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Discovery of Wild Amphibians Infected with Ranavirus in Brazil

Joice Ruggeri,[1,6] Luisa P. Ribeiro,[1,2] Mariana R. Pontes,[1,2] Carlos Toffolo,[3] Marcelo Candido,[4] Mateus M. Carriero,[5] Noeli Zanella,[3] Ricardo L. M. Sousa,[4] and Luís Felipe Toledo[1] [1]Laboratório de História Natural de Anfíbios Brasileiros, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil, CEP 13083-862; [2]Programa de Pós-Graduação em Ecologia, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil, CEP 13083-862; [3]Programa de Pós-Graduação em Ciências Ambientais, Instituto de Ciências Biológicas, Universidade de Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil, CEP 99052-900; [4]Laboratório de Higiene Zootécnica, Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, São Paulo, Brazil, CEP 13635-900; [5]Laboratório de Imunologia de Parasitas, Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, São Paulo, Brazil, CEP 13635-900; [6]Corresponding author (email: joice.ruggeri@gmail.com)

ABSTRACT: Ranavirus is a double-stranded DNA virus associated with amphibian, fish and reptile die-offs worldwide. International trade of live animals farmed for human consumption, such as the American bullfrog (*Lithobates catesbeianus*), plays a key role in spreading the pathogen. In Brazil, ranavirus has only been reported in captive bullfrog farms. We found infected tadpoles of both native species and the American bullfrog in the wild, and a case of mass mortality of amphibians and fish potentially associated with ranavirus. Dead animals presented skin ulcerations, hemorrhages, and edemas. We also found an overall prevalence of 37% of the amphibian chytrid in the area, and two bullfrog tadpoles were co-infected with both pathogens. We suggest that the interaction between the two pathogens should be investigated to improve global conservation of ectothermic vertebrates.

Key words: Clinical signs, conservation, die-off, ectotherm vertebrates, infection, North American bullfrog, ranaviruses.

Ranavirus is an emergent virus in the Iridoviridae family associated with die-offs of ectothermic vertebrates worldwide (Chinchar 2002; Gray and Chinchar 2015). Introduction of this pathogen into different regions may be due to the pet trade, initially ornamental fish (Jankovich et al. 2015), and currently with the international movement of live amphibians (Schloegel et al. 2009; Kolby et al. 2014) such as the North American Bullfrog (*Lithobates catesbeianus*). The bullfrog can be infected with ranavirus without clinical disease (Hoverman et al. 2011), thereby acting as a vector for viral dissemination (Mazzoni et al. 2009; Schloegel et al. 2009). *Lithobates catesbeianus* is one of the main species farmed for human consumption (Schlaepfer et al. 2005), and Brazil is a major producer (Warkentin et al. 2009). However, in the early 1990s, several farms were abandoned, resulting in bullfrogs escaping into natural environments (Both et al. 2011). Such escapes are a risk of transmission of ranavirus to naïve and endemic ectothermic vertebrate populations, which could be catastrophic (Berger et al. 1998; Price et al. 2014). Because ranavirus was linked to die-offs in bullfrog farms in Brazil (Galli et al. 2006; Mazzoni et al. 2009), we hypothesized that this virus would be found in natural habitats, especially in the south of the Atlantic Forest, where feral populations of bullfrogs are widespread (Both et al. 2011).

On 17 November 2017 in the state of Rio Grande do Sul, southern Brazil (28°15′44″S, 52°24′22″W), we found a pond with several dying and dead American bullfrog tadpoles and a few dead fish, all showing signs of ranaviral disease, such as skin ulcerations, hemorrhages, and edemas (Fox et al. 2006; Miller et al. 2015). We collected samples for disease screening for ranavirus and *Batrachochytrium dendrobatidis*.

We collected tadpoles in nets of various sizes in two ponds 12 km apart, both located in highly disturbed habitats (Fig. 1A, B). We used different nets between ponds, but not between animals. Pond A (580 m elevation) is a natural pond where we found adult bullfrogs and tadpoles of native species of the families Bufonidae and Hylidae (not identified to species level), and pond B (724 m elevation) is an artificial pond where we only found bullfrog tadpoles and a few unidentified fish. We collected a total of 18

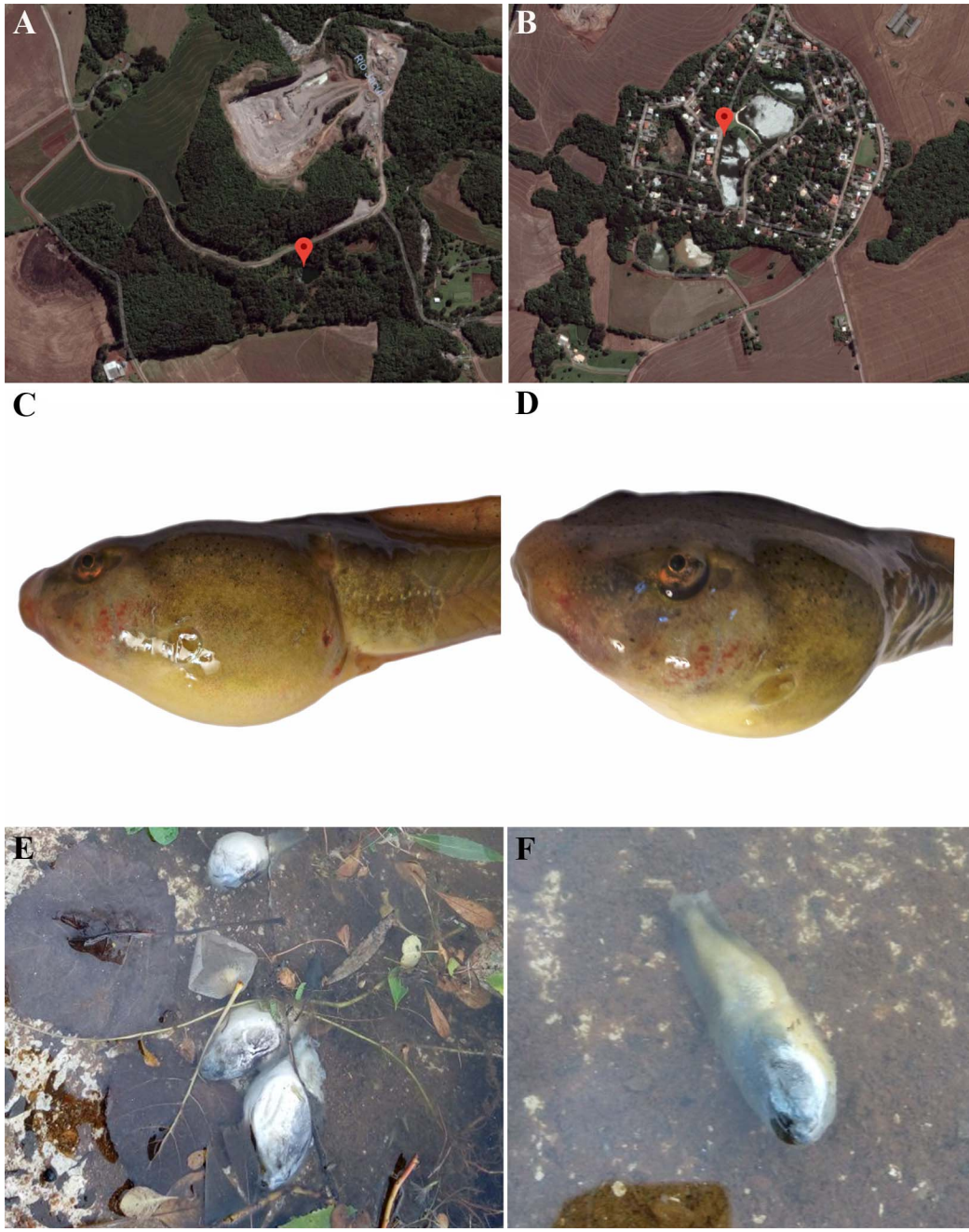

FIGURE 1. Sampling locations, lesions, and dead animals in the municipality of Passo Fundo, state of Rio Grande do Sul, southern Brazil, where sampling of tadpoles occurred in November 2017. (A) A natural pond, (B) an artificial pond, (C) skin ulcerations and (D) hemorrhage in a North American bullfrog (*Lithobates catesbeianus*) tadpole, (E) dead unidentified fish, and (F) dead bullfrog tadpoles in the artificial pond.

tadpoles of native species in pond A, and four bullfrog tadpoles (two dead and two lethargic individuals) in pond B. We collected a smaller number of specimens in pond B, because most dead animals were either in a state of advanced decomposition or in very deep, difficult to access parts of the pond. Each bullfrog tadpole was placed in an individual plastic bag. Native species were placed in plastic bags containing four or five individuals. Live individuals were anaesthetized and euthanized according to Brazilian regulations (Conselho Nacional de Controle de Experimentação Animal 2016). To avoid cross-contamination, a new pair of gloves was used before handling each tadpole. Kidneys, livers, and spleens were removed from all animals with sterile scissors. Pools of organ samples were stored in individual cryotubes with 1 mL of DNAzol® Reagent (Invitrogen®, Thermo Fisher Scientific, São Paulo, Brazil) for later DNA extraction following product specifications. After extraction, we used a spectrophotometer (Take3 Plate, BioTek Instruments, Winooski, Vermont, USA) to determine the amount of nucleic acid in each sample and diluted the samples to a final concentration of 7.5 ng/μL. We analyzed the presence or absence of viral DNA with a TaqMan real-time quantitative (q)PCR assay (Allender et al. 2013) using the 7500 ABI Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA). The threshold was set manually to 0.168 ΔRn (Rn value of an experimental reaction minus the Rn value of the baseline signal) to be comparable to the standard curve. We used two positive templates and two no-template controls in the assay. Mean cycle threshold for positive templates was 37. Therefore, we set the cycle threshold cutoff of the assay to 37.5 (10 DNA viral copies/μL), and samples with more than 10 DNA copies were considered positive for ranavirus. Although amphibians did not show clinical signs of chytridiomycosis, *B. dendrobatidis* is prevalent in the southern Atlantic Forest (James et al. 2015; Jenkinson et al. 2016); therefore, we also tested for the presence of this pathogen in the mouthparts of tadpoles with a qPCR assay (Boyle et al. 2004).

We did not observe bullfrog tadpoles in pond A, and all native specimens seemed healthy. Contrarily, we found more than 20 dead bullfrog tadpoles in pond B (Fig. 1E, F) and no native anuran species. All observed individuals in this pond had severe skin lesions (Fig. 1C, D), and were positive for ranavirus. The two live tadpoles collected in pond B presented low ranavirus infection loads (15 and 21 copies/μL). The qPCR of dead tadpoles revealed higher infection loads (188 and 1,914 copies/μL). In pond A, five samples from native tadpoles amplified for the presence of ranavirus, but with small numbers of viral copies (between 10 and 12 genomic DNA/μL). The chytrid fungus was detected in seven of 19 tadpoles, including the native and invasive species (Table 1). However, infection load was lower than that expected for chytridiomycosis (Vredenburg et al. 2010), and we discarded chytridiomycosis as the potential cause of death of anurans in the pond.

Despite bullfrogs being tolerant to ranavirus (Hoverman et al. 2011), we detected high loads of ranavirus and apparent signs of disease, and most dead animals were in advanced stages of decomposition, which is characteristic of death by ranavirus (Brunner et al. 2015; Miller et al. 2015). Studies on susceptibility to ranavirus across life stages have shown that tadpoles often die of infection whereas adults might clear the virus (Lesbarrères et al. 2012), which could explain the mortality of tadpoles observed here. However, without histopathologic examination, we do not have evidence to attribute these deaths to this pathogen.

Although ranavirus is known to occur in natural populations in South America (Zupanovic et al. 1998b; Fox et al. 2006), it has not previously been described in wild populations, as well as in native amphibian species, in Brazil. Because the susceptibility of native amphibian hosts to ranavirus in Brazil has not been investigated, the low infection loads that we detected (<12 copies/μL) could either represent the beginning stage of infection

TABLE 1. Results for the TaqMan quantitative (q)PCR assay for ranavirus detection in tadpole samples collected from two ponds in the municipality of Passo Fundo, state of Rio Grande do Sul, southern Brazil. Cycle threshold (C_t) for positive templates was C_t=37. We considered positive for ranavirus samples at $C_t \geq 37.5$ (bold). The presence or absence of *Batrachochytrium dendrobatidis* is also given, and infection load as the number of zoospore genome equivalent (GE) detected in the qPCR assay is provided for positive samples.

			Ranavirus			*B. dendrobatidis*	
No.	Family	Pond	C_t	Results	DNA (ng/μL)	Results	Load (GE)
Rv177	Hylidae	A	38	Negative	7	Negative	—
Rv178	**Hylidae**	**A**	**37**	**Positive**	**11**	Negative	—
Rv179	Hylidae	A	Undetermined	Negative	0	Positive	2.5
Rv180	Hylidae	A	39	Negative	4	Negative	—
Rv181	**Hylidae**	**A**	**37**	**Positive**	**10**	Negative	—
Rv182	Hylidae	A	Undetermined	Negative	0	Positive	7.6
Rv183	Hylidae	A	39	Negative	3	Positive	2.3
Rv184	Bufonidae	A	39	Negative	4	Negative	—
Rv185	Bufonidae	A	39	Negative	5	Positive	2.2
Rv186	Bufonidae	A	39	Negative	4	Negative	—
Rv187	Bufonidae	A	39	Negative	5	Positive	3.0
Rv188	Bufonidae	A	38	Negative	6	Negative	—
Rv189	**Bufonidae**	**A**	**37**	**Positive**	**12**	Negative	—
Rv190	Bufonidae	A	39	Negative	5	Negative	—
Rv191	Bufonidae	A	38	Negative	6	Negative	—
Rv194	Bufonidae	A	38	Negative	9	Not sampled	—
Rv195	**Bufonidae**	**A**	**37**	**Positive**	**10**	Not sampled	—
Rv196	Bufonidae	A	39	Negative	3	Not sampled	—
Rv197	**Ranidae**	**B**	**37**	**Positive**	**15**	Positive	7.7
Rv198	**Ranidae**	**B**	**36**	**Positive**	**21**	Negative	—
Rv199	**Ranidae**	**B**	**29**	**Positive**	**1,914**	Positive	13.1
Rv200	**Ranidae**	**B**	**33**	**Positive**	**188**	Negative	—

(Brunner et al. 2005) or be related to host tolerance to this pathogen (Zupanovic et al. 1998a; Roy and Kirchner 2000).

Studies on ranaviruses in Brazil are incipient (Mazzoni et al. 2009; Neves et al. 2016), so we are not aware of how long ranavirus has been present in Brazil, its geographic range and distribution, which strains are present in the wild, and the consequences of infection to native hosts. More studies on ranavirus infection among native species in Brazil must be carried out to clarify these questions, particularly in the Atlantic Forest of Brazil, which is a biodiversity hotspot (Myers et al. 2000) with recognized historic amphibian declines associated with pandemic pathogens (Carvalho et al. 2017).

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