

Competency of Reptiles and Amphibians for Eastern Equine Encephalitis Virus

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Abstract. Eastern equine encephalitis virus (EEEV) is endemic throughout most of the eastern United States. Although it is transmitted year round in Florida, transmission elsewhere is seasonal. The mechanism that enables EEEV to overwinter in seasonal foci remains obscure. In previous field studies, early season EEEV activity was detected in mosquito species that feed primarily upon ectothermic hosts, suggesting that reptiles and amphibians might represent overwintering reservoir hosts for EEEV. To determine if this might be possible, two commonly fed upon amphibian and reptile species were evaluated as hosts for the North American subtype I strain of EEEV. Neither amphibian species was a competent host. However, circulating viremias were detected in both reptile species examined. Hibernating infected garter snakes remained viremic after exiting hibernation. These data suggest that snakes may represent an overwintering host for North American EEEV.

INTRODUCTION

Eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) is the most pathogenic arbovirus endemic to the United States. The case fatality rate among individuals with eastern equine encephalitis is in the range of 30–70%, and the majority of the survivors of the disease suffer severe long-term neurological complications.¹ Eastern equine encephalitis virus is endemic throughout the eastern half of the United States, from New England south to Florida, extending west to Michigan.² It is also endemic to Latin America, although recent studies have suggested that the North American and South American strains of the virus may actually represent distinct viruses.³

In North America, EEEV is endemic to hardwood swamps, and is primarily considered an enzootic infection of passerine birds. Among birds, the primary vector is thought to be the ornithophilic mosquito *Culiseta melanura*.⁴ The virus escapes the enzootic cycle to periodically infect horses and humans through the intercession of bridge vectors such as *Aedes vexans*, *Coquillettidia perturbans*, and *Uranotaenia sapphirina*,⁵ although recent evidence suggests that *Cs. melanura* may also occasionally act as a bridge vector and as the primary enzootic vector.⁶ Mammals are generally considered dead-end hosts for the virus, though small mammals have recently been implicated as potential amplification hosts for North and South American strains of EEEV.⁷

The EEEV circulates year round in Florida, but its transmission is seasonal outside of this state. In the Northeastern United States, recent studies have suggested that the virus is periodically introduced from Florida, where it establishes itself in defined foci.^{8–11} The virus then continues to circulate in these foci for several years. However, the mechanism that the virus uses to overwinter in these foci remains obscure. In contrast to the flaviviruses, EEEV does not appear to be transovarially transmitted to the progeny of an infected mosquito,¹² suggesting that the virus does not overwinter in the mosquito vector. Field-based studies on the ecology of EEEV

in the Southeastern United States conducted in the Tuskegee National Forest (TNF) in East Central Alabama have documented the presence of EEEV in pools of *Culex peccator* and *Culex territans*, with some EEEV-positive pools detected early in the transmission season.^{13,14} Both of these mosquito species feed almost exclusively upon ectothermic hosts, with *Cx. territans* primarily feeding upon amphibians and *Cx. peccator* primarily feeding upon reptiles.¹⁴

Several previous studies have implicated ectothermic vertebrates as potential hosts for a variety of arboviruses. For example, western equine encephalitis virus (WEEV) can infect garter snakes (*Thamnophis* spp.) in the laboratory^{15,16} and can persist for prolonged periods in the Texas tortoise (*Gopherus berlandieri*).¹⁷ EEEV has also been recovered from a number of wild ectotherms,^{18,19} while alligators have been implicated as potential amplifying hosts for West Nile virus.²⁰ These studies suggest that ectothermic hosts might serve as reservoir hosts for EEEV, and may provide a mechanism for overwintering in some areas or transmission foci of the virus.

In the current study, four ectothermic species (two amphibians and two reptiles) were studied in the laboratory for their ability to serve as hosts for EEEV. The species were chosen because data obtained from long-term field studies of the ecology of EEEV transmission at the TNF site suggested that they were among the most common and frequently fed upon ectotherms present.^{13,14,21–23}

MATERIALS AND METHODS

Four ectothermic species (two amphibians and two reptiles) were included in this study. The amphibians examined were the bullfrog (*Rana catesbeiana*) and the green tree frog (*Hyla cinerea*). Both amphibian species were among the most frequently targeted hosts by *Cx. territans* at the TNF site.¹⁴ The reptile species studied were the green anole (*Anolis carolinensis*) and the garter snake (*Thamnophis sirtalis*). Both of these species were among the more common reptile species found at the TNF site¹⁴ and the green anole was the most commonly targeted lizard species by both *Cx. peccator* and *Cx. territans*.¹⁴ The cottonmouth (*Agkistrodon piscivorus*) is the most common snake fed upon by *Cx. peccator* at the TNF site.^{13,14} However, it is a venomous species that bites readily, and it was judged too dangerous to manipulate EEEV-infected cottonmouths in the laboratory. For this reason, the garter snake was

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chosen as a model to replace the cottonmouth in the laboratory infection studies.

All animals were inoculated intravenously with 1.5×10^4 plaque-forming units (PFU) of the M05-316 strain of EEEV in 50 μ L of MEM. The M05-316 strain of EEEV was originally isolated from a pool of *Cs. melanura* mosquitoes collected in 2005 from Volusia County, Florida, and was passaged once in Vero cells. The M05-316 strain was provided by Dr. Lillian Stark of the Florida Department of Health, Bureau of Laboratories, Tampa. Initially, inoculated animals were held for 10 days in incubators maintaining light and temperature conditions replicating those typically found at the TNF site during the height of the EEEV transmission season (14 hours of light at 30°C followed by 10 hours of dark at 25°C). Subsequently, snakes were held at both higher and lower temperatures, and were also induced to enter and exit hibernation. To induce hibernation, the temperature was lowered from 25°C over a period of 4 days in 4–6°C increments, reaching a final temperature of 7°C. The animals were maintained for 30 days at 7°C, and induced to exit hibernation by raising the temperature to 20°C over a period of 2 days in 6–7°C increments. Once the animals had exited hibernation, they were maintained at 20°C for up to six days post-hibernation.

Blood samples (100–500 μ L) were collected from infected amphibians using cardiac puncture, and from infected reptiles from the caudal vein. The blood was subjected to centrifugation at $850 \times g$ for 10 minutes at 4°C to pellet the erythrocytes and the serum collected and stored at –80°C until assayed for the presence of EEEV. Each sample was initially assayed for the presence of EEEV using a real-time polymerase chain reaction (PCR) assay as previously described.²⁴ The sensitivity of the RT-PCR assay was estimated to be < 10 PFU/mL, by comparison to a standard curve produced from serial dilutions of a viral stock with a known viral titer. The amount of virus in 10 μ L of any positive sample was then quantified by plaque assay on Vero cells, as previously described.²⁵ The sensitivity of the plaque assay was estimated to be ~100 PFU/mL. The studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of South Florida.

RESULTS

The results of the initial studies testing the susceptibility of ectotherms to EEEV are summarized in Table 1; neither of the frog species tested developed detectable viremia. In contrast, both the green anole and garter snake developed detectable circulating EEEV. Viremias in the snakes were approximately two orders of magnitude higher than the anoles (Table 1). All of the infected snakes were viremic at both 2 d and 7 d

post-infection (dpi) (Table 1). In contrast, only a small proportion of the anoles remained viremic at 10 dpi. Furthermore, the majority of the snakes exhibited viral titers that were equal to or greater than \log_{10} 4.0 PFU/mL, a titer that has been suggested to be the minimum viremia necessary to infect *Cs. melanura*.²⁶ Taken together, these data suggest that the reptile species tested were permissive hosts for EEEV, whereas the frog species tested were not.

To further characterize the kinetics and duration of viremia in the reptile species, additional post-inoculation time points and environmental conditions were examined. When held under the conditions replicating those typically found during the transmission season at the TNF, the anoles maintained a stable, low viral titer for ~7 dpi, which then began to decline (Figure 1, panel A). Garter snakes held under these conditions exhibited a similar pattern of viremia, maintaining viral titers in the range of \log_{10} 4–5 from Day 2 to Day 7 pi, at which point the viremias declined, reaching undetectable levels 21 dpi (Figure 1, panel B). When the garter snakes were held at 32°C, the course of viremia was similar to that in animals cycled between 30 and 25°C (Figure 1, panel B). However, the viremia time course differed in garter snakes kept at 18°C. Here, the viral titer remained steady from 2 to 21 dpi (Figure 1, panel B). Two of the animals maintained at 18°C produced a viremia that exceeded \log_{10} 4.0, with one maintaining a viremia at or above this level for 7 dpi, and the other for 14 dpi. This finding suggests that snakes may remain infectious for mosquitoes for a prolonged period at low temperatures, such as might be expected to exist early in the transmission season.

For an ectothermic species to serve as an overwintering host for EEEV, it must be able to remain viremic during hibernation. To determine if infected animals might remain viremic following hibernation, 15 garter snakes were infected and maintained for 2 days to permit the development of a circulating viremia, along with four sham-infected negative control snakes. Animals were bled and assayed for circulating virus at 2 dpi and then induced to enter and exit hibernation as described in the Materials and Methods section. One of the four sham-infected animals and 8 of 15 of the infected animals survived the hibernation period. Six of the eight surviving infected garter snakes remained viremic when exiting hibernation, exhibiting titers ranging from \log_{10} 2.2 to \log_{10} 6.1 (Figure 2). The six viremic animals were then maintained at 20°C (ambient temperature in the animal facility). Three were sacrificed and assayed for circulating viremia 2 days after exiting hibernation, and the remaining three were sacrificed and assayed for viremia 6 days after exiting hibernation. In each case, two of the three animals examined had circulating

TABLE 1
Development of EEEV in different species of reptiles and amphibians*

Species	Common name	dpi*	Proportion viremic	Mean viremia titer (\log_{10} PFU/mL)	Proportion with titer > 4.0 \log_{10} PFU/mL
<i>Hyla cinerea</i>	Green tree frog	3	0/6 (0%)	nd	nd
<i>Hyla cinerea</i>	Green tree frog	10	0/6 (0%)	nd	nd
<i>Rana catesbeiana</i>	Bullfrog	3	0/6 (0%)	nd	nd
<i>Rana catesbeiana</i>	Bullfrog	10	0/5 (0%)	nd	nd
<i>Anolis carolinensis</i>	Green anole	3	11/12 (92%)	2.44	0/11 (0%)
<i>Anolis carolinensis</i>	Green anole	10	2/12 (17%)	1.75	0/2 (0%)
<i>Thamnophis sirtalis</i>	Garter snake	2	4/4 (100%)	5.19	3/4 (75%)
<i>Thamnophis sirtalis</i>	Garter snake	7	4/4 (100%)	4.59	3/4 (75%)

* dpi = days post-infection; PFU = plaque-forming units; nd = not determined.

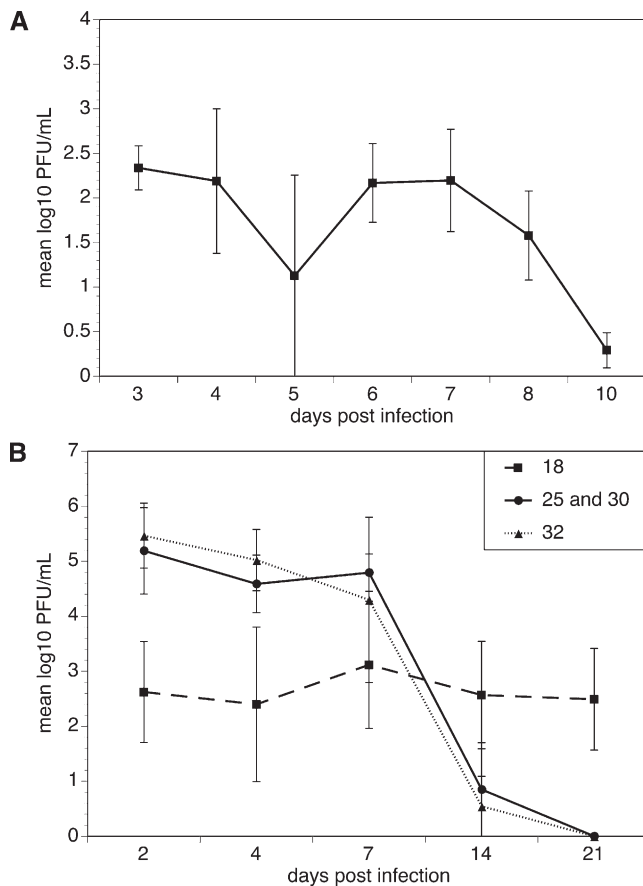


FIGURE 1. Time course of viremias in green anoles and garter snakes. Panel **A**: Kinetics of EEEV viremia in green anoles. Animals were infected with EEEV and maintained under conditions mimicking those present during the middle of the transmission season at the Tuskegee National Forest (TNF), as described in the text. Blood was drawn from the infected animals on 3–8 dpi and 10 dpi. Panel **B**: Kinetics of EEEV viremia in garter snakes. Animals were infected with EEEV and held under various temperatures as indicated in the figure. “25 and 30” = conditions mimicking those at TNF (30°C day time and 25°C night time temperature). Blood was drawn from these animals on 2, 4, 7, 14, and 21 dpi. In each panel, the points represent the mean, and the error bars the SEM, of the viral titer in four individual animals.

viremia, although the viral titers declined by Day 6 post-hibernation (Figure 2).

DISCUSSION

These studies suggest that reptiles are susceptible to infection with North American EEEV and that snakes in particular have the potential to serve as a reservoir host for the virus. The garter snake was shown to be a competent host for EEEV, maintaining circulating levels of the virus that would be expected to be infectious for a mosquito for up to 14 DPI. The duration of the viremia in the snakes was also found to be temperature dependent. Circulating virus levels reached lower levels, but were maintained for longer periods, in animals held at lower temperatures. This might be related to the observation that the kinetics of an immune response to a challenge in reptiles is known to be temperature dependent.²⁷ In support of this hypothesis, antibodies to EEEV were not detected

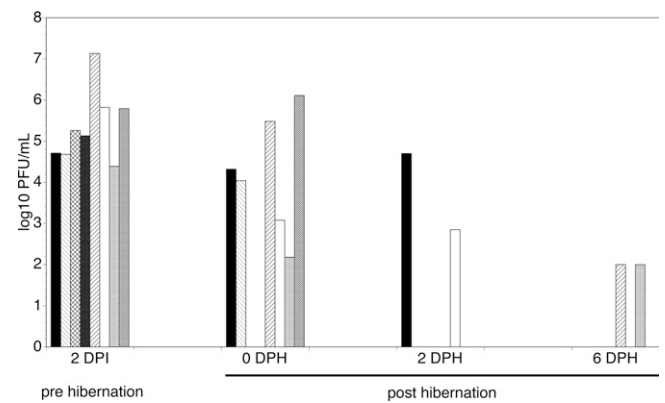


FIGURE 2. Viremia of garter snakes induced to hibernate: Snakes were infected, induced to hibernate, and to exit hibernation as described in the text. Bars with identical shading indicate viral titers from individual animals sampled at different times pre- and post-hibernation (dpi = days post-infection; dph = days post-hibernation).

in snakes exiting hibernation at 30 dpi when assayed using a plaque reduction neutralization test.²⁵

Recent studies have demonstrated that cotton rats may serve as reservoirs for both North American and South American strains of EEEV.⁷ Interestingly, the South American strains of the virus seemed to replicate to higher titers in cotton rats than did the North American strain and the South American strains were less pathogenic to cotton rats than the North American strain.⁷ These data suggested that while both North American and South American strains of the virus were capable of using cotton rats as a reservoir, the South American strain might be better adapted to a small mammal reservoir than North American strain. In this regard, we noted no significant pathology in the reptiles infected with the North American subtype I strain of the virus, suggesting that this strain of the virus may be fairly well adapted to reptile hosts. It would be of interest to determine if other strains of EEEV are equally capable of replicating in reptiles and if so do they induce any significant pathology in infected animals.

Although the viral titers reached in the snakes were lower than those observed in birds, the infectious viremic period was rather prolonged. Garter snakes were able to maintain a potentially infectious viremia for up to 7 dpi. This is longer than the period that avian hosts for the virus usually maintain an infectious EEEV titer, which is in the range of 2–3 days.²⁶ This finding suggests that although snakes might be a less efficient reservoir for EEEV than birds, they could remain infectious to mosquitoes for a longer period of time. This might have been expected, given that the metabolic rate of ectothermic animals and presumably their ability to clear the virus will vary depending upon the temperature of their environment.

The data presented in this study show that EEEV-infected garter snakes can remain viremic during hibernation. This finding is in concordance with previous studies of garter snakes infected with WEEV, another alphavirus related to EEEV, where it was found that WEEV-infected garter snakes remain viremic during hibernation.¹⁶ The persistence of EEEV viremia during hibernation lends support to the hypothesis that these animals might serve as overwintering hosts for EEEV.

For snakes to serve as an efficient overwintering host for EEEV, it is necessary that they be fed upon by mosquitoes that can serve as vectors for the virus. The three most common

mosquito species that feed frequently upon ectothermic hosts at TNF are *Cx. peccator*, *Cx. territans*, and *Ur. sappharina*.²¹ EEEV-positive pools from all these species have been collected from the TNF site, indicating that all three species have come into contact with EEEV-infected hosts. *Uranotaenia sappharina* has previously been implicated as a potential bridge vector for EEEV.⁵ However, the competency of *Cx. peccator* and *Cx. territans* for EEEV is unknown, and attempts to colonize these species to conduct such vector competency studies have not been successful (Unnasch TR, unpublished). Thus, the role that these mosquito species play in the transmission of EEEV remains to be determined. The role these species play in the dynamics of EEEV transmission will also be determined in part on their feeding preferences. In light of these experiments, which indicate that reptiles may be much more competent hosts of EEEV than the amphibians, *Cx. peccator*, which feeds primarily upon reptiles,¹⁴ may contribute more to EEEV transmission than *Cx. territans*, which feeds primarily upon amphibians.¹⁴ In addition, *Cx. erraticus* has been shown to feed upon reptiles at the TNF site. This is the most common species found at the TNF site and throughout the Southeastern United States^{21,28–30} and it is believed to represent a major potential vector of EEEV in this region.^{21,28} It is therefore possible that any or all of these four mosquito species might be responsible for initiating the enzootic transmission cycle through feeding upon EEEV-infected snakes exiting hibernation in the spring.

In conclusion, the data presented above suggest that garter snakes can serve as competent hosts for North American EEEV and that these animals, when infected, can remain viremic through hibernation. This finding, together with the discovery of early season EEEV infections in pools of mosquitoes that feed primarily upon ectothermic hosts provides support to the hypothesis that EEEV may overwinter in seasonal foci in ectothermic vertebrates. These data also reinforce recent studies⁷ that suggest other animals in addition to birds may play an important role in the dynamics of the EEEV enzootic transmission cycle.

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