



## ANIMAL SCIENCE

# Mutagenic effect of a commercial fungicide on *Rana catesbeiana* and *Leptodactylus latrans* tadpoles

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**Abstract:** We have examined the mutagenic effects of the fungicide Elatus® on tadpoles of *Rana catesbeiana* and *Leptodactylus latrans*. Sixty-four tadpoles of each species have been exposed to three concentrations of Elatus® (10, 20, and 50 µg/L-1) during 96 hours. We've carried out the micronucleus test (MN) and erythrocyte nuclear abnormalities (ENAs) in 32 tadpoles of each species, the others 32 tadpoles of each species remained in a solution free of Elatus® during 96 hours, in order to assess the ability to recover from the damage caused by the fungicide. There was significant difference in MNs frequency between the treatment exposed to 50µg/L-1 and the control groups for *R. catesbeiana*, while for *L. latrans*, we've found difference between the treatment of 20 µg/L-1, followed by a period without exposure to the contaminant and the control group when all ENAs were analyzed. When we compared the two species, *R. catesbeiana* presented a higher frequency of MNs than *L. latrans* in the treatment exposed to 50 µg/L-1 of the fungicide. Our findings highlight the need to monitor amphibians in places where this product is widely used.

**Key words:** anuran, Elatus®, micronucleus test, pesticides.

## INTRODUCTION

Pesticides used in conventional agricultural can contaminate the environment through drift, runoff, or percolation (Pérez-Iglesias et al. 2020). This fraction lost to the environment contaminates the soil (Cruz-Esquivel et al. 2017), bodies of water (Lajmanovich et al. 2014), and the surrounding fauna (Schmutzer et al. 2008, Cruz-Esquivel et al. 2017). Amphibians are particularly affected by this contamination due to specific characteristics of this group, such as dependence on aquatic habitats for reproduction, skin permeability, and unprotected eggs (Burlibaşa & Gavrilă 2011, Fanali et al. 2018, Gregorio et al. 2019, Gonçalves et al. 2019). Several studies have been pointed out the effects of pesticide

contamination on the health of these animals (Gonçalves et al. 2019, Borges et al. 2019, Pérez-Iglesias et al. 2020), and for this reason, these pollutants are recognized as one of the causes of the population decline of amphibians observed worldwide in the last 30 years (Blaustein & Kiesecker 2002, Benvindo-Souza et al. 2020).

The use of biomarkers in ecotoxicological studies aiming to recognize the impacts of pesticides, helps the detection and measurement of the effects of environmental pollutants in biological models (Adams et al. 2001, Gregorio et al. 2019), leading to the assessment of environmental health. Among these biomarkers, the micronucleus test is widely used because it is considered effective in detecting mutagenic

damage (Udroiu et al. 2015, Borges et al. 2019, Gregorio et al. 2019, Carvalho et al. 2019). This test consists of detecting micronuclei, which are chromosomal fragments that are not incorporated into the main nucleus of the cell (Udroiu et al. 2015) and can be observed as a smaller additional nucleus (Al-Sabti & Metcalfe 1995, Gregorio et al. 2019). In amphibians, more specifically for tadpoles, this biomarker has been applied to erythrocytes ecotoxicology studies for over 30 years and, in addition to micronuclei, other nuclear erythrocyte abnormalities, such as anucleated, apoptotic and binucleated cells, have been detected in response to xenobiotic agents exposure (Benvindo-Souza et al. 2020).

The micronucleus test has already been applied in a series of studies with tadpoles to measure the response to pesticide toxicity (Arcaute et al. 2014, Babini et al. 2015, 2016, Borges et al. 2019, Carvalho et al. 2019). Despite the widespread occurrence of fungicides in aquatic environments, ecotoxicological data for these chemicals are scarce when compared to other types of pesticides (Wightwick et al. 2012, Bernabò et al. 2015). Elatus®, whose contains two active ingredients: azoxystrobin and benzovindiflupyr, is a fungicide used in preventive spraying, to control diseases of the aerial part of cotton, peanut, oat, sugar cane, coffee, barley, beans, maize, soybean, and wheat, and has toxicological classification I (extremely toxic) and class II (product very dangerous to the environment, regarding environmental hazard) according to the commercial product instructions. So far, there is no information about the toxicity of Elatus® in anurans, though some studies have already reported genotoxic damage caused by azoxystrobin in fishes (Bony et al. 2008, 2010, Han et al. 2016). Thus, to assess the toxic effect of Elatus® on anurans, we selected two species as biological models in this study. The *Rana catesbeiana* (Shaw 1802), a species

of anurans highly tolerant to diseases and infections, is considered a good experimental model in toxicological studies (Benvindo-Souza et al. 2020). Also, it is an invasive species that can cause problems in ecosystems in Brazil (Silva et al. 2011). We also studied *Leptodactylus latrans* (Steffen 1815), a species that occurs naturally in Brazil and has an extensive neotropical distribution (Heyer et al. 2010). Therefore, the objective of this work was to evaluate the mutagenicity of a commercial formulation based on azoxystrobin and benzovindiflupyr (Elatus®) in tadpoles through the micronucleus test and the analysis of other erythrocyte nuclear abnormalities. The data from this study are an important contribution to the scientific effort to assess the impact of fungicides on natural ecosystems.

## MATERIALS AND METHODS

### Sampling of animals

One hundred and twenty-eight tadpoles, sixty-four of the species *R. catesbeiana* and sixty-four of the species *L. latrans*, were used in the experiment. The project was approved by the Animal Use Ethics Committee of the Federal Goiano Institute (CEUA / IF Goiano, process number 1458170317) and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, 34485-1). The tadpoles of *R. catesbeiana* were obtained through an authorized commercial ranch. *L. latrans* tadpoles were collected in the egg phase in a body of water, far from agricultural areas, in the municipality of Rio Verde, State of Goiás, Brazil. The tadpoles were kept in aquariums containing tap water dechlorinated with artificial aeration and natural photoperiod and fed with commercial fish feed twice a week until the beginning of the experiment (Pérez-Iglesias et al. 2019) when all animals were at the stage 25G (Gosner 1960).

### Exposure to Elatus®

Four treatments were used for each species. Each experimental group had 16 individuals divided into four glass test aquariums (N = 4 tadpoles per aquarium) with four liters of water containing the appropriate solution, in addition to constant aeration forming one quadruplicate per group. The solutions were prepared by dissolving the commercial formulation of Elatus® in dechlorinated tap water. Three fungicide concentrations were used: 10 µg/L<sup>-1</sup>, 20 µg/L<sup>-1</sup>, and 50 µg/L<sup>-1</sup>, in addition to the control group, kept in clean, dechlorinated water. To date, there are no studies on the concentrations of Elatus® in a natural environment. Previous studies with the active ingredient Azoxystrobin, considered concentrations of 20 and 200 µg/L<sup>-1</sup> (Ortiz-Cañavate et al. 2019), 440, 44 and 4.4 µg/L<sup>-1</sup> (Belden et al. 2010) with the environmentally relevant for this fungicide. For tadpoles, 82.46 µg/L<sup>-1</sup> was considered a lethal concentration of 10% and 196.59 µg/L<sup>-1</sup> was considered an average lethal concentration (Li et al. 2016). However, in aquatic environments, the maximum concentrations ever found were almost 30 µg/L<sup>-1</sup> in France (Berenzen et al. 2005) and more than 11 µg/L<sup>-1</sup> in Germany (Liess & Von Der Ohe 2005). For benzovindiflupyr, we did not find information about concentrations in bodies of water. However, it is worth considering that Elatus® has benzovindiflupyr in its composition and that other ingredients contained in the commercial formulation of pesticides can contribute to the final toxicity of the product in amphibians (Jones & Relyea 2009, Belden et al. 2010). The animals were kept on exposure for 96h, after that period, half of the individuals of each species (n = 32) were anesthetized in cold water and euthanized by a section behind the operculum to obtain blood (Carvalho et al. 2019). The other half (n = 32 individuals) remained for another 96 hours in clean, dechlorinated water,

in a “post-exposure” period, to assess the ability to recover from the damage caused by Elatus®. The water temperature was monitored daily during the experiment.

### Micronucleus analysis

Two slides of blood cell smear were made for each tadpole. The slides were fixed in cold methanol for 20 minutes and stained with 5% Giemsa solution for 12 minutes (Nikoloff et al. 2014, Pérez-Iglesias et al. 2019). The criteria for the identification of micronuclei (MNs) applied were: a diameter less than 1/3 of the diameter of the main nucleus, the similar intensity of color, not refractile, with no connection with the main nucleus, and no overlap with the main nucleus (Al-Sabti & Metcalf 1995, Fenech 2000, Ferreira et al. 2004, Cabagna et al. 2006). The analyzes of the slides were performed by a single researcher under an optical microscope in a blind analysis. We analyzed 1000 cells from each tadpole, using 100x magnification as suggested by Cabagna et al. (2006). In addition to the MNs, other erythrocyte nuclear abnormalities (ENAs) were recorded (binucleated cells, apoptotic cells, and anucleated cells), as suggested in a recent review performed by Benvindo-Souza et al. (2020).

### Statistical analysis

Data from MN and other ENAs are presented as mean ± standard deviation. The variation in the frequency of MN and other ENAs for each species between the different treatments was evaluated using the factorial analysis of variance (using the factors “treatment”: 10, 20 and 50 µg/L<sup>-1</sup> and “period”: exposure and post-exposure) followed by Fisher’s post-hoc test. Friedman test was used for non-parametric data. The normality and homogeneity of the data were tested through the Shapiro-Wilk and Levene tests, respectively and when necessary to satisfy the criteria of

homogeneity of variances, the original data were transformed using the square root of  $x$  ( $\sqrt{x}$ ) in which  $x$  represents the unit value of each measure. For comparisons between the two species for each treatment, Student's T test was used for parametric data and Mann-Whitney U test was used for nonparametric data. All values were considered significant when  $p < 0.05$ .

## RESULTS

### Evaluation of genotoxic damage to the species

For *R. catesbeiana* species, a significant difference for the frequency of MN was found between treatments ( $F_{3,56} = 0.3047$ ,  $p < 0.05$ ), with a frequency of MN increase of 1.40-fold in animals exposed to  $50 \mu\text{g L}^{-1}$  in comparison with animals in the exposure and post-exposure control groups (See Figure 1). No difference was found between treatments for this species as to the sum of the other abnormalities ( $p > 0.05$ ).

For *L. latrans*, no significant difference was observed for micronucleated cells between treatments ( $p > 0.05$ ). However, when the ENAs were added, a significant difference was found, and the post-exposure treatment at  $20 \mu\text{g L}^{-1}$  of

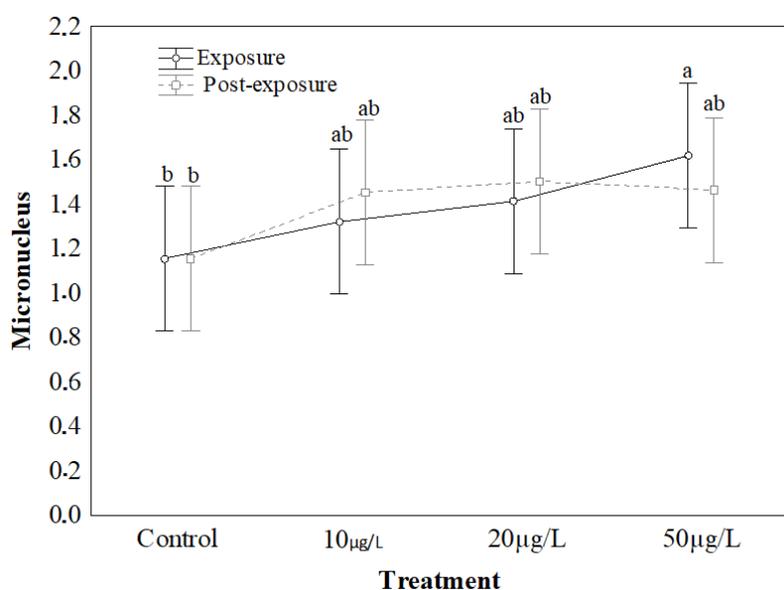
the product showed a 1.92-fold increase in the frequency of ENAs compared to the post-exposure control ( $F_{3,42} = 0.6573$ ;  $p < 0.05$ ; See Figure 2).

### Comparison between species

Finally, when the two species were compared, *R. catesbeiana* showed a frequency of MN 1.46 times higher ( $t = 2.5172$ ;  $p < 0.05$ ) than *L. latrans* in exposure to the highest dose of  $50 \mu\text{g L}^{-1}$  (See Figure 3). No significant difference was found between the two species for the other ENAs and other treatments ( $p > 0.05$ ).

## DISCUSSION

In the present study, we observed that the fungicide Elatus® showed a mutagenic effect in erythrocytes of *R. catesbeiana* tadpoles at a concentration of  $50 \mu\text{g/L}^{-1}$ . This North American species is widely used in genotoxicity assessments through the MN test (Benvindo-Souza et al. 2020), and some characteristics such as resistance to xenobiotic agents, efficiency as a bioindicator and, mainly, the higher availability of tadpoles in commercial



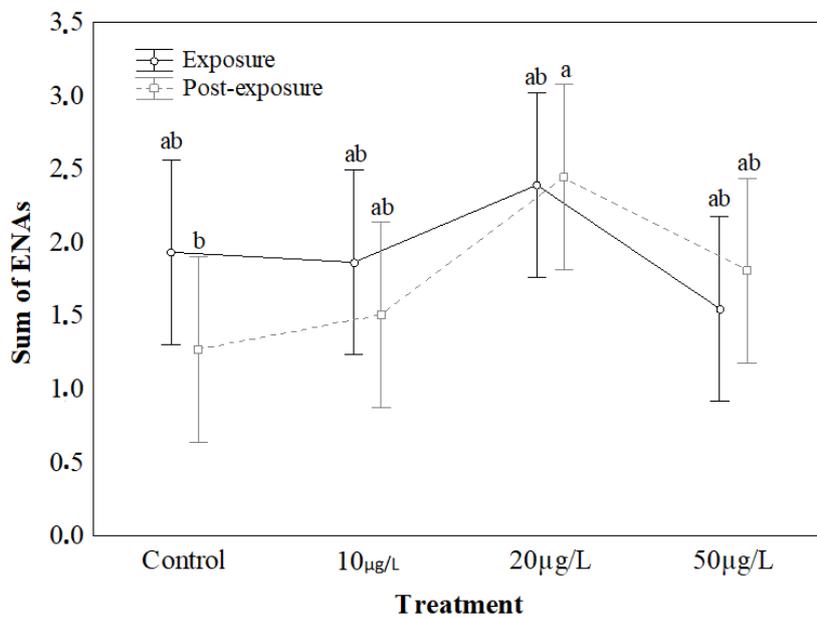
**Figure 1.** Mean frequency of micronuclei in *R. catesbeiana* after 96 hours of exposure to Elatus® (Exposure) and 96 hours after the end of exposure (Post-exposure). Different letters indicate a significant difference, while values marked with the same letters are statistically similar according to the factorial ANOVA test. The data are presented as mean (circles) and standard deviation (vertical bars).

ranches over the entire year, turn this species an excellent model for ecotoxicological studies. Thus, studies with this species exposed to other pesticides such as pyrethroid insecticide lambda-cyhalothrin (Campana et al. 2003), the herbicide phenoxaprop-ethyl (Jing et al. 2017), among others, have already detected an increase in micronucleated cells in tadpoles, which reinforces our findings.

The increase in the frequency of MNs observed in the present study indicates genetic damage, since MNs originate in the condensation of chromosomal fragments or whole chromosomes that were not incorporated into the main nucleus of the cell during cell division, and maybe a product of DNA fragmentation or of alteration of the mitotic system (Balmus et al. 2015, Hayashi 2016, Pérez-Iglesias et al. 2020). Studies that evaluated the effects of azoxystrobin, the main active compound in Elatus®, also showed an increase in MNs (Bony et al. 2010) and DNA damage (Bony et al. 2008, Han et al. 2016) in fish, in addition to tadpole mortality (Johansson et al. 2006, Hooser et al. 2012). These studies are important, mainly

because this compound has already been detected in water in natural environments in several countries, including Brazil (Rodrigues et al. 2013). Thus, we present the first evidence that the commercial formulation of the fungicide Elatus® can also cause genetic damage in tadpoles.

In *L. latrans*, we observed an increase in the frequency of cells with ENAs after the recovering time of 96 hours (without exposure to a concentration of 20 µg/L<sup>-1</sup> of the commercial fungicide), which suggests that the toxic effect of Elatus® for this species can be late and manifest even after the end of the exposure. The frequency of ENAs has been shown to increase in tadpoles exposed to pesticides (Nikoloff et al. 2014, Perez-Iglesias et al. 2016, 2018). Among them, apoptotic cells, recognized for their intense chromatin condensation, represent a process of nuclear fragmentation (Fenech et al. 2003), indicating serious DNA damage induction by a chemical (Gregório et al. 2019). Binucleated cells may in turn be a result of blocking cytokinesis by abnormal cell division (Çavaş & Ergene-Gözükara 2005, Pollo et al. 2015). Anucleated cells, on the



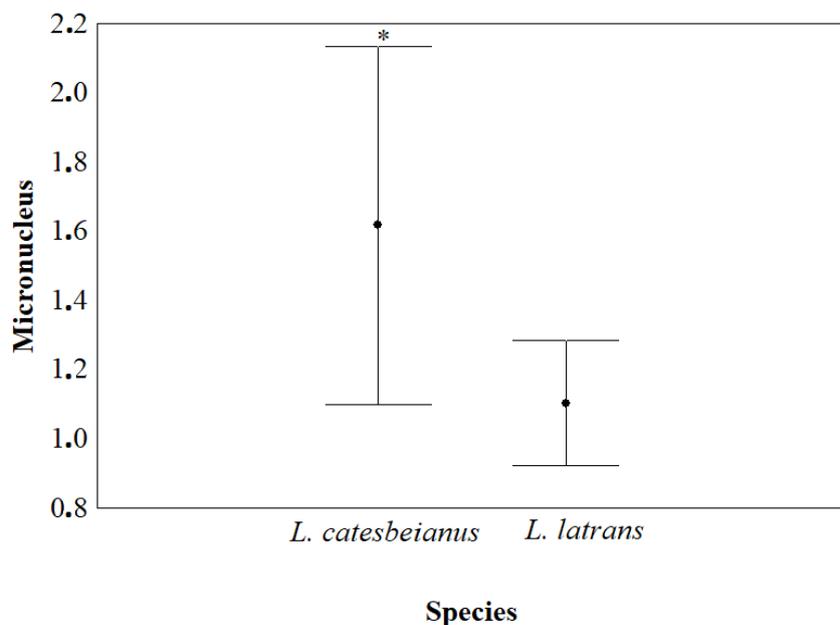
**Figure 2.** Sum of erythrocyte nuclear abnormalities (ENAs) in *Leptodactylus latrans* after 96 hours of exposure to Elatus® (Exposure) and 96 hours after the end of exposure (Post-exposure). Different letters indicate a significant difference, while values marked with the same letters are statistically similar according to the factorial ANOVA test. The data are presented as mean (circles) and standard deviation (vertical bars).

other hand, can be related to mechanisms of increased oxygen transport (Glomski et al. 1997) increasing in stressful situations (Lajmanovich et al. 2014).

Thus, our results indicate the harmful effects of Elatus® also on *L. latrans*, notwithstanding no damage was observed in animals exposed to 50 µg/L<sup>-1</sup> of the product. A study with this species indicated that the insecticide Introban® caused an increase in the frequency of ENAs in animals exposed to 2.5 mg/L, 5 mg/L, and 10 mg/L of the product compared to the control group, but animals exposed to the highest concentration, 20 mg/L, showed less damage than the negative control (Lajmanovich et al. 2015). This response can be explained by the fact that at higher toxic doses, the rate of cell division decreases, and this can reduce the frequency of ENAs (Lajmanovich et al. 2014). However, only the post-exposure treatment at 20 µg/L<sup>-1</sup> showed an increase in the frequency of damage for this species, indicating that more studies are needed to clarify the toxic effects of Elatus® in native species.

Animals of both species showed no difference in the frequency of damage during

exposure and after the exposure ceased. Some studies have already demonstrated the ability to recover genetic damage in other species of anurans in the larval phase in the post-exposure phase, after the interruption of treatments. However, there is a discussion about the minimum time necessary for this recovery, since this time can vary greatly depending on the species (Pérez-Iglesias et al. 2018). Morse et al. (1996) found that tadpoles of *Xenopus laevis* showed a reduction in the frequency of MNs after 24 hours free from exposure to benzo[a]pyrene. Mouchet et al. (2015) in a study with the same species exposed to cadmium and zinc pointed out the ability to recover from damage after 7 days free from exposure. Pérez-Iglesias et al. (2018) in a study with the species *Boana pulchella* pointed out the ability to recover DNA damage, by returning to baseline levels of MNs and ENAs of the tadpoles 7 days after the end of exposure to the herbicide Pivot®. Hence, the 96-hour period may not have been enough for the action of the DNA repair mechanisms of the two species in this study, and future work testing longer post-exposure times is encouraged.



**Figure 3.** Mean frequency of micronuclei in *Rana catesbeiana* and *Leptodactylus latrans* after 96 hours of exposure to 50 µg L<sup>-1</sup> of Elatus®. The asterisk (\*) indicates a significant difference ( $p < 0.05$ ) between the two species according to the Student's T test. The data are presented as mean (circles) and standard deviation (vertical bars).

A small difference was found in *R. catesbeiana* and *L. latrans* responses to exposure, being that the first species presented a higher MNs frequency than the second only for 50 µg/L<sup>-1</sup> exposure. Notably, most studies using the MN test in tadpoles evaluate the response for only one species, reinforcing the knowledge gap about the differences between species (Benvido-Souza et al. 2020). Notwithstanding, Araújo et al. (2014a) in a study with *R. catesbeiana* and *L. latrans* exposed to copper, showed similar sensitivity between them to detect and avoid sublethal concentrations of the product. Araújo et al. (2014b) also detected that both species were able to detect sublethal concentrations of the fungicide pyrimethanil, demonstrating similarity in sensitivity to pesticides between the two species. Also, although *R. catesbeiana* is considered a resistant species due to its generalist characteristic and ease of adaptation to different habitats, which turns it an invasive species in Brazil (Silva et al. 2011), *L. latrans* is also a species considered tolerant to habitat modifications since it is commonly found in areas altered by human influence such as pastures and agricultural areas (Heyer et al. 2010). This may explain the similarity in the exposure responses to the fungicide of *L. latrans* and *R. catesbeiana*.

## CONCLUSIONS

This study demonstrated the mutagenic responses of two anuran species exposed to the commercial formulation of fungicide Elatus®. Both species showed sensitivity to product exposure, either through an increase in the frequency of micronuclei (*R. catesbeiana*) or other erythrocyte nuclear abnormalities (*L. latrans*). The possibility of recovering the damage caused by the product in these species within 96 hours was not evidenced in this study. Thus, we

bring the first evidence of the damage caused by this fungicide in anurans and encourage new studies with other native species, increasing the concentrations of the contaminant and the time after exposure.

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RAA and MBS conducted the experiment, analyzed the data and wrote the manuscript. CGAS, REB, IDSF and LCO, assisted in conducting the experiment and analyzing the data. MACM and LRSS reviewed the manuscript.

