January 2021

Vector-Host Interactions Of Culex Pipiens Mosquitoes And Their Role In Arbovirus Transmission In The Mid-Atlantic United States

Karen Jane Yuan
kjy118@gmail.com

Vector-host Interactions of *Culex pipiens* Mosquitoes and Their Role in Arbovirus Transmission in the Mid-Atlantic United States

Karen Jane Yuan

May 2021

Master of Public Health Thesis

Department of Epidemiology of Microbial Diseases

Yale School of Public Health

Primary Reader: Dr. Goudarz Molaei, PhD

Secondary Reader: Dr. Brian Weiss, PhD

ABSTRACT

As the primary vector for the West Nile virus in the United States, members of the *Culex pipiens* complex have caused illnesses and deaths in the human population since its discovery in 1937. The potential contribution of *Cx. pipiens* to transmission and enzootic amplification of WNV can be influenced by multiple aspects, including their interactions with various vertebrate hosts as an important factor. By using blood meal analysis and virus testing, this study further investigated the role of *Cx. pipiens* in WNV transmission and amplification. With 91% of blood meals identified from avian species, birds remain the most frequent source of blood meals for *Cx. pipiens*. However, the primarily ornithophilic *Cx. pipiens* also obtained blood meals from mammals (4.6%) and reptiles (0.4%), albeit at lower frequencies. This study also found 4.3% of mixed avian and mammalian blood meals from *Cx. pipiens*, indicating the possibility of their contribution to the epidemic and epizootic transmission of WNV while maintaining enzootic cycles.

Keywords: West Nile virus, *Culex pipiens*, vector-host interactions, enzootic vector, epidemic and epizootic transmission, Virginia.

ACKNOWLEDGMENTS

I would like to thank Charles Abadam, Karen Akaratovic, and Jay Kiser at the Suffolk Mosquito Control agency for collecting mosquitoes analyzed in this project and for providing valuable information on mosquito activity, abundance, and infection status in the region. I would also like to thank the Connecticut Agricultural Experimental Station (CAES) for providing the equipment and opportunity to conduct this project. Additional thanks to Dr. Philip Armstrong, Michael Misencik and Angela Bransfield at the CAES Biosafety Level 3 laboratory for testing mosquitoes for arboviruses.

I would like to extend my gratitude to my thesis advisors, Dr. Goudarz Molaei and Dr. Brian Weiss, for providing guidance throughout this project. I am grateful to Noelle Khalil, Fiona Quigley, and Alyssa Marini at CAES for their assistance. I would also like to thank my friends at YSPH for their support in getting me through these two years, and Michael Budianto at Vanderbilt University for his coding help. I'm deeply grateful to Lily Cao at Harvard Medical School for her love and support, encouragement, and humor that gets me through the day.

Lastly, I would like to thank my family for always being there for me. Thank you for all the love, support, and guidance for getting me through hard and hopeless times. I wouldn't have gone this far without you all. Thank you. Thanks to my cats, Toast (2011-2021) and Merlot (2011-2020), who gave me comfort during this COVID-19 pandemic. I am grateful to my current cats, Truffle and Muffin, for their constant mental support and getting me through these hard times.

To everyone who have supported me in my public health journey, I thank you from the bottom of my heart.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Initially isolated from the blood of a febrile patient in 1937 in the West Nile district of Uganda (Sejvar, 2003; Chancey et al., 2015), the West Nile virus (WNV) was discovered as a neurotropic flavivirus that poses public health threats globally (Suthar et al., 2013). Between the years 1950 through the 1980s, the WNV occasionally caused febrile illness outbreaks in India, South Africa, France, Egypt, and Israel (Chancey et al., 2015). Following reports of an unusual cluster of cases to the New York City Department of Health, WNV was introduced into the United States in the summer of 1999 (Nash et al., 2001). Subsequently, WNV has spread rapidly throughout the United States (*Figure 1*). Between 1999 and 2019, 51,801 cases were reported to the Centers for Disease Control and Prevention (CDC) from all 50 states (CDC, 2020). Of these disease cases, 2,390 deaths were reported since 1999, including deaths from neuroinvasive and non-neuroinvasive diseases (CDC, 2020).

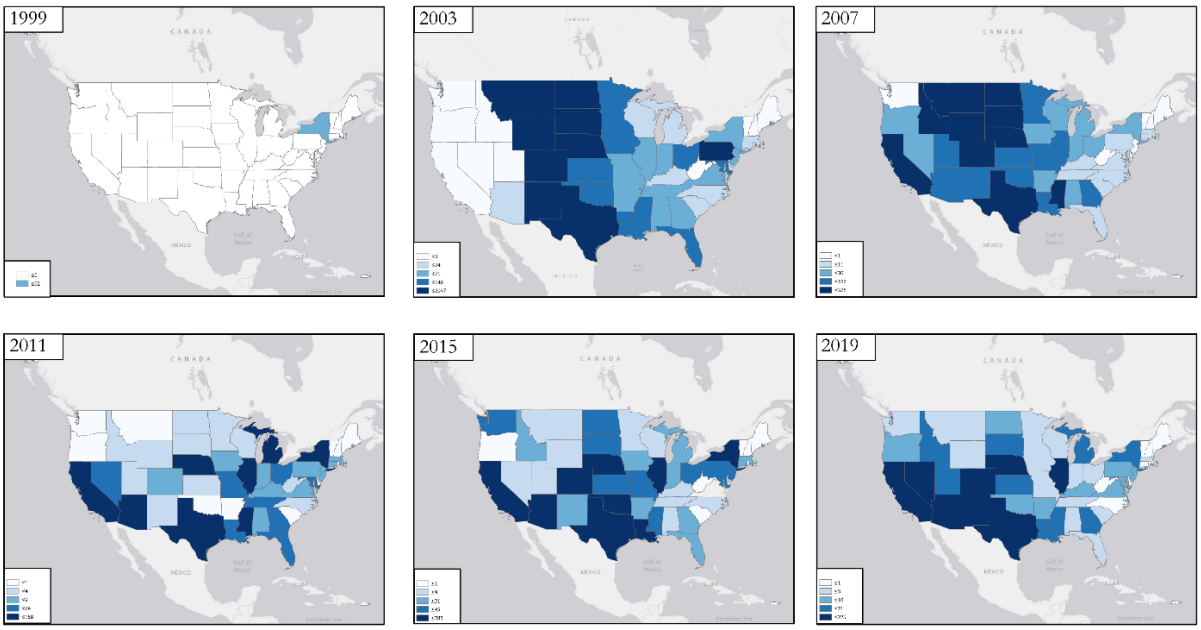

Figure 1. WNV in the United States. Progression of WNV in the United States from 1999 to 2019. Data Source: CDC. Service Layer Credits: Esri, HERE, Garmin, © OpenStreetMap contributors, and the GIS user community.

WNV is maintained in an enzootic transmission cycle with the involvement of ornithophilic mosquitoes and reservoir-competent avian species (Kilpatrick et al., 2007). Some mosquito species that feed on both birds and mammals can act as bridge vectors to transmit the virus to incidental hosts (e.g., humans and/or horses), potentially leading to development of the disease (CDC, 2018) (*Figure 2*).

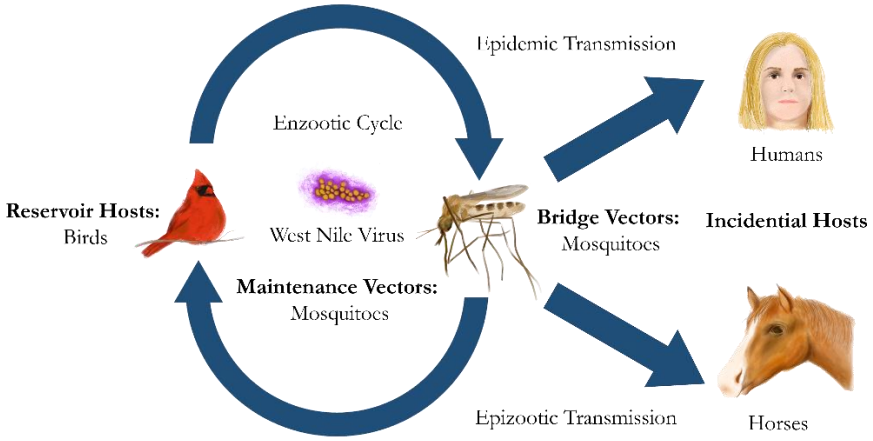

Figure 2. WNV transmission cycle. WNV is transmitted and maintained in an enzootic cycle between mosquitoes (bridge vectors) and birds (reservoir hosts). Mammals such as humans and horses can be incidental hosts of WNV through epidemic and epizootic transmission, respectively. Credits to Lily Cao for her illustration.

While over 65 mosquito species have tested positive for WNV, not all species are competent for virus transmission or to act as bridge vectors outside the enzootic cycle (Colpitts et al., 2012; Rückert & Ebel, 2018; Kain & Bolker, 2019). Results of investigations indicated that more than 23 mosquito species can serve as potential bridge vectors (Hamer et al., 2008). Of these species, *Culex pipiens* mosquitoes are considered as primary vectors of WNV in the United States (Andreadis, 2012; Colpitts et al., 2012; Koenraadt et al., 2019). The *Cx. pipiens* complex consists of three primary forms: *Cx. p. pipiens* form pipiens, *Cx. pipiens* f. molestus, and *Cx. p. quinquefasciatus* (also known as *Cx. quinquefasciatus*), with the potential of hybridizations between the three forms (Barr, 1957; Molaei et al., 2012). Since members of the *Cx. pipiens* complex may vary in their feeding behavior,

physiology, and geographic range (Molaei et al., 2012; Chaulk et al., 2016), understanding host feeding habits and vector-host interaction of the members in this mosquito complex are essential.

Mosquito blood-feeding is influenced by several factors, including genetics, host availability and abundance, defensive behavior of potential hosts, vector flight behavior, among many others (Renshaw et al., 1994; Chaves et al., 2010; Takken & Verhulst, 2013; Börstler et al., 2016; Brugman et al., 2017). The mosquito blood-feeding patterns are classified into three categories: restricted, unrestricted, and opportunistic (Takken & Verhulst, 2013). Mosquitoes with a restricted blood-feeding behavior will only feed on selected host species, whereas those with unrestricted blood-feeding behavior would feed on a broader range of hosts (Takken & Verhulst, 2013). Mosquitoes with an opportunistic blood-feeding behavior would feed on the less frequently attacked hosts when the more frequently attacked are absent (Chaves et al., 2010; Takken & Verhulst, 2013).

A number of studies have examined the blood-feeding behaviors of *Cx. pipiens* mosquitoes. For instance, *Cx. p. pipiens* f. pipiens, or the northern house mosquito, feed on avian hosts, predominantly the American robin, gray catbird, and the house sparrow in Connecticut (Molaei et al., 2006; 2012). Although humans are considered as an infrequent source of blood meals for *Cx. p. pipiens* f. pipiens (Molaei et al., 2007; 2012), this member of the complex may feed on mammals, including humans (Hamer et al., 2008). A study conducted in Chicago, Illinois showed that the typically avian-feeding *Cx. pipiens* exhibited an inclination for mammalian hosts. Microsatellite analysis on mosquitoes that fed on birds *vs* those fed on mammals showed that specimens of *Cx. pipiens* with mammalian-derived blood meals appeared to have a significantly higher ancestry and proportion of hybrid from *Cx. p. pipiens* f. molestus, which are mammalian feeders (Huang et al., 2009; Molaei et al., 2012). These studies concluded that the probability of genetic ancestry from the underground *Cx. pipiens* f. molestus could predispose hybrid *Cx. pipiens* mosquitoes to feed on mammals, including humans (Huang et al., 2009). Conversely, *Cx. quinquefasciatus,* or the southern

house mosquito, is an opportunistic feeder that feeds indiscriminately on both avian and mammalian hosts throughout its distribution range, including Texas and southern California (Molaei et al., 2007; 2010).

Although *Cx. pipiens* are mainly ornithophilic, regional differences exist, so they may also feed on mammalian hosts at lower frequencies. Several studies on blood meal behavior of *Cx. pipiens* have been conducted in various geographic locations in the United States, but such studies have not been conducted in Virginia (VA). Since the 2002 outbreak of WNV in VA, the number of human cases has fluctuated (CDC, 2020) (*Figure 3*).

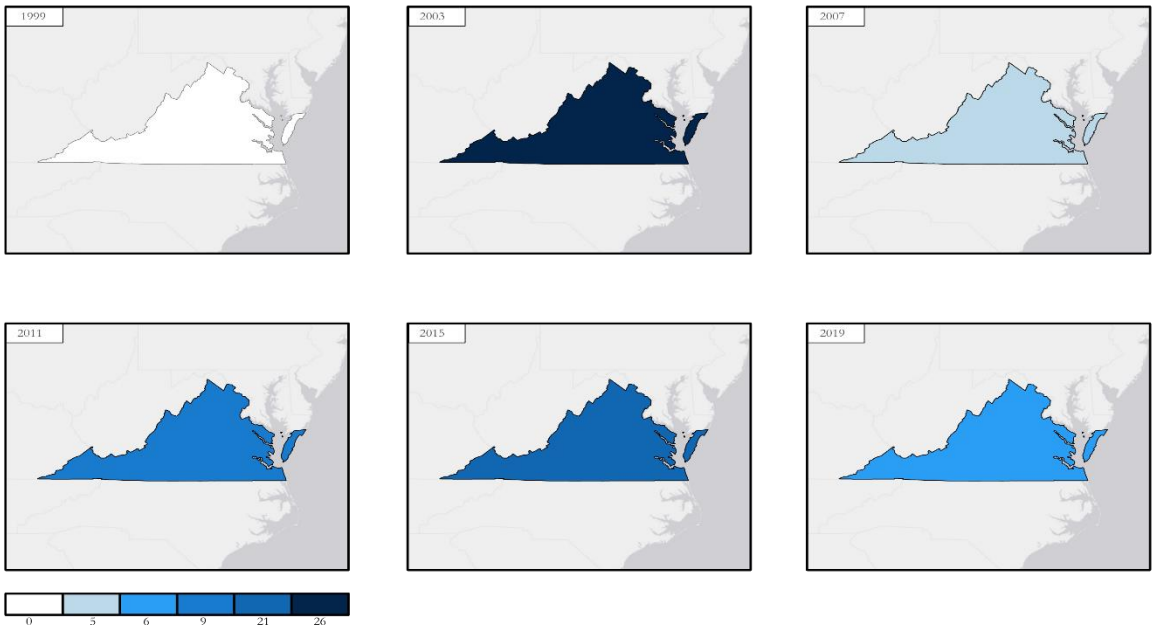

Figure 3. WNV in VA. Progression of WNV in VA from 1999 to 2019. Data Source: CDC. Service Layer Credits: Esri, HERE, Garmin, © OpenStreetMap contributors, and the GIS user community.

It is important to understand the factors contributing to the maintenance and amplification of WNV and other arboviruses (Huang et al., 2009). The goal of this study is to investigate blood-meal analysis and infection status in field-collected mosquitoes with the specific objectives to (I) quantify the degrees of *Cx. pipiens* interactions with hosts and spatial differences in vector-host interactions, (II) identify vertebrate species that serve as sources of blood meals for *Cx. pipiens* and

reservoir and amplification host for WNV, and (III) investigate the potential of *Cx. pipiens* to serve as a "bridge vector" of arboviruses and the risk to infect the human population in VA.

METHODS

Study area

The City of Suffolk (36°44' 29" N 76° 36' 36" W) is part of the Hampton Roads in the southeastern corner of VA (*Figure 4A*). The Chesapeake Bay is 15km to the north, while the Atlantic Ocean lies 50 km east of Suffolk. Suffolk hosts three watersheds: The Great Dismal Swamp watershed in the east, James River watershed in the north, and Chowan River watershed in the southwest. Suffolk is the largest city by land area in VA with 1,036km^2 and the second-lowest human population density of VA's independent cities of 92,108 in 2019 (United States Census Bureau, 2019). Nearly 59% of the land in Suffolk is zoned as agriculture, 26% zoned as mixed urban, suburban, and commercial, while 15% is zoned as conservation (*Figure 4B*).

Mosquito collection

Mosquitoes were collected using various traps from April to November each year at 50 sites throughout the city of Suffolk, VA, on a weekly schedule (*Figure 4C*). CDC light traps (BioQuip Products, Rancho Dominguez, CA), BG-Sentinel 2 traps (Biogents, Regensburg, Germany), and modified Reiter gravid traps (Reiter, 1987) were set most commonly at 32, 19, and 11 traps per week, respectively. With the modified CDC-Collection Bottle Rotators (BioQuip Products) attached to the CDC light trap, mosquitoes were baited with carbon dioxide from a gas cylinder. BG-Sentinel 2 traps utilized human skin non-toxic chemical lures and carbon dioxide from a gas cylinder to bait. The self-constructed modified Reiter gravid traps with a mixture of chicken manure, alfalfa, yeast, and water were fermented for seven to fourteen days to bait. Traps were set between 11:00 AM and 3:00 PM, and picked up the following morning between 07:00 AM and 09:00 AM.

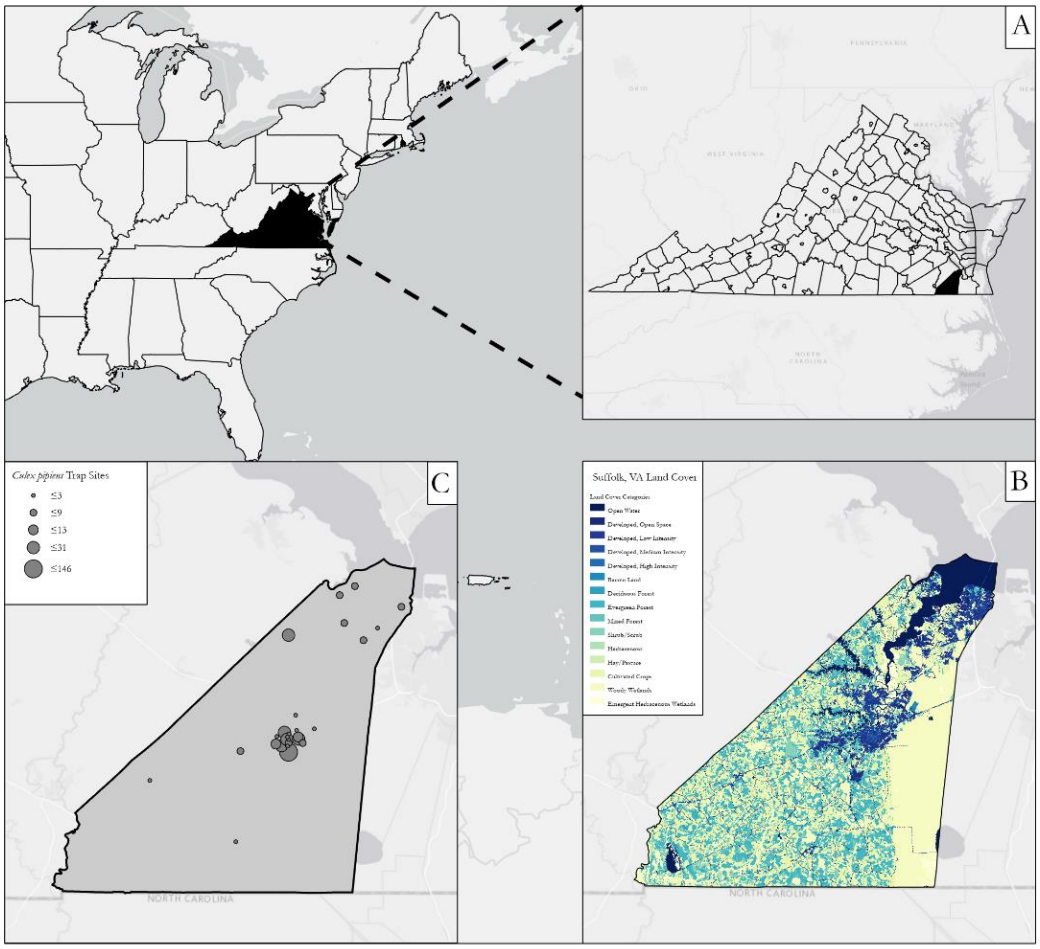

Figure 4. Map of study area. A. Geographic location of Suffolk, VA. **B.** Suffolk land cover. **C.** *Cx. pipiens* trap sites in Suffolk, VA 2019-2020.

Mosquito specimen processing, identification, and transportation

After mosquito collection, the trap chambers with live mosquitoes were transported to the City of Suffolk Mosquito Control laboratory. Except for specimens from the CDC-Collection Bottle Rotator traps, where a dichlorvos compound was used, specimens from other collection methods were sedated with triethylamine. Mosquitoes were transferred to Petri dishes for species identification using a dissecting microscope and regional taxonomic key (Slaff & Apperson, 1989). Mosquitoes with fresh or visible blood remnants were transferred into individual 2-mL tubes, stored at -80°C, and later transported to the Connecticut Agricultural Experimental Station (CAES) for blood meal analyses and virus testing.

DNA isolation from blood-fed mosquitoes and blood meal analysis

Mosquito dissection

Dissection of mosquitoes was performed on dry ice to maintain a cold chain to preserve DNA and RNA integrity. The mosquitoes were placed on microscope slides under a dissecting microscope, and the abdomens were examined to determine the engorgement status. Then, the mosquitoes were dissected into the head & thorax, and abdomen using clean individual razor blades to avoid cross-contamination. The abdomens containing blood were placed into a separate tube for DNA isolation, and the head and thorax were stored at -80°C and later used for virus testing.

DNA extraction

DNA was extracted from the abdomen of blood-fed mosquitoes individually by using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) and DNA-zol BD reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendation with modifications (Molaei et al., 2008).

DNA extraction using Qiagen Dneasy Blood & Tissue Kit

Each mosquito abdomen was homogenized with a disposable polycarbonate micropestle (USA Scientific, Ocala, FL) in a 1.5-mL microcentrifuge tube containing 180 µl of Buffer ATL Tissue Lysis Buffer, followed by adding 20 µl Proteinase K and mixing for three-second on a vortex. The procedure was continued by adding 200 µl Buffer AL and mixing on a vortex for three additional seconds. The tubes were placed in a 56°C water bath for 10 minutes. Then, 200 µl of 100% ethanol were added into samples and vortexed for three seconds. Individual samples were added into columns and centrifuged for two minutes at 8,000 RPM. The flow through was disposed of, and 500 µl of AW1 Buffer was added into the columns and centrifuged for two minutes at 8,000 RPM. After discarding the flow through, individual columns were put in new collection tubes and

500 µl of Buffer AW2 were added into the columns and centrifuged for four minutes at 14,000 RPM. Columns were placed on new 1.5-mL tubes, and DNA was eluted by adding 42 µl of Buffer AE and centrifugating for two minutes at 8,000 RPM. The latter step was repeated once in order to increase the DNA yield. The 1.5-mL tubes containing DNA were stored at -20°C for blood meal analyses.

DNA extraction using DNA-zol BD reagent

The abdomen of individual mosquitoes was homogenized with a micropestle in a 1.5-mL microcentrifuge tube with 400 µl DNA-zol BD. After adding 15 µl of proteinase K (Qiagen, Valencia, CA), the homogenate was mixed, incubated for 10 minutes at 70°C, and centrifuged at 14,000 RPM for 10 minutes. The supernatant was then transferred to a new microcentrifuge tube. By adding 3 µl of Poly Acryl Carrier (Molecular Research Center) and 200 µl of 100% ethanol, the DNA was precipitated after incubating on ice for 10 minutes. The supernatant was discarded after the tube containing the precipitated DNA was centrifuged for 10,000 RPM for 10 minutes. The DNA pellets were washed twice with 75% ethanol, air-dried for four minutes, reconstituted in TE buffer, and stored at -20°C for blood meal analyses.

Blood meal identification

PCR was performed to amplify portions of the mitochondrial *Cytochrome b* gene in the mosquito blood meals using avian-, and mammalian-specific primers or universal primers targeting vertebrate groups. Avian-specific primer pairs were 5'-GAC TGT GAC AAA ATC CCN TTC CA-3' (forward) and 5'-GGT CTT CAT CTY HGG YTT ACA AGA C-3' (reverse). Mammalian-specific primer pairs were 5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G'-3 (forward) and 5'-TGT AGT TRT CWG GGT CHC CTA-3' (reverse) (Molaei et al., 2006). Universal primers pairs were 5'-TGT AAA ACG ACG GCC AGT TCT CAA CCA ACC ACA ARG AYA TYG G'-3

(forward) and 5'-CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CRA ARA AYC A-3' (reverse).

The *Taq*PCR core kit (Qiagen) was utilized for PCR assays following the manufacturer's recommendation. PCR assays were performed with the Veriti or GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). DNA isolated from the mosquitoes' blood meal served as a template in the PCR assays. DNAs isolated from sheep, house finch, and common grackle blood were used as positive controls in PCR assays. Using the QIAquick PCR purification kit (Qiagen), PCR amplified products were purified and then sequenced by Sanger sequencing method using the sequencer, 3730xl DNA Analyzer (Applied Biosystems) at the Keck Sequencing Facility, Yale University, New Haven, CT. DNA sequences were annotated and analyzed using ChromasPro (Technelysium Pty Ltd, South Brisbane, Australia). Annotated sequences were compared to publicly available sequences at the GenBank sequence database, the National Center for Biotechnology Information, using the BLASTn search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to determine the host identity. Positive identification was made when >95% similarity was attained between the query and subject sequence.

Mosquito virus testing

Engorged mosquitoes were screened individually for evidence of infection with arboviruses at the CAES Biosafety Level 3 laboratory. The head and thorax of individual mosquitoes were placed into a 2-mL microcentrifuge tube containing a copper BB and 500 µl of phosphate-buffered saline, 0.5% gelatin, 30% rabbit serum, and 1% antibiotic-antimycotic and homogenized at 25 cycles per second for four minutes with the aid of a MM 300 Mixer Mill (Retsch, Newtown, PA). The homogenates were then centrifuged for seven minutes at 7,000 RPM at 4 °C. The tubes were kept on ice blocks until inoculation.

Vero cells were grown in essential media supplemented with 5% fetal bovine serum, 2% sodium bicarbonate, 1% antibiotic-antimycotic, and 1% L-glutamine (Gibco, ThermoFisher Scientific, Waltham, MA) after seeded into 25-cm^2 flasks and grown overnight at 37°C. Individual flasks were inoculated with 100 μL mosquito homogenate and shaken for 10 minutes after the media was decanted from the flasks. Four mL of the media was then added and placed in an incubator for a week at 37°C. Starting on day three till day seven post-inoculation, flasks were checked daily for cytopathic effects. Infectious supernatant displaying cytopathic effect were harvested and stored at -80°C.

RNA was extracted from positive mosquitoes and cell cultures using QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD). Viruses were identified using the real-time RT-PCR assays set up by using the TaqMan RNA-to-Ct 1-Step Kit (Applied Biosystems, Foster City, CA) and run on a SmartCycler II system (Cepheid, Sunnyvale, CA). WNV RNA was amplified using primer-probe set WNV10533fwd, WNV10625rev, and WNV10560-FAM (Herman, 2015). Eastern Equine Encephalitis virus (EEEV) RNA was amplified using a multiplex of primer-probe sets EEE1858, EEE 1926c, and EEE1881probe and EEE411F, EEE527R, and EEE463probe (Armstrong et al., 2012). The thermal cycling conditions were: 50°C for 30 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for one minute.

Avian population abundance estimates

The average frequency estimates of avian species in Suffolk, VA and surrounding cities/counties (City of Portsmouth, City of Chesapeake, Southampton County, and Isle of Wight county) were obtained from eBird, a database developed by the Cornell Laboratory of Ornithology and the national Audubon Society to track bird distribution and abundance. The estimates were obtained through checklist data collected from professional and amateur birdwatchers and were

accessed through the World Wide Web (https://ebird.org/). The term "frequency" refers to how often a species is reported on checklists within a particular date range and region.

RESULTS

Mosquito abundance

Between 2019-2020, 195,558 female mosquitoes representing 32 species were collected in Suffolk, VA. The most frequently-collected mosquito species was *Aedes albopictus*, comprising 30.2% (n=59,073) of total mosquitoes collected, followed by *Culiseta melanura* with 27.8% (n=54,431), and *Cx. pipiens* with 20.4% (n=39,859) (Table 1). A total of 365 slightly, partially, or fully engorged *Cx. pipiens* were collected at 28 traps during 2,392 trap nights (946 BG-Sentinel traps, 238 CDC miniature light traps, and 1,208 modified Reiter gravid traps) between 2019 to 2020.

Table 1. Number and percentage of adult female mosquitoes (by species) collected from the 28 sites in Suffolk, VA, 2019-2020.

	2019 to 2020	
Species of Mosquito	**No.**	**(%)**
Aedes albopictus	59,073	30.2%
Culiseta melanura	54,431	27.8%
*Culex pipiens**	39,859	20.4%
*Culex restuans**	12,511	6.4%
Culex salinarius	9,663	4.9%
Aedes canadensis	5,463	2.8%
Psorophora ferox	4,316	2.2%
Culex erraticus	2,206	1.1%
Anopheles quadrimaculatus	2,014	1.0%
Aedes vexans	1,193	0.6%
Aedes atlanticus	747	0.4%
Other**	4,082	2.1%
TOTAL	195,558	

**Culex pipiens* and *Culex restuans* have been combined in many studies due to the difficulty of separating them by morphological characteristics. During 2010-2020, we identified specimens to the species level, but in the later half new morphological characteristics were used to separate these two species with greater certainty.

**Other includes an additional 21 species in 2019-2020 collected at the sites with less frequency, and specimens that were not able to be identified to species by morphological characteristics due to severe damage from environmental conditions and/or trapping equipment.

Proportion of avian and mammalian blood meal from Cx. pipiens

All 365 *Cx. pipiens* were subjected to blood meal analysis. Of these, 77% (n=281) produced conclusive host feeding results. Results indicated that 90.7% (n=255) of the *Cx. pipiens* fed on avian hosts, 4.6% (n=13) fed on mammalian hosts, 0.4% (n=1) fed on reptilian hosts, and 4.3% (n=12) fed on both avian and mammalian hosts in mixed bloods. The four most common hosts were all avian species: American robin (50.18%, n=141), Northern cardinal (20.28%, n=57), Carolina wren (4.98%, n=14), and House finch (3.2%, n=9), together representing 87.8% of all identified hosts (Table 2). American robin and Northern cardinal comprised 55.3% and 22.4% of all avian species, respectively, in mosquito blood meals. With mammalian species, Virginia opossum and domestic cats each composed of 46.2% of all mammalian hosts (*Figure 5*).

Table 2. Number and percentage of avian-, mammalian-, and reptilian-derived blood meals from *Cx. pipiens* collected in Suffolk, VA, 2019-2020.

Vertebrate Hosts Common Name (Species Name)	Frequency of Blood Meals No. (%)
Avian	
American robin (*Turdus migratorius*)	141 (50.18%)
Northern cardinal (*Cardinalis cardinalis*)	57 (20.28%)
Carolina wren (*Thryothorus ludovicianus*)	14 (4.98%)
House finch (*Carpodacus mexicanus*)	9 (3.20%)
Gray catbird (*Dumetella carolinensis*)	6 (2.14%)
Northern mockingbird (*Mimus polyglottos*)	6 (2.14%)
Brown thrasher (*Toxostoma rufum*)	3 (1.07%)
Common grackle (*Quiscalus quiscula*)	3 (1.07%)
European starling (*Sturnus vulgaris*)	3 (1.07%)
Mourning dove (*Zenaida macroura*)	3 (1.07%)
House wren (*Troglodytes aedon*)	2 (0.71%)
Barred owl (*Strix varia*)	1 (0.36%)
Black-and-white warbler (*Mniotilta varia*)	1 (0.36%)
Blue jay (*Cyanocitta cristata*)	1 (0.36%)

Carolina chickadee (*Poecile carolinensis*)	1 (0.36%)
Eastern bluebird (*Sialia sialis*)	1 (0.36%)
Tufted titmouse (*Baeolophus bicolor*)	1 (0.36%)
White-eyed vireo (*Vireo griseus*)	1 (0.36%)
Yellow-billed cuckoo (*Coccyzus americanus*)	1 (0.36%)
Mammalian	
Domestic cat (*Felis catus*)	6 (2.14%)
Virginia opossum (*Didelphis virginiana*)	6 (2.14%)
Dog (*Canis lupus familiaris*)	1 (0.36%)
Reptilian	
Brown snake (*Storeria dekayi*)	1 (0.36%)
Mixed	
American robin & Virginia opossum (*Turdus migratorius* & *Didelphis virginiana*)	6 (2.14%)
Northern cardinal & Virginia opossum (*Cardinalis cardinalis* & *Didelphis virginiana*)	2 (0.71%)
American robin & Domestic cat (*Turdus migratorius* & *Felis catus*)	1 (0.36%)
Northern cardinal & Domestic cat (*Cardinalis cardinalis* & *Felis catus*)	1 (0.36%)
Mourning dove & Virginia opossum (*Zenaida macroura* & *Didelphis virginiana*)	1 (0.36%)
Northern mockingbird & Virginia opossum (*Mimus polyglottos* & *Didelphis virginiana*)	1 (0.36%)
Total	281 (100%)

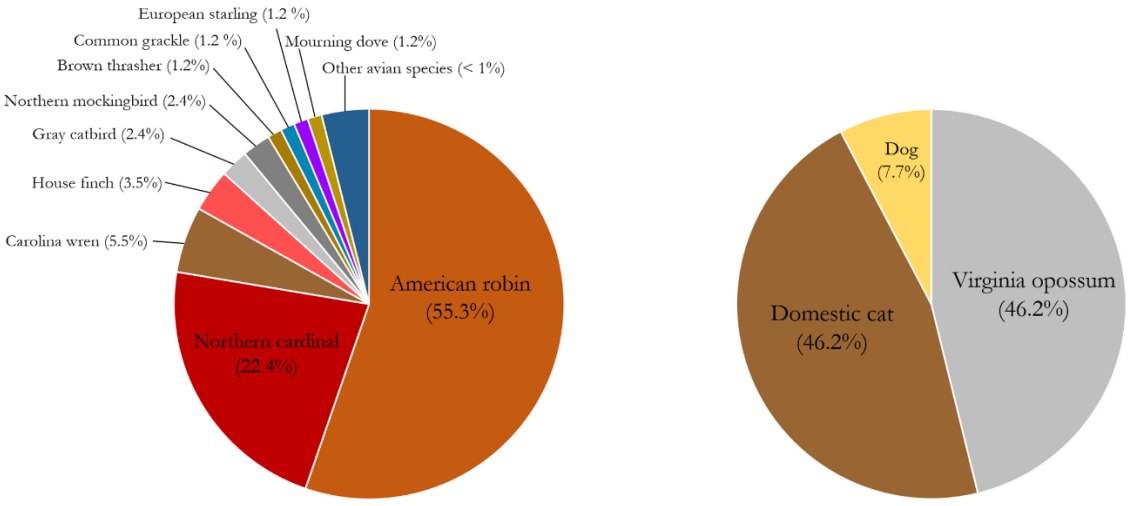

Figure 5. Species identified in blood meal analyses. Left: avian species identified in blood meal analyses. Right: mammalian species identified in blood meal analyses.

Temporal differences in avian blood feeding

Table 3 shows the seasonal frequencies of *Cx. pipiens* avian blood meals acquired from four birds individually and the remaining avian species collectively. In the early season, May-June, 23 avian-derived blood meals were collected, of which 39.1% (n=9) were from American robin, followed by other avian species at 30.4% (n=7), Northern cardinal 21.7% (n=5), and Carolina wren and House finch each at 4.3% (n=1). In mid-season, July-August, the frequency of blood meal acquired from American robin increased to 57.8% (n=85), while the other avian species declined to 13.6% (n=20), Northern cardinal with a slight decrease to 20.4% (n=30), and Carolina wren and House finch at 5.4% (n=8) and 2.7% (n=4), respectively. In the late season, September-November, the American robin remained the most frequent source of blood meal at 55.3% (n=47), followed by Northern cardinal at 25.9% (n=22), other avian species at 8.2% (n=7), and Carolina wren and House finch at 5.9% (n=5) and 4.7% (n=4), respectively. There was no significance temporal difference observed in the proportion of blood meals acquired from two species ($p > 0.05$, Chi-square test), indicating that *Cx. pipiens* did not acquire more or less blood from one particular avian species progressively throughout the seasons.

Table 3. Seasonal frequencies of avian-derived blood meals of *Cx. pipiens* collected from Suffolk, VA and its surrounding cities and counties from 2019-2020.

	Early (May-Jun)	Mid (July-Aug)	Late (Sep-Nov)	Total
American robin	9 (39.1%)	85 (57.8%)	47 (55.3%)	141
Northern cardinal	5 (21.7%)	30 (20.4%)	22 (25.9%)	57
Carolina wren	1 (4.3%)	8 (5.4%)	5 (5.9%)	14
House finch	1 (4.3%)	4 (2.7%)	4 (4.7%)	9
Other avian species	7 (30.4%)	20 (13.6%)	7 (8.2%)	34
Total	23	147	85	255

Avian abundance and frequency of avian-derived bloodmeals in *Culex pipiens*

The monthly frequencies of avian species from May through November of 2019-2020 are depicted in Figure 6. Northern cardinal, Carolina wren, Mourning dove, Blue jay, and Carolina chickadee had a relatively high abundance throughout the collection period in the region. The percentage of *Cx. pipiens* that acquired blood meal from Northern cardinal and Carolina wren were as expected based on their abundance. However, it was not expected that Mourning dove, Blue jay, and Carolina chickadee had a lower percentage of blood meal from *Cx. pipiens* despite their high abundance. It was also unexpected that American robin has the highest percentage of *Cx. pipiens* blood meals with a relatively lower abundance.

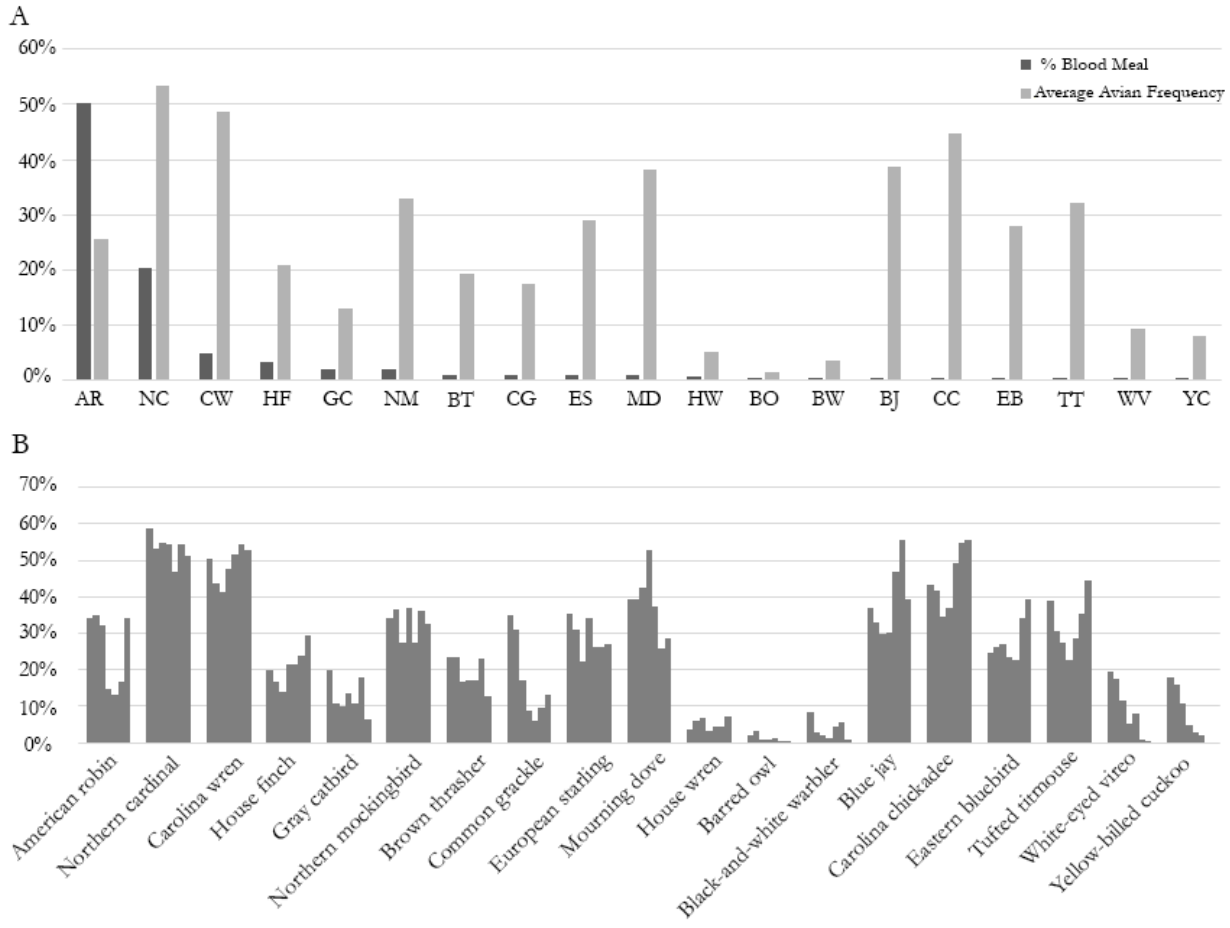

Figure 6. Avian abundance and frequency of avian-derived blood meals in *Cx. pipiens*. A. Comparison between average avian frequencies and percentage of avian-derived blood meals in *Cx.*

pipiens observed in Suffolk, VA and surrounding cities/counties (City of Portsmouth, City of Chesapeake, Southampton County, and Isle of Wight County), May through November 2019-2020. **B.** Monthly frequencies of avian species based on point count data in Suffolk, VA and surrounding cities/counties (City of Portsmouth, City of Chesapeake, Southampton County, and Isle of Wight County), May through November 2019-2020.

Arbovirus infection in *Culex pipiens*

Screening of head and thorax of engorged *Cx. pipiens* for arboviruses resulted in two viral isolates. The virus isolates were identified as WNV and EEEV. The WNV-positive specimen was collected on 08/06/2019 from Lakeside, a collection site near Lake Kilby, and the source of the blood meal was a Virginia opossum. The EEEV-positive specimen was collected on 10/01/2019 from Riddick, a collection site around the Great Dismal Swamp National Wildlife Refuge, and the source of blood meal was a Black-and-white warbler.

A total of 28,311 pools of 12 mosquito species, including *Cs. melanura*, *Cx. pipiens/restuans*, and *Aedes albopictus* were screened for WNV and EEEV by a combination of Vectests, Vector Tests, and RT-PCR during routine mosquito and arbovirus surveillance in Suffolk, VA, from 2010 to 2020. Out of the 28,311 mosquito pools screened for WNV and EEEV, 222 and 610 pools tested positive for WNV and EEEV, respectively. In addition, a total of 18,852 pools of *Cs. melanura* screened for WNV and EEEV and resulted in 58 positive pools for WNV, and 606 positive pools for EEEV. For *Cx. pipiens*, 3,419 pools were tested, and 164 pools tested positive for WNV (Table 4).

Table 4. Number of pools tested positive for WNV and EEEV in Suffolk, VA, 2010-2020.

Species of Mosquito	No. of Pools Tested	WNV+ pools	EEE+ pools
*Culiseta melanura**	18,852	58	606
*Culex pipiens/restuans**	3,419	164	0
*Aedes albopictus**	2,556	0	0
*Culex salinarius**	1,228	0	0
*Culex erraticus**	1,032	0	1
*Aedes vexans**	568	0	0
*Coquillettidia perturbans**	306	0	0
*Uranotaenia sapphirina***	271	0	3

*Aedes triseriatus**	70	0	0
*Anopheles quadrimaculatus***	4	0	0
*Aedes japonicus****	4	0	0
*Culex territans****	1	0	0
Total	28,311	222	610

*A combination of Vectests, Vector Tests, and RT-PCR were used to test these mosquitoes
**Only RT-PCR was used to test these mosquitoes
***Only Vector Test was used to test these mosquitoes

DISCUSSION

The results of this study showed that *Cx. pipiens* primarily feed on passerine birds, particularly American robin, Northern cardinal, Carolina wren, and House finch in Suffolk, VA. As 90.75% of *Cx. pipiens* blood meals were from avian hosts, this study is concordant with previous studies on blood meal analysis of *Cx. pipiens* in the United States that have shown that 93 % and 80% of the blood meals from *Cx. pipiens* were derived from avian hosts in Connecticut and Illinois, respectively (Molaei et al., 2006; Hamer et al., 2009).

In our study, we have identified American robin as the most frequent source of blood meal (50.18%), and this result is consistent with previous studies throughout the United States (Apperson et al., 2002; 2004; Molaei et al., 2006; Hamer et al., 2009). American robins are active year-round in parks, deciduous woodlands, shrublands backyards, and regenerating forests after fires throughout the continental United States (https://www.nwf.org/Educational-Resources/Wildlife-Guide/Birds/American-Robin; https://birdsoftheworld.org/bow/home). As the most prominent tree-roosting bird in woodland habitats, American robins tend to roost in large flocks and spend more time in trees during fall and winter (Molaei et al., 2013; https://birdsoftheworld.org/bow/home). Their roosting behavior creates more opportunities for mosquitoes, including *Cx. pipiens* to acquire blood meal. Spatial overlap is another potential factor for American robins to serve as the primary host of blood meal for *Cx. pipiens*. As American robins roost at tree canopies (Shaw et al., 2002), *Cx. pipiens* also fly around tree canopy height (Anderson et

al., 2004). Despite a relatively lower frequency of American robins observed in this study (Figure 6), the high percentage of *Cx. pipiens* blood meals could be due to roosting behavior and habitat of this bird species. Laboratory studies have shown that infection with high doses of WNV leads to a relatively high viremia titer in American robins, which suggested that these birds likely play a prominent role in the amplification of WNV and to give rise to spill over of the virus into human populations (Komar et al., 2003; VanDalen et al., 2013).

Other avian hosts for *Cx. pipiens* with relatively higher percentage of blood meals in our study were Northern cardinal and Carolina wren. As shown in Figure 6, Northern cardinal has an average avian frequency of more than 50%, and Carolina wren with almost 50% during this study period in the region. Northern cardinals serving as one of the most frequent hosts could play significant roles in maintenance and amplification of WNV and other arboviruses. Previous studies have identified Northern cardinals as a WNV "super suppressor" species, which slows the transmission of WNV by drawing infectious bites but fails to amplify viruses due to its moderate host competencies (Levine et al., 2016). Despite showing relatively low host competencies, a study suggested that the Northern cardinal was one of the avian species exhibiting the highest WNV seroprevalence rates (Beveroth et al., 2006).

As 4.6% of the blood meal originated from mammalian hosts and 4.3% mixed blood meals from avian and mammalian hosts in our study, this indicates that the predominately ornithophilic *Cx. pipiens* exhibited some tendencies to feed on mammalian hosts. Other studies have also shown similar or higher proclivity for *Cx. pipiens* to feed on mammalian host in various regions in the United States: Connecticut-2.5% mammalian and 3.9% mixed blood meals (Molaei et al., 2006), Chicago, Illinois-16% mammalian with 2% mixed bloods (Hamer et al., 2008) and New Jersey-38% mammalian (Apperson et al., 2004). These results could be due to the potential hybridization between *Cx. pipiens* form pipiens and the *Cx. pipiens* form molestus, as form molestus is known to be

mammalophilic (Huang et al., 2009). Although the potential of hybridization or ancestry genetic variation is unknown among *Cx. pipiens* population in this study, the introgression may have led to the mammalophilic behaviors. The tendency for mammalophilic behavior suggests that *Cx. pipiens* may not only be involved in the transmission of arboviruses such as WNV and EEEV among avian hosts in enzootic cycling, but it could also play a role in the epidemic and epizootic transmission to mammalian hosts including humans.

Because some of the engorged mosquitoes examined in our study had been collected from urban settings in Suffolk, VA with an estimate human population of 92,108 in 2019 (United States Census Bureau, 2019), it was expected to identify human-derived blood meals from *Cx. pipiens* as reported in previous studies (Apperson et al., 2004; Molaei et al., 2006; Hamer et al., 2009). However, none of the blood meals from this study was identified to be from a human host. While the exact reason is unknown, the absence of human-derived blood meals could be due to the city's effort in mosquito control by trapping and spraying the city, and releasing information and tips on mosquito bite preventions, among other factors. The City of Suffolk also offered free mosquito dunks to the city residents to control larval population and prevent/reduce the frequency of mosquito bites (Haugdahl, 2020).

In addition to blood meals from avian and mammalian hosts, 0.4% (n=1) of *Cx. pipiens* fed on a reptilian host, specifically the Brown Snake. Previous studies have similarly discovered that reptilian species also served as sources of blood meals for *Cx. pipiens*. One study showed that 10% and 7.7% of *Cx. pipiens* fed on reptiles in New Jersey and New York, respectively (Apperson et al., 2004). Another study conducted in Pennsylvania found two Eastern Garter Snakes (1.62%) tested positive for WNV (Dahlin et al., 2016). These findings indicate the possibility for reptilian species to play a role in the transmission cycle of WNV.

Of the 365 *Cx. pipiens* that were screened for viruses, one tested positive for WNV. Studies from other regions of the United States have found varying results- 1.5% in Chicago, Illinois in 2005 (Hamer et al., 2009) and 0.99% and 0.22% in Stratford, Connecticut during 2002-2003 (Anderson et al., 2004). The variation in the results could be due to the differences in geographic regions and availability of competent reservoir hosts, as well as the years that *Cx. pipiens* mosquitoes were collected and tested. Chicago's relatively high percentage of positive virus cases may be due to the 2005 WNV epidemic and epizootic (Hamer et al., 2008), while there were no epidemics or epizootics declared in the region and the detected WNV maintained under 1% for the studies conducted in 2002 and 2003 in Stratford, Connecticut.

CONCLUSION

Our study has found that while *Cx. pipiens* fed on a wide variety of species, and they appeared to focus their feeding on avian species (90.7%), particularly American robins, throughout the season. This finding suggests that *Cx. pipiens* is involved in the enzootic transmission cycle of WNV among reservoir-competent avian species. The ornithophilic *Cx. pipiens* also obtained blood meals from mammalian, and reptilian species, as well as mixed blood of avian and mammalian. As a primary enzootic vector for WNV, *Cx. pipiens* may also contribute to the epidemic or epizootic transmission of the virus and poses a major risk of human and equine health in Virginia.

REFERENCES

Anderson, J. F., Kline, D. L., Main, A. J., & Andreadis, T. G. (2004). Prevalence of West Nile Virus in Tree Canopy–Inhabiting *Culex pipiens* and Associated Mosquitoes. *The American Journal of Tropical Medicine and Hygiene*, 71(1), 112-119.

Andreadis T. G. (2012). The Contribution of *Culex pipiens* complex Mosquitoes to Transmission and Persistence of West Nile Virus in North America. *Journal of the American Mosquito Control Association*, *28*(4 Suppl), 137–151.

Apperson, C. S., Harrison, B. A., Unnasch, T. R., Hassan, H. K., Irby, W. S., Savage, H. M., et al. (2002). Host-Feeding Habits of *Culex* and Other Mosquitoes (Diptera: Culicidae) in the Borough of

Queens in New York City, with Characters and Techniques for Identification of *Culex* mosquitoes. *Journal of Medical Entomology*, 39(5), 777–78.

Apperson, C. S., Hassan, H. K., Harrison, B. A., Savage, H. M., Aspen, S. E., Farajollahi, A., et al (2004). Host Feeding Patterns of Established and Potential Mosquito Vectors of West Nile Virus in the Eastern United States. *Vector-Borne and Zoonotic Diseases*, 4(1), 71–82.

Armstrong, P. M., Prince, N., & Andreadis, T. G. (2012). Development of a Multi-Target TaqMan Assay to Detect Eastern Equine Encephalitis Virus Variants in Mosquitoes. *Vector-Borne and Zoonotic Diseases, 12*(10), 872-876.

Barr A. R. (1957). The Distribution of *Culex p. pipiens* and *C.P. quinquefasciatus* in North America. *The American Journal of Tropical Medicine and Hygiene*, *6*(1), 153–165.

Beveroth, T. A., Ward, M. P., Lampman, R. L., Ringia, A. M., & Novak, R. J. (2006). Changes in Seroprevalence of West Nile Virus Across Illinois in Free-Ranging Birds From 2001 Through 2004. *The American Journal of Tropical Medicine and Hygiene*, 74(1), 174–179.

Börstler, J., Jöst, H., Garms, R., Krüger, A., Tannich, E., Becker, N., et al (2016). Host-Feeding Patterns of Mosquito Species in Germany. *Parasites & Vectors*, *9*(1), 1-14.

Brugman, V. A., Hernández-Triana, L. M., England, M. E., Medlock, J. M., Mertens, P. P., Logan, J. G., et al (2017). Blood-Feeding Patterns of Native Mosquitoes and Insights Into Their Potential Role as Pathogen Vectors in the Thames Estuary Region of the United Kingdom. *Parasites & Vectors*, *10*(1), 1-12.

Centers for Disease Control and Prevention (CDC). (2018). *Transmission*. https://www.cdc.gov/westnile/transmission/index.html.

Centers for Disease Control and Prevention (CDC). (2020). *Final Cumulative Maps & Data for 1999–2019*. https://www.cdc.gov/westnile/statsmaps/cumMapsData.html.

Chancey, C., Grinev, A., Volkova, E., & Rios, M. (2015). The Global Ecology and Epidemiology of West Nile Virus. *Biomed Research International, 2015*, 1-20.

Chaulk, A. C., Carson, P. K., Whitney, H. G., Fonseca, D. M., & Chapman, T. W. (2016). The Arrival of the Northern House Mosquito *Culex pipiens* (Diptera: Culicidae) on Newfoundland's Avalon Peninsula. *Journal of Medical Entomology*, *53*(6), 1364–1369.

Chaves, L. F., Harrington, L. C., Keogh, C. L., Nguyen, A. M., & Kitron, U. D. (2010). Blood Feeding Patterns of Mosquitoes: Random or Structured? *Frontiers in Zoology*, *7(1)*, 1-11.

Colpitts, T. M., Conway, M. J., Montgomery, R. R., & Fikrig, E. (2012). West Nile Virus: Biology, Transmission, and Human Infection. *Clinical Microbiology Reviews*, *25*(4), 635–648.

Dahlin, C. R., Hughes, D. F., Meshaka, W. E., Jr, Coleman, C., & Henning, J. D. (2016). Wild Snakes Harbor West Nile Virus. *One Health, 2*, 136–138.

Hamer, G. L., Kitron, U. D., Brawn, J. D., Loss, S. R., Ruiz, M. O., Goldberg, T. L., & Walker, E. D. (2008). *Culex pipiens* (Diptera: Culicidae): A Bridge Vector of West Nile Virus to humans. *Journal of Medical Entomology*, 45(1), 125-128.

Hamer, G. L., Kitron, U. D., Goldberg, T. L., Brawn, J. D., Loss, S. R., Ruiz, M. O., et al (2009). Host Selection by *Culex pipiens* Mosquitoes and West Nile Virus Amplification. *The American Journal of Tropical Medicine and Hygiene*, 80(2), 268–278.

Haugdahl, S. (2020). Suffolk Gears Up for Mosquito Season. Retrieved from https://www.13newsnow.com/article/sports/outdoors/suffolk-gears-up-for-mosquito-season/291-5b88176d-acec-4f21-b45a-1b3044742355.

Herman, N. M. (2015). *Real-Time Rt-Pcr Assays To Detect West Nile Virus In Mosquito Pools* (Master's thesis, Yale School of Public Health, 2015) (pp. 1-24). New Haven: Yale University.

Huang, S., Hamer, G. L., Molaei, G., Walker, E. D., Goldberg, T. L., Kitron, U. D., & Andreadis, T. G. (2009). Genetic Variation Associated With Mammalian Feeding in *Culex pipiens* From a West Nile Virus Epidemic Region in Chicago, Illinois. *Vector-Borne and Zoonotic Diseases*, *9*(6), 637–642.

Kain, M. P., & Bolker, B. M. (2019). Predicting West Nile Virus Transmission in North American Bird Communities Using Phylogenetic Mixed Effects Models and Ebird Citizen Science Data. *Parasites & Vectors*, *12*(1), 395, 1-22.

Kilpatrick, A. M., LaDeau, S. L., & Marra, P. P. (2007). Ecology of West Nile Virus Transmission and Its Impact on Birds in the Western Hemisphere. *The Auk, 124*(4), 1121-1136.

Koenraadt, C., Möhlmann, T., Verhulst, N. O., Spitzen, J., & Vogels, C. (2019). Effect of Overwintering on Survival and Vector Competence of the West Nile Virus Vector *Culex pipiens*. *Parasites & Vectors*, *12*(1), 1-9.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D.,et al (2003). Experimental Infection of North American Birds With the New York 1999 Strain of West Nile Virus. *Emerging Infectious Diseases*, 9(3), 311–322.

Levine, R. S., Mead, D. G., Hamer, G. L., Brosi, B. J., Hedeen, D. L., Hedeen, M. W., et al (2016). Supersuppression: Reservoir Competency and Timing of Mosquito Host Shifts Combine to Reduce Spillover of West Nile Virus. *The American Journal of Tropical Medicine and Hygiene*, 95(5), 1174–1184.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Anderson, J. F., & Vossbrinck, C. R. (2006). Host Feeding Patterns of *Culex* Mosquitoes and West Nile Virus Transmission, Northeastern United States. *Emerging Infectious Diseases, 12*(3), 468-474.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Bueno, R., Jr, Dennett, J. A., Real, S. V., et al (2007). Host Feeding Pattern of *Culex quinquefasciatus* (Diptera: Culicidae) and Its Role in Transmission of West Nile Virus in Harris County, Texas. *The American Journal of Tropical Medicine and Hygiene*, *77*(1), 73–81.

Molaei, G., Andreadis, T. G., Armstrong, P. M., & Diuk-Wasser, M. (2008). Host-Feeding Patterns of Potential Mosquito Vectors in Connecticut, U.S.A.: Molecular Analysis of Bloodmeals From 23 Species of *Aedes*, *Anopheles*, *Culex*, *Coquillettidia*, *Psorophora*, and *Uranotaenia*. *Journal of Medical Entomology*, *45*(6), 1143–1151.

Molaei, G., Cummings, R. F., Su, T., Armstrong, P. M., Williams, G. A., Cheng, M. L., et al (2010). Vector-Host Interactions Governing Epidemiology of West Nile Virus in Southern California. *The American Journal of Tropical Medicine and Hygiene*, *83*(6), 1269–1282.

Molaei, G., Huang, S., & Andreadis, T. G. (2012). Vector-Host Interactions of *Culex pipiens* Complex in Northeastern and Southwestern USA. *Journal of the American Mosquito Control Association*, *28*(4 Suppl), 127–136.

Molaei, G., Andreadis, T. G., Armstrong, P. M., Thomas, M. C., Deschamps, T., Cuebas-Incle, E., et al (2013). Vector-Host Interactions and Epizootiology of Eastern Equine Encephalitis Virus in Massachusetts. *Vector-Borne and Zoonotic Diseases*, *13*(5), 312–323.

Nash, D., Mostashari, F., Fine, A., Miller, J., O'Leary, D., Murray, K., et al (2001). The Outbreak of West Nile Virus Infection in the New York City Area in 1999. *The New England Journal of Medicine*, *344*(24), 1807–1814.

Reiter P. (1987). A Revised Version of the CDC Gravid Mosquito Trap. *Journal of the American Mosquito Control Association*, *3*(2), 325–327.

Renshaw, M., Service, M. W., & Birley, M. H. (1994). Host Finding, Feeding Patterns and Evidence for a Memorized Home Range of the Mosquito *Aedes cantans*. *Medical and Veterinary Entomology*, *8*(2), 187–193.

Rückert, C., & Ebel, G. D. (2018). How Do Virus-Mosquito Interactions Lead to Viral Emergence?. *Trends in Parasitology*, *34*(4), 310–321.

Sejvar, J. J. (2003). West Nile Virus: An Historical Overview. *Ochsner Journal, 5*(3), 6-10.

Shaw, D. C., Freeman, E. A., & Flick, C. (2002). The Vertical Occurrence of Small Birds in an Old-growth Douglas-fir-Western Hemlock Forest Stand. *Northwest Science*, 76(4), 322-344.

Slaff, M., & Apperson, C. S. (1989). A Key to the Mosquitoes of North Carolina and the Mid-Atlantic States. Raleigh, NC, NC: North Carolina Agricultural Extension Service, 1-38.

United States Census Bureau. (2019). Suffolk city, Virginia (County). Retrieved from https://www.census.gov/quickfacts/suffolkcityvirginiacounty.

Suthar, M. S., Diamond, M. S., & Gale, M., Jr (2013). West Nile Virus Infection and Immunity. *Nature Reviews Microbiology*, *11*(2), 115–128.

Takken, W., & Verhulst, N. O. (2013). Host Preferences of Blood-Feeding Mosquitoes. *Annual Review of Entomology*, *58*, 433–453.

VanDalen, K. K., Hall, J. S., Clark, L., McLean, R. G., & Smeraski, C. (2013). West Nile Virus Infection in American robins: New Insights on Dose Response. *PLoS ONE, 8*(7).

APPENDIX

Table 5. Primer-probe set sequences for amplification of WNV and EEEV RNA.

Primer/Probe	Sequence
WNV10533fwd	5'-AAGTTGAGTAGACGGTGCTG-3'
WNV10625rev	5'-AGACGGTTCTGAGGGCTTAC-3'
WNV10560probe	5'- [6~FAM]CTCAACCCCAGGAGGACTGG[BHQ1a~Q]-3'
EEE1858	5'-ACACCGCACCCTGATTTTACA-3'
EEE1926c	5'-CTTCCAAGTGACCTGGTCGTC-3'
EEE1881probe	5'- [6~FAM]TGCACCCGGACCATCCGACCT[BHQ1a~Q]-3'
EEE411F	5'-GAACGGACAGGTGAATGGTT-3'
EEE527R	5'-CTGGCCTTCTTCAGCTTGAT-3'
EEE463probe	5'- [6~FAM]CCGCTGCACGTAGAAGGCAGA[BHQ1a~Q]-3'