# MOSQUITO DIVERSITY, ARBOVIRAL RISKS, AND BLOOD FEEDING PATTERNS AT THE NASHVILLE ZOO AT GRASSMERE

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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May 2021

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ABSTRACT

The epidemiological triad is a framework to conceptualize interactions between the environment, a disease agent, and wildlife/human hosts that result in disease transmission. Zoological parks provide a unique opportunity to study the epidemiological triad because they are areas where exotic animals, free-roaming native animals, humans, and mosquito habitats are located in close proximity. The Nashville Zoo at Grassmere in Tennessee previously experienced arboviral transmission, and as such, became a prime research site for the increasing knowledge of arboviral disease transmission dynamics within a zoo setting.

I sampled mosquitoes over four months in 2020 within and outside of the Nashville zoo using four mosquito trap methods and 12 sampling locations. Mosquitoes were identified to species, *Culex* mosquitoes were analyzed for arboviruses, and engorged mosquitoes were preserved for host feeding analysis to determine zoonotic feeding risk. I captured over 9,000 mosquitoes representing 24 different species, including a new species record for Davidson County, TN (*Cx. nigripalpus*). Minimum infection rates (MIR)s for WNV, SLEV and FLAV were 0.79, 0, and 4.14, respectively. Host DNA from 60 engorged mosquitoes was matched to 18 host species, including four species belonging to the zoo. Overall, wild birds were the preferred host species. Northern cardinals, which are competent reservoirs of WNV, were the most commonly fed upon wild bird. Further research is needed to determine if the northern cardinals are serving as zooprophylaxis for WNV transmission in the zoo or if the presence and utilization of these competent reservoirs present a higher risk of infection to the zoo animals. These results collectively demonstrate the utility of zoological parks as sentinels for both emerging pathogens, human and wildlife risk, and vector diversity.

# BIOGRAPHICAL SKETCH

Cierra was born in raised in Flower Mound, Texas, where she attended Flower Mound High School. She went on to attend Texas A&M University. During this time, she was introduced to One Health and found herself determined to find a career that would enable her to work at the intersection of human and animal health. This led her to become an undergraduate research assistant in Dr. Gabe Hamer's arthropod research laboratory. While working in the laboratory, Cierra became aware of the vector-borne disease risks within Texas and the vulnerability of Texas to the introduction of arboviruses. Following the completion of a Bachelor of Science in Biomedical Sciences and Entomology from Texas A&M, Cierra decided to pursue a Master of Science in Vector-Borne Disease Biology at Cornell University to increase her knowledge of how entomology can be used for the betterment of public health. As a result, Cierra joined Dr. Laura Harrington's laboratory in the fall of 2019. During this time, Cierra developed additional interests in science communication and science policy advancement while exploring her original interests through collaborating with the Tennessee Department of health and the Nashville Zoo at Grassmere.

**In dedication to my parents, Leslie and Andie Briggs, who have shown me nothing but unwavering support no matter my dreams and aspirations.**

# ACKNOWLEDGMENTS

First and foremost, I want to thank Dr. Laura Harrington for her constant support, guidance, and expertise. Without her mentorship, none of this study would have been possible. Not to mention, her advocacy for the importance of this study enabled me to complete my field work despite a university wide research pause brought about by COVID-19. I would also like to thank Dr. Abelardo Moncayo for the oppurtunity to collaborate with him and the Tennessee Department of Health. Additionally, his guidance and expertise were invaluable to the implementation of my field work. I would also like to thank him for the use of his laboratory facilities.

The completion of my data collection would not have been possible without the assistance of several individuals. Thank you to Rayan Osman for her willingness to get dirty to help me check mosquito traps each day. I also thank her for the countless hours spent sitting at the microscopes assisting me with mosquito identifications. I thank Brent Newman for testing the bulk of my mosquitoes collected for viruses. Their assistance allowed me to complete my field work in a timely and efficient manner.

While I want to thank all of the members of the Harrington Lab for their support during this process, a few deserve special mentions. Many thanks are owed to Sylvie Pitcher for making sure I always had the necessary supplies to complete my work and for working with me to conduct molecular species identifications. Thank you to Kara Fikrig for your guidance provided while conducting the bloodmeal analysis and assistance species identifications. Thank you to Dr. Alex Amaro for sharing your protocols and for answering my many questions along the way.

This project would not have been possible without the cooperation of the Nashville Zoo. Thank you Dr. Margarita Woc Colburn for advocating for this project and for being a valuable resource throughout this whole process. Thank you to Justin Collins for providing your inside knowledge related to mosquitoes within the zoo and for making time for us in your weekly

schedule. Thank you to Jayne Hardwick for providing information about the zoo. Many thanks are also due to the rest of the zoo staff for their support and encouragement of this project.

Furthermore, thank you to Emily Mader from the Northeast Regional Center for Excellence in Vector-Borne Diseases for your assistance through the research process. I also want to thank Erika Mudrak from the Cornell Statistical Consulting Unit for your expertise and assistance with the data analysis process. Thank you to Dr. Elizabeth Buckles for being a part of my committee and providing your valuable input.

Last but not least, I am thankful for the funding provided through the Northeast Regional Center for Excellence in Vector-Borne Diseases. Additionally, I am thankful for the support and friendship found within my follow cohort members.

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## The Importance of Mosquito-Borne Diseases in the USA

Despite fluctuations in the number of mosquito-borne disease cases from year to year, mosquito-borne infections are a considerable cause of morbidity in the United States. For instance, over 7,000 cases of domestically acquired cases of mosquito-borne disease were reported to the CDC from 2016 to 2018 (Burakoff et al., 2018; Curren et al., 2018; McDonald et al., 2019). Since at least 2004, the most commonly acquired mosquito-borne virus in the continental United States was WNV, but sporadic outbreaks of La Crosse, California serogroup, St. Louis encephalitis, and eastern equine encephalitis viruses have occurred in the continental United States as well (Rosenberg et al., 2018; Foster and Walker, 2009). Outbreaks of dengue, chikungunya, and Zika viruses occurred in U.S. territories with occasional cases of autochthonous transmission in Texas, Florida, and Hawaii (Thomas et al., 2016; Lew et al., 2018; Teets et al., 2014; Texas Department of State Health Services, 2016; Kendrick et al., 2014; Likos et al., 2016; Texas Department of State Health Services, 2020). Despite limited continental spread of these viruses, the presence of competent vectors makes these viruses continual threats. Additional intrinsic and extrinsic factors relevant to the epidemiology of arboviruses make it difficult to predict the emergence or reemergence of future outbreaks.

## Mosquito-Borne Diseases in Tennessee, USA

A national survey of vector control programs published in 2017 highlighted the challenges for vector-borne disease control in Tennessee (NACCHO, 2017). In that report, less than 20% of vector control programs in Tennessee were classified as competent to address vector

control needs. Additionally, arboviral-related illness is likely under-reported by health care providers in the state (Shaffner et al., 2016).

West Nile, La Crosse encephalitis, Eastern Equine Encephalitis, St, Louis encephalitis, dengue, and chikungunya are all reportable arboviruses in Tennessee (https://www.tn.gov/health/cedep/reportable-diseases.html). Since 2004, the number of reported human mosquito-borne disease cases has increased from 40 in 2004 to 112 cases in 2016 (Figure 1). These numbers represent both locally acquired cases and those from travel-related infections.

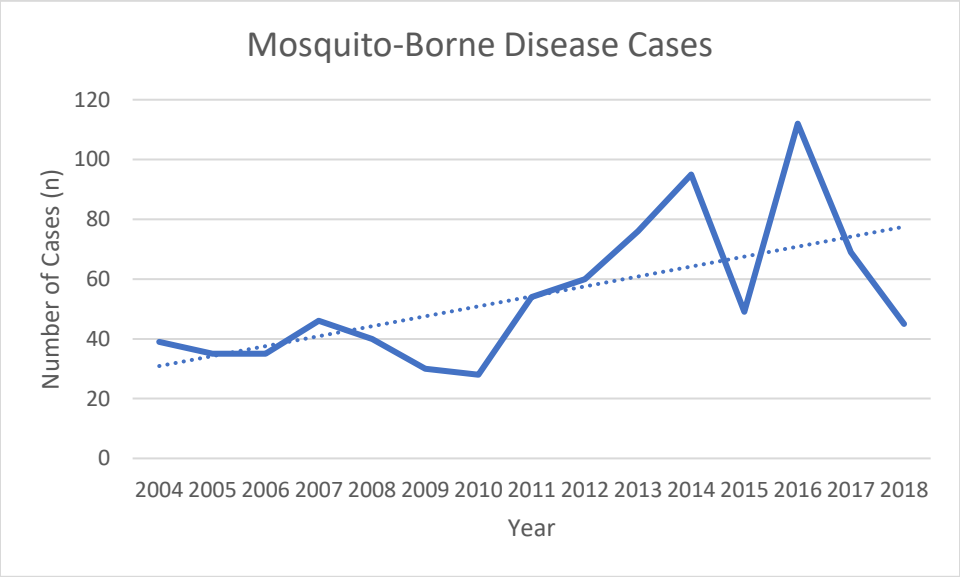



**Figure 1**. The number of reported human mosquito-borne disease cases each year in Tennessee (solid line) plotted with linear trend line (data reported by the CDC: https://www.cdc.gov/ncezid/dvbd/vital-signs/tennessee.html).

From 2010 - 2019, 111 cases of La Crosse encephalitis virus, 2 cases of Jamestown Canyon virus, and 155 cases of human West Nile virus were reported in Tennessee (CDC, 2020a; CDC, 2020b; CDC, 2020c). In 2018, the most common human mosquito-borne disease in the state was

La crosse encephalitis virus and Jamestown Canyon virus (collectively referred to as California Serogroup viruses by CDC). Despite diagnosis of the first human case of Eastern Equine encephalitis in Tennessee in 2019, there has been a history of equine cases in the state since 2002 (CDC, 2020d; Cohen et al., 2009).

## Flaviviruses

Flaviviruses are single-stranded positive-sense RNA viruses with an envelope. Presently, there are 39 known mosquito-borne flaviviruses (Theil et al., 2005) Flaviviruses such as yellow fever, dengue, and Japanese encephalitis are of global epidemiological importance. Two flaviviruses of zoonotic importance in the United include West Nile and St. Louis Encephalitis viruses (Theil et al., 2005).

### *West Nile virus history, ecology, and transmission*

West Nile virus (WNV) was first described from the West Nile District of Uganda in 1937 (Smithburn et al., 1940) and was detected in Africa, the Middle East, Europe, and Asia up to the 1960s (Kramer et al., 2019; Komar, 2003). From 1975 to 1996, there was little documented WNV activity (Kramer et al., 2019). However, in 1996, cases of WNV began to occur more frequently in the Mediterranean Basin, Russia, and Australia (Komar, 2003). In the following year, a new strain, WN-Israel 1998, emerged in Israel that resulted in the death of geese (Lanciotti et al., 1996; Weinberger et al., 2001). This was the first time avian fatalities had been connected to WNV (Hubálek and Halouzka, 1999; Malkinson and Banet, 2002). This same strain was documented in the Western Hemisphere for the first time in the United States in 1999

(Jia et al., 1999; Lanciotti et al., 1999). By 2003, WNV spread across most of the United States (Roehrig, 2013). Within three years, WNV was also detected throughout most of Central American and as far south as Argentina (Komar and Clark, 2006; Morales et al., 2006).

Despite the similarities in the outbreaks in Israel and the United States, it took a thorough investigation to determine that WNV had been introduced to the United States. The outbreak began during the beginning of the summer of 1999 with the discovery that dead crows (*Corvus brachyrhynchos and Corvus ossifragus*) were found in unusual numbers near Queens, New York. Additionally, Chilean flamingos (*Phoenicopterus chilensis*) and a snowy owl (*Bubo scandiacus*) died from an unknown illness at the Bronx Zoo (Roehrig, 2013). Horses were diagnosed with an equine encephalitis in Long Island at the same time (Roehrig, 2013). However, these unusual incidents did not receive a full investigation by the Centers for Disease Control and Prevention (CDC) until a cluster of cases of encephalitis in humans were reported in Queens during August of 1999 (Roehrig, 2013). Initial investigations determined the causative agent in humans was St. Louis Encephalitis virus (Roehrig, 2013). SLE, a virus spread by mosquitoes, had previously been reported in the United States but never in New York or the Northeast (Reisen, 2003). Mosquito management strategies were implemented in New York City (NYC) as further investigation of the outbreak was conducted. However, the head veterinary pathologist for zoos in the NYC Area, Dr. Tracy McNamara, objected to this identification because the bird die-offs at the zoos did not match typical SLE epidemiology and clinical patterns (Wilson and McNamara, 2020). McNamara sent samples to the National Veterinary Services Laboratory (NVSL) at the United States Department of Agriculture in Ames, Iowa for further analysis. The NVSL identified the agent as a flavivirus, which was concerning because no flaviviruses had been known to cause symptomatic animal disease in the Western

Hemisphere (Wilson and McNamara, 2003). Samples were submitted to the CDC for further testing, while additional samples were sent to the U.S. Army Medical Institute for Infectious Diseases (USAMRIID). The CDC and USAMRIID confirmed the flavivirus identification and ruled out St. Louis encephalitis virus (GOA, 2000; Wilson and McNamara, 2003). The CDC identified WNV in the animal samples, while an academic researcher identified WNV in human samples (GOA, 2000; Wilson and McNamara, 2003). In the initial attempts at identifying the agent, serologic testing was performed (CDC, 1999). St, Louis encephalitis virus and WNV are antigenically related, leading to cross-reactions on the serologic tests. Furthermore, since WNV was not a previously known agent in the US, testing reagents were not included in initial PCR assays (CDC, 1999). The discovery was significant because it was the first time West Nile virus had been detected in the Western Hemisphere. The initial outbreak and consequent spread of WNV across the U.S. exposed weaknesses in free-ranging wildlife surveillance, agency collaboration, multi-species analyses, and a lack of valuable "non-traditional" health partners (GOA, 2000).

To this day, it is not clear how West Nile virus was introduced to the United States, but it is likely through the importation of infected birds into the United States. Phenology studies have shown a close relationship between New York City virus isolates and one from a goose in Israel collected in 1998 (Lanciotti et al., 1999). In 1999, the Bronx Zoo only imported six birds. All of these birds were WNV antibody negative during the outbreak, indicating that they were not the initial WNV outbreak source in New York (Ludwig et al., 2002). Birds imported for pet shops, would have most likely died during a quarantine period, so it is possible illegal importations could be the source (Johnson and Conly, 2000). Another possible explanation is the introduction of infected adults through transportation from other countries (Roehrig, 2013). Live mosquitoes

have been found on both international flights and shipping containers on boats (Kilpatrick et al., 2006a). Infected humans are not suspected as a source because humans typically do not have a high enough WNV titer load to infect mosquitoes (Roehrig, 2013; Kilpatrick et al., 2006a).

As of 2016, WNV has been detected in 67 mosquito species in the United States (https://www.cdc.gov/westnile/resources/pdfs/MosquitoSpecies1999-2016.pdf). However, not all of these species are competent vectors. Additionally, some mosquitoes have host blood feeding patterns that reduce the likelihood that they would be involved in the transmission cycles of WNV (Turell et al., 2005). WNV is maintained in a bird-mosquito cycle with occasional spillover over into mammals and humans (Turell et al., 2005). Therefore, species that primarily feed on birds are the most effective enzootic vectors, while species considered to be opportunistic feeders are the most effective bridge vectors (Turell et al., 2005). Important vectors of WNV in the U.S. are *Culex p. pipiens*, *Culex p. quinquefasciatus*, *Culex restuans*, and *Culex tarsalis* (Hayes, 2006; Turell et al., 2005; Kilpatrick et al., 2005). *Culex p. pipiens*, is typically found north of U.S. 30-40°N latitude, while *Culex p. quinquefasciatus* is found in the southern US, with a zone of hybridization in between (Barr, 1957). *Culex restuans* is a common species in the eastern and central United States (Darsie and Ward, 2005). *Culex tarsalis* is common west of the Mississippi River with occasional populations in the eastern US (Darsie and Ward, 2005). Passerine birds, especially corvids (such as crows and jays), are the reservoir for WNV (Hayes, 2006; Murray, 2010). However, American robins (*Turdus migratorius*) are suspected to be super spreaders of WNV. Despite representing a small proportion of bird species in urban settings, they are favored for feeding (Kilpatrick et al., 2006b). While, mammals such as humans and horses, are dead end hosts (Murray, 2010).

Most WNV infections in humans are asymptomatic, while approximately 20% of infections result in symptoms similar to those of flu, such as fever, fatigue, headache, weakness, and muscle pains (Hayes, 2006). In an estimated 1% of infections, the virus infiltrates the central nervous system resulting in neuroinvasive disease (Hayes, 2006). WNV neuroinvasive disease symptoms can range from meningitis to severe encephalitis. Long term effects, as well as mortality, varies with the neuroinvasive form (Murray, 2010). The majority of human cases are reported from July to September (Lindsey et al., 2010). As of 2018, over 50,000 cases of West Nile virus have been reported in the U.S., with approximately half of those cases reported as neuroinvasive. The neuroinvasive case fatality rate is approximately 9%, while the non-invasive case fatality rate is 0.5%. Overall, WNV has a case fatality rate of 4.6% in the United States (CDC, 2020c).

***St. Louis encephalitis history, ecology and transmission***

In 1933, there was an epidemic of over 1,000 encephalitis cases in St. Louis, Missouri (Lumsden, 1985). Following the inoculation of mice and monkeys with infected brain tissues from patients, the infectious agent, now named St. Louis encephalitis virus (SLEV), was isolated and determined to be distinct from other known causes of encephalitis (Armstrong and Lillie, 1934). Although this was the first time the agent was identified, in the previous year there was an encephalitis epidemic in Paris, Illinois now suspected to have been caused by SLEV (Leake, 1933). Since 1933, SLEV outbreaks have occurred sporadically across the United States with a concentration among major cities and smaller towns (Tsai and Mitchell, 1989; Diaz et al., 2018). SLE has also been detected in South America (Diaz et al., 2018).

Much like WNV, the primary vectors of SLEV are *Culex* mosquitoes (Tsai and Mitchell, 1989). *Culex p. pipiens* is the main vector from Ontario to the Midwest. *Culex p. quinquefasciatus* is a competent vector from the Midwest to Mexico, while *Culex nigripalpus* is the primary vector in Florida. *Culex tarsalis* is a vector in western states. *Culex salinarius* and *Culex restuans* might be secondary vectors of SLEV (Tsai and Mitchell, 1989). The transmission cycle is often perpetuated through passeriform and columbiform birds, such as mourning doves (*Zenaida macroura*), pigeons (*Columba* sp.), and house sparrows (*Passer domesticus*) (Reeves et al., 1962; Lord et al., 1974; Smith et al., 1983). Despite the detection of SLEV virus in a variety of mammals and birds, clinical disease in these animals has not been documented (Tsai and Mitchell, 1989).

The majority of cases in humans are asymptomatic (Tsai and Mitchell, 1989). However, the case fatality rate can reach up to 23% in elderly populations (Day, 2001). Those that develop symptoms typically experience fever, headaches, dizziness, nausea, or malaise. These symptoms can intensify and show signs of CNS infection through stiff neck, altered levels of consciousness, more pronounced dizziness, cranial nerve palsy, tremors, seizures, paralysis, or coma (Tsai and Mitchell, 1989). Human cases of SLEV tend to occur in the late summer and into early fall (Tsai and Mitchell, 1989). Even though there were nearly 2,000 human cases of SLEV from the 1950s to the 1990s, only 97 cases of human SLEV, of which 6 were fatal, were reported from 2010 to 2019 in the United States (Day, 2001; CDC, 2020e).

## Alphaviruses

Alphaviruses are positive strand RNA viruses with an envelope. The alphavirus serocomplexes of medical importance are eastern equine encephalitis, western equine encephalitis, Venezuelan equine encephalitis, and Semliki Forest viruses (Powers and Roehrig, 2011). The Semliki Forest serocomplex includes chikungunya, which has the potential to become an established pathogen in the United States (Escobar et al., 2016). Despite frequent exposure in Florida to the Venezuelan equine encephalitis serocomplex, these viruses are rare (Coffey et al, 2006). The lack of human cases of western equine encephalitis virus in the United States since 1998 and the reduced detection of western equine encephalitis virus in mosquitoes has decreased concern for this virus as a health concern (Robb et al, 2019). Therefore, the primary endemic alphavirus concern in the United States is eastern equine encephalitis.

### *Eastern equine encephalitis history, ecology, and transmission*

Eastern equine encephalitis virus (EEEV) was first isolated in 1933, following outbreaks of equine encephalitis in coastal areas of New Jersey and Virginia (Ten Broeck and Merrill, 1933). Retrospective work suggests that equine encephalitis in North American horses has been occurring since at least 1831 (Hanson, 1957; Morris, 2019). The first identification of EEEV in humans was made in 1938 in Massachusetts (Fothergill et al., 1938). Since discovery, EEEV human and nonhuman outbreaks have occurred primarily in states along the Gulf of Mexico, the Atlantic Coast, and the Great Lakes (Morris, 1989; Lindsey et al., 2018; Foster and Walker, 2009). All the remaining states east of the Mississippi River, Oklahoma, and Arkansas have

occasionally documented human and nonhuman EEEV cases (Lindsay et al., 2018). EEEV also occurs in Central and South America (Foster and Walker, 2009)

EEEV is maintained in a bird-mosquito enzootic cycle in swamps and wetlands (Foster and Walker, 2009; Morris, 2019). This is primarily due to swamps and wetlands being the preferred oviposition environment for the primary enzootic vector, *Culiseta melanura* (Joseph and Bickley, 1969). *Culiseta melanura* is highly ornithophilic and commonly feeds on passeriformes (Morris, 1989). The infection of humans and other mammals is facilitated through occasional mammal feeding by *Culiseta melanura* and bridge vectors such as *Aedes canadensis*, *Aedes cinereus*, *Aedes sollicitans*, *Coquillettidia perturbans*, *Culex nigripalpus*, *Culex restuans*, and *Culex erraticus* (Armstrong and Andreadis, 2010; Crans and Schulze, 1986; Nayer, 1982; Chamberlain et al., 1954). In Tennessee, it is suspected that *Culex erraticus* is replacing *Culiseta melanura* in wetlands, and as such, could be the species maintaining EEEV transmission (Mukherjee et al, 2012).

Approximately 5% of infected humans develop symptoms of EEEV, which present as either a systemic or encephalitic infection (Goldfield et al., 1968, Morris, 1989). Symptoms of systemic infection include malaise, myalgia, fever, and shaking (Morris, 1989). Encephalitic infection symptoms include fever, restlessness, drowsiness, vomiting, diarrhea, headache, convulsions, tremors, neck stiffness, and coma. Fatality can result from the encephalic form (Morris, 1989). In humans, the EEEV mortality rate is approximately 70% with the majority that recover suffering long term sequelae (Morris, 1989). From 2010-2019, there were 107 human cases of EEEV in the United States (CDC, 2020d). In addition to horses, a variety of birds, including pheasants and sparrows, show signs of infection (Morris, 1989). A number of other animals such as snakes, rodents, and white-tailed deer (*Odocoileus virginianus*) show symptoms

of EEEV infection in nature or are susceptible to infection in laboratory settings (Morris, 1989; Schmitt et al., 2007).

## Bunyaviruses

Bunyaviruses are tri-segmented, negative sense, and single stranded RNA viruses with an envelope. Within the *Peribunyaviridae* family is the California serogroup (CSG). The CSG currently includes 18 viruses, all of which are arboviruses (Evans and Peterson, 2019). Although the CSG viruses are distributed globally, only 11 are found in North America (Evans and Peterson, 2019). In North America (and Tennessee), the main CSG viruses of concern are La Crosse virus and Jamestown Canyon virus (Rosenberg et al., 2018; Foster and Walker, 2009; CDC, 2020a; CDC, 2020b).

### *La Crosse virus history, ecology, and transmission*

In 1960, La Crosse virus (LACV) was isolated following the death of a child from meningoencephalitis in La Crosse, Wisconsin (Thompson et al., 1965). LACV infections were traditionally associated with the forested areas of the upper-midwestern United States (Grimstad, 1989a). However, the Appalachian region has experienced a rise in LACV infections since the 1990s and often surpasses the yearly number of cases in the midwestern United States (Jones et al., 1999; Nasci et al., 2000; Leisnham and Juliano, 2012). The reason for this geographic shift of LACV is unclear, but it could be linked to the expansion of humans into the hardwood forests of the Appalachian region or the interactions of multiple competent vectors (Bewick et al., 2016; Leisnham and Juliano, 2012).

The distribution of cases of LACV overlaps with the distribution of *Aedes triseriatus* and suitable hardwood forest environments (Grimstad, 1989a; Darsie and Ward; 2005). *Aedes triseriatus* is a competent vector of LACV and commonly feeds on chipmunks and tree squirrels, which are reservoirs of LACV (Watts et al., 1972; Grimstad, 1989a; Moulton and Thompson, 1971). *Aedes triseriatus* also feeds on humans frequently. Therefore, *Aedes triseriatus* is considered the primary vector of LACV. Additionally, LACV can be maintained in *Aedes triseriatus* through transovarial transmission (Watts et al., 1975). LACV infected *Aedes japonicus* and *Aedes albopictus* have been collected during routine surveillance, making these species potential secondary vectors of LACV (Westby et al., 2015). Both species are competent vectors of LACV in the laboratory (Sardelis et al., 2002; Grimstad, 1989b).

Most severe cases of LACV occur in children under age 16 (Grimstad, 1989b). As a result, LACV is one of the leading causes of neuroinvasive arboviral infections in children in the United States (Gaensbauer et al., 2014). Initial symptoms of LACV can include fever, chills, headaches abdominal pain, and upper respiratory issues. More severe symptoms can develop such as vomiting, nick stiffness, lethargy, and coma (Calisher, 1994). The case fatality rate in the eastern United States is 1.9% (Haddow and Odoi., 2009). Most cases of LACV occur in the summer months when mosquito activity is high (Calisher, 1994; Haddow and Odoi, 2009). In addition to the 111 cases of LACV in Tennessee, there were 630 cases of LACV in the United States from 2010-2019 (CDC, 2020a)

***Jamestown Canyon virus history, epidemiology, and transmission***

Jamestown Canyon virus (JCV) was first isolated from *Culiseta inornata* collected in Jamestown Canyon, Colorado, in 1961 (https://wwwn.cdc.gov/arbocat/VirusDetails.aspx?ID=206&SID=2). Since that time, JCV has been detected in mosquitoes, humans, and animals across the United States (Pastula et al., 2015). However, since 2000, most cases of JCV have occurred in the midwestern United States (Pastula et al., 2015; CDC, 2020b).

JCV has been isolated from at last 26 different species of mosquitoes, including a variety of *Aedes*, *Anopheles*, *Culex*, *Ochlerotatus*, and *Psorophora* (Andreadis et al., 2008). White-tailed deer are considered to be an important reservoir, but JCV has been detected in moose (*Alces alces*), elk (*Cervus canadensis*), bison (*Bison bison*), and mule deer (*Odocoileus hemionus*) as well (Grimstad, 1989a).

Symptomatic human infections with JCV can present as a range from fever, headache, and respiratory disease to signs of meningitis or encephalitis (Grimstad, 1989a). Most cases occur during spring to early fall (Pastula et al., 2015). JCV reported cases have increased in recent years. From 2010 to 2019, there were 225 cases of JCV in the United States. During this time, the case fatality rate was 2% (CDC, 2020b). However, JCV may be underreported as demonstrated by an increase in the number of cases since 2013 when routine JCV IgM antibody testing was implemented by the CDC (Pastula et al., 2015).

## The Relationship Between Mosquitoes and Zoological Institutions

The presence of multiple types of larval habitats ranging from artificial containers to natural pools in zoological parks supports the mosquito life cycle. This variance in microhabitats within allows for mosquitoes with a variety of larval habitat preferences to coexist. Additionally, the incorporation of plants, such as bromeliads or bamboo, into animal enclosures unintentionally creates more natural larval habitats (Nelder, 2007; Shimonski, 2010). The collection of larval mosquitoes from multiple zoos demonstrates that mosquitoes are taking advantage of the presence of these environments (Beier and Trpis, 1981; Derraik, 2004a; Derraik, 2005b; Derraik et al., 2008; Tuten, 2011a; Heym et al., 2018). The hesitancy of zoos to use chemical control methods or the lack of control efficacy assessments can lead to continued development of mosquitoes (Adler et al., 2011). In addition to providing unique larval habitats for mosquitoes, zoos represent unique aggregations of animals for host seeking adult mosquitoes. Zoological institutions create a complicated network of animal interactions through the presence of wild native animals, clustering of exotic species, and the movement of captive species between zoological institutions. Often valuable and rare animals kept at zoos are susceptible to infections transmitted by mosquitoes from native wildlife reservoirs (see Table 1). This animal network in combination with the presence of humans and larval mosquito habitats within a zoo creates opportunities for transmission of mosquito-borne diseases to humans as well.

Mosquitoes are the leading arthropod of medical and veterinary importance in zoos as evidenced by the number of and variety of mosquito-borne disease infections in animals housed in zoological parks (Adler et al., 2011). Despite, the knowledge that medically important arthropods could be abundant in zoos, few studies involving these arthropods are proactive. Rather, most of the knowledge about medically important arthropods in zoos is the result of

retroactive studies following an epizootic outbreak at a zoo (Adler et al., 2011). The potential utility of studying medically important arthropods and associated disease transmission factors in zoos are numerous. Studies could provide insight into host specificity, potential reservoir species, and environmental characteristics related to arthropod life cycles (Adler et al., 2011). Zoos also have the potential to serve as biosurveillance sites due to the daily monitoring of the health status of captive animals is and the full investigation of any death of a captive animal. As demonstrated through the use of the Bronx Zoo during the investigation of the 1999 WNV outbreak, this information was useful in the identification of WNV as the causative agent of infection (Ludwig et al., 2002; McNamara, 2007). As arboviral threats continue to change due to factors such as invasive and expanding species, climate change, and habitat modification, biosurveillance at zoos represents a significant way to monitor these changes and contribute to our growing knowledge of One Health and emerging pathogens.

Table 1. Examples of mosquito-borne infections impacting captive zoo and aquarium animals in the USA

| Year/location | Pathogen | Animals Infected | Vector Surveillance Program in Place | Reference |
|---|---|---|---|---|
| 1999/Bronx NY | WNV | 38 avian species, red panda (*Ailurus fulgens fulgens*), snow leopard (*Panthera uncia*), ring-tailed lemur (*Lemur catta*), Indian rhinoceros (*Rhinoceros unicornis*), Indian elephant (*Elephas maximus indicus*), babirusa (*Babyrousa babyrousa*) | No | Ludwig et al., 2002 |
| 2002,2003/ Miami FL, Powell OH, Tampa FL, Albuquerque NM, Aurora, OH | WNV | Cougar (*Puma concolor*), lion (*Panthera leo*), tiger (*Panthera tigris*), Asian elephant (*Elephas maximus*), alpaca | unknown | Keller, 2005* |

| | | | | |
|---|---|---|---|---|
| | | (*Vicugna pacos*), box turtle (*Terrapene* sp.) | | |
| 2002/ Camden NJ, Detroit, MI | WNV | Harbor seal (*Phoca vitulina*) | unknown | Del Piero et al., 2006 |
| 2002/ Washington D.C. | WNV | Gray seal (*Halichoerus grypus*) | unknown | Wilkins and Del Piero, 2004 |
| 2002/ Milwaukee, WI | WNV | Humboldt penguin (*Spheniscus humboldti*), satyr tragopan (*Tragopan satyra*), snowy owl (*Bubo scandiacus*) | unknown | The Journal Times, 2002 |
| 2004/Albuquerque, NM | WNV | Harbor seal (*Phoca vitulina*), thick-billed parrot (*Rhynchopsitta pachyrhyncha*), Impeyan pheasant (*Lophophorus impejanus*), rainbow lory (*Trichoglossus moluccanus*) | unknown | Gentz and Richard, 2004 |
| 2007/ San Antonio, TX | WNV | Killer whale (*Orcinus orca*) | Yes, enhanced following infection | St. Leger et al., 2011 |
| 2017/ Nashville, TN | WNV | Bontebok (*Damaliscus pygargus*) | None prior to 2017 | Moore et al., 2018 |
| 1990/ Orlando, FL | SLEV | Killer whale (*Orcinus orca*) | Yes, enhanced following infection | Buck et al., 1993 |
| 2002,2003/ Miami FL, Powell OH, Tampa FL, Albuquerque NM, Aurora, OH | SLEV | Camels (*Camelus* sp.), Asian elephants (*Elephas maximus*), lion (*Panthera leo*), tiger (*Panthera tigris*) | unknown | Keller, 2005* |
| 2006/ MA | EEEV | Harbor seal (*Phoca vitulina*) | unknown | McBride et al., 2008 |
| 2019/ Battle Creek. MI | EEEV | Mexican gray wolves (*Canis lupus baileyi*) | CDC light trap sampling following infections | Thompson et al., 2020 |
| 2003/ Mystic, CT | EEEV | African penguins (*Spheniscus demersus*) | unknown | Tuttle et al., 2005 |
| 2014/ Norfolk, VA | EEEV | Southern cassowary (*Casuarius casuarius*) | unknown | Guthrie et al., 2016 |

*A list of zoos from which samples were obtained was provided, but specifics about each animal's location were not provided.

# Chapter 2:

# Mosquito Diversity, Arboviral Risks, and Blood Feeding Patterns at the Nashville Zoo at Grassmere

## Introduction

The epidemiological triad is a commonly used framework for conceptualizing how interactions between the environment, hosts, and the agent result in disease. In the case of vector-borne diseases, vectors are added to the center of the epidemiological triad given their direct link with each component of the triad. In the United States, these interactions have resulted in outbreaks such as West Nile virus (WNV) infections in over 100 animals at the Bronx Zoo, the death of two Mexican grey wolves (*Canis lupus baileyi*) from Eastern equine encephalitis virus in a Michigan zoo, and the death of a killer whale (*Orcinus orca*) from St. Louis encephalitis virus (SLEV) at a park in Florida (Ludwig et al., 2002; Thompson et al., 2020; Buck et al., 1993). Some of these examples are the product of the exposure of naïve animals to newly introduced mosquito pathogens. Other examples demonstrate the increased vulnerability of animals, such as marine mammals, to mosquito-borne disease when captivity can lead to alternations in normal behavior. Some examples also demonstrate how prior health issues can make captive animals more susceptible to infection with mosquito-borne diseases (Buck et al., 1993; Thompson et al., 2020). However, these examples also demonstrate the untapped potential zoos could have as important biosurveillance sites if a more proactive approach is made (McNamara, 2007).

In addition to biosurveillance, zoos represent valuable environments to further study basic mosquito biology such as habitat associations or feeding behavior (Adler et al., 2007; Tuten, 2011b). In this study, I investigated several factors related to mosquito-borne disease ecology. My objectives were to compare species diversity inside and outside of the zoo, determine blood feeding patterns, and conduct surveillance for mosquito-borne viruses. To investigate these objectives, I used CDC miniature light traps and BG Sentinel traps to attract a wide variety of

species. I also employed resting boxes to increase the chances of collected engorged mosquitoes and gravid traps to target mosquitoes of interest for viral testing.

High densities of mosquitoes can be collected from zoos and it is likely that the abundance of hosts at zoos support larger mosquito populations (Derriack et al., 2003). The abundance of a variety of larval habitat types in zoos could support the establishment of a wide variety of mosquito species, which could lead to the maintenance of multiple mosquito-borne diseases. Therefore, I expected that a wider diversity of species will be collected from within the zoo when compared to the surrounding area.

The results of previous US studies on mosquito feeding patterns from zoos have generally aligned with host-class usage trends, but novel variations in host usage and reports of mosquito-borne diseases in non-typical captive animals support further investigation (Tuten et al., 2012). I expected to find similar classes of animals utilized as blood hosts by mosquito species at the zoo, but I also expected some variation based on the regional availability of exotic hosts.

In 2017, a bontebok (*Damaliscus pygargus*) from the Nashville Zoo at Grassmere died from an infection with WNV (Thomas et al., 2018). As a result, I tested mosquito pools for WNV to explore factors related to the year-to-year persistence of WNV within the Nashville Zoo at Grassmere. In Tennessee, Flanders virus (FLAV) causes benign infections, but detection can signal future WNV activity. FLAV infection rates typically peak in mosquitoes approximately 10 weeks prior to WNV peak infection rates (Lucero et al., 2016). I tested specimens for FLAV to further evaluate its value as a sentinel virus. In addition, I tested our collected mosquitoes for SLEV because the unpredictability and historical presence of SLEV in Tennessee makes it an important focus of surveillance for my study (Day 2001, Levy et al 1978).

## Methods

### Field site

The Nashville Zoo at Grassmere (36°05'21.1" N, 86°44'37.4" W) is an Association of Zoos and Aquariums-accredited institution located approximately 10 km south of downtown Nashville. The 76 ha zoo, which lies within the Outer Nashville Basin ecoregion (Griffith *et al*., 1997), is surrounded by neighborhoods and industrial buildings. In 2019, over 1.2 million people visited the zoo (Nashville Zoo Annual Report, 2019). It is currently home to 2,764 animals representing 365 different species (https://www.nashvillezoo.org/our-animals; J. Hardwick, personal communication, November 6, 2020). Throughout the zoo, deciduous forest has been modified to mimic habitats such as savannas, Indonesian forest, Peruvian forest, and bamboo forest. The 2020 average temperature during the collection months was as follows: June 25.3°C (range 11.7°C to 35°C); July 28.3°C (range 19.4°C to 36.7°C); August 26.4°C (range18.9°C to 36.1°C); September 22.6 °C (range 8.9°C to 33.3°C); October 16.9°C (range 3.3°C to 30°C). Total rainfall during the study period was as follows: June 84.58 mm, July 112.27mm, August 149.10mm, September 96.52mm, and October 89.15mm (https://w2.weather.gov/climate/xmacis.php?wfo=ohx).

### Mosquito Collecting

I collected adult mosquitoes within the Nashville Zoo from 10 June to 1 October 2020. Ten sites were selected using prior knowledge of mosquito activity noted by zoo colleagues and with the consideration of minimizing direct viewing by the public (Fig 1). Two additional sites outside of the zoo were selected based on environmental factors and proximity to the zoo (Fig 1).

Residence A was two kilometers from the zoo, had a small creek flowing through the yard, and was adjacent to a wooded area. Residence B was a half kilometer from the closest zoo border and was adjacent to a drainage ditch. I divided the sites within the zoo into two groups of five. I alternated the collection group each week so that collections were made at each site every other week. The residential sites were sampled every week from 24 June to 1 October 2020. At each site, I placed a $CO_2$-baited miniature CDC light trap, BG sentinel trap baited with a BG lure, CDC gravid trap baited with a grass infusion, and a black wooden resting box (45.72 cm x 31.75 cm x 24.13 cm). The first 3 traps were obtained from BioQuip Products (Rancho Dominguez, CA, USA). The resting boxes were created by purchasing wooden boxes with the beforementioned dimensions at a hardware store, lined with felt to seal any openings large enough for mosquitoes to escape, and painted black. Five of the most productive trapping sites were selected for placement of a larger additional resting box with an expanded opening (45.72 cm x 30.48 cm x 76.2 cm). The larger resting boxes were custom built of plywood which was painted a flat black color. Traps were set in the morning between 07:00 to 10:00 hrs and left in place for 48 hrs each week weather permitting. Approximately 1.15 kgs of dry ice was added to the CDC light traps between 14:00 to 15:30 hrs on the day of set up. An additional 4 kgs of dry ice was added when replacing mosquito collection nets on the second day. Mosquito samples were collected on the following two mornings from 07:00 to 10:00 hrs. Collections were placed in plastic bags, labeled by trap location and date, and transported to the Tennessee Department of Health laboratory in a cooler on ice. They were stored at -20 °C for sorting and identification.

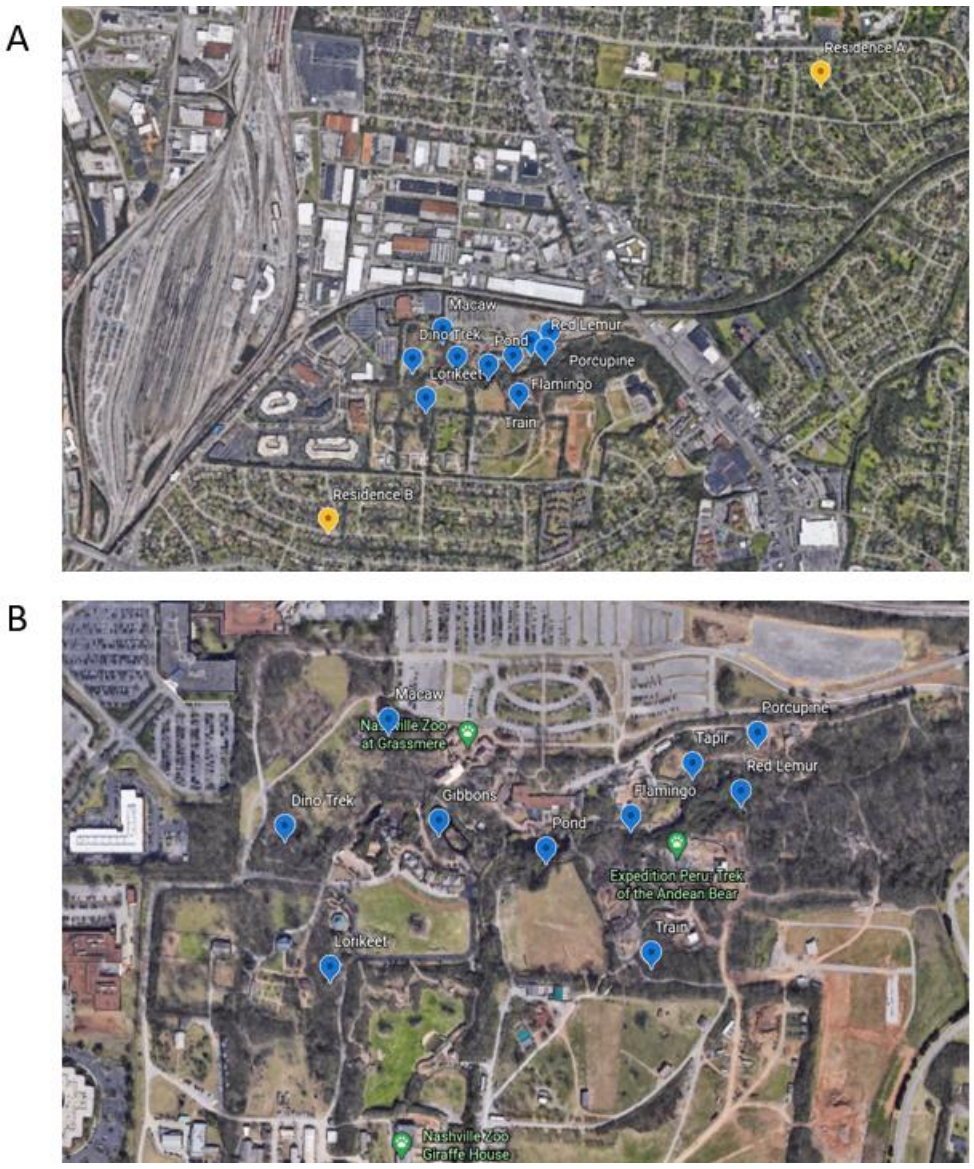



**Figure 2**. (A) Map showing the location of the ten mosquito sampling sites inside the zoo (blue) and location of two sites outside of the zoo (yellow). (B) A closer view of mosquito sampling sites inside of the zoo.

## Mosquito sorting and identification

Mosquitoes were sorted on a chill table under a stereo microscope and identified according to published keys (Darsie and Ward, 2005; Burkett-Cadena, 2013; Harrison et al., 2016 ). Males were discarded, while females were examined for physiological status and

considered blood engorged if a bloodmeal was visible in the abdomen. Engorged mosquitoes were placed individually in sterile labeled microcentrifuge tubes and stored at -80°C for transport back to Cornell University for bloodmeal identification. The remaining female mosquitoes were placed in pools of up to 50 individuals by trap date, location, trap type, and species in microcentrifuge tubes. Pools were stored at -80°C for virus testing. Mosquito data was recorded by species, engorgement status, collection date, trap type, and location in a master spreadsheet.

**Virus Testing**

*Culex* mosquitoes were tested for WNV, FLAV, and SLEV according to the Tennessee Department of Health protocols (Westby et al., 2015), with modifications. Briefly, three copper BBs (Crosman, Fairport, NY, USA) were added to each microcentrifuge tube containing pooled mosquitoes, followed by the addition of one ml of Eagle's Minimum Essential Medium (Mediatech, Inc., Manassas, VA) with 2% Fetal Bovine Serum (Life Technologies Corporation, Grand Island, NY), 0.5% Sodium Bicarbonate (Mediatech, Inc., Manassas, VA), and 1% Antibiotic-Antimycotic Solution (Mediatech, Inc., Manassas, VA). Samples were homogenized using a MM300 Mixer Mill (Retsch, Haan, Germany) at 30/s for 90 sec. The microcentrifuge tubes were removed from the Mixer Mill and left to rest for at least a minute. The microcentrifuge tubes were centrifuged at room temperature for 7 mins at 12,000 rpm. Afterward, 140 ml of mosquito supernatant was transferred from each tube into the corresponding well of a 96 well S-block. The S-block was then placed in a BioRobot Universal System 9604 (Qiagen Sciences, Germantown, MD, USA) for RNA extraction using the QIAmp Viral Isolation 96-well protocol. Tubes containing the remaining supernatant were stored at -80°C.

A 20 uM reaction mixture was used containing 6.25 ul of 4X TaqMan Fast Virus 1 Step Master Mix (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), 0.4 ul of WNV_#3 1160 forward primer (25uM), 0.4 ul of WNV_#3 1229c reverse primer (25uM), 0.1 ul of probe WNV_#3 p1186-FAM/BHQ1 (25uM), 0.4 ul of FLD f_16 forward primer (25uM), 0.4 ul of FLD r_94 reverse primer (25uM), 0.1 ul of probe FLD p41 JOE/BHQ1 (25uM), 0.4 ul of SLE_834 forward primer (25uM), 0.4 ul of SLE_905c reverse primer (25uM), 0.1 ul of probe SLE p857 CY-5/ZEN/ (25uM), and 6.25 ul of RT-PCR grade water (Table 2). For each multiplex RT-PCR, RT-PCR grade water was used as a negative control; WNV, FLAV, and SLE dilutions were used as positive controls. Pools were considered positive if the control threshold score was less than or equal to 37. The following cycling conditions were used: 50°C for 5 min, 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec.

**Table 2**. Primers sequences used to amplify WNV, FLAV, and SLE RNA in mosquito samples.

| Type | Name | Sequence |
|---|---|---|
| Primer | WNV_1160 fwd | 5'- TCA GCG ATC TCT CCA CCA AAG -3' |
| Primer | WNV_1229 rev | 5'- GGG TCA GCA CGT TTG TCA TTG -3' |
| Probe | WNV 1186 | 5'- /56-FAM/TGC CCG ACC /ZEN/ATG GGA GAA GCT C/31ABkFQ/ -3' |
| Primer | FLD f_16 fwd | 5'- AAG TCA ATA AGA AAT GGC AAG CAA -3' |
| Primer | FLD r_94 rev | 5'- AGA AGG CTT TTG GAT ACT GTG GTT -3' |
| Probe | FLD p41 zen | 5'-/56-JOEN/TTC GCT TTT /ZEN/TGG CAC CTG CAG ATA AGG t/31ABkFQ/ -3' |

| | | |
|---|---|---|
| Primer | SLE_834 fwd | 5'- GAA AAC TGG GTT CTG CGC A -3' |
| Primer | SLE_905c rev | 5'- GTT GCT GCC TAG CAT CCA TCC -3' |
| Probe | SLE 857 | 5'- /5Cy5/TGG ATA TGC /TAO/CCT AGT TGC GGC /31ABRQSp/ -3' |

**Blood meal analysis**

Mosquitoes were maintained on ice and abdomens from engorged specimens were removed and transferred to a sterile microcentrifuge tube. The head and thorax were returned to the original vial for later molecular identification (*Culex* spp. complex). The degree of blood engorgement was scored using the Sella's score (Sella, 1919). Forceps were cleaned between samples by dipping in 80% ethanol and passing through a flame. DNA extractions were performed using Qiagen Puregene kits (Qiagen Sciences, Germantown, MD, USA). To identify bloodmeals, medium size primer sets were used to amplify a 395 base pair vertebrate-specific region of cytochrome c oxidase subunit I (Table 3) (Reeves et al., 2018). To increase amplification success for failed PCR reactions, small-sized primers used by Reeves et al. (2018) and cytochrome b primers used by Townzen et al. (2008) were used on all failed samples from the first round of PCR.

A 20 uM reaction mixture was used containing 10 ul of 2.0X Apex Taq RED Master Mix (Genesee Scientific Corp., San Diego, CA), 0.75 ul of VertCOI_7194_F forward primer (10 uM), 0.75 ul of Mod_RepCOI_R reverse primer (10 uM), 7.5 ul sterile nuclease-free water, and 1 ul of extracted DNA. The following thermocycler conditions were used: 94°C for 3 min, followed by 40 cycles of 94°C for 40 s, 53.5°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 7 min. All reactions included a positive animal fed control and a negative water

control. PCR products were loaded onto a 1% agarose gel stained with gelRED, electrophoresed for 45 min, and visualized with BioRad Gel Doc XRS. Samples with positive bands after gel electrophoresis were excised and cleaned with ExoSap-IT then submitted to Cornell Biotechnology Resources Center for sequencing. Sequences were compared to BOLD and NCBI databases and identified to a host if the matches were greater than 98%.

**Table 3**. Primer sequences used to amplify a 395 bp segment of COI gene from host blood meals (Reeves et al., 2018).

| Primer | Sequence | Specificity |
|---|---|---|
| VertCOI_7196_F | 5'- CGM ATR AAY ATR AGC TTC TGA Y -3' | Vertebrate universal |
| Mod_RepCOI_R | 5'- TTC DGG RTG NCC RAA RAA TCA -3' | Universal |

**Host availability data**

Zoo census of captive species and visitors was determined weekly for the duration of the mosquito collection period (Appendix 1, Appendix 2). There were at least 500 captive animals at the zoo with outdoor access and potential exposure to blood feeding mosquitoes. With capacity limitations due to COVID-19 restrictions, an average 15,225 guests visited the zoo per week during the collection period. In addition, I checked the BOLD (http://www.boldsystems.org/) and NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases to ensure that DNA was available for these captive species.

**Data analysis and reporting**

Data cleaning and analysis were performed in RStudio using the following packages: dplyr (Wickam et al., 2021), ggplot2 (Wickham, 2016), tidyr (Wickham and Henry, 2019), vegan (Oksanen et al., 2020), lubridate (Grolemund and Wickham, 2011), devtools (Wickham et al., 2020), pmartinezarbizu/pairwiseAdonis/pairwiseAdonis (Martinez Arbizu, 2020), pairwiseAdonis (Martinez Arbizu, 2017), and ggthemes (Arnold, 2021). Data collected from the Rhino, Farmhouse, and Spider Monkey sites were not included as they were discontinued following low mosquito collections. Vegetation aspirator collections with Prokopak style aspirators were not included in analysis because they were not productive and discontinued. Both dimensions of resting boxes were combined into the same category.

Mosquito diversity was determined using the Shannon - Weiner index (Barnes et al., 1998), which considers species richness and evenness for each site. Comparison of the Shannon-Weiner indexes was performed using a Wilcoxon rank-sum test. Multivariate analysis of variance was used to determine factors contributing to differences in collections at each site. Minimum infection rates (MIR) were determined for WNV, FLAV, and SLEV activity using calculator tools in TennSurv (https://vectorsurv.org/). MIRs were calculated as the number of virus specific positive pools per number of mosquitoes tested (CDC, 2013) and expressed as the number of positive pools per 1,000 tested. The Metro Public Health Department conducted mosquito surveillance across Davidson County. Their information was also stored in TennSurv, where it was accessed for MIR calculations. The Metro Public Health Department does not maintain trapping sites within the zoo. A final report of the results with recommendations for larval and adult management within the zoo will be provided to zoo personnel.

## Results

### Adult Mosquito Collections

A total of 9,141 adult mosquitoes from 24 species were collected at the 12 trapping sites (Table 4). A proportionally large number of mosquitoes were collected at the two residential sites representing 22 % (2,044 individuals) of total mosquitoes collected. The remaining 78% were collected from the ten sites within the zoo. Mosquito species diversity was higher within the zoo; fourteen species were collected at the residential sites, whereas 24 species were collected from the zoo sites. A total of 100 blood-fed mosquitoes were collected, with the majority (n= 96, 96%) from within the zoo compared to the residential sites. Nine species were collected with blood meals. The top species collected, defined as over 100 specimens, were *Culex* sp., *Culex pipiens/quinquefasciatus/restuans, Aedes albopictus, Aedes vexans,* and *Culex erraticus.*

**Table 4**. Mosquito species collected at the Nashville Zoo and nearby houses. Numbers represent the total number of each species collected by site grouping from June 10 – October 1.

| Species | Within the Zoo Sites Totals (%) | Outside of the Zoo Sites Totals (%) |
|---|---|---|
| *Culex* sp. | 2499 (34.51) | 781 (37.80) |
| *Culex pipiens/quinquefasciatus/restuans* | 2352 (32.48) | 693 (33.54) |
| *Aedes albopictus* | 1170 (16.16) | 492 (23.81) |
| *Aedes vexans* | 467 (6.45) | 19 (0.92) |
| *Culex erraticus* | 150 (2.07) | 22 (1.06) |
| *Anopheles punctipennis* | 140 (1.93) | 13 (0.63) |
| *Aedes triseriatus* | 73 (1.01) | 10 (0.48) |
| *Aedes* sp. | 69 (0.95) | 15 (0.73) |
| *Anopheles quadrimaculatus s.l.* | 68 (0.94) | 2 (0.10) |

| | | |
|---|---|---|
| *Culex territans* | 59 (0.81) | 4 (0.19) |
| *Culex nigripalpus* | 51 (0.70) | 2 (0.10) |
| *Psorophora columbiae* | 48 (0.66) | 0 (0) |
| *Psorophora ferox* | 32 (0.44) | 4 (0.19) |
| *Uranotaenia sapphirina* | 16 (0.22) | 0 (0) |
| *Aedes japonicus* | 15 (0.21) | 0 (0) |
| *Aedes trivittatus* | 6 (0.08) | 0 (0) |
| *Aedes atlanticus* | 5 (0.07) | 0 (0) |
| *Orthopodomyia* sp. | 4 (0.06) | 2 (0.10) |
| *Anopheles perplexans* | 3 (0.04) | 0 (0) |
| *Psorophora* sp. | 3 (0.04) | 0 (0) |
| *Psorophora cyanscens* | 3 (0.04) | 0 (0) |
| *Aedes infirmatus* | 2 (0.03) | 0 (0) |
| *Psorophora mathesoni* | 2 (0.03) | 1 (0.05) |
| *Aedes fulvus pallens* | 1 (0.01) | 0 (0) |
| *Anopheles crucians* | 1 (0.01) | 0 (0) |
| *Orthopodomyia signifera* | 1 (0.01) | 3 (0.15) |
| *Psorophora howardii* | 1 (0.01) | 1 (0.05) |
| *Toxorhynchites rutilus* | 1 (0.01) | 2 (0.10) |

The most mosquitoes were collected at the macaw site, and the least were collected at the pond site (see Fig 1, Table 5). The CDC miniature light traps collected the most species (Table 6). The gravid traps consistently yielded more mosquitoes per trap night than other traps (Fig 3), likely due to the gravid traps' attractiveness to *Culex* sp. such as *Culex pipiens/quinquefasciatus/restuans*, which represented the bulk of my collections in gravid traps (Fig 4). *Culex erraticus* was most frequently collected in CDC miniature light traps, followed closely by resting boxes (Fig 4). *Aedes vexans* and *Anopheles punctipennis* were most frequently

collected in CDC miniature light traps (Fig 4). *Aedes albopictus* was commonly collected in CDC miniature light traps but was also well represented by in the BG Sentinel traps and gravid traps (Fig 4). There was an increase in the number of mosquitoes collected for most species during the second half of the collection period (Fig 5 and 6).

**Table 5**. The total number of mosquitoes collected at each site (by trap type) and mean number of mosquitoes collected at each site (by trap type) expressed per trap night.

| Site | Trap Type | Total mosquitoes (total trap nights) | Mean no. mosquitoes/trap night |
|---|---|---|---|
| Residence A | BG Sentinel | 95 (18) | 5.28 |
| | CDC mini light trap | 141 (22) | 6.41 |
| | Gravid | 946 (24) | 39.42 |
| | Resting Box | 0 (34) | 0 |
| Residence B | BG Sentinel | 178 (22) | 8.10 |
| | CDC mini light trap | 79 (24) | 3.29 |
| | Gravid | 599 (23) | 26.05 |
| | Resting Box | 6 (34) | 0.18 |
| Dino Trek | BG Sentinel | 23 (11) | 2.10 |
| | CDC mini light trap | 234 (13) | 18.0 |
| | Gravid | 414 (13) | 31.85 |

| | | | |
|---|---|---|---|
| | Resting Box | 21 (16) | 1.31 |
| Flamingo | BG Sentinel | 20 (13) | 1.54 |
| | CDC mini light trap | 70 (15) | 4.67 |
| | Gravid | 325 (14) | 23.21 |
| | Resting Box | 13 (20) | 0.65 |
| Gibbons | BG Sentinel | 86 (12) | 7.17 |
| | CDC mini light trap | 319 (13) | 24.54 |
| | Gravid | 747 (12) | 62.25 |
| | Resting Box | 43 (24) | 1.79 |
| Lorikeet | BG Sentinel | 21 (9) | 2.33 |
| | CDC mini light trap | 197 (11) | 17.91 |
| | Gravid | 450 (11) | 40.91 |
| | Resting Box | 21 (16) | 1.31 |
| Macaw | BG Sentinel | 12 (11) | 1.10 |
| | CDC mini light trap | 220 (13) | 16.92 |
| | Gravid | 957 (13) | 73.62 |
| | Resting Box | 30 (25) | 1.20 |
| Pond | BG Sentinel | 18 (14) | 1.29 |

| | | | |
|---|---|---|---|
| | CDC mini light trap | 108 (15) | 7.20 |
| | Gravid | 217 (15) | 14.47 |
| | Resting Box | 18 (20) | 0.90 |
| Porcupine | BG Sentinel | 46 (13) | 3.54 |
| | CDC mini light trap | 139 (13) | 10.69 |
| | Gravid | 417 (13) | 32.08 |
| | Resting Box | 3 (18) | 0.17 |
| Red Lemur | BG Sentinel | 21 (13) | 1.62 |
| | CDC mini light trap | 92 (12) | 7.67 |
| | Gravid | 252 (13) | 19.38 |
| | Resting Box | 66 (24) | 2.75 |
| Tapir | BG Sentinel | 20 (13) | 1.54 |
| | CDC mini light trap | 81 (15) | 5.40 |
| | Gravid | 425 (15) | 28.33 |
| | Resting Box | 13 (26) | 0.50 |
| Train | BG Sentinel | 39 (12) | 3.25 |
| | CDC mini light trap | 245 (12) | 20.42 |
| | Gravid | 629 (12) | 57.18 |

| | | | |
|---|---|---|---|
| | Resting Box | 23 (24) | 0.96 |

**Table 6.** The number of species collected by each trap type.

| Trap Type | Number of Species |
|---|---|
| BG Sentinel | 10 |
| CDC Miniature Light Trap | 22 |
| Gravid Trap | 13 |
| Resting Box | 9 |

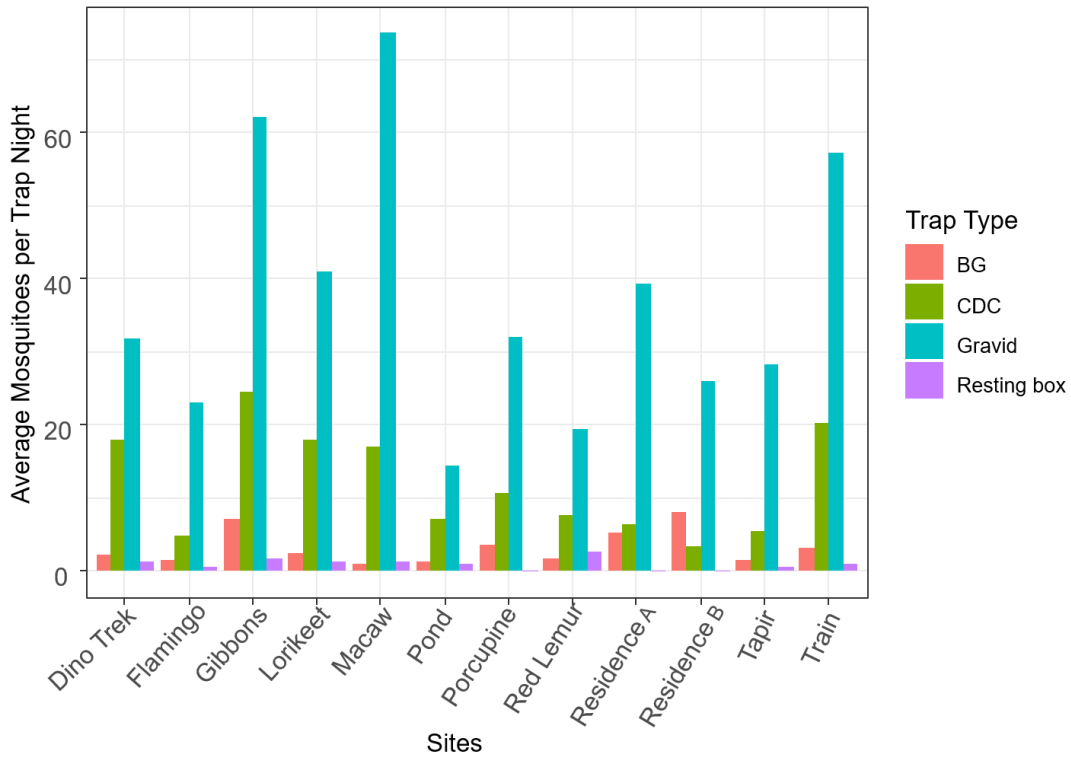



**Figure 3**. The average number of mosquitoes trapped per night by collection site and trap type.

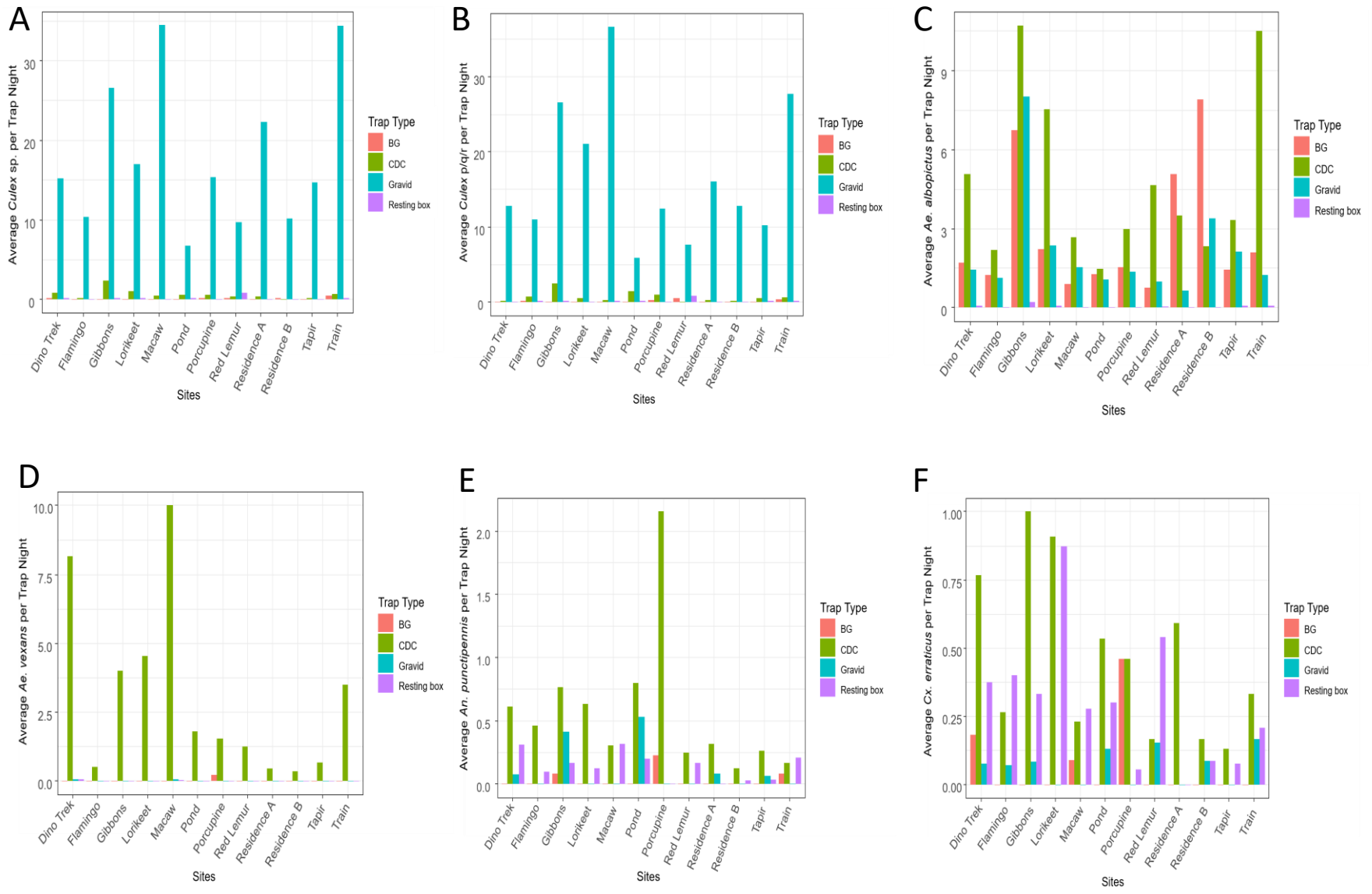



**Figure 4**. The average number of six most abundant species of mosquitoes trapped per night by site and trap type: *Culex* sp. (A), *Cx. pipiens/quinquefasciatus/restuans* (B), *Ae. albopictus* (C), *Ae. vexans* (D), *An. punctipennis* (E), and *Cx. erraticus* (F).

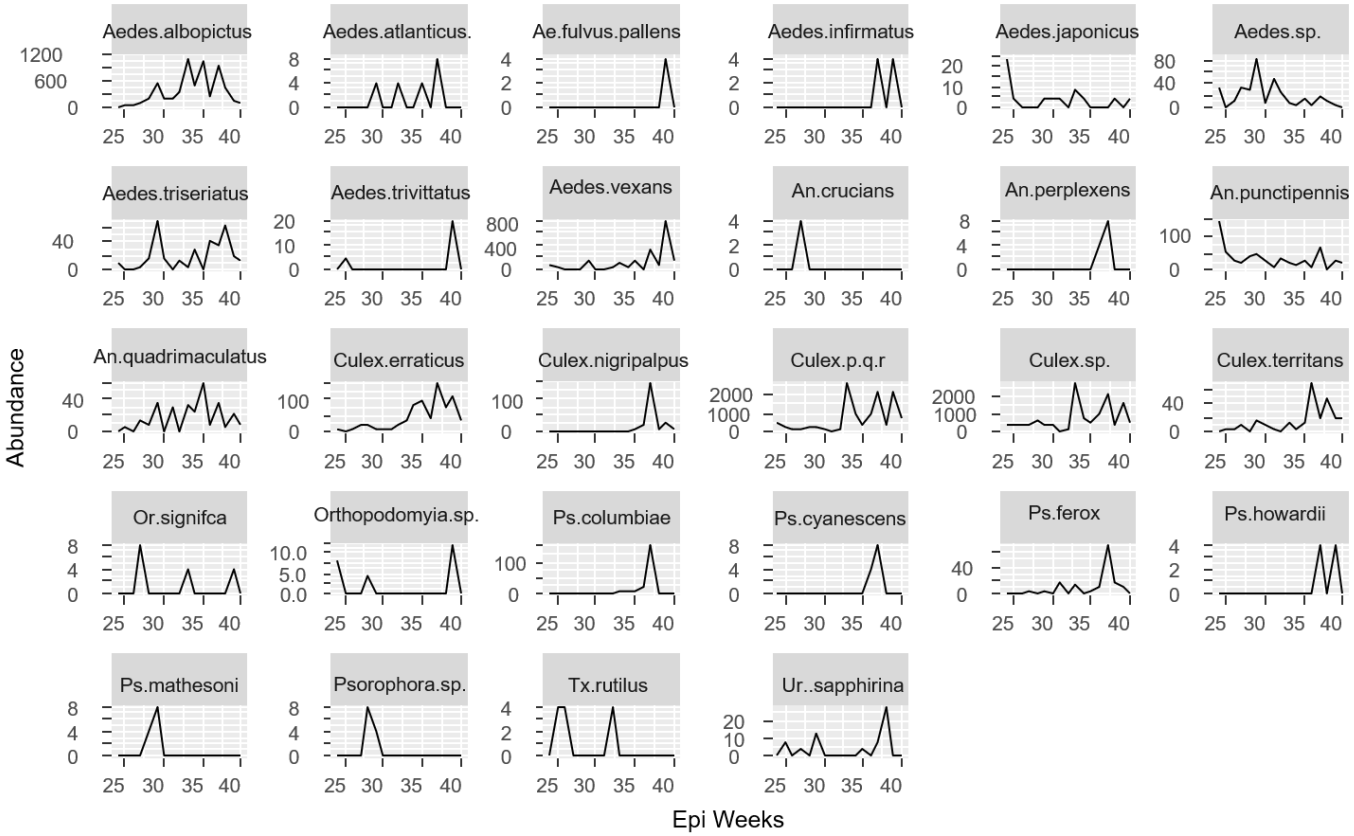



**Figure 5.** The number of each species collected per epi week from all collection sites.

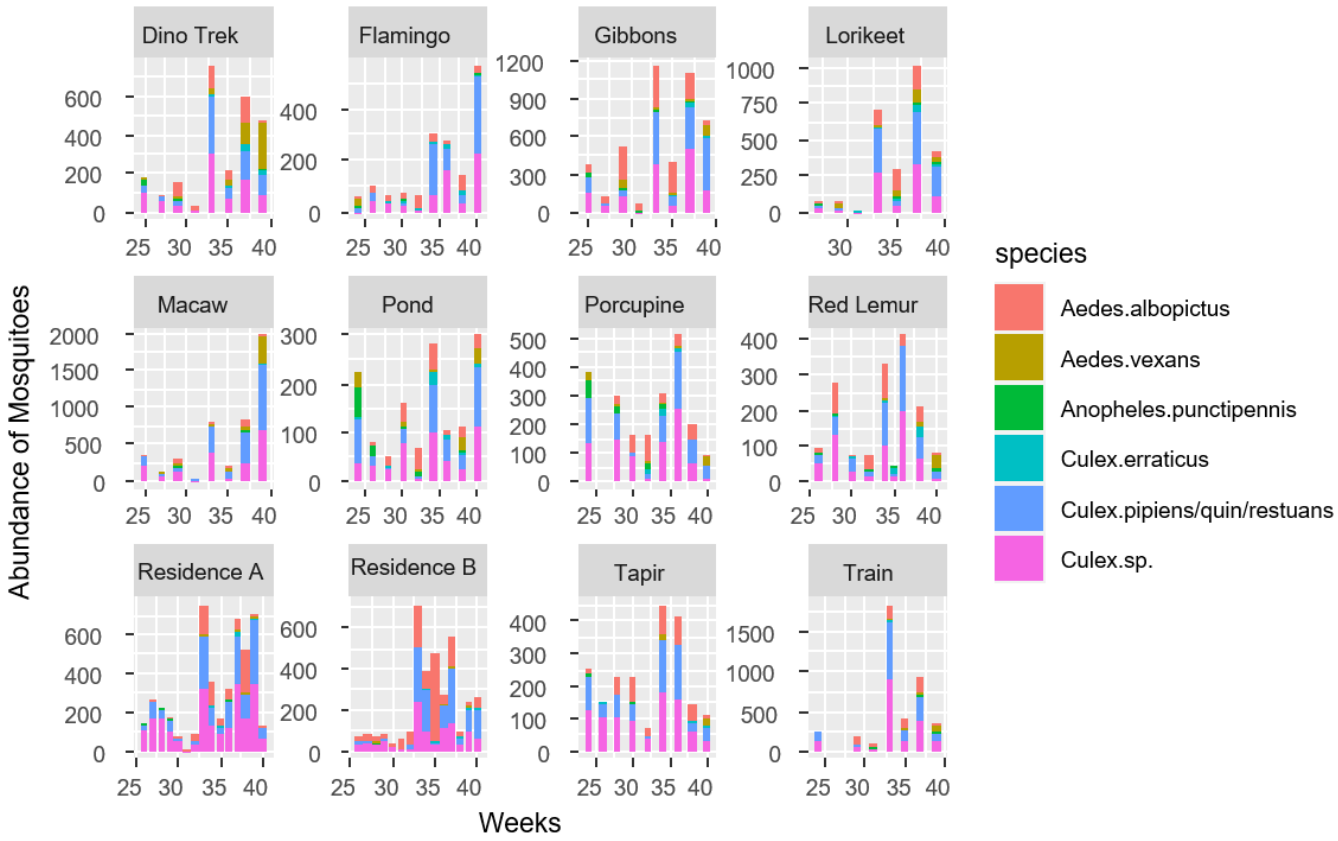



**Figure 6.** The weekly abundance of the six most abundant mosquito species by trap site**.**

## Mosquito Diversity Analysis

The highest diversity of mosquitoes was collected from the dino trek site, with a Shannon-Weiner index of 1.91. Residence B had the lowest mosquito diversity with a Shannon-Weiner index of 1.24 (Fig 7). Diversity was significantly higher from sites inside the zoo compared with those outside the zoo, potentially due to greater larval habitat within the zoo (Wilcoxon rank-sum test; $p = 0.0303$). Collection week attributed to 45% of the variance between sites ($p = 0.001$); while the site location itself attributed 15% of the variance ($p = 0.001$).

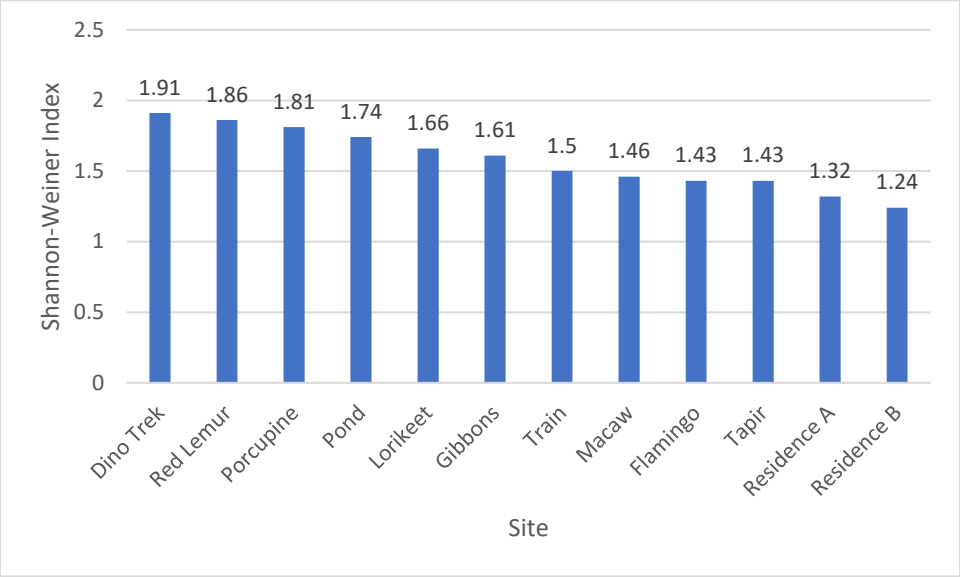



**Figure 7**. The Shannon-Weiner index values for all collection sites.

## Viral Testing Results

A total of 5,072 *Culex* mosquitoes were tested in 575 pools from the ten sites within the zoo. None of the pools were positive for SLEV, while four pools were positive for WNV and 21 pools were positive for FLAV. FLAV was detected at eight sites within the zoo (porcupine, red

lemur, flamingo, pond, train, gibbons, lorikeet, and dino trek), and WNV was detected at four sites (red lemur, tapir, flamingo, and macaw) (Fig 8). At two of the sites (red lemur and flamingo), WNV and FLAV were both detected (Fig 8). The MIR for FLAV in the zoo was comparable to that reported for the rest of Davidson County (Table 7). The MIR for WNV in the zoo was higher when compared with the MIR for the rest of Davidson County (Table 4). The peak in FLAV MIR preceded the peak in WNV MIR by 12 weeks, demonstrating the potential of FLV as a sentinel virus for WNV infections (Fig 9).


**Figure 8.** A map of where Flanders and WNV positive pools were collected from the zoo.

**Table 7.** The MIRs for *Culex* mosquitoes collected inside the Nashville Zoo and across Davidson County.

| | Nashville Zoo (MIR) | Davidson County (MIR) |
|---|---|---|
| Flanders Virus | 4.14 | 3.99 |
| WNV | 0.79 | 0.39 |
| St. Louis Encephalitis Virus | 0 | 0 |

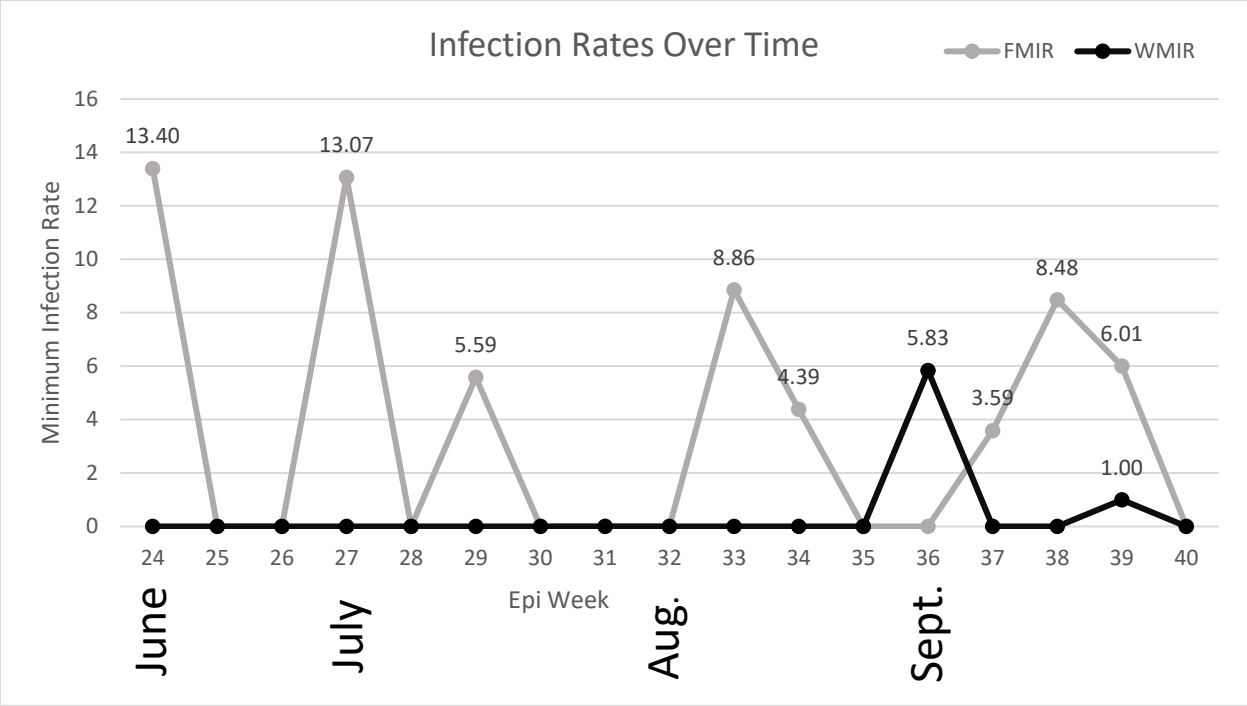


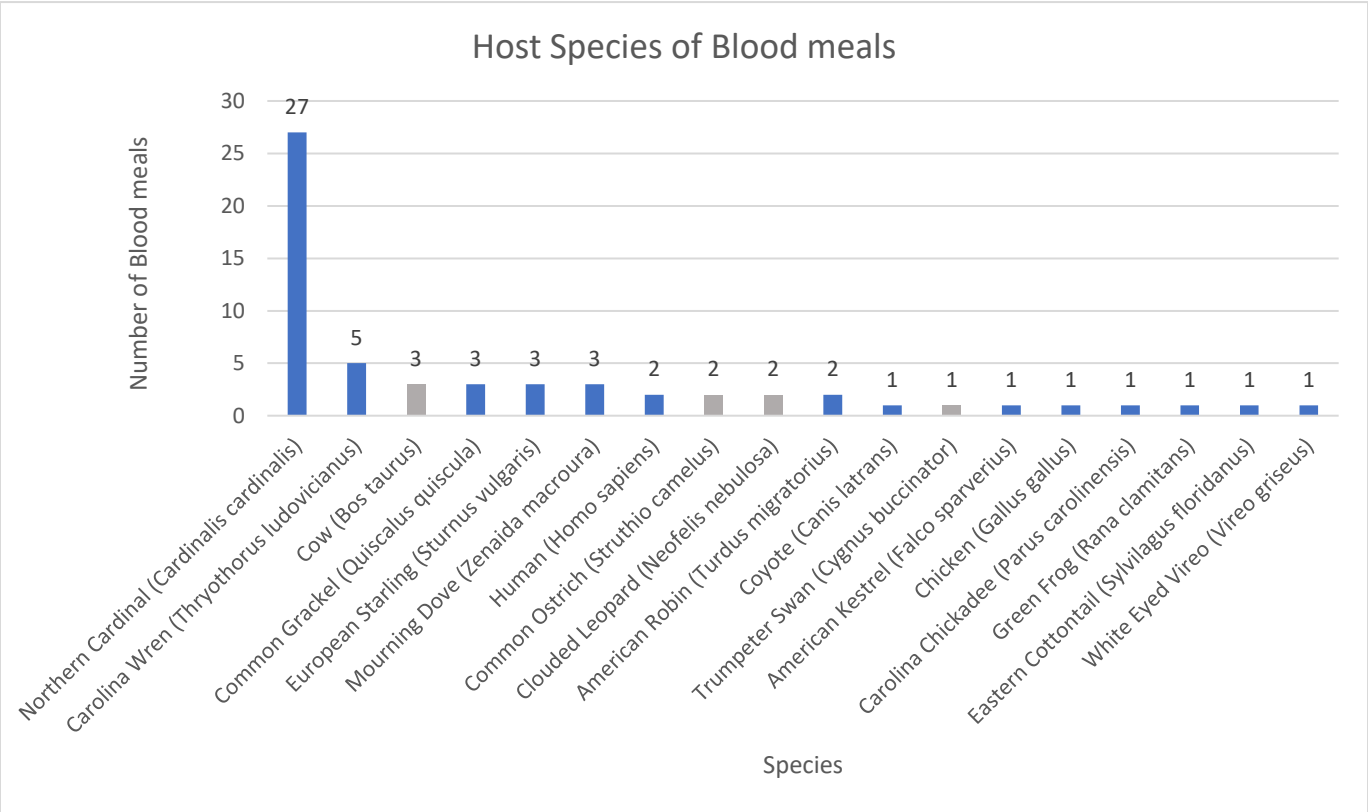

**Figure 9**. A comparison of the chronological trends in minimum infection rates for Flanders virus and WNV detected in the zoo.

## Blood meal analysis

Of the 100 engorged mosquitoes, 60 were matched to a host species. An additional four mosquitoes have mixed blood meals that will require further analysis to identify host DNA sources. Of the 18 host species identified, only four were from captive zoo species (cow [*Bos taurus*], common ostrich [*Struthio camelus*], trumpeter swan [*Cygnus buccinator*], and clouded leopard [*Neofelis nebulosa*]) (Fig 10). The blood meal from a coyote (*Canis latrans*) was below the threshold of 98% (93.5%), but this could be attributed to variations in coyote genetics from breeding with feral dogs or degradation of the sample. Coyotes have been previously sighted within the zoo. The origin of the blood meal from a chicken (*Gallus gallus*) is unknown but could be from chickens kept at one of the residences bordering the zoo. However, we did not

survey animals on residential properties surrounding the zoo. Surprisingly, the most blood meals were from northern cardinals (*Cardinalis cardinalis*), followed by other wild birds at the zoo, rather than meals from the approximately 500 zoo animals on display or the more than 15,000 human visitors per week. Two host identifications were from residence B (a human, *Homo sapiens*, and an American robin, *Turdus migratorius*). Due to a low number of blood meals matched to zoo animals, I did not calculate host forage ratios.

A




B

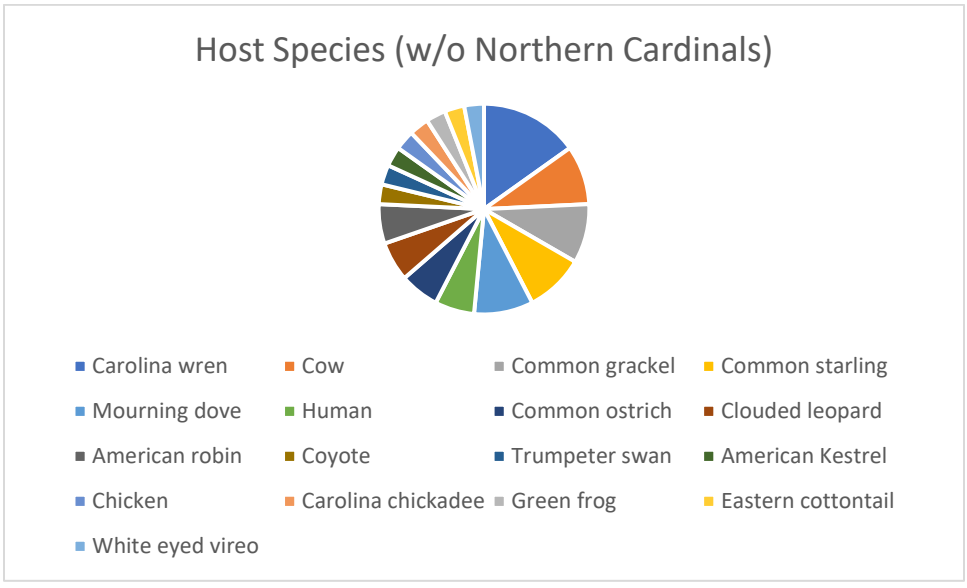



**Figure 10.** (A) The number of blood meals for each host species. (B) A representation of the blood meals after removing the most common host, northern cardinals.

Mosquitoes with more than five successful blood meal identifications were *Cx. erraticus* and *Culex* sp.. The majority of blood meals from *Cx. erraticus* were from avian hosts (Table 8). The majority of blood meals from the *Culex* sp. were avian (Table 8). The most blood meals were collected from the Red lemur site, which was the only site where WNV was detected and the predominant blood meal host was avian (Fig 11).

**Table 8**. The number of blood meals (by mosquito species) categorized by the type of host species.

| Species | Number Collected | Successful Host Match | Human | Avian | Mammal | Amphibian |
|---|---|---|---|---|---|---|
| *Aedes albopictus* | 1 | 1 | 1 | 0 | 0 | 0 |
| *Aedes trivittatus* | 1 | 0 | 0 | 0 | 0 | 0 |
| *Aedes vexans* | 1 | 1 | 0 | 0 | 1 | 0 |
| *Anopheles punctipennis* | 5 | 2 | 0 | 1 | 1 | 0 |
| *Anopheles quadrimaculatus* | 7 | 4 | 0 | 1 | 3 | 0 |
| *Culex erraticus* | 22 | 9 | 1 | 7 | 1 | 0 |
| *Culex* sp. | 61 | 43 | 0 | 41 | 1 | 1 |
| *Culex territans* | 2 | 0 | 0 | 0 | 0 | 0 |
| Total | 100 | 60 | 2 | 50 | 7 | 1 |

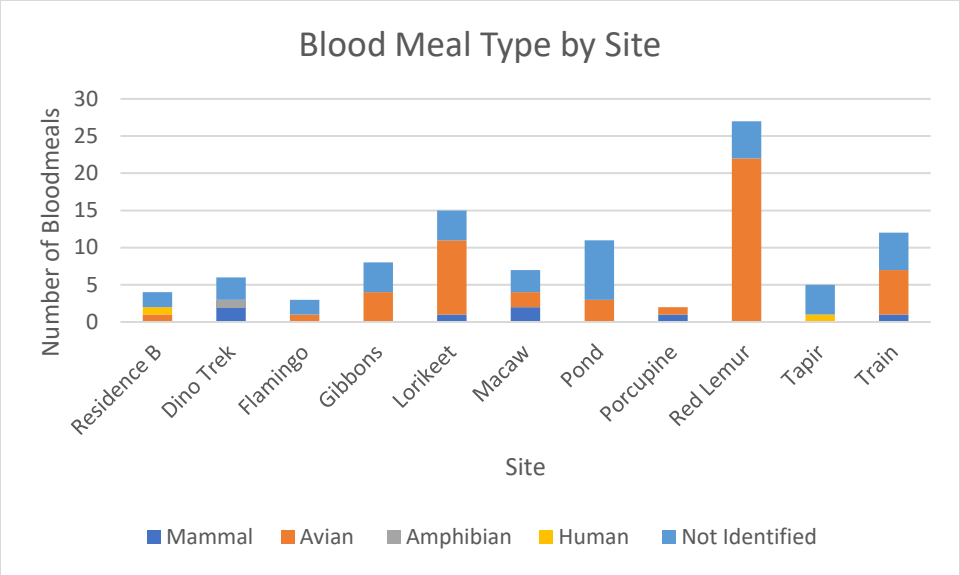



**Figure 11**. The distribution of the type of blood meals from each site.

## Discussion

This study represents one of the most detailed assessments of mosquito diversity, host use patterns, and arbovirus infection at a zoo setting in the United States. We demonstrated greater WNV infection rates within the Nashville zoo compared to surveillance outside of the zoo. Our results to date support the notion that zoos can be hotspots for high mosquito diversity and may pose zoonotic transmission risks as well as threats to valuable and rate captive zoo species. However, we noted lower than expected feeding on exotic animals compared to native birds.

Adult collections revealed that mosquito diversity was high at the zoo, despite the use of routine mosquito control interventions such as *Bti* larvicide applications and deployment of In2Care traps (http://www.in2care.org/mosquito-trap/) for adult container breeding mosquito control. The CDC miniature light traps collected the greatest number of mosquito species (22), supporting their use for diverse mosquito surveillance. BG Sentinel traps only collected ten

species. Two *Toxorhychites rutilus* was captured in the BG trap. The breadth of mosquito species collected in the zoo indicates the presence of a variety of larval habitats ranging from man-made containers to natural pools within or in close proximity to the zoo. Although only 15% of the variance in diversity was attributed to the site of collection, my model did not factor into account quantifiable habitat similarities or dissimilarities for each site. While there was a significantly higher level of species diversity collected inside the zoo, additional surveillance for larval habitats and analysis of associated environmental factors would provide insight into factors driving diversity.

Many of the species collected were of medical and veterinary concern. Several of the *Culex* species I captured are known maintenance vectors of WNV in avian populations, while species like *Aedes vexans* could transmit WNV to mammals. *Culex nigripalpus* can transmit SLEV and EEE virus. In addition, the collection of *Culex nigripalpus* at the Nashville Zoo is significant because it was the first time *Culex nigripalpus* has been documented in Davidson County, this species has been documented in the far western part of Tennessee previously (Darsie and Ward, 2005). My results suggest the possible expansion of *Culex nigripalpus* into new regions of Tennessee.

The detection of a FLAV MIR peak before a WNV MIR supports the use of FLAV virus as an indicator of future WNV activity. The 12-week gap in the MIR peaks is similar to a previous study from Tennessee which detected an average 10-week gap between peaks (Lucero et al., 2016). However, the FLAV peak was detected during the first series of collections, so it is possible that a higher weekly MIR occurred earlier in the season. Poh et al. (2018) detected a one- and three-week gap between infection rate peaks in mosquitoes collected in Chicago, IL, supporting the possibility that there are regional differences in timing of FLAV detections.

The detection of WNV in pools of *Culex* sp. and *Culex pipiens/quinquefasciatus/restuans* is consistent with patterns seen in the southeastern United States (Godsey et al., 2005). All but one pool of WNV and FLAV infected mosquitoes was collected by gravid traps. The gravid traps also resulted in more pools of *Culex* mosquitoes when compared to the other trap types. Gravid traps are ideal tools for WNV surveillance because they capture females after they have blood fed and potentially ingested an infectious meal (CDC, 2013). The WNV MIR for the Nashville Zoo in 2017 was 9.72 (Moore et al., 2018), a value lower than prior data taken from that zoo in 2017. This result is not surprising considering that WNV case incidence was higher across the United States in 2017 compared to 2020 (CDC, 2020c).

The presence of the Nashville Zoo in the middle of an urbanized area could lend the zoo to being an oasis for wild birds. As a result, the Nashville Zoo could be prone to higher WNV MIR when compared to Davidson County due to the increased concentration of a variety of wild bird species. These birds could serve as introduction points for WNV into the area following migration as well as providing a continual presence of reservoirs. Most blood meals in this study were from wild birds supporting the notion that wild birds at the zoo could play an important role in mosquito infection and transmission dynamics. Interestingly, Levine et al. (2016), found the Atlanta Zoo had lower WNV MIR when compared to nearby forested areas. However, approximately 40% of the blood meals of mosquitoes collected at the Atlanta Zoo were from wild birds as opposed to 77% of the blood meals in my study. This difference alone does not justify the differences in infection rates in mosquitoes because not all wild birds are equally competent reservoirs of WNV. Some of these differences could be attributed to differences in collection methods as Levine et al. (2016) only deployed three gravid traps and one light trap within the zoo.

The success rate (60%) of blood meal identification is similar to the success rates of previous studies using similar methods and with similar species compositions (Tuten et al., 2012). A decrease in successful identifications as the level of digestion increased was expected and has been reported previously (Tuten et al., 2012; Ejiri et al., 2011). The number of captive species fed on (four) and the percentage of blood meals from captive animals (23%) was lower than other studies conducted at the Greenville Zoo (SC), Riverbanks Zoo (SC), and Rio Grande Zoo (NM) (Tuten et al., 2012; Greenburg et al., 2012). These differences could be due to different sampling methods. Tuten et al. (2012) relied primarily on aspiration of vegetation and structures, while Greenburg et al. (2012) used gravid and CDC light traps. Due to the low number of captive animal feedings and unknown wild bird population numbers, flight distances and host foraging ratios were not calculated. These studies also reported bloodmeals from coyotes, cottontail rabbits, frogs, cows, ostriches, northern cardinals, common starlings, mourning doves, American robins, and Carolina chickadees.

Northern cardinals were the most commonly fed upon animal at the Nashville Zoo, which was also reported as a common avian host at both zoos in South Carolina (Tuten et al., 2012). Previous studies have indicated that house sparrows and corvids play a smaller role in WNV amplification than previously thought, but these studies also indicated that American robins could be important WNV amplification hosts in urban areas (Apperson et al., 2004; Molaei et al., 2006; Kilpatrick et al., 2006b; Savage et al., 2007) However, northern cardinals have also been a common host in blood feeding studies on *Culex* mosquitoes conducted in Tennessee and New Jersey, as well as the most common host in New York and Atlanta, GA ( Apperson et al., 2004; Savage et al., 2007; Patrican et al., 2007; Levine et al., 2016). In Georgia, WNV seroprevalence is consistently higher in northern cardinals than other avian species (Levine et al., 2016).

Northern cardinals are moderately competent reservoirs of WNV and could be playing an important role as enzootic vectors of WNV (Kilpatrick et al., 2007; Levine et al., 2016). Kilpatrick et al. (2006c) documented a host switching trend that was defined as a decrease in the occurrence of feeding on American robins and a simultaneous increase in feeding on mammals beginning in July. Levine et al. (2016) also noted that *Culex* mosquitoes were demonstrating host switching patterns away from American robins beginning in early summer (July), but rather than switching to mammals later in the season, *Culex* switched to feed on northern cardinals. This could be a possible explanation for the high prevalence of northern cardinal meals at the Nashville Zoo because I did not begin trapping mosquitoes until midsummer and most engorged mosquitoes were collected beginning in July. Additionally, since a wild bird census was not taken, it is unclear how common northern cardinals were during the collection period, making it difficult to determine if the proportion of blood meals from northern cardinals is proportional to the population. The lack of a bird census also made it difficult to determine if the population of American robins in the area is migratory, which is considered a factor as to why host switching occurs (Kilpatrick et al., 2006c). It is worth noting though, that the tendency of *Culex* mosquitoes to feed on northern cardinals in the latter half of the summer could be a factor contributing to lower incidence of WNV in mammals or humans (Levine et al., 2016).

There is still much to be learned about the nuances of blood feeding patterns and the role of these patterns in the transmission of WNV. Further research should be performed to investigate the wild bird populations at the Nashville Zoo would provide more insight into the repeated detection of WNV within the Nashville Zoo. Despite a low number of blood meals taken from captive species, the continuous circulation of WNV puts these animals at risk for infection. The variety of mosquito species collected within the zoo could indicate the captive animals are at risk

of infection beyond WNV if proper preventative steps are not taken. Humans in and near the zoo could also be at risk for WNV and other arbovirus infections and should be cautioned to wear repellants. The detection of WNV and *Culex nigripalpus* within the zoo further demonstrates the value of zoological institutions as biosurveillance sites for existing and potential arboviral threats.

# APPENDICES

Appendix 1.

The Nashville Zoo takes weekly census of the approximately 3,000 organisms at the zoo. Below is a subset of the weekly total from the middle of the summer that lists the number of individuals with outdoor access at the zoo. Animals housed inside were not included in this list. There are approximately 500 individuals without outdoor access. These individuals could be at risk for infection with mosquito-borne diseases.

| Scientific Name | Common Name | Number |
|---|---|---|
| *Alligator mississippiensis* | American alligator | 1 |
| *Varanus komodoensis* | Komodo dragon | 3 |
| *Chelonoidis nigra* | Galapagos tortoise | 2 |
| *Geochelone gigantea* | Aldabra giant tortoise | 4 |
| *Struthio camelus* | Common ostrich | 3 |
| *Casuarius casuarius* | Southern cassowary | 4 |
| *Crax alberti* | Blue-billed curassow | 5 |
| *Chauna torquata* | Southern screamer | 2 |
| *Dendrocygna viduata* | White-faced whistling duck | 11 |
| *Cygnus buccinator* | Trumpeter swan | 2 |
| *Aix galericulata* | Mandarin duck | 1 |
| *Tadorna tadorna* | Common shelduck | 4 |
| *Phoenicopterus chilensis* | Chilean flamingo | 12 |
| *Phoenicopterus ruber* | American flamingo | 24 |
| *Ptilinopus porphyreus* | Pink-headed Fruit-dove | 1 |
| *Crinifer piscator* | Western grey plantain-eater | 1 |
| *Corythaeola cristata* | Great blue turaco | 2 |
| *Anthropoides paradiseus* | Stanley crane | 2 |
| *Ephippiorhynchus senegalensis* | Saddle-billed stork | 2 |
| *Tyto alba* | Common barn owl | 4 |
| *Bubo bubo* | Eurasian eagle owl | 1 |
| *Bubo scandiacus* | Snowy owl | 2 |
| *Megascops asio* | Eastern screech owl | 2 |
| *Cathartes aura* | Turkey vulture | 1 |
| *Buteo augur* | Augur buzzard | 1 |
| *Parabuteo unicinctus* | Harris' hawk/bay-winged hawk | 1 |
| *Tockus deckeni* | Von der Decken's hornbill* | 2 |

| | | |
|---|---|---|
| *Buceros rhinoceros* | Rhinoceros hornbill | 5 |
| *Bycanistes brevis* | Silvery-cheeked hornbill | 1 |
| *Dacelo novaeguineae* | Laughing kookaburra | 1 |
| *Pteroglossus viridis* | Green aracari* | 1 |
| *Ramphastos toco* | Toco toucan | 2 |
| *Trichoglossus ornatus* | Ornate lorikeet | 3 |
| *Trichoglossus haematodus haematodus* | Green-naped lorikeet | 30 |
| *Trichoglossus rosenbergii* | Rosenberg's lorikeet | 9 |
| *Trichoglossus moluccanus* | Swainson's lorikeet | 21 |
| *Trichoglossus weberi* | Weber's lorikeet | 6 |
| *Trichoglossus euteles* | Olive-headed lorikeet | 10 |
| *Psitteuteles iris* | Iris lorikeet | 1 |
| *Psitteuteles goldiei* | Goldie's lorikeet | 9 |
| *Anodorhynchus hyacinthinus* | Hyacinth macaw | 5 |
| *Amazona oratrix* | Yellow-headed amazon | 1 |
| *Probosciger aterrimus* | Palm cockatoo | 1 |
| *Cacatua moluccensis* | Salmon-crested cockatoo* | 1 |
| *Cotinga cayana* | Spangled cotinga | 1 |
| *Querula purpurata* | Purple-throated fruitcrow | 1 |
| *Lanius ludovicianus* | Loggerhead shrike | 6 |
| *Calocitta formosa* | Magpie jay | 8 |
| *Corvus brachyrhynchos* | Common crow | 1 |
| *Cyanocorax chrysops* | Plush-crested jay | 1 |
| *Paradisaea minor* | Lesser bird-of-paradise | 2 |
| *Icterus galbula* | Northern oriole | 4 |
| *Sturnella bellicosa* | Greater red-breasted blackbird | 1 |
| *Ammodramus savannarum* | Grasshopper sparrow | 1 |
| *Tangara chilensis* | Paradise tanager | 3 |
| *Didelphis virginiana* | Virginia opossum | 2 |
| *Macropus rufus* | Red kangaroo | 20 |
| *Orycteropus afer* | Aardvark* | 1 |
| *Chaetophractus vellerosus* | Screaming hairy armadillo* | 1 |
| *Tolypeutes matacus* | Southern three-banded armadillo* | 1 |
| *Myrmecophaga tridactyla* | Giant anteater | 9 |
| *Tamandua tetradactyla* | Southern tamandua | 2 |
| *Lemur catta* | Ring-tailed lemur | 4 |
| *Varecia rubra* | Red ruffed lemur | 5 |
| *Saguinus Oedipus* | Cotton-top tamarin | 5 |
| *Ateles geoffroyi* | Black-handed spider monkey | 4 |
| *Nomascus leucogenys* | White-cheeked gibbon | 4 |
| *Symphalangus syndactylus* | Siamang | 2 |
| *Hystrix africaeaustralis* | Cape porcupine | 2 |
| *Coendou prehensilis* | Prehensile-tailed porcupine* | 1 |
| *Chinchilla lanigera* | Long-tailed chinchilla* | 2 |
| *Cavia porcellus* | Domestic guinea pig (breed unspecified) | 35 |
| *Octodon degus* | Degu | 4 |

| | | |
|---|---|---|
| *Oryctolagus cuniculus* | European rabbit* | 3 |
| *Atelerix albiventris* | Four-toed hedgehog* | 1 |
| *Caracal* | Caracal lynx | 6 |
| *Leptailurus serval* | Serval | 1 |
| *Puma concolor* | Cougar | 2 |
| *Neofelis nebulosa* | Clouded leopard | 14 |
| *Panthera tigris* | Tiger | 2 |
| *Arctictis binturong* | Binturong | 6 |
| *Hemigalus derbyanus* | Banded palm civet | 9 |
| *Suricata suricatta* | Slender-tailed meerkat | 7 |
| *Tremarctos ornatus* | Spectacled bear | 2 |
| *Mephitis mephitis* | Striped skunk | 3 |
| *Potos flavus* | Kinkajou | 1 |
| *Ailurus fulgens* | Red panda | 2 |
| *Equus asinus* | Donkey | 3 |
| *Equus caballus* | Horse | 1 |
| *Equus quagga* | Plains zebra | 2 |
| *Tapirus bairdii* | Baird's tapir | 2 |
| *Ceratotherium simum* | White rhinoceros | 5 |
| *Potamochoerus porcus* | Red River hog | 2 |
| *Sus scrofa* | Wild boar | 4 |
| *Vicugna pacos* | Alpaca | 6 |
| *Odocoileus virginianus* | White-tailed deer | 2 |
| *Pudu puda* | Southern pudu | 1 |
| *Giraffa camelopardalis* | Giraffe | 2 |
| *Okapia johnstoni* | Okapi | 1 |
| *Damaliscus pygargus* | Bontebok/blesbok | 3 |
| *Bos taurus* | Domestic cow/ox (breed unspecified) | 5 |
| *Tragelaphus eurycerus* | Bongo | 3 |
| *Tragelaphus oryx* | Common eland | 3 |
| *Capra hircus* | Domestic goat (breed unspecified) | 11 |
| *Ovis aries* | Domestic sheep/mouflon (breed unspecified) | 4 |
| *Cephalophus silvicultor* | Yellow-backed duiker | 1 |

*indicates animals with both indoor and access or animals that are brought outside for shows

Appendix 2.

The Nashville Zoo keeps weekly records of the guest attendance counts. Daily capacity was limited due to COVID-19 restrictions. These restrictions combined with general caution related to COVID-19 probably decreased typical attendance over the summer. Additionally, during the first week, I trapped mosquitoes the zoo was closed to visitors. The total number of visitors during the study period was 258,827.

| Week | Total Attendance |
|---|---|
| 6/7-6/14 | 0 |
| 6/15-6/21 | 18,855 |
| 6/22-6/28 | 16,309 |
| 6/30-7/5 | 11,551 |
| 7/6-7/12 | 9,630 |
| 7/13-7/19 | 9,256 |
| 7/20-7/26 | 9,256 |
| 7/27-8/2 | 11,545 |
| 8/3-8/9 | 18,756 |
| 8/10-8/16 | 13,369 |
| 8/17-8/23 | 16,053 |
| 8/24-8/30 | 21,942 |
| 8/31-9/6 | 20,483 |
| 9/7-9/13 | 24,314 |
| 9/14-9/20 | 20,814 |
| 9/21-9/27 | 19,614 |
| 9/28-10/4 | 17,080 |

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