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PREVALENCE OF INFECTION BY *BATRACHOCHYTRIUM DENDROBATIDIS* AND *RANAVIRUS* IN EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*) IN EASTERN TENNESSEE

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ABSTRACT: Hellbenders ($n=97$) were collected from the Little and Hiwassee Rivers in eastern Tennessee, USA, during 2009 and 2010. Location and morphometrics for each animal were recorded, and nonlethal tissue samples were collected to estimate the prevalence of infection with *Batrachochytrium dendrobatidis* (*Bd*) and *Ranavirus* in each watershed and year. Real-time polymerase chain reaction was performed on skin swabs for *Bd* and on tail clips for ranaviruses. Overall prevalences of DNA of *Bd*, *Ranavirus*, and coinfections (i.e., detectable DNA of both pathogens in the same individual) were 26%, 19%, and 5%, respectively. Differences in infection prevalence were detected between watersheds and years. Gross lesions were observed in 31 animals (32%), but the types of lesions were not consistent with chytridiomycosis or ranaviral disease. This is the first report of infection of eastern hellbenders with *Bd* and *Ranavirus*. Despite infection by both pathogens, it is unclear whether chytridiomycosis or ranaviral disease develops in wild populations of hellbenders. More research is needed to determine the susceptibility of hellbenders to *Bd* and ranaviruses and their role in the epidemiology of these pathogens.

Key words: *Batrachochytrium dendrobatidis*, chytrid, *Cryptobranchus alleganiensis*, hellbender, ranavirus, Tennessee.

INTRODUCTION

The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is one of two subspecies of hellbenders found in the United States, and over most of its range, its abundances are decreasing (Foster et al., 2009; Burgmeier et al., 2011). Several factors have been hypothesized for contributing to hellbender declines (Mayasich et al., 2003), including pathogens (Irwin, 2008). In Tennessee, USA, hellbenders are considered "in need of management" according to the State Wildlife Action Plan (Tennessee Wildlife Resources Agency, 2005).

Two infectious agents, *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses, have been implicated in massive or localized mortality of amphibians (Gray et al., 2009a; Kilpatrick et al., 2010). Although *Bd* is a fungal pathogen that has been associated with mortality predominately in adult frogs (Voyles et al., 2011), experimental infection of some species of salamanders has demonstrated that morbidity and mortality from this pathogen are possible (Chinnadurai et al., 2009; Vazquez et al., 2009; Rosenblum et al., 2010). Recent studies have shown that *Bd* is present throughout the southeastern USA, and commonly found in red-spotted newts (*Notophthalmus viridescens*; Rothermel et al., 2008; Keitzer et al., 2011). The pathogen also has been detected in several species of lungless salamander (Plethodontidae) in the southern Appalachian Mountains (Miller and Gray, unpubl.). There is one published report of *Bd* prevalence in an eastern hellbender population in Georgia (Gonynor et al., 2011), and it has been detected in Ozark hellbenders (*Cryptobranchus alleganiensis bishop*; Irwin, 2008). Researchers were unable to determine if *Bd* led to negative health effects or if Ozark hellbenders were acting as carriers of the pathogen (Irwin, 2008).

Ranaviruses are a group of pathogens belonging to the genus *Ranavirus* (family *Iridoviridae*) and have been associated with mortality of frogs and salamanders (Miller et al., 2011). A frog virus 3–like ranavirus was responsible for mortality of Chinese giant salamanders (*Andrias davidianus*), which are in the same family (Cryptobranchidae) as hellbenders (Geng et al., 2011). A ranavirus also has been identified in lungless salamanders in the southern Appalachian Mountains, with greater prevalence noted in those species whose larvae are aquatic (Gray et al., 2009b).

Tributaries containing lungless salamanders and other potential host species of *Bd* and ranaviruses typically are interconnected with lower elevation streams containing hellbenders, and some species (e.g., Blue Ridge two-lined salamander, *Eurycea wilderae*) can be syntopic (Petranka, 1998). Thus, it is likely that hellbenders are exposed to *Bd* and ranaviruses in the wild, yet to our knowledge, no studies have been published examining the co-occurrence of these pathogens in eastern hellbender populations. The purpose of this study was to estimate the prevalences of *Bd* and *Ranavirus* in eastern hellbenders in two major southern Appalachian watersheds in eastern Tennessee.

MATERIALS AND METHODS

Field collection and sample analysis

Eastern hellbenders were collected from two watersheds (Little River [LR] and Hiwassee River [HR]) in eastern Tennessee from May to September 2009 and May to August 2010. The LR and HR watersheds are located northwest and southwest of the Great Smoky Mountains National Park (GSMNP). Hellbenders were sampled from the LR in the GSMNP and from the HR in the Cherokee National Forest. The only other amphibian species commonly encountered during sampling was the mudpuppy (*Necturus maculosus*).

Hellbenders were located under rocks during daylight, caught by hand, and placed into a cloth mesh bag in the water or a plastic bag filled with river water. Mesh bags were used only once per day. At the end of each day of collection, mesh bags were soaked in a dilute (1%) solution of chlorhexidine (Vedco, Inc., St. Joseph, Missouri, USA) for approximately 12 hr, then laundered with detergent and bleach in hot water (approximately 55 C). This method of disinfection should have been adequate to inactivate both *Bd* and ranaviruses (Johnson et al., 2003; Bryan et al., 2009). If plastic bags were used, each animal was placed into a new bag. Because previous research on hellbenders had been performed at some of the sampling sites, each animal was scanned for a passive integrated transponder (PIT) tag. If no PIT tag was detected and the animal was >50 g, a PIT tag was placed subcutaneously over the dorsum at the base of the tail (Biomark, Inc., Boise, Idaho, USA) following swab and tissue sample collection. In addition to contributing to a long-term survival data set on hellbenders at these sites, PIT tags allowed identification of recaptured animals. Swab and tissue samples for pathogen testing were collected only once per individual each year. Total length, snout-to-vent length, mass, approximate age, date, time, location of capture, and any physical abnormalities were recorded. Animals were returned to the location where they were captured after sampling. Disposable nitrile gloves were worn while handling animals and were changed between animals.

To sample for *Bd*, the ventral surfaces of the animals were swabbed with a fine tipped rayon swab (Dryswabs™ MW113, Medical Wire & Equipment Co., Corsham, Wiltshire, England) at least 20 times as described previously (Hyatt et al., 2007). Swabs were placed into a plastic vial and kept on ice packs (<6 hr) until placed into a −80 C freezer for storage. At the end of each sampling year, all samples were shipped overnight on dry ice to the Amphibian Disease Laboratory at the San Diego Zoo's Institute for Conservation (San Diego, California, USA). The PCR protocol used by the San Diego Zoo has been described (Hyatt et al., 2007; Keitzer et al., 2011). Briefly, DNA template for real-time Taqman PCR was prepared using PrepMan® Ultra (Applied Biosystems, Foster City, California, USA). Reactions were run in triplicate; positive controls and standard curves were constructed using 10-fold serial dilutions of cultured *Bd* zoospores (Keitzer et al., 2011).

For ranavirus testing, an oval (1×0.5-cm diameter) section of skin (epidermis and dermis) was collected using a sterile scalpel blade and thumb forceps from the middorsal tail. The skin sample was placed into 70% ethanol in a plastic vial and kept at room temperature until analysis. Genomic DNA was extracted from the tail skin using commercially

available kits (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, California, USA). Conventional PCR was performed in duplicate using the protocol and primer sets (MCP4 and MCP5) described by Mao et al. (1997) that targeted an approximately 450 base-pair sequence of the major capsid protein (MCP) gene of *Ranavirus*. The PCR products were resolved via electrophoresis on a 1.0% agarose gel. Four controls were included for each PCR run and included two negative controls (water and tissue from a *Ranavirus*-negative tadpole) and two positive controls (cultured *Ranavirus* and tissue from a *Ranavirus*-positive tadpole). For positive samples, a TaqMan real-time qPCR was performed following Picco et al. (2007) to verify the PCR products as *Ranavirus* DNA.

Statistical analysis

Infection prevalence with 95% confidence intervals was calculated for each pathogen and coinfection with both pathogens. A chi-square test of homogeneity was used to test the difference in prevalence between watersheds and years. A Fisher's exact test was used if less than five animals were present in a category. All analyses were performed using Epi Info™ statistical software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA) at $P<0.05$.

RESULTS

Samples were collected from 97 hellbenders over 2 yr. During 2009, 45 animals were sampled (HR=25; LR=20), and in 2010, 52 animals were sampled (HR=30; LR=22). The overall prevalences of detectable DNA of *Bd*, *Ranavirus*, and coinfection (i.e., detectable DNA of both pathogens in the same individual) were 25% (17–36%; 95% CI), 19% (11–28%; 95% CI), and 5% (2–12%; 95% CI), respectively (Table 1). No animals were positive for *Ranavirus* DNA in 2010. There was only one recapture in 2010 from 2009. This animal was positive for *Ranavirus* DNA in 2009 but negative in 2010; it was negative for *Bd* DNA both years.

There was no significant difference in *Bd* infection between years in either river (Table 1). The prevalence of *Bd* was greater in the HR than in the LR in 2009 ($P=0.01$) and 2010 ($P=0.04$). The prevalence of *Ranavirus* was greater in 2009 than in 2010 in both rivers ($P<0.01$), and in 2009 was greater in the LR compared to the HR ($P=0.01$). There was no difference in coinfection prevalence between watersheds, but it was greater in 2009 than in 2010 ($P=0.02$, Table 1). Various physical lesions were noted on 31 animals (Table 2); similar lesions were seen on animals with and without presence of an infectious agent.

DISCUSSION

The prevalence of *Bd* infection in hellbenders in the HR was greater than in the LR, and this trend was consistent both years. Qualitatively, the density of hellbenders appeared to be greater in the HR compared to the LR because catch-per-unit effort was lower for the latter. If transmission of *Bd* is dependent on the population density of infected hellbenders (Rachowicz and Briggs, 2007; Briggs et al., 2010), a greater number of individuals could facilitate conspecific interactions and spread of the pathogen. Additionally, a higher density of animals and higher prevalence of *Bd* in those animals may

TABLE 1. Prevalence with 95% confidence interval of *Batrachochytrium dendrobatidis*, *Ranavirus*, and coinfection by year and river in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*), Tennessee, USA.

Sample year	River	*Batrachochytrium dendrobatidis* prevalence (%)	*Ranavirus* prevalence (%)	Coinfection prevalence (%)
2009	Hiwassee ($n=25$)	44 (24–65)	24 (9–45)	12 (3–31)
	Little ($n=20$)	10 (1–32)	60 (36–81)	10 (1–32)
2010	Hiwassee ($n=30$)	33 (17–53)	0 (0–12)	0 (0–12)
	Little ($n=22$)	9 (1.1–30)	0 (0–15)	0 (0–15)

TABLE 2. Lesions observed and results of polymerase chain reaction for *Batrachocyhtrium dendrobatidis* or *Ranavirus* infection associated with 31 eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in the Hiwassee (H) and Little (L) Rivers, Tennessee, 2009 and 2010.

ID	River	*Batrachochytrium dendrobatidis*[a]	*Ranavirus*[a]	Lesion
09-02	H	N	N	Healed wound on tail, missing digits
09-03	H	P	P	Healed abrasions on face and other parts of body
09-05	H	P	N	Missing digits, scars on tail
09-09	H	N	N	Cloudy left cornea, missing digits
09-11	H	P	N	Tail abrasions
09-12	H	N	P	Healed wounds on front R limb
09-13	H	P	N	Missing digits
09-14	H	N	N	Numerous healed wounds
09-15	H	P	N	Healed abrasions
09-16	H	N	N	Healed wounds on toes
09-18	H	N	N	Shortened tail, missing and fused toes
09-19	H	N	N	Healed scar on nose
09-20	H	P	P	Tail lesions, right front missing digit
09-22	L	P	P	Fused toes
09-30	L	N	P	Missing foot
09-35	L	N	P	Healed abrasion on tail
09-43	H	N	N	Healed wound on tail
10-01	H	N	N	Healed scars and abrasions on tail and body
10-02	H	P	N	Numerous subcutaneous worms
10-05	H	P	N	Healed scars on ventral body
10-12	H	N	N	A few toes missing
10-14	H	P	N	Numerous subcutaneous worms
10-19	H	N	N	Subcutaneous worms, healed wound on left lower jaw
10-20	H	P	N	Missing numerous digits
10-28	H	P	N	Missing digits
10-30	H	N	N	Subcutaneous worms, missing digit
10-31	L	N	N	Missing digits and right eye
10-36	L	N	N	Missing digit
10-38	L	N	N	Missing digits
10-39	L	N	N	Extra toes on one foot
10-48	L	P	N	Healed wounds

[a] N = negative and P = positive PCR result for *Batrachochytrium dendrobatidis* or *Ranavirus* DNA.

lead to more *Bd* zoospores being present in the environment, leading to additional infections. These hypotheses need to be investigated further for the hellbender populations in these watersheds.

The prevalence of *Ranavirus* infection in hellbenders was greater in the LR than in the HR in 2009. Ranaviruses have been reported in populations of lungless salamanders that inhabit tributaries that flow into the LR (Gray et al., 2009b). The mechanisms driving the greater prevalence in the LR in 2009 are unknown. However, this geographic trend in *Ranavirus* infection prevalence was not observed in 2010, as *Ranavirus* infection was not detected in either watershed. Seasonal and annual fluctuations in *Ranavirus* infection prevalence are common (Hoverman et al., 2011), and are likely related to changes in abiotic or biotic mechanisms that facilitate transmission or induce stress. The recaptured animal that was positive in 2009 and negative in 2010 suggests that this animal may have cleared the virus. Sublethal infections and clearing of ranaviruses has been observed in experimental challenges (Tweedle and Granoff, 1968; Gray et al., in press) and captive colonies (Miller, unpubl.), and suspected in free-ranging amphibians

(Zupanovic et al., 1998). It is important to note that testing of skin samples for *Ranavirus* infections underestimates prevalence compared to testing samples from internal organs (St-Amour and Lesbarreres, 2007; Gray et al., in press). Thus, prevalence was likely underestimated in our study: however, trends between years and watersheds should not have been affected because the same procedures were used.

Five animals were coinfected with *Bd* and a ranavirus. Researchers are just beginning to investigate coinfections with these pathogens, which occur in the wild and captivity (Fox et al., 2006; Miller et al., 2008; Rothermel and Travis, 2011). In the field, the significance of detectable coinfections remains unclear, primarily because concurrent histopathology has not been performed. However, at least one morbidity event at a field site (Rothermel and Travis, 2011) and one mortality event in a captive colony (Miller et al., 2008) have been documented with coinfection by these pathogens. In both the morbidity and mortality event, one pathogen appeared as the primary (more severe) pathogen, and this varied by amphibian host. Given the lack of supportive histology, gross lesions, or other evidence of clinical disease, the significance of the detection of both pathogens in the hellbenders remains unclear. It is possible that eastern hellbenders are not very susceptible to these pathogens or perhaps they serve as subclinical carriers (i.e., reservoirs) for the pathogens. Determining the susceptibility of hellbenders to *Bd* and ranaviruses and defining the role hellbenders might play in the epidemiology of these pathogens needs further investigation before management strategies can be developed.

Physical abnormalities were observed on animals with and without infection. None of the lesions noted were specifically characteristic of either *Bd* or *Ranavirus* infections in amphibians, and most were likely due to previous trauma. Loss of digits and feet has been observed in Ozark hellbender populations, but the etiology and effect of lesions in hellbenders needs further investigation (Irwin, 2008; Nickerson et al., 2011).

This is the first published report of detection of *Bd* and *Ranavirus* in eastern hellbenders. Similar surveillance studies should be performed in other areas of eastern hellbender distribution, and population levels monitored simultaneously. Additionally, prevalence of infection with *Bd* and ranaviruses needs to be monitored several times per year to increase the likelihood of detecting an outbreak (Hoverman et al. 2011), and improve understanding of the seasonal and annual fluctuations in prevalence of these pathogens. Field studies should be combined with controlled experimental challenges that test the relative susceptibility of hellbenders to these pathogens exposed singly and in combination.

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