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Influence of temperature on the antioxidant responses and lipid peroxidation of two species of tadpoles (*Rhinella schneideri* and *Physalaemus nattereri*) exposed to the herbicide sulfentrazone (Boral 500SC®)

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ABSTRACT

Amphibians can experience large temperature fluctuations in their habitats, especially during the larval stage, when tadpoles are restricted to small and ephemeral ponds. Changes in water temperature can alter development, metabolism and behaviour of cold-blooded animals but also the toxicokinetics of chemicals in the environment. In Brazil, pesticides application is intensified during the rainy season, which is the period of reproduction for many amphibian species. We evaluated here the influence of temperature (28, 32, and 36 °C) on the toxicity of the herbicide sulfentrazone (Boral®SC) in tadpoles of *Physalaemus nattereri* and *Rhinella schneideri*, by analysis of oxidative stress biomarkers. Exposure of tadpoles to sulfentrazone altered the antioxidant enzymes activities and induced lipid peroxidation with temperature-associated responses. Catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase (G6PDH) were impaired by combined effect of temperature and sulfentrazone in both species. G6PDH was increased in most groups exposed to 36 °C. Biotransformation enzyme glutathione-*S*-transferase had more evident alterations in *P. nattereri* at higher temperatures and changes in *t*GSH contents presented different patterns between the species. Lipid peroxidation was particularly induced in tadpoles of *P. nattereri.* Integrated biomarker response (IBR) index indicated a synergic effect of temperature and sulfentrazone for tadpoles of *P. nattereri*, while the IBR was mainly influenced by temperature in *R. schneideri*. Our study showed that temperature modulates biochemical responses in tadpoles exposed to sulfentrazone with a species-specific pattern. These findings imply that the effects of abiotic factors should be taken into account to evaluate the real risks of exposure of amphibians to commonly used pesticides.

1. Introduction

Increased agriculture activity in Brazil has caused extensive impact on several natural ecosystems and non-target organisms, especially due to the intensified use of pesticides. Brazil is the largest consumer of pesticides in the world, and sugarcane is one of the main crops contributing to the expansion of cultivated areas in recent years (UNICA, 2015). In south-eastern Brazil, the state of São Paulo is responsible for the usage of approximately 20% of all Brazilian pesticides and has the highest rates of conversion of natural habitats to agricultural land (IPT, 2000). Several species of amphibians have been recorded in this area (Bernarde and Kokubum, 1999; Prado et al., 2008; Provete et al., 2011) and many of them are distributed in areas common to agriculture practice. Considering the accelerated expansion of sugarcane in Brazil, mainly due to its use in biofuel production, there is a great concern that several amphibian species are threatened by exposure to local pesticides.

Sulfentrazone (2′,4′-dichloro-5-(4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl) methanesulfonanilide) is an herbicide widely used in sugarcane crops in Brazil and other areas of the world. It is also applied in crops of great commercial importance, such as soy, coffee, citrus and eucalyptus (Embrapa, 2013). Sulfentrazone inhibits protoporphyrinogen oxidase, an important enzyme in chlorophyll biosynthesis, causing extreme dehydration of foliage due to the loss of membrane integrity (Dayan and Watson, 2011). Little is known about the transport and fate of sulfentrazone in the environment, however it is known that its first route of dissipation includes microbial degradation (Hatzios, 1998). This herbicide belongs to the aryl

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triazolinones group and is considered a potential contaminant due to its long residual effect in soil (110–280 days) and relatively high water solubility (Hirata, 2010; Melo et al., 2010). Following application to soil, sulfentrazone can runoff into surface water, potentially leading to negative effects to aquatic organisms. Concentrations of sulfentrazone found in freshwater environments such as rivers, lagoons or even temporary ponds used by amphibians as a transitory environment during larval development, are still unknown due to a lack of studies in the literature. However, evidence has shown that sulfentrazone has been found in both soil and water in agriculture areas of Sao Paulo (Dutra De Armas et al., 2005). Concentrations of this and other herbicides may be particularly high in small and ephemeral ponds used by amphibians as habitats, since they are formed by flooding of soil during the rainy season.

Larval stages of anurans are especially susceptible to pesticides and other chemical compounds (Greulich and Pflugmacher, 2003) because tadpoles are strictly aquatic and have high skin permeability (Yan et al., 2008). In tropical areas with well-defined seasons, the reproduction of most amphibian species, and the subsequent occurrence of tadpoles, is restricted to the rainy season due to the availability of water (Mcdiarmid and Altig, 1999; Provete et al., 2011). It is also during this season that the contamination of lakes and streams as result of herbicide application is usually increased, especially due to the use of pre-emergent herbicides, such as sulfentrazone, which are better absorbed in moist soils. In Brazil, the rainy season occurs predominantly during the summer and is accompanied by the highest temperature averages of the year. Due to the small volume of water in ephemeral ponds, the water temperature is easily elevated, increasing the average temperatures and the daily thermal fluctuations. Previous studies have shown that the water temperature can exceed 40 °C during the summer in ponds where several species of tadpoles have been found in Brazil (Freitas et al., 2016). The deforestation in nearby areas of amphibian habitats and the loss of vegetation has also favored the increase of solar incidence in these small ponds, thus contributing to the water heating. Therefore, it should be considered that Neotropical tadpoles inhabiting agricultural areas are not only threatened by pesticide exposure, but also by the interaction effects of these substances and the environmental temperature variations.

Temperature is mentioned as one of the main abiotic factors influencing the toxicity of chemical compounds in the environment (Hooper et al., 2013; Noyes et al., 2009). Chemical and biological reactions become faster in warmer waters, and therefore, the effect of a toxic agent may be more pronounced at higher temperatures (Middlebrooks et al., 1973). For poikilothermic animals, thermal variation of the environment is directly translated into the variation of the physiological performance (Fontenot and Lutterschmidt, 2011). Temperature affects virtually all physiological aspects of amphibians, including digestion, vision, locomotion, growth and metabolism (Rome, 2007; Gatten et al., 1992). Thus, changes in environmental temperature can interfere with many natural biological responses in different species, making the organisms more susceptible to the action of xenobiotics (Blaustein et al., 2010; Blaustein and Kiesecker, 2002; Moe et al., 2013). Previous studies have shown that temperature plays an important role in the toxicity of several pesticides for amphibians (Boone and Bridges, 1999; Freitas et al., 2016; Hammond et al., 2015). For example, the mortality of tadpoles of *Rana clamitans* exposed to the insecticide carbaryl was increased at lower concentrations when animals were exposed to higher temperatures (Boone and Bridges, 1999). Exposure of tadpoles of the American bullfrog (*Lithobates catesbeianus*) to the herbicide diuron and its metabolite 3,4-DCA also showed that thyroidogenic effects of both compounds were more pronounced in animals exposed at higher temperatures (Freitas et al., 2016).

Despite the increasing number of studies exploring the effects of temperature on the toxicity of chemical compounds (Bao et al., 2008; Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009; Rohr and Palmer, 2005; Schiedek et al., 2007), the data collected to date are not comprehensive enough to routinely support integrated risk assessments for biological communities. Cold-blooded animals, such as amphibians, have inter- and intra-specific variations in their thermal tolerances (Katzenberger et al., 2012), so studying the toxicity of a chemical at one standard temperature may not be adequate to estimate negative effects to different species in environmental systems. Biomarkers of oxidative stress have been widely used to assess negative effects of pesticides on amphibians (e.g., Dornelles and Oliveira, 2014; Gripp et al., 2017; Stefani Margarido et al., 2013), since they may provide valuable information about amphibian health status (Peltzer et al., 2013). They are also relevant to assess the effects of thermal stress on poikilotherms, since temperature is an important factor affecting antioxidant enzymes in these organisms (e.g., Bagnyukova et al., 2003; Madeira et al., 2013; Vinagre et al., 2012). So far, no studies have investigated the effects of sulfentrazone on tadpoles and very few studies report its effects in non-target organisms, especially considering temperature variations. Therefore, this study aimed to investigate the effects caused by the interaction between temperature and the herbicide sulfentrazone in two native species of tadpoles in Brazil, *Physalaemus nattereri* and *Rhinella schneideri*, using oxidative stress markers. For this, we evaluated enzymes involved in antioxidant responses, including activities of superoxide dismutase (SOD), catalase (CAT), glucose-6-phosphate dehydrogenase (G6PDH), the biotransformation enzyme glutathione-S transferase (GST) and levels of total glutathione (*t*GSH). Lipid peroxidation was also evaluated by measuring the levels of malondialdehyde (MDA). Biological responses in tadpoles of both species were assessed based on individual biomarkers and on an integrative index, the integrated biomarker response (IBR, sensu Beliaeff and Burgeot, 2002). We hypothesized that a temperature increase may enhance the effects of sulfentrazone; with alterations to antioxidant defence systems, and induction of lipid peroxidation as a response of increased generation of reactive oxygen species (ROS) by stimulation of the metabolic processes in both species. Also, sulfentrazone may cause distinct effects in different tadpole species, indicating that they can be distinctly susceptible to negative effects caused by this herbicide.

2. Material and methods

2.1. Animals

Laboratory tests were conducted using two species of tadpoles, since species- or family-level sensitivity has been increasingly considered for use in laboratory tests evaluating toxicity of pesticides to amphibian populations (Egea-Serrano et al., 2012; Relyea, 2005; Shinn et al., 2008; Snodgrass et al., 2008). *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) are common anuran species with a wide geographical distribution in the northwest of São Paulo, Brazil. They can be found in different habitats, including pastures and sites of intense agriculture (Provete et al., 2011). Tadpoles of the selected species have different habitats in water bodies, with *P. nattereri* preferring benthic habitats and *R. schneideri* neustonic habitat (Prado et al., 2008; Provete et al., 2011).

2.2. Tadpole collection and acclimation

Spawns of the anurans *P. nattereri* and *R. schneideri* were collected in temporary ponds in non-agricultural areas in the region of Sao Jose do Rio Preto, Sao Paulo, Brazil (20°47′07.05″ S, 49°02′42.09″ W). After hatching, the larvae were maintained in the laboratory under ideal conditions of temperature (28 °C) and pH (7.5–8.0) in aerated aquariums during four weeks, until they reached stages 27–30 (Gosner, 1960), just before the development of legs. The animals were collected under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).