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Cardiac biomarkers as sensitive tools to evaluate the impact of xenobiotics on amphibians: the effects of anionic surfactant linear alkylbenzene sulfonate (LAS)

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ABSTRACT

Amphibian populations have been experiencing a drastic decline worldwide. Aquatic contaminants are among the main factors responsible for this decline, especially in the aquatic environment. The linear alkylbenzene sulfonate (LAS) is of particular concern, since it represents 84% of the anionic surfactants' trade. In Brazil, the maximal LAS concentration allowed in fresh waters is 0.5 mg L^{-1}, but its potential harmful effects in amphibians remain unknown. Therefore, this study aimed to analyze the effects of a sublethal concentration of LAS (0.5 mg L^{-1}) for 96 h on sensitive cardiac biomarkers of bullfrog tadpoles, *Lithobates catesbeianus* (Shaw, 1802). For this, we measured the activity level (AL - % of animals), *in situ* heart rate (f_H - bpm), relative ventricular mass (RVM - % of body mass), *in vitro* myocardial contractility and cardiac histology of the ventricles. Tadpoles' AL and f_H decreased in LAS group. In contrast, the RVM increased, as a result of a hypertrophy of the myocardium, which was corroborated by the enlargement of the nuclear measures and the increase of myocytes' diameters. These cellular effects resulted in an elevation of the *in vitro* contractile force of ventricle strips. Acceleration in the contraction (TPT - ms) also occurred, although no alterations in the time to relaxation (THR -ms) were observed. Therefore, it can be concluded that even when exposed to an environmentally safe concentration, this surfactant promotes several alterations in the cardiac function of bullfrog tadpoles that can impair their development, making them more susceptible to predators and less competitive in terms of reproduction success. Thus, LAS concentrations that are considered safe by Brazilian by regulatory agencies must be revised in order to minimize a drastic impact over amphibian populations. This study demonstrates the relevance of employing cardiac biomarkers at different levels (*e.g.,* morphological, physiological and cellular) to evaluate effects of xenobiotics in tadpoles.

1. Introduction

Amphibians have long suffered from a serious decline in their populations (Collins and Storfer, 2003; Wake and Vredenburg, 2008; Collins, 2010;). These declines occur in different regions of the world and result from various different factors such as the loss and

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fragmentation of their habitats, increased ultraviolet radiation, global warming, emerging infectious diseases and the presence of contaminants, notably those in aquatic environments (Blaustein et al., 1994; Collins and Storfer, 2003; Roe et al., 2005; Collins, 2010; Hof et al., 2011; Abdalla et al., 2013; Van Meter et al., 2014; Costa et al., 2015, 2016; Carvalho et al., 2017). Indeed, several studies have provided evidence that aquatic contaminants are among the main causes of amphibian declines (Collins, 2010; Blaustein et al., 2011). Acting alone or combined in the environment, the contaminants can cause changes ranging from cellular and physiological to ecological and population (Collins, 2010).

In order to maximize cutaneous respiration and osmotic balance mechanisms, amphibians present a particularly thin and highly permeable skin (Heatwole and Wilkinson, 2009). This makes them especially susceptible to xenobiotics due to the lack of a hydrophobic barrier and its high porosity to water molecules (Van Meter et al., 2014). Tadpoles, in turn, are even more susceptible to contaminants as they are born and develop in superficial waters, have highly permeable skin and undergo complex morpho-physiological changes controlled by many hormonal pathways (Shi, 2000). Tadpoles can also be exposed to contaminants through either food (Sparling et al., 2000) or gills (Bueno-Guimarães et al., 2001), but to a lesser extent. Moreover, as in most other ectothermic vertebrates, the metabolism of anuran larvae is relatively lower than that observed in avians and mammals (McDiarmid and Altig, 1999). As a consequence, lower catabolism and depuration rates are also observed in these animals when compared with birds and mammals, which increases the bioaccumulation of xenobiotics (Lillywhite et al., 1999).

In this context, it is worth emphasizing the participation of surfactants as potential pollutants. These compounds are widely used in washing products and cosmetic products (Ivankovi and Hrenović, 2010) such as emulsifying agents and foaming promoters (Brasil, 2014). They are classified as anionic, cationic and nonionic according to the load of its hydrophilic head (Penteado et al., 2006). In 2012, anionic surfactants represented 28% of the detergent's global market, with growth prospects of 2.5% by the year 2020 (Brasil, 2014). Linear alkylbenzene sulfonate (LAS) represents 84% of the anionic market. Brazil's participation in the surfactant market reached 1.5 billion dollars with a growth of 7% per year between 2009 and 2012 and growth prospects of around 2.1 billion dollars by 2018 (Brasil, 2014).

It is important to mention that the maximum LAS concentration allowed for fresh waters in Brazil is 2.5 mg L^{-1}. However, after its usage, the entire range of products containing LAS is discharged into sewers, and consequently reaches the environment (Ivankovi and Hrenović, 2010).

In the aquatic environment, LAS is known to damage fish gills and cause excessive mucus secretion (Venhuis and Mehrvar, 2004). Although there is strong evidence that LAS is taken up *via* gills rather than the skin in fish (Kikuchi et al., 1980; Tolls et al., 1994), it has been demonstrated that surfactants lead to marked alterations in the skin morphology of amphibians, causing hyperplasia or hypertrophy of the epithelium (Rissoli et al., 2016). Another alteration of LAS in fish is related to increasing liver vacuolization (Priya et al., 2016) and scattering inflammation (Kumar et al., 2007).

Regarding the effects of LAS on Ca^{2+} homeostasis, a decay in the electrophysiological potential of the abdominal skin in *Rana pipiens* was observed (Kulkarni and Goddard, 1980). Additionally, LAS induces an increase in intracellular Ca^{2+} concentration in cultured epithelial cells of *Xenopus laevis* kidney tissues (Bjerregaard et al., 2001). Alterations in cellular membrane permeability by surfactants, which interferes with intracellular Ca^{2+} balance, were already reported for *Xenopus laevis* when exposed to the other surfactant (POEA - polyethoxylated tallow amine; Hedberg and Wallin, 2010). These observations are particularly relevant, especially with respect to the maintenance of mechanisms and physiological functions, notably the cardiac function, which directly requires refined calcium supply for the maintenance of contractile mechanisms. Cardiac function is directly related to various important functions such as oxygenation, circulation and distribution of nutrients, which, once unbalanced, can aggravate other directly dependent functions, such as growth and metamorphosis, metabolism, among other functions (Lillywhite et al., 1999).

Thus, considering the previous findings about the effects of LAS on Ca^{2+} management and the lack of studies analyzing the effects of surfactants on amphibians' physiology and morphology, this study aimed to evaluate the effects of exposure to a sublethal concentration of the surfactant LAS on cardiac biomarkers of bullfrog tadpoles. By recognizing the physiological responses to environmental contaminants, adaptive strategies of species can be identified in order to determine the survival of these organisms in their habitats. Cardiac biomarkers have been used as a determinant and quite a sensitive tool to detect responses of the organism to environmental changes (Dal-Medico et al., 2014; Salla et al., 2016). By using these biomarkers, more effective responses to environmental monitoring can be proposed.

2. Material and methods

2.1. Animal care

Ninety-six tadpoles (*Lithobates catesbeianus*, Shaw 1802) were acquired from a frog farm in São Roque, São Paulo State, Southeast Brazil (22°78'S, 47°40'W). Tadpoles were placed in 60 L aquaria (*i.e.* closed system) equipped with a continuous supply of aerated (1.2 L h^{-1}) and dechlorinated water at a constant temperature (25 ± 1 °C), and under natural photoperiod (~ 12 h light/dark), until they reached Gosner developmental stage 25 (Gosner, 1960). The tadpoles were fed every two days with cooked organic lettuce *ad libitum*, which was stopped 48 h before exposure. All leftover food and feces remaining in the aquaria were removed. Only after reaching Gosner developmental stage 25 (Gosner, 1960), the ecotoxicological procedures were performed.

2.2. Ecotoxicological experiments

During the whole experimental period, water was monitored daily to ensure that the physical and chemical parameters were maintained at acceptable levels (pH 7.5–7.6; hardness as $CaCO_3$ 51–58 mg L^{-1}; dissolved oxygen 7.0–7.5 mg L^{-1}; conductivity 97.56 ± 0.02 µS cm^{-1} – TDS Digital®), similar to most Brazilian inland waters (CONAMA, 2011). Ammonia concentrations in water were checked every day (K-1510;CHEMets) and remained under 1 mg L^{-1}. All parameters were kept within acceptable (ASTM, 2014) guidelines.

For the exposure procedures, tadpoles (N = 72) with body mass between 2.39 and 3.37 g (2.81 ± 0.17 g – mean ± SE), were randomly divided into two experimental groups (triplicated): controls (CT; N = 12 each aquarium), and LAS-exposed at the sublethal concentration of 0.5 mg L^{-1} for 96 h (LAS; N = 12 each) in a static-system. To date, there are no studies of lethal doses of this contaminant in amphibian populations, therefore the concentration used was based on the reference values of LC_{50} of LAS for fish (Coelho and Rocha, 2010). Moreover, the maximum LAS concentration allowed in Brazilian fresh waters is 0.5 mg L^{-1} (CONAMA, 2011). Thus, it is important to verify the possible consequences of such concentrations.

The anionic surfactant sodium dodecylbenzene sulfonate (LAS, linear formula $CH_3\ (CH_2)_{11}\ C_6H_4SO_3Na$; molecular weight: 348.48 g), with 80% purity, was provided by Sigma Aldrich®. According to the Brazilian environmental council (CONAMA, 2011), this concentration is considered suitable for inland waters intended for recreational fishing, supply for humans (after treatment), livestock consumption and watering crops.

All experimental groups were placed in 12 L glass aquaria filled with dechlorinated and aerated water (≥ 6.0 mg O_2. L^{-1}) with controlled temperature (25 ± 1 °C) on a 12:12 h light: dark cycle. The sides of the aquaria were covered with dark plastic to prevent external