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Assessment *in situ* of genotoxicity in tadpoles and adults of frog *Hypsiboas cordobae* (Barrio 1965) inhabiting aquatic ecosystems associated to fluorite mine

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ABSTRACT

Non-lethal biological techniques such as blood biomarkers have gained attention due to their value as early signals of anthropic effects of contamination representing significant tools to evaluate ecosystems health. We evaluate and characterize *in situ* genotoxicity of water samples collected from aquatic ecosystems around a fluorite mine using amphibian frogs *Hypsiboas cordobae* as bioindicator species complemented with 16 physicochemical parameters. Four stations associated with fluorite mine sampling were sampled: a stream running on granitic rock with natural high fluorite content; two streams both running on metamorphic rock with low fluorite content; and an artificial decantation pond containing sediments produced by fluorite flotation process with high variation in physicochemical parameters. We analyses the blood of tadpoles and adults of *H. Cordobae*, calculated frequencies of micronuclei, erythrocyte nuclear abnormalities, mitosis, immature and enucleated erythrocytes. Individuals were measured and weighed and body condition was calculated. The results of this study indicate that individuals of decantation pond are exposed to compounds or mixtures which are causing cell damage when compared to those that were collected of stream. Larval stage was more vulnerable than the adult phase and it could be related mainly to the higher exposure time to xenobiotics, which can penetrate easily by skin, mouth and gills; additionally this site offers a reduced availability of food than other sites. Therefore, chronic exposure to pollutants could derive in degenerative and neoplastic diseases in target organs. Moreover these individuals may experience reproductive and behavioral disturbances which could lead to population decline in the long term.

1. Introduction

Degradation of freshwater resources is a world-wide growing concern (Antunes et al., 2007; Marques et al., 2008). Many are the causes of such degradation that go from agricultural practices (Bionda et al., 2011, 2013; Babini et al., 2015) to industrial activities such as mining (Castro et al., 2003; Marques et al., 2008; Antunes et al., 2008). Mining activity is a source of physical, chemical, biological and landscape alterations. Evaluation of environmental quality, particularly in aquatic ecosystems, has traditionally been based on physicochemical measurements of water, but not necessarily provides adequate information on exposure and response of living organisms to pollution (Antunes et al., 2008; Lavoie et al., 2012). Therefore, the development of complementary monitoring methods is a priority. In this sense, the use of non-lethal biological techniques such as analysis of blood biomarkers have gained attention due to their unquestionable value as early signals of adverse effects of contamination, because provide an estimation of biological exposure to genotoxic pollutants (Vera Candioti et al., 2010). These effects can be monitored using a broad range of assays, including analysis of micronuclei frequency and nuclear abnormalities, which are the most frequently used methods for detecting cytogenetic and genotoxic effects in nucleated erythrocytes (Aylon and Garcia-Vazquez, 2000; da Silva Souza and Fontanetti, 2006; Machado da Rocha, 2011; Pollo et al., 2012; de Arcaute et al., 2014).

Hence, changes in biological endpoints and blood biomarkers as responses of multiple changes occurred in the test organisms can turn into a consistent warning signal of environmental

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modification level, and have been considered a priority in the characterization of environmental risk for amphibian (Lajmanovich et al., 2010; Peltzer et al., 2013; Babini et al., 2015; Pollo et al., 2015a). It is well-known that amphibians have a great potential as bioindicators, and especially their aquatic early-life stages are very sensitive to contaminants (Rowe et al., 1992; Marques et al., 2013; Babini et al., 2015). Furthermore, anurans have a permeable skin that can more easily absorb moisture and substances dissolved in water. On the other hand, amphibians are one of the groups extremely important in the trophic chain. Depending on habitat and life stage, amphibian may occupy both the role of prey and top predators being a key element in the accumulation and transfer of toxic substances between aquatic and terrestrial environments (Marques et al., 2013).

Hypsiboas cordobae (Barrio, 1965) has a distribution restricted to highlands of Córdoba and San Luis provinces, in central Argentina, generally associated to slightly disturbed habitat. This species presents ecological characteristics that are essential for the election of a sentinel species to ensure the detection of local perturbations: present in abundance in the study area, have a low rate of migration, and be limited to a small space (Flickinger and Nichols, 1990).

The aim of the current study was to determine and characterize *in situ* the genotoxicity in natural and artificial surface waters associated with a fluorite mine from central Argentina, using *Hypsiboas cordobae* as bioindicator species. Because of absence of other important sources of contaminants (e.g. agrochemicals, sewage, livestock breeding) the study area can be regarded as a "field laboratory" offering an opportunity for the assessment of toxicity under realistic conditions.

2. Materials and methods

2.1. Study area and site selection criteria

The study area is located in a large granitic batholith, Cerro Áspero (440 km^2, altitude 1200 m.a.s.l) in the centre-south region of Sierra de Comechingones, Córdoba, Argentina. In this area the main deposits of epithermal fluorite of Sierras Pampeanas are located (Coniglio, 2006). The Sierras Pampeanas are constituted mostly by metamorphic plutonic basement, composed mainly of coarse-grained metamorphic rocks (gneisses and migmatites), and intruded into the Lower Paleozoic by granitic batholiths (Cantú and Degiovanni, 1984). These batholiths have an average content of F^- of 1.210 ppm, which is two times higher than the host metamorphic rocks and that of other non-mineralized granites of the Sierras de Córdoba (Coniglio et al., 2006).

This natural formation allowed the installation of mines in the area being the Los Cerros Negros mine the only active in Argentina since 1991. The effluent from the treatment of mineral ending in a series of artificial ponds (earth dams) of approximately 15 m by 25 m, vegetated with *Typha* sp. In these artificial ponds precipitate sediments produced by fluorite flotation process.

Associated with this area, the basin of stream "Los Cerros Negros" with an area of 10 km^2 circulates on granitic rock from west to east. Near the fluorite mine, it receives the stream "Los Vallecitos" that is born and runs through metamorphic rock and as well as without number of small streams that finally flows into the river "Guacha Corral", the most important water course in this area (Fig. 1).

The chemical characterization of surface water made in this area indicate that the fluoride ion is found in concentrations less than 0.35 mg/l for streams belonging to metamorphic environments, while streams circulating in granitic environments have an average concentration of 0.90 mg/l (Coniglio, 2006). This is due to, fluoride can be transferred from these granitic rocks to water through dissolution (Chuah et al., 2016).

Considering the data presented above, four sampling stations were selected: (I) Las Hylas (LH) stream and (II) Los Vallecitos stream (LV), both which runs on metamorphic rock with low fluorite content; (III) Los Cerros Negros stream (CN), which runs on granitic rock with a high fluorite content and (IV) artificial decantation ponds (DP), containing sediments produced by fluorite flotation process. In all sites the presence of populations of *Hypsiboas cordobae* was previously detected (Fig. 1).

2.2. Organism's collection

15–20 individuals (adults and tadpoles) of *H. cordobae* (Anura, Hylidae) were collected in each site during the period of activity of the species (September to April).

The adult individuals were found by visual encounter surveys (Heyer et al., 1994) and captured by hand. In these individuals were recorded: sex, using external secondary sexual characters (black vocal sacs and vocalizations for males, and eggs readily visible through the abdomen skin for females); total length (Snout-vent length – SVL) using a manual Somet Inox Extra Vernier caliper (0.01 mm); and weight, using a Mettler balance (P11N0–1000 g).

Tadpoles of *H. cordobae* were collected using a hand net. Subsequently, tadpoles were anesthetized by immersion in a solution at 0.05% of MS222 or Methanesulfonate Salt (3-Aminobenzoic Acid Ethyl Ester Sigma-Aldrich™), and were recorded: development stage (following Gosner, 1960); total length (TL; length from the snout to the tail end), using a manual Somet Inox Extra Vernier caliper (0.01 mm); and weight, using a Mettler balance (P11N0–1000 g).

The body condition (BC) of all individuals collected was calculated according to Jakob et al. (1996) that relates weight and total length, giving an estimate of nutritional state.

2.3. Blood sampling

Previously before release, blood samples were obtained from the angularis vein (Nöller, 1959; Martino and Sinsch, 2002) of each adult specimen. In tadpoles, the blood was obtained by cardiac puncture (Babini et al., 2015). Smears of fresh blood were air-dried, fixed and stained using May Grunwald-Giemsa (Dacie and Lewis, 1984).

2.4. Blood cell morphology

Two thousand erythrocytes per individual (adult and tadpoles) were examined by a single observer using a microscope at 1000 × magnification (Zeiss Primo Star iLED) and the results were expressed per 1000 cells (‰).

Genotoxicity was tested using micronuclei (Mn) and erythrocyte nuclear abnormalities (ENA), carried out in mature peripheral erythrocytes according to the procedures of Fenech (2000) and Carrasco et al. (1990) respectively. Four ENAs were considered: notched, binucleated, lobed and blebbed (Pollo et al., 2015a). The results were expressed as ENA mean frequency (‰) of the sum of all abnormalities observed (Guilherme et al., 2008; Lajmanovich et al., 2014). In addition, frequencies of enucleated erythrocytes (EN) and in mitotic division (M) were calculated.

Immature erythrocyte frequency (IE) was estimated in order to assess alterations on the haematological dynamics. The distinction between mature erythrocyte (ME) and immature erythrocyte (IE) was made following Ghillerme et al. (2008): IE have a bluish-grey cytoplasm and the nucleus is rounder and larger than ME.

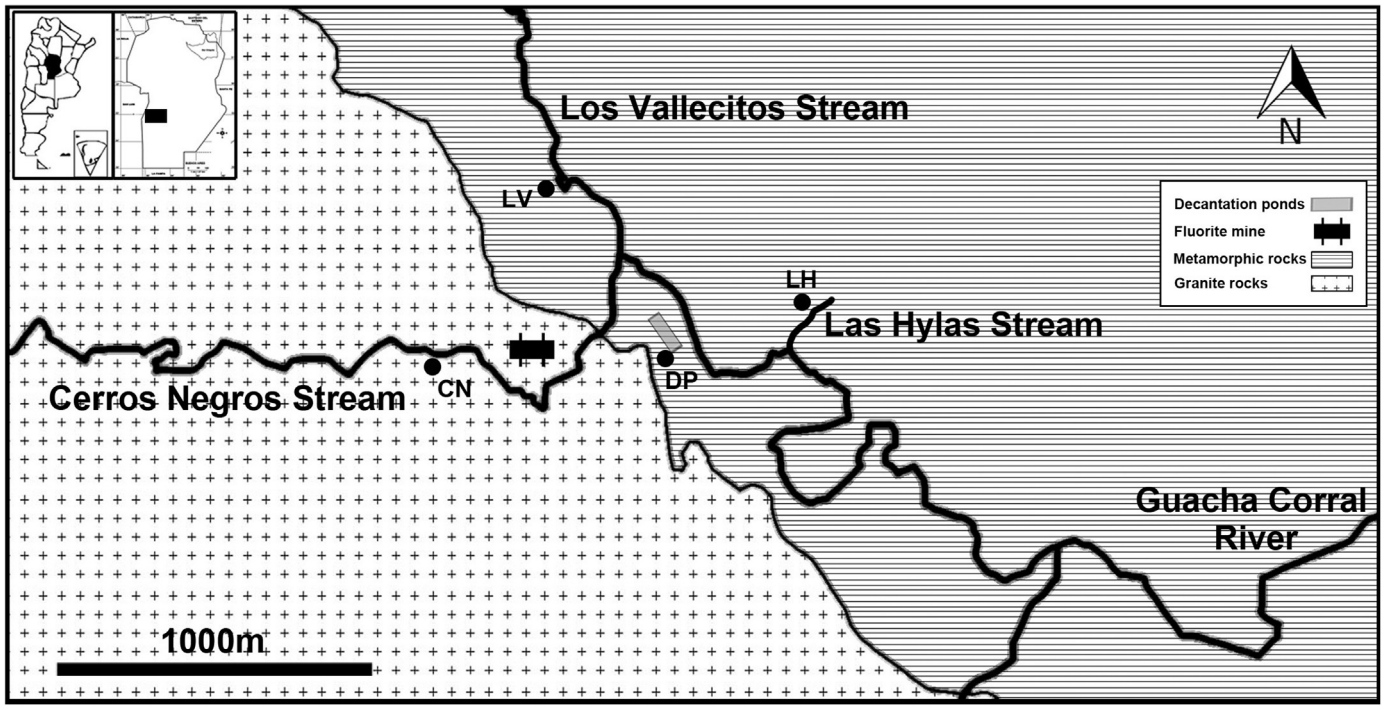

Fig. 1. Location of sampling sites in the centre-south region of Sierra de Comechingones, Córdoba, Argentina. Black point indicates the sampling sites.

2.5. Water parameters

Water samples for physical and chemical analyses were collected from all sites in 0.5 L plastic bottles. Samples were analyzed for Cl, Na, K, Ca, Mg and F and hardness (based on calcium and magnesium total content) by the area of Hydrology, Department of Geology, National University of Río Cuarto, using standard methods (APHA-AWWA, 1999). Furthermore, water temperature, pH, electrical conductivity, total dissolved solids and salinity were measured *in situ*, using a digital multiparameter 35-Series 35425–10 tests (Oakton Instruments 625E Bunker Court Vernon Hills, IL 60,061, USA). Dissolved oxygen was measured using a meter HD3030.

2.6. Statistical analysis

All data were expressed as mean ± standard deviation. Data set from different treatments were checked for normality (Shapiro-Wilks test) and homogeneity of variances (Levene test). A comparative analysis of environmental variables was performed. Water temperature, pH and dissolved oxygen (%) were compared using one-way ANOVAs. Conductivity, Salinity and TDS were compared between sites using a non parametric Kruskal–Wallis test, because these variables did not meet the assumptions of the ANOVA.

Weight (W) and snout–vent length (SVL) were log-transformed. Measures of tadpole and adult weights were regressed on SVL and the residual distances of individual points from the regression line were taken as an estimator of body condition index (Jakob et al., 1996; Wood and Richardson, 2009). Then, differences in body condition (BC) of tadpoles and adults between treatments were analyzed by ANOVA. All differences were considered significant at $p < 0.05$. Statistical analyses were performed using InfoStat (Di Rienzo et al., 2012).

Canonical correspondence analysis based on water parameters, genotoxicity and body condition of tadpoles and adults was performed following Babini et al. (2016). The analysis was performed using Canoco for Windows 4.5, and the TRIPLOT was performed with Canodraw for Windows (ter Braak and Smilauer, 2002).

3. Results

3.1. Physicochemical analysis of surface water

Physicochemical variables and ion concentration in water of each sampling site are shown in Table 1. Water temperature ($p < 0.001$), pH ($p < 0.001$) and dissolved oxygen ($p < 0.05$) showed significant differences between sites. Water pH values were similar in streams circulating in metamorphic environments (LH and LV) while CN, which runs on granitic rock, showed a neutral pH. Conductivity was extremely high in the decantation pond samples (Kruskal–Wallis, $p < 0.0001$) in comparison with other sites (Table 1). Water salinity (Kruskal–Wallis $p < 0.0001$) and TDS (Kruskal-Wallis, $p < 0.001$) also varied among sites (Table 1).

3.2. Blood biomarkers

The mature erythrocytes of *H. cordobae* adults were elliptical with a centrally located oblong nucleus while in tadpoles a spherical shape was observed. Micronuclei, ENAs, mitotic, immature and enucleated erythrocytes were observed in all the sites (Fig. 2).

Sex influence on micronuclei (Mn), nuclear abnormalities (ENA), mitotic erythroid (M), immature (IE) and enucleated erythrocytes (EN) frequencies, using SVL as covariable was evaluated but it was not statistically significant for any site ($p > 0.05$). Consequently, for subsequent analyses we combined males and females in a single sample per site.

Frequencies of Mn, ENA, M, IM and EN (Table 2) were compared between adult and tadpoles within each site.

In adults, Mn and IM frequencies were higher to DP and CN in relation to LV and LH sites. This difference was not significant for IM ($p = 0.30$, Table 2), Mn ($p = 0.19$). This relationship was also found in tadpoles and the difference was statistically significant

Table 1
Chemical, physical and ion concentration data for each sampling site.

	Sites			
	LH	LV	CN	DP
Temperature Water (T°W)	18,67 ± 2,63	19.17 ± 2.73	20.01 ± 2.60	23.20 ± 4.15*
pH	8.20 ± 0.25	8.30 ± 0.24	7.70 ± 0.34	8.50 ± 0.32*
SDT (ppm)	106.55 ± 20.58	78.57 ± 16.16	35.51 ± 17.43	251.19 ± 374.78*
Salinity (S) ppm	72.24 ± 13.7	54.75 ± 10.61	26.46 ± 8.92	476.23 ± 382.01*
Conductivity (Cond) μS/cm	153.91 ± 25.22	112.50 ± 24.50	49.04 ± 23.48	1495.81 ± 643.99*
Dissolved Oxygen (O) %	71.40 ± 9.01	92.30 ± 17.18	94.90 ± 12.16	69.93 ± 9.07
Hardness CO_3Ca (ppm)	82.00 ± 2.83	54.00 ± 5.66	20.00 ± 5.70	74.00 ± 8.50
CO_3 mg/l	0.00 ± 0.00	1.80 ± 2.55	0.00 ± 0.00	0.00 ± 0.00
HCO_3 mg/l	90.25 ± 24.40	70.65 ± 7.99	22.50 ± 7.10	375.00 ± 240.40
Sulphates ($SO4^{=}$) mg/l	15.70 ± 9.48	25.00 ± 0.28	8.25 ± 1.60	102.05 ± 30.20
Chloride (Cl^-) mg/l	5.00 ± 0.99	3.60 ± 0.99	2.9 ± 0.00	378.6 ± 232.40
Sodium (Na^+) mg/l	5.55 ± 2.90	7.30 ± 0.99	5.65 ± 1.50	422.65 ± 277.40
Potassium (K^+) mg/l	0.85 ± 0.21	0.85 ± 0.21	0.30 ± 0.00	9.60 ± 2.10
Calcium (Ca^{++}) mg/l	22.40 ± 0.00	15.6 ± 5.09	4.40 ± 0.60	18.40 ± 1.10
Magnesium (Mg^{++}) mg/l	4.40 ± 3.39	3.65 ± 1.77	2.20 ± 1.00	6.85 ± 2.80
Fluoride (F^-) mg/l	0.20 ± 0.00	0.25 ± 0.07	1.90 ± 1.00	14.20 ± 3.70

LH = stream Las Hylas; LV = stream Los Vallecitos; CN = stream Los Cerros Negros; DP = decantation pond. Mean ± standard deviation.
* $p < 0.05$.

for both biomarkers (Kruskal-Wallis, Mn $p < 0.01$; IM $p < 0.001$; Table 2).

ENA frequency of *H. cordobae* adults increased significantly in DP respect to the remaining sites (Kruskal-Wallis, $p < 0.01$). This relation was also found for tadpoles (Kruskal- Wallis, $p < 0.0001$). Enucleate cell frequency in tadpoles was higher in DP but this difference was not statistically significant (Kruskal-Wallis, $p = 0.43$).

3.3. Morphological analysis in tadpoles and adults

Mean SVL and weight of males, females and tadpoles are shown in Table 3. We found significant differences in weight of males among sites ($F_{3,51} = 23.91$ $p < 0.001$) but not in females ($F_{2,10} = 1.34$ $p = 0.30$). Snout–vent length differed between sites in both females ($F_{2,10} = 4.65$ $p < 0.05$) and males ($F_{3,51} = 23.91$ $p < 0.01$) (Table 3). Body condition (BC) was statistically significant between sites for males ($F_{3,51} = 7.65$ $p < 0.01$) but there was no differences in females ($F_{2,10} = 2.15$ $p > 0.05$). The smaller BC was observed in individuals from LH stream while individual with higher BC were found in DP.

All tadpoles were between stages 27–31 (Gosner, 1960). There were significant differences between sites for total length ($F_{3,73} = 31.78$ $p < 0.001$) and weight ($F_{3,73} = 32.51$ $p < 0.001$). Tadpoles from DP showed the lower total length, weight (Table 3), and body condition ($F_{3,73} = 6.14$ $p < 0.001$).

3.4. CCA

Canonical correspondence analysis (CCA) indicated that the accumulated inertia of ratio between water parameters and biological variables of tadpoles in the first axis was 49.9%. Collinearity was detected between conductivity, salinity, and TDS, so TDS was removed from the analysis. Na^+, K^+ and CO_3 were removed from the analysis because did not contribute significantly in a previous analysis.

The TRIPLOT (Fig. 3) shows that the first axis separates the LV and LH sites of the CN and DP sites. Magnesium, pH, Calcium and water hardness had an inverse relation to the rest of water parameters.

DP was related with water temperature and Cl- and F^- concentrations and these were associated with enucleated and mitotic erythrocytes in tadpoles and micronucleus in tadpoles and adults. LH and LV streams had relation to calcium concentration and hardness and these variables were associated principally with erythrocyte nuclear abnormalities in tadpoles and adults.

4. Discussion

This work provides the first data on the genotoxicity *in situ* on anurans inhabiting water surfaces associated with a fluorite mine. Toxicity data on fluoride effluents are limited, and most information is only available from laboratory assays with fluoride solutions at different concentrations (Goh and Neff, 2003; Lihong et al., 2011; Cao et al., 2013; Chai et al., 2016). *In situ* assays have become popular tools in aquatic toxicology and their use as providers of site-specific toxicological information is widely recommended (Castro et al., 2003; Antunes et al., 2008). Furthermore, the evaluation of pollutant effects on natural exposed organisms give information about the health of the environment (Hoffman et al., 2010) generating more realistic and toxicologically relevant data (Marques et al., 2013).

4.1. Physicochemical characterization of sites and the influence on organisms

Decantation ponds (DP) are artificial environments that receive effluents from the industrial process of fluorite flotation. During this process the water is heated to 28 °C, and its pH is modified using calcium carbonate. After the sediment is decanted, the water returns to the factory to restart the process, without making contact with any natural water course. As expected, DP water samples showed the highest ion concentration and conductivity, which may be explained by the formation of complex ionic forms that precipitate on the sludge. The frog population inhabiting the DP was chronically exposed to a complex mixture of compound and high pH values by direct contact with the effluent.

In our study, pH differed among sites, with DP showing the most basic values (8.5), near of optimal limits to growth, survival and normal development reported for amphibians ($6 <$ pH $9 <$) (Addy et al., 2004; García and Fontúrbel, 2003;Gauthier et al., 2004). Dissolved oxygen showed normal values for development of amphibians in all sites ($\geq 60\%$; Gauthier et al., 2004).

Fluoride is an essential element for all animals. However, it is also recognized worldwide as serious inorganic contaminant in

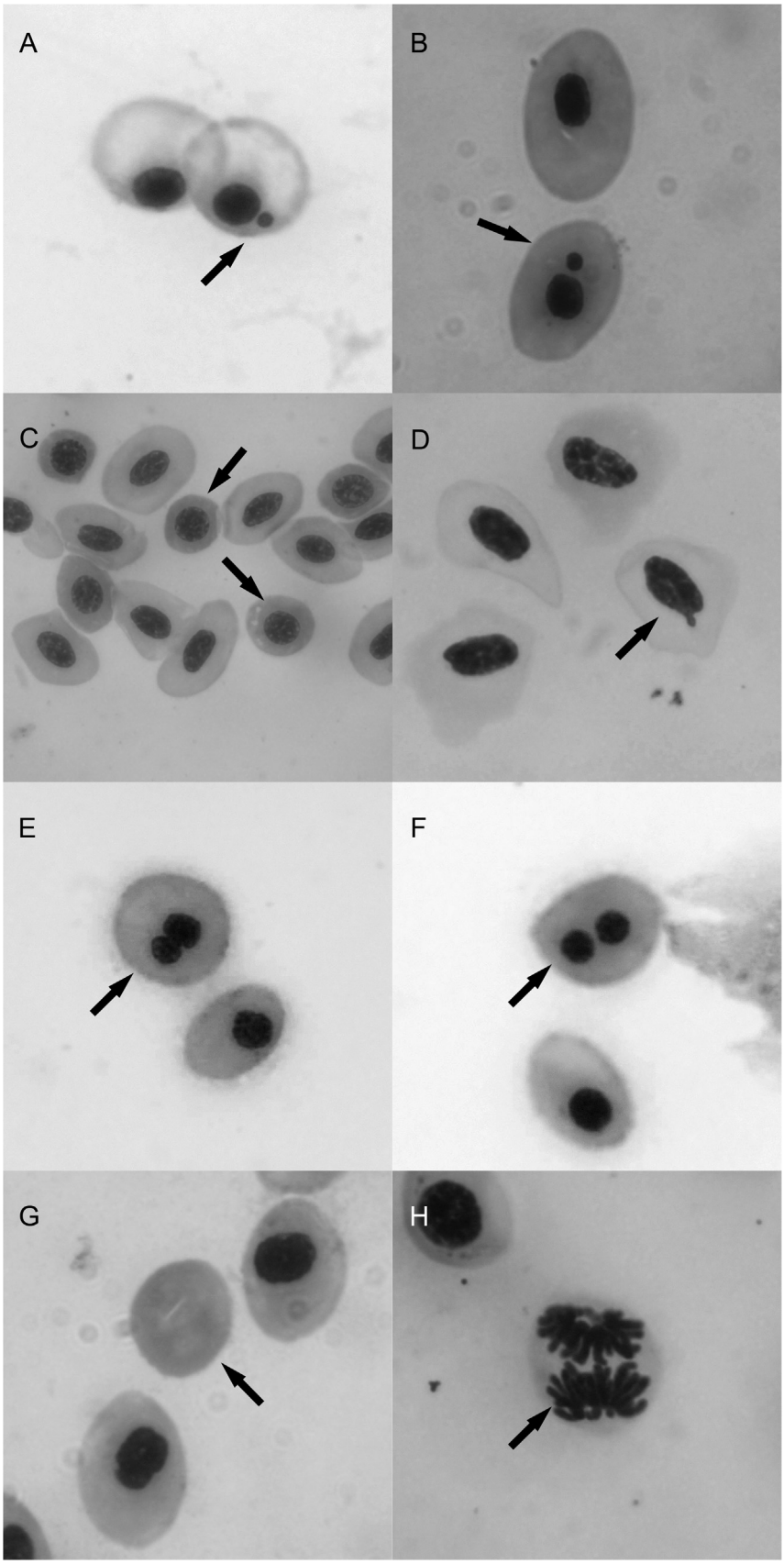

Fig. 2. Erythrocytes in blood of *Hypsiboas cordobae*: (A) tadpole erythrocyte micronucleus and (B) adults erythrocyte micronucleus; (C) mature and immature erythrocyte (D) lobed nuclei (E) blebbed nuclei (F) binucleated cell; (G) enucleated (H) mitotic erythrocyte. May Grünwald-Giemsa, 100 ×.

Table 2
Frequencies of micronuclei (Mn), nuclear abnormalities (ENAs), mitotic erythroid (M), immature erythrocytes (IM) and enucleate cells (EN) in tadpoles (*Ta*) and adults (*Ad*) of *Hypsiboas cordobae*.

Sites	n	‰ Mn	‰ ENAs	‰ M	‰ IM	‰ EN
LH	20 *Ta*	0.32 ± 0.51	8.53 ± 5.64	2.80 ± 5.09	14.85 ± 7.17	3.42 ± 3.30
	21 *Ad*	0.08 ± 0.20**	3.91 ± 2.85	0.12 ± 0.43	4.52 ± 5.95	0.54 ± 0.89
DP	20 *Ta*	1.40 ± 1.35	10.66 ± 7.04	1.77 ± 1.69	13.5 ± 10.27	5.64 ± 6.44
	20 *Ad*	0.36 ± 0.59	8.60 ± 5.34**	1.17 ± 1.59	14.86 ± 19.85	1.07 ± 1.77
CN	17 *Ta*	1.42 ± 1.09	1.74 ± 2.59**	0.86 ± 1.13	15.32 ± 10.9	4.09 ± 4.40
	14 *Ad*	0.39 ± 0.85	3.56 ± 2.08	1.39 ± 1.33**	43.41 ± 46.25	0.44 ± 0.68
LV	20 *Ta*	0.90 ± 1.36	10.14 ± 6.49	0.82 ± 1.39	7.69 ± 4.52**	2.36 ± 1.84
	13 *Ad*	0.21 ± 0.61	4.50 ± 3.27	0.45 ± 0.95	22.36 ± 26.09	0.21 ± 0.39

LH=Las Hylas stream; DP=Decantation Ponds; CN=Los Cerros Negros stream; LV=Los Vallecitos stream; n=sample size, ‰=frequency per one thousand.
** < 0.01.

Table 3
Mean values ± standard deviation of weigh and snout–vent length of adults and tadpoles of *Hypsiboas cordobae*.

Sites	n	Weigh (g)	Snout–vent length (mm)
LH	20 *Ta*	1.22 ± 0.70	47.09 ± 11.31
	4♀	9.50 ± 5.45**	45.79 ± 6.36
	17♂	7.06 ± 1.25***	42.32 ± 3.57***
DP	20 *Ta*	0.42 ± 0.15***	37.16 ± 5.93***
	5♀	13.60 ± 1.52	52.40 ± 2.07
	15♂	10.53 ± 1.96	48.20 ± 3.06
CN	17 *Ta*	1.18 ± 0.40	41.89 ± 8.02
	0♀	–	–
	14♂	9.00 ± 2.45	51.49 ± 3.61
LV	20 *Ta*	2.14 ± 0.73	63.99 ± 10.47
	4♀	13.00 ± 1.83	53.95 ± 2.95
	16♂	8.94 ± 1.98	50.12 ± 2.39

LH=Las Hylas stream; DP=Decantation Ponds; CN=Los Cerros Negros stream; LV=Los Vallecitos stream; n=sample size, *Ta*=tadpoles. *p < 0.05
** p < 0.01.
*** p < 0.001.

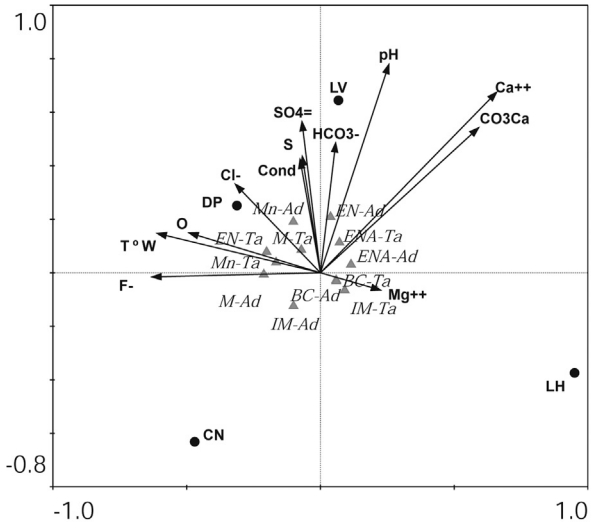

Fig. 3. TRIPLOT (first two CCA axes) showing the sites (circles), the biological variables (triangles), and the water parameters (arrows represent the correlation of the physicochemical variables with the canonical axes). See Table 1 and Table 2 for definition of variables.

water and cause of toxicity for many aquatic organisms (Camargo, 2003). F^- content at all sites were above the limit suggested (0.12 mg/l) by the Canadian Council of Ministers of Environment (CCME, 2002). CN stream and DP showed higher concentrations than the value proposed by Camargo (2003) for protection of aquatic biota in freshwater ecosystems (0.5 mg/l) and the value suggested by the Argentinian government (1.4 mg/l - Law 24,051, 1992).

On the other hand, the high concentration of SO_4^{2-} found in DP could be a direct outcome of the mining activity, which can increase 30–40 times this value, impacting on salinity and conductivity (Cañedo Argüelles et al., 2013). A high salinity directly influence on freshwater organisms because they need to maintain an internal osmotic pressure relative to the medium in which they live. Therefore, when the salt concentration of the medium becomes too high the osmoregulatory mechanisms will collapse resulting in cellular damage and possibly death (Cañedo Argüelles et al., 2013).

High concentrations of Cl^- (378.6 mg/l) in DP were recorded. These values exceeded the levels recommended by the US Environmental Protection Agency (230 mg/l) for the protection of aquatic life (USEPA, 1988). Prolonged exposure to Cl^- concentrations above 220 mg/l is harmful to aquatic species causing developmental abnormalities such as decreased growth and failure or delays in the metamorphosis (Christy and Dickman, 2002; Sanzo and Hecnar, 2006; Kaushal et al., 2005; Collins and Russell, 2009).

4.2. Blood biomarkers

The erythrocyte micronucleus test is used in several amphibian species to monitor aquatic pollutants displaying genotoxic potential (Peltzer et al., 2013; Babini et al., 2015; Pollo et al., 2015a). Consequently, the increase of micronucleated erythrocytes in peripheral blood of individuals collected in more perturbed environment indicates that some pollutant, or a combination of them, are inducing genotoxic effects. The higher micronucleated erythrocyte frequency in tadpoles respect to adults may be related to the highest cell division rate and to the varying degree and time of exposure to water pollutants during this phase of life cycle (Barni et al., 2007).

Additionally, ENA assays in adults and tadpoles showed the existence of a significantly higher value of nuclear abnormalities in erythrocytes of *H. cordobae* from the mine pond compared to individuals inhabiting in natural streams. In general, ANE frequencies were consistently higher than the Mn frequencies. Since both blood markers have been interpreted as analogous nuclear lesions (Aylon and Garcia-Vazquez, 2000; Serrano García and Montero Montoya, 2001; Guilherme et al., 2008) this result suggest that ANEs could indicate a wider spectrum of DNA damage than the micronuclei formation (Gómez Meda et al., 2006). These results demonstrate that there would be a higher degree of genetic instability in *H. cordobae* individuals inhabiting in the decantation

ponds and that this environment would represent a hostile habitat for the species. However, to our knowledge, this is the first report on the incidence of genotoxic damage in *H. cordobae* and there is no previous data on the basal threshold of micronucleus and nuclear abnormalities of this species obtained in laboratory. In this case, the reference values were obtained from individuals of LH stream, which was the natural site farther from the mine.

One of the most important functions of erythrocytes is to carry oxygen and carbon dioxide. The presence of enucleated forms of circulating red cells in DP may represent a special device for increasing oxygen carrying efficiency, particularly in conditions of water contamination, by improving the cell surface/volume ratio (Barni et al., 2007). The high appearance of enucleated erythrocytes in tadpoles in comparison to adults may be attributed to the absolute dependence of this stage of aquatic environment. Although, the eccentric or peripheral splitting plane of bilobed cells can result to the formation of enucleus (Anbumani and Mohankumar, 2012), this cell type have also been reported in situations of diet change, diseases and metabolic stress (Fijan, 2002). Some of these conditions could explain their occurrence in adults.

The increase in inmature erythrocyte frequency in tadpoles observed in DP and CN stream would reflect an increase in erythropoiesis (Peltzer et al., 2013), which could be a response to stress caused by high concentrations of ions (Valenzuela et al., 2006; Prieto et al., 2008). Furthermore, fluorine is considered an effective anabolic agent because it promotes cell proliferation (Barbier et al., 2010) affecting the oxygen availability in this site. Consequently, the presence of mitotic erythroid cells in polluted conditions indicates that erythropoiesis is directly stimulated in peripheral circulation of adults and could represent a short-term means for increasing the oxygen carrying capacity of blood in amphibian species (Barni et al., 2007).

4.3. Body condition

Of the two variables used to calculate body condition, only the weight can both increase and decrease rapidly in response to a change in stress levels (Reading and Clarke, 1995). The loss of body condition in tadpoles from DP may result from food scarcity, because food availability may be directly affected by environmental conditions. Pollo et al. (2015b) analyzed the diet of *Rhinella arenarum* and *H. cordobae* tadpoles from this site, and reported that the quantity of food available for tadpoles was significantly lower compared to natural sites. This outcome could be related to water characteristics such as turbidity, depth and substrate type. Furthermore, strong variations in conductivity in short time periods and high concentrations of salinity could affect periphyton growth (Cañedo Argüelles et al., 2013).

Comparing the biometric measures, weight and size, adult individuals from DP were significantly larger and heavier and showed a higher body condition than individuals from streams. This increased value does not necessarily mean that individuals have better state of health (Polo Cavia et al., 2010). A habitat may induce phenotypic differences in natural populations, resulting in apparently healthy traits, as a larger body size that could not necessarily correlate with the true health condition of population. Consequently, individuals from DP may have a higher body condition because the environment presents a high availability of food resources and warmer temperatures that promote the digestion and increase the metabolic rate. Thus, the election of this site is explained by the ecological and reproductive features of this species, which prefers vegetated ponds as breeding sites, favoring the calling site availability. It is common to find this species basking on rocks or perched on vegetation (Barrio, 1962). These features could be associated with a higher water temperature and an increase of food and shelter availability. However, this habitat with increased food availability could not be necessarily the more appropriate considering not only the mixture of compounds to which the individual are exposed but also other biological stressors as parasites, competitors and the human activity.

4.4. Conclusion

In this work, an increased genotoxicity in DP was found in both adults and tadpoles, the last being the most affected. This could be explained by the high values of Na^+, Cl^- and F^-. Previous studies have shown that Cl^- and Na^+ ions have an acute and chronic toxic effect on amphibians at environmentally realistic concentrations (Christy and Dickman, 2002; Sanzo and Hecnar, 2006) while fluorine ions act to cellular level as an inhibitor of enzymatic activities causing cell death by apoptosis (Camargo, 2003; Barbier et al., 2010). Therefore, it is difficult to establish a direct cause–effect relationship with a specific element, but, in such complex mixtures, synergistic and/or antagonistic interactions between substances could lead to biological effects that are not easily predictable (Gauthier et al., 2004).

Finally, biomarker responses indicate that individuals of *H. cordobae* are being affected by exposure to pollution in artificial decantation ponds. However, this environment appears to be preferred by this species compared to other surrounding natural environments. Chronic exposure to pollutants could derive in degenerative and neoplastic diseases in target organs (Barni et al., 2007). Although we did not detect an increased mortality in this site, individuals inhabiting in this environment may experience reproductive and behavioral alterations, which could lead to long-term population declines.

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