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Genotoxic evaluation in two amphibian species from Brazilian subtropical wetlands

Marcelo Estrella Josende [a], Alexandro Marques Tozetti [b], Marcelo Tavares Alalan [a], Volnei Mathies Filho [a], Simone da Silva Ximenez [a], Flavio Manoel Rodrigues da Silva Júnior [a], Samantha Eslava Martins [a,*]

[a] Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Av. Itália km 8, Rio Grande, RS 96203-900, Brazil
[b] Universidade do Vale do Rio dos Sinos, Avenida Unisinos, 950, São Leopoldo, RS 93022-000, Brazil

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ABSTRACT

Biomarkers analysis serves as an early warning system for the presence of pollutants because their responses appear before irreversible damage to the ecosystem takes place. The genotoxic effects of pollutants may occur at cellular pollutant concentrations that are well below levels that would cause gross cytotoxicity, making this a useful tool to detect early effects of toxic environmental agents. Combining the importance of Brazilian wetlands to the conservation of amphibian biodiversity with the potential negative impacts of irrigated rice fields in the surrounding areas, the aim of the present study was to evaluate genotoxic damage in two amphibian species, *Pseudis minuta*, and *Leptodactylus* gr *latrans*, from the southern Brazilian wetlands. Adult specimens from both Anuran species were captured from preserved (Taim Ecological Station = TAIM) and non-preserved (Senandes) wetlands. Nuclear abnormalities were quantified in erythrocytes, and the results were compared using the Mann–Whitney U test. There was a higher incidence of micronucleated erythrocytes in *P. minuta*, and of notched nuclei in *L.* gr *latrans* that were collected in TAIM when compared to those that were collected in Senandes, despite the fact that TAIM is a conservation unit. These findings indicate that Anurans are coping with genotoxic substances in their habitats, and underscore the need to implement monitoring programs in TAIM to determine which compounds or mixtures might be causing cell damage and to investigate the effects of such compounds on other anuran species and animal groups.

1. Introduction

The world decline in amphibian communities was first detected during the 1990s (Mackey and Boone, 2009), but the reasons for this decline remain controversial (Stuart et al., 2008). Generally, the decline has been associated with habitat loss and pollution. Both factors are related to the growing development of agricultural activities that replace the natural vegetation cover and generate habitat pollution with fertilizers and pesticides (Relyea and Mills, 2001). These toxic substances cause the animals to be borne and develop with malformations and/or develop behavior alterations, which may limit their reproductive success (Bridges, 1999; Stuart et al., 2008).

As a function of their biology and life cycle, amphibians dwell in water and on land and feed on plants and animals that may serve as important routes of pollutants uptake. Furthermore, they possess a semi-permeable skin, which could advance the entry of harmful organic substances such as pesticides and hydrocarbons (Wells, 1977). These features make amphibians to be appropriate models for ecotoxicological studies (Burlibaşa and Gavrilă, 2011).

Despite the fact that Brazil shelters the highest anuran biodiversity in the world (SBH, 2013), there have been few studies concerning amphibian population trends in wetland habitats (Oliveira et al., 2013). Most of the southern Brazilian wetlands have been diminished in size due to the expansion of agricultural areas, primarily irrigated rice fields (Maltchik et al., 2003). This situation is problematic because these wetlands store water, and they are essential hotspots of biodiversity (Guadagnin, 1999).

One of the most important continuous area of wetland in southern Brazil is the Taim Ecological Station (TAIM), which is protected as a conservation unit. This area shelters high animal and plant biodiversity and many migratory birds use this area to rest and feed. Extensive areas with irrigated rice fields, in which large amount of pesticides are applied, cattle farms, and the exploitation

* Corresponding author. Tel.: +55 5332935162.
E-mail address: samanthamartins@furg.br (S.E. Martins).

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of non-native plants such as *Pinus* spp. and *Eucalyptus* spp., have occurred in the surroundings of TAIM. These activities could disrupt ecological processes and increase the risk of vulnerability and extinction of many species, so it is crucial to assess the health of such ecosystems and their resident organisms. With regard to wetlands, even the maintenance of large areas under the protection of the law does not assure a high-quality ecosystem because of the inputs of pollutants from allochthonous sources of water (Ramsar Convention Secretariat., 2007). This scenario reinforces the urgency of studies on species associated with wetlands, particularly assessments of the health of wild populations (Oliveira et al., 2013).

Biomarker analysis has been successfully used in biomonitoring programs. It serves as an early warning system for the presence of pollutant compounds because biomarker responses appear before irreversible damage to the ecosystem takes place. The genotoxic effects of pollutants may occur at cellular pollutant concentrations well below those that cause severe cytotoxicity (Al-Sabti and Metcalfe, 1995), which makes this tool particularly useful to detect the early effects of environmental toxic agents. The micronucleus (MN) test is a rapid, effective, low-cost assay to determine mutagenicity in several cell types (Ferrier et al., 1998; Gauthier, 1996). Micronuclei are derived from chromosomal fragments or whole chromosomes that do not migrate to the main nucleus during cell division (Schmid, 1975). Other nuclear abnormalities result from analogous damage (Serrano-Garcia and Montero-Montoya, 2001) and may be taken into account to assess the exposure of organisms to genotoxic contaminants (Marques et al., 2009).

In the present study, we evaluated wild populations of two anuran species, *Leptodactylus* sp. (gr *latrans*) and *Pseudis minuta*. These species occupy either preserved or disturbed habitats, suggesting that they present plasticity in coping with changes in their habitats. *L.* gr *latrans* distributes along the Pampa biome in South America, being a relative large semiaquatic anuran that feeds on a wide variety of prey (Heyer et al., 1990). *P. minuta* inhabits the Pampa biome in South America, being a mostly aquatic anuran, small in size, which feeds on aquatic invertebrates (Kwet, 2000). Considering these ecological features and the relative high abundance of these species in Brazilian wetlands, we believe that they depict good models to evaluate genotoxicity in wetland anuran populations.

Combining the importance of subtropical Brazilian wetlands to the conservation of amphibian biodiversity, and the potential negative impacts of irrigated rice fields in the surrounding areas, the main goal of the present study was to verify the suitability of using genotoxic damages as biomarkers of pollutant effects in amphibian as bioindicators of such effects. Also, we verified if the anthropogenic activities in the surroundings of TAIM affect the anuran population in regards of nuclear abnormalities.

2. Material and methods

2.1. Study area

The study was conducted in a region of the Pampa biome, which is characterized by an extensive area of wetlands, temporary lagoons, lakes, and associated marshes (Waechter, 1985). To determine the possible relationship between the intensification of agricultural activities and genotoxicity in Anurans, amphibians were collected in two sampling sites: (1) the Taim Ecological Station (TAIM) (32°50′S; 52°26′W), which is an extension of the lowlands that are protected as a conservation unit. Most of the protected area (approximately 33,000 ha) is surrounded by farms, so this habitat is indirectly affected by agricultural activities. It should be noted that TAIM is the most important conservation unit in southern Brazil and encompasses the last remains of relatively well-preserved subtropical Brazilian wetlands; and (2) the wetlands in Senandes neighborhood (32°08′S; 52°11′W), which is composed of permanent and temporary flooded grasslands (Carvalho and Ozorio, 2007) in a rural and urban areas that is composed of a mosaic of natural wetlands, rice fields, and cattle fields.

2.2. Sample collections

From March 2011 to August 2012, adult specimens from the anuran species *P. minuta* ($n = 30$; average body length = 29 ± 3 mm; average body weight = 2.9 ± 1.1 g) and *L.* gr *latrans* ($n = 30$; average body length = 65.4 ± 11.8 mm; average body weight = 32.4 ± 14.1 g) were captured from wetlands after a visual search (Tozetti and Toledo, 2005). Sample collections were in accordance to Brazilian regulations (SISBIO license number 27,755-1).

2.3. Nuclear abnormalities assays

After length, weighting, and sex determination, Anurans were anesthetized and blood was obtained by heart puncture. Two peripheral blood smears for each sampled specimen were prepared on clean slides, fixed with methanol for 10 min and dyed with Giemsa stain (10% v/v). Slides were viewed under an optic microscope (1000× magnification). A total of 1000 erythrocytes per slide were scored. For MN quantification, two slides per animal were scored. The criteria for MN determinations followed those proposed by Lajmanovich et al. (2005). Kidney shaped nuclei (KS), lobed nuclei (LB), and notched nuclei (NT) were identified following Carrasco et al. (1990) and blebbed nuclei (BL) was quantified according to the criteria adopted by Strunjak-Perovic et al. (2009). All experimental procedures involving animal handling were approved by the Ethics Committee on Animal Use of FURG (permit number 23,116.005645/2012-89).

2.4. Statistical analysis

Individuals of each species from TAIM were compared to those that were captured in Senandes. Moreover, the incidence of micronuclei in *P. minuta* was compared to that of *L.* gr *latrans* for the same sampling area. Data were fitted to a non-parametric statistical analysis, so the Mann–Whitney U test was used. A value of $p < 0.05$ was adopted to indicate significance.

3. Results and discussion

Similarly to other Anurans (Lajmanovich et al., 2005), both *P. minuta* and *L.* gr *latrans* have oblong-oval shaped mature erythrocytes with a centric nucleus (Figs. 1 and 2A). The MN incidence ranged from 0 to 22 in 2000 analyzed cells for *P. minuta* and from 0 to 15 in 2000 analyzed cells for *L.* gr *latrans.* The observed MN were spherical fragments that were smaller and separated from the principal nucleus (Figs. 1 and 2B). Although, individual MN were common in the analyzed cells, multiple MN were rarely observed. In regard of the other nuclear abnormalities investigated, incidence in 1000 analyzed cells from *P. minuta* were as follows: NT ranged from 0 to 16.4; LB ranged from 0 to 7.8; KS ranged from 0 to 2.9; and BL ranged from 0 to 3.9. In *L. latrans* erythrocytes (1000 per individual), the anomalies were found as following: NT ranged from 0 to 7; LB ranged from 0 to 3.9; KS ranged from 0 to 2; and BL ranged from 0 to 2. Figs. 1 and 2 show the nuclear morphology in any of the abnormality found.

Previous studies have shown a very low incidence of MN in Anurans that were not exposed to contaminants, with a frequency