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A glyphosate-based herbicide reduces fertility, embryonic upper thermal tolerance and alters embryonic diapause of the threatened annual fish *Austrolebias nigrofasciatus*

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HIGHLIGHTS

- *A. nigrofasciatus* couples exposed to Roundup produced less but larger embryos.
- Roundup exposure alters the diapausing pattern of *A. nigrofasciatus* embryos.
- Roundup exposure diminished *A. nigrofasciatus* embryonic upper thermal tolerance.
- Roundup disrupts key-adaptations of annual fish and may jeopardize its populations.

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ABSTRACT

Roundup is the most popular glyphosate-based herbicide (GBH) worldwide. These formulations kill a wide range of plants. Despite that, non-target species can be jeopardized by GBH, such as the annual fish *Austrolebias nigrofasciatus*. This species occurs in wetlands that dries annually. Key-adaptations permit them to live in such harsh habitats, *e. i.* Elevated fertility, drought-tolerant diapausing embryos and elevated thermal tolerance. We aimed to evaluate acute (96 h) effects of Roundup exposure (0.36 or 3.62 mg a. e./L) in reproduction, diapause pattern and embryonic upper thermal tolerance (EUTT) of *A. nigrofasciatus.* For such, we evaluated the number and diameter of embryos produced by exposed fish. Also, recently fertilized embryos were exposed and its diapause pattern was evaluated. Following 15 post exposure days (PED), we evaluated the number of somite pairs and following 30, 35 and 40 PED we evaluated the proportion of pigmented embryos (PPE). Finally, the critical thermal maximum (CTMax) of exposed embryos was assessed. Results demonstrated that couples exposed to 0.36 mg a. e./L Roundup produced less but larger embryos. Similarly, embryos exposed to 3.62 mg a. e./L Roundup had a reduced PPE following 30 PED. Finally, embryos exposed to 0.32 mg a. e./L Roundup had a CTMax reduction of 2.6 °C and were more sensitive to minor increases in heating rates. These results indicate that Roundup have negative outcomes in fish reproduction, embryonic development and EUTT. This information is of particular interest to the conservation of annual fish, considering that those are key-adaptations that permit these animals to survive the harsh impositions of ephemeral wetlands.

1. Introduction

Roundup formulations are among the most popular glyphosate based herbicides (GBH) used. With total volume applied of approximately 830 million kg in 2014, glyphosate is the most used pesticide in the world (Benbrook, 2016). Used for agricultural and non-agricultural weed control, GBH acts as post-emergent herbicides that kills a wide range of plants (EPA; WHO). Roundup are commercial formulations that consist in a mixture of the active ingredient glyphosate, that produce the herbicide desired effect, and surfactants such as polyethoxylated amines (POEA) (Annett

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et al., 2014), that increase glyphosate bioavailability (Richard et al., 2005). Glyphosate herbicide effects are produced by inhibition of the 5-enolpiruvilshikimate-3-phosphate synthetase (EPSPS), an enzyme of the shikimate pathway, present in plants. Ultimately, glyphosate leads to protein shortage and plant death (Boocock and Coggins, 1983). GBH are commonly used in association with genetically engineered plants that are resistant to glyphosate, such as soybean (Benbrook, 2016). In 2014, the global soybean production was 315.4 million metric tons, with Brazil accounting for the production of 94.5 million metric tons, of which 94% belonged to genetically engineered herbicide-tolerant variants (Benbrook, 2016). Considering this data, Brazil was the second soybean producer country in the word, behind the U.S. that produced 108 million metric tons and followed by Argentina, that produced 56 million metric tons (Benbrook, 2016).

Glyphosate is highly soluble in water (10,000–15,700 mg/L at 25 °C) (Annett et al., 2014). Therefore, during rainfall seasons, this herbicide can be easily lixiviated from plantation zones to surrounding water bodies (Botta et al., 2009; Giesy et al., 2000). Indeed, glyphosate has already been found in water bodies from agricultural areas in southern Brazil at concentrations ranging from 0.36 to 2.16 mg/L (Almeida, 1992) and in Argentinian water bodies located near agricultural plantations at concentrations ranging from 0.10 to 0.70 mg/L (Peruzzo et al., 2008). Despite that, predicted worst-case scenarios for glyphosate contamination of surface water ranges in concentrations of 1.7–5.2 mg/L (for review: Annett et al., 2014). A great number of studies have already evaluated the half-life of this herbicide in water and it was usually demonstrated to be of proximally 8 days (Giesy et al., 2000; Pérez et al., 2007; Vera et al., 2010; Iummato et al., 2013).

Once in the water, glyphosate can jeopardize non-target species (Mesnage et al., 2015), such as fish. Classical well stablished toxic effects of these herbicides, in concentrations ranging from 0.12 to 7.2 mg a. e./L, include the induction of oxidative stress (Guilherme et al., 2010; Cattaneo et al., 2011); Glusczak et al., 2011), genotoxicity (Guilherme et al., 2010; Ghisi and Cestari, 2013), histological alterations (Jiraungkoorskul et al., 2003) and changes in acetylcholinesterase activity (Glusczak et al., 2007; Cattaneo et al., 2011). Despite that, other toxic effects of these herbicides have already been described. For example, it has been shown that GBH can cause negative effects in reproduction. It has already been demonstrated that GBH may inhibit steroidogenesis (Walsh et al., 2000) and aromatase activity (Richard et al., 2005). Indeed, Soso et al. (2007) shown that the fish *Rhamdia quelen* chronically exposed to Roundup (3.6 mg a. e./L) had diminished levels of 17β-estradiol. In accordance, many studies shown that GBH may reduce fish sperm quality (Harayashiki et al., 2013; Lopes et al., 2014; Sánchez et al., 2017) in concentrations of 0.13 and 0.7 mg a. e./L; 5 and 10 mg a. e./L and 0.5 mg a. e./L, respectively. Moreover, Uren Webster at al. (2014) demonstrated that exposure to 10 mg/L glyphosate reduced fertility in zebrafish. Also, some studies have already shown that GBH can cause embryotoxicity. Paganelli et al. (2010) shown that these herbicides (1.35–2.03 mg a. e./L) disrupt the retinoic acid signalization, leading to craniofacial deformities in chicken and *Xenopus laevis* embryos. Similarly, it has already been shown that exposure to GBH (3.07–7.50 mg a. e./L) induce craniofacial alterations in *Scinax nasicus* tadpoles (Lajmanovich et al., 2003) and in embryos of the fish *Odontesthes humensis* (0.36–5.43 mg a. e./L) (Zebral et al., 2017).

Despite the extensive literature describing GBH toxic effects and its intense use in Brazil (ANVISA, 2010), just a few studies evaluated the deleterious outcomes of the exposure to these herbicides in Brazilian native species (Albinati et al., 2007). In response, we evaluated the effects of acute exposure to Roundup on a suite of organismal endpoints in the annual fish *Austrolebias nigrofasciatus*, an endangered species from Rivulidae family. There are many characteristics of this species that makes it a good experimental model. These animals can be easily maintained and reproduced under laboratory conditions (Podrabsky, 1999). Also, this species naturally occurs in agricultural areas from Uruguay to Rio Grande do Sul state (southern Brazil) where GBH are greatly used (ICMBio, 2013; Benbrook, 2016). Consequently, *A. nigrofasciatus* can be considered as an environmental-realistic experimental model for ecotoxicological studies. Lastly, considering that most of the annual fishes in Brazil are threatened with extinction (Costa, 2002; Rosa and Lima, 2008; ICMBio, 2013), *A. nigrofasciatus* has been recently chosen as a key species for protection of this group in a national plan instated by the Brazilian government (ICMBio, 2013).

American annual fishes occur from Mexico to Argentina in ephemeral wetlands that completely dry out annually (ICMBio, 2013). To survive in such harsh environment, annual fishes developed a suite of key-adaptations. During the periods of drought, all the adult individuals die and the fish populations are maintained by drought-tolerant diapausing embryos that survives desiccation buried in the substrate (Wourms, 1972; Hand et al., 2016). Beyond that, these animals also have to tolerate great variabilities in ambient physical-chemical parameters, such as temperature (Podrabsky et al., 1998). With the start of the rainfall season, annual fishes have to break its diapause and hatch into fast growing larvae that will rapidly reach sexual maturity (Walford and Liu, 1965; Sakakura and Noakes, 2000; Lee et al., 2008). Once mature, these animals will continually and greatly reproduce until they die in the next dry season (Costa, 1998; Arenzon et al., 1999, 2001; Errea and Danulat, 2001). Therefore, high fertility, embryonic diapause and thermal tolerance are key-adaptations that permits annual fishes to tolerate the harsh environments in which they occur.

In light of the background described above, the objective of our study was to evaluate the effects of acute (96 h) exposure to environmental realistic Roundup concentrations (0.36 and 3.62 mg a. e./L) in a suite of organismal endpoints that could indicate deleterious outcomes in reproduction, embryonic diapause and embryonic upper thermal tolerance in the annual fish *A. nigrofasciatus*. To accomplish our goal, Roundup effects in fish reproduction were assessed by the number and the diameter of produced embryos. Moreover, we evaluated the effects of the exposure to this herbicide in embryonic diapause patterns by tracking the number of somite pairs and the percentage of pigmented embryos. Finally, possible negative effects of Roundup exposition in embryonic upper thermal tolerance was assessed by the critical thermal maximum (CTMax) method.

2. Materials and methods

2.1. Animal collection and rearing

A total of 12 *A. nigrofasciatus* mating pairs were collected (license: IBAMA/ICMBio n^{o}. 41907-1) in a wetland located in the floodplains of the Padre Doutor stream (Patos-Mirim lagoon system, Rio Grande do Sul state, southern Brazil) and transferred to the aquatic laboratory located at UFPel (Universidade Federal de Pelotas), where the experiments were performed. Mating pairs were reared in 20 L aquaria. An artificial spawning nest containing peat moss was added to each aquaria and animals reproduced freely (Arenzon et al., 2002). Animals were acclimated to laboratory conditions for 3 weeks. During this period, fertilized embryos were manually collected once a week and reared according to literature (Arenzon et al., 2002). Following embryo collection or adult acclimation period, animals were distributed among treatment groups as described in experimental design section. During acclimation period and during the different experimental periods, animals were