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Combined toxicological effects of pesticides: A fish multi-biomarker approach

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ABSTRACT

The combined effects of two synthetic insecticides on a wide array of biomarkers in the freshwater fish *Piaractus mesopotamicus* were studied. Fish were exposed to sublethal concentrations of endosulfan (ED), lambda-cyhalothrin (LC), and the combination of both pesticides for 96 h. The set of analyzed biomarkers included morphometric and hematological parameters, transaminases and alkaline phosphatase activities, antioxidant enzymes activities and oxidative damage biomarkers measured in gills, liver, kidney, brain and muscle. According to the principal component analysis, the most significant effects were produced by the ED–LC combination. So, the mixture of both insecticides produced an increase in the liver-somatic index, hematological changes related to immunological biomarkers (increased white blood cells count, and alterations in the differential leukocytes count), decreased liver transaminases activity, antioxidant enzymes induction in almost every tissue, and lipid peroxidation levels increases in liver, kidney and brain of exposed fish. Our results suggest deleterious effects of ED and LC insecticides in combination, and support the usefulness of the multi-biomarker approach for the characterization of toxicological mechanisms induced by pesticides.

1. Introduction

Pesticides are widely used in agricultural and urban areas, and their mixtures are frequently used to improve efficacy and reduce cost of crop pest treatment. The methods employed for pesticide application (spraying and dusting) enable them to enter the aquatic ecosystem (Elezović et al., 1994). Thus, aquatic organisms are inevitably exposed to a multitude of toxicologically and structurally different pesticides. Although toxicity of individual pesticides has been extensively studied in fish (Joseph and Raj, 2011), the toxicological impact of their combinations is relatively less understood. So, more studies are necessary in order to understand their interactions on the living system and to evaluate their risk assessment.

The combined use of a set complementary biomarkers that can both signal exposure to contaminants and quantify their impact on living organisms, enables a more comprehensive and integrative assessment of biochemical and cellular effects induced by environmental pollutants (Linde-Arias et al., 2008; Cazenave et al., 2009). Consequently, the multi-biomarker approach has gained considerable interest in ecotoxicological research, and has been recently applied in both field and laboratory studies (He et al., 2012; Jolly et al., 2012; Matozzo et al., 2012; Liang et al., 2013).

Organochlorines and pyrethroid insecticides are among the most frequently used classes of pesticides, overlapping in soybean crops. In Argentina, Endosulfan (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 $\mu g\,L^{-1}$ have been found on water bodies near rice fields in neotropical wetlands, and concentrations from 0.1 to 0.7 $\mu g\,L^{-1}$ on mountain rivers (Baudino et al., 2003; Silva et al., 2005). Similar to ED, the pyrethroid lambda-cyhalothrin (LC) is used in a wide range of crops. Although residues of other pyrethroids have been detected in water and sediment samples from streams and rivers in Argentina (Jergentz et al., 2005; Marino and Ronco, 2005), no data about environmental LC levels are available. The lack of information about this insecticide is likely related to its recent registration and current use (US EPA, 2001).

Due to its effects, both insecticides are considered highly toxic to fish and aquatic invertebrates (US EPA, 2002; Gu et al., 2007). It has been shown that individual ED and LC exposure can significantly induce antioxidant enzymes and oxidative stress (Ballesteros et al.,

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2009; Piner and Üner, 2012), and alter hematological parameters, transaminases, and alkaline phosfatase (ALP) activities in fish (Chandrasekar and Jayabalan, 1993; Hii et al., 2007; Girón-Pérez et al., 2008). In spite of this, no data on the effect of these insecticides together in aquatic organisms are available.

Piaractus mesopotamicus (Pisces, Characidae) is a South American omnivorous fish, widely used in fish farming due to their good consumer acceptance and high growth rate (Jomori et al., 2003). Thus, this study is aimed at evaluating multi-biomarker responses in *P. mesopotamicus*, after controlled exposure to commercial formulations of the organochlorine ED and the pyrethroid LC alone and in combination. The multi-biomarker approach proposed in the present study was focused simultaneously on morphometric parameters, antioxidant defenses, tissue damage, and hematological responses in fish.

2. Materials and methods

2.1. Fish

Juvenile *P. mesopotamicus* ($n = 160$; 8.1 ± 0.5 cm standard length; 23.9 ± 4.4 g) were obtained from a local fish farm. For acclimation purpose, fish were held in 150-L tanks containing well aerated dechlorinated water for two weeks, and fed once daily with dry commercial pellets. Fish feeding was suspended 24 h before the beginning of the tests.

2.2. Chemicals

ED (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-ethano-2,4,3-benzodioxathiepin-3-oxide) and LC [(RS)-alphacyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2,-dimethylcyclopropanecarboxylate] test solutions were prepared from commercial formulations containing 35% (Zebra Ciagro®, CAS No. 115-29-7, Ciagro Argentina S.A.) and 5% (Cilambda®, CAS No. RN [71751-41-2], Ciagro Argentina S.A.) active ingredient, respectively. Since technical-grade ED has xylene as vehicle that contribute to its water solubility, stock solution was prepared by the direct addition of technical-grade solution to 25 ml of ultrapure water. A stock solution of technical-grade LC was prepared dissolving it in 25 ml of acetone. Necessary volumes of stock solutions were added to the aquaria to achieve the desired final concentrations (solvent = 0.003% per aquaria). Stock solutions used in this study were always freshly prepared when needed.

2.3. Experimental design

Tests were conducted in 25-L glass aquaria under semi-static conditions. The experiments were carried out in 12:12 h light–dark cycles, and the test water conditions were: pH 6.9 ± 0.2, total hardness 48 ± 0.1 ppmCO_3Ca, and temperature 25 ± 1 °C. The aquarium solutions were renewed daily by transferring the fish to another aquarium. Experimental insecticide concentrations were calculated according to the active ingredient percentage present in the commercial formulation; and quantified at the beginning of each chemical renewal period by GC-ECD; showing recoveries >90% of the nominal value for both insecticides.

In order to determine insecticide sublethal concentrations, static 96-h acute toxicity tests were performed in accordance with OECD Guidelines for Testing of Chemicals (OECD, 1992). Fish ($n = 10$ per treatment) were individually exposed to five different nominal concentrations of ED (2.2, 2.8, 3.5, 4.4, and 5.5 $\mu g\,L^{-1}$) and LC (1.8, 2.2, 2.8, 3.5, and 4.4 $\mu g\,L^{-1}$). An additional group, which was kept in tap water, served as the control one. Mortality of test organisms was recorded when opercular movements stopped, and dead individuals were removed instantly. Accurate records of mortality counts were maintained at a regular interval of 12–96 h. LC_1 values were calculated for 96 h using US EPA probit software 1.5 free version (US EPA, 1992). At the end of the experiments, 96-h LC_1 values were calculated as 2.2 for ED and 1.4 $\mu g\,L^{-1}$ for LC.

In the sublethal toxicity test, 50% LC_1 values of both insecticides were applied. The selection of the 50% LC_1 was based on the methodology used in previous studies (Bacchetta et al., 2011a,b). Fish ($n = 10$ per treatment) were individually exposed to the following treatments: 0 (control), ED (1.1 $\mu g\,L^{-1}$), LC (0.7 $\mu g\,L^{-1}$) and a combination of both insecticides (1.1 μg ED L^{-1} + 0.7 μg LC L^{-1}, ED + LC), during 96 h. Fish were anesthetized and measured, weighted, sacrificed and dissected. Brain, gills, kidney, liver, and muscle were immediately frozen in liquid nitrogen and stored at −80 °C until biochemical determinations were carried out.

2.4. Biomarkers

2.4.1. Morphometric and hematological parameters

The condition factor (CF) and the liver somatic index (LSI), were calculated according to Goede and Barton (1990).

Blood was extracted from the caudal vessel by dissection of the caudal peduncle (Reichenbach-Klinke, 1980). Red blood cells (RBC) counts were performed with a Neubauer chamber and hematocrit (Ht) values were determined by the micromethod. Hemoglobin concentration (Hb) was measured by the cyanomethemoglobin method at wavelength of 546 nm (Houston, 1990). 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 cells (WBC) counts and for determination of leukocyte frequency according to Tavares-Dias and de Moraes (2007).

2.4.2. Transaminases and alkaline phosphatase

Samples of liver and kidney 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; EC 2.6.1.1) and alanine aminotransferase (ALT) (L-alanine-2-oxaloglutarate aminotransferase; EC 2.6.1.2) activities were estimated according to Reitman and Frankel (1957). Alkaline phosphatase (ALP) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity was determined colorimetrically using a commercial kit (REF 1361003 Wiener Lab®, Argentina). Each sample was measured by triplicate and the enzymatic activity was calculated in terms of protein content (Bradford, 1976).

2.4.3. Antioxidant defenses

Enzyme extracts from each tissue (brain, gills, kidney, liver, and muscle) were prepared from each individual (not pooled). Briefly, tissues were homogenized using 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE). The homogenate was centrifuged at 20,000 × *g* (4 °C) for 30 min, and the supernatant was collected and stored at −80 °C for enzyme measurement.

The activity of glutathione-*S*-transferase (GST, EC 2.5.1.18) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR, EC 1.6.4.2) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase activity (CAT, EC 1.11.1.6) was determined according to Beutler (1982). The enzymatic activity was calculated

in terms of the sample protein content (Bradford, 1976), and is reported in mU mg prot^{-1} except for the CAT which was expressed in U mg prot^{-1}. All enzymatic assays were carried out in triplicate.

2.4.4. Lipid peroxidation

Brain, gills, kidney, liver, and muscle LPO levels were determined by measuring the formation of thiobarbituric reactive substances (TBARS), according to Fatima et al. (2000). The rate LPO was expressed as nanomoles of TBARS formed per hour, per milligram of proteins (nmol TBARS mg prot^{-1}). Protein content of each sample was determined according to Bradford (1976).

2.5. Statistical analysis

All data are reported as mean ± standard error. Shapiro-Wilks test was applied to evaluate normality while Levene test was used to test the homogeneity of variance. For statistical comparisons of data among treatments, one way analysis of variance (ANOVA) followed by a Multiple Comparison Test (Tukey) were performed. Kruskal–Wallis test was applied to those variables with non-normal distribution or variance heterogeneity. p-Values below 0.05 were regarded as significant. In addition, principal component analysis (PCA) was performed in order to get a comprehensive view of the results, and to define the most important parameters involved in pesticides toxicity. To minimize the number of empty spaces in the dataset, multivariate analysis was carried out taking into account four cases (individuals with 44 variables measured). All statistical analysis was performed by the InfoStat software (Di Rienzo et al., 2012).

3. Results

3.1. Morphometric and hematological parameters

Morphometric and hematological biomarkers in control and exposed fish are summarized in Table 1. Fish exposed to a combination of ED (1.1 μg L^{-1}) and LC (0.7 μg L^{-1}) (ED + LC) showed a significant increase in the LSI ($p = 0.0102$, $F_{3,34} = 4.4$) and WBC ($p = 0.0004$, $F_{3,23} = 9.0$), which was not observed in fish exposed to both individual pesticides. All treatments produced an alteration in differential leucocytes counts, when compared with the control group.

3.2. Transaminases and alkaline phosphatase

ED + LC produced a significant inhibition of both transaminases in fish liver (AST: $p < 0.0001$, $F_{3,48} = 14.1$; ALT: $p = 0.0137$, $H = 10.7$) (Fig. 1A). On the contrary, an induction of AST ($p = 0.001$, $H = 16.3$) and ALT ($p = <0.0001$, $H = 32.5$) was observed in the kidney of fish exposed to both insecticides individually (Fig. 1B).

3.3. Oxidative stress biomarkers

Fish exposed to ED alone showed antioxidant enzymes induction in almost every tissue (Table 2). In this way, enzyme activity was increased in brain (GR: $p = 0.0089$, $F_{3,28} = 4.7$; CAT: $p = 0.0072$, $F_{3,36} = 4.7$; GST: $p = 0.0375$, $H = 8.4$), gills (GPx: $p = 0.0002$, $F_{3,29} = 9.4$; CAT: $p = 0.0158$, $F_{3,30} = 4.0$), and muscle (GST: $p = 0.0365$, $H = 8.51$; GPx: $p = 0.0163$, $F_{3,29} = 4.0$) of the exposed fish. In contrast, hepatic CAT ($p < 0.0001$, $F_{3,38} = 14.77$) and gill GST ($p = <0.0001$, $F_{3,44} = 13.4$) activities were significantly lower in ED-treated fish than in the control group.

On the other hand, LC exposed fish showed a different antioxidant response, which varied according to the tissue (Table 2). The enzyme activity was inhibited in gills (GR: $p = 0.0004$, $F_{3,28} = 8.5$), kidney (GST: $p = 0.0018$, $H = 15.0$; GR: $p = 0.0313$, H = 8.8), and liver

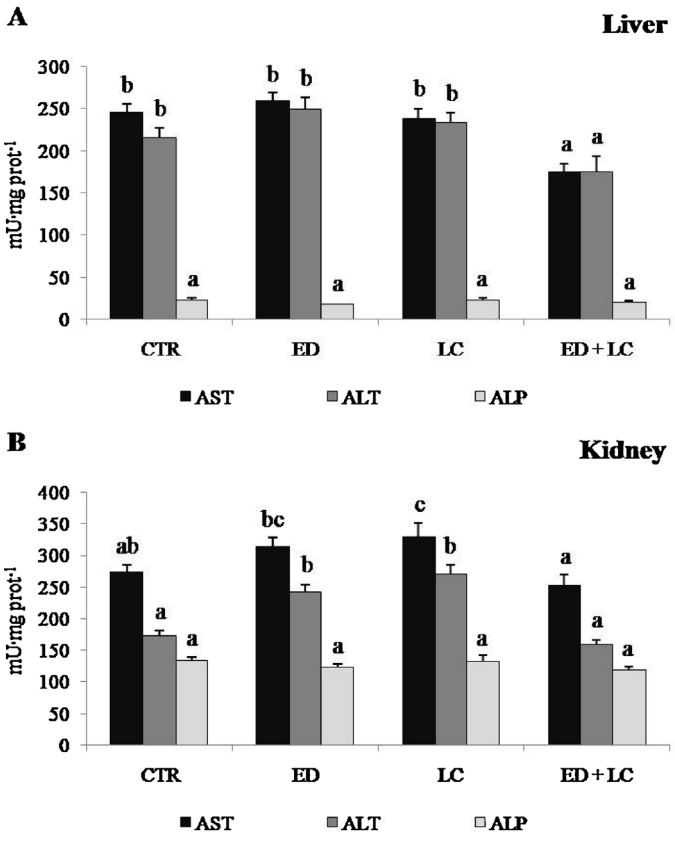

Fig. 1. Aspartate aminotransferase (AST), alanine aminotranferase (ALT), and alkaline phosphatase (ALP) activities in (A) liver and (B) kidney of *Piaractus mesopotamicus* exposed to 1.1 μg L^{-1} endosulfan (ED) and 0.7 μg L^{-1} lambda-cyhalothrin (LC) individually, and in combination (ED + LC), for 96 h. Means not sharing the same letter (a, b, or c) are significantly different at $p < 0.05$.

(GST: $p = 0.0004$, $H = 18.2$) whereas an increased enzyme activity was observed in brain and muscle.

Last, the antioxidant response of fish exposed to ED + LC was characterized by an enzyme induction in liver, kidney (GPx: $p = 0.0031$, $H = 13.9$, CAT: $p = 0.0007$, $H = 16.9$), brain, and muscle. Only in gills was registered an inhibition of GST and GR activity in ED + LC exposed fish (Table 2).

Related to the lipid oxidative damage, LPO levels increased in liver ($p = 0.0335$, $F_{3,13} = 3.9$), kidney ($p = 0.0097$, $H = 11.4$) and brain ($p = 0.0111$, $F_{3,14} = 5.4$) of fish exposed to ED + LC; whereas both individual insecticides caused an increment only in brain (Fig. 2).

3.4. Multi-biomarker approach

Two components were extracted by applying the principal component analysis (PCA) (Fig. 3). According to Legendre and Legendre (1979), interpretation of principal components may be done for eigenvalues of the data matrix higher than 1. The PCA indicated that 11 eigenvalues were higher than 1; moreover correlation coefficients are significant when they are higher than $\sqrt{d/n}$, d being the number of principal components and n the number of variables. Therefore, correlation coefficients >0.5 were indicative of a good representation of the variables with principal component axes. The components accounted for 39.3% of the original dataset variance. The first principal component (PC1) explained 25.3% of the variance, and showed significant positive loadings for the LSI, hematological parameters associated to white blood cells, antioxidant enzymes in both liver and kidney and LPO in liver, kidney and brain. On the contrary, a negative correlation was found mainly for transaminases in both tissues. PC2 (14.0% of the total variance) showed no strong

Table 1
Morphometric and hematological parameters of *Piaractus mesopotamicus* exposed to 1.1 μg L^{-1} endosulfan (ED) and 0.7 μg L^{-1} lambda-cyhalothrin (LC) individually, and in combination (ED + LC), for 96 h.

Parameter	Control	ED	LC	ED + LC
LSI	0.77 ± 0.03^{a}	0.80 ± 0.02^{a}	0.76 ± 0.03^{a}	0.94 ± 0.06^{b}
CF	4.62 ± 0.09	4.34 ± 0.13	4.62 ± 0.12	4.56 ± 0.08
RBC (10^6 μL^{-1})	2.15 ± 0.06	1.93 ± 0.11	2.19 ± 0.11	2.35 ± 0.08
Ht (%)	38.72 ± 1.00	35.80 ± 0.94	39.78 ± 1.08	39.26 ± 1.08
Hb (g dL^{-1})	7.43 ± 0.21	7.12 ± 0.28	7.54 ± 0.31	7.74 ± 0.25
MCH (pg)	36.10 ± 0.97	38.71 ± 2.89	33.49 ± 1.45	34.30 ± 1.23
MCV (μm^3)	185.00 ± 7.33	177.08 ± 11.88	173.20 ± 12.19	164.06 ± 10.78
MCHC (%)	19.86 ± 0.69	21.14 ± 0.73	19.58 ± 0.69	20.14 ± 0.78
WBC (μL)	4559 ± 612^{a}	4559 ± 612^{a}	5417 ± 524^{a}	9837 ± 1030^{b}
Lymphocytes (%)	29.19 ± 6.46^{b}	43.62 ± 5.87^{b}	36.57 ± 6.23^{b}	9.34 ± 3.32^{a}
Neutrophils (%)	54.66 ± 5.75^{b}	35.31 ± 5.08^{a}	40.83 ± 8.16^{a}	66.81 ± 8.55^{b}
Eosinophils (%)	0.93 ± 0.77^{a}	1.61 ± 0.78^{a}	0.46 ± 0,23^{a}	9.59 ± 2.63^{b}
Monocytes (%)	15.09 ± 3.19	19.43 ± 3.61	22.13 ± 3.76	14.24 ± 3.46

The values are expressed as means ± SE.
Means not sharing the same superscript (a or b) in each column are significantly different at $p < 0.05$.

positive loadings, but negative correlations mainly for the antioxidant enzyme GPx in brain, kidney and liver, and for CAT in gills.

4. Discussion

4.1. Morphometric and hematological parameters

Fish exposed to a combination of ED and LC (ED + LC) showed a significant increase in the LSI. As we also found an increase in the GST enzyme activity in liver of *P. mesopotamicus* exposed to ED + LC, is likely that the enlargement of the liver may increase its capacity to biotransform xenobiotics (Martin and Black, 1998; Almeida et al., 2005). These results are in agreement with Arnold et al. (1995), who observed an increase in the LSI and the GST activity of *Oncorhynchus mykiss* exposed to a mixture of ED and disulfoton.

The evaluation of fish hematological characteristics has become an important means of understanding normal and pathological processes, as well as toxicological impacts of insecticides (Singh and Srivastava, 2010; Li et al., 2011). Nonetheless, we found no significant responses in most hematological parameters of exposed fish. Only fish exposed to ED + LC showed a significant increase in WBC counts. It is likely that such increment can be due to the addition of both insecticides concentrations, which individually failed to cause significant effects on the treated fish. Taking into account the significant increase in brain, kidney, and liver LPO levels in ED + LC-exposed fish (Fig. 2A, C and D), WBC response may be explained by an increased leukocyte mobilization to protect tissues suffering oxidative damage.

Table 2
Activity of glutathione *S*-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT) in different tissues of *Piaractus mesopotamicus* after exposure to 1.1 μg L^{-1} endosulfan (ED) and 0.7 μg L^{-1} lambda-cyhalothrin (LC) individually, and in combination (ED + LC), for 96 h.

Enzyme[a]	Control	ED	LC	ED + LC
Brain				
GST	164.51 ± 10.63^{a}	194.81 ± 5.72^{b}	155.94 ± 13.04^{a}	164.00 ± 8.00^{a}
GR	19.51 ± 2.10^{a}	26.65 ± 2.06^{b}	29.54 ± 2.49^{b}	22.08 ± 1.55^{a}
GPx	53.18 ± 6.57	70.82 ± 7.32	65.82 ± 6.24	46.04 ± 5.68
CAT	11.24 ± 2.78^{a}	23.11 ± 2.23^{b}	21.33 ± 1.65^{b}	21.66 ± 3.10^{b}
Gills				
GST	118.53 ± 7.47^{c}	95.38 ± 4.32^{b}	69.01 ± 1.47^{a}	80.05 ± 7.79^{a}
GR	19.11 ± 2.07^{b}	18.81 ± 0.78^{b}	11.06 ± 0.93^{a}	14.03 ± 1.16^{a}
GPx	18.90 ± 2.64^{a}	25.70 ± 1.58^{b}	12.51 ± 0.46^{a}	16.79 ± 1.96^{a}
CAT	5.15 ± 0.86^{a}	8.10 ± 0.60^{b}	5.37 ± 0.52^{a}	6.02 ± 0.45^{aa}
Kidney				
GST	51.53 ± 3.21^{b}	72.24 ± 14.79^{bc}	40.21 ± 2.50^{a}	65.60 ± 3.18^{c}
GR	8.68 ± 0.80^{b}	12.97 ± 3.28^{c}	6.71 ± 0.77^{a}	11.13 ± 0.79^{c}
GPx	4.61 ± 0.84^{a}	10.09 ± 1.03^{c}	5.41 ± 0.59^{ab}	11.95 ± 2.40^{bc}
CAT	2.38 ± 0.32^{a}	2.44 ± 0.66^{a}	1.31 ± 0.10^{a}	4.80 ± 0.34^{b}
Liver				
GST	171.21 ± 6.37^{ab}	204.91 ± 23.86^{b}	145.64 ± 3.36^{a}	256.38 ± 15.33^{c}
GR	12.81 ± 1.31	14.11 ± 1.17	10.33 ± 1.03	12.49 ± 0.79
GPx	11.28 ± 1.93	15.80 ± 1.83	9.78 ± 1.31	14.13 ± 1.06
CAT	29.77 ± 1.34^{b}	23.76 ± 2.97^{a}	18.19 ± 1.00^{a}	42.09 ± 4.13^{c}
Muscle				
GST	24.16 ± 1.01^{a}	28.03 ± 1.76^{ab}	26.65 ± 1.46^{ab}	38.19 ± 4.48^{b}
GR	3.92 ± 0.26	4.87 ± 0.76	4.51 ± 0.53	3.44 ± 0.34
GPx	8.03 ± 0.82^{a}	12.00 ± 1.21^{b}	12.05 ± 1.03^{b}	10.28 ± 0.33^{b}
CAT	3.21 ± 0.29	4.03 ± 0.28	3.56 ± 0.30	3.47 ± 0.17

The values are expressed as means ± SE.
Means not sharing the same superscript (a or b) in each column are significantly different at $p < 0.05$.
[a] Activity expressed in mU mg prot^{-1} (GST, GR and GPx) or U mg prot^{-1} (CAT).

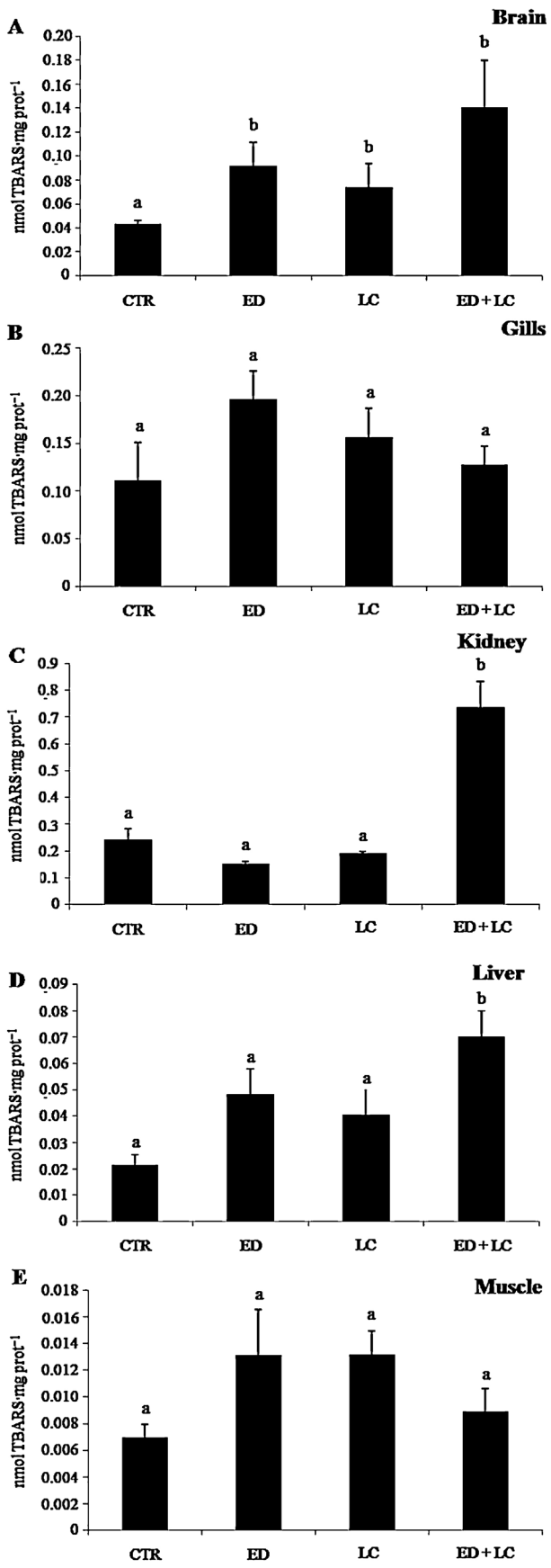

Fig. 2. TBARS levels in (A) brain, (B) gills, (C) kidney, (D) liver, and (E) muscle of *Piaractus mesopotamicus* exposed to 1.1 $\mu g\,L^{-1}$ endosulfan (ED) and 0.7 $\mu g\,L^{-1}$ lambda-cyhalothrin (LC) individually, and in combination (ED + LC), for 96 h. Means not sharing the same letter (a or b) are significantly different at $p < 0.05$.

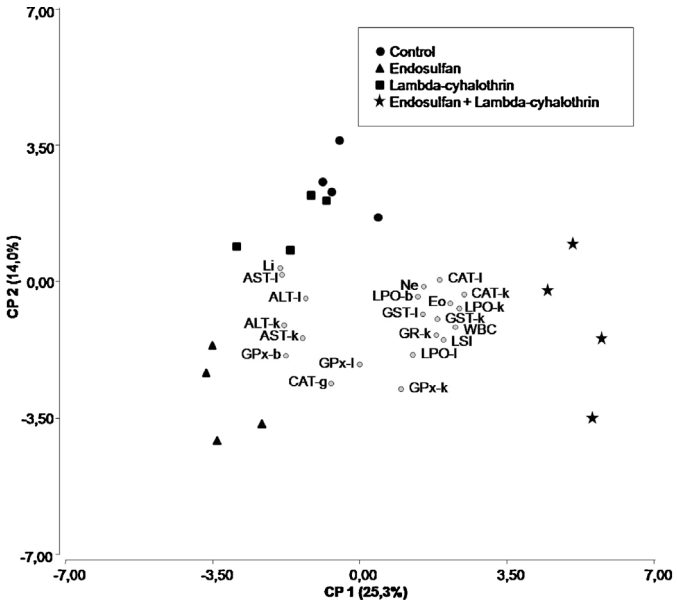

Fig. 3. Representation of the biomarkers (in letters) and individuals (in forms) onto the first factorial plane of the principal component analysis. Biomarkers with correlation coefficients >0.5 were represented in the PCA. Biomarkers abbreviations are explained in the text. Enzymes and lipid peroxidation abbreviations for different organs were followed by the corresponding letter. l: liver; k: kidney; g: gills; b: brain.

4.2. Transaminases activity

Aspartate and alanine aminotransferase 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 (Ramaiah, 2007). In our study, transaminases responses were different according to the analyzed organ. Liver AST and ALT activities fell significantly below the control levels in fish exposed to ED + LC, which was probably related to cytolysis and enzymes leakage into the blood. In a recent study, we found the same response in liver of *Cichlasoma dimerus* exposed to individual ED for 96 h (Bacchetta et al., 2011b). On the contrary, fish exposed to individual ED and LC showed an increase in the kidney AST and ALT activities, but not in combination. Elevation in the transaminases indicates the utilization of amino acids for the oxidation or for glucogenesis and it is used to determine tissue damage, although it is not clear why this response was not observed in the ED + LC treatment. According to our results, antioxidant enzymes activities and LPO levels showed a strong increase in kidney of the ED + LC-exposed fish. Some metabolites produced through antioxidant reactions and oxidative processes may be affecting amino acid metabolism, and/or interfering with transaminases, neutralizing their activity. Further studies are needed to know why enzyme activities remain unchanged.

4.3. Oxidative stress biomarkers

ED + LC significantly induced liver and kidney antioxidant enzymes which are crucial in the detoxification from oxyradicals to non-reactive molecules. These results suggest that the combination of both insecticides leads to the production of superoxide anions and lipid peroxides, triggering the activation of the enzymatic antioxidant defense system in order to prevent oxidative damage. However, the activation of detoxification and antioxidant mechanisms failed to counteract oxidative damage, since liver and kidney LPO levels significantly increased in fish exposed to ED + LC.

Induction of GST might indicate that phase II conjugation reactions would be one way in the metabolism of pesticides in *P. mesopotamicus*. It is well known that these reactions can generate ROS and damage cellular components such as lipids, which may be related to the increase in CAT activity and LPO levels. An increased activity of multiple enzymes in kidney was reported by Oruç et al. (2004) in *Oreochromis niloticus* exposed to the herbicide 2,4-D and the insecticide azinphos-methyl alone, and in combination. In our study, ED-exposed fish showed a similar response to fish exposed to ED + LC. Such response was characterized by an increase in GST, GR and GPx activity; whereas the opposite was observed in kidney of the pyrethroid exposed fish. Therefore, we suggest that ED could be the determinant of the antioxidant response in kidney of *P. mesopotamicus*. In spite of the differential responses obtained in both ED and LC treatments, none of them caused alterations in LPO levels, whereas their combination clearly increased this biomarker. These results suggest that ED and LC could have some interactive effects on the fish oxidative metabolism capacity, and highlight the importance of kidney in detoxification and antioxidant activity during pesticides exposure.

Antioxidant enzymes responses in gills were the opposite of those observed in liver and kidney. Exposure to ED + LC resulted in a decreased activity of the antioxidant enzymes GST and GR. It is well known that both diminished enzymes are sensitive to products of the Haber–Weiss and Fenton reactions (Hermes-Lima, 2004). This response was also observed in *Carassius auratus* exposed to three organophosphate and carbamate insecticides, individually and in mixture, showing an enzymatic inhibition in all treatments (Wang et al., 2009). Moreover, only ED exposed fish showed an activation of GPx and CAT probably related to the increase in LPO levels in such treatment even if this change was no statistically significant (Fig. 2B). In spite of the differential response in antioxidant enzymes, LPO levels were unaffected by insecticides treatments. Possibly, LPO increase may be masked by overcompensation through active stimulation of cellular regeneration. Additional investigation is needed to know the actual cause of the reported response.

Although the detoxification functions occur primarily in the liver, skeletal muscle is also involved in these processes. Skeletal muscle cells have been shown to express different types of xenobiotic-metabolizing enzymes, including cytochrome P450 and GST (Bainy et al., 1999; Hussey et al., 1991). In our study, ED and LC alone and in combination produced an antioxidant induction (GST and GPx) in muscle of exposed fish. Although the relative amount of enzymes in skeletal muscle is lower than in the liver, its importance in the detoxification processes may be considered since it represents a high body mass percentage (Monteiro et al., 2006). Thus, our results suggest that the activation of antioxidant mechanisms in gills and muscle was effective against oxidative damage.

Brain CAT activity and LPO levels were significantly increased in all treatments tested. CAT is the first enzyme to show alterations following induction of oxidative stress (Jin et al., 2010). Then, the direct relationship between CAT and LPO increases in fish brain is corroborated by our results.

4.4. Multi-biomarker approach

It is known the importance of the use of a wide battery of biomarkers when assessing the biological effects in impacted environments, since a single biomarker may not reflect the health status of an organism. Some authors suggest that the selection of an appropriate battery of biomarkers can avoid false-negative responses obtained with a single biomarker (Beliaeff and Burgeot, 2002; Linde-Arias et al., 2008). The main goal of the present work was to evaluate the toxicological effects of a combination of pesticides using a multi-biomarker approach. The principal component analysis (Fig. 3) showed that the set of selected biomarkers was able to separate ED + LC from control and individual insecticides treatments. Biomarkers which responded in a significant way were mainly the LSI, hematological parameters associated to white blood cells, transaminases, antioxidant enzymes and lipid peroxidation. Overall, our current and previous results (Cazenave et al., 2009) demonstrate the utility of most of those biomarkers to assess biological effects of the exposure to complex pollutants mixtures.

5. Conclusions

Our results revealed different effects in *P. mesopotamicus* exposed to individual insecticides and their combination, pointing out that only the ED + LC treatment produced an increase in the LSI, an activation of the immune system and oxidative damage in many tissues. The most affected organs were the liver, kidney and brain, offering further support for the hepato and neurotoxicity induced by both insecticides in combination. Thus, we suggest that single-chemical risk assessment is likely to underestimate the impact of insecticides on fish inhabiting aquatic systems where mixtures occur.

We propose that the LSI, WBC, antioxidant enzymes activity, and LPO levels can be used as a set of biomarkers for the assessment of fish health condition and toxicological effects of pesticides. To the best of our knowledge, this is the first report of a multi-biomarker assessment carried out on fish exposed to combined insecticides. This work underscores the need for new studies to explore how complex mixtures of pesticides can affect physiological responses of aquatic organisms.

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