Chemosphere 240 (2020) 124910

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Review

Micronucleus test in tadpole erythrocytes: Trends in studies and new paths

Marcelino Benvindo-Souza [a, b], Eliane Andreia Santos Oliveira [a], Rhayane Alves Assis [a], Cirley Gomes Araújo Santos [a], Rinneu Elias Borges [c], Daniela de Melo e Silva [b], Lia Raquel de Souza Santos [a, *]

[a] Laboratório de Biologia Animal - Instituto Federal Goiano — IF Goiano, Rodovia Sul Goiana, Km 01, Zona Rural, Rio Verde, Goiás, CEP 75.901-970, Brazil
[b] Laboratório de Mutagênese, Instituto de Ciências Biológicas, ICB I - Universidade Federal de Goiás, Goiânia, Goiás, CEP: 74690-900, Brazil
[c] Universidade de Rio Verde — UniRV, Fazenda Fontes do Saber, Rio Verde, Goiás, CEP: 75.901-970, Brazil

HIGHLIGHTS

- The micronucleus test was reviewed for tadpoles (anuran).
- Its application has been taking place for more than three decades.
- In addition to micronucleus a number of other abnormalities have been reported.
- Genotoxic damage in the medium term may impact the survival of these animals.

ARTICLE INFO

Article history:
Received 14 April 2019
Received in revised form
16 September 2019
Accepted 18 September 2019
Available online 18 September 2019

Handling Editor: Jian-Ying Hu

Keywords:
Amphibians
Anurans
Contaminants
Genotoxicity
Pesticides

ABSTRACT

The micronucleus test has been applied for more than three decades in tadpoles, generating an early warning of environmental quality. In this study, we reviewed 48 articles on the micronucleus test in tadpoles, published between 1987 and 2018. The findings reveal that pesticides have been the main topic discussed in the induction of micronucleus and other nuclear abnormalities in anuran larvae to the detriment of the widespread use of compounds used in agriculture. In addition to pesticides, a number of other xenobiotic agents have been targeted for genotoxic damage, such as heavy metals, radiation and wastewater. An appeal is reported to environmental contaminants, which when released naturally into the environment or because of human activities may contaminate aquatic habitats, threatening populations of tadpoles that depend on these environments for their survival. Larvae can bioaccumulate these contaminants that cause progressive impacts, ranging from DNA damage to metamorphosis delays, as well as malformations. We found that Argentina is the main driving force for the application of this test in anuran larvae along with Brazil. Different erythrocyte malformations have been reported for the erythrocyte nuclear abnormalities test, binucleated cells, nuclear buds, notched, lobed, reniform, nuclear bebbled, anucleated, picnotic and apoptotic cells are the most cited. In summary, the presence of chemical or physical agents, along with other disturbances of the habitat, can have a significant impact on the life history of the species, contributing to the decline of anuran populations.

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* Corresponding author.
E-mail addresses: lia.santos@ifgoiano.edu.br, lirabio@yahoo.com.br (L. Raquel de Souza Santos).

https://doi.org/10.1016/j.chemosphere.2019.124910
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1. Introduction

A significant decline in amphibian populations has been observed in many regions of the world since the 1990s (Hayes et al., 2010; Pérez-Iglesias et al., 2016). As amphibians are commonly found in agroecosystems, pollution by pesticides is thought to be one of the primary determinants of this process (Gonçalves et al., 2015; Pérez-Iglesias et al., 2016). Other chemical contaminants, such as industrial effluents containing heavy metals and pesticides, have also been implicated, given their toxic effects on aquatic organisms, in particular amphibians (Ossana et al., 2013; Fernando et al., 2016; Patar et al., 2016). In this context, characteristics such as the biphasic life cycle of these organisms, in which they are exposed to both aquatic and terrestrial environments, the limitations on their dispersal capacity, their semi-permeable skin, and their physiological adaptations for life in highly specific microhabitats (Hayes et al., 2010; Burlibasa and Gravila, 2011; Gonçalves et al., 2015, 2017a; 2019), make amphibians one of the most sensitive vertebrate groups to contamination by xenobiotic compounds.

Given these characteristics, together with the cutaneous sensitivity of these organisms and their tolerance of experimental conditions (Mikó et al., 2017), tadpoles are frequently used in toxicological research, and are excellent bioindicators, besides being more vulnerable than the adult phase. The micronucleus (MN) test has been used to verify the possible existence of DNA damage resulting from environmental impacts. A significant increase in this damage, relative to a control sample, can provide a systematic measure of environmental alterations in a microhabitat (Ossana et al., 2013; Pérez-Iglesias et al., 2016; Fernando et al., 2016).

The micronucleus test is simple, sensitive, and reliable, and provides an immediate result for the examination of genetic damage caused by the presence of chemical agents in a given environment (Pollo et al., 2015). The micronucleus test is based on the analysis of erythrocytes, according to specific criteria, such as the diameter of the micronucleus (which must be between 1/16 and 1/3 that of the principal nucleus), and its refringence and color, which must be the same as those of the principal nucleus (Babini et al., 2016). The MN is an important marker for environmental biomonitoring, being used to evaluate the initial effects of chronic exposure to xenobiotic compounds in the target species, used either in the laboratory or in the field (Udroiu et al., 2015).

The present study aimed to review the scientific literature up until the end of 2018 about the micronucleus test involving tadpoles. The interest is to elucidate the number of published papers so far, evidencing the main genotoxic agents and the most common erythrocyte nuclear abnormalities (ENAs) in studies with tadpoles on a world scale.

2. Configuration of data collection and analysis

We reviewed papers published in the ISI Web of Science database (www.isiknowledge.com), Scopus (<www.scopus.com/>) and Scielo (<www.isiknowledge.com>) until the end of 2018. For each tracked paper, variables such as year of publication, country of the institution to which the first author belong during the study, number of animals, stages of development, number of cells and the main ENAs were compiled. This trend study was based on our recent report for the micronucleus test on mammalian buccal mucosa (Benvindo-Souza et al., 2017), as well as in other trends (Bayat et al., 2014; Zukal et al., 2015).

Pearson's correlation coefficient was used to test whether a relationship exists ($p < 0.05$) between the number of papers published (dependent variable) and the year of publication. The geographic data (origin of the author) were organized by continent and presented as percentages. Variables such as number of animals, stages of development of tadpoles and number of cells are highlighted by the proportion of citations, and the main ENAs were evaluated by a Pearson's multiple correction analysis ($p < 0.05$).

3. Chronology and geography of the application of the micronucleus test in tadpoles

The micronucleus test as a biomarker of genotoxic damage has been applied to tadpoles for more than 30 years. Our survey of scientific collaboration shows that studies on this subject has been carried out since 1987 (Krauter et al., 1987). Although the researches have selected only 48 published papers on this theme (indexed in the Web of Science, Scopus and Scielo), the data presented a significant correlation for the growth of the studies over time (Fig. 1), which indicates a progress in application. The vast majority of the research was conducted at institutions in South America (65%) and Asia (19%), while a much smaller number of studies originated from Europe (12%) and North America 4% (Fig. 2). In South America, Argentina leads the research followed by Brazil. In Argentina, as in many South American countries, the expansion of agricultural land caused extensive losses and degradation in habitats (Lajmanovich et al., 2013); perhaps this is the great promoter of research with the micronucleus test in the region. Brazil is a world leader in the production and export of grains, mainly soybeans (Araujo et al., 2019) and since 2008 the country has excelled in the consumption of agrochemicals (Guida et al., 2018). In this sense, the increase in studies in Brazil and Argentina (33.33% investigating the action of pesticides) in the last decade is justified due to a concern about the growth of agricultural expansion.

We emphasize the larval phase 33 was the most used in the studies, when considering that the studies approached stages 24–47, according to Gosner (1960). Gonçalves et al. (2017b) suggested that phases 24–33 are good choices for tadpoles biomonitoring in environmental health diagnosis. The larval stage of anurans is more vulnerable than the adult phase, mainly due to the greater exposure of tadpoles to xenobiotics, which can easily penetrate the skin, mouth and gills in the aquatic environment (Pollo et al., 2016), depending on the stages of larval development.

4. Total cells analyzed and synthesis of the main ENAs

The 1000 cells is the most common in the micronucleus test

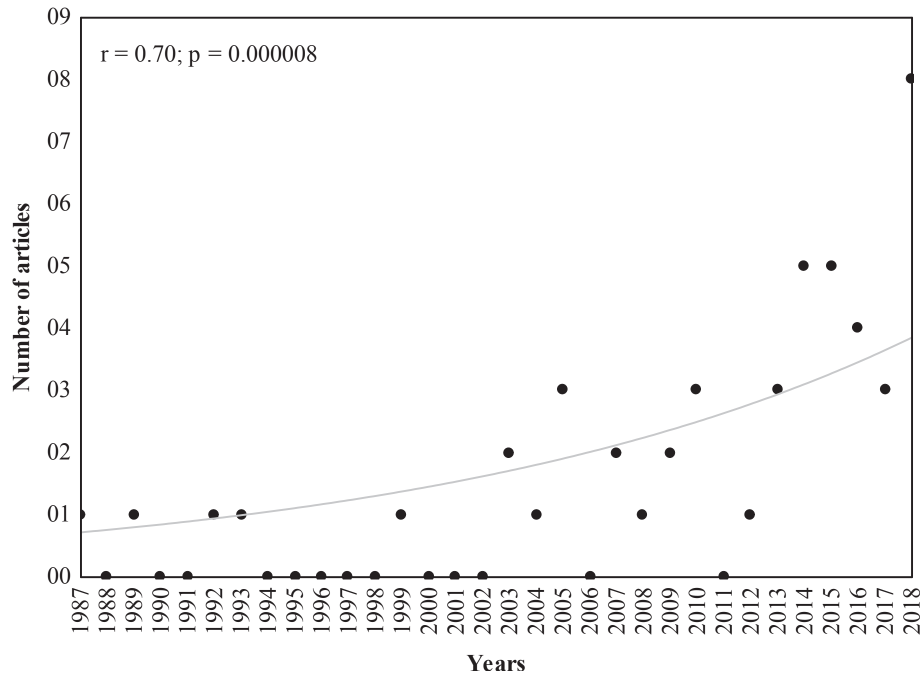

Fig. 1. Number of papers on the micronucleus test in tadpoles between 1987 and December 2018.

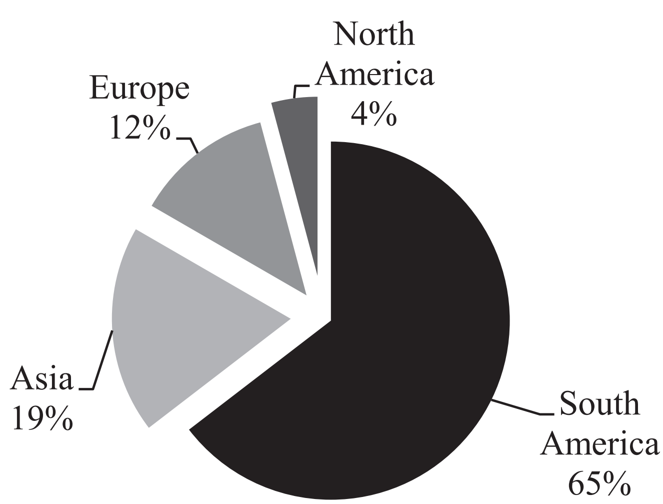

Fig. 2. Geographical distribution by continent of studies evaluating MNs in tadpoles as published between 1987 and December 2018.

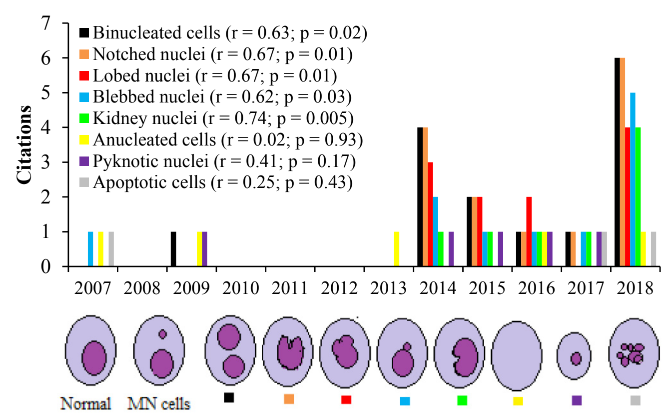

Fig. 3. The correlation of the main ENAs scored in the MN-ENAs test in tadpoles.

(work number = 29; 58%), followed by 2000 cells (n = 13; 29%), whereas analyzes above 2000 are poorly documented (n = 6; 13%). Although the micronucleus test has been applied to tadpoles for approximately three decades, it has been only a little over ten years, since other ENAs are being scored, as complementary alternatives to MN analysis. A series of ENAs have been scored, however, only eight have been frequently selected in the studies. Significant growth was observed for binucleated cells, notched nucleus, lobed nucleus, blebbed nucleus or nuclear bud and kidney nuclei, when we observed the ENAs most cited over time (Fig. 3). Among these, cells with two nuclei are called binucleate and result from the blockade of cytokinesis (Çavaş and Ergene-Gözükara, 2005; Mahboob et al., 2014) by an abnormal cell division. Pollo et al. (2015) discussed that its origin along with the origin of MN is related to cell division, while other abnormalities as the nuclear bud may be related to DNA amplification.

However, the nuclear bud is classified as a MN-like structure attached to the nucleus of the cell by a chromatin (Prieto et al., 2008), its origin probably is the same propellant of MN. Nuclear bud and blebbed nuclei probably have the same nuclear cytomorphology, being observed the same characteristic in several studies (Amaral et al., 2018a, 2018b; Borges et al., 2019). However, the nuclei with vacuoles and appreciable depth in a nucleus without containing nuclear material were recorded as notched nuclei (Arcaute et al., 2014). Nuclei with larger nuclear membrane evaginations that could have several lobes were considered lobed (Arcaute et al., 2014).

Other abnormalities, such as reniform nuclei, with a kidney-like morphological feature may represent a precursor to the formation of MNs or binucleation (Harabawy and Mosleh, 2014). Pyknotic cells with reduced nuclear structure are very common in human studies (Bolognesi et al., 2013) and are associated with cell death. The occurrence of anucleated erythrocytes in amphibians may represent a specific mechanism that increases the efficiency of oxygen transport (Glomski et al., 1997), particularly where water is polluted (Barni et al., 2007). Finally, apoptotic cells exhibit nuclear fragmentation in smaller nuclear bodies within an intact cytoplasm (Fenech, 2000), which is indicative of cell death processes. Thus, apoptosis plays an important role in the regulation of cell numbers during tissue development and homeostasis (Kiechle and Zhang, 2002).

The mechanisms responsible for such abnormalities have not yet been fully recognized (Muranli and Guner, 2011). The scheme of the abnormalities shown in Fig. 3 (MN and ENAs) were schematized according to personal observations (Fig. 4; Borges et al., 2019) and in others studies with anurans (Çavaş and Ergene-Gözükara, 2003; Lajmanovich et al., 2014).

5. Overview of the main genotoxic factors

During 31 years of studying tadpoles as bioindicators of environmental quality through the application of the micronucleus test, several different types of xenobiotic agents were investigated for the genotoxic effect, among the most cited are pesticides (56%), followed by residues of mining (19%), combined analyzes for metals and pesticides (8%), while other genotoxic agents accounted for 17%. Anurans from agricultural areas are exposed to mixtures of pesticides, and a number of studies primarily analyzing herbicide and insecticide action has warned about the effects on larvae. According to Mesak et al. (2018), the 2,4-D herbicide has a mutagenic effect on *Rana catesbeiana* tadpoles, even at low concentrations and for a short period of time. Tadpoles of *Dendropsophus minutus*, one of the most common species in South America, exposed to atrazine 18 mg/L showed higher frequencies of MNs regardless of the stage of development (Gonçalves et al., 2017b).

Fenoxaprop-ethyl, a herbicide widely used in weed control, was used in pre-metamorphic tadpoles (*R. catesbeiana*) at 10 μg/mL and MN rates were 2.8, 2.4 and 1.7 times higher than the control after 96 h of exposure (Jing et al., 2017). The imazethapyr Pivot H® herbicide at a dose of 1.17 mg/L for 48 h also caused an increase in the frequency of MNs in *Boana pulchella* (Pérez-Iglesias et al., 2015). In addition, a significant rate of blebbed and notched nuclei was detected for tadpoles exposed to 96 h of imidazolinone imazethapyr (Pérez-Iglesias et al., 2015). Recently, reports of acute exposure based on imazethapyr have indicated induction of cytogenetic effects (eg increased MN frequency, other ENAs and single strand breaks of DNA) in *B. pulchella* tadpoles (Pérez-Iglesias et al., 2018).

Low concentrations of Roundup (1, 2 and 3 mg ae/L) induced the formation of MN in tadpoles erythrocytes at 24, 48, 72 and 96 h in a concentration-dependent manner (Yadav et al., 2013). Carvalho et al. (2018) did not find statistical significance for the frequency of MNs in erythrocytes of *D. minutus* after 96 h of exposure to Roundup Original ® glyphosate (0.28 mg ai/L, 1.0 mg ai/L, 2.0 mg ai/L, 4.0 mg/L). Lajmanovich et al. (2014) evaluated *Rhinella arenarum* tadpoles exposed for 48 and 96 h at three sublethal concentrations (3.75, 7.5 and 15 mg/L) of a commercial formulation of the herbicide

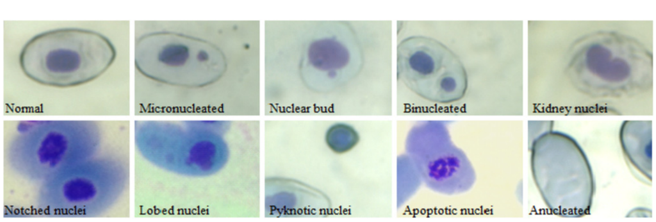

Fig. 4. Photomicrography of micronucleus and other nuclear abnormalities of erythrocytes of tadpoles, stained with Giemsa and magnified in $1000\times$.

glufosinate-ammonium (GLA)-based herbicide (Liberty®, LY), concluding that the inert ingredients of the commercial formulation performed genotoxic damage on erythrocytes of tadpoles. The commercial formulation of the herbicide flurochloridone, Rainbow (®) (25 percenta.i.) increased the MN frequency only when the lowest concentration (0.71 mg/L) was tested on *R. arenarum* tadpoles (Nikoloff et al., 2014).

For the insecticide effects scenario, FASTAC 10 CE induced mutagenicity with MN formation in temporary frog tadpoles, demonstrating that these larvae are more sensitive than *Xenopus laevis* (Rudek and Rozek, 1992). A high frequency of MNs was observed when tadpoles of *B. pulchella* were exposed to15 and 30 mg/L of imidacloprid for 48 h, and in 96 h of exposure only 15 mg/L promoted an increase in the frequency of micronucleated cells (Arcaute et al., 2014). Pérez-Iglesias et al. (2014) demonstrated that tadpoles exposed to the neonicotinoid insecticide imidacloprid (25.0 mg/L) for 96 h showed an increased frequency of MNs, while no other ENA was induced. In the evaluation of Lajmanovich et al. (2005) of the endosulfan insecticide, the MN frequency measured in erythrocytes of *B. pulchella* tadpoles showed no dose dependence at different concentrations (2.5, 5 and 10 μg/L).

A pioneering study of genotoxic and/or cytotoxic agents in amphibians exposed to abamectin (36 μg/L and 72 μga.i./L) revealed the frequency of MNs influenced by exposure time (72 h), while other ENAs (sum of multiloculated nucleus, binucleate cell, notched nucleus, kidney-shaped nuclei, bubble nucleus, apoptotic cell, picnotic nuclei and erythroplastídeos) were influenced by the treatments (Montalvão and Malafaia, 2017). *R. catesbeiana* tadpoles at abamectin exposure even at the lowest concentration (12.5% of LC50) presented higher frequency of NM and other ENAs when compared to the control group (Amaral et al., 2018a), confirming the cytotoxic potential of this pesticide in amphibians. A concentration range of pirimicarb 0.005–0.39 mg/L. Aficida ® induced damage to DNA at the chromosome level by increasing the frequency of MNs and other ENAs, ie, lobed and carved nuclei and binucleate cells (Natale et al., 2018). In the report by Giri et al. (2012) lower concentrations of malathion that extend into the sublethal range (0.5, 1.0 and 2.0 mg/L) induce MNs in erythrocytes of tadpoles at 24 h, 48 h, 72 h and 96 h in a manner concentration dependent.

It is worth mentioned that the pesticides based on *in situ* studies, since agricultural chemicals can contaminate water from permanent ponds, temporary lakes, rivers, streams or streams in agroecosystems due to runoff. Gonçalves et al. (2015) report a significant increase in the frequency of MNs in *D. minutus* from disturbed areas compared to those in the preserved area matrix. Another significant frequency of MN and ENAs was evidenced in the study by Babini et al. (2015) for tadpoles obtained in areas with agricultural activity, indicating the sensitivity of these animals in these environments.

In addition to pesticide studies, investigations into the mining activity have gained prominence. Although mining is important for industrial demands, it is also a source of physical, chemical, biological and landscape changes (Pollo et al., 2016) and an analysis of organisms in these natural environments provides information on the health of the environment, generating data that are toxicologically relevant (Marques et al., 2013). In contrast, it is difficult to establish a direct cause-and-effect relationship with a specific element because of environmental complexity and synergistic and/or antagonistic interactions between substances (Gauthier et al., 2004; Pollo et al., 2016). Fluorite mine work, for example, indicates that settling pond tadpoles are exposed to compounds or mixtures that are damaging the cells when compared to those collected in contamination-free streams (Pollo et al., 2016). Ores such as iron and manganese can be dispersed during extraction, transport and storage and have the potential to induce biological impacts.

These heavy metals raised the frequency (~15-fold) of MN in tadpoles of *R. catesbeiana* in an experimental study (Veronez et al., 2016). Ferreira et al. (2003), pointed out that different toxic concentrations of copper led to increased MN frequency in a dose-dependent manner. Copper toxicity parameters were tested for premetamorphic larvae of *R. catesbeiana*, however, there was only a modest increase in the frequency of MNs (Ossana et al., 2010). The effect of Cadmium and Lead was explored for induction of MN in tadpoles (*Bufo raddei*), and a significantly high rate was observed (Zhang et al., 2007). In addition to the larval phase, Alimba et al. (2018) reported that cadmium and lead were able to induce NM and ENAs as binucleate cells, nuclear bud, notched nucleus, lobed nucleus, vacuolated erythrocytes, apoptotic cells in adult anurans of the species *Amietophrynus regularis.* The cadmium was also tested (Patar et al., 2016) in *Fejervarya limnocharis* tadpoles, whose time of exposure (48 h treated with 0.2 mg/L and 72 h at 0.3 mg/L) induced the incidence of MN. In the recent study by Monteiro et al. (2018) using Chromium, a significant difference in MN frequency was detected between the negative control and all the groups treated with dose-dependent effect.

Radiation was curiously the first xenobiotic evaluated by the test of MN in anurans (*R. catesbeiana* tadpoles), where it was found that the gamma ray favors the linear increase in the frequency of MNs in tadpoles depending on the doses 0.5 to 3.0 gamma rays (Krauter et al., 1987). Later, Marquis et al. (2009) pointed out that high-altitude populations appear to be locally adapted to better resist UV-B genotoxicity, as they showed the lowest MN numbers. Already, Schuch et al. (2015) emphasize that the number of MNs resulting from UVB exposure was much higher when compared to UVA at 48 h and 7 days after treatments, demonstrating the high mutagenic potential of these lower wavelengths of UV solar radiation. On the other hand, the increased incidence of solar ultraviolet (UV) radiation due to ozone depletion has affected terrestrial and aquatic ecosystems and may explain the enigmatic decline of amphibian populations at specific locations (Schuch et al., 2015).

Finally, residual waters from tanneries (tanning effluents), led to increased MNs, notched cells, kidney cells, nuclear bubbles and multiloculated nuclei when compared to control (Amaral et al., 2018b), as well as the chemical agent formaldehyde is also genotoxic for larvae of *R. catesbeiana* (Santana et al., 2015).

6. Gap of knowledge and new paths

16 species of anurans distributed in five families were used in the research (Table 1), which corresponds to approximately 0.23% of the total number of species described around the world (Frost, 2019). Considering the great diversity of anurans in the world, and although our data demonstrate an increase in the researches with the micronucleus test in tadpoles, it is evident the scarcity of researches with this subject. Much of the research has been directed at *R. catesbeiana*, a North American species, and few studies have been carried out on native species elsewhere. *R. catesbeiana* is commonly study for being resistant to xenobiotic agents, considered as a good bioindicator. However, the possibility of obtaining these animals at any time of the year through commercial ranches seems to be the most likely hypothesis of the use of this species in experimental studies. Another possible explanation may be related to the unawareness of larvae of native species, whose identification requires specialists in tadpoles. In this paper, researchers should take into account that species respond differently to xenobiotic agents, producing unequal sensitivity among species because of differences in metabolic rates, physiological conditions and target organs used in the analysis (Monteiro et al., 2018). Therefore, studies with more than one species are encouraged to confirm the response to genotoxic under experimental conditions (Campana et al., 2003) and finally to generate strong conclusions.

Table 1
Species of anuran larvae studied for the micronucleus test.

Family/Species	N	Status of IUCN
Pipidae		
Xenopus laevis (Daudin, 1802)	4	LC
Ranidae		
Rana catesbeiana (Shaw, 1802)	15	LC
Rana temporaria Linnaeus, 1758	1	LC
Pelophylax nigromaculatus (Hallowell, 1861)	1	–
Pelophylax kl. esculentus (Linnaeus, 1758)	1	LC
Hylidae		
Boana pulchella (Duméril & Bibron, 1841)	7	LC
Boana cordobae (Barrio, 1965)	1	DD
Scinax nasicus (Cope, 1862)	1	LC
Dendropsophus minutus (Peters, 1872)	3	LC
Trachycephalus typhonius (Linnaeus, 1758)	1	LC
Bufonidae		
Rhinella arenarum (Hensel, 1867)	6	LC
Strauchbufo raddei (Strauch, 1876)	1	LC
Bufo gargarizans Cantor, 1842	1	LC
Dicroglossidae		
Quasipaa spinosa (David, 1875)	1	VU
Euphlyctis cyanophlyctis (Schneider, 1799)	2	LC
Fejervarya limnocharis (Gravenhorst, 1829)	2	LC

N = Citation number. LC = Least Concern, DD = Data Deficient and VU = Vulnerable.

We also call the attention to Brazil, which despite having the largest biodiversity of anurans in the world (Segalla et al., 2016), has few studies with amphibian populations in natural habitats. Of course, the few research groups that work with anuran ecotoxicology mainly test *R. catesbeiana* leaving many native species unassessed, such as those classified as data deficient and which could disappear without knowledge of their environmental sensitivity. In this same thought, we shed light on other South American countries with hot zones for species of anurans that may face the same problem.

Finally, future studies may also perform a cytomorphometric analysis in erythrocytes of anurans in order to better characterize ENAs, in view of the current disagreement in the punctuation of these among researchers in the field. Another point that we draw attention to is *in situ* analyzes, whose researches are still reduced with tadpoles. In addition, it is important to note that in this phase, these organisms are more sensitive than the adult phase, an environment (ponds) located in or around agricultural areas can receive nutrients and pesticides from neighboring agricultural lands (Peltzer et al., 2008) and compromise the health of these organisms. Screening of vulnerable populations or those with data deficiency, can also be a focus of investigation in ecotoxicology of anurans larvae, especially aim of detecting indicators of chemical and physical disturbance.

7. Conclusion

In synthesis, we detected that the micronucleus test has been applied for 31 years in anuran larvae. Although only 48 articles were found in the databases (Web Of Science, Scopus and Scielo), these findings showed a significant increase over the years, implying that the test tends to be more used in the future. We report that in addition to micronucleus, other erythrocyte nuclear abnormalities have been investigated and gained increasing attention in recent years. As for the genotoxic agents that drive the scientific interest, pesticides followed by heavy metals are the most investigated themes as a result of the expansion of agriculture and its strong use of chemicals. It was found that South America leads the publications in particular due to the contribution of Argentina and Brazil. Finally, based on the premise that the larval stage is vulnerable to genotoxic damage attributed to chemical and physical agents, the broader spectrum of the assay, taking into account a larger range of species and xenobiotics, may illuminate the understanding of the population decline of anurans worldwide.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

To the Foundation for Research Support of the State of Goiás (FAPEG), and the Coordination of Improvement of Higher Education Personnel, CAPES, Brazil for the support given to these authors. D.M.S acknowledges National Council for Scientific and Technological Development (CNPq) for her scholarship.

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