# Is the Chytrid Fungus Really Responsible for Amphibian Decline?

Lycia de Brito-Gitirana*[,1], Flavia Abreu Felsemburgh[1], Sérgio Potsch de Carvalho e Silva[2] and Pablo Germano de Almeida[1]

[1]*Laboratory of Animal and Comparative Histology, ICB – UFRJ, Av. Trompowsky s/nº, Ilha do Fundão – Cidade Universitária – RJ, 21940-970, Brazil*

[2]*Laboratory of Amphibian and Reptiles, IB – UFRJ, Av. Trompowsky s/nº, Ilha do Fundão – Cidade Universitária – RJ, 21940-970, Brazil*

**Abstract:** Besides other pathogens, the chytrid fungus *Batrachochytrium dendrobatidis* has been considered the main etiologic agent that causes chytridiomycosis and associated with amphibian die-offs. Chytrid fungus was first described in a living specimen of a *Physalaemus signifer* population (Anura, Leiuperidae), a common frog in natural environment in Rio de Janeiro, Brazil. The *P. signifier* integument is formed by a poorly keratinized epidermis supported by a dermis, which is subdivided into a spongious and compact layers. The granular and mucous glands are located in the spongy dermis. Although *P. signifer* showed no macroscopic lesions, microscopic analysis revealed hyperkeratosis (epidermal disruptions associated with thickening of horny layer), and some oval to spherical sporangia in the horny layer. The occurrence of chytrid fungus indicates that this pathogen is still active in Brazilian anurans and can be spreading. Considering the few sporangia in the epidermis and the strong epidermal disruption, the results suggest that the integument lesion occurs before the colonization by the chytrid fungus. Thus, the chytrid may be an opportunist fungus and not the main cause of amphibian decline.

**Keywords:** Physalaemus signifier, chytrid fungus, integument, decline.

In the past 25 years dramatic declines in amphibian populations have been reported all over the world [1-3]. Although natural population fluctuations (including amphibians) are not unusual, the general decline in amphibians – even in protected areas - is persistent and represents a real threat to biodiversity [3]. A number of factors, including destruction and modification of habitat, over-exploitation, and water-borne pollution, introduction of xenofauna, climate changes, and diseases are implicated as causes of amphibian decline [1, 2].

Four important pathogens have been identified in amphibian populations that can infect the integument. The chytrid fungus, *Batrachochytrium dendrobatidis*, has been associated with recent amphibian die-offs [2, 4-8]. A second fungus, *Basidiobolus ranarum,* has been isolated from clinically ill individuals from declining populations of *Bufo baxteri* [9-13]. A third pathogen is an iridovirus, a lethal virus from the tiger salamander *Ambystoma tigrinum stebbinsi* [14, 15]. A fourth pathogen associated with amphibian declines is the "red leg" bacterium *Aeromonas hydrophila* isolated from wild-caught frogs and tadpoles of *Rana pipiens* [16].

The chytridiomycosis has been characterized through cutaneous lesions, resulting in hyperkeratosis which together with the fungal plaques causes death by preventing cutaneous respiration [17, 18].

Chytridiomycosis in amphibians was diagnosed in Africa [19, 20], Australia [4], Europe [21], North America [22], Central America [5, 23], and South America [18, 24]. Although the chytrid fungus has been identified only in amphibian collections in Brazil [25], this fungus has not been demonstrated in living anurans. Thus, this work intended to identify the chytrid fungus in frogs living thewild.

Three adult males of *Physalaemus signifer* (Girard, 1853) with an average weight of 1.3g and average length of 2.8cm were collected next to Reserva Biológica do Tinguá (Nova Iguaçu, Rio de Janeiro State) (license no. 128/05 – IBAMA/RAN). For the light microscopic (LM) analysis, integument fragments of the body ventrolateral region near the inguinal glands were fixed with 10% buffered formaldehyde, processed according to standard histological techniques for paraffin embedding, and 5µm thick serial slices were stained with haematoxylin-eosin (HE) and Gomori´s trichrome [26]. Periodic acid Schiff (PAS) staining was employed to diagnose of chytridiomycosis, as recommended by Berger *et al.* [27].

Histological observations of the integument showed a normal anuran morphology, i.e., the integument is formed by an epidermis and a dermis supported by a thin hypodermis. The epidermis is formed by four cellular layers (Fig. **1**). The outmost layer, the horny layer, is composed by a thin layer of keratinized cells where nuclear profile is still visualized, and so, the epidermis is a parakeratinized stratified squamous epithelium. The dermis is subdivided into a spongious layer of loose connective tissue rich in blood vessels and a compact layer formed by collagenous fibers arranged compactly in a criss-crossed manner (Fig. **1**). Pigmented cells and mucous and granular glands occupy almost the entire spongious dermis. Between the spongious

*Address correspondence to this author at the Laboratory of Animal and Comparative Histology, ICB – UFRJ, Av. Trompowsky s/nº, Ilha do Fundão – Cidade Universitária – RJ, 21940-970, Brazil; E-mail: lyciabg@ufrj.br

1874-3366/09 

and compact layers a poorly developed basophilic layer (Eberth-Katschenko layer) is observed. As a result, the structure of the *Physalaemus signifer* integument is similar to other anurans as reported by other investigators [28-33].


**Fig. (1).** Normal morphology of the integument. Note the epidermis (E) supported by the dermis. Note granular (G) and mucous (MG) glands in the spongious dermis. CD = compact dermis; V = blood vessel in the hypodermis. Bar = 0.05 µm. Gomori´s trichrome staining.

Although the external surface of the *P. signifer* integument had no evidence of cutaneous lesions, light microscopy revealed significant epidermal disruptions are near the inguinal glands. The epidermal morphology is completely destroyed, the number of epidermal cellular layers is increased, and the outmost layer is sloughed off. The epidermal lesions exhibit marked thickening of horny layer (hyperkeratosis) (Fig. **2**). Deeper epidermal changes are observed and consist of moderate morphological disruption of intermediary and basal layer. Few immature sporangia are visualized by HE-staining (Figs. **2**, **3** and **5**) in the outmost layer of the epidermis and exhibit PAS-positive reaction (Fig. **4**). The occurrence of oval to spherical sporangia in the horny layer associated with hyperkeratosis provides the positive diagnosis of chytridiomycosis.

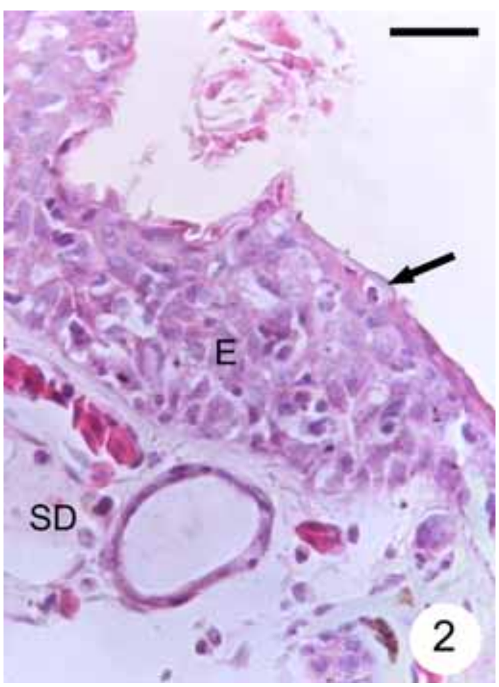


**Fig. (2).** Section of the integument with focal hyperkeratosis and the epidermis has heavily destroyed morphology. Note marked thickening of horny layer and a sporangium (➝). E = epidermis; SD = spongious dermis. Bar = 0.02µm. HE staining.

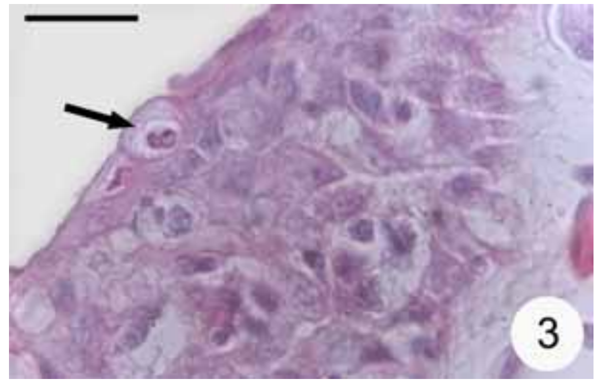


**Fig. (3).** The epidermis morphology is destroyed. A single chytrid (→) appears on the horny layer, possible representing the initial infection stage. Bar = 0.02µm. HE staining.

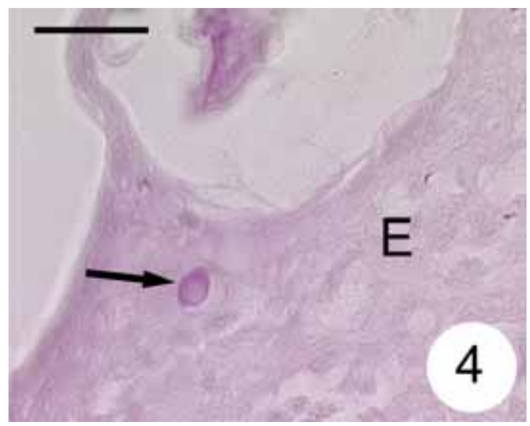


**Fig. (4).** The immature sporangia exhibit PAS-positive reaction (→) in the outmost layer of the epidermis. Bar = 0.02µm. PAS staining.

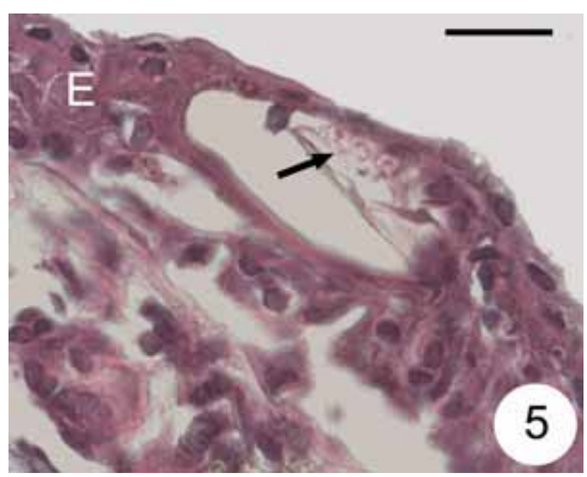


**Fig. (5).** Note sporangia in the epidermis (E). Bar = 0.02 µm. HE staining.

This work provides the first record of the chytrid fungus in living frogs in Brazil. In these individuals, the degree of infection was not severe, and the animals did not exhibit external lesions characteristic of advanced disease. *P. signifer* were apparently healthy when they were captured. According to some authors, the frogs *Taudactylus eugellensis* [34] and *Litoria wilcoxii* [35] appear to be a vector and reservoir of the fungus, since they have fungus sporangia in the integument but do not exhibit severe cutaneous lesions. Furthermore, the authors commented that in the natural environment they appear to be living as healthy frogs.

In South America, this fungus has previously been found in Uruguay in captive bullfrogs (*Lithobates catesbeianus*) [7], and in Argentina in *Leptodactylus ocellatus* [18]. Brazil however, in, the chytrid fungus until now only has been identified in preserved anuran specimens from zoological collections: *Colostethus olfersioides* from Nova Iguaçu (Reserva Biológica do Tinguá, RJ), collected in 1981, *Bokermannohyla gouvai* from Itamonte (Parque Nacional de Itatiaia, Pedra da Lapa, MG), collected in 2005, *Hypsiboas freicnecae* from Jaqueira (Reserva Particular do Patrimônio Natural Frei Caneca, PE), collected in 2001, *Crossodactylus caramaschii* from Apiaí (SP), collected in 2003, *Toropa miliaris* from Peruíbe (Estação Ecológica Juréia-Itatins, SP), collected in 1998 [25]. However, no histological sections indicative of chytridiomycosis were shown.

Only few sporangia of the chytrid fungus in the epidermis of wild *P. signifer* were visualized in association to strong epithelial disruptions with characteristic of hyperkeratosis. This morphological evidence suggests that the integument lesion occurs before the chytrid fungus colonizes the epidermis.

Is well know that stratospheric ozone is Earth´s natural protection for all life forms, shielding our planet from harmful ultraviolet-B (UV-B) radiation, which causes skin cancer, cataracts and immune suppression both in wild animals and in humans [36-38].

The keratosis, as a pathological condition in which the natural immunity of the organism is depleted, is usually induced by sun exposure [39] and, in humans, is characterized by dysplastic epidermal lesions that can involve the dermis. If an amphibian has a cutaneous lesion, probably caused UV radiation, an opportunist fungus (as chytrid fungus) can break the horny layer and replicate in the epithelial cells. Thus, infection may spread from integument to the entire body causing death.

While chytridiomycosis has been reported as the main cause of amphibian mass mortality associated with population declines [2], the origin of this virulent pathogen is unknown. It is mentioned that the chytrid fungus colonizes keratinized epithelium of adult amphibians [5, 6]. According to Rollins-Smith and Conlon [40], the ability of this fungus to replicate in the outermost layer some distance away from the vascularized layers of the integument may protect the pathogen from the cells of the immune system (antigen presenting cells, B and T lymphocytes).

Thus, the chytrid fungus may be an opportunist pathogen that can parasitize the anuran integument. In addition, the presence of the chytrid fungus in *P. signifer* also suggests that this frog can survive a low level of the disease, and the fungus may be spreading throughout the environment.

Since *P. signifier* is a common frog in natural environment in Rio de Janeiro, Brazil, further studies are necessary in order to establish if this frog represents a natural reservoir and/or is a vector of the chytrid fungus. Research is also necessary to investigate the possible infection of other anuran species within the same local community.

Although the study points out that chytrid fungus is the pathogen that causes amphibian death, our results led us to consider the possibility that the chytrid fungus is not the main agent responsible for the amphibian death. Furthermore, due to the depleted immunity caused by keratosis, the chytrid fungus can infect the integument and cause the disruption of normal homeostasis. Thus, the amphibian declines can be caused by the hyperkeratosis that becomes the integument barrier less efficient. This integumentary disruption may be due to global changes caused by the UV-ray increase, and only making and enforcing of laws by all Governments around the World can prevent people and animals from the UV-rays and other harmul environmental agents.

Since the first Global Environment Summit in 1992, national strategies are now generally in place to integrate plans for conservation management within and between industrial sectors and communities to meet appropriate environmental, economic and social objectives. The goal is to provide the principles and tools to soften the clash between Earth's ability to sustain life and the effects of its human occupancy. This means developing methods for biological conservation management together with organization of softer technology for production (natural economy) as well as 'green' legislative actions.

## REFERENCES


[1] Carey C, Cohen N, Rollins-Smith L. Amphibian declines: an immunological perspective. Dev Comp Immunol 1999; 23: 459-72.

[2] Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R. Emerging infectious diseases and amphibian population declines. Emerg Infect Dis 1999; 5: 735-48.

[3] Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. Quantitative evidence for global amphibian population declines. Nature 2000; 404: 752-75.

[4] Berger L, Speare R, Daszak P, *et al*. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 1998; 95: 9031-6.

[5] Longore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. et sp Nov, a chytrid pathogenic to amphibians. Mycologia 1999; 91: 219-27.

[6] Pessier AP, Nichols DK, Longcore JE, Fuller MS. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). J Vet Diag Invest 1999; 11: 194-9.

[7] Mazzoni R, Cunningham AC, Daszak P, Apolo A, Perdomo E, Speranza G. Emerging pathogen of wild amphibians in frogs *(Rana catesbeiana)* farmed for international trade. Emerg Infect Dis 2003; 9: 995-8.

[8] Muths E, Corn PS, Pessier AP, Green DE. Evidence for disease-related amphibian decline in Colorado. Biol Conserv 2003; 110: 357-65.

[9] Gugnani HC, Okafor JI. Mycotic flora of the intestine and other internal organs of certain reptiles and amphibians with special reference to characterization of *Basidiobolus* isolates. Mykosen 1980; 23: 260-8.

[10] Okafor JI, Testrake D, Mushinky HR, Yangco BG. A *Basidiobolus* sp. and its association with reptiles and amphibians in southern Florida. Sabouraudia 1984; 22: 47-51.

[11] Feio CL, Bauwens L, Swinne D, De Meurichy W. Isolation of *Basidiobolus ranarum* from ectotherms in Antwerp zoo with special reference to characterization of the isolated strains. Mycoses 1999; 42: 291-6.

[12] Taylor SK, Williams ES, Mills KW. Experimental exposure of Canadian toads to *Basidiobolus ranarum*. J Wild Dis 1999a; 35: 58-63.

[13] Taylor SK, Williams ES, Thorne ET, Mills KW, Withers DI, Peir AC. Causes of mortality of the Wyoming toad. J Wild Dis 1999b; 35: 49-57.

[14] Cunninggham AA, Langton TES, Bennet PM, *et al*. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philos Trans R Soc Lond Series B Biol Sci 1996; 351: 1537-9.

[15] Jancovich JK, Davidson EW, Morado JF, Jacobs BL, Collins JP. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Dis Aquatic Organ 1997; 31: 161-7.

[16] Hird DW, Diesch SL, McKinnel RG, *et al*. *Aeromonas hydrophila* in wildcaught frogs and tadpoles (*Rana pipiens*) in Minnesolta. Lab Anim Sci 1981; 31: 166-9.

[17] Berger L, Speare R, Hyatt AD. Chytrid Fungi and Amphibian Declines: Overview, Implications and Future Directions. In: Campbell A, Ed. Declines and Disappearances of Australian Frogs. Canberra: Environment Australia 1999a; pp. 23-33.

[18] Herrera RA, Steciow MM, Natale GS. Chytrid fungus parasiting the wild amphibian *Leptodactylus ocellatus* (Anura: leptodactylidae) in Argentina. Dis Aquatic Organ 2005; 64: 247-52.

[19] Hopkins S, Channing A. Chytrid fungus in Northern and Western Cape frog populations, South Africa. Herpetol Rev 2003; 34: 4.

[20] Lane EP, Weldon C, Bingham J. Histological evidence of Chytridiomycosis in a free-ranging amphibian *Afrana fuscigula* (Anura: ranidae) in South Africa. J S Afr Vet Assoc 2003; 74: 20-1.

[21] Bosch J, Martinez-Solano I, Garcia-Paris M. Evidence of chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of Central Spain. Biol Conserv 2001; 97: 331-7.

[22] Daszak P, Cunningham AA, Hyatt AD. Infectious disease and amphibian population declines. Divers Distrib 2003; 9: 141-50.

[23] Lips KR. Mass mortality and population declines of anurans at upland site in weatern Panama. Conserv Biol 1999; 13: 117-25.

[24] Lötters S, Schulte R, Duellman WE. A new and critically endangered species of *Atelopus* from the Andes of northern Peru (Anura: bufonidae). Rev Esp Herpetol 2004; 17: 101-9.

[25] Carnaval ACOQ, Puschendorf R, Peixoto OL, Verdade VK, Rodrigues MT. Amphibian Chytrid fungus distributed in the Brazilian Atlantic Rain Forest. EcoHealth 2006; doi: 10.1007/sl 0393-005-008-2.

[26] Lillie RD, Fullmer HM. Histopathologic Technique and Practical Histochemistry. 4th ed. New York: MacGraw-Hill Book Co1976.

[27] Berger L, Speare R, Kent A. Diagnosis of chytridiomycosis in amphibians by histologic examination. 1999b. [updated 2007 february 13]. Available from: http://www.jcu.edu.au/school/phtm/PHTM/frogs/histo/chhisto.htm

[28] Duellman WR, Trueb L. Biology of Amphibians. Baltimore and London: Johns Hopkins University Press 1994.

[29] Fox H. Epidermis. In: Bereiter-Hahn J, Matoltsy AG, Richards S, Eds. Biology of the Integument. 2. Vertebrates. Berlin, New York: Springer-Verlag 1986a; pp. 78-110.

[30] Fox H. Dermis. In: Bereiter-Hahn J, Matoltsy AG, Richards S, Eds. Biology of the Integument. 2. Vertebrates. Berlin, New York: Springer-Verlag 1986b; pp. 111-149.

[31] Azevedo RA, Pelli AA, Ferreira-Pereira A, Santana ASJ, Felsemburgh FA, de Brito-Gitirana L. Structural aspects of the Eberth-Katschenko layer of *Bufo ictericus* integument: histochemical characterization and biochemical analysis of the cutaneous calcium (Amphibian, Bufonidae). Micron 2005; 36: 61-5. Erratum in Micron 2005; 36(4): 385-6.

[32] de Brito-Gitirana L, Azevedo RA. Morphology of *Bufo ictericus* integument (Amphibia, Bufonidae). Micron 2005; 36(6): 532-8.

[33] Felsemburgh FA, Carvalho-e-Silva SP, de Brito-Gitirana L. Morphological characterization of the anuran integument of the *Proceratophrys* and *Odontophrynus* genera (Amphibia, Anura, Leptodactylidae). Micron 2007; 37: 439-45.

[34] Retallick R, McCallum HI, Speare R. Apud fungus knocks a frog down but not out, raising questions about amphibian declines. [updated 2007 february 13; cited 2004 oct 5]. Available from: http://www.sciencedaily.com/releases/2004/10/ 041004191756.htm

[35] Kerry MK, Hero JM. Survivorship in wild frogs infected with chytridiomycosis. EcoHealth 2006; doi: 10.1007/s10393-006-0027-7.

[36] Madrinich S, McKenenzi RL, Björn LO, Caldwell MM. Changes in biologically active ultraviolet radiation reaching the Earth´s surface. J Photochem Photobiol B 1998; 46: 5-19.

[37] Gruijil FR. Skin cancer and solar UV radiation. Eur J Can 1999; 35: 2003-9.

[38] Collins JP, Storfer A. Global amphibian declines: sorting the hypothese. Divers Distrib 2003; 9: 89-98.

[39] Baba T, Yaoita H. UV Radiation and *Keratosis folicularis*. Arch Dermatol 1984; 120: 1484-7.

[40] Rollins-Smith LA, Colon JM. Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Dev Comp Immunol 2005; 29: 589-98.

Received: August 02, 2008 Revised: December 05, 2008 Accepted: March 17, 2009