AMPHIBIAN DISEASES

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First Report of Ranavirus in Plethodontid Salamanders from the Mount Rogers National Recreation Area, Virginia, USA

Ranaviruses are an emerging pathogen responsible for numerous amphibian die-offs throughout Europe and North America (Miller et al. 2011). In the southeastern United States, local anuran die-offs due to ranavirus have been observed (Green et al. 2002; Hoverman et al. 2012; Todd-Thompson 2010), and suggested to contribute to local species declines (Gray et al. 2009a). The southern Appalachian Mountains have one of the greatest global diversities of plethodontid salamanders (Dodd 2004), and disease emergence could have devastating impacts on community structure and ecosystem function (Whiles et al. 2006). Ranavirus infections have been reported in 14 species of plethodontid salamanders occurring in the southern Appalachian Mountains (Miller et al. 2011). Initial surveys for ranavirus infections have focused on the Great Smoky Mountains National Park (81% prevalence; Gray et al. 2009b), which is known for high species richness of plethodontid salamanders (Dodd 2004). However, much of the remainder of the southern Appalachian Mountains has not been investigated except for a single study in the Ridge and Valley physiographic province of Wise County, Virginia (33% prevalence; Davidson and Chambers 2011). Many other Appalachian peaks have high plethodontid salamander richness and include many species with limited distributions, but the occurrence of ranavirus in these communities is unknown.

We sampled salamanders in the Mount Rogers National Recreation Area (MRNA), Virginia from Whitetop and Beech Mountains (Grayson, Smyth, and Washington counties). Many species reach the extreme northern limit of their range on these mountains with 15 species of plethodontid salamanders found on Whitetop Mountain alone (Organ 1991). Our sampling focused on five species with aquatic larval stages (i.e., complex life cycle), which have shown higher prevalence of ranavirus infection (Gray et al. 2009b), and four terrestrial-breeding species with direct development. Nine species that we targeted for sampling included: Northern Dusky (*Desmognathus fuscus*), Seal (*D. monticola*), Blue Ridge Dusky (*D. orestes*), Northern Pygmy (*D. organi*), Black-bellied (*D. quadramaculatus*), Blue Ridge Two-lined (*Eurycea wilderae*), Northern Gray-cheeked (*Plethodon montanus*), Ravine (*P. richmondi*), and Weller's (*P. welleri*) salamanders. In Virginia, Weller's, Northern Pygmy, Blue Ridge Dusky, and Blue Ridge Two-lined salamanders are listed as species of greatest conservation need (Virginia Department of Game and Inland Fisheries 2005).

Salamanders were captured along transects from June to August 2008 and May to August 2009 from Byars Creek (1158–1311 m elev.; 36.62755°N, 81.58903°W), Whitetop Creek (1158–1615 m elev.; 36.64016°N, 81.60040°W), Dells Branch (945–1585 m elev.; 36.64774°N, 81.60254°W), Beech Mountain (956–1097 m elev.; 36.63362°N, 81.63030°W), and Daves Ridge (1128–1433 m elev.; 36.6475°N, 81.59185°W). Each transect was positioned perpendicular to the elevational gradient centered on either a stream or ridge line. We searched for salamanders on each side of the transect for one person-hour (~150 m) at every 30.5 m of elevation by turning all cover objects. We placed each captured salamander in individual 1.2-liter plastic bags. We used sterile forceps to remove a small tail section along the natural break point and placed each sample in a sterile microcentrifuge tube with 99% reagent grade isopropyl alcohol. We used a different set of sterilized forceps for each individual tail sample. Between sampling events, forceps were autoclaved to destroy remnant DNA.

We extracted genomic DNA from tail samples using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California), and used a Qubit™ fluorometer to quantify the concentration of genomic nuclear DNA from tail samples. Samples from 2008 (N = 99) were extracted and quantified at the Diagnostic and Investigational Laboratory in the College of Veterinary Medicine at the University of Georgia (UGA). In 2009, we extracted and quantified the 2009 samples (N = 320) in the Center for Wildlife Health at the University Tennessee (UT). Real-time qPCR was performed using the identical primers and protocol of Gray et al. (2012). The qPCR was conducted using a SmartCycler system (Cepheid, Sunnyvale, California) for 2008 samples and an ABI 7900HT PCR system (Live Technologies Corp., Carlsbad, California) was used for 2009 samples. Controls included two positive controls (cultured virus and known positive tadpoles) and two negative controls (water and known negative animal). The qPCR was run in

M. KEVIN HAMED*
Center for Wildlife Health, Department of Forestry, Wildlife, and Fisheries, Institute of Agriculture, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37966, USA; and Virginia Highlands Community College, 100 VHCC Drive, Abingdon, Virginia 24210, USA
MATTHEW J. GRAY
DEBRA L. MILLER
Center for Wildlife Health, Department of Forestry, Wildlife, and Fisheries, Institute of Agriculture, University of Tennessee, 274 Ellington Plant Sciences Building, Knoxville, Tennessee 37966, USA
**Corresponding author; e-mail: khamed@vhcc.edu*

TABLE 1. Ranavirus prevalence in salamander communities inhabiting Whitetop Mountain (Grayson, Smyth, Washington counties), Virginia, USA.

Species	No. infected / total no. sampled	Prevalence of infection (95% confidence interval)
Desmognathus fuscus	1 / 11	0.09 (0.02–0.37)
Desmognathus monticola	2 / 22	0.09 (0.03–0.28)
Desmognathus orestes	3 / 145	0.02 (0.01–0.05)
Desmognathus organi	3 / 70	0.04 (0.01–0.12)
Desmognathus quadramaculatus	1 / 32	0.03 (0.01–0.16)
Eurycea wilderae	0 / 5	0 (0–0.44)
Plethodon montanus	3 / 101	0.03 (0.01–0.08)
Plethodon richmondi	0 / 3	0 (0–0.56)
Plethodon welleri	1 / 30	0.03 (0.01–0.17)
Total	14 / 419	0.03 (0.02–0.06)

duplicate and a C_T value ≤ 30 was deemed positive, according to equipment optimization at UGA and UT.

Our results document the first known presence of ranavirus infection in amphibians of the MRNA. Salamanders sampled and all other salamanders observed did not display external signs of ranavirus infection such as swelling or ulcerations (Miller et al. 2011). We detected ranavirus in seven salamander species (Table 1)—four of the species were first-time detections: Weller's, Northern Pygmy, Blue Ridge Dusky, and Northern Gray-cheeked (Miller et al. 2011). Ranavirus prevalence ranged from 3–9% within species, and was 3% among all species that tested positive (Table 1). Infected salamanders were found between elevations of 945–1554 m, and along all transects except Daves Ridge. Species with a more aquatic life history (*D. fuscus* and *D. monticola*) had the highest prevalence of infection (9%), as reported by Gray et al. (2009b). However, there was no association between larval life history (aquatic larvae or terrestrial direct development) and infection status ($\chi^2 = 0.01$, $p = 0.920$), which may be a consequence of low overall prevalence. Additionally, there was no association between sampling years ($\chi^2 = 0.196$, $p = 0.658$).

Our low detection of ranavirus infection could be partly influenced by the type of tissue collected. Gray et al. (2012) demonstrated that tail clips produced false-negative results for 20% of tail samples in American Bullfrog (*Lithobates catebeianus*) tadpoles when compared to liver tissue samples and suggested that false negatives could relate to the sensitivity of the amphibian host. Even with the possibility of a 20% false detection rate, salamanders from the MRNA appear to have lower ranavirus prevalence than salamanders from the Great Smoky Mountains National Park (81%; Gray et al. 2009b) and Wise County, Virginia (33%; Davidson and Chambers 2011). Future research could include histological analyses to reduce the potential of false negatives (Gray et al. 2012) as well as genetic sequencing to identify the strain(s) of ranavirus present in the MRNA.

We documented ranavirus infections occurring in secluded areas (often ≥ 300 m from trails, roads, or other areas of public activities) of the MRNA, indicating that this pathogen is not always associated with human-impacted sites (Gray et al. 2007). We documented infections in four additional species of plethodontid salamanders, including rare species. Weller's Salamanders are an IUCN (2008) Red List species, and our positive results from this species are a concern suggesting that further monitoring may be warranted. These results provide additional evidence that ranavirus could be distributed throughout the Southern Appalachian Mountains. In the MRNA, ranavirus was found to infect both rare and more abundant salamanders as well as those with aquatic or terrestrial embryonic development, suggesting that ranavirus is not selective and any southern Appalachian salamander could be at risk. Additionally, six other species of plethodontid salamanders, Eastern Newts (*Notophthalmus viridescens*), and five anuran species are found in the MRNA, but were not part of this study (Hamed, unpubl. data). Given the tendency for anurans to be more susceptible to ranavirus than salamanders (Hoverman et al. 2011), frogs may be important reservoirs and amplifying hosts for ranavirus (Paull et al. 2012). It would be prudent to include all amphibian species in future monitoring designs to provide a more comprehensive understanding of ranavirus prevalence in the MRNA, and to increase the likelihood of identifying amplifying species and disease hotspots (Paull et al. 2012).

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