Antioxidant, phase II and III responses induced by lipoic acid in the fish *Jenynsia multidentata* (Anablapidae) and its influence on endolsulfan accumulation and toxicity

J.M. Monserrat [a,b,*], M.L. Garcia [a,b], J. Ventura-Lima [a,b], M. González [c], M.L. Ballesteros [c], K.S.B. Miglioranza [c], M.V. Amé [d], D.A. Wunderlin [d]

[a] Curso de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Universidade Federal do Rio Grande – FURG, Cx. P. 474, CEP 96.201-900, Rio Grande, RS, Brazil
[b] Instituto de Ciências Biológicas (ICB), FURG, Brazil
[c] Laboratório de Ecotoxicología, Universidad Nacional de Mar del Plata, Instituto de Investigaciones Marinas y Costeras (IIMyC)-CONICET, Buenos Aires, Argentina
[d] Universidad Nacional de Córdoba – CONICET, Facultad Ciencias Químicas, Córdoba, Argentina

ABSTRACT

Antioxidants like lipoic acid (LA) are known to trigger augmented antioxidant and phase II and III responses. This study aimed to evaluate the effect of LA in P-glycoprotein (Pgp) expression, glutathione-S-transferase (GST) activity, total antioxidant competence, levels of lipid peroxides (TBARS) and accumulation of the organochlorine insecticide endosulfan (Endo: α-, β-isomers and sulfate metabolite) in different organs of the fish *Jenynsia multidentata*. One hundred and twenty females (1.55 ± 0.07 g) were fed during 8 days with (n = 60) or without (n = 60) a LA enriched ration (6000 mg/kg). Four experimental groups were defined: −LA/−Endo; +LA/−Endo; −LA/+Endo; and +LA/+Endo. Endo groups were exposed during 24 h to 1.4 μg of insecticide/L. Results showed that only LA induced a significant increment in liver Pgp expression. GST activity was augmented in liver after exposure to LA or Endo. TBARS levels were lowered in liver and gills after LA pre-treatment. Total antioxidant capacity was lowered in liver of Endo exposed fish, a result that was reversed by LA pre-treatment. It is concluded that LA induced the expected effects in terms of Pgp expression, GST activity and reduced TBARS levels although favored α-Endo accumulation in brain. However, the Endo metabolism to the more persistent endosulfan sulfate was not facilitated by LA pre-treatment.

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1. Introduction

Lipoic acid (LA) is a well-known cofactor of mitochondrial dehydrogenases with excellent antioxidant properties [1–3]. In fish, many authors showed biochemical effects of LA in terms of circulating aminoacids levels, lipid metabolism and ascorbic acid content [4–6]. *Corydoras paleatus* fed with a LA enriched diet, showed lower levels of carbonylated proteins in liver and muscle, demonstrating the antioxidant effect of this molecule [7]. Other aquatic organisms like the gastropod *Haliotis discus hannai* showed augmented levels of glutathione (GSH) in the hepatopancreas when fed with LA added to diet (up to 3200 mg/kg) [8].

Suh et al. [3] showed that LA induces the antioxidant system through the control of the expression of key genes as, for example, those that code for both subunits of glutamate–cysteine ligase (GCL), the rate-limiting enzyme for the synthesis of glutathione (GSH). Antioxidant genes and other involved in phase II and III reactions present motifs known as antioxidant response elements (ARE) at the promoters or upstream of them. Transcription factors like Nrf2 together with partners like Jun or Fos can bind to ARE regions, regulating the constitutive and inducible expression of genes like GCL subunits, glutathione-S-transferase (GST) and multidrug resistance proteins [3,9]. The influence of LA on the antioxidant and detoxification systems has prompted its use as a chemoprotectant. In fish, Amado et al. [10] reported that carp *Cyprinus carpio* injected i.p. with LA (40 mg/kg) augmented the expression of several GST forms. Moreover, LA treatment showed to be effective to counteract liver GST inhibition elicited by a cyanotoxin, microcystin. As several molecules induce toxicity by direct or indirect generation of oxidative stress [11], the pre-treatment with LA should protect or ameliorate these toxic effects.

As mentioned above, Nrf2-reponsive genes include multidrug resistance-associated proteins (Mrp) and multidrug resistance proteins (Mdr) [9]. Amé et al. [12] have characterized the P-glycoprotein multidrug transporter protein (Pgp) in the fish

Jenynsia multidentata (Anablapidae), a protein known to be involved in the protection against toxins like microcystin. Sreeramulu et el. [13] verified that Pgp activity in reconstituted proteoliposomes was inhibited by several pesticides, including the organochlorine endosulfan, meaning that pesticides can affect Pgp functionality, in turn affecting the clearing of xenobiotics from the cells, an effect that should augment the toxicity of these molecules.

Endosulfan is a widespread insecticide that has been used in many countries in different crops such as cotton, fruits, tomato and cereals. Due to its massive use and volatility it occurs on several environmental compartments [14]. Moreover, endosulfan represents one of the main pesticide used in Argentina, being their residues (i.e., α- and β-isomers and endosulfan sulfate) found in ground and stream water at concentration that in some cases are above international limits for the protection of aquatic organisms (3 ng/L) [14]. Toxic effects induced by this chemical on fish at biochemical (enzyme induction) and hematological levels [15] as well as on reproduction and behavior, are well known. Particularly, previous results, using the model fish, *J. multidentata*, showed that sub-lethal concentrations of technical endosulfan (1.4 μg/L) augmented lipid peroxidation in liver and brain [16].

Thus, considering the modulation of LA in the antioxidant and detoxifying systems, the present study aimed to evaluate its potential effect as chemoprotectant agent against endosulfan toxicity in *J. multidentata* by measuring several parameters associated with antioxidant defense system and oxidative damage in conjunction with changes in the expression of Pgp protein induced by LA, as well as the effect on endosulfan accumulation pattern.

2. Materials and methods

2.1. Fish sampling

Female adult fish (1.55 ± 0.07 g; $n = 120$) were sampled using a backpack electrofisher equipment from a non-polluted area at San Antonio River (64°13′20″W, 31°12′80″S-Córdoba, Argentina), as in previous studies [12,16,17]. Fish were transported to the laboratory in water tanks (20 L) within 4 h after capture. Once in laboratory, fish were maintained during 8 days at 21 ± 1 °C with photoperiod fixed in 12 L:12 D in 40 L tanks with 100% oxygen saturated water and pH of 7.85 ± 0.02.

2.2. Preparation of lipoic acid-enriched ration

The one hundred and twenty fish were randomly divided in two groups: fed with (+LA) or without (−LA) lipoic acid enriched diet during eight days. Racemic lipoic acid (ACROS Organics, purity >98%) was added to a commercial fish diet (TetraColor, 47.5% of protein), mixed with ultrapure water (Millipore, Milli Q system) and dried at 50 °C to obtain pellets. Fish were fed twice a day (morning: 8:30 h; afternoon: 15:30 h), in order to get a daily ingestion of 2% of body weight.

In order to measure the effective dose of LA in the ration, 0.25 g were dissolved in 2 ml of methanol (HPLC grade) and extracted overnight at room temperature. LA was quantified by HPLC–ESI-MS using a Varian 1200 triple quadrupole, equipped with an ESI ion source operated in negative mode with nitrogen as main gas (74 psi), drying gas (21 psi, 250 °C) and synthetic air (5.0 grade, 99.9999%) as nebulizing gas (58 psi); needle 5000 V, shield at 600 V, capillary −50 V. The detector was set at 1900 V, with a requested scan of 0.5 s. LA was separated using a column Varian Polaris 5 μm C18-A (50 × 2.0 mm). Solvent delivery was performed at 0.25 ml/min by two pumps Varian Prostar 210 Dynamax using ammonium acetate (4 mM) (A) and methanol (B), starting with 50% B, changing to 90% B within 8 min, held by 2 min, returning to 50% B in one minute and keeping this condition for four additional minutes to achieve column stabilization before next run (total run time 15 min). Quantification was performed using external standard method, using pure LA dissolved in the starting mobile phase (50%A: 50%B). Both samples and standard solutions were analyzed by triplicate.

The −LA group was fed only with the commercial ration, which was prepared with the same procedure cited above but omitting the adding of LA. Visual examination confirmed that fishes accepted very well both kinds of rations, feeding even when the pellets sank to the bottom of the aquariums.

2.3. Exposure to technical endosulfan (Endo)

After eight days of feeding fish with (+LA) or without (−LA) enriched ration, fish were split into four groups: −LA/−Endo; +LA/−Endo; −LA/+Endo; and +LA/+Endo. +Endo groups were exposed to a commercial formulation of endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-:benzodioxathiepine-3-oxide), Galgofan 35 EC®, with a α-/β-ratio equal to 70:30. A concentration of 1.4 μg/L during 24 h exposure was selected on the basis of Ballesteros et al. [16], that demonstrated antioxidant and oxidative damage responses. In a posterior study, Ballesteros et al. [18] determined that the measured total endosulfan concentration in water represented almost 23–45% of the nominal assayed concentration (1.4 μg/L) after 24 h exposure. Fish exposure of +Endo group was performed by immersion and the organisms were starved during exposure.

2.4. Biochemical analysis

After Endo exposure, fish were sacrificed and organs (brain, gills and liver) dissected and homogenized (1:5 for brain, 1:10 for the other organs) in Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) [7]. Samples were centrifuged at $10,000 \times g$ during 20 min at 4 °C and the supernatant used for antioxidant and oxidative damage measurements. Previously, total protein content was determined by the Biuret method (550 nm), in triplicate, using a microplate reader (BioTek LX 800).

The activity of the enzyme glutathione S-transferase (GST) was determined following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm [19,20]. Total antioxidant competence against peroxyl radicals was determined through reactive oxygen species (ROS) determination in sample tissues treated or not with a peroxyl radical generator. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2,2′-azobis(2-methylpropionamidine)dihydrochloride (ABAP; 4 mM; Aldrich) [21]. ROS concentration was measured along 30 min with the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) at a final concentration of 40 μM, in according to the methodology reported by Amado et al. [22]. These authors integrated the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were expressed as area difference of FU min^{-1} in the same sample, with and without ABAP addition, and standardized to the ROS area without ABAP as follows:

$$(\text{ROS area}_{\text{with ABAP}} - \text{ROS area}_{\text{without ABAP}})/\text{ROS area}_{\text{without ABAP}}$$

However, data analysis using this methodology is cumbersome and time-consuming. A new approach was defined to calculate the relative area, using only FU data for 30 min measurement and the following expression:

$$(\text{FU 30min}_{\text{with ABAP}} - \text{FU 30min}_{\text{without ABAP}})/\text{FU 30min}_{\text{without ABAP}}$$

Both approaches were then compared using correlation analysis.

Oxidative damage was measured, considering the peroxidation of lipids, using the TBARS method [23]. This methodology involves the reaction of malondialdehyde (MDA), a degradation product of the lipid peroxidation, with thiobarbituric acid (TBA) at high temperature and acidity, producing a fluorophore that was quantified by fluorometry (excitation: 520 nm, emission: 580 nm). Briefly, aliquots of homogenized organs were incubated at 95 °C during 30 min with 35 µM of butylated hydroxytoluene (BHT), 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid and 0.8% TBA. After cooling to room temperature, n-butanol was added, followed by centrifugation at $3000 \times g$ during 10 min at 15 °C. Tetramethoxypropane (ACROS Organics) was used as standard. Fluorescence was read at room temperature using a plate reader fluorimeter (Victor 2, Perkin Elmer).

2.5. Expression of Pgp

After Endo exposure, fish were sacrificed and organs dissected (brain, gills and liver). Total RNA was extracted in according to Amé et al. [12], using a solution of guanidine thiocyanate–phenol–chloroform (40 µl/mg of tissue). Then, the RNA pellet was resuspended in nuclease-free water. RNA purity and concentration were determined by spectrophotometry at 260 and 280 nm. RNA integrity was assured after running a non-denaturing agarose gel electrophoresis. A complementary DNA (cDNA) strand was synthesized by the M-MLV reverse transcriptase (Invitrogen, Carlsbad, USA) using oligo (dT)$_{15}$ primer (Biodynamics S.R.L, Buenos Aires, Argentina). For mRNA relative expression levels assessment, real-time PCR was performed on a Bio-Rad iQ cycler (Bio-Rad, Hercules, USA). Twenty-five nanograms of total RNA transcribed were mixed with SYBR Green PCR Master Mix, spiked with fluorescein 10 nM (Applied Biosystems, Foster City, USA) and 7.5 pmol primers to a final volume of 25 µL. Primers sequences, for Pgp (NCBI accession number: EF362745), were: Sense 5′-CTGCACGCTAGCGGAAAAC-3′ and Anti-sense 5′-CCTCTATCTCCTCCATGGTCACA-3′ [12]. Pgp expression was relativized to the β-actin gene (NCBI accession number: EF362747), which expression was quantified employing the following primers: Sense 5′-AAAGCCAACAGGGAGAAGATGAC-3′ and Anti-sense 5′-GCCTGGATGGCAACGTACA-3′. Cycling conditions were the same to those established by Amé et al. [12]: 1 run at 95 °C during 3 min and 45 runs at 95 °C during 10 s and 58.7 °C during 45 s. Real-time PCR reactions were run in triplicate for each sample; amplification products were quantified by comparison of experimental Ct (the PCR cycle where an increase in fluorescence over background levels first occurred). Amplification efficiency on qRTPCR was higher than 95%, with variation coefficient varying from 1% to 3%. The relative expression levels of Pgp were based on mean Ct differences between the tested groups (+LA/−Endo; −LA/+Endo and +LA/+Endo) and −LA/−Endo used as the control group [24].

2.6. Measurement of α- and β-endosulfan and endosulfan sulfate in fish organs

Organs were ground in a mortar with addition of anhydrous sodium sulfate in relation 1:5 (w/w), Soxhlet-extracted during 8 h with a mixture of hexane and dichlorometane (50:50). Extracts were concentrated using a flow of nitrogen to ca 3 ml. Lipids were removed from the extracts by gel permeation chromatography (GPC) using Bio Beads S-X3 (200–400 mesh) (Bio-Rads Laboratory, Hercules, California). Fatty-free extracts were fractionated by column chromatography using silica gel [25].

Endosulfan isomers (α- and β-) and its metabolite (endosulfan sulfate) were identified and quantified by gas chromatography (Shimadzu 17-A gas equipped with a ^{63}Ni Electron Capture Detector, GC-ECD), using a capillary column coated with SPB-5 [(5% phenyl)-methyl polysiloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco Inc.]. One microliter extract was injected using the splitless mode at 275 °C. The ECD temperature was 290 °C. The oven temperature program was: starting at 100 °C and held for 1 min, increasing 5 °C/min up to 150 °C, held for 1 min, further increase at 1.5 °C/min up to 240 °C, followed by a ramp at 10 °C/min until reach 300 °C, held for 10 min. Ultra-high purity Helium (5.0 grade) was used as carrier gas (1.5 ml/min), while nitrogen (5.0 grade) was used as make-up gas [26]. Quantification of both endosulfan isomers and endosulfan sulfate was done using both pure compounds as external standards and PCB #103 as internal standard. The sum of α, β isomers and endosulfan sulfate is named as Endo through the manuscript.

Laboratory and instrumental blanks were frequently analyzed to ensure the absence of contaminants, or interference arising from samples or laboratory handling. Recoveries, calculated from spiked matrixes, were greater than 90%. Detection limits (LOD), ranged between 0.08–0.33 ng/mL in agreement with values reported in the literature [27].

2.7. Statistical analysis

All results were expressed as mean + 1 standard error. Significant differences were assessed by analysis of variance (ANOVA), being the treatments the four experimental groups (−LA/−Endo; +LA/−Endo; −LA/+Endo; and +LA/+Endo). *Post hoc* comparisons were performed using the Newman–Keuls test or orthogonal contrasts. ANOVA assumptions (normality and variance homogeneity) were previously checked [28]. As mentioned in Section 2.4, correlation analysis was employed for the two ways of estimation of total antioxidant capacity. Non-parametric Kruskall–Wallis and Friedman test were employed in order to analyze the accumulation of endosulfan metabolites. In all cases, type I error probability (α) was fixed in 5%.

3. Results

The content of LA in the TetraColor diet was 6000 mg/kg as determined by HPLC–ESI-MS. No mortality was registered after 24 h neither in control groups (−LA/−Endo and +LA/−Endo) nor in endosulfan-exposed groups (−LA/+Endo and +LA/+Endo). It was observed no differences in terms of altered swimming behaviour of fish exposed to endosulfan (with or without lipoic acid pre-exposure).

As Fig. 1 shows all analyzed organs (liver, brain and gills) showed an excellent linear relationship, for the calculation of total antioxidant capacity against peroxyl radicals, between the method described by Amado et al. [22] and the method proposed in the present work. Taking into account this good relationship, the total antioxidant capacity was calculated using only the fluorescence data after 30 min.

Total antioxidant capacity against peroxyl radicals was not altered by LA pre-treatment in none of three organs analyzed since no differences between −LA/−Endo and +LA/−Endo groups were detected ($p > 0.05$; Fig. 2). However, pre-treatment with LA did induced changes in the antioxidant response after exposure to Endo. In livers from −LA/+Endo group, total antioxidant capacity diminished (higher relative area) when compared to the control group ($p < 0.05$; Fig. 2a), a result not observed in fish pre-treated with LA (+LA/+Endo group) ($p > 0.05$; Fig. 2a). In brain, an opposite result was observed: in fish not exposed to LA, Endo exposure (−LA/+Endo group) triggers an augmented antioxidant capacity (lower relative area) when compared with the control group ($p < 0.05$;
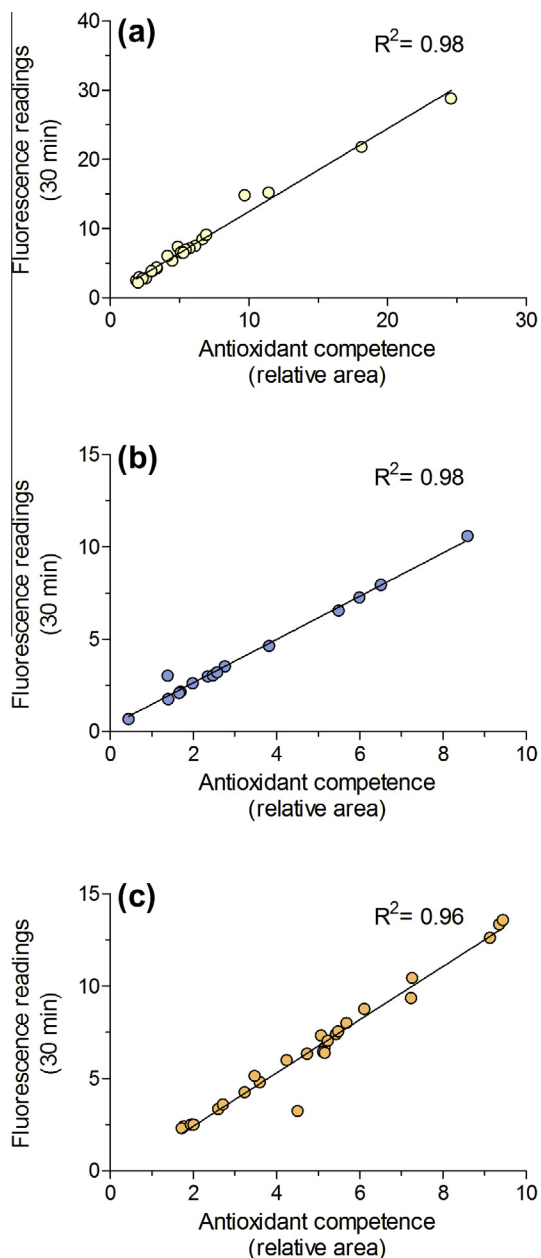
Fig. 1. Correlation in liver (a), brain (b) and gills (c) samples employing the methodology for measurement of total antioxidant capacity described by Amado et al. (2009) (relative area in the x-axis) and the modification proposed in present study (fluorescence readings; y-axis).
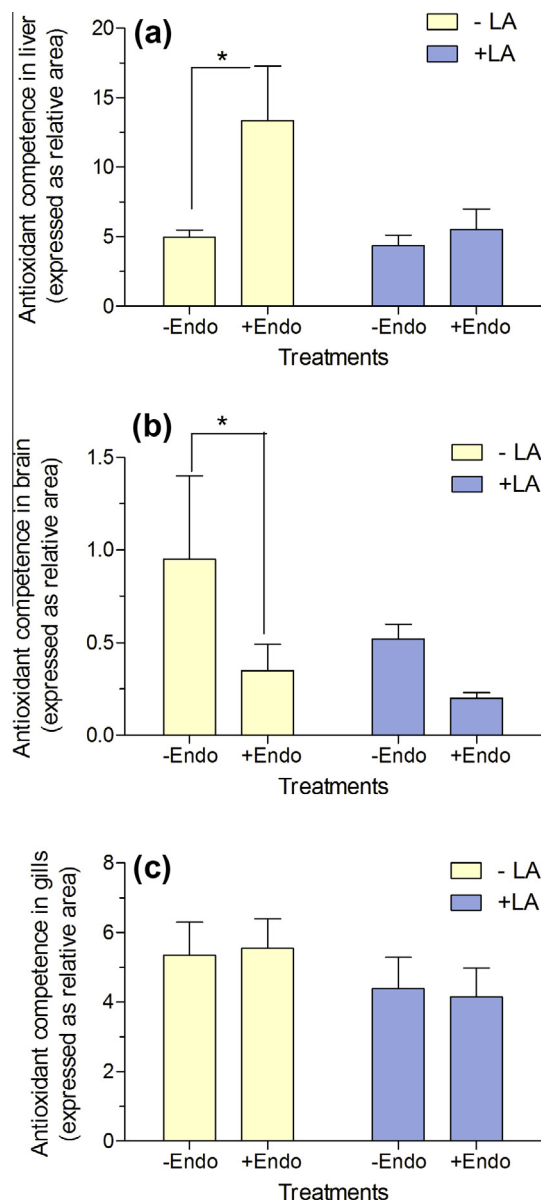

Fig. 2. Total antioxidant capacity against peroxyl radicals in liver (a), brain (b) and gills (c) from *J. multidentata*. Data are expressed as mean ± 1 SE (n = 3–6). Antioxidant competence was calculated as depicted in the Section 2 (readings of fluorescence after 30 min, with or without ABAP addition). Asterisk ([*]) indicates significant differences ($p < 0.05$) in the treatments connected by lines. ABAP: 2,2′-azobis 2 methylpropionamidine dihydrochloride. −LA: fish that were fed with standard ration. +LA: fish fed with lipoic acid-enriched ration. −Endo: fish not exposed to technical endosulfan. +Endo: fish exposed to technical endosulfan.

Fig. 2b). In brain no differences between −Endo and +Endo groups were observed in the LA-treated fish (+LA groups) ($p > 0.05$; Fig. 2b). Neither LA pre-exposure nor Endo treatment affected the total antioxidant capacity in gills ($p > 0.05$; Fig. 2c).

Fish fed with enriched LA diet induced an increase in GST activity in liver ($p < 0.05$), and a reduction in gills ($p < 0.05$) both compared with −LA/−Endo group. Additionally, Endo exposure induced an augmented GST activity in the liver of fish without LA pretreatment ($p < 0.05$; Fig. 3a). Conversely, no differences between −Endo and +Endo groups were observed, both presenting higher activity than the corresponding to −LA/−Endo group ($p < 0.05$; Fig. 3a). LA pre-treatment was not effective in brain, where Endo exposure prompted an augmented GST activity ($p < 0.05$; Fig. 3b). In gills, GST was diminished in the −LA/+Endo group when compared with −LA/−Endo ($p < 0.05$; Fig. 3c), whereas in the +LA groups, Endo exposure did not alter the enzyme activity ($p < 0.05$; Fig. 3c).

In liver, Pgp expression was augmented in the +LA groups when compared with −LA groups, irrespective of Endo exposure ($p < 0.05$; Fig. 4a). No effects were registered in brain ($p > 0.05$; Fig. 4b).Conversely, in gills, Endo exposure diminished Pgp expression in the −LA group when compared with the −LA/−Endo group ($p < 0.05$; Fig. 4b). Moreover, groups receiving LA-diet remained with a lower expression than the −LA/−Endo group ($p < 0.05$), but the expression was not modified by Endo exposure ($p < 0.05$; Fig. 4c).

Lipid peroxidation, as measured by the concentration of TBARS, was reduced as consequence of LA pre-treatment in the liver ($p < 0.05$; Fig. 5a). However no effect related to Endo exposure
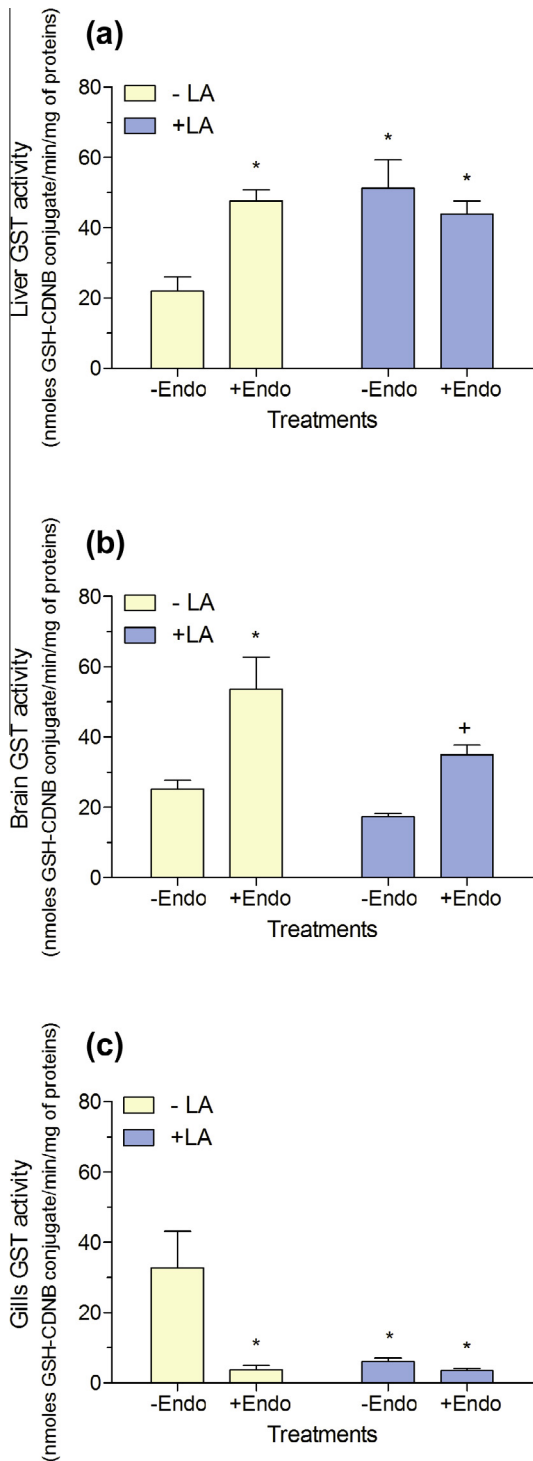
Fig. 3. Glutathione-S-transferase activity (in nmol CDNB-GSH conjugate/min/mg of proteins) in liver (a), brain (b) and gills (c) from *Jenynsia multidentata*. Data are expressed as mean ± 1 SE ($n = 3$–6). Asterisk (*) indicates significant differences ($p < 0.05$) when compared with −LA/−Endo treatment. Plus (+) sign indicates significant differences ($p < 0.05$) when compared with +LA/−Endo treatment. −LA: fish that were fed with standard ration. +LA: fish fed with lipoic acid-enriched ration. −Endo: fish not exposed to technical endosulfan. +Endo: fish exposed to technical endosulfan. GSH: reduced glutathione. CDNB: 1-chloro-2,4-dinitrobenzene.

Fig. 4. Relative P-gp expression in liver (a), brain (b) and gills (c) from *Jenynsia multidentata*. Data are expressed as mean ± 1 SE ($n = 3$). Asterisk (*) indicates significant differences ($p < 0.05$) when compared with −LA/−Endo treatment. Plus (+) sign indicates significant differences ($p < 0.05$) when compared with −LA/+Endo treatment. −LA: fish that were fed with standard ration. +LA: fish fed with lipoic acid-enriched ration. −Endo: fish not exposed to technical endosulfan. +Endo: fish exposed to technical endosulfan.

was observed in this organ ($p > 0.05$; Fig. 5a). Furthermore, no influences of LA or Endo were registered in the brain ($p > 0.05$; Fig. 5b). However, in gills, LA pr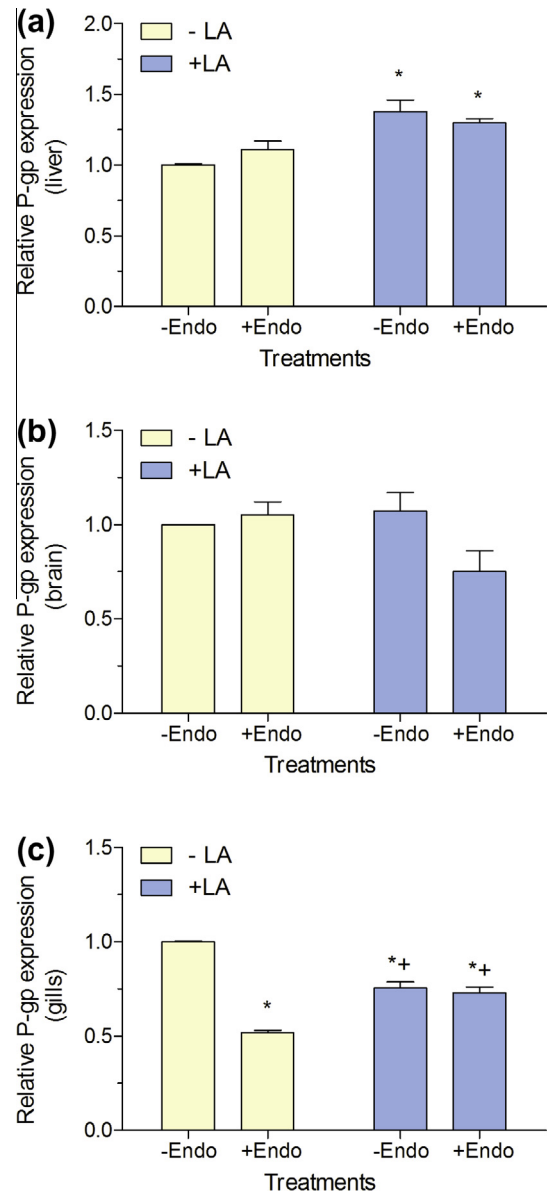e-treatment induced a reduction of TBARS concentration when comparing −LA/−Endo and +LA/−Endo groups ($p < 0.05$; Fig. 5c). Nevertheless Endo did not induced lipid peroxidation when compared with −Endo groups ($p > 0.05$).

Although pre-treatment with LA seemed to augment accumulation of endosulfan isomers and their metabolites (Fig. 6), statistical differences ($p < 0.05$) were detected only for brain from fish fed with LA, where higher level of α-endosulfan was observed (Fig. 6b). In each organ, no differences ($p > 0.05$) were detected for β-endosulfan, endosulfan sulfate and total endosulfan level. In brain from fish from both treatments did not accumulate β-isomer (Fig. 6b). A significant higher endosulfan sulfate concentration ($p < 0.05$) was observed in liver when compared with the other organs (compared the scales in Fig. 6), whereas the highest levels of total endosulfan were registered in liver and gills (Fig. 6).
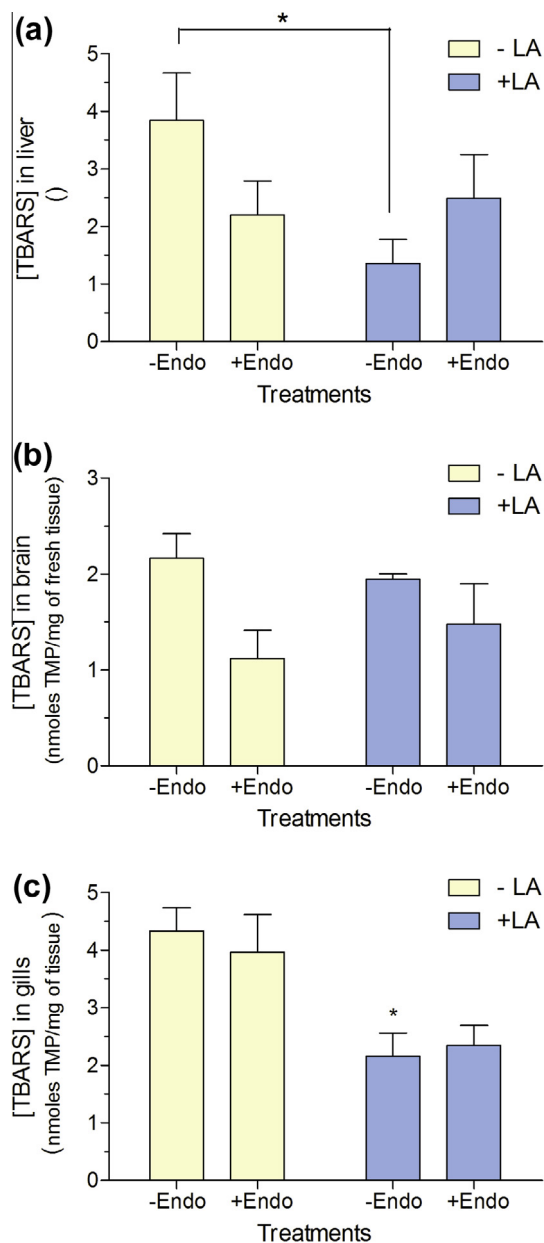
Fig. 5. Concentration of thiobarbituric reactive substances (TBARS) in liver (a), brain (b) and gills (c) from *Jenynsia multidentata*. Data are expressed as mean ± 1 SE (n = 3–6). Asterisk (*) indicates significant differences ($p < 0.05$) when compared with −LA/−Endo treatment or in the treatments connected by lines. −LA: fish that were fed with standard ration. +LA: fish fed with lipoic acid-enriched ration. −Endo: fish not exposed to technical endosulfan. +Endo: fish exposed to technical endosulfan.

Fig. 6. Concentration (ng g^{-1} of wet weight) of technical endosulfan in liver (a), brain (b) and gills (c) from *Jenynsia multidentata*. Data are expressed as mean ± 1 SE (n = 5). −LA: fish that were fed with standard ration. +LA: fish fed with lipoic acid-enriched ration. −Endo: fish not exposed to technical endosulfan. +Endo: fish exposed to technical endosulfan. α-E: α isomer of technical endosulfan. β-E: β isomer of technical endosulfan. E-S: endosulfan sulfate. Σ-E: total endosulfan. LD: lower than the detection limit. Asterisk (*) indicates significant differences ($p < 0.05$) between −LA and +LA treatment for a particular endosulfan metabolite.

4. Discussion

The fish *J. multidentata* has been employed as animal model in virtue that this species inhabits both polluted and non-polluted areas in relative high number of individuals in the Neotropical region of South America [12]. Recent papers have employed this species in molecular biology studies, describing partial nucleotides sequences for *Pgp* and *CYP 1A* genes [12,29] and also in biochemical studies, aiming to estimate total antioxidant competence [22].

Although the importance of LA as antioxidant has been reported several years ago [1,2], only few aquatic species have been analyzed in terms of their antioxidant responses after the administration of this molecule.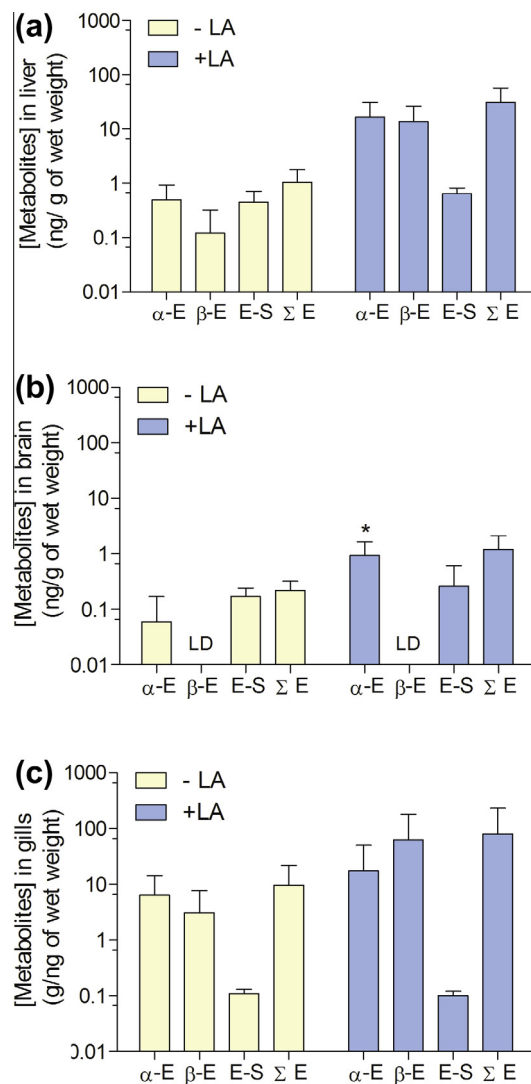 In the present study, several biochemical responses indicated better antioxidant and detoxification competence after LA administration, including: (1) higher liver GST activity; (2) higher liver Pgp expression; and (3) lower TBARS levels in liver and gills. It is worthy to remark that a clear reduction of GST activity was observed in gills. A high dose of LA (3200 mg/kg), even lower to that employed in present study (6000 mg/kg) was found to reduce glutathione peroxidase activity in hepatopancreas of the snail *Haliotis discus hannai* [8] and also in the fish *Trachinotus marginatus* fed with 524 mg LA/kg it was observed a reduction of gills GST activity [30]. Aleksunes and Manautou [9] and Kwak et al. [31] postulated that transcription factor Nrf2 is sequestered by the protein Keap1 in the cytoplasm, being the oxidation of −SH residues in Keap1 protein one of the signals that trigger Nrf2 migration to the nucleus. It is possible that an organ with high concentration of an antioxidant like LA could have a reductive redox state, thus down-regulating ARE-controlled genes.

Endosulfan is an organochlorine pesticide used not only for agriculture but also in shrimp farms [32]. The last use has

prompted the interest to study the toxicological effects induced by this pesticide in aquatic organisms. Higher HSP90 content in muscle of the black tiger shrimp *Penaeus monodon* was registered after exposure to 0.1 or 1.0 μg/L endosulfan, although no evidences of oxidative stress, measured by TBARS, was observed [32]. Previous studies analyzed biochemical parameters in *J. multidentata*, showing that 1.4 μg/L endosulfan altered activity of antioxidant enzymes (lower GST activity in gills and liver, higher in brain) and augmented TBARS levels in liver and brain [16].

Chemoprotection is considered a strategy to the prevention of degenerative diseases through the use of specific diets or by the administration of dietetic supplements [33]. Antioxidants are between the chemicals able to induce chemoprotection against a broad variety of toxic molecules. Because of different toxicants can directly or indirectly augment ROS generation, induce antioxidant responses and/or oxidative damage [11], exists a consensus that antioxidant administration can protect against these toxicants and several kinds of diseases, including those of degenerative nature [34]. In the present study, LA pre-treatment reverted the total loss of antioxidant capacity against peroxyl radicals in liver after endosulfan exposure and cancelled the antioxidant response in brain of the +LA/+Endo group, that was registered in the +Endo group.

The higher GST activity observed in the present study in liver and brain after endosulfan exposure was also reported in amphibian, where low concentrations of this pesticide (0.01–0.04 μg/L) were enough to strongly augment GST activity [35]. In *J. multidentata* the GST response was reverted in LA-pretreated fish. At the gene level, LA induced a lowering of gills Pgp expression in the +LA/−Endo group. However, endosulfan *per se* lower gills Pgp expression in −LA/+Endo group, a result not observed in +LA/+Endo group.

A previous study performed in *J. multidentata* at the same endosulfan concentration and exposure time, determined that liver, brain and gills presented high capacity to accumulate both α and β isomers, as well as endosulfan sulfate. In general present results are in accordance with this previous study with the exception of the absence of accumulation of the β isomer of endosulfan in brain [18]. Levels of endosulfan sulfate were similar in the three organs of fish fed with or without LA added. Endosulfan sulfate is one of the metabolites that posses residual activity after application [36]. The study of Wan et al. [37] estimated the 96 h-LC$_{50}$ for the fish *Oncorhynchus mykiss* and showed a higher toxicity of the (α + β) endosulfan mixture (LC$_{50}$: 0.7 μg/L) than for the endosulfan sulfate (LC$_{50}$: 1.4 μg/L). For *J. multidentata* it was observed that LA pre-treatment did not altered the accumulated levels of endosulfan sulfate. These results suggest that, although LA promotes higher accumulation of the α-isomer in brain, it did not affect endosulfan metabolization to the more persistent sulfated form. As lipoic acid is a molecule derived from octanoic acid, it remains to establish the endosulfan solubility in this molecule in order to study a potential dual effect of LA: by one side triggering antioxidant and detoxifying responses but at the same time favoring pesticide accumulation. This last point can be discussed taking into account that, according to Kenneth and Willem [38], the log Kow of endosulfan is 3.25, a similar value to the log Kow of lipoic acid (3.40) as reported previously [39]. This suggests that if some organs can accumulate lipoic acid, this in turn favor endosulfan accumulation. In fact we tried to measure lipoic acid accumulation in the evaluated organs, but no signals were observed in the HPLC–EIS-MS. We hypothesized that as the weight of the organs of this fish are very low (mean weight of liver, gills and brain samples: 15.5; 24.5 and 8.9 mg, respectively), lipoic acid content was not high enough to show a clear signal.

Is also important to remark that even in the liver, where LA elevated Pgp expression, this not resulted in a reduction of endosulfan

accumulation, as stated above. Sreeramulu et al. [13] (2007) reported that Pgp transport was inhibited by pesticides like endosulfan, a result that opens the possibility to consider that not necessarily a chemoprotective agent will render higher phase III activity if Pgp inhibitors (chemosensitizers) are present.

As general conclusions it can be stated that LA pretreatment modulated the antioxidant competence of *J. multidentata*, lowering the liver TBARS levels, as well as augmenting phase II and phase III capabilities in this organ. After endosulfan exposure, higher accumulation of the α-isomer was observed in LA pre-exposed group, pointing to future studies in order to elucidate LA-endosulfan interaction. However, this higher accumulation did not resulted in higher toxicity. Instead, LA pre-treatment prevented the lowering of total antioxidant capacity registered in liver and the opposite effect in brain.

Acknowledgments

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