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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.