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Trace Amounts of Ranavirus in Eastern Worm Snakes (*Carphophis amoenus*) in Central Virginia, USA

Ranaviruses (family Iridoviridae, genus *Ranavirus*) are pathogens that infect and can cause mass mortality events in amphibians, reptiles, and fish (Green et al. 2002; Whittington et al. 2010; Brunner et al. 2015; Gray et al. 2017). The occurrence and impact of ranaviruses in squamates is understudied, and most reports in the suborder Serpentes have come from captive animals who present signs of infection, including: *Morelia viridis* imported into Australia from Indonesia (Hyatt et al. 2002), *Python brongersmai* imported into Germany from Indonesia (Stöhr et al. 2015), and *Bothrops moojeni* in Florida that was originally from Brazil (Johnsrude et al. 1997). One exceptional study detected ranavirus DNA in 19 species of colubrid snakes (*Carphophis amoenus*, *Cemophora coccinea*, *Diadophis punctatus*, *Farancia abacura*, *Heterodon platirhinos*, *Lampropeltis calligaster*, *L. getula*, *Masticophis flagellum*, *Nerodia erythrogaster*, *N. fasciata*, *N. sipedon*, *Opheodrys aestivus*, *Pantherophis alleghaniensis*, *P. guttatus*, *Pituophis melanoleucus*, *Storeria dekayi*, *S. occipitomaculata*, *Tantilla coronata*, and *Virginia valeriae*) and three species of viperid snakes (*Agkistrodon contortrix*, *Crotalus horridus*, *Sistrurus miliarius*) from several wild populations in North Carolina, USA (Lentz et al. 2021). Antibodies have been detected against a ranavirus (Bohle iridovirus, BIV) in five species of free-ranging Australian snakes (*Boiga irregularis*, *Dendrelaphis punctulatus*, *Morelia spilota*, *Liasis childreni*, and *Tropidonophis mairii*; Ariel et al. 2017). A population of *Natrix maura* in Galicia, Spain, had a high prevalence of ranavirus DNA

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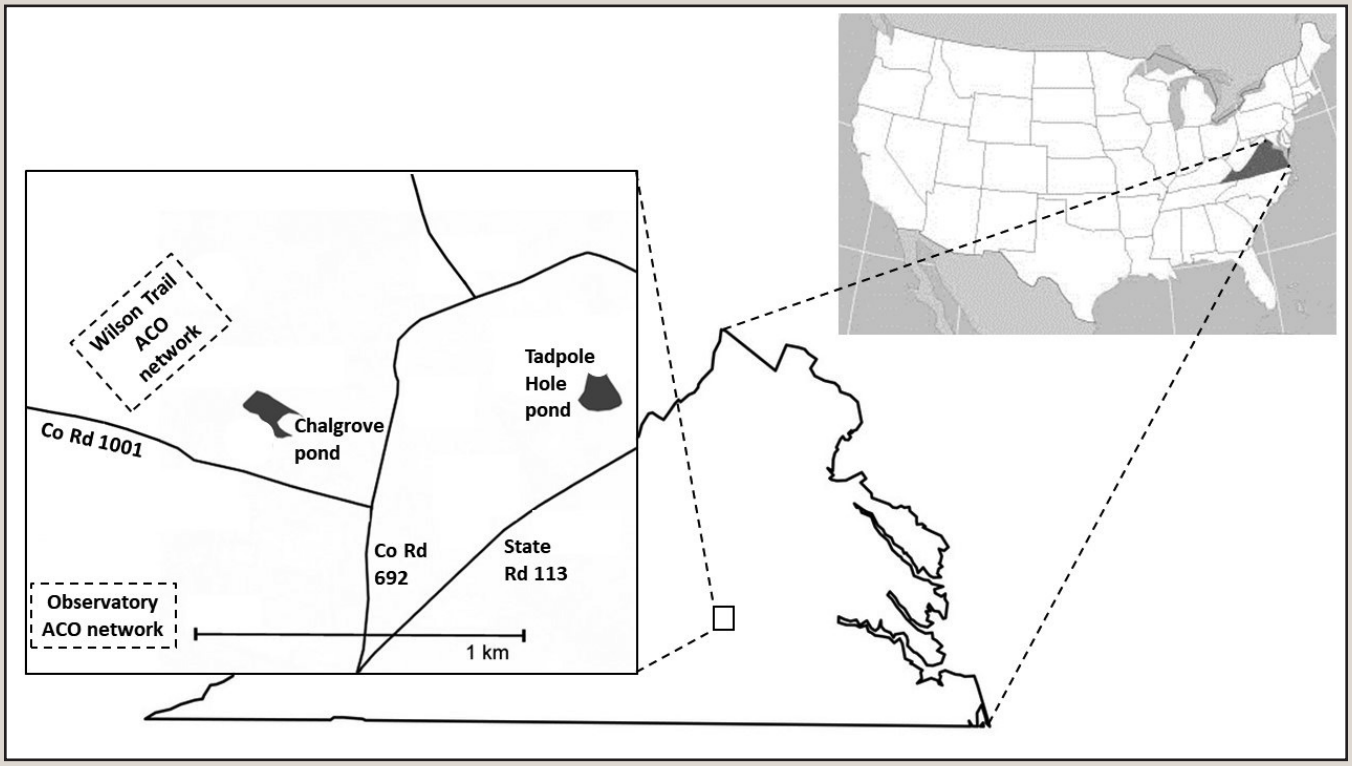

FIG. 1. Map of the study area, the campus of Hampden-Sydney College in Prince Edward County, Virginia, USA. The Wilson Trail and Observatory survey networks contained 201 wood and tin artificial cover objects (ACOs) which were used to capture snakes.

(common midwife toad virus, CMTV; 70% of 10 snakes sampled), but individuals displayed no symptoms of ranavirosis.

The current study surveyed terrestrial snakes for presence of ranavirus DNA on the campus of Hampden-Sydney College in central Virginia, USA, where previous studies detected FV3-like (Frog virus 3) ranavirus in three species of turtles and lizards (Goodman et al. 2013; 2018). Snakes were captured mostly in two survey sites in temperate deciduous woods (Fig. 1; Observatory: 37.23741°N, 78.47088°W; Wilson Trail: 37.24490°N, 78.46862°W). A total of 201 artificial cover objects (ACOs, 0.6 × 1.2 m, plywood and corrugated tin roofing material) were distributed evenly between the two sites. Within each 3- to 4-ha site, wood and tin ACOs were placed 20-m apart in alternating occurrence. ACOs were checked 1–2 times per week for 22 weeks total during June–July 2021, September–October 2021, March–April 2022, and May–July 2022. We also caught a few snakes opportunistically when they were observed on or near trails and near buildings on the college campus.

Snakes were transported in individual plastic containers to the laboratory, where they were marked or tagged for identification, weighed, measured, and examined externally, and tail tips were collected for tissue samples. We removed 3–4 mm of the tail tip using a sterile scalpel, disinfected the site with 10% povidone-iodine, and applied Kwikstop® Styptic Powder for local anesthesia and to aid in healing. We collected tissues rather than swabs because swabs failed to detect ranavirus in turtles in a previous study at our site (Goodman et al. 2013). In summer 2021, we used cautery of ventral scales for marking, but we switched to passive integrated transponder devices (PIT tags) in the fall for more reliable identification of individuals (various brands; 1.25 × 7 mm). Orasol® was applied to the injection site 3 min before insertion, and 3M Vetbond® tissue adhesive was applied to the wound site afterward. All animals were checked 10 min after processing to ensure the integrity of wound sealing methods, and they were released at their trapping site within 12 h of capture. Animals were handled with nitrile gloves from the time of trapping to release. Gloves were changed and all laboratory equipment was washed and then disinfected for at least 1 min with 70% ethanol or 1% Nolvasan (Zoetis Inc., Kalamazoo, Michigan, USA; Bryan et al. 2009) between snakes. Field equipment and shoes had either only been used on the college campus, or were disinfected with 1% Nolvasan before use in this study (with the exception of the ACOs, which were purchased from a local hardware store).

Upon collection, tissue samples were stored at -80°C. We later extracted DNA using DNeasy Blood and Tissue kits (QIAGEN, Valencia, California, USA) and quantified DNA concentrations using an Epoch spectrophotometer (Biotek). We tested for ranavirus using 75 ng of sample DNA in quantitative PCR targeting a 70 base-pair region of the MCP gene that detects FV3 and FV3-like ranaviruses, following Gray et al. (2012). We used qPCR rather than conventional PCR because it enables real-time quantification of target DNA with higher sensitivity and accuracy. Reactions were tested on a StepOne Real-Time PCR machine (Applied Biosystems, Foster City, California, USA) using water as a negative control and ranavirus gBlocks gene fragments (Integrated DNA Technologies, Inc., Coralville, Iowa, USA; Standish et al. 2018) in serial dilutions of 10^0 to 10^5 as positive controls. We initially processed all samples twice and ran any sample that resulted in any numeric CT-value two more times. Because of our low detection rates, we also extracted DNA and ran qPCR on samples of liver and kidney tissue from four FV3-infected Wood Frogs (*Lithobates sylvaticus*) from another laboratory to verify that reagents and procedures were working properly, to reduce uncertainty in laboratory results.

TABLE 1. Snake species sampled for ranavirus during a 2021–2022 survey focused on artificial cover objects in wooded regions on a college campus in central Virginia, USA. Two snakes (*Storeria dekayi* and *Heterodon platirhinos*) were captured elsewhere on campus and are not counted in either network. "Neg" means negative and "Pos" means positive for trace amounts of ranavirus DNA; see text for details. Detection rate is only calculated for *Carphophis amoenus* and all species combined, because sample sizes are too small in other species.

	Observatory			Wilson Trail			All locations combined			
	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total	Detection rate
Carphophis amoenus	0	26	26	6	37	43	6	63	69	8.7%
Diadophis punctatus	0	1	1	0	5	5	0	6	6	–
Coluber constrictor	0	1	1	0	1	1	0	2	2	–
Heterodon platirhinos	0	0	0	0	0	0	0	1	1	–
Opheodrys aestivus	0	0	0	0	1	1	0	1	1	–
Pantherophis alleghaniensis	0	1	1	0	0	0	0	1	1	–
Storeria dekayi	0	0	0	0	0	0	0	1	1	–
All species combined	0	29	29	6	44	50	6	75	81	7.4%

We captured and tested tissues from 69 Eastern Worm Snakes (*Carphophis amoenus*; N = 22 in 2021; N = 47 in 2022), six Ring-necked Snakes (*Diadophis punctatus*), two Eastern Racers (*Coluber constrictor*), one Eastern Hognose Snake (*Heterodon platirhinos*), one Rough Green Snake (*Opheodrys aestivus*), one Eastern Rat Snake (*Pantherophis alleghaniensis*), and one Dekay's Brown Snake (*Storeria dekayi*). In total, using mostly ACO checks and our few opportunistic captures, we captured 81 snakes (N = 26 in 2021; N = 55 in 2022; Table 1), however the Eastern Worm Snake was the only species with an adequate sample size to calculate a detection rate for ranavirus. A minimum of thirty individuals per taxon per site is advised for detecting low levels of pathogens in small populations (Gray et al. 2017). For example, if an estimated total population size is 50 individuals, then 35 individuals would be required to detect ranavirus if it occurs at 5% prevalence, and all 50 would have to be sampled to detect a prevalence of 2% in the population (Gray et al. 2017).

We detected ranavirus in 8.7% of Eastern Worm Snakes (6 individuals; 95% CI = 4.1–17.7%, N = 69) and 7.4% of all snakes (95% CI = 3.4–15.2%, N = 81; Table 1). Tissue samples from six snakes yielded CT-values consistent with 1 copy/µL of ranavirus DNA on at least two of four qPCR tests. For comparison, tissue samples from the four ranavirus-infected Wood Frogs from an unrelated study (used for additional positive controls) yielded CT-values corresponding to concentrations of 1000-10,000 copies/µL of ranavirus DNA (same primers and protocol detecting FV3 and FV3-like ranaviruses).

Ranavirus was only detected in Eastern Worm Snakes in 2022, and only in the Wilson Trail survey site. Interestingly, this ACO network was adjacent to Chalgrove Pond, in which ranavirus was detected in a parallel study of turtles in 2022 but not 2021 (Goodman et al. 2023). While there were significant differences in sample sizes between years (snakes: N = 26 in 2021; N = 55 in 2022; turtles: N = 99 in 2021; N = 150 in 2022), the sample size for turtles in 2021 was robust and the lack of ranavirus in both taxa probably reflects an absence or senescence of ranavirus in the local ecosystem in 2021. The trace amounts (1 copy/µL of ranavirus DNA) and low detection rate of ranavirus in the current study mirror the trace amounts and low detection rate of ranavirus in the parallel turtle study (0.0% in Eastern Painted Turtles, *Chrysemys picta picta* and 2.8% in Eastern Musk Turtles, *Sternotherus odoratus*, for both years combined; Goodman et al. 2023). These low levels of ranavirus stand in contrast to previous surveys on the College campus in 2010 and 2013-2014, when samples yielded lower CT-values representing higher viral loads and prevalences were much higher (23.8% in Eastern Painted Turtles, 20.0% in Eastern Box Turtles, *Terrapene carolina carolina*; 36.1% in Eastern Fence Lizards, *Sceloporus undulatus*; Goodman et al. 2013; 2018).

This study represents the first report of ranavirus DNA in Eastern Worm Snakes and supports more-frequent surveillance for ranavirus in snakes. As with lizards, there are now only two published surveys of ranavirus in free-living snakes, and both have found ranavirus prevalences similar to those in turtles in nearby areas (Ariel et al. 2017). No animals in the historic studies or current study displayed any signs of the disease ranavirosis, and we have not received any reports of herpetofaunal die-offs in these areas which are frequented by students, faculty, staff, and visitors (RMG, pers. observ.). However, a lack of observed morbidity and mortality does not mean there is an absence thereof, especially for snakes, which are more difficult to detect and observe. Also, snakes may serve as reservoirs for ranavirus that impacts other, potentially more susceptible species in the ecosystem. The type of ranavirus detected in both snakes here and in turtles and lizards from earlier work (Goodman et al. 2013, 2018, 2023) warrants further analyses. We encourage more research on ranavirus in squamates generally and emphasize the need for large sample sizes and repeated sampling to detect ranavirus that may occur at low prevalence, noting that prevalence varied dramatically between years in our study.

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