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Molecular and Biological Epidemiology of Mosquito Vecors for Canine Heartworm Infection in the Cumberland Gap Region

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MOLECULAR AND BIOLOGICAL EPIDEMIOLOGY OF MOSQUITO VECTORS FOR CANINE HEARTWORM INFECTION IN THE CUMBERLAND GAP REGION

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I am submitting herewith a thesis written by Michelle Norden entitled "*Molecular and Biological Epidemiology of Mosquito Vectors for Canine Heartworm Infection in the Cumberland Gap Region.*" I have examined the final electronic copy of this submission for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science in the program of Life Science Research conferred by Lincoln Memorial University.

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BIOGRAPHIC SKETCH OF THE AUTHOR

Michelle Norden was born in Miami, Florida, and raised in Tampa. She worked as a Lead veterinary technician for Banfield on and off for 9 years and a zookeeper at Lowry Park for 3 years. During that time, she was married and had 2 sons. She started working in lab animal medicine and was recruited into the lab of Huntington Potter at USF/Byrd Alzheimer's Institute in Tampa. Michelle maintained a vast transgenic mouse colony with over 15 transgenes and was responsible for the animal behavior and surgical experiments. In 2012 the lab was moved to Aurora, Colorado to start the Linda Crnic Institute for Down Syndrome along with the Rocky Mountain Alzheimer's Institute.

She pursued her undergraduate degree at the Metropolitan State University of Denver in Denver, Colorado where she obtained her Bachelor of Science degree in Biology and a minor in Chemistry and decided to move back into veterinary medicine and apply for a DVM.
In 2017, Michelle came to Lincoln Memorial University to pursue her Master of Science degree in Veterinary Biomedical Science and transferred to the thesis program. She started in Dr. Charles Faulkner's parasitology laboratory in 2018 to begin work on her master's thesis project.

Now, Michelle is headed to Ross University School of Veterinary Medicine. She has professional interests in public health, providing biomedical research support to investigators, research staff, and lab animal medicine.

Outside of school, Michelle enjoys scuba diving in various areas of archeological interest and treasure hunting.

DEDICATION

To my father Doug Norden and stepmother Claudia Matthews, who taught me it's never too late to follow your dreams. Thank you for your unwavering support.

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ABSTRACT

Canine heartworm (*Dirofilaria immitis*), is a nematode parasite that infects canines, both wild and domestic and other vertebrate hosts like ferrets, otters, and cats worldwide. The disease, dirofilariasis, can be fatal in advanced stages if untreated and is recognized as a significant concern in veterinary medicine. Canine heartworm has been reported in 49 states of the United States (U.S.) and is considered enzootic. The nematode lifecycle is dependent upon canines infected with adult worms that produce microfilaria, the vermiform embryonic stage, that circulate in the peripheral blood of the host and the mosquito vectors that ingest them taking a blood meal from the infected canine. The three-fold objective of this research was to:

1. Conduct a biological survey and inventory of the mosquito population in the Cumberland Gap Region (CGR) of southern Appalachia.
2. To determine the mosquito species infected with *Dirofilaria immitis* larvae
3. Estimate the prevalence of *D. immitis* in the mosquito population for its transmission to pet dogs in communities within the CGR.

Adult female questing and gravid mosquitos (n=2455) (representing 778 pools) were collected each year from May to September during 2017, 2018, and 2019 in an area encompassing the campus of Lincoln Memorial University in Harrogate, TN focused in Claiborne county. This was accomplished using CO2 baited CDC gravid and questing traps. Three collection zones were the focus of this research.

1. Collection area A had high residential density, with ~42 homes per km2. It had sparse forest cover and a relatively high number of free-roaming dogs observed during the study.
2. Collection area B had moderate residential density, with ~13 homes per km2 with dense forest cover and frequent free-roaming dogs were observed during the study.
3. Collection area C had the lowest residential density, with ~7 homes per km2 with a mix of dense forest cover proximal to a local dog park. This location has transient dog visitation and no free-roaming dogs were noted during the study.

Collected mosquitoes were euthanized by freezing overnight at -20°C and sorted into pools by species based on collection site and date. There were 2455 collected specimens representing 5 main species: *Culex pipiens* (n=521, pools=175), *Aedes albopictus* (n=460, pools=138), *Aedes japonicus* (n=417, pools=111), *Anopheles punctipennis* (n=186, pools=74) and A*edes vexans* (n=172, pools=62). Another species significant to this study but found less frequently was *Anopheles quadrimaculatus* (n=13, pools=7). *Culex pipiens* was the most prevalent mosquito species accounting for 24.05% of the species collected overall. Pools of sorted mosquitoes were assayed via PCR to detect the presence of *D. immitis* DNA. Five (0.57%) of 778 assayed pools were positive. The positive pooled species identified were: *An. quadrimaculatus* (2 pools), *Ae. albopictus*, *Ae japonicus*, and *Ae. vexans.* All these species are recognized as established vectors of CHW in Tennessee. The identification of *Ae. japonicus* in this study is the 1st report of its vector potential for transmission of CHW in Eastern Tennessee.

KEYWORD LIST

Heartworm, *Dirofilaria immitis*, DNA, prevalence, microfilaria, mosquito, diversity, vector, *Aedes albopictus*, *Aedes japonicus*, *Culex pipiens*, *Anopheles quadramaculatis*, *Aedes vexans*, heartworm development units, polymerase chain reaction (PCR)

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LIST OF ABBREVIATIONS

Ae	*Aedes*
AHS	American Heartworm Society
An	*Anopheles*
B	Blank
CAPC	Companion Animal Parasite Council
CDC	Centers for Disease Control
CGR	Cumberland Gap Region
CHW	Canine Heartworm
Coq	*Coquillettidia*
cm	Centimeter
Cu	*Culiseta*
Cx	*Culex*
CVM	College of Veterinary Medicine
D. immitis	*Dirofilaria immitis*
DNA	Deoxyribose Nucleic Acid
GNIS	Global Names Info System
GPS	Global Positioning System
HABRI	Human Animal Bond Research Initiative
HDU	Heartworm Development Unit
km	kilometer
L	Ladder
LMU	Lincoln Memorial University
m	meter
mL	milliliter
ng	nanogram
NOAA	National Oceanic and Atmospheric Administration
Or	*Orthopodomyia*
PCR	Polymerase Chain Reaction
Ps	*Psorophora*
Tox	*Toxorhynchites*
U.S.	*United States*
µL	microliter

CHAPTER 1

INTRODUCTION

The canine heartworm (CHW), *Dirofilaria immitis* (Filarioidea, Onchocercidae), is a mosquito transmitted filarial nematode that primarily affects domestic dogs (*Canis lupus familiaris*) and other wild Canidae world-wide. Infection with the parasite is comparatively more prevalent in geographic areas and regions areas where temperate or tropic climates support mosquito populations that facilitate transmission between hosts and completion of the lifecycle (AHS, 2010). CHW is endemic in the United States (U.S.) and can cause life-threatening cardio-pulmonary disease in dogs and cats that can be an economic burden to pet owners.

According to the Companion Animal Parasite Council (CAPC), the average cost of treating heartworm disease is approximately $1000 per case. Prevalence estimates derived from the CAPC published statistics indicate that approximately 1.8% of animals in Kentucky, Tennessee, and Virginia tested positive for heartworm infection in 2017 (https://capcvet.org/maps/#2017/all/heartworm-canine/dog/united-states). However, the prevalence of CHW in Bell County, Kentucky was closer to 7% based on examination of blood samples collected from dogs confined in the local animal shelter (Watlington, 2018). According the American Heartworm Society (AHS), reports of CHW infections are increasing every year despite pet owners having effective prophylaxis available for prevention (https://www.heartwormsociety.org/veterinary-resources/veterinary-education/ahs-board-speaks-out/368-ahs-survey-finds-increasein-heartworm-cases). There is interest in determining what factors are causing this trend.

The presence of female mosquito vectors with *D. immitis* larva is the number one factor in forecasting CHW risk in a region, but information at local levels is often limited (Brown 2012). Canine heartworm is under-reported due to a lack of data, or because of limited data from people seeking treatment for domestic canids after infection has occurred. Testing mosquito pools for the prevalence of *D. immitis* is a commonly used method to obtain an estimate of their vector potential and local heartworm prevalence.

Serologic examination of pet dogs from communities within the Cumberland Gap Region (CGR) carried out in conjunction with *Healthy Pet Healthy People* screening events funded by a grant from the Human Animal Bond Research Initiative (HABRI) to Lincoln Memorial University (LMU) failed to identify any CHW positive canines even though only 45% of pet caretakers were compliant with a veterinary approved heartworm prophylaxis protocol (Watlington, 2018). Most recently, a door-to-door neighborhood survey of households in the CGR community of Harrogate TN estimated the prevalence of CHW in pet dogs to be 2.4% with 39% of pet caretakers acknowledging non-compliance with heartworm prophylaxis measures (Cappiello, 2019). The discrepancy between the relatively high prevalence of CHW positive cases observed in Bell County shelter dogs and the low prevalence of CHW in companion dogs from CGR communities led us to investigate the prevalence of CHW in mosquito vectors and their role in local transmission.

This paper presents the results of a three-fold study designed to obtain information about the different species of mosquitoes and their vector potential for transmission of CHW in pet dogs of CGR communities. The objectives of this study are:

1. Conduct a biological survey and inventory of the mosquito population in the Cumberland Gap Region (CGR) of southern Appalachia.
2. To determine the mosquito species infected with *Dirofilaria immitis* larvae
3. Estimate the prevalence of *D. immitis* in the mosquito population for its transmission to pet dogs in communities within the CGR.

Selected trapping locations in Claiborne County, Tennessee were monitored from May-September 2017, 2018 and 2019. Little is known about the distribution of mosquito species in the CGR and this data is important for further research to better understand the environmental and host relationships that affect mosquito populations and their role as vectors for transmission of CHW in communities within the CGR. The majority of CAPC and AHS sponsored efforts to identify principal vector species and forecast CHW risk to pet dogs in the U.S. are based on relatively few collections and studies of mosquito populations (Ledesma and Harrington, 2011, 2015). This research contributes to that limited dataset to provide a better understanding of the vector potential of mosquito species involved in CHW transmission and may be helpful in efforts to reduce infections in pet dogs.

CHAPTER 2

LITERATURE REVIEW

Introduction

Dirofilaria immitis, a filarial nematode, is the causative agent of heartworm disease in pet dogs and endemic to Tennessee. This parasite has an obligate relationship with mosquitoes that feed on microfilaremic dogs. The disease is prevalent in areas of the world with temperate or tropic climates; consistently high temperatures allow year-round mosquito reproduction. *D. immitis* is transmitted by several Culicid mosquito species belonging to a wide range of genera, including *Culex*, *Aedes* and *Anopheles*, (Brown et al, 2012).

Although the biology of the parasite in its mosquito and canine host has been well researched since its initial description in 1856 (Leidy 1856; Kartman 1953), the relationship between *D. immitis* infected dogs and mosquito vectors and the environmental factors that sustain the host-parasite relationship in the U.S. has been studied less (Ledesma and Harrington, 2011). Comparatively few studies of mosquito vectors and heartworm prevalence have been undertaken at the community level despite their value for forecasting trends and predicting infection risk in canine populations (Paras et al 2014; Ledesma 2019; Brown et al 2012). This review of published literature is provided as foundational context for the research described in this thesis and to assist future research studies of mosquito populations and their role as vectors for transmission of canine heartworm in the southern Appalachia Region.

Mosquito biology

Of the more than 3500 different species of mosquitos known to biology, only a limited number appear to be important in the transmission of canine heartworm disease (Ledesma and

Harrington 2011). Female mosquitoes vary in feeding and breeding habits, travel abilities, and habitat preferences, and these differences affect their efficiency as heartworm intermediate hosts and vectors (McCall, 2019). More than 25 species of mosquitoes with infective heartworm larvae have been collected in field studies all over the world, and this number continues to rise. In the past several years, new species of mosquitoes known to transmit *D. immitis* have been discovered in the U. S. (McCall, 2019).

Tempelis (1975) described nine host-feeding patterns exhibited by mosquitoes based on their preference for a particular vertebrate host class or classes, the seasonal availability of preferred host and adaptability to utilize alternate host(s) via host-switching, and eco-regional vertebrate specialization. Accordingly, all mosquito species are susceptible to classification into ecological groups based on their predilection to feed:

1. almost exclusively on birds,
2. almost exclusively on mammals,
3. readily on birds and mammals,
4. almost exclusively on amphibians,
5. almost exclusively on reptiles,
6. exclusively on fish,
7. readily on all four classes of terrestrial vertebrates,
8. preferentially on birds in spring then shift to mammals seasonally, and
9. exclusively on birds in one geo-region and on mammals in a different region.

Given these feeding preferences, only female mosquitos that either feed exclusively or partly on mammals are relevant as potential vectors of CHW (Ledesma, 2019). For example, *Aedes albopictus*, also known as the Asian tiger mosquito, has spread throughout the eastern US and

breeds in containers and commonly inhabits areas near human dwellings (Barker, 2003). As an indiscriminate feeder, this species has become dominate in many communities to the extent it is displacing native species like *Culex* spp. throughout the U.S. South and Midwest (Spielman & D'Antonio, 2001). Moreover, male *Ae. albopictus* are able to breed with female mosquitos of other species and render them unable to oviposit (Spielman & D'Antonio, 2001). These behaviors, indiscriminate host feeding, daytime biting activity, and aggressive breeding have helped to make female Ae. albopictus a locally important vector for CHW throughout the U.S. wherever it occurs (Spielman & D'Antonio, 2001).

Other important CHW mosquito species include *Anopheles* spp., *Ae. japonicus*, and *Cx. pipiens* which can feed on hoofed ruminant mammals (Cervidae), cloven-hoofed ruminant vertebrates (Bovidae) and humans (Homo sapiens sapiens) (Burkett-Cadena, 2013). *An. quadrimaculatus* is a native species of Tennessee and can be found in permanent bodies of water that support emergent aquatic vegetation and is known for often being found inside homes, biting any time of day in a 24 hour period as they are not clock-regulated (Burkett-Cadena, 2013). *Aedes japonicus* is also known for being a diurnal feeder, and commonly oviposits in water-filled automobile tires and other man-made and natural containers (Burkett-Cadena, 2013). Many of the *Ae. japonicus* native range characteristics, i.e., use of diverse larval habitats, host feeding preferences, propensity for forested areas, and cold tolerance, are important components of its success as an invasive species and disease (Ledesma and Kaufman, 2015). *Ae. japonicus* is native to eastern Asia and was introduced to the U.S. in 1998 and continues to spread throughout the eastern U.S. (Kaufman and Fonseca 2014). Ward (2005) experimentally infected *Ae. japonicus* and demonstrated that it is an efficient vector of *D. immitis* (Sigheli et al., 2017). These data suggest that *Ae. japonicus* has potential to contribute to transmission of *D. immitis* in

mountainous habitats characterized by cooler average temperatures. *Culex pipiens* (the House Mosquito) is a primarily avian host feeder, but it readily feeds on mammals (Burkett-Cadena, 2013). It is considered an important vector for CHW in the U.S. (Huang, 2013). Because *D. immitis* is transmitted by such a broad range of mosquito species, all mammal biting mosquitoes could be candidate vectors (Ledesma, 2019). This supports the widespread observation that the key vectors of *D. immitis* are quite variable locally and regionally based on their distribution. (Ledesma, 2019).

Mosquito Vectors for Canine Heartworm

Over 60 species of mosquitoes have been shown to be competent vectors for transmission of CHW, and many of these are widespread in the U.S. (Lok et al, 1988). Ideal vectors are capable of feeding on infected hosts, becoming infected, accommodating the necessary life stages within the mosquito, and transmitting infectious heartworm to another host (Ledesma et al, 2011). If a species of mosquito is endemic to an area, it is likely that the species is a transmission vector for *D. immitis*. A study in Tennessee that trapped and tested mosquitoes for *D. immitis* detected its genomic DNA in 1.3% of sampled pools in eastern Tennessee and in 8% of pooled samples in western Tennessee (Fryxell et al, 2014). In eastern Tennessee, *Cx. nigripalpus*, *Cx. pipiens*, *Ae. vexans*, and an unknown species of *Culex* were identified as vectors for CHW. These species were also identified as vectors in the western Tennessee collections along with 2 additional species, *An. quadrimaculatus*, and *Ae. japonicus* (Fryxell et al 2014). Prior to the Fryxell et al (2014) study, the only documented vector for CHW in Tennessee was *Ae. trvittatus* based on recovery of infective larvae dissected from a single wild - caught mosquito (Hribar and Gerhardt, 1985).

Lifecycle biology of *Dirofilaria immitis*

Heartworm disease is associated with significant cardiovascular disease and may result in premature death. The complete life cycle of *D. immitis* in the mosquito consists of three developmental stages, L_1-L_3. The embryonic stage of the parasite, microfilaria, circulate in the peripheral of the infected canine host and are ingested by the female mosquito during her obligate bloodmeal to facilitate reproductive maturity and oviposition. Ingested microfilariae move into the Malpighian tubules where they undergo a molt to develop into the first larval stage (L_1) (Palmer & Whittrock, 1986). The L_1 migrate to the abdomen and molt into their second larval stage (L_2), following a short period of development the 3rd larval stage (L_3) is found in the head of the mosquito where they are positioned for transmission in the hemolymph via the proboscis (feeding tube) during a feeding activity. The mosquito feeding on a susceptible dog transfers the L_3 in hemolymph deposited onto the skin of the dog when the feeding tube is withdrawn at the completion of the bloodmeal (Montarsi, 2015). The L_3 enter through the wound created by the feeding mosquito, and development from L_3 to L_4 occurs in the musculature and submuscular layers of the dog approximately 3 to 8 days later. The developing larvae mature in the canine host and migrate to the pulmonary vasculature to become reproductively active adult worms. The entire lifecycle, from initial infection with the L_3 to detection of circulating microfilariae in the peripheral blood, takes 6 to 8 months. Adult heartworms reside primarily in the right side of the heart and pulmonary vasculature where they incite an inflammatory response that results in clinical disease (American Heartworm Society, 2017).

Transmission of *D. immitis* is dependent on infected female mosquitos competently hosting the infective L_3. Because *D. immitis* is transmitted by the bite of an infected mosquito, the simultaneous presence of both microfilaremic dogs and the appropriate vector species (a

mosquito that feeds on dogs) are required for transmission (Tzipory et al, 2010). The prevalence of heartworm disease in canids is dependent on the existence and distribution of competent vectors (Ledesma and Harrington 2011).

Development in the Mosquito and Environmental Constraints

For a mosquito to become infective and for *D. immitis* to reach the infective stage, an ambient temperature range of 18-34° C (64.4 - 93.2 F°) needs to be maintained for between 8 and 29 days (Watts, 1999). The ability of female mosquitoes to successfully transmit heartworm among the vertebrate populations is causally related to biological fitness. Biological fitness means the female mosquito had the ability to survive to reproductive age, find a mate and reproduce and in order to do this the female mosquito needs; steady temperatures above 14°C, water, and blood in order to oviposit. Water sources used by mosquitoes for oviposition and larval development vary from permanent structures like ponds, lakes and marshes to more transient water sources such as tree holes, puddles or manmade containers such as tires, pots and pools. Container breeding mosquitoes are especially affected by their environment and temperature due to the small size of their aquatic habitats (Alto, 2008). The Heartworm Development Unit (HDU) was developed to measure when the environment is most conducive to finding mosquitoes positive for *D. immitis* (Slocombe, 1989). The HDU describes a linear relationship between the time requited for maturation of the parasite larvae and the number of days above the 14° C threshold. As previously described, microfilariae ingested by a mosquito undergo a series of molts to become infective L_3 for transmission to the susceptible host and completion of the lifecycle. Microfilariae are able to develop into the infective L_3 stage in as little time as two weeks or may require as much as four weeks, depending on the warmth of the climate. However, this process may cease entirely at ambient temperatures below 14°C.

Estimating the timeframe required for development of the infective stage involves counting the degrees above 14°C that are reached each day. This is expressed in the following equation:

$$\sum Average\ daily\ temp\ \text{-}14°C = Accumulated\ HDUs$$

As demonstrated by Slocombe, (1989) and affirmed by Ledesma and Harrington (2015), the accumulation of 130 HDUs, or 234 HDUs measured on the F° scale, within a 30-day period facilitates the successful development of infective L_3 CHW in the mosquito host required for subsequent transmission and completion of the parasite lifecycle in the susceptible canine host.

Knowledge of the temporal and spatial occurrence of the primary mosquito species responsible for the transmission of *D. immitis* is essential to the success of decreasing the frequency of the disease in domestic pet dogs and limiting the socioeconomic impact for pet owners and the local community in the CGR. Distributional data of mosquito vectors can be used to map geographic areas and months throughout the season(s) with increased hazardous risk transmission (Brown et al, 2012).

The temperature dependent nature of heartworm development as predicted by the HDUs, supports the observation that transmission can likely occur year-round in selected geographical regions of the U.S. (Bowman et al, 2009). Although cooler temperatures associated with seasonal periodicity and local weather may be detrimental to survival, mosquitos are able to undergo brief periods of quiescence, where they can rest and survive until the return of warmer temperatures, and resume activity (Lok et al, 1998). In the mosquito, *D. immitis* larvae pause development when cooled and resume development once appropriate temperature has been reached (Lok et al, 1998). Moreover, cooling mosquitoes to 11.67°C (57 F°) does not affect the viability of L3 larvae after the temperature is increased (Ernst et al, 1983). Additional studies on the overwintering mosquito are needed to fully understand this effect. It is essential to consider the

preference of mosquito vectors for breeding sites and geographic areas when discussing the epidemiology of dog heartworm (Ledesma et al, 2011). In addition to a competent vector having access to a susceptible host, it is necessary to consider average temperature, rainfall, humidity, and the availability of viable breeding sites in a geographical area affect the development and successful transmission of *D. immitis* (Ledesma et al, 2011).

The annual pattern of temperature and precipitation for the CGR is described from data collected from the National Weather Service cooperative observer reporting station in Tazewell TN (Figure 2.1). Average monthly temperature ranges from 13.3 to 32°C (90-92°F) during the summer months between June and August (US Climate Data 2017, 2018). Lower temperatures occur during the winter months between December and February and range from 10 to 13°C (50-55.4°F) (US Climate Data 2017, 2018). The average rainfall in the area is approximately 5-13 centimeters with humidity ranging from 50-100%, highest during June and July (Figure 2.1). These data indicate transmission of CHW in the CGR is likely seasonal in nature and predicable based on the concept of HDU accumulation.

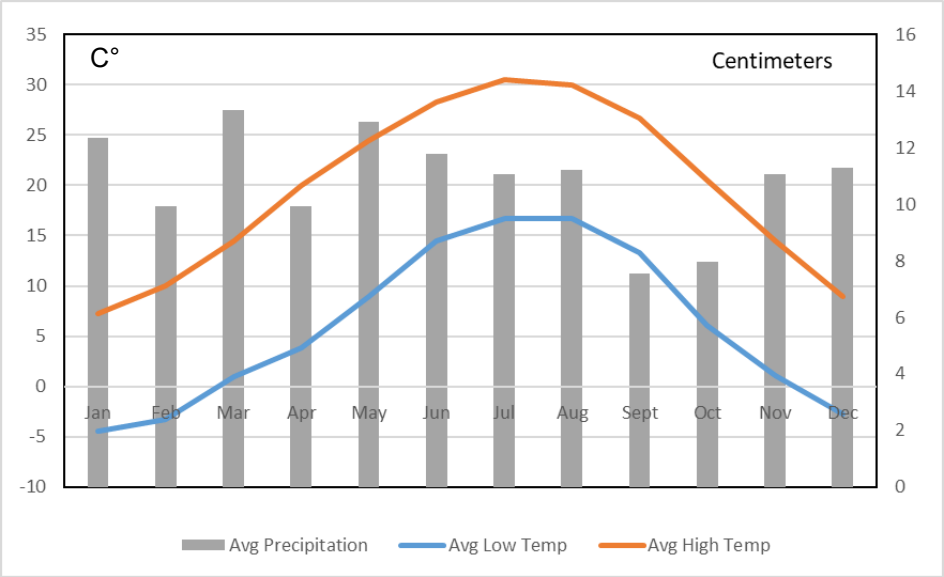

Figure 2.1. Average monthly high and low temperatures and monthly precipitation for Claiborne Co TN based on data collection from National Weather Service cooperative observer reporting station in Tazewell, TN. Data available online at https://www.usclimatedata.com/climate/tazewell/tennessee/united-states/ustn0498

It is well established that CHW has seasonal trends and develops above 26.7°C (80.1°F), and studies performed on mosquitoes found an increase in pools of mosquitoes positive for *D. immitis* during the summer months of June and July, when temperature and humidity are at an all-time high for the region (Fryxell et al, 2014; Bowman et al, 2009).

The higher temperatures that are predicted to accompany climate change may cause higher prevalence of *D. immitis* due to range expansion of vectors and faster parasite development with increasing temperatures (Genchi, 2009). Climate change could also lead to the introduction of invasive mosquito species such as *Ae. albopictus* which has been hypothesized to be important in the changing distribution patterns of CHW and its introduction into new habitats (Otranto, 2009). A study conducted in Italy confirmed a higher rate of infection in *Ae. albopicutus* compared with native *Cx. pipiens* and raised concerns over the role this species plays in newly developing CHW infection rates in previously nonendemic areas (Cancrini, 2007). *Ae. albopictus* in not native to North America, but was introduced in the mid-1980's. Since its introduction, this species has spread throughout the eastern half of the U.S. south of the Great Lakes (Burkett-Cadena, 2013). Another short-term effect of climate change could be increased mosquito population densities after prolonged periods of rainfall, which have been shown to be predictive of increased CHW prevalence (Ledesma, Harrington, 2011).

Community and Mosquito Diversity

Paras and colleagues (2014) conducted a community wide survey of mosquito populations in Stillwater, Oklahoma to evaluate the relationship between landscape and social factors on the prevalence of canine heartworm in the vector population. This research demonstrated that urbanization and habitat fragmentation are important predictors for the spread of mosquito borne diseases including CHW (Paras et al 2014). Moreover, urban areas supported

greater numbers of *Ae. albopictus* and a higher overall likelihood of *D. immitis* infection rates compared to non-urban areas (Paras et al 2014). Factors correlated with the community maintenance of mosquito populations, canine heartworm spread of epizootic mosquito-borne diseases, such as landscape (microenvironments such as pots, tree holes, etc.), population socioeconomic status, and the number of species of heartworm positive mosquitoes in the area.

A recent study in Florida examined two communities St. Augustine, Fl., and Lake City, Fl., both having active mosquito control programs and different socioeconomic characteristics with proximity to specific mosquito habitats (Ledesma, 2019). In St. Augustine, the community with a higher socioeconomic status, *Anopheline* species were mostly absent. Although the number of species represented in both collections were similar (27 in Lake City and 26 in St Augustine south) abundance was more evenly distributed among 6 species in Lake City in contrast to the predominance of *Ae. albopictus* in the St Augustine south collections *Ae. albopictus* and *An. quadrimaculatis* together represented equal parts of most of the collected species. These species depend on a variety of ecological niches such as woodland areas, lakes and temporary pools with submerged vegetation for successful breeding and larval development. The differences found between St Augustine South and Lake City in mosquito species distribution emphasizes the importance of evidence-based ecological approaches to vector research (Ledesma, 2019).

Mosquito trapping and identification are necessary to identify the pattern of transmission responsible for the annual incidence (new cases) and overall prevalence (all cases) of canine heartworm infection in the CGR. Understanding the geographic distribution of mosquito species, the environmental variables that account for their abundance, and how mosquito populations are connected across different landscapes in the CGR should help to identify areas that are likely to

have a higher risk of CHW transmission. Although the CGR is primarily rural, certain mosquitoes are more likely to be present in more populated residential areas due to container oviposition site availability (Brown, 2012).

Wild Canid Populations as Reservoirs for Infection

Otto (1974) suggested that heartworm in wild canids was more of a natural curiosity than a veterinary health issue and that wild canids were not a significant reservoir of infection for pet dogs (Weinmann, 1980). Although this may be true for red (*Vulpes vuples*) and grey fox (*Urocyon cinereoargentus*) whose infections are generally single sex with low intensity, and are typically amicrofilaremic, studies of CHW in coyote (*Canis latrans*) demonstrate their suitability as competent reservoir hosts. At a local level (Crawford, 1992) estimated that the population density for coyotes in the Smoky Mountains in the early 90's was one coyote in every $13k^2$ to $39k^2$. It is now known that wild canid populations play a role as microfilaremic reservoirs for sustaining *D. immitis* infected mosquitoes (Brown et al, 2012). A study in Arkansas examined coyotes and foxes and out of 193 coyotes, 127 (65.8%) were heartworm positive. Areas along the Mississippi River had the highest incidence of HW positive coyotes. This suggests that these coyotes are the primary reservoir for infection in northeast Arkansas (King, 1984). Another study in neighborhoods of northeastern Arkansas found that 73.7% of the mosquitoes tested in the vicinity of an HW positive dog were positive for *D. immitis*. This suggests that a single HW positive canid can potentially increase the likelihood of infections of susceptible animals in that canid's area (McKay, 2013).

In California, sentinel coyotes sampled at the county level in the Sierra-Nevada foothills had a wide range of prevalence (~25%), the highest in northern California counties. The most heartworm infected coyotes were in woodland areas. Researchers predicted that the dense

canopy provided a good environment for the primary vector (*Ochloerotatus sierrensis*) to rapidly reproduce and subsequently become infected with *D. immitis* (Sacks et al, 2004). .

A study of coyotes in South Carolina reported 40% of adult coyotes were positive for microfilaria (Miller et al, 2009). In central Georgia, 52% of coyotes tested were heartworm positive (Gates et al, 2014). In Tennessee, Van Den Bussche et. al, (1987) reported 38.5% statewide prevalence based on 267 coyotes sampled from 29 counties over 4 years. Faulkner and Donnell (2011) also found 37% of coyotes from Knox County, TN were microfilaremic hosts for *D. immitis*. This body of research suggests coyotes play a major role in the geographic spread of heartworm as they migrate to establish territorial ranges. Moreover, where they co-exist with pet dogs, coyotes can be an important source of microfilaria infecting mosquitoes and reservoir hosts.

Detection of *Dirofilaria immitis* in mosquitoes

Early detection and identification of mosquito-borne helminths, protozoa, bacteria, and viruses is essential for effective disease management globally and locally. Conventional methods to detect and identify pathogens have often relied on isolating the pathogen onto selective media or using biochemical/immunological analyses. Early efforts to survey mosquito vectors of canine heartworm infection were based on identification of larvae by filarial worms when mosquitoes were dissected in saline and examined microscopically for worms within 24-hours of collection (Buxton and Mullen, 1980; Hribar, 1985). These studies were fundamental to understanding the geographic dispersion of heartworm in mosquito populations, but they relied on time-consuming and labor-intensive lab techniques and on skilled taxonomical expertise and created an obstacle that served to discourage vector studies and few were conducted by only the most dedicated researchers. Moreover, questions regarding specificity of

results arose because mosquitoes potentially host other filarial species besides *D. immitis* and thus, its presence may have been overestimated (Scoles and Kambhampati, 1995). Molecular-based techniques were proposed to overcome many of the shortcomings of the conventional taxonomic assays. These molecular-based techniques utilize polymerase chain reaction (PCR) assays. PCR-based assays are better at identifying different parasites and are much faster than conventional techniques. Moreover, the techniques could also be applied on non-culturable microorganisms, as the organism does not need to be isolated to be identified by PCR. This means that mosquitoes could be identified and pooled into like-species and tested faster and more accurately for many pathogens. Scoles and Kambhampati (1995) designed a set of primers based on a surface antigen gene in *D. immitis*; the predicted product was a 378 base-pair DNA fragment. Experimental validation of the method with pools of 30 mosquitoes and whole blood from a microfilaremic dog demonstrated strong specificity of the primers to identify CHW. This technique is now routinely used for molecular identification of *D. immitis* in mosquito populations.

CHAPTER 3

EXPERIMENTAL METHODS

Mosquito Specimen Collection

Study Area

This study occurred in an approximately 31 square km area surrounding the Lincoln Memorial University campus located in Harrogate TN, USA (36.5823° N, 83.6569°W). The area is characterized by mixed residential development with agricultural clearing, and moderate to dense mixed hardwood-coniferous forest cover at its edges. The study area was subdivided into three general trapping areas defined by residential density and proximity to vegetation cover.

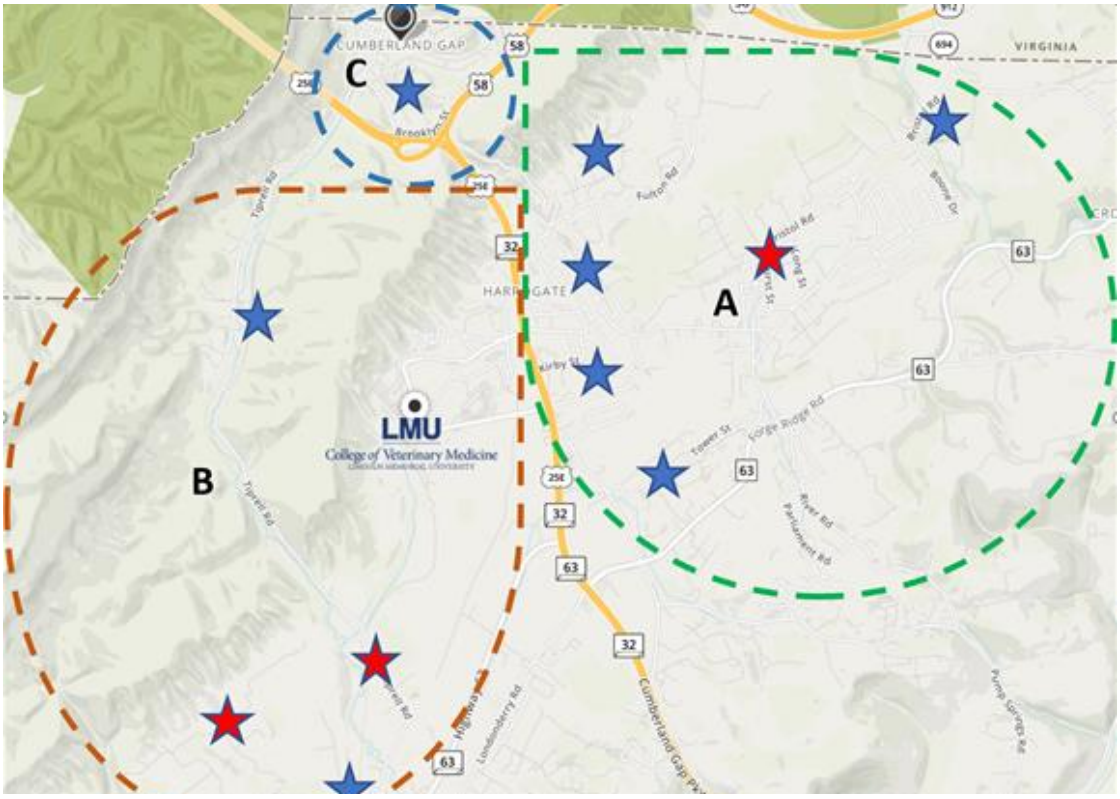

Figure 3.1: Map of the study area and proximity to Lincoln Memorial campus. Subdivided areas A, B, and C with blue stars denote mosquito collection sites from 2017 to 2019. Collection sites with red star provided at least 1 positive mosquito pool positive for DNA of *Dirofilaria immitis* by PCR assay.

These trapping areas are identifiable by the Claiborne County Property Tax Accessor's Office as the unincorporated community of Shawanee (A), the unincorporated communities of Tiprell, and Arthur (B), and the town of Cumberland Gap (C) (Figure 3.1). Trapping locations within each of these three areas were selected opportunistically, based on convenience and familiarity with cooperating household residents and landowners (Table 3.1). In all cases, trapping locations were selected for accessibility to neighborhood pet dogs and wild canid populations, specifically coyotes, known to frequent the edges of human residential development, agricultural clearing and vegetation cover.

Table 3.1. Mosquito collection sites and GPS coordinates identified with trapping areas A, B, and Claiborne County TN communities. Total number of "Trap Nights" for 2017, 2018, and 2019 are listed for each collection site.

Trapping Location	GPS Coordinates		Total Trapping nights		
			2017	2018	2019
Area A					
1	36.588738	-83.653686			14
2	36.581199	-83.639333		5	
3	36.569664	-83.645152	6	1	5
4	36.583551	-83.640403	8	3	7
5	36.585573	-83.653984		9	1
6	36.581672	-83.650203	5	2	
Area B					
1	36.553710	-83.686316		7	9
2	36.557562	-83.666962			2
3	36.580621	-83.679394		3	9
Area C					
1	36.595489	-83.666939		13	21
2	36.598301	-83.663270			1
Total			**19**	**43**	**69**

Trapping area A (Figure 3.2), located in the unincorporated community of Shawanee, is characterized as the densest residential area in which the trapping effort was conducted with approximately 42 houses per km^2 based on visual examination of satellite imagery available with

Google Maps (https://www.google.com/maps/place/Harrogate,+TN/@36.5720542,-83.6540543). Vegetation cover is sparse, and predominately composed of shrubbery associated with backyards. Backyards provide many opportunities for container breeding mosquitoes in parked

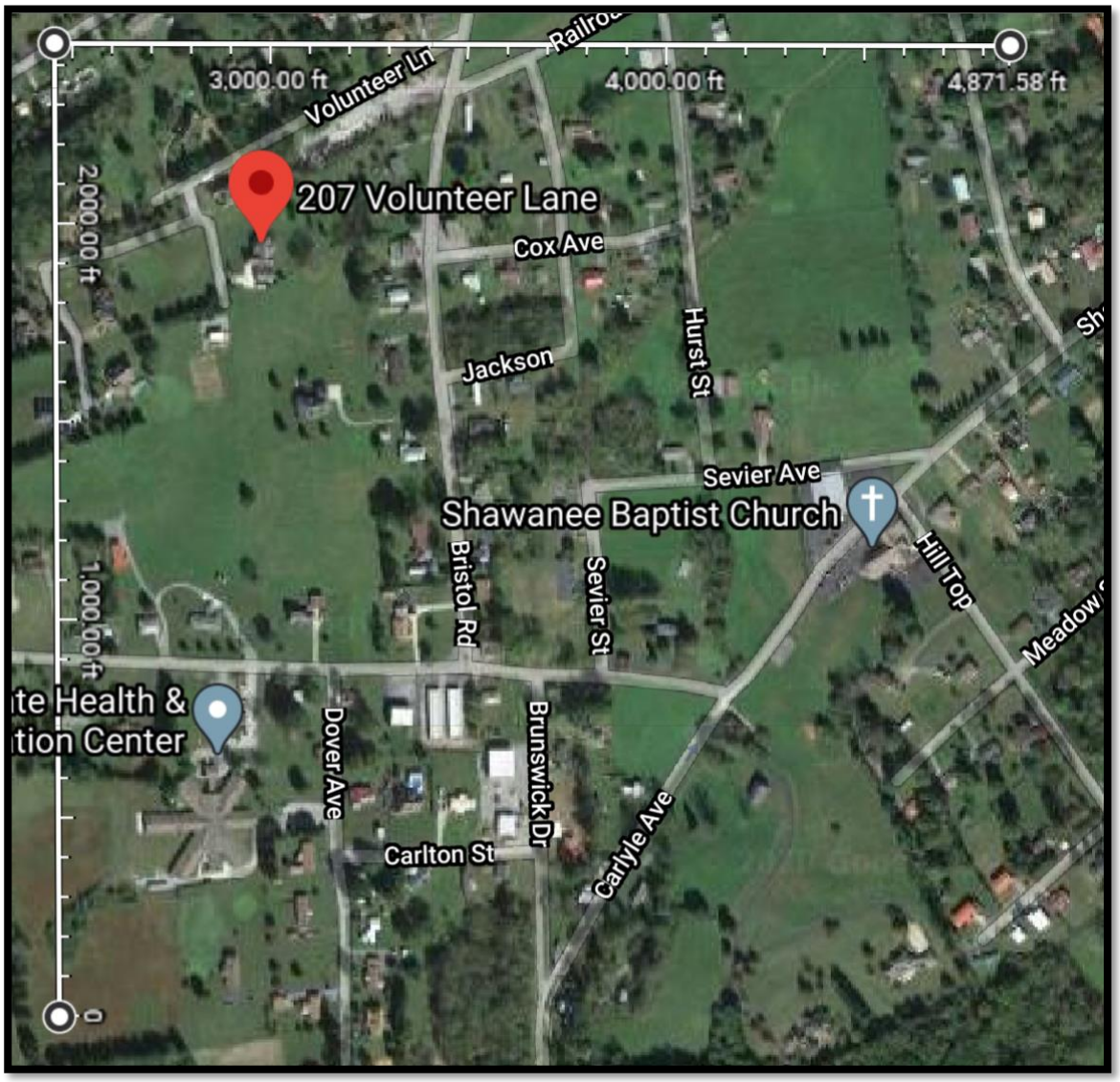

Figure 3.2. Satellite image of Trapping Area A in unincorporated community of Shawanee, Claiborne County, TN illustrating high residential density and sparse vegetation cover.

automobiles, empty flowerpots, buckets, and other accumulated items and debris. Free roaming pet dogs are numerous and were frequently encountered by members of the research team.

Mosquito collections were undertaken at six trapping locations for a total of 66 trapping nights (Table 3.1).

Trapping area B (Figure 3.3) was located east and southeast of the Lincoln Memorial University (LMU) campus. It was geographically separated from the campus by an elevated ridge with dense forest cover and several bodies of water. Area B includes the unincorporated

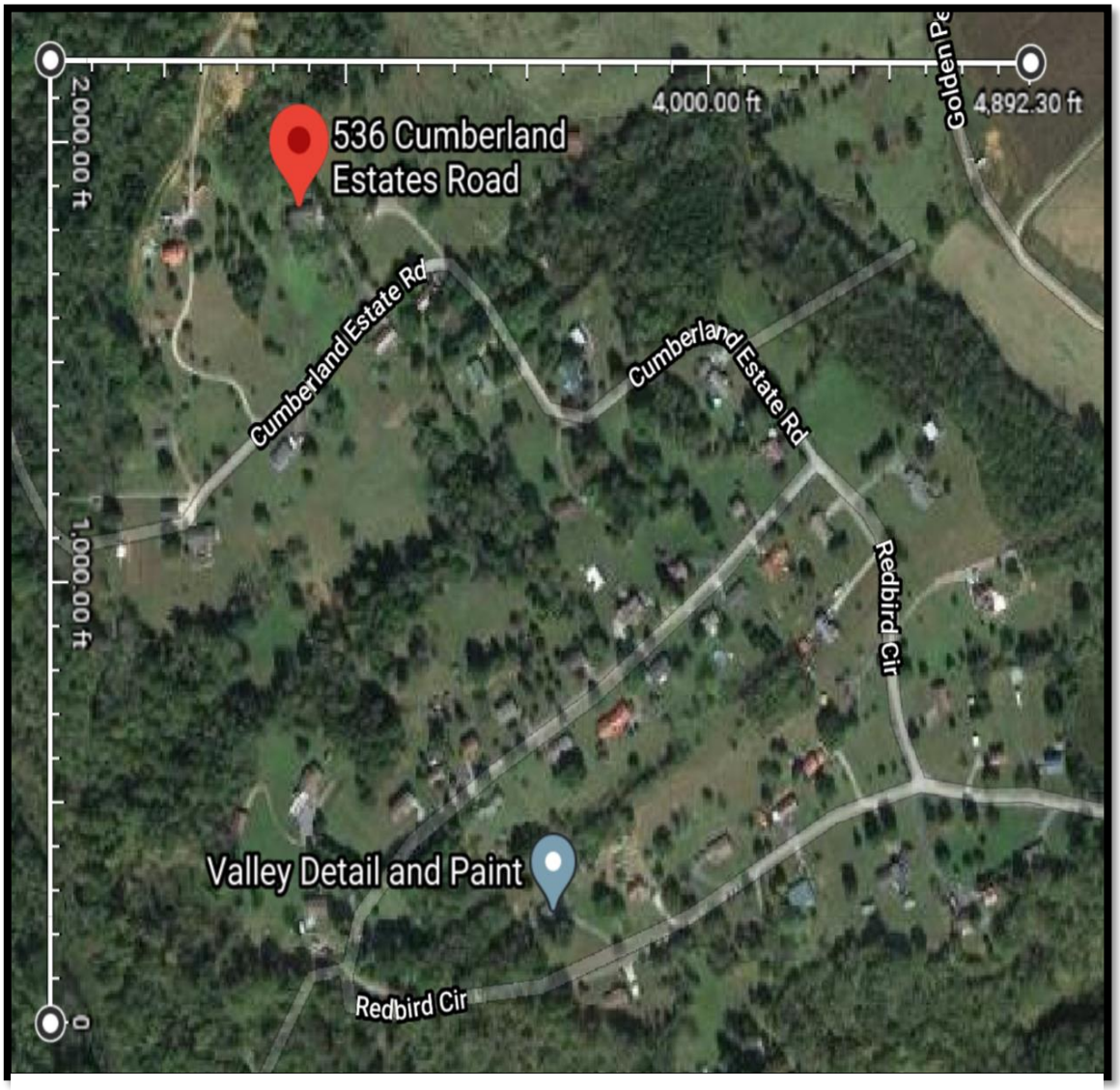

Figure 3.3. Satellite image of portion of Trapping Area B in unincorporated community of Arthur, Claiborne County, TN illustrating moderate residential density and proximity to surrounding dense vegetation cover.

communities of Tiprell and Arthur. Residential density in Tiprell and Arthur is approximately 13 houses per km^2 based on visual examination of satellite imagery available with Google Maps (https://www.google.com/maps/place/Harrogate,+TN/@36.5720542,-83.6540543). Yards are typically small with accumulated materials and debris that provide opportunities for water collection and mosquito breeding. Free ranging domestic dogs are frequent based on observations of the researcher and co-workers. The area is surrounded by dense forest cover and offers significant protection and opportunity for wild canids. Mosquito collection occurred at the three trapping locations for a total of 30 trapping nights (Table 3.1).

Trapping area C (Figure 3.4) located in the incorporated town of Cumberland Gap, TN, is characterized as having low to moderate residential density of approximately 7 homes per km^2.

Figure 3.4. Satellite image of portion of Trapping Area C showing location of Kaitlyn DeVries Memorial Dog Park and proximity to dense vegetation cover.

This location is surrounded by dense forest cover and numerous bodies of water providing many opportunities to support breeding mosquitos. Only two locations, a residential backyard, and a municipal dog park (Kaitlyn DeVries Memorial Dog Park) were the focus of trapping activity in area B. The dog park location is unique among the other collection sites used during the study because of its transient population frequented by pet caretakers and their pets from communities throughout the greater Tennessee, Kentucky, and Virginia area surrounding the Cumberland Gap. Mosquito collections were made at these locations for a total for 31 trapping nights. (Table 3.1).

Mosquito Collection

Adult host-seeking female mosquitoes were collected using CO2-baited CDC light traps and CDC gravid traps (John W. Hock Co.) from May until September in 2017, 2018, and 2019 for a total of 131 successful trapping nights. Traps were suspended approximately one meter above

Figure 3.5. CO_2 baited CDC light trap designed to collect questing (host-seeking) female mosquitos.

the ground surface at each location with an Igloo® cooler containing approximately 2 kg of dry ice, which was allowed to sublimate for 12 hours overnight (Figure 3.5). Collected mosquitos were recovered the following morning, transported to the laboratory and euthanized by freezing for 24 hours at -20°C. Euthanized mosquitos were sorted and separated into pools of like species containing 1-10 individual specimens. Species identification utilized the nomenclature of Burkett-Cadena (2013). Identified mosquito pools were stored frozen at -20° C until processing for molecular assay.

Molecular Methods

Experimentally infected mosquitoes

Experimentally infected and negative *Ae. aegypti* mosquitos were obtained from TRS Laboratories, Athens, GA., dissected, and used as controls to establish the validity of the assay to identify L3 CHW in the genomic DNA of mosquitos. The TRS lab infected mosquitoes, and frozen wild caught mosquito pools (of 1-10) were homogenized using the Qiagen TissueLyser LT at 40 Hz for 1-minute following the manufactures instructions. Genomic DNA was extracted with the DNeasy™ Blood and Tissue kit (Qiagen) using the general insect purification protocol described in the product insert. A negative extraction control was included every 24 samples. The DNA concentration of the sample was quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific) for single sample nucleic acid at 260/280nm following the manufacturer's protocol. Results were recorded graphically and numerically, to confirm successful extraction of DNA from the sample. A 2µl sample of extracted DNA was assayed by polymerase chain reaction (PCR) to amplify a 378-bp segment of a *Dirofilaria immitis*. PCR was used to detect *D. immitis* DNA in each of the mosquito pools with primers that amplify a tandemly repeated *D. immitis* surface antigen gene: F 5'-ACG TAT CTG AGC TGG CTC AC -

3’ and R 5’-ATG ATC ATT CCG CTT ACG CC-3’ (Scoles, 1995). Initial denaturation was performed at 95°C for 2 min, annealing temperature optimized at 60°C for 30s for 45 cycles with final extension, to clean up any partial fragments, at 72°C for 10 m. A positive control, the extraction negative controls, and a PCR negative control were included in each PCR. DNA extraction and PCR were conducted in separate dedicated areas to prevent contamination.

DreamTaq® Hot Start Green PCR Master Mix (Thermofisher) was used to prevent the amplification of non-specific products prior to the amplification step. PCR amplification was confirmed using a 1.5 % agarose gel with added SYBR® Safe DNA Gel Stain (Invitrogen) up to pool 760 and the remaining sample gels were stained and confirmed using GelGreen® (Biotium). Standard electrophoresis was conducted at 99V for 1 hour for full gels and 30 min for double run sample gels. PCR products were illuminated in the lab using the Safe Imager® 2.0 Blue-Light Transilluminator (Invitrogen) and bands were compared to the ladder to determine the size of the PCR product and subsequent success of the PCR. Results were recorded using a camera on an iPhone 7Plus (Figure 3.6). In some instances, PCR products were illuminated using the UVP ChemiDoc-It®2 510 Imager. Gel electrophoresis was conducted in a separate, dedicated area from PCR and DNA extraction to prevent contamination.

Genomic DNA Extraction from adult heartworms

Genomic DNA from a single *D. immitis* adult female worm collected from the right ventricle of a euthanized dog from the Bell County shelter was extracted using the DNeasy™ Blood and Tissue kit (Qiagen) to establish primer sensitivity and validity. The manufacturer’s protocol for *Caenorhabditis elegans*, a free-living nematode used as model organism in molecular research, was utilized with some modification for extraction of DNA for *D. immitis*. The worm was removed from the -20°C freezer and allowed to thaw for 5-10 minutes on ice.

The worm was cut into three pieces, each segment weighed (to ensure no sample over 25mg by weight per the manufacturer's recommendation) and placed in a 1.5mL microcentrifuge tube with the appropriate identifying data. Pieces of the worm were centrifuged for 30 seconds at 2500 rpm, and 180µL Buffer ATL and 20µL proteinase K was added to the tube and incubated at -80 overnight and three cycles of freeze thaws (-20°C)/thaw (37°C) were performed. A general protocol for blood and tissue was used to complete the extraction. The DNA concentration of the sample was then quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific) according to the manufacturer's protocol. The "nucleic acid" and "single sample" settings at 260nm/280nm were selected, and sample IDs manually entered. After the water sample step and blanking with buffer AE, 1 µL of sample was placed in the sample well and measured. PCR was performed using the primers listed above, and all samples were run on a 1.5% gel using Ultrapure™ Agarose. (Figure 3.6)

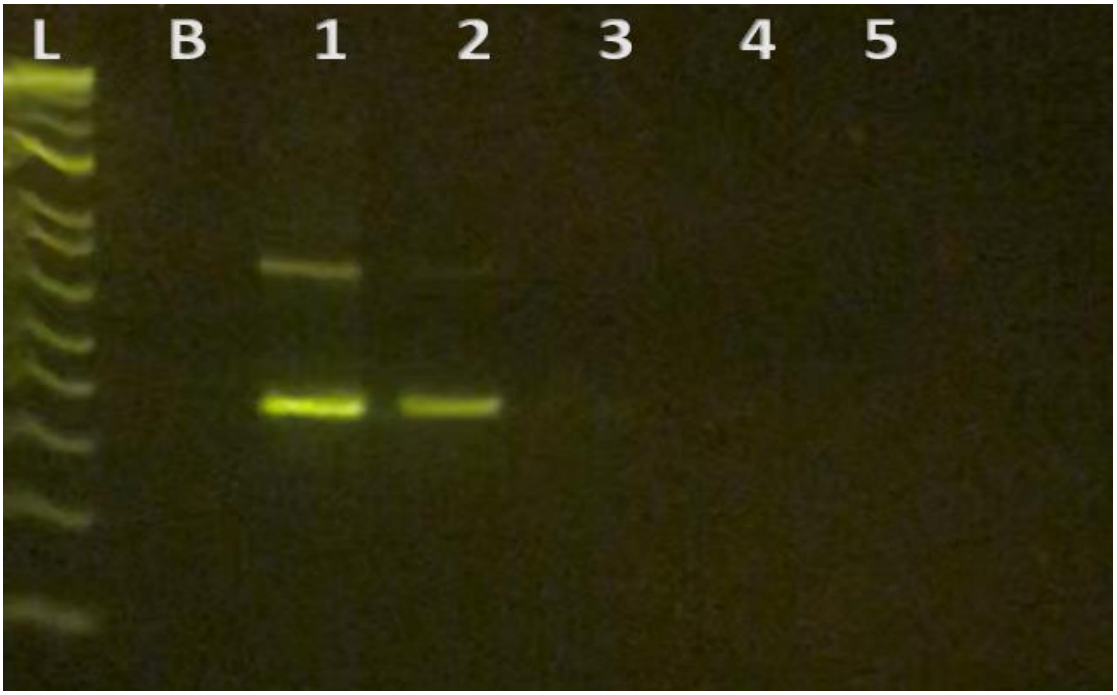

Figure 3.6. PCR gel of gradient for *Dirofilaria immitis* primers captured on an iPhone 7+. L=Ladder, B=blank, 1=Sample, 2-Sample 2, 3=Sample 3, 4= Sample 4, 5=Sample 5. Illuminated band in lanes 1 and 2 indicate 100,000 and 10, 000 DNA copies, respectively

Dilution assay of the samples confirmed that ~10,000 copies of *D. immitis* DNA was needed to acquire a positive result with optimal illumination of the 378 product (Table 3.2).

Table 3.2. Dilution assay of samples used in PCR gradient to identify 378 bp DNA fragment of *Dirofilaria immitis* with specified primers (Scoles, 1995)

Copy number ng/µL	100,000	10,000	1,000	100	10
Sample	1	2	3	4	5
H20	88.25µL	90µL	90 µL	90µL	90µL
DNA from stocks	11.75µL	<10µL	<10µL	<10µL	<10µL
Concentration ng/ µL	4.695	.4695	.04695	.004695	.0004695

Genomic DNA extraction from whole blood from microfilaremic dog

Approximately 5 mL of blood was taken from the jugular vein using a 22-gauge needle of a shelter dog in the care of LMU-CVM for clinical assessment. Blood was transferred to a treated EDTA tube and inverted to mix. Diagnostic assays were conducted with ZippTest Canine Heartworm Antigen tests (SafePath Laboratories, LLC, Carlsbad, California). Following the product protocol, it was determined the dog was heartworm positive. The positive result was confirmed by re-testing using another ZippTest. Additionally, a modified Knott's test was performed to visualize the presence of microfilaria (Knott 1939). The blood sample was stored at 4°C in the parasitology lab at Lincoln Memorial University (Harrogate TN) for further testing and analysis. The whole blood containing confirmed microfilaria from a CHW seropositive dog, at LMU for clinical assessment, was also assayed to additionally validate the PCR primers. The blood was extracted with a DNeasy™ Blood and Tissue kit (Qiagen) using the general recommended purification protocol to establish validity. The DNA

concentration of the sample was quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific) according to the manufacturer's protocol using the same setting previously listed. This was followed by polymerase chain reaction (PCR) amplification using the primers and gel electrophoresis confirmation previously listed.

DNA purification

The QIAquick Spin Kit (Qiagen) was used to purify PCR products of dNTPs and excess primers according to the manufacturer's protocol in preparation for DNA sequencing at a later date. The DNA concentration was then quantified using the NanoDrop 8000 Spectrophotometer, per the protocol outlined above. The purified PCR product was frozen -80 and will be sent to the University of Tennessee Genomics Core for sequencing prior to final publication.

CHAPTER 4

PREVALENCE OF CANINE HEARTWORM IN THE MOSQUITO POPULATION IN THE CUMBERLAND GAP REGION

Introduction

Environmental characteristics, such as temperature, precipitation, and aquatic communities that are natural and manmade, are well known to influence the presence and population size of adult mosquitoes. In this study, geographically separated populations of mosquitoes were trapped to confirm the presence of *D. immitis* and to determine primary mosquito species involved in transmission of CHW in the CGR.

Mosquito Collections

Adult host seeking mosquitoes (n=2455) mosquitoes collected from May to September annually between 2017 and 2019 were sorted into pools (n=778) and tested by PCR for the presence of *D. immitis* based on detection of the 378bp DNA fragment using the primers described in Chapter 3. The trapping effort demonstrated that the most prevalent species in the area were *Culex pipiens*, *Aedes albopictus*, and *Ae. japonicus*, *Anopheles punctipennis*, and *Ae. vexans* (Table 4.1). All of these species feed indiscriminately on humans, dogs, cats, and various wildlife hosts. All have been previously incriminated as competent vectors for transmission of *D. immitis* (Ledesma and Harrington 2011; Silaghi et al 2019).

Table 4.1. Mosquito species trapped and pools examined from Claiborne County locations in the Cumberland Gap study area during 2017, 2018, and 2019. Pooled mosquitos testing positive for *Dirofilaria immitis* DNA by PCR are indicated with "+".

Mosquito Species	***2017 specimens***	***2017 pools***	***PCR +-***	***2018 specimens***	***2018 pools***	***PCR +-***	***2019 specimens***	***2019 pools***	***PCR +-***	***Total specimens***
Aedes										
Ae. albopictus	201	40		165	46	+	94	52		460
Ae. cantator							1	1		1
Ae. hendersoni				1	1		2	2		3
Ae. japonicus	169	30	+	175	43		74	38		417
Ae. sticticus							1	1		1
Ae. triseriatus	24	14		31	15		25	16		80
Ae. trivittatus	7	3		13	9		2	2		22
Ae. vexans	83	18		39	18		50	26	+	172
Anopheles										
An. crucians							1	1		1
An. punctipennis	30	10		54	19		102	45		186
An. quadrimaculatus	1	1		9	5	+	1	1	+	13
Coquillettidia										
Coq. perturbans							20	9		20
Culex										
Cx. pipiens	25	10		189	47		307	118		521
Cx. spp.	422	70		101	42		29	20		7
Culiseta.										
Cu. melanura							1	1		1
Orthopodomyia										
Or. signifera							1	1		1
Psorophora										
Ps. ferox	2	1		2	1					4
Toxorhynchites										
Tox. rutilis	1	1								1
Total	**965**	**198**	**1**	**779**	**246**	**2**	**711**	**334**	**2**	**2455**

Spatial distribution of collected mosquitoes

Mosquito collection efforts were focused on three broadly defined habitat types thought to be influential for structuring mosquito communities in the study area and representative of the Cumberland Gap region. The premise of this effort was that these trapping areas presented varied breeding habitats and conditions that supported different communities of mosquito species. Once identified, pooled mosquito species representative of these trapping areas could be evaluated for their vector potential as likely sources for CHW transmission to pet dogs in the study area.

Trapping Area A

This area was the highest residential area of dogs and people, providing the most opportunities for container breeding mosquitoes. The trapping effort in area A consisted of a total of 66 trapping nights between May and September each year from 2017-2019. This collection effort resulted in identification of 16 mosquito species (n=1671) summarized in Table 4.2. The mosquito community for this area was dominated by 4 mosquito species: *Ae. albopictus*, *Culex pipens*, *Ae. japonicus,* and *Ae. vexans* with combined prevalence values that accounted for 65% of the component community (Figure 4.1). These species are predominately container breeding mosquitos and commonly distributed around human habitations where they feed indiscriminately on humans, dogs, cats, and a variety of other mammals. All have been incriminated as competent vectors for transmission of CHW (Ledesma and Harrington 2011; Silaghi et al 2019).

Table 4.2. Mosquito species and percent representation collected in Trapping Area A during 2017, 2018, and 2019

Mosquito Species	***Specimens Collected***	***Percent Represented***
Aedes.		
Ae. albopictus	387	23.15
Ae. hendersoni	1	0.06
Ae. japonicus	286	17.11
Ae. triseriatus	59	3.53
Ae. trivittatus	17	1.02
Ae. vexans	113	6.76
Anopheles.		
An. punctipennis	47	2.81
An. quadrimaculatus	2	0.12
Coquillettidia.		
Coq. perturbans	2	0.12
Culex		
Cx. pipiens	293	17.52
Cx. spp.	460	27.51
Psorophora.		
Ps. ferox	4	0.24
Toxorhynchites		
Tox. rutilis	1	0.06
Total	**1672**	**100.00**

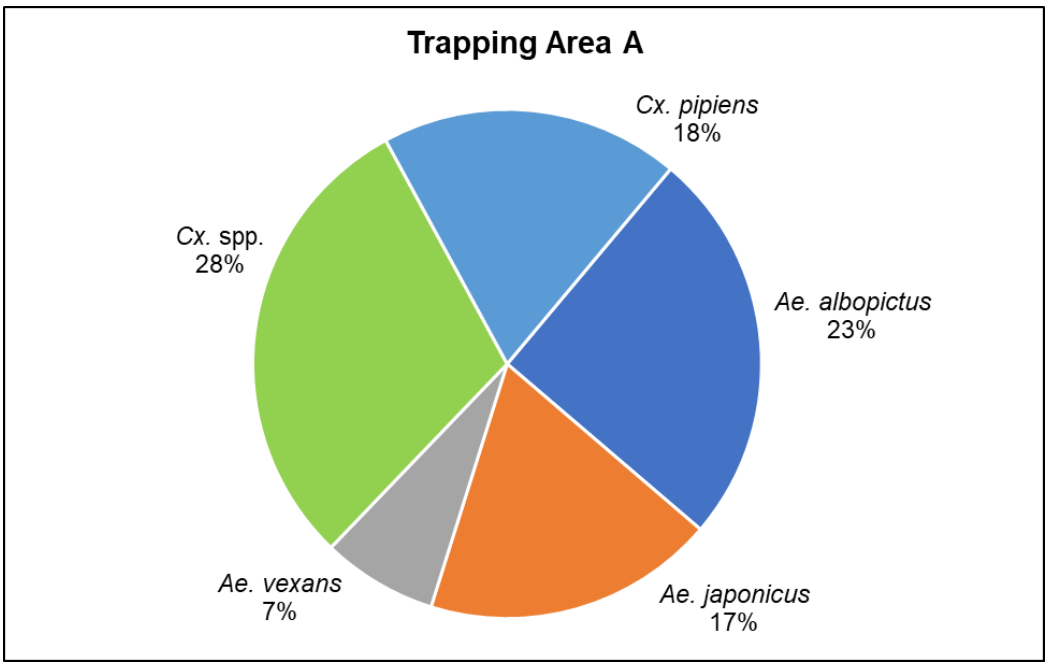

Figure 4.1. Principle mosquito species from Trapping Area A based on representation greater than 5% of collected total between 2017 and 2019,

Trapping Area B

Trapping area B was a moderately populated residential area with dense forest cover. The area is transected by Gap Creek which is flash-flood prone during episodic rainstorms and overflows its banks throughout the summer months to create damp grassy habitats conducive to mosquito breeding. Wet weather depressions that hold water throughout the area also provide breeding habitat for mosquitos. Homes in this location were spread farther apart with fewer free-roaming pet dogs based on casual observation. Given its proximity to dense forest cover and fewer backyard pet dogs, it appears well suited for coyote visitation. The trapping effort in Area B consisted of 30 trapping nights that resulted in collection of 527 individual mosquitos representing at least 13 different species (Table 4.3). The mosquito community for this area was dominated by 5 mosquito species: *Cx. pipens*, *An. punctipennis*,, *Ae. japonicus*, *Ae. vexans*, and *Ae. albopictus* with combined prevalence values that accounted for 87% of the component community (Figure 4.2). Although these species have been characterized as container breeding mosquitos, *An punctipennis*

and *Ae. vexans* are associated with wet weather habitats such as ephemeral floodwater pools and ditches (Burkett-Cadena 2013). *Ae. japonicus* is commonly known as the *Asian Rock pool* mosquito and successfully breeds where small quantities of water accumulate in the axils of trees and small depressions in rock beds (Kaufman and Fonseca 2014). *Cx. pipens* and *Ae. albopictus* are commonly distributed around human habitations where they feed indiscriminately on humans, dogs, cats, and a variety of other mammals. All have been incriminated as competent vectors for transmission of CHW (Ledesma and Harrington 2011; Silaghi et al 2019

Table 4.3. Mosquito species and percent representation collected in Trapping Area B during 2017, 2018, and 2019

Mosquito Species	***Specimens Collected***	***Percent Represented***
Aedes		
Ae. albopictus	35	6.64
Ae.cantator	1	0.19
Ae. hendersoni	1	0.06
Ae. japonicus	96	18.22
Ae. stictius	1	0.19
Ae. triseriatus	15	2.85
Ae. trivittatus	1	0.19
Ae. vexans	45	8.54
Anopheles		
An.crucians	1	0.19
An. punctipennis	91	17.27
An. quadrimaculatus	7	1.33
Coquillettidia		
Coq. perturbans	1	0.19
Culex		
Cx. pipiens	189	35.86
Cx. spp.	42	7.97
Orthopodomyia		
Or. signifera	1	0.19
Total	**527**	**100.00**

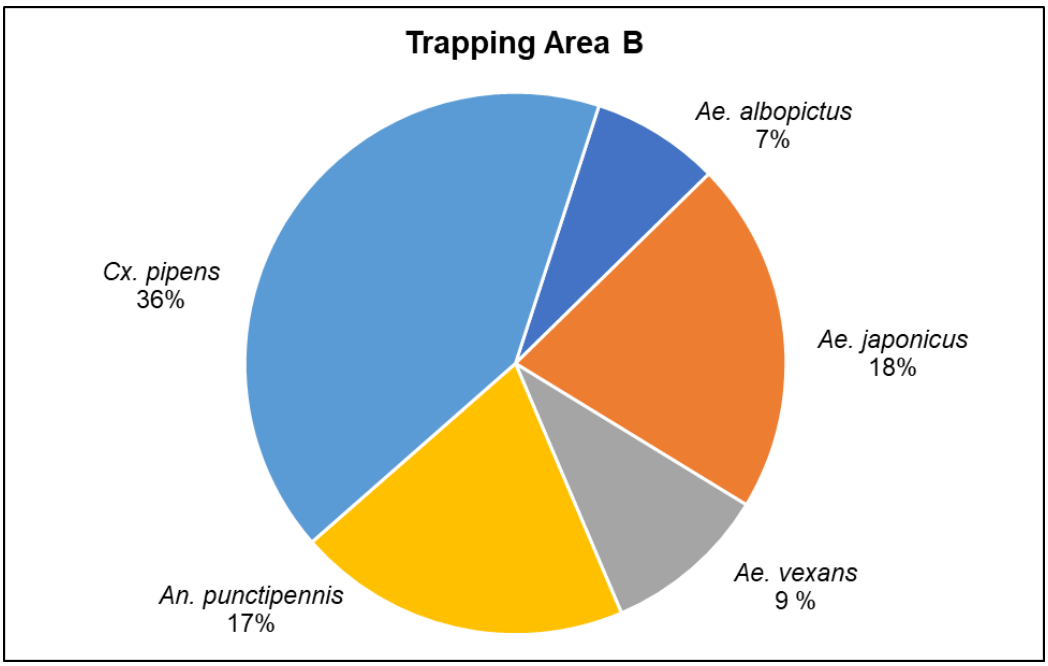

Figure 4.2. Principle mosquito species from Trapping Area B based on representation greater than 5% of collected total between 2017 and 2019.

Trapping area C

Trapping area C was a moderate to low density residential area that included a municipal dog park for the trapping location. This area is surrounded by dense forest cover and transected by Gap Creek. The town of Cumberland Gap is commercially developed and relatively free of accumulated debris adjacent to shops. Backyards of residences are small and confined by dense vegetation and forest cover. Although infrequent in comparison to other study area communities, discarded and unused flower pots, spare tires, and other material accumulation in the backyards of dwellings provide habitat opportunities for mosquito breeding. Free-roaming domestic dogs are prohibited by city ordinance. The municipal dog park is frequented by residents of Cumberland Gap and communities in the surrounding area of Claiborne, Bell, and Lee counties of Tennessee, Kentucky, and Virginia, respectively.

The trapping effort in Area C consisted of 35 trapping nights that resulted in collection of 256 individual mosquitos representing at least 12 different species (Table 4.3). The mosquito community for this area was dominated by 7 mosquito species: *An. punctipennis, Cx. pipens, Ae. japonicus, Ae. albopictus, Coq perturbans,* and *Ae. vexans,* with combined prevalence values that accounted for 75% of the component community (Figure 4.4). The occurrence of these species reflects the combination of natural and container breeding opportunities throughout the trapping area. *An. punctipennis, Coq perturbans,* and *Ae. vexans* represented 35% of the trapped mosquitos in the area and are likely associated with the breeding habitat facilitated by wet weather accumulation of rain runoff adjacent to the dog park. *Ae. japonicus* commonly known as the *Asian rock pool* or *Asian bush* mosquito and successfully breeds where small quantities of water accumulate in the axils of trees and small depressions in rock beds (Kaufman and Fonseca 2014). *Cx. pipens* and *Ae. albopictus* are commonly distributed around human habitations where they feed indiscriminately on humans, dogs, cats, and a variety of other mammals. All have been incriminated as competent vectors for transmission of CHW (Ledesma and Harrington 2011; Silaghi et al 2019).

Table 4.4. Mosquito species and percent representation collected in Trapping Area C during 2017, 2018, and 2019

Mosquito Species	***Specimens Collected***	***Percent Represented***
Aedes.		
Ae. albopictus	38	14.84
Ae. hendersoni	1	0.39
Ae. japonicus	36	14.06
Ae. triseriatus	6	2.34
Ae. trivittatus	4	1.56
Ae. vexans	14	5.46
Anopheles.		
An. punctipennis	48	18.75
An. quadrimaculatus	2	0.78
Coquillettidia.		
Coq. perturbans	17	6.64
Culex		
Cx. pipiens	39	15.23
Cx. spp.	50	19.53
Culiseta		
Cu.melanura	1	0.39
Total	**256**	**100.00**

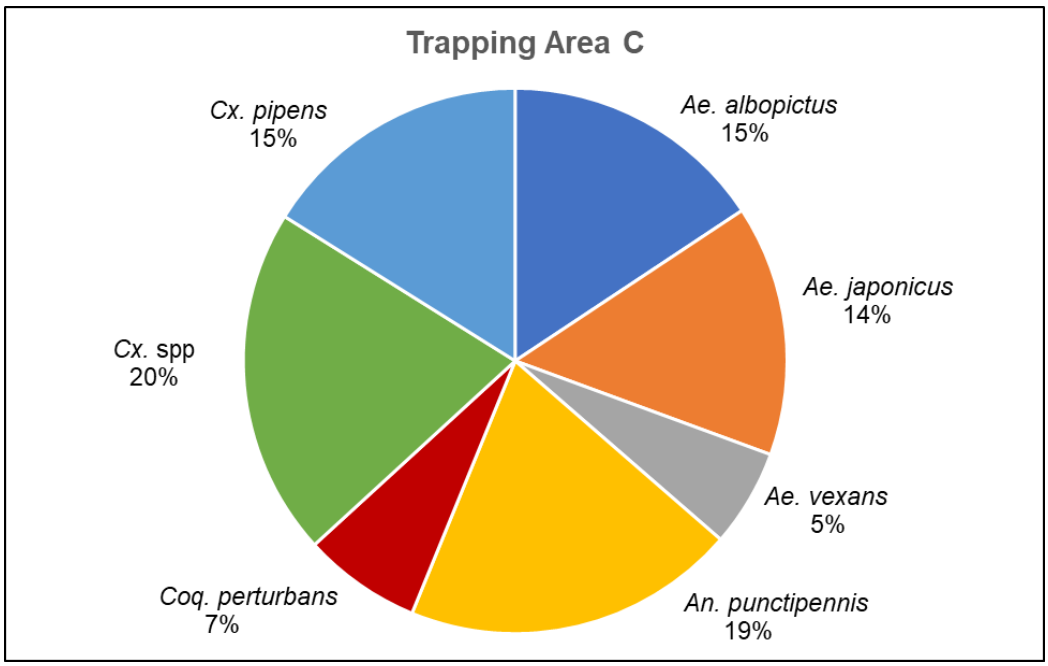

Figure 4.3. Principle mosquito species from Trapping Area C based on representation greater than 5% of collected total between 2017 and 2019.

PCR assay of wild caught mosquitoes

DNA from *D. immitis* larval stages was identified by the PCR assay described in Chapter 3 for 5 of the pooled species of mosquitos collected from traps throughout the study area (Table 4.5). CHW positivity was demonstrated by gel electrophoresis isolation and illumination of the 378bp fragment of DNA specific for *D. immitis* (Figure 4.5)

Table 4.5. Pooled mosquito species testing positive by PCR for *Dirofilaria immitis* DNA identified by pool number, collection date trapping area and calculated *Minimum Infection*

Pool number	Mosquitoes per pool	Date of collection	Area trapped	Mosquito Species	Minimum infection rate per 1000 mosquitoes
143	6	7/12/2017	A	*Aedes japonicus*	1.77
472	3	7/3/2018	B	*Anopheles quadrimaculatus*	158.8
566	1	7/19/2018	B	*Aedes albopictus*	1.97
830	3	7/7/2019	B	*Aedes vexans*	5.26
953	1	8/7/2019	B	*Anopheles quadrimaculatus*	158.8

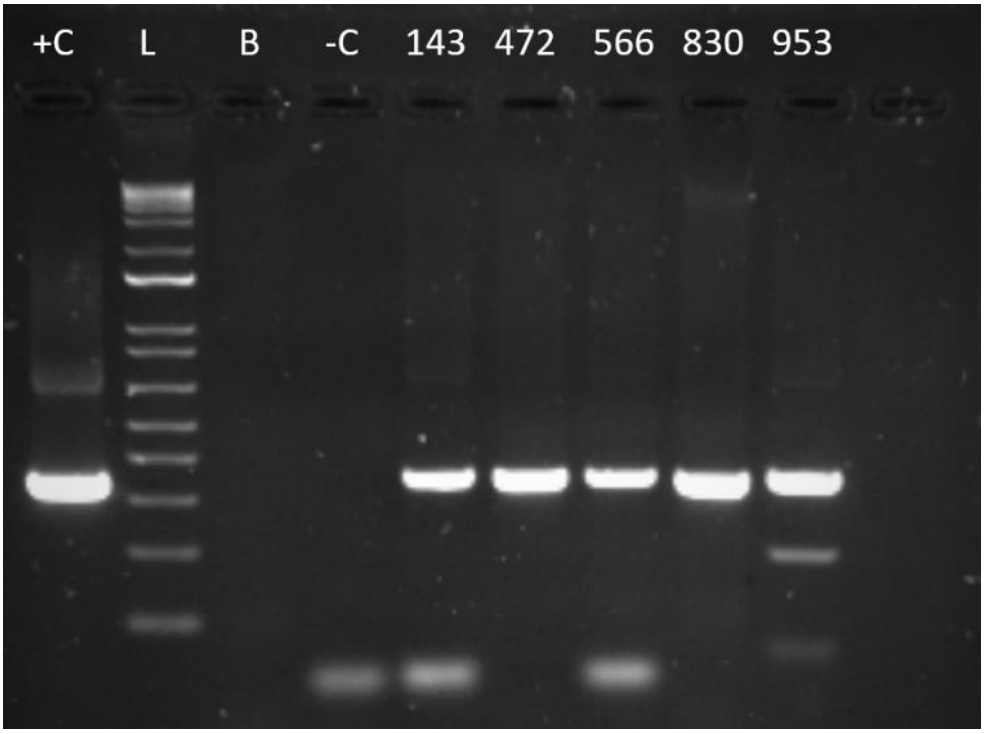

Figure 4.3. 1.5% agarose gel illuminating 378bp fragment of *Dirofilaria immitis* DNA amplified by PCR. PCR products illuminated from left to right showing positive control (+C), ladder (L), Blank (B), negative control (-C), positive pooled mosquito samples 143, 472, 566, 830, and 953. Image captured on the 510-imager.

The overall infection rate (IR) for the occurrence of *D. immitis* in the mosquito vector population based on the 5 positive mosquito pools was 0.57% (Figure 4. 5)

$$Infection\ Rate\ = \frac{\text{positive mosquito pools}\ (5)}{\text{pools of mosquitoes tested}\ (870)} = 0.57\%$$

Figure 4.5. Infection rate as a measure of the occurrence of *D. immitis* in pooled mosquitos collected from Claiborne County TN locations in the Cumberland Gap region.

Calculated minimum infection rates (MIR) were used to determine which of the 5 pooled species has the greater vector potential for transmission of CHW to canines in the study area

(Figure 4.6). These results indicated that *An quadrimaculatus*, (MIR 158.8) based on positivity for 2 tested pools has the greatest vector potential for CHW transmission among the 5 pooled species identified (Table 4.5)

$$Minimum\ Infection\ Rate = \frac{\text{positive mosquito pools}}{\text{total mosquitoes collected}} X1000$$

Figure 4.6. Minimum Infection Rate as a measure of the vector potential for transmission of *D. immitis* in pooled mosquito species collected from Claiborne County TN locations in the Cumberland Gap region.

Validation of the assay

The PCR assay was validated with purified DNA obtained from a morphologically identified *D. immitis* adult female worm collected from the right ventricle by necropsy of a canine euthanized at a local animal shelter. Whole blood containing microfilaria of *D. immitis* was also collected from a live canine by LMU clinical staff during routine physical examination to determine the animal's health status. The microfilariae were confirmed morphologically as *D. immitis* based on the criteria of Newton and Wright (1956, 1957) following concentration and microscopic examination of the blood sample using the technique of Knott (1939). Moreover, the dog's heartworm positive status was further confirmed serologically with a positive test result using a commercially available canine heartworm antigen detection assay (VETSCAN Heartworm Rapid Test®, Abaxsis, Inc). The illumination at the target 378bp location validated the specificity of the primer function for identification of *D. immitis* DNA in (1) experimentally infected mosquitos used as positive control, (2) whole blood from a *D. immitis* microfilaremic, serologically confirmed canine, and (3) purified DNA extracted from an adult female worm obtained post-mortem from an infected canine (Figure 4.9)

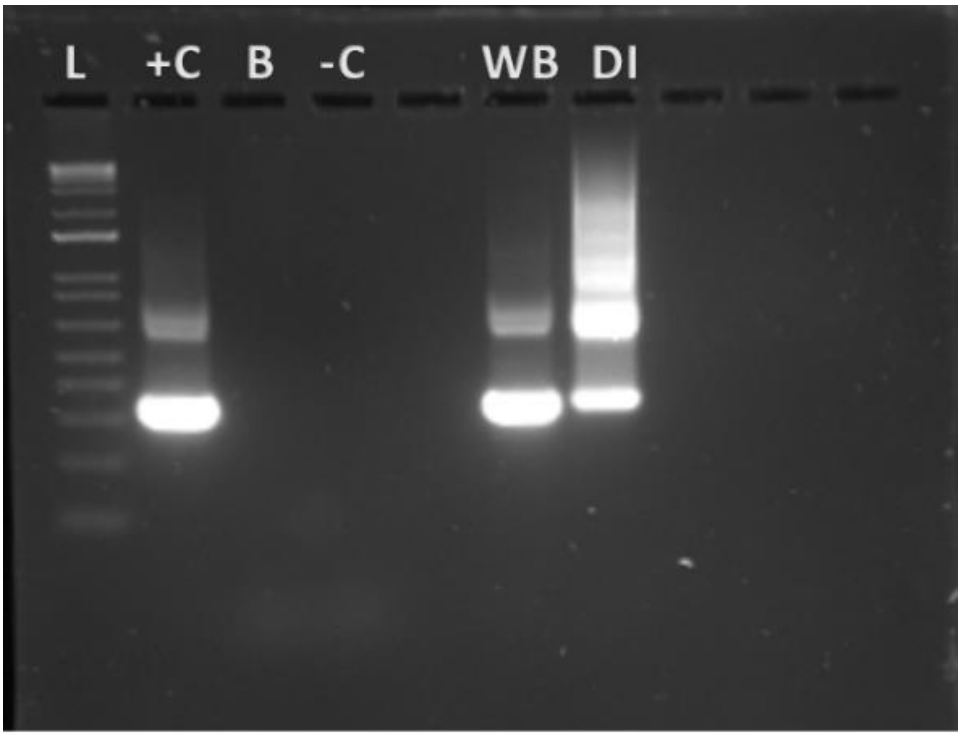

Figure 4.7. Results of PCR analysis to validate specificity of *D. immitis* primers. (L) Ladder, (C+) positive control from extracted lab infected mosquito, (B) Blank, (C-) non-infected lab raised mosquito, (DI) *D. immitis* worm acquired post mortem from infected canine, and (WB) whole blood from microfilaremic dog verified heartworm positive by serologic test for CHW antigen. Image captured on the 510-imager.

Infected mosquito experiments

Experimentally infected mosquitoes from TRS laboratories in Athens, GA were used to determine the preferred pool size as well as serve as controls for assays. Different pool numbers of mosquitoes were used to determine the maximum number of mosquitoes that should be used during DNA extraction processing. It was determined that three (one positive and two negative) mosquitoes were the optimal amount per tube, but positive results could be seen with up to seven samples per tube using GelGreen® stain. Dissection of a laboratory infected *Ae. aegypti* mosquito shows an L3 stage larvae emerging from the proboscis confirming infection in the mosquitoes. (Figure 4.8).

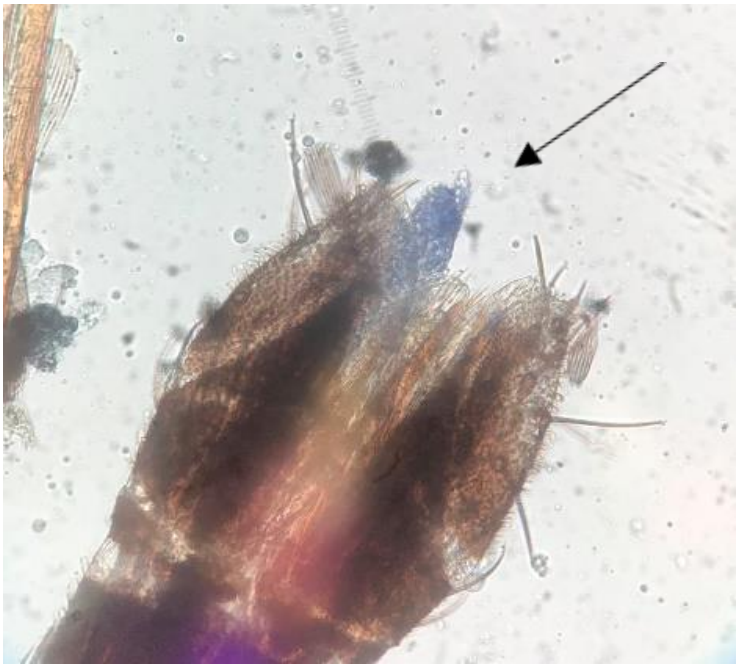

Figure 4.8. Experimentally infected *Aedes aegypti* mosquito shown stained with methylene blue at 40x with larval stage *D. immitis* emerging from the proboscis (arrow).

Heartworm Development Unit (HDU) Accumulation

HDU accumulation for local climatic conditions representative of the CGR demonstrate the occurrence of steady temperatures above 14°C (57.°F) from late May through late August each year and coincide with availabilty of mosquito vectors. The chart below starts at a zero-accumulation day (May 26, 2017) and ends with a zero-accumulation day on August 25, 2017 (Figure 4.11). Accordingly, the 238 HDU threshold for degrees Fahrenheit was 1st achieved on June 17, 2017 and supports recognition of a seasonal pattern for CHW transmission between June and September based on survival and longevity of infected mosquito vectors.

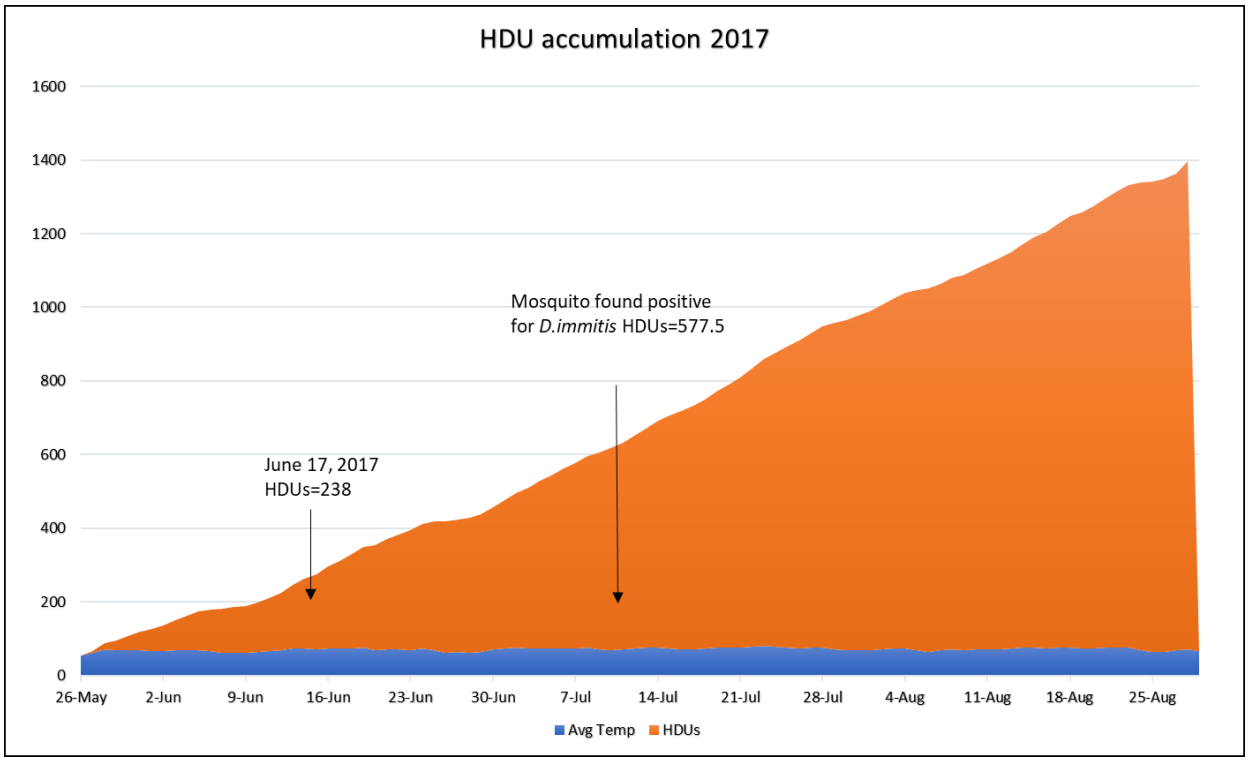

Figure 4.9. Cumulative Heartworm Developmental Units (HDU) for CGR study area based on average daily temperature data for May through August 2017 collected from National Weather Service reporting station in Tazewell, TN U.S. USC00408868. Data depicted indicate accumulation of 238 HDU threshold required for development of infective *D. immitis* larvae and CHW transmission by mosquito vectors achieved on June 17, 2017. Graph data based on temperature recording by degrees Fahrenheit.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

The purpose of this research was to gain as complete a picture as possible of the prevalence of canine CHW in the Cumberland Gap Region. This was done by collecting and testing the source of the disease, the mosquito. Information about the rate of infection in the mosquito population allows us to more accurately estimate the overall incidence of heartworm disease. This data can be used to educate pet owners, veterinary professionals, and other researchers interested in mosquitoes or canine health. To the best of our knowledge, there are currently no long-term studies on the species of mosquitoes serving as a vector of *D. immitis* in any area of the US. The species information can also be used in many areas of vector biology that concern diseases related to species found in the area; such as equine encephalitis virus.

CHW vectors are breeding and producing enough offspring to spread canine heartworm infection in the CGR. Furthermore, the study reinforces the importance of adding a body of knowledge of mosquito ecology in eastern Tennessee and southern Appalachia.

The prevalence of vector-borne diseases like CHW is dependent upon the distribution of competent vectors. A total of 2455 mosquitoes, representing at least 17 different species, were collected in this study (Table 4.1). The three most abundant genera; *Aedes*, *Culex*, and *Anopheles*, are established vectors of CHW (Vector Disease Control International, 2019). Importantly, one pool of *Ae. japonicus* in Trapping Area B was positive for *D.immitis*. This is the first report of a wild caught *Ae. japonicus* to be PCR positive for *D. immitis* in Eastern Tennessee.

Cx. pipiens (aka *Cx. quinquiefasciatus*/Southern house mosquito) was the highest in abundance in the mosquito collection. It represented 21% of all mosquitoes collected in the CGR. It feeds mostly on songbirds but will feed on large mammals and humans at night, dusk and dawn and has a flight range of up to 1.6 kilometers (Burkett-Cadena, 2013). It breeds in water with high organic content (such as sewage ditches) and artificial containers. It is a well-known vector of CHW but, in this study, was not found as a species of concern in the CGR (Burkett-Cadena, 2013). However, *An. quadrimaculatus* comprised only 0.52% of the mosquitoes collected provided 40% of the positive results for *D. immitis* infected mosquitoes. The few specimens collected were all found in Trapping Area B, which contained hollow trees, swamps and marshes all of which this species uses for breeding (Burkett-Cadena, 2013). The large number of infected mosquitoes in such a small collection pool indicates the species could play a major role in the transmission of heartworm disease in CGR. It also has a large flight range of 1.6 kilometers and is a native species. All five species in the *Anopheles* complex are present and native in the CGR, and because of their propensity for feeding on mammalian hosts and their crepuscular to nocturnal activity, they should be further investigated as a source of CHW infection.

Ae. albopictus (Asian tiger mosquito) has the ability to take over an entire region and displace native species (Spielman, 2001) and has a short flight range of 500-1000 meters(Burkett-Cadena, 2013). These mosquitoes are one of the few that will bite in direct sunlight and seem to prefer humans; however, it is known to feed on any mammal (Spielman, 2001). This mosquito species represented the third most abundant specimen pool in our collection (19.7%). It was the primary species captured in Trapping Area A, making up 23% of the collection from that site. This mosquito is a container-breeding mosquito originally found in

East Asia (Hawley, 1988) and is well known to be an important vector of *D. immitis* in Singapore, Japan, China and Korea (Chellappah and Chellappah, 1968, Konishi, 1989, Lai et al., 2000, Lee et al., 2007). Also, the spread of *D. immitis* from northern into southern areas of Italy may have been facilitated solely by *Ae. albopictus* (Otranto, 2009). Finding a positive mosquito in the collection suggests the further increase and spread of the disease by this species in the CGR is likely.

Ae. japonicus (Asian bush mosquito) is a cool temperate mosquito with a short flight range of 1600 meters and could be a significant vector in the area of CGR. They are persistent biters and attack when the vegetation in which they are resting is disturbed. They feed frequently during the early evening but can bite any time of day. They mainly feed on the blood of mammals, including humans. Of the species collected, 19.7% were *Ae. japonicus*: 23% in Trapping Area A; 22.4% in Trapping Area B; and 14.6% in Trapping Area C. This species has been artificially infected with *D. immitis* and shown to be vector competent (Silaghi, 2017).

Aedes vexans is also a native species in the CGR and it suited well for woodlands. They have a flight range of up to 24.1 kilometers and feed on a wide variety of hosts including humans, large mammals and birds. They tend to rest in grassy vegetation and feed in the twilight hours. It is a well know vector for canine heartworm (Burkett-Cadena, 2013). *Aedes vexans* made up 7.3% of the species in the collection and was positive for *D. immitis* in Trapping Area B. *Aedes vexans* numbers remained consistent in the collection over all three years and in all three locations: 6.7% of Trapping Area A; 6.2% in Trapping Area B; and 4.1% in Trapping Area C. The adaptability of this species in each location makes it another significant vector for CHW in the CGR. With its flight range it could feed on wild canids and easily facilitate the spread of CHW into many locations in the CGR.

No *D. immitis* positive mosquitoes were found in Trapping Area C, even though this site contained a local dog park. It is possible that the owners who bring their pet dogs to the dog park have a higher level of vet care and maintain their dogs on heartworm prophylaxis. Or, that the microhabitat is not suitable for the vectors.

Characteristics of mosquitoes in the CGR show that the non-native species have a very short flight range compared to the native species. It is possible that the native species have the ability to bring CHW into an area and it is proliferated by the invasive species that have short flight ranges and compete for territory (**Table 5.1**).

Table 5.1. General characteristics of HW positive mosquitoes trapped in the CGR

Species	Flight range	landscape	Biting activity	Host preference	Origin
Aedes japonicus	1.6km	Tree holes and artificial containers, preferring shade and water with abundant organic matter	Day, dusk, dawn	Humans, mammals, birds	Non-native, Japan, invasive
Anopheles quadrimaculatus	1.6km	Grassy pools, lake margins	Dusk, dawn, night	Humans, large mammals	Native to the CGR
Aedes albopictus	0.5-1.0km	Tree holes and artificial containers such as tires	daytime	Humans, mammals, birds	Non-native, Japan, invasive
Aedes vexans	24.1km	Floodwaters, grassy pools, woodland pools, temporary pools in sunlight or shady areas	Dusk, Night	Humans, large mammals	Native to the CGR

Some of the of the limitations with this study that are readily identifiable include potential false negatives, validity of testing, and application of HDUs. False negatives were a problem with this assay as selected primers used for PCR need at least 10,000 copies of the amplicon to illuminate on the gel product. Although sequencing was not performed in and outside lab due to the SARS-CoV2 pandemic, this study did a series of experiments to provide

validity. Those tests were on TRS lab infected mosquitoes, the whole blood from a microfilaremic dog, and a heartworm collected from a necropsied shelter dog (Figure 4.7, Figure 4.8).

Another limitation of this study resulted from the difficulty in separating mosquito heads, where L_3 stage infectious larvae reside, from the thorax. Although the testing of heads alone was attempted on a small sample, the amount of DNA necessary for the chosen assay was not consistently extracted. Therefore, we cannot state with certainty that the positive results were infective to dogs.

The temperatures remain too low in the month of June to accumulate the necessary HDU's for *D. immitis* to become infective until the later part of the month. This means *D. immitis* will have difficulty developing into the third stage larvae (L3) until the beginning of July. This limited window of infectivity may be why other studies have seen low numbers of positive CHW dogs living in the CGA without HW prevention. By September the set traps no longer have viable collection numbers for testing as a freeze has usually occurred in the first part of September and reduced the temperature to under 14°C (57°F) and started HDU's back to zero-accumulation (Figure 4.9).

Taking into consideration how relatively small the sample sizes are compared to the overall mosquito population is in the CGR (Claiborne County, TN), it can be concluded that the prevalence of heartworm-positive vectors is likely high. This study should be continued over a much greater length of time for a true representation of mosquito diversity and heartworm vector diversity present in the Cumberland Gap Region.

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