ECOEPIDEMIOLOGY OF *TRYPANOSOMA CRUZI* in TEXAS

A Dissertation

by

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ABSTRACT

This dissertation focused on elucidating factors affecting *Trypanosoma cruzi* transmission in the southern US, using triatomine, canine, and wildlife samples.

Collection of triatomine vectors from 2012-2015 included standard entomological sampling, as well as submissions through a citizen science program. The insects were identified to species, dissected, and tested for *T. cruzi* infection. *T. gerstaeckeri* and *T. sanguisuga* were the most abundant species in the collection. Kissing bugs were captured primarily April-October, and peak activity varied by species. A *T. cruzi* infection prevalence of 58.9% was found in 1,226 triatomines of 6 species, and infection prevalence varied by species. Amplification and sequencing of the TcSC5D gene revealed *Triatoma gerstaeckeri* was approximately equally infected with TcI and TcIV, and 10 individuals showed mixed TcI/TcIV infections. In contrast, *Triatoma sanguisuga* was more frequently found infected with TcIV than TcI. Relative abundance of parasite DTUs varied spatially, with both TcI and TcIV co-circulating nearly equally in vectors in central Texas, while TcIV predominated in northern Texas.

A study of *T. cruzi* infection in dogs in south central Texas using paired IFA and Chagas Stat-Pak serological testing showed a seroprevalence of 57.6%. The odds of being seropositive were greater for dogs older than 6 years of age than dogs less than 2 years of age. PCR analyses of blood revealed 26.7% of dogs, including both seronegative and seropositive dogs, harbored parasite DNA in their blood. Sequencing of the TcSC5D gene from blood and tissue samples showed TcI and TcIV were present, including a co-occurrence of both DTUs in an individual dog.

Cardiac tissue and blood were collected from wildlife—including raccoons (*Procyon lotor*), coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), and bobcats (*Lynx rufus*)—from central Texas. PCR analyses found 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot). Strain typing revealed raccoons infected with DTU TcIV, and a single raccoon with TcI/TcIV mixed infection.

DEDICATION

To those at risk of developing Chagas disease, with the hope that sustained research of *Trypanosoma cruzi* will continue to reduce the global burden of Chagas disease and the suffering it causes.

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NOMENCLATURE

PCR	Polymerase Chain Reaction
DTU	Discrete Typing Unit
DNA	Deoxyribonucleic Acid

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1. INTRODUCTION

Carlos Chagas first described the protozoan parasite *Trypanosoma* (*Schizotrypanum*) *cruzi* from an insect in Minas Geraes, Brazil in 1909 (Chagas, 1909). In 1916, Kofoid and McCulloch described a similar organism from an insect captured in a woodrat nest in California, likening it to Chagas' *T. cruzi*, but describing it as a different species (Kofoid and McCulloch, 1916). Not until 17 years later did Kofoid and Donat publish the realization that it was in fact the same species that Chagas had described, and the first report of *T. cruzi* in the US was in fact in 1916 (Kofoid and Donat, 1933). In the 100 years subsequent to the discovery of *T. cruzi* in the US, this parasite has captured the research attention of a number of scientists.

Trypanosoma cruzi, the causative agent of Chagas disease in humans and dogs, is a vector-borne parasite transmitted by triatomine insects, commonly known as kissing bugs. Infected vectors are found throughout the Americas, where the parasite causes both acute and chronic disease in animals and humans. Infection with *T. cruzi* is recognized in a variety of wild animal hosts (Bern et al., 2011), and is a source of concern for dog owners throughout Texas and other states, where Chagas disease in dogs has been reported (Beard et al., 2003; Kjos et al., 2008; Tenney et al., 2014; Williams et al., 1977). Although *T. cruzi* is known to be harbored in wild and domestic animals throughout the southern United States (Bern et al., 2011; Brown et al., 2010; Burkholder et al., 1980), the most intensive research has been conducted in countries where Chagas disease has historically been considered endemic (i.e. Central and South America), where an estimated 6 million people may be infected (World Health Organization, 2015). Lack of awareness in the US health provider community and absence of reporting requirements in many states have likely led to underestimates of disease risk and burden across the US.

Texas was the site of the first documentation of locally-acquired human Chagas disease in the US, in the 1950s (Woody and Woody, 1955), and also claims the first documentation of canine Chagas disease in the 1970s (Williams et al., 1977). Although

there is infrequent documentation of autochonthonous cases of human Chagas disease in the US (Cantey et al., 2012; Herwaldt et al., 2000), infected wildlife, domestic dogs, and insect vectors demonstrate an established disease system in Texas and throughout the southern US (Cantey et al., 2012; Charles et al., 2013; Kagan et al., 1966; Kjos et al., 2009; Maloney et al., 2010). As of 2013, Texas became only the fourth state to require reporting of Chagas disease in humans, and the first and only state to require reporting of animal cases (from 2013-2015), which may offer a more informative view of the burden in the state in the future.

Triatomine insects—more commonly known in the US as 'kissing bugs', 'cone-nose bugs', and other names—are the nocturnal, blood-feeding vectors of *T. cruzi*. Eleven different species of kissing bugs have been documented in 29 states across the southern US (Bern et al., 2011; Swanson, 2011), with the highest diversity and density found in Texas, New Mexico, and Arizona (Bern et al., 2011). Historical records document triatomine species in the US as early as 1855 (Le Conte, 1855), with all eleven species described by 1939 (Ryckman, 1986). Seven species have been reported from Texas: *Triatoma gerstaeckeri*, *T. indictiva*, *T. lecticularia*, *T. neotomae*, *T. sanguisuga*, *T. protracta*, *T. rubida* (Kjos et al., 2009; Lent and Wygodzinsky, 1979). In fact, Texas's central geographic location makes it home to the most western limit of *T. sanguisuga* ('eastern conenose bug'), the most eastern distribution of *T. protracta* ('western conenose bug'), and the most northern distribution of *T. gerstaeckeri* (Bern et al., 2011; Lent and Wygodzinsky, 1979). The most commonly documented and studied species in the state have been *T. gerstaeckeri* and *T. sanguisuga* (Davis et al., 1943; Kjos et al., 2009; Sullivan et al., 1949; Wozniak et al., 2015). Previous studies of Texas triatomines have found *T. cruzi* infection in all species, with infection prevalence varying by study, region collected, and triatomine species (Buhaya et al., 2015; Burkholder et al., 1980; Eads et al., 1963; Kjos et al., 2009).

Little is known about the optimal climatic conditions for survival and reproduction of each of the several species of triatomines that exist in the US. Suitable habitat would have to consider conditions supporting bug populations, conditions

supporting potential blood meal host populations, and climate conditions that would be likely to influence ecological characteristics of the landscape. Wood and Wood (Wood and Wood, 1937, 1961) published the earliest maps of kissing bug distribution in the southwestern United States. Although crude by today's standards, these first mapping efforts in southwestern US were certainly informative for their time. A variety of modeling approaches have been used since the development of computer programs (Beard et al., 2003; Lescure et al., 2010; Ramsey et al., 2005). More recent publications include county-level maps (Bern et al., 2011; Kjos et al., 2009) and a Chagas disease risk map spanning Mexico and the southern US (Sarkar et al., 2010). Factors that may influence the habitat suitability for kissing bugs includes a number of factors: vegetation, precipitation, temperature, elevations, populations of natural predators, populations of blood meal sources, soil type, and human alterations to the landscape. Although there have been studies investigating triatomine bug activity in varying thermal conditions (Lazzari et al., 1991; Schofield et al., 1992; Sjogren and Ryckman, 1966), these have mainly focused on dispersal and flying. In order to better direct public health initiatives and build complex Chagas disk spatial risk models, it is imperative to successfully delineate suitable vector habitat.

The genetic complexity of *T. cruzi* has been the focus of many studies, mainly in South America (Lewis et al., 2011, 2009; Rocha et al., 2013; Roellig et al., 2013, 2008; Yeo et al., 2011), and the taxonomy of the genetically, ecologically, and epidemiologically-relevant parasite sub-classifications is under constant revision (Barnabé et al., 2000; Brisse et al., 2001; Westenberger et al., 2005; Zingales et al., 2012, 2009). The current understanding is that there exist six discrete typing units (DTUs) of *T. cruzi*, TcI through TcVI. These DTUs are categorized based similarities in ecological and epidemiological scope, but not necessarily in the context of their biological evolutionary relationships (Zingales et al., 2012). For example, TcI, TcII, TcV, and TcVI are responsible for most human Chagas disease cases in South America, while TcIII is associated with burrowing mammals, and little is known about TcIV (Messenger et al., 2015b). Three DTUs—TcI, TcIV, and most recently, TcII—have been

found in various sample types from the US (Barnabé et al., 2001; Beard et al., 1988; Herrera et al., 2015; Roellig et al., 2008). The five cases of human Chagas disease in the US that have been strain-typed were all TcI, although other samples (mainly raccoons) have been typed TcIV (Roellig et al., 2008). Much remains to be understood about the evolutionary biology of the DTUs, particularly the North American TcIV, which has preliminarily been shown to differ from TcIV in Central and South America (Roellig et al., 2013; Zingales et al., 2012). In many respects, the ecoepidemiology of *T. cruzi* in the US has been relatively understudied in comparison to regions of Central and South America. Little is known about the strain types circulating in the US, and much stands to be gained from the combination of an ecological sampling approach and enhanced molecular biological understanding of circulating *T. cruzi* strains in Texas. Even the most extensive strain-typing study included only three samples from Texas (Roellig et al., 2008), despite Chagas disease being well-documented in bugs, dogs, and wildlife throughout the state (Burkholder et al., 1980; Kjos et al., 2008, 2009; Williams et al., 1977).

A number of untested hypotheses have been suggested to explain why the Chagas disease system is largely enzootic (i.e., parasite readily circulates among wild and domestic reservoirs and kissing bug vectors), but has yet to emerge as zoonotic (i.e. infect humans) in the US. For example, vector competence, behavior, and environmental preferences may result in less risk of human infection in the US when compared to the rest of the Americas (Klotz et al., 2009). Additionally, the genetic strains of *T. cruzi* found in wildlife populations in the US may not be as effective at establishing disease in humans as those in regions where human infection is endemic—previous studies found different strains were maintained in non-domestic and domestic populations of vectors and hosts (Miles et al., 2009). However, before researchers can begin to address such hypotheses, we must first establish an understanding of the basis of disease transmission in the US. In order to address this need, a combination of field, lab, and modeling methods were used to exam the ecological factors affecting the transmission of *T. cruzi* throughout the southern United States.

2. COMBINING PUBLIC HEALTH EDUCATION AND DISEASE ECOLOGY RESEARCH: USING CITIZEN SCIENCE TO ASSESS CHAGAS DISEASE ENTOMOLOGICAL RISK IN TEXAS[*]

2.1 Introduction

Citizen science—the engagement of non-scientists in collecting scientific data—has long been used in ecological and wildlife research (Bonney et al., 2014, 2009; Dickinson et al., 2010), resulting in an engaged public and providing researchers access to data from large geographic and temporal scales. We pose that citizen science is a powerful yet underutilized tool in public health, given that community engagement is recognized as a core component of many successful public health programs (*Principles of Community Engagement*, 2011).

Chagas disease is a vector-borne zoonotic disease caused by the parasitic protozoan Trypanosoma cruzi. Infection with *T. cruzi* can result in cardiac and digestive disease in humans and dogs that may not manifest until years after infection. Disease in humans is well-documented throughout the Americas (Bern et al., 2011; World Health Organization, 2015), and canine Chagas disease is well-documented in Texas (Kjos et al., 2008; Tenney et al., 2014). In 2013 and 2014, the first two years in which Chagas disease was a notifiable disease in Texas, a total of 351 canine cases and 39 human cases were reported; the latter including 12 locally-acquired cases (Texas Department of State Health Services, 2015a, 2015b). Colloquially referred to as 'kissing bugs', triatomine insects (Figure 2.1) are vectors of *Trypanosoma cruzi*. In the US, the highest species diversity of triatomines is found in Texas (Bern et al., 2011; Kjos et al., 2009). Triatomine bugs feed on blood during all stages of their lives, and may acquire the parasite from feeding on an infected mammal. The parasite *T. cruzi* is spread through the feces of the insect.

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[*] Reprinted from (Curtis-Robles et al., 2015), with permission from authors under Creative Commons license CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/legalcode). Minor grammatical and syntactical changes have been made.

Figure 2.1. Three species of kissing bugs commonly found in Texas. (Left to right) *Triatoma protracta*, the most common species in the western US; *Triatoma gerstaeckeri*, the most common species in Texas; *Triatoma sanguisuga*, the most commonly species in the eastern US. Scale bar represents 25mm, or approximately 1 inch.

Community-based vector surveillance has been widespread for decades as an approach to manage Chagas disease in South and Central America, through which householders monitor kissing bug presence within the home to allow for timely response with insecticide treatment. In these regions, some species of kissing bugs occupy a domestic niche (i.e., they successfully establish colonies in houses) (Zeledón and

Vargas, 1984). Diverse approaches have been employed in community-based vector surveillance programs, including the use of sensor boxes for passive detection of triatomines (Chuit et al., 1992) and training of community leaders in monitoring for reinfestation and insecticide spraying (Cardinal et al., 2007; Cecere et al., 2002). Community-based collections were found to be more sensitive than the gold standard of timed manual searches for triatomine recoveries (Dumonteil et al., 2009). A systematic review of Chagas disease vector control interventions across South and Central America concluded that community participatory surveillance significantly boosted vector detection probabilities above those found by vector control program staff using active searches or vector detection devices (Abad-Franch et al., 2011). Further, retrospective analyses of data from Argentina revealed that vector control strategies that incorporate community participation avert more human cases of disease and cost less than vertical or centralized strategies that consist of insecticide application by program staff only (Vazquez-Prokopec et al., 2009).

Community engagement has less commonly been used in the southern US for kissing bug research and control, likely because the vector species in the southern US tend not to colonize homes in the same manner as in Latin America, and insecticide spraying within the home is therefore not a widely used tool for public health protection. The first recruitment of the public in the US to help collect kissing bugs was in 1941, when Dr. Sherwin F. Wood of Los Angeles City College encouraged Arizona miners to collect insects from their sleeping quarters with the recruitment slogan 'Nab that bug at one cent each for Dr. Wood at City College to keep.' (Wood, 1942); this was followed by other similar efforts in the 1940s (Davis et al., 1943; Sullivan et al., 1949). Subsequently, community participation in kissing bug surveillance in Tucson, Arizona (Reisenman et al., 2012, 2010) and New Orleans, Louisiana (Moudy et al., 2014) has provided unprecedented information on vector phenology and infection in these regions. For public health and research purposes, the recruitment of submissions of kissing bugs from citizens who incidentally encounter them is an attractive option for collection given that kissing bugs are nocturnal, elusive, and difficult to collect using standardized

entomological traps (Kjos et al., 2013, 2009; Sjogren and Ryckman, 1966). Akin to the widespread community-based vector surveillance programs in areas where Chagas disease is endemic across Latin America, here, we describe the implementation and results from a two-year citizen science program in Texas that provides public health education and encourages citizens to aid in the collection of kissing bugs across Texas.

2.2 Materials and Methods

In early 2013, we developed a citizen science program for Chagas disease research with priority interest in Texas. Our program provides resources for people seeking information about Chagas disease and kissing bugs in the US, while also requesting kissing bug samples through a variety of media: printed pamphlets (Curtis-Robles et al., 2015), phone communication, an educational website (http://kissingbug.tamu.edu), solicitations on news stations, and a dedicated e-mail address (kissingbug@cvm.tamu.edu). The public may submit insect photos through the website or email to be identified by our team, and are invited to submit kissing bug specimens along with associated information. Submitters are informed about Chagas disease transmission, and cautioned to not touch the insects with bare hands. We request that the bugs be captured in bags and stored in a freezer, to kill the insect before shipping. The minimum requested dataset to accompany each bug includes: date, time and location of capture and whether the bug was alive or dead. Location data were validated and geo-coded in a geographic information system (ArcMap, ESRI, Redlands, CA).

In the laboratory, kissing bugs were identified to species (Lent and Wygodzinsky, 1979), measured, sexed, and dissected. Following DNA extraction (Omega Bio-tek, Norcross, GA; Qiagen, Germantown, MD), bug hindguts were tested for infection through amplification of *T. cruzi* satellite DNA quantitative real-time PCR that is selective for *T. cruzi* (Duffy et al., 2013) for which our internal validations defined a positive sample as one with a cycle threshold value of 33 or less. This qPCR is highly sensitive with a limit of detection that approximates 0.5 parasite equivalents of

DNA (Duffy et al., 2013). Bugs known to have fed on humans were sent to the Centers for Disease Control via Texas Department of State Health Services for testing so that submitters can be in immediate contact with those who can make medical recommendations. We provided submitters with the species identification and preliminary *T. cruzi* infection status of their submission, which is shared with a statement about potential false positive or false negative results. Our website includes an interactive map to allow submitters to see their data contributions and examine the spatial and temporal distribution of kissing bugs submitted by the public Texas. We provided citizens with information on reducing kissing bug occurrence in dog kennels or patios outside the home, as these were the primary areas from which bugs were collected. These recommendations included turning off the outdoor lights at night, housing dogs indoors when possible, reducing woody debris or other potential bug harborage sites within the vicinity of the home/kennel, and the use of commercially-available insecticides, although none available in the United States are labeled for the control of triatomines.

2.3 Results

From May 2013 through December 2014, we received approximately 4,000 emails that resulted in a total of 1,980 kissing bug submissions. The triatomines submitted to the program comprised at least seven species, of which *T. gerstaeckeri* and *T. sanguisuga* were most abundant (71.3 and 14.4% of submissions, respectively; Figure 2.1, Table 2). Locations from which triatomines were most commonly reported to be collected include dog kennels (24.6%), patios/porches (19%), and inside homes (10.8%), followed by other locations including outside walls of homes, garages, cat sleeping areas, inside buildings, barns, pools, tents, and chicken coops. Overall, 10.8% of triatomines representing 5 species were collected from inside homes (Table 2) and the majority of submissions of adults from inside homes consisted of a single bug that was encountered. As a proportion of the number of collections of each species, *T. rubida*, *T. lecticularia*, and *T. sanguisuga* were most commonly captured inside homes. Regarding

nymphs, 20 of the 56 (36.7%) nymphs submitted to our program were collected from inside the home (Table 2), including three submissions of more than one nymph (two submissions with two nymphs; one submission with five nymphs).

Over 99% of submissions were from Texas (1,968 kissing bugs), although we also received kissing bugs from Arizona, Florida, Louisiana, Oklahoma, and Virginia (Figure 2.2). In our initial program year in 2013, we received 881 kissing bugs submitted by 145 citizens. Our expanded program in 2014 resulted in the submission of 1,099 kissing bugs by 243 citizens, 13 of which had submitted bugs to our laboratory the preceding year. Whereas the majority of citizens submitted a single bug (200 individuals), many individuals submitted multiple bugs. There were 21 individuals who submitted 20 or more bugs over two years, including one individual who submitted 271 bugs. The majority of these large quantity submitters (90.5%) found bugs in the sleeping quarters of their dogs and expressed concerns about canine Chagas disease risk.

Table 2. Triatomine species. Proportion encountered inside homes, and *T. cruzi* infection prevalence in bugs submitted to the Texas citizen science kissing bug program, 2013-2014.

Species	No. submitted (% of total)	No. from inside house (% of species)	No. tested	No. *T. cruzi* positive (% of species)
Adults				
T. gerstaeckeri	1412 (71.3)	95 (6.7)	487	330 (67.8)
T. indictiva	108 (5.5)	15 (13.9)	40	17 (42.5)
T. lecticularia	49 (2.5)	12 (24.5)	20	15 (75)
T. neotomae	1 (0.1)	0	0	
T. protracta	2 (0.1)	0	0	
T. rubida	13 (0.7)	6 (46.2)	6	1 (16.7)
T. sanguisuga	286 (14.4)	65 (22.7)	120	70 (58.3)
Unknown[a]	53 (2.7)	1 (1.9)	6	3 (50)
Nymphs				
Unknown[a]	56 (2.8)	20 (35.7)	15	3 (20)
Total	1980	214 (10.8)	694	439 (63.3)

[a]Specimen could not be identified to species due to poor quality (smashed bug; missing key morphologic features) or nymphs (no key exists for nymphs)

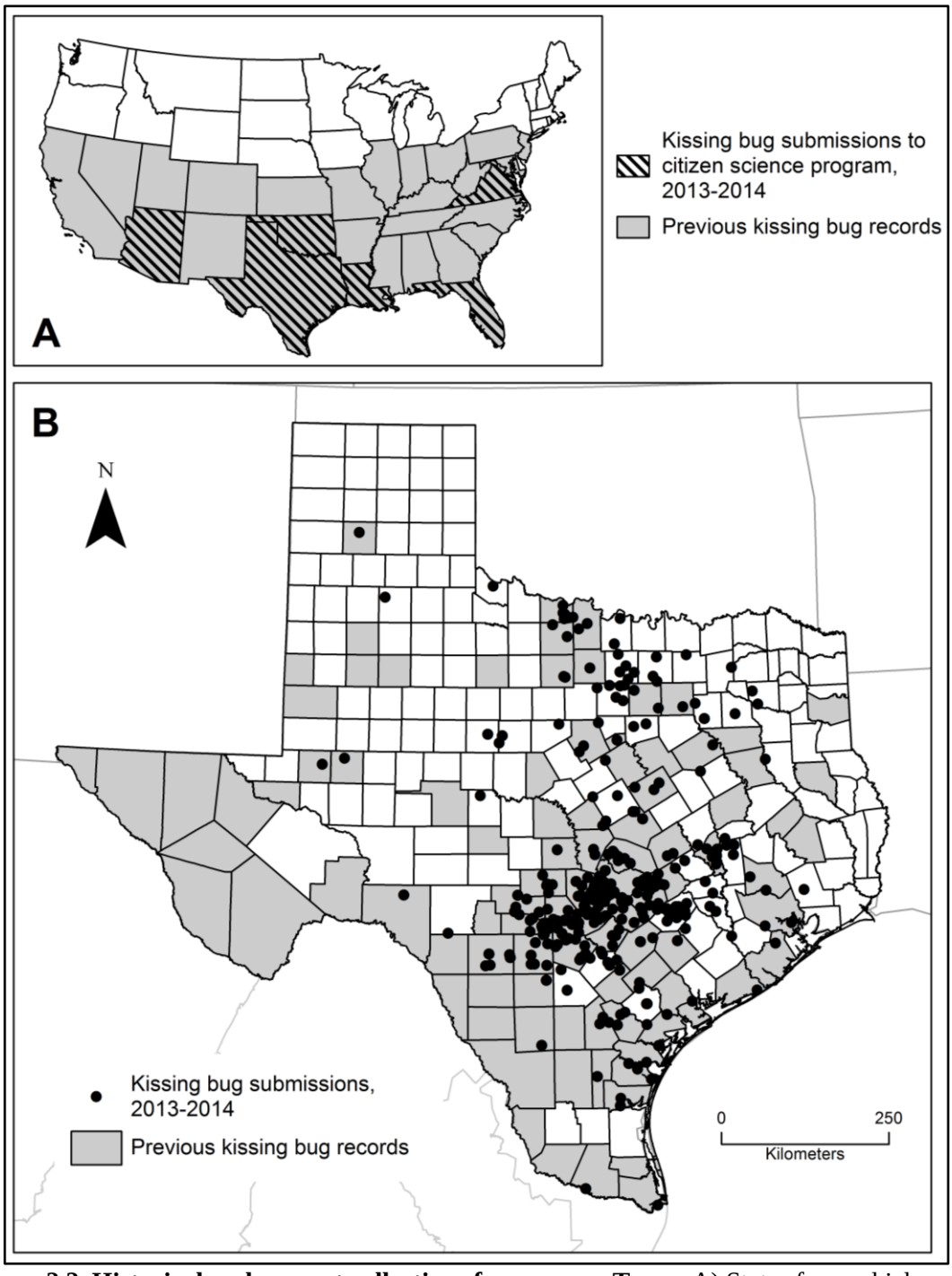


Figure 2.2. Historical and current collections from across Texas. A) States from which our program received kissing bugs in 2013-2014 overlaid on historical state-level records of kissing bugs throughout the US (Bern et al., 2011; Swanson, 2011); B) Historical county-level records of kissing bugs in Texas (1928-2006, as from (Kjos et al., 2009)) and submission of kissing bugs through our citizen science program (2013-2014).

Kissing bugs received by our laboratory were captured primarily throughout April-October, with the highest number of captures in June-July (Figure 2.3). The small number of kissing bugs (n=11) that were captured in the winter months (November-March) were mainly collected from indoors (63.6%) and were comprised of a higher percentage of nymphs (36.4%) than submissions throughout the summer months (26.3%). In the subset of 694 kissing bugs submitted through this program that we subjected to molecular detection of *T. cruzi*, we detected infection in 493 bugs (63.3%). In all counties from which at least two bugs were submitted, at least one infected bug was detected.

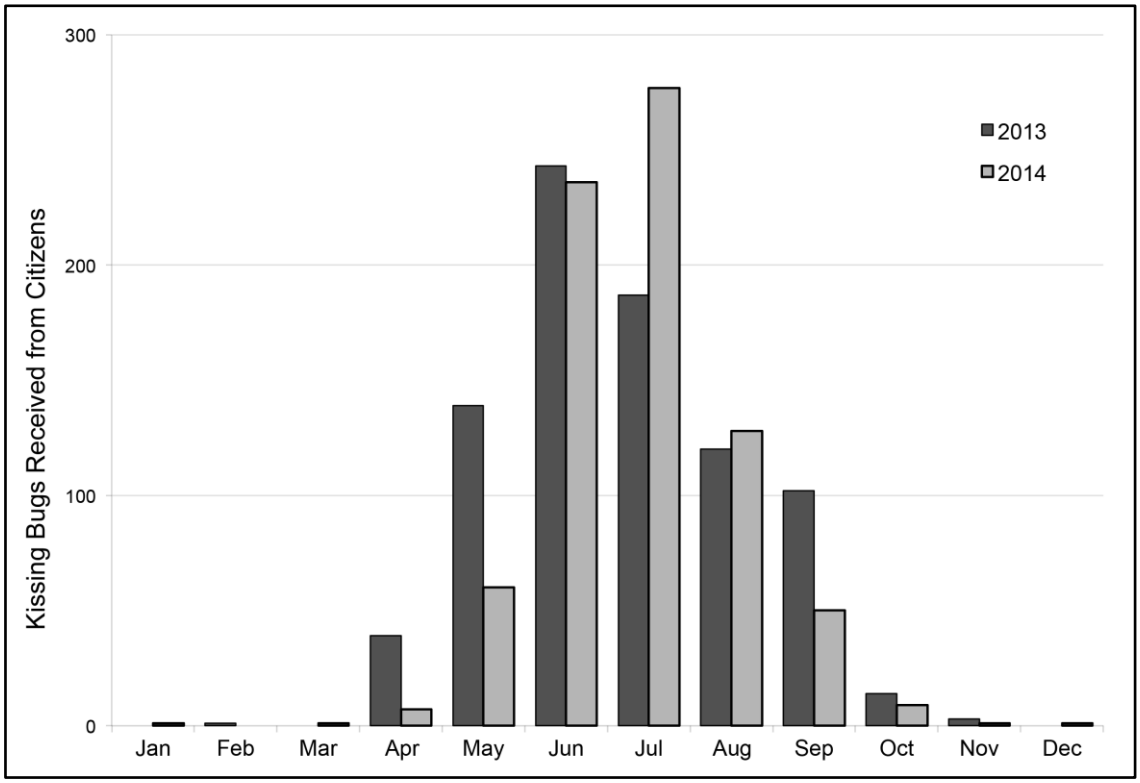


Figure 2.3. Kissing bug collection phenology. Seasonal occurrence of the collection of kissing bugs by citizens, 2013-2014.

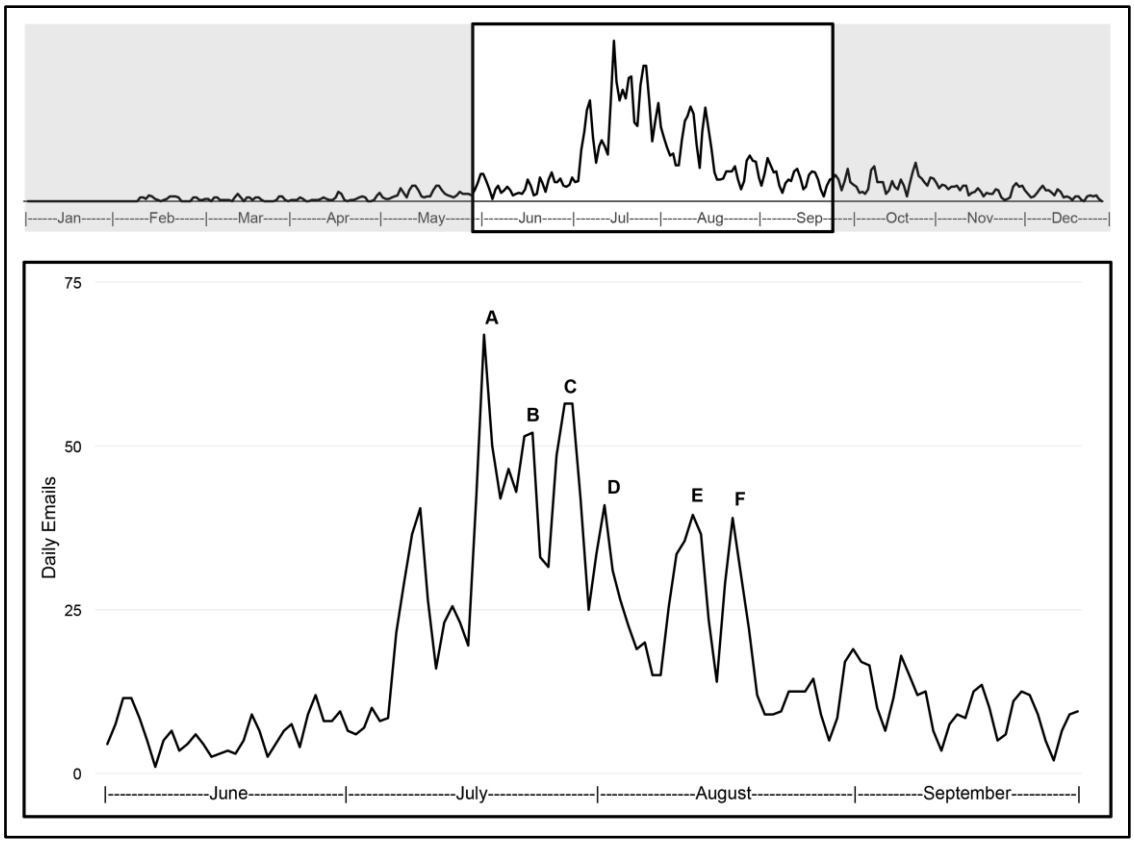


Figure 2.4. Email activity. Number of daily emails to kissingbug@cvm.tamu.edu from the public, 2014. Peaks generally correspond with a media event featuring Chagas disease and/or kissing bugs: A) July 17/18, Amarillo, Texas newscast and National Public Radio website article; B) July 24, articles about Chagas disease in Virginia; C) July 27, USA Today online article; D) August 2, Arkansas newscast; E) August 11, Cat Channel online article; F) August 18, Lubbock, Texas newscast. The regular pattern of decreasing and increasing activity (most noticeable throughout September) corresponds with weekends and weekdays, respectively.

Since establishing a dedicated email account in late 2013, emails regarding kissing bugs were more frequent in the summer months (June-August) than the rest of the year. Periods of exceptionally high email traffic were frequently associated with newscasts and releases of online media related to Chagas disease and kissing bugs (Figure 2.4). Captures of non-kissing bugs (mainly reduvvids and other hemipterans; Figure 2.5) represented approximately 10% of photo submissions to our program. We occasionally received emails from citizens concerned about a bug bite that may be from a triatomine, sometimes accompanied by photos of the bite site. Rarely, these citizens

also have collected a kissing bug. In all these cases, we put the citizen in contact with the local contact of the Texas Department of State Health Services who can investigate further and provide medical recommendations when warranted.

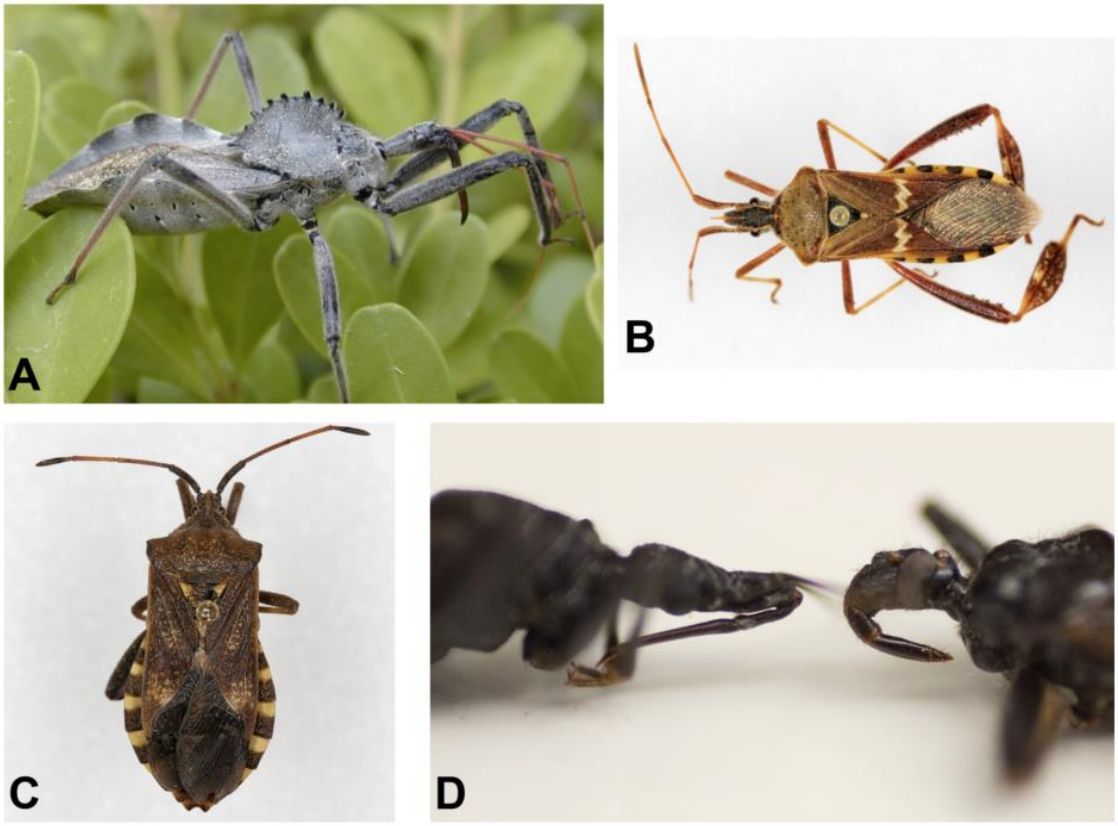


Figure 2.5. Key features of non-kissing bugs. Key morphologic features distinguish similar-looking insects: (A) Gray color and dorsal crest of wheel bugs (*Arilus cristatus*) (B) Wide, flattened back legs of leaf-footed bugs (*Leptoglossus sp.*) (C) Short head of squash bugs (*Mozena sp.*) (D) Close view of mouthparts of a kissing bug (left; thin and straight) and non-kissing bugs (right; tick and curved); Photos courtesy of M. Merchant (A), P. Porter (B, C) and R. Bardin (D).

2.4 Discussion

We used a citizen science approach to establish a collection of triatomine vectors nearing 2,000 specimens in order to define key periods of kissing bug activity, expand the county-level known range of kissing bugs in Texas, and ascertain infection

prevalence with *T. cruzi*. This method of sampling provides unique insight into the specific subset of bugs in nature that are epidemiologically relevant—that is, those bugs that people are encountering during daily activities and that potentially pose the highest risk for transmission of *T. cruzi* (Sjogren and Ryckman, 1966). Further, the educational campaign and community engagement at the core of this initiative allow people to take an active role in understanding how to improve their health.

This two-year citizen science program has revealed a similar geographic distribution of kissing bugs in Texas as was previously documented over almost eight decades (Figure 2.2). Our results highlight kissing bug occurrence in central and south Texas, which were predicted to be the highest Chagas disease risk zones in a statewide risk map (Sarkar et al., 2010) and further extend potential risk zones to include north Texas. Further, the expansive occurrence data from the citizen science initiative can provide unprecedented spatial resolution to complement the limited data used in a previous state-wide effort (108 kissing bugs from 63 unique spatial cells) (Sarkar et al., 2010). We received vector submissions from six of the seven focal areas across Texas from which *T. cruzi* seropositive dogs were recently detected (Tenney et al., 2014).

The peak in the collection of adult bugs occurred in June-July, with most activity occurring between April-October. While this apparent phenology certainly reflects periods of human outdoor activity given the collection method, it is congruent with the only other study of triatomine phenology in Texas which employed blacklight traps (a collection method that is independent of public outdoor activity) in a central Texas county in April-September to conclude that 83.4% of adult triatomines collections occurred in May-July (Pippin, 1970). Because vector activity is a key component of human risk, detailed phenology data are useful for public health initiatives.

The detected *T. cruzi* infection prevalence in citizen-submitted Texas kissing bugs was 63%, and is similar to that found in previous studies of kissing bugs from Texas that were collected using other means. For example, a sample of 241 bugs, including those collected from wildlife nests and by health department employees, was

characterized by 50.7% infection prevalence (Kjos et al., 2009), and 69-82% of bugs collected from houses and dog kennels in central Texas were infected (Kjos et al., 2013).

The analysis of citizen-collected data presents unique challenges due to observer error and sampling bias (Dickinson et al., 2010). For example, public submission programs result non-target bug species (Dias and Garcia, 1978; Yoshioka, 2013); however this potential source of observer error is controlled for in our program by laboratory identification of all kissing bugs. The geographic breadth of submissions reflects the area over which citizens are aware of the program and able to contribute to it. While areas from which no bugs were submitted cannot be interpreted as an absence of kissing bugs, the occurrence data are useful for increasing medical and veterinary awareness for Chagas disease over an expanded region.

The longitudinal pattern of inquiries from the public revealed that emails to our citizen science account peaked after media events (Figure 2.3); many of these emails concerned inaccurate information on television, internet, or social media. The most common cause of confusion resulted when pictures of common bug species that share some similarity in appearance to kissing bugs, but are not vectors of *T. cruzi*, were displayed while discussing Chagas disease on the news. Our data demonstrate the influence of the media for increasing awareness for the citizen science initiative to contribute to the growing field of digital epidemiology (Brownstein et al., 2014).

This citizen science program has resulted in strengthened relationships among university researchers, state health departments, the Centers for Disease Control and Prevention, clinical veterinarians, medical practitioners, and the general public. Such coordinated efforts among stakeholders—including the public—for insect surveillance offer opportunities for integrated pest management, research, and the protection of human health (e.g., The Collaborative Strategy on Bed Bugs (United States Environmental Protection Agency, 2015) and nuisance black fly reporting (Wilson et al., 2014)). The collection of samples generated through this program will be available for analyses of triatomine population genetics, blood meal analyses, genetic typing of *T. cruzi*, and additional research pursuits to enhance our understanding of vector ecology,

allowing us to further build upon state-wide and regional models of triatomine distributions and disease risk (Garza et al., 2014; Sarkar et al., 2010). Given the demonstrated public health benefit of community engagement in vector surveillance and control in areas of Chagas disease endemicity across Latin America, citizen science should be promoted as a key approach for enhancing vector-borne disease research and public health protection efforts in the United States.

3. PHENOLOGY AND SPATIAL PATTERNS OF TRIATOMINE VECTORS OF *TRYPANOSOMA CRUZI* IN TEXAS

3.1 Introduction

Triatomine insects (Reduviidae: Triatomine) are vectors of *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease in over 5.7 million people throughout the Americas (World Health Organization, 2015). Triatomines are obligatory hematophagous arthropods and become infected with *T. cruzi* when blood feeding on infected mammalian hosts. The parasite replicates in the gut of the insect, and the insect passes the parasite through fecal material. The study of these vectors has been key to public health initiatives aimed at reducing risk of Chagas disease.

Eleven species of triatomine insects have been recorded across the southern US, where they are colloquially known as 'kissing bugs' or 'conenose bugs' (Bern et al., 2011). Texas has the highest triatomine species richness of any state and is home to the most western limit of *Triatoma sanguisuga* ('eastern conenose bug'), the most eastern distribution of *T. protracta* ('western conenose bug'), and the most northern distribution of *T. gerstaeckeri* (Bern et al., 2011; Lent and Wygodzinsky, 1979). In contrast to areas of Central and South America where domestic and peridomestic populations of triatomines exist, the species in the US are almost exclusively sylvatic (Bern et al., 2011; Kribs-Zaleta, 2010). Nymphs are rarely documented in human domiciles (Wozniak et al., 2015), and the triatomines found in peridomestic and domestic settings are mainly dispersing adults. Although human contact with such dispersing adults poses potential risk of spill-over transmission of *T. cruzi* to humans, studies of triatomine dispersal phenology in the US are limited (Ekkens, 1981; Pippin, 1970; Reisenman et al., 2012; Wozniak et al., 2015).

Historical records of kissing bugs show distributions of triatomine species in 29 US states (Bern et al., 2011; Swanson, 2011); however, occurrence maps do not reflect the complexity of unique species distributions, particularly in areas where there is high species diversity, like central/southwestern Texas (Bern et al., 2011; Kjos et al., 2009).

Species distribution modeling efforts of US triatomines are frequently limited by small sample sizes from broad geographic areas (Garza et al., 2014; Sarkar et al., 2010). Since infection prevalence (Curtis-Robles et al., 2015) and host associations (Ibarra-Cerdeña et al., 2009) are known to vary among triatomine species, risk of parasite transmission to humans and domestic animals likely varies according to the triatomines found in a local area. A more detailed understanding of unique occurrences of individual triatomine species in Texas is needed as a basis for robust studies analyzing habitat suitability and the potential for range expansion due to climate change. The objective of this study was to determine temporal variation of triatomine activity and define contemporary and unique distributions of *Triatoma* spp. across Texas.

3.2 Materials and Methods

3.2.1 Specimen collection

From December 2012 to December 2015, we acquired kissing bug specimens using a citizen science program as previously reported (Curtis-Robles et al., 2015), and standard entomological trapping techniques. The collection techniques employed by members of our research group included black lights and mercury vapor lights, dry ice baited white sheets, manual searching with lights at night, and active search/destructive sampling of woodrat (*Neotoma* spp.) nests and other nidicolous habitats as described by others (Eads et al., 1963; Ekkens, 1984; Kjos et al., 2013; McPhatter et al., 2012). In addition, stand-alone traps were set for overnight captures, including a standard universal black light trap (Product #2851A, BioQuip Products, Rancho Dominuez, CA, USA) and the MegaCatch ULTRA Mosquito Trap (EnviroSafe Technologies International Limited, Albany, Auckland, NZ). The MegaCatch ULTRA included an octenol-based pheromone attractant. Additional captures were attempted using yeast-baited traps consisting of a baker's yeast/water chamber set on a sticky trap and partially covered with an inverted container, similar to descriptions from previous studies (Guerenstein et al., 1995; Lorenzo et al., 1999).

3.2.2 Laboratory processing

Adult kissing bugs were morphologically identified to species (Lent and Wygodzinsky, 1979), sexed, and measured. To mitigate risk of exogenous DNA contamination from the external exoskeleton, bugs were soaked in 50% bleach for 15 seconds and then rinsed in distilled water (Graham et al., 2012). Then hindguts were dissected using sterile forceps and dissecting scissors. The relative amount of blood was scored (1=no blood, desiccated guts; 2=no blood, guts visible; 3=traces of blood in gut; 4=blood present, but not much or not fresh [dried]; 5=large amount of fresh blood).

A subset of bugs was unable to be identified using morphological characteristics, which included nymphs, incomplete or damaged specimens, and those with aberrant morphological characteristics. These specimens were subjected to molecular determination of species. DNA from leg or hindgut tissue was extracted using the Omega E.Z.N.A Tissue DNA kit (Omega Bio-Tek, Norcross, GA), and PCR amplification of the mitochondrial *cytochrome b* gene (Pfeiler et al., 2006). *Cytochrome b* PCR reactions consisted of 1uL DNA, 0.6 μL of each 10 μM primer, and FailSafe PCR Enzyme Mix in PreMix E (Epicentre, Madison, WI, USA) in a total volume of 15μL. PCR amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide. Target amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and bi-directionally sequenced (Eton Bioscience, Inc., San Diego, CA, USA). Sequences were visually inspected for quality in Geneious version 8 (http://www.geneious.com) (Kearse et al., 2012); unknown species identifications were inferred based on alignment with specimens of known species in a boot-strap consensus phylogenetic tree using 1000 replicates and the neighbor-joining method using MEGA5 software (Tamura et al., 2011).

3.2.3 Spatial modeling

Locations of citizen submitted specimens were geocoded and vetted for accuracy and precision using GoogleEarth. ArcMap 10.1 (ESRI, Redlands, CA, USA) was used to create distribution maps of locations of *Triatoma* spp.

Spatial point pattern analysis was completed in Program R (R Development Core Team, 2008). The Kelsall and Diggle method of analysis (Kelsall and Diggle, 1995; Mabud et al., 2014) was used to determine locations of significant occurrence for individual *Triatoma* species by comparing expected density ratios of each 'species of interest' (i.e., *T. gerstaeckeri*, *T. sanguisuga*, *T. indictiva*, or *T. lecticularia*) to all the other species in the database ('non-interest *Triatoma* spp.'). Kernel density estimates for collection locations of the species of interest were calculated along a two-dimensional grid within Texas. Kernel density estimates for the collection locations of non-interest species were calculated along the same grid, and the ratio of the species of interest and non-interest species estimates was calculated at each grid point. The natural log of the ratio at each point was used as the "observed data set." A permutation test was used to assess significance of the observed data set. The collection locations of all species were randomly assigned to 'species of interest' or 'non-species of interest' categories, and kernel densities, ratios, and natural logs were computed as above. This was repeated for 1,000 simulations, and the observed data set for the species of interest was compared to the permutated datasets. Observed data set calculations greater than 99.95% of the permutated data were classified as significant, indicating higher than expected observations of the species of interest compared to the non-interest species. Observed data set calculations lower than 0.05% were also classified as significant, indicating lower than expected observations of the species of interest. Areas with less than 0.0005 probability of finding both a species of interest and a non-interest species were classified as not having enough information to perform a calculation.

3.3 Results

3.3.1 Specimen collection

From July 2012 to December 2015, we collected 2638 kissing bug specimens. The majority (2318) were obtained through citizen collections, and we collected 320 bugs in the field using multiple collection techniques. Specimens came from more than 465 unique locations although some bugs submitted by citizens did not include exact

locations. Most of the bugs were from Texas (2574) but additional bugs were submitted from Arizona (43), Florida (7), Virginia (8), California (3), Louisiana (2), and Oklahoma (1) (Table 3.1). All of our field-collected bugs were captured in Texas; citizen scientists captured all of the non-Texas bugs.

Table 3.1. Triatomine specimens collected from multiple states, 2012-2015. *Triatoma* spp. were identified using morphological characteristics. *Specimen morphological identification was not possible (missing key morphological features) **No morphological key for nymphs exists

	TX	AZ	FL	VA	CA	LA	OK	Total
T. gerstaeckeri	1894							1894
T. sanguisuga	336		7	7		2	1	353
T. indictiva	119							119
T. lecticularia	76							76
T. rubida	6	33						39
T. protracta	7	8			3			18
T. neotomae	3							3
Unknown (adults)*	46							46
Unknown (nymphs)**	87	2		1				90
Total	2574	43	7	8	3	2	1	2638

Of the 320 bugs that were collected by our team, 219 were alive at time of capture, and 208 were categorized by method of search (Table 3.2). Despite attempts over multiple nights in different areas using stand-alone collection traps with black and/or mercury vapor lights, added carbon dioxide attractants, and synthetic pheromone attractants, no triatomines were captured in such unattended devices. Capture success varied by collection technique. At one south Texas site in June 2013, a team of six people collected 86 *T. gerstaeckeri* and 2 *T. neotomae* in a matter of hours; these collections were made in an area under a high, bright security-type light, in an open area surrounded by dense brushy vegetation. In other areas across Texas, there were also at least 34 instances of bugs collected after being attracted towards a person or other carbon dioxide source, including one on a sunny afternoon. Thirty-two of the 34 were *T.*

gerstaeckeri, one was *T. indictiva*, and one was a nymph. There were two instances of *T. sanguisuga* flying and landing on a collector, as well as one *T. protracta* discovered on the vest of a collector at night. Four bugs were collected during early morning hours from the outside of the tent where we camped. Bugs were most commonly collected from the immediate vicinities of dog kennels and dog sleeping areas; 1275 bugs (48.3%) collected were found in association with canine environments.

Table 3.2. Average bugs per collection method. Triatomines collected live by members of our research team (219) were categorized by capture method, total amount of effort was estimated, and an estimated number of bugs resulting per dedicated man hour was calculated. Eleven bugs did not have sufficient collection information to categorize. *These bugs were collected from caves, as by-catch of baiting Argasidae ticks.

Time	Method	Total bugs	Unit Effort (man hours)	Number of Efforts	Total Effort (man hrs)	Average bugs/ man hr
Day						
	Active/destructive searching of nidicolous habitats	17	1.5	3	4.5	3.7
	Active searching around a dog kennel	14	0.5	5	2.5	5.6
	Attracted to lab members (no light)	7	0.5	5	2.5	2.8
	Active searching around a residential building	1	0.5	1	0.5	2
	Day total	*39*			*10*	*3.9*
Night						
	Attracted to carbon dioxide produced by dry ice (no lights)*	9	0.5	4	2.0	4.5
	Black light, carbon dioxide, white sheet, and monitoring	24	1	11	11.0	2.2
	Active searching around a lighted residential building	118	3	13	39.0	3
	Attracted to lab members (no light)	18	3.5	2	7.0	2.6
	Night total	*169*			*59.0*	*2.9*
Total		*208*			*69.0*	*3.0*

3.3.2 Characterization of specimens

A total of 2338 bugs were scored for blood meal; 1056 (45.2%) had evidence of blood in the gut (score of 3-5), with 647 having a recent blood meal (score of 4-5). Of 2082 bugs received from citizen submitters and scored, 938 (45.1%) had evidence of blood in the gut (scores 3-5), with 594 having a recent blood meal (score 4-5). Of 256 bugs collected by our research team and scored, 138 (53.9%) were starved (blood meal score of 1 or 2), 118 (46%) had evidence of blood in the gut (scores of 3-5), including 53 with evidence of a recent blood meal (score of 4-5). Bugs with evidence of recent blood meal had mainly been found associated with dog living/sleeping area or in a wildlife nest (42 of 53, 79.2%). Of the subset of 34 triatomines captured by our team while the bugs were approaching a person or carbon dioxide source (without light) (Table 3.2), 22 (64.7%) were starved (score of 1 or 2), 12 (35.3%) had evidence of blood in the gut (scores 3-5), but only one (2.9%) bug had evidence of a recent blood meal (score of 4).

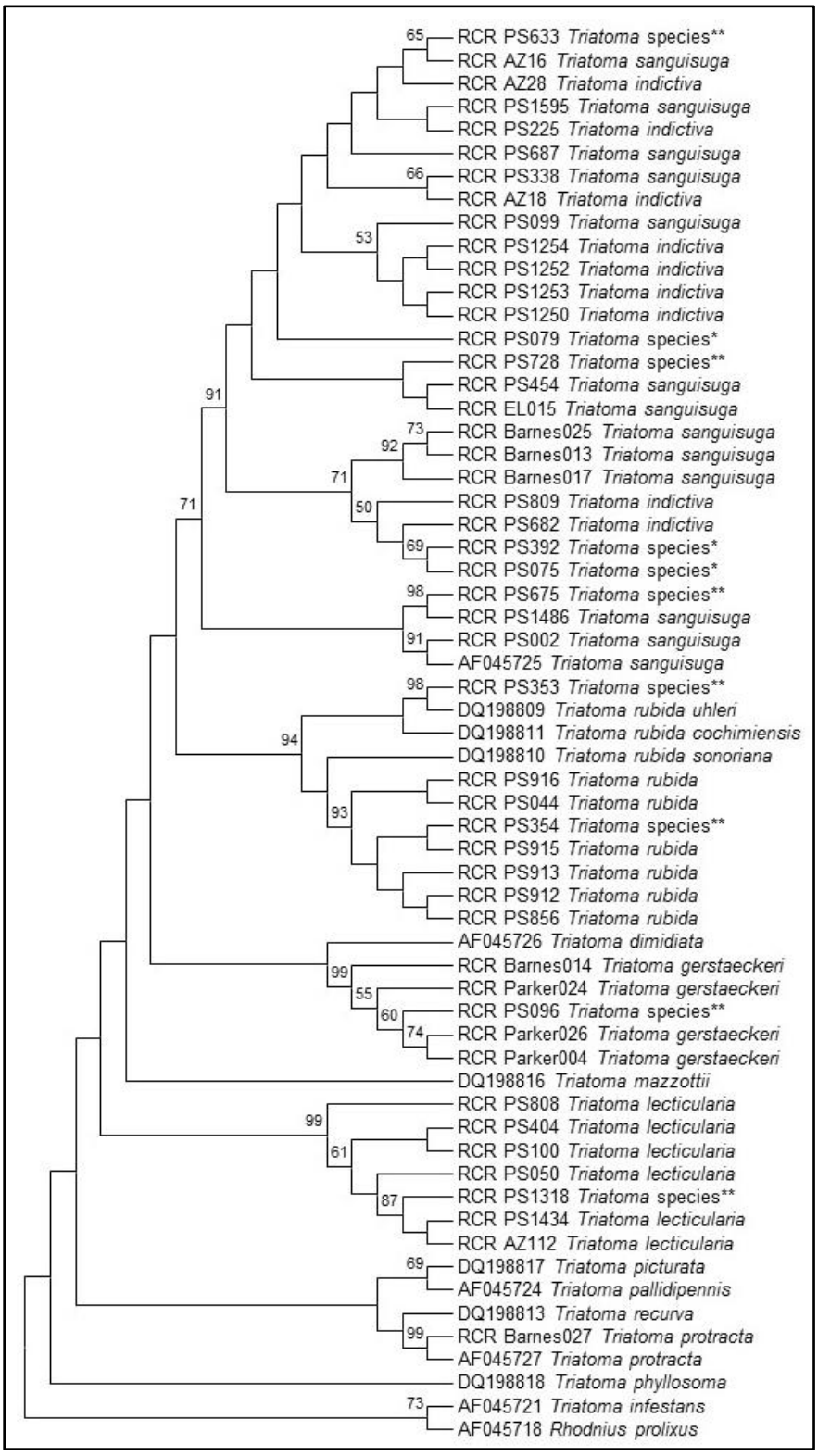
Collections consisted of more females than males for *T. gerstaeckeri*, *T. sanguisuga*, and *T. indictiva*, but fewer females than males of *T. lecticularia* were collected (Table 3.3). *T. gerstaeckeri* was the largest size species included in the collection. Sexes of nymphs were not determined. A total of 90 nymphs were collected, of which 74 were measured, with a mean length of 15.8 ± 5.7mm. Specimens measuring outside of the range of expected lengths (Lent and Wygodzinsky, 1979) were rare.

Table 3.3. Sex and length distributions of triatomines. *Triatoma* spp. proportions of males and females, and lengths in millimeters with standard deviations are shown. Percentages of females, males, and unknowns are calculated out of the total number of each species collected. The lengths of females included ovipositors.

	Females			Males			Unknown sex	Total
Species	Total no.	No. measured	Mean Length ± SD	Total no.	No. measured	Mean Length ± SD	Total no.	
T. gerstaeckeri	1079 (57.0%)	908	27.7 ± 1.68	777 (41.0%)	652	26.1 ± 1.50	38 (2.0%)	1894
T. sanguisuga	219 (62.0%)	197	22.2 ± 1.65	125 (35.4%)	106	20.9 ± 1.63	9 (2.6%)	353
T. indictiva	71 (59.7%)	70	21.3 ± 1.14	42 (35.3%)	40	20.1 ± 1.16	6 (5.0%)	119
T. lecticularia	36 (47.4%)	34	20.5 ± 1.48	39 (51.3%)	32	19.5 ± 1.65	1 (1.3%)	76
T. rubida	29 (74.4%)	28	21.5 ± 0.84	9 (23.1%)	9	18.1 ± 1.36	1 (2.6%)	39
T. protracta	8 (44.4%)	7	17.9 ± 1.35	10 (55.6%)	9	15.6 ± 0.88	-	18
T. neotomae	-	-	-	2 (66.7%)	1	16	1 (33.3%)	3
nymph	-	-	-	-	-	-	90 (100%)	90
Total	1442	-	-	1004	-	-	146	2592

Figure 3.1. Evolutionary relationships among *cytochrome b* sequences. Sequences from field samples (with RCR prefix) and existing sequences (with GenBank accession numbers) were used to construct a phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method, and the optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches; only those with values of greater than 50% are displayed. Evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The analysis involved 61 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 197 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). *adult specimen **nymph specimen

Figure 3.1 Continued.



3.3.3 Species identification using genetic markers

Morphological identification of 48 specimens was confirmed by amplification and sequencing of the *cytochrome B* gene (Figure 3.1). The consensus tree revealed distinct clades for all included species, except for *T. sanguisuga* and *T. indictiva*, which appeared indistinguishable based on this genetic region.

3.3.4 Adult dispersal

Adult triatomine specimens noted as alive at time of collection by citizen scientists were considered to likely be dispersing (Schweigmann et al., 1988; Sjogren and Ryckman, 1966). The number of bugs collected alive per week varied by species (Figure 3.2). Collections of *T. gerstaeckeri* were mainly from early May to late September, peaking in June, while collections of *T. sanguisuga* were mainly from mid-June to early October, peaking from August-September (Figure 3.3). Although *T. indictiva* and *T. lecticularia* were collected throughout April – October, there were insufficient collections of live specimens to determine peak activity periods.

3.3.5 Geographic distribution and spatial analyses

Specimens were collected from 117 counties in Texas, 4 counties in each Virginia and Florida, 2 counties in each California, Louisiana, Arizona, and 1 county in Oklahoma.

The Kelsall and Diggle method of analysis was used to determine locations of significant occurrence in Texas for each *Triatoma* species by comparing expected density ratios of the species of interest to all the other species ("non-interest species"). The spatial analysis was performed for *T. gerstaeckeri* (n=1437), *T. sanguisuga* (n=246), *T. lecticularia* (n=46), and *T. indictiva* (n=106). The resulting maps (Figure 3.4) indicate areas of higher expected encounters in west Texas for *T. gerstaeckeri*, in northeast Texas for *T. sanguisuga*, in north central Texas for *T. lecticularia*, and the northwest Texas for *T. indictiva*.

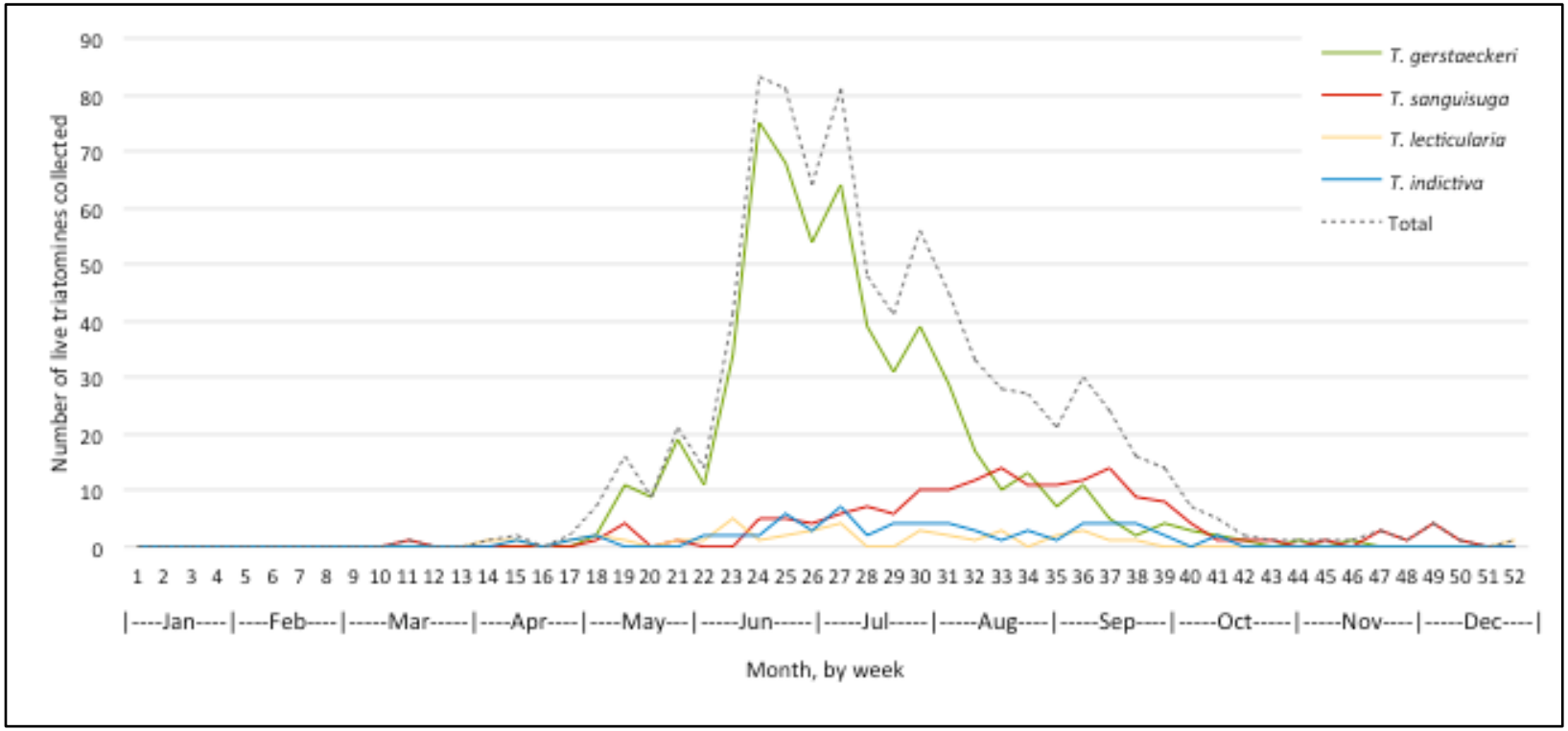


Figure 3.2. Phenology of collection of live triatomine species. Seasonal occurrence of four species of triatomines collected alive by citizen scientists in Texas, 2013-2015. Specimens collected prior to May and after October were mainly found indoors, or in animal housing (dog house, guinea fowl coop), where temperatures allowed for bugs to be active.

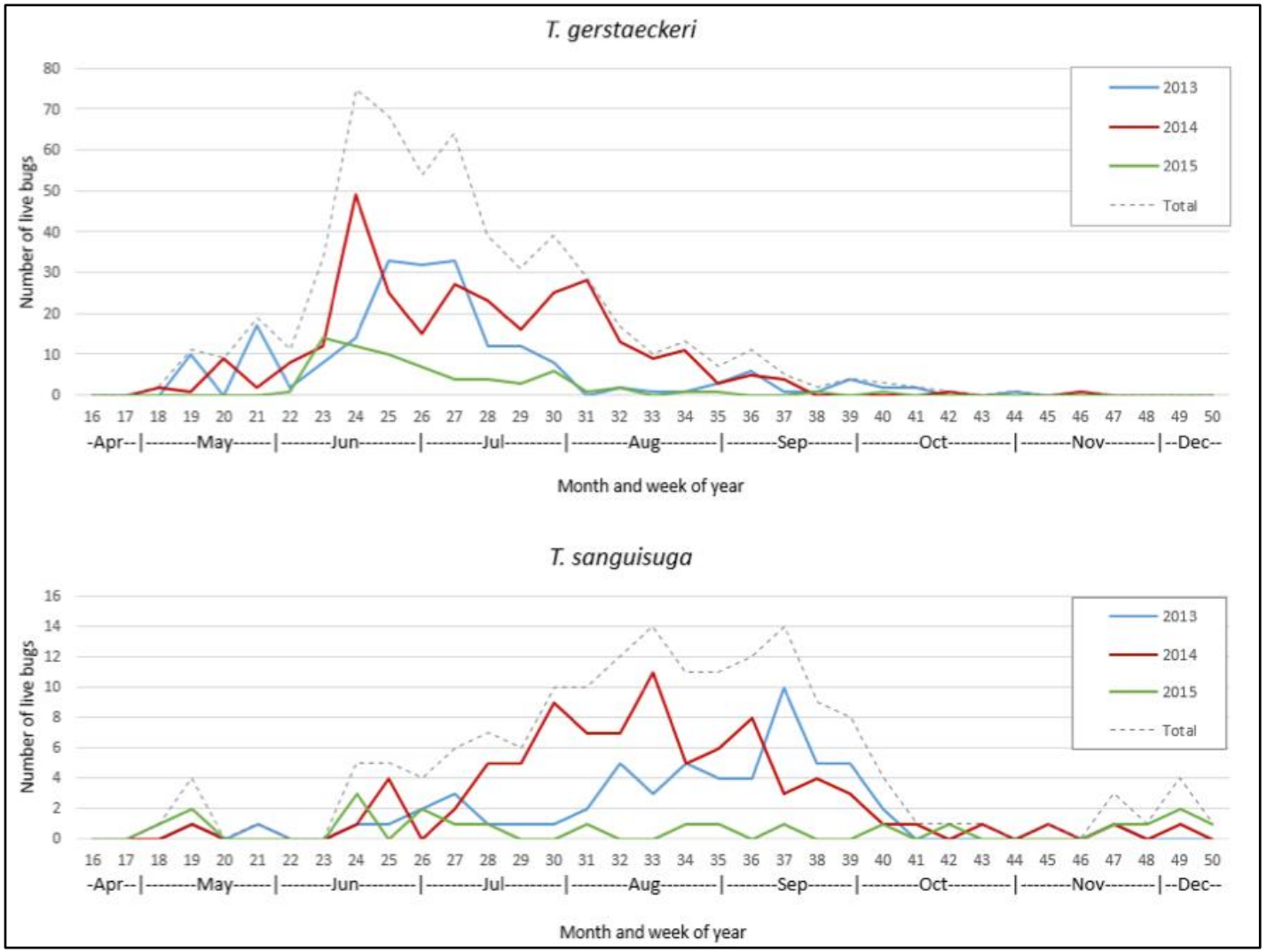


Figure 3.3. Yearly phenology of collection of live triatomine species from Texas. *T. gerstaeckeri* and *T. sanguisuga* were collected alive by citizen scientists in Texas, 2013-2015, with a focus on the season of main activity.

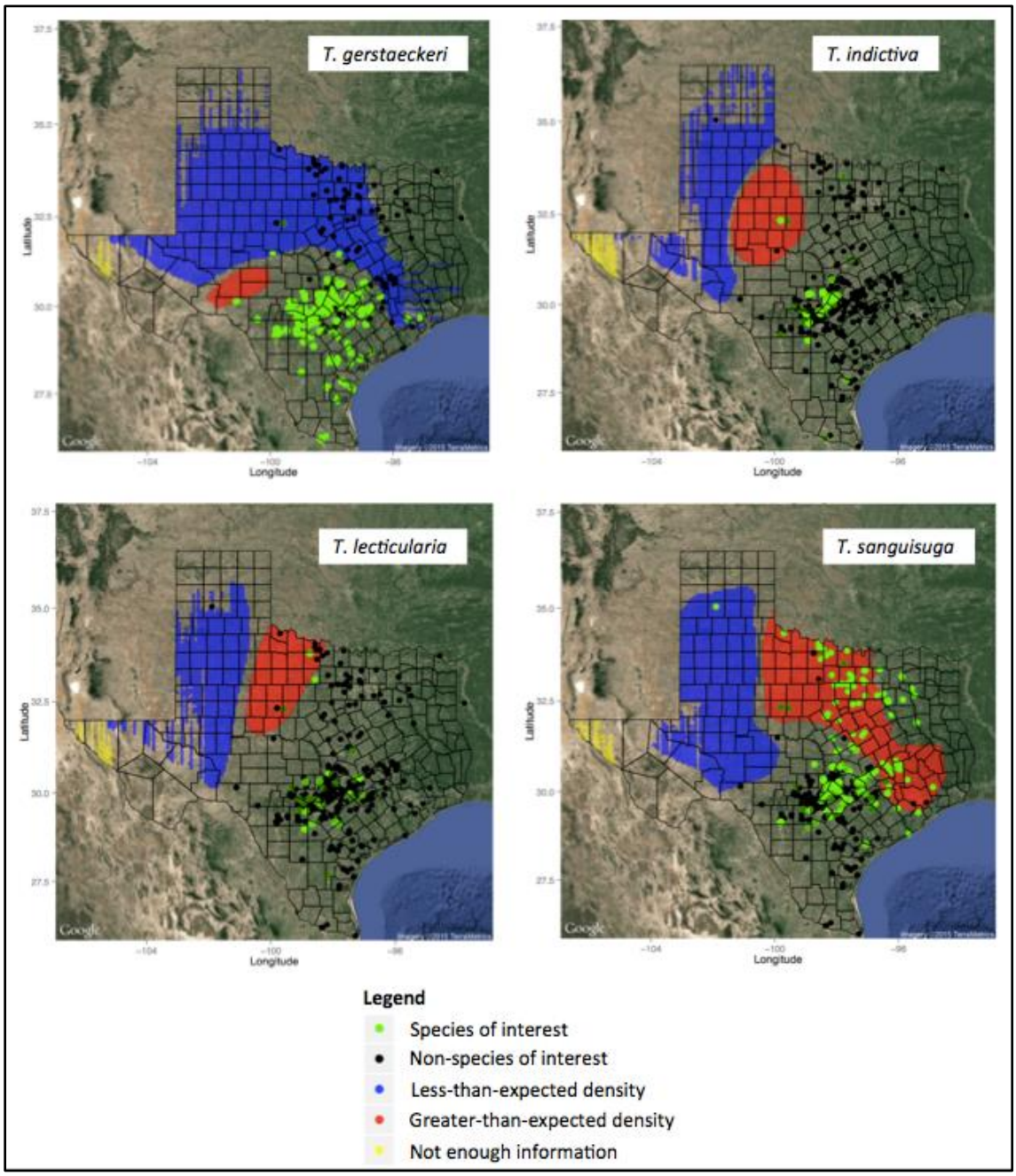


Figure 3.4. Maps indicating areas of relatively unique distributions. Higher than expected densities of *Triatoma* spp. in Texas are highlighted in red, lower than expected densities are highlighted in blue, and zones lacking sufficient information for calculations are highlighted in yellow.

3.4 Discussion

Although Chagas disease is increasingly recognized as a disease of human and veterinary significance in the southern US, there have been relatively few studies of the phenology and spatial patterns of triatomine vectors in the US. We established a collection of 2638 bugs from 7 states to examine unique phenological and spatial patterns.

Triatomines collected were predominantly *T. gerstaeckeri* (71.7%), with *T. gerstaeckeri* and three other species—*T. indictiva*, *T. lecticularia*, and *T. sanguisuga*—comprising 92.6% of all collected specimens. These four most commonly encountered species are consistent with other studies in Texas (Kjos et al., 2009; Sullivan et al., 1949; Wozniak et al., 2015). The preponderance of *T. gerstaeckeri* may not necessarily reflect a higher relative density of this species in nature, since citizen collected bugs were usually encountered in peridomestic habitats, where *T. gerstaeckeri* are known to be found (Wozniak et al., 2015). As noted by others (Miles et al., 2009), the relatively low burden of human Chagas disease in the US is partially due to vectors unlikely to colonize human residences. The majority of US species of triatomines appear to be mainly sylvatic and are rarely reported as having established populations in domestic settings (Wozniak et al., 2015). However, many of the bugs in the current study were encountered in or near houses or kennels and represent a risk of spill-over transmission of *T. cruzi* to humans and domestic animals. Therefore, although triatomine egg laying, nymphal development, and infection with *T. cruzi* may mainly occur in the sylvatic environment in the US, dispersing adult triatomines found in the peridomestic setting are an important risk to human/animal health. Although *T. gerstaeckeri* are frequently encountered in peridomestic settings, rare findings of nymphs suggests that these bugs are dispersing adults, rather than bugs with established life cycles in the domestic setting. However, it is also possible that adult bugs are more easily noticed and submitted by citizen scientists, compared to smaller, less conspicuous nymphs. *T. sanguisuga* nymphs have been described as “especially shy in the nymphal stages”

(Pippin, 1970). Rigorous field studies of host associations are needed in order to understand the sylvatic sources of US triatomines.

Differences of behavior by sex in US triatomines have not been well studied, and few prior studies of US triatomines document sex ratios of collections. In one study, *T. rubida* females were likely to defecate during a blood meal, though males were not (Reisenman et al., 2011), which would affect risk of *T. cruzi* transmission to the host. Although no studies of effect of sex on flighted dispersal of US triatomine species have been done, one dispersal study of the South American triatomine *Triatoma infestans* found more females than males flew when released, and that females likely flew farther than males (Schofield et al., 1992). For almost all species included in this study, more females were collected than males. One study of dispersing *T. gerstaeckeri* attracted to light traps in central Texas found a higher female:male ratio at one site, but lower ratio at another site (Pippin, 1970). A study of dispersing *T. protracta* in California captured more males than females at light traps (Sjogren and Ryckman, 1966), and a study of non-dispersing *T. sanguisuga* collected from a residence in Louisiana found approximately equal numbers of male and female bugs (Cesa et al., 2011).

We found distinctive periods of peak activity for *T. gerstaeckeri* and *T. sanguisuga*. *T. sanguisuga* activity peaked from August-September, and *T. gerstaeckeri* was more active during May-August. This is in agreement with previous findings of activity from May-August (Pippin, 1970; Wozniak et al., 2015). Although limited, systematic collections have documented potential climatic and biological variables that induce flight dispersal of adult triatomines in the southern US. Low nutritional status, lack of nearby blood meal sources, and temperature have been proposed as the main drivers of dispersal for *T. protracta* (Sjogren and Ryckman, 1966), although similar studies have not yet been done for other US species. Relative to the more northern distribution of *T. sanguisuga* in Texas, it might also be possible that the more southern distribution of *T. gerstaeckeri* would have earlier warmer temperatures that would induce flighted dispersal (Sjogren and Ryckman, 1966). One prior phenology study found that *T. rubida* primarily overwinter as 5th instar nymphs, with males molting to

dispersing adults earlier than females (Ekkens, 1984). The well-studied *Neotoma* spp. rodent/*T. protracta* relationship led Sjogren and Ryckman (Sjogren and Ryckman, 1966) to hypothesize that death of a blood source would result in nutritional deprivation and subsequent dispersal of triatomines. Little is known about the natural hosts of *T. gerstaeckeri* and *T. sanguisuga*; perhaps they have similarly unique natural histories that lead to distinct dispersal timings. Many of the adult specimens collected had evidence of recent blood meals; however, it is likely they had fed after a dispersal event, since they were found in environments near dogs and sources of blood. Triatomines captured by us while approaching a potential blood meal were often starved (64.7%, 22 of 34). Others have also found that dispersing adults attracted to traps are more likely to be starved (Pippin, 1970; Sjogren and Ryckman, 1966), suggesting that need for a blood meal is a main driver of flighted dispersal of adult triatomines in the US. Understanding local factors affecting dispersal, including habitat modification and subsequent dispersal, needs further study.

When comparing the phenology and total submissions across the three years of the study, we noted an overall lower number of triatomine submissions in 2015 compared to the previous two seasons (Figure 3.3). Although the passive sampling technique of citizen science submissions does not allow for conclusions regarding population dynamics, the collection efforts by members of our research team and other scientists with experience collecting kissing bugs converged on the observation that fewer bug sighting occurred in 2015 relative to the two prior years. Studies in other disease systems have shown that temperature and precipitation can influence vector abundance and risk of disease transmission (Karki et al., 2016; Shand et al., 2016). Factors regulating population sizes and kissing bug activity are poorly understood, and these observations warrant future studies.

The use of molecular methods for triatomine species barcoding is valuable, particularly for low-quality specimens and nymphs. The sequences generated using the mitochondrial cytochrome b target were able to differentiate all species in Texas, except for *T. sanguisuga* and *T. indictiva*. This genetic similarity of these two species is

consistent with a previous report examining the same genetic region (Kjos et al., 2013). There has been conflicting classification of *T. indictiva*, with some including it as a subspecies of *T. sanguisuga* (Usinger, 1944), and others separating it as its own species (Lent and Wygodzinsky, 1979). Additionally, *T. sanguisuga* is also divided into subspecies by Usinger (Usinger, 1944), but not by Lent and Wygodznisky (Lent and Wygodzinsky, 1979). In this study, *T. indictiva* was found in a more focal region than *T. sanguisuga* (Figure 3.4), although *T. indictiva* has also been recorded throughout additional southwestern states (Lent and Wygodzinsky, 1979). A study of *T. sanguisuga* collected from a focal location in Louisiana revealed distinct groups of the triatomine species based on two genetic regions (de la Rua et al., 2011). More investigation—including targeting of other genetic markers, field studies of host preferences, laboratory studies of cross-breeding success—is warranted regarding the classification of *T. indictiva* and *T. sanguisuga*.

Our investigation of spatial distribution revealed unique occurrences of different triatomine species across Texas. Point pattern analyses were specifically used to compare the collection localities of *T. gerstaeckeri*, *T. sanguisuga*, *T. indictiva*, and *T. lecticularia*. Each individual species of interest was compared to the collective locations of the three other species, revealing locations where the species of interest was more likely to be encountered than the other three species. While the unique occurrences in north and east Texas were expected of *T. sanguisuga* (the 'eastern cone nose bug'), we also identified potentially unique occurrence locations of *T. lecticularia* and *T. indictiva* in an area just southeast of the Texas panhandle, as well as a unique occurrence for *T. gerstaeckeri* in west Texas. A limitation of the dataset is non-uniform citizen science sampling, and several regions of Texas had very few submissions, although triatomines are known to occur in those regions (Buhaya et al., 2015; Kjos et al., 2009). The areas with few submissions in west and south Texas are likely due to low human population densities and/or lack of outreach to those areas. Our current analysis attempted to compensate for the regions of low submissions by including calculations of areas for which there were insufficient data. Future research could target citizens living in those

areas to increase submissions. The data generated in this modeling exercise could be used to improve the specificity of models attempting to delineate species distributions and expansions (Garza et al., 2014; Sarkar et al., 2010). This study demonstrates widespread occurrence of triatomine bugs in Texas with unique spatial occurrences of different species. Further investigation of these areas should include habitat suitability modeling efforts to determine specific environmental factors that affect the distribution of US triatomine species.

4. SPATIAL DISTRIBUTION OF *TRYPANOSOMA CRUZI* INFECTION AND DISCRETE TYPING UNITS IN TRIATOMINE VECTORS ACROSS THE SOUTHERN US

4.1 Introduction

Throughout the Americas, the protozoan parasite *Trypanosoma cruzi* is responsible for an estimated burden of Chagas disease exceeding 5.7 million people (World Health Organization, 2015). Transmission is primarily through the infective feces of triatomine insects, though transmission can also occur congenitally, through organ transplant and blood transfusion, and through consumption of contaminated food and drink (Bern et al., 2011). Most people are diagnosed during the chronic stage of disease, which is characterized by parasite infection and associated dysfunction of the heart, digestive tract, and other organs, although many infected individuals may remain asymptomatic for life.

Trypanosoma cruzi exhibits remarkable genetic variation (Miles et al., 2009; Zingales et al., 2012, 2009), and current classification delineates six major discrete typing units (DTUs), TcI - TcVI, each with particular ecological and epidemiological associations (Zingales et al., 2012). In South America, for example, TcII, TcV, and TcVI have been mainly documented in domestic transmission cycles in the Southern Cone region; in contrast, TcIII and TcIV are more typically associated with sylvatic transmission cycles in Brazil and northern South America (Miles et al., 2009; Zingales et al., 2012). TcI is the most genetically diverse DTU and is found throughout the Americas with variable domestic and sylvatic associations (Zingales et al., 2012). The effect of DTU on exact clinical manifestations of disease progression is not well understood, and is complicated by co-infections, transmission mode, and individual host immune function (Messenger et al., 2015a; Zingales et al., 2012).

Trypanosoma cruzi actively circulates through vector, wildlife, and domestic dog populations in the southern US (Brown et al., 2010; Burkholder et al., 1980; Curtis-Robles et al., 2016, 2015; Kjos et al., 2008, 2009), and autochthonous human Chagas

disease is documented in the US as well (Cantey et al., 2012; Dorn et al., 2007; Garcia et al., 2015). The five human cases in the US that have been typed were all TcI (Roellig et al., 2008), and limited studies of dog and wildlife populations have revealed an association of TcI with opossums, and TcIV with dogs and raccoons (Curtis-Robles et al., 2016; Roellig et al., 2008). Recently, TcII was found in small rodents from Louisiana (Herrera et al., 2015). Studies of DTUs in triatomine vectors have documented TcI in limited sample sizes from California, Florida, Georgia, Louisiana, and Texas (Barnabé et al., 2001; Beard et al., 1988; Buhaya et al., 2015; Herrera et al., 2015; Roellig et al., 2008; Shender et al., 2016); TcIV has been documented rarely in California, Georgia, and Texas (Barnabé et al., 2001; Roellig et al., 2008; Shender et al., 2016).

Despite the recognized ecological and epidemiological importance of *T. cruzi* genetic variation, few studies have examined the DTUs that exist in the US, and none have examined relative spatial distribution across a broad area. Knowledge of the prevalence and distribution of *T. cruzi* distribution in US triatomine vectors—particularly those encountered by humans in peridomestic settings—would allow a greater understanding of disease risk and potential consequences. The aim of this study was to determine *T. cruzi* infection prevalence and DTUs in vector species and to map occurrences of *T. cruzi* DTUs.

4.2 Materials and Methods

Triatomine specimens were collected from July 2012-June 2016 via a citizen science program (Curtis-Robles et al., 2015) and traditional entomological techniques (black light, mercury vapor light, and active searching of environments) (see Section III of this dissertation). Almost all bugs were dead prior to arriving to the laboratory for processing, which precluded culture-based methodologies for detecting infection.

Triatomines were morphologically identified to species (Lent and Wygodzinsky, 1979), sexed, and dissected using sterile instruments after bugs were cleaned with 10% bleach solution and rinsed with distilled water. DNA from bug hindguts was extracted

using commercially-available extraction kits (Omega Bio-tek, Norcross, GA; Qiagen, Germantown, MD). *T. cruzi* infection status was determined by amplification of a 166-bp region of repetitive nuclear satellite DNA using a Taqman qPCR reaction with Cruzi 1, 2, and 3 primers and probe (Duffy et al., 2013; Piron et al., 2007). This approach has previously been shown as both sensitive and specific for *T. cruzi* (Schijman et al., 2011). Reactions consisted of 5 μL of template DNA, primers at a final concentration of 0.75 μM each, 0.25 μM of probe, and iTaq Universal Probes Supermix (Bio- Rad Laboratories, Hercules, CA), in a total volume of 20 μL in a Stratagene MxPro3000 instrument (Agilent Technologies, Santa Clara, CA), following previously described thermocycling parameters (Duffy et al., 2013), except with a reduced, 3-min, initial denaturation. Our internal laboratory validations defined samples with cycle threshold (Ct) values of 33 or less as positive, and samples with Ct values of 35 or greater as negative. Samples with Ct values of 33-35 were considered equivocal and subjected to additional testing using the 121/122 primer set to amplify a 330 bp region of kinetoplast DNA and determine status (Virreira et al., 2003; Wincker et al., 1994). Reactions consisted of 1 μL DNA, primers at final concentration of 0.75 μM each, and FailSafe PCR Enzyme Mix in PreMix E (Epicentre, Madison, WI) in a total volume of 15 μL. PCR products were visualized on 1.5% agarose gel stained with ethidium bromide. No-template controls were included in each set of DNA extractions, and molecular grade water was included as negative controls in all PCRs. All PCRs were run with a *T. cruzi* positive control, including DNA extracted from Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection) or positive field-collected samples.

Amplification of the TcSC5D putative sterol oxidase gene and subsequent DNA sequencing was used to determine the discrete typing units (DTU) of *T. cruzi* isolates (Cosentino and Agüero, 2012). Initially, we attempted amplification of the TcSC5D gene of any sample generating a positive infection result in prior PCRs. However, *T. cruzi*-positive samples with relatively low parasite burdens were not successfully amplified with the TcSD5D assay, reflecting its lower sensitivity on mixed DNA samples. Thereafter, we focused on TcSC5D gene amplification from samples yielding

Ct values of less than 17 on the TaqMan qPCR. Reactions consisted of 1 μL extracted DNA, 0.75 μM of each primer, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a total volume of 15 μL. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. Samples that did not produce a band were diluted 1:10 and TcSC5D gene amplification was attempted a second time. Amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and bi-directionally sequenced (Eton Bioscience, Inc., San Diego, SA). Sequences and chromatographs were viewed with Geneious version R7 (http://www.geneious.com) (Kearse et al., 2012) for quality, forward and reverse sequence alignment, and examination of key SNPs to designate DTU and discern double nucleotide peaks at SNPs (Cosentino and Agüero, 2012).

A logistic regression model was built using Program R (R Development Core Team, 2008) to determine the effect that species had on the *T. cruzi* PCR status of a bug, in which predictor variables included the six species found in Texas that had at least 10 bugs tested for *T. cruzi* status. Odds ratios and 95% confidence intervals were calculated. All bug location data were geo-coded in a geographic information system (ArcMap, ESRI, Redlands, CA).

4.3 Results

A total of 1226 triatomine bugs collected from 8 states via the citizen science program and standard entomological collecting techniques were tested for the presence of *T. cruzi* parasites using PCR, of which 722 (58.9%) were positive. Of 29 samples that gave equivocal results on the qPCR, 20 samples generated a band of appropriate size using the 121/122 primers, and were considered positive for determination of infection prevalence. We found 32.6% infection prevalence in bugs from states other than Texas (n=46) (Table 4.1), whereas infection prevalence was 59.9% in bugs from Texas (n=1180) (Table 4.2). Infection prevalence varied in different triatomine species, ranging from 0% in *T. rubida* to 71.1% in *T. lecticularia*. A logistic regression of infection on

species revealed all tested species except *T. lecticularia* had lower risks of infection than *T. gerstaeckeri* (Table 4.3).

Table 4.1. Localities of *T. cruzi* in triatomines. Triatomines were collected from across the southern US through citizen science submission and tested for *T. cruzi* infection. **T. cruzi* was typed to TcIV.

State	County	Species	Infected/Tested
Arizona	Pima	nymph	0/1
	Yavapai	*T. protracta*	1/3
		T. rubida	7/19
		nymph	0/1
California	Los Angeles	*T. protracta*	0/1
	San Diego	*T. protracta*	0/3
Florida	Indian River	*T. sanguisuga*	0/1
	Lake	*T. sanguisuga*	1/1
	Polk	*T. sanguisuga*	0/3
	Suwannee	*T. sanguisuga*	1/1
Missouri	Christian	*T. sanguisuga*	0/1
Oklahoma	McCurtain	*T. sanguisuga*	1/1
	Oklahoma	nymph	0/1
Tennessee	Warren	*T. sanguisuga*	1/1
Virginia	Albemarle	*T. sanguisuga*	1/2
	Fairfax	*T. sanguisuga*	1*/2
	Nelson	*T. sanguisuga*	1*/2
		nymph	0/1
	Prince William	*T. sanguisuga*	0/1
			15/46 (32.6%)

Table 4.2. *T. cruzi* infection prevalence and DTUs. Triatomines were collected from 2012 – 2016 in Texas through citizen science submission and standard entomological sampling. ND=No Data

	Sex/Life Stage	Positive/Tested (Infection prevalence)	*T. cruzi* DTU		
			TcI	TcIV	Mixed TcI and TcIV
T. gerstaeckeri	Female	283/446 (63.5%)	37	49	8
	Male	223/333 (67.0%)	33	35	2
	Unknown	8/15 (53.3%)	0	3	0
	Total	514/794 (64.7%)	70	87	10
T. sanguisuga	Female	69/124 (55.6%)	1	20	0
	Male	42/79 (53.2%)	4	14	0
	Unknown	0/2 (0.0%)	ND	ND	ND
	Total	111/205 (54.1%)	5	34	0
T. indictiva	Female	19/38 (50.0%)	1	6	0
	Male	9/18 (50.0%)	ND	ND	ND
	Unknown	1/1 (100%)	0	1	0
	Total	29/57 (50.9%)	1	7	0
T. lecticularia	Female	18/27 (66.7%)	1	2	0
	Male	19/25 (76.0%)	1	3	0
	Total	37/52 (71.2%)	2	5	0
T. rubida	Female	0/4 (0.0%)	ND	ND	ND
	Male	0/1 (0.0%)	ND	ND	ND
	Total	0/5 (0.0%)	ND	ND	ND
T. protracta	Female	0/1 (0.0%)	ND	ND	ND
	Male	1/3 (33.3%)	1	0	0
	Total	1/4 (25.0%)	1	0	0
Triatoma sp.	Female	8/20 (40.0%)	0	2	0
	Male	2/8 (25.0%)	ND	ND	ND
	Unknown	0/1 (0%)	ND	ND	ND
	Total (adults)	10/29 (34.5%)	0	2	0
	Nymphs	5/34 (14.7%)	ND	ND	ND
Subtotals	Female	397/660 (60.2%)	50	79	8
	Male	296/467 (63.4%)	39	52	2
	Unknown	9/19 (47.4%)	0	4	0
Grand total		707/1180 (59.9%)	79	135	10

Table 4.3. *T. cruzi* infection prevalence statistics. A logistic regression model was used to determine the odds ratio (OR) of infection of triatomine species from across the southern US, compared to the referent group of *T. gerstaeckeri*.

Species	Total tested	Infection Prevalence	OR	95% CI	p-value
T. gerstaeckeri	794	64.7%	Referent	NA	NA
T. indictiva	57	50.9%	0.56	0.34-0.97	0.038
T. lecticularia	52	71.1%	1.34	0.74-2.56	0.348
T. protracta	11	18.2%	0.12	0.02-0.47	0.007
T. rubida	24	29.2%	0.22	0.09-0.53	0.001
T. sanguisuga	221	53.4%	0.62	0.46-0.84	0.002

In Texas bugs, there was no significant difference ($\chi 2=1.07$, df=1, P>0.30) in *T. cruzi* infection prevalence in females (60.2%) and males (63.4%). A Fisher's exact test calculated that the percentage of infected bugs differed significantly by life stage (OR=9.15, 95% CI 3.47-30.54), with adults (61.3%) more likely to be infected than nymphs (14.7%).

We found the Ct value on the qPCR to be a good indicator of whether a sample would be likely to be successfully amplified, sequenced, and typed using the TcSC5D primer set. When Ct values were greater than 17, we successfully typed 13.1% (n=61), but when Ct values were less than 17, we successfully typed 76.2% (n=286). Parasite DTU was successfully ascertained for a total of 226 triatomines. Of the 224 Texas bugs, inspection of key SNPs (Cosentino and Agüero, 2012) revealed 79 were TcI and 135 were TcIV, and double nucleotide peaks indicating possible mixed infection occurred in an additional 10 samples (Table 4.2). *T. gerstaeckeri* was found to be approximately equally infected with TcI and TcIV DTUs, while *T. sanguisuga* had a higher number of TcIV infections. To compare TcI and TcIV infection ratio in *T. gerstaeckeri* and *T. sanguisuga*, a Fisher's exact test calculated that *T. sanguisuga* more likely to be infected with TcIV than *T. gerstaeckeri* (OR=5.49, 95% CI 1.99-18.95). All potential mixed infections were from *T. gerstaeckeri.*

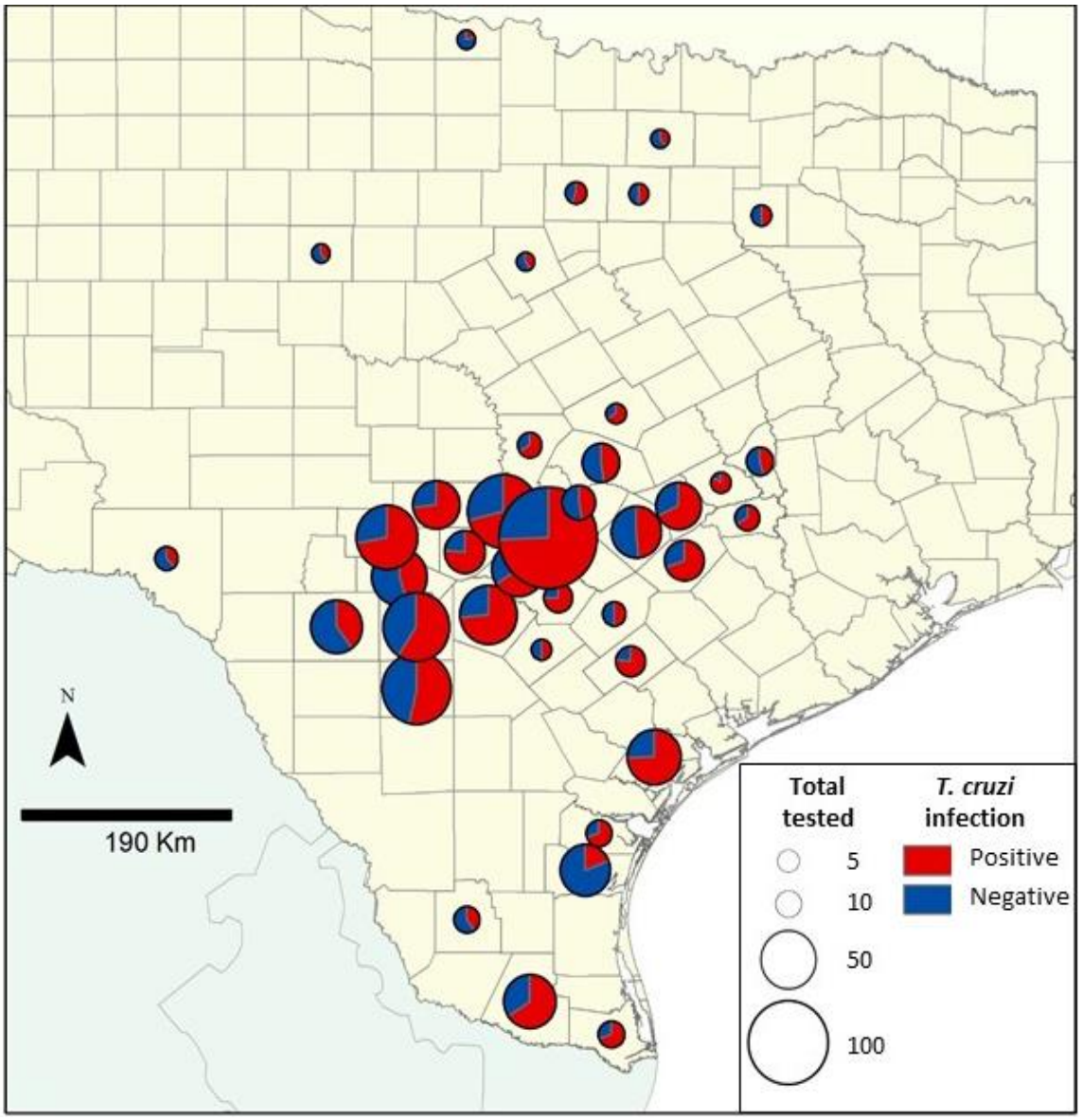


Figure 4.1. County distribution of *T. cruzi*-infected triatomines in Texas. The map includes all counties from which at least five bugs were tested. Circle size is proportional to the number of bugs tested from each county.

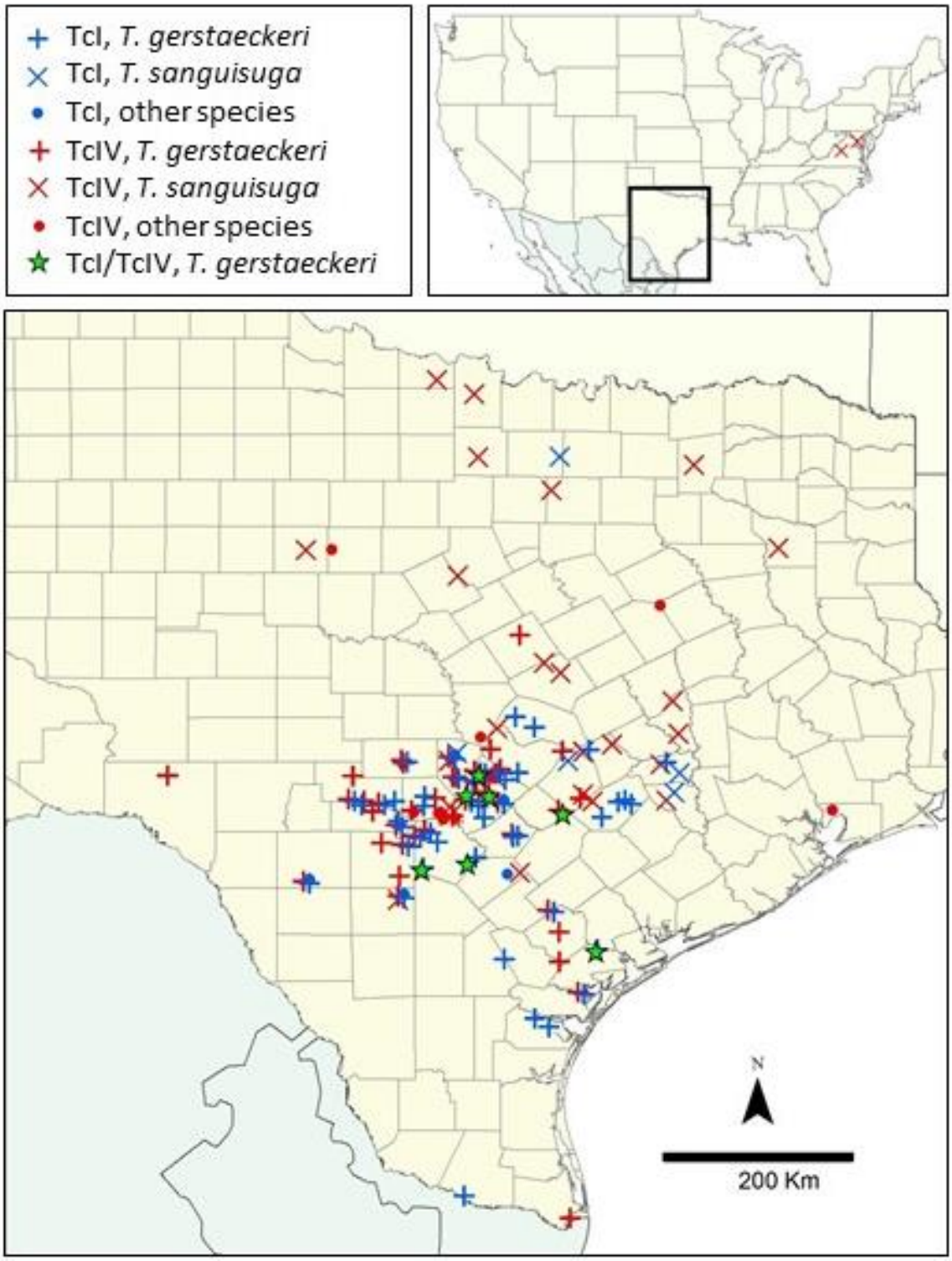


Figure 4.2. *T. cruzi* isolates from triatomine bugs across Texas and the US. *T. cruzi* isolates were typed to DTU using the TcSC5D primer set and mapped by exact location of where each bug was found.

Infected triatomines were found across the state (Figure 4.1); any county with at least 6 bugs tested was found to have at least one positive bug. Both TcI and TcIV were found in 'sylvatic' adult bugs collected in remote areas using standard entomological techniques, as well as in bugs from peridomestic settings and captured in houses. Both TcI and TcIV were found across the sampling area in Texas, although there were more TcIV than TcI isolates in north Texas (Figure 4.2). In addition to the Texas triatomines, parasite typing from triatomines from other areas revealed DTU TcIV in 2 *T. sanguisuga* from Virginia. Although bugs from other states were positive for *T. cruzi* DNA, the Ct values they generated on the qPCR were greater than 17, and attempted amplification of the TcSC5D gene was unsuccessful.

4.4 Discussion

We tested triatomine insects collected from across the southern US for *T. cruzi* infection, and typed samples from infected bugs to determine parasite DTUs. We found an overall infection prevalence of 58.9% (n=1,226), with infected bugs coming from across the southern US. Amplification and sequencing of the TcSC5D gene from *T. cruzi* samples revealed DTUs TcI and TcIV.

In Texas, where the majority of bugs were from, we found an overall infection prevalence of 59.9% (n=1,180); this is in alignment with a recent study that found an infection prevalence of 50.7% in 241 tested bugs (Kjos et al., 2009). In all Texas counties from which at least 6 bugs were tested, *T. cruzi* DNA was detected in at least one bug (Figure 4.1), suggesting that uninfected populations of vectors are rare. Infection prevalence varied widely across insect life stage and species. Adult bugs collected from Texas were more likely to be infected with *T. cruzi* than nymphs. Lower infection prevalence in nymphs than adults is expected, considering vertical transmission of *T. cruzi* in the triatomine vector does not occur (Kirchhoff, 2011; Ryckman and Olsen, 1965), and triatomines take multiple blood meals throughout their lives, each potentially exposing them to *T. cruzi* infection. Similar to previous findings in Texas bugs (Kjos et al., 2009; Pippin, 1970), *T. gerstaeckeri* adults had a higher infection

prevalence (64.8%) than *T. sanguisuga* adults (54.1%). The odds of infection in *T. gerstaeckeri* were greater than for all other species, except *T. lecticularia* (Table 4.3). These significant differences may be due to different bug-host associations, including differences in where eggs are laid and nymphs feed until molting to adults with potential to disperse, as well as differing *T. cruzi* infection and potential transmission dynamics in different species of hosts.

Since citizen science submissions and our collection techniques encountered mainly dispersing adult bugs (as opposed to flightless nymphs or non-blood seeking adults), these samples offer a perspective of landscape-level infection prevalence and DTU diversity. Sylvatic hosts are known to play an important role in triatomine infection, especially for US triatomine species not frequently encountered in peridomestic settings. Previous studies of US wildlife found predominantly TcI in opossums (Barnabé et al., 2001; Roellig et al., 2008) and mainly TcIV in raccoons (Barnabé et al., 2001; Curtis-Robles et al., 2016; Roellig et al., 2008). One study in a focal area in west Texas found TcIV in a cotton rat and rock squirrel, as well as TcI and TcIV in woodrats and striped skunks (Charles et al., 2013). In addition to wildlife, canines have been shown to be infected with TcI and TcIV (Patel et al., 2012; Roellig et al., 2008). The degree to which each host species plays a role in sustaining triatomine populations and serving as infection sources is unknown. Although there is a well-documented association between *T. protracta* and *Neotoma* spp. woodrats in the southwestern US (Charles et al., 2013; Packchanian, 1942; Ryckman et al., 1981), host utilization by other US triatomines species is not well known (Ibarra-Cerdeña et al., 2009). Many wildlife species are known to sustain infection with *T. cruzi* in Texas (Burkholder et al., 1980; Charles et al., 2013; Comeaux et al., 2016; Curtis-Robles et al., 2016; Eads and Hightower, 1952; Grögl et al., 1984; Ikenga and Richerson, 1984; Lathrop and Ominsky, 1965; Packchanian, 1942; Pinto et al., 2010); more research on the natural associations of triatomine species and their sylvatic hosts is needed to elucidate vector/host associations and reveal the array of mammalian hosts that serve as reservoirs of *T. cruzi* DTUs TcI and TcIV in US.

Both TcI and TcIV were documented across the state of Texas. In Texas, *T. gerstaeckeri* was found to be equally infected with TcI and TcIV, including possibly mixed TcI/TcIV infections, while *T. sanguisuga* were more likely to be infected with TcIV. Samples typed from the northern part of Texas were mainly TcIV; however this apparent geographic bias may also reflect that the samples were from *T. sanguisuga*, which is more likely to be found in that part of the state (Kjos et al., 2009)(see Section III of this dissertation). Limited studies of DTUs in triatomine vectors have documented TcI in *T. sanguisuga* in Louisiana (Barnabé et al., 2001; Herrera et al., 2015), Florida (Barnabé et al., 2001; Beard et al., 1988; Roellig et al., 2008), Georgia (Roellig et al., 2008) and California (Shender et al., 2016), in *T. rubida* from west Texas (Buhaya et al., 2015), and in *T. gerstaeckeri* from Texas (Roellig et al., 2008). TcIV has been documented in *T. sanguisuga* from Georgia (Barnabé et al., 2001), and *T. protracta* from California (Shender et al., 2016). Mixed TcI/TcIV *T. cruzi* infections have been documented in one *T. protracta* from California, and one *T. gerstaeckeri* from Texas (Roellig et al., 2008). Much remains to be investigated regarding mixed infections: are triatomines becoming infected by sequential feeding events on hosts infected with single DTUs, or from feeding on a host simultaneously infected with two DTUs? Mammalian hosts co-infected with more than one DTU have been documented in multiple species the southern US (Curtis-Robles et al., 2016; Herrera et al., 2015; Roellig et al., 2008). A recent study in Venezuela revealed distinct patterns of DTU findings in human, vector, and wild hosts, suggesting that multi-host assessments of circulating DTUs are needed to better understand human and animal risk of infection with specific DTUs (Carrasco et al., 2012). There is a need for concurrent investigations of vectors and hosts in the same geographic area to elucidate host sources of infection with different parasite DTUs.

Many *T. cruzi* strain-typing methods involve the propagation of the parasite in pure culture followed by multiple PCRs, with differentiation of strains based on amplicon presence/absence, amplicon size, and or multi-locus sequence typing (Messenger et al., 2015b). While these methods are valuable—given extensive prior use, potential usefulness for elucidating evolutionary relationships, and relative ease of

application in areas where sequencing facilities are not available or cost-effective—we sought an approach based on a single PCR and DNA sequencing to allow simple, rapid classification to DTU-level. We have found the TcSC5D primer set an attractive option for DTU-level classification of *T. cruzi* from mixed DNA samples with high loads of parasite, as well as its usefulness in determining potential mixed DTU infections based on examination of sequence chromatographs. However, although targeting the TcSC5D gene can be efficient method to determine DTU, it was originally developed using pure parasite culture containing high concentrations of *T. cruzi* DNA (Cosentino and Agüero, 2012). Subsequent studies have had variable success amplifying the TcSC5D locus of DNA from field samples with low concentrations of *T. cruzi* DNA (Cominetti et al., 2014; Curtis-Robles et al., 2016)(also see Section V of this dissertation). In addition, we first determined infection using a qPCR targeting a region of parasite DNA with varying copy numbers per DTU (Duffy et al., 2013), and it is possible that our sampling was biased towards a DTU with higher copy numbers and more likely to generate a lower Ct value and be selected for amplification of the TcSC5D gene. It is also possible different DTUs of *T. cruzi* establish different parasite densities in triatomines, potentially leading to bias if our methods preferentially amplified DTUs that were more abundant. Visualization of parasite density in vector hindguts was not possible in this study. Although potential biases restrict interpretation of DTU ratios, we found the TcSC5D gene—originally developed using pure cultured parasite DNA—a useful amplification target for determining *T. cruzi* DTU in a subset of samples from field-collected vectors.

This study represents the largest number of typed *T. cruzi* samples US triatomines to-date, and provides a base for future studies investigating the within-DTU genetic variations of *T. cruzi*. The few *T. cruzi* isolates from human cases of Chagas disease in the US were all TcI (Roellig et al., 2008), and our findings of TcI in vectors encountered by the public are therefore directly important for assessing human health risk. Although TcIV is known to infect dogs and wildlife (Curtis-Robles et al., 2016; Patel et al., 2012; Roellig et al., 2008), whether triatomines infected with TcIV are of risk to human health is unknown. Much remains to be learned about the genetic variation

of US isolates of *T. cruzi*; such studies will be important to understanding potential disease progressions and protecting veterinary and public health.

5. EPIDEMIOLOGY AND MOLECULAR TYPING OF *TRYPANOSOMA CRUZI* IN NATURALLY-INFECTED HOUND DOGS AND ASSOCIATED TRIATOMINE VECTORS IN TEXAS, USA

5.1 Introduction

Chagas disease in humans and dogs is caused by the vector-borne hemoflagellate protozoan *Trypanosoma cruzi*. Active transmission cycles of the parasite occur across the southern US, where infected triatomine 'kissing bug' vectors and wildlife co-occur (Burkholder et al., 1980; Charles et al., 2013; Pietrzak and Pung, 1998; Pung et al., 1995). Although epidemiological studies of canine Chagas disease in the southern US are limited (Beard et al., 2003; Burkholder et al., 1980; Kjos et al., 2008; Williams et al., 1977), canine infection with *T. cruzi* is widespread, especially in Texas. The first cases of canine *T. cruzi* infection in the US were documented in Texas (Williams et al., 1977), and a recent retrospective study reported cases from across the state (Kjos et al., 2008). Studies have revealed anti-*T. cruzi* seroprevalences of 7.5% in stray dogs and 8.8% in shelter dogs across Texas (Beard et al., 2003; Tenney et al., 2014). However, given variation in clinical presentation in infected dogs, which ranges from asymptomatic to acute death or chronic heart disease (Barr, 2009), the veterinary implications of canine *T. cruzi* infections are uncertain.

Infection with *T. cruzi* can occur through the introduction of infected triatomine insect feces to skin lesions as the bug defecates on the host during or shortly after blood feeding. Oral transmission to dogs and wildlife may result from consumption of infected bugs or infected rodents (Bradley et al., 2000; Gürtler et al., 1986a; Montenegro et al., 2002). Although congenital transmission in canines has been documented (Barr et al., 1995; Mazza, 1935; Rodríguez-Morales et al., 2011), the frequency with which this occurs is unknown. Accordingly, owners of seropositive breeding bitches are left with little information to guide breeding programs, except for the option of removal of positive females from breeding roles (Snowden and Kjos, 2011).

Although serologic testing is a common tool for diagnosing *T. cruzi* infections in dogs, little is known about the relationship among positive serostatus, infectiousness, and disease outcome. In dogs, limited experimental studies indicate that parasitemia occurs within days to four weeks after initial infection (Andrade et al., 1997; Barr et al., 1991b; Veloso et al., 2008), with development of anti-*T. cruzi* antibodies detected at 15 days to 4 weeks post infection (Barbabosa-Pliego et al., 2009; Barr et al., 2005). Further, *T. cruzi* genetic strain differences are known to play a role in disease outcomes in humans, with genetic variation occurring across geographic regions (Zingales et al., 2012), yet there has been limited investigation of which strains infect dogs in the US (Roellig et al., 2008) and how this relates to disease outcome. In the absence of a canine vaccination or canine antiparasitic treatments against *T. cruzi*, veterinarians and dogs owners are faced with increasing diagnoses of canine *T. cruzi* infections, yet a limited ability to understand the veterinary and public health consequences. Our objective was to compare multiple serological and molecular biology techniques to detect and characterize *T. cruzi* infections in a cross-sectional analysis of working hound dogs in a parasite-endemic region.

5.2 Materials and Methods

5.2.1 Study design and sample collection

This study was motivated by the sudden and unexplained deaths of several dogs from a network of government-owned working hound dogs used for various tracking and detection functions, mainly across Texas. The dogs all died within a short time period, and postmortem findings indicated that canine *T. cruzi* infection was the probable cause of death in all cases.

Using a cross-sectional study design, we sampled 86 working dogs from three multi-dog kennels in the network: 26 dogs from kennel A, 31 dogs from kennel B, and 29 dogs from kennel C, which comprised all dogs in residence at these kennels. Animals were housed in indoor-outdoor, open air, cement/concrete kennels located in Bee and Karnes counties in south central Texas. All dogs were Coonhounds, most were bred by

the facilities, and ages ranged from approximately 6 months to 13 years. Dogs had limited travel history, mainly within Texas. Research use of all samples from these dogs was secondary to collection for diagnostic purposes; this research was granted exemption by the Texas A&M Institutional Animal Care and Use Committee.

Blood samples were collected between July and September, 2013. Opportunistic postmortem samples of blood and other tissues (heart, mammary gland, testicle, uterus) were collected from dogs euthanized for reasons unrelated to this study. Pedigree lineage records were analyzed to determine relationships among sampled dogs (i.e., dams and littermates). Triatomine bugs were opportunistically collected from kennels in the network by kennel staff and pest control operators in summer 2013.

5.2.2 Serology

Serum aliquots were tested for anti-*T. cruzi* antibodies using indirect fluorescent antibody (IFA) testing at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL; College Station, TX). All samples were screened for the presence of anti-*T. cruzi* antibodies at a 1:20, 1:80 and 1:160 dilutions. According to TVMDL protocols, titer values of 20 or greater were considered positive.

The remaining serum was stored at -20 °C until analyzed using the Chagas Stat-Pak chromatographic dipstick test (ChemBio, NY). The Chagas Stat-Pak test has been validated as an antibody-detection test in the dog (Nieto et al., 2009) and shows high specificity and sensitivity when compared with other serological techniques (Luquetti et al., 2003). Stored serum samples were tested according to manufacturer's instructions, including strict adherence to the incubation time, and any development of a band at 15 minutes was considered positive. Band strength was noted as faint, medium, or bold. Samples positive using both IFA and Chagas Stat-Pak dipstick tests were considered seropositive in the calculation of population-level seroprevalence.

5.2.3 Detection of T. cruzi *DNA*

An extraction kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA) was used to extract DNA from 250 µL of clotted blood from dogs for which serology testing was also performed. Extracted DNA was analyzed using qPCR and conventional PCR to detect parasite DNA.

Samples were first screened for presence of *T. cruzi* DNA using the probe-based real-time PCR Cruzi I/II primer set and Cruzi III probe (Duffy et al., 2013; Piron et al., 2007). This PCR amplifies a 166-bp region of a repetitive nuclear DNA sequence, and is sensitive and specific for *T. cruzi* when compared to other PCR techniques (Schijman et al., 2011). A Stratagene MxPro3000 instrument (Agilent Technologies, Santa Clara, CA) was used to amplify DNA under previously described thermocycling parameters (Duffy et al., 2013), except with a 3-minute initial denaturation. Reactions consisted of 5 µL of template DNA, primers at a final concentration of 0.75 µM each, 0.25 µM of probe, and iTaq University Probes Supermix (BioRad Laboratories, Hercules, CA), in a total volume of 20 µL. Machine-calculated thresholds and reaction curves were visually checked to assure successful amplification. Internal laboratory validation tests have determined cycle threshold (Ct) values indicating positive (<31), negative (>34), and equivocal status (31-34). Negative controls were included in each set of DNA extractions and PCR reactions. Positive controls included *T. cruzi* DNA extracted from isolate Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection [ATCC]), an isolate cultured from a published canine case (Nabity et al., 2006), an isolate from an infected Texas raccoon (Curtis-Robles et al., 2016), and isolates from triatomine bugs (*T. sanguisuga* and *T. indictiva*).

Samples classified by qPCR as equivocal were subjected to a conventional PCR using *T. cruzi* 121/122 primers to amplify a 330-bp region of kinetoplast DNA (Virreira et al., 2003; Wincker et al., 1994). Reactions included 1µL template DNA, primers at final concentrations of 0.75 µM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final reaction volume of 15 µL. Amplicons were

visualized on 1.5% agarose gels stained with ethidium bromide. Samples that yielded a band of the appropriate size were interpreted as positive in our analyses.

5.2.4 Microscopic and molecular analysis of tissues

Tissues collected opportunistically from euthanized dogs were preserved in 10% neutral buffered formalin. Formalin-preserved samples were submitted for histopathologic examination with routine H&E staining at the TVMDL and reviewed by a pathologist. Additionally, DNA was extracted from an approximately 1 cm3 piece of various fresh tissues using the same methods as the above molecular processing of dog blood samples as described above.

5.2.5 Determination of T. cruzi *strain types*

To determine *T. cruzi* discrete typing unit (DTU) strain types circulating in the dog population, samples were amplified using a primer set that amplifies a region of the TcSC5D gene, a putative lathosterol/episterol oxidase (Cosentino and Agüero, 2012). The DNA extracted from isolate Sylvio X10 CL4 (ATCC 50800, ATCC), and DNA from an isolate cultured from a published canine case (Nabity et al., 2006) were used as positive controls. The 832-bp amplicons were visualized on 1.5% agarose gel with ethidium bromide, and sequenced using Sanger sequencing (Eton Bioscience Inc., San Diego, CA, USA). Geneious version 8 [http://www.geneious.com (Kearse et al., 2012)] was used to visually review chromatographs and sequences, align forward and reverse sequences, and examine locations of key SNPs to determine DTU (Cosentino and Agüero, 2012).

5.2.6 Vector morphologic and molecular characterization

Bugs were identified to species using morphologic features (Lent and Wygodzinsky, 1979); sex and evidence of a recent blood meal were noted. After bugs were washed in 10% bleach solution and rinsed in distilled water, sterile instruments were used to dissect the bugs and isolate hindgut material. DNA was extracted from

hindguts and tested for *T. cruzi* DNA and determination of *T. cruzi* DTU using the same methods as the above testing of dog samples. In order to determine the source of recent blood meals, hindgut DNA was subjected to PCR amplification of host cytochrome B sequences using previously published primers and cycling conditions (Cupp et al., 2004; Hamer et al., 2009). Reactions included 3 μL template DNA, primers at final concentrations of 0.66 μM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final reaction volume of 50 μL. Amplicons were visualized on 1.5% agarose gel with ethidium bromide, and sequenced using Sanger sequencing (Eton Bioscience Inc., San Diego, CA, USA). Resulting sequences were compared to existing sequences using Basic Local Alignment Search Tool (National Center for Biotechnology Information, US National Library of Medicine).

5.2.7 Statistical testing

Samples positive using both IFA and Chagas Stat-Pak dipstick tests were considered seropositive in the calculation of population-level seroprevalence. Blood samples classified positive by qPCR and those that were equivocal by qPCR and positive by conventional PCR, were considered positive in calculation of population-prevalence of *T. cruzi* DNA in blood samples. Separate multiple logistic regression models were built using Program R (R Development Core Team, 2008) to determine predictors of anti-*T. cruzi* antibody status and blood *T. cruzi* PCR status, in which predictor variables included kennel (A, B, or C), canine age (<2 years, 2-6 years, or >6 years), and canine sex. Odds ratios and 95% confidence intervals were calculated.

5.3 Results

Blood samples from 86 dogs in three kennels were analyzed using a variety of serologic and molecular techniques to detect *T. cruzi* exposure and infection. Additionally, tissue samples were tested from 11 dogs. A total of 44 triatomine insects were recovered from the kennels for testing and analyses.

5.3.1 Population data

The birthdate was known for 80 of the 86 dogs in the three kennels. Ages ranged from 6 months to 13 years, with a mean and median of 3.96 years and 3.58 years, respectively. There were 15 dogs less than 1 year old (18.6% of 80). There were 39 males (45.3%) and 47 females (54.7%). At the time of the cross-sectional blood sampling, there were seven dams that had a total of seventeen offspring that were included in the study (Figure 5).

5.3.2 Serological results

A total of 55 of 86 (64.0%) dogs had a titer value of 20 or greater on IFA, and 54 of 85 (63.5%) dogs were reactive on the Chagas Stat-Pak (Table 5.1). Combined, 49 of 85 dogs were positive on both antibody detection tests yielding a seroprevalence of 57.6%. Additionally, a single sample was positive with a titer of 160 on the IFA, but was not tested on the Chagas Stat-Pak. There were 10 dogs positive on only one test and negative on the other; these dogs with discordant results were considered seronegative for the purpose of this study. Of these ten discordant samples: 4 dogs were negative on IFA but had faint (positive) lines on the Chagas Stat-Pak, and 6 dogs were negative on Chagas Stat-Pak but had IFA titer values of 20 (3 dogs), 80 (1 dog), and 160 (2 dogs). Overall seroprevalences at each kennel were: 46.2% at kennel A (C) (n=26), 71.0% at kennel B (G) (n=31), and 53.6% at kennel C (M) (n=28).

Analysis of canine serostatus in relation to lineage revealed both positive and negative littermates born to positive and negative dams. Four 20-month old pups were seropositive, despite the concurrently tested dam being seronegative (Figure 5).

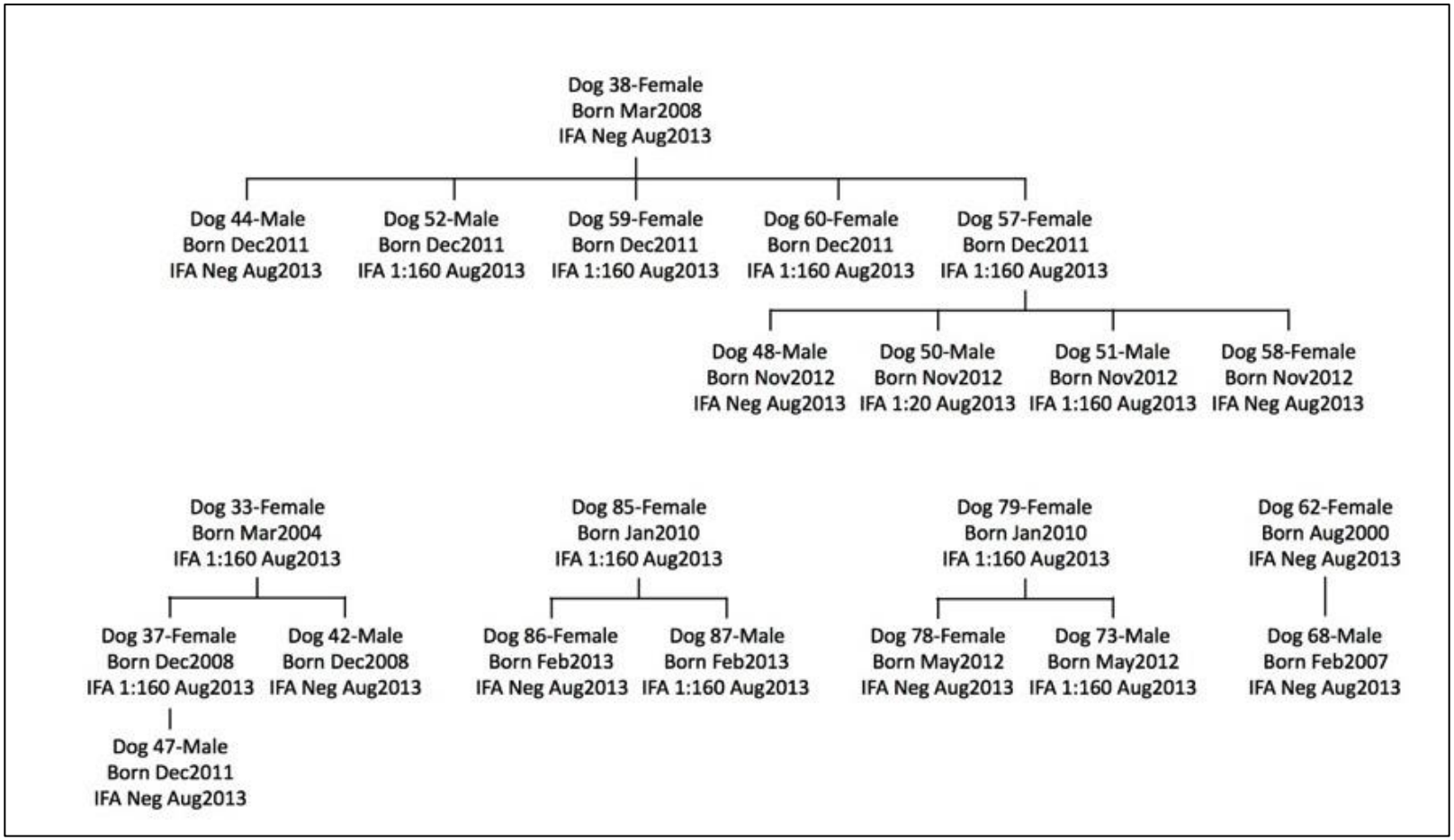


Figure 5. Lineages of five groups of related dogs. Each dog is represented by its number, sex, date of birth, and IFA status at time of August 2013 testing. Dams Dog 62, Dog 79, and Dog 85 were from kennel A, dams Dog 33, Dog 37, Dog 38, and Dog 57 were from kennel C.

Table 5.1. Serology results of tested dogs. Blood samples from working dogs were tested for anti-*T. cruzi* antibodies using IFA and Chagas Stat-Pak. Only those samples positive on both assays were considered positive for calculation of seroprevalence

		IFA		
		Positive	**Negative**	**Total**
Stat-Pak	**Positive**	49	4	53
	Negative	6	26	32
	Total	55	30	85

A litter of six pups was born to a female who tested serologically positive (≥1:160) nine months previously, and these pups were serially sampled twice over 4 weeks. The dam accidentally smothered one of the pups one day after birth. Blood samples from that pup were PCR negative for parasite, although testing on Chagas Stat-Pak gave a faint (positive) band. The other five pups had blood sampled two weeks after birth and tested on Chagas Stat-Pak: two gave very faint (positive) bands and three were negative. At one month of age, all five were negative on Chagas Stat-Pak. None of the two week or one month samples were PCR positive.

5.3.3 Molecular detection of parasite DNA

PCR analysis of 86 DNA extracts of blood clots revealed 23 (26.7%) positive samples, including 18 samples that were positive in the initial qPCR, and all five samples that were categorized as equivocal in the qPCR and subsequently tested positive using the conventional PCR assay. Infection prevalences at each kennel were: 15.4% at kennel A (n=26), 35.5% at kennel B (n=31), and 27.6% at kennel C (n=29).

5.3.4 Comparison of serology and PCR

Using the serological positivity criterion of being positive on both IFA and Stat-Pak assay, serology and PCR findings categorized 19 of 85 dogs (22.4%) as both

seropositive and PCR positive (Table 5.2), 30 dogs (35.3%) as seropositive and PCR negative; 4 dogs (4.7%) as PCR positive and seronegative; and 32 dogs (37.6%) as both seronegative and PCR negative. One dog sample with a 160 IFA titer was not run on Chagas Stat-Pak; that dog was PCR negative. Two dogs that were PCR positive but did not meet the positivity criterion on both serological assays were positive on IFA with titers of 20 and 80.

Table 5.2. Serology and PCR results of blood tested from dogs. Blood samples from working dogs were tested for anti-*T. cruzi* antibodies using IFA and Chagas Stat-Pak; DNA extracted from the blood clots was tested for presence of *T. cruzi* DNA using PCR. Samples were considered serologically positive if positive on both IFA and Chagas Stat-Pak.

		Serology		
		Positive	**Negative**	**Total**
PCR	**Positive**	19	4	23
	Negative	30	32	62
	Total	49	36	85

Table 5.3. Opportunistic additional testing of serologically positive dogs. Serologic, molecular, and histologic results of tissue samples opportunistically collected from *T. cruzi*-infected dogs.

Dog ID	Sex	Age at time of sampling	Molecular (PCR) results	Histopathology results
Dog 4	F	5 y	uterus - negative	NA
Dog 5	F	5 y	uterus - negative	NA
Dog 7	F	8 y	uterus - negative	NA
Dog 50	M	15 mo	heart - positive testicle - negative	heart - chronic lesions consistent with canine trypanosomiasis
Dog 51	M	15 mo	heart - negative testicle - negative blood - negative	heart - no significant lesions
Dog 53	M	3 y	heart - negative testicle - negative	NA
Dog 75	M	2 y	heart - positive testicle - negative	heart - chronic lesions consistent with canine trypanosomiasis
Dog 88	F	13 mo	heart - positive (4/4) blood - positive (2/5) uterus - positive (4/5) mammary gland - positive (2/2)	heart - chronic lesions consistent with canine trypanosomiasis uterus - no significant lesions
Dog 432	M	6 mo	heart - positive blood - positive	NA

5.3.5 Microscopic and molecular analysis of tissues

A total of five tissue samples opportunistically collected from four IFA-positive dogs were examined histologically. Three of four cardiac samples had lesions consistent with chronic canine trypanosomiasis (Table 5.3), although no amastigotes were observed in any of the sections. Lesions included cardiomyofiber degeneration (ranging from minimal to moderate), accumulations of lymphocytes, plasma cells, and rare macrophages. One uterine tissue section was viewed; no amastigotes or significant lesions were observed (Table 5.3).

T. cruzi DNA was detected in heart, blood, uterus, and mammary gland tissues collected opportunistically from multiple serologically-positive dogs (Table 5.3). Three dogs did not have detectable parasite DNA in tested uterine tissue, whereas four of the

five samples from the body of the uterus of one dog were PCR positive. Three dogs did not have detectable parasite DNA in tested testicular tissue. One dog had multiple parasite positive tissues, including heart, blood, uterus, and mammary gland.

5.3.6 Determination of T. cruzi *strain types*

Of the 23 PCR positive blood samples, amplification and sequencing of the TcSC5D gene DNA target was successful in five blood samples. Three samples were of DTU TcI and two samples were of DTU TcIV. Cardiac and uterine samples from one dog (Dog 88) revealed TcIV in uterine tissue and TcI in cardiac tissue. *T. cruzi* sequences were deposited to GenBank (Accession nos. KX594832-KX594838).

5.3.7 Bugs

A total of 44 bugs (Table 5.4) were opportunistically collected in summer 2013 from the network of working dog kennels, including the three kennels that housed dogs tested in the cross-sectional serological study. Bugs included 16 adult *Triatoma gerstaeckeri* and 28 adult *T. sanguisuga*. Of the 36 insects that were tested for *T. cruzi*, 29 (80.6%) were positive, including all tested *T. gerstaeckeri* (n=16) and 13 of 20 (65%) *T. sanguisuga*. Of the seven infected *T. sanguisuga* for which parasite DTU was determined, DTU TcIV was evident in all seven, with a single bug harboring a mixed DTU TcI and TcIV infection. In contrast, of the seven infected *T. gerstaeckeri* for which parasite DTU was determined, DTU TcI was evident in six, with a single bug infected with TcIV (Table 5.4). Representative *T. cruzi* sequences were deposited to GenBank (Accession nos. KX594839-KX594840). Based on visual examination, 30 of the 44 bugs had evidence of a recent blood meal in their guts. Of 24 bugs with sufficient blood meal volume for successful blood meal PCR and Sanger sequencing, all 24 had ≥97% identity to *Canis lupus familiaris* (domestic dog).

Table 5.4. Triatomine insects collected from kennels. Bugs collected from kennels were tested for *T. cruzi*. *T. cruzi* DTUs and blood meal sources were determined.

	Submitted	Positive / Tested (Infection prevalence)	*T. cruzi* DTU(s)	Blood meal source
T. sanguisuga	28 (10M, 18F)	11/18 (61%)	TcIV (6 bugs) TcI/TcIV mix (1 bug)	*Canis lupus familiaris* (15/15 bugs)
T. gerstaeckeri	14 (4M, 10F)	14/14 (100%)	TcI (6 bugs) TcIV (1 bug)	*Canis lupus familiaris* (9/9 bugs)

Table 5.5. Logistic regression. Model output for *Trypanosoma cruzi* seropositivity and PCR positivity from 86 working hound dogs in central Texas, 2013.

Risk factor	No. tested for antibodies	No. seropositive (%)	Odds ratio	95% CI	p value	No. tested for parasite DNA	No. positive (%)	Odds ratio	95% CI	p value
Kennel										
A	26	12 (46.15)		referent		26	4 (15.38)		referent	
B	31	22 (70.99)	6.06	1.67-25.60	0.0088	31	11 (35.48)	3.50	0.94-15.25	0.0723
C	28	15 (53.57)	1.29	0.41-4.14	0.6656	29	8 (27.59)	2.18	0.57-9.38	0.2646
Age										
<2 years	30	13 (43.33)		referent		30	8 (26.67)		referent	
2-6 years	32	22 (68.75)	2.71	0.90-8.57	0.0799	32	9 (28.13)	0.94	0.29-3.05	0.9120
>6 years	17	12 (70.59)	4.12	1.10-17.42	0.0418	18	6 (33.33)	1.44	0.37-5.55	0.5891
Sex										
female	47	29 (61.70)		referent		47	16 (34.04)		referent	
male	38	19 (48.72)	1.22	0.44-3.54	0.7095	39	7 (17.95)	0.56	0.18-1.61	0.2902

5.3.8 Statistics

In logistic regression model to predict serostatus, both kennel and age were significant predictors whereas sex was not (Table 5.5). The odds of being seropositive were 6.06 (95% CI 1.67-25.60) times greater for dogs at kennel B than at kennel A (p=0.0088). Seropositivity increased with dog age, in which the odds of being seropositive were 4.12 (95% CI 1.10-17.42) times greater for dogs greater than 6 years of age than dogs less than 2 years of age (p=0.0418). In the logistic regression model to predict blood PCR status, no predictor variables were significant.

5.4 Discussion

Over half (57.6%) of a population of working hound dogs in this study were seropositive for *T. cruzi*, and over one quarter (26.7%) harbored parasite DNA in their blood, indicating active infections (Table 5.2). Additionally, we documented parasite DNA in opportunistically collected heart, mammary, and uterine tissues in dogs from this network. A remarkably high (80.6%) prevalence of infection was found in triatomines recovered from premises around the dog kennels. Infected young dogs and the presence of infected kissing bug vectors in the same kennels suggest these kennels of working dogs may be important nidi of *T. cruzi* transmission.

The 57.6% seroprevalence in these kenneled working dogs is much higher than the 8.8% seroprevalence found in a more general population of dogs across Texas (Tenney et al., 2014)(. This difference is similar to findings in Louisiana, in which kenneled hunting dogs had a seroprevalence of 51.6%, which was higher than the 22.1% seroprevalence reflected in a more general population of dogs in the surrounding area (Nieto et al., 2009). This population of working dogs could be considered at high risk to acquiring *T. cruzi* infection due to the presence of infected vectors in the kennel environment as well as in the outdoor working settings.

Antibody-detecting indirect fluorescent antibody (IFA) testing and immunochromatographic ‘dipstick’ tests have been used in dogs to provide evidence of circulating antibodies and history of exposure to *T. cruzi* (Cardinal et al., 2006; Nieto et al., 2009; A. Rosypal et al., 2010). In this study, both serological diagnostic approaches

(IFA and Chagas Stat-Pak) resulted in similar population-level estimates of seroprevalence (64.0% vs. 63.5%, (Table 5.1), however there was variation in the apparent status of some individual dogs. Of ten samples with discordant Chagas Stat-Pak and IFA results, seven were negative on one test and only faintly positive (faint band or 20 titer) on the other; three dogs were negative on Chagas Stat-Pak with titers of 80 and 160. Whereas we interpreted any development of color to indicate a positive result for the test, others have considered faint band development as negative (Nieto et al., 2009). Additionally, in this study, sera dilutions for IFA began at 1:20, and any titer value was considered positive, as per TVMDL reporting standards. In other studies, dilutions of ≥1:128 or ≥1:160 were interpreted as serologically positive (Burkholder et al., 1980; Kjos et al., 2008); however, a dilution as low as 1:16 has also been considered positive (Malan et al., 2006). A previous study found titer values from 120 to 320 in chronically infected dogs (Andrade et al., 1997). Low titers on the IFA may result from *T. cruzi* strain type variation, weak immune response, or an early, rising antibody response to a recent infection. Considering these variations and potential shortcoming, we chose to use two lines of evidence to consider a dog seropositive. However, it may be that the ten dogs with discordant serological results do reflect true infections (Tarleton et al., 2014); therefore, the population-level seroprevalence estimate we present is conservative. When testing dogs in a diagnostic veterinary setting, it would be prudent to consider retesting dogs that present with a low titer or equivocal serostatus at first testing.

There have been limited studies detecting *T. cruzi* in US dog populations, and there is a lack of information regarding the use and interpretation of concurrent serologic and molecular testing. One survey found evidence of *T. cruzi* DNA in 6% of dog blood samples and a seroprevalence of 8.8% in dogs from shelters across Texas (Tenney et al., 2014). Prior research in chronically-infected experimental dogs has demonstrated that multiple extractions and PCRs are needed to ensure detection of *T. cruzi* DNA from whole blood samples (Araújo et al., 2002), although another study found that *T. cruzi* DNA was more likely to be detected the blood clot (which was used in this study) than buffy coat or whole blood samples (Fitzwater et al., 2008). One study conducting

controlled experimental reinfection in dogs found that parasitemia was not as characteristic in reinfections as initial infection, and that parasitemia profile varied depending upon the individual dog (Machado et al., 2001). While PCR of samples does not confirm the presence of whole, viable parasites in the blood, findings of parasite DNA in the blood suggest that positive dogs could potentially be infectious to blood-feeding insect vectors. It is likely the high prevalence of *T. cruzi* DNA found in this study (26.7%) reflects the timing of the blood sampling (late July) corresponding with the time of year kissing bugs are most likely to be encountered in Texas (Curtis-Robles et al., 2015). With potential for continued exposure to kissing bugs and repeat infections with *T. cruzi*, it is possible that dogs with positive serological and positive PCR results (Table 5.2) could have been recently reinfected. Parasite DNA was detected in samples of two dogs that were considered seronegative, based on our definition of serologic positivity, but did have low IFA titers (20 and 80); it is possible that these dogs were acutely infected and just starting to develop detectable levels of antibodies. An additional diagnostic difficulty is that detection likely depends upon *T. cruzi* strain type and whether the individual is in the acute, indeterminate, or chronic stage of disease. More research is needed regarding the effect of *T. cruzi* strain and reinfection on canine antibody response, parasite DNA detection, and infectiousness.

The data presented here offer insight to an important concern among dog owners and veterinarians—the risk of blood-borne parasite transmission from an infected dog to other dogs. While serological tests are the most frequently used to diagnose *T. cruzi* infection in canines, we found parasite DNA present in blood samples from four dogs that were not seropositive. In addition, two dogs had low titers, no development of band on Chagas Stat-Pak and yet were positive by PCR, indicating a possible acute, recent infection. Based on this evidence, we echo previous suggestions for preventing infection (Bradley et al., 2000); it is prudent for anyone handling potentially infected dog blood to use personal protective equipment to avoid contact with parasites in blood.

In comparing molecular and histology results, we found that all four PCR-positive hearts subjected to histology were associated with lesions consistent with

chronic canine Chagas disease, however no amastigotes were seen in heart samples (Table 5.3). The lack of apparent amastigotes is not surprising, however, given that experimental studies have shown that parasites are not always histologically detected in cardiac tissue of chronically infected dogs (Andrade et al., 1997; Machado et al., 2001). Further, *T. cruzi* strain type can also influence level of cardiac damage and presence of amastigotes (Veloso et al., 2008).

There is an interest, particularly in the canine breeding community, in whether *T. cruzi* can be sexually transmitted between dogs. In order to investigate whether *T. cruzi* might be found in reproductive tissues, we used PCR testing to evaluate testicle and uterine samples from seropositive dogs. None of four testicle samples were positive, but small sample size and conflicting reports in previous literature (Junqueira de Alvarenga, 1960; Lamano Carvalho et al., 1991; Tavares et al., 1994) leave us unable to draw any conclusions. Of four uteri tested, we detected a single positive uterus in which four of the five samples taken from the body of the uterus were positive. The mix of positive and negative samples suggests that *T. cruzi* distribution in the tissue is not uniform, and additional testing of apparently negative tissues may reveal infection. The detection of parasite DNA in uterine tissue supports previous reports of transplacental transmission of this parasite in dogs and in humans (Rodríguez-Morales et al., 2011; Torrico et al., 2004). In addition to reproductive tissues, the potential for transmammary transmission has been suggested by others (Barr et al., 1995; Gürtler et al., 1986a). Our finding of *T. cruzi* DNA in mammary gland tissue was in a dog that also had evidence of parasites in heart and uterine tissue.

Congenital transmission of *T. cruzi* in dogs has been shown previously (Rodríguez-Morales et al., 2011) and is a concern to breeders in *T. cruzi* endemic regions. Although the cross-sectional nature of this study does not allow us to know the infection status of dams at time of whelping, we reviewed seroprevalence records in the context of relationships among dams and offspring. Five litters had both seropositive and seronegative siblings (Figure 5). Our observation of seropositive and seronegative siblings from a litter of a dam that was seronegative at time of sampling may be most

readily explained by local vector-borne transmission rather than congenital transmission. In contrast, we also observed several bitches that were seropositive at the time of testing and had both seropositive and seronegative offspring. The possibility of transplacental or transmammary transmission of parasites in these dogs is unknown because the serostatus at the time of whelping was not determined. Although no parasite DNA was detected in six young pups from a seropositive dam, faint bands produced on the Chagas Stat-Pak test on early blood draws might be the result of maternal antibodies circulating in the pups.

Our finding that older dogs (>6 years old) were more likely to be seropositive than young dogs (<2 years old) (Table 5.5) is expected and has been previously detected (Gürtler et al., 1986a; Rowland et al., 2010), since older dogs have had longer opportunity to be exposed to *T. cruzi* and develop life-long seropositivity. It is unclear why dogs in kennel B were more likely to be seropositive than dogs in kennel A; one potential risk factor not examined in this study was additional outdoor kennels at kennel B that possibly served as refugia for triatomine bugs.

We found a high prevalence (>80%) of infection in kissing bugs collected from kennels. This level of infection is higher than recent statewide estimates of 63% and 51% (Curtis-Robles et al., 2015; Kjos et al., 2009). Blood meal analysis of the guts of triatomines collected from the kennels revealed that all bugs that were evaluated had fed on dogs (Table 5.4). Other studies in the US have found evidence of dog blood in triatomines, including bugs associated with houses and dog kennels (Gorchakov et al., 2016; Kjos et al., 2013; Stevens et al., 2012; Waleckx et al., 2014). High infection prevalence in vectors collected from canine quarters in combination with evidence of canine feeding in these bugs confirms vector-host contact and supports vector-based parasite transmission to dogs.

Based on a limited subset of samples for which strain-types were determined, we found dogs infected with DTU TcI and dogs infected with TcIV in this study, and one dog with evidence of TcI in cardiac tissue and TcIV in uterine tissue. Previous strain typing efforts of limited dog samples from the US have shown almost exclusively TcIV

infections (Patel et al., 2012; Roellig et al., 2008), although one TcI/TcIV mixed infection was documented in a US dog (Roellig et al., 2008), and mixed strain infections have been documented in dogs in Columbia (Ramírez et al., 2013). Consistent with the strain-typing in the dog samples, we found both TcI and TcIV circulating in insect vectors collected from kennels (Table 5.4). *T. gerstaeckeri* were primarily infected with TcI, and *T. sanguisuga* were primarily infected with TcIV, in contrast to previous findings of only TcI in limited *T. sanguisuga* samples from the eastern US (Roellig et al., 2008). Differing geographic distribution of these species (Kjos et al., 2009) may put geographically disparate kennels and dog populations at risk of acquiring different strains of *T. cruzi*. Previous research suggests that parasitemia, antibody development, and disease vary according to strain type of *T. cruzi*, as well as length of infection and infected host species (Barr et al., 1991b; Coura et al., 1984; Roellig et al., 2009a; Veloso et al., 2008). It is also important to note that our success of amplification of the TcSC5D locus was limited to only five of the twenty-three positive blood samples. The TcSC5D gene target assay was originally developed using pure parasite culture (Cosentino and Agüero, 2012), and its usefulness for field-collected samples may be limited (Cominetti et al., 2014; Curtis-Robles et al., 2016). Much work remains to be done concerning the pathogenicity of strain types circulating in Texas, as well as whether dogs are more susceptible to and/or sustain a particular strain type.

In the absence of available antiparasitic treatments for infected dogs, prevention of canine Chagas disease relies on vector control. Integrated pest management strategies consisting of pesticide use, barrier methods (netting or mesh around kennels), and physical management of dogs (moving dogs to indoor facilities at night) have been employed in different areas around the state. Selected pesticide-impregnated collars marketed for other pests have been shown to reduce populations of triatomines in Argentina (Reithinger et al., 2009, 2005), and may also show promise to reduce the degree to which triatomines feed on dogs in the southern US. However, the use of pesticide-impregnated collars would be unlikely to prevent transmission from canine consumption of bugs. A key contrast in the role of canines in the *T. cruzi* transmission

cycle in central Texas versus the role in areas throughout Latin America is tied to the differences in the human-dog relationship as well as socioeconomic/housing differences and bug ecology. Although dogs have been shown to be important *T. cruzi* reservoir hosts in areas of Latin America (Estrada-Franco et al., 2006; Gürtler et al., 1986b), dogs in central Texas are typically housed either in a kennel separate and somewhat distant from the human dwelling or indoors in a house constructed with screens and doors that limit bug entry. Although parasitemic dogs in a kennel setting may not play a key reservoir role in relation to human infection, they do serve as reservoirs within the kennel setting: positive dogs can be fed on by kissing bugs that can then transmit *T. cruzi* to other dogs in the kennel. Canine kennels likely represent a high-risk environment for *T. cruzi* transmission, in which both vectors and infected dogs play important roles in maintaining parasite infection in the population.

6. HIGH *TRYPANOSOMA CRUZI* INFECTION PREVALENCE ASSOCIATED WITH MINIMAL CARDIAC PATHOLOGY AMONG WILD CARNIVORES IN CENTRAL TEXAS*

6.1 Introduction

The vector-borne protozoal parasite *Trypanosoma cruzi* is the etiologic agent of Chagas disease in humans and domestic canines. Vectors of *T. cruzi*, blood-feeding triatomine insects also called 'kissing bugs', are found throughout the Americas, including Texas (Kjos et al., 2009). Infection with parasites may occur after introduction of infected triatomine insect fecal material into a wound or mucous membrane, as well as orally (consumption of kissing bugs or their feces), congenitally, or through transfusion/transplantation (Bern et al., 2011).

Diverse wildlife species serve as reservoirs of *T. cruzi* across the parasite's range, and our understanding of the relative importance of wildlife reservoirs likely reflects not only vertebrate life history, especially as it relates to vector contact, but has likely been limited by the difficulty to collect large sample sizes from across broad geographic areas. With respect to life histories, although any mammalian species can potentially become infected with *T. cruzi* (Bern et al., 2011), the species that interact most frequently with kissing bugs have the opportunity to become infected or serve as the source of an infection. For example, in the southern United States, *Neotoma spp.* woodrats are well-recognized wild *T. cruzi* reservoirs, reflecting their association with nests commonly infested by triatomine nymphs and adults (Eads et al., 1963; Kjos et al., 2009). In South America, palm trees are an important ecological niche for contact between the *Rhodnius* genus of triatomines and opossums, which are a recognized reservoir of *T. cruzi* (Gaunt and Miles, 2000). With respect to the search effort, the ideal assessment of wildlife species' contributions to the enzootic transmission cycle would be

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to first have an unbiased assessment of triatomine feeding patterns, and then study those vertebrate species known to provide blood meals to the vector. A recent metaanalysis of triatomine feeding patterns suggests, however, that rather than innate preference for host species, host utilization by kissing bugs is dictated by the habitat they colonize (Rabinovich et al., 2011). Finally, with few exceptions (Barr et al., 1991a; Pietrzak and Pung, 1998; Roellig et al., 2009a), wildlife reservoirs studies do not typically address how infection may relate to disease within infected individuals, likely because of sampling limitations.

The purpose of this study was to survey populations of raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*) in central Texas to determine infection prevalence and circulating *T. cruzi* strain types. Further, we aimed to study the relationships among parasite infection in blood, infection in different areas of the heart, and the manifestation of cardiac pathology. Each of these species has previously been shown to be exposed to or infected with *T. cruzi* (Brown et al., 2010; Burkholder et al., 1980; Charles et al., 2013; Kribs-Zaleta, 2010; A. C. Rosypal et al., 2010). Central Texas is a region where at least four species of infected triatomine species occur (Curtis-Robles et al., 2015; Kjos et al., 2009) and where human and canine Chagas disease have been diagnosed (Texas Department of State Health Services, 2015a, 2015b). These particular wildlife species were selected for study due to their high population densities across Texas and 'varmint' status among ranch owners, which results in efforts to reduce population size through recreational hunting--hunting potentially posing a human health risk from hunter contact with infected wildlife tissues (Pung et al., 1995; Yeager, 1961).

6.2 Materials and Methods

6.2.1 Sampling

We conducted a cross-sectional wildlife study in January 2014 at a hunting check station for a recreational nuisance predator hunt in central Texas. This event was organized by a group of private landowners as a predator calling competition of animals

considered pests to Texas ranches. Animals legally harvested by teams of hunters over a 24-hour period were brought to a central check station where teams were awarded for their harvest. Our team collected samples from animals in which harvest location information was available. Animals in our study were harvested from 27 counties in central Texas and included: raccoons, coyotes, foxes, and bobcats. We performed a field necropsy, at which time we removed the heart from each animal within 24 hours of death and stored at -20°C until further processing.

In the laboratory, hearts were examined grossly and dissected in order to obtain an approximate 1cm3 section of each of the apex and right ventricular free wall for molecular testing. In some cases, portions of the cardiac tissue had been destroyed during hunter harvest, and so paired samples of right ventricle and apex were not always possible. When present, blood clots were collected from within the chambers of the heart during the dissection and frozen until further processing occurred. A section of right ventricular free wall was prepared in 10% formalin for histological examination. Additionally, any gross lesions were described and preserved as above for histological examination.

6.2.2 Molecular work

6.2.2.1 DNA extraction and *T. cruzi* detection

DNA was extracted from heart apex, right ventricle, and blood clot samples using the Omega E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA). No-template controls were included in each set of DNA extractions and molecular grade water was included as negative controls in PCR reactions. In order to detect presence of *T. cruzi* DNA, a 166-bp segment of the *T. cruzi* 195-bp repetitive satellite DNA was amplified using a Taqman qPCR reaction with Cruzi 1, 2, and 3 primers and probe (Duffy et al., 2013; Piron et al., 2007). This approach has previously been shown as both sensitive and specific for *T. cruzi* (Schijman et al., 2011). Reactions consisted of 5µL of template DNA, primers at a final concentration of 0.75µM each, 0.25µM of probe, and iTaq Universal Probes Supermix (BioRad Laboratories, Hercules, CA), in a total volume

of 20µL in a Stratagene MxPro3000 instrument (Agilent Technologies, Santa Clara, CA), following previously described thermocycling parameters (Duffy et al., 2013), except with a reduced, 3-minute, initial denaturation. The DNA extracted from *T. cruzi* strain Sylvio X10 (American Type Culture Collection, Manassas, VA), as well as DNA extracted from *T. cruzi*-positive kissing bugs collected in Texas (*Triatoma gerstaeckeri*, *T. lecticularia*, and *T. sanguisuga*), served as positive controls. After each reaction, the machine-calculated threshold was visually confirmed as reliable, and all reaction curves were visually checked for appropriate shape indicating successful amplification. Internal laboratory validation tests have defined cycle threshold (Ct) values indicative of positive (<31), negative (>33), and equivocal (between 31-33) status.

Samples classified as equivocal status after qPCR were subjected to confirmatory testing to determine sample status using the *T. cruzi* 121/122 primers to amplify a 330bp region of kinetoplast DNA (Virreira et al., 2003; Wincker et al., 1994). Reactions consisted of 1µL template DNA, primers at a final concentration of 0.75µM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final volume of 15µL. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Any sample that yielded a band at the appropriate fragment size (330bp) was interpreted as a positive sample, and those with no target fragments were considered negative in our calculations.

6.2.2.2 Strain-typing using TcSC5D PCR

All positive samples that yielded Ct values lower than approximately 25 on the qPCR were subjected to an additional PCR and subsequent DNA sequencing in order to determine the *T. cruzi* discrete typing unit (DTU). We performed a PCR to amplify the TcSC5D putative sterol oxidase gene (Cosentino and Agüero, 2012). Reactions consisted of 1µL extracted DNA, 0.75µM of each primer, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a total volume of 15µL. PCR products were visualized with gel electrophoresis as described above. Target amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and bi-directionally sequenced

on either an ABI Prism® 3130 Genetic Analyzer or ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Florida DNA Sequencing Core Laboratory (Gainesville, FL). Resulting sequences and chromatographs were reviewed visually using Geneious version R7 (Kearse et al., 2012) and MEGA version 6 (Tamura et al., 2013) to confirm quality, align forward and reverse sequences, and examine the locations of key SNPs used to designate DTU (Cosentino and Agüero, 2012). Representative *T. cruzi* sequences were deposited to Genbank (Accession nos. KU705713-KU705715).

6.2.3 Histology

Histopathologic examination of right ventricle sections was performed on a total of 51 animals, including a random subset of 19 animals in which *T. cruzi* DNA was detected in heart tissue, and 32 animals in which *T. cruzi* was not detected in heart tissue was performed at the Texas A&M Veterinary Medical Diagnostic Laboratory. Formalin-fixed samples were routinely processed, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin and eosin. All sections were scanned in their entirety at low power (20-40x), and areas of inflammation were examined at high power (200-400x) in an attempt to detect amastigotes. The viewer did not know the PCR status of the tissues examined.

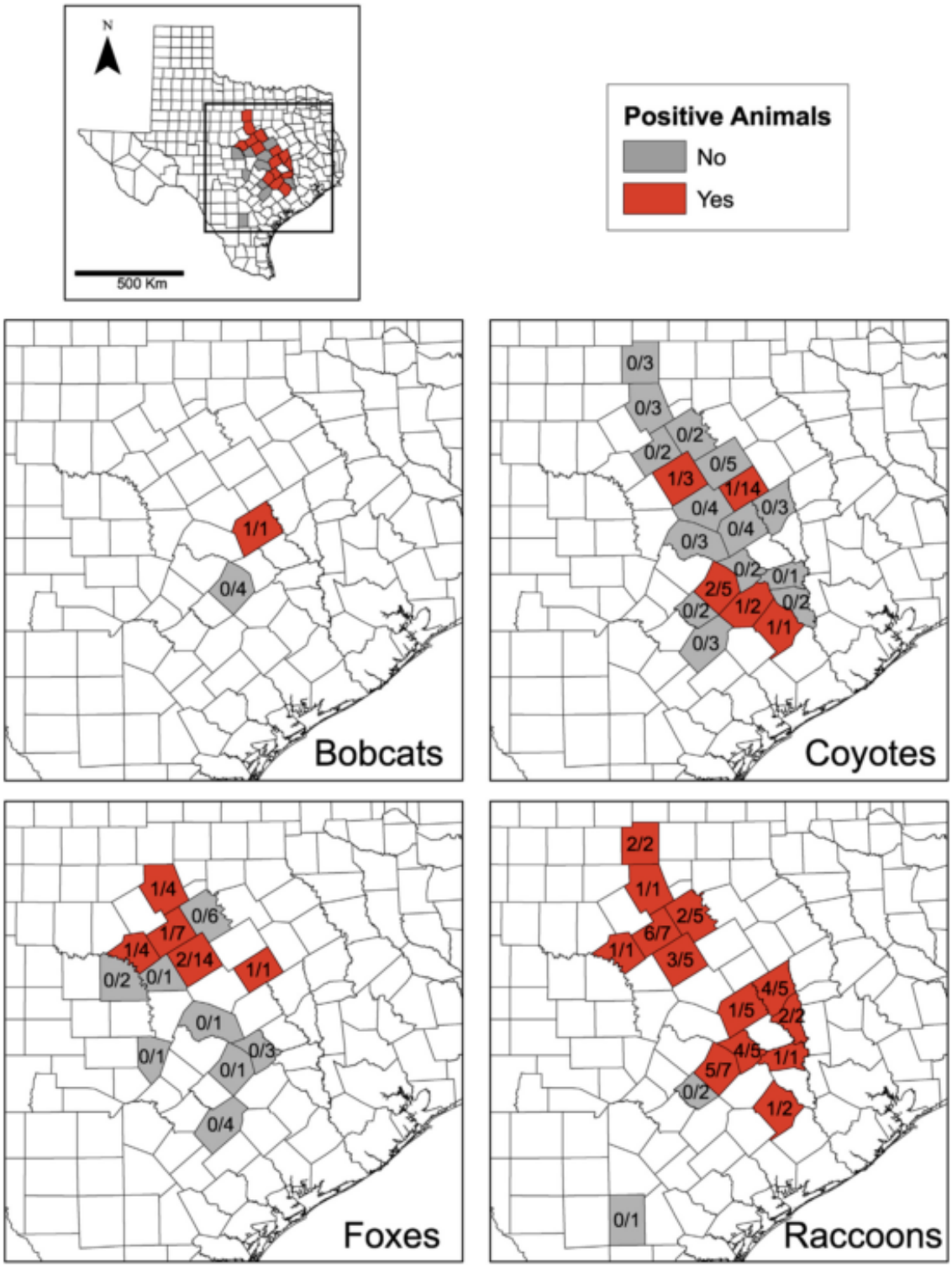

Figure 6.1. Spatial occurrence and distribution of *T. cruzi* infected, hunter-harvested wildlife, 2014. Number of infected over total number of that species tested are shown by county.

6.3 Results

6.3.1 T. cruzi *detection*

A total of 226 wild animals were included in our study, including bobcats (n=14), coyotes (n=84), foxes (n=58), and raccoons (n=70) from 25 counties in central and south Texas (Figure 6.1). Paired right ventricle and apex samples were available from 219 animals, right ventricle only from 3 animals, and apex only from 4 samples. Blood clots from within heart chambers were recovered from 56 individuals. After the initial screening with qPCR, 45 samples were assigned an equivocal status and therefore subjected to additional, independent PCR, in which 41 (91.1%) were determined to be positive. Based on the results of our diagnostic algorithm, we determined that 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot; Table 6). Fifteen of the twenty-five sampled counties had at least one animal test positive for *T. cruzi* (Figure 6.1).

Table 6. *T. cruzi* infection prevalence as determined by PCR in hunter-harvested wildlife of central Texas, 2014. Number of positive samples over total number of that type of sample is shown with infection prevalence in parenthesis.

	Bobcat	Coyote	Fox	Raccoon
Apex	2/13 (15.4%)	8/83 (9.6%)	1/58 (1.7%)	43/69 (62.3%)
Right Ventricle	1/14 (7.1%)	8/82 (7.3%)	7/57 (12.3%)	34/68 (50.0%)
Clot	0/2 (0.0%)	4/25 (16.0%)	1/11 (9.1%)	14/18 (77.8%)
Individuals	2/14 (14.3%)	12/84 (14.3%)	8/58 (13.8%)	49/70 (70.0%)

6.3.2 T. cruzi *tropism in hearts and blood*

In comparisons of parasite molecular detection among multiple sample types from the same individuals (Figure 6.2), across 219 individuals in which both right ventricle and apex were tested, we found that analysis of the two different tissues yielded the same result for 194 animals (88.6%), whereas the right ventricle tested positive and apex tested negative for 9 animals (4.1%) and the right ventricle tested negative and

apex tested positive for 16 animals (7.3%). In 54 individuals, blood clot was also tested in addition to right ventricle and apex. We found that the blood clot tested positive when both right ventricle and apex tested positive in 7 of 8 animals (87.5%), whereas the blood clot tested positive when both right ventricle and apex tested negative in 7 of 38 animals (18.4%).

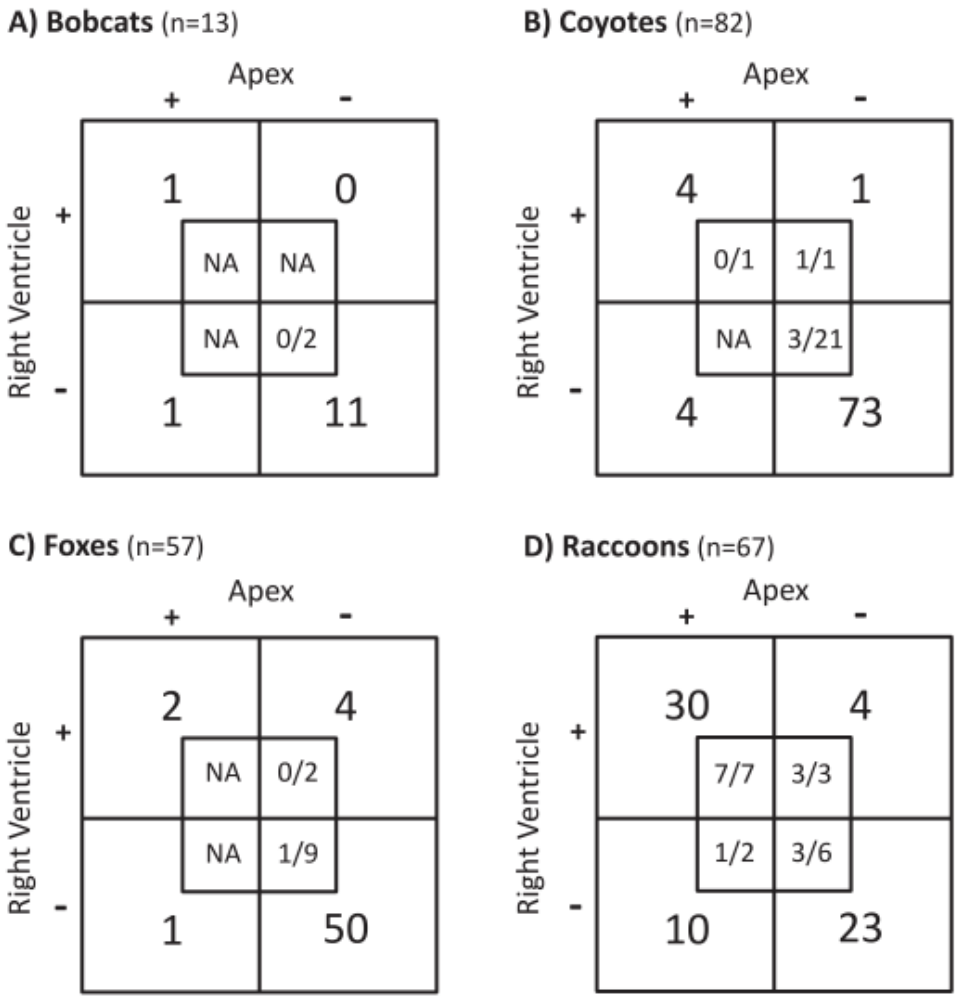

Figure 6.2. Tissue-specific PCR results of *T. cruzi* testing. For each species, the outer 2 x 2 table cell values represent the number of animals with all possibilities of results based on analysis of right ventricle and apex. The inner cell data include the *T. cruzi* infection status of blood clots (number positive/number tested) stratified by the infection status of the RV and apex. Not all animals had matched samples form each apex, right ventricle, and clot.

6.3.3 T. cruzi *strain determination*

Amplification by PCR using the TcSC5D primer pair was attempted on 31 samples. This included 17 apex, 2 clot, and 12 right ventricle samples from a total of 21 animals, of which 13 samples (9 apex, 1 clot, and 3 right ventricle) from 11 raccoons were successfully sequenced at the TcSC5D locus. Visual inspection of key SNPs (Cosentino and Agüero, 2012) classified ten as TcIV and one as mixed TcI and TcIV.

6.3.4 Gross and histopathology

Grossly, approximately half of all hearts exhibited one more pale or tan focal areas of discoloration that extended approximately 1mm into the myocardium; these were not associated with any histologic lesions and were determined to represent autolysis or freeze/thaw artifact that did not affect overall histologic evaluation. In a single fox heart, a nematode was present. No other gross abnormalities were noted. Histologically, of the 32 right ventricle samples examined that came from PCR-negative hearts (1 bobcat, 14 coyote, 8 fox, 9 raccoon), 31 were found to have no significant lesions and a single raccoon (3.1% of uninfected animals) was characterized by minimal, multifocal myofiber mineralization with no inflammation or cysts (an incidental finding). Of the 19 right ventricle samples examined that came from PCR-positive hearts (4 coyote, 3 fox, 12 raccoon), 15 (78.9% of histologically examined infected animals) were found to have no significant lesions and 4 animals (21.1% of histologically examined infected animals) were associated with some cardiac pathology. One infected coyote and three infected raccoons were characterized by minimal, focal lymphoplasmacytic myocarditis. Two of these raccoons were infected with TcIV, whereas the strain of *T. cruzi* in the coyote or third raccoon with noted cardiac pathology was not determined.

6.4 Discussion

We found widespread *T. cruzi* infection across a central Texas wildlife community (n=226 individuals of 4 species), in which bobcat, coyote, and fox shared a

similar prevalence of infection between 13-14%, whereas over 70% of sympatric raccoons were infected. Infected animals occurred in 60% of the sampled counties within the 25-county central Texas study region. Previous raccoon studies in Texas have ranged from 24% hemoculture-positive of 25 raccoons sampled in west-central Texas (Schaffer et al., 1978) to 0% seroprevalence in nine raccoons sampled in southern Texas (Burkholder et al., 1980). However, in other southern states, studies based on hemoculture, analysis of blood smears, or serology have revealed infection rates in raccoons as high as 68% (Brown et al., 2010; Kribs-Zaleta, 2010). Similar to our findings, previous coyote studies in Texas, have found 12.8%-14% seropositive (Burkholder et al., 1980; Grögl et al., 1984). Previous bobcat testing in Texas included 2 bobcats, one of which was seropositive (Burkholder et al., 1980). Although *T. cruzi* antibodies have been documented in foxes from other states (A. C. Rosypal et al., 2010; Rosypal et al., 2007), we are not aware of previous testing in foxes in Texas.

In contrast to recent wildlife serological studies that identified evidence of exposure, but not active infection, our approach of direct parasite detection confirms the current infection of these individuals. Although our methods do not confirm the viability or transmissibility of the parasite in this wildlife community and do not quantify the reservoir status of each species (Gürtler and Cardinal, 2015) the high number of animals with infected blood suggests they may be infectious to triatomine vectors. Recent blood meal analysis studies have detected the presence of raccoon blood within kissing bugs in west central Texas (Kjos et al., 2013), and we recently amplified raccoon DNA from the dissected hindgut of a central Texas *T. gerstaeckeri* bug (unpublished data), suggesting further that these wildlife species and triatomines interact in nature, with the potential for *T. cruzi* transmission.

As shown previously in raccoons, parasite distribution may not be uniform throughout the heart, although up to five sampled sites within the heart, including the right ventricle, were likely to test positive by PCR in infected animals (James et al., 2002). With over 88% congruence in infection status across two sample types, we found that both right ventricle and apex were nearly equally suitable samples for PCR-based

detection of *T. cruzi* in naturally-infected wildlife. Further, we found that testing of blood revealed infection in over 18% of animals with negative heart tissues, suggesting that these animals were acutely-infected or parasitemic.

Although *T. cruzi* DNA was readily detected in heart tissue of the wildlife community, no amastigotes were seen in the survey of right ventricle sections, and only 21.1% of animals with histologically examined infected heart tissue were histologically characterized by minimal to mild lymphoplasmacytic myocarditis. The scarce cardiac pathology may certainly reflect that only a limited section of the heart was histologically evaluated, with only single section of right ventricle trimmed in. Additionally, a limited number of samples were histologically examined. One previous study of infected raccoons also failed to detect more than "mild, multifocal, and interstitial inflammation", possibly suggesting that the *T. cruzi* strain may not cause severe lesions in raccoons (Pietrzak and Pung, 1998). In contrast, a study of experimentally infected raccoons showed that a small sample of acutely infected raccoons displayed more severe lesions than chronically infected raccoons, and the authors suggested that wild-trapped raccoons displaying mild lesions may be in the chronic stage of infection (Roellig et al., 2009a). Previous experimental infection of mice with a *T. cruzi* isolate from a raccoon from South Carolina resulted in PCR-detectable DNA in some tissue samples, but no parasites were observed in histological sections (Yabsley and Noblet, 2002). Additionally, our molecular results may reflect the presence of acutely infected, parasitemic animals with positive blood within vessels in the heart and absence of infection of the cardiac myocytes. However, at least four animals in our study had negative blood clot samples and positive heart tissue, suggesting that the cardiac muscle itself was infected. The relatively high infection prevalence in raccoons could be explained if they are able to sustain infections without severe chronic pathological implications, which is supported by the lack of cardiac lesions in our histological investigation. This has been shown for dogs (Barr et al., 1991b) and may therefore extend to other Canidae members, such as coyotes and foxes, which similarly failed to show cardiac lesions in our study. One previous study of experimentally infected raccoons showed that raccoons in the chronic

stage of infection has less severe lesions in various tissue types than acutely infected raccoons, suggesting raccoons as host species able to sustain infection without long term severe lesions (Roellig et al., 2009a). In contrast, the animals with severe myocarditis and those that died as a result of infection would be unlikely to be harvested by recreational hunters and therefore are not represented in our sample. However, it could also be possible that infected animals become ill and more susceptible to hunting; in which case, they would be overrepresented in this sampling effort. The clinical effects of *T. cruzi* infection in wildlife species require further study.

The differences in infection prevalence in the four species included in this study may reflect life histories that lead to differential contact with vectors and the parasite. We suspect that in the same way that triatomines are known to co-habit the nests of woodrats and squirrels (Grijalva and Villacis, 2009; Kjos et al., 2009; Kofoid and McCulloch, 1916), that they may also co-habit raccoon nests and dens, leading to high risk of vector contact. It has been shown that woodrats perform 'vector control' in their nests by eating kissing bugs (Ryckman and Olsen, 1965; Wood, 1942), and the omnivorous diet of raccoons suggests the possibility that they may behave similarly. In fact, raccoon infection by *T. cruzi* after ingesting an infected triatomine has been demonstrated experimentally (Roellig et al., 2009b). In contrast, the generally more carnivorous diets of bobcat, fox, and coyote might lower their likelihood of ingesting a *T. cruzi* infected triatomine, although the likelihood of ingesting an infected small mammal may be greater. The degree to which predation of infected vectors or infected prey species contributes to the relative levels of infection in this wildlife community remains to be studied.

Current classification of the within-species genetic diversity of *T. cruzi* distinguishes six main discrete typing units (DTUs), TcI through TcVI (Zingales et al., 2009), which appear to have different ecological and epidemiological associations, as well as different geographical distributions (Zingales et al., 2012). Until recently, previous work in the southern United States has revealed exclusively TcI and TcIV in vector and wildlife samples (Roellig et al., 2013, 2008), and the small number of

infected humans that have been typed were all TcI (Roellig et al., 2008), although a recent study of small rodents in Louisiana has detected a low prevalence of TcII (Herrera et al., 2015). In our study the vast majority of *T. cruzi* that infected the raccoons was TcIV (90.1% of animals), and one individual harbored a mixed TcI/TcIV infection. Similarly, a previous study has shown TcIV circulating in raccoons (n=5) in the western part of Texas (Charles et al., 2013), and TcIV was the predominant strain found in raccoons in Georgia, Florida, and other eastern states (Roellig et al., 2013). The strain-typing assay we employed was based upon the amplification and sequencing of a single gene target (the TcSd5d gene;(Cosentino and Agüero, 2012)) and therefore is an economical and efficient method relative to strain-typing approaches based upon multilocus sequence typing. However, this method was developed for typing of pure *T. cruzi* cultures, and does not appear to be very sensitive for field-collected samples that contain mixed populations of DNA (i.e., a majority population of host DNA and minority population of parasite DNA). Among our samples, some were determined to be positive for *T. cruzi* on the basis of qPCR or conventional PCR to target a small gene fragment, yet negative on the strain-typing assay. The degree to which the proportion of TcI, TcIV, and mixed infections that we detected in raccoons reflects any preferential amplification in this assay is unknown. It has previously been shown that laboratory mice and rats infected with what is now known as TcIV from wildlife the US did not experience severe morbidity or mortality (Roellig and Yabsley, 2010), which may indicate a less virulent strain that could more easily be sustained in natural cycles. Nonetheless, while the significance of TcIV from a human and canine health perspective in the southern states is yet to be established, our identification of the raccoon as a reservoir of TcIV provides increased understanding of the natural history of this parasite strain.

Although the exact risk of *T. cruzi* transmission from infected wildlife to hunters is unknown, it has already been suggested by others that infected wildlife do pose a risk to hunters during field-dressing and skinning due to potential direct contact with infected materials (Bern et al., 2011; Garcia et al., 2015; Yeager, 1961). This study documents

infection in a variety of wildlife species that are commonly hunted in Texas and other areas, and public health outreach efforts—including education about Chagas disease, triatomine bugs, and use of personal protective equipment when handing animals—directed to hunters should be undertaken in order to reduce risk to human public health.

7. SUMMARY

This dissertation work has resulted in a large collection of triatomines from across Texas and the southern US, as well as generated additional knowledge of *T. cruzi* infection in dog and wildlife populations in Texas.

Submissions of kissing bugs encountered by the public in Texas and other states from 2013-2014 were accepted to the lab, while providing educational literature about Chagas disease. In the laboratory, kissing bugs were identified to species, dissected, and tested for *T. cruzi* infection. A total of 1,980 triatomines were submitted to the program comprised at least seven species, of which *T. gerstaeckeri* and *T. sanguisuga* were the most abundant (85.7% of submissions). Triatomines were most commonly collected from dog kennels and outdoor patios; overall 10.5% of triatomines were collected from inside the home. Triatomines were submitted from across Texas, including many counties which were not previously known to harbor kissing bugs. Kissing bugs were captured primarily throughout April-October, and peak activity occurred in June-July. Emails to our dedicated account regarding kissing bugs were more frequent in the summer months (June-August) than the rest of the year. *T. cruzi* was detected in 63.3% of tested bugs. Citizen science was found to be an efficient approach for generating data on the distribution, phenology, and infection prevalence of kissing bugs—vectors of the Chagas disease parasite—while educating the public and medical community.

In order to define spatial and temporal patterns of triatomines, a citizen science program and field collections from 2013-2015 resulted in a collection of 2638 kissing bugs from diverse ecological regions in Texas, as well as 64 additional bugs from 6 additional southern US states. Seven different species of *Triatoma* were identified, with adults most commonly encountered between May and October. The two most common species, *T. gerstaeckeri* and *T. sanguisuga*, exhibited activity peaks in mid-summer and early fall, respectively. Point pattern analyses were conducted to compare the occurrence of each triatomine species against all other triatomine species collected. This analysis revealed unique occurrences of *T. sanguisuga* in north Texas, *T. gerstaeckeri* in west

Texas, *T. indictiva* in north central Texas, and *T. lecticularia* in north central Texas. These unique geographic occurrences of the different *Triatoma* species suggest associations with different suitable habitats, and serve as a basis for future models evaluating the ecological niche for the different vector species. Consideration of local temporal and spatial heterogeneity of *Triatoma* species occurring in Texas will allow targeting of vector control and medical/veterinary outreach initiatives to reduce human and animal vector contact.

T. cruzi DNA amplified from triatomine insects (*Triatoma* spp.) from across the southern US was used to determine infection prevalence and relative abundance of parasite DTUs. A *T. cruzi* infection prevalence of 58.9% was found in 1,226 triatomines of 6 species. In Texas, the state with the greatest number of specimens collected, infection prevalence varied by species: 64.8% in *T. gerstaeckeri* (n=795), 54.1% in *T. sanguisuga* (n=205), 50.0% in *T. indictiva* (n=56), 71.2% in *T. lecticularia* (n=52), no infection in *T. rubida* (n=5), and 25.0% infection in *T. protracta* (n=4). Adults were more likely than nymphs to be infected. Amplification and sequencing of the TcSC5D gene revealed *T. cruzi* DTUs TcI and TcIV present in triatomine vectors across Texas. *Triatoma gerstaeckeri*, the species with the greatest number of typed samples (n=167), was found approximately equally infected with TcI (42.0%) and TcIV (52.0%), and 10 individuals (6.0%) showed mixed TcI/TcIV infections. In contrast, *Triatoma sanguisuga* (n=39) was more frequently found infected with TcIV (87.2%) than TcI (12.8%). *T. sanguisuga* was significantly more likely (OR=5.49, 95% CI 1.99-18.95) to be infected with TcI than *T. gerstaeckeri*. Relative abundance of parasite DTUs varied spatially, with both TcI and TcIV co-circulating nearly equally in vectors in central Texas, while TcIV predominated in northern Texas. Given prior findings implicating TcI in US human Chagas disease cases, knowledge of the spatial distribution of *T. cruzi* infection and DTUs in vector populations is important to understanding the risk of *T. cruzi* infection to public health.

A cross-sectional study of *T. cruzi* infection in working hound dogs in south central Texas, a hotspot for local transmission, included analysis of triatomine vectors

collected within kennel environments. Paired IFA and Chagas Stat-Pak serological testing showed an overall seroprevalence of 57.6% (n=85). The odds of being seropositive were 4.12 (95% CI 1.10-17.42) times greater for dogs older than 6 years of age than dogs less than 2 years of age (p=0.0418). PCR analyses of blood revealed 26.7% of dogs, including both seronegative and seropositive dogs, harbored parasite DNA in their blood, suggesting active infections. Opportunistic post-mortem sampling and molecular screening of organs from seropositive dogs revealed parasite DNA in heart, uterus, and mammary tissues. Sequencing of the TcSC5D gene from blood and tissue samples showed parasite discrete typing units (DTU) TcI and TcIV were present, including a co-occurrence of both DTUs in an individual dog. Bloodmeal analysis of *Triatoma gerstaeckeri* and *Triatoma sanguisuga* triatomine insects collected from the kennels revealed exclusively dog DNA. Vector infection with *T. cruzi* was 80.6% (n=36), including DTUs TcI and TcIV. Tracing of infection status across litters of dogs showed serologically positive offspring of serologically negative dams without clear evidence of congenital transmission. Integrated canine and vector surveillance is an effective method for detecting robust enzootic transmission cycles.

Using a cross-sectional study design, cardiac tissue and blood were collected from hunter-donated wildlife carcasses—including raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*)—from central Texas, a region with established populations of infected triatomine vectors and increasing diagnoses of Chagas disease in domestic dogs. PCR analyses found that 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot). Although a histologic survey of right ventricles showed that 21.1% of 19 PCR-positive hearts were characterized by mild lymphoplasmocytic infiltration, no other lesions and no amastigotes were observed in any histologic section. DNA sequencing of the TcSC5D gene revealed that raccoons were infected with *T. cruzi* strain TcIV, and single raccoon harbored a TcI/TcIV mixed infection. Relative to other wildlife species tested here, these data suggest that raccoons may be important reservoirs of TcIV in Texas and

a source of infection for indigenous triatomine bugs. The overall high level of infection in this wildlife community likely reflects high levels of vector contact, including ingestion of bugs. Although the relationship between the sylvatic cycle of *T. cruzi* transmission and human disease risk in the United States has yet to be defined, these data suggest that hunters and wildlife professionals should take precautions to avoid direct contact with potentially infected wildlife tissues.

The research presented here advances our knowledge of *T. cruzi* ecoepidemiology in the southern US, most specifically in Texas. The results also lead to additional questions regarding *T. cruzi* host-vector transmission dynamics. A sampling of future research efforts that would be useful to pursue include: determination of dispersal drivers and potential of triatomine species, sampling of other wildlife species to determine the main sylvatic host(s) of TcI, study of disease progression in dogs infected with *T. cruzi*.

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