AMPHIBIAN AND REPTILE DISEASES

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Prevalence of *Ranavirus*, *Batrachochytrium dendrobatidis*, *B. salamandrivorans*, and *Ophidiomyces ophiodiicola* in Amphibians and Reptiles of North Carolina, USA

The viral pathogen *Ranavirus* (*Rv*) and the fungal pathogens *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*), and *Ophidiomyces ophiodiicola* (*Oo*) infect ectothermic vertebrates. In recent years, there has been increased interest in reporting the occurrences of these pathogens (e.g., Olson et al. 2013; Allender et al. 2015a, 2020; Duffus et al. 2015; Lorch et al. 2016). All have been found in wild animals on multiple continents and have contributed to mortality events, yet their impacts on population health remain poorly understood. Many amphibian populations and species are undergoing rapid decline worldwide due to habitat loss and a variety of other causes, including diseases from infections by *Rv*, *Bd*, and *Bsal* (Pimm et al. 2014; Ceballos et al. 2017). Because these pathogens can result in host mortality and have been associated with population level die-off events (Skerratt et al. 2007; Blaustein et al. 2012; Price et al. 2017), their spread into new regions may threaten naïve populations.

Rv infection has been detected in a broad range of ectothermic hosts including fishes, amphibians and reptiles (Duffus et al. 2015). Infection in amphibians presents as lethargy, loss of swimming ability, swelling (edema) and internal hemorrhaging, ulcers, and friable (necrotic) tissues (Miller et al. 2015). Infection in reptiles presents as behavioral changes in the animal (e.g., lethargy, anorexia, respiratory distress, and oral and nasal ulcers), and histopathology may reveal hemorrhaging, edema and tissue necrosis. High mortality rates associated with *Rv* infection have been described predominantly in amphibians but are reported in reptiles and are similarly influenced by temperature, life stage, and other contributing factors (Rivas et al. 2014; Kimble et al. 2017; Adamovicz et al. 2018).

Bd and *Bsal* produce dermal infections associated with skin lesions and hyperkeratosis, among other dysplastic features in the epidermis (Berger et al. 2005; Martel et al. 2013). Both *Rv* and

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Bd have been detected in a broad range of amphibian and non-amphibian hosts (McMahon et al. 2013; Olson et al. 2013; Duffus et al. 2015). *Bsal* primarily infects salamanders, but frogs may also harbor this pathogen (Martel et al. 2014; Stegen et al. 2017).

The disease associated with *Oo* infection, termed ophidiomycosis or "Snake Fungal Disease," has only been reported in snakes (Allender et al. 2015a, 2020; Franklinos et al. 2017; Thompson et al. 2018). The pathogen invades cutaneous tissues of the host, causing scale abnormalities, lesions, and disfiguration (Allender et al. 2015b; Lorch et al. 2015; Baker et al. 2019). Infection can result in mortality but may also resolve following loss of the infected dermal layers through ecdysis (Allender et al. 2011).

North Carolina, USA, has a rich diversity of amphibians and reptiles (Palmer and Braswell 1995; Beane et al. 2010) and is notably the most species-rich U.S. state with respect to salamanders (AmphibiaWeb 2020). *Rv* and *Bd* are both present in North Carolina at levels that suggest they are endemic (Rothermel et al. 2008; Duffus et al. 2015; Williams and Groves 2014; Moffitt et al. 2015). At present, *Bsal* has not been detected in wild amphibians in North Carolina, or elsewhere in the U.S. (AmphibiaWeb 2020; Waddle et al. 2020). Due to high levels of diversity and endemism in salamanders, several models have predicted that North Carolina could be particularly impacted if *Bsal* became established in the wild (Yap et al. 2015; Richgels et al. 2016; Yap et al. 2017). *Oo* has been detected in reptiles in the southeastern United States (Guthrie et al. 2016; Lind et al. 2018; Allender et al. 2020), yet the full breadth of species in which it may occur, and endemic prevalence across taxonomic groups, is not well understood.

The outcome of infection for all of these pathogens is host-specific, and it is unclear how they may be impacting populations of amphibians and reptiles in North Carolina. In addition, little is known about the interaction of these pathogens and their rates of coinfection. The complexity engendered by having diverse host species, including susceptible and reservoir hosts (Brunner et al. 2015; Brannelly et al. 2018), makes it difficult to assess potential for disease spread without first understanding which species can serve as hosts in a given geographic area. In this report, we assess prevalence of *Rv*, *Bd*, *Bsal*, and *Oo* in a broad taxonomic and geographic representation of amphibians and reptiles in North Carolina.

Samples were obtained by swabbing animals that were captured from the wild or being maintained in captivity during the spring and summer of 2016. Project participants were provided sampling kits that contained disposable, powderless nitrile gloves; sterile Peel Pouch Dryswab Fine Tip (MWE, product no. MW113) rayon-budded swabs; sterile cryogenic tubes and cryogenic storage boxes; vial label markers; plastic and cloth bags; and detailed instructions. After capture, animals were temporarily housed individually in fresh bags (new plastic bag or machine-washed cloth bag) and handled separately with gloves to avoid cross contamination. The swabbing method for each animal type (i.e., frog, salamander, lizard, snake or turtle) was standardized to specify a certain number of strokes over particular regions of the body that maximized the chances of detecting these pathogens (Berger et al. 2005; Retallick et al. 2006; Van Rooij et al. 2011; Gray et al. 2012; Hileman et al. 2017). In post-metamorphic frogs, a total of 30 strokes was taken with a swab, consisting of five strokes on the belly, five strokes on each plantar surface, five strokes on the ventral surface of each thigh, and five strokes on the vent. In larval frogs, the swab was twirled using thumb and forefinger inside the oral cavity a total of five times. In salamanders, a total of 40 strokes was taken, consisting of 10 strokes on the belly (30 for eel-like salamanders lacking robust limbs), five strokes on each palmar and plantar surface, five strokes on the vent, and five strokes on the ventral surface of the tail. In lizards, a total of 45 strokes was taken, consisting of five strokes each on the gular region, left and right supralabial scales, left and right infralabial scales, and each palmar and plantar surface (substituted for 10 strokes in each of the left and right lateral body groove in the limbless genus *Ophisaurus*). In snakes, a total of 35 strokes was taken, consisting of five strokes each on the gular region, left and right eye, left and right supralabial scales, and left and right infralabial scales. In turtles, all of which were injured in the wild and rehabilitating under the care of the Turtle Rescue Team at North Carolina State University's College of Veterinary Medicine, Raleigh, North Carolina, USA, a total of 10 strokes was taken inside the oral cavity by trained veterinary staff. Swabs were stored dry on ice or refrigerated as quickly as possible during collection and transit, after which they were stored at -20°C or -80°C until processing. Following study, swabs and DNA extractions were transferred to -80°C for long-term archival storage in the herpetology genetic resources collection at the North Carolina Museum of Natural Sciences, Raleigh, North Carolina.

DNA isolation from swabs was performed using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer recommendations. The presence and quantity of *Rv*, *Bd*, *Bsal,* or *Oo* DNA in the swabs was determined by quantitative polymerase chain reaction (qPCR; Boyle et al. 2004; Allender et al. 2013; Blooi et al. 2013; Allender et al. 2015c). *Rv* was detected with forward (5'-AACGCCGACCGAAAACTG-3') and reverse (5'-GCTGCCAAGATGTCGGGTAA-3') primers, and the probe 5' 6FAM-CCGGCTTTCGGGC-MGBNFQ 3' (Allender et al., 2013). *Bd* was detected with the primers ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC-3') and 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3'), and the probe 5' 6FAM-CGAGTCGAACAAAAT-MGBNFQ 3' (Boyle et al., 2004). *Bsal* was detected with primers STerF (5'-TGCTCCATCTCCCCCTCTTCA-3') and STerR (5'-TGAACGCACATTGCACTCTAC-3'), and the probe STerC 5' 6FAM-ACAAGAAAATACTATTGATTCTCAAACAGGCA-MGBNFQ 3' (Blooi et al., 2013). *Oo* was detected with forward (5'-TGTTTCTGTCTCGCTCGAAGAC-3') and reverse (5'-AGGTCAAACCGGAAAGAATGG-3') primers, and the probe 5'6FAM-CGATCGGGCGCCCGTCGTC-MGBNFQ 3' (Allender et al. 2015c). Cycling parameters were as described in the primer and probe reference for each assay. Reactions were prepared by hand or an epMotion 5075 liquid handler (Eppendorf) in 25-µl volumes consisting of 12.5-µl Platinum qPCR SuperMix-UDG (Invitrogen), 0.5-µl forward primer (10µM), 0.5-µl reverse primer (10µM), 0.25-µl TaqMan probe (10µM), and 2.5-µl sample DNA. Pathogens were tested in individual rather than multiplexed reactions. Each sample was tested in duplicate. For each assay, a 10-fold dilution curve of a plasmid DNA standard containing the target sequence was used to determine limit of sensitivity and absolute quantitation of genome equivalents of target (Allender et al. 2015c, Fritch et al. 2017). For *Rv,* a positive control of total DNA was isolated from fathead minnow (*Pimephales promelas*) cell cultures infected with Frog Virus 3 and negative controls of total DNA were isolated from naïve *P. promelas* cell cultures. H_2O and AE buffer were included as negative controls in all runs.

Sample results (i.e., positive or negative detection) were determined by consensus of two data reviewers. Lead author TBL evaluated the resulting qPCR curves unblinded to the sample information, and second author MCA made an independent evaluation blinded to sample information and TBL's determination.

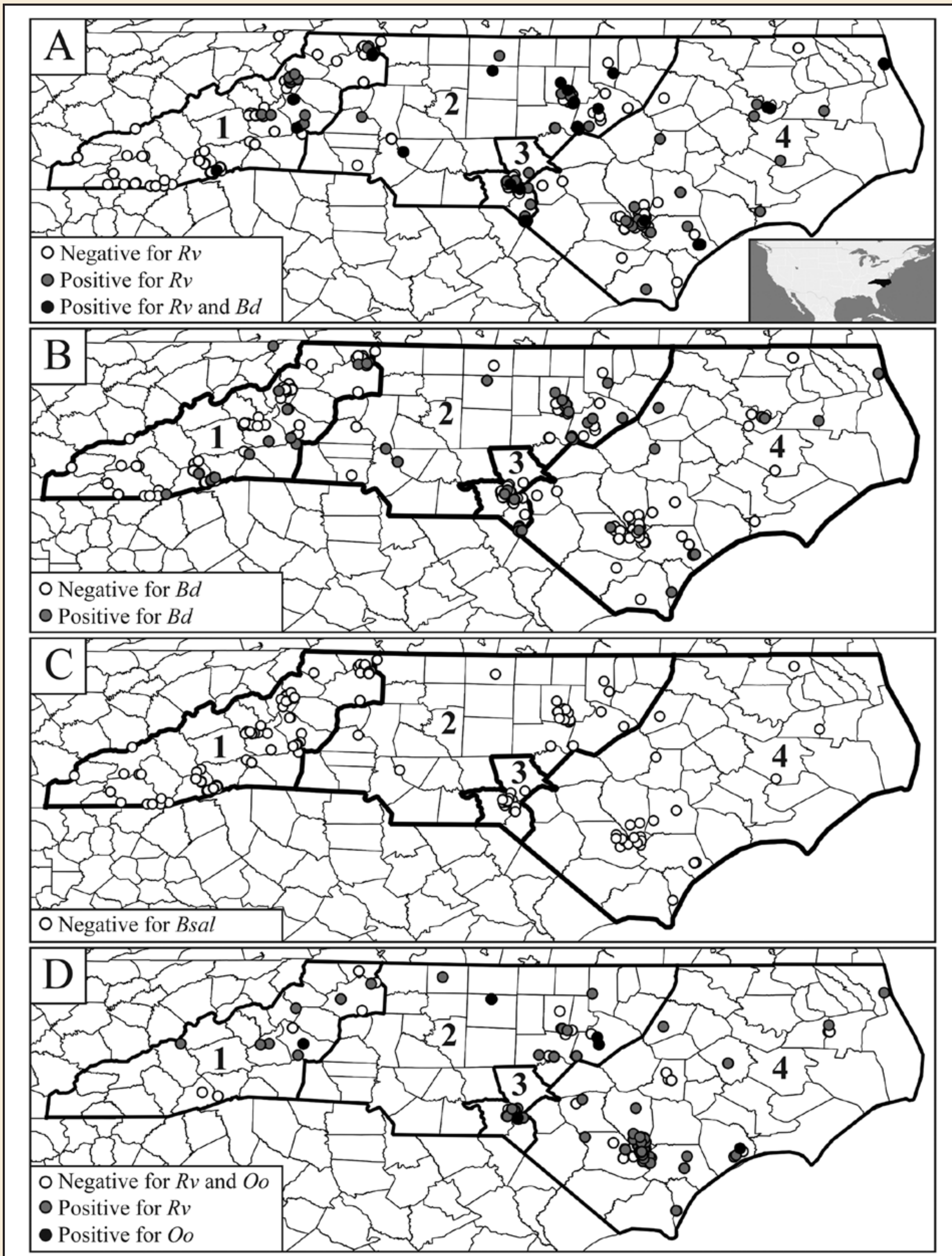

Fig. 1. Distribution of sampling and detection of (A) *Ranavirus* (*Rv*), (B) *Batrachochytrium dendrobatidis* (*Bd*), and (C) *Batrachochytrium salamandrivorans* (*Bsal*) from skin swabs of wild amphibians, and (D) *Ranavirus* (*Rv*) and *Ophidiomyces ophiodiicola* (*Oo*) from skin swabs of wild reptiles in North Carolina, USA. White dots indicate negative samples, gray dots indicate positive samples, and black dots indicate samples that are positive for both *Rv* and *Bd* (panel A) or *Oo* (panel D). Physiographic regions are indicated as Mountains (1), Piedmont (2), Sandhills (3), and Coastal Plain (4). Overlapping samples may appear as a single dot.

Only samples determined to be positive by both reviewers were reported as positives in this study. To be determined as positive, sample replicates had to amplify earlier than the limit of sensitivity determined by the plasmid standard for the pathogen included on the same plate and earlier than all negative control reactions included on the same plate. Three samples that initially tested positive for *Bsal* DNA by qPCR were subjected to additional tests before determining the final result. These additional tests included 1) retesting these samples for *Bsal* by the qPCR assay described above, 2) testing these samples for amplification of *Bsal* DNA sequences outside of the qPCR primer set by using endpoint PCR with the primers Bsal 01 (5'-AGGGAGACGAAAAAGATCAAG-3') and Bsal 02 (5'-GGAGTAAAATCCCAACACAGTG-3'), which were designed to detect *Bsal* 5.8S rRNA (GenBank accession number KC762295), in combination with the primer STerF, 3) testing these samples for sequences present in the plasmid DNA standard by endpoint PCR, and 4) sampling additional individuals collected from the same site. Sample data (locality, date, collector, museum voucher, if applicable) and results were deposited in Dryad Digital Repository [https://doi.org/10.5061/dryad.x69p8czhj], except that locality details below the county level were withheld for sensitive species (available upon request from the corresponding author).

For each pathogen, rate of detection in animals (i.e., prevalence) and 95% confidence intervals (CI) were determined using the online tool available at http://vassarstats.net/prop1.html. Chi-square tests were used to test for differences in prevalence between two subject groups. Odds ratios (OR) were calculated using the online tool available at https://www.scistat.com/statisticaltests/odds_ratio.php.

A total of 718 individual amphibians was sampled, representing 12 families and 68 species. Of these, 666 (93%) were wild individuals sampled from across North Carolina (Table 1, Fig. 1A-C) and 52 (7%) were captive individuals from the live holdings in the North Carolina Museum of Natural Sciences' Educational Living Collections (Table 2). All captive individuals had been originally captured in the wild in North Carolina, except for three individuals of the non-native *Ambystoma mexicanum* and one individual of the non-native *A. texanum*. All 718 amphibians were screened for *Rv*, with 688 frogs and salamanders also screened for *Bd*, and 471 salamanders also screened for *Bsal*.

A total of 254 individual reptiles was sampled, representing eight families and 36 species. Of these, 221 (87%) were wild individuals sampled from across North Carolina (Table 3; Fig. 1D), and 33 (13%) were captive individuals in the live holdings in the North Carolina Museum of Natural Sciences' Educational Living Collections or the Turtle Rescue Team at the North Carolina State University's College of Veterinary Medicine (Table 4). All 254 individual reptiles were screened for *Rv* and a subset of 103 snakes was tested for *Oo*.

Rv was detected in 284 of 972 (29%) individual amphibians and reptiles tested. Plotting detection events across North Carolina's four physiographic regions indicated *Rv* is broadly distributed in the state (Figs. 1A & 1D). In amphibians, *Rv* was detected in 174 of 718 (24%; 95% CI of 21–28%) animals tested, representing 37 of 61 (61%) species sampled (Tables 1–2). In wild amphibians, *Rv* was detected in 161 of 666 (24%; 95% CI of 21–28%) animals tested (Table 1), and in captive amphibians, *Rv* was detected in 13 of 52 (25%; 95% CI of 15–38%) animals tested (Table 2). No difference in rate of detection of *Rv* was found between wild frogs, 28% (63 of 223 animals tested; 95% CI of 23–34%), and wild salamanders, 22% (98 of 444 animals tested; 95% CI of 18–26%), as determined by a chi-square test. In reptiles, *Rv* was detected in 110 of 254 (43%; 95% CI of 37–49%) animals tested, representing 28 of 39 (72%) species sampled (Tables 3–4). In wild reptiles, *Rv* was detected in 109 of 221 (49%; 95% CI of 43–56%) animals tested (Table 3), and in captive reptiles, *Rv* was detected in 1 of 33 (3%; 95% CI of 1–15%) animals tested (Table 4).

Bd was detected in 98 of 688 (14%; 95% CI of 12–17%) individual amphibians tested, representing 25 of 68 (37%) species sampled (Tables 1–2). Plotting detection events across North Carolina's four physiographic regions indicated *Bd* is broadly distributed in the state (Fig. 1B). In wild amphibians, *Bd* was detected in 98 of 636 (15%; 95% CI of 13–18%) animals tested (Table 1), and in captive amphibians, *Bd* was detected in 0 of 52 (0%) animals tested (Table 2). Rate of detection was greater in wild frogs, 22% (46 of 212 animals tested; 95% CI of 17–28%), than in wild salamanders, 12% (52 of 424 animals tested; 95% CI of 9–16%) as determined by a chi-square test.

Coincidence of *Rv* and *Bd* occurred in 40 of 636 (6%; 95% CI of 5–8%) individual amphibians tested for both pathogens (Table 1; Fig. 1A). Using an OR test, presence of *Bd* was positively associated with presence of *Rv* in frogs (OR 5.5263, 95% CI of 2.7668–11.0380, $P < 0.0001$). Rate of detection was greater for *Rv* in *Bd*-positive frogs, 59% (27 of 46 *Bd*-positive frogs; 95% CI of 44–72%), and *Bd* in *Rv*-positive frogs, 43% (27 of 63 *Rv*-positive frogs; 95% CI of 31–55%), than for either pathogen in total frogs tested. The same association was not observed in salamanders (OR 1.2039, 95% CI of 0.6148–2.3577, $P = 0.5884$), for which rate of detection of *Rv* in *Bd*-positive salamanders, 25% (13 of 52 *Bd*-positive salamanders; 95% CI of 15–38%), and *Bd* in *Rv*-positive salamanders, 13% (13 of 98 *Rv*-positive salamanders; 95% CI of 8–21%), were not different from the prevalence of these pathogens in all salamanders tested for each. Thus, frogs positive for one pathogen had a greater likelihood of also being positive for the second pathogen than did frogs from the total sampled population, but this was not the case for salamanders. Results from captive amphibians suggested that *Rv* detection was lower than that observed in wild animals, except for *P. jordani* (71%; 10 of 14 captive individuals tested; Table 2). The high prevalence in these captive *P. jordani* may be due to the circumstances of their origin, as these were confiscated by law enforcement from captive conditions where they had been in contact with other amphibian species.

Bsal was not detected in any of the 471 salamanders tested, wild-caught or captive (Fig. 1C; Tables 1–2). During laboratory analyses, three positive results for *Bsal* DNA were observed. However, in all three cases, these initial positive results were determined to be caused by contamination with the plasmid standard and were not true positive events, following test verification as described above. In all cases, these samples tested negative for additional *Bsal* DNA sequences outside of the qPCR target sequence but positive for DNA sequences present in the plasmid standard. In addition to molecular assay verification, we collected additional samples of the same species from the site where the "positive" animals had been collected. None of these secondary animal samples tested positive for *Bsal*. This result is consistent with the absence of reports detecting this pathogen in the wild on the North American continent (Waddle et al. 2020) and emphasizes the value of proactive mitigation efforts (Hopkins et al. 2018).

Oo was detected in 10 of 103 (10%; 95% CI of 5.4–17.0%) individual snakes tested, representing 6 of 23 (26%) snake species sampled (Tables 3–4). In wild snakes, *Oo* was detected in 9 of 101 animals tested (9%; 95% CI of 5–16%; Table 3), and in

Table 1. qPCR detection of *Ranavirus* (*Rv*), *Batrachochytrium dendrobatidis* (*Bd*), and *B. salamandrivorans* (*Bsal*) from skin swabs of wild amphibians in North Carolina, USA. An "–" indicates samples that were not tested for the pathogen or were not coinfected with *Rv* and *Bd* (*Rv+Bd*).

Family	Species	No. positive/total no. tested			
		Rv	*Bd*	*Rv+Bd*	*Bsal*
Ambystomatidae	*Ambystoma maculatum*	5/6	1/6	1/6	0/6
	Ambystoma opacum	0/25	1/25	–	0/25
Amphiumidae	*Amphiuma means*	0/5	1/5	–	0/5
Bufonidae	*Anaxyrus americanus*	3/21	1/18	1/18	–
	Anaxyrus fowleri	2/13	1/12	–	–
	Anaxyrus quercicus	0/3	0/3	–	–
	Anaxyrus terrestris	9/22	1/22	1/22	–
Cryptobranchidae	*Cryptobranchus alleganiensis*	0/6	0/6	–	0/6
Hylidae	*Acris crepitans*	12/20	7/18	6/18	–
	Acris gryllus	5/8	5/8	3/8	–
	Hyla andersonii	0/9	0/9	–	–
	Hyla chrysoscelis	0/8	0/8	–	–
	Hyla cinerea	2/4	2/4	2/4	–
	Hyla femoralis	0/6	0/6	–	–
	Hyla gratiosa	0/6	0/6	–	–
	Hyla squirella	0/1	0/1	–	–
	Hyla cf. versicolor	0/1	0/1	–	–
	Pseudacris crucifer	3/5	3/5	2/5	–
	Pseudacris ocularis	1/1	0/1	–	–
Microhylidae	*Gastrophryne carolinensis*	1/6	0/6	–	–
Plethodontidae	*Aneides aeneus*	0/31	5/31	–	0/31
	Desmognathus cf. auriculatus	3/3	0/3	–	0/3
	Desmognathus carolinensis	2/20	0/14	–	0/14
	Desmognathus fuscus	16/51	8/51	3/51	0/51
	Desmognathus marmoratus	2/2	0/2	–	0/2
	Desmognathus monticola	4/12	0/12	–	0/12
	Desmognathus ocoee	0/10	0/7	–	0/7
	Desmognathus orestes	3/16	1/16	–	0/16
	Desmognathus organi	0/4	0/4	–	0/4
	Desmognathus quadramaculatus	4/16	0/12	–	0/12
	Eurycea arenicola	1/4	0/4	–	0/4
	Eurycea chamberlaini	1/3	2/3	1/3	0/3
	Eurycea cirrigera	1/3	0/3	–	0/3
	Eurycea guttolineata	2/5	1/5	1/5	0/5
	Eurycea wilderae	8/17	1/17	–	0/17
	Gyrinophilus porphyriticus	3/5	0/4	–	0/4
	Hemidactylium scutatum	1/1	0/1	–	0/1
	Plethodon chlorobryonis	7/16	0/16	–	0/16
	Plethodon cinereus	5/9	0/9	–	0/9
	Plethodon cylindraceus	2/6	0/6	–	0/6
	Plethodon jordani	0/4	0/2	–	0/2
	Plethodon longicrus	0/5	0/5	–	0/5
	Plethodon metcalfi	0/9	0/9	–	0/8
	Plethodon montanus	3/16	0/16	–	0/16
	Plethodon serratus	0/2	0/2	–	0/2
	Plethodon teyahalee	0/4	0/2	–	0/2
	Plethodon teyahalee x *shermani*	0/2	–	–	–
	Plethodon wehrlei	0/8	0/8	–	0/8
	Plethodon yonahlossee	2/6	0/6	–	0/6
	Pseudotriton montanus	0/4	0/4	–	0/4
	Pseudotriton ruber	0/12	1/12	–	0/12
Proteidae	*Necturus punctatus*	0/3	1/3	–	0/3
Ranidae	*Lithobates capito* (transformed)	0/2	1/2	–	–
	Lithobates capito (larvae)	0/2	0/2	–	–
	Lithobates catesbeianus (transformed)	1/19	4/18	1/18	–
	Lithobates catesbeianus (larvae)	0/3	2/3	–	–
	Lithobates clamitans (transformed)	3/10	2/10	2/10	–
	Lithobates clamitans (larvae)	3/3	1/3	1/3	–
	Lithobates kauffeldi	1/2	2/2	1/2	–
	Lithobates palustris	4/13	5/10	3/10	–
	Lithobates sphenocephalus (transformed)	11/26	9/26	4/26	–
	Lithobates sphenocephalus (larvae)	0/4	0/4	–	–
	Lithobates virgatipes	1/2	0/2	–	–
Salamandridae	*Notophthalmus viridescens*	23/93	29/93	7/93	0/93
Scaphiopodidae	*Scaphiopus holbrookii*	1/2	0/2	–	–
	Total:	161/666	98/636	40/636	0/423

TABLE 2. qPCR detection of *Ranavirus* (*Rv*), *Batrachochytrium dendrobatidis* (*Bd*), and *B. salamandrivorans* (*Bsal*) from skin swabs of captive amphibians in North Carolina, USA. An "–" indicates samples that were not tested for the pathogen.

Family	Species	No. positive/total no. tested		
		Rv	*Bd*	*Bsal*
Ambystomatidae				
	Ambystoma mabeei	0/1	0/1	0/1
	Ambystoma maculatum	0/4	0/4	0/4
	Ambystoma mexicanum	0/3	0/3	0/3
	Ambystoma opacum	1/4	0/4	0/4
	Ambystoma texanum	0/1	0/1	0/1
	Ambystoma tigrinum	0/6	0/6	0/6
Bufonidae				
	Anaxyrus americanus	0/1	0/1	–
Hylidae				
	Hyla gratiosa	1/1	0/1	–
Plethodontidae				
	Desmognathus orestes	0/1	0/1	0/1
	Desmognathus wrighti	0/1	0/1	0/1
	Gyrinophilus porphyriticus	0/1	0/1	0/1
	Plethodon cylindraceus	0/1	0/1	0/1
	Plethodon jordani	10/14	0/14	0/14
	Plethodon montanus	1/2	0/2	0/2
	Plethodon shermani	0/1	0/1	0/1
	Plethodon yonahlossee	0/4	0/4	0/4
	Pseudotriton ruber	0/2	0/2	0/2
Ranidae				
	Lithobates catesbeianus	0/2	0/2	–
Salamandridae				
	Notophthalmus viridescens	0/1	0/1	0/1
Sirenidae				
	Siren lacertina	0/1	0/1	0/1
Total:		13/52	0/52	0/48

captive snakes, *Oo* was detected in 1 of only 2 individuals tested (Table 4). Plotting detection events across North Carolina's four physiographic regions indicated *Oo* is broadly distributed in the state (Fig. 1D).

Overall, this study expanded or verified known host range and geographic distributions of these pathogens in amphibians and reptiles in North Carolina. Prevalence of *Rv* in amphibians and reptiles and *Bd* in amphibians occurs statewide and at levels that are overall consistent with previous reports from the southeastern USA (e.g., Rothermel et al. 2008; Duffus et al. 2015; Williams and Groves 2014; Moffitt et al. 2015; but see Keitzer et al. 2011). Our *Rv*-positive and *Bd*-positive samples were all collected from sites that appeared to be in a 'normal' state of pathogen burden, without apparent evidence of disease outbreak or significant die-off events, and from captive animals that otherwise appeared healthy with no indication of disease at the time of sampling. Thus, these data support the hypotheses that *Rv* and *Bd* are endemic to North Carolina. Similar observations have been made in prior reports, in which populations were found to maintain endemic levels of pathogen and appear to experience infection as a result of local, temporal re-introduction of pathogen or to changes in

TABLE 3. qPCR detection of *Ranavirus* (*Rv*) and *Ophidiomyces ophiodiicola* (*Oo*) from skin swabs of wild reptiles in North Carolina, USA. An "–" indicates samples that were not tested for *Oo* or were not coinfected with *Rv* and *Oo* (*Rv*+*Oo*).

Family	Species	No. positive/total no. tested		
		Rv	*Oo*	*Rv+Oo*
Anguidae				
	Ophisaurus ventralis	3/4	–	–
Colubridae				
	Carphophis amoenus	9/11	0/11	–
	Cemophora coccinea	1/1	0/1	–
	Coluber constrictor	0/18	3/18	–
	Diadophis punctatus	1/8	0/1	–
	Farancia abacura	4/4	0/4	–
	Heterodon platirhinos	3/4	0/4	–
	Lampropeltis elapsoides	0/1	–	–
	Lampropeltis calligaster	2/4	0/2	–
	Lampropeltis getula	1/5	0/1	–
	Masticophis flagellum	2/2	0/2	–
	Nerodia erythrogaster	7/9	0/9	–
	Nerodia fasciata	6/7	0/7	–
	Nerodia sipedon	5/6	1/6	1/6
	Opheodrys aestivus	2/4	0/2	–
	Pantherophis alleghaniensis	8/23	2/8	2/8
	Pantherophis guttatus	8/11	0/8	–
	Pituophis melanoleucus	2/2	1/2	1/2
	Rhadinaea flavilata	0/1	–	–
	Storeria dekayi	2/3	1/2	1/2
	Storeria occipitomaculata	3/4	0/3	–
	Tantilla coronata	1/1	0/1	–
	Thamnophis sirtalis	0/1	–	–
	Virginia valeriae	3/3	0/3	–
Dactyloidae				
	Anolis carolinensis	3/15	–	–
	Anolis sagrei	4/4	–	–
Scincidae				
	Plestiodon fasciatus	4/8	–	–
	Plestiodon inexpectatus	17/18	–	–
	Plestiodon laticeps	0/1	–	–
	Plestiodon sp.	0/1	–	–
	Scincella lateralis	0/17	–	–
Phrynosomatidae				
	Sceloporus undulatus	2/11	–	–
Teiidae				
	Aspidoscelis sexlineatus	0/3	–	–
Viperidae				
	Agkistrodon contortrix	3/3	0/3	–
	Crotalus horridus	2/2	0/2	–
	Sistrurus miliarius	1/1	1/1	1/1
Total:		109/221	9/101	6/101

environmental factors that alter host susceptibility (Briggs et al. 2010; Hoverman et al. 2012). We infer that conservation efforts aimed at improving and maintaining environmental health in North Carolina (e.g., reducing habitat loss, pollution, and

Table 4. qPCR detection of *Ranavirus* (*Rv*) and *Ophidiomyces ophiodiicola* (*Oo*) from skin swabs of captive reptiles in North Carolina, USA. An "–" indicates samples that were not tested for *Oo*.

Family	Species	No. positive/total no. tested	
		Rv	*Oo*
Colubridae			
	Nerodia sipedon	0/1	1/1
	Pantherophis alleghaniensis	1/1	0/1
Emydidae			
	Terrapene carolina	0/31	–
Total:		1/33	1/2

other environmental stressors) should have greater impact in preventing outbreaks than would efforts to specifically contain or mitigate pathogen infections at any single site. Our identification of geographic areas and taxa in the state that have coinfections of *Rv* and *Bd* should facilitate further investigation into possible consequences on individuals and populations that may differ from infection by only one pathogen. Coinfection by *Rv* and *Bd* has been identified in *Cryptobranchus alleganiensis* in Tennessee (Souza et al., 2012) as well as frogs in the tropical Andes and Costa Rica (Warne et al., 2016; Whitfield et al., 2013). Mechanisms of coinfection in wild amphibian populations, including both antagonistic and facilitative interactions between these pathogens, are unknown, and will be important in understanding vulnerability of amphibians to disease risk across life stages, as well as approaches for containing and mitigating disease-induced population declines (Warne et al., 2016).

Fortunately, we found no evidence for the presence of *Bsal* in wild amphibians in North Carolina, consistent with the current paradigm that *Bsal* remains absent from wild North American amphibians (Waddle et al. 2020). Due to the remarkably high levels of diversity and endemism of salamander species in the state (Beane et al. 2010; AmphibiaWeb 2020), we recommend ongoing monitoring of *Bsal* in wild and captive North Carolina amphibians (including frogs; Martel et al. 2014; Stegen et al. 2017) to maximize the early detection and successful implementation of a response should *Bsal* become established (Gray et al. 2015; AmphibiaWeb 2020; Waddle et al. 2020).

All six snake species that tested positive for *Oo* in this study, *Coluber constrictor*, *Nerodia sipedon*, *Pantherophis alleghaniensis*, *Pituophis melanoleucus*, *Sistrurus miliarius*, and *Storeria dekayi*, were previously reported to be susceptible to the pathogen (Rajeev et al. 2009; Guthrie et al. 2016; Burbrink et al. 2017; Lind et al. 2018; Licitra et al. 2019; Allender et al. 2020). Positive samples were detected state-wide (Fig. 1D); however, the natural history, ecology and epidemiology of *Oo* remain poorly understood (Allender et al. 2015a), and further study on the distribution and prevalence of *Oo* in the state is warranted.

From a methodological perspective, we have identified criteria to aid in eliminating false positive detection events, including independent evaluations of qPCR results, with at least one blinded data reviewer; verifying positive samples using endpoint PCR primers outside of the qPCR primer set; and testing positive samples for sequences present in the plasmid DNA standard by endpoint PCR. These or similar criteria should be useful in reducing erroneous reports of pathogen occurrences.

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A Shell Disease in an Iowa, USA Population of Yellow Mud Turtles, *Kinosternon flavescens*: Evidence for an Isolated Local Distribution

Studies of an Iowa, USA population of *Kinosternon flavescens* (Yellow Mud Turtle) began in 1973 on the south edge of Muscatine, in Muscatine and Louisa counties. This location, known as Big Sand Mound (BSM), is where Dodge and Miller (1955) found the largest of the three Iowa populations. Further described by Christiansen et al. (1984, 1985, 2012), the BSM population lies within the northeastern extent of the species' range, and *K. flavescens* is currently an Iowa-endangered species.

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During the studies in the 1970s, we observed a shell disease in *K. flavescens* characterized by depigmentation, deterioration of the carapacial scutes and sometimes the underlying dermal bone, leaving the plastron unaffected. In 1995, JLC photographed and biopsied portions of the shell for histological examination, but no inflammatory cells or causative agents were observed at that time using special stains for fungi (Gomori's methenamine silver) and bacteria (Brown and Brenn Gram), and the technology had not been developed to pursue molecular diagnostics. As no supporting information was found regarding a pathogenic cause of this disease, we ceased trying to identify a cause and only noted the shell disease morphology during continued monitoring of the *K. flavescens* BSM population.

Despite the carapacial disease, the population grew substantially until 1993 when the area experienced severe flooding. Following the flooding, *K. flavescens* populations throughout Iowa, Missouri, and Illinois, USA declined