PREVALENCE OF *TRYPANOSOMA CRUZI* IN FREE-RANGING MAMMALIAN POPULATIONS IN SOUTH TEXAS

A Dissertation

by

MATHEW M. KRAMM III

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

DOCTOR OF PHILOSOPHY

Chair of Committee,	Roel R. Lopez
Co-Chair of Committee,	Donald S. Davis
Committee Members,	Susan M. Cooper
	Nova J. Silvy
Head of Department,	Michael P. Masser

December 2015

Major Subject: Wildlife and Fisheries Sciences

ABSTRACT

Chagas disease, also known as American trypanosomiasis, is caused by the etiological flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). It is a significant health concern in South and Central America where millions of people are infected or at risk of infection, and an emerging health concern in the United States. Kissing bugs (*Triatoma sp*.) are vectors for Chagas disease and feed upon a variety of taxa including humans and sylvatic free-ranging mammals. It is likely that free-ranging mammals are important in the maintenance and transmission of Chagas in the environment, but solid empirical support is lacking. In an effort to address this information gap, I conducted a field study to determine *T. cruzi* prevalence in free-ranging mammals in Bexar and Val Verde Counties, Texas. My research objectives were to (1) determine disease prevalence of *T. cruzi* parasites in free-ranging mammals and species population densities in various vegetation communities, (2) determine if the use of immunoassay lateral rapid flow diagnostic devices is feasible in field environments to detect Chagas antibodies in meso-mammals, and (3) determine the behavior of *Peromyscus pectoralis* and communal tolerance to collective triatomine insects in the burrow, and (4) to measure vector defecation intervals for pathogen potential.

The study analyzed 483 whole blood and tissue samples from free-ranging meso-mammal species using immunochomatographic assay strips and polymerase chain

reaction (PCR) methodologies to screen for *T. cruzi*. I documented overall infection presence in 60% of animal species. *T. cruzi* prevalence was determined through whole-blood and tissue analysis to ensure identification of the protozoan parasite that could be occurring in either the acute or chronic infection stage. I further evaluated cave dwelling *Peromyscus pectoralis* for behavioral response to hematophagous triatomine insects, and found the communal species to possibly be codependent. Meso-mammals tolerated insect blood feeding activity and routinely bite or consume insect vectors as an antagonistic response or nutritional requirement. Triatomine insect defecation intervals occurred 11 – 24 minutes after a blood meal and away from the host mammal. Delayed triatomine insect defecation indicates pathogen occurs from direct insect ingestion or meso-mammal grooming activities rather than at the site of the bite. My results indicate that cave and burrow dwelling free-ranging meso-mammals serve as primary pathogen hosts, and facilitate Chagas disease prevalence. Findings support the emerging disease as a major zoonotic and public health concern for south Texas Counties.

DEDICATION

To my family Anna, Jessica and Max

ACKNOWLEDGEMENTS

I would like to thank my committee chair Dr. Lopez, committee co-chair Dr. Davis and committee members, Dr. Cooper, and Dr. Silvy for their guidance, patience, and support throughout the course of this research…you were great!

I want to extend my gratitude to Dr. Maria Gutierrez, Troy Luepke, Dr. Israel Parker, Cynthia Soria, Carrie Merson, John Wildie, Dr. Thomas Leo Cropper, Dr. David Bowles, Don Lauer, Chad Grantham, Andrea Montalvo, Bethany Friesenhahn, Jaime Agudelo, Dr. Angelica Lopez, Dr. Sarah Hamer, Lisa Auckland, Dr. Candelaria Daniels, Angel Osuna, and Lauren Hoffman, who provided outstanding support throughout this endeavor.

Thanks also go to my friends, colleagues and the department faculty and staff for their assistance, and for making my time at Texas A&M University a great experience.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

INTRODUCTION

Previous studies report that *Trypanosoma cruzi* (*T. cruzi*) infection of meso-mammal and canine species in Southern Texas may be endemic (Beard et al. 2003). Serology monitoring of military working dogs at Joint Base San Antonio (JBSA) - Lackland facilities revealed *T. cruzi* antibody prevalence of 8% (McPhatter et al. 2012) The investment that comes with training military working dogs and the possibility of *T. cruzi* infection resulting in life long chronic health issues, warrants research to aid in the identification and management of the parasite pathogen at Joint Base San Antonio (JBSA) and surrounding areas. The role of sylvatic free-ranging mammalian species as reservoir hosts and prevalence of *T. cruzi* infection at JBSA is unknown. Effective preventative strategies for military working dog personnel and public health to consider when assessing disease potential would need to include, information of the triatomine insect life cycle (e.g. surrounding areas of kennels, host species, vegetation composition, etc.).

American trypanosomiasis disease is considered a significant human health problem in Central and South America, where 16-18 million people are infected (Center for Disease Control and Prevention [CDC]). In the United States, although there have been few reported cases, the CDC estimated in 2009 that 300,000 emigrant people in the United States are infected. *T. cruzi* parasites evolutionarily adapted and depend on triatomine insects as vectors, and over 130 species are known to carry the parasite

(Iowa State Center for Food Security and Public Health 2009). Eleven species of triatomines, or 'kissing bugs' as the common name occur in the United States, with the most numerous vector species being *Triatoma rubida* and *Triatoma protacta* in Arizona and California, and *Triatoma gerstaeckeri*, *Triatoma sanguisuga*, *Triatoma protracta*, and *Triatoma invicta* in Texas (Bern et al. 2011).

Kissing bugs are blood feeding insects and obtain the protozoan parasite *T. cruzi* from an infected mammalian host. The parasite carries out part of its life cycle in the insect's digestive tract, and is later transmitted to blood meal hosts when the insect defecates during or after feeding. Alternatively, *T. cruzi* is also transmitted when the triatomine insect is directly ingested by a mammalian host. Most species of triatomine are associated with sylvatic mammalian species, which usually serve as reservoir hosts for *T. cruzi* (Miles et al. 1981, Rozas et al. 2005). A recent study in Mexico identified the blood meal origins of 47 triatomines and found that raccoons (*Procyon lotor*) and armadillos (*Dasypus* spp.) were the main blood meal hosts (Bosseno et al. 2009). Another study in the U.S. reported *T. cruzi* infection among 11 reservoir vertebrate species from 6 southern states, and detected a higher incidence of the infection in Virginia opossums (*Didelphis virginiana),* raccoons, skunks (*Mephitis* spp.) and rodents (*Neotoma spp*.) species (Bern et al. 2011).

RESEARCH OBJECTIVES

The studies suggest *T. cruzi* prevalence varies between host species and geographic regions. Control and risk management of *T. cruzi* will require an understanding of the reservoir host species and their geographic distribution at the local level to aid in developing prevention programs (Brown et al. 2010). It is possible to investigate pathogen anthropology and sylvatic mammalian species association, and methods for identifying and suppressing disease transmission. As noted above, strategies are proposed to understand the diversity, abundance and geographic distribution of free-ranging mammals related to physiological life cycles that encourage parasitic protozoan routes of transmission (Noireau et al. 2009). My dissertation was divided into chapters designed as individual publications with some repetition of material between chapters. Chapter titles are as follows:

1. Prevalence of *Trypanosoma cruzi* in free-ranging mammalian populations in Bexar County, Texas (Chapter II)
2. Immunochromatographic antibody screening comparison for diagnosis of *Trypanosoma cruzi* in free-ranging mammals in Bexar and Val Verde Counties, Texas (Chapter III)
3. Meso-mammal Behavior to hematophagous triatomines at Joint Base San Antonio – Camp Bullis, Bexar County, Texas (Chapter IV)
4. Conclusion and Implications (Chapter V)

STUDY AREA

The project study area was conducted in Texas. Study sites included JBSA – Lackland Annex, and JBSA –Camp Bullis in Bexar County, and Laughlin Air Force Base in Val Verde County, with each having different environmental regimes that influence vector triatomine and host free-ranging mammal density (Fig. 1.1). JBSA-Lackland Annex is located within the Blackland Prairie ecological region of gently rolling plain with short and tall grasses, and woody vegetation limited to arroyos and scarps in elevations ranging from sea level to 304.8 meters (Lackland Air Force Base, Integrated Natural Resources Management Plan. 2007). JBSA – Camp Bullis encompasses approximately 11,735 hectares of the Edwards Plateau. Variable soils and plant species make up the patchy shrub lands and open wood lands throughout the landscape, with vegetation generally extending from the ground level to about 1.8m, and covering approximately 60% of the total. Numerous avian and karst species are found on the property and protected by the Endangered Species Act (U.S. Fish and Wildlife Service Programmatic Biological Opinion. 2009). Laughlin Air Force Base covers approximately 2,168 hectares and is primarily developed to support pilot training. Vegetation community is primarily comprised of open shrub-lands, mesquite grasslands with mesquite trees randomly distributed, small woody vegetation 1.2 – 1.8m in height with dense canopies and riparian regimes of thick understory.

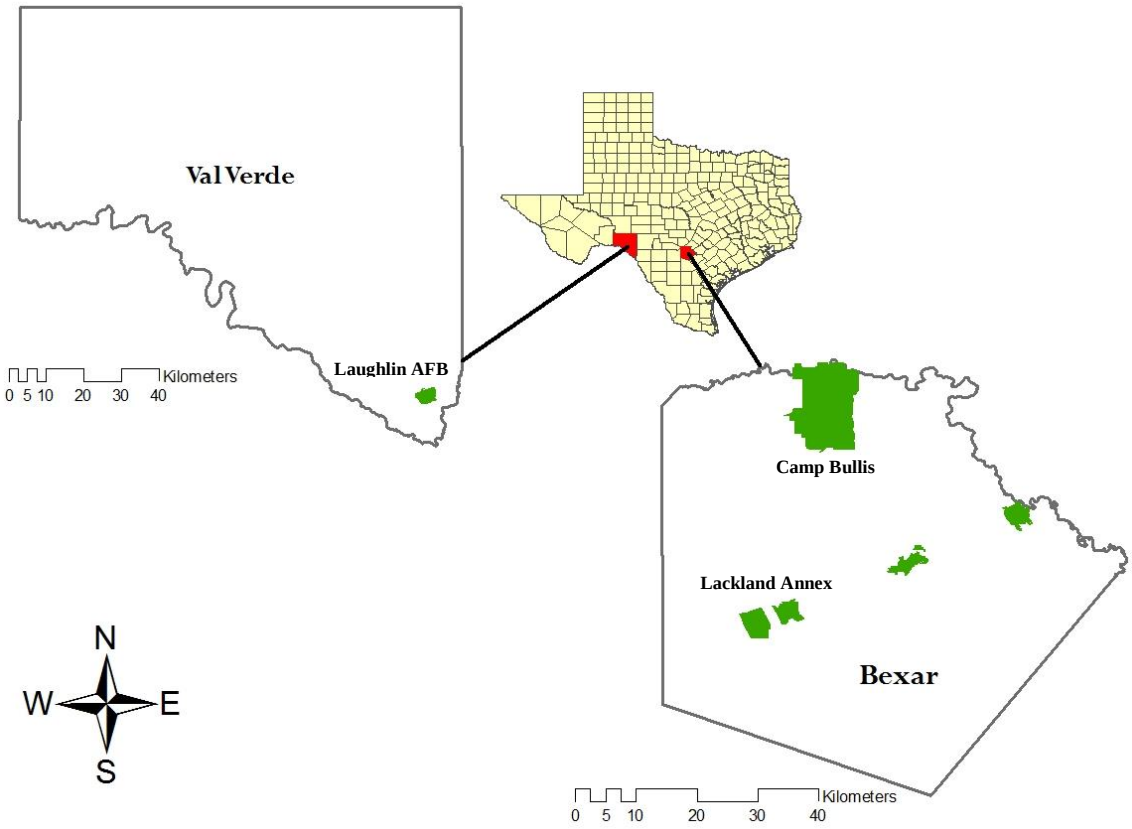

Figure 1.1. Joint Base San Antonio - Lackland Annex, Camp Bullis, Bexar County, and Laughlin Air Force Base, Val Verde County, Texas. 2011 – 2015.

CHAPTER II

PREVALENCE OF *TRYPANOSOMA CRUZI* IN FREE-RANGING MAMMALIAN POPULATIONS IN SOUTH TEXAS

SYNOPSIS

Chagas disease, caused by the flagellate protozoan *Trypanosoma cruzi*, is considered a significant human health problem in Central and South America where 8-11 million people are infected (Center for Disease Control and Prevention [CDC] 2011). In the United States, although there have been few reported cases, the CDC estimated that ≥300,000 immigrant and homeless people are infected (Bern et al. 2011). The primary vector of Chagas transmission is kissing bugs (*Triatoma* spp.), which are found from South America through the southern United States. Eleven species of triatomines are known to occur in the North America (Bern et al. 2011), with the most common vector species being *Triatoma rubida* and *Triatoma protacta* in Arizona and California, and *Triatoma gerstaeckeri* and *Triatoma sanguisuga* in Texas and New Mexico (Sarkar et al. 2010). Transmission occurs when fecal material from infected kissing bugs (Hemiptera: Reduviidae, Triatominae, Triatomine), containing infective *T. cruzi* protozoa trypomastigotes is rubbed or introduced into the feeding bite wound or mucous membranes, or when infected feces contaminates food or water (Lent and Wygodzinky 1979). Additionally, the disease pathogen can be transmitted through contact with infected blood and tissue, transplacentally, through carnivory, and possibly through consumption of infected triatomines by mammals (Roellig et al. 2009).

INTRODUCTION

Recent research suggests that Chagas disease may be more wide-spread in the United States than previously reported (Sarkar et al. 2010). Additionally, the role of free-ranging mammalian populations as host preference species for *T. cruzi* is poorly understood (Bosseno et al. 2009, Brown et al. 2010), particularly in the United States. (Sarkar et al. 2010). Control and risk management of *T. cruzi* requires an understanding of the reservoir host species and their geographic distribution at the local level to aid in developing prevention programs (Noireau et al. 2009, Brown et al. 2010). The goal of the current project was to better understand the current parasite-vector-host association in Southern Texas. My study objectives were to (1) determine disease prevalence of *T. cruzi* parasite in free-ranging mammalian species in various vegetative communities, and (2) calculate mammal population densities to define *T. cruzi* prevalence in those populations. I hypothesized that pathogenesis by *T. cruzi* is influenced by assemblages of free-ranging mammal species and associated triatomines. This information can be used to provide understanding into the potential disease risk transmission to South Texas communities.

MATERIALS AND METHODS

Study Area

My study was conducted on Joint Base San Antonio (JBSA) – Lackland Annex located in San Antonio, Bexar County, Texas (Fig. 2.1). The 1,619 hectare military installation supports multiple uses that include military mission and training activities, and is comprised of grasslands, managed woodlands, and deciduous riparian upland

woodlands. Herbaceous cover primarily includes King Ranch bluestem (*Bothriochloa ischaemum* var. *songarica*), buffalograss (*Buchloe dactyloides*), and Texas wintergrass (*Stipa leucotricha*). Managed woodlands are similar to deciduous woodlands but differ in the relative openness of the areas due to periodic mowing and selective harvest of canopy species (JBSA INRMP 2013). Woodland plant species include mesquite (*Prosopis glandulosa*), sugarberry (*Celtis laevigata*), cedar elm (*Ulmus crassifolia*), annual sunflower (*Helianthus annuus*), and ashy sunflower (*Helianthus mollis*). Riparian woodland plant species include black willow (*Salix nigra*), green ash (*Fraxinus pennsylvanica*), basswood (*Tilia caroliniana*), sugarberry (*Celtis laevigata*), chinaberry (*Melia azedarach*), giant ragweed (*Ambrosia trifida*), and morning glory (*Ipomoea* sp.). The climate of this areas is subtropical with mild winters and hot summers (mean temperature = 14 and 27^{o} C, respectively). Precipitation is variable but averages 835 mm annually with peaks in spring and fall (Joint Base San Antonio Integrated Natural Resource Management Plan. 2013).

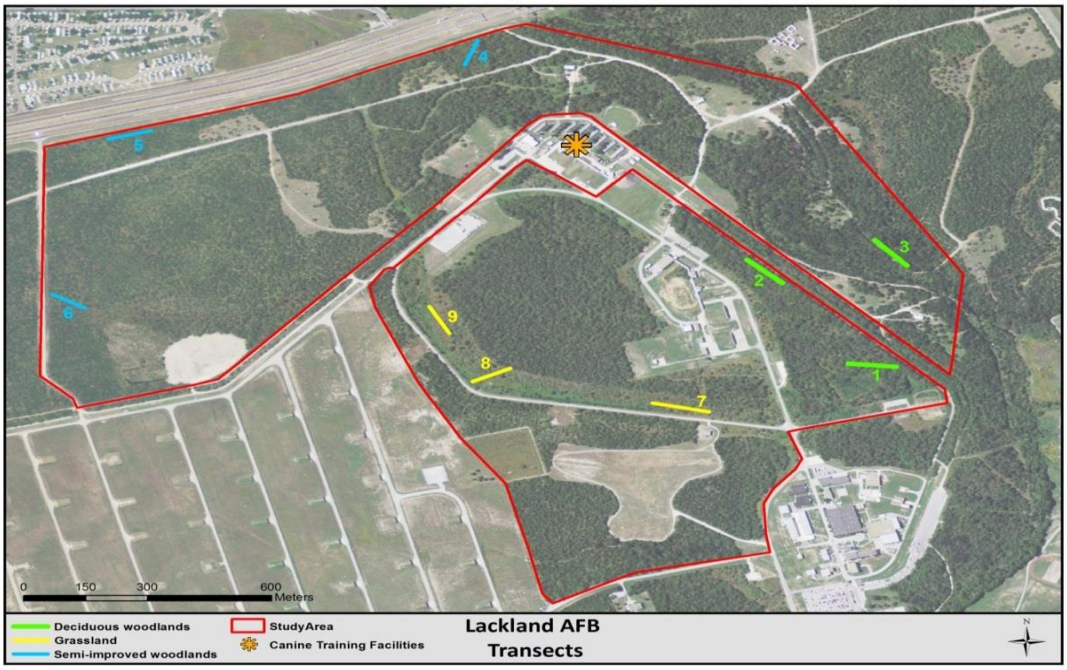

Figure 2.1. Transect locations at Joint Base San Antonio-Lackland Annex, Bexar County, Texas, USA. 2011 – 2014.

Species Abundances

We conducted seasonal (December 2011–2013) free-ranging large and meso-mammal (body mass of 2.5-25 kg) population surveys and trapping to estimate mammal densities in various vegetative community types (i.e., grasslands, managed woodlands, etc.) and to determine *T. cruzi* incidence. In our study, we defined seasons as fall (September-November), winter (December-February), spring (March-May), and summer (June-August). Population estimates derived from mark-recapture data were limited to fall seasons due to sample sizes. Mammalian population density data were collected using 2 primary approaches: (1) digital infrared-triggered cameras (DITC), and (2) mark-recapture analyses derived from camera and live-capture data. First, 9 Cuddeback DITC (Non-typical Inc., Park Falls, WI) were placed at transect locations (one randomly placed along each transect at 0.5 m above ground, $n = 9$ total cameras) to determine presence of mammal species and provide relative abundance of large and mid-size mammals. I placed cameras along 9 different 100-m transects for approximately 60 days prior to mammal trapping efforts (3 transects in each of the 3 vegetative communities). Cameras were set to take one picture followed by a 30 second video on a 15 second delay. Data were collected on 2GB standard digital cards and animals were individually identified when possible to help determine frequency of use. Each camera was located using a handheld global positioning system and then uploaded into a Geographic Information System. DITC were baited with aromatic lures, to include, skunk scent, liquid apple scent, oats and wet or dry cat food to attract diverse mammal species.

Second, I calculated densities of small and meso-mammals using mark-recapture methods (White et al. 1982). We trapped small mammals using 10 Sherman live traps (7.6 x 9 x 23 cm; H.B. Sherman Traps, Tallahassee, FL) placed at 10-m intervals along the 9 individual 100-m transects (n = 90 total traps) described previously. Meso-mammals were trapped using 5 Tomahawk Live Traps (48 cm x 15 cm x 15 cm; Tomahawk, WI) randomly placed along each 100-m transect (n = 15 total traps). Traps were baited with oats and cat food and set between dusk and dawn for 4-5 consecutive nights. Trapping events were conducted 2-4 times per season annually during the 2 year study. I identified mammals by species, sex, age, weight, vegetation type captured in, and transect/trap number. Individuals were ear-tagged or paint marked for recapture identification.

Vegetation Communities

I measured and characterized the vegetation at 3 randomly-selected points along each transect. Visual obstruction and vegetation height was measured using a Robel pole (Robel et al. 1970) to characterize the understory within each treatment. Ground cover (%) was measured within a $1m^2$ quadrat (soil, litter, herbaceous, woody). Mid-story vegetation was measured using the line intercept method by stretching a measuring tape between stakes (10 meters) and measuring the canopy width of all plants touching the tape (Silvy 2012). The point-centered-quarter method was used to measure over-story tree density. The distance to the nearest tree in each of the 4 cardinal directions from the center point was measured, and mean area calculated by squaring the mean distance between points (Silvy 2012).

Disease Data Collection and Prevalence Analysis

I collected blood and tissue samples from small and meso-mammals during density estimation capture and additional lethal captures (medical-grade CO_2, Institutional Animal Care and Use Committee (IACUC) #2011-294). Additionally, I secured blood and tissue samples from white-tailed deer (*Odocoileus virginianus*) research and feral hog (*Sus scrofa*) control efforts conducted on JBSA. I extracted femoral artery blood from live-captured and released white-tailed deer. I collected whole blood and tissue samples from euthanized feral hogs caught in circular style corral traps, baited with corn dispensed from a game feeder, (JBSA- Lackland Pest Management Plan 2014). Collected whole blood specimens were placed in BD Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and tissue excisions, which included heart, liver, lung and muscle, were placed in Ziploc® plastic bags; specimens were stored in a cooler with dry ice for transport to the laboratory.

Parasitic behavior and different parasitemia levels occur between the trypomastigote (extracellular non-dividing form) acute and amastigote (binary fission replicative form) chronic disease phases, make detecting protozoan gene DNA sequences or host antibodies challenging depending on analysis techniques (Brasil et al. 2010). Polymerase chain reaction (PCR) investigations were applied in our study to detect specific gene sequence (parasite) molecules from meso-mammal blood and tissue samples, and to serve in further validation. To determine *T. cruzi* infection, both mammal whole blood and tissue samples were submitted to determine acute versus chronic infection. I extracted whole blood and tissue from mammalian samples.

PCR analyses were performed by the Department of the Army Public Health Command Region – South, JBSA – Ft. Sam Houston, San Antonio, Texas. Samples were processed for nucleic acid extraction according to Quiagen DNeasy manufacturer instructions (Quiagen, Valencia, California, USA). All DNA samples were screened for two *T. cruzi* genomic targets using conventional PCR methods (Reisenman et al. 2010). All samples were first screened for *T. cruzi* minivaccircle kDNA, followed by a second conformity PCR to amplify a nuclear nDNA repetitive *T. cruzi* specific sequence using Eppendorf Mastercycler (Eppendorf, Hauppauge, New York, USA). PCR products were separated by gel electrophoresis on 2% agarose gel and visualized by UV light on BioRad ChemiDoc (BioRad, Hercules, California, USA). Samples were designated as *T. cruzi* positive based off 2 separate PCRs coinciding with expected 330 bp kDNA and 188 bp nDNA (Reisenman et al. 2010).

Data Analyses

I calculated species presence and selection of vegetation communities using DITC photographs (white-tailed deer, feral hogs) and trapping (small and meso-mammals). Densities of small and meso-mammals were calculated using the Schumacher-Eschmeyer mark-recapture analysis (Silvy et al. 2005), and species densities among vegetation community selections were compared using a Chi-squared test ($\alpha = 0.05$). I compared *T. cruzi* prevalence rates between vegetative communities using Kruskal-Wallis test ($\alpha = 0.05$).

RESULTS

Species Relative Abundances

DITCs recorded 15 mammal species in a total of 2,065 photographs (Fig.2.2). I found that white-tailed deer had higher relative abundances in photographs compared to other mammalian species (Fig.2.2). I also found that white-tailed deer were photographed in much higher numbers than raccoons throughout all vegetative communities (white-tailed deer, n = 620 total photographs; raccoons, n = 493 total photographs), but selection of vegetative communities differed significantly between the 2 species ($\chi^2 = 44.008$, $P < 0.001$). Other species recorded from DITCs included coyote (10%), eastern cottontail rabbit (*Sylvilagus floridanus*) (8%), Virginia opossum (*Didelphis virginiana*) (6 %), javelina (*Tayassu tajacu*) (5%), striped skunk (*Mephitis* spp.) (4%), nine-banded armadillo (*Dasypus novemcinctus*) (4%), wild hog (*Sus scrofa*) (3%), gray fox (*Urocyon cinereoargenteus*) (3%), bobcat (*Lynx rufus*) (2%), eastern fox squirrel (*Sciurus niger*) (0.04%), ringtail (*Bassariscus astutus*) (0.2%), and wood rat (*Neotoma micropus*) (0.05) (Fig. 2.2).

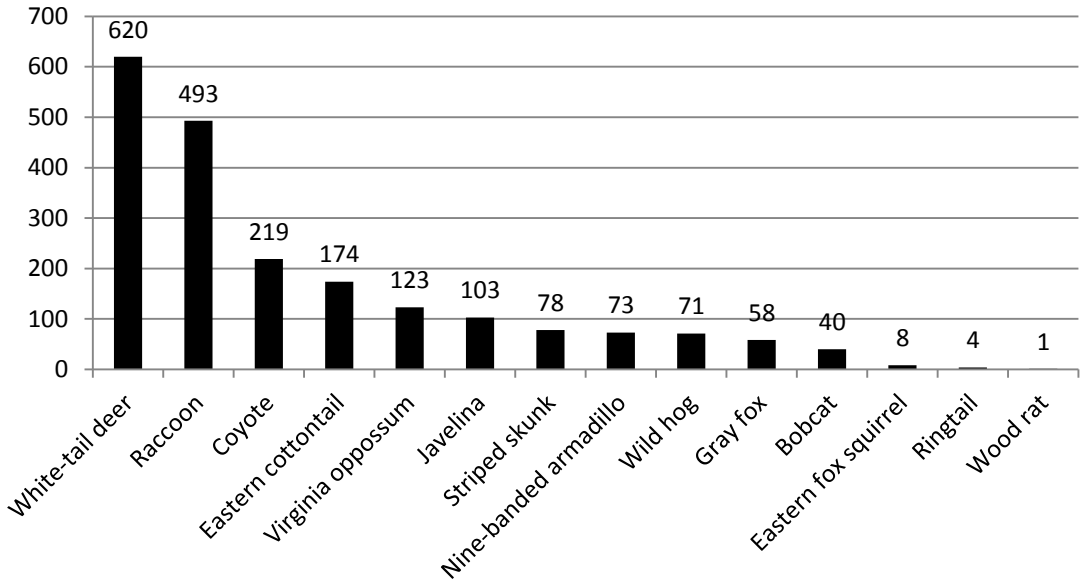

Figure 2.2. Photographs of species recorded via digital infrared-triggered cameras at Joint Base San Antonio - Lackland Annex, Bexar County, Texas. USA. 2011 - 2014.

I captured a total of 166 individual small and meso-mammals in live traps. Free-ranging mammal species included 28 raccoons, 29 Virginia opossums, 19 skunks (*Mephitis mephitis*), 18 white-tailed deer, 27 feral hogs, 15 hispid cotton rats, 5 southern plains wood rats (*Neotoma micropus*), and 4 white-ankled mouse (*Peromyscus pectoralis*), 4 plains harvest mouse (*Reithrodontomys montanus*), 2 northern pygmy mouse (*Baiomys taylori*) and 2 fox squirrels (*Sciurus niger*). I pooled mark-recapture data into 12-16 week trap periods due to acceptable mark retention and increased data collection. I found that densities of species with confirmed *T. cruzi* infections were comparable amongst seasons, with fall counts providing the most stable trapping estimates with the lowest variation (Table 2.1). The majority of DITC photographs identified mammalian species that foraged between different vegetation regimes with greater abundance with photograms, I had insufficient data to calculate densities for hispid cotton rats.

Table 2.1. Meso-mammal density estimates derived from mark-recapture data (9 transects) for Joint Base San Antonio – Lackland Annex, San Antonio, Texas, 2012 – 2013.

Species	Season	Density (km^2)	CI-Low (95%)	CI-High (95%)
Raccoon	Fall 2012	41.9	28.6	78.9
Virginia Opossum	Fall 2012	43.5	30.5	75.7
Virginia Opossum	Fall 2013	42.8	29.6	77.6
Striped Skunk	Fall 2012	49.9	31.3	123.0
Striped Skunk	Fall 2013	55.7	33.6	161.4

Parasite Prevalence

I found *T. cruzi* in 4 species (Virginia opossums, *n* = 29; 61% of total sampled; striped skunks, *n* = 19; 25% of total sampled; raccoons, *n* = 28; 23% of total sampled; and cotton rats, *n* = 15; 13% of total sampled). I found that parasite prevalence differed significantly (H=11.04, df = 2, *P*=0.004) based on vegetation community with grasslands demonstrating far less test-positive mammals (*n* = 4 positives, 24% of total sampled) than deciduous woodlands (*n* = 11 positives, 85% of total sampled) and semi-improved woodlands (*n* = 6 positives, 60% of total sampled) (Fig.2.3).

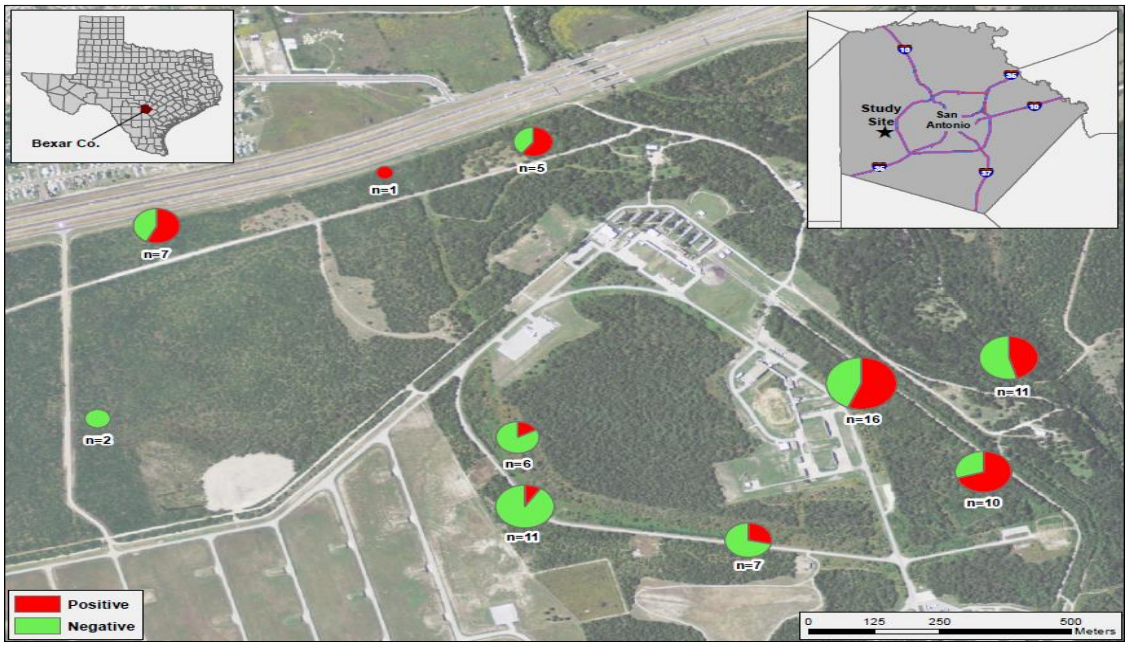

Figure 2.3. *Trypanosoma cruzi* sampling transects and prevalence study results (*n*=total sample size, red=positive, green=negative) at Joint Base San Antonio-Lackland Annex, Bexar County, Texas, USA. 2011 – 2014.

Table 2.2. Animals tested and incidence rate of *Trypanosoma cruzi*, Joint Base San Antonio – Lackland Annex, San Antonio, Texas, 2012- 2013.

Mammal Species	Total Animals	# PCR Samples	# Positive Samples	% *T. cruzi* Prevalence
Hispid cotton rat *Sigmodon hispidus*	15	15	2	13.3
White-ankled mouse *Peromyscus pectoralis*	4	2	0	0.0
Deer mouse *Peromyscus maniculatus*	13	36	0	0.0
Wood rat *Neotoma micropus*	5	0	0	0.0
Plains harvest mouse *Reithrodontomys montanus*	4	2	0	0.0
Pygmy mouse *Baiomys taylori*	2	3	0	0.0
Fox squirrel *Sciurus niger*	2	5	0	0.0
White-tail deer *Odocoileus virginianus*	18	7	0	0.0
Wild pig *Sus scrofa*	27	85	0	0.0
Virginia opossum *Didelphis virginiana*	29	85	52	61.2
Raccoon *Procyon lotor*	28	93	22	23.7
Skunk *Memphitis memphitis*	19	59	15	25.4
Total	166	392	91	10.3 ($\bar{x}$)

Bar X ($\bar{x}$) = percent average total

DISCUSSION

Raccoons, Virginia opossums, striped skunks, and hispid cotton rats are typically generalists with the ability to live in human-dominated areas. My investigation recognized these species as localized primary *T. cruzi* mammal reservoirs, which supports the hypothesis that these common mammals help maintain and transmit these parasites in association with vector triatomines. I suspect animals tested were the primary hosts for the pathogen and the low numbers of test-positive animals in grasslands (Fig 2.3) is reflective of habitat preferences for these species. This is supported by research that indicates triatomines are located in a variety of habitats including grasslands, woodlands, and human-dominated areas such as houses and often prey on mammals (Bern et al. 2011). Furthermore, despite the different characteristics of the two types of woodlands I studied, they still had significantly higher rates of mammal infection than grasslands; thus, lending credence that these species (woodland generalists) are important in pathogen persistence. The lack of evidence of *T. cruzi* in all rodents except in hispid cotton rats is contrary to the findings of Pinto et al. (2010), and may be attributed to phylogenetic lineage differences between the protozoan pathogen and diversified mammal composition found within specific vegetation communities (Roellig et al. 2009). Similarly, I found no evidence that white-tailed deer and feral hogs are major contributors in transmission of the disease, and can probably be attributed to the lack of burrowing activity (where insects are commonly found) and different resting locations. My research largely supports much of the available evidence of mammal host roles in *T. cruzi* persistence. Beard et al. (2003) reported that *T. cruzi* infection of canine

species in South Texas may be endemic. A recent study in Mexico identified the blood meal origins of 47 triatomines and found that raccoons and armadillos (*Dasypus* spp.) were the main blood meal hosts (Bosseno et al. 2009). Another study in the U.S. reported *T. cruzi* infection among 11 reservoir species from 6 southern states and detected a higher incidence of the infection in Virginia opossums and raccoons (Brown et al. 2010). These studies suggest that *T. cruzi* prevalence varies between host preference species and geographic regions.

The distribution of *T. cruzi* prevalence in mammal species has implications for managing wildlife populations to limit transmission to people. No action is needed against the majority of rodent species and larger game species. Instead, management should focus on meso-mammals, specifically raccoons, Virginia opossums, and skunks. However, due to the high incidence of *T. cruzi* among these animals, their removal could be problematic because the kissing bugs typically associated with them could become displaced and search for alternative blood meals including pets and people (Barr 2009). Modeling of meso-mammal population dynamics is proposed to evaluate various management actions *a priori* to include management of vegetative communities having relatively high prevalence of *T. cruzi* infection. Peridomestic meso-mammals are no longer constrained by natural predators, and are occupying microhabitat regimes that increase the human interactions threat of zoonotic diseases. Managing vector and reservoir movement requires a simultaneous integrated control approach that includes, identifying harborage locations, spatial insecticide applications, mechanical or chemical vegetation management to discourage vectors and reservoirs, and Environmental

Protection Agency (EPA) approved mammal bait formulations impregnated with systemic insecticide (McPhatter et al. 2012) that produces hematophagous triatomine mortality without deleterious effects on vertebrate hosts.

CHAPTER III

IMMUNOCHROMATOGRAPHIC ANTIBODY SCREENING COMPARISON FOR DIAGNOSIS OF *TRYPANOSOMA CRUZI* IN FREE-RANGING MAMMALS IN BEXAR AND VAL VERDE COUNTIES, TEXAS

SYNOPSIS

American trypanosomiasis (Chagas disease) is caused by the flagellated protozoan *Trypansoma cruzi* (*T. cruzi*), a zoonotic parasite in meso-mammal species in South Texas. The emerging disease is of growing concern in vertebrate species, and not well understood. Approved mammalian *T. cruzi* xenodiagnoses and serological procedures are available to detect infection, but are extremely sensitive, require specialized training by clinicians, and do not provide immediate disease confirmation. My objectives were to evaluate commercially available single-use immunochromatographic rapid detection dipstick assay devices for the detection of antibodies to *T. cruzi* in meso-mammal species, and quantify data findings. I tested 3 brands of devices in blood from white-tail deer (*Odocoileus virginianus*), white-ankled mouse (*Peromyscus pectoralis*), raccoon (*Procyon lotor*), and fox squirrel (*Sciurus niger*) mammal species, and research results determined rapid antibody dipsticks were difficult to evaluate effectiveness because manufacturers' recombinant antigen to detect antibodies to *T. cruzi* was not specific for meso-mammal species. Immunochromatographic rapid detect assay systems can be an effective first-line device to detect and mass screen for disease infection, but will require precise antigen to detect antibodies to *T. cruzi* in meso-mammals.

INTRODUCTION

Previous studies have reported that woodrats (*Neotoma micropus*), are the primary host of *T. cruzi* due to location preference of vectors in the burrows of these mammals. Otherhost species in Texas include hole-dwelling species like skunks and raccoons (Charles et al. 2013). There is little information about Chagas disease infecting sylvatic mammals, in Bexar or Val Verde Counties, Texas, and my research has found peridomestic and free-ranging animals to be significant reservoirs for the disease. Currently, there are no chemotherapies available to prevent the disease, but trial treatments are being used with marginal success during the acute phase of the infection (Bern et al. 2007).

The genetic lifecycle for the pathogen *T. cruzi* with contributing phenotypic and genotypic diversity suggests that genetic exchange of the protozoan DNA occurs during the sylvatic transmission with different phylogenetic lineages. The two main discreet phylogenetic lineages are; *T. cruzi* I (TcI) and *T. cruzi* I (TcII), which is further subdivided into five sub-lineages (IIa, IIb, IIc, IId, IIe) that share similar clonal properties and exchange genetic material, but with differing geographic distribution within specific mammal host species (Roellig et al. 2009). Phylogenetic lineages TcI and TcII genotypes have the largest distribution in the southern United States, and evidence suggest that the two different genotypes are evolving and adapting to colonize new hosts as an emerging south Texas disease. Parasitic behavior and different parasitemia levels occur between the trypomastigote (extracellular non-dividing form) acute and amastigote (binary fission replicative form) chronic disease phases, make detecting protozoan gene

DNA sequences or host antibodies challenging depending on analysis techniques (Brasil et al. 2011). Chagas disease detection commonly involves indirect immunofluorescence assay (IFA), enzyme-linked immunosorbant assay (ELISA), or polymerase chain reaction (PCR) investigations as laboratory confirmation methods. Due to different protozoan life stages and parasitemia levels during the acute and chronic disease stage, there is debate between researchers when applying different investigative protocols and base-line threshold defining disease documentation. Disease diagnostic literature is diverse, so there is concern when testing reliability is not universally established and standardized, and necessitates two or more different protocols to validate the disease pathogen *T. cruzi*. Diagnosis of the parasite in the acute stage is particularly important when administering treatment therapies, but is difficult for clinicians to microscopically isolate or visualize trypomastigotes in anti-coagulated blood smears stained with Giemsa dyes.

My study confirmed that triatomine insect species have a host preference for sylvatic and domestic animals, but will readily feed on any mammal species to facilitate growth development and reproduction (Reisenman et al. 2010). Due to host species specificity, triatomine insect assemblages are adapting to climate change, promoting colonization of new habitats, and a zoonotic and public health concern. Trypomastigote parasitemia density found in mammal blood (acute stage) may be difficult to determine through IFA techniques, and due to antigen response, infection rate, and health symptoms are different in host species (Salomone et al. 2003). When approved detection methodologies such as, PCR confirms *T. cruzi* DNA, there are differences expressed

with different pathogen genotypes and host species (Britto et al. 1999). This suggests that evolutionarily, the pathogen has evolved host specificity, and the identification of the pathogen through different analysis protocols could yield differing testing results. Anthropogenic disturbance is forcing triatomine species to seek alternative hosts, and due to the emerging disease, screening processes using immunochromatographic assay protocols was implemented for this study as a potential first-line rapid evaluation process to detect antibodies to *T. cruzi*. The goal of analytical processes is to measure Chagas disease through a rapid assay detection system that provides visual confirmation of the parasite antibodies through control line reagents using testing methodologies that are cost-effective, reliable and easy to administer by clinicians. I applied three different immunochromatogaphic assay systems (Chembios Diagnostics Systems Chagas STAT-PAK™ Assay, Chembios Diagnostics Systems DPP® Chagas Assay, and InBios *Trypanosoma* DETECT™) to confirm assay recombinant antigen reliability to *T. cruzi* antibodies in forty four free-ranging mammal species between April and July 2015. Due to the variation of sensitivity that differentiates between a true-positive and a false-positive for the presence of mammal antibodies, I had to thoroughly understand different clinical characteristics and testing methodologies. Mammal whole blood samples (n =36) were obtained, and I found immunochromatographic screening assay test STAT-PAK™, DPP® , and *Trypanosoma* DETECT™ were 0.0 % effective at isolating parasitological *T. cruzi* antibodies in free-ranging meso-mammal species. To confirm immunochromatographic rapid assay strip test results, real time PCR was applied to target protozoan DNA in mammalian tissue samples. The current combination

of specific recombinant antigens employed by the different detection systems was unsuccessful at detecting *T. cruzi* antibodies from whole blood in free-ranging meso-mammal species.

MATERIALS AND METHODS

Study Area

The study was conducted at Joint Base San Antonio (JBSA) – Lackland Annex (N29.3794° W98.66924°), JBSA – Camp Bullis (N29.68689° W98.55835°), Bexar County, Texas and Laughlin Air Force Base (N29.355659° W100.783836°), Val Verde County, Texas. Investigation was to evaluate the effectiveness of

immunochromatographic recombinant antigen screening assays specific for detection of antibodies to *Trypanosoma cruzi* (*T. cruzi*) in free-ranging meso-mammals occupying different communities to include, woodlands, grassland savannas, upland woodlands, caves, burrows and successional areas. JBSA – Lackland Annex in Bexar County land features and vegetation regimes have been previously described.

JBSA - Camp Bullis is in Bexar County, and on the edge of the Edwards Plateau in an area called the Texas Hill Country or the Balcones Canyonlands. Topography of the site consists of numerous hills and valleys that are drained by intermittent streams. Formations that occur in the area include limestone, shoreline, reefs and lagoon deposits. The Balcones Escarpment, a fault zone that developed during the Cretaceous and Miocene extends from Uvalde to Austin, and forms the southeastern edge of the Edwards Plateau and intersects the southeastern corner of JBSA-Camp Bullis. The Edwards Plateau rises approximately 1,006 ft (305 m) above the Gulf Coast Plain. The

elevation of the Edwards Plateau is approximately 2,000 ft (606 m) above mean sea level.

JBSA-Camp Bullis is part of the juniper-oak-mesquite savanna portion of the Prairie Brushland Province. Historically, vegetation grasslands and savanna cover types were likely spatially and temporally dynamic, and reflect a complex interaction between fires and climatic change. Vegetation cover includes, 61% (6,658 ha) live oak-juniper woodland, 32% (3,507 ha) oak/grassland, and 7% (735 ha) grassland (Fig. 3.1). Dominant grassland areas are little bluestem (*Schizachyrium scoparim*), Texas wintergrass (*Nassella leucogtricha*), big bluestem (*Andropogon gerardi*), Sideoats gramma (*Bouteloua curtipendula*), Indiangrass (*Sorghastrum nutans*) and switchgrass (*Panicum virgatum*). Similar grasses are found mottes of oak species (*Quercus sp.*), cedar elm (*Ulmus* crassifolia), hackberry (*Celtis sp.*), Ashe juniper (*Juniperus* ashei), and black cherry (*Prunus serotina*). Grasses in woodlands are sparse and principally bluestem (Ft Sam Houston Integrated Natural Resource Management Plan. 2008). Additionally, savannahs include live oak (*Quercus virginiana*), Texas oak (*Quercus buckleyi*), cedar elm (*Ulmus crassifolia*), escarpment black cherry (*Prunus serotina*), post oak (*Quercus stellata*), blackjack oak (*Quercus merlandica*), hackberry (*Celtis* sp.), and Ashe juniper (*Juniperus ashei*). Woodlands are predominantly juniper and live oak interspersed with little bluestem and muhlys (*Muhlenbergia* sp.) (Cooksey 2009).

Laughlin Air Force Base (LAFB) is in Val Verde County, and located on the Edwards Plateau and Rio Grande Plain physiographic province. The western section of the installation is upper cretaceous Buda limestone and Del Rio clay. The Buda

limestone is light gray to pale orange in color and consists of fine grained poorly bedded nodular material. The Del Rio clay is calcareous light gray material with significant oil and gas deposits. The soil found on LAFB is very shallow beds of caliche with 20 percent of the surface being covered with well drained limestone. Zorro Creek drainage basin flows south to southwest through the installation, and meets the Rio Grande approximately 19.3 kilometers downstream. A majority of Laughlin AFB property is urbanized to support flying mission requirements, with unimproved vegetation locations consisting of upland shrublands with grassland mosaic in undeveloped and dry slope locations. Tall shrubs such as, guajillo (A*cacia berlanderia*), Texas colubrine (*Colubrina texensis*), littleleaf sumac (*Rhus microphylla*), calderona (*Krameria ramosissima*), Texas paloverde (*Parkinsonia texana*), and amargods (*Castela erecta*). Deciduous woodlands support small trees and larger shrubs of honey mesquite (*Prosopis glandulosa*), Texas persimmon (*Diospyros texana*), netleaf hackberry (*Celtis reticulata*), netleaf hackberry, granjeno (*Celtis pallida*), honey mesquite, huisache (*Acacia smallii*), and western soapberry (Sapindus saponaria). Grasses are limited due to regular mowing but include, cane bluestem (*Bothriochloa barbinodis*), plains lovegrass (*Eragrostis intermedia*), pink pappusgrass (*Pappophorum bicolor*), and Texas winter grass. (Laughlin Air Force Base Integrated Natural Resource Management Plan. 2010).

Bexar County temperatures range from an average high of 17° degrees Celsius (C) in January to 36°C in July and August. Normal mean temperature ranges from 10°C in January to a high of 29ºC in July. Although the summers are hot, with daily maximum temperatures are above 32ºC. Situated between a semiarid area to the west

and a coastal area of heavy precipitation to the southeast, the average annual rainfall is 84.6 centimeters (cm) but has ranged from a low of 3.6 cm to a high of 164.3 cm over the past 100 years. Normally, the heaviest amount of rainfall is during the months of May and June; much of this rainfall is accountable in sizeable downpours.

Val Verde County weather is characterized as semi-arid with dry winters and hot summers. Sunshine occurs 80 percent of the time during the summer months and 53 percent in the winter. Strong dusty winds cause temperatures to average between 11° degrees Celsius (C) in winter to 38°C during the summer months. Precipitation average annual rainfall is 45.7 cm, with most rain occurring during the months of April through October. Relative humidity is higher at night and averages 79 percent, while mid-afternoon humidity is typically 54 percent.

Meso-mammal Trapping

Meso-mammal species were captured using Sherman box traps (7.6 x 9 x 23 cm; H.B. Sherman Traps, Tallahassee, FL), and Tomahawk (48 cm x 15 cm x 15 cm; Tomahawk, WI) or Havaheart (Lititz, PA) live traps, baited with sardines, wet and dry cat food, or milo sorghum, depending on target species. Twenty-one trapping grids were identified with known meso-mammal activity from photos captured by digital infrared-triggered cameras (DITC). All live traps were pre-baited to attract meso-mammals, and set weekly from April – July. Traps were checked the following morning, and captured mammals were recorded for species, sex, weight, overall health condition information.

Whole blood and tissue samples from euthanized white-ankled mouse (*Peromyscus pectoralis*) (n = 8), raccoon (*Procyon lotor*) (n = 8), fox squirrel (*Sciurus*

niger) (*n* = 2), white-tailed deer (*Odocoileus virginianus*) (*n* = 22), and opossums (*Didelphis virginiana*) (*n* = 4) were placed in BD Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and tissue excisions, which included heart, liver, lung and muscle, were placed in Ziploc® plastic bags; stored in a cooler with dry ice for transport to the laboratory per, (Animal Care and Use Committee [IACUC] #2015-0032) and Texas Parks Wildlife Department Permit SPR-1211-394. Each meso-mammal whole blood sample was immunochromatographic assay tested for reactive results for the presence of antibodies to *T. cruzi*.

Immunochromatographic Rapid Detection Assay Evaluation

Three different immunochromatographic rapid assay systems derived from *T. cruzi* antigens were applied to the study to identify potential antibodies for the presence of protozoa infection (Cardinal et al. 2006). According to manufacturer's assay instructions, Chembio Diagnostic Systems, Inc. STAT-PAK® (Medford, NY), Chembio Diagonstic Systems, Inc. DPP® (Medford, NY), and InBios *Trypanosoma* DETECT™ (Seattle, WA), antibody evaluation systems "are for research purposes only" and were evaluated to detect antibodies to *T. cruzi* in meso-mammals in Bexar and Val Verde Counties, Texas.

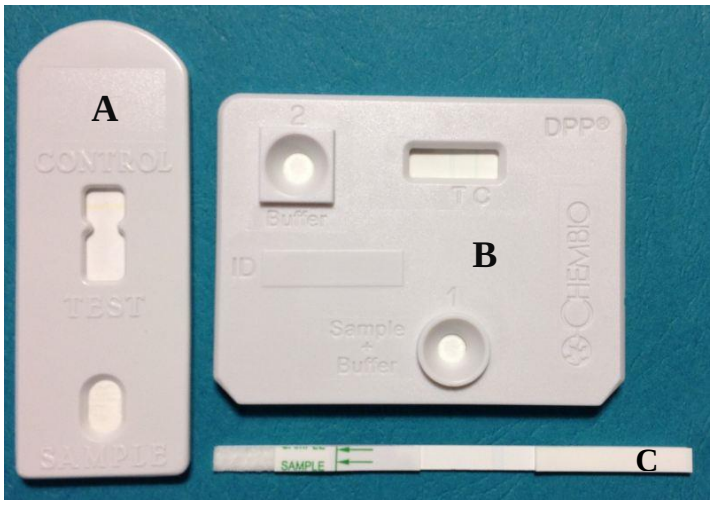

Figure 3.1. Chembio Diagnostics Systems, Inc. STAT-PAK® (A), Chembio Diagnostics Systems, Inc. DPP® Chagas Assay (B), and InBios DETECT™ (C) immunochromatographic screening devices for the detection of antibodies to *Trypanosoma cruzi.*

Immunochromatographic rapid strip assays are intended for the qualitative detection of antibodies to *T cruzi* in vertebrate whole blood specimens, and used as an initial screening detection test for antibodies to *T cruzi* infection. Each rapid assay system contained 25–100 individual pouched dipsticks and was stored at 8-30°C. For assay product evaluation, we labeled the device with the animal species, sex and identification number. I collected meso-mammal whole blood using a Microsafe® Tube (Medford, NY, USA) held in a horizontal position to allow capillary action to draw the blood sample to the tube fill line. Chembio Diagnostic STAT-PAK™ and DPP® systems required 10 µl of animal whole blood that was added to the sample well using a the Microsafe® Tube. 240 µl or 6 drops of diluent was added to the sample well and allowed to react for 15 minutes. A single pink or purple line represents no detection of antibodies to *T. cruzi* or a negative result of the control area location, and a second pink or purple line appearing in the test location represents detection to antibodies and a positive *T. cruzi* infection result. The InBios *Trypanosoma* DETECT™ system requires 20 µl of whole blood to the test strip down arrow, and adding 120-200 µl Chase Buffer solution diluent and the results read in 15 minutes. The single line represents the negative control, while a second line appearing parallel to negative control line signifies visual confirmation to *T. cruzi* antibodies. The test consists of a proprietary gold mix containing Gold Conjugant that binds to antibodies found in specimen whole blood (Neito, et al. 2009).

Immunochromatographic rapid detection systems results visually detecting antibodies to *T. cruzi* were compared to whole blood and tissue quantitative real time

polymerase chain reaction (PCR) laboratory analysis. PCR testing to detect *T. cruzi* deoxyribonucleic acid (DNA) was performed by Texas A&M University, Department of Veterinary Integrative Biosciences and Interdisciplinary Program in Ecology and Evolutionary Biology, College Station, Texas. In order to detect *T. cruz* DNA within meso-mammal hosts, 250 µl of whole blood and 1g of heart apex tissue was subjected to DNA extraction using E.Z.N.A® Tissue Extraction (Omega BioTek, USA) per manufacturer's instructions. Quantitative real-time PCR (PCR) was performed with the Cruzi 1/2 primer set with 6-carboxyfluorescein (FAM)-labeled Cruzi 3 probe to amplify satellite DNA as described, but with initial denaturation time of three minutes (Piron et al. 2007; Duffy et al. 2013). Based on laboratory validations, samples with cycle threshold (Ct) value of <32 were interpreted as positive for *T. cruzi* infection. DNA extracted from the Sylvio X10 strain of *T. cruzi* (American Type Culture Collection, Manassas, VA) was used as a positive control, and both water and no template negative controls were included in each reaction.

RESULTS

Biodiversity and anthropogenic expansion has allowed for increased American trypanosomiasis (Chagas disease) throughout Bexar and Val Verde Counties, causing an increased zoonotic and public health concern. Visual diagnostics from the immunochromatographic rapid detection assays revealed negative results for all sample

White-tailed deer was the most common mammal tested, followed by White-ankled mouse, Virginia opossum, raccoon and fox squirrel. Results using the immunochromatographic screening device assays were unable to confirm meso-mammal

antibodies to *T. cruzi*, or validate as a visual first line rapid detection test in support of Center for Disease Control (CDC) approved PCR laboratory analysis for *T. cruzi* DNA confirmation. I trapped a total of 36 meso-mammals and the rapid immunochromatographic rapid detect dipstick samples resulted in negative detection of antibodies to *T. cruzi* for all samples of white-tailed deer (0.0%), white-ankled mouse (0.0%), Virginia opossum (0.0%), raccoon (0.0 %) and fox squirrel (0.0%), while real time PCR analysis of submitted tissue identified *T. cruzi* DNA in Virginia opossum only.

Quantitative real time PCR analysis confirmed *T. cruzi* DNA in (8.3%) of whole blood and tissue sample submitted (Table 3.1). Despite negative *T. cruzi* confirmation in all animals tested using immunochromatographic assay strips; I did identify and confirm *T. cruzi* DNA presence using real-time PCR in Virginia opossum heart apex tissue. I revealed that meso-mammal species inhabiting caves or burrows are important contributors to the emergence of the pathogen. The significance of an approved immunochromatographic assay strip as an initial first-line detection system will contribute researchers with disease identification across all susceptible mammal species, and provide management strategies to protect non-infected mammal species. If manufacturers will need to develop immunochromatographic rapid detect assays that are able to identify specific mammal antibodies to detect *T. cruzi* before advantages are recognized as a first-line detect system in conjunction with other approved laboratory analysis methodologies.

Table 3.1. Summary of immunochromatographic rapid assay systems and PCR sample comparison for meso-mammal *Trypanosoma cruzi* in Bexar and Val Verde Counties, Texas, 2015.

Mammal Species	Total Animals	ChemBio DPP®	ChemBio Stat Pak®	InBios Detect™	PCR
White-tail deer *Odocoileus virginianus*	22	Negative 0/22	Negative 0/22	Negative 0/22	0/22
White-ankled mouse *Peromyscus pectoralis*	8	Negative 0/8	Negative 0/8	Negative 0/8	0/8
Virginia opossum *Didelphis virginiana*	4	Negative 0/4	Negative 0/4	Negative 0/4	3/4
Fox squirrel *Sciurus niger*	2	Negative 0/2	Negative 0/2	Negative 0/2	0/2
Total	36	0/36	0/36	0/36	3/36

DISCUSSION

I determined that Chembio Diagnostic Systems STAT-PAK ®Assay, Chembio Diagnostics Systems DPP® Chagas Assay, and InBios DETECT ™ immunochromatographic rapid detection devices with current recombinant antigens for detection of antibodies to *T. cruzi* in meso-mammals are not effective as a first-line infection recognition system for *T. cruzi* in a variety of mammal species. These quick detection test strips were developed to identify possible *T. cruzi* infection in human blood (Roddy et al. 2008) and may not cover a wide enough spectrum of *T. cruzi* variants to provide reliable readings for non-human samples. While immunochromatographic rapid assay strips did not identify antibodies to *T. cruzi* infection in the 5 mammal species tested, the study using real-time PCR did confirm pathogen disease concentration in Virginia opossum.

It is unfortunate that of the 36 animals trapped for testing only 4 individuals tested positive for *T. cruzi* by standard recognized PCR methods. I recommend that further testing with samples of blood known to be infected with *T. cruzi* in both stages of its life cycle (trypomastigote and amastigote infection phases) be conducted before any definitive assessment of the immunochromatographic assay strips is made.

The value of an approved and effective immunochromatographic assay strip as an initial first-line detection system would be to aid researchers with disease identification across all susceptible mammal species that could be used under field conditions or in areas with limited laboratory resources. Based on my preliminary results, manufacturers will need to develop immunochromatographic rapid detect assays

that are able to identify specific mammal antibodies to detect *T. cruzi* before advantages are recognized as a first-line detect system in conjunction with other approved laboratory analysis methodologies (Reithinger et al. 2010). My research suggests that manufacturers should first develop test strips for Virginia opossums, raccoons and skunks, which were the main carriers of *T. cruzi* in my study. It is unlikely that there would be a large enough demand for such strips for wildlife species to create a commercial incentive for their development. However, there is value in developing easy use test strips to detect *T. cruzi* in dogs (Cardinal et al. 2006), which have a close association with humans as pets and a high value as working animals for military and law enforcement agencies.

CHAPTER IV

MESO-MAMMAL BEHAVIOR TO COLLECTIVE HEMATOPHAGOUS TRIATOMINES AT JOINT BASE SAN ANTONIO – CAMP BULLIS, TEXAS

SYNOPSIS

An investigation of local triatomine insect species and their communal association with cave or burrow dwelling mammals that facilitate transmission of American trypanosomiasis (Chagas disease) is of concern as an emerging disease at Joint Base San Antonio – Camp Bullis, Bexar County, Texas. The behavior and aggregate response of captured mammals related to active sylvatic hematophagous triatomine species that transmit *Trypanosoma cruzi* (*T. cruzi*) disease has not been described in literature, and is poorly understood. My objectives were to evaluate the behavior of cave and burrow dwelling mammal species in response to hematophagous triatomine insects and to investigate their communal association. I observed the behavioral response of white-ankled mice (*Peromyscus pectoralis*), raccoons, and fox squirrels to feeding triatomine insects and monitored the defecation patterns of the insects after their blood meal to determine species interaction in a shared environment and thus, predict *T. cruzi* infection pathways. I found that cave and burrow dwelling meso-mammals species tolerate insect aggregation and blood-feeding activity, but white-ankled mice responded to the insects and bit them. The insects rarely defecated at the site of the bite, so it may be that grooming and ingestion of triatomines is an important pathway that facilitates Chagas disease prevalence.

INTRODUCTION

Hematophagous triatomines aggregate within mammalian microenvironments such as burrows, dens and caves for shelter and as a food source (Marshall 1982). Thus, mammals using burrows or caves will have a greater contact with these insects than animals which remain on the surface. Unless the animals have a way of deterring the insects from biting, burrow dwelling animals will be at increased risk of being infected with *T. cruzi*.

Another factor affecting rates of transmission of *T. cruzi* from triatomines to burrow dwelling mammals is the defecation pattern of insects. In studies of human infection, the insects tend to feed around the lips, hence the common name "kissing bug." They also defecate while or immediately after feeding, which allows the parasite entry to the host body through the bite wound (Iowa State Center for Food Security and Public Health 2009). Whether the insects feed on the oral mucosa of smaller mammalian hosts is unknown. Similarly, it is unknown when the insect is on a small animal or active host if defecation occurs directly on the host. Answers to these questions are important in the understanding of transmission of *T. cruzi* in non-human hosts..

My study objectives were to (1) determine small and meso-mammal innate behavior in response to collective hematophagous triatomines inhabiting burrows or caves, and (2) measure, and categorize blood-engorged triatomine vector feeding and defecation intervals and potential to disperse *T. cruzi* infection to reservoir vertebrates. We hypothesized that pathogenesis by *T. cruzi* is influenced by the behavior of burrow or cave dwelling mammal species habituating to assemblages of triatomine species.

Further, we hypothesize that disease transmission prevalence is influenced by mammals actively feeding on vector insects as a nutritional source or a response to eliminate bite wound irritation. This information can be used to provide understanding and predicting mammal species most susceptible to host-parasite dynamics and disease mitigation strategies within a shared environment.

MATERIALS AND METHODS

Study Area

Joint Base San Antonio (JBSA) – Camp Bullis is a located on the northern edge of the San Antonio city limits, Bexar County, Texas, and provides military training (~18,000/annual) to mission partners in order to sustain operational readiness. The principal function at JBSA – Camp Bullis is to support field training for the U.S. Army Medical Department Center and School (AMEDDC&S), Defense Medical Readiness, 502d Training Wing, 937th Training Group, and Ground Combat Skills School (Joint Base San Antonio Integrated Natural Resource Management Plan. 2015).

Completely surrounded by private and commercial urbanization, the 11,361 ha military installation is comprised of karst geology terrain characterized by dissolving of rock, sinkholes, sinking streams, closed depressions, subterranean drainage, and caves (JBSA Integrated Natural Resource Management Plan. 2015). JBSA – Camp Bullis has documented over 100 caves throughout the installation that are predominately found in the Lower Glen Rose formation and Kainer formation of the Edwards group (Ft Sam Houston Integrated Natural Resource Management Plan, 2008). The installation also encompasses the Lewis, Salado and Panther Creeks, and 35 ha of semi-permanent

springs or manmade wetlands that complement the grasslands, managed woodlands, and deciduous riparian upland woodlands. Woodlands along the intermittent creeks are composed primarily of live oak (*Quercus virginiana*), ash juniper (*Juniperus ashei*), cedar elm (*Ulmus crassifolia*), hackberry (*Celtis* spp.), Spanish oak (*Quercus falcata*), sycamore (*Platanus occidentalis*), buttonbush (*Cephalanthus occidentalis*), with eastern cottonwood (*Populus deltoides*) and poison hemlock (*Conium* spp.). Upland plant communities are dominated with Spanish oak, Texas persimmon (*Diospyros texana*), live oak and escarpment black cherry (*Prunus serotine*). Along the creeks, riparian communities are comprised of cedar elm, hackberry, Texas red oak (*Quercus buckleyi*), Texas black walnut (*Juglans microcarpa*), and live oak. Vegetation found in these areas include, Eastern gamagrass, frogfruit (*Phyla nodiflora*), sedges (*Carex* spp), bushy bluestem (*Andropogon glomeratus*), switchgrass, and cattails (*Typha domingensis*). Predominant grassland species includes, big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scoparium*), big bluestem (*Andropogon gerardii*), Lindheimer muhly (*Muhlenbergia lindheimeri*), green sprangletop (*Leptochloa dubia*), Texas cupgrass (*Eriochloa sericea*), plains lovegrass (*Eragrostis intermedia*), and tall dropseed (*Sporobolus asper*).

The regional climate in central Texas is classified as modified subtropical with long hot summers and short cool winters. Temperatures range from an average high of 17°C in January to 35°C in July and August. Normal mean temperature ranges from 10.3 degrees Celsius (ºC) in January to a high of 29.2ºC in July. Although the summers are hot, with daily maximum temperatures above 32ºC greater than 80 percent of the time,

extremely high temperatures (>38ºC) are rare. The average annual rainfall is 835.6 millimeters but has ranged from a low of 35.6 inches to a high of 1643.4 millimeters over the past 100 years (U.S. Climate Data 2015).

Meso-mammal and Triatomine Species Abundance

I conducted free-ranging meso-mammal (body mass of 0.05 - 25 kg) and triatomine insect surveys and captures each weekend during April through July 2015, using relative abundances in various karst cave features and burrow locations to determine presence or absence of vertebrate species. Meso-mammal and triatomine insect populations are expected to utilize and accumulate at burrow or cave locations as suitable habitat, and associated with each other as a constant food source (Conditt et al. 1997). Data for trapping purposes was collected using two primary methods: (1) digital infrared-triggered cameras (DITC) to identify mammal species present and (2) visual sampling techniques to identify triatomine species. Cuddeback DITC (Non-typical Inc., Park Falls, WI) were placed at cantonment burrow and cave locations (two each randomly placed at 0.5 m above ground, n = 10 total cameras) to determine presence of mammals and collective triatomine species. DITC will provide relative abundance of host and vector activity in favorable environmental habitat locations (Schwanz et al. 2011) to support future live-trapping and monitor behavioral interaction. DITCs were placed along different locations adjacent to training sites for approximately 2 weeks prior to mammal and insect trapping efforts. Cameras were set to take one picture followed by a 30 second video on a 15 second delay. Data were collected on 2GB standard digital cards an mammal species were individually identified when possible to

help determine frequency of use. DITCs were baited with aromatic lures that included peanut butter, grain, milo-sorghum, oats, sardines and wet and dry cat food to attract and determine mammal species found within the habitat.

During the study, I trapped and collected meso-mammals and triatomine insects associated with burrows and caves. I calculated host-vector species presence and selection of burrow and cave communities using DITC photographs and trapping meso-mammals. I used logistic regression to determine feeding and defecation timeline differences in triatomine species. White-ankle mouse (*Peromyscus pectoralis*), fox squirrel (*Sciurus niger*) and raccoon (*Procyon lotor*) meso-mammal species were live-trapped from burrows and lime stone caves on JBSA – Camp Bullis, and placed in an artificial clear aquarium type container box to characterize innate behavior when hematophagous triatomine species were introduced, and recorded animal habituation behavior. Triatomine species that were released into a clear artificial container and allowed to actively feed on mammal hosts, were immediately removed to measure defecation timelines and determine how the pathogen protozoa from triatomine feces causes animal infection. Finally, I compared meso-mammal behavioral response to feeding triatomines to quantify as a learned or innate behavior interaction within the burrow or cave communities, and determine *T. cruzi* mode of transmission from vector to host.

Host Ethology and Vector Feeding and Defecation Timelines

Mammals and triatomine species associated with burrows or caves were trapped within cantonment areas and cave locations to characterize host-vector interaction. Prior

to trapping and collecting meso-mammals or triatomine insects, DTIC confirmed activity, and Sherman live traps (7.6 x 9 x 23 cm; H.B. Sherman Traps, Tallahassee, FL) or Tomahawk live traps (48 cm x 15 cm x 15 cm; Tomahawk, WI) were used depending on mammal species size, and pre-baited and placed adjacent to cave or burrow entrances (n = 31 total traps) to pre-determine trap acceptance. Vertebrate live traps were baited with milo sorghum and set each Friday evening and checked Saturday morning over an 8 week period. Collected mammals were according to Institutional Animal Care and Use Committee #2011-294 guidelines.

Triatomine insects were passively or actively hand collected from burrow and cave locations and placed in Tupperware® (Tupperware Brands Corporation, Orlando, FL) clear snack size containers with locking lids, and a moist paper towel inserted inside to ensure extended survival. Trapped White-ankle mice (n = 8), fox squirrel (n= 2) were placed in a clear rectangular ten gallon vivarium (Perfecto Manufacturing, Noblesville, IN) and allowed to acclimate for 30 minutes prior to triatomine insects (n = 5) being released to blood-feed, and describe meso-mammal host behavior during hematophagous activity, and measure insect defecation rates. Trapped raccoons (n = 2) were placed in a wooden box painted white inside and triatomine insects (n = 10) released to record animal behavior and insect defecation timeline after blood-meal. Meso-mammals were visually evaluated to characterize innate behavior from feeding arthropods, and time-monitored blood engorged insects removed and placed in clear Tupperware® cups to measure defecation time intervals and determine pathogen transmission mode.

RESULTS

Digital infrared-triggered cameras (DITC) recorded 5130 mammal species photographs around JBSA-Camp Bullis caves. I found that *Peromyscus pectoralis* had higher relative abundance in photographs compared to other mammalian species. I also found that porcupines (*Erethizon dorsatum*) were photographed in much higher numbers (n=2755) than expected throughout most cave communities, but were not considered for this study due to their diet. Selection of burrow and cave or karst communities differed significantly depending on adjacent vegetation land cover. Other species recorded from DITCs included Virginia opossum (23%), raccoon (16%), armadillo (6%), ringtail (3%), eastern fox squirrel (1%), cottontail rabbit (0.05%), and red fox (0.001%).

To evaluate meso-mammal behavior towards hematophagous triatomines, and determine if insect feeding occurs, I targeted aggregate mammal species and trapped eight white-ankled mice (*Peromyscus pectoralis*), two fox squirrels (*Sciurus niger*), four Virginia opossums *(Didelphis virginiana*) and two raccoons (*Procyon lotor*), using Sherman and Tomahawk traps. I determined collective hematophagous triatomine species are mutually associated with meso-mammals inhabiting burrows or caves, and are dependent on the hosts and their distribution as a blood-meal source.

I found triatomines would aggressively pursue and feed on white-ankled mice when the animal was quiet, but were deterred and forced to retreat from approaching and feeding when animal movement occurred. White-ankled mice would routinely bite insects while they were approaching to blood-feed. Insects not bitten and killed by the mice were forced to retreat multiple times and re-approach to feed when the animal

became quiet. Four Raccoons and two fox squirrels were placed into artificial environment to allow triatomines to feed and monitor mammal response and triatomine feeding and defecation timeline. Both raccoons and fox squirrels appeared to recognize approaching insects, and did not attempt to bite or move away from triatomine during blood-meal feeding activity. Triatomines did not move towards or feed around the face (mucosal locations) of meso-mammals species tested, but would move to locations without fur, such as ventral side, legs, and tail to locate capillaries for blood-meal feeding. By introducing triatomines to meso-mammals, I determined that insect feeding activity timeline range was 11 - 24 minutes (n=7, $\bar{x}$=11) before insect would release and retreat from animal.

After a blood-meal, triatomines had a tendency to retreat away from the meso-mammal and defecation timelines ranged from 2–58 minutes (n=11, $\bar{x}$=28). Results from this study demonstrate meso-mammal *T. cruzi* parasite transmission occurring through insect ingestion and grooming activities within the burrow or cave. It is important to understand meso-mammal host ethology and hematophagous triatomines when predicting Chagas disease or zoonotic disease potential, and further studies are warranted to understand hollow residing vertebrate species, associated hematophagous triatomines, and strategies to diminish disease transmission.

The relationship between hosts and vector found in burrows or caves is mutually beneficial to meet nutritional requirements and facilitate pathogen incidence and distribution through collective vector defecation. Triatomine defecation did not occur simultaneous with feeding activity, but away from the mammal with a timeline range

2 – 36 minutes. First signs of defecation were after feeding activity away from bite location based on observed initial fecal material. Initial defecation was sometimes difficult to determine because it was clear and easier to observe as fecal material become darker in color. Insects were removed once feeding activities were complete and visibly engorged. All defecation events were single clear drops except two which were rusty colored.

DISCUSSION

My investigation found that JBSA – Camp Bullis karst and cave habitats are unique, and collective meso-mammal and triatiomine species have adapted to co-exist and probably sustaining Chagas disease on the site. I found meso-mammal behavior response to hematophagous triatomines was recognizable with white-ankled mice, but unnoticeable with raccoons and fox squirrels. *Trypanosoma cruzi* (*T. cruzi*) zoonosis is supported by meso-mammal hosts that are actively feeding on triatomine insects or are ingesting parasitic infected feces during grooming activities within the burrow or cave.

I determined that meso-mammal species such as raccoons and fox squirrels did not respond to hematophagous triatomine activity, and would allow the insects to blood-feed until fully engorged and retreat. White-ankled mice would routinely bite at approaching triatomines, causing insect mortality or insects having to retreat until the animal settled down to re-approach to obtain a full blood-meal. Meso-mammal response to triatomine feeding supports predictions on how the protozoa are ingested by the animal and causes infection. The relationship between hosts and vector found in burrows

or caves is mutually beneficial to meet nutritional requirements and facilitate pathogen incidence and distribution through collective vector defecation.

Triatomine Species Feeding and Defecation Timelines

I found *Triatoma gerstaeckeri* were routinely identified and associated with cave location environments during white-ankled mouse trapping activities. I also found that parasite potential and prevalence differed between burrow and cave environments as opposed to woodland environments, demonstrating far less meso-mammal disease incidence in karst locations.

Furthermore, triatomine defecation timeline is important related to protozoan pathogen opportunities to infect the mammalian host. Literature research documents simultaneous triatomine feeding and defecation around mucosal membranes on the face, allowing for parasite ingestion and disease persistence (Barr 2009). My study demonstrated blood engorged triatomines would retreat after feeding and defecate away from the host animal. Defecation away from the host animal lessens the opportunity for disease transmission through mucosal membranes, unless insect defecation occurs within the burrow and protozoan pathogen is viable for extended periods of time and animal ingests parasite through grooming activities. The ecology of meso-mammal species should be considered when evaluating burrow or cave environments.

The distribution of *T. cruzi* prevalence in mammal species has implications for managing wildlife populations to limit transmission to people. Action is needed against the white-ankled mouse, Virginia opossum, raccoons, and skunks since they have been identified as primary reservoir species responsible for *T. cruzi* persistence. Management

and mitigation actions should focus on the primary reservoir species; however, removal of these common peridomestic animals may be detrimental due to the high disease incidence rate without simultaneous control of triatomines. Disruption of cave and burrow dwelling mammal populations could cause triatomine insects to relocate to alternative blood-meal food sources, causing an increased disease risk to other animal species (Barr 2009).

My research contributes to pathogen protection in Bexar and Val Verde Counties, and will eventually support diminishing disease incidence in humans and other animal species. I recommend continued Chagas disease research with a primary focus on habitat management, host ecology and vector controls.

CHAPTER V

CONCLUSION AND IMPLICATIONS

This chapter is to analyze the role of meso-mammals as primary reservoirs for *Trypanosoma cruzi* (*T. cruzi*) in Bexar and Val Verde Counties, Texas, and potential zoonotic and public health concern. This study emphasizes the significance of accounting for the density, behavior and preferred environmental regimes of meso-mammal species when defining Chagas disease incidence. When comparing pathogen analysis protocols for meso-mammal *T. cruzi* confirmation, variables need to be fully understood between clinicians, manufacturer characteristics, variations to confirmation sensitivities, and testing methodologies. Understanding interactions between meso-mammal behavior and triatomine communal association allowed me to predict variation in parasitism (Altizer et al. 2003). Each chapter summarizes and highlights previous chapters, and attempts to quantify the parasite-host-vector interaction, pathogen prevalence and the importance of an emerging disease threat impacting the Department of Defense and surrounding communities.

In Chapter II, I determined that meso-mammals were a significant disease (*T. cruzi*) reservoir threat throughout the test study range. I found different vegetation regimes and fragmentation reduces the risk of meso-mammal reservoir species that are *T. cruzi* infected, due to reservoir population size or carrying capacity falling below a threshold required for adequate parasitemia to exist (Renwick et al. 2012).

Promoting vegetation fragmentation may reduce the rate of contact between infected and susceptible reservoirs until a threshold in the host population density is reached, below which the disease has difficulty persisting (Renwick et al. 2012).

In Chapter III, the goal of the immunochromatographic process was to quantify *T.cruzi* parasites using testing methodologies that are cost-effective with no errors, and standardized thresholds that are acceptable by the clinicians doing similar work. Since the pathogen progresses through different life-cycles with eventual migration to tissue (organs), PCR isolating the pathogen DNA genetic material is the preferred method and was used for this study to confirm immunochromatographic detection of antibodies to *T. cruzi* in mammal blood samples. Since no assay has adequate sensitivity and specificity incorporated when confirming pathogen presence, approved protocol requires two or more methodologies for defining disease confirmation. Variables occur between commercially manufactured analysis processes, and required the study to implement DNA analysis, but the process involves a high level of pathogen and antigen response understanding by scientists and clinicians for disease validation. In support of the study, it was determined that immunochromatographic assay strips are not sufficient to definitively state absence or presence of antibodies to *T. cruzi* infection, and PCR that isolated *T. cruzi* specific DNA genetic material in meso-mammal blood and tissue samples was the preferred method for surveillance.

In Chapter IV, I determined that burrow and cave dwelling meso-mammals and associated triatomine insects are important contributors to Chagas disease persistence. My studies revealed the communal existence of collective meso-mammals and

triatomines in the same habitat regime promotes pathogen transmission throughout the animal and arthropod community. Further research is warranted to develop management strategies that influence pathogen incidence through habitat manipulation. The influence of anthropogenic disturbances such as prescribed burning and land management alterations contribute to meso-mammal and triatomine density control and pathogen reduction.

My research identified elevated levels of Chagas disease prevalence within the meso-mammal population residing in South Texas, and supports aggressive mitigation strategies to reduce mammalian infection and suppress the emerging disease migration. Without meso-mammal and triatomine control initiatives, disease adaptation and incidence could occur within other mammal species not considered primary hosts, and cause increased concern to public health. The research contributes to pathogen protection in Bexar and Val Verde Counties, and will eventually support diminishing disease incidence in humans and other animal species. I recommend continued Chagas disease research with a primary focus on habitat management, host ecology and vector controls.

LITERATURE CITED

Barr SC 2009. Canine Chagas' Disease (American Trypanosomiasis) in North America. *Vet Clin Small Anim* 39:1055-1064.

Brasil PE, DeCastro L, Jasslocher-Moreno AM, Sanginis HC, Braga JU. 2010. ELISA versus PCR for diagnosis of chronic Chagas disease: Systemic review and meta-analysis. BMC *Infect Dis* 10:337.

Beard CB, Pye G, Steurer FJ, Rodriguez R, Campman, Townsend A, Ramsey JJ, Wirtz RA, Robinson LE. 2003. Chagas disease in a domestic transmission cycle in Southern Texas, USA. *Emerg Infect Dis* 9:103-105.

Bern C, Kjos S, Yabsley MJ, Montgomery SP. 2011. *Trypanosomiasis cruzi* and Chagas' Disease in the United States. *Clin Microbiol Rev* 24(4): 655–681.

Bern C, Montgomery SP, Herwaldt BL, Rassi A, Marin-Neto JA, Dantas RO, Maguire JH, Acquatella H, Morillo C, Kirchhoff LV, Gilman RH, Reyes PA, Salvatella R, Moore AC. 2007. Evaluation and treatment of Chagas disease in the United States: A Systematic Review. *J Am Med Assoc* 298 (18):2171-2181.

Bosseno MF, Barnabe C, Ramirez-Sierra MJ, Kengne P, Guerrero S, Lozano F, Ezequiel K, Gastelum M, Breniere SF. 2009. Short Report: Wild ecotopes and food habits of *Triatoma longipennis* infected by *Trypanosoma cruzi* lineages I and II in Mexico. *Am J Trop Med Hyg* 80:988-991.

Brasil PE, DeCastro L, Jasslocher-Moreno AM, Sanginis LHC, Braga JU. 2010. ELISA versus PCR for diagnosis of chronic Chagas disease: Systemic review and meta-analysis. BMC *Infect Dis* 10:337.

Britto C, Cardoso MA, Marques P, Fernandez O, MorelCM. 1999. Polymerase Chain Reaction Direction: New Insights into the diagnosis of chronic Chagas disease. BMC *Infect Dis* 94(1):305 – 306.

Brown EL, Roellig DM, Gompper ME, Monello RJ, Wenning KM, Gabriel MW, Yabsley MJ. 2010. Seroprevalence of *Trypanosoma cruzi* among eleven potential reservoir species from six states across the Southern United States. *Vector Borne Zoonot Dis* 10(8):757-63.

Cardinal MV, Reithinger R, Gurtler RE. 2006. Use of an Immunochromatographic dipstick test for rapid detection of *Trypanosoma Cruzi* in sera from animal reservoir hosts. *J Clin Microbiol* 44(8):3005-3007.

Centers for Disease Control and Prevention. 1998. National Center for Infectious Diseases. http://www.cdc.gov/ncidod/dpd/chagas.htm. Accessed 30 August 2013

Charles RA, Kjos S, Angela S, Ellis E, Barnes JC, Yabsley MJ. 2013. Southern plains woodrats (*Neotoma micropus*) from Southern Texas are important reservoirs of two genotypes of and host of a putative novel species. *Vector-Born and Zoonotic Diseases* 13(1):22–30.

Conditt SA, Ribble DO. 1997. Social organization of *Neotoma Micropus*, the Southern Plains Woodrat. *Am Midl Nat* 137:290-297.

Cooksey ML. 2009. Biological assessment of the programmatic golden-cheeked warbler (*Dendroica chrysoparia*) habitat mitigation process for Camp Bullis Training Site, TX. U.S. Fish and Wildlife Service.

Duffy T, Cura CI, Ramirez JC, Abate T, Cayo NM, Parrado R, Bello ZD, Velazquez E, Muñoz-Calderon A, Juiz NA, Basile J, Garcia L, Riarte A, Nasser JR, Ocampo SB, Yadon ZE, Torrico F, de Noya BA, Ribeiro I, Schijman AG (2013) Analytical Performance of a Multiplex Real-Time PCR assay using TaqMan Probes for quantification of *Trypanosoma cruzi* satellite DNA in blood samples. *PLoS Neg Trop Dis* 7(1):e2000.

Faulhaber CA, Silvy NJ, Lopez RR, Porter BA, Frank PA, M.J. Peterson MJ. 2005. From the Field: Use of drift fences to capture Lower Keys marsh rabbits. *Wildlife Soc Bull* 33:1160-1163.

Fryxell JM, Falls JB, Falls EA, Brooks RJ. 1998. Long-term dynamics of small-mammal populations in Ontario. *Ecology* 79:213-225.

Ft Sam Houston. 2008. Integrated Natural Resource Management Plan. Prepared by MACTEC Development Corporation.

Hecht MM, Nitz N, Araujo PF, Sousa AO, Rosa AC, Gomes DA, Leonardecz E. 2010. Inheritance of DNA transferred from American Trypanosomiasis to human hosts. *PLoS One* 5(2):e9181.

Iowa State University, Center for Food Security & Public Health, 2009.

Jacobson HA, Kroll JC, Browning RW, Koerth BH, Conway MH. 1997. Infrared-triggered cameras for censusing white-tailed deer. *Wildlife Soc Bull* 25:547-556.

Joint Base San Antonio. 2015. Integrated Natural Resource Management Plan. Prepared by Weston Solutions.

Kjos SA. 2007. Biography and genetic variation of triatomine Chagas disease vectors and *Trypanosoma cruzi* isolates from Texas. Ph.D. Dissertation. Texas A&M University Press, College Station Texas.

Klotz J, Dorn P, Logan J, Stevens L, Pinnas J, Schmidt J, Klotz S. 2010. Kissing bugs: Potential disease vectors and cause of anaphylaxis. *Clin Infect Dis* 50(12):1629-1634.

Krebs CJ, Wilson DJ, Sinclair T. 1999. Limitation of collared lemming populations during a population cycle. *Oiko*s. 87:382-398.

Kribs-Zaleta C. 2010. Estimating contact process saturation in sylvatic transmission of *Trypanosoma Cruzi* in the United States. *PLoS Neg Trop Dis* 4(4): e656.

Lackland Air Force Base. 2007. Integrated Natural Resources Management Plan. Prepared by John Wildie and updates 2008, 2009, 2013 (Matt Kramm).

Laughlin Air Force Base. 2012. Integrated Natural Resource Management Plan. Prepared by Tetra Tech Inc.

Lent H, Wygodzinky P. 1979. Revision of the triatominae (Hemiptera, Reduvidae), and their significance as vectors of Chagas disease. *Bull Am Museum Nat Hist* vol. 163, art. 3.

Lescure FX, LeLoup G, Freilij H, Develoux M, Paris L, Brutus L, Pialoux G. 2010. Chagas Disease: Changes in knowledge and management. *Lancet Infect Dis* 10: 556-70.

Marshall, AG. 1981. *The Ecology of Ectoparasitic Insects*. London: Academic Press, 392 pp.

McPhatter L, Roachell W, Lockwood N, Osuna A, Mahmood F, Lopez J, Hoffman L, Lockwood N, Osuna A, Lopez J, Debboun M. 2012. Vector surveillance to determine species composition and occurrence of *Trypanosoma cruzi* infection at three military installations in San Antonio, Texas. United States Medical Department Journal. PB8-12-7/8/9:12-21.

Miles MA, de Souza AA, Povoa M. 1981. Chagas' disease in the Amazon Basin: III. Ecotopes of ten triatomine bug species (Hemiptera:Reduviidae) from the vicinity of Belem, Para State, Brazil. *J Med Entomol* 18:266-278.

Mills JN, Gage KL, Khan AS. 2010. Potential influence of climate change on vector-borne and zoonotic diseases: A Review and Proposed Research Plan. *Environ Health Perspect* 118(11):1507-1514.

Nieto PD, Boughton R, Dorn PL, Steurer F, Raychaudhuri S, Esfandiari J, Gonçalves E, Diaz J, Malone JB. 2009. Comparison of two immunochromatographic assays and the indirect immunofluorscence antibody test for diagnosis of Trypanosoma Cruzi infection in dogs in South Central Louisiana. *Vet Para* 165:241-247.

Noireau F, Diosque P, Jensen A. 2009. *Trypanosoma cruzi*: adaptation to its vectors and its hosts. *Vet. Res* 40(2):26.

Pinto CJ, Baxter BD, Hanson JD, Mendez-Harclerode FM, Suchecki JR, Grijalva MJ, Fulhorst CF, Bradley RJ. 2010. Using museum collections to detect pathogens. *Em Infect Dis* 16:356-357.

Piron M, Fisa R, Casamitjana N, López-Chejade P, Puig L, Vergés M, Gascón J, Prat JG, Portús M, Sauleda S. 2007. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. *Acta tropica* 103(3):195-200.

Reisenman CE, Lawrence G, Guerenstein PG, Gregory T, Dotson E, Heldebrand JG. 2010. Infection of kissing bugs with *Trypanosoma cruzi* Tucson, Arizona, USA. *Emerg Infect Dis* 16:400–405.

Renwick AR, Lambin X. 2012. Host–parasite interactions in a fragmented landscape. *Int J Parasitol* 43:27-35.

Reithinger R, Grijalva MJ, Chiriboga RF, Alarcon De Noya B, Torres JR, Pavia-Ruz N, Manrique-Saide P, Cardinal MV, Gurtler RE. 2010. Rapid detection of *Trypanosoma cruzi* in human Serum by Use of an Immunochromatographic dipstick test. *J Clin Microbiol* 48(8):3003–3007.

Robel RJ, Briggs JN, Dayton AD, Hulbert LC. 1970. Relationships between visual obstruction measurements and weight of grassland vegetation. *J Range Manage* 23:295-297.

Roellig DM, Ellis AE, Yabsley MJ. 2009. Oral transmission of *Trypanosoma cruzi* with opposing evidence for the theory of carnivory. *J Parasitol* 95(2):360-364.

Roddy P, Goiri J, Flevaud L, Palma PP, Morote S, Lima N, Villa L, Torrico F, Albajar-Vinas P. 2008. Field evaluation of a rapid immunochromatographic assay for detection of *Trypanosoma cruzi* infection by use of whole blood. *J Clin Microbiol* 80:2022-2027.

Roellig DM, Ellis AE, Yabsley MJ. 2009. Genetically different isolates of *Trypanosoma Cruzi* elicit different infection dynamics in raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*). *Int J Parasitol* 39:1603-1610.

Rozas M, Botto-Mahan C, Coronado X, Ortiz S, Cattan PE, Solari A. 2005. Short Report: Trypanosoma Cruzi infection in wild mammals from a Chagasic area of Chile. *Am J Trop Med Hyg* 73:517-519.

Salomone OA, Basquiera AL, Sembaj A, Aguerri AM, Reyes ME, Omelianuk M, Fernández RA, Enders J, Palma A, Barral JM, Madoery RJ. 2003. *Trypanosoma cruzi* in persons without serologic evidence of disease, Argentina. *Emerg Infect Dis* 9:1558-1562.

Sarkar S, Strutz SE, Frank DM, Rivaldi C, Sissel B. 2010. Chagas disease risk in Texas. *PLoS Neg Trop Dis* 4:1-14.

Schwanz L E, Brisson D, Gomes-Solecki M, Ostfeld RS. 2011. Linking disease and community ecology through behavioural indicators: immunochallenge of white-footed mice and its ecological impacts. *J Anim Ecol* 80:204-214.

Silvy NJ, Lopez RR, Peterson MJ. 2005. Wildlife marking techniques. Pages 339-376 in C.E. Braun, editor. Techniques for Wildlife Investigations and Management. Sixth edition. The Wildlife Society, Bethesda, Maryland, USA.

Silvy NJ. 2012. The Wildlife Techniques Manual. Seventh edition. John Hopkins University Press.

Smeins FE, Slack RD. 1982. Fundamentals of Ecology Laboratory Manual, 2nd Edition. Kendall/Hunt Publishing Company, Dubuque, Iowa, 140 pp.

Souza-Moraes H, Bordin J. 1996. Strategies for prevention of transfusion-associated Chagas disease. *Transfusion Med Rev* 35(9):161-170.

Texas Department of Health. 2013. http://www.dshs.state.tx.us/IDCU/investigation/Texas-Notifiable-Conditions.doc . Accessed 30 August 2013.

U.S. Climate Data. 2015. http://www.usclimatedata.com/climate/php?location-USTX1200. Accessed 4 April 2015.

U.S. Fish and Wildlife Service. 2009. Department of the Army Ft Sam Houston, Texas Programmatic Biological Opinion. White GC, Anderson DR, Burnham KP, Otis DL. 1982. Capture-recapture and removal methods for sampling closed populations. Los Alamos National Laboratory, Report LA-8787-NERP, 235 pp.