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Effects of a fungicide formulation on embryo-larval development, metamorphosis, and gonadogenesis of the South American toad *Rhinella arenarum*

Gabriela Svartz[a,c], Fernando Meijide[b,c], Cristina Pérez Coll[a,c,*]

[a] Instituto de Investigación e Ingeniería Ambiental (3iA) y Escuela de Ciencia y Tecnología (ECyT), Universidad Nacional de San Martín (UNSAM), Av 25 de Mayo y Francia, C1650, San Martín, Buenos Aires, Argentina
[b] Departamento de Biodiversidad y Biología Experimental (FCEN-UBA), Ciudad Universitaria, C1428, Buenos Aires, Argentina
[c] CONICET, Argentina

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ABSTRACT

Sublethal toxicity of the formulated fungicide Maxim® XL on embryonic, larval and juvenile development of *Rhinella arenarum* was evaluated by means of standardized bioassays. Maxim® XL, one of the most used fungicides in Argentina, is based on a mixture of two active ingredients: Fludioxonil and Metalaxyl-M. Maxim® XL exposure induced severe sublethal effects on the embryos, expressed as general underdevelopment, axial flexures, microcephaly, cellular dissociation, abnormal pigmentation, underdeveloped gills, marked edema and wavy tail. As the embryo development advanced, alterations in behavior as spasmodic contractions, general weakness and inanition were observed. Maxim® XL did not affect neither the time required to complete metamorphosis nor sex proportions, but gonadal development and differentiation were impaired. Gross gonadal analysis revealed a significant proportion of exposed individuals with underdevelopment of one or both gonads. Histological analysis confirmed that 18% and 10% of the individuals exposed to 0.25 and 2 mg/L Maxim® XL, respectively, exhibited undifferentiated gonads characterized by a reduced number (or absence) of germ cells. Taking into account the risk evaluation performed by means of Hazard Quotients, this fungicide could be a threat to *R. arenarum* populations under chronic exposure. This study represents the first evidence of toxic effects exerted by Maxim® XL on amphibians. Finally, our findings highlight the properties of this fungicide that might jeopardize non-target living species exposed to it in agricultural environments.

1. Introduction

Maxim® XL is a broad spectrum therapeutic fungicide for seed treatment, used to protect germination and the early development of crops (Syngenta, 2016). Maxim® XL, based on a mixture of two active ingredients: 2.5% Fludioxonil and 1% Metalaxyl-M, is one of the most used formulated fungicides in Argentina, with annual application of 290,400 L (CASAFE, 2009). Despite its intensive use, there are few studies reporting environmental levels of these two active ingredients in surface water. In United States, it was reported a high occurrence of metalaxyl levels in water bodies, detecting about 0.67 μg/L (Battaglin et al., 2011). According to Syngenta, Maxim® XL is considered to be highly toxic to aquatic organisms. Fludioxonil, 4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile, belongs to the chemical class of phenylpyrroles. It is a chemical compound closely related to a natural bioactive molecule produced by a soil bacterium, *Pseudomonas* sp. Fludioxonil acts by contact and partial penetration, disrupting the intercellular fungus exchange at membrane level and reducing absorption of amino acids and sugars, and thereby inhibiting the fungal mycelium development. According to WHO, fludioxonil toxicity belongs to class III, moderately dangerous, while the European classification 67/548/EEC or 1999/45/EC considers it very toxic to aquatic organisms, causing long-term adverse effects. It is slightly persistent in soil and it is degraded by microbial activity under aerobic conditions, persisting under anaerobic conditions and absence of light. Due to its high resistance to hydrolysis and long persistence, residues in aquatic environments near grapevine crops were reported (NRAAVC, 2000). Although the toxicity data of this fungicide on non-target species is scarce,

* Corresponding author at: Av. 25 de Mayo y Francia, C1650, San Martín, Buenos Aires, Argentina.
E-mail addresses: fmeiji@gmail.com (F. Meijide), perezcoll@unsam.edu.ar, cris060957@yahoo.com.ar (C. Pérez Coll).

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it is known that algae show higher sensitivity to the fungicide (IC 50 = 4.55 mg/L for *Scenedesmus acutus*) than vascular plants (IC 50 > 100 mg/L for *Lemna minor*) (Verdisson et al., 2001). In humans, fludioxonil is considered a potential endocrine disruptor, with antiestrogenic activity in mammary cancer cells (Orton et al., 2011).

Metalaxyl-M, *N*-(2,6-dimethylphenyl)-*N*-(2′-methoxyacetyl)-D-alanine methyl ester, belongs to the chemical class of acylalanines and is the most biologically active isomer of metalaxyl. It is used not only in agriculture but also in grasses and ornamental plant crops. Metalaxyl inhibits mycelial growth and the formation of spores penetrating the seed coat and translocating systemically throughout the growing seedling. Regarding its effects on non-target species, Sakr et al. (2011) reported that metalaxyl induced nephrotoxicity in mice. Demsia et al. (2007) found that metalaxyl induced *in vitro* micronucleus formation and sister-chromatid exchange induction in human lymphocytes. Metalaxyl interacts with the RNA polymerase-I-template complex, inhibiting the incorporation of ribonucleotide triphosphates into ribosomal RNA (Hassall, 1990). Its very low absorption in soil, high solubility in water, and high persistence in water, with a half life of 47.5 days, make metalaxyl-m a potential contaminant in water bodies. In amphibians, lethal and sublethal effects of metalaxyl-M were reported only for *Rana pipiens* larvae. Hayes et al. (2006) reported 35% mortality of larvae exposed to 0.1 μg/L at the beginning of metamorphosis.

Taking into account the wide use of this product for fungal control in soybean and the monoculture model being printed in the Argentinean agro-ecosystems, it is critical to evaluate the toxicity of this fungicide in key native species as amphibians during the most sensitive life cycle stage such as the embryo-larval development. The aim of the present study was to assess the sublethal effects of Maxim® XL on embryonic, larval and juvenile stages of the South American toad *Rhinella arenarum*, using the standardized AMPHITOX bioassay (Herkovits and Perez-Coll, 2003). The endpoints included teratogenesis, behavioural alterations, and impairment of metamorphosis and gonadogenesis. An ecological risk assessment of Maxim® XL for *R. arenarum* was also performed using the Hazard Quotient approach (USEPA, 1998).

2. Materials and methods

2.1. Acquisition of R. arenarum embryos

To examine the potential effects of the commercial formulation Maxim® XL on the embryo-larval development of *R. arenarum*, 3 mating pairs of adults weighing 200–250 g per animal were acquired in a non-impacted site, Lobos (Buenos Aires province, Argentina: 35°11′S, 59°05′W). Toad care, breeding, embryo acquisition and analysis were conducted according to the methods described in the AMPHITOX protocols (Herkovits and Perez-Coll, 2003). Briefly, ovulation of females was induced by means of an intraperitoneal injection of a suspension of one homogenized toad pituitary gland in 1 mL AMPHITOX solution (AS) per female preserved according to Pisanó (1956), plus 2500 IU human chorionic gonadotropin (hCG). The composition of AS was NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L, and $NaHCO_3$ 2 mg/L prepared in distilled water. Oocytes were fertilized *in vitro* using a testicular macerate homogenate suspended in AS, resulting in a spermatozoid suspension of 10%. The sperm viability was confirmed by observing the spermatozoid morphology and movements under an optical microscope. The eggs were inspected for quality and fertility and were considered acceptable if the fertility rate was greater than 75%, and embryo survival at the neurula stage was greater than 70%. The jelly coat was dissolved by immersing egg ribbons in a solution of 2% thioglycolic acid at pH 7.2 containing 1.35 mL saturated NaOH solution in 100 mL AS. This step was followed by a thorough wash of embryos. Embryos were staged according to Del Conte and Sirlin (1951) and larval stages according to Echeverria and Fiorito de Lopez (1981). Embryos were kept in AS and maintained at 20 ± 2 °C. The AS was replaced entirely every three days and monitored weekly to ensure that the pH was at acceptable levels (7 ± 0.5).

2.2. Preparation of test solutions

Toxicity tests were performed using the commercial formulation Maxim® XL which is commercialized in Argentina by Syngenta. MAXIM® XL is a water-based flowable seed treatment for control of fungal diseases in registered crops. This formulation contains a mixture of two active ingredients (a.i): 2.5 g/100 mL (2.5% w/v) of Fludioxonil (CAS N° 131341-86-1) and 1 g/100 mL (1% w/v) of Metalaxil-M (CAS N° 70630-17-0). Concentrations tested were: 0.25, 0.5, 1, 2, 3, 5 and 10 mg/L Maxim® XL. Test solutions were prepared in AS and they were used immediately after their preparation.

2.3. Toxicity bioassays

Ten embryos at early blastula stage (S.4) were randomly placed in triplicate 10 cm-diameter glass Petri dishes containing 40 mL of test solution. The toxicity bioassays were performed by continuous exposure of embryos to Maxim® XL, maintained at 20 ± 2 °C and a 12:12 h photoperiod, up to metamorphosis (75 days). When specimens reached the complete operculum stage (S.25, free swimming larvae), they were fed daily with 3 granules (6 ± 0.5 mg) of balanced fish food TetraColor® per Petri-dish. Test solutions were entirely replaced every 48 h. Control groups were simultaneously maintained in AS without additions.

The analysis of the endpoints in post-metamorphic individuals were performed with larvae surviving at 0.25, 0.5, 1, 2 and 3 mg/L Maxim® XL and their respective controls, which were maintained without exposure up to 60 days post-metamorphosis that is the estimated time to reach sexual differentiation. Metamorphosed juveniles (complete tail resorption) were transferred to glass flasks with 100 g of sand embedded on AS and maintained wet by spraying with AS daily. Juveniles were fed with micro-crickets, *Acheta domestica* (~3 mm).

2.4. Effects assessment

Throughout the study, mortality was recorded daily. The teratogenic and neurotoxic effects of Maxim® XL were evaluated daily, comparing the alterations observed in exposed individuals with the normal development and behavior of controls. Feeding behavior was qualitatively assessed. Abnormalities were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed and recorded with a Sony DSC-S90 digital camera, and identified according to Bantle et al. (1998). Embryos with significant adverse effects and control embryos were fixed in 4% formalin, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy (SEM) by means of the critical point drying technique and observed in a Philips XL-30 operated at 10 kW for ultrastructure evaluation.

At 60 days post-metamorphosis, juveniles were euthanized by submersion in a lethal concentration of benzocaine solution and their snout-vent lengths (SVL) were measured. Individuals were dissected under a Zeiss Stemi DV4 stereo microscope and their gonads were measured and examined for sex classification and gross gonadal anomalies. Digital photographs were taken of each specimen. A subset of the juveniles examined for gross morphology of the gonads was also examined for gonadal histology.

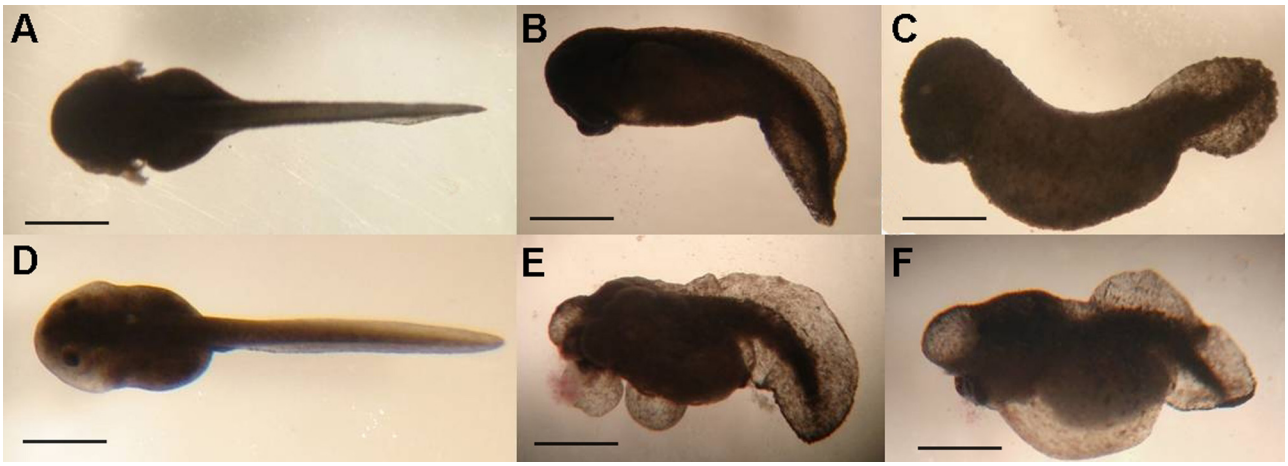

Fig. 1. Stereomicroscope photographs of *R. arenarum* exposed to Maxim® XL at different concentrations starting at blastula stage (S.4): (A) control embryo (S.23), 96 h; (B) specimen exposed to 5 mg/L Maxim® XL, 96 h, (C) specimen exposed to 10 mg/L Maxim® XL, 96 h; (D) control larva (S.25), 216 h; (E, F) specimens exposed to 5 mg/L Maxim® XL, 216 h. All exposed specimens exhibited general underdevelopment, microcephaly, marked edema and axial curvature.

Gonad-kidney complexes were dissected and fixed in Bouin's solution for 24 h. Fixed organs were dehydrated in a graded ethanol series and embedded in paraffin. Serial cross-sections (7 μm) were made and stained with hematoxylin and eosin. All slides of sectioned ovaries and testes were observed and photographed under a Nikon Microphot FX microscope.

2.5. Data analysis

Effective and lethal concentrations (EC10 and LC10) were statistically estimated for each larval stage by using the USEPA Probit program (USEPA, 1988). The Teratogenic Index (TI) was calculated as the LC10 divided by the EC10, establishing a TI > 1.5 as a high risk for embryos to be malformed in the absence of significant embryonic lethality (American Society for Testing and Materials, 1993). Median time to complete metamorphosis at each treatment was analyzed by Kruskal-Wallis test. Median body length (SVL) values were statistically compared by one-way ANOVA. Sex ratios were statistically analyzed by Fisherís exact test, and Pearson's chi-square was used to test for significant differences in the incidences of gonadal anomalies. Analyses were performed using GraphPad Prism software version 6.03 and differences were considered to be significant when $p < 0.05$.

2.6. Ecological risk evaluation

The Hazard Quotient (HQ) is the ratio of the expected environmental concentration (EEC) (Boutin et al., 1993, 1995) and the level at which no adverse effects are expected (EC10). The use of EC10s has been advocated for deriving protective concentrations in lieu of no-effect concentrations (NOECs) estimated using hypothesis-testing techniques, which are usually driven by experimental design issues (Carriger et al., 2011). The EEC of a pesticide is a theoretical concentration based on a worst-case scenario for exposure of non-target aquatic and terrestrial habitats interspersed within or adjacent to proposed use areas. The expected environmental concentration (EEC) for Maxim® XL was based on 10% of the maximum application rate given on manufacturer labels. The maximum concentration applied of this commercial formulation is 175 g/L/ha (Syngenta, 2016). The EEC was calculated assuming a water depth of 15 cm and an area of 1 m^2 (Boutin et al., 1993, 1995). The hazard quotient (HQ) was calculated as EEC/EC10 and compared with the USEPA level of concern (LOC) (USEPA, 1998). The LOC is a policy tool that the USEPA uses to interpret the hazard quotient, analyze the potential risk to non-target organisms, and evaluate the need to consider regulatory action. The LOC value for risk is 1. If the hazard quotient is greater than 1, harmful effects are likely to occur due to the contaminant in question.

Table 1
Initial and final sample size (n) of metamorphosed individuals, and mortality percentages recorded during the post-metamorphic period.

Treatment	Initial sample size (number of metamorphosed individuals)	Mortality (%)	Final sample size (60 days post metamorphosis)
Control	20	20.00	16
0.25 mg/L Maxim® XL	16	31.25	11
0.5 mg/L Maxim® XL	14	50.00	7
1.0 mg/L Maxim® XL	12	50.00	6
2.0 mg/L Maxim® XL	21	52.38	10
3.0 mg/L Maxim® XL	4	25.00	3

3. Results

3.1. Effects on embryo-larval development

Concentrations over 2 mg/L Maxim® XL caused highly relevant sublethal effects from 48 h of exposure onwards; EC10 at 48 h was 1.12 mg/L Maxim® XL. The percentage of affected embryos was proportional to Maxim® XL concentration reaching a maximum of 75% at 10 mg/L. At acute time (96 h), embryos exposed to 5 and 10 mg/L Maxim® XL were significantly delayed (S.21) with respect to the control group (opercular folds stage, S.23), exhibiting malformations such as axial curvatures, microcephaly, and underdeveloped gills (Fig. 1). LC10 and EC10 at 168 h were 3.52 and 0.43 mg/L Maxim® XL respectively, so the TI for Maxim® XL was 8.19. As development progressed, marked edema and wavy tail were observed (Figs. 1 and 2). Different cell surface dissociation degrees and general epithelium disorganization were also observed in larvae exposed to 5 mg/L Maxim® XL (Fig. 2). Larvae at complete operculum stage (S.25, free swimming larvae) exhibited neurotoxic signs such as spasmodic contractions, weak movements, erratic swimming and non-feeding behavior

No significant differences were observed in the time to complete metamorphosis between Maxim® XL-exposed and control larvae. Once metamorphosis was completed, mortality was high both in control and exposed individuals due to captivity conditions (Table 1).

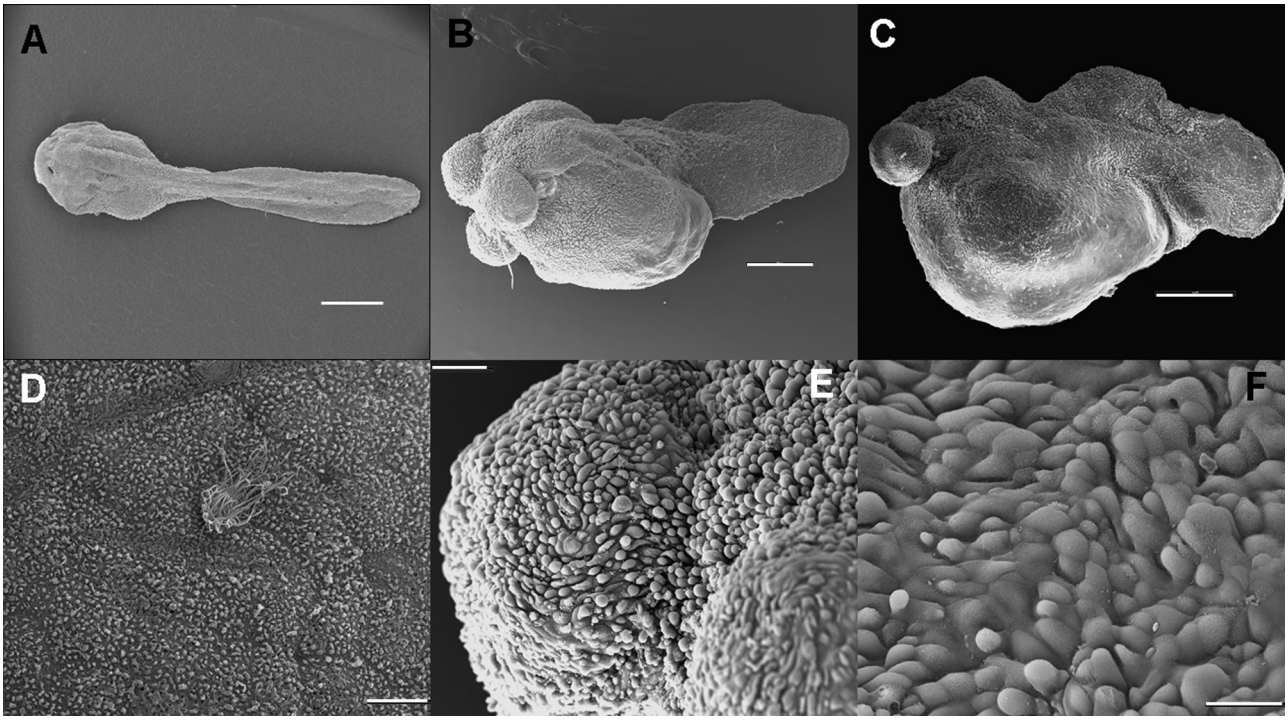

Fig. 2. SEM photographs of *R. arenarum* continuously exposed to Maxim® XL starting at the blastula stage (S.4) and fixed at 240 h: (A) control larva (S.25); (B, C) specimens exposed to 5 mg/L Maxim® XL exhibited general underdevelopment, microcephaly, wavy tail and edema; (D) detail of control larva epithelium; (E, F) detail of the epithelium of specimens exposed to 5 mg/L Maxim® XL showing different cell surface dissociation degrees and general disorganization of the epithelium. Scale bars: 1000 μm (A); 500 μm (B, C); 10 μm (D); 100 μm (E); 50 μm (F).

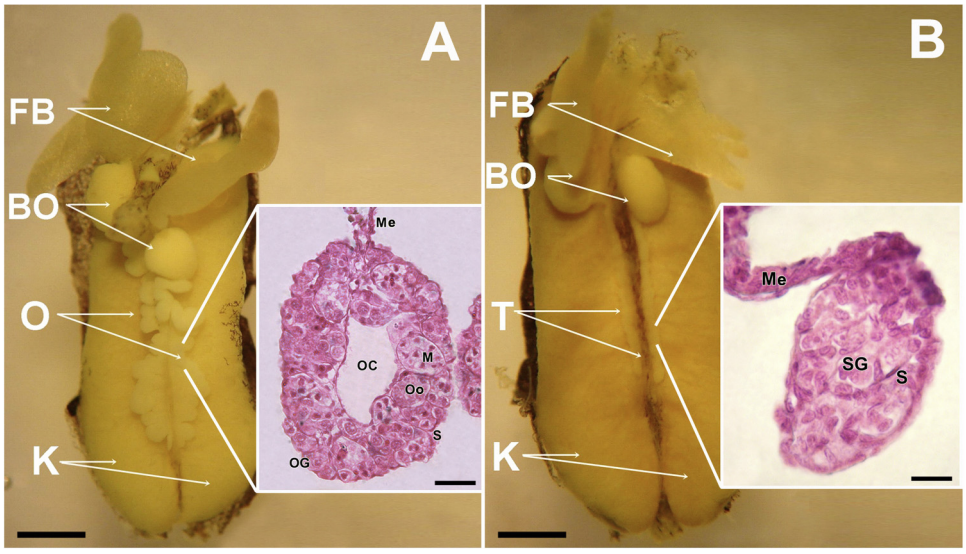

Fig. 3. Gonadal development in 60 day post-metamorphic *R. arenarum* from control treatments. Stereomicroscope photographs of the kidney-gonad complex and insets showing light-microscope photographs of representative histological sections of the gonads. (A) Female; (B) Male. Scale bars: 500 μm (A, B), 50 μm (inset A), 20 μm (inset B). BO: Bidder's organ; FB: fat body; K: kidney; M: mitotic figures; Me: gonadal mesentery; O: ovary; OC: ovarian cavity; OG: oogonia; Oo: oocytes in early meiosis; S: somatic cells; SG: spermatogonia; T: testis.

3.2. *Effects on gonads of post-metamorphic juveniles*

Sex of the individuals was determined during gross examination for gonadal anomalies. The sex ratios of juveniles from control and Maxim® XL treatments were not significantly different from an expected 50:50 ratio. Likewise, there were no significant differences in juvenile size (SVL) and gonadal length between control and Maxim® XL-exposed individuals. After 60 days post-metamorphosis, gonads of control animals were observed as paired organs located ventrally to the kidney. Ovaries were thicker than testes and constituted by multiple lobes of irregular contour. Bidder's organs, characteristic of Bufonidae, were observed between the gonads and fatty bodies (Fig. 3A, B). The macroscopic analysis showed a relatively high proportion of exposed individuals exhibiting underdevelopment of one (Fig. 4A, B) or both (Fig. 4C) gonads, these gonads being thinner than ovaries or testes of control specimens. Gonadal alterations observed under stereoscopic microscopy were confirmed by histological analysis. Using Pearson's chi-square

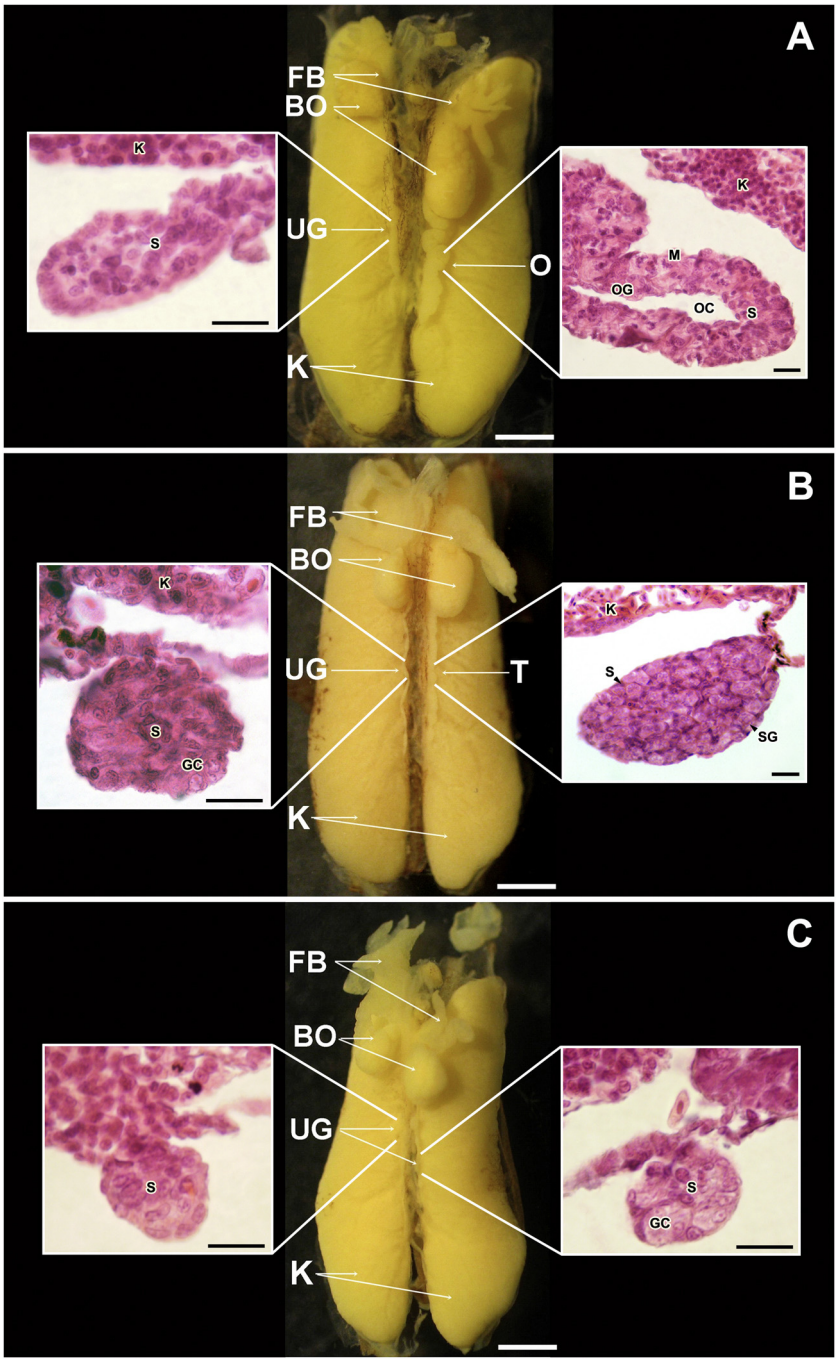

Fig. 4. Gonadal development in 60 day post-metamorphic *R. arenarum* from Maxim® XL treatments. Stereomicroscope photographs of the kidney-gonad complex and inset showing light-microscope photographs of representative histological sections of the gonads. (A) Female from 0.25 mg/L Maxim® XL treatment with underdeveloped right gonad; (B) Male from 2 mg/L Maxim® XL treatment with underdeveloped right gonad; (C) Juvenile from 0.25 mg/L Maxim® XL treatment showing underdevelopment of both gonads. Underdeveloped gonads exhibited scarce to complete absence of germ cells, so that frequently only somatic cells were observed in cross sections. Scale bars: 500 μm (A–C), 20 μm (insets A–C). BO: Bidder's organ; FB: fat body; GC: germ cells; K: kidney; M: mitotic figures; O: ovary; OC: ovarian cavity; OG: oogonia; S: somatic cells; SG: spermatogonia; T: testis; UG: underdeveloped gonad.

test, significant differences in the incidence of gonadal anomalies were found for 0.25 and 2 mg/L Maxim® XL treatments compared to the control group. One or both gonads showed impaired development in 18% of the individuals exposed to 0.25 mg/L Maxim® XL (the lowest concentration tested) and 10% of those exposed to 2 mg/L Maxim® XL. In most histological sections, gonads contained only somatic cells (left insets Fig. 4A, C), while in some sections germ cells occasionally occurred and the gonad exhibited an undifferentiated appearance (left inset Fig. 4B, right inset Fig. 4C). These features contrasted with the more advanced degree of development displayed by ovaries and testes from control individuals (insets Fig. 3). In control females, the ovaries were distinguished by the presence of the ovarian cavity, surrounded by a germinal tissue in which oogonia associated to supporting somatic cells (pre-follicle cells), mitotic figures corresponding to proliferating oogonia, and oocytes at the onset of meiosis, were observed (inset Fig. 3A). In control males, the testes contained numerous spermatogonia surrounded by supporting somatic cells (pre-Sertoli cells) (inset Fig. 3B)

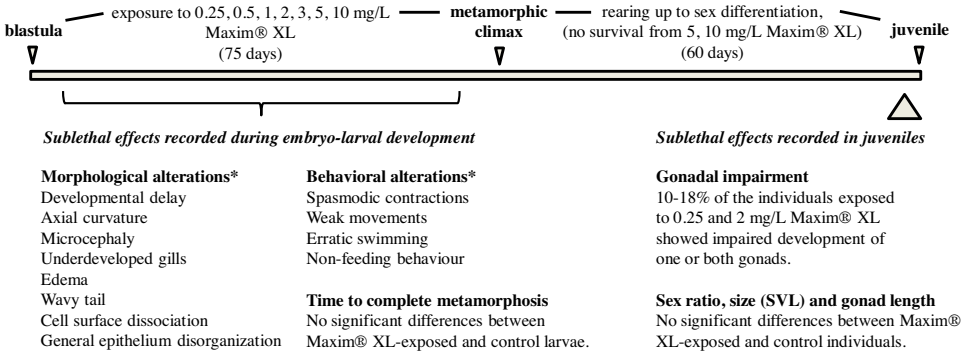

Fig. 5. Conditions of Maxim® XL treatments and sublethal effects observed in *Rhinella arenarum* early life stages after exposure throughout embryo-larval development. *Effects observed at concentrations ≥ 2 mg/L Maxim® XL.

Fig. 5 summarizes the treatment conditions and the sublethal effects observed in Rhinella arenarum early life stages after exposure to Maxim® XL throughout embryo-larval development.

3.3. Ecological risk evaluation

The expected environmental concentration (EEC) for Maxim® XL was calculated as 10% of the maximum application rate allowed (175 g/L/ha), so the EEC was 1.75 mg/L/m2. The EC10 at 168 h for embryo development was 0.43 mg/L Maxim® XL, so the hazard quotient (HQ = EEC/EC10) was 4.07, i. e. over the LOC value.

4. Discussion

The results obtained in this study demonstrate that Maxim® XL causes severe sublethal effects both in *R. arenarum* embryo-larval development and juveniles. Maxim® XL was highly teratogenic, reaching a Teratogenic Index (TI) of 8.19. This value might be considered relevant, since a TI higher than 1.5 implies a high risk for embryos to be malformed in the absence of significant embryo lethality (American Society for Testing and Materials, 1993). The difference between Maxim® XL concentrations that cause lethal and sublethal effects suggests to consider malformations as a relevant endpoint to assess population viability that might be affected by reduced fitness of individuals.

The sublethal effects observed on embryos after Maxim® XL exposure are usually exerted by other pesticides, such as insecticides (Svartz et al., 2016) and other fungicides (Bernabò et al., 2015). Thus, they can be considered as non-specific effects. The reduced food intake, probably associated with a metabolic disruption, also contributed to the reduced growth in Maxim® XL exposed embryos. Edema, also a noteworthy sublethal effect observed in amphibian embryos exposed to Maxim® XL, could be related to a disturbance in the ionic balance (Nieves-Puigdoller et al., 2007) and disruption of the endocrine system (Herkovits et al., 1980), as reported for other pesticides.

By extending the exposure period during larval development, the time to complete metamorphosis was not altered. Also, no significant differences were found neither in juvenile size nor in sex ratios between treated and control animals. However, upon reaching the period of sex differentiation, individuals exposed to 0.25 and 2 mg/L Maxim® XL showed underdevelopment of one or both gonads, which was confirmed by histological analysis. These gonadal alterations may be attributed to an endocrine disrupting effect of Maxim® XL on gonad development. It would be important to continue the assessment of the effects of this and other fungicides on the endocrine system, because a recent study demonstrated that many fungicides with previously unknown endocrine activity were revealed as endocrine-disrupting chemicals (Orton et al., 2011). Moreover, the assessment of the histological alterations could be considered an "early warning" signal to assess environmental quality because they commonly occur at lower exposure concentrations than other endpoints, such as behavioral alterations and mortality (Leino et al., 2005).

Although Maxim® XL is a fungicide especially appropriate for seed treatment, the results obtained in this study provide useful information about the effects of the formulation on amphibian development in the case of, for example, spill events. Developmental disorders can make amphibians more vulnerable, having decreased ability to escape from unfavorable conditions such as predators, infectious agents, invasive species, and changes in physical and chemical environmental characteristics, influencing the physical condition of animals or their reproductive success (Egea-Serrano et al., 2012), thus causing an indirect risk to the population continuity. The HQ approach provides a possibility to assess the risk for adverse effects of Maxim® XL to *R. arenarum*. Based on the results obtained from the risk assessment, the HQ could take values higher than the LOC value highlighting that Maxim® XL represents a threat for the viability of the populations of this native amphibian.

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