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High Prevalence of the Amphibian Pathogen *Batrachochytrium dendrobatidis* in Plethodontid Salamanders in Protected Areas in New Brunswick, Canada

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Abstract.—**Amphibian fungal diseases have been implicated in a number of extinctions and declines worldwide. To establish the presence/absence of the amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*) on conservation lands in northern and south-central New Brunswick, Canada, we sampled eight species of anurans and six species of caudate amphibians (n = 390). Six species, including *Rana catesbieana* (American Bullfrog), *R. clamitans* (Green Frog), *R. sylvatica* (Wood Frog), *Desmognathus fuscus* (Northern Dusky Salamander), *Plethodon cinereus* (Eastern Red-backed Salamander) and *Notopthalmus viridescens* (Eastern Newt) tested positive for *Bd*. Prevalence for *Bd* in New Brunswick amphibians was low overall (4.6%) relative to those reported from surrounding states and provinces; however, two plethodontid salamanders, *D. fuscus* and *P. cinereus*, had the highest *Bd* prevalence among any of the amphibians sampled (9.1% and 12.9% respectively). In contrast, other studies have generally found plethodontids to have *Bd* prevalence of ≤ 2%. This survey for *Bd*, while not comprehensive, can serve as baseline and help direct future research in the province of New Brunswick.**

Key Words.—amphibians; chytrid fungus; disease; *Plethodon cinereus*; qPCR; *Rana catesbeiana*; salamander; North America

Introduction

Amphibian populations are declining worldwide (Houlahan et al. 2000). Since the 1980s, the emergence of the amphibian fungal disease, chytridiomycosis, has been implicated in the decline or extinction of more than 200 amphibian species (Olson et al. 2013). *Batrachochytrium dendrobatidis* (*Bd*), one causative agent of chytridiomycosis, is now known to infect more than 500 amphibian species worldwide (Olson et al. 2013). Also, chytrid has been detected on frogs in museum collections dating back to 1888 (Talley et al. 2015).

There is evidence that *Bd* infection loads and prevalence vary latitudinally (Kriger et al. 2007) and seasonally (Longo et al. 2010). Sapsford et al. (2013) found that *Bd* grew optimally between 15° to 25° C and that the fungus died at temperatures above 30° C. Surveys conducted across much of the U.S. have found *Bd* to be widespread (Fisher et al. 2009). In the northeastern U.S., *Bd* occurs in Maine, Massachusetts, New Hampshire, New York, and Vermont (Longcore et al. 2007). In eastern Canada, *Bd* has been detected in Quebec and New Brunswick (Ouellet et al. 2005) and on Prince Edward Island (Forzán et al. 2010); however, the occurrence of *Bd* in New Brunswick is based on histological samples taken from a museum specimen collected in the 1960s (Ouellet et al. 2005). No studies have explored the current presence or prevalence of *Bd* in New Brunswick amphibians.

There have not been any observed declines in amphibians associated with *Bd* in eastern North America; however, we are unaware of any ongoing monitoring projects for this region. Because of the complex relationship between environment and host-pathogen interactions, it is important to track *Bd*, particularly in the context of latitude, seasonality, and predicted climate change (Pounds et al. 2006). Such data are especially valuable to management strategies for wildlife populations occupying conservation lands. The purpose of this study is to help establish a baseline of prevalence of *Bd* infection for amphibian populations in New Brunswick, with emphasis on two Protected Natural Areas (PNAs), so that changes in disease prevalence can be monitored into the future and a mitigation plan designed if needed.

Materials and Methods

***Field surveys*.**—We sampled amphibians during BiotaNB, an all-taxa biological inventory of selected Protected Natural Areas (PNAs) in New Brunswick, Canada. We collected amphibians during visual encounter surveys by hand or with dip-nets and held them in individual plastic bags. We swabbed live amphibians using sterile medical swabs (MW113; Medical Wire and Equipment Co., Wiltshire, UK) following the protocols described by Hyatt et al. (2007). We stored swabs in 1.5-ml snap-cap tubes with 95% EtOH and maintained in a refrigerator (about 7° C). Because they were to be used for additional projects, we euthanized amphibians after swabbing them. Following euthanasia, we removed liver tissue for genetic work, fixed each individual in formalin, and deposited the specimens in the herpetological collections of the New Brunswick Museum (Saint John, New Brunswick, Canada).

***Site descriptions*.**—We surveyed for *Bd* in New Brunswick, Canada, focusing on two PNAs and adjacent conservation lands. We sampled Grand Lake PNA (45.8500°N, 66.1833°W) from 8 to 18 August 2014 (one or two searchers) and Nepisiguit PNA-Mount Carleton Provincial Park (47.3818°N, 66.6929°W) from 26 to 30 June 2015 (one to four searchers). The Grand Lake region supports 15 amphibian species, of which we swabbed 14 for *Bd*; whereas, the Nepisiguit PNA-Mount Carleton Provincial Park region supports 10 species, eight of which we swabbed (McAlpine 2010; Table 1).

Grand Lake PNA is located in central New Brunswick and consists of 10,321 h spread over 21 individual parcels of land with elevations that range from just above sea level to 160 m (Doucet et al. 2010). Wetlands and waterways that comprise Grand Lake, the Saint John River, and the Oromocto River occupy much of the PNA. Spring flooding covers bottomland forests, fields, and marshes. Floodwaters recede between May and June, with soil deposits contributing to wetland and riparian biodiversity (Zelazny et al. 2003). The lowland region of New Brunswick that includes the Grand Lake PNA supports the warmest temperatures in the province; 5° C mean annual, with a summer mean of 15.5° C (Ecological Stratification Working Group [ESWG] 1995). During the same sampling period, we also acquired swabs from the adjacent Portobello National Wildlife Refuge, Odell Park (an old-growth municipal park in the core of the city of Fredericton), and the University of New Brunswick Woodlot (Fig. 1B).

Nepisiguit PNA is located in the highland region of north-central New Brunswick and encompasses 11,895 h. It shares its western boundary with Mount Carleton Provincial Park and together the two areas comprise 29,290 h (Doucet et. 2010; Fig. 1C). Elevations range from 248 to 687 m in the PNA. Mount Carleton, at 820 m, is the highest peak in New Brunswick and is encompassed by Mount Carleton Provincial Park. Mean annual temperature in the region is 3° C, with a summer mean annual temperature of 14.5° C (ESWG 1995).

***Extractions and qPCR*.**—We extracted samples following the PrepMan® Ultra extraction protocol

Table 1. Summary of *Batrachochytrium dendrobatidis* prevalence by species (See common names in Results section) and site in New Brunswick, Canada. Abbreviations are GL = Grand Lake Protected Natural Area-Portobello National Wildlife Refuge (south), FR = Fredericton area (south), including Odell Park and the University of New Brunswick Woodlot, NE = Nepisiguit Protected Natural Area-Mount Carleton Provincial Park (north), and GE = Genomic equivalent, presented with one standard deviation.

Species	GL	FR	NE	*Bd* + / total	Total % infected	GE average
Anaxyrus americanus	2	—	12	0/14	0.0	—
Hyla versicolor	1	—	—	0/1	0.0	—
Pseudacris crucifer	1	—	—	0/1	0.0	—
Rana catesbeiana	27	—	—	2/27	7.4	17.8 ± 23.2
R. clamitans	62	13	42	4/117	3.4	32.4 ± 57.4
R. pipiens	49	1	—	0/50	0.0	—
R. septentrionalis	—	—	23	0/23	0.0	—
R. sylvatica	11	—	20	1/31	3.2	2.5
Ambystoma laterale	9	—	—	0/9	0.0	—
A. maculatum	1	—	—	0/1	0.0	—
Desmognathus fuscus	—	11	—	1/11	9.1	0.9
Eurycea bislineata	—	5	9	0/14	0.0	—
Plethodon cinereus	23	12	35	9/70	12.9	13.4 ± 19.8
Notophthalmus viridescens	7	—	14	1/21	4.8	0.8
TOTAL	193	42	155	18/390	$\bar{X}$ = 4.6	—

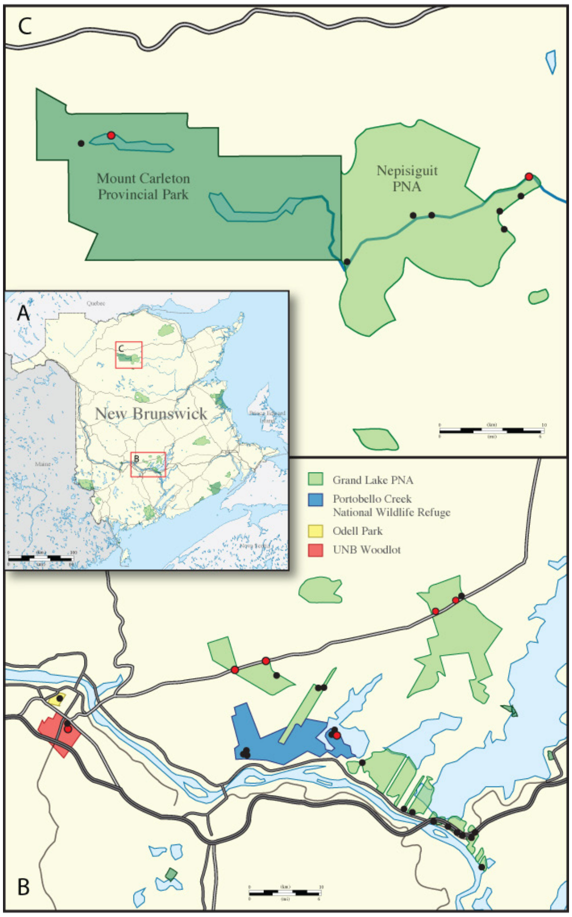

Figure 1. (A) New Brunswick locations sampled for *Bd* prevalence in amphibians showing (B) principal sampling sites in the Grand Lake Protected Natural Area and region, and (C) the Nepisiguit Protected Natural Area-Mount Carleton Provincial Park. Red circles mark sites positive for *Bd*, closed circles mark sites negative for *Bd*.

(Hyatt et al. 2007) and diluted 1:10 with 0.25 TE buffer (Tris-EDTA and sterile water) to reduce potential inhibition during quantitative polymerase chain reaction (qPCR) analysis. For the quantitative PCR assays, we incorporated an 18 base pair insert into the reference sequence presented in Boyle et al. (2004) for use as a synthetic positive control (see Wilson et al. 2016, Table 2). The inclusion of the 18 base pair insert reduces the risk of false positive detections due to lab contamination when amplifying field samples (Wilson et al. 2016). The assay uses *Bd*-specific genetic markers and compares each sample to a set of standards to calculate a genomic equivalent. Synthetic controls were manufactured by Life Technologies (now Thermo Fisher Scientific, Waltham, Massachusetts, USA) using GeneArt®StringsTM DNA fragments. As per manufacturer guidelines, we re-suspended GeneArt®StringsTM in sterile water and diluted 10-fold to create a dilution series of standards from 10^{10} down to one target copy per 5 µL volume. We assessed the qPCR assay using this dilution series to ensure positive controls were amplifying as expected, and to assess reaction efficiency and limit of detection.

We prepared Quantitative PCR assays containing 5 µL of sample, 1X TaqMan® Universal PCR Master Mix, 0.9 µM each of ITS1-3 Chytr and 5.8S Chytr primers, 0.15 Chytr-MGB2 probe for *B. dendrobatidis* (Boyle et al. 2004), 0.2 µM synthetic control MGB probe (Life Technologies) and sterile distilled water (Life Technologies) up to a total volume of 20 µL. For all assays, we used a StepOnePlusTM Real-time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) to detect DNA. Reaction conditions consisted of 2 min at 50° C, 10 min at 95° C, followed by 50 cycles of 15 sec at 95° C and 1 min at 60° C (following Boyle et al. 2004). We used the default settings on StepOneTM software v.2.2.2 (Applied Biosystems) to analyze all run data. We amplified all samples using the above conditions with two replicates of synthetic control dilution series and two negative controls with no DNA template per qPCR run. We used a 0.1 Δ Rn threshold of detection, as per Boyle et al. (2014). We ran each sample in duplicate and compared the data between runs to ensure accuracy. We considered all detections over the threshold limit repeated in both qPCR runs positive for *Bd*.

Results

We sampled 390 individuals, representing eight species of anurans and six species of caudate amphibians. Overall, the prevalence for *Bd* in New Brunswick is low (18/390; 4.6%). *Rana catesbeiana* (American Bullfrog), *R. clamitans* (Green Frog), *R. sylvatica* (Wood Frog), *Plethodon cinereus* (Eastern Red-backed Salamander), *Notophthalmus viridescens* (Eastern Newt), and *Desmognathus fuscus* (Northern Dusky Salamander) tested positive for *Bd* (Table 1). *Anaxyrus americanus* (American Toad), *Hyla versicolor* (Grey Treefrog), *Pseudacris crucifer* (Spring Peeper), *R. pipiens* (Northern Leopard Frog), *R. septentrionalis* (Northern Mink Frog), *Ambystoma laterale* (Blue Spotted Salamander), *A. maculatum* (Yellow Spotted Salamander), and *Eurycea bislineata* (Northern Two-lined Salamander) all tested negative (Table 1). Overall, *Rana catesbeiana* had the highest prevalence among frogs (2/27; 7.4%). *Rana clamitans* and *R. sylvatica* each had positive samples (4/117; 3.4% and 1/31; 3.2%, respectively). Among the other anurans, toads (*Anaxyrus americanus*, n = 14) and treefrogs (*Hyla versicolor*, n = 1 and *Pseudacris crucifer*, n = 1) did not test positive for *Bd*. Two plethodontids had the highest *Bd* prevalence among any of the amphibians we sampled. We recorded a *Bd* prevalence of 9.1% (1/11) from *Desmognathus fuscus* and a prevalence of 12.9% (9/70) from *Plethodon cinereus*.

Discussion

Overall, the prevalence for *Bd* in New Brunswick is low (18/390; 4.6%) relative to reports from surrounding states and provinces. For example, in adjacent Prince Edward Island, prevalence was 26.9% (Forzán et al. 2010), and 26.4% in Maine (Longcore et al. 2007). *Bd* prevalence in Nova Scotia is unknown. Although we did not detect *Bd* in 8 of the 14 species sampled, sample sizes of some of these species are possibly too small to detect *Bd*. Skerratt et al. (2008) suggest that 59 individuals should be sampled to detect *Bd* when prevalence is low. Further sampling will likely increase the number of amphibian species in New Brunswick that are *Bd*-positive. Nonetheless, even for cases where our New Brunswick sampling is sufficiently large (> 60), the prevalence for *Bd* is low (3.4% for *R. clamitans*; 12.9% for *P. cinereus*).

Remarkably, two plethodontids had the highest *Bd* prevalence among any of the amphibians sampled. Previous research has remarked on the low (≤ 2%) *Bd* prevalence in plethodontid salamanders generally (Gratwicke et al. 2011; Krynak et al. 2012; Muletz et al. 2014). Muletz et al. (2014) report that only 0.7% (n = 2,728 individuals; 95% CI = 0.4–1.1%) of plethodontids tested for *Bd* provided positive results. Ouellet et al. (2005), report 0% prevalence (n = 35) for *P. cinereus*. Muletz et al. (2014) report 0% (n = 561) and 1% (n = 396) prevalence for *P. cinereus* for the period 1957–1987 and 2011, respectively. The low prevalence of *Bd* contrasts sharply with the 12.3% (n = 81) prevalence detected in these New Brunswick populations.

Plethodon cinereus exhibited markedly higher infection loads at the Nepisiguit sites than the Grand Lake Meadow sites, Odell Park or UNB Woodlot. Disentangling whether this is the influence of latitude, seasonality, or some other variable is not possible with this dataset because the sites were sampled at different times of the year (June and August). More surveys at these sites and others across New Brunswick will help reveal the causes of variation in *Bd* prevalence. Both the influences of latitude (Kriger et al. 2007) and seasonal variation (Kriger and Hero 2007; Lenker et al. 2013) should be considered as potential causes.

The goal of this work was to gather baseline data on *Bd* prevalence in New Brunswick. In some cases, sample sizes are too small to infer prevalence of *Bd* in some species; however, *Bd* detection was geographically widespread in New Brunswick and occurred in a variety of both aquatic and terrestrial anurans and salamanders. The unusually high *Bd* prevalence for plethodontid salamanders requires further investigation to determine if these results are consistent across the Atlantic region or elsewhere in New Brunswick. Examination of museum specimens following the protocols of Cheng et al. (2011) could confirm if plethodontids have historically had higher *Bd* prevalence in New Brunswick than in other regions. This survey of nearly 400 amphibian individuals has contributed to the establishment of a baseline for *Bd* in New Brunswick. Such information will help to inform management strategies for amphibian populations both on and off conservation lands and in the face of changing climate in the region.

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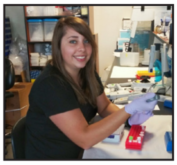

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Donald F. McAlpine has spent the past 38 y investigating the biodiversity of Atlantic Canada. His previously published research ranges from worms to whales (including frogs!). In 2015 he received the Roland Michener Award for Conservation Research from the Canadian Wildlife Federation. He is currently Research Curator of Zoology and Head of the Department of Natural History at the New Brunswick Museum and an Adjunct Professor in the Department of Biology at the University of New Brunswick, Saint John, Canada. (Photographed by Donald McAlpine).