DISTRIBUTION PATTERNS OF THE *ANOPHELES QUADRIMACULATUS* (DIPTERA: CULICIDAE) SPECIES COMPLEX IN TEXAS

A Thesis

by

JENNIFER ANN MURRELL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Entomology

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Approved by:

Co-Chairs of Committee,	Jimmy K. Olson
	Craig J. Coates
Committee Member,	Leon H. Russell
Head of Department,	Kevin M. Heinz

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ABSTRACT

Distribution Patterns of the *Anopheles quadrimaculatus* (Diptera: Culicidae) Species Complex in Texas.

(December 2005)

Jennifer Ann Murrell, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Jimmy K. Olson
Dr. Craig J. Coates

The primary vector of malaria in the eastern United States, *Anopheles quadrimaculatus* (Say), was recently discovered to be a complex of five different cryptic species: A - *An. quadrimaculatus*, B - *An. smaragdinus*, C1 - *An. diluvialis*, C2 - *An. Inundatus*, D - *An. maverlius* (Reinert et al. 1997). In this research project, the goals were to determine which species were found in Texas, establish overall distribution patterns of those species, and observe the dates in which each specimens were collected so that any seasonal changes in species could be observed.

Both *An. quadrimaculatus* (A) and *An. smaragdinus* (B) were identified from collections made throughout Texas from September 2002 through January 2005. *Anopheles smaragdinus* only made up 3% of the total specimens collected and neither *An. inundatus* nor *An. maverlius* were collected in Texas, even though they have both been collected in neighboring Parishes in Louisiana.

Anopheles. quadrimaculatus' habitat and geographic range was found to be more extensive than *An. smaragdinus*. While *An. smaragdinus* was found only in the eastern half of Texas with no collection south of Fort Bend County, *An. quadrimaculatus* was

found throughout the eastern half of Texas, many of the southern Golf coast counties, and a few counties in far west Texas. The most common land cover where *An. quadrimaculatus* specimens were collected was on pasture/hay fields. This is very different from *An. smaragdinus* specimens in that pasture/hay was one of the least common land covers and the dominant land cover was woody wetlands. Overall, *An. smaragdinus* was usually associated with land covers that could provide shelter, while *An. quadrimaculatus* could be found among habitat that was more open and urban.

There was no observed change in the species composition over time in this study. In fact, when *An. smaragdinus* was collected, *An. quadrimaculatus* was usually collected at the same time. Both *An. quadrimaculatus* and *An. smaragdinus* were collected throughout late spring, summer and early fall. Of course, the collection times of these species could have been an artifact of when most of the collectors were looking for *An. quadrimaculatus* (Say) specimens.

DEDICATION

I dedicate this thesis to my husband, Jason Murrell. His support and endless patience has allowed me the opportunity to complete my master's degree and become a stronger person in the process. I also dedicate this thesis to my family who never doubted that I would finish and who have made me the person that I am today.

ACKNOWLEDGEMENTS

I would like to start by thanking Dr. Jimmy K. Olson for helping me develop this project and tweak it along the way. Thanks also to Dr. Craig Coates for his support and input about my genetics work and also for use of his wonderful lab facilities. My committee would not have been as well rounded as it was without the help of my third committee member, so thank you also Dr. Russell. Many graduate students along the way have also given me their support and taught me a great deal about research. Thanks to Darren Hagan for teaching me how to perform PCR and clone DNA. I appreciate that you always answered my questions and made sure that I understood all of the processes involved with my work. Thanks also to all of Dr. Coates Lab, especially Lara Lewey, Christine Gray, and Garret Hagan. I cannot forget to also thank everyone in the Mosquito Research Lab for the help with my research as well as for your help relaxing after work. I would like to especially thank Cat Zindler from the Mosquito Lab for all of her help with my GIS analysis. Thank you, everyone in the Entomology Department for the wonderful opportunities that I have had over the years.

I must also thank the Texas Department of State Health Services (TDSHS), Harris County Mosquito Control District (HCMCD), and all of the other Mosquito Control Districts and individuals that sent mosquito samples to TDSHS. Without these organizations and the people in them, I would not have had the quality and quantity of information that I have today. A special thanks to Glenna Teltow, Robyn Seiferth, and

Susan Real for sending me all of the *An. quadrimaculatus* (Say) mosquitoes that came in with the West Nile Virus testing.

This project could not have been completed without the monetary and emotional support of the Hispanic Leadership Fellowship in Agriculture and Natural Resources. Through this program, I learned what it means to be a leader and how important it is to help minority students enroll in higher education. Thank you to all of the staff of HLPANR and my fellow students.

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CHAPTER I

GENERAL INTRODUCTION

Malaria is a protozoan disease that accounts for approximately 500 million clinical cases of human disease in the world every year. The World Health Organization has placed malaria among the top ten most devastating diseases that the world faces today. Each year, this disease costs the world 42.28 million disability-adjusted life years (DALY), and 40% of the world is currently at risk of contracting malaria (WHO 2002). Most of the tropical and subtropical areas of the world have at least one species of the malaria parasite and one species of *Anopheles* that can vector malaria, but some areas in temperate climates also have problems with locally-transmitted malaria.

In each region of the world, different species of *Anopheles* mosquitoes are involved as vectors for malaria. The primary vector of malaria in the eastern United States, *Anopheles quadrimaculatus* (Say), was recently discovered to be a complex of five different cryptic species: A – *An. quadrimaculatus*, B – *An. smaragdinus*, C1 – *An. diluvialis*, C2 – *An. inundatus*, D – *An. maverlius* (Reinert et al. 1997). Researchers believe that some of the species may be more likely to vector malaria than others, so it is important that the geographic, habitat and temporal distribution of these species are known.

This thesis follows the format of the *Journal of the American Mosquito Control Association*.

In the United States, it has been fifty years since malaria was last considered to be an established disease. The eradication of malaria was due in large part to a population shift to urban areas, improved drainage and housing, improved human nutrition and an increased understanding and implementation of vector control. However, malaria still occur in the United States in isolated instances. Fortunately, most of these cases occur in individuals who travel to an endemic area in a foreign country and become infected while there (Causer et al. 2002). However, each year, several cases of locally-transmitted malaria occur with native *Anopheles* populations serving as vectors. From 1957-1994, 76 cases were documented as having been acquired via mosquito-born transmission in the United States (Zucker 1996). Most recently, seven documented cases of locally-transmitted *Plasmodium vivax* were reported in Palm Beach County, Florida. (*MMWR* 2003).

Many experts believe that malaria could become endemic in the United States once again, based on several current trends. Firstly, mosquitoes are becoming resistant to various pesticides, and the *Plasmodium* parasites are becoming resistant to different drugs used to prevent and treat malaria (Causer et al. 2002, Mallet and Porter 1993). Also, the increase and overall frequency and ease of travel abroad has created a serious cause for concern. It is now easier than ever to travel overseas, become infected with a disease like malaria, travel back to the United States, and then infect the local mosquitoes. Legal and illegal immigration of people from malarias areas into the United States also could cause an increase in the risk for local *Anopheles* populations to become infected with malaria. Further, the current trend of global warming has allowed many

tropical organisms, including mosquitoes, to increase their distribution range and reproduce more efficiently (Causer et al. 2002). Due to these and other factors, the probability of malaria becoming re-established in *Anopheles* mosquitoes in the United States has increased.

This project was intended to gather pertinent information about the *An. quadrimaculatus* mosquitoes in Texas so that a malaria outbreak can be controlled or even prevented in the future. The goals of this project were to discover which species are found in Texas, establish the geographic and environmental distribution of those species, and to observe any shifts in species occurrence that may happen over the span of the study, due to seasonal changes. The species in this complex are virtually impossible to discern using morphological characteristics; so, PCR primers designed by A. J. Cornel et al. (1996) based on ribosomal DNA ITS2 region are currently being used to differentiate the particular species in the *An. quadrimaculatus* species complex (e.g., Rutledge and Meek 1998, Rutledge et al. 1996, 1999).

CHAPTER II

LITERATURE REVIEW

Malaria in the United States

In the United States, it has been fifty years since malaria was last considered to be an established disease. The "eradication" of malaria was due in large part to a population shift to urban areas, improved drainage and housing, improved human nutrition, and an increased understanding and implementation of vector control. However, malaria still occurs in the United States in isolated instances. Fortunately, most of these cases occur in individuals who travel to an endemic area in a foreign country and become infected while there (Causer et al. 2002). However, each year, several cases of malaria in non-endemic foci (locally transmitted) occur with native *Anopheles* populations serving as vectors. From 1957-1994, 76 cases were documented as having been acquired via mosquito-born transmission in the United States (Zucker 1996). Most recently, seven documented cases of locally-transmitted *Plasmodium vivax* were reported in Palm Beach County, Florida in July and August of 2003. (*MMWR* 2003)**.** Loudoun County, Virginia reported two cases of locally transmitted *Plasmodium vivax* in August and March 2002. Four *An. quadrimaculatus* pools and one *An. punctipennis* pool, collected from September 23rd through October 11th, tested positive for *Plasmodium vivax* (*MMWR* 2002).

Many experts believe that malaria could become endemic in the United States once again, based on several current trends. Firstly, mosquitoes are becoming resistant to various pesticides, and the *Plasmodium* parasites are becoming resistant to different

drugs used to prevent and treat malaria (Causer et al. 2002, Mallet and Porter 1993). In addition, the increase and overall ease of travel abroad has created a serious cause of concern. It is now easier than ever to travel overseas, become infected with a disease like malaria, travel back to the United States, and then infect the local mosquitoes. Further, the current trend of global warming has allowed many tropical organisms, including mosquitoes, to increase their distribution range and reproduce more efficiently in previously temperate regions (Causer et al. 2002). Due to these, and other factors, the probability of malaria becoming re-established in *Anopheles* mosquitoes in the United States has increased.

Migrant workers from Mexico and immigrants from countries in Central and South America also provide a means that the Plasmodium parasite could become reestablished in the *Anopheles* mosquitoes found here in Texas. Since malaria is still endemic in Mexico and most other countries south of Texas a migrant farm worker who is infected could infect the local *An. quadrimaculatus* mosquitoes and start a malaria epidemic in that region of Texas. Migrant farm workers are a potential reservoir for malaria because they work outdoors and are, as a result, more likely to be bitten by a mosquito than people who work indoors. Unfortunately many migrant workers, as well as some immigrants, live in poor conditions and a mosquito could take advantage of any cracks, and open windows that would allow them to enter the house and feed upon the inhabitants while they sleep.

***Anopheles quadrimaculatus* biology**

As previously mentioned, *Anopheles quadrimaculatus* was recently discovered to be a complex of five different cryptic species: A – *An. quadrimaculatus*, B – *An. smaragdinus*, C1 – *An. diluvialis*, C2 – *An. inundatus* and D – *An. maverlius* (Kaiser 1994, Reinert et al. 1997). The most widely-distributed species is *An. quadrimaculatus quadrimaculatus* (A). This species can be found eight months out of the year, usually March through October, south of isotherm 18^0. Between isotherm 14^0 and 18^0, the adults are only active six months out of the year, usually April through September. In warmer climates, like southern Florida, the adults can breed throughout the year (Kaiser 1994).

Adults in the *An. quadrimaculatus* species complex can be found most commonly resting on dark surfaces during the day. An optimal resting site for *An. quadrimaculatus* adults is near a suitable oviposition site. This usually entails an area with ideal climate conditions and with a blood source nearby. The adults prefer that the average temperature in the resting site be 4^0 F lower than outside, the relative humidity about 8% higher, and an evaporation rate that is lower than outside. Many common natural resting sites are barns, hollow trees, stumps, under houses, and under bridges. *Anopheles* are usually collected by aspirating them out of their natural resting sites. The flight range of this mosquito is usually around one mile under normal conditions, however, there have been mark and release studies that have shown adults to travel three miles from a release sight (Horsfall 1955).

This species complex overwinters as fertilized adult females. As soon as ambient temperatures begin to increase following the winter months, they are ready to take a blood meal and proceed with ovipositing the first brood of the season. The female mosquito lays about 194 to 263 eggs singly, per gonotrophic cycle, and a female can go through 9 to 12 gonotrophic cycles in her life-time (Horsfall 1955). The female mosquito feeds mainly on large mammals such as bovines, horses, pigs, and deer but will also readily feed on humans if they are near their breeding sights (Kaiser 1994). A study in Arkansas found that horses were the preferred host among their known mammalian hosts (Williams and Meisch 1981).

Blood feeding rates of *An. quadrimaculatus* species A, B, and C_1 sibling species were compared in an article by Jensen et al. in 1996. The mosquitoes were collected from woodland and campground sites. As expected, there were an extremely low percentage of mosquitoes that contained human blood (1.6% of species B and 0% of A and C_1) in the woodland sites. The wooded sites probably had little if no human habitation, making the possibility that one would find a mosquito engorged with human blood very small. There was a significant difference between the human blood feeding rates of the species studied from the campground site. Species A had the highest rate with 10.7%, then species C1 with a rate of 1.2%, while there were no species B individuals that had fed on humans in the campground sites. These findings should not be taken out of context, however. Even Jensen et al. (1996) believe that the distance between the different breeding areas of the species from humans, competition for non-human blood meals, and position of resting sights also may play a role in the difference

in percentage of human blood meals among the sibling species (Jensen et al. 1996). In contrast, other studies have shown no significant difference in the host-feeding patterns of species A and species B (Apperson and Lanzaro 1991).

Anopheles quadrimaculatus will readily enter houses and feed on the residents within. Additionally, Jensen et.al. (1996) found that *An. quadrimaculatus* females collected in domiciles feed on humans at a rate of 36% to 93.4%, while females collected from non-domicile resting areas only fed on humans at a rate of 1.1% to 17.9%. These feeding rates obviously have to do with host availability within the mosquito's immediate environment.

If there are blood meals available to an *An. quadrimaculatus* female when she emerges, then oviposition can take place as soon as three days after emergence (Carpenter and LaCasse 1955). The female mosquito lays her eggs in the menisci created by vegetation in the water. Larvae in this species complex are most frequently found in permanent fresh water in slow moving streams, ponds, canals, and lakes that contain vegetation or debris on the surface (Carpenter and LaCasse 1955). Larvae feed on the surface of the water by rotating their head 180^0 so that their body is oriented with the dorsal side of the thorax and abdomen toward the surface, and the ventral side of the head facing the surface. This allows the larvae to breath while it is feeding off the surface of the water.

Temperature, population density, nature and amount of food, and depth of water all have an effect on the developmental time of *An. quadrimaculatus* larvae. Larvae develop best when the water surface is between 27^0 and 28^0 C and each larva has about

15 cm^2 surface available to them. *Anopheles quadrimaculatus* should take between two and five weeks to complete a generation in the wild (Horsfall 1955).

Anopheles quadrimaculatus species complex discovery

The first reference to a division *of An. quadrimaculatus* into cryptic species was performed by Gregory Lanzaro et al. in 1986 when he established that there were two sympatric sibling species of *An. quadrimaculatus* indicated by hybrid sterility in males produced from crosses and the analysis of isozyme frequencies of twenty loci among these two sibling species. Nine field populations spanning across Louisiana, Arkansas, Mississippi, Alabama, and Florida were collected for analysis. The F1 adults from these collections were crossed to ORLANDO (ORL) adults that have been laboratory-raised for over forty years. Six of the nine populations produced normal progeny with normal survival rate and sex ratios. The remaining populations were named A/B populations because some females produced normal offspring, while other females from these matings produced sterile males, lower survival rate, and/or a sex ratio skewed towards females. Isofemale lines formed two of the three A/B populations that were created, resulting in four different lines. Each population had an A and B line and all combinations of matings were performed among them as well as with the OPL strain. The results showed that all matings between A and B individual always produced only sterile males. In every mating between a B male to an A female, the sex ratio was skewed toward females because of the high mortality rate of male pupa and the survival rate to adult was lowered, except in one population (KBG – A female x KBG – B male). Backcrosses were also made from the F1 hybrid progeny to both parents and the males

produced from this mating were all sterile. There was no evidence that supports hybrid matings occuring in nature because no sterile males were found among the males collected from sites that contained both species A and B (Lanzaro et al. 1988).

Lanzaro (1986) also discovered a way to genetically distinguish the two known sibling species when they were electrophoresed and the gels stained for isocitrate dehydrogenase (IDH). Twenty loci were analyzed and the allele frequencies for species A and B were compared. At two loci, (Idh-1 and Idh-2 loci) species B males were found to contain a single allele, while species A males were polymorphic at these loci. By observing the allele frequencies at both of these loci, rapid identification of species could be established (Lanzaro 1986).

Kaiser et al. (1988a) also published data on a hybridization study of species A and B. Isofemale lines were created from two different locations, Montgomery, AL and Gainsville, FL, and these were then crossed with a lab strain known to be species A (ORL). With the results from this cross, species A and B strains were created and then mated to each strain and to the ORL strain. Both of the collection sites contained species A and B and all of the B-lines produced sterile males or no males when crossed to all strains of species A. The sex ratio was also often skewed toward the females due to high male pupal mortality rate, and the females that were produced from the hybrid matings were often at least semi-sterile. Crosses between the same species from one location and crosses between the same species from different locations yielded fertile progeny and all lines of the same species were considered conspecific (Kaiser et al.

1988a). These results supported the theory that there is an *An. quadrimaculatus* species complex of at least two sibling species.

In 1987, Kaiser and Seawright published their data on chromosome polymorphism in species A that, further supported the idea of a species complex. Several inversions were discovered when both species A and B ovarian polytene chromosomes were observed, however, the most diagnostic inversion was a fixed inversion found on the X chromosome that was found only in species B and includes a large, diffuse puff. This inversion is believed to be homozygous in all species B individuals and was one of the first diagnostic tools discussed in the literature (Kaiser and Seawright 1987). The results of this study confirmed what Lanzaro found in 1986 and further proved that *An. quadrimaculatus* is, in fact, a species complex of at least two different species.

A third species, species C, was discovered in Florida in 1988 (Kiaser et al. 1988b). The new species was confirmed using hybrid sterility tests, chromosomal differences, and diagnostic allozymes. Reciprocal crosses between A and C produced sterile males and no females in the F1 progeny. Crosses between C males and B females had three different results. Some of the crosses resulted in low survivability, with only sterile females produced. The second type also had a low survivability, but only sterile males were produced. Finally, there were also F1 generations that had a 43% survival rate and produced fertile males and females with ovaries that were smaller than normal. The reciprocal cross resulted in F1 progeny having an extremely low survival rate and only sterile males produced, or the F1 progeny had a survival rate around 40% and

fertile males were produced as well as females with slightly smaller ovaries than normal. Although the method used by Kaiser and Seawright in 1987 to prepare the ovarian nurse cell polytene chromosomes of species A and B did not work for species C, Kiaser et al. (1998b) were able to differentiate species C by cytological markers such as diffuse bands. The electrophoretic patterns of species C at the loci Idh-1 and Idh-2 could be distinguished from species B and usually from species A. Analysis of additional loci (Acon-1) was needed for the correct differentiation of the 3 species (Kiaser et al. 1988b).

A dichotomous electrophoretic taxonomic key for the sibling species A, B, and C was published by Narang et al. (1989b). This key uses genetic variability at 33 enzyme loci that are present in both male and female mosquitoes (Narang et al. 1989b). About this same time, Narang et al. (1989a) also discovered species D in Mississippi and Florida and developed another eloctrophoretic taxonomic key for distinguishing species D from A, B, and C (Narang et al. 1989a). Species C was split into two different species by Narang et al. (1990) when they discovered there were significant differences in allelic frequencies in five different loci. It is suggested that the genetic differences of the two species is due to spatial subdivision of species C (Naranag et al. 1990).

Further genetic experimentation was performed to differentiate the species more rapidly. Mitchell et al. (1992) found that the restriction enzymes *Ava1*, *HindIII*, and *PvuII* would each digest the mitochondrial DNA into species-specific DNA restriction patterns, and *Ana1* and *HindIII* could produce unique restriction patterns in the ribosomal DNA. Although these restriction enzymes could not be used to distinguish between C1 and C2, this was still a very helpful discovery.

Using PCR and species-specific primers that target the highly conserved ribosomal DNA ITS2 region, Cornel et al. (1996) were able to discern between all but the C species quickly and accurately. A researcher only needed to sequence the DNA that was amplified during PCR to differentiate between species C1 and C2. Rutledge et al. (1996) found Cornel's method for identifying wild caught mosquitoes in Louisiana and Mississippi to be 100% specific and 95% sensitive. This study further showed the effectiveness of using PCR as a method for species identification.

Identifying immature stages of the *An. quadrimaculatus* complex accurately can save both time and money for the researcher. Rutledge et al. (1999) explored the identification of all the immature stages of species A and C2 using PCR. Using the protocol described by Cornel et al. in their 1996 paper, Rutledge was able to identify all of the immature stages using primers that targets the ITS2 region on the mosquitoes ribosomal DNA (Rutledge et al. 1999).

The first morphological key that distinguishes between all five species was produced by Reinert et al. in 1997. Their article in the *Journal of the American Mosquito Control Association* contains keys for males, females, pupae, fourth-instar larvae, and eggs. Reinert et al. (1997) also modified Narang et al (1989b) electrophoretic taxonomic key to include all five species. In this article, all of the species are given names for the first time, but many scientists still refer to them with their corresponding letters and numbers for convenience.

The use of PCR has increased dramatically in the last few years, because, if done properly, results can be fast, accurate, and precise. Many taxonomists are finding that

PCR can be a rapid way to identify species. In 2002, Rafferty et al. (2002) developed a quick method to identify *Anopheles* mosquitoes with a 96-pin bacterial replicator. To accomplish this they modified a buffer used to extract *Drosophila* DNA for *Anopheles* mosquitoes. The buffer and method they used to extract the mosquito DNA provides a crude but effective DNA extraction procedure. Using the method described by Rafferty et al. (2002), DNA can be extracted from a mosquito in less than 30 minutes.

Anopheles quadrimaculatus **distribution studies**

The most recent published distribution of *An. quadrimaculatus* (Say) in Texas was performed in 1977 by Fournier and Snyder. This publication, shows *An. quadrimaculatus* collected from almost every county in eastern Texas and the southern portion of the state which extends from the tip of Texas in Cameron County along the Mexico-US border to Maverick county and almost all of the counties to the east of that line. There are only a few counties that were known to contain *An. quadrimaculatus* in the western part of the state. El Paso, Culberson, Lubbock, Upton, Val Verde, Nolan, Runnel, Taylor, and Childress Counties are the mid to upper western counties that are known to contain *An. quadrimaculatus* (Fournier and Snyder 1977).

The most wide spread distribution study on the *An. quadrimaculatus* species complex was performed in 1992 by J. A. Seawright et al. This study spanned over 16 states and 94 different counties. The mosquitoes were collected by aspirating them from their natural and artificial resting sites using power aspirators. Seawright distinguished species A, B, C, and D using the electrophoretic taxonomic key of Narang et al. (1989b) and by Kaiser's and Seawright's (1987) protocol which includes the examination of the

polytene chromosomes found in the ovarian nurse cells. Species A was collected in all of the counties sampled and was the dominant species in all of the sites, except for Hamilton Co., Florida and Camden Co., Georgia and Montgomery Co., Alabama, where species B was the dominant species found. Species B was found from Louisiana to Florida, North to North Carolina and then West to Kentucky and Tennessee. Species C was found only in Florida and Georgia, and species D was found from Mississippi to Florida and up to South Carolina and Tennessee (Seawright et al. 1992).

While Seawright et al. (1992) did not differentiate between Species C1 and C2, the study done in 1998 by Rutledge and Meek differentiated between them in their study on the distribution of this complex in Louisiana. Collections in the Louisiana study were performed by aspirating adult mosquitoes out of natural and artificial resting sights. The identification of the sibling species were conducted using the biochemical keys that were published by Naranag et al. in 1989b and *An. inundatus* (C2) and *An. diluvialis* (C1) were identified using ribosomal DNA analysis. Rutledge and Meek (1998) collected mosquitoes from 31 different parishes in Louisiana and found 23 positive for *An. quadrimaculatus* (Say). All of these positive parishes contained *An. quadrimaculatus* (A). The second most common sibling species found, *An. smaragdinus* (B), was found in 70% of the parishes. This species was almost always found in combination with *An. quadrimaculatus* (A). *Anopheles maverlius* (D) was found in 39% of the parishes sampled. This species was always found with other members of the species complex, and never reached above 10% of the total adult mosquito population. *Anopheles inundatus* (C2) was found in only two parishes, one of those parishes being Cameron,

which is located on the Gulf Coast and borders Texas. All of the species identified in Louisiana can be found in at least one of the parishes that border Texas (Rutledge and Meek 1998).

A predictive approach to determining the distribution of the *An. quadrimaculatus* (Say) complex was taken in 2004 by Levine et al. To predict the distribution of the species in the *An. quadrimaculatus* complex, Levine used a genetic algorithm developed by Stockwell and Peters (1999) called the Genetic Algorithm for Rule-set Prediction (GARP). This predictive model uses environmental data and species point-occurrence data to create a predicted distribution of each species of interest. The final distribution maps had Texas, because of it ecological components, containing all five species in the complex. *Anopheles diluvialis* and *An. inundatus* were confined to the coastal region and along some of the major rivers in Texas. *Anopheles maverlius* was predicted to occur mainly in the coastal regions and east Texas. The predicted area for *An. quadrimaculatus* contained all of Texas, but the areas that were most likely to contain them were south, central, and east Texas. *Anopheles smaragdinus* had a slightly different predicted distribution in that the area that they are more likely to occur was east and most of central Texas. From the overall distribution predictions of all species, Levine et al. (2004) concluded that "*An. quadrimaculatus* was the only species in the complex capable of vectoring malaria in the United States throughout the area in which malaria occurred." Levine does point out, however, that the other four species could have been regionally important (Levine et al. 2004). The problem with this conclusion is that Levine does not take into account the resistance study that Mallet and Porter

performed in 1993 which showed that *An. quadrimaculatus* specimens that were collected in Mississippi were highly resistant to malathion and some populations were also acquiring a permethrin resistance. Mallet and Porter's study did not find any resistance in *An. smaragdinus* or *An. maverlius* (Mallet and Porter 1993). This resistance evidence creates another theory that *An. quadrimaculatus* has been able to keep a wide distribution throughout the eastern half of the United States because the main pesticides used in mosquito control are not reducing their populations. Both *An. smaragdinus* and *An. maverlius*, on the other hand, have had their numbers greatly decreased and their distribution narrowed because of the adulticiding efforts made by mosquito control districts.

Anopheles quadrimaculatus **habitat studies**

Rutledge and Meek (1998) conducted a habitat study in Louisiana by selecting different sites and sampling them biweekly for two years. A wooded habitat, artificial resting sites from rice fields, and a heavily shaded swamp were sampled for the study. *Anopheles quadrimaculatus* (A) was found in association with rice fields, the wooded site, and in livestock holding facilities. In cattle barns, *An. quadrimaculatus* (A) was the dominant species found and the only sibling species found in sheep and pig facilities. *Anopheles inundatus* (C2) was only found in fresh water swamps containing cypress stands, white oak trees, and palmetto. Riceland habitat was compatible with *An. maverlius* (D) as well as the wooded habitat sampled. *Anopheles smaragdinus* (B) was also found in the wooded sight and was the only member to be found associated with chicken coops (Rutledge and Meek 1998).

In Seawright et al.'s 1992 article, they state that species A was the dominant species found in every reservoir that was sampled and that Species B and D were also found at low levels in a few of the reservoirs sampled. Unfortunately, Seawright et al. (1992) did not report habitat data for all of their sites; however, the above information might provide insight into where the species prefer to breed.

CHAPTER III

DISCOVERY OF SPECIES IN THE *AN. QUADRIMACULATUS* SPECIES COMPLEX IN TEXAS

Introduction

Anopheles quadrimaculatus mosquitoes are the primary vector of malaria in Texas and the rest of the eastern United States. Since the discovery of the *An. quadrimaculatus* species complex, made up of *An. quadrimaculatus*, *An. smaragdinus*, *An. diluvialis*, *An. inundatus* and *An. maverlius*, many eastern states have tried to establish a distribution pattern of these new species in the interest of reassessing the vector potential for malaria in their regions. In the United States, it has been fifty years since malaria was last considered to be an established disease. The "eradication" of malaria was due in large part to a population shift to urban areas, improved drainage and housing, improved human nutrition and an increased understanding and implementation of vector control. However, malaria still occurs in the United States in isolated instances. Fortunately, most of these cases occur in individuals who travel to an endemic area in a foreign country and become infected while there (Causer et al. 2002). Yet, each year, several cases of locally-transmitted malaria occur with native *Anopheles* populations serving as vectors. From 1957-1994, 76 cases were documented as having been acquired via mosquito-born transmission in the United States (Zucker 1996). The last case of locally transmitted malaria that occurred in Texas was in 1994 when three cases of malaria were diagnosed in homeless people living in the Houston area (*MMWR* 1995).

Determining the distribution of new species of the *An. quadrimaculatus* complex is interesting from an ecological perspective and it could also aid in mosquito control and disease prevention. It has been suspected that *An. quadrimaculatus* (A) could be more resistant to pesticides, hence its wider distribution than the other members of the complex. Vector competency testing has not been performed on these five species, and scientists still do not know if some of the species are more competent that others. Once this information is established and the distribution is determined, effective mosquito control programs can be implemented to control and even prevent malaria in the United States.

Collecting *An. quadrimaculatus* mosquitoes can be time consuming and difficult, especially if the area of interest is as large as the state of Texas. *Anopheles quadrimaculatus* is not as attracted to light as most mosquitoes, so light traps are not very effective collection tools. Gravid traps are even less effective because the females like to lay their eggs in large bodies of permanent water with thick stands of aquatic vegetation. The best way to collect these mosquitoes is to aspirate them out of their day-time resting sites which include hollow logs, tree stumps, under bridges and houses, barns, and any other dark, cool surface (Horsfall 1995). Another well documented collection technique is to create an artificial resting site trap as described by Weathersbee and Meish (1988) (Fig. 1). The artificial resting site trap works best when the researcher knows where high populations of *Anopheles* occur. This can make collecting difficult in areas that are unknown to the researcher.

Figure1. Picture depicting an artificial adult mosquito resting site trap out of which adults can be aspirated during the day. This trap consists of a brown or red trash can, wire mesh covered lid, and black trash bag extended out from the opening that is held up by a tomato cage.

Presently there are morphological keys produced by Reinert et al. (1997) for male genitalia, females, pupae, fourth-instar larvae and eggs of the species in the *An quadrimaculatus* complex (Reinert et al. 1997). While these keys are well thought out and detailed, molecular tools like PCR can result in faster and more accurate and precise identification information, allowing species determination, if done properly.

The focus of this research was to collect as many *An. quadrimaculatus* (Say) adults as possible within the state of Texas. Different ecological habitats and areas were chosen to be sites of collecting trips, but most of the mosquitoes were collected with the help of the Texas Department of State Health Services (TDSHS) and Harris County Mosquito Control District (HCMCD). In addition, all of the mosquitoes collected were identified using a PCR based protocol.

Materials and methods

Collection Sites

Initial collecting trips made in Texas by this investigator proved less successful than needed for this project. While this did not stop further collecting trips, it did precipitate the need to find a means of eliciting help from different parts of Texas. TDSHS provided *An. quadrimaculatus* specimens found in the samples sent to them for West Nile virus testing in 2003 and 2004. Counties from all over Texas supplied mosquito samples to TDHSH, which resulted in mosquito samples with a wide range of habitat and geographical diversity. HCMCD sent TAMU *An. quadrimaculatus* specimens that were found in their collections made in 2004, since they did their own identification and West Nile testing for Harris County. Brazos County collections were performed by this investigator from 2002- 2004.

Additional collection trips were made in the Lower Rio Grande Valley, Jefferson County, Chambers County, the San Antonio area, Corpus Christi, and Kinney County. These areas were chosen because they are known to contain *An. quadrimaculatus* mosquitoes, few if any collection had been made there, and most of them have a high population of Hispanic residents. Since some of this research was funded by the Hispanic Leadership Program in Agriculture and Natural Resources (HLPANR), it was important that areas of Texas with higher percentages of Hispanics be sampled. This turned out to be very important because past *An. quadrimaculatus* collections have been made in most of the areas of Texas that have a high population of Hispanic residents. Collections in these areas are also important because most Latin countries have endemic

malaria and if some of the Hispanic residents of Texas visit their home country or have visitors from endemic areas, then the chances of a malaria outbreak in these areas of Texas increases. This also increases the risk that the areas that are more likely to have an outbreak of malaria are locations with a large Hispanic population (Appendix C).

Collections in the Lower Rio Grande Valley (Cameron and Hidalgo County, Texas) were taken from August 2 – 4 in 2003. Each day, two or three sites were picked which had an environment that was suitable for *Anopheles* to rest during the day, blood feed, and lay eggs. At each site, an artificial resting site trap and a light trap was set up and then picked up the following morning. All mosquitoes collected were aspirated into containers labeled with the GPS data, date, and trap type. The containers then were placed into a cooler until they were brought back to Texas A&M, identified, and then frozen at -20^0C.

Jefferson and Chambers counties, Texas, were sampled in the summer of 2004. July 7th and 8th were spent aspirating *Anopheles* mosquitoes out of various resting sites. The artificial resting site traps were not used because an abundance of mosquitoes were found in many different man-made structures such as old barns and wood sheds. After the specimens were collected, they were transferred into adult containers that were placed into a cooler to keep them cool enough to survive the transport back to College Station. Once the mosquitoes reached Texas A&M University, they were processed using the same procedures as the mosquitoes collected in the Lower Rio Grande Valley.

The area around San Antonio was sampled July 20th through the 22nd. Natural and man-made resting sites were sampled, and a light trap was set up at three locations

that possessed good habitats for *Anopheles* adults. Fort Clark in San Antonio was searched for the presence of mosquitoes by setting up light traps, artificial light traps, as well as making a thorough search of any structures that might harbor day-time resting adults. Several light traps were set up the week prior to July 20th and they were searched for any presence of *Anopheles* adults. Any *Anopheles* found were transferred, and processed using the same procedures as were the mosquitoes captured from the previous collecting trips.

Collecting was performed in the Corpus Christi area on August 9th through the 11th and samples of *An. quadrimaculatus* were found in Nueces, Victoria and Live Oak County. Again, it was determined that the most effective way to collect *Anopheles* mosquitoes was to aspirate them out of resting sites found while surveying the Corpus Christi area. The resulting mosquito specimens were treated the in the same manner as described above until they could be identified and frozen at -20^{0}C.

Species Identification

DNA extraction was accomplished by following the protocol used by Rafferty et al. (2002) for PCR identification of *Anopheles* mosquitoes. Each mosquito was individually placed in a microfuge tube with 50µl of denaturing buffer made up of 10 mM Tris-HCL 8.2, 1mM ethylenediaminetetraacetic acid, 50 mM NaCl, 0.1% Triton-X 100. Each mosquito was then ground with a sterile pestle and heated for 15 minutes at 94^{0}C. To ensure that no DNA sample was contaminated, each pestle was soaked in a 2 molar solution of hydrochloric acid for 2 hours. The pestles were then rinsed with distilled water, dried off and autoclaved. Each mosquito was ground up using one of

these sterile pestles and after one use; each pestle was sterilized again the same way before it was used again.

Species identification was accomplished by following the PCR protocol established by Cornel et al. (1996). This protocol used specific primers that targeted the ribosomal DNA ITS2 region for species A, B, C, and D. Five primers were developed by Cornel et al. (1996); four are species-specific primers and the fifth is the universal primer. Each sample contained 10.9µl of ddH2O, 2µl of 10xbuffer, 0.8µl of MgCl2 (50 molar solution), 1.0µl of all five primers, 1.0µl of dNTP's, 0.2µl of taq, and 0.1µl of template DNA. Amplification conditions consisted of 25 cycles with denaturing at 94^0C for 1 minute, annealing at 50^0C for 2 minutes, and extension at 72^0C for 2 minutes. Although differences between C1 and C2 were not sufficient in this region to differentiate them by gel electophoresis, the amplified region can be sequenced and specific repeated regions would have differentiated the two species had either of these species been collected. The remaining species were separated by adding 4µl of loading dye to the PCR product and then running it out on a 2.5% agarose gel to determining the length of the product. *Anopheles quadrimaculatus* samples result in an amplification product of 319 base pairs, *An. smaragdinus* samples result in an amplification product of 227 base pairs, *An. diluvialis* and *An. inundatus* samples result in an amplification product of 293 base pairs, and *An. maverlius* samples result in an amplification product of 141 base pairs.

To verify the results of the species identification, the DNA sequence of the PCR product was determined or each of the different species that were found. Five PCR

amplification products of *An. quadrimaculatus* mosquitoes and five representatives from *An. smaragdinus* had their DNA sequence determined. In order determine the DNA sequence, the PCR product was then purified using a kit made by Qiagen (Valencia, California). A spectrometer was used to determine the absorption at 260nm and 280nm with a 20 fold dilution (5µl of PCR product to 95µl of ddH2O). This test determined the concentration level of DNA in the sample. The concentration levels need to be high enough to ensure accurate sequencing data. Then, 7.5µl of the purified DNA was added to 0.5µl M13 reverse primer and 2.0µl of Big Dye. This solution was then placed in a thermal cycler and the following protocol was run in sequential order:1) 96^{o} C for 2 minutes, 2) 96^{o} C for 0.3 minutes, 3) 55^{o} C for 0.15 minutes, 4) 69^{o} C for 4 minutes, 5) steps 2 through 4 were repeated 34 times, and 6) 60^{o} C for 5 minutes.

Each PCR product was then purified before submission to the Institute for Plant Genomic and Biotechnology (TAMU) where the DNA was sequenced using an ABI 3100 Capillary Sequencer. To accomplish this, 500µl of sephadex (g50/fine) were placed into centrifuge columns which were inserted into centerphuge tubes. These columns were then placed into the centrifuge and spun at 3,500rpm for 3 minutes, then placed into larger centrifuge tubes. Twenty (20) µl of ddH_2O was added to the PCR product and then all of the solution was pipetted onto the sephadex columns. These columns, which were still inside the centrifuge tubes, were then centrifuged for 3 minutes at 3,500 rpm. The columns were then removed and the tubes were placed into the speed vacuum dryer for 20 minutes with the tops open. This allowed the DNA to dry. The dried DNA was then taken to a sequencing lab to be analyzed. Unfortunately,

the sequencing data from these reactions was not clean and many nucleotides were unknown (N). When the sequences were analyzed, however, the unique attributes of *An. quadrimaculatus* that separate it from the other 4 species were present in all of the species A specimens tested and the unique attributes of *An. smaragdinus* were also seen in the 5 specimens of species B tested. Although this data proves that the specimens were originally identified correctly, a different and cleaner approach was attempted to create an accurate sequence for at least one specimens of each species.

To create a clearer and more precise sequence, the amplified DNA was cloned into a TA vector. Five (5) µl of each PCR reaction was then used to run out on an electrophoesis gel in order to ensure that the specimens chosen were the correct species. A ligation reaction for each of the specimens was then set up containing 7µl of the PCR product, 1µl of a TA cloning vector called pGEMT (Promega, St. Louis, Missouri), 1ul of 10x T4 ligase buffer, and one 1µl of T4 ligase. The reaction sat at room temperature overnight. One (1) µl of the ligation reaction was then added to 45µl of competent cells and 40µl of that mixture was placed in electroporation cuvettes. This step was performed over ice to ensure that the cells remained viable.

The cuvettes were then placed in an electroporation machine so that the competent cells could be electroporated at 2.25kV with 186 ohms of resistance timing and thus incorporate the plasmids created from the ligation reaction. The cells were immediately placed into SOC medium and allowed to incubate for 1 hour with shaking at 200 rpm and 37^0 C. After 1 hour, 50µl of each cell solution was placed on an AMP/IPTG/x-Gal media plate, while the rest of the cell solution was spun down in

microphuge tubes for 2 minutes at 10,000rpm. Most of the media was then poured off and the concentrated cell solution was then plated onto an AMP/IPTG/x-gal media plate. All of the plates were then incubated overnight at 37ºC.

All white colonies were picked off the plates and placed on a master plate. A PCR amplification was also performed on each of the white colonies. Each reaction contained 11.9µl of ddH_2O, 1.5µl of 10x buffer, 0.75µl of 15mm MgCl2, 0.75µl M13 forward primer, 0.75µl of M13 reverse primer, 0.25µl of 10mm dNTP's, 0.1µl Taq, and bacteria from a white colony. An electrophoresis gel was then run for each of the PCR products to determine which colonies actually had the DNA insert from the original amplification.

The colonies that contained the correct insert were then inoculated into liquid media to grow up overnight with shaking at 300rpm and 37^0 C. A mini prep by Promega (St. Louis, Missouri) for DNA purification was then performed on each of the colonies that were grown up over-night. A spectrometer was then used to test absorption at 260nm and 280nm with a 20 fold dilution (5µl of PCR product to 95µl of ddH_2O). This test determined the concentration levels of DNA in the sample. This step is important because concentration levels needed to be high enough to ensure accurate sequencing data.

Once the concentration levels were found to be high enough, 7.5µl of the purified DNA was added to 0.5µl M13 reverse primer and 2.0µl of Big Dye. This solution was then placed in a thermal cycler and the following protocol was run in sequential order:1)

96^{o} C for 2 minutes, 2) 96^{o} C for 0.3 minutes, 3) 55^{o} C for 0.15 minutes, 4) 69^{o} C for 4 minutes, 5) steps 2 through 4 were repeated 34 times, and 6) 60^{o} C for 5 minutes.

Each PCR product was then purified before submitting it to the sequencing laboratory. To accomplish this, 500 µl of sephadex (g50/fine) were placed into centrifuge columns which were inserted into centerphuge tubes. These columns were placed into the centrifuge and spun at 3,500rpm for 3 minutes, then placed into larger centrifuge tubes. Twenty (20) µl of ddH_2O was added to the PCR product and then all of the solution was pipetted onto the sephadex columns. These columns, which were still inside the centrifuge tubes, were then centrifuged for 3 minutes at 3,500 rpm. The columns were then removed and the tubes were placed into the speed vacuum dryer for 20 minutes with the tops open. This allowed the DNA to dry. The dried DNA was then taken to the Institute for Plant Genomic and Biotechnology (TAMU) where the DNA was sequenced using an ABI 3100 Capillary Sequencer.

Results

Each *An. quadrimaculatus* (Say) adult that was captured by or sent to this researcher was individually tested using the Cornel et al. (1996) PCR protocol to determine the species. A photograph was taken of each agarose gel so that the amplification product that resulted from each mosquito could be measured and stored for future reference (Fig. 2).

In order to prove that the *Anopheles* mosquitoes were being accurately identified, the fragments produced through PCR amplification for *An. quadrimaculatus* mosquitoes and *An. smaragdinus* mosquitoes had their amplified DNA sequenced and then

compared to the DNA sequences published by Cornel et al. (1996). When the DNA sequences were compared, they all contained the unique attributes associated with their species that Cornel et al. (1996) identified, supporting the identifications made by the amplified fragment lengths on the agarose gels. The only problem with this alignment was a C found in the published sequence at the end of the amplified ITS2 section that had a transversion into a G on the *An. quadrimaculatus* specimen sequenced during this project (Fig. 3).

Figure 2. An example of an agarose gel used for species identification. A 50 base pair ladder in lane 1 and a 100 base pair ladder in lane 2. Lanes 3-14 and 16-17 have a band length of 319 and represents specimens of *An. quadrimaculatus*. Lane 15 has a band length of 227 and represents an *An. smaragdinus* specimen.

The first difference in the sequences of *An. quadrimaculatus* and *An. smaragdinus* observed was a transition from a T to a G indicated at nucleotide 102 in Figure 4. *Anopheles smaragdinus* shows a 4 base-pair frameshift mutation after the 106th nucleotide. Three more transitions are shown in bold in figure 4 at *An.*

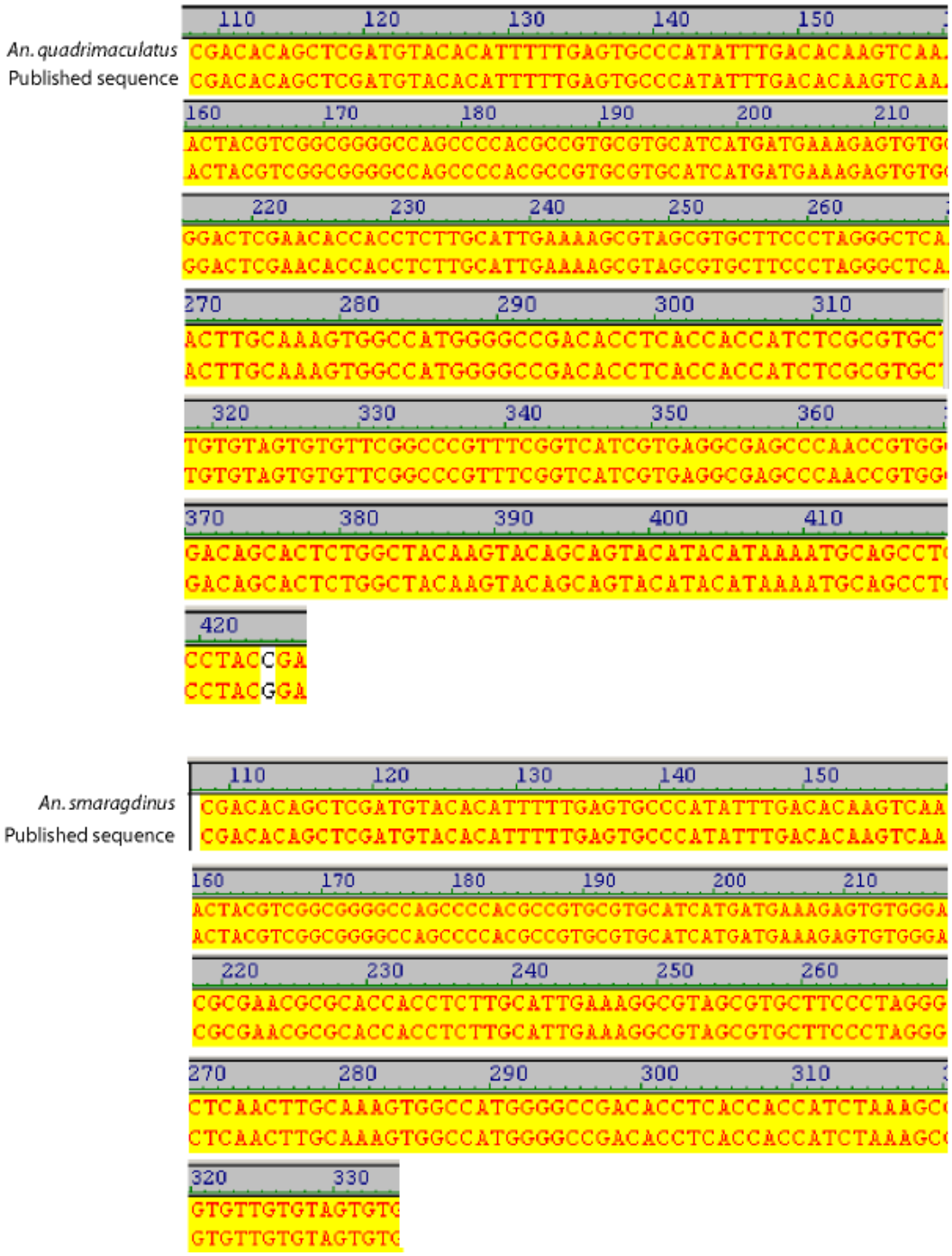

Figure 3. Alignment of the rDNA ITS2 amplified region of an *An. quadrimaculatus* specimen and an *An. smaragdinus* specimen with the sequence published by Cornel et al. (1996). The single difference is indicated by a bolding of the nucleotide.

A CGACACAGCT CGATGTACAC ATTTTTGAGT GCCCATATTT 40
B CGACACAGCT CGATGTACAC ATTTTTGAGT GCCCATATTT 40

A GACACAAGTC AAAC TACGTC GGCGGGGCCA GCCCCACGCC 80
B GACACAAGTC AAAC TACGTC GGCGGGGCCA GCCCCACGCC 80

A GTGCGTGCAT CATGATGAAA GAGTGTGGGA C**T**CGAA - - - - 106

B GTGCGTGCAT CATGATGAAA GAGTGTGGGA C**G**CGAA**CGCG** 110

A CACCACCTCT TGCATTGAAA **A**GCGTAGCGT GCTTCCCTAG 146
B CACCACCTCT TGCATTGAAA **G**GCGTAGCGT GCTTCCCTAG 150

A GGCTCAACTT GCAAAGTGGC CATGGGGCCG ACACCTCACC 186
B GGCTCAACTT GCAAAGTGGC CATGGGGCCG ACACCTCACC 190

A ACCATCT**C - -** GCGTG**C**TGTG TAGTGTG 211
B ACCATCT**AAA** GCGTG**T**TGTG TAGTGTG 217

Figure 4. Alignment of the ITS2 region amplified using the species-specific primers for the *An. quadrimaculatus* species complex developed by Cornel et al. (1996). The emboldened text indicates differences in the sequences of specimens of *An. quadrimaculatus* and *An. smaragdinus*. The underlined portions indicate the primers used for the PCR assay.

quadrimaculatus' nucleotide numbers 131, 198, and 206. Finally, a two base pair insertion can be found at *An. smaragdinus* nucleotide number 199 and 200 (Fig. 4). These sequence data are a strong indication that the *Anopheles* mosquitoes identified using the above method is correct because these are also the same differences observed by Cornel et al. (1996).

From September 2002 through January 2005, 1,377 *An. quadrimaculatus* (Say) mosquitoes were captured and identified. The collections were made in a variety of habitats and areas of Texas to obtain the most diverse *Anopheles* populations as possible. A total of 1,326 were identified as *An. quadrimaculatus* and 51 were identified as *An.*

smaragdinus. TDSHS sent 63 *An. quadrimaculatus* (Say) adults in 2003 and 57 were identified as *An. quadrimaculatus*, while 5 were identified as *An. smaragdinus*. During 2004, TDSHS sent 703 mosquitoes and 43 which were *An. smaragdinus*, while 646 were identified as *An. quadrimaculatus* (Appendix A).

In the summer of 2003, a collecting tip was made to the Lower Rio Grande Valley in which four *An. quadrimaculatus* specimens were collected. Three were collected in Cameron County and one was collected in Hidalgo County. The next collecting trip made was in the Beaumont area. A total of 116 *An. quadrimaculatus* adults were collected by aspirating them out of their resting sites. These mosquitoes were collected in three different counties. In Jefferson County, 42 *An. quadrimaculatus* adults were identified, in Chambers County there were 68 *An. quadrimaculatus* adults found and identified, and in Liberty County the last 6 *An. quadrimaculatus* adults were collected.

An unsuccessful collecting trip was made in July of 2004 to the San Antonio area in hopes that a greater number of *An. quadrimaculatus* (Say) adults could be collected in west Texas where the habitat is very different from the east Texas piney woods region of the state. Unfortunately, only one *An. quadrimaculatus* adult was collected on Fort Sam Houston in Bexar County. The collecting trip made in August of that same year to Kinney and Edwards County yielded no *An. quadrimaculatus* (Say) adults, but it was late in the season and the dry habitat would have put a strain on any surviving adults.

A more successful collecting trip was made in early August 2004 to the area of Corpus Christi. During this trip a total of 42 *Anopheles* adults were captured and all of

them were identified as *An. quadrimaculatus*. The largest collection was made from Live Oak County where 31 adults were collected. A total of 9 adults and 1 larvae were collected from Nueces County and 1 was collected from Victoria County.

A High School Science Fair student, Megan Mock, collected mosquitoes from Grimes County around the city of Stoneham. These collections were made using a light trap in January of 2005 and 10 of the *An. quadrimaculatus* adults were sent to this researcher for identification. All 10 of the mosquitoes were identified as *An. quadrimaculatus*.

Collections were made in the Brazos Valley starting in September of 2002 through October of 2004. In the 2002 Season, 48 *Anopheles* adults were collected using artificial resting site traps and all of them were identified as *An. quadrimaculatus*. All of the specimens were collected from the Texas A&M University Equine Center located on George Bush Drive. During the 2003 *Anopheles* season, collections were made from June through November and 117 *Anopheles* adults were collected and identified. While a majority of the mosquitoes were identified as *An. quadrimaculatus*, 3 were confirmed to be *An. smaragdinus*. All three of the An. *smaragdinus* adults, and the majority of the *An.* quadrimaculatus specimens were collected from the Texas A&M Equine Center (Table 1).

Collections in 2004 were made from March through October and a total of 118 Anopheles specimens were identified. There were no *An. smaragdinus* specimens found, all of the *Anopheles* adults were identified as *An. quadrimaculatus*. Again, the majority of the specimens were collected on George Bush Drive (Table 2).

Table 1: List of all *An. quadrimaculatus* (Say) specimen identifications made in Bryan/College Station (Brazos County), Texas in 2003 organized by date and street name.

Brazos County 2003 *An. quadrimaculatus* (Say) Collections			
Date	**Street**	**# of species A**	**# of species B**
06/16/03	George Bush Dr.	2	0
06/18/03	George Bush Dr.	16	1
06/21/03	George Bush Dr.	13	0
06/21/03	Gilchrist Ave.	1	0
07/09/03	Copper Falls Dr.	1	0
07/14/03	George Bush Dr.	10	0
07/16/03	George Bush Dr.	5	1
07/22/03	George Bush Dr.	7	0
07/25/03	George Bush Dr.	7	0
07/25/03	Copper Falls Dr.	1	0
07/25/03	Camalot Dr.	1	0
07/28/03	George Bush Dr.	5	0
07/30/03	George Bush Dr.	10	0
07/30/03	Camalot Dr.	4	0
09/25/03	George Bush Dr.	15	0
09/30/03	George Bush Dr.	1	0
10/03/03	George Bush Dr.	2	0
10/03/03	Baker St.	1	0
10/08/03	Deer Trail	1	0
10/22/03	George Bush Dr.	3	0
10/24/03	George Bush Dr.	4	0
10/28/03	George Bush Dr.	1	0
11/06/03	George Bush Dr.	3	1
11/30/03	George Bush Dr.	1	0
Total		115	3

Table 2: List of all *An. quadrimaculatus* (Say) specimen identifications made in Bryan/College Station (Brazos County), Texas in 2004, organized by date and street name.

Brazos County 2004 *An. quarimaculatus* (Say) Collection			
Date	**Street**	**# of species A**	**# of species B**
03/30/04	George Bush Dr.	4	0
04/01/04	George Bush Dr.	1	0
04/07/04	George Bush Dr.	7	0
04/14/04	George Bush Dr.	3	0
04/15/04	George Bush Dr.	3	0
04/20/04	George Bush Dr.	4	0
05/24/04	George Bush Dr.	3	0
05/28/04	George Bush Dr.	9	0
06/02/04	George Bush Dr.	4	0
06/05/04	George Bush Dr.	2	0
07/01/04	George Bush Dr.	3	0
07/08/04	Vine St.	1	0
07/08/04	Morningside	1	0
07/08/04	Medow	1	0
07/08/04	Edgewood	1	0
07/06/04	George Bush Dr.	4	0
07/13/04	Vine St.	3	0
07/13/04	Lyndhurst	3	0
07/14/05	George Bush Dr.	3	0
07/18/04	Lyndhurst	3	0
07/27/04	Vine St.	4	0
07/27/04	Sharon	7	0
07/27/04	Lyndhurst	2	0
08/04/04	George Bush Dr.	6	0
08/10/04	Vine St.	1	0
08/10/04	Morningside	2	0
08/17/04	George Bush Dr.	2	0
09/22/04	George Bush Dr.	3	0
09/25/04	George Bush Dr.	15	0
10/06/04	George Bush Dr.	6	0
10/07/04	George Bush Dr.	7	0
Total		118	0

Discussion

This study demonstrated that *An. quadrimaculatus* was by far the most common species in the *An. quadrimaculatus* species complex found in Texas. *Anopheles smaragdinus* made up less than 3% of the total number of *An. quadrimaculatus* (Say) adults collected and identified. During this study, a record of only the species of the *An. quadrimaculatus* (Say) complex were recorded and all other species caught at the collection sites were discarded. Because of this, there was a selection bias for the five species in the *An. quadrimaculatus* (Say) complex and this data should not be used to compare to other species in the location of the collection sites.

It is interesting that only *An. quadrimaculatus* and *An. smaragdinus* were found in Texas, since, according to Levine et al. (2004), Texas has the habitat for all five species in the *An. quadrimaculatus* complex. This situation is not unique. Even with an environment that is suitable for a certain species, some areas harbor unsuitable living conditions that do not allow the species to be found in that location. Many barriers could keep a species from reaching and establishing themselves in a certain area. Geographic barriers such as mountains, dense forest, wind currents, and bodies of water could exclude a species from a certain location. Even if a species manages to invade an area, they could be out-competed by a species already living there, or a predator could wipe out the invading individuals.

Anopheles inundatus was found in the Cameron Parish of Louisiana, which is located directly across the Texas border from Orange and Jefferson County. Orange County is also very near to the Louisiana Parish called Calcasieu, which contains *An.*

maverlius (Rutledge and Meek 1998). Only three specimens of *An. maverlius* were collected in Calcasieu Parish and one specimen of *An. inundatus* was found in Cameron County, so the populations of both do not appear to be very high. To put these number in perspective, 60 specimens of *An. quadrimaculatus* were collected in Calcasieu Parish at the same location and 56 specimens of *An. smaragdinus* specimens were collected in Cameron Parish at the same location where one *An. inundatus* specimen was found. There is no known geological barrier that would keep *An. inundatus* and *An. maverlius* from entering into Texas, so there may be a competing species that is keeping these two species from establishing in Texas. Further testing will have to be done in this area to make sure that neither of these two species are found in Texas and to discover the reasons behind their exclusion. It was no surprise that *An. diluvialis* was not found in Texas since it has only previously been found in Florida, Georgia, and South Carolina.

The high percentage of *An. quadrimaculatus* specimens collected could have been because their numbers are greater than *An. smaragdinus* or maybe the habitat of the collections sites were targeted more toward *An. quadrimaculatus*. Most of the collections made by TDSHS were around large cities because that is where West Nile Virus problems were most severe. The other collection sites chosen for the current study were located in a more rural setting, and yielded only 3 *An. smaragdinus* specimens collected from a horse ranch in Brazos County.

Collection methods could have also had an effect on the large percentage of *An. quadrimaculatus* specimens. TDSHS collections were all made with gravid and light traps which are the most effective traps for *Culex quiquefaciatus* (Say), the vector of

West Nile virus. While neither of these traps is very effective at collecting *An. quadrimaculatus* (Say) specimens, they will catch a few, as was shown in this study. There is always the possibility that these traps are slightly more attractive to *An. quadrimaculatus* than *An. smaragdinus*. Of course, most of the other collections conducted in this study were done using either an artificial resting site trap or by aspirating the mosquitoes out of their natural resting sites. This method of collecting specimens was performed in numerous other studies, such as Rutledge and Meek (1998), Williams and Meisch (1981, Jensen (1996), and many others. While Rutledge and Meek used aspiration of artificial and natural resting sites and discovered 4 of the 5 species in the complex, only two species in the *An. quadrimaculatus* complex were found in the current study. With this precedent, it is interesting that the collection trips did not find a larger variety of species. Perhaps *An. diluvialis*, *An. inundatus*, and *An. maverlius* have not traveled into Texas and become established to date.

CHAPTER IV

THE GEOGRAPHIC AND ENVIRONMENTAL DISTRIBUTION OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX IN TEXAS

Introduction

The primary vector of malaria in Texas and the rest of the eastern half of the United States has always been *An. quadrimaculatus* (Say). Since the discovery of the *An. quadrimaculatus* species complex, made up of *An. quadrimaculatus, An. smaragdinus, An. diluvialis, An. inundatus* and *An. maverlius,* many eastern states have tried to determine a distribution and habitat of these new species. Fifty years has passed since malaria was last considered an established disease in the United States. Malaria still occurs in the United States in isolated instances, but fortunately, most of these cases occurred in individuals who traveled to an endemic area in a foreign country and become infected while there (Causer et al. 2002). Yet, each year, several cases of locally transmitted malaria occur with native *Anopheles* populations serving as vectors. To ensure that Health officials and mosquito control districts are prepared for a malaria outbreak, the distribution and habitat range of the An. quadrimaculatus species complex must be determined.

Global positioning has allowed researchers the opportunity to accurately assess the spatial distribution and habitat of organisms. Geographic Information System (GIS) technology is now being used for a wide array of tasks, from road directions, protecting the nation from terrorist attacks, and plotting out the distribution of an endangered animal. GIS applications are endless and will remain invaluable in the future. Many

Entomologists have turned to this technology to show current distribution and habitat and to even predict the present or future distribution of insect species.

Several studies have been performed on the distribution of the *An. quadrimaculatus* species complex, but only one has applied GIS technology. Levine et al. (2004) employed a computer program called Genetic Algorithm for Rule-set Prediction (GARP), which bases the prediction output on known Global Positioning System (GPS) coordinates of collection sites. For the current study, coordinates taken from each collection site were entered into a GIS computer program known as ArcVeiw 8.3 (ESRI, Redlands, California) and distribution patterns were observed across Texas.

While over half of the collection sites for the *An. quadrimaculatus* species complex used in this study also had habitat information provided, not all the reported sties contained these vital data and some habitat information was at best, very vague. A Texas map containing the collection sites was thus overlaid onto a map of the Texas Ecological Regions to determine the general habitat for both *An. quadrimaculatus* and *An. smaragdinus* since they were identified as being present in Texas, as described in the previous chapter. In order to get a more detailed picture of the habitat for these two species, the 1992 National Land Cover data set was overlaid onto the map of the collection sites. The known habitat information available for certain of the collections sites could then be used as a verification device.

Materials and methods

The latitudinal and longitudinal coordinates were recorded for each collection site that was surveyed by this investigator. Information data sheets were included along

with each *Anopheles* specimen sent by TDSHS. These data sheets contained the longitudinal and latitudinal coordinates or address, trap, and comments about the given specimen's collection site. If the information sheets only had an address of the collection site, then the GPS coordinates were obtained by entering the address into a program on the Geocode web site. These coordinates are as accurate as obtaining the coordinates at the collection site.

Once all of the coordinates were obtained, they were entered into a geographic information system (GIS) computer program, ArcView 8.3. The collection points were first overlaid onto a map of Texas with all of the counties shown in outline form. This map and all layers that were added to it were always projected in Albers Conical Equal Area to ensure that the all of the layers aligned properly and were as accurate as possible. The overall distribution across the state could then be observed on this map. Next, the 1992 Texas Ecological regions were overlaid onto a map containing the outline of Texas and all of the collection sites. Once it was discovered that the *Anopheles* collections were located in almost all of the ecological regions found in Texas, the coordinates were then overlaid onto the 1992 National Land Cover data set, enabling more specific habitat information to be observed. This data set used the Anderson et al. (1976) classification system with a 21-class legend. When Anderson et al. (1976) developed their classification system, they stated that the accuracy of using this system should be no lower than 85% (Anderson et al. 1976). Unfortunately, the 1992 data set is the most recent data available for land cover. An accuracy assessment made in 2004 by Wickham et al. (2004) of the South Central United States (which

includes Texas) concluded that this data set was 74% accurate. There were 21 different land cover categories found in Texas on the 1992 National Land Cover data set and *An. quadrimaculatus* (Say) specimens were collected in 15 of them (Table 3).

Table 3: Land cover numbers used by the 1992 National Land Cover data set that identify the habitat types where *An. quadrimaculatus* (Say) specimens were collected and the ecological description that corresponds to each number.

Land Cover #	**Ecological description**
21	Low intensity residential
22	High intensity residential
23	Commercial/industrial/transportation
32	Quarries/strip mine/gravel pits
41	Deciduous forest
42	Evergreen forest
43	Mixed forest
51	Shrub land
71	Grassland/herbaceous
81	Pasture/hay
82	Row crops
83	Small grains
85	Urban/recreational grasses
91	Woody wetlands
92	Emergent herbaceous wetlands

Table 3 contains all of the land cover descriptions where *An. quadrimaculatus* (Say) specimens were found. The land cover designated as low intensity residential includes locations with 30-80 percent of the area covered by human construction, with vegetation covering from 20 – 70 percent of the area. Low intensity residential land cover usually includes single-family homes with the human population density lower than the high residential areas. The high intensity residential areas include locations

with human construction accounting for 80 – 100 percent of the area and vegetation accounting for 0 – 20 percent of the designated area. These areas are usually highly-populated with multiple family homes and are intensely developed.

The land cover known as commercial/industrial/transportation includes all areas not designated as high intensity residential, roads, railroads, and other modes of transportation. Another urban land cover is the urban/recreational grass cover which includes vegetation planted in urban areas for erosion control, recreation, or aesthetic purposes. While the vegetation planted usually is species of grasses, this is not always the case. Some of the examples of this land cover include parks, airport, golf courses, and lawns.

Some of the less urbanized land covers include quarries/strip mine/gravel pits, deciduous forest, evergreen forest, mixed forest, and shrub land. Areas designated as quarries/strip mines/gravel pits are any locations of extractive mining activities with surface expression. For a region to be designated as deciduous forest it must be dominated by trees and at least 75 percent of those tree species must shed foliage simultaneously because of seasonal changes. Evergreen-forested areas are locations where at least 75 percent of the tree species maintain their leaves throughout the year. A forested area is determined to be mixed if neither deciduous nor evergreen species make up 75 percent of the tree species. Shrub land areas are designated where shrub canopy accounts for 25 – 100 percent of the cover. Shrubs include natural to semi-natural woody vegetation that are usually less than 6 meters tall and can include both deciduous

and evergreen species of true shrubs or they could be trees that are young or stunted because of environmental conditions.

Grasslands and herbaceous locations that are not cultivated include the land cover area known as grassland/herbaceous. This area mainly contains upland grasses and forbs. These areas can be utilized for grazing, but they are not heavily managed by humans. Regions that are heavily managed by human for food or feed for animals include pasture/hay, row crops, and small grains. Pasture/hay land cover areas contain grasses, legumes, or grass-legume mixtures that are planted for livestock grazing, for hay, or seed production. Areas that contain crops such as corn, soybeans, vegetables, tobacco, and cotton are designated as row crops. Locations where grainoid crops are produced such as wheat, barley, oats, and rice are said to have “small grain cover”.

Finally, there are two types of wetlands found in Texas: woody wetlands and emergent herbaceous wetlands. A wetland includes areas where the soil or substrate is periodically saturated with or covered by water. Woody wetland cover encompases areas that have 25 – 100 percent of the land covered by forest or scrubland and the soil is periodically saturated or covered by water. A region where perennial herbaceous vegetation accounts for 75 – 100 percent of the land cover and is also considered a wetland by the standards written above, is defined as an emergent herbaceous wetland.

The land cover data was separated into four different data sets. The first was looking at the land cover percentages for all of the collections sites of the *An. quadrimaculatus* (Say) specimens. Then the land cover percentages for all *An.*

quadrimaculatus (Say) specimens were observed. Finally, the land cover percentages were calculated for the *An. quadrimaculatus* specimens and *An. smaragdinus* separately.

Results

When the overall distribution of the *An. quadrimaculatus* (Say) collection sites in Texas were observed it became apparent that the majority of the sites were located in the eastern half of the state. One collection site in Potter County, which is located in the pan handle of Texas, and one collection site in El Paso County are the only two collections that were truly in far west Texas (Fig. 5). There are several collection sites on the edge of west Texas in Bexar, Hidalgo, and in Live Oak County, but in the majority of west Texas either no collections were attempted, or no *An. quadrimaculatus* (Say) specimens were found. Collections were attempted in the counties of Bandera, Medina, Kerr, and Kendall, which are all west of San Antonio. A collection trip was also made to Kinney and Edwards counties, which are on the border of Texas and Mexico, but again no specimens of *An. quadrimaculatus* (Say) mosquitoes were found. The fact that few collections were attempted in the western half of Texas needs to be taken into account when these data are discussed.

The distribution of *An. smaragdinus'* collection sites in Texas is similar to the overall distribution of *An. quadrimaculatus* in the eastern third of the state (Fig. 5 and 6). However, none of the collection sites where *An. smaragdinus* specimens were found are located in west Texas. The only collection sites that are even close to west Texas are either on the Texas Gulf Coast, near a large city, or both. These areas would provide water that is essential to mosquito survival. In south Texas, *An. smaragdinus* was

collected around the Corpus Christi area, but no counties further south were shown to contain this species (Fig. 6).

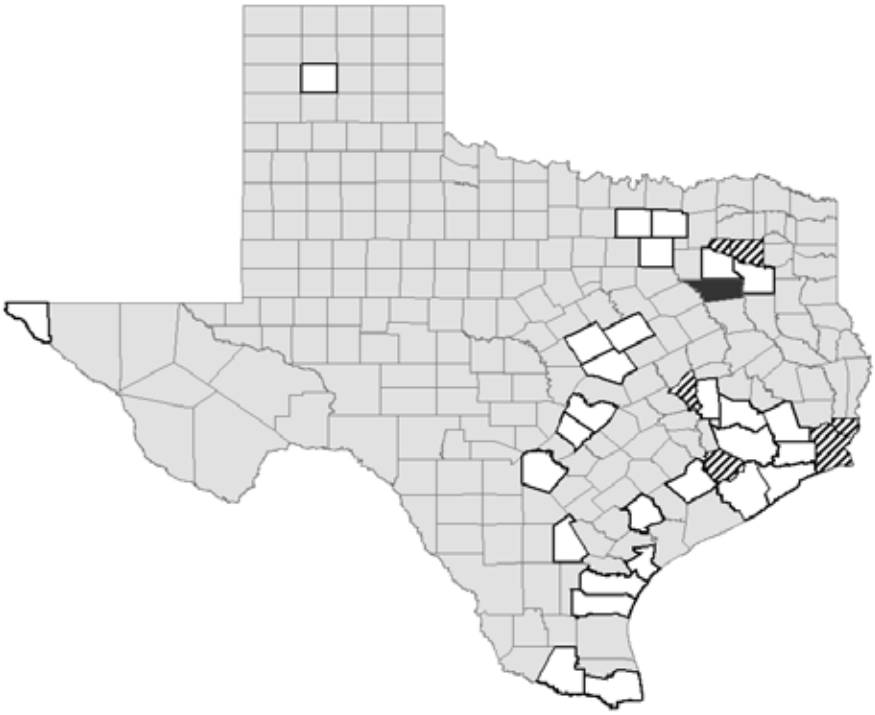

Figure 5. Texas map depicting the counties where collections of *An. quadrimaculatus* (Say) specimens were made in Texas and the species found in them. The gray counties represent no *An. quadrimaculatus* (Say) mosquitoes collections, white counties are where only *An. quadrimaculatus* specimens were collected, striped counties are those where both *An. quadrimaculatus* and *An. smargdinus* were collected, and the black county is where only *An. smargdinus* specimens were collected.

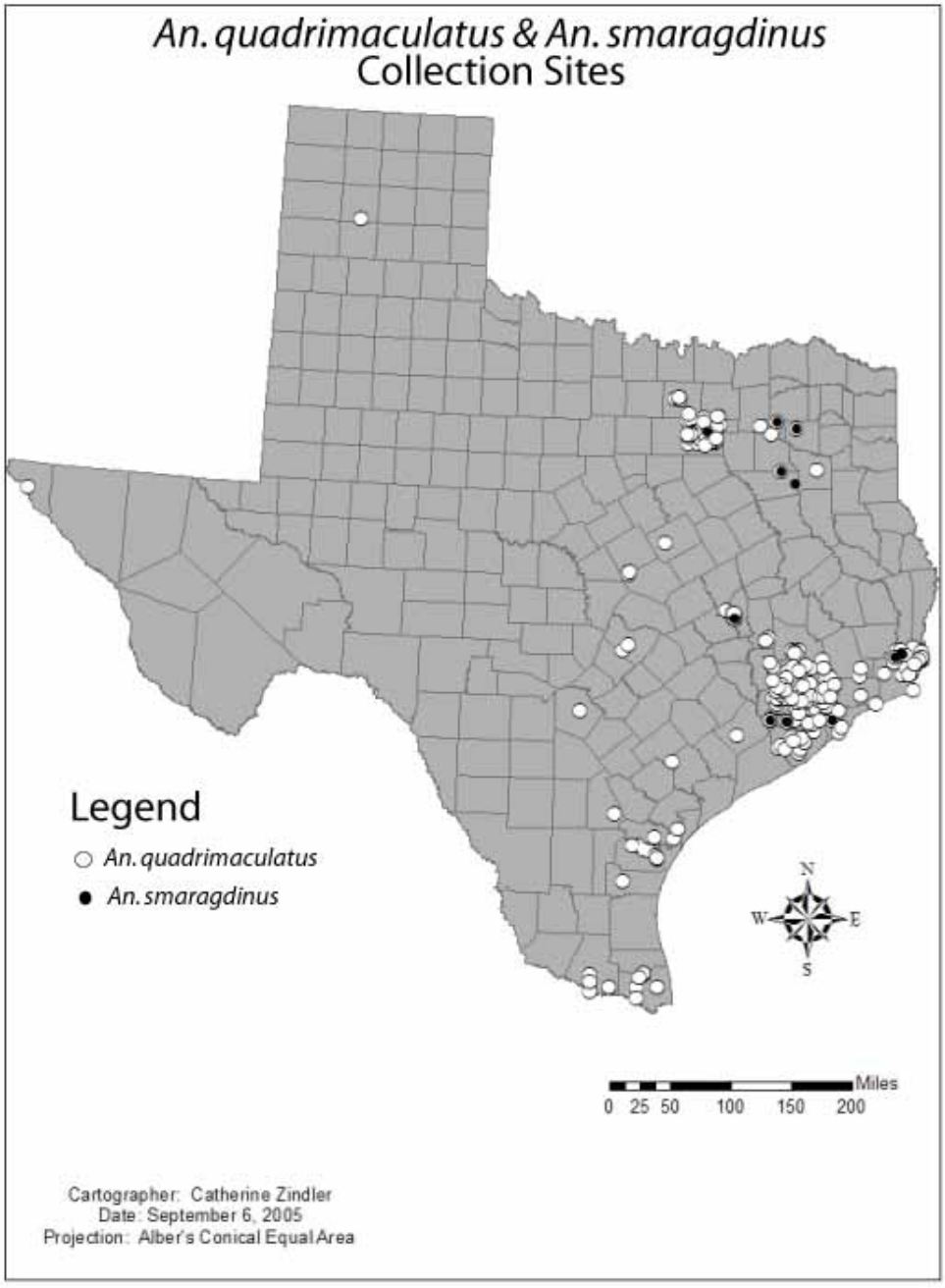

Figure 6. Map depicting all *An. quadrimaculatus* collection sites with a white circle and all of the *An. smaragdinus* collection sites with a black circle.

There are 11 ecological regions in Texas and *Anopheles* collections were made in 9 of these regions (Fig 7). *Anopheles quadrimaculatus* specimens were collected from blackland prairie, coastal sand plains, Edwards Plateau, Gulf Coast prairies and marshes, high plains, oak woods and prairies, piney woods, south Texas country, and Trans Pecos. Although the high plains and Trans Pecos are represented, there was only one collection site from these two ecological zones. The only two ecological zones that were excluded were the Llano uplift and the rolling plains. *Anopheles smaragdinus* was found in the blackland prairie, gulf coast prairies and marshes, oak woods and prairies, and piney woods (Fig. 7).

When the land cover data was observed for the mosquitoes identified as *An. smaragdinus* using ArcView 8.3, 43% of them were collected in woody wetlands, 23% of them were collected in deciduous forest, 17% were captured from low intensity residential, 15% came from pastureland/hay, and 2% were collected from shrub land (Fig. 8). These percentages are interesting because the most common land cover for *An. quadrimaculatus* specimens, all of the collection sites, and all of the *An quadrimaculatus* (Say) specimens was pasture/hay, not woody wet lands. In fact, woody wetlands only made up between 4% to 7% of the land covers for all three data sets (Figs. 9, 10, 11). *Anopheles quadrimaculatus* was collected on all of the 15 land covers, but there was no land cover that came close to the percentage of pasture/hay (32%) for all of the *An. quadrimaculatus* specimens collected in this study. The next highest percentage was low intensity residential at 16%, then deciduous forest at 11%, followed by high intensity at 8% (Fig. 10).

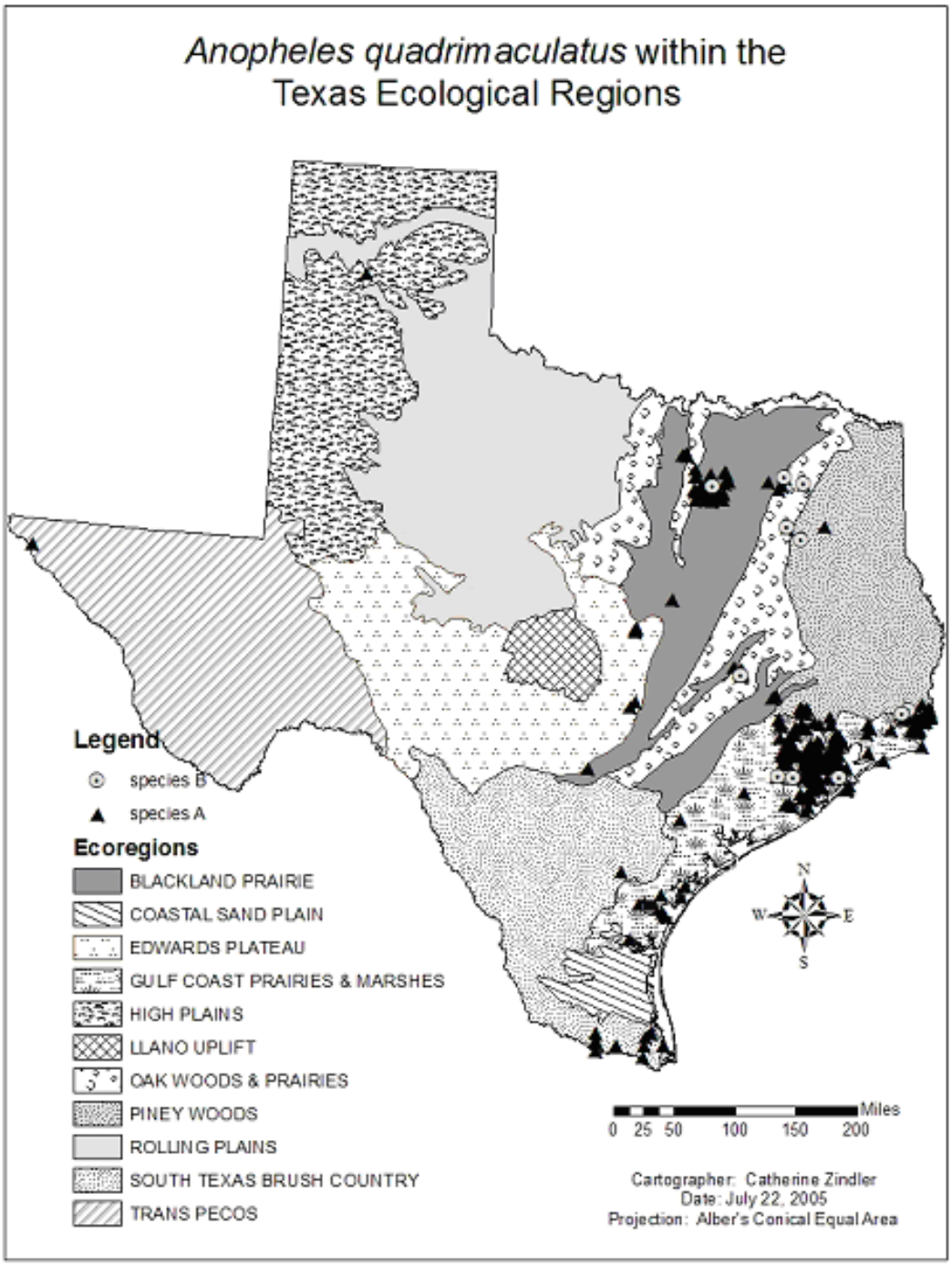

Figure 7. Map of Texas with the 11 different ecological zones. The collection sites of *An. quadrimaculatus* represented on it as black triangles, while *An. smaragdinus* is represented by a grey bull's eye.

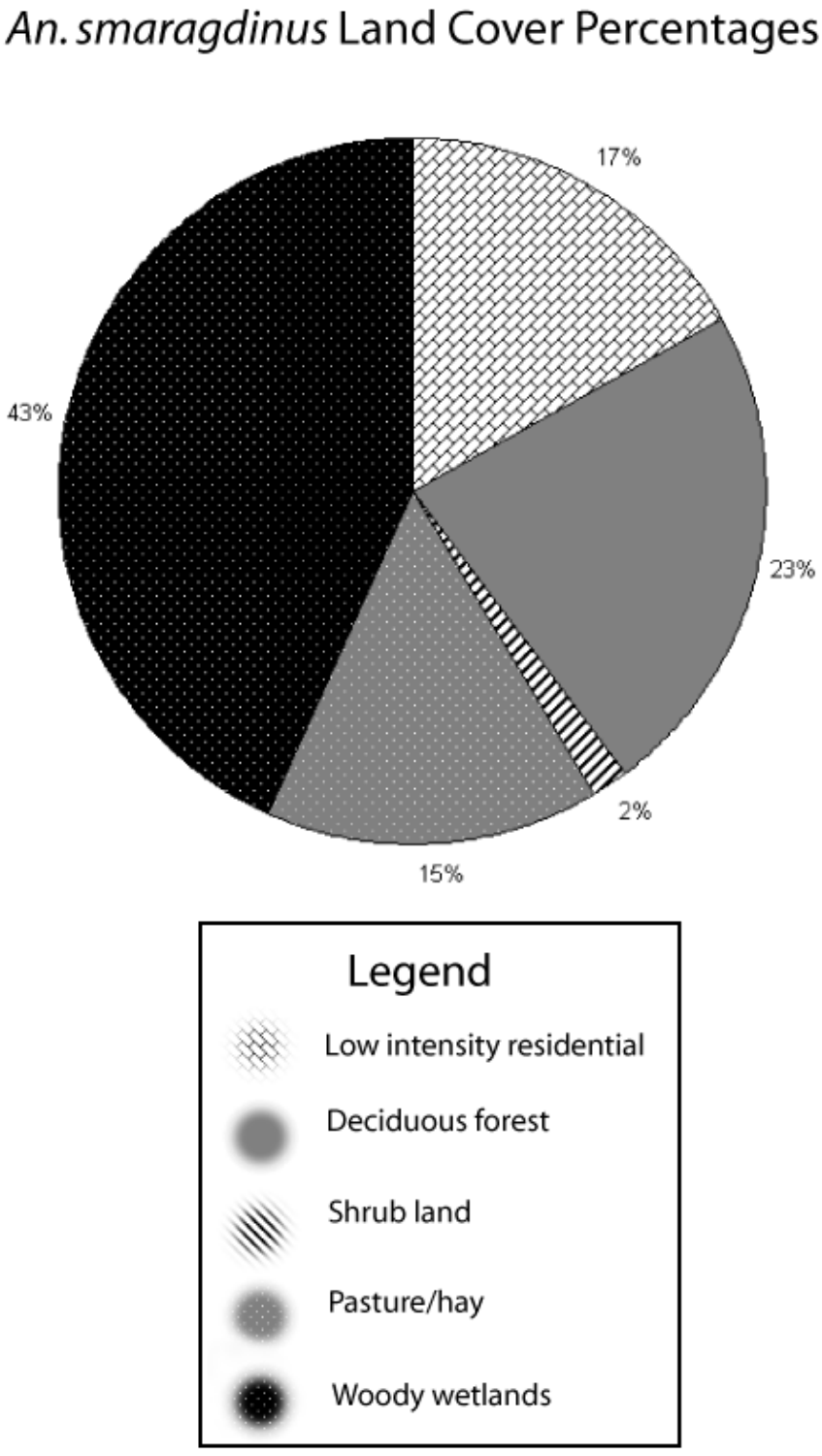

Figure 8. Pie chart depicting the percentage of *An. smaragdinus* specimens collected on 5 different land covers.

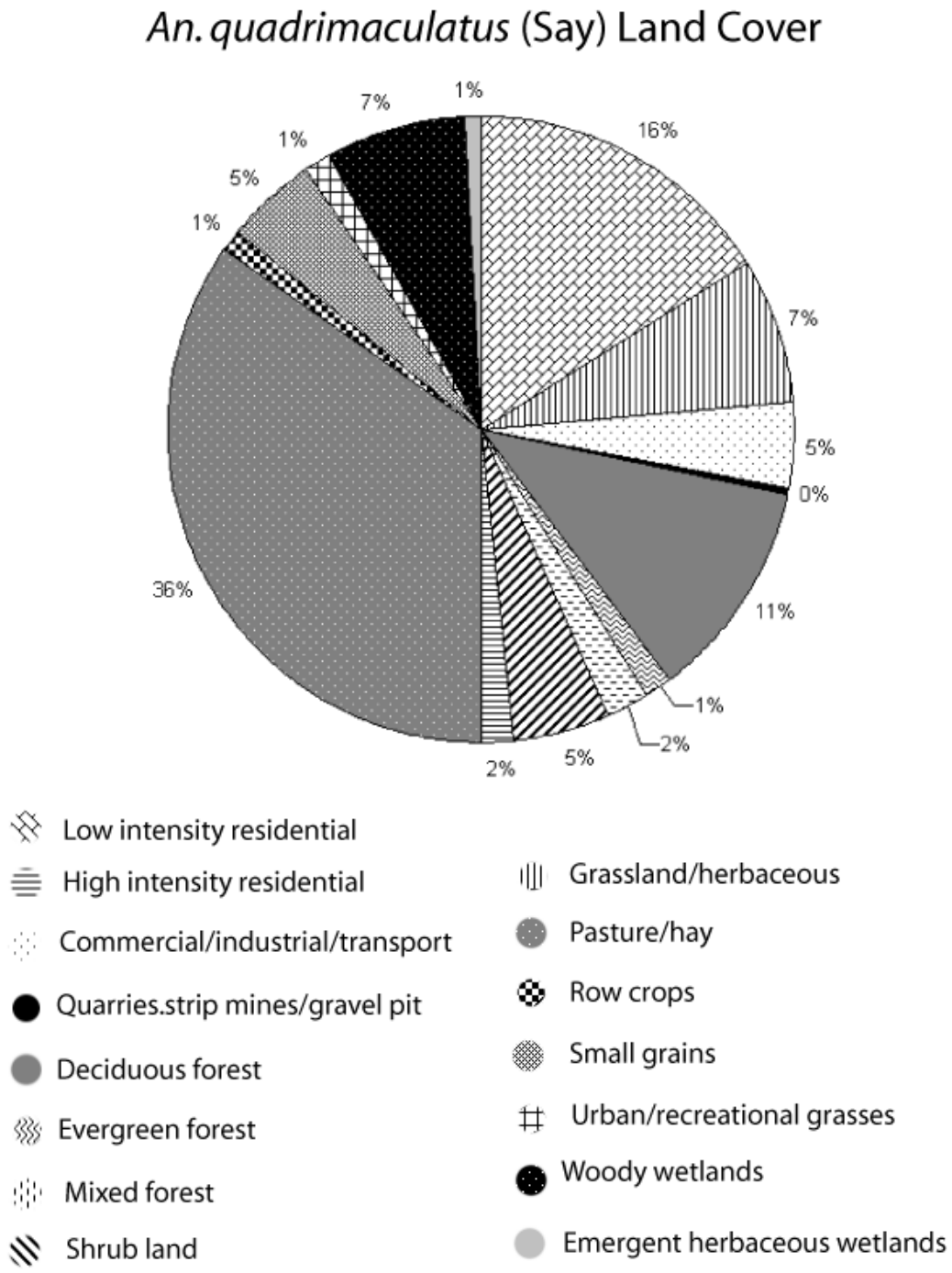

Figure 9. Pie chart depicting the percentage of *An. quadrimaculatus* (Say) specimens collected on 15 different land covers.

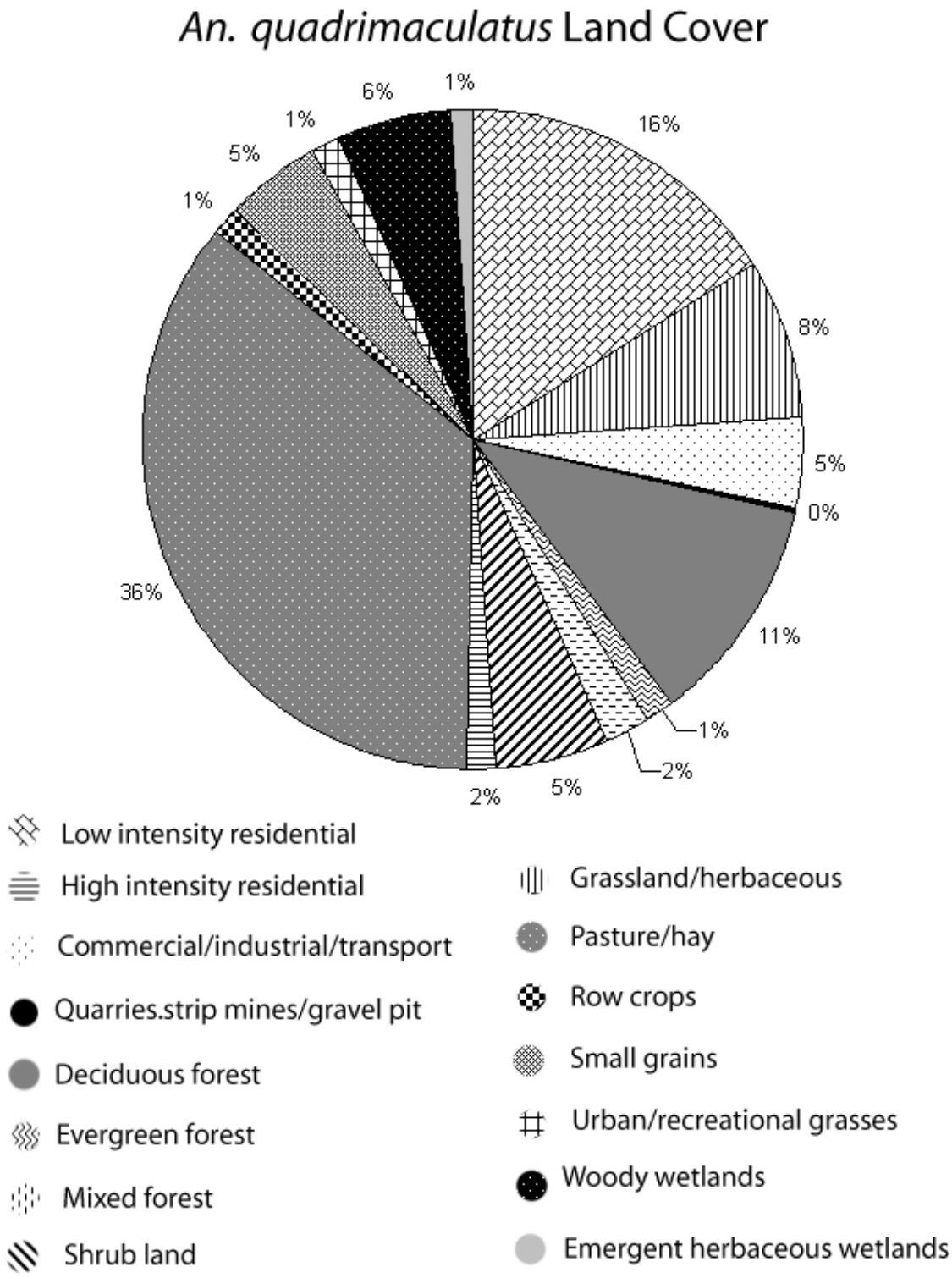

Figure 10. Pie chart depicting the percentage of *An. quadrimaculatus* specimens collected on 15 different land covers.

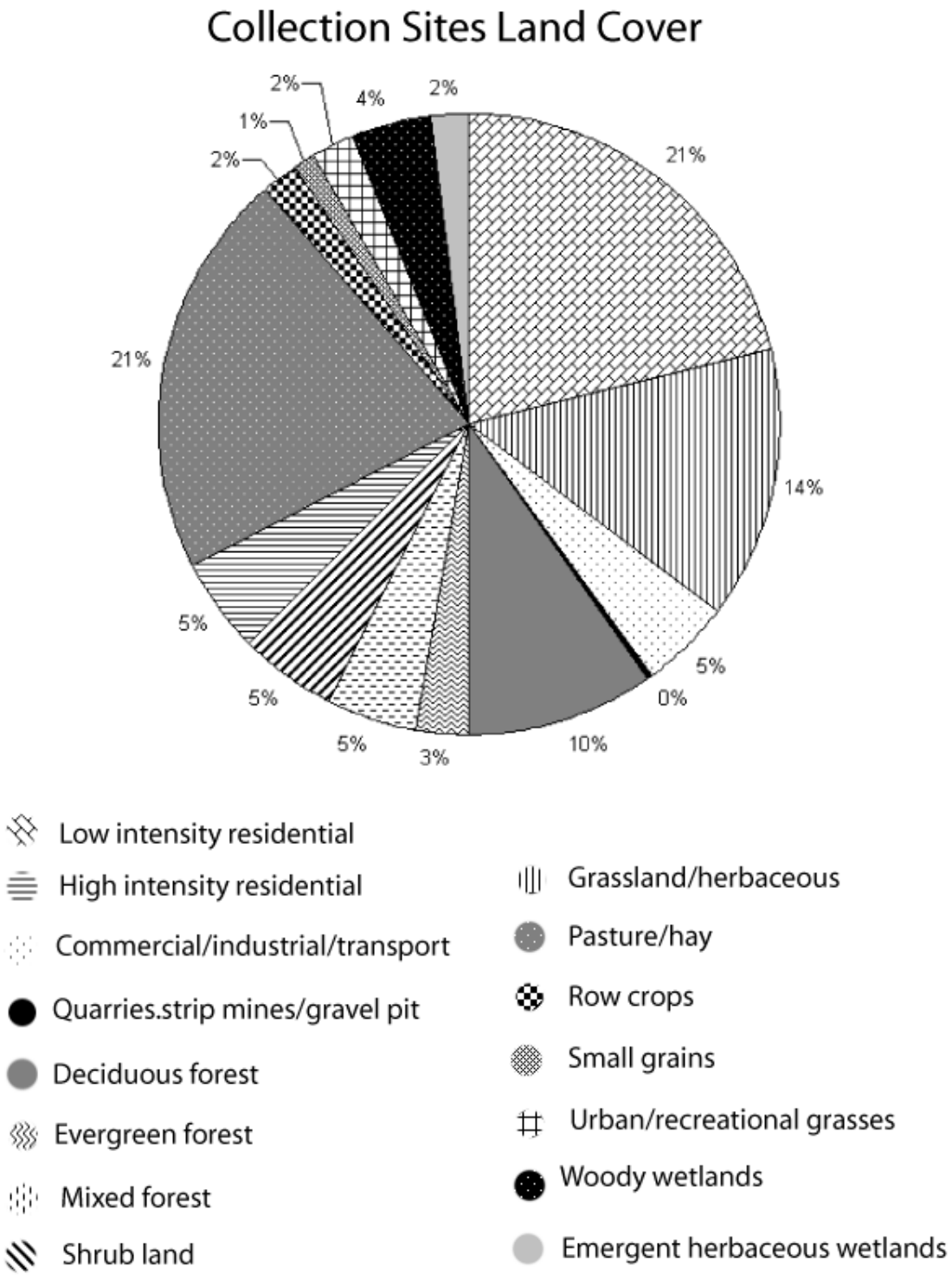

Figure 11. Pie chart depicting percentages of all collection sites found on 15 different land covers.

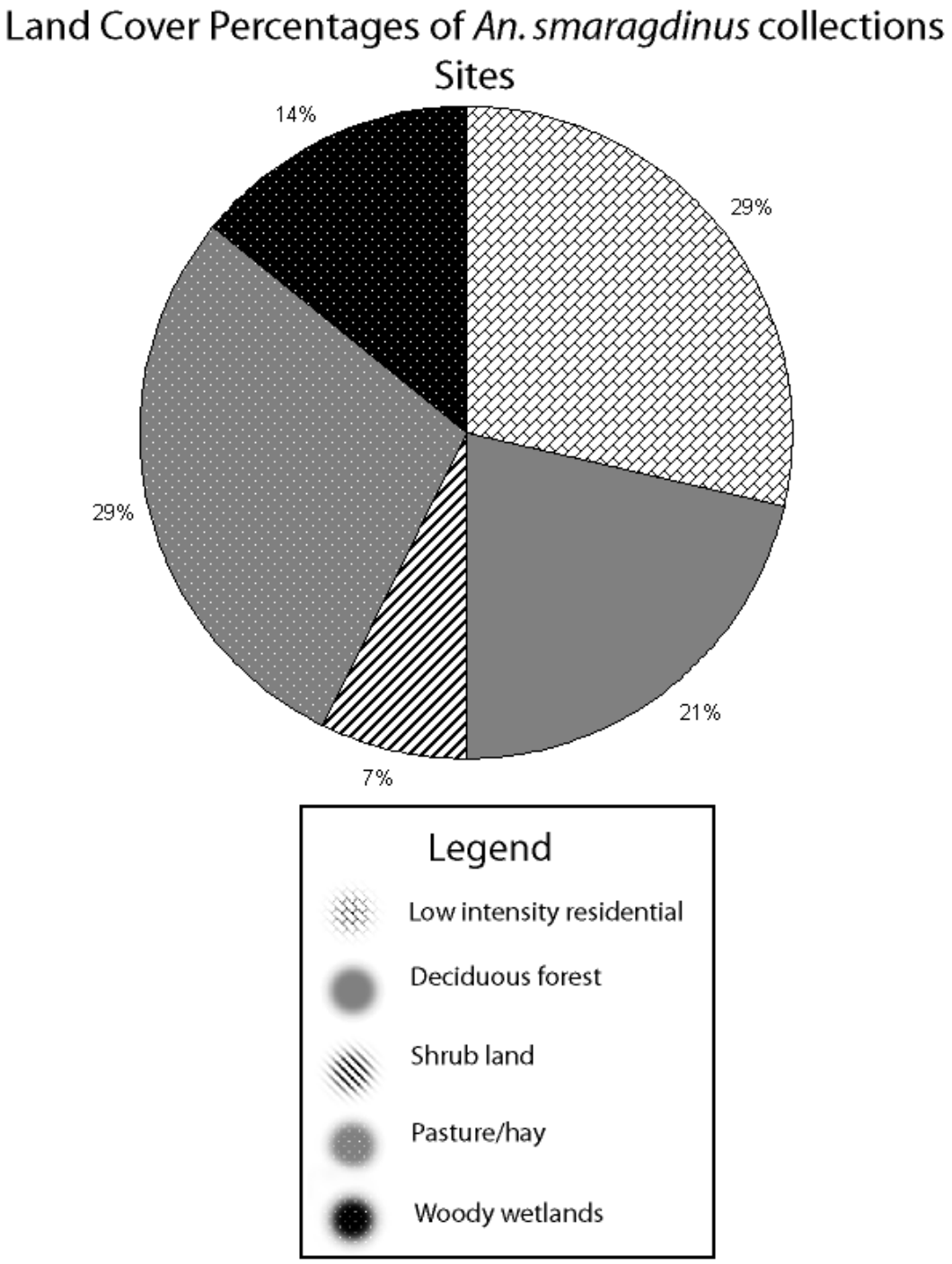

Figure 12. Pie chart depicting percentages of *An. smaragdinus* collection sites found on 5 different land covers.

When the land cover data for all of the individual collection sites were analyzed, it was found that both pasture/hay and low intensity residential had the highest percentage of collection sites at 21%. The next highest was high intensity residential at 14% and then deciduous forest at 11% (Fig. 11). When looking at the percentages of *An. quadrimaculatus* (Say) specimens found on the different land covers, it was discovered that the only land cover percentage that was different from those observed just for *An. quadrimaculatus* by itself was the woody wetlands frequencies that changed from 6% for *An. quadrimaculatus* specimens to 7% of the total number of specimens found (Fig. 9 and 10).

When just the land cover percentages of *An. smaragdinus* collection sites were looked at the amount of specimens caught at each site was not taken into account the results were very different from the land cover percentages of all *An. smaragdinus* specimens. In fact, the *An. smaragdinus* collection site land cover percentages resembled the combined collection sites of *An. quadrimaculatus* and *An. smaragdinus* percentages. The most common land cover for *An. smaragdinus* collection sites was pasture/hay and low intensity residential. Although woody wetland still made up 14% of the collection sites, it was only the fourth most common land cover out of five (Fig. 12).

Many of the specimens collected by TDSHS had habitat data provided for the location in which they were collected. The original plan was to compare the habitat information provided by the specimens' collector, but most of the descriptions were either too vague or only pertained to the microhabitat of the collection site. Some

examples: Four (4) collections sites were set up “in a bird roost”, one collection site was set up “on a trail”, and a few reoccurring habitat descriptions were: “rural”, “residential”, “pond”, “field”, and “storm culvert” (Appendix B). While any description of the habitat is informative, it was hard to use most of the habitat information provided with the TDSHS collections to validate the land cover data that were collected from the ArcView 8.3 GIS program.

Nine of the thirteen different sites where *An. smaragdinus* was found had habitat descriptions provided (Appendix B). While it is still hard to compare the TDSHS habitat descriptions with the land cover data, the additional information for the TDSHS collections is informative about the microhabitat, especially when the collection sites were low intensity residential. The TDSHS habitat description at one of the low intensity residential sites said that at the site there were trees and brush, which could provide the mosquitoes with shelter. Another TDSHS collection site description where the land cover was low intensity residential said that the trap was placed in a storm culvert”. This structure could have also provided adult mosquitoes with shelter during the day as well as a possible larval habitat. Among all 9 collection sites the additional habitat information provided by the TDSHS described some form of adult resting site. Tree, brush, river bottom (which is almost always lined with trees), storm culvert, and woods were the potential resting sites described in the TDSHS habitat information. Taking all this information into account, the typical collection site for *An. smaragdinus* in a residential area of Texas is a zone with oak and/or pine trees with some type of water source near-by, often a ditch or small pond (Fig. 13).

Figure 13. Typical collection site of *An. smaragdinus* in an urban setting in Texas. This collection site was in Beaumont, Texas, in a residential area and both *An. smaragdinus* and *An. quadrimaculatus* specimens were collected here on two different dates in 2004.

One of the collection sites for *An. smaragdinus* that contained a slightly atypical habitat was one found in Sabine Pass, just south of Port Arthur. This location contained mostly coastal prairies and a few oak trees (Fig. 14). The limited amount of resting sites for the adult mosquitoes at this location was interesting, however, at the collection site, there was a small stand of oak trees where the adults probably sought shelter during the day. This location was only a few hundred yards away from the Gulf of Mexico and the area is most likely very marshy when it rains. This, along with the small clump of trees, is what allowed the *An. smaragdinus* mosquitoes to survive.

Figure 14. Atypical coastal prairies habitat where *An. smaragdinus* and *An. quadrimaculatus* specimens were collected several times throughout the 2004 season.

Anopheles quadrimaculatus, on the other hand, have several recurring TDSHS habitat descriptions that did not note any forms of adult resting sites. Some of the descriptions included: “field”, “yard”, and “trails”. There are not many differences in the types of habitat where *An. quadrimaculatus* specimens were collected and where *An. smaragdinus* specimens were collected. There are several collections of *An. quadrimaculatus* that were made from bird roosts, and no *An. smaragdinus* collection were noted in the TDSHS information on being made from bird roosts. Another interesting observation is that several TDSHS *An. quadrimaculatus* collection site descriptions were residential and no *An. smaragdinus* descriptions were simply labeled as “residential”. Another TDSHS habitat description of some *An. quadrimaculatus* collection sites that was not among the descriptions of *An. smaragdinus* collection sites was that there was “sewage at the site”. The final difference in the TDSHS collection site description data between *An. quadrimaculatus* and *An. smaragdinus* is that the former was collected in barns, but the later was never collected in a barn during this study (Appendix B).

Discussion

When the overall distribution of all of the collection sites for *An. quadrimaculatus* (Say) in this study is observed, the distribution is very similar to that found by Fournier and Snyder in 1977. There are a few counties that are represented by Fournier and Snyder (1977) that the current study does not include, and there are a few counties identified in the current study as having *An quadrimaculatus* (Say) that were not represented by Fournier and Snyder (1977). This may not mean that the distribution

has changed from 1977. Researchers may have used different resources to obtain their information and used varying techniques to collect their specimens and data points. A combined map of both the distributions is probably best in this case, especially since not all of the counties in Texas were sampled in this study or even perhaps in Fournier and Snyder's (1977) research.

It is also interesting to note that the distribution of *An quadrimaculatus* (Say) found in the current study is very similar to that of the areas in Texas where malaria was thought to be endemic in 1912 (Zucher 1996, Fig. 15). This should not be surprising however, because if the distribution of *An. quadrimaculatus* mosquitoes has not changed, then the disease that can be vectored by these mosquitoes would have the same distribution as long as that disease is endemic to the area where the vector is found. While malaria has been removed from Texas, the susceptible host and reservoir (humans) and the vector (*An. quadrimaculatus*) are still present. If the malaria parasite is ever reintroduced into Texas, then the distribution of the disease could again reach the 1912 estimation.

As noted in data collected during the current study, the distribution of *An. smaragdinus'* appears to be limited to the eastern half of Texas. When collections were attempted in west Texas, either no *An. quadrimaculatus* (Say) specimens were found, or only a few *An. quadrimaculatus* mosquitoes were collected. The habitat in west Texas is very different from the eastern half of the state. In west Texas, the elevation increases in some areas, and the climate becomes much dryer. These conditions would stress any species of the *An. quadrimaculatus* (Say) complex. It may be that *An. smaragdinus* can

not handle these conditions and thereby have difficulty finding habitat to prevent them from dehydrating during hot, dry days. *Anopheles quadrimaculatus* would also be stressed under these conditions; hence, the possible reason for the decreased number of collections recorded for the western region of Texas. However, it appears that a few individuals can survive, thus allowing for a theory that *An. quadrimaculatus* is a heartier species than is *An smaragdinus*. Out of all of the species in this complex, *An. quadrimaculatus* has the widest distribution. This would lead one to believe that this species is the most adaptable of the *An quadrimaculatus* (Say) species to habitat changes and can perhaps adapt to changing climates and habitats at a faster rate than can other species in the complex. Future study needs to be done to support this theory.

Anopheles smaragdinus was found in lower numbers and appears to be more restricted in the type of habitat that it can occupy. Comparable research in the distribution of this species complex was performed in Louisiana by Rutledge and Meek (1998) where they also found that *An. smaragdinus* had a narrow habitat range while *An. quadrimaculatus* could be found in higher numbers and in a large variety of land covers. This holds true even on a larger scale; Seawright et al. (1992) found that, out of the 16 states they sampled, all of them contained *An. quadrimaculatus* while only 9 of them contained *An. smaragdinus*.

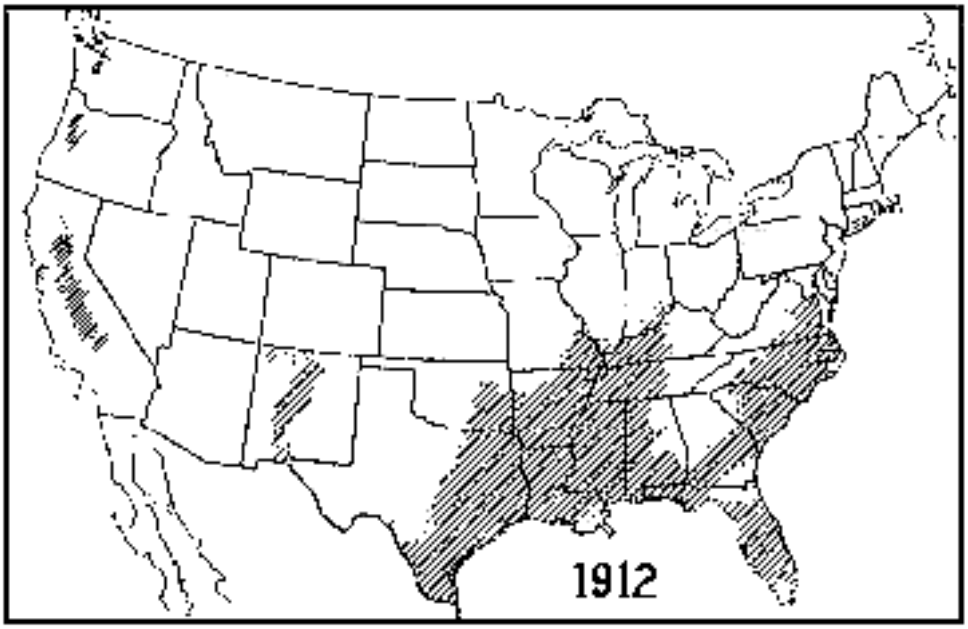

Figure 15. Map showing areas of the United States where malaria was thought to be endemic in 1912 (Zucher 1996).

The majority of *An. smaragdinus* specimens, collected in Texas, were mostly found in woody wetlands which also corresponds to results described by Rutledge and Meek (1998) for Louisiana. However, Rutledge and Meek (1998) found that *An. smaragdinus* was always the dominant species when collections were made from chicken coops. In the case of chicken coops sampled during the current study, the only species collected was *An. quadrimaculatus*. In four different sites, it was stated by TDSHS descriptors that birds were present at the time of collection or that the collection was made in a bird coop. Multiple TDSHS collections were made at the site in Ft. Bend and Dallas County, which would suggest that no species that occurred at the site were missed. The other two collection sites were single collections and were from Cameron

and Galveston counties. When the positions of these collection sites was examined, northeastern, southeastern, and southern Texas all had TDSHS collections made where birds were present. A large geographical range was thus represented and collection were made from a variety of habitats; so, most likely, if *An. smaragdinus* was the most dominate species in Texas bird coops then at least one of these collection sites would have shown that. Further investigation needs to be made as to why *An. smaragdinus* was not found in these bird coops, while, in the neighboring state, *An. smaragdinus* was always found to be the dominate species in the same habitat.

While the majority of individual *An. smaragdinus* specimens were found on woody wetlands, the majority of the collection sites were found on pasture/hay and low intensity residential. This shows similarity *to An. quadrimaculatus* collection sites and individual specimens; however, the sampling bias needs to be taken into account when discussing this result. When individuals are sampling for mosquitoes, they look for a site that is easy to reach and requires only a small amount of walking. As a result, many traps are set on the edge of a field near a clump of trees or woods. The trees supply a place to tie the trap to and few mosquito collectors will hike through the woods. This sampling bias may be why most of the collections are from savannah type habitats. A greater amount of *An. smaragdinus* specimens were collected in the woody wetlands habitats, so this may suggest that it is a preferred and more prolific habitat for this species. Another fact that must be taken into account is that *An. quadrimaculatus* (Say) species can fly around 1 mile in the course of one day and so the habitat that the

specimens were collected on my not have been where the mosquito spent most of its time.

CHAPTER V

OBSERVATIONS ON SHIFTS IN *ANOPHELES QUADRIMACULATUS* (SAY) SPECIES COMPOSITION OVER TIME IN TEXAS

Introduction

The *An. quadrimaculatus* (Say) species complex, made up of *An. quadrimaculatus, An. smaragdinus, An. diluvialis, An. inundatus* and *An. maverlius,* includes the primary vectors for malaria in the eastern half of the United States. Since scientists still do not know all of the biological differences between these species, it is important to continue research on this complex. While malaria is no longer endemic in the United states, it does still occur in isolated instances. For this reason it is important to give as much information to public health officials and mosquito control districts about where to treat, when to treat and at what times of the year each of the members of the *An. quadrimaculatus* (Say) species complex are most active.

During the course of a year, temperature, humidity, and precipitation changes along with the biotic environment can impact the survival rate of local mosquito populations. In many cases, an animal does not stay active all year around and it has been determined that mosquitoes, as well as many other animal species, have seasonal preference. As an example: during the winter months in Brazos County, Texas, the primary winter pest is *Aedes vexans,* while *Culex quinquefasciatus* and *Aedes albopictus* take over in the summer. While conducting the current study of the distribution of the *An. quadrimaculatus* (Say) species complex in Texas, the date of specimen collection was always recorded so any seasonal preferences by the species discovered in Texas

could be observed and recorded. This information could be very important, because we still do not know if some of the species in the *An. quadrimaculatus* complex are more competent malaria vectors than others. If it is discovered that some species are more competent malaria vectors, then public heath officials and mosquito control districts need to know when the species is most likely to occur in any given area.

Materials and methods

The collection dates for each site included in the current study were linked to the coordinates in the ArcView 8.3 GIS computer program to allow the distribution over time to be analyzed. The computer program could then show the density pattern each month in Texas for both *An. quadrimaculatus* and *An. smaragdinus*.

The data were analyzed further by dividing each month into three parts and looking at when and where only *An quadrimaculatus* was collected, only *An. smaragdinus*, and where *An. quadrimaculatus* and *An. smaragdinus* were collected in combination. The number of mosquito specimens collected was also observed in this analysis. By looking at how much overlap occurred, it could be determined if the species were usually found in combination, or if one species became less frequently collected while the other was colleted in higher numbers as time progressed.

Results

The density levels of *An. smaragdinus* were observed to occur first during the month of May in south Texas around the Corpus Christi area and in southeast Texas around the Houston and Beaumont area (Fig. 16). In June, specimens of this species were only collected in southeast Texas, but in July they could be found throughout

eastern Texas, mainly around the Beaumont area and east of Dallas. While *An. smaragdinus* was still be found in the Houston area in August, the majority were located in northeast Texas, just east of Dallas. The density then became higher in the southeastern part of the state again and could only be found in the Houston area in September and October. In November, at the end of the *Anopheles* season, *An. smaragdinus* was still found with the highest density around the Houston area, with a few also found in Nueces County (Fig. 16).

Anopheles quadrimaculatus distribution over time was very similar to *An. smaragdinus*, but they were collected two months earlier and two months later than were any specimens of *An. smaragdinus* (Fig. 17). Specimens of *An. quadrimaculatus* were first collected in March and were located in southeast Texas, mainly in Brazos County. In April, specimens of *An. quadrimaculatus* were also collected in southeast Texas, but the highest density of specimens during this month were collected from Cameron County, the most southern county in Texas. Specimens were collected throughout eastern Texas in the month of May and in Cameron County. The densities levels are relatively even throughout east Texas during this month with, the highest density was occurring in the Dallas area. During the month of June, specimens of *An. quadrimaculatus* were found throughout eastern Texas, but some collection sites appeared in central Texas and a few more sites were found along the southern coast of Texas. The highest density areas during the month of June were in the southeastern areas around Beaumont, Houston and College Station (Fig. 17).

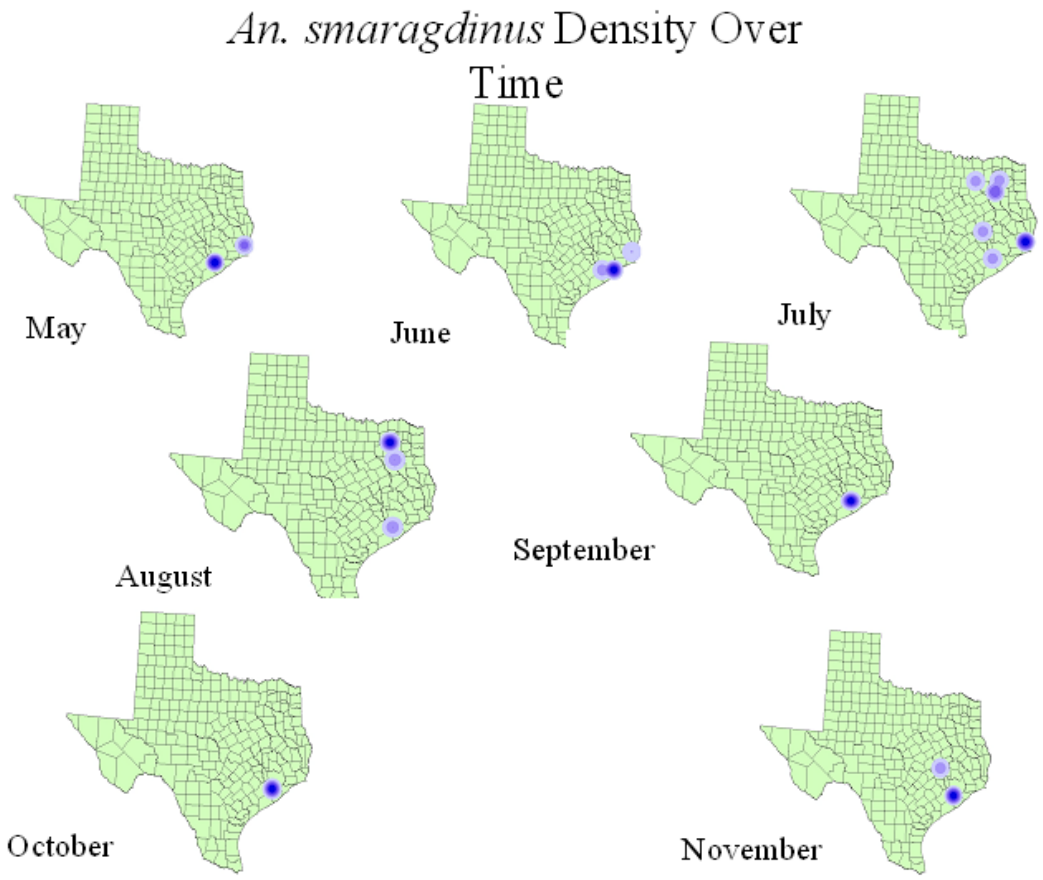

Figure 16. Density of *An. smaragdinus* in Texas from May to November. As the density of *An. smaragdinus* specimens collected increases, the shaded circles get darker.

The distribution of specimens identified as *An. quadrimaculatus* during July was very similar to June, except that 2 specimens were found in El Paso. During the month of August, the distribution of *An. quadrimaculatus* was again found throughout eastern and central Texas, but areas of highest density included the areas surrounding Dallas, College Station, Houston, Beaumont, and Corpus Christi. *Anopheles quadrimaculatus*

specimens were predominantly collected in east Texas during September, but a few specimens were collected in south Texas as well.

The distribution of *An. quadrimaculatus* collections in October was almost identical to the distributions found in September. The difference found between these two months is that sites with the highest number of specimens during October were found only in southeast Texas while the highest number of specimens was found in south and northeast Texas during September. *Anopheles quadrimaculatus* specimens were also found in November, but the collection sites were confined to southeast Texas and one site along the southern coastal region. No species A specimens were collected in December, but a few were collected during January in Grimes County, which is located east of Brazos County and northwest of Harris County (Fig. 13).

County collections of *An. quadrimaculatus* (Say) specimens were also observed by year and month. Only 2003 and 2004 collections were analyzed this way because in both 2002 and 2005, collections were only made from one county. In 2003, collections were made from ten counties. *Anopheles smaragdinus* was collected in four of the counties. Fort Bend, Henderson, and Rains Counties all had collections of *An. smaragdinus* in the month of August, while Brazos County had collections of *An. smaragdinus* in June, July, and November. Three out of the four counties had collections of *An. smaragdinus* in combination with *An. quadrimaculatus*. *Anopheles smaragdinus* was the only species collected in Henderson County in 2003, but since only one specimen was collected in that county, this was not a true representation of its species composition (Table 4).

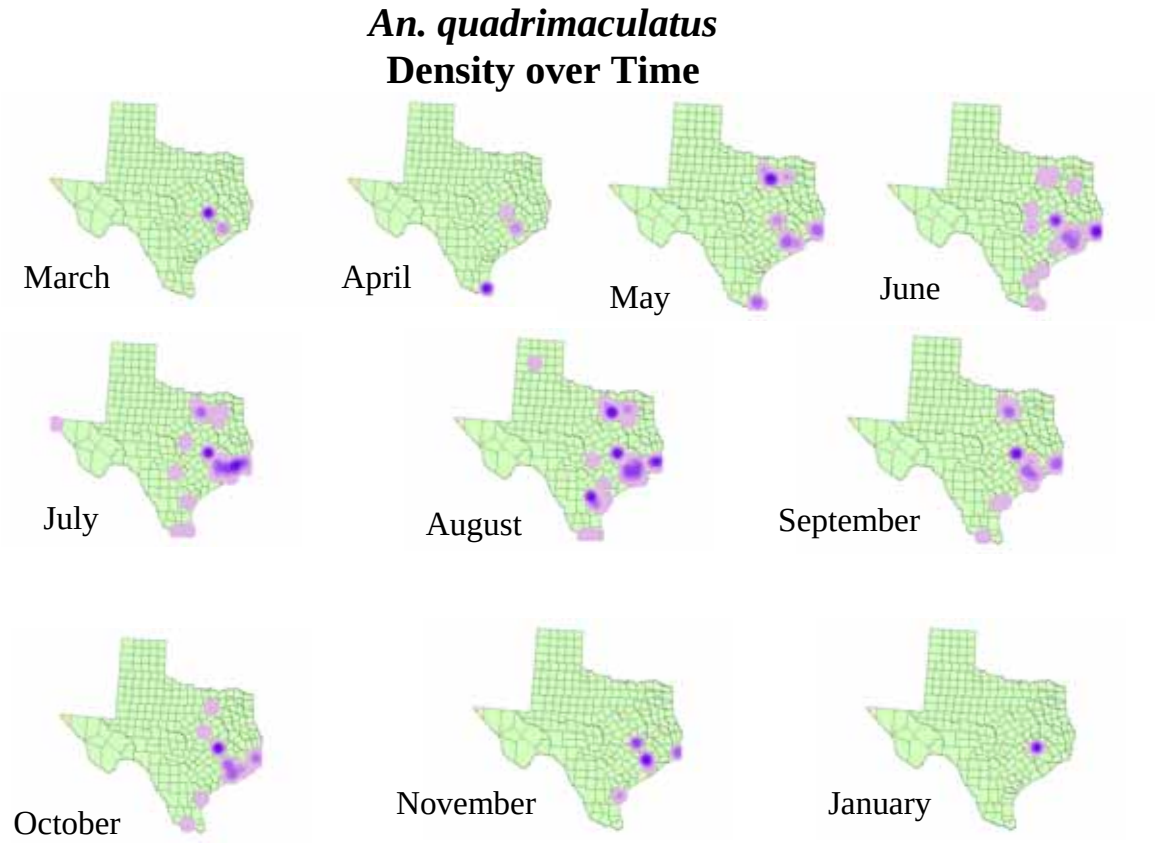

Figure 17. Density of *An. quadrimaculatus* in Texas from May to November. As the density of *An. quadrimaculatus* specimens collected increases, the shaded circles get darker.

Table 4. The number of *An. quadrimaculatus* (Say) mosquitoes collected in ten Texas counties during six months in 2003. Each month is separated out into three sections. The first box is for day 1-10, the second box is for day 11-20, and the last box under each month represents day 21-31. A blank box means that no mosquitoes were collected, a gray box with a white number means only *An. quadrimaculatus* were collected, a black box with a white number means only *An. smaragdinus* were collected, and a gray box containing black dots and a black number mean that both *An. quadrimaculatus* and *An. smaragdinus* were collected during that time. The numbers in the boxes represent the number of mosquitoes collected and identified during that time.

County	**June**			**July**			**August**			**Sept.**			**October**			**November**		
Brazos		19	14	1	16	35						16	4		8	4		1
Brazoria														4				
Dallas						8		4						3				
Fort Bend						7		7										
Galveston														4				
Henderson								1										
Hidalgo						1												
Jefferson														7				
Nueces														3				
Rains								9										

Table 5. The number of *An. quadrimaculatus* (Say) mosquitoes collected in thirty-four counties during nine months in 2004. Each month is separated out into three sections. The first box is for day 1-10, the second box is for day 11-20, and the last box under each month represents day 21-31. A blank box means that no mosquitoes were collected, a gray box with a white number means only *An. quadrimaculatus* were collected and a gray box containing black dots and a black number mean that box *An. quadrimaculatus* and *An. smaragdinus* were collected during that time. The numbers in the boxes represent the number of mosquitoes collected and identified during that time.

County	March			April			May			June			July			August			September			October			November		
Aransas											1					1					1						
Bell										3					1												
Bexar														1													
Brazos			4	8	10				12	6			11	14	13	9	2				18	13					
Brazoria									1	14	1	4		2			9		2	6	1	6	9	1			
Cameron					75		2		6	1	1																
Chambers													68														
Collin												1							1								
Coryell							3							1													
Dallas							7	11	5	1		3	11	14	7	5	14	10	17	7	2						
Denton										2				3	2		3	1		3							
El Paso													4														
Fort Bend						24		10	2	9	5	12		5			1		4		29	1	17	5	8	4	
Galveston									2				1	9	2		1	4			6		4				
Harris			1							4	10	10	26	39	45	27	7										
Hays																	1										
Henderson														5													
Hidalgo																							1				
Jefferson								2	1	4		21	42	42			1			7	11						
Kleberg											1																
Liberty													6														
Live Oak																31											
McLennan																							1				
Montgomery													2	3			3	1		2	4						
Nueces														2		12											
Orange								4	3	25	8						11	11	4		10	2	3	1	1	2	
Potter																	2										
Rains								2																			
Smith												1															
Travis										5																	
Wharton												4															
Wood														3													
Victoria																1											
VanZault															2												

A more extensive collection was made in 2004 due to an increase in the amount of mosquitoes sent by TDSHS, HCMCD, and collecting trips made by this investigator. Collections were made from 34 counties during 2004 and while all of them contained *An. quadrimaculatus*, only 5 of them contained *An. smaragdinus*. Fort Bend County had the most collections of *An. smaragdinus*, but they were only found sporadically in the. beginning of the *Anopheles* season, while they were more consistently found toward the end of their season during October and November. In all of the other counties where *An Smaragdinus* was collected, the collections all occurred at the same time or within only one month.

Anopheles quadrimaculatus (Say) was consistently collected throughout the *Anopheles* season in at least six of the counties and three of those counties also had at least one collection of *An. smaragdinus* (Table 5). This information will to be discussed in the next section.

Discussion

Density information of mosquitoes for each month of the year is interesting and could be of some use in determining where and when to target control efforts, but further research needs to be done on this aspect of the study. The density level might be an artifact of where the collections were taken by the mosquito control districts or other agencies collecting mosquitoes and submitting them to TDSHS. Many collections came from agencies that were sampling mosquitoes for West Nile Virus and this could have given a bias to the density levels of *An. smaragdinus* during certain times of the year.

The fact that July and August appeared to be the peak months of the year to find *An. smaragdinus* still holds true and is supported in the literature as well.

When observing the *Anopheles* collections for just 2003 and 2004, no apparent pattern emerged. *Anopheles smaragdinus* was almost always collected in combination with *An. quadrimaculatus* and both of the species were collected throughout the *Anopheles* season (May – September). Of course, there were far fewer collections made of *An. smaragdinus* than *An. quadrimaculatus,* so this could have had an effect on the results. Mosquito control agencies collected most of the mosquitoes and they were collecting for *Cx. Quinquefasciatus,* which live in the city very well and thrives in storm sewers and culverts. The collection sites could have skewed the number of *An. smaragdinus* mosquitoes collected, since in the previous chapter it was observed that this species prefers a more rural setting.

Of course, in light of this information, one must look at the collections that were purposely made in rural areas. Almost all of the collections made during the colleting trips by this investigator in Beaumont, the Lower Rio Grande Valley, and Corpus Christi were made in rural areas. Unfortunately none of the specimens caught on these collecting trips were identified as *An. smaragdinus*. It could, therefore be proposed that *An. smaragdinus* is not found in as large of numbers as *An. quadrimaculatus* in the state of Texas at any time of the year.

CHAPTER VI

GENERAL CONCLUSIONS

Adults of the *An. quadrimaculatus* species complex were collected from September 2002 through January 2005 throughout the state of Texas. A total of 1,372 specimens were collected and identified from a variety of habitats and areas of Texas. Of the total, 1,321 specimens were identified as *An. quadrimaculatus* and 51 of them were identified as *An. smaragdinus*. All of the *An. smaragdinus* specimens were collected in the eastern half of Texas, but a few *An. quadrimaculatus* specimens were found as far west as El Paso and Potter County. The arid climate and lack of shelter in west Texas is probably what is preventing a larger population of *An. quadrimaculatus* from occurring there and excluding *An. smaragdinus*.

Land cover data analysis using the ArcView GIS program and the 1992 Land cover data, determined that the most popular land cover for the *An. smaragdinus* specimens collected in Texas during this study was woody wet land, while *An. quadrimaculatus* was found predominately in grass/ hay habitat. Overall, *An. quadrimaculatus* was found in a greater variety of land covers and could be found more on land covers that did not provide as much shelter and in more urbanized areas than was the case for *An. smaragdinus*. An interesting discovery was that all of the habitats that were known to contain birds (chicken coops) were all sites where only *An. quadrimaculatus* was found even though other states had found that *An smaragdinus* was the most common species in the *An. quadrimaculatus* complex to be found among birds.

June, July, and August provided the most *An. quadrimaculatus* and *An. smaragdinus* collections. Past research has also shown that these summer months are when *An. quadrimaculatus* (Say) adults are at their highest populations, so it was no surprise when more specimens were collected during this time. There was no observed change in the species composition over time for any of the collection sites. When *An. smaragdinus* specimens were collected, there were almost always at least a few *An. quadrimaculatus* specimens collected as well. This was probably because *An. quadrimaculatus* was found to inhabit all of the *An. smaragdinus* habitats, but *An. smaragdinus* did not inhabit all the same habitats as did *An. quadrimaculatus*.

In the future, Texas needs to be continually survived for the presence of additional species in the *An. quadrimaculatus* (Say) complex and to accumulate a more extensive knowledge of the geographic ranges of these species in the western half of Texas. To better understand how transmission of malaria occurs in the United States, vector competency tests need to be conducted comparing all five of the species found in the *An. quadrimaculatus* (Say) complex to the four species of malaria. Finally, a study looking at the adaptation rates and environmental extremes for all of the species in this complex needs to be performed. With this information, Malaria outbreaks in the eastern United States can be more accurately prevented and controlled.

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APPENDIX A

TABLE OF ALL *AN. QUADRIMACULATUS* (SAY) SPECIMENS SENT TO TDSHS IN 2003 AND 2004

Table 6: *An. quadrimaculatus* (Say) specimens sent to the TDSHS during the 2003 and 2004 West Nile Virus season.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections			
Date of Collection	**County**	**# of Species A**	**# of Species B**
07/29/03	Dallas	8	0
07/31/03	Hidalgo	1	0
07/31/03	Fort Bend	7	0
08/12/03	Dallas	2	0
08/12/03	Dallas	1	0
08/13/03	Fort Bend	0	1
08/13/03	Fort Bend	1	0
08/13/03	Fort Bend	5	0
08/13/03	Rains	1	0
08/13/03	Rains	5	3
08/13/03	Henderson	0	1
08/13/03	Dallas	1	0
08/14/03	Orange	1	0
10/13/03	Dallas	1	0
10/13/03	Dallas	2	0
10/14/03	Brazoria	1	0
10/14/03	Jefferson	3	0
10/14/03	Jefferson	1	0
10/14/03	Jefferson	2	0
10/14/03	Jefferson	1	0
10/15/03	Brazoria	3	0
10/15/03	Chambers	1	0
10/16/03	Galveston	4	0
10/20/03	Nueces	3	0
04/20/04	Cameron	75	0
04/23/04	Fort Bend	19	0
04/28/04	Fort Bend	0	5
05/03/04	Dallas	1	0
05/04/04	Cameron	2	0
05/06/04	Dallas	6	0
05/12/04	Fort Bend	2	0
05/12/04	Dallas	6	0
05/13/04	Dallas	4	0
05/18/04	Jefferson	2	0

Table 6: Continued.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections (Cont.)			
Date of Collection	**County**	**# of Species A**	**# of Species B**
05/19/04	Rains	2	0
05/20/04	Fort Bend	5	3
05/20/04	Dallas	1	0
05/20/04	Orange	4	0
05/25/04	Denton	2	0
05/25/04	Cameron	6	0
05/25/04	Jefferson	1	0
05/25/04	Dallas	2	0
05/26/04	Brazoria	1	0
05/26/04	Dallas	1	0
05/27/04	Dallas	2	0
05/27/04	Fort Bend	2	0
05/27/04	Galveston	2	0
05/27/04	Orange	2	1
06/02/04	Orange	19	0
06/02/04	Brazoria	14	0
06/02/04	Dallas	1	0
06/02/04	Travis	5	0
06/03/04	Bell	3	0
06/03/04	Fort Bend	1	0
06/06/04	Cameron	1	0
06/08/04	Jefferson	4	0
06/09/04	Orange	5	1
06/10/04	Fort Bend	8	0
06/15/04	Aransas	1	0
06/15/04	Kleberg	1	0
06/15/04	Cameron	1	0
06/16/04	Orange	8	0
06/16/04	Brazoria	1	0
06/17/04	Fort Bend	5	0
06/22/04	Wharton	4	0
06/22/04	Jefferson	21	0
06/23/04	Dallas	1	0
06/23/04	Brazoria	2	0
06/23/04	Collin	1	0
06/23/04	Dallas	1	0

Table 6: Continued.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections (Cont.)			
Date of Collection	**County**	**# of Species A**	**# of Species B**
06/23/04	Fort Bend	1	8
06/29/04	Dallas	1	0
06/30/04	Fort Bend	1	2
06/30/04	Smith	1	0
06/30/04	Brazoria	2	0
07/01/04	Galveston	1	0
07/01/04	Dallas	3	0
07/05/04	Montgomery	1	0
07/06/04	Dallas	3	0
07/06/04	El Paso	4	0
07/07/04	Brazoria	2	0
07/07/04	Dallas	2	0
07/07/04	Montgomery	1	0
07/08/04	Dallas	3	0
07/12/04	Dallas	1	0
07/13/04	Nueces	1	0
07/13/04	Jefferson	27	7
07/13/04	Denton	1	0
07/13/04	Dallas	1	0
07/14/04	Jefferson	1	0
07/14/04	Henderson	2	3
07/14/04	Wood	2	1
07/14/04	Dallas	2	0
07/14/04	Fort Bend	5	0
07/14/04	Brazoria	2	0
07/15/04	Dallas	1	0
07/15/04	Galveston	1	0
07/18/04	Montgomery	3	0
07/19/04	Dallas	9	0
07/20/04	Galveston	2	0
07/20/04	Nueces	1	0
07/20/04	Galveston	6	0
07/20/04	Denton	2	0
07/20/04	Jefferson	7	0
07/29/04	Bell	1	0
07/21/04	Van Zaudt	2	0

Table 6: Continued.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections (Cont.)			
Date of Collection	**County**	**# of Species A**	**# of Species B**
07/21/04	Dallas	1	0
07/22/04	Galveston	2	0
07/22/04	Dallas	3	0
07/26/04	Dallas	3	0
07/27/04	Denton	2	0
08/03/04	Aransas	1	0
08/05/04	Fort Bend	5	0
08/10/04	Nueces	2	0
08/10/04	Dallas	5	0
08/11/04	Orange	1	0
08/12/04	Galveston	1	0
08/13/04	Hays	1	0
08/13/04	Denton	1	0
08/16/04	Montgomery	3	0
08/16/04	Potter	2	0
08/17/04	Denton	2	0
08/17/04	Dallas	1	0
08/17/04	Jefferson	1	0
08/18/04	Dallas	13	0
08/18/04	Fort Bend	1	0
08/18/04	Orange	10	0
08/18/04	Brazoria	9	0
08/25/04	Orange	11	0
08/26/04	Dallas	2	0
08/26/04	Galveston	4	0
08/30/04	Montgomery	1	0
08/31/04	Denton	1	0
08/31/04	Dallas	8	0
09/01/04	Orange	2	0
09/02/04	Dallas	5	0
09/02/04	Fort Bend	2	0
09/08/04	Orange	2	0
09/08/04	Dallas	12	0
09/08/04	Fort Bend	1	0
09/08/04	Brazoria	2	0
09/09/04	Fort Bend	1	0

Table 6: Continued.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections (Cont.)			
Date of Collection	**County**	**# of Species A**	**# of Species B**
09/09/04	Collin	1	0
09/13/04	Montgomery	1	0
09/14/04	Jefferson	7	0
09/14/04	Denton	3	0
09/15/04	Brazoria	6	0
09/15/04	Dallas	1	0
09/16/04	Dallas	6	0
09/29/04	Montgomery	1	0
09/21/04	Aransas	1	0
09/21/04	Jefferson	10	0
09/21/04	Galveston	1	0
09/22/04	Fort Bend	7	3
09/22/04	Orange	6	0
09/22/04	Montgomery	3	0
09/28/04	Jefferson	1	0
09/29/04	Fort Bend	19	0
09/29/04	Orange	4	0
09/29/04	Brazoria	1	0
09/29/04	Montgomery	1	0
09/30/04	Dallas	2	0
09/30/04	Galveston	5	0
10/06/04	Orange	2	0
10/06/04	Brazoria	6	0
10/07/04	Fort Bend	1	0
10/13/04	Galveston	4	0
10/13/04	Orange	1	0
10/13/04	Brazoria	7	0
10/14/04	Fort Bend	8	1
10/14/04	McLennan	1	0
10/20/04	Orange	2	0
10/20/04	Hidalgo	1	0
10/20/04	Fort Bend	7	1
10/20/04	Brazoria	2	0
10/27/04	Orange	1	0
10/27/04	Brazoria	1	0
10/28/04	Fort Bend	3	2

Table 6: Continued.

Texas DSHS 2003 and 2004 *An. quadrimaculatus* (Say) Collections (Cont.)			
Date of Collection	**County**	**# of Species A**	**# of Species B**
11/03/04	Fort Bend	0	1
11/04/04	Orange	1	0
11/10/04	Fort Bend	7	0
11/17/04	Orange	2	0
11/19/04	Fort Bend	1	3

APPENDIX B

TABLE OF ALL COLLECTION SITES, THE SPECIES FOUND THERE, LAND COVER, AND HABITAT INFORMATION (IF KNOWN)

Table 7: Depiction of all the collection sites, the species found there, their land cover, and habitat information (if known).

Habitat information for collection sites of *An. quadrimaculatus* (Say)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
0	70	0	deciduous forest	brush
1	5	0	shrubland	brush
2,62,146,185,224	7	0	shrubland	bird roost by pond
3	18	0	pasture/hay	wooded wetlands next to homes
4,15,49,80,173,212,218,227,230, 236,331	3	22	woody wetlands	Brazos River bottom by lakes
5	7	0	low intensity residential	
6	1	0	emergent herbaceous wetlands	trails
7	2	0	grassland/herbaceous	brush
8,44	7	0	low intensity residential	
9,184,210,220,231	9	0	row crop	wooded swamp (sometimes dry)
10	1	0	deciduous forest	
11	4	0	pasture/hay	
12,149,246	23	0	high intensity residential	bird roost by pond
13,48,239,244	12	1	low intensity residential	storm culvert
14	2	0	pasture/hay	
16,229	12	1	pasture/hay	treeline by ditch and homes
17	2	0	pasture/hay	
18	1	0	shrubland	yard
19	4	0	deciduous forest	brush
20	1	0	shrubland	brush
21,321	3	0	high intensity residential	
22,64,65,66,68,69,113,114,133,155,157,158,159,267,269	30	0	low intensity residential	storm culvert - fence line
23	1	0	pasture/hay	rural
24	1	0	mixed forest	
25	2	0	low intensity residential	

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
26	2	0	grassland/herbaceous	
27	1	0	grassland/herbaceous	wooded homesite North of rice fields
28,54,85,123,186,211,217,232,249	44	0	woody wetlands	wooded wetlands
29,23,72,38,252	10	0	deciduous forest	brush; wooded wetlands next to homes
30	2	0	mixed forest	trees/brush
31	0	1	low intensity residential	trees/brush
32,37,93,80	8	0	deciduous forest	
33	12	0	woody wetlands	trees/brush
34	1	0	deciduous forest	wooded area
35	3	0	pasture/hay	sewage/brush
36	3	0	deciduous forest	trees/bayou
37	1	0	high intensity residential	
38	2	0	deciduous forest	residential
39	2	0	pasture/hay	rural
40	5	0	pasture/hay	rural
41	2	0	evergreen forest	residential
42	1	0	evergreen forest	rural
43	2	0	grassland/herbaceous	rural
45	1	0	high intensity residential	creek/wooded line/ heavy brush
46	2	0	grassland/herbaceous	creek/wooded line/ heavy brush
47,79,153,392	4	0	low intensity residential	storm sewer in field by ditch and homes
50	1	0	shrubland	
51	1	0	mixed forest	sewage/brush
52	0	1	woody wetlands	sewage/wooded area
53	4	0	mixed forest	brush/trees
55	1	0	grassland/herbaceous	
56	1	0	shrubland	marshy
57	1	0	grassland/herbaceous	brush
58	8	0	emergent herbaceous wetlands	sewage/trees
59	1	0	pasture/hay	residential
60	3	0	deciduous forest	wooded homesite

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
61,171,245	3	0	quarries/strip mines/gravel pits	wooded wetlands next to homes
63,265	7	0	low intensity residential	storm culvert
67	1	0	high intensity residential	tree canopy
70,72	5	0	low intensity residential	storm culvert
71	1	0	low intensity residential	storm culvert
73	1	0	pasture/hay	field
74	1	0	woody wetlands	residential
75	1	0	low intensity residential	rural
76	1	0	grassland/herbaceous	lift station
77,166	2	0	urban/recreational	
78	1	8	deciduous forest	Brazos River bottom by lakes
81	1	0	pasture/hay	rural
82	1	0	pasture/hay	rural
83	1	0	pasture/hay	rural
84	1	0	low intensity residential	wooded
86	1	0	shrubland	brush
87	2	0	commercial/industrial/transportation	rural
88,150	2	0	low intensity residential	
89,191	2	0	high intensity residential	
90	1	0	high intensity residential	
91	1	0	high intensity residential	
92	4	0	shrubland	pond
93	1	0	low intensity residential	heavily wooded area behind house
94	1	0	urban/recreational	
95,96,98,99, 100,101	17	6	low intensity residential	storm culvert
97,168,169	14	0	low intensity residential	storm culvert
102	1	0	grassland/herbaceous	over grown lot
103	2	3	pasture/hay	rural - woods

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
104	2	1	pasture/hay	rural
105	1	0	low intensity residential	field
106	3	0	low intensity residential	wooded homesite
107	1	0	pasture/hay	homesite backing up to brush and tall grass
108	1	0	deciduous forest	wooded homesite
109	2	0	row crop	residential
110,200	3	0	low intensity residential	field
111,198	2	0	high intensity residential	
112,383	1	1	shrubland	
115	1	0	high intensity residential	marsh.wooded line/creek
116	2	0	pasture/hay	rural
117	1	0	high intensity residential	
118	1	0	high intensity residential	
119,240	5	0	high intensity residential	
120	2	0	deciduous forest	
121,167	2	0	low intensity residential	
122,219,225	3	0	pasture/hay	wooded wetlands (sometimes dry)
124	1	0	pasture/hay	
125	1	0	high intensity residential	
126	1	0	grassland/herbaceous	park (woods)
127,143	8	0	deciduous forest	
128	1	0	evergreen forest	
129	1	0	pasture/hay	sewage/wooded area
130	1	0	deciduous forest	brush
131	2	0	low intensity residential	Playa lake
132	1	0	low intensity residential	residential area middle of island in roadway
134	1	0	emergent herbaceous wetlands	wooded homesite (trash cans and tires with water and larvae)
135	6	0	mixed forest	sewage/brush
136	4	0	deciduous forest	pasture/wooded area

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
137	2	0	pasture/hay	rural
138	7	0	pasture/hay	rural
139	10	0	evergreen forest	brush/wooded area
140	1	0	woody wetlands	brush
141	2	0	high intensity residential	
142	1	0	mixed forest	sewage/brush
144,165	8	0	low intensity residential	
145,241	7	0	commercial/industrial/transportation	
147	4	0	pasture/hay	barn
148	2	0	pasture/hay	residential
151	1	0	low intensity residential	
152	1	0	urban/recreational	
154	1	0	row crop	storm drain
156	1	0	low intensity residential	storm culvert
160	1	0	pasture/hay	rural
161	2	0	grassland/herbaceous	rural
162	1	0	pasture/hay	rural
163	2	0	pasture/hay	rural
164	1	0	high intensity residential	
170	0	1	deciduous forest	
172	1	0	low intensity residential	overgrown cemetary by ditch
174	1	0	deciduous forest	wooded wetlands next to sewer plant
175,209	4	0	pasture/hay	prairies wetlands by retention pond
176	4	0	woody wetlands	wooded area
177	5	3	deciduous forest	
178,179	2	0	mixed forest	pasture/wooded area
180	1	0	mixed forest	heavily wooded
181	2	0	evergreen forest	heavily wooded
182	1	0	commercial/industrial/transportation	storm culvert
183	1	0	small grains	wooded thicket
187	1	0	low intensity residential	pasture/wooded area
188	2	0	pasture/hay	pasture/wooded area
189	1	0	low intensity residential	sewage/trees
190	1	0	deciduous forest	residential

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
192	1	0	emergent herbaceous wetlands	
193	2	0	mixed forest	sewage/wooded area
194	1	0	high intensity residential	residential
195	1	0	pasture/hay	rural
196,207	3	0	woody wetlands	residential
197,205	4	0	pasture/hay	residential
199	1	0	pasture/hay	wooded residential lot
201	1	0	pasture/hay	standing water/brush
202	1	0	commercial/industrial/transportation	
203	1	0	pasture/hay	residential
204	1	0	evergreen forest	rural
206	1	0	high intensity residential	residential
208	1	0	grassland/herbaceous	residential
213	1	0	deciduous forest	woody vegitation, moist ground
214	1	0	low intensity residential	sewage/brush
215	1	0	mixed forest	sewage/brush
216	1	0	shrubland	
221	2	0	deciduous forest	rural
222	1	0	mixed forest	sewage/wooded area
223	1	0	deciduous forest	rural
226	1	0	pasture/hay	treeline by ditch and homes
228	1	0	woody wetlands	wooded area
233	2	0	high intensity residential	brush
234	8	1	low intensity residential	
235	1	0	low intensity residential	wooded backyard, 2 dirty ponds
242	2	0	low intensity residential	
243	1	0	evergreen forest	
247	1	0	low intensity residential	sewage/brush
248	1	0	low intensity residential	
250	2	0	woody wetlands	sewage/wooded area
251	1	0	low intensity residential	

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
253	7	0	low intensity residential	collected in old tin barn in a pasture with 3 cows on the edge of town
254	8	0	pasture/hay	collected in wood shed behind a single family home with oaks and pine trees surrounding; horses fenced next door
255,332	32	0	pasture/hay	collected in red wooden barn surrounded by coastal prairie
256	27	0	small grains	collected in old wooded barn at the edge of a rice field
257,333	36	0	small grains	collected in delapidated house surrounded by coastal prairies
258	6	0	pasture/hay	collected in tin shed with dirt floors surrounded by pine trees and coastal prairie
259	10	0	shrubland	collected from old red, wooden barn on the edgeof cotton fields
260	31	0	shrubland	collected from abandoned, white barn in wooded areas near a lake and shrubland
261	1	0	grassland/herbaceous	collection from old well house with oak trees behined it and grassland all around
262	2	0	high intensity residential	
263	1	0	low intensity residential	
264	3	0	high intensity residential	
266	2	0	pasture/hay	
268	1	0	pasture/hay	
270,305,334-378	242	3	pasture/hay	stand of oak trees on the edge of pasture land containing horses
271	1	0	high intensity residential	
272	1	0	pasture/hay	collected along fence line with an open fieldon one side and houses on the other; chickens on other side of fence
273	1	0	pasture/hay	mixed stands of trees near water canal and open fields
274	1	0	pasture/hay	small stand of oaks trees next to a hotel and on the edge of an open field

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
275	1	0	shrubland	line of mixed trees near crops and on the edge of pasture/shrubland with cows near by
276	1	0	low intensity residential	treeline cement ditch with open fields and barricks near by
277,381	5	0	low intensity residential	
278	1	0	high intensity residential	
279	1	0	pasture/hay	
280	5	0	high intensity residential	
281	1	0	low intensity residential	
282	2	0	pasture/hay	wooded
283,330	2	0	woody wetlands	yard
284	1	0	mixed forest	heavily wooded hear drainage ditch
285	1	0	pasture/hay	yard
286	1	0	high intensity residential	storm drain
287	1	0	row crop	storm drain
288	1	0	high intensity residential	wooded
289	1	0	deciduous forest	wooded
290	3	0	commercial/industrial/transportation	
291	4	0	shrubland	
292	1	0	commercial/industrial/transportation	
293	1	0	low intensity residential	
294,325,326,327,328,329	10	0	pasture/hay	
295	1	0	low intensity residential	
296	1	0	emergent herbaceous wetlands	storm drain
297	3	0	mixed forest	wooded backyard
298	1	0	high intensity residential	
299	1	0	pasture/hay	chicken pen
300	4	0	low intensity residential	wooded

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
301	9	0	high intensity residential	woods and water East
302	1	0	high intensity residential	wooded
303	1	0	commercial/industrial/transportation	wooded
304	3	0	low intensity residential	Alley
306	1	0	emergent herbaceous wetlands	farm
307	1	0	high intensity residential	wooded residential lot
308	3	0	low intensity residential	heavily wooded area near parcking lot and tennis courts
309	4	0	deciduous forest	
310	1	0	evergreen forest	lightly wooded, water is standing in puddles near by
311	1	0	low intensity residential	heavily wooded
312	1	0	pasture/hay	storm drain
313	1	0	pasture/hay	
314	1	0	pasture/hay	park, woody, lots of water
315	1	0	low intensity residential	brush
316	3	0	urban/recreational	brush
317	2	0	pasture/hay	farm
318	2	0	woody wetlands	
319	1	0	low intensity residential	
320	2	0	high intensity residential	
322	4	0	pasture/hay	
323	3	0	mixed forest	
324	3	0	deciduous forest	
382	2	0	deciduous forest	
384	1	0	commercial/industrial/transportation	
385	1	0	row crop	
386	1	0	pasture/hay	
387	1	0	high intensity residential	
388	1	0	low intensity residential	
389	1	0	deciduous forest	

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
390	2	0	high intensity residential	
391	1	0	pasture/hay	
393	1	0	commercial/industrial/transportation	
394	2	0	shrubland	
395	1	0	low intensity residential	
396	1	0	low intensity residential	
397,400	3	0	deciduous forest	
398	1	0	high intensity residential	
399,423	2	0	high intensity residential	
401,444	11	0	commercial/industrial/transportation	
402	2	0	high intensity residential	
403,425,435	8	0	urban/recreational	
404,410,413,428,443	17	0	pasture/hay	
405,415,420	6	0	commercial/industrial/transportation	
406,417,432	11	0	pasture/hay	
407	2	0	high intensity residential	
408,421,458,466	12	0	pasture/hay	
409,419,452,459	6	0	pasture/hay	
411,414,430	3	0	row crop	
41,-442	10	0	low intensity residential	
416	1	0	low intensity residential	
418,461	5	0	high intensity residential	
422-437	2	0	urban/recreational	
424	2	0	high intensity residential	
426,438,446,455	25	0	commercial/industrial/transportation	
427,451	5	0	low intensity residential	

Table 7: Continued.

Habitat information for collection sites of *An. quadrimaculatus* (Say) (Cont.)				
FID	**# of A**	**# of B**	**landcover**	**TDH Comments**
429	1	0	low intensity residential	
431	1	0	low intensity residential	
433	1	0	commercial/industrial/transportation	
434	3	0	low intensity residential	
436	3	0	grassland/herbaceous	
439	1	0	high intensity residential	
440	1	0	commercial/industrial/transportation	
441,456	3	0	high intensity residential	
445	1	0	low intensity residential	
447	1	0	low intensity residential	
448	1	0	low intensity residential	
449	2	0	urban/recreational	
450	1	0	pasture/hay	
453,465	4	0	low intensity residential	
454	3	0	deciduous forest	
457	2	0	pasture/hay	
460	1	0	low intensity residential	
462	1	0	high intensity residential	
463	1	0	low intensity residential	
464	1	0	grassland/herbaceous	

APPENDIX C

RELATIVE IMPORTANCE TO THE HISPANIC COMMUNITY

Collection sites were chosen for this study because they were known to contain *An. quadrimaculatus* mosquitoes, few if any collection had been made there, and most of them have a high population of Hispanic residents. Since some of this research was funded by the Hispanic Leadership Program in Agriculture and Natural Resources (HLPANR), it was important that areas of Texas with higher percentages of Hispanics be sampled. This turned out to be very important since past *An. quadrimaculatus* collections have been made in most of the areas of Texas that have a high population of Hispanic residents. Collections in these areas are also important because most Latin countries have endemic malaria and if some of the Hispanic residents of Texas visit their home country or have visitors from endemic areas, then the chances of a malaria outbreak in these areas of Texas increases. This also means that the areas that are more likely to have an outbreak of malaria are locations with a high population of Hispanic residents.

VITA

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