GEOGRAPHIC AND INDIVIDUAL DETERMINANTS OF IMPORTANT AMPHIBIAN PATHOGENS IN HELLBENDERS (CRYPTOBRANCHUS ALLEGANIENSIS) IN TENNESSEE AND ARKANSAS, USA

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ABSTRACT: Wildlife diseases are a major threat for species conservation and there is a growing need to implement disease surveillance programs to protect species of concern. Globally, amphibian populations have suffered considerable losses from disease, particularly from chytrid fungi (*Batrachochytrium dendrobatidis* [*Bd*] and *Batrachochytrium salamandrivorans* [*Bsal*]) and ranavirus. Hellbenders (*Cryptobranchus alleganiensis*) are large riverine salamanders historically found throughout several watersheds of the eastern and midwestern US. Populations of both subspecies (Ozark hellbender, *Cryptobranchus alleganiensis bishopi*; eastern hellbender, *Cryptobranchus alleganiensis alleganiensis*) have experienced precipitous declines over at least the past five decades, and emerging pathogens are hypothesized to play a role. We surveyed Ozark hellbender populations in Arkansas (AR) and eastern hellbender populations in Middle Tennessee (MTN) and East Tennessee (ETN) for both chytrid fungi and ranavirus from swabs and tail tissue, respectively, from 2011 to 2017. Overall, we detected *Bd* on hellbenders from nine out of 15 rivers, with total prevalence of 26.7% (54/202) that varied regionally (AR: 33%, 28/86; MTN: 11%, 4/36; ETN: 28%, 22/80). Ranavirus prevalence (9.0%, 18/200) was comparatively lower than *Bd*, with less regional variation in prevalence (AR: 6%, 5/85; MTN: 11%, 4/36; ETN: 10%, 8/79). We did not detect *Bsal* in any hellbender populations. We detected a significant negative correlation between body condition score and probability of ranavirus infection ($\beta=-0.13$, SE$=0.06$, 95% confidence interval: -0.24, -0.02). Evaluation of infection load of positive individuals revealed different trends than prevalence alone for both ranavirus and *Bd*, with MTN having a significantly greater average ranaviral load than both other regions. We documented a variety of lesions that likely have multiple etiologies on hellbenders located within all geographic regions. Our data represent a multiyear pathogen dataset across several regions of *C. alleganiensis*, and we emphasize the need for continued pathogen surveillance.

Key words: Amphibian disease, *Bd*, *Bsal*, chytrid, *Cryptobranchus*, hellbender, pathogen surveillance, ranavirus.

INTRODUCTION

Infectious diseases are major threats to wildlife conservation (Cunningham et al. 2017). Precipitous population declines have occurred after outbreaks of emerging patho- gens, including white nose syndrome in North American bats (Frick et al. 2010), nidovirus infection in Bellinger River snapping turtles (*Myuchelys georgesi*; Zhang et al. 2018), and bacterial septicemia in steppe saiga antelope (*Saiga tatarica*; Kock et al. 2018).

Amphibians have experienced particularly high mortality rates from disease. Worldwide, chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is implicated in at least 90 amphibian species extinctions (Scheele et al. 2019). Ranavirus is an amphibian pathogen of concern with potential to cause extinctions in both North America (Green at al. 2002; Earl et al. 2016) and Europe (Duffus and Cunningham 2010). Furthermore, a novel chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), has been discovered in Europe and, in contrast to *Bd*, is highly lethal to species of salamanders from several families (Martel et al. 2014). Wildlife diseases can be difficult to control and little epidemiologic data exist for a majority of emerging pathogens (Ryser-Degiorgis 2013). Disease monitoring programs have historically been minimal, and mortality events have been hard to predict, which makes it difficult for researchers and conservation agencies to implement preventative strategies (MacPhee and Greenwood 2013). Successful conservation management plans for at-risk amphibian species should include disease mitigation, surveillance programs, and strategies to prevent emerging pathogens from affecting population health (Kuiken et al. 2005).

The hellbender (*Cryptobranchus alleganiensis*) is a large, fully aquatic salamander that inhabits cool, rocky, well-oxygenated rivers and streams in the eastern and midwestern US (Nickerson and Mays 1973). Populations of both subspecies (eastern hellbender, *C. alleganiensis alleganiensis*; Ozark hellbender, *C. alleganiensis bishopi*) have been greatly reduced throughout their range (Wheeler et al. 2003; Graham et al. 2011; US Fish and Wildlife Service 2018).

Streamside disturbances that result in erosion of adjacent riparian zones with subsequent sedimentation have been suggested as primary causes of declines of hellbenders (Humphries and Pauley 2005; Hopkins and DuRant 2011), but the mechanism by which these disturbances affect population growth are not fully understood. For eastern hellbender populations, decreased riparian forest cover has been linked to decreased juvenile recruitment (Bodinof-Jachowski and Hopkins 2018). However, within the same study area, current land use was not a strong predictor of hellbender presence (Bodinof-Jachowski et al. 2016). Other factors that affect hellbender health and survival are, therefore, likely to be involved in declines. Additionally, populations of Ozark hellbenders, which have experienced rapid declines, are affected by high prevalence of ulcerative lesions of distal extremities (Hiler et al. 2005; Nickerson et al. 2011; Hardman et al. 2020). An etiology for this syndrome has not been established, which highlights the need for range-wide disease monitoring in hellbenders.

Batrachochytrium dendrobatidis is thought to have been introduced into hellbender populations as early as 1969 (Bodinof et al. 2011), and infection is reported in several hellbender populations with no reports of chytridiomycosis (Briggler et al. 2008; Souza et al. 2012; Bales et al. 2015). However, *Bd* has caused mortality in captive adults (Dusick et al. 2016) and caused >50% mortality in captive propagation (head-starting) programs during juvenile releases (Utrup and Mitchell 2008; Dean et al. 2016). Ranaviruses have caused catastrophic mortality events in farms of the Chinese giant salamander (*Andrias davidianus*; Geng et al. 2011), and affected animals present with lesions similar to those seen in wild Ozark hellbenders. Despite these reports, data on ranavirus epidemiology for wild hellbenders are limited to one survey that reports infections with no clinical signs of disease (Souza et al. 2012). *Batrachochytrium salamandrivorans* has not been reported in the US, and all hellbenders tested have been negative for this pathogen (Bales et al. 2015). Although the effects of *Bsal* on hellbenders are not known, susceptibility to this fungus presumably would be similar to or greater than *Bd*.

Previous studies on pathogen surveillance of hellbenders provide a necessary first step, but most are restricted to small temporal and spatial scales and focus on *Bd* prevalence. No long-term datasets occur for all three globally important amphibian pathogens in hellben-

ders, and no studies have evaluated populations in Middle Tennessee and Arkansas. Furthermore, although we have evaluated toe lesion prevalence and epidemiology in Arkansas Ozark hellbenders (Hardman et al. 2020), data are lacking on disease and other health parameters associated with pathogen presence from multiple pathogens across both hellbender subspecies. To this end, we evaluated long-term trends in amphibian pathogen prevalence in wild hellbender populations, including previously unsurveyed portions of the geographic range. We acquired a large sampling dataset by coupling disease surveillance programs with ongoing population surveys in Tennessee and Arkansas over 7 yr (2011–17).

MATERIALS AND METHODS

We sampled hellbenders from 2011 to 2017 in 15 streams and rivers from the Blue Ridge ecoregion of East Tennessee (ETN), Interior Plateau of Middle Tennessee (MTN), and Ozark Highlands of Arkansas (AR). We sampled in both Tennessee ecoregions for the eastern hellbender and in AR for the Ozark hellbender. All sampling in AR was conducted under observation of the US Fish and Wildlife Service (permit TE66039A-0 the Arkansas Game and Fish Commission) and with approval from the Tennessee Wildlife Resources Agency (permits 1525, 1529, 1505, 1450, and 1877). Most of our sampling efforts were opportunistic on the basis of areas of population survey history, team availability, and stream conditions from late May to early August. We performed limited sampling between late August and mid-October to prevent disturbance of breeding and nesting. In Tennessee and in shallower water in AR, we used standard snorkeling techniques to locate individual hellbenders. In AR, we also sampled hellbenders with artificial nest boxes (Briggler and Ackerson 2012) and in deep-water habitats up to 4 m with a hookah dive system consisting of a gasoline-powered air compressor with tethered air supply lines to dive regulators. We captured any animals under cover objects and nest boxes and placed them in a clean, soft cotton or mesh bag. We kept bags submerged in the river before and after animals were processed (University of Tennessee Institutional Animal Care and Use Committee [IACUC] protocol 2481-0916). We changed dive gloves between animal captures.

We recorded presence of lesions and external abnormalities and documented abnormal behaviors, such as increased lethargy or slow righting response. To sample for chytrids (*Bd* and *Bsal*), we ran a sterile cotton swab (Fisherbrand 23400111, Thermo Fisher Scientific, Waltham, Massachusetts, USA) five times over each body region in the following order: dorsal, ventral, and inguinal areas and all portions of the feet, including toe tips (Brem et al. 2007). Although the standard *Bd* swabbing protocol in frogs generally does not include the dorsum (see Brem et al. 2007), we included it because of anecdotal reports of suspicious dorsal skin lesions associated with *Bd* infection in hellbenders. We collected tissue samples for ranavirus with a sterile punch biopsy or forceps and blade to remove a 6×3-mm tissue section of the dorsal tailfin. Tissue can be a reliable nonlethal alternative to liver samples for ranavirus detection (St-Amour and Lesbarrères 2007), is more sensitive than skin swabs (Gray et al. 2012), and has been used as a method for ranavirus surveillance in North American salamanders (Souza et al. 2012; Blackburn et al. 2015; Sutton et al. 2015). We immediately placed swabs and tissue in sterile 1.5-mL microcentrifuge tubes with 70% ethanol for field storage and transport.

After pathogen sampling, we recorded total length (TL), snout-vent length, and mass. For Ozark hellbenders, we marked every new capture with passive integrated transponder (PIT) tags (Biomark, Inc., Boise, Idaho, USA) and recorded tag numbers of previously captured animals. Application of PIT tags was not part of all sampling protocols in Tennessee; however, all available PIT tag data were recorded when available (Lee University IACUC protocol 2011-02). After collection of biological data, we released hellbenders at their capture locations. We used new containers, bags, and gloves between individuals and cleaned equipment with soap, followed by a soaking in a 5% solution of either bleach or chlorhexidine for at least 10 min. We then rinsed equipment thoroughly before use at another site.

For DNA extraction and quantitative PCR (qPCR) detection of *Bd*, *Bsal*, and ranavirus, we followed protocols described in Hardman et al. (2020). In short, we extracted DNA with DNeasy Blood and Tissue Kits (Qiagen Inc., Valencia, California, USA) and stored DNA at −80 C. We performed separate qPCR singleplex assays for each target pathogen (ranavirus, *Bd*, and *Bsal*) with primer and probe combinations from Picco et al. (2007) for ranavirus and from Blooi et al. (2013) for *Bd* and *Bsal* reactions.

We established standard curves from pure cultures for all three pathogens. For chytrid fungi (*Bd* and *Bsal*), we determined zoospore genomic equivalents (GEs) from DNA extracted from cultures of known zoospore concentrations pro-

vided by the Rollins-Smith laboratory (Vanderbilt University, Nashville, Tennessee, USA). For ranavirus, recently titrated culture was not available; thus, we could not quantify plaque-forming units. Instead, we created relative load scores that represented average cycle threshold (Ct) values, where a Ct of 45 received a score of 1, with score increases of 1 for every single-cycle decrease, resulting in a relativized load score range of 1–21 that represented Ct values from 45 to 25. A cutoff of 45 was determined for all three assays on the basis of positive control standard curves (10 serial dilutions for ranavirus standards and 1 zoospore GE for Bd or Bsal).

We performed generalized linear mixed models (GLMM) with a binomial distribution in RStudio (R Core Team 2013) with the lme4 package (Bates et al. 2015) to test for effects of measured parameters on animals being positive or negative for either Bd or ranavirus by fixed effects of region (ETN, MTN, AR), julian day, mass, and body condition score (BCS), with year as a random effect. We did not include TL or snout-vent length in our models because they were highly correlated with mass. We calculated BCS from residuals of the fitted polynomial plot of TL and mass in Microsoft Excel (Redmond, Washington, USA). We did not include sex as a predictor variable in the GLMM because sexing hellbenders is unreliable either in immature individuals or in mature individuals outside of the breeding season. We calculated z-scores for all numeric predictor variables before analysis with the exception of BCS.

We evaluated relative model fit using Akaike information criterion adjusted for small sample size (AIC_c) for all possible combinations of fixed effects to determine the top-performing model with the AICcmodavg package (Mazerolle 2019), and considered models competitive when ΔAIC_c was <2.0. We model-averaged variables that appeared in more than one of the top models, as recommended in Burnham and Anderson (2002).

We calculated infection intensity of pathogen-positive animals for each region across all years on the basis of zoospore GEs and load scores for Bd and ranavirus, respectively. We performed linear mixed model regressions to test for effects of the same predictor variables listed for the infection status models on infection intensity of both Bd-and ranavirus-positive individuals. We log- and square root–transformed zoospore GEs and ranavirus load scores, respectively, to obtain normal distributions before analysis.

RESULTS

We surveyed 15 rivers (one from AR, seven from MTN, and seven from ETN) over 7 yr, with 10 of those rivers surveyed at least three times. We sampled 202 individual hellbenders (86 Ozark hellbenders from AR, 36 eastern hellbenders from MTN, and 80 eastern hellbenders from ETN). We obtained a Bd/Bsal swab from 202 animals and tail tissue from 200 hellbenders (85 from AR, 36 from MTN, and 79 from ETN). We detected ranavirus and Bd in all three regions. We did not detect Bsal in any samples.

Overall, prevalence of Bd across all sampled populations (Fig. 1) was 26.7% (54/202, 95% confidence interval [CI]: 20.6, 32.8). By region, we found that AR had greatest prevalence (33%, 28/86, 95% CI: 22.7, 42.5), followed by ETN (28%, 22/80, 95% CI: 17.7, 37.3) and MTN (11%, 4/36, 95% CI: 0.8, 21.4). We detected Bd in nine of the 10 rivers where we conducted repeated sampling. We did not detect Bd in any of the five single-visit river sites. We observed yearly fluctuations in Bd prevalence across all regions, with greatest prevalence in 2011, when 39% (15/39, 95% CI: 23.2, 53.7) of individuals were positive for Bd. Other years with sampling in all three regions (2012, 2014, and 2017) had prevalence rates of 15% (9/59, 95% CI: 6.1, 24.4), 26% (12/46, 95% CI: 13.4, 38.8), and 32% (10/31, 95% CI: 15.8, 48.7), respectively.

We detected ranavirus sporadically throughout all three regions, with total prevalence of 9.0% (17/200, 95% CI: 4.6, 12.4) across all years. We found the greatest prevalence in MTN at 11% (4/36, 95% CI: 0.8, 21.4) and ETN at 10% (8/79, 95% CI: 3.5, 16.8), followed by 6% prevalence in AR (5/85, 95% CI: 0.9, 10.9; Fig. 1). We found ranavirus-positive animals to be spatiotemporally clustered. The greatest cluster of positive animals (71%, 5/7) were sampled from a single site in ETN during early July 2016. This one sampling event accounted for half of all positive individuals in ETN across all years. Similarly, all four ranavirus-positive animals from MTN in 2012 were from a sampling effort of 14 animals (29%) in one river. Year 2012 represented the only year in which ranavirus-positive animals were detected from all three regions (16%, 9/58, 95% CI: 6.2, 24.8). Of the 200 hellbenders sampled for
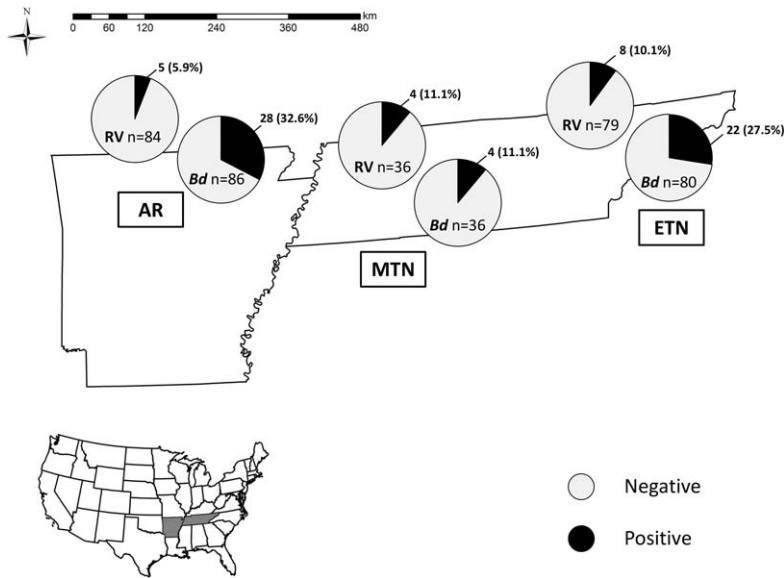
FIGURE 1. Map of Tennessee and Arkansas (USA) including overall prevalence of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus from hellbenders (*Cryptobranchus alleganiensis*) sampled from 2011 to 2017 in the following ecoregions: 1) Ozark Highlands in Arkansas (AR), 2) Interior Plateau of Middle Tennessee (MTN), and 3) Blue Ridge ecoregion of East Tennessee (ETN). Ozark hellbenders (*C. a. bishopi*) were sampled in AR, and eastern hellbenders (*C. a. alleganiensis*) were sampled in MTN and ETN. Black shaded portion represents percent of total animals positive for each pathogen by quantitative (q)PCR of skin swabs for *Bd* and tail biopsy tissue for ranavirus. Sample sizes are noted for each pathogen tested by region from a total of 202 swabs for *Bd* and 200 tissue samples for ranavirus. All swabs were also tested by qPCR for *Batrachochytrium salamandrivorans* (*Bsal*), and all were negative.

both pathogens, six (3%) were coinfected with *Bd* and ranavirus.

Of the models evaluated to explain *Bd* presence, we found greatest support ($\Delta AIC_c < 2.0$) for the following models: 1) region + BCS, 2) BCS only, and 3) BCS + region + julian (Table 1). We did not find statistical support for fixed effects that explained *Bd* infection status.

Two models ($\Delta AIC_c < 2.0$) explained ranavirus prevalence and included 1) BCS + mass and 2) BCS + julian (Table 1). The BCS model term was included in both top ranavirus models and was negatively associated with ranavirus presence ($\beta = -0.13$, SE=0.06, 95% CI: −0.24, −0.02), whereas mass was only included in the top ranavirus model and was positively associated with ranavirus presence ($\beta = 0.92$, SE=0.42, 95% CI: 0.1, 1.73). The random effect of year explained considerable variance, which likely reflects the site-year–specific variation in prevalence (variance 6.29±2.51 SD).

Of the 54 individuals that tested positive for *Bd*, mean zoospore GEs varied regionally (AR: 116.4, SE=34.9; MTN: 114.1, SE=45.7; ETN: 59.5, SE=16.3; Fig. 2A), with a total range of 2.1–2,592.5 zoospore GEs. Five individuals had a zoospore GE ≥1,000. Of these five individuals, two were captured in AR, and both individuals had moderately severe lesions compared with others seen in that river (Hardman et al. 2020). The other three individuals were from ETN, one of which had active toe ulcerations. Linear mixed model evaluation for *Bd* zoospore GEs of positive individuals included three top models: 1) BCS-only, 2) mass-only, and 3) mass + region (see Table 1). As was the case with *Bd* infection status models, we did not find clear effects that explained zoospore load.

Mean load score for ranavirus-positive individuals (Fig. 2B) was greatest in MTN

TABLE 1.   We used generalized linear mixed models (GLMMs) fitted with a binomial distribution to test for influence of fixed effects on hellbender (*Cryptobranchus alleganiensis*) infection status (0/1) of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus. Ozark hellbenders (*C. a. bishopi*) were sampled in Arkansas, and eastern hellbenders (*C. a. alleganiensis*) were sampled in Middle Tennessee and East Tennessee (USA). Fixed effects included sampling Region (Arkansas, Middle Tennessee, East Tennessee), julian day, mass, and body condition score (BCS). Year was used as a random effect. Linear mixed models (LMMs) with normal distribution were created to test the same effects on infection load of *Bd*-positive individuals on the basis of zoospore genomic equivalents (GEs). Models were ranked by Akaike information criterion (AIC) accounting for small sample size (AIC_c). We did not perform AIC_c model evaluation for LMMs created for ranavirus load scores because of the low number of ranavirus-positive individuals (n=17). Highest supported predictive models ($\Delta AIC_c < 2.0$) are listed in descending order for each response variable. *Batrachochytrium dendrobatidis* infection status and zoospore GEs were calculated from quantitative (q)PCR of skin swabs. Ranavirus infection status was determined from qPCR of tail tissue biopsies.[a]

Pathogen infection (status or load)	Model	K	AIC_c	ΔAIC_c	AIC_cWt	CumWt	LL
Bd infection status (GLMM); n=202	Region + BCS + year	5	207.37	0.00	0.33	0.33	−99
	BCS + year	3	208.65	1.28	0.18	0.51	−101
	Region + BCS + julian + year	6	209.09	1.72	0.14	0.65	−98
Ranavirus infection status (GLMM); n=200	BCS + mass + year	4	96.87	0.00	0.41	0.41	−44
	BCS + julian + year	4	98.75	1.88	0.16	0.57	−45
Bd zoospore GEs of positive individuals (LMM); n=54	BCS + year	4	99.41	0.00	0.27	0.27	−45
	Mass + year	4	99.51	0.10	0.26	0.53	−45
	Region + mass + year	6	101.42	2.00	0.10	0.62	−44

[a] K = number of parameters in each model; AIC_cWt = AIC_c adjusted for small sample sizes; CumWt = cumulative AIC_c adjusted for small sample sizes; LL = log-likelihood estimate.

(score=14.0, SE=1.4; Ct=32.0), followed by AR (score=8.0, SE=0.1; Ct=38.0), and ETN (score=6.9, SE=1.3; Ct=39.1). We did not perform all possible models or AIC_c model evaluation for ranavirus intensity because of small sample size (n=17). Instead, we performed a linear mixed model of the global model and found MTN to be positively associated with ranavirus load score, where load scores for both ETN ($\beta=-7.96\pm2.17$ SE, CI: −3.70, −12.2) and AR ($\beta=-5.87\pm1.59$ SE, CI: −2.75, −8.99) were less than MTN.

In AR, we found 93% (80/86) of animals had distal limb lesions characterized by swollen, ulcerative and missing toes (Fig. 3F; see Hardman et al. [2020] for full AR lesion descriptions). In eastern hellbenders of Tennessee, 10.3% (12/116) had abnormal toes or missing toes, but only two had toe lesions similar to those observed on Ozark hellbenders in AR (one from MTN and one from ETN; Fig. 3B, C). The remaining missing toes observed from eastern hellbenders appeared healed, with thickened, slightly fibrotic overlying skin. Additionally, one hellbender from MTN had two missing feet and was positive for ranavirus (Fig. 3D). During 2017, four of the ETN animals with toe lesions were sampled at the same site with greatest ranavirus site prevalence during 2016. This group included the one lesion observed from ETN that was similar to lesions seen in AR hellbenders (Fig. 3C). We observed skin lesions on the body on 14 individuals in Tennessee, seven of which appeared to be scarring from previous trauma, four with open wounds or ulcerations and three with extensive areas of graying on the dorsal skin (Fig. 3E).

DISCUSSION

We found *Bd* was present in all regions sampled. Our data indicated a *Bd* prevalence of 27% for eastern hellbenders inhabiting streams of ETN, which is consistent with prevalence of 26.0% reported previously for this subspecies in this region (Souza et al.
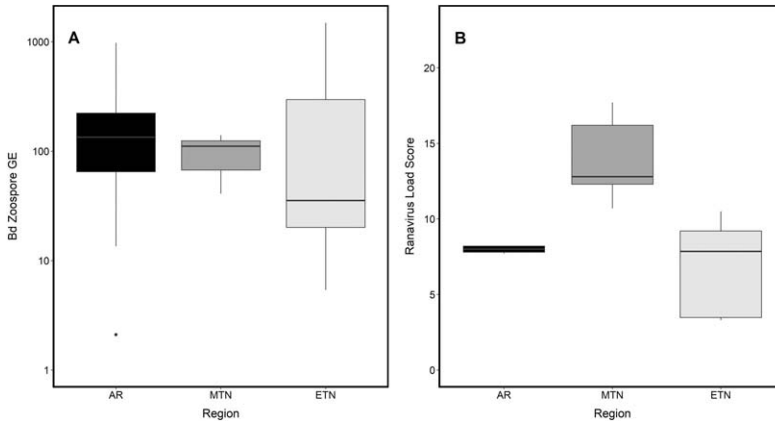
FIGURE 2. Boxplots of pathogen load of positive hellbenders (*Cryptobranchus alleganiensis*) for (A) *Batrachochytrium dendrobatidis* (*Bd*), *n*=54 and (B) ranavirus, *n*=17. Pathogen loads displayed as zoospore genomic equivalents (GEs) for *Bd*-positive individuals (A) and load scores (1–25) for ranavirus-positive individuals (B). Pathogen loads determined by quantitative (q)PCR of skin swabs for *Bd* and tail biopsy tissue for ranavirus. All swabs were also tested by qPCR for *Batrachochytrium salamandrivorans* (*Bsal*), and all were negative. Plots are grouped by region of Ozark Highlands of Arkansas (AR), Middle Tennessee (MTN), and East Tennessee (ETN), USA. Ozark hellbenders (*C. a. bishopi*) were sampled in AR and eastern hellbenders (*C. a. alleganiensis*) were sampled in MTN and ETN. Mean (first, second, third quartiles) zoospore GEs for *Bd*-positive individuals were 116 (55, 142, 280), 114 (41, 111, 140), and 60 (18, 36, 366) for AR, MTN, and ETN, respectively. Mean load scores and quartiles for ranavirus-positive individuals were 8.0 (7.8, 8.0, 8.2), 14.0 (11.5, 12.8, 17.0), and 6.9 (3.4, 7.9, 9.8) for the same respective regions.

2012). Furthermore, these values were similar to reports of 27.9% prevalence for eastern hellbenders in streams in western North Carolina (Williams and Groves 2014) and of 24.0% prevalence from a study that encompassed the eastern US (Bales et al. 2015). A slightly greater prevalence of 33% occurred in northern Georgia (Gonynor et al. 2011). We also report the first *Bd* prevalence data for Ozark hellbenders; previous reports only noted the presence of *Bd*-positive animals (Briggler et al. 2008). Our 11.1% prevalence for hellbenders inhabiting streams of MTN was the first report of *Bd* in eastern hellbenders from that region. Both MTN and AR represent rapidly declining populations (Wheeler et al. 2003; Miller 2013) and did not have significantly different *Bd* prevalence from ETN, where populations are still considered relatively stable. Although not significant, *Bd* zoospore loads were greater in both MTN and AR. Furthermore, AR populations, which harbored a high prevalence of skin lesions (93.2%), had the greatest overall *Bd* prevalence and zoospore GEs of positive

animals. Zoospore GEs from eastern hellbenders in MTN and ETN were also greater than loads reported previously for eastern hellbenders: 12.7 (Bales et al. 2015) and 12 (Williams and Groves 2014). However, we included the dorsum in our swabbing protocol, which makes direct comparisons with these studies difficult to interpret. Still, our zoospore GEs were generally low across all populations, with more than half of all positive individuals having fewer than 100 zoospore GEs and no correlation with lesions and relative *Bd* load. Although zoospore loads between regional groups overlapped considerably, these trends warrant investigation into potential factors that can affect zoospore load in amphibians where chytridiomycosis from *Bd* infection may be dependent on both host and environmental factors.

Our AR recapture data indicated that individual hellbenders can clear *Bd* infection at least to below detectable levels and become reinfected, which demonstrates the dynamic nature of *Bd* in AR populations. We sampled AR populations only in late summer, which

FIGURE 3.   Examples of lesions observed during disease surveys, the majority of which were observed on feet. (A) Normal, apparently healthy eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) foot from East Tennessee (ETN), USA for comparison. (B) Left front foot from an individual in Middle Tennessee (MTN), USA with one missing toe, two slightly swollen toes, a firm nodule developing on the plantar aspect of foot (black arrow), and gray skin instead of normal dark brown on the remaining limb (demarcated by white arrows). (C) Right front foot of individual in ETN with all four toes shortened and with active hemorrhage (white arrows). (D) Individual from MTN with missing front (black arrow) and rear left feet with healed skin. This individual was also positive for ranavirus by quantitative (q)PCR. (E) Individual from ETN with extensive graying of dorsal skin from neck to tail base (demarcation at neck shown with white arrows). This individual was positive for *Bd* by qPCR. (F) Four out of five toes very swollen and erythematous (white arrows) on rear right foot in an Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) from Arkansas (AR), USA. The majority of individuals encountered in AR had multiple swollen toes in each foot that often progressed to ulceration and necrosis (Hardman et al. 2020).

represents a time of low *Bd* infection prevalence in other species of amphibians in North America, such as crawfish frogs (*Lithobates areolatus*; Kinney et al. 2011) and northern leopard frogs (*Lithobates pipiens*; Voordouw et al. 2010). Therefore, *Bd* may be affecting a greater proportion of Ozark hellbenders in AR than our prevalence data suggest. Although we do not hypothesize *Bd* as a primary pathogen for the lesions that we observed in AR, we caution that *Bd* may play a role in lesion pathophysiology, especially in areas with high prevalence.

We detected ranavirus in all regions sampled. There are no previous reports of ranavirus in Ozark hellbenders as well as the MTN region for eastern hellbenders. Overall, we noticed sporadic temporospatial clusters of ranavirus infection, which corroborates Souza et al. (2012) and other studies of ranavirus epidemiology in amphibians (Brunner et al. 2015; Sutton et al. 2015). We did not notice signs of ranaviral disease in positive individuals; however, we recorded several swollen toes and one hemorrhagic toe lesion at an ETN site the year after a spike in prevalence at the same location. Pathogenesis of ranavirus occurs largely through vascular and epithelial damage (Miller et al. 2011), and digital swelling and necrosis is a prominent lesion documented in ranavirus outbreaks of the closely related Chinese giant salamander (Geng et al. 2011). Therefore, the lesions we observed in hellbenders a year later after a documented ranavirus event possibly represented long-term sequelae from vascular damage acquired during previous infection. Detection of disease consequences of a pathogen and detection of infection may be temporally separated; therefore, we suggest repeated sampling is required to understand the effects of ranavirus. We also did not observe a clear link between ranavirus and toe lesions on animals from the MTN or AR regions. However, relatively low occurrence—and unbalanced sampling in MTN—confounds attempts to interpret the effect of this pathogen on these populations. In AR, the majority of individuals encountered had lesions (>90%; Hardman et al. 2020), making

delayed effects of previous infections such as ranavirus difficult to determine. Furthermore, the lesions we observed in AR appeared to be progressive and more severe than those observed in MTN and ETN. Still, although these lesions in Ozark hellbenders from AR likely have a different pathogenesis and etiology than the more sporadic lesions seen in Tennessee, ranavirus is also considered one of many potential etiologic agents (Hardman et al. 2020). The GLMM also supports the hypothesis that ranavirus could have negative sublethal effects on hellbender health, as suggested by the negative correlation between ranavirus infection and BCS. Although these results are strictly correlative, they warrant further investigation of ranavirus disease dynamics in stream systems to better understand the potential population-level effects of this virus.

Our findings emphasize the importance of repeated disease monitoring in hellbenders. We did not detect *Bd* in any of the streams that had a single sampling event, and ranavirus was only detected sporadically, illustrating that pathogen detection may be low, especially in single-season studies. This point is important because hellbender populations are considered to be relatively stable in certain areas such as in the Blue Ridge Ecoregion of ETN, where both *Bd* and ranavirus were present in a majority of streams surveyed. Both pathogens likely play subtle roles in hellbender declines and detailed disease data are required to understand fully the multifactorial threat to hellbender populations. We also described a spectrum of distal limb lesion severity, with multiple likely etiologies. Some lesions were healed, others were ulcerated, some had swollen and missing toes, and others had toes intact. Future hellbender research must describe and document all lesions observed in the field and pair these observations with disease sampling to help properly diagnose morbidities that may affect population persistence. Additionally, we recommend researchers use qPCR in their studies to allow evaluation of relative pathogen loads between regions and health parameters of interest. Infection burden is important to determine subsequent individual and popula-

tion health consequences (Wilber et al. 2017) and can have independent and sometimes opposite trends of prevalence in response to environmental fluctuations (Terrel et al. 2014; Ruggeri et al. 2018). We discovered different trends in pathogen load versus prevalence and showed that prevalence-only data may not best describe how these pathogens affect hellbenders.

Continued disease monitoring will be important to detect emerging or unknown disease threats, such as *Bsal*. A recent outbreak of a previously unknown virus almost resulted in extinction of the endangered Bellinger River snapping turtle (Zhang et al. 2018), but because of early detection and prepared wildlife disease teams, that species persists today. Our data demonstrated the sporadic and easily missed occurrence of important amphibian pathogens and the presence of lesions in both subspecies of hellbenders, which should motivate increased disease monitoring efforts for this important and rapidly declining amphibian species.

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