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IDENTIFICATION OF REPTILIAN AND AMPHIBIAN BLOOD MEALS FROM MOSQUITOES IN AN EASTERN EQUINE ENCEPHALOMYELITIS VIRUS FOCUS IN CENTRAL ALABAMA

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Abstract. *Uranotaenia sapphirina*, *Culex erraticus*, and *Cx. peccator* were collected in an enzootic eastern equine encephalomyelitis (EEE) virus focus in central Alabama (Tuskegee National Forest) from 2001 to 2003 and analyzed for virus as well as host selection. EEE virus was detected in each species every year except 2003, when pools of *Cx. peccator* were negative. Most (97%) of the 130 *Cx. peccator* blood meals identified were from ectothermic hosts; 3% were from birds. Among blood meals from reptiles (approximately 75% of the total), 81% were from *Agkistrodon piscivorus* (cottonmouth); all amphibian blood meals (approximately 25%) were from *Rana* spp. with > 50% taken from the bullfrog *R. catesbeiana*. Host identifications were made from 131 of 197 *Cx. erraticus*, but only 3 (2%) were derived from ectothermic species. Identification of *Ur. sapphirina* blood meals proved difficult and only 2 of 35 hosts were determined. Both were from *R. catesbeiana.* Ectothermic species are possible EEE virus reservoirs in the southeastern United States where species such as *Cx. peccator* and *Ur. sapphirina* occur with large, diverse reptilian, amphibian, and avian populations such as those at the Tuskegee site.

INTRODUCTION

Eastern equine encephalomyelitis (EEE) virus is endemic in Alabama, with enzootic transmission occurring in 6–10 months of the year in the central and southern portions of the state.[1] In an analysis of a focus near Tuskegee, Alabama in 2001 and 2002, EEE virus was detected at various times in *Culiseta melanura*, *Aedes vexans*, *Coquillettidia perturbans*, *Uranotaenia sapphirina*, and *Culex erraticus* from early May to early October.[1,2] *Uranotaenia sapphirina*, a species generally assumed to blood feed on amphibians and reptiles, had minimum EEE virus infection rates in 2001 and 2002 of 5.6 and 2.65 females per 1,000, respectively, with virus-positive pools occurring over a four-month period in 2001. These findings were unusual because reports of EEE virus infection in this mosquito are relatively rare.[3,4] These data also suggested that ectothermic vertebrates might be serving as virus reservoirs at the Tuskegee site, a possibility that had been raised previously for EEE virus in enzootic foci in Georgia and Massachusetts.[5,6]

To investigate if amphibians and reptiles were likely virus hosts in central Alabama, *Cx. peccator* and *Ur. sapphirina* females, collected during three transmission seasons (April–October 2001–2003) at the Tuskegee focus, were examined for EEE virus. *Culex peccator*, as does *Ur. sapphirina*, primarily selects cold-blooded animals as hosts.[7,8] *Culex peccator* is also a member of the subgenus *Melanoconion*, a largely Neotropical group of mosquitoes that contains a number of taxa that are vectors of alphaviruses.[4] *Culex erraticus*, also a member of the subgenus *Melanoconion* and a frequently infected species at the site during 2001[1] and 2002,[2] was evaluated for EEE virus during 2003. Blood-engorged *Cx. peccator* and *Ur. sapphirina* females were also collected simultaneously and all were analyzed using a polymerase chain reaction (PCR)–based method to identify host source to the species level.[2,9] Blood-engorged *Cx. erraticus* that had been avian-negative in previous host analyses[2] were tested again for possible selection of reptile/amphibian hosts. We report here the occurrence of EEE virus in another mosquito species that readily blood feeds on reptiles and amphibians and identify several vertebrate species that may be involved as part of the EEE virus reservoir in the southeastern United States and the subtropics.

MATERIALS AND METHODS

Study site. The study site is located in the Tuskegee National Forest in Macon County, Alabama, and has been described previously.[1] There has been extensive re-forestation over depleted farmland within the 10–15-acre site that was abandoned for agricultural use in the early 1900s. Five beaver ponds that are interconnected and fluctuate in size and depth provide standing water for much of the year. Reptiles and amphibians are abundant at the site, with ≥ 62 species commonly occurring in or along the periphery of the forested wetland,[10] i.e., 1 crocodilian, 5 turtle, 11 salamander, 2 toad, 16 frog, 8 lizard, and 19 snake species. Greatest activity begins in March and continues through October.[10] Amphibian winter breeding activity also includes chorus frogs and *Rana sphenocephala* (October–March) and tree frogs (Hylidae) that chorus sporadically from March to August.

Collections. Mosquitoes were collected using portable Centers for Disease Control light-traps baited with CO_2 and by vacuum collection. Light-traps ran from dusk to dawn and were positioned approximately two meters above ground. Sampling (twice a week) began during the first week of April and was concluded during the first week of October. Live material was returned to the laboratory, sorted, and identified using a chill table and binocular microscope, and then frozen at −70°C. Vacuum collections were made twice a week from resting boxes,[11,12] and natural resting sites during this same time period and mosquitoes having what appeared to be blood in their midguts were identified to the species level and preserved as noted earlier.

Virus identification. The methods described previously were used to detect EEE virus.[1] Pools of *Ur. sapphirina*, *Cx. peccator*, and *Cx. erraticus* containing up to 50 individuals were homogenized in 1.5 mL of BA-1 tissue culture medium and subjected to centrifugation at 13,000 × *g* for five minutes at room temperature. A total of 140 μL of the resulting su-

pernatant was removed and RNA was purified from the aliquot using the QiaAMP viral RNA extraction kit (Qiagen, Valencia, CA). The RNA was purified following the manufacturer's instructions, with the exception that the number of washes with buffers AW1 and AW2 were increased from one to two.

EEE viral RNA was detected in the RNA prepared from pools of mosquitoes using a nested reverse transcriptase (RT)–PCR assay. This assay was a modification of a previously published protocol[13] that included a nested amplification step to increase the limit of detection of the assay. Briefly, 4 μL of RNA prepared as described earlier was used in a 50-μL total volume one-step RT-PCR amplification reaction using reagents provided by Qiagen (one-step RT-PCR) and the EEE virus-specific primers EEE c7601 (5′-TACCCTACACTTAACTAYCCGC-3′ where Y = C or T) and EEE nc7873 (5′-TGTCGTTTGCCTGGTTTAGGT-3′). The amplification reactions contained 1× Qiagen Onestep RT-PCR buffer, 400 μM each of dATP, dGTP, dCTP, and dTTP, 0.6 μM of each primer, and 2 μL of Qiagen OneStep RT-PCR enzyme mixture. Reaction conditions were at 50°C for 30 minutes and 95°C for 15 minutes, followed by 40 cycles each at 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 2 minutes. Reactions were completed with a final extension at 72°C for 10 minutes. Nested PCRs were carried out in a total volume of 50 μL, using 0.5 μL of the first step PCR product as a template. The nested PCRs contained 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dGTP, dCTP, and dTTP, 0.5 μM of each primer, and 2.5 units of *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). The primers used in the nested PCRs were EEE c7643 (5′-ATGGCYTACCGGGATCCTAATC-3′, where Y = C or T) and EEE nc7848 (5′-ACGTTTTTGTTTCTTGGCAGGT-3′). Cycling conditions consisted of 40 cycles at 95°C for 45 seconds, 58°C for 1 minute, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. Products were visualized by electrophoresis on a 1.5% agarose gel, followed by staining with 1 μg/mL of ethidium bromide. Each experiment was conducted with a series of positive and negative controls. The positive control for each experiment consisted of RNA extracted from inactivated EEE virus culture supernatants kindly provided by the Centers for Disease Control and Prevention (Fort Collins, CO). Negative controls consisted of sham extractions done with each set of 24 samples at the time of sample RNA preparation, and RT-PCR-negative samples set up on each plate, which contained water instead of RNA. Samples producing an amplicon of the expected size (228 basepairs) were retested with a second independent RT-PCR. Samples giving amplicons of the predicted size in both independent reactions were scored as putative positive samples, and the identity of the amplicons in the putative positive samples were then confirmed by DNA sequencing.

Preparation and identification of blood meals. Genomic DNA prepared from blood fed mosquitoes[2] was used as a template in a nested PCR using primers that were designed to preferentially amplify cytochrome B sequences of ectothermic species. Primers were designed to amplify cytochrome b sequences of reptile and amphibian but not mosquito DNA and were validated in control experiments. The sequences of the primers were 5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′ and 5′-GCH GAY ACH WVH HYH GCH TTY TCH TC-3′, where H = A, C, or T, Y = C or T, and V = A, C, or G. The PCR amplifications were carried out in a volume of 50 μL containing 60 mM Tris-HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 1.25 units of *Taq* DNA polymerase (Roche Biochemicals, Indianapolis, IN) and 2.5 μL of DNA template. Cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes, followed by 55 cycles at 94°C for 45 seconds, 50°C for 50 seconds, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The PCR amplification products were analyzed by agarose gel electrophoresis. The products from reactions producing an amplicon of the expected size were purified using the Sephaglas BandPrep kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and the purified products subjected to direct DNA sequence analysis.

RESULTS

EEE virus was detected in each species under consideration during the three-year sampling period except 2003, when pools of *Cx. peccator* were negative (Table 1). A total of 803 females of this species were collected, with EEE virus detected in 2001 (5 positive pools) and 2002 (1 positive pool). Because of the relatively low numbers, minimum infection rates (MIRs)[14] for *Cx. peccator* were high for both years, i.e., 21.5 (per 1,000 females) for 2001 and 16.9 for 2002. *Uranotaenia sapphirina* was virus positive each year: 6 positive pools in 2001 and 1 positive pool in each of the 2002 and 2003 collection seasons. The MIRs of this species varied from 9.3 in 2001 to 0.44 in 2003. Five pools of *Cx. erraticus* were positive for EEE virus in 2003; this species had an MIR of 0.36. Interestingly, there was a slow decrease in virus activity over the three-year period in all three species.

Fifty-four of a possible 62 reptile/amphibian species known to inhabit the area were collected in and around the study site and specific cytochrome B sequences were amplified for each

TABLE 1
Virus isolation data from three mosquitoes blood-feeding on cold-blooded vertebrates at the Tuskegee National Forest site, 2001–2003*

	2001			2002			2003		
	Total	+Pools	MIR	Total	+Pools	MIR	Total	+Pools	MIR
1. *Cx. peccator*	232	5 (13)	21.5	62	1 (11)	16.9	509	0 (25)	0
2. *Ur. sapphirina*	992	6 (68)	9.3	377	1 (10)	2.7	2,265	1 (64)	0.44
3. *Cx. erraticus*	11,369	37 (248)	3.2†	9,998	8 (215)	0.8‡	13,785	5 (294)	0.36

* MIR = minimum infection rate per 1,000 females. Values in parentheses are the total number of pools screened. *Cx. = Culex; Ur. = Uranotaenia.*
† 2001 data from Cupp and others.[1]
‡ 2002 data from Hassan and others.[2]

species (Table 2). Taxa represented included 20 species in the Class Amphibia and 34 species in the Class Reptilia. DNA sequence data from the cytochrome B gene used in the blood meal analysis was confirmed in the GenBank database for 37 of these species and new DNA sequence data were generated for 17 additional species known to be present in and around the study site. This cytochrome B sequence data permitted the identification of reptilian and amphibian derived blood meals to the species level.

Culex peccator fed readily on reptiles and amphibians (Table 3 and 4). One hundred thirty of 210 blood meals (positive identification rate = 62%) from this mosquito were identified to the host species level, with the majority (n = 126; 97%) taken from ectothermic taxa. Among reptiles, *Agkistrodon piscivorus* (cottonmouth) was selected 81% (77 of 95) of the time with *Nerodia erythrogaster* (red-bellied watersnake) and *Thamnophis sauritus* (Eastern ribbonsnake) occasionally bitten (13.6%; 13 of 95). Only species belonging to the genus *Rana* were selected among amphibians. More than half (58%) of those were taken from *R. catesbeiana* (bullfrog), with the remainder from *R. clamitans* (green frog) and *R. sphenocephala* (Southern leopard frog). *Culex peccator* also occasionally took blood from *Nyctanassa violacea* (yellow-crowned night heron) and *Ardea herodias* (great blue heron).

Of 197 blooded *Cx. erraticus* examined, it was possible to identify the blood meal to the species level in 131(66%). *Culex erraticus* was found to feed primarily upon birds and mammals at the Tuskegee site (Tables 3 and 4). Only 3 (2%) of 131 blood meals were found to be derived from reptile species. Two of these were from *Terrapene carolina* (Eastern box turtle) and one was from *Trachemys scripta* (pond slider).

Attempts to identify blood meals from *Ur. sapphirina* proved difficult and only 2 species identifications were made from a total of 35 blooded mosquitoes tested. Both blood meals were obtained from *Rana catesbeiana*.

The temporal feeding pattern by *Cx. peccator* during the three-year study period is shown in Figure 1. This species displayed a predilection for reptiles which peaked in the month of July. During June–August, *Ag. piscivorus* was the predominant species selected, almost doubling all the other identified hosts in two of the three months.

DISCUSSION

EEE virus was found in each of the three mosquito species during each year of the study except 2003, when only *Cx. peccator* was uninfected. These data extend our previous observation at the Tuskegee site to include multi-year prevalence of EEE virus infection in *Ur. sapphirina* and is, to our knowledge, the first report of detection of this virus from *Cx. peccator*. Both mosquito species occur throughout the southeastern United States, with *Ur. sapphirina* extending into the central and eastern States, southeastern Canada, Mexico, and parts of the West Indies.[15] *Culex peccator* is also found in the southcentral and southeastern states as far west as Texas, with southern extensions into the Caribbean as far as Puerto Rico.[16] Because of the constrained host range of each mosquito species for amphibians and reptiles, these data incriminate ectothermic species as possible EEE virus reservoirs in the southeastern United States.

Culex erraticus was infected with EEE virus each year and was also the most abundant species at the site during the three year study. However, analysis of non-avian blood meals not identified to the species level in a previous study[2] indicated that more than 90% of these were from the white-tailed deer instead of reptiles or amphibians. This feeding pattern is interesting in light of recent data from a study in Georgia demonstrating that deer in the coastal plain of that state have seropositive rates to EEE virus as high as 55% (Mead D, unpublished data). The remaining blood meals from *Cx. erraticus* were from two turtle species.

Identification of blood meals from *Ur. sapphirina* proved problematic with only 2 of 35 identified to host species level. Both were from *R. catesbeiana*, the bullfrog. These limited specific identifications support the general assumption that similar to *Ur. lowii*, a closely related species in the same subgenus, *Ur. sapphirina* chooses frogs and other amphibians as hosts.[17] However, the range of host selection by this species

TABLE 2

Amphibian/reptile species present in the Tuskegee National Forest (TNF) site with identified cytochrome B sequences versus number of resident species

Class	Order	No. of species with identified cytochrome B sequences	(No. of species at the TNF site)
Amphibia	Caudata (Salamanders)	5	(11)
	Anura (Frogs and toads)	15	(18)
Reptilia	Crocodilia (Crocodilians)	1	(1)
	Squamata (Snakes and lizards)	25	(27)
	Testudines (Turtles)	8	(5)

TABLE 3

Identification of blood meals from mosquitoes collected at the Tuskegee National Forest site, 2001–2003*

Species	Total screened by PCR	Number feeding on			
		Mammals	Birds	Reptiles	Amphibians
Ur. sapphirina	35	–	–	–	2†
Cx. erraticus	197	41‡	87§	3	0
Cx. peccator	210	0	4¶	95	31

* PCR = polymerase chain reaction; *Ur.* = *Uranotaenia; Cx.* = *Culex.*

† Both blood meals were obtained from *Rana catesbeiana* (bull frog).

‡ Forty blood meals were obtained from *Odocoileus virginianus* (white-tailed deer) and one blood meal from *Canis familiaris* (domestic dog).

§ Avian blood meals were obtained from 19 species. Breakdown by species may be found in Hassan and others.[2]

¶ Three blood meals were obtained from *Nyctanassa violacea* (yellow-crowned night-heron) and one blood meal from *Ardea herodias* (great blue-heron).

TABLE 4

Host-specific identification of reptile/amphibian blood meals taken by *Culex erraticus* and *Cx. peccator* at the Tuskegee National Forest Site, 2001–2003

Mosquito species	Hosts			
	Agkistrodon piscivorus	Other reptiles	*Rana catesbeiana*	Other amphibians
Cx. erraticus	0	3*	0	0
Cx. peccator	77	18†	18	13‡

* Two blood meals were obtained from *Terrapene carolina* (Eastern box turtle) and one blood meal from *Trachemys scripta* (pond slider).

† Eight blood meals were obtained from *Nerodia erythrogaster* (plain-bellied watersnake), five from *Thamnophis sauritus* (Eastern ribbonsnake), two from *Elaphe obsoleta* (Eastern ratsnake), two from *Regina rigida* (glassy crayfish snake), and one from *Trachemys scripta* (pond slider).

‡ Ten blood meals were obtained from *Rana clamitans* (green frog) and three from *Rana sphenocephala* (Southern leopard frog).

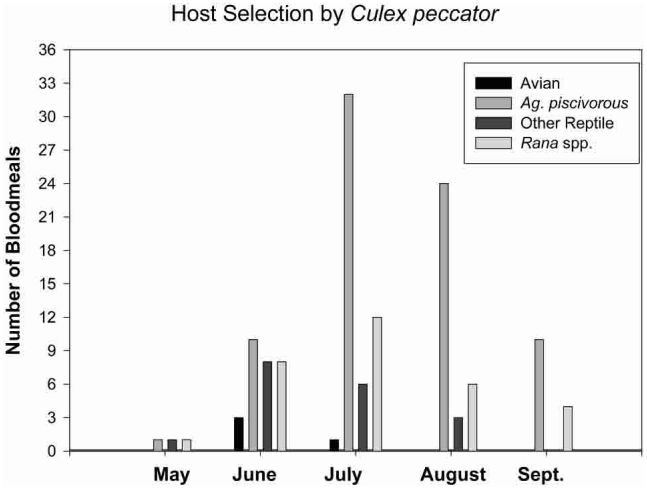

FIGURE 1. Host selection by *Culex peccator* by month at the Tuskegee National Forest Study Site, 2001–2003. Avian = *Nyctanassa violacea* and *Ardea herodias*; *Ag. piscivorus* = *Agkistrodon piscivorus* (cottonmouth); Other Reptile = *Terrapene carolina*, *Trachemys scripta*, *Nerodia erythrogaster*, *Thamnophis sauritus*, *Elaphe obsoleta*, and *Regina rigida*; *Rana* spp. = *R. catesbeiana*, *R. clamitans*, and *R. sphenocephala*.

may be broader. For example, Irby and Apperson[8] identified 2 of 120 blood meals from *Ur. sapphirina* collected in North Carolina and resolved the host of both to an unknown reptile by using a pool of antisera made to the sera of four snake species. Further work is clearly required to determine the optimum state of the *Ur. sapphirina* blood meal for host identification, using either a serologic or PCR-based method.

Culex peccator fed primarily on reptiles and amphibians, a general selection pattern reported previously.[8] The temporal pattern of feeding by this mosquito also demonstrated a relatively broad set of hosts with selection of two species of ciconiiform birds occurring in June and July, and seven reptile and three amphibian species attacked during the five-month season. A striking feature was the large number of blood meals taken from *Ag. piscivorus*, the cottonmouth. This species was selected 62% of the time from the 10 reptile/amphibian species identified. The cottonmouth population at the Tuskegee site is relatively large and stable and therefore provides ample opportunity for host selection by mosquitoes. Cottonmouths are opportunist predators that may remain motionless for relatively long periods of time to ambush prey.[18] This quiescent behavior, particularly at night, likely contributes to successful mosquito feeding as well. Species of *Rana,* which were selected approximately 25% of the time, are also ambush predators and therefore likely to be readily available for blood feeding.

Culex peccator fed at low levels on the yellow-crowned night heron (*N. violacea)* and the great blue heron (*A. herodias)* in the early to mid-portion of the virus transmission season. This finding supports previous reports in Florida[7] and North Carolina[8] that this mosquito occasionally selects avian hosts for blood meals. Interestingly, as is the case for *Ag. piscivorus* and the three *Rana* species, the yellow-crowned night heron is quiescent and may stand motionless for long periods of time. Stamm[19] observed at an endemic EEE virus focus in Louisiana that nestlings of this species stood quietly and allowed scores of mosquitoes to feed on them. He also noted that the yellow-crowned night heron had antibody prevalences to EEE virus that were the highest of any avian species collected in significant numbers at that site. In a very recent study, it was shown that this species is a preferred host by *Cx. erraticus*,[2] an enzootic vector of EEE virus at the Tuskegee site and in other locations in the mid-southern United States.[1,20] The yellow-crowned night heron could therefore provide a nexus for movement of EEE virus from avian to reptile/amphibian reservoirs or vice-versa.

All three mosquitoes analyzed in this study hibernate as inseminated females,[21] suggesting that this survival behavior could serve as a possible over-wintering mechanism for the virus. This activity is clearly different from that of *Cs. melanura*, the enzootic vector of EEE along the east coast of the United States, which over-winters in the larval stage. *Agkistrodon piscivorus* also hibernates after producing young in the mid-to-late summer. Early experimental studies[6] demonstrated that reptiles (snakes and turtles) held either outside during winter or in a refrigerator to simulate that season maintained viremias for six months and could therefore serve as over-wintering reservoir hosts of EEE virus. Thus, the likelihood that *Ag. piscivorus* may be maintaining the virus for significant time periods in the southeastern United States requires further investigation, since previous observations in Georgia indicated that this species frequently had antibodies to EEE virus.[5]

EEE virus is distributed in wet forest habitats southward through the Caribbean and Central America into Brazil and northern Argentina. *Culex* (*Melanoconion*) species are considered to be the principal enzootic vectors throughout this area,[4] with various vertebrate species involved as amplifying hosts. Among these, birds are considered to be the main hosts,[22] although there is evidence that some reptiles such as lizards may be important as well. For example, studies in Panama demonstrated that 13% of *Ameiva* and *Cnemidophorus* spp. and 30% of *Basiliscus* spp. had antibodies to EEE virus.[23]

Berezin[24] also isolated EEE virus from the blood of an iguana in Cuba, as well as 15 strains of this virus from 8 species of birds. Feeding patterns of several *Cx.* (*Melanoconion*) species in the region indicated that reptiles (particularly lizards) and amphibians were frequently selected as hosts.[25] Thus, it is likely that a similar enzootic pattern of EEE virus transmission occurs throughout the southeastern United States where species such as *Cx. peccator* and *Cx. erraticus* occur in conjunction with large, diverse reptilian, amphibian, and avian populations such as those at the Tuskegee site.

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