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## Presence of *Dirofilaria immitis* in mosquitoes in Southeastern Georgia

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Presence of *Dirofilaria immitis* in mosquitoes in Southeastern Georgia

An Honors Thesis submitted in partial fulfillment of the requirements for

Honors in the Department of Biology

by

Angelica C. Tumminello

Under the mentorship of Dr. William Irby, PhD

### ABSTRACT

Canine heartworm disease is caused by the filarial nematode *Dirofilaria immitis*, which is transmitted by at least 25 known species of mosquito vectors. This study sought to understand which species of mosquitoes are present in Bulloch County, Georgia, and which species are transmitting canine heartworm disease. This study also investigated whether particular canine demographics correlated with a greater risk of heartworm disease. Surveillance of mosquitoes was conducted in known heartworm-positive canine locations using traditional gravid trapping and vacuum sampling. Mosquito samples were frozen until deemed inactive, then identified by species and sex. The mosquitoes were then either dissected under a microscope and/or set aside for detection of heartworm DNA using TaqMan Polymerase Chain Reaction (PCR). Species, sex, date collected, trap type, feeding status, and heartworm presence were recorded for each specimen in the lab. A primer specific to *Dirofilaria immitis* was used for TaqMan PCR. Shelter intakes at the Humane Society for Greater Savannah provided additional data on how heartworm affects dogs of varying demographics. Two species of mosquito, *Culex quinquefasciatus* (CQ) and *Culex nigripalpus* (CN), were identified as heartworm vectors in Bulloch County. No correlations between heartworm status and dog demographics were found; however, a more pertinent issue was discovered – the lack of publicly available data for heartworm status of companion animals.

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November 2019  
Department of Biology  
University Honors Program  
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## ACKNOWLEDGEMENTS

*I would like to express my gratitude to Dr. William Irby for his guidance, encouragement, and support throughout the duration of this project as well as for species identification of the mosquitoes. Thank you to Dr. Stephen Greiman for guiding me throughout the molecular portion of this project and allowing me to utilize his equipment. Thank you to Ansleigh Banks, who trained me in the field work involved with the project and spent countless hours dissecting mosquitoes with me. I would also like to thank my peers Lizzie Reigleman, Tyler Prichard, Caden Boyette, Ally Love, Devin Abbott, and Erin Pearson for their contributions to the field work involved. I would like to thank the Chandler Family, the Georgia Southern Biology Department, and TriBeta Biological Honor Society for providing funding and making this project a possibility.*

## INTRODUCTION

Canine heartworm disease is considered to be the most important vector-borne disease of dogs in the United States. It is caused by the filarial nematode, *Dirofilaria immitis*, that causes filariasis in domestic and wild canids. *Dirofilaria immitis* requires two hosts – a mosquito intermediate host and canine definitive host – to complete its life cycle development (Fig. 1).

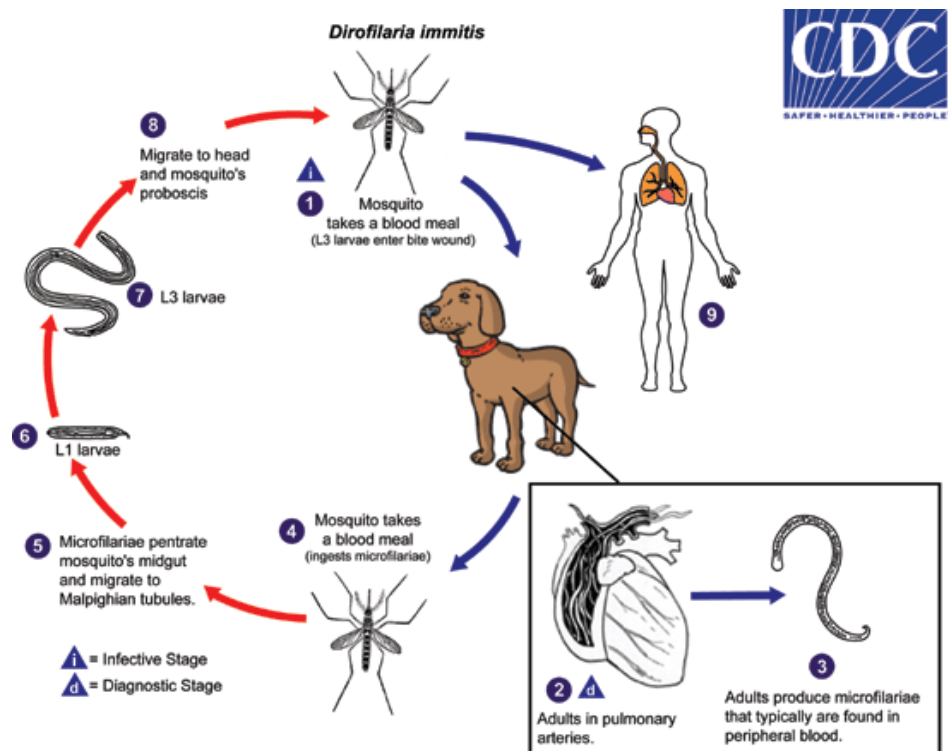


Figure 1. The canine heartworm lifecycle (CDC, 2012).

Mosquitoes act as vectors of the disease by ingesting microfilariae from infected dogs. The microfilariae develop into infective larvae that are then transmitted when the mosquito feeds again. Dogs and other canids are the primary and definitive natural host for heartworm, which means that over the course of 6-7 months, the nematodes can develop into their adult stage and reproduce inside the dog. Developmentally mature

nematodes are between seven and fourteen inches long and have the potential to clog the dog's heart, lungs, and arteries.

Sexually mature worms can reproduce by releasing microfilariae into the dog's bloodstream. Microfilariae are not able to mature in the dog; however, they are able to live in the animal's bloodstream for up to three years. Within those three years, many mosquitoes ingest blood meals from the dog and are thereby infected with the microfilariae, continuing the host cycle and infecting more dogs. If left untreated, dogs with heartworm can develop severe lung disease, suffer heart failure, and sustain lasting damage to internal organs, all of which will ultimately affect the dog's health, quality of life, and longevity. If the heartworm burden increases, blood flow to the heart can be compromised and lead to a condition known as Caval Syndrome, which can ultimately result in heart failure and death (Serrano-Parrenó et al., 2017).

Fast and cost-effective heartworm blood tests are available at veterinary clinics, as are monthly prophylactic medications to prevent the disease. However, many owners decline these preventative tests and medications. By the time clinical signs such as a mild and persistent cough, fatigue after moderate activity, decreased appetite, and weight loss arise in pets, the worms have developed into adults, and the disease has progressed (Serrano-Parrenó et al., 2017). This leaves many owners facing large upfront costs for treatment with no alternative options other than palliative care till end of life.

Mosquitoes are a developmental host and vector for canine heartworm and provide a model organism for this study, allowing for readily available collection of data. At least 25 species of mosquito present in the United States are able to transmit heartworm in nature including *Aedes*, *Anopheles*, *Culex*, and *Mansonia* species (Ledesma

and Harrington, 2011). After the mosquito ingests the microfilariae from an infected canine, the microfilariae migrate from the midgut to the Malpighian tubule cells, which are the equivalent of a kidney in mosquitoes. The microfilariae develop into their “sausage” form, which is shorter, thicker, and immobile, and also known as the L1 or first larval stage. After eight days, the first molt occurs in the Malpighian tubules, which results in the second larval stage (L2). During the L2 stage, the internal organs of the nematode form. Between the eleventh and twelfth day, the third larval stage (L3) forms, which resembles a small adult worm (Montarsi et al., 2015). These worms puncture the Malpighian tubules and move through the hemocoel to the mosquito head. The nematodes aggregate in the mouth parts of the mosquito and are considered infective larvae because they are able to be transferred to other hosts. The infective larvae can be transferred from the mosquito to the blood stream of the host through the puncture wound that results from a mosquito feeding, thus continuing the heartworm life cycle.

Heartworm has been reported in all U.S. states except Alaska and is particularly common in the Southeastern United States (Fig. 2). Due to the region rarely experiencing below-freezing temperatures, mosquito biting activity is present year-round in Georgia, a problem exacerbated by environmental and man-made changes leading to the spread and increased prevalence of heartworms. Exposure risk increases in warm climates, in regions with increased mosquito populations, and in regions with high numbers of infected dogs (Wang et al., 2014).

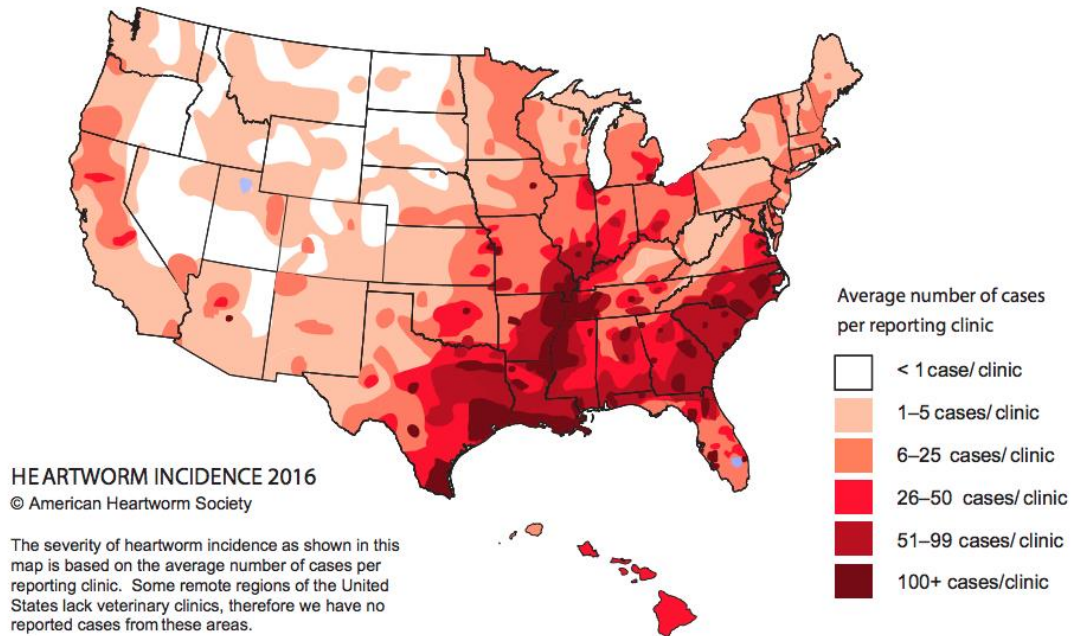


Figure 2. Average number of positive heartworm tests per veterinary clinic in the U.S. in 2016. Bulloch County, Georgia falls into the 100+ cases per clinic range (American Heartworm Society, 2016).

Data provided by IDEXX and ANTECH laboratories gathered from veterinary clinics across the U.S. shows that Bulloch County, Georgia represents one of the highest infection rates in the country: 5.4 percent compared to a state rate of 2.2 percent and a countrywide rate of 1.3 percent (Companion Animal Parasite Council, 2019). Therefore, our location provided an ideal opportunity to study the mosquito vectors present in our county and to determine which species are transmitting canine heartworm.

## METHODS

### *Field Work*

Field work was conducted in Bulloch County, due to the area's proximity to my institution of study and the high prevalence of canine heartworm disease in the area (Fig. 2). This study was two-and-a-half years in length, lasting from April of 2017 through November of 2019. Traditional gravid (Fig. 3) and vacuum trapping (Fig. 4) from vegetation or from under bridges were used for collection of mosquitoes. Gravid trapping was used in warm months beginning mid-March until October. This method employs the use of "stink bait" consisting of chicken manure mixed with hay fermented in water to specifically attract female mosquitoes that have blood-fed and are attracted to egg-laying substrates and are therefore more likely to contain infected mosquitoes. Likewise, vacuum sampling selectively collects higher proportions of blood-fed and gravid mosquitoes than other techniques, such as light traps.



Figure 3. Gravid trap set-up.





Figure 4. Vacuum trapping set-up.

Traps typically were put out weekly at dusk and collected early the next morning. During cooler months, vacuum trapping was conducted to continue data collection. Sampling locations were placed in the general area of known heartworm-positive canine locations (personal communication with local veterinarians indicated general areas of heartworm cases) – Mill Creek Regional Park, the Bulloch County Animal Shelter, the power substation at Azalea Drive, Mill Creek at Akins Pond Road bridge, Mill Creek at North Main/Lakeview Road bridge, and Mill Creek at 301 bridge (Fig. 5).

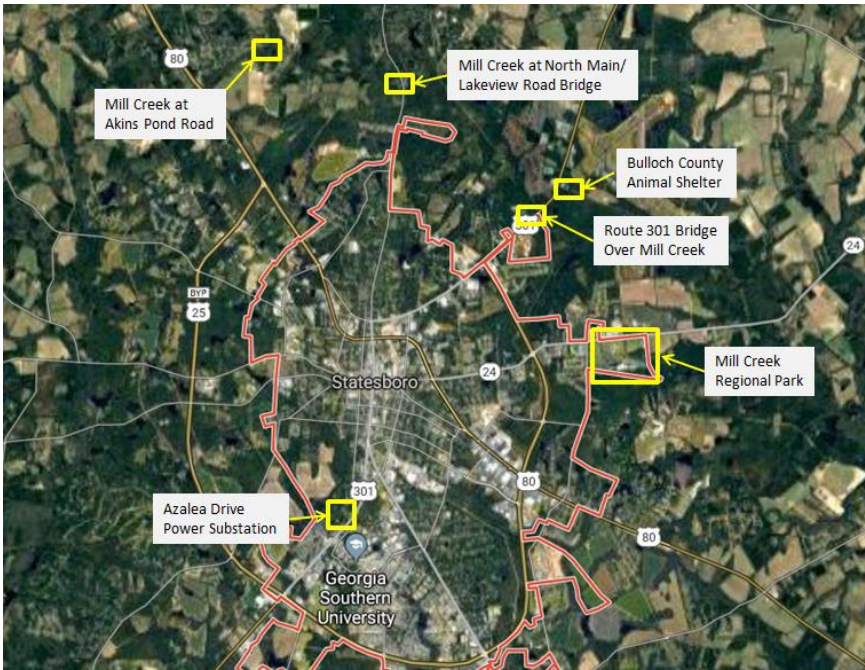


Figure 5. Sampling locations at known heartworm-positive canine locations.

Samples were taken to the lab for identification and either dissected or used in DNA analysis by Polymerase Chain Reaction (PCR) to identify heartworm larval stages present in the mosquito (Fig 6 and 7).

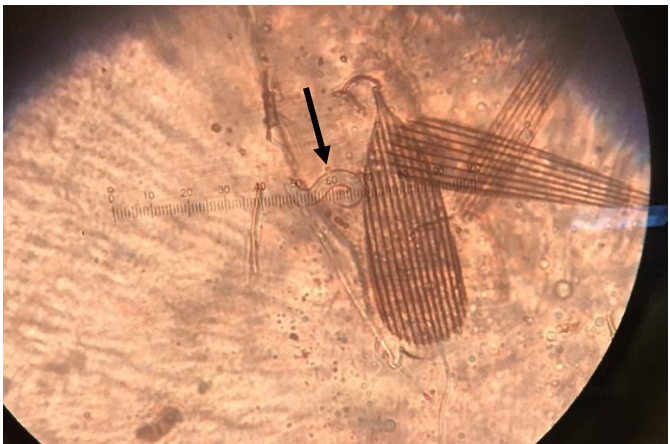


Figure 6. Possible heartworm larvae identified by visual detection under the microscope at 100X.



Figure 7. Possible melanized heartworm larvae visually identified by microscopic detection at 100X.

#### *Dissection*

For each mosquito, the species, sex, reproductive state (for females, unfed, bloodfed, or gravid), and type and location of collection were documented prior to dissection or PCR. For dissection, each mosquito was frozen until deemed inactive then placed in 0.8% physiological saline on a compound microscope slide under a dissecting scope. The mosquito was placed on a glass slide and pulled apart between the thorax and abdomen and then between the thorax and head. The hindgut and Malpighian tubules were teased out of the posterior of the abdomen. These body parts were gently smashed, and a coverslip was placed on top. This allowed for optimal viewing of the internal contents of the mosquito under a compound microscope at 100 and 400X magnification. The contents then were searched for the presence of live or melanized heartworm larvae in their L1 or L2 stages in the Malpighian tubules and in the L3 stage in other parts of the mosquito. Positive and negative specimens were recorded accordingly.

### *Polymerase Chain Reaction*

DNA analysis has been shown to be effective for heartworm detection, and TaqMan PCR with appropriate primers and probes is specific enough to rule out the possibility of other roundworm species. For DNA analysis, quantitative Norgen Biotek TaqMan PCR was utilized with primers specific to the mitochondrial DNA of *D. immitis*, which was visualized with fluorophore-labeled probes. Not only is PCR often more accurate, but it also provides a real-time map of the increase in the amount of material. Taqman PCR utilizes double-stranded DNA, a forward primer, a reverse primer, and a probe. The primers function to extend the DNA via Taq Polymerase. The probe has a reporter on the 5' end and a quencher on the 3' end. When the reporter and quencher are in close proximity to one another, the quencher prevents the reporter from fluorescing. When Taq Polymerase begins to replicate the DNA strand by attaching to a primer, it runs into the probe. The probe cannot be extended by Taq Polymerase, so it is cleaved in the DNA replication process. When the probe is cleaved, the reporter and quencher are separated, allowing the reporter to fluoresce, which indicates a positive result – the presence of *D. immitis* in this experiment (Fig. 8).

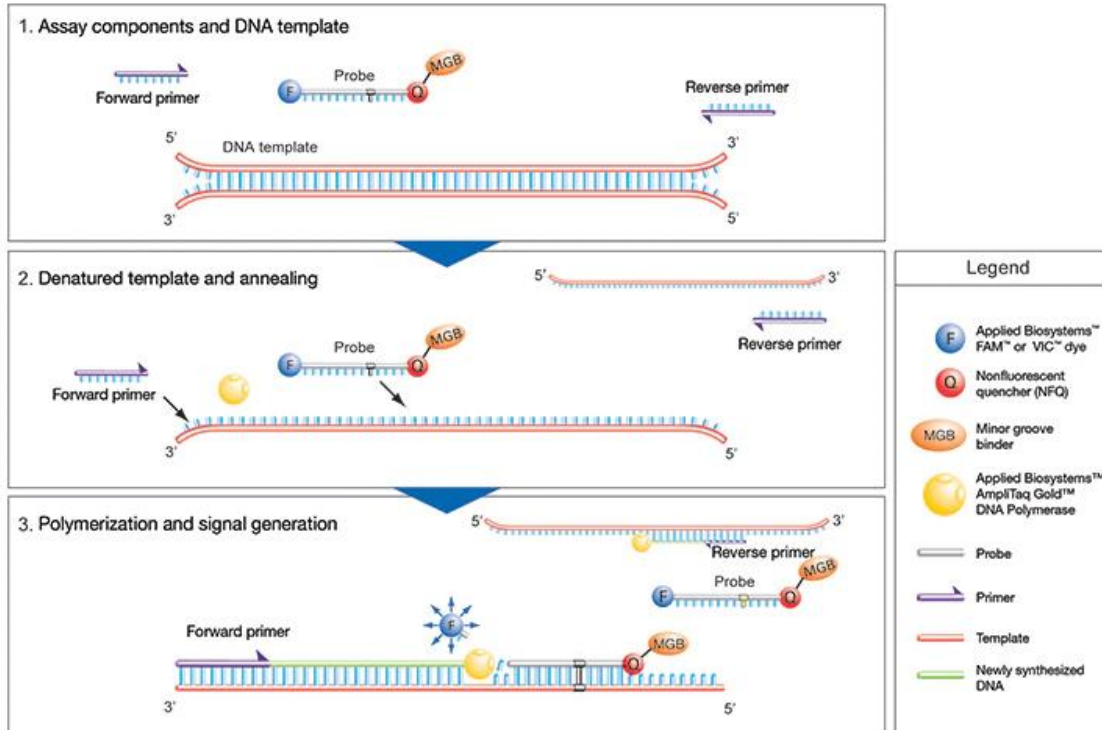


Figure 8. TaqMan PCR gene expression assay process (Thermo-Fisher Scientific, 2019).

### Preparation of Lysate

Mosquitoes from the same location, sample date, and species were set aside for PCR, stored in pools of up to 10 in 95% ethanol at  $-20^{\circ}\text{C}$ . Pools of five were then used for DNA isolation using Norgen Biotek's Cells and Tissue DNA Isolation Kit. Ethanol was removed from each sample, first using a pipette and then a heating block at  $96^{\circ}\text{C}$ , as ethanol is known to be a strong inhibitor of PCR. Each pool of five mosquitoes was homogenized using a microfuge-sized pestle in the provided Lysis Buffer B. Once the batch was homogenized,  $20\ \mu\text{L}$  of Proteinase K was added to each sample and incubated at  $56^{\circ}\text{C}$  for at least 1 hour. Each tube was then briefly spun in a centrifuge at 8,000 RPM to collect any drops of liquid from the lid before  $300\ \mu\text{L}$  of nuclease-free water was

added to each sample and mixed by vortexing. The tubes were then briefly spun again in a centrifuge to collect any liquid from inside the lid before 110  $\mu\text{L}$  of ethanol was added. Then, each sample was mixed by vortexing and again spun briefly in the centrifuge.

#### *DNA Purification from Lysate*

Each sample was assigned a spin column and collection tube. Then, 700  $\mu\text{L}$  of lysate was applied to the spin column assembly, which was then capped and centrifuged for 2 minutes at 8,000 RPM. Flow-through into the collection tube was discarded, and the spin column was reassembled so that 500  $\mu\text{L}$  of Solution WN could be applied to each column and centrifuged for 1 minute at 8,000 RPM. Flow-through into the collection tube was again discarded and the spin column reassembled so that 500  $\mu\text{L}$  of Wash Solution A could be applied to the column and centrifuged for 1 minute at 14,000 RPM. Flow-through into the collection tube was discarded, and the spin column was reassembled. Another 500  $\mu\text{L}$  of Wash Solution A was applied to the column and centrifuged for 1 minute at 14,000 RPM. Flow-through into the collection tube was discarded, and the spin column was reassembled. The column was spun for an additional 2 minutes at 14,000 RPM to thoroughly dry the column. The collection tube was discarded.

#### *Elution of Clean DNA*

Each column was placed into a 1.7-mL elution tube, and 200  $\mu\text{L}$  of Elution Buffer B was added to the column. Each tube was incubated at room temperature for at least one minute and then centrifuged at 8,000 RPM followed by one minute at 14,000 RPM. Each purified DNA sample was then stored at  $-20^{\circ}\text{C}$ .

### TaqMan PCR

All TaqMan PCR components were thawed at room temperature, mixed by gentle vortexing, and centrifuged briefly. A negative control consisting of 8  $\mu\text{L}$  of nuclease-free water, 10  $\mu\text{L}$  of Applied Biosystems Universal TapMan PCR MasterMix, 2  $\mu\text{L}$  of Primer, and Probe mix was placed in PCR plate well 1A. A positive control consisting of 10  $\mu\text{L}$  of Applied Biosystems Universal TapMan PCR MasterMix, 2  $\mu\text{L}$  of primer and probe mix, and 8  $\mu\text{L}$  of positive control was placed in PCR late well 1B. Sample wells each received 5  $\mu\text{L}$  of nuclease-free water and 10  $\mu\text{L}$  of Applied Biosystems Universal TapMan PCR MasterMix. Then, 4  $\mu\text{L}$  of each sample was placed consecutively in its own individual well 1C-12H.

TaqMan PCR Assay Programming

A Fisher Scientific StepOnePlus thermocycler was programmed to run the steps in the table below (Table 1).

Table 1. Thermal cycler profile.

<b>PCR Cycle</b>	<b>Step</b>	<b>Temperature</b>	<b>Duration</b>
<b>Cycle 1</b>	Step 1	50°C	2 min
	Step 2	95°C	15 sec
<b>Cycle 2</b>	Step 3 x 40X	60°C	1 min

TaqMan PCR Assay Interpretation

Data was recorded using the StepOnePlus software system. Assay results were interpreted based on the table below (Table 2).

Table 2. Assay interpretation.

<b>FAM (Target detection)</b>	<b>HEX (PCR validation)</b>	<b>Result</b>
+	+	Positive
-	+	Negative
-	-	PCR Inhibited



### *Canine Demographics*

Communication and cooperation with the Humane Society of Greater Savannah provided access to domestic dog population demographics and heartworm test results. Dog demographics were recorded for each humane society intake including sex, spay/neuter status, and breed and compared with heartworm status data to investigate correlations.

Communication was also attempted with the Bulloch County Animal Shelter, the Humane Society of Statesboro and Bulloch County, Augusta Animal Services, CSRA Humane Society, Columbia County Animal Services, Columbia County Humane Society, Screven County Animal Control, Jenkins County K911 Rescue, Burke County Animal Control, Old Fella Burke County Animal Rescue, Girard Lifesaver Dog Rescue, Effingham County Animal Shelter, Humane Society of Effingham County, Bryan County Animal Control, Humane Society of Evans County, Metter Animal Shelter, Emanuel County Animal Shelter, Emanuel County Humane Society, Washington County Animal Shelter, Chatham County Animal Services, McDuffie County Animal Shelter, Soperton Animal Shelter, Diamonds in the Ruff Dog Rescue, Laurens County Animal Control, Humane Society of Laurens County, Vidalia Animal Control, Liberty County Humane Shelter, Liberty County Animal Control, Twiggs County Animal Control, and Southern Comfort Animal Rescue. The organizations, however, were often unresponsive, could not afford heartworm testing, or did not keep records.

## RESULTS

Overall, a total of over 4,000 mosquitoes representing 25 species in Bulloch County were identified during this study (Fig. 9). Over 70 percent of mosquitoes gathered represented the species *Culex quinquefasciatus* (CQ). Other numerous species included *Culex nigripalpus* (CN) representing 13 percent, *Culex erraticus* (CE) representing 4.7 percent, and *Aedes atlanticus* (EL) representing 3.8 percent (Fig. 10 and Table 3). The highest numbers of mosquitoes collected were between the months of April through October (Fig. 11). Over 65 percent of mosquitoes collected were from the Azalea Drive power substation location (Fig. 12). Over half of the mosquitoes collected were gravid females useful for our heartworm study, but bloodfed and unfed mosquitoes were also dissected; 2,862 mosquitoes were gravid, 908 were unfed, and 273 were bloodfed (Fig. 13).

Possible positive species found during the dissection process included *Culex quinquefasciatus* (CQ), *Culex nigripalpus* (CN), *Culex erraticus* (CE), and *Aedes atlanticus* (EL) (Table 4). Out of 82 pooled samples used in TaqMan PCR, 5 samples were positive for *D. immitis*, and 77 samples were negative for *D. immitis* (Fig. 14). Positive species included *Culex quinquefasciatus* (CQ) and *Culex nigripalpus* (CN). Positive locations included Azalea Drive, Mill Creek Park, and the Bulloch County Animal Shelter (Table 4).

Data for 869 canine intakes at the Humane Society of Greater Savannah provided us with demographical information including sex, spay/neuter status, breed, and heartworm status. Data analysis indicated that 10.9 percent of intakes were heartworm positive, 53.4 percent of intakes were heartworm negative, and 35.7 percent of intakes

were not tested (Fig. 15). Comparison of heartworm status to sex indicated that there were 251 heartworm-negative males, 213 heartworm-negative females, 50 heartworm-positive males, 45 heartworm-positive females, 119 not tested males, and 94 not tested females (Fig. 16). Comparison of heartworm status to spay/neuter status indicated that 207 of the heartworm-negative dogs were spayed, 249 of the heartworm-negative dogs were neutered, 8 of the heartworm-negative dogs were intact, 45 of the heartworm-positive dogs were spayed, 50 of the heartworm-positive dogs were neutered, no heartworm-positive dogs were intact, 87 of the not tested dogs were spayed, 107 of the not tested dogs were neutered, and 116 of the not tested dogs were intact (Fig. 17). Within the heartworm-positive group, 59 percent were mixed breed; 8.4 percent were pit bulls; 7.4 percent were retrievers; 6.3 percent were hounds; and 18.9 percent were other various breeds, each representing less than 5 percent (Fig. 18).

Table 3. Species diversity of female mosquitoes collected at each heartworm case location in Bulloch County (n = 2,862).

Species	Mill Creek at Akins Pond Road Bridge N= 12	Mill Creek at Lakeview Road Bridge N=8	Route 301 Bridge over Mill Creek N=10	Bulloch County Animal Shelter N=45	Mill Creek Regional Park N=46	Azalea Drive Power Substation N=53
<i>Aedes albopictus</i> (EB)	0	0	0	8	17	12
<i>Aedes atlanticus</i> (EL)	0	0	0	110	1	1
<i>Aedes triseriatus</i> (ET)	0	0	0	1	0	0
<i>Anopheles crucians</i> (AC)	5	5	1	1	1	0
<i>Anopheles inundatus</i> (AI)	3	0	1	0	0	0
<i>Anopheles maverlius</i> (AM)	13	0	0	0	0	0
<i>Anopheles punctipennis</i> (AP)	3	0	0	0	0	1
<i>Anopheles quadrimaculatus</i> (AQ)	28	2	4	0	0	2
<i>Anopheles smaragdinus</i> (ASM)	0	1	3	0	0	0
<i>Anopheles sp.</i> (AN)	11	12	20	0	0	0
<i>Culex coronator</i> (CC)	0	0	0	3	3	13
<i>Culex erraticus</i> (CE)	32	44	66	0	0	0
<i>Culiseta melanura</i> (CM)	0	0	0	1	0	0
<i>Culex nigripalpus</i> (CN)	2	0	0	16	62	311
<i>Culex pilosus</i> (CO)	3	0	0	0	0	1
<i>Culex quinquefasciatus</i> (CQ)	0	0	0	298	171	1517
<i>Culex restuans</i> (CR)	1	0	1	2	8	13
<i>Culex salinarius</i> (CL)	0	0	1	0	0	0
<i>Culex territans</i> (CT)	0	2	0	0	4	2
<i>Toxorhynchites rutilus</i> (TR)	0	0	0	0	0	1
<i>Uranotaenia lowii</i> (UL)	0	0	0	0	8	0
<i>Uranotaenia sapphirina</i> (US)	1	0	0	0	6	0
<i>Orthopodomyia signifera</i> (OS)	0	0	0	2	0	0
<b>Total</b>	102	66	97	442	281	1874

Table 4. Positive sample species, date, location, and identification type. “Yes” indicates heartworm positive; “no” indicates heartworm negative; “not tested” indicates that detection method was not utilized. \*PCR samples were pooled in batches of five of the same species, date, and location.

<b>Species</b>	<b>Date</b>	<b>Location</b>	<b>Dissection</b>	<b>PCR*</b>
<i>Culex quinquefasciatus</i>	13-VI-2017	Azalea Drive	Yes	Not Tested
<i>Culex quinquefasciatus</i>	7-V-2018	Mill Creek Park	Yes	Not Tested
<i>Culex quinquefasciatus</i>	25-V-2018	Mill Creek Park	Yes	Not Tested
<i>Culex quinquefasciatus</i>	11-VII-2019	Azalea Drive	Not Tested	Yes
<i>Culex quinquefasciatus</i>	9-IX-2018	Azalea Drive	Not Tested	Yes
<i>Culex quinquefasciatus</i>	16-X-2018	Mill Creek Park	Not Tested	Yes
<i>Culex quinquefasciatus</i>	1-XI-2018	Azalea Drive	Not Tested	Yes
<i>Culex nigripalpus</i>	20-VIII-2018	Animal Shelter	Yes	No
<i>Culex nigripalpus</i>	9-X-2018	Mill Creek Park	Yes	Yes
<i>Culex erraticus</i>	15-III-2019	Akins Pond Road	Yes	No
<i>Aedes atlanticus</i>	5-X-2017	Animal Shelter	Yes	Not Tested

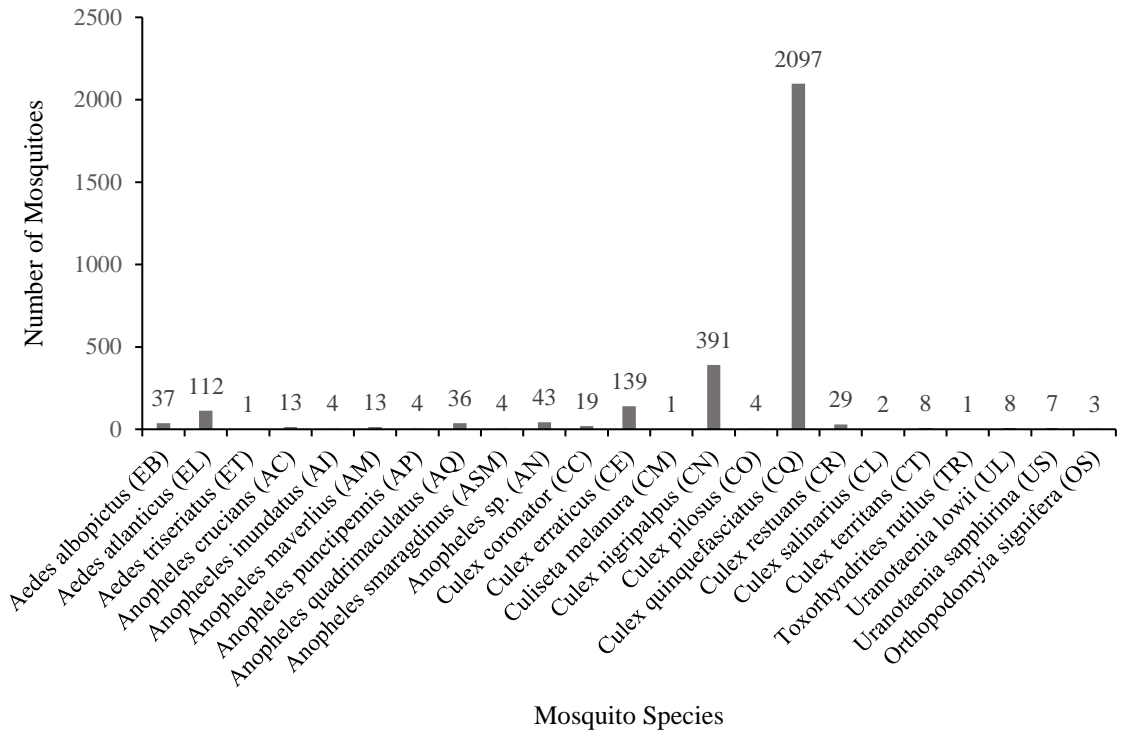


Figure 9. Mosquito species diversity present in Bulloch County, Georgia at heartworm case locations.

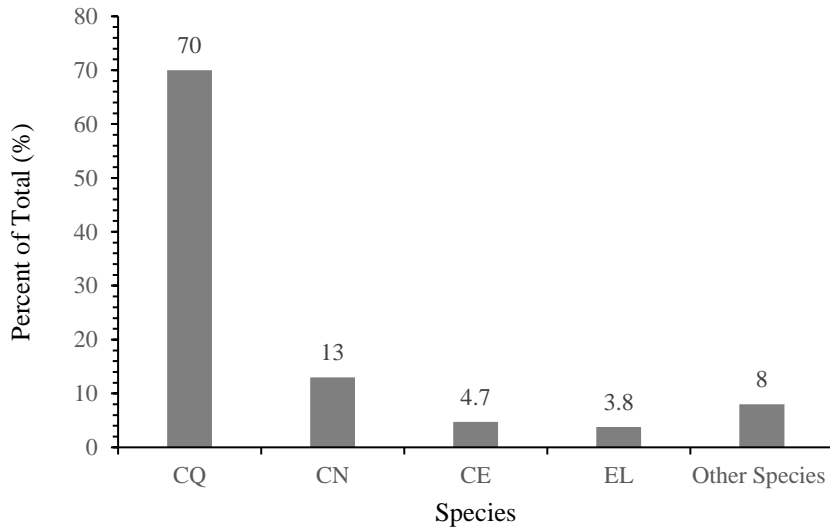


Figure 10. The most abundant species of mosquitoes collected and the percent of total mosquitoes collected that they represent at heartworm case locations in Bulloch County.

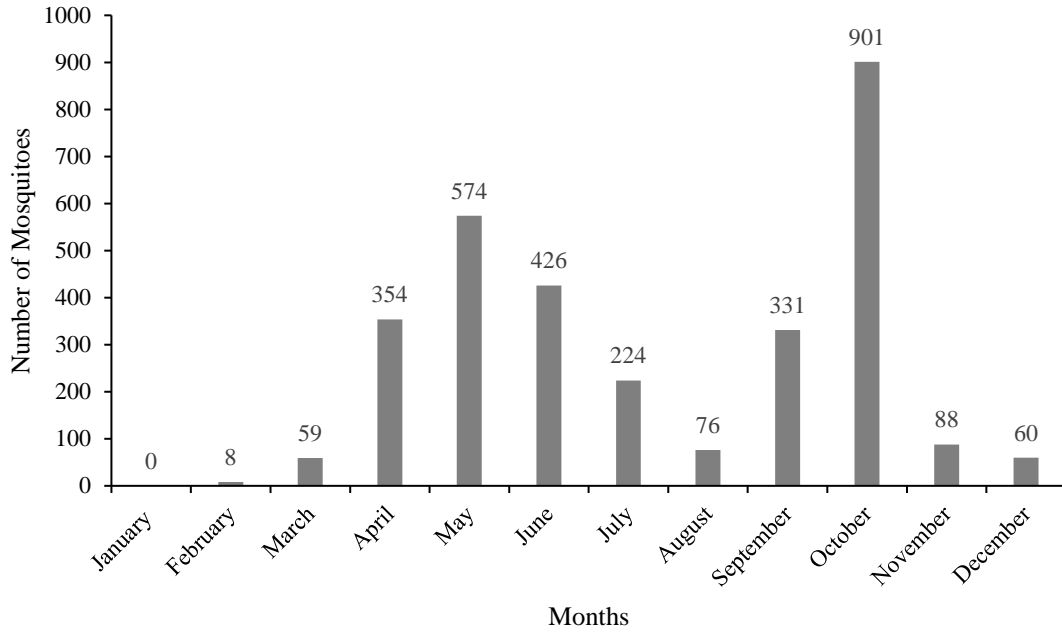


Figure 11. The abundance of mosquitoes collected during both vacuum sampling and gravid trapping by month at heartworm case locations in Bulloch County.

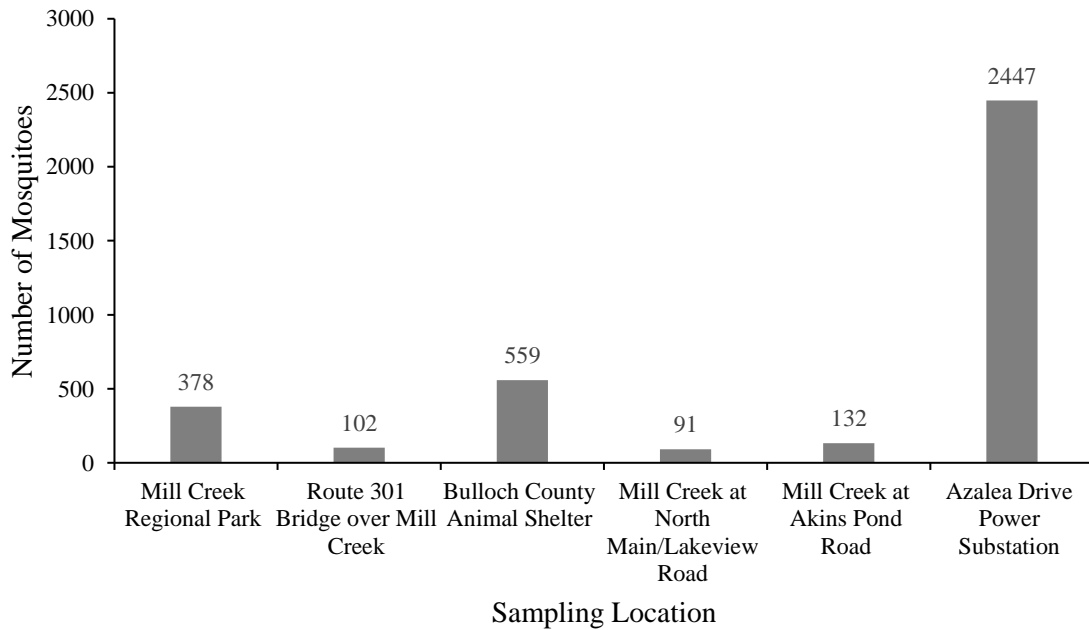


Figure 12. Number of mosquitoes collected at each heartworm case location in Bulloch County over the course of this study.

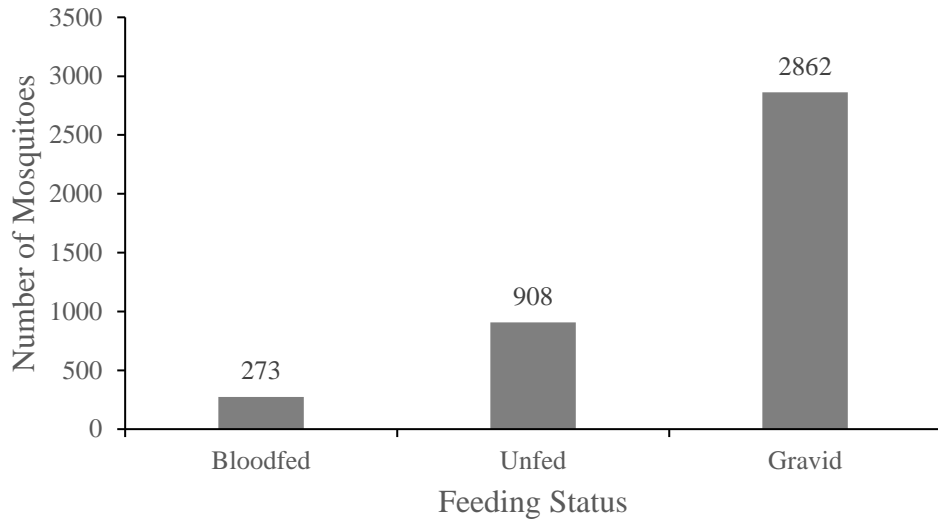


Figure 13. Number of bloodfed, unfed, and gravid mosquitoes collected at heartworm case locations in Bulloch County (n = 4,043).

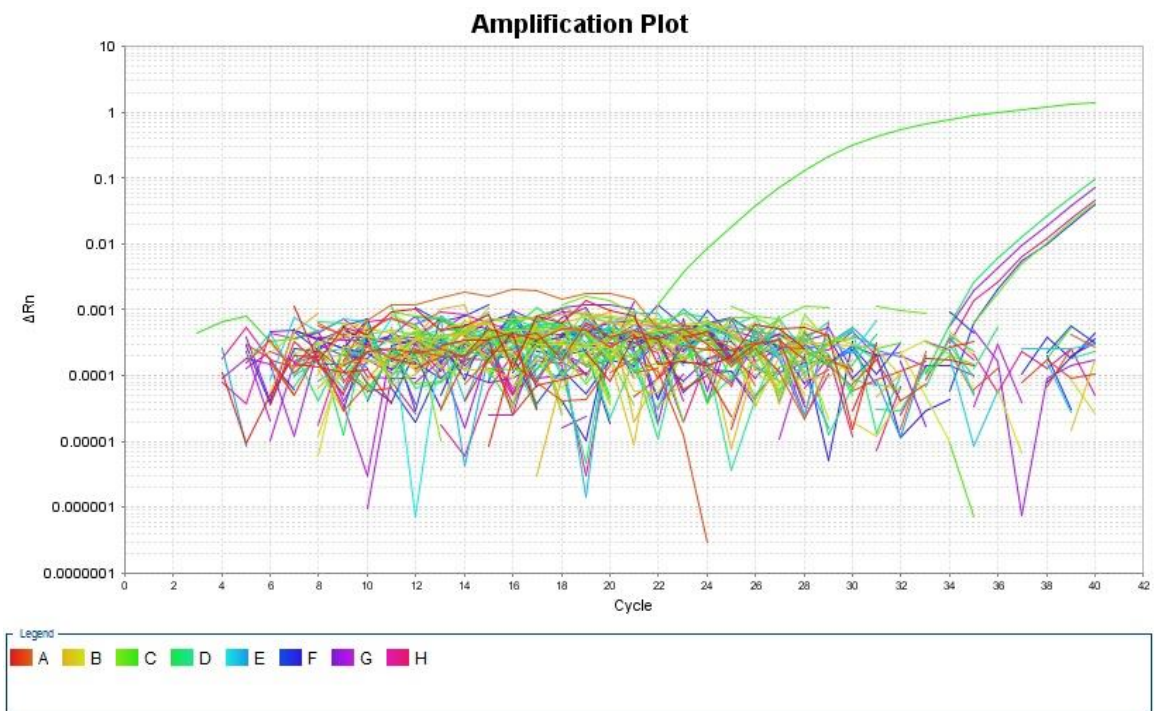


Figure 14. TaqMan PCR amplification plot indicating the positive control (first green curve) and positive samples 19, 20, 38, 47, and 64 (subsequent curves).



### Percentage of Canine Intakes

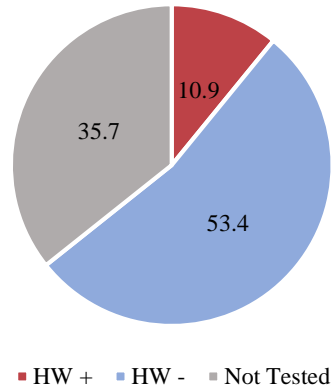


Figure 15. Percentage of canine intakes that were heartworm-positive, heartworm-negative or not tested at the Humane Society of Greater Savannah.

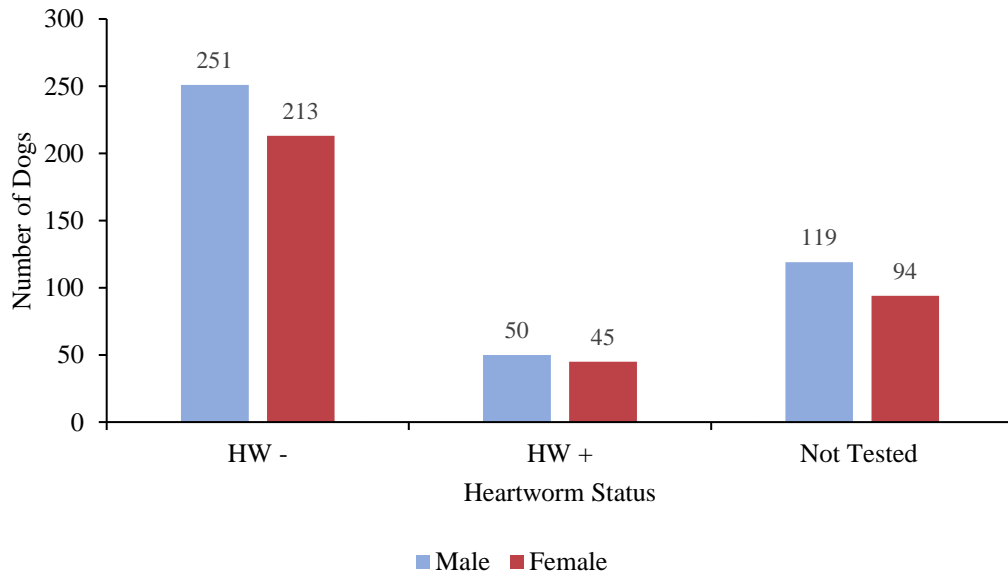


Figure 16. Comparison of heartworm-negative, heartworm-positive, and untested dogs based on sex at the Humane Society of Greater Savannah.

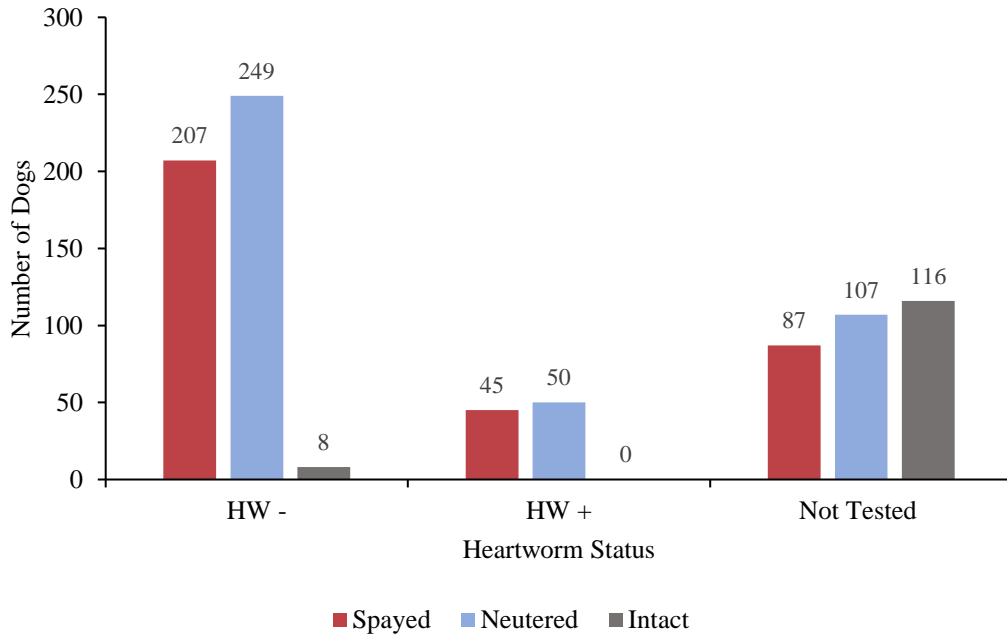


Figure 17. Comparison of heartworm-negative, heartworm-positive, and untested dogs based on spay/neuter status at the Humane Society of Greater Savannah.

Percentage of total HW+ dogs

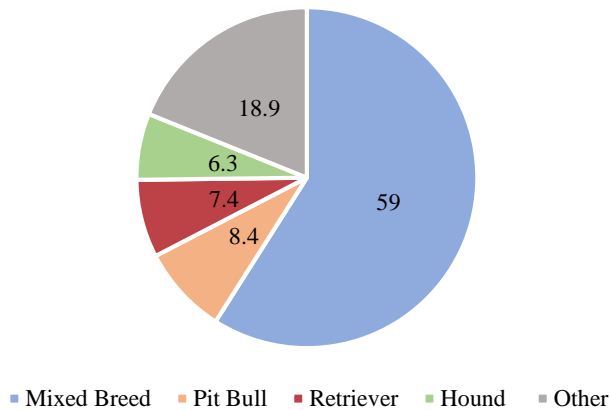


Figure 18. The four most common heartworm-positive breeds and the percentage of the total number of heartworm-positive dogs that they represent at the Humane Society of Greater Savannah.

## DISCUSSION

### *Mosquitoes*

The four most abundant species collected were *Culex quinquefasciatus* (70%), *Culex nigripalpus* (13%), *Culex erraticus* (4.7%), and *Aedes atlanticus* (3.8%), all representing the heartworm positive species found in this study (Fig. 10). *Aedes*, *Anopheles*, *Culex*, and *Mansonia* species are all known vectors of canine heartworm disease (Vector Disease Control International, 2019). Therefore, this study did not reveal any new species of heartworm vector. However, of interesting note, 70 percent of mosquitoes collected during this study were *Culex quinquefasciatus* (CQ), indicating this species plays a major role in the transmission of heartworm disease in Bulloch County, Georgia.

Significant mosquito species in Bulloch County to note are members of the *Anopheles quadrimaculatus* complex: *Anopheles quadrimaculatus s.s.*, *Anopheles diluvialis*, *Anopheles inundatus*, *Anopheles maverlius*, and *Anopheles smaragdinus*. All of the species in the complex are present in our region (Levine, Peterson, and Benedict, 2004). This study represents the first systematic attempt to determine the potential role of the members of this species complex in heartworm epizootiology. Although this study discovered no heartworm-positive *Anopheles* mosquitoes, because of their propensity for feeding on mammalian hosts and their year-round biting activity in our area, this deserves further attention.

Only one of the mosquitoes that was identified as a possible heartworm positive specimen during the dissection process was PCR-positive – the *Culex nigripalpus* collected in September of 2018 at Mill Creek Park. At least one sample was a melanized

larvae, which may have contributed to the sample being PCR-negative. Some of the samples visually identified may have been other filarial nematode species, which would not have been identified by the species-specific probe used in PCR. Also, some samples were stored for longer than a year in ethanol at -20°C before being tested using PCR.

Taking into consideration how large the mosquito population is in Bulloch County and how relatively small our sample sizes were implies that the prevalence of heartworm-positive vectors is likely very 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 Bulloch County, Georgia.

### *Canines*

No significant differences were present when comparing sexes and spay/neuter status to the heartworm status of canines (Fig. 16 and 17). However, dogs labeled as “mixed breed” represented 59 percent of all heartworm-positive cases (Fig. 18). This could be due to an array of confounding variables including the popularity of mixed breeds, intakes by the humane society of mainly mixed breeds, and the possibility of owners being less likely to provide veterinary care for “mutts.” Future, more thorough studies of greater scale and length would be valuable in determining how canine heartworm affects dogs of varying demographics.

Before these studies can take place, however, a standardized way of collecting data for the infectious diseases affecting companion animals – similar to data collection of human infectious diseases by the Centers for Disease Control and Prevention – needs to be developed and made accessible to the public. The main challenge faced while

undertaking this project was the lack of data availability. After personally contacting over 30 county animal shelters, humane societies, and animal control groups, the most significant data obtained was from the Humane Society of Greater Savannah. Rural shelters were typically short-staffed with minimal record-keeping and no funding available for heartworm testing. Throughout the process, it was found that many rescue groups that did not have adequate funds to heartworm test the dogs in their care. During this study, many e-mails and phone numbers were found that no longer worked due to rescue groups lacking staffing and funding.

Initially, this study was also to include how the Carolina dog – a wild dog native to South Carolina and Georgia – is affected by heartworm disease in comparison with domestic canines. The Carolina dog, *Canus lupis familiaris*, was once a naturally selected wild dog. Data show that the ancestors of these dogs were present in pre-Columbian America. This entails that their ancestors were brought here across the Bering Land Bridge 8,000 to 11,000 years ago by Paleo-Indians traveling to North America from Asia (Van Asch et al., 2013). Today, the breed exists in small populations in the wild.

In the 1970s, Dr. I. Lehr Brisbin rediscovered a small, wild, genetically distinct colony still present along the South Carolina-Georgia border. Locals thought they were unwanted mutts wandering around due to their varying coloration. Carolina dogs come in many hues and combinations, including pale yellow buff and spotted black and tan; however, the most common color is a reddish tan. They all have pointed ears, a foxlike snout, a white stripe at the base of the neck above the shoulders and a curved tail (Fig. 18). They have often been compared to Australian dingoes or the description of the dog in Old Yeller and are referred to as “yallers” by the locals.



Figure 19. Standard Carolina Dog breed appearance (American Kennel Club, 2019).

Small colonies still exist – and are known to be present around the Savannah River Site at the Georgia-Carolina border due to the secluded nature of the area. The 310-square-mile area is remote because of the presence of the Savannah River Site nuclear facility run by the Department of Energy. This land has remained isolated from domestic dogs and, until recently, coyotes. This has allowed the Carolina dog population to remain genetically distinct from the remainder of the canine family. Carolina dogs often turn up in shelters throughout the Southeast due to being mistaken as feral or lost dogs.

DNA analysis of these wild dogs indicates that Carolina dogs fall at the base of the phylogenetic tree, making them primitive. The dogs in the wild are not a breed; however, there are a select group of people who domesticate and breed Carolina dogs. This breed has now officially been recognized by the American Rare Breed Association and the United Kennel Club (UKC) and categorized as a pariah dog. They have been categorized as pariah dogs not only because of their similarity to other primitive breeds but also due to a couple of distinct behaviors not presented by other domesticated dogs.

Carolina dogs – especially females – often dig small pits with their noses only in specific areas during the fall. The reason behind this behavior is still a point of interest in study. Carolina dogs also display pack behavior when hunting for food. Most interesting, is that their breeding cycles are regulated by the challenges of wilderness survival. Carolina dogs begin breeding at a young age, whelping litters multiple times per year. This is theorized to be an adaptation to fight the powers of natural selection. By having pups at a young age multiple times per year, the population ensures that the next generation is born before the existing population is infected with diseases, such as heartworm.

While looking for access to Carolina Dog populations, Dr. I. Lehr Brisbin, Senior Research Ecologist Emeritus of the Savannah River Ecology Lab (SREL), who discovered the breed while working for SREL was contacted. Dr. Brisbin studied a captive pack of Carolina dogs toward the end of his career. Upon retiring, he took the dogs into his personal care. They now reside on his property and are cared for as pets – including being on monthly heartworm medication. Dr. Brisbin was not able to provide access to wild Carolina dog populations other than offering that he still believes wild populations exist in wilderness portions of the Savannah River Site and other secluded areas in South Carolina and Georgia. Due to the inability to access these populations, the Carolina dogs were not included in this study; however, wild, feral, and domestic canines should be considered for future heartworm studies.

A large problem is present – especially in Southern states – where enormous numbers of stray animals exist yet adequate programs and funding are inadequate to manage these populations. Many Southern states still lack laws for registering or

licensing animals, enforcing spay/neuter regulations, and addressing the tethering of animals. This lack of regulation puts no moral responsibility on pet owners and greatly contributes to enormous numbers of roaming animals that continue to breed and act as reservoirs for infectious disease. States need to develop standardized reporting venues and authorities for stray animals instead of leaving the issue to county and local levels where adequate funding is not present.

The data obtained from the Humane Society of Greater Savannah represents animals that were deemed adoptable and worthy of further veterinary care and effort to place into forever homes. This data does not account for the numerous stray animals and unadoptable animals that are euthanized each year. Approximately 6.5 million animals enter U.S. shelters each year, with approximately 1.5 million of those animals being euthanized. Southern states like Georgia, Mississippi, Louisiana, and Texas have the highest kill rates in the country (Fox 24 WGXA, 2017). Common-sense laws and regulating bodies with adequate funding need to be implemented in these states as well as a standard data collection body so that animal welfare can become a priority and devastating diseases such as canine heartworm can be studied and controlled.



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