Stage-Dependent Toxicity of Bisphenol A on *Rhinella arenarum* (Anura, Bufonidae) Embryos and Larvae

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ABSTRACT: The acute and chronic toxicity of bisphenol A (BPA) was evaluated on the common South American toad *Rhinella arenarum* embryos and larvae by means of continuous and pulse exposure treatments. Embryos were treated continuously from early blastula (S.4) up to complete operculum (S.25), during early larval stages and by means of 24 h pulse exposures of BPA in concentrations ranging between 1.25 and 40 mg L^{-1}, in order to evaluate the susceptibility to this compound in different developmental stages. For lethal effects, S.25 was the most sensitive and gastrula was the most resistant to BPA. The Teratogenic Index for neurula, the most sensitive embryonic stage for sublethal effects was 4.7. The main morphological alterations during early stages were: delayed or arrested development, reduced body size, persistent yolk plug, microcephaly, axial/tail flexures, edemas, blisters, waving fin, underdeveloped gills, mouth malformations, and cellular dissociation. BPA caused a remarkable narcotic effect from gill circulation stage (S.20) onwards in all the organisms exposed after 3 h of treatment with 10 mg L^{-1} BPA. After recovering, the embryos exhibited scarce response to stimuli, erratic or circular swimming, and spasmodic contractions from 5 mg L^{-1} onwards. Our results highlight the lethal and sublethal effects of BPA on *R. arenarum* embryos and larvae, in the last case both at structural and functional levels.
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Keywords: bisphenol A; amphibian embryos; amphibian larvae; stage-dependent toxicity; teratogenesis; neurotoxicity; narcosis; evoecotoxicology; evolution

INTRODUCTION

Bisphenol A (BPA, 2, 2-bis (4-hydroxyphenyl) propane, CAS Registry No. 80-05-7) is an organic compound mainly used in polymer applications as monomer of polycarbonate plastic and epoxy resins. BPA is also used in nonpolymers as an additive for a number of purposes such as brake fluids, thermal paper, and as flame retardant in the form of tetrabromobisphenol A (TBBPA) (Kitamura et al., 2005). Both diffuse sources (products in use, rest, and waste products) and point sources (accidental spills, industrial wastewater discharges) may contribute to the emission of BPA to the environment. Releases of BPA to the environment exceed one million pounds per year (EPA, 2010).

Rivers, lakes, and estuaries are major sinks for BPA and wildlife exposure may occur via water (Euling and Sonawane, 2005). BPA was reported in different environmental

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scenarios like water (surface and underground), sediments, and landfill leachate in concentrations between 0.01 μg L^{-1} and 17 mg L^{-1} (González-Casado et al., 1998; Crain et al., 2007; Klecka et al., 2009; Staples et al., 2011). The BPA acute toxicity (LC50/EC50) recorded for some aquatic organisms ranges between 1 and 10 mg L^{-1}, whereas for chronic exposure, the NOEC values range from 0.1 to 1 mg L^{-1} (MVW, 2001). BPA is degraded primarily by bacteria (Kang and Kondo, 2002) and has a half-life of 4.5–4.7 days depending on the medium of release (Cousins et al., 2002). However, the biodegradation of BPA under anaerobic conditions is limited. This leads to concern about the BPA accumulation in anaerobic sediments such as estuaries, because of its physicochemical characteristics as moderate hydrophobicity and low-bioconcentration factor (Euling and Sonawane, 2005). BPA is relevant for public health because humans are daily exposed to it. This exposure occurs primarily via hydrolysis of polycarbonate plastics and epoxy resins, resulting in low-concentrations of free BPA in food and liquids (Biles et al., 1997).

Reproductive and developmental effects for BPA were reported for 16 species from seven animal taxa: gastropods, crustaceans, insects, amphibians, fish, birds, and mammals (Euling and Sonawane, 2005). Among sublethal effects, previous studies in mice reported that BPA causes abnormalities in oocytes (Hunt et al., 2003), affects reproductive tissue morphogenesis (Markey et al., 2005), and alters behavior (Vom Saal et al., 1998). In amphibians, BPA induces feminization in *Xenopus laevis* tadpoles (Levy et al., 2004), causes microcephaly (Sone et al., 2004), induces apoptosis in central neural cells (Oka et al., 2003) and interferes metamorphosis by disrupting TH-signaling pathways even at very low-concentrations of TBBPA (2.3 μg L^{-1}) (Kitamura et al., 2005).

The decline and extinction of amphibians is a major concern for biodiversity protection worldwide already alerted since the 1960's (Simms, 1969). Frogs and toads are more susceptible than most vertebrates to physicochemical agents because their eggs are not protected by a semi impervious shell, and hence, are readily exposed to pollutants. Early life stages involve very complex cellular differentiation and morphogenetic processes, and are usually more susceptible to noxious agents than adult organisms. *Rhinella arenarum* is native anuran specie from South America found from the coastal southern Brazil and from the east Bolivia to the south of Argentina (Gallardo, 1964). Their high sensitivity to a wide diversity of environmental contaminants mainly at embryonic and larval stages, make them good indicators of environmental quality. For this reason, they are widely used in hazard assessment and ecotoxicological studies (Herkovits and Pérez-Coll, 2003). The main aim of this study was to assess the acute and chronic toxicity of bisphenol A on *Rhinella arenarum* embryos and larvae and the stage-dependent susceptibility to this compound. Among sublethal effects, we report BPA neurotoxicity and its impact on cell differentiation and morphogenesis, observed by means of optical and electron microscopy.

MATERIALS AND METHODS

Obtention of *Rhinella arenarum* Embryos and Larvae

R. arenarum adult females weighing around 200–250 g were obtained in Lobos (Buenos Aires province, Argentina: 35° 11′ S; 59° 05′ W). Ovulation of females was induced by an intraperitoneal injection of suspension of homologs hypophysis in 1 mL of AMPHITOX solution (AS) (Herkovits and Pérez-Coll, 2003) per female preserved according to Pisanó (1956). Oocytes were fertilized *in vitro* with a sperm suspensions in AS. Embryos were kept at 20 ± 2°C in AS until reaching the stage (Del Conte and Sirlin, 1951) required by each experimental protocol. Larvae were fed with Tetra Color Fin Sinking Granules for Goldfish *ad libitum* every other day. For early life stage studies (embryos up till neurula stage), the jelly coats were dejellied by a 2-min treatment with 2% thioglycolic acid at pH 7.2–7.4 with 1.35 mL of saturated NaOH solution every 100 mL in AS followed by a thorough wash of the embryos.

Toxicity Bioassays

Batches of 10 embryos of *R. arenarum* (by triplicate) providing from three different couples were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of AS with BPA in at least five different concentrations in the following experimental conditions:

1. Continuous treatment ranging between 3 and 30 mg L^{-1} BPA from early blastula (S.4) up to complete operculum stage (S.25), the end of embryonic development.
2. Continuous treatments in concentrations between 1.25–10 mg L^{-1} BPA starting from the complete operculum stage (S.25) onwards during 168 h. For NOEC values, *R. arenarum* larvae were maintained in BPA until 336 h.
3. Twenty-four hours pulse exposure in concentration from 3 to 40 mg L^{-1} BPA starting at: blastula (S.4), gastrula (S.10–S.12), neurula (S.13–S.16), muscular activity (S.18), gill circulation (S.20), opercular folds (S.23), and complete operculum (S.25). After exposure, embryos were thoroughly washed and kept in AS media until 240 h.

Survival, malformations and neurotoxicity (narcosis, scarce response to stimuli, shortening, erratic or circular, swimming and spasmodic contractions) were evaluated each 24 h and dead organisms were removed. Sublethal effects were recorded in a table with each LOAEL (Lowest Observed Adverse Effect Level) values. Teratogenic effects

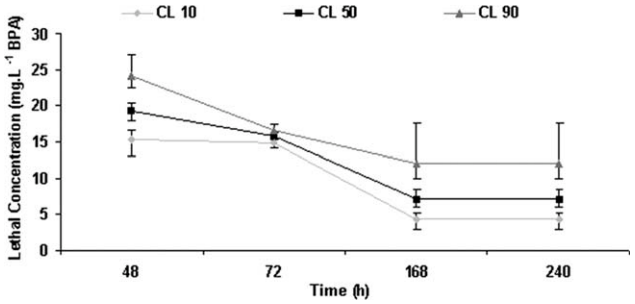

Fig. 1. Toxicity profile (TOP) curves of BPA on *Rhinella arenarum* embryos continuously exposed from S.4 with the LCs10, 50, and 90 values and their corresponding confidence limits (95%). Note the overlapping between confidence intervals at 72 h, the neurula stage.

were studied with stereoscopic microscopy (SM) and identified according to the ''Atlas of Abnormalities'' (Bantle et al., 1998). The teratogenic index (TI) was calculated for the most sensitive embryonic stage as the ratio between the NOEC value for lethality and EC for teratogenesis within the initial 48 h. This index reflects the hazard of a test agent to produce malformations during embryonic development. In addition, other embryos were fixed in 4% formol, dehydrated in a gradient of acetone, prepared for scanning electron microscopy (SEM) by means of the critical-point technique, coated with gold and observed in a JEOL 5800LV microscope operated at 5 kW.

Test Solutions

A commercial formulation of bisphenol A was a generous gift from INDUR SACIFI. Stock solution (2,2-bis(4-hydroxyphenyl)propane) was prepared in acetone to a final concentration of 5000 mg L^{-1} BPA. The experimental solutions were obtained by diluting BPA stock solution with AS. Control embryos were kept simultaneously in AS and AS plus acetone in the highest concentration employed for BPA treated organisms. The media were replaced every other day. BPA purity was checked by fourier transform infrared spectroscopy (FTIR) and supported with analytical-grade BPA (Purity: 99.99% from Sigma Aldrich) spectra in the NIST (National Institute of Standards and Technology) data base. BPA concentrations were measured from an aliquot of the experimental solutions with a UV–VIS spectrophotometer at 276 nm (Li et al., 2008), establishing that the difference between the nominal and real values was below 0.2%.

Statistical Analyses

Lethality data were statistically analyzed by means of US EPA Probit Program (EPA, 1988) and R. Cinto's Probit method modified by E.M. Rodríguez, with Abbot's correction and Lichfield's and Wilcoxon's alternative method; version April 1994 (Rodriguez and Amin, 1991). To establish statistical differences between the LC50 values obtained, a comparison was made, considering the difference statistically significant when the higher LC50/lower LC50 ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association (APHA, 1980). Toxicity Profile's (TOP's) curves were plotted based on LC10, 50, and 90 values obtained by Probits.

RESULTS

Continuous Exposure to BPA from Early Blastula Stage (S.4) Onwards

The toxicity profile (TOP) curves of BPA and their correspondent confidence limits (95%) obtained from blastula stage onwards is plotted in Figure 1. During the initial 24 h, no lethality was recorded even with 30 mg L^{-1}, the highest concentration evaluated. From 48 h onwards a gradual increase in toxicity was recorded with 15 mg L^{-1} as a NOEC value. The LC10, 50, and 90 values at 48 h were, in mg L^{-1}, 15.25 (13–16.6); 19.2 (18–20.3), and 24.2 (22.6–27.2), respectively, whereas for 168 h (coincident with the end of the embryonic development) were 4.2 (2.8–5.2); 7.1 (6–8.3), and 12 (9.9–17.6) mg L^{-1} and continued with the same values until 240 h. This gradual increase in the toxicity was significant from 48 to 168 h ($P < 0.05$). In control and acetone-treated embryos, lethality was less than 10%.

Continuous Exposure to BPA from S.25 Onwards

The results obtained by means of continuous treatments with BPA from complete operculum stage onwards plotted as TOP curves are depicted in Figure 2. As in the case of continuous treatment from S.4, no lethality was recorded up to 48 h. The LC10, 50, and 90 values at 48 h were, in

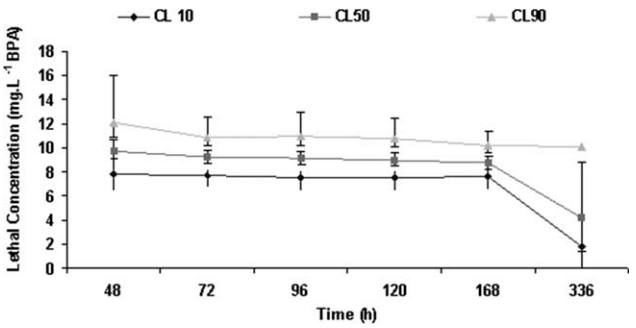

Fig. 2. Toxicity profile (TOP) curves of BPA on *Rhinella arenarum* larvae exposed from S.25 onwards. The LCs 10, 50, and 90 values and their corresponding confidence limits (95%) from 48 to 336 h.

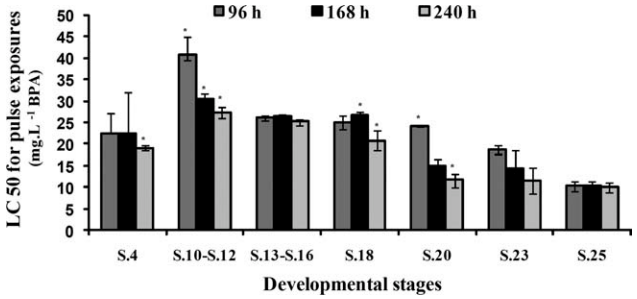

Fig. 3. The stage-dependent susceptibility of *Rhinella arenarum* embryos to BPA plotted as the LC 50 and its corresponding confident limit (95%) for seven developmental stages. Asterisks indicate statistically significant differences ($P < 0.05$).

mg L^{-1}, 7.8 (6.5–8.4), 9.7 (9.1–10.7), and 12.1 (10.9–16), respectively and did not change significantly ($P > 0.05$) during the remaining short term chronic exposure period. However, by expanding the exposure to 336 h, based on NOEC values (1.8 mg L^{-1}), the BPA toxicity increased more than three times ($P < 0.05$). No death occurred in S25 controls.

In comparison, S.25 was significantly more susceptible than S.4 ($P < 0.05$) within the initial 48 h. However, at 168 h of treatment, as the embryonic development approaches S.25 the susceptibility to BPA achieved similar values than those found in larvae exposed from S.25 onwards.

Twenty-Four Hour Pulse Exposure to BPA in Different Developmental Stages

The stage-dependent susceptibility of *R. arenarum* embryos to BPA obtained by means of short exposures at seven developmental stages is represented in Figure 3. Within the initial 96 h, the complete operculum stage (S.25) was the most susceptible to BPA, while gastrula (S10–12) was at least 3 times more resistant to this chemical ($P < 0.05$). Furthermore, compared with the other five developmental stages (S.4, S.13–16, S.18, S.20, and S.23) gastrula was also significantly more resistant ($P < 0.05$). However, as the post exposure period was extended, embryos treated at gastrula and S.20 exhibited a significantly higher susceptibility ($P < 0.05$) (Fig. 3). An average of 90% of the control embryos from the stage-dependent susceptibility study developed normally.

Sublethal Effects

The main sublethal effects recorded in BPA treated embryos are summarized in Table I. The lowest adverse effect level (LOAEL) was established as the BPA concentration resulting in any adverse effect in acute treatments, considering that in control or acetone-treated embryos, 10% was the maximum of spontaneous abnormalities; but

TABLE I. LOAEL values and most common sublethal effects produced by bisphenol A at different developmental stages of *Rhinella arenarum*

Developmental Stage	LOAEL (mg L^{-1})	Sublethal Effects
S.4 continuous	3	Delayed/arrested development. Reduced body size. Spina bifida. Axial/tail flexures. Microcephaly. Edemas/blisters. Mouth malformations. Underdeveloped gills. Tumors.
S.4 (24 h)	10	Delayed/arrested development. Reduced body size. Spina bifida. Axial/tail flexures. Microcephaly. Edemas/blisters. Mouth malformations. Underdeveloped gills.
S.10–S.12 (24 h)	10	Delayed/arrested development. Reduced body size. Spina bifida. Axial/tail flexures. Microcephaly. Edemas/blisters. Mouth malformations. Underdeveloped gills. Waving fin.
S.13–S.16 (24 h)	5	Delayed/arrested development. Reduced body size. Axial/tail flexures. Microcephaly. Edemas/blisters. Mouth malformations. Underdeveloped gills. Waving fin.
S.18 (24 h)	15	Delayed/arrested development. Reduced body size. Axial/tail flexures. Microcephaly. Edemas/blisters. Underdeveloped gills. Neurotoxicity.
S.20 (24 h)	10	Delayed/arrested development. Underdeveloped gills. Neurotoxicity including narcosis.
S.23 (24 h)	7.5	Delayed/arrested development. Axial/tail flexures. Microcephaly. Edemas/blisters. Abnormal gut coiling. Neurotoxicity including narcosis.
S.25 (24 h)	5	Delayed/arrested development. Neurotoxicity including narcosis.
S.25 continuous	5	Delayed/arrested development. Neurotoxicity including narcosis.

Neurotoxicity includes: scarce response to stimuli, shortening, erratic or circular swimming and spasmodic contractions.

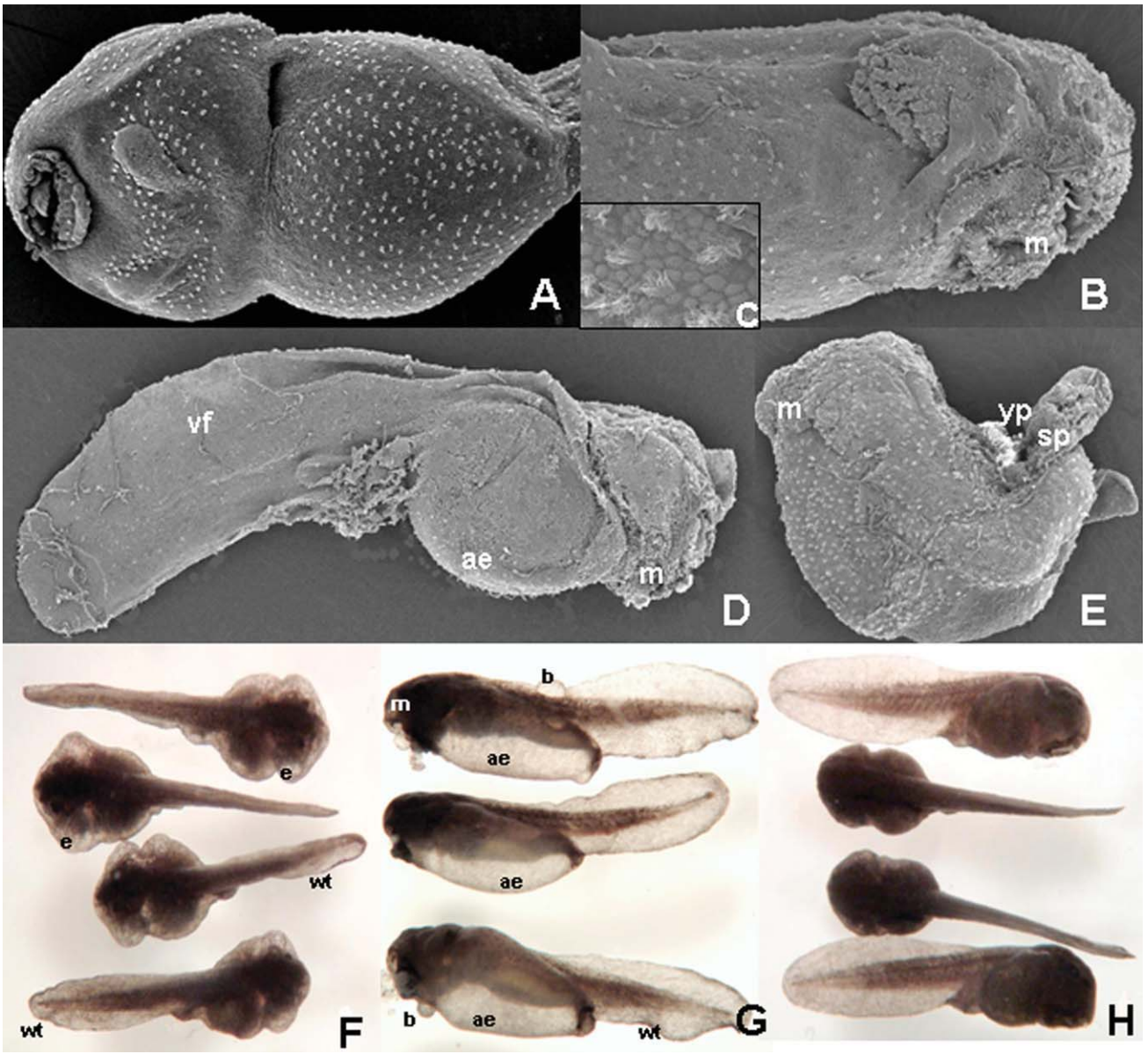

Fig. 4. Scanning Electron Microscopy (SEM) images of *Rhinella arenarum* embryos exposed to bisphenol A. (A) Control embryo at S.24 (B) Embryo exposed to 7.5 mg L^{-1} from S.4 onwards exhibiting microcephaly (m), undeveloped gills, incomplete opercular fold, malformed mouth, and adhesive apparatus. (C) Detail of the tegument showing ciliary cells which indicates delayed development. (D) Panoramic embryo exposed to 3 mg L^{-1} from early blastula onwards with delayed development, reduced body size, ventral flexure (vf), microcephaly, undeveloped gills, malformed mouth, and adhesive apparatus, abdominal edema (ae) and irregular outline, mainly in the dorsal side. (E) Embryo exposed for 24 h in S.4 to 10 mg L^{-1} with delayed development, stunted body, ventral flexure, spina bifida (sp), general underdevelopment, microcephaly (m), and persistent yolk plug (yp). Stereoscopic Microscopy (SM) images: (F) Dorsal view of embryos exposed in gastrula to 20 mg L^{-1} for 24 h with reduced body size, localized edemas (e), and waving tail (wt). (G) Lateral views embryos exposed 24 h in S.23 to 20 mg L^{-1} with abdominal edema (ae), blisters (b), microcephaly (m), axial flexures. (H) Control embryos in S.25. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in control or acetone-treated larvae from S.25, no malformations or neurotoxic effects were observed. In the case of 24 h pulse exposure, neurula was the most sensitive embryonic stage. Adverse effects were observed in 50% of embryos from 5 mg L^{-1}, resulting in a TI-48 h value of 4.7. Figure 4 shows some of the most common malformations produced by BPA on *R. arenarum* embryos. As it is common in teratogenesis, different degrees of one or multiple malformations were recorded in a particular concentration. Certain adverse effects like delayed development, reduced body size, hydropsy and axial flexures affected over 80% of the embryos, while others, like underdeveloped fin and gills, affected between 30 and 70% of the embryos. Glandular and ciliated cells were observed by means of SEM in BPA treated embryos reflecting that although the cells exhibited vaulted surface, the cellular differentiation was not significantly affected. From the muscular activity stage (S.18) onwards the most common alterations were associated with neurotoxicity such as scarce response to stimuli, shortening, erratic or circular swimming, and spasmodic contractions. S.25 was the most sensitive stage for neurotoxic effects, recorded from 5 mg L^{-1} BPA onwards in 100% of individuals. From the gill circulation stage (S.20) onwards, 10 mg L^{-1} BPA caused narcosis in all embryos after 3 h of exposure. It is noteworthy that by washing the embryos in SA, they recovered normal behavior after few minutes.

DISCUSSION

The results of a commercial formulation of BPA on *Rhinella arenarum* embryos and larvae reported in this study point out the toxicity of this organic compound. In continuous treatments, BPA toxicity increased about three times by the end of the embryonic period (Fig. 1) and conversely remained about constant during the initial 240 h of early larval stages (Fig. 2). This fact might indicate a gradual increase in embryo susceptibility to BPA from muscular activity stage onwards, reaching a maximum level when the organisms get an advanced maturation of the nervous system, which might be related to the well-known neurotoxicity of this chemical (Oka et al., 2003; Negishi et al., 2004; Kunz et al., 2011) as it will be discussed for pulse exposure results.

LC50 values for a specific exposure time is the usual parameter reported for lethal effects. TOP curves provide the possibility to understand the concentration/magnitude of a chemical or physical agent to produce the same adverse effect (e.g., LC10, 50, or 90) at different exposure periods providing by this means a curve representing isotoxicity (Herkovits and Helguero, 1998; Castañaga et al., 2009; Aronzon et al., 2010; Sztrum et al., 2011). Other valuable information derived from the TOP curves is the range of concentrations in which the minimum and maximum effects of a substance (LC10 and LC90) are obtained. In the case of BPA, at neurula stage, the confidence intervals for LC50 overlapped with those of LC10 and LC90 reflecting that the concentration producing 10% of lethality could affect 50% of the embryos, whereas the concentration producing 50% lethality could imply the mortality of 90% of the embryos (Fig. 1). Taking into account that 1.8 mg BPA L^{-1} was the NOEC value obtained in this study for *R. arenarum* embryo-larval development, it seems that this specie is about 2.5 times more susceptible than *X. laevis* (Oka et al., 2003).

For sublethal toxicity, embryos treated with BPA exhibited teratogenic and neurotoxic effects while larvae showed only neurotoxicity. This might be related to the relatively high resistance of *R. arenarum* embryos at early stages to BPA, providing the condition for the development of severe teratogenic effects. In fact, as development progresses, the susceptibility for lethal effects increases and all malformed embryos die around the last embryonic or early larval stages. Thus, during the embryonic development, 3 mg L^{-1} of BPA resulted as NOEC both for malformations and survival. Some teratogenic effects of BPA in *R. arenarum* embryos like scoliosis reduced body size, microcephaly, flexures, edema, and abnormal gut coiling were previously reported in *X. laevis* at very low doses (Sone et al., 2004; Baba et al., 2009). In addition, we report blisters, spina bifida, underdeveloped gills, tumors, warts, waving fin, and tail flexures that highlight the multiple teratogenic effects of this organic compound. A combination of different malformations was frequently observed. For instance, embryos treated with 30 mg L^{-1} BPA during gastrula exhibited 100% of malformations. Conversely S.25 showed no malformations, but 100% of the embryos had neurotoxic effects with 10 mg L^{-1}. The delayed development was a general adverse effect recorded also at cellular level as the persistence of ciliated cells in the epithelial surface of experimental embryos, whereas in controls embryos at S.25 these cells have regressed [Fig. 4(c)]. The potential mechanisms related to the teratogenic effects of BPA might be its capability to induce epigenetic changes by altering patterns of DNA methylation and/or by activating or silencing genes during critical periods of development (Gross, 2007). These mechanisms of toxicity might provide a general explanation of the diverse and severe malformations reported in this study. The edema and blisters caused by BPA may be associated with osmoregulation failures as reported for other estrogen mimics like some pesticides, alkylphenolic, phthalates, and 4-nonylphenol (Jobling et al., 1998; Lerner et al., 2007). It has been recently suggested that BPA increases cancer susceptibility through developmental reprogramming, potentially involving changes in target organ morphogenesis as a result of epigenetic alterations (Keri et al., 2007). Taking into account that carcinogens usually cause teratogenesis, the multiple and severe malformations found in BPA treated *R. arenarum* embryos contribute to the concept that teratogenicity studies might provide the

carcinogenic potential of environmental agents. Indeed, the epidermal tumors and warts observed in BPA treated embryos, allow associating this organic compound directly to its carcinogenic potential.

One of the main neurotoxic effects of BPA on *R. arenarum* embryos was narcosis, observed from gill circulation stage onwards. The sudden narcotic effect recorded after BPA exposure implies that its uptake and impact on the nervous system occurs rapidly. Narcosis was observed in *R. arenarum* embryos with other agents like naringenin (Pérez-Coll and Herkovits, 2004) highlighting its value as a rapid possibility to evaluate neurotoxic effects. Narcosis had a transient duration whereas other neurotoxic effects such as erratic swimming, spasm, and low-response to stimuli continued during short term chronic or chronic exposure periods. These results may indicate that the central nervous system is one of the most affected by BPA during embryonic development, as it was also considered for *X. laevis* by Oka et al. (2003). The neurological effects might have a histological basis. Recently, studies in rats reported that exposure to BPA may cause neuronal cell death during development, which would irreversibly lead to an abnormal neural network (Negishi et al., 2004). Furthermore, 1 mg L^{-1} BPA administered in rats during gestation and lactation leads to significant neuronal and glial developmental alterations (Kunz et al., 2011). It is noteworthy that the malformations produced by BPA also interfered with the swimming performance as it was reported by Cooke (1981) for different toxicants.

By means of 24 h pulse exposure to BPA at seven developmental stages, a remarkable stage-dependent susceptibility was recorded with gastrula as the most resistant and complete operculum (S.25) as the most susceptible stage for lethality (Fig. 3). The pulse exposures provide the possibility to report that BPA exerts teratogenesis at early and subsequent developmental stages. This suggests that BPA may affect certain presumptive embryonic territories interfering with the onset of structures and functions that take place at later developmental stages. Additionally, it was possible to establish that neurula stage had a teratogenic index of 4.7, the highest value when compared with other developmental stages. This value means that BPA is highly teratogenic (ASTM, 1993) specially in case of short term exposures that might be potentially associated with BPA disruption capacity in the onset of gene expression (Gross, 2007). Stage-dependent susceptibility of *R. arenarum* embryos was previously reported for metals like cadmium (Herkovits et al., 1997a), physical agents as UVB irradiation (Castañaga et al., 2009), and pesticides like 2,4-D (Aronzon et al., 2010). Usually, the most susceptible stages to environmental agents are within the organogenic period (S.15–S.21) because of the conspicuous cell differentiation and morphogenetic processes, which may be potentially disrupted by noxious agents. An evolutionary perspective could also contribute to a rational explanation of the stage-dependent susceptibility phenomenon (Herkovits et al., 1997b; Castañaga et al., 2009). For environmental agents in place and contact with the ancestor of living organisms, there is a clear correlation between the susceptibility in different ontogenic stages and the exposure conditions during the evolutionary process. For instance in the case of UVB, the high resistance to this physical agent at early developmental stages of *R. arenarum* embryos, was correlated with the absence of the ozone layer during the anoxic period of the Earth (Castañaga et al., 2009). Moreover, based on geochemical studies during Earth history, the environmental features in ancient times could be related to the stage-dependent susceptibility to Ni during the ontogenic period (Sztrum et al., 2011). In such cases, the organogenic period was the most susceptible to noxious agents, including teratogenic effects, indicating that those morphogenetic and cellular differentiation processes occurred during an evolutionary period of environmental conditions with very low-toxicity (Herkovits, 2006). In the case of BPA, as a new industrial chemical, living organisms have no previous evolutionary experience and therefore its adverse effects during ontogenesis are directly related to its capacity to interfere with mechanisms involved in the complexity of cellular functions at different developmental stages. In this context, based on pulse exposures, the most susceptible stages for lethality were blastula and complete operculum, while neurula was the most labile for teratogenic effects. For neurotoxicity, adverse effects might be directly related to the maturation of the nervous system, affecting in a similar way from gill circulation stage onwards. These facts contribute to sustain that the susceptibility to noxious agents are not related to the complexity achieved at different developmental stages but the evolutionary experience and mechanisms of actions involved in adverse effects.

The continuous increase in the use of BPA-based products contrasts with the lack of consensus regarding the exposure levels that represents a risk for environmental health (Bondessona et al., 2009). In aqueous media, BPA was reported in concentrations around 20 μg L^{-1} in stream/river water samples and 17 mg L^{-1} in landfill leachate (Crain et al., 2007; Staples et al., 2011), indicating that BPA concentrations in some environmental scenarios may be toxic for *R. arenarum* embryos and larvae. Sublethal effects like teratogenesis and neurotoxicity may have great ecological relevance by reducing the ability of organisms to avoid predators and then, contributing to the decline in amphibian population. In that context, the information reported in this study is relevant for amphibian protection purposes.

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