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Extremely Low Prevalence of *Batrachochytrium dendrobatidis* Infection in Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Southwest Virginia, USA

The large, fully aquatic Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) is a phylogenetically unique member of North America's salamander diversity and a focus of increasing conservation concern. Substantial population declines have been reported across much of the Eastern Hellbender's range (Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011b), and this subspecies is now apparently extirpated from some historical localities (Graham et al. 2011). Exact causes for population declines are unclear, although habitat degradation is often suspected to play a significant role (Wheeler et al. 2003; Foster et al. 2009; Graham et al. 2011). Recently, however, studies have documented *Batrachochytrium dendrobatidis* (*Bd*) infection in both captive and wild hellbender populations (Briggler et al. 2007; Briggler et al. 2008; Gonynor et al. 2011; Bodinof et al. 2012; Regester et al. 2012; Tominaga et al. 2013). This fungal pathogen has been detected on hellbender museum specimens collected as far back as 1969 (Bodinof et al. 2011). *Bd* causes the devastating amphibian disease chytridiomycosis (Berger et al. 1998; Kilpatrick et al. 2010; Eskew and Todd 2013), and evidence from the tropics suggests that disease-driven amphibian declines may have wide-ranging effects on ecosystem functioning in lotic systems (Whiles et al. 2006). However, the prevalence of *Bd* in river- and stream-associated amphibians from temperate latitudes is understudied; the limited data currently available suggests that *Bd* infection is at relatively low prevalence in low-order stream systems in the US (Hossack et al. 2010).

Although many studies suggest that wild hellbenders do not typically manifest clinical symptoms of chytridiomycosis or suffer mortality from the disease (Gonynor et al. 2011; Souza et al.

2012; Tominaga et al. 2013), there is at least one report of hellbender mortality associated with chytridiomycosis (Bodinof et al. 2012). In addition, even if chytridiomycosis rarely causes hellbender mortality in isolation, *Bd* infection might still negatively affect hellbender populations by allowing for detrimental co-infections (Bodinof et al. 2011) or through interactions with other stressors such as habitat alteration (Hopkins and DuRant 2011). In at least some systems, however, the relationship between *Bd* prevalence and habitat disturbance is counterintuitive, with the highest infection risk occurring in the most pristine habitats (Becker and Zamudio 2011). Therefore, we sought to evaluate *Bd* prevalence in Eastern Hellbenders occupying a single stream predominately surrounded by forest but with a gradient of surrounding habitat disturbance. We also report *Bd* infection status of another fully aquatic salamander, the Common Mudpuppy (*Necturus maculosus*), captured incidentally during our study.

We sampled 14 different stream reaches across a 57-km expanse of a single stream (3rd–4th order) within the Tennessee River Basin of southwest Virginia, USA where hellbenders are known to be relatively abundant (Hopkins and DuRant 2011). The name and location of the stream is withheld because of the sensitive conservation status of our study species in Virginia. The watershed is predominately forested (65–76% forest cover across our sampling range), but progressive encroachment of agricultural activity, suburban development, and increased road density pose threats to downstream habitat quality and generate a disturbance gradient within the watershed. For additional information on a subset of the study reaches, including data on land use within the watershed, see Hopkins and DuRant (2011).

Sampling occurred in the summer months between 27 June 2011 and 2 September 2011, and between 9 July 2012 and 6 September 2012. The sex of adult hellbenders is distinguishable at this time due to the swollen cloacae of males. We located animals during visual encounter searches by lifting and searching beneath large rocks while snorkeling. After capture, we swabbed hellbenders and incidentally captured Common Mudpuppies for *Bd* by rubbing a single cotton-tipped swab five times over the dorsal and ventral surfaces, five times on each side, and five times on each foot for a total of 40 swabs per animal. We used disposable vinyl gloves when swabbing animals. We stored swabs in 70% ethanol at room temperature until extractions took place.

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We isolated DNA following the swab extraction protocol of Retallick et al. (2006) with the exception that we removed ethanol from swabs by placing them on a heat block at 55°C until all standing ethanol was evaporated (approximately 48 h). To detect *Bd* DNA, we used the real-time PCR assay described by Boyle et al. (2004). We ran samples on a Step-One Plus Real-time PCR machine (Applied Biosystems, Foster City, California) using the “Presence/Absence” mode as we were only interested in *Bd* prevalence within the hellbender population. For real-time PCR, we used 2.5 µl of diluted DNA in a total reaction volume of 12.5 µl. We ran samples singly (Kriger et al. 2006), and included positive (100 zoospore equivalent *Bd* standards) and negative (molecular-grade water) controls on all plates. All positive and negative controls behaved as expected, producing no false negatives or false positives. To validate our laboratory methods, we also ran seven samples collected in August 2013 from a *Bd*-positive captive population of hellbenders (Virginia Dept. of Game and Inland Fisheries, unpubl. data); results obtained using our methodology (one *Bd* positive hellbender out of seven tested) corresponded with those independently generated by Virginia state biologists.

A total of 230 unique individual hellbenders, 113 from 2011 (76 adults, 37 juveniles) and 117 from 2012 (103 adults, 14 juveniles), were captured and swabbed for *Bd* infection during our study. Captured hellbenders averaged 36.81 ± 0.57 (mean \pm SE) cm in total length, 23.81 ± 0.37 cm in snout–vent length (SVL), and 337.30 ± 14.25 g in mass. Only a single hellbender collected in 2011 tested positive for *Bd*, resulting in a prevalence of 0.88% that year. The sample from this individual was re-run and again tested positive, further confirming *Bd* presence. The positive individual was an adult male captured on 11 July 2011 in apparently good condition in a stream reach lying in the middle of the sampled watershed and thus in the middle of the habitat gradient described above and in Hopkins and DuRant (2011). A total of 76 other individuals were sampled from this stream reach in 2011; these samples all tested negative. All hellbenders sampled in 2012 tested negative for *Bd* infection. A total of six Common Mudpuppies were incidentally captured and swabbed during sampling events in 2012. Captured mudpuppies averaged 21.63 ± 1.14 (mean \pm SE) cm in total length, 14.52 ± 0.80 cm SVL, and 48.33 ± 7.62 g in mass. All mudpuppies tested negative for *Bd*.

Overall, our study suggests there was an extremely low prevalence of *Bd* infection within an Eastern Hellbender population in southwest Virginia at the time animals were sampled. Only a single hellbender of 230 sampled during the summers of 2011 and 2012 tested positive for *Bd*. The low overall prevalence of *Bd* precluded us from making any general inferences about factors (e.g., age/size, sex, surrounding land use) that might influence *Bd* prevalence in this hellbender population. However, it is important to note that the stream reaches examined in this study are all predominately forested and lack major industrial inputs, and even the most disturbed reaches of this study system have not yet experienced the level of habitat degradation of some other historical hellbender habitats. Thus, sampling across a larger range of stream habitat quality might provide greater insight as to whether anthropogenic factors influence *Bd* prevalence in hellbender populations. Although our sample size for Common Mudpuppies was small, our report of *Bd* absence in this species mirrors the result of Ouellet et al. (2005) who also failed to find evidence of infection in 12 individuals of this species collected from Québec, Canada. However, *Bd* infection

has been reported from other fully aquatic *Necturus* species collected in Florida and Louisiana (Chatfield et al. 2012), and more comprehensive studies are needed to elucidate *Bd* infection dynamics in this salamander genus.

Prior reports of *Bd* prevalence within hellbender populations have found the pathogen to be common in some populations (within-population prevalence of approximately 20–50%; see Gonynor et al. 2011; Souza et al. 2012; Tominaga et al. 2013) but rare in others (1.3% prevalence in Burgmeier et al. 2011a). Our results are similar to several other studies of stream-associated salamanders that also found low prevalence of *Bd* (summarized in Hossack et al. 2010). Hossack et al. (2010) hypothesized that their findings of low *Bd* prevalence in many North American stream-associated amphibians might be driven by seasonally cold in-stream temperatures that limit *Bd* growth during cooler winter months. Optimal temperatures for the growth of *Bd* range from 17–25°C (Piotrowski et al. 2004). In our stream system in Virginia, water temperatures are cold (< 6–8°C) during winter months, but at the time we sampled animals, water temperatures typically exceeded the lower limit for optimal *Bd* growth (average during surveys = $18.6 \pm 0.3^\circ\text{C}$; range 14.5–22.0°C). Thus, the low prevalence of *Bd* infection in hellbenders in the present study could partially reflect an inability of *Bd* to thrive in areas that experience suboptimal, cold winter temperatures. However, in light of the fact that other streams throughout the hellbender's range likely have comparable winter temperatures but *Bd* can be prevalent in these systems (Gonynor et al. 2011; Souza et al. 2012; Tominaga et al. 2013), other factors related to surrounding land use, in-stream habitat quality, pollution, community assemblage, and the general health of hellbenders are more likely determinants of *Bd* prevalence that deserve further investigation.

The results of this study add to our basic understanding of the prevalence of *Bd* infection in hellbender populations in one of the remnant strongholds within the range of this imperiled amphibian species. The extremely low prevalence of *Bd* reported here and elsewhere (Burgmeier et al. 2011a), contrasts with the higher prevalence of *Bd* infections in other hellbender populations (Gonynor et al. 2011; Souza et al. 2012; Tominaga et al. 2013). It remains unclear whether *Bd* infections regularly result in disease (chytridiomycosis) in wild hellbenders to a degree that negatively affects hellbender health, reproduction, and survival. However, chytridiomycosis has been a problem in captive populations and may have caused mortality in at least one translocated animal (Bodinof et al. 2012). Broader spatial and temporal monitoring is needed to fully assess the risk that *Bd* poses to wild hellbender populations and to facilitate the early detection and mitigation of any potential chytridiomycosis outbreaks in this unique freshwater amphibian. Additionally, the recent description of another pathogenic fungus, *Batrachochytrium salamandrivorans* (*Bs*), that can cause lethal chytridiomycosis in northwestern European salamanders suggests that pathogen surveillance efforts focused solely on *Bd* may underestimate the threat of fungal disease (Martel et al. 2013). Consequently, future work should use newly developed molecular methods that enable the simultaneous detection of both pathogens in amphibian host populations (Bloom et al. 2013).

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