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NOTE

Prevalence of *Batrachochytrium dendrobatidis* in immature eastern hellbenders *Cryptobranchus alleganiensis* from North Carolina, USA

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) has been detected in wild hellbender *Cryptobranchus alleganiensis* populations, with rare instances of chytridiomycosis and *Bd*-induced mortality. To date, *Bd* surveillance in hellbender populations has been disproportionately focused on adult age classes. A lingering question is whether *Bd* might contribute to hellbender declines through disproportionate negative effects on immature age classes. The objective of this study was to quantify *Bd* prevalence and load in immature hellbenders in western North Carolina, USA. We conducted field surveys during 2018 and 2019 and collected 88 skin swabs from 84 hellbenders spanning 3 age classes. *Bd* was detected on 11 % of individuals, including 8 larvae and 1 juvenile. We did not detect symptoms of chytridiomycosis or a decline in body condition in *Bd*-positive hellbenders. Load varied from approximately 1–153 zoospore equivalents for the 9 *Bd*-positive hellbenders and was not associated with size class of the individual. While hellbenders appeared to be abundant in each survey reach, more work is needed to determine whether *Bd* may increase the vulnerability of immature hellbenders to anthropogenic stressors.

KEY WORDS: Chytrid · Hellbender · Amphibian diseases · *Batrachochytrium dendrobatidis*

1. INTRODUCTION

Batrachochytrium dendrobatidis, commonly known as amphibian chytrid fungus or *Bd*, is a fungal pathogen that infects keratinized skin tissues of amphibians, sometimes causing a clinical infection known as chytridiomycosis (Ouellet et al. 2005). While chytridiomycosis is sometimes lethal, development of and severity of infection is often context dependent and varies with host and environment (Retallick & Miera 2007). *Bd* has been detected on every continent where amphibians exist since it was first described in 1998, and is suspected to have spread via the global movement of common frog species, such as *Xenopus laevis* and *Lithobates catesbeianus* for industrial and consumer uses (Berger et al. 1998, Rosenblum et al. 2010). Along with habitat loss, *Bd* is considered a leading cause of amphibian declines worldwide and has led to several species extinctions (Ouellet et al. 2005, Lips 2016).

The eastern hellbender *Cryptobranchus alleganiensis alleganiensis* is a large (up to 74 cm), long-lived, fully aquatic salamander in the Family Cryptobranchidae (Taber et al. 1975). *Bd* is present in many hellbender populations, and *Bd*-induced mortality, although rare, has been occasionally documented in captive and free-ranging adults (Burgmeier et al. 2011, Gonynor et al. 2011, Souza et al. 2012). *Bd*

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prevalence in wild populations ranges from 15–50% and has been detected in most states where hellbenders still occur (Gonynor et al. 2011, Souza et al. 2012, Williams & Groves 2014, Bales et al. 2015). Although in general, adult hellbenders seem to carry the pathogen with few to no symptoms of infection (i.e. lethargy, skin sloughing, etc.), it is unknown whether larval and juvenile hellbenders possess the same immune capacity to tolerate *Bd* (Retallick & Miera 2007, Tominaga et al. 2013, Williams & Groves 2014). It is widely believed that larval amphibians are less likely to be infected by *Bd* relative to their adult counterparts, as *Bd* infects keratinized tissues, which tend to be restricted to the mouthparts in larval amphibians (Berger et al. 1998, Ouellet et al. 2005). However, some studies on anurans have revealed that *Bd* infections have the potential to impact survivorship in young life stages (pre-metamorphic larvae, juveniles of direct-developing species) and thereby reduce overall population recruitment (Longo & Burrowes 2010, Fernández-Loras et al. 2017). Hellbender populations have experienced precipitous declines throughout the last several decades, and the demographic structure of declining populations often reflects a decline in recruitment (Wheeler et al. 2003, Pitt et al. 2017). The loss of suitable habitat for breeding and to support larvae and juveniles due to increased sedimentation is suspected as a leading driver of losses in recruitment. However, specific mechanisms of most hellbender declines remain poorly understood (Wheeler et al. 2003). *Bd* may be contributing to declines, specifically through negative effects on young age classes.

To date, *Bd* has rarely been detected in hellbender larvae (Williams & Groves 2014), and no study has focused solely on quantifying prevalence and infection loads in immature age classes. Since vulnerability to disease may be exacerbated by anthropogenic stressors, it is important to continue to monitor the pathogen and understand how it may affect each hellbender age class (Rosenblum et al. 2010, Williams & Groves 2014). Our objective was to quantify *Bd* prevalence (proportion of individuals carrying the pathogen) and infection load (zoospore equivalents, ZEs) for immature eastern hellbenders in North Carolina, USA.

2. MATERIALS AND METHODS

2.1. Field surveys

Our study area included 8 stream reaches from 7 streams in western North Carolina. Study streams were in the Hiwassee (n = 1), Little Tennessee (n = 1), and French Broad (n = 5) river basins. Although precise abundance estimates for each hellbender population are lacking, the populations sampled are considered amongst the most robust in the state and are within protected, forested catchments (North Carolina Wildlife Commission unpubl. data). We have withheld site names and locations due to the protected status of our study species and risk of poaching and harassment.

We used cobbling surveys to locate and capture immature hellbenders. Briefly, cobbling surveys involve 2 to 4 snorkelers moving upstream along zigzag transects while searching the stream substrate. Cobbling surveys took place from July–August 2018 and May–August 2019. While we were not focusing on adult age classes, we opportunistically hand captured adults when encountered during surveys and processed them similar to younger age classes.

To assess *Bd* prevalence and load in hellbenders, we first rinsed each hellbender in sterile water (100 ml for larvae and juveniles and 1 l for adults) immediately following capture. We then swabbed all animals 5–10 times by rotating a sterile rayon-tipped swab (MW113 Peel Pouch Dryswab™ Fine Tip, Medical Wire & Equipment) on the skin throughout the body while applying pressure. We swabbed gilled larvae on the mouth parts as well, as there is more keratin in the mouthparts of larval amphibians than elsewhere on the body, although this has not been evaluated for hellbenders specifically (Davis et al. 2010). We then checked hellbenders for physical abnormalities, measured total length (mm), snout–vent length (mm), and mass (g), and gave animals individual marks. Animals <200 mm received a visible implant elastomer mark (Northwest Marine Technology), injected under the skin on the ventral surface of all 4 feet. Animals ≥200 mm received a passive integrated transponder tag (Biomark), which was inserted into the dorsal tail musculature. We released all hellbenders in their original capture location. We placed swabs in 1.5 ml microcentrifuge tubes, which were stored on ice for up to 5 d prior to being moved to a −80°C freezer for long-term storage. Once ready for assay, we shipped frozen samples on dry ice to the University of California, Berkeley, for processing. Due to shipping errors outside of our control, shipment of our samples was delayed, resulting in sample exposure to ambient (room) temperature conditions for up to 7 d.

2.2. Laboratory methods

We isolated DNA from skin swabs using the DNeasy Blood and Tissue Kit (Qiagen) following the animal tissues protocol. Because DNA extractions were performed in a lab where *Bd* cultures have been grown in the past, we included negative extraction controls (extraction of DNA from new and clean swabs) to evaluate for sample contamination during DNA extraction. To detect and quantify *Bd* infection, we performed real-time quantitative PCR (qPCR) reactions in triplicate following the protocol of Boyle et al. (2004) plus a few modifications. Each qPCR reaction consisted of 5 µl of template DNA, 12.5 µl of 2× TaqMan Fast Advanced Master Mix (ThermoFisher), 900 nM forward/reverse primers ITS1-3 Chytr and 5.8S Chytr (Boyle et al. 2004), 250 nM minor groove binder probe MGB2 (Boyle et al. 2004), 400 ng $µl^{-1}$ of BSA, and 2.75 µl of molecular-grade water for a total volume of 25 µl. In each 96-well qPCR plate, we included 3 replicates of *Bd* ITS gBlock (Integrated DNA Technologies) synthetic oligonucleotide in standard dilutions of 100 000 to 0.1 ZEs. In addition, we included triplicates of negative extraction controls and negative reaction controls to evaluate contamination during extraction and qPCR reaction setup, respectively. qPCR reactions were run and analyzed on an Applied Biosystems StepOnePlus Real-Time PCR system (ThermoFisher). We classified negative samples as those whose average qPCR quantification fell below 1 ZE.

2.3. Statistical analysis

We used a chi-squared goodness-of-fit test to determine the strength of relationships between prevalence and age class followed by a Fisher's exact pairwise test between age classes, and linear regression to determine the strength of the relationship between load and total length of the hellbender. To evaluate potential sublethal effects on body condition, we used a *t*-test to compare the measured residual distances between observed and predicted $\log_{10}$-transformed mass (body condition scores) between *Bd*-positive and -negative individuals (Peterson et al. 1983). Negative values of our body condition index indicate relatively low body mass and positive values indicate relatively high body mass given length. We analyzed our data using program R version 3.5.2 (R Core Team 2018).

3. RESULTS

We collected a total of 88 skin swabs from 84 individual hellbenders (larvae: n = 41; juveniles: n = 36; adults: n = 11), 7 streams, and 3 watersheds. All of the negative extraction and negative reaction controls were negative. We detected *Bd* DNA across all triplicates in all positive samples and the standard deviation of the cycle threshold (C_t) across positive sample triplicates was <1. If substantive DNA degradation had occurred, one would expect to see only 1 or 2 of the triplicates amplifying as well as more varied C_t scores (Jackson et al. 2012). Therefore, our results suggest that DNA degradation was minimal during transportation of samples and did not influence our results.

Of the 84 hellbenders sampled, 9 were positive for *Bd* (11%), none of which were recaptures (Table 1). Approximately 21% of larvae tested positive for *Bd* (8 of 39 larvae), compared to 3% of juveniles (1 of 34 juveniles). None of the 11 adult hellbenders tested positive for the pathogen. There was a significant difference in prevalence among age classes ($\chi^2 = 7.28$, df = 2, p = 0.03). We detected a significant difference in prevalence between larvae and juveniles (Fisher's exact test, p = 0.03), but not between adults and larvae (Fisher's exact test, p = 0.17) or adults and juveniles (Fisher's exact test, p > 0.99). Seven positive individuals came from 1 tributary in the Hiwassee watershed, 1 came from the French Broad watershed, and 1 came from the Little Tennessee watershed (Table 1).

Among the 9 positive individuals, *Bd* load varied (1.12–153.47 ZEs; Fig. 1) and was not correlated with size (p = 0.14). None of the *Bd*-positive individuals

Table 1. *Batrachochytrium dendrobatidis* prevalence (percent *Bd*-positive) among sites and age classes of *Cryptobranchus alleganiensis alleganiensis* in western North Carolina, USA, 2018–2019. Sites identified only by codes to protect the host species. Numbers in parentheses represent the number of *Bd*-positive individuals over the total number of individuals; (–): age class not caught at a site

Watershed	Site	Age class		
		Larva	Juvenile	Adult
French Broad	NF	0 (0/5)	0 (0/4)	–
	WF	20 (1/5)	0 (0/8)	0 (0/3)
	ST	–	–	0 (0/7)
	LC	0 (0/1)	0 (0/3)	–
	DR	0 (0/5)	0 (0/3)	–
Hiwassee	FC	35 (6/17)	6 (1/16)	0 (0/1)
Little Tennessee	SC	20 (1/6)	–	–
Total		21 (8/39)	3 (1/34)	0 (0/11)

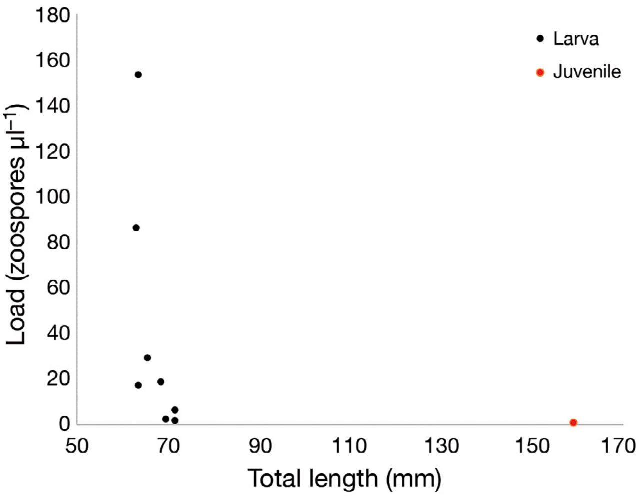

Fig. 1. Zoospore equivalent (ZE) counts (load) in relation to total length of *Cryptobranchus alleganiensis alleganiensis* larvae (n = 8) and juveniles (n = 1) carrying *Batrachochytrium dendrobatidis* in western North Carolina, USA, 2018–2019. Load ranged from 1.21 to 153.47 ZEs

exhibited abnormalities or symptoms indicative of clinical disease. Length and mass were highly correlated (Intercept = −1.59 ± 0.07 SE, $\log_{10}$length = 2.46 ± 0.07, R^2 = 0.93, p < 0.001). Body condition did not differ between *Bd*-positive and -negative individuals (*Bd*-positive mean residual distance = −0.03 ± 0.01, *Bd*-negative mean residual distance = 0.01 ± 0.01, df = 19.47, p = 0.14).

4. DISCUSSION

To our knowledge, our study marks the most robust attempt to investigate *Bd* prevalence in immature hellbenders specifically. Our results are consistent with those of other studies in that *Bd* is widespread in southern Appalachian streams, and hellbenders of all age classes carry the pathogen (Bodinof et al. 2011, Gonynor et al. 2011, Souza et al. 2012, Williams & Groves 2014). The relatively high prevalence of *Bd* among gilled larvae in our study (21% of individuals carrying the pathogen) contradicts the hypothesis that larval amphibians are less likely than adults to harbor the pathogen due to a rarity of keratinized tissue (Keitzer et al. 2011).

Chytridiomycosis is considered rare in free-ranging hellbenders, and factors that contribute to progression from *Bd* infection to disease remain unclear. *Bd*-induced mortality in amphibians is often load dependent (Wilber et al. 2017), and the load threshold where *Bd* would lead to chytridiomycosis is often context-dependent and host-specific (Retallick & Miera 2007, Wilber et al. 2017). Our highest load value was 153.47 ZEs in a larval hellbender with no detectable symptoms, and our range of load values was lower than those reported by Williams & Groves (2014), who also detected no symptoms of chytridiomycosis (<1 to 380 ZEs among 38 hellbenders from all age classes in North Carolina). Further experimental research is needed to understand how load influences the likelihood of developing a clinical infection from *Bd* in hellbenders specifically.

It is important to note that our study was seasonally limited, as we only sampled from May–August and did not have the sample size in certain months to detect seasonal shifts (Table 2). Rates of *Bd* infection may change seasonally, and there may be peaks of infection that were missed by our sampling frame, as *Bd* often thrives in cooler temperatures (Lips 2016). Several studies of anurans have detected seasonal differences in *Bd* prevalence, with higher winter infection and mortality rates (Berger et al. 2004, Ruggeri et al. 2015). However, Williams & Groves (2014) did not detect an effect of water temperature on *Bd* presence or prevalence in hellbenders in western North Carolina, despite sampling wide temperature range (12.8–27.8°C) that included the temperatures most optimal for *Bd* growth. Further sampling of *Bd* prevalence in hellbender populations during the winter months would fill significant gaps in our understanding of *Bd* dynamics among hellbender life stages.

It remains unclear how *Bd* prevalence in larval and juvenile hellbenders compares to that of adults. Because we did not target adults in our surveys, our sample of opportunistically caught adult hellbenders was small (n = 11), which prevented us from detecting pairwise differences between adults and larvae or adults and juveniles. However, we know from a variety of range-wide studies that adult hellbenders commonly carry *Bd*. Other studies in southern

Table 2. *Batrachochytrium dendrobatidis* prevalence (percent *Bd*-negative and *Bd*-positive) in *Cryptobranchus alleganiensis alleganiensis* across sampled months in western North Carolina, USA, 2018–2019. Numbers in parentheses represent the number of *Bd*-positive individuals over the total number of individuals

Month	% Negative	% Positive
May	100 (9/9)	0 (0/9)
June	100 (2/2)	0 (0/2)
July	84 (41/49)	16 (8/49)
August	94 (16/17)	6 (1/17)

Appalachian watersheds have reported *Bd* prevalence in adult hellbenders between 28 and 50% (Gonynor et al. 2011, Williams & Groves 2014, Bales et al. 2015). Future investigations of *Bd* prevalence in hellbender populations should target all size and age classes equally in order to effectively draw comparisons.

Although we did not detect any visible symptoms of chytridiomycosis or differences in body condition between positive and negative individuals, it is possible that infected individuals may be more susceptible to chytridiomycosis when faced with additional stressors, or that carriers of the pathogen may experience detrimental sub-lethal effects such as immunosuppression (Fernández-Loras et al. 2017, Campbell et al. 2019). Carrying and fighting the pathogen has been shown to be energetically costly in other species, diverting resources away from behaviors such as breeding, foraging, and evading predation (Campbell et al. 2019). The global devastation in amphibian populations driven by *Bd*, our findings, and the relatively dire conservation status of hellbenders throughout most of the species' range collectively warrant further studies on the effects of *Bd* infection in larval and juvenile hellbenders specifically, especially in the context of stressors such as climate change and reduced habitat quality.

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