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# A Brazilian anuran (*Hylodes magalhaesi*: Leptodactylidae) infected by *Batrachochytrium dendrobatidis*: a conservation concern

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***Abstract.*—Several studies have associated the chytrid fungus *Batrachochytrium dendrobatidis* with anuran population declines worldwide. To date, the fungus has been found in Africa, the Americas, Australia, and Europe. However, it has never been reported to occur in the Atlantic forest or Brazil. Based on morphological, histological, and molecular data, we encountered evidence of *B. dendrobatidis* infection in a high-altitude stream-dwelling Brazilian anuran species, *Hylodes magalhaesi* (Leptodactylidae). One population (Municipality of Camanducaia, State of Minas Gerais) was surveyed from 2001 to 2005. Tadpoles lacking teeth were observed and collected in 2004. Histological and molecular analyses identified infection by *B. dendrobatidis*. Although infected tadpoles seem nowadays to co-exist with the disease, our results are alarming due to the highly endangered situation of the Brazilian Atlantic forest and its fauna. Effects of the chytrid infection on the studied population are still unknown. Further investigations are needed to provide information on its distribution in relation to other populations of *H. magalhaesi*.**

**Key words.** *Batrachochytrium dendrobatidis, Hylodes, anuran decline*, *conservation*, *Atlantic forest*, *Brazil*

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## Introduction

Brazil currently figures as the most species-rich country in the world with regard to amphibian diversity (Young et al. 2004; Silvano and Segalla 2005), encompassing two biomes of conservation concern: the Cerrado and the Atlantic forest (Myers et al. 2000). Notwithstanding its striking percentage of endemic species, the Atlantic forest has been seriously threatened by human intervention, reduced to approximately 7% of its original distribution (Morellato and Haddad 2000), and therefore, considered one of the most important biodiversity hotspots for conservation (Myers et al. 2000, Conservation International 2005). Such intense habitat change and fragmentation have been tied to local amphibian population fluctuations and declines (for a recent review, see Eterovick et al. 2005). However, at a global scale, several factors other than habitat disturbance have been associated to amphibian population crashes (Collins and Storfer 2003), particularly emerging infectious diseases such as chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (review in Daszak et al. 2003). This chytrid fungus is globally widespread (Speare and Berger 2000) and has been tied to amphibian declines in Australia, the Americas, and Europe (Berger et al. 1998, 1999a; Bosch et al. 2001). To date, there have been no reports of *B. dendrobatidis* occurring in the Atlantic forest or in Brazil. Based on morphological, histological, and molecular data, we document infection in *Hylodes magalhaesi*—a high-altitude stream-dwelling leptodactylid endemic to the Brazilian Atlantic rainforest.

## Methods

### Field survey

From 2001 to 2005, we followed a population of *H. magalhaesi* through yearly 10-day trips to Vila de Monte Verde, Municipality of Camanducaia, State of Minas Gerais (22°52'37.9"S, 46°02'01.8"W, ca. 1,600 m above sea level). Adults and tadpoles of *H. magalhaesi* were observed along a fast rivulet in this montane forest. Males were observed during the day while calling from the water margin (Fig. 1); tadpoles were observed under debris or in crevices under water. Adults were collected by hand during the day. Tadpoles (stage 25 *sensu* Gosner 1960) were collected with wicker fish traps, using raw meat as bait. Specimens were deposited in Célio F. B. Haddad anuran collection, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, State of São Paulo, Brazil (CFBH 8287: tadpole lot for molecular analysis, collected in January 2005; CFBH 8288: tadpole lot for histological analysis, collected in

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**Figure 1.** Adult male of *Hylodes magalhaesi*. Municipality of Camanducaia, State of Minas Gerais, Brazil. Photo: Célio F. B. Haddad.
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January 2002). A collecting permit was provided by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA 02001.002792/98-03).

### *Histology*

Four tadpoles with deformed oral disks were fixed in 10% formaldehyde solution for 24 hours (h), and their mouthparts were then transferred to a buffer sodium phosphate solution (pH 7.4), where they were kept for an additional 24 h. Mouthparts were dehydrated through 30-minute (min) immersion in each ethanol solution of a series of increasing concentrations (70, 80, 90, and 95%), then embedded in Leica resin for 24 h at 4°C before inclusion. Resin polymerization was performed in a 37°C chamber. Sections (5 Ìm thick) were stained with two techniques: hematoxylin and eosin (HE) were first used to document the general morphologic aspects of the tissue, and periodic acid Schiff (PAS) was subsequently used to enable better visualization of glycogen, mucin, basal membranes, and the fungus (Junqueira and Junqueira 1983).

### *Molecular analysis*

We used specific DNA-based assay of Annis et al. (2004) to detect infection by *B. dendrobatidis* in two tadpoles preserved in ethanol (100%). From each tadpole, we extracted DNA from a 2 x 2 mm piece of mouth tissue corresponding to approximately half of the oral disk. We dried the material for 1 h at 55°C to allow for ethanol evaporation, then added 20 µl of polymerase chain reaction (PCR) buffer (Perkin-Elmer), and 2 µl of proteinase-K (10 mg/ml), crushing the tissue with a pipette tip to increase the contact surface between the material and the solution. We incubated this mixture for 3 h at 55°C, mixing occasionally. After centrifuging for a few seconds (s) at 6,000 RPM, we incubated the material for 5 min at 100°C as described in Annis et al. (2004). For DNA extraction, we added 20 µl of GeneReleaser (BioVentures) and followed a thermocycle program per manufacturer's protocols on a Peltier Thermal Cycler 200 (PTC-200, MJ Research). After centrifuging samples for 1 min at 6,000 RPM, the DNA-bearing supernatant was transferred to a new tube. To control for contamination, a blank extraction was carried out in a tube containing no sample.

PCR was performed to determine the presence of *B. dendrobatidis* in the extracted material, using primers Bd1a and Bd2a of Annis et al. (2004). We added 3 µl of extracted DNA, 19 µl of water, and 1.5 µl of each primer (10 µM solution) to a tube of PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences). These primers are known to bind specifically to *B. dendrobatidis* DNA as opposed to that of other Chytridiomycota and are expected to result in the amplification of a fragment of approximately 300 base pairs (bp) containing *B. dendrobatidis* 5.8S ribosomal RNA and flanking internal transcriber spacer regions ITS1 and ITS2 (Annis et al. 2004). For amplification, we used a PCR thermocycle program comprising a denaturation step at 95°C for 5 min, 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, and final extension at 72°C for 10 min. To ensure that no false

positives were generated due to contamination during the amplification step, we used the blank extraction as a negative control.

We evaporated all amplification products to bring them to final volumes of 10 µl, which were run on a 1% low-melt low-EDTA agarose gel for band visualization. We cut and incubated the bands of interest with 1 µl of GELase (Epicentre Technologies) for 5 min at 50°C, subsequently leaving them at 45°C overnight. We performed cycle sequencing reactions using 2 µl of template, 3 µl of water, 1 µl of BigDye Terminators ver. 3.0 (ABI Prism), 3 µl of BigDye Buffer, and 1 µl of primer. Cycle sequencing profiles comprised a denaturation step at 96°C for 1 min and 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. We precipitated final products in ethanol and resuspended in 10 µl of Hi-Di formamide (ABI Prism) per manufacturer's protocols. Sequencing reactions were run on a 3730 Genetic Analyzer (Applied Biosystems). We used Sequencer version 4.1.2 (Gene Codes Corporation) to visualize sequences and to align them to partial sequences of ITS1 and adjacent 5.8S ribosomal RNA sequence of *B. dendrobatidis* available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html; accession numbers AY598034 and AY997031). We used MegaBLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to align and compare them to sequences of all fungi species available in GenBank. To that end, we retained all alignments with identity percentage higher than 60%, setting log-odd match and mismatch scores to 1 and -1, respectively. Only alignments larger than 40 bp in length and with E-scores equal or smaller than e-04 were considered for the purposes of sequence comparison.

## Results

### *Field survey*

The population did not seem to vary in abundance along the rivulet where calling males were found, or over the years of observation. However, no count data were collected to document these observations. Adults showed no apparent physical or behavioral abnormalities. Five tadpoles were collected in 2004, all showing deformed or incomplete mouthparts, with total or partial teeth loss. Most tadpoles also lacked keratinized jaw sheaths or showed partially keratinized jaw sheaths. Gut content observation nonetheless suggests that the lack of keratinized mouthparts seemed not to interfere with feeding ability. All four tadpoles collected in 2005 had completely keratinized mouthparts.

### *Histology*

Fungal structures consistent with *B. dendrobatidis* occurred in the oral region of all four tadpoles, in the epidermis adjacent to tooth rows. Four stages of *B. dendrobatidis* were identified (Fig. 2A and 2B); an early phase containing a central spherical basophilic mass, a zoospore-filled phase with 4-10 round or oval basophilic zoosphores in cross section, empty spherical zoosporangia with internal septa, as well as later stage where the empty zoosporangium had collapsed into an irregular shape. These structures stained strongly with PAS. Zoosporangia were present at greater density in the areas of the epidermis of tooth rows and jaw sheaths that showed the most abnormal histology, consisting of hyperkeratosis and loss of superficial epidermis (Fig. 2C). The prominent keratin plate that forms the jaw sheath in healthy tadpoles was almost completely destroyed (Fig. 2D).

### *Molecular Analysis*

DNA was successfully extracted from the material sampled. No contamination was found in the blank extraction. As expected under the scenario of infection by *B. dendrobatidis*, the PCR procedure resulted in a bright band of approximately 300 bp in both tadpoles (Fig. 3). No amplification was obtained in the negative control tube, thus excluding the possibility of contamination. Direct sequencing of the amplified band resulted in a chromatogram with several multiple peaks, which is not surprising given that these are tandem repeats of ribosomal DNA that are not identical. Yet, a 114-bp piece located at the 5' end of the sequence, excluding primer Bd1a, did not include multiple peaks and was easily aligned to portions of the ITS1+5.8S *B. dendrobatidis* sequences available in GenBank. In total, MegaBLAST encountered 21 GenBank records that aligned to this 114-bp query sequence. All of them correspond to fungi species. The obtained sequence was 100% identical to known partial sequences *B. dendrobatidis* and differed from all remaining fungi species by more than 9% in sequence composition (91% to 67% sequence identity). Based on data available in GenBank, sequences of congeneric species of Chytridiomycota can differ by approximately 5% in composition at this locus.

## Discussion

The identification of *B. dendrobatidis* was confirmed by the fact that the morphology of the fungal structures was consistent with previous descriptions, particularly the presence of zoosporangia in four stages of development and the occurrence of colonial morphology in the zoosporangia (Berger et al. 1998, 1999b; Longcore et al. 1999; Pessier et al. 1999; Fellers et al. 2001). The morphological identification as *B. dendrobatidis* was confirmed by PCR showing a match with a partial sequence of the ITS1+5.8S sequence in GenBank.

*Hylodes magalhaesi* is an Atlantic forest endemic frog restricted to areas of high elevations in southeastern Brazil (> 1,500 m above sea level). To date, it has been known solely from its type locality (Municipality of Campos do Jordão, State of São Paulo; Bokermann 1964; Frost 2004). Our observations provide the second population record of the species, which extends its distribution approximately 50 km northward. They also provide the first record of this species in the State of Minas Gerais. The data represent the first report of the presence of *B. dendrobatidis* in Brazil and in the Atlantic forest. Furthermore, this is one of the southernmost reports of a natural population infected by *B. dendrobatidis* in the Americas (see also Herrera et al. 2005).

Our data are alarming with regard to the status of *H. magalhaesi* given the lack of information about this species (*H. magalhaesi* is classified under the World Conservation Union (IUCN) "Data Deficient" category, AmphibiaWeb

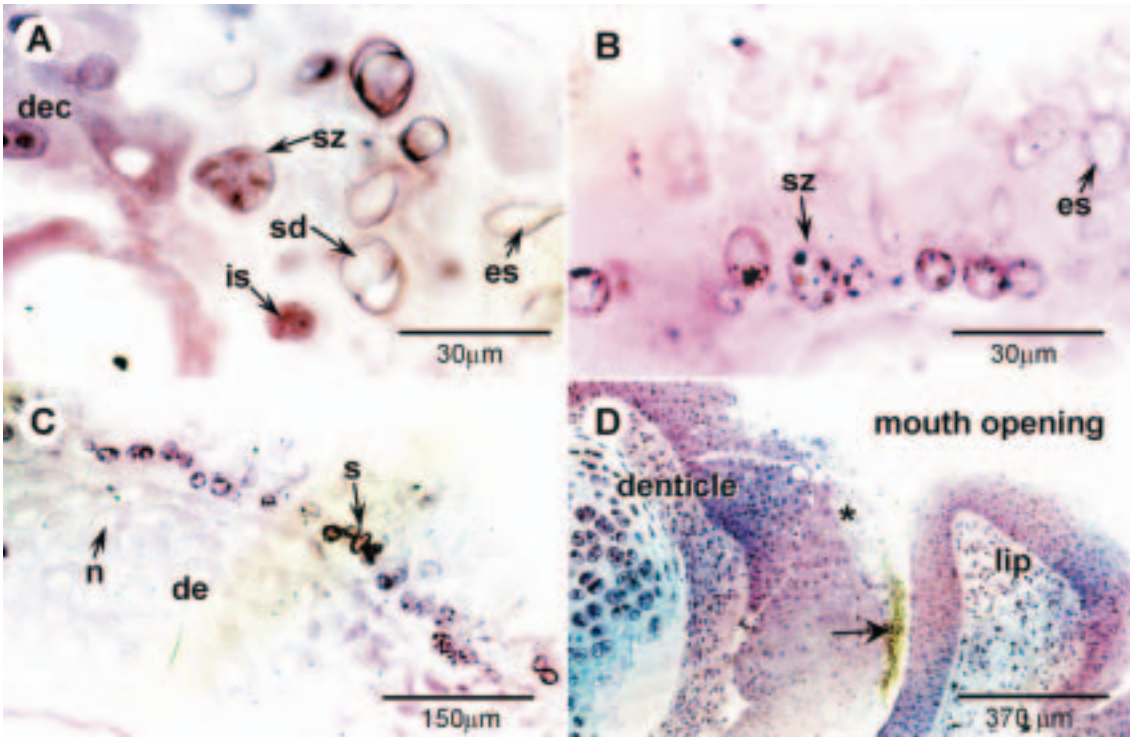


**Figure 2.** Mouthpart section of *H. magalhaesi* tadpole; keratinized epidermis with various stages of *Bd*. (**A**) Detail of sporangia as shown by HE technique: immature sporangium (**is**), sporangium with zoospores (**sz**), empty sporangium (**es**), and sporangium with internal septum (**sd**); **dec** = denticle epithelial cell. (**B**) Detail of sporangia as shown by PAS technique. (**C**) Fungus infection as shown by PAS technique, arrows point to sporangia (**s**); **de** = denticle epidermis; **n** = nucleus of epithelial cells. (**D**) Region of irregular epidermal surface (*) evidenced by HE technique. Arrow shows remaining keratinized denticle surface.
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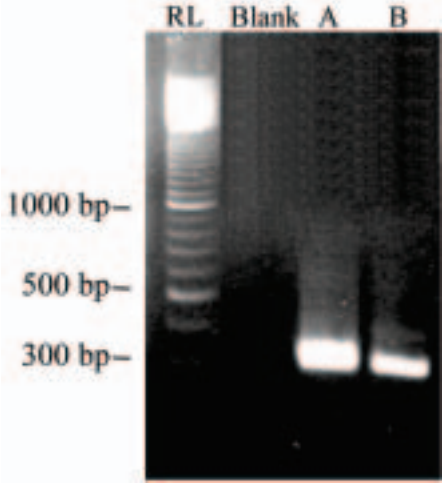


**Figure 3.** Gel image of PCR results using *Bd*-specific primers Bd1a and Bd2a. A reference DNA ladder is presented (**RL**). Results obtained with a negative control (extraction blank), DNA template from specimen CFBH 8287.1 (**A**), and DNA template from specimen CFBH 8287.2 (**B**) are provided.
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2005), particularly given the increased rate of deforestation faced by the Brazilian Atlantic forest (Myers et al. 2000; Morellato and Haddad 2000). Population declines have been reported for other species of *Hylodes* endemic to this biome (*H. asper*, *H. babax*, *H. phyllodes*, and *H. lateristrigatus*). For *H. asper* and *H. phyllodes*, local extinctions have been attributed to climate change (Heyer et al. 1988; Bertoluci and Heyer 1995). Other decline-leading factors have been suggested for the remaining species of *Hylodes*, as well as for other mountain-stream frogs (cycloramphines, centrolenids, hylids, and dendrobatids), including infectious diseases (Weygoldt 1989). Observations by Weygoldt (1989) of the metamorphosis of individuals of *H. babax* led him to suggest the occurrence of bacterial infections in this species. However, there is a great probability that they could not complete their development due to *B. dendrobatidis* infection, as observed in other infected species (see Berger et al. 1998, 1999a).

Even though we have noticed no apparent decline in the abundance of adult *H. magalhaesi* over the last five years, additional and more intensive studies are necessary to determine the dynamics of the infected population. Tadpoles of *H. magalhaesi* seem to be able to feed normally with unkeratinized tooth rows, which could be caused by the presence of the chytrid (see also Berger et al. 1999a) and may represent a pathogen reservoir (Collins and Storfer 2003), with potential consequences for other less resistant anuran hosts in Brazil (see Daszak et al. 2004). The following questions shall be answered in future studies: Do apparently normal tadpoles also carry *B. dendrobatidis*? Are there differences concerning the trophic ecology of toothless and normal tadpoles? Does *B.*

*dendrobatidis* infect other stages of tadpole development? Do infected tadpoles metamorphose and reach adult sizes? Are post-metamorphic individuals infected? Are populations of *H. magalhaesi* declining at the study site? What other syntopic high-altitude stream-dwellers are also infected?

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