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CHRONIC TOXICITY OF ARSENIC DURING *RHINELLA ARENARUM* EMBRYONIC AND LARVAL DEVELOPMENT: POTENTIAL BIOMARKERS OF OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE

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Abstract: The Argentinean autochthonous toad *Rhinella arenarum* was selected to study the chronic toxicity of arsenic (As) and the biochemical responses elicited by exposure to As in water during embryonic and larval development. Significant decreases in the total reactive antioxidant potential and in catalase activity were observed in individuals exposed chronically to sublethal concentrations of As, which is indicative of an oxidative stress situation. However, an antioxidant response was elicited during chronic exposure to As, as evidenced by the increase in endogenous reduced glutathione content and glutathione-related enzymatic activities such as glutathione *S*-transferase (GST) and glutathione reductase. This protective response might prevent a deeper decline in the antioxidant system and further oxidative damage. Alternatively, it might be linked to As conjugation with reduced glutathione for its excretion. Considering the sustained increase in GST activity and the decrease in the total antioxidant reactive potential observed, the authors propose them as good candidates to be used as biomarkers during As exposure. Interestingly, glutathione reductase activity was inhibited at a very low concentration of As considered safe for aquatic life. *Environ Toxicol Chem* 2017;36:1614–1621. © 2016 SETAC

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INTRODUCTION

Arsenic (As) is one of the most abundant hazardous elements in the environment. It can be found in water, soil, and air from natural and anthropogenic sources, existing both as inorganic and organic forms with different oxidation states and differential toxicity. Inorganic As is the predominant form in surface and underground water reservoirs [1]. The US Environmental Protection Agency's recommended aquatic life ambient water quality criteria for toxics are levels of a pollutant or other measurable parameter that allow for protection of aquatic life in the United States. Regarding freshwater, maximum values of 340 μg As/L for acute exposure and 150 μg As/L for chronic exposure have been established. Other countries have set lower limits to protect freshwater aquatic life [2]. In Canada, a guideline of 5 μg/L for total As was established [3]; and in Argentina, a maximum guide value of 15 μg As/L was set for protection of freshwater aquatic life [4].

Like many metals and metalloids, As can trigger reactive oxygen species (ROS) production, disrupt signal transduction, alter gene expression, and induce lipid and deoxyribonucleic acid (DNA) damage [5]. Major As-induced ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen, and peroxyl radicals. Oxygen-derived radicals are the most important class of radical species generated in living systems as a result of the particular electronic configuration of molecular oxygen, which allows it to form singlet oxygen by addition of 1 electron. Superoxide anions, arising through metabolic processes or after oxygen "activation" by physical irradiation, are considered "primary" ROS. They can further interact directly, through enzyme-catalyzed or metal-catalyzed processes, with other molecules to generate "secondary" ROS. Reactive oxygen species adversely affect cellular proteins, DNA, and membrane lipids and stimulate an increase in antioxidant defense. Experimental results have shown the generation of $O_2^{\bullet -}$ and H_2O_2 after As exposure in some cell lines such as human vascular smooth muscle cells, human–hamster hybrid cells, and vascular endothelial cells, whereas other cell lines such as HEL30, NB4, and CHOK1 have shown induction of H_2O_2. Furthermore, As-induced $^{\bullet}OH$ generation has been reported too in the striatum of rats. Apart from direct evidence of As-induced ROS, indirect evidence too has been reported. For instance, changes in antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) have been shown to suppress As-induced sister chromatid exchanges in human lymphocytes. Similarly, H_2O_2-resistant Chinese hamster ovary cells were resistant to arsenite, whereas CAT-deficient Chinese hamster ovary cells were hypersensitive to arsenite, demonstrating As-mediated ROS (reviewed by Flora [6]). Many vertebrates, including amphibians, try to counteract oxidative stress using the first-line defense system including reduced glutathione (GSH), vitamin C or E, carotenoids [7], or radical-scavenging enzymes like SOD, CAT, and glutathione peroxidase (GPx) [8,9]. Arsenic-induced oxidative stress has been the focus of toxicological research for the last decade to evaluate its possible mechanisms of toxicity.

Arsenic is also an element of ecological significance because of its persistence, bioaccumulation, and toxicity [10,11]. The presence of As in water is a potential threat to aquatic ecosystems. Amphibians are good bioindicators of

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environmental pollution because of their susceptibility to chemicals during their freshwater cycles. However, toxicological research on amphibians has been rather scarce compared with other aquatic organisms. Chemical analysis of water and soils represents the nature and degree of contamination, but sensitive organisms provide an indication of ecological effects. Biomarkers of exposure and effect are needed to connect the presence of pollutants in the environment with their action in an organism [12]. We previously reported that the main effect observed at acute exposure to sublethal concentrations of As in *Rhinella arenarum* embryos was oxidative stress [13], requiring high concentrations of this toxic element to elicit mortality.

We studied the effects at biochemical levels of chronic As exposure during embryonic and larval development of the common Argentinean toad, *R. arenarum*, with the aim of establishing possible biomarkers of As-induced oxidative stress at environmental concentrations considered safe for the preservation of freshwater aquatic life (10 μg As/L) [4] as well as at concentrations 10 to 1000 times higher.

MATERIALS AND METHODS

Chemicals

Sodium meta-arsenite ($NaAsO_2$) was purchased from Anedra Argentina (purity >99.95%). Bovine serum albumin (BSA), GSH, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5-dithio-bis-nitrobenzoate (DTNB), glutathione reductase (GR; lot 78H74301, 173 U/mg), β-nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt (NADPH), and luminol sodium salt were purchased from Sigma. Trolox, sodium hydroxide, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Aldrich. Oxidized glutathione (GSSG) was purchased from ICN Biomedicals. Triethanolamine and diethanolamine (analytical grade, 99% purity) were obtained from Riedel-de Haën. All other reagents used were also of analytical grade.

Toad embryo development and assessment of As toxicity

Adult *R. arenarum* males and females were collected in Los Barreales Lake (38.45344° S, 68.72918°W), a pristine environment, during the breeding season (spring). Animals were maintained and treated with regard for the alleviation of suffering according to recommended guidelines [14–16]. On collection, adults were transported to an outdoor terrarium. Ovulation was induced by intraperitoneal injection of 2500 IU of human chorionic gonadotrophin (ELEA Laboratory). Freshly extruded eggs were fertilized in vitro with a testicular homogenate. Thirty minutes after fertilization, groups of 800 embryos were transferred to glass receptacles containing 800 mL of amphibian Ringer's solution (0.65 g/L sodium chloride, 0.01 g/L potassium chloride, 0.02 g/L calcium chloride) either alone (control group) or with different concentrations of As solution (0.01 mg/L, 0.1 mg/L, 1 mg/L, and 10 mg/L) to assess As chronic effects [17]. Treatments were carried out in duplicate. Exposures were semistatic, with solution renewals every 48 h, spanning the complete embryonic development period (9 d) and 14 d of the first larval stage, until day 23 of development. Three independent experiments were performed at 18 °C to 20 °C and a 12:12-h light:dark photoperiod. Embryos were fed boiled lettuce from the open mouth stage until the end of the assay.

Biochemical analysis

Sampling and homogenization. Embryonic stages were assessed according to Del Conte and Sirlin [18]. Samples consisting of 20 embryos of the control, 1 mg As/L, and 10 mg As/L groups were collected in the following embryonic stages: tail bud, muscular response, gill circulation, caudal fin circulation, operculum fold, and operculum closed at right, corresponding to 3 d, 4 d, 5.5 d, 6.5 d, 7 d, and 8 d of development, respectively. In the first larval stage (complete operculum), samples consisting of 20 larvae were collected at 9 d, 16 d, and 23 d of development from the control, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L groups. Samples were thoroughly washed with cold Ringer's solution, briefly cooled on ice, and homogenized in 1 mL of 143 mM potassium phosphate buffer (pH 7.5) with 6.3 mM ethylenediaminetetraacetic acid (EDTA). Homogenates were divided into aliquots for immediate determination of GSH, lipid peroxidation, and total reactive antioxidant potential or centrifuged at 10 000 *g* or 20 000 *g* for 30 min at 4 °C; the resulting supernatants were divided into aliquots and kept frozen until enzymatic activity determinations were performed.

Total reactive antioxidant potential assay. The reaction mixture contained the free radical–generating compound AAPH 10 mM and 200 μM luminol in 50 mM sodium phosphate buffer (pH 7.4). Aliquots of 10 μL to 20 μL of the crude homogenates were added to 3 mL of the reaction mixture to assess their antioxidant potential. The mixture was incubated at room temperature to generate luminescence once the antioxidant potential was overcome. The induction time for chemiluminescence was measured in a liquid scintillation counter (Wallac LC1010). Trolox was used as a standard reference for the induction time [19].

Reduced glutathione content. Aliquots of 200 μL of the crude homogenates were added to 200 μL of 10% trichloroacetic acid, and the mixture was centrifuged at 10 000 *g* at 4 °C for 15 min. The GSH content was immediately measured in duplicate as acid-soluble thiols, using 100 μL of the supernatant and 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer (pH 8.0). The mixture was incubated for 10 min, and the absorbance at 412 nm was measured. Acid-soluble thiols were quantified using a calibration curve with pure GSH as a standard [20,21].

Lipid peroxidation. Aliquots of 100 μL of the crude homogenates were mixed with 250 μL of 0.72% thiobarbituric acid, 250 μL of 25% trichloroacetic acid, 25 μL of 2.78% ferrous sulfate heptahydrate, and 25 μL of 0.22% butylated hydroxy toluene in ethanol. The tubes containing the reaction mixture were heated in a boiling water bath for 25 min. Then, the samples were cooled, and fluorescence was measured in a Hitachi F7000 fluorescence spectrophotometer using an excitation wavelength of 517 nm and an emission wavelength of 550 nm. Malondialdehyde (MDA) was used as an index of lipid peroxidation by comparison with a standard curve prepared with 1,1′,3,3′-tetramethoxypropane (modified from Venturino et al. [21]).

Enzymatic determinations

CAT. Catalase (EC 1.11.1.6) activity was determined according to Beers and Sizer [22] with slight modifications [23]. The reaction was performed in 1 mL sodium phosphate buffer 50 mM (pH 7.0) containing 25 mM H_2O_2. Baseline absorbance was controlled to be stable and equal to 1 unit of absorbance, and 20 μL of 10 000 *g* supernatant were added to initiate the reaction. Specific activity (at 25 mM H_2O_2) was expressed as international units per milligram protein using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

SOD. Superoxide dismutase (EC 1.15.1.1) activity was measured through enzyme competition with reduced

nicotinamide adenine dinucleotide (NADH) oxidation by superoxide anion, following the consumption of NADH at 340 nm as described [23]. The reaction was performed in a final volume of 1.065 mL of 100 mM triethanolamine–100 mM diethanolamine buffer (pH 7.4), containing 0.28 mM NADH, 1.17 mM $MnCl_2$, 2.35 mM EDTA-Na_2, and 0.95 mM β-mercaptoethanol to trigger the chemical generation of superoxide anion. Maximum (control) NADH oxidization was competed with different volumes of 20 000 *g* supernatant (20–100 μL) to determine the SOD activity. One unit of SOD activity is defined as the amount of SOD leading to a 50% inhibition of NADH chemical oxidation by superoxide anion.

Glutathione S-transferase. The activity of glutathione *S*-transferase (GST; EC 2.5.1.18) was assayed in a final volume of 1 mL of 100 mM phosphate buffer (pH 6.5) containing 0.5 mM CDNB dissolved in 1% v/v acetonitrile and 2.5 mM GSH as substrate. Baseline (nonenzymatic reaction) was continuously recorded at 340 nm, and 10 μL of the 10 000 *g* supernatant were added. Changes in absorbance were recorded and corrected for spontaneous reaction, and the milli-international units of enzymatic activity were calculated using a molar extinction coefficient of 9.6 $mM^{-1}\,cm^{-1}$ [24].

GR. Glutathione reductase (EC 1.6.4.2) activity was determined in 1 mL of 143 mM potassium phosphate buffer (pH 7.5) with 6.3 mM EDTA, containing 0.21 mM NADPH and 0.5 mM GSSG as substrates and up to 0.2 mL embryo/larvae 10 000 *g* supernatant [23]. A continuous decrease in absorbance as a result of NADPH oxidation was recorded at 340 nm. Activity values were corrected by unspecific NADPH oxidation (i.e., omitting GSSG in the reaction mixture) and converted to milli-international units using a molar extinction coefficient of 5.886 $mM^{-1}\,cm^{-1}$ according to our experimental conditions.

Selenium-dependent GPx. Selenium (Se)–dependent GPx (EC 1.11.1.9) activity was determined using 0.3 mM H_2O_2 as substrate in 1 mL (final volume) of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM GSH as cosubstrate, 0.5 IU/mL GR plus 0.21 mM NADPH as a recycling system, and 1 mM sodium azide as a CAT inhibitor [23]. Up to 100 μL of 10 000 *g* supernatants was used to measure activity by continuously recording NADPH oxidation at 340 nm. Values were corrected by unspecific NADPH oxidation and converted to milli-international units using the same extinction coefficient as for GR.

Protein determination

Protein content was determined according to Lowry et al. [25] using BSA as a standard.

Data analysis

A block design was applied to include clutch effects on the results, and statistical differences between clutches and between treatments were assessed by analysis of variance (ANOVA) and Tukey's post hoc test using Statistica, Ver 7.1 (StatSoft). The factors used for the analysis were "treatment" and "stage" of development in which the determinations were performed, including analysis of the interactions between factors. Statistical analysis by ANOVA in block design indicated that there were no significant effects between the different clutches (experiment replicates, $p > 0.05$). However, the interaction between the factors "treatment × stage" was significant, so we proceeded to apply a one-way ANOVA analysis for the "treatment" factor within each stage. Tukey's post hoc test was applied to evaluate the differences between the different treatments in each stage.

RESULTS

Arsenic toxicity during embryonic and larval development

Exposure to As at the concentrations tested did not cause significant macroscopic alterations on the development or survival of *R. arenarum* embryos and larvae along day 23 of the assay. No malformations were observed at any of the As concentrations and times of exposure evaluated.

Biochemical responses to arsenic

GSH content. Endogenous GSH measured as acid-soluble thiols content in controls showed a continuous and significant increase during embryonic and larval development ($p < 0.0001$). Embryos exposed to 10 mg As/L showed a significant decrease during the muscular response stage (Figure 1). No differences in GSH content were found between

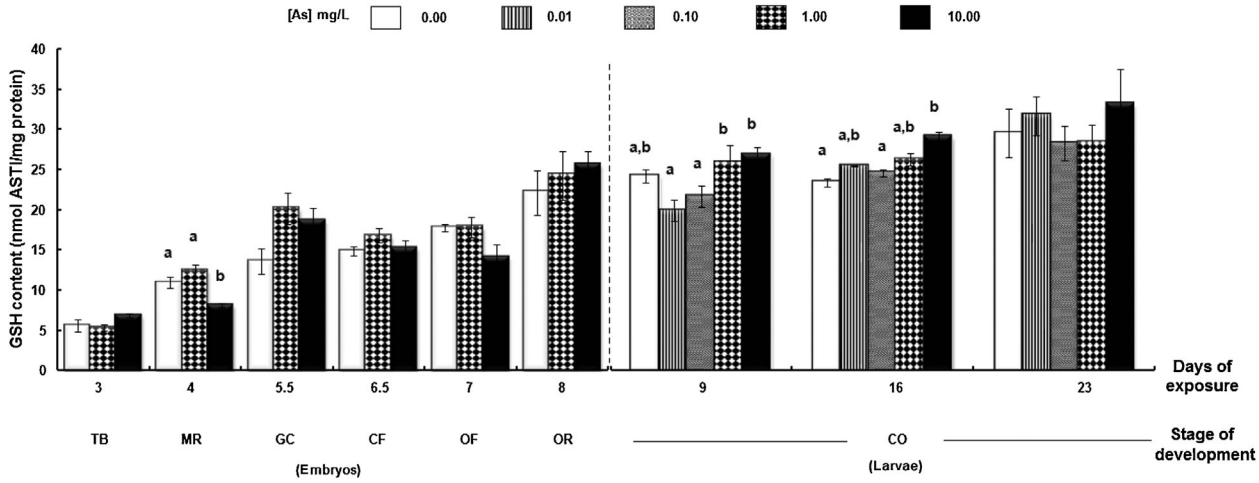

Figure 1. Effects of arsenic (As) on the reduced glutathione content measured in embryos and larvae of *Rhinella arenarum* chronically exposed to As. The exposure was carried out from fertilization up to 23 d to 0 mg As/L, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. ASTI = acid-soluble thiols; CF = caudal fin circulation; CO = complete operculum; GC = gill circulation; GSH = reduced glutathione; MR = muscular response; OF = operculum fold; OR = operculum closed at right; TB = tail bud.

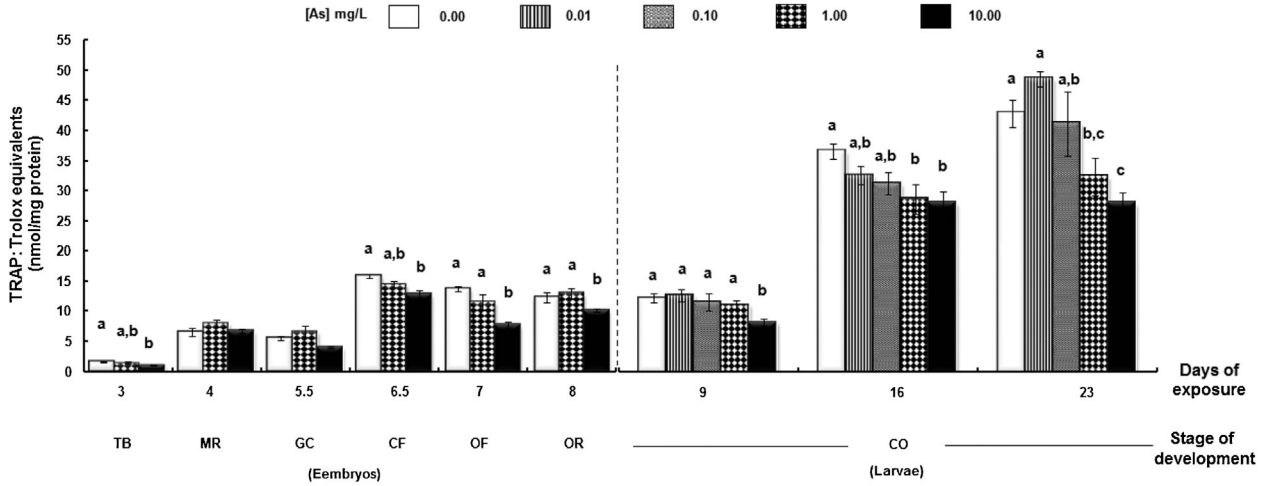

Figure 2. Effects of arsenic (As) on the antioxidant capacity (total reactive antioxidant potential) measured in embryos and larvae of *Rhinella arenarum* chronically exposed to As. Exposure from fertilization up to 23 d to 0 mg As/L, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. CF = caudal fin circulation; CO = complete operculum; GC = gill circulation; MR = muscular response; OF = operculum fold; OR = operculum closed at right; TB = tail bud; TRAP = total reactive antioxidant potential.

treatments in all of the other embryonic stages evaluated. Once the larval stage was reached, GSH content was significantly increased after 16 d of exposure to 10 mg As/L compared with the control group (Tukey's post hoc test, $p = 0.0017$; Figure 1). Of note, there were no significant effects on GSH content for any treatment after 23 d of exposure.

Total reactive antioxidant potential. Total reactive antioxidant potential levels continuously and significantly increased from embryonic to larval stages in controls. Exposure to the sublethal concentration of 10 mg As/L caused a significant decrease of total reactive antioxidant potential values compared to control groups during the entire period of exposure, from the beginning of embryonic development until the last day evaluated, except for muscular response and gill circulation embryonic stages, where no significant differences were found between control and exposed groups (Figure 2). At larval stages, there was also a significant decrease of total reactive antioxidant potential in the groups exposed to 1 mg As/L at 16 d and 23 d ($p < 0.01$), whereas no differences were observed in the groups exposed to 0.01 mg As/L and 0.1 mg As/L when compared to the corresponding control group (Figure 2).

Lipid peroxidation. Lipid peroxidation levels were not significantly modified by exposure to 0.01 mg As/L to 10 mg As/L up to 23 d of treatment. Lipid peroxidation levels varied between 0.045 nmol MDA/mg protein to 0.090 nmol MDA/mg protein in control larvae (data not shown).

CAT activity. Exposure to 1 mg As/L caused a significant inhibition of CAT activity compared to control groups in muscular response, caudal fin circulation, operculum fold, and operculum closed at right embryo stages ($p < 0.05$), whereas exposure to 10 mg As/L had the same effect in muscular response, operculum fold, and operculum closed at right stages ($p < 0.05$; Figure 3). On the contrary, As did not significantly

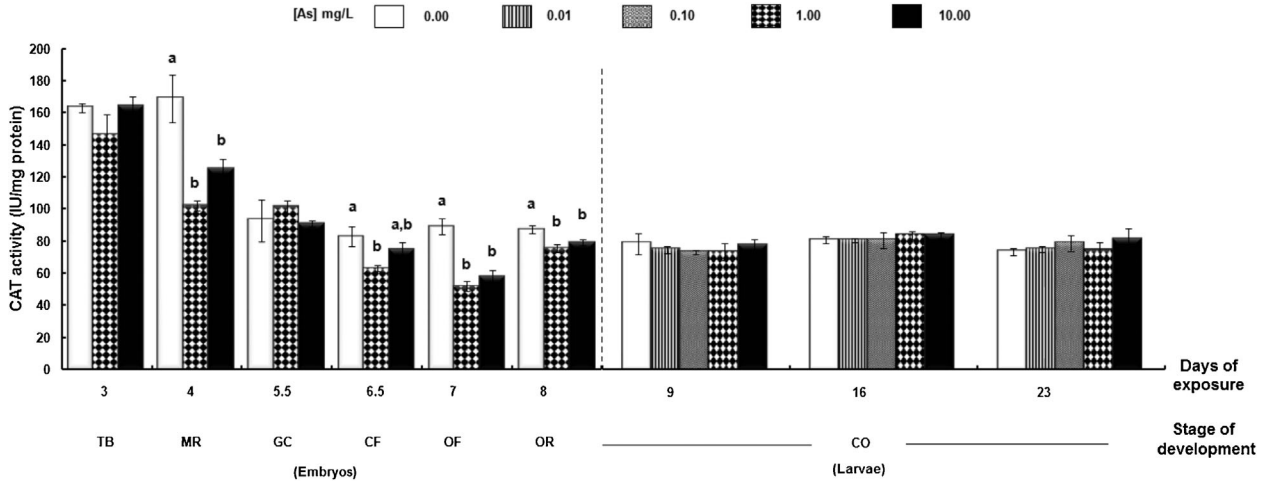

Figure 3. Effects of arsenic (As) on catalase activity measured in embryos and larvae of *Rhinella arenarum* chronically exposed to As. Exposure from fertilization up to 23 d to 0 mg As/L, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. CAT = catalase; CF = caudal fin circulation; CO = complete operculum; GC = gill circulation; MR = muscular response; OF = operculum fold; OR = operculum closed at right; TB = tail bud.

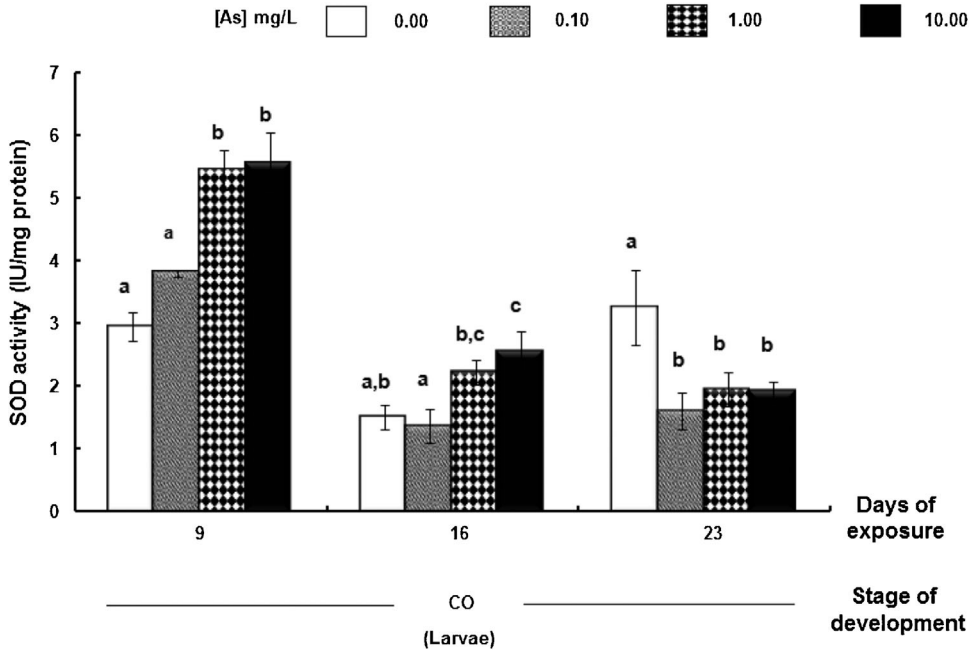

Figure 4. Effects of arsenic (As) on superoxide dismutase activity measured in larvae of *Rhinella arenarum* chronically exposed to As. Exposure from fertilization up to 23 d to 0 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. CO = complete operculum; SOD = superoxide dismutase.

affect CAT activity once the larval stage was reached and up to 23 d of exposure (Figure 3).

SOD activity. Significant increases of 84% and 88% in SOD activity were observed in embryos exposed to 1 mg As/L and 10 mg As/L, respectively, compared with the control group when embryos reached the first larval stage at 9 d of development (Tukey's post hoc test, $p = 0.005$ and $p = 0.004$, respectively). After 16 d of exposure to 10 mg As/L, SOD activity increased 69% compared to the control group ($p = 0.028$), whereas lower concentrations did not cause significant effects. After 23 d, larvae exposed to the 3 As concentrations evaluated (0.1 mg/L, 1 mg/L, and 10 mg/L) showed a significant inhibition of SOD activity of approximately 40% to 50% (ANOVA, $p = 0.02$; Figure 4).

GST activity. The activity of GST was variable during embryonic and larval stages, reaching a maximum value of 153.3 mIU/mg protein at the muscular response stage and a minimum of 86.88 mIU/mg protein value at the gill circulation stage. Continuous exposure to 10 mg As/L caused a significant increase in activity in all embryo and larval stages ($p < 0.001$; Figure 5). In turn, continuous exposure to 1 mg As/L caused a significant increase in GST activity from the gill circulation stage on ($p < 0.05$) compared to the control group. No significant effects were detected in larval GST activity at the lowest As concentrations assayed (0.01 mg/L and 0.1 mg/L).

GR activity. Control GR–specific activity increased continuously and significantly during toad larval development, from 7.28 mIU/mg protein at day 9 to 18 mIU/mg protein at day 23

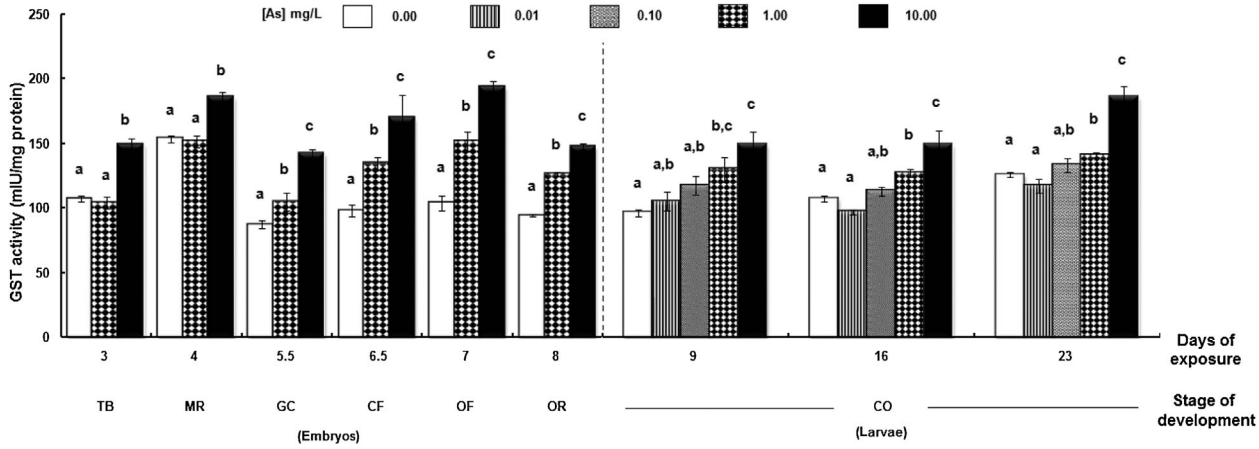

Figure 5. Effects of arsenic (As) on glutathione *S*-transferase activity measured in embryos and larvae of *Rhinella arenarum* chronically exposed to As. Exposure from fertilization up to 23 d to 0 mg As/L, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. CF = caudal fin circulation; CO = complete operculum; GC = gill circulation; GST = glutathione *S*-transferase; MR = muscular response; OF = operculum fold; OR = operculum closed at right; TB = tail bud.

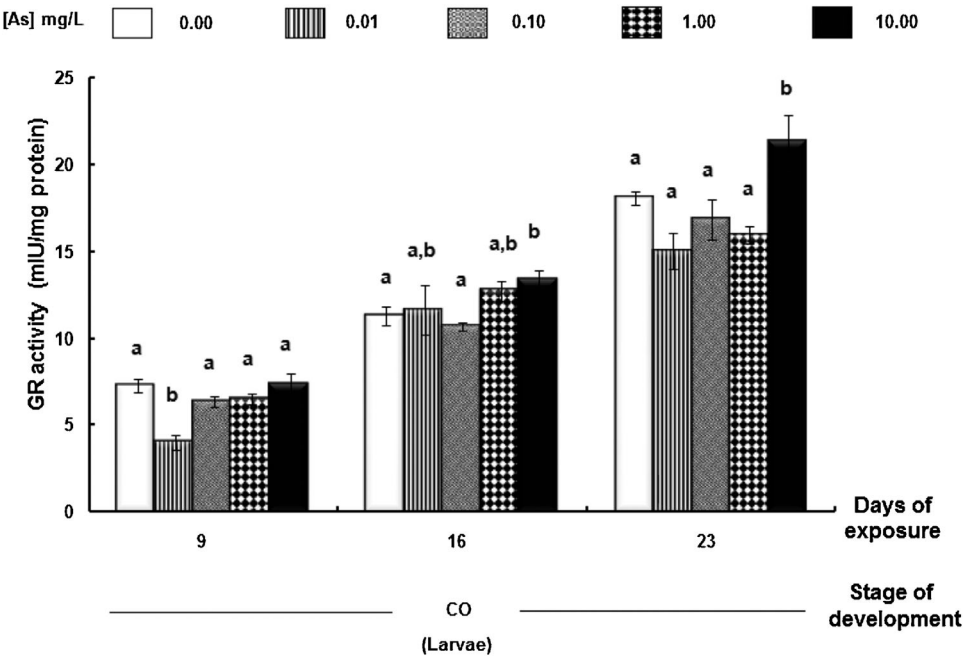

Figure 6. Effects of arsenic (As) on glutathione reductase activity measured in larvae of *Rhinella arenarum* chronically exposed to As. Exposure from fertilization up to 23 d to 0 mg As/L, 0.01 mg As/L, 0.1 mg As/L, 1 mg As/L, and 10 mg As/L. Data represent mean ± standard error of 3 independent experiments, with treatments in duplicate ($n = 6$). Different letters indicate significant differences between different treatments within each stage: $p < 0.05$ determined by analysis of variance and Tukey's post hoc test. CO = complete operculum; GR = glutathione reductase.

(2.5 times, $p < 0.001$). Glutathione reductase activity decreased significantly by 45% in individuals exposed for 9 d to the lowest concentration of As (0.01 mg/L, $p = 0.000001$), but there were no significant decreases at higher As exposures. On the contrary, after 16 d and 23 d of exposure to 10 mg As/L, GR activity increased significantly by 18% (Tukey's post hoc test, $p = 0.024$ and $p = 0.03$, respectively) when compared to control groups (Figure 6). Glutathione reductase activity was not significantly increased at the other As concentrations after 16 d or 23 d.

Selenium-dependent GPx activity. Similarly to GR activity, control Se–GPx activity also showed an increase during toad larvae development, being approximately 58 mIU/mg protein at 9 d of development and reaching approximately 94 mIU/mg protein after 23 d of development ($p < 0.01$). No significant differences in Se–GPx activity were detected between controls and larvae exposed to all of the As concentrations up to 23 d (data not shown).

DISCUSSION

In the present study, we assess the effects of chronic exposure to sublethal concentrations of As along *R. arenarum* embryonic and larval development, determining the oxidative stress and the activity of antioxidant enzymes. Considering that oxidative stress–induced damage in vital targets would be the main mode of action of As in many organisms [1,5,6,13,26,27], we selected the study of oxidative stress and antioxidant response endpoints as potential biomarkers. The advantage of counting with biochemical biomarkers that respond early to toxicants in a chronic exposure is that they may anticipate irreversible damage at later developmental stages as a result of constant oxidative challenge. The biochemical biomarkers of effect analyzed in the present study might reveal oxidative stress from As toxicity anticipating irreversible damage related to ROS imbalance because a delicate equilibrium is required for normal processes such as programmed cell death in tissue remodeling during larvae progression and metamorphosis. Nevertheless, we are aware that these biomarkers are not exclusive for As effects because oxidative stress is a very common process elicited by toxicants. In this sense, the use of a battery of endpoints as biomarkers is always recommended, including, if possible, more specific ones. This allows overcoming a high variability in the responses depending on seasonal aspects, duration and frequency of contamination episodes, and many other factors including the organisms themselves. Even those biomarkers considered specific for a group of toxicants may show diverging responses, as reported for the biomarker of neurotoxic effects acetylcholinesterase, which was induced in *R. arenarum* larvae exposed in situ for 2 wk in an orchard receiving organophosphate pesticide applications [28].

We effectively found in the present study that chronic exposure to As of *R. arenarum* at developmental stages generated oxidative stress and an antioxidant response, as indicated by different endpoints at varying stages and As concentrations. The decreasing levels of total reactive antioxidant potential clearly indicate a pro-oxidant situation generated by chronic exposure to As. However, GSH levels are slightly affected at the beginning of the exposure and show an increasing response at the larval stage, suggesting a partial recovery, with no significant effect on GSH at As concentrations up to 10 mg/L after 23 d. The negative effect on total reactive antioxidant potential would thus indicate that other nonenzymatic antioxidants are negatively affected by As. A similar situation was previously reported for acute exposure to high concentrations of As during the early development in toads [13]. We observed that the As effect on total reactive antioxidant potential is manifested at very early stages (from day 3) and at concentrations at least 10 times lower than the no-observed-effect concentration (NOEC) value for long-term exposures. This decrease was sustained during the whole assay. While in the acute exposure a decrease was observed at 10 mg/L [13], in the present study we

observed an underlying effect at lower concentrations that was manifested after 16 d of exposure. Ferrari et al. [23] studied changes in antioxidant metabolism during *R. arenarum* embryonic development. They showed that CAT and SOD play an important role in *R. arenarum* embryos to detoxify hydrogen peroxide and superoxide anion, respectively, being the main pathway for oxidative stress control in oocyte and early stages of *R. arenarum*. The importance of these enzymes is evidenced by the high control activity levels along *R. arenarum* embryonic development [23]. They also suggested that the GSH-dependent antioxidant system increases its participation in oxidative control when the gills are fully active in the toad embryos [23]. Catalase activity is also inhibited by the presence of As only in embryonic stages, suggesting an impact of ROS on the active site of the enzyme [29]; but it recovers in the larval stage. The antioxidant response toward As exposure generated at the larval stage thus seems adequate because also lipid peroxidation levels remain at control values. Moreover, As significantly increases SOD activity in larvae exposed for 9 d and 16 d at 1 mg/L and 10 mg/L. Nevertheless, a significant decrease in SOD activity is observed after 23 d of exposure to concentrations 1000 times lower than the NOEC, suggesting that SOD protein may be particularly susceptible to ROS because of its cysteine residues [30].

Inorganic As is known as a human carcinogen that acts as a tumor promoter by inducing a quick burst of ROS [31]. This might create an oxidative stress scenario with a subsequent increase in GSH levels, which is involved in the detoxification of ROS and whose increase may be part of an adaptive response to oxidative stress. Glutathione-related enzymes, including GST and GR, also play a key role in these processes [26], participating in the detoxification of As, among other xenobiotics [32,33], and in the recycling of GSSG to GSH [34], respectively. The GSH content in embryos was not affected by exposure to As, suggesting a proper recycling of GSSG to GSH by GR. In larval stages, GSH levels increased in organisms exposed to 1 mg As/L and 10 mg As/L (at day 9 but not day 23), along with an increase in GR activity in larvae exposed to 10 mg As/L at day 16 and day 23. Schuliga et al. [26] detected a significant increase in GR activity of 100% above control activity levels and in GR protein expression parallel to the increase in enzymatic activity in fibroblasts and keratinocytes treated with As (III) concentrations of 75 μg/L, 225 μg/L, and 750 μg/L for 24 h. Sarkar et al. [5] exposed zebrafish to a sublethal concentration of As that caused a gradual increase in GSH content after 60 d of exposure. However, this increase of GSH did not correlate with GR activity. The present results show a strong induction of GST activity in embryos and larvae chronically exposed to concentrations 10 times lower than the NOEC [13], 1 mg As/L and 10 mg As/L, from a very early embryonic stage (TB); and this answer was sustained during the whole assay. Ventura-Lima et al. [35] reported that low levels of As affect the antioxidant response in zebrafish by increasing the activity of the enzyme glutamate-cysteine-ligase and GSH levels, even at a concentration of As considered safe for the environment. Schuliga et al. [26] observed an increase of 25% in GST activity of HaCaT cells and human keratinocytes exposed to 750 μg As/L for 24 h compared with control cells. Similarly to what happens with As, *R. arenarum* embryos exposed to sublethal concentrations of the organophosphorus pesticide azinphos-methyl show an induction of GST activity [36]. Increased GST activity in *R. arenarum* embryos and/or larvae exposed to As from the beginning of embryonic development could indicate an adaptive response that would protect them from As toxicity in chronic exposures. On the other hand, such an effect on GR activity was not observed up to 9 d of exposure to As, or it was counterbalanced by enzyme inactivation by ROS as suggested by its lower activity at very low As concentrations. Instead, an adaptive increase in GR activity was observed during the chronic exposure and when toads were exposed to the highest concentration. Both enzymes are potential good biomarkers of response in developing toad embryos environmentally exposed to As.

Although control Se–GPx enzyme activity levels are high and increase during larval development, the activity of this enzyme is not affected by As. This suggests that Se–GPx may not have an important role in the detoxification of ROS generated by As during toad embryonic development or that its activity would not be regulated by the presence of this toxicant. Furthermore, the Se–GPx protein structure would not be affected by ROS generated by As exposure. In contrast to the present results, Sarkar et al. [5] observed that GPx and CAT levels increased in zebrafish brain after 15 d of exposure to 50 μg/L of As trioxide, helping to detoxify superoxide radicals from the cell.

The GSH system also plays an important role in protecting cells against exposure to inorganic As. This is supported by other studies showing that resistance to inorganic As in mammalian cells is correlated with increased intracellular GSH levels and higher activities of GSH-related enzymes [26]. In addition to improving redox balance, GSH is in direct competition with thiol groups of proteins to form complexes with As. Thiol groups of proteins are susceptible to oxidation by trivalent As and may be a critical target in As toxicity [37]. Sakurai et al. [38] also suggested that GSH offers protection against As toxicity. Exposure of epithelial cells from rat liver with organic As was not cytotoxic even at concentrations above 10 mM but became weakly cytotoxic and induced cell death after depleting endogenous GSH by treating cells with a GSH synthase inhibitor or a GR inhibitor. These data indicate that As toxicity is, at some point, related to GSH levels and that, when this antioxidant is depleted, As toxicity is increased. Oketani et al. [39] reported that As trioxide induced apoptosis in hepatoma cells and proposed that the sensitivity of these cells to As trioxide was inversely related to its intracellular GSH content and GSH synthesis. Therefore, GSH seems to have a dual role against As toxicity because it acts as an antioxidant defense and at the same time is involved in As biotransformation. Nowadays, the role of GSH in As toxicity and biotransformation mechanisms in aquatic animals is not completely understood. Ventura-Lima et al. [27] observed high levels of GSH in *Danio rerio* gills after exposure to arsenate and in *Cyprinus carpio* liver after exposure to arsenite and arsenate. Both GST and GSH may participate in As conjugation prior to excretion [40].

In conclusion, chronic exposure of developing *R. arenarum* toad embryos to sublethal concentrations of As causes oxidative stress that is evidenced early by the decrease of total reactive antioxidant potential and sensitive enzymes such as CAT. However, there is an important antioxidant response that develops toward As, generating an increase in GSH content and GSH-related enzymatic activities of GST and GR as an adaptive response that gives cells protection from As toxic effects. Therefore, in view of their sustained response during toad aquatic development, we propose GST activity and total reactive antioxidant potential as appropriate candidates to be used as biomarkers of effect for As exposure. Remarkably, GR activity showed a transient effect at a very low concentration considered safe for aquatic life.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (aventu1@yahoo.com.ar).

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