First evidence of the effects of agricultural activities on gonadal form and function in *Rhinella fernandezae* and *Dendropsophus sanborni* (Amphibia: Anura) from Entre Ríos Province, Argentina

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Abstract. The relationship between male gonadal abnormalities and habitats with different degrees of agricultural activities was quantified in two anuran species, *Rhinella fernandezae* and *Dendropsophus sanborni*. The study sites were selected along a gradient of increasing agricultural land use in south-western Entre Ríos province (Argentina): an agroecosystem, a natural wetland (a non-agricultural site adjacent to monoculture zones), and a natural forest (not associated with agriculture). *Rhinella fernandezae* and *D. sanborni* were manually captured from each environment during field surveys. A scaled mass index (MI) was evaluated for each animal. Specimens of *R. fernandezae* from the agroecosystem and the natural wetland site presented poorly developed seminiferous tubules, lower testicular volume, and a lower number of seminiferous tubules, primary spermatogonia, and spermatids than specimens from the natural forest site. Additionally, we observed fewer primary spermatocytes in the agroecosystem group than in the natural forest group. Individuals of *D. sanborni* from the agroecosystem and the natural wetland site presented poorly developed tubules, higher proportions of irregularly shaped testes, and a reduced number of primary and secondary spermatogonia compared with specimens from natural forest sites. Consequently, the affected anurans are likely to have reduced reproductive success. We suggest that agrochemical use may be associated with decreased testicular development and function in both *R. fernandezae* and *D. sanborni* occurring in agroecosystems and nearby environments. Buffer zones are needed to prevent contamination, preserve wildlife, and enhance the conservation value of pristine natural forests.

Keywords. Agriculture, anomalies, testicular histology, germ cells, *Rhinella fernandezae*, *Dendropsophus sanborni*.

INTRODUCTION

Intensification of agricultural activities has become a major threat to biodiversity in different parts of the world. Agriculture can alter natural systems in different ways, such as habitat loss and the creation of isolated fragments by conversion of natural habitats to arable land (e.g., Joly et al., 2001; Grau et al., 2005; Morton et al., 2006), as well as through the possible deleterious impacts

of chemicals on native flora and fauna (e.g., Smith et al., 2000; Khan and Law, 2005; Peltzer et al., 2011; Amaral et al., 2012).

Exposure of amphibians to endocrine-disrupting chemicals affects the functioning of endocrine systems involved in reproduction, development, and metamorphosis (Crump, 2001; McDaniel et al., 2008; Trachantong et al., 2013). As a result of abnormal development of reproductive organs or structures, recruitment can

decrease, which has been as noted a proximate (direct) cause of amphibian declines (Hayes et al., 2010). Estrogenic chemicals, such as pesticides, are extensively used in natural environments, being distributed through sewage effluents, agricultural runoff, aerial drift, and possibly rainfall (Colborn et al., 1993; Storrs-Méndez and Semlitsch, 2010). There is increasing evidence of endocrine disruption and reproductive abnormalities in amphibian populations inhabiting agricultural areas (e.g., Hayes et al., 2003; McCoy et al., 2008; McDaniel et al., 2008; Orton and Routledge, 2011).

In Latin America, the use of genetically modified soybean crops has been largely increased, with the associated increase in pesticide application. Soybean crop expansion has been driven by: (1) crop prices; (2) government and agro-industrial support; and (3) increasing demand from countries such as China (Pengue, 2005). In Argentina, a great expansion of soybean planting has occurred since 2005 (Altieri and Pengue, 2006). Almost 100% of this increase involves the use of genetically modified "Roundup Ready" soybeans, resistant to glyphosate-based herbicides; therefore, the use of these chemicals has also increased (Lajmanovich et al., 2010). Several studies have suggested that glyphosate-based herbicides affect development (Paganelli et al., 2010) and reproduction (Oliveira et al., 2007; Soso et al., 2007; Romano et al., 2010) in wild fauna. In addition, glyphosate herbicides are rarely applied alone, but in combination with other biocides (herbicides, insecticides, and fungicides) that may have their own effects and interact with glyphosate in various ways (Jergentz et al., 2005).

According to laboratory experiments, the most widely used insecticides in Argentina (chlorpyrifos, cypermethrin and endosulfan) also have negative effects on the reproductive system (Usha and Harikrishnan, 2005; Jeong et al., 2006; Singh and Singh, 2008; Rey et al., 2009). However, the possible associations between agricultural practices and changes in histoarchitecture or gonadal function of wild animals have been poorly explored in Argentina, with no records of the effect on amphibians being reported despite the high susceptibility of this group. Indeed, amphibians have biphasic life cycle, which makes them especially vulnerable to perturbations of both aquatic and terrestrial environments; highly permeable skin at all life history stages; restricted home range and limited dispersal abilities (Jansen and Healey, 2003). Accordingly, these kinds of field studies are necessary because of the rapid agricultural expansion and intensification and would be very important for amphibian conservation and evaluation of the ecological risks associated with agriculture.

In the present study we quantified the relationship between male anuran gonadal abnormalities and habitats with different degrees of agricultural activity. Specifically,

we hypothesized that gonadal form and function would be altered by agricultural activity in soybean fields.

## MATERIALS AND METHODS

Site selection

We selected three sites (an agroecosystem, a continental natural wetland, and a natural forest of island) located in southwestern Entre Ríos province (Fig. 1). The agroecosystem site (AG) was a soybean field (*Glycine max* (L.) Merril) situated in Diamante department (32º 06′12.3′′S; 60º 37′17.5′′W; 23 ha). It was cultivated by direct seeding (soybean in spring – summer, and wheat in autumn – winter). Specifically, soybean is usually sown in November/December and harvested in March/April. A natural water body flows across the field, forming a small wetland. Several agrochemicals were applied at different doses during the study period. Before sowing, two herbicides were used to remove weeds (glyphosate 66% 2 L/ha and 2-4 D 1 L/ha). After soybean emergence and at the beginning of bloom, glyphosate 48% 3.5 to 4 L/ha was applied twice. Moreover, cypermethrin 0.1 L/ha and endosulfan 1L/ha were applied once or twice to eliminate caterpillars and bugs (Cooperativa Agrícola de Diamante, pers. comm.).

The natural wetland site (NW) is located between the agricultural and natural forest zones; this site is directly exposed to pluvial runoff from soybean fields due to the slope. The vegetation in the NW site was dominated by wooded and shrubby species, such as *Phytolacca dioica*, *Rapanea laetevirens*, *Fagara hyemalis*, and *Celtis spinosa*. The vegetation in water bodies and flooding areas was characterized by *Eichhornia azurea*, *Lemna gibba*, *Ludwigia peploides*, *Panicum elephantipes*, and *Polygonum punctatum*. This site is located in the continental zone of Pre-Delta National Park (PDNP) (32º 07′17.6′′S; 60º 38′02.2′′W). PDNP is a wetland reserve (2458 ha) located 2 km away from the agroecosystem, in the Paraná River floodplain close to the starting point of Paraná Delta, which includes a continental zone as well as several islands (APN, 2003; Aceñolaza et al., 2004). This reserve presents the typical vegetation of fluvial gallery forest (Bó, 2006).

The natural forest (NF) was located in the island region of the PDNP (32º 07′30.7′′S; 60º 38′11.6′′W). This is a pristine sector preserved from human impact and not exposed to direct runoff carrying agricultural chemicals. The dominant woody vegetation in NF site was *Salix humboldtiana, Tessaria integrifolia,* and *Albizia inundata.* Herbaceous vegetation was mainly composed by *Panicum prionitis.* Vegetation in the lower zones is the typical of areas prone to flooding, such as *Aspilia silphiodes, Eichhornia azurea, Enydra anagallis, Eringium pandanifolium,* and *Pontederia rotundifolia.*

Field surveys

According to Sanchez et al. (2013) anuran species richness in AG site was S = 16, whereas in NW and NF sites it was S = 21 and S = 20, respectively. The most abundant species in
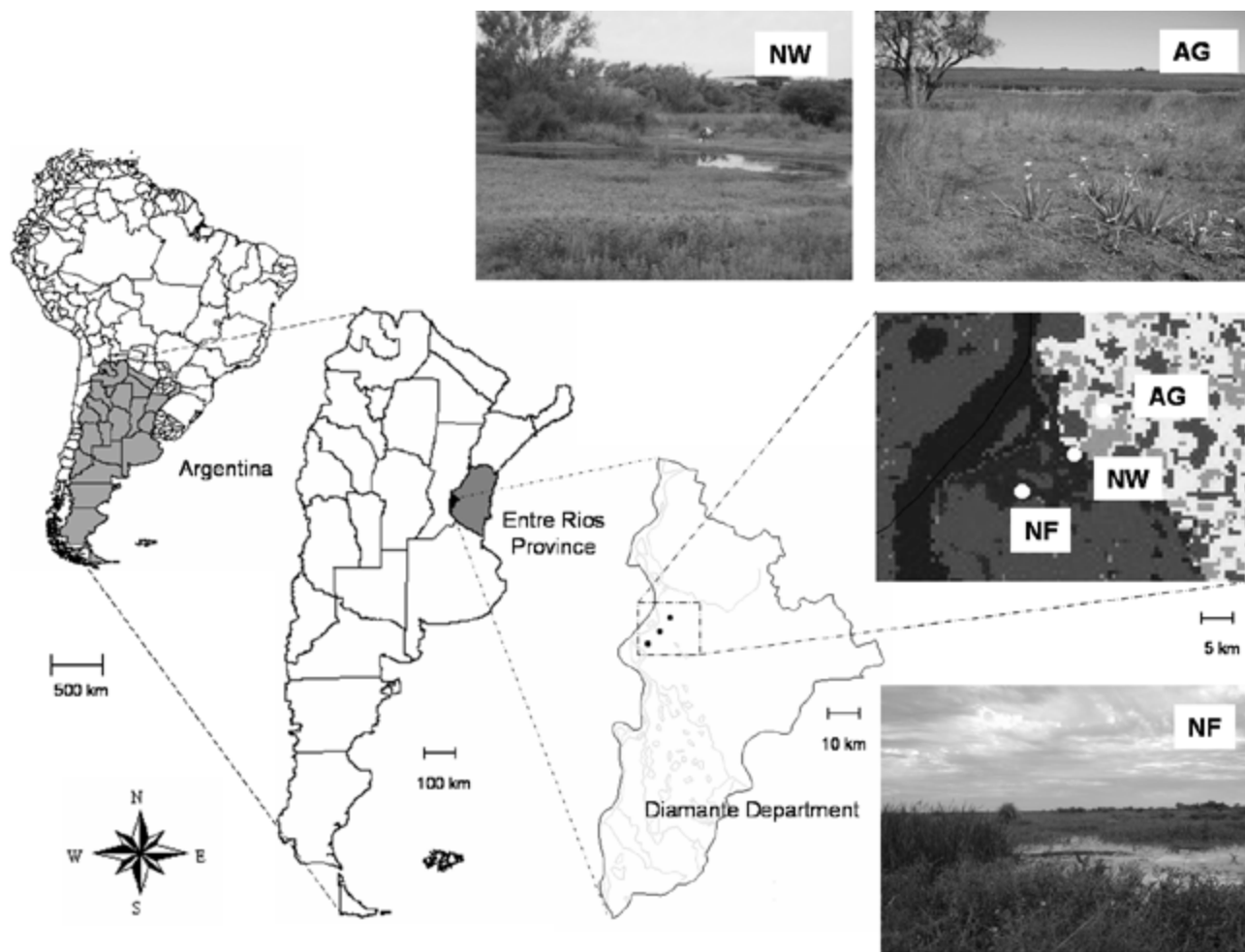
Fig. 1. Location of study sites in Diamante, south-western Entre Ríos province, central-eastern Argentina, southern South America. (AG) Agroecosystem, (NW) natural wetland, and (NF) natural forest. Pictures: (*AG*) agroecosystem (back) and wetland formed from the natural water body (at the front); (*NW*) pond in the natural wetland where the agroecosystem can be observed on the hill (at the bottom); (*NF*) island pond in the natural forest site with surrounding vegetation and, at the bottom, levees with remnants of riparian forest.

AG were *Hypsiboas pulchellus* (Hylidae), *Leptodactylus gracilis, L. latinasus, L. mystacinus* (Leptodactylidae) and *Odontophrynus americanus* (Odontophrynidae). The dominant species both in NW and NF were *R. fernandezae* (Bufonidae), *Dendropsophus nanus, D. sanborni, H. pulchellus* (Hylidae), and *L. latrans* (Leptodactylidae). *Rhinella fernandezae* (Gallardo, 1957) and *Dendropsophus sanborni* (Schmidt, 1944) were selected based on their high representation in amphibian communities of the study area. Estimated total abundance was 251 individuals in *R. fernandezae* ($N_{AG} = 45$, $N_{NW} = 130$, $N_{NF} = 76$) and 226 individuals in *D. sanborni* ($N_{AG} = 66$, $N_{NW} = 95$, $N_{NF} = 65$). These values included adults, juveniles and tadpoles (Sanchez et al., 2013). We used these species to test our hypothesis, as these wild populations were less likely to be adversely affected by the removal of a few individuals for the study. Both species are found in natural and anthropogenic ecosystems and are widely distributed in northeastern Argentina, southern Paraguay, Uruguay and southern Brazil (IUCN, 2011).

We conducted field surveys during the anuran reproductive period in this region (Peltzer and Lajmanovich, 2007) from December 2007 to April 2008. This period coincides with the sowing and harvest of soybean crops. In each environment, we recorded amphibians using nocturnal searches. Nocturnal searches combine visual encounter surveys (Crump and Scott, 1994) and audio strip transects (Zimmerman, 1994). We conducted four searches per month, inspecting all sites during the same night and spending at least 90 minutes per person per site. We captured individuals of the selected species by hand. Only individuals with evident secondary sexual characters (following Cei, 1980) were captured (*R. fernadezae*: external vocal sac and dark thumb pads in the first and second fingers; *D. sanborni*: external vocal sac).

General measurements and dissection of reproductive organs

Individuals of *R. fernandezae* (RF) and *D. sanborni* (DS) were euthanized following the NRC (1996) guide, and according to the requirements of the Ethical Committee of our institution. Specifically, a noninhaled agent (ethanol 20%) was used (AVMA, 2013). We weighed each individual using a digital balance (Means ± SE in g: RF = 13.179 ± 1.323; DS = 0.345 ± 0.008) and measured their snout-vent length (SVL) with a digital caliper (Means ± SE in mm: RF = 48.896 ± 1.327; DS = 17.622 ± 0.141). To compare the nutritional condition of individuals among populations, we calculated the scaled mass index (MI) for each animal (Peig and Green, 2009, 2010), which was computed as follows:

$$MI = Mi \, (L0/Li)^{\,bSMA}$$

where Mi and Li are body weight (g) and length (mm), respectively, of individual i; bSMA is the scaling exponent estimated by the SMA regression of M on L; L0 is the arithmetic mean value of body length for the whole dataset; and MI is the predicted body weight for individual i when the linear body measurement is standardized to L0. We removed the testes and immediately measured their length (Means ± SE in mm: RF = 5.304 ± 0.203; DS = 1.193 ± 0.031) and width (Means ± SE in mm: RF = 1.818 ± 0.059; DS = 0.755 ± 0.016) with a digital caliper, under binocular stereo microscope. We estimated testicular volume using the formula for the volume of a prolate spheroid (Dunham, 1981). Additionally, the presence of vitellogenic oocytes in Bidder´s organs in *R. fernandezae* was explored, following McCoy et al. (2008). Although both testes are of the same size, we analyzed the right testis in order to standardize the procedure. The right testis of each species was fixed in 4% formaldehyde solution for 1 to 8 hours, depending upon size (which varies widely between species) because 4% formaldehyde penetrates at an approximated rate of 1 mm per hour (Fox et al., 1985). Afterwards, testes were transferred to 70% alcohol for histological processing. The specimens were deposited in the herpetological collection of Centre of Scientific Investigation and Transference of Technology to the Production (CICYTTP-CONICET), Diamante, Entre Ríos, Argentina.

Histological evaluation

We evaluated testis histology using light microscopy. After dehydration in increasing concentrations of ethanol, and clearing with xylene, testes were embedded in paraffin (Bancroft and Gamble, 2002). Transverse sections (7 μm) were cut and stained with hematoxylin–eosin (Bancroft and Gamble, 2002). For each male, we examined 10 seminiferous tubules randomly selected from five different sections in the central part of each testis (Díaz-Páez and Ortiz, 2001; Ferreira et al., 2008). In turn, the five sections analyzed were separated by at least five sections of distance, to avoid evaluating the same tubules in different sections. For each section, testicular area was measured, the total number of seminiferous tubules was determined, and the shape of the testis was classified as round or irregular, according to Gyllenhammar et al. (2009). In addition, we measured the total area of each of the 10 seminiferous tubules selected per individual. We classified all cysts, depending on the maturation stage of the germ cells, into one of the six categories: primary spermatogonia (I SG), secondary spermatogonia (II SG), primary spermatocytes (I SC), secondary spermatocytes (II SC), spermatids (SP), and spermatozoa (SZ), following Tsai et al. (2005) and Gyllenhammar et al. (2009).

The number of spermatozoa in the lumen of the seminiferous tubules was estimated and ranked from zero to three, with zero corresponding to the seminiferous tubules with no spermatozoa and three, to those with the highest number of spermatozoa (Gyllenhammar et al., 2009). In addition, we analyzed sections to detect possible testicular anomalies (Hecker et al., 2006), such as lack of development of the seminiferous tubules (e.g., Sower et al., 2000; Hayes et al., 2003), numerous pigment-containing cells (e.g., Patiño et al., 2006; Kloas et al., 2009), testicular oocytes (e.g., Coady et al., 2005; McDaniel et al., 2008), and lack of elongated spermatids (e.g., Murata et al., 2002; Edwards et al., 2006).

All histological evaluations were made by one person to prevent observer bias. Prior to evaluation, we randomized slides so that the observer was unaware (blind) of the origin site of each specimen. We used an Arcano L 1200B HTG microscope equipped with a Sony DSC-W55 digital camera for taking photographs. Histomorphometric measurements were made using Image J software, version 1.32j (ImageJ, National Institutes of Health, Bethesda, USA).

Data analysis

We performed all statistical analyses with STATISTICA software, version 6.0 (Statistica for Windows, Statsoft Inc., Tulsa, USA). For each parametric analysis, we assessed normality of data distribution (Kolmogorov–Smirnov test) and homogeneity of variances (Barlett test). We performed natural logarithmic transformations when it was necessary to meet the assumptions of the parametric tests.

To compare testicular volume of individuals from different sites, the effect of SVL had to be removed because, in general, larger individuals have greater testicular volume (e.g., Ortega et al., 2005; Sanabria et al., 2007). Thus, we ran an ANCOVA with site group as the fixed effect, testicular volume as the dependent factor, and SVL as the covariate (McCoy et al., 2008). To test for significant differences between study sites, post-hoc comparisons within the ANCOVA were made using the Tukey's test. Before running the test, we checked the assumption of parallelism (homogeneity of slopes).

MI, testicular area, total number of seminiferous tubules, and seminiferous tubular area were compared among study sites using a one-way analysis of variance (ANOVA) combined with a Tukey's test. Number of I SG cysts, II SG cysts, I SC cysts, II SC cysts, SP cysts, SZ cysts per seminiferous tubule, and spermatozoa rank in the lumen are not independent of one another (since a cell type gives rise to another) and this can make interpretation difficult. Therefore, these data were analyzed using a multivariate analysis of variance (MANOVA) to assess if there were group differences in all the response variables considered simultaneously. After performing the MANOVA, we used uni-

variate ANOVA on each response variable to assess which variables were responsible for significant main effects. This was followed by post hoc comparisons (Tukey's tests) to test for significant differences among study sites (Quinn and Keough, 2002). In addition, we compared frequencies of irregularly shaped testes and testes with anomalies among sites using the test of difference between proportions (Martínez-González et al., 2006).

RESULTS

General measurements

The manual captures of adult males resulted in n = 28 individuals of *R. fernandezae* and n = 29 individuals of *D. sanborni*. There were no differences in scaled mass index (MI) for both species among study sites (RF: $F_{2,25}$ = 0.245, P = 0.784; DS: $F_{2,26}$ = 2.567, P = 0.096). Nevertheless, in *R. fernandezae*, the testicular volume was significantly different among site groups ($F_{2,24}$ = 10.706, P = 0.0005). AG and NW groups had significantly reduced testicular volume than the NF group (Tukey's post-hoc test: P = 0.025 and P = 0.0005, respectively). *Dendropsophus sanborni* showed no variation in testicular volume among sites ($F_{2,25}$ = 0.116, P = 0.891).

Histological evaluation

In *R. fernandezae* the seminiferous tubules/testis was significantly different among sites ($F_{2,25}$ = 3.476, P = 0.046). There were significantly fewer seminiferous tubules/testis in the AG and NW groups than in NF group (Tukey's post-hoc test: P = 0.043 and P = 0.047 respectively). Testicular area and $\log_e$ (seminiferous tubular area) were not different among sites ($F_{2,25}$ = 1.696, P = 0.204 and $F_{2,25}$ = 2.476, P = 0.104, respectively; Table 1).

Dendropsophus sanborni showed no significant differences among sites in these variables (Testicular area: $F_{2,26}$ = 0.066, P = 0.936; seminiferous tubules/testis $F_{2,26}$ = 0.828, P = 0.448; and seminiferous tubular area $F_{2,26}$ = 1.085, P = 0.353; Table 1). Numbers of germ cell cysts were significantly different among sites in both species (Table 2). In *R. fernandezae*, primary spermatogonia, primary spermatocytes, and spermatid cysts per seminiferous tubule were different among sites (Fig. 2A, Table 2). Tukey's post-hoc test showed that there were significantly fewer I SG and SP cysts per seminiferous tubule in the AG and NW groups than in NF group (I SG: P = 0.027 and P = 0.002 respectively; SP: P = 0.02 in both cases) and that there were significantly fewer I SC cysts per seminiferous tubule in the AG group than in the NF group (Tukey's post-hoc test: P = 0.008). Number of secondary spermatogonias cysts, secondary spermatocytes cysts, spermatozoa cysts, and spermatozoa rank in the lumen were not different among sites (Fig. 2A, Table 2). In *D. sanborni* the primary and secondary spermatogonia cysts per seminiferous tubule were different among sites (Fig. 2B, Table 2). There were significantly fewer I SG cysts per seminiferous tubule in the AG and NW groups than in NF group (Tukey's post-hoc test: P = 0.044 and P = 0.01 respectively), and there were significantly fewer II SG cysts per seminiferous tubule in the NW group than in the NF group (Tukey's post-hoc test: P = 0.049). Cyst number of primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa, and the spermatozoa rank in the lumen were not different among sites (Fig. 2B, Table 2).

In *R. fernandezae*, the incidence of irregularly shaped testes did not differ among groups (NF = 33.3%, NW = 63.6%, AG = 62.5%). However, in *D. sanborni*, the incidence of irregularly shaped testes increased with agricultural intensity (NF = 30.0%, NW = 50.0%, AG = 72.7%),

Table 1. Histological evaluation of testes of adult males of *Rhinella fernandezae* and *Dendropsophus sanborni* in agroecosystem (AG), natural wetland (NW), and natural forest (NF) sites in south-western Entre Ríos province, Argentina. Data are expressed as means ± SE. Means followed by different superscript letters (A or B) are significantly different from each other (p < 0.05, ANOVA test combined with Tukey's test).

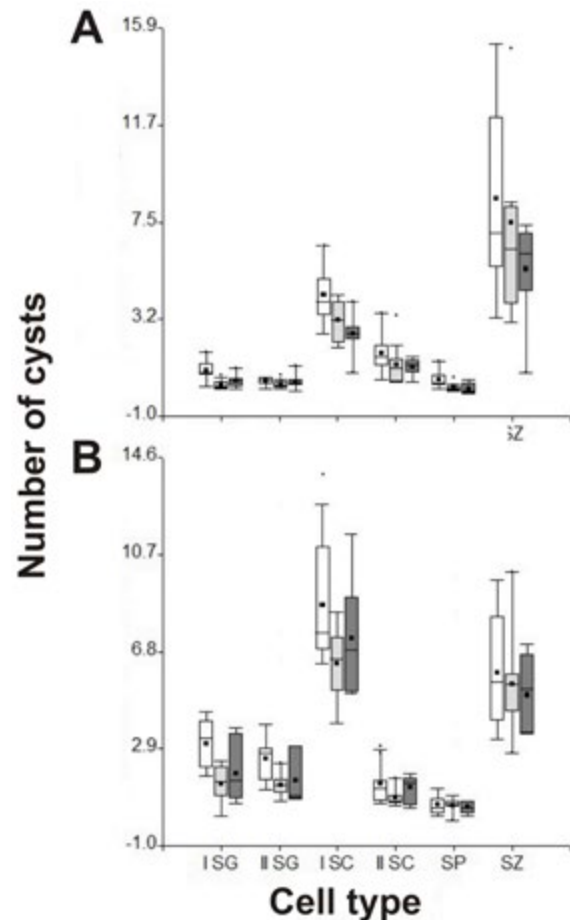|	Site	Testicular area mm²	Number of seminiferous tubules/testis	* Seminiferous tubular area mm²
R. fernandezae	AG *n = 8*	0.946 ± 0.254	52.775 [A] ± 4.455	0.015 ± 0.003
	NW *n = 11*	1.035 ± 0.172	53.545 [A] ± 4.045	0.020 ± 0.003
	NF *n = 9*	1.431 ± 0.162	65.756 [B] ± 2.762	0.023 ± 0.002
D. sanborni	AG *n = 11*	0.308 ± 0.027	22.564 ± 1.880	0.016 ± 0.002
	NW *n = 8*	0.304 ± 0.025	26.975 ± 1.919	0.012 ± 0.001
	NF *n = 10*	0.319 ± 0.034	24.460 ± 3.018	0.015 ± 0.002

* Log transformed variable in *Rhinella fernandezae* before performing the parametric significance test. However, for clarity, non-transformed data are presented.

Table 2. Results of MANOVA for overall effects of habitat types on numbers of cysts and ANOVAs for each response variable. (I SG) Primary spermatogonia, (II SG) secondary spermatogonia, (I SC) primary spermatocytes, (II SC) secondary spermatocytes, (SP) spermatids, (SZ) spermatozoa, (SZ in lumen) spermatozoa rank in the lumen.

|	F	d.f.	P
R. fernandezae			
MANOVA	2.105	14,38	0.035
ANOVA s			
I SG	8.372	2,25	0.002
II SG	1.098	2,25	0.349
I SC	5.722	2,25	0.009
II SC	1.465	2,25	0.250
SP	5.516	2,25	0.010
SZ	0.835	2,25	0.446
SZ in lumen	0.485	2,25	0.622
D. sanborni			
MANOVA	2.025	14,40	0.041
ANOVA s			
I SG	5.754	2,26	0.009
II SG	3.713	2,26	0.038
I SC	2.371	2,26	0.113
II SC	1.825	2,26	0.181
SP	0.197	2,26	0.823
SZ	0.464	2,26	0.634
SZ in lumen	3.223	2,26	0.056

Fig. 2. Histological evaluation of germ cell cysts in the testes of adult males of *Rhinella fernandezae* (A) and *Dendropsophus sanborni* (B) collected from the agroecosystem (dark gray), natural wetland (light gray), and natural forest (white) sites, in south-western Entre Ríos province, Argentina. (I SG) Primary spermatogonia, (II SG) secondary spermatogonia, (I SC) primary spermatocytes, (II SC) secondary spermatocytes, (SP) spermatids, (SZ) spermatozoa. Box-plot diagram shows the quartiles, median (band inside the box), mean (square inside the box), minimum and maximum (ends of the whiskers), and presence of extreme values (black dots).

with statistically significant differences being found only between AG and NF (Table 3). The analysis of testicular anomalies revealed cases of poorly developed seminiferous tubules, pigmented cells, testicular oocytes, and lack of elongated spermatids in both species. In *R. fernandezae* the incidence of poorly developed tubules (Fig. 3A, B) increased with agricultural intensity (NF = 0.0%, NW = 27.3%, AG = 50.0%). This anomaly was significantly increased in AG and NW groups compared to the NF group, but no difference was found between AG and NW groups. The presence of pigmented cells distributed in the testicular interstitium (Fig. 3C) was greater in AG (50%) and NW groups (63.6%) than in NF group (22.2%), and the incidence of this anomaly was significantly increased in NW compared to NF. Only one individual with testicular oocytes was found; it belonged to the NW group (Fig. 3D). Additionally, only one individual with vitellogenic Bidder´s organ was recorded. The specimen was collected in the NW site. In both cases, because of the small number of records, no statistical analyses were performed. The presence of specimens with lack of elongated spermatids increased with agricultural intensity (NF =

0.0%, NW = 18.2%, AG = 62.5%). AG group had a significantly higher number of individuals with no elongated spermatids than NW and NF groups; however, no differences were found between NW and NF groups (Table 3). On the other hand, in *D. sanborni* the incidence of poorly developed tubules (Fig. 3E, F) increased with agricultural intensity (NF = 0.0%, NW = 25.0%, AG = 27.3%). This anomaly was significantly increased in AG group compared to the NF group; however, no difference was found either between AG and NW groups or between NW and NF groups. The presence of pigmented cells distributed in the testicular interstitium (Fig. 3G) was similar

Table 3. Summary of proportion tests for variables analyzed by means its frequency in *Rhinella fernandezae* and *Dendropsophus sanborni*. (AG) Agroecosystem, (NW) natural wetland, and (NF) natural forest.

Variable	Comparison	R. fernandezae		D. sanborni	
		Z	P	Z	P
Irregularly shaped testes	NF – NW	1.417	0.078	0.875	0.192
	NF – AG	1.255	0.104	2.163	0.015
	NW - AG	0.051	0.480	1.024	0.154
Poorly developed tubules	NF – NW	2.031	0.021	1.633	0.052
	NF – AG	2.828	0.002	2.031	0.021
	NW - AG	1.024	0.154	0.112	0.456
Pigmented cells	NF – NW	2.064	0.020	0.108	0.456
	NF – AG	1.237	0.108	0.171	0.433
	NW - AG	0.596	0.274	0.051	0.480
Testicular oocytes	NF – NW	_	_	0.166	0.433
	NF – AG	_	_	0.071	0.472
	NW - AG	_	_	0.234	0.409
Lack of elongated spermatids	NF – NW	1.564	0.059	_	_
	NF – AG	3.652	0.0002	_	_
	NW - AG	2.142	0.016	_	_
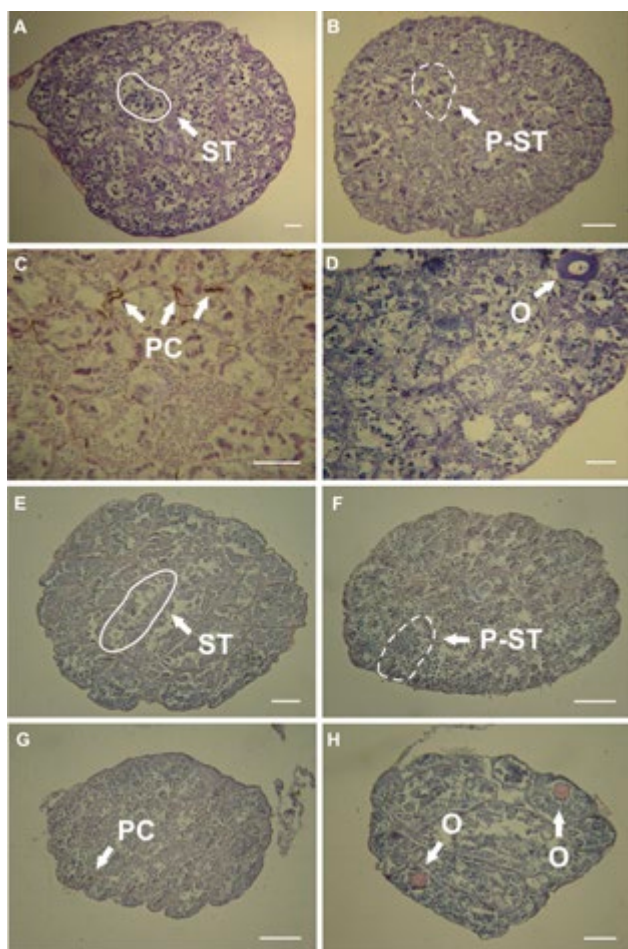
Fig. 3. Histological sections of anuran testes. *Rhinella fernandezae*: (*A*) testis with normal seminiferous tubules, (*B*) testis with poorly developed tubules, (*C*) testis showing pigmented cells, (*D*) testis with a single oocyte. *Dendropsophus sanborni*: (*E*) testis with normal seminiferous tubules, (*F*) testis with poorly developed tubules, (G) testis showing pigmented cells, (H) testis with multiple oocytes. (ST, solid line) normal seminiferous tubules, (P-ST, dotted line) poorly developed seminiferous tubules, (PC) pigmented cells, (O) oocytes. In all images, individuals from the natural wetland are shown. Bar represents 100 μm.

in the three study sites (NF = 40.0%, NW = 37.5%, AG = 36.4%). Three individuals with testicular oocytes were recorded, one in each environment (NF = 10.0%, NW = 12.5%, AG = 9.1%; Fig. 3H). As a result, the observed proportions were not different among sites. We found only one individual with lack of elongated spermatids, which belonged to the NW group. Because we recorded a small number of cases, we did not perform a statistical analysis on this testicular anomaly (Table 3).

DISCUSSION

This is the first work exploring the association of abnormal gonadal form and function with agricultural activities in wild *R. fernandezae* and *D. sanborni*. Specifically, testicular volume and shape, development of seminiferous tubules, and presence of several spermatogenic stages were affected with increasing agricultural intensity.

Gonadal abnormalities, such as those reported here, are likely to reduce the reproductive success of affected individuals and could help explain the declines in amphibian populations reported in environments exposed to pesticides (Davidson et al., 2002; Davidson, 2004; McCoy et al., 2008).

General measurements

The scaled mass index (MI) for adult males of *R. fernandezae* and *D. sanborni* did not differ significantly among the environments studied, indicating that the three populations of each species would not present differences in their nutritional state or energy capital accumulated in the body as a result of feeding (Peig and Green, 2009). On the other hand, we observed a decrease in testis volume of *R. fernandezae* in environments with greater exposure to agricultural activities (AG and NW). These observations might be influenced by the amplitude of the time window in which the *R. fernandezae* specimens were collected (December 2007 to April 2008). The time window might have influenced the testis volume measured if individuals had been captured from each site at different times within that period. However, the species were collected simultaneously from the three sites, and we did not expect differences in the mean values of any reproductive parameter among sites. Additionally, Martori et al. (2005) investigated possible changes in testicular volume of *R. fernandezae* between September 1999 and April 2000 in a field in Córdoba province, Argentina. As a result, the authors found no significant differences in that variable between those months, and they interpreted that testes are potentially active and are ready for reproduction throughout the spring-summer period.

An alternative explanation is provided by Tavera-Mendoza et al. (2002). They reported that exposure to 21 μg/L atrazine for 48 h during development resulted in decreased testicular volume (57%) and decreased numbers of primary germ cells among male *Xenopus laevis* (Amphibia, Pipidae) tadpoles (70%). Accordingly, in the present study, individuals from AG may have been exposed to various pesticides at some time during their development, since the agroecosystem was sprayed with glyphosate, 2-4 D, cypermethrin and endosulfan, and the main implementation period coincides with the breeding season of amphibians (Lorenzatti et al., 2004; Peltzer and Lajmanovich, 2007). During the time that these chemicals are used, rainfall can cause intense runoff, carrying agrochemicals to other nearby water bodies (Peltzer et al., 2008), such as that located in NW; this fact might explain the observation of decreased testicular volume both in AG and NW anurans.

On the other hand, *D. sanborni* showed no variation in testicular volume among sites. One explanation for the apparently greater vulnerability of *R. fernandezae* would be associated with terrestrial habitat use (Sanchez and Busch, 2008; Sanchez et al., 2010). Both species breed in ponds, flooded areas, temporary swamps, and the periphery of permanent lakes, where tadpoles live (Sanchez et al., 2009, 2010), but newly metamorphosed *R. fernandezae* individuals build caves in the moist soil of the periphery of the water body (Gallardo, 1969). Consequently, despite having left the aquatic environment, they are potentially exposed to chemicals that may be in the water, because the sediment that forms the cave is embedded in water. In addition, unlike the specimens of *D. sanborni*, which can be frequently observed in the vegetation at a height of up to 1.5 m (Toledo et al., 2003; Conte and Machado, 2005), *R. fernandezae* would be more directly exposed to pesticide sprays and the related surface runoff due to its burrowing-terrestrial habits.

Histological evaluation

Testes have many different vital structures (Ogielska and Bartmańska, 2009) involved with their main function: spermatozoa production. Seminiferous tubules are of primary importance because they hold and release the sperm that is necessary to fertilize eggs (Dutta and Meijer, 2003). There are no data available on the gametogenic cycles of *R. fernandezae* and *D. sanborni*; however, studies in related species of the Bufonidae and Hylidae families in the region (e.g., Bufonidae: *Rhinella arenarum, R. major*; Hylidae: *Dendropsophus minutus, Hypsiboas riojanus, Lysapsus limellum*) indicate the presence of potentially uninterrupted gonadal activity and continuous annual spermatogenesis (Cei, 1949; Santos and Oliveira, 2007; Ferreira et al., 2008). These species are characterized by keeping constant values of various morphometric parameters of the testis (e.g., testicular weight, area and diameter of the seminiferous tubules) and almost constant presence of all spermatogenic stages throughout the year (Santos and Oliveira, 2007; Ferreira et al., 2008). Nevertheless, Basso (1990) suggests that females of *D. sanborni* have a seasonal cycle, with breeding taking place only in spring and summer.

In our study, in the anuran testes from the NF site, the overall structure showed a regular pattern of seminiferous tubules surrounded by a layer of interstitial tissue. By contrast, the overall structure of the AG (*R. fernandezae* = 50.0%; *D. sanborni* = 27.3%) and NW testes (*R. fernandezae* = 27.3%; *D. sanborni* = 25.0%) was disrupted and the shape of the tubules was not well defined. The potential exposure of anuran individuals to agrochemi-

cals could induce damage to the layers of interstitial tissue surrounding the seminiferous tubules, making them less visible (Dutta and Meijer, 2003). This could explain the lower number of seminiferous tubules recorded in both AG and NW *R. fernandezae* groups. The structure of the seminiferous tubules might also be severely disrupted. Similar results were found by Hayes et al. (2003) in anurans exposed to Atrazine (field and laboratory experiments), by Dutta and Meijer (2003) in bluegills exposed to diazinon (laboratory experiences), and by Al-Jahdali and Bin Bisher (2007) in rats treated with Sumithion insecticide (laboratory experiments). In addition, the number of cysts of germ cells inside the seminiferous tubules was generally decreased in *R. fernandezae* and *D. sanborni* collected from environments with some level of influence of agricultural activities (AG and NW). Accordingly, Sower et al. (2000) reported a lack of development in the seminiferous tubules and reduced numbers of spermatogonias in individuals of *Lithobates clamitans* and *L. catesbeianus* (Amphibia, Ranidae), and associated these results with endocrine disrupting chemicals. Those results reinforce our observations that agricultural pesticides might be driving the abnormalities observed in gonadal form and function.

In addition, other testicular anomalies were recorded, such as pigmented cells and testicular oocytes. Pigmented cells increased in AG and NW *R. fernandezae* toads, whereas they were recorded in similar proportions in *D. sanborni* in the three sites. Many studies suggest that the general function of these cells is focalization of destruction, detoxification, or recycling of endogenous and exogenous materials (Ellis, 1980; Herraez and Zapata, 1986). There are several examples of the increase of pigmented cells in gonads, kidney or spleen in relation to pollutants, and they have been promoted as biomarkers of environmental exposure to pollutant chemicals both in frogs and fishes (Wolke et al., 1995; Couillard and Hodson, 1996; Patiño et al., 2006; Kloas et al., 2009). In addition, a recent study shows that actin filaments in pigmented cells of *X. laevis* are affected by Roundup formulations (glyphosate-based herbicide), and that, consequently, intracellular transport of pigment and aggregation of melanin to the cell centre are inhibited, rendering the cells a dark color (Hedberg and Wallin, 2010). Accordingly, pigmented cells may be present in higher numbers in environments with greater agricultural exposure, or may not be more numerous but may have become more conspicuous because of damage in the transport mechanism of melanin. On the other hand, only one *R. fernandezae* with testicular oocytes was found and it was in NW group, whereas similar proportions of this anomaly were recorded for *D. sanborni* in the three environments.

There has been some still unresolved debate in the literature regarding the relevance of testicular oocytes in amphibians and whether there may be a background rate resulting from natural processes in development, at least in juvenile individuals (e.g., Jooste et al., 2005; Storrs-Méndez and Semlitsch, 2010), or may be induced by exposure to pesticides or other estrogenic endocrine disrupting compounds (e.g., Hayes et al., 2003; McDaniel et al., 2008; Tompsett et al., 2012, 2013; Trachantong et al., 2013). With regard to vitellogenic Bidder's organ, only one individual of *R. fernandezae* with this condition was recorded and it was in NW site. Vitellogenic oocytes within Bidder's organs in *R. marina* (Amphibia, Bufonidae) have been recently associated with agricultural sites in Florida, United States (McCoy et al., 2008). These authors suggest that the testes of these toads may not function normally to suppress Bidder's organ oogenesis (McCoy et al., 2008).

In general, pathological conditions in the testes, such as those reported here, may be associated with nutritional or environmental factors that contribute to these abnormalities (Siddiqui et al., 2005). However, in this case, the nutritional status of the amphibian populations (analyzed by means of the scaled mass index) of each studied species did not differ among sites (Peig and Green, 2009). However, the frequency of testicular anomalies was found to increase, in general, with agricultural activity and was very low or absent in the site with zero agriculture, suggesting that these anomalies would not occur at a high frequency in areas not affected by agriculture (McCoy et al., 2008).

Evidence suggests an increased vulnerability of *R. fernandezae* compared with *D. sanborni* (based on greater evidence of affected morphology and gonadal histoarchitecture). This agrees with the results reported by Lajmanovich et al. (2010), who indicate this bufonid as one of the species at highest ecological risk due to conversion of native environments to soybean crops.

Conclusions

The reduced presence of germ cells and the high numbers of testicular anomalies in *R. fernandezae* and *D. sanborni* from environments with great agricultural exposure indicate an overall negative effect of agricultural land use upon testes (both abnormal testicular development and function) in these wild anurans from the southwestern Entre Ríos province, Argentina. These animals are exposed to pesticides in soybean crops (Jergentz et al., 2005). Previous works have detected residues of organochlorine pesticides (e.g., chlordane, heptachlor, and endosulfan) in tissues of native anurans from the region (Laj-

manovich et al., 2002), and the concentrations recorded were strongly influenced by the use of intensively cropped lands (Lajmanovich et al., 2005). Therefore, the establishment of buffer zones around pristine natural forests could help reduce runoff from surrounding agricultural fields, increasing the conservation value of such areas. Several factors should be taken into account in the design of buffer zones, such as type of vegetation in the buffer, the slope, infiltration rate and soil texture (Hawes and Smith, 2005). Structurally diverse vegetation (combined presence of trees, shrubs and grasses) is much more effective at capturing a wide range of pollutants than a buffer with only trees or grass. In this sense, native plants are able to acquit better benefit than alien ones (Essien, 2012). In addition, the speed of water flow increases with increasing slope. Therefore, the steeper the land within the buffer, the wider it needs to be to have time to slow the flow of water and absorb the pollutants within it (Wenger, 1999). On the other hand, it is important to investigate the roles of agricultural activities and the effectiveness of regulations on agrochemicals, particularly in Latin American countries, in causing ecological risk for amphibians as native ecosystems are converted to soybean cultivations (Lajmanovich et al., 2010). Although the sample size is low, the extensive global use of pesticides and conversion of land use into agriculture, affecting amphibians, stresses the importance of the present findings. The data obtained in this study can help assess the ecological risk of land use for agriculture to Argentine anurans.

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