BATRACHOCHYTRIUM DENDROBATIDIS OCCURRENCE IN DEAD AMPHIBIANS OF CENTRAL MEXICO: A REPORT OF *AMBYSTOMA ALTAMIRANI* AND *LITHOBATES MONTEZUMAE*

PRESENCIA DE *BATRACHOCHYTRIUM DENDROBATIDIS* EN ANFIBIOS MUERTOS DEL CENTRO DE MÉXICO: UN INFORME DE *AMBYSTOMA ALTAMIRANI* Y *LITHOBATES MONTEZUMAE*

M. Delia Basanta[1,2], Omar Betancourt-León[3], Oscar L. Chávez[4], Armando Pérez-Torres[3], Eria A. Rebollar[5], Emanuel Martínez-Ugalde[5], Víctor D. Ávila-Akerberg[6], Tanya M. González Martínez[6,7,8], Montserrat Vázquez Trejo[8] and Gabriela Parra-Olea[1*]

[1]*Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, AP 70-153, Ciudad Universitaria, Ciudad de México 04510, México.*
[2]*Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México, México.*
[3]*Departamento de Biología Celular y Tisular, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México 04510, México.*
[4]*Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlanepantla, Estado de México 54090, México.*
[5]*Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, México.*
[6]*Instituto de Ciencias Agropecuarias y Rurales, Universidad Autónoma del Estado de México, Toluca, Estado de México, México.*
[7]*Posgrado en Ciencias Agropecuarias y Recursos Naturales, Universidad Autónoma del Estado de México, Toluca, Estado de México, México.*
[8]*Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad Universitaria, Ciudad de México 04510, México.*
Correspondence: gparra@ib.unam.mx
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Resumen.— La quitridiomicosis, causada por los hongos *Batrachochytrium dendrobatidis* (*Bd*) y *B. salamandrivorans* (*Bsal*), es una enfermedad infecciosa relacionada con la muerte masiva de anfibios en todo el mundo. En este estudio, se analizaron cuatro individuos muertos y moribundos de *Ambystoma altamirani* y *Lithobates montezumae* para detectar la presencia de *Bd* y *Bsal*. Mediante el uso de PCR en tiempo real (qPCR) e histopatología, se detectó la presencia de *Bd* y la ausencia de *Bsal* en todos los individuos analizados. Estos resultados indican que la quitridiomicosis puede representar una amenaza para estas especies, y sugieren la urgencia de realizar futuros estudios que evalúen la infección por *Bd* en las poblaciones de *A. altamirani* y *L. montezumae*.

Palabras clave.— Quitridiomicosis, anfibios, declives, enfermedades infecciosas.

Abstract.— Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), is an infectious disease of amphibians linked to mass amphibian die-offs worldwide. In this study, we sampled four dead and dying individuals of *Ambystoma altamirani* and *Lithobates montezumae* to detect the presence of *Bd* and *Bsal*. By real-time PCR (qPCR) and histopathology methods, we found the presence of *Bd* and the absence of *Bsal* in all individuals sampled. Our study indicates that chytridiomycosis may act as a threat for these species and highlight that future surveys are urgently needed to evaluate the *Bd* infection on populations of *A. altamirani* and *L. montezumae*.

Keywords.— Chytridiomycosis, amphibians, declines, infectious disease.

Chytridiomycosis is cataloged as the worst infectious disease in vertebrates due to the great extent of affected species and the mass amphibian die-offs caused worldwide over the last century (Gascon et al., 2007). The disease is caused by two fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). Chytridiomycosis causes hyperkeratosis and hyperplasia, which can cause death and even catastrophic declines in susceptible species' populations (Voyles et al., 2009; Martel et al., 2013). In Mexico, *Bd* has been found in 83 amphibian species (Basanta et al., 2019; Bolom-Huet et al.,

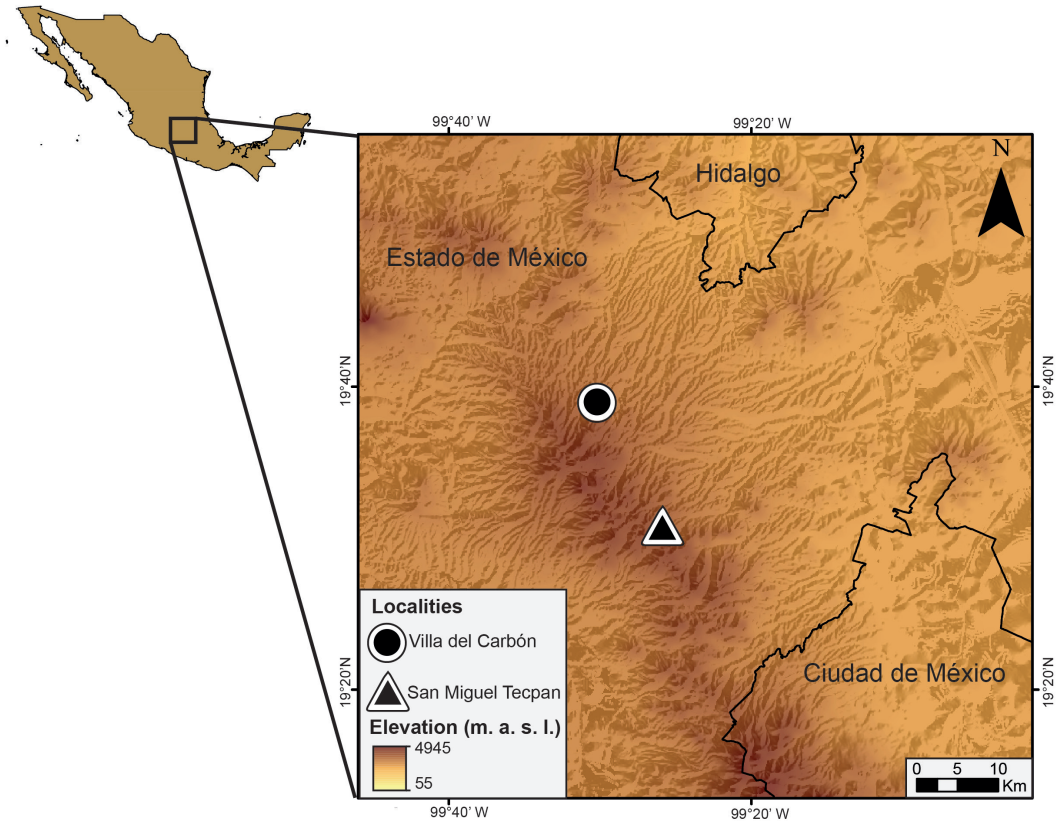

Figura 1. Mapa de las localidades Villa del Carbón y San Miguel Tecpan donde fueron encontrados los individuos moribundos o muertos de *A. altamirani* y *L. montezumae* infectados por *Bd*.
Figure 1. Map of the localities Villa del Carbón and San Miguel Tecpan where individuals of *A. altamirani* and *L. montezumae* were found moribund or dead, and infected by *Bd*.

2019; Hernández-Martínez et al., 2019), while the *Bsal* has not been detected yet in the country (Olivares-Miranda et al., 2020; Waddle et al., 2020).

Between January-July 2019 (winter and summer seasons) we found two dead and one dying individuals of *Ambystoma altamirani*, and one dead individual of *Lithobates montezumae* in the municipalities of Villa del Carbón, and Jilotzingo (at San Miguel Tecpan locality), Estado de México, all located in the northern part of Sierra de las Cruces in Central Mexico (Fig. 1). All specimens were found without obvious external causes of death or damage (e.g., predation or injury), and the dying individual showed chytridiomycosis signs such as lack of reflexes, stiffness, and extreme skin shedding (Fig. 2). The specimens of *A. altamirani* (N = 3) and *L. montezumae* (N = 1) were swabbed with a synthetic cotton swab following the protocol by Hyatt et al. (2007). All the individuals were fixed and stored in neutral 10% formalin. In the laboratory, DNA extraction from swab samples was performed using Prepman or Qiagen Blood and Tissue Kit DNA extraction (Table 1). Then, samples were assayed using real-time TaqMan PCR assays according to Boyle et al. (2004) and Martel et al. (2013) to detect *Bd* and *Bsal* presence, respectively. Each sample was run in duplicate with a negative control (5 uL sterile water) and four standards of DNA Gblocks (1, 100, 1000, and 10000 genome equivalents, GE) for separate assays of *Bd* and *Bsal*.

Multiple skin samples from fixed individuals were obtained for histological examination according to Berger et al. (1999). Briefly, skin samples were dehydrated in ethanol of increasing gradation, from 40% to 100%, clearing with xylene, and embedded in paraffin using a Tissue Embedding Center-Tissue-Tek®. Microtomy with disposable blades was carried out in a microtome Leica RM2125RT to obtain 4-6 µm thick sections which were stained with hematoxylin and eosin (Berger et al., 1999) or Schiff periodic acid histochemistry (PAS) and analyzed with an Olympus BX50 microscope equipped with a Lumenera digital camera and Infinity Analyze 6.3.0 software. The search

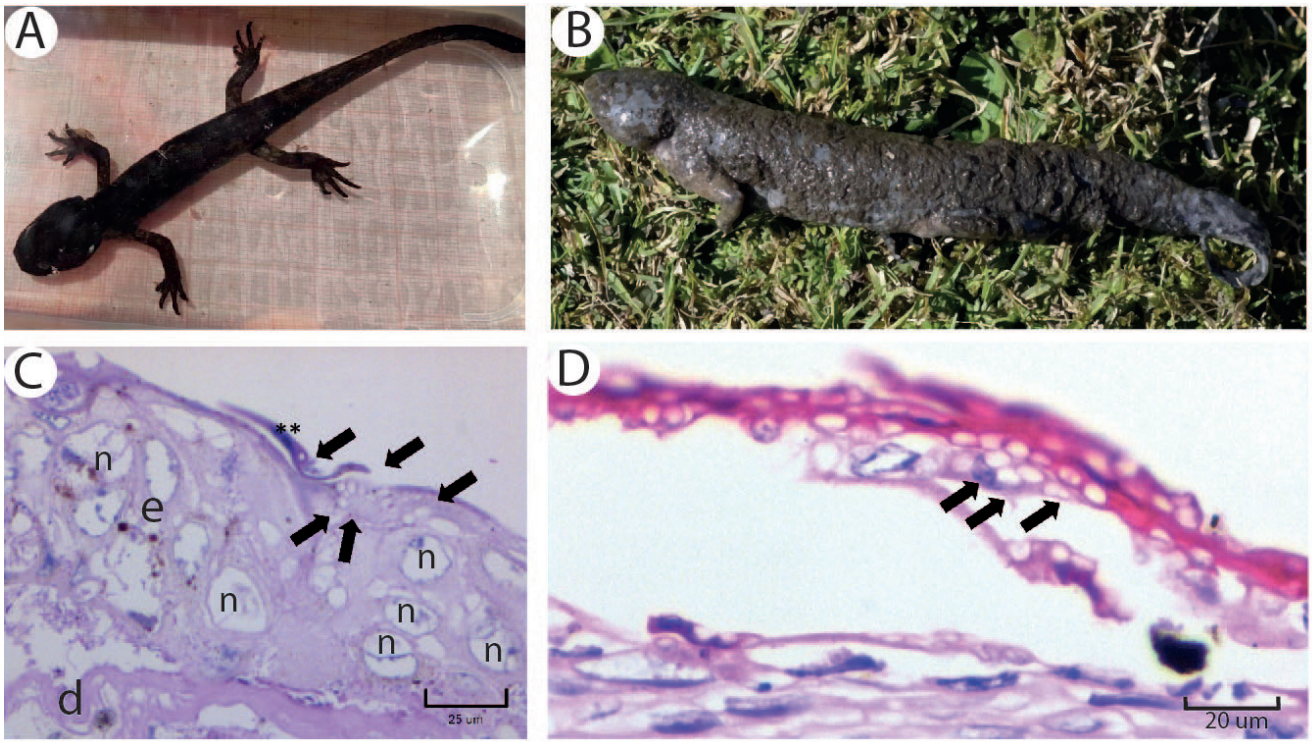

Figura 2. Individuos de *Ambystoma altamirani* infectados por *Bd*. A) individuo moribundo con desprendimiento de piel extremo, B) individuo muerto, C) piel con zoosporangios de *Bd*, D) fragmento de exfoliación epidérmica con zoosporangio incrustado. Dermis (d), epidermis (e), núcleo de una célula epitelial (n), zoosporangio (flechas), exfoliación epidérmica (**). Fotos de Eria A. Rebollar (A), Oscar L. Chávez (B), y microfotografías de Omar Betancourt y Armando Pérez-Torres (C-D).

Figure 2. Individuals of *Ambystoma altamirani* infected by *Bd*. A) dying individual with extreme skin shedding, B) dead individual, C) skin with *Bd* zoosporangia, D) fragment of epidermal exfoliation with an embedded zoosporangium. Dermis (d), epidermis (e), nucleus of an epithelial cell (n), zoosporangia (arrows), epidermal exfoliation (**). Photographs by Eria A. Rebollar (A), Oscar L. Chávez (B), and photomicrographs by Omar Betancourt and Armando Pérez-Torres (C-D).

for *Bd* zoosporangia was carried out at a total magnification of 400X. All specimens were deposited in the Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).

The analyses of qPCR showed *Bd* presence and *Bsal* absence in all swab samples of *A. altamirani* and *L. montezumae* (Table 1). Dead individuals showed lower *Bd* infection loads than the dying individual (Table 1). Histopathology of skin showed evidence of fungal infection in all *A. altamirani* individuals. Spherical and ovoid zoosporangia, empty or containing zoospores, were identified in the superficial and partially detached keratinized cell layers of the epidermis (Fig. 2) with irregular thickening of the epidermis due to hyperplasia. The infected areas included zoosporangia ranging from 5 µm to 10 µm in diameter, mild to moderate hyperkeratosis, and areas of focal erosion adjacent to the infection. These observations agree with *Bd* infection as described by Berger et al. (1999). Skin histopathology of *L. montezumae* skin showed diffuse epidermal detachment related to postmortem changes, so the search for fungal infection was not feasible.

Our finding constitutes the first record of *Bd* in dead or dying amphibians of Central Mexico. The presence of dead and moribund specimens on different occasions and seasons of the year of *A. altamirani* suggests that these species could be susceptible to *Bd* infection. The low *Bd* infection load found in dead individuals may have been due to DNA degradation as a cause of the deteriorating state of the specimen, while the high infection load found in the two dying individual suggests that *Bd* may be one of its causes of death. *Ambystoma altamirani* and *L. montezumae* are threatened and endemic species of Mexico. The axolotl *A. altamirani* has a restricted distribution in Central Mexico, considered "Endangered" by the IUCN (IUCN, 2020a) and identified as "Threatened" by the Mexican law (NOM-059; SEMARNAT 2015). Meanwhile, the frog *L. montezumae* has a wide distribution in Central Mexico and it is considered a species of "Least Concern" by the IUCN (IUCN, 2020b) and subject to

Tabla 1. Individuos analizados para la detección de *Bd* y *Bsal* y depositados en la Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).
Table 1. Individuals sampled for Bd and *Bsal* detection and deposited in the Colección Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM (IBH).

Species	Individual status	Collection date	Locality	Extraction method	Histological examination	Bd load GE	Bsal load GE	Voucher number
Ambystoma altamirani	Dead	January 2019	Villa del Carbón	Not evaluated	Presence of zoosporangia	Not evaluated	Not evaluated	IBH32583
Ambystoma altamirani	Dead	April 2019	Villa del Carbón	Prepman	Presence of zoosporangia	1.4	0	IBH32584
Ambystoma altamirani	Moribund	July 2019	San Miguel Tecpan	Qiagen	Presence of zoosporangia	337,927	0	IBH32585
Lithobates montezumae	Dead	January 2019	Villa del Carbón	Prepman	Not evaluated	329.4	0	IBH32582

"Special Protection" by Mexican law (NOM-059; SEMARNAT, 2015). The main threats to both species are habitat loss, pollution of the streams where these species are distributed, and the presence of invasive fish species (Lemos-Espinal et al., 1999). Our study indicates that chytridiomycosis is an additional threat for these native species from Central Mexico. Previous studies of *Bd* detection on wild *Ambystoma* and *Lithobates* species in Mexico have found medium to high *Bd* prevalence, but without any dead individuals or those with signs of the disease chytridiomycosis (Frías-Alvarez et al., 2008; García-Feria et al., 2017; Peralta-García et al., 2018; Basanta et al., 2019).

Based on our results, future surveys are urgently needed to evaluate the prevalence and infection intensity in populations of *A. altamirani* and *L. montezumae* across their respective distributions, so that proper conservation strategies can be implemented for these species.

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