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Ecotoxicological Methodologies to Evaluate Biomarkers at Different Scales in Neotropical Anurans

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Abstract

The new questions in ecotoxicology highlight the importance of applying a battery of biomarkers, as this results in ecotoxicological predictions that improve not only the interpretation of the effects of environmental stressors on organisms but also the determination of their possible impact. It is well known that the use of ecotoxicological biomarkers at different levels of organization allows for the prediction of the biological responses of organisms to environmental stressors, which is useful in environmental risk assessment.

Nevertheless, it is necessary to consider the optimization of basic procedures, to generate historical data in control groups, and to employ specific bioassays to evaluate responses in organs and tissues in order to elucidate the nature and variation of the effects observed. Therefore, the present work aims to describe several ecotoxicological methodologies employed in all stages of neotropical anurans at different ecological levels and to validate them as useful biomarkers to be used both in wildlife and in laboratory conditions. In this work, these biomarkers were applied at the individual/organismic level (body condition index), histological/physiological level (histopathology, histometric, and pigmentary analyses), biochemical level (oxidative stress enzymes), and genetic level (direct and oxidative damage in DNA by comet assay).

Although these methodologies have small variations or modifications depending on the species, these techniques provide effective biomarkers for evaluating the effect of xenobiotics on anurans, which possess certain characteristics that make them

useful indicator species of aquatic and terrestrial ecosystems. In conclusion, the battery of biomarkers employed in the present study has proven to be adequate for estimating toxic responses in Neotropical anurans and can be further recommended as bioindicators for identifying the impact of pollutants on the aquatic ecosystems of the region. Finally, it is recommended to achieve the standardization of these important biomarkers for anurans in specific regions as well as to possibly include them in risk assessments and decision-making.

Introduction

The input of environmental stressors into natural water bodies can affect the health of the aquatic ecosystem[1]. Exposure to these environmental stressors can affect the survival or fitness of aquatic organisms through different toxicity mechanisms, including direct exposure (both short- and long-term)[2]. Hence, standardized laboratory bioassays to assess toxicological endpoints related to fitness and survival may be an unreliable estimate of the many indirect effects of stress in the field. Furthermore, alterations in normal physiological levels and effects on individuals, such as in terms of prey capture, may be better long-term indicators of the impact on survival and reproductive fitness in organisms and, ultimately, on the health of the ecosystem[1,3]. Predicting changes in ecosystem composition and function, as well as organism health, based on a known set of environmental parameters and contaminant concentrations, is important for improving pollution management[1].

Biomarkers are defined as biochemical, physiological, or histological changes due to either exposure to or the effects of xenobiotic chemicals[4,5]. Biomarkers have proven to be very useful as early warning signals[4,5]. An important question that biomarkers help answer is whether certain stressors are present in high enough concentrations in the environment to cause adverse effects. This information contributes to the assessment of whether it is worth investigating the nature and extent of the damage and the causative agents or whether no more resources should be invested in that case[6,7,8]. Moreover, since the concept of evaluating a single biomarker as a bioindicator may not be adequate[5,7,8,9,10], there is a growing trend toward performing a comprehensive evaluation of multiple biomarkers in order to detect early warning signs and, thus, prevent irreversible effects on ecosystems.

It is very important to note that all toxic effects begin with the interaction of a stressor with biomolecules. In this sense, effects can cascade through the biochemical, subcellular, cellular, tissue, organ, individual, population, community, ecosystem, landscape, and biospheric levels of organization. Cells are the primary site of interaction between environmental stressors and biological systems. Thus, understanding molecular and genetic effects allows researchers to associate low and high levels of ecological organization and helps them to predict the effect of environmental pollutants, for example, on human health, that have not yet been tested[5]. Moreover, due to the high specificity of cells, they are not only useful for evaluating environmental pollutants but also human health[5,11]. Therefore, understanding the effects of stressors at the biochemical level may provide insights into the

causes of the observed effects and allow them to be connected with those at the next higher level[5]. In addition, by understanding the biochemical mechanisms of stressors, the effects of new stressors that have not yet been toxicologically evaluated may be predicted with respect to other well-known contaminants based on their similarities in function. In the presence of various environmental stressors, genetic and biochemical biomarkers may provide valuable information on the specific effects observed. In addition to this, histochemical evaluations related to biochemical changes can provide information on toxicodynamics[5]. In short, a comprehensive analysis of cellular, biochemical, and histological biomarkers is necessary[10,12], and this type of analysis, in turn, should be included in biomonitoring programs for local species[5,13,14].

The study of biomarkers under laboratory conditions may, nonetheless, present some difficulties, including difficulties in the detection of sublethal effects and chronic impacts after exposure to pollutants and in the validation and standardization of the methods employed, as well as the complex time- or dose-dependent responses, the unclear or undetermined links to fitness, and the lack of integrated mechanistic models[1,4]. To solve these problems, the solution is not to increase the number of biomarkers measured but to carefully design studies and testable hypotheses that contribute to explaining the mechanistic bases of chemical effects on whole organisms[4].

The new questions in ecotoxicology highlight the importance of applying a battery of biomarkers to generate ecotoxicological predictions that improve the interpretation of the effects of environmental stressors on organisms, as well as decision-making about their possible impact. Moreover, the importance of combining both concepts-biomarkers and bioindicators-in environmental risk assessments and biomonitoring is that this will allow researchers to determine whether organisms in a specific environment of interest are physiologically normal or stressed. The approach taken in this study resembles that of the biochemical analysis that is carried out in humans. In this sense, a battery of biomarkers may be analyzed to see if an organism is healthy both in the field and in the laboratory[6]. Finally, biomarkers will contribute to ecological risk assessments in two ways: (1) assessing the exposure of rare and/or long-lived species, and (2) testing hypotheses about the mechanisms of chemical impacts at different levels of biological organization[4].

In the last decade, biomarkers have been used in anurans for biomonitoring the exposure to cytotoxic and genotoxic contaminants. Among these, the techniques that have been used most frequently are the micronucleus (MN) assay and the comet assay or the induction of single-stranded DNA breaks by single-cell gel electrophoresis (SCGE) assay. In addition, those techniques have been successfully used to estimate the DNA damage induced by various environmental stressors in several neotropical anurans[14,15,16,17,18,19]. Other biomarkers can used to examine changes in the oxidative status in organisms exposed to environmental pollutants[16,17,18,19]. Oxidative stress is a response to exposure to different xenobiotics, leading to several detrimental effects, including on the antioxidant capacity of the exposed individuals[5,6,7,19,20].

In ecotoxicological studies, bioindicator species are used because they are organisms that identify the long-term interactions and adverse effects of environmental stressors at higher organizational levels (e.g., organism, population, community, and ecosystem levels)[10,20,21]. By integrating the two concepts-biomarkers and bioindicators-species can be screened to broadly define biochemical, physiological,

or ecological structures or processes that are correlated or linked with measured biological effects at one or more levels of biological organization. Finally, the great challenge of utilizing both concepts to improve the estimates of the toxicity of a stressor relates to analyzing biomarkers and bioindicators that have high utility in the evaluation of ecological risks[20]. In this sense, there is consensus on the relevance of employing biomarkers and bioindicators as early warning signs, as they offer relevant information about the response of a test organism to environmental stressors[12,20,21].

Amphibians are one of the most threatened and rapidly declining groups of organisms worldwide. One of the main reasons for this decline is pollutants that enter their habitat, such as pesticides, metals, and emerging pollutants[22,23,24,25]. Anurans have several characteristics that make them useful as bioindicator species, such as their permeable skin, close relationship with water, and sensitivity to environmental pollution[2,23,24]. These characteristics make amphibians effective bioindicators of environmental health[7,8,22,23,24,26].

Nevertheless, it is necessary not only to consider the optimization of basic procedures and the generation of historical data in control groups but also to employ specific bioassays to evaluate responses in organs and tissues to elucidate the nature and variation of effects observed in bioindicators. In this sense, the present work aims to describe several ecotoxicological methodologies to be employed in all stages of neotropical anurans at different ecological levels and validate them as useful biomarkers to be employed both in wildlife and laboratory conditions. This work presents a battery of biomarkers that may be integrated and that have been proven for laboratory and wildlife biomonitoring in anurans exposed to environmental stressors.

Protocol

The following techniques include the previous sacrifice of the animal, which was carried out in accordance with international ethical standards[46,47,48], and the subsequent dissection and ablation of the organs. The animals were captured under the authorization of the Ministry of Environment, Agriculture and Production of San Luis Province (Resolution 49-PMA2019). The methods of sacrifice and euthanasia of the animals were duly approved by the protocols of the Institutional Animal Care and Use Committee (CICUA, protocol Q-322/19) from the National University of San Luis. The procedures with anuran organisms were carried out according to guidelines detailed in Garber et al.[46], CONICET[47], and INTA[48]. In addition, all the protocols presented here are for neotropical anuran species in their larval and adult life stages; they have already been widely accepted by local researchers and are carried out under a strict protocol and with authorization from the "Comité Institucional de Cuidado y Uso de Animales (CICUA)" of each university involved. A list of the materials and solutions used is presented in the **Table of Materials** and **Table 1**.

1. Individual level: Body condition and hepatic and gonadal indexes

1. Body condition index: Scaled mass index

   NOTE: This index can be used both with adults and with juveniles and larvae. This methodology is based on Peig and Green[27], with minor modifications for anurans according to Brodeur et al.[28,30] and MacCracken and Stebbings[29].

   1. Record the body mass of each specimen using a precision analytical scale.

2. Place each specimen on a millimeter sheet, and take a photograph at a distance of ~15 cm.

   NOTE: It is important to always take photographs from the same distance.

3. Photograph analysis:

   NOTE: The photographs can be analyzed with the ImageJ program, which is freely available online (https://imagej.nih.gov/ij/index.html), or with any other image analysis program that allows measurements to be made from a scale included in the photograph.

   1. Measure the snout-vent length (SVL) of each specimen using the **measure tool** from the ImageJ program, and first refer to a known measure in the millimeter sheet.

      NOTE: In tadpoles, the measurement of the body length must be made up to the cloacal tube, disregarding the caudal fin.

4. Scaled mass index (S)

   1. With the data obtained from the body mass and SVL of each anuran, construct a non-linear power function regression line of body mass (y-axis) against SVL (x-axis).

   2. Calculate the mean length with the SVL data of all the individuals measured from the studied population.

   3. Finally, calculate the scaled mass index (S) according to Peig and Green[27] and Brodeur et al.[28] using equation (**1**):

      $S = M_i(L_0/L_i)^b$ (**1**)

      where M_i and L_i are body mass and SVL from the individual i, respectively; b is the scaling exponent estimated by a non-linear power function regression[28]; L_0 is the mean length for the studied population; and S is the predicted body mass for the individual i when its SVL is standardized to the L_0 value.

      NOTE: It is important to note that b is a species-specific constant that could also be sex-specific in sexually dimorphic species.

2. Hepatosomatic index: Scaled liver index

   NOTE: As for the body condition index, this index can be used with individuals at all stages of development. The methodology is based on Brodeur et al.[28].

   1. Record the SVL of each specimen according to step 1.1.2 and step 1.1.3.

   2. Euthanize individuals according to ethical standards for anuran amphibians[46,47,48].

   3. Remove the entire liver, and record its mass on a precision analytical scale to the nearest milligram.

   4. With the data obtained from the liver mass and SVL of each anuran, construct a non-linear power function regression line of liver mass (y-axis) against SVL (x-axis).

   5. Calculate the mean length with the SVL data of all the individuals measured from the studied population.

   6. Calculate the scaled liver index (SLI) according to Brodeur et al.[28] using equation (**2**):

      $SLI = Lm_i (L_0/L_i)^b$ (**2**)

      where Lm_i and L_i are the liver mass and SVL from the individual i, respectively; b is the scaling exponent estimated by a non-linear power function

regression[28]; L_0 is the mean length for the studied population; and SLI is the predicted liver mass for the individual i when its SVL is standardized to the L_0.

3. Gonadal index: Scaled gonadal index

NOTE: This methodology is based on Brodeur et al.[30] and is useful only with adult individuals. It is important to note that male and female indices must be analyzed separately as they vary on distinct scales.

1. Record the SVL of each specimen according to step 1.1.2 and step 1.1.3.
2. Euthanize individuals according to ethical standards for anuran amphibians[46,47,48].
3. Remove the right and left gonads, and record the mass on a precision analytical scale to the nearest milligram.
4. With the data obtained from the gonad mass and SVL of each anuran, construct a non-linear power function regression line of gonad mass (y-axis) against SVL (x-axis), similar to step 1.1.4.1 and step 1.2.3.
5. Calculate the mean length with the SVL data of all the individuals measured from the studied population.
6. Estimate the scaled gonadal index (SGI) according to Brodeur et al.[30] for each animal using equation (**3**):

$$SGI = Gm_i(L_0/L_i)^b \quad (3)$$

where Gm_i and L_i are the gonad mass and SVL from the individual i, respectively, b is the scaling exponent estimated by a non-linear power function regression by Brodeur et al.[30], L_0 is the mean length for the studied population, and SGI is the predicted gonad mass for an individual i when its SVL is standardized to the L_0.

2. Morphological-histological level

NOTE: For this analysis, it is necessary to use histological sections. The first step is to collect the tissue.

1. Fixing and dehydration
   1. Use a fixative solution to preserve the structures. For anuran tissues, preferably use methacarn or Bouin solution, recommended over the rest of the fixing solutions (**Table 1**).
      NOTE: All substances must be mixed at the time of use.
   2. Place tadpoles or a tissue fragment of 50-100 mg in a conical tube with 1-2 mL of fixative solution at 4 °C for 3 h. Wash the tissue in the same volume of 70% ethyl alcohol for 30 min.
   3. Place the tissue in a fresh 70% ethyl alcohol solution.
      NOTE: It is possible to stop the procedure at this point for some days before continuing with the dehydration.
   4. Dehydrate the tissue in a series of alcohol solutions: ethyl alcohol 80% (10 min), alcohol 90% (10 min), ethyl alcohol 100% (10 min), and ethyl alcohol 100% (10 min).
   5. Perform diaphanization (clearing) 2 x 10 min in xylene.
   6. Melt the paraffin in a beaker. Soak the tissue for 10 min. Remove the tissue, and place in a histological mold with melted paraffin. Wait for it to solidify at room temperature.

7. Place 3 µm sections made on a rotating microtome on a glass slide.

2. Histometric analysis

1. Stain the tissue sections in hematoxylin-eosin[31].

2. Use 10-20 photomicrographs for measurement with a 100x objective in a light microscope. Analyze 5-10 hepatic cells in each photomicrograph.

NOTE: Here, liver was used as a model to describe the technique. It is possible to use other tissues; ensure that the objective of the light microscope to be used is suitable for the type of tissue. It is necessary to be able to see and identify the cells to be measured.

3. Histopathology

1. After staining the tissue sections in hematoxylin-eosin[32], examine 5-10 histological sections under 10x, 20x, 40x, and 100x objectives.

2. Look for tissue changes and estimate the degree of tissue changes (DTC) index as described by Bernet et al.[32] using equation (**4**):

$$DTC = \Sigma alt.(a \times w) \quad (4)$$

where Σalt is the sum of alteration; a represents the damage stage (0, absent; 1, low; 2, moderate; 3, high); and w represents the degree of damage reversibility (1, reversible; 2, partially reversible; 3, irreversible).

NOTE: Here, liver was used as a model to describe the technique. It is possible to use other tissues; ensure that the objective of the light microscope to be used is suitable for the type of tissue.

4. Pigmentary system

1. After staining the slides in hematoxylin-eosin, examine 10-20 photomicrographs with a 20x objective.

2. Quantify the area occupied by melanin by measuring differences in the staining intensity using Image Pro-Plus software according to Santos et al.[33].

3. Open the software Image Pro Plus 6.0®.

4. Select **Menu** and **Toolbar: Biological**.

5. Select the **Spatial calibration of magnification** used to take the photomicrographs.

6. Open a histological image.

7. Select **Tool count | Measure objects**.

8. Click on the **Select colors** button.

9. Select the **dropper tool**, and mark the coloring to be measured in the image.

10. Close the window, and click on **Count**.

11. Select **View | Statistics** to see the results.

3. Biochemical level: Reactive oxygen species (ROS) and cholinergic enzymes

1. Sample homogenization

NOTE: Once the experiment is over, preserve the samples (tissues or tadpoles) in phosphate-buffered saline (PBS) if they are not going to be processed at that time by freezing at −20 °C (for 2 months) or −80 °C (for 6 months). Alternatively, they can be homogenized at the time according to the recommendations proposed below.

1. Tadpoles

1. Weigh 1 g of a tadpole or pool of tadpoles on a precision analytical balance.

2. Place the 1 g of tadpoles in a 15 mL conical tube containing 1 mL of PBS, and immerse it in an ice bath (4 °C).
3. Homogenize with a teflon-tipped homogenizer adapted to a conical tube, and work in cold conditions (4 °C).
   CAUTION: Avoid using a high number of revolutions, as this may destroy proteins and raise the temperature.
4. Transfer the same volume of the liquid homogenate of each sample into two labeled 2 mL microcentrifuge tubes, and work in cold conditions (4 °C).
5. Centrifuge the samples with the liquid homogenate for 10 min at 4 °C and 9,520 × *g*.
6. Finally, extract the supernatant, place it in microcentrifuge tubes, label it in aliquots of 0.5 mL to 1 mL, and preserve in a freezer at −80 °C (for 6 months) or −20 °C (for 2 months) until the enzyme assay.

2. Adults
   1. Remove the tissue of interest (e.g., liver, muscle, kidney), and weigh 1 g on a precision analytical balance.
   2. Place the tissue in a 50 mL conical tube containing 1 mL of PBS, and immerse it in an ice bath (4 °C).
   3. Homogenize with a teflon-tipped homogenizer adapted to a conical tube, and work cold (4 °C).
      CAUTION: Avoid using a high rpm, as this may destroy proteins and raise the temperature.
   4. Transfer the same volume of the liquid homogenate of each sample into two labeled 2 mL microcentrifuge tubes, and work cold (4 °C).
   5. Centrifuge the tubes with the liquid homogenate for 10 min at 4 °C and 9,520 × *g*.
   6. Finally, extract the supernatant, place it in microcentrifuge tubes, label in aliquots of 0.5 mL to 1 mL, and preserve in a freezer at −80 °C (for 6 months) or −20 °C (for 2 months) until the enzyme assay.

2. Protein determination

   NOTE: The protein value is obtained to estimate the enzymatic activity in relation to the total amount of protein while avoiding the underestimation or overestimation of the enzymatic activity values. This methodology is based on Bradford[34] with minor modifications for anurans:

   1. Prepare Bradford reagent (see **Table 1**).
   2. Add 100 mL of H_3PO_4 at 85% (p/v) to Coomassie G-250 + ethanol, and bring to a volume of 1 L with distilled water.
   3. Prepare the calibration curve with bovine serum albumin (BSA) as a known protein standard (**Table 1**).
   4. Prepare albumin standards in increasing concentrations for a 3 mL spectrophotometer cuvette. For an example of the standard curve, see **Table 2**.
   5. Measure the absorbance using a spectrophotometer at 590 nm (this is the wavelength at which the complex of the reagent and the protein is formed).
      NOTE: Performing the Bradford reaction with the sample for reading in the spectrophotometer using

a glass cuvette with a maximum volume of 3 mL and an optical path of 1 cm helps the protein determination.

6. Place the following reactants in a cuvette in this order: 2,000 µL of the Bradford reagent + X µL of the sample (X = 20 µL of the homogenate sample of the organ of interest from an adult anuran, or 40 µL of the homogenate sample from a tadpole).

7. Finally, measure the absorbance in the spectrophotometer at 590 nm.
   NOTE: It is not necessary to work at 4 °C.

3. Catalase

NOTE: This methodology is based on Aebi[35] with minor modifications for anurans.

1. Incubate 1,900 µL of PBS + 40 µL of hydrogen peroxide (H_20_2) + 20 µL of the sample (pure in tadpoles and diluted 1/25 for adult tissue) in a 3 mL quartz cuvette with a 1 cm path length.
   CAUTION: Follow the order given above for the addition of the reagents and the sample during the incubation. The reactants can be incubated at room temperature (25 °C) since anurans are physiologically ectothermic.

2. Read the catalase kinetics at 240 nm for 2 min in a UV spectrophotometer.

3. Calculate the enzyme activity with the following equation (**5**)[35]:
   k (mmol·min^{-1}·mg^{-1} Prt) = (Δ absorbance/1,000)/ protein concentration (**5**)
   NOTE: In studies with purified enzyme preparations, the specific activity *k'0* is obtained by dividing k by the molar extinction coefficient, (e = 43,6); k' = (k/e).

4. Glutathione S-transferase (GST)

NOTE: This methodology is based on Habig et al.[36] with minor modifications for anurans.

1. Incubate 300 µL of GST + 10 µL of 1-choro-2, 4-dinitrobenzeno (CDNB) (0.1 M) + 10 µL of the sample (pure in tadpoles and diluted 1/25 for adult tissue) in the cuvette.
   CAUTION: Respect the order of addition of the reagents and the sample given above during the incubation. The reagents can be incubated at room temperature (25 °C) since anurans are physiologically ectothermic.

2. Prepare the GST (**Table 1**).

3. Read the GST kinetics at 340 nm for 2 min in the spectrophotometer.

4. Calculate the enzyme activity with the following equation (**6**):
   Activity rate of GST (mmol/per minute/mg protein) = ([Δ absorbance/1,000]/9.6 × 10^4)/protein concentration (**6**)
   Where 9.6 mM^{-1} cm^{-1} = molar extinction coefficient.

5. Lipid peroxidation (TBARS)

NOTE: This methodology is based on Buege and Aust[37] with minor modifications for anurans.

1. Construct a calibration curve with MDA as the standard and 0.7% TBA (**Table 3**).

2. Prepare the standard MDA solution (10 mM) (**Table 1**). Carry out THP hydrolysis to MDA by placing the solution in a 50 °C water bath for 1 h.

3. Prepare a 0.7% (w/v) solution of TBA (**Table 1**) using a magnetic hot plate stirrer.

4. Then, centrifuge the sample homogenates for 30 min at 9,520 × *g* and 4 °C.
5. Extract 500 µL of the supernatant, add 100 µL of 6 M NaOH, and place in a thermal bath at 60 °C for 30 min.
6. After the thermal bath, add 250 µL of 35% $HClO_4$, and then place in an ice bath (4 °C) for 30 min.
7. After that time, centrifuge at 9,520 × *g* for 12 min, and extract 300 µL of the supernatant.
8. Add 300 µL of 0.7% TBA to the supernatant (sample), and place in a thermal bath (97.5 °C) for 30 min.
9. Read the absorbance of the complex formed by the sample and TBA at 532 nm, and determine the MDA concentration in the samples by substituting the absorbance values in the equation of the curve (**Figure 1**).

6. Acetylcholinesterase (AChE)

NOTE: This methodology is based on that proposed by Ellman et al.[38] with minor modifications for anurans.

1. At the moment of measurement, set up the DTNB and acetylthiocholine iodide (ATCh) reagents.
2. Prepare the DTNB reaction (**Table 1**).
3. Prepare the AChE reaction (**Table 1**).
4. In a glass cell with an optical path of 1 cm, prepare the AChE reaction by placing these reagents in the following order: 150 µL of PBS buffer (pH = 8) + 150 µL of DTNB + 50 µL of ATCh + 10 µL of the sample (pure in tadpoles and diluted 1/10 in adult anurans).
5. Read the AChE kinetics at 412 nm for 2 min at room temperature.
6. Obtain the value of AChE activity using the following equation (**7**):

   Activity rate of AChE ($mmol \cdot min^{-1} \cdot mg^{-1}$ protein) = ([Δ absorbance/1,000]/1.36)/protein concentration (**7**)

4. Genetic and cellular level: Micronucleus and comet assay

1. Micronucleus (MN) assay

   NOTE: The methodology is in agreement with that proposed by Fenech[39] with slight modifications for neotropical anurans.

   1. Anesthetize the specimens by immersion in ice water (4 °C), and obtain blood samples by sectioning behind the operculum in tadpoles or by cardiac puncture in adults.
   2. Smear two drops of peripheral blood from the specimens onto precleaned slides.
   3. Afterward, air-dry the slides, fix with 100% (v/v) cold methanol (4 °C) for 20 min, and then stain with 5% Giemsa solution for 12 min.
   4. Have one researcher code and blind-code the slides at 1,000x magnification.
   5. Determine the frequency of MNs by analyzing 1,000 mature erythrocytes from each specimen, and express as the total number of MNs per 1,000 cells.
   6. Use the following criteria for the correct identification of MNs:
      1. Look for MNs that have diameters smaller than 1/3 of that of the main nucleus.

2. Ensure that the coloration of the MN is not refractable but has a staining intensity that is the same or lighter than that of the main nucleus.
3. Look for an MN boundary that is distinguishable from the main nucleus boundary without any connection to the core or overlapping with the main nucleus.
4. Ensure that the number of MNs in the cells does not exceed more than four MNs associated with the nucleus.

2. Comet assay or single-cell gel electrophoresis (SCGE)
1. Anesthetize specimens by immersion in ice water, and obtain blood samples by sectioning behind the operculum in tadpoles or by cardiac puncture in adults.
2. Dilute the blood with 1 mL of PBS, centrifuge (381 × *g*, 9 min), and resuspend in a final volume of 50 mL of PBS.
3. Mix 30 µL of the blood sample in 70 µL of low-melting point agarose (LMPA) at a 0.5% concentration of agarose to form layer 2. Subsequently, place 50 µL of layer 2 on a slide previously coated with layer 1 containing 100 µL of 0.5% normal-melting point agarose.
4. Cover the slide with a coverslip, and place in the cold (4 °C) for 10 min to solidify layer 2.
5. After solidification, remove the coverslip, and form the third layer by laying down 100 µL of 0.5% LMPA.
6. When the third layer solidifies, remove the coverslip, and immerse each slide in a 100 mL Coplin jar containing lysis solution (**Table 1**) that has been prepared on the same day and kept at 4 °C. Place the Coplin jars in a cold bath for 1 h in the dark (4 °C) for lysis to occur.
NOTE: The experiment can be paused and restarted later. The lysis period has been reported to last from 1 h to 1 month.
7. At the end of the lysis, remove the slides, and immerse in the electrophoresis buffer solution (**Table 1**) in the electrophoresis tank. Perform this step in the dark for 15 min at 4 °C to allow the DNA to unwind. After nuclear DNA unwinding, perform electrophoresis on the same buffer at a temperature of 4 °C for 10 min at 25 V and 250 mA.
8. At the end of the electrophoresis, neutralize the samples contained in the slides 3 x 5 min by adding 2 mL of Tris-HCl solution (**Table 1**).
9. In the last step of the comet assay technique, dehydrate the samples by placing the samples in 100 mL Coplin jars containing alcohol (95%) at 4 °C.
10. Finally, stain the samples by placing 10 µL of 4′,6-diamino-2-phenylindole (DAPI) in the center of the slide, and place a coverslip that spreads the dye throughout the samples. Examine the slides under a fluorescence photomicroscope with a WB filter.
CAUTION: The whole process must be carried out in the dark to avoid DNA photodegradation.
11. Quantify the DNA damage by evaluating the length of DNA migration from the nucleoid. In this case, visually determine it on 100 randomly selected cells without overlap. Classify the DNA damage into four classes: 0-I (no damage), II (minimum damage), III (medium damage), and IV (maximum damage). Express the data as the mean number of damaged cells (sum of classes II-IV) and the mean comet

score for each treatment group. From these data, calculate the genetic damage index (GDI) for each test organism using the following equation (**8**):

GDI = (1[I] + 2[II] + 3[III] + 4[IV])/N[I-IV]) (**8**)

where I-IV represent the type of nucleoid damage, and NI-NIV represent the total number of nucleoids scored according to Pitarque et al.[40].

5. Correlated biomarkers

NOTE: In recent times, biomarkers can be integrated at all levels by using the biomarker response (IBR) index proposed by Beliaeff and Burgeot[49] and adapted for neotropical anurans. The IBR provides a numeric value that integrates all biomarker responses; higher IBR values indicate higher stress levels[49]. In terms of the IBR estimation for a given station or treatment of a given survey, the successive data-processing steps to determine the final score are as follows:

1. Compute the mean estimate (*X*) when individual results are available; otherwise, use the value from the pooled sample at each sampling station.
2. For each biomarker, compute the general mean (m) and standard deviation (s) of *X* for all the stations and/or surveys, depending on the comparisons to be made.
3. Standardize *X* to obtain *Y.* Calculate a value called *Y*: *Y* = (*X*- m)/s.
4. Compute the *Z* value as follows: *Z* = *Y* or *Z* = −*Y*, in the case of a biological effect corresponding, respectively, to inhibition or activation in response to a stressor.
5. Finally, compute the score (S), with S = Z + |Min|, where S ≥ 0 and |Min| is the minimum absolute value determined.
6. Plot a spider or star diagram after calculating the value of S for each biomarker (Si) and performing the summatory to obtained the IBR.

   NOTE: refer to the original paper[49] for any details of the calculations.

Representative Results

All the biomarker techniques presented here are simple, rapid, convenient, sensitive, low cost, and accurate methods. For each biomarker, it is important to note the following.

Individual level

Scaled mass index

Taking photographs on the millimeter scale is of great importance since this value will be used to calibrate the software, and this results in better objectivity with respect to the caliper measurement when taking the SVL variable. In addition, using a photograph for later analysis saves processing time during the handling of individuals, either during laboratory or field studies, where the time of sampling is always critical. In order to carry out the analysis of the scaled mass index, it is important to perform a non-linear regression analysis that demonstrates the power function relationship between the SVL and body mass for the species to determine the scaling exponent (b) (**Figure 2**). The scaled mass index is useful for comparing groups of adults or juveniles from the same species.

Scaled liver index

The SLI gives an idea of the relative mass of the liver in relation to the SVL. Although it can be used both with adult individuals and with larvae, the study of the SLI with adult anurans is more convenient due to the smaller size of the larvae. Alterations in this parameter indicate possible effects

at lower levels (histological, biochemical). The SLI is a useful biomarker for making comparisons between groups.

Scaled gonadal index

The scaled gonadal index provides a measure of the gonad size relative to the SVL and can indicate the degree of gonadal development. It is an index applicable only to adult anurans. Alterations in this parameter could indicate effects on gonadogenesis, among others, and the scaled gonadal index effectively complements studies at the histological and physiological levels. This is a useful index for comparative studies.

Morphological-histological level

Histological biomarkers are sensitive and easy to analyze. However, these markers require prior tissue processing, meaning it can take some time to obtain the results. Here, we use liver sections to exemplify the techniques (**Figure 3**). **Figure 3** demonstrates how hepatocytes can be measured. The interpretation of the results is based on interspecific or intraspecific comparisons.

Histometric data

Here, data from the area or length measurements of the selected structures are used. With these data, it is possible to compare effects between treatments or compare species. **Table 4** shows a pattern for *Boana raniceps*. To calculate nucleus:cytoplasm ratio (RNC), we used the following equation: RNC = (nucleus area/cytoplasm area × 100). The nucleus volume was calculated using the following equation: nucleus volume = $4/3\pi r^3$. The cytoplasm volume was calculated indirectly using the following equation: cytoplasm volume = (hepatocyte area × nucleus volume)/ nucleus area. Here, a correct delimitation of the desired structures is important for the precision of the technique. In tissues that do not have a good delimitation of cells and/or structures, the measurements may be wrong.

Histopathology

Histopathology allows for a comparative analysis regarding the degree of tissue alteration. When a degree of tissue change (DTC) is present, it is possible to make a table comparing groups. To apply this technique with precision, personnel training is necessary to eliminate subjectivity. The best results may be achieved through double-blind analysis.

Pigmentary system

Tissue pigmentation is associated with the presence of melanin in a given area (**Table 4**). With these data, it is possible to perform a comparative analysis between the same organ of different species or between experimental groups. The difficulty of this technique is the accurate selection of the color to be measured. Here, double-blind analysis is also advisable.

Biochemical level

The correct reading of the enzymatic activity shows a drop in the values during the 120 s of measurement time, which indicates consumption of the substrate (**Figure 4A**). The enzymatic reaction should preferably not be carried out at 4 °C, since the cold temperature of both the reagents and the sample delays the reaction, and it will take more time for the drop to show (**Figure 4B**). For the correct calculation of the enzyme activity, the molar extinction coefficient must be considered, and the protein value of that sample should be referred to in order to avoid underestimating or overestimating the enzymatic values. However, it is important to use the absorbance difference as a selection criterion in this case, we select the greatest difference in absorbance in the enzymes in which the kinetics are measured.

Catalase

The underlying principle of the method is based on the fact that catalase exerts a dual function: (1) the decomposition of H_2O_2 to give H_2O and O_2 (catalytic activity, equation [**9**]), and (2) the oxidation of H donors (e.g., methanol, ethanol, formic acid, phenols) with the consumption of mol of peroxide (peroxidic activity, equation [**10**])[35]:

$$2H_2O_2 \rightarrow H_2O + O_2 \quad (\mathbf{9})$$

where "→" represents reacting catalase.

$$ROOH + AH_2 \rightarrow H_2O + ROH + A \quad (\mathbf{10})$$

where "→" represents reacting catalase.

The formation of bubbles resulting from the release of oxygen in the reaction must be taken into account. This bubble formation may interfere with the reading signal when the light beam falls on the spectrum and may generate erroneous readings (**Figure 5**). The quartz cell (not glass or plastic) is essential for reading catalase because it absorbs the wavelengths that are below UV-visible range, thus avoiding interference with the material.

Colorimetric methods: Proteins, TBARS, GST (Table 5), and AChE

An important point between each measurement is to clean the cuvette as much as possible, first with 95% alcohol and then with distilled water.

Phase II enzymes conjugate glutathione with a xenobiotic or its metabolite to reduce lipid hydroperoxides. Glutathione *S*-transferases (GSTs) are cytosolic enzymes that attach glutathione (GSH, a tripeptide comprising cysteine, glutamate, and glycine) to the xenobiotic or its transformation products. They can also be recognized by specific transporter systems[5].

The foundation of the technique is based on the decomposition of polyunsaturated fatty acids such as malondialdehyde (MDA). This process serves as a convenient index for determining the extent of the peroxidation reaction. In turn, MDA, a product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to yield a red species that absorbs at 535 nm[37].

The method is based on the rate of production of thiocholine during the hydrolysis of acetylthiocholine. Particularly, this reaction is accomplished by the continuous reaction of the thiol with dithiobis-nitrobenzoic acid (DTNB) (I) to produce a yellow anion (II) (equation **11**). The kinetics of the reaction production are measured by a spectrophotometer at 412 nm. As the reaction is sufficiently rapid, care should be taken to avoid enzymatic hydrolysis by carefully setting the time taken to measure the enzyme I concentration.

$$H_2O + (CH_3)_3N^+CH_2CH_2SCOCH_3 \xrightarrow{\text{(enzyme)}} (CH_3)_3N^+CH_2CH_2S + CH_3^+COO^- + 2H^+$$

$$(CH_3)_3N^+CH_2CH_2S^- + RSSR\ (I) \rightarrow (CH_3)_3N^+CH_2CH_2SSR + RS^-\ (II)] \quad (\mathbf{11})$$

Genetic and cellular level: Micronucleus and comet assay

The time of exposure and the concentration should always be considered as a factor in the protocol, since a particular time or concentration may not be enough to induce damage, or on the contrary, the damage peak may have already passed, the health status of the organism may have worsened, or the organism may be employing its recovery mechanisms (**Figure 6**).

Correlated biomarkers

Figure 7 shows how the biomarkers are related to the IBR and indicates that catalase is an effective biomarker for stress situations in the highest concentrations of the stressor (or the stress situation). In addition, there is a response of the biomarkers at increasing ecotoxicological levels. This indicates which is the most effective biomarker to use for that evaluated stress situation.

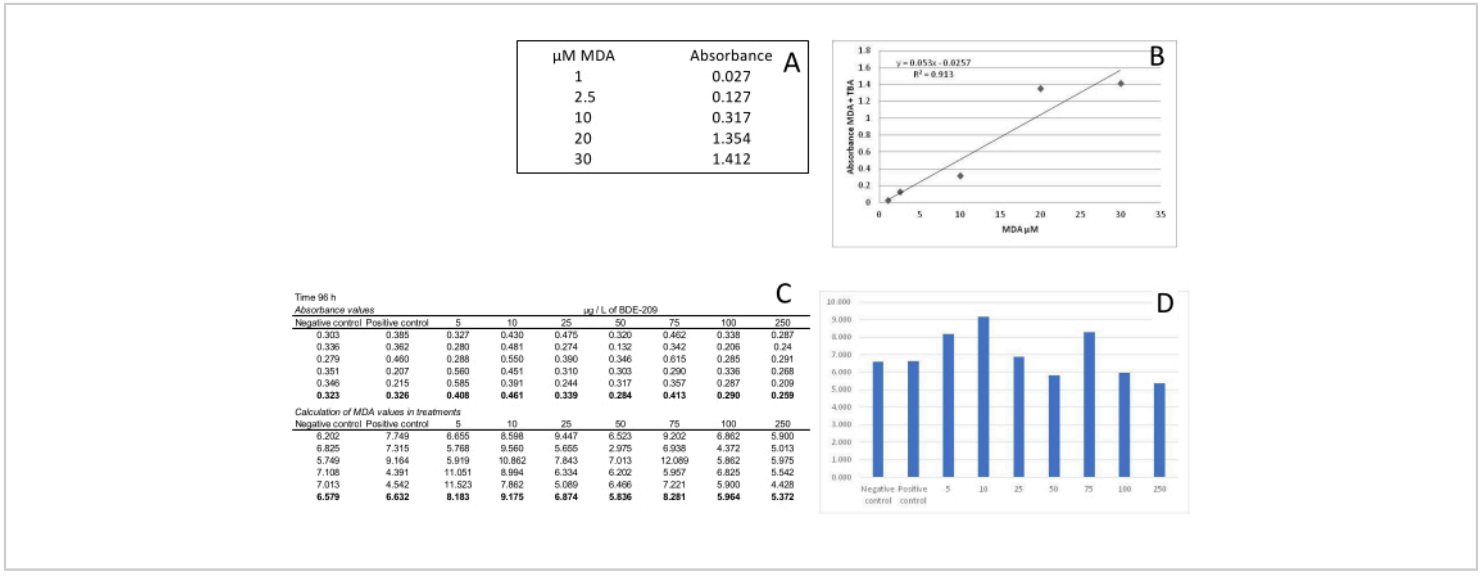


Figure 1: Data obtained from the analysis of TBARS in tadpoles of *Rhinella arenarum* exposed to BDE-209. (**A**) Constructed curve represented by the absorbance of the reaction of MDA and TBA, (**B**) the calculation of the equation of the curve, (**C**) obtaining the values in each tadpole sample, and (**D**) plotting the average values of MDA in each treatment. Please click here to view a larger version of this figure.

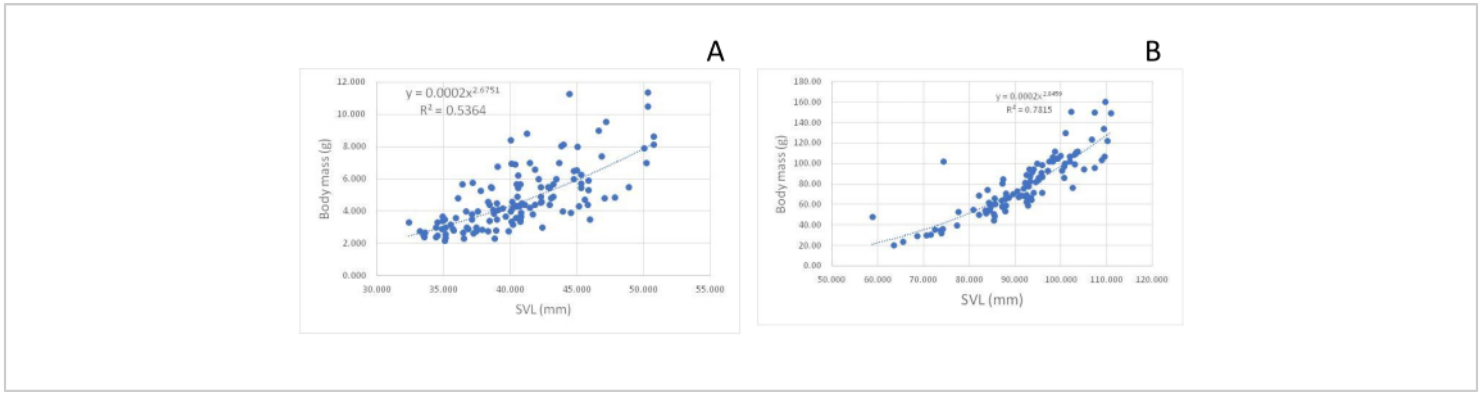


Figure 2: Scaled mass index. (**A**) *Boana pulchella* and (**B**) *Leptodactylus luctator* adults. Please click here to view a larger version of this figure.

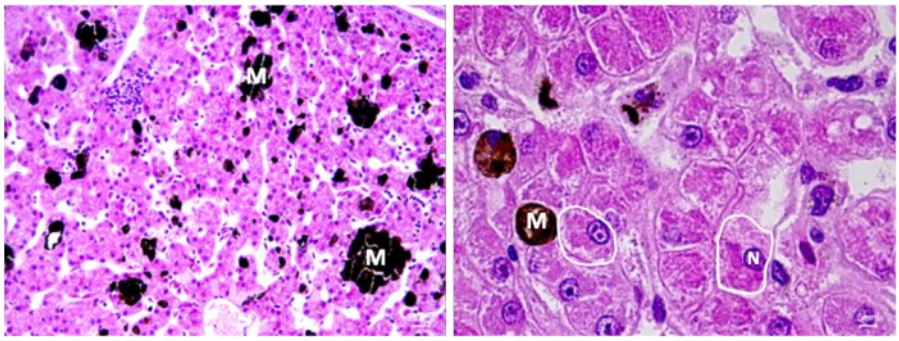


Figure 3: Histological sections of the liver of *Boana raniceps*. A greater amount of melanomacrophages and greater dimensions of the hepatocyte (white outlines) and nucleus (N) of the hepatocytes. Scale bars = 25 µm (left) and 5 µm (right). Abbreviations: M = melanomacrophages; N = nucleus. Please click here to view a larger version of this figure.

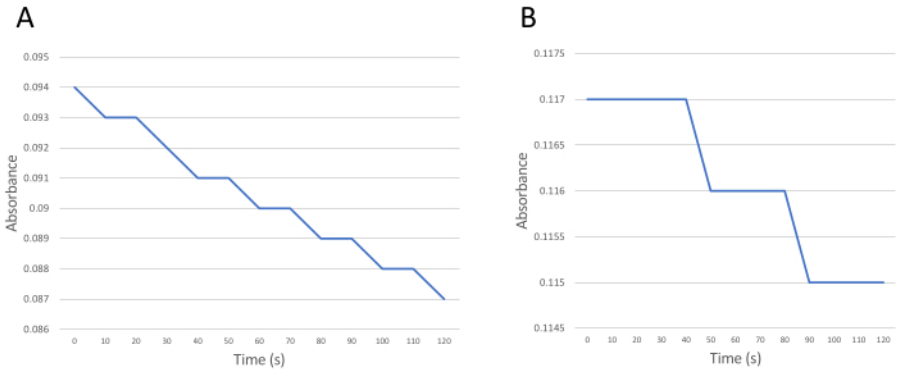


Figure 4: Acetylcholine absorbance in *Rhinella arenarum* tadpoles. The tadpoles were exposed to polybrominated BDE-209 (unpublished data). (**A**) The correct decrease in the reaction with acetylcholine and (**B**) the delay in the AChE reaction due to an inadequate temperature. Please click here to view a larger version of this figure.

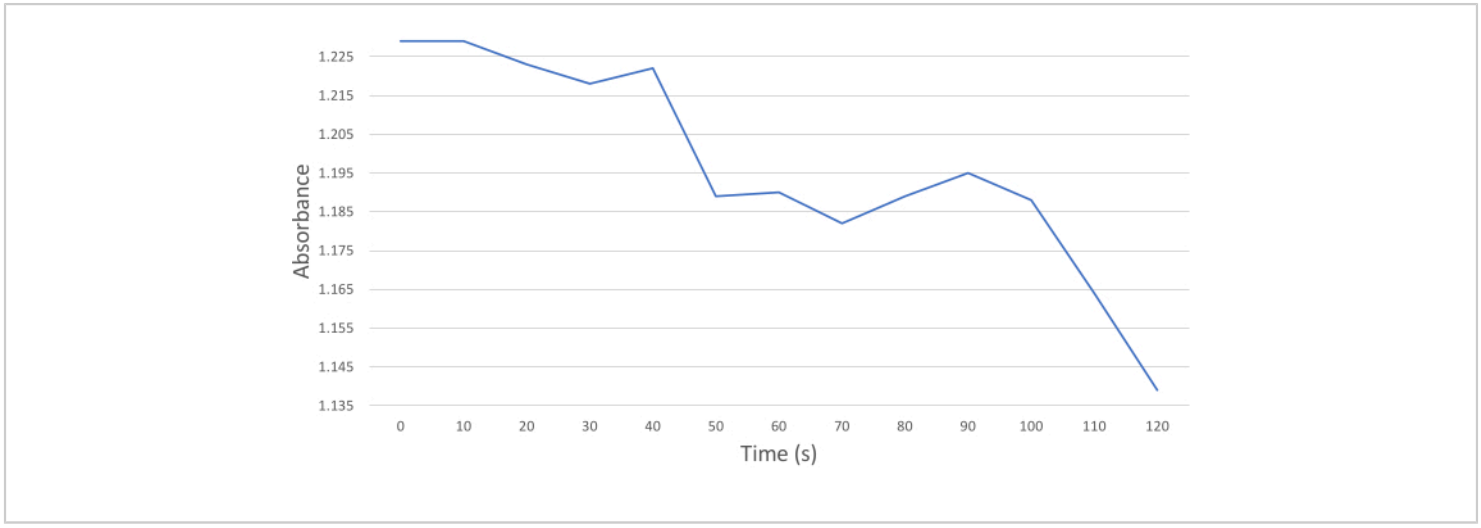


Figure 5: Irregular reading of catalase due to the addition of too much sample and an interruption of the signal due to bubbles. Please click here to view a larger version of this figure.

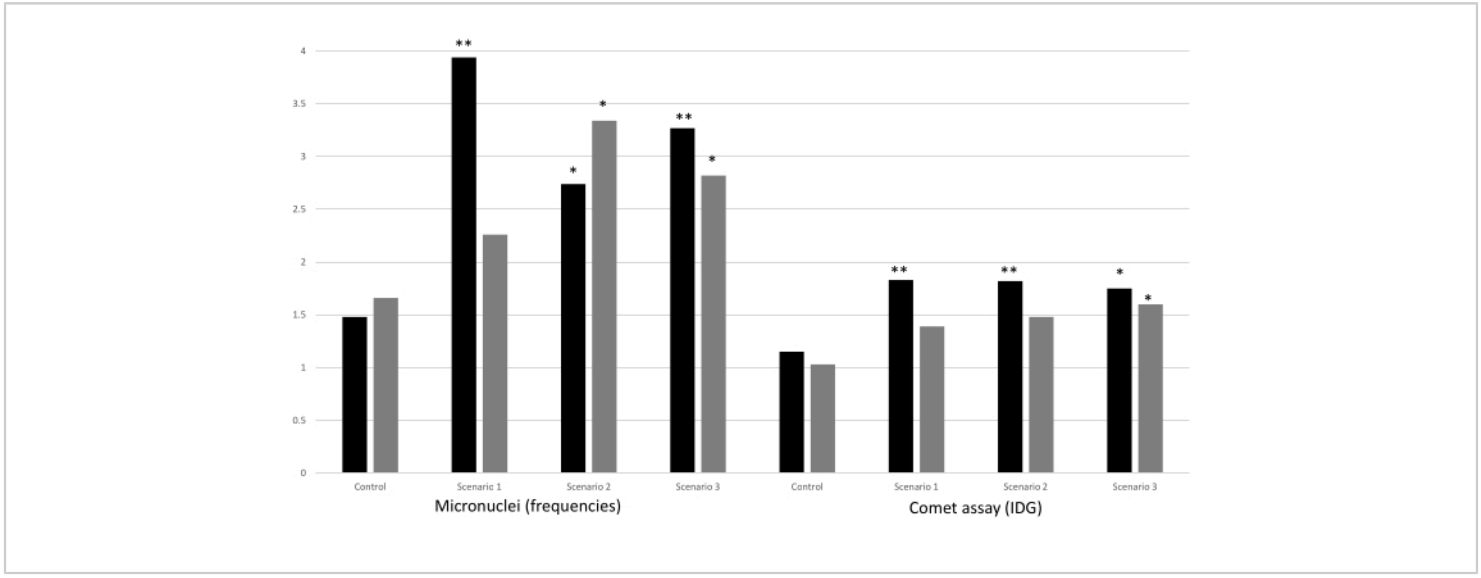
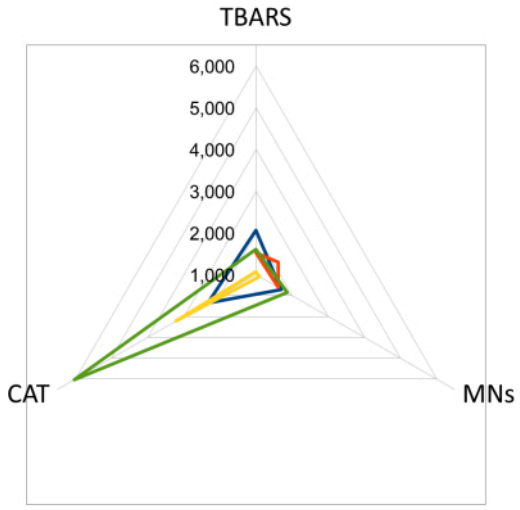


Figure 6: Different and complementary responses of cytogenetic biomarkers in an herbicide scenario exposure in *Boana pulchella*. The black bars indicate 48 h, and the gray bars indicate 96 h. Please click here to view a larger version of this figure.



	BDE-209 concentrations (µg/L)			
	0	25	50	75
TBARS	2.071	1.531	1.072	1.611
CAT	2.342	0.391	3.223	6.032
MNs	1.098	1.608	1.084	1.859
S-VALUE	**5.511**	**3.530**	**5.379**	**9.502**

Figure 7: IBR responses of several biomarkers in a gradient of BDE-209 concentrations in a neotropical anuran, *Leptodactylus luctator*. The blue color indicates the control group, while the orange (25 µg/L), yellow (50 µg/L), and green (75 µg/L) colors indicate the exposure scenarios to BDE-209. Please click here to view a larger version of this figure.

Table 1: Compositions of the reagents and solutions. Please click here to download this Table.

Table 2: Calibration curve with BSA for the quantification of proteins using the method of Bradford[34]. Please click here to download this Table.

Table 3: Calibration curve for the quantification of MDA using TBA as a control. Please click here to download this Table.

Table 4: Histometric data of the area and volume of the hepatocytes and the liver melanin pigmentation of *Boana raniceps*. Please click here to download this Table.

Table 5: Data of GST activity in two stages (tadpoles and adults) of Neotropical anurans obtained through the technique mentioned after 96 h of exposure. Please click here to download this Table.

Discussion

The biomarkers at the individual level are very simple to determine and very low-cost, as examining these biomarkers requires only a few pieces of equipment that are usually available in any research laboratory. In addition, these biomarkers provide general information on the health and fitness of the animals. The number of animals employed in each protocol is critical for obtaining reliable results. Due to the variability of data, a minimum of five animals (N = 5) is necessary for each treatment. In detail, a critical step for biomarkers at the individual level is measuring the SVL for the body condition index, since variations in the angle of the camera position could generate variations in the SVL measurement. It is essential that the images of all the individuals in the same study are taken in the same way, with the same angle and at the same distance from the object to be photographed. However, measuring the SVL by the

image analysis method is preferable to caliper measurement for time-saving reasons. Using this image analysis method would make it possible, therefore, to increase the number of studied animals, resulting in more robust and reliable results.

The hepatic index is useful for comparing groups of adults, juveniles, and even larvae from the same species, and as the liver is the main detoxifying organ, significant variations would indicate alterations in the histological and biochemical levels. The gonadal index is useful for comparing groups of adult anurans from the same species, and, as could be expected, female and male comparisons must be made separately due to the scale variations between ovaries and testes. Significant variations in this index could indicate alterations in gonadogenesis, which may affect the reproduction of the organisms.

Histological biomarkers are relatively low cost, although they require laboratories that have the required equipment. Biomarkers at morphological levels are important since they integrate responses from the biochemical and physiological levels. Here, we demonstrate examples of liver histological processing. However, we emphasize that the procedure may be applied to other organs such as the kidneys, heart, gonads, skin, spleen, and intestine.

The critical points for histological biomarkers are the identification of the cells and structures that are to be evaluated. It takes some training to apply the technique correctly. When analyzing the samples, methacarn is suggested because it is a fixative that allows for the better treatment of the tissues and also prevents the rapid deterioration of the histological equipment. In addition, fixatives based on methacarn have the advantages of preserving the tissues with good hydration and have a lower chance of contaminating the sample and the operator. In addition, for the biochemical biomarkers, a critical point during the enzymatic determination is maintaining the temperature at 4 °C, as well as storing enough samples in case the repetition of the enzyme determination is required. The technique modifications in the protocols depend on the species employed. It is necessary to take into consideration that for smaller species (or for tadpoles), the sacrifice of the organisms will be required to obtain the samples. Briefly, some modifications at the individual level (e.g., morphometric indexes) could be the use of mathematical transformations to improve the data interpretation.

Recently, Brodeur et al.[28] compared two different methods for expressing body condition: the scaled mass index (SMI) and the residuals methods. After the comparative analysis, the authors concluded that the determination of the body condition using SMI during monitoring programs with local anurans could be of great value, since it would provide accurate information on the health status of these anurans in conjunction with estimates of the population abundance. Modifications in histological biomarkers can be principally determined by employing either specific software for histological counting or visual determination.

All the biomarkers presented in this section have a relatively low cost, can quickly detect adverse effects caused by environmental stressors, and are highly reproducible in laboratories. In addition, they may be used with a small or large amount of sample (diluted), from 5 µL to more than 100 µL. Further, they have been widely applied not only for anurans but also for a large number of organisms ranging from plants to mammals. In anurans, these biomarkers are extremely important because they are the main defense system against environmental changes and, thus, reflect their health status. However, the techniques have slight

modifications that depend mainly on the species and the equipment. In this sense, for example, the volume of sample added in each technique will depend on the amount of anuran protein. In tadpoles, there is little protein, so more sample volume must be added for protein determination; then, in the calculations, this volume must be considered. Modifications at a biochemical level are usually related to the use of microplate incubators, incubation at a temperature of 37 °C, and the use of specific software to measure each enzyme.

Troubleshooting in the colorimetric quantifications (proteins and TBARS) will ensure that the absorbances of the samples fall within the range of the standard curve; if not, it is necessary to adjust the standard concentrations accordingly. At the cytogenetic level, a slight modification may involve the addition of a staining step in the MN assay. In the SCGE assay, modifications may involve changing the length of the lysis period, the concentrations of the agarose, the unwinding and electrophoresis times, the fluorescent dyes, and the employed analysis methodology.

The main limitation of employing biomarkers is that results usually do not bring conclusive information when analyzed separately. Therefore, it is necessary to analyze them jointly. This is because several biomarkers respond monotonously and not according to the dose-response concept in toxicology.

The comet and MN assays are rapid and sensitive methods that are commonly employed to detect genetic damage. The MN assay detects clastogenic or aneugenic damage through the examination of cells in interphase, whereas the SCGE assay detects direct DNA damage (strand breaks, DNA adducts, excision repair sites, cross-links) at the single-cell level. Both methods are fast to perform, with a relatively low cost and an easy and simple analysis. Another advantage is that both require a small sample to be performed (15 µL to over 100 µL). Given the characteristics of the methodology, the analysis of the frequency of MNs should be restricted to cells that have undergone the first mitotic division after treatment with the agent under study, since the induced lesions in the DNA are excluded from the nuclei of new cells as small pieces of extranuclear material after mitotic metaphase-anaphase[39]. This is an advantage of the SCGE assay, in which cells do not need to go through a cycle of cell duplication to manifest the damage caused to them. In addition, the number of cells analyzed per sample is lower in the SCGE assay than in the MN assay.

Among the difficulties SCGE presents are the costs of some reagents and equipment involved (mainly for the SCGE assay) and the variations in the times of lysis, unwinding, and/or electrophoresis, which have to be determined for the species employed. In addition, the analysis procedure (qualitative or quantitative) is another source of variability in the technique. Misleading results obtained with these techniques are usually due to not knowing the basal levels of cytogenetic damage in the studied species or, during the analysis, considering undamaged cells as damaged. However, erroneous results can be obtained by not following the established criteria, for example, to determine a micronucleus. The staining technique is one factor to take into consideration, since non-specific stains can be associated with false positive and false negative results due to underestimation or overestimation of the micronucleus count[41]. Finally, the critical points of the MN assay are fixing the material at 4 °C and ensuring the observation is performed by a trained analyst in order to avoid the underestimation or overestimation of the damage. Moreover, the critical points of the SCGE assay are the manipulation of numerous slides with delicate agarose gels and the challenge of performing the

assay in a dark environment to avoid inducing extra damage in the samples.

Another challenge is employing these studies in new species and under acute exposures, which sometimes are not long enough to produce a measurable response. Moreover, the number of individuals employed (sometimes N < 5) produces high variability, and statistical analysis reduces the statistical robustness. A limitation of the SCGE assay is that after the exposure, the samples must be processed within 36 h. Finally, and according to the recommendations of an ecotoxicologist, it is necessary to establish standard or control values (or normal distributions) for each biomarker and species employed.

These methods provide reliable results that are easy to interpret and contribute to explaining the mechanisms of toxicity of an environmental stressor. From a single organism, it is possible to perform a comprehensive analysis, which is necessary for biomonitoring and risk assessment[5,6,44]. In an organism, a stressor may produce apoptosis (detected by cytogenetic biomarkers), cause DNA damage that may be repaired (detected by cytogenetic biomarkers), trigger an altered response in the cell or organ (cytogenetic, biochemical, histological, and/or individual biomarkers), and also induce oxidative damage (biochemical and cytogenetic biomarkers), which, if not repaired, could lead to cancer or death, thus decreasing the quality of the ecosystem.

Most of the individual, biochemical, and cytogenetic biomarkers presented here are non-invasive techniques (no sacrifice of animals is required) that may be performed both in a laboratory as well as in biomonitoring studies to detect the effects induced by contaminants in bioindicator species before the damage to the ecosystem becomes irreversible. In addition, the biomarkers presented here have recently gained interest and are currently employed in investigations several Neotropical anurans exposed to different environmental pollutants.

Finally, it should be noted that the IBR has been used in recent years[51] in several stress situations for different species of neotropical anurans. This analysis allows for the evaluation of the impact of environmental pollutants in an integrated way and for performing a more exhaustive evaluation of the effects that pesticides induce on different native species. This would allow for taking measures to minimize the anthropogenic impact on non-target organisms. However, there is still a long way to go to establish an accurate and complete risk assessment and evaluation protocol with Neotropical anurans. This study aims to give an overview of several available strategies and offer a base guide to facilitate future research.

Disclosures

The authors declare no competing interests.

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