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Living organisms influence on environmental conditions: pH modulation by amphibian embryos versus aluminum toxicity

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HIGHLIGHTS

- Aluminum toxicity is more related to pH than the metal concentration.
- Amphibian embryos reduce Al toxicity by modifying the pH in the maintaining media.
- Natural selection and resilience are illustrated in one experiment.
- Ecotox should evaluate toxicity and resilience simultaneously.
- Ontogenic stages provide the opportunity to explore ancient environmental conditions.

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ABSTRACT

The LC10, 50 and 90/24 h of aluminum for *Rhinella arenarum* embryos at complete operculum stage were 0.55, 0.75 and 1 mgAl^{3+}/L respectively. Those values did not change significantly by expanding the exposure period till 168 h. The aluminum toxicity was evaluated in different pH conditions by means of a citrate buffer resulting for instance, 1 mgAl^{3+}/L at pH 4, 4.1, 5 and 6 in 100%, 70%, 35% and 0% of lethality respectively. As an outstanding feature, the embryos changed the pH of the maintaining media both in the case of Al^{3+} or citrate buffer treatments toward neutral. 10 embryos in 40 mL of AMPHITOX solution were able to increase the pH from 4.2 to 7.05, a fact related with a metabolic shift resulting in an increase in nitrogen loss as ammonia. Our study point out the natural selection of the most resistant amphibian embryos both for pH or aluminum as well as the capacity of living organisms (as a population) to alter their chemical environment toward optimal conditions for their survival. As these facts occur at early life stages, it expand the concept that living organisms at ontogenic stages are biomarker of environmental signatures of the evolutionary process (Herkovits, 2006) to a global Onto-Evo concept which imply also the feedback mechanisms from living organisms to shape environmental conditions in a way that benefits them.

1. Introduction

The worldwide decline of amphibian populations (Blaustein and Wake, 1990; Mackey and Boone, 2009) results as a junction of many factors in a complex scenario. In the American continent, Young et al. (2004) reported that the main causes affecting amphibians were: habitat loss (89% of all threatened species); chytrid fungal disease (47% of all critically endangered amphibians) and environmental pollutants (26% of species). Ecotoxicological studies conducted by standardized protocols, FETAX (Dumont et al., 1983) and AMPHITOX (Herkovits and Pérez-Coll, 2003), as well as, bioassays focused on stage dependent susceptibility (Herkovits et al., 1997a,b; Castañaga et al., 2009; Aronzon et al., 2011) point out the high susceptibility of amphibians to noxious agents at certain early-life stages. In line with this fact, water quality-based toxicity of different environmental scenarios conducted with amphibian embryos was reported in rivers and streams (Herkovits et al., 1996; Linder, 2003; Griffis-Kyle and Ritchie, 2007).

Aluminum, the most abundant metal in Earth's crust is mostly present in nature combined with other elements. Its LC50 reported for *Rhinella arenarum* was 0.3 mg/L at 96 h and it is noteworthy that its toxicity does not increased by expanding the exposure time till 168 h (Herkovits et al., 1997a,b). Al^{3+} solubility is highly pH-dependent and therefore, its concentration as a free ion in fresh waters is lower than 0.1 mgAl/L in the 95% of surface water with pH values above 5.5 (Sorenson et al., 1974; Filipek et al., 1987). Under acidic or alkaline conditions or in the presence of appropriate ligands, soluble species are formed, but in the range of circumneutral pH values Al^{3+} is generally insoluble (Suwalsky et al.,

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2002). Thus, as the solubility of aluminum increases as water pH drops, the events of Al-induced negative effects in natural environments are related to acidification of water bodies (Blaustein et al., 2003). Acidic environments were reported for a large number of places both natural and associated to anthropogenic inputs (Henriksen, 1979; Wells, 2007), resulting in amphibian embryos mortality (Clark and La Zerte, 1985). The influence of pH on the toxicity of aluminum was also reported for other aquatic organisms such as zebra fish (Dave, 1985) and rainbow trout (Gundersen et al., 1994). In amphibian tadpoles, changes in water pH were associated with developmental delays, malformations and erratic swim which can result in mortality (Muñoz and Bautista, 2011). At water pH levels lower than 5.0, hatching is sometimes inhibited, but at pH levels below 4.5 hatching is completely abolished in *Bufo americanus* (Tattersall and Wright, 1996) as well as in *L. peronii* embryos. More extreme acidic conditions with pH values below 4 were associated to lethality (Pierce, 1985; Herkovits et al., 2001). In this contribution the dose–response of pH and aluminum toxicity will be reported.

Global environmental conditions can be altered both, from abiotic and biotic inputs (Herkovits, 2006). The biota has a significant effect on Earth's environment, e.g. the rise of free oxygen during the evolutionary process (Anbar and Knoll, 2002) and oxygen production and consumption from that time onwards. According to the Gaia hypothesis the whole ecosystem can be seen as a giant organism in which life tend to optimizes physical and chemical environment to best fit their needs (Lovelock, 1986; Lenton, 1998), while Kirchner (2002), considering natural selection as a well documented theory, point out that claims that life alters the environment to its benefit are fundamentally misleading. In the case of acidic conditions produced by glyphosate, it was observed that the amphibian embryo modulated the maintaining media toward neutral pH which imply a reduction in the herbicide toxicity (Piazuelo et al., 2011). Thus it seems meaningful to evaluate the embryo-pH interaction in the case of aluminum toxicity as a contribution from an ecotoxicological perspective for these global conjectures. The main aim of this study is to report Al^{3+} toxicity in the embryo of *R. arenarum*, a South American toad, the role of pH on Al toxicity and to assess the potential of amphibian embryos to alter pH toward less harmful condition. The results will be considered from the Gaia hypothesis within an Onto-Evo perspective.

2. Materials and methods

2.1. Test organisms

R. arenarum adults weighting 200–250 g were collected in the surroundings of Lobos, (S35°11′10.15″, W59°12′09.02″) province of Buenos Aires and handled in accordance with institutional guidelines of animal welfare. Ovulation was induced by means of an intra-peritoneal injection of a macerated homologous hypophysis. Oocytes were fertilized *in vitro* with a sperm suspension in Amphitox Solution (AS) (Herkovits and Perez-Coll, 1999). Jelly coats were removed by a 2% solution of thyoglicolic acid in AS neutralized with NaOH. The development of embryos was staged according to the table of Del Conte and Sirlin (1951). Test organisms were maintained in AS at 20 ± 2 °C up till complete operculum stage (S.25).

2.2. Test solutions

A stock solution of 10 $mgAl^{3+}/L/L(AlCl_3{\cdot}6H_2O$, Mallinckrodt, USA) *was* diluted in AS to nominal concentrations of: 0.1, 0.25, 0.3, 0.4, 0.5, 0.55, 0.70, 0.85, 1, 1.15, 1.30 and 1.45 mgAl/L. Samples from each nominal concentration were analyzed and confirmed (within a range of 7%) by Buck Graphite Furnace Atomic Absorption Spectroscopy (GF/AAS). For controls embryos were maintained in AS. The experiments conducted to study Al toxicity at different pH values were prepared with citrate buffer. Stock solutions of citric acid 0.1 M (Biopack, Argentina) and sodium citrate 0.1 M (Anedra, Argentina) were mixed in different proportions to obtain buffer solutions resulting in pH values: 4, 5 and 6. Toxic effects of citrate buffer dilutions: 0.1%, 1%, 10% and 100% v/v, as well as their buffer capacity were tested to assess optimal conditions for the maintenance of *R. arenarum* embryos. As 1% v/v dilution of citrate buffer resulted in no adverse effects while the buffer capacity was still in place, this condition was selected for the experiments assessing Al – pH dependent toxicity.

2.3. Exposure conditions

Bioassays were conducted with *R. arenarum* embryos at S.25 (complete operculum) following the AMPHITOX protocol (Herkovits and Pérez-Coll, 1999, 2003), room temperature was controlled at 20 ± 2 °C with a 12-h light/12-h dark cycle. To evaluate Al^{3+} toxicity, a 168 h exposure period with semi-static flow with test solution renewal each 24 h was conducted. Groups of 10 embryos by triplicate were placed in glass 10 cm diameter, covered Petri dishes containing 40 ml of AS (as a control condition) or AS with Al^{3+} from 0.1 to 1.45 mg/L. Lethality was evaluated daily up to the end of the experimental period, tests were validated when the survival of control organisms resulted equal or above 85%. For the case of the experiments conducted with different pH conditions, a 24 h exposure period was employed, taking into account previous results, in which the evaluation of Al-induced lethal effects over amphibian embryos did not suffer significant alterations from 24 to 168 h. Test organisms were maintained, under the same exposure conditions (test chamber and volume of the test solution), in citrate buffer adjusted to: 4 (4.12), 5 (4.85) and 6 (5.97) nominal and (determined) pH values respectively, which were registered with an Extech pH220 portable pH meter. Al (nominal) concentrations tested in AS were: 0.3, 0.5 and 1 $mgAl^{3+}/L$; these Al concentrations were also diluted in citrate buffer maintaining the pH values referred above. To evaluate *R. arenarum* embryos impact on the pH in the maintaining media, 10 organisms were exposed to 0.3, 0.5 and 1 $mgAl^{3+}/L$ in AS (with their spontaneous pH values) and to the same concentrations of the metal with citrate buffer solutions adjusted to pH values: 4, 5 and 6. Lethality and pH values of the maintaining media were registered each 24 h up to 96 h.

2.4. Data analysis

Survival data obtained from Al exposure with *R. arenarum* embryos was analyzed by means of EPA Probit program (version 1.5 EPA, 1991). To test for statistical significance differences between the LC_{50} values among different exposure times, the division between the major LC_{50} and the minor LC_{50} exceeds a critic value established by the American Public Health Association (APHA, 1980). Normal distribution of the data was studied by means of Kolmogorov–Smirnov test; significant differences between experimental groups were studied by ANOVA one-way test and in the case of percentage data, by means of Chi-Square analysis. All the analyses were performed with SYSTAT 8.0 statistical package (SPSS Inc, Microsoft Co, WA, USA).

3. Results

Aluminum toxicity for *R. arenarum* embryos resulted in a LC_{50} 24 h value of 0.754 $mgAl^{3+}/L$ [95% Confidence interval (CI) 0.708–

0.798 mgAl^{3+}/L], diminished to 0.709 mgAl^{3+}/L (CI 0.669–0.747 mgAl^{3+}/L) at 96 h, and to 0.695 mgAl^{3+}/L (CI 0.654–0.734) at 168 h, which represent a slight increment (7.8%) in the toxicity of the metal during the exposure period (Fig. 1). In average, an increase in 0.35 mgAl^{3+}/L in the maintaining media resulted in a increase in lethality from LC$_{10}$ to LC$_{90}$.

As a general pattern Al^{3+} toxicity is directly related to the pH in the maintaining media. *R. arenarum* embryos response to 0.3 mgAl^{3+}/L exposure at different pH values can be observed in Fig. 2. The results show that only 5% of the organisms survived after a 24 h in the case of pH 4.2. Conversely, 0.3 mgAl^{3+}/L with a pH 4.8 or higher, did not produce lethality. The exposure to 0.5 mg Al^{3+}/L at different pH values, resulted in: 0% survival at pH 4.05; 80% at pH 4.4, 95% at pH 4.6 and a 100% at pH 5.95 (Fig. 2). The most illustrative dose response relationship between pH and Al^{3+} is depicted Fig. 2.

1 mgAl^{3+}/L at pH 3.9 resulted in a 0% of survival; at pH 4.05 in 30%; at pH 4.43 in 65% and at pH 5.6 in 100%. Thus, although 1 mgAl^{3+}/L is a LC$_{90}$ 24 h for *R. arenarum* embryos (Fig. 1), its toxicity can be modulated from 0% to 100% of survival by means of pH changes in the maintaining media. The effect of *R. arenarum* embryos on pH in the AS maintaining media plus citrate buffer is reported in Fig. 3. Although the pH of solutions with citrate buffer in AS did not change significantly from 0 to 96 h, with embryos it changed within 96 h from an initial 4.12, 4.85 and 5.97 to 7.05, 7.25 and 7.5 respectively. The effect of *R. arenarum* embryos on pH in the AS maintaining media plus Al^{3+} in different concentration is depicted in Fig. 3. Embryos exposed to 1 mgAl$^{3+/}$L died and no change in the pH was registered. The embryos exposed to 0.3 mgAl$^{3+/}$L and 0.5 mgAl$^{3+/}$L survived and the pH changed from initial 4.41 and 4 to 6.35 and 5.5 respectively.

4. Discussion

4.1. The role of pH in aluminum toxicity

The results obtained in this study with *R. arenarum* embryos confirm their high sensibility to aluminum (Herkovits et al., 1997a,b) with a CL50 around 0.7 mgAl^{3+}/L from 24 till 168 h of exposure. The fact that by expanding the exposure period did not result in enhanced toxicity (Fig. 1) could be related to a maximal intake of Al within the initial 24 h of exposure and/or the constant susceptibility to this noxious agents at the last embryonic stage of *R. arenarum* as it was reported for other metals like Cu (Herkovits and Helguero, 1998). Our results are in line with those reported for the American toad embryos (*B. americanus*) with a LC$_{50}$ 96 h value of 0.627 mgAl^{3+}/L and the leopard frog (*Rana pipiens*), with a LC$_{50}$ 96 h values of 0.811 and 0.403 mgAl^{3+}/L, depending pH values (Freda and McDonald, 1990) and with general data reported by EPA for aluminum sensitivity in amphibian embryos (EPA, 1996). The mechanism for this toxic effect in fish was related to interference with ionic and osmotic balance as well as the coagulation of mucus on the gills (Förstner and Wittmann, 1981; Lacroix et al., 1993; Barabasz et al., 2002).

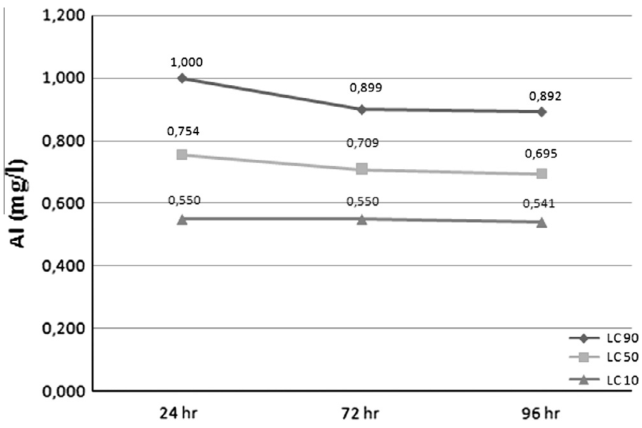

Fig. 1. Aluminum toxicity profile (LC10, LC50 and LC90) for *Rhinella arenarum* embryos evaluated at 24, 96 and 168 h of exposure.

Our study confirms that aluminum toxicity is pH dependent also in the case of *R. arenarum* as it was reported for eggs, larvae, and postlarvae stages of white suckers (Catostomus commersoni), brook trout (Salvelinus fontinalis) (Baker and Schofield, 1982). Conversely, Göran (1985) reported that in the case of zebra fish (*Brachydanio rerio*) high pH conditions resulted more toxic. Aluminum in acidified waters is particularly harmful also for invertebrates such as snails, bivalves and crustaceans because it replaces calcium cation in their bodies. In fish, aluminum accumulates in gills which cause the blockade of ion exchange and respiration, while in frogs, reproductive processes are disturbed (Barabasz et al., 2002). This fact is related to the speciation and hence the solubility and bio-availability of this metal (Suwalsky et al., 2002). Although it was reported that aluminum is generally more toxic over the pH range of 4.4–5.4 (EPA, 1996), our study point out that at least in the case of the amphibian embryos, the maximal toxicity produced by Al is around pH4 (100% of lethality). Acidic conditions as a very relevant parameter for synergy with Al is documented in this study by employing citrate buffer resulting in a dose response relationship from sublethal to 100% of lethality. Considering that environmental monitoring reports of Al concentrations in surface water under acidification processes range from 1.09 to 53.96 mgAl/L. Keller et al. (2003) and Álvarez et al. (1993), ecotox data obtained with amphibians in early life stages are relevant for habitat conditions surveys as they reflect the synergy of pH and Al toxicity.

4.2. The incidence of amphibian embryos in the pH of their media

The exposure of *R. arenarum* embryos to a wide range of low pH conditions by means of aluminum treatments or citrate buffer resulted in a gradual but sharp increase in the pH in the maintaining media toward neutral condition. This is a remarkable incidence of the embryos in their media, beneficial for embryo survival. In our laboratory it was found a similar result in front of glyphosate which toxicity diminishes also significantly in neutral pH conditions (Piazuelo et al., 2011). A different line of evidence that amphibian larvae could increase the pH in their media was provided by Barth and Wilson (2010); in physiological studies they had to acidify the maintaining media every 4 h in order to evaluate the performance of amphibian larvae at constant pH condition. The capacity of amphibian embryo to increase the pH in their media seems to be directly related with an increase in nitrogen loss as ammonia with no compensatory decrease in urea excretion as it was reported in the case of *B. americanus* embryos exposed to moderately acid waters (Tattersall and Wright, 1996). The authors reported that ammonia loss was strictly related to low pH in the maintaining media. This feature seems to be common for other aquatic vertebrate species. The removal of the acid gill water boundary layer, either by increasing the buffering capacity of the water or by reducing CO$_2$ excretion, decreases ammonia transfer in the isolated head of rainbow trout (Randall and Wright, 1987). Conversely, Tilapia (*Oreochromisalcalicus grahami*) in Lake Magadi, Kenya, at a water pH of 10, excrete nitrogenous wastes as urea, avoiding by this means to increase alkalinity by ammonia excretion in those alkaline conditions (Walsh et al., 2001). The amphibian embryos seem to adapt their metabolism in order to modify environmental pH toward neutral which reduce or even

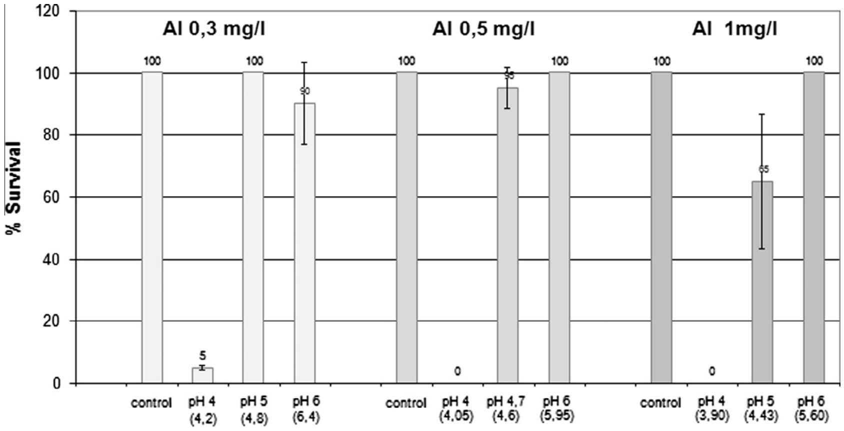

Fig. 2. Survival of *Rhinella arenarum* embryos after 24 h of exposure to 0.3, 0.4 and 0.5 $mgAl^{3+}/L$ at different pH values in citrate buffer.

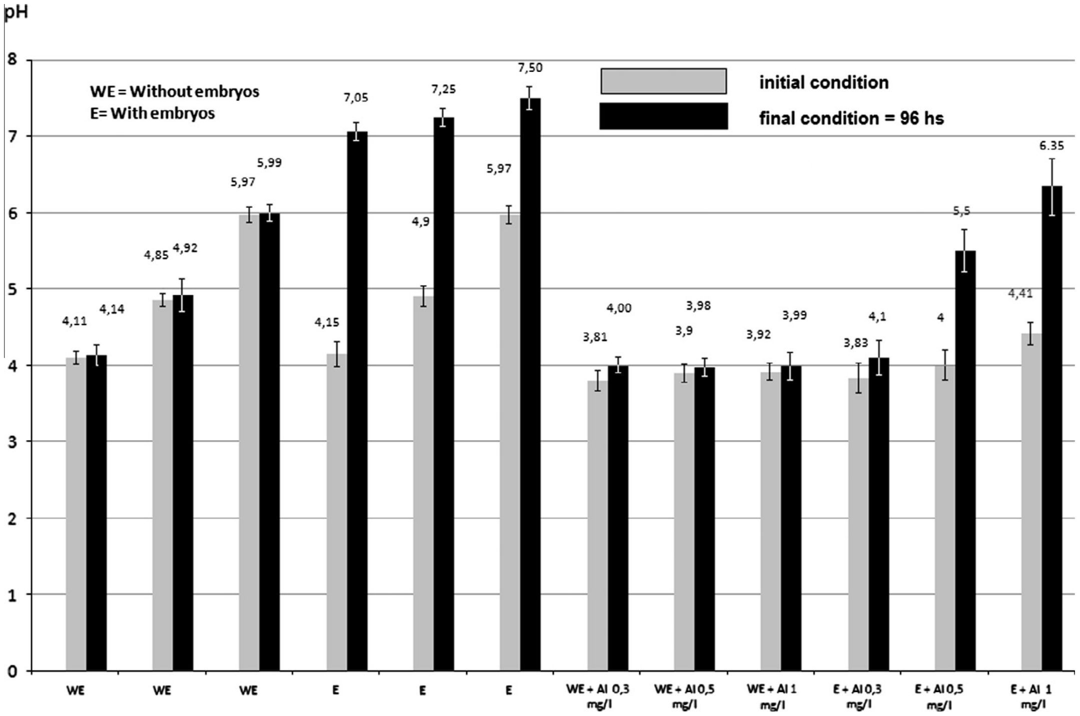

Fig. 3. The effect of *Rhinella arenarum* embryos on different pH maintained with citrate buffer or 0.3, 0.5 and 1 $mgAl^{3+}/L$ after 96 h of exposure.

neutralize aluminum toxicity. Thus, evidence from different studies, including our results, contributes to understand the complexity of the interactions between living organisms and environmental stress produced by chemical agents.

Resilience was associated to the capacity for an ecosystem to absorb disturbances and/or to return to equilibrium (Holling, 1973; Gunderson, 2000). Our results provide the opportunity to evidence the organisms' activity to build environmental conditions for their benefit. Thus, surface water under acidification processes will recover as soon as the balances of living organisms' activity will surplice the input of acidic compounds in those environments. As a whole, our results support the Gaia hypothesis which states that the environmental conditions have somehow been adjusted to the needs of living organisms, contributing to regulate their planetary environment (Lovelock and Margulis, 1974; Lenton, 1998) as well as the well documented fact that dominant organisms will be best suited to the local environment, in this case, the obvious natural selection is the survival of the more resistant embryos to low pH and/or aluminum toxicity. It is noteworthy that even the less resistant embryos which eventually die due to the low pH condition and/or the Al toxicity, contributed for the surviving of the best suited to this particular adverse condition. In fact, before dying they actively participated elevating the pH in the maintaining media.

Considering that the pH of the solution was maintained by the embryos around neutral values, the eventual inconsistency in the Gaia hypothesis raised by Kirchner (2002) based on the assumption that the same organisms cannot be involved in altering as well as stabilizing their environments, seems not to be endorsed. During the last years have emerged several model systems for addressing the interconnectedness between an organism's environment, its development responses, and its ecological interactions in natural populations (e.g. Ledon-Retting and Pfenning, 2011). Based on our results, resilience is a byproduct of a continuous activity by living organisms toward optimal environmental conditions, eventually registered as the capacity to absorb disturbances and/or to return to equilibrium.

4.3. The evolutionary relevance of environmental pH regulation capacity by embryos

Could the remarkable embryo's-mediated pH modulation in the maintaining media reflect environmental features during the evolutionary process? Considering ontogenic features such as stage-dependent biochemical or physiological features (Sivori et al., 1986; Castane et al., 1987) and stage-dependent susceptibility to noxious agents (Pérez-Coll and Herkovits, 1990; Herkovits et al., 1996) as biomarkers of the coevolution between living forms ancestors and environmental signatures during Earth's history, a new Onto-Evo synthesis was provided (Herkovits, 2006), confirmed by subsequent studies focusing on stage dependent susceptibility both to physical (Castañaga et al., 2009) and chemical agents (Sztrum et al., 2011). The theory includes a new datation method, e.g. the shift from anaerobic to aerobic metabolisms during early embryonic stages, allowed us to anticipate that multicellular organisms flourished over 2 billion years ago (Herkovits, 2006); they were discovered in 2010 (El Albani et al., 2010). The rise of O_2 in the water and atmosphere initiated by photosynthetic cyanobacteria about 2.4 billion years ago represents an example of the magnitude of the impact of living forms in the Earth environmental features. In this context, the remarkable capacity of amphibian embryos to neutralize acidic environmental condition produced by aluminum and citrate buffer reported in this paper as well as in front of other agents like glyphosate (Piazuelo et al., 2011), point out that this phenomena is not specific for a certain chemical but to pH itself. As acidic conditions were documented in ancient environmental scenarios (Knoll et al., 1996), the capacity of amphibian embryos to neutralize acidic pH could be considered as a biomarker of ancestral organisms actively adjusting environmental condition to their needs. The Onto-Evo hypothesis expands evoecotoxicology (Herkovits, 2006) to include also the possibility to study the incidence of living organisms on ancestral environmental conditions. It could contribute to a better understanding of the coevolution of living organisms and their environment, providing the possibility to obtain experimental data on the participation of individual species or a set of species in the buildup of environmental condition that benefit life. As a whole, based on the Onto-Evo synthesis, our study provide some evidence that pH 4 probably was the lower pH condition in the habitat of this South American amphibian ancestors, it is the limit for embryo survival, their capacity to modify environmental pH and thus their lower limit within the resilience phenomena. Our results point out that beside internal mechanisms of defense against toxicity, living organisms could contribute to modify environmental pH toward their benefit.

5. Conclusions

The results of this study allow us to conclude: (i) the high toxicity of aluminum to amphibian embryos is more related to pH than the metal concentration in the aquatic media; (ii) the isotoxicity curves provide the range of aluminum concentrations exerting adverse effects from acute to chronic exposure conditions; (iii) the remarkable capacity of amphibian embryos to modify the pH in their maintaining media, reducing by this means aluminum or pH toxicity, point out that toxicity studies should evaluate not only the adverse effects of noxious agents on living organisms but also the capacity of living organisms to modify environmental condition; (iv) the study, in one experiment, highlight the natural selection process affecting individuals and the capacity of a population to adjust environmental condition in their benefit; (v) resilience is the manifestation of a constant incidence of living organisms on their environment which became visible in front of major perturbations, in this study, acidic pH and/or aluminum toxicity; (vi) the fact that living organisms from at least late embryonic stages exhibit capacity to modify the environment on their benefit point out from an Onto-Evo perspective that this capacity was in place in living organisms ancestors; (vii) our study support the Gaia theory providing a new avenue to explore it experimentally.

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