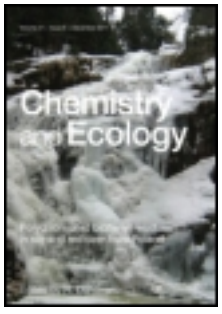

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Considering that amphibians are good sentinels of environmental conditions, *Rhinella arenarum* embryos were used to investigate the effects of sublethal concentrations of the organophosphorus insecticide azinphos-methyl, focusing on its anticholinesterasic or pro-oxidant actions and its possible connection with the appearance of morphological alterations. Early amphibian embryos exposed to azinphos-methyl displayed a protective response through glutathione *S*-transferase induction, along with superoxide dismutase inhibition. At intermediate embryonic stages, embryos exposed to azinphos-methyl displayed superoxide dismutase inhibition and morphological alterations, although cholinesterase activity was not altered, suggesting that molecular targets other than cholinesterase were involved in the development of morphological alterations. At the end of embryonic development, decreases in reduced glutathione and cholinesterase inhibition were observed, along with a significant increase in the number of malformed embryos. The connection between biochemical alterations and the appearance of malformations was not evident in *R. arenarum* embryos. However, increased glutathione *S*-transferase and decreased superoxide dismutase activities could be considered as early markers of exposure to azinphos-methyl. The results obtained demonstrate that sublethal concentrations of azinphos-methyl are a serious threat to toad embryos in their natural habitats because biochemical and morphological alterations could impair their ability to deal with environmental stresses.

Keywords: organophosphorus pesticide; cholinesterase; glutathione *S*-transferase; superoxide dismutase; reduced glutathione; amphibian

1. Introduction

Azinphos-methyl (phosphorodithioic acid, *O*,*O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4H)-yl)methyl] ester) (AM; CAS RN 86-50-0) is an organophosphorus pesticide intensively applied in the Valley of Rio Negro and Neuquén, northern Patagonia, Argentina. Of the major organophosphorus pesticides applied to foliage, AM is one of the most persistent in water. It is moderately

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persistent at acid and neutral pH, while its half-life in alkaline water is in the order of several days [1]. The fact that 0.6 kg of AM is applied per hectare every 15 days during the productive season and 600 tons of product are released annually into the environment [2], makes this insecticide a threat for nontarget species such as the amphibians that inhabit the area. In this regard, amphibian populations are experiencing widespread declines due to several factors, including pesticide exposure [3–5]. Although the concentrations of AM detected (in the order of $\mu g \cdot L^{-1}$) in surface water and groundwater of the Valley of Rio Negro and Neuquén, generally range below lethal concentrations for aquatic vertebrates [6,7], samplings revealed that the AM concentration exceeded criteria for the protection of aquatic life and remained high throughout the growing season [7]. Moreover, in studies carried out in channels in the region and in the Negro river, toad larvae showed significant responses in biomarkers such as cholinesterase, carboxylesterase, glutathione *S*-transferase (GST) and reduced glutathione. These results suggest that field concentrations, immediately after pesticide application, must temporarily exceed those reported for surface water [8]. The organophosphorus pesticide AM exerts its insecticidal action by inhibition of acetylcholinesterase [9], which is considered a biomarker of exposure to this class of pesticides [10]. Several authors have reported a relationship between cholinesterase (ChE) inhibition and teratogenesis in amphibian embryos and larvae exposed to organophosphorus and carbamate insecticides [11–13]. In addition to the anticholinesterasic action of AM, it is well documented that exposure of aquatic species to this insecticide elicits alterations in cellular redox status, which may lead to modification of the antioxidant defence system [14–17]. Likewise, a critical involvement of reactive oxygen species (ROS) in the appearance of developmental alterations by means of oxidative damage to macromolecules in animal models and cell cultures exposed to drugs, environmental chemicals and gamma irradiation has been noted [18]. In this regard, we previously analysed the effects of lethal concentrations of organophosphorus pesticides, including AM, and carbamates on antioxidant defences in *Rhinella arenarum* embryos [16]. *Rhinella arenarum* is a native toad of Argentina and inhabits the productive area of the Valley of Río Negro and Neuquén, spending its embryonic and larval stages in water bodies in the region. Accordingly, water quality greatly influences growth and development of this species. At the end of embryonic development, significant effects driven by oxidative stress were proportionally related to the appearance of malformations and death in *R. arenarum* embryos chronically exposed to organophosphorus and carbamate insecticides [16]. Recent studies on the effects of acute exposure to AM using premetamorphic toad larvae showed that an adaptive response of GST occurs earlier than morphological alterations or mortality [19].

The antioxidant defence system present in aerobic organisms is complex and includes enzymatic and nonenzymatic defences to counteract ROS actions [20]. The enzymes superoxide dismutase (SOD) and catalase (CAT) constitute the first line of defence against ROS in toad embryos [21]. The antioxidant defence system also comprises a set of enzymes which make use of reduced glutathione (GSH) as a substrate, including glutathione peroxidases (GPox), glutathione reductases (GR) and GSTs. Development of the antioxidant system in amphibian embryos protects them against prooxidant environments because GSH levels and the activities of GSH-related enzymes increase after hatching and gill development [21].

Although previous studies in *R. arenarum* embryos and larvae have contributed to greater knowledge of AM-induced oxidative stress and mechanisms of toxicity [16,19], experiments involving sublethal concentrations of the organophosphorus pesticide would allow more sensitive evaluation of toxicological mechanisms and would provide useful information on the assessment of AM pollution in aquatic ecosystems. In this study, we investigate the effects of sublethal concentrations of AM, in the order of those transiently expected in irrigation channels and ponds, in *R. arenarum* embryos. We focus on the anticholinesterasic or pro-oxidant actions and their possible connection with the appearance of morphological alterations.

2. Materials and methods

2.1. *Chemicals*

Azinphos-methyl (AM; 99.0% purity) was purchased from Chem Service (West Chester, PA, USA). Acetylthiocholine iodide, 5,5′-dithio-2-bis-nitrobenzoate (DTNB), GSH, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase (GR) (lot 78H74301, 173 U/mg) and β-NADPH were purchased from Sigma Co. (St. Louis, MO, USA). GSSG was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Sodium azide was purchased from Mallinckrodt (Phillipsburg, NJ, USA). All the other reagents used were of analytical grade.

2.2. *Toad embryo culture and insecticide exposure*

Three independent experiments were performed using three different pairs of parents. Ovulation of females was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotropin. *Rhinella arenarum* embryos were obtained by *in vitro* fertilisation, as described previously [22]. Groups of 1000 newly fertilised embryos were transferred to glass recipients containing either amphibian Ringer's solution ($0.65\,g \cdot L^{-1}$ NaCl; $0.01\,g \cdot L^{-1}$ KCl; $0.02\,g \cdot L^{-1}$ $CaCl_2$) (control group) or pesticide solution, keeping a ratio of 1 embryo · mL solution^{-1}. Pesticide solutions of 0.5 and $2\,mg \cdot L^{-1}$ AM were prepared by diluting the insecticide standard solution, prepared in acetone, with an appropriate amount of amphibian Ringer's solution, keeping acetone to 0.3%. The exact concentration of the insecticide in the standard solution was checked by gas chromatography with a nitrogen and phosphorus detector (GC-NPD). Controls of 0.3% acetone were also performed because this solvent concentration has no adverse effects on embryonic development [19]. Both concentrations tested were sublethal (96 h LC_{50} value: $15.6\,mg \cdot L^{-1}$) [23]. In addition, a lethal concentration of $9\,mg \cdot L^{-1}$ AM was used in parallel to confirm data reported previously [16]. Treatments were carried out by duplicate. Solutions were renewed every 48 h until embryos reached the stage of complete operculum (CO; 10 days of development) [24]. Embryos were maintained at 18–20°C in a 12:12 h light/dark photoperiod. The viability of individuals was monitored using a stereoscopic microscope [16]. Morphological alterations were recorded according to the *Atlas of Abnormalities* [25]. Control embryos at the end of development displayed $<5\%$ of mortality and $<15\%$ of malformations under laboratory conditions. Embryonic development was assessed in samples of nontreated embryos and embryonic stages were determined according to Del Conte and Sirlin [24]. Samples were taken at three different embryonic stages: tail bud (TB), open mouth (OM) and CO, which correspond to 3, 7 and 10 days of development, respectively.

All the methodologies employed were in accordance with the bioethical normative of the University Committee for Care and Use of Laboratory Animals, Universidad Nacional del Comahue.

2.3. *Sampling and homogenisation for enzymatic determinations*

Each sample consisted of a total of 50 embryos at TB stage or 25 embryos at OM and CO stages. Embryos were thoroughly washed with cold Ringer's solution, briefly cooled on ice and homogenised in 1 mL of 143 mM potassium phosphate buffer pH 7.5 with 6.3 mM EDTA. The homogenates were centrifuged at 10,000 ***g*** for 20 min at 4°C and the resulting supernatants were divided into aliquots and kept frozen until the enzymatic activity determinations. For superoxide dismutase (SOD) determination, embryos were homogenised in the same buffer and centrifuged at 20,000 ***g*** for 20 min at 4°C.

2.4. *Cholinesterase activity*

Cholinesterase activity was determined according to Ellman *et al*. [26]. Reactions were performed in 1.03 mL of 100 mM phosphate buffer, pH 8.0 containing 0.2 mM DTNB and 0.75 mM acetylthiocholine iodide as substrate and 0.1 mL of 10,000 ***g*** supernatant were added to initiate the reaction. Activity was recorded continuously at 412 nm. Specific activity was expressed as mIU · mg protein^{-1} using a molar extinction coefficient of 13.6 mM^{-1} · cm^{-1}.

2.5. *Antioxidant enzyme determinations*

2.5.1. *Glutathione* S-*transferase*

GST (EC 2.5.1.18) activity 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 substrates. Baseline (nonenzymatic reaction) was continuously recorded at 340 nm and 30 µL of the supernatant was added [27]. The international units of enzymatic activity were calculated using a molar extinction coefficient of 9.6 mM^{-1} · cm^{-1}.

2.5.2. *Glutathione reductase*

GR (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 0.1 mL embryo supernatant (modified from Schaedle and Bassham [28]). NADPH oxidation was recorded at 340 nm. Activity values were corrected by unspecific NADPH oxidation omitting GSSG in the reaction mixture, and converted to mIU using a molar extinction coefficient of 5.886 mM^{-1} · cm^{-1} according to our experimental conditions.

2.5.3. *Selenium-dependent glutathione peroxidase*

Se-GPox (EC 1.11.1.9) activity was determined using 0.3 mM H_2O_2 as substrate in a final volume of 1 mL of 50 mM potassium phosphate buffer pH 7.4 containing 1 mM GSH as cosubstrate, 0.5 IU · mL^{-1} GR plus 0.21 mM NADPH as a recycling system, and 1 mM sodium azide as a CAT inhibitor [29]. Fifty microlitres of supernatant were used to measure Se-GPox activity by continuously recording NADPH oxidation at 340 nm. The values were corrected by unspecific NADPH oxidation and were converted to mIU using the same extinction coefficient as for GR.

2.5.4. *Catalase*

CAT (EC 1.11.1.6) activity was determined according to Beers and Sizer [30], with slight modifications. The reaction was performed in 3 mL sodium phosphate buffer 50 mM pH 7.0 containing 25 mM H_2O_2. Baseline absorbance was controlled to be equal to one unit of absorbance and stable and 20 µL of supernatant were added to initiate the reaction. Specific activity (at 25 mM H_2O_2) was expressed as IU · mg protein^{-1} using a molar extinction coefficient of 40 M^{-1} · cm^{-1}.

2.5.5. *Superoxide dismutase*

SOD (EC 1.15.1.1) activity was measured through enzyme competition of NADH oxidation by superoxide anion, recording the consumption of NADH at 340 nm [31]. The reaction was performed in a final volume of 1.065 mL of 100 mM triethanolamine (TEA)–100 mM diethanolamine

(DEA) buffer pH 7.4 containing 0.28 mM NADH, 1.17 mM $MnCl_2$, 2.35 mM EDTA and 0.95 mM β-mercaptoethanol to initiate the chemical generation of superoxide anion. Control (maximum) NADH oxidation was competed with 10, 20, 40 and 60 µL of 20,000 ***g*** supernatant to determine SOD activity in each sample [21]. One unit of SOD activity is defined as the amount of SOD leading to a 50% inhibition of NADH chemical oxidation by superoxide anion.

2.6. *GSH content determination*

Fifty TB embryos or 25 OM and CO embryos from each duplicate treatment were randomly separated. Embryos were rinsed with cold amphibian Ringer's solution and homogenised in 5% trichloroacetic acid at 4°C. The homogenates were centrifuged 10 min at 10,000 ***g*** and GSH content was immediately measured as acid-soluble thiols in triplicate in 0.1 mL of the supernatant, using 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer, pH 8.0 [21]. 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 standard.

2.7. *Protein determination*

Protein content was determined according to Lowry *et al*. [32] using BSA as standard.

2.8. *Data analysis*

Three independent experiments were performed. Within each assay, different treatments were tested in duplicate. For the statistical analysis, data from the three experiments were pooled ($n = 6$ for each embryo stage and treatment) because no significant differences were observed between experiments. Statistical differences between treatments were assessed by ANOVA and Fisher's lowest significant differences (LSD) *post hoc* test.

3. Results

3.1. *Effects of azinphos-methyl on embryonic development*

At the early embryonic stage of TB (3 days of development), embryos exposed to both concentrations of AM displayed no significant differences in the percentage of malformations when compared with controls. However, when they reached the OM stage, the percentage of malformed embryos increased significantly at exposure to 2 mg $\cdot$ L^{-1} AM when compared with control values (10.3 vs. 6.5% respectively; $p \leq 0.05$) (Figure 1). Finally, at the end of embryonic development, CO embryos exposed to 2 mg $\cdot$ L^{-1} AM displayed a significant increase in the frequency of malformations when compared with control values (23.5 vs. 14.4% respectively; $p \leq 0.001$) (Figure 1). The morphological abnormalities commonly found included gills atrophy, axial shortening, body blistering, dorsal tail flexure and oedema. Control OM embryos were 7 mm long and displayed external gills (Figure 2A). At this stage, the embryonic mouth opens and the embryo begins to feed on external substrates. The CO stage is the last embryonic stage in *R. arenarum* and is characterised by complete reabsorption of the external gills (Figure 2D,E). Control CO embryos were 10 mm long and showed a rounded anterior area well differentiated from their caudal fin. In this study, a generalised shortening of the embryonic axis was frequently observed in *R. arenarum* embryos exposed to 2 mg L^{-1} AM and was usually associated with other abnormalities such as oedema (Figure 2B,C,F). Abdominal oedema appeared as a transparent and swollen fluid-filled

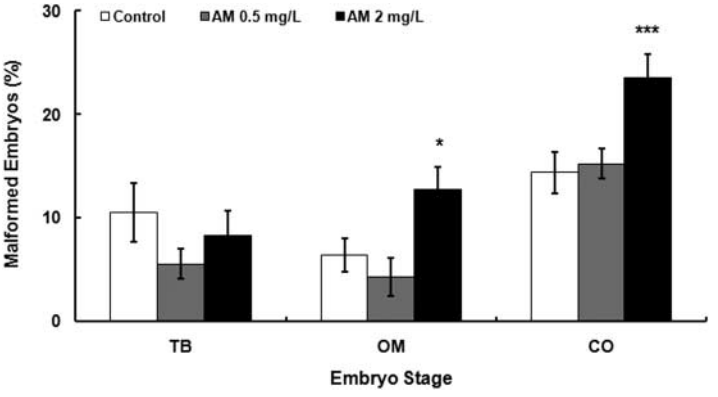

Figure 1. Percentage of malformed *Rhinella arenarum* embryos exposed to 0, 0.5 and 2 mg · L^{-1} AM from fertilization until the CO stage. Data represent mean ± SE from six replicates. Asterisks indicate significant differences with respect to control values (*$p \leq 0.05$; ***$p \leq 0.001$) determined by ANOVA and Fisher's LSD test. AM, azinphos-methyl; TB, tail bud; OM, open mouth; CO, complete operculum.

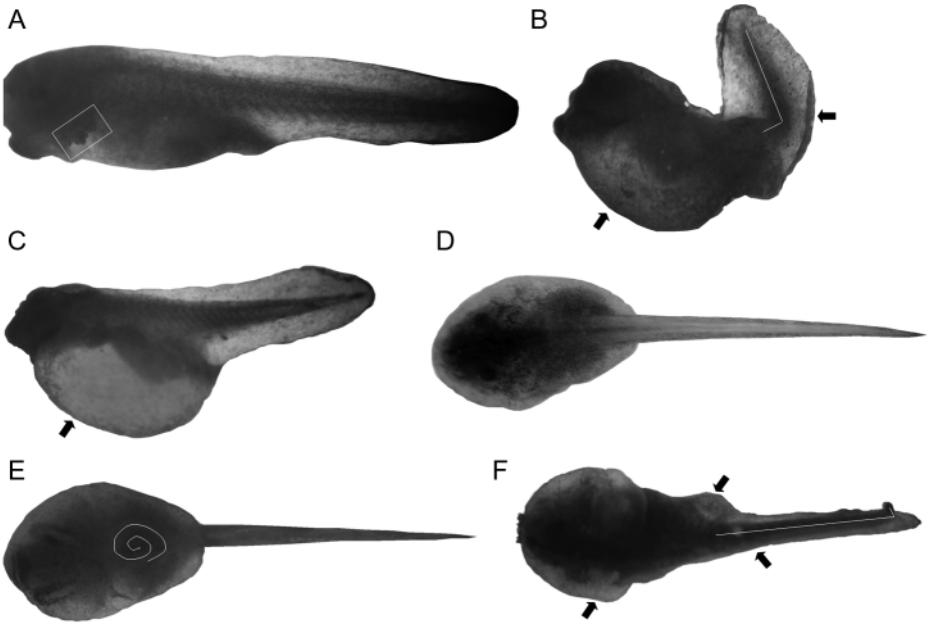

Figure 2. Malformations observed in *Rhinella arenarum* embryos exposed to 2 mg · L^{-1} AM. The arrows indicate the different morphological abnormalities observed. (A) Lateral view of a control OM embryo (length: 7 mm; × 16). Normally developed gills are evident. (B) Lateral view of a malformed OM embryo showing severe abdominal oedema along with developmental failure of the gut. (C) Lateral view of a malformed OM embryo showing severe abdominal oedema and dorsal tail flexure. (D) Dorsal view of a control CO embryo (length: 10 mm; × 4). Gills have been completely reabsorbed. (E) Ventral view of a control CO embryo. The line indicates the normal coiling of the gut. (F) Ventral view of a malformed CO embryo showing severe oedema, body blistering and decreased body size. Malformed embryos fail to develop their gut normally.

area and was often accompanied by an impairment in gut development (Figure 2C,F). Pronounced dorsal tail flexure was observed in OM embryos exposed to AM, and epidermal oedema (blistering) was also evident in CO embryos exposed to this insecticide (Figure 2F). We also observed that the external gills of OM *R. arenarum* embryos exposed to AM were atrophied when compared with control embryos and lacked from their characteristic ramifications. No significant increase in the number of malformations was found in embryos exposed to 0.5 mg · L^{-1} AM. As

Table 1. GSH-dependent enzymes and GSH levels in *Rhinella arenarum* embryos exposed to sublethal concentrations of azinphos-methyl.

Embryo stage	Treatment ($mg \cdot L^{-1}$ AM)	GST ($mIU \cdot mg\ protein^{-1}$)	GR ($mIU \cdot mg\ protein^{-1}$)	Se-GPox ($mIU \cdot mg\ protein^{-1}$)	GSH ($nmol \cdot mg\ protein^{-1}$)
TB	0	90.17 ± 6.47^{a}	2.87 ± 0.27^{a}	37.53 ± 5.48^{a}	1.27 ± 0.19^{a}
	0.5	102.01 ± 9.51	3.5 ± 0.35	38.08 ± 14.42	1.28 ± 0.02
	2	$133.80 \pm 10.9^{*}$	3.65 ± 0.98	26.53 ± 8.12	1.42 ± 0.06
OM	0	65.27 ± 1.68^{b}	2.84 ± 0.08^{a}	86.15 ± 3.10^{b}	6.55 ± 0.21^{b}
	0.5	69.42 ± 3.18	2.3 ± 0.66	66.96 ± 21.73	6.98 ± 0.46
	2	78.42 ± 0.18	4.04 ± 0.67	81.95 ± 15.68	6.40 ± 0.40
CO	0	157.42 ± 8.26^{c}	7.06 ± 0.67^{b}	98.91 ± 1.07^{c}	9.56 ± 0.84^{c}
	0.5	131.21 ± 9.87	8.01 ± 1.05	80.78 ± 6.18	8.79 ± 0.79
	2	153.30 ± 7.80	7.83 ± 1.68	104.02 ± 20.93	$7.06 \pm 0.53^{*}$

Notes: Data represent mean ± SE from six replicates. Different letters denote significant differences between control embryos of different embryonic stages ($p \leq 0.01$). Asterisks indicate significant differences between azinphos-methyl-exposed (AM) embryos and the corresponding control ($p \leq 0.05$). TB, tail bud stage; OM, open mouth stage; CO, complete operculum stage.

expected, no significant mortality resulted from continuous exposure to both sublethal concentrations of AM during *R. arenarum* embryonic development. Besides, in malformed OM and CO exposed embryos, some changes in behavioural responses could be observed, such as quietness, over-reaction to stimuli and altered locomotion.

3.2. *GSH-dependent enzymes and GSH levels*

Among GSH-dependent enzymes, TB embryos exposed to the sublethal concentration of $2\,mg \cdot L^{-1}$ AM showed a significant increase of 48% in GST activity when compared with control values. In the OM and CO stages, no significant differences were detected between control and AM-exposed embryos. Control GST activity decreased significantly during the OM stage with respect to the TB and CO stages (Table 1).

GR and Se-GPox activities showed no significant differences in embryos exposed to sublethal concentrations of AM when compared with control values (Table 1). Control GR activity was similar between the TB and OM stages, and increased significantly when embryos reached the CO stage, whereas control Se-GPox activity increased along embryonic development.

Exposure to the insecticide did not affect GSH levels in either TB or OM embryos exposed to sublethal concentrations of AM. However, GSH levels decreased significantly by 26% in CO embryos exposed to $2\,mg \cdot L^{-1}$ AM compared with control values. GSH levels increased continuously in control embryos throughout embryonic development (Table 1).

3.3. *Catalase and superoxide dismutase activity*

In TB embryos exposed to $2\,mg \cdot L^{-1}$ AM, SOD activity was significantly decreased by 39% compared with control values (Figure 3A). The effect on SOD inhibition was accentuated when embryos exposed to AM reached the OM stage. When compared with control values, SOD was inhibited by 41 and 47% in OM embryos exposed to 0.5 and $2\,mg \cdot L^{-1}$ AM, respectively. When embryos reached the end of embryonic development, SOD activity recovered control values. Control SOD activities were $\sim 33\,IU \cdot mg\ protein^{-1}$ in the TB stage, $6\,IU \cdot mg\ protein^{-1}$ in the OM stage and $4\,IU \cdot mg\ protein^{-1}$ in the CO stage. However, no significant differences were detected in CAT activity in *R. arenarum* embryos exposed to sublethal concentrations of AM during embryonic development. Control CAT activity was high and remained constant during embryonic development, averaging $110\,IU \cdot mg\ protein^{-1}$. (Figure 3B).

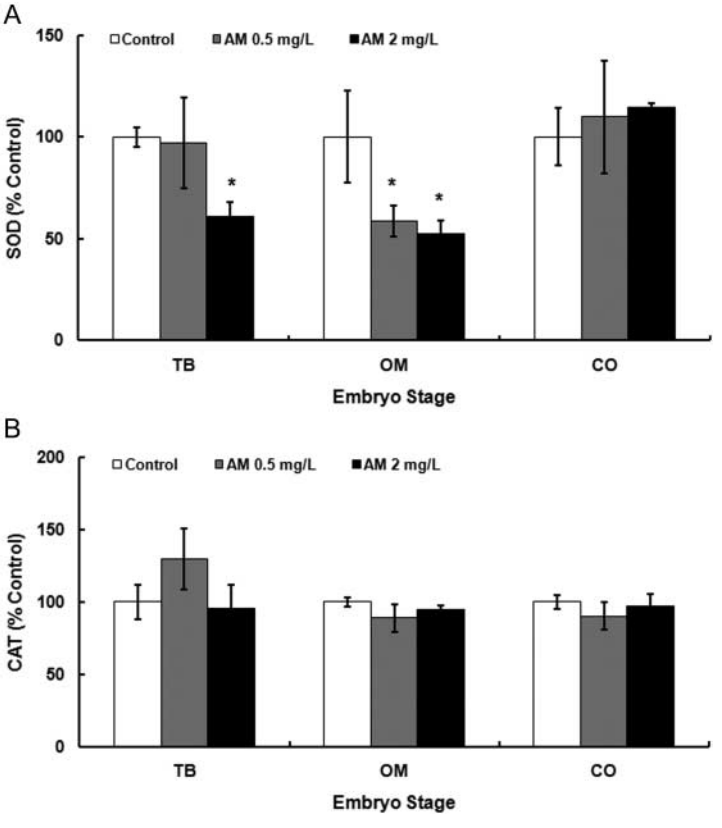

Figure 3. CAT and SOD activity in *Rhinella arenarum* embryos expressed as percentage of control activity. Embryos were exposed to 0, 0.5 and 2 mg · L^{-1} AM from fertilization until the CO stage. (A) CAT activity in *R. arenarum* embryos. (B) SOD activity in *R. arenarum* embryos. Data represent mean ± SE from six replicates. Asterisks indicate significant differences with respect to control values (*$p \leq 0.05$) determined by ANOVA and Fisher's LSD test.

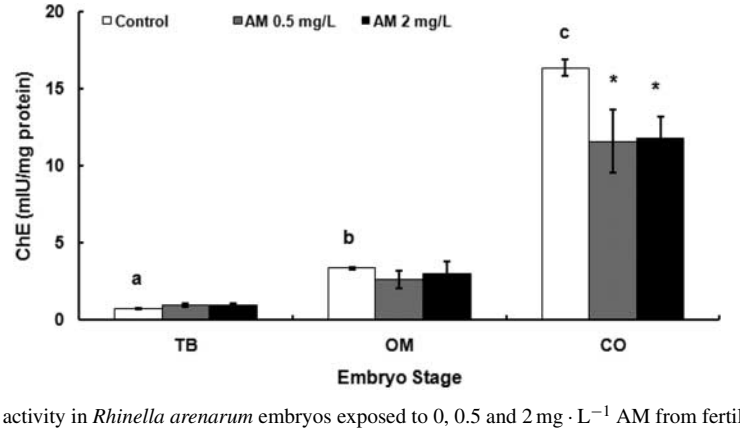

Figure 4. ChE activity in *Rhinella arenarum* embryos exposed to 0, 0.5 and 2 mg · L^{-1} AM from fertilization until the CO stage. Data represent mean ± SE from six replicates. Asterisks indicate significant differences with respect to control values (*$p \leq 0.05$) and different letters denote significant differences between control values among different embryonic stages ($p \leq 0.0001$) determined by ANOVA and Fisher's LSD test.

3.4. *Cholinesterase activity*

The primary target of organophosphorus pesticides, ChE activity, was also evaluated after AM exposure. No significant differences were observed between control embryos and those exposed to sublethal concentrations of AM, at either the TB or OM stage. However, CO embryos continuously exposed to 0.5 and 2 mg · L^{-1} AM showed a significant decrease of 29 and 27% in ChE activity, respectively (Figure 4). Control ChE activity increased significantly throughout embryonic development.

4. Discussion

It has been documented that exposure of amphibian embryos or larvae to organophosphorus pesticides may induce diverse alterations that affect normal development [11,12,33]. In this study, we observed that 2 mg · L^{-1} AM was capable of inducing morphological changes in *R. arenarum* embryos and affecting antioxidant defences and cholinesterase activity, whereas 0.5 mg · L^{-1} AM transiently altered SOD activity at the OM stage and decreased ChE activity at the CO stage. It is important to mention that the highest sublethal concentration evaluated in this study, 2 mg · L^{-1} AM, was 4.5 times lower than the concentration of 9 mg · L^{-1} AM previously reported to cause 30% mortality in *R. arenarum* embryos at the end of their embryonic development [16].

Several authors have reported the appearance of malformations in amphibian embryos and larvae exposed to anticholinesterasic pesticides, indicating a connection between teratogenesis and ChE inhibition [11–13]. In this study, despite the fact that ChE is the primary target of AM action, its activity was only significantly inhibited by sublethal concentrations of AM at the end of embryonic development (CO stage), when control ChE specific activity reached its highest value. A lack of inhibition in early stages of *R. arenarum* development due to organophosphorus exposure has been observed previously by Caballero de Castro *et al*. [34]. Although ChE inhibition in the CO stage was not associated with mortality, it could affect vital behavioural responses of tadpoles, as noted in some studies in aquatic species [35]. In amphibians exposed to organophosphorus, the relation between behavioural alterations and ChE activity has not yet been fully assessed. Nevertheless, several studies report modifications in amphibian behaviour such as impaired swimming capacity and a decrease in food consumption due to organophosphorus exposure [36,37]. It is important to mention that the significant ChE inhibition in CO *R. arenarum* embryos exposed to 2 mg · L^{-1} AM was accompanied by an increase in the number of malformed individuals. The malformations observed in individuals exposed to AM could be due to the inhibition of ChE activity which is crucial for neuromuscular development, as reported previously [11–13]. However, morphological alterations in *R. arenarum* embryos were also evident in earlier stages when ChE activity was not inhibited, suggesting that other molecular targets of organophosphorus are involved in teratogenesis.

Teratogenesis has also been associated with an increase in ROS or alteration in the antioxidant system [38,18]. Also, oxidation of DNA may be particularly critical in situations of oxidative stress and/or low antioxidant defences, leading to generalised teratogenic processes in several tissues. Both concentrations of AM tested in this study had diverse effects on the antioxidant system during *R. arenarum* embryonic development. At the TB stage, a significant increase in GST activity might represent an attempt to protect early embryos from insecticide toxicity by stimulation of pesticide detoxification. It is well known that the enzyme GST plays an important role in the detoxification of AM [39] and its expression is controlled by the transcription factor Nrf2, which binds to the antioxidant response element (ARE) present in the promoter region of the target gene [40,41]. An increase in GST activity has been suggested as an adaptive response to other pesticides in toad embryos [33]. As embryos exposed to sublethal concentrations of AM continue their development,

GST activity returns to control levels, reflecting the fact that continuous exposure to AM prevents the maintenance of the protective response established in TB embryos. Moreover, a significant decrease in GST activity at the end of *R. arenarum* development, caused by a lethal concentration of AM ($9\,mg \cdot L^{-1}$), has been reported previously [16]. In this study, GSH levels were diminished in CO embryos as a consequence of their sustained exposure to the sublethal concentration of $2\,mg \cdot L^{-1}$ AM. Similarly, GSH was depleted in CO embryos exposed to $9\,mg \cdot L^{-1}$ AM [16]. A decrease in GSH is considered a valuable biomarker of oxidative stress in fish [42], as well as amphibians [16]. Besides, the significant decrease of GSH observed in exposed CO embryos might seriously affect their antioxidant response [39]. GSH levels are low in cells that accumulate ROS, which in turn, can damage macromolecules and impair correct embryonic development [18]. Teratogens such as hydroxyurea cause oxidative stress and induce tail and limb malformations in mouse embryos [43]. In this sense, oxidative stress caused by AM in amphibian embryos could be related to caudal alterations such as those observed in this study. At lethal concentrations, anticholinesterasic insecticides caused a situation of oxidative stress in toad embryos, which correlated with the appearance of a high percentage of malformed individuals [16]. Despite the effect on GSH levels, sublethal concentrations of AM caused no significant effects on the GSH-related enzymes GR and Se-GPox in this study. Conversely, whereas GR was significantly inhibited by the lethal concentration of $9\,mg \cdot L^{-1}$ AM, Se-GPox activity was induced by AM at the CO stage [16]. Besides, different organophosphorus pesticides were reported to cause no effect as well as both induction and inhibition of these enzymes in aquatic vertebrates [14,35,42].

CAT and SOD are antioxidant enzymes also involved in the protection against oxidative injuries. In *R. arenarum* embryos, CAT maintained an elevated activity which was not affected by sublethal concentrations of AM along embryonic development. Similar results in *R. arenarum* embryos acutely exposed to the herbicide paraquat, whose mode of action is the generation of ROS, have been reported [44]. In this study, the enzyme SOD was in turn inhibited by sublethal concentrations of AM at the TB and OM stages. SOD inhibition could be due to the oxidation of –SH groups of cysteine residues of the enzyme [45] as a result of protein damage. The inhibition of SOD activity in *R. arenarum* embryos would lead to the accumulation of superoxide anion. Besides, in individuals exposed to $2\,mg \cdot L^{-1}$ AM, SOD inhibition was accompanied by a significant increase in the percentage of malformations. When embryos reached the stage of CO, the previous inhibition of SOD activity was overcome and control values recovered. Exposure of toad embryos to lethal concentrations of AM showed in turn an inhibition in SOD activity followed by its recovery at the CO stage [16]. Similarly, it has been reported that freshwater snails exposed to AM were capable of activating compensatory mechanisms to overcome oxidative stress after an initial inhibition of SOD activity [17].

In *R. arenarum* embryos exposed to sublethal concentrations of AM, morphological abnormalities were not observed at the beginning of embryonic development but were evident from intermediate (OM) to final stages (CO). Although *R. arenarum* embryos were able to survive to $2\,mg \cdot L^{-1}$ AM under laboratory conditions, the malformations observed might impair their performance in their habitat and also affect their progression to metamorphosis [46]. In this study, the ability of embryos to adopt a correct position was impaired as a result of the morphological alterations observed, such as oedema, which forced the embryos to rest either on their dorsal surface or on their sides. Under these circumstances, malformed embryos exposed to AM displaced with difficulty and moreover, severe impairment in swimming capacity was evident. In addition, the dorsal tail flexure documented in this study also contributed to embryonic swimming impairment and has also been observed in *Xenopus laevis* larvae exposed to other anticholinesterasic compounds [11–13]. This has been noted in studies performed with different species of amphibians inhabiting agricultural areas or their surroundings [47,48].

Summarising, the results indicate that antioxidant defences were altered in early *R. arenarum* embryos exposed to sublethal concentrations of AM. These effects were followed by transcendent

morphological alterations, while GSH content and ChE activity decreased as a late effect. The connection between these biochemical alterations and the appearance of malformations at sublethal concentrations of AM was not evident in *R. arenarum* embryos. However, increased GST and decreased SOD activities could be considered as early AM expositional markers.

Taken together, these data demonstrate that sublethal concentrations of AM are a serious threat for toad embryos in their natural habitats since biochemical and morphological alterations could impair their ability to deal with an environmental stress situation.

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References

[1] US Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances. *Interim Reregistration Eligibility Decision for Azinphos methyl Case No. 0235,* (2006). Available at http://www.epa.gov/pesticides/reregistration/REDs/azinphosmethyl_ired.pdf.

[2] O.L. Anguiano and A.M. Pechen de D'Angelo, *Provincia de Río Negro y Provincia de Neuquén, in La problemática de los agroquímicos y sus envases, la incidencia en la salud de los trabajadores, la población expuesta y el ambiente*, Ministerio de Salud de la Nación, 2007, organophosphorusS/OMS, Buenos Aires, pp. 181–201.

[3] D.W. Sparling, G.M. Fellers, and L.L. McConell, *Pesticides and amphibian population declines in California, USA*, Environ. Toxicol. Chem. 20 (2001), pp. 1591–1595.

[4] M.D. Boone, R.D. Semlitsch, E.E. Little, and M.C. Doyle, *Multiple stressors in amphibian communities: Effects of chemical contamination, bullfrogs, and fish*, Ecol. Appl. 17 (2007), pp. 291–301.

[5] C. Davidson and R.A. Knapp, *Multiple stressors and amphibian declines: Dual impacts of pesticides and fish on yellow-legged frogs*, Ecol. Appl. 17 (2007), pp. 587–597.

[6] M. Loewy, V. Kirs, G. Carvajal, A. Venturino, and A.M. Pechen de D'Angelo, *Groundwater contamination by azinphos methyl in the Northern Patagonic Region (Argentina)*, Sci. Total Environ. 225 (1999), pp. 211–218.

[7] A.P. Tosi, A.M. Pechen de D'Angelo, M.C. Savini, and R.M. Loewy, *Assesing pesticide hazards on surface water from the northern patagonian region Argentina*, Acta Toxicol. Arg. 17 (2009), pp. 1–7.

[8] L. Duboscq, J. Soleño, E. Rosenbaum, M. Montagna, A. Venturino, and A.M. Pechen de D'Angelo, *Larvas de Bfo arenarum como sistema de biomonitoreo en aguas provenientes de explotaciones frutícolas*, Acta Toxicol. Arg. 15 (2007), p. 44.

[9] F. Matsumura, *Toxicology of Insecticides*, 2nd ed., Plenum Press, New York, 1985.

[10] C.A. Edwards and S.W. Fisher, *The use of cholinesterase measurement in assessing the impact of pesticides on terrestrial and aquatic invertebrates,* in *Cholinesterase Inhibiting Insecticides. Their Impact on Wildlife and the Environment, Vol 2. Chemicals in Agriculture*, P. Mineau, ed., Elsevier, New York, 1991, pp. 255–275.

[11] P. Bonfanti, A. Colombo, F. Orsi, I. Nizzetto, M. Andrioletti, R. Bacchetta, P. Mantecca, U. Fascio, G. Vailati, and C. Vismara, *Comparative teratogenicity of chlorpyrifos and malathion on Xenopus laevis development*, Aquat. Toxicol. 70 (2004), pp. 189–200.

[12] A. Colombo, F. Orsi, and P. Bonfanti, *Exposure to the organophosphorus pesticide chlorpyrifos inhibits acetylcholinesterase activity and affects muscular integrity in Xenopus laevis larvae*, Chemosphere 61 (2005), pp. 1665–1671.

[13] R. Bacchetta, P. Mantecca, M. Andrioletti, C. Vismara, and G. Vailati, *Axial-skeletal defects caused by carbaryl in Xenopus laevis embryos*, Sci. Total Environ. 392 (2008), pp. 110–118.

[14] E.O. Oruç, Y. Sevgiler, and N. Uner, *Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl*, Comp. Biochem. Physiol. C 137 (2004), pp. 43–51.

[15] A. Ferrari, A. Venturino, and A.M. Pechen de D'Angelo, *Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Oncorhynchus mykiss*) detoxifying enzymes*, Pest. Biochem. Physiol. 88 (2007), pp. 134–142.

[16] A. Ferrari, C.I. Lascano, O.L. Anguiano, A.M. Pechen de D'Angelo, and A. Venturino, *Antioxidant responses to azinphos methyl and carbaryl during the embryonic development of the toad* Rhinella (Bufo) arenarum *Hensel*, Aquat. Toxicol. 93 (2009), pp. 37–44.

[17] G. Kristoff, N.R. Verrengia Guerrero, and A.C. Cochón, *Effects of azinphos-methyl exposure on enzymatic and non-enzymatic antioxidant defenses in* Biomphalaria glabrata *and* Lumbriculus variegatus, Chemosphere 72 (2008), pp. 1333–1339.

[18] P.G. Wells, G.P. McCallum, C.S. Chen, J.T. Henderson, C.J.J. Lee, J. Perstin, T.J. Preston, M.J. Wiley, and A.W. Wong, *Oxidative stress in developmental origins of disease: Teratogenesis, neurodevelopmental deficits, and cancer*, Toxicol. Sci. 108 (2009), pp. 4–18.

[19] A. Ferrari, L. Anguiano, C. Lascano, V. Sotomayor, E. Rosenbaum, and A. Venturino, *Changes in the antioxidant metabolism in the embryonic development of the common South American toad* Bufo *arenarum: Differential responses to pesticide in early embryos and autonomous-feeding larvae*, J. Biochem. Mol. Toxicol. 22 (2008), pp. 259–267.
[20] B. Halliwell and J.M.C. Gutteridge, *Free Radicals in Biology and Medicine*, 4th ed., Oxford University Press, New York, 2007.
[21] A. Ferrari, C. Lascano, A.M. Pechen de D'Angelo, and A. Venturino, *Effects of azinphos methyl and carbaryl on* Rhinella arenarum *larvae esterases and antioxidant enzymes*, Comp. Biochem. Physiol. C 153 (2011), pp. 34–39.
[22] T. Fonovich Schroeder and A.M. Pechen de D'Angelo, *Dieldrin effects on phospholipid and phosphoinositide metabolism in* Bufo *arenarum oocytes*, Comp. Biochem. Physiol. C 98 (1991), pp. 287–292.
[23] J. Soleño, *Mecanismos comunes de toxicidad de plaguicidas: Interacción entre carbaril y metilazinfos*, Graduate thesis, Universidad Nacional del Comahue, 2000.
[24] E. Del Conte and J.L. Sirlin, *Pattern series of the first embryonary stages in* Bufo arenarum, Anat. Rec. 112 (1952), pp. 125–130.
[25] J.A. Bantle, J.N. Dumont, R.A. Finch, and G. Linder, *Atlas of Abnormalities: A Guide for the Performance of FETAX*, Oklahoma State Publications, Stillwater, 1991.
[26] G.L. Ellman, K.D. Courtney, V. Andres Jr, and R.M. Featherstone, *A new and rapid colorimetric determination of acetylcholinesterase activity*, Biochem. Pharmacol. 7 (1961), pp. 88–95.
[27] W.H. Habig, M.J. Pabst, and W.B. Jacoby, *Glutathione* S*-transferases. The first enzymatic step in mercapturic acid formation*, J. Biol. Chem. 249 (1974), pp. 7130–7139.
[28] M. Schaedle and J.A. Bassham, *Chloroplast glutathione reductase*, Plant Physiol. 59 (1977), pp. 1011–1012.
[29] C. Di Ilio, G. Polidoro, A. Arduini, A. Muccini, and G. Federici, *Glutathione peroxidase, glutathione reductase, glutathione* S*-transferase and* γ*-glutamyl transpeptidase activities in human early pregnancy placenta*, Biochem. Med. 29 (1983), pp. 143–148.
[30] R.F. Beers and I.W. Sizer, *A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase*, J. Biol. Chem. 195 (1952), pp. 133–140.
[31] F. Paoletti, D. Aldinucci, A. Mocali, and A. Caparrini, *A sensitive spectrophotometric method for determination of superoxide dismutase activity in tissue extract*, Anal. Biochem. 154 (1985), pp. 536–541.
[32] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *Protein measurement with the Folin phenol reagent*, J. Biol. Chem. 193 (1951), pp. 265–275.
[33] O.L. Anguiano, A. Caballero Castro, and A.M. Pechen de D'Angelo, *The role of glutathione conjugation in the regulation of early toad embryos' tolerance to pesticides*, Comp. Biochem. Physiol. C 128 (2001), pp. 35–43.
[34] A. Caballero Castro, E.A. Rosenbaum, and A.M. Pechen de D'Angelo, *Effect of malathion on* Bufo arenarum *Hensel development – I. Esterase inhibition and recovery*, Biochem. Pharmacol. 41 (1991), pp. 491–495.
[35] P. Kavitha and J. Venkateswara Rao, *Oxidative stress and locomotor behaviour response as biomarkers for assessing recovery status of mosquito fish,* Gambusia affinis *after lethal effect of an organophosphate pesticide, monocrotophos*, Pestic. Biochem. Physiol. 87 (2007), pp. 182–188.
[36] H.P. Gurushankara, S.V. Krishnamurthy, and V. Vasudev, *Effect of malathion on survival, growth, and food consumption of Indian cricket frog (*Limnonectus limnocharis*) tadpoles*, Arch. Environ. Contam. Toxicol. 52 (2007), pp. 251–256.
[37] M.R. Wijesinghe, M.G. Bandara, W.D. Ratnasooriya, and G.P. Lakraj, *Chlorpyrifos-induced toxicity in* Duttaphrynus melanostictus *(Schneider 1799) larvae*, Arch. Environ. Contam. Toxicol. 17 (2010), pp. 1411–1421.
[38] P.A. Dennery, *Effects of oxidative stress on embryonic development*, Birth Defects Res. C Embryo Today 81 (2007), pp. 155–162.
[39] M. Jokanović, *Biotransformation of organophosphorus compounds*, Toxicology 166 (2001), pp. 139–160.
[40] A.K. Jaiswal, *Nrf2 signalling in coordinated activation of antioxidant gene expression*, Free Radical Biol. Med. 36 (2004), pp. 1199–1207.
[41] C.J. Harvey, R.K. Thimmulappa, A. Singh, D.J. Blake, G. Ling, N. Wakabayashi, J. Fujii, A. Myers, and S. Biswal, *Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress*, Free Radical Biol. Med. 46 (2009), pp. 443–453.
[42] S. Peña-Lopis, M.D. Ferrando, and J.B. Peña, *Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by N-acetylcysteine*, Aquat. Toxicol. 65 (2003), pp. 337–360.
[43] A.E. Schlisser, J. Yan, and B.F. Hales, *Teratogen-induced oxidative stress targets glyceraldehyde-3-phosphate dehydrogenase in the organogenesis stage mouse embryo*, Toxicol. Sci. 118(2) (2010), pp. 686–695.
[44] M.A. Mussi and N.B. Calcaterra, *Paraquat-induced oxidative stress response during amphibian early embryonic development*, Comp. Biochem. Physiol. C 151 (2010), pp. 240–247.
[45] M.S.T. Dimitrova, V. Tsinova, and V. Velcheva, *Combined effect of zinc and lead on the hepatic superoxide dismutase-catalase system in carp (*Cyprinus carpio*)*, Comp. Biochem. Physiol. C 108 (1994), pp. 43–46.
[46] D.W. Sparling and G.M. Fellers, *Toxicity of two insecticides to California, USA, anurans and its relevance to declining amphibian populations*, Environ. Toxicol. Chem. 28 (2009), pp. 1696–1703.
[47] M.L. Harris, C.A. Bishop, J. Struger, B. Ripley, and J.P. Bogart, *The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II. Effects of pesticides and eutrophic conditions on early life stage development*, Environ. Toxicol. Chem. 17 (1998), pp. 1351–1363.
[48] P.A. Spear, M. Boily, I. Giroux, C. Deblois, M.H. Leclair, M. Levasseur, and R. Leclair, *Study design, water quality, morphometrics and age of the bullfrog,* Rana catesbeiana*, in sub-watersheds of the Yamaska River drainage basin, Québec, Canada*, Aquat. Toxicol. 91 (2009), pp. 110–117.