Transactions of the Illinois State Academy of Science
(2008), Volume 101, #1&2, pp. 87-94

received 9/13/07
accepted 2/17/08

West Nile Virus: A Serosurvey of Ranid Frogs Across Illinois

Bradley A. Danner and Christopher A. Phillips
Illinois Natural History Survey
Division of Biodiversity and Ecological Entomology
1816 Oak Street, Champaign, IL 61820, USA

ABSTRACT

West Nile virus (WNV) has been isolated in many amphibian feeding species of mosquitoes. Based on this evidence, this study sought to evaluate if amphibians and reptiles, in addition to birds and mammals, play a role in the transmission of West Nile virus (WNV). Bullfrogs, Leopard frogs, and Green frogs were collected from selected sites across the state of Illinois. Blood, and in some individuals tissue, was collected from all individuals (n=239) and tested for the presence of WNV antibodies using epitope-blocking enzyme-linked immunosorbent assay (ELISA). No WNV antibody positive frogs were found of the 239 tested. This suggests that frogs may not build up immunity to the virus. However, the results of this study do not address whether frogs die or become refractory from exposure to the virus, indicating that further research is needed to address the role amphibians play in the WNV transmission cycle.

INTRODUCTION

Past research has suggested that amphibians and reptiles may play a small role in the West Nile Virus (WNV) cycle (Kostyukov et al. 1986). A study conducted by Klenk et al. (2004) on farm raised American Alligators (*Alligator mississippiensis*) showed that after being injected with WNV, alligators exhibited viremia for up to 14 days before building antibodies. This study also suggests that predators may contract WNV by consuming prey that has been infected with the virus. American Alligators that consumed mice inoculated with the virus developed viremia 3-6 days after feeding. The alligators maintained viremia for 9-14 days (Klenk et al. 2004). Another study found that Lake Frogs (*Rana ridibunda*) could be a reservoir host for the virus (Hubalek and Halouzka 1999). Both of these studies suggest that not only can amphibians and reptiles contract the virus, but may also amplify and transmit it back to the mosquitoes that feed on them. Because frogs and mosquitoes are in close proximity to one another, it would be expected that they interact, thus frogs could play a significant role in the amplification and transmission of WNV. This study investigated wild populations of ranid frogs across the state of Illinois to determine their susceptibility of contracting WNV.

METHODOLOGY

Selection of Sites

Frogs were sampled in three regions (north, central, and south) throughout the state of Illinois (Fig. 1). Sites were selected to coincide with ongoing studies (Illinois Natural History Survey and the Medical Entomology Department of the University of Illinois Urbana-Champaign) of WNV in mosquitoes and birds. In the northern region, collection was done in the Cook County Forest Preserve District of Chicago. The Cook County area has been well known for its WNV-positive mosquito pools based on mosquito sampling performed by the various mosquito abatement districts in the area. Frogs were also sampled from the I&M Canal in Will County. Ongoing bird sampling has demonstrated high rates of WNV seroprevalence throughout much of the Chicago area (Beveroth et al. 2006.) Sampling sites in the central region were in Champaign (The Japanese Gardens and Aquatic Fisheries Ponds on the University of Illinois Urbana-Champaign campus), Piatt (Robert Allerton Park and Conference Center), and Effingham (Ballard Nature Center) counties. WNV positive mosquito pools and seropositive birds have been found in Champaign and Piatt counties (Beveroth et al. 2006.) Effingham County is not part of the ongoing bird sampling. In the southern region Horseshoe Lake, in Alexander County, was sampled in coordination with the ongoing bird study. Frogs were collected along the spillway located at the southwestern end of the lake.

Frog Collecting

Species of ranid frogs including Bullfrogs, Leopard frogs, and Green frogs were selected for this study due to their abundant populations and larger body size. Species of smaller frogs, such as Cricket frogs and Spring Peepers, were not included in this study because of their small size. At least 2 ml of blood was needed from each individual to test for WNV antibodies. Blood sampling techniques made collecting enough blood from smaller sized frogs difficult. Two species of *Bufo* adults were also sampled opportunistically.

Frog collecting began in the spring/summer of 2005 and continued into the summer of 2006. Collecting began in late March/early April and continued through early September. Frogs were captured with a dip-net, or by hand. Frogs were placed in plastic zip-lock bags with a small amount of water until returning to the lab (no more than 24 hours and as little as 2-3 hours in most cases). Upon returning to the lab frogs were placed together by species into holding tanks that were set up with appropriate

Figure 1: Locations of collecting sites for frogs checked for West Nile Virus, 2005 - 2006.

temperature and humidity until they could be processed. Often frogs were processed the day following capture.

Blood Serum Collection

Blood was collected from each frog under Animal Care and Use Protocol # 03183 approved by the University of Illinois Institutional Animal Care and Use Committee. Frogs were placed in a general anesthetic mixture of MS-222 and distilled water until the individual was unresponsive to moderate handling. This took 5-10 minutes for smaller individuals and up to 30 minutes for larger individuals.

Two different methods were used to collect blood. The preferred method was a cardiac puncture with an insulin syringe or an equivalent small gauge needle. The second method of blood extraction was through the anterior abdominal vein. In individuals where this method was used, the purpose was to withdraw blood with intent of releasing the individual once awake and fully mobile. This method was best performed on larger individuals, such as adult Bullfrogs, because the vein is easily seen through the skin on the ventral side.

Serum Testing

Blood serum analysis for WNV antibodies was completed using Epitope Blocking ELISA (Enzyme-Linked Immunosorbent Assay; Blitvich et al. 2003). Three flavivirus monoclonal antibodies (MAbs) for the detection of WNV antibodies were used. Two of the MAbs (2B2 and 6B6C-1) react with multiple flaviviruses including WNV. The third MAb (3.1112G) reacts more specifically to WNV. See Blitvich et al. (2003) for details on the MAbs used in this study.

Samples were considered positive if all three MAbs had a >30% inhibition. Positives were based on optical density output readings by a Multiskan mcc/340 from Thermal Laboratory Systems. The data output was then imported into an Excel spreadsheet and percent inhibition of WNV antibodies was calculated based on a standard optical density. See Blitvich et al. (2003) for details on ELISA methodology.

RESULTS

A total of 239 anuran blood samples were taken over the course of two summers. Of the 239 samples, there were two recaptures totaling 237 individual frogs and toads tested across the state of Illinois. In the northern region a total of 33 frogs were collected from four sites in Cook County and two sites in Will County. In the southern region a total of 67 frogs were collected from one site. In the central region a total of 139 frogs were collected from six sites in four counties (Champaign, Piatt, Scott, and Vermillion.) Scott County consisted of one random sample brought into the lab and was not part of the project's sampling schedule. Table 1 summarizes the number of captures per site.

As shown in Table 2, no individuals tested positive for WNV antibodies. For a sample to be considered positive for WNV antibody, all three MAbs needed to yield >30% inhibition. Some samples were positive for 2B2 indicating they could possibly have been infected with another flavivirus.

Table 1. Summary of frog sample totals from 2005 through 2006.

Region	County	Site	Species	N
North	Cook	Axehead Lake	*R. catesbeiana*	2
		Beck Lake	*R. catesbeiana*	5
		Big Bend Lake	*R. catesbeiana*	9
		Tuma Lake	*R. clamitans*	2
	Will	I&M Canal	*R. catesbeiana*	10
		Quarry Pond	*R. clamitans*	5
Central	Champaign	Cottonwoods	*B. americanus*	3
			R. catesbeiana	20
		Japanese Gardens	*B. americanus*	1
			R. catesbeiana	36
	Effingham	Ballard Nature Center	*R. catesbeiana*	9
	Piatt	Allerton Park	*B. fowleri*	5
			R. catesbeiana	56
	Scott	Random*	*R. clamitans*	1
	Vermillion	Larimores Cabin	*R. catesbeiana*	8
South	Alexander	Horseshoe Lake	*B. fowleri*	7
			R. blairi	1
			R. catesbeiana	7
			R. spenocephala	52
Total				239

* Sample was taken from a frog that was caught at a site that was not part of the regular sampling site.

Table 2. Sample portion of Blocking ELISA antibody test results. To be considered positive, the sample's optical density (OD) reading must be less than 0.300 (>30% inhibiton).

		Sample Information				Monoclonal Antibody OD			
Sample#	Species	Date Collected	County	Site	Serial Vial#	ELISA#	3.1112G	2B2	6B6C-1
83	*R. blairi*	3/31/2006	Scott	Random	rd-1	06-0495	0.399	0.343	0.643
84	*B. americanus*	4/13/2006	Champaign	Cottonwoods	cwp-11	06-0496	0.389	0.243	0.728
85	*B. americanus*	4/13/2006	Champaign	Cottonwoods	cwp-12	06-0497	0.530	0.304	0.755
86	*B. americanus*	4/13/2006	Champaign	Cottonwoods	cwp-13	06-0498	0.491	0.312	0.796
87	*R. catesbeiana*	4/13/2006	Champaign	Cottonwoods	cwp-14	06-0499	0.457	0.298	0.371
88	*R. catesbeiana*	4/13/2006	Champaign	Cottonwoods	cwp-15	06-0500	0.489	0.310	0.709
89	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	cwp-16	06-0501	0.490	0.339	0.744
90	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	cwp-17	06-0502	0.450	0.345	0.672
91	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	cwp-18	06-0503	0.401	0.370	0.676
92	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	cwp-19	06-0504	0.362	0.350	0.655
93	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	cwp-21	06-0505	0.577	0.364	0.928
94	*R. catesbeiana*	4/21/2006	Champaign	Cottonwoods	jh-17	06-506	0.530	0.326	0.845
95	*R. catesbeiana*	5/3/2006	Champaign	Japan House	jh-18	06-0519	0.380	0.332	0.765
96	*R. catesbeiana*	5/3/2006	Champaign	Japan House	jh-19	06-0520	0.397	0.312	0.697
97	*R. catesbeiana*	5/3/2006	Champaign	Japan House	jh-20	06-0521	0.414	0.330	0.710
98	*R. catesbeiana*	5/3/2006	Champaign	Japan House	cwp-22	06-0522	0.394	0.363	0.752
99	*R. catesbeiana*	5/3/2006	Champaign	Cottonwoods	cwp-23	06-0507	0.501	0.356	0.914
100	*R. catesbeiana*	5/3/2006	Champaign	Cottonwoods	cwp-14	06-0508	0.518	0.321	0.415

DISCUSSION

The lack of positive tests for WNV in frog serum and tissue is a precursor for further investigation. The USGS National Wildlife Health Center has been testing amphibians for WNV and has yet to find any viral isolation for WNV in frogs (Green 2006 unpublished data.) However, some amphibian feeding mosquitoes have been isolated with the virus (South Dakota Mosquitoes, CDC 2005.) One example is *Culex territans*. This mosquito is primarily an amphibian feeder and has been isolated with WNV. Why anurans have not been isolated with the virus is not yet known. One theory suggests that replication of WNV in reptiles and amphibians is poor (Klenk and Komar 2003.) The authors of this paper experimentally injected green iguanas, American bullfrogs, red-ear sliders, and Florida garter snakes subcutaneously with the virus. They tested individuals every three days for viremia. The maximum viremia detected was $10^{3.2}$ pfu/mL of serum, approximately 60-fold lower than necessary for transmission back to the vector host *Culex pipiens*. The authors stated that relatively low titers may be infectious for other species and that threshold viremias are not known for mosquitoes that feed on reptiles and amphibians. They also suggested that slow humoral response times in cold-blooded vertebrates may explain the low seroconversion rates. This study illustrates that the virus can be replicated at low levels in amphibians and reptiles.

Because frogs and other amphibians and reptiles exhibit a relatively low titer response to WNV, alternative methods of antibody detection may be useful in our investigation of the role, if any, that amphibians play in the WNV transmission cycle. One suggested method is the Plaque Reduction Neutralization Test (PRNT.) This method is sometimes used to confirm ELISA positive samples for various flaviviruses, including WNV (Prince and Hogrefe 2003). PRNT may be the solution to investigating the presence or absence of WNV in those species that exhibit relatively low titers, such as frogs.

A second theory suggests that frogs are not as prone to mosquito feedings as previously thought. Recent research on Australian frog species has demonstrated that chemicals secreted by frog skin may act as a natural mosquito repellent (Williams et al. 2006.) The study covered the tails of mice with a frog-skin formula and exposed them to mosquitoes. Mice receiving the control treatment were probed and bitten earlier and more often than the mice receiving the frog formula. This illustrates the possibility that frogs, though still susceptible to mosquito feedings, would be less likely to be fed upon by a WNV carrying mosquito due to the natural repellent in their chemical secretions. Future research investigating natural chemical repellents in frog species of North America would provide valuable information to more accurately understand if frogs play a role in WNV transmission.

There are three possible explanations for the negative findings of our study. First, frogs are not susceptible to the virus based on limited exposure to mosquitoes that carry WNV. Second, frogs may be contracting the virus but cannot mount a sufficient immune response and die, removing them from the potential sample. Lastly, WNV carrying mosquitoes may feed on frogs but the virus has no effect because of poor replication. The later scenario is based on the findings of Klenk and Komar 2003. Some tissue sampling to test for viremia was also done in this study as a step toward isolating live virus in indi-

vidual frogs. However, the window of opportunity for catching an individual in a viremic state is not very long, 1-4 days in birds (CDC 2003) and up to 14 days, perhaps longer, in *Alligator mississipiensis* (Klenk et al. 2004). These short viremic periods increase the difficulty of isolating the virus from tissue samples and there were no positive tissues found in this study. Periodic blood sampling from mark and re-capture may be a better way of monitoring WNV activity in wild populations of frogs. In this situation, testing the blood for antibody response over time may hold more information concerning how the virus affects wild frog populations than euthanizing them and testing their tissues for viremia.

Some WNV bird studies theorize that vertical stratification may play an important role in the location of feeding mosquitoes that carry the virus and thus in the virus's transmission cycle (Anderson et al. 2004). In an assessment of the prevalence of WNV in the canopy, these authors found that capture rates of the primary mosquito vector, *Culex pipiens,* of WNV was greater higher in the canopy than in traps placed closer to the ground. In addition, the authors found a greater number of WNV isolations higher in the canopy. They attribute the higher number of virus isolations to the greater number of mosquitoes found at the higher strata. This follows with the hypothesis that most birds roost in trees and while roosting they are most susceptible to being fed upon by mosquitoes. The theory that birds are contracting WNV at higher strata such as in the canopy of trees or bushes could explain why the ground dwelling frogs in this study do not appear to be exposed to WNV. One approach to answer this would be to sample arboreal frogs. Tree frogs, such as the Eastern Gray Treefrog (*Hyla versicolor*) and the Green Treefrog (*Hyla cinerea*), are generally found off the ground in small shrubs or trees where viremic feeding mosquitoes may occur. The Eastern Gray Treefrog can be found statewide in Illinois and the Green Treefrog can be found in the southern tip of the state. Tree frog blood and tissue could be tested using the same methods applied here to investigate their susceptibility, if any, to WNV. This could potentially be an important follow-up study to the results presented here.

This study may be a starting point to future research on WNV and its effects on amphibian and reptile populations. There is still much information we do not understand on the interactions, if any, between amphibians and reptiles and WNV. The findings of this research are meant to encourage others to build upon what we already know and to take the next step toward finding the relationship between these animals and the virus. The Cook County Forest Preserve has already taken the initiative to collect a wide variety of fauna for blood testing. Their collections included a wide number of turtle bloods that were examined using the same methods applied to the frog blood for WNV testing. As of now, there have been no positives identified for antibodies but none of these individuals have been tested for viremia. Shared habitat between amphibians, reptiles, and WNV vector mosquitoes suggests that these animals would have some susceptibility to contracting the virus. Further research with revised methods should help researchers understand what part amphibians and reptiles play in the transmission cycle of WNV.

ACKNOWLEDGEMENTS

This project would not have been possible with out the support of many people. Thanks to the Illinois Natural History Survey for providing the funding and supplies for the pro-

ject. Thanks to the Medical Entomology Lab and Robert J. Novak for providing laboratory equipment and testing of the serum samples. Thanks to Tara A. Danner (Illinois Natural History Survey) for running the laboratory samples and assisting in frog collection. Thanks to Christopher Anchor (the Forest Preserve District of Cook County), Nathan J. Beccue (Robert Allerton Park, University of Illinois), Steven R. Buck (Natural Areas Committee, University of Illinois), the Illinois Department of Natural Resources (Horseshoe Lake State Park), and the folks at Ballard Nature Center (Effingham County) for allowing access to sites for frog collecting. Thanks to Mark G. Alessi (University of Illinois Urbana-Champaign), John E. Petzing and Whitney J. Banning (Illinois Natural History Survey) for assisting with the collection of frogs.

LITERATURE CITED

Anderson, J. F. Andreadis, T. G. Main, A. J. and Kline, D. L. 2004. Prevalence of West Nile Virus in Tree Canopy–Inhabiting *Culex pipiens* and Associated Mosquitoes. The American Journal of Tropical Medicine and Hygiene. 71:112-119.

Beveroth, T. A. Ward, M. P. Lampman, R. L. Ringia, A. M. and Novak, R. J. 2006 Changes in Seroprevalence of West Nile Virus Across Illinois in Free-Ranging Birds from 2001 Through 2004. The American Journal of Tropical Medicine and Hygiene. 74:174-179.

Blitvitch, B. J. Marlenee, N. L. Hall, R. A. Calisher, C. H. Bowen, R. A. Roehrig, J. T. Komar, N. Langevin, S. A. and Beaty, B. J. 2003. Epitope-Blocking Enzyme-Linked Immunosorbent Assays for the Detection of Serum Antibodies to West Nile Virus in Multiple Avian Species. Journal of Clinical Microbiology. 41:1041-1047.

Boyce, W. Kreuder, C. Anderson, R. and Barker, C. 2004. Potential Impacts of West Nile Virus on Wildlife in California. Wildlife Health Center, University of California, Davis, CA 95616. 1-17.

Centers for Disease Control and Prevention. Division of Vector- Borne Infectious Dieases. 2003. West Nile Virus. Vertebrate Ecology. http://www.cdc.gov/ncidod/dvbid/westnile/birds& mammals.htm. Accessed 10/08/05

Centers for Disease Control and Prevention. Division of Vector- Borne Infectious Dieases. 2004. West Nile Virus. Entomology. http://www.cdc.gov/ncidod/dvbid/westnile/insects.htm. Accessed 10/08/05

Centers for Disease Control and Prevention. Division of Vector- Borne Infectious Dieases. 2005. West Nile Virus. Entomology. http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm. Accessed 10/08/05

Green, D. E. 2006. Collection of Blood Samples from Adult Amphibians. USGS, National Wildlife Health Center. 6006 Schroeder Road. Madison, WI 53711-6223.

Hubalek, Z. and Halouzka, J. 1999. West Nile Fever- A Reemerging Mosquito-Borne Viral Disease in Europe. Emerging Infectious Diseases. 5:643-650.

Klenk, K. and Komar, N. 2003. Poor Replication of West Nile Virus (New York 1999 Strain) in Three Reptilian and One Amphibian Species. The American Journal of Tropical Medicine and Hygiene. 69:260-262.

Klenk, K. Snow, J. Morgan, K. Bowen, R. Stephens, M. Foster, F. Gordy, P. Beckett, S. Komar, N. Gubler, D. and Bunning, M. 2004. Alligators as West Nile Virus Amplifiers. Emerging Infectious Diseases [serial on the internet]. Available from http://www.cdc.gov/ncidod/EID/vol10no12/04-0264.htm

Kostyukov, M. A. Alekseev, A. N. Bul'chev, V. P. and Gordeeva, Z. E. 1986. Experimentally Proven Infection of *Culex pipiens* L. Mosquitoes with West Nile Fever Virus via the Lake Pallas *Rana ridibunda* Frog and its Transmission via Bites. Med Parazitol (Mosk) 3:76-78.

Lampman, R. L. Krasavin, N. M. Szyska, M. and Novak, R. J. 2006. A Comparison of Two West Nile Virus Detection Assays (Taqman Reverse Transcriptase Polymerase Chain Reaction and Vectest Antigen Assay) During Three Consecutive Outbreaks in Northern Illinois. Journal of the American Mosquito Control Association. 22:76-86.

Lanciotti, R. S. Kerst, A. J. Nasci, M. S. Godsey, M. S. Mitchell, C. J. Savage, H. M. Komar, N. Panella, N. A. Allen, B. C. Volpe, K. E. Davis, B. S. and Roehrig, J. T. 2000. Rapid Detection of West Nile Virus from Human Clinical Specimens, Field-Collected Mosquitoes, and Avian Sam-

ples by a Taqman Reverse Transcriptase-PCR Assay. Journal of Clinical Microbiology. 38:4066-4071.

Prince, H. E. and Hogrefe, W. R. 2003. Detection of West Nile Virus (WNV)-Specific Immunoglobulin M in a Reference Laboratory Setting during the 2002 WNV Season in the United States. Clinical and Diagnostic Laboratory Immunology. 10:764-768.

South Dakota Mosquitoes. Identified Species During 2001-2004. http://biomicro.sdstate.edu/ Hildrethm/mosquito/sdmosquito.html. Accessed 10/08/05

Van der Meulen, K. M. Pensaert, M. B. and Nauwynck, H. J. 2005. West Nile Virus in the Vertebrate World. Archives of Virology. 150:637-657.

Williams, C.R., Smith, B.P.C., Best, S.M., & Tyler, M.J. (2006). Mosquito repellents in frog skin. Biology Letters 2:242-245.

Wilkins, P. A. 2004, West Nile Virus: Lessons From the 21st Century. Journal of Veterinary Emergency and Critical Care. 14:2-14.

Yaremych, S. A. Warner, R. E. Van de Wyngaerde, M. T. Ringia, A. M. Lampman, R. and Novak, R. J. 2003. West Nile Virus Detection in American Crows. Emerging Infectious Diseases. 9:1319-1321.