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Low Prevalence of Chytrid Fungus (*Batrachochytrium dendrobatidis*) in Amphibians of U.S. Headwater Streams

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ABSTRACT.—**Many declines of amphibian populations have been associated with chytridiomycosis, a disease caused by the aquatic fungus *Batrachochytrium dendrobatidis* (Bd). Despite the relatively high prevalence of chytridiomycosis in stream amphibians globally, most surveys in North America have focused primarily on wetland-associated species, which are frequently infected. To better understand the distribution and prevalence of Bd in headwater amphibian communities, we sampled 452 tailed frogs (*Ascaphus truei* and *Ascaphus montanus*) and 304 stream salamanders (seven species in the Dicamptodontidae and Plethodontidae) for Bd in 38, first- to third-order streams in five montane areas across the United States. We tested for presence of Bd by using PCR on skin swabs from salamanders and metamorphosed tailed frogs or the oral disc of frog larvae. We detected Bd on only seven individuals (0.93%) in four streams. Based on our study and results from five other studies that have sampled headwater- or seep-associated amphibians in the United States, Bd has been detected on only 3% of 1,322 individuals from 21 species. These results differ strongly from surveys in Central America and Australia, where Bd is more prevalent on stream-breeding species, as well as results from wetland-associated anurans in the same regions of the United States that we sampled. Differences in the prevalence of Bd between stream- and wetland-associated amphibians in the United States may be related to species-specific variation in susceptibility to chytridiomycosis or habitat differences.**

Infectious diseases are a growing threat to global biodiversity, including several species of amphibians (Daszak et al., 2003; Skerratt et al., 2007). Amphibian declines on several continents have been attributed to chytridiomycosis, a directly transmitted disease caused by the aquatic fungus *Batrachochytrium dendrobatidis* (Bd; Berger et al., 1998; Bosch et al., 2001; Muths et al., 2003). This parasitic fungus colonizes the keratinized epidermis and mouthparts of amphibians, often resulting in death of juveniles and adults. The effects of Bd on amphibian larvae are less well known. In some species, infection with Bd does not seem to cause mortality during the larval stage, although larvae may transmit disease to other amphibians or die from chytridiomycosis after metamorphosis (Rachowicz et al., 2006; Rachowicz and Briggs, 2007).

Globally, forest-associated amphibians that live in or along streams are more likely to have declined than species in other assemblages, particularly in Central America and Australia (Woodhams and Alford, 2005; Ryan et al., 2008; Stuart et al., 2008). Despite the relatively high prevalence of chytridiomycosis in stream amphibians in other regions of the world (Berger et al., 1998; Woodhams and Alford, 2005; Smith et al., 2007), most surveys in North America have focused primarily on wetland-associated species, which are frequently infected (e.g., Ouellet et al., 2005; Longcore et al., 2007; Pearl et al.,

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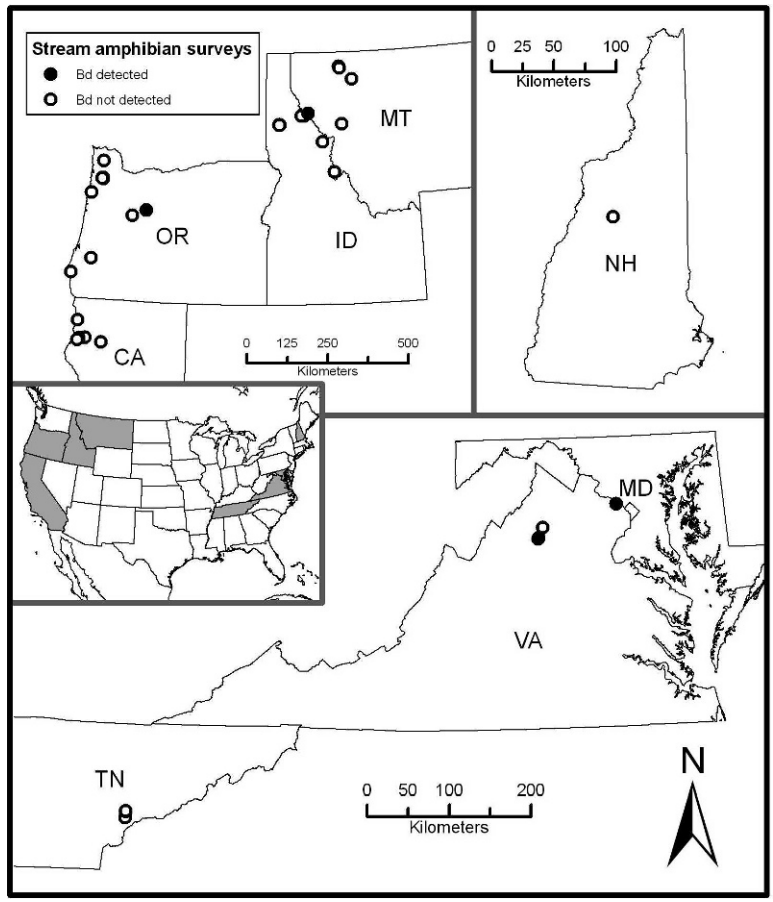

FIG. 1. Location of the 38 headwater streams in seven states (in grey) of the United States that we sampled for the presence of the pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). We sampled 452 tailed frogs (Ascaphidae) and 304 salamanders (Dicamptodontidae and Plethodontidae). The fungus was detected on only seven individuals from four streams (solid black circles). In some cases, circles overlap for neighboring streams. For example, we sampled three adjacent streams in both New Hampshire and Tennessee.

2007; Muths et al., 2008; Rothermel et al., 2008). Approximately one-third of amphibian species in the United States and Canada are associated with cold streams, where they are a major component of watershed biodiversity and biomass and are often the top predators (Hairston, 1987; Corn et al., 2003; Meyer et al., 2007). Several ecological characteristics of amphibians in cold headwater streams may predispose them to infection by Bd: larvae often have overlapping generations and occur in high densities, potentially facilitating transmission; and adults tend to be aquatic or semiaquatic and often share habitats with larvae (Petranka, 1998; McCallum et al., 2001; Corn et al., 2003; Rachowicz and Briggs, 2007). Information on the distribution and prevalence of Bd in stream-associated amphibians in the United States is important because of its potential threat to populations and because variation in a pathogen across habitats and landscape can affect transmission rates and disease dynamics (e.g., McCallum, 2008).

To better understand the distribution and prevalence of Bd in cold headwater streams, we sampled two species of frogs and seven species of salamanders in the Pacific Northwest, northern Rocky Mountains, and Appalachian Mountains in the United States (Fig. 1). Most of the areas we sampled have been surveyed previously for Bd on wetland-associated anurans. Also, we review other studies of Bd in headwater amphibian communities in the United States to help place our results in the context of existing knowledge and to compare the prevalence of Bd in wetland- and stream-associated species.

TABLE 1. Locations and sampling effort for amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on headwater amphibians in five regions of the United States. Parenthetic numbers indicate the number of positive samples. For *Dicamptodon* spp., the larvae column includes any paedomorphs captured. One stream in California and one stream in Idaho were sampled for both *Ascaphus* spp. and *Dicamptodon* spp.

Location	No. streams	Elevation range (m)	Months sampled	Species	No. larvae	No. metamorphosed
Northern Coast, CA	3	122–349	Jun–Dec	*Ascaphus truei*	60 (0)	0
Northern Coast, CA	3	30–541	Jul–Oct	*Dicamptodon tenebrosus*	60 (0)	0
Cascade and Coast Ranges, OR	11	47–1,165	Jun–Aug	*Ascaphus truei*	128 (0)	38 (**3**)
Northern Rocky Mountains, ID and MT	9	1,035–1,637	Jun–Sep	*Ascaphus montanus*	198 (0)	28 (0)
Northern Rocky Mountains, ID and MT	3	1,035–1,410	Jun–Sep	*Dicamptodon aterrimus*	57 (**1**)	3 (0)
Southern Appalachian Mountains, TN	3	480–590	Jul	*Desmognathus monticola*	0	22 (0)
				D. ochrophaeus	0	3 (0)
				D. quadramaculatus	0	34 (0)
				Gyrinophilus porphyriticus	0	1 (0)
Central Appalachian Mountains, MD and VA	5	100–750	Jun–Jul	*Desmognathus fuscus*	0	12 (**1**)
				D. monticola	0	50 (**2**)
Northern Appalachian Mountains, NH	3	300–600	Jul	*Gyrinophilus porphyriticus*	38 (0)	24 (0)

MATERIALS AND METHODS

Site Selection.—We sampled headwater amphibians in the areas of the United States with the greatest diversity of these species (Fig. 1). Stream selection was based upon historical population data, presence of museum collections, or because they were already included in current studies. All streams were first to third order with rocky substrates and were surrounded by conifer or mixed forests.

In the Pacific Northwest and northern Rocky Mountains, we sampled two species of tailed frogs (*Ascaphus truei*, *Ascaphus montanus*) and two species of giant salamanders (*Dicamptodon tenebrosus*, *Dicamptodon aterrimus*; Table 1). Tailed frogs and giant salamanders are the most widespread and common stream amphibians in the northwestern United States. We hypothesized that tailed frog larvae would be likely candidates for Bd because they have a large, keratinized oral disc and have long larval periods (1 to $\geq$ 3 yr). Giant salamanders are the largest resident amphibians in western streams and have long larval periods (1 to $\geq$ 3 yr). Many giant salamanders never leave the stream because paedomorphy is common in this genus (Petranka, 1998). We collected samples from the Pacific Northwest and Northern Rockies between June and December 2008 (Table 1).

In the Appalachians, we targeted the largest, common salamander in each stream because larger species tend to be the most aquatic (Hairston, 1987); thus, we expected to have a greater chance of detecting Bd if it was present (Table 1). All of the eastern salamanders we sampled were in the family Plethodontidae, which includes most seep and headwater salamanders in the region. In New Hampshire, all samples were from the Spring Salamander (*Gyrinophilus porphyriticus*). In the central and southern Appalachians, samples were primarily from Dusky Salamanders (*Desmognathus* spp.; Table 1). The Appalachian species we sampled have larval periods that range from two weeks (*Desmognatha ochrophaeus*) to 5 years (*G. porphyriticus*) and remain strongly associated with streams after metamorphosis (Hairston, 1987; Petranka, 1988). There are no anurans in the eastern United States that are dependent upon headwater streams for breeding. We collected samples from the Appalachians during June and July 2008 (Table 1).

Field Methods.—We captured animals by turning rocks and logs (in water and on stream banks) or electro-fishing, using freshly disinfected aquarium nets and new gloves. We exchanged equipment after each capture to prevent cross-contamination. Each animal was stored individually and kept cool until it was sampled for Bd. All tailed frog samples were dominated by larvae because juveniles and adults were encountered infrequently. Samples of salamanders included larvae, suspected paedomorphs, and metamorphosed individuals that were in or near streams (Table 1).

Our goal was to test 20 individuals per sampling event, which would allow reasonable ability to detect Bd where it was present. Furthermore, our goal for salamanders was to sample three streams in each region. Assuming there were ≥230 individuals per stream, infected individuals were distributed randomly, and a sampling efficiency and specificity for Bd of 100%, 20 individuals per stream would provide 95% probability of detecting Bd at least once if the true prevalence was ≥14% (Cameron and Baldock, 1998). In some Appalachian streams, we collected individuals of more than one species, because no species was abundant; for the same reason, we had to sample five streams in the central Appalachians to capture 60 salamanders (Table 1). To evaluate potential temporal patterns in the detection of Bd, we sampled two-tailed frog streams in each of Oregon and Montana during early summer and again later in the season. Presence of Bd in amphibians is often correlated with temperature (Retallick et al., 2004, Woodhams and Alford 2005), and we hypothesized that prevalence may increase during summer after streams warmed.

Laboratory Methods.—We used different sampling and laboratory methods to test for Bd on tailed frogs and salamanders. We sampled tailed frog larvae by rubbing a cotton-tipped swab (Puritan Medical Products 25-806-1WC) around their oral disc ≥20 times. Metamorphosed frogs were sampled by rubbing their ventral surface, rear legs, and webbing ≥20 times, total. Each swab was placed into a sterile 1.5-ml microcentrifuge tube with 70% ethanol until analysis at the U.S. Fish and Wildlife Service California-Nevada Fish Health Center. DNA was extracted from air-dried swabs using the Qiagen DNEasy Blood and Tissue Kit (Germantown, MD). Extraction followed the manufacturer's protocol including a single DNA elution volume of 200 µl. Extracted DNA was stored at −20°C until assayed. Testing was conducted with a validated real-time PCR assay that used a minor grove binding (MGB) probe and ITS1-3 Chytr and 5.8S Chytr primers specific to Bd DNA sequences (Boyle et al., 2004). Standardized DNA controls, with known genome copy, were provided by Dr. Boyle of the Australian Animal Health Laboratory and used to generate the assay standard curve. The sensitivity of this assay is <0.1 zoospore equivalent per 5 µl PCR reaction.

Quantitative PCR assays were conducted using an Applied Biosystems Prism 7300 Sequence Detection System under the following conditions: 50°C for 2 min, 95°C for 10 min, 40–45 cycles of 95°C for 15 sec, and 60°C for 1 min. Test samples were run in duplicate 30 µl reaction volumes containing 1× Universal master mix (Applied Biosystems, Foster City, CA), 900-nM concentration of each primer, 250-nM concentration Taqman MGB Probe, and 5-µl DNA template (approximately 300ng/reaction). Positive control standards were prepared by 10-fold serial dilution of a 2.5×10^7 stock concentration of zoospores diluted in 30 ng/µl calf thymus DNA. Three standards of 100, 10, and 1 zoospore per 5-µl reaction volume were run in replicate on each assay plate, along with no template controls consisting of molecular grade water.

We sampled salamanders for Bd by rubbing their ventral surface ≥20 times with a cotton-tipped swab (Medical Wire Equipment Company MW100), placing particular emphasis on the feet and the area around the cloaca. Each swab was returned to its tube and refrigerated at ~4°C until it was tested at Virginia Commonwealth University. DNA was extracted from each swab using Prepman Ultra (Applied Biosystems). We used the Bd primers Bd1a and Bd2a developed by Annis et al. (2004) to conduct amplification and PCR analysis of the extracts. Amplification reactions consisted of 1 µM of each primer Bd1a and Bd2a, 25 µl Hotstart Plus Master Mix (Qiagen), 22 µl of water, and 1 µl of DNA from each swab extract. The amplifications were performed on a thermocycler by initial denaturation at 95°C for 15 min, followed by 45 cycles of 45 sec at 93°C, 45 sec at 60°C, and 1 min at 72°C. A final extension at 72°C for 10 min completed the amplifications. We used Bd-positive DNA provided by John Wood from Pisces Molecular LLC, for the positive control and used water as the negative control. Amplified DNA fragments were separated by electrophoresis in 1.2% agarose gel and positive reactions produced a 300 bp band. The expected sensitivity of this assay is ~2 zoospore equivalents per PCR reaction.

After sampling for Bd, we examined each animal for gross abnormalities such as darkened or sloughing skin or other signs of chytridiomycosis. We used a 10× hand lens to examine tailed frog larvae for oral disc abnormalities such as discoloration or loss of tooth rows, which can be correlated with presence of chytridiomycosis (Smith et al., 2007; Symonds et al., 2007).

Results

We sampled 452 tailed frogs and 304 stream salamanders in five mountainous areas of the United States and detected Bd on only seven individuals (0.93%; Table 1). We detected Bd on three adult *A. truei* from one stream in Oregon,

TABLE 2. Summary and location of five other studies that have sampled headwater and seep salamanders for *Batrachochytrium dendrobatidis* (Bd) in the United States. Parenthetic numbers indicate the number of positive samples. The superscripted numbers indicate the source of the data. Chatfield et al. (2009) did not distinguish whether their samples came from the North Carolina or Tennessee portion of their study area. Byrne et al. (2008) also reported results for pooled samples that are excluded here.

Species (States)	Sampled
Desmognathus apalachicolae[3] (GA)	29 (0)
D. conanti[3] (GA)	86 (**2**)
D. marmoratus[3] (GA)	7 (0)
D. monticola[2, 3] (GA, NC)	123 (0)
D. imitator/ocoee[3, 5] (GA, NC/TN)	57 (0)
D. quadramaculatus[2, 3] (GA)	23 (0)
D. fuscus[1] (MD)	66 (**9**)
Eurycea bislineata[1] (MD)	14 (**1**)
E. chamberlaini[3] (GA)	1 (0)
E. cirrigera[3, 4] (AL, GA)	97 (**21**)
E. guttolineata[3, 4] (AL, GA)	18 (0)
E. longicauda[3] (GA)	8 (0)
E. lucifiga[3] (GA)	3 (0)
E. wilderae[3, 5] (GA, NC/TN)	15 (0)
Gyrinophilus porphyriticus[2, 3, 4] (AL, GA, TN)	8 (0)
Pseudotriton ruber[1, 4, 5] (AL, MD, NC/TN)	3 (0)

[1] Grant et al., 2008;
[2] Rothermel et al., 2008;
[3] Timpe et al., 2008;
[4] Byrne et al., 2008;
[5] Chatfield et al., 2009.

one larval *Dicamptodon aterrimus* in Montana, one terrestrial *Desmognathus fuscus* in Maryland, and two *Desmognathus monticola* from 1 stream in Virginia (Table 1). Two of the three Bd-positive *A. truei* had low estimated zoospore loads (0.34, 0.36, 5.62 zoospore equivalents/5-μL reaction). None of the tailed frogs from the four streams that were sampled during both early and late summer tested positive for Bd.

DISCUSSION

Our data suggest that Bd is rare on headwater amphibians in the United States. No previous studies have surveyed stream amphibians for Bd in the Pacific Northwest or Rocky Mountains, although a dead *D. aterrimus* collected in Idaho was confirmed to have chytridiomycosis (Cleary, 2001). *Desmognathus fuscus* have tested positive in Maryland (Grant et al., 2008), but to our knowledge, this is the first report of Bd on wild *D. monticola*. None of the Bd-positive animals showed signs of chytridiomycosis, such as lethargy or sloughing of skin. Only six tailed frog larvae had missing or incomplete toothrows, which appeared to be the result of physical damage rather than the manifestation of disease; none of these larvae tested positive for Bd.

Based on our study and results from five other studies of Bd in amphibians that inhabit cold headwater streams or seeps in the United States, Bd has been detected on only 3% of 1,322 individuals representing 21 species (Byrne et al., 2008; Grant et al., 2008; Rothermel et al., 2008; Timpe et al., 2008; Chatfield et al., 2009; Tables 1 and 2). Many of the species in these studies have been sampled sparingly, but these results are a marked contrast to those in tropical and subtropical ecosystems, where Bd is prevalent on stream anurans and has been associated with extensive declines of diverse amphibian communities (Berger et al., 1998; Woodhams and Alford, 2005; Smith et al., 2007; Symonds et al., 2007; Ryan et al., 2008). Some stream-breeding amphibians in Europe are also susceptible to chytridiomycosis, including species with similar life-history traits and shared phylogenies with species in the United States (Bosch et al., 2001; Bosch and Martínez-Solano, 2006; Bovero et al., 2009). In Queensland, Bd was also more prevalent on amphibians that were associated with permanent streams than those in permanent ponds (Kriger and Hero, 2007).

Our results also differ strongly from surveys for Bd on wetland-associated amphibians in the some of the same regions we sampled. For example, 24–39% of postmetamorphic anurans from wetlands tested positive for Bd in extensive surveys of the Southeast (Rothermel et al., 2008), Pacific Northwest (Pearl et al., 2007), and Rocky Mountains (Muths et al., 2008). Differences in habitat characteristics between wetlands and streams (e.g., flow, temperature) could affect the prevalence of Bd in these communities, as well as taxonomic variation in susceptibility to Bd. Cold temperatures may limit Bd populations in some of the streams we sampled. In the laboratory, Bd grows fastest between 17°C and 24°C and growth ceases <4°C (Piotrowski et al. 2004). Stream temperatures during summer samples from Oregon, the northern Rockies, New Hampshire, and Virginia ranged from 7–14°C. Lower-elevation streams in California, Maryland, and the southern Appalachians were warmer (≤19°C when sampled), but all of the streams we sampled are likely <10°C for much of the year, and streams with snow-dominated hydrology are likely <4°C most of the year (Vannote and Sweeney, 1980). In contrast, most streams in the Neotropics and Australia, where Bd is common, do not experience extended seasonal periods where cold water temperatures would likely inhibit growth of the fungus, although there is evidence that warm water temperatures may be

limiting (Ranvestel et al., 2004; Kriger et al., 2007).

We primarily sampled salamanders, the predominant stream amphibians in North America. Studies that have documented a high prevalence of Bd in streams are mostly from areas where anurans are the primary (or only) stream amphibian. Several wetland-associated and even strictly terrestrial salamanders have been diagnosed with chytridiomycosis (summarized in Byrne et al., 2008), but in general, they seem less susceptible to chytridiomycosis than anurans (Rothermel et al., 2008; Timpe et al., 2008). Several amphibian species, including some plethodontid salamanders that we did not sample, also have bacterial flora or produce antimicrobial peptides that can inhibit growth of Bd (Rollins-Smith et al., 2002; Harris et al., 2006).

Bd could have been rare in our tailed frog samples ($\leq$0.66%) because we sampled larvae primarily. Detection of Bd can be rare on lentic amphibian larvae even in populations where adults are infected (Adams et al., 2010); however, it is commonly found on lentic amphibian larvae that require $\geq$1 year to metamorphose (Fellers et al., 2001; Rachowicz et al., 2006; Rothermel et al., 2008). Chytridiomycosis is common in larvae of stream frogs in eastern Australia (*Mixophyes* spp.) and southern Africa (*Heleophryne natalensis*, *Strongylopus hymenopus*) that have many similarities with tailed frogs: they inhabit rocky, montane streams; many have large oral discs; and they have long developmental periods ($\geq$6 months; Woodhams and Alford, 2005; Smith et al., 2007; Symonds et al., 2007).

Two studies of Bd on amphibians from headwater habitats in the United States have reported significantly higher prevalence rates than we found, which suggests we may have failed to detect pathogen hotspots that occur across the landscape. Bd was detected on 21 of 50 Southern Two-Lined Salamanders (*Eurycea cirrigera*) in headwater streams in a park in Alabama; 26 individuals from five other species in the same park were all negative (Byrne et al. 2008). Grant et al. (2008) detected Bd on 14% of salamanders in Maryland streams and found a higher prevalence of Bd in May than in October. The Grant et al. (2008) data may be exceptional because they sampled an area where streams and wetlands were in close proximity, and species associated with both habitats were likely to come in contact, suggesting that local topography, hydrology, and community dynamics may be important to the distribution of Bd.

In some cases, we did not detect Bd on individuals that inhabited streams where the pathogen was known to occur. None of the salamanders from the three streams we sampled in the southern Appalachians tested positive for Bd, even though the pathogen was detected during concurrent water sampling in all three streams (WJB, unpubl. data). Also, we know Bd is common in some of the watersheds we sampled in the West (Pearl et al., 2007; Muths et al., 2008; Adams et al., 2010). For example, two Montana streams where we sampled *Ascaphus montanus* flowed through an area where Boreal Toads (*Bufo boreas*) have a high prevalence of Bd and have been captured in the sampled streams (BRH and PSC, unpubl. data). The rarity of Bd-positive animals from streams in areas where Bd is common (or even in the streams themselves) suggests that at least some headwater species have resistance to chytridiomycosis (e.g., Vasquez et al., 2009).

By sampling a large number of headwater amphibians from five areas in the United States, we provide further evidence that, overall, Bd seems rare in these species. Although we rarely detected Bd, our data may be used to measure future changes in its distribution and prevalence. However, Bd is not rare in all headwater streams, and its detection may depend upon seasonal influences such as changes in water temperature or proximity to wetland-associated species (Byrne et al., 2008; Grant et al., 2008). How spatial variation in the distribution and prevalence of Bd and interactions between wetland- and stream-associated species may affect the spread of this pathogen is an important issue that has not been explored.

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