First Report of Ranavirus and Batrachochytrium dendrobatidis in Green Salamanders (Aneides aeneus) from Virginia, USA.

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First Report of Ranavirus and *Batrachochytrium dendrobatidis* in Green Salamanders (*Aneides aeneus*) from Virginia, USA

The Green Salamander (*Aneides aeneus*) is distributed from extreme southwest Pennsylvania, USA to northern Alabama and Mississippi with a disjunct population in southern North Carolina, northeastern Georgia, and northern South Carolina (Petranka 1998). Because of unique habitat requirements, Green Salamanders are thought to be at risk of range-wide declines and extirpations (Corser 2001). Green Salamanders primarily dwell in rock crevices within rock outcrops (Gordon 1952; Rossell et al. 2009). Like its western congener, Green Salamanders also inhabit arboreal habitats (Petranka 1998; Waldron and Humphries 2005) as well as decaying logs and stumps on the forest floor (Fowler 1947).

Green Salamander populations, especially those in the disjunct Carolina populations, have experienced declines and extirpations (Snyder 1991; Corser 2001). From the 1970s to 1980s local extirpations occurred in once-abundant North Carolina populations (Snyder 1991), and since the 1980s reductions of up to 98% have occurred in remaining populations (Corser 2001). One of the most-studied Blue Ridge escarpment populations was located at Biscuit Rock (Highlands, North Carolina; Gordon 1952; Snyder 1991; Corser 2001), and in the past five years this population became extirpated without a definitive cause (Lori Williams, unpubl. data). Various hypotheses have been proposed for Green Salamander declines such as disease and loss of surrounding timber (Snyder 1991; Petranka 1998; Corser 2001). Diseases are one of the leading causes of worldwide amphibian declines (Stuart et al. 2004; Young et al. 2004; Sodhi et al. 2008). Currently the Green Salamander is listed as threatened or endangered in Indiana, Maryland, Mississippi, North Carolina, Ohio, and Pennsylvania (MDNR 2005; MMNS 2005; PGCBFSC 2005; IDNR 2006; NCWRC 2014; ODNR 2014) and as a species of greatest conservation need in all other inhabited states (GDNR 2005; SCDNR 2005; TWRA 2005; VDGIF 2005; WFFDADCNR 2005; WVDNR 2005; KDFWR 2013). However, the potential impact of diseases on Green Salamanders is unknown, especially in Virginia.

Diseases caused by ranaviruses are responsible for amphibian die-offs throughout Europe and North America, including the southeastern United States (Green et al. 2002; Miller et al. 2011; Hoverman et al. 2012), and may contribute to population declines (Gray et al. 2009a). In the southern Appalachian Mountains, ranavirus infections have been reported in 18 species of plethodontid salamanders, but Green Salamanders have not been sampled (Miller et al. 2011; Hamed et al. 2013). Overall ranavirus prevalence in plethodontid salamanders has varied from 3–81% throughout the southern Appalachian Mountains (Gray et al. 2009b; Hamed et al. 2013) with salamanders from the genus *Desmognathus* having highest individual prevalence (Sutton et al. 2014). Only a single past study has sampled plethodontid salamanders for ranavirus within the Virginia portion of the Green Salamander’s range (Wise Co.; Davidson and Chambers 2011a); overall ranavirus prevalence in this study was 33%, although Green Salamanders were not sampled.

Chytridiomycosis, the disease caused by *Batrachochytrium dendrobatidis* (*Bd*), has also been responsible for numerous amphibian declines and extirpations worldwide, especially in Central American anurans (Lips et al. 2006; Lötters et al. 2009). Infection rates of *Bd* vary throughout the southeastern U.S., with 17 species of plethodontid salamanders testing positive for *Bd* in past studies, and an additional 23 species not infected (Hughes et al. 2014). However, *Bd* is thought to have played a role in the declines of plethodontid salamanders in Central America and has been shown to be lethal to plethodontid salamanders in the western United States (Lips et al. 2006; Weinstein 2009; Cheng et al. 2011). Only a single prior study with a limited sampled size (*N* = 3) has surveyed Green Salamanders for *Bd* with no positive results (Hill et al. 2011). Our goal was to determine ranavirus and *Bd* prevalence in Green Salamander populations from southwestern Virginia.

To determine the potential impact of ranaviruses and *Bd*, we sampled Green Salamanders from known historic locations as well as new locations with ideal habitat in southwestern Virginia (Dickenson, Scott, Washington, and Wise counties; Fig. 1; VDGIF 2014) and tested them for infection by these pathogens. Multiple observers searched rock crevices and surrounding trees and mid-story vegetation for Green Salamanders from May–August 2013. We also used burlap bands at three sites to intercept Green Salamanders climbing trees (Thigpen et al. 2010), but burlap proved to be ineffective due to repetitive damage from mammals, presumed to be Black Bears (*Ursus americanus*). Once located, we captured Green Salamanders by hand, while wearing nitrile gloves (Fisher Scientific, Pittsburg, Pennsylvania USA), and placed salamanders in individual 1.2-liter plastic bags, where they remained throughout processing and until released. We measured both snout–vent length (SVL) and total length (TL) using dial calipers to assess life stage (adult, subadult, juvenile; Waldron and Humphries 2005). To sample for ranavirus (*N* = 38), we followed procedures of Hamed et al. (2013) and used sterilized stainless steel forceps to collect a small tail section...
from a natural break point. Each sample was placed in a sterile, 2-ml microcentrifuge tube with 99% reagent grade isopropyl alcohol. We did not sample for ranavirus if a salamander had tail damage, and we also excluded female salamanders guarding eggs. To sample for \(Bd\) infection (N = 41), we swabbed each Green Salamander with a Dryswab™ with wire shaft and rayon bud swab (MW&E, England). First, we swabbed each flank 5 times, then the ventral surface 10 times, and finally the bottom of each foot 5 times (Chatfield et al. 2012). Swab tips were placed in separate microcentrifuge tubes with 99% reagent grade isopropyl alcohol. We released all salamanders at their original location of capture. Bags and gloves were changed and forceps autoclaved after each use.

Genomic DNA was extracted from tail samples and swabs using Qiagen DNeasy Blood and Tissue Kits (Qiagen Inc., Valencia, California, USA) and then quantified utilizing a Qubit™ Fluorometer (Life Technologies Corp., Carlsbad, California, USA). We used quantitative real-time PCR (qPCR) utilizing an ABI 7900HT PCR system (Life Technologies Corp.). For ranavirus testing, we used identical primers and protocol of Gray et al. (2009b) and ran samples in duplicate. We utilized two positive (cultured virus and DNA from a confirmed positive animal) and two negative (water and DNA from a known negative animal) controls. We deemed samples to be positive if \(C_v\) value \(\leq 30\), based on a 95% confidence interval that we derived from a standard curve originating from runs with known concentrations of virus (Caraguel et al. 2011). For \(Bd\) testing, we also used qPCR following the procedure and primers of Boyle et al. (2004) and ran samples in duplicate. We repeated analysis if \(C_v\) values differed by more than 1 \(C_v\) value. We used two positive (DNA from a \(Bd\) culture and DNA from a confirmed positive animal) and two negative (water and DNA from a known negative animal) controls. We considered samples to be positive for \(Bd\) infection if \(C_v\) value \(\leq 35\), which was similarly based on a 95% confidence interval derived from a standard curve (Caraguel et al. 2011). All DNA extraction and PCR testing was conducted in the Center for Wildlife Health at the University of Tennessee. We established 95% confidence intervals (CIs) for prevalence using Lowry (2014) 2-sided CIs from a single proportion.

Our survey is the first known to document ranavirus and \(Bd\) infections in Green Salamanders (Miller et al. 2011; Hughey et al. 2014). We did not observe co-occurrence of pathogens in a single individual, but we did within the same site (Breaks Interstate Park; Fig. 1). We sampled a total of 42 Green Salamanders (21 adults, 15 subadults, and 6 juveniles), but due to tail damage, escape, or females guarding eggs we did not collect a tail sample or swab from every individual. We sampled 38 Green Salamanders for ranavirus, and salamanders averaged 41.22 ± 1.90 (mean ± SE) mm in SVL. Ranavirus prevalence was 8% (3/38), and no salamanders testing positive for ranavirus displayed external signs of infections (Table 1; Miller et al. 2011). Only one Green Salamander testing positive for ranavirus was an adult (50 mm SVL) while the remaining two salamanders were both subadults (40 mm SVL each). Green Salamanders infected with ranavirus were collected only from the Breaks Interstate Park (Dickenson Co.; Fig. 1), which had the greatest number of Green Salamanders encountered during the survey.

We swabbed 41 Green Salamanders for \(Bd\), and salamanders averaged 42.70 ± 1.89 mm SVL. We detected \(Bd\) in 6 of 41 (15%) sampled Green Salamanders (Table 1). Two Green Salamanders infected with \(Bd\) were adults (52 and 57 mm SVL), whereas the other four were subadults (32–42 mm SVL). Only one salamander that tested positive for \(Bd\) displayed external signs of illness (e.g., emaciation). However, another Green Salamander was thin but not emaciated and did not test positive for either pathogen. Plethodontid salamanders from Mexico that were \(Bd\)-positive also lacked clinical external signs, thus suggesting positive salamanders do not always exhibit external signs of disease (Rooij et al. 2011). Green Salamanders infected with \(Bd\) were collected from Breaks Interstate Park (N = 4), Flag Rock (N = 1; Wise Co.), and Brumley Cove Camp (N = 1; Washington Co; Table 1; Fig. 1).

Due to the cryptic behavior of Green Salamanders and the presence of nesting females, we were only able to sample a single individual at six locations and are therefore cautious about inferring the potential disease status of these populations. However, two locations (Breaks Interstate Park, N = 22; Flag Rock, N = 13) provided a sufficient sample size to evaluate disease status. Only the Breaks Interstate Park had ranavirus-positive individuals, and this location was much lower in elevation (553 m) than Flag Rock (983 m), where no Green Salamanders tested positive for ranavirus. A similar trend was observed in the Great Smoky Mountains National Park where ranavirus prevalence was greater in salamander communities at lower elevation sites (Gray et al. 2009b; Sutton et al. 2014). However, our two sites were separated by almost 50 km and could have been influenced by other factors, including variation in temperature and moisture that have also been linked with ranavirus prevalence (Sutton et al. 2014). The Breaks Interstate Park also had the highest prevalence for \(Bd\). A seep and subsequent stream were adjacent to three rock crevices at the Breaks Interstate Park. Both ranavirus and \(Bd\) are associated with streams and aquatic environments (Lips et al. 2006; Sutton et al. 2014).

Green Salamanders are not typically associated with aquatic habitats, but other hosts (i.e., those associated with aquatic habitats) may be transmitting one or both of these pathogens. For example, ranavirus has been demonstrated to be transmitted by an infected individual to an uninfected individual following a short contact time (Brunner et al. 2007). Also, \(Bd\) can be passed via water containing zoospores (Carey et al. 2006) and can survive for up to three months in river sands, thus suggesting moist rock ledges could harbor \(Bd\) (Johnson and Speare 2005). Brumley...
Cove Camp had a Bd-positive individual and a large stream (Brumley Creek) that drains a reservoir (Hidden Valley Lake) is within a few meters of the rock face. We observed Desmognathus ochrophaeus (Alleghany Mountain Dusky Salamander) on the Brumley Cove Camp rock faces. In southwest Virginia, two sister species, D. ochrophaeus and D. orestes (Blue Ridge Dusky Salamander), have tested positive for ranavirus (Davidson and Chambers 2011a; Hamed et al. 2013) and the former also has tested positive for Bd (Davidson and Chambers 2011b). Thus, it is possible that salamanders of the genus Desmognathus could be moving both ranavirus and Bd out of aquatic systems to rock crevices. Flag Rock had no flowing or standing water, and only dry rock outcrops. Lastly, on rock faces where other amphibians are utilizing the same rock ledges or those in close proximity to known Green Salamander crevices, a broader sampling effort would determine if other amphibians are potentially spreading ranavirus and/or Bd from aquatic environments to rock faces inhabited by Green Salamanders.

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partment of Game and Inland Fisheries, Richmond, Virginia.
A Report of Ranavirus Infecting Midland Painted Turtles with Novel Localities for Frog Infection in Northwest Ohio

Amphibian die-offs attributed to ranaviruses (genus *Ranavirus*, *Family Iridoviridae*) continue to be reported from around the world (Gray et al. 2009; Duffus et al. 2015), and constitute one of the single most important factors in mass mortalities in the United States in recent years (Green et al. 2002). Continued reporting is important as it affords an opportunity to monitor the spread and distribution of the pathogen (Muths et al. 2006), identify affected species (Duffus et al. 2015), potentially compare viral genomes (e.g., Tan et al. 2004), and study the long-term effects of viral infections on host survival in local populations (Gray et al. 2009).

In September 2011, during the third year of a routine, biweekly sampling of frogs at a five-year old, 1-ha, mitigated wetland named Spurgat’s Wetland in Hancock County in northwest Ohio, USA (40.9905°N, 83.7596°W), dozens of tadpoles and recently transformed larvae, or adult frogs, all exhibiting physical signs of disease were noted. Skin swab samples were collected using sterile, single use, cotton-tipped transport swabs transported to the laboratory where DNA extraction was performed. Extracted DNA was used to perform conventional PCR of two targets in the major capsid protein (MCP) gene using previously published *MCP1* / 6R primer set) or *600 bp* amplicon size of 1300 bp (MCP1 / 6R primer set) or 600 bp (MCP1 / 6R primer set) or 600 bp (MCP1 / 6R primer set) after gel electrophoresis were sent to the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility for Sanger sequencing.

Whole dead or moribund frogs were collected and transported in sterile, plastic bags to the laboratory for both gross examination and genetic testing for the presence of ranavirus (Table 1). Liver samples (~ 5 mm in diameter) were taken from 13 whole frog specimens and immediately frozen at -20°C for use in molecular analysis before the whole specimen was fixed in formalin. All tools used for liver sample collection were rinsed between each specimen in 50% bleach solution for 20 minutes and thoroughly rinsed in sterile distilled water. The life stage of whole frog specimens was determined as described by (Gosner 1960) and physical signs of disease were noted. Skin swab samples were collected using sterile, single use, cotton-tipped transport swabs with a plastic shaft. Skin swab samples were transported on wet ice to the laboratory where DNA extraction was performed.

DNA was extracted from both liver and skin swab samples for molecular analysis using DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) following the manufacturer's protocol. Extracted DNA was used to perform conventional PCR of two targets in the major capsid protein (MCP) gene using previously published *gene specific primers*, including: *MCP1, MCP6R, M153, and M154* (Hyatt el al. 2000, Marsh et al. 2002). Each PCR reaction was carried out using established methodology (Hyatt el al. 2000, Marsh et al. 2002). Samples showing an approximate *amplicon size* of 1300 bp (*MCP1 / 6R primer set*) or ~600 bp (*M153 / 154 primer set*) after gel electrophoresis were sent to the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility for Sanger sequencing.

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