BRINGING GENOMICS INTO WILDLIFE ECOTOXICOLOGY: MICRORNA SEQUENCING IN ANNUAL KILLIFISH EXPOSED TO ROUNDUP® HERBICIDE

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ABSTRACT

MicroRNAs (miRNAs) are short noncoding RNA molecules that regulate gene expression post-transcriptionally. Environmental factors, such as pesticide pollution, can influence miRNA biogenesis and target gene expression, making miRNAs valuable in ecotoxicology to monitor stress responses in exposed organisms. Annual killifishes are the most endangered group of fish in Brazil, with the species *Austrolebias charrua* inhabiting temporary wetlands from southern Brazil to eastern Uruguay. The main threat to *A. charrua* is extensive agriculture, as these fish inhabit ponds near agricultural systems that heavily use glyphosate-based herbicides like Roundup®. This investigation aims to profile global miRNA expression in the gonads of *A. charrua* exposed to Roundup®. Following acute exposure, fish gonads were excised, RNA was extracted, and miRNA sequencing and bioinformatics analyses were performed. Sequencing revealed 205 and 150 mature miRNAs in ovarian and testicular tissues from *A. charrua*, respectively. This pioneering study on miRNA sequencing in annual killifish exposed to pollution enhances understanding of the (epi)genomics and adaptive responses of this endangered species. Differential expression analysis identified miRNAs associated with herbicide toxicity. As miRNAs are being innovatively used as biomarkers in ecotoxicology, this study paves the way for developing molecular tools for annual killifish biomonitoring and environmental health assessment.

Keywords: Chemical Pollution; Biodiversity; Biomonitoring; Next-Generation Sequencing; Bioinformatics.

1 INTRODUCTION

Biomonitoring involves the systematic use of biological responses from living organisms to assess changes in an ecosystem. Fish are the primary bioindicators used to evaluate the ecotoxicity of pollutants.[1] Ecotoxicogenomics integrates genomics with ecotoxicology, studying gene expression in bioindicators exposed to environmental pollution.[2] Among the molecular biomarkers used to monitor water quality, microRNAs (miRNAs) stand out due to their stability, specificity, and ability to reflect early responses to environmental stressors.[3] The microRNA sequencing technique (miRNA-Seq) is employed to catalog all miRNA species in an organism and quantify changes in their expression levels.[4] This methodology has been applied in ecotoxicology to identify epigenetic markers associated with pollutant exposure. For example, miRNA expression has been shown to change in fish exposed to pharmaceuticals, heavy metals, plastics, and herbicides.[5, 6, 7, 8] The miRNA-Seq technology offers sensitivity and comprehensiveness, making it a powerful tool for environmental genomics. Bioinformatics tools, such as interactome mapping, can elucidate the molecular interactions between miRNAs and their target genes, providing insights into how environmental stressors affect gene expression networks.

Annual killifish are habitat specialists and are considered the most extremophilic vertebrates. In Brazil, they are the most endangered group of fish, inhabiting extreme environments with unique biological characteristics.[9] The species *Austrolebias charrua* inhabits temporary wetlands from southern Brazil to eastern Uruguay. The primary anthropogenic threat to this species is extensive agriculture, as *A. charrua* lives in flooded areas adjacent to monocultures that intensively use pesticides, especially glyphosate-based herbicides like Roundup®.[10] The loss of such species not only impacts biodiversity but also disrupts ecosystem function and stability. Glyphosate and its formulations are known to impact fish reproduction in various ways, including reducing fertility, inhibiting steroidogenic enzyme activity, decreasing sperm quality, causing morphological changes in gonads, and increasing oxidative stress.[11] These reproductive changes are concerning in an environmental context, as they reduce species fitness, leading to population decline or even extinction.

This study aimed to investigate the epigenetic effects of Roundup® on *A. charrua* reproductive tissues (ovaries and testicles) by profiling global miRNA expression using miRNA-seq. Finding the miRNAs biomarkers for Roundup® exposure can be useful for developing biotechnological tools for the environmental biomonitoring of annual killifishes. Additionally, interactomics analysis was used as a bioinformatics approach to unveil miRNA-gene interactions potentially impaired by pesticide stress.

2 MATERIAL & METHODS

A total of 24 adult *A. charrua* specimens (12 males and 12 females) were collected from a temporary pond in Rio Grande city, Rio Grande do Sul state, Brazil, under permit IBAMA/ICMBio Nº 71072-4. Fish were housed in 10 L tanks, fed twice daily with live food, and maintained at 21 ± 0.5°C under a 12 h light/dark cycle. Water quality parameters, including temperature, pH, ammonia, nitrite, and nitrate, were monitored daily. The fish were acclimated for ten days under these conditions. The exposure protocol followed our previous studies[12], using a single concentration of Roundup®: 5 mg/ L, simulating peak environmental contamination. Fish were exposed for 96 hours. Each experimental group (control and exposed to Roundup®) comprised six tanks containing

one mating pair (1 male and 1 female). Following acute exposure, fish were euthanized, their gonads excised, snap-frozen, and stored at -80°C for RNA extraction and miRNA sequencing.

Total RNA was extracted from 50 mg of ovarian and testicular tissues using the PureLink™ RNA Mini Kit (ThermoFisher Scientific, USA) and treated with DNase using the TURBO DNA-Free® Kit (ThermoFisher Scientific, USA). RNA integrity and purity were assessed using the 4200 TapeStation System (Agilent Technologies, USA). Small RNA libraries were constructed using the NEBNext® Small RNA Library Prep Set for Illumina. The gonadal tissues samples were used, forming six libraries per experimental group. Libraries were sequenced on the Illumina NovaSeq 6000 System.

Raw sequencing data were processed to remove contaminants and low-quality reads. miRNA profiling was performed using sRNAtoolbox, with reference genomes from *Kryptolebias marmoratus, Xiphophorus couchianus, and Nothobranchius furzeri*. miRNAs were annotated using miRBase. Differential expression analysis was conducted with DESeq2 package. Pathway enrichment for differentially expressed miRNAs was performed using mirPath (version 3.0), and target gene prediction was done with microT-CDS (version 5.0). Interactomes were constructed and visualized using Cytoscape (version 3.9.1).

3 RESULTS & DISCUSSION

Chemical pollution stands as a major driver for the loss of biodiversity and ecosystem services.[13] Exposure to environmental pollutants can negatively impact the health of living organisms, posing significant risks to freshwater biodiversity fitness and maintenance. Annual killifish are rare, endangered species highly specialized for life in ephemeral environments. Given that natural habitats are being encroached upon by monocultures reliant on the use of Roundup® in Rio Grande do Sul, the hypothesis that annual killifish reproductive events may no longer be as productive as before is extremely concerning.[10] Developing novel environmental diagnostic methods aimed at species conservation and the maintenance of the physical integrity and hydrological cycle of temporary wetlands becomes urgent.

In this study, miRNA transcriptome sequencing of ovarian and testicular tissues from *A. charrua* revealed expressions of 205 and 150 mature miRNAs, respectively. The data from sequencing is available in NCBI datase under the SRA accession number SRP434236. This study is a pioneer in sequencing miRNA in annual killifish exposed to environmental pollution and presents valuable scope for biomonitoring measures of this species that compose the endangered Brazilian fauna. The use of miRNA-Seq allowed for the characterization of functional elements of the genome of annual killifish, non-model species where there is no availability of a reference genome. Through high-throughput sequencing techniques, this work stimulates the development of new biomonitoring technologies based on miRNAs (Figure 1). miRNAs are sensitive and specific biomarkers for the toxicity status of the environment and provide a more comprehensive approach to the toxicological effects of pollutants on aquatic biota. Effectively, through biomarker analysis in fish, it is possible to diagnose water quality and analyze the ecological integrity of resident fauna, as chemical pollutants alter the physiology of organisms at different levels, such as cellular, biochemical, behavioral, and reproductive. Considering the numerous effects caused by the Roundup® herbicide on aquatic species, studies that seek to unravel the molecular effects of exposure to this xenobiotic become important and necessary.

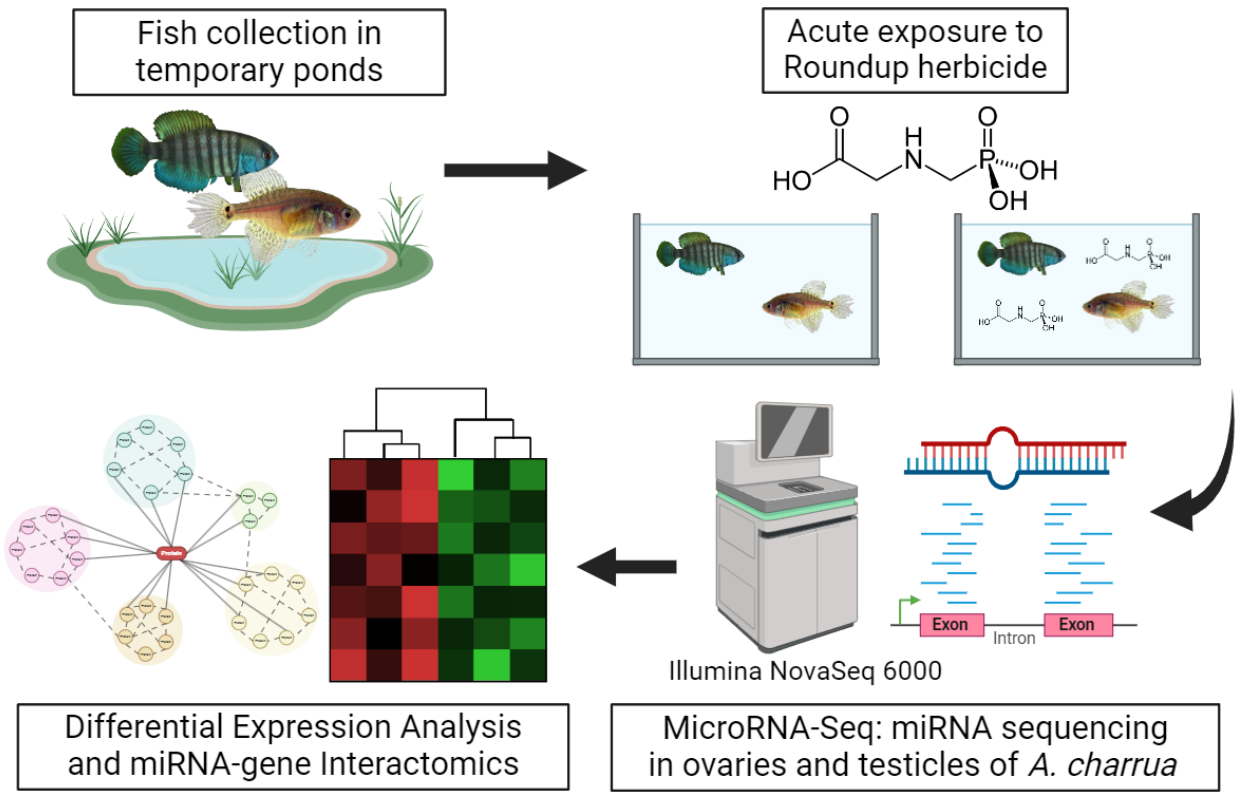

Figure 1. Flowchart summarizing the present study

4 CONCLUSION

This study is pioneering in miRNA sequencing in annual killifish exposed to a pesticide, significantly enhancing our understanding of epigenetic responses in a species that is part of the threatened Brazilian fauna. The exploration of the microRNAome of *A. charrua* gonads allowed the global identification of miRNAs. Between the perspectives of this project is the analysis of differential expression, which will enable the identification of miRNAs related to herbicide toxicity in this endagered species. Since miRNAs have been innovatively used as biomarkers in ecotoxicology, this study provides scope for the development of new biotechnologies for annual killifish biomonitoring and ecotoxicological assessment measures.

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ACKNOWLEDGEMENTS

This study was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS ARD 04/2019 #19/2551-0001323-0, FAPERGS/CNPq 07/2022 #23/2551-0000181-0, FAPERGS/SICT INOVA AGRO #22/2551-0001645-6), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/MCTI/CT-BIOTEC #31/2022, #440636/2022-1), and CAPES (Finance Code 001, AUXPE #2537/2018).