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SHORT NOTE

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Endangered amphibians infected with the chytrid fungus *Batrachochytrium dendrobatidis* in austral temperate wetlands from Argentina

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Infections caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) have been highlighted as a driver of worldwide amphibian declines. In austral temperate wetlands of South America, anurans are exposed to stressors that can increase the negative effects of emerging infectious diseases. We assessed the presence of *Bd* infecting anurans from different environments in Patagonia through molecular analysis of skin swabs from 122 individuals belonging to six species. We report *Bd* in four species, two of which are categorised as endangered, and extend the known distribution of the fungus.

Key words: anurans, Argentina, chytridiomycosis, conservation, Patagonia

Infection caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been highlighted as one of the suspected drivers of worldwide amphibian declines (Lötters et al., 2010). Globally, there have been calls to map the distribution of *Bd* (http://www.bd-maps.net/) to identify sources and potential sinks for this disease (Skerratt et al., 2007; Gower et al., 2012), and studies based on niche models analysed its potential distribution (Ron, 2005; Rödder et al., 2009; Ghirardi et al., 2011). Actual and potential maps reveal that *Bd* is either absent or its presence has yet to be assessed in a wide area of the Neotropics, including the temperate wetlands from Argentinian Patagonia region.

Patagonia harbours unique ecosystems with high numbers of endemic flora and fauna species (Perotti et al., 2005). The highest diversity of amphibians occurs in north-western Patagonia. The amphibian diversity of the colder Patagonian steppe is lower, but characterised by anurans considered to be important indicators of environmental health (Úbeda & Grigera, 2007) and harbouring the highest proportion of endangered amphibians of Argentina (Vaira et al., 2012). Major threats include habitat alteration, exotic fish introduction and climate change, with the role of emerging infectious disease only assessed in the field for one species (*Atelognathus patagonicus*; Fox et al., 2006).

The first case of chytridiomycosis in Argentina was detected in a specimen of *Leptodactylus latrans* found dead in 2002 (Herrera et al., 2005). Since then, new records of *Bd* have been reported in a growing number of species and regions (Barrionuevo & Mangione, 2006; Arellano et al., 2009; Ghirardi et al., 2009; Gutierrez et

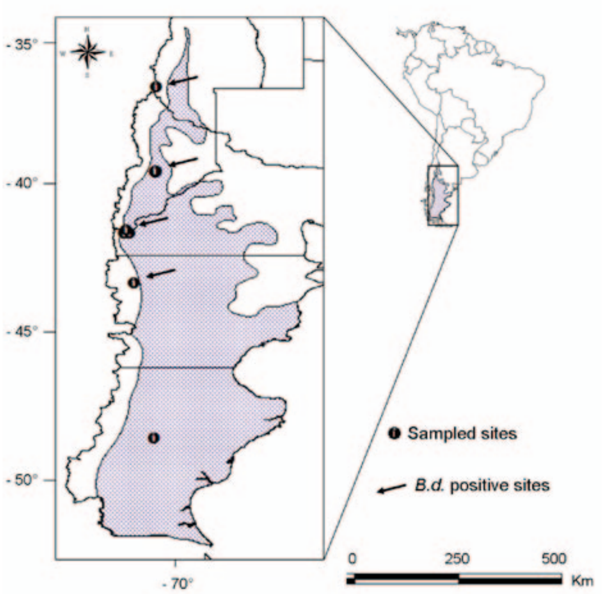

Fig. 1. Sampled sites in Patagonia Argentina. Circles indicate sampled sites and arrows *Bd* positive sites.

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Table 1. Species of Patagonian anurans tested for *Bd* and details of sampling sites. N_{Bd}: number of individuals infected by *Bd*. N_{total}: total number of individuals sampled. [d]: dead or dying individuals. Ct: cycle threshold (mean±standard deviation).

Species	Date	Localities	Coordinates	N_{Bd}/N_{total}	Ct
Alsodes gargola[d]	02/2008	Laguna Toncek (Río Negro province)	71°19'S 41°12'W	0/1	
	02/2008	Refugio Frey (Río Negro province)	71°18'S 41°13'W	0/1	
Alsodes pehuenche	12/2008 04/2010	Paso Internacional Pehuenche (Mendoza province)	70°22'53''S 35°58'35''W	2/31	41
Pleurodema thaul	10/2008 09/2009 01/2010	Laguna Fantasma, Nahuel Huapi National Park (Río Negro province)	71°27'00''S 41°05'33''W	2/7	40.65±1.06
	01/2010	Teleférico, Nahuel Huapi National Park (Río Negro province)	71°22'07''S 41°06'47''W	0/2	
Rhinella spinulosa papillosa	09/2009	Route 40 Km 294 (Río Negro province)	71°19'13''S 41°10'51''W	0/7	
Atelognathus patagonicus[d]	03/2008	Laguna Blanca National Park (Neuquén province)	39°00'S 70°23''W	31/48	38.27±3.29
Pleurodema bufoninum	12/2009	Laguna Blanca National Park (Neuquen province)	39°00'S 70°23''W	0/1	
	10/2008 01/2010	Ñireco river (Río Negro province)	71°19'13''S 41°10'52''W	0/19	
	12/2009	Route 40 Km 543 (Chubut province)	71°09'50.5''S 42°58'08''W	1/2	38.33
	12/2009	Route 40 Km 166 (Santa Cruz province)	70°26'10''S 48°31'37''W	0/3	
Total				36/122	38.57 ±3.13

al., 2010; Delgado et al., 2012; Ghirardi, 2012; Lescano et al., 2013), but a systematic survey for Patagonia was yet lacking. We here report on such a survey, focusing on endemic and endangered species. We discuss potential threats to, and consequences for recently evaluated species (Vaira et al., 2012).

Sampling was carried out between February 2008 and April 2010 (Table 1, Fig. 1). Amphibians were caught by hand and handled using latex rubber examination gloves. Individuals were gently but firmly swabbed 10 times using a sterile polyester-tipped applicator on the ventral surface, hind limbs and interdigital membrane following the techniques of Livo (2004) and Hyatt et al. (2007); tadpoles were swabbed several times at the mouthpart region. We followed sampling protocols from the DAPTF Fieldwork Code of Practice (DAPTF, 1998) and Livo (2004) to avoid possible cross contamination of pathogens between ponds. Each swab was placed in a numbered plastic cryogenic vial for storage. Samples were dried or preserved in ETOH and shipped to a laboratory at the North Carolina State University School of Veterinary Medicine.

Taqman PCR detection for chytrid fungus was performed according to the method of Boyle et al. (2004). Dry swabs were transferred to a sterile 2 ml cryovial (Fisher, USA) and approximately 50 mg of 0.5 mm glass beads (Biospec Products, USA) was added along with 100 µl PrepMan Ultra (Applied Biosystems, California, USA). Following 1 min processing in a Beadbeater (Biospec Products) the vials were placed in a boiling water bath for 10 min, cooled for 1 min and centrifuged at 16,000 x g for 3 min. Ten to 20 µl of sample was removed and stored either at 4°C or frozen at -20°C for later analysis. The reaction mixture was composed of 25 µl Master Mix (Applied Biosystems Taqman Universal PCR Master Mix 4324018l), 4.5 µl forward primer (5'CCTTGATATAATACAGTGTGCCATATGTC), 4.5 µl reverse primer (5'AGCCAAGAGATCCGTTGTCAAA), 1.25 µl probe (5' 6FAMCGAGTCGAACAAAAT MGBNFQ), 13.75 µl water, 1 µl DNA template (1:10 dilution of evaluate from swab). Reaction conditions consisted of 95°C for 10 min, 95°C 15s, 60°C 1 min, repeated 49 times. Appropriate positive and negative controls were included with each run. We analysed the cycle threshold (Ct) of positive results and

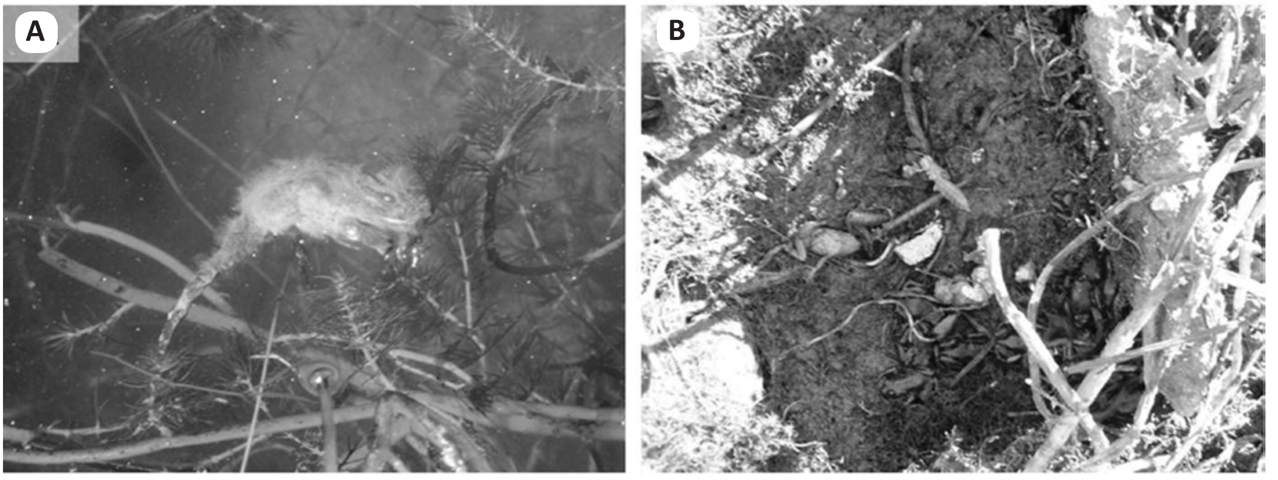

Fig. 2. *Atelognathus patagonicus* from Laguna Blanca National Park. (A) dead and (B) dying individuals.

evaluated differences among species with a Kruskal-Wallis test.

We analysed 122 individuals belonging to six anuran species inhabiting different environments from Patagonia (Table 1). We found chytrid skin infection in 29.5% of individuals, corresponding to four species. Cycle threshold for *Bd* identification were between 33.1 and 44.6, indicating moderate to minimal amounts of *Bd* DNA in the samples. There were no statistical differences for Ct between species (p=0.759; KW=1.175, Table 1).

Every studied Patagonian region had at least one individual infected by *Bd*, and 40% of the wetlands sampled contained positive amphibians (Table 1). The mountain toad *Rhinella spinulosa papillosa* (Bufonidae) was negative for *Bd.* In the same locality, *Pleurodema thaul* (Leiuperidae) was *Bd* positive. In the Patagonian steppe we found a single *Bd*-positive *P. bufoninum*, which is currently the most southern record of *Bd* in the Neotropics. One of the analysed *Alsodes pehuenche* (Cycloramphidae) individuals was captured and swabbed twice, being *Bd*-positive in December 2008 and *Bd*-negative in April 2010; the other positive individual of this species was a tadpole with no clinical signs of infection. Specimens of the sister species *A. gargola*, inhabiting similar environments, were found dead albeit negative for *Bd* (Table 1). We found dead and dying individuals of the endemic *Atelognathus patagonicus* (Ceratophryidae) in endorheic lagoons of the Laguna Blanca National Park, Patagonian steppe (Fig. 2), with all swabbed individuals being *Bd* positive (Table 1).

Previous work on biological communities in the Argentinean Patagonian wetlands has resulted in a consensus that they are strongly affected by habitat alteration, climate change, contamination, species introduction and emerging diseases (Perotti et al., 2005; Vaira et al., 2012). Considering that amphibians tend to have high endemism in most of the Patagonian wetlands (Úbeda & Grigera, 2007; Vaira et al., 2012), chytridiomycosis has the potential to pose a rather immediate risk to entire species. Given our results, *Bd* is a potentially severe threat to endemic and endangered frogs of Patagonia.

Different species are however differentially threatened by *Bd*. *Rhinella spinulosa papillosa* and *P. bufoninum* have a wide distribution including protected areas, and are listed as Least Concern at national and international level (Vaira et al., 2012). Following the arguments of Bielby et al. (2008), they have a low probability to decline following a *Bd* outbreak. Although *P. thaul* occurs in protected areas and covers further areas in Chile, it occupies only a narrow range along the eastern slopes of the Argentinean Andes (Ferraro & Casagranda, 2009), and is the only aquatic vertebrate inhabiting suburban wetlands of San Carlos de Bariloche, Nahuel Huapi National Park (Jara & Perotti, 2009), where infected specimens were found. Notwithstanding the low probability of decline (0.22) by an outbreak of *Bd* reported by Bielby et al. (2008), local monitoring and conservation programs for this species should have high priority.

According to Bielby et al. (2008), *A. gargola* and *A. pehuenche* have a high probability of *Bd*-related declines (0.78 and 1, respectively), suggesting that it is necessary to increase survey efforts and long term monitoring programmes. *Alsodes gargola* inhabits northern Patagonia and is classified as vulnerable, whereas *A. pehuenche* is listed as endangered (Vaira et al., 2012), inhabiting only a 10 km^2 area in Argentina strongly affected by human impacts (Corbalán et al., 2010). However, our data imply that low susceptibility cycles of infection and disinfection are possible (such as described in other amphibians, Kriger & Hero, 2007; Briggs et al., 2005). We found substantially higher *Bd* prevalence for *A. patagonicus* than reported previously (87.5%, this study, and 25%, Fox et al., 2006), including new locations. We lack histopathological examinations of tissues of diseased individuals to conclusively attribute the observed cases of morbidity and mortality to *Bd*, and additional samples and long term monitoring are needed. However, our sampling was too limited for robust interpretations about species susceptibilities, despite pronounced differences in *Bd* prevalence between taxa. This is also supported by the lack of statistical differences between species in the cycle threshold, considering that the results are according to the standard curve for the determination of the pathogen in experimental samples used by Boyle et al. (2004).

Considering the enlarged range of *Bd* reservoirs and the growing evidence of non-amphibian hosts (i.e.

Johnson & Speare, 2003; Garmyn et al., 2012; Kilburn et al., 2011; Shapard et al., 2012), there is a high probability of dispersal of *Bd* to distant wetlands. Numerous regions of Argentinian Patagonia remain unsurveyed for *Bd*, but occurrence in areas adjacent to Patagonia elsewhere in Argentina and Chile (Solís et al., 2010) suggests that this pathogen is locally widespread. More data are essential to evaluate infection rates and risks to amphibian populations inhabiting this region.

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