

## Detection of Ranavirus in Eastern Fence Lizards and Eastern Box Turtles in Central Virginia

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**Abstract** - Ranaviruses are a group of emerging infectious pathogens that threaten reptiles around the world; however, their geographic and taxonomic distribution in wild reptiles is understudied relative to amphibians. We sampled tissues from 2 reptile species: *Terrapene carolina carolina* (Eastern Box Turtle) and *Sceloporus undulatus* (Eastern Fence Lizard) in central Virginia to determine if they carried these pathogens. We found moderate prevalence of a ranavirus in these 2 species (36.1% and 20.0%, respectively). This study supplements the existing survey information for Eastern Box Turtles, which are known to carry and suffer mortality from ranaviruses. We also report on the first documentation of ranaviruses in the family Phrynosomatidae, and the first systematic screening for ranavirus in a wild, terrestrial squamate population.

### Introduction

Ranaviruses (family Iridoviridae, genus *Ranavirus*) are emerging infectious diseases that have gained attention in the last 2 decades with increasing reports of associated infections and mass-mortality events in reptiles, amphibians, and fishes (Gray and Chinchar 2015). The impact of ranaviruses on wild reptilian population dynamics is unknown, although several cases of morbidity and mortality in captive and natural populations have been attributed to these pathogens (de Voe et al. 2004, Hyatt et al. 2002, Marschang et al. 1999). Surveillance for ranaviruses in natural reptile populations has been mostly limited to chelonians, with at least 10 events of mass infection and die-offs documented in *Terrapene carolina carolina* (L.) (Eastern Box Turtle) (Allender 2012, de Voe et al. 2004, Farnsworth and Seigel 2013, Johnson et al. 2008). Disease surveillance of Eastern Box Turtles is important because this species is listed as vulnerable by the International Union for the Conservation of Nature due to population declines throughout its range caused by habitat destruction and fragmentation, road mortality, pollution, and pathogens (van Dijk 2016). The distribution of ranavirus in wild reptiles other than turtles, however, is largely unknown despite evidence that these pathogens can cause disease in several species of squamates (Duffus et al. 2015, Goodman 2013, Stöhr et al. 2013).

In a study of ranavirus in central Virginia reptiles, Goodman et al. (2013) detected ranavirus DNA in populations of *Chrysemys picta picta* (Schneider) (Eastern Painted Turtle) that displayed no signs of disease. In the current study, we

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extended the survey of ranavirus to include Eastern Box Turtles and *Sceloporus undulatus* (Bosc & Daudin) (Eastern Fence Lizard) in the same region. Our survey in Eastern Box Turtles contributes to knowledge of the geographic distribution of ranavirus in a terrestrial chelonian species that makes frequent use of aquatic habitats. Ours is the first survey of ranavirus in a wild population of Eastern Fence Lizards in the US.

### Methods

We sampled animals from 5 sites in and bordering the campus of Hampden-Sydney College in Prince Edward County in central Virginia (site 1: 37°14'17"N, 78°28'15" W; site 2: 37°14'30"N, 078°27'51"W; site 3: 37°14'45"N, 078°28'05"W; site 4: 37°15'04"N, 078°27'55"W; site 5: 37°14'44"N, 078°27'12"W; Fig. 1). All sites were located within 2 km of each other. We captured most animals in mixed *Pinus* (pine) and hardwood forests that contain multiple streams and small ponds. During the period 5 June–9 July 2013, we collected 35 Eastern Fence Lizards by noosing and hand-catching. During the periods 4 June–17 July 2013 and 21 May–27 July 2014, we captured 26 Eastern Box Turtles by hand. We caught 2 additional turtles opportunistically from 6 to 7 October 2013. Demographic summaries for these animals are available in Goodman and Carter (2017). When collecting and handling animals, researchers wore disposable nitrile gloves that were changed between handling individuals. We permanently marked each animal for identification upon recapture and to prevent repeat sampling of ranavirus from individuals. We marked Turtles with unique combinations of scute notches and lizards via toe

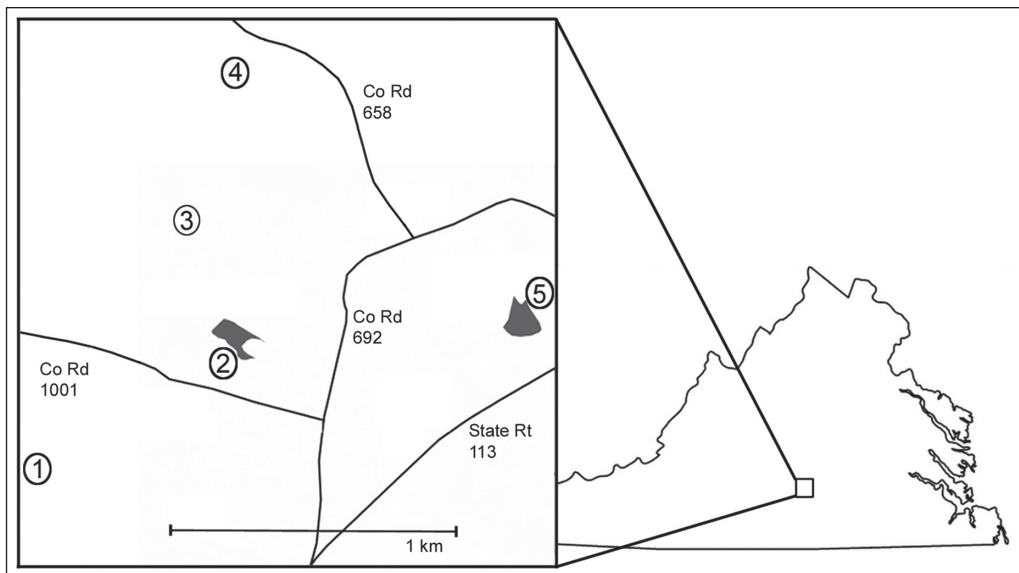


Figure 1. Locations of ranavirus survey sites for Eastern Fence Lizards (*Sceloporus undulatus*) and Eastern Box Turtles (*Terrapene carolina carolina*) in central Virginia. Sites are labeled with numbers within circles, and corresponding GPS locations are included in the text. The dark shaded areas are ponds.

clipping. We released all animals at their exact capture location within 24 h of capture, except 1 turtle that was sick and unresponsive when captured and died within 24 h (details below).

Tissues are more effective than oral–cloacal swabs for detecting ranavirus and we sought non-lethal sampling; thus, we removed a 5-mm distal portion of tail tip from each individual using a sterile, disposable scalpel blade (Goodman et al. 2013, Gray et al. 2012). We preserved tissue samples by freezing at  $-80^{\circ}\text{C}$  until processing. We disinfected all non-disposable materials using a Nolvasan solution (2% chlorhexidine diacetate, diluted 1:100 with water). We used Qiagen DNeasy Blood and Tissue Kits (Qiagen, Venlo, Netherlands) for DNA extraction and standardized the amount of genomic DNA for all individuals using an Epoch spectrophotometer (Biotek, Winooski, VT). We tested for presence of ranavirus DNA using quantitative polymerase chain reaction (qPCR) targeting a 70-bp region of the MCP gene, following the protocols of Gray et al. (2012) and Picco et al. (2007). Each 25- $\mu\text{L}$  PCR reaction contained the following: a 7- $\mu\text{L}$  volume of combined nuclease-free water and genomic DNA (volume-specific to each individual for 50 ng DNA); 12.5  $\mu\text{L}$  of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA); 1.5  $\mu\text{L}$  each of 10- $\mu\text{M}$  primers F 5'-ACA CCA CCG CCC AAA AGT AC -3' and R 5'- CCG TTC ATG ATG CGG ATA ATG -3'; and 2.5  $\mu\text{L}$  of 2.5- $\mu\text{M}$  probe 5'- /56-FAM/CCT CAT CGT /ZEN/TCT GGC CAT CAA CCA /3IABkFQ/ -3' (Integrated DNA Technologies, Coralville, IA). We employed an Applied Biosystems StepOne Real-time PCR machine with both negative and positive controls (pure water and DNA extracted from cultured FV3 ranavirus) to test all samples in duplicate. We considered as positive for ranavirus all samples with CT values  $< 30$  for both runs, based on standards established for this machine using known negative and positive controls from water, cultured ranavirus, and experimentally infected and uninfected reptiles.

## Results

Thirteen out of 36 Eastern Fence Lizards tested positive for the presence of ranavirus DNA (prevalence = 36.1%, 95% CI: 22.5–52.4%). Six out of 30 Eastern Box Turtles tested positive for presence of ranavirus DNA (prevalence = 20.0%, 95% CI: 9.5–37.3%).

## Discussion

We present the first estimate of ranavirus prevalence in a wild population of lizards and the first report of ranavirus occurrence in the squamate family Phrynosomatidae. Ranavirus infection has been previously reported in 2 families of snakes—Pythonidae: *Morelia viridis* (Schlegel) (Green Tree Python; Hyatt et al. 2002) and *Python brongersmai* Stull (Brongersma's Short-tailed Python; Stöhr et al. 2015), and Viperidae: *Bothrops moojeni* Hoge (Brazilian Lancehead; Johnsrude et al. 1997). Ranavirus has also been detected in 6 families of lizards—Agamidae: *Pogona vitticeps* Ahl (Central Beared Dragon; Stöhr et al. 2013) and *Japalura*

*splendida* Barbour & Dunn (Japalura Tree Dragon; Behncke et al. 2013, Stöhr et al. 2013); Anguillidae: *Dopasia gracilis* (Gray) (Asian Glass Lizard) Stöhr et al. 2013); Dactyloidae: *Anolis sagrei* Duméril and Bibron (Brown Anole) and *A. carolinensis* Voight (Carolina Anole; Stöhr et al. 2013); Gekkonidae: *Uroplatus fimbriatus* (Schneider) (Common Flat-tail Gecko; Marschang et al. 2005); Iberolacerta: *Lacerta agiles* L. (Sand Lizard; Marschang et al. 2013); *Iberolacerta monticola* (Boulenger) (Iberian Mountain Lizard; Alves de Matos et al. 2011); and Iguanidae: *Iguana iguana* L. (Green Iguana; Stöhr et al. 2013). However, most of these studies documented sick, captive animals brought in to a medical facility for treatment or animals shipped in the husbandry trade, which may experience high levels of stress and contact rates that enhance disease transmission and susceptibility. An exception is a report of ranavirus in 1 wild-caught, asymptomatic Iberian Mountain Lizard in Portugal (Alves de Matos et al. 2011). One study found ranavirus DNA in esophageal tissue of a *Natrix maura* (L.) (Viperine Watersnake); however, this animal was found dead after ingesting ranavirus-infected amphibians, so the ranavirus DNA may have come from either the host or its prey, or both (Price et al. 2014).

No studies have been published that estimate ranavirus prevalence in any free-ranging population of squamates. Although we are unable to compare the prevalence in our population of Eastern Fence Lizards (36.1%) to other wild squamates, it is noteworthy that this rate is higher than that found in 2 species of turtles at our study site and several populations of turtles sampled elsewhere (reviewed below). Squamates may be under-sampled with respect to ranavirus, as compared to other reptiles, especially in the case of terrestrial and arboreal species that spend little to no time in water. While the link between frogs, fish, and turtles in ranavirus community dynamics is more obvious due to time spent in shared aquatic habitat, where contact with water, ingestion of water, and consumption of infected prey allow for transmission of virions, terrestrial species may play a larger role than previously thought. Kimble et al. (2015) recently suggested that mosquitoes may serve as a potential source of transmission, based on presence of ranavirus DNA in 2 species of mosquitoes autochthonous with, and 1 individual feeding on, ranavirus-infected Eastern Box Turtles.

Prevalence of 20.0% in Eastern Box Turtles collected for this study is comparable to a previous study by Goodman et al. (2013) from the same study site, wherein Eastern Painted Turtles had ranavirus prevalence of 17.5% (11 of 63 turtles collected in 3 ponds) based on tail tissue samples taken in late May–June of 2010. None of a subset of those same individuals (50 of 63) tested positive for ranavirus based on oral–cloacal swabs and a different assay (500-base pair MCP gene and conventional PCR as in Mao et al. [1996, 1997]). Goodman et al. (2013) suggested low confidence in the 0% prevalence detected among 43 *Sternotherus odoratus* (Latreille in Sonnini & Latreille) (Eastern Mud Turtle) at the same site, from which only oral–cloacal swabs were taken because the tail tips of that species are cornified. However, the possibility of false positives in tail-tip samples cannot be excluded because that tissue may be subject to environmental contamination. Prevalence of 36.1%, 20.0%, and 17.0% for Eastern Fence Lizards, Eastern Box

Turtles, and Eastern Painted Turtles, respectively suggest that this study site had a continued presence of ranavirus in reptiles from 2010 to 2014. In contrast, Allender et al. (2009) found no ranavirus DNA in 47 Eastern Painted Turtles and 58 *Emydoidea blandingii* (Holbrook) (Blanding's Turtle) in Illinois, based on oral swabs and blood sampling.

For Eastern Box Turtles specifically, several epizootic events have been reported in the literature; however, most of these studies did not sample an entire population to determine prevalence of ranavirus (reviewed in Duffus et al. 2015). In a captive population experiencing a ranavirus outbreak concurrent with *Mycoplasma* and Herpesvirus infection, ranavirus prevalence was 86% based on organ tissue collected at necropsy, although only 77% prevalence was detected using cloacal swabs (Sim et al. 2016). We know of only 3 studies that have surveyed ranavirus in free-ranging populations of Eastern Box Turtles with no indication of previous or current infection. In a free-ranging population of Eastern Box Turtles in suburban wetland habitat of middle Tennessee, ranavirus prevalence was only 1% (1 of 102 turtles) based on PCR assay of blood samples (Vannatta 2015). Only 3% of Eastern Box Turtles (4 of 132) tested positive for ranavirus DNA in blood samples in a wild population occurring in and around 3 semi-ephemeral ponds in south-central Indiana (Currylow et al. 2014). Prevalence of ranavirus DNA in blood samples was 3.4%, 0.0%, and 2.7%, respectively, for Eastern Box Turtles brought into wildlife rehabilitation centers in Tennessee, Virginia, and North Carolina (29, 34, 36 turtles sampled, respectively; Allender et al. 2011). Although these individuals do not represent random samples of populations near these centers, prevalence of ranavirus is typically overestimated by sampling sick and injured turtles presented for medical care (Allender 2012). Allender et al. (2011) also sampled 1 free-ranging population of Eastern Box Turtles in Oak Ridge, TN and found no ranavirus (0% prevalence) in blood samples from 39 turtles.

Despite the occurrence of ranavirus among several reptile species at our study site, we never observed die-offs during herpetofaunal sampling and research projects conducted in 2010–2015 (summary of efforts is detailed in Goodman and Carter 2017). We only observed 1 moribund reptile in our studies, which exhibited lethargy, blepharitis, and sinusitis when found on the side of the Wilson Trail on 13 June 2013. This male Eastern Box Turtle died in the lab within 24 h of capture. We obtained a tail-tissue sample for ranavirus testing and sent the body overnight on ice to the University of Tennessee Center for Wildlife Health (Knoxville, TN) for an extensive necropsy, molecular testing, and histopathological analysis to determine the cause of death. The suggested cause of death was respiratory compromise due to severe mycoplasma pneumonia, with a positive qPCR result for mycoplasma and a negative qPCR result for ranavirus (based on liver, kidney, and intestinal tissue samples). Our in-house qPCR test of tail tissue for this individual also tested negative for ranavirus.

Ranavirus is an emerging wildlife disease that merits further surveillance in reptiles, amphibians, and fishes, in addition to experimental studies of susceptibility, transmission dynamics, and treatment that may inform management. Ranaviruses

can cause rapid die-offs and persist sub-lethally in populations; thus, we should continue to try to understand the distribution and prevalence of these viruses in their diverse host species. In particular, we encourage enhanced taxonomic and geographic surveys of ranaviruses, since we have now documented the presence of ranaviruses in the family Phrynosomatidae, and more generally in a terrestrial squamate population.

### Acknowledgments

We are grateful to Hampden-Sydney College and the Honors Program for providing funding and support for this research. We thank Debra Miller at the University of Tennessee Center for Wildlife Health for her assistance in investigating the cause of death in 1 turtle. All work in this study was approved by the Hampden-Sydney College Animal Care and Use Committee and performed under scientific collection permit 044820 from the Virginia Department of Game and Inland Fisheries.

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