Herpetological Conservation and Biology 17(3):573–581.
Submitted: 26 January 2022; Accepted: 7 November 2022; Published: 16 December 2022.

MORPHOLOGICAL ABNORMALITIES IN AMPHIBIANS IN AND ADJACENT TO ESTEROS DE FARRAPOS E ISLAS DEL RÍO URUGUAY NATIONAL PARK, URUGUAY

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***Abstract*.—Morphological abnormalities in amphibians, defined as a lack of symmetry or an imbalance in structure, color, or other characteristics, are usually caused by genetic, epigenetic, or traumatic factors. Here, we report the first study of morphological abnormalities in Uruguayan amphibians. We evaluated 378 post-metamorphic anurans representing 11 species, in and around the Esteros de Farrapos e Islas del Río Uruguay National Park (EFIRU) and surrounding areas, Uruguay. We observed morphological abnormalities in 8.8% of the assemblage, which was significantly higher than the baseline for comparison (5%) suggested in the literature. Based on the observed prevalence, we classified the studied area as an amphibian abnormality hotspot as defined in the literature. The prevalence of abnormalities varied in relation to their microhabitat association: ground-dwelling species (13.2%) were more affected than aquatic (5.4%) and arboreal (4.3%) species. We consider that the high frequency of morphological abnormalities in the amphibians within and adjacent to a protected area should be a wake-up call to the environmental authorities. Although the causes are unknown, researchers should explore the evidence that suggests a connection with agricultural intensification in the area, particularly the presence of agrochemicals in the environment.**

Key Words.—agroecosystems; amphibian disease; anurans; conservation; *Leptodactylus latinasus*; malformations

INTRODUCTION

Amphibians are one of the most threatened vertebrates, with more than 40% of their species under some degree of conservation risk, according to the global red lists of the International Union for Conservation of Nature (IUCN; Hoffmann et al. 2010). We are therefore facing an evident global decline in amphibians, which has been associated with multiple causes such as habitat loss, climate change, ultraviolet radiation, environmental pollutants, harvest, diseases, and alien species invasions (Blaustein and Kiesecker 2002; Wren et al. 2015). These factors have had different effects on amphibian populations, evidenced by alterations in their morphological attributes, such as body condition or abnormalities, and/or on their habitat use and geographical distributions (Collins et al. 2009).

Abnormalities are deviations in the range of morphological variation of an individual that appear as a lack of symmetry or an imbalance in structure, color, or other characters (Lannoo 2008; Johnson et al. 2010). Among factors proposed as the cause of abnormalities in amphibians are damages produced by predation, parasitic infection, UV-B radiation, and chemical contaminants, as well as interactions among some of these factors (Kiesecker 2002; Johnson et al. 2002, 2010; Taylor et al. 2005; Johnson and Bowerman 2010; Haas et al. 2018). Carrying morphological abnormalities has significant costs in performance and survival (Goodman and Johnson 2011). For instance, bilateral limb symmetry is essential for locomotion in vertebrates. The evidence suggests that deviations from bilaterality, no matter how small, can strongly affect individual performance (Martín and López 2001). Many researchers have observed that abnormal amphibians have less endurance and less jumping and swimming ability than normal individuals (e.g., Goodman and Johnson 2011; Zamora-Camacho and Aragón 2019). In addition, abnormal individuals can have reduced foraging time and efficiency (Goodman and Johnson 2011; Tolledo et al. 2014), which would limit their access to certain prey and specific resources. Despite this evidence, the effects of morphological abnormalities on individual growth have been scarcely explored. Understanding whether the possession of a certain abnormality affects individual body size is crucial, due to the numerous ecological processes and mechanisms dependent on this trait (Brown et al. 2004).

There has been a chronic difficulty in linking high rates of amphibian morphological abnormalities observed in the field to local causes (Johnson et al. 2010). This could be due to the complexity of natural ecosystems, and the scarce knowledge of the dynamics of the potential causes. Moreover, comparing studies is complex because of the lack of standardization. There are differences in ontogenic stages, abnormalities, and assemblages to consider, as well as differences in the type of ecosystems sampled (e.g., ponds, streams, and wetlands) and in baseline information availability (Lunde and Johnson 2012). Nevertheless, there is a consensus among researchers about the relationship between the high incidence of amphibians malformations with the presence of chemical pollutants in the environment, especially agrochemicals applied for agriculture (e.g., Taylor et al. 2005; Agostini et al. 2013; Haas et al. 2018).

The Uruguayan categorization by IUCN criteria assigns a conservation risk to various species that are associated with the development of agriculture (Carreira and Maneyro 2015). The recent expansion of intensive agriculture in the east coast region of the Uruguay River (i.e., soybean, wheat, sorghum, corn, and eucalyptus afforestation) could be affecting native ecosystems (Baldi and Paruelo 2008). Surveying morphological abnormalities is a tool that could strongly contribute to understanding of the status of amphibians in this region.

Our objective was to explore the occurrence of external morphological abnormalities in the anuran assemblage in and adjacent to Esteros de Farrapos e Islas del Río Uruguay (EFIRU) National Park, Uruguay (Sistema Nacional de Áreas Protegidas [SNAP], a national protected areas system). In particular, we tested two hypotheses. First, we hypothesized that abnormalities would differentially affect arboreal, aquatic, and terrestrial species. We predicted that arboreal and aquatic species would have a lower abnormality frequency than ground-dwelling species. Individuals with morphological abnormalities, especially in their limbs, would likely have mobility restrictions. This would affect their ability to forage, as well as their chance of escaping from predators. Morphological abnormalities would strongly reduce survival in certain microhabitats that require fine movements in locomotion involving specialized limb structures, such as adhesive patches for arboreal species and interdigital membranes for aquatic species. Therefore, we expected that arboreal and aquatic species would have lower survival rates than ground-dwelling species, resulting in a lower abnormality frequencies for the arboreal and aquatic species. Second, we hypothesized that abnormalities would affect body size. We predicted that abnormal individuals would reach smaller body sizes than normal ones. The aforementioned foraging restriction would likely negatively affect the access of individuals with abnormalities to certain resources (i.e., reducing the quantity and quality of captured prey). This lower energy acquisition would reduce their growth rate, such that abnormal individuals would reach smaller body sizes than normal ones.

Materials and Methods

Study area.—We sampled amphibians at EFIRU National Park and its surrounding area (i.e., within about 5 km from the border of the Park), in the Río Negro Department, on the eastern side of the Uruguay River, Uruguay. This lowland area (15 m elevation) is the longest longitudinal riverine wetland in Uruguay (Gazzano and Achkar 2014), with fluvial islands located on the margin of the Uruguay River (Fig. 1). A riparian forest occurs on the islands and on the borders of the river and creeks. This Park was designated as a Ramsar Site in 2004, and a national protected area (SNAP program) in 2008 (Ministerio de Vivienda Ordenamiento Territorial y Medio Aambiente [MVOTMA] 2018). This protected area lacks peripheral buffer zones and is surrounded by private land and productive farms.

In 2016, the SNAP program incorporated a new protected area adjacent to EFIRU, Esteros y Algarrobales del Río Uruguay (EARU; Fig. 1). In addition to the wetlands, EARU also includes a small fraction of the typical, relict Savanna Woodlands (i.e., *Algarrobal*), composed mainly of *Ñandubay* (*Prosopis affinis*) and *Algarrobo Negro* (*P. nigra*), and patches with alkaline soils (i.e., *blanqueal*; Brussa and Grela 2007). The EFIRU and EARU together cover a surface of 18,037 ha, which amounts to 5% of the total national protected areas in Uruguay (MVOTMA 2018). During the last two decades, the region where these protected areas are located has undergone the greatest agricultural intensification in Uruguay (Soutullo et al. 2020). Soybean monoculture and eucalyptus (*Eucalyptus* sp.) afforestation replaced an extensive amount of the natural Grasslands and Savannah Woodlands that were previously used for cattle and dairy farms (Gazzano and Achkar 2014). This novel land use changed the landscape by direct loss of native vegetation and environmental homogenization (Medan et al. 2011).

Native amphibian assemblages in these protected areas include species widely distributed in Uruguay, along with species restricted to the northern Uruguayan coast of the Uruguay River (Arrieta et al. 2013). For several of those amphibian species, this area is the southern distribution limit in Uruguay (Laufer et al. 2021). These amphibians inhabit medium-sized lentic

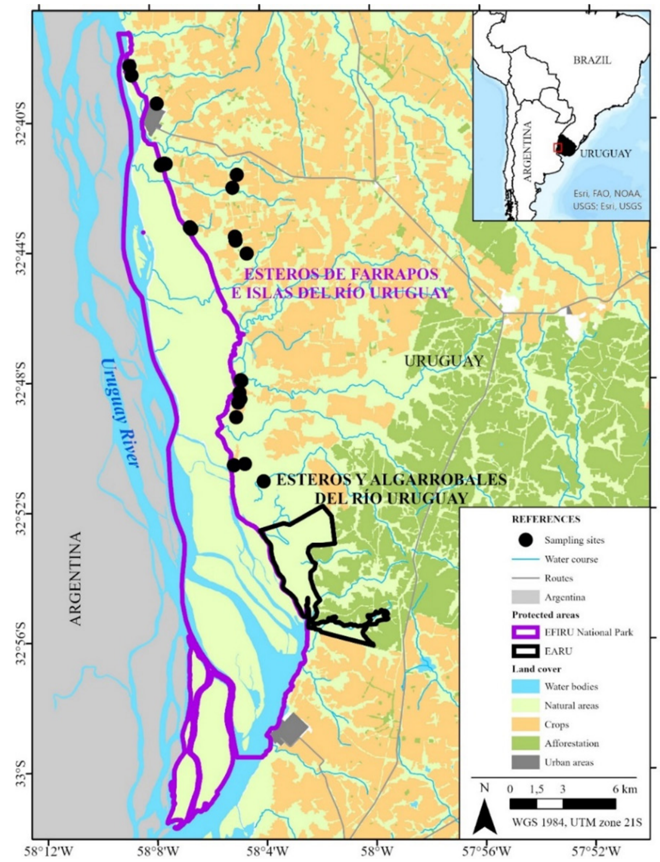

Figure 1. Sampled sites for amphibian morphological abnormalities, all located east of the Uruguay River, Uruguay. Protected areas and land usages are indicated in the map. Acronyms and designations are EFIRU = Esteros de Farrapos e Islas del Río Uruguay (national park); EARU = Esteros y Algarrobales del Río Uruguay; Water bodies = river, creeks, and lagoons; Natural areas = native grasslands, forest, and wetlands; Crops = soybean, corn, and sorghum; Afforestation = eucalyptus (*Eucalyptus* sp.) plantations; and Urban areas = towns and cities (Sistema de información territorial. 2015. Mapa de cobertura del suelo. Available from https://sit.mvotma.gub.uy/js/cobertura [Accessed 24 October 2021]).

water bodies that naturally occur in the ecotone between wetlands and Grasslands with Savannah Woodlands (Arrieta et al. 2013).

Fieldwork.—We conducted three field campaigns in February, May, and November of 2018, sampling for morphological abnormalities in anurans. We selected 23 small permanent or semi-permanent water bodies, avoiding the major watercourses associated with the river (Fig. 1) where predatory fish remain and likely limit the abundance of amphibians (Hecnar and M'Closkey 1997; Semlitsch et al. 2015). At night (from 2000 to 0000), experts conducted walking surveys on the perimeter of each water body for 20 to 30 min, manually collecting post-metamorphic anurans.

We euthanized the collected individuals with an overdose of eugenol. The euthanasia of the specimens and the transfer to the laboratory allowed us to better study the abnormalities in detail, especially in those species of small body size. We measured snout-vent length (SVL) of amphibians to the nearest 0.01 mm in the laboratory using a digital caliper and examined them under a magnifying glass to determine the presence of external morphological abnormalities. We classified the observed abnormalities following Meteyer (2000) for the limbs, Johnson et al. (2002) for the cephalic and axial region, and Agostini et al. (2013) for the eyes. We deposited all the collected specimens in the herpetological collection of the Museo Nacional de Historia Natural (MNHN), Montevideo, Uruguay as voucher specimens for the region. Finally, we calculated the frequency of abnormalities by dividing the number of individuals with any abnormality by the total sample size.

Data analysis.—We evaluated whether the observed frequency of abnormalities was higher than the reference value of 5%, using the Two-way Chi-square test (Rayat 2018). Lunde and Johnson (2012) suggested that 5% should be the baseline value for morphological abnormalities and emphasized the need for adequate sample sizes (i.e., 100 individuals per species/group/

developmental stage) to define real prevalences. The ability of the Chi-square test to detect if an observed frequency is > 5% increases with sample size and with the proportion of abnormal individuals. With a higher prevalence (> 10%), however, the need for a large sample size drops dramatically (Lunde and Johnson 2012). Using the Chi-square test, we determined whether the whole assemblage and the five most frequent species (those with > 40 individuals collected in the three field campaigns) showed a prevalence of abnormalities significantly > 5% (Stocum 2000). Then we explored the differences in abnormalities related to species with different microhabitat use during the post-metamorphic phase: aquatic (surface and water column of ponds), arboreal (plants, shrubs, and trees surrounding the ponds) and ground-dwelling (grasslands and depressions in the ground surrounding the ponds). We classified the species as follows: (1) arboreal (three species), Montevideo Treefrog (*Boana pulchella*), Sanborn's Treefrog (*Dendropsophus sanborni*), and *Scinax granulatus* (no common name); (2) aquatic (one species), Lesser Swimming Frog (*Pseudis minuta*); and (3) ground-dwelling (seven species), Dumeril's Striped Frog (*Leptodactylus gracilis*), Oven Frog (*L. latinasus*), Wrestler Frog (*L. luctator*), Weeping Frog (*Physalaemus biligonigerus*), Hensel's Swamp Frog (*Pseudopaludicola falcipes*), Dorbigny's Toad (*Rhinella dorbignyi*), and Cururu Toad (*R. diptycha*). We tested whether these microhabitat association groups differed in the frequency of abnormalities, using a null hypothesis for a Two-way Chi-square test with no differences between groups (Rayat 2018). We used this test because samples were random and of sufficient size (i.e., > 5) in each group. Likewise, we explored whether the prevalence of abnormalities observed in each group was significantly higher than the 5% expected baseline value using the Two-way Chi-squared test.

To assess the individual cost of abnormalities, we tested whether the presence of abnormalities was related to body size (SVL) using a Generalized Linear Model (GLM) for a binomial distribution. We included species identity (and its interaction with body size) in the model as a factor (Zuur et al. 2007). Finally, we performed a residual analysis to confirm the homoscedasticity and normality assumptions of the residuals (Zuur et al. 2007). We analyzed all data in R open software, using α = 0.05 for significance (R Development Core Team 2020).

RESULTS

We collected 378 post-metamorphic native anurans, belonging to 11 species (Table 1). We observed morphological abnormalities in 8.8% of the collected anurans. This prevalence was significantly greater than the baseline value of 5% ($X^2 = 4.56$, df = 1, $P = 0.033$). We detected seven types of abnormalities: brachydactyly, ectromelia, polyphalangy, amelia, distally complete but malformed limbs, mandibular dysplasia, and malformed eye (Table 1). Most of the morphological abnormalities occurred in the limbs. Brachydactyly was the most frequent (5.4%), followed

TABLE 1. Anuran abnormalities observed in and adjacent to Esteros de Farrapos e Islas del Río Uruguay National Park, Uruguay. Each row shows the observed frequency (%) of each type of abnormality, with the number of individuals with the abnormality in parentheses, for each species and the complete assemblage. The bottom rows summarize the total number of individuals analyzed, the total number of individuals with abnormalities, and frequency by species and the entire assemblage. Species abbreviations, separated into microhabitat association groups, are Arboreal (AR): *B. pul*: Montevideo Treefrog (*Boana pulchella*); *D. san*: Sanborn's Treefrog (*Dendropsophus sanborni*); *S. gra*: *Scinax granulatus* (no common name). Aquatic (AQ): *P. min*: Lesser Swimming Frog (*Pseudis minuta*); Ground-dwelling (GD): *L. gra*: Dumeril's Striped Frog (*Leptodactylus gracilis*); *L. lat*: Oven Frog (*L. latinasus*); *L. luc*: Wrestler Frog (*L. luctator*); *B. bil*: Weeping Frog (*Physalaemus biligonigerus*); *P. fal*: Hensel's Swamp Frog (*Pseudopaludicola falcipes*); *R. dor*: Dorbigny's Toad (*Rhinella dorbignyi*); *R. dip*: Cururu Toad (*Rhinella diptycha*). A dash (−) means no abnormality was detected.

	B. pul	*D. san*	*S. gra*	*P. min*	*L. gra*	*L. lat*	*L. luc*	*P. bil*	*P. fal*	*R. dor*	*R. dip*	
abnormality	AR	AR	AR	AQ	GD	GD	GD	GD	GD	GD	GD	Assemblage
Brachydactyly	4.5 (4)	–	–	3.6 (4)	–	9.0 (7)	6.5 (3)	–	6.8 (3)	–	–	5.4 (21)
Ectromelia	–	–	–	0.9 (1)	–	3.8 (3)	–	–	4.5 (2)	–	–	1.6 (6)
Polyphalangy	–	–	–	–	–	1.3 (1)	–	–	–	–	–	0.3 (1)
Amelia	–	–	–	–	–	–	2.2 (1)	–	–	–	–	0.3 (1)
Distally complete but malformed limbs	–	–	–	0.9 (1)	–	–	4.3 (2)	–	–	–	–	0.8 (3)
Mandibular dysplasia	–	–	–	–	–	1.3 (1)	–	–	–	–	–	0.3 (1)
Malformed eye	–	–	–	–	–	–	–	–	–	11.1 (1)	–	0.3 (1)
Total sampled	88	2	3	112	1	78	46	1	44	9	3	387
Total individuals with abnormalities	4	–	–	6	–	12	6	–	5	1	–	34
Frequency of abnormalities (%)	4.5	–	–	5.4	–	15.4	13.0	–	11.4	11.1	–	8.8

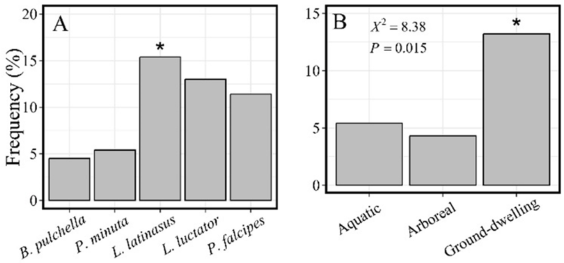

Figure 2. Frequencies of abnormalities of (A) the most frequent anuran species, and (B) the trait groups of the complete assemblage (aquatic, arboreal and ground-dwelling), in Esteros de Farrapos e Islas del Río Uruguay National Park and its surroundings. Each species or trait group (A, B) was tested (Chi-square) for differences with a baseline 5%; significant differences are marked with an asterisk (*). In B, differences between the groups were also tested by a Chi-square test, and the statistic and P value are provided.

by ectromelia (1.6%). Frequencies of the other types of abnormalities were lower than 1% (Table 1). In all cases, a single morphological abnormality was observed per individual.

Among the five most frequent species (n > 40), *L. latinasus*, *L. luctator*, and *P. falcipes* showed the highest prevalence of abnormalities, over 11% (Table 1; Fig. 2). The prevalence of abnormalities for *L. latinasus* was significantly higher than the baseline ($X^2 = 4.46$, df = 1, $P = 0.035$), whereas prevalence did not differ significantly from the baseline for *L. luctator* ($X^2 = 2.19$, df = 1, $P = 0.139$) and *P. falcipes* ($X^2 = 0.60$, df = 1, $P = 0.439$). Likewise, the frequencies of abnormalities for the other two of these five species, *B. pulchella* and *P. minuta*, also did not differ significantly from the baseline ($X^2 = 0.00$, df = 1, $P = 1.000$ for both species; Table 1; Fig. 2).

We observed differences in the prevalence of abnormalities between microhabitat association groups ($X^2 = 8.38$, df = 2, $P = 0.015$, Fig. 2). The observed prevalence in aquatic (5.4%; $X^2 = 0.00$, df = 1, $P = 1.000$) and arboreal (4.3%; $X^2 = 0.12$, df = 1, $P = 0.733$) groups did not differ from the 5% baseline. The ground-dwelling group, however, had a significantly higher prevalence than the baseline (13.2%; $X^2 = 7.50$, df = 1, $P < 0.006$). The GLM binomial model for the probability of occurrence of abnormalities in relation to SVL was not significant (Residual Deviance, *ResDev* = 221.75, df = 366, $P = 0.550$). Species identity (*ResDev* = 213.08, df = 362, $P = 0.070$) and its interaction with SVL (*ResDev* = 203.93, df = 358, $P = 0.057$) also were not significant.

Discussion

Our results are the first evidence of morphological abnormalities in Uruguayan native amphibians. Evaluations of abnormalities were previously conducted in the Pampas region, in Argentina (e.g., Peltzer et al. 2011; Agostini et al. 2013) and in southern Brazil (e.g., Ascoli-Morrete et al. 2019). We found elevated frequencies of abnormalities for the complete anuran assemblage (8.8%) and for the ground-dwelling species (13.2%), in the EFIRU National Park and its surrounding area. Although the design and the way of reporting the prevalences differed among the above authors, we can make some comparisons with these previous studies. In our case we found a higher prevalence of abnormalities than those found by Ascoli-Morrete and collaborators (2019) in Rio Grande do Sul, Brazil (5.38% for the entire ensemble). By contrast, the prevalences of morphological abnormalities reported at the species level by Agostini and collaborators (2013) in the Province of Buenos Aires, Argentina, were higher (i.e., 37.1% in *R. dorbignyi* and 28.1% in *L. luctator*) than those we observed. Based on the prevalences we observed, the studied site can be classified as an amphibian abnormality hotspot (i.e., prevalences > 5% following Reeves et al. 2013). The most frequent abnormalities in the present study were brachydactyly and ectromelia, which is consistent with other studies (e.g., Meteyer et al. 2000; Agostini et al. 2013; Rebouças et al. 2019). Like Agostini et al. (2013), we did not observe any case of multiple limbs or multiple segments, which have been reported in other regions (Meteyer et al. 2000).

As we predicted, we found a higher prevalence of abnormalities in ground-dwelling species than in arboreal or aquatic species. A high cost of a morphological abnormality on the limbs of a climbing frog could explain the low prevalence of abnormalities observed in the hylid treefrog *B. pulchella* (Goodman and Johnson 2011). Two studies in the Pampas biome (where our studied site is located) in Argentina reported higher limb abnormalities in ground-dwelling than in arboreal or aquatic anurans (Peltzer et al. 2011; Agostini et al. 2013). These authors suggested that differences in the probability of survival of abnormal individuals in different microhabitats could pertain to the prevalence of morphological abnormalities. In this context, we suggest that the evaluation of morphological abnormalities in ground-dwelling amphibians is a potentially good indicator of local ecosystem health.

Despite reports showing that the factors that induce morphological abnormalities could also delay growth and development in amphibians (e.g., Greulich and Pflugmacher 2003), we did not find a difference in body size between normal and abnormal individuals. Contrary to our findings, Guerra and Aráoz (2016) reported a positive association between the probability of carrying abnormalities and body size. The lack of knowledge about the individual effects of morphological abnormalities and the need to generate more empirical data is evident. As it is a preliminary survey, we were unable to assess the cause of these observed results.

Although our objective was not to determine the causes of the morphological abnormalities, we cannot ignore that agricultural intensification has highly altered the surveyed landscape, and agricultural activities have affected abnormality rates in amphibian assemblages in other areas (Taylor et al. 2005). Haas et al. (2018) analyzed multiple databases, concluding that a frequency of abnormality > 5% in tadpoles could be related to the presence of agricultural pesticides in the environment. Researchers who previously evaluated abnormalities in southern South America highlighted agricultural intensification (e.g., Agostini et al. 2013; Guerra and Aráoz 2016; Ascioli-Morrete et al. 2019). In addition, several experimental studies have identified toxicity, epigenetic, and enzymatic effects on local anurans as a result of exposure to the most frequently used chemicals in intensive regional agriculture (e.g., Lajmanovich et al. 2003, 2011, 2019). Finally, we must consider another possible cause of abnormalities in amphibians, the parasite Frog-mutating Flatworm (*Ribeiroia ondatrae*), although it has not yet been reported in the region (Johnson and Sutherland 2003).

Agroecosystems in southern South America are being strongly affected by the increasing development of rainfed crops, especially soybean (Baldi and Paruelo 2008; Modernel et al. 2016), with high use of chemical products that are affecting native amphibians (Guerra and Aráoz 2016; Agostini et al. 2020). Researchers have reported the presence of pesticides in the EFIRU region in natural ecosystems (Soutullo et al. 2020), in fish tissue for human consumption (Ernst et al. 2018; Soutullo et al. 2020), and in beehive products (Niell et al. 2015, 2017). Our findings create the need to evaluate if the high levels of amphibian abnormalities observed in the EFIRU region are caused by local agricultural activity or other factors. Finding the causes of this abnormality hotspot should be a priority for amphibian conservation and the management of the protected areas in Uruguay.

Acknowledgments.—We thank local producers for allowing us to sample on their farms. Thanks to Lucía Bartesaghi and Sebastián Horta from Sistema Nacional de Áreas Protegidas (SNAP), and to the Museo Nacional de Historia Natural (MNHN) team for their support during field sampling. We thank Claudio Borteiro for his generous support and encouragement during the preparation of this manuscript. NG and NK thank the Postgraduate Grants from the Agencia Nacional de Investigación e Innovación (ANII) and the Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Uruguay. GL is a member of the Sistema Nacional de Investigadores (SNI) and PEDECIBA. This research was funded by SNAP, within the EFIRU protected area framework and was performed by MNHN. The Committee for Ethics in Animal Research of the Museo Nacional de Historia Natural authorized the field and laboratory procedures (CEUA-MNHN, N° 02/2018).

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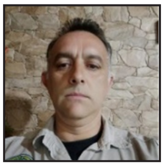

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