



## Phospholipid changes in *Rhinella arenarum* embryos under different acclimation conditions to copper



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### ABSTRACT

We report phospholipid changes in *Rhinella arenarum* embryos after applying three acclimation protocols to copper between 40 and 420 ng L<sup>-1</sup>. The lower and higher acclimation treatments resulted in embryos' enhanced resistance to this metal. Phospholipid remodeling activity, evident through arachidonic acid radioactivity incorporation increase in phosphatidylcholine (PC) and sphingomyelin (SPH) fractions, was registered in embryos acclimated to the intermediate exposure condition. Concomitantly, a decrease in phosphatidic acid fraction (PA) was registered in the higher acclimation condition. PC/PE radioactivity ratio increased both for medium and high acclimation conditions from 0.493 in control embryos to 1.378 and 1.032 respectively. Phospholipid changes could be relevant for changes in membrane features associated with low level exposures to copper, preparing the embryo for a higher resistance to this metal. The increased resistance to copper could also be associated with both an increase in metallothioneins concentration, as registered with HPLC in all the acclimation conditions, and an increase in the copper bound to the third fraction of metallothioneins separated by this method. Our results point out that even very low level exposure to copper results in phospholipid metabolism changes that could be relevant for the acclimation phenomena.

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### 1. Introduction

The environment contains a base level of chemical toxicants. Living organisms have developed a series of protective and repair mechanisms in order to respond to noxious agents. Copper is an essential trace element for all living systems and crucial for many cellular processes and metabolism, as it is a cofactor of a number of metalloenzymes and cupro-proteins, some of which are related to cell growth and proliferation (Leary et al., 2009). However, copper concentrations as low as 1 to 20 µg/L, slightly higher than the pristine concentrations, might produce adverse effects on aquatic organisms, including both invertebrates and vertebrates (Flynn et al., 2015). Copper exposure in fish has been shown to affect reproduction (Cazan and Klerks, 2015), behaviour (Sommers et al., 2016), ionic regulation (Saglam et al., 2013), oxidant enzyme activities and epithelial cells in gills, hepatopancreas and intestines

(Jiang et al., 2011). Studies in our laboratory have shown high copper toxicity in *Rhinella arenarum* embryos and larvae, one of the most sensitive amphibian species, even at normal environmental concentrations (Aronzon et al., 2011). The amount of this metal in water continuously increases as a result of anthropogenic activities, such as mining, domestic and industrial discharges, agricultural applications, animal feed additives and soil erosion. Other sources of contamination include the textile industry, petroleum refinery, the manufacturing of copper compounds, the siding and roofs of buildings, automobile brakes, tires and oil leakage, and road surface materials.

Documenting the biological effect of low-level exposures has been hampered by the difficulty either to conduct statistically robust experiments or to collect sufficient epidemiological data. Environmental conditions involve a large range of low level exposure scenarios, which seems to be a matter of particular importance for restoration purposes (Paustenbach et al., 2006), phenotypic plasticity (Calabrese and Mattson, 2011) and criteria for human health risk assessment (Vandenberg et al., 2012; Lim et al., 2016). In the case of low level exposure, within two orders of magnitude below the no-observed-effect level (NOEL) value, the Arndt-Schulz law predicts the acquirement of a dose-response beta curve with a low dose stimulation-high dose inhibition effect sometimes referred to as hormesis (Calabrese and Mattson, 2011).

**Abbreviations:** PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; SPH, sphingomyelin; CL, cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

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Thus, exposure to low levels of specific harmful physical or chemical agents could exert a beneficial effect, such as an enhanced resistance to subsequent challenges of the same environmental agents at toxic doses/concentrations (van Straalen and Vaal, 1993; Herkovits and Pérez-Coll, 2007; Brinkman and Woodling, 2014).

A decreased uptake of the toxic agent, possibly due to changes in the membrane permeability, could also be associated with this acclimation phenomenon (Pane et al., 2006; Herkovits and Pérez-Coll, 2007). Transportation activities across cell membranes strongly depend on the phospholipid composition and the microenvironment. Lipid rafts, which consist of plasma membrane regions enriched with phospholipids and cholesterol, have been closely related to different membrane functions that sometimes involve structural transport proteins. Different authors have reported alterations in transport mechanisms through cell membranes due to lipid content modifications, sometimes as the result of the phospholipids' fatty acids remodeling (Garg et al., 1990). Zeng et al. (1998) have suggested that fatty acid-mediated ion transport contributes to the leak currently present in many cell types. They also reported that cellular responses during signal transduction are modulated by intracellular content of fatty acids. Alterations in calcium flux have recently been associated with fatty acid interactions with proteins in an evaluation of myocyte apoptosis (Fang et al., 2008), store-dependent and store-independent calcium rise in VSMCs and HEK293 cells (X. Zhang et al., 2014) and permeabilization of liposomes and mitochondria (Belosludtsev et al., 2014).

The balance between the deacylation and reacylation processes on cellular membrane phospholipids plays an important role in multiple physiological and pathological processes (Fonovich de Schroeder and Pechén de D'Angelo, 2000; Seleznev et al., 2006; Gijón et al., 2008; Imae et al., 2010; Bridges et al., 2010; Zachman et al., 2010; Astudillo et al., 2011). Mason and Jenkins (1995) described a decreased influx of metals due to alterations in the lipids content of the membranes from marine invertebrates in the Arctic region. Alterations in membrane fluidity and raft regions order have been reported as the result of cold acclimation in poikilothermic animals (Hayward et al., 2014; Zehmer and Hazel, 2004). Different authors have reported that the exposure of different organisms to copper, as well as cadmium and methyl-mercury, rendered changes in their fatty acid composition through activation of phospholipase A2 (Verity et al., 1994), peroxidation (Bindesbøl et al., 2009) and *de novo* biosynthesis (Maazouzi et al., 2008; Song et al., 2014).

Acclimation processes include the increased synthesis of "stress proteins" such as heat shock proteins (Franzellitti and Fabri, 2005) and metallothioneins, the latter of which are usually associated with metal exposure (Thirumoorthy et al., 2011). Metallothioneins are characterized by a low molecular weight (6000–7000 Da) and one-third of their residues are cysteines, which bind and store metal ions and have no aromatic amino acids or histidines in their composition (Thirumoorthy et al., 2011). Under physiological conditions, metallothioneins bind copper and zinc, but they also link xenobiotic metals like cadmium, mercury and silver and protect against oxidative damage (Thirumoorthy et al., 2011). The induction of these proteins has been proposed as an important adaptive and protective mechanism in response to environmental injury. They were also identified as acute phase proteins in the first phase defense system against environmental stressors (Gabay and Kushner, 1999). Thus, these proteins are considered useful biomarkers which enable the detection of environmental stress primarily produced by metal exposure (Valavanidis et al., 2006). Metallothionein genes are expressed during early development (Vergani, 2009). Different numbers of native and metal-inducible metallothioneins were identified in amphibians, such as one metallothionein form in the liver of copper-treated adult *Xenopus laevis*, two other isoforms in the liver of both larvae and adults of *Rana catesbeiana* (Hidalgo et al., 2009) and three cadmium-binding proteins in the liver of *Rhinella arenarum* (*Bufo arenarum*) adults (Pérez-Coll et al., 1997) and embryos (Pérez-Coll et al., 1999). In the case of the latter, the metallothioneins were induced as a consequence of a cadmium acclimation process.

Amphibian embryos are increasingly employed to evaluate chemical stress in different environmental conditions. A standardized test with amphibian embryos such as AMPHITOX allows a customized toxicity evaluation according to specific purposes (Herkovits et al., 2002; Herkovits and Pérez-Coll, 2003). We have previously employed this test to evaluate probable changes in metal toxicity after exposing acclimated embryos (Herkovits and Pérez-Coll, 2007). The main purposes of this study were 1) to evaluate the possibility that certain acclimation conditions to copper could result in enhanced resistance to a subsequent challenge of a lethal copper concentration in *Rhinella arenarum* embryos and 2) to report the effect of a wide range of low level exposure to copper on membrane phospholipids-arachidonic acid turnover and metallothioneins.

## 2. Materials and methods

### 2.1. Ethical procedures

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

### 2.2. Acquisition of *Rhinella arenarum* embryos

*R. arenarum* adults weighing approximately 200 to 250 g were obtained in Lobos (Buenos Aires Province, Argentina: 35°11'S; 59° 05' W), a presumably pristine region. Ovulation of *R. arenarum* females was induced by i.p. injection of a suspension of one homologous hypophysis in 1 mL of AMPHITOX solution (AS) per female. Oocytes were fertilized *in vitro* with sperm suspensions in AS. The AS contained Na<sup>+</sup> 14.75 mg L<sup>-1</sup>; Cl<sup>-</sup> 22.71 mg L<sup>-1</sup>; K<sup>+</sup> 0.26 mg L<sup>-1</sup>; Ca<sup>2+</sup> 0.3 mg L<sup>-1</sup> and HCO<sub>3</sub><sup>-</sup> 1.45 mg L<sup>-1</sup>. After fertilization, embryos were kept in AS at 20 ± 1 °C until they reached the complete operculum stage, which is the end of embryonic development (Del Conte and Sirlin, 1951).

### 2.3. Acclimation protocol

In order to set-up the acclimation protocol and the appropriate copper (Cu) concentrations for the challenge experiments, Cu 24-h LC/90, 50 and 10 were previously obtained, before acclimation onset and prior to the challenge, by means of PROBIT analysis applied to results provided by an acute toxicity test (AMPHITOX) and previous results (Aronzon et al., 2011). Then, 3 batches containing 300 embryos each, at stage 25, were maintained in 3-L aquaria under different copper exposure protocols during fourteen days as follows: a) the treatment started with 40 (A); 115 (B) and 190 (C) ng L<sup>-1</sup> Cu<sup>2+</sup> (CuCl<sub>2</sub>·2H<sub>2</sub>O) respectively for each batch. b) Concentrations were gradually increased up to final concentrations of 270 (A); 350 (B); and 420 (C) ng L<sup>-1</sup> Cu<sup>2+</sup> respectively (Table 1). This last copper concentration was 240 times lower than the LC100/24-h of copper for these embryos (0.1 mg L<sup>-1</sup> Cu<sup>2+</sup>) and 125 times lower than the NOEC value (0.05 mg L<sup>-1</sup> Cu<sup>2+</sup>) (Aronzon et al., 2011). A fourth batch with 300 embryos was simultaneously maintained in AS without additions as

**Table 1**

The exposure protocol for the acclimation of the amphibian embryos to copper.

Acclimation treatment day	A (ng L <sup>-1</sup> Cu)	B (ng L <sup>-1</sup> Cu)	C (ng L <sup>-1</sup> Cu)
1st	40	115	190
3rd	80	155	230
5th	120	195	270
7th	160	235	310
9th	200	275	350
11th	240	315	390
13th	270	350	420

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