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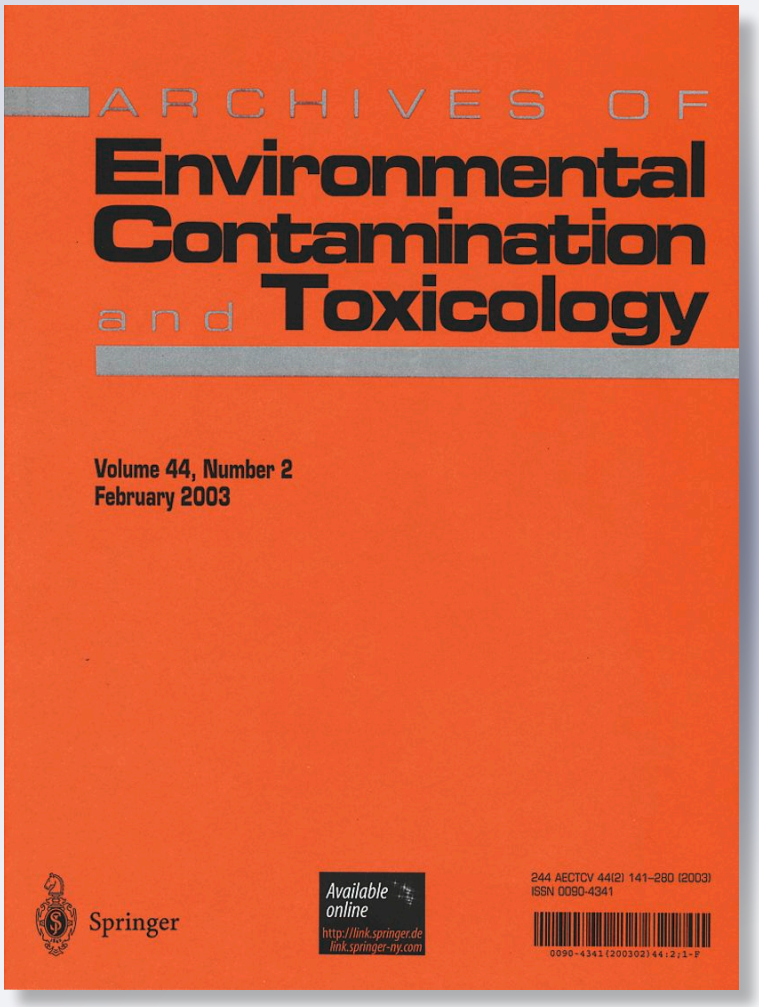

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Biochemical Stress Responses in Tissues of the Cichlid Fish *Cichlasoma dimerus* Exposed to a Commercial Formulation of Endosulfan

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Abstract Median lethal concentration (LC_{50}) and sublethal effects of the commercial endosulfan formulation Zebra Ciagro® on the fish *Cichlasoma dimerus* were studied. The 96-h LC_{50} was estimated as 17.7 μg/L. In order to investigate sublethal effects, fish were exposed to 25% and 50% LC_1 (3.4 and 6.8 μg/L, respectively). Endosulfan (ED) significantly increased the hemoglobin concentration and white blood cell count after 96 h. Differential leukocytes count was also altered, due to an increase in the percentage of neutrophils in exposed fish. The hepatopancreatic tissue of fish under ED treatment showed a decrease in aspartate aminotransferase and alanine aminotransferase and an increase in alkaline phosphatase. Lipid peroxidation levels in the 6.8-μg/L ED-containing group were higher than those in control fish for all organs tested (gills, hepatopancreas, and brain).

Non-point-source pesticide pollution from agricultural areas is widely regarded as one of the greatest causes of surface waters contamination (Loague et al. 1998; Schulz 2004). Endosulfan (ED) (6,7,8,9,10,10-hexachloro-1,5,5*a*,6,9,9*a*-hexahydro-6,9-ethano-2,4,3-benzodioxathiepin-3-oxide) is a nonsystemic insecticide and acaricide that acts by ingestion and contact. It is used to control sucking, chewing, and boring insects on a very wide range of crops, including fruits, cotton, tea, coffee, rice, cereals, oilseed crops, sugar cane, tobacco, alfalfa, mushrooms, and ornamental plants. In addition to its agricultural use, ED is used as a wood preservative and to control home and garden pests (EFSA 2005). The active ingredients of the technical ED used for commercial product formulations consist of a mixture of α- and β-endosulfan isomers in a ratio of 2:1 to 7:3 (Wan et al. 2005). ED is moderately persistent on aquatic systems and wild fish (Naqvi and Vaishnavi 1993).

Due to its toxic effects, this insecticide has been classified by the US Environmental Protection Agency (USEPA 2002) as highly toxic to both marine and freshwater fish. Although it has been phased out in most countries of the European Union and North America, it is still being used in tropical and subtropical regions (EFSA 2005). Data for ED in the southern hemisphere are limited to a smaller number of studies compared to the northern hemisphere. In Argentina, ED is one of the remaining organochlorine pesticides registered and widely used for control of a large spectrum of insect pests (Miglioranza et al. 2003). ED concentrations from 0.2 to 13.5 μg/L have been found on water bodies near rice fields in neotropical wetlands and concentrations from 0.1 to 0.7 μg/L have been found on mountain rivers (Baudino et al. 2003; Silva et al. 2005). These concentrations exceed those established by the US EPA (2002) for the protection of freshwater aquatic life (0.22 μg/L). In addition, Jergentz et al. (2005) reported values of 318 μg/kg in suspended particles of the stream Horqueta, located in the main area of soybean production in Argentina.

Endosulfan median lethal concentration (LC_{50}) values of 0.4–12.8 μg/L have been reported for marine and freshwater fish species (Bacchetta et al. 2010; Ballesteros

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et al. 2007; Capkin et al. 2006; Hii et al. 2007; Jonsson and Toledo 1993; Magesh and Kumaraguru 2006; Pandey et al. 2006; Tellez-Bañuelos et al. 2009; Vittozzi and De Angelis 1991). Current water quality criteria for priority pollutants were mainly derived from laboratory toxicity tests using standard test species (e.g., zebrafish, medakafish, rainbow trout). However, the setting of realistic water quality criteria requires information on the response of native endangered species to pollutants. Despite the fact that ED is widely used in neotropical regions, little is known about its toxicity on neotropical fish species.

Exposure to sublethal concentrations of waterborne and dietary ED has induced a wide array of effects, including neurotoxicity and genotoxicity (Ballesteros et al. 2009a; Dutta and Arends 2003), liver toxicity (Glover et al. 2007; Mishra and Shukla 1994), and metabolical and histopathological changes (Nowak 1996; Tripathi and Verma 2004). Alterations in the immune and endocrine systems and behavioral changes have been also reported in fish exposed to ED (Ballesteros et al. 2009a; Bisson and Hontela 2002; Coimbra et al. 2005; Harford et al. 2005; Stanley et al. 2009; Thangavel et al. 2010).

Cichlasoma dimerus (Pisces, Cichlidae) is a South American perciform teleost, commonly inhabiting quiet shallow waters of the Paraguay and Paraná River basins (Kullander 1983). This freshwater species adapts easily to captivity and shows notable reproductive features such as a high spawning frequency and acceptable survival rates, providing an appropriate model for laboratory studies (Rey Vázquez and Guerrero 2007).

The present study was designed to evaluate the 96-h median lethal concentration (96-h LC_{50}) and sublethal changes on hematological, enzymatic, and oxidative stress markers in different tissues of *C. dimerus* exposed to a commercial formulation of ED. Moreover, this work is aimed at enlarging the ED toxicity database of aquatic vertebrates.

Materials and Methods

Experimental Design

Adult *C. dimerus* ($n = 90$) were collected from an unpolluted area of the Paraná River (31°42′ S, 60°45′ W, Argentina). Fish were measured (total length = 9.9 ± 1.1 cm) and weighed (23.6 ± 7.7 g). For acclimation purpose, fish were held in 150-L tanks containing well-aerated dechlorinated tap water for 2 weeks and were fed once daily with dry commercial pellets.

Tests were conducted in 25-L glass aquaria under static conditions following the recommendations of the OECD Guidelines for Testing of Chemicals (OECD 1992). The experiments were carried out in 12:12-h light–dark cycles, and the test water conditions were as follows: dechlorinated tap water, pH 6.89 ± 0.2, total hardness = 48 ± 0.1 ppm CO_3Ca, and temperature = 24 ± 1°C. Fish feeding was suspended 24 h before the beginning of the tests. ED test solutions were prepared from a commercial formulation containing 35% active ingredient (Zebra Ciagro®; Ciagro S.A. Argentina), as such a grade of ED is frequently employed in field practices. ED concentrations were expressed as active ingredient and were quantified in water at the beginning of each experiment by gas chromatograph-electron capture detector, according to the USEPA (1989), showing recoveries >95% of the nominal value. For the lethal 96-h toxicity test, preliminary range-finding tests were performed to establish a mortality range and to define test concentrations.

The lethal 96-h toxicity test consisted of exposing fish to five concentrations of ED (14.0, 15.6, 17.3, 19.3, and 21.4 μg/L). An additional group served as the control and was kept in tap water. Each concentration and the control group consisted of five individuals and were tested in duplicate. The mortality of test organisms was recorded when opercular movements stopped, and the dead individuals were removed instantly. Accurate records of mortality counts were maintained at a regular interval of 12–96 h. As an estimate of relative lethal toxicity, LC_{50} values and their corresponding 95% confidence limits were calculated for 24, 48, 72, and 96 h using US EPA probit software 1.5 free version (USEPA 1992).

For the sublethal toxicity test, five fish were exposed to the following ED concentrations: 0 (control), 3.4 (25% 96-h LC_1), and 6.8 μg/L (50% 96-h LC_1) for 96 h. Test and control groups were tested in duplicate. Fish were first anesthetized with 0.1% benzocaine (Vissio et al. 2008), and after blood sampling, they were sacrificed and dissected. Morphometric parameters, the condition factor (CF), and the liver somatic index (LSI) were calculated according to Goede and Barton (1990). Gills, hepatopancreas, and brain were immediately frozen in liquid nitrogen and stored at −80°C until biochemical determinations were conducted.

Hematological Parameters

Blood was rapidly extracted from the caudal vessel by dissection of the caudal peduncle (Reichenbach-Klinke 1980; Roberts 1981), using heparinized syringes. Red blood cell (RBC) counts were performed with a Neubauer chamber, using a physiological solution for dilution. Hematocrit (Ht) values were determined by the micromethod using capillary tubes and centrifuged at 1409×*g* for 10 min. Hemoglobin concentration (Hb) was measured by the cyanomethemoglobin method at wavelength of 546 nm on a spectrophotometer (Houston 1990).

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Mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were calculated from primary indices.

A drop of freshly collected blood was smeared on clean slides to estimate the total white blood cell (WBC) counts and for determination of leukocyte frequency according to Tavares-Dias and de Moraes (2007). The air-dried blood smears were fixed in absolute methanol for 10 min and stained by May-Grünwald-Giemsa (Houston 1990). The total WBC count was performed in relation to the number of erythrocytes counted in randomly selected fields and recalculated per unit volume: WBC/μL = number of WBCs in blood smear × (RBCs/μL/4000 RBCs counted in smear). Differential leukocytes counts were performed by identifying 100 WBCs in each blood smear.

Additionally, plasma was separated from whole blood by centrifugation at 1409×*g* for 10 min. Glucose and total protein concentrations were determined colorimetrically using commercial kits (Wiener Lab®).

Transaminases and Alkaline Phosphatase

Samples of hepatopancreas from each individual fish were homogenized in phosphate buffer (pH 7.4). The homogenate was centrifuged at 25,000×*g* at 4°C for 10 min, supernatant collected, and stored at −80°C for enzymatic studies. Aspartate aminotransferase (AST) (L-aspartate-2-oxaloglutarate aminotransferase; E.C. 2.6.1.1) and alanine aminotransferase (ALT) (L-alanine-2-oxaloglutarate aminotransferase; E.C. 2.6.1.2) activities were estimated according to Reitman and Frankel (1957). The reaction mixture contained 2 mmol/L α-ketoglutarate, AST and ALT specific substrates (100 and 200 mmol/L of aspartate and alanine, respectively) in buffer phosphate (100 mM, pH 7.4). The reaction was started by adding aliquots of the homogenate; after 30 min of incubation, the 2.4-dinitrophenylhydrazine reagent was added and the colored product was measured spectrophotometrically at 505 nm. Alkaline phosphatase (ALP) (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) activity was determined colorimetrically using a commercial kit (Wiener Lab®). Each sample was measured in triplicate and the enzymatic activity was calculated in terms of protein content (Bradford 1976).

Lipid Peroxidation

Liquid peroxidation (LPO) in gills, hepatopancreas, and brain was estimated by measuring the formation of thiobarbituric reactive substances (TBARS), according to Fatima et al. (2000). Tissues were individually homogenized with 0.15 M potassium chloride solution using a glass homogenizer. Then 1.0 mL of homogenate was incubated for 1 h at 37°C with continuous shaking. Afterward, 1.0 mL of 5% trichloroacetic acid and 1.0 mL of 0.67% thiobarbituric acid were added to each sample and mixed. Then each vial was centrifuged at 1409×*g* for 10 min. The supernatant was separated and placed in a boiling water bath for 10 min, cooled to room temperature, and measured spectrophotometrically at 535 nm. The rate of LPO was expressed as nanomoles of TBARS formed per hour per milligram of proteins (nmol TBARS/mg prot). Protein content of each sample was determined according to Bradford (1976).

Statistical Analysis

All data are reported as mean ± standard error. The statistical analyses were carried out using the PAST Software Package (Hammer et al. 2001). We carried out an analysis of variance (ANOVA) test to check for statistical differences in the measured variables between sexes (males and females). We did not find significant variations. Thus, we decided to pool data from both sexes during further statistical analysis. Data obtained for different biochemical parameters were first tested for normality using the Shapiro–Wilks test. Variables with no normal distribution were analyzed by the Kruskal–Wallis test. One-way ANOVA followed by an a posteriori Tukey test were performed to evaluate changes in measured variables between treatment and control groups. Differences were considered statistically significant when $p < 0.05$.

Results

The 24-, 48-, 72-, and 96-h LC_{50} values (95% confidence limit) were 18.4 (17.5–19.6), 18.1 (17.1–19.2), 17.9 (16.8–19.1), and 17.7 (16.7–18.8) μg/L, respectively. No mortality occurred in the control and the 14.0- μg/L ED-containing groups. One hundred percent mortality was recorded after 24 h at the highest ED concentration (21.4 μg/L) tested.

The results showed that there were no changes on morphometric biomarkers (CF and LSI) in fish exposed to sublethal concentrations of ED compared to the control group (Table 1). Conversely, ED exposure changed some of the measured hematological parameters (Table 1). A significant increase in Hb was only registered at the highest concentration tested (6.8 μg/L), whereas an increase in WBCs was observed in fish exposed to both ED concentrations. Differential leukocyte count was also affected, showing a higher proportion of neutrophils in exposed fish. Basophils were not found in the prepared smears and eosinophils were the rarest WBCs.

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Table 1 Morphometric and hematological parameters of *Cichlasoma dimerus* exposed to sublethal concentrations of the commercial ED formulation, Zebra Ciagro

Parameter	Control group	Endosulfan concentration (μg/L)	
		3.4	6.8
LSI	0.40 ± 0.06	0.39 ± 0.04	0.29 ± 0.07
CF	5.83 ± 0.47	5.54 ± 0.64	6.04 ± 0.48
RBC (10^6 cells/μL)	2.57 ± 0.26	2.98 ± 0.41	2.98 ± 0.36
Ht (%)	54.80 ± 2.88	54.63 ± 3.27	53.60 ± 5.07
Hb (g/dL)	9.07 ± 1.29[a]	8.00 ± 0.89[a]	10.38 ± 1.01[b]
MCH (pg)	34.81 ± 2.85[a]	27.02 ± 1.87[b]	34.96 ± 2.35[a]
MCV (μm^3)	164.30 ± 21.82	166.96 ± 10.77	186.90 ± 6.61
MCHC (%)	18.26 ± 3.58	15.66 ± 0.24	19.24 ± 1.26
WBC (cells/μL)	14816 ± 5368[a]	44257 ± 600[b]	40520 ± 13925[b]
Lymphocytes (%)	94.46 ± 2.24	95.42 ± 2.24	86.06 ± 11.16
Neutrophils (%)	1.90 ± 1.56[a]	0.70 ± 0.91[a]	8.47 ± 7.78[b]
Eocinophils (%)	3.45 ± 1.92	2.94 ± 1.15	5.27 ± 5.90
Monocytes (%)	0	0.23 ± 0.45	0.20 ± 0.45
Thrombocytes (%)	0.19 ± 0.42	0.71 ± 0.91	0
Glucose (g/L)	0.71 ± 0.10	0.88 ± 0.06	0.99 ± 0.36
Total protein (g/dL)	3.52 ± 0.79	4.14 ± 0.60	4.95 ± 0.14

Note: The values are expressed as means ± standard error

Means not sharing the same superscript (a or b) in each column are significantly different at $p < 0.05$

In fish exposed to 6.8 μg/L, a significant decrease in hepatopancreas ALT and AST was observed after 96 h of exposure (Fig. 1). Conversely, ALP was increased in the 3.4-μg/L ED-containing group.

Fish exposed to 6.8 μg/L ED showed a significant increase of LPO levels in gills, hepatopancreas, and brain (Fig. 2). At the 3.4-μg/L concentration LPO decreased in gills but increased in the brain of exposed fish.

Discussion

Chichlasoma dimerus 96-h LC_{50} was found to be 17.7 μg/L in the present study. Tellez-Bañuelos et al. (2009) recorded similar 96-h LC_{50} values for the cichlid *Oreochromis niloticus* (12.8 μg/L) exposed to a commercial ED formulation. Comparing these results with those found in the literature about other fish species (Table 2), we observed that *C. dimerus* and *O. niloticus* are less sensitive to ED than *Monopterus albus* (96-h LC_{50}:0.4 μg/L), *Prochilodus lineatus* (3.7 μg/L) and *Oncorhynchus mykiss* (4.6 μg/L).

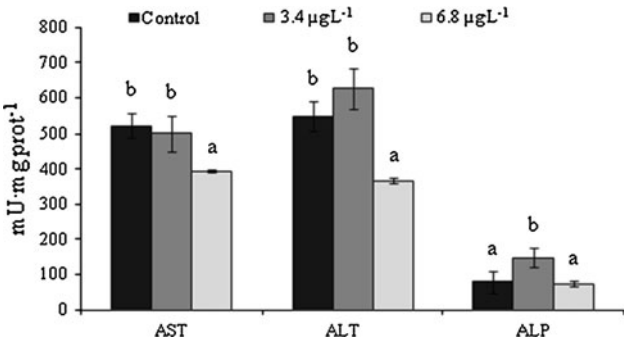

Fig. 1 AST, ALT and ALP activities in hepatopancreas of *C. dimerus* exposed to sublethal concentrations of the commercial ED formulation Zebra Ciagro. Means not sharing the same letter (a or b) are significantly different at $p < 0.05$

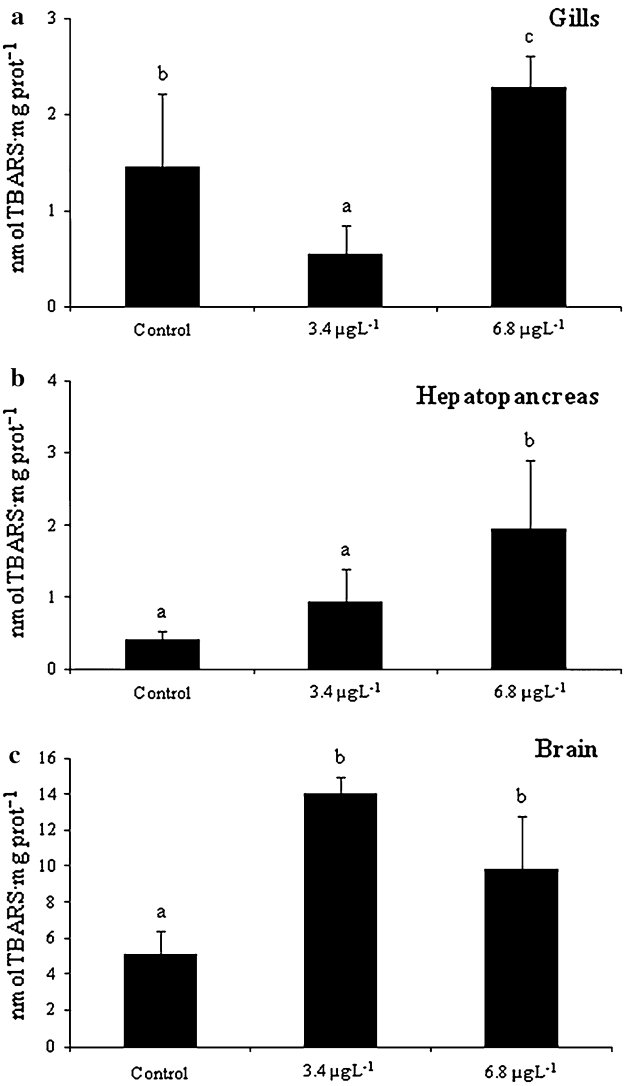

Fig. 2 TBARs in **a** gills, **b** hepatopancreas, and **c** brain of *C. dimerus* exposed to sublethal concentrations of the commercial ED formulation Zebra Ciagro. The values are expressed as means ± standard error. Means not sharing the same letter (a, b or c) are significantly different at $p < 0.05$

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Table 2 Median lethal concentration of different commercial ED formulations in some fish species

Species	Duration (h)	Purity (%)	Commercial product name	System	LC_{50} (μg/L)	References
Monopterus albus	96	33	CH endosulfan®	Static	0.4*	Hii et al. (2007)
Chanos chanos	96	35	Thiodan®	Static-renewal	0.6	Magesh and Kumaraguru (2006)
Jenynsia multidentata ♂	96	35	Galgofan® EC	Static	0.7	Ballesteros et al. (2007)
Jenynsia multidentata ♀	96	35	Galgofan® EC	Static	1.3	Ballesteros et al. (2007)
Oncorhynchus mykiss	96	33	Thiodan®	Static-renewal	1.7*	Capkin et al. (2006)
Prochilodus lineatus	96	35	Zebra Ciagro® EC	Static	3.7*	Bacchetta et al. (2010)
Oncorhynchus mykiss	96	40	Thiodan® 4EC	Static	4.6*	Wan et al. (2005)
Channa punctatus	96	35	EC	Flow-through	7.7	Pandey et al. (2006)
Oreochromis niloticus	96	35	EC	Static	12.8	Tellez-Bañuelos et al. (2009)
Cichlasoma dimerus	96	35	Zebra Ciagro® EC	Static	17.7*	Present study
Hyphessobrycon bifasciatus	24	97	Thiodan®	Static-renewal	2.6*	Jonsson and Toledo (1993)
Brachydanio rerio	24	97	Thiodan®	Static-renewal	1.6*	Jonsson and Toledo (1993)

*ED concentration expressed as active ingredient

In spite of this, data obtained in our study reinforce the fact that ED must be classified as a highly toxic substance for fish (USEPA 2002).

Organochlorine insecticides are usually highly lipophilic and can be kept out of circulation into lipid deposits. These metabolic and dispositional influences will determine how much active toxicant is available for interaction with its targets (Chambers and Carr 1995). ED is less lipophilic than other organochlorines (e.g., dichlorodiphenyltrichloroethane, polychlorinated biphenyls). However, Ballesteros et al. (2007) found lower LC_{50} values in males than females of *Jenynsia multidentata*, attributed by the authors to the higher lipid content of females in this species. In addition, metabolism might be an important determinant of pesticide toxicity. Biotransformation of α- and β-endosulfan occurs in the liver, through the oxidation of cytochrome P450 (Lee et al. 2006). This metabolic pathway leads to the formation of ED sulfate, which is the main biotransformation product of both isomers in aquatic organisms (Wan et al. 2005). Carriger et al. (2010) conducted flow-through toxicity tests in two indigenous fish from South Florida exposed to ED sulfate and they found 96-h LC_{50} values of 2.1 and 2.7 μg/L for *Heterandria formosa* and *Gambusia affinis*, respectively, showing lethal concentrations similar to those reported for technical ED.

Hematological parameters in control *C. dimerus* fell within the normal values range previously reported by Rey Vázquez and Guerrero (2007). Sublethal concentrations of ED had adverse effects on some hematological biomarkers of *C. dimerus*. At the end of our experiment (96 h), fish exposed to 6.8 μg/L showed higher Hb values than the control group, which potentially represents an increased requirement of oxygen transportation as a result of toxic acclimation processes. This increase in Hb contrasts with previous studies that reported a decrease in Hb content in other fish species exposed to ED for 96 h. Shafiq-ur-Rehman (2006) and Hii et al. (2007) found a decrease in Hb content in *C. carpio* and *M. albus*, respectively, exposed to 1 μg/L ED, whereas Bacchetta et al. (2010) registered the same response in *P. lineatus* exposed to 2.4 μg/L.

The immune response of fish owing to ED has been previously studied; for instance, exposure to ED sublethal concentrations significantly diminished the phagocytic activity in *O. niloticus* (Girón-Pérez et al. 2008) and leukocyte count in *M. albus* (Hii et al. 2007). In contrast, we observed immunostimulation, which was evidenced by a significant increase at both ED concentrations tested. A similar immune response was found by Bacchetta et al. (2010), who observed an increase in WBCs of *P. lineatus* exposed for 96 h to 2.4 μg/L of the same commercial ED formulation used in the present study. In addition, Tellez-Bañuelos et al. (2009) registered an activation of spleen macrophages in Nile tilapia exposed to 7 μg/L ED for 96 h. An increased leukocyte mobilization to protect the body against infections in the damaged tissues might account for the leucocytosis reported in the present study.

Differential leukocytes count showed that lymphocytes were the most frequent WBCs in the control *C. dimerus*. There was an increase in the percentage of neutrophils in exposed fish, whereas lymphocytes, eosinophils, monocytes, and thrombocytes remained the same. Neutrophils are the primary phagocytic leukocyte, and their capability to carry out their function is associated with the activation state of the innate immune response, which is highly influenced by xenobiotics (Ayub et al. 2003).

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Aspartate aminotransferase (AST), ALT, and ALP are enzymes involved in the metabolism of amino acids, and their alterations allow the identification of tissue damage in organs such as the liver and kidney (Begum 2004; Borges et al. 2007; de la Torre et al. 2007; Karan et al. 1998; Peri et al. 2003; Ramaiah 2007). In our study, hepatopancreas AST and ALT activities fell significantly below the control levels in fish exposed to the highest ED concentration, which is probably related to cytolysis and the enzyme leakage into the blood. Some authors have observed increased levels of liver transaminases as a consequence of pesticide exposure (Crestani et al. 2007; David et al. 2004; Philip and Rajasree 1996: Sancho et al. 2009, 2010). However, a decrease in transaminases levels has been observed in liver of fish exposed to organophosphorus and nonylphenol (Bhattacharya et al. 2008; de Aguiar et al. 2004). ALP activity increased significantly in the hepatopancreas of *C. dimerus* exposed to 3.5 μg/L ED. Generally, elevated ALP is observed as a result of increased osteoblastic activity or due to intrahepatic and extrahepatic obstruction to the biliary passage (Jyothi and Narayan 1999). Saravana Bhavan and Geraldine (2001) found the same results in the freshwater shrimp *Macrobrachium malcolmsonii* exposed to ED for 21 days, at a concentration of 0.032 μg/L.

The important role of lipids in cellular components emphasizes the significance of understanding the mechanisms and consequences of LPO in biological systems (Kelly et al. 1998). Significant increases in LPO were observed in the hepatopancreas, gills, and brain of *C. dimerus* exposed to sublethal concentrations of ED. Changes in hepatopancreas and brain LPO reported in *C. dimerus* are similar to those found in *J. multidentata* (Ballesteros et al. 2009b) and *P. lineatus* (Bacchetta et al. 2010) after 24 and 96 h of ED exposure, respectively.

The increase in gills LPO levels of *C. dimerus* exposed to 6.8 μg/L has been also observed by Pandey et al. (2001) and Atif et al. (2005) in *Channa punctatus* exposed to ED. Oxidative stress in gills might lead to deterioration in vital functions, such as gas exchange, ion regulation, and nitrogen excretion. Additionally, the increase in Hb content observed in blood reinforces the hypothesis of some respiratory ability failure of exposed fish. To the contrary, fish exposed to 3.4 μg/L showed a significant decrease in gills LPO level, which might be due to the activation of the antioxidant system at low ED levels.

To conclude, the commercial ED formulation Zebra Ciagro exposure alters hematological parameters and affects *C. dimerus* vital organs via metabolical disorders and oxidative damage of tissues (hepatopancreas, gills, and brain). It should be emphasized that although the changes found are reversible, they could affect fish health by making them more sensitive to environmental variations and less resistant to diseases. Even though significant changes were observed in several biochemical markers at 96 h of exposure to this commercial ED formulation, no clear concentration–response was observed for most of the responding biomarkers. This highlights the need of further work to fully understand the metabolism and biotransformation pathways of ED in neotropical fish. Therefore, the present study on the acute and sublethal ED toxicity in *C. dimerus* is an approach to assess nonlethal effects of pesticides on fish and to establish water quality criteria for control policies and conservation strategies in neotropical regions.

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