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Biomarkers of genotoxicity and health status of *Rhinella fernandezae* populations from the lower Paraná River Basin, Argentina

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ABSTRACT

Anthropogenic activities may affect wild animal populations. Among them, amphibians are particularly sensitive to environmental change. The aim of this study was to evaluate the health condition of *Rhinella fernandezae* populations at three sites in the Buenos Aires Province, Argentina: a site inside a natural reserve (S1), a site surrounded mainly by industrial activities (S2) and a site surrounded principally by agriculture (S3). Sex and body condition (health status indicators), and micronuclei frequency (genotoxicity biomarker) of *R. fernandezae* adults were recorded to determine the health status of the local populations. Water samples were also analyzed for metals and pesticides to identify potential correlations with *R. fernandezae* malformations. Metals and pesticides were detected in the water samples of all three sites, with Al, Cu and Se exceeding the relevant limits for the protection of aquatic life. S1 had the highest overall environmental quality, and S2 had the greatest variety of pesticides. Individuals from S3 had significantly higher micronuclei frequency and females from S3 significantly lower body condition than females from S1, while females from S2 were significantly smaller than those from S1. Worst amphibian-based biological quality was assessed for S3, followed by S2 and S1. The low health status-fitness of females from the impacted sites (especially of the agriculture-surrounded S3 site), indicated by the low body condition and the high micronuclei frequency, may compromise the long-term health of the local amphibian populations. We conclude that action needs to be taken towards the long-term conservation of local amphibian populations from human-induced ecosystem degradation.

1. Introduction

Over the last decades, natural habitats have been altered by human activities, which pose a constant pressure on wild populations (Peters and Lovejoy, 1992). Among them, agriculture affects ecosystems through the use of pesticides and fertilizers that may benefit humans but they are also expanding agricultural barriers that constantly shrink natural habitats and biota (Tilman et al., 2001). Cattle fields also release high amounts of nutrients and pollutants that may reach the surrounding water bodies damaging native populations (Schmutzer et al., 2008). Likewise, the global urbanization replaces large natural landscapes by buildings and industries, causing severe ecosystem changes (Wang et al., 2013).

Argentina has followed the global trend of the green revolution and the cultivated areas have increased exponentially (Satorre, 2011). The lowlands of the Paraná River Basin comprise a highly productive area dominated by crop fields and industries that deteriorate the environmental quality. The systems of wetlands in this area constitute vital biodiversity reservoirs providing important socioeconomic benefits (Baigún et al., 2008). Yet, this area is highly affected by the surrounding human activities that degrade the ecosystems.

Amphibians are very sensitive to pollutants due to their permeable skin. In addition, they reproduce in freshwater bodies during spring-summer, a period that coincides with the release of pesticides and fertilizers from agriculture. They exploit agricultural areas due to less predation pressure and the availability of water bodies (Mann et al., 2009). Over the last decades, a high frequency of malformations and declining amphibian populations has been reported all over the world (Arntzen et al., 2017; Wake, 1991). The main reasons for these findings are habitat fragmentation/loss and pollution. Both factors are related to the expansion of agricultural land and the increased use of pesticides and fertilizers (Hamer et al., 2004), that reach the surrounding water

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bodies causing negative effects on these organisms. Additionally, the emissions of heavy metals from industry also alter considerably the amphibian health and survival (Croteau et al., 2008). Besides, amphibians from agricultural areas have shown a great variety of histological, biochemical and physiological abnormalities (Boily et al., 2009; Spear et al., 2009). However, there are few studies on abnormalities and genotoxicity in amphibian populations from South America (Josende et al., 2015). Studies on anuran populations from the crop-dominated mid-eastern region of Argentina indicate high rate of alterations (Agostini et al., 2013; Peltzer et al., 2011). Still, these studies are mainly focused on external abnormalities and to our knowledge, no information on genotoxicity in anurans from agricultural and suburban sites of Buenos Aires is available.

Rhinella fernandezae is a native species from South America. In Argentina, it can be found in agricultural, suburban and forested areas of Entre Ríos, Santa Fe and Córdoba showing high incidence of external malformations (Peltzer et al., 2011). It is a terrestrial toad that lives in burrows and only goes to water bodies to reproduce during spring and summer (Cei, 1980).

The use of non-lethal and non-invasive biological techniques such as the analysis of blood biomarkers has gained attention due to the integrated information they provide about the adverse effects of exposure to genotoxic pollutants. These effects can be monitored with the micronucleus frequency test. High micronuclei frequency occurs from irreparable DNA damage caused by the exposure to toxic environmental agents. This test based on the nucleated amphibian red blood cells is one of the most frequently employed (Lajmanovich et al., 2005).

The purpose of this study was to analyze the health condition of *Rhinella fernandezae* populations in the lower Paraná River Basin. Three sites with various human activities were sampled for metals and pesticides, and *R. fernandezae* individuals from these sites were analyzed using biomarkers of health status and genotoxicity to examine relationships between metals/pesticides, human activities and *R. fernandezae* health. Within a sustainable framework for managing freshwater biological resources, this study offers valuable information on the current status of *R. fernandezae* populations in the lower Paraná River Basin that could help towards the application of relevant ecological protective measures.

2. Materials and methods

2.1. Study area

The study area belongs to the pampean region in the north west of Buenos Aires Province, Argentina (Fig. 1). This region is one of the most important areas of human production in Argentina and is characterized by the presence of wetlands, streams, lagoons and rivers. The Paraná River is the second most important river in South America. From its origin in southern Brazil to its mouth in the Río de la Plata (Buenos Aires, Argentina) goes through approximately 2,570 km. In the lower section, the main course has numerous islands forming a delta. Three sampling sites were selected within the floodplain of the lower Paraná River: (1) S1; in the El Morejón Natural Reserve, a 300-ha wide protected area in Campana. This reserve was founded in 2012 and is one of the few in the lower Paraná Basin (SAyDS, 2015). (2) S2; an area dominated by industrial and urban activities in Campana near the De la Cruz stream. (3) S3; an agricultural area in Baradero, near the Arrecifes River, dominated by crops and cattle grazing fields. S3 is located further north than the other sites, in a distance of approx. 65 km from S2. The distance between S1 and S2 is approx. 6 km.

2.2. Determination of metals and pesticides in surface water samples

Three sampling campaigns were carried out, two in spring (September 2017 and October 2018) and one in summer (February 2018). On each sampling campaign, surface water samples of 1 L were collected at each site in clean plastic bottles.

For metals determination, samples were filtered through Millipore APFF, 0.45 μm pore diameter, acidified with HNO_3 and measured by inductively coupled plasma mass spectrometry (Agilent 7500 cx ICP-MS). Aluminum (Al), Arsenic (As), Copper (Cu), Lead (Pb) and Selenium (Se) were measured. For the calibration curve a multi-element standard solution XXI (0.5–300 μg/L, Merck ®) in 2% v/v HNO_3 medium was used. Each analyte was quantified by normalizing to one of the internal standards according to the proximity of the mass.

Pesticides were measured by gas chromatography-mass spectrometry (PerkinElmer Clarus 600) with a single quadrupole mass detector. Samples (2 mL) were injected into the programmable split/splitless injector with a DB-5MS capillary column (Agilent Technologies). Also, a liquid chromatography-mass spectrometry (Waters Acquity UPLC-SQD) with a single quadrupole mass detector with a column XBridge BEH C18 was employed. The detection and quantification limits were 1 ng/L and 4 ng/L respectively.

2.3. Sampling of Rhinella fernandezae adults

At each site, following the chemical sampling, *R. fernandezae* adults were collected at night by visual encounter surveys and captured by hand. The same effort of sampling was performed in the three sampling sites (3 h of visual surveys per site). Sex (by external secondary sexual characteristics), total length (snout-vent length, measured with a manual caliper Ruhlmann 0–150 mm) and weight (measured with a balance Quiltech Q30001) were recorded. Also, external malformations were evaluated. All animal management was conducted according to the guidelines for use of live amphibians and reptiles in field and laboratory research (Beaupre et al., 2004) and were controlled and approved by the Institutional Committee for the Care and Use of Animals in Experimentation (CICUAE) of the National University of San Martín (UNSAM).

2.4. Body condition

The body condition of the individuals was analyzed by examining the residuals from a regression of body mass on snout-vent length (Schulte-Hostedde et al., 2005). Even though S1 cannot be considered a reference site, it presented the best environmental quality in comparison with the other two sites. The S1 site had increased environmental quality compared to S2 and S3, and thus, the *R. fernandezae* individuals from S1 were used as a control sample for relevant comparisons. Males and females were separately analyzed due to sexual dimorphism. All data were pooled and analyzed together; no seasonal grouping was applied.

2.5. Genotoxicity assessment by micronucleus test in Rhinella fernandezae adults

Peripheral blood samples were withdrawn from each *R. fernandezae* adult by cardiac puncture with heparinized needles. This method was considered adequate for amphibians (Tyler, 1999). The volume extracted was the minimum required for experimental purposes. Then, smears on glass slides were air dried, fixed with May-Grünwald (Biopack ®) and stained with filtered 10% Giemsa solution (Biopack ®). For the micronuclei (MN) frequency, 1000 mature erythrocytes were analyzed following the established criteria by Fenech (2000)with a Microscope Leica DFC7000 T, at 1000 × magnification and oil immersion lens. Since toads were released in their natural habitats, they were toe-clipped (Bionda et al., 2011) in order to identify them and avoid recapture.

2.6. Data analysis

The software GraphPad Prism 6 was employed to perform the

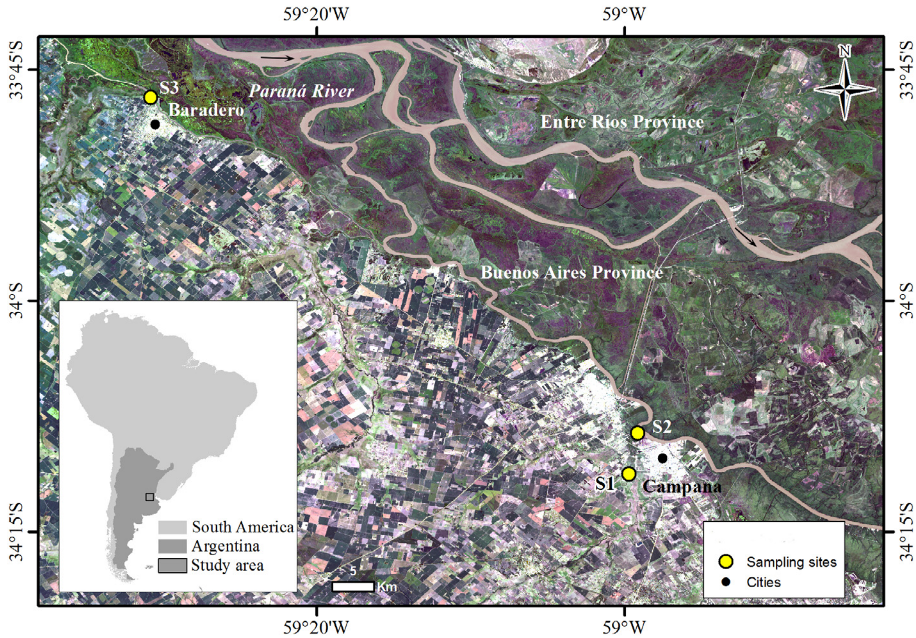

Fig. 1. The study area; the lower Paraná River Basin in which three sampling sites were selected. Image made in QGIS using freely available Landsat 8 satellite images.

statistical analysis. The residuals of body condition and the MN frequency were compared among animals from different sites using either a one-way ANOVA when the assumptions of normality and homoscedasticity were fulfilled, or a Kruskal-Wallis test followed by the Dunn's post-hoc test when the above assumptions were not met. A principal component analysis (PCA) was performed in order to correlate the parameters measured in water samples with the means of micronucleus frequency for each site using R version 3.6.2.

3. Results

3.1. Pesticide and metal concentrations in water samples

The average concentrations of metals and pesticides differed among sampling sites (Tables 1 and 2) but only the average As levels were significantly lower in S2, compared to the other sites. The levels of Al, Cu and Se exceeded the limits for the protection of aquatic life according to the Argentine Law 24051, decree 831/93 (5, 2, 1 µg/L respectively) in all water samples. The concentration of Pb also exceeded the limit (1 µg/L) in water samples from S2 and S3, while As was near the limit in water samples from S3 (50 µg/L).

Table 1
Average pesticide concentrations of surface water samples collected from three sites in the lower Paraná River Basin in three sampling periods. nd: not detectable.

Concentration µg/L	Sampling site S1	S2	S3
2,4-D	nd	nd	1–4
Acetochlor	nd	7.1	10.3
AMPA	5.20	1–4	nd
Atrazine	0.53	0.71	0.0054
Azoxystrobin	0.017	0.016	nd
Glyphosate	3.4	1–4	nd
Metalaxyl	1–4	7.30	nd
Tebuconazole	nd	68.40	nd

Table 2
Minimum and maximum metal concentrations in surface water samples collected from three sites in the lower Paraná River Basin in all sampling campaigns. nd: not detectable. * Differences are statistically significant at the $p < 0.05$ level.

µg/L	S1 Min	Max	S2 Min	Max	S3 Min	Max
Al	24.6	289	53.6	333	54.6	162
As	15.5	35.8	2.7*	3.86*	21.6	81.2
Cu	2.8	8.2	3.7	6.27	4.1	5.6
Pb	nd	0.8	nd	2.9	nd	1
Se	0.6	29.3	0.7	32.3	1.48	40.1

3.2. Sampling of *Rhinella fernandezae* adults and body condition

The mean weights and lengths of the captured males and females are shown in Table 3. Females captured at S2 and S3 were significantly smaller than the ones from S1. One animal with no hind limb was found in S2 (4.76%) while one animal with partial front leg was captured in S3 (4.54%) (Fig. 2).

The weight, length and body condition did not differ between males from different sites (Table 3, Fig. 3). However, the females captured at S2 and S3 were significantly smaller than the females from S1 (Table 3). Also, the body condition of females from S3 was significantly lower than the body condition of females captured in S1 (Fig. 3).

3.3. Genotoxicity: Micronucleus test results

In spring 2017, the MN frequencies for S1, S2 and S3 were: 2.16 (± 0.75), 2.57 (± 1.51) and 4 (± 0.70), respectively. In summer 2018, the MN frequencies were 2.18 (± 1.17), 2.71 (± 1.11) and 4.25 (± 0.70), for S1, S2 and S3. Finally, in spring 2018, the MN frequencies were: 1.5 (± 0.55), 3.6 (± 0.89) and 4.25 (± 1.26) for S1, S2 and S3 respectively. The MN frequencies of individuals from S2 collected in spring 2018 and from S3 in all sampling campaigns were significantly higher than values of the MN frequency in individuals from S1 (Fig. 4). In Fig. 5, a control erythrocyte of *Rhinella fernandezae* (Fig. 5 A) and an erythrocyte with a micronucleus (Fig. 5 B) are shown.

Table 3
Mean (± SD) weight and length of *R. fernandezae* females and males captured at each of the three sampling sites in the lower Paraná River Basin. * Differences are statistically significant at the p < 0.05 level.

Site	Females n	Weight (g)	Length (mm)	Males n	Weight (g)	Length (mm)
S1	16	15,92 ± 1,75	53,6 ± 3,68	7	12,72 ± 0,58	51,17 ± 5,25
S2	7	9,08 ± 10,53*	33,28 ± 29,64*	14	16,02 ± 0,58	53,8 ± 5,13
S3	15	13,76 ± 2,20*	51,30 ± 5,39*	7	11,90 ± 1,53	48,75 ± 0,90

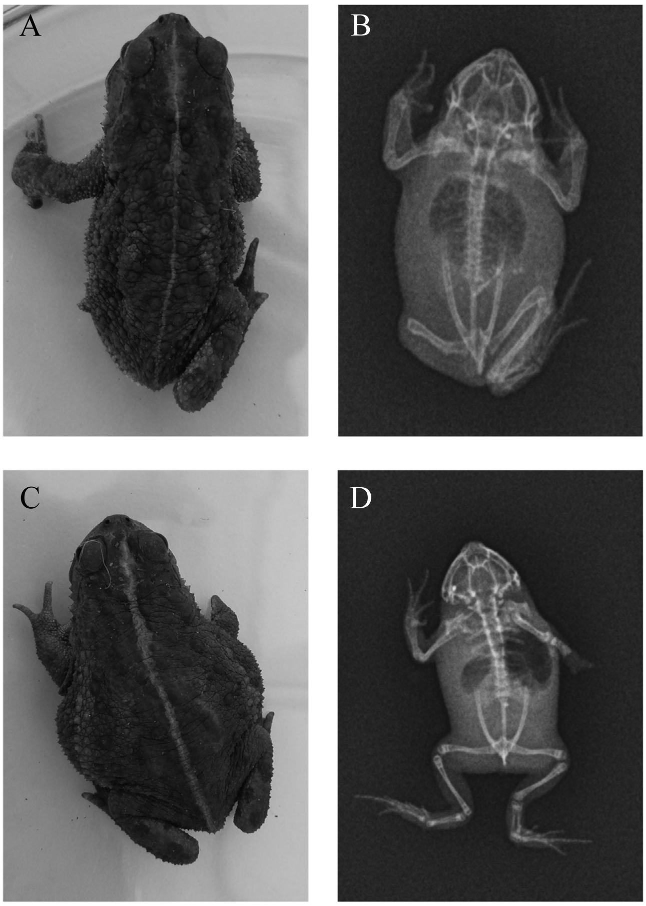

Fig. 2. Photographs and x-ray images of malformed *Rhinella fernandezae* adults captured at sites S2 (A-B) and S3 (C-D) of the study area in the lower Paraná River Basin.

3.4. Principal component analysis (PCA)

The PCA allowed to group the data and find some relationships among measured parameters. The 2,4-D and AMPA data were removed from the analysis due to the high correlation with other variables. The PCA allowed distinguishing the sites according to their characteristics. A 50.34% of the total variability was explained by the PC1 and PC2 axes (Fig. 6). The PC1 was positively correlated with the concentrations of azoxystrobin, acetochlor and metalaxyl and negatively with Al, Cu, Pb and glyphosate. On the other hand, PC2 was mainly positively related with the concentrations of As, atrazine, Se and the MN frequency, and negatively with Al, Cu, Pb and glyphosate. In all the campaigns, S1 had low correlation with metals and pesticides concentrations. Data from S2 presented a higher variability among the sampling campaigns while S3 correlated with the concentrations of As, atrazine, Se and the MN frequency.

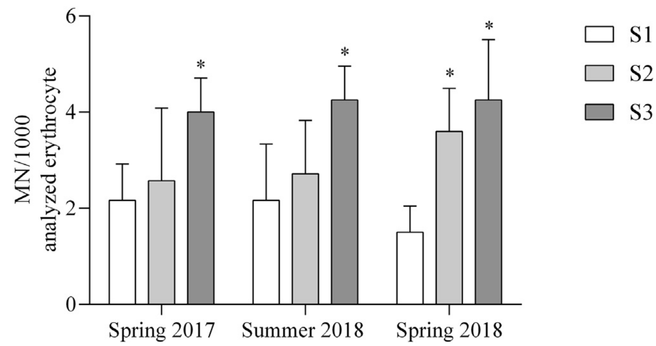

Fig. 4. Mean (± SD) of the micronucleus frequency of *Rhinella fernandezae* adults captured atS1 (inside the natural reserve) vs that of S2 (dominated by urban/industrial activities) and S3 (surrounded by agricultural land use). * Differences are statistically significant at the p < 0.05 level.

4. Discussion

Agriculture, urban and industrial pollution are substantial sources of contamination that contribute to environmental degradation (Davis and Birch, 2010). The lower Paraná River Basin is a complex environment where such activities are combined (Ronco et al., 2016). In this context, field studies are highly valuable because the effects observed in the organisms are the integrated results of multiple stressors from various anthropogenic activities (Preston and Shackelford, 2002).

The concentration of pesticides and metals varied among sites. In all

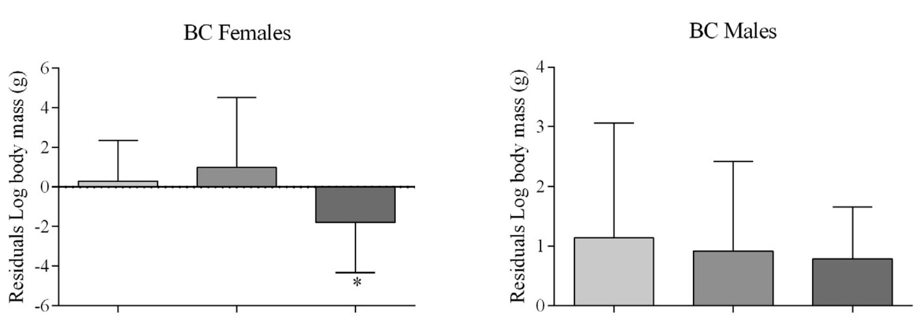

Fig. 3. Mean (± SD) of the residuals between measured and theoretical body mass of *Rhinella fernandezae* males and females captured at S1 (inside the natural reserve), S2 (dominated by urban/industrial activities) and S3 (surrounded by agricultural land use). * Differences are statistically significant at the p < 0.05 level.

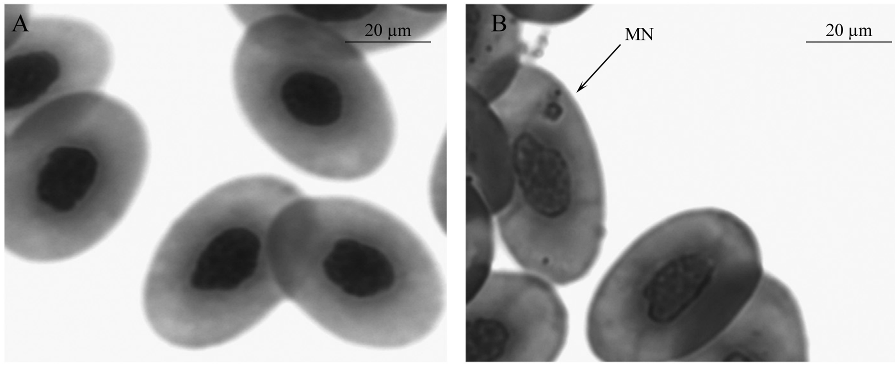

Fig. 5. Images of erythrocytes from *Rhinella fernandezae* blood samples. A: control erythrocytes of a toad from the natural reserve (S1); B: erythrocyte of a toad from S3 exhibiting a micronucleus.

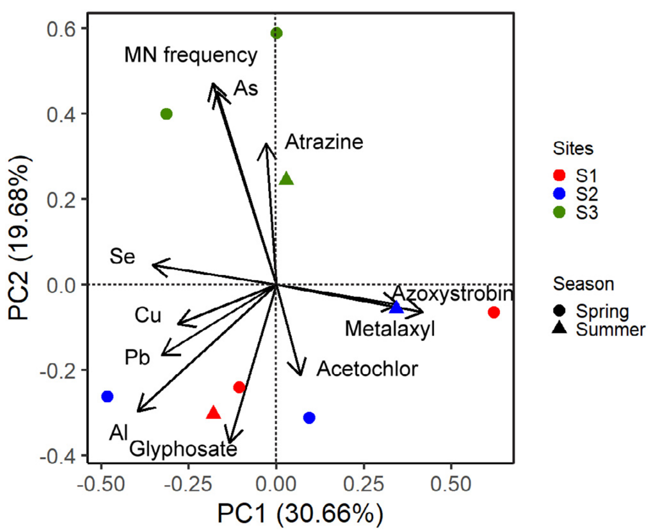

Fig. 6. Principal components analysis biplot illustrating the distribution of sampling sites in relation to metals, pesticides concentrations and the MN frequency.

water samples, the levels of Al, Cu and Se were higher than the limits for the protection of aquatic life (Argentina Law 24051, decree 831/93). The levels of As were highest in S3, probably due to the natural geochemistry of the basin of the Arrecifes River (Castro et al., 2017). In particular, the concentration of Se in all water samples exceeded the natural levels reported for freshwater bodies which range between 0.1 and 0.4 µg/L (Ohlendorf, 2003). There are few studies in this area that report similar concentrations of As (5.7–56 µg/L), Cu (3–4 µg/L) and glyphosate (0.7–12 µg/L) (Peluso et al., 2020). Also, previous studies show higher levels of Cu (20–40 µg/L), Pb (30–80 µg/L and Cd (4–6 µg/L) (Ronco et al., 2011; Ronco et al., 2016). Moreover, Marino and Ronco (2005) reported concentrations of cypermethrin (0.46–194 µg/L) and chlorpyrifos (0.4–10.8 µg/L) in water bodies near the study area. Considering that S2 is an industry-dominated site, the high pesticide variety was unexpected. However, the S2 site is near the De la Cruz Stream that flows through agricultural fields and could be affected by soil runoff. Metal pollution is very common in suburban, industrial and agricultural areas, so increased concentrations of metals could be expected in water bodies as reported by Davis and Birch (2010). Metals and pesticides were also detected in S1, which despite being located in an urban natural reserve, it is influenced by surrounding crops and urban settlements. Although previous studies suggest that it is rather well-preserved with very low anthropogenic impact (SAyDS, 2015), the concentrations of metals and pesticides measured in this study showed that it has better environmental quality compared to S2 and S3 but not high enough to be considered not impacted. The high levels of Al, Cu, Pb, Se, atrazine and azoxystrobin are a concern for environment and species that inhabit these ecosystems. These parameters had greater temporal variation in S1 and S2, while the ones from S3 remained mostly constant among sampling campaigns. S2 had the highest variety of pesticides, while metal concentrations differed among sites and sampling campaigns. These sites are affected by floods and low flows of the Paraná River, which may explain the differences observed among parameters (Kandus et al., 1999). On the other hand, the higher variability of data from S1 and S2 among samplings can be attributed to seasonal fumigations and effluent discharges.

Body condition is an indicator of the energy reserve of the individuals that influences growth, survival and reproduction (Bachman and Widemo, 1999). Animals with increased energy reserves have better *fitness* than those with poor body condition. Thus, the lower body condition of females from S3 indicates a reduced *fitness* suggesting that the reproductive parameters may be altered in this population (Brodeur et al., 2011), and if this alteration persists, a decrease in *R. fernandezae* populations of the area can be expected. Brodeur et al. (2011) also found decreased body condition in amphibians from cultivated sites. The reduced body condition of animals from polluted areas may be a consequence of decreased food intake by the reduction of habitats and the decline in insects' abundance, as a consequence of pesticide applications. In addition, the exposure to toxic compounds and the consequent activation of detoxification systems may increase energetic costs and reduce body condition (Brodeur et al., 2011).

Although the limb malformation in the toad from S2 can be due to an injury rather than to a congenital malformation, the observed in the toad from S3 appears to be ectromelia, a congenital abnormality where long bones are missing or underdeveloped. Despite that this type of malformation is not uncommon to find in frogs from agricultural sites (Huang et al., 2010), the frequency of malformations observed in toads from S3 (4.54%) was within the acceptable range of spontaneous malformations (0–5%) (Johnson and Lunde, 2005). These results are just illustrative and preliminary and more field studies are required to have a clearer idea of the malformation frequency in wild populations of this species. However, some of the agrochemicals and metals found in water samples are well known teratogens that can affect the normal development of animals (Li et al., 2016; Morgan, 1996; Pérez-Coll and Herkovits, 1990). Acetochlor concentrations in water samples from S2 and S3 were higher than the NOEC of this herbicide (2.7 µg/L) for *Rana catesbeiana* (Helbing et al., 2006). Besides, the levels of Cu in all water samples were near the NOEC (7.5 µg/L) for *Rhinella arenarum* embryos (Aronzon et al., 2011b). The levels of the remaining substances were below the concentrations that cause effects in amphibians (Aronzon et al., 2011a; Calevro et al., 1998; Hayes et al., 2006; Mann and Bidwell, 1999; Svartz et al., 2012). In spite of these facts, the observed effects may be due to the complex mixtures of contaminants and stressors present in all compartments of the environment. Also, other stressors such as viruses and parasites beside the soil and water contaminants, can increase the intrinsic malformations (Johnson and Lunde, 2005). Generally, malformed animals do not reach the adulthood, and the incidence of malformations is higher in juveniles. It is noteworthy that the rate of malformed frogs in nature has increased over the last decades (Blaustein and Johnson, 2003) indicating important degradation of environmental health (Roy, 2002). So, it is highlighted the need for ecotoxicological field studies to reveal adverse environmental conditions that may affect the normal development of species.

In the present study, the analysis of weight, length and malformations provide general information on the physical condition of toads. These data integrated with the measurement of a biomarker of genotoxicity present relevant information on sublethal effects. The increase in the frequency of MN in individuals collected in S3 highlights the presence of compounds capable of producing genotoxic damage in native populations. Also, the physicochemical characteristics of S3, such as the levels of As, Se, atrazine and 2,4-D, correlate with the MN frequency. Besides, other studies that measured DNA damage in tadpoles collected from agricultural areas also showed higher DNA damage in comparison to the reference site (Ralph and Petras, 1997). Some chemicals detected in water samples are known genotoxic agents, such as atrazine (Cavas, 2011), azoxystrobin (Yingnan et al., 2016), acetochlor (Li et al., 2011), metalaxyl (Demsia et al., 2007), glyphosate (Bolognesi et al., 1997) and Cu (Prá et al., 2008). The exposure to these substances can cause unstable chromosomal phenotypes leading to increased teratogenesis and tumors, and in consequence a decreased species *fitness*. Since amphibians are good ecosystem health indicators, these results indicate a loss in the environmental quality floodplain of the lower Paraná River.

5. Conclusions

The health condition of *Rhinella fernandezae* populations from different sites of the floodplain of the lower Paraná River measured by biomarkers of health status and genotoxicity indicated the degraded quality of the surrounding areas. The biological parameters of the amphibians and the physicochemical characteristics of the area near the Arrecifes River (S3) showed the lowest environmental quality, followed by the industrial and urban area near the De la Cruz stream (S2) and finally the area within a natural reserve (S1). It is highlighted that the presence of metals and pesticides in S1 was not expected and may be a consequence of the crops and urban areas that surround the natural reserve.

We conclude that the degraded water quality of the lower Paraná River Basin due to anthropogenic activities (unacceptably increased concentrations of metals and pesticides) has a negative impact on native amphibian populations (lower body condition and increased MN frequency), which may critically threaten their long-term health. Thus, the current study highlights the need for continuous environmental monitoring of these ecosystems to ensure sustainable amphibian populations in the lower Paraná River Basin.

CRediT authorship contribution statement

Julieta Peluso: Data curation, Formal analysis, Investigation, Resources, Validation, Writing - original draft, Writing - review & editing. **Carolina M. Aronzon:** Conceptualization, Methodology, Investigation, Resources, Validation, Writing - original draft, Writing - review & editing. **Mercedes Acquaroni:** Investigation. **Cristina S. Pérez Coll:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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