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Short Communication

Prevalence of *Batrachochytrium dendrobatidis* in 120 Archived Specimens of *Lithobates catesbeianus* (American Bullfrog) Collected in California, 1924–2007

Monika Huss,[1,4] Laura Huntley,[2] Vance Vredenburg,[3] Jennifer Johns,[1] and Sherril Green[1]

[1]*Department of Comparative Medicine, Stanford University, Edwards Building R321, 300 Pasteur Drive, Stanford, CA 94305-5342*
[2]*Department of Electrical Engineering, Stanford University, David Packard Building, 350 Serra Mall, Stanford, CA 94305-9505*
[3]*Department of Biology, San Francisco State University, San Francisco, CA*
[4]*287 Campus Dr, RAF 1 Quad 7 Bldg 330, Stanford, CA 94305-5101*

Abstract: The chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been identified as a major cause of the recent worldwide amphibian decline. Numerous species in North America alone are under threat or have succumbed to *Bd*-driven population extinctions. The American bullfrog (*Lithobates catesbeianus*) has been reported as a tolerant carrier of *Bd*. In this report, we used a qPCR assay to test 120 archived American bullfrog specimens collected between 1924 and 2007 in California, USA and Baja California, Mexico. The overall prevalence of *Bd* infection in this archived population of *L. catesbeianus* was 19.2%. The earliest positive specimen was collected in Sacramento County, California, USA in 1928 and is to date the earliest positive archived *Bd* specimen reported globally. These data demonstrate that *Bd*-infected wild amphibians have been present in California longer than previously known.

Keywords: *Batrachochytrium dendrobatidis* (*Bd*), American bullfrog (*Lithobates catesbeianus*), emerging pathogen, chytridiomycosis

Chytridiomycosis, an infectious disease caused by *Batrachochytrium dendrobatidis* (*Bd*), represents one of the most significant threats to native amphibian populations and is the most lethal disease affecting vertebrates documented in history (Skerratt et al. 2007). *Bd* colonizes the keratinous tissue in amphibian skin causing hyperkeratosis and excessive skin sloughing which subsequently impairs cutaneous respiration and osmoregulation, and can ultimately result in death (Berger et al. 1998; Voyles et al. 2009, 2012). Some species appear to be tolerant carriers of the fungus, and maintain a subclinical infection (Daszak et al. 2004; Fisher and Garner 2007). California is home to two invasive species of frogs known to be tolerant carriers of *Bd*—the research model organism, *Xenopus laevis*, and the American bullfrog, *Lithobates catesbeianus* (Weldon et al. 2004; Garner et al. 2006). Such *Bd*-tolerant species have been identified as likely causes of *Bd* epizootics in susceptible native amphibians (Cheng et al. 2011; Greenspan et al. 2012; Reeder et al. 2012; Gervasi et al. 2013; Vredenburg et al. 2013).

The study of the distribution and prevalence of *Bd*-tolerant carriers is valuable for understanding the mecha-

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Correspondence to: Monika Huss, e-mail: monikag@stanford.edu

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nisms of disease spread and evaluating the likelihood of future outbreaks. One hypothesis proposes *Bd* originated out of Africa and spread via the release of African clawed frogs *X. laevis* (Weldon et al. 2004) into non-native areas, including North America, Europe, and Australia. Soto-Azat et al. (2010) examined archived specimens collected in South Africa from 1844 to 1994 and detected the earliest confirmed *Bd*-positive specimens in *Xenopus fraseri* in 1933 and *X. laevis* in 1934 both samples were collected within the native African geographical range of these species. By the mid-twentieth century, *X. laevis* was being exported worldwide for use in human pregnancy tests (Gurdon and Hopwood 2000) and later in the pet and laboratory animal trade (Tinsley and Kobel (1996); Daszak et al. 2004; Fisher and Garner 2007).

The American bullfrog is native to eastern North America and spread to California in the late 1800s (Hayes and Jennings 1986; Fisher and Garner 2007; Vredenburg et al. 2010). Introduced populations grew rapidly as they were highly mobile, possessed broad eating habits and had a high reproductive capacity (Moyle 1973; Adams and Pearl 2007). By the early 1900s, established feral populations existed across much of North America (Storer 1922). Recent work by Gervasi et al. (2013) indicates that the American bullfrog may not be tolerant carrier of *Bd.* They found that the American bullfrog can suffer from clinical signs of the disease with some *Bd* strains and with other strains they maintain low infection loads asymptomatically (Gervasi et al. 2013). Furthermore, recent work has suggested that non-amphibian carriers such as the crayfish and birds may have played an important role in the emergence of *Bd* (Liu et al. 2013; McMahon et al. 2013). We sought to answer if *Bd* could be detected on the CAS archived American bullfrog specimens and determine the earliest date at which it existed.

In this report, a *Bd*-PCR assay was used to test for the presence of *Bd* in the entire collection of 120 archived specimens of *L. catesbeianus* from the California Academy of Sciences (CAS). These archival specimens were collected at various locations in California, USA and Baja California, Mexico between 1924 and 2007 by several research groups. Specimens were stored in either individual jars or jars with specimens also collected at the same time and locality. The specimens, preserved in either ethanol or formalin, were sampled using the swabbing techniques and *Bd*-PCR assay previously described by Cheng et al. (2011). To prevent possible cross contamination from multiple specimens, each specimen was rinsed with 70% ethanol. Each specimen was swabbed 30 times using a MW100 sterile synthetic cotton-tipped swab: ten strokes along each side of the ventral surface (including abdomen, pelvis, and thighs) and five strokes on each space of interdigital webbing. Swabs were stored in 1.5 mL microcentrifuge vials and refrigerated at 4°C until extraction. Samples were shipped to the Amphibian Disease Laboratory (ADL) of the San Diego Zoo for PCR testing, using methods described by Boyle et al. (2004) and revised for museum specimens by Cheng et al. (2011). All samples were run in triplicate and reported as positive when replicates showed *Bd* DNA product within 50 cycles in at least two wells (Cheng et al. 2011). Samples that were only positive in one well were rerun in five replicates to confirm results and were reported as positive when product replicated in at least three of the five replicates.

The overall prevalence of *Bd* in archived *L. catesbeianus* specimens from California and Baja California Sur, Mexico was 19.2% (23 positives out of 120). *Bd*-positive specimens were found in 13 of the 27 California counties represented (Table 1). The earliest *Bd*-positive American bullfrog specimen was collected in Sacramento County in 1928 (see Supplemental Table SI). *Bd*-positive specimens were identified in each of the past nine decades with the exception of the 1960s (Table 2). Geographically, the majority of the positive specimens were originally collected in northern California (14/23) near rivers, creeks, or lakes (Pepperwood Creek, San Joaquin River, Capell Creek, Webber Creek, Plumas National Forest Pond, Matanzas Reservoir, Kings River, Hume Lake, Lake Lagunita). A minority of the *Bd*-positive specimens were from southern California in San Bernardino (1/23) and San Diego counties (1/23); central California in Fresno (5/23) and Madera (1/23) counties; and Baja California Sur, Mexico (1/23) (Table 1). *Bd* prevalence was calculated for each county and time interval with the Bayesian probability for 95% credible intervals using the reported positives and total number of specimens sampled under the assumption that all detection probabilities were equal (Cheng et al. 2011). In instances of zero prevalence or low sample size, detection probability could not be calculated without guessing at a false negative *Bd* detection rate.

The findings reported here confirm the historical presence of *Bd*-infected wild American bullfrog (*L. catesbeianus*) populations throughout California, with an overall prevalence rate of 19.2% between 1920 and 2007. In addition, we identified the first archived *Bd*-positive specimen collected from Baja California, Mexico in 1977. This

Table 1. Prevalence of chytridiomycosis in North American archived *Rana catesbeiana*, by county of origin in California or state of origin in Mexico.

County of origin	No. examined	No. positives	% Positive (95% CI[a])
Alameda, CA	6	0	0.0 (0.0, 52.0)
Butte, CA	11	2	18.2 (0.5, 52.8)
El Dorado, CA	5	3	60.0 (12.0, 99.1)
Fresno, CA	9	5	44.4 (13.6, 78.7)
Kern, CA	1	0	N/A
Madera, CA	8	1	12.5 (0.0, 55.0)
Mendocino, CA	1	0	N/A
Merced, CA	1	0	N/A
Monterey, CA	2	0	0.0 (0.0, 98.7)
Napa, CA	4	1	25.0 (0.0, 84.9)
Placer, CA	2	0	0.0 (0.0, 98.7)
Plumas, CA	7	1	14.3 (0.0, 60.3)
Sacramento, CA	4	1	25.0 (0.0, 84.9)
San Bernardino, CA	8	1	12.5 (0.0, 55.0)
San Diego, CA	2	1	N/A
San Joaquin, CA	1	1	N/A
San Luis Obispo, CA	1	0	N/A
San Mateo, CA	2	0	0.0 (0.0, 98.7)
Santa Clara, CA	10	2	20.0 (0.6, 56.6)
Sierra, CA	1	0	N/A
Siskiyou, CA	1	0	N/A
Sonoma, CA	5	2	40 (0.9, 88)
Stanislaus, CA	5	0	0.0 (0.0, 58.7)
Trinity, CA	1	0	N/A
Tuolumne, CA	4	0	0.0 (0, 67.4)
Yolo, CA	5	1	20.0 (0.0, 74.7)
Yuba, CA	2	0	0.0 (0.0, 98.7)
Baja California Sur, MX	11	1	9.09 (0.0, 43.6)

CI confidence interval.

Table 2. Prevalence of chytridiomycosis in archived *Rana catesbeiana* by time intervals, North America.

Time interval	No. examined	No. positives	% positive (95% CI)
1920–1929[a]	3	2	66.7 (1.1, 100.0)
1930–1939	28	3	10.7 (2.2, 29.1)
1940–1949	4	1	25.0 (0.0, 84.9)
1950–1959	12	2	16.7 (0.5, 49.5)
1960–1969	12	0	0.0 (0.0, 31.2)
1970–1979	13	1	7.7 (0.0, 38.4)
1980–1989	8	2	25.0 (0.7, 66.0)
1990–1999	23	5	21.7 (8.0, 43.8)
2000–2009[b]	17	7	41.2 (19.3, 66.4)

CI confidence interval.
[a]Earliest specimen dates from 1924; earliest positive dates from 1928.
[b]Latest specimen dates from 2007.

study also describes the earliest *Bd*-positive specimen recorded in the literature to date, a *L. catesbeianus* collected in 1928 in Sacramento County, California. Previously, the earliest confirmed *Bd*-positive archival specimens were reported in *X. fraseri* collected in South Africa in 1933 (Soto-Azat et al. 2010), *Xenopus borealis* collected in Kenya in 1934 (Vredenburg et al. 2013), and *X. laevis* collected in South Africa in 1935 (Weldon et al. 2004).

Prior to this report, the earliest confirmed *Bd*-positive specimen in the American bullfrog originated in Palo Alto, California in 1961 (Padgett-Flohr and Hopkins 2009). Padgett-Flohr and Hopkins (2009) examined a subset of CAS bullfrog specimens using histological methods to test for *Bd*. However, this method can be insensitive as in many cases *Bd* infections may be localized (Berger and Speare 1998). For example, Reeder et al. (2012) described how the histological changes seen in *Pseudacris regilla*, as a result of *Bd*, are patchy. This patchy distribution permits *P. regilla* to have enough functionally normal skin to avoid the osmotic imbalances that ultimately lead to the death of most species (Reeder et al. 2012). In contrast, the PCR swabbing samples a greater surface area of the skin; furthermore several recent studies show that the *Bd*-PCR assay is an effective method to detect *Bd* on formalin-preserved specimens (Cheng et al. 2011; Richards-Hrdlicka 2012).

There are multiple hypotheses describing how *Bd* may have emerged in California. Our discovery of a positive specimen prior to the first identified specimen in southern Africa and the start of the international trade of *X. laevis* suggests that the *Xenopus* introduction route was not the only potential pathway for the introduction of *Bd*. It is therefore possible that *Bd* was introduced to California earlier than previously thought, perhaps with the importation of American bullfrogs that began in the late 1800s. It is also possible that neither *X. laevis* nor *L. catesbeianus* were the first or primary means of introducing *Bd* into North America.

Our results indicate that *L. catesbeianus* were *Bd*-infected, but the strain of *Bd* in these specimens remains unknown. It is important to consider that when using qPCR, different strains yield different infection intensity due to variability in the ITS copy number (Boyle et al. 2004; Longo et al. 2013). Our PCR test was developed using the

global pandemic lineage of *Bd*, so it is possible we may have lower detection probabilities for other strains. Given that we used PCR only to test for positive or negative results, our results are less likely to be impacted by strain variability. Additionally, as a retrospective study, we do not know the exact history of each specimen or how it was stored. We could also not sequence enough *Bd* DNA to determine *Bd* strain, because formalin preservation prohibits isolation of high quality DNA. Future work may consider using new technology to sequence isolated *Bd* DNA and determine individual *Bd* strains. This would certainly provide further valuable insight into the epidemiology and spread of the disease. Despite the absence of strain information, this retrospective study gives important insight into the epidemiology and historical perspective of *Bd* infection in California. Our data indicate that *Bd* was present in the American bullfrog in California much earlier than previously known.

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