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# A Pilot Study of Gene Expression Modulation from Antioxidant System of Killifish *Austrolebias charrua* After Exposure to Roundup Transorb®

**Natiéli M. Gonçalves[1] · Tony L. R. Silveira[1,2] · Amanda W. S. Martins[1] · Eduardo N. Dellagostin[1] · Eduardo B. Blödorn[1] · Leandro S. Nunes[1] · William B. Domingues[1] · Luana C. Salvi[1] · Patrícia G. Costa[2] · Adalto Bianchini[2] · Mateus T. Kütter[2] · Luis F. F. Marins[2] · Matheus V. Volcan[3] · Mariana H. Remião[1,4] · Vinicius F. Campos[1,4]**

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## Abstract

Roundup Transorb® (RDT) is the most popular glyphosate-based herbicide (GHB) used in agriculture, and its impact extends to non-target organisms. The annual killifish *Austrolebias charrua* is an endangered species endemic to southern South America and inhabits temporary ponds. This study evaluates the effects of RDT concentrations (0.065 and 5 mg/L GAE) on *A. charrua* exposed for 96 h. Gene expression of *cat*, *sod2*, *gsta*, *gclc*, and *ucp1* was evaluated on the liver and gills. Highlighting that even at low concentrations permitted by Brazilian legislation, the RDT can have adverse effects on *A. charrua*.

**Keywords** Gene Expression · Oxidative Stress · Killifish · Herbicide · Roundup · Glyphosate

## Introduction

Roundup® utilizes glyphosate (N-[phosphonomethyl] glycine), a non-selective herbicide, as an active ingredient. The damage caused by glyphosate-based herbicides (GBHs) to non-target organisms, including aquatic animals, is well documented (Martins et al. 2021). Among the harmful consequences, the accumulation of reactive oxygen species (ROS) stands out, often leading to oxidative stress by dysregulating the enzymatic antioxidant defense system, consisting of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Lushchak 2016). Biomarkers, such as genes involved in antioxidant pathways, can help us understand the adverse effects caused by aquatic contaminants like herbicides (Velasques et al. 2016; Kronberg et al. 2018; Martins et al. 2021; Pagano et al. 2024b).

*Austrolebias charrua*, is an endangered species from the Rivulidae family, endemic to the southern region of South America. These species are confined to ponds in the vicinity of the Patos-Mirim lagoon system in southern Brazil and Uruguay (de Oliveira Fernandes et al. 2021) where crops using GBHs are often cultivated. These fishes inhabit temporary ponds and wetlands, with eggs that overwinter in the mud. This might make them especially vulnerable to drainage from agricultural lands. This study aimed to evaluate the effects of RDT exposure on the gene expression of the antioxidant system in the liver and gills of the killifish *A. charrua*. Furthermore, due to the limited understanding of *A. charrua* at the molecular level, it was essential the sequence and characterize some genes to assess the potential toxicity of RDT on gene expression.

Natiéli M. Gonçalves and Tony L. R. Silveira contributed equally to this work.

✉ Mariana H. Remião
mariana.remiao@ufpel.edu.br

✉ Vinicius F. Campos
fariascampos@gmail.com

[1] Laboratório de Genômica Estrutural, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brasil

[2] Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, Rio Grande, RS, Brasil

[3] Laboratório de Ictiologia, Instituto Pró-Pampa (IPPampa), Pelotas, RS, Brasil

[4] Laboratório de Genômica Estrutural, Programa de Pós-Graduação em Biotecnologia, CDTec, Campus Universitário Capão do Leão s/ nº - Prédio 20, Jardim América, Capão do Leão, RS CEP: 96.010-900, Brasil

## Materials and methods

Adult *A. charrua*, sourced from temporary ponds in the coastal fields of the Pampa Biome (Rio Grande city, RS, Brazil) under IBAMA/SISBIO permit number 71,072, had an average length of 3.34 ± 0.5 cm (standard deviation, SD) and a mass of 0.42 ± 0.15 g (SD). They underwent a 10-day acclimation period at 19 °C, with dissolved oxygen levels maintained above 6 mg/L, pH levels at 7 ± 0.1 (SD), ammonia levels below 0.25 mg/L, water hardness of ≤ 1 °dH, and a photoperiod of 12 h light and 12 h dark. The tanks had 50% of their water renewed weekly, and the fish were fed twice daily with live *Artemia salina* nauplii *ad libitum*.

The 27 animals were distributed in 9 aquaria, with three animals by aquaria. So, the experimental design consisted in three groups of three aquaria each: a control group without exposure, and two groups exposed to RDT at 0.065 mg/L and 5 mg/L (glyphosate acid equivalent, GAE) for 96 h, followed by 96 h of non-contaminated water. The concentration of 0.065 mg/L represents the maximum limit of glyphosate allowed in the natural water bodies according to Brazilian legislation (CONAMA 2015), while 5 mg/L is a challenge for fish and an environmentally relevant concentration (Topal et al. 2015). At the same time of exposure time, three aquaria, also with three fish by aquaria, were maintained for each group, to be used in case of replacement necessity. The data of mortality were calculated considering the total number of aquaria (18 animals per group). Glyphosate concentration in aquarium water samples ($n = 9$) was quantified by gas chromatography following extraction using Oasis-MAX 200 mg cartridges (Waters Corporation), as described by Zebral et al. (2018).

After 96 h in clean water, 5 randomly chosen survival animals from each group were euthanized by immersion in iced water (2 –4 °C) for 10 min. Subsequently, they were measured, and samples of gills and liver tissue ($n = 5$) were collected RNA extraction from gills and liver sample and cDNA synthesis were conducted as described by Martins et al. (2021). Primers were designed in the PriFi tool (https://services.birc.au.dk/prifi/) for genes (*cat*, *sod2*, *gsta*, *gclc*, and *ucp1*) using the conserved regions of the orthologs of other fish species [Supplementary Information (SI) 1 and 2]. The gene fragments were amplified by polymerase chain reaction (PCR) using these primers and subsequently sequenced using Applied Biosystems 3500 Genetic Analyzer® (Life Technologies, USA). Phylogenetic analyses were performed using the Bayesian inference method implemented in Mr. Bayes v.3.2.6 (Ronquist et al. 2012) and available in the web portal Phylogeny.fr (phylogeny.fr/) (Dereeper et al. 2008).

Primers for the *cat*, *sod2*, *gstα*, *gclc*, and *ucp1* genes were designed using the Primer3web v.4.1.0 tool (primer3.ut.ee) (SI 2). Quantitative reverse transcription PCR (qRT-PCR) was performed on the CFX96T Real-Time PCR Detection System (Bio-Rad Laboratories, EUA) and the results were analyzed using the $2^{-\Delta\Delta CT}$ method (Pagano et al. 2024a).

The normality distribution of quantitative data was evaluated by the Shapiro-Wilk test. Levene's test, O'Brien's test, and Brown and Forsythe's test were used to evaluate the homogeneity of variances. Thus, gene expression data were submitted to the one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test, with a significance level of 5%. The survival analysis data were assessed using the Log-rank (Mantel-Cox) test for trend., in the entire population of each aquarium. Gene expression results were expressed as mean ± standard error of the mean (SEM).

After the 96 h exposure period, the water glyphosate concentration was 4.36 ± 0.39 mg/L$^{-1}$ for 5 mg/L group, 0.059 ± 0.001 mg/L$^{-1}$ for 0.065 g/L group, and < 0.015 for the control group. The period of non-contaminated water maintenance glyphosate concentration was < 0.015 in all aquariums. Significant lethality ($p < 0.05$) was observed during the experimental period when fish were exposed to 5 mg/L (SI 3).

## Results

Sequencing and molecular characterization were performed on fragments of *cat*, *eef1α*, *gclc*, *gstα*, *sod2*, and *ucp1* genes from *A. charrua*, and the sequences were deposited on GenBank (SI 1). The molecular characterization of the cDNA fragments sequenced of *A. charrua* is presented in (SI 4). Information regarding the phylogenetic tree constructed from *A. charrua* sequences for *cat* (SI 5), *eef1α* (SI 6), *gclc* (SI 7), *gst* (SI 8), *sod2* (SI 9), and *ucp1* (SI 10) is provided. These analyses confirmed that the characterized fragments belong to the genes of *A. charrua*.

On the gills, the relative expression of the *cat* gene exhibited a significant down-regulation ($p < 0.05$) in fish exposed to both concentrations of RDT (Fig. 1A). Regarding the *ucp1* gene expression, fish exposed to 0.065 mg/L of RDT showed significantly higher ($p < 0.05$) expression than those exposed to 5 mg/L of RDT on the control group (Fig. 1E). No significant differences ($p > 0.05$) were observed in *gclc*, *sod2*, and *gstα* gene expressions between the groups (Fig. 1B, C, and D).

On the liver, the relative expression of *cat* and *sod2* genes showed no significant difference ($p > 0.05$) between any tested group (Fig. 2A and C). However, the mRNA expression of *gclc* and *gstα* was significantly up-regulated ($p < 0.05$) in fish exposed to 5 mg/L of RDT (Fig. 2B and D). Additionally, the gene expression of *ucp1* was decreased

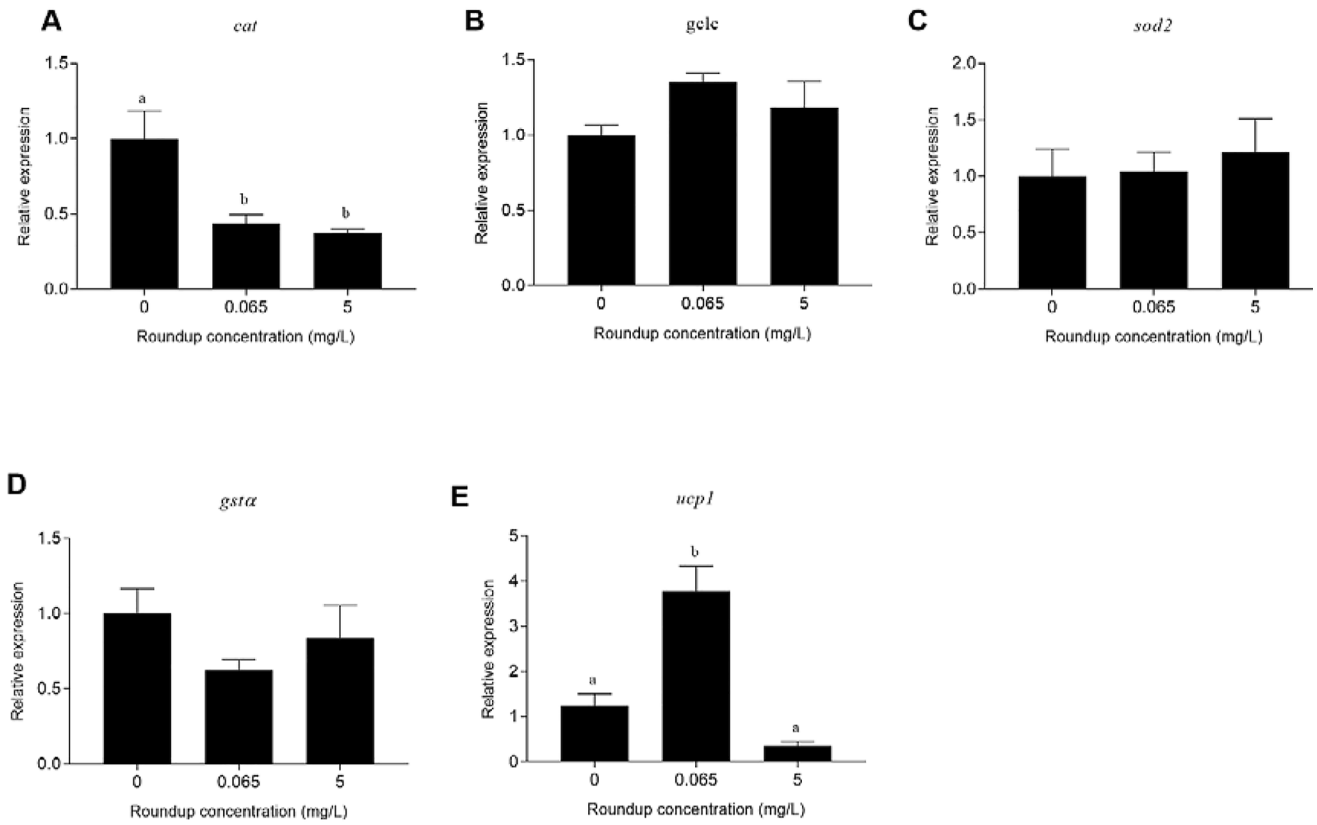


**Fig. 1** Analysis of gene expression in gills of *A. charrua* of (**A**) *cat*, (**B**) *gclc*, (**C**) *sod2*, (**D**) *gstα*, and (**E**) *ucp1* evaluated by qRT-PCR, $n = 5$

($p < 0.05$) in fish exposed to both 0.065 mg/L and 5 mg/L of RDT concentrations (Fig. 2E).

## Discussion

Contrary to findings in prior studies, we observed a down-regulation of *cat* expression in the gill tissue of fish exposed to RDT. The observed down-regulation of *cat* expression suggests impaired catalytic activity, potentially resulting in cellular damage due to the accumulation of $H_2O_2$ (Sepasi Tehrani and Moosavi-Movahedi 2018).

Moreover, our findings unveiled differential expression of the *ucp1* gene in the gills of fish exposed to 0.065 mg/L of RDT. *A. charrua* appears to exhibit heightened sensitivity to RDT compared to zebrafish, as evidenced by the up-regulation of *ucp1* even at a lower concentration (0.065 mg/L). Understanding the role of *ucp1*, integral to the coupling mechanism of the electron transport chain to ATP synthesis (Tine et al. 2012), may prove crucial in safeguarding against oxidative stress damage (Cadenas 2018).

In our study, we observed an up-regulation of *gclc* and *gstα* gene expression in the liver following exposure to 5 mg/L of RDT, alongside a down-regulation of *ucp1* in both RDT-exposed groups. So, our results suggest that RDT may act as a pro-oxidant agent in *A. charrua*, as evidenced by the up-regulation of *gclc* expression observed at 5 mg/L of RDT exposure. Opposed results for *ucp1* and *gclc* gene expression were found by Velasques et al. (2016). On the other hand, the *gstα* gene codes for a protein involved in detoxification processes, targeting both endogenous and exogenous compounds (Modesto and Martinez 2010). Interestingly, even after 96 h in uncontaminated water, *A. charrua* continued to exhibit up-regulation of *gstα*, indicating ongoing activation of this gene in response to pollutants. This observation aligns with the findings of Ma et al. (2018), further supporting the notion of *gstα* activation in response to environmental contaminants. However, chronic exposure to glyphosate did not result in differential *gstα* expression (Zheng et al. 2021), suggesting that the observed response may be specific to the acute exposure to RDT.

Additionally, our study unveiled that the initial 48 h of exposure to RDT posed the highest stress for *A. charrua*, resulting in the highest lethality rate. Evidence suggests that comparable concentrations of RDT yield different effects depending on the fish species (Velasques et al. 2016; Modesto and Martinez 2010; Martins et al. 2021), highlighting the heightened sensitivity of *A. charrua* to RDT

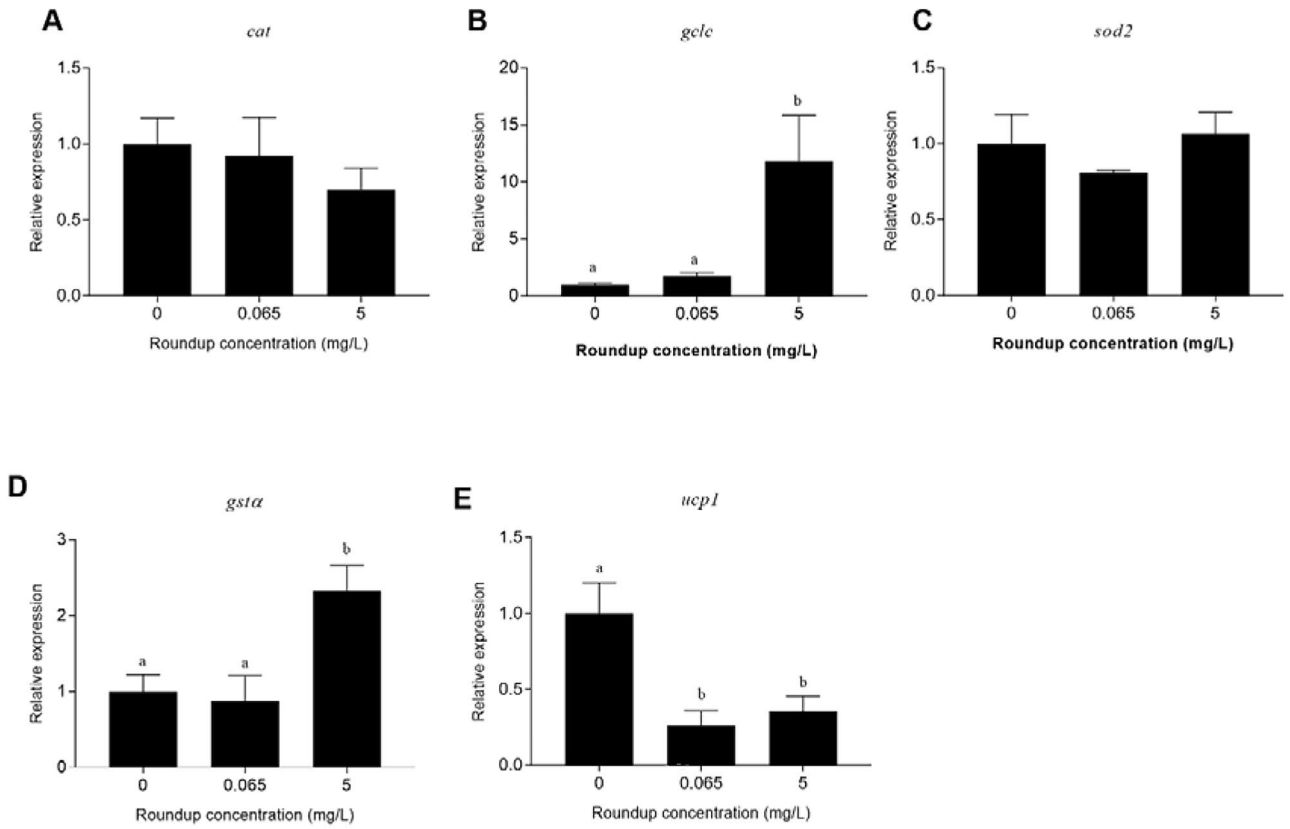


**Fig. 2** Analysis of gene expression in liver of A. charrua of (**A**) *cat*, (**B**) *gclc*, (**C**) *sod2*, (**D**) *gstα*, and (**E**) *ucp1* evaluated by qRT-PCR, $n = 5$

compared to other fish species. The disruption caused by exposure to GBHs in genes associated with the antioxidant system can adversely affect the health of adult individuals, potentially altering their behavior, growth, and reproduction rates, consequently leading to a decline in the population's genetic variability in future generations. However, it is important to recognize that these data may vary according to the sex and developmental stage of the fish, for example. Therefore, future studies could be conducted to better elucidate the effect of RDT on the species *A. charrua*.

In conclusion, our findings indicate that a permissive concentration, according to Brazilian legislation, can still have a significant impact on sensitive species like *A. charrua*. Considering *A. charrua*'s rapid life cycle and generational alternation, comprehending its physiological responses to environmental xenobiotics is imperative for the conservation of this species and the formulation of effective governmental policies. Furthermore, study alternative pathways and assessment methodologies in future studies will enhance understanding of how environmental contaminants affect *A. charrua*.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00128-024-03930-w.

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## Declarations

**Conflict of interest** The authors declare not having conflict of interest.

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