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Seasonal Dynamics of Mosquito-Borne Viruses in the Southwestern Florida Everglades, 2016, 2017

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Abstract. Mosquitoes were collected for 12 consecutive months beginning June 2016, from 11 locations in the Florida Everglades, Collier County, and tested for viruses by isolation in Vero cells and subsequent identification. One species complex and 31 species of mosquitoes were identified from 668,809 specimens. *Ochlerotatus taeniorhynchus* comprised 72.2% of the collection. Other notable species were *Anopheles crucians* complex, *Culex nigripalpus*, *Cx. erraticus*, and *Cx. cedecei*. Seven species of virus were identified from 110 isolations: Everglades, Gumbo Limbo, Mahogany Hammock, Pahayokee, Shark River, Tensaw, and West Nile viruses. Everglades, West Nile, Tensaw, and Mahogany Hammock viruses were most frequently isolated. Largest numbers of viruses were identified from *Cx. cedecei*, *Cx. nigripalpus*, and *An. crucians* complex. Five species of virus were isolated from *Cx. cedecei*. Viruses were isolated from mangrove, cypress swamp, hardwood hammock, and sawgrass habitats. West Nile virus was isolated August through October when *Cx. nigripalpus* was most abundant. Everglades virus was the most frequently isolated virus from nine species of mosquitoes collected from June through August. Tensaw virus was isolated primarily from *Anopheles* species. Isolations were made in July, August, January, February, and April, suggesting that this virus may be present in host-seeking mosquitoes throughout the year. Mahogany Hammock, Shark River, Gumbo Limbo, and Pahayokee viruses were isolated primarily from *Cx. cedecei* from June through December. Shotgun metagenomic sequencing was used to document that seven pools of *Cx. cedecei* were infected with two arboviruses. As communities expand into the Everglades, more humans will become exposed to arboviruses.

INTRODUCTION

A diversity of arboviruses in south and central Florida was first recognized in the 1960s.[1–4] The report of Venezuelan equine encephalitis virus (VEEV; Togaviridae: Alphavirus),[2,3] now designated as subtype II and named Everglades virus (EVEV; Togaviridae: Alphavirus),[5] in the Brighton Reservation and Big Cypress Reservation in the Florida Everglades, prompted other arbovirus studies, resulting in the isolation and naming of five previously unknown species of Orthobunyavirus from mosquitoes. These were Gumbo Limbo (GLV; Peribunyaviridae: Orthobunyavirus),[6,7] Pahayokee (PAHV; Peribunyaviridae: Orthobunyavirus),[7,8] Shark River (SRV: Peribunyaviridae: Orthobunyavirus),[7,8] Mahogany Hammock (MHV; Peribunyaviridae: Orthobunyavirus,[7,9] and Tensaw (TENV; Peribunyaviridae: Orthobunyavirus).[10,11] None of these viruses has been associated with outbreaks of human disease in Florida, with the exception of infrequent reports of infection with EVEV.[12] Additionally, Keystone virus (KEYV; Peribunyaviridae: Orthobunyavirus) was isolated in the Tampa, FL, area in 1964.[4] This virus was recently reported to cause disease in one human.[13] Mosquito-borne viruses associated with outbreaks of human disease in Florida include St. Louis encephalitis virus (SLEV; Flaviviridae: Flavivirus),[7,14] eastern equine encephalitis virus (EEEV; Togaviridae: Alphavirus),[7] West Nile virus (WNV; Flaviviridae: Flavivirus),[15] Zika virus (ZIKV; Flaviviridae: Flavivirus),[16] dengue virus (DENV; Flaviviridae: Flavivirus),[17] and chikungunya virus (CHIKV; Togaviridae: Alphavirus).[18]

The subgenus *Melanoconion* of the genus *Culex*, from which the majority of tropical New World arboviruses have been isolated,[19] consists of at least 160 designated species in tropical and subtropical areas.[20] These species are divided into two sections: Melanoconion and Spissipes.[21,22] Eight species within this subgenus have been collected in the Everglades: *Culex atratus, Cx. cedecei, Cx. erraticus, Cx. iolambdis, Cx. mulrennani, Cx. panocossa, Cx. peccator*, and *Cx. pilosus*.[23,24] *Culex cedecei* and *Cx. panocossa* are in the Spissipes section, and the remaining six species have been placed in the Melanoconion section. The Spissipes section contains almost all of the enzootic VEEV vectors in the tropics.[19] Adults are primarily found in tropical forests or swamps feeding in the evening or during night on small mammals and opportunistically on other mammals including humans. This behavior of feeding on presumed reservoir hosts of small mammals may contribute to their effectiveness as vectors of VEEV.

Six viruses (EVEV, GLV, MHV, SRV, PAHV, and KEYV) have been isolated from *Melanoconion* species collected in the Everglades. One or more *Melanoconion* species are likely enzootic vectors of EVEV and four Orthobunyaviruses. Everglades virus has been reported to be restricted to the sylvatic habitats of the Everglades in south Florida,[7,19] but serologic studies of dogs indicate this virus may be more widespread.[25] This virus occasionally has been reported to cause human disease,[2,12,26,27] but infections may often not be severe enough for an accurate diagnosis to be made, and thus disease is likely underreported.[25]

Melanoconion species are the important vectors of EVEV.[3,7,28] The isolations or detections of EVEV from *Cx. cedecei*,[7,28] the relatively frequent feeding of *Cx. cedecei*

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on the presumed important mammalian reservoirs (hispid cotton rat, *Sigmodon hispidus* and cotton mouse, *Peromyscus gossypinus*),[28–32] and the demonstrated competency of *Cx. cedecei* to transmit EVEV,[33] indicate that *Cx. cedecei* is the principal vector. However, viremias in Virginia opossum, *Didelphis virginiana*, were reported to last 3–5 days, and this medium-sized mammal along with raccoon, *Procyon lotor*, cotton mice and hispid cotton rats were identified as the only four mammalian species in hammocks in the Everglades to be numerous enough to adequately maintain EVEV.[34]

Tensaw virus was first isolated in Alabama, Georgia, and Florida and has been isolated or detected from mammalian-biting mosquitoes from April through December, including *Aedes vexans*,[35] three species of *Anopheles*,[10,36–39] five species of *Ochlerotatus*,[10,36–38] two species of *Culex* and a *Melanoconion* species,[10,36,37,40] *Psorophora columbiae*,[10,36,37] and *Coquillettidia perturbans*.[10,36–38] *Anopheles crucians* complex (*An*. *crucians*, *An*. *bradleyi*, and *An. georgianus*) is the most frequently infected. It has been isolated from rabbits, *Sylvilagus*, and from small rodents, and a dog,[10,36,37,40,41] and antibody has been detected in medium- and large-sized mammals.[40,42,43] Rabbits, raccoons, cotton rats, and dogs developed viremias after experimental infection.[40] *Anopheles crucians* complex has been reported to feed disproportionately on rabbits.[44] Mammalian reservoirs have not been conclusively identified.[36,45] There has been one reported human case,[46] and antibody has been detected in humans.[10,47,48]

Three Orthobunyaviruses (GLV, MHV, and PAHV)[6,8,9] are known only from the Everglades. Shark River virus also has been isolated in Manatee County, FL, Mexico, and Guatemala.[49,50] These four viruses are not known to cause human or veterinary disease, but some are closely related to species that cause a self-limited, dengue-like illness that may not be easily diagnosed.[51]

Gumbo Limbo virus was first collected in the Everglades National Park in 1963 and described in 1969 and is the only Group C Orthobunyavirus in the United States.[6,7,52] It was isolated from 13 pools of *Culex (Melanoconion*) and from one pool of *Ochlerotatus taeniorhynchus*. It was also isolated from two hispid cotton rats. This virus was again isolated from the same National Park in 1966–1969 from 48 pools of *Culex (Melanoconion)* and one pool of *Oc*. *taeniorhynchus*,[40] and it has been reported to circulate among *Culex (Melanoconion*) mosquitoes, hispid cotton rats, and likely other rodents.[7]

Mahogany Hammock virus, a member of the Guama antigenic group, was isolated initially in 1964 in the Everglades National Park and described in 1969.[7,9] This virus was isolated from 13 pools of *Melanoconion* specimens and from one hispid cotton rat in 1964 and 1965 and from 32 pools of *Melanoconion* collected in 1966–1969.[40] Maintenance of MHV was reported to involve rodents and *Melanoconion* mosquitoes.[7]

Pahayokee virus, belonging to the antigenic group Patois, was isolated from *Culex* (*Melanoconion*) mosquitoes in the Florida Everglades National Park in 1963 and 1964 and named and characterized in 1969.[7,8] A subsequent study suggested that PAHV and SRV were separate species.[53] An isolation also was made from one pool of *Cx*. *nigripalpus*.[40]

Shark River virus also belongs to the Patois antigenic group. It was isolated from 66 pools of *Melanoconion* mosquitoes and from one hispid cotton rat in Everglades National Park in Florida in the 1960s and was characterized and named in 1969.[7,8,40] Single isolations were made from *Cx. nigripalpus* and *An. crucians* complex.

Keystone virus, originally isolated in Keystone, FL, in 1964, is distributed from Connecticut to Florida westward to Texas.[40] Vertically infected *Oc. atlanticus* are competent to transmit KEYV and more likely to transmit than females horizontally acquiring virus from gray squirrels, *Sciurus carolinensis*, which are often viremic.[54]

The Everglades is a region of tropical wetlands that originally covered about one-third of the Florida Peninsula. It is subject to frequent flooding in the wet season, usually during June through September, and drought in the dry season. Sawgrass prairie is its primary feature, which is interspersed with cypress swamps, estuarine mangrove forests, tropical hardwood hammocks, pine forest, and the marine environment of Florida Bay. Beginning in 1882 to the present, 50% of the original land was developed into agricultural or urban areas.[55] While the Everglades is sparsely populated, about seven million people live near the Florida Everglades, an area that receives 60 million annual tourists. Currently, efforts are being made to restore wetland ecosystems for purposes of habitat and wildlife conservation.[56] These past and extensive current efforts have affected and will affect mosquito populations as well as the viruses they carry. With the large human population that lives nearby, the large number of tourists that visit throughout the year, and the continued encroachment, viruses associated with mosquitoes could cause outbreaks of human disease in or near the Everglades. Orthobunyavirus infections are common elsewhere in the world, but actual diagnosis of acute illness is often rare because of lack of laboratory diagnosis.[57] With six known species of Orthobunyavirus in the Everglades, one or more of these poorly studied viruses and EVEV may cause disease and may have emerging disease potential.

We therefore initiated a study of mosquitoes and documented their monthly abundance and their viruses in a relatively small portion of southwestern Everglades during a 1-year timeframe in 2016 and 2017 and compared our findings with those previously published.

MATERIALS AND METHODS

Weather. Data for Naples, FL, obtained from https://www.usclimatedata.com/.

Mosquito collections and identification. Mosquitoes were trapped in the Everglades in Collier County, FL, in 2016 and 2017. Monthly collections were made from six sites beginning June 2016 and an additional four sites beginning July 2016 through May 2017 (Table 1, Figure 1). Our trapping area was centered near the residential area known as Port of the Islands, a community in the eastern end of Naples, FL. It extended over an 11 mile (17.7 km) stretch along Highway 41 east and west of Port of the Islands on both the north and south sides of the highway. Port of the Islands is bisected by the Faka Union Canal, which extends south into the Ten Thousand Islands and the Gulf of Mexico. Our trapping area covered approximately 0.01% of the 2.5 million acres (1,011,736 hectares) of the Everglades. Areas where traps

TABLE 1
Specific locations and landscapes of mosquito trapping sites, southwestern Everglades, Collier County, Florida

Site number	Latitude degrees north	Longitude degrees west	Landscape
1	25.9493	81.5065	Mangrove
2	25.9720	81.5068	Cypress swamp
3	25.9522	81.5119	Residential
4	25.9429	81.4701	Cypress swamp
5	25.9797	81.5047	Hardwood hammock
6	25.9781	81.4942	Sawgrass
7	25.9851	81.5062	Hardwood hammock
8	25.9781	81.5008	Cypress swamp
9	25.9782	81.4883	Sawgrass
10	25.9590	81.5149	Mangrove
13	26.0196	81.6318	Hardwood hammock/tires

were placed usually had been disturbed and included the building of roads, water pumping stations, residential areas, Native American community, canals, the diversion of water, land clearing, and trail building. Habitats included mangrove, residential, hardwood hammock, sawgrass, and cypress swamps. Additionally, mosquitoes were trapped on a single night on August 11, 2016 in a hardwood hammock with discarded tires on Lake Park BLD relatively near a convenience store, immediately south of Route 41 (Site 13, Table 1, Figure 1).

Mosquitoes were collected with CDC miniature light traps (Model 512; John W. Hock Co., Gainesville, FL) baited with compressed CO_2, released at a rate of 500 mL/minute. Traps were hung from a branch of a tree at a height of 0.9 m above the ground in the afternoon or evening and retrieved the following morning. Collected mosquitoes were placed in an insulated container with cold packs, brought to the laboratory, knocked down with freezing temperatures, and transferred into glass or plastic containers sealed with water and gas-proof tape. Containers were appropriately labeled and immediately stored on dry ice or in a −80°C freezer until packaged in dry ice within a Styrofoam shipping container and sent by overnight mail to The Connecticut Agriculture Experiment Station (CAES) in New Haven, CT. Mosquitoes were stored at CAES in a −80°C freezer until processing.

Mosquitoes were removed from the freezer, placed on a cold platform, and each specimen was identified to species using a dissecting microscope.[23,58,59] Specimens were pooled according to species, date, and trap location. Pools contained from one to 50 mosquitoes. Mosquitoes were transferred to a 2 mL plastic microcentrifuge vial containing a copper BB pellet and frozen at −80°C until attempted isolation of virus. All mosquitoes were tested, except during the outbreak of *Oc. taeniorhynchus* in April and May 2017, when a maximum of 1,000 specimens of this species was often tested from each site.

Virus isolation. Upon removal from −80°C, each vial of mosquitoes received 1 mL of phosphate-buffered saline containing 0.5% gelatin, 30% rabbit serum, and 1% 100× antibiotic-antimycotic (10,000 units ug/mL of streptomycin sulfate, and 25 μg/mL of amphotericin B, in 0.85% saline [Invitrogen, Carlsbad, CA]). Mosquitoes were triturated by placing vials containing a copper BB in a Vibration Mill MM 300 (Retsch Laboratory, Irvine, CA) set at 25 cycles per second for 4 minutes. This mill was operated inside a biosafety hood.

Following centrifugation at 4°C for 7 minutes at 7,000 rpm, a 100 μL sample of supernatant was inoculated onto a 1- or 2-day old monolayer of Vero cells. Cells were grown in a 25 cm^2 flask containing 4 mL of Minimum Essential

FIGURE 1. Mosquito trapping locations in or near Port of the Islands, Florida Everglades, Collier County, Florida. Google Earth map. This figure appears in color at www.ajtmh.org.

Medium (MEM) supplemented with fetal bovine serum, L-glutamine, and antibiotic-antimycotic, and buffered with sodium bicarbonate (Invitrogen). Flasks were stored in an incubator set at 37°C and 5% CO_2. Growth medium was decanted from each flask prior to addition of the inoculum. After inoculation, flasks were rocked for 5 minutes, after which 4 mL of new growth medium, supplemented with 2× as much antibiotic-antimycotic as original MEM, was added to each flask. Inoculated flasks were placed into the CO_2 incubator, and cells were examined for cytopathogenic effects 3–7 days after inoculation.

Virus identification. RNA was extracted from primary viral isolates using the viral RNA Kit (Qiagen, Valencia, CA). West Nile virus was identified by a TaqMan reverse transcriptase-polymerase chain reaction (RT-PCR) assay using primers:

WNV10533 AAGTTGAGTAGACGGTGCTG and WNV10 625 AGACGGTTCTGAGGGCTTAC with WNV10560probe [FAM]CTCAACCCCAGGAGGACTGG[BHQ1] by procedures described previously.[60]

To identify EVEV, a portion of the nonstructural polyprotein gene was amplified by primers ALPHAfwd (TCCACACT CTGTTTGAYATGTC) and ALPHArev (GCATCGATGATCTT-CACYTCCAT) using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). Amplification was performed using the following conditions: One cycle of 50°C for 30 minutes and 94°C for 30 seconds, 10 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 1 minute, followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 1 minute + 5 seconds per cycle, and 1 cycle of 68°C for 7 minutes. The amplification product (~460 nts) was then commercially sequenced at the DNA Facility on Science Hill (New Haven, CT) using an Applied Biosystems 3730xl 96-capillary genetic analyzer (Foster City, CA).

To identify TENV, the entire S segment was amplified by primers BUNS+new: 5-TGACCAGTAGTGTACTCCAC-3′_ and BUNS-new: 5_-CAAGCAGTAGTGTGCTCCAC-3′ using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN), as previously described.[61,62] The amplification product (~950 bps) was then commercially sequenced at the DNA Analysis Facility on Science Hill.

The other species of Orthobunyavirus (GLV, MHV, PAHV, and SRV) were initially identified by shotgun metagenomic sequencing of RNA from mosquito pools and virus isolates. Shotgun libraries from total RNA were prepared using the Kapa RNA Hyper Prep kit (Roche Diagnostics, Indianapolis, IN). Libraries were sequenced using paired-end 2x75 sequencing on the NextSeq system using mid output run (Illumina, San Diego, CA). Datasets were processed by: 1) removing low quality and adapter sequences, 2) removing PCR duplicates, 3) removing host sequences (mosquito or African green monkey sequences, using the *Cx. pipiens* and African green monkey genomes), 4) de novo assembling remaining reads, 5) taxonomically assessing the assembled contigs, first by comparison to nucleotide sequences in Genbank, then by comparison to protein sequences. Sequences were then manually inspected and validated putative virus and virus-like contigs.

Polymerase chain reaction primers were subsequently designed to target a conserved region of the M segment and used to identify the remaining isolates of GLV, MHV, PAHV, and SRV. Primers FL-BUNMfwd (ATGGAGCATGGKATAGC TGA) and FL-BUNrev2 (CCACATACACAATTTTTCCCR-CATTT) were used to amplify a portion of the M polyprotein gene. Reactions were set up using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN). Amplification was performed as described above for EVEV. The amplification product (~712 nts) was then commercially sequenced at the DNA Facility on Science Hill. The edited nucleotide sequences of GLV, MHV, PAHV, and SRV were submitted to GenBank (accession nos. MT476027-MT47 6041 and MW287555-MW287561).

Phylogenetic analysis. Phylogenetic relationships among Orthobunyavirus sequences were evaluated by maximum likelihood analysis in MEGA X version 10.1. The general time reversible substitution model was selected for phylogenetic analysis after performing maximum likelihood fits of 24 different models in Mega. Support for individual nodes was evaluated by performing 1,000 bootstrap replicates.

Statistics. The field infection rates for each species of mosquito infected with a specific virus for each month was determined per 1,000 specimens using the bias-corrected maximum likelihood estimation (MLE) method.[63] Spearmen rank-correlation analysis ($P < 0.05$; Systat 11, Systat Software, Inc., Point Richmond, CA) was used to compare numbers of collected mosquitoes at different habitat sites.

RESULTS

Weather. The average yearly rainfall in Naples, Fl, is 55.56 inches (141.1 cm). Total rainfall during our study was 57.03 inches (144.9 cm). The largest and smallest monthly rainfalls occurred in August 2016 (17.86 inches [45.4 cm]) and March 2017 (0.7 inches [1.8 cm]). A total of 43.97 inches (111.7 cm) fell during June–September 2016 ("wet season") and 13.06 inches (33.2 cm) fell during October 2016–May 2017 ("dry season"). Average monthly high temperatures ranged from 91.6°F (33.1°C) in July 2016 to 76.8°F (24.9°C) in January 2017.

Mosquito species. Thirty-two species or species complexes were identified from 668,809 specimens collected in 2016 and 2017 (Supplemental Table 1). *Ochlerotatus taeniorhynchus* was collected in all months and represented 72.2% of the collection. The majority was collected in April and May 2017 following a massive emergence (Figure 2). This species was abundant at all collecting sites with significantly larger numbers at sawgrass landscapes (Table 2).

Other notable species or species complexes included *An. crucians* complex (9.6%), *Cx. nigripalpus* (8.2%), *Cx. erraticus* (4.7%), *An. atropos* (1.4%), *Cx. iolambdis* (1.0%), *Ps. columbiae* (1%), and *Cx. cedecei* (0.8%) (Supplemental Table 1). These species were found at most sites where collections were made monthly. Numbers of *An. crucians* complex, *Cx. nigripalpus*, and *Cx. pilosus* were not significantly different among habitats (Table 2). Significantly fewer *Cx. cedecei* and *Cx. iolambdis* were collected at sawgrass habitats. *Mansonia dyari* were more abundant at cypress swamp and hardwood hammock than sawgrass landscapes, and *Ps. columbiae* were least abundant in mangrove habitat.

Viruses. Viruses were isolated during all months except March and May 2017 (Table 3). The largest numbers of isolations were made in July through October 2016 when 38, 29, 13, and 11 were made, respectively. Seven species of viruses were identified among the 110 isolates (Table 4). Everglades (31.8%), WN (22.7%), TEN (15.5%), and MH (10.9%) viruses were the most prevalent. The largest number

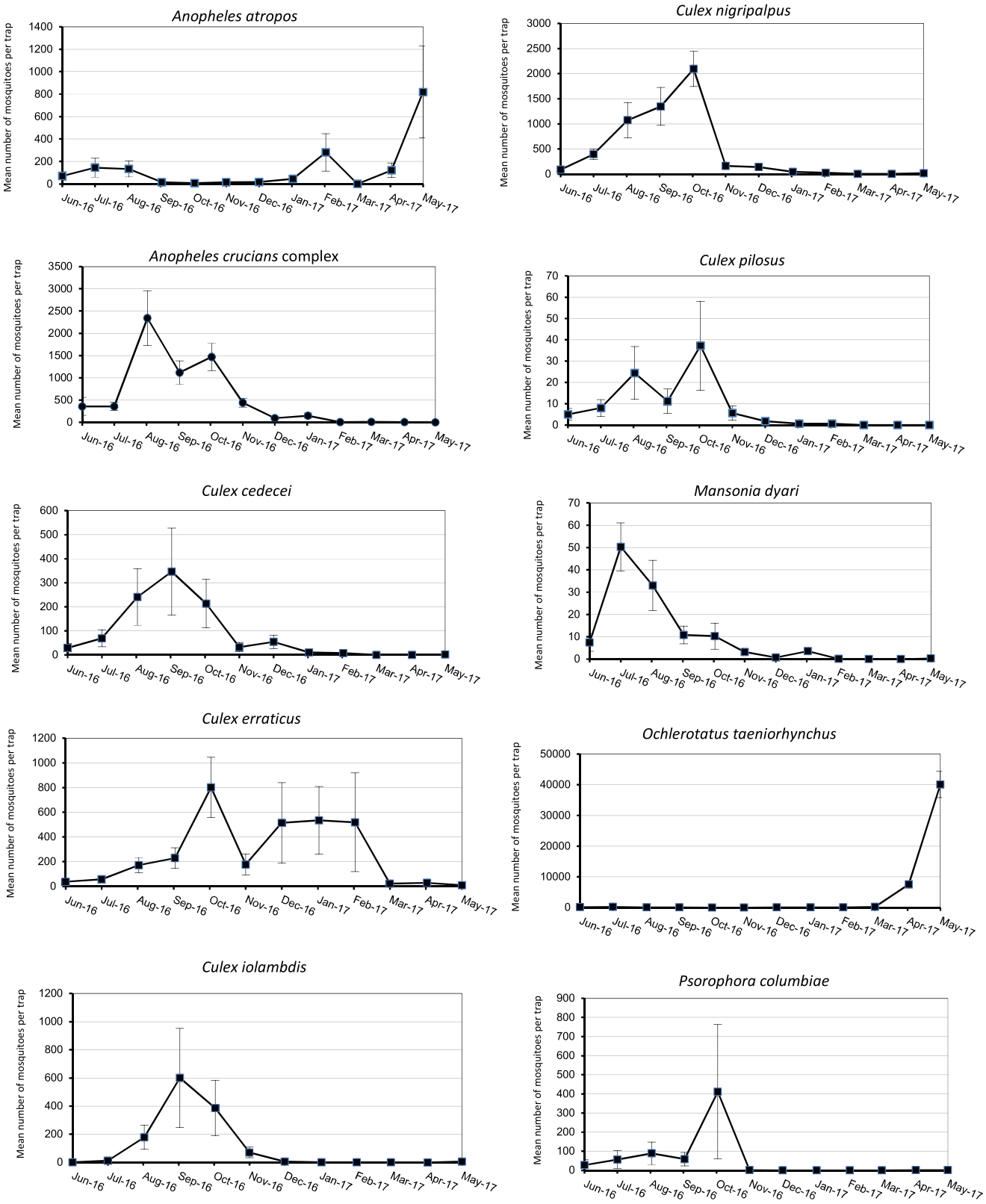

FIGURE 2. Mean monthly numbers per trap (±SEM) of *Anopheles crucians* complex and nine species of mosquitoes, from which viruses were isolated, southwestern Everglades, 2016, 2017. This figure appears in color at www.ajtmh.org.

of isolates were from *Cx. cedecei* ($N = 41$), *Cx. nigripalpus* ($N = 28$), and *An. crucians* complex ($N = 24$) (Table 5). Five different viruses (EVEV, GLV, MHV, PAHV, and SRV) were isolated from *Cx. cedecei*, whereas one or two different viruses were isolated from *An. crucians* complex, *Anopheles* sp., and each of the other eight species of mosquitoes.

Viruses were isolated from all habitats except residential (Table 4). Two viruses were associated with sawgrass

TABLE 2
Mean numbers of *Anopheles crucians* complex and nine species of mosquitoes, from which viruses were isolated, by habitat, southwestern Everglades, Florida, 2016–2017

Habitat	Species									
	Anopheles crucians complex	*Culex cedecei*	*Culex erraticus*	*Culex nigripalpus*	*Ochlerotatus taeniorhynchus*	*Anopheles atropos*	*Culex iolambdis*	*Culex pilosus*	*Mansonia dyari*	*Psorophora columbiae*
Cypress swamp	5,496 a*	1,037 a	3501 a	6,648 a	48,279 b	29 c	516 ab	43 a	150 a	84 b
Hardwood hammock	7,423 a	545 a	7,698 ab	5,635 a	40,412 bc	1,634 abc	160 ab	39 a	151 a	367 ab
Mangrove	5,747 a	534 a	1,356 b	5,709 a	3,7201 c	2,153 a	2,514 a	98 a	100 ab	34 c
Saw grass	1,0203 a	19 b	1,129 b	5,191 a	70,588 a	369 b	22 c	36 a	60 b	2,682 a
Residential†	909	9	157	668	4,1585	881	34	0	46	64

* Numbers within each column with the same letter are not significantly different.
† There was no replicate for residential.

habitat: WNV and TENV. Everglades virus, TENV, MHV, SRV, and WNV were isolated at mangrove sites. All seven different viruses were obtained at cypress swamp and hardwood hammock habitats.

West Nile virus. This introduced Flavivirus was widely distributed and isolated from mosquitoes collected from eight locations and from four different habitats (Table 4). The 25 isolates were from *Cx. nigripalpus* (96%) and *Ps. columbiae* (4%) (Table 5). Isolations were made in August through October 2016 (Table 3), when populations of *Cx. nigripalpus* were most abundant (Figure 2). Monthly infection rates in *Cx. nigripalpus* were 0.99 per 1,000 mosquitoes in September but lower in August and October (Table 3). The infection rate of *Ps. columbiae* in October was comparable to that of *Cx. nigripalpus*.

Everglades virus. This Alphavirus was the most frequently isolated virus. The 35 isolates were obtained from *An. crucians*

TABLE 3
Monthly infection rates for seven viruses, southwestern Florida Everglades, 2016, 2017

Virus	Mosquito species	Year	Month	Total no. of mosquitoes (pools)	No. of virus isolations	Infection rate (95% CI)
West Nile	*Culex nigripalpus*	2016	August	11,745 (255)	6	0.52 (0.21–1.07)
	Cx. nigripalpus	2016	September	13,406 (300)	13	0.99 (0.55–1.65)
	Cx. nigripalpus	2016	October	20,900 (449)	5	0.24 (0.09–0.53)
	Psorophora columbiae	2016	October	4,121 (113)	1	0.24 (0.01–1.18)
Everglades	*Anopheles atropos*	2016	July	798 (18)	3	4.02 (1.07–11.09)
	An. crucians complex	2016	June	2,155 (48)	5	2.43 (0.91–5.41)
	An. crucians complex	2016	July	3,572 (80)	2	0.56 (0.10–1.85)
	An. crucians complex	2016	August	25,736 (536)	3	0.12 (0.03–0.32)
	An. sp.	2016	June	148 (4)	1	5.86 (0.41–29.45)
	Cx. cedecei	2016	July	403 (27)	6	17.64 (7.77–36.14)
	Cx. cedecei	2016	August	1,317 (60)	3	2.34 (0.62–6.37)
	Cx. erraticus	2016	July	561 (26)	1	1.72 (0.10–8.23)
	Cx. iolambdis	2016	July	68 (13)	1	14.93 (0.87–72.79)
	Cx. nigripalpus	2016	July	3,948 (94)	4	1.03 (0.34–2.48)
	Cx. pilosus	2016	July	40 (13)	2	51.89 (9.82–163.27)
	Mansonia dyari	2016	July	503 (22)	1	1.92 (0.12–9.26)
	Ochlerotatus taeniorhynchus	2016	June	1,015 (25)	1	0.99 (0.06–4.83)
	Oc. taeniorhynchus	2016	July	1,726 (44)	2	1.18 (0.21–3.87)
Gumbo Limbo	*Cx. cedecei*	2016	July	403 (27)	2	5.10 (0.93–16.87)
	Cx. cedecei	2016	August	1,317 (60)	2	1.55 (0.28–5.12)
	Cx. cedecei	2016	October	964 (70)	3	3.28 (0.86–9.01)
	Cx. erraticus	2016	October	7,403 (200)	1	0.14 (0.01–0.65)
Mahogany Hammock	*Cx. cedecei*	2016	June	156 (9)	1	6.64 (0.40–36.1)
	Cx. cedecei	2016	July	403 (27)	2	5.10 (0.93–16.87)
	Cx. cedecei	2016	August	1,317 (60)	7	5.56 (2.49–10.91)
	Cx. cedecei	2016	November	178 (17)	1	5.76 (0.34–29.32)
	Cx. cedecei	2016	December	296 (16)	1	3.26 (0.20–15.76)
Pahayokee	*Cx. cedecei*	2016	August	1,317 (60)	2	1.54 (0.28–5.05)
	Cx. cedecei	2016	October	964 (70)	1	1.04 (0.06—5.11)
	Cx. cedecei	2016	November	178 (17)	1	5.82 (0.34—29.89)
Shark River	*Cx. cedecei*	2016	July	403 (27)	1	2.39 (0.14—11.41)
	Cx. cedecei	2016	August	1,317 (60)	4	3.16 (1.03—7.62)
	Cx. cedecei	2016	November	178 (17)	2	11.78 (2.23—39.34)
	Cx. cedecei	2016	December	296 (16)	2	6.53 (1.28—20.95)
Tensaw	*An. atropos*	2016	July	798 (18)	2	2.56 (0.47—8.46)
	An. crucians complex	2016	July	3,572 (80)	9	2.66 (1.31—4.88)
	An. crucians complex	2016	August	25,736 (536)	2	0.08 (0.01—0.25)
	An. crucians complex	2017	January	1,499 (37)	2	1.36 (0.24—4.48)
	An. crucians complex	2017	February	57 (11)	1	18.46 (1.05—92.82)
	Oc. taeniorhynchus	2017	April	12,671 (255)	1	0.08 (0.00—0.38)

TABLE 4
Viruses isolated by collecting site and habitat, Collier County, Florida, 2016, 2017

Site number	Habitat	Everglades	Gumbo Limbo	Mahogany Hammock	Pahayokee	Shark River	Tensaw	West Nile	Total
1	Mangrove	17	0	0	0	0	3	0	20
2	Cypress swamp	2	0	1	0	1	0	2	6
3	Residential	0	0	0	0	0	0	0	0
4	Cypress swamp	13	1	5	1	3	3	3	29
5	Hardwood hammock	2	3	1	1	2	0	1	10
6	Sawgrass	0	0	0	0	0	4	5	9
7	Hardwood hammock	0	0	1	0	0	1	3	5
8	Cypress swamp	1	4	2	2	1	1	4	15
9	Sawgrass	0	0	0	0	0	3	0	3
10	Mangrove	0	0	2	0	2	2	5	11
13	Hardwood hammock/tires	0	0	0	0	0	0	2	2
Total		35	8	12	4	9	17	25	110

complex, *Anopheles* species, and eight different species of mosquitoes, primarily collected in mangrove and cypress swamp habitats (Tables 3–5). Largest numbers of isolates were from *An. crucians* complex and *Cx. cedecei*. Virus was isolated during June through August 2016 with the largest numbers isolated in July ($N = 22$) (Table 3). High infection rates were recorded in July 2016 for *Cx. cedecei*, *Cx. iolambdis*, and *Cx. pilosus* of 17.64, 14.93, and 51.89 per 1,000 mosquitoes, respectively (Table 3). Largest numbers of viruses were isolated before peak numbers of these three species occurred in August through October (Table 3, Figure 2). Lower infections rates were recorded for other species (Table 3).

Tensaw virus. The 17 isolates of this Orthobunyavirus were primarily from *An. crucians* complex (82%) (Table 5). One and two isolations were from *Oc. taeniorhynchus* and *An. atropos*, respectively. Infected mosquitoes were collected most frequently from cypress swamp, mangrove, and sawgrass habitats (Table 4). One isolation was from a hardwood hammock. Virus was isolated in 2016 and 2017. The largest number of isolates were in July (65%) before numbers of *An. crucians* complex peaked (Figure 2, Table 3), when infection rates were 2.66 per 1,000 mosquitoes. The virus was also isolated from *An. crucians* complex in August, January, and February. The infection rate for *An. crucians* complex in February was 18.46 per 1,000 specimens when populations were low (Table 3, Figure 2).

Mahogany Hammock virus. Twelve virus isolates from *Cx. cedecei* collected from six locations in mangrove, cypress swamp, and hardwood hammock habitats were identified as MHV (Tables 4 and 5). Two-thirds of the isolations were from mosquitoes collected in cypress swamps. They were made in June through August and in November and December 2016 with monthly infection rates ranging from 3.26 to 6.64 per 1,000 specimens (Table 3). Seven of the isolates were made in August when mosquitoes were increasing but below their peak in September (Figure 2). Five of the positive pools contained an additional Orthobunyavirus. Mosquito pools containing 50, 34, 1, 35, and 15 specimens were infected also with GLV, PAHV, SRV, SRV, and SRV, respectively (Table 5). Seven isolates belonged to a single clade of genetically related strains closely allied to the MHV prototype isolated in 1964 (Figure 3) and were distinctly different from *Guajara* virus and *Bimiti* virus, other members of the Guama antigenic group.

Shark River virus. Nine isolates were made from *Cx. cedecei* collected in July, August, November, and December, primarily in cypress swamps but also from hardwood hammock and mangrove habitats (Tables 3–5). Infection rates exceeded two and three per 1,000 specimens in July and August when mosquitoes were increasing (Table 3, Figure 2), but they were higher when mosquitoes were less abundant in November and December. Five pools containing up to 30 specimens were dually infected with MHV or GLV (Table 5). Five isolates were phylogenetically similar to SRV isolate 64U80 isolated from *Melanoconion* species in 1964 and distinctly different from the 16 other Orthobunyaviruses isolated in 2016 (Figure 3).

TABLE 5
Seven viruses isolated from *Anopheles crucians* complex, *Anopheles* species, and nine species of mosquitoes collected in southwestern Everglades, Florida, 2016–2017

Species	Everglades	Gumbo Limbo	Mahogany Hammock	Pahayokee	Shark River	Tensaw	West Nile	Total
Anopheles atropos	3	0	0	0	0	2	0	5
An. crucians complex	10	0	0	0	0	14	0	24
An. species	1	0	0	0	0	0	0	1
Culex cedecei	9	4, 2*, 1†	7, 3‡, 1§, 1†	3, 1§	4, 3‡, 2*	0	0	41
Cx. erraticus	1	1	0	0	0	0	0	2
Cx. iolambdis	1	0	0	0	0	0	0	1
Cx. nigripalpus	4	0	0	0	0	0	24	28
Cx. pilosus	2	0	0	0	0	0	0	2
Mansonia dyari	1	0	0	0	0	0	0	1
Ochlerotatus taeniorhynchus	3	0	0	0	0	1	0	4
Psorophora columbiae	0	0	0	0	0	0	1	1
Total	35	8	12	4	9	17	25	110

* Gumbo Limbo and Shark River viruses present in same pool.
† Mahogany Hammock and Gumbo Limbo viruses present in same pool.
‡ Mahogany Hammock and Shark River viruses present in same pool.
§ Mahogany Hammock and Pahayokee viruses present in same pool.

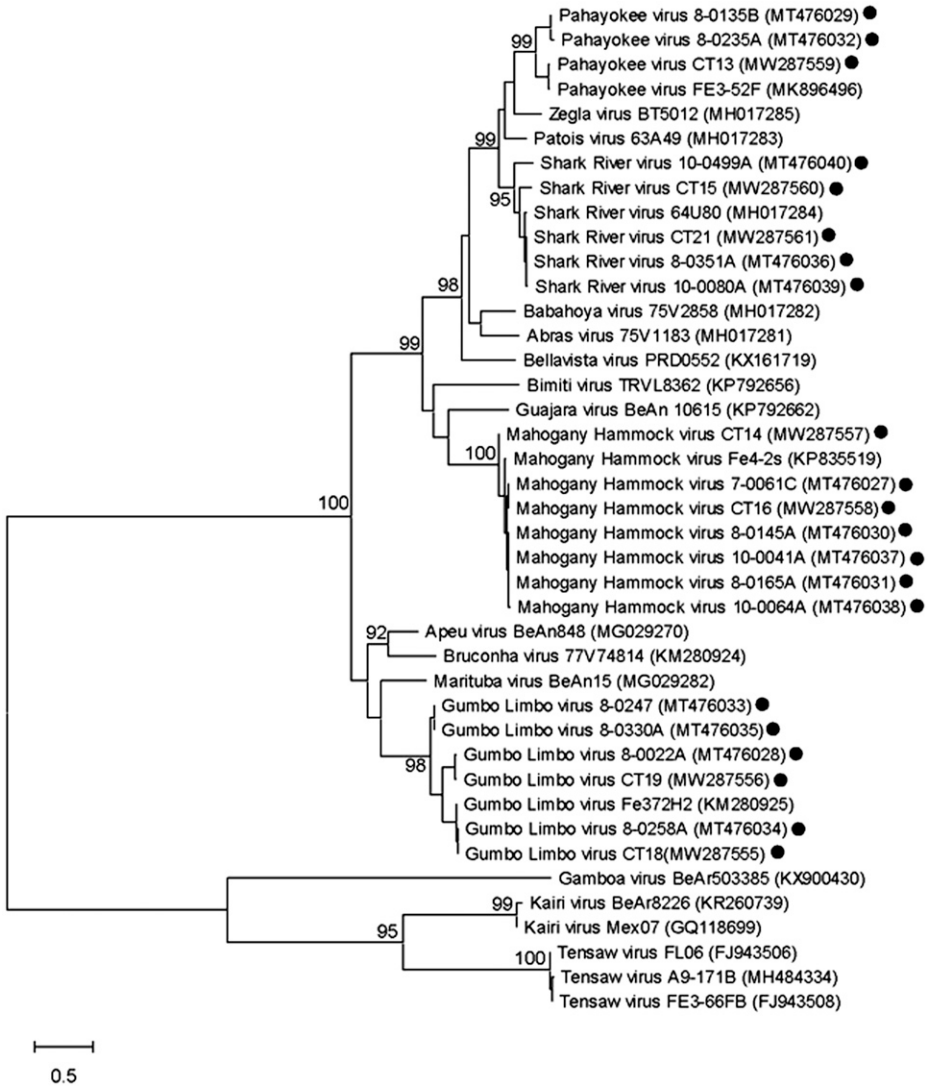

FIGURE 3. Phylogenetic tree showing relationships of Gumbo Limbo, Mahogany Hammock, Pahayokee, and Shark River viruses that were isolated from mosquitoes collected in southwestern Everglades, Florida, 2016. Relationships are based on ML analysis of M-segment nucleotide sequences. Virus strains from this study are highlighted with a black circle. Branch lengths are proportional to the number of nucleotide substitutions per site. Bootstrap values ≥ 90% are presented only at major nodes for clarity.

Gumbo Limbo virus. This Orthobunyavirus was isolated from seven *Cx. cedecei* (87.5%) and one *Cx. erraticus* (12.5%) collected in cypress swamp and hardwood hammock habitats during July, August, and October 2016 (Tables 3–5). Monthly infection rates for *Cx. cedecei* were as high as 5.1 per 1,000 mosquitoes in July prior to their peak abundance in September (Table 3, Figure 2). Three of the infected pools containing 35, 50, and 50 specimens also were infected with SRV, SRV, and MHV, respectively (Table 5). The infection rate for *Cx. erraticus* was 0.14 per 1,000 specimens in October when mosquitoes had reached peak numbers (Table 3, Figure 2). Six isolates were phylogenetically similar to GLV strain FE372H2 isolated in the Everglades in 1963 (Figure 3). These six isolates were distinctly different from the 15 other Orthobunyaviruses isolated in 2016 but clustered with three other Group C viruses, Marituba, Bruconha, and Apeu.

Pahayokee virus. This virus was isolated from four pools of *Cx. cedecei* collected in cypress swamp and hardwood hammock habitats in August, October, and November 2016 (Tables 3–5). Infection rates of 1.54 and 1.04 per 1,000 mosquitoes were recorded in August and October but reached 5.82 in November when few *Cx. cedecei* were collected (Table 3, Figure 2). One pool with 34 specimens also contained MHV (Table 5). Three isolates were phylogenetically similar to the Pahayokee virus prototype isolated in 1963 and distinctly different from the 18 other Orthobunyaviruses isolated in 2016 (Figure 3).

DISCUSSION

Our small sampling area may be representative of disturbed areas of the Everglades near coastal Gulf of Mexico, but it is not representative of vast undisturbed areas away from the coast or disturbed areas in eastern Florida. We collected 42% of the reported species of mosquitoes in Florida and 76% reported from Collier County but did not collect significant numbers of mosquito species associated with

human disease reported elsewhere in Florida, such as *Ae. aegypti*, *Ae. albopictus*, *Culiseta melanura*, and *Cx. quinquefasciatus*. We also clarify that our study encompassed a time frame of 12 months with an uneventful weather pattern. Had we sampled over a much longer period-of-time with different weather patterns, we may have encountered additional mosquito species and viruses. Nonetheless, we isolated six different viruses reported to occur elsewhere in the Everglades in the 1960s and a more recent invasive virus, WNV.

Culex cedecei is a prominent mosquito in our sampling area of the Everglades, even though it comprised only 0.8% of the mosquitoes collected. This percentage would have been higher if not for the outbreak of *Oc. taeniorhynchus* in April and May 2017, the last two months of our study. Although previous studies have emphasized hardwood hammock habitats for *Cx. cedecei*,[7,28] we found it equally abundant in mangrove, cypress swamps, and hardwood hammocks primarily in August through October. Thirty-seven percent of viral isolations were from *Cx. cedecei*. Isolations were made from June to December, although no isolations were made from this species in September. Monthly infection rates were as high as 17.64 per thousand specimens in July for EVEV and 11.78 in November for SRV. This mammalian feeding species obtains blood from small-, medium-, and large-sized mammals, including humans, and is the enzootic vector of EVEV and likely Orthobunyaviruses.[28,29,33]

Eighty-five percent of the viral isolations were made from *An. crucians* complex, *Cx. cedecei*, and *Cx. nigripalpus* (Table 5). Everglades virus and TENV were isolated from *An. crucians* complex, EVEV and WNV were isolated from *Cx. nigripalpus*, and five different viruses, EVEV, GLV, MHV, PAHV, and SRV, were isolated from *Cx. cedecei*.

Viruses were isolated from *An. crucians* complex, *Anopheles* sp., and nine different species of mosquitoes at sawgrass, hardwood hammock, cypress swamp, and mangrove habitats. West Nile virus and TENV were isolated from sawgrass habitat where *Cx. nigripalpus* and *An. crucians* complex were abundant (Tables 2 and 4). Everglades virus, WNV, MHV, SRV, and TENV were isolated from hardwood hammock, cypress swamp, and mangrove landscapes where *Cx. nigripalpus*, *An. crucians* complex, and *Cx. cedecei* were relatively abundant. Gumbo Limbo and PAHV were isolated in cypress swamp and hardwood hammock habitats.

Viruses tended to be isolated during distinct times of the year, though there were exceptions. Two viruses, WNV and EVEV, were each isolated during a 3-month period from August to October and from June to August, respectively. In contrast, the Orthobunyaviruses were isolated over a 4-month or longer period (Table 3). Gumbo Limbo virus was isolated from July into October, and TENV was isolated in July and August 2017 and from January to April 2017. Keystone virus was not isolated, probably because few *Oc. atlanticus/tormentor* were collected.

Competence,[64] or the ability of a mosquito species to acquire and later transmit a virus through bite or vertical transmission to a naïve host, has been determined for one or more species infected with WNV, EVEV, and TENV.[33,65,66] Isolation of a specific virus, as we have reported here, does not prove competency, but repeated isolation and consistently high virus infection rates may suggest that the mosquito species is important in transmission.[10,67]

West Nile virus is a relatively new addition in the Everglades and was first reported in Florida in 2001, with virus activity confirmed in 65 of the state's 67 counties, including Collier County where this study was conducted.[15] Focal outbreaks and not widespread epidemics have characterized WNV amplification in Florida.[68] A total of 402 cases were reported in Florida from 2001 to 2018, with four cases occurring in Collier County, but none was reported in 2016 during our study.[69]

Culex nigripalpus was the important enzootic vector of WNV in the southwestern Everglades. Ninety-six percent of the 25 isolates of WNV were obtained from this species. All isolations were made during August through October when *Cx. nigripalpus* were abundant, averaging more than 1,000 specimens per trap-night. Infected specimens were collected in mangrove, hardwood hammock, sawgrass, and cypress swamp habitats. Monthly infection rate in September was 0.99 infected mosquitoes per 1,000 specimens (Table 3). This infection rate compares to minimum infection rates for *Cx nigripalpus* elsewhere in Florida, 2005 of 0.12–2.93 per 1,000 mosquitoes.[70] *Culex quinquefasciatus*, a minor species in our study, is considered to be the primary enzootic and epizootic vector of WNV in southeastern United States.[71–74] However, *Cx. nigripalpus*, which is not as competent as *Cx. quinquefasciatus*,[65] is likely the most important vector in Florida.[68] The influence of temperature, drought, and rainfall on blood-feeding on avian hosts, oviposition behavior of *Cx. nigripalpus*, along with its marginal vector competence have likely limited WNV amplification to local sites in Florida. Our numerous isolations of WNV from *Cx. nigripalpus* in the Everglades support previous studies identifying this species to be the important vector in Florida.[68,70,71,73]

We isolated EVEV from *An. crucians* complex and eight species of mosquitoes, the most diverse group of species infected with EVEV to date (Table 5). The largest numbers were from *An. crucians* complex and *Cx. cedecei*. Multiple isolates were also from *An. atropos, Cx. nigripalpus, Cx. pilosus*, and *Oc. taeniorhynchus*.

Everglades virus was isolated from *Cx. cedecei* during June to August from mangrove, cypress swamps, and hardwood hammock habitats. Previously, RNA of EVEV was detected in four *Cx. cedecei* collected in June and August in hammock, mangrove, and pine rockland habitats within Everglades National Park.[28] Monthly infection rates in our studies were as high as 9.07 per 1,000 specimens in July, an infection rate comparable to that reported earlier for *Melanoconion* species.[7] No isolations were made during September when numbers of *Cx. cedecei* were at their peak averaging 190 specimens per trap-night. These findings, along with its reported feeding on humans and rodents, particularly hispid cotton rats, a primary reservoir host, and its competency, support the conclusion that *Cx. cedecei* is the most important vector of EVEV.[28,33]

We also isolated EVEV in July from three other species of *Melanoconion*, *Cx. erraticus*, *Cx. pilosus*, and *Cx. iolambdis*. Infection rates in July were 14.93, 51.89, and 1.72 per 1,000 specimens for *Cx. iolambdis*, *Cx. pilosus*, and *Cx. erraticus*, respectively. All three feed on mammals, including humans,[29,75–77] but none feed extensively on small sylvatic mammals as does *Cx. cedecei*. *Cx. pilosus* feeds primarily on reptiles in Florida,[29,77] particularly *Anolis* sp., and *Cx. iolambdis* and *Cx. erraticus* feed more extensively on vertebrate

classes other than mammals.[29,75,76,78,79] Our collection of relatively large numbers of *Cx. iolambdis* in mangrove habitat supports the findings of Blosser et al.,[75] who reported 13.1% feeding on mammals, particularly raccoons. We do not know how these three species became infected with EVEV. Venezuelan equine encephalitis virus has been isolated from *Cx. iolambdis* in the Mexican State of Veracruz.[80] Although competency to transmit EVEV has not been demonstrated, these three species need further study to determine their possible role in transmitting EVEV. Unlike *Cx. cedecei,* which belongs to the Spissipes section of subgenus *Melanoconion,* these three species belong to the Melanoconion section. It is the Spissipes section that contains species known to be vectors of VEEV and are most likely to be vectors of other arboviruses.

Three and four isolations of EVEV were from *Oc. taeniorhynchus* and *Cx. nigripalpus*, respectively. Previous isolations were reported from these species,[7] but both species are incompetent to transmit EVEV.[81]

Fourteen isolations of EVEV were made from *Anopheles*, primarily from *An. crucians* complex. Two isolations were reported previously from this complex,[40] a group of mosquitoes known to feed extensively on rabbits and ruminants and to a lesser extent on other mammals in Florida.[44] Although *An. crucians* complex were infected with EVEV, with monthly infection rates ranging from 0.12 to 5.86 per 1,000 mosquitoes, competence of *Anopheles* to transmit is not known.

The remaining five viruses isolated from mosquitoes in the southwestern Florida Everglades belong to the genus Orthobunyavirus. This genus is the largest of the five genera comprising the Peribunyaviridae, with more than 170 named viruses.[82] Serologic studies grouped the species into 18 serogroups. Thirty-nine Orthobunyaviruses have been isolated from mosquitoes or Culicoides midges on the North America continent.

Tensaw virus has been isolated from several mammal feeding mosquitoes but seems to be associated with *An. crucians* complex. Its numerous isolations from *An. crucians* complex,[10,36] its corresponding geographical distribution with that of *An. crucians* complex,[36] its likely competency,[66] and its isolation from small- and medium-sized mammals suggest this complex to be the important enzootic vector among mammals.[40] Our 14 isolations from *An. crucians* complex from mangrove, cypress swamp, sawgrass, and hardwood hammock habitats during several months, but largely during July when infections rates were 2.66 per thousand specimens and when numbers of the *Anopheles* complex were increasing in the field (Table 3, Figure 2), support previous studies that show this virus to be relatively common in the southwestern Everglades. Isolations from mosquitoes collected in January, February, and April have not been reported previously and suggest this virus to be present in host-seeking mosquitoes throughout the year. Although not considered a major health threat, TENV may be associated with congenital defects in humans.[83]

Our phylogenetic analysis, using M segment sequences, shows MHV, GLV, SRV, and PAHV to be distinctly different and to align with their serogroups (Figure 3). Seven isolates of MHV belonged to a single clade of genetically related strains closely allied to the prototype and that formed a separate lineage from Guajara virus and Bimiti virus, other members of the Guama antigenic group. Little genetic variation was noted among the isolates. The grouping of six of our GLV isolates around a 1963 isolate demonstrates the uniqueness of this virus compared with other Orthobunyaviruses. All the GLV M segment sequences we included in the tree formed a monophyletic cluster within the Group C viruses and shared a most recent ancestor with Marituba virus, also a member of the Marituba antigenic complex.[52] Shark River virus and PAHV sorted out with the Patois antigenic group close to Patois virus and Zegla virus. Three isolates of SRV were indistinguishable from an earlier isolate, 64U80, and two showed some variation from this isolate. Shark River virus has been collected outside of the Everglades, and these differences may reflect a flow of strains from elsewhere. Phylogenetic analysis clearly shows one of our isolates to be highly similar to the prototype strain of PAHV, but two others showed variation.

Culex cedecei is the likely enzootic vector of GLV. We isolated GLV in hardwood hammocks and cypress swamps from seven *Cx. cedecei*, a species that feeds on small, medium, and large mammals including humans,[28,29] from the Everglades, but outside Everglades National Park where the original isolations were made from *Melanoconion* species and from two hispid cotton rats captured in 1963–1965.[6,7] Monthly infection rates were as high as 5.10 per 1,000 specimens. Our findings document this virus to be present in the Everglades 53 years after its original isolation. The isolations of SRV and MHV in the same mosquito pools positive for GLV show these three viruses circulate in the same mosquito populations. It is worth noting that 10 of the 13 different viruses within the Group C arboviruses are associated with human disease that is described as a self-limited, dengue-like illness that is not easily diagnosed.[52] Gumbo Limbo virus has not been reported to cause human illness.

Culex cedecei also is likely the important enzootic vector of MHV, PAHV, and SRV. Almost all isolates were from *Cx. cedecei*, the monthly infection rates varied from 1.04 to 11.78 per 1,000 specimens, the earlier numerous isolations in the 1960s were from *Melanoconion* sp., and the isolations of MHV and SRV from hispid cotton rats suggest that *Cx. cedecei* is the most important enzootic vector for these three viruses.[8,9] While collections in the 1960s were primarily from hammocks,[7] we isolated viruses from hardwood hammocks, cypress swamp, and mangrove habitats, indicating these viruses to be widespread among different habitats in the southwestern Everglades. No human involvement of MHV, PAHV, or SRV has been reported.

We identified seven mosquito pools that were infected with two arboviruses. All coinfections were from pools of *Cx. cedecei* containing 1–50 individuals. This finding is not entirely surprising given that these arboviruses circulate in the same sites and share a common vector and possibly vertebrate hosts. One pool of a single specimen was simultaneously infected with MHV and SRV. This mosquito may have acquired its two different viruses from a single feeding. Alternatively, this mosquito could have fed on more than one host or have acquired infection vertically.

It is also worth noting that all coinfected pools were identified by shotgun metagenomic sequencing of mosquito pools and virus cultures. This technique produces millions of short sequences reads that results in high coverage to identify rare or minority viral sequences. The other positive pools

were identified by PCR amplification from virus cultures using conserved primers and standard sequencing of PCR products. This later technique will typically only identify the most abundant virus sequence in the sample (the consensus sequence) and may miss coinfected mosquito pools. The adoption of unbiased deep sequencing techniques during arbovirus surveys will likely reveal a greater diversity of viruses and more instances of coinfection than previously recognized.

Efforts need to be undertaken to determine if unexplained mild or severe febrile illness, encephalitis, or hemorrhagic fever in humans associated with the Florida Everglades might be caused by SRV, MHV, PAHV, GLV, or other viruses. At least 30 of the more than 170 named viruses of the genus Orthobunyavirus are associated with human disease.[82] Extensive and sustained serological testing using antigens of these and other viruses as previously reported,[84] isolation attempts from blood, urine, or cerebrospinal fluid, or detection of viral RNA might implicate an arbovirus in human disease as recently reported for KEYV.[13] Currently, there is limited clinical or research interest with these viruses.

We isolated seven different viruses from one mosquito complex and nine species of mosquitoes during a 1-year study in a small section of the Everglades in southwestern Florida. Eighty-five percent of all viruses were isolated from the human biting *Cx. cedecei*, *Cx nigripalpus*, and *An. crucians* complex. Two viruses, EVEV and WNV, cause human disease and were isolated from four different habitats. The remaining five viruses, TENV, GLV, SRV, MHV, and PAHV, belong to the genus Orthobunyavirus, of which the latter four were initially isolated in the Everglades in the early 1960s. Our isolations of these viruses in 2016 show these viruses to be present after 50 years in this semitropical environment. Our repeated isolations of five different viruses from *Cx. cedecei*, including EVEV and four Orthobunyaviruses, suggest this mosquito to be an important enzootic vector of these viruses and possibly an epizootic vector as well. As Naples and other communities continue to expand into the Everglades and as new invasive mosquito species and viruses become established in Florida, more humans likely will encounter mosquitoes and the viruses they carry. This increased contact may result in more arthropod-borne infections than have been recognized in the past.

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