

## Prevalence of Ranavirus in Virginia Turtles as Detected by Tail-Clip Sampling versus Oral-Cloacal Swabbing

Rachel M. Goodman<sup>1,\*</sup>, Debra L. Miller<sup>2,3</sup>, and Yonathan T. Ararso<sup>1</sup>

**Abstract** - Ranaviruses are emerging infectious diseases that infect amphibians, fish, and reptiles. Several cases of morbidity and mortality in captive and natural populations of reptiles have been attributed to ranaviruses, but research in this taxon has been limited. We used oral-cloacal swabs and tail clips to survey two species, *Chrysemys picta picta* (Eastern Painted Turtles) and *Sternotherus odoratus* (Common Musk Turtles), in three water bodies in central Virginia to determine if ranaviruses were present. Prevalence of ranavirus in *C. p. picta* ranged from 4.8–31.6% at the three sites. Ranavirus was not detected in *S. odoratus*, but only oral-cloacal swabs were used in this species because of the cornified tail tip. While tail-tip tissues from all three study sites indicated presence of ranavirus in *C. p. picta*, no oral-cloacal swabs from these same turtles tested positive. We therefore suggest that oral-cloacal swabbing may yield false negatives when ranavirus is present in turtles, and that tissue sampling may be more appropriate for monitoring ranavirus in turtles.

### Introduction

Biodiversity is declining worldwide, and many biologists believe we are witnessing the sixth mass extinction in the history of life (Barnosky et al. 2011, Wake and Vredenburg 2008). Nearly half of all amphibian populations are in decline (IUCN et al. 2008), and reptiles may face similar levels of endangerment (Gibbons et al. 2000, IUCN 2010, Reading et al. 2010). Many factors have contributed to population declines and extirpations: habitat destruction and degradation, pollution, global climate change, introduction of non-native species, and emerging infectious diseases (Wells 2007, Wilcove et al. 1998). Globally, two thirds of freshwater turtle and tortoise species are considered threatened or endangered (IUCN 2010), and infectious diseases may be a contributing factor (Ernst and Lovich 2009). Emerging infectious diseases contribute to population declines, and ranaviruses (family Iridoviridae; genus Ranavirus) are of concern because they infect multiple taxa, including fish, reptiles, and amphibians (Chinchar 2002). Currently we have limited research on the susceptibility of this wide range of potential hosts and the potential for transfer among species.

Ranaviruses are double-stranded DNA viruses that infect reptiles, amphibians, and fish and have caused mortality events in each taxon (reviewed in Chinchar 2002). The importance of ranaviruses in amphibian population

<sup>1</sup>Biology Department, Box 74, Hampden-Sydney College, Hampden-Sydney, VA 23943.

<sup>2</sup>Veterinary Diagnostic and Investigational Laboratory, University of Georgia, Tifton, GA

31793. <sup>3</sup>Current address - University of Tennessee, Center for Wildlife Health, Knoxville, TN 37996. \*Corresponding author - rgoodman@hsc.edu.

declines has only recently been recognized, although they have caused more die-offs in North America than the more-studied fungal pathogen *Batrachochytrium dendrobatidis* (Daszak et al. 1999, Duffus 2009, Gray et al. 2009). Among fish, iridovirus infections have been reported on several continents and can cause economic damage in commercial freshwater fisheries (Ahne et al. 1997, Whittington et al. 2010). The importance of ranaviruses for reptilian population dynamics is unknown, but several cases of morbidity and mortality in captive and natural populations have been attributed to the pathogen (De Voe et al. 2004, Hyatt et al. 2002, Marschang et al. 2011). Research thus far has been limited to description and isolation of viruses from infections in captive and wild species (Chen et al. 1999, De Voe et al. 2004, Johnson et al. 2008, Marschang et al. 1999, Westhouse et al. 1996), and clinical challenges of two North American species, *Terrapene ornata ornata* Agassiz (Ornate Box Turtle) and *Trachemys scripta elegans* Weid-Neuwied (Red-eared Slider), and two Australian species, *Emydura krefftii* Gray (Kreffft's River Turtle) and *Eiseya latisternum* Gray (Saw-shelled Turtle) (Ariel 1997, Johnson et al. 2007). Signs of ranavirus infection in turtles reported in these studies include lethargy, respiratory distress, anorexia, cutaneous erythema, ocular and nasal discharge, and oral ulceration and plaques. Surveillance of ranavirus in reptile populations is important to determine whether associated disease threatens persistence, and whether sub-lethally infected reptiles may serve as reservoirs for the pathogen that threatens co-occurring species. Also, this work in reptiles is necessary to gain an understanding of the complete epidemiology, including interspecific transmission, of ranaviruses. In the current study, we used and compared oral-cloacal swabbing and tissue sampling for ranavirus surveillance in two species of turtles, *Chrysemys picta picta* Schneider (Eastern Painted Turtles) and *Sternotherus odoratus* Latreille (Common Musk Turtles), in three water bodies in Virginia.

### Field Site Description

The study was conducted at three sites in Prince Edward County, VA: Briery Creek Lake in Briery Creek Wildlife Management Area (north end; 37°12.0'N, 78°27.0'W), and two ponds on the campus of Hampden-Sydney College (HSC), Chalgrove (37°14.5'N, 78°27.8'W) and Tadpole Hole (37°14.7'N, 78°27.2'W). Chalgrove and Tadpole Hole are both approximately 1 ha and located 0.8 km apart. Briery Creek Lake is a 342-ha lake managed by the Virginia Department of Game and Inland Fisheries and is located 4.5 km south of the HSC ponds (Fig. 1).

### Methods

Turtles were collected during 24 May–1 July 2010. We changed trapping sites every week, and trapped at each site twice, with 6–10 visits per site. Traps were set 1–2 m from shore and included four Promar collapsible crab/fish traps with dual-ring entrance, a Sundeck turtle trap with a bait tower (Item #840876, Heinsohn's Country Store, <http://www.texastastes.com/outdoors>).

htm), and a floating turtle tunnel (Item#840460, Heinsohn's Country Store). Because all turtle traps could capture more than one turtle at a time, there was a small risk that pathogen transmission could occur among individuals within the traps.

Upon removal from traps, turtles were weighed, measured for mass and length, and individually marked using combinations of notches filed into scutes. We used and compared two methods of sampling for ranavirus, oral-cloacal swabbing and tail clips, for use in the polymerase chain reaction (PCR). We swabbed turtles with plastic, sterile, cotton-tipped applicators (Puritan model 25-806 2PC), first rolling it inside the mouth and then inside the cloaca for 3–5 seconds each. The distal-most 0.5 cm of the tip of the tail was collected only from species not possessing cornified tail tips (i.e., *C. p. picta*) using a new, sterile scalpel blade for each animal. Both tissue samples and swabs were stored in 1-ml vials containing 70% ethanol. Turtles were released at the site of capture immediately after sampling.

A total of 106 turtles, including *C. p. picta* ( $n = 63$ ) and *S. odoratus* ( $n = 43$ ), were captured, and all turtles appeared clinically normal. *Chrysemys picta picta*

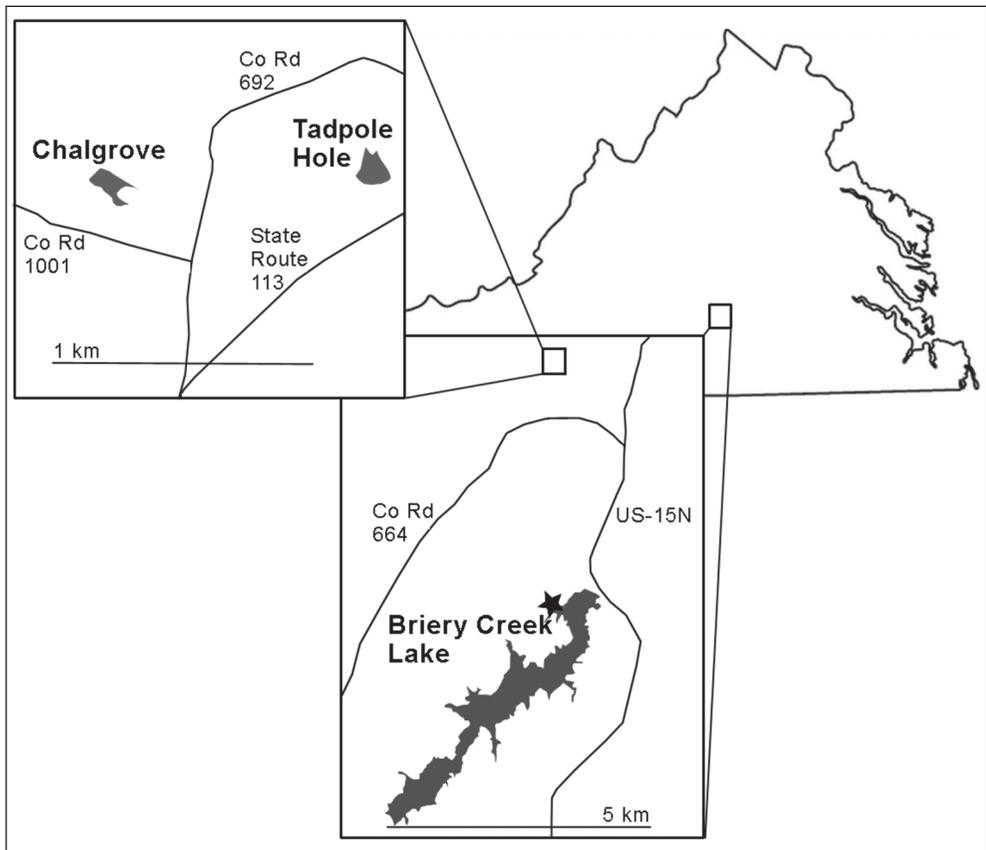


Figure 1. Map of three water bodies in central Virginia where turtles were sampled for ranavirus: Chalgrove, Tadpole Hole, and Briery Creek Lake. The star indicates the area at Briery Creek Lake where turtle trapping was conducted (across most of shoreline at other sites). GPS coordinates are given in the Methods section.

were collected at all sites, whereas *S. odoratus* were only collected from Briery and Chalgrove (Table 1). Among the samples collected, only those from species and sites with sample sizes of approximately 20 were tested. All traps, rubber boots and waders, and other gear were scrubbed, soaked in a 1% chlorhexidine diacetate (Fort Dodge Nolvasan Solution) for at least one minute, and rinsed in water between use at different water bodies.

Genomic DNA was extracted from the tissues or swabs using a commercially available kit (DNeasy Blood and Tissue Kit, Qiagen, Inc., Valencia, CA). Negative and positive extraction controls were included. Conventional PCR was performed using the protocol and primer sets (MCP4 and MCP5) found in Mao et al. (1996, 1997) and targeting an approximately 500-base pair sequence of the major capsid protein (MCP) gene. The PCR products were resolved via electrophoresis on a 1.0% agarose gel. Controls for each PCR run included two negative controls (water and gDNA extracted from a ranavirus-negative tadpole) and two positive controls (cultured ranavirus and gDNA extracted from a ranavirus-positive tadpole). The PCR protocol was repeated once more on all samples to verify results.

## Results

Only oral-cloacal swabs were tested for *S. odoratus*, and none were positive for ranavirus (Table 1). While tail tips from all three study sites indicated presence of ranavirus among *C. p. picta*, none of the oral-cloacal swabs from these same turtles tested positive (Table 1). However, two of the eight ranavirus-positive individuals that tested positive for ranavirus via tissue samples did not have accompanying oral-cloacal swabs because they were too small for effective use of technique (i.e., juveniles). Based on tail-tissue sampling, prevalence of ranavirus in *C. p. picta* was 4.8% in Briery, 31.6% in Chalgrove, and 17.4% in Tadpole Hole.

## Discussion

We found evidence of ranavirus infection in *C. p. picta* in our three study sites using tail-tissue sampling; however, oral-cloacal swab sampling failed to detect the pathogen. These findings suggest that oral-cloacal swabbing may yield false negatives when ranavirus is present in turtles, and that tissue sampling may be more appropriate. Gray et al. (2012) conducted a controlled infection study with *Lithobates catesbeianus* Shaw (American Bullfrog) tadpoles and found

Table 1. Ranavirus infections in turtles from three water bodies in central Virginia.

| Water body   | <i>Chrysemys picta picta</i> |             |          |             | <i>Sternotherus odoratus</i> |             |
|--------------|------------------------------|-------------|----------|-------------|------------------------------|-------------|
|              | Tissues                      |             | Swabs    |             | Swabs                        |             |
|              | <i>n</i>                     | Ranavirus + | <i>n</i> | Ranavirus + | <i>n</i>                     | Ranavirus + |
| Briery       | 21                           | 1 (4.8%)    | 21       | 0 (0.0%)    | 21                           | 0 (0.0%)    |
| Chalgrove    | 19                           | 6 (31.6%)   | 8        | 0 (0.0%)    | 22                           | 0 (0.0%)    |
| Tadpole Hole | 23                           | 4 (17.4%)   | 21       | 0 (0.0%)    | -                            | -           |

false-negative and false-positive rates of 20% and 6% for tail samples, and 22% and 12% for swabs, respectively, using liver samples as the standard for virus infection. Those results suggest a similar rate of false negatives for tail and swab samples in an amphibian, whereas our field surveillance study suggests a difference between the methods in a reptile. Further comparisons in additional species may be warranted.

Necropsy and histology provide the most certain evidence for ranaviral disease (Miller and Gray 2010); however, lethal sampling is not desirable in the absence of morbidity or mortality events. Oral-cloacal swabbing is the least invasive method of sampling, but the current study indicates the sensitivity of this testing method may be low. While not compared to testing internal organs, tail-tip sampling appears to be more sensitive than oral-cloacal swabbing and was able to detect ranavirus in *C. p. picta* using moderate sample sizes of around twenty individuals. Future research may investigate other potential areas for superficial tissue sampling on catch-and-release specimens, particularly for species with a cornified or boney tail tip that is used in courtship and copulation (Ernst and Lovich 2009). In such species, we recommend an approximately 5-mm diameter skin (epidermal and dermal) biopsy taken from the mid-dorsal tail.

Compared to common rates for amphibians, prevalence of ranavirus infection in turtles was low in the three water bodies sampled. Research in amphibians indicates that prevalence can vary widely, depending on the species and date of sampling. Using tissue collected from all major organs, Gray et al. (2007) found ranavirus prevalence of 15–57% in tadpoles in undisturbed and cattle accessed ponds, depending on the species and sampling period. Using tail tips and liver samples, Brunner et al. (2004) found prevalence of 46–100% in dispersing metamorph salamanders following an epidemic, but only 7% prevalence in adults returning to ponds in the following spring. A recent survey of injured/rehab and free-ranging *Terrapene carolina carolina* L. (Eastern Box Turtle) found prevalence of ca. 3% from blood samples collected from injured/sick turtles submitted to rehab centers/medical facilities in the southeastern US (Allender et al. 2011). This same study was able to detect ranavirus in swab samples collected from injured/sick *T. c. carolina*, a species that spends large amounts of time on dry land, submitted to the medical facility in Tennessee. Our study differs from Allender et al. (2011) in that we surveyed a heavily aquatic species and, if it holds true that water is an excellent medium for ranavirus (Chinchar 2002), one would expect greater prevalence in turtles spending more time in water. However, a recent survey of free-ranging *C. picta* and *Emydoidea blandingii* Holbrook (Blanding's Turtle) in Illinois found 0% prevalence for ranavirus using blood samples and oral swabs (Allender et al. 2009). Explanations for the low prevalence and lack of ranavirus in the two species we studied include possible resistance to infection or ability to clear infection in these species. Infection rates and ability to clear ranavirus vary among amphibian species exposed to standardized virus treatments, and also according to ranavirus isolate (Hoverman et al. 2010, 2011). Thus far, this

comparative analysis of infection rates has not been investigated in turtles or any reptile. Given our findings, the marked declines of turtle populations, and the fact that many turtle species are syntopic with amphibians and fish (potential hosts of ranaviruses), further investigation, including controlled laboratory studies, is needed to determine the impact of ranaviruses on turtles.

### Acknowledgments

We thank Hampden-Sydney College, the Biology Department, and the Honors Program for providing funding and support for this research. All work in this study was approved by the Hampden-Sydney College Animal Care and Use Committee and performed under scientific collection permit 38354 from Virginia Department of Game and Inland Fisheries. We thank Briery Creek Wildlife Management for permitting us to work on the site, Lisa Whittington for assistance conducting laboratory tests at the University of Georgia, and Zach Harrelson, Allen Luck, Sam Smith, and Erica Rutherford for assistance in the field.

### Literature Cited

- Ahne, W., M. Bremont, R.P. Hedrick, A.D. Hyatt, and R.J. Whittington. 1997. Iridoviruses associated with epizootic haematopoietic necrosis (EHN) in aquaculture. *World Journal of Microbiology and Biotechnology* 13:367–373.
- Allender, M.C., M. Abd-Eldaim, A. Kuhns, and M. Kennedy. 2009. Absence of ranavirus and herpesvirus in a survey of two aquatic turtle species in Illinois. *Journal of Herpetological Medicine and Surgery* 19:16–20.
- Allender, M.C., M. Abd-Eldaim, J. Schumacher, D. McRuer, L.S. Christian, and M. Kennedy. 2011. PCR prevalence of ranavirus in free-ranging Eastern Box Turtles (*Terrapene carolina carolina*) at rehabilitation centers in three southeastern US states. *Journal of Wildlife Diseases* 47:759–764.
- Ariel, E. 1997. Pathology and serological aspects of Bohle Iridovirus infections in six selected water-related reptiles in north Queensland. Ph.D. Dissertation. James Cook University of North Queensland, Australia. 176 pp.
- Barnosky, A.D., N. Matzke, S. Tomiya, G.O.U. Wogan, O.U. Wogan, B. Swartz, T.B. Quental, C. Marshall, J.L. McGuire, E.L. Lindsey, K.C. Maguire, B. Mersey, and E.A. Ferrer. 2011. Has the Earth's sixth mass extinction already arrived? *Nature* 471:51–57.
- Brunner, J.L., D.M. Schock, E.W. Davidson, and J.P. Collins. 2004. Intraspecific reservoirs: Complex life history and the persistence of a lethal ranavirus. *Ecology* 85:560–566.
- Chen, Z.X., J.C. Zheng, and Y.L. Jiang. 1999. A new iridovirus isolated from soft-shelled turtle. *Virus Research* 63:147–151.
- Chinchar, V.G. 2002. Ranaviruses (family Iridoviridae): Emerging cold-blooded killers. *Archives of Virology* 147:447–470.
- Daszak, P.L., A.A. Berger, A.D. Cunningham, A.D. Hyatt, D.E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* 5:735–748.
- De Voe, R., K. Geissler, S. Elmore, D. Rotstein, G. Lewbart, and J. Guy. 2004. Ranavirus-associated morbidity and mortality in a group of captive Eastern Box Turtles (*Terrapene carolina carolina*). *Journal of Zoo and Wildlife Medicine* 35:534–543.

- Duffus, A. 2009. Chytrid blinders: What other disease risks to amphibians are we missing? *EcoHealth* 6:335–339.
- Ernst, C.H., and J. Lovich. 2009. *Turtles of the United States and Canada*. 2nd Edition. Johns Hopkins University Press, Baltimore, MD. 840 pp.
- Gibbons, J.W., D.E. Scott, T.J. Ryan, K.A. Buhmann, T.D. Tuberville, B.S. Metts, J.L. Greene, T. Mills, Y. Leiden, S. Poppy, and C.T. Winne. 2000. The global decline of reptiles, déjà vu amphibians. *BioScience* 50:653–666.
- Gray, M.J., D.L. Miller, A.C. Schmutzer, and C.A. Baldwin. 2007. Frog virus 3 prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA. *Diseases of Aquatic Organisms* 77:97–103.
- Gray, M.J., D.L. Miller, and J.T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. *Diseases of Aquatic Organisms* 87:245–266.
- Gray, M.J., D.L. Miller, and J.T. Hoverman. 2012. Reliability of non-lethal surveillance methods for detecting ranavirus infection. *Diseases of Aquatic Organisms* 99:1–6.
- Hoverman J.T., M.J. Gray, and D. L. Miller. 2010. Anuran susceptibilities to ranaviruses: Role of species identity, exposure route, and a novel virus isolate. *Diseases of Aquatic Organisms* 89:97–107.
- Hoverman, J.T., M.J. Gray, N.A. Haislip, and D.L. Miller. 2011. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. *EcoHealth* 8:301–319.
- Hyatt, A., M. Williamson, B.E. Coupar, D. Middleton, S.G. Hengstberger, A.R. Gould, P. Selleck, T.G. Wise, J. Kattenbelt, A.A. Cunningham, and J. Lee. 2002. First identification of a ranavirus from Green Pythons (*Chondropython viridis*). *Journal of Wildlife Diseases* 38:239–252.
- International Union for Conservation of Nature (IUCN), Conservation International, and NatureServe. 2008. *Global Amphibian Assessment*. Available online at <http://www.globalamphibians.org>. Accessed 1 October 2011.
- IUCN. 2010. *IUCN Red list of threatened species, version 2010*. Available online at <http://www.iucnredlist.org>. Accessed 1 October 2011.
- Johnson, A.J., A.P. Pessier, and E.R. Jacobson. 2007. Experimental transmission and induction of ranaviral disease in Western Ornate Box Turtles (*Terrapene ornata ornata*) and Red-Eared Sliders (*Trachemys scripta elegans*). *Veterinary Pathology* 44:285–297.
- Johnson, A.J., A.P. Pessier, and J.F. Wellehan. 2008. Ranavirus infection of free-ranging and captive box turtles and tortoises in the United States. *Journal of Wildlife Diseases* 44:851–863.
- Mao J., T.N. Tham, G.A. Gentry, A. Aubertin, and V.G. Chinchar. 1996. Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. *Virology* 216:431–436.
- Mao, J., R.P. Hedrick, and V.G. Chinchar. 1997. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. *Virology* 229:212–220.
- Marschang, R.E., P. Becher, H. Posthaus, P. Wild, H.J. Thiel, U. Müller-Doblies, E.F. Kalet, and L.N. Bacciarini. 1999. Isolation and characterization of an iridovirus from Hermann's Tortoises (*Testudo hermanni*). *Archives of Virology* 144:1909–1922.
- Marschang, R.E., S. Braun, and P. Becher. 2011. Isolation of a ranavirus from a gecko (*Uroplatus fimbriatus*). *Journal of Zoo and Wildlife Medicine* 36:295–300.
- Miller, D.L., and M.J. Gray. 2010. Amphibian decline and mass mortality: The value of visualizing ranavirus in tissue sections. *The Veterinary Journal* 186:133–134.

- Reading, C.J., L.M. Luiselli, G.C. Akani, X. Bonnet, G. Amori, J.M. Ballouard, E. Filippi, G. Naulleau, D. Pearson, and L. Rugiero. 2010. Are snake populations in widespread decline? *Biology Letters* 6:777–780.
- Wake, D.B., and V.T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America* 105:11467–11473.
- Wells, K.D. 2007. *The Ecology and Behavior of Amphibians*. University of Chicago Press, Chicago, IL. 1400 pp.
- Westhouse, R., E.R. Jacobson, R.K. Harris, K.R. Winter, and B.L. Homer. 1996. Respiratory and pharyngo-esophageal iridovirus infection in a Gopher Tortoise (*Gopherus polyphemus*). *Journal of Wildlife Diseases* 32:682–686.
- Whittington, R.J., J.A. Becker, and M.M. Dennis. 2010. Iridovirus infections in finfish: Critical review with emphasis on ranaviruses. *Journal of Fish Diseases* 33:95–122.
- Wilcove, D.S., D. Rothstein, J. Dubow, A. Phillips, and E. Losos. 1998. Quantifying threats to imperiled species in the United States. *Bioscience* 48:607–615.