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First Report of *Ranavirus* Infecting Lungless Salamanders

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Ranaviruses are a group of pathogens belonging to the genus *Ranavirus* (Family Iridoviridae) that have been linked to catastrophic die-offs of larval amphibians in North America and elsewhere (Daszak et al. 2003). They also have been identified as the etiologic agent in the mass mortality of adult Common Frogs (*Rana temporaria*) and Common Toads (*Bufo bufo*) in the United Kingdom (Cunningham et al. 1996; 2007a,b). In the United States, ranaviruses are responsible for the majority of disease-related mortality events in amphibians (Green et al. 2002; Muths et al. 2006). There is evidence that ranaviruses may be an emerging infectious disease (Storfer et al. 2007), possibly due to novel strain introduction (Picco and Collins 2008) or increased occurrence of anthropogenic stressors on the landscape (Forson and Storfer 2006; Gray et al. 2007). Recognizing the potential threat of ranaviruses to global amphibian biodiversity, the World Organization for Animal Health (OIE) recently listed this pathogen as a notifiable disease, requiring proof of *Ranavirus*-negative results before commercial shipment of amphibians (OIE 2008). The OIE identifies field surveillance as a critical component of risk assessment for ranaviruses (OIE 2008). Although surveillance for the amphibian pathogen *Batrachochytrium dendrobatidis* has become widespread (e.g., Chatfield et al. 2009; Rothermel et al. 2008), testing amphibians for *Ranavirus* occurs less frequently.

The Southern Appalachian Mountain Range of North America represents a global hotspot for salamander biodiversity (Dodd 2004; Petranka 1998). In particular, lungless salamanders (Family Plethodontidae) occur in high abundance and biomass (Peterman et al. 2008; Petranka and Murray 2001) and are important components of the ecosystem (Davic and Welsh 2004). Known die-offs of Eastern Newts (*Notophthalmus viridescens*), Spotted Salamanders (*Ambystoma maculatum*), Pickerel Frogs (*Lithobates palustris*), and Wood Frogs (*L. sylvaticus*) occurred in the Southern Appalachian Mountains due to ranaviruses in 1999 and 2001 (Converse and Green 2005; Green et al. 2002). Despite these mortality events, surveillance for *Ranavirus* in Southern Appalachian amphibians has been nonexistent. Our goal was to test for the presence of *Ranavirus* in lungless salamander communities located in the Southern Appalachian Mountains. We tested for *Ranavirus* in salamander communities at three sites that differed in elevation and report prevalence by species and for each site.

Methods.—We captured adult lungless salamanders for *Ranavirus* testing on 21 April 2007 at three locations in the Great Smoky Mountains National Park, Tennessee: 1) Ash Hopper Branch (456 m elev.; 35.6836°N, 83.5375°W); 2) Chimney Tops Seeps (831 m elev.; 35.6367°N, 83.4928°W); and 3) Indian Gap Seeps (1537 m elev.; 35.61°N, 83.45°W). At all sites, we searched 1 h for salamanders in streams, seeps, and under terrestrial ground cover items (e.g., logs, rocks). We placed captured salamanders in individual 1-L plastic containers and processed up to 10 individuals per species per site. We swabbed the oral cavity and the cloaca twice each. We wore disposable gloves and changed them between animals to minimize the likelihood of cross contamination among samples. We put each swab in separate microcentrifuge tubes, placed the tubes on dry ice, and froze the swabs at -70°C within 10 h of collection. We swabbed 21, 21, and 27 salamanders at Ash Hopper Branch, Chimney Tops, and Indian Gap sites, respectively. All salamanders were released at their approximate capture location within 1 h of capture, and containers, footwear, and equipment were disinfected with 2% Nolvasan® prior to moving between sites (Bryan et al. 2009). We shipped frozen swabs overnight on dry ice to the University of Georgia Veterinary Diagnostic and Investigational Laboratory for *Ranavirus* testing.

We used conventional polymerase chain reaction (PCR) to test for the occurrence of *Ranavirus*. We extracted genomic DNA from swabs using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, California). A heminested PCR targeting a 500-bp region of the major capsid protein (MCP) gene was used following the protocol by Kattenbelt et al. (2000). The PCR products were resolved by gel electrophoresis for determination of *Ranavirus* occurrence. We randomly chose one sample per species with a distinct PCR-positive band, cut the band from the gel, and submitted the isolated product to SeqWright DNA Technology Services, Houston, Texas, for automated sequencing in the forward and reverse directions. We then performed a GenBank BLAST search (http://www.ncbi.nlm.nih.gov/Genbank.html) on the sequences to verify that positive PCR results were *Ranavirus*. Additionally, real-time quantitative PCR (qPCR) was performed following the procedure by Picco et al. (2007) on samples used for sequencing to further support our findings.

We summarized the positive test results for each species, and tallied *Ranavirus* prevalence among species for each sampling

TABLE 1. Prevalence of *Ranavirus* detected from oral and cloacal swabs using PCR for 10 species of adult lungless salamander, Plethodontidae, in the Great Smoky Mountains National Park, Tennessee, USA, April 2007.

Species	No. Infected/ Tested	Prevalence	Accession Number[1]
Desmognathus conanti	13/14	0.93	GQ326559
D. imitator	11/13	0.85	GQ326560
D. monticola	1/1	1.0	GQ326561
D. ocoee	2/2	1.0	GQ326562
D. quadramaculatus	7/8	0.88	GQ326563
D. santeetlah	11/13	0.85	GQ326564
D. wrighti	1/2	0.5	NS
Eurycea wilderae	8/12	0.67	GQ326565
Gyrinophilus porphyriticus	1/2	0.5	GQ326566
Plethodon jordani	1/2	0.5	NS
Total	56/69	0.81	

[1]GenBank accession number of the DNA sequence used for *Ranavirus* determination; NS = not sequenced because insufficient genomic DNA was amplified for sequencing.

site. We tested for the differences in *Ranavirus* prevalence among sites using logistic analysis and calculated odds-ratio statistics to estimate the likelihood of *Ranavirus* infection for each site (Stokes et al. 2000). All analyses were performed using the SAS® system (SAS Institute, Cary, North Carolina) with $\alpha = 0.05$.

Results.—We captured 10 species of lungless salamanders and all species included individuals that tested positive for *Ranavirus*. The qPCR results matched the conventional PCR results. We were able to sequence the PCR products from eight of the infected species. GenBank BLAST searches on the sequences revealed identities of 94–96% and 97–100% with ATV and FV3, respectively. Overall prevalence was 81% among species and sites (Table 1). *Ranavirus* prevalence differed among sites ($\chi^2 = 7.14$, $P = 0.028$; Fig. 1). Salamanders were 10 and 3 times more likely to be infected at Ash Hopper Branch (odds ratio = 0.1) and Chimney Tops Seeps (odds ratio = 0.3), respectively, compared to the Indian Gap Seeps.

Discussion.—We report the first documentation of *Ranavirus* infecting salamander species in the family Plethodontidae. We documented *Ranavirus* infecting 10 species of lungless salamander at an overall prevalence of 81% among species. The high prevalence of *Ranavirus* in adult lungless salamanders may indicate that they are tolerant of infection. Brunner et al. (2004) reported that postmetamorphic Tiger Salamanders (*Ambystoma tigrinum*) could be asymptomatic carriers of ranaviruses. However, no studies have quantified the pathogenicity of ranaviruses for lungless salamanders or whether virulence differs among developmental stages. Studies with anurans and ambystomatid salamanders suggest that adult stages are less susceptible to *Ranavirus* than larval stages (Collins et al. 2004; Gantress et al. 2003), thus adult amphibians have the greatest likelihood of serving as a *Ranavirus* reservoir. It also is possible that the high *Ranavirus* prevalence was evidence that a die-off was occurring or imminent. Laboratory studies with ranaviruses have shown that infection and mortality rates typically track each other in highly susceptible species (Brunner et al. 2007; Cunningham et al. 2007b). None of the individuals that we swabbed had gross signs of ranaviral disease (e.g., edema, erythema) and dead salamanders were not observed. However, it is important to note that diseased individuals often do not exhibit external signs and die-offs in the wild are rarely observed (Green et al. 2009). Inasmuch as this is the first documentation of ranaviruses infecting lungless salamanders, more research is needed to determine the threat that ranaviruses pose to this diverse group of amphibians.

Although positive results from swab specimens can be an indication of virus infection (Driskell et al. 2009), they also may reflect virions attached to skin surfaces (Green et al. 2009). In a separate controlled study with tadpoles, we determined that PCR test results for *Ranavirus* from swabs have about a 10% false-positive rate for systemic infection when compared with test results from liver tissue (Miller, Gray, and Hoverman, unpubl.

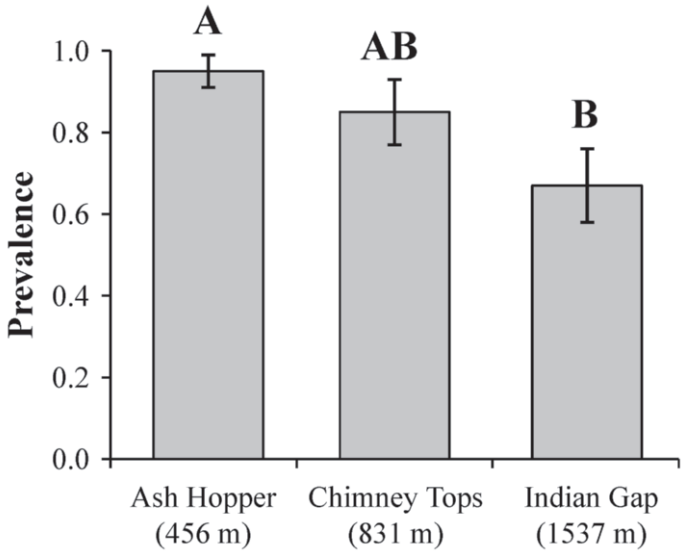

FIG. 1. *Ranavirus* prevalence in lungless salamanders (Plethodontidae) among three sampling locations that differed in elevation, Great Smoky Mountains National Park, Tennessee, USA, April 2007. Bars with unlike letters are different ($P = 0.03$) by Wald's chi-square test; standard error bars calculated as: $\hat{p} \pm \sqrt{\hat{p}(1-\hat{p})/n}$

data). We did not euthanize salamanders in our study so it is unknown if positive swabbed individuals had systemic infections. Nonetheless, given the high prevalence of *Ranavirus* across species, systemic infection of some individuals was possible.

Pathogen prevalence differed among sites, with prevalence increasing with decreasing site elevation. Salamanders at 456 m above sea level were 10 times more likely to be infected with *Ranavirus* than those at 1537 m elev. Greater pathogen prevalence at lower elevation is opposite of the trends reported by previous studies with *Ranavirus* and *Batrachochytrium dendrobatidis* (Brem and Lips 2008; Gahl and Calhoun 2008), which have related differences in infection among elevations to changes in temperature. Inasmuch as stream temperature is relatively constant among elevations in the Southern Appalachian Mountains (Swift and Clinton 1997), more optimal temperature for virus replication or temperature-induced stress probably were not factors responsible for higher prevalence at lower elevation. Higher prevalence at low-elevation sites may reflect greater human access. All sites could be accessed by trails but the sampling location for Ash Hopper Branch was <200 m from the U.S. National Park Service Sugarlands Visitor Center and a campground was <100 m from the Chimney Tops Seeps. In contrast, the Indian Gap seeps were approximately 400 m from a minor parking lot. Greater human access could increase the likelihood of infection by increasing the level of stressors in the environment or by introducing *Ranavirus* virions that are attached to footwear or recreational gear (Green et al. 2009). It also is possible that greater infection at low-elevation sites was a result of watershed position. Most of the salamanders that we collected were in or adjacent to flowing streams. *Ranavirus* virions transmit effectively in water (Harp and Petranka 2006), and they probably remain active outside the host for several months (Langdon 1989). Thus, greater prevalence of *Ranavirus* at lower elevation may be a consequence of cumulative downstream transport of virions and higher contact rates with the pathogen. The possible impacts of factors associated with elevation and human access on exposure frequency and susceptibility of lungless salamanders to ranaviruses need to be explored.

Our results indicate that plethodontid salamanders are suitable hosts for ranaviruses. The pathogenicity of ranaviruses to plethodontid salamanders remains unknown and is a part of ongoing experimental challenges (Gray, Miller, and Hoverman, unpubl. data). Given the high prevalence of *Ranavirus* in Southern Appalachian salamanders, we recommend that surveillance efforts be expanded in this region and perhaps elsewhere. Future efforts should include isolating and molecularly characterizing ranaviruses infecting plethodontid salamanders to determine if they are phylogenetically similar to previous isolates. Given the possible threat of ranaviruses to lungless salamanders, we recommend that researchers take biosecurity precautions to reduce the likelihood of transporting *Ranavirus* virions among watersheds. Bryan et al. (2009) reported that 1% Nolvasan®, 1% Virkon S®, and 3% bleach were effective at inactivating *Ranavirus* after 1 min contact duration. Mud and debris should be removed from footwear and gear prior to applying disinfectant (Green et al. 2009).

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