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

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Richard Harris West

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This Thesis, "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," by Richard H. West, is approved by:

Thesis Committee Chair

Eric W. Chambers, Ph.D.
Associate Professor of Biology

Committee Members

Mark S. Blackmore, Ph.D.
Professor of Biology

Donna L. Gosnell, Ph.D.
Professor of Chemistry

Dean of the Graduate School

James T. LaPlant, Ph.D.
Professor of Political Science

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ABSTRACT

Anopheles crucians s.l. includes several understudied cryptic mosquito species. This species complex is comprised of six morphologically indistinguishable species. Female mosquitoes were obtained from 14 collection sites in Lowndes County, GA, USA during the 2014 trapping season. The internal transcribed spacer two (ITS2) gene was amplified in individual mosquitoes and used to identify them at the species level. Of the six species within the cryptic species complex, only three species (A, C, and D) were identified within Lowndes County, GA, USA. Sympatry was observed between the species at different collection sites, with four sites having all three species in sympatry and other sites having only two species in sympatry. *Anopheles crucians s.l.* has been implicated as a vector of *Dirofilaria immitis*, as well as other pathogens, but its competency has not been fully confirmed. The collected mosquitoes were screened for the presence of *D. immitis* using a polymerase chain reaction (PCR) protocol to amplify the 16S rRNA gene from dog heartworm within the host mosquitoes. The heads and thoraces were separated from the abdomens of the mosquitoes, and pools of each were screened for *D. immitis* DNA by PCR. *Dirofilaria immits* DNA was detected in a single pool of *An. crucians* abdomens. Future studies could be aimed toward screening the *An. crucians* cryptic species complex for competency in vectoring other pathogens and arboviruses.

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Chapter I

INTRODUCTION

Arthropod-borne diseases have impacted mankind throughout history, yet it was not until the late 19th century that humans finally became aware of the role that mosquitoes played in disease transmission. The first pathogen identified to be transmitted by mosquitoes was *Wuchereria bancrofti* (Manson, 1878). Manson (1914) demonstrated that the filarial parasites exhibited a diurnal periodicity which coincided with the feeding time of their competent mosquito vectors.

In particular, arthropod-borne viruses (arboviruses) have historically caused high morbidity and mortality throughout the tropics and subtropics. Yellow fever virus caused major epidemics during the 17th, 18th, 19th, and early 20th centuries (Gubler, 2004). Yellow fever initially causes fever, and general malaise, but in more severe cases it causes liver damage as well as hemorrhagic symptoms, and can become fatal. Carlos Finlay first proposed the idea in 1881 that yellow fever was transmitted by arthropods, namely mosquitoes. Finlay's theory was eventually confirmed by Major Walter Reed's Yellow Fever Commission in Havana, Cuba (1900). The Reed Commission performed meticulous experiments establishing that mosquitoes transmitted the disease and that the agent was able to be successfully filtered through a Berkefeld filter (Frierson, 2010). The primary vector for yellow fever is the highly domesticated mosquito, *Aedes aegypti*,

which is widely distributed in tropical regions (Gubler, 2004). Since this early work with yellow fever by Walter Reed, numerous arboviruses, viruses transmitted by arthropods, have now been identified.

Dengue virus is another common arbovirus transmitted by mosquitoes and is recognized as the most common human arbovirus infection (Tassaneetrithep et al., 2003). It is estimated that there are over 390 million cases of dengue fever annually, the majority of which are transmitted by *Ae. aegypti*. Most dengue infections are mild, but a secondary infection with a heterologous strain of the dengue virus can result in the development of dengue hemorrhagic fever and/or dengue shock syndrome (Tassaneetrithep et al., 2003). In addition to dengue virus, there have been more recent outbreaks of arboviruses such as Chikungunya and Zika viruses in the Western hemisphere. Chikungunya virus causes a condition known as chikungunya fever, which is an arthralgic disease (Charrel et al., 2007). Zika virus infections resemble dengue and chikungunya infections, but has also been linked to microcephaly in newborn children (Mlakar, 2016).

West Nile virus is an arbovirus that is transmitted primarily by mosquitoes in the genus *Culex*, and has its natural reservoir in avian hosts. The mosquito-bird-mosquito transmission cycle, primarily involving *Culex* species, is how the West Nile virus is maintained in nature (Campbell et al., 2002). Most individuals infected with West Nile virus are asymptomatic, but when patients do exhibit symptoms they are akin to the symptoms associated with the flu, a condition referred to as West Nile fever (Kramer et al., 2007). In more serious cases West Nile virus can cause encephalitis and meningitis, both of which can be fatal.

Arboviruses can be transmitted by a myriad of different genera of mosquitoes. The genus *Anopheles* is one of the genera which can vector a variety of arboviruses, as well as other parasites. Arboviruses and parasites isolated from anopheline mosquitoes include malaria (*Plasmodium spp.*), Eastern Equine Encephalitis virus (EEEV), Venezuelan Encephalitis virus (VEEV), St. Louis Encephalitis virus (SLEV), *Wuchereria bancrofti*, and *Dirofilaria immitis*. The transmission of a variety of *Plasmodium spp.*, the parasite that causes malaria, in particular makes Anopheline mosquitoes one of the most medically important genera. *Anopheles gambiae* is one of the major vectors of human malaria and is occasionally referred to as the "malaria mosquito." In Africa, *Anopheles gambiae* is the primary vector of *Plasmodium falciparum* and is considered the most efficient malaria vector in the world (Holt et al., 2003).

In North America a variety of viruses that cause encephalitis are vectored by anopheline mosquitoes. Many of these viruses are specific to one geographic region within the country. There are more than 20 arboviruses which cause encephalitis, and chief among these are Eastern Equine Encephalitis virus (EEEV), St. Louis Encephalitis virus (SLEV), and Venezuelan Equine Encephalitis virus (VEEV), all which are found in North America (Johnson, 1996). *Dirofilaria immitis* is a parasitic nematode and is the cause of dog heartworm, and is a major veterinary problem in many tropical and temperate countries (Chambers et al., 2009). *Wuchereria bancrofti* and *Brugia malayi*, like *Dirofilaria immitis*, are parasitic filarial nematode worms that are vectored by mosquitoes and cause lymphatic filariasis in humans. Lymphatic filariasis in more severe cases can lead to the debilitating condition known as elephantiasis. An estimated 120

million people worldwide are affected by lymphatic filariasis annually (Michael et al., 1996).

One understudied species of anopheline mosquitoes that is distributed throughout the Western hemisphere is *Anopheles crucians*. *Anopheles crucians s.l.* has been implicated as a competent vector for malaria (Floore et al., 1976). It is also a potential vector for a variety of arboviruses, including West Nile virus, EEEV, VEEV, SLEV, Keystone virus, and LaCrosse virus (Wilkerson et al., 2004). *Anopheles crucians* has also been implicated as a vector of *Dirofilaria immitis*, the causative agent of dog heartworm, in the Southeastern United States (Licitra et al., 2010).

Anopheles crucians (Wiedemann) is distributed throughout the temperate zone of North America and in the tropical zone of Central America, and the Caribbean islands (Wilkerson et al., 2004). Females of this species prefer to lay their eggs in lentic, slow moving fresh water, as well as ponds, lakes, and swamps (Wilkerson et al., 2004). A species of *An. crucians*, *An. crucians bradleyi*, breeds in brackish waters along the Atlantic and Gulf coasts and has been found as far south as Nicaragua (Wilkerson et al., 2004). The females are zoophilic, nocturnal feeders, but have been known to feed on humans at sunset (Floore et al., 1976), during the day on particularly cloudy days, and in the shade (www.wrbu.org).

Some anopheline species, including *An. crucians*, form cryptic species complexes, where all of the species in the complex are morphologically indistinguishable, but can be separated genetically (Collins & Paskewitz, 1996). Often, cryptic species complexes include species that vector pathogens and species that do not. These species may be sympatric, with two or more species in a geographical area.

Identification of vectors and non-vectors within a cryptic species complex is important because more efficient control methods can be enacted to prevent disease transmission once a species habitat, feeding habits, and vector status are confirmed.

Identification of species within a cryptic species complex can be performed using different types of genetic markers such as mitochondrial DNA, microsatellites, and ribosomal DNA. Mitochondrial DNA is useful for identifying the genetic relationships of individuals within a species. A plethora of anophelines and anopheline species complexes have been investigated using mitochondrial DNA (Foley et al., 1998; Thelwell et al., 2000; Besansky et al., 1997) and microsatellites (Zheng et al., 1996; Conn et al., 2001; Wang et al., 2001).

Microsatellites are regions of repetitive DNA in which short sequences of DNA (2-5 bp) are repeated up to 50 times. This form of genetic marker DNA, is commonly used in the genetic studies of anophelines (Norris, 2002). Ribosomal DNA studies involve three target regions: the intergenic spacer (IGS), the internal transcribed spacer one (ITS1), and the internal transcribed spacer two (ITS2) to investigate differences within cryptic species complexes. Taxonomic studies of anopheline cryptic species complexes have used the nuclear ribosomal genome, particularly the (IGS) and (ITS1 and ITS2) genes (Norris, 2002). ITS2 sequencing of ribosomal DNA has been used as an identification tool and Hackett et al. (2000) developed a rapid Polymerase Chain Reaction (PCR) based method for differentiating the ITS2 sequences between two species in Africa (*Anopheles funestus* and *Anopheles rivulorum*). Walton et al. (2007) also successfully developed a PCR based method for differentiating the ITS2 sequences in the *Anopheles maculatus* cryptic species complex.

The aim of the present study is to utilize a species-specific PCR assay developed by Wilkerson et al. (2004) to identify species within the *Anopheles crucians* cryptic species complex in Lowndes County, GA, USA, using the ribosomal internal transcribed spacer two (ITS2) gene.

Materials and Methods

Specimen Collection

Anopheles crucians were obtained from arbovirus surveillance collections at 14 separate collection sites in Lowndes County, Georgia (Figure 1 in Appendix A) during the 2014 trapping season. The collection sites include both developed and undeveloped areas (Figure 2a-2n). Mosquitoes were caught using CDC miniature light traps (John W. Hock Co., Gainesville, FL, USA) baited with dry ice between 10 June 2014 and 4 November 2014. Mosquitoes sorted by trap location and date collected were identified to species using keys (Burkett-Cadena, 2013), and then stored individually at -80°C. Eppendorf tubes containing individuals were removed from the -80°C freezer, and 4 individuals were randomly selected from each sample site for screening.

DNA Extraction

DNA extractions for the identification of sub-species in the *An. crucians* cryptic species complex were conducted using a modified alkaline extraction method (Rudbeck & Dissing, 1998). Mosquitoes were placed in individual 2mL Eppendorf tubes with a zinc bb (Walmart Corp., Bentonville, AR, USA) and homogenized in a solution of 80µL of 0.2N NaOH for 10 min using a vortex adapter (MoBio, Carlsbad, CA, USA). The samples were then incubated at 75°C for 10 min. After incubation, 28.8µl of 1M TRIS at (pH 8.0) and 891.2µL of double distilled water were added to bring the final volume in

the tube to 1,000µL. Permission from the Institutional Animal Care and Use Committee (IACUC) was not required for this study.

PCR Amplification

The ITS2 region of the 5.8S and the 28S ribosomal subunits was amplified using a multiplex PCR developed by Wilkerson et al. (2004). Each 25-µL reaction contained 1 X Go-Taq buffer (Promega, Madison, WI, USA), 2-mM $MgCl_2$ (Promega, Madison, WI, USA), 0.25 mM each of dATP, dCTP, dGTP, and dTTP (Fisher Scientific, Pittsburgh, PA, USA), 0.2 µM of the universal ITS2-forward, universal ITS2- reverse, ITS2-reverse bradleyi, and ITS2-reverse C primers (IDT, Coralville, IA, USA), and 0.05 U of Go-Taq®, DNA polymerase (Promega, Madison, WI, USA). The primer sequences were developed by Wilkerson et al. (2004). PCR reactions were run on a Bio-Rad I-Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR thermal cycling conditions consisted of 35 cycles of 90°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 5 min. The PCR products were then size fractionated on a 2.5% agarose gel (Fisher Scientific, Pittsburgh, PA, USA) stained with Gel Red (Biotium, Hayward, CA, USA). Agarose gels were run at 70 V for 1.5 h and visualized under UV light using an ImageQuant LAS 4000 imager (General Electric, Schenectady, NY, USA).

Results

A total of 3,585 female *Anopheles crucians* individuals were collected from 14 sites in Lowndes County, Georgia, USA (Table 1 in Appendix B). Of the 3,585 mosquitoes, 239 individuals were screened using a multiplex PCR to identify the individuals to the species level within the *An. crucians* species complex. The six species within the *An. crucians* complex were identified by their ITS2 fragment sizes in base

pairs (bp). In this study, three size classes of PCR products were identified as belonging to species "A," "C," and "D," (Figures 3 and 4). Of the six species within the complex, three species were not found in any of the individual screenings of any of the trap locations. There were six sites from which no *An. crucians* were collected during this study (Table 1). The three species which were found in the study area were species "A," "C," and "D," which were identified from eight sample locations (Figure 5). Of the eight sites which did yield *An. crucians* individuals, four sites contained three sympatric species "A," "C," and "D" (Deloach, Masonic Lodge, Old Clyattville Rd., and Thomas) (Table 1), two sites contained two sympatric species "A" and "C" (Baytree, and Plantation) (Table 1), and the last two sites contained two sympatric species "A" and "D" (Brown Rd., and Hammock) (Table 1). Temporal data also shows an early emergence of species "A," with a late emergence of species "D," and species "C" having little abundance during the trapping season (Figure 6).

Discussion

A cryptic species complex is a group of distinct species that cannot be distinguished based upon their morphology but can be separated using molecular genetic analysis. Species differentiation is important because cryptic species complexes can contain both vector and non-vector species. The use of rDNA ITS2 sequencing in decrypting a cryptic species complex is important because, although species within the complex may be morphologically indistinguishable, species specific ITS2 primers allow for the identification of individual species within the complex. Molecular analysis has been used to identify several cryptic mosquito species complexes including *An. gambiae s.l.* complex (Green, 1981), *An. quadrimaculatus s.l.* (Cornel et al., 1996), *An.*

punctulatus s.l. (Beebe & Saul, 1999), and *Culex pipiens s.l.* (Dumas et al., 2016). *Anopheles gambiae s.l.* includes vector and non-vector species within the complex, and species which vary in insecticide resistance. Two species within this complex, *An. arabiensis* and *An. quadriannulatus*, are sympatric, with *An. arabiensis* being an anthropophilic species and a vector of malaria, and *An. quadriannulatus* being a zoophilic, non-vector species. *Anopheles arabiensis* exhibits high levels of insecticide resistance, while *An. quadriannulatus* shows no resistance to the insecticide (Green, 1981).

Anopheles crucians is considered a medically important species because it is a potential vector for arboviruses and other pathogens, including malaria. In previous work by Wilkerson et al. (2004), the rDNA ITS2 gene was sequenced in individual *An. crucians* mosquitoes within the *An. crucians* cryptic species complex and the sequence data were then used to develop primers to amplify the target DNA in a multiplex PCR for the identification mosquitoes. Wilkerson et al. (2004) had 28 collection sites throughout six states: Alabama, Florida, Georgia, Louisiana, North Carolina, and Mississippi. Those collection sites yielded 245 *An. crucians* individuals for identification. Of those 28 collection sites, six sites were in GA with one site being in Lowndes County, GA. The data reported in my study were obtained from mosquitoes collected at 14 locations across Lowndes County, GA. The present study screened 239 individual *An. crucians* mosquitoes and also corroborated the Wilkerson study in terms of species composition. As in the Wilkerson study, species A, D, and C were present, but species B, E, and *An. bradleyi* were not found. This was not unexpected because species B was previously found primarily in the bayou swamps of Louisiana, while species E has only been

collected at one site in a mountainous region of North Carolina, and *An. crucians bradleyi* has been shown to only breed in coastal brackish waters (Floore et al., 1976; Wilkerson et al. 2004).

The *An. crucians* cryptic species identified in this study are sympatric, but the microhabitats from which they were collected correspond with subhabitats associated with the species described by Wilkerson et al. (2004). All identified species were collected from undeveloped or less-developed areas, mostly woods and fields, which would provide a greater variety of breeding habitats than the more developed areas of the county where much of the area is paved and has efficient drainage.

Extensive sympatry among species within the *An. gambiae* cryptic species complex has also been reported (Green, 1981; Sougoufara et al., 2016). Species within the *An. gambiae* cryptic species complex vary behaviorally in ways that affect their ability to transmit malaria. *Anopheles gambiae s.s.* and *An. coluzzii* are anthropophilic, endophagic vector species, while *An. arabiensis* is an exophilic vector species (Sougoufara et al., 2016) and *An. quadriannulatus* is an exophilic, non-vector species (Green, 1981) within the *An. gambiae* cryptic species complex. Just as there are differences among the *An. gambiae* cryptic species with regard to behavior and vector status, similar differences may exist within the *An. crucians* cryptic species complex described here. Following the work set forth in this study, future studies should be aimed toward the surveillance of different pathogens within the vector species and monitoring areas where both the vector and non-vector species are sympatric for the presence of pathogens.

Chapter II

INTRODUCTION

Dog heartworm is a disease caused by infection with *Dirofilaria immitis*, a filarial nematode that is vectored by multiple mosquito species and transmitted to canine hosts (Acha & Szyfres, 2003). Canines are the definitive host of *D. immitis*, while mosquitoes are the intermediate hosts. Sixty-three species of mosquitoes have been implicated as potential vectors of dog heartworm with most of the species belonging to the genera *Aedes*, *Anopheles*, and *Culex* (Ludlam et al., 1970). In 2011, 1.2% (56,612 out of 4,769,403 dogs tested by commercial laboratories in the United States) tested positive for dog heartworm antigen, which indicates an active *D. immitis* infection (Brown et al., 2012). As the name implies, adult heartworms aggregate in the heart and associated blood vessels. Light heartworm infections generally produce few or no symptoms, whereas heavy infections cause coughing, dyspnea, weight loss, and eventual congestive heart failure leading to death (Bowman & Atkins, 2009). *Dirofilaria immitis* is closely related to two other nematode species, *Wuchereria bancrofti* and *Brugia malayi*, which cause lymphatic filariasis in humans. These nematodes are also vectored by multiple mosquito species, many within the genera *Culex*, *Anopheles*, *Aedes*, and *Mansonia* (Erickson et al., 2009). Annually, it is estimated that more than 120 million people are infected with either *W. bancrofti* or *B. malayi* (Erickson et al., 2009). Lymphatic

filariasis can produce more severe syndromes such as lymphedema and the more serious condition elephantiasis.

Dirofilaria immitis is a parasite that exhibits a complex life cycle (Figure 7). Dog heartworm is not limited to canines, as it is capable of infecting other animals such as cats, coyotes, foxes, penguins, and even humans (Ledesma & Harrington, 2011). The *D. immitis* life cycle contains multiple developmental life stages, both within the invertebrate and vertebrate hosts. During a blood meal, a competent infective adult female mosquito infects a definitive host with *D. immitis*. Once in the definitive host, the L3 larvae undergo two molts to become adults. After mating, females produce microfilariae which migrate to the peripheral blood of the infected canine. When an uninfected female mosquito takes a blood meal from the infected canine, she ingests the microfilariae and becomes infected. The microfilariae then migrate to the malpighian tubules and undergo two molts to become L3 larvae (Bradley & Nayar, 1987). The L3 infective larvae then migrate to the infected mosquito's head and proboscis. The infected mosquito is now infective and ready to transmit the L3 larvae to a naïve host upon its next blood meal (www.cdc.gov).

For many years the preferred method for the detection of *D. immitis* involved dissecting freshly euthanized individual female mosquitoes (Scoles & Kambhampati, 1995). This method was laborious, and very time consuming so other methods were developed. A bulk processing method using a Baermann funnel apparatus (Muller & Denham, 1974) was developed to detect *D. immitis* by testing homogenized pools of multiple mosquitoes instead of dissecting individuals. Although this method allows for the processing of a large number of mosquitoes, infection rate estimates are not always

accurate and it is impossible to determine the site of infection (Scoles & Kambhampati, 1995). Determining the site of the infection is important because the mosquito is considered "infected" if the larvae are found in the abdomen, but "infective" if the larvae occur in the head and thorax. A PCR assay was developed by Scoles and Kambhampati (1995) to more quickly and accurately identify infections within competent mosquito vectors.

The aim of the present study is to utilize the species-specific PCR assay developed by Vezzani et al. (2011) to determine the prevalence rate of *D. immitis* infection within *An. crucians* mosquitoes in Lowndes County, GA, USA. This assay will be used to identify both infected and infective mosquitoes.

Materials and Methods

Specimen Collection

Anopheles crucians were obtained from arbovirus surveillance collections at 14 separate collection sites in Lowndes County, Georgia (Figure 1) during the 2014 trapping season. Mosquitoes were caught using CDC Miniature Light traps (John W. Hock Co., Gainesville, FL, USA) baited with dry ice, between June 10 and November 4. Mosquitoes sorted by trap location and date collected were identified to species using keys (Burkett-Cadena, 2013), and then stored individually at -80°C. Eppendorf tubes containing individuals were removed from the -80°C freezer. Ten randomly chosen mosquitoes from a collection site were pooled. Heads and thoraces were then separated from the abdomens of the ten mosquitoes and placed in separate Eppendorf tubes (10 abdomens in one tube; 10 heads and thoraces in another). The pooled heads and thoraces and the pooled abdomens were then screened for the presence of *Dirofilaria immitis*.

DNA Extraction

DNA was extracted from the pooled heads and thoraces and abdomens using a modification of the Qiagen DNEasy Blood and Tissue Kit (Qiagen Corp., Hilden, Germany) to test for the presence of *D. immitis*. Ten heads and thoraces were placed in an Eppendorf tube with a zinc bb (Walmart Corp., Bentonville, AR, USA), and 10 abdomens (from the same 10 mosquitoes) were placed in a separate Eppendorf tube with a zinc bb (Walmart Corp., Bentonville, AR, USA). Each Eppendorf tube received 180µL of 1X phosphate-buffered saline (PBS) and the lids were wrapped in Parafilm (BemisNA, Neenah, WI, USA). The pooled mosquitoes within the Eppendorf tubes were then homogenized for 15 min using a vortex mixer (Fisher Scientific, Hampton, New Hampshire, USA) fitted with a horizontal adapter (MoBio, Carlsbad, CA, USA). After homogenization, 200µL of lysis buffer (Buffer AL) and 20µL of proteinase K were added to each Eppendorf tube. The tubes were briefly vortexed and incubated at 56°C for 1 h in a hot water bath. The tubes were spun at 13,000 rpm for 5 min, and the supernatant of each tube was transferred to separate tubes containing 200µL of 98% ethanol. The supernatant and ethanol mixtures of each tube were transferred to separate Qiagen DNEasy spin columns and spun at 8,000 rpm for 1 minute, and the supernatant was discarded. A fresh collecting tube was added to each spin column, and was washed twice with 500µL of Buffer AW1 then spun at 8,000 rpm for 1 min. The tubes were then washed with 500µL of Buffer AW2 and spun at 13,000 rpm for 3 min. The two spin columns were then placed in separate 1.5 mL Eppendorf tubes with 200 µL of buffer AE, incubated for 1 min, then spun at 8,000 rpm for 1 min and repeated. The samples were

then stored in a freezer at -20°C. Permission from the Institutional Animal Care and Use Committee (IACUC) was not required for this study.

PCR Amplification

PCR amplification of the *D. immitis* 16S rRNA gene was performed using the protocol developed by Vezzani *et al.* 2011. Each 25µL reaction tube contained 1X Go-Taq buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$ (Promega, Madison, WI, USA), 100 µM each of dATP, dCTP, dGTP, and dTTP (Fisher Scientific, Pittsburgh, PA, USA), .2uM of the DiF-forward and DiR-reverse primers (IDT, Coralville, IA, USA), and 1/ U reaction of Go-Taq®, DNA Polymerase (Promega, Madison, WI, USA). PCR reactions were run on a Bio-RAD I-Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling conditions consisted of 1 cycle of 95°C for 10 min, 35 cycles of 95°C for 1 min, 55°C for 45 sec, 72°C for 1 min, and terminated by cooling to 4°C. The PCR products were then size fractionated on a 2.5% agarose gel (Fisher Scientific, Pittsburgh, PA, USA) stained with Gel Red (Biotium, Hayward, CA, USA). Agarose gels were run at 70 V for 1 h and visualized under UV light using an ImageQuant LAS 4000 imager (General Electric, Schenectady, NY, USA).

Statistical Analysis

The prevalence of *D. immitis* infection was determined for the subset of *An. crucians* mosquitoes using PoolScreen2 statistical software. PoolScreen2 provides the maximum likelihood estimate (MLE) of infection along with confidence intervals based upon pool size, the number of pools examined, and the number of negative pools (Barker 2000).

Results

A total of 594 female *Anopheles crucians* individuals from eight sites in Lowndes County, Georgia, USA (Figure 1) were screened for the presence of *Dirofilaria immitis* using PCR amplification of the 16S rRNA gene. The PCR amplification of the 16S rRNA genes was performed on 60 pools of mosquito head and thoraces and 60 pools of mosquito abdomens, with each pool containing an average of 10 *An. crucians* mosquitoes. No head and thorax pools showed signs of infection, and the maximum likelihood estimate of infection (MLE) for the pooled heads and thoraces was determined to be 0, with a 95% confidence interval between 0 and .003 (Table 2). The MLE for the pooled abdomens was .00169, with a 95% confidence interval between .00005 and .0087 (Table 2). This reflected infection of a single abdomen pool from mosquitoes collected at the Masonic Lodge site (Figures 1 and 2m), between June 10 and June 16, 2014.

Discussion

Although *An. crucians* has been implicated as a vector for *D. immitis* (Watts et al., 2001; Licitra et al., 2010), limited research regarding its competency as an efficient vector for dog heartworm has been completed. PCR protocols have been developed that accurately screen mosquitoes for the presence of *D. immitis* DNA. These protocols amplify certain target genes (e.g., a surface cuticular antigen (Scoles & Kambhampati, 1995), the mitochondrial cytochrome c oxidase subunit I (COI) gene (Tahir et al., 2017), or the 16S rRNA gene (Watts et al., 1999.) to identify *D. immitis*.

PCR has been successfully used to identify different vectors of *D. immitis*, and has become an important tool for real-time measurement of the presence of the parasite within multiple species of mosquito in a given area. PCR has made the detection of *D.*

immitis much more efficient on a large scale when coupled with a technique such as molecular xenomonitoring (Chambers et al., 2009; Latrofa et al., 2012; Schmaedick et al., 2014). Xenomonitoring paired with PCR allows for the identification of *D. immitis* within vector species to be more efficient and less time consuming.

The protocol used in this study amplifies the 16S rRNA gene of *D. immitis* to determine the presence of infected and infective female *An. crucians*. Mosquitoes were identified as infected or infective depending on whether the 16S rRNA gene could be amplified in the head and thorax pools (=infective) or only in the abdomen pools (=infected). The results of this study indicate *An. crucians* is a potential vector of *D. immitis* because the presence of DNA from the 16S rRNA in this mosquito indicates that it has fed on an infected host. Because *D. immitis* DNA was only found in one pool of abdomens and there were no DNA positive head-thorax pools, the vector status of *An. crucians* remains in question. *Anopheles crucians* is a potential vector for *D. immitis*, but other mosquito species, including, *Aedes albopictus* (Gratz, 2004), *Aedes vexans* (Ledesma & Harrington, 2011) *Culex quinquefasciatus* (Ledsma & Harrington, 2011), and *Anopheles quadrimaculatus* (Ledesma & Harrington, 2011), all of which are found within the *An. crucians* geographic range should also be monitored, because multiple species within a given geographic region can serve as intermediate hosts for infection. Monitoring of the many potential vectors of *D. immitis* can help to map infection patterns and explain the distribution of dog heartworm throughout *An. crucians* range. Because different mosquito species emerge at different times of year, an understanding of which mosquitoes are competent vectors as well as their phenology, could facilitate the development of multiple preventative measures to prevent *D. immits* infections.

Understanding the distribution of *D. immitis* can help in assessing the epidemiology of *D. immitis* in South Georgia and assessing the threat of infection to domesticated animals.

Chapter III

CONCLUSION

Cryptic species complexes are problematic because of the difficulty in identifying individual species. Additionally, it may be even more difficult to distinguish vector species from non-vector species. The ability to identify medically important species within a cryptic species complex has been greatly aided by the development of a number of PCR techniques. The use of PCR protocols that amplify the ITS2 gene has become an important tool for identifying cryptic species because the ITS2 gene is highly conserved between closely related species (Wilkerson et al., 2004). In this study, three species within the *An. crucians* cryptic species complex were identified using PCR of the ITS2 rDNA gene. *Anopheles crucians s.l.* has been implicated as a potential vector of malaria and other arboviruses, as well as for *Dirofilaria immitis*. PCR amplification using primers that target the *D. immitis* 16S rRNA gene of L3 larvae allowed for the identification of infection. Because the species within the *An. crucians* complex can now be determined, future studies should attempt to determine which species within the *An. crucians* cryptic species complex can competently vector *D. immitis*. The spatial and temporal presence of each species should also be taken into account so precautions can be taken to prevent transmission of *D. immitis* by implementing vector control strategies and administering prophylactic treatment.

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APPENDIX A:

Figures for Chapters 1-3

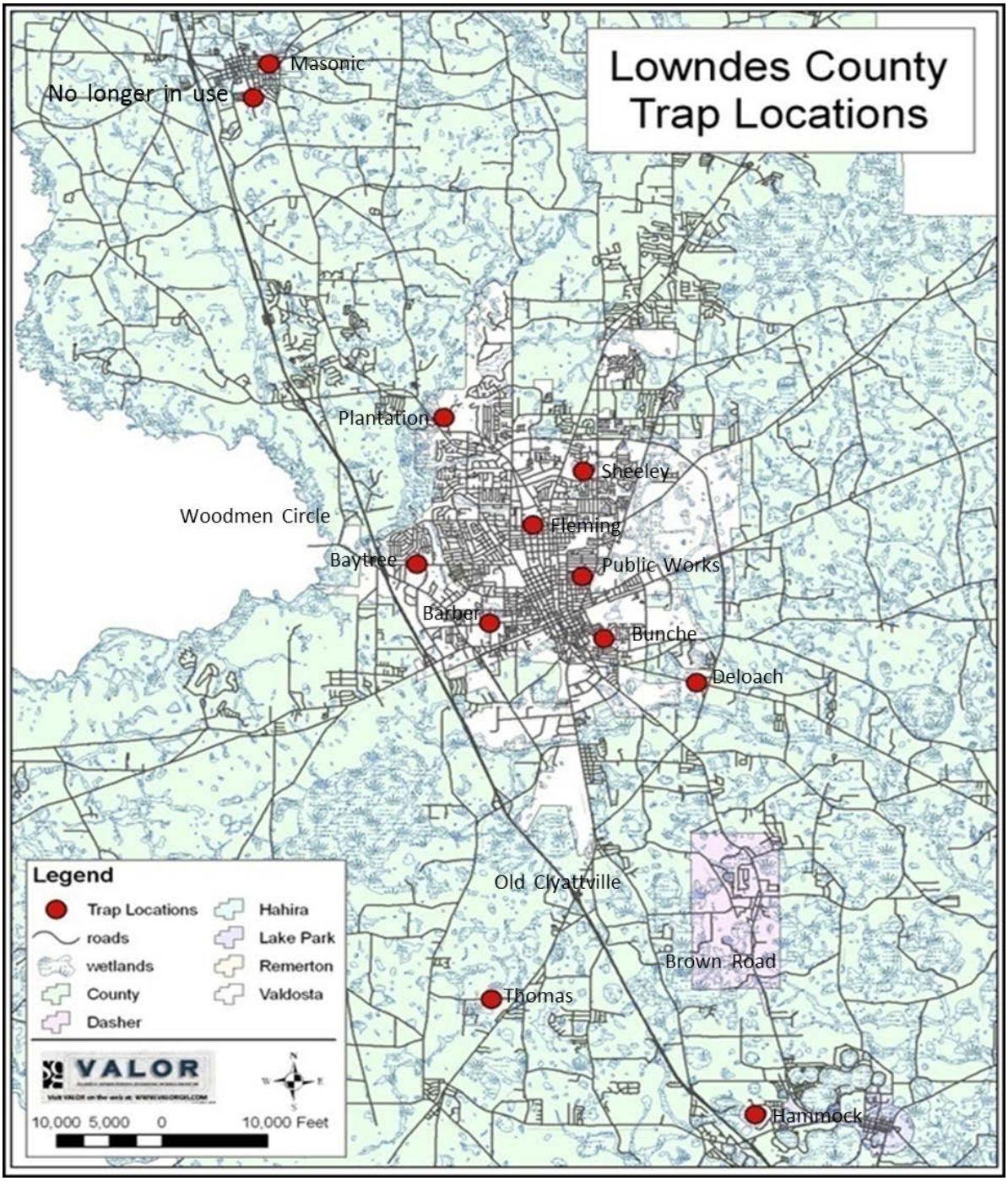

Figure 1. Trap locations in the study area of Lowndes, County, GA

Figure 2a. Fleming collection site.

Figure 2b. Sheeley collection site

Figure 2c. Public Works collection site

Figure 2d. Barber collection site

Figure 2e. Bethune collection site

Figure 2f. Deloach collection site

Figure 2g. Brown Rd. collection site

Figure 2h. Hammock collection site

Figure 2i. Thomas collection site

Figure 2j. Old Clyattville collection site

Figure 2k. Baytree collection site

Figure 2l. Woodmen Circle collection site

Figure 2m. Masonic Lodge collection site

Figure 2n. Plantation collection site

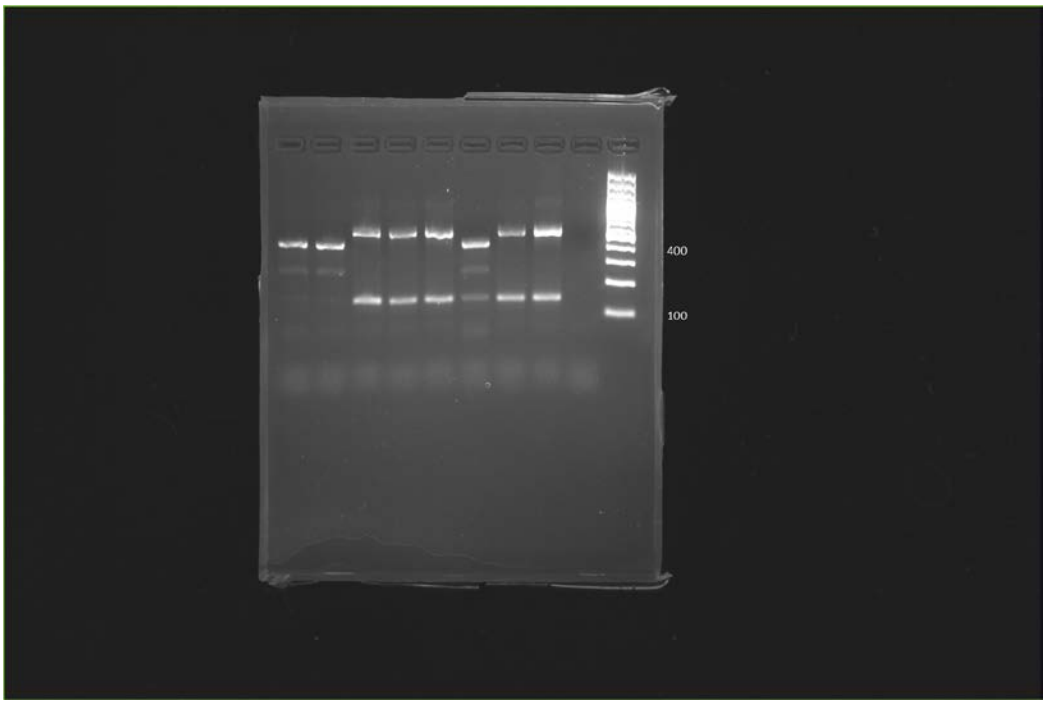

Figure 3. rDNA-ITS2 amplification products of individual mosquitoes following multiplex PCR. Lanes 1-2 and 6, Species D; Lanes 3-5, 7-8, Species A; Lane 9, negative control, Lane 10, 100-bp molecular weight marker. Labeled bands are in base pairs (bp).

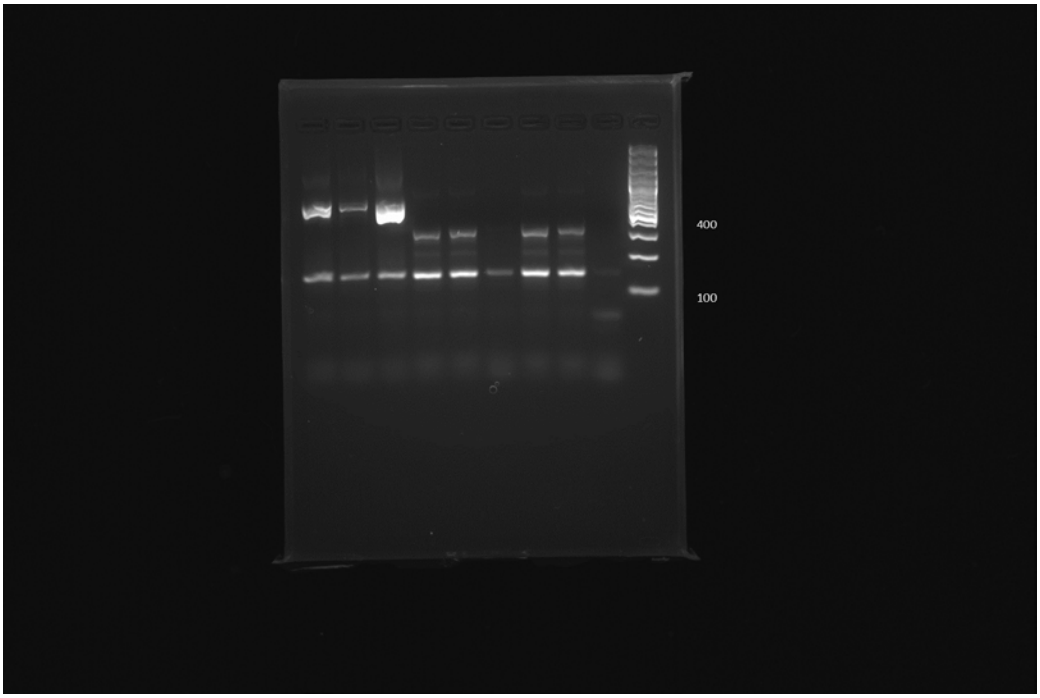

Figure 4. rDNA-ITS2 amplification products of individual mosquitoes following multiplex PCR. Lanes 1-3, Species A; Lanes 4-5, 7-8, Species C; Lane 9, negative control, Lane 10, 100-bp molecular weight marker. Labeled bands are in base pairs (bp).

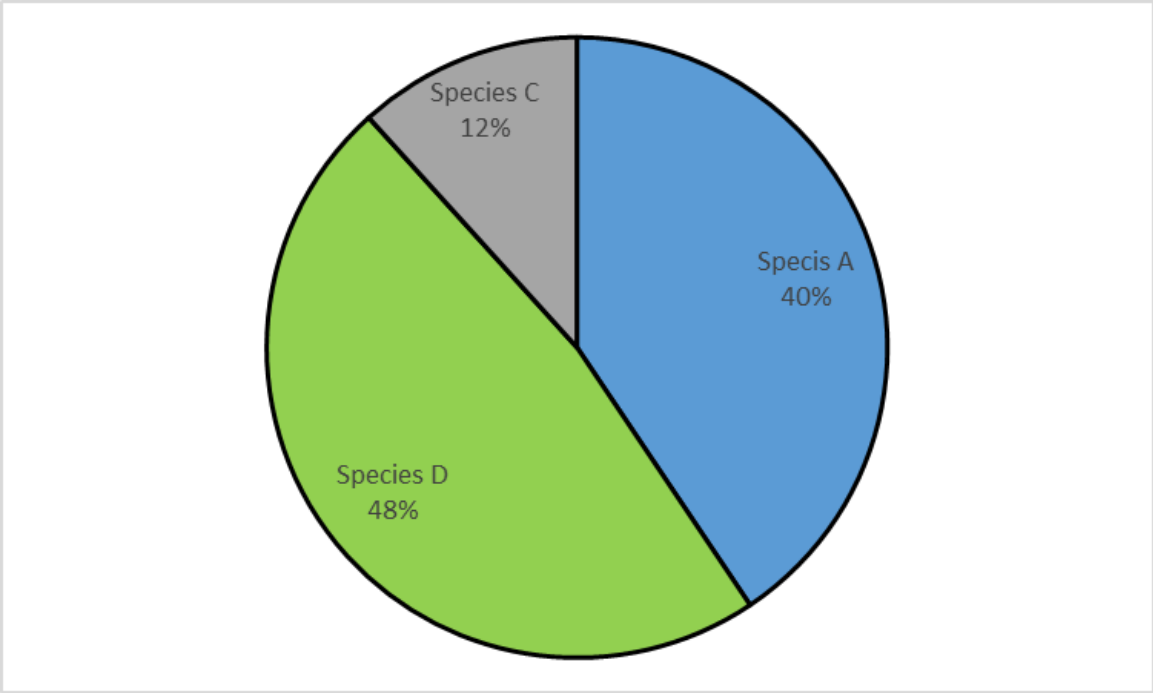

Figure 5. Percentage of species from the *An. crucians* complex found throughout Lowndes County, GA.

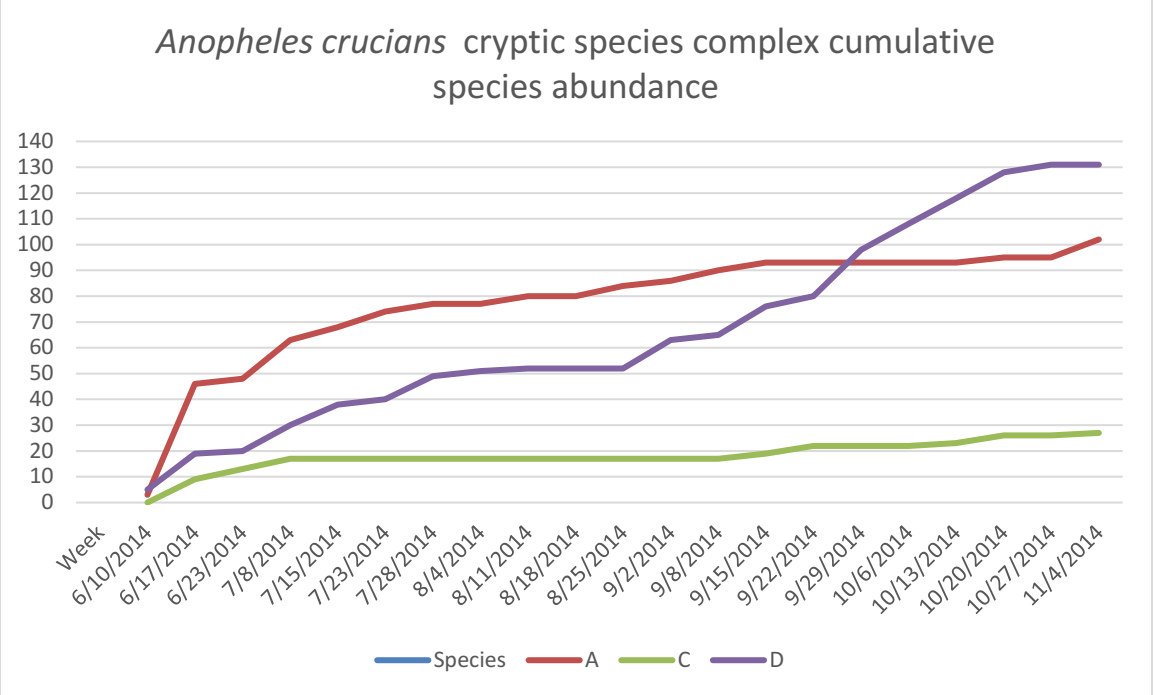

Figure 6. *Anopheles crucians* cryptic species complex cumulative species abundance by week during the 2014 trapping season.

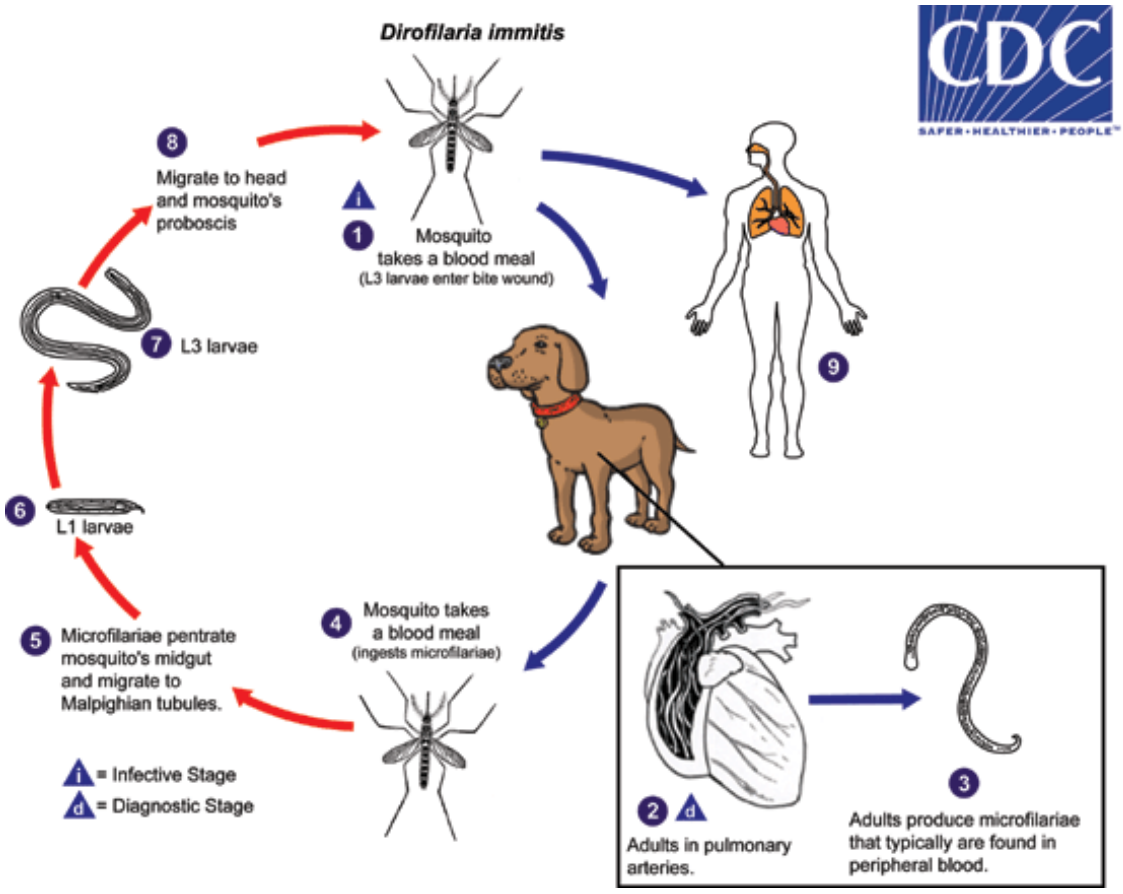

Figure 7. The life cycle of the filarial nematode worm, *Dirofilaria immitis*

APPENDIX B:

Tables for Chapters 1-3

Table 1. Total number of *An. crucians* mosquitoes identified by PCR amplification of the rDNA- ITS2 sorted by species and collection site in Lowndes County, GA.

Site	Species A	Species B	Species C	Species D	Species E	Species brad	Total
Baytree	3	0	8	0	0	0	11
Brown Rd.	7	0	0	17	0	0	24
Deloach	3	0	1	19	0	0	23
Hammock	4	0	0	2	0	0	6
Masonic Lodge	9	0	1	22	0	0	32
Old Clyattville Rd.	48	0	13	35	0	0	96
Plantation	2	0	3	0	0	0	5
Thomas	21	0	2	19	0	0	42
Fleming	0	0	0	0	0	0	0
Sheeley	0	0	0	0	0	0	0
Public Works	0	0	0	0	0	0	0
Barber	0	0	0	0	0	0	0
Bunche	0	0	0	0	0	0	0
Woodmen Circle	0	0	0	0	0	0	0
	97	0	28	114	0	0	239

Table 2. Maximum Likelihood of infection Estimate of *D. immitis* within the *An. crucians* cryptic species complex in Lowndes County, GA, USA

53 pooled heads/thoraces, 0 positive		
Point Estimators:		
Maximum Likelihood Estimate:	0.00E+00	
95% Confidence Intervals:		
Likelihood Ratio Method:	0.00E+00	3.66E-03
53 pooled abdomens, 1 positive		
Point Estimators:		
Maximum Likelihood Estimate:	1.92E-03	
95% Confidence Intervals:		
Likelihood Ratio Method:	5.94E-05	9.87E-03