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PREVALENCE OF THE AMPHIBIAN PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* IN EASTERN HELLBENDERS (*CRYPTOBRANCHUS A. ALLEGANIENSIS*) IN WESTERN NORTH CAROLINA, USA

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***Abstract.*—Due to recent documented declines of Eastern Hellbender (*Cryptobranchus a. alleganiensis*) populations, in 2008–2012 we surveyed both wild-caught and captive hellbenders in Western North Carolina for the prevalence of a common amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*). We sampled 165 wild and 15 captive animals of North Carolina origin. We collected morphometric data from hellbenders, noted the presence of any physical anomalies or injuries, and recorded water temperature and site elevation. We found *Bd* to be widespread in Western North Carolina waterways and in all river basins and sub-basins sampled. *Batrachochytrium dendrobatidis* prevalence was 27.9% for wild-caught animals and 26.7% for captives. Adult female hellbenders had a significantly higher prevalence of *Bd* (38.8%) than adult males (19.7%; $P = 0.0207$). All age classes tested positive for *Bd*, including gilled larvae. From examining hellbender body condition, we found no physical evidence of acute infection or compromised immunity due to disease. There was no significant relationship between the presence of *Bd* and physical injuries or anomalies ($P = 0.1196$). We also found that water temperature ($P = 0.5038$) and elevation ($P = 0.5100$) were not significant for predicting the presence of *Bd*. Ours is the first report of *Bd* for Eastern Hellbenders in North Carolina. Although it does not appear that hellbenders in North Carolina are in crisis due to disease, future monitoring and surveillance efforts within populations should continue, particularly across a variety of habitat disturbance regimes.**

Key Words.—amphibian disease; body condition; chytrid; hellbenders; environmental conditions

INTRODUCTION

Numerous anuran and caudate studies in the Eastern United States have documented the presence of the amphibian pathogen *Batrachochytrium dendrobatidis* (*Bd*), an aquatic fungus that causes chytridiomycosis, a severe skin disease (e.g., Grant et al. 2008; Rothermel et al. 2008; Pullen et al. 2010; Hill et al. 2011; Huang and Wilson 2013). In the Southern Appalachian Mountain region of Western North Carolina and Eastern Tennessee, *Bd* has either been detected at low prevalence (< 1–6%; Chatfield et al. 2009; Chinnadurai et al. 2009; Caruso and Lips 2012; Kiemnec-Tyburczy et al. 2012; Rothermel et al. 2013) or not at all in a variety of terrestrial and aquatic amphibian habitats and species (Hossack et al. 2010; Keitzer et al. 2011; Moffitt 2012). Although few amphibian mortality events have been linked to the pathogen in the Southern Appalachians, disease surveillance efforts for the majority of the region's diverse salamander species, particularly those in large riverine systems, are lacking.

Disease could be one of many factors affecting the hellbender (*Cryptobranchus alleganiensis*), a giant, riverine salamander, one of the more imperiled salamanders in the world (IUCN. 2013. IUCN Red List of Threatened Species. Available from http://www.iucnredlist.org [Accessed 3 December 2013]). Hellbenders have declined dramatically throughout their historic range, particularly in recent decades (Mayasich et al. 2003). Population declines have been documented for Ozark Hellbender (*Cryptobranchus a. bishopi*) in Missouri and both Ozark and Eastern Hellbender (*Cryptobranchus a. alleganiensis*) in Arkansas (Trauth et al. 1992; Wheeler et al. 2003; Nickerson and Briggler 2007; Hiler et al. 2013). Other areas of documented Eastern Hellbender decline include the Tennessee River drainage in Alabama (Graham et al. 2011), the Ohio River drainage of Indiana (Burgmeier et al. 2011a; Olson et al. 2013), Ohio (Pfingsten 1990), West Virginia (Keitzer et al. 2013), and Maryland (Gates et al. 1985), the upper Allegheny River drainage of New York (Foster et al. 2009), and the Susquehanna River drainage in Maryland (Gates et al. 1985) and New York (Quinn et al. 2013). Hellbender populations are under threat from multiple factors including habitat degradation, habitat and natural systems disturbance (Williams et al. 1981; Mayasich et al. 2003; Phillips and Humphries 2005; Briggler et al. 2007b), overharvesting and over-collecting (Nickerson and Briggler 2007), toxins and pollutants, and possibly disease as well (Solis et al. 2007; Hopkins and DuRant 2011; Unger et al. 2013a).

Of the numerous threats to hellbenders, disease is one of the more poorly understood, and researchers are just beginning to document the scope and severity of the pathogen *Bd*. Initial work indicates *Bd* may be widespread in aquatic systems but may have a low prevalence in some headwater streams and protected drainages (Timpe et al. 2008; Hossack et al. 2010; Gratwicke et al. 2011), or some salamanders occupying streams in upper watersheds might have the ability to live with these pathogens at sub-clinical levels (Chinnadurai et al. 2009; Vazquez et al. 2009). Prior to 1969, there was no evidence that *Bd* was present in Ozark Hellbenders (Bodinof et al. 2011), suggesting the exotic fungal pathogen was introduced several decades ago but has since become well established. Genetic analysis of *Bd* found on Ozark and Eastern Hellbenders revealed the same fungal types that are found on amphibians around the world, providing more evidence that *Bd* is a novel pathogen in the United States that has spread widely and invasively since its introduction (Tominaga et al. 2013).

Batrachochytrium dendrobatidis in cryptobranchids has been documented in many locations. The fungus has been found on the Japanese Giant Salamander (*Andrias japonica*; Goka et al. 2009), in captive populations of both Ozark and Eastern Hellbenders in Missouri (Briggler et al. 2007a), and in wild hellbenders in individual watersheds or streams in numerous states (e.g., Briggler et al. 2008; Burgmeier et al. 2011b; Gonynor et al. 2011; Regester et al. 2012; Souza et al. 2012). However, our study is the first widespread disease surveillance effort for Eastern Hellbender at the state level and the first in North Carolina. In this project, our main objectives were to examine the prevalence and distribution of *Bd* in hellbenders in Western North Carolina, sampling within a variety of water temperatures and elevations, to assess hellbender body condition looking for any visible signs of disease, and to establish baselines for future surveillance and monitoring.

MATERIALS AND METHODS

From 2008–2012, primarily in the months of May-August, we collected 165 *Bd* samples from wild populations of Eastern Hellbenders in Western North Carolina (n = 57 sites). Samples came from 33 individual river systems in 13 counties, eight river sub-basins (HUC 8), and across all four river basins (HUC 6), where hellbenders occur: the Kanawha, the French Broad-Holston, the Upper Tennessee, and the Middle Tennessee-Hiwassee Basins. All watersheds surveyed drain to the Tennessee or Ohio Rivers, then the Mississippi River, and eventually to the Gulf of Mexico. Major waterways in these basins in Western North Carolina include the Hiwassee, Little Tennessee, Tuckasegee, Pigeon, French Broad, Nolichucky, Watauga, and New Rivers. Due to the sensitivity of capture locations and the threat of site disturbance and illegal harassment or collection of hellbenders, a protected, Special Concern Species in North Carolina (North Carolina Wildlife Resources Commission 2005), we have withheld specific site details on file with the North Carolina Wildlife Resources Commission.

We collected skin swab samples for disease surveillance opportunistically and concurrent with ongoing hellbender population monitoring, inventories, and research from May through August each year. Hellbender surveys consisted of snorkeling and visual encounter techniques (Nickerson and Krysko 2003; Browne et al. 2011). We recorded morphometric data, age class, and sex of captured hellbenders and noted any visible wounds, scars, injuries, or anomalies as part of routine data collection methods. We laundered mesh bags used for holding, handling, and processing hellbenders after use in hot water, detergent, and diluted bleach. Unless hellbender secondary sex characteristics were present, as a general rule, we considered animals with total lengths less than 25 cm to be juveniles; however, suggested minimum adult sizes vary widely in the literature from approximately 20 cm up to 39 cm total length (e.g., Nickerson and Mays 1973; Taber et al. 1975; Peterson et al. 1983; Hecht-Kardasz et al. 2012; Pugh et al. 2013). At all capture locations, we used a standard bulb thermometer or a dissolved oxygen and temperature meter to record water temperature and a Garmin™ 60CSx Global Positioning System (GPS; Garmin International, Inc, Olathe, Kansas, USA) unit to record latitude and longitude coordinates and an estimate of site elevation.

Batrachochytrium dendrobatidis has been documented in captive hellbenders (Briggler et al. 2007a), so we tested 15 captive hellbenders (six adults, nine juveniles) of North Carolina origin, all from the French Broad-Holston River Basin. At the time of swab collections, these animals were housed at the Como Park Zoo (St. Paul, Minnesota, USA), the North Carolina Zoological Park (Asheboro, North Carolina, USA), the North Carolina Wildlife Resources Commission's Marion Fish Hatchery and Aquaculture Center (Marion, North Carolina, USA), the North Carolina Wildlife Resources Commission's Pisgah Wildlife Education Center (Pisgah Forest, North Carolina, USA), and Earthshine Nature Programs (Lake Toxaway, North Carolina, USA). The Amphibian Disease Laboratory at the San Diego Zoo's Institute for Conservation Research (Escondido, California, USA) offered sampling protocol and supplies for collecting *Bd* swabs in the field. We used sterile, plastic-handled, fine-tipped, rayon swabs (Dryswab ™) from Advantage Bundling SP (catalog number MW113,

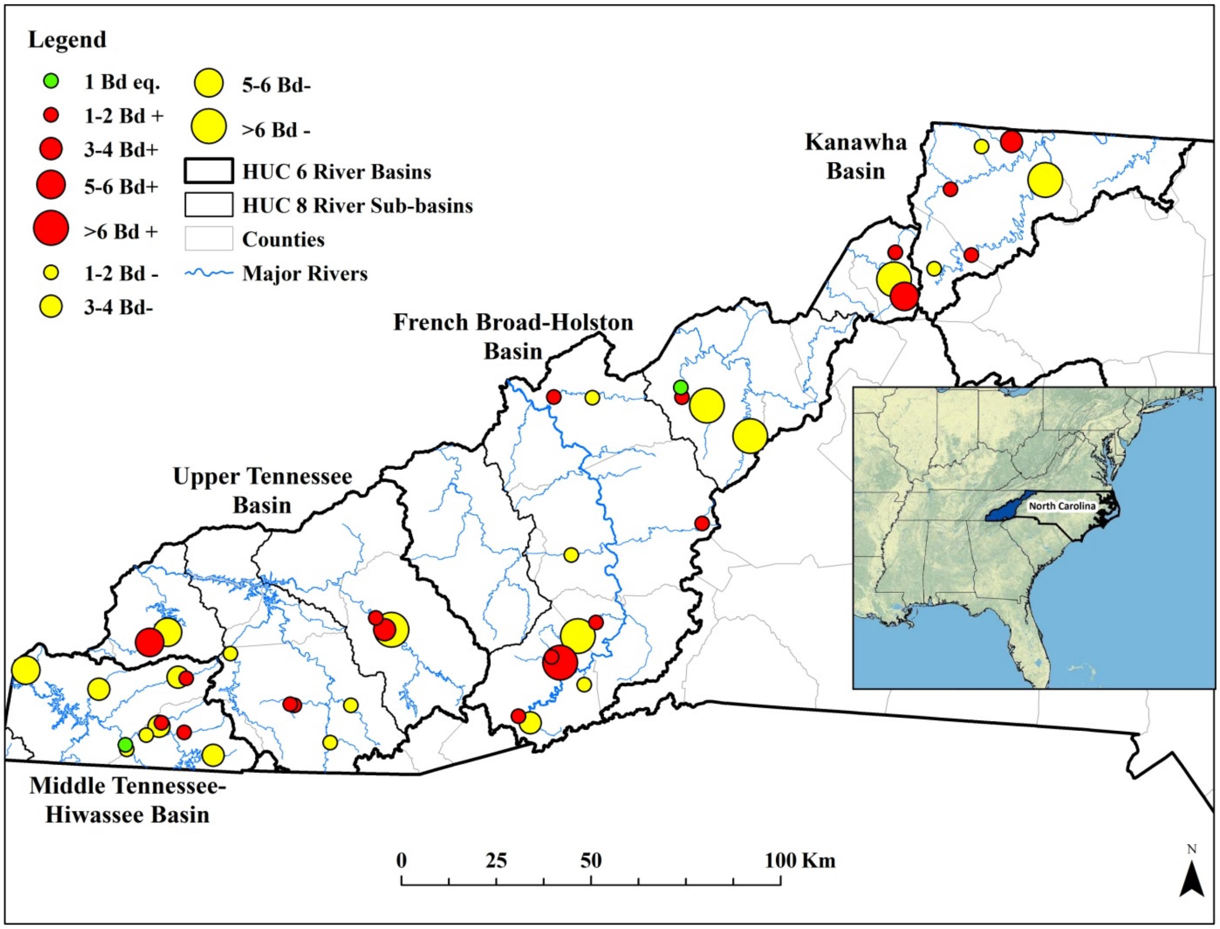

FIGURE 1. Number of *Batrachochytrium dendrobatidis* equivocal (*Bd* eq.), positive (*Bd*+), and negative (*Bd*-) samples from wild-caught Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) per individual stream across all HUC 6 river basins in Western North Carolina.

Medical Wire & Equipment Co., Corsham, Wiltshire, England) and screw-top, 2.0 mL plastic vials. For *Bd* samples, we used a single swab in approximately 20 passes along the skin on the ventral surface of hellbenders, focusing around the pelvis, inner thighs, foot pads, and between the toes (Brem et al. 2007; Gonynor et al. 2011; Regester et al. 2012; Souza et al. 2012). We cut swabs 2–3 cm from the bottom and stored them dry in individually labeled vials kept in a cooler until they could be transferred to a freezer for longer storage throughout the field season.

Each fall, we shipped the frozen vials overnight on dry ice to The Amphibian Disease Laboratory at the San Diego Zoo's Institute for Conservation Research for testing. Lab staff conducted *Bd* assays with isolated DNA on a prepared template using Prep-Man® Ultra Sample Preparation Agent (catalog number 4318930, Applied Biosystems, Foster City, California, USA) for use in TaqMan® Real-Time Polymerase Chain Reaction (PCR) procedures (Skerratt et al. 2011) based on published protocol (Annis et al. 2004; Boyle et al. 2004; Hyatt et al. 2007) and used with similar amphibian disease surveillance studies (Keitzer et al. 2011; Souza et al. 2012). Diluted, cultured *Bd* zoospores were used for positive controls, and reactions were run in triplicate (Keitzer et al. 2011; Souza et al. 2012).

We used Epi Info™ statistical software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA) to calculate summary statistics of percent prevalence, with 95% confidence intervals, as demonstrated by Souza et al. (2012). We used included Pearson's Chi-squared or Fisher's exact test to examine differences in *Bd* prevalence among river basins, hellbender age classes, and hellbender sexes. We also used Pearson's Chi-squared or Fisher's exact test to investigate differences in the frequency of physical injuries or anomalies in *Bd*-positive versus *Bd*-negative hellbenders, all samples pooled, and across sexes, age classes, and river basins. We tested normality of site elevation and temperature data with a Shapiro-Wilk goodness-of-fit test and used ANOVA and logistic regression to analyze the relationship between those metrics for sites where *Bd* was detected. Statistical analyses were conducted using Rcmdr package, v.2.0.3 in R, v.3.0.2 statistical software (The R Foundation for Statistical Computing, Vienna, Austria) at $P < 0.05$.

RESULTS

We found *Bd* to be widespread among North Carolina's hellbender populations. From 2008–2012, we found *Bd* in all HUC 6 river basins sampled (n = 4), in all HUC 8 sub-basins sampled (n = 8), in 12 of 13 (92.3%) counties sampled, and in 19 of 33 (57.6%) individual stream systems sampled (Table 1, Fig. 1). Where zoospore equivalent data were available for *Bd*-positive samples (n = 38), zoospore counts across replicates averaged 12 with a maximum of 380 zoospores/µL. A majority of positive samples (52.6%) had average zoospore equivalents < 1, 34.2% had an average of 1–20, and 13.2% averaged > 20 zoospores/µL.

Of the 19 *Bd*-positive streams, 13 (68.4%) had both negative and positive *Bd* samples, while six (31.6%) only had positive *Bd* samples with no negative or equivocal results. Of 165 samples total, 46 (27.9%) were positive for *Bd*, 117 (70.9%) negative, and two (1.2%) equivocal. The equivocal results occurred in adult hellbenders from two different streams, one where all other samples were negative and one that had both positive and negative results.

Of the 140 adults sampled, 40 (28.6%) were positive for *Bd*, while six (24.0%) of the 25 juveniles sampled were positive, including two gilled larvae (Table 2). Prevalence of *Bd* between adults and juveniles was not significantly different (Fisher's exact test, $P = 0.8096$). Not including adult hellbenders whose sex was undetermined in the field, the number of adult males and females tested was almost identical (66M, 67F). However, females showed a significantly higher prevalence of *Bd* than males, with 26 females (38.8%) positive compared to 13 males (19.7%; $\chi^2 = 5.35$, df = 1, $P = 0.021$; Table 2). Hellbenders from the Kanawha River Basin showed the highest prevalence of *Bd* (35.3%), while the Middle Tennessee-Hiwassee River Basin had the lowest (18.5%; Table 2). There were no significant differences in *Bd* prevalence among river basins ($\chi^2 = 1.90$, df = 3, $P = 0.593$). The 15 captive hellbenders of North Carolina origin we tested showed a similar prevalence of *Bd* as did wild populations. Two of six adults and two of nine juveniles tested positive for *Bd* for a combined prevalence of 26.7% (95% C.I. = 7.8–55.1%).

None of the hellbenders tested for *Bd* showed any noticeable physical or behavioral symptoms that would indicate severe skin infection, lethargy, emaciation, morbidity, or other evidence of compromised immunology or outright disease. Overall we noted only minor abrasions, head, body, or tail scarring, healed or healing wounds, and regenerating tissue (tail tips, digits, limbs; see Appendix A). Not counting one *Bd*-negative animal whose body condition was not recorded, 46.9% of all captures had some type of minor injury or former wound. Of the animals that tested negative for *Bd*, 50.9% had minor physical anomalies compared to 37.0% of animals positive for *Bd*. On very few occasions did we note the presence of suspicious-looking tissue such as inflamed masses or a white-colored fungus at the site of an old wound. Subsequent laboratory tests at the North Carolina Zoological Park determined the white-colored coating to be a common type of fish fungus in the genus *Saprolegnia*. There were no significant differences in the frequency of physical injuries or anomalies in *Bd*-positive hellbenders versus *Bd*-negative hellbenders (Fisher's exact test, $P = 0.120$) or in adult males versus adult females (Fisher's exact test, $P = 0.116$). There were no significant differences in hellbender physical condition across river basins ($\chi^2 = 2.769$, df = 3, $P = 0.429$). However, as to be expected, there were significantly fewer injuries or anomalies in juveniles compared to adults (Fisher's exact test, $P < 0.001$).

Site elevations and water temperatures, collected at the time of sampling, were normally distributed ($W = 0.969$, $P = 0.303$; $W = 0.978$, $P = 0.705$, respectively). Elevations ranged from 361 m to 960 m (mean = 658.3 ± 136.4 m), and water temperatures ranged from 12.8° C to 27.8° C (mean = 20.7 ± 3.1° C). There was a significant, inverse relationship between site elevation and water temperature, as would be expected with typically cooler temperatures at higher elevations ($F_{1,43} = 6.61$, $P = 0.014$). However, neither water temperature ($r^2 = 0.007$, df = 1, $P = 0.504$) nor elevation ($r^2 = 0.006$, df = 1, $P = 0.510$) were significant for predicting the presence of *Bd* at field sites.

DISCUSSION

Results of our disease surveillance in hellbenders reiterate what similar studies have found, that *Bd* may be well established and widespread in aquatic systems and can be detected in both juvenile and adult hellbenders (Regester et al. 2012; Souza et al. 2012). We also detected *Bd* in two gilled hellbender larvae. This finding is contrary to a previous study (Keitzer et al. 2011) suggesting that larval aquatic salamanders may not be susceptible to *Bd* due to the limited amount of epidermal keratin. The overall *Bd* prevalence we documented for pooled samples across all river basins was 27.9%, which was comparable to the 26% prevalence (Souza et al. 2012) and within the range of 16–50% prevalence (Tominaga et al. 2013) reported in East Tennessee across multiple watersheds shared with Western North Carolina. Our results show a lower prevalence than what was found in Pennsylvania's hellbenders across two drainages, 43% (Regester et al. 2012), or in Georgia's hellbenders in one stream across early and late spring sampling seasons, 48% (Gonynor et al. 2011).

TABLE 1. Number (percentage frequency [# detected/total], 95% confidence interval) of individual Eastern Hellbender (*Cryptobranchus a. alleganiensis*) streams where *Batrachochytrium dendrobatidis* (*Bd*) was detected in at least one sample (*Bd*+) vs. streams with no detection (*Bd*-), per HUC 6 river basin in Western North Carolina, from 2008–2012.

HUC 6 River Basin	n	No. Streams *Bd*+	No. Streams *Bd*-
Middle Tennessee-Hiwassee	8	3 (37.5; 8.5–75.5)	5 (62.5[a]; 24.5–91.5)
Upper Tennessee	8	5 (62.5; 24.5–91.5)	3 (37.5; 8.5–75.5)
French Broad-Holston	12	8 (66.7[b]; 34.9–90.1)	4 (33.3; 9.9–65.1)
Kanawha	5	3 (60.0; 14.7–94.7)	2 (40.0; 5.27–85.34)
Total	33	19 (57.6; 39.22–74.52)	14 (42.4; 25.5–60.8)

[a]one equivocal result occurred in a stream with only negative results otherwise.
[b]one equivocal result occurred in a stream with both positive and negative results.

Assays of *Bd* in Florida, Mississippi, and Louisiana for other large-bodied, fully aquatic salamanders such as *Amphiuma* sp., *Necturus* sp., *Pseudobranchus* sp., and *Siren* sp. showed a similar infection prevalence of 34% (Chatfield et al. 2012), but in a separate study in Florida, *Bd* was not detected in *Siren* sp. or *Amphiuma* sp. (Rizkalla 2010).

Despite a smaller sample size than that of wild-caught animals in North Carolina, the 26.7% prevalence of *Bd* in captive hellbenders was relatively the same. This result supports other studies that indicate hellbenders raised in captivity are also susceptible to *Bd* infection, as reported for captive Ozark and Eastern Hellbenders in Missouri (Briggler et al. 2007a). Our 2 y-old juveniles, hatched in captivity at Ft. Worth Zoo, originated as eggs collected from a North Carolina river known to be positive for *Bd*. These animals were *Bd*-negative upon arrival back to North Carolina at age 20 mo. When swabbed again for *Bd*, six months after arrival to the fish hatchery, two were *Bd*-positive but apparently experiencing a sub-clinical level of infection. It is unknown when these captive animals contracted *Bd*, but it is possible the fungus could be in the hatchery's water system that includes a spring-fed pond. *Batrachochytrium dendrobatidis* has been documented in fish hatcheries and natural or artificial ponds throughout the Eastern United States (e.g., Green and Dodd 2007; Rothermel et al. 2008; Groner and Relyea 2010; Goodman and Ararso 2012; Bletz and Harris 2013), so it is likely the captive juveniles contracted the fungus once housed at the fish hatchery. Future surveillance of captive stock and baseline assays of *Bd* in resident pond-dwelling amphibians at the hatchery may reveal whether these pathogens pose a serious health risk to the captive hellbenders in this setting.

Hellbenders positive for *Bd* in North Carolina appeared to have no clinical symptoms or indications of full-blown disease, nor did they show signs of poor immunity or excessive stress that might make them more susceptible to illness (Tominaga et al. 2013). Rather, our animals seemed to be living with low-level fungal infections and likely have been doing so for some time. For positive samples, zoospore equivalents were quite low compared to thresholds established for chytridiomycosis in ranid frogs (> 10,000 zoospores/μL; Vrendenburg et al. 2010; Kinney et al. 2011), demonstrating that hellbenders were not suffering from outright disease despite possessing sub-clinical infections of *Bd*. The physical conditions we observed are commonly seen in hellbenders and can often be attributed to interactions with each other and predators (Nickerson and Mays 1973; Miller and Miller 2005). We did not see similar gross physical abnormalities, severe fungal infections, unhealed wounds, tumors, lesions, or other signs of poor immunity that have been reported for declining Ozark hellbender populations (Wheeler et al. 2002; Hiler et al. 2005). Our observations of physical condition were consistent with those of other regional hellbender disease surveillance efforts and not indicative of chytridiomycosis (Gonynor et al. 2011; Regester et al. 2012; Souza et al. 2012).

The significantly higher prevalence of *Bd* in adult female hellbenders (38.8%) compared to adult males (19.7%) in our results should be explored in future surveillance efforts and with larger sample sizes. Despite a similar prevalence of physical injuries or anomalies between males and females, future studies should examine whether differences among male and female activity levels and interactions with other hellbenders or predators have any effect on the amount or type of cutaneous bacteria available for disease resistance, or whether life-history strategies, movement patterns, or home range sizes may increase the exposure of females to pathogens, increasing disease susceptibility. If differential infection rates exist between males and females, then a greater risk of female mortality may have broad implications for declining population health, structure, and reproductive success (Unger et al. 2013b).

Further, Grant et al. (2008) suggested a link between frequency of skin shedding and disease resistance in aquatic, desmognathine salamanders, and Meyer et al. (2012) examined the role of skin sloughing to reduce fungal spores and microbes in Cane Toads (*Rhinella marina*). Nickerson et al. (2011) studied the suite of microbes available on hellbender skin that aid in tissue

Table 2. Number (percentage frequency [# detected/total], 95% confidence interval) of *Batrachochytrium dendrobatidis* positive (*Bd*+), negative (*Bd*-), and equivocal (*Bd* eq.) samples from wild-caught Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) by age class and sex, per HUC 6 river basin in Western North Carolina, 2008–2012.

Age Class / Sex	n	*Bd*+	*Bd*-	*Bd* eq.
Middle Tennessee-Hiwassee				
Adults	24	5 (20.8; 7.1–42.2)	18 (75.0; 53.3–90.2)	1 (4.17; 0.1–21.1)
Males	11	3 (27.3; 6.0–61.0)	7 (63.6; 30.8–89.1)	1 (9.1; 0.2–41.3)
Females	12	2 (16.7; 2.1–48.4)	10 (83.3; 51.6–97.9)	0
Unknown	1	0	1 (100)	0
Juveniles	3	0	3 (100)	0
Basin Total	27	5 (18.5; 6.3–38.1)	21 (77.8; 57.7–91.4)	1 (3.7; 0.1–19.0)
Upper Tennessee				
Adults	32	11 (34.4; 18.6–53.2)	21 (65.6; 46.8–81.4)	0
Males	14	3 (21.4; 4.7–50.1)	11 (78.6; 49.2–95.3)	0
Females	18	8 (44.4; 21.5–69.2)	10 (55.6; 30.8–78.5)	0
Unknown	0	0	0	0
Juveniles	1	0	1 (100)	0
Basin Total	33	11 (33.3; 18.0–51.8)	22 (66.7; 48.2–82.0)	0
French Broad-Holston				
Adults	68	18 (26.5; 16.5–38.6)	49 (72.1; 59.9–82.3)	1 (1.5; 0.0–7.9)
Males	34	5 (14.7; 5.0–31.1)	28 (82.4; 65.5–93.2)	1 (2.9; 0.1–15.3)
Females	28	12 (42.9; 24.5–62.8)	16 (57.1; 37.2–75.5)	0
Unknown	6	1 (16.7; 0.4–64.1)	5 (83.3; 35.9–99.6)	0
Juveniles	20	6 (30.0; 11.9–54.3)	14 (70.0; 45.7–88.1)	0
Basin Total	88	24 (27.3; 18.3–37.8)	63 (71.6; 61.0–80.1)	1 (1.1; 0.0–6.2)
Kanawha				
Adults	16	6 (37.5; 15.2–64.6)	10 (62.5; 35.4–84.8)	0
Males	7	2 (28.6; 3.7–71.0)	5 (71.4; 29.0–96.3)	0
Females	9	4 (44.4; 13.7–78.8)	5 (55.6; 21.2–86.3)	0
Unknown	0	0	0	0
Juveniles	1	0	1 (100)	0
Basin Total	17	6 (35.3; 14.2–61.7)	11 (64.7; 38.3–85.8)	0
TOTALS				
Adults	140	40 (28.6; 21.3–36.8)	98 (70.0; 61.7–77.5)	2 (1.43; 0.2–5.1)
Males	66	13 (19.7; 10.9–31.3)	51 (77.3; 65.3–86.7)	2 (3.00; 0.4–10.5)
Females	67	26 (38.8; 27.1–51.5)	41 (61.2; 48.5–72.9)	0
Unknown	7	1 (14.3; 0.4–57.9)	6 (85.7; 42.1–99.6)	0
Juveniles	25	6 (24.0; 9.4–45.1)	19 (76.0; 54.9–90.6)	0
Overall Total	165	46 (27.9; 21.2–35.4)	117 (70.9; 63.3–77.7)	2 (1.20; 0.2–4.3)

regeneration and combat infection, but the direct role of hellbender skin and slime components as a natural barrier and defense to pathogens like *Bd* remains largely unknown (Nickerson and Mays 1973; Mayasich et al. 2003). The relationship between activity levels and behaviors, frequency of skin shedding (or slime production), and natural disease resistance has not been adequately studied in hellbenders.

We found no significant relationship between site elevation and *Bd* presence or between water temperature and *Bd* presence, despite the fact that a majority of sampled sites fell within the temperature threshold most optimal for *Bd* growth, 17–25° C (Daszak et al. 2003; Piotrowski et al. 2004). Fisher et al. (2009) discussed the potential increased risk of *Bd* for sites with cooler temperatures and higher altitudes but noted that many studies have detected *Bd* in low-elevation, warmer locations as well. *Batrachochytrium dendrobatidis* can be rare or occur in low prevalence in cooler, headwater streams (Hossack et al. 2010), but the effect of seasonality on *Bd* prevalence in these cooler, high-elevation habitats has not been examined thoroughly (Chatfield et al. 2012). However, differential prevalence and virulence of *Bd* has been documented across seasons in a variety of other habitats, with fungal loads increasing in the spring and early summer (Kriger and Hero 2007; Kinney et al. 2011; Lannoo et al. 2011) or decreasing after episodic, dramatic environmental change such as drought (Terrell et al. 2014). These factors have not been examined in North Carolina, particularly in riverine systems.

Although this study provided a baseline of *Bd* prevalence in North Carolina's hellbenders, researchers must continue to monitor population health long-term, given the reality that the fungus is widespread and, by now, likely well established in hellbender waterways. A long-term disease surveillance effort at the population level and across a variety of river habitat conditions, landscape positions, elevations, and seasons may help us understand what factors or stressors could exacerbate the spread and virulence of this pathogen. These disease dynamics could ultimately affect hellbender populations and influence conservation efforts in North Carolina and throughout the Southern Appalachian region.

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John D. Groves has been in the zoo field for 48 y. He began his career at the Baltimore Zoo where he worked as a Keeper and Senior Keeper of Reptiles for seven years. He then went to the Philadelphia Zoological Park as Head Keeper of Reptiles in 1973, and then as Assistant Curator and Curator of Reptiles. He added Curator of Birds at the Philadelphia Zoo to his list of titles in 1986. John continued in these positions until 1993 when he took the position as Curator of Amphibians and Reptiles at the North Carolina Zoological Park. He has recently retired and is currently Curator Emeritus at the North Carolina Zoological Park. John has led and/or participated in research and conservation trips to Bolivia, Peru, Venezuela, Costa Rica, Mexico, Hawaii, and the Mariana Islands. John has also done much research in various parts of the United States. He has been a Research Associate at the Academy of Natural Sciences of Philadelphia (Vertebrate Zoology), a Research Associate (Ornithology) at the North Carolina Museum of Natural Sciences in Raleigh, and a Conservation Associate at the Philadelphia Zoological Garden. (Photographed by Lori Williams).

APPENDIX A. Field notes of physical condition (e.g., injuries, anomalies, scarring, etc.) and results of *Batrachochytrium dendrobatidis* (*Bd*) testing of wild-caught Eastern Hellbenders (*Cryptobranchus a. alleganiensis*) by age class, sex, and HUC 6 river basin in Western North Carolina, 2008–2012; one animal where body condition was not recorded is not included. A = Adult; J = Juvenile; F = Female; M = Male; U = Undetermined Sex; FB = French Broad; UpTN = Upper Tennessee; MidTn = Middle Tennessee; Hiw = Hiwassee; LF = Left Front Foot; LR = Left Rear Foot; RR = Right Rear Foot; RF = Right Front Foot.

Hellbender Sample	Age Class	Sex	HUC 6 River Basin	*Bd* Result	Physical Condition (injuries or anomalies)
2008_01	A	F	FB-Holston	negative	none
2008_02	A	F	FB-Holston	positive	none
2008_03	A	M	UpTN	negative	none
2008_04	A	M	UpTN	negative	missing LF digits1, 3, 4
2008_05	A	F	UpTN	positive	none
2008_06	A	M	UpTN	negative	no toes on rear feet, 3–4 cm of tail gone, open sore on head over left eye
2008_07	A	F	UpTN	positive	none
2008_08	A	F	UpTN	positive	none
2008_09	A	F	UpTN	negative	none
2008_10	A	M	UpTN	negative	scar on head, bite marks on end of tail
2008_11	J	U	UpTN	negative	none
2008_12	A	M	UpTN	negative	missing RR; LF digit 3 missing
2008_13	A	F	UpTN	negative	missing RR4, RF digit 2
2008_14	A	M	UpTN	negative	none
2008_15	A	F	UpTN	negative	RR digits 4,5 reduced; tissue missing from tail
2008_16	A	F	UpTN	negative	notch on tail
2008_17	A	F	UpTN	positive	LF digits 1,3,4 missing
2008_18	A	M	UpTN	negative	none
2008_19	A	F	UpTN	negative	tail notches, injury on LF elbow
2008_20	A	M	UpTN	negative	missing RR digits 1,4
2008_21	A	F	FB-Holston	negative	bite mark on top and bottom of head
2008_22	A	F	FB-Holston	positive	injury on top of head, bottom jaw, cloaca, right eye infected
2008_23	J	U	FB-Holston	negative	LF missing two toes; tail tip split; RR digits1, 2 fused
2008_24	A	F	FB-Holston	positive	none
2008_25	A	M	FB-Holston	negative	RR foot has extra digit
2008_26	J	U	FB-Holston	positive	none
2008_27	A	M	FB-Holston	positive	none
2008_28	A	M	FB-Holston	positive	bite on RR leg; tumor-like mass on tail; toe missing
2008_29	A	F	FB-Holston	positive	none
2008_30	A	F	FB-Holston	positive	none
2008_31	J	U	FB-Holston	positive	none
2008_32	A	F	FB-Holston	positive	exposed bone/infectious tissue on RR; stunted toe RF
2008_33	J	U	FB-Holston	negative	none
2008_34	J	U	FB-Holston	positive	old scars on head; missing RF
2009_01	A	F	Kanawha	positive	half of LF digit 2 missing; bump on tail
2009_02	A	F	Kanawha	positive	none
2009_03	J	U	FB-Holston	negative	cleft pallet
2009_04	A	F	FB-Holston	positive	none
2009_05	J	U	FB-Holston	positive	notch at top of tail
2009_06	J	U	FB-Holston	positive	none
2009_07	J	U	FB-Holston	negative	none
2009_08	A	U	FB-Holston	positive	none
2009_09	A	M	FB-Holston	negative	head scar
2009_10	A	M	FB-Holston	negative	none
2009_11	A	F	FB-Holston	positive	none
2009_12	A	M	FB-Holston	positive	none
2009_13	A	M	FB-Holston	negative	old wounds on head
2009_14	A	M	FB-Holston	negative	none
2009_15	A	M	FB-Holston	negative	none
2009_16	A	F	FB-Holston	negative	notch on LF elbow
2009_17	J	U	FB-Holston	positive	none
2009_18	J	U	FB-Holston	negative	none
2009_19	A	F	FB-Holston	positive	none
2009_20	A	M	FB-Holston	negative	old bite marks on head, tail; RF, LF, LR all with missing toes

2009_21	A	M	FB-Holston	negative	LR3,4,5 missing/regenerating
2009_22	A	F	FB-Holston	negative	old bite on tail; missing portions of RF, LF, LR feet, toes
2009_23	J	U	FB-Holston	negative	none
2009_24	A	F	Kanawha	negative	none
2010_01	J	U	FB-Holston	negative	none
2010_02	J	U	FB-Holston	negative	none
2010_03	J	U	FB-Holston	negative	none
2010_04	A	F	Kanawha	negative	none
2010_05	A	M	Kanawha	negative	none
2010_06	J	U	Kanawha	negative	none
2010_07	A	F	Kanawha	positive	none
2010_08	A	F	Kanawha	negative	none
2010_09	A	M	Kanawha	negative	none
2010_10	A	M	Kanawha	positive	old bite or cut on LR
2010_11	A	M	Kanawha	positive	none
2010_12	A	F	FB-Holston	negative	none
2010_13	A	U	FB-Holston	negative	none
2010_14	A	F	FB-Holston	negative	none
2010_15	A	M	FB-Holston	negative	none
2010_16	J	U	FB-Holston	negative	none
2010_17	A	M	FB-Holston	negative	LF has fused toes
2010_18	A	M	FB-Holston	negative	old wound on tail
2010_19	A	M	FB-Holston	negative	old notch on tail
2010_20	A	U	FB-Holston	negative	LF toes1–3 missing; notch on bottom of tail
2010_21	A	U	FB-Holston	negative	notch on tail
2010_22	A	F	FB-Holston	negative	none
2010_23	A	M	FB-Holston	negative	none
2010_24	A	F	FB-Holston	negative	none
2010_25	A	F	Kanawha	negative	LF all toes missing; nares inflamed; wound on R side of neck near gill slit
2010_26	A	F	FB-Holston	negative	yellow spot on snout
2010_27	A	M	Kanawha	negative	LR1 missing
2010_28	A	M	Kanawha	negative	none
2010_29	A	M	Kanawha	negative	LF digit 3 no toe pad
2010_30	A	F	UpTN	negative	LR foot has white fungus
2010_31	A	F	Kanawha	positive	LF digit 1 missing; o ld scar on forehead and chin
2010_32	A	F	MidTN-Hiw	negative	notch on tail
2010_33	A	M	MidTN-Hiw	positive	none
2010_34	J	U	MidTN-Hiw	negative	none
2010_35	A	F	FB-Holston	negative	notch on top of tail regenerating
2010_36	A	U	FB-Holston	negative	none
2010_37	A	M	FB-Holston	negative	old scar on forehead; missing RR digit 3
2010_38	A	M	FB-Holston	negative	old scar on forehead; RF2 stub toe; notch on keel of tail
2010_39	A	M	FB-Holston	negative	old scar on forehead; LR digit 3 missing; LR digits 1, 2 short and regenerating
2010_40	A	F	FB-Holston	negative	notch on tail tip
2010_41	A	M	FB-Holston	negative	old bite on tail; notch on tail; LR toes regenerating; dark pigment on center of head
2010_42	A	M	FB-Holston	negative	RF digit 3 missing; all other digits on RF foot regenerating
2010_43	A	F	FB-Holston	negative	none
2010_44	A	M	FB-Holston	negative	RR4 missing
2010_45	A	M	FB-Holston	negative	notch on tail; RF digit 1 missing
2010_46	A	M	FB-Holston	negative	none
2010_47	A	M	FB-Holston	negative	none
2010_48	A	M	FB-Holston	negative	bite on tail
2010_49	A	M	FB-Holston	negative	puncture wound on Right hip; white fungus on wound and venter
2010_50	J	U	FB-Holston	negative	none
2010_51	A	F	FB-Holston	positive	none
2010_52	A	M	FB-Holston	negative	laceration on R forearm; missing one rear toe
2010_53	J	U	FB-Holston	negative	none
2010_54	J	U	FB-Holston	negative	none
2010_55	J	U	FB-Holston	negative	none

2011_01	A	F	MidTN-Hiw	positive	old notch on left side
2011_02	A	U	MidTN-Hiw	negative	LR toes short and regenerating
2011_03	A	M	MidTN-Hiw	positive	none
2011_04	A	M	MidTN-Hiw	negative	none
2011_05	A	F	MidTN-Hiw	negative	scar on LF foot
2011_06	J	U	MidTN-Hiw	negative	none
2011_07	A	F	MidTN-Hiw	negative	none
2011_08	A	F	MidTN-Hiw	positive	notches on posterior tail keel
2011_09	A	F	MidTN-Hiw	negative	none
2011_10	A	M	MidTN-Hiw	negative	notch on bottom of tail tip
2011_11	A	M	MidTN-Hiw	negative	none
2011_12	A	F	MidTN-Hiw	negative	lower chin has abrasion, raw area
2011_13	A	M	MidTN-Hiw	negative	none
2011_14	A	F	MidTN-Hiw	negative	RF foot all toes missing, starting to regenerate 1, 2; LR digit 1 old abscess; four skin tags on ventral side of tail
2011_15	A	F	MidTN-Hiw	negative	none
2011_16	A	F	MidTN-Hiw	negative	none
2011_17	A	M	MidTN-Hiw	negative	LR has a double toe; old injury on tail, possibly from fishing line; RF has red, swollen footpad
2011_18	A	F	MidTN-Hiw	negative	skin tag on RR5
2011_19	A	M	MidTN-Hiw	negative	LR digit 5 regenerating; bloody lower lip
2011_20	A	M	MidTN-Hiw	negative	RF digit 3 regenerating; old bite mark both sides of tail
2011_21	A	F	MidTN-Hiw	negative	none
2011_22	A	M	MidTN-Hiw	equivocal	none
2011_23	A	M	MidTN-Hiw	positive	none
2011_24	A	M	MidTN-Hiw	negative	scar near R eye; divot in tail keel
2011_25	A	M	UpTN	positive	LF digits 1, 2 regenerating, 3–5 missing; RF digits1,2,3 missing; right eye damage; old wounds on left side, base of tail, end of tail
2011_26	A	F	UpTN	positive	none
2011_27	A	F	UpTN	negative	RR limb missing (trying to regenerate with 1–2 toes); LR digit 4 regenerating; skin tag on tail tip
2011_28	A	M	UpTN	negative	notches on tail; old discoloration on anterior tail keel
2011_29	A	M	UpTN	negative	none
2011_30	A	F	UpTN	positive	none
2011_31	A	F	UpTN	positive	scrape L side of neck; RR digits 2,3,4 bitten off
2011_32	A	M	UpTN	positive	divot in anterior tail keel
2011_33	A	F	UpTN	negative	none
2011_34	A	F	UpTN	positive	none
2011_35	A	M	UpTN	negative	RR4, 5 regenerating
2011_36	A	M	UpTN	positive	RR1 missing; LR foot all toes missing and single toe pad regenerating
2011_37	A	F	UpTN	negative	divot on anterior tail keel; joints of leg fused with skin tag; two toes regenerating
2011_38	J	U	MidTN-Hiw	negative	none
2011_39	A	F	MidTN-Hiw	negative	none
2011_40	A	F	MidTN-Hiw	negative	none
2011_41	A	F	FB-Holston	negative	none
2011_42	A	M	FB-Holston	equivocal	old bite marks/scars on RR foot and tail
2011_43	A	F	FB-Holston	positive	old bite mark/scar on top of tail
2012_01	A	F	FB-Holston	negative	none
2012_02	A	F	FB-Holston	negative	RR1–4 missing, regenerating
2012_03	A	M	FB-Holston	positive	none
2012_04	A	M	FB-Holston	negative	old scar from bite on venter, lower R
2012_05	A	M	FB-Holston	negative	none
2012_06	A	M	MidTN-Hiw	positive	ragged tail tip
2012_07	A	F	FB-Holston	positive	none
2012_08	A	M	FB-Holston	negative	none