substances (both prescribed and over the counter) has generated a significant discharge of pharmaceuticals and their metabolites into sewerages systems, leading to their detection in water -even in drinking water- in the last few years due to the improved sensitivity of analytical methods (World Health Organization, 2011). In this context, many of these substances are today considered to be contaminants of emerging concern (CECs), according to Sauvé and Desrosiers (2014), since these chemicals or

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materials -whether naturally occurring, manufactured or man-made- have now been discovered in the environment, and their toxicity and/or persistence can potentially alter the metabolism of organisms. Once a pharmaceutical is consumed and excreted, it remains in sewage as it cannot be completely removed from effluents at wastewater treatment plants (WWTPs) (Blair et al., 2013). In consequence, these pollutants can reach aquatic environments, where they can affect non-target organisms, such as fish, amphibians and invertebrates (Segura et al., 2009).

One of these pharmaceuticals, Fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI), is the active ingredient of commercial antidepressants such as Prozac®, which are commonly prescribed not only for treatment of major depression but also for other psychological disorders such as obsessive compulsive disorder, bulimia nervosa and panic disorder (Burt et al., 2007). The mode of action of SSRIs in the human central nervous system (CNS) was characterized based on their effects on several biological mammalian models (Bymaster et al., 2002). In brain serotoninergic synapses, the neurotransmitter serotonin (5-hydroxytriptamine, 5-HT) is released into the synaptic cleft, where it interacts with a variety of membrane receptors on the postsynaptic membrane, provoking the corresponding response. The stimulus is ended by 5-HT re-uptake on the presynaptic membrane by the serotonin transporter (SERT). All SSRIs act by blocking SERT, hence increasing brain serotonergic activity (Koch et al., 2002; Wong et al., 1995). Since SERT is blocked by FLX, less 5-HT is taken up by the presynaptic neuron and metabolized by monoamine oxidase (MAO), resulting in an altered ratio between the levels of 5-hydroxyindoleacetic acid (5-HIAA), the main 5-HT metabolite, and 5HT in the brain (5-HIAA/5-HT ratio), a parameter which is known as brain 5-HT turnover (McDonald et al., 2011).

Serotonin-producing neurons in the CNS have been extensively studied and characterized in every group of vertebrates, including bony fish. In teleosts, three different populations of serotonergic neurons have been identified: pretectal, hypothalamic and raphe nuclei (Lillesaar, 2011). These neurons not only differ on their location in the brain but also on their physiological role (Ekström and Van Veen, 1984; Lillesaar et al., 2009). The pretectal area is known to be involved in the integration of visual inputs and motor behaviour (Wullimann, 1998), whereas hypothalamic serotonergic neurons are suggested to sense cerebrospinal fluid (CSF) and be involved in the release of substances into the circulation due to their proximity to the ventricular system (Lillesaar, 2011), some authors even including this population in the group of CSF-contacting cells, which were described in several species (Vigh and Vigh-Teichmann, 1998). The raphe contains a population of serotonergic neurons whose projections reach all areas in the brain, including the olfactory bulb, telencephalon, hypothalamus and spinal cord (Lillesaar et al., 2009). In humans, 5-HT is related to mood, aggression, sexual drive and appetite (Tops et al., 2009), and there is evidence that it also exerts control over hypothalamic regulation of pituitary secretion of several hormones, such as adrenocorticotrophin, prolactin, growth hormone, and the gonadotrophins (GtHs): Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) (Frazer and Henzler, 1999).

As in other vertebrates, reproduction in fish is highly regulated by the Hypothalamic Pituitary Gonadal (HPG) axis (Hachfi et al., 2012). Regulation of reproductive anatomy, physiology and behaviour in fish are closely related to the serotonergic system (Khan and Thomas, 1993; Winberg et al., 1997), mainly through the releasing of Gonadotrophin Releasing Hormones (GnRH) from the hypothalamus (Senthilkumaran et al., 2001; Yu et al., 1991). In zebrafish, *Danio rerio*, 5-HT receptors are expressed in the hypothalamus (Norton et al., 2008), suggesting a possible co-expression with GnRH, as was described in mammals (Bhattarai et al., 2013). Khan and Thomas (1993) demonstrated in Atlantic croakers, *Micropogonias undulates*, injected with 5-HT an increase in LH levels through potentiation of GnRH effects. Moreover, perfusion of goldfish, *Carassius auratus*, pituitary fragments with 5-HT, resulted in a dose-dependent increase of LH and decrease of GH release (Somoza and Peter, 1991), and this action seemed to be mediated by 5-HT2 subtype receptors, as it was blocked by a specific antagonist (Prasad et al., 2015).

Previous studies indicate that FLX can alter reproductive parameters in fish. Exposure to FLX generated an increase on plasmatic levels of 17β-estradiol (E2) in males of *C. auratus* (Mennigen et al., 2010) and females of Japanese medaka, *Oryzias latipes* (Foran et al., 2004). However, other authors observed a reduction of E2 levels, and a decrease on gene expression of aromatase and gonadotrophin receptors in the ovaries of *D. rerio*, which inhibited egg production (Lister et al., 2009). With regard to the effect of FLX on vitellogenin (VTG) synthesis, while one study reported the abnormal presence of plasmatic VTG in males and alterations in secondary sex characteristics of fathead minnow, *Pimephales promelas* (Schultz et al., 2011), another study reported that plasmatic VTG was not induced by the presence of waterborne FLX in *O. latipes* males (Foran et al., 2004). Additionally, a wide variety of behaviour alterations were observed due to FLX, which include either a decrease (Barry, 2013; Dzieweczynski and Hebert, 2012; Kania et al., 2012) or an increase in territorial aggression (McDonald et al., 2011), an anxiolytic effect (Ansai et al., 2016), a lower predator avoidance (Painter et al., 2009; Weinberger II and Klaper, 2014), and a decrease in swimming patterns, such as swimming speed and distance to the nearest neighbour (Barry, 2013).

In this context, cichlid fish, a diverse family of teleosts are commonly used as an experimental model since they have a distinctive pattern of social hierarchies and territorial aggression, which regulates several physiological processes: fertility, gonadal maturation and even neurogenesis and stress levels (Maruska and Fernald, 2013; Ramallo et al., 2014). Particularly, *Cichlasoma dimerus* (Heckel, 1840) (Cichliformes; Cichlidae) (Nelson et al., 2016), a Neotropical freshwater fish endemic to the Paraná basin, is easy to keep in captivity under controlled environmental conditions. Having *C. dimerus* been successfully used as a model in aquatic ecotoxicology testing (Da Cuña et al., 2013, 2016; Genovese et al., 2012, 2014; Meijide et al., 2016; Piazza et al., 2015; Rey Vázquez et al., 2016), it is included on local environmental regulations for developing acute toxicity tests in freshwater fish (IRAM, 2008).

Based on the aforementioned evidence, we hypothesized that FLX has the ability to alter serotoninergic brain activity, which in turn impacts on the reproductive physiology of the cichlid fish *C. dimerus*.

2. Materials and methods

2.1. Animals

Sexually mature male and female *Cichlasoma dimerus*, were captured at Esteros del Riachuelo, Corrientes, Argentina (27°35′S, 58°45′O). Fish were transferred to the laboratory facilities, where they were held in 200 L aquaria for at least 4 weeks under controlled conditions (aeration, light and temperature). Animals were fed daily with commercial food (Tetra food® sticks).

All experiments were conducted in accordance to international standards on the care and use of fish in research and testing according to the guide for the care and use of laboratory animals (National Research Council, 2011), as well as being in compliance with the local Ethical Committee (CICUAL, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires).

2.2. Fluoxetine solution

Fluoxetine hydrochloride (99.9% of purity; Saporiti, Argentina) was dissolved in physiological saline solution (0.6% NaCl) to achieve a 6 mg mL^{-1} FLX stock solution; the final work solution was obtained by diluting 1:10.

2.3. Experimental design

Male and female *C. dimerus* (total body weight 30 ± 5 g) were placed in individual 10 L tanks for a 5-day period prior to the start of the exposure, without any visual contact in order to eliminate social interactions between animals. Fish were injected intraperitoneally (i.p.; injection volume 100 μL per 30 g body weight) every third day during 15 days, for a total of 5 injections of 2 or 20 μg g^{-1} FLX (N = 8 for each dose). These doses were chosen based on published data on the field (Mennigen et al., 2008). The control group (N = 8) was injected with vehicle (saline solution). No animal from the 20 μg/g survived till the end of the experiment therefore no endpoints were analyzed for this group.

Throughout the experiment, animals were fed with commercial food daily (5 pellets per animal). After 24 h from the last injection, fish were sedated by immersion in commercial sedative solution (Fish Calmer, Jungle Hypno, USA), weighed and then euthanized by decapitation. Sampling was conducted by standardized methods, as follows.

2.4. Blood sampling

Blood was collected at the onset and at the end of the experiment by caudal vein puncture, using a 1 mL syringe and 27G x 0.5″ needle which were previously heparinized.

For quantification of plasma sexual steroids (estradiol and testosterone) and detection of plasma vitellogenin (VTG), samples were centrifuged at 3000 rpm for 15 min at 4 °C and supernatant plasma was stored at −20 °C for posterior analysis.

2.5. Brain tissue and monoamines

At the end of the experiment, brains were removed and divided into 3 anatomical portions: the hypothalamus (HPT), the anterior region (ANT; containing the olfactory bulb and the telencephalon), and the posterior region (POST; composed by the optic *tectum*, the cerebellum and the medulla oblongata). These sections effectively separate the three populations of serotonergic neurons which were previously identified in *C. dimerus* (Morandini et al., 2015). The three regions were separately homogenized in 300 μL cold 4% hydrochloric acid, using a motorized homogenizer (PRO200, PRO Scientific, USA). Homogenates were centrifuged at 10,000 rpm for 2 min and supernatants were stored at −80 °C until posterior analysis.

Brain 5-HT and 5-HIAA content were quantified by high performance liquid chromatography with electrochemical detection (HPLC-EC), which consisted on a Phenomenex Luna 5-μm, C18, 150 × 4.60 mm column (Phenomenex, USA) and a LC-4C electrochemical detector with glassy carbon electrode (BAS, USA). The working electrode was set at +0.70 V respect to an Ag/AgCl reference electrode. The mobile phase contained 74 mM $NaH_2PO_4 \cdot H_2O$, 0.5 mM EDTA, 1.2 mM 1-octane sulfonic acid, and 5% acetonitrile, with pH adjusted to 2.8. The detection limit was 1.5 ng mL^{-1} for 5-HT and 1.8 ng mL^{-1} for 5-HIAA.

The serotonergic turnover was expressed as the 5-HIAA/5-HT ratio (Dahlbom et al., 2012). Each concentration was normalized to the protein content of each brain region measured by the Lowry assay (Gaworecki and Klaine, 2008; Lowry et al., 1951).

2.6. Gonadotrophins β−subunit pituitary content

Pituitaries were homogenized in 100 μL of 0.1 mM Tris-HCl buffer, pH 7.4 with a manual homogenizer with a teflon tip. Samples were kept at −20 °C until determination. Gonadotrophins β−subunit pituitary content (βFSH and βLH) were semi-quantified by SDS-PAGE followed with Western blot.

Briefly, denatured protein samples were separated in polyacrylamide gels by electrophoresis using a 15% separating gel (Mini-Protean III, Bio-Rad, USA). Proteins were transferred to nitrocellulose membranes (Hybond® ECL, Amersham Biosciences, UK), and non-specific binding sites were blocked with 3% skimmed milk and 3% BSA in TTBS (100 mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5) overnight (ON) at 4 °C.

Immunodetection was performed using heterologous antiserum anti-βFSH (1:1000) and anti-βLH (1:2000) of mummichog (*Fundulus heteroclitus*, Cyprinodontiformes; Shimizu and Yamashita, 2002; Shimizu et al., 2003), which were already successfully tested in *C. dimerus* (Pandolfi et al., 2006). After incubation, membranes were washed with TTBS and incubated with the secondary antibody (biotinylated anti-rabbit IgG, 1:10,000; Sigma, USA). The signal was amplified with Streptavidin/HRP (1:5000; Dako, USA) and immunoreactive bands were detected by incubation of the membranes with 0.1% 3,3'-diaminobencidine and 1% hydrogen peroxide (Dako, USA). Each band was normalized by the simultaneous detection of β-tubulin, a constitutively synthesized protein (anti-tubulin E7, 1:1000; Hybridoma Bank). Immunological reactive protein levels were evaluated through optic densitometry (OD) with *Image Pro Plus* software (Media Cybernetics, USA). For both gonadotrophins, omission of the primary antibody was also performed (not shown).

2.7. Sex steroid levels

Sex steroid levels, Testosterone (T) and estradiol (E2), were measured by RIA and electrochemiluminescence, respectively, using commercial kits: Active® Testosterone RIA DSL-4000 (Diagnostic Systems Laboratories, USA; limit of detection of 0.08 ng mL^{-1}; crossreaction with 5α-Dihydrotestosterone 5.8%, 11-Oxotestosterone 4.2%, Androstenedione 2.3%, other related steroids below 2%), and Cobas® Estradiol II (Roche Diagnostics, Germany; detection limit of 5 pg mL^{-1}; crossreaction with other related steroids below 0.5%). All analyses were carried out according to the manufacturer's instructions and a standard curve was run for each steroid. Final steroid levels were normalized to their respective initial value to account for interindividual differences.

2.8. VTG immunodetection

Plasma samples with equal amounts of protein (40 μg as measured by the Lowry assay) were separated by SDS-PAGE at 100 V using an 8% separating gel (Mini-Protean III, Bio-Rad, USA). After transference to nitrocellulose membranes (ECL Amersham Biosciences, UK) at 100 V for 90 min, endogenous peroxidases were blocked by 2% 30 v/v hydrogen peroxide in TTBS for 5 min. Non-specific binding sites were blocked with 3% skimmed milk and 3% BSA in TTBS ON at 4 °C.

Immunodetection of VTG was conducted using rabbit anti-perch VTG antiserum 1:10,000 (*Perca fluviatilis*, Perciformes; generously donated by Dr. B. Allner, Hessische Landesanstalt für Umwelt und Geologie, Germany) for 90 min at room temperature (RT). The specificity and cross-reactivity of the primary antibody has been previously tested in *C. dimerus* (Moncaut et al., 2003). Membranes were then sequentially incubated with biotinylated anti-rabbit IgG secondary antibody 1:15,000 (Sigma, USA) for 1 h at RT,

streptavidin-HRP (Dako, USA) 1:3000 for 1 h, and 0.1% 3,3-diaminobenzidine (Dako, USA) for 5 min, for amplification and detection of the signal. Omission of the primary antibody was also performed. Fish injected with E2, in order to produce a strong induction of VTG, were used as positive controls (Genovese et al., 2012).

2.9. Gonadal histology

Gonads were fixed in Bouin's solution for histological processing. Samples were dehydrated, embedded in Paraplast® and sectioned at 7 μm thickness. Sections were stained using topographical -Hematoxylin and Eosin or Masson's Trichrome stains- or histochemical techniques -Periodic acid Schiff (PAS) or Alcian-Blue (AB) stains- and analyzed blind to treatment through a light microscope (Nikon Microphot FX) coupled to a digital camera for images capture (Coolpix 5400, Nikon, Japan). The incidence of any abnormality was established considering the number of animals in which they were observed.

2.10. Statistical analysis

Data was analyzed using two-way ANOVA, followed by post-hoc Tukey HSD comparisons. When assumptions were not met, data was log transformed. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Serotonergic turnover

No significant differences were found on the serotonergic turnover (5-HIAA/5-HT ratio) between the different brain regions (Table 1; $p = 0.17$). When comparing data discriminating between sexes, no differences were seen between treatments (data not shown).

3.2. Pituitary gonadotrophin β-subunits content

Pituitary βLH content in females treated with 2 μg g^{-1} FLX was significantly higher respect to control females ($p = 0.03$; Fig. 1). In addition, females injected with FLX exhibited higher βLH pituitary levels when compared to both control and FLX-injected males ($p = 0.02$; Fig. 1). No differences were seen between control and injected males.

No statistical differences were observed as regards βFSH levels in pituitaries of *C. dimerus* treated with 2 μg g^{-1} FLX ($p > 0.05$; Fig. 2).

3.3. Plasma steroid levels

The relationship between initial and final E2 plasma levels were not statistically significant, neither for males ($p = 0.49$; Fig. 3) nor for females ($p = 0.76$; Fig. 3) i.p. injected with 2 μg g^{-1} FLX for 2 weeks, when compared to control groups.

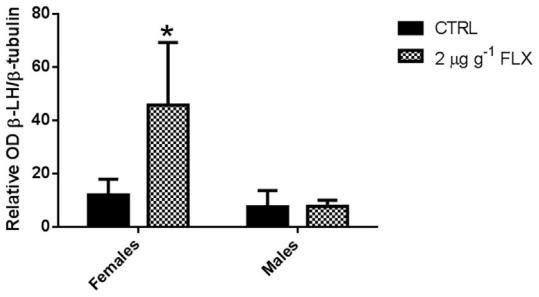

Fig. 1. Relative Optical Density (OD) of βLH immunoreactive bands in pituitary homogenates of *C. dimerus* using SDS-PAGE followed by Western blot. Data is presented as mean ± SEM. * Significant differences between FLX i.p. injected females and the other experimental groups. N = 4 for each group. FLX, 2 μg g^{-1} FLX; CTRL, control (saline 0.6%).

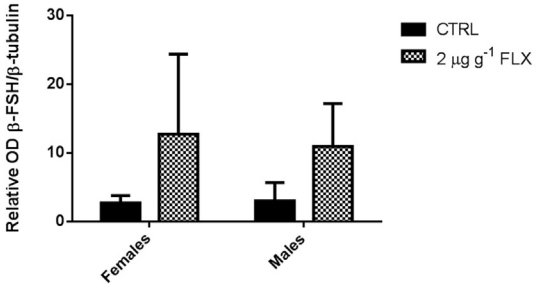

Fig. 2. Relative Optical Density (OD) of βFSH immunoreactive bands in pituitary homogenates of *C. dimerus* using SDS-PAGE followed by Western blot. Data is presented as mean ± SEM. N = 4 for each group. FLX, 2 μg g^{-1} FLX; CTRL, control (saline 0.6%).

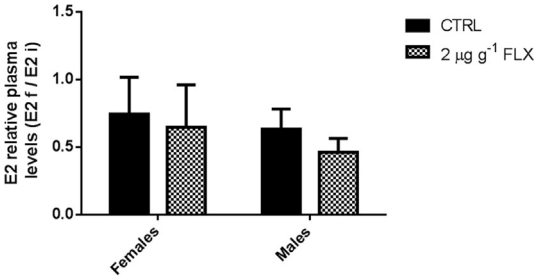

Fig. 3. Relative plasma levels of the sexual steroid estradiol (E2) in males and females of *C. dimerus* measured by electrochemiluminescence. Final levels were normalized to each respective initial level of E2 and are presented as mean ± SEM. N = 4 for each group. FLX, 2 μg g^{-1} FLX; CTRL, control (saline 0.6%).

Circulating T plasma levels did not present significant differences between control and FLX-treated fish in neither females nor males (Fig. 4).

3.4. Vitellogenin detection

No induction of VTG synthesis was observed in plasma of males

Table 1
Serotonergic turnover in hypothalamus (HPT), anterior brain region (ANT), and posterior brain region (POST) of *C. dimerus* injected i.p. with 2 μg g^{-1} FLX or saline 0.6% (CTRL). 5-HT and 5-HIAA concentrations were normalized to the protein content of each brain region measured by the Lowry assay and expressed as ng per g of protein. Data is shown as mean ± SEM. N = 8 for each treatment.

	HPT		ANT		POST	
	CTRL	2 μg g^{-1} FLX	CTRL	2 μg g^{-1} FLX	CTRL	2 μg g^{-1} FLX
5-HT (ng g^{-1})	105704 ± 20836	71848 ± 12702	93384 ± 19220	37266 ± 9972	34968 ± 4383	20333 ± 4798
5-HIAA (ng g^{-1})	6357 ± 1884	8868 ± 5476	5670 ± 1111	2415 ± 911	1306 ± 376	304 ± 111
5-HIAA/5-HT	0.071 ± 0.022	0.098 ± 0.057	0.070 ± 0.016	0.134 ± 0.089	0.032 ± 0.006	0.018 ± 0.006

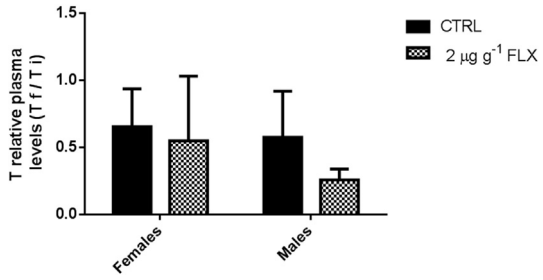

Fig. 4. Relative plasma levels of the sexual steroid testosterone (T) in females and males of *C. dimerus* measured by RIA. Final levels were normalized to each respective initial level of T and are presented as mean ± SEM. N = 4 for each group. FLX, 2 μg g^{-1} FLX; CTRL, control (saline 0.6%).

of *C. dimerus* i.p. injected with 2 μg g^{-1} FLX for 2 weeks (data not shown).

3.5. Gonadal histology

Gonads from control animals showed the typical morphological arrangement previously described for *C. dimerus*: unrestricted lobular testes type and a cystovarian type.

When analyzing the gonad sections of the exposed animals under light microscope, the ovarian tissue of females treated with FLX did not reveal distinguishable changes (Fig. 5A). Similarly, control males did not show any abnormal features (Fig. 5B). However, testes histology of males injected i.p. with FLX revealed the presence of a weakly acidophilic PAS positive, AB negative secretion within the lobular lumen interspersed between the sperm (Fig. 5C, D and E). In addition a high preponderance of "foam" cells -PAS positive, AB highly positive- in the lobular lumen were seen in the FLX exposed males (Fig. 5D and E). The incidence of these alterations was very high in treated males, being 100% for foam cells and 75% for the acidophilic secretion, whereas no control fish showed these changes.

4. Discussion

The long list of CECs includes substances such as pesticides, personal care products, fragrances, plasticizers, hormones, flame retardants, nanoparticles, perfluoroalkyl compounds, chlorinated paraffins, siloxanes, algal toxins, various trace elements including rare earths and radionuclides, etc. (Sauvé and Desrosiers, 2014). Among CECs, pharmaceuticals are of high interest for research due to their widespread consumption around the world, and their potential as Endocrine Disrupting Chemicals (EDCs).

Since its approval for human consumption in 1987, FLX remains one of the most prescribed SSRIs worldwide, mainly because of its low incidence of side effects, compared to other types of antidepressants such as tricyclics (Wong et al., 1995). Environmental studies suggest that FLX is released to the environment from WWTPs, and could potentially generate sublethal effects on aquatic biota, especially fish (Brooks et al., 2003). According to the "Read-Across Hypothesis" first articulated by Huggett et al. (2003), the molecular target of pharmaceuticals would be conserved between vertebrate groups and a pharmacological response will require a similar plasma concentration in non-target species than in humans. Several studies in fish have shown evidence that supports this hypothesis (see Rand-Weaver et al. (2013) for review). In agreement with the Read-Across Hypothesis, Margiotta-Casaluci et al. (2014) found that FLX caused an anxiolytic response only at predicted fish plasma concentrations above the Human Therapeutic Plasma Concentration Range (HTR: 91–302 ng mL^{-1}). Using the volume of distribution (Vd) of FLX in humans (between 20 and 42 L kg^{-1}, Moffat et al., 2011), as transportability into tissues from plasma has been shown to be similar in fish and humans yielding comparable Vd values (Tanoue et al., 2015), the plasma concentration of FLX resulting from the administration of the sublethal dose of 2 μg/g used in this study could be estimated, using the equation Plasma Concentration = Dose/Vd, between 48 and 100 μg/L, below or at the lower limit of the HTR. The dose of 20 μg g^{-1} would derive in a plasma concentration between 476 and 1000 μg L^{-1}, well above the HTR, which would potentially explain why this dose proved lethal to fish.

After treatment with 20 μg g^{-1} FLX, fish died almost immediately (from 0 to 30 min after injection). In most cases they showed fast erratic swimming when returned to the aquaria following injection and then remained at the bottom of the aquaria, usually in a vertical position, snout down, until death. This lethal effect was unexpected based on published data. In a study conducted in gulf toadfish (*Opsanus beta*), the effect of intraperitoneal FLX on fish survival was evaluated, and only the highest doses assayed, 75 and 100 μg g^{-1}, exhibited any mortality (Morando et al., 2009). The dose of 25 μg g^{-1} FLX, higher than the highest used in our case, showed no differences in survival from control treatment. A recent study using this same species, again injected fish with a 25 μg g^{-1} FLX dose to evaluate change in cardiovascular and ventilatory parameters and did not report lethality (Panlilio et al., 2016). When considering the plasma concentration, even though the toxic level for humans is reported at 1000 μg L^{-1} FLX (Schulz and Schmoldt, 2003), Margiotta-Casaluci et al. (2014) found that fish exceeding this value, in some cases doubling it, did not show any evident symptom of toxicity. Exposure to waterborne FLX concentrations that would result in plasma steady state concentrations well above the HTR (water concentrations of 150 or 300 μg L^{-1} FLX equal to 266 or 532 μg L^{-1} plasma FLX, respectively) also resulted in no mortality of fish (Gaworecki and Klaine, 2008; Winder et al., 2012). Fish lethal median concentrations for waterborne FLX are even higher than the aforementioned, being 546 μg L^{-1} for mosquitofish neonates (Henry and Black, 2008).

Being FLX an SSRI, an alteration on serotonergic turnover in the brain because of treatment with this drug was expected (Clotfelter et al., 2007; Fuller et al., 1974; Gaworecki and Klaine, 2008; Malagié et al., 1995). However, in this work, when analyzing the results in *C. dimerus*, no significant differences were seen on serotonergic turnover neither between the three analyzed brain regions nor between treatment groups. This result is consistent with a previous work carried on in females of *C. auratus*, where the i.p. injection of 5 μg g^{-1} FLX for 2 weeks did not produce significant changes in serotonergic turnover (Mennigen et al., 2008).

In mammals, the location and spatial distribution of serotonergic neurons and its major projections throughout the brain, which virtually reach all brain areas, have been widely studied (Lam and Heisler, 2007). This same analysis was performed in teleost fish, leading to the description of three different serotonergic neurons populations and a wide projections network (Lillesaar, 2011). It is known that the regulation of the serotonergic system is more complex than previously thought and that there are multiple factors that could interfere with it, so an increase on 5-HT levels would not be the univocal answer to the treatment of depression symptoms (Koch et al., 2002; Martín-García et al., 2007; Valentino and Commons, 2005). Moreover, recent studies focusing on major depressive disorder determined that other monoamines, such as dopamine (DA) and norepinephrine, could be involved in 5-HT metabolism in the human brain, given the close relationship between the 5-HT and the DA systems (Porcelli et al., 2011). For instance, 5-HT receptors in the midbrain are expressed by dopaminergic neurons, and some 5-HT terminals were shown to be

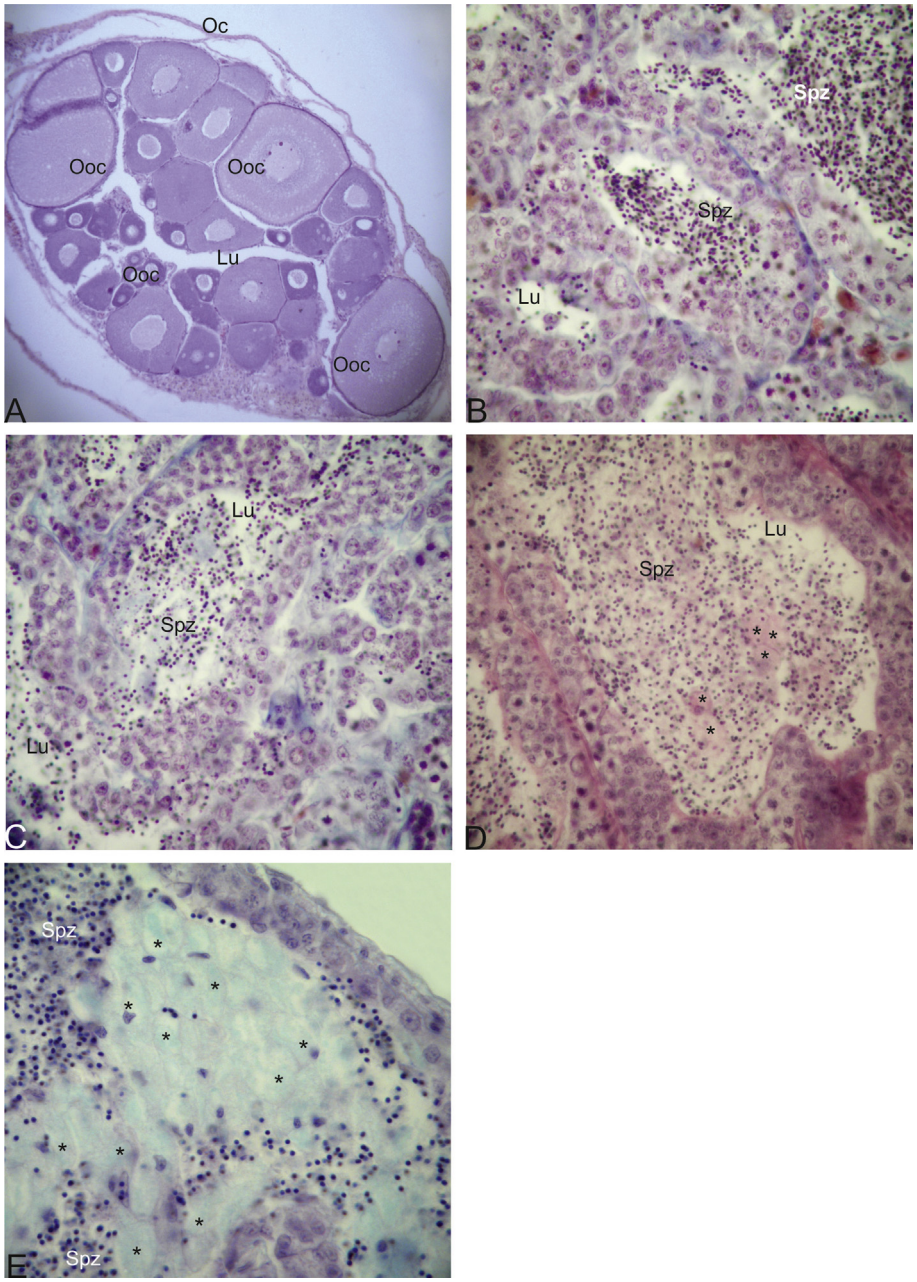

Fig. 5. Microphotographs of gonads from *Cichlasoma dimerus* in cross section. A) Female injected i.p. with 2 μg g^{-1} FLX for 2 weeks presented a normal ovarian tissue organization with different oocytes stages in the ovarian lamellae. B) Control male. Testis presented normal cytoarchitecture with lobules containing cysts with all the stages of spermatogenesis and lobular lumen filled with sperm. C – E) Males injected i.p. with 2 μg g^{-1} FLX for 2 weeks. C) An acidophilic secretion can be observed within the lumen, which is positive to PAS staining (negative to AB staining; not shown). D) Notice the PAS positive stain of the luminal secretion and the presence of foam cells (asterisk) in the lobular lumen in FLX-exposed male fish. E) Detail of foam cells stained with AB (asterisk) within the lobular lumen and interspersed between sperm. Asterisk: foam cells; lumen (Lu); Oocytes (Ooc); Ovarian capsule (OC); sperm (Spz). (A) Hematoxylin and Eosin stain. (B and C) Masson's Trichrome stain. (D) PAS stain. (D) Alcian-Blue stain. (A) 40X. (B, C and D) 400X; (E) 600X.

interconnected to DA release through interneurons (Herve et al., 1987; Nedergaard, 1988).

The relationship between 5-HT and gonadotrophins has been well studied over the last decades, and it is well known that fish reproductive function is regulated by 5-HT through multiple pathways, at both central and peripheral levels (Prasad et al., 2015). In the CNS of teleost species, 5-HT stimulates GnRH release from the hypothalamus (Senthilkumaran et al., 2001; Yu et al., 1991), one of the brain areas on which a serotonergic neurons nucleus has been identified (Lillesaar, 2011). Moreover, it is possible that 5-HT receptors are expressed in GnRH neurons (Bhattarai et al., 2013). Despite this, contradictory results have been found regarding FLX administration and GtHs in fish. In the present study, when analyzing GtHs content, higher pituitary βLH levels in females treated with 2 μg g^{-1} FLX were observed, but non-appreciable differences appeared between males. This result is in accordance with the results obtained by Somoza et al. (1988), where the i.p. co-administration of 5-HT and FLX, produced an increase in serum LH levels in *C. auratus* L. However, Mennigen et al. (2008, 2010) observed no changes in circulating levels of LH when administrating a 5 μg g^{-1} i.p. injection or exposed of FLX in females or males respectively of the same species. Interestingly, in these latter

studies, even though no changes were seen for LH, circulating E2 levels showed a differential response to FLX in both sexes, decreasing in females and increasing in males. These apparent sex-specific effects found in the present study for LH and by Mennigen et al. (2008, 2010) for E2 requires further study and should be an additional cause of concern when analysing effects. With regards to the HPG axis, plasma steroid levels (E2 and T) did not exhibit changes due to FLX administration in both males and females of *C. dimerus*. Contrarily, an increase on plasma E2 levels and a decrease in T levels due to FLX exposure were observed in males of *C. auratus* (Mennigen et al., 2010). However, females of the same species injected i.p. with a 5 $\mu g\ g^{-1}$ FLX dose for 2 weeks presented a decrease in plasma E2 levels (Mennigen et al., 2008). Recently, Bain et al. (2016) found a decrease in plasma E2 coupled with a 10-fold downregulation of aromatase A mRNA levels in the ovary, when exposing female Murray-Darling rainbowfish to 100 $\mu g\ L^{-1}$ of waterborne FLX for 96 h. Similarly, exposure to FLX resulted in a significant reduction in ovarian E2 levels and gonadotrophin receptors expression in *D. rerio*, which was followed by a lower egg production (Lister et al., 2009). Alternatively, Foran et al. (2004) observed an increase in plasmatic E2 levels in females of *O. latipes* exposed to FLX. This latter work however, did not report differences in other reproductive endpoints, such as T or VTG plasma levels, in agreement with the results presented in this work. On the other hand, Schultz et al. (2011) did find VTG induction in males of *P. promelas* after a 21-day exposure to 28 $ng\ L^{-1}$ FLX, a water concentration that would result in a plasma level well below the HTR. In the liver of female Murray-Darling rainbowfish, Bain et al. (2016) found a strong and significant decrease of the hepatic estrogen-responsive transcripts VTG and choriogenin L, after exposing fish to 100 $\mu g\ L^{-1}$ of waterborne FLX for a short-term period (96 h). According to our previous research (Genovese et al., 2011, 2012; Moncaut et al., 2003) the presence of VTG in plasma of *C. dimerus* males is a useful tool for the detection of xenoestrogen exposure since unexposed males do not produce VTG. This is not the case for female *C. dimerus*. In this work, since the impact of FLX in the HPG axis seems not to be sufficiently potent, the absence of plasma VTG induction in males was expected.

Regarding gonadal cytoarchitecture, as in all vertebrates, the testis in fish is composed of two main compartments, the germinal and the interstitial compartments. The basic functional unit of the spermatogenic epithelium is a cyst, formed by a group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone (Parenti and Grier, 2004; Schulz et al., 2010). Testicular structure in *C. dimerus* corresponds to an unrestricted lobular type where the spermatogonial distribution is characterized by the occurrence of spermatogonia all along the germinal compartment (Grier et al., 2016). In some teleost species, histological changes in the morphology of the testicular and ovarian germinal epithelium have been used to document pathological features produced by pollutants.

In the present study, some alterations were seen in testes from males injected with FLX, such as the presence of foam cells and an acidophilic secretion in the lumen of treated males. Foam cells are macrophages, with lipid-full cytoplasm, which are well studied due to its high presence in atherosclerosis (Shashkin et al., 2005). Their presence in the testicular lobular lumen suggests the development of degenerative or necrotic processes, as a consequence of the exposure to toxic substances. These type of cells were previously reported in gonads of male *C. dimerus*, upon exposure to an estrogenic compound, 4-*tert*-octylphenol (Rey Vázquez et al., 2009) and a pesticide, endosulfan (Da Cuña et al., 2013). In fish, macrophages constitutes the most important cell in the immune response, not only because of being, as it happens in mammals, the primary antigen-presenting cell in teleost, but also as they are involved in phagocytosis and the killing of pathogens upon first recognition and subsequent infection (Kum and Sekkin, 2011). Immune cells infiltration into the testicular lobular lumen was reported during sex reversal of hermaphroditic fish, as granulocytes could be seen in the germinal compartment of the protogynous swamp eel *Synbranchus marmoratus* (Lo Nostro et al., 2004); or the protandrous gilthead seabream, *Sparus aurata* L., in the post-spawning period, in which a degenerative process occurs and testis organization is disrupted (Chaves-Pozo et al., 2003).

The acidophilic secretion observed among sperm cells in the lumen, which proved positive to PAS staining, but negative to AB staining, indicative of presence of neutral glycoconjugates, could also be related to an inflammatory process in the tissue. Several studies describe 5-HT as a key modulator of the immune system. Mössner and Lesch (1998) define 4 main functions of 5-HT as regards immunity, which includes T-cell activation, delayed-type hypersensitivity responses, production of chemotactic factors, and natural immunity delivered by macrophages.

Schultz et al. (2011) also showed alterations in gonadal histology of males exposed for 21 days to a concentration of 28 $ng\ L^{-1}$ of FLX, observing an increase on interstitial cell prominence and interstitial space in the testes of *P. promelas*. On the contrary, Mennigen et al. (2010) found non-appreciable histomorphological changes in the testis of *C. auratus*, following a 2 week exposure to environmentally relevant concentrations of FLX.

The discrepancy in the range of effects caused by FLX on the HPG axis could be partly related to the duration of the exposures. It is known that the action of SSRIs in humans requires a period of 30 days, with daily administration, before plasmatic concentrations of the drug remain stable and the SSRI is able to elicit its effect diminishing depression symptoms (Altamura et al., 1994). When assessing pollutants effects, it is also important to consider the route of exposure in order to analyze results. Intraperitoneal injection, though of low environmental relevance to test potential aquatic EDCs, constitutes a valuable tool to identify mechanisms of action of substances whose effects on the reproductive physiology are still unclear (Karami et al., 2011).

Taken together, these results lead to the conclusion that FLX can act as a mild EDC in *C. dimerus* at the experimental dose assayed, especially when considering the amount of information that has accumulated in the last 10 years about this topic. Nevertheless, there is still no clear consensus about the effect of this drug over reproductive physiology, which is supported by the variability in the sensitivity of different aquatic species (Sumpter et al., 2014). Further research is needed in order to evaluate if other physiological parameters, such as food intake, energy metabolism, growth or even brain functioning could be altered due to FLX exposure, which could indirectly impact the HPG axis and hence reproductive success.

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