Environmental Toxicology and Chemistry

Environmental Toxicology

DEVELOPMENTAL TOXICITY OF BISPHENOL A DIGLYCIDYL ETHER (EPOXIDE RESIN BADGE) DURING THE EARLY LIFE CYCLE OF A NATIVE AMPHIBIAN SPECIES

IANINA HUTLER WOLKOWICZ, GABRIELA V. SVARTZ, CAROLINA M. ARONZON, and CHRISTINA PÉREZ COLL

Environ Toxicol Chem., **Accepted Article** • DOI: 10.1002/etc.3491

Environmental Toxicology **Environmental Toxicology and Chemistry**
DOI 10.1002/etc.3491

DEVELOPMENTAL TOXICITY OF BISPHENOL A DIGLYCIDYL ETHER (EPOXIDE RESIN BADGE) DURING THE EARLY LIFE CYCLE OF A NATIVE AMPHIBIAN SPECIES

Running title: Toxicity of Badge on *Rhinella Arenarum*

IANINA HUTLER WOLKOWICZ,†‡ GABRIELA V. SVARTZ,†‡ CAROLINA M. ARONZON,†‡ and CHRISTINA PÉREZ COLL*†‡

†Instituto de Investigación e Ingeniería Ambiental (3iA), Universidad Nacional de San Martín (UNSAM). Av 25 de Mayo y Francia (C1650), San Martín, Buenos Aires, Argentina.

‡CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) Buenos Aires, Argentina

* Address correspondence to perezcoll@unsam.edu.ar

Submitted 9 April 2015; Returned for Revision 14 May 2015; Accepted 10 May 2016

Abstract: Bisphenol A, diglycidyl ether (BADGE) is used in packaging materials, epoxy adhesives, additive for plastics but is also a potential industrial wastewater contaminant. The aim of this study was to evaluate the adverse effects of BADGE on *Rhinella arenarum*, by means of standardized bioassays at embryo-larval development. The results showed that BADGE was more toxic to embryos than to larvae at all exposure times. At acute exposure, lethality rates of embryos exposed to concentrations from 0.0005 mg/L BADGE were significantly higher than vehicle control, whereas lethality rates of larvae were significantly higher from 10 mg/L BADGE. Then, the toxicity increased significantly, with a LC50-96 h of 0.13 and 6.9 mg/L BADGE for embryos and larvae respectively. By the end of the chronic period the LC50-336 h were 0.04 and 2.2 mg/L BADGE for embryos and larvae respectively. This differential sensitivity was also ascertained by the 24-h pulse exposure experiments, in which embryos showed a stage-dependent toxicity, being blastula the most sensitive stage and S.23 the most resistant one. The most important sublethal effects in embryos were cell dissociation and delayed development, whereas the main abnormalities observed in larvae were related to neurotoxicity, as scare response to stimuli and narcotic effect.

Keywords: Bisphenol A diglycidyl ether, Standardized toxicity bioassays, Stage-dependent toxicity, Teratogenesis, Neurotoxicity

INTRODUCTION

Bisphenol A diglycidyl ether (2, 20-bis (4-hydroxyphenyl) propane bis (2, 3-epoxypropyl) ether, commonly known as BADGE (CAS No. 1675-54-3), is a synthetic chemical obtained by the reaction of one mole of bisphenol A (BPA) with two moles of epichlorohydrin (ECH) [1]. BADGE is mainly used in packaging materials such as storage vessels and lacquer coatings on food cans, to protect the food from metal contamination and to prevent metal corrosion. Levels of bisphenols and their diglycidyl ethers as BADGE were reported in wastewater influents at concentrations from 0.00096 to 0.0016 mg/L [2]. The total annual production of bisphenol A based epoxy resins grows annually implying a concern for public health owing to impurities present in faulty formulations that can migrate into canned food, representing a toxicity risk [1]. In fact, BADGE and its derivatives were found in concentrations between 1 mg/kg and 12.5 mg/kg in many canned foods [3,4,5]. It is also a concern that BPA was reported as an endocrine disruptor by reversing gonadal sex and altering gonadal histoarchitecture [6, 7]. However, the estrogenic activity of BADGE is 100 times lower than BPA [8].

Nevertheless, BADGE can increase the proliferation of MCF7 breast cancer cells [9]. Due to this, the European Legislation has established that the sum of the migration levels of BADGE, its hydrolysis and chlorohydroxy derivatives to food or food simulants should not exceed 9 mg/kg [10]. The Shell Tunstall Toxicology Laboratories [11] reported an increase in the frequency of chromosomal aberrations of *in vitro* rat liver cells exposed to BADGE. In 1986, the Scientific Committee on Food (SCF) from the EU evaluated BADGE as a monomer used in the production of plastic food contact materials and it was classified into List 4A [12]. Then, in 2000 it was reported that BADGE and its hydrolysis products can induce micronuclei in cultured human lymphocytes from 0.0125 mg/mL [13].

Because of BADGE´s different uses and its high production worldwide, it is relevant to know the risk to the wildlife after epoxy resins reach the environment. Toxicological bioassays can provide

information about the potential hazards of synthetic products in living organisms. Amphibians are frequently being used for toxicity screening purposes due to their high sensitivity to physico-chemical stressors [14]. Moreover, standardized tests employing amphibian embryos are successfully used to evaluate the toxicity of hazardous substances and environmental samples [15]. In contrast to bioassays that only evaluate acute toxicity of chemicals by a unique endpoint, such as 48 or 96 hours median lethal concentration (LC50), AMPHITOX assesses toxicity by using different endpoints (exposure times and developmental stages), giving more complete information about the toxicity profile of the substance. This test employs *Rhinella arenarum* (Fam. Bufonidae) embryos and larvae as the appropriate biological material to perform toxicity tests [16,17]. Besides its extensive neotropical distribution, which includes Argentina, Bolivia, Brazil, Uruguay, and Paraguay, this species is easy to handle, produces large clutches (up to 40,000 eggs) and has a short life cycle, reaching the prometamorphic stage in about 7–8 days after egg laying [15].

The aim of this study was to evaluate the toxic effects of BADGE on *Rhinella arenarum* development by means of the standardized AMPHITOX test at different developmental stages and exposure times by characterizing lethal and sublethal effects involving teratogenesis and ethological disorders.

MATERIALS AND METHODS

Obtaining Rhinella arenarum embryos and larvae

Healthy *Rhinella arenarum* adults weighing approximately 200-250 g were collected in Lobos (Buenos Aires Province, Argentina: 35º 11´ S; 59º 05´ W) from a local provider. Ovulation of *R. arenarum* females was induced by means of an intraperitoneal injection of one homologous hypophysis in 1 mL of AMPHITOX solution (AS) per female [18], plus 5000 IU human chorionic gonadotropin [19]. The AS composition was (in mg/L): Na^+14.75, Cl^- 22.71, K^+ 0.26, Ca^{2+} 0.36, HCO_3^- 1.45, prepared in distilled water. Oocytes were fertilized *in vitro* using a testicular macerate homogenate

suspended in AMPHITOX solution, resulting in a 10% spermatozoid suspension. Embryos were kept in this physiological solution at 20±2°C until they reached the stage required for each experimental protocol. For early life stage studies, (embryos up till S.17) the jelly coat was dissolved by a 2-min treatment with 2.5% thioglycolic acid solution neutralized at pH 7.2-7.4 with 1.35 mL of saturated NaOH solution every 100 mL in AS, and then thoroughly washed with AS. Although the jelly coats can provide protection against toxics [20] in some cases it is not relevant for protection purposes [21]. In our study, we dejellied the embryos to select a homogeneous, high-quality biological material (round shape embryos with non-cellular dissociation) both for control and experimental groups. The eggs were inspected for quality and fertility. This biological material was considered acceptable if the fertility rate was greater than 75% per female, and embryo survival at the neurula stage was greater than 70%.

Chemicals and test solutions

Technical-grade BADGE (99.9%, CAS No 1675-54-3) was obtained from Sigma Chemical Co. Stock solutions were prepared in analytical grade acetone to a final concentration of 10 g/L, and experimental solutions were prepared by diluting it with AS. Acetone concentrations were always lower than 1.1% [22]. Both AS and acetone treatments, were simultaneously maintained as controls and they did not differ statistically so both treatments were combined and reported as the 'control' in the rest of the manuscript.

Toxicity bioassays

Rhinella arenarum embryos and larvae were used in the standardized semi-static bioassays following the AMPHITOX protocol [16,17]. Ten embryos were randomly placed in triplicate 10 cm glass Petri dishes containing 40 mL of AS with or without BADGE (controls). The toxicity bioassays were performed under the conditions summarized in Table 1. Embryos and larvae were maintained at 20±2ºC. Experiments were replicated three times.

In order to evaluate the stage-dependent sensitivity, different experimental conditions were performed as follow: i) continuous exposure of embryos from early blastula stage (S.3-4) and larvae from complete operculum stage (S.25) onwards for 336 h; ii) 24-h pulse exposure of embryos starting at: early blastula (S.3-S.4), gastrula (S.10-12), rotation (S.15), tail bud (S.17), muscular activity (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23) and complete operculum (S.25) stages. After exposure, embryos were thoroughly washed, kept in AS and evaluated up to 336 h. The developmental stages were defined according to Del Conte and Sirlin [23].

Lethal effects were evaluated and dead individuals were removed every 24 h. Larvae were fed with 3 granules of balanced fish food TetraColor® per Petri dish every other day. Sublethal effects were studied under a stereoscopic microscope (Zeiss Stemi DV4),. Teratogenic effects were identified according to the "Atlas of Abnormalities" [24] and organisms were photographed with an Olympus X-42 digital camera mounted on the microscope objective. A teratogenic index (TI) was calculated as LC50/EC50 at 96 h in embryos exposed from blastula. This index reflects the hazard of a test agent to produce malformations during embryonic development without significant lethality [22]. Behavioral alterations including narcosis, spasmodic contractions, abnormal fast rotations, lateral or dorsal side lying, abnormal breathing, feeding and swimming patterns were evaluated as they are typical signs of neurotoxic stress [25]. General weakness was defined as lower and slower movements than control larvae, Narcosis was particularly evaluated as the lack of sudden swimming response to gentle touching with a glass rod compared with control organisms, and finally heartbeat was checked under the microscope. Starvation was determined by observing the granules after 24 hours, while in control larvae, we found the feces instead. Abnormal skin pigmentation was defined as irregular distribution of somatic pigmentation compared with normal pigmentation found in the control organism's skin. Cellular dissociation was determined by observing detached cells floating in the perivitelline fluid as well as in the maintaining media. Delayed development was determined when the developmental stage

of each embryo was different from the control in each concentration group. Larvae snout-vent lengths were recorded as a measure of the body size.

Data analysis

Lethal and sublethal effects were analyzed as LC50 and EC50 respectively with their upper and lower 95% confidence limits, by the US EPA Spearman-Karber program version 1.5 [26]. Toxicity profile (TOP), or isotoxicity curves [27] were plotted based on LC50 values at different times. The LC50 were considered to be substantially different when the higher/lower ratio exceeded the corresponding critical value established by the American Public Health Association et al. [28]. We conducted generalized linear mixed models (GLMMs) assuming a binomial distribution of the error to evaluate the effect of concentration and exposure time on lethality and on the frequency of sublethal endpoints. Di Rienzo, Guzmán and Casanoves (DGC) test [29] was used to compare treatment means at a significance level of $p<0.05$. This analysis was conducted using InfoStat statistical software [30]. LOAEL (lowest observed adverse effect level) values were determined by the lowest concentration that has a statistically significant deleterious effect compared with the control group.

RESULTS

Lethal effects

Continuous exposure of embryos from early blastula stage (S.3-4) and larvae from complete operculum stage (S.25) onwards for 336 hours. Following BADGE treatment lethality rates of embryos and larvae gradually increased with the concentration and the exposure time (Table 2). Lethality rates of embryos exposed from 0.0005 mg/L BADGE and larvae exposed from 10 mg/L were significantly higher than vehicle control from 96 h onward. At 2.5 mg/L BADGE, larvae lethality was significantly increased compared with vehicle control at 168 h onward. BADGE toxicity was substantially higher in embryos than larvae at all exposure times (Table 2). The toxicity of BADGE in embryos exposed from

blastula stage increased considerably from 24 to 48 h with LC50 values of 0.35 (0.25-0.5) and 0.15 (0.09-0.23) mg/L BADGE respectively (Fig. 1). However from that time forward, toxicity did not substantially increase with LC50-336 h of 0.04 (0.02-0.08) mg/L BADGE. In contrast, the toxicity of BADGE to larvae did not vary considerably during acute exposure, with the LC50 values at 24 and 48 h of 11.3 (11.1-12.5) and 10.90 (10.70-11.10) mg/L BADGE respectively. The LC50-96 h decreased from 6.9 (6.6-7.1) mg/L BADGE to 2.2 (2.1-2.4) mg/L BADGE at the end of the chronic exposure.

24-hour pulse exposure. No embryos were affected after being exposed to the lowest concentrations: 0.0001 to 0.001 mg/L. The toxicity profile obtained shows a clearly stage-dependent sensitivity to the epoxy resin with early blastula (S.3-4) the most sensitive stage, with a LC50-24 h of 0.58 mg/L BADGE, and S.23 the most resistant one, with a LC50-24 h of 14.9 mg/L BADGE (Fig. 2). The remaining developmental stages had LC50s between 8 and 11.9 mg/L BADGE.

Sublethal effects

Continuous exposure of embryos from early blastula stage (S.3-4) and larvae from complete operculum stage (S.25) for 336 hours. All embryos exposed at 0.5 mg/L BADGE (LOAEL value) and above showed at 24 h cell dissociation and delayed development (exposed embryos were in early gastrula stage, while controls were in late gastrula or rotation stage). At 96 h the LOAEL remained constant but other sublethal effects as reduced body size, hydropsy, acephaly and axial flexure were also observed (Table 3a). The EC50-96 h was 0.17, so the teratogenic index was 0.76. At chronic exposure the LOAEL dropped to 0.1 mg/L BADGE, and behavioural alterations such as starvation, scare response to stimuli and spasmodic contractions were recorded.

All early larvae exposed from 10 mg/L BADGE exhibited neurological alterations few hours after exposure started. These effects were general weakness, spasmodic contractions and shortening, erratic or circular swimming. Moreover, all larvae exposed up to 15 mg/L BADGE developed narcosis after few hours of exposure followed by death. At 168h all larvae exposed to 5 mg/L showed

starvation, abnormal skin pigmentation, scare response to stimuli and tail/axial flexures and then death, whereas those exposed to the lowest concentration (1 mg/L) developed hydropsy and abnormal skin pigmentation but no neurotoxic effects (Table 3b). By the end of the bioassay the LOAEL was 1 mg/L BADGE.

24-hour pulse exposure. Table 4 summarizes the LOAEL values and the most conspicuous teratogenic and neurotoxic effects caused by BADGE in embryos exposed at different developmental stages. Blastula was the most sensitive stage to BADGE, whereas S.23 was the most resistant (LOAEL = 0.1 and 10 mg/L respectively). The rate of malformations in control embryos was always less than 10% during all the bioassay.

Abnormal embryos treated in blastula exhibited several teratogenic effects such as bifid spine, oral desquamation, tumors, delayed development, microcephaly, acephaly and axial flexures even at BADGE post-exposure (Table 4, Fig. 4).

All embryos exposed in gastrula developed malformations and delayed on their development 24 h after being washed from 1 mg/L onwards, highlighting cellular dissociation , and as well as persistent yolk plug, but only in 30% of the individuals.

The main sublethal effects observed in embryos exposed in rotation stage were microcephaly, hydropsy, axial flexures and reduced body size, with a LOAEL of 1 mg/L BADGE at 24 h. Those effects were also conspicuous in those embryos exposed in S.18 from 5 mg/L. These embryos also developed tail flexures.

Embryos at stages between S.20 and S.25 developed neurotoxic effects such as narcosis few hours after the beginning of exposure. This narcotic effect was irreversible for those embryos exposed at 7.5 mg/L BADGE in S.20 and S.21 and were dead after a few hours. On the other hand, the LOAEL values at 24 h for embryos at S.23 and S.25 were 10 mg/L, but at 336 h these values increased upward to 17.5 mg/L and 15 mg/L respectively. This fact points out the recovery capacity from malformations

as well as from neurotoxic effects caused by BADGE, because larvae exposed to 10 mg/L BADGE at 336 h did not show any sublethal effect and were not significant different from control. By the end of the bioassay only larvae exposed at S.25 showed reduced body size in 20% of the individuals. No control larvae showed neurotoxicity at 336 hours.

DISCUSSION

The present results provide the first description of the lethal and sublethal effects of BADGE epoxy resin on the early life cycle of an amphibian species, *Rhinella arenarum*. In continuous exposure bioassays, the beginning of the early development (blastula) was the most sensitive stage to the resin with toxicity being highest during the acute period. At chronic period (336 h), only embryos exposed to BADGE in concentrations lower than 0.5 mg/L survived, but developed morphological alterations. On the other hand, the toxicity profile of BADGE in larvae was time-dependent with LC50 values that decreased five times from acute to chronic exposure period. Our findings suggest there may be an increased susceptibility as the central nervous system matures rather than a bioaccumulation because it is known that BADGE is metabolized to non-toxic substances [31]. The low potency of BADGE to cause teratogenicity in larvae is coincident with the slowing rate of morphogenetic changes toward the latter developmental stages associated with the higher teratogenic index for embryos than the one in larvae. It is well established that a TI higher than 1.5 implies a high risk for embryos to be malformed in absence of significant lethality [22]. In our study, embryo lethality occurred above 0.0005 mg/L BADGE exposure, concentrations lower than the reported values in wastewater influents (0.00096-0.0016 mg/L) [2].

Even though the 24-hour pulse exposure concentrations were relatively high, this experimental design allowed us to simulate environmental emergency conditions such as accidental spills. This information has important value in risk assessment analysis of industrial contaminants such as BADGE. Our experimental design also allows associating certain effects with characteristic

morphogenetic events of the development. According to our findings, early blastula was the most susceptible stage whereas S.23 was at least 25 times more resistant. Moreover, the highest incidence of malformations was at the beginning of the development, particularly blastula and gastrula stages. In this last stage, just 24 h post-exposure sublethal effects as yolk plug persistence were observed in embryos exposed to the highest concentrations. It is known that epoxy resins induce cytotoxic action, specifically in those tissues with high cellular division rates [32]. Also, Steiner et al. [33] reported that glycidaldehyde, a BADGE metabolite, binds to adenine nucleotides. Therefore, all early developmental adverse effects might be related to the capacity of BADGE to alter DNA. This has also been identified in yeast, rat and *in vitro* human studies, and even in human workers [13,31,32,34].

It is noteworthy that morphological abnormalities also affected the swimming ability of larvae, which is likely to interfere with their general performance in the natural environment. These observations confirm the importance of reporting not only lethality but also developmental disorders, which make organisms more vulnerable to predation or other environmental stressors such as infectious agents, invasive species, and changes in physical and chemical parameters of the environment, influencing the physical condition of animals or their reproductive success [35].

There is only one previous study on the neurotoxic effects of BADGE but in rodents [36] in which it was demonstrated that exposed organisms significantly reduced water and food consumption. In present study, embryos exposed to BADGE from S.20 expressed behavioral alterations such narcosis, just a few hours after the beginning of treatment. It is noteworthy that these behavioral markers have relevance as early warning systems when other toxicity parameters such as lethality are absent.

Narcosis is an interesting effect with potential ecotoxicological consequences that might be brought about by numerous structurally unrelated chemicals in relation to their high octanol/water partition coefficients [37]. Bisphenol A and epichlorohydrin, chemicals used to synthesize the BADGE epoxy resin, can also cause induced narcosis on *R. arenarum* larvae [38,39].

Developmental effects of BADGE were only previously described in rats receiving oral doses [40]. Hence, the results obtained in this study provide real, valuable ecotoxicological information about its toxicity as well as details of the main malformations and behavioral alterations of this epoxy resin in non-mammal species.

The increase of industrial wastes is just one of the many factors that can contribute in the decline of many amphibian populations [41], Industrial wastewaters containing BADGE, as well as its migration substances and metabolites, represent potential sources of aquatic contamination, which may disrupt populations of this native amphibian.

Acknowledgment—The authors thank Massone Institute Argentina for providing hCG hormones. Ianina Hutler Wolkowicz was a fellow of CONICET. Cristina Pérez Coll, Carolina Aronzon and Gabriela Svartz are scientists of CONICET. This study was supported by ANPCyT PICT 0891 and Universidad Nacional de San Martin.

Data Availability—Data are available upon request from the corresponding author at perezcoll@unsam.edu.ar

REFERENCES

1. Terasaki M, Kazama T, Shiraishi F, Makino M. 2006. Identification and estrogenic characterization of impurities in commercial bisphenol A diglycidyl ether (BADGE). *Chemosphere* 65:873-880.

2. Ballesteros-Gómez A, Ruiz FJ, Rubio S, Pérez-Bendito D. 2007. Determination of bisphenols A and F and their diglycidyl ethers in wastewater and river water by coacervative extraction and liquid chromatography-fluorimetry. *Anal Chem Acta* 603:51-59.

3. Hammarling L, Gustavsson H, Svensson K, Oskarsson A. 2000. Migration of bisphenol-A diglycidyl ether (BADGE) and its reaction products in canned foods. *Food Addit Contam* 17:937-943.

4. Biedermann M, Grob K. 1998. Food contamination from epoxy resins and organosols used as can coatings: analysis by gradient NPLC. *Food Addit Contam* 15:609–618.

5. Uematsu Y, Hirata K, Suzuki K, Iida K, Saito K. 2001. Chlorohydrins of bisphenol A diglycigyl ether (BADGE) and of bisphenol F diglycigyl ether (BFDGE) in canned foods and ready-to drink coffees from the Japanese market. *Food Addit Contam* 18:177–185.

6. Stoker C, Rey F, Rodriguez H, Ramos JG, Sirosky P, Larriera A, Luque EH, Muñoz-de-Toro M. 2003. Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of the xenoestrogen bisphenol A. *Gen Comp Endocrinol* 133(3):287-96.

7. Durando M, Cocito L, Rodríguez HA, Varayoud J, Ramos JG, Luque EH, Muñoz-de-Toro M. 2013. Neonatal expression of amh, sox9 and sf-1 mRNA in *Caiman latirostris* and effects of *in ovo* exposure to endocrine disrupting chemicals. *Gen Comp Endocrinol* 15:31-38

8. Lyons G. 2000. Bisphenol A: A known endocrine Disruptor. Godalming (Surrey): WWF European Toxics Programme.

9. Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Sonnenschein C. 1996. Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Persp* 104:298-305.

10. European Commission. 2002. Commission Directive 2002/16/EC of 20 February 2002 on the use of certain epoxy derivatives in materials and articles intended to come into contact with foodstuffs, Official Journal of the European Communities L51, 27–31.

11. Shell Tunstall Toxicology Laboratory. 1981. Toxicity studies with epoxy resins: *in vitro* genotoxicity studies with diglydicyl ether of Bisphenol A, EPITOKE 828, EPITOKE 1001, EPITOKE 1007, EPA Document No. 878210037.

12. Scientific Committee on Food (SCF). 1988. Nineteenth Series of Reports, EUR 11322, Commission of the European Communities, Luxembourg, 1988.

13. Suárez S, Sueiro RA, Garrido J. 2000. Genotoxicity of the coating lacquer on food cans, bisphenol A diglycidyl ether (BADGE), its hydrolysis products and a chlorohydrin of BADGE. *Mutat Res* 470:221-228.

14. Pérez-Coll C, Herkovits J. 1990. Stage dependent susceptibility to lead in *Bufo arenarum* embryos. *Environ Poll* 63:239-245.

15. Ferrari A, Anguiano L, Lascano C, Sotomayor V, Rosenbaum E, Venturino A. 2008. 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* 22:259-267.

16. Herkovits J, Pérez-Coll CS, Herkovits FD. 2002. Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX). *Environ Pollut* 116:177-183.

17. Herkovits J, Pérez-Coll CS. 2003. AMPHITOX. A customized set of toxicity tests employing amphibian embryos, in: Linder, G.L., Krest, S., Sparling, D., Little, E.E. (Eds.). Symposium on multiple stressor effects in relation to declining amphibian populations, ASTM International STP 1443, USA, pp. 46–60.

18. Pisanó A. 1957. Efficienza funzionale e structura dell' ipofisi di anfibio. *Arch Zool Ital* 42:221-227.

19. Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquat Toxicol* 51:19-29.

20. Edginton AN, Rouleau C, Stephenson GR, Boermans HJ. 2007. 2,4-D butoxyethyl ester kinetics in embryos of *Xenopus laevis*: the role of the embryonic jelly coat in reducing chemical absorption. *Arch Environ Contam Toxicol* 52:113-120.

21. Räsänen K, Pahkala M, Laurila A, Merilä J. 2003. Does jelly envelope protect the common frog *Rana temporaria* embryos from UV-B radiation? *Herpetologica* 59(3): 293-300.

22. ASTM. 1993. Standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (FETAX). ASTM. Standards on aquatic toxicology and hazard evaluation, Philadelphia, PA, 1199–1209.

23. Del Conte E, Sirlin L. 1951. The first stages of *Bufo arenarum* development. *Acta Zool Lillo* 12:495-499.

24. Bantle JA, Dumont JN, Finch RA, Linder G, Fort DJ. 1998. Atlas of abnormalities. A guide for the performance of FETAX, 2nd ed. Oklahoma State University, Stillwater.

25. Denoël M, D'hooghe B, Ficetola GF, Brasseur C, De Pauw E, Thomé JP, Kestemont P. 2012. Using sets of behavioral biomarkers to assess short-term effects of pesticide: a study case with endosulfan on frog tadpoles. *Ecotoxicology* 21:1240–1250.

26. U.S. Environmental Protection Agency. 1999. Trimmed Spearman–Karber estimation of LC50 Values Users' Manual, Office of Research and Development, National Exposure Research Laboratory-Ecosystems Research Division, Center for Exposure Assessment Modeling (CEAM), Athens, Georgia.

27. Herkovits J, Helguero LA. 1998. Copper toxicity and copper-zinc interactions in amphibian embryos. *Sci Total Environ* 221:1-10.

28. APHA. 2005. Standard methods for the examination of water and wastewater, 21st Ed., 15 ed. American Public HealthAssociation, American Water Works Association, Water Pollution. Control Federation, Washington, DC.

29. Di Rienzo JA, Guzmán AW, Casanoves F. 2002. A multiple comparisons method based on the distribution of the root node distance of a binary tree. *J Agric Biol Environ Stat* 7:1-14.

30. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. 2015. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar

31. Poole A, Van Herwijnen P, Weideli H, Thomas MC, Ransbotyn G, Vance C. 2004. Review of the toxicology, human exposure and safety assessment for bisphenol A diglycidylether (BADGE). *Food Addit Contam* 21:905-919.

32. Andersen M, Kiel P, Larsen H, Haxild J. 1978. Mutagenic action of aromatic epoxy resins. *Nature* 276:391-392.

33. Steiner S, Crane AE, Watson WP. 1992. Molecular dosimetry of DNA adducts in C3H mice treated with glycidaldehyde. *Carcinogenesis* 13:119-124.

34. Scientific Committee on Food (SCF). 1999. Opinion on bisphenol A diglycidyl ether (BADGE), Commission of the European Communities. Available from: URL:http://eceuropa.eu./food/fs/sc/saf/out28_en.pdf

35. Semlitsch RD. 1990. Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. *Can J Zool* 68:1027-1030.

36. Yang YJ, Lee SY, Kim KY, Hong YP. 2010. Acute testis toxicity of bisphenol A diglycidyl ether in Sprague-Dawley rats. *J Prev Med Public Health* 43:131-137.

37. Sandermann H. 2008. Ecotoxicology of narcosis: Stereoselectivity and potential target sites. *Chemosphere* 72:1256-1259.

38. Hutler Wolkowicz IR, Aronzon CM, Pérez Coll CS. 2013. Lethal and sublethal toxicity of the industrial chemical epichlorohydrin on *Rhinella arenarum* (Anura, Bufonidae) embryos and larvae. *J Hazar Mater* 263:784-791.

39. Hutler Wolkowicz I, Herkovits J, Pérez-Coll CS. 2014. Stage-dependent toxicity of Bisphenol A on *Rhinella arenarum* (Anura, Bufonidae) embryos and larvae. *Environ Toxicol* 29:146–154

40. Un-jun H, Yun-jung Y, Su-kyoung K, Jae-hyoung Y, Soon-chul M, Sae-chul K, Yeon-pyo H. 2007. Developmental toxicity by exposure to bisphenol A diglycidyl ether during gestation and lactation period in Sprague-Dawley male rats. *J Prev Med Public Health* 40:155-161.

41. Beebee TJC, Griffiths RA. 2005. The amphibian decline crisis: a watershed for conservation biology? *Biol Conserv* 125:271-285.

Figure 1. BADGE Toxicity Profile (TOP) curves based on LC50 and their confidence limits for *R. arenarum* embryos (S.3-4) and larvae (S.25) continuously exposed for 336 h. Note that S.25 was significantly more resistant than S.3-4 during the whole bioassay ($P<0.05$).

Figure 2. BADGE Stage-dependent sensitivity assessed by 24-h pulse exposure of *R. arenarum*. Different letters indicate significant differences among stages ($P<0.05$).

Figure 3. Stereoscopic Microscopy of larvae continuously exposed to BADGE from S.25. (a) Control larvae. (b) Larvae exposed to 5 mg/L BADGE at 48 h. (c) Larva exposed to 5 mg/L BADGE at 168 h. Scale bar 1 mm. af: axial flexure; asp: abnormal skin pigmentation; cd: cellular dissociation; ud: underdeveloped caudal fin.

Figure 4. Stereoscopic Microscopy of 24-h pulse exposed *R. arenarum* embryos to BADGE at different developmental stages: (a) Control embryo in S.25; (b) S.17 (1 mg/L) at 120 h; (c) S.17 (2.5 mg/L) at 120 h.;(d) S.17 (5 mg/L) at 168 h; (e) S.18 (5 mg/L) at 96 h; (f) S.18 (5 mg/L) at 96 h; (g) S.21 (15 mg/L) at 24 hs; (h) S.23 (5 mg/L) at 24 h. Scale bar 1 mm.
a: acephaly; af: axial flexure; m: microcephaly; h: hidropsy; lgc: lack of gut coiling; od: oral desquamation, ssi: skin surface irregularities; t: tumors, ucf: underdeveloped caudal fin.

Table 1. Conditions of BADGE bioassays

Developmental stage	Treatment	Exposure concentrations (mg/L BADGE)
Blastula (S.3-S.4)	Continuous exposure	0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5
Complete Operculum (S.25)		1, 5, 7.5, 10, 12.5, 15
Blastula (S.3-S.4)	24-h pulse exposure	0.0001 - 10
Gastrula (S.10-S.12)		0.5 - 10
Rotation (S.15)		1 - 15
Tail Bud (S.17)		1 - 15
Muscular Activity (S.18)		0.1 - 15
Gill Circulation (S.20)		0.5 - 15
Open Mouth (S.21)		0.5 - 15
Opercular Folds (S.23)		5 - 17.5
Complete Operculum (S.25)		10 - 25

Table 2. Mortality rates (%) of *R. arenarum* embryos and larvae continuously exposed to BADGE. Data represent the percentage of mortality (mean ± SEM), n = 3. *Significantly different from vehicle control by DGC test ($p<0.05$).

Developmental stage	Concentration (mg/L BADGE)	Exposure time (h)		
		96	168	336
Embryos (S.3-4)	0	3.33 ± 3.33	6.67 ± 6.67	6.67 ± 6.67
	0.0001	3.33 ± 3.33	3.33 ± 3.33	13.33 ± 3.33
	0.0005	23.33 ± 8.82*	26.67 ± 6.67*	30.00 ± 5.77*
	0.001	23.33 ± 8.82*	23.33 ± 8.82*	30.00 ± 10.00*
	0.005	20.00 ± 10*	23.33 ± 8.82*	30.00 ± 5.77*
	0.01	16.67 ± 6.67*	16.67 ± 6.67*	26.67 ± 3.33*
	0.05	16.67 ± 6.67*	16.67 ± 6.67*	30.00 ± 0.00*
	0.10	30.00 ± 10.00*	36.67 ± 6.67*	43.33 ± 8.82*
	0.50	26.67 ± 3.33*	26.67 ± 3.33*	83.33 ± 12.02*
	1.00	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
	5.00	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
Larvae (S.25)	0	0.00 ± 0.00	0 .00 ± 0.00	0.00 ± 0.00
	1.00	0.00 ± 0.00	3.33 ± 3.33	3.33 ± 3.33
	2.50	3.33 ± 3.33	10.00 ± 10.00*	10.00 ± 10.00*
	5.00	0.00 ± 0.00	93.33 ± 6.67*	100.00 ± 0.00*
	7.50	0.00 ± 0.00	100.00 ± 0.00*	100.00 ± 0.00*
	10.00	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
	12.20	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
	15.00	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*

Table 3. Frequency (%) of embryos (a) and larvae (b) with sublethal effects after 96 h and 168 h BADGE treatment respectively. Data represent the percentage of sublethal effects (mean ± SEM), n = 3. *Significantly different from vehicle control by DGC test ($p<0.05$).

a)

Sublethal effects	Concentration (mg/L BADGE)						
	0	**0.0005**	**0.001**	**0.005**	**0.05**	**0.10**	**0.50**
Acephaly	-	4.3 ± 4.3	3.8 ± 3.8	4.2 ± 4.2	-	-	-
Reduced body size	-	-	-	-	-	-	100.0 ± 0.0*
Hydropsy	-	-	-	4.2 ± 4.2	8.0 ± 3.7	10.0 ± 4.2	100.0 ± 0.0*
Axial flexures	6.9 ± 3.5	8.7 ± 4.9	7.7 ± 3.9	-	-	-	-
Total abnormal embryos (%)	6.9 ± 3.5	8.7 ± 4.9	7.7 ± 3.9	8.3 ± 4.9	8.0 ± 3.7	10.0 ± 4.2	100.0 ± 0.0*

b)

Sublethal effects	Concentration (mg/L BADGE)		
	0	**1**	**5**
Hydropsy	14.3 ± 5.0	51.8 ± 5.3*	-
Abnormal skin pigmentation	-	51.8 ± 5.3*	100.0 ± 0.0*
Tail/axial flexure	-	-	100.0 ± 0.0*
Scare response to stimuli	-	-	100.0 ± 0.0*
Starvation	-	-	100.0 ± 0.0*
Total abnormal larvae (%)	14.3 ± 5.0	51.8 ± 5.3*	100.0 ± 0.0*

Table 4. LOAEL values and most common sublethal effects produced by BADGE at different developmental stages of *Rhinella arenarum*.

Developmental stage	Observation time (h)	Sublethal effects	LOAEL (mg/L BADGE)
Blastula (S.3-S.4)	24	Bifid spine Oral desquamation Tumors Microcephaly/acephaly Delayed development Axial flexures	0.1
	96-168	Tumors Microcephaly/acephaly Axial flexures Reduced body size Scare response to stimuli	0.1
	336	Reduced body size Scare response to stimuli	0.1
Gastrula (S.10-S.12)	24	Delayed development Persistent yolk plug Cellular dissociation Bifid spine Tumors Microcephaly/acephaly Reduced body size	1
	96-168	Cellular dissociation Hydropsy Microcephaly/acephaly Skin surface irregularities Axial flexures Abnormal skin pigmentation Reduced body size Scare response to stimuli	1
	336	Hydropsy Axial flexures Reduced body size Scare response to stimuli	1
Rotation (S.15)	24	Reduced body size Microcephaly Hydropsy Axial flexures	1
	96-168	Delayed development	1
	336	Reduced body size	1
Tail Bud (S.17)	24	Delayed development	1
	96-168	Cellular disociation Hydropsy Microcephaly/acephaly Mouth malformations Scare response to stimuli	1
	336	Reduced body size Hydropsy Scare response to stimuli	1

Muscular Activity (S.18)	24	Reduced body size Axial flexures	5
	96-168	Hydropsy Abnormal skin pigmentation Scare response to stimuli	5
	336	Hydropsy Abnormal skin pigmentation Tail flexures	5
Gill Circulation (S.20)	24	Delayed development	0.5
	96-168	Scare response to stimuli	0.5
	336	Starvation Scare response to stimuli	0.5
Open Mouth (S.21)	24	Abnormal skin pigmentation Scare response to stimuli	5
	96-168	Hydropsy Abnormal skin pigmentation	5
	336	Reduced body size	5
Opercular Folds (S.23)	24	Delayed development Scare response to stimuli	10
	96-168	Delayed development	10
	336	-	>17.5
Complete Operculum (S.25)	24	Scare response to stimuli	10
	96-168	Axial flexures	10
	336	Reduced body size	15

(-) There were no sublethal effects.

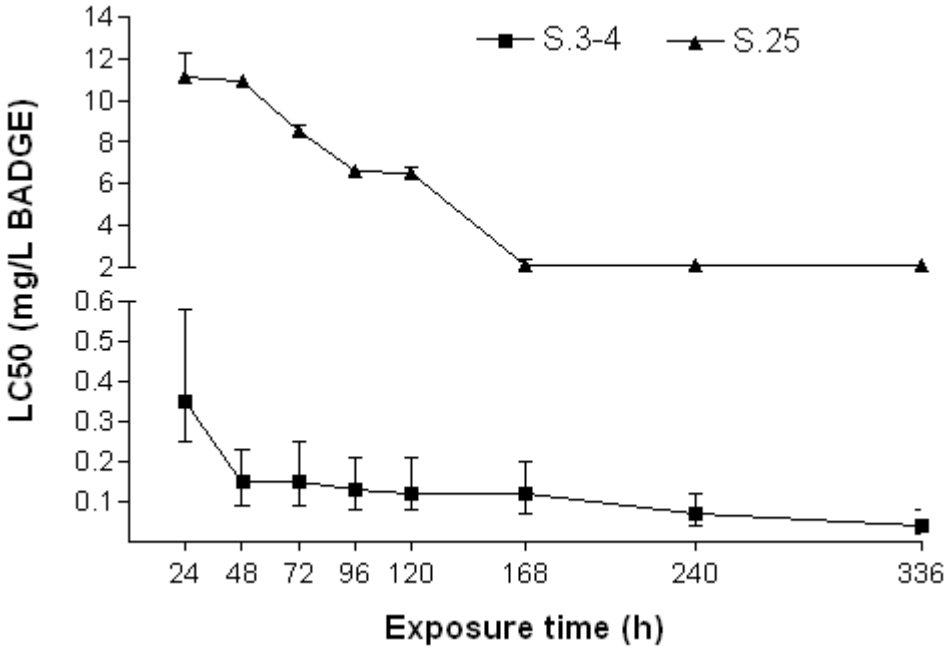

Figure 1

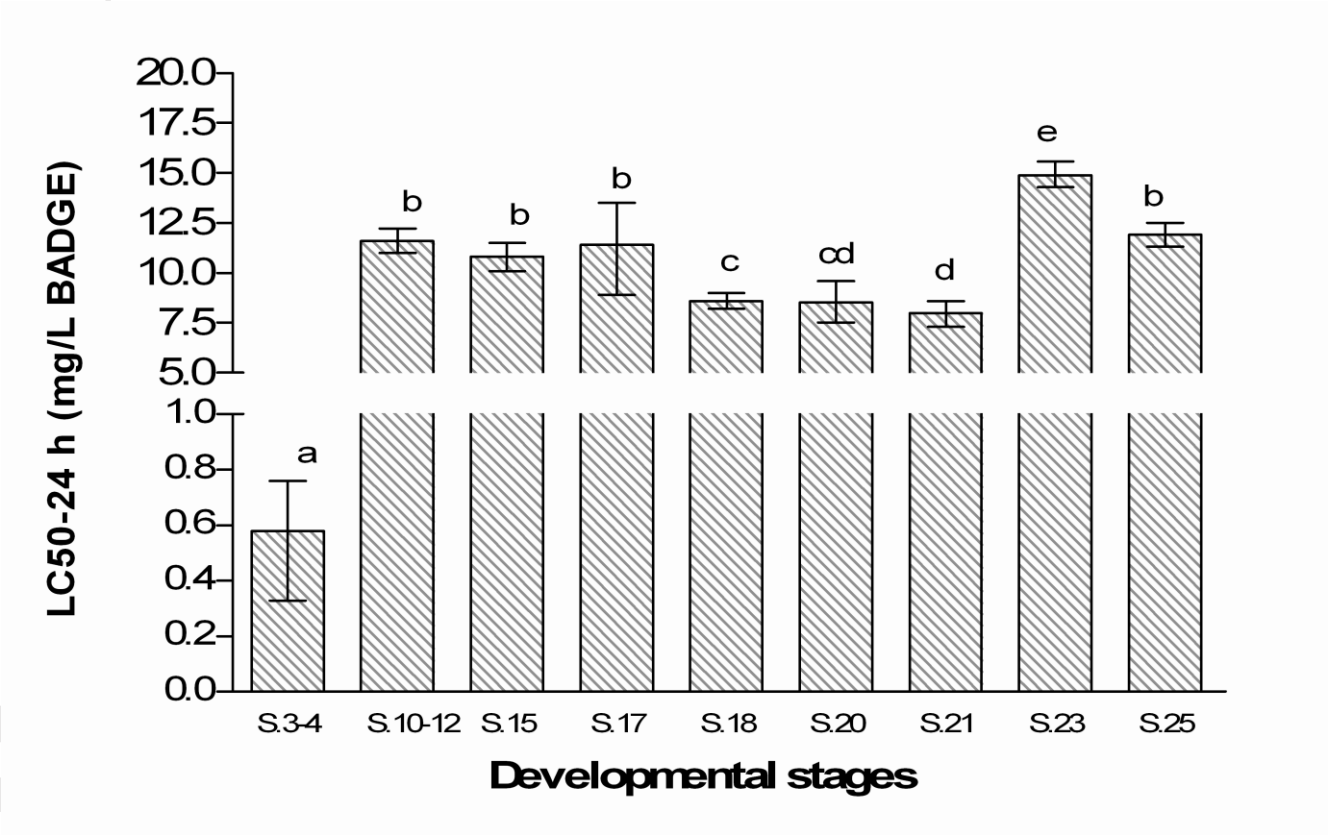

Figure 2

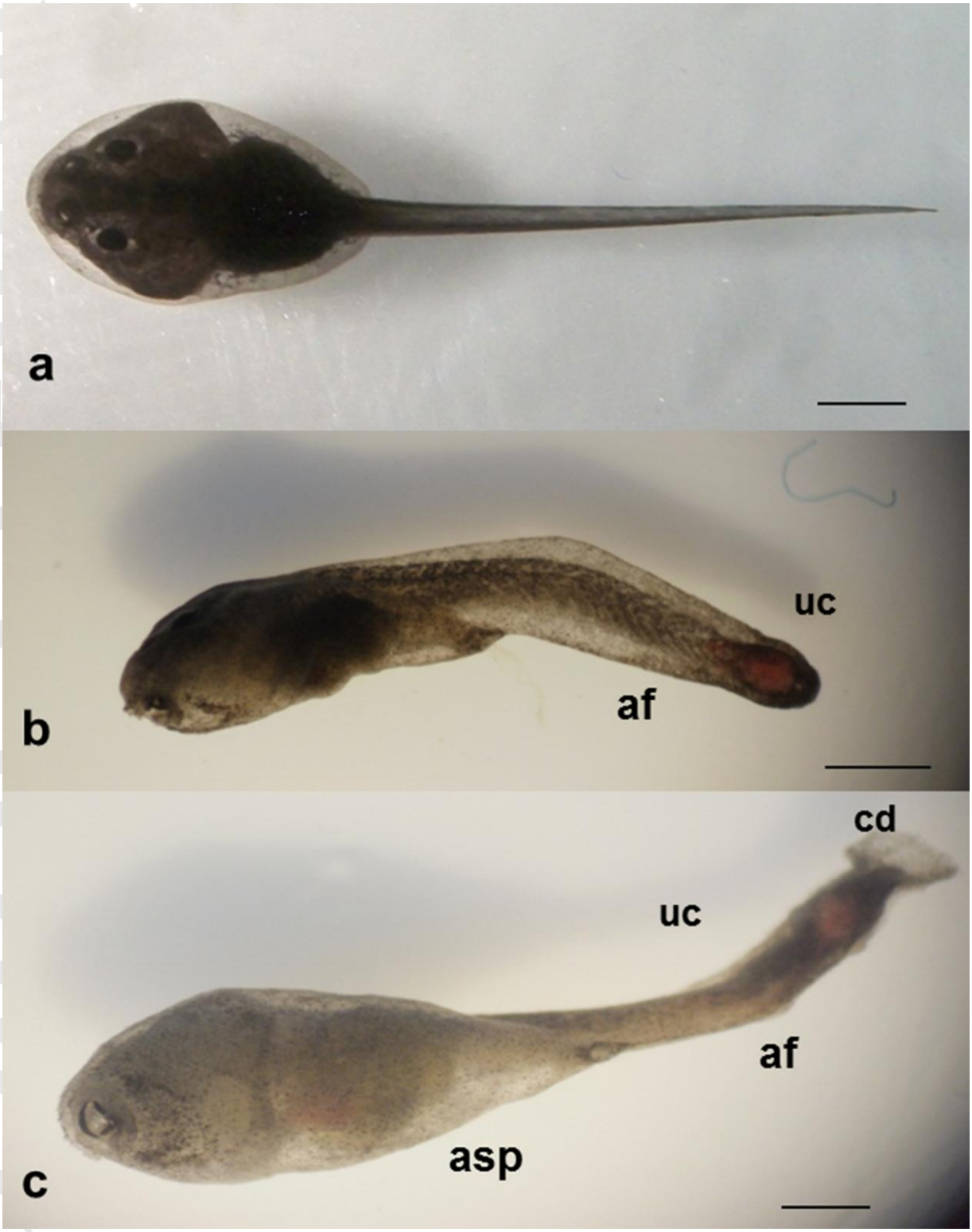

Figure 3

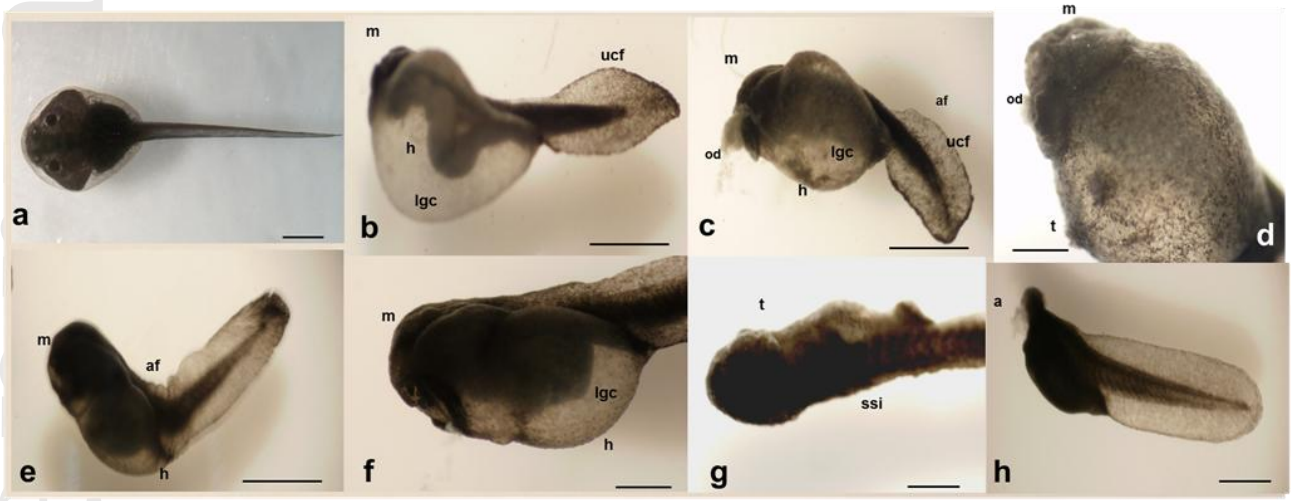

Figure 4