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Environmental relevant concentrations of a chlorpyrifos commercial formulation affect two neotropical fish species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus*

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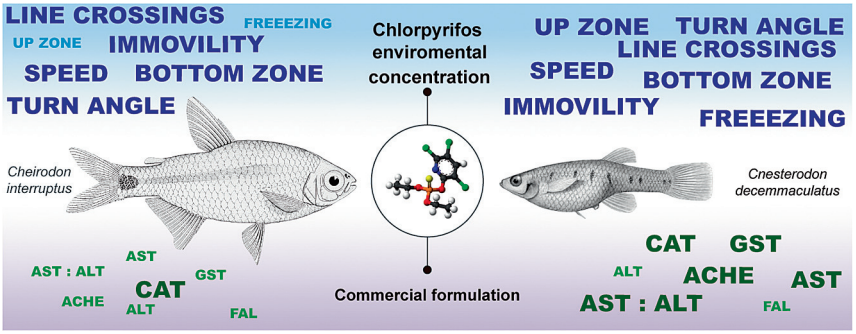
HIGHLIGHTS

- Chlorpyrifos caused different responses in the studied species.
- Behavioral parameters significantly changed under chlorpyrifos exposure.
- Enzyme activities were affected by chlorpyrifos.

GRAPHICAL ABSTRACT



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ABSTRACT

The increase of cultivated areas together with the intensive use of pesticides have greatly contributed to impair the quality of aquatic systems along different areas of South America. The main goal of the present study was to assess the effects of a commercial formulation of chlorpyrifos at environmentally relevant concentrations on two native fish species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus*. Adult individuals were exposed during 48 h to the following concentrations: 0.084 nl/l (*Ci*-Cf 1) and 0.84 nl/l (*Ci*-CF 2) in *C. interruptus* (*Ci*) of Clorfox (CF), and 0.84 nl/l (*Cd*-CF 1) and 8.4 nl/l (*Cd*-CF 2) in *C. decemmaculatus* (*Cd*). Fish behavior was evaluated through locomotor activity and space usage variables. The activity of acetylcholinesterase (AChE) in brain and muscle, catalase (CAT) and glutathione-S-transferase (GST) in brain, liver, muscle and gills, and aspartate amino-transferase (AST), alanine amino-transferase (ALT), AST/ALT ratio and alkaline phosphatase (ALP) in liver, were measured. Both locomotor activity and space usage varied between the two species studied and between CF treatments. The enzyme activities showed significant variations in CAT for *C. interruptus* and

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in CAT, GST, AChE, AST, and AST/ALT for *C. decemmaculatus* under the exposure conditions. Given that both species responded to CF and the concentrations we tested are environmentally relevant, the presence of this pesticide in freshwater systems could impose a risk for populations of both native fish studied at field.

1. Introduction

An extensive area located in South America called the Pampean region covers 750,000 km^2 and includes territories of three countries: Argentina, Uruguay and Brazil. The high demographic and agricultural production in this area become the Pampean basins into severely affected water bodies (Rodrigues Capítulo et al., 2010). Particularly, in the recent decades the increase of cultivated areas together with the intensive use of pesticides, have greatly contributed to impair the quality of aquatic systems.

The presence of agrochemicals has been indicated recently in several studies of water resources of the Argentinean Pampean region (Bonansea et al., 2013; Brodeur et al., 2011; De Gerónimo et al., 2014; Jergentz et al., 2005; Marino and Ronco, 2005; Peruzzo et al., 2008). Particularly, chlorpyrifos (CPF) (O, O-dimethyl O-(3, 5, 6-trichloro-2-pyridinyl) phosphorothioate), an organophosphate insecticide, is used to control a wide variety of insects populations in over 100 different crops (Benedetto et al., 2010). It has been registered in Argentinean streams up to 10 μg CPF/l in water and 19 μg CPF/Kg in sediments (Brodeur et al., 2011; Marino and Ronco, 2005). Its application to agricultural fields can lead to accidental spill, spray drift, and runoff from the terrestrial applications (CCME, 2008). In freshwater bodies chlorpyrifos has a half-life of 16 h–77 days according to the environmental conditions (De Silva and Samayawardhena, 2005). Aquatic organisms could accumulate it, therefore the presence of chlorpyrifos in streams and lakes has gained great interest due to its toxic effects on fish species, causing alterations in behavioral, neurological and reproductive aspects (Botté et al., 2012; De Silva and Samayawardhena, 2005; Özcan Oruç, 2010).

Two relevant Pampean fish species by its occurrence and distribution are: *Cnesterodon decemmaculatus* (Poeciliidae) and *Cheirodon interruptus* (Characidae) (Liotta, 2005). These species inhabit shallow and vegetated freshwaters. However, they present some specific differences. *C. decemmaculatus,* commonly known as “Ten spotted live-bearer” is a small fish that belongs to the cyprinodontiform order, a group who evolved in the sea (Bone and Moore, 2008). It feeds upon detritus, algae from periphyton and plankton, and benthic and planktonic invertebrates when they are available (Quintans et al., 2009). According to Young et al. (2016) females produce between 10 and 25 live young several time per reproductive season. On the other hand, *C. interruptus,* commonly known as “Uruguay tetra” reaches a bigger size than *C. decemmaculatus*; and belongs to the characiform order, group that evolved in freshwater systems (Bone and Moore, 2008). It feeds mainly upon insect larvae and microcrustaceans (Fernández et al., 2012) and females produce a mean fecundity of 448 oocytes (Ferriz et al., 2011). Rosso (2006) considered the Uruguay tetras as small migratory fish because they perform migratory movements associated with exploration of diverse habitats (Levin and González, 1994), whereas species like *C. decemmaculatus* are considered non-migratory fish.

Different sensitivity between both species has been reported in several field studies in which *C. decemmaculatus* is classified as a tolerant species and *C. interruptus* as a sensitive fish to stressful conditions (Hued and Bistoni, 2005; Maggioni et al., 2012; Merlo et al., 2011). Furthermore, both species are likely to be exposed to agrochemicals due to their wide distribution across the Pampean region. *C. decemmaculatus* has been used as a laboratory model to evaluate the effect of toxic substances through the application of different biomarkers under acute or chronic toxicity test (Bonifacio et al., 2016; Mastrángelo and Ferrari, 2013; Menéndez-Helman et al., 2012; Ossana et al., 2016; Vera-Candioti et al., 2014), instead, *C. interruptus* has been scarcely exposed to toxicant assays (Campana et al., 1999).

To understand how an organism responds to a given toxic situation in both natural and control laboratory conditions, biomarkers arise as useful tools. The best situation is to make an approach where a multiplicity of biomarkers is measured at different levels of biological organization. The more biomarkers are taken into account, the better the integrated vision of the toxic substance effects on an organism. Biomarkers such as the behavioral parameters are observable at individual level and have the advantage of summarize several cellular processes that are essential for individual and population survival. On the other hand, biochemical parameters like enzyme activities have the advantage of showing early responses due to the effects of the substance assess.

The main goal of the present work was to assess the effects of a commercial formulation of chlorpyrifos at environmentally relevant concentrations on two native species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus*, through a set of biomarkers at different level of biological organization.

2. Materials and methods

2.1. Bioindicators

Adults of *C. interruptus* and *C. decemmaculatus* were chosen for laboratory studies due to their high abundance across the Pampean region and their suitability for laboratory conditions. Individuals were collected using a dip net of 1 mm mesh size from a site on Yuspe River (31°14′S; 64°27′ W) (Córdoba, Argentina). This site has been used as a reference location for fish collection according to previous water quality assessments (Hued and Bistoni, 2005; Maggioni et al., 2012; Valdés et al., 2014).

Fish were transported to the laboratory in water tanks (20-L) and acclimated to laboratory conditions (12/12 light/dark; 21 ± 1 °C) for at least 15 days in 120 l tanks, one for each species. During this period fish were fed ad libitum twice a day with commercial fish food (Tetra Min®) and chironomidae larvae (Tetra Bloodworms®).

2.2. Chemicals

Toxicity tests were performed to evaluate the toxicity of commercial chlorpyrifos based insecticide on *C. interruptus* (*Ci*) and *C. decemmaculatus* (*Cd*). Commercial chlorpyrifos formulation was Clorfox® (CF) (Gleba, SA, Argentina) which contains 48% of the

active ingredient (0,0-diethyl 0-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) and 52% as solvent and emulsifier. To perform the bioassay, a stock solution of CF was made in acetone, because of the moderate hydrophilic nature of chlorpyrifos, (log K_{ow} = 3.31–5.27) (CCME, 2008), and more diluted solutions were prepared with distilled water for daily changes of water.

The concentrations of chlorpyrifos were measured in the aquarium water at 0 and 24 h by triplicate (after filling and before replacement of water, respectively). Chlorpyrifos was extracted from water samples by solid phase extraction as described by Bonansea et al. (2013). The extracts were analyzed by an Agilent 6890 gas chromatograph (Santa Clara, CA, USA) equipped with a microelectron capture detector and a Varian VF-5 ms 30 m × 0.25 mm × 0.25 μm capillary column (Palo Alto, CA, USA) to separate and identify the pesticide residue (Maggioni et al., 2012). The quantification of the pesticide was performed using external standards (Sigma–Aldrich). Recoveries of the complete analytical technique were obtained by the laboratory fortified sample method reaching 98%. The obtained limits of the detection and quantification in aquarium water were 0.001 and 0.002 μg/l.

2.3. Bioassays

Fish were exposed to Clorfox (CF) for 48 h. The selection of exposure concentrations was made according a previous work carried out by Bonifacio et al. (2016) which are environmentally relevant since they are lower than the maximum concentrations found for the region (Marino and Ronco, 2005). Since our main goal was to assess the effect of a commercial formulation we decided to express exposure concentrations in nl/l, because it is a liquid formulated. Individuals of *C. decemmaculatus* were exposed to 0 nl CF/l (*Cd*-Ctrl), 0.84 nl CF/l (*Cd*-CF 1) and 8.4 nl CF/l (*Cd*-CF 2). On the other hand, *C. interruptus* was exposed to one magnitude order less than *C. decemmaculatus*: 0 nl CF/l (*Ci*-Ctrl), 0.084 nl CF/l (*Ci*-CF 1) and 0.84 nl CF/l (*Ci*-CF 2), due to previous works that describe this fish as sensitive to environmental alterations (Hued and Bistoni, 2005; Maggioni et al., 2012; Merlo et al., 2011).

For both species five individuals per treatment were randomly assigned in 10-L (for *C. interruptus*) in and 5-L (for *C. decemmaculatus*) aerated glass aquaria. All the bioassay was performed by duplicate. Individuals were not fed along the experiment. Water of each aquarium was completely renewed every 24 h. The wastewaters generated from bioassays were dismissed by activated carbon filtration.

2.4. Behavioral parameters

After the exposure period, each fish was transferred individually into a recording aquarium (30 cm width × 9 cm depth × 25 cm height), containing 4 l of dechlorinated tap water. Four minutes after the fish was introduced into the tank, the activity of each fish was continuously registered along six minutes. Parameters were obtained at the end of each trial from video films, through the video-tracking software (ANY-Maze® Stoelting Co, USA). In order to assess if CF affect the locomotor activity, it was determined the mean speed of mobile episodes (m/s), maximum speed (m/s), absolute turn angle (radians), number of episodes of freezing and number of episodes and duration of immobility of each individual. To assess if CF affects the space usage the tanks were divided into equal sections with three vertical lines and two horizontal lines by the software; these divisions allowed us to measure the number of line crossings (vertical and horizontal lines) and the entries in each zone (up and bottom).

2.5. Enzyme activities

After the locomotor activity assessment each individual was killed by severing the spinal cord behind the opercula and dissected. This method of euthanasia is chosen in order to avoid factors that may confuse the enzymatic response and it follows the recommendation of NIH Guidelines (American Veterinary Medical Association, 2013). Enzyme extracts from each organ were prepared from individual fish according to Cazenave et al. (2006). Briefly, organs were homogenized in 0.1 M potassium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE) using a glass homogenizer (Potter Elvehjem), affording a tissue weight of ca. 10% per volume. The samples were centrifuged at 6900 × g and 4 °C for 10 min to separate cell debris and the supernatant, using refrigerated centrifuge. Acetylcholinesterase activity (AChE) was measured only in brain and muscle homogenates at 412 nm according to Ellman et al. (1961) using acetylthiocholine iodide as substrate and dithiobisnitrobenzoic acid (DTNB). Catalase activity (CAT) was determined according to Beutler (1982) at 240 nm using H2O2 as substrate. The activity of glutathione-S-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate at 340 nm, according to Habig et al. (1974). All the determinations were carried out by triplicate using a multi-plate reader Biotek®, equipped with a multiple cell holder and temperature control. The enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976), and is reported in nkat (mg prot)$^{-1}$. Each enzymatic assay was carried out by triplicate.

For transaminases and alkaline phosphatase liver from each individual fish was homogenized in phosphate buffer (pH 7.4). The homogenate was centrifuged at 15,000 × 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) and alanine aminotransferase (ALT) (L-Alanine-2-oxaloglutarate aminotransferase) activities were estimated according to Reitman and Frankel (1957). The reaction mixture contained 2 mmol l−1 of α-ketoglutarate and AST and ALT specific substrates (100 and 200 mmol l^{-1} of aspartate and alanine l-1, respectively), in buffer phosphate (100 mM pH 7.4). The reaction was started by adding aliquots of the supernatant; 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 activity was determined colorimetrically using a commercial kit (Wiener Lab) (Bacchetta et al., 2011). The enzymatic activity was calculated in terms of the protein content of the sample (Bradford, 1976), and is reported in nkat (mg prot)$^{-1}$.

2.6. Statistical analysis

Data are expressed as mean ± standard error. Data distributions were analyzed using the Shapiro–Wilks index (Sokal and Rohlf, 1999). To compare the biological parameters among different treatments, Kruskal–Wallis test (Sokal and Rohlf, 1999) was performed followed by a Dunn's multiple comparison test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using Infostat Software Package (Infostat, 2014).

3. Results

3.1. Analytic concentrations of chlorpyrifos

Chlorpyrifos levels of in water from control groups were below the detection and quantification limits (LOD) at 0 and 24 h. Concentrations of chlorpyrifos showed a drop of 100% (*Ci*-CF 1), 100%

Table 1
Chlorpyrifos concentration in exposure media at 0 h and 24 h (T0 and T24) (n = 3). The values are expressed as means ± standard deviation.

Pesticide value	Chlorpyrifos (μg/l)	
	T0	T24
Control	<DL	<DL
0.084 nl/l of Clorfox	0.014 ± 0.009	<DL
0.84 nl/l of Clorfox	0.196 ± 0.020	<DL
8.4 nl/l of Clorfox	1.022 ± 0.031	0.135 ± 0.004

(*Ci*-CF 2, and *Cd*-CF 1) and 87% (*Cd*-CF 2) at 24 h (Table 1). The drops in CF levels justified the renewal of the aquarium media 24 h after the beginning of the test.

3.2. Behavioral parameters

The normal behavioral parameters of both species are significantly different. Individuals of *C. interruptus* from the control group showed higher mean speed of mobile episodes, maximum speed, absolute turn angle, line crossings and bottom zone entries than *C. decemmaculatus* (Fig. 1).

Clorfox altered the locomotor activity of both fish species. The time immobile of *C. interruptus* was significantly increased at both exposure concentrations (*Ci*-CF 1 and *Ci*-CF 2); on the other hand, the *Ci*-CF 1 treatment also caused a decrease of the mean speed of mobile episodes, maximum speed, absolute turn angle and an increase of immobile episodes. For *C. decemmaculatus* the mean speed, line crossings and absolute turn angle were significantly decreased at both Clorfox concentrations. On the other hand, significantly increases were registered for time immobile, immobile episodes and freezing episodes, at *Cd*-CF 2 exposure concentrations. (Fig. 1a).

Line crossings was the only space usage variable affected in *C. interruptus*, whereas *C. decemmaculatus* showed a decrease in line crossings and in the number of entries in upper zone at both Clorfox concentrations; and a decrease of entries in the bottom zone at *Cd*-CF 2 exposure concentration only (Fig. 1b).

3.3. Enzyme activities

The brain and muscle AChE activities in *C. interruptus* were not

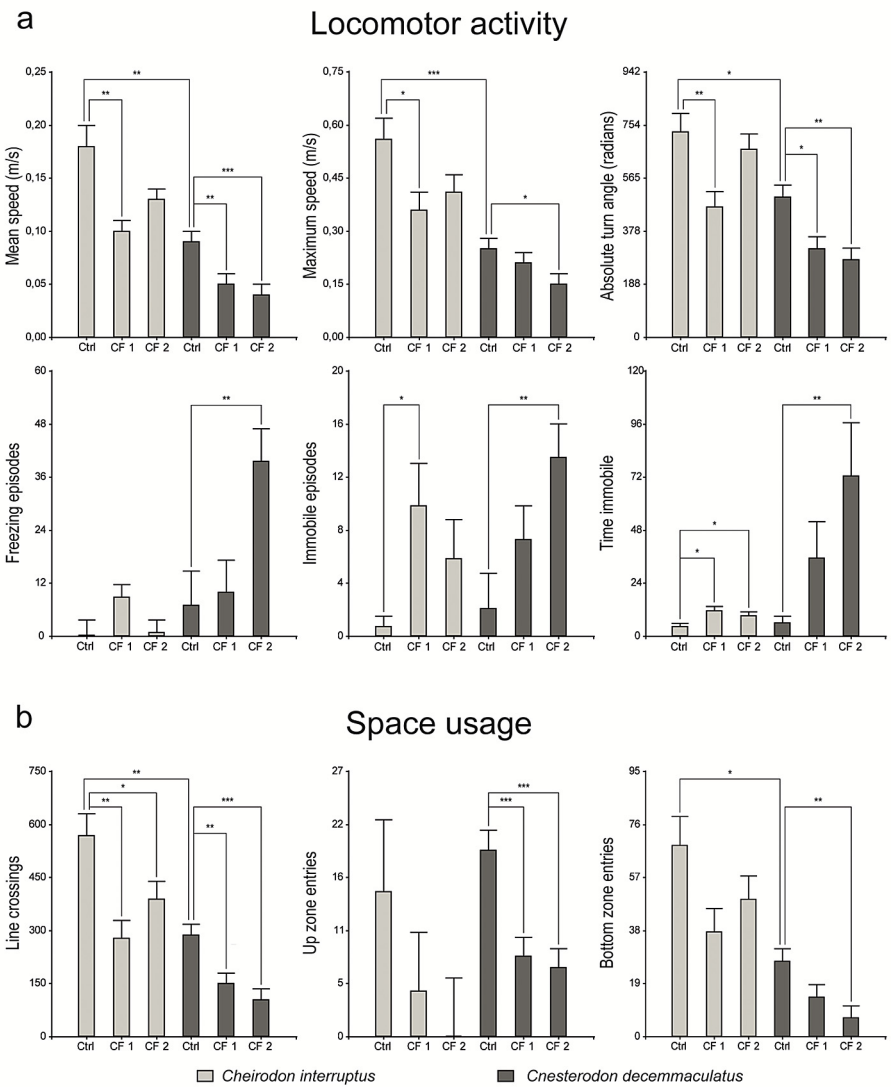

Fig. 1. Behavioral parameters of locomotor activity (a) and space usage (b) recorded in *C. interruptus* and *C. decemmaculatus* exposed to Clorfox (n = 10). References for *C. interruptus* (light grey): Ctrl – control group, CF 1–0.084 nl CF/l, CF 2–0.84 nl CF/l; for *C. decemmaculatus* (dark grey): Ctrl – control group, CF 1–0.84 nl CF/l, CF 2–8.4 nl CF/l. The values are expressed as means ± standard error. Asterisks indicate significant differences, * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

affected under Clorfox exposure, whereas a significantly decrease were registered in *C. decemmaculatus* muscle at both concentrations tested (Table 2).

The CAT activity did not change significantly among concentrations in gills of both species and it was not detected in muscle of *C. interruptus* nor in brain of *C. decemmaculatus*. On the other hand, the liver CAT activity did not change in *C. interruptus*, whereas in *C. decemmaculatus*, it was significantly inhibited at higher CF treatment (*Cd*-CF 2). Also, there was an inhibition of CAT activity in brain of *C. interruptus* and in muscle of *C. decemmaculatus* at both concentrations of CF (Table 2).

The activity of GST was significantly decreased only in muscle of *C. decemmaculatus* exposed to *Cd*-CF 1 concentration. There were no significant changes in gills, liver and brain activity in *C. interruptus* nor in gills and liver of *C. decemmaculatus*. Finally, the activity of GST was not detected in muscle of *C. interruptus* nor in brain of *C. decemmaculatus* (Table 2).

No significant differences were registered in AST, ALT, AST/ALT and ALP activity in *C. interruptus* exposed to CF. Conversely, in *C. decemmaculatus* AST activity show significant changes. The *Cd*-CF 1 concentration elicit a decrease, whereas an increase was registered at the *Cd*-CF 2. The AST/ALT ratio showed significant increase only at the *Cd*-CF 2 exposure concentration (Table 2).

4. Discussion

Our results are relevant due to the wide distribution and abundance of *C. interruptus* and *C. decemmaculatus* in the Argentinean Pampean region, where the coexistence between these fish species and the insecticide occurs. Marino and Ronco (2005) and Brodeur et al. (2011) have recorded up to 10 μg CPF/l in waters of aquatic systems of the Argentinean Pampean region. According to our results, the environmentally relevant concentrations we used, were enough to elicit impairment health condition of the fishes exposed to them.

Behavioral parameters comprises a set of variables that provides information about how much an individual moves and how it makes use of the available space. The fish swimming ability is directly associated with morphological traits. The body of *C. interruptus* is compressed and high on its major axis. Its general morphology denotes that it is an active swimmer, which moves in shoals in lentic and lotic environments. On the other hand, *C. decemmaculatus* is a small fish with a circular body section whose morphology makes it appropriate for inhabiting lentic environments or lentic zones in lotic environments, always associated to low speed waters with vegetation where fish find shelter. Thus, behavioral parameters of both species analyzed in the control individuals showed marked differences. Variables such as mean speed of mobile episodes, maximum speed, absolute turn angle, line crossings and bottom zone entries presented significantly higher values in *C. interruptus* respect to *C. decemmaculatus*. These results together with body morphological characteristics demonstrated that under normal water conditions *C. interruptus* is an active swimmer whereas *C. decemmaculatus* is a less active fish which prefer to live in quiet waters.

Behavioral parameters are also effective tools to assess the effects of any deleterious substance on fish and other animals (Bonansea et al., 2016; Bonifacio et al., 2016; Garcia et al., 2008; Jensen and Baatrup, 1997; Jin et al., 2015; Lee et al., 2015; Topal et al., 2015). From our work, under exposure conditions, interesting findings were made in *C. interruptus*. This species showed more sensitivity to the low exposure concentration (*Ci*-CF 1). This response was registered for several variables such as absolute turn angle, mean speed and number of lines crossings. Similar pattern of responses has been found in *Danio rerio* exposed to neurotoxic substances like ethanol and taurine (Rosemberg et al., 2012). Furthermore, it is important to note that the *Ci*-CF 1 concentration that causes the more important behavioral changes in *C. interruptus*, is several orders of magnitude below than those reported for *Puntius chola* exposed also to a commercial formulation (Verma and Saxena, 2013) and *Danio rerio* and *Jenynsia multidentata* (Bonansea et al., 2016; Jin et al., 2015) exposed to analytical grade chlorpyrifos. Thus, the results obtained showed that *C. interruptus* is a sensitive fish that is affected by very low CF concentrations. On the other hand, although *C. decemmaculatus* responded to the both concentrations used, exposure to the high Clorfox concentration (*Cd*-CF 2) caused the highest number of significant differences in locomotor activity as well as in space use. De Silva and Samayawardhena (2002) founded, in the related species *Poecilia reticulata*, increases in paralysis time of early life stages individuals at concentrations of 0.5 μg/l of Lorsban (another chlorpyrifos formulation) which is comparable with our analytical concentrations assayed. Also, Verma and Saxena (2013) indicated that individuals of *Puntius chola* exposed to 100 μg/l of a commercial grade

Table 2
Enzyme activities (nkat/mg prot) in different tissues of *C. interruptus* and *C. decemmaculatus* exposed to Clorfox (n = 10). References for *C. interruptus*: *Ci*-Ctrl – control group, *Ci*-CF 1–0.084 nl CF/l, *Ci*-CF 2–0.84 nl CF/l; for *C. decemmaculatus*: *Cd*-Ctrl – control group, *Cd*-CF 1–0.84 nl CF/l, *Cd*-CF 2–8.4 nl CF/l. The values are expressed as means ± standard error. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$).

C. interruptus					*C. decemmaculatus*		
Treatment		*Ci*-Ctrl	*Ci*-CF 1	*Ci*-CF 2	*Cd*-Ctrl	*Cd*-CF 1	*Cd*-CF 2
Biomarker	Organ						
AChE	Brain	2,06 ± 0,67	2,73 ± 0,73	2,51 ± 0,67	3,88 ± 1,13	5,41 ± 0,75	4,92 ± 0,62
	Muscle	1,31 ± 0,24	1,19 ± 0,24	1,51 ± 0,26	1.40 ± 0.30$^{(a)}$	0.5 ± 0.08$^{(b)}$	0.42 ± 0.10$^{(b)}$
CAT	Gills	59,49 ± 10,14	42,09 ± 7,10	50,11 ± 6,34	355,42 ± 111,29	231,35 ± 26,44	233,05 ± 15,32
	Liver	530,78 ± 154,26	558,04 ± 52,92	472,28 ± 63,03	2916,74 ± 391,99$^{(a)}$	2585,67 ± 82,47$^{(a)}$	1947,60 ± 127,37$^{(b)}$
	Brain	221,06 ± 40,88$^{(a)}$	58,64 ± 8,12$^{(b)}$	73,40 ± 8,53$^{(b)}$	±	±	±
	Muscle	±	±	±	510,71 ± 45,52$^{(a)}$	319,13 ± 41,68$^{(b)}$	245,96 ± 22,84$^{(b)}$
GST	Gills	0,23 ± 0,02	0,20 ± 0,02	0,23 ± 0,02	0,73 ± 0,12	0,69 ± 0,07	0,78 ± 0,11
	Liver	0,42 ± 0,05	0,42 ± 0,07	0,41 ± 0,06	2,32 ± 0,31	2,34 ± 0,20	2,27 ± 0,17
	Brain	0,16 ± 0,04	0,09 ± 0,04	0,13 ± 0,04	±	±	±
	Muscle	±	±	±	0,75 ± 0,05$^{(a)}$	0,56 ± 0,03$^{(b)}$	0,87 ± 0,07$^{(a)}$
AST	Liver	0,079 ± 0012	0,093 ± 0018	0,110 ± 0013	0,122 ± 0,020$^{(b)}$	0,064 ± 0,009$^{(a)}$	0,184 ± 0,009$^{(c)}$
ALT	Liver	0,039 ± 0009	0,036 ± 0006	0,038 ± 0007	0,065 ± 0012	0,050 ± 0005	0,046 ± 0010
FAL	Liver	0,017 ± 0006	0,015 ± 0004	0,011 ± 0004	0,022 ± 0005	0,018 ± 0006	0,024 ± 0006
AST:ALT*	Liver	2,61 ± 0,42	2,87 ± 0,42	2,81 ± 0,42	1,76 ± 0,37$^{(a)}$	1,27 ± 0,47$^{(a)}$	3,60 ± 0,58$^{(b)}$

* Ratio values are adimensional.

chlorpyrifos showed slower swimming speed than control individuals. *Jenynsia multidentata*, exposed to 0.4 and 4 μg/l of chlorpyrifos (the same order of magnitude of the analytic concentrations we used) for 24 h and 96 h, presented a decrease in locomotor activity variables only at the highest exposure concentration (Bonansea et al., 2016). However, unlike our work, these authors registered the mentioned decreases in individuals exposed to the active ingredient of chlorpyrifos only. It is important to note that these changes in behavioral performance after the exposure to CF environmental concentrations could strongly affects the ability of a fish to obtain food, find a mate and/or avoid unfavorable conditions in their natural environments (Plaut, 2000; Scott and Sloman, 2004).

Although previous works have related changes in AChE activity with variations in locomotor parameters (Kavitha and Rao, 2008; Sandahl et al., 2005), in *C. interruptus* AChE activities were not sensitive to CF exposures. Detrimental effects in locomotor activities could be due to the non-cholinergic effects of CF. It has been proposed possible effects of organophosphates related to the phosphorylation of active sites in different substrates than AChE (Pope, 2010), as well as, effects in muscarinic and nicotinic receptors in *Danio rerio* exposed to chlorpyrifos (Watson et al., 2014). On the contrary, only in muscle of *C. decemmaculatus,* the AChE activity was decreased by CF exposures. Rendón-von Osten et al. (2005) found AChE activity inhibitions at muscle in *Gambusia yucatana* exposed to 50 μg/l of commercial formulation of chlorpyrifos for 96 h. The concentration they used was at one magnitude order higher than we used in the present work. Nevertheless, a similar response of muscle AChE, has also been detected in *J. multidentata* exposed to a mixture of chlorpyrifos and cypermethrin for 96 h (Bonansea et al., 2016) in concentrations quite similar to those used in the present work. The relationship between behavioral parameters and AChE activities showed by *C. decemmaculatus* is in agreement with Kavitha and Rao (2008). These authors observed the same pattern in *Gambusia affinis* after the exposure to 297 μg/l analytical grade chlorpyrifos, and also with Sandahl et al. (2005), who demonstrated the same relationship in individuals of *Oncorhynchus kisutch* exposed to 0.6 μg/l of analytical grade chlorpyrifos. According to our results, we suggest that behavioral parameters are more sensitive than AChE activity to the concentrations tested.

Under normal physiological conditions antioxidant enzymes and nonspecific antioxidants are responsible to maintain a dynamic balance between oxidant products and antioxidant responses. When an imbalance between both mechanisms occurs, oxidative stress effects results (Oruc, 2012). Particularly, catalase (CAT) scavenges oxyradicals and transform them into water. In our study the CAT activity was inhibited in brain of *C. interruptus* and in liver and muscle of *C. decemmaculatus*. Similar results have been registered by individuals of *Cyprinus carpio* exposed to 40 μg/l of a chlorpyrifos commercial formulation (Ural, 2013) and in *Carassius auratus* exposed to 15.3 μg/l of an analytical grade chlorpyrifos (Ma et al., 2013). However, both works found significant effects at one and two orders of magnitude higher than our exposure concentrations. These authors pointed out that a decrease in CAT activity changes the redox capacity status of the cells where radicals are not totally scavenged by the antioxidant enzyme. Thus, a CAT inhibition could produces an excess of reactive oxygen species which finally damage the membrane lipids through lipid peroxidation (Leaver et al., 1992; Winston and Di Giulio, 1991). This kind of response suggest that both species could become especially vulnerable to oxidants in presence of CF.

Glutathione S-transferase (GST) plays an important role in the detoxification and excretion of xenobiotics by adding a molecule of glutathione to a metabolite or to the original compound and promoting its elimination from the organism (Leaver et al., 1992; Winston and Di Giulio, 1991). GSTs have been used as a biomarker in various tissues of different fish species both under laboratory and under natural conditions (Aksoy et al., 2015). In the present study, none of the studied organs in *C. interruptus* responded to the concentrations of CF whereas the muscle of *C. decemmaculatus* showed an activity increase at both CF treatments, which denotes the activation of a detoxifying process in this tissue. Al-Harbi et al. (2014), founded that a commercial formulation of chlorpyrifos increase GST activity in *Oreochromis spilurus* in a 14 days assay, however they exposed individuals at exposure concentrations two orders of magnitude higher than our work. On the other hand, Rendón-von Osten et al. (2005), showed no significant changes in *Gambusia yucatana* (species belonging to the same family than *C. decemmaculatus*) exposed during 96 h to a same order of magnitude concentration of a commercial chlorpyrifos formulation. Hayes et al. (2005) showed through their review that the activity and expression of GST is variable according to the type of organism, having a multiplicity of functions, such as the xenobiotic detoxification processes. In this sense, the abundant literature shows the disparity of the response of this enzyme, according to the species, toxic and time of exposure, which is easily observable through the different GST activity responses obtained from the two species that we have studied in the present work. Furthermore, our results demonstrated that *C. decemmaculatus* shows defense reactions at least in muscle with GST to deal with CF toxicity.

The changes in the activity of transaminases enzymes indicate liver tissue damages (Bhattacharya et al., 2008). These enzymes did not show significant changes respect to the control group in *C. interruptus*. However, in *C. decemmaculatus* the AST activity showed significant changes. The later species showed a decreased activity in liver transaminases at the *Cd*-CF 1 exposure concentration. This change could be due to an increase in intracellular levels of ROS which can lead to lipid peroxidation resulting in an increased permeability of hepatocyte cell membrane, and finally ending in a transaminase release out of the liver (Banaee et al., 2013). On the contrary, the *Cd*-CF 2 concentration elicit an increase in the AST activity. This kind of response suggests the shunting of amino acids into tricarboxylic acid cycle through oxidative deamination and active transamination, in order to face the energy demand caused by toxic stress (David et al., 2004). In a previous work, *C. decemmaculatus* exposed to the same concentrations of CF showed no significant effects in AST activities at 42 days of exposure denoting adaptation processes (Bonifacio et al., 2016) whereas Narra et al. (2015), found increases of AST in *Clarias batrachus* exposed to a commercial formulation of chlorpyrifos during 7 days, but at concentrations three orders of magnitude higher than our work. AST/ALT ratio was affected only in *C. decemmaculatus* individuals exposed to the *Cd*-CF 2 concentration, showing a ratio increase. The AST/ALT ratio is a sensitive biomarker of liver dysfunction and has been used in previous works carried out in both laboratory and field stressful conditions (Bonifacio et al., 2016; De La Torre et al., 2007). This ratio allowed us to detect that the higher concentration of exposure causes a greater effect on transaminases, as has been shown in individual of *C. decemmaculatus* exposure to CF during 42 days (Bonifacio et al., 2016).

In summary, behavioral parameters, acetylcholinesterase, detoxification enzymes and enzymes indicative of hepatic tissue damages were affected by the insecticide commercial formulation tested. Both species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus* showed to be sensitive to the exposure to Clorfox although they responded quite different. According to our results, environmental concentrations of chlorpyrifos threaten the health of wild populations of these species in their natural environment.

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