



# Acute exposition to Roundup Transorb® induces systemic oxidative stress and alterations in the expression of newly sequenced genes in silverside fish (*Odontesthes humensis*)

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## Abstract

Roundup Transorb® (RDT) is a glyphosate-based herbicide commonly used in agricultural practices worldwide. This herbicide exerts negative effects on the aquatic ecosystem and affects bioenergetic and detoxification pathways, oxidative stress, and cell damage in marine organisms. These effects might also occur at the transcriptional level; however, the expression of genes associated with oxidative stress has not been studied well. *Odontesthes humensis* is a native Brazilian aquatic species naturally distributed in the habitats affected by pesticides, including Roundup Transorb® (RDT). This study evaluated the toxic effects of short-term exposure to RDT on *O. humensis*. Moreover, the genes related to oxidative stress were sequenced and characterized, and their expressions in the gills, hepatopancreas, kidneys, and brain of the fish were quantified by quantitative reverse transcription-polymerase chain reaction. The animals were exposed to two environmentally relevant concentrations of RDT (2.07 and 3.68 mg L<sup>-1</sup>) for 24 h. Lipid peroxidation, reactive oxygen species (ROS), DNA damage, and apoptosis in erythrocytes were quantified by flow cytometry. The expression of the target genes was modulated in most tissues in the presence of the highest tested concentration of RDT. In erythrocytes, the levels of lipid peroxidation, ROS, and DNA damage were increased in the presence of both the concentrations of RDT, whereas cell apoptosis was increased in the group exposed to 3.68 mg L<sup>-1</sup> RDT. In conclusion, acute exposure to RDT caused oxidative stress in the fish, induced negative effects on cells, and modulated the expression of genes related to the enzymatic antioxidant system in *O. humensis*.

**Keywords** Reactive oxygen species · Aquatic ecosystem · Glyphosate · Herbicide · Oxidative damage · Gene expression

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## Introduction

Herbicides are widely used in agriculture. Among them, glyphosate [N-(phosphonomethyl)glycine] is a broad-spectrum, systemic, non-selective, and post-emergence herbicide. Recent studies have reported its toxic effects on humans and other animals (Van Bruggen et al. 2018; Hussain et al. 2019; Ma et al. 2019).

Among glyphosate-based herbicides, Roundup® (Monsanto, Saint-Louis, MO, USA) formulations are most widely used. The emergence and release of Roundup Ready® (RR) in the market have increased the use of glyphosate-based herbicides for glyphosate-tolerant seeds (Pokhrel and Karsai 2015; Tapkir et al. 2019; Van Meter et al. 2019). Roundup Transorb® (RDT) formulation consists of glyphosate (isopropylamine salt), surfactant mixture of amino polyoxyethylene (POEA), and other unspecified surfactants (Howe et al. 2004). This formulation is more toxic than others because of the presence of POEA, a substance responsible for increasing the penetration of glyphosate in the cuticle of plants (de Brito Rodrigues et al. 2017).

Agrochemicals sprayed on plants finally reach the aquatic ecosystems (Wagner et al. 2013; Yang et al. 2015). The detection of glyphosate-based herbicides in water bodies is difficult due to the variability of formulations. Therefore, glyphosate concentration is used as a measure of contamination of water bodies with herbicides (Wagner et al. 2013). In the areas cultivated with genetically modified soybeans in the USA, 2–430  $\mu\text{g L}^{-1}$  of glyphosate was detected in rivers and streams reaching the sea. Glyphosate was also detected in water bodies in southern Brazil and Argentina (Silva et al. 2003; Peruzzo et al. 2008; Castro Berman et al. 2018). A concentration of 0.1–165  $\mu\text{g L}^{-1}$  of glyphosate was detected in water bodies in European countries, such as France, Germany, Spain, and Switzerland, where the cultivation of genetically modified seeds is prohibited (Van Bruggen et al. 2018). Some studies considered the glyphosate concentration of up to 10  $\text{mg L}^{-1}$  as environmentally realistic; thus, relatively high concentrations of glyphosate might be present in these environments immediately after Roundup® application (Langiano and Martinez 2008; Topal et al. 2015; Silveira et al. 2019b). The presence of herbicides in aquatic ecosystems is associated with negative effects on various organisms, such as aquatic plants (Zhong et al. 2018), micro-organisms (Wu et al. 2016), and fish (Bridi et al. 2017; Silveira et al. 2019b). Commercial glyphosate formulations can induce changes in bioenergetic and detoxification pathways, including the antioxidant defense system, leading to the accumulation of reactive oxygen species (ROS). Oxidative stress induced by excess ROS can cause damage to cell components and even cell death (Lushchak 2011, 2014; Braz-Mota et al. 2015). The disturbance in the homeostasis of these organisms can be quantified using biomarkers, which can help understand and evaluate the potential toxicological effects of different contaminants in the aquatic

environment (Valavanidis et al. 2006; Hedayati 2018). These alterations caused by exposure to contaminants also occur at the transcriptional level; however, the expression of genes associated with oxidative stress has not been studied well (Topal et al. 2015).

*Odontesthes* sp., popularly known as silversides, is a genus of fish naturally endemic to South America. This is the most diverse genus in the Atherinopsidae group, with species inhabiting marine coastal waters, coastal lagoons, and estuarine regions in the southern region of South America (Bemvenuti 2006; Brian and Dyer 2006; Moresco and Bemvenuti 2006). Some of these species are economically important in these regions for production, flesh commercialization, and sport fishing (Somoza et al. 2008). In the extreme southern region of Brazil, *Odontesthes humensis* inhabits the lakes and large water bodies of the drainages of the dos Patos-Mirim lagoon system, which represents the two largest lagoons in Brazil (Brian and Dyer 2006). These water bodies are surrounded by agricultural-productive areas, which mainly cultivate soy and rice monocultures with the use of RDT de Brito Rodrigues et al. 2017. Furthermore, *O. humensis* is considered a bioindicator in environmental studies because of its demands for good water quality and survival only in a narrow range of water parameters (Zebal et al. 2017; Silveira et al. 2018a, b; Silveira et al. 2019b).

The aim of this study was to evaluate the possible toxic effects of RDT exposure on *O. humensis*, a native Brazilian aquatic species naturally distributed in the habitats affected by pesticides, including glyphosate. Moreover, the genes related to oxidative stress were sequenced and characterized; their expressions in the gills, hepatopancreas, kidneys, and brain of the fish were quantified. Furthermore, cytological and biochemical parameters in the erythrocytes affected by oxidative stress were also evaluated.

## Materials and methods

### Animals and conditions

*O. humensis* were obtained from the Laboratory of Pisciculture of the Barragem do Chasqueiro Fish Farming (Arroio Grande, Brazil, 32°14'15"S/53°05'13"W). The specimens were born in captivity and were 1.5-year-old with the mean weight and length of  $31.4 \pm 8.3$  g and  $16.5 \pm 5.3$  cm, respectively, at the beginning of the experiment. The animals were fed three times a day with commercial feed (Supra, 38% crude protein) and zooplankton until satiety. The fish were maintained in plastic tanks with a nominal capacity of 1000 L and a working volume of 650 L. The tanks used were opaque to reduce visual stress. Approximately two-thirds of the water in each tank was renewed once a week. The water parameters from the beginning of acclimation until the end of the experimental period were as follows: the temperature of  $13.8 \pm 0.34^\circ\text{C}$ ; dissolved

oxygen level of  $9.85 \pm 0.1 \text{ mg L}^{-1}$ ; salinity of  $3.2 \pm 0.21 \text{ ppt}$ ; pH of  $7.04 \pm 0.19$ ; and ammonia level lower than  $0.6 \text{ mg L}^{-1}$ . The use of animals and all handling practices were approved by the ethics committee of Animal Experimentation of the Federal University of Pelotas (# 23110.007018/2015–85).

### Experimental design and sample collection

After 4 weeks of the acclimation period, the specimens were randomly categorized into three groups: two experimental groups exposed to RDT diluted in water at the nominal concentrations of 2 and  $4 \text{ mg L}^{-1}$  (glyphosate acid equivalent (GAE)) and a control group without RDT. These concentrations were selected because they are considered environmentally realistic. The experimental groups were exposed to RDT for 24 h, whereas the control group was exposed to water without herbicide. The acute exposition of all the groups was performed in plastic tanks in duplicate (6 tanks), with three animals in each tank (18 animals in total) maintained under the same conditions. At the end of the experiment, all animals were anesthetized by submersion into  $50 \text{ mg L}^{-1}$  benzocaine and then euthanized by cranial spinal section and excision of the brain. The brain, hepatopancreas, gills, and kidneys of all the animals were collected. The tissues collected from one animal per tank were used for the sequencing and molecular characterization of target genes, whereas the tissues from the other two animals from each tank were used for gene expression analysis. These tissues were preserved in liquid nitrogen until use for molecular biology processing and analyses. Moreover,  $20 \mu\text{L}$  of peripheral blood was collected from two animals from each tank (the animals used for gene expression analysis) by puncturing the caudal fin. Each blood sample was added to 1 mL of fetal bovine serum (FBS), stored at  $4^\circ\text{C}$  in the dark until use for flow cytometry analysis.

A commercial formulation of glyphosate [N-(phosphonomethyl)glycine], commercially known as Roundup Transorb®, and containing  $588 \text{ g L}^{-1}$  potassium salt of glyphosate as the active ingredient ( $480 \text{ g L}^{-1}$  or 48% acid equivalent of glyphosate), was used. The real concentration of glyphosate in the water contaminated with RDT (expressed as

GAE) was measured in triplicate from the water samples collected from each tank as previously described (Zebral et al. 2018; Silveira et al. 2019b). For this analysis, Shimadzu 2010 Plus equipped with a flame photometric detector and DB-17 fused silica capillary column ( $30 \text{ m} \times 0.25 \text{ mm I.D.}$ ,  $0.25 \mu\text{m}$  film thickness) (J & W, USA) was used. A calibration curve ( $0.15\text{--}10 \text{ mg L}^{-1}$  glyphosate) was plotted and used for the determination of glyphosate concentration in water samples. This method showed adequate linearity for the calibration curve ( $r^2 = 0.999$ ). The concentrations of glyphosate in the water samples measured after experimental exposition to the nominal concentrations of 2 and  $4 \text{ mg L}^{-1}$  were found to be  $2.07 \pm 0.15$  and  $3.68 \pm 0.31 \text{ mg L}^{-1}$  (GAE), respectively, and glyphosate concentration in the control group was less than the limit of detection of the method ( $<0.05 \text{ mg L}^{-1}$ ).

### RNA extraction and cDNA synthesis

Total RNA was extracted from the samples using the RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. RNA concentration and purity were measured by UV-light spectrophotometry using the NanoVue™ equipment (GE Healthcare Life Sciences, USA). Subsequently, the samples were standardized by concentration, and only the samples presenting high purity ( $\text{OD}_{260/280} \geq 2.0$  nm) were used in the following steps. The samples were treated with DNase using DNA-free™ Kit (Invitrogen™, USA) according to the manufacturer's recommendation. First-strand cDNA synthesis was performed with  $2 \mu\text{g}$  of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's recommendation. The synthesized cDNA was stored at  $-20^\circ\text{C}$  until further use.

### Target genes

The target genes related to antioxidant defense and oxidative stress were as follows: carbonic anhydrase (*ca1*), catalase (*cat*), glutathione peroxidase (*gpx1*), NADH dehydrogenase subunit 2 (*nd2*), and Cu/Zn superoxide dismutase (*sod1*) (Table 1).

**Table 1** Target genes

Gene symbol	Gene name	Function
<i>ca1</i>	Carbonic anhydrase	Enzyme for acid-base balance, respiration, carbon dioxide, and ion transport
<i>cat</i>	Catalase	Enzyme of the antioxidant pathway
<i>gpx1</i>	Glutathione peroxidase	Enzyme of the antioxidant pathway
<i>nd2</i>	NADH:ubiquinone oxidoreductase	First enzyme complex of the mitochondrial oxygen respiratory chain
<i>sod1</i>	Superoxide dismutase (Cu/Zn)	Enzyme of the antioxidant pathway

### Sequencing and molecular characterization

The gene fragments of *ca1*, *cat*, *gpx1*, *nd2*, and *sod1* were amplified by polymerase chain reaction (PCR) using primers designed by the PriFi online tool (<https://services.birc.au.dk/prifi/>) after the alignment of known sequences of each gene deposited in GenBank.

The PCR parameters were as follows: an initial denaturation step for 1 min at 94°C, followed by 35 cycles at 94°C for 30 s, 55.2–65.5°C (depending on the primer sequence given in Table 2) for 30 s, and 72°C for 1 min, and a final extension for 5 min at 72°C. The PCR products were inserted into the pCR@4-TOPO® TA cloning vector and transformed into the electrocompetent *Escherichia coli* strain DH5α. The characterized fragments were sequenced using the Applied Biosystems 3500 Genetic Analyzer® automatic sequencer (Life Technologies, USA).

### Sequence and phylogenetic analysis

The partial sequences of *ca1*, *cat*, *gpx1*, *nd2*, and *sod1* genes identified in this study were deposited in GenBank (see in

“Results”). The translation of the sequenced nucleotides to amino acid sequences and the identification of open reading frame (ORF) were performed using the ExPASy bioinformatics resource portal (<https://www.expasy.org/>). The conserved domains and sites were mapped using the UniProt database (<https://www.uniprot.org/>). The amino acid sequences of CA, CAT, GPX, ND2, and SOD from various group species were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) or UniProt database and aligned to the newly deduced amino acid sequences of *O. humensis* using default parameters of ClustalX v.2 (Larkin et al. 2007).

Phylogenetic trees were constructed using the Bayesian inference method, according to Mr. Bayes v.3.2.6 (Ronquist et al. 2012) available in the web portal Phylogeny.fr ([http://www.phylogeny.fr/one\\_task.cgi?task\\_type=mrbayes](http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes)). Model selection tests for each sequence were performed using the maximum likelihood method available in MEGA v.6.06. The models available in Phylogeny.fr that had the lowest Bayesian information criterion value were selected. The selected models were the WAG model with a discrete Gamma distribution (WAG+G) to CA, CAT, and GPX alignments, and the mtREV model with a discrete Gamma

**Table 2** Primer sequences used in this study

Gene symbol	Primer sequence 5'→3'	Annealing temperature (°C)	Efficiency (%)	Objective
<i>ca1</i>	CAGTCTCCCATG YATCBTACC TGCGRAATTTRGCCATCTG	60.7	-	Characterization
	CCTCAACAACGGACATTCCT GTCATCAGATGCTCCCCAGT	60.0	109.2%	qRT-PCR
<i>cat</i>	TGCAYCAGGTGTCBTTCT GGAGCACTGAAGCT GTTGGG	55.2	-	Characterization
	TGCTAGGTGTTCTGCCTCCT GCTACGGCTCTCACACCTTC	60.0	105.3%	qRT-PCR
<i>gpx1</i>	CATCAGGAGAACTGCAAGA AGGAACTTCTCAAAGTTCCA GG	59.3	-	Characterization
	GCTTCGAGCCAAAGTTTCAG GATCAGCTTGGGGTCGTTT	60.0	97.4%	qRT-PCR
<i>nd2</i>	AGCTTCTACTGGCTYCTTGC GGGGAKAGGGTAAG AGTTATTGCG	60.0	-	Characterization
	AAATTTCTACCCCGTTCCT TCAGCCCTGTGGTAAGGTCT	60.0	107.1%	qRT-PCR
<i>sod1</i>	ACAAACGGSTGCATCAGTGC AGG CYTTCTCATGRATCACCATG G	57.6	-	Characterization
	GGTTCTGCCAATGATGGAGT AGATTTGGGAACG TGACTG	60.0	106.0%	qRT-PCR
<i>h3a</i>	CGTGGCTCTGAGAGAGATCC TCGGTCTTGAAGTCTGAGC	60.0	105.3%	qRT-PCR Reference gene

distribution (mtREV+G) to ND2 and SOD alignments. The parameters of the best-fit model were subsequently used for Bayesian analysis in [Phylogeny.fr](http://Phylogeny.fr) (Dereeper et al. 2008). The first tree was constructed by the random addition of sequences. The Markov chain Monte Carlo algorithm was applied, and two simultaneous, completely independent analyses starting from different random tree runs of 100,000 generations, were performed, sampling one tree at every ten generations. The runs were treated as burn-in, and the first 1000 trees sampled were discarded. The maximum clade credibility tree was displayed and viewed using the FigTree software v.1.4.3 (Rambaut 2016) and vectorized using the Inkscape software v.0.92.3 (<https://inkscape.org/>).

### Gene expression analysis

Gene expression was analyzed by quantitative reverse transcription PCR (qRT-PCR). The primers used in this study (Table 2) were designed using the Primer3 online tool (<https://primer3.ut.ee/http://bioinfo.ut.ee/primer3-0.4.0/>) and the sequences of the identified fragments. The qRT-PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) and SYBR® Green PCR Master Mix (Applied Biosystems, USA). The amplification conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 60 s, followed by the conditions needed to calculate the melting curve. The  $2^{-\Delta\Delta CT}$  method was used to normalize the fold change in the gene expression (Livak and Schmittgen 2001) using Histone h3a (h3a) as the reference gene (GenBank accession No. KX060037) as described previously (Silveira et al. 2018a).

### Flow cytometry analysis

Flow cytometry analysis was performed using Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems, USA) to evaluate the effect of RDT exposure on the systemic physiology of silversides by analyzing the erythrocytes. The blood was washed with 500  $\mu\text{L}$  of FBS. Erythrocytes were assayed for apoptosis, ROS, lipid peroxidation, and DNA fragmentation using a flow cytometer (Attune Acoustic Focusing®, Life Technologies) equipped with violet laser (UV 405 nm-450/40, VL-1). Erythrocytes were stained with Hoechst 33342 (16.2 mM) (Martinez-Alborcia et al. 2012). Cell debris was excluded based on forward scatter  $\times$  side scatter plot and negative fluorescence of Hoechst 33342. In order to read all parameters, the fluorophore-stained cells were added into calcium- and magnesium-free phosphate-buffered saline (80  $\text{g L}^{-1}$  of NaCl, 11.5  $\text{g L}^{-1}$  of KCl, 24  $\text{g L}^{-1}$  of  $\text{Na}_2\text{HPO}_4$ , and 2  $\text{g L}^{-1}$  of  $\text{KH}_2\text{PO}_4$  dissolved in deionized water). A total of 20,000 erythrocytes were analyzed during each analysis.

For the evaluation of ROS produced by the erythrocytes, 10  $\mu\text{L}$  of previously collected and stored blood sample was added to 20  $\mu\text{L}$  of saline solution containing 2  $\mu\text{M}$  2',7'-dichlorofluorescein diacetate and 5  $\mu\text{M}$  propidium iodide (PI) fluorescent probes (Sigma-Aldrich Co., USA). The samples were analyzed after incubation at 22°C for 60 min in the dark. Only live cells (PI negative) were selected and measured for ROS production based on the median intensity of the emitted green fluorescence.

The erythrocyte lipid peroxidation (LPO) was quantified using the final concentration of 1  $\mu\text{M}$  of the lipid peroxidation sensor 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY) in 100  $\mu\text{L}$  of the sample, which was incubated for 2 h at room temperature (20°C). The rate of lipoperoxidation was calculated as the median intensity of green fluorescence (peroxidized lipid) / median green fluorescence intensity + median red fluorescence (non-peroxidized lipid)  $\times$  100 (Hagedorn et al. 2012).

In order to evaluate the DNA damage in erythrocytes, 10  $\mu\text{L}$  of blood sample was added to 5  $\mu\text{L}$  of 0.01 M Tris-HCl, 0.15 M NaCl, and 0.001 M EDTA (pH 7.2), followed by the addition of 10  $\mu\text{L}$  of Triton 1X (Triton X-100, 1%, v/v) 30 s later. Then, 50  $\mu\text{L}$  of acridine orange dye (2  $\text{mg mL}^{-1}$ , #A6014, Sigma-Aldrich, USA) was added to the sample, followed by incubation from 30 s up to 2 min before each reading. The DNA of erythrocytes was classified as integrated (green fluorescence emission) or damaged (orange/red fluorescence emission). The percentage of DNA fragmentation index (DFI) was calculated as the median of red fluorescence intensity / (median of the red + green fluorescence intensities)  $\times$  100.

Apoptosis was studied using a combination of fluorescent dyes, namely, Annexin V-FITC conjugate (AnV, Sigma A9210) and propidium iodide (PI) (7.3  $\mu\text{M}$ ). In order to detect apoptosis, phosphatidylserine that translocates to the outside of the cell membrane was stained. The erythrocytes were classified as live (AnV- and PI-), necrotic (AnV+ and PI+), or apoptotic (AnV+ and PI-). The results are expressed as the total number of apoptosis / total number of erythrocytes  $\times$  100.

### Statistical analysis

The normality distribution of quantitative data was evaluated by the Shapiro–Wilk test. The homogeneity of variances was evaluated by Levene's test, O'Brien's test, and Brown and Forsythe test. The data not showing normal distribution were transformed using the log function. The evaluated parameters that presented normal distribution and homogeneous variance were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-test with a significance level of 5%. The results from the analysis of gene expression, ROS,

lipid peroxidation, DFI, and apoptosis were expressed as mean  $\pm$  standard error of the mean (SEM).

## Results

### Sequencing and molecular characterization

The length of *cal*, *cat*, *gpx1*, *nd2*, and *sod1* fragments from *O. humensis* was 537, 591, 254, 552, and 204 bp, respectively. The fragments were sequenced and deposited under the GenBank accession numbers KX035017, KX184718, KX060036, KX184716, and KX184717, respectively. The fragment of *cal* cDNA from *O. humensis* belongs to the middle of ORF + 1 and codes for 179 amino acids that belong to the alpha carbonic anhydrase family. The fragment contains the alpha carbonic anhydrase conserved domain from amino acids 1 to 179. The alpha carbonic anhydrase conserved site is also present in the fragment between 73 and 89 amino acids.

The fragment of *cat* cDNA was found in ORF + 1 and encoded 197 amino acids that belong to the catalase family. The fragment between amino acids 1 and 197 contained a catalase core domain, whereas the catalase heme-binding site was found between amino acids 157 and 165.

The fragment of *gpx1* cDNA belongs to the middle region of ORF + 1 and codes for 84 amino acids belonging to the glutathione peroxidase family. This fragment contains a thioredoxin-like fold domain in the region between amino acids 1 and 84.

The fragment of *nd2* cDNA codes for 184 amino acids on ORF + 1. The fragment belongs to NADH:ubiquinone oxidoreductase and has a membrane subunit domain of NADH:quinone oxidoreductase between amino acids 8 and 179.

The fragment of *sod1* cDNA codes for 67 amino acids on ORF + 2. The fragment belongs to the superoxide dismutase (Cu/Zn) family and has a copper/zinc-binding domain between amino acids 1 and 67.

### Phylogenetic analysis

The cDNA fragments of *O. humensis* were translated into amino acids. Furthermore, the amino acid sequences of CA, CAT, GPX, ND2, and SOD from various species were considered references for the phylogenetic analysis. The *O. humensis* CA sequence (Supplementary Fig. S1) was found to be clustered in the CA1 cluster, which comprises only bony fishes, showing that this sequence was more related to subunit 1 than CA2, CA3, CA4, or CA13. The phylogenetic tree divided the catalase family into two main groups, mammals and teleosts, under which the CAT sequence of *O. humensis* was grouped (Supplementary Fig. S2). The phylogenetic tree constructed based on the alignment of GPX amino acid sequences

(Supplementary Fig. S3) formed different subunits, such as 1, 2, and 4a and b. GPX1 was further divided into two clusters with mammalian and fish sequences. The silverside was grouped in the cluster next to the bony fish species, showing that it was more closely related to GPX1b than the other subunits. The NADH phylogenetic tree (Supplementary Fig. S4) was divided into four large groups based on the NADH subunits. The analyzed sequence was grouped into the cluster related to the NADH2 subunit, close to other fish species. The SOD amino acid sequences also formed different clusters based on different SOD subunits (Supplementary Fig. S5). *O. humensis* was grouped in the cluster related to SOD1, as expected. Based on these analyses, the characterized fragments were confirmed as *cal*, *cat*, *gpx1b*, *nd2*, and *sod1*.

### Gene expression analysis

#### Gills

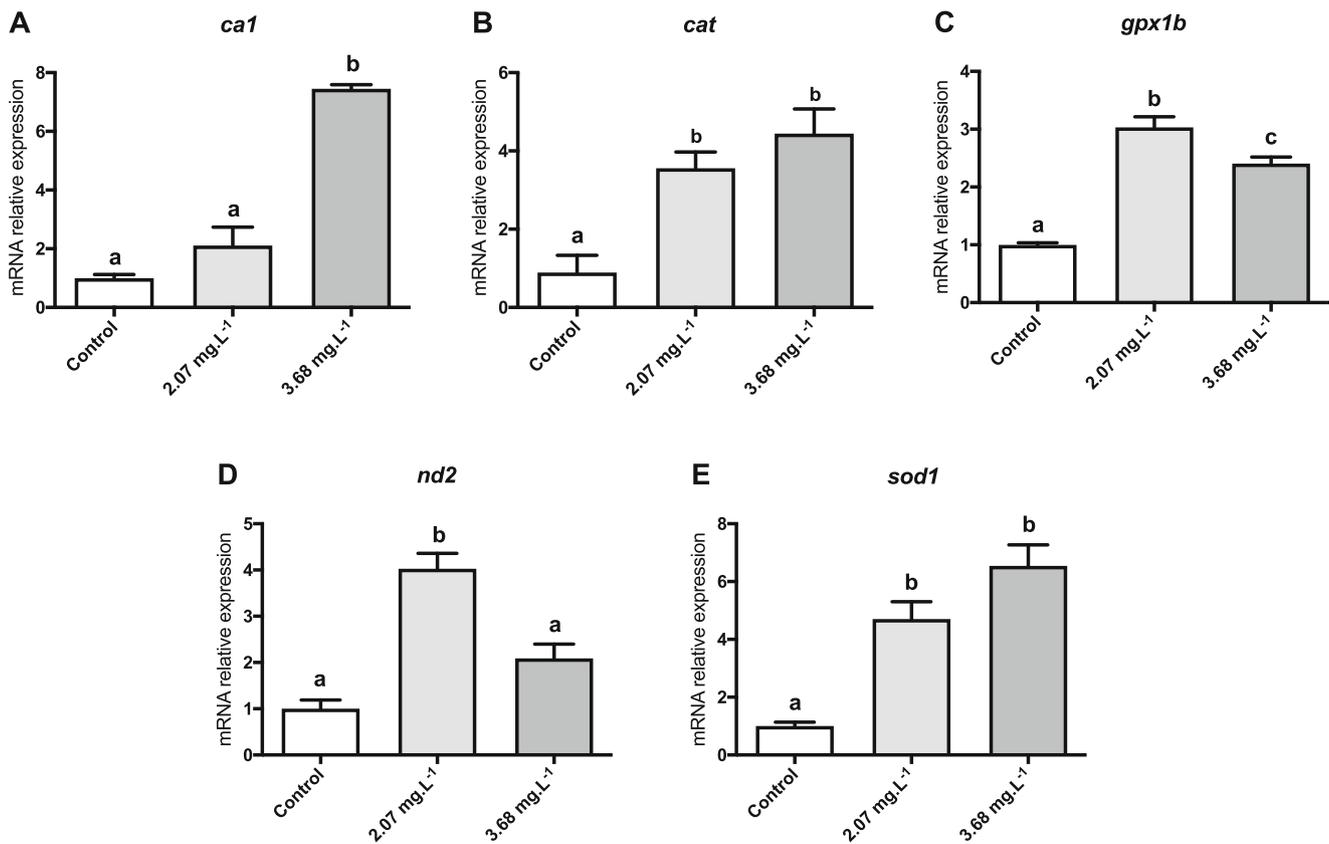
The silversides exposed to 3.68 mg L<sup>-1</sup> (GAE) of RDT exhibited higher *cal* mRNA expression in the gills ( $p < 0.05$ ) compared to the animals in the control group (Fig. 1A). Compared to the control group, the relative mRNA expression of *cat*, *gpx1b*, and *sod1* in the gills of the fish exposed to 2.07 and 3.68 mg L<sup>-1</sup> (GAE) of RDT was significantly higher ( $p < 0.05$ ) (Fig. 1B, C, and E). *Nd2* mRNA expression in the gills was significantly higher ( $p < 0.05$ ) in the fish exposed to 2.07 mg L<sup>-1</sup> (GAE) of RDT (Fig. 1D).

#### Hepatopancreas

*Cal*, *cat*, and *gpx1b* mRNA expression in the hepatopancreas of the fish exposed to 2.07 and 3.68 mg L<sup>-1</sup> (GAE) of RDT showed a significant decrease ( $p < 0.05$ ) compared to that of the control group (Fig. 2A–C). The relative expression of *nd2* mRNA did not show a significant difference ( $p > 0.05$ ) between the groups (Fig. 2D). The *sod1* mRNA expression in hepatopancreas was significantly lower ( $p < 0.05$ ) in fish exposed to 2.07 mg L<sup>-1</sup> (GAE) of RDT than in those from the control group and those exposed to 3.68 mg L<sup>-1</sup> (GAE) of RDT (Fig. 2E).

#### Kidney

The *cal* mRNA expression in the kidney of silversides exposed to 2.07 and 3.68 mg L<sup>-1</sup> (GAE) of RDT was significantly lower ( $p < 0.05$ ) than that in the fish from the control group (Fig. 3A). No statistical difference ( $p > 0.05$ ) was observed in *cat*, *gpx1b*, and *sod1* expressions after 24 h (Fig. 3B, C, and E). The *nd2* mRNA expression in the kidney of fish exposed to 3.68 mg L<sup>-1</sup> (GAE) of RDT was decreased ( $p > 0.05$ ) (Fig. 3D).



**Fig. 1** Gene expression in the gills of *Odontesthes humensis* fish in the control group and those exposed to Roundup Transorb® at the concentrations of 2.07 and 3.68 mg L<sup>-1</sup> (glyphosate acid equivalent) for 24 h. The mRNA relative expression of *ca1* (A), *cat* (B), *gpx1b* (C), *nd2* (D), and *sod1* (E) was evaluated by quantitative reverse transcription-

polymerase chain reaction and normalized using the *h3a* gene. The values are expressed as mean ± standard error of the mean. Different letters indicate significant differences between the experimental groups (one-way analysis of variance; n = 4, p < 0.05)

### Brain

The mRNA expression of *ca1* in the brain of the fish exposed to 2.07 and 3.68 mg L<sup>-1</sup> (GAE) of RDT was higher (p < 0.05) than that in the fish from the control group (Fig. 4A). The mRNA expression of *cat* and *gpx1b* in the brain of the fish exposed to 3.68 mg L<sup>-1</sup> (GAE) of RDT was significantly higher (p < 0.05) than that in the fish from the control group and those exposed to 2.07 mg L<sup>-1</sup> (GAE) of RDT (Fig. 4B, C). Furthermore, the *nd2* mRNA expression in the brain of the fish exposed to both the concentrations of RDT showed a significant decrease (p < 0.05) compared to that in the fish from the control group (Fig. 4D). No statistical difference was observed (p > 0.05) in *sod1* mRNA expression in the brain of silversides of any group (Fig. 4E).

### Flow cytometry analysis

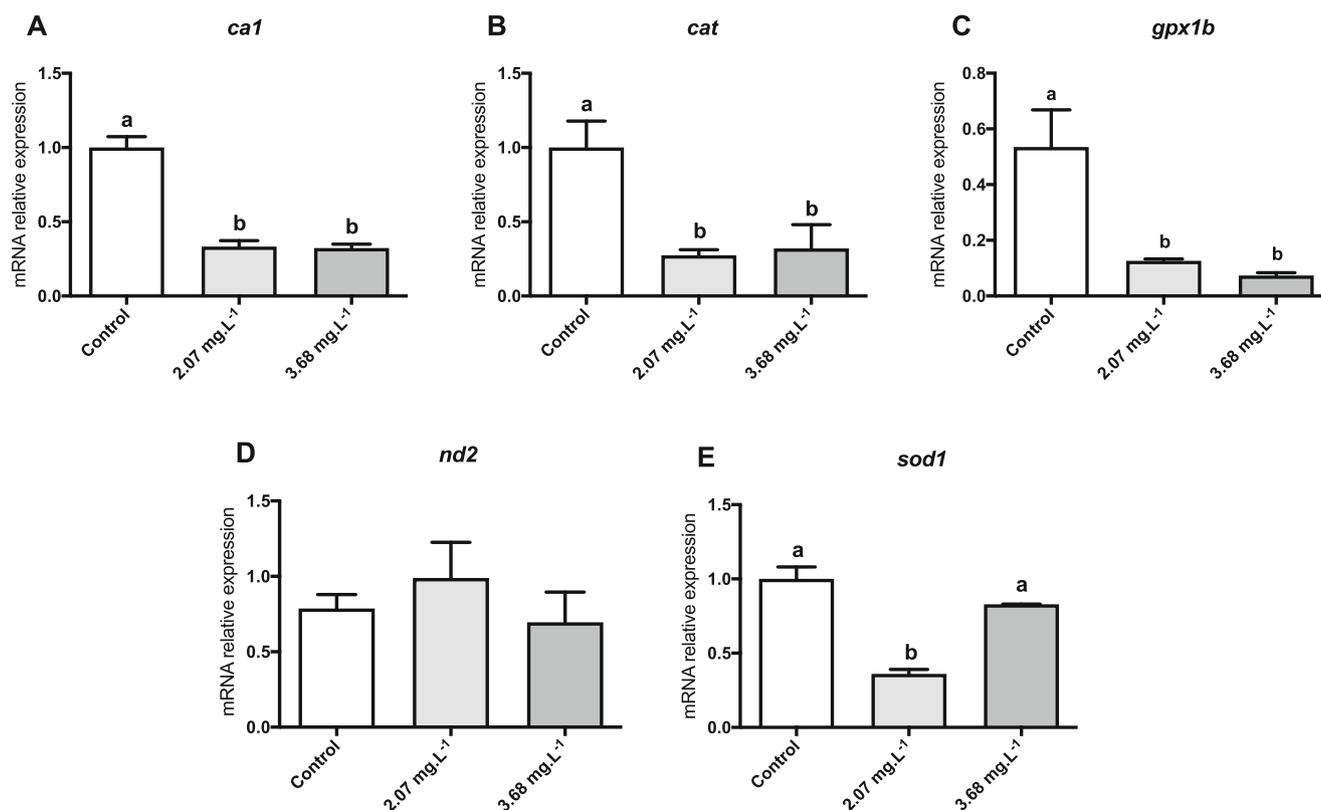
The acute exposure of silversides to RDT for 24 h significantly increased (p < 0.05) the ROS levels (Fig. 5A) and LPO levels in erythrocytes (Fig. 5B). Furthermore, both the concentrations of RDT induced a significant increase (p < 0.05) in

the DFI of erythrocytes compared to the control group (Fig. 5C). The apoptosis levels in the erythrocytes of silversides exposed to 3.68 mg L<sup>-1</sup> (GAE) of RDT showed a significant increase (p < 0.05) (Fig. 5D).

### Discussion

The present study is, to the best of our knowledge, the first one to demonstrate the isolation and characterization of mRNA sequences of *ca1*, *cat*, *gpx1b*, *nd2*, and *sod1* from *O. humensis*. Although this is not a large-scale study due to the limited number of animals that can be hatched and raised in captivity, it was nonetheless possible to demonstrate the harmful effects of exposure to RDT herbicide on the expression of the genes involved in antioxidant pathways in a silverside fish species. Other studies that have included a similar number of animals have also reported adverse effects of glyphosate-based herbicides on fishes (Moresco and Bemvenuti 2006; Çavaş and Könen 2007; Moreno et al. 2014).

After 24 h of RDT exposure, the increase in the relative expression of *sod1*, *cat*, and *gpx1b* mRNA in the gills may



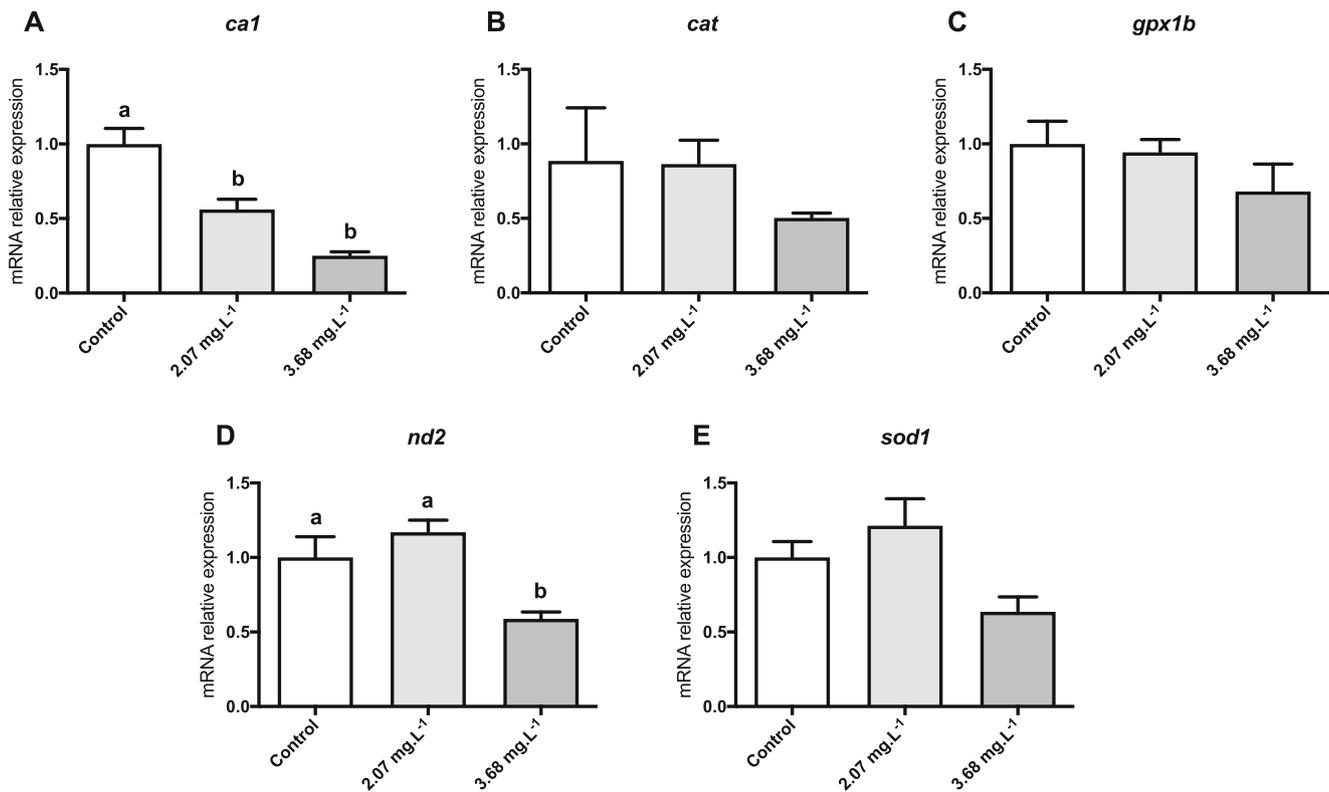
**Fig. 2** Gene expression in the hepatopancreas of *Odontesthes humensis* fish from the control group and those exposed to 2.07 and 3.68 mg L<sup>-1</sup> (glyphosate acid equivalent) of Roundup Transorb® for 24 h. The relative expression of *ca1* (A), *cat* (B), *gpx1b* (C), *nd2* (D), and *sod1* (E) mRNA was evaluated by quantitative reverse transcription-polymerase chain

reaction and normalized using the *h3a* gene. The values are expressed as mean ± standard error of the mean. Different letters indicate significant differences between the experimental groups (one-way analysis of variance; n = 4; p < 0.05)

have occurred via the activation of the NRF2/KEAP pathway due to stress. Several types of enzymes have been reported to be involved in the regulation of antioxidant pathways, including the transcription factor NRF2, which is dissociated from the KEAP1 protein and activates gene transcription in the presence of high oxidative stress (Chun et al. 2014; Sies and Jones 2020). Recent studies suggest that carbonic anhydrase is involved in the important protective mechanism against oxidative damage and ROS-induced cell death. Therefore, the increase in the expression of *ca1* gene observed in the present study could indicate the activation of different exposure-induced antioxidant pathways, which might have occurred for balancing the redox status of cells. The transcription factor NRF2 also regulates genes, such as *nd2*, that encode the subunits involved in the electron transport chain and oxidative phosphorylation (Kelly and Scarpulla 2004). The increase in the *nd2* expression upon RDT exposure indicates that RDT possibly interferes with cellular respiration and can reduce the productivity and growth of the fish (Pereira et al. 2018).

Xenobiotics reach the blood after entering the gills (Heath 1995). The present study showed the systemic effects of RDT exposure on the erythrocytes, which represent a cell type that

circulates through all systems of an organism. Oxidative parameters in the blood have been proposed as potential biomarkers for the detection of contaminants and are frequently used to assess fish health (Bojarski and Witeska 2020). We observed an increase in the ROS levels in the erythrocytes of silversides exposed to RDT. The majority of the environmental pollutants are capable of enhancing ROS production in fish, causing an accumulation of reactive intermediates, inactivation of antioxidant enzymes, and/or depletion of non-enzymatic antioxidants, leading to disturbed redox status (Lushchak 2011; Dumitru et al. 2019). ROS can interact virtually with all cellular components, such as proteins, nucleic acids, or lipids, and cause severe oxidative damage (Valavanidis et al. 2006; Birnie-Gauvin et al. 2017). Erythrocytes are relatively vulnerable to lipid peroxidation, and hence, LPO levels are considered potential markers for detecting oxidative stress induced by xenobiotics. The increased levels of LPO observed in the RDT-exposed fish in the present study might be a consequence of the high ROS levels generated by RDT. High LPO levels are extremely harmful to the organism as they can alter the integrity of the plasma membrane, affecting its permeability, and may also



**Fig. 3** Gene expression in the kidney of *Odontesthes humensis* fish in the control group and those exposed to 2.07 and 3.68 mg L<sup>-1</sup> (glyphosate acid equivalent) of Roundup Transorb® for 24 h. The relative expression of *cal* (A), *cat* (B), *gpx1b* (C), *nd2* (D), and *sod1* (E) mRNA was evaluated by quantitative reverse transcription-polymerase chain reaction

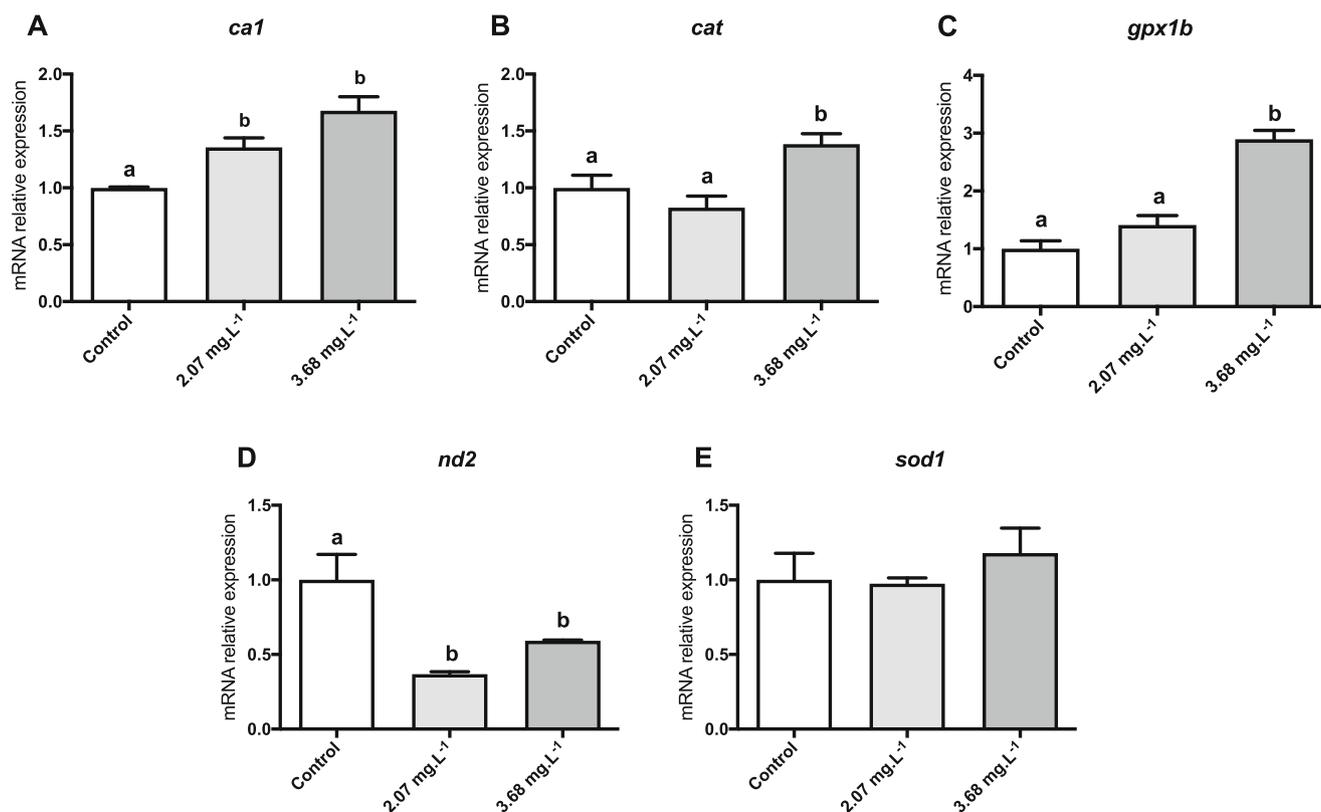
and normalized using the *h3a* gene. The values are expressed as mean  $\pm$  standard error of the mean. Different letters indicate significant differences between the experimental groups (one-way analysis of variance;  $n = 4$ ;  $p < 0.05$ )

cause DNA damage (Slaninova et al. 2009; Silveira et al. 2019a). The increased DFI observed in the fish exposed to RDT in the present study might be an indirect consequence of the oxidative stress induced by lipid peroxidation, as mentioned before, or a direct result of excess ROS. We also observed high levels of apoptosis in the fish exposed to RDT, which may have occurred because of the changes in the plasma membrane or generation of excess ROS, as these play critical roles in signaling pathways that induce apoptosis (Chaufan et al. 2014). Certain previous studies have reported similar results, showing an increase in the level of cellular apoptosis in animals exposed to herbicides (Topal et al. 2015; Sulukan et al. 2017).

The hepatopancreas is the key organ in fish that is affected by chemical pollutants. A significant decrease in *sod1*, *cat*, *gpx1b*, and *cal* expression was observed in hepatopancreas upon RDT exposure to fish. In some tissues, xenobiotics can exclude or inhibit transcription factors, which could explain the results obtained in this study. Our results are in agreement with those of Topal et al. (2015), who also observed a decrease in the expression of *cat* and *sod2*. Conversely, SOD, CAT, and GPX enzymes were reported to be activated in *Oncorhynchus mykiss* exposed to glyphosate (Topal et al. 2015).

In fish, the kidney receives a large proportion of post-branchial blood, as it serves as an important route of xenobiotic excretion. Owing to the function of the kidney and its direct contact with the blood, acute herbicide intoxication in this organ can potentially cause a decrease in the antioxidant defense and an increase in ROS. In addition, it can induce histopathological and biochemical changes in the kidney of fish (Jiraungkoorskul et al. 2002; Husak et al. 2014). A significant modulation of genes encoding the main antioxidant enzymes was expected because of the high ROS levels in the erythrocytes; however, we found no differences in the expression of *cal*, *cat*, *gpx1b*, and *sod1* genes. This could be attributed to the pre-existing enzymes or non-enzymatic defense pathways that were probably activated in the hepatopancreas and kidney after 24 h of exposure to RDT. In addition, a decrease in the *nd2* mRNA expression was observed. Therefore, it was inferred that acute exposure to RDT might have changed the functioning of the electron transport chain by the partial inhibition of mitochondrial complexes (Scandalios 2005; Velasques et al. 2016).

The neurotoxic effects of glyphosate-based herbicides have been studied in fish (Roy et al. 2016). The brain is particularly susceptible to oxidative stress because of high oxygen consumption and the presence of metals such as Fe and Cu (Santo



**Fig. 4** Gene expression in the brain of *Odontesthes humensis* fish in the control group and those exposed to 2.07 and 3.68 mg L<sup>-1</sup> (glyphosate acid equivalent) of Roundup Transorb® for 24 h. The relative expression of *ca1* (A), *cat* (B), *gpx1b* (C), *nd2* (D), and *sod1* (E) mRNA was evaluated by quantitative reverse transcription-polymerase chain reaction

and normalized using the *h3a* gene. The values are expressed as mean  $\pm$  standard error of the mean. Different letters indicate significant differences between the experimental groups (one-way analysis of variance;  $n = 4$ ;  $p < 0.05$ )

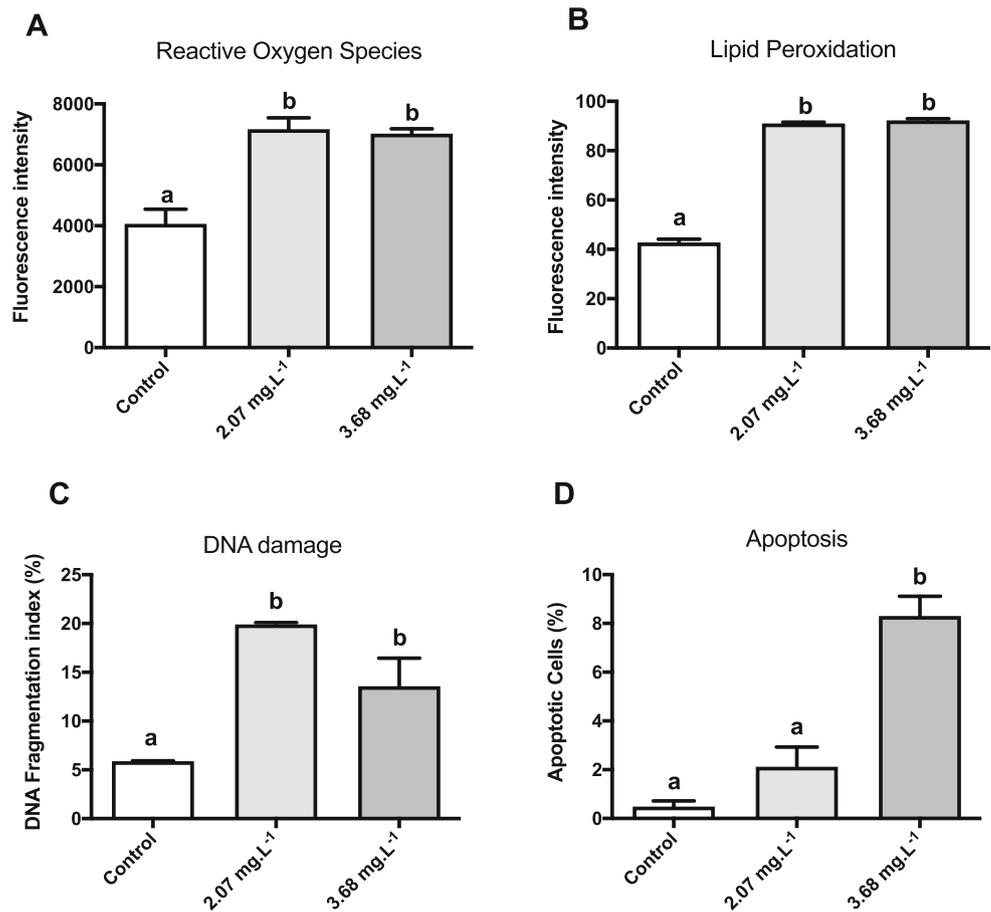
et al. 2018). In the present study, a decrease in the expression of the *nd2* gene was observed upon RDT exposure. This could lead to mitochondrial dysfunction, interference with electron transport, and consequently ROS accumulation. Hydrogen peroxide has a high potential of crossing the cell membrane and is, therefore, potentially toxic to the cells. This toxicity may be increased up to ten thousand times in the presence of iron (Scandalios 2005; Barbosa et al. 2010). According to previous reports, hydrogen peroxide can induce the activity of NF-Kb, a transcription factor that participates in the regulation of several genes involved in stress response and cellular defense mechanisms (Bowie and O'Neill 2000; Scandalios 2005; Tönnies and Trushina 2017; Sies and Jones 2020). The activation of these signaling pathways might have caused the observed increase in the *ca1*, *cat*, and *gpx1b* expression in the brain.

In this study, the acute exposure to RDT disturbed the oxidative status of silverside tissues and organs, even at the lowest concentrations of RDT evaluated. The molecular mechanisms of the antioxidant defense pathways in aquatic organisms are only at the initial stages of being deciphered. However, the responses to oxidative stress may not

necessarily be associated with the transcriptional variations of genes or with the post-translational modifications of proteins (Rhee et al. 2005; Regoli and Giuliani 2014; Xu et al. 2016). The transcriptional responses observed in this study were variable depending on the tissue investigated, which can be because of the different activities performed by these tissues.

In South America, the glyphosate-based herbicides reach the water bodies containing *O. humensis* mainly through soil surface run-off. Besides this one, many other fish species also share the same natural habitat and may be exposed to similar risks. Our results indicate the toxicological effects of RDT at environmentally relevant concentrations on silversides. An increase in ROS, lipid peroxidation, DNA damage, and apoptosis was observed in the fish exposed to RDT. In addition, the herbicide also stimulated the expression of genes associated with antioxidant responses in the gills and brain. In contrast, a contrary response was observed in the hepatopancreas, which exhibited a decrease in the expression of these genes. These findings are concerning for the ecotoxicology and conservation of aquatic communities in the environment close to monocultures. Therefore, the genomic and molecular

**Fig. 5** The oxidative effects in terms of the production of reactive oxygen species (fluorescence intensity) (A), lipid peroxidation (fluorescence intensity) (B), DNA fragmentation index (%) (C), and apoptotic cells (%) (D) in the erythrocytes of *Odontesthes humensis* fish in the control group and those exposed to 2.07 and 3.68 mg L<sup>-1</sup> (glyphosate acid equivalent) of Roundup Transorb® for 24 h, as evaluated by flow cytometry. The values are expressed as mean ± standard error of the mean. Different letters indicate significant differences between the experimental groups (one-way analysis of variance; n = 4; p < 0.05)



knowledge of sparsely studied native species, such as silversides, is important to determine the effects of xenobiotics on the natural environment and the resident species. This would help develop policies for a further responsible use of pesticides considering the environment and native species.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11356-021-15239-w>.

**Availability of data and materials** Not applicable

**Authors' contribution** AWSM and TLRS conducted all experiments and were major contributors in writing the manuscript. MHR, WBD, and END conducted the gene cloning and qRT-PCR experiments. ASVJ and CDC conducted and analyzed flow cytometry experiments. PGC and AB performed an evaluation of real concentrations of glyphosate. GMS and RBR designed the experiments and analyzed data. VFC was responsible for project administration, funding acquisition, and writing the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Ethics approval** The methodology used in this study was approved by the Ethics Committee of the Federal University of Pelotas/RS, Brazil, (number 23110.007018/2015–85).

**Consent to participate** Not applicable

**Consent for publication** Not applicable

**Conflict of interest** The authors declare no competing interests.

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