Disease Surveillance of *Dirofilaria immitis* and West Nile Virus in Mosquitoes in Lowndes County, Georgia

A Thesis submitted

to the Graduate School

Valdosta State University

in partial fulfillment of requirements

for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
of the College of Science and Mathematics

July 2023

Amber Nichol Holley

BS, Valdosta State University, 2020

Abstract

Dirofilaria immitis, the causative agent of canine heartworm disease, and West Nile virus (WNV) are both mosquito-borne pathogens. Mosquito surveillance and vector determination are crucial in understanding the transmission of these pathogens which helps implement methods of control to prevent and predict outbreaks. In this study I assessed the ability of the *Anopheles quadrimaculatus* complex to transmit *D. immitis* and investigated the influence climate and land use and land changes (LULC) have on WNV transmission in *Culex quinquefasciatus* mosquitoes, the primary vector in the Southeastern United States. Standard PCR and an L3-specific RT-PCR protocol were used to *assess D. immitis* infection rates in *An. quadrimaculatus* mosquito samples. To determine the influence of climate and LULC on WNV from 2012-2021, data were collected from Weather Underground and the VSU WNV Mosquito Surveillance lab. Results from this study suggest that the *An. quadrimaculatus* complex is a potential vector of *D. immitis* and that urbanization and temperature influence WNV transmission by mosquitoes in Lowndes Co., GA.

This thesis, "Disease Surveillance of *Dirofilaria immitis* and West Nile Virus in Mosquitoes in Lowndes County, Georgia" by Amber Nichol Holley, is approved by:

Thesis Committee Chair

DocuSigned by:
A9B07B3A216741E...
Eric Chambers, Ph.D.
Associate Professor of Biology

Committee Member

DocuSigned by:
4C2154A2587A4FF...
Mark Blackmore, Ph.D.
Emeritus Professor of Biology

DocuSigned by:
5265380C8EC44E8...
Erin Grabarczyk, Ph.D.
Assistant Professor of Biology

DocuSigned by:
B918D0B5E8BA45E...
Jack Lockhart, Ph.D.
Professor of Biology

Associate Provost for Graduate Studies and Research

DocuSigned by:
84AFF646370449F...
Becky K. da Cruz, Ph.D., J.D.
Professor of Criminal Justice

Defense Date 07/03/2023

FAIR USE

DUPLICATION

I authorize the Head of Interlibrary Loan or the Head of Archives at the Odum Library at Valdosta State University to arrange for duplication of this thesis for educational or scholarly purposes when so requested by a library user. The duplication shall be at the user's expense.

Signature DocuSigned by: Amber Holley A9A93EA9CB8D40F

I refuse permission for this thesis to be duplicated in whole or in part.

Signature _____

Table of Contents

ACKNOWLEDGEMENTS

I cannot express enough gratitude to my committee for their support and encouragement: Dr. Eric Chambers, my committee chair; Dr. Mark Blackmore, Dr. Erin Grabarczyk, and Dr. Jack Lockhart. The advice and knowledge I have gained from them is invaluable and I will carry it with me throughout my career.

I would like to acknowledge the NIH/NIAID Filariasis Research Reagent Resource Center (www.filariasiscenter.org) for providing us with the *Ae. aegypti* mosquitoes infected *D. immitis*. I would also like to acknowledge Dr. Daniel Mead and Kayla Adcock for their help with WNV detection at Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia.

This could not have been accomplished without the help of the graduate students and undergraduate students that have helped throughout this process. Thank you to the undergraduate students that have helped me in the lab and thank you to the graduate students that listened and encouraged me when my experiments were unsuccessful. I would especially like to thank the friends that I spent many late nights in Bailey Science Center with and the friends I have spent long days with in the VSU Mosquito Surveillance Lab.

Finally, I would like to thank my family for their unwavering support in my decisions and career choices.

Chapter I

Introduction

Canine Heartworm

Canine heartworm disease is a veterinary health risk that poses a threat to canine populations (Smith et al. 2022). Foxes, wolves (Gomes-de-Sá et al. 2022), coyotes (Sobotyk et al. 2022), and feline species also are infected, but serve mostly as reservoir hosts (Alberigi et al. 2022; Smith et al. 2022). Worldwide, outbreaks persist in wild and domestic populations. Without appropriate treatment, infected canines and felines succumb to the disease due to congestive heart failure (Simon et al. 2012). The American Heartworm Society provides evidence that the Southeast is a hotspot for canine heartworm infection within the United States (American Heartworm Society 2019) which highlights the need for implementation of control methods in these areas.

Dirofilaria immitis is the vector-borne filarial nematode that causes canine heartworm disease. Microfilariae are produced by mature adult worms and travel through the bloodstream of vertebrate hosts. Microfilariae ingested by a female mosquito during feeding unsheathe to become L1. L1 then migrate to the Malpighian tubules of the mosquito and undergo two molts to become L3 (Simon et al. 2012). This process can take anywhere from 10 days to several weeks depending on temperature: as temperature increases, the time it takes the nematode to develop decreases (Fortin and Slocombe 1981; Silaghi et al. 2017). L3 then migrate to the proboscis of the mosquito. Once on the proboscis, the mosquito deposits the L3 onto a vertebrate host during a blood meal and L3 enter through the wound created by the mosquito. L3 is the infective stage of the pathogen because it is at this life stage that the pathogen can cause infection in the host (Simon et al. 2012). Once in the vertebrate host, L3 molt to L4 and migrate into the surrounding tissues. A final molt occurs and the adult worms infiltrate the

bloodstream. Adult worms then migrate to the heart and surrounding vessels where they congregate and reproduce (Mullen and Durden 2009). Congestion causes coughing, difficulty breathing, and eventually congestive heart failure (Chikweto et al. 2014; Simon et al. 2012).

Currently, the main mechanism of control is through treatment of canines with drugs containing macrocyclic lactones. These drugs are safe, highly efficient, and specifically target the L3 and L4 stages of *D. immitis* (Diakou and Prichard 2021). Although it has been largely successful, there have been claims of loss of efficiency and resistance development in the parasite in the Lower Mississippi region of the United States (Diakou and Prichard 2021). These intensive year-round treatments of dogs may create a selective pressure that aids in parasite resistance development. While resistance has not been observed elsewhere, it is possible for resistance to spread to new areas through vector mobility, host mobility, and even an independent evolutionary event (Diakou and Prichard 2021). Because *D. immitis* have traits favorable to resistance, it is imperative to look to other forms of control like vector reduction.

Although *D. immitis* is transmitted by mosquitoes, not all mosquito species are efficient vectors. Some species of mosquitoes are unable to facilitate development of pathogens due to mechanical defenses, immune defenses, and climatic factors. These factors affecting pathogen development may prevent infected mosquitoes from transmitting parasites to a new host. The first barrier encountered most often is the cibarial armature located in the cibaria of female mosquitoes. These are teeth-like structures designed to cause mechanical damage to ingested pathogens. The presence, shape, and number of teeth varies from species to species which can affect a pathogen's likelihood of survival within a mosquito (Lee and Craig 1983). Additional armature is present in the bucco-pharyngeal region which causes mechanical damage to the cuticle of a pathogen (Manfredi et al. 2007). Some mosquitoes also have immune defenses like encapsulation that halts all development of a foreign pathogen (Beernsten et al. 2000). Salivary anticoagulants are secreted during a blood meal to increase the rate of blood flow from the host. The duration of this can vary depending on the species. Prolonged duration

can aid a pathogen when migrating to the Malpighian tubules. Without the production of this coagulant, blood clotting could prevent pathogen migration therefore reducing vector ability (Nayar and Sauerman 1975). While some mosquitoes are capable of transmitting *D. immitis* in cooler climates, Fortin and Slocombe (1981) found that there is a 14°C threshold for extrinsic incubation in *Aedes triseriatus* and *Ae. vexans* mosquitoes which inhibits their vectoral capacity in cooler climates.

Not all mosquitoes effectively transmit *D. immitis* to new hosts, so many studies have been conducted to identify efficient vectors. Over 60 species have been reported as being infected with *D. immitis* (Huang et al. 2013). Within the United States, 25 of these species can be found (Ledesma and Harrington 2011). These studies only indicate harboring, not transmitting the pathogen. Other studies implicate the following genera as potential vectors: *Aedes*, *Anopheles*, *Culex*, *Culiseta*, *and Psorophora* (Huang et al. 2013; Licitra et al. 2010; Watts et al. 2001). Licitra et al. (2010) conducted a study that implicated 3 species as being infectious and capable of transmitting *D. immitis* in GA; *Aedes albopictus*, *Anopheles crucians s.l.*, and *Anopheles punctipennis*. Recent studies conducted in Lowndes Co., GA also identified *Anopheles crucians sensu latu (s.l.)* as a vector of *D. immitis*, particularly in rural regions (Slaton 2020; West 2017). Based on these findings, my study focused on analyzing the role of *Anopheles quadrimaculatus sensu lato (s.l.)* mosquitoes in local *D. immitis* transmission through standard PCR and L3 specific RT-PCR.

West Nile Virus

` West Nile virus (WNV) belongs to the genus Flavivirus and is the leading cause for arboviral disease in the United States. WNV was first discovered circulating in the blood of an African woman in Uganda in 1937 (Smithburn et al. 1940). A new strain of WNV emerged in the late 20th century in Romania, Russia, and Israel that resulted in neurological infections (May et al. 2011; Tsai et al. 1999). In the United States, WNV was first observed in exotic and domestic birds within and near the Bronx Zoo in New York City, NY in 1999. Subsequently, human cases of WNV were identified in New York City. Not

only were these the first recorded infections within the United States, but they were also the first cases in the Western Hemisphere (Nash et al. 2001). Since its introduction, WNV has spread to all 48 contiguous states (CDC 2023). Since its introduction to the United States, there has been at least 56,569 human cases of WNV and 2,773 deaths (CDC 2023).

While most humans infected with WNV never develop symptoms, some experience febrile illness with symptoms such as headaches, body aches, vomiting, diarrhea, or rash. A small portion of those infected experience neuroinvasive disease causing encephalitis and meningitis (Sejvar et al. 2003). Infections resulting in mild symptoms are known as West Nile fever (WNF) while infections resulting in more severe neurological symptoms are described as West Nile neuroinvasive disease (WNND). The neuroinvasion of WNV is hypothesized to be due to hematogenous or transneural entry of the virus. Both proposed pathways involve the disruption of the blood-brain barrier to gain access to the central nervous system (Suen et al. 2014).

The primary route of WNV transmission is through the bite of an infected *Culex* mosquito, but intrauterine and blood transfusion transmission has been observed (Hayes and O'Leary 2004; MMWR Nov 1, 2002). The virus is maintained in the environment in an enzootic cycle involving birds and ornithophilic mosquitoes. WNV has been found to infect approximately 59 species of mosquitoes and 284 species of birds in North America (Hayes et al. 2005). Surveys conducted in the Southeastern United States implicated passerine birds as good reservoir hosts (Gibbs et al. 2006; Godsey et al. 2005). Both surveys found high antibody prevalence rates in northern cardinals, common ground doves, and northern mockingbirds.

Dead-end hosts like humans, horses, and other mammals are infected through the bite of a bridge vector. *Culex pipiens* has been implicated as the most important bridge vector of WNV in some northern parts of the United States due to its autogenous and anautogenous feeding patterns. Spielman

hypothesized that this allows *Cx. pipiens* to amplify the virus while feeding on birds alone in the anautogenous form during the early season and then act as bridge vectors when autogenous-anautogenous hybrids that feed on birds and mammals are present (Spielman 2001). Studies like these highlight the importance and the need for insight into the ecological factors that could be influencing WNV transmission. The purpose of this study is to examine the environmental factors influencing WNV transmission to improve management and mitigation of transmission.

Chapter II

L_3 Specific RT-PCR and Standard PCR Analysis of *D. immitis* presence in the *Anopheles quadrimaculatus* Mosquito Complex

Literature Review

Anopheles mosquitoes are vectors of a multitude of pathogens including *Plasmodium* species, O'nyong-nyong arbovirus, and *D. immitis* (Minkeu and Vernick 2018). They also have been implicated as potential vectors of multiple arboviruses (Minkeu and Vernick 2018). Studies indicate a variety of arboviruses, both DNA and RNA, have been found in *Anopheles* species globally (Minkeu and Vernick 2018). While extensive research has been conducted regarding their role in malaria transmission, there is a knowledge gap in their role as a veterinary health risk.

Anopheles quadrimaculatus s.l. is a species complex comprised of 5 sibling species that are closely related but morphologically indistinguishable (Kaiser et al. 1988a, 1988b; Narang et al. 1989, 1990). The inability to mate is what distinguishes them as individual species. The genus *Anopheles* has a number of species complexes. The first complex discovered within the genus was *Anopheles maculipennis s.l.*, discovered in Europe during efforts to control malaria transmission. *Anopheles maculipennis s.l.* was assumed to be the sole vector and a single species but was later determined to be a complex of several species. These cryptic species can vary in ecology, behavior and host preferences leading to both vector and non-vector species within the same complex. This can also affect approaches to vector control for different populations (Collins and Paskewitz 1996). One of the most studied complexes is *Anopheles gambiae s.l.* due to its competency to vector the blood parasite that causes malaria and its distribution throughout endemic areas. It is comprised of at least 6 sibling species, some of which have a higher vectoral capacity than others. Along with vectoral capacity, sibling species also

differ in habitat preference, host preference, breeding site preference, and feeding times, as well as geographical distribution (White 1974).

The *An. quadrimaculatus s.l.* complex is composed of 5 cryptic species characterized by dark scaled wings and palps unlike others in the genus (Kaiser et al. 1988a, 1988b; Narang et al. 1989, 1990). These species include *An. quadrimaculatus Say, An. diluvialis* Reinert*, An. inundatus* Reinert*, An. maverlius* Reinert*,* and *An. smaragdinus* Reinert. Reinert et al. (1997) described and created keys for each species within the complex during the following stages of life: adults, pupae, 4th instar larva, and egg. Reinert's first descriptions and depictions were later updated in 1999. He identified variation in adults, pupae, and 4th instar larvae. The most abnormalities were observed in the pupal stage, 4th-instar larval stage and in male genitalia (Reinert 1999). All five species within this complex were described as having gregarious adults and immatures associated with swamp-like habitats. Reinert et al. (1997) collected adult specimens from shared habitats, but in a more recent study Levine et al. (2004) described the sibling species as being allopatric. Levine used a genetic algorithm called GARP to predict distributions. GARP uses species point-occurrences and environmental data to make rules and then permutes and refines them until optimized models are produced that predict species distributions (Stockwell and Peters 1999). This difference could be due to the difference in location of specimen collection or the six-year time difference. These predictions show the presence of all five cryptic species within Lowndes Co., GA (Levine et al. 2004). Vector studies like these are important because they allow for a more accurate prediction of prevalence of a pathogen and contribute to species composition maps (Bowman et al. 2016; Brown et al. 2012).

Anopheles quadrimaculatus s.l. includes the principal vector of malaria in North America (Mullen and Durden 2009). Blackmore et al. (1998) implicate them as potential vectors of Cache Valley Virus and Dieme et al. (2022) found that they exhibit an increased vector competence compared to others in the genus. *An. quadrimaculatus s.l.* mosquitoes of the United States have also been found to exhibit the

ability to vector the Mayaro virus which can lead to risk of transmission of this virus in North America (Dieme et al. 2020). While extensive research has been conducted regarding *An. quadrmiaculatus s.l.* mosquitoes' role in malaria transmission, there is a knowledge gap in their role as a veterinary health risk.

Studies have been conducted on the infection of *An. quadrimaculatus s.l.* by *D. immitis* and the factors that could affect its vector ability (Bradley and Nayar 1987; Kartmen 1953; Nayar and Sauerman 1975; Newton 1957). Kartmen (1953) conducted studies comparing the different factors influencing *D. immitis* infection in different species of mosquitoes. Infections of *An. quadrimaculatus s.l.* and *An. freeborni* were compared and showed similar rates of development. Microfilariae migration to the Malphigian tubules is inhibited by the formation of blood clots in *Aedes aegypti* and *Ae. albopictus*. This does not occur in *An. quadrimaculatus s.l.* and it was found that about 91% of the parasites ingested were in the tubules by the 24-hour mark. This was significantly higher than what was found in *Aedes* (Kartmen 1953). Another study (Newton 1957) demonstrated that *An. quadrimaculatus s.l.* developed high levels of infection in the head and proboscis region that potentially inhibits them from feeding. Nayar and Sauerman (1975) also found that *An. quadrimaculatus s.l.* mosquitoes were highly susceptible to *D. immitis* infection, but infected mosquitoes did not survive long enough for the L3 stage to migrate from the Malphigian tubules to the proboscis. Bradley and Nayar (1987) conducted an ultrastructural study of *D. immitis* infection in the Malphigian tubules and observed that infection causes major damage to the apical microvilli, mitochondria, and basal membranes of the cells that make up the Malphigian tubules. Transmission of *D. immitis* is dependent upon these structures remaining intact long enough for the migration to the proboscis to occur (Bradley and Nayar 1987). The evidence suggests that *An. quadrimaculatus s.l.* could play a role in transmission under the right circumstances, which is why vector studies are needed across different regions.

Vector determination can be done in different ways. A technique that has been used in the past involves dissecting mosquitoes to look for the development of L3 *D. immitis*. However, this method is labor intensive and leaves more room for error. This method is also impractical when handling large sample sizes (Sauerman and Nayar 1983). A more recent technique is determination through amplification of pathogen DNA using polymerase chain reaction (PCR) (Scoles and Kambhampati 1995; Watts et al. 2001). Chambers et al. (2009) compared PCR and dissection when xenomonitoring *Wuchereria bancrofti* and *D. immitis*. They found PCR to be the more efficient technique when assessing transmission of filariasis. While this technique is more time efficient and less error prone, it does not allow for differentiation between life stages of the parasite, a crucial distinction needed for determining vector ability. Filarial DNA does not allow this differentiation, but filarial RNA does. The most recent method of identifying potential vectors does allow for this differentiation through reverse transcriptase polymerase chain reaction (RT-PCR). A protocol was developed by Laney et al. (2010) that detects *Wuchereria bancrofti* infective larvae in mosquitoes using RT-PCR. This allows for simultaneous detection of infection and infectivity to evaluate transmission potential. Recently, Thompson (2016) developed a protocol that detects the infective stage by amplification of an L3 stage-active collagen gene expressed in *D. immitis*. Tumor protein homolog is constitutive and is expressed in all life stages and is used to detect presence of infection (Thompson 2016). Protocols like Laney's and Thompson's provide a more accurate representation of transmission rates and allow for better surveillance and control of vector populations.

Methods

Specimen Collection

Two different types of traps were used to collect specimens: Reiter gravid traps (JW Hock Company, Gainesville, FL) and CDC miniature light traps (JW Hock Company, Gainesville, FL). Gravid traps were baited with water which was infused with hay to attract gravid female mosquitoes (primarily

Culex species) attempting to oviposit after a blood meal. Mosquitoes that have already taken a blood meal have an increased likelihood of being infected with *D. immitis*. CDC light traps were baited with approximately 2 kg of dry ice, which sublimates to produce gaseous carbon dioxide, attracting female mosquitoes seeking a blood meal. *D. immitis* positive mosquitoes caught in these traps could potentially transmit heartworm to new hosts.

Mosquitoes were collected for this study from May 2021 to July 2022. Each week during this time, gravid traps were set three nights a week and light traps were set once a week. Traps were set between 16:00 – 18:00 EST and retrieved between 07:00 -09:00 EST the next morning. The 2 h range in times reflects travel time between trap locations. 14 different trap sites were located within Lowndes Co., GA and included both rural and urban areas with varying socioeconomic conditions. During peak trapping months (March-October), traps were set at all 14 locations, while only 8 locations were used during the months of November-February.

Once collected, mosquitoes were transported alive to the lab where they were anesthetized by triethylamine fumes within a hood. After they were immobilized, mosquitoes were examined using dissecting microscopes and identified to species using standard morphological keys (Burkett-Cadena 2013; Darsie and Morris 2003). All intact female *An. quadrimaculatus s.l.* specimens identified were pooled by date, location, and trap type. Pools were stored at -80°C until RNA or DNA extraction was performed.

RNA Extraction and Conversion to cDNA

RNA was extracted from pools of mosquitoes using Trizol LS Reagent (Invitrogen Corp., Waltham, MA, USA). Total RNA extractions were performed according to manufacturer's instructions. Pools of mosquitoes based on location were transferred to a 1.5 mL tube containing 250 µl RNase-free water and 750 µl TRIzol LS Reagent. Tissue was homogenized using a pestle. Samples were allowed to

incubate for 10 min at room temperature. This allowed for complete dissociation of nucleoprotein complexes. Following incubation, 200 µL of chloroform was added followed by vigorous shaking. After 10 min of incubation at room temperature, samples were centrifuged at 11,000 rpm for 15 minutes at 4 °C. This induced phase separation. RNA remained in the aqueous phase. The aqueous phase was transferred to a new tube along with 1 µL of RNase inhibitor. To precipitate the RNA from the aqueous phase, 600 µL of cold isopropyl alcohol was added. Tubes were inverted to mix thoroughly and then allowed to incubate for 15 min. Samples were then centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant was removed leaving the RNA precipitate. The remaining RNA pellet was then washed with 1 mL of 75% cold ethanol and vortexed gently. The sample was then centrifuged at 9,000 rpm for 5 min at 4 °C. The RNA pellet was washed a second time with 500 µL of cold 100% ethanol and centrifuged at 9,000 rpm for 2 min at 4 °C.

The RNA pellet was allowed to air-dry for 10 min after removing the ethanol with a pipet. RNA was then dissolved by incubating samples for 10 min at 60 °C with RNase-free TE, pH 8.0. Both purity and quantity of RNA extractions were evaluated using a NanoDrop 20000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Samples were then converted to cDNA using SuperScript® IV First-Strand Synthesis System kit (Invitrogen Life Technologies, Grand Island, NY) following the manufacturer's protocol. Each 20 µL reaction consisted of 50 µM Oligo d(T)20 primer, 10mM dNTP mix, DEPC-treated water, 5x SSIV Buffer, 100 mM DTT, 100 mM RNaseOUT™ Recombinant RNase Inhibitor, and 100 mMSuperScript® IV Reverse Transcriptase.

DNA Extraction

Specimens were thawed to room temperature and then individual mosquitoes were transferred to 2 mL microfuge tubes with a zinc grinding bead. DNA was extracted using the DNeasy Blood and

Tissue Kit (Qiagen, Hilden, Germany). The manufacturer's protocol was slightly modified. After adding 180 µl of 1 X phosphate buffered saline and a layer of Parafilm, mosquitoes were homogenized using a vortex mixer fitted with an attached horizontal adapter (Scientific Industries, Bohemia, NY) for 15 min. Once homogenized, 180 µL of Qiagen Buffer AL (lysis buffer) and 20 µL of proteinase K were added. Samples were then incubated in a water bath at a temperature of 56°C for at least 2 h. Tubes were then centrifuged for 5 min at 13,000 rpm. The supernatant was then combined with 200 µL of ethanol, transferred to a Qiagen DNeasy spin column, and centrifuged at 8,000 rpm for 1 min. The collecting tube was replaced and 500 µL of buffer AW1 was added to the column. The tube was then centrifuged again at 8,000 rpm for 1 min. This same step was repeated, but with buffer AW2 and at an rpm of 13,000 for 3 min. The spin column was placed in a new 1.5 mL tube and 125 µL of AE eluting buffer was applied to the column. The spin column was allowed to sit for 1 min and then centrifuged at 8,000 rpm for 1 min. After extraction, DNA samples were evaluated for DNA concentration and quality using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extracted DNA was stored at 20°C until used for PCR.

RT-PCR Analysis and Gel Electrophoresis

Aedes aegypti mosquitoes of known infection were used to confirm the viability of the assay. These were provided by the NIH/NIAID Filariasis Resource Reagent Resource (FR3) Center at the University of Georgia. These mosquitoes were fed *D. immitis* infected blood, monitored, collected at 2- and 11-days post infection (d.p.i), and then immediately frozen. The different time points represent the infective stage (11 d.p.i) and non-infective stage (2 d.p.i). Uninfected mosquitoes were also sent by the FR3 center to serve as negative controls. RNA extraction, conversion to cDNA, and RT-PCR were performed on pools of 4 female mosquitoes of 2 and 11 d.p.i.

Aedes aegypti mosquitoes were screened for L3 stage *D. immitis* by RT-PCR using the Platinum ® Taq DNA Polymerase kit (Invitrogen Life Technologies, Grand Island, NY). The Platinum ® Taq DNA

Polymerase kit was used according to manufacturer's instructions. The CK855471A primer set developed by Thompson (2016) and the Ditph primer set were used.

Each 25 µL reaction consisted of buffer, varying concentrations of $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP (Fisher Scientific, Pittsburgh, PA, USA), 0.2 µM of both forward and reverse primers, 0.2 µM Platinum ® Taq DNA Polymerase, and 2 µL template cDNA. Various concentrations ranging from 0 to 1.5 mM of MgCl2 were used to determine the appropriate concentration for the reaction. Conditions were as follows: 95°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and an extension of 72°C for 10 min. PCR products were examined using ethidium bromide (Biotium, Hayward, CA) stained 1.0% agarose gels. Gels were run at 70 V for 1.5 h and then imaged under UV light using an ImageQuant LAS 4000 (General Electric, Schenectady, NY). Mosquitoes were considered infected if there was amplification of a 152 bp fragment. Mosquitoes were considered infective if there was amplification of a 286 bp fragment.

PCR Analysis and Gel Electrophoresis

PCR assays were used to determine the presence of *D. immitis* DNA. The following protocol was adapted from Vezzani et al. (2011). Two primers were used to amplify *D. immitis* DNA: Di_16S_RNA-F and DI_16S_RNA-R. A 25 ul reaction was composed of 1XµL Green GoTaq Flexi buffer, 2 mM of $MgCl_2$, 400 uM of dNTPs, 200 uM of each primer, 1 unit of GoTaq DNA polymerase and 2 µL of purified DNA PCR reactions were completed using a Bio-Rad I-Cycler (Bio Rad Laboratories, Hercules, CA). The PCR conditions were as follows: 95°C for 10 min, 95°C for 60 s, 55°C for 45 s, 72°C for 60 seconds, and 72°C for 10 min. The middle three steps were repeated for 35 cycles.

PCR products were examined using 1.0% agarose gels stained with ethidium bromide. Gels were run at 80 V for 60 min and then imaged under UV light using an ImageQuant LAS 4000 (General Electric, Schenectady, NY). With each set of samples, a known positive extraction control was run as well as a

negative control with no DNA. Mosquitoes were considered positive if there was amplification of a 453 bp fragment.

Statistical Analysis

PoolScreen2.0 was used to calculate maximum likelihood estimates (MLE) and 95% confidence intervals of infection rates of the *Anopheles quadrimaculatus* complex. This calculation provides an estimate of the number of infected mosquitoes within a population. These results were expressed as the number of infected *An. quadrimaculatus s.l.* for every 1000.

Results

Standard PCR and Gel Electrophoresis

A total of 352 *An. quadrimaculatus s.l.* were collected over the course of the three trapping periods. Out of the 70 *An. quadrimaculatus s.l.* individuals screened for *D. immitis* using the PCR protocol, 1 specimen was found to be positive (Figure 4). This preliminary study yielded an MLE of 14.3/1000 mosquitoes (95% CI: 0.44-71.4).

L3 Specific RT-PCR

RNA extractions used for RT-PCR had concentrations between 150 and 210 ng/µl. While all 260/280 ratios for extractions were above 2.0, the 260/230 ratios were all under 1.5 (Table 1). After multiple attempts, the *ditph* gene was the only band to amplify clearly and consistently using control samples. Amplification of both bands occurred once, but those results could not be replicated (Figure 5).

Discussion

The results of this study implicate *An. quadrimaculatus s.l.* as a potential vector of *D. immitis*. Their feeding preference includes canines which further implicates them as a potential vector (Jensen et al. 1996). In comparison to another study conducted in Lowndes Co., the *An. quadrimaculatus s.l.* MLE falls within the middle range of MLEs of other vector species (Slaton, 2020). Slaton (2020) found no *An.*

quadrimaculatus s.l. individuals to be infective with *D. immitis*, but this was probably due to the limited number of individuals tested (n <5). Slaton (2020) used only the heads and thoraces of mosquitoes in order to detect the L3 stage of *D. immitis*, but with such a small sample size this could have resulted in an insufficient amount of DNA needed for PCR amplification for this species complex. More studies need be conducted to understand their vector status and assess the extent of their involvement in *D. immitis* transmission within Lowndes Co.. The 95% confidence interval for the MLE of the *An. quadrimaculatus s.l.* is wide, but with a larger sample size of 1,000 individuals or more I expect this range to decrease in size.

This study also shows that the RT-PCR protocol developed by Thompson (2016) may not be practical when applied to field collected mosquitoes. Because the purpose of this study was to determine the sensitivity of the assay, our pools only consisted of 5 control mosquitoes, while the pools used in Thompson (2016) consisted of 25. The difference in the amount of RNA in our pools may have prevented us from obtaining clear and consistent amplification using standard RT-PCR and gel electrophoresis. I still believe the sensitivity should be determined and this could be done in future studies by increasing the amount of control mosquitoes used in pools or by developing a qRT-PCR assay. qRT-PCR assays have been shown to be highly sensitive and reproducible which may yield better results than the standard RT-PCR assay.

Chapter III

Analysis of Temperature and Habitat on West Nile Virus Infection of Bridge Vectors

Literature Review

The predominant mosquito vectors involved in WNV transmission vary geographically. While *Cx. pipiens* and *Cx. restuans* have been implicated as principal vectors in the northeastern United States (Molaei et al. 2006), *Cx. quinquefasciatus* (Say) has been implicated as the predominant vector within the southeastern United States because they thrive in urban and suburban areas (Molaei et al. 2007). *Culex quinquefasciatus* are predominantly ornithophilic but have been shown to feed on mammals including humans. Because of their opportunistic feeding habits, they have been implicated as playing a major role in both avian and human transmission of WNV (Molaei et al. 2007).

Culex nigripalpus is the predominant vector of Saint Louis Encephalitis (SLE) virus in Florida (Chamberlain et al. 1964) and has been implicated as a vector of WNV in the southeastern United States as well (Rutledge et al. 2003). Like *Cx. quinquefasciatus* mosquitoes, *Cx. nigripalpus* feed predominantly on avian species but are opportunistic and will feed on mammals, reptiles, and amphibians (Edman 1974). While gravid *Cx. nigripalpus* females prefer temporary freshwater habitats as breeding sites like pastures and roadside ditches (Nayar 1982), they are opportunistic in breeding site selection after flooding events. Due to this oviposition preference, it is hypothesized that the viral extrinsic incubation period has a higher likelihood of finishing within a single gonotrophic cycle when compared to *Cx. quinquefasciatus* mosquitoes that oviposit more frequently (Day and Curtis 1994).

WNV transmission is affected by habitat, temperature, and rainfall. Land use and land cover (LULC) has an influence on vector distribution which therefore affects pathogen transmission. Regional differences have been found in the associations between LULC and human WNV incidence (Bowden et al. 2011). In northeastern regions of the United States, a positive association was found between urban

land covers and WNV disease incidence, but in western regions disease incidence was positively associated with agricultural areas (Bowden et al. 2011). The differences observed may be due to different WNV vectors in these regions and their habitat preferences (Bowden et al. 2011). Changes in LULC have been shown to influence pathogen transmission indirectly by impacting vector distribution. Some mosquitoes are able to adapt to areas that have been urbanized or altered for agriculture. A number of vector species of the genera *Aedes* (Flaibani et al. 2020), *Anopheles* (Hamza and Rayah 2020), and *Culex* (Weitzel et al. 2014) utilize man-made artificial breeding sites produced through these LULC changes which has allowed them to spread to regions that were once uninhabitable (Amerasinghe and Ariyasena 1990).

Increased urbanization is associated with a rise in temperature due to the formation of urban heat islands through loss of canopy shading (Lindblade et al. 2000). Soverow and colleagues (2009) found that an increase in weekly maximum temperature and weekly cumulative temperature was associated with an increase in WNV incidence in humans. This phenomenon was also observed when monthly average temperature was used instead of weekly average temperature specifically in the months preceding the WNV season (Hahn et al. 2015). This could be due to mosquito being temperature dependent (Kweka et al. 2016). Increased temperatures speed up the development of mosquitoes and increase the number of eggs laid in a gonotrophic cycle (Kweka et al. 2016).

There is a positive association with WNV disease outbreaks and increased rainfall in preceding months (Takeda et al. 2003). The increased rainfall could create additional breeding sites for mosquitoes and result in higher abundance of potential vector mosquitoes overwintering (Nasci et al. 2001). Landesman et al. (2007) found this association to vary geographically. While they found WNV incidence in humans in the eastern United States tended to increase when above average rainfall occurred in the year prior, they found the opposite to be true in the western United States. The varying hypotheses may be due to environmental responses depending on the ecology of the different mosquito species.

The objective of this study was to analyze the habitat and temperature trends associated with positive WNV detection in *Culex* species in Lowndes Co., GA. I used mosquito surveillance data and weekly average temperature from 2012-2021 to discern patterns. I hypothesized that a positive association exists between temperature, WNV infection rates, and impervious surface area.

Methods

Specimen Collection and Processing

Two different types of traps were used to collect specimens: Reiter gravid traps (JW Hock Company, Gainesville, FL) and CDC miniature light traps (JW Hock Company, Gainesville, FL). The gravid traps were baited with water which was infused with hay to attract gravid female mosquitoes (primarily *Culex* species) attempting to oviposit after a blood meal. Mosquitoes that have already taken a blood meal have an increased likelihood of being infected with WNV. The CDC light traps were baited with approximately 2 kg of dry ice, which sublimates to produce gaseous carbon dioxide, attracting female mosquitoes seeking a blood meal. This may include mosquitoes that have fed at least one time as well as those that are seeking a first host (nulliparous females).

Trapping occurred weekly from March-November from 2012 to 2021. Gravid traps were set three nights a week and CDC miniature light traps were set one night a week. Traps were set between 16:00 – 18:00 EST and retrieved between 07:00 -09:00 EST the next morning. The 2 h range in times reflects travel time between trap locations. The 14 different trap sites were all located within Lowndes Co., GA.

Once collected, mosquitoes were transported alive to the lab where they were anesthetized by triethylamine fumes within a hood. After they were immobilized, the mosquitoes were examined using dissecting microscopes and identified to species using standard morphological keys (Burkett-Cadena 2013; Darsie and Morris 2003). All intact female *Cx. quinquefasciatus* specimens identified were then

pooled by date, location, and trap type. Each pool consisted of at least 5 specimens and no more than 25. Pools were then stored at -80°C until they were shipped on dry ice at the end of each week to Dr. Daniel Mead's lab at the College of Veterinary Medicine of University of Georgia where WNV detection takes place.

All specimen data were recorded, and trap index was calculated weekly. Trap index is the average number of female mosquitoes collected per trap night. The total number of *Cx. quinquefasciatus* mosquitoes collected each week were divided by the number of traps set that week to obtain the trap index.

WNV Detection

All testing was completed by Dr. Daniel Mead and Kayla Guinn Adcock at Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia. Mosquito pools were tested for cytopathic effect using the protocol found in Kunkel et al. (2021). 1 ml of BA-1 media was added to each pool. Samples were then mixed for 2 min for 5 cycles per s (Retsch MM 300, Haan, Germany) and centrifuged at 10,000 x g for 10 min. 100 ml of supernatant was then plated for virus isolation and monitored for cytopathic effect.

A VectorTest ® WNV Antigen Assay (VecTOR Test Systems, Inc, Thousand Oaks, CA) was performed on samples where cytopathic effect was observed as a preliminary analysis of WNV presence. After a positive VectorTest, viral isolates are extracted using the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA) following manufacture's protocol. RT-PCR is then conducted on the RNA extractions using the protocol published in Allison et al. 2004. Gel electrophoresis is then performed on the PCR products to confirm WNV presence following the method in (Adcock et al. 2023).

Trap Index Calculation

The trap indices for *Cx. quinquefasciatus* mosquitoes were calculated for every week trapped to represent mosquito distribution. These were calculated by dividing the total number of female *Cx. quinquefasciatus* mosquitoes collected that week by the number of traps set multiplied by the number of nights running. This is a standard representation for mosquito surveillance that allows for comparison between weeks that have a different number of traps set nightly or number of nights trapped.

Climate and LULC Data Collection

Climate data for 2012-2021 were obtained from historical reports from Weather Underground. The weekly average temperature was recorded for every week trapping occurred. Next, I determined habitat composition within a 1 km radius at each of our 14 surveillance sites. I then downloaded LULC imagery for 2012 from the USDA National Agricultural Statistics Service Cropland Data Layer (2012). In ArcGIS (version 10.5, Esri, Redlands, CA), I classified habitats of interest including wetlands and urbanization (e.g., impervious surface) and extracted percent habitat from 1 km buffers around each surveillance site.

Results

From 2012-2021, 124 pools of *Cx. quinquefasciatus*, 15 pools of *Cx. nigripalpus*, and 1 pool of *Cx. restuans* tested positive for WNV. 2018 had the highest number of confirmed WNV positive pools (n=57) while the years 2015, 2016, and 2021 had the lowest number of confirmed WNV positive pools with 0 (Table 2; Figure 6).

Yearly trap indices were calculated for every species collected in the lab over the 10-year study (Table 3). Further analyses were conducted using only data regarding pools of *Cx. quinquefasciatus*. Weekly *Cx. quinquefasciatus* trap indexes ranged from 3.3 to 68.5 with an average of 25.6 (Table 4). The weekly average temperatures when WNV positive pools were collected range from 18.89°C to 29.79°C,

with an average of 26.14°C (Table 4). After plotting these values, a linear relationship was not seen between trap index and weekly average temperature (Figure 7).

The total number of WNV positive pools collected at the 14 different locations ranged from 0 to 32. The most WNV positive pools came from the Fleming trap site with a total of 32 positive pools. The Woodmen trap site was the only location where no WNV positive pools were collected (Table 5; Figure 8). The proportion of impervious surface for the trap sites ranged from 0.03 to 0.93. Impervious surface was plotted against the number of WNV positive pools collected at each site and a positive association can be seen between the two (Figure 9). The Hammock trap site had the lowest proportion, and the Fleming trap site had the highest. The proportion of wetland for the trap sites ranged from 0.012 to 0.324. The Fleming trap site had the lowest proportion, and the Thomas trap site had the highest proportion (Table 6). The proportion of wetland at each site exhibits a linear relationship with the proportion of impervious surface (Figure 10).

Discussion

While most WNV positive pools collected were *Cx. quinquefasciatus,* WNV has been detected in *Cx. nigripalpus* and *Cx. restuans* in Lowndes Co. During the period covered in this study, the first *Cx. nigripalpus* WNV pool was not collected until 2017 and the only *Cx. restuans* WNV positive pool was collected in 2019. Since 2019, if WNV was detected, it was detected in both *Cx. quinquefasciatus* and *Cx. nigripalpus.*

Urbanization requires transformations in land use and land cover that are more adapted for handling higher concentrations of humans. According to the United States Environmental Protection Agency (EPA), one way to quantify urbanization is through identifying the proportion of impervious surface (e.g., roads, parking lots, roofs) in a given area. Although habitat type was not significantly associated with presence or number of *Cx. quinquefasciatus* collected in Lowndes Co., the results from

this study do suggest a positive association between urbanization and the number of WNV positive *Cx. quinquefasciatus* pools collected. The 3 sites with the highest number of WNV positive *Cx. quinquefasciatus* pools include 3 of the 4 sites with the highest proportions of impervious surface. The sites with no WNV positive *Cx. quinquefasciatus* pools had a relatively low proportion of impervious surface. Because of the impervious surface-wetland relationship, it can be inferred that if there is a positive association between urbanization and the number of WNV positive *Cx. quinquefasciatus* pools, there is a negative association between wetland and the number of WNV positive *Cx. quinquefasciatus* pools. A positive association with urbanization was also found by Bowden et al. (2011) in northeastern regions of the United States where the primary WNV vector is *Cx. pipiens*, which are within the same species complex as *Cx. quinquefasciatus* (Joyce et al. 2018). This association also coincides with the idea that the preferred habitats of the primary WNV vectors of each region drive the geographical differences in habitat influence on transmission hypothesized by Bowden et al. (2011).

One limitation to this study is that weekly average temperature was only analyzed for weeks when WNV positive pools were collected. Even with this limitation, I was able to observe that most of the weeks in which positive pools were collected had a weekly average temperature of 23.9°C. Only 3 weeks fell below this threshold (Figure 6). This observation could also be due to the seasonality of WNV. The CDC (2023) reports that most human WNV incidence occurs between the months of July and October. To further understand the influence of temperature on WNV transmission in *Cx. quinquefasciatus*, a study could be performed comparing weekly average temperatures of weeks where WNV positive pools were collected and weeks where they were not.

This is the first study to analyze the relationship between West Nile virus mosquito infection data and land use and land coverage (LULC) data from the Valdosta State University Mosquito Surveillance Program. While this is just an initial dive into data from 2012-2021, there are records dating back to 2005 that can be analyzed as well. I examined the influence of temperature, trap index, and

habitat on transmission of WNV, but did not examine the influence of the avifauna at the different sites. This is a critical component of the WNV transmission cycle which should be assessed. This study highlights the importance of local mosquito surveillance and molecular xenomonitoring of mosquito populations. Long term surveillance programs like these generate data sets which can provide insight into external factors influencing disease transmission. This information can be used to predict local transmission risk and better understanding of vector ecology.

Chapter IV

Conclusion

In summary, I found that the *An. quadrimaculatus s.l.* is a potential vector of *D. immitis* and that there is a regional association between urbanization and WNV transmission in *Cx. quinquefasciatus* mosquitoes. These results are supported by other studies reporting laboratory infection of *An. quadrimaculatus s.l.* and land cover associations being driven by vector mosquito ecology. Regional differences in vector populations and factors influencing disease transmission emphasize the importance of local mosquito surveillance programs. Research identifying vectors of *D. immitis* and WNV and their distributions aid in the development of prevalence maps and predictive models of pathogens and mosquitoes which are crucial in disease management and the implementation of control methods.

References

Adcock, K. G., R. D. Berghaus, C. C. Goodwin, M. G. Ruder, M. J. Yabsley, D. G. Mead, and N. M. Nemeth. 2023. Lymphoproliferative disease virus and reticuloendotheliosis virus detection and disease in wild turkeys (*Meleagris gallopavo*). J. Wildl. Dis. (in press).

Alberigi, B., D. R. Campos, A. S. Branco, A. Bendas, R. P. Brum, R. Calixto, L. C. Alves, J. W. Pinheiro Júnior, F. B. Knackfuss, N. Labarthe, J. K. Levy, and F. Mendes-de-Almeida. 2022. Feline Heartworm in Clinical Settings in a High Canine Prevalence Area. Front. Vet. Sci. 9:819082.

Amerasinghe, F. P., and T. G. Ariyasena. 1990. Larval Survey of Surface Water-Breeding Mosquitoes During Irrigation Development in the Mahaweli Project, Sri Lanka. J of Med Ent. 27(5): 789-802.

American Heartworm Society. 2019. Heartworm Incidence Maps. American Heartworm Society. Retrieved December 2, 2022, from https://www.heartwormsociety.org/veterinary-resources/incidence-maps.

Beernsten, B. T., A. A. James, and B. M. Christensen. 2000. Genetics of Mosquito Vector Competence. Microbiology and Molecular Biology Reviews. 64(1).

Blackmore, C. G. M., M. S. Blackmore, and P. R. Grimstad. 1998. Role of Anopheles quadrimaculatus and (diptera: Culicidae) in the transmis- sion cycle of Cache Valley virus (Bunyaviridae: Bunyavirus) in the midwest, U.S.A. Journal of Medical Entomology, 35, 660–664, 197.

Bowden, S. E., K. Magori, and J. M. Drake. 2011. Regional Differences in the Association Between Land Cover and West Nile Virus Disease Incidence in Humans in the United States. Am J Trop Med Hyg. 84(2): 234-238.

Bowman, D. D., Y. Liu, C. S. McMahan, S. K. Nordone, M. J. Yabsley, and R. B. Lund. 2016. Forecasting United States heartworm *Dirofilaria immitis* prevalence in dogs. Parasit. Vectors. 9: 940.

Bradley, T. J., and J. K. Nayar. 1987. An Ultrastructural Study of *Dirofilaria immitis* Infection in the Malphigian Tubules of *Anopheles quadrimaculatus*. J. Parisitol. 73(5): 1035-1043.

Brown, H. E., L. C. Harrington, P. E. Kaufman, T. McKay, D. D. Bowman, C. T. Nelson, D. Wang, and R. Lund. 2012. Key Factors influencing canine heartworm, *Dirofilaria immitis*, in the United States. Parasit. Vectors. 5: 245.

Burkett-Cadena, N. D. 2013. Mosquitoes of the Southeastern United States. University of Alabama Press, Tuscaloosa, AL. pp. 123-141.

CDC. 2023. Centers for Disease Control and Prevention. [modified June 13, 2023; accessed June 17, 2023]. https://www.cdc.gov/westnile/statsmaps/historic-data.html.

Chamberlain, R. W., W. D. Sudia, P. H. Coleman and l. D. Beadle. 1964. Vector Studies in the St. Louis Encephalitis Epidemic, Tampa Bay Area, Florida, 1962. Am. J. Trop. Med. Hyg. 13(3):456-61.

Chambers, E. W., S. K. McClintock, M. F. Avery, J. D. King, M. H. Bradley, M. A. Schmaedick, P. J. Lammie, and T. R. Burkot. 2009. Xenomonitoring of *Wuchereria bancrofti* and *Dirofilaria immitis* infections in mosquitoes from American Samoa: trapping considerations and a comparison of polymerase chain reaction assays with dissection. Am. J. Trop. Med. Hyg. 80(5): 774-781.

Chikweto, A., M. I. Bhaiyat, M. Lanza-Perea, S. Veytsman, K. Tiwari, C. De Allie, and R. N. Sharma. 2014. Retrospective study of canine heartworm disease with caval syndrome in Grenada, West Indies. Vet. Parasitol. 205: 721-724.

Collins, F. H. and S. M. Paskewitz. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol. Biol. 5(1): 1-9.

Darsie, R. F., and C. D. Morris. 2003. Keys to the adult females and fourth instar larvae of the mosquitoes of Florida (Diptera, Culicidae). Technical Bulletin of the Florida Mosquito Control Association 1: 1–159.

Day, J. F. and G. A. Curtis. 1994. When It Rains, They Soar—and That Makes Culex Nigripalpus a Dangerous Mosquito. Am. Entomologist. 40(3): 162–167.

Diakou, A. and Prichard, R.K. 2021. Concern for *Dirofilaria immitis* and Macrocyclic Lactone Loss of Efficacy: Current Situation in the USA and Europe, and Future Scenarios. Pathogens, 10: 1323.

Dieme, C., A. T. Ciota, and L. D. Kramer. 2020. Transmission potential of *Mayaro virus* by *Aedes albopictus*, and *Anopheles quadrimaculatus* from the USA. Parasites Vectors. 313: 613.

Dieme, C. A., K. A. Ngo, S. Tyler, J. G. Maffei, S. D. Zink, A. P. Dupuis, C. A. Koetzner, C. Shultis, J. Stout, A. F. Payne, P. B. Backenson, L. Kuo, M. A. Drebot, A. T. Ciota, and L. D. Kramer. 2022. Role of *Anopheles* Mosquitoes in Cache Valley Virus Lineage Displacement, New York, USA. Emerg Infect Dis. 28(2): 303-313.

Edman, J. D., L. A. Webber, and A. A. Schmid. 1974. Effect of Host Defenses on the Feeding Pattern of Culex nigripalpus When Offered a Choice of Blood Sources. The Journal of Parasitology, 60(5), 874–883.

Flaibani, N., A. A. Pérez, I. M. Barbero, and N. E. Burroni. 2020. Different approaches to characterize artificial breeding sites of *Aedes aegypti* using generalized linear mixed models. Infect Dis Poverty. 9:107.

Fortin, J. F. and J. O. D. Slocombe. 1981. Temperature requirements for the development of *Dirofilaria immitis* in *Aedes triseriatus* and *Ae. vexans*. Mosq. News. 41: 625-633.

Gibbs, S. E. J., A. B. Allison, M. J. Yabsley, D. G. Mead, B. R. Wilcox, and D. E. Stallknecht. 2006. West Nile Virus Antibodies in Avian Species of Georgia, USA: 2000–2004.Vector-Borne and Zoonotic Diseases. 6(1): 57-72.

Godsey Jr., M. S., M. S. Blackmore, N. A. Panella, K. Burkhalter, K. Gottfried, L. A. Halsey, R. Rutledge, S. A. Langevin, R. Gates, K. M. Lamonte, et al. 2005. West Nile Virus Epizootiology in the Southeastern United States, 2001. Vector-Borne and Zoonotic Diseases. 5(1):82-89.

Gomes-de-Sá, S., S. Santos-Silva, A. d. Moreira, P. F. Barradas, I. Amorim, L. Cardoso, and J. R. Mesquita. 2022. *Dirofilaria immitis* antigenemia and microfilaremia in Iberian wolves and red foxes from Portugal. Parasites Vectors 15: 119.

Hahn, M. B., A. J. Monaghan, M. H. Hayden, R. J. Eisen, M. J. Delorey, N. P. Lindsey, R. S. Nasci, and M. Fischer. 2015. Meteorological conditions associated with increased incidence of West Nile virus disease in the United States, 2004-2012. Am. J. Trop Med. Hyg. 92(5): 1013-22.

Hamza, A. M. and E. A. E. Rayah. 2020. A Qualitative Evidence of the Breeding Sites of *Anopheles arabiensis* Patton (Diptera: Culicidae) in and Around Kassala Town, Eastern Sudan. International J of Insect Science. 8(1).

Hayes, E. B., N. Komar, R. S. Nasci, S. P. Montgomery, D. R. O'Leary, and G. L. Campbell. 2005. Epidemiology and Transmission Dynamics of West Nile Virus Disease. Emerg Infect Dis. 11(8): 1167-1173.

Hayes, E. B. and D. R. O'Leary. 2004. West Nile Virus Infection: A Pediatric Perspective. Pediatrics. 113 (5): 1375–1381.

Huang, S., D. J. Smith, G. Molaei, T. G. Andreadis, S. E. Larsen, and E. F. Lucchesi. 2013. Prevalence of *Dirofilaria immitis* (Spirurida: Onchocercidae) infection in *Aedes*, *Culex*, and *Culiseta* mosquitoes from north San Joaquin Valley, CA. J Med Entomol. 50(6): 1315-23.

Jensen, T., A. F. Cockburn, P. E. Kaiser, and D. R. Barnard. Human Blood-Feeding Rates Among Sympatric Sibling Species of *Anopheles quadrimaculatus* Mosquitoes in Northern Florida. The American Journal of Tropical Medicine and Hygiene. 1996;54(5):523-525.

Joyce, A.L., E. Melese, PT. Ha, and A. Inman. 2018. Population genetic structure of the *Culex pipiens* (Diptera: Culicidae) complex, vectors of West Nile virus, in five habitats. Parasites Vectors. 11(10).

Kaiser, P. E., S. E. Mitchell, G. C. Lanzaro and J. A. Seawright. 1988a. Hybridization of laboratory strains of sibling species A and B of *Anopheles quadrimaculatus*. J. Am. Mosq. Control Assoc. 4: 34-38.

Kaiser, P. E., S. K. Narang, J. A. Seawright and D. L. Kline. 1988b. A new member of the *Anopheles quadrimaculatus* complex, species C. J. Am. Mosq. Control Assoc. 4: 494-255.

Kartmen, L. 1953. Factors influencing infection of the mosquito with *Dirofilaria immitis* (Leidy, 1856). Ex. Parasitol. 2(1): 27-78.

Kunkel, M. R., D. G. Mead, R. D. Berghaus, K. G. Adcock, M. G. Ruder, and N. M. Nemeth. 2021. Experimental West Nile Virus Infection in Northern Bobwhite Quail (*Colinus virginianus*). Avian Dis. 65(4):523-529.

Kweka, E. J., E. E. Kimaro, and S. Munga. 2016. Effect of Deforestation and Land Use Changes on Mosquito Productivity and Development in Western Kenya Highlands: Implication for Malaria Risk. Front Public Health. 4.

Laney, S. J., R. M. R. Ramzy, H. H. Helmy, H. A. Farid, A. A. Ashour, G. J. Weil, and S. A. Williams. 2010. Detection of Wuchereria bancrofti L3 Larvae in Mosquitoes: A Reverse Transcriptase PCR Assay Evaluating Infection and Infectivity. PLoS. Negl. Trop. Dis. 4(2): e602.

Ledesma, N., and L. Harrington. 2011. Mosquito vectors of dog heartworm in the United States: vector status and factors influencing transmission efficiency. Top. Companion Anim. Med. 26: 178-185.

Lee, R. M. K. W., and D. A. Craig. 1983. Cibarial sensilla and armature in mosquito adults (Diptera: Culicidae). Can. J. Zoo. 61: 633-646.

Levine, R. S., A. T. Peterson, and M. Q. Benedict. 2004. Distribution of Members of *Anopheles quadrimaculatus* Say s.l. (Diptera: Culicidae) and Implications for Their Roles in Malaria Transmission in the United States. Journal of Medical Entomology, 41(4), 607-613.

Licitra, B., E. W. Chambers, R. Kelly, and T. R. Burkot. 2010. Detection of *Dirofilaria immitis* (Nematoda: Filarioidea) by polymerase chain reaction in *Aedes albopictus*, *Anopheles punctipennis*, and *Anopheles crucians* (Diptera: Culicidae) from GA, USA. J. Med. Entomol. 47: 634-638.

Lindblade, K. A., E. D. Walker, A. W. Onapa, J. Katungu, and M. L. Wilson. 2000. Land use change alters malaria transmission parameters by modifying temperature in a highland area of Uganda. Trop Med Int Health. 5(4):263–74.

Manfredi, M. T., A. Di Cerbo, and M. Genchi. 2007. Biology of filarial worms parasitizing dogs and cats, pp. 39–47 In C. Genchi, L. Rinaldi, and G. Cringoli (1), *Dirofilaria immitis* and *D. repens* in dog and cat and human infections. Mappe parassitologiche, vol. 8. Università degli Studi di Napoli Federico II, Naples, Italy.

May F. J., C. T. Davis, R. B. Tesh, and A. D. Barrett. 2011. Phylogeography of West Nile virus: from the cradle of evolution in Africa to Eurasia, Australia, and the Americas. J Virol. 85(6):2964–74.

Minkeu, F. N. and K. Vernick. 2018. A Systematic Review of the Natural Virome of Anopheles Mosquitoes. Viruses. 10(222).

Molaei, G., T. G. Andreadis, P. M. Armstrong, J. F. Anderson, and C. R. Vossbrinck. 2006. Host feeding patterns of Culex mosquitoes and West Nile virus transmission, northeastern United States. Emerg. Infect. Dis. 12(3): 468–474.

Molaei, G., T. G. Andreadis, P. M. Armstrong, R. Bueno Jr., J. A. Dennett, S. V. Real, C. Sargent, A. Bala, Y. Randle, H. Guzman, A. T. da Rosa, T. Wuithiranyagool, and R. B. Tesh. 2007. Host Feeding Pattern of Culex quinquefasciatus (Diptera: Culicidae) and Its Role in Transmission of West Nile Virus in Harris County, Texas. Am. J. Trop. Med. Hyg. 77(1): 73–81.

Mullen, G. R., and L. A. Durden. 2009. Mosquitoes (Culicidae), pp. 245-246. In G. R. Mullen and L. A. Durden (2), Medical and veterinary entomology. Elsevier.

Narang, S. K., P. E. Kaiser and J. A. Seawright. 1989. Identification of species D, a new member of the *Anopheles quadrimaculatus* complex: a biochemical key. J. Am. Mosq. Control Assoc. 5: 317-324.

Narang, S. K., J. A. Seawright and P. E. Kaiser. 1990. Evidence for microgeographic genetic subdivision of *Anopheles quadrimaculatus* species C. J. Am. Mosq. Control Assoc. 6: 179-187.

Nasci, R. S., H. M. Savage, D. J. White, J. R. Miller, B. C. Cropp, M. S. Godsey, A. J. Kerst, P. Bennett, K. Gottfried, and R. S. Lanciotti. 2001. West Nile virus in overwintering Culex mosquitoes, New York City, 2000. Emerg Infect Dis. 7(4):742-4. doi: 10.3201/eid0704.010426

Nash D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, et al. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med. 344(24):1807–14.

Nayar, J. K., and D. M. Sauerman. 1975. Physiological basis of host susceptibility of Florida mosquitoes to *Dirofilaria immitis*. J. Insect Physiol. 21: 1965-1975.

Newton, W. L. 1957. Experimental Transmission of the Dog Heartworm, *Dirofilaria immitis*, by *Anopheles quadrimaculatus*. J. Parasitol. 43(5): 589.

Reinert, J. F., P. E. Kaiser, and J. A. Seawright. 1997. Analysis of the *Anopheles (Anopheles) quadrimaculatus* complex of sibling species (Diptera: Culicidae) using morphological, cytological, molecular, genetic, biochemical, and ecological techniques in an integrated approach. Journal of the American Mosquito Control Association, 13, 1-102.

Reinert, J. F. 1999. Morphological abnormalities in species of the *quadrimaculatus* complex of *Anopheles* (Diptera: Culicidae). Journal of the American Mosquito Control Association. 15(1), 8-14.

Rutledge, C. R., J. F. Day, C. C. Lord, L. M. Stark, and W. J. Tabachnick. 2003. West Nile Virus Infection Rates in *Culex nigripalpus* (Diptera: Culicidae) Do Not Reflect Transmission Rates in Florida. J. Med. Entomol. 40(3): 253-258.

Sauerman, D.M., and J. K. Nayar. 1983. A survey for natural potential vectors of *Dirofilaria immitis* in Vero Beach, Florida. Mosq. News 43: 222-225.

Scoles, A. G., and S. Kambhampati. 1995. Polymerase chain reaction-based method for the detection of canine heartworm (Filarioidea: Onchocercidae) in mosquitoes (Diptera: Culicidae) and vertebrate hosts. J. Med. Entomol. 32.6: 864-869.

Sejvar, J. J., M. B. Haddad, B. C. Tierney, et al. 2003. Neurologic Manifestations and Outcome of West Nile Virus Infection. J Am Med Assoc. 290(4):511–515.

Silaghi, C., R. Beck, G. Capelli, F. Montarsi, and A. Mathis. 2017. Development of *Dirofilaria immitis* and *Dirofilaria repens* in *Aedes japonicus* and *Aedes geniculatus*. Parasit. Vectors. 10: 94.

Simon, F., M. Siles-Lucas, R. Morchon, J. Gonzalez-Miguel, I. Mellado, E. Carreton, and J. A. Montoya-Alonso. 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin. Microbiol. Rev. 25: 507-544.

Slaton, C. A. 2020. *Dirofilaria immitis* (Rhabditida: Onchocercidae) Infection in Mosquitoes (Diptera: Culicidae) from Urban and Rural Habitats in South GA and North Florida [Unpublished master's thesis]. Valdosta State University.

Smith, R., D. F. B. Murillo, K. Chenoweth, S. Barua, P. J. Kelly, L. Starkey, B. Blagburn, T. wood, and C. Wang. 2022. Nationwide molecular survey of *Dirofilaria immitis* and *Dirofilaria repens* in companion dogs and cats, United States of America. Parasites Vectors 15: 367.

Smithburn K. C., T. P. Hughes, A. W., and J. H. Paul. 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med. 20:471–92.

Sobotyk, C., N. Nguyen, V. Negrón, A. Varner, M. N. Saleh, C. Hilton, J. M. Tomeček, M. D. Esteve-Gasent, and G. G. Verocai. 2022. Detection of *Dirofilaria immitis* via integrated serological and molecular analyses in coyotes from Texas, United States. Int. J. Parasitol. Parasites Wildl. 18: 20-24.

Soverow, J. E., G. A. Wellenius, D. N. Fisman, and M. A. Mittleman. 2009. Infectious Disease in a Warming World: How Weather Influenced West Nile Virus in the United States (2001–2005). Environmental Health Perspective. 117(7).

Spielman, A. 2001. Structure and Seasonality of Nearctic *Culex pipiens* Populations. Annals of the New York Academy of Sciences, 951: 220-234.

Stockwell, D. R. B. and D. Peters. 1999. The GARP modelling system: problems and solutions to automated spatial prediction. Int. J. Geographical Information Science. 13(2):143-158.

Suen, W.W., N. A Prow, R. A. Hall, and H. Bielefeldt-Ohmann. 2014. Mechanism of West Nile Virus Neuroinvasion: A Critical Appraisal. Viruses. 6: 2796-2825.

Takeda, T., C. A. Whitehouse, M. Brewer, A. D. Gettman, and T. N. Mather. 2003. Arbovirus surveillance in Rhode Island: assessing potential ecologic and climatic correlates. Journal of the American Mosquito Control Association. 19(3):179-189.

Thompson, S. J. 2016. Determination of a Gene Expressed only in the Infective Larval Stage of *Dirofilaria immitis* [Unpublished master's thesis]. Valdosta State University.

Tsai T. F., F. Popovici, C. Cernescu, G. L. Campbell, and N. I. Nedelcu. 1999. West Nile encephalitis epidemic in south- eastern Romania. Lancet. 352(9130):767–71.

USDA National Agricultural Statistics Service Cropland Data Layer. 2012. Published crop-specific data layer [Online]. Available at http://nassgeodata.gmu.edu/CropScape/. USDA-NASS, Washington, DC.

Vezzani, D., M. Mesplet, D. F. Eiras, M. F. Fontanarrosa, and L. Schnittger. 2011. PCR detection of *Dirofilaria immitis* in *Aedes aegypti* and *Culex pipiens* from urban temperate Argentina. Parasitol. Res.108: 985-989.

Watts, K. J., G. R. Reddy, R. A. Holmes, J. B. Lok, D. J. Knight, G. Smith, and C. H. Courtney. 2001. Seasonal prevalence of third-stage larvae of *Dirofilaria immitis* in mosquitoes from Florida and Louisiana. J. Parasitol. 87: 322.

Weitzel, T., P. Jawień, K. Rydzanicz, E. Lonc, and N. Becker. 2014. *Culex pipiens s.l.* and *Culex torrentium* (Culicidae) in Wrocław area (Poland): occurrence and breeding site preferences of mosquito vectors. Parasitol Res. 114:289-295.

West, R. H. 2017. Identification of the distribution and species composition of the *Anopheles crucians* cryptic species complex in Lowndes County, GA, USA, and the prevalence of *Dirofilaria immitis* within the *An. crucians* complex in Lowndes County, GA, USA. M.S. thesis, Valdosta State University, Valdosta.

White, G. B. 1974. Anopheles gambiae complex and disease transmission in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene. 68(4): 278–298.

Appendix A: Figures for Chapters 1-4

Figure 1. Gravid trap set at a site in Lowndes Co.

Figure 2. CDC miniature light trap set at a site in Lowndes Co.

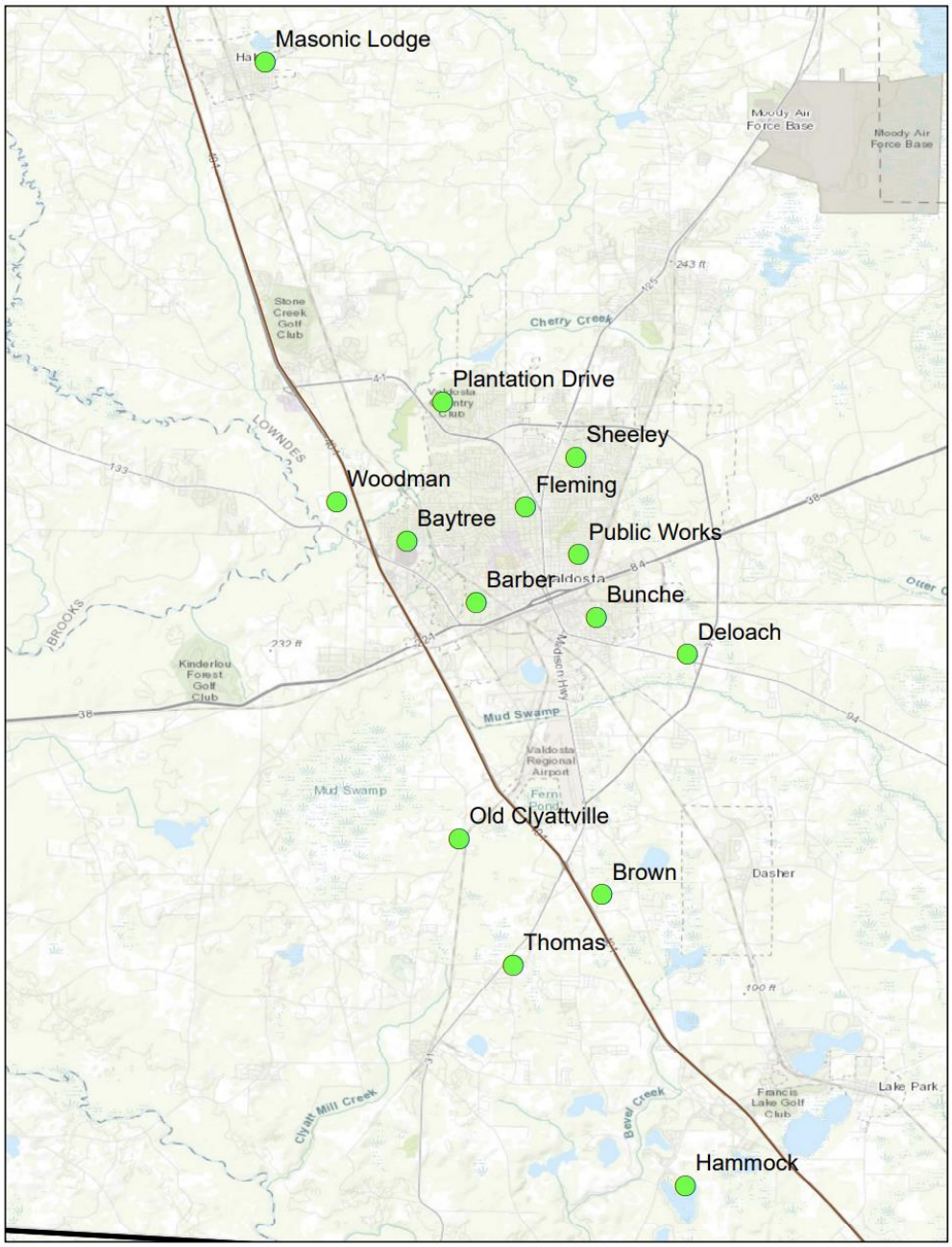

Figure 3. Trap site locations in Lowndes Co., GA.

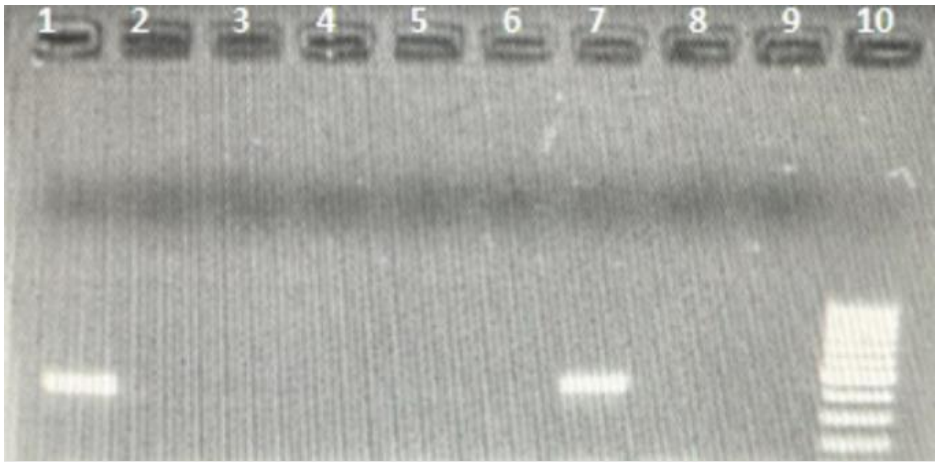

Figure 4. Agarose gel of individual *Anopheles quadrimaculatus s.l.* after standard PCR amplification with *Dirofilaria immitis* primers, Di_16S_RNA-F and DI_16S_RNA-R. Lane 1: positive control (*Aedes aegypti* 5 days post infection?); Lane 2-5 and Lanes 8-9: *An. quadrimaculatus s.l.* individuals negative for *D. immitis*; Lane 7: *An. quadrimaculatus s.l.* individual positive for *D. immitis*; Lane 10: 100-basepair molecular weight gene ladder.

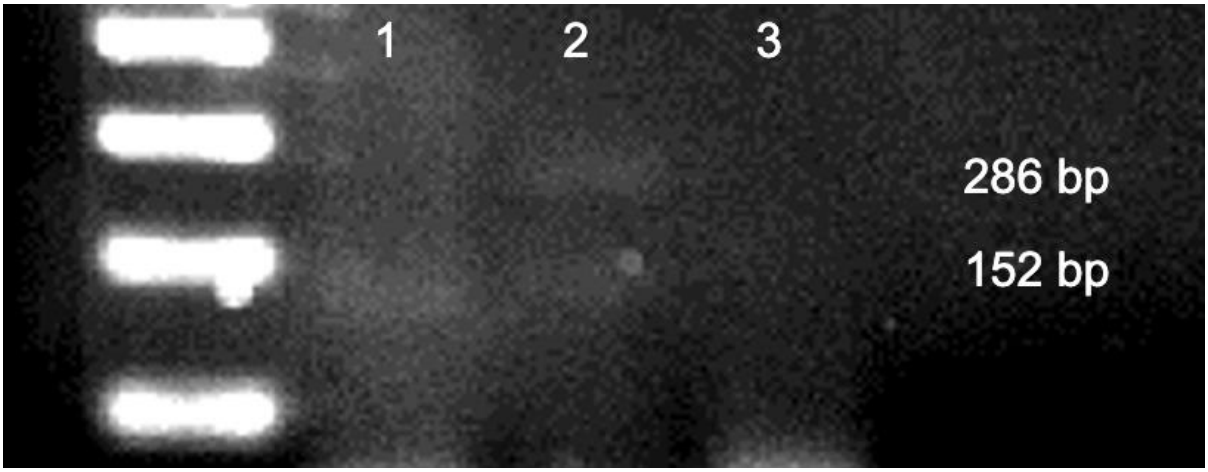

Figure 5. Agarose gel of pooled control *Aedes aegypti* mosquitoes after standard RT-PCR amplification using *Ditph* and CK855471A primers. Far left lane: 100-basepair molecular weight gene ladder; Lane 1: 2 days post *Dirofilaria immitis* infection *Ae. aegypti* mosquitoes; Lane 2: 11 days post *D. immitis* infection *Ae. aegypti* mosquitoes; Lane 3: uninfected *Ae. aegypti* mosquitoes.

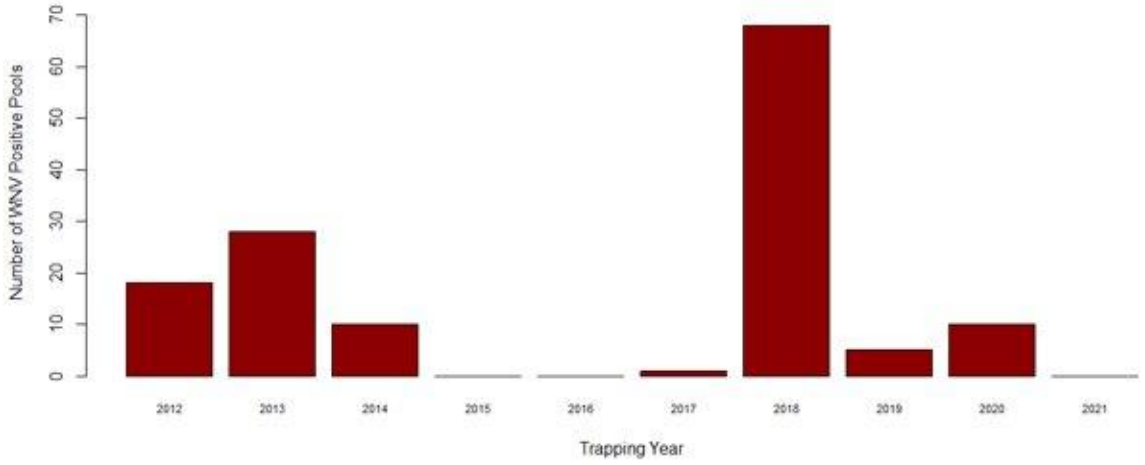

Figure 6. This bar chart shows the total number of positive WNV pools collected during each year. Pools of *Cx. quinquefasciatus*, *Cx. nigripalpus*, and *Cx. restuans* were included in this analysis. The most WNV positive pools were detected in 2018. No positive pools were detected in 2015, 2016, or 2021.

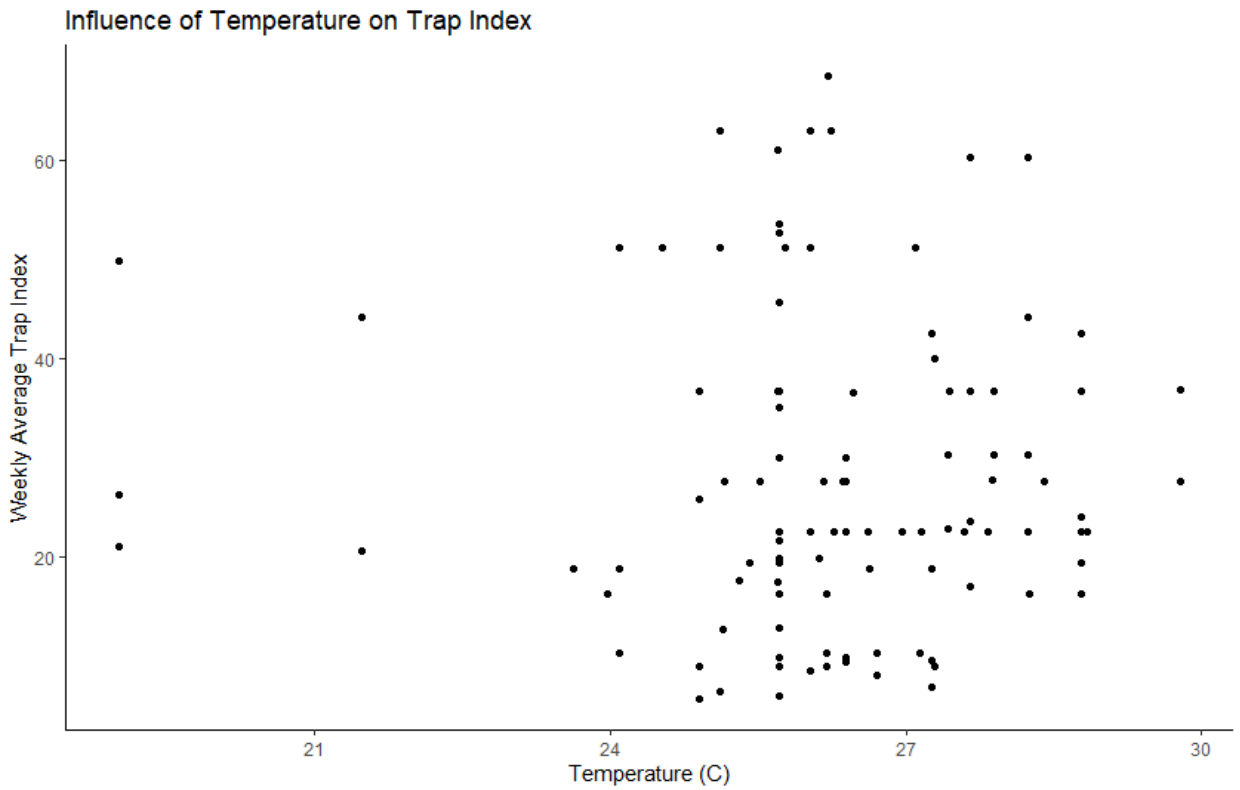

Figure 7. Relationship between weekly average temperature and weekly *Cx. quinquefasciatus* trap index of weeks WNV positive pools were collected. No linear relationship is present, but most temperatures fall above 24°C.

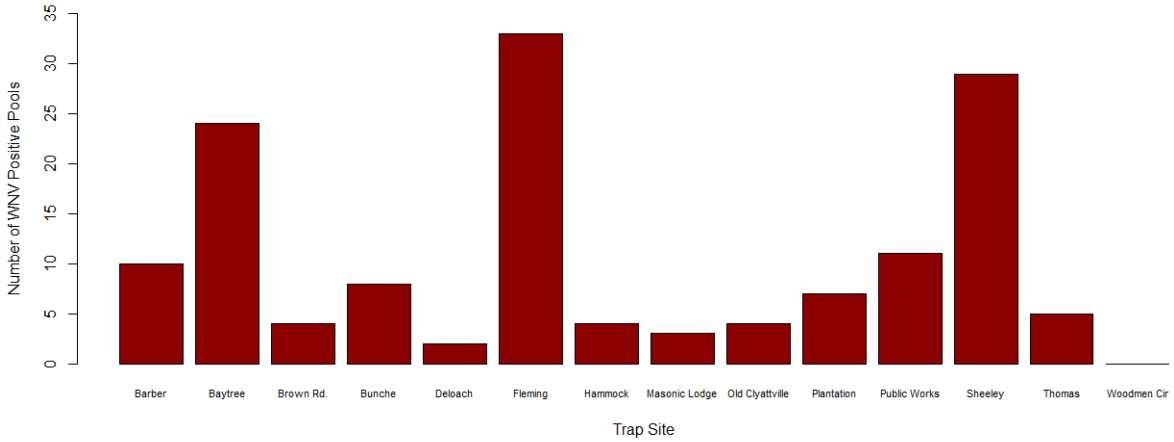

Figure 8. This bar chart shows the total number of positive WNV pools collected at the different trap sites from 2012-2021. Pools of *Cx. quinquefasciatus*, *Cx. nigripalpus*, and *Cx. restuans* were included in this analysis. The 3 sites with the most WNV positive pools were Fleming, Sheeley, and Baytree. The Woodmen trap site was the only site with 0 WNV positive pools.

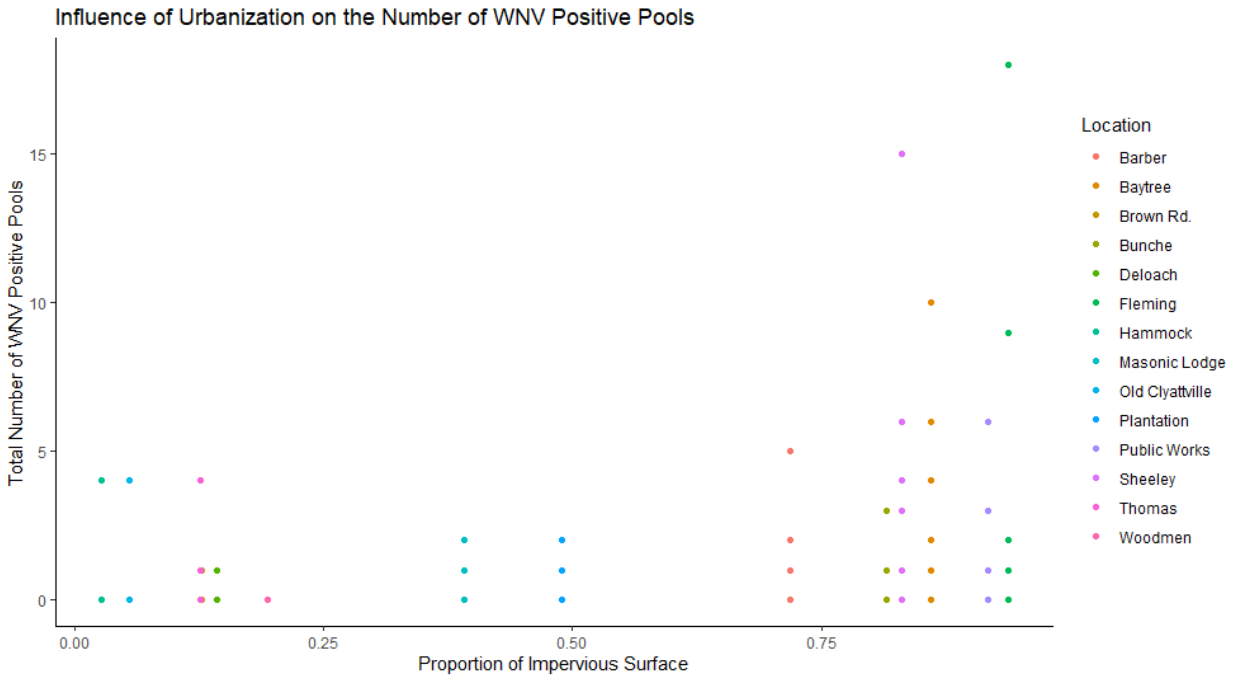

Figure 9. This scatterplot shows the relationship between the total number of WNV positive pools collected and the proportion of impervious surface (urbanization) found at each location. Locations seem to fall into three groups when looking at proportion of impervious surface. This plot shows that as the proportion of impervious surface increases, the total number of *Cx. quinquefasciatus* WNV positive pools collected increases as well. The coloration represents the different trap sites.

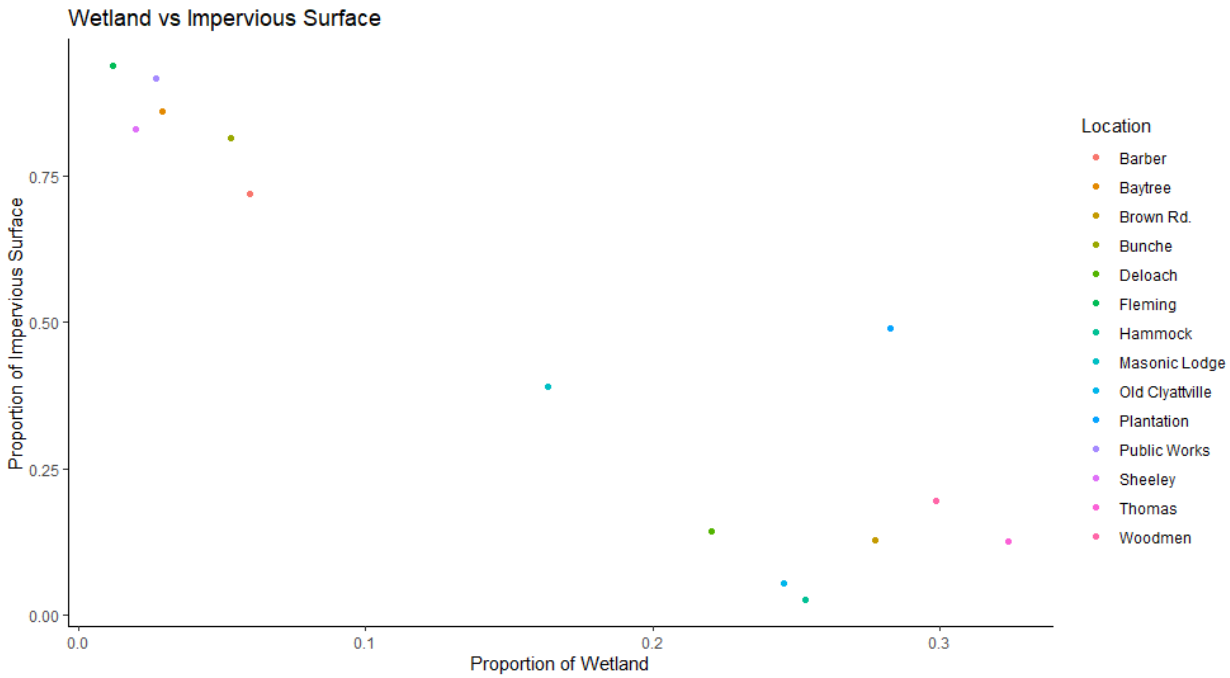

Figure 10. This scatterplot shows the linear relationship between the proportion of wetland habitat and the proportion of impervious surface at each trap site. As impervious surface increases, wetland habitat decreases. The coloration represents the different trap sites.

Appendix B: Tables for Chapters 1-4

Table 1: Nanodrop readings of *Aedes aegypti* RNA extractions.

Sample	260/280	260/230	Concentration (ng/ul)
X	2.14	0.73	148.6
2 d.p.i.	2.19	1.27	201.4
11 d.p.i.	2.06	0.79	140.6

Table 2: Number of WNV positive pools collected of each species. Total number of WNV positive pools collected each year and values other than 0 are bolded.

Species	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**
Cx. nigripalpus	0	0	0	0	0	**1**	**11**	**1**	**2**	0
Cx. quinquefasciatus	**18**	**28**	**10**	0	0	0	**57**	**3**	**8**	0
Cx. restuans	0	0	0	0	0	0	0	**1**	0	0
Total	**18**	**28**	**10**	**0**	**0**	**1**	**68**	**5**	**10**	**0**

Table 3: Trap indices of the species collected in the VSU Mosquito Surveillance Lab for each year. Trap indices of species that tested positive for WNV in this study are bolded.

Species	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Aedes albopictus	1.2	0.9	0.9	1.7	1.1	1.4	1	0.8	1.4	1
Ae. atlanticus	1.5	0	0	0.4	1.1	5.3	0.9	0.5	3.2	2.3
Ae. canadensis	5.9	0.2	0.2	0.4	0.3	0.8	0.2	0	1.3	5.1
Ae. cinereus	0	0	0	0	0	0	0	0	0	0
Ae. fulvus pallens	0.3	0	0	0	0.1	0	0.1	0.1	0.2	0.1
Ae. infirmatus	5.5	0.6	0.6	1	1.3	6.1	1.2	0.1	1.7	1.1
Ae. mitchellae	0	0	0	0	0	0	0	0	0.1	0
Ae. sticticus	2.7	0.9	1	0.1	0.1	0	0	0	0.2	0.2
Ae. triseriatus	0.1	0	0	0.1	0	0	0	0	0	0
Ae. unidentified	0.1	0	0	0	0	0	0.1	0.3	0.2	0.1
Ae. vexans	6.5	0.4	0.5	0.8	1.4	1.3	0.9	7.3	1.6	0.8
Anopheles crucians	12.3	6.9	7.2	7.9	9.6	4.6	8.1	0.2	10.7	8.8
An. punctipennis	0.2	0.1	0.1	0.2	0.3	0.2	0.1	0.3	0.6	0.3
An. quadrimaculatus	0.4	0.4	0.4	0.2	0.4	0.2	0.3	0	0.4	0.5
An. unidentified	0.1	0	0	0	0	0	0	0	0	0.1
Coquillettidia perturbans	3.7	3.2	3.3	2	2.9	1.9	4.2	1.4	2.6	4.4
Culiseta inornata	0	0	0	0	0	0	0	0	0	0
Cs. melanura	5.2	1.5	1.6	1.3	1.3	2.1	2.9	1.2	4.8	4.5
Cx. coronator	0	0	0	0.1	0.1	0.4	0.1	0.1	0.1	0.1
Cx. erraticus	2.9	1.4	1.4	1.2	1.9	1	1.2	1.5	1.7	1.2
Culex nigripalpus	**7**	**9.6**	**10**	**5.3**	**8.4**	**28.6**	**23.9**	**9.7**	**17.8**	**26.4**
Cx. quinquefasciatus	**22.4**	**11.7**	**12.2**	**11**	**14.9**	**11.4**	**13.5**	**6.8**	**10.6**	**7.1**
Cx. restuans	**0.7**	**2.9**	**3.1**	**1.2**	**1.7**	**0.3**	**2.8**	**1.4**	**3.2**	**0.8**
Cx. salinarius	4.5	4.2	4.3	4.5	1.7	4.4	7.6	2.6	4.6	3.9
Cx. territans	0	0.1	0.1	0	0.1	0.1	0	0	0.1	0.1
Cx. unidentified	5	3.5	3.6	3.5	4.2	1.6	4.7	1.9	5.1	4.4
Mansonia titillans	0	0	0.1	0.1	0.1	0.2	0.1	0.1	0.3	0.1
Orthopodomyia signifera	0	0	0	0	0	0	0	0	0	0
Psorophora ciliata	0.1	0	0	0	0	0.1	0.1	0	0	0
Ps. columbiae	2.9	0.1	0.1	3	1.3	0.6	1.5	0.2	0.1	2.9
Ps. cyanescens	0	0	0	0	0	0	0	0	0	0
Ps. ferox	0.7	0.2	0.2	0.4	0.6	0.9	0.6	0.3	1.3	0.8
Ps. howardii	0	0	0	0	0	0	0	0	0.1	0
Ps. mathesoni	0	0	0	0	0	0	0	0	0	0
Toxorhynchites rutilus	0	0	0	0	0	0	0	0	0	0
Uranotaenia lowii	0	0	0	0	0.1	0.1	0	0	0	0
Ur. sapphirina	0.1	0.1	0.1	0.3	0.2	0.2	0.1	0.2	0.2	0.1

Table 4: Weekly average temperatures and *Culex quinquefasciatus* trap indexes of weeks WNV positive pools were collected. Weeks with multiple positive pools are indicated by an asterisk.

Year	Week	Weekly Average Temperature (C)	Trap Index
2012	19	23.6	53.6
2012	23	25.5	61.1
2012	24	26.2	68.5
2012	25	26.4	40
2012	27	28.8	36.5
2012	30*	27.6	18.7
2012	31*	28.2	22.5
2012	33	27.9	26.3
2012	38	25.1	21
2012	41*	21.5	19.8
2012	41	21.5	19.8
2013	27*	25.7	30.3
2013	28*	26.7	44.1
2013	29	27.3	20.6
2013	30	26.2	23.5
2013	32*	28.8	36.7
2013	33*	27.4	21.7
2013	34*	26.2	30
2013	35*	27.1	60.3
2013	36	27.8	52.7
2013	37	27	45.6
2013	38	26.6	17
2013	39	24	35
2014	31	28.4	22.9
2014	32*	29.8	42.6
2014	33	27.1	27.7
2014	34	27.6	17.5
2014	35	26.2	24
2014	36	26.2	17.6
2014	37	25.8	12.6
2014	38	25.3	8.5
2018	23	25.2	36.8
2018	24*	24.1	51.2
2018	25*	27.3	63
2018	26	26.3	49.9

2018	27*	25.7	27.5
2018	28*	26.4	22.5
2018	29*	25.7	16.2
2018	30	26.1	9.3
2018	31*	24.9	8.9
2018	32	26.5	12.7
2018	33*	26	10.3
2018	34	26.6	8
2018	35*	25.1	10.2
2018	39	26.4	5.9
2019	17*	19	19.3
2020	25	24.5	9.5
2020	24	25.4	25.8
2020	29	28.3	6.3
2020	30*	27.9	9.8
2020	32	27.3	5.6
2020	35	28.5	6.9

Table 5: Number of WNV positive pools collected at each site. The total number of WNV positive pools collected at each site and values other than 0 are bolded.

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Cx. nigripalpus	**1**	0	**1**	0	0	0	**4**	0	**4**	0	**1**	**1**	**3**	0
Cx. quinquefasciatus	**8**	**23**	**1**	**8**	**1**	**32**	0	**3**	0	**7**	**9**	**28**	**2**	0
Cx. restuans	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0
Total	**9**	**23**	**2**	**8**	**1**	**32**	**4**	**3**	**4**	**7**	**11**	**29**	**5**	**0**

Key to sites: 1, Barber; 2, Baytree; 3, Brown; 4, Bunche; 5, Deloach; 6, Fleming; 7, Hammock; 8, Masonic Lodge; 9, Old Clyattville; 10, Plantation; 11, Public Works; 12, Sheeley; 13, Thomas; 14, Woodmen.

Table 6: Proportion of impervious surface and wetland at each site in the year 2012 with 1km buffer.

Location	Lattitude	Longitude	Imperious Surface	Wetland
Barber	30.82	-83.30	0.71	0.05
Baytree	30.84	-83.32	0.85	0.02
Bunche	30.82	-83.26	0.81	0.05
Deloach	30.81	-83.23	0.14	0.22
Fleming	30.85	-83.28	0.93	0.01
Hammock	30.65	-83.23	0.02	0.25
Masonic Lodge	30.99	-83.36	0.39	0.16
Old Clyattville	30.75	-83.30	0.05	0.24
Plantation Drive	30.88	-83.31	0.48	0.28
Public Works	30.84	-83.26	0.91	0.02
Sheeley	30.87	-83.27	0.82	0.01
Thomas	30.72	-83.28	0.12	0.32
Brown	30.74	-83.26	0.12	0.27
Woodman	30.85	-83.34	0.19	0.29