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Arsenic absorption and excretion in chronically exposed developing toad *Rhinella arenarum*

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ABSTRACT

We assessed the toxicodynamics of As in developing *Rhinella arenarum* toad embryos and larvae exposed from fertilization to 0.01–10 mg As L^{-1}. We determined As content in toad embryos and larvae by X-ray fluorescence spectrometry. Toad embryos and larvae actively bioaccumulated As, reaching tissue concentrations more than one-thousand higher than control levels after 23d-exposure to 10 mg As L^{-1}. The bioconcentration factors also increased up to fifty times higher levels in toad larvae respect to media levels. Once recovered in As-free media, the larvae rapidly excreted the bioaccumulated As with a half-life of 1.6 d. By calcein transport competition assays, we infer that As is excreted through ABCC-like transporters, probably conjugated with GSH. These results are relevant for comprehending the risks posed by As exposure in this autochthonous aquatic species that develops in water courses from Argentina, that may contain As levels ranging between 10–15,000 μg L^{-1}.

1. Introduction

Arsenic contamination of the environment from both natural and anthropogenic sources is nowadays a major environmental concern in various parts of the world, due to its persistence and toxic effects on living organisms at certain concentrations (Arslan et al., 2017). It can be found in water, soil or air, existing both as inorganic as well as organic species with different oxidation states and differential toxicity. Inorganic As is the predominant form in surface and underground water reservoirs (Lu et al., 2014). Whereas humans acquire arsenic *via* food and water, aquatic animals are particularly vulnerable to As in the environment because they can also take up this toxicant through the gills or skin (Miller et al., 2007). Arsenic, like many metals and metalloids, can trigger reactive oxygen species (ROS) production, disrupt signal transduction, alter gene expression and induce lipid and DNA damage (Mardirosian et al., 2017; Sarkar et al., 2014), and many vertebrates try to counteract oxidative stress using the first-line defense system including reduced glutathione (GSH), vitamin C or E, carotenoids (Martínez-Álvarez et al., 2005) or radical-scavenging enzymes like superoxide dismutase, catalase, and glutathione peroxidase (Feidantsis et al., 2013; Valavanidis et al., 2006).

Inorganic As in water is mostly in the form of arsenate. In a pH range of 4–10, trivalent arsenic compounds have neutral charge, while pentavalent forms are negatively charged. Therefore, at physiological pH, trivalent arsenic species can more easily cross the cell membrane through their transporters than the pentavalent species (Cohen et al., 2006; Tseng, 2009). Arsenate is rapidly reduced *in vivo* to arsenite, mainly in the blood and liver (Aposhian, 1997; Gregus and Németi, 2005; Herbel et al., 2002; Vahter and Marafante, 1985). Because arsenite is taken up by cells much more rapidly than arsenate, it is preferred in many studies to evaluate the toxicity of inorganic As *in vivo* and *in vitro* (Cohen et al., 2006).

The uptake and efflux of arsenicals occur mainly through transport proteins. Transporters are critical for regulating the body burden of

Abbreviations: ABC, ATP-binding cassette; Abcb1, p-glycoprotein; Abcc, multidrug resistance-associated proteins; As, arsenic; BCF, bioconcentration factor; Bcrp, breast cancer resistance protein; BSA, bovine seroalbumine; calcein-AM, calcein acetoxymethyl ester; DW, dry weight; FW, fresh weight; GSH, reduced glutathione; GST, glutathione-S transferase; ROS, reactive oxygen species

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arsenic and potentially play both protective (through preventing cellular and tissue accumulation as well as facilitating elimination) and harmful roles (allowing the entry of arsenic across epithelial layers) (Roggenbeck et al., 2016). Different species of aquatic organisms are capable of taking up and accumulating As (Chen et al., 2009; Erickson et al., 2011; Farombi et al., 2007; Guidi et al., 2010; Heyes et al., 2014; Liu et al., 2006; Miller et al., 2007; Rahman et al., 2012; Roe et al., 2005; Suhendrayatna et al., 2002) and different members of the aquaglyceroporin family have been identified as responsible for As uptake through gills and intestine in fish (Hamdi et al., 2009).

Arsenic is excreted mainly in the form of conjugates. The enzyme glutathione S-transferase (GST) catalyzes the conjugation of As with GSH, giving rise to glutathionyl derivatives that are eliminated from the cell through ATP-binding cassette (ABC) proteins (Miller et al., 2007). However, there are still very few studies about the way in which As or its metabolites are excreted from the cells. The ABC superfamily contains membrane proteins that hydrolyze ATP to actively translocate a wide variety of substrates across plasmatic and intracellular membranes (Chan et al., 2004; Ishikawa, 1992; Klaassen and Aleksunes, 2010). The proteins ABCB1 (P-glycoprotein, Pgp), ABCC2 (multidrug resistance protein type 2, MRP2) and ABCG2 (breast cancer resistance protein, BCRP) are located in the apical membrane of polarized cells and participate in the efflux of toxic compounds. Other ABCC transporters are located in basolateral membranes, and thus are involved in absorption of toxic compounds by the organism (Chan et al., 2004). Several authors have reported the involvement of ABCC1, ABCC2 and ABCB1 in As detoxification in mammals (Gregus et al., 2000; Kala et al., 2000; Liu et al., 2002, 2001). Arsenic enhances ABCC2 expression and xenobiotic transport activity in killifish renal proximal tubules (Miller et al., 2007), leading to the acquisition of tolerance to acute toxicity. Arsenic upregulation of ABCC2 expression was also described in the liver of the same species (Shaw et al., 2007).

Amphibians are considered good bioindicators of aquatic pollution due to their susceptibility to chemicals during their freshwater cycles as embryos and larvae. Toxicants accumulated during the aquatic period and not depurated before or during metamorphosis to terrestrial juveniles may be transferred to organisms that feed on juvenile and adult amphibians (Heyes et al., 2014). *Rhinella arenarum*, the common South American toad, is widely distributed in Argentina and may be threatened by exposure to agrochemicals and other toxicants such as arsenic, which is present in concentrations between 0.01 and 15 mg L^{-1} in natural water sources throughout Argentina (Bundschuh et al., 2012). We have previously described the biochemical effects and the molecular mechanisms of action of As during the embryonic and larval development of *R. arenarum*, and reported that acute and chronic exposure to As can trigger oxidative stress in *R. arenarum* embryos and larvae (Mardirosian et al., 2016, 2015). In the present study, we focused on the uptake, accumulation and excretion of As along the embryonic and first larval stage of development of the common toad, *R. arenarum*.

2. Materials and methods

2.1. Chemicals

Sodium metaarsenite ($NaAsO_2$) was purchased from Anedra Argentina (purity > 99.95%). Calcein acetoxymethyl ester (calcein-AM, Calbiochem, San Diego, CA) was a generous gift from Dr. Amro M. Hamdoun (Scripps Institution of Oceanography, UCSD). All used chemicals and reagents were commercial products of analytical grade purity and were used as supplied. Calcein-AM was previously dissolved in DMSO. Final concentration of these solvents was always below 0.3%.

2.2. Toad embryo and larval growth

Adult *R. arenarum* males and females were collected during the breeding season (Spring) at Los Barreales Lake (S38.45344 W68.72918), a pristine environment. Animals used in this study were maintained and treated with regard for the alleviation of suffering according to recommended guidelines (Committee on Pain and Distress in Laboratory Animals et al., 2009; National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011; Schad et al., 2007). Upon collection, adults were transported to an outdoor terrarium. Ovulation was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotrophin (ELEA Laboratory, Buenos Aires, Argentina). Freshly extruded eggs were fertilized *in vitro* with a testicular homogenate and depending on the assay, they were immediately exposed to As or developed in glass dishes with amphibian Ringer's solution (0.65 g L^{-1} NaCl; 0.01 g L^{-1} KCl; 0.02 g L^{-1} $CaCl_2$) and maintained at 20 ± 2 °C in a 12 h light- 12 h dark photoperiod until the exposure.

2.3. Contents of As and other elements in embryos and larvae exposed to As

The bioaccumulation of As and the effects of As exposure on Fe, Zn and Cu content in embryo and larval tissues were analyzed. For this assay, thirty minutes after fertilization, groups of 200 embryos were transferred to glass receptacles containing 200 mL of amphibian Ringer's solution either alone (control group) or with different concentrations of As solution (0.01, 0.1, 1 and 10 mg L^{-1}) (Lascano et al., 2011). The treatments were carried out in duplicate. The exposures were semi-static with solution renewals every 48 h, spanning the complete embryonic development period (9 d) and 14 d of the first larval stage, until d 23 of development, at 18–20 °C and a 12:12 h light: dark photoperiod. Embryos were fed with boiled lettuce from open mouth stage on until the end of the assay. Three independent experiments were performed. Measurements were performed by X-ray fluorescence spectrometry under total reflection conditions using synchrotron radiation (SR-TXRF) on the DO09B line at the Laboratorio Nacional de Luz Sincrotron (LNLS) of Campinas, Brazil. Samples consisting of 15 embryos from each treatment batch were collected at 5, 9, 16 and 23 d of development, washed with ultrapure water and lyophilized for transport to the LNLS. Subsequently, samples were resuspended in Milli-Q water with 0.1% Triton X100 and incubated 15 min in an ultrasonic bath for reconstitution. An aliquot of 5 µL of each sample was dispensed onto the center of a Si carrier with its mirror polished surface to achieve reflection of the incident beam. The excitation geometry of the collimated white beam (3 mm × 300 µm) was 0.1–0.2° respect to samples and the measurements were performed under conditions of total reflection of the beam. X-ray fluorescence spectra were recorded with a Si (Li) detector placed at 90° of the incident beam. The X-ray fluorescence intensities of the k-α peak of each element were recorded for quantification ($16 < Z < 42$), and the resulting spectra were analyzed with the AXIL program. The fluorescence intensity was normalized by using 10 mg L^{-1} of Ga as an internal standard and converted to element mass content using calibration parameter algorithms.

2.4. Bioconcentration of arsenic

Bioconcentration Factors (BCF) were estimated considering that larval dry weight (DW) was about 10% of the fresh weight (FW) and the exposure ratio of one larva in one mL of media. Considering an approximate fresh weight of 10 mg per larva (Lascano et al., 2011), then one g of larval dry weight would have been exposed to one L of media. Then, BCF may be expressed in L kg^{-1} from As accumulation values in mg As kg^{-1} DW divided by the corresponding media concentration in mg As L^{-1}.

2.5. Arsenic excretion assay

In order to determine the ability of *R. arenarum* larvae to excrete As,

embryos were separated in three different groups (A, B and C) and developed in Ringer's solution without As (group A: control), with 1 mg L^{-1} As immediately after fertilization (group B), or with 1 mg L^{-1} As at 5 d of embryonic development at the heart beat (HB) stage (group C). After 14 d of development in these conditions, the three groups of larvae were incubated in Arsenic-free Ringeŕs solution for recovery and As net excretion, and samples were collected at 0, 1 d and 2 d. Arsenic content in larvae was measured in the three groups by X-ray fluorescence spectrometry as described above (Section 2.3). Simple exponential model equations were fitted to the experimental data to estimate the initial As content (accumulation level at d 14 of development) and the half-life of the excretion process, using a non linear regression procedure (Venturino et al., 2007).

2.6. Arsenic transport assays

In order to determine if As efflux transport involves ABCC proteins in *R. arenarum* larvae, we performed two experiments using calcein-AM, a cell permeating and non-fluorescent lipophilic compound. The non-fluorescent calcein-AM is removed by ABCB1 transporter. Once inside the cell, esterases hydrolyze the ester bond producing calcein, which is a fluorescent substrate of ABCC transporters (Evers et al., 2000; Luckenbach et al., 2014). For both experiments, larvae at d 28 of development in amphibian Ringer's solution were used, once they were large enough to allow the removal of their skin and intestine. Three independent experiments were performed for each design.

2.6.1. Efflux competition assays in control larvae

Control *R. arenarum* larvae were sacrificed to remove intestine and skin. Three portions of approximately 5 mm in length were cut from sections of both tissues to prepare strips. Each strip was weighed and randomly placed in microcentrifuge tubes with 300 µL of Cortland's solution (159.9 mM NaCl, 2.55 mM KCl, 1.56 mM $CaCl_2$, 0.93 mM $MgSO_4$, 5 mM $NaHCO_3$, 2.97 mM NaH_2PO_4, 5.55 mM glucose, pH 7.4), containing 0.25 µM calcein-AM alone, calcein-AM with 1 mg L^{-1} As, or calcein-AM plus 10 mg L^{-1} As. The samples were placed in a bath at 20 °C, and 200 µL-aliquots were taken every 10 min along 90 min to read calcein fluorescence in a Qubit fluorometer (Invitrogen-Molecular Probes, Oregon; excitation/emission wavelengths 485/530 nm). Immediately after each reading, the aliquots were returned to the tube to maintain constant volumes. Calcein efflux rate was calculated in fluorescence arbitrary units (AU) min^{-1} and referred to tissue mass. Calcein-AM solution without tissue was used as baseline solution control, and readings of intestinal and skin strips incubated in Cortland's solution were used as tissue controls (Bieczynski et al., 2014).

2.6.2. Effects of arsenic pre-exposure in calcein efflux and its competition with Arsenic

R. arenarum larvae were pre-exposed to 0, 1 and 10 mg L^{-1} As during 3.5 d in the conditions indicated above (Section 2.2). After the exposure, three larvae from each group were sacrificed and sections of the intestine and skin were removed. Two portions of approximately 5 mm in length were cut from these sections for strip preparation. Each strip was weighed and randomly placed in microcentrifuge tubes with 300 µL of Cortland's solution with 0.25 µM calcein-AM alone or with 10 mg L^{-1} As. Calcein efflux was measured as described above (2.6.1).

2.7. Data analysis

Statistical differences between treatments were assessed by ANOVA and Fisher-LSD *post hoc* test. For the statistical analysis of the excretion assay, data from the three experiments were used (n = 3 for each treatment) and a Student's *T*-test was applied.

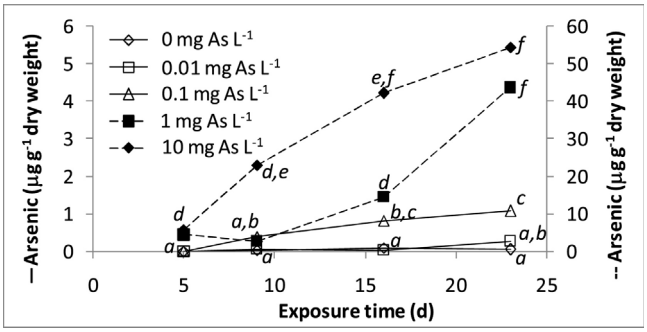


Fig. 1. Arsenic bioaccumulation in developing toad *R. arenarum*.Toad embryos and larvae were continuously exposed to As from fertilization (d0). Data represent means from duplicate exposure treatments and 3 independent experiments. Different lowercase letters indicate significant differences ($p < 0.05$, ANOVA and Fisher's LSD).

3. Results

3.1. Arsenic bioconcentration in toad embryo and larvae

Arsenic concentration in developing toad embryo and larvae was dependent on the concentration in the media and the time of exposure (Fig. 1). Control values of As in embryos and larvae remained constant along the experiment, with a mean value of 0.0415 µg As g DW^{-1}, and a standard deviation of 0.0371 µg As g DW^{-1}. The lowest concentration of As in water (0.01 mg L^{-1}) caused an accumulation of As that was observable in larvae at 23 d, of 6.6X respect to control values. In turn, 0.1 mg L^{-1} As in water caused As bioconcentration from 9 d of exposure, once reached larval stage, that gradually increased at 16 d and 23 d, reaching values of 9.5X, 20X and 26X respect to controls, respectively. At the end of the exposure to 0.1 mg L^{-1}, As levels in larvae were about 1 µg As g DW^{-1}. The higher As concentrations in the media caused a significant accumulation from the earliest embryonic stage evaluated, with values bordering 4.5–5.8 µg As g DW^{-1}. These values represented an increment of 107X and 140X of the control As values for 1 mg L^{-1} and 10 mg L^{-1}, respectively. Arsenic bioconcentration remained stable in larvae exposed to 1 mg L^{-1} As in water at 9 d of exposure, and then rapidly increasing to reach 1052X accumulation at 23 d of exposure. For 10 mg L^{-1} As, the accumulation was continuous along larval development, tending to stabilize after 23 d of exposure, with As accumulation values 553X, 1017X and 1310X of controls at 9, 16 and 23 d, respectively. At the end of the exposure to the highest As concentration in water, As in larvae accumulated to 54.4 µg g DW^{-1}.

Bioconcentration Factors (BCF) were estimated from the accumulation values of As in embryo and larvae and the corresponding As concentration values of exposure media. In general, BCF increased with the time of exposure (Fig. 2). Bioconcentration indicated that there was

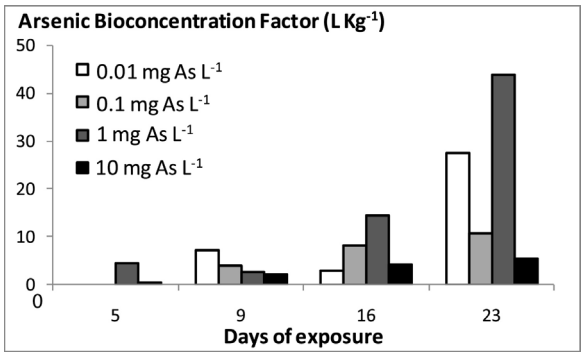


Fig. 2. Bioconcentration Factors (BCF) in developing toad *R. arenarum* exposed to As.BCF were calculated from bioaccumulation data in Fig. 1 and the exposure concentrations, considering 1 g of dry weight larvae exposed in 1 L of media.

Table 1
Estimation of As mass removal from the media.

[As] in water mg/L	Daily removal µg As/g DW/d	R^2	2d-As removal µg	As mass remaining in water µg	As mass remaining in water %
0.01	0.013	0.694	0.026	9.974	99.74
0.1	0.058	0.960	0.116	99.884	99.88
1	2.213	0.857	4.426	995.574	99.56
10	2.653	0.969	5.306	9,994.694	99.95

Data from As accumulation was used to estimate daily As mass removed by developing toads by linear regression. From the estimated slope, the remaining As in water could be in turn calculated.

an active uptake of As by the embryos at 5 d of exposure from the higher concentrations in the media up to a value of 4.5 for 1 mg L^{-1} As. Larvae showed the active uptake at all the concentrations; at 9 d of exposure there was an inverse relationship of BCF with As concentration in the media, with a maximum value of 7.2 for 0.01 mg L^{-1} As. This relationship was lost at longer times of exposure, and the maximum BCF was of 44 L kg^{-1} at 23 d in larvae exposed to 1 mg L^{-1} As. In turn, the highest exposure concentration of 10 mg L^{-1} As showed in general the lowest BCF values, tending to a maximum of 5.5 L kg^{-1}.

The remaining As in the different exposure media could be also estimated from the concentration data in developing toads (Table 1). All the treatments lead to As removal below 1% of the concentration media in 48 h, that was the renewal period used for the semistatic exposure experiments. In particular, in 0.01–1 mg L^{-1} As, the developing toad embryos and larvae only removed 0.12%–0.44% of the media As concentration. For the highest concentration of 10 mg L^{-1} As, the estimated removal was the lowest, of 0.05%.

3.2. Arsenic excretion in developing toad larvae

Arsenic excretion from toad embryos exposed to 1 mg L^{-1} As and recovered in Arsenic-free media was determined (Fig. 3). The two exposure designs performed, starting from d 0 (14 d of exposure) and d 5 (9 d of exposure) respectively, showed different accumulation values at the end of exposure; d 0 caused an accumulation of 3.75 ± 0.34 µg As g DW^{-1}, while d 5 showed a significantly higher accumulation of 6.88 ± 0.93 µg As g DW^{-1} ($p < 0.03$, Student's *T*-test). Nevertheless, both designs presented quite similar half-life times for the excretion process, being 1.61 ± 0.40 d and 1.77 ± 0.58 d for

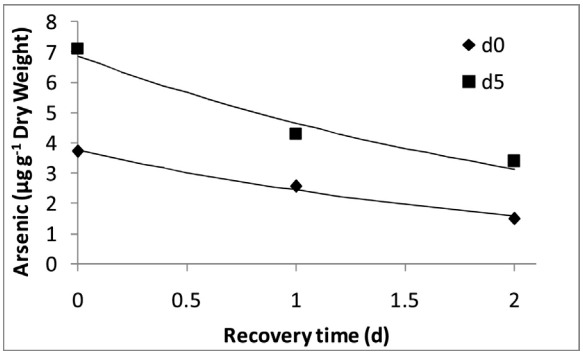
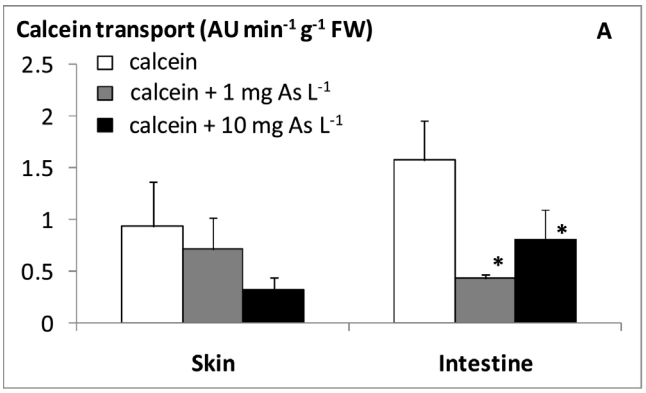
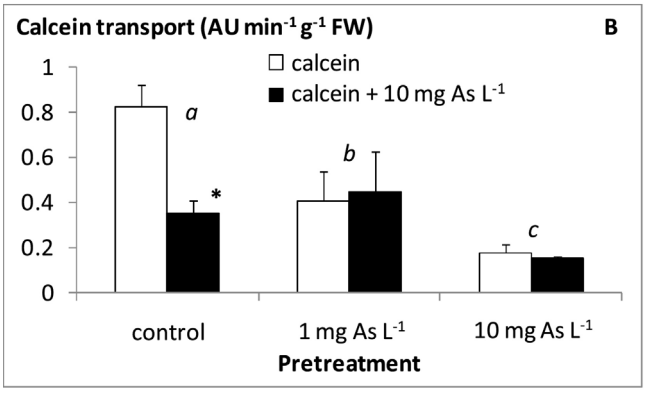


Fig. 3. Arsenic excretion in pre-exposed developing toad larvae.Larvae were continuously exposed from fertilization (d0) or from "heart beat" embryonic stage on (d5), and then at 14 d of development in both designs, larvae were recovered in an As-free medium during 2 d. Control larvae showed As contents in the same range as indicated in Fig. 1. Simple exponential decay model fitting to data let parameter estimations: Accumulated As = 3.75 ± 0.34 µg As g^{-1} dry weight, Excretion half-life = 1.61 ± 0.40 d for "d0" exposure design; Accumulated As = 6.88 ± 0.93 µg As g^{-1} dry weight ($p < 0.03$, Student's *T*-test *vs.* d0), Excretion half-life = 1.77 ± 0.58 d for "d5" (NS).





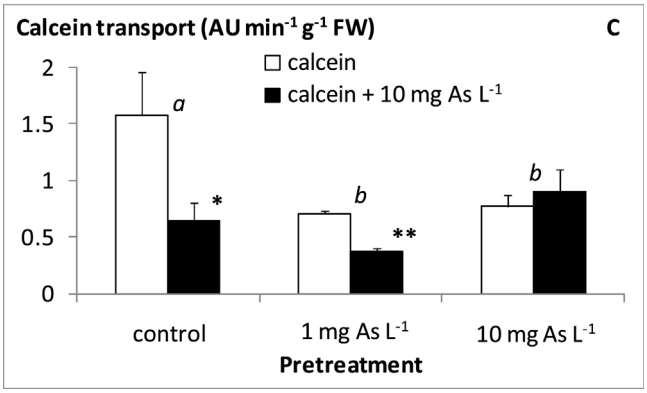


Fig. 4. Effects of As on ABCC-like transport in developing toad larvae.Calcein efflux was assessed in (A) skin and intestine from control larvae, alone or under competence of As in the assay media; (B) in skin or (C) in intestine from larvae pre-exposed during 3.5 d to different concentrations of As in the developing media and then assayed with calcein alone or with 10 mg L^{-1} As in the transport media. AU: Arbitrary Units of fluorescence; FW: fresh weight. Significant differences by ANOVA-Fisher's LSD test: *$p < 0.05$ or **$p < 0.01$ in As-calcein competence; different lowercases $p < 0.01$ in skin, $p < 0.05$ in intestine.

d 0 and d 5 respectively (not significant, Student's *T*-test).

In order to analyze possible mechanisms involved in As excretion, competition experiments were developed in *ex vivo* skin and intestine segments using calcein to measure ABCC-mediated transport (Fig. 4). We first analyzed the competition of different As concentrations with calcein transport in non-pretreated larvae (Fig. 4A). Calcein transport showed the tendency to a gradual competition by 1 and 10 mg L^{-1} As in the skin, although not in a significant way. In turn, intestine showed a significant competition of calcein transport ($p < 0.05$), that was comparable for both As concentrations (50%–70% of inhibition). Secondly, we pre-treated toad larvae with 1 and 10 mg L^{-1} As and then performed competition assays for calcein transport with 10 mg L^{-1} As in the media. Larval skin showed a progressive and

significant reduction in calcein transport due to the pretreatment with As (Fig. 4B; $p < 0.01$), which fell to 50% and 20% of control values for 1 and 10 mg L^{-1} As pretreatments, respectively. Competition of calcein transport with As in the medium was only significant for skin from non-treated larvae, diminishing the control value by 57% ($p < 0.03$), while no competition effects were observed in skin from As-pretreated larvae. Calcein transport in the intestine was also significantly affected by As pretreatment (Fig. 4C) and was equally reduced by 52%–55% by both concentrations ($p < 0.05$). Competition of As in calcein transport could be significantly observed in the intestine from control (non treated) larvae (60%, $p = 0.05$) and from 1 mg L^{-1} As pretreated larvae (50%, $p < 0.01$).

3.3. Effects of chronic exposure to arsenic in larval metal content

We also analyzed the effects of the exposure to different As concentrations on Cu, Zn and Fe contents along toad development. No variations were observed in the first embryonic stages, *i.e.* at 5 d of exposure (data not shown). Control values of the 3 elements remained constant along the development until 23 d of the assay. Copper control values averaged 5.00 ± 1.73 µg g DW^{-1}, and they were significantly augmented in larvae at 9 d of exposure to 1 mg L^{-1} As by 4.9X and to 10 mg L^{-1} As by 2.6X ($p < 0.05$, ANOVA-Fisher LSD test) (Fig. 5A). At 16 d and 23 d, only 1 mg L^{-1} As caused a significant increase in larval Cu content, of 4.1X and 8.8X, respectively ($p < 0.05$). Zinc levels in controls were 13.87 ± 3.83 µg g DW^{-1}. Exposures to 0.1, 1 and 10 mg L^{-1} As caused an increment in Zn levels at 9-d larvae; the effect was maximal for 0.1 mg L^{-1} As (3.5X increment), while it was quite similar for the other As concentrations (1.8–1.9X) ($p < 0.05$, ANOVA-Fisher LSD test; Fig. 5B). Other significant effects were observed only for 23 d of exposure to 1 mg L^{-1} As, which incremented Zn content in larvae by 3.5X ($p < 0.0005$). Iron content in control larvae was 253.4 ± 95.8 µg g DW^{-1}; As caused significant effects of Fe content only at 9 d of exposure, at 0.1 mg L^{-1} As (2.4X), 1 mg L^{-1} As (3.3X) and 10 mg L^{-1} As (2.3X) ($p < 0.05$; Fig. 5C). We also analyzed the possible correlations between As bioconcentration and the concentrations of these elements in the exposed toad larvae (Table 2). Copper concentration showed a positive correlation with As bionconcentration (R = 0.60) and Zn also did, although to a lesser extent (R = 0.37) ($p = 0.05$). Iron content did not correlate with As bioconcentration, while it showed low but significant correlations with Cu and Zn contents. Finally, Cu and Zn contents were also significantly correlated (R = 0.57, $p = 0.05$).

4. Discussion

We demonstrated in this study that *R. arenarum* embryos continuously exposed to As until 23 d in the first larval developmental stage were able to bioaccumulate this element in an active way. Arsenic was bioconcentrated from the exposure media up to about fifty times in the developing larvae. Considering the passive nature of the transporters described for As uptake for other aquatic species (Hamdi et al., 2009), a very active and fast biotransformation of arsenite to other chemical species would be expected, enabling the continue entrance of the toxicant in favor of its electrochemical gradient. Bioaccumulation magnitude may vary in a wide range depending on the chemical nature and properties of contaminants (Rahman et al., 2012). Arsenic may be accumulated by amphibian larvae and adults from contaminated areas, reaching values in the order of µg g of $tissue^{-1}$ (Moriarty et al., 2013). We found that As bioconcentration was increasing with the exposure time and in a variable range for concentrations in the media between 0.01–1 mg L^{-1} As. However, 10 mg L^{-1} As caused the lowest BCF in toad larvae among the concentrations tested, indicating that there were other factors influencing the absorption kinetics, probably compartment saturation effects. Even though body levels of As in the larvae exposed to 10 mg L^{-1} As were high, it must be noted that this concentration corresponds to the NOEC (lethality) value in *R. arenarum* embryos (Mardirosian et al., 2015).

Arsenic bioconcentration seems to increase during *R. arenarum* development. Similarly, *Rana perezi* tadpoles are able to uptake and accumulate As about 10–15 times higher than those in control larvae,

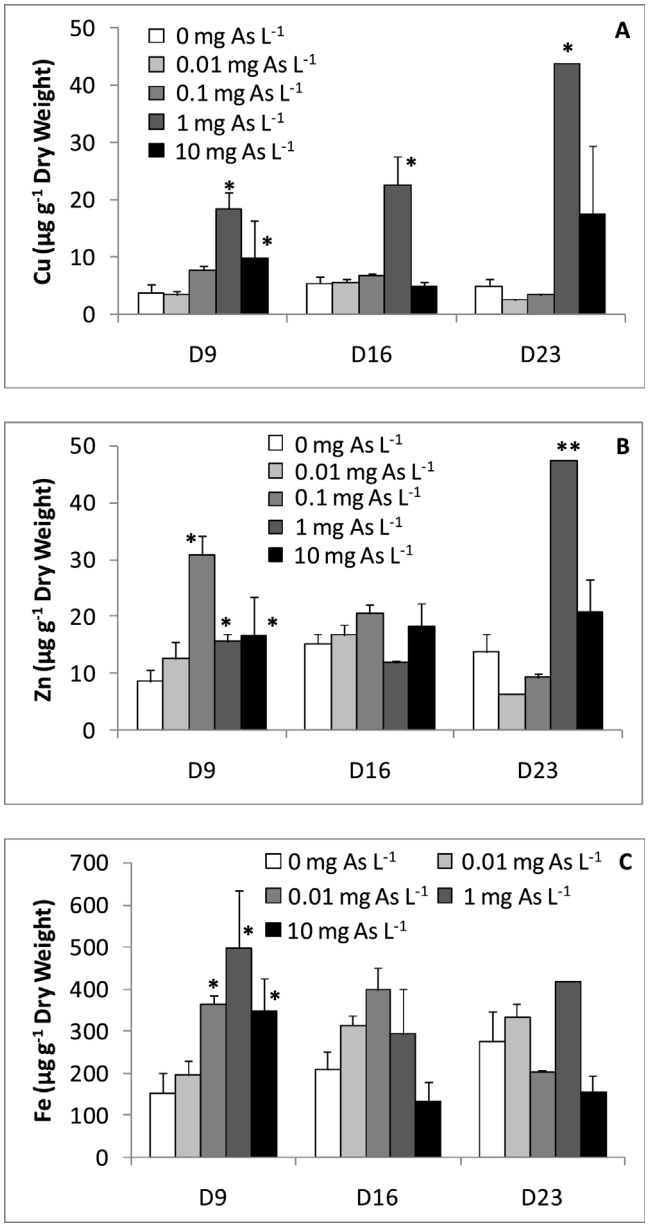


Fig. 5. Effects of As on metal contents in developing toad (*R. arenarum*) larvae.Metal contents of (A) Cu, (B) Zn and (C) Fe were measured in toad larvae exposed to different concentrations of As along their development. Significant differences by ANOVA-Fisher's LSD test: *$p < 0.05$ or **$p < 0.0005$.

Table 2
Correlations between As bioaccumulation and Fe, Cu and Zn contents in toad larvae.

Element	As	Fe	Cu	Zn
As	1.00			
Fe	−0.08 (NS)	1.00		
Cu	0.60*	0.34*	1.00	
Zn	0.37*	0.40*	0.57*	1.00

The contents of the different elements along the embryonic and larval development were subjected to correlation analysis (N = 45), with a statistical significance level of $p = 0.05$ (*).

depending on the developmental stage and time of exposure (Bryszewska et al., 2011). In another study, *Rana pipiens* larvae exposed up to 1 mg L^{-1} As(V) during 113 d accumulated the toxicant in a range of 0.6–5 μg g DW^{-1} (Chen et al., 2009), one order of magnitude lower than in our study, without causing evident changes in survival or metamorphosis rates. Environmental exposures to As in contaminated areas also lead to its accumulation in developing anurans, as described in metamorphosing *R. pipiens* collected at Green Bay in USA, which also showed 3.2 μg g DW^{-1} (Karasov et al., 2005). In turn, tadpoles from 2 lakes in Bryan, Texas, showed As concentrations reaching 23.6–51.3 μg g DW^{-1} (Clark et al., 1998), close to the levels found by us in the laboratory studies. Arsenic may be introduced in aquatic organisms such as fish and amphibian by food or water intake, or directly through the epithelium in skin and gills. The highly permeable skin is a very important site for the absorption of contaminants such as Arsenic in amphibian embryos and larvae, and in general for small organisms with an important surface-to-volume ratio due to their size (Moriarty et al., 2013; Rahman et al., 2012).

We determined in this study that the exposure to As caused also an increase in Cu, Zn and Fe levels, mainly at 9 d when the developing toad reached the first larval stage. There was a positive correlation between As accumulation and the increases of Cu and Zn. This suggests that As could be affecting normal metal elimination or modifying particularly Cu or Zn conjugation with cellular components. These elements are necessary cofactors of enzymes such as Cu/Zn superoxide dismutase which is also affected by As-induced oxidative stress (Mardirosian et al., 2016, 2017). Copper accumulation due to As intake has been previously reported in kidney from rat and hamster (Hunder et al., 1999), as well as in pig liver (Wang et al., 2006). In pigs, As accumulation also caused Fe and Zn accumulation in certain organs, and a decrease in their levels in others. Thus, an elevated intake of As in the diet may alter metals retention in specific organs (Wang et al., 2006).

We could observe that the stationary levels of As coming from the balance between accumulation, biotransformation and excretion were different if toad embryos were exposed from d 0 or d 5. The objective of this part of the study was to analyze if toad embryos were undergoing changes in As toxicodynamics that could explain the dramatic changes in its toxicity (Mardirosian et al., 2015). In fact, when *R. arenarum* embryos were exposed to As from d 5 of development, almost no toxicity was observed at As levels that caused 100% mortality in embryos exposed from fertilization (d0), about twice their LC50 (Mardirosian et al., 2015). Surprisingly, the level of total As bioconcentrated by larvae from d 5 of development to d 14 was two times higher than the level in larvae exposed since fertilization throughout 15 d. The fact that the half-lives of As depuration were similar in both treatments rules out possible adaptive effects on As excretion processes involving conjugating enzymes and transporters. It is possible that embryonic exposure from d 5 on, enables a higher uptake through aquaporins to explain a higher bioconcentration in a shorter time. Nevertheless, it still does not explain the higher tolerance to As. Instead, there may be a better biotransformation/detoxification battery of enzymes at 5 d of development in As-free media that lets the embryo challenge the toxicant at this stage even at higher rates of bioaccumulation. Arsenic depuration seems to be fast in aquatic animals, as suggested by studies in fish. Tilapia and Japanese Medaka fish (*Oryzias latipes*) exposed to 1 mg L^{-1} As, eliminated 90% in 1 d after being transferred to As-free media (Suhendrayatna et al., 2002). Zebrafish excreted 30%, 55% and 100% of accumulated As in 1, 3 and 5 d of recovery, respectively, after exposure to 1 mg L^{-1} arseniate (Liu et al., 2006). Our data are quite close to these values, as it can be estimated that 33%, 75% and 88% of the accumulated As in toad larvae is excreted after 1, 3 and 5 d of recovery in our experimental system.

We studied in turn the *ex vivo* transport through ABCC-like proteins in skin and intestine using calcein as a tracer. Arsenic is actively excreted through ABC transporters, particularly through ABCB1 and ABCC1-2 (Kala et al., 2000; Liu et al., 2002; Miller et al., 2007). Thus, As may be conjugated to GSH by glutathione-S-transferases (GST) before being excreted through ABCC transporters (Ventura-Lima et al., 2011). We have established that GST is induced by chronic As treatment in *R. arenarum* embryos and larvae (Mardirosian et al., 2016, 2017), thus favoring As excretion. Arsenite induces ABCC2 expression and activity in intestine of the killifish (Miller et al., 2007). The resistance to As citotoxicity has been associated with an increased expression of genes encoding GST-II, ABCC1 and 2 and ABCB1 in a rat cell line (Liu et al., 2002). We first established that As is effectively excreted by ABCC-like transport in toad larvae epithelia, as indicated by competition experiments with calcein. In this sense, calcein transport in larval skin showed a descending trend, caused by As in the media in a concentration-dependent way. Similarly, calcein transport was partially decreased by As in larval intestine. These effects suggest that As is effectively exported from epithelial cells by ABCC-like proteins in the developing toad larvae. However, the *in vivo* exposure to As caused significant reductions in ABCC-like transport, as it could be determined in *ex vivo* epithelia dissected from larvae previously exposed to 1 and 10 mg As L^{-1}. In fact, the remaining calcein transport activity observed after As pre-treatment could not be further competed by the presence of As in the media, as observed in Fig. 5B and C. Our results are in contrast with those reported by Miller et al. (2007) in renal tubules of killifish exposed to sodium arsenite, who observed an increase in both ABCC2 protein expression and ABCC2-mediated transport activity. The significant inhibitory effect of As pretreatment on calcein efflux in larval epithellia is sustained once As is removed from the media. Considering that As is highly bioaccumulated in the developing toad larvae, we may assume that As levels remain high in the studied tissues after the pretreatments, and thus directly compete with calcein from inside the cells even in the As-free media, determining the observed lower control values. Moreover, the high levels of As reached in larvae (about 4.4 and 5.8 μg g larval DW^{-1} for 1 and 10 mg As L^{-1} pretreatments) would amply exceed As uptake during the short competence assays with 10 mg As L^{-1} in the media and maximally displace calcein from ABCC transporter. An alternative hypothesis explaining our results would imply a direct inhibitory effect of As on ABCC transporters. Considering that As is rapidly excreted from the larvae as demonstrated by the recovery assays, there may be also other alternative transporters such as ABCB1 that may also account for As export from the cells, not evaluated in the present study.

In conclusion, As is bioconcentrated in developing embryos and larvae of the toad *R. arenarum*, up to fifty times the concentrations of the toxicant in the developing media. When exposed larvae are transferred to an As-free medium, the toxicant is rapidly excreted with a half life of 1.6 d. Arsenic would be metabolized by conjugation with GSH in *R. arenarum*, followed by its active excretion through an ABCC-like apical transporters.

Conflicts of interest

None.

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