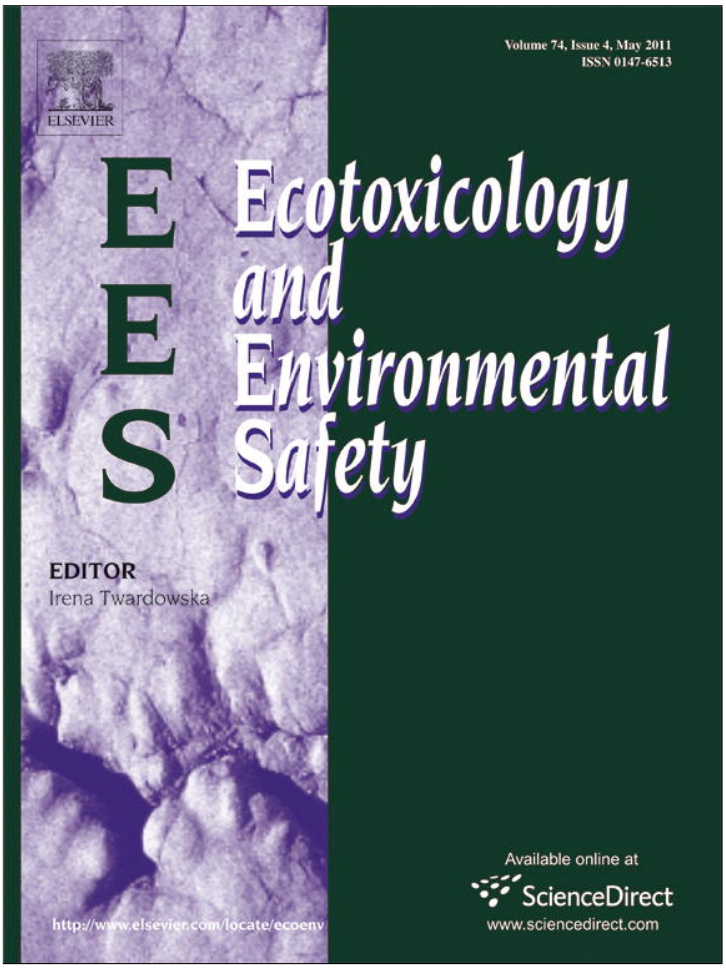

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Assessment of the acute toxicity of the organochlorine pesticide endosulfan in *Cichlasoma dimerus* (Teleostei, Perciformes)

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ABSTRACT

The organochlorine insecticide endosulfan (ES) is widely used despite its high toxicity to fish (96-h LC_{50} median value of 2.6 μg L^{-1}). This study aimed to assess the acute toxicity, histological and physiological parameters after exposure to 0; 0.25; 1; 2; 3; 4 and 16 μg L^{-1} ES for 96 h under semi-static conditions in a freshwater perciform fish, *Cichlasoma dimerus*. Prior to death, fish exhibited behavior indicative of neurotoxicity. No difference was found in brain AChE activity. A decrease in erythrocyte mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration in exposed fish suggests a state of anemia. Histological alterations observed in exposed fish included hyperplasia of the interlamellar epithelium, blood congestion in secondary lamellae, and mucous cells hyperplasia and hypertrophy in gills; pycnotic nuclei and hydropic degeneration in liver; testicular damage. These moderate pathological responses in major organs could become crucial during reproduction and under prolonged exposure periods.

1. Introduction

Based on the recognition of their long term negative impact on the global environment, the use of organochlorine pesticides in agriculture has been largely banned. However, many pesticides such as endosulfan (ES) are still widely employed in developing countries despite their discontinued or restricted use in European and North American countries. ES (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is a broad spectrum organochlorine insecticide of the cyclodiene subgroup, used to control insects and mites in crops of high commercial value (soy, cotton, tea, coffee, maize, fruits). Following application it can reach water bodies through surface runoff and air drift from nearby agricultural fields (Miglioranza et al., 2002) and cause adverse effects to non-target aquatic animals. Despite its low persistence in water, it is capable of associating to the organic matter present in both suspended and bottom particles, remaining in sediments for several years (Weber et al., 2010). Reported concentrations of ES α and β isomers in surface and ground water range from 0.05 to 2.5 μg L^{-1} (Dalvie et al., 2003; Leong et al., 2007). In Argentina, this pesticide has been detected in bivalves and fish at relatively high concentrations (Lanfranchi et al., 2006; Menone et al., 2000; Miglioranza et al., 2002) possibly due to bioaccumulation in fatty tissues (Jonsson and Toledo, 1993).

ES is a known neurotoxic and is very highly toxic to fish (US EPA Class I; US Environmental Protection Agency, 2002) with a reported 96-h LC_{50} median value of 2.6 μg L^{-1} for teleost fish (Kegley et al., 2009). Several negative effects have been reported as a result of ES exposure. Chronic effects on fish include oxidative damage (Ballesteros et al., 2009a), genotoxicity (Neuparth et al., 2006), damage to testes (Dutta et al., 2006), changes in circulating thyroid hormones (Coimbra et al., 2005) and alteration of acetylcholinesterase activity in the brain (Dutta and Arends, 2003). Lowered levels of testosterone and estradiol have been found in fish with elevated residues of ES (Singh et al., 2008). Other reported effects are reduced feeding behavior (Giusi et al., 2005), alterations on development (Willey and Krone, 2001), sexual and escape behavior, and reproductive physiology (Balasubramani and Pandian, 2008; Gormley and Teather, 2003).

One of the most frequently used biomarkers of exposure to pesticides in fish, in particular organophosphorus and carbamates,

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is the activity of the enzyme acetylcholinesterase (AChE). In the central nervous system of vertebrates, AChE degrades the neurotransmitter acetylcholine (ACh) into choline and acetic acid causing the termination of the synaptic transmission. Its inhibition by toxicants can lead to an excess of ACh levels resulting in neuromuscular paralysis and eventually death (Fulton and Key, 2001; Pope et al., 2005). Alterations in fish behavior caused by AChE inhibition upon pesticide exposure include decreased locomotor activity (Rao et al., 2005a; Sismeiro-Vivas et al., 2007), inability to capture prey (Gaworecki et al., 2009), and impaired swimming performance (Tierney et al., 2007).

The study of hematological changes can be used as a diagnostic tool to assess health status, since blood is an indicator of the physiological condition of animals. Hematological parameters can be altered due to environmental, nutritional and/or pathological stress. Several studies have used hematology as a biomarker of pesticide exposure, reporting decreased hemoglobin and hematocrit (Velisek et al., 2009), and changes in phagocytic activity (Girón-Pérez et al., 2008).

A sensitive tool to detect direct effects of chemical compounds on target organs of freshwater fish in laboratory experiments is the histopathological investigation of said organs following exposure (Schwaiger et al., 1997). Gills interact directly with the aquatic environment and are therefore useful for evaluating toxic effects (Hinton et al., 1987; Evans et al., 2005). The liver, due to its function in toxic substances biotransformation, is the major target organ for xenobiotics, and thus one of the most affected (Au, 2004). The spleen as one of the main immune organs in fish is also subject to suffer histomorphological changes due to the action of toxicants (Grinwis et al., 2000; Agius and Roberts, 2003). Alterations in the gonads can adversely affect the reproductive performance of fish, with implications in overall populations (Hinck et al., 2007; Scholz and Klüver, 2009).

The South American cichlid fish *Cichlasoma dimerus*, a perciform teleost, is common in 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 teleosts in the La Plata River basin and relevant to the Argentinean riverine ecosystems. *C. dimerus* 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) and acceptable survival rates, providing an amenable model for laboratory studies. This species has been successfully utilized in ecotoxicological testing (Moncaut et al., 2003; Rey Vázquez et al., 2009), prompting its inclusion 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).

Despite cichlids being the most species-rich non-Ostariophysan family in freshwaters worldwide (Kullander, 2003), few studies have dealt with the toxic effect of ES on fishes of this group. Therefore, the aim of the present study was to determine the acute toxicity and to assess various histological, physiological and biochemical parameters representative of the exposure to ES in the native freshwater cichlid species, *C. dimerus*.

2. Materials and methods

2.1. Animals

Adult fish of the native freshwater species *Cichlasoma dimerus* were captured in Esteros del Riachuelo, Corrientes, Argentina (27°35′S 58°45′W), and held in 100 L well aerated aquaria with external filtration and a layer of gravel on the bottom, with filtered tap water at 25 ± 1 °C, pH 7.3, and 14:10 h photoperiod until the time of the experiment. Fish were allowed to acclimate to laboratory conditions for a month prior to the start of experimentation. During the acclimation period fish were fed daily with pelleted commercial food (Tetra food® sticks). Guidelines on the care and use of fish in research and testing from the Canadian Council on Animal Care (2005) were followed.

2.2. Acute toxicity test

Sixty three fish (mean weight ± SD = 21.2 ± 1.5 g; mean standard length ± SD = 7.5 ± 0.2 cm) were randomly transferred to bare 20 L aquaria for one week. 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, they were exposed to ES nominal concentrations of 0, 0.25, 1, 2, 3, 4 and 16 μg L^{-1} for 96 h under semi-static conditions. 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 daily for 4 days. Fish were not fed during the experiment. Mortality was recorded at 24, 48, 72 and 96 h. Median lethal concentrations (LC_{50}) were estimated for 24, 48, 72 and 96 h by Probit transformation of the mortality dose curve (Finney, 1952). At the end of the 96 h exposure period, surviving fish were anesthetized with Fish Calmer (active ingredients: acetone, dimethylketone alpha methyl quinoline, Jungle Laboratories, Cibolo, TX, USA) and processed. The actual concentrations of ES were measured by gas chromatography-electron capture detector (GC/ECD, EPA SW-846 M8081A; US Environmental Protection Agency, 1996).

2.3. Brain AChE activity

The activity of AChE was determined following the colorimetric assay described by Ellman et al. (1961). Brains were homogenized in 20 mM Tris–HCl buffer (pH 7.5), 1% Triton X, and 0.5 mM EDTA (5 mL buffer per 1 g tissue). Homogenates were centrifuged at 10,000*g* for 25 min at 4 °C. Supernatants were used to measure activity of the enzyme by incubating 15 μL of the sample at 25 °C with 1.2 mL of a 0.2 mM solution of the reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and 0.1 mL of a 10 mM solution of the substrate acetylthiocholine iodide. Absorbance was measured at 412 nm for 2 min (Shimadzu UV-1603, Shimadzu Corporation, Japan). The assay is based on the reaction of DTNB with thiocoline—product of the degradation of the substrate acetylthiocoline by AChE—which forms 5-thio-2-nitrobenzoic acid characterized by strong yellow coloring. Each homogenate was run by duplicate, and enzymatic activities were calculated relative to the total amount of protein in the sample, as measured by the method described by Lowry et al. (1951).

2.4. Hematological parameters

Peripheral blood was collected by puncture of the caudal vein using a heparin-coated 25 gauge × ½″ in needle attached to a 1 mL syringe (Terumo®, Terumo Medical Corporation, NJ, USA). Glucose levels were estimated using a blood glucose meter (Accu-Chek® Performa, Roche Diagnostics, Switzerland). An aliquot of blood was diluted 1:200 with 0.4% formaldehyde and 3% trisodium citrate, to determine the red blood cell count (RBC) in a Neubauer counting chamber (hemocytometer). The hematocrit value or packed cell volume (PCV) was determined by centrifuging the blood in a capillary or micro-hematocrit tube at 12,000 rpm for 5 min (Routine RM24 microcentrifuge, Argentina). The hemoglobin concentration (Hb) was obtained using the cyanmethaemoglobin method (hemogloWiener® reactive, Wiener Lab., Argentina); blood was diluted (1:250) in the provided buffer, centrifuged to remove dispersed nuclear material, and optical density was measured at 540 nm (Shimadzu UV-1603, Shimadzu Corporation, Japan). Erythrocyte mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Seiverd (1964): MCV (fL L^{-1}) = PCV (L L^{-1})/RBC (cell L^{-1}); MCH (pg) = Hb (g L^{-1})/RBC (cell L^{-1}); MCHC (g dL^{-1}) = Hb (g L^{-1})/PCV (L L^{-1}).

2.5. Histological analysis

Gills (second left arch), liver, spleen and gonads were dissected, weighed, fixed in Bouin's solution for 24 h at room temperature, dehydrated, embedded in paraffin (Paraplast®, Fisher, WA, USA), and transversally sectioned at 7 μm (Leitz Wetzlar 1212 Leica microtome, Germany). Sections were stained with Masson's trichrome, Hematoxilyn-Eosin, Periodic Acid Schiff (PAS) or Alcian Blue (AB) stains. Photographs were taken in a Nikon Microphot FX microscope coupled with a Coolpix 5400 digital camera (Nikon, Japan). Area and number per section per total organ area of melanomacrophage centers (MMC) were measured in spleen serial sections (Image Pro Plus® 4.5 software, Media Cybernetics).

2.6. Statistical analysis

One way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons, was used to establish differences between weight, length, somatic indices (gonad, liver and spleen), hematological parameters, AChE activity, and number and area of MMCs (nested design) between control and ES exposed fish (Statistica 7.0, StatSoft, Inc., 2004). Comparison of LC_{50} values was achieved using the statistic proposed by APHA et al. (1992). Differences were considered significant at $P<0.05$.

3. Results

3.1. Acute toxicity

The results of the acute toxicity test for the pesticide ES on *C. dimerus* are presented in Table 1. The 96-h LC50 value obtained for this species was 3.34 $\mu g\,L^{-1}$ (confidence interval 2.97–3.80 $\mu g\,L^{-1}$). As expected, the LC_{50} values decreased with the time of exposure. The LC_{50} at 24 h was significantly higher than at all other exposure times evaluated ($P<0.05$), which did not differ statistically between them. No control fish died during the toxicity test. Prior to death fish exhibited hypo-activity, erratic swimming, loss of balance, hyper-excitability and darkening of the skin.

At the end of the experiment, no differences were found on weight, length or somatic indices between surviving ES exposed and control fish. The concentrations measured by GC were 0.23; 0.96; 1.87; 2.80; 3.74 and 14.94 $\mu g\,L^{-1}$, showing recoveries higher than 90% of the nominal value; the ratio α:β corresponded to a 70:30 proportion.

Table 1
Median lethal concentrations (LC_{50}) of ES ($\mu g\,L^{-1}$) at 24, 48, 72 and 96 h for *C. dimerus*.

Time of exposure (h)	LC_{50} ($\mu g\,L^{-1}$)	Confidence interval	
		Lower limit	Upper limit
24	13.60[a]	9.90	18.92
48	4.98[b]	4.03	13.75
72	3.90[b]	3.40	5.58
96	3.34[b]	2.97	3.80

Different letters denote significant differences ($P<0.05$).

Table 2
Analysis of brain AChE activity in control and ES exposed *C. dimerus*. Data expressed as mean ± SD. $P>0.05$; no significant differences between tested concentrations.

ES concentration ($\mu g\,L^{-1}$)	Brain AChE activity (μ mol mg prot^{-1})
Control	0.22 ± 0.03
0.25	0.24 ± 0.01
1.00	0.25 ± 0.01
2.00	0.24 ± 0.02
3.00	0.26 ± 0.05
4.00	0.25 ± 0.02

3.2. Brain AChE activity

No significant differences in brain AChE activity in control and ES exposed *C. dimerus* were observed at any concentration tested (Table 2).

3.3. Hematological parameters

Hematological parameters measured for control and ES exposed fish are summarized in Table 3. No statistical differences were found for PCV, RBC, Hb, and glucose between experimental groups. Though not statistically different due to a large inter-individual variation between ES exposed fish, glucose levels tended to be higher for exposed fish, except for the highest ES concentration tested. Fish exposed to ES concentrations of 2 $\mu g\,L^{-1}$ and/or higher showed a decrease in MCV (3 and 4 $\mu g\,L^{-1}$), MCH (2, 3 and 4 $\mu g\,L^{-1}$) and MCHC (2 and 3 $\mu g\,L^{-1}$) values when compared to control fish.

3.4. Histological analysis

3.4.1. Gills

At the light microscopy level, gills of control *C. dimerus* showed the typical filaments or primary lamella with a central cartilaginous support, afferent and efferent arterioles, and a thin epithelial covering of no more than three strata. The thin simple epithelial covering of the secondary lamellae laid on a basement membrane supported by pillar cells. Blood elements could be seen in the spaces between pillar cells or lacunae. Other cell types found on primary and secondary lamellae included vacuolated mucous cells (positive PAS and AB reaction), macrophages, chloride cells, and mast cells (Fig. 1A, B). Hyperplasia of the interlamellar epithelium and blood congestion in secondary lamellae were observed in fish exposed to 2 $\mu g\,L^{-1}$ ES or higher (Fig. 1C). Both PAS (not shown) and AB stainings revealed mucous cells hyperplasia and hypertrophy in treated fish (Fig. 1D).

3.4.2. Liver

Under light microscopy, the liver tissue of control *C. dimerus* was composed of hepatocytes supported by lattice fibers, located around blood sinusoids and organized in poorly defined cord-like structures. This species possesses a hepatopancreas, with pancreatic acinar tissue interspersed with the hepatic tissue, mainly around the major blood vessels, a common feature among certain teleost families. In control fish, the hepatic cells had a polyhedric

Table 3
Hematological parameters of *Cichlasoma dimerus* under ES exposure. Values are expressed as mean ± SD.

	ES Concentration ($\mu g\,L^{-1}$)					
	Control	0.25	1.0	2.0	3.0	4.0
RBC ($\times 10^6\,\mu L^{-1}$)	2.5 ± 0.6	2.5 ± 0.2	2.6 ± 0.1	3.0 ± 0.8	3.5 ± 0.7	3.7 ± 1.4
PCV (%)	30.2 ± 6.3	33.3 ± 1.5	32.7 ± 1.1	33.0 ± 6.9	32.5 ± 6.4	37.0 ± 7.1
Hemoglobin ($g\,dL^{-1}$)	6.3 ± 1.9	6.0 ± 0.8	5.6 ± 1.2	4.6 ± 0.8	4.8 ± 1.2	4.9 ± 0.2
MCV (fL)	123.0 ± 5.2	130.9 ± 4.9	127.1 ± 6.8	112.1 ± 9.5	101.8 ± 4.9*	98.6 ± 1.4*
MCH (pg)	25.1 ± 2.2	23.4 ± 1.2	21.9 ± 5.3	15.9 ± 1.7*	13.9 ± 1.5*	13.8 ± 4.7*
MCHC ($g\,dL^{-1}$)	20.5 ± 2.4	17.9 ± 1.4	17.3 ± 4.3	14.2 ± 0.5*	13.0 ± 0.8*	14.1 ± 5.0
Glucose ($mg\,dL^{-1}$)	67.0 ± 5.6	134.3 ± 48.6	102.0 ± 74.2	102.3 ± 28.9	110.3 ± 8.5	56.5 ± 13.4

* Significantly different than control fish ($P<0.05$).

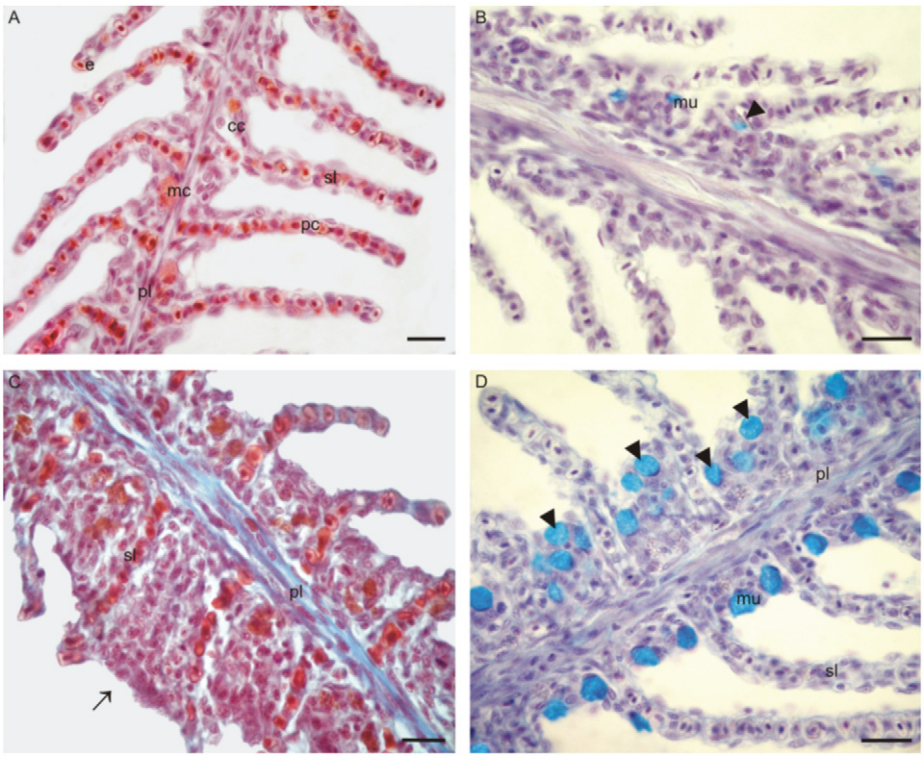


Fig. 1. Gills cross sections of *C. dimerus*. (A) and (B) Control fish. The typical organization of the gills can be seen: primary lamella (pl), secondary lamella (sl), pillar cells (pc), mast cells (mc), chloride cells (cc), erythrocytes (e) and vacuolated mucous cells (mc; arrowheads). (C) and (D) Fish exposed to 2 $\mu g\,L^{-1}$ ES. Hyperplasia of the interlamellar epithelium (arrow) and an important mucous cells hyperplasia and hypertrophy (arrowheads) can be observed. (A) and (C) Masson Trichrome staining. (B) and (D) Alcian Blue staining. Scale bars = 15 μm.

shape with a basal nucleus and usually one nucleolus. The nuclear membrane was weakly basophilic, while the cytoplasm had primarily a vacuolated aspect and contained eosinophilic material (Fig. 2A). Melanomacrophage centers (MMC) could be seen near blood vessels, and stained positive with PAS (not shown) and AB (Fig. 2A, inset). In exposed fish, pycnotic nuclei, indicating a necrotic process, and hydropic degeneration were observed at 2 $\mu g\,L^{-1}$ ES and upwards (Fig. 2B).

3.4.3. Spleen

The spleen of control *C. dimerus* was composed of blood vessels, red and white pulp, MMCs and ellipsoids. The red pulp, an extensive, interconnecting system of splenic cords – a mesh of fibroblast-like cells and blood cells (erythroid cells and thrombocytes) – and sinusoid capillaries, comprised most of the parenchyma of the organ. White pulp consisted of lymphoid cells surrounding arterial vessels, MMCs and ellipsoids, or forming small clusters. The number per section per total organ area, and the cell area of MMCs showed no difference between control and exposed fish (Table 4). Ellipsoids, which are periarterial sheaths of macrophages and fibrocytes at the end of splenic arterioles, were also observed in all experimental groups. Control and exposed fish showed similar cytoarchitecture (Fig. 2C).

3.4.4. Gonads

Testes of control *C. dimerus* were a pair of elongated structures composed of branching seminiferous lobules lined by the germinal epithelium, embedded in the stroma. The thin-walled lobules contained isogenic germ cells enclosed by Sertoli cells forming cysts (unrestricted lobular testis type). In testes of control fish, all stages of spermatogenesis were present. Testicular lobules contained spermatogonia as well as numerous cysts with early (spermatocytes) and late (spermatids) stages of spermatogenesis. Mature sperm was usually observed in the lobular lumen, with spermatogonia and cysts of spermatocytes remaining in the lobule walls. Stroma consisted of connective tissue, blood vessels and Leydig cells (Fig. 3A). Males exposed to a concentration of 2 $\mu g\,L^{-1}$ ES and higher showed an abnormal predominance of sperm. Release of immature germ cells into the lobular lumen (Fig. 3B) and presence of aggregative foam cells in the lumen (Fig. 3C) could be observed in fish exposed to 4 $\mu g\,L^{-1}$ of ES.

The ovary, as in all teleost fishes, was a hollow sac-like organ into which extended numerous ovigerous folds lined by germinal epithelium, comprised of oogonia, primary or pre-vitellogenic oocytes, and secondary or vitellogenic oocytes, surrounded by follicular cells. Ovaries of ES exposed females did not differ histologically from those of control fish (Fig. 3D)

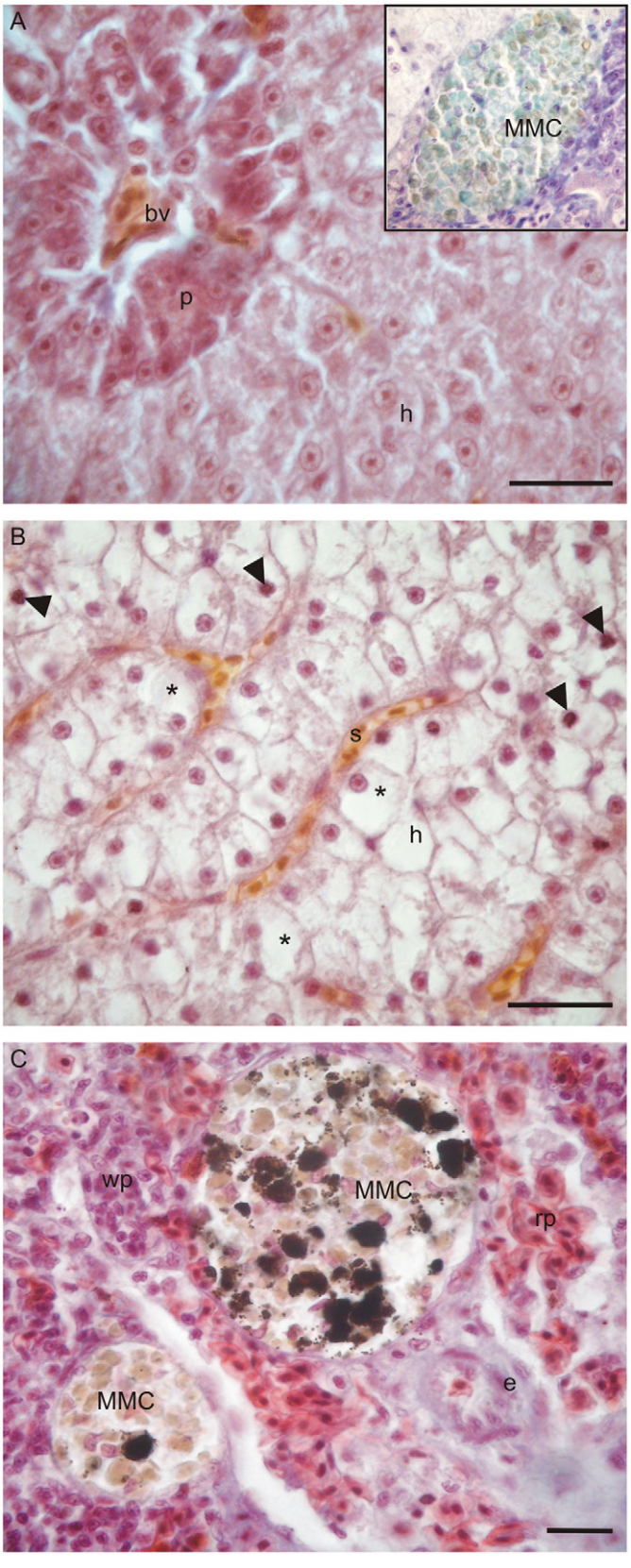
4. Discussion

4.1. Acute toxicity

Reported 96-h LC_{50} values for ES under static renewal conditions for other teleost species cover a wide range, from 0.42 $\mu g\,L^{-1}$ for the Asian swamp eel (*Monopterus albus*, Siang et al., 2007) to 41 $\mu g\,L^{-1}$ for the European eel (*Anguilla anguilla*; Ferrando et al., 1991), with a median value of 2.6 $\mu g\,L^{-1}$ (Kegley et al., 2009). According to these data, ES classifies as a very highly toxic substance to fish (Kegley et al., 2009). The 96-h LC_{50} for the perciform *C. dimerus* reported in the present study (3.34 $\mu g\,L^{-1}$; Table 1), is in agreement with the aforementioned results for other freshwater fish orders. In addition, in terms of its acute toxicity, *C. dimerus* could be considered a sensitive species to ES.

Prior to death, exposed fish exhibited a suppression of spontaneous locomotor activity, hyper-excitability to external stimuli,

darkening of the skin, spasms and scoliosis. Fish would remain on their side on the bottom of the aquaria and occasionally swim rapidly and erratically around it, colliding with the tank walls. When compared to other non-symptomatic fish, a higher frequency of opercular movements could be observed in dying fish.



The symptoms exhibited correspond to the physical deformity syndrome (PDS), an indicator of neurotoxicity described by Drummond and Russom (1990) in fathead minnows (*Pimephales promelas*) exposed to several toxic agents. The exact pathway that leads ES to exert its neurotoxic effect on fish is still unknown. ES exposure was found to upregulate histamine receptors subtypes 3 and 1 in localized areas of the brain (preoptic and hypothalamic areas) involved predominantly on endocrine dependent activities in the ornate wrasse (*Thalassoma pavo*) leading to a higher hyperventilation activity and decreased feeding behavior (Giusi et al., 2005). The primary site of action of some cyclodienes insecticides, including ES, is the interference with GABA receptors in the brain of target insects (Bloomquist, 2003). An inhibition of the GABA-induced Cl flux was also observed in cultured cerebellar granule cells of mice treated with αES, probably due to binding to picrotoxinin sites (Vale et al., 2003), so it is possible that the neurotoxic effect of ES in fish also follows this pathway.

4.2. Brain AChE

One way toxicants can induce a PDS is through the inhibition of acetylcholinesterase (AChE) (Drummond and Russom, 1990). This enzyme is involved in several key optomotor behaviors of fish, including locomotion, orientation, feeding, and predator evasion (Scott and Sloman, 2004). Reduction of brain AChE enzyme activity leads to an accumulation of acetylcholine in the brain tissue, interfering with energy metabolism in the nervous system and preventing transmission of nerve impulses, thereby causing behavioral alterations (Dutta et al., 1992; Rao et al., 2005b). We did not find an inhibition of this enzyme in ES exposed fish in this study (Table 2). Similarly, Ballesteros et al. (2009b) reported that brain AChE did not differ in onesided livebearer (*J. multidentata*) between control and fish subjected to a sublethal ES exposure, suggesting the neurotoxic action of the pesticide follows other pathways. In the same study, muscle AChE was susceptible of being inhibited by ES, and could therefore be responsible for some of the locomotor symptoms described in the present study through muscular dysfunction. However, Dutta and Arends (2003) and Sarma et al. (2010) reported brain AChE inhibition caused by exposure to high concentrations of ES for 96 h in bluegill sunfish (*Lepomis macrochirus*) and spotted murrel (*Channa punctatus*), respectively, so involvement of this enzyme in the neurotoxic effect of ES cannot be ruled out.

4.3. Hematological parameters

The values for the hematological parameters obtained in this study for control fish (Table 3) were in agreement with those previously reported for this species (Rey Vázquez and Guerrero, 2007).

Hematology has been used in recent years as a convenient and easy method to screen the effects of pesticides in fish, although these parameters can be highly variable owing to the influence of various intrinsic and extrinsic factors (Groff and Zinkl, 1999). One of the most reported consequences is that of an anemic state

Fig. 2. Liver and spleen cross sections of *C. dimerus*. (A) Control fish. The liver is composed of polyhedric hepatocytes (h) with a basal nucleus, arranged around sinusoids (s), and interspersed with pancreatic tissue (p) associated to blood vessels (bv). The cytoplasm showed a vacuolated aspect. Inset: Detail of a melanomacrophage center (MMC) within the liver parenchyma. (B) Fish exposed to 2 μg L^{-1} ES. Hydropic degeneration (asterisks) and pycnotic nuclei (arrowheads) of hepatocytes (h) were observed. (C) Detail of spleen from a control fish showing MMC, red pulp (rp), white pulp (wp), and ellipsoids (e). The spleen of exposed fish did not differ from control *C. dimerus*. (A, B, C) Masson Trichrome staining; scale bar=15 μm. Inset: Alcian Blue staining.

Table 4
Melanomacrophage centers area and number (relative to total organ area) in spleen of control and ES-exposed fish. Values are expressed as mean ± SD. $P > 0.05$; no significant differences between tested concentrations.

	ES concentration (μg L^{-1})					
	Control	**0.25**	**1.0**	**2.0**	**3.0**	**4.0**
Area (μm)	242 ± 24	191 ± 17	196 ± 18	198 ± 15	152 ± 15	178 ± 12
Number	8.8 ± 1.6	9.2 ± 2.5	8.6 ± 3.0	13.2 ± 7.2	15.0 ± 2.0	14.8 ± 1.4

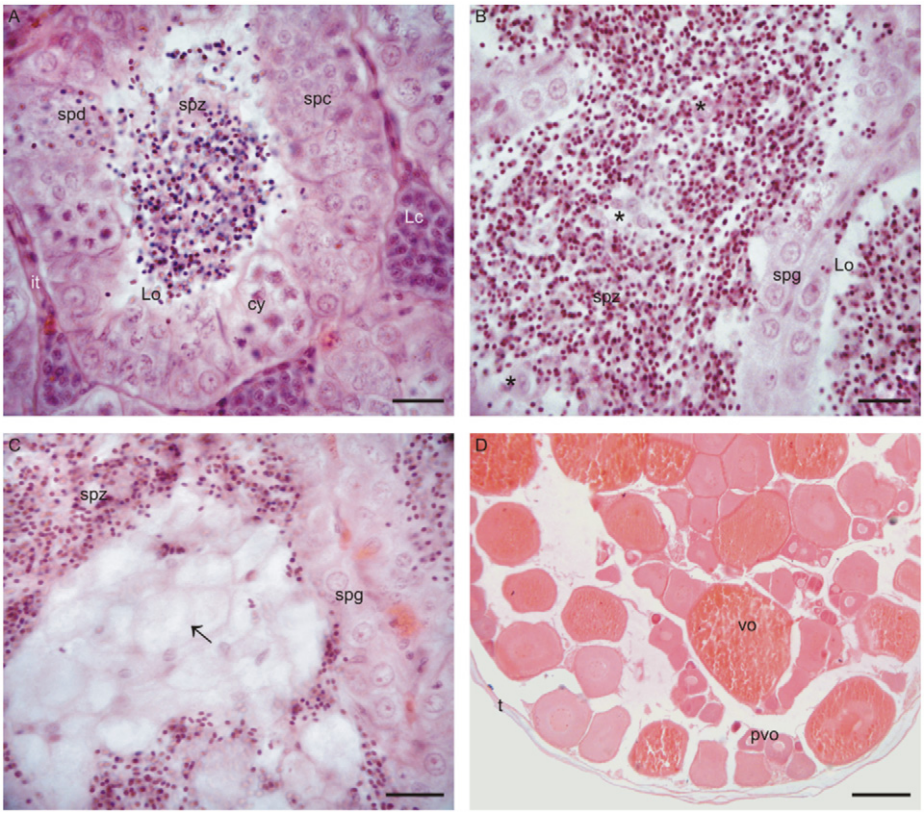


Fig. 3. Testis cross sections of *C. dimerus*.(A) Control fish. This species possesses an unrestricted lobular testis type. The normal cytoarchitecture of lobules (Lo) immersed in interstitial tissue (it) and Leydig cells (Lc) could be observed. Cysts (cy) containing all spermatogenesis stages were present: spermatocytes (spc), spermatids (spd), spermatogonia (spg) and a moderate amount of sperm (spz) within the lumen. (B and C) Males exposed to 4 μg L^{-1} ES. Pathologies observed include disarrangement of the lobular structure, an abnormal predominance of spermatozoa, scarce intermediate germ cell stages, presence of immature germ cells (asterisk) and of foam cells (arrow) in the lobular lumen. (D) Female exposed to 4 μg L^{-1} ES. Ovaries were surrounded by an interstitial theca (t) and contained pre-vitellogenic (pvo) and vitellogenic oocytes (vo). No alterations were observed in ES treated fish. (A, B and C) Masson Trichrome staining; scale bar=15 μm. (D) Hematoxylin-Eosin staining; scale bar=250 μm.

following exposure. *C. dimerus* exposed to ES showed a decrease in the size and hemoglobin content of erythrocytes, as measured by the MCV, MCH and MCHC indices, which would signal a state of microcytic, hypochromic anemia (Table 3). This condition can be produced by any number of stressors, from bacterial infection (Silveira-Coffigny et al., 2004) to agrochemicals exposure (Mikula et al., 2008). ES also caused decreased erythrocyte count, hemoglobin content and hematocrit values in carp (*C. carpio*, Jenkins et al., 2003) and swamp eel (*M. albus*, Siang et al., 2007). In addition, this pesticide had an immunosuppressive effect in Nile tilapia (*O. niloticus*), reducing the phagocytic index and the percentage of active phagocytic cells in peripheral blood (Girón-Pérez et al., 2008); though no changes in hematological parameters were reported. An anemic status in fish could lead to a higher susceptibility to infection, and diminished feeding and breeding behaviors.

Stress related physiological changes include osmotic disturbances, increase in the concentration of energetic substrates and in the activity of certain enzymes, decrease in humoral immune factors (Wendelaar Bonga, 1997). An increase in blood glucose in a fish submitted to a stressor, such as exposure to waterborne toxicants, is due to the elevated energetic demand by the organism (Morgan and Iwama, 1997), and is a widely reported consequence of stress in fish (Abreu et al., 2009; Miller et al., 2009). Though not statistically significant, a notable increase in blood glucose could be observed in most exposed fish, even at the lowest concentration of ES. Differences in glucose levels were evident between exposed fish, with some showing a marked increase in glucose whereas others exhibited similar levels than control fish.

In conclusion, ES acted as a stressor in *C. dimerus*, inducing a state of anemia (as evidenced by the decrease in MCV, MCH and

MCHC indices) and, in some individuals, an increase in blood glucose levels.

4.4. Histological analysis

4.4.1. Gills

The fish gill is a multifunctional organ involved in a wide variety of basic functions, including oxygen uptake, carbon dioxide release, osmoregulation, acid–base regulation, nitrogen excretion, hormone metabolism, and sensing. Gills consist of thin filaments which help increase the surface area for gas and ion exchange. A large respiratory surface area means increased ion and water fluxes over the gills, which result in considerable osmoregulatory costs, and it makes the fish more accessible to toxic substances and pathogens. Due to its constant and intimate contact with water, gills are one of the most sensitive organs to the actions of pollutants. Injury to fish gills can result in a reduction of the oxygen consumption and disruption of the osmoregulatory function. Some pathologies observed in rainbow trout (*O. mykiss*) exposed to ES were lifting of the lamellar epithelium, distal hyperplasia of lamellas and fusion of lamellas (Altinok and Capkin, 2007). In addition to extensive epithelial lifting and hypertrophy of lamellar epithelium, an increase in the thickness of secondary lamella was proved in onesided livebearer (*J. multidentata*, Ballesteros et al., 2007). Though the changes were not widespread, *C. dimerus* also responded to the presence of ES with hyperplasia of the interlamellar epithelium (Fig. 1C). This alteration – which could lead to fusion of lamella–, as well as hypertrophy or hyperplasia of the lamellar epithelium – evidenced by an increase in lamellar thickness – are widely reported defense responses of fish to increase the respiratory diffusion distance and therefore prevent toxicants from reaching the blood stream. However, these strategies also prevent the normal diffusion of oxygen, thus fish have to reach equilibrium between protection against toxicants and normal physiological needs to be able to tolerate toxicants.

Proliferation of mucus cells and increased mucus secretion are also reported responses to stress and the presence of toxics or pathogens in the environment (Cengiz and Ünlü, 2002). Based on gill histology, our results (mucus cells hyperplasia and hypertrophy) also suggest that mucus secretion is increased in *C. dimerus* exposed to ES (Fig. 1D).

4.4.2. Liver

The exposure of fish to even sublethal concentrations of chemical contaminants can induce damage to different organs. The major functions of the liver involve protein, lipid and carbohydrate metabolism, as well as detoxification of foreign substances. However, elevated concentrations of these compounds can overwhelm hepatic detoxification, which could lead to structural damage. Several studies have dealt with liver alterations in fish caused by aquatic pollutants (Fishelson, 2006; Miranda et al., 2008). In mosquitofish (*G. affinis*) exposed to Thiodan® (33.7% ES) a significant dose and time-dependent increase in liver hypertrophy, dilatation of sinusoids and hepatocytes vacuolization was observed (Cengiz et al., 2001). Increased hepatocyte vacuolization and eosinophil granular cell aggregates were also reported after ES dietary exposure in Nile tilapia (*O. niloticus*, Coimbra et al., 2007). Similar vacuolar distrophy and hypertrophy of hepatocytes was the result of the exposure to sublethal concentrations of ES in rainbow trout (*O. mykiss*, Altinok and Capkin, 2007). In onesided livebearer (*J. multidentata*) exposed to ES, liver alterations included hydropic degeneration, sinusoids dilation and necrosis (Ballesteros et al., 2007). Additionally, rainbow trout exposed to ES for 96 h presented spleen, liver and kidney necroses (Capkin et al., 2006). In keeping with these results, in the present work, *C. dimerus* exposed for 96 h to ES also exhibited signs of hepatotoxicity, such as necrosis and hydropic degeneration of hepatocytes (Fig. 2B).

4.4.3. Spleen

In teleost fish, the spleen, along with the kidney, serves as one of the primary hematopoietic organs, and constitutes a major site of phagocytosis of particulate matter and decaying blood cells. As fish have no lymph nodes, the spleen alone plays an essential role in antigen trapping. Unlike that of mammals, the spleen of fish has no clear distinction between red and white pulp (Genten et al., 2009). MMCs are a characteristic immune structure in the spleen of teleosts, also found in liver and kidney, with phagocytic function and containing varying amounts of pigments (melanin, hemosiderin, and lipofuscin). Stressed and unhealthy fish tend to have more and larger MMCs in the spleen, though size and number can also increase with fish age (De Vico et al., 2008). ES exposure caused oxidative stress in macrophages of spleen in Nile tilapia (*O. niloticus*) (Tellez-Bañuelos et al., 2009), and exudate and necrosis in white pulp and larger number and size of MMC in rainbow trout (*O. mykiss*) (Altinok and Capkin, 2007). In the present study, no histological alterations, including no variations in the number and area of MMCs, were observed in an acute exposure to ES in spleen of *C. dimerus* (Fig. 2C; Table 4).

4.4.4. Gonads

The histological examination of the testes of ES-exposed male fish revealed dose-related testicular damage. Exposure to concentrations up to $1\ \mu g\ L^{-1}$ did not produce apparent effects assessed at the light microscopic level, whereas exposure to $2\ \mu g\ L^{-1}$ ES and upward resulted in an increase in the amount of sperm found, and in the release of immature germ cells into the lumen of lobules (Fig. 3B, C). These changes are indicative of impairment of spermatogenesis and possibly of testis functionality. In addition to the effects observed in *C. dimerus*, disruption of lobules and damaged Sertoli cells were also reported as a result of ES exposure in bluegill fish (*L. macrochirus*, Dutta et al., 2006). Though no other studies on fish testes are available, related effects regarding an alteration of spermatogenesis upon ES exposure have also been reported for mammals (rats), including reduction of testosterone levels in plasma, reduced spermatozoa motility and diminished sperm count in cauda epididymis (Choudhary and Joshi, 2003; Sinha et al., 2001). The mechanisms whereby ES causes the effects observed on the testes of *C. dimerus* are not clear and need further investigation. Some preliminary results of our work group indicate an endocrine disruptive effect of ES on the hypothalamus–pituitary–gonad axis in larvae of this species during development (Piazza et al., 2010).

In summary, histological examination of gills, liver and gonads revealed several pathologies caused by the exposure to ES. Gills exhibited hyperplasia of the interlamellar epithelium, as well as hypertrophy and hyperplasia of mucus cells, as general responses to prevent the toxicant from reaching the circulation. Necrosis and hydropic degeneration of hepatocytes in liver were indicative of hepatotoxicity. The alterations observed in testes, including the release of immature germ cells into the testicular lumen, the presence of foam cells, and an increase in the amount of sperm in the lobules, suggest that ES is able to impair testis function in *C. dimerus*.

5. Conclusion

This study combined different approaches (histology, physiology, cell biology) to achieve an integrative analysis of ES exposure in

freshwater fish, useful for assessing possible environmental risk to fish species. The changes found in ES exposed fish could be considered moderate pathological responses. The intensity of the histological and physiological alterations found was not of such a degree that the function of any vital organ could be seriously affected. However, such pathological changes in these major organs could become crucial during reproduction and under prolonged exposure periods.

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