Alterations in Gonads and Liver Tissue in Two Neotropical Anuran Species Commonly Occurring in Rice Fields Crops

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Abstract Amphibians find a suitable habitat in rice agroecosystems, but their viability is affected by the sublethal effects of agrochemicals. Animal condition, external morphology, and liver and male gonad tissue histology were examined on *Lysapsus limellum* and *Rhinella bergi*, common species that occurs in rice fields (RF) and reference sites (RS) from Santa Fe and Chaco provinces, Argentina, respectively. Animal condition factor showed no significant differences between environments in either species. Males of both species from RF showed asymmetrical testis size. A higher proportion of cysts with early spermatogenesis cell stages were observed in RF specimens than in RS individuals. The proportion of tubules with free spermatozoa differed between environments for *L. limellum*. Bidder organ presented vacuoles, atretic follicles, and pigmentary cells in *R. bergi* from Chaco RF. Liver tissues of both species from RF exhibited sinusoid enlargement, hypervascularization, hepatocyte vacuolization, and dilated blood vessels. *R. bergi* had a higher number of hepatic melanomacrophages in RF than in RS. The histological biomarker was more sensitive than the morphological one to evaluate chronic pesticide exposure

using anuran non-target organs (liver, gonads) and Bidder organ. The potential effect of those alterations on the reproductive performance of anurans that commonly occur in agroecosystems needs further research.

Keywords Agroecosystems · Amphibians · Histological testis alteration · Hepatic melanomacrophages

1 Introduction

Intensive land uses, such as agriculture, modify the ecosystems and cause loss of wild habitats, nutrient runoff, greenhouse gas emissions, and contamination by pesticides in humans and non-target organisms (Power & Jetz, 2019). Rice (*Oryza sativa* L.) is one of the most extensively cultivated cereals in the world, occupying more than 11% of arable land (Kole et al., 2010). In Argentina, more than 50% of the land is cultivated with soybean and rice, with rice production has reached 1.1 million tons in the 2018/2019 crop season and covering 190,800 hectares (SIIA, 2019).

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This rice agroecosystem provides temporary food and niche resources for many vertebrates, which are exposed to several pesticides (Attademo, Lorenzón, et al., 2018a; Lorenzón et al., 2020). The main herbicides used for rice crops are bentazone, molinate, propanil, clomazone, 2,4-D Amina, and glyphosate (Quayle et al., 2006; Terrado et al., 2007), whereas dimethoate, parathion, imidacloprid, and carbofuran are the most widely used insecticides (Parsons et al., 2016). Pesticides and contaminants of emerging concern accumulate in aquatic environments, negatively affecting ecology, physiology, ethology, and metabolism; therefore, they are an important driver of global amphibian decline (Egea-Serrano et al., 2012; Tamschick et al., 2016). Ecotoxicological studies provide evidence of the adverse effects of agrochemicals worldwide (Relyea, 2005; Sievers et al., 2019).

Amphibians play important roles, such as pest biological control in agricultural systems (Attademo et al., 2005; Khatiwada et al., 2016) and energy flow from aquatic to terrestrial habitats (Sánchez-Hernandez, 2020). Anuran species were found to differ in their sensibility within the field crops (Pyrón, 2018). Agrochemicals cause reproductive dysfunction and abnormal development in anurans via interference with the endocrine system (Trudeau et al., 2020). In addition, evidence of endocrine disruptions in other vertebrates exposed to agrochemicals was investigated (Matthiessen et al., 2018; Mnif et al., 2011).

Gonadal dysgenesis and morphological alterations, presence of intersex gonads, and testicular oocytes have been frequently reported worldwide in frogs (Hayes et al. 2006a, b; McCoy et al., 2017). Laboratory experiments demonstrated deleterious effects of glyphosate and atrazine exposure on amphibian reproduction (Hayes et al., 2010; Hayes, Stuart, et al., 2006a; Howe et al., 2004; Tavera-Mendoza et al., 2002). The majority of studies that reported the prevalence of intersex are referred to anuran species from North America (reviewed by Abdel-moneim et al., 2015). However, a causal relationship between environmental concentrations of endocrine disruptors and the prevalence and severity of intersex on wildlife individuals was unable to establish (Abdel-moneim et al., 2015). In South America, the effects of agrochemicals on anuran reproduction have been scarcely studied at the field scale, and few reports suggested morphological or histological gonad abnormalities (Rojas-Hucks et al., 2019; Sánchez et al., 2014). Further evidence regarding gonad

histology, endocrine disruption, and effects on reproduction is needed to characterize the biological risk of anurans exposed to compounds with endocrine effect (Cevasco et al., 2008; Matthiessen et al., 2018).

On the other hand, the liver is widely used in ecotoxicological studies in different vertebrates due to its important role in biotransformation processes of xenobiotics, which involves both hepatocytes and Kupffer cells (also called melanomacrophages/MMs) through enzymatic and nonenzymatic mechanisms (Fenoglio et al., 2005; Loumbourdis, 2007). MMs are pigmented cells located in hematopoietic organs, such as the liver in frogs, which participate in immunological response and detoxification of pollutants (Bach et al., 2018; Steinel & Bolnick, 2017). The abundance of MMs in the liver parenchyma after anuran exposure to pesticides was recorded in laboratory assays (Bernabó et al., 2014; Cakici, 2015; Curi et al., 2019; De Oliveira et al., 2016); however, few studies reported similar effects on frogs occurring in agroecosystems (Huespe et al., 2017; Jantawongsri et al., 2015).

In the present study, adults of two anuran species (*Lysapsus limellum* and *Rhinella bergi*) with different ecological characteristics (reproductive mode, microhabitat use) inhabiting rice fields were selected as bioindicators. Three hypotheses were tested: (a) the morphological parameters measured in adults (length, weight, and animal condition factor) differ between rice and reference sites, (b) the morpho-histology of gonads (testicular volume, spermatogenic cyst proportion, and quantity of free spermatozoa) is altered due to chronic exposure to agrochemicals in rice fields, and (c) MMs increase in the liver of anurans from rice fields.

2 Materials and Methods

2.1 Study Sites

Anuran surveys were carried out in two rice fields (RF1 and RF2); the natural environments where these anurans occur were used as reference sites (RS1 and RS2) in the central-eastern of Argentina (Fig. 1). RS1 is a wetland site associated with the Paraná River, near forest fragments in the locality of Garay (Santa Fe province, 31° 10' 21.1" S, 60° 15' 31.73" W, 700 ha). This area has no agricultural activities (Attademo et al., 2011; Huespe et al., 2017). The RF1 was a rice (*Oryza sativa*) cropland situated in the locality of San Javier (Santa Fe province,

Fig. 1 Location and general view of sampling sites in Santa Fe (RF1 and RS1) and Chaco (RF2 and RS2) provinces, Argentina. References: RF1, rice field located in San Javier department; RS1,

reference site located in Garay department; RF2, rice field located in Bermejo department; RS2, reference site situated in Presidencia de la Plaza department

30° 05' 13.56" S, 59° 53' 19.98" W, 287 ha). The distance between the RF1 and RS1 is approximately 50 km. Rice fields are surrounded by several forest fragments with native vegetation of the Paraná River Delta and islands and Espinal ecoregions. The average annual temperature ranges between 15 and 20 °C (Apodaca et al., 2015), and the rainy season is from October to March.

The other two sites, RF2 and RS2, were located in the Humid Chaco ecoregion. These two sites were characterized by a warm subtropical climate with a mean annual temperature of 26 °C and rains concentrated in summer months (Burkart et al., 1999). RS2 was located in the Presidencia de la Plaza department (Chaco province, 27° 07' 36.80" S, 59° 36' 7.39" W, 300 ha). This site presented native vegetation where agricultural activities or direct agrochemical runoff is far away (10 km); non-intensive cattle settlements are performed in adjacent areas. RF2 was a rice field located in the locality of Bermejo (Chaco province, 27° 5' 35.45" S, 58° 45' 19.81" W, >500 ha). The distance between RF2 and RS2 is approximately 90 km.

2.2 Selection of Anuran Species

Lysapsus limellum (Amphibia, Anura, Hylidae) was selected because it is frequently found in the rice field (Duré et al., 2008), and it was previously used as a bioindicator in another ecotoxicological study (Attademo et al., 2015). This is a small-sized frog species that can reach 20 mm of snout-vent length (SVL) at adult stages and has an aquatic lifestyle. It inhabits permanent or semi-permanent water bodies with abundant submerged or floating vegetation (Zaracho et al., 2011). It is found in contact with water or associated with aquatic vegetation (Attademo et al., 2015; Duré et al., 2008). In Argentina, it is widely distributed in the Chaco, Espinal, and Paranaense ecoregions and is categorized as “not threatened” (Vaira et al., 2012; Zaracho et al., 2011). It feeds on dipterans, orthoptera, arachnids, and fish (Duré & Kehr, 2001).

Rhinella bergi (Amphibia, Anura, Bufonidae) was also selected because it frequently occurs on secondary unpaved roads crossing rice crops. Other similar bufonids species (*Rhinella arenarum*, *R. fernandezae*, and *R. dypticha*) have been used as bioindicators in laboratory and field studies (Lajmanovich et al., 2004, 2019; Sánchez et al., 2014). This frog can reach 50 mm in total length, has a terrestrial lifestyle (burrowing

habitats), and is distributed throughout the Chaco and Espinal ecoregions in Argentina (Zaracho et al., 2011). It is categorized as “not threatened” (Vaira et al., 2012). It is insectivorous, feeding mainly on ants and beetles (Duré et al., 2008).

2.3 Anuran Field Sampling

Both *L. limellum* and *Rhinella bergi* were sampled in two environment types (RF and RS), the former in Santa Fe province and the latter in Chaco province. Both species were abundant in each rice crop field (Attademo, Lorenzón, et al., 2018a; Duré et al., 2008). Sampling was performed during two consecutive annual periods (2015–2016 and 2016–2017) simultaneously with the anuran breeding season (Curi et al., 2014) between November and March. The samplings were made between 19:00 and 01:00 once a month accounting $n = 10$ sampling dates in each survey site during the entire studied period. The rice period covers the entire crop cycle, from sowing to harvest. Visual and auditory encounter techniques (Crump & Scott, 2001; Zimmerman, 2001) were used during day and night surveys (a minimum of 6 h a day). A total area of 80.06 ha was surveyed in each environment. We captured the individuals by hand, collecting a maximum number of 10 adult males per species from each site on each sampling date. Only individuals with evident secondary sexual characters or calling males were captured (Sánchez et al., 2014). Collection permissions were granted by the Wildlife and Natural Areas department of Chaco Province and the Environment Ministry of Santa Fe Province (N°02101-0018519-1).

2.4 Measurements of External Morphology and Organ Dissection in Males

A total of $n = 26$ and 13 *L. limellum* individuals were collected from RF1 and RS1, respectively, whereas $n=24$ and 13 *R. bergi* individuals were collected from RF2 and RS2, respectively. They were anesthetized individually by immersion in a buffered solution of 0.1% tricaine methanesulfonate (MS-222), following the guidelines of ASIH (2004) and with the approval of the Committee of Bioethics of the Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral (Santa Fe, Argentina; Res. CD. N°388/06). Individuals were weighed (WE) using a digital balance (0.01 g precision) and their snout-vent

length (SVL) was measured with digital calipers (0.01 mm precision). Animal body condition factor (CF) was calculated using the formula of Bagenal and Tesch (1978) as follows: $CF = [WE (g) / SVL (mm)]^3 * 100$. Each individual was dissected along the mid-ventral line (longitudinal incision), and digestive organs (stomach, intestine) were removed to photograph reproductive organs under a stereoscopic magnifying glass. Subsequently, the reproductive organs were fixed in Bouin solution (24 h) and preserved in alcohol (70%). The specimens were numbered and deposited in the Universidad Nacional del Nordeste (UNNE) Herpetological Collection (Corrientes, Argentina).

2.5 Morphological and Histological Gonadal Assessment

2.5.1 Assessment of External Gonadal Morphology

The length and width of the testes of both species were measured before the Bouin fixation using digital calipers (0.001 mm) under a binocular stereomicroscope (Leica Ez4W). Testicular volume (TV) was calculated with the spheroid formula (Dunham, 1981). Additionally, the external morphology of testes was examined for scored abnormalities (Coady et al., 2005; Hecker et al., 2006; Sánchez et al., 2014).

2.5.2 Histological Assessment of Male Gonads

The urogenital system (testes and kidneys in *L. limellum*, testes + Bidder organs (BO), and kidneys in *R. bergi*) was removed from $n=6$ male individuals from RF1-2 and RS1-2 for histological procedures. The tissues were dehydrated in increasing ethanol concentrations, cleared in butyl-paraffin, and embedded in paraffin. Transverse sections of the central regions of gonads were cut (4–5 μ M) with a rotary microtome and stained with hematoxylin-eosin (H-E) and Gomory Trichrome (GT). Sections were analyzed and photographed with a digital camera (Leica LCC50HD) that was mounted on a light microscope (Leica DM500).

The testicular cells present in the seminiferous tubules were classified as spermatogonia (Sg), primary spermatocytes (ISc), secondary spermatocytes (IISc), spermatids at different stages of maturation (Sp), spermatozoa bound to Sertoli cells (Sz), or free spermatozoa

(FSz) (De Souza Santos & De Oliveira, 2008; Ferreira et al., 2008). In each testis, 10 seminiferous tubules of a medial section of the right gonad were examined (Sánchez et al., 2014). The number of cysts with spermatogenic cells at early stages (CES) (Sg, ISc, and IISc) and with spermatogenic cells at late stages (CLS) (Sp, Sz) were counted (adapted from Tsai et al., 2005). The number of spermatozoa in the lumen of the seminiferous tubules was estimated and converted to score numbers between zero and three, with zero corresponding to seminiferous tubules with no spermatozoa and three representing those with the highest number of spermatozoa (Gyllenhammar et al., 2009).

In addition, testicular anomalies were identified in histological sections under a light microscope (Kloas et al., 2009; McCoy et al., 2017). For *L. limellum*, the intensity of external gonad pigmentation was categorized following the classification of Franco-Belussi et al. (2017), as 0 (absence of pigment cells on organ surface), 1 (a few scattered of pigment cells), 2 (a large number of pigment cells), and 3 (a massive amount of pigment cells).

In *R. bergi*, the BO was histologically characterized using specific literature such as Scaia et al. (2011), Piprek et al. (2014), and Freitas et al. (2015).

2.6 Liver Histological Evaluation

The liver from six individuals per site (RF1-2 and RS1-2) was used for the histological procedures. Transverse histological sections (4–5 μ M) of the central region of the liver were cut with a rotary microtome and stained with H-E and GT. They were observed and photographed under a light microscope (Leica DM500) equipped with a digital camera (Leica LCC50HD).

Six photographs ($\times 40$ field of magnification) of non-consecutive liver sections of each individual were observed and digitized (Chai et al., 2017). The number of MMs in each section was quantified using Image J software (adapted from De Oliveira et al., 2016 and Bach et al., 2018). Furthermore, the same histological sections were analyzed under a light microscope to identify the presence of hypervascularization (congestion), sinusoid enlargement, blood vessel dilation, hepatocyte vacuolation, or necrosis, among others (Cakici, 2015; Sayed & Younes, 2017). One person made all histological assessments.

2.7 Statistical Analyses

Before each statistical analysis, normality and homogeneity of variances were tested using the Kolmogorov-Smirnov and Levene tests, respectively (Zar, 1999). A one-way analysis of variance (ANOVA (F)) was used to compare SVL, CF, and WE of individuals between sites. TV did not meet the assumptions of parametric tests; therefore, it was natural-log transformed. Then, an ANCOVA (F) was used with the SVL as a covariate. The mean number of cysts with CES (Sg, ISc, and IISc) and cysts with CLS (Sp, Sz) was compared between sites using a KW test due to the non-parametric nature of the variables. Additionally, a chi-square (χ^2) test was used to check for significant differences in the proportion of seminiferous tubules with abundant, moderate, small, and no amount of free spermatozoa in the lumen between sites. Thus, number of MMs in liver sections was compared between sites using a KW test. All the statistical analyses were performed with Infostat/P version 1.1 software (Infostat Professional Group, Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Argentina). The level of significance used was $P < 0.05$.

3 Results

3.1 Body Measurements

L. limellum showed no differences in SVL, WE, or CF between environments (RF1 and RS1) ($F_{SVL}=2.0$, $P>0.05$; $F_{WE}=1.80$; $P>0.05$; $F_{CF}=2.44$; $P>0.05$). *R. bergi* did not show differences in WE or CF ($F_{WE}=3.91$, $P>0.05$; $F_{CF}=1.09$, $P>0.05$) between environments; however, SVL was larger in individuals from the rice field than in those from the reference site ($F_{SVL}=4.46$, $P<0.05$, Tukey post-test $P<0.05$) (Table 1).

3.2 Gonadal Morphology

Testicular volume did not show significant differences between sites either in *L. limellum* ($F_{TV}=0.05$, $P>0.05$) or in *R. bergi* ($F_{TV}=2.09$, $P>0.05$) (Table 1).

The male reproductive system of both anurans species consisted of testes located in the anterior portion of the kidneys and covered by a tunica albuginea (Fig. 2). They were ovoid and were associated with fat bodies

(Fig. 2). The BO was located in the anterior pole of the testes in *R. bergi* (Fig. 2e).

In *L. limellum*, a great variation in testicular pigmentation was observed in RF1 individuals (Fig. 2b–d, Fig. 3b–d). Four different pigmentation categories were observed in males from this site (category 3, with $n=2$; category 2, $n=3$; and categories 0 and 1, $n=2$) (Fig. 2b–d). In males from RS1, two categories were recorded (category 2 $n=6$, category 1 $n=1$) as shown in Fig. 2a. Morphologically, the presence of asymmetrical testis size was mostly observed in *L. limellum* from RF1 (18.51%) (Fig. 2b). On the other hand, in *R. bergi*, testes of irregular size (16.66%, fig. 2 g), BO with enlarged follicles (8.33%), and bilobed BO (8.33%, Fig. 2f) were remarkable also in RF2.

3.3 Histology of Male Gonads

The testes of *L. limellum* and *R. bergi* had seminiferous tubules that contained numerous cysts with the germ cell line (Sg, ISc, IISc, Sp, Sz, and FSz) (Fig. 3 and Fig. 5).

3.3.1 *Lysapsus limellum*

The histological section of testes revealed connective tissue containing collagen fibers (slightly blue in GT stained cuts) and melanin among the seminiferous tubules (Fig. 3a). The mean number of cysts with spermatogenic cells at the early stages was significantly different between sites (KW=11.32, $P<0.05$), being higher in testes of individuals from the rice field than in those from the reference site (Fig. 4a, Table 1). The mean number of cysts with cells in late stages did not show significant differences between sites (Fig. 4a, Table 1). The proportion of seminiferous tubules with each score of FSz is summarized in Fig. 4c. *L. limellum* had a higher proportion of score zero tubules (without FSz) and a smaller proportion of score 3 tubules (with abundant FZs) in rice field than in reference site ($\chi^2 = P < 0.05$).

3.3.2 *Rhinella bergi*

The histological configuration of testes was similar to that described for *L. limellum*. The seminiferous tubules were well defined, although, unlike in this species, pigment cells were absent. The germ cell cysts were visible, and the Sertoli cell was observed in some cases (Fig. 5a, b). The mean number of cysts with spermatogenic cells in the early stages was significantly different

Table 1 Biomarkers measured in *Lysapsus limellum* and *Rhinella bergi* from rice fields (RF 1 and RF 2) and reference sites (RS 1 and RS 2) in Santa Fe and Chaco provinces, respectively. References: Snout-vent length (SVL), animal body condition factor (CF), testicular volume (TV), cysts with spermatogenic cells in

early stages (CES) and with spermatogenic cells in late stages (CLS), melanomacrophages (MMs), sinusoid enlargement (SE), dilation of blood vessels (DBV), hepatocyte vacuolization (HV), hypervascularization (H)

Biomarkers	<i>Lysapsus limellum</i>		<i>Rhinella bergi</i>	
	RF 1	RS 1	RF 2	RS 2
Morphological measurements				
SVL (mm)	16.81±1.81	16.84±2.15	36.5±3.62	33.03±5.0
Weight (g)	0.60±0.10	0.65±0.12	3.78±0.95	2.95±1.26
CF	0.005±0.001	0.006±0.003	0.14±0.15	0.10±0.09
TV (cm ³)	0.93±0.77	0.80±0.44	4.91±3.28	1.44±0.83
Testicular histological analyses				
Number of CES	16.61±8.10	12.21±6.59	6.23±2.44	4.65± 2.07
Number of CLS	9.21±7.33	9.51±5.64	10.95±7.10	13.15±7.85
Liver histological analysis				
Histological alterations	SE, DBV		HV, SE, H, DBV	
MM number	43.33±19.69	42.19±17.63	20.19±7.0	17.08±6.63

*Numbers in bold indicate significant differences ($P<0.05$)

Fig. 2 Urogenital system morphology of *Lysapsus limellum* (a–d) and *Rhinella bergi* (e–g) observed under a stereoscopic magnifying glass. Individual from RS1 (a); individual from RF1 with irregular testis size and a massive amount of pigment cells (b), with absence of pigment cells (c) and a large amount of pigment

cells (d); individual from RS2 (e); individuals from RF2 with bilobed BO (f) and with irregular testis size (g). References: testis (T), kidney (K), fat body (FB), liver (L), Bidder organ (BO), scale bar: 1 mm

Fig. 3 Histological sections of *Lysapsus limellum* testes ($\times 40$). Testis of an individual from RS1 showing the seminiferous tubules and ISc cyst (black cut line) (a); testis of an individual from RF1 with few germ cell cysts and pigmented testes (category 3) (b),

with pigmentation category 2 (c) and pigmentation category 0 (d). References: spermatids (Sp), primary spermatocyte (ISc), free spermatozoa (FSz), spermatozoa (Sz), melanocytes (arrow head)

between sites (KW=11.04, $P < 0.05$), being higher in testes of individuals from the rice field than in those from the reference site (Fig. 4c, Table 1). The mean number of cysts with cells in late stages did not show significant differences between sites (Fig. 4c, Table 1). The proportion of seminiferous tubules with each FSz score is summarized in Fig. 4d. The proportion of tubules with FSz scores 0 and 2 was significantly different between sites ($\chi^2 = P < 0.05$; Fig. 4d).

Histologically, BO consisted of two well-defined regions, cortex, and a medullary region (Fig. 5c). In the cortex, Bidderian oocytes in previtellogenic stages, consisting of oocytes surrounded by follicular cells, were identified. In the medullary region, blood vessels and connective tissue with presence of fiber cells were observed (Fig. 5c). Vacuoles in BO (33.33%, atretic follicles in BO (66.66%) and pigmentary cells in the medullary region of BO (16.66%) were observed in *R. bergi*

Fig. 4 Proportion of cell spermatogenic types in *Lysapsus limellum* and *Rhinella bergi* between sites. Mean number of cysts with early (Sg, ISc, and IISc) and late (Sp, Sz) spermatogenic

stages in *L. limellum* (a) and *R. bergi* (b); number (scores) of free spermatozoa (FSz) in *L. limellum* (c) and *Rhinella bergi* (d). The asterisk (*) indicates a significant difference

from the rice field (Fig. 5f, i). Three individuals with testicular oocytes were recorded from the reference site (Fig. 5e). Testicular tissue and BO connections in certain histological sections were also distinguished (Figs. 5f–h).

3.4 Liver Histology

In *L. limellum* and *R. bergi* from RS, the liver tissue presented hepatocytes in cordons surrounded by sinusoid

capillaries, veins, and MMs dispersed throughout the parenchyma were also identified (Fig. 6). Sinusoid enlargement (37.71%) and dilated blood vessels (5.55%) were observed (Fig. 6b, c), in the liver tissue of *L. limellum* from RF1 (Table 1). On the other hand, hepatocyte vacuolization (41.66%, Fig. 6e), sinusoid enlargement (33.33%, Fig. 6f), hypervascularization (11.11%, Fig. 6e), and enlarged blood vessels (5.55%) were recorded in *R. bergi* from RF 2 (Table 1). The number of MMs

Fig. 5 Histological sections of testes and BO in *Rhinella bergi*. Testis section (a), detail of seminiferous tubules and ISc cyst (b), and BO structure in an individual from RS2 (c); testis with predominance of spermatogenic cell in early stages in an individual from RF2 (d); testicular oocytes in the testis of an individual from RS2 (e); testis and BO containing vacuoles in the Bidderian follicle and pigment cells in medullary region in an individual from RF 2 (f), presence of testicular oocytes in the testis near BO

(g); details of testis and BO connections (h) and atretic follicles of BO in an individual from RF2 (i). References: testis (T), Bidder organ (BO), cortex (c), medullary region of BO (m), ISc (primary spermatocyte), Sg (spermatogonia), Sp (spermatid), Sz (spermatozoa), FSz (free spermatozoa), atretic follicle (Af), previtellogenic follicle (PVf), testicular oocyte (red arrow), vacuoles (asterisk), cyst of ISc (white cut line). a, c, d, e, i ($\times 40$); b ($\times 100$); g, h ($\times 10$)

recorded in the liver tissue of *L. limellum* was similar between sites, but it was significantly higher (KW= 4.13, $P<0.05$) in *R. bergi* from the rice field than in those collected from the reference site (Table 1).

4 Discussion

Two anuran species common in the agroecosystems, *L. limellum* and *R. bergi*, presented morphological and

Fig. 6 Liver histological sections of *L. limellum* and *R. bergi* in $\times 40$. Liver section of *L. limellum* from RS1 (a); liver section with sinusoid enlargement (b) and dilated veins (c) in an individual from RF1; liver section of *R. bergi* from RS 2 (d); liver section

with vacuolization of hepatocytes (e) and sinusoid enlargement (f) of an individual from RF2. References: hepatocytes (h), blood vessel (bv), sinusoid (s), melanomacrophages (white arrow), vacuolization (white asterisk), red blood cells (red circles)

histological abnormalities in male gonads and liver tissue alterations of different intensity. These results highlight the importance of testing various anuran species under the same condition because species vary in sensitivity to agrochemicals within the community (Relyea & Jones, 2009). A high number of pesticides is applied in Mideastern Argentina, the main rice production area (Attademo et al., 2015; Rossi et al., 2020), where *L. limellum* and *R. bergi* commonly occur (Vizgarra, 2018). Within this area, several studies demonstrated the presence of pesticide residues in dairy farm water (Demonte et al., 2018) and rainfall water samples (Alonso et al., 2018). Pesticide residues are frequently found to account for more than 80% of paddy rice monitored during the pre-harvest season (October and November 2018) where concentrations ranged between 11.6 and 43.6 times being higher than the maximum

residues limits (see Medina et al., 2020). The presence of insecticides such as imidacloprid was detected in sediment samples from the RF1 (Attademo, Tamburi, et al., 2018b) as well as the presence of glyphosate, bifenthrin, azoxystrobin, and cyproconazole detected in water and sediment samples, bioaccumulating in fish species (Rossi et al., 2020).

The animal CF of *L. limellum* and *R. bergi* did not differ significantly between the rice fields and the reference sites. Similar results were found in *R. fernandezae* and *Dendropsophus sanborni* in three environments that differed in disturbance degrees (Sánchez, 2011). This result may be explained by the consumption of invertebrates with high nutrient value in agroecosystems (Bambaradeniya et al., 2004; Peltzer et al., 2010). In contrast, other studies recorded a lower CF in *Pelophylax ridibundus* occurring in contaminated areas

(domestic wastewater and heavy metal contamination) compared to populations of uncontaminated areas (Zhelev et al., 2015, 2017). According to our results, only SVL differed in *R. bergi*, being higher in individuals from the rice field than in those from the reference site. However, this result may be attributed to the low number of males collected in the reference site from Chaco province.

The TV of *L. limellum* and *R. bergi* did not differ between environments. Martori et al. (2005) pointed out that the similarities in testicular volume of *R. fernandezae* from different environments may be due to the time of collection, which coincides with the species breeding season (spring-summer period). Conversely, Sánchez et al. (2014) found a decrease in testicular volume of *R. fernandezae* collected from environments with agricultural activities. Thus, external and internal causes (climatic conditions, intraspecific variations, reproductive stage, and age) may affect this parameter (Huang et al., 1997). In our study, it was not possible to relate agrochemicals to testicular volume. Difference in size between the right and left testes was also recorded in both species from rice fields, but the proportion was low. A similar result was recorded for *R. fernandezae* and *D. sanborni* in agricultural areas (Sánchez, 2011). Jooste et al. (2005) explained that the cause of these anomalies might be due to a delay in the testis maturation. Gonad abnormalities were few registered in our study. These controversial results are exposed by other authors who demonstrated that there were no significant effects on amphibian gonad morphology between agriculture and non-agriculture environments (Du Preez et al., 2005; Smith et al., 2005; Spolyarich et al., 2011).

Testicular pigmentation is part of an extracutaneous pigmentary system whose function is poorly understood (De Oliveira & Zieri, 2005). In *L. limellum*, variation in pigmentation was much greater in individuals from the rice field than in those from the reference site. However, these variations cannot be linked with a specific cause of environmental contamination. Few studies investigated how environmental variables influence the coloration of testes (Franco-Belussi et al., 2017). In addition, intracellular transport and the aggregation of melanin were inhibited after Roundup formulation exposure in *Xenopus laevis* (Hedberg & Wallin, 2010). The existence of an intraspecific variation between individuals may play a role in pigmentation expression, as noted for *Dendropsophus* and *Scinax* (Franco-Belussi et al.,

2017). Melanocytes present in gonads would be expected to protect the tissues from oxidative damage, toxic agents, DNA damage, or UV, similarly to extracutaneous pigment types (Franco-Belussi et al., 2016; Zieri et al., 2015). The testicular pigmentation pattern in gonads should be analyzed under different environmental conditions and also at different life stages (tadpoles, juveniles, adults) for to have a more comprehensive analysis of other factors that may influence.

Histological analysis of testes revealed that *L. limellum* and *R. bergi* have a similar structure and cellular composition to those of other anurans species (De Oliveira et al., 2003; De Souza Santos & De Oliveira, 2008). Spermatogenic activity in *L. limellum* is classified as continuous in Pantanal (Mato Grosso do Sul, Brazil) by Ferreira et al. (2008); i.e., the testes contain cells in all stages of maturation in seminiferous tubules throughout the year. A higher proportion of cysts with cells in the early spermatogenesis stages were observed in *L. limellum* from the rice field than in those from the reference site. Moderate and abundant number of free spermatozoa in the lumen of tubules was observed in testes of frogs from the reference site. More studies are necessary to relate hormonal disruptions and alteration of the spermatogenic process in amphibians from agroecosystems. Plasma testosterone concentration in males from agricultural areas was also recorded in *B. marinus*, a species that is frequent in agricultural areas (McCoy et al., 2008); it has been recently hypothesized that the reduction in the spermatogenic cell number is due to some alterations in the functioning of the hypothalamus or pituitary gland in these species (McCoy et al., 2017). Similarly, the number of spermatogonial cell nests decreased in atrazine-exposed tadpoles compared to control; it represented a 70% reduction in spermatogonial nests (Tavera-Mendoza et al., 2002). The proportion of cysts with cells in the early spermatogenesis stage was higher in *R. bergi* from the rice field than in those from the reference site. In contrast, the number of free spermatozoa was similar in both sites. This toad has an explosive reproduction that occurs in a few days (Ceí, 1980), which may explain the scarce number of free spermatozoa in the collection days. Since *L. limellum* is an extremely aquatic frog, it is expected that this species would be more exposed to agrochemicals due to high transdermal exposure during metamorphosis in water altering as consequence the spermatogenic processes. Attademo et al. (2015) documented approximately 3–4 *L. limellum* dead frogs per

m² after aircraft chlorpyrifos application in the same rice field.

On the other hand, the histological analysis revealed the presence of testicular oocytes embedded in the testicular tissue in three *R. bergi* individuals from the reference site. The cause of the presence of testicular oocytes in anurans has not been completely elucidated (Lent et al., 2019; Orton & Tyler, 2014). Sánchez (2011) also observed this structure in *R. fernandezae* and *D. sanborni* and found that it was not exclusive of contaminated sites. Skelly et al. (2010) reported a high frequency of testicular oocytes in *Lithobates clamitans* from the suburban environment (21%) and 6% in the agricultural environment. Therefore, they did not mention an association between this structure and agricultural cover. However, McCoy et al. (2008) found that the number and frequency of gonadal abnormalities, such as intersex individuals (gonads containing ovarian and testicular tissue) of *Rhinella marina* increased along a gradient of increasing agricultural activity. The presence of testicular oocytes found in *R. bergi* testes cannot be considered a histological alteration. The oocytes observed inside the testicular tissues probably correspond to an adjacent Bidder organ follicle due to the nearest between these organs and the fact that transverse cuts were made.

The BO structure is similar to that described for the species, such as *Rhinella dypticha* (Freitas et al., 2015), *Bufo bufo* (Falconi et al., 2007), and *Rhinella icterica* (Farias et al., 2002). Pigmentary cells were observed in *R. bergi* from the rice field. As suggested by Freitas (2013), these cells may have phagocytic activity and be an adaptive characteristic of *R. dypticha* to the environment (Freitas et al., 2015). Furthermore, toads from RF presented atretic follicles and cortical vacuoles. The cortical vacuoles have been also reported in *R. schneideri* males exposed to 17 β estradiol for 3 to 7 days (Freitas, 2013).

No significant differences were found in the number of MMs in *L. limellum* frogs between sites. Nevertheless, in *R. bergi*, a higher number of MMs was recorded in the rice field than in reference site. In this sense, the use of MMs is a useful histological biomarker of oxidative stress in some anuran tadpoles (Bach et al., 2018; Curi et al., 2019; Pérez Iglesias et al., 2019) and adult anurans exposed to different agrochemicals under laboratory conditions (Cakici, 2015; De Gregorio et al., 2016; Paunescu et al., 2010; Pérez Iglesias et al., 2016). Nevertheless, studies about individuals collected

from natural environments are scarce. Huespe et al. (2017) found a greater number of MMs, which were occupying a larger area in livers of *Leptodactylus macrosternum* adults from a rice crop collected seven days after chlorpyrifos application compared to a reference site. The presence of different compounds like contaminants of emerging concern in aquatic environments where anurans develop could be another possible explanation of the MMs presence in anurans from the forest environment. Additionally, toads from rice fields presented other histological alterations (sinusoid enlargement, dilated blood vessels, hepatocyte vacuolization). These alterations were also observed in livers of *Physalaemus albonotatus* and *Leptodactylus luctatur* tadpoles exposed to 2,4-D Amina Zamba® and Roundup Ultramax and glyphosate, respectively (Bach et al., 2018; Curi et al., 2019). Recently, Pérez Iglesias et al. (2019) found an increase in vascularization and enlargement of hepatic sinusoids in *R. schneideri* tadpoles after exposure to atrazine (SIPTRAN 500SC®) for 48 and 96 h and concluded that this herbicide has hepatotoxic capacity.

5 Conclusions

The assessment of chronic agrochemical exposure in amphibians occurring in rice fields should include morphological parameters and histological analysis of target organs (gonad and liver) to characterize the risk at different biological levels. The result of the histological analysis of testes varied between anuran species. An altered spermatogenic pattern was observed only in *L. limellum* males from the rice field. Further analysis is needed to determine if amphibian species are suffering reduced fertility or other reproductive health disorders at population level (McCoy et al., 2017). Furthermore, the Bidder organ is a useful target organ to be used in the evaluation of pesticide effects on Bufonids. Histopathological liver lesions in anurans may be the result of long-term exposure to agrochemicals in rice fields. The increase in MMs indicates high oxidative stress in *R. bergi* from the rice field in contrast to the reference site. We also confirmed the use of liver MMs as a biomarker of agrochemical exposure on some adult anurans.

The sublethal effects analyzed in natural populations of *L. limellum* and *R. bergi* at the histological level give a relevant and realistic scenario and highlight the

dissimilarities in species sensitivity and responses. The rice fields environments imply a high risk in these to anuran species, which frequently occur in those environments. Moreover, these two species are useful as bioindicator species of pesticide contamination and disruption of reproduction. Further, a battery of biomarkers at different levels (morphological, histological, but also physiological, biochemical, molecular, etc.) is needed to understand the pathways evolved in reproductive abnormalities (Abdel-moneim et al., 2015).

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Author Contribution All authors contributed to the study conception and design. Field work, laboratory activities, and analysis were performed by Curi, L.M. The first draft of the manuscript was written by Curi, L.M. The field work and the commented on the previous version of manuscript were performed by Peltzer, P.M., Attademo, M.A., and Lajmanovich R.C. All authors read and approved the final manuscript.

Declarations

Ethics Approval Animal used in this research was treated following the American Society of Ichthyologists and Herpetologists (2004) guidelines and with the approval of the Committee of Bioethics of the Facultad de Bioquímica y Ciencias Biológicas of the Universidad Nacional del Litoral, Santa Fe, Argentina (Res. CD. N°388/06), <http://www.fbc.unl.edu.ar/pages/investigacion/comite-de-etica.php>.

Consent to Participate All authors are consent to participate.

Consent for Publication All authors are consent for publication.

Conflict of Interest The authors declare no competing interests.

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