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Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles

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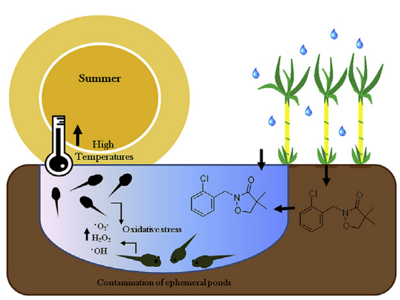
HIGHLIGHTS

- Clomazone effects at 28, 32 and 36 °C were evaluated in two tadpole species.
- Temperature enhanced oxidative stress in tadpoles exposed to clomazone.
- Carboxilesterase, but not acetylcholinesterase, was altered by clomazone.
- Neotropical tadpoles have different vulnerability to the heat stress and chemicals.
- Temperature effect was more evident than clomazone effects by the IBR analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Temperature is an important factor influencing the toxicity of chemicals in aquatic environments. Neotropical tadpoles experience large temperature fluctuations in their habitats and many species are distributed in areas impacted by agriculture. This study evaluated the effects caused by the exposure to clomazone (Gamit®) at different temperatures (28, 32 and 36 °C) on biochemical stress responses and esterase activities in *Physalaemus nattereri* and *Rhinella schneideri* tadpoles. Results evidenced that temperature modulates the effects of clomazone on biochemical response of tadpoles. Antioxidant enzymes, including catalase, superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase had their activities increased by clomazone in *P. nattereri* treated at higher temperatures. The biotransformation enzyme glutathione-*S*-transferase (GST) was also induced by clomazone at 32 and 36 °C. In *R. schneideri*, clomazone failed to alter antioxidant enzymes at 28 °C, but SOD and GST were increased by clomazone at higher temperatures after three days. All enzymes had their activities returned to the control levels after eight days in *R. schneideri*. Lipid peroxidation was induced in both species exposed to

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clomazone at 32 and 36 °C, but not at 28 °C. Acetylcholinesterase was not sensitive to clomazone and temperature, while most treatments impaired carboxylesterase activity. Integrated biomarker response (IBR) was notably induced by temperature in both species, and a synergic effect of temperature and clomazone was mostly observed after three days of exposure. These findings imply that tadpoles from tropical areas may present differential responses in their physiological mechanism linked to antioxidant defense to deal with temperature fluctuations and agrochemicals presence in their habitats.

1. Introduction

Temperature is an important variable that has direct impacts for aquatic ectotherms, such as amphibians. Changes on environmental temperature can alter behavior, metabolism, development, digestion and vision of amphibians, resulting in altered physiological performance in the environment (Murata and Yamauchi, 2005; Rome, 2007). Indeed, temperature can also interfere in the amphibian's responses triggered by environmental contaminants in natural systems (Freitas et al., 2017, 2016; Hooper et al., 2013; Noyes et al., 2009), and one of the current challenges in environmental toxicology is to understand how abiotic factors, such as temperature fluctuations, can interfere on the effects of chemical compounds in aquatic organisms.

Amphibians can experience large temperature fluctuations in their habitats, especially during the larval stages, in which they are restricted to small and ephemeral ponds, where the water occupies small volumes and then can be rapidly heated due to sun incidence (Wilbur, 1990). The process of water heating is also favored by deforestation and loss of vegetation in nearby areas of amphibian habitats. In tropical areas of the world, the water of these ponds can reach high temperatures (up to 40 °C) during the summer, which is the period of reproduction for many amphibian species (Freitas et al., 2016). Higher water temperature diminishes the availability of dissolved oxygen, accelerates ventilation and the metabolism, enhancing the oxygen demand of cold-blooded animals (Kaur et al., 2011), and possibly increasing the susceptibility to the effects of xenobiotics (Blaustein et al., 2010; Moe et al., 2013). Gradual change in temperature can be physiologically compensated by the organism, however a rapid change disturbs homeostasis and may become a stress (Kaur et al., 2011), especially in the presence of environmental pollutants.

In Brazil, agriculture has gradually invaded open areas used by amphibians for breeding (Da Silva et al., 2011). After intense periods of rain, soils of agriculture areas are flooded forming ephemeral ponds used by these animals to spawn. However, rainwater usually carries several agrochemicals, such as insecticides and herbicides, which leach from the soil to these water fractions, making them available to tadpoles (Bridges et al., 2004). Moreover, concentrations of agricultural compounds in these habitats are also usually higher than in other aquatic environments (e.g. rivers and lakes), due their smaller size. Considering these factors, the biological consequences caused by exposure to pesticides to anurans have a high potential to be significant (Mann et al., 2009).

The rapid expansion of sugarcane plantations due to the increased biofuel demand has contributed significantly to the use of pesticides (Kissmann, 1991) in Brazil. Clomazone (2-(2-clorofenil) metil-4-dimetil-3-isoxazolidinone) is an herbicide widely used in sugarcane crops, but also widely applied in rice, potato and cotton plantations. In 2009, clomazone ranked among the top ten herbicides most widely used in Brazil (IBAMA, 2010). Although very effective, clomazone causes extensive environmental contamination due to its high solubility in water (1100 $mg.L^{-1}$), besides a half-life that can reach 84 days (Zanella et al., 2002). This herbicide is often applied in sugarcane culture from September to March, corresponding to the spring/summer period of the year. This period coincides with the higher rain incidence in some areas, such as the northwest region of the Sao Paulo state, in which 36 species of anuran were registered (Provete et al., 2011). Therefore, the intensive use of clomazone in sugarcane during the reproductive period of anurans in the northwest of Sao Paulo State would represent a risk for their populations due to this herbicide runoff to the aquatic environment, especially considering higher temperatures influence recorded in this period of the year.

Environmental contaminants can lead organisms to oxidative stress, a condition that is also triggered by increases of temperature (Freitas and Almeida, 2016; Gripp et al., 2017; Madeira et al., 2013; Stefani Margarido et al., 2013; Vinagre et al., 2012). Studies have indicated that changes on environmental temperature can alter physiological stress response in aquatic organisms, and that ROS production is usually increased at higher temperatures as a consequence of an increased metabolism which leads to higher oxygen consumption (Almeida and Mascio, 2011; Freitas and Almeida, 2016). Moreover, it is also known that temperature can also modulate neurotoxicity of acetylcholinesterase (AChE) inhibitors (Beauvais et al., 2002; Durieux et al., 2011). Previous studies have shown that clomazone cause suppression of the antioxidant enzyme catalase (CAT) and enhance lipid peroxidation levels in fish (*Rhamdia quelen*) (Crestani et al., 2007), besides activating the activity of biotransformation enzyme glutathione-*S*-tranferase (GST) in *Prochilodus lineatus* (Pereira et al., 2013). Moreover, clomazone was also reported as an inhibitor of AChE in brain and muscles of *P. lineatus* (Pereira et al., 2013), suggesting a neurotoxic effect of this herbicide despite at relatively high concentration (10 $mg.L^{-1}$). Yet, the implication of temperature elevation on oxidative stress parameters and AChE inhibition caused by clomazone remains unknown, especially for amphibians. In fact, the toxic effects of clomazone for amphibians still need clarification, especially considering studies with field species. There is only a recent study showing that acute exposure (96 hrs) to clomazone changed the hepatic response such as, melanomacrophages contents, accumulation of eosinophils and occurrence of lipidosis in the hepatocytes in tadpoles of the American bullfrog (*Lithobates catesbeianus*) at environmental concentrations (0.5 $mg.L^{-1}$) (Oliveira et al., 2016).

Considering these points, this study aimed to investigate the effects of a commercial formulation of the herbicide clomazone (Gamit® 360CS) in two species of tadpoles, *Physalaemus nattereri* and *Rhinella schneideri,* under different thermal conditions (28, 32 and 36 °C). Both species have wide geographical distribution in Brazil, including sites of intense agriculture practice and sugar cane plantation (Provete et al., 2011). *P. nattereri* has the current population trend designed as decreasing and its major threats is the spread of intensive agriculture in the Cerrado biome. *R. schneideri* is generally a common and increasing species, although its range appears to be contracting in some areas from South America (Stuart