



First report of chytridiomycosis in the Southern Yungas Andean forest: a threat to the endangered La Banderita marsupial frog *Gastrotheca gracilis*

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This work reports the first record of *Batrachochytrium dendrobatidis* (*Bd*) infection in the endangered La Banderita marsupial frog *Gastrotheca gracilis* from the Southern Yungas Andean forest. We analysed swab samples from the oral discs of 20 tadpoles and histological sections from two post-metamorphic individuals. We found 60 % of the tadpoles to be infected, and the histological sections revealed the presence of zoosporangia of *Bd* in different maturation stages. The signs of infection confirm the presence of *Bd*, which may pose a threat to the endangered La Banderita marsupial frog populations.

Keywords: conservation, *Batrachochytrium dendrobatidis*, chytridfungus infection, Hemiphractidae, tadpoles

Globally, amphibians are experiencing a major decline, rendering 60 % of the amphibian species being Endangered (IUCN, 2022). There is a consensus among scientists and conservationists that emerging infectious diseases, such as chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans*, are among the main factors that contribute to this global decline (Martel et al., 2013; O'Hanlon et al., 2018; Scheele et al., 2019; Fisher & Garner, 2020).

La Banderita marsupial frog *Gastrotheca gracilis* Laurent 1969, is the southernmost species of the family Hemiphractidae (Duellman, 2015). *Gastrotheca gracilis* is a threatened species listed as Endangered on both National and International Red Lists (Vaira et al., 2012; IUCN, 2019). The main threats to this marsupial frog are habitat loss by clear-cutting of primary forests, wildfires and reproductive habitat alteration by domestic animals and human activities (IUCN, 2019). After the rediscovery of this species in 2011 (Akmentins et al., 2012), the three

known populations of *G. gracilis* have been monitored and seem stable despite the active threats (pers. obs.). In 2018, a population supplementation program of *G. gracilis* was established with the support of the Amphibian Ark in the ex-situ facilities of the HorcoMolle Experimental Reserve of the National University of Tucumán (REHM-UNT), with the target of recovering the population of this threatened species in Los Sosa Provincial Reserve in Tucumán province, Argentina (Akmentins, 2019).

Chytridiomycosis is a potential threat to the amphibians of the Southern Yungas Andean forest (Akmentins et al., 2012; 2014). In Bolivia, *Bd* prevalence values of up to 30 % were reported for the Hemiphractidae family (Burrowes & De la Riva, 2017). In this work, we combine molecular and histological analysis to evaluate the presence of *Bd* in a wild population of *G. gracilis*.

On 12 January 2019, we captured 67 tadpoles of *G. gracilis* in Los Sosa Provincial Reserve (27° 00' S; 65° 40' W, 1455 m.a.s.l.), Tucumán Province, Argentina. Following the swabbing protocol proposed by Fisher et al. (2018), we collected samples with swabs (DELTA LAB-300201) from the oral disc of the largest 20 tadpoles and released them immediately after. Swabs were stored in labelled refrigerated cryogenic vials until the laboratory processing. We extracted DNA using the DNeasy Blood & Tissue commercial kit (Qiagen®), and we quantified *Bd* DNA following the protocol proposed by Boyle et al. (2004). We conducted a real-time amplification (qPCR) using a StepOnePlus thermal cycler (Applied Biosystems®). Thermal profiling of the reaction was performed at 95 °C for 20 seconds, then 50 cycles (90 °C for 1 second, followed by 60 °C for 20 seconds). Each plate included a negative control (UltraPure™ DNase/RNase-free distilled water) and standard curve from 0.01–1000 zoospore genome equivalents as positive control for the qPCR.

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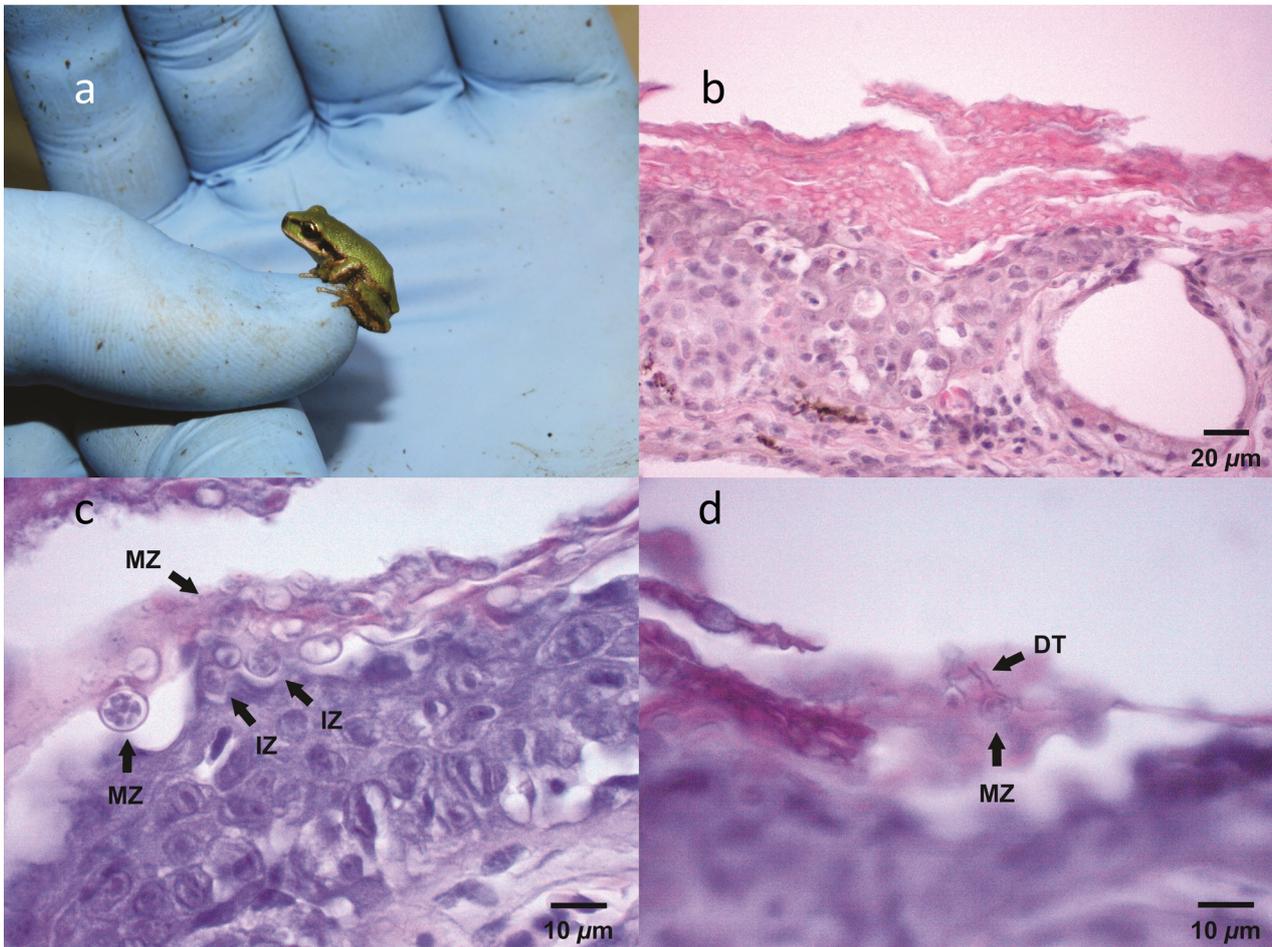


Figure 1. Post-metamorphic individual (a) and photomicrographs of the epidermis of La Banderita marsupial frog *Gastrotheca gracilis* maintained in the ex-situ facilities of the HorcoMolle Experimental Reserve, Tucumán, Argentina. Epidermis sections showing (b) signs of hyperplasia and hyperkeratosis of the stratum corneum; (c) zoosporangia of *Batrachochytrium dendrobatidis* in mature (MZ) and immature (IZ) stages; and (d) detail of a mature zoosporangium (MZ) containing discrete basophilic zoospores and discharge tube (DT).

All samples were run duplicate, and we used StepOne v2.3 software (Applied Biosystems®) to estimate *Bd* DNA loads in zoospores equivalents from the amplification curves. We considered a *Bd* positive sample if *Bd* DNA was amplified.

The remaining 47 tadpoles were kept in captivity in the ex-situ facilities of the HorcoMolle Experimental Reserve of the National University of Tucumán (REHM-UNT) until they completed metamorphosis. The tadpoles were maintained in a fish tank (dimensions: 100 x 50 x 40 cm), with a density of one tadpole per two litres of dechlorinated tap water. Food was provided every other day, alternated between dry and fresh food. Food rations per tadpole were 0.05 g of flake fish food (i.e. dry food) and 1 g of pumpkin (i.e. fresh food). Room temperature was maintained at 24 °C, with a 12 h light cycle. Swab samples from the oral disc in these 47 tadpoles were not collected. We performed a post-mortem histopathological analysis from the skin of the pelvic patch in two 12-day-old post-metamorphic individuals. The skin samples were formalin-fixed and paraffin-embedded. A 5 µm histological sections of the skin was obtained, used hematoxylin and eosin stain, and determined the life cycle stages of *Bd* (Berger et al., 1999; 2005).

The percentage of infected individuals with *Bd* in the 20 analysed tadpoles of *G. gracilis* was 60%. Loads of *Bd* were between 0.05 and 42.17 zoospore equivalents. In total, 12 (25.5%) of the 47 post-metamorphic individuals of *G. gracilis* died after showing a combination of signs of infection compatible with chytridiomycosis infection (i.e. poor body condition, lethargy and inappetence). We observed hyperplasia and hyperkeratosis of the stratum corneum (a sign of *Bd* infection) and the presence of zoosporangia of *Bd*. The zoosporangia were detected in at least three different maturation stages: immature, mature containing numerous basophilic zoospores and with formed discharge tubes, and empty mature (Fig. 1).

Our observations represent the first record of *Bd* in the endangered *G. gracilis* and the first record of *Bd* in the Southern Yungas Andean forest. *Bd* has been reported in other Andean ecoregions of the Neotropics, such as the Northern Yungas forest, Puna, and Inter-Andean Mesothermy Valley. In many locations, the amphibian population decline and local extinction has been associated with the emergence of *Bd* in the Southern Andes (Barrionuevo & Mangione, 2006; Burrowes & De la Riva, 2017). The presence of *Bd* in the Southern Yungas Andean forest, the ecoregion with the highest percentage

of endemic and threatened species of Argentina, is concerning (Lavilla & Heatwole, 2010; Vaira et al., 2017). The three southernmost marsupial frog species inhabit Argentina, and all of them have suffered substantial population declines since the 1990s (Akmentins et al., 2012). Our results suggest that *G. gracilis* is susceptible to infection by *Bd*. Monitoring local amphibian populations in combination with *Bd* surveillance is desirable to assess the impact of this pathogen on the persistence of populations of this and other frog species.

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REFERENCES

- Akmentins, M.S. (2019). Rescuing the southernmost Marsupial Frog species in Argentina. *AArk Newsletter*, 46, 8–9.
- Akmentins, M.S., Bonduri, Y.V., Contreras, P., Francisconi, L., Massabie, E.P.J. & Santillán, J. (2014). Redescrición del canto de anuncio de *Gastrotheca gracilis* Laurent, 1969 (Anura: Hemiphraclidae) y primer registro para el Parque Nacional Campo de Los Alisos, Tucumán, Argentina. *Cuadernos de Herpetología*, 28, 147–152.
- Akmentins, M.S., Pereyra, L.C. & Vaira, M. (2012). Using sighting records to infer extinction in three endemic Argentinean marsupial frogs. *Animal Conservation*, 15, 142–151.
- Barrionuevo, S. & Mangione, S. (2006). Chytridiomycosis in two species of *Telmatobius* (Anura: Leptodactylidae) from Argentina. *Diseases of Aquatic Organisms*, 73, 171–174.
- Berger, L., Speare, R. & Kent, A. (1999). Diagnosis of chytridiomycosis of amphibians by histological examination. *Zoos' Print Journal*, 15(1), 184–190.
- Berger, L., Hyatt, A.D., Speare, R. & Longcore, J.E. (2005). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of aquatic organisms*, 68(1), 51–63.
- Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T. & Hyatt, A.D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of aquatic organisms*, 60(2), 141–148.
- Burrowes, P.A. & De la Riva, I. (2017). Unraveling the historical prevalence of the invasive chytrid fungus in the Bolivian Andes: implications in recent amphibian declines. *Biological Invasions* 19(6), 1781–1794.
- Duellman, W.E. (2015). *Marsupial Frogs: Gastrotheca and Allied Genera*. JHU Press, Baltimore: EEUU.
- Fisher, M.C. & Garner, T.W. (2020). Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology*, 18(6), 332–343.
- Fisher, M.C., Ghosh, P., Shelton, J.M.G, Bates, K., Brookes, L., Wierzbicki, C., Rosa, G.M., Farrer, R.A., Aanensen, D.M., Garner, T.W.J. et al. (2018). Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, 8(1), 1–8.
- IUCN SSC Amphibian Specialist Group. (2019). *Gastrotheca gracilis*. The IUCN Red List of Threatened Species 2019: e.T55336A101425007. <https://dx.doi.org/10.2305/IUCN.UK.2019-1.RLTS.T55336A101425007.en>. Accessed on 28 June 2022.
- IUCN (2022) Summary Statistics. <https://www.iucnredlist.org/resources/summary-statistics>. Accessed on 28 June 2022.
- Laurent, R.F. (1969). Una segunda especie del género *Gastrotheca* Fitzinger en Argentina. *Acta Zoológica Lilloana*, 25, 143–150.
- Lavilla, E.O. & Heatwole H. (2010). Status of amphibian conservation and decline in Argentina. In: *Amphibian Biology. Volume 9. Status of Decline of Amphibians: Western Hemisphere. Part 1. Paraguay, Chile and Argentina*. Heatwole, H. (Ed.). Surrey Beatty & Sons. Australia.
- O’Hanlon, S.J., Rieux, A., Farrer, R.A., Rosa, G.M. & Fisher, M.C. (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* 360(80), 621–627.
- Martel, A., Spitzen-van der Sluijsb, A., Blooia, M., Bertc, W., Ducatellea, R., Fisher, M.C., Woeltjesb, A., Bosmanb, W., Chiersa, K., Bossuyte, F. & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences* 110(38), 15325–15329.
- Scheele, B.C., Pasmans, F., Skerratt, L.F., Berger, L., Martel, A., Beukema, W., Acevedo, A.A., Burrowes, P.A., Carvalho, T., Catenazzi, A. et al. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, 363(6434), 1459–1463.
- Vaira, M., Akmentins, M.S., Attademo, A., Baldo, D., Barrasso, D., Barrionuevo, S., Basso, N., Blotto, B., Cairo, S., Cajade, R. et al. (2012). Categorización del estado de conservación de los Anfíbios de la República Argentina. *Cuadernos de Herpetología*, 26, 131–159.
- Vaira, M., Pereyra, L.C., Akmentins, M.S. & Bielby, J. (2017). Conservation status of amphibians of Argentina: An update and evaluation of national assessments. *Amphibian & Reptile Conservation*, 11(1), 36–44.

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