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Contrasting Patterns of DNA Damage by the Comet Assay in Four Species of the Hylidae Family (Amphibia-Anura)

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Abstract

Amphibians are components of many ecological communities and they can be used as indicators of environmental quality. Thus, in this study we evaluated the genome sensitivity of four species of anuran amphibians of the Hylidae family in a cattle raising area (Bela Vista de Goiás) and in a nickel area (Barro Alto) both at Goiás state, Central Brazil. The species analyzed are: *Dendropsophus minutus*, *Hypsiboas albopunctatus*, *Hypsiboas paranaiba* and *Scinax fuscomarginatus*. The comet assay was used as a biomarker to estimate DNA damage in these four Hylidae species. The genomic analyses were realized using the software "*Comet Score*" v1.5. An analysis of variances (ANOVA) and t-test were carried out to verify the genomic damage among and between all the four species analyzed. In the three comet assay parameters used, the species sampled from the nickel areas showed the largest stretches of DNA damage when compared to the same species sampled in the cattle raising area. So, we can suggest that all evaluated species may be used for eco-genotoxicity of aquatic environments.

***Keywords**: Anuran, Comet Assay, Biomonitoring, Environmental Quality*

1. Introduction

Nickel (Ni) is a ubiquitous, naturally occurring element in soil and a recognized environmental and industrial pollutant (Wozniak & Blasiak, 2002). Elevated levels of nickel concentrations in the soil, either caused by the presence of serpentine soils (Mesjasz-Przybylowicz et al, 2001) or by anthropogenic Ni discharges, may lead to accumulation of the metal in soil-dwelling organisms, such as anurans. Compounds of nickel are known human carcinogens. However, their genotoxic potentials in mammalian cells are rather weak and/or restricted to cytotoxic concentrations (Hiraku & Kawanishi, 1996).

The anurans belong to the amphibian class and present behavioural and physiological characteristics, such as permeable skin, low mobility and life cycle with simultaneous dependence on terrestrial and aquatic environments. These animals are considered important bioindicators of environmental quality because they are sensitive to changes occurring in the environment (Beiswe-nger, 1988; Weigoldt, 1989; Vitt et al, 1990; Blaustein & Wake, 1995 and Skelly, 1996).

To detect DNA damage Östling & Johanson (1984) developed the technique of single-cell gel electrophoresis (SCGE). Subsequently, Singh et al (1988) presented a similar technique under alkaline conditions (pH = 13). The alkaline pH substantially increased the sensitivity of the assay to identify genotoxic agents. Tice et al (2000) and Collins (2004) demonstrated not only double-strand breaks and cross-links, but also single-strand breaks, alkali-labile

sites and incomplete excision repair sites. The comet assay is a simple, rapid, sensitive and relatively inexpensive technique and can be performed in any nucleated eukaryotic cells, including plant cells (Olive et al 1990 and Mitchelmore & Chipman, 1998). It is used as a detector of genotoxicity and contrary to the Micronucleus Test is highly sensitive to various types of DNA damage (Bücker et al, 2006).

The use of biological indicators in cytogenetic tests not only helps in evaluating the physico-chemical integrity of environment but also the responses of these organisms to environmental changes resulting from pollution (Moraes, 2000). Thus, the comet assay may also be used in biomonitoring programs to localize potential sources of pollution and in environmental impact studies (Zagatto & Bertoletti, 2006).

So, the aim of our study was to evaluate the genome sensitivity of four species of anuran amphibians of the Hylidae family in a cattle raising area (Bela Vista de Goiás) and in a nickel area (Barro Alto) both at Goiás state, Central Brazil.

2. Material and Methods

2.1. Collection Area and Sampling

Adult's anuran of four amphibian species, *D. minutus*, *H. albopunctatus*, *H. paranaiba and S. fuscomarginatus* were selected as the test organisms. 41 individuals were sampled in the municipalities of Barro Alto (14º 58' 15'' S and 48º 54' 57'' W) and Bela Vista de Goiás, Goiás state (Table 1), from October 2010 to April 2011, all of them in water bodies. Barro Alto area is characterized by anthropic Cerrado, and is recognized for biodiversity conservation. The region presents a semi-humid tropical climate. The average rainfall is 1,400 mm, with a rainy period from October to April and the dry season from May to September. The mean temperature is 27 ºC in the rainy period and 25 ºC in the dry period. The relative humidity during the rainy period is 77 % and in the dry period it is 51 %. As there are natural nickel-mining areas, nickel could be observed in serpentine soils, with adapted vegetation which can accumulate such metal (Cempel & Nikel, 2006).

The area of Bela Vista de Goiás (16º 58' 00'' S and 48º 57' 00'' W) is characterized as a cattle raising area, without the usage of agrochemicals. The mean temperature is 29 ºC in the rainy period and 26 ºC in the dry period. The relative humidity during the rainy period is 81 % and in the dry period it is 58 %.Voucher individuals are housed at the Coleção Zoológica of the Universidade Federal de Goiás (ZUFG).

2.2. Comet Assay

Table 1. Species, Number of Individuals and Municipalities Evaluated in this Study

Species	Number	Municipality
D. minutes (Peters, 1872)	6	Barro Alto
	7	Bela Vista
H. albopunctatus (Spix, 1824)	5	Barro Alto
	5	Bela Vista
H. paranaiba (Günther, 1859)	5	Barro Alto
	5	Bela Vista
S. fuscomarginatus (Lutz, 1925)	4	Barro Alto
	4	Bela Vista
Total	41	

The comet assay was performed following the protocol described by Singh et al (1988), an alkaline method with some modifications. The anurans were euthanized with xylocaine (5 %). Blood samples were collected by cardiac puncture and erythrocytes were chosen because they are nucleated in amphibians. The slides were coated with agarose of normal melting point (1.5 %) and 15 µl of blood, diluted in 1ml of PBS buffer (pH 7.0), was dropped on each slide. Then, 130 µl agarose low melting points (0.5%) was pipetted onto the precoated microscope slides. Those were covered with cover slips and kept at 4 °C for 10 minutes—and then kept in cuvettes (protected from light) containing cold lysis solution (Triton X-100, DMSO and Stock Lysis Solution of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, NaOH to pH 10 $\pm$ 0.5, and 1% Triton X-100) for 4 hours. Electrophoresis was carried at 25V and the current was adjusted to 300 mA. The slides were routinely exposed to this current in the dark for 30 minutes. After electrophoresis, the slides were placed in a staining tray and covered with a neutralizing buffer (0.4 M, Tris-HCL, pH 7.5) in the dark for 5 minutes. For analysis, the slides were stained with 20 µl solution of ethidium bromide (0.02 mg/ mL) and covered with cover slip. 50 nucleoids per slide and 100 nucleoids per sample were analyzed. The analysis was performed by fluorescence microscopy system *Axioplan-Imaging*®, using the software Isis with excitation filter of 510-560 nm and a barrier filter of 590 nm, in an increase of 200X.

2.3. Software "Comet Score"

For genomic damage assessment, we used the program TriTek Comet ScoreTM, version 1.5. DNA damage was expressed as Arbitrary Units (AU). Cells with the core completely fragmented, that is, in apoptosis process, were not accounted for during the analysis. In software analyses, the intensity of pixels to provide values corresponding to genomic damage estimates is given as AU. Of the 17

parameters provided by the program, we selected three for the quantification of DNA damage, as described: tail length, percentage of DNA in tail, and olive tail moment.

2.4. Statistical Analysis

The 100 nucleoids assessed per individual were considered replicates. After obtaining the results of genotoxicity, we first performed the Kolmogorov-Smirnov test to verify whether the data followed a normal distribution, together with the Levene's and Brown-Forsythe tests, to test samples' homogeneity of variance. The comparison between the estimates of genomic damage between different anuran species was based on Analysis of Variance (ANOVA) followed by Tukey's post-hoc tests. In all situations we adopted a significance level of $p<0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS) 20.0.

3. Results

Erythrocytes from the four species of anuran amphibians were used to evaluate DNA damage using the comet assay. Tail length, % of DNA in tail and Olive tail moment, all of them in arbitrary units (AU), were measured (Table 2). According to the t-test, regarding the tail length parameter in the species *Hypsibos paranaiba* (Figure 1 and Table 2), we found statistically significant differences in all the three parameters (Figures 1, Table 2) evaluated, among the species analyzed in natural and degraded areas. The species *Scinax fuscomarginatus* showed the greatest extent of all the three parameters evaluated (Figure 1).

4. Discussion

There are few studies involving comet assay in adult amphibians (Bosch et al, 2011). In this context, our study was carried out in adult amphibians, of very wide-spread and abundant species in the Cerrado Biome, Central Brazil, to evaluate the genomic sensibility of hylids to natural nickel exposition. As reported by Agostini et al (2010), frequent reproduction, large numbers of eggs in nests and easiness in sampling make the species analysed in our paper interesting candidates for genotoxicity evaluation, especially with a tool used for the assessment of DNA damages, the comet assay (Dhawan et al, 2009).

The analysis of the comet assay generates a large number of parameters, which reflect the amount of damage to DNA and reduces the subjectivity of visual analysis. However, the parameters most frequently used in scientific research are estimates of the tail length, percentage of DNA in the tail and Olive tail moment (Kumaravel & Jha, 2006) which were the ones used in this study. Collins et al (2008) discussed that tail length parameter varies linearly according to the frequency of DNA breaks, showing a positive correlation when compared to manual measurements.

In this study, *S. fuscomarginatus* presented the most extensive DNA damage found in areas with anthropogenic disturbance (Aquino et al, 2010). Such species is listed as least concern in view of its wide distribution, tolerance of a broad range of habitats, presumed large population, and because it is unlikely to be declining fast enough to qualify for listing in a more threatened category (Azevedo-Ramos et al, 2004). On the other hand, the only species that prese-

Table 2. Estimates of DNA Damage in Four Anuran Species in Two Municipalities of Goias State, Central Brazil

Species	Municipality	TL*		% DNA*		OTM*	
		Mean +SD	Probability	Mean +SD	Probability	Mean +SD	Probability
D. minutes	Barro Alto	6.05 ± 2.58	*P* <0.01	4.21 ± 3.26	*P*<0.01	2.97 ± 2.84	*P* <0.01
	Bela Vista	3.31 ± 3.21		0.05 ± 0.08		0.04 ± 0.09	
H. albopunctatus	Barro Alto	5.80 ± 2.85	*P*<0.01	5.32 ± 2.86	*P*<0.01	3.84 ± 2.57	*P* <0.01
	Bela Vista	2.71 ± 2.08		0.69 ± 0.16		0.43 ± 0.06	
H. paranaiba	Barro Alto	5.20 ± 3.16	*P* >0.05	2.96 ± 2.74	*P*<0.01	1.71 ± 2.30	*P* <0.01
	Bela Vista	4.18 ± 2.92		6.48 ± 2.32		5.12 ± 2.96	
S. fuscomarginatus	Barro Alto	6.43 ± 2.68	*P* <0.01	6.48 ± 2.32	*P*<0.01	5.12 ± 2.96	*P* <0.01
	Bela Vista	2.87 ± 2.08		0.10 ± 0.22		0.06 ± 0.05	

** TL: Tail Length; %DNA: Percentage of DNA in the tail ;OTM: Olive Tail Moment*

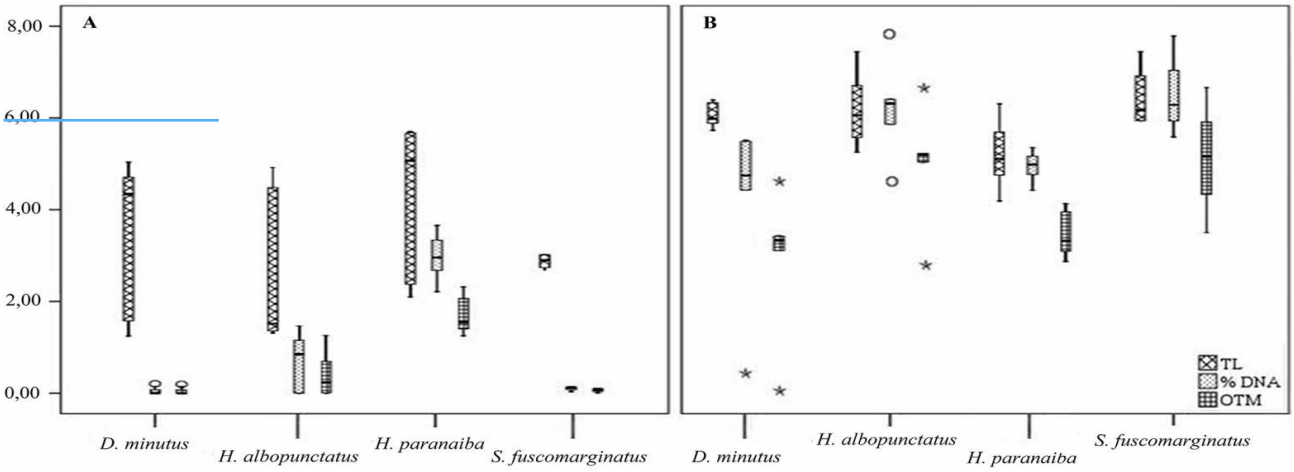

Figure 1. Tail Length (TL), DNA in Tail (% DNA) and Olive Tail Moment (OTM) of Four Hylid Species from Municipalities of Goias State, Central Brazil

(A) Bela Vista, cattle raising area (B) Barro Alt, nickel-mining area

In this context, nickel could explain the increase of genomic damages in all the analyzed species. It is known that nickel is easily accumulated in the biota, particularly in the phytoplankton or other aquatic organisms, which are sensitive bioindicators of water pollution. It can be deposited in the sediment by such processes as precipitation, and ad-sorption on clay particles and via uptake by biota (Cempel & Nikel, 2006).

Many amphibian populations are declining in number worldwide. This phenomenon being, in most cases, associated with pollution of anthropized areas, due to an excessive amount of heavy metals in nature. However, other factors such as over-exploitation, diseases, habitat loss and/or modification, introduced species, climate change, can also contribute with this situation (Mann et al, 2009). Since amphibian skins are highly absorptive, contaminants have the potential to permeate their epidermis, leading to, for example, reproductive failure, which has been determined to be an important contributory factor to the decline of several species (Nystrom et al, 2007).

However, in the studied area (Barro Alto), which presents a natural nickel concentration in the soil, the number of registered amphibian species (39, unpublished data) is similar (or even higher) to those found in other localities of the Cerrado Biome: 27 at Parque Estadual das Furnas de Bom Jesus (Araújo et al, 2009), 33 species at Floresta Nacional de Silvânia (Morais et al, 2012), 34 at Niquelandia (Nomura et al, 2012), 37 at João Pinheiro municipality (Silveira, 2006) and 39 at Estação Ecológica Serra Geral do Tocantins (Valdujo et al, 2010). Nomura et al (2012) found that the abundance of anuran species on another locality that also presents higher amounts of nickel did not differ of distinct spots of the Cerrado. So, some anuran species are adapted to natural environmentals that present high levels of nickel, as observed to other biological groups (Boyd et al, 2006 and Kazakou, 2010).

5. Conclusion

In conclusion, we can suggest that all the evaluated species may be used for eco-genotoxicity of aquatic environments. However, further studies are necessary in many aspects. The levels of environmental exposure concentrations which cause DNA damage need to be determined, as well as what concentration can cause DNA damage repair.

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References

Agostini, M.G., Natale, G.S., and Ronco, A.E. (2010) Lethal and sublethal effects of cypermethrin to *Hypsiboas pulchellus* tadpoles. **Ecotoxicology**, 19(8), pp. 1545-1550.

Aquino, L., Bastos, R., Kwet, A., Reichle, S., Silvano, D., Azevedo-Ramos, C., Scott, N., and Baldo, D. (2010) ***Hypsiboas albopunctatus*** [Internet], IUCN 2012. IUCN Red List of Threatened Species. Version 2012. 2. Available from: <http://goo.gl/IU16Q>

[Accessed 26 March 2012].

Araújo, C.O., Condez, T.H., and Sawaya, R. (2009) **Anfíbios anuros do Parque Estadual das Furnas do Bom Jesus, sudeste do Brasil, e suas relações com outras taxocenoses no Brasil** [Internet], Biota Neotropica, 9(2). Available from: <http://goo.gl/sKPAu> [Accessed 26 March 2012].

Azevedo-Ramos, C., La Marca, E., Andrade, G., and Hoogmoed, M. (2004) ***Hypsiboas multifasciatus*** [Interne-t], IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available from: <http://goo.gl/EfqDm> [Accessed 26 March 2012].

Blaustein, A.R., and Wake, D.B. (1995) The puzzle of declining amphibian populations. **Scientific American**, 272(4), pp. 52-57.

Beiswenger, R.E. (1988) Integranting anuran amphibian species into environmental assessment programs. In: **Proceedings Symposium Management of Amphibians, Reptiles and Small Mammals in North American**. Genetics Technology Reproduction RM-166, USDA Forest Serv., Fort Collins, CO. pp. 159-165.

Bosch, B., Mañas, F., Gorla, N., and Aiassa, D. (2011) Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. **Journal of Toxicology and Environmental Health Sciences**, 3(5), pp. 155-163.

Boyd, R.S., Wall, M.A., and Jaffré, T. (2006) Nickel levels in arthropods associated with Ni hyperaccumulator plants from and ultramafic site in New Caledonia. **Insect Science**, 13(4), pp. 271-277.

Brianezi, G., de Camargo, J.L.V., and Miot, H.A. (2009) Desenvolvimento e validação de técnica quantitativa de análise de imagem para avaliação do teste do cometa corado pela prata. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, 45(4), pp. 325-334.

Bücker, A., Carvalho, W., and Alves-Gomes, J.A. (2006) Avaliação da mutagênese e genotoxicidade em *Eigenmannia virescens* (Teleostei: Gymnotiformes) expostos ao benzeno. **Acta Amazonica**, 36(3), pp. 357-364.

Cempel, M., and Nikel, G. (2006) Nickel, A review of its sources and Environmental Toxicology. **Polish Journal of Environmental Studies**, 15(3), pp. 375-382.

Colli, G., Aquino, L., Azevedo-Ramos, C., Silvano, D., Scott, N., and Langone, J. (2004) ***Scinax fuscomarginatus*** [Internet], IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. Available from: < http://goo.gl/mUbDY> [Accessed 26 March 2012].

Collins, A.R. (2004) The comet assay for DNA damage and repair: principles, applications and limitations. **Molecular Biotechnology**, 26(3), pp. 249-261.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., and Stetina, R. (2008) The comet assay: topical issues. **Mutagenesis**, 23(3), pp. 143-151.

Dhawan, A., Bajpayee, M., and Parmar, D. (2009) Comet assay: a reliable tool for the assessment of DNA damage in different models. **Cell Biology and Toxicology**, 25, pp. 5-32.

Hiraku, Y., and Kawanishi, S. (1996) Mechanism of oxidative DNA damage induced by delta-aminolevulinic acid in the presence of copper ion. **Cancer Research**, 56(8), pp. 1786-1793.

Kazakou, E., Adamidis, G.C., Baker, A.J.M., Reeves, R.D., Godino, M., and Dimitrakopoulos, P.G. (2010) Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. **Plant and Soil**, 332 (1-2) pp. 369-385.

Kumaravel, T.S., and Jha, A.N. (2006) Reliable comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. **Mutation Research**, 605(1-2), pp. 7-16.

Mesjasz-Przybylowicz, J., Przybylowicz, W.J., Rama, D.B.K., and Pineda, C.A. (2001) Elemental distributions in *Senecio anomalochrous*-nickel hyperaccumulator from South Africa. **South African Journal of Science**, 97(11/12), pp. 593-595.

Mitchelmore, C.L., and Chipman, J.K. (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. **Mutation Research**, 399(2), pp. 135-147.

Moraes, D.S.L. (2000) **Avaliação dos potenciais tóxico, citotóxico e genotóxico de águas ambientais de Corumbá-MS em raízes de *Allium cepa***. 158 f. Dissertação, Mestrado em Genética e Melhoramento, Universidade Estadual de Londrina, Londrina.

Mann, R.M., Hyne, R.V., Choung, C.B., and Wilson, S.P. (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. **Environmental Pollutants**, 157(11), pp. 2903-2927.

Morais, A.R., Bastos, R.P., Vieira, R., and Signorelli, L. (2012) Herpetofauna da Floresta Nacional de Silvânia, um remanescente de Cerrado no Brasil Central. **Neotropical Biology and Conservation**, 7(2), pp. 114-121.

Nomura, F., Maciel, N.M., Pereira, E.B., and Bastos, R.P. (2012) Diversidade de anuros (Amphibia) em áreas recuperadas de atividade mineradora e de plantio de *Eucalyptus urophyla*, no Brasil Central. **Bioscience Journal**, 28(2), pp. 312-324.

Nystrom, P., Hansson, J., Mansson, J., Sundstedt, M., Reslow, C., and Broström, A. (2007) A documented amphibian decline over 40 years: possible causes and implications for species recovery. **Biological Conservation**, 138(3-4), pp. 399-411.

Östling, O., and Johanson, K.J. (1984) Microelectrophoretic study of radiationinduced DNA damages in individual mammalian cells. **Biochemical and Biophysical Research Communications**, 123, pp. 291-298.

Olive, P.L., Banath, J.P., and Durand, R.E. (1990) Heterogeneity in radiation induced DNA damage and repair in tumor and normal cells measured using the comet assay. **Radiation Research**, 122, pp. 86-94.

Silveira, A. (2006) Anfíbios do município de João Pinheiro, uma área de Cerrado do noroeste de Minas Gerais, Brasil. **Arquivos Do Museu Nacional**, 64(2), pp. 131-139.

Singh, N.P., McCoy, M.T., Tice, R.R., and Scheider, E.L. (1988) A simple technique for quantification of levels of DNA damage in individual cells. **Experimental Cell Research**, 175, pp. 184-191.

Skelly, D.K. (1996) Pond drying, predators and the distribution of *Pseudacris* tadpoles. **Copeia**, 1996(3), pp. 599-605.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., and Sasaki, Y.F. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. **Environmental and Molecular Mutagenesis**, 35(3), pp. 206-221.

Valdujo, P.H., Camacho, A., Recoder, R.S.,Teixeira Jr., M., Ghellere, J.M., Mott, T., Nunes, P.M.S., Nogueira, C., and Rodrigues, M.T. (2011) **Anfíbios da Estação Ecológica Serra Geral do Tocantins, região do Jalapão, Estados do Tocantins e Bahia** [Internet], Biota Neotropica 11(1), pp. 251-262.

Vitt, L.J., Caldwell, J.P., Wilbur, H.M., and Smith, D.C. (1990) Amphibians as harbingers of decay. **Bioscience**, 40(6), p. 418.

Weygoldt, P. (1989) Changes in the composition of mountain stream frog communities in the Atlantic mountains of Brazil: frogs as indicators of environmental deteriorations? **Studies on Neotropical Fauna and Environment**, 243, pp. 249-255.

Wozniak, K., and Blasiak, J. (2002) Free radicals-mediated induction of oxidized DNA bases and DNA-protein cross-links by nickel chloride. **Mutation Research**, 514(1-2), pp. 233-243.

Zagatto, P.A., and Bertoletti, E. (2006) **Ecotoxicologia Aquática Princípios e Aplicações**. São Carlos, SP.