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Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae)

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ABSTRACT

Endosulfan (ES), a persistent organochlorine pesticide, is widely used despite its toxicity to non-target animals. Upon reaching water bodies, ES can cause negative effects on aquatic animals, including disruption of hormonal systems. However, the action of ES on fish reproductive axis has been hardly studied thus far. The aim of the present work was to assess the endocrine disruptive potential of endosulfan on the pituitary gonadotropins levels and on the testes function due to ES in the South American freshwater fish *Cichlasoma dimerus*, using *in vitro* and *in vivo* approaches. *In vitro* experiments showed that ES inhibited the LH-stimulated steroidogenesis in gonads; no change was observed in gonadotropins release from pituitaries in culture. Laboratory waterborne ES (0.1, 0.3 and 1 μg/L) exposure for two months caused decrease in βFSH pituitary content and γGT activity in the testes (Sertoli cell function marker). Testicular histology revealed pathologies such as scarce intermediate stages of spermatogenesis, release of immature germ cells into the lobular lumen, presence of foam cells and interstitial fibrosis. As FSH and FSH-mediated steroidogenesis regulate spermatogenesis and Sertoli cell function, the effect of ES on FSH could be responsible for the morphological alterations observed in testes. *In vitro*, ES disrupted steroidogenesis in gonads, therefore similar effects *in vivo* cannot be ruled out. Based on this evidence, ES exhibits an endocrine disruptive action on the reproductive axis of *C. dimerus*, causing disruption at the pituitary and/or at the gonad level. These effects could acquire ecological significance under prolonged exposure to the pesticide in nature.

1. Introduction

Water ecosystem pollution is, together with loss of habitat, one of the main factors endangering wildlife species (Wilcove et al., 1998). As pesticide runoff constitutes a significant contribution to aquatic pollution, their use in agriculture is under constant screening to ensure non-target animals' welfare, leading to the ban and restriction of a large number of products. The use of the organochlorine pesticide endosulfan (ES; 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) has been limited or discontinued in recent years in many European and North American countries due to its persistence in the environment and toxic effects (Sutherland et al., 2004). Endosulfan has been recently classified as a Persistent Organic Pollutant (POP) by the Stockholm Convention on POPs, supporting the ban on its use and production (POPRC, 2010). However, it is still widely used, particularly in developing countries, as a broad spectrum insecticide to control insects and mites in crops of high commercial value (soy, cotton, tea, coffee, maize, fruits) (Capkin et al., 2006). Following application, ES can reach non-target aquatic animals through groundwater, surface runoff and air drift from nearby agricultural fields (Miglioranza et al., 2002). Half life in water and soil for α isomer has been reported as 7–75 days, whereas for β isomer and endosulfan sulfate, the equally toxic main metabolite of ES degradation, half lives can exceed 300 days, depending on environmental conditions (Singh et al., 2000; Weber et al., 2010). Isomers have been detected in surface and groundwater in concentrations ranging from 0.05 to 2.5 μg/L (Dalvie et al., 2003; Leong et al., 2007) as well as in fish at

Abbreviations: γGT, gamma-glutamyl transpeptidase; ES, endosulfan; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

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relatively high concentrations (1–120 ng/g wet tissue, Lanfranchi et al., 2006; Menone et al., 2000; Singh et al., 2008). Endosulfan is a known neurotoxin and is very highly toxic to fish (LC_{50} for aquatic species ≤100 μg/L, 96-h LC_{50} median value of 2.6 μg/L for teleost fish, Kegley et al., 2011) with oxidative damage (Ballesteros et al., 2009), genotoxicity (Neuparth et al., 2006), damage to testes (Dutta et al., 2006) and changes in circulating thyroid hormones (Coimbra et al., 2005) among the negative effects reported upon exposure.

As is the case with many pesticides, ES has also been suggested as an endocrine disrupting chemical, capable of interfering with the normal functions of the endocrine system of animals (Mills and Chichester, 2005). Evidences for the action of ES on the reproductive axis of fish include: decreased clutch size (Gormley and Teather, 2003) and sex ratio skew toward females (Teather et al., 2005) in exposed Japanese medaka (*Oryzias latipes*); lowered vitellogenin plasma levels in females (Chakravorty et al., 1992) and altered expression of steroidogenic enzymes, gonad-related transcription factors and *cfGnRH* mRNAs in larvae (Rajakumar et al., 2012; Chakrabarty et al., 2012) of the Asian catfish (*Clarias batrachus*); sex ratio skew toward females and delayed sexual maturity following discrete immersion of fry (Balasubramani and Pandian, 2008) and decreased hatching rate, reduced gonadosomatic index in females, vitellogenin levels increase in males and histological gonadal alterations (Han et al., 2011) in exposed zebrafish (*Danio rerio*).

Despite cichlids being the most species-rich non-Ostariophysan family of freshwater fishes worldwide (Kullander, 2003), few studies have dealt with the toxic effect of ES on members of this group. The South American cichlid fish *Cichlasoma dimerus* inhabits quiet shallow waters of the Paraguay and most of the Paraná Rivers basins (Kullander, 1983), including some heavily agricultural areas. This freshwater species is representative of perciform teleosts in the La Plata River basin and relevant to the Argentinean riverine ecosystems. *C. dimerus* can be considered an amenable model for laboratory studies, as it acclimates easily to captivity and shows notable reproductive features such as a complex social and breeding behavior, which includes parental care, a high spawning frequency (Meijide and Guerrero, 2000) coupled with acceptable survival rates. Considering its successful utilization in ecotoxicological testing (Genovese et al., 2011, 2012; Moncaut et al., 2003; Rey Vázquez et al., 2009), this species has been included as one of the suitable native fish species for the determination of the lethal acute toxicity of xenobiotics by the Argentinean Institute of Standardization and Certification (IRAM, 2008).

The aim of the present study was to evaluate the potential of ES to act as an endocrine disruptor of the reproductive axis in the native freshwater cichlid species *C. dimerus* by using chronic exposures and *in vitro* approaches to better understand the mechanisms underlying ES toxicity and assess its possible long term impact on fish populations.

2. Materials and methods

2.1. Animals

Adult fish of the native freshwater species *C. dimerus* were captured in Esteros del Riachuelo, Corrientes, Argentina (27°35′S 58°45′W). Fish were held in 100 L well aerated aquaria with external filtration and a layer of gravel on the bottom, with filtered tap water (pH 7.3, alkalinity 36.5 mg/L, hardness 55 mg/L, conductivity 219 μS/cm, dissolved oxygen 8 mg/L, sodium 20 mg/L) at 25 ± 1 °C and 14:10 h photoperiod. They were allowed to acclimate to laboratory conditions for a month prior to experimentation. During the acclimation period fish were fed daily with pelleted commercial food (Tetra food® sticks). All experiments were conducted in accordance to international standards on animal welfare (Canadian Council on Animal Care, 2005).

2.2. Pituitary culture

To study the effect of ES on pituitary gonadotropins secretion *in vitro*, pituitary explants from control fish ($N = 12$) were cultured individually at 25 °C in 100 μL Leibovitz L15 (pH 7.6, 80% (v/v) medium (Gibco®, USA) supplemented with 10% fetal bovine serum, 10 mM HEPES, 100 IU/mL penicillin and 100 mg/mL streptomycin) following a modified protocol from Pandolfi et al. (2009) for this species. Glands were pre-incubated in media alone for 1 h to allow for the stabilization of hormone release following loss of neuromodulatory transmitters upon dissection. Media were discarded and fresh medium was added for 24 h to obtain basal hormone release levels for each pituitary (day 1 medium). Over the next 24 h of incubation, medium was renewed and randomly supplemented with DMSO (solvent control) or 100 μM ES (day 2 medium) – concentration in the range below reported LC_{50} for cell viability and above EC_{50} for hormone secretion (Bisson and Hontela, 2002; Dorval et al., 2003; Je et al., 2005). After collection, media were stored at −80 °C until analysis.

Semi-quantification of gonadotropins present in the culture media was achieved by SDS-PAGE followed by Western blot and densitometry of immunoreactive(ir)-bands following Pandolfi et al. (2006). Media (16 μL) was mixed with loading buffer (120 mM Tris–HCl, pH 6.8, 3% sodium dodecyl sulfate, 10% glycerol, 2% bromophenol blue and 1% β-mercaptoethanol), boiled for 5 min and loaded into polyacrylamide gels. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) at constant 100 V using 4% stacking and 15% separating gel (Mini-Protean III, Bio-Rad, USA) and 124 mM Tris–HCl, pH 8.8 running buffer. Transference to nitrocellulose membranes (ECL Amersham Biosciences, UK) was achieved at 75 V for 60 min, in 25 mM Tris, 187 mM glycine and 20% methanol. 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 at 4 °C. βLH and βFSH were immunodetected using heterologous antibodies raised against synthetic fragment peptides of *Fundulus heteroclitus* (mummichog) gonadotropins subunits (Dr. A. Shimizu, Fisheries Research Agency, Japan; Shimizu and Yamashita, 2002) at a 1:1000 dilution for 2 h at RT, the specificity of which has been previously established for *C. dimerus* and other acanthopterygians species by preadsortion tests, western blot and immunohistochemistry (Pandolfi et al., 2006; Shimizu et al., 2003). Membranes were washed with TTBS and incubated with biotinylated secondary anti-rabbit IgG antibodies diluted to 1:1000 (Sigma–Aldrich Inc., USA) for 1 h at RT. Immunodetection signal was amplified by incubating with Streptavidin alkaline phosphatase (Dako, USA) 1:3000 for 1 h and developed by a commercial kit (Dako, USA). Omission of the primary antibody was also performed. Membranes were scanned, molecular weights were estimated using SigmaGel software (Jandel Scientific software 1.0, USA). βLH was evidenced as a single ir-band of 24 kDa, whereas βFSH revealed two ir-bands of 19 and 15 kDa, the latter was chosen for analysis as both showed the same response in previous studies in the same species (Pandolfi et al., 2009). Ir-levels were estimated by densitometry using Image Gauge software (Fuji Photo Film Co.). A 148 kDa protein only present in the culture medium, visualized by Ponceau S solution, was used to normalize data after Canepa et al. (2008). Day 2 values were also normalized by the basal value (day 1 value) for each pituitary.

2.3. Gonad culture

To investigate the effect of ES on gonadal sex steroid release *in vitro*, testes and ovaries from control fish were incubated at RT under constant shaking as follows. Whole testes ($N = 10$) were sectioned into 5 even fragments and cultured separately in

150 μL Krebs–Ringer-glucose-HEPES medium (pH 7.4, NaCl 0.9%, KCl 1.15%, $CaCl_2$ 1.22%, KH_2PO_4 2.11%, $AgSO_4{\cdot}7H_2O$ 3.8%, glucose 5.4%, HEPES 0.026 M). Pieces of 45–50 mg were taken from each ovary ($N = 10$) and incubated in 200 μL Krebs–Ringer medium. In both cases, caudal portions (ducts area) of each gonad were discarded. Gonad fragments were mechanically disgregated using fine needles. Following a 30 min pre-incubation, medium was renewed and fragments from the same gonad were randomly supplemented with DMSO, 100 μM ES, 0.05 mg/L mummichog βLH (positive control) or both 100 μM ES and 0.05 mg/L βLH simultaneously. After incubation at RT for 4 h, media were collected and androgens (A) and estradiol (E_2) were measured using commercial kits (Active® Testosterone RIA DSL-4000, Diagnostic Systems Laboratories, Inc. USA; Cobas® Estradiol II, Roche Diagnostics GmbH, Germany, respectively). Androgen levels rather than testosterone levels were used since the heterologous detection system shows a 4.5% cross-reaction with 11-ketotestosterone, steroid usually present in high levels in fish.

2.4. Exposure experiment

To evaluate the effect of ES on the reproductive axis of *C. dimerus in vivo*, adult fish ($N = 36$; mean weight ± SD = 33.8 ± 7.7 g; mean standard length ± SD = 8.8 ± 0.7 cm) were randomly transferred to 20 L aquaria devoid of ornamentations. Due to the lack of evident external sexual dimorphism in this species, no sex distinction was made. Once fish were accustomed to this new environment for one week, they were exposed to nominal concentrations of 0 (solvent control), 0.1, 0.3 and 1 μg/L ES for two months under semi-static conditions. Concentrations were chosen according to the 96-h LC_{50} value previously established for this species (Da Cuña et al., 2011), also considering that they were within the environmental range reported for this pesticide. Each concentration was tested by triplicate with 3 individuals per test group. A stock solution of ES (94.99% purity, technical grade, 70:30 α:β stereo-isomers mixture) was prepared dissolving it in acetone; the necessary volume of stock solution was added to the aquaria to achieve the desired final concentrations (solvent = 0.005% per aquaria); water and the test chemical solutions were renewed every 48 h. Fish were fed daily; on the days of water renewal, feeding took place an hour before to minimize chemical adsorption to the pelleted food. The actual concentrations of ES were measured 15 min after water renewal by gas chromatography-electron capture detector (GC/ECD, EPA SW-846 M8081A; US Environmental Protection Agency, 1996; Detection limit: 0.1 ng/L for isomer ESI; 0.9 ng/L for ESII) and showed on average a 10% decrease of the nominal value (0.09, 0.25 and 0.96 μg/L respectively for nominal concentrations of 0.1, 0.3 and 1 μg/L). At the end of the exposure period, fish were anesthetized with Fish Calmer® (active ingredients: acetone, dimethylketone alpha methyl quinoline; Jungle Laboratories, USA), weighed, measured, and sacrificed by decapitation for sample collection.

2.4.1. Gonadotropins pituitary content

Pituitaries from ES-exposed fish were collected and homogenized individually in 100 μL Tris–HCl 0.1 mM pH 7.4 buffer and 5 μL protease inhibitor cocktail (Sigma) for gonadotropin content semi-quantification by SDS-PAGE followed by Western blot. GtHs from pituitary homogenates (16 μL) were immunodetected as described for the *in vitro* experiment. The E7 antibody (Developmental Studies Hybridoma Bank, The University of Iowa, USA) at a 1:200 dilution overnight at 4 °C was used for simultaneous β-tubuline immunodetection for normalization purposes. Ir-levels were estimated by densitometry using Image Gauge software (Fuji Photo Film Co.).

2.4.2. Gamma-glutamyl transpeptidase activity (γGT)

Testes were collected and weighed; samples were homogenized in Tris–HCl 50 mM pH 7.5 at a 1:10 organ weight:buffer volume ratio for quantification of γGT activity, a useful Sertoli cell function marker in fish. Homogenates were centrifuged at 20,000 × *g* for 10 min. Enzyme activity was assayed on the resulting supernatant at 25 °C using a commercial kit (modified Szasz et al. (1974); Wiener Lab, Argentina).

2.4.3. Histological analysis

Gonads were fixed in Bouin's solution for histological processing. Following dehydration, testes were embedded in Paraplast® (Oxford Labware, USA). Cross-sections (7 μm) were slide-mounted and stained with Masson's trichrome. Photomicrographs were taken with a Nikon-Microphot FX microscope.

2.5. Statistical analysis

For gonadotropins semi-quantification, relative optical density of ir-bands was analyzed using one way ANOVA, followed by Tukey multiple comparisons. Steroid production was analyzed by means of a randomized blocks design ANOVA. Differences were considered statistically significant if $p < 0.05$. Statistica 7.0 software (StatSoft®, Inc.) was employed for all tests.

3. Results

3.1. Pituitary culture

No statistical differences were found when comparing hormone release to the medium between control, solvent-exposed and ES-exposed cultured pituitaries, for neither gonadotropin (Fig. 1).

3.2. Gonad culture

When gonads were cultured with ES alone, steroid hormones release to the medium – androgens for testes, estradiol for ovaries – did not differ from control values. As expected, βLH caused a marked increase in hormone release from both gonad types. Co-administration of ES and βLH inhibited the stimulatory effect of the gonadotropin on cultured gonads as sex steroid values were consistent with basal values (Fig. 2).

3.3. Exposure experiment

3.3.1. Gonadotropins pituitary content

Pituitary content of βFSH in exposed fish at the two highest ES concentrations assayed (0.3 and 1 μg/L) was on average 65%

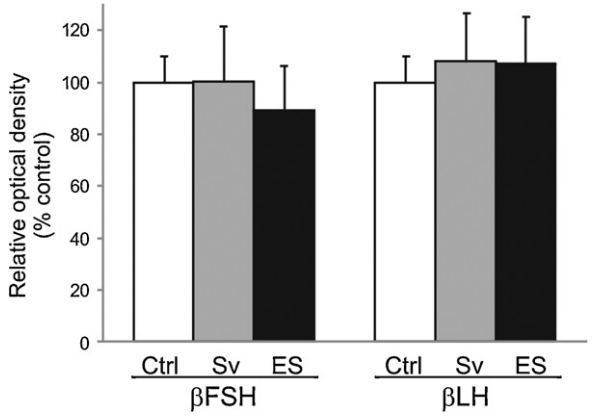

Fig. 1. Relative optical density of βFSH and βLH ir-bands in media from *C. dimerus* pituitary cultures using SDS-PAGE followed by Western blot. Data were normalized to the optical density of a culture medium protein and to the basal value (day 1) of each corresponding pituitary. Data are expressed as percentage of control values. Ctrl, control; Sv, solvent (DMSO); ES, endosulfan 100 μM.

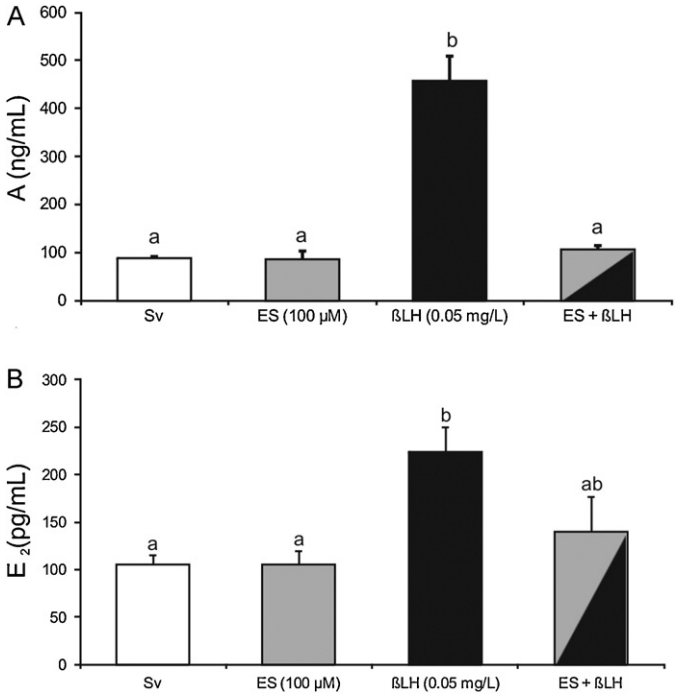

Fig. 2. Androgens (A; A) and estradiol (E_2; B) levels released to the medium in *C. dimerus* testis and ovary cultures, respectively. Data are expressed as mean ± SD and normalized to the corresponding control treatment. Different letters indicate significant differences ($p < 0.05$) using a Randomized-block design ANOVA and Tukey tests. Ctrl, control; Sv, solvent (DMSO); ES, endosulfan 100 µM; bLH, beta luteinizing hormone 0.05 mg/L.

lower than that of control fish and those exposed to the lowest concentration (Fig. 3A). Though βLH content at the same ES concentrations was on average 36% lower than control fish, no statistical differences were found (Fig. 3B).

3.3.2. γGT activity in testes

In testes homogenates, γGT activity decreased in males exposed to the two lowest ES concentrations (0.1 and 0.3 µg/L) when compared to control values (Table 1). Though not statistically different, probably due to the high inter-individual variation, a trend towards decrease in γGT activity was observed for the highest ES concentration tested.

3.3.3. Gonad histology

Control males showed the typical cytoarchitecture of an unrestricted lobular type testis (Grier, 1993). All spermatogenic stages could be identified: spermatogonia, spermatocytes and spermatids within spermatocysts close to the lobular wall, spermatozoa within the lobular lumen (Fig. 4A). An abnormal preponderance of spermatozoa and cysts with spermatogonia and scarce cells in intermediate stages of spermatogenesis could be seen in testes of ES exposed fish at the two highest concentrations (Fig. 4B and C). Immature germ cells and foam cells were commonly present in the lumen of treated males (Fig. 4B). Interstitial fibrosis was also observed upon ES exposure at the highest concentration (1 µg/L; Fig. 4D). No alterations were observed for ovaries (not shown).

Table 1
γGT activity in testes homogenates of ES exposed *C. dimerus* (mUnits/g wet tissue). Values are expressed as mean ± SD.

ES concentration	γGT activity (mUnits/g wet tissue)
Control	181.4 ± 86.7
0.1 µg/L	37.3 ± 9.3*
0.3 µg/L	52.5 ± 36.3*
1.0 µg/L	76.7 ± 26.4

* Statistically different than control (one way ANOVA).

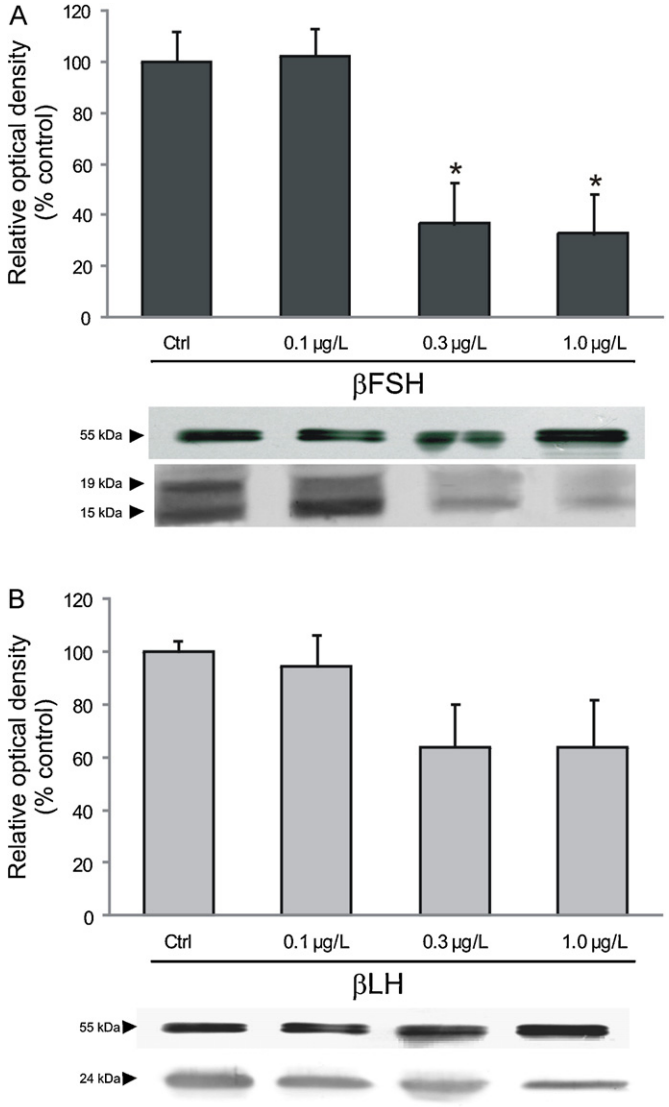

Fig. 3. Relative optical density of βFSH (A) and βLH (B) in pituitary homogenates of ES-exposed *C. dimerus*. Data were normalized to the optical density of simultaneously detected β-tubuline and expressed as percentage of control fish (Ctrl). Photographs below graphs are representative ir-bands (β-tubuline 55 kDa; βFSH 19 and 15 kDa (the latter was chosen for densitometric analysis); βLH 24 kDa). *Significantly different from Ctrl ($p < 0.05$) (one way ANOVA, followed by Tukey test). Results from male and female fish were pooled together due to the lack of differences between sexes.

4. Discussion

Hormones of the reproductive axis of vertebrates – pituitary gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH); sex steroids: androgens, estrogens and progestagens levels – play central roles in regulating gametogenesis and steroidogenesis required for the development and maintenance of sexual behavior and secondary sex characters (Swanson et al., 2003). Several environmental toxicants can disrupt the normal function of these hormones. However, the role of the organochlorine insecticide ES on this vertebrate endocrine system has been scantily studied, particularly in fish (Balasubramani and Pandian, 2008; Chakrabarty et al., 2012; Rajakumar et al., 2012).

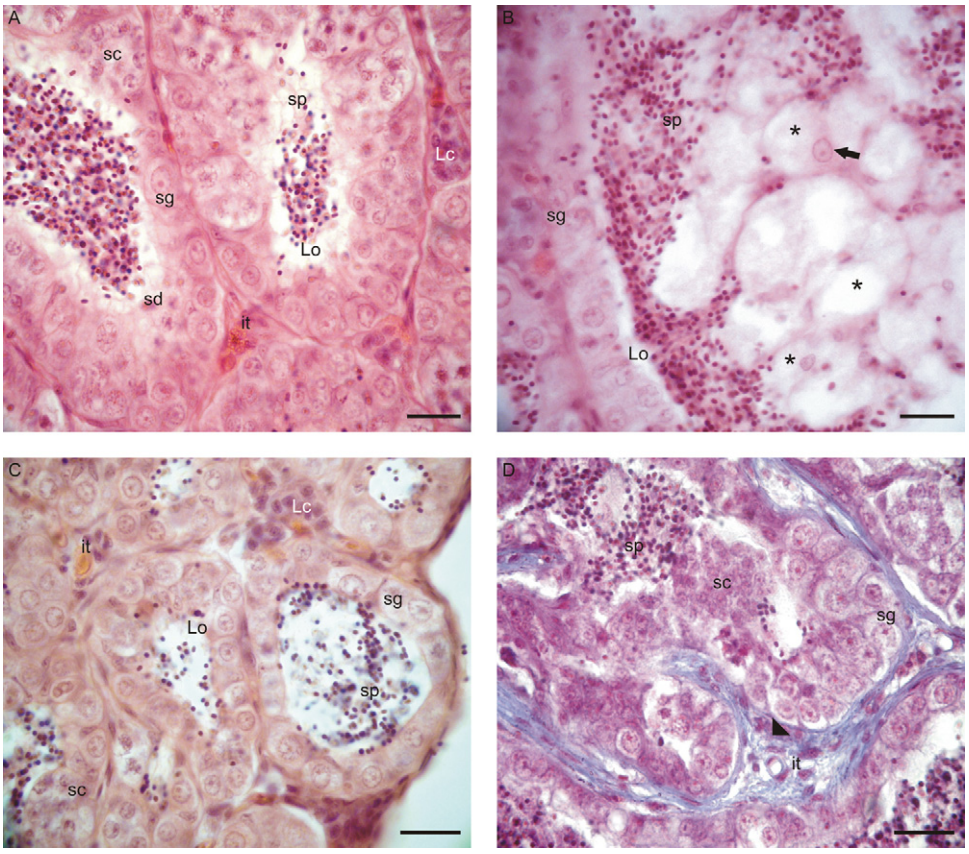

Fig. 4. Testis cross sections of ES-exposed *C. dimerus*. (A) Control fish. The normal cytoarchitecture of lobules (Lo) immersed in interstitial tissue (it) and Leydig cells (Lc) could be observed. Cysts (cy) containing all spermatogenesis stages were present: spermatocytes (spc), spermatids (spd), spermatogonia (spg) and a moderate amount of sperm (spz) within the lumen. (B) and (C) Males exposed to 0.3 μg/L ES. Pathologies observed include disarrangement of the lobular structure, an abnormal predominance of spermatozoa, scarce intermediate germ cell stages, presence of immature germ cells (arrow) and of foam cells (asterisk) in the lobular lumen. (D) Male exposed to 1 μg/L ES. In addition to the aforementioned alterations, interstitial fibrosis (arrow head) could be observed. Masson Trichrome staining; scale bar = 15 μm.

Pituitary gonadotropin levels have been shown to alter due to exposure to other organochlorine pesticides (Armenti et al., 2008; Beard and Rawlings, 1999; Singh and Singh, 1981). Evidence for the action of ES on the pituitary was revealed in ES-fed rats by a marked decrease in plasma levels of both LH and FSH (Singh and Pandey, 1990), as well as altered gene expression and serum levels of other pituitary hormones such as GH, prolactin and TSH (Caride et al., 2010). The present study showed lower βFSH content in pituitaries of ES-exposed fish than that of controls. Whether this translates into decreased release, and therefore decreased serum levels, remains unclear. Caride et al. (2010) found loss of linear positive correlation between gene expression and plasma levels of rat pituitary hormones with ES treatment, suggesting that synthesis and secretion might be differently affected by the pesticide. Additionally, the *in vitro* study did not show differences in βFSH release by cultured pituitaries upon ES exposure. Though the ES concentration tested *in vitro* (100 μM) could not have been enough to induce changes in gonadotropin release, preliminary results with a higher dose (500 μM) also showed no effect, indicating an indirect effect of ES on the pituitary upon exposure. On the other hand, no effect on synthesis or release of βLH was observed in *C. dimerus*. In accordance, Piazza et al. (2011) also found altered morphometry of FSH-producing gonadotrops but not of LH-synthesizing cells in larvae of the same species exposed to waterborne ES. Therefore, FSH appears to exhibit enhanced sensitivity than LH to the presence of the toxicant in this species.

In several teleosts, FSH not only regulates spermatogenesis *via* Sertoli cell function, but also exerts a steroidogenic function on Leydig cells (Kagawa et al., 1998; Kamei et al., 2005; Planas and Swanson, 1995), possibly related in part to the constitutive activity of LH receptors reported in some fish species (Kumar et al., 2001; Kwok et al., 2005; Vischer and Bogerd, 2003). Assuming a similar role of FSH as the driving force regulating testes function on *C. dimerus*, alterations in this gonadotropin by ES are more likely to lead to reproductive failure. Symptoms of altered spermatogenesis could be seen through analysis of testes histology of ES-exposed males. Dose-related alterations included release of immature germ cells into the lobular lumen and abnormal preponderance of sperm and spermatogonia over intermediate germ cell stages. Similar alterations were observed for acute exposure to ES (Da Cuña et al., 2011), and are indicative of impairment of spermatogenesis. Related reported effects regarding altered spermatogenesis caused by ES include disruption of testicular lobules and damaged Sertoli cells in bluegill fish *Lepomis macrochirus* (Dutta et al., 2006), reduced sperm motility and sperm count in rats (Choudhary and Joshi, 2003; Sinha et al., 2001).

Presence of foam cells in the lobular lumen and interstitial fibrosis constitute signs of degenerative or necrotic processes. Tissue injury by the presence of ES could lead to accumulation of cell debris including plasma membrane fragments, rich in cholesterol (Kisilevsky and Tam, 2003). With the consequent inflammatory infiltration that follows, macrophages ingest these fragments and acquire foam cells characteristics, whereas chemical messengers from inflammatory response cells are responsible for extracellular matrix elements production – collagen, glycoproteins, proteoglycans – that result in fibrosis (Highleyman and Franciscus, 2011).

Altered spermatogenesis evidenced in testes could be related to changes in Sertoli cell function. Sertoli cells play a critical

role in testicular function – *e.g.* germ cells support and nutrition, phagocytosis of residual bodies, inhibin secretion, regulation of spermatogenesis and spermiation. A useful Sertoli cell function marker in fish is the activity of the γ-glutamyl transpeptidase enzyme (γGT), given that it is primarily found in this cell type in the testes (Carreau et al., 1996; Christiansen et al., 2000). Different xenobiotics have been shown to cause alterations in Sertoli γGT activity in fish (Christiansen et al., 1998; Rasmussen et al., 2005; Sun et al., 2011). Consequently, reduced γGT activity in ES-exposed *C. dimerus* in the present study signals cell dysfunction. Though direct effects cannot be ruled out, decreased FSH levels induced by ES could be responsible for the decrease in Sertoli cell function, as this gonadotropin is the main regulator of γGT activity (Caston and Sanborn, 1988; Schteingart et al., 2002).

As FSH and FSH-mediated steroidogenesis regulate entry into meiosis and initiation of spermatogenesis (Abel et al., 2008; Ohta et al., 2007), a decrease in the number of intermediate germ cell stages (spermatocytes and spermatids) was to be expected in ES-exposed males considering not only the decrease in FSH pituitary content but also the inhibitory effect of ES on LH-stimulated steroidogenesis *in vitro*. In the Asian catfish (*C. batrachus*), waterborne ES also reduced the number of spermatocytes in male testes with the concomitant increase in number of spermatogonia. This effect was accompanied by decreased gene expression of 11βHSD2, 17βHSD and P450c17 in testes, though plasma testosterone and 11-KT levels were not affected (Rajakumar et al., 2012). In mammals, ES was capable of reducing the activity of the steroidogenic enzymes 3βHSD and 17βHSD in testes and hence the serum level of testosterone in rats (Singh and Pandey, 1990).

Taking together these results, ES exhibits an endocrine disruptive action on the reproductive axis of *C. dimerus*, causing disruption, whether directly or indirectly, at the pituitary level – FSH content – and/or at the testes level – Sertoli cell function, steroidogenesis. Planned further studies investigating gene expression of key factors involved in reproduction in fish would help better elucidate ES mode of action. These effects could acquire greater significance under prolonged exposure to the pesticide in nature.

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