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RESEARCH ARTICLE

The agricultural impact of pesticides on *Physalaemus cuvieri* tadpoles (Amphibia, Anura) ascertained by comet assay

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ABSTRACT. Amphibians inhabiting agricultural areas are constantly exposed to large amounts of chemicals, which reach the aquatic environment during the rainy season through runoff, drainage, and leaching. We performed a comet assay on the erythrocytes of tadpoles found in the surroundings of agricultural fields (soybean and corn crops), where there is an intense release of several kinds of pesticides in different quantities. We aimed to detect differences in the genotoxic parameters between populations collected from soybeans and cornfields, and between them and tadpoles sampled from non-agricultural areas (control group). Tadpoles collected from ponds located at soybean fields had significantly more DNA damage, followed by tadpoles collected from cornfields. In contrast, animals sampled from non-agricultural areas had the lowest incidence of DNA damage. In addition, we found a negative correlation between the parameters of the comet assay and the area of the ponds surrounding soybean. This correlation indicates a possible dilution effect in the concentration of pesticides. Finally, *Physalaemus cuvieri* Fitzinger, 1826 seems to be a good bioindicator for detecting the genotoxic effects of field agricultural insecticides; therefore, we suggest that this species should be used in environmental biomonitoring studies, since it is common and abundant where it occurs.

KEY WORDS. Amphibians, bioindicators, exposure, genotoxicity, pesticides.

INTRODUCTION

In Brazil, agriculture is an important economic activity, and in view of this, the country has developed a large-scale commercial agricultural system. Brazil accounts for approximately 50% of the agricultural pesticides consumed in Latin America (ANVISA 2005). However, the success of this sector has been associated with the widespread destruction of Brazilian ecosystems, especially the Cerrado. In 2009, the world's soybean production was 216.8 million tons, which is almost twice as much as in 1990. In Brazil, soybean covers the largest cropped area (23 million ha), followed by corn (12 million ha) (SoyStats 2015, OECD/FAO 2015). After the implementation of financing programs for agriculture in Brazil, the use of pesticides such as insecticides and herbicides has increased (Waissmann 2007) at an unprecedented rate (3 to 9.6 l/ha). Of special concern is the fact that several pesticides that have been banned from use in most countries are still allowed in Brazil (Cançado et al. 2006,

Pignati et al. 2014).

Amphibians, especially anurans, are broadly used as test animals and bioindicators in evaluating the effects of pollutants in aquatic and agricultural ecosystems (Linder and Grillitsch 2000, Camargo and Alonso 2006, Marquis et al. 2009). Amphibians are among the most sensitive organisms to environmental changes, mainly due to their behavioral and physiological characteristics, such as a highly permeable skin, little mobility, complex life cycle, and simultaneous dependence on aquatic and terrestrial environments (Pollet and Bendell-Young 2000, Gonzalez-Mille et al. 2013). All of these characteristics facilitate the accumulation of environmental contaminants in their bodies (Carey and Bryant 1995, Linder and Grillitsch 2000, Henry 2000). In addition, pollution in the water and in the air, are the main causes of mortality in amphibian populations, often contributing to local and global extinction of species (Stuart et al. 2004).

Over the past decade, the comet assay or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage, with applications in genotoxicity testing, biomonitoring and molecular epidemiology, as well as fundamental research in ecogenotoxicology (Collins 2004). The comet assay is now considered one of the most promising genotoxicity tools to detect a broad spectrum of DNA lesions, with very high sensitivity in aquatic species (Jha 2008, Frenzilli et al. 2009, Frenzilli and Lyons 2013, Bolognesi and Cirillo 2014).

Physalaemus cuvieri Fitzinger, 1826, known commonly as barker frog, belongs to Leptodactylidae. The reproductive activity of the species begins in late September and extends through March (Bastos et al. 2003). Individuals lay eggs in foam nests directly on the water (Mijares et al. 2010). This species is widely spread in the east-central region of South America, from northeastern Brazil to eastern Paraguay and northern Argentina, including several protected areas (Mijares et al. 2010). The aim of the present study was to evaluate the potential genotoxicity of the environmental matrix (agricultural areas associated to pesticide use) using *P. cuvieri* tadpoles as bioindicators. We selected this species as a sentinel organism (bioindicator) in view of its wide geographic distribution, dependence on the aquatic environmental and ease of handling.

MATERIAL AND METHODS

This study was conducted during the rainy season in the Brazilian Cerrado biome from November 2013 to January 2014, in the municipalities of Bela Vista (16°58′24″S, 48°57′35″W), Bonfinópolis (16°37′2″S, 48°57′36″W), Caldazinha (16°42′17″S, 48°59′43″W), Leopoldo de Bulhões (16°42′17″S, 48°59′43″W), and Silvânia (16°38′35″S, 48°36′15″W), all of which are situated in the state of Goiás (Fig. 1). We sampled 177 tadpoles (Table 1) of *P. cuvieri* in stage 37 (sensu Gosner 1960). All specimens were placed in plastic bags containing water samples from the same pond from which they had been captured. They were kept alive until they were brought to the laboratory. The permission for collecting was granted by ICMBio, a Brazilian Environmental Institute linked to the Ministry of Environment (code 18163-1). In all experiments, animal care was performed following the guidelines of the Ethical Committee on Animal Use (CEUA-UFG), in accordance with the National Council for Animal Experiments Control (CONCEA). Voucher specimens were deposited in the zoological collection of the Universidade Federal de Goiás (ZUFG). For the test group, 83 tadpoles were collected from nine permanent ponds from agricultural lands (four in soybean and five in corn). To improve the accuracy of the sampling design, areas where there was only soybean or corn were selected. All samples were performed during pesticide application campaigns. For the control group (non-agricultural areas) we sampled from eleven permanent ponds (94 tadpoles) in areas where no agri-

Table 1. Sample areas of the *Physalaemus cuvieri* tadpoles (pond, city), number of individuals (n) and type of pesticides (related by the farmers), from November/2013 to January/2014.

Pond	City	Treatment	Pesticide	n	Geographical Coordinates
P01	Leopoldo de Bulhões	Control	No pesticide	7	16°57′80″S, 48°93′36″W
P02	Silvânia	Control	No pesticide	9	16°67′38″S, 48°83′07″W
P03	Bela Vista de Goiás	Control	No pesticide	11	16°75′30″S, 48°83′38″W
P04	Leopoldo de Bulhões	Control	No pesticide	5	16°56′67″S, 48°93′15″W
P05	Silvânia	Control	No pesticide	8	16°65′96″S, 48°81′28″W
P06	Caldazinha	Control	No pesticide	10	16°72′69″S, 48°84′16″W
P07	Leopoldo de Bulhões	Corn	Atrazine	10	16°59′93″S, 48°87′53″W
P08	Leopoldo de Bulhões	Control	No pesticide	10	16°57′34″S, 48°95′46″W
P09	Caldazinha	Control	No pesticide	7	16°73′17″S, 48°95′10″W
P10	Silvânia	Corn	Malathion; Furadan 350	11	16°68′80″S, 48°93′60″W
P11	Silvânia	Control	No pesticide	10	16°65′96″S, 48°81′28″W
P12	Bela Vista de Goiás	Control	No pesticide	9	16°79′69″S, 48°87′35″W
P13	Leopoldo de Bulhões	Corn	Atrazine; Malathion	10	16°53′68″S, 48°83′00″W
P14	Leopoldo de Bulhões	Control	No pesticide	8	16°62′98″S, 48°79′85″S
P15	Leopoldo de Bulhões	Corn	Furadan 350	10	16°58′08″S, 48°89′50″W
P16	Caldazinha	Soybean	Alto-100; Glyphosate	8	16°71′31″S, 48°83′42″W
P17	Leopoldo de Bulhões	Soybean	Glyphosate; Lannate	6	16°59′86″S, 48°87′88″W
P18	Leopoldo de Bulhões	Soybean	Dimethoate; Alto-100	8	16°59′25″S, 48°84′04″W
P19	Silvânia	Soybean	Dimethoate; Glyphosate	9	16°54′99″S, 48°80′59″W
P20	Bonfinópolis	Soybean	Glyphosate; Lannate	11	16°60′37″S, 48°96′00″W

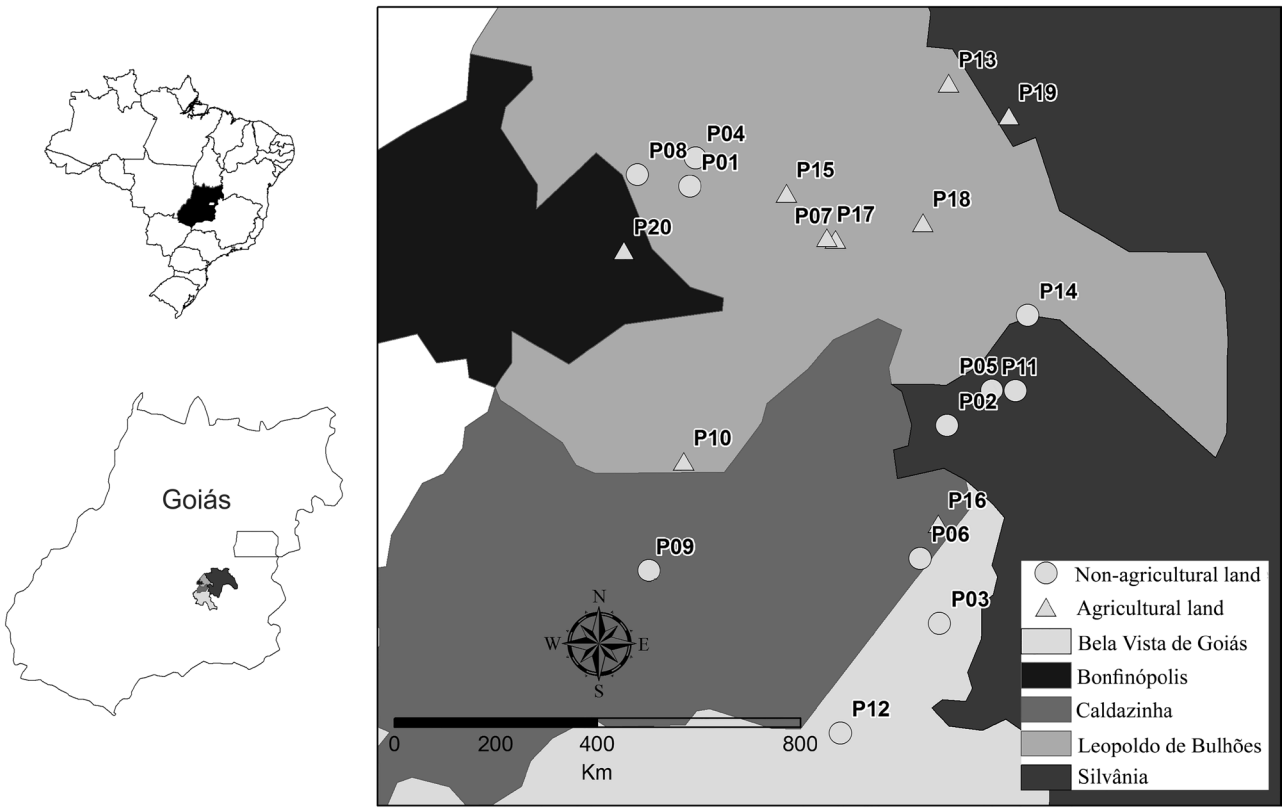


Figure 1. Spatial distribution of the ponds sampled at five municipalities in the state of Goiás.

cultural activities had taken place in the last six months. Table 1 represents the sampled areas for tadpoles, and the pesticides used according to information provided by the farmers, from November 2013 to January 2014. We quantified the percentage of non-natural areas around each pond with a buffer radius of 480m, using the ArcGIS (10).

Tadpoles were not fed, and four hours after the sampling they were anesthetized for approximately two minutes in a 5% benzocaine solution. Blood samples were obtained by a transversal cut in the tail. We performed the alkaline comet assay method described by Singh et al. (1988) with a few modifications. Fifteen µl erythrocytes mixed with 0.5% LMP agarose were placed on normal 1.5% agarose microscope slides. The essential steps of comet assay involved at least three hours of cell lysis by detergent at a high salt concentration (1% Triton X-100, 10% DMSO, Stock Lysis Solution pH = 10; at 4 °C). Electrophoresis under alkaline conditions (300 mM NaOH, 1 mM EDTA, pH > 13, 25 min unwinding, 25 min electrophoresis at 300 mA and 25V, at 4 °C). Nucleoids were stained with 20 µg/ml ethidium bromide (EB). We analyzed 50 nucleoids per slide, totaling 100 nucleoids per sample. The analysis was performed by a fluorescence microscopy system called Axioplan-Imaging® using the Isis software with an excitation filter of 510–560 nm and a barrier filter of 590 nm, with 20× objective.

For the evaluation of genomic damage, we used the TriTek Comet ScoreTM program, version 1.5. This software evaluated pixel intensity to provide corresponding values to estimate genomic damage, as arbitrary units (AU). We quantified genomic damages with tail length (TL), the percentage of DNA in the tail (% DNA), and the Olive tail moment (OTM) (Collins 2004).

Statistical analyses were based on the average of TL parameters,% DNA, and OTM analyzed for each individual. Previously, we performed the Kolmogorov-Smirnov (K-S) test in order to verify the normality of the three comet parameters. To test the discriminative power of the comet parameters we performed a discriminant function analysis using agricultural and non-agricultural areas as grouping variables and the comet parameters TL,% DNA, and OTM as explanatory variables. We also performed an analysis of variance (ANOVA) among soybean, corn, and non-agricultural areas also considering the three comet parameters. The principal components analysis (PCA) was performed to observe the dispersal patterns of locations and their relationship to the three comet variables using the pond area (in square meters) and the percentage of non-natural area as covariates. Therefore, we used the method based on the cor-

relation matrix. Finally, we also carried out a simple regression analysis in order to verify the relationship between genomic damage and the pond area occupied by the tadpoles. All statistical analyses were performed using the statistical package SPSS 23.0 and STATISTICA 10, with a 5% significance level.

RESULTS

All the points associated with the agricultural areas showed positive scores, in contrast with non-agricultural areas, which had negative scores. We found that the% DNA presented the highest contribution ($F = 180.3$, $p = 8.82e^{-29}$), followed by OTM ($F = 178.54$, $p = 1.59e^{-28}$) revealing DNA damage. In addition, statistically significant differences were found between the agricultural and non-agricultural areas for all parameters of the comet (Fig. 2).

Tadpoles located in soybean areas had the highest stretches of DNA damage estimated by the TL parameter (9.39 ± 1.08), followed by tadpoles in corn fields (7.95 ± 0.23), differing significantly from the damage suffered by the tadpoles in the non-agricultural areas (7.25 ± 0.60) (Fig. 2). Regarding% DNA, we also found significant differences between sites. Tadpoles sampled from ponds next to soybean fields showed the highest values of this parameter (7.54 ± 0.47), while tadpoles occupying ponds surrounding corn fields presented an average of 6.73 ± 0.55, differing from the values found in the non-exposed areas (5.04 ± 1.39). Tadpoles occupying areas surrounding soybean presented the greatest genomic damage for OTM (35.67 ± 6.14).

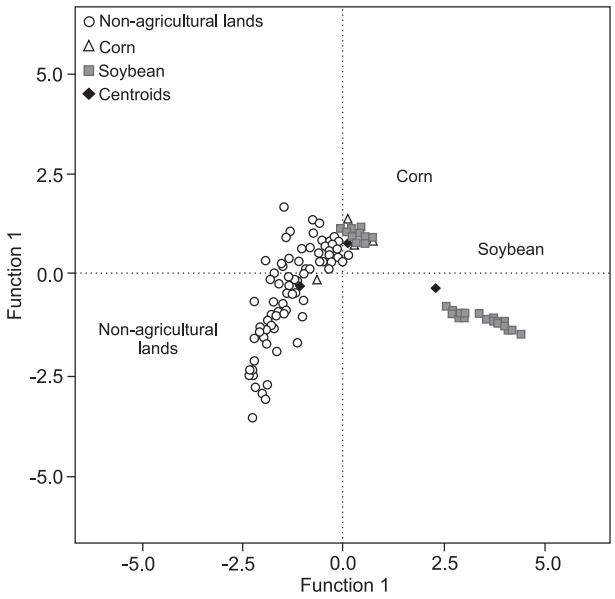


Figure 2. Differences between the average scores of Soybean, Corn and Non-agricultural lands, generated by the discriminant function scores.

Table 2. Results from a principal component analysis of comet assay parameters, related to pond area and remnant native vegetation (RNV). The factors loading for all three comet assay parameters, pond area and RNV and those higher than 0.05 are in bold. (TL) Tail length, (% DNA) % DNA in tail, (OTM) Olive tail moment, (EV) eigenvalue, (V) variance (%), (CV) Cumulative variance (%).

PCA FACTOR	Comet assay parameters, pond area and RNV					EV	V	CV
	TL	% DNA	OTM	Pond area	RNV			
Factor 1	0.551	0.542	0.584	-0.023	-0.111	2.872	57.436	57.436
Factor 2	-0.012	-0.056	-0.019	-0.607	0.792	1.074	21.481	78.917
Factor 3	0.000	0.274	0.143	0.744	0.592	0.822	16.434	95.350
Factor 4	0.752	-0.629	-0.043	0.166	0.094	0.227	4.538	99.888
Factor 5	0.362	0.481	-0.798	-0.025	0.001	0.006	0.112	100.000

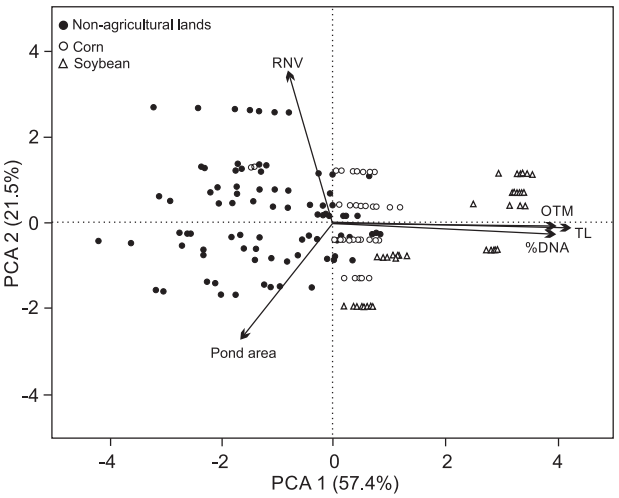
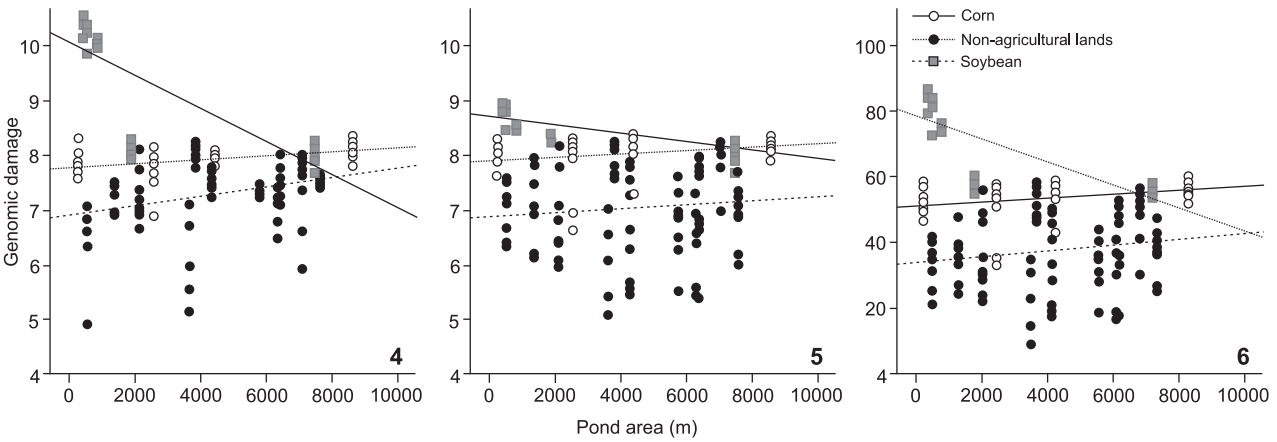


Figure 3. Principal component analysis (PCA) of the descriptor variables and the covariate pond area. Relationship between the PCA 1 and PCA 2, points were grouped according to the soybean (white triangle), corn (white circles) and non-agricultural lands (black circles). (TL) Tail Length, (% DNA) percentage of DNA in Tail, (OTM) Olive tail moment, (RNV) Remnant native vegetation.

In corn, the average value was 26.92 ± 2.64, whereas in the non-agricultural areas the average was 18.90 ± 5.97.

We observed a separation between the points associated with the soybean, corn, and non-agricultural areas (Table 2, Fig. 3). The PCA 1 accounts for 57.4% of the total variance, whereas the PCA 2 explains 21.5%. Thus, there was a negative correlation between the three comet assay variables and the pond area. The OTM parameter had the higher contribution in the separation between points (Table 2, Fig. 3). On the second axis, the% DNA had a greater negative association with the area of the pond. In addition, in relation to the first axis we observed a negative



Figures 4–6. Relationship between comet assay parameters and the pond area according to soybean (gray squares), corn (white circles) and non-agricultural lands (black circles); 4) TL with pond area; 5)% DNA; 6) OTM with pond area. (TL) Tail Length, (% DNA) percentage of DNA in Tail, (OTM) Olive tail moment.

association of genomic damage to the percentage of natural area within the buffer (Fig. 3). We observed that the points associated with soybean were clearly separated from the other points, as shown by the first canonical axis in Fig. 3.

Considering the soybean crop, we found a negative correlation between the TL and the ponds area ($r = -0.75$, $p = 6.0e^{-05}$), i.e., the smaller the area of the pond, the greater the genomic damage. Conversely we observed a positive correlation in areas where corn was cultivated ($r = 0.44$, $p = 4.0e^{-04}$) or the non-agricultural areas ($r = 0.45$; $p = 6.0e^{-05}$) (Fig. 4a). In the association of the pond area with% DNA, we found that only in the soybean crops there was a negative correlation ($r = -0.73$, $p = 4{,}0e^{-06}$), while the corn crop and non-agricultural areas had positive correlations ($r = 0.28$, $p = 0.036$ and $r = 0.19$, $p = 0.092$, respectively) (Fig. 4b). We still found a negative correlation between the OTM parameter and the area of the ponds located in soybean fields ($r = -0.76$, $p = 3.0e^{-06}$), in contrast with corn areas and non-agricultural areas that showed a positive correlation ($r = 0.35$, $p = 0.036$ and $r = 0.28$, $p = 0.015$, respectively) (Fig. 4c).

DISCUSSION

We found significant differences in DNA damage among specimens collected from soybean, corn and non-agricultural fields in all parameters of the comet assay. Tadpoles in ponds surrounded by soybeans presented more DNA damages, followed by tadpoles collected from ponds in corn fields. In contrast, the tadpoles sampled from non-agricultural areas had the lowest rates of DNA damage. In addition, in soybean fields alone, we found a negative correlation between the parameters of the comet assay and the area of the ponds. That is, the smaller the area of the pond the more extensive the genomic damage was. This correlation indicates that, since the concentration of pesticides is more diluted in larger pools, the number of genomic lesions they cause would be mitigated by dilution. In soybean fields, the pesticide more commonly used is glyphosate. A number of studies have demonstrated that this pesticide can cause the generation of free radicals and reactive oxygen species in bullfrogs (Costa el al. 2008) and other organisms, such as fish species (Nwani et al. 2013), increasing DNA damage.

According to Giesy et al. (2000) an aggravating factor in the use of glyphosate, mainly in soybean fields, is its long life in water bodies, reaching up to 70 days. Due to its high solubility in water and extensive use in the environment, the harmful effects of glyphosate on aquatic organisms are of great concern (Cavas and Könen 2007). However, several studies have indicated that the toxic effect of glyphosate is not only in its active principle, but mainly in polyoxyethylene amine surfactant (POEA), which is present in its most common commercial formulation, Roundup® (Mann and Bidwell 1999, Perkins et al. 2000, Howe et al. 2004, Cox and Surgan 2006, Brausch and Smith 2007).

The results found in our study justify the growing concern over the continued increase in the use of different classes of pesticides in agriculture (Cerejeira et al. 2003, Konradsen2007, Alavanja 2009). Unfortunately, the indiscriminate use of a wide range of pesticides, in order to improve agricultural production, ends up generating a negative impact on non-target organisms, especially anurans that depend on the water until the adult stage (See review of Sparling et al. 2010). It is worth mentioning that the main period of application of pesticides in the area usually occurs during the rainy season, coinciding with the breeding season of amphibians, from November to March (Mijares et al. 2010) which could cause developmental and reproductive failures, as described by many authors worldwide (Carey and Bryant 1995, Lowcock et al. 1997, Ralph and Petras 1997, Thomson et al. 2004, Meza-Joya et al. 2013).

Although we did not measure the concentration of pesticides in the ponds, we sampled the tadpoles during the pesticide application campaigns, in all sites. Our study did not attempt to sort out which pesticide and/or concentrations are responsible for genomic damage; the purpose was to ascertain the stress caused by the agricultural activities associated with the use of complex mixtures of pesticides, as those we observed, including herbicides, insecticides and fungicides. Farmers use this combination to decrease the total time of insecticide applications (Pedlowski et al. 2012). Among the pesticides applied during the sample collection were glyphosate – Roundup® (herbicide), alto-100® (fungicide); dimethoate, MD (insecticide and acaricide); atrazine (herbicide), carbofuran – Furadan® (insecticide), Lannate (insecticide) and malathion (pesticide). Indeed, there are several studies with tadpoles, under controlled laboratory conditions, that have drawn attention to the cytotoxic, mutagenic and genotoxic effects of these pesticides and their susceptibility may vary depending on the studied species (Howe et al. 2004, Relyea 2005, Relyea and Jones 2009, Bernal et al. 2009, Bosch et al. 2011, Meza-Joya et al. 2013,; Yadav et al. 2013). Our study, however, differs from those in that it measured genomic damages in animals exposed in their natural habitat, in real conditions of contamination.

It is worth mentioning that if genomic damages are not repaired, the DNA damage may be fixed, after, at least, one cell cycle. In this case, mutations arise and may impact the survival of the affected animals. It is known that the effects of pesticides are especially concerning in aquatic environments, which are particularly vulnerable as they have several exposure routes for the influx of chemicals. These effects are of particular concern as biodiversity loss reaches unprecedented rates. This includes recent declines in amphibian populations and loss of amphibian species (Makkimane and Krishnamurthy 2013).

Finally, our results indicate that the tadpoles of *P. cuvieri* are good bioindicators when the alkaline comet assay is used, and that the combination of the two can be used for biomonitoring studies of agricultural areas. However, the methodology for field studies needs to be standardized, so that the results of different surveys can be compared. In summary, genotoxicity studies involving amphibian tadpoles may be more informative and applied routinely to assess the impact of anthropogenic environments and/or exposure to pesticides.

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