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High Prevalence of Ranavirus Infection in Permanent Constructed Wetlands in Eastern Kentucky, USA

Amphibians are declining globally, and both land-use change and infectious diseases are major drivers (Miller et al. 2011; Stuart et al. 2004). Because most wetlands have been destroyed or altered throughout the United States (e.g., Kentucky has lost >81% of its historic wetlands; Dahl 2000), wetlands have been created for mitigation or wildlife management (Brown and Richter 2012; Dahl 2000). Hundreds of closely spaced permanent wetlands have been constructed on ridge tops in eastern Kentucky for wildlife management within the same landscape as natural, ephemeral wetlands (Brown and Richter 2012). Although constructed wetlands provide breeding habitat for amphibians, they might not replace the function of natural wetlands, supporting different amphibian communities than natural ponds (Denton and Richter 2013; Drayer 2011). Moreover, constructed ponds have been associated with ranavirus outbreaks (Harp and Petranka 2006; Petranka et al. 2007). Our objective was to test for the occurrence of ranavirus and amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), in amphibian populations inhabiting natural and constructed ridge-top wetlands of eastern Kentucky, USA.

Field surveys were conducted in five constructed and one natural ridge-top wetland located in the Daniel Boone National Forest (DBNF), Kentucky. All boots, dipnets, and other field supplies were disinfected with a 1% solution of Nolvasan® to prevent spread of pathogens between sampling sites (Bryan et al. 2009). Dipnet sampling was used to capture up to 10 adult Eastern Newts (*Notopthalmus viridescens*) in each constructed wetland and 10 Wood Frog (*Lithobates sylvaticus*) larvae in the natural wetland from 21 to 27 May 2012 (Table 1). No Eastern Newts were detected in natural wetlands, and no Wood Frog larvae were detected in constructed wetlands. These species were chosen because they have been associated with ranaviral disease die-offs in eastern North America (Green et al. 2002; Greer et al. 2005; Harp and Petranka 2006). None of the individuals that were collected had signs of ranaviral disease (Miller et al. 2011). Each Eastern Newt was swabbed using a BBL™ CultureSwab™ (Beckton, Dickinson, and Company, Franklin Lakes, New Jersey, USA) and had a 10-mm portion of its tail clipped. Swabs and tail clips were stored in 70% ethanol. Each *L. sylvaticus* larva was euthanized in 10% ethanol and stored in 95% ethanol (EKU Institutional Animal Care and Use Committee; protocol #04-2012).

Ranavirus and *Bd* testing was performed at the University of Tennessee Center for Wildlife Health following published standardized procedures (Hoverman et al. 2011a; Souza et al. 2012). Genomic DNA (gDNA) was extracted from a homogenate of liver and kidney tissue (Wood Frogs), tail clips (Eastern Newts), and swabs (Eastern Newts) using a commercially available kit (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, California, USA) with molecular-grade water as the extraction control. We measured the concentration of gDNA in each sample and standardized the amount of gDNA (0.25 µg) used for PCR among samples. Quantitative real-time PCR (i.e., TaqMan® PCR) was performed following Boyle et al. (2004) for *Bd* assays and following Picco et al. (2007) for ranavirus assays. Positive controls were similar for each assay, and included DNA extracted from culture and a positive animal for each pathogen. Negative controls included molecular-grade water and DNA extracted from an animal that was known to be negative for each pathogen. Each assay was run for 40 cycles on an ABI 7900 Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, California, USA). Each sample was run in duplicate and considered positive only if the PCR cycle threshold (CT) was < 30 for both samples. The CT value was determined by developing a standard curve for our PCR equipment using serial dilutions of known pathogen quantities. When samples were positive, we used the standard curve to predict virus concentration (i.e., plaque forming units, PFU) using the average CT for the sample.

We did not detect *Bd* in any samples; however, nine samples from two constructed wetlands were positive for ranavirus

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TABLE 1. Prevalence with 95% confidence intervals of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus infection in Eastern Newts (*Notophthalmus viridescens*; *Nv*) in five constructed wetlands and Wood Frogs (*Lithobates sylvaticus*; *Ls*) from one natural wetland in the Daniel Boone National Forest, Kentucky. For each *Nv*, tail clips were tested for ranavirus, and swabs were tested for *Bd*. For each *Ls*, a homogenate of liver and kidney tissue was tested for ranavirus. Confidence intervals were calculated for small sample size using Wilson Score method with continuity correction (Newcombe 1998).

Study Site	Coordinates	Sample Size	*Bd* Prevalence (95% CI)	Ranavirus Prevalence (95% CI)
Gas Line Natural	38.284583°N 83.368972°W	*Ls*: N = 10	not tested	0 (0–0.345)
Gas line Artificial 2	38.285556°N 83.371778°W	*Nv*: N = 10	0 (0–0.345)	0 (0–0.345)
P5	38.087889°N 83.425278°W	*Nv*: N = 10	0 (0–0.345)	0 (0–0.345)
P5 Algae	38.087911°N 83.423889°W	*Nv*: N = 6	0 (0–0.483)	0.333 (0.060–0.759)
Jones Ridge Artificial	38.092306°N 83.354722°W	*Nv*: N = 10	0 (0–0.345)	0 (0–0.345)
Elk Lick Artificial Large	38.329806°N 83.364472°W	*Nv*: N = 10	0 (0–0.345)	0.700 (0.354–0.919)

infection (prevalence = 70% and 33%; Table 1). Eight of nine positive samples had titers < 100 PFU and the other had a titer of 4114 PFU. The lower titers are consistent with these newts being sublethally infected (Miller and Gray, unpubl. data). From our controlled research (e.g., Hoverman et al. 2011a), individuals with titers > 4000 PFU frequently develop ranaviral disease (Miller and Gray, unpubl. data). Given that adult newts are known to move among wetlands in close proximity (Porej et al. 2004) and use ephemeral and permanent wetlands (Hunsinger and Lannoo 2005), it is possible that this species could transport ranavirus overland among sites similar to Tiger Salamanders (*Ambystoma tigrinum*, Brunner et al. 2004) into amphibian communities composed of highly susceptible species (e.g., Wood Frogs; Hoverman et al. 2011a). The role of Eastern Newts in the epidemiology of ranavirus needs greater attention.

While our sample sizes do not allow for meaningful comparisons of ranavirus prevalence between natural and constructed wetlands, there are several reasons we think that constructed ponds might have important consequences for ranavirus epidemiology. First, the constructed wetlands where ranavirus was detected are permanent compared to the ephemeral hydroperiod (< 200 days) of natural wetlands in the ecosystem. Because ranavirus virions are inactivated faster in dry soil compared to water (Nazir et al. 2012), the long hydroperiods might increase the persistence of ranavirus outside the host. Second, constructed wetlands tend to have deeper littoral zones, which might be important sites for ectotherms to warm body temperatures and inactivate pathogens (Raffel et al. 2010). The absence of *Bd* in the constructed wetlands might be attributed to lack of substrate complexity and shade (Raffel et al. 2010), or it could be because *Bd* has not arrived to the ecosystem or was simply not detected. Lastly, the constructed wetlands were inhabited by amphibian species that require a longer hydroperiod for development and may function as reservoirs for ranavirus, including Eastern Newts, American Bullfrogs (*L. catesbeianus*), and Green Frogs (*L. clamitans*; Daszak et al. 2004; Gahl et al. 2012; Hoverman et al. 2011b).

Ranavirus has been previously documented in two wetlands in Kentucky (J. MacGregor, Kentucky Department of Fish and Wildlife Resources, pers. comm.). We recommend more intensive studies in the future that examine a larger geographic area and larger sample size per wetland type. Additionally, post-metamorphic stages should be tested to determine if terrestrial stages of amphibians are important reservoirs as hypothesized by Brunner et al. (2004).

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