Serosurveillance of Eastern Equine Encephalitis Virus in Amphibians and Reptiles from Alabama, USA

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Abstract. Eastern equine encephalitis virus (EEEV) is among the most medically important arboviruses in North America, and studies suggest a role for amphibians and reptiles in its transmission cycle. Serum samples collected from 351 amphibians and reptiles (27 species) from Alabama, USA, were tested for the presence of antibodies against EEEV. Frogs, turtles, and lizards showed little or no seropositivity, and snakes had high seropositivity rates. Most seropositive species were preferred or abundant hosts of *Culex* spp. mosquitoes at Tuskegee National Forest, that target ectothermic hosts. The cottonmouth, the most abundant ectotherm sampled, displayed a high prevalence of seropositivity, indicating its possible role as an amplification and/or over-wintering reservoir for EEEV.

INTRODUCTION

Because of its high virulence and case-fatality rate, eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) is considered one of the most medically important viral encephalitides in eastern North America.^{1,2} Birds are considered the primary reservoir host for EEEV,^{1,3–5} and the mosquito *Culiseta melanura*, its primary vector,³ while other mosquito species serve as bridge vectors for transmission of EEEV to dead end hosts, such as horses and humans.^{6,7} A complete understanding of the transmission dynamics of this arbovirus requires knowledge of the availability and abundance of competent and incompetent reservoir hosts, vector feeding preferences and behavior, and the interactions between these species.⁸ To date, most of this research has focused on a few mosquito and host species acknowledged to play a key role in EEEV transmission. However, a more comprehensive understanding will involve the inclusion of understudied taxa in transmission models.

Amphibians and reptiles may play a larger role in the transmission cycle of arboviruses than previously assumed. Because of their high energy conversion efficiency, ectotherms typically represent a large portion of the vertebrate biomass in many ecosystems,^{9–13} and may therefore greatly influence transmission dynamics, either as virus amplifiers or dilution hosts. This feature may be especially true of EEEV because it is found in hardwood swamps where amphibians and reptiles are particularly abundant.¹ In addition, the prolonged viremia observed in experimentally infected reptiles relative to what is found in mammals and birds^{14,15} may lead to an increased probability of transmission to mosquitoes. Scattered studies indicate that some ectothermic hosts potentially serve as competent reservoirs for arboviruses,^{15–20} and support their potential role as over-wintering hosts for arboviruses.^{20–22} Certain mosquito species appear to prefer ectothermic hosts, and some generalist species feed upon ectothermic and endothermic taxa.^{23–26} This finding indicates that arboviruses can potentially navigate through entire ecto-parasite–vertebrate host communities, an observation supported by a network analysis, which demon-

strated that these communities have nested structure and are highly centralized.²⁷

We report the results of a three-year surveillance for EEEV in amphibians and reptiles from our study site in Tuskegee National Forest in Alabama, USA. A total of 351 animals individuals representing 9 amphibian and 18 reptile species were tested for antibodies against EEEV. In general, patterns of EEEV seropositivity supported results from our previous research on mosquito host preference²⁵ and laboratory studies inducing viremia in amphibian and reptile hosts,²⁰ suggesting that these species are commonly exposed to EEEV at Tuskegee National Forest.

MATERIALS AND METHODS

Study area. The study area was the 75.54-km² Tuskegee National Forest. At the time of its designation as a national forest, Tuskegee National Forest was composed largely of abandoned farmland. Since that time, ecological succession has proceeded and the forest is now a mosaic of lowland hardwood, mixed pine-hardwood, and wetland habitats. Tuskegee National Forest has 81 documented reptile and amphibian species.²⁵ This study area has been thoroughly investigated as a focus for EEEV zoonotic transmission.^{23–27}

Field sampling. During April–October 2007–2009, a herpetofaunal census was conducted in Tuskegee National Forest wetlands.²⁵ Two to four researchers conducted visual encounter surveys²⁸ at five Tuskegee National Forest wetlands (four beaver marshes and an oxbow lake) twice a week (once during the day and the following night) for each week of the active season (April through October). Visitation to these ponds was rotated so that each was sampled twice per month. During these surveys, observers walked slowly along the wetland margin and counted each individual of each amphibian and reptile species encountered. Visual encounter surveys were primarily conducted to determine the relative abundance of amphibian and reptile species at the study area for a previous study that determined host feeding patterns of mosquitoes at Tuskegee National Forest; for this previous study, numbers of individual amphibians and reptiles were adjusted for survey effort.²⁸

During this study one of the observers (S.P.G.) also collected blood samples from a subset of the animals. Consistent with the hypotheses we intended to test, we attempted to obtain multiple samples from a number of species across a wide range

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of dates. During 2007–2009, hoop and crayfish traps were set for one night each week at one of the above ponds, such that each pond was trapped once per month. Traps were checked the next day for captured amphibians and reptiles. Crayfish traps were placed in 0.5-meter deep water along the pond margin, usually in emergent vegetation. Hoop traps were placed in 1-meter deep water, usually along the deep channel of beaver ponds along the dam. Limited minnow trap and drift fence sampling was also conducted once a week in 2008. Minnow traps were placed in emergent vegetation in beaver ponds, and 15-meter drift fences with two bucket traps and minnow traps were erected within 5 meters of each beaver pond parallel to its margin.

We obtained a blood sample (125–1,000 μL) by cardiac puncture (frogs) or from the caudal sinus (turtles, lizards, and snakes) by using a 26-gauge, heparinized syringe from most species and individuals of a size enabling blood sampling. Blood sample volume varied by species, such that blood samples represented no more than 5% of the sampled individual's mass. Samples were placed in a labeled 1.5-mL microcentrifuge tube, placed on ice packs, and transported to the laboratory. Samples were then centrifuged, and plasma was drawn off and placed in a new labeled tube, frozen, and stored (-20°C) until assayed. Processed turtles and snakes received a unique mark and were released at their point of capture, to prevent multiple sampling of a single individual. Frogs and lizards did not receive a mark and were released at their point of capture because preliminary mark-recapture data collected at this study site indicated a low level of recapture probability for these species (Graham SP, unpublished data). The procedures used in this study were approved by the Institutional Review Board for Animal Use and Care of Auburn University.

Assay for antibodies against EEEV. Reptile and amphibian serum samples were assayed for antibodies against EEEV by using a Luminex-based species-independent antibody assay.²⁹ To produce antigen for this assay, confluent monolayers of Vero cells were infected with EEEV (strain M05-316²⁰) at a multiplicity of infection of approximately 0.01. When a cytopathic effect was evident, the cells were removed from the flask, pelleted by centrifugation, washed twice, and resuspended in 0.9 mL of borate saline (0.5 M Na_3BO_4 , pH 9.0) containing 0.1% (w/v) sodium dodecyl sulfate and 10% Triton X 100 (v/v). The cells were homogenized in a glass-glass homogenizer until the solution cleared. The sample was then subject to centrifugation at $12,000 \times g$ for 3 minutes at 4°C to clear the homogenate. The supernatant was recovered and β -propiolactone added to a final concentration of 0.3% (w/v). The antigen preparation was stored at 4°C for 24 hours and viral inactivation was confirmed by plaque assay.³⁰

Once viral inactivation was confirmed, the protein concentration in the antigen preparation was determined by using the Bradford method.³¹ Set 15 Luminex beads coated with monoclonal antibody 2A2C-3 against alphaviruses (Radix Biosolutions, Georgetown, TX) were then coated with the EEEV antigen preparation by mixing 50 μL of the beads with 1 μg of EEEV antigen in a final volume of 500 μL of phosphate-buffered saline (PBS). The mixture was placed on a shaker at room temperature for 1 hour. The bead solution was added to 9.5 mL of PBS containing 1% BSA (bovine serum albumin (w/v) and stored on ice.

Serum samples (1.5 μL per reaction) were biotinylated with approximately a 50-fold molar excess of biotin by using

the EZ-Link Sulfo-LC-Biotin Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The biotinylated antibodies were passed through a 100-kD MW cutoff filter (Acroprep 96 Omega 100K; VWR Scientific, San Francisco, CA). The 12.5 μL of retentate was washed twice with PBS and diluted with 62.5 μL of PBS, 0.2% BSA to produce a final dilution of 1/50 relative to the original serum sample.

To bind the biotin-labeled antibody preparations, 100 μL per well of the antigen-coated bead preparation was placed into each well of a 96-well 1.2- μm filter plate (MultiScreen-BV, 1.2 μm , Millipore, Billerica, MA). The beads were washed twice in PBS, 1% BSA by vacuum filtration and resuspended in 50 μL of the biotinylated serum sample. The plate was shaken for 45 minutes at room temperature. The plate was removed from the shaker, the supernatant was removed by vacuum filtration, and the retained beads were washed twice PBS, 1% BSA. The beads were resuspended in 50 μL of a solution consisting of 4 $\mu\text{g}/\text{mL}$ of streptavidin-phycoerythrin (Jackson ImmunoResearch, West Grove, PA) in PBS, 1% BSA and the plate shaken for 15 minutes at room temperature. The solution was removed by vacuum filtration, and the beads were washed twice in PBS, 1% BSA and resuspended in 100 μL of PBS, 1% BSA. The samples were analyzed by using a Bio-Rad (Hercules, CA) BioPlex instrument. Results were expressed as mean fluorescent intensity (MFI) of two replicates per sample tested. All plates tested included a series of three known positive and negative serum samples. Positive serum samples were derived from garter snakes (*Thamnophis sirtalis*) and green anoles (*Anolis carolinensis*) experimentally inoculated with EEEV as described.²⁰ Negative samples were obtained from commercially reared individuals of the same species not exposed to EEEV. Samples were considered to contain antibodies against EEEV if the MFI obtained was greater than the mean plus three SD of the MFI obtained from the negative control serum samples processed in parallel with the experimental serum samples.

RESULTS

A total of 351 individuals representing 9 amphibian and 18 reptile species were examined during the course of this study (Table 1). Antibodies reacting with EEEV were detected in 12 of 26 amphibian and reptile species sampled, with an overall individual seropositivity rate of 19%. Seroprevalence was low in frogs, lizards, and turtles, and the prevalence of antibodies against EEEV in snakes was fairly high. Of the nine frog species examined, individuals of two species were seropositive for EEEV, the green treefrog (*Hyla cinerea*) and the gray treefrog (*Hyla chrysoscelis*). Similarly, 3 of 7 of the turtle species examined contained EEEV-seropositive individuals (Table 1). In contrast, 8 of 9 snake species and 35% of the individual snakes overall contained antibodies against EEEV antibodies in their serum samples (Table 1).

When we compared the seropositivity rates over the three years of the study, the proportion of seropositive individuals did not differ significantly among the three years ($\chi^2 = 3.83$, $P = 0.14$). Thus, for a seasonal analysis of the prevalence of seropositivity, data from all three years were combined and compared across months. When all amphibian and reptile species were combined and compared across months, the proportion of seropositive individuals was relatively steady from

TABLE 1

Serosurveillance for antibodies against eastern equine encephalitis virus in amphibians and reptiles of Tuskegee National Forest, Alabama*

Common name	Latin name	No.	No. seropositive	% Positive $\pm$ 95% CI
Frogs				
Green frog	<i>Lithobates clamitans</i>	10		
Bullfrog	<i>Lithobates catesbeianus</i>	6		
Green treefrog	<i>Hyla cinerea</i>	33	1	3 $\pm$ 5.7
Gray treefrog	<i>Hyla chrysoscelis</i>	12	1	8 $\pm$ 14.5
Barking treefrog	<i>Hyla gratiosa</i>	4		
Bird-voiced treefrog	<i>Hyla avivoca</i>	2		
Fowler's toad	<i>Anaxyrus fowleri</i>	4		
Southern toad	<i>Anaxyrus terrestris</i>	7		
Eastern spadefoot	<i>Scaphiopus holbrookii</i>	4		
Total		82	2	3 $\pm$ 3.3
Turtles				
Pond slider	<i>Trachemys scripta</i>	67	4	6 $\pm$ 5.36
Stinkpot	<i>Sternotherus odoratus</i>	6	1	16 $\pm$ 26
Southern painted turtle	<i>Chrysemys dorsalis</i>	5		
River cooter	<i>Pseudemys concinna</i>	2		
Chicken turtle	<i>Deirochelys reticularia</i>	3		
Mud turtle	<i>Kinosternon subrubrum</i>	7	1	14 $\pm$ 22.9
Box turtle	<i>Terrapene carolinensis</i>	2		
Total		92	6	7 $\pm$ 4.75
Lizards				
Green anole	<i>Anolis carolinensis</i>	3		
Five-lined skink	<i>Plestiodon fasciatus</i>	1		
Total		4	0	0
Snakes				
Ringneck snake	<i>Diadophis punctatus</i>	1	1	100 $\pm$ NA
Mud snake	<i>Farancia abacura</i>	1		
Black racer	<i>Coluber constrictor</i>	5	3	6 $\pm$ 34
Plainbelly watersnake	<i>Nerodia erythrogaster</i>	13	2	15 $\pm$ 15.07
Midland watersnake	<i>Nerodia sipedon</i>	2		
Dekay's brownsnake	<i>Storeria dekayi</i>	1	1	100 $\pm$ NA
Rattlesnake	<i>Crotalus horridus</i>	2	1	5 $\pm$ 70
Copperhead	<i>Agkistrodon contortrix</i>	4	1	25 $\pm$ 38
Cottonmouth	<i>Agkistrodon piscivorus</i>	144	51	35 $\pm$ 6.17
Total		173	60	35 $\pm$ 5.61

*CI = confidence interval; NA = not applicable. CIs were calculated as described.³⁴

April through July and then increased in August and September (Figure 1). In contrast, when cottonmouths were considered alone, the seasonal pattern of the proportion of seropositive individuals exhibited a bimodal pattern, with peaks in the early spring and the fall (Figure 1).

FIGURE 1. Monthly variation in Eastern equine encephalitis virus seroprevalence of tested amphibians and reptiles from Tuskegee National Forest, Alabama.

DISCUSSION

The data presented show that antibodies against EEEV are commonly found in some ectothermic species, particularly snakes residing at Tuskegee National Forest. These findings corroborate previous studies on the reservoir competence of these species for EEEV.²⁰ In the reservoir competency studies, green treefrogs (*Hyla cinerea*) and bullfrogs appeared refractory to EEEV infection. Green anoles (*Anolis carolinensis*) were susceptible to infection with the virus, but produced low viremias.²⁰ In contrast, garter snakes experimentally infected with EEEV produced relatively high viremias (levels capable of infecting mosquitoes) that were maintained for a prolonged period.²⁰ Viremias have also been experimentally induced in garter snakes exposed to western equine encephalitis virus,^{20–22} an alphavirus related to EEEV. A previous study in Massachusetts did not identify antibodies against EEEV in most amphibians and reptiles sampled, including several of the same amphibian and reptile species that we found to contain antibodies against EEEV (e.g., *Coluber constrictor*, *Sternotherus odoratus*, and *Storeria dekayi*).¹⁷ This difference could be caused by several factors, including temporal or site effects, differences in the total number of individuals sampled, and differences in assay sensitivity.

Host preferences of mosquitoes involved in EEEV transmission at Tuskegee National Forest also corroborate the general pattern of seropositivity that we observed. Amphibian

and reptile species with antibodies against EEEV were often preferred or abundant hosts of the mosquitoes *Culex territans*, *Cx. erraticus* and *Cx. peccator*, which feed upon ectothermic hosts, and which have been found to be infected with EEEV at the Tuskegee National Forest site where this study was conducted.²⁵ Other species that had detectable antibodies against EEEV were not among those that we previously reported to be commonly fed upon by mosquitoes at Tuskegee National Forest,²⁵ including the snake *Coluber constrictor*, and the turtles *Trachemys scripta*, *Kinosternon subrubrum*, and *Sternotherus odoratus*. However, we have subsequently identified blood meals of *C. constrictor* and *T. scripta* taken from *Cx. erraticus* collected at the Tuskegee National Forest TNF site (Graham SP, unpublished data).

The serologic data presented suggest that seroprevalence in frogs was quite low. There may be two explanations for this finding. First, because frogs are refractory to infection,²⁰ it is possible that the virus does not replicate much, if at all, in these hosts, resulting in a lack of any antibody response. If this is the case, frogs may serve as a dilution host, particularly early in the year, when frogs are an important host for mosquitoes at Tuskegee National Forest.²⁵ Alternatively, this finding may also reflect a lack of exposure to these species. *Culex peccator* and *Cx. erraticus* feed upon a variety of ectothermic hosts, but seem to prefer snakes over amphibians.²⁵ *Culex territans* feeds preferentially upon amphibians and targets other host classes much less frequently.²⁵ If snakes are indeed the major competent ectothermic reservoir hosts for EEEV at Tuskegee National Forest, this partitioning of feeding by the different mosquito species may limit the exposure of frogs to EEEV.

Because of their relatively low body temperatures and inefficient antibody responses,¹⁴ ectotherms can support prolonged viremias compared with birds and mammals,¹⁵ and previous laboratory studies have suggested a role for ectotherms as overwintering hosts for arboviruses.^{20–22} These studies suggest that ectotherms may represent an over-looked reservoir host, and perhaps an important over-wintering refuge for EEEV. The high proportion of seropositive animals (particularly cottonmouths) provides indirect evidence to support this hypothesis, suggesting that these animals are indeed commonly exposed to EEEV in their natural environment. However, seropositivity may simply indicate exposure to the virus and not necessarily a patent infection. In addition, the Luminex assay does not detect a particular antibody class, and thus cannot distinguish recent from more distant exposure or infection. Mark-release-recapture sequential serosurveys to assess incidence of exposure to EEEV and studies to detect circulating viremia in animals emerging from hibernation would be useful in more carefully defining the role cottonmouths may play as amplification and over-wintering hosts for the EEEV.

The hibernation and emergence habitats of female mosquitoes and cottonmouths are similar enough that during early spring, cottonmouths are probably readily available hosts for emerging mosquitoes. For example, Burkett-Cadena and others³² found that several species of *Culex* mosquitoes overwinter in underground burrows and root holes, sites also favored as hibernacula by cottonmouths and other snakes.³³ If snakes support viremias at this time, the early emergence period for mosquitoes and their ectothermic hosts could be a crucial window for the persistence of EEEV from year to year. Although this time period (e.g., March) is considerably earlier than

mosquito control programs typically operate, it is possible that the targeted use of pesticides near over-wintering sites during this period may succeed in interrupting the annual zoonotic cycle of EEEV.

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