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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^{-1}. 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.

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 Fabbri, 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^+ 14.75 mg L^{-1}; Cl^- 22.71 mg L^{-1}; K^+ 0.26 mg L^{-1}; Ca^{2+} 0.3 mg L^{-1} and HCO_3^- 1.45 mg L^{-1}. 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^{-1} Cu^{2+} ($CuCl_2 \cdot 2H_2O$) respectively for each batch. b) Concentrations were gradually increased up to final concentrations of 270 (A); 350 (B); and 420 (C) ng L^{-1} Cu^{2+} 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^{-1} Cu^{2+}) and 125 times lower than the NOEC value (0.05 mg L^{-1} Cu^{2+}) (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^{-1} Cu)	B (ng L^{-1} Cu)	C (ng L^{-1} 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

control. Embryos were fed with balanced fish food Tetra Color *ad libitum* for 24 h every 48 h. The maintaining media were changed every other day coinciding with the increase of the copper concentration in the solution. Experiments were carried out at 20 ± 1 °C. By the 15th day, batches of the acclimated embryos exposed to the different low level copper concentrations were selected to conduct the following studies:

2.4. Challenge experiments

Batches of 10 embryos at complete operculum stage (by triplicate) from each acclimation condition were challenged with the following copper LC: 0.075 (A1, B1 and C1), 0.115 (A2, B2 and C2) and 0.155 mg Cu^{2+} L^{-1} (A3, B3 and C3) in Petri dishes with 40 mL of solution at 20 ± 1 °C. Control embryos were maintained in AS and exposed to the different Cu^{2+} LCs employed: 0.075 (E); 0.115 (F) and 0.155 mg Cu^{2+} L^{-1} (G) and (D) absolute control (no copper exposure). Embryo survival was evaluated each hour up to 8 h and then at 24 h of exposure.

2.5. Membrane phospholipids-arachidonic acid turnover studies

The purpose of [3H]-arachidonic acid labelling experiments was to assess this fatty acid turnover in membrane phospholipids of *Rhinella arenarum* embryos, without interference of *de novo* synthesis of these compounds. Batches of 25 embryos per acclimation experiment (by duplicate) A, B and C were processed. They were washed and incubated in the presence of 1 μCi of [3H] arachidonic acid during 2 h. Then, they were homogenized in 7.5% trichloroacetic acid containing bovine seroalbumin as a carrier. After centrifugation, major phospholipids were extracted from the pellets using chloroform:methanol solution (2:1). The extracts were washed and evaporated under an N_2 stream and phospholipidic phosphorus was measured in each extract. Individual phospholipids were separated by thin layer chromatography according to the method of Rouser et al. (1970). Spots were scrapped and 3H radioactivity of the samples was counted by Liquid Scintillation.

2.6. Copper bound to metallothioneins

a) Embryo extracts for metallothioneins detection by reverse phase-High Pressure Liquid Chromatography (HPLC).

Three hundred embryos provided from each acclimation treatment and from control pools were processed for metallothionein isolation as follows. The embryos were homogenized in 1 vol of ice-cold 20 mM Tris-HCl buffer solution (pH 8.6, 0.25 M glucose) using a glass-teflon homogenizer under an atmosphere of nitrogen gas. Ethanol and chloroform (at −30 °C) were added to the homogenate in a 1.05:0.08:1.00 (Ethanol:Chloroform:Homogenate) ratio.

The supernatants were recovered by centrifugation at 27,000 g for 15 min at −20 °C. The soluble proteins were precipitated during 10 h at −30 °C adding 3 vol of ethanol. The pellets were collected by centrifugation described above and dissolved in water, freeze-dried and redissolved in the buffer A for HPLC (see below).

b) Reverse phase-HPLC metallothionein separation and copper content

Embryo extracts (200 μL) were chromatographed during 60 min at 1 mL/min, using a mobile phase composed by two buffers. Buffer A: 50 mM Tris-HCl, pH: 7.5 and buffer B: 50 mM Tris-HCl pH: 7.5 40%, acetonitrile 60% v/v, in a gradient system from 100% buffer A at 0 min to 100% buffer B at 50 min. The UV absorbances at 220, 254 and 280 nm were registered. The results were analyzed comparing UV chromatograms from control and acclimated embryos at three different wavelengths. Standard metallothioneins I and II from rabbit liver were also chromatographed using the same gradient, filtered and degassed before the chromatography. The chromatography techniques were performed with an HPLC equipment with a UV Vis detector on an UltropacLichrosorb RP-18 column (250 × 4.6 mm I.D., 18.5 μm particle size) obtained from Pharmacia (Uppsala, Sweden). Copper concentrations from the HPLC eluted fractions were quantified by atomic emission spectroscopy with inductively coupled argon plasma excitation (ICP-AES, Baird ICP 2070). Detection limit: 1 μg/L. Metal concentrations were expressed as μg/mL extract. Before analysis, acetonitrile was evaporated to dryness and the residue digested in 5 M HNO_3.

2.7. Solutions and reagents

A Cu^{2+} stock solution of 1.5 g L^{-1} was prepared by directly weighing and dissolving the corresponding mass of $CuCl_2 \cdot 2H_2O$ (purity 99%, Riedel-de Haen) in distilled water. Hydrochloric acid was added until reaching pH 1.9 for conservation purposes. Test solutions were prepared by diluting secondary stock solutions of Cu^{2+} in AS. Experimental Cu^{2+} solutions were measured four times with a Perkin Elmer atomic absorption spectrophotometer. The error between nominal and measured concentrations did not exceed 5%. Trifluoroacetic acid and TrisHCl and HPLCgrade acetonitrile were obtained from Sigma Chem Co, USA. Mobile phase solutions were filtered. All other chemicals used were of analytical purity.

3. Results

3.1. Survival

The 24-h LC10, 50 and 90 estimated by probit analysis were 0.035, 0.050 and 0.075 mg Cu^{2+} L^{-1} respectively while 0.03 mg Cu^{2+} L^{-1} did not exert lethal effect (NOEC). From LC90 value, three higher concentrations were selected for challenge, 0.075, 0115 and 0.155 mg Cu^{2+} L^{-1}.

Table 2 shows, as a general pattern, that the copper acclimation protocol exerted a transient beneficial effect on survival of *Rhinella arenarum* embryos exposed to different high copper concentrations. For certain cases a significant ($p < 0.05$) protective effect in embryo for up to 8 h of exposure was registered. However, results at 24 h of exposure revealed no differences in survival among acclimated and control embryos (data not presented).

3.2. Phospholipid arachidonic acid turnover

Fig. 1 shows that while phosphatidylethanolamine radioactivity was not modified as a consequence of acclimation treatments, phosphatidylcholine radioactivity increased from 130 in control embryos to 343.33 cpm/μg of phospholipidic Pi in embryos acclimated according to the low level Cu concentrations (A) and decreased to 170 cpm/μg of phospholipidic Pi in embryos acclimated according to the high level Cu concentrations (C). (ANOVA *post hoc t*-test: $p < 0.0315$ between Control and B and $p < 0.0254$ between A and B. Sphingomyelin radioactivity tended to increase (from 1.87 in control embryos to 7.50 cpm/μg phospholipidic Pi in B ones and to decrease in C embryos) and other phospholipids as PI, PS, LPC and LPE also showed similar patterns (Fig. 2a). Cardiolipin and phosphatidic acid labelling did not increase as a consequence of embryo acclimation. On the other hand, phosphatidic acid radioactivity decreased in treated embryos (ANOVA *post hoc t*-test: $p < 0.0467$ between Control and B, $p < 0.0278$ between Control and C) (Fig. 2B).

PC/PE ratio increased from 0.493 in control embryos to 1.378 in B and 1.032 in C acclimated ones (see Table 3). Table 3 also shows that elevated PC/PE ratio was a consequence of the increased turnover of arachidonic acid in PC, which rendered in B embryos similar PC and PE radioactivities and increased PC + PE. Although PC labelling was not elevated in C acclimated embryos, PC/PE ratio was indeed higher in those embryos than in control ones because PC and PE were also labelled at a similar extent under these treatment conditions, in contrast with the elevated PE labelling observed in Control and group A acclimated embryos.

Table 2
Survival percentages of *Rhinella arenarum* embryos acclimated to low copper concentrations when challenged with different copper concentrations for 8 h. (LC: lethal concentration).

Challenge Cu^{2+} ($mg\ L^{-1}$)	Acclimation condition												
	A1	B1	C1	E (not acclimated exposed to low Cu LC)	A2	B2	C2	F (not acclimated exposed to medium Cu LC)	A3	B3	C3	G (not acclimated exposed to high Cu LC)	D (AMPHITOX Solution)
0.075	53.3	53.3	70.0[a]	33.3									
0.115					70.0	50.0	50.0	43.3					
0.155									50.0[a]	46.7	43.3	20.0	
AS													100.0
SD	6.7	12.0	15.3	6.7	17.3	0.0	15.3	3.3	5.8	3.3	8.8	10.0	

[a] mg per liter of solution.

3.3. Copper bound to metallothioneins

The absorbance profiles of the extracts prepared from control embryos and embryos acclimated to different levels of copper were obtained at 254 nm. At 280 nm there was no absorbance peak. The UV absorbance peaks obtained at 254 nm could be related to the prosthetic group of metallothioneins, (thiolates) which selectively absorbs at 254 nm. The absence of absorbance peaks at 280 nm wavelength, which is selectively absorbed by proteins containing tyrosine and tryptophane, is in agreement with the essential feature of metallothioneins, which is the absence of aromatic amino acids in their composition. The chromatograms from different acclimated embryos (low, medium and high copper acclimation protocols), showed similar profiles but with areas proportionally increasing along with metal concentration in the acclimation protocol. By comparing the UV absorbance profiles at the 3 λ, 3 metallothionein-like protein fractions were detected at the following retention times: metallothionein 1 (MT1) between 20 and 40 min, metallothionein 2 (MT2) between 41 and 45 min and metallothionein 3 (MT3) between 46 and 50 min. Total metallothionein induction took place in embryos acclimated to all conditions. Fig. 3 shows the percentages of total metallothionein induction for each acclimation treatment, calculated through comparison of each chromatogram area with the one from control embryos. Fig. 4 shows copper contents in chromatographed extracts obtained from control and acclimated embryos (A, B and C). MT1, MT2 and MT3 fractions from control and acclimated embryos corresponded to fractions from 10 to 20, 21 and 22, and 23 to 25 respectively, which represent retention times from 20 to 40, 41 to 45 and 46 to 50 because this analysis was done in pools of two samples each. A trend can be observed in copper binding to MT3 at a greater extent in C acclimated embryos than in control ones.

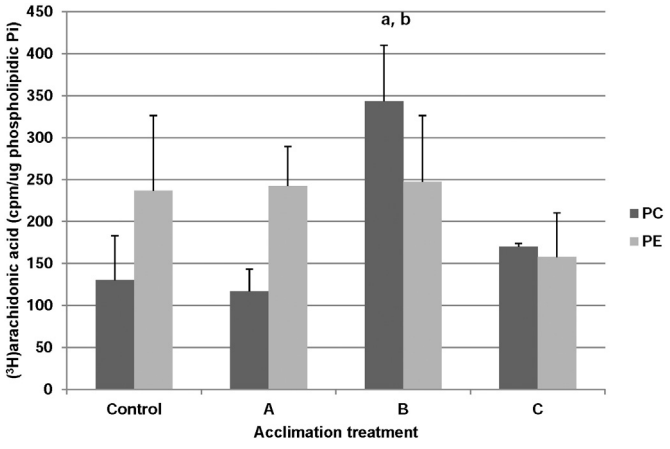

Fig. 1. Major phospholipids radioactivity incorporation in control and copper acclimated *Rhinella arenarum* embryos exposed to a solution containing [3H] arachidonic acid for 2 h. Acclimation treatments were described in the Materials and Methods section. PC: phosphatidylcholine, PE: phosphatidylethanolamine. Results are expressed in cpm/μg of phospholipidic phosphorus content (Pi). ANOVA: a) $p < 0.0315$ between Control and A, b) $p < 0.0254$ between A and B.

4 Discussion

Present results confirm those obtained from our previous work showing that the protocol used for copper acclimation exerts a transient beneficial effect on the survival of *Rhinella arenarum* embryos upon exposure to different lethal copper concentrations. That significant protective phenomenon for certain acclimation/challenge conditions was evident for up to 8 h of exposure (Herkovits and Pérez-Coll, 2007). Among other protective mechanisms, effects exerted on transport mechanisms through cell membranes can be responsible by decreasing the influx or by increasing the efflux of copper. The results presented here show significant increased turnover of arachidonic acid specific for choline derived phospholipids (mainly PC) as well as decreased incorporation of the label in phosphatidic acid in acclimated embryos from groups B and C. Thus, a deacylation/reacylation process was evident as a consequence of

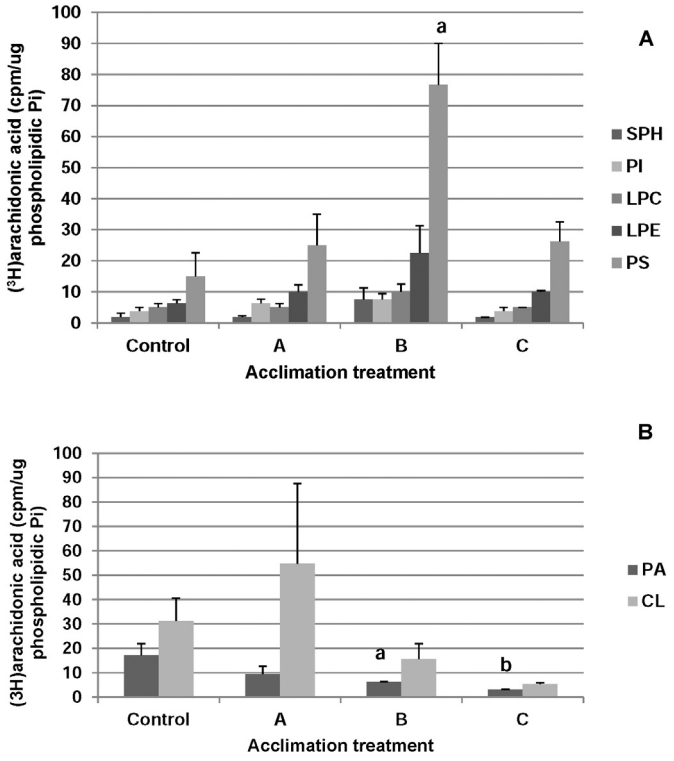

Fig. 2. a - Minor phospholipids radioactivity incorporation in control and copper acclimated *Rhinella arenarum* embryos exposed to a solution containing [3H] arachidonic acid for 2 h. Acclimation treatments were described in the Materials and Methods section. SPH: sphingomyelin, PI: phosphatidylinositol, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, PS: phosphatidylserine. Results are expressed in cpm/μg of phospholipidic phosphorus content (Pi). ANOVA: a) $p < 0.05$ between Control and B. b - Minor acid phospholipids radioactivity incorporation in control and copper acclimated *Rhinella arenarum* embryos exposed to a solution containing [3H] arachidonic acid for 2 h. Acclimation treatments were described in the Materials and Methods section. PA: phosphatidic acid, PICL: cardiolipin. Results are expressed in cpm/μg of phospholipidic phosphorus content (Pi). ANOVA: a) $p < 0.0467$ between control and B and b) $p < 0.0278$ between Control and C.

Table 3
PC/PE and PC + PE radioactivity in control embryos and embryos acclimated to copper. Embryos acclimated to copper were groups A, B and C. ANOVA: $P < 0.0106$ between treatments. PC: phosphatidylcholine, PE: phosphatidylethanolamine. Posthoc *t*-test: a) $p < 0.005$ between control and B b) $p < 0.0272$ between control and C, c) $p < 0.0041$ between A and B, d) $p < 0.0204$ between A and C.

	Control	A	B	C
PC/PE	0.49 ± 0.05[a,b]	0.44 ± 0.03 c,d	1.38 ± 0.24	1.03 ± 0.20
PC + PE	363.9 ± 198.8	349.2 ± 100.7	581.6 ± 206.7	315.2 ± 85.3

treating embryos with low level concentrations of copper. Arachidonic acid is a highly unsaturated fatty acid containing 20 carbon atoms which confers fluidity to biological membranes. According to the literature, the deacylation/reacylation process that we report here is in line with the increase in the unsaturation index reported by Słaba et al. (2013) and may affect copper transport (Wang et al., 2014). This suggestion is in agreement with the results we found in a previous study, where acclimated embryos accumulated lower copper concentration than control ones (Herkovits and Pérez-Coll, 2007).

Results from studies on phospholipid mixtures provided strong evidence for the existence of phase transitions in biological membranes, from the bilayer lamellar arrangement to micelles, inverted micelles or hexagonal arrangements. Phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA) are phospholipids that favor the formation of non-bilayer arrangements of the plasmatic membrane, (hexagonal arrangements and inverted micelles) which are necessary for pinocytosis, exocytosis and flip-flop processes. On the contrary, choline derived lipids (phosphatidylcholine (PC) and sphingomyelin (SPH)) are responsible for the stabilisation of the bilayer structure of the membrane (Cullis and Kruijff, 1979; Hidalgo et al., 1996; Tessier et al. (2004). The abundance of different phospholipids in certain regions of the membrane provides different arrangement possibilities; the abundance of the unsaturated fatty acids in membrane phospholipids, including arachidonic acid, contributes to the increase in the fluidity of the bilayer structure, thus allowing enhanced rotational and translational movements of the phospholipids (Hidalgo et al., 1996). Alexandre et al. (1994) described an increment in plasma membrane fluidity of *Saccharomyces cerevisiae* and *Kloeckeraapiculata* as the consequence of an increase in insaturation index. Gasser et al. (1990) reported that an increment in the fluidity of secretory granules from several unstimulated glands (rat pancreas and parotid, rabbit gastric glands) was associated with higher Cl^- transport rates. Gasser and Holda (1994) demonstrated that exogenously added phospholipase A2 (PLA2) caused a huge increase in the Cl^- specific

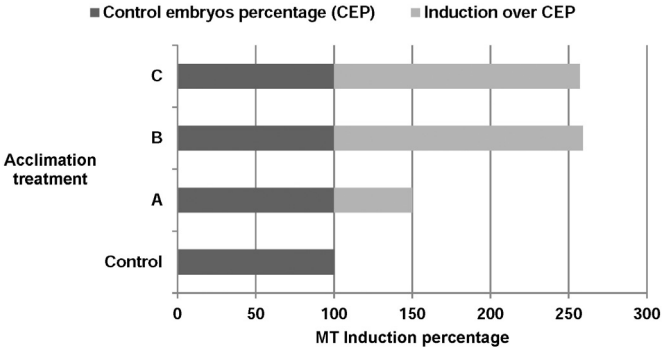

Fig. 3. Metallothionein induction in *Rhinella arenarum* embryos following the acclimation protocol. Acclimation treatments were A, B and C, as described in the Materials and Methods section. Chromatogram area measurements were used to estimate metallothionein concentrations and calculate induction percentages. Results were expressed as percentages corresponding to the metallothionein concentrations in control embryos.

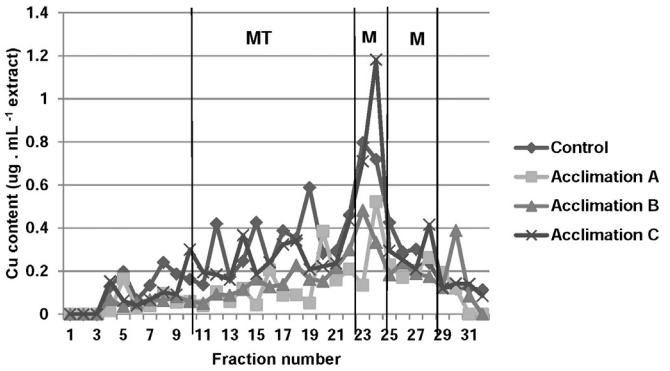

Fig. 4. Copper content in HPLC metallothionein eluted fractions quantified by atomic emission spectroscopy with inductively coupled argon plasma excitation. Results are expressed as μg mL^{-1} elution fraction pools (2 retention time fractions per copper concentration determination fraction number).

transport by the isolated pancreatic granules. They also reported that PLA2 hydrolysis products, lysophospholipids and unesterified fatty acids, directly increased the rate of Cl^- transport when they were incubated with granules *in vitro*.

Our results also show that PC/PE radioactivities ratio increased in acclimated group B of embryos and did not decrease in acclimated group C, regardless of the decreased sum of PC + PE radioactivity. These conditions provide major phospholipid acyl group remodeling activities different from those found in control and acclimated group A embryos. Therefore, PC and PE arachidonic acid incorporation seems to be related, as reported in different adult mouse tissues and heart development for the sn1 18:0, sn2 20:4 (arachidonic acid), and sn2 22:6 marginals (Zarringhalam et al., 2012). All 3 of these marginals tracked closely between PC and PE, suggesting that they are substrates of the two phospholipids' shared regulatory processes. It is noteworthy that sometimes acclimation could result in less tolerance to certain challenges. Bindesbøl et al. (2009) reported that exposing worms to sublethal copper concentrations produced changes in their fatty acid composition associated with a decreased tolerance to low temperature.

On the other hand, metallothioneins were usually associated with metal exposure (Thirumoorthy et al., 2011). Different native and metal-inducible metallothioneins were reported in amphibians (Hidalgo et al., 2009), including the ones identified in cadmium-treated *Rhinella arenarum* (*Bufo arenarum*) adults (Pérez-Coll et al., 1997) and embryos (Pérez-Coll et al., 1999). Under the current copper acclimation protocol, metallothioneins induction was near 160% in embryos acclimated to medium (B) and high (C) copper concentrations. Thus, the increase in metallothioneins content obtained in these acclimated embryos could be related to the transient protection observed against some lethal copper concentrations (Herkovits and Pérez-Coll, 2007). In line with the protective role of MTs against metal toxicity, copper binding to MT3 fraction was evident in extracts from all the studied embryos and tended to increase in C acclimated ones.

Embryo survival results presented here are in accordance with previous findings from our group and others who found different levels of protection resulting from acclimation treatments. Both phospholipids remodeling activity, through phospholipase A changes in membrane fluidity and metallothionein induction, showed responses to copper exposure but seem to have different metal concentration thresholds, acclimation condition 2 for phospholipids and 3 for metallothioneins. These results were associated with different cell responses to low level contaminant exposure as reported also by Q. Zhang et al. (2014). While regulation of phospholipase A activity may take place through serine phosphorylation (Buschbeck et al., 1999; Casas Requena, 2008) and this mechanism fits well with an ultrasensitive motif (Q. Zhang et al., 2014), metallothionein induction maintains cellular homeostasis and may probably comply with a negative feedback motif (Q. Zhang et al.,

2014). Nevertheless, it can be seen that our results from acclimation condition C rendered a deacylation/reacylation response together with a metallothionein induction and copper content increase tendency, as well as increased survival percentage at 0.075 mg L^{-1} copper challenge concentration. Taken together, these findings may suggest a possible relationship between the biochemical parameters evaluated and acclimated embryo survival after challenged with higher doses of the same xenobiotic. Our results emphasize the high sensitivity of amphibian embryos to a wide range of low exposure conditions to copper resulting in different phospholipid and metallothionein responses, in some conditions associated with an increased resistance to this essential and toxic metal.

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