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PRESENCE AND PREVALENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN COMMERCIAL AMPHIBIANS IN MEXICO CITY

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Abstract: In México City, native and exotic amphibians are commonly sold through the pet trade. This study investigates the presence of *Batrachochytrium dendrobatidis* (*Bd*) in native amphibians being sold at two commercial markets and at a herpetarium in Mexico City. A total of 238 individuals (6 genera and 12 species) were tested for *Bd* using real-time polymerase chain reaction (PCR) analysis. There were 197 *Bd*-positive individuals (prevalence 82%) from five species of amphibians. *Hyla eximia* from the markets had very high *Bd* prevalence (100%; 76/76 and 99%; 88/89) but those from the herpetarium were *Bd* negative (0/12). *Ambystoma mexicanum* from the herpetarium also had a high *Bd*-positive prevalence (80%; 28/35). Though *A. mexicanum* is nearly extinct in the wild, a commercial market continues to flourish through the pet trade. Now that captive colonies of *A. mexicanum* are currently used for reintroduction programs, the authors recommend quarantine to reduce spread of *Bd* via movement of infected animals in the trade and between colonies and via disposal of wastewater from captive collections.

Key words: Amphibians, *Batrachochytrium dendrobatidis*, chytridiomycosis, commercial trade, México.

INTRODUCTION

Amphibian species around the world are in crisis, with nearly one-fourth at risk of extinction.[36,41] Although the causes of these declines vary, their most recent declines have been attributed to a deadly pathogenic chytridiomycete fungus, *Batrachochytrium dendrobatidis* (*Bd*), which causes the disease chytridiomycosis in amphibians,[4,22] This newly emerged pathogen has been implicated in the decline of over 200 amphibian species worldwide,[39] leading to species extinction in the most extreme cases.[40]

Mexico hosts the fifth highest amphibian diversity on the American continent, with 376 species and a high degree of endemic species (67%).[25] Recent declines have threatened a majority of Mexican amphibians such that 71 are critically endangered, 85 threatened, and 42 vulnerable.[16] In recent years there has been strong evidence linking *Bd* with declines in Mexican anurans.[8,12,31,35] Past and recent surveys have found *Bd* to be widely distributed throughout México from the northern states of Sonora,[29] Baja California,[23] and Central México[12,38] to the southern states of Oaxaca and Chiapas.[21]

The global trade in amphibians for human consumption is suspected to have facilitated the emergence of *Bd*. Many commercial bullfrogs are infected with *Bd*–global panzootic lineage (GPL) genotypes,[30,34] but novel highly divergent *Bd* genotypes (*Bd*-Brazil) were also detected from a live bullfrog in a United States market, in native Brazilian anurans in the Atlantic Forest where bullfrogs are widely farmed, and in Japan, primarily on invasive *Rana* (*Lithobates*) *catesbeiana*.[34] Evidence of a hybrid genotype produced via sexual reproduction between the *Bd*-GPL and *Bd*-Brazil lineages in the Atlantic Forest[30,34] has also been reported, and it is hypothesized that anthropogenic mixing of allopatric lineages of *Bd* has led to the generation of the hypervirulent *Bd*-GPL.[9] The finding that *Bd* is capable of sexual reproduction between panzootic and endemic genotypes emphasizes the risk of international wildlife trade as a source of additional *Bd* epizootics owing to hybridization.

The international spread of this infectious disease has been mediated through the trade and movement of other amphibian hosts,[19] such as the American bullfrog, *R.* (*L.*) *catesbeiana*.[33] *Bd*-infected commercial bullfrogs have been found in a number of countries such as the United States, Canada, Brazil, Uruguay, United Kingdom, France, Italy and China.[3,13,28] In Mexico City,

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Table 1. List of species and localities surveyed for *Batrachochytrium dendrobatidis*. Prevalence of infection is given for each species.

Species	No. of infected and examined organisms (prevalence %)	International Union for Conservation of Nature category[a]
Locality: Herpetarium		
Ambystoma mexicanum	28/35 (80)	CR
Rhinella marina	0/3 (0)	
Incilius perplexus	0/2 (0)	EN
Incilius sp.	0/1 (0)	
Incilius valliceps	0/3 (0)	LC
Hyla arenicolor	0/6 (0)	LC
Hyla eximia	0/12 (0)	LC
Hyla sp.	1/1 (100)	
Pachymedusa dacnicolor	0/3 (0)	LC
Rana (*Lithobates*) *catesbeiana*	0/1 (0)	LC
Locality: Nuevo San Lazaro market		
H. eximia	76/76 (100)	LC
R. (*L.*) *catesbeiana*	1/3 (33)	LC
Rana (*Lithobates*) *montezumae*	1/1 (100)	LC
Rana sp.	2/2 (100)	
Locality: Emilio Carranza market		
H. eximia	88/89 (98)	LC

[a] CR indicates critically endangered; EN, endangered; LC, least concern.

native and exotic amphibians are commonly sold through the pet trade. *Batrachochytrium dendrobatidis* is a waterborne and highly infectious agent that could easily spread without proper holding and handling regulations in amphibian markets. The potential for these animals to harbor *Bd* and spread it to wild amphibian populations is of great concern for Mexican amphibians. This study investigates the presence of *Bd* in commercial amphibians being sold in two markets and a herpetarium in México City. Documenting the presence of *Bd* in commercial amphibians is critical for controlling the further spread of novel *Bd* strains in Mexico and preventing further devastation caused by this emerging infectious disease.

Determining the presence of this pathogen in commercial amphibians will help document the spread of *Bd* in Mexico and inform decisions on managing the trade of amphibians in an effort to control this emerging disease.

MATERIAL AND METHODS

Sampling for *Bd* was conducted at two of the most popular pet markets in Mexico City where native and exotic amphibians are sold: San Lazaro and Emilio Carranza markets. Amphibians at the herpetarium of the National Autonomous University of Mexico campus Iztacala, which houses native amphibians for commercial as well as education and research purposes, were also surveyed.

Each frog was sampled for *Bd* infection using sterile cotton swabs run over the body of each animal following the protocols of Kriger et al.[20] In order to prevent cross contamination, new latex gloves were used for each individual, and once sampled, the frog was transferred to a different container. For *Ambystoma mexicanum*, a neotenic salamander, swabs were taken from keratinized mouthparts. Swabs were stored in 96% ethanol and kept at 4°C until processed. Markets were sampled five times only between November and December of 2008 because of permit constraints (Table 1). The San Lazaro market sells both native and imported amphibians; however, Emilio Carranza market housed only the native anuran, *Hyla eximia*. A sample equivalent to 10% of the total number of specimens of *H. eximia* (76 at San Lazaro and 89 at Emilio Carranza) was sampled. Three other Mexican anuran species, *Rana* (*Lithobates*) *montezumae* ($n = 1$) and *Lithobates* sp. ($n = 2$) plus, *R.* (*L.*) *catesbeiana* ($n = 5$) were also swabbed. The herpetarium was sampled once in November 2008 (Table 1). Because of herpetarium protocol, only 10% of *A. mexicanum* were sampled ($n = 35$). Nine additional species, *Rhinella marina* ($n = 3$), *R.* (*L.*) *catesbeiana* ($n = 1$), *Incilius perplexus* ($n = 3$), *Incilius valliceps* ($n = 3$), *Incilius* sp. ($n = 1$), *Hyla*

arenicolor ($n = 6$), *H. eximia* ($n = 12$), *Hyla* sp. ($n = 1$), and *Pachymedusa dacnicolor* ($n = 3$), were also sampled for *Bd*.

Ethanol from sample swabs was evaporated in a vacuum centrifuge at room temperature. Dried swabs were extracted using 160 µl PrepMan Ultra (Applied Biosystems, Foster City, California 94404, USA) following the manufacturer's protocols. Extracts were run using quantitative (real-time) Taqman (Applied Biosystems) PCR assays for the detection of *Bd* following protocol of Boyle et al.[5] DNA standards (provided by A.D. Hyatt) were diluted to give 100, 10, and 1 zoospore genome equivalents for use in the Taqman assay.[5]

Because DNA extracts were diluted by a factor of 1/320 the scores were corrected by the same factor and considered a genomic equivalent of 0.1 as evidence of the presence of *Bd*.

The assay results are presented quantitatively as the number of zoospore genomic equivalents (derived from the standard curves) recovered from the skin swab assay for each specimen. Prevalence was estimated as the proportion of individuals per species and per locality that tested positive for *Bd*, and derived the 95% confidence interval (CI) using the two-tailed CI for proportions, or where prevalence was zero, with a one-tailed CI.[14]

RESULTS

A total of 238 individuals (6 genera and 12 species) were tested for *Bd* using real-time PCR analysis. There were 197 *Bd*-positive individuals (prevalence 82%) from five species from all sites tested (Table 1). Despite the presence of *Bd* in these animals, all individuals appeared healthy and did not exhibit any of the clinical signs associated with chytridiomycosis, such as lethargy, weight loss, and skin shedding.[10,27]

At the herpetarium, 80% of *A. mexicanum* (28/35) and one Hyla sp. (1/1) were positive for *Bd*. The other eight species were negative for *Bd* infection. At the San Lazaro market, *Bd* was found in 98% (80/82) of all samples. *Hyla eximia* comprised a majority of these positives with 100% (76/76) of animals positive for *Bd*, but *R.* (*L.*) *montezumae* (1/1), *Rana* sp. (2/2) and one of three specimens of *R.* (*L.*) *catesbeiana* were also positive. At the Emilio Carranza market, 99% of *H. eximia* (88/89) tested positive for *Bd*.

DISCUSSION

The amphibians tested from Mexico City commercial markets and the herpetarium had a high prevalence of *Bd*-positive individuals (82%; 197/239). The native Mexican tree frog (*H. eximia*) had the highest prevalence of *Bd* (92%; 164/177) out of all species tested. Infection in this species was most prevalent in the two market samples whereas the herpetarium samples were negative. This difference in prevalence is likely due to the fact that the two market colonies are large and held in close contact with poor husbandry, whereas the herpetarium frogs are housed separately and in better conditions as part of a live collection.

Hyla eximia has been categorized by the IUCN as Least Concern because it has a wide distribution in central Mexico and it is quite abundant in the rainy season.[16] However, it is also known to carry *Bd* in the wild, so the pathogen is probably being transferred to captivity, where it is the most abundant amphibian sold as pet or as food for reptiles and other amphibians.[12] The use of *H. eximia* as food for other amphibians and its high *Bd* infection prevalence creates high potential for this species to serve as a vector in spreading *Bd* to other amphibians. It has been shown that *Bd*-positive larvae of *Bufo boreas* can transmit the pathogen to uninfected larvae when housed together.[7]

Presence of *Bd* was previously reported in 84% (76/90) of a captive population of the endangered axolotl, *A. mexicanum,*[12] which concurs with these findings of 80% *Bd* infection prevalence in *A. mexicanum* sampled from the herpetarium. *Ambystoma mexicanum* has been listed as critically endangered since 2006, and its dwindling remaining population continues to be threatened by pollution,[16] human harvesting for pet trade, and introduced fish that compete directly for resources and predate on juveniles.[44] Though it is nearly extinct in the wild, a commercial market for *A. mexicanum* continues to flourish through the pet trade. Captive colonies of *A. mexicanum* are currently used for reintroduction programs. However, the high prevalence of *Bd* in this species requires special attention now that these animals are being reintroduced in the wild. In central Spain, an outbreak of *Bd* that resulted in mass mortality of *Alytes obstetricans* may have come as a result of the reintroduction of *Bd*-infected *Alytes muletensis* that originated from a captive-bred colony.[42] On the other hand, infected frogs imported into zoos have caused epidemics of chytridiomycosis in established amphibian collections.[24,26] Special attention needs to be paid to verify the health of *Ambystoma* colonies if individuals are to be transposed to different colonies

in order to increase the genetic pool of the offspring.

Furthermore, it is not known if *Bd* is lethal in *A. mexicanum*; however, it has been reported that *A. mexicanum* can carry relatively high loads of *Bd* infection (1726.29 zoospore equivalents),[12] which have been associated with mortality in other species.[39] Thus, it is advised that any reintroduction program should practice careful periodic screening of *Bd* in captive *A. mexicanum* before reintroduction.

The number of species found for sale during this study was not very large, which may be because the sampling was conducted in November, which is the time of the year when amphibians are least active in the wild. Because many of these amphibians are potentially harvested from the field, a larger abundance and diversity of amphibians would most likely be found at these markets during the rainy season. It is therefore important to conduct further sampling throughout the year in order to sample more commercial amphibians, and also to investigate potential seasonal variation in *Bd* prevalence.

Treatment of *Bd* infection with an antifungal[11,18,24,27] may be a viable method of clearing infection in *A. mexicanum* prior to reintroduction; however, further investigation should be made to test the rigor and safety of this method for this neotenic salamander. Some studies made on anurans show that a treatment of itraconazole at different concentrations and different treatments reliably cures *Bd* infection, but there are also some reports of toxicity.[2,6,15,37]

Evaluation of effectiveness of different treatments and antifungals need to be performed before treating the entire colony.

Amphibian markets and herpetariums potentially serve as hotbeds for housing *Bd* and as a potential spreading source to wild populations. Although the mechanisms of spread for this pathogen are not well known, *Bd* can potentially be transmitted to wild populations in a couple of ways: 1) The release of *Bd*-positive captive amphibians to the wild could spread the pathogen to other wild amphibians, and 2) dead amphibians and wastewater from these facilities are typically discarded through the public sewage system, which could spread the pathogen by means of waterways.

In the international trade, the World Organization for Animal Health (OIE) in the Aquatic Animal Health Code establishes that the competent authority of the importing country of live aquatic animals intended for use in animal feed, or for agricultural, laboratory, zoo, pet trade, industrial, or pharmaceutical use, whose origin is from a country not declared free from *Bd*, should require an international aquatic animal health certificate issued by the competent authority of the exporting country attesting that the aquatic animals have been appropriately treated to eradicate infection and have been subsequently tested to confirm absence of the disease.[43] However, dealing with local fauna and specimens collected within Mexico and sold at markets, there are no specific standards and specifications for husbandry. Because of the huge number of animals sold, the short time remaining in the store, and the limited space for display, traders keep the animals in plastic bags.

Proper management of all animals and wastewater must be regulated to prevent the spread of *Bd* to wild populations.[18,19] Proper disposal of dead animals and treatment of wastewater should follow OIE standards.[32] In order to prevent further spread of invasive *Bd* strains to wild amphibian populations, it is critical for conservation and governmental authorities such as Secretaría del Medio Ambiente y Recursos Naturales and Secretaría de Agricultura Ganadería Desarrollo Rural, Pesca y Alimentación to implement proper handling and disposal procedures. Furthermore, efforts should be made to educate both amphibian merchants and amphibian pet owners to promote safe practices in an effort to mitigate spread of *Bd*.

Conservation efforts to control the impact of *Bd* on wild amphibian populations are still under investigation. However, because of the rapid movement of this disease across the landscape and devastating mass mortality events associated with this disease, strategies to mitigate effects of *Bd* in infected populations may prove challenging. The authors thus recommend continued sampling and implementation of handling and sanitary regulations at amphibian markets as a preemptive act for the conservation of Mexican anurans.

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