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Morphological abnormalities in natural populations of the common South American toad *Rhinella arenarum* inhabiting fluoride-rich environments

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A B S T R A C T

Morphological abnormalities in amphibians may be attributed to contaminants, ultraviolet radiation and trematode parasites, or a synergistic effect between them. In the present study, morphological abnormalities in *Rhinella arenarum* adults from natural and artificial fluoride-rich environments were identified and evaluated. Three sites were sampled in central Argentina: Los Vallecitos stream (LF-LV), Los Cerros Negros stream (MF-CN), and Decantation ponds (HF-DP), with low (0.33 mg/L), middle (2.03 mg/L) and high (14.0 mg/L) fluoride levels respectively; the latter site is associated with a fluoride mine. Abnormal individuals were photographed and then standard radiographs were taken. Abnormality frequencies and relative percentage of abnormal individuals were calculated for each site. In addition, skeletochronology was used to estimate toad's age. Five abnormality types were identified: syndactyly, ectrodactyly, polydactyly, microphthalmia and ectromelia. Percentages of abnormal individuals per site were: LF-LV = 4%, MF-CN = 21.2% and HF-DP = 6.4%. The MF-CN and HF-DP populations had morphological abnormality frequencies that exceeded the reference value (5%) reported in the literature. The average age did not differ between sites. The results of this study indicate that there is an association between frequency of morphological abnormalities and high fluoride levels.

1. Introduction

Sentinel organisms were defined by Stahl (1997) as "… any non-human organism that can react to an environmental pollutant before the contaminant impacts humans …" For detection of local perturbations, these organisms should be abundant in the study area, have a low rate of migration and be limited to a small space (Flickinger and Nichols, 1990). Therefore, the use of biological indicators helps to evaluate not only the physico-chemical integrity of an environment but also the responses of these organisms to pollution-induced environmental changes. Accordingly, some features of anuran amphibians make them sensitive to environmental contaminants and maximize their exposure: a permeable skin and a complex life cycle that alternates aquatic and terrestrial life stages, and the consumption of plants and animals at different stages of their life cycle both in water and on land (Duellman and Trueb, 1994; Beebee, 1996; Young et al., 2004). In recent years, concerns arising about amphibian malformations have led to an increase in ecotoxicological studies of amphibians (Burlibaşa and Gavrilă, 2011), principally the role of chemical contaminants as the top stressor (Ankley et al., 2004; Gurushankara et al., 2007; Peltzer et al., 2011). Contributions on this topic are mainly based on concerns about possible adverse effects that might also occur in humans (Ankley et al., 2004).

Fluoride is an element that occurs naturally in the earth's crust and freshwater (Camargo, 2003; Rosso et al., 2011). Nevertheless, in humans, a high concentration of fluoride, causes adverse effects on different organs such as liver and kidney (Guan et al., 1998; Xiong et al., 2007), neurotoxicological alterations (Choi et al., 2012), dental and skeletal fluorosis (World Health Organization (WHO), 2011). In amphibians, negative effects of high fluoride concentrations, such as growth inhibition, behavioral changes, metabolism alterations, bone abnormalities, generalized edema, stunted growth, and ruffled dorsal and/or ventral fin were found in laboratory (Camargo, 2003; Chen et al., 2016; Chai et al. 2016, 2017).

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In situ studies have ecological relevance because they provide real exposure scenarios, which cannot be replicated in the laboratory (Crane et al., 2007). However, *in situ* studies identifying morphological abnormalities in amphibians that inhabiting environments with high fluoride content are absent. The aim of this study was to identify and evaluate the types of morphological abnormalities in adults of *Rhinella arenarum* from natural and artificial environments rich in fluoride. Also, we determine age of the toad's because the literature suggests that age of amphibian populations could change significantly with the environmental pollution (Spear et al., 2009; Zhelev et al., 2014; Kaczmarski et al., 2016; Bionda et al., 2018; Otero et al., 2018). This is a native species of anuran with a wide distribution in the Neotropics (present in Argentina, Bolivia, Brazil, Uruguay and most likely in Paraguay) (Frost, 2018) widely used as a sentinel organism in the laboratory and in the field studies for the monitoring of the aquatic environment (Vera Candioti et al., 2010; Pollo et al. 2015, 2017), its sensitivity has been assessed in several studies (e.g. Herkovits et al., 2002; Brodeur et al., 2009; Bosch et al., 2011; Pollo et al., 2015; Otero et al., 2018). Adult individuals generally form large breeding groups at lentic and lotic water bodies. Eggs are deposited in large gelatinous strings along the edges of ponds (Kehr, 1994; Bionda et al., 2011).

2. Materials and methods

2.1. Study area and field work

The sampling area was located in the central-southern Sierra de Comechingones, Córdoba, Argentina (Cerro Áspero batholith: 32° 50′ 22.85´´ S; 64° 79′40.60´´ W; altitude 1200 m.a.s.l). This batholith contains 1.210 mg fluoride/kg rock. Fluoride is extracted from an open pit and is recovered physically by a flotation process. The wastewater generated by flotation process is deposited on a series of artificial earth dams, two of which have vegetation, generating a suitable environment for anuran development.

The landscape corresponds to a mountain environment, dry with grasslands typical of xerophilous forest (Oggero and Arana, 2012). We select two environments with a high concentration of fluoride (> 0.5 mg F/L; Camargo (2003): Cerros Negros stream (MF-CN), running on granitic rock with medium natural fluoride content (Fig. 1) and, artificial Decantation ponds (HF-DP) where the waste generated by the fluorite process precipitates (Fig. 1). Furthermore, we selected a reference site following to Hawkins (2007) that is areas ecologically and environmentally similar to the site or area of interest, with the exception of the stressor or environmental condition of interest. The reference site selected was Los Vallecitos stream (LF-LV), running on metamorphic rock with low natural fluoride content (≤0.5 mg F/L) suggested by Camargo (2003) for the protection of aquatic biota in freshwater ecosystems.

These sites were surveyed on the same day, systematically, during the breeding season (September to March, Bionda et al., 2015), for three consecutive years (2014–2016). These months coincide with a season of rainfall and warmer temperatures. In each survey, we measure *in situ* the following physicochemical parameters: water temperature, pH, total dissolved solids (TDS), conductivity (EC) and salinity (S), using a digital equipment 35-Series 35425-10 Oakton® Multi-Parameter Testr™ (precision of ± 0.01 for pH; ± 1% full scale for EC/TDS/S). Dissolved oxygen was measured using an oxygen meter HD3030 (± 1.5%).

Individuals were captured by hand through visual encounter surveys (Heyer et al., 1994). After the capture, each individual was anesthetized for a few minutes by immersion in a solution at 0.05% of MS 222 or Methanesulfonate Salt. A phalanx of each toad was clipped, using an identification pattern specific for each site, following Donnelly (1989) systems: LF-LV: second toe forefeet right; MF-DP: fourth toe hind left and HF−CN: second toe forefeet left, to avoid resampling (Bionda et al., 2013), and it was preserved in a solution of 70% alcohol. Antifungal or antibacterial and healing agents were added at the puncture site to prevent infections. Before the release in the capture site individuals were visually examined to determine sex (Duellman and Trueb, 1994) and those showing any type of physical abnormalities were transported to the laboratory in 10-L plastic containers. Morphological abnormalities were classified following Meteyer (2000) and Lannoo (2008). The term "morphological abnormality" was used according to the recommendations of Bionda et al. (2012).

2.2. Laboratory analysis

For physico-chemical analysis of major ions (F^-, Na^+, Ca^{++}, Mg^{++}, K^+, Cl^-, $SO4^=$, and HCO_3^-), one water sample per year and sampling site was collected in 1 L plastic bottles; this coincided with the period of major activity of the toads. The samples were immediately transported to the laboratory of the Department of Geology, Hydrology Area of National University of Río Cuarto for analysis.

In the laboratory, abnormal toads were photographed with a digital camera and then radiographs were taken using Vetter REMS 40–50 KV, 100 mA with 0.01 and 0.05 s exposures at 80 cm distance. Prior to

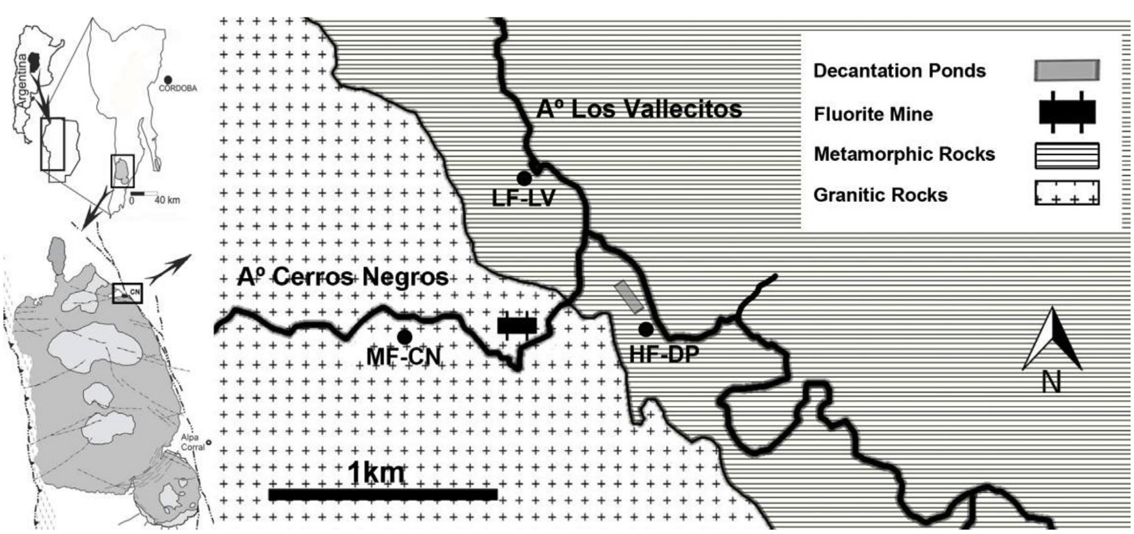

Fig. 1. Location of sampling sites in the central-southern region of Sierra de Comechingones, Córdoba, Argentina. Black point indicates sampling sites, *LF-LV*: Los Vallecitos stream, *MF-CN*: Cerros Negros stream and *HF-DP*: Decantation ponds.

Table 1
Physico-chemical parameters of water from three study sites. Mean ± SD, ranges (in parentheses) and statistical analysis of the chemical, physical and ion concentration for each sampling site are given. LF-LV = Los Vallecitos stream; MF-CN = Los Cerros Negros stream; HF-DP = Decantation pond.

	Sites			
	LF-LV	MF-CN	HF-DP	
Water Temperature (T°W)	18.24 ± 3.14 ^{A} (12.2–23.5)	18.91 ± 3.46 ^{A} (11.5–24.2)	22.02 ± 4.68 ^{B} (12.2–29.5)	$F_{2, 72}$ = 7.29; P < 0.01
pH	8.38 ± 0.28 ^{A} (7.73–8.98)	7.81 ± 0.30 ^{B} (7.24–8.36)	8.62 ± 0.42 ^{C} (7.35–9.4)	$F_{2, 72}$ = 33.64 P < 0.001
Total dissolved solids TDS mg/L	85.03 ± 17.8 ^{A} (40.6–128.5)	35.66 ± 14.35 ^{A} 19.80–76.40	259.75 ± 333.63 ^{B} (1.05–887.0)	H = 15.27 P < 0.001
Salinity mg/L	55.86 ± 10.65 (29.7–67.6)	26.48 ± 7.33 (17.60–54.7)	421.84 ± 362.11 (1.0–984.0)	H = 28.29 P < 0.0001
Conductivity (C) µS/cm	117.80 ± 22.66 ^{A} (57.0–148.2)	49.87 ± 20.66 ^{A} (27.9–108.30)	1000.55 ± 709.27 ^{B} (2.06–1991.0)	H = 28.18 P < 0.0001
Dissolved Oxygen (DO) %	90.88 ± 10.25 ^{A} (78.2–112)	91.02 ± 11.48 ^{A} (75.7–110)	69.92 ± 9.07 ^{B} (62.5–83.1)	H = 6.76 P < 0.05
HCO_3^- mg/L	77.83 ± 9.48 ^{A} (65–85)	38.75 ± 29.55 ^{A} (17.5–82.5)	302.50 ± 169.34 ^{B} (170.0–545.0)	H = 8.77 P < 0.001
Sulphate ($SO4^{=}$) mg/L	23.25 ± 4.70 ^{A} (16.3–26.7)	17.3 ± 12.24 ^{A} (7.10–32.6)	112.45 ± 30.95 ^{B} (80.7–150.50)	H = 8.0 P < 0.001
Chloride (Cl^-) mg/L	3.25 ± 0.70 ^{A} (2.9–4.3)	7.9 ± 10.00 ^{A} (2.9–22.9)	352.18 ± 161.18 ^{B} (214.3–542.9)	H = 7.39 P < 0.001
Sodium (Na^+) mg/L	7.93 ± 0.96 ^{A} (6.6–8.9)	9.75 ± 8.34 ^{A} (4.6–22.2)	378.43 ± 186.74 ^{B} (226.5–618.8)	H = 7.73 P < 0.001
Potassium (K^+) mg/L	0.75 ± 0.26 ^{A} (0.4–1)	0.53 ± 0.45 ^{A} (0.3–1.2)	11.18 ± 4.10 ^{B} (8.1–17.0)	H = 8.0 P < 0.01
Calcium (Ca^{++}) mg/L	15.8 ± 2.95 ^{A} (12–19.2)	8.20 ± 6.35 ^{B} (4.0–17.6)	22.2 ± 7.63 ^{A} (17.6–33.6)	H = 6.26 P < 0.05
Magnesium (Mg^{++}) mg/L	5.0 ± 1.9^{A} (2.4–6.8)	2.45 ± 1.53 ^{B} (1.0–4.4)	6.73 ± 2.12 ^{A} (1.9–8.8)	H = 6.27 P < 0.05
Fluoride (F^-) mg/L	0.33 ± 0.13 ^{A} (0.2–0.5)	2.03 ± 0.66 ^{B} (1.2–2.6)	14.0 ± 2.62 ^{C} (11.6–16.8)	H = 9.85 P < 0.001

Different letters show statistically significant differences, according to post hoc DGC and Wilcoxon test p < 0.05.

radiographic examination the individuals were anesthetized by immersion in a solution at 0.05% of MS 222 or Methanesulfonate Salt. Individuals were then kept in glass tanks containing water and after 48 h all anurans were returned to the collection sites.

2.3. Age determination

For age assessment standard methods of skeletochronology were followed (Sinsch et al., 2001; Bionda et al., 2015; Sinsch, 2015; Otero et al., 2017): (1) fixation in formol 4% (at least 12 h), (2) decalcification of bones (5–10% formic acid, 24 h), (3) paraffin embedding, (4) cross sectioning of the diaphysis at 10–12 µm using on a rotary microtome (5) staining with Ehrlich's haematoxylin (3 min, sample), (6) light microscopic count of the number of lines of arrested growth (=LAG). Periosteal lines of arrested growth (LAGs) were registered by at least two authors using a light microscope, Zeiss AxioPhot-Axio Lab (100 ×) equipped with digital camera Canon G10. We identified double and false lines following Sinsch et al. (2007) and endosteal resorption was assessed based on the presence of the Kastschenko line (KL; section between endosteal and periosteal zones; Rozenblut and Ogielska, 2005). The complete resorption was also defined by the diameter difference between LAGs and KL (sensu Liao and Lu, 2010; Li et al., 2013).

2.4. Statistical analysis

Descriptive statistics as mean ± standard deviation and range is included. All parameters measured were verified for normality (Shapiro-Wilks test) and homogeneity of variances (Levene test). When these assumptions were met, the parametric ANOVA analysis was performed, and the post-hoc DGC test (Test of Di Rienzo, Guzmán and Casanoves) was used to test differences between means (Di Rienzo et al., 2002). A comparative analysis of environmental variables was performed. Water temperature and pH were compared using one-way ANOVAs. The variables conductivity, salinity, TDS, dissolved oxygen and ion concentrations did not meet the assumptions of the ANOVA and were therefore compared between sites using a non-parametric Kruskal-Wallis test and the post-hoc Wilcoxon test. The comparisons of age between sites were performed by separating sexes, because sexual size dimorphism has been demonstrated in *R. arenarum* (Bionda et al., 2015).

A Principal Component Analysis (PCA) including water parameters of sites was performed using InfoStat 2017 (Di Rienzo et al., 2017). We standardized the data set before plotting the PCA because the variables were expressed in different units. The variables with a correlation coefficient major than 97% were removed from the analysis and a new PCA was performed to find water parameters with the highest positive and negative weights of PC1 and PC2.

The mean proportion of total morphological abnormalities and mean proportion for each type of morphological abnormality per site were calculated considering the total number of individuals examined (Johnson et al., 2002). The data were adjusted to a generalized linear mixed model (GLMM) for the analysis of the amount of morphological abnormalities using R 3.3.2 (R Core Team, 2016) and InfoStat 2017 (Di Rienzo et al., 2017). The best model was selected using the Akaike information criterion (AIC) and Bayesian information criterion (BIC) methods. The response variable “mean proportion of morphological abnormalities recorded in one year per site” was adjusted to a binomial distribution and *logit* link function (Nelder and Wendderburn, 1972; Myers et al., 2002). The variable site was considered a fixed factor, and the variables dissolved oxygen, total dissolved solids (TDS) and fluoride were considered random factors. These variables were the water parameters with the highest positive and negative weights of PC1 and PC2. This test uses the multivariate technique of cluster analysis on a distance matrix using the values predicted in the scale of the *logit* link function (Di Rienzo et al., 2002; Balzarini et al., 2015).

3. Results

Water temperature, pH and dissolved oxygen showed significant differences among sites. Conductivity, salinity and TDS were highest in HF-DP site compared with the other sites (Table 1). Ion concentrations showed values extremely high with great variation in HF-DP (Table 1).

The PCA indicates that the two principal components accounted for 88% of the variations in the original variables (Fig. 2). PC1 represents the variability introduced by dissolved oxygen in water, being the only variable with negative weight. Variables pH and calcium contributed the highest weights on PC2 (Table 2).

PC1 distinguished LF-LV and MF-CN sites from HF-DP site (Fig. 2). Dissolved oxygen was positively associated with LF-LV and MF-CN sites, whereas it was negatively associated with HF-DP. LF-LV site was negatively associated with fluoride (Fig. 2).

A total of 105 individuals were recorded (LF-LV: male = 6, female = 19; MF-CN: male = 19, female 14; HF-DP: male = 40, female 7). Five types of morphological abnormalities were identified (Table 3), with all of them being recorded in individuals from MF-CN site (Fig. 3). Individuals from HF-DP exhibited polydactyly, ectrodactyly and sindactyly, whereas LF-LV individuals only exhibited polydactyly. The proportion of total morphological abnormalities recorded in individuals

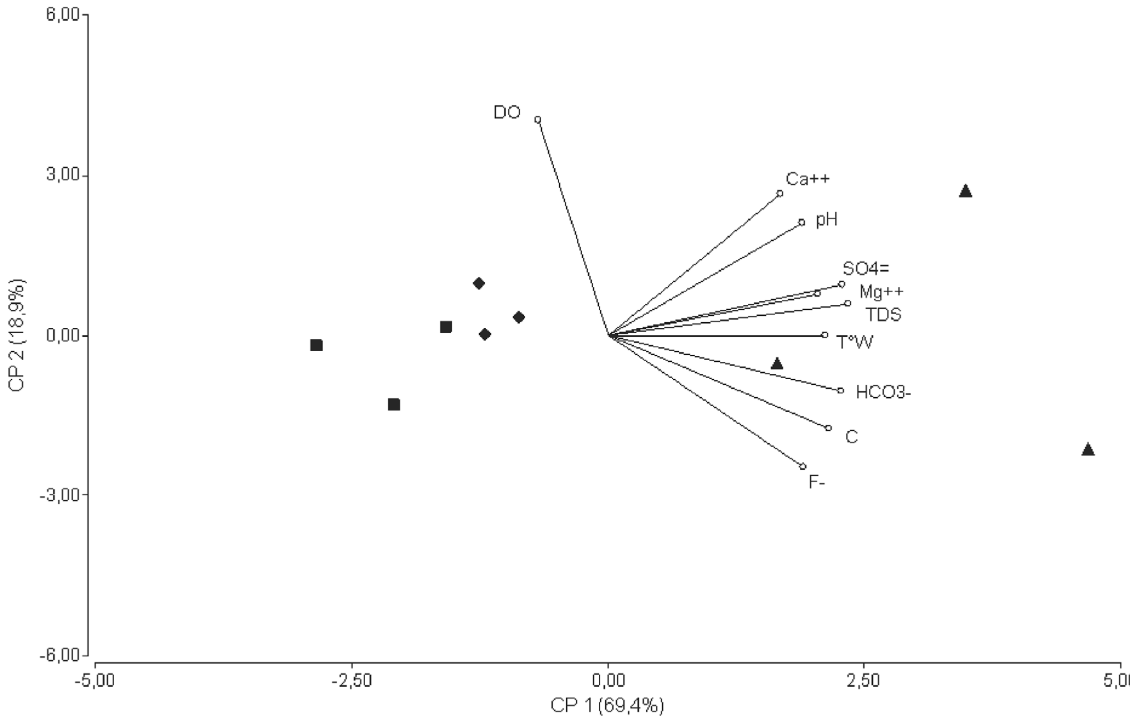

Fig. 2. Biplot of physico-chemical parameters and ion concentration in water. Triangle (▲): Decantation pond, square (■): Cerros Negros stream and diamond (◆): Los Vallecitos stream. *DO* (Dissolved Oxygen), Ca^{++} (Calcium), *pH*, $SO4^{=}$ (Sulphates), Mg^{++} (Magnesium), *TDS* (total dissolved solids), *T°W* (Temperature Water), HCO_3 (Bicarbonate), *C* (Conductivity) and F^{-} (Fluoride).

Table 2
Principal component analysis. Factor loading matrix in the two components.

Variables	CP 1	CP 2
Water Temperature (T°W)	0.89	2.2E-03
pH	0.79	0.46
Total dissolved solids (TDS)	0.98	0.13
Conductivity (C)	0.90	−0.38
Dissolved oxygen (DO)	- 0.28	0.88
HCO_3^{-}	0.95	−0.23
Sulphate ($SO4^{=}$) mg/L	0.96	0.21
Calcium (Ca^{++}) mg/L	0.70	0.58
Magnesium (Mg^{++}) mg/L	0.85	0.17
Fluoride (F^{-}) mg/L	0.80	−0.54

was 21.2% in MF-CN, 6.4% in HF-DP and 4% in LF-LV, with statistically significant differences between individuals from MF-CN with respect to those from HF-DP and LF-LV (DGC *post-hoc* test, $p < 0.05$; Fig. 4).

The radiologic examination revealed malformations that were not detected by examining external anatomy. For example, the hind limb (Fig. 3A) of individuals from MF-CN site showed normal tarsal bones and skeletal remains, but the lateral metatarsal was not present. This image would be compatible with a bone osteolysis process (infection or tumour). Fig. 3B shows skeletal remains that may correspond to a foreign body or to a remnant of a badly consolidated fracture. Part of the bone is missing, which can be due to an articular or bone infection or osteomyelitis and skeletal remains that may correspond to a phalangeal malformation.

The age of 87 individuals of the 105 collected for this study was determined (Table 3). The mean age by sex between sites was not statistically significant both in males ($H = 0.49$; $p = 0.512$. MF-CN = 3.13 ± 0.63; LF-LV = 3.2 ± 0.84; HF-DP = 3.37 ± 0.83) and females ($H = 0.47$; $p = 0.459$. MF-CN = 3.18 ± 0.71; LF-LV = 3.28 ± 1.04; HF-DP = 3.4 ± 0.55).

4. Discussion

To our knowledge, this is the first *in situ* study that reports morphological abnormalities in adult amphibians as a possible effect of natural and artificial concentrations of fluoride in surface water. Our

Table 3
Anuran morphological abnormalities detected at sites from the central-southern region of Argentina. Values indicate percentage of each type of abnormality with respect to the number of individuals recorded per site.

Type of Abnormality	Description	Percentage of morphological abnormalities (n)		
		LF-LV	MF-CN	HF-DP
Syndactyly	Fused digits	0.0	6.1 (2)	2.1 (1)
Ectrodactyly	Entire toe missing	0.0	3.0 (1)	2.1 (1)
Polydactyly	Extra digit	4.0 (1)	3.0 (1)	2.1 (1)
Microphthalmia	Fusion of eye	0.0	6.1 (2)	0.0
Ectromelia	Missing limb segments	0.0	3.0 (1)	0.0
Total of morphological abnormalities		4.0	21.2	6.3
Total amphibians sampled		25	33	47
Average and standard deviation age of toads analyzed		3.11 ± 0.96	3.14 ± 0.71	3.38 ± 0.73

LF-LV = Los Vallecitos stream; MF-CN = Cerros Negros stream; HF-DP = Decantation ponds.

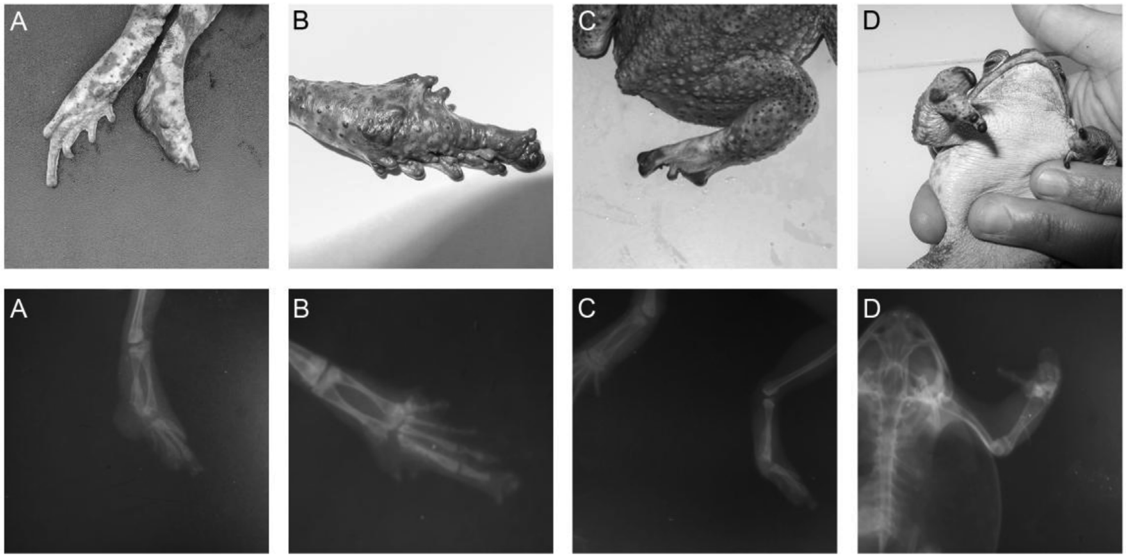

Fig. 3. Morphological abnormalities detected in individuals of Cerros Negros stream. A = Ectrodactyly, entire toe missing. B = Polydactyly, extra digit C = Ectromelia, missing limb segments; D = Syndactyly, fused digits.

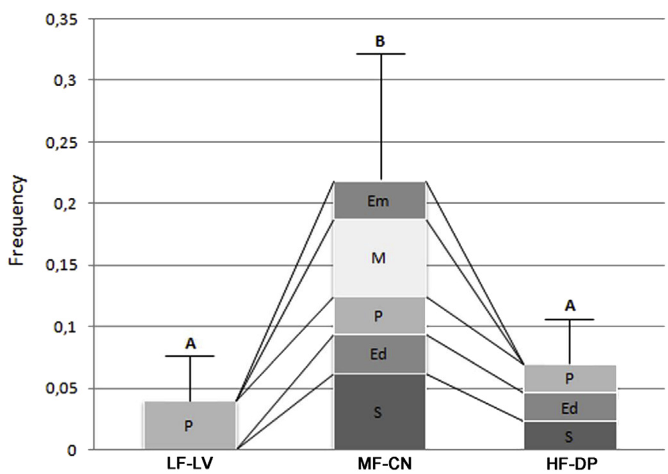

Fig. 4. Stacked bar graph of frequency of morphological abnormalities (Mean and Standard Error) per site. Means with a different letter are significantly different (DGC *post-hoc* test, $p < 0.05$). S: Syndactyly; Ed: Ectrodactyly; P: Polydactyly; M: Microphthalmia; Em: Ectromelia. *LF-LV*: Los Vallecitos stream, *MF-CN*: Cerros Negros stream and *HF-DP*: Decantation ponds.

results showed that the frequency of morphological abnormalities of amphibian populations from Cerros Negros stream (MF-CN) and Decantation ponds (HF-DP) largely exceed the bibliographic reference value (5%) reported in the literature (Piha et al., 2006; Johnson and Bowerman, 2010). Indeed, MF-CN and HF-DP showed the highest concentrations of fluoride content, exceeding the reference level (0.8 mg/L) proposed by Argentine Food Code and the value (0.5 mg/L) suggested by Camargo (2003) for the protection of the aquatic biota. Accordingly, values of F^- ion analyzed in the water samples were lower in MF-CN than in HF-DP. However, these values are still higher than those recommended for protection of aquatic organisms in freshwater ecosystems (Camargo, 2003).

Fluoride is a natural element in the environment and has been recognized to be essential and beneficial for organisms at normal concentrations. However, concentrations exceeding 0.5 mg F/L may have multiple effects (Camargo, 2003). Accordingly, in recent years abundant information on fluoride toxicity to aquatic organisms and even humans has been reported (e.g. Camargo, 2003; Rosso et al., 2011; Buchhamer et al., 2012; Chai et al., 2016; Pollo et al., 2016; Reddy, 2017; Otero et al., 2018). For example, it has been demonstrated in laboratory assays that fluoride can cause developmental toxicity in frog embryos (Goh and Neff, 2003). Chai et al. (2016) reported that values above 4.1 mg F^-/L could significantly inhibit embryo growth and delay metamorphosis in *Rana chensinensis*, decreasing size at metamorphosis and inducing malformations in embryos. Similar results were found by Goh and Neff (2003), who applied Xenopus Assay (FETAX). These studies clearly demonstrate that fluoride has teratogenic effect on the development of amphibians; moreover, the results and analysis of the present work reveal high incidence of morphological abnormalities in fluoride-contaminated areas.

Values of pH differed among sites, with HF-DP water showing alkaline values (7.35 < 9.4 <), close to optimal limits for normal development for amphibians (Addy et al., 2004; Gauthier et al., 2004). Drastic and abrupt exposure of amphibians to basic pH (≈9) has been found to cause mortality, whereas slight changes in pH (7 < pH 9 <) have sublethal effects, such as embryonic abnormalities, malformations and delay in tadpole development (Henao Muñoz and Bernal Bautista, 2011). Therefore, the alkaline pH could be the cause of the abnormalities observed in this site.

Salinity and conductivity were high in Decantation ponds due to high concentrations of major ions (Na^+, Ca^{2+}, Mg^{2+}, Cl^-, ${SO_4}^{2-}$, $CO3^{2-}$ and HCO^{3-}). This mixture could be responsible for the limb malformations observed in amphibians captured in HF-DP (Karraker, 2007). Interestingly, Decantation ponds showed a lower frequency of morphological abnormalities than Cerros Negros stream. This result could be attributed to the fact that HF-DP is an environment with complex ionic mixtures; in such an environment, the interpretation of its effects can be extremely challenging due the multiple possible synergistic and/or antagonistic interactions between different substances, resulting in biological effects that are not easily predictable (Eggen et al., 2004; Gauthier et al., 2004). Thus, when ions are combined, they can not only have cumulative additive or synergistic effects on the organism, but also minimize the effects (Little et al., 2003).

In addition to chemical contaminants, we must also consider several plausible causes of morphological abnormalities, such as environmental factors, pathogenic organisms, UV-B radiation, and aquatic predators (Ankley et al., 2002; Johnson et al., 2002; Johnson and Bowerman, 2010). No data were collected on pathogenic organisms, predators and UV-B radiation in these systems, which are other areas requiring further research. However, these causes were discarded in this study because, for example, UV-B radiation is rapidly attenuated in aquatic ecosystems in few centimeters of depth (Diamond et al., 2002), in addition, produce symmetrical deformities (Piha et al., 2006) and in our study, they were unilateral. Predators usually cause brachydactyly and ectromelia in the individuals that survive predation attempts (Bacon et al., 2013) and these abnormalities were not the most frequent in this study.

Most of the abnormalities recorded in our study concerned the hind limbs (syndactyly; ectrodactyly; polydactyly; ectromelia). This is consistent with most studies (Ouellet et al., 1997; Johnson et al., 2001, 2002; Agostini et al., 2013), possibly because the hind limbs develop in contact with substances environment, whereas the forelimbs develop within the defense of the gill chamber until metamorphosis occurs (McDiarmid and Altig, 1999). Abnormalities caused by fluoride exposure often represent failures in primary developmental stages (Goh and Neff, 2003; Chai et al. 2016, 2017; Chen et al., 2016; Zhang et al., 2018). Accordingly, the abnormalities found in our study probably occurred in the early stages of development and their presence in the adult stage means that they were not lethal (Goodman and Johnson, 2011). In adults we do not observe abnormalities in the bones, pathology commonly called skeletal fluorosis, which have been associated with chronic exposition to fluoride in frogs (Shaw et al., 2012). However, the morphological abnormalities found in our study would affect toad fitness and survival (Goodman and Johnson, 2011). In addition, amphibians are highly phylopatric; therefore, adults with abnormalities analyzed at each site have probably gone through their larval cycle at that site (Sinsch, 1990).

Average age of individuals was not different between sites. In this sense, recently Otero et al. (2018) in the same study sites reported that *R. arenarum* individuals that inhabit disturbed sites (MF-CN and HF-DP) present a delayed sexual maturity and reduced longevity while populations that inhabit the less disturbed site (LF-LV) maintain a long growth period and consequently reach a larger body size at older ages than populations from sites disturbed. Therefore, life history traits obtained by Otero et al. (2018) in addition to the higher incidence of morphological abnormalities that present populations of sites with high fluoride concentrations could affect the future trend of the population.

5. Conclusion

In summary, due to the small sample size we cannot be certain about the proximate cause(s) of abnormalities. However, the findings of this study indicate an association between morphological abnormalities in adult of *R. arenarum* and the composition of landscape. Therefore, we confirmed that *R. arenarum* is a true environmental sentinel suggesting that high concentration of major ions in aquatic ecosystems may pose a threat to the fitness of common South American toad.

This study increases the limited number of studies *in situ*, exploring the impacts of natural fluoride on amphibian. However, further monitoring over time and examination of incidences of abnormalities in *R. arenarum* and on other amphibian species would be needed before broader generalizations are attempted, especially because results on fluoride and its effect on many organisms, including human, remain controversial.

Ethics in publishing

The care, treatment and sampling of animals used in this study followed the Animal Care Regulations of University National of Río Cuarto and state law “Protection and Conservation of Wild Fauna” (Argentina National Law N° 22.421).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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