# THE ECOLOGY OF *Trypanosoma cruzi* AND ITS MAMMALIAN HOSTS IN TEXAS, USA

A Dissertation

by

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# ABSTRACT

*Trypanosoma cruzi*, agent of Chagas disease, is a zoonotic vector-borne protozoan that infects mammalian hosts throughout the Americas. Spillover from sylvatic cycles occurs when triatomine vectors bridge the parasite from wildlife to humans, dogs, or captive NHPs. Although knowledge of reservoir capacity of diverse wildlife could provide an ecological basis for disease management, most wildlife studies document exposure with little attention to infectiousness to vectors. Additionally, pathology investigations can provide key information on pathogenesis and population impacts.

To investigate *T. cruzi* infection prevalence, strain types, and risk factors in dogs, we collected blood from 611 dogs at shelters across Texas. 18.2% of dogs were seropositive for *T. cruzi*. Six (1.1%) dogs harbored parasite DNA in their blood, of which 5 were DTU TcI and 1 was TcIV.

To determine the trypanosome prevalence in Texas bats, we collected hearts and blood from bats across Texas. Of 593 bats, 1 was positive for *T. cruzi* (0.17%), 9 for *T. dionisii* (1.5%), and 5 for *Blastocrithidia* spp. (0.8%), a group of insect trypanosomes. The *T. cruzi*-infected bat was carrying TcI. In the *T. dionisii*-infected bats, we detected three unique variants associated with the three infected bat species.

To characterize the *T. cruzi* reservoir status and associated pathology in coyotes and raccoons in Texas, we collected hearts and/or blood from animals in Central and South Texas. Infection prevalence was greater in raccoons (66.7%) than coyotes (7.5%; $Z=-6.8$, $P<0.0002$). Most raccoons with *T. cruzi*-infected hearts also had positive blood (83%), in contrast to coyotes (17%). Histologic lesions were more severe in coyotes, with 4/6 PCR-

positive coyotes exhibiting mild to moderate lymphoplasmacytic inflammation and occasional myodegeneration and fibrosis. In contrast, raccoons had only very mild inflammation.

At a NHP facility in San Antonio, we tested 145 rats for *T. cruzi* infection and found no positives. Limited vector surveillance yielded no kissing bugs. At a NHP facility in Bastrop, we tested blood from NHPs, trapped free-ranging wild mammals, and collected kissing bugs. 80% of NHPs were PCR-positive, though parasite concentrations were low and intermittent. Raccoons, opossums, and skunks were highly infected with *T. cruzi*, while rodents were uninfected. Few kissing bugs were collected, but most were from a building very close to NHP housing.

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# NOMENCLATURE

| | |
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| CF | complement fixation |
| DTU | discrete typing unit (strain type) |
| ELISA | enzyme-linked immunosorbent assay |
| ICT | immunochromatographic test |
| IFA | indirect fluorescent antibody |
| IHA | indirect hemagglutination assay |
| IIF | indirect immunofluorescence |
| NHP | non-human primate |

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# 1. INTRODUCTION: TOWARD AN ECOLOGICAL FRAMEWORK FOR ASSESSING RESERVOIRS OF VECTOR-BORNE PATHOGENS: WILDLIFE RESERVOIRS OF *TRYPANOSOMA CRUZI* ACROSS THE SOUTHERN UNITED STATES[*]

## 1.1 Introduction

Identifying and characterizing reservoirs of vector-borne zoonotic pathogens is critical for disease management interventions that aim to dampen transmission in natural disease cycles in order to reduce spillover to humans. However, these pathogens are usually maintained in complex transmission cycles involving diverse vertebrate taxa and multiple arthropod vector species. Additionally, these systems are often heterogeneous across space and time, creating challenges for characterizing the wild reservoirs of vector-borne zoonoses (Figure 1.1). The purpose of our review is to provide a framework and highlight gaps in knowledge for the evaluation of candidate wildlife reservoirs of *Trypanosoma cruzi*, agent of Chagas disease (American trypanosomiasis), in the United States, although the approach we use is broadly applicable to any multihost vector-borne pathogen. *T. cruzi* is maintained in a complex multihost transmission system at enzootic levels in the Southern US. Despite the first report 100 years ago (Kofoid and Donat, 1933; Kofoid and McCulloch, 1916), the transmission cycles in the US and relative importance of different reservoir species have been relatively understudied. In the US, the disease poses a major threat to the health of domestic dogs and captive non-human primates (Dorn et al., 2012; Kjos et al., 2008), and

[*]Reprinted with permission from: Hodo, C.L., Hamer, S.A., 2017. Toward an ecological framework for assessing reservoirs of vector-borne pathogens: wildlife reservoirs of *Trypanosoma cruzi* across the southern United States. ILAR J. *in press*.

autochthonous transmission has been demonstrated in humans as well (Bern et al., 2011; Curtis-Robles et al., 2017b; Garcia et al., 2015; N. C. Woody and H. B. Woody, 1955). Domestic dogs are key reservoirs of *T. cruzi* in South America where the parasite is transmitted in domestic cycles with vector species that colonize the home (Gürtler and Cardinal, 2015). However, given ecological differences in *T. cruzi* vectors and transmission cycles in the southern United States (discussed below), we pose that wild species are critical

**Challenges in characterizing wildlife species as reservoirs of zoonotic pathogens**

- Diagnostic limitations
  - Diagnostic tests are rarely validated for wildlife species and species-specific controls are largely unavailable.
  - Even published serosurveys must be interpreted with caution in the absence of reliable sensitivity and specificity data.
- Sample size limitations
  - Large sample sizes are difficult to achieve
  - Studies are often biased toward species that are relatively easy to capture, leaving voids in understanding of more elusive species.
  - Study designs often utilize convenience sampling, and results may not be applicable to a broader population.
- Longitudinal studies are rarely feasible
  - Most wildlife sampling is done on a cross-sectional basis.
  - Individuals can be tracked over time using mark-recapture studies, but these are labor-intensive and associated with biases in trappability of animals.
- Permit requirements for wildlife research are daunting
  - Institutional animal use and care committees may be unfamiliar with wildlife field studies (Sikes and Bryan 2016).
  - *A priori* estimates of sample size or infection prevalence are often unknown.
  - Protocols for anesthesia and sampling are often optimized for laboratory animals and not translatable to wildlife.
- Statuses of host exposure or infection in relation to disease are ill-defined
  - Serosurveys demonstrate exposure and alone should not be used to evaluate reservoir potential
  - Wildlife pathology investigations can provide key information on pathogenesis and population impacts, but are rarely conducted across populations
- Limited experimental infection data
  - Wildlife species are not commonly used as animal models
  - Expectations for progression of disease and time course of infectiousness are typically extracted from domestic and laboratory species

**Figure 1.1 Challenges in characterizing wildlife species as reservoirs of zoonotic pathogens.** Reprinted with permission from (Hodo and Hamer, 2017).

for maintaining sylvatic transmission cycles and as a source of spillover to target hosts. Here, we outline a framework for assessing the relative importance of reservoir species, which will aid in the development of interventions that limit spillover to humans and domestic animals.

## 1.2 Ecological Framework for Defining and Characterizing Reservoirs

### *1.2.1 Definition of a Reservoir*

The definition of a reservoir is much discussed and has been refined in recent years. Haydon et al (Haydon et al., 2002) defined a reservoir as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population." This definition is appropriate for the multihost transmission system of *T. cruzi* (Gürtler and Cardinal, 2015). The target population is defined as the population or host species of interest or concern. Here, we discuss candidate wildlife reservoirs of *T. cruzi* in the context of the target populations of humans, dogs, and nonhuman primates, all of which are associated with increasing diagnoses of Chagas disease in the southern US (Figure 1.2).

In previous definitions, a criterion for a reservoir host is that it does not develop disease as a result of infection with the pathogen (Keane and Miller, 2003). However, it is clear from many systems that this is not a requirement for reservoir status. For example, rabies, Hendra, and Nipah viruses all have some pathogenicity to their reservoir host populations (Haydon et al., 2002). Degree and duration of disease can certainly influence the reservoir capacity of a host, however, by directly affecting the time during which it is available to pass the disease on to vectors or other hosts.

It is important to note that ability to be infected by the pathogen does not alone qualify a species as a reservoir. Thus, serological studies on their own are of limited use in determining reservoir potential, as they merely indicate exposure to the pathogen, and give little to no information about the ability of the host to infect vectors or other hosts. They can, however, be used in combination with other data to calculate reservoir competence. Because infection with *T. cruzi* is generally considered to be life-long, hosts that harbor anti-*T. cruzi* antibodies are also interpreted to be currently infected (Hall et al., 2007), though more research is needed in wildlife species. For this reason, in contrast to many other zoonotic pathogen systems, *T. cruzi* seroprevalence estimates can be considered interchangeable with infection prevalence estimates. Additionally, in vector-borne diseases, the presence of infected reservoirs alone does not pose a risk to the target host; the vector must be present and must come into contact with both the reservoirs and target hosts.

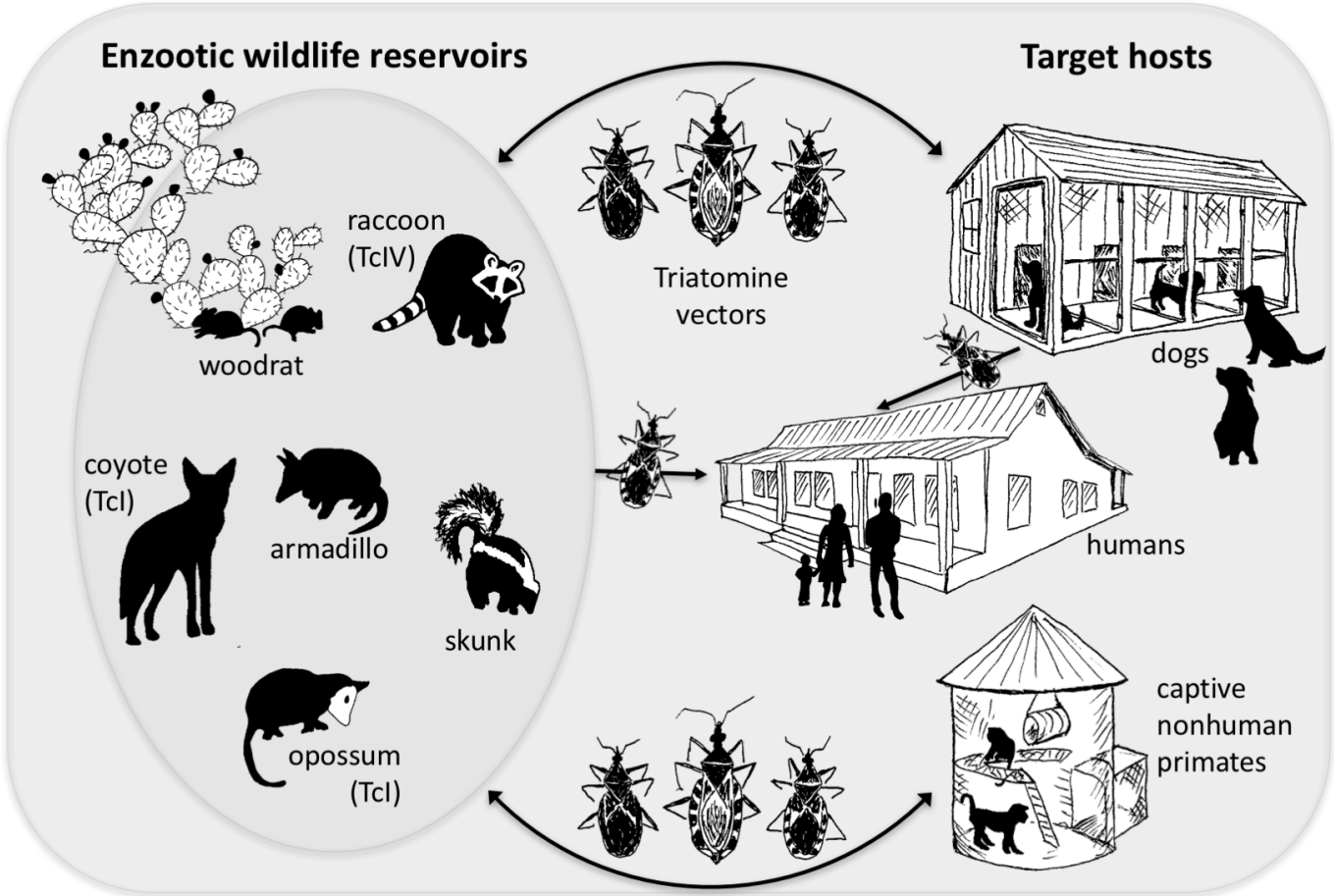


**Figure 1.2** ***Trypanosoma cruzi*** **transmission cycles in the southern US.** Current understanding of transmission cycles with wildlife hosts and well-characterized strain-type associations. In contrast to transmission settings across South and Central America and Mexico, in the southern US, exclusive domestic cycles appear less important in terms of risk to target hosts than does spillover from enzootic transmission. Original artwork by C. Hodo. Reprinted with permission from (Hodo and Hamer, 2017).

### *1.2.2 Importance of Identifying Reservoirs*

The identification of reservoirs is imperative for guiding intervention strategies to reduce transmission in multihost pathogen systems. While it is unlikely that any intervention into the sylvatic cycle could block transmission completely, identifying which species are the most important reservoirs serving to infect those vectors most likely to contact humans or other target hosts (pet dogs, non-human primates) could help to guide strategies to reduce spillover. For example, field vaccination of white-footed mice (*Peromyscus leucopus)*, the principle reservoir of *Borrelia burgdorferi* in the northeastern US, reduced the infection prevalence of the tick vector in the study sites (Richer et al., 2014). Additionally, aerial distribution of oral baits laden with a rabies vaccine targeted to raccoons has created an immune barrier to halt the westward spread of raccoon strain rabies in the northeast, and a similar program targeted to coyotes helped eliminate the canine strain of rabies from Texas (Slate et al., 2005).

### *1.2.3 Heterogeneity in Pathogen Transmission*

The rate of pathogen transmission (basic reproductive number, $R_0$) is not homogeneous across individuals or host species. Study of heterogeneities in transmission of vector-borne diseases and human sexually transmitted diseases led to the empirical 20/80 rule, which states that in general, 20% of the host population contributes to 80% of the net transmission potential (Woolhouse et al., 1997). Thus, interventions that do not completely block transmission from the most important 20% of the population would be much less effective than predicted given homogeneity of transmission potential. In extreme cases of transmission heterogeneity, only a few key individuals, known as “superspreaders,”

contribute disproportionately to the number of transmission events (Lloyd-Smith et al., 2005). This concept of superspreaders has been applied to whole species within multihost transmission systems; for example, American Robins serve as a superspreader of West Nile virus (Kilpatrick et al., 2006). Within a reservoir species, heterogeneity in contribution to transmission have been noted for guinea pig (*Cavia porcellus*) reservoirs of *T. cruzi* in urban Peru, where most individuals quickly control parasitaemia, but a subset of animals remains highly infectious to vectors for many months (Levy et al., 2015). Conversely, certain host species may have a relatively lower transmission potential and act to dampen the spread of pathogens, termed supersuppressors or dilution hosts. Examples here include Virginia opossum (*Didelphis virginiana*) which consume the ticks that vector the Lyme disease pathogen *Borrelia burgdorferi* and therefore serve as an ecological trap, and Northern Cardinals and Mimidae spp. that are fed upon by a disproportionate number of mosquitoes but are only moderately competent hosts for West Nile virus (Levine et al., 2016; Ostfeld and Keesing, 2000). However, some of these incompetent hosts still contribute to the overall transmission system by serving as bloodmeal sources and amplifying vector populations, as has been shown for deer in the Lyme disease system (Dobson and Randolph, 2011), and for chickens with *T. cruzi* (Gürtler and Cardinal, 2015).

### *1.2.4 Measures of Reservoir Importance*

Measures of the relative importance of different host species as reservoirs of a pathogen have been refined over the years in various disease systems. These have been expressed in terms of reservoir potential, reservoir competence, and reservoir capacity. The concept of *reservoir potential* was first introduced in the Lyme disease system and defined as

the relative contribution made by a host species to the horizontal infection of a vector population (Mather et al., 1989). Reservoir potential (Figure 1.3) is calculated as the product of the number of vectors fed by an individual of a given species and "realized reservoir competence," the probability that a vector feeding on a host species becomes infected (Brunner et al., 2008). *Reservoir competence* is therefore the product of the prevalence of host infection and host infectiousness. Considering pathogens for which the reservoir is composed of a group of connected populations (metapopulation), *reservoir capacity* is defined as a weighted measure of the potential of a host metapopulation to support long-term persistence of a pathogen in the absence of external imports (Viana et al., 2014). Using these concepts, Gürtler and Cardinal (2015) explored the relative contribution of certain domestic and peridomestic reservoirs of *T. cruzi* in light of three parameters: 1) host susceptibility, infection, and survival; 2) host infectiousness; and 3) host-vector contact. Although the terminology and mathematics surrounding these concepts vary, it is clear that evaluating reservoirs of vector-borne pathogens necessitates quantitative measures of the vertebrate species and their interactions with vectors, and very few studies are designed to fill this knowledge gap.

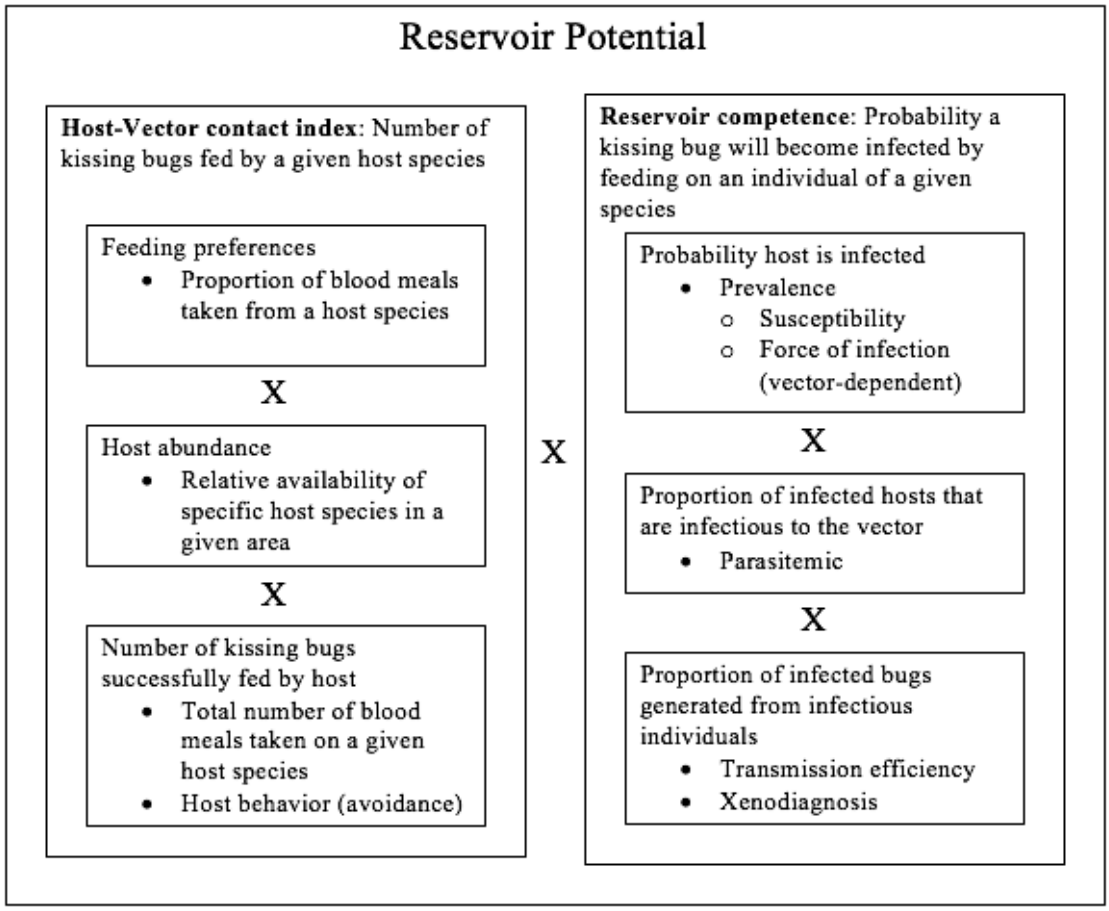


**Figure 1.3 Reservoir potential.** A conceptual framework for evaluating wildlife reservoirs of T. cruzi by determining reservoir potential (Mather et al., 1989; Brunner et al., 2008), an index of the relative importance of a reservoir host as a source of infection to vectors. Reprinted with permission from (Hodo and Hamer, 2017).

## 1.3 Trypanosoma cruzi Background

*T. cruzi* is a zoonotic vector-borne protozoan capable of infecting animals from virtually all mammalian orders (Gaunt and Miles, 2000). An estimated 6 million people are infected worldwide (World Health Organization, 2015), of which an estimated 240,000-300,000 reside in the US, though the true burden of human disease in the US is unknown due to a lack of recognition and reporting (Bern and Montgomery, 2009; Manne-Goehler et al., 2016). The disease is enzootic in triatomine insect vectors, wild mammals, and dogs in the

southern US (Bern et al., 2011). Autochthonous transmission to humans was first reported in the US in 1955 (N. C. Woody and H. B. Woody, 1955), and is increasingly recognized as a public health threat (Cantey et al., 2012; Garcia et al., 2015). In addition to the human health burden, *T. cruzi* infection is also a significant veterinary health problem in the southern US, with studies documenting 10-25% of dogs (Kjos et al., 2008; Tenney et al., 2014); Hamer SA, unpublished data) and a significant number of non-human primates (Bern et al., 2011; Dorn et al., 2012) being seropositive, the latter posing a threat to biomedical science initiatives that use non-human primate models.

There are 11 species of kissing bugs in the US, and the highest species diversity of triatomines is found in Texas (Bern et al., 2011). The insect vector acquires the trypomastigote stage of the *T. cruzi* parasite during blood feeding on an infected host, and the parasite replicates as epimastigotes in the digestive tract of the bug, maturing to infective metacyclic trypomastigotes in the hindgut, which are passed in the feces. The parasite can be transmitted through the stercorarian route when the insect defecates the infectious stage of the parasite onto the host during or shortly after blood feeding, which is then rubbed into the bite wound, broken skin, or a mucous membrane. Oral transmission has been implicated in outbreaks of acute human Chagas disease following consumption of contaminated juices, and oral transmission through the consumption of vectors is likely very important in sylvatic cycles, especially for omnivorous or insectivorous wildlife. In an experimental infection study, four striped skunks (*Mephitis mephitis*) were infected with *T. cruzi* intravenously or *per os* (Davis et al., 1980). Ingestion of infected insects was shown to cause infection in opossums, raccoons, and woodrats and is a probable route of infection in dogs (Montenegro et al., 2002; Roellig et al., 2009a; Ryckman, 1965; Yaeger, 1971). Transmission may also

occur via ingestion of a parasitemic animal (Rocha et al., 2013; Thomas et al., 2007). Additional alternative routes of transmission are transplacental and through blood transfusion or organ transplant.

*T. cruzi* is a genetically heterogeneous species and is comprised of 7 strain types or discrete typing units (DTUs), TcI-VI and TcBat. TcI has been divided into TcIdom and TcIsyl, representing domestic and sylvatic isolates (J. D. Ramírez et al., 2013). These strain types are associated with different geographical locations, reservoir host species, and reportedly, clinical manifestations (Jansen et al., 2015; J. D. Ramírez et al., 2010). TcI and TcIV are the most commonly reported DTUs in the US (Bern et al., 2011; Roellig et al., 2013), though TcII has been isolated from a small number of rodents (C. P. Herrera et al., 2015). More research is needed on the specific importance of these strain types in the US and their relevance to outcome of infection.

Across Latin America, *T. cruzi* is maintained in distinct transmission settings of domestic/peridomestic cycles - defined by vector species that are adapted to live predominantly in and around human dwellings and feed on inhabitants - and sylvatic cycles, with different vectors, reservoirs, and strain types associated with each (Zingales et al., 2012). Dogs, cats, commensal rodents and domesticated guinea pigs serve as predominant reservoirs in the peridomestic and domestic settings, whereas opossums, armadillos and rodents are major sylvatic reservoir hosts (Gürtler and Cardinal, 2015; Jansen et al., 2015). In the US, however, although there have been infrequent recent reports of both adult and nymphal kissing bugs found within homes in the US (Curtis-Robles et al., 2015; Klotz et al., 2016; Navin et al., 1985; Wozniak et al., 2015), truly domestic transmission cycles are rare, owing in part to different standards of housing and different species of triatomines.

Peridomestic and sylvatic bug activity is much more common and transmission to humans and other target taxa results from spillover from the enzootic cycles (Figure 1.2). Wildlife are important in the maintenance of the parasite in these sylvatic cycles, and better characterizing their relative importance as reservoirs is important in understanding the transmission of *T. cruzi* in the US.

## 1.4 Characterizing Reservoirs of *T. cruzi* in the US

### *1.4.1 Framework for Characterizing Reservoir Potential*

Reservoir potential, introduced by Mather et al. (1989), is an index of the relative importance of a reservoir host as a source of infection to vectors, and provides a useful framework for evaluating host species in multihost pathogen transmission systems. There are numerous reports of *T. cruzi* infection in various wildlife species in the US, but with little attention to the degree to which each species serves as a reservoir. Models of contact processes between triatomines and wildlife hosts concluded that the limiting factors of stercorarian transmission to hosts was dependent upon host species. In particular, the population density of vectors limited transmission to woodrats, whereas the population density of raccoons and opossums limited transmission to these hosts (Kribs-Zaleta, 2010). However, the author acknowledged a severe lack of data underlying parameter estimates and did not attempt to quantify a reservoir potential for the hosts discussed. While there is indeed a significant paucity of data on some criteria necessary for calculating the reservoir potential of candidate species in the US, we will discuss the available data to attempt to inform the following parameters, as outlined previously (Gürtler and Cardinal, 2015): 1) host susceptibility (proportion of exposed hosts that get infected); 2) host infectiousness to

triatomine vectors; and 3) vector-host contact (considering relative abundance of vectors and hosts and vector feeding preferences). The first two parameters can be combined to calculate a numerical index of reservoir competence. This, combined with measures of vector-host contact, informs reservoir potential (Figure 1.3). Further, we will discuss the additional consideration of host-strain type associations.

### *1.4.2 Candidate Species*

We reviewed all published studies of *T. cruzi*-infected wildlife species in the US to tabulate parameters to input into the reservoir potential conceptual framework to evaluate the relative importance of each species (Table 1.1). In total, we reviewed 77 published estimates of anti-*T. cruzi* antibodies or *T. cruzi* parasite infection in at least 26 wildlife species across 15 southern states., expanding upon those previously reviewed across the US (Bern et al., 2011) and in Texas (Gunter et al., 2016). In Table 1.1, we combined reports of seroprevalence with direct parasite detection to calculate the *overall prevalence* (including seropositive animals and animals with evidence of parasite anywhere) because infection with *T. cruzi* is considered life-long such that hosts harboring anti-*T. cruzi* antibodies are also currently infected. Recognizing that not all infected hosts will be *infectious* to vectors at any given time, we then compiled reports that utilized PCR of blood, hemoculture, or microscopic methods (i.e., measures of parasitemia) to calculate an *infectiousness index*. For each wildlife host species, we then summarized the total number of positive animals over the total number of tested animals across all published reports to present species-specific *aggregate overall prevalence* and *aggregate infectiousness indices* for comparative purposes. It must be recognized, however, that each individual study is associated with its own biases

and so the aggregate measures we computed are not intended to be representative of all populations of a particular wildlife species across the southern US. Further, some relatively understudied species may also have key ecological roles, but logistics of sampling have led to them being underrepresented. Below, we comment specifically on some of the key wildlife species most well represented in the literature in the context of the available data to address some of the key parameters in the reservoir potential equation.

**Table 1.1 Summary of *Trypanosoma cruzi* studies in wildlife in the US.** Results are compiled as overall prevalence (including as positive animals harboring anti-*T. cruzi* antibodies and animals with evidence of parasite anywhere) and infectiousness index (including as positive animals with measures of parasitemia). For each wildlife species, an aggregate infection prevalence and aggregate infectiousness index was calculated for comparative purposes, although each individual study is associated with its own biases and so these metrics are not intended to represent all wildlife populations in the southern US. Reprinted with permission from (Hodo and Hamer, 2017).

| | | Overall Prevalence [b] | | | Infectiousness Index[c] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **State[a]** | **No. tested** | **No. positive** | **Prev.** | **No. tested** | **No. positive** | **% Infectious** | **Method(s)[a]** | **References** |
| **Raccoon (*Procyon lotor*)** | | | | | | | | | |
| | AL | 35 | 5 | 14.3% | 35 | 2 | 5.7% | Culture (heart and blood) | (Olsen et al., 1964) |
| | FL | 33 | 4 | 12.1% | 33 | 4 | 12.1% | Culture (blood) | (Schaffer et al., 1978) |
| | GA | 10 | 5 | 50.0% | 10 | 5 | 50.0% | Culture (blood) | (Schaffer et al., 1978) |
| | TX | 25 | 6 | 24.0% | 25 | 6 | 24.0% | Culture (blood) | (Schaffer et al., 1978) |
| | TX | 9 | 0 | 0.0% | | | | Serology (IHA) | (Burkholder et al., 1980) |
| | OK | 8 | 5 | 62.5% | 8 | 5 | 62.5% | Culture (blood) | (John and Hoppe, 1986) |
| | NC | 20 | 3 | 15.0% | 20 | 3 | 15.0% | Culture (blood) | (Karsten et al., 1992) |
| | GA | 54 | 12 | 22.2% | 54 | 12 | 22.2% | Culture (blood) | (Pung et al., 1995) |
| | GA | 30 | 13 | 43.3% | 30 | 13 | 43.3% | Culture (blood), blood smear | (Pietrzak and Pung, 1998) |
| | TN | 3 | 2 | 66.7% | 3 | 2 | 66.7% | Culture (blood) | (Herwaldt et al., 2000) |
| | GA, SC | 221 | 104 | 47.1% | | | | Serology (IFA) | (Yabsley and Noblet, 2002) |
| | VA | 464 | 153 | 33.0% | | | | Serology (IFA) | (Hancock et al., 2005) |
| | KY | 44 | 19 | 43.2% | 44 | 17 | 38.6% | Serology (IFA), culture (blood) | (Groce, 2008) |
| | AZ | 5 | 1 | 20.0% | | | | Serology (IFA) | (Brown et al., 2010) |
| | FL | 70 | 38 | 54.3% | | | | Serology (IFA) | (Brown et al., 2010) |
| | GA | 510 | 167 | 32.7% | 168 | 50 | 29.8% | Serology (IFA), culture (blood) | (Brown et al., 2010) |
| | MO | 109 | 74 | 67.9% | | | | Serology (IFA) | (Brown et al., 2010) |
| | TN | 706 | 206 | 29.2% | | | | Serology (IFA) | (Maloney et al., 2010) |
| | TX | 20 | 18 | 90.0% | 20 | 12 | 60.0% | Culture (blood), PCR | (Charles et al., 2012) |
| | TX | 70 | 49 | 70.0% | 18 | 14 | 77.8% | PCR (heart, blood) | (Curtis-Robles et al., 2016) |
| | TX | 24 | 15 | 62.5% | 18 | 9 | 50.0% | PCR (heart, blood) | Hodo, unpublished data |
| | TX | 2 | 2 | 100% | 2 | 2 | 100% | PCR (heart, blood) | Hodo, unpublished data |
| **Raccoon aggregate** | | **2472** | **901** | **36.4%** | **488** | **156** | **32.0%** | | |
| **Woodrat (*Neotoma* spp.)** | | | | | | | | | |
| *Neotoma micropus* | TX | 100 | 32 | 32.0% | 100 | 31 | 31.0% | Culture (blood), xenodiagnosis | (Packchanian, 1942) |
| *Neotoma micropus* | TX | 30 | 7 | 23.3% | 30 | 7 | 23.3% | Culture (blood), blood smear | (Burkholder et al., 1980) |
| *Neotoma micropus* | TX | 159 | 42 | 26.4% | | | | PCR (liver) | (Pinto et al., 2009) |
| *Neotoma micropus* | TX | 104 | 50 | 48.1% | 104 | 35 | 33.7% | Serology (IFA, ICT), blood smear, culture (blood), PCR (blood) | (Charles et al., 2012) |

**Table 1.1 Continued**

| Species | State[a] | Overall Prevalence[b] No. tested | No. positive | Prev. | Infectiousness Index[c] No. tested | No. positive | % Infectious | Method(s)[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| | LA | | | | | | | PCR (heart, liver, skeletal | (C. P. Herrera et al., 2015) |
| *Neotoma floridana* | | 15 | 11 | 73.3% | | | | muscle, spleen) | |
| *Neotoma macrotis* | CA | 49 | 7 | 14.3% | 49 | 7 | 14.3% | PCR (blood) | (Shender et al., 2016) |
| *Neotoma floridana* | TX | 1 | 0 | 0.0% | 1 | 0 | 0.0% | PCR (heart, blood) | Hodo, unpublished data |
| **Woodrat aggregate** | | **458** | **149** | **32.5%** | **284** | **80** | **28.2%** | | |
| **Opossum (*Didelphis virginiana*)** | | | | | | | | | |
| | TX | 8 | 8 | 100% | 8 | 8 | 100.0% | Culture (blood), xenodiagnosis | (Packchanian, 1942) |
| | TX | 391 | 63 | 16.1% | 391 | 63 | 16.1% | Blood smear | (Eads et al., 1963) |
| | AL | 126 | 17 | 13.5% | 126 | 14 | 11.1% | Culture (heart and blood) | (Olsen et al., 1964) |
| | OK | 10 | 0 | 0.0% | 10 | 0 | 0.0% | Culture (blood) | (John and Hoppe, 1986) |
| | LA | 48 | 18 | 37.5% | 48 | 16 | 33.3% | Culture (blood), histopathology | (Barr et al., 1991a) |
| | NC | 12 | 1 | 8.3% | 12 | 1 | 8.3% | Culture (blood) | (Karsten et al., 1992) |
| | GA | 39 | 6 | 15.4% | 39 | 6 | 15.4% | Culture (blood) | (Pung et al., 1995) |
| | KY | 48 | 15 | 31.3% | 48 | 0 | 0.0% | Serology (IFA), culture (blood) | (Groce, 2008) |
| | FL | 27 | 14 | 51.9% | | | | Serology (IFA) | (Brown et al., 2010) |
| | GA | 421 | 118 | 28.0% | 83 | 11 | 13.3% | Serology (IFA), culture (blood) | (Brown et al., 2010) |
| | GA | 29 | 3 | 10.3% | | | | PCR (heart) | (Parrish and Mead, 2010) |
| | VA | 6 | 1 | 16.7% | | | | Serology (IFA) | (Brown et al., 2010) |
| | TX | 5 | 4 | 80.0% | 5 | 4 | 80.0% | PCR (heart, blood) | Hodo, unpublished data |
| **Opossum aggregate** | | **1170** | **268** | **22.9%** | **770** | **123** | **16.0%** | | |
| **Striped skunk (*Mephitis mephitis*)** | | | | | | | | | |
| | CA | 1 | 1 | 100% | | | | Serology, histology | (Ryan et al., 1985) |
| | AZ | 34 | 3 | 8.8% | | | | Serology (IFA) | (Brown et al., 2010) |
| | GA | 1 | 1 | 100% | | | | Serology (IFA) | (Brown et al., 2010) |
| | TX | 4 | 4 | 100% | 4 | 3 | 75.0% | Culture (blood), PCR (blood) | (Charles et al., 2012) |
| | TX | 3 | 2 | 66.7% | 3 | 2 | 66.7% | PCR (heart, blood) | Hodo, unpublished data |
| **Striped skunk aggregate** | | **43** | **11** | **25.6%** | **7** | **5** | **71.4%** | | |
| **Nine-banded armadillo** | | | | | | | | | |
| **(*Dasypus* | TX | | | | | | | | (Packchanian, 1942) |
| ***novemcinctus*)** | | 15 | 1 | 6.7% | 15 | 1 | 6.7% | Culture (blood), xenodiagnosis | |
| | LA | 80 | 30 | 37.5% | 80 | 23 | 28.8% | Culture (blood); Serology (direct agglutination) | (Yaeger, 1988) |
| | LA | 98 | 1 | 1.0% | 98 | 1 | 1.0% | Culture (blood) | (Barr et al., 1991a) |
| **Armadillo aggregate** | | **193** | **32** | **16.6%** | **193** | **25** | **13.0%** | | |
| **Coyote (*Canis latrans*)** | | | | | | | | | |
| | TX | 156 | 20 | 12.8% | | | | Serology (IHA) | (Burkholder et al., 1980) |
| | TX | 134 | 19 | 14.2% | | | | Serology (IFA) | (Grögl et al., 1984) |

**Table 1.1 Continued**

| Species | State[a] | Overall Prevalence[b] No. tested | No. positive | Prev. | Infectiousness Index[c] No. tested | No. positive | % Infectious | Method(s)[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| | GA | 23 | 1 | 4.3% | | | | Serology (IFA) | (Brown et al., 2010) |
| | VA | 26 | 1 | 3.8% | | | | Serology (IFA) | (Brown et al., 2010) |
| | GA | 27 | 2 | 7.4% | | | | Serology (IFA) | (Gates et al., 2014) |
| | TN | 21 | 2 | 9.5% | | | | Serology (ICT) | (Rosypal et al., 2014) |
| | TX | 84 | 12 | 14.3% | 23 | 4 | 17.4% | PCR (heart, blood) | (Curtis-Robles et al., 2016) |
| | TX | 199 | 16 | 8.0% | | | | Serology (ICT) | (Garcia et al., 2016) |
| | TX | 97 | 8 | 8.2% | 92 | 3 | 3.3% | PCR (heart, blood) | Hodo, unpublished data |
| **Coyote aggregate** | | **767** | **81** | **10.6%** | **115** | **7** | **6.1%** | | |
| **Gray fox** | | | | | | | | | |
| **(*Urocyon*** | SC | | | | | | | | (Rosypal et al., 2007) |
| ***cinereoargenteus*)** | | 26 | 2 | 7.7% | | | | Serology (IFA) | |
| | GA | 21 | 0 | 0.0% | | | | Serology (IFA) | (Brown et al., 2010) |
| | NC | 43 | 4 | 9.3% | | | | Serology (ICT) | (Rosypal et al., 2010) |
| | VA | 11 | 2 | 18.2% | | | | Serology (ICT) | (Rosypal et al., 2010) |
| | TX | 58 | 8 | 13.8% | 11 | 1 | 9.1% | PCR (heart, blood) | (Curtis-Robles et al., 2016) |
| **Gray fox aggregate** | | **159** | **16** | **10.1%** | **11** | **1** | **9.1%** | | |
| **Bobcat *(Lynx rufus*)** | | | | | | | | | |
| | GA | 62 | 2 | 3.2% | | | | Serology (IFA) | (Brown et al., 2010) |
| | TX | 14 | 2 | 14.3% | 2 | 0 | 0.0% | PCR (heart, blood) | (Curtis-Robles et al., 2016) |
| **Bobcat aggregate** | | **76** | **4** | **5.3%** | **2** | **0** | **0.0%** | | |
| **Feral swine (*Sus scrofa*)** | | | | | | | | | |
| | GA | 110 | 0 | 0.0% | | | | Serology (IFA) | (Brown et al., 2010) |
| | TX | 64 | 3 | 4.7% | 64 | 0 | 0.0% | PCR (heart, blood) | (Comeaux et al., 2016) |
| **Feral swine aggregate** | | **174** | **3** | **1.7%** | **64** | **0** | **0.0%** | | |
| **Other rodents** | | | | | | | | | |
| *Perognathus hispidus, Liomys irrorattus, Onychomys leucogaster* | TX | 45 | 6 | 13.3% | 45 | 6 | 13.3% | Culture (blood), blood smear | (Burkholder et al., 1980) |
| *Otospermophilus beecheyi, Peromyscus maniculatus* | CA | 23 | 2 | 8.7% | 23 | 2 | 8.7% | Serology (CF, IIF), culture | (Navin et al., 1985) |
| *Mus musculus, P. pectoralis laceianus, P. leucopus, Sigmodon hispidus, Rattus rattus, Ictidomys mexicanus, Otospermophilus variegatus* | TX | 28 | 5 | 17.9% | 28 | 5 | 17.9% | PCR (blood), culture (blood) | (Charles et al., 2012) |

**Table 1.1 Continued**

| Species | State[a] | Overall Prevalence[b] No. tested | No. positive | Prev. | Infectiousness Index[c] No. tested | No. positive | % Infectious | Method(s)[a] | References |
|---|---|---|---|---|---|---|---|---|---|
| *Mus musculus, Peromyscus gossypinus* | LA | 44 | 34 | 77.3% | | | | PCR (heart, liver, skeletal muscle, spleen) | (C. P. Herrera et al., 2015) |
| *Rattus rattus* | TX | 145 | 0 | 0.0% | 61 | 0 | 0.0% | PCR (heart, blood) | (Hodo, Bertolini et al., 2016) |
| *Sigmodon hispidus* | TX | 27 | 0 | 0.0% | 27 | 0 | 0.0% | PCR (heart, blood) | Hodo, unpublished data |
| **Other rodents aggregate** | | **312** | **47** | **15.1%** | **184** | **13** | **7.1%** | | |
| **Other species** | | | | | | | | | |
| Ringtail (*Bassariscus astutus*) | AZ | 1 | 1 | 100.0 % | | | | Serology (IFA) | (Brown et al., 2010) |
| Badger (*Taxidea taxus*) | TX | 8 | 2 | 25.0% | | | | Serology (IHA) | (Burkholder et al., 1980) |
| Bats (various species) | TX | 593 | 1 | 0.2% | | | | PCR (heart) | (Hodo et al., 2016) |

CF, complement fixation; ELISA, enzyme-linked immunosorbent assay; ICT, immunochromatographic test; IFA, indirect fluorescent antibody; IHA, indirect hemagglutination assay; IIF, indirect immunofluorescence

[a]Excluding results from nonendemic states (e.g. Maryland, Pennsylvania), or from studies using samples considered nondiagnostic for *T. cruzi* (e.g. kidney culture). Data from negative populations are shown when the same study also reported positive data for different states or species, or when a large sample size of animals was involved.

[b]Overall prevalence includes all measures of *T. cruzi* detection: serology, whole parasite detection (blood smear or culture), and PCR. In *T. cruzi*, self cure is considered extremely rare, so seropositive animals are considered to be infected.

[c]Measures that detect parasite in the blood (culture, blood smear, PCR of blood) are used to calculate the infectiousness index, acknowledging that PCR may not necessarily represent live intact parasite.

*1.4.3 Host Susceptibility*

The gold standard methodology for elucidating host susceptibility to infection is through experimental infection, but such studies have only been conducted with *T. cruzi* on a limited number of wildlife species with small sample sizes (Davis et al., 1980; Roellig et al., 2009b), discussed below. Additionally, infection studies may be limited in generalizability because of the marked heterogeneity in both the pathogen and hosts. Relative susceptibility can be inferred from reports of seroprevalence, when considering infection prevalence of vectors as well as that of other mammalian hosts in the same environment. A major limitation, however, are the numerous different methods used to determine infection, many of which have not been properly validated for use in wildlife species, or even in domestic species, given the absence of a gold standard diagnostic test. Because sensitivity and specificity of different existing diagnostic tests may vary widely across tests and species, it is difficult to compare or combine data from different studies. Further, because dynamics of local transmission vary by geographic location and lower prevalence of infection is expected in northern regions where vectors are not abundant, the positive predictive value of diagnostic tests is not uniform across studies. Despite these challenges, the available literature can be used to draw some conclusions about relative susceptibility of the wildlife community, and below we comment on some of the most well studied species in the US.

Raccoons (*Procyon lotor*) are the most frequently studied candidate *T. cruzi* reservoir species in the US and have been studied across at least 13 states. Raccoons across the southern US are consistently highly infected, with an aggregate overall prevalence of 36.4% and many individual studies showing overall prevalence in excess of 60% (Table 1.1); variation within geographic areas is likely an artifact of diagnostic method (Bern et al., 2011;

Curtis-Robles et al., 2016). Raccoons have been experimentally inoculated with *T. cruzi* intravenously, *par os*, or though ingestion of infected bugs, and in two studies all of the inoculated raccoons became infected (Roellig et al., 2009b; 2009a). The next most frequently studied species in the US, the Virginia opossum, *Didelphis virginiana*, is the only opossum species in the US. Many other *Didelphis* spp. and *Philander opossum* are recognized as key *T. cruzi* reservoirs across South America, Central America, and Mexico (Jansen and Roque, 2010). The aggregate overall prevalence from 11 studies of naturally infected opossums is 22.9% (Table 1.1). Experimental infections with strain type TcI have yielded infected opossums, but attempts to inoculate opossums with TcIV did not result in a patent infection (Roellig et al., 2009b). In another study, 3/7 opossums became infected after eating infected triatomine bugs (Yaeger, 1971). Woodrats (*Neotoma* spp.) are recognized as key hosts for triatomine vectors, especially in the western United States, where triatomines infest the nests of the rats (Kjos et al., 2013; Kofoid and McCulloch, 1916; Packchanian, 1942; Ryckman et al., 1965; Shender et al., 2016). The 7 studies of *T. cruzi* in woodrats show an aggregate overall prevalence of 32.5% (Table 1.1). Among the other less-studied candidate wildlife reservoir species in the southern US that have shown some level of infection are coyotes, striped skunk, nine-banded armadillo, and gray fox, with aggregate infection prevalences of 10.6%, 26%, 17%, and 10%, respectively (Table 1.1).

### *1.4.4 Host Infectiousness*

Xenodiagnosis, or the feeding of pathogen-free vectors on hosts in order to quantify the incidence of vector infection, is a gold standard method for determining host infectiousness. Xenodiagnosis of naturally infected *T. cruzi* reservoirs has only been

performed on a very limited basis in the US, with 2/2 woodrats and 5/8 opossums infecting xenodiagnostic triatomines (Packchanian, 1942). Less direct indicators of host infectiousness include the presence of parasite in the blood, which can be detected via microscopy, hemoculture, or PCR. While PCR results do not necessarily reflect the presence of viable parasite, PCR positivity has been correlated with parasitemia in experimental studies (Caldas et al., 2012).

Of the 77 estimates of wildlife *T. cruzi* infection that we reviewed, 49 (63%) used methods that can inform the potential infectivity of the host. The aggregate infectiousness index for raccoons and opossums is 32% and 16%, respectively (Table 1.1). Experimental infections showed short duration of parasitemia in opossums compared with raccoons (Roellig et al., 2009b). Supporting this, surveys of wild raccoons and opossums in GA and FL showed increased blood culture-based parasite detection in raccoons compared with opossums, despite similar seroprevalence rates between the two species (Brown et al., 2010). Woodrats have an aggregate infectiousness index of 28.2% (Table 1.1). Only two studies have assessed the presence of parasite in the blood of coyotes, and these both used PCR (Curtis-Robles et al., 2016); Hodo CL, Hamer SA, unpublished data) and were located in central Texas, with an aggregate infectiousness index of 6%. Both of these studies were conducted in the winter, and may not reflect the parasitemia status of coyotes throughout the year. The two studies from which skunk infectiousness can be inferred both have a very small sample size (total n=7) but have an aggregate infectiousness index of 71%. Finally, armadillos in 3 studies were associated with aggregate infectiousness index of 13%, while foxes had an infectiousness index of 9% in one study (Table 1.1).

*1.4.5 Vector-Host Contact*

Although a host species may be highly infected and infectious, it only serves as an important reservoir if triatomine vectors feed on it, become infected, and subsequently transmit the parasite to the target hosts. Assessment of vector-host interactions is limited by a number of factors (Figure 1.4), including opportunistic rather than systematic sampling of triatomines in the US, limited blood meal analysis studies, and lack of information on the relative population densities of the host community. The primary means for quantifying vector-host contact in arthropod-borne disease studies is through blood meal analysis of vectors, through which the residual traces of a host bloodmeal in a vector's digestive tract are identified to the genus or species level using immunologic or molecular methods. Extreme flexibility in triatomine feeding behavior has been demonstrated, with insects feeding opportunistically based on host availability (Gürtler et al., 2009; Rabinovich et al., 2011). We generated a qualitative indication of the generalist feeding behavior of kissing bugs in the southern US by reviewing the four published triatomine bloodmeal analysis studies from this region (Table 1.2), but we caution that these data alone cannot be interpreted as a measure of kissing bug feeding preferences due to the aforementioned biases (Figure 1.4).

**Complexities of triatomine vectors and *Trypanosoma cruzi* transmission that limit the ability to define vector-host interactions**

- Generalist vector feeding behavior results in large pool of candidate hosts
  - Determining feeding preferences necessitates large-scale biodiversity survey encompassing multiple classes (mammals, reptiles, amphibians, birds).
- Opportunistic vector collection leads to biases in the apparent host community
  - Systematic collection of triatomines has proved more difficult relative to that of ticks, mosquitoes, or other vectors.
  - Triatomines are most commonly collected opportunistically (e.g., dispersing adults seen in areas frequented by humans) or through manual searches of known harborage sites such as wildlife dens and dog kennels, where hosts are obvious
- Vectors may feed on many different hosts during their life cycle, which limits ability to pinpoint infection source
  - *T. cruzi* infection is maintained transstadially, complicating the ability to incriminate which host species was the source of infection
- Stercorarian transmission of the parasite results in dissociation of the transmission event from the act of blood feeding
  - Vectorial capacity is difficult to calculate when transmission pathway is unknown.
- Molecular bloodmeal analysis of triatomine hindguts is challenging
  - Status quo methods based on PCR and Sanger sequencing likely reveal only the most recently utilized host species
  - Human contamination may be intractable
  - Freshly engorged insects have the highest chance of success for incriminating host species

**Figure 1.4. Complexities of triatomine vectors and *Trypanosoma cruzi* transmission that limit the ability to define vector-host interactions.** Reprinted with permission from (Hodo and Hamer, 2017).

Raccoon blood has commonly been detected in the gut contents of triatomine bugs in the southern states. In one report of blood meals from triatomine bugs collected in rural peridomestic settings in Texas, raccoon blood was detected in 5/62 bugs (Gorchakov et al., 2016). Another study of bugs in residential settings in Texas also identified a raccoon blood meal in a single *Triatoma gerstaeckeri* (Kjos et al., 2013). In Louisiana, 12 of 49 *Triatoma sanguisuga* were found to contain a raccoon blood meal (Waleckx et al., 2014). Our own unpublished data include 4 raccoon blood meals in citizen-collected triatomines collected from central, south, and west Texas (Hamer SA, unpublished data). Additionally, there are 3

reports of raccoon blood being detected in the same bug which had also fed on a human (Gorchakov et al., 2016), creating a scenario of spillover risk. Canids are the second most common blood meal source detected in triatomines in the US (Table 1.2) but unfortunately, most blood meal analysis studies do not use methods capable of differentiating between *Canis* species so distinguishing coyote from dog blood meals is not feasible. Opossum blood meals were detected in a *Triatoma protracta* and two *Triatoma recurva* in a zoological park in Arizona (Klotz et al., 2009), and in a *Triatoma indictiva* found within a bedroom in Texas (Hamer SA, unpublished data). Two of the opossum-fed bugs from Arizona also had evidence of human blood-feeding (Table 1.2). Blood from woodrats unsurprisingly comprised the majority of blood meals detected in triatomines collected in or around woodrat nests (Kjos et al., 2013), and woodrat blood was also detected in three other blood meal analysis studies (Gorchakov et al., 2016; Klotz et al., 2014; Waleckx et al., 2014). Woodrat blood co-occured with a human blood meal in a bug found inside a house in Texas (Gorchakov et al., 2016). Other wildlife species represented in triatomine blood meals include armadillo, cottontail rabbit, gray fox, porcupine, house mouse, roof rat, and skunk, as well as a number of species refractory to *T. cruzi* infection (e.g., insects, birds, reptiles, and amphibians) (Table 1.2; Hamer SA, unpublished data).

**Table 1.2 Host species detected in triatomine blood meal analysis studies in the United States.** Reprinted with permission from (Hodo and Hamer, 2017).

| **Study location (reference)** | TX (Gorchakov et al., 2016) | LA (Waleckx et al., 2014) | AZ (Klotz et al., 2014) | TX (Kjos et al., 2013) | CA, AZ (Stevens et al., 2012) | |
|---|---|---|---|---|---|---|
| **Bug collection sites** | ih, oh, ru | ih, oh | z | dk, ih, oh, wr | CA:sy; AZ:sy, z | |
| **Species detected in blood meal** | Number of bugs with blood meal from each species | | | | | **Total** |
| Human (*Homo sapiens*) | 40 | 21 | 10 | 1 | 5 | 77 |
| Woodrat (*Neotoma* spp.) | 2 | 1 | 1 | 47 | | 51 |
| Dog/wolf/coyote (*Canis* spp.) | 20 | 3 | 3[a] | 19 | 4[a] | 49 |
| Green tree frog (*Hyla cinerea*) | | 23 | | | | 23 |
| Raccoon (*Procyon lotor*) | 5 | 12 | | 1 | | 18 |
| Cricket (*Gryllus texensis/rubens*) | | | | 15 | | 15 |
| Cow (*Bos taurus*) | 2 | 6 | | 5 | | 13 |
| Pig (*Sus scrofa*) | 2 | | 6 | 1 | 2 | 11 |
| Cat (*Felis catus*) | 2 | 1 | | 6 | | 9 |
| Squirrel (*Sciurus* spp.) | 4 | 2 | | | | 6 |
| Cottontail (*Sylvilagus* spp.) | 4 | | | | | 4 |
| Mouse (*Mus musculus*) | 1 | | 2 | | | 3 |
| Opossum (*Didelphis virginiana*) | | | 3 | | | 3 |
| Rat (*Rattus* spp.) | | | 1 | | 1 | 2 |
| Gray fox (*Urocyon cinereoargenteus*) | 2 | | | | | 2 |
| Armadillo (*Dasypus novemcinctus*) | 2 | | | | | 2 |
| Bighorn sheep (*Ovis canadensis*) | | | 2[a] | | | 2 |
| Chicken (*Gallus gallus*) | | | | | 1 | 1 |
| Deer (*Odocoileus virginianus*) | 1 | | | | | 1 |
| Black Vulture (*Coragyps atratus*) | | | | 1 | | 1 |
| Turkey Vulture (*Cathartes aura*) | | | | 1 | | 1 |
| Evening bat (*Nyctceius humeralis*) | 1 | | | | | 1 |
| Mustelid | 1 | | | | | 1 |
| Porcupine (*Erythizon dorsatum*) | 1 | | | | | 1 |
| **Total bugs with blood meal**[b] | 62 | 43 | 11 | 96 | 10 | 222 |

dk, dog kennel; ih, inside home; oh, outside home; ru, rural; sy, sylvatic habitat; wr, woodrat nest; z, zoological park

[a]Blood meal may be from captive zoo animal. [b]In some cases, multiple host blood meals were detected in single bugs, so the sum of individual blood meals is greater than the total number of bugs tested.

*1.4.6 Host-Strain Type Associations*

Growing evidence suggests that certain *T. cruzi* strain types are associated with particular host species as well as different clinical outcomes in humans (Gürtler and Cardinal, 2015; J. D. Ramírez et al., 2010; Zingales et al., 2012). Experimental studies in dogs have demonstrated differing clinical, pathologic, and immunologic outcomes resulting from infection with different strains. For example, dogs infected with *T. cruzi* isolates from an armadillo and opossum developed acute and chronic myocarditis, while dogs infected with an isolate from a dog did not develop disease (Barr et al., 1991b). Increased numbers of inflammatory cells were observed in the heart of dogs infected with TcI compared to TcII (Duz et al., 2014). Strain types TcI and TcIV are enzootic in the US (Bern et al., 2011) and TcII has recently been detected in a small number of rodents in Louisiana (C. P. Herrera et al., 2015). While the sample size is admittedly small (n=5), thus far the only locally-infected humans in the US that have been definitively strain typed have been infected with TcI (Roellig et al., 2008). Similarly, while domestic dogs are infected with both TcI and TcIV, preliminary evidence suggests the majority of dogs suffering from chronic heart disease are infected with TcI (Hodo CL, Hamer SA, unpublished data). Therefore, it is possible that reservoir hosts harboring TcI may be more important in the context of spillover risk to humans and dogs than those carrying TcIV. TcI and TcIV infections have been documented in non-human primates at facilities throughout the US, but strain type has not yet been associated with disease status (Bern et al., 2011; Hodo CL, Hamer SA, unpublished data). Opossums throughout the Americas are predominantly infected with TcI (Bern et al., 2011; Zingales et al., 2012), while raccoons are almost exclusively infected with TcIV (Bern et al., 2011; Curtis-Robles et al., 2016; Roellig et al., 2008). Attempts to experimentally infect

opossums with a TcIV isolate from a raccoon did not result in infection (Roellig et al., 2009b). Both TcI and TcIV have been detected in skunks and armadillos (Charles et al., 2012; Roellig et al., 2008); Hodo CL, Hamer SA, unpublished data), while only TcI has been detected in coyotes (Curtis-Robles et al. 2016; Hodo CL, Hamer SA, unpublished data). Woodrats in Texas (*Neotoma micropus*) were infected with either TcI or TcIV, and two *Neotoma floridana* in Louisiana were infected with TcI, while a third was co-infected with TcI and TcII (C. P. Herrera et al., 2015).

## 1.5 Summary and Conclusion

Reservoir potential is heterogeneous across space, given changes in the composition of wildlife, vector, and parasite communities. Accordingly, the biological relevance of the reservoir potential framework depends upon the spatial scale of the empirical data. As a starting point, we have reviewed and aggregated the available data on candidate wildlife *T. cruzi* reservoirs from across 15 states that encompass vastly diverse ecosystems, and future studies at a finer spatial resolution will be useful in identifying key reservoirs in different epidemiological settings. Our review highlights three key knowledge gaps that remain before reservoir potential can more comprehensively be evaluated and filling these gaps should form the framework for future study.

### *1.5.1 Knowledge Gap #1: Measuring Host Infectiousness and Infection Dynamics*

Diagnostics for *T. cruzi* exposure or infection in wildlife rarely involve methods that directly inform infectiousness to kissing bug vectors - a key parameter for understanding reservoir potential. This knowledge gap could be addressed with more xenodiagnosis studies

in the US, which have routinely been done in Central and South America (Carrasco et al., 2012; Gürtler et al., 2007; L. Herrera and Urdaneta-Morales, 1997). However, laboratory colonies of uninfected kissing bugs in the US are rare and high maintenance, and Institutional Biosafety Committee approval of xenodiagnoses protocols is challenging. To resolve this, one approach would be to concurrently conduct xenodiagnoses along with quantitative PCR, which determines genome copies of *T. cruzi* relative to a house-keeping gene. This approach could determine a 'threshold' of parasitemic infectiousness that once determined, could be used in place of xenodiagnoses.

Infectiousness may not be constant over time, depending on host-level factors or infectious dose. Therefore, aside from measuring infectiousness of naturally-infected animals in a cross-sectional fashion, important knowledge could be gained from studies designed to measure susceptibility, dynamics of infectiousness over time, and pathology in wildlife species. Some experimental infection studies have been performed in wildlife species such as raccoon (Roellig et al., 2009b), opossum (Roellig et al., 2009b; Yaeger, 1971), and skunk (Davis et al., 1980), but sample sizes are so small that it is difficult to draw conclusions about susceptibility across the entire species. Longitudinal studies in naturally-infected wildlife are logistically difficult and labor-intensive (Figure 1.1), but could provide invaluable data on dynamics of infectiousness over time. Pathology studies of *T. cruzi*-infected wildlife have been conducted on a limited basis (Barr et al., 1991a; Charles et al., 2012; Curtis-Robles et al., 2016; Packchanian, 1942; Pietrzak and Pung, 1998; Ryan et al., 1985), but more thorough investigation could shed light on infection dynamics, tissue tropisms, and population-level effects of infection.

### *1.5.2 Knowledge Gap #2: Measuring Vector-Host Contact*

Understanding triatomine feeding patterns, and thus host-vector contact, through the use of blood meal analysis presents several challenges (Figure 1.4). Because each triatomine may feed dozens of times throughout the nymphal instars and in the adult life stage, future blood meal analysis studies should use methods that allow the detection of mixed species and historic bloodmeals, and should incorporate estimates of the relative abundance of available vertebrate hosts in the area sampled. Additionally, bugs found within and directly around human housing with wildlife blood meals are of interest and can help to indicate the risk of spillover from these sylvatic transmission cycles. Finally, when vector infection data are combined with bloodmeal identification, the infective bloodmeal index (Gürtler et al., 2007; Zárate et al., 1980) can be calculated, although the infective host may not definitively be identified given transstadial passage of *T. cruzi* that could have been acquired from one or more hosts.

### *1.5.3 Knowledge Gap #3: Determining Epidemiological Relevance of T. cruzi Strains in Enzootic Transmission*

Molecular epidemiological investigations to source-track transmission of the most pathogenic strains in target hosts could incriminate enzootic reservoirs that could be targeted in control interventions, and this field of study applied to *T. cruzi* transmission in the US is not as advanced as that in South America (Fernández et al., 2014). While raccoons are associated with the highest aggregate overall *T. cruzi* prevalence (36.4%), the available studies reveal that they are disproportionately infected TcIV. The significance of this strain for human health is unknown relative to TcI which has been more frequently implicated in

human and canine disease. For this reason, wildlife reservoirs that are infected with TcI such as opossums and coyotes, despite the lower aggregate overall prevalence in the latter (10%), may play a greater role as reservoirs of the strain that is pathogenic to target populations of humans and dogs. Further, from a wildlife health perspective, the pathogenic effects of *T. cruzi* in general, and specific *T. cruzi* strains in particular, on individual wildlife hosts is largely unknown. Future work should include studies designed to determine differences in clinical outcome between parasite strain types in target hosts, as well as in infection dynamics in reservoirs.

This review has illuminated the significant gaps in knowledge that will need to be addressed in future research in order to better characterize the reservoir potential of wildlife species for *T. cruzi* and other vector-borne diseases. While raccoons, opossums, woodrats and skunks appear to rise to the top in importance as reservoirs of *T. cruzi* in the US, other understudied species may have similar or even greater importance. Additionally, more data are needed on the association of particular strain types with disease outcomes. In light of the increasing human and veterinary health burden of vector-borne zoonotic disease, detailed understanding of wildlife reservoirs will provide necessary data for protecting human and animal health.

# 2. *TRYPANOSOMA CRUZI* EXPOSURE IS WIDESPREAD AMONG DOGS IN TEXAS SHELTERS

## 2.1 Introduction

American trypanosomiasis (Chagas disease), caused by the protozoan parasite *Trypanosoma cruzi*, is a vector-borne disease most commonly affecting humans and dogs. *T. cruzi* is endemic in Latin America and increasingly recognized as an important threat to canine and human health in the southern United States, where Texas is a hotspot for active transmission (Beard et al., 2002; Bern et al., 2011; Curtis-Robles et al., 2017b; Kjos et al., 2009b). Insects of the family Reduviidae, sub-family Triatominae (known colloquially as 'kissing bugs' or 'cone-nose bugs') are the vectors of the parasite and are widespread across the southern U.S. (Bern et al., 2011; Curtis-Robles et al., 2015; Kjos et al., 2009b). *T. cruzi* is transmitted via introduction of an infected bug's feces into a wound or mucous membrane or through ingestion of the infected bug or its feces. The oral route is highly efficient and is likely the most important route in dogs (Barr, 2009). The parasite may also be transmitted via blood transfusion, congenitally, or through ingestion of parasitemic hosts (Bern et al., 2011; Gürtler and Cardinal, 2015).

In dogs, infection may result in myocarditis with associated conduction abnormalities, dilation, and/or reduced contractility of the heart, leading to sudden death or development of congestive heart failure (Snowden and Kjos, 2013). The infection has three phases: acute, indeterminate, and chronic (Barr, 2009). The acute phase is often subclinical, with an initial rise and fall of parasitemia documented in experimentally infected dogs (Barr et al., 1991b; Lana et al., 1992). However, severe myocarditis and death during the acute phase has been

described (Vitt et al., 2016) and may be more common in young dogs (Barr, 2009; Kjos et al., 2008). During the indeterminate stage, infected dogs are seropositive but show no clinical signs, and parasitemia is infrequent (Veloso et al., 2008) although in some cases can be demonstrated via hemoculture or xenodiagnosis (Barr, 2009). Many infected dogs remain in the indeterminate stage for life (Barr, 2009), but an unknown proportion will progress to the chronic stage, developing cardiac lesions and heart failure. The details of parasitemia dynamics in naturally-infected dogs during the chronic stage of infection are poorly understood.

*T. cruzi* is a genetically heterogeneous species, divided into DTUs TcI-TcVI, and a seventh bat-associated strain TcBat (Zingales et al., 2012). Different strain types are associated with different geographical regions and reservoir hosts and may be associated with different disease manifestations (Barr et al., 1991b; J. D. Ramírez et al., 2010; Zingales et al., 2012). TcI and TcIV are the most common strain types documented in animals in the U.S. (Bern et al., 2011; Hodo and S. A. Hamer, 2017), and of these, only TcI has been isolated from autochthonous human cases (Roellig et al., 2013).

The first published canine Chagas disease cases in the U.S. occurred in Texas in the 1970s (Tippit, 1978). Since then, *T. cruzi* infection or exposure has been documented in dogs throughout many of the southern states (Beard et al., 2002; Bern et al., 2011; Bradley et al., 2000; Kjos et al., 2008; Rowland et al., 2010; Tenney et al., 2014). In 2013, Texas became the first state to institute mandatory Chagas disease reporting for veterinary cases through the Texas Department of State Health Services, and the fourth state (along with Arizona, Massachusetts, and Tennessee) in which human cases must also be reported. Veterinary reporting was discontinued in 2015, but from 2013–2015, a total of 439 canine cases were

reported from across 58 counties (Figure 2.1) (Texas Department of State Health Services, 2016a). For the same time period, a total of 64 human cases were reported (15 locally-acquired and 44 imported from endemic zones, with 5 of undetermined origin of infection) (Texas Department of State Health Services, 2016a). Given the limitations in scope of these reports and previous seroprevalence studies, the overall seroprevalence among dogs in the U.S. and the relevant risk factors associated with infection remain largely unknown.

Knowledge of risk factors for exposure of dogs to *T. cruzi* is imperative for safeguarding canine health. Additionally, defining the current disease burden is essential for driving efforts to develop vaccines, therapeutics, and improved diagnostics. Further, in some regions, dogs are considered sentinels of human risk of Chagas disease (Castanera et al., 2016; Castillo-Neyra et al., 2015; Estrada-Franco et al., 2006; Tenney et al., 2014), and therefore information on the distribution of disease in dogs may be useful in guiding broader public health efforts. Shelter dogs in particular are considered representative of the canine population across a large geographic area as they comprise both stray dogs and household pets (Torrence et al., 1990). The objectives of this study were to determine the seroprevalence of *T. cruzi* in shelter dogs across Texas and to identify potential risk factors for canine infection. We hypothesized that *T. cruzi* seroprevalence would differ among shelters based on their geographic location and would reflect the risk of transmission in those areas. We suspected that demographic factors such as age, breed, sex, and origin (stray vs. owner surrender) would also be associated with *T. cruzi* exposure. Here, we present our findings of widespread *T. cruzi* seroprevalence in Texas shelter dogs, discuss limitations of current diagnostic testing options, and give recommendations for prevention of *T. cruzi* infection and future research directions.

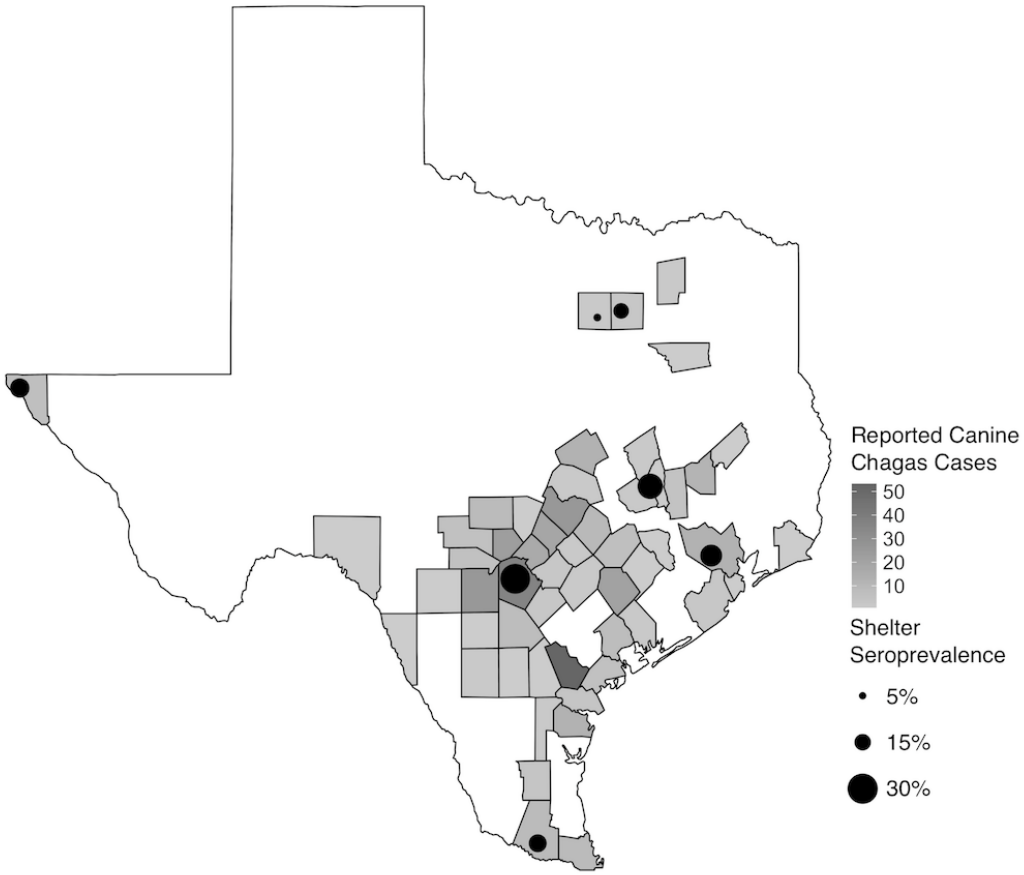


**Figure 2.1 Map of Texas with counties shaded according to number of canine Chagas cases reported to the Texas Department of State Health Services from 2013-2015.** Circles mark sampled shelter locations, with circle size relative to T. cruzi seroprevalence found in our study. The map was created in R.

## 2.2 Methods

### *2.2.1 Study Design and Sample Collection*

In this repeated cross-sectional study, we sampled dogs at 7 shelters across 7 of the 10 Gould ecoregions of Texas (Gould et al., 1960), visiting each shelter 3 times over an 18-month period (Summer, Winter, and Fall) from May 2013 through December 2014. Shelters located in the cities of Bryan/College Station, Dallas, Edinburg, El Paso, Fort Worth, Houston, and San Antonio (Figure 2.1) responded to a request for participation and were

selected for inclusion in the study. To estimate the prevalence within +/- 2.5% with 95% confidence (using an estimated true prevalence of 10% (Tenney et al., 2014)), the target was > 550 samples. Thus, approximately 30 dogs were sampled from each shelter during each visit. During each visit, up to 5 ml of blood was collected from each dog in accordance with client-owned animal use protocols approved by the Texas A&M University Institutional Animal Care and Use Committee. Criteria for inclusion were: dogs 6 months of age and older, preferentially admitted to the shelter within the last 3 days, with fresh feces available at time of sampling (for a separate project (Leahy et al., 2017; 2016)). Demographic data (age, sex, breed) were recorded based on shelter records and/or based on the investigators' assessments of the animal. Blood from each dog was collected into a tube with no additive and a tube containing EDTA. In the laboratory, the tubes were centrifuged and the blood was separated into the following components: serum and clot (from no additive tubes), and plasma, buffy coat, and packed cells (EDTA tubes). Serum was refrigerated for up to 3 days before completing the initial serologic test. Remaining serum and other blood components were frozen at -20 or -80°C for up to 3 years with molecular and additional serologic testing performed on aliquots throughout this time.

### *2.2.2 Serology*

To detect anti-*T. cruzi* antibodies, serum samples were first tested using a commercially available rapid immunochromatographic test ('Stat-Pak'; Chagas Stat-Pak, Chembio Diagnostic Systems, Inc., Medford, NY, USA) that was developed for use in humans using 3 recombinant antigens and validated using human sera from South and Central America (Luquetti et al., 2003). The Stat-Pak has also been used in dogs, with

reported high sensitivity and specificity when compared to IFAT (Nieto et al., 2009). We ran the test according to the manufacturer's instructions. As per the instructions, all samples that generated a band were considered positive.

All of the Stat-Pak positive samples and 81 of the negatives (randomly selected 10% of total dogs [n=61] plus 50% [n=20] of the remaining suspect PCR-positive dogs [see below]) were subjected to another a rapid immunochromatographic test ('CDP'; Chagas Detect Plus Rapid Test, InBios International, Inc., Seattle, WA) that uses a multi-epitope recombinant antigen derived from antigens specific to North American *T. cruzi* strains as well as those from Central and South America (Houghton et al., 2009). Tests were run according to manufacturer's instructions. Samples positive on both the Stat-Pak and the CDP tests were considered seropositive in the calculation of seroprevalence and in statistical analyses for identification of risk factors.

*2.2.3 Molecular Detection of Parasite DNA*

DNA was extracted from approximately 250 µl of clotted blood using a commercial spin-column based kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA). Each set of DNA extractions included a no-template negative control. Clots from all sampled dogs were tested except for rounds 1 and 2 from the Bryan/College Station shelter, which were not retained for this analysis. We performed an initial screening with a real-time qPCR using Cruzi 1, 2 primer set and Cruzi 3 probe as previously reported (Piron et al., 2007). This qPCR amplifies a 166-bp region of a repetitive satellite DNA sequence, and is sensitive and specific for *T. cruzi* when compared to other PCR techniques (Schijman et al., 2011). Positive (*T. cruzi* pure culture[d]) and negative (water) controls were included in each PCR

reaction. Based on internal laboratory validations, samples with a cycle threshold (Ct) value of <34 were considered suspect positive. Suspect positive samples were subjected to a multiplex probe-based qPCR targeting the spliced leader intergenic region (SL-IR) to confirm positivity and for determination of strain type, according to previously described protocols (Cura et al., 2015; Curtis-Robles et al., 2017a) with positive and negative controls for each reaction.

### *2.2.4 Statistical Methods*

Statistical analyses were performed in R (R Core Team, 2014). Assessed variables were dog age (< 2-year-old or ≥ 2-year-old), sex, origin (stray or surrendered), round of sampling (as a proxy for season of year), and breed group. Dogs were classified into American Kennel Club breed groups based on the most dominant breed features, and some breed categories were combined due to small sample sizes in some groups. Bivariable analysis using the chi-squared or Fisher's exact tests was performed to evaluate the relationship between the putative risk factors and *T. cruzi* seroprevalence, excluding dogs with unknown status for each variable. Additionally, a chi-squared test was performed to evaluate the relationship between shelter and serologic status. All risk factors with $P$ value ≤ 0.25 in bivariable analysis were further investigated with logistic regression using a mixed-effects model, controlling for shelter as a random effect. Values of $P \leq 0.05$ were considered significant. Odds ratios and their 95% confidence intervals were calculated for the risk factors included in the final model. To determine variation in seroprevalence among shelters, a logistic regression model was created using the GLM (generalized linear model) method in which the shelter with the lowest seroprevalence served as the referent to which the other 6

shelters were compared. An exact binomial test was used to compare the proportion of males to females, and a chi-squared test was used to compare serologic status with PCR status.

## 2.3 Results

### *2.3.1 Population Data*

The study included 611 dogs, and demographic data are reported in Table 2.1. Sampling took place over 3 rounds: Summer 2013 (May-Aug), Winter 2013-14 (Dec-Feb), and Fall 2014 (Sep- Dec). Number of dogs sampled from each shelter ranged from 65 (10.6%, Bryan/College Station) to 95 (15.5%, San Antonio). The proportion of males to females was approximately equal (*P* value=0.49) with 295 (48.3%) females and 313 (51.2%) males, while sex was not recorded for 3 dogs (0.5%). A total of 369 (60.4%) were ≥ 2 years old and 236 (38.6%) were < 2 years old, with 6 (0.98%) dogs of unknown age. The origin of 342 (56.0%) dogs was classified as stray, 93 (15.2%) dogs were surrendered by their owners, and origin was not known for 176 (28.8%) dogs. The Terrier breed group was the most numerous, with 188 (30.7%) dogs, of which 74% were pit bull-type dogs. Breed was not recorded for 30 (4.9%) dogs.

### *2.3.2 Serologic Results*

Of 120 dogs positive on the Stat-Pak, 111 were also positive on the CDP. Of 81 dogs negative on the Stat-Pak, 39 (48%) were positive on the CDP. Using the criterion of being positive on both tests for the purposes of statistical analysis, 111 of 611 dogs were therefore considered seropositive, yielding an overall seroprevalence of 18.2%. Within-shelter seroprevalence ranged from 5.4% in Fort Worth to 29.5% in San Antonio (Table 2.1, Figure 2.1).

**Table 2.1. Demographic data and results of bivariable analysis of potential risk factors for *T. cruzi* seropositive status among 611 dogs at 7 animal shelters across Texas.**

| Risk factor | No. | No. Seropositive (%) | *P* value |
|---|---|---|---|
| Shelter location | | | 0.01 |
| Bryan/College Station | 65 | 16 (24.6) | |
| Dallas | 93 | 13 (14.0) | |
| Edinburg | 91 | 15 (16.5) | |
| El Paso | 89 | 16 (18.0) | |
| Fort Worth | 92 | 5 (5.4) | |
| Houston | 86 | 18 (20.9) | |
| San Antonio | 95 | 28 (29.5) | |
| Age group | | | 0.06 |
| <2 y | 236 | 31 (13.1) | |
| ≥2 y | 369 | 78 (21.1) | |
| Unknown* | 6 | 2 (33.3) | |
| Sex | | | 0.98 |
| F | 295 | 54 (18.3) | |
| M | 313 | 56 (17.9) | |
| Unknown* | 3 | 1 (33.3) | |
| Origin | | | 0.84 |
| Stray | 342 | 50 (14.6) | |
| Owner surrender | 93 | 15 (16.1) | |
| Unknown* | 176 | 46 (26.1) | |
| Sampling round (months) | | | 0.84 |
| 1 (May-Aug) | 205 | 37 (18.0) | |
| 2 (Dec-Feb) | 207 | 40 (19.3) | |
| 3 (Sep-Dec) | 199 | 34 (17.1) | |
| Breed group | | | 0.160 |
| Herding and Working | 173 | 28 (16.2) | |
| Hound, nonsporting, toy | 108 | 25 (23.1) | |
| Sporting | 112 | 26 (23.2) | |
| Terrier | 188 | 28 (14.9) | |
| Unknown* | 30 | 4 (13.3) | |

*Unknowns for each risk factor were excluded from bivariable analysis of that risk factor

*2.3.3 Molecular Detection of Parasite DNA*

DNA extracted from blood clots of 559 dogs was tested on the *T. cruzi* screening qPCR, and 53 samples were considered suspect-positive with a Ct value < 34 and were subjected to further testing. Of these, 6 (1.1%) were confirmed positive by the strain typing qPCR. Five of these dogs were infected with DTU TcI, and 1 with TcIV. Two of the positive

dogs were from Dallas, both sampled in May; 3 from San Antonio, one sampled in each of July, December, and September; and 1 from El Paso, sampled in January. Only 1 of these 6 PCR-positive dogs (San Antonio, December) was seropositive on both the Stat-Pak and CDP serologic tests; an additional 3 of the dogs were positive on the CDP only, and 2 PCR-positive dogs were negative on both serologic tests. Of the remaining unconfirmed suspect positive dogs, 6/47 (11.3%) were seropositive on both Stat-Pak and CDP. Seropositivity was not statistically related to suspect PCR-positivity ($P$=0.43).

*2.3.4 Risk Factor Assessment*

In bivariable analysis to determine significant predictors of canine seropositive status, age group and breed group both had $P$ values < 0.25 (Table 2.1) and were included in the mixed-effects model for logistic regression. As estimated by logistic regression, only age group was significantly associated with seropositive status (Table 2.2). The odds of seropositivity were 1.6 times (95% C.I. 1.0–2.6) greater in older dogs (≥ 2 years old) than in dogs less than 2 years old. Shelter was also significantly associated with seropositive status in bivariable analysis ($P$ value = 0.01). In the shelter-level logistic regression model, when compared to the shelter with the lowest seroprevalence (Fort Worth), dogs at the Bryan/College Station, El Paso, Edinburg, Houston, and San Antonio shelters had 3.4-7.2 times greater odds of seropositivity whereas seroprevalence in Dallas was not statistically different (Table 2.3).

**Table 2.2. Association between *T. cruzi* seropositive status and the potential risk factors age and breed group, assessed using a linear mixed-effects model.**

| Risk factor | Odds ratio | 95% CI | *P* value |
|---|---|---|---|
| Age group | | | |
| <2 y | Referent | Referent | Referent |
| ≥2 y | 1.6 | 1.0-2.6 | 0.049 |
| Breed Group | | | |
| Herding and Working | Referent | Referent | Referent |
| Hound, nonsporting, toy | 1.3 | 0.7-2.5 | 0.307 |
| Sporting | 1.4 | 0.7-2.6 | 0.257 |
| Terrier | 0.94 | 0.5-1.7 | 0.848 |

**Table 2.3. Association between *T. cruzi* seropositive status and shelter, assessed using a logistic regression model.**

| Shelter location | Odds ratio | 95% CI | *P* value |
|---|---|---|---|
| Bryan/College Station | 5.7 | 2.1-18.2 | 0.001 |
| Dallas | 2.8 | 1.0-9.1 | 0.058 |
| Edinburg | 3.4 | 1.3-11.0 | 0.022 |
| El Paso | 3.8 | 1.4-12.1 | 0.013 |
| Fort Worth | Referent | Referent | Referent |
| Houston | 4.6 | 1.7-14.5 | 0.004 |
| San Antonio | 7.2 | 2.9-22.3 | 0.0001 |

## 2.4 Discussion

We found an overall *T. cruzi* seroprevalence of 18.2% in dogs in shelters across Texas, with prevalence estimates in individual shelters ranging from 5.4 – 29.5%. These results indicate that dogs across Texas are frequently exposed to *T. cruzi* and reinforce the need for better options for diagnosis and treatment of infected animals. The seroprevalence obtained in this study (18.2%) is more than twice the estimated seroprevalence (8.8%, n=205) we previously reported from initial sampling at these shelters (Tenney et al., 2014), with the difference explained largely by differences in test interpretation. In that study, the

Stat-Pak was used and very faint lines were conservatively scored as negative, with no additional confirmatory serologic test. In the current study, we scored any level of serological band as positive, even very faint bands, and used an expanded diagnostic approach with the requirement that samples test positive on two independent assays. Evidence of positivity on at least two tests that employ different *T. cruzi* antigens reflects the lack of certainty in sample status based on a single result with an imperfect test, and is in keeping with current guidelines for human diagnostics (Afonso et al., 2012). With our new methods, additional dogs from the first report were re-defined as seropositive, resulting in no statistical difference between sampling rounds. A small serologic survey of stray dogs in one county in the Rio Grande Valley of South Texas in 2002 found 7.5% (n=375) seropositivity using IFAT (Beard et al., 2002), while a recent study across multiple counties in the same region using methods comparable to ours reported that 19.6% of 209 dogs were seropositive.(Curtis-Robles et al., 2017b) Our seroprevalence results are also similar to those of a study in Louisiana, which found 22% seroprevalence in 122 tested dogs using IFAT, Stat-Pak, and Trypanosoma Detect (an earlier version of the CDP) (Nieto et al., 2009). In contrast, a serosurvey from three counties in Oklahoma showed 3.6% of 301 dogs were exposed (Bradley et al., 2000). A retrospective study of serum samples submitted to the Texas Veterinary Medical Diagnostic Lab for *T. cruzi* IFAT from 1993-2007 reported that 22% of submitted samples were positive (Kjos et al., 2008). Other, more targeted studies in Texas have found even higher seroprevalence: a group of working dogs housed in outdoor kennels in south Texas (n= 85) had a seroprevalence of 57.6% (Curtis-Robles et al., 2017a), indicating that multi-dog kennels may serve as niduses of *T. cruzi* transmission.

Dogs aged 2 years and older were 1.6 times more likely to be seropositive than dogs younger than 2 years, though the statistical significance of this finding was marginal (95% C.I. 1.0-2.6). Previous canine studies in other regions also reported higher seroprevalence in older dogs (Gürtler and Cardinal, 2015; Rowland et al., 2010). This finding was expected because *T. cruzi* is a cumulative infection in which infected dogs presumably develop life-long seropositivity, and older dogs have had a longer duration of potential exposure to the parasite. We also found variation in canine exposure across geographic regions. Because our study design specifically targeted the sampling of dogs within 3 days of admittance to the shelter, the difference noted in seroprevalence among shelters likely reflects regional differences in triatomine vector distribution and infection prevalence, rather than any unmeasured factors within the shelter. The patterns of canine exposure generally fit with previous estimates of *T. cruzi* risk distribution in Texas (Kjos et al., 2008; Sarkar et al., 2010). An exception is the El Paso shelter where dogs had a relatively high seroprevalence in our study (18.0%) despite predicted low transmission risk since the main vector in the region (*Triatoma rubida*) generally has a low infection prevalence (Buhaya et al., 2015). We did not detect a significant difference in seropositivity between stray and owner-surrendered dogs (Table 2.1), however, origin was unknown for many of the dogs (n=176). While the sporting and working breed groups were overrepresented among *T. cruzi*-infected dogs in a previous study (Kjos et al., 2008), we did not find any statistical difference in seropositivity between breed groups.

A small percentage (1.1%) of dogs had evidence of parasite DNA in their blood confirmed by 2 qPCR assays. While PCR does not demonstrate the presence of whole, viable parasites, PCR-positive blood samples suggest that the dog could be parasitemic and thus

serve as a source of infection to blood-feeding kissing bug vectors. Only 1 of the 6 confirmed PCR-positive dogs was seropositive on both the Stat-Pak and CDP, though 3 were positive on the CDP only. The low frequency of PCR-positivity among seropositive dogs may suggest that this population of dogs does not sustain high parasitemia levels during the chronic stage of infection. Additionally, relatively high Ct values in all suspect positive dogs are consistent with low levels of circulating parasite. Our results indicate that PCR status does not correlate well with serologic status, but the number of seronegative, PCR-suspect positive dogs also highlights the possibility that the serologic testing method is underestimating the true prevalence. Some seronegative PCR-positive dogs may be explained as acute infections, as during the early stages of infection a dog may be parasitemic, but not yet have developed antibodies. However, this scenario is unlikely to explain dogs with circulating parasite DNA during the winter months, when vector activity is greatly reduced. Further, we extracted DNA from a small volume (~250 µl) of blood clot, which may have limited our ability to detect circulating parasite DNA, especially if the parasite burden was low. While the qPCR we used has been shown to be highly sensitive for detection of *T. cruzi* in blood samples (Schijman et al., 2011), extraction volumes and methods differ across studies. While extracting from a larger volume of blood may have improved our limit of detection for samples with an even lower concentration of parasite DNA, the epidemiologic significance of dogs with a very low level of circulating parasite DNA is uncertain.

Most of the PCR-positive dogs were infected with strain type TcI, with only 1 dog infected with TcIV. While historic reports documented primarily TcIV infection in dogs,(Roellig et al., 2008) our findings are consistent with recent studies in Texas that found predominantly TcI with fewer TcIV infections (Curtis-Robles et al., 2017b; 2017a). There is

some indication that different strain types may have different pathologic effects in dogs (Barr et al., 1991b; 1991c; Duz et al., 2014) and in humans (J. D. Ramírez et al., 2010), and thus determination of which strain type a dog is infected with may provide some clinical insight or be relevant for zoonotic concerns. Thus far, autochthonous human cases of *T. cruzi* infection in the U.S. for which the DTU has been determined have consisted of TcI or TcII/V/VI group, with no finding of TcIV in humans (Garcia et al., 2017; Roellig et al., 2013). An unknown proportion of seropositive dogs will remain subclinical for life. The predictive factors for whether a dog will develop disease have not been determined, and it is possible that strain type is a factor.

A major obstacle in Chagas disease diagnostics in both humans and dogs is discordance between serologic test results, as we observed in our study. It is generally recommended that at least two different tests be used to confirm a diagnosis, reflecting the uncertainty among experts about the accuracy of any single test (Afonso et al., 2012). There is no gold standard against which to compare existing or new tests, and while several studies have attempted to validate different tests, these are of varying quality and differ in their handling of discordant or borderline results (Afonso et al., 2012). Discordance between serologic tests may be related to parasite genetic differences that vary over geographic areas (Guzmán-Gómez et al., 2015; Houghton et al., 2009; Verani et al., 2009), and thus certain tests may be better suited to a particular area than others. One study with human sera reported Stat-Pak sensitivity to be as low as 26.6% in Peru but as high as 87.5% in Bolivia (Verani et al., 2009). In our study, only 9 (7.5%) dogs that were positive on the Stat-Pak were negative on the CDP. However, an additional 39 of 81 dogs that were Stat-Pak negative developed faint to strong bands on the CDP. Furthermore, several PCR-positive and Stat-Pak

negative dogs were CDP positive, suggesting that the CDP may have a higher sensitivity in this population of dogs. Thus, our reported seroprevalence may be a conservative estimate of the true prevalence, and improved diagnostic tests are critical for more precisely determining the true canine *T. cruzi* seroprevalence in the U.S. and for improving monitoring and development of interventions to reduce transmission.

No specific therapy against *T. cruzi* is approved for dogs in the United States. In the absence of treatment options or vaccines, efforts to prevent disease transmission must be aimed at limiting contact with kissing bug vectors. Kissing bug nymphal stages utilize harborage sites such as brush and wood piles, so debris should be cleared from the areas surrounding residences and dog kennels. Ideally, dogs should be housed inside a bug-proof residence at night, though this does not completely eliminate the risk, as kissing bugs can be active during the day as well (particularly dawn and dusk) and bugs have also been found within homes (Curtis-Robles et al., 2015; Kjos et al., 2009b; Klotz et al., 2016). Additional measures that can be taken to reduce bug exposure include: fully screening open areas of kennels to exclude bugs; reducing cracks, crevices, and other dark areas where bugs can hide within the kennel and/or around dog resting areas; clearing vegetation in the immediate vicinity; and treating perimeters with pesticides. Risk of direct transmission from dog to human is considered extremely low, but infected dogs may serve as a source of infection to kissing bug vectors in the local environment, which could increase transmission risk to other human or animal hosts (Gürtler et al., 2007; Gürtler and Cardinal, 2015). Perhaps more importantly, infected dogs serve as a warning of the local transmission environment (e.g. presence of infected vectors) that could pose a risk to humans or other dogs in the same locale.

## 2.5 Conclusion

Shelter dogs across Texas show widespread *T. cruzi* seroprevalence, with relatively lower risk associated with shelters in the more northern part of the state (Fort Worth, Dallas). Older dogs (> 2 years) had higher odds of seropositivity. Only 1.1% of dogs were confirmed PCR-positive, and strain types TcI (n=5) and TcIV (n=1) were detected in these dogs. Discordance between diagnostic tests was observed, and our reported seroprevalence is likely a conservative estimate of the true prevalence. American trypanosomiasis is an under-recognized threat to canine health in the United States. *T. cruzi* infection should be considered as a differential in dogs with cardiac signs that reside in or have a travel history to the southern United States. Improved diagnostics are necessary, even before advances can be made in development of drugs or vaccines, because accurate testing is required for determination of efficacy. Additionally, from a One Health perspective, any advances in our understanding of canine Chagas disease have the potential to also advance human health given the shared risk factors of triatomines in the environment, similar disease progression between humans and dogs, and parallel challenges with respect to suboptimal diagnostics.

# 3. TRYPANOSOME SPECIES, INCLUDING *TRYPANOSOMA CRUZI*, IN SYLVATIC AND PERIDOMESTIC BATS OF TEXAS, USA*

## 3.1 Introduction

Bats are associated with a number of zoonotic pathogens (Calisher et al., 2006), and their reservoir potential may be heightened relative to other mammals due to their ability to fly, highly gregarious social structures, and long life spans (Luis et al., 2013). Long migration distances of some bat species may play a role in the circulation and spread of pathogens, as has been demonstrated for neotropical migratory birds (Cohen et al., 2015; Mukherjee et al., 2014). The vector-borne protozoal parasite *Trypanosoma cruzi*, agent of Chagas disease, is of major public health importance and infects animals of virtually all mammalian orders (Gaunt and Miles, 2000). It is transmitted via the feces of hematophagous insects of the subfamily Triatominae (kissing bugs), and wildlife reservoirs appear to play an important role in the maintenance and transmission of the parasite in sylvatic transmission cycles (Bern et al., 2011). *T. cruzi* is a genotypically heterogeneous species that has been divided into six discrete typing units (DTUs), TcI – TcVI (Zingales et al., 2012), and a seventh recently discovered bat-associated type TcBat (L. Lima et al., 2015a; Marcili et al., 2009a). The DTUs TcI and TcIV are enzootic in the southern United States. Evidence now suggests that *T. cruzi* and related parasites likely evolved originally from a bat trypanosome lineage, rather than evolving in isolation in mammals of South America, Antarctica, and Australia as previously theorized (Hamilton et al., 2012b; L. Lima et al., 2013; 2012).

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*Reprinted with permission from: Hodo, C.L., Goodwin, C.C., Mayes, B.C., Mariscal, J.A., Waldrup, K.A., Hamer, S.A., 2016. Trypanosome species, including *Trypanosoma cruzi*, in sylvatic and peridomestic bats of Texas, USA. Acta Tropica 164, 259–266. doi:10.1016/j.actatropica.2016.09.013

The *T. cruzi* clade of trypanosomes is divided into two main sister phylogenetic lineages: the sugbenus *Schizotrypanum* and the *T. rangeli/T. conorhini* clades (L. Lima et al., 2015b). Bats have long been associated with trypanosomes of the *Schizotrypanum* subgenus, of which *T. cruzi* (*sensu stricto*) is the only member not restricted to bats (Barnabe et al., 2003; Molyneux, 1991). Other members of *Schizotrypanum* include *T. dionisii* in Old and New World bats, *T. cruzi marinkellei* in bats of Central and South America, and *T. erneyi* in African bats (Baker et al., 1978; Barnabe et al., 2003; Gardner and Molyneux, 1988; L. Lima et al., 2015a; 2012; Molyneux, 1991). Genetic similarities between strains of *T. dionisii* isolated from Europe and South America suggest the movement of this parasite via bats between the Old and New worlds (Hamilton et al., 2012a). Other species within the *T. cruzi* clade include: *T. vespertilionis*, *T. conorhini*, *T. rangeli*, *T. livingstonei*, and a number of others isolated from bats and other mammals or marsupials in Africa and Australia (L. Lima et al., 2015b).

The most common trypanosomes detected in neotropical bats are *T. cruzi*, *T. c. marinkellei*, *T. dionisii*, *T. rangeli*, and *T. conorhini*, with apparent prevalences ranging from 10 to 80% (Cottontail et al., 2009; García et al., 2012; Marcili et al., 2009a; 2009b; Pinto et al., 2012; J. D. Ramírez et al., 2014). Despite the migration of some bat species between South, Central, and North America, and local presence of large numbers of *T. cruzi*-infected triatomine vectors across Mexico and the Southern US (Bern et al., 2011; Curtis-Robles et al., 2015; Ramsey et al., 2000), no study has reported the presence of *T. cruzi* or any trypanosome species in bats in North America. Our objective was to quantify the frequency at which bats were infected with trypanosomes and compare the genetic diversity of these parasites in bats from both peridomestic and sylvatic habitats across Texas.

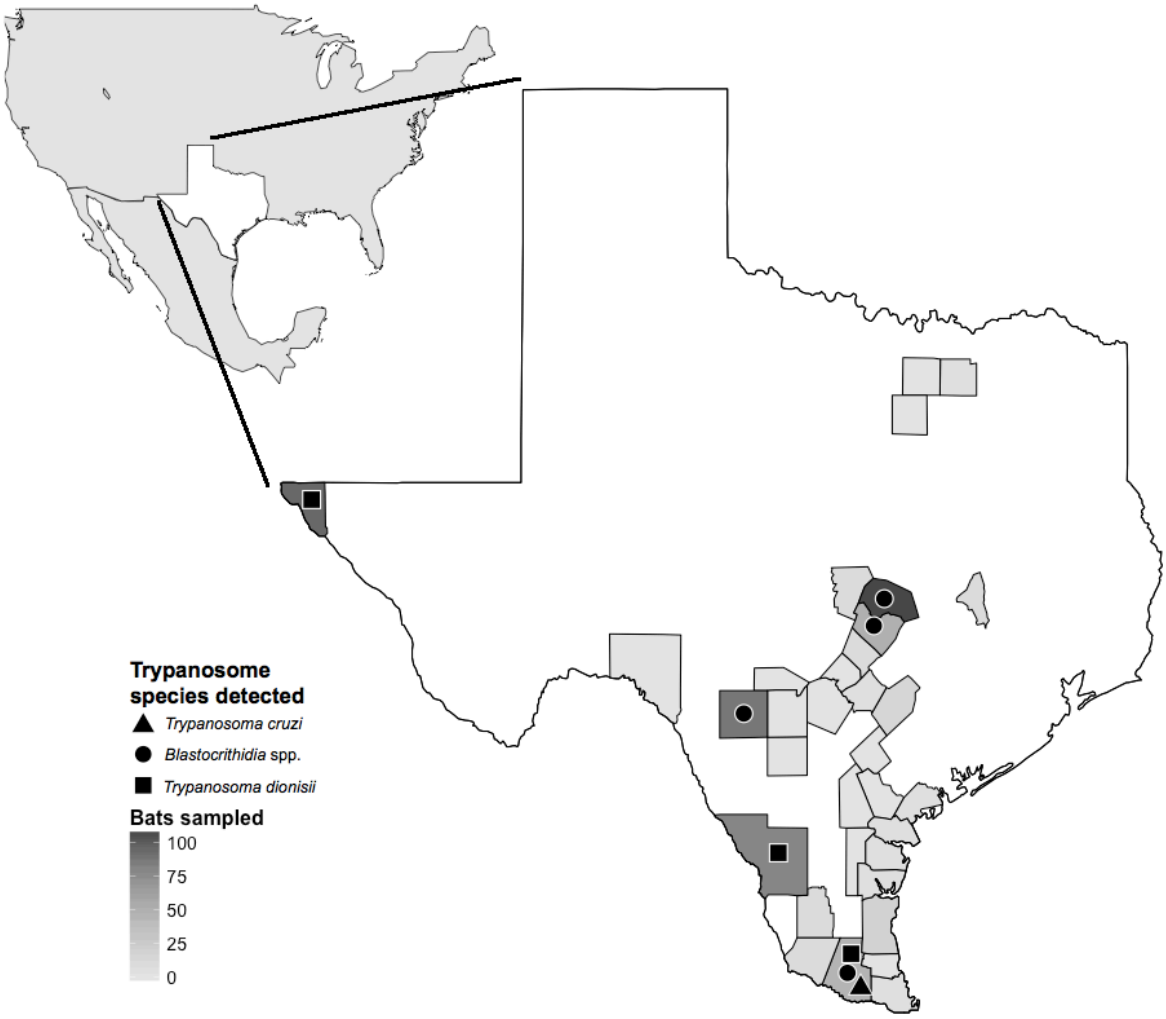


**Figure 3.1. Map of Texas with sampled counties shaded according to sample size and shapes marking counties from which trypanosome-positive bats originated.** Reprinted with permission from (Hodo et al., 2016).

## 3.2 Materials and Methods

### *3.2.1 Peridomestic Bats*

Through collaboration with the Texas Department of State Health Services (DSHS), we acquired carcasses of bats previously submitted by the public and determined to be negative for rabies by state laboratories in Austin or El Paso. These bats were considered

peridomestic because they were encountered directly by members of the public, often in homes or places of work. The majority (87%) of bats submitted for rabies testing in Texas are submitted because of concerns that they potentially exposed a person or domestic animal to rabies (Mayes et al., 2013a). Bats were identified to species by personnel at the DSHS labs using morphological characteristics, including standard measurements such as antebrachium length (Ammerman et al., 2012). Bats had been stored in a freezer for up to three years prior to our study, but the majority (85%) were stored from 3-9 months. Each animal's species, sex, and degree of autolysis were recorded, and the heart was collected and bisected in a biosafety level 2 cabinet. The apex of the heart was minced in preparation for DNA extraction.

*3.2.2 Sylvatic Bats*

To represent sylvatic populations of bats that are less likely to be encountered directly by the public, bats were captured at three field sites in South Texas in Kenedy (27.174N, 97.864W), Jim Hogg (26.965N, 98.852W and 26.908N, 98.758W), Starr (26.737N, 98.774W), and Uvalde (29.435N, 99.685W) counties. In Kenedy, Jim Hogg, and Starr counties, bats were captured on large cattle ranches using mist nets set over low water tanks. In Uvalde county, bats were captured during emergence and return to a cave using hand-held mist nets (Waldien and Hayes, 1999). Bats were removed from mist nets, weighed, evaluated for species and sex identification, and manually restrained for blood collection. Species was determined without difficulty by morphologic features using a field guide of bats in Texas (Ammerman et al., 2012). A 25g needle was used to puncture one of the interfemoral veins, and capillary tubes were used to collect a volume equal to no more than 1% of the animal's

body weight. Pressure was applied to the puncture site until bleeding had stopped and bats were then released directly or returned to a cloth bag to recover for up to 10 minutes then released. The capture of animals and all subsequent procedures were conducted according to the recommendations and approval of Texas A&M University IACUC (Institutional Animal Care and Use Committee) Animal Use Protocol 2015-0088 and Texas Parks and Wildlife Department scientific collections permit SPR-0512-917. Additionally, in collaboration with researchers performing a biodiversity study, we obtained hearts from bats collected as museum specimens from the ranch properties. These bats were captured in mist nets and euthanized via an overdose of halothane or isoflurane in accordance with IACUC permit 2015-0126 and Texas collections permit SPR-0409-082.

### *3.2.3 Trypanosome Detection*

DNA was extracted from blood and heart tissue using a commercial kit (E.Z.N.A Tissue DNA Kit; Omega Bio-Tek, Norcross, GA) following manufacturer's instructions with an overnight lysis period. Extracted DNA was subjected to two separate PCR protocols for the detection of *T. cruzi* and other trypanosomes. First, a sensitive quantitative, real-time PCR for the specific detection of *T. cruzi* was performed using the Cruzi 1/2 primers and a 6-carboxyfluorescein (FAM)-labeled probe, Cruzi 3, as previously described (Piron et al., 2007; J. C. Ramírez et al., 2015), but with an initial denaturation time of 3 minutes. Based on internal laboratory validations, the cutoff for positive samples was determined to be a quantification cycle value of 33 or less. Next, all samples were subjected to a nested PCR targeting an 18S (SSU) rRNA-encoding gene fragment of trypanosomes, as previously described (Noyes et al., 1999; Pinto et al., 2015). Additionally, *T. cruzi* positive samples

were subjected to a multiplex probe-based qPCR for determination of strain type (Cura et al., 2015). DNA extractions, primary and secondary amplifications, and product analyses were performed in separate dedicated laboratory areas. A negative control was included in each set of DNA extractions and a water negative control was used in PCR reactions as contamination controls. The DNA from *T. cruzi* Sylvio X10 clone4 (American Type Culture Collection, Manassas, VA) served as a positive control. Samples that gave positive results on the nested PCR were repeated on the same assay one or two more times for confirmation in consistency of results. Amplification products were separated on agarose gels, purified (ExoSAP-IT; Affymetrix, Santa Clara, CA), and sequenced in both forward and reverse at Eton Biosciences Inc. Resulting sequences were analyzed and aligned using MEGA7 software (Kumar et al., 2016), and compared to a national sequence database (GenBank) using the BLAST program (Altschul et al., 1990). We created alignments for each separate species group generated in this study (*T. cruzi*, *T. dionisii*, and *Blastocrithidia*) including representative reference sequences, as well as aligning all of the species together with additional reference sequences from other trypanosome species. Neighbor Joining trees were created in Mega7 with 1000 bootstrap replicates to compare sequences generated in the current study to representative sequences from GenBank.

### *3.2.4 Confirmatory PCRs*

For the purpose of confirming our nested PCR findings, attempts were made to amplify and sequence additional genetic markers several months after the initial molecular work. The *Blastocrithidia* positive samples were subjected to a PCR targeting the 24Sα rRNA gene using primers D75 and D76 as described previously (Schijman et al., 2006; Souto

et al., 1999). The remaining positive samples were subjected to a PCR previously used in the description of bat trypanosomes, targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Da Silva et al., 2004).

Additionally, to assess the probability of *T. cruzi* detection from these mixed DNA samples in which the majority of DNA is from the bat host, 5% of the negative bat samples (n=30), selected across a variety of autolysis scores and dates of extraction, were spiked with a low concentration (1:10^5 dilution, equivalent to <4 parasites) of *T. cruzi* positive control DNA and run on the nested PCR negative water control and positive control of the same concentration as that in the spiked samples but without the vertebrate DNA.

## 3.3 Results

### *3.3.1 Peridomestic Bats*

A total of 487 peridomestic bats were received from the Austin and El Paso DSHS labs, and we collected the heart from 474. Sampled bats originated from 29 counties across Texas (Figure 3.1), and represented 8 insectivorous species of the family Vespertilionidae. The majority of bats sampled were *Tadarida brasiliensis* (82.5%, n=391), followed by *Nycticeius humeralis* (7.8%, n=37), *Parastrellus hesperus* (3.2%, n=15), *Lasiurus borealis* (2.9%, n=10), *Antrozous pallidus* (1.9%, n=9), *Lasiurus intermedius* (1.5%, n=7), *Myotis velifer* (0.8%, n=4), and *Perimyotis subflavus* (0.8%, n=1). There were 296 males (61.8%), 166 females (35%), and 15 for which sex could not be determined due to the condition of the carcass.

*3.3.2 Sylvatic Bats*

We captured 105 bats in mist nets in 4 counties in south Texas (Figure 3.1) and collected blood from 103. We obtained 16 hearts from animals collected for museum specimens. These sylvatic bats were of 3 species: *T. brasiliensis* (71%, n=85), *N. humeralis* (29%, n=35), and *L. intermedius* (0.8%, n=1). There were 75 females (63%) and 44 males (37%).

*3.3.3 Trypanosome Detection*

Samples from 593 bats were tested for trypanosomes using both nested PCR (for generic *Trypanosoma* detection) and qPCR (for specific *T. cruzi* detection). A single male peridomestic *N. humeralis* bat was positive for *T. cruzi* on both the qPCR and nested PCR; 9 peridomestic bats were positive for *T. dionisii* via nested PCR; and 4 peridomestic bats (3 *T. brasiliensis*, 1 *N. humeralis*) and 1 sylvatic bat (*T. brasiliensis*) were positive for *Blastocrithidia* spp. via nested PCR (Tables 3.1 and 3.2). The *T. cruzi* positive sample was determined to be TcI on the multiplex qPCR. The *T. cruzi* positive bat was from Hidalgo county; *T. dionisii* positive bats were from Hidalgo, El Paso, and Webb counties; and *Blastocrithidia* spp. were from Hidalgo, Travis, Williamson, and Uvalde counties (Table 3.2; Figure 3.1).

**Table 3.1 Species distribution and apparent prevalence of trypanosomes in bats tested.** Reprinted with permission from Reprinted with permission from (Hodo et al., 2016).

| | | *T. cruzi* | | *T. dionisii* | | *Blastocrithidia* spp. | |
|---|---|---|---|---|---|---|---|
| Species | # Tested | # Positive | Apparent Prevalence | # Positive | Apparent Prevalence | # Positive | Apparent Prevalence |
| *Tadarida brasiliensis* | 476 | 0 | 0.0% | 5 | 1.1% | 4 | 0.8% |
| *Nycticeius humeralis* | 70 | 1 | 1.4% | 0 | 0.0% | 1 | 1.4% |
| *Parastrellus hesperus* | 15 | 0 | 0.0% | 2 | 13.3% | 0 | 0.0% |
| *Antrozous pallidus* | 9 | 0 | 0.0% | 2 | 22.2% | 0 | 0.0% |
| Others* | 23 | 0 | 0.0% | 0 | 0.0% | 0 | 0.0% |
| Total | 593 | 1 | 0.2% | 9 | 1.5% | 5 | 0.0% |

*Other species include: *Lasiurus borealis, Lasiurus intermedius, Myotis velifer, Perimyotis subflavus*

**Table 3.2. Demographic details of bats that tested positive for trypanosomes.** Reprinted with permission from (Hodo et al., 2016).

| Sample ID | Species | Sex | County | Trypanosome ID |
|---|---|---|---|---|
| A14-6132 | *Nycticeius humeralis* | M | Hidalgo | *T. cruzi* |
| A14-6383 | *Tadarida brasiliensis* | M | Hidalgo | *T. dionisii* |
| A15-1338 | *Tadarida brasiliensis* | F | Webb | *T. dionisii* |
| A15-1726 | *Tadarida brasiliensis* | M | Hidalgo | *T. dionisii* |
| A15-1920 | *Tadarida brasiliensis* | F | Webb | *T. dionisii* |
| R15-053 | *Tadarida brasiliensis* | M | El Paso | *T. dionisii* |
| R12-302 | *Antrozous pallidus* | M | El Paso | *T. dionisii* |
| R14-230 | *Antrozous pallidus* | M | El Paso | *T. dionisii* |
| R15-094 | *Parastrellus hesperus* | F | El Paso | *T. dionisii* |
| R15-092 | *Parastrellus hesperus* | F | El Paso | *T. dionisii* |
| A14-5860 | *Tadarida brasiliensis* | M | Williamson | *Blastocrithidia* spp. |
| A14-6260 | *Tadarida brasiliensis* | M | Travis | *Blastocrithidia* spp. |
| A14-6629 | *Tadarida brasiliensis* | F | Travis | *Blastocrithidia* spp. |
| FC1507-03 | *Tadarida brasiliensis* | F | Uvalde | *Blastocrithidia* spp. |
| 2015AU-3671 | *Nycticeius humeralis* | F | Hidalgo | *Blastocrithidia* spp. |

### *3.3.4 Confirmatory PCRs*

For the PCR targeting the 24Sα region, 4/5 of the samples that were positive for *Blastocrithidia* on the nested PCR yielded a band on gel electrophoresis, and sequences were obtained from 3 of these. These sequences had 98-99% homology with a sequence of *Blastocrithidia sp*. from a *Triatoma guasayana* (AY820895). We obtained a partial GAPDH

sequence with 96% homology to *T. dionisii* (GQ140363) from sample A14-1338. The remaining samples showed nonspecific amplification likely resulting from the mixed DNA template (i.e., not cultured parasite) that was studied. Of the subset of samples that were negative for trypanosomes in the nested PCR, none were determined to be positive in any other assay.

We detected *T. cruzi* in all 30 of the spiked samples with intensity of bands indistinguishable to that of the positive control which contained the same concentration of *T. cruzi* DNA but without bat host DNA.

### *3.3.5 Phylogenetic Analysis*

A phylogenetic tree was constructed including all of the 18S rRNA sequences generated in this study together with representative reference sequences from GenBank (Figure 3.2). The *T. cruzi*-positive bat from the current study (GenBank accession KX227594) was grouped together with other TcI isolates, supporting the results from the strain typing qPCR.

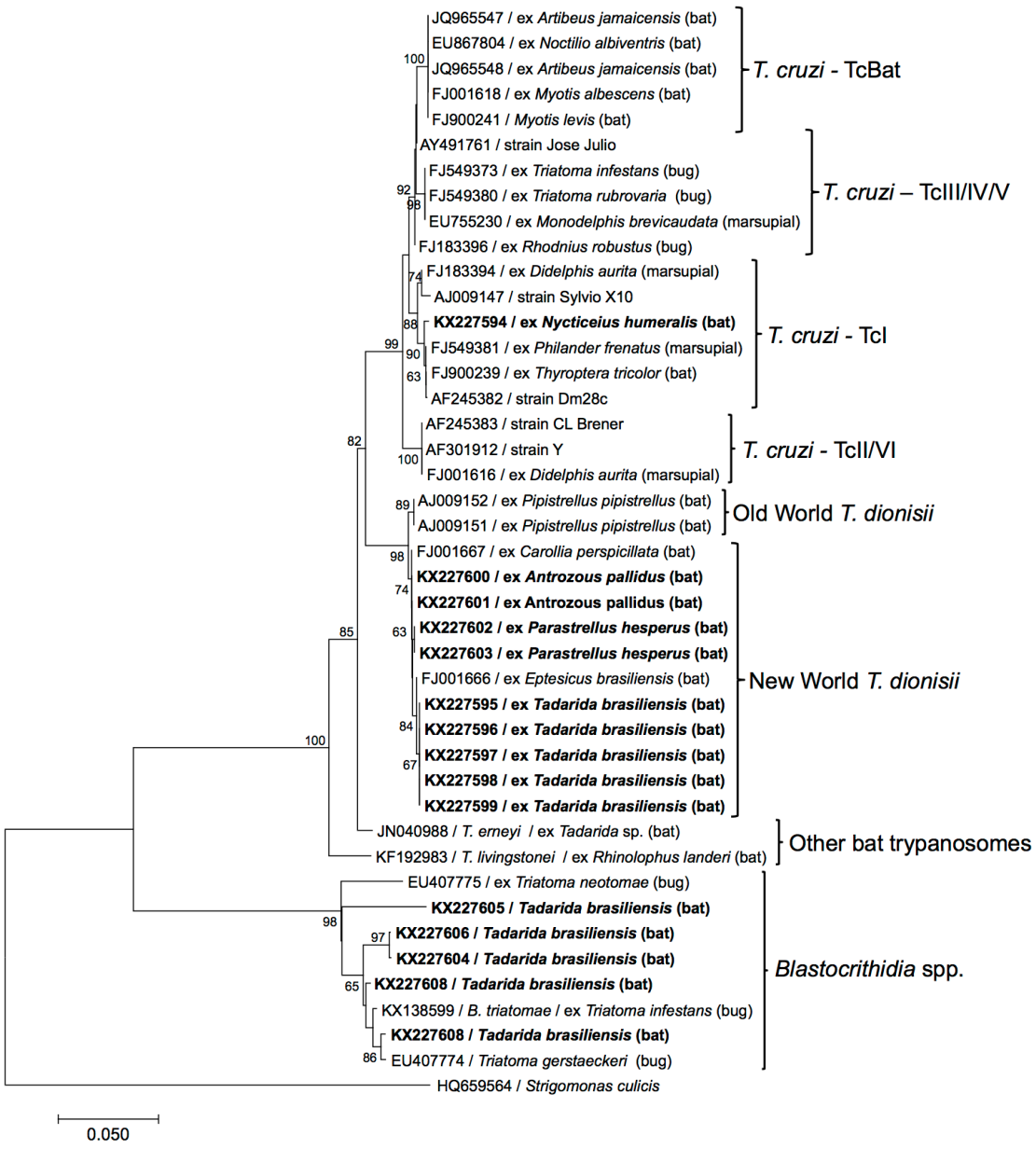


**Figure 3.2. Phylogenetic tree comparing a 532 bp segment of the 18S rRNA gene of trypanosomes, constructed using the Neighbor-Joining method in Mega7. The sequences in bold were generated during this study.** Reprinted with permission from (Hodo et al., 2016).

The nine *T. dionisii* sequences from the current study represented three unique sequence variants, grouped within the clade of New World *T. dionisii* isolates. Within the 493 bp region of analysis, a total of 6 single nucleotide polymorphisms (SNPs) or indel

events distinguished these variants from each other and from the most similar *T. dionisii* sequences in Genbank. All sequences obtained from the same bat species were identical to each other, yet differed among species, with the sequences obtained from *A. pallidus* (GenBank accessions KX227600-1) and *P. hesperus* (KX227602-3) being more similar to each other than to the sequences from *T. brasiliensis* (KX227595-9; Figure 3.2).

The five *Blastocrithidia* sequences from our study (KX227604-8) all differed from each other and from previously published *Blastocrithidia* sequences, which have exclusively been reported from insects (Figure 3.2). When compared to sequences in GenBank, the closest matches were with *Blastocrithidia* spp. isolated from true bugs (Hemiptera), including *Blastocrithidia triatomae* from a *Triatoma protracta* in Argentina (AF153037) and *Blastocrithidia* spp. isolated from *Triatoma* spp. in Texas (EU407774-6). Among the 9 *Blastocrithidia* sequences of 592 base pairs included in the alignment, there were 38 indel events and 86 SNPs. One sequence we generated, from a *Tadarida brasiliensis* in Travis county (KX227605), was especially different from the others, forming a separate clade on the phylogenetic tree (Figure 3.2).

### 3.4 Discussion

Trypanosomes were detected in 15 of 593 (2.6%) bats, with a single bat positive for *T. cruzi*. The overall level of trypanosome detection in bats across Texas is lower than that found in studies of bats from Brazil, Panama, Ecuador, and Colombia (Cavazzana et al., 2010; Cottontail et al., 2014; Pinto et al., 2015; J. D. Ramírez et al., 2014), which ranged from 11-37%. Meanwhile, a survey of bats in Mexico specifically for *T. cruzi* found that none of 116 were infected (Ramsey et al., 2012). However, the species of bats we sampled

are different from those represented in previous studies, with the exception of a small number (2/6) of *T. brasiliensis* reported to be infected with uncharacterized trypanosomes in Brazil (Cavazzana et al., 2010).

Our findings likely represent a conservative estimate of the true prevalence, given the aged nature of some carcasses. Additionally, the degree to which cardiac tissue analysis reflects bat-level trypanosome infection status is not known. *T. cruzi* is well known to localize in heart muscle cells, but tissue tropism has not been established for *T. dionisii* or *Blastocrithidia* species. Previous surveys of trypanosomes in bats have almost exclusively used peripheral blood samples, and it is possible that the use of heart tissue is less sensitive for the detection of some species of trypanosomes. However, it should be noted that a small amount of cardiac blood was likely included with heart tissue of most samples subjected to PCR. Because our study design and use of predominantly bat carcasses did not allow for the use of hemoculture to isolate any of the detected trypanosomes in culture, we were unable to perform extensive genetic characterization.

### *3.4.1. Detection of T. cruzi and Epidemiological Importance*

Assessing the epidemiological importance of *T. cruzi* in wild bats in the United States must consider many ecological factors. Although we detected *T. cruzi* in only a single evening bat (*Nycticeius humeralis*), this must be considered in the context of the overall population size of these bats and how they move across the landscape. Estimates of population sizes of most North American bats are challenging due to their small body size, nocturnal behavior, and cryptic roost sites (Kunz, 2003), and population size of *N. humeralis* is estimated to be between 100,000 and 1 million (NatureServe, 2015). Given our observed

frequency of infection of 1/72 (1.4%) in *N. humeralis*, extrapolation suggests between 1,388 and 13,880 *T. cruzi*-infected evening bats across the US.

Although *T. cruzi* infection in bats was rare, bats may nonetheless serve as reservoirs if they are part of a community in which the pathogen can be permanently maintained and transmitted (Haydon et al., 2002). Texas is home to 32 species of bats and at least 7 species of triatomines (Ammerman et al., 2012; Curtis-Robles et al., 2015). The two most common triatomine species encountered in Texas are *Triatoma gerstaeckeri* and *T. sanguisuga* and approximately 50-70% of these are infected with *T. cruzi* (Curtis-Robles et al., 2015; Kjos et al., 2009b). Bats have the opportunity to encounter triatomines during foraging and feeding at night when both are active, as well as potentially being fed upon by the bugs during the day when roosting in trees or caves. While there has been no specific research into whether North American bats feed on triatomines, a significant proportion of the diet of many species includes Hemipterans (Carter et al., 2004; McWilliams, 2005). An experimental trial documented infection of Phyllostomid bats with *T. cruzi* after feeding on infected triatomines (Thomas et al., 2007). Triatomines are notoriously found in nests and resting areas of terrestrial mammals (Lent and Wygodzinsky, 1979), and have been found in and around caves in Texas (Hamer et al, unpublished data) as well as under loose bark of trees (Lent and Wygodzinsky, 1979), sites similar to those where many species of bats roost during the day. Further, a blood-meal analysis study revealed the blood of an evening bat (*N. humeralis*) in a *T. gerstaeckeri* from Texas (Gorchakov et al., 2016), demonstrating triatomine-bat contact. The degree to which bats maintain parasitemia and thus are infectious to vectors has not been well-studied, however, the isolation via hemoculture of *T. cruzi* from blood of bats in Central

and South America supports their status as a reservoir in those areas (Cavazzana et al., 2010; L. Lima et al., 2012; Pinto et al., 2015).

Further, assessing the epidemiological significance of *T. cruzi*-infected wildlife must also consider the parasite genetic strain and the degree to which it is infective to humans. Two main strain types of *T. cruzi* are endemic in the US, TcI and TcIV, with one report of TcII from rodents in Louisiana (C. P. Herrera et al., 2015). The *T. cruzi*-infected bat in our study harbored TcI, the only strain type associated with disease in humans in the US thus far (Roellig et al., 2008).

Finally, evaluating bats in the epidemiology of Chagas disease requires an understanding of the overall ecology of the wildlife reservoir system of *T. cruzi* in the US. Nearly all other wildlife species that have been evaluated and reported for infection with *T. cruzi* in the southern US are associated with a higher frequency of infection that what we found in bats. For example, reported apparent prevalence of *T. cruzi* infection is 75% in striped skunks (*Mephitis mephitis*), 60-70% in raccoons (*Procyon lotor*), 14% in bobcats (*Lynx rufus*), 14% in coyotes (*Canis latrans*), 14% in gray foxes (*Urocyon cinereoargenteus*), 34% in woodrats (*Neotoma micropus*), and 18% in other rodents (Charles et al., 2012; Curtis-Robles et al., 2016). Bats may play a unique role in this host community because of their ability to transport the pathogen over long distances during foraging or migration.

### *3.4.2. Detection of Other Trypanosomes*

We detected *T. dionisii* in 9/593 bats (1.5%) of 3 species (*T. brasiliensis*, *A. pallidus*, *P. hesperus*). *T. dionisii* is a well-known trypanosome of bats in South America and Europe

(Molyneux, 1991), but has not before been detected in North America. Although this parasite can enter cells and form pseudocysts in cardiac myocytes (Cavazzana et al., 2010; Gardner and Molyneux, 1988), there is no evidence that *T. dionisii* or other trypanosomes are pathogenic to bats. Based on the 18S rRNA gene fragment we sequenced, the Texas bat *T. dionisii* sequences all grouped with the New World isolates of *T. dionisii*. Further, we detected three unique variants that were uniform within each of the three infected bat species. Variants differed among species even within the same geographical area (Figure 3.2). Additional genetic analyses may further characterize the ecological importance of these host-parasite associations.

As an unexpected finding, based on sequencing of two gene regions (18S rRNA and 24Sα rRNA), we detected bats infected with *Blastocrithidia* sp., a genus of trypanosome associated with the alimentary tract of insects of the order Heteroptera. They are considered monoxenous, restricted to a single host during their life cycle, and are closely related to other similar insect trypanosomes that infect other insect orders, and to the dixenous parasite *Leishmania*, a mammalian pathogen (Maslov et al., 2013). *Blastocrithidia triatomae* was isolated from a laboratory reared colony of *Triatoma infestans* (vectors of *T. cruzi*) in Argentina (Cerisola et al., 1971) and genetically similar organisms were isolated from several species of *Triatoma* in Texas (Kjos et al., 2009a). The PCR-based approach we used does not allow us to evaluate whether the parasite was alive or dead; further, the presence of this parasite in the heart tissue samples could reflect either infection of the cardiac myocytes or infection of the blood. It is possible that *Blastocrithidia* spp. are capable of travelling systemically within bats following the consumption of an infected insect, but the degree of transience and outcome of such an event is unknown.

*3.4.3. Ecology of Bat Species Infected with Trypanosomes*

*T. brasiliensis* is the most numerous species of bat in Texas, is commonly encountered by humans (Mayes et al., 2013b), and was also the most well-represented species in our sample (80% of all bats). Both *T. dionisii* and *Blastocrithidia* spp. infected this species at a low frequency. *T. brasiliensis* are highly gregarious and migratory, and while the full range of the species extends from Argentina to the central US, the bats found in Texas in the warm months are thought to spend the winter in central Mexico (Villa and Cockrum, 1962). The county with the highest number of trypanosome-positive bats was El Paso, and two of the species in which *T. dionisii* was detected (*A. pallidus* and *P. hesperus*), were not collected from any other county. *A. pallidus* and *P. hesperus* are found throughout the western US down to Mexico and do not migrate (Ammerman et al., 2012). Due to the lack of migration, the trypanosome detections in these species likely reflect infection in the west Texas region of El Paso. *N. humeralis*, the only species in which *T. cruzi* was detected in our study, is found across the eastern US, west to central Texas and south to northern Mexico. Females are thought to be migratory, while males may remain in the southern part of the range through the summer (Ammerman et al., 2012). The single *N. humeralis* positive for *T. cruzi* was male, suggesting that infection was most likely acquired in south Texas. The *T. cruzi* and *T. dionisii* positive bats were all from counties along the Texas-Mexico border, a region of increasing concern for local transmission of the Chagas parasite to humans and dogs (Beard et al., 2002; Esteve-Gassent et al., 2014; Sarkar et al., 2010; Tenney et al., 2014).

### *3.4.4. Future Directions*

Future work to explore the trypanosomes of bats of the US should focus on acquiring a larger sample size of diverse species of bats, especially from counties along the US-Mexico border, and include sequencing of additional gene segments for more detailed phylogenetic analysis, as well as attempts to culture isolates. Through these efforts, advances could be made to expand the knowledge base of host associations, genetic diversity, and geographical range of bat-associated trypanosomes.

# 4. STRAIN TYPE ASSOCIATIONS AND PATHOLOGY OF *TRYPANOSOMA CRUZI* INFECTION IN COYOTES (*CANIS LATRANS*) AND RACCOONS (*PROCYON LOTOR*) OF TEXAS, USA

## 4.1 Introduction

*Trypanosoma cruzi*, the vector-borne protozoal agent of Chagas disease, is endemic across much of Latin America and is capable of infecting over 200 mammalian species (Hoare, 1972). The parasite multiplies in the hindgut of triatomine insect vectors (family Reduviidae, subfamily Triatominae), which pass infectious trypomastigote forms in their feces. Chagas disease is a major public health problem in endemic areas and is increasingly recognized as a threat to human and veterinary public health across the southern US, where sylvatic transmission cycles among vectors and wildlife reservoirs have been recognized for decades (Kofoid and Donat, 1933; Kofoid and McCulloch, 1916). While many infected hosts may remain asymptomatic, some infected humans, dogs, and non-human primates develop cardiac disease leading to sudden death or the development of congestive heart failure (Bern et al., 2011; Rassi et al., 2010; Snowden and Kjos, 2013). Meanwhile, few studies have investigated disease outcomes in wildlife reservoirs.

Across the Americas, *T. cruzi* is maintained in complex transmission cycles involving diverse mammalian reservoir species and triatomine vector species. The complexity of these cycles and regional heterogeneity is one of the major challenges in Chagas disease control and prevention. In the US, over 30 wildlife species have been identified as susceptible hosts, but the relative importance of these species as reservoirs (i.e., their contribution to the transmission and maintenance of the parasite in nature by serving as sources of infection to

vectors) has been understudied (Hodo and S. A. Hamer, 2017). Additionally, investigations into the pathology of *T. cruzi* in naturally-infected wildlife have been conducted only on a limited basis and in only a few species (Barr et al., 1991a; Packchanian, 1942; Pietrzak and Pung, 1998; Ryan et al., 1985). An understanding of the degree to which various wildlife reservoirs are clinically impacted by *T. cruzi* infection; i.e., their position on the spectrum from unaffected carriers of the parasite to severely diseased hosts, and the extent to which they develop parasitemia is necessary for predicting population-level impacts of infection as well as targeting interventions to manage zoonotic risk.

*T. cruzi* is genotypically heterogeneous and is divided into 7 discrete typing units (DTUs): TcI-TcVI and TcBat, which are associated with different geographical regions (Marcili et al., 2009a; Zingales et al., 2012), mammalian hosts (Jansen et al., 2017), and vector species (Brenière et al., 2016). Further, there is evidence for associations between DTU and varying clinical outcomes in humans (J. D. Ramírez et al., 2010) and dogs (Barr et al., 1991b; Duz et al., 2014), as well as in other experimental animal models (Lisboa et al., 2007; Roellig et al., 2009b). Strains TcI and TcIV predominate in the United States (Bern et al., 2011; Hodo and S. A. Hamer, 2017; Roellig et al., 2008), and TcII was identified in a small number of rodents in Louisiana (C. P. Herrera et al., 2015). In the US, only TcI and TcII/V/VI group have been associated with autochthonous human infection (Garcia et al., 2017; Roellig et al., 2013). Limited studies have revealed that most sampled raccoons (*Procyon lotor*) were infected with TcIV, while opossums of *Didelphis* spp. were almost exclusively infected with TcI (Bern et al., 2011; Roellig et al., 2008). Both TcI and TcIV have been identified in skunks (*Mephitis mephitis*), armadillos (*Dasypus novemcinctus*), woodrats (*Neotoma* sp.), and domestic dogs (Charles et al., 2012; Roellig et al., 2008). Strain

type associations may be important in classifying wildlife species as important reservoirs of infection. Additionally, the apparent associations between strain type and pathology or clinical outcome in wildlife may be translatable for human and domestic animal health.

In this study, we investigated *T. cruzi* infection and cardiac pathology in raccoons (*P. lotor*) and coyotes (*Canis latrans*) from Central and South Texas. Raccoons are perhaps the most well-studied reservoir species in the US, and *T. cruzi* infection dynamics and pathology have been described in both naturally infected and experimentally infected animals (Bern et al., 2011; Pietrzak and Pung, 1998; Roellig et al., 2009b). In contrast, coyotes are a relatively understudied candidate reservoir in which the majority of previous studies report only seroprevalence and for which only a single limited pathology study exists (Curtis-Robles et al., 2016; Hodo and S. A. Hamer, 2017). Raccoons and coyotes are abundant in both rural and urban settings, having the potential to bridge parasite infections from sylvatic to peridomestic habitats. Our objectives were to perform a detailed comparative cardiac pathology study of naturally infected coyotes and raccoons sampled simultaneously from overlapping geographical areas, as well as to characterize the overall *T. cruzi* infection prevalence in heart and blood of each species via PCR and identify infecting DTU. This study furthers our understanding of these two species as reservoirs of *T. cruzi*, and describes significant lesions associated with *T. cruzi* infection in coyotes for the first time.

## 4.2. Materials and Methods

### *4.2.1 Sampling*

We conducted a cross-sectional sampling effort in January 2016 at a hunting check station for a recreational nuisance animal hunt in central Texas. Animals from this hunt in a

prior year have previously been studied in the context of *T. cruzi* infection such that we anticipated finding infected animals (Curtis-Robles et al, 2016). Animals legally harvested under recreational permits by teams of hunters over a 24-h period were brought to a central check station. The area of harvest encompassed 25 counties in central Texas spanning 5 different ecoregions (Gould et al., 1960). Our team collected samples from coyotes and raccoons for which the county of harvest was known. For a few animals, county of origin was known to be one of two different counties. In these cases, animals were split between the two possible counties for mapping. Hearts and blood (when available) were collected in the field within 24 h of death. At the time of collection, each heart was briefly examined for evidence of gross pathology. Blood was collected from the axillary vasculature or the thoracic cavity as available during heart collection. Blood and hearts were transported to the laboratory on ice. In the laboratory, blood samples were centrifuged and serum was collected from those samples that were not extensively hemolyzed or autolyzed. A 500 ul volume of blood clot or whole hemolyzed blood was subsampled from each available blood sample and stored at -20°C until DNA extraction. Hearts were stored at -80°C for 4-5 months, then thawed, dissected along the lines of blood flow, and right and left atria and ventricles were examined. Two tissue samples were taken from each of the four chambers (right and left atria, right and left ventricles). One section from each chamber was stored in 10% neutral buffered formalin for histology, and the other section was minced, then pooled together with the samples from the other chambers for DNA extraction.

We also received samples from coyotes collected during oral rabies vaccine program surveillance in Webb county (South Texas) by the Texas Department of State Health Services (DSHS) in coordination with USDA APHIS Wildlife Services Texas branch from

Feb 29 - Mar 1st, 2016. Heart apex, blood soaked filter paper (Nobuto strips) and whole blood (when available) were collected by DSHS personnel in the field. Hearts were processed as described above, except for gross examination of the individual chambers, since only the apex of the heart was available.

*4.2.2 Molecular Work*

From each animal, as available, one approximately 0.5 $cm^3$ sample from each heart (representing all four chambers of Central TX animals and apex of South TX animals), 500 ul of blood, and one Nobuto strip was subjected to DNA extraction using the Omega® E.Z.N.A. ® Tissue Extraction Kit according to the manufacturer's protocol for tissue extraction with an overnight lysis for hearts and Nobuto strips and ≥3 hour lysis for blood.

After DNA extraction, samples were tested for infection with *T. cruzi* using a highly sensitive quantitative PCR to amplify a 166-base-pair fragment of *T. cruzi* satellite DNA as previously described (Curtis-Robles et al., 2016; Piron et al., 2007). DNA-negative controls and a positive control of DNA extracted from pure culture of Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection, Manassas, VA; DTU TcI) were included in all reactions. Samples with a cycle threshold (Ct) value less than 36 were considered suspect positive and subjected to a multiplex probe-based qPCR targeting the spliced leader intergenic region (SL-IR) to confirm positivity and for determination of strain type, according to previously described protocols (Cura et al., 2015; Curtis-Robles et al., 2017a). The criteria for considering a sample positive on this assay was the detection of specific fluorescence to one or more DTU-specific probes within 40 cycles in a 45 cycle assay. Negative controls (water) and positive controls of DNA extracted from *T. cruzi* strain Sylvio X10 CL4 (DTU TcI, details above) and *T. cruzi*-infected *Triatoma sanguisuga* from Texas (DTU TcIV) were

included in all reactions, and positive control of DNA extracted from *T. cruzi* Y-strain (ATCC 50832, American Type Culture Collection, Manassas, VA; DTU TcII) was added for reactions run later in the study.

### *4.2.3 Histopathology*

Formalin-fixed heart tissue from Central TX coyotes and raccoons was processed routinely for histopathology and stained with hematoxylin and eosin (H&E). Two slides from each animal, representing right and left heart, were examined using light microscopy by a board-certified veterinary pathologist (CLH) who was blinded to the PCR-status. Inflammation was semiquantitatively scored for each heart chamber on a numeric scale as normal (0), minimal (1), mild (2), moderate (3), or severe/marked (4). Additionally, the presence of fibrosis, cardiomyocyte degeneration or necrosis, and the distribution (focal, multifocal, focally extensive) and location (interstitial, myocardial, epicardial) of lesions were recorded. An inflammation index for each animal was calculated by combining the inflammation scores for each chamber. For analysis, animals were dichotomized by pathology status (significant lesions present or absent), in which significant was defined as an inflammation score ($\geq 3$). The inflammation cutoff of 3 was chosen because it represented at least minimal inflammation in 3 heart chambers, at least mild inflammation in one section and minimal in another, or at least moderate inflammation in any one heart chamber. Slides from animals with an inflammation score $\geq 3$ were re-examined, and a descriptive morphologic diagnosis was recorded. Animals with lymphoplasmacytic myocarditis (consistent with reported lesions of *T. cruzi* infection) were included in statistical analyses.

*4.2.4 Statistical Analysis*

We tested for significant differences between the presence of *T. cruzi* DNA in samples (PCR status of heart and blood) and host attributes of species and sex using a Fisher's exact test. Further, we used the Fisher's exact text to compare the presence of T. cruzi DNA in samples with the presence of lymphoplasmacytic myocarditis for each species separately. Finally, the Mann-Whitney-Wilcoxon test was used to determine whether the inflammation scores differed between *T. cruzi*-positive coyotes and raccoons and whether the Ct values of PCR-positive blood samples were different between species. Statistical analyses were performed in R (R Core Team, 2014).

## 4.3. Results

*4.3.1 Sample Population*

We sampled 120 coyotes from 24 Texas counties, and 24 raccoons from 14 counties (Figure 4.1). Both males and females of each species were sampled (Table 4.1). We collected hearts from 97 Central Texas coyotes and 23 raccoons, and heart apex from 23 South Texas coyotes. Blood was available for 92 coyotes and 18 raccoons from Central Texas and 21 coyotes from South Texas. We also received blood-soaked Nobuto filter paper strips from all 23 South Texas coyotes.

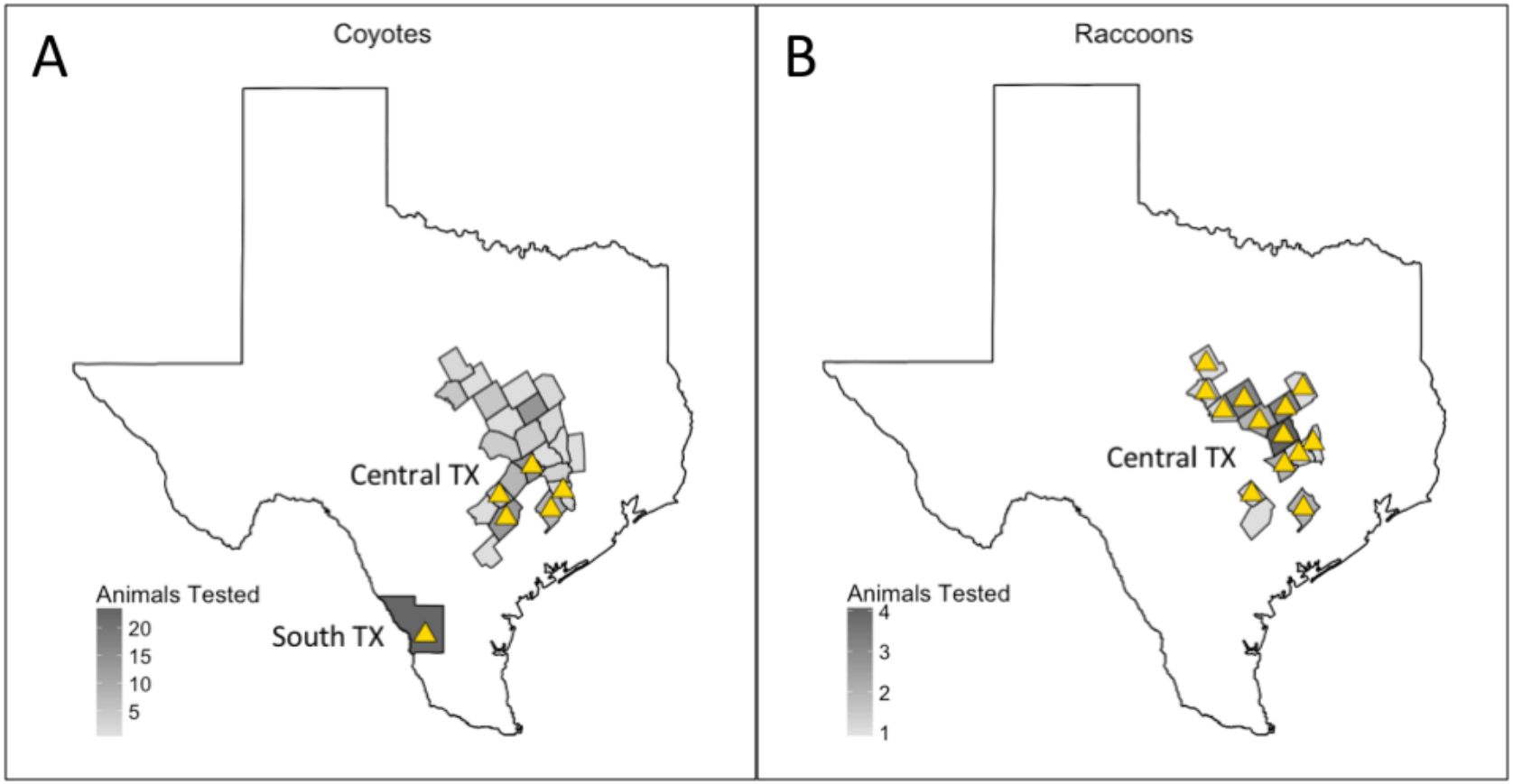


**Figure 4.1. Maps showing distribution of sampled coyotes (A) and raccoons (B) by county, with gold triangles marking counties from which PCR-positive animals were collected.**

*4.3.2 PCR Results*

Ten of 120 (8.3%) coyotes and 15 of 24 (62.5%) raccoons were confirmed PCR-positive for *T. cruzi* on 2 separate qPCRs of either heart or blood. Raccoons were significantly more likely to be PCR-positive than coyotes ($p$-value= $2.48x10^{-08}$). Sex was not associated with PCR status within either species. Of the 10 PCR-positive coyotes, 5 had PCR-positive blood (4 with whole blood and 1 with Nobuto strip). Of the 11 PCR-positive raccoons for which blood was available, 8 had PCR-positive blood. Ct values in positive blood were significantly lower for raccoons than coyotes ($p$-value = 0.0016); thus infected raccoons had a higher concentration of parasite DNA in blood than coyotes. All infected coyotes harbored DTU TcI, while all infected raccoons harbored TcIV, based on both blood and heart for individuals in which both tissues were positive. Positive raccoons were identified in 13/14 sampled counties, and positive coyotes originated from 5/25 sampled counties (Figure 4.1).

*4.3.3 Pathology*

No significant gross lesions (aside from trauma due to gunshot) were observed in any of the hearts. Incidental gross findings included adult heartworms (Dirofilaria immitis) in several animals. On histopathology, 62/120 animals had no lesions, 41/120 had minimal findings that were not considered pathologically significant (inflammation score < 3), and 15/120 animals (11 coyotes and 4 raccoons) had lesions of pathologic significance. One coyote was excluded from the histopathology analysis because of severe autolysis. Four other coyotes that had severe autolysis of all sections except the left ventricle were retained in the analysis, and had no significant lesions in the assessed region of left ventricle. The full

morphologic diagnoses for the 15 animals with significant histologic lesions are listed in Table 4.2.

**Table 4.1. Demographic data and PCR results for coyotes and raccoons sampled from across Texas.**

| | Coyotes | | | | Raccoons | | | |
|---|---|---|---|---|---|---|---|---|
| | *N* | PCR+ heart (% of *N*) | PCR+ blood (% of *N*) | Total PCR+ (% of *N*) | *N* | PCR+ heart (% of *N*) | PCR+ blood (% of *N*) | Total PCR+ (% of *N*) |
| Total | 120 | 5/120 (4.2%) | 5/113 (4.4%) | 10/120 (8.3%) | 24 | 12/23 (52%) | 8/18 (44%) | 15/24 (63%) |
| Sex | | | | | | | | |
| F | 53 | 4/53 (7.5%) | 2/50 (4.0%) | 7/53 (13.2%) | 9 | 4/9 (44%) | 1/4 (25%) | 5/9 (56%) |
| M | 59 | 1/59 (1.7%) | 2/57 (8.8%) | 3/59 (5.1%) | 14 | 7/13 (54%) | 6/13 (46%) | 9/14 (64%) |
| Unknown | 8 | 0/8 | 0/5 | 0/8 | 1 | 1/1 | 1/1 | 1/1 |
| Location | | | | | | | | |
| Central | 97 | 5/97 (5.2%) | 1/92 (1.1%) | 6/97 (6.2%) | | | | |
| South | 23 | 0/23 (0%) | 4/21 (19.0%) | 4/19 (21.2%) | | | | |

**Table 4.2. Morphologic diagnoses for coyotes and raccoons with significant histologic lesions.**

| ID | Species | Morphologic Diagnosis |
|---|---|---|
| C16-44 | Coyote | Mild, multifocal, subacute, lymphoplasmacytic myocarditis |
| C16-62 | Coyote | Mild, multifocal, subacute, lymphoplasmacytic myocarditis with myocellular degeneration and necrosis |
| C16-19 | Coyote | Moderate, multifocal, subacute, lymphoplasmacytic myocarditis with multifocal myocardial necrosis |
| C16-38 | Coyote | Moderate to severe, multifocal, subacute, lymphoplasmacytic myocarditis with myocellular degeneration and necrosis |
| C16-59 | Coyote | Moderate, multifocal, chronic lymphoplasmacytic myocarditis with fibrosis |
| C16-75 | Coyote | Moderate, multifocal, subacute lymphoplasmacytic myocarditis with myocellular degeneration and necrosis |
| C16-05 | Coyote | Mild, multifocal, subacute, histiocytic and lymphoplasmacytic myocarditis with numerous microfilariae |
| C16-34 | Coyote | Focal, mild, subacute, histiocytic and lymphocytic perivascular myocarditis with suspect intrahistiocytic protozoa |
| C16-66 | Coyote | Severe, focally extensive, subacute, pyogranulomatous myocarditis |
| C16-71 | Coyote | Severe, focally extensive, subacute, pyogranulomatous myocarditis with *Hepatozoon canis* cysts |
| C16-76 | Coyote | Severe, focally extensive, subacute, histiocytic and lymphoplasmacytic myocarditis |
| R16-12 | Raccoon | Minimal, multifocal, subacute to chronic lymphoplasmacytic myocarditis with fibrosis |
| R16-17 | Raccoon | Mild, multifocal, subacute lymphoplasmacytic myocarditis |
| R16-14 | Raccoon | Mild, multifocal, subacute eosinophilic and lymphoplasmacytic myocarditis |
| R16-16 | Raccoon | Moderate to severe, multifocal, subacute, histiocytic and lymphoplasmacytic perivascular myocarditis and epicarditis; *Sarcocystis* sp. cyst |
| R16-23 | Raccoon | Mild, multifocal, subacute, lymphoplasmacytic myocarditis. |

Of the 11 coyotes with significant lesions, 6 (including 4 *T. cruzi* PCR-positive animals) had mild to moderate, multifocal, lymphoplasmacytic myocarditis with varying degrees of myocardial degeneration and fibrosis (Figure 4.2A-C), consistent with that described for *T. cruzi* infection in other species. Another 4 of the 11 coyotes with significant lesions had severe focally extensive inflammation that was primarily histiocytic or pyogranulomatous, occasionally with visible intraleukocytic zoites, most consistent with *Hepatozoon americanum* infection (Davis et al., 1978). None of these were PCR-positive for *T. cruzi*. Mature cysts of *H. americanum* were observed in 2 of these animals, as well as in other coyotes without inflammation. Finally, 1 coyote had mild, multifocal pyogranulomatous inflammation associated with microfilariae of *D. immitis*. Cysts of *Sarcocystis* sp. were observed in 2 coyote hearts, with no accompanying inflammation.

Of the 4 raccoons with significant histologic lesions, 2 had minimal to mild lymphoplasmacytic myocarditis (Figure 4.2D), which was accompanied by fibrosis in 1 animal. In a third raccoon, lymphoplasmacytic inflammation was accompanied by abundant eosinophils. The fourth raccoon had moderate to severe multifocal histiocytic and lymphoplasmacytic perivascular myocarditis and epicarditis. A *Sarcocystis* sp. cyst was also observed in this animal, but not in the area of inflammation. *T.cruzi* amastigotes were not observed in any of the sections examined for either species.

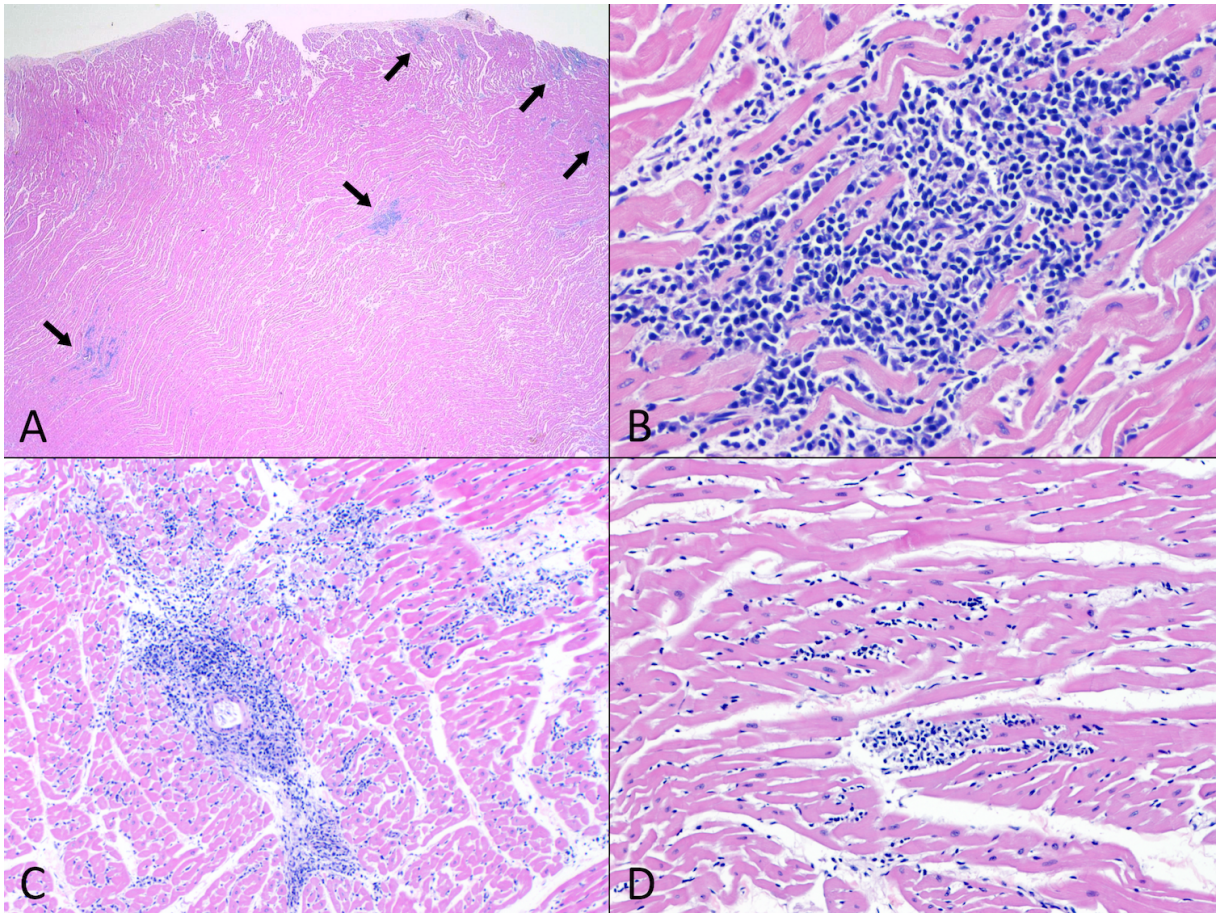


**Figure 4.2. Photomicrographs showing myocarditis in Texas coyotes and raccoons, hematoxylin and eosin stain.** A) 2x objective, coyote C16-38, left ventricle; inflammation is present in multiple areas across the section. B) 20x objective, higher magnification view of A; cardiac myofibers are separated by a moderate amount of inflammation composed primarily of lymphocytes and plasma cells, with myocellular degeneration and loss. C) 4x objective, right ventricle, coyote C16-75; moderate lymphoplasmacytic inflammation is perivascular and infiltrates between cardiac myofibers, disrupting normal architecture. D) 10x objective, raccoon R16-23, left ventricle; mild lymphoplasmacytic myocarditis.

**Table 4.3. Two-by-two tables comparing T. cruzi PCR status and presence or absence of lymphoplasmacytic myocarditis in coyotes and raccoons of Central Texas).**

| | Coyotes | | | Raccoons | | |
|---|---|---|---|---|---|---|
| | Species total | Num. with lympho-plasmacytic myocarditis | No myocarditis, or other etiology | Species total | Num. with lympho-plasmacytic myocarditis | No myocarditis, or other etiology |
| T. cruzi PCR+ | 6 | 4/6 (67%) | 2/6 (33%) | 15 | 3/15 (20%) | 12/15 (80%) |
| T. cruzi PCR- | 90 | 2/90 (2.2%) | 88/90 (98%) | 8 | 2/8 (25%) | 6/8 (75%) |
| Total | 96 | 6/96 (6.3%) | 90/96 (94%) | 23 | 5/23 (22%) | 18/23 (78%) |

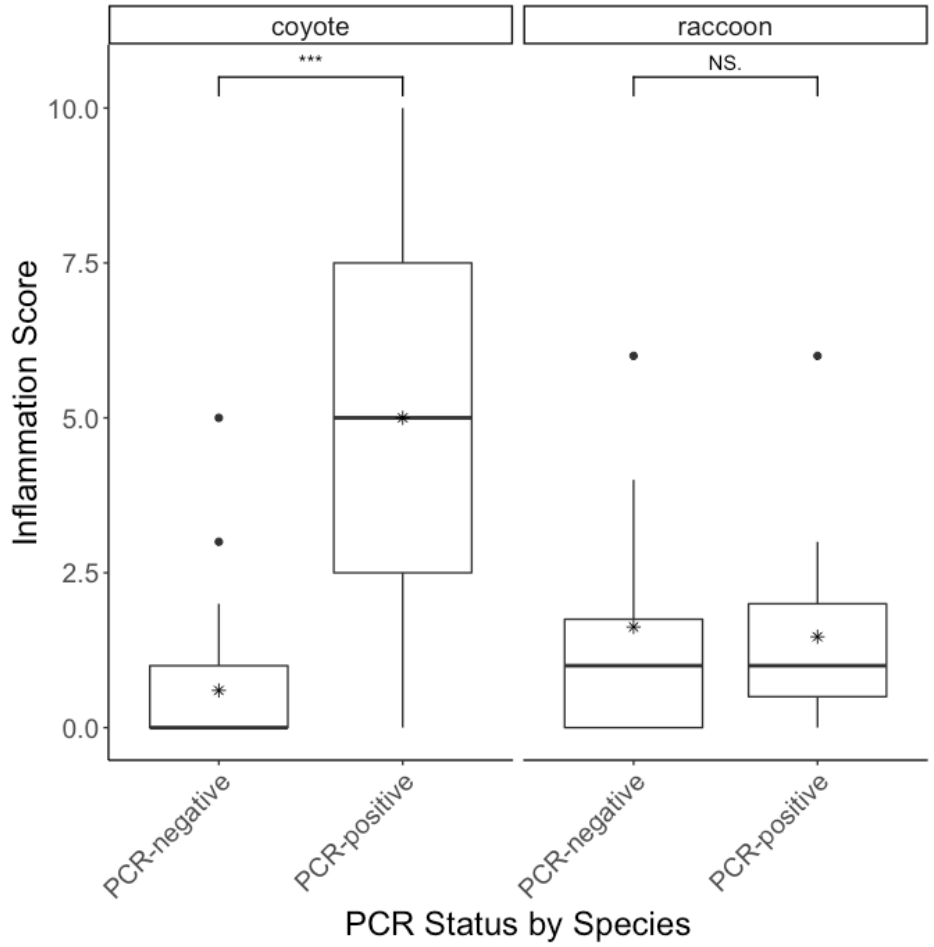


**Figure 4.3 Boxplot demonstrating the distribution of inflammation scores in *T. cruzi* PCR-positive and PCR-negative coyotes and raccoons, excluding coyotes which had inflammation attributed to other etiologic agents.** Horizontal lines represent median values, while asterisks represent means. *** = statistically significant using Wilcoxon test, NS = not significant.

The presence of lymphoplasmacytic myocarditis was significantly associated (Figure 4.3) with *T. cruzi* PCR-status in coyotes ($p$-value= $6.539 \times 10^{-5}$) but not in raccoons ($p$-value=0.7). Additionally, the severity of lymphoplasmacytic myocarditis (as measured by combined inflammation score) was greater for *T. cruzi* PCR-positive coyotes than for PCR-negative coyotes ($p$-value=0.0007) when other types of inflammation (attributed to other etiologic agents) were excluded (Figure 4.3).

**4.4. Discussion**

In a region of central and south Texas known to harbor triatomine vectors, we demonstrated that both raccoons and coyotes have *T. cruzi*- infected blood and cardiac tissue, sometimes associated with myocarditis, suggesting not only that these species are involved in the sylvatic cycle as parasite reservoirs, but in some cases are also negatively impacted by the infection, especially coyotes. Although the overall *T. cruzi* infection prevalence was significantly higher in raccoons (62.5%) than in coyotes (8.3%), the infected coyotes exhibited more severe histopathologic lesions than raccoons. Finally, we found strong parasite strain type associations with host taxa, in which all 10 coyotes for which DTU was determined harbored TcI, while all 15 raccoons harbored TcIV.

The infection prevalence we found is consistent with previous reports of infection prevalence or seroprevalence in both raccoons and coyotes, which range from 20-90% in raccoons across the southern states (Brown et al., 2010; Charles et al., 2012; Pietrzak and Pung, 1998; Yabsley and Noblet, 2002), and from 4-14% in coyotes in several southern states (Brown et al., 2010; Burkholder et al., 1980; Garcia et al., 2016; Gates et al., 2014; Grögl et al., 1984; Rosypal et al., 2014). Specifically, a study that sampled animals during a

previous year of the recreational hunt across many of the same central TX counties reported prevalences of 70.0% in raccoons and 14.3% in coyotes (Curtis-Robles et al., 2016).

Only minimal to mild lesions have previously been reported as a result of natural infection with *T. cruzi* in raccoons (Charles et al., 2012; Curtis-Robles et al., 2016; Pietrzak and Pung, 1998), and this was consistent with our findings. Experimentally infected raccoons exhibited mild to severe lesions that varied with acuteness of infection and infecting DTU, exhibiting more severe cardiac lesions during the acute stages of infection with TcI and TcII than with TcIV (Roellig et al., 2009b). The specific lesions described in previous studies are very similar to those we observed: inflammation composed primarily of lymphocytes and plasma cells, with occasional myocardial degeneration or necrosis, with infrequent observation of intramyocellular *T. cruzi* amastigotes. A study from the same region in central Texas reported minimal inflammation in 1/4 infected coyotes and 3/12 infected raccoons examined histologically, though this was based on small tissue sections from right ventricle only (Curtis-Robles et al., 2016).

Autolysis hindered interpretation of some histologic sections, but less than 5% of examined sections were affected to the extent that we could not determine the presence or absence of inflammation. Autolysis most likely resulted from the varying duration between death and heart collection for the hunter-harvested animals. Overall, only minimal artifactual change resulted from the single freeze-thaw cycle, which was manifest mainly by artifactual separation of myofibers. The relative lack of freeze-thaw artifact observed should be considered in future sampling efforts for which histopathologic examination may have otherwise been discounted because logistics would require freezing of samples.

In our study, fewer positive coyotes than raccoons were available for histologic examination, but myocarditis was more severe in coyotes. Only 4 raccoons had inflammation scores above the threshold we determined to be significant, and even in these lesions were only minimal to mild, and likely of low clinical significance. The presence of inflammation in raccoons was not associated with *T. cruzi* infection status. In contrast, inflammation in coyotes ranged from mild to moderate, was more often accompanied by cardiomyocyte damage, and more widely distributed throughout multiple areas of the heart. *T. cruzi* infected coyotes were more likely to exhibit lymphoplasmacytic inflammation that PCR-negative coyotes. Significant histopathologic lesions in *T. cruzi*-infected coyotes have not previously been described, but our findings of multifocal lymphoplasmacytic inflammation with destruction of cardiomyocytes are consistent with lesions reported in infected dogs (Barr et al., 1991c; Snowden and Kjos, 2013).

Definitive diagnosis of *T. cruzi* based solely on histopathology is often difficult because the intracellular amastigote form is not commonly observed in chronic infections. However, while not pathognomonic, the multifocal and infiltrative pattern of lymphoplasmacytic myocarditis is highly suggestive of *T. cruzi* infection, especially when accompanied by positive PCR or serologic results. Studies of nonspecific lymphocytic myocarditis in NHPs identified an association with the presence of *T. cruzi* DNA (Andrade et al., 2009; Mubiru et al., 2014).

Inflammation was observed in the hearts of 7 PCR-negative coyotes. However, five of these can likely be explained by infection with other parasites, as determined by the nature of the inflammation or by direct observation of organisms. In one of these animals, microfilaria from *D. immitis* were associated with granulomatous inflammation that was

distinct from the lymphoplasmacytic inflammation consistent with *T. cruzi* infection. Lesions in the other 4 were focally extensive rather than multifocal as expected with *T. cruzi*, and were characterized by abundant macrophages and neutrophils, consistent with the pyogranulomatous inflammation described in *H. americanum* infection. In support of this diagnosis, mature *H. americanum* cysts were identified in one of these 4 animals, and presumed zoites were observed within macrophages in two others. Quiescent cysts do not elicit an inflammatory response, but the release of zoites from the cysts causes an intense pyogranulomatous inflammatory response (Baneth, 2011). *H. americanum* is an apicomplexan parasite of canids in the US, transmitted by ingestion of the gulf coast tick *Amblyomma maculatum*, or directly via carnivory of infected hosts (Baneth, 2011). It can cause severe disease in dogs, characterized by severe muscle and bone lesions, and was first reported in coyotes in Texas in 1978 (Davis et al., 1978). Other organisms, primarily the dimorphic fungi, can also cause pyogranulomatous lesions in canines.

Similarly, one raccoon had inflammation that was moderate to severe but characterized by abundant eosinophils and histiocytes, not consistent with the lymphoplasmacytic myocarditis expected with *T. cruzi* infection. While some *Sarcocystis* sp. cysts can frequently be observed without inflammation, myocarditis associated with *Sarcocystis neurona* was reported in 2 raccoons in Oregon (Hamir and Dubey, 2001).

Besides an unidentified infection with another pathogen, another explanation for PCR-negative status in the face of lesions suggestive of *T. cruzi* is the possibility of false negative PCR-results. The results of *T. cruzi* testing of a single blood sample or small pieces of heart tissue do not necessarily reflect the true infection status of the individual. Thus, false negatives could have resulted from sampling error, because of the multifocal nature of

parasite distribution within the heart. This is supported by our histologic findings in which inflammation was focal to multifocal and not diffusely distributed throughout all sections, and also by the lack of PCR-positivity in the hearts of the South Texas coyotes, for which only apex was available. The multifocal nature of parasite distribution has been supported by other studies in raccoons which reported PCR positivity in some sections of heart but not others within the same animal (Curtis-Robles et al., 2016; James et al., 2002). Additionally, while the PCRs we used are reported to be highly specific, there can be inter-laboratory variation in extraction methods, setting of cut-off limits for Ct values, etc. Conservatively, we only called animals PCR-positive if confirmed by the second qPCR. While highly specific, the second assay is in fact less sensitive than the screening test and therefore we may have misclassified some truly infected samples.

We found distinct associations between host taxa and parasite DTU, in which raccoons were only infected with TcIV and coyotes only with TcI. The difference in degree of pathology observed between coyotes and raccoons could be explained by species-level or DTU-level differences. Raccoons are almost exclusively infected with TcIV across multiple studies, with only a handful of reports of natural TcI infection (Bern et al., 2011; Curtis-Robles et al., 2016; Roellig et al., 2008). Experimentally, raccoons were successfully infected with DTUs TcIV, TcI, and TcII, but developed longer-lasting parasitemia with TcIV, and more severe cardiac lesions with TcI and TcII infections (Roellig et al., 2013). It has been proposed that *T. cruzi* DTU TcIV is host-adapted to raccoons, supported by their high infection prevalence and lack of obvious pathology (Roellig et al., 2009b). No such association has been suggested for coyotes, and our findings of TcI infection with resulting cardiac pathology may be evidence that TcI is fundamentally a more cardiopathogenic strain

than TcIV, or simply that coyotes are more susceptible to cardiac disease resulting from *T. cruzi* infection. Of interest is the fact that TcIV has not been detected in autochthonous human infections of *T. cruzi* in the US (Garcia et al., 2017; Roellig et al., 2008). More research is needed on the comparative pathology of the different DTUs in canines as well as in humans. While part of the response to *T. cruzi* infection is likely individual host-dependent, more and more evidence is accumulating that DTU may play a role as well.

Eight of 11 *T. cruzi* infected raccoons had PCR-positive blood, and all but one of these also had positive heart, suggesting chronic infection. While PCR cannot confirm the presence of whole, viable parasite, the presence of parasite DNA is suggestive of parasitemia and likely infectiousness to vectors. In contrast, we only detected *T. cruzi* DNA in 5/10 of the infected coyotes, and only 1 of these also had PCR-positive heart tissue. This suggests the possibility that while raccoons maintain long-term parasitemia with TcIV, coyotes may only have circulating TcI during the acute stage of infection, before the parasite localizes in the heart. Interestingly, 4/5 of the coyotes with PCR-positive blood were from the South Texas population, and may reflect acute infections explained by seasonal differences, as these coyotes were collected later in the year, when kissing bugs are more likely to be active. Additionally, only a small section of heart was available for PCR in these animals, so it is possible that *T. cruzi* DNA would have been identified in the heart if other sections were available for testing.

In conclusion, we report that coyotes, while less likely to be infected with *T. cruzi* than raccoons, are associated with more severe pathology and with DTU TcI. The findings in this study may have important implications for the association of *T. cruzi* DTU with resulting pathology, as well as for the reservoir potential of coyotes and raccoons. We also provide

further support for the association of DTU TcIV with raccoons in the US. While raccoons are known to maintain high levels of parasitemia into the chronic stages of infection, more research into parasitemia dynamics of coyotes is needed to determine their contribution to the reservoir system of *T. cruzi* in the US. Additional considerations necessitating further research are the risks posed to hunters exposed to infectious wildlife as well as the impact of infection on wildlife populations.

# 5. APPARENT LACK OF *TRYPANOSOMA CRUZI* INFECTION IN URBAN ROOF RATS (*RATTUS RATTUS*) AT A TEXAS NON-HUMAN PRIMATE FACILITY WITH NATURALLY INFECTED PRIMATES[*]

## 5.1 Introduction

Maintenance of biosecurity and prevention of disease transmission at non-human primate facilities involves intensive efforts to limit contact between primates and wildlife species. Rodent control in particular represents an ongoing challenge, especially in outdoor or indoor/outdoor facilities (Kelley and Crockett, 2012). Rodents can enter primate enclosures, consume and contaminate primate feed, and travel between enclosures and nearby sylvatic habitats. Primates with access to the outdoors are at increased risk of exposure to wildlife reservoirs of disease as well as to arthropod vectors of pathogens. Transmission of wildlife and vector-borne diseases to nonhuman primates, including West Nile virus, tularemia, and leptospirosis, have all been reported in primate facilities (Ferrecchia et al., 2012; Ratterree et al., 2003; Szonyi et al., 2011).

Chagas disease is a vector-borne disease that primarily affects humans and dogs, and is endemic throughout much of Latin America. Active transmission of the causative parasite, *Trypanosoma cruzi,* is increasingly recognized as a major public health issue in the southern United States. Entomological surveillance has identified infected triatomine insect vectors (kissing bugs) across Texas (Sarkar et al., 2010). *T. cruzi* is maintained in nature by diverse

[*] Reprinted with permission from: Hodo, C.L., Bertolini, N.R., Bernal, J.C., VandeBerg, J.L., Hamer, S.A., 2017. Lack of Trypanosoma cruzi Infection in Urban Roof Rats (Rattus rattus) at a Texas Facility Housing Naturally Infected Nonhuman Primates. J Am Assoc Lab Anim Sci 56, 1–6.

species of wildlife which serve as reservoirs (Bern et al., 2011). In areas where the vectors and parasite are found, Chagas disease has emerged as a major concern in non-human primate facilities. There are at least 14 published reports of primate infection with *T. cruzi* in the US, and all of the affected primates originated from southern states (Dorn et al., 2012). Texas is home to a number of non-human primate facilities, including one of seven national primate research centers, and sporadic natural cases of Chagas disease in these primates have been reported for decades in areas where kissing bugs are established (Gleiser et al., 1986; Grieves et al., 2008; Williams et al., 2009). While reports of infected primates continue to increase with increased testing, few centers currently conduct routine comprehensive surveillance. Infection of primates with *T. cruzi* can diminish their value as appropriate models in research and can lead to health problems and death, resulting in significant scientific and economic losses. An undiagnosed infection in a primate enrolled in a research study could potentially confound results of that study. Primates housed with outdoor access are at risk of encountering kissing bugs, and transmission may occur either through the traditional route of contamination of a bite wound or mucous membrane with feces from the bug following blood-feeding, or by direct ingestion of the bug by the primates (Pung et al., 1998). While the pathological manifestations of Chagas disease in primates have been well described (Andrade et al., 2009; Carvalho et al., 2003; Zabalgoitia et al., 2003), the specific details of transmission and the role of wildlife reservoirs in these facilities are relatively unknown.

Identifying reservoirs is crucial to devising effective interventions in a complex multi-host system such as Chagas disease (Viana et al., 2014). Southern plains woodrats have been repeatedly implicated as important wildlife reservoirs of *T. cruzi* in the US (Charles et

al., 2012; Eads et al., 1963). Other species of rodents, such as urban rats, have been less thoroughly investigated in this country, though they have been shown to harbor *T. cruzi* in highly endemic areas of Latin America (Edgcomb and Johnson, 1970; Galuppo et al., 2009; L. Herrera and Urdaneta-Morales, 1997; M. M. Lima et al., 2012; Pinto et al., 2006; Ramsey et al., 2012). A recent survey of potential *T. cruzi* reservoirs in Texas found an infection prevalence of 34% in woodrats (*Neotoma micropus*), 75% in striped skunks (*Mephitis mephitis*), 60% in raccoons (*Procyon lotor*), and 18% in other rodents which included a single infected black/roof rat (*R. rattus*) and two house mice (*Mus musculus*) (Charles et al., 2012). We investigated the presence and *T. cruzi* infection status in kissing bugs and roof rats - the most abundant nuisance wildlife species - at a non-human primate facility with endemic Chagas disease.

## 5.2 Materials and Methods

### *5.2.1 Non-human Primate Facility*

The Southwest National Primate Research Center (SNPRC), located at the Texas Biomedical Research Institute in San Antonio, TX, houses ~2,500 non-human primates, including baboons, chimpanzees, and two species of macaques housed in indoor and outdoor cages, as well as common marmosets housed exclusively in indoor cages. The 200-acre property is partially surrounded by dense brushy vegetation with a small dry creek, and is bordered by three major highways (Figure 5.1). Chagas disease was first detected in primates at this facility in 1984 (Gleiser et al., 1986) and has since been well-characterized (Andrade et al., 2009; Grieves et al., 2008; Mubiru et al., 2014; Williams et al., 2009; Zabalgoitia et al.,

2003). Roof rats (*Rattus rattus*), also known as ship rats, black rats, and house rats, are the most predominant rodent pest species identified by pest control personnel at the facility.

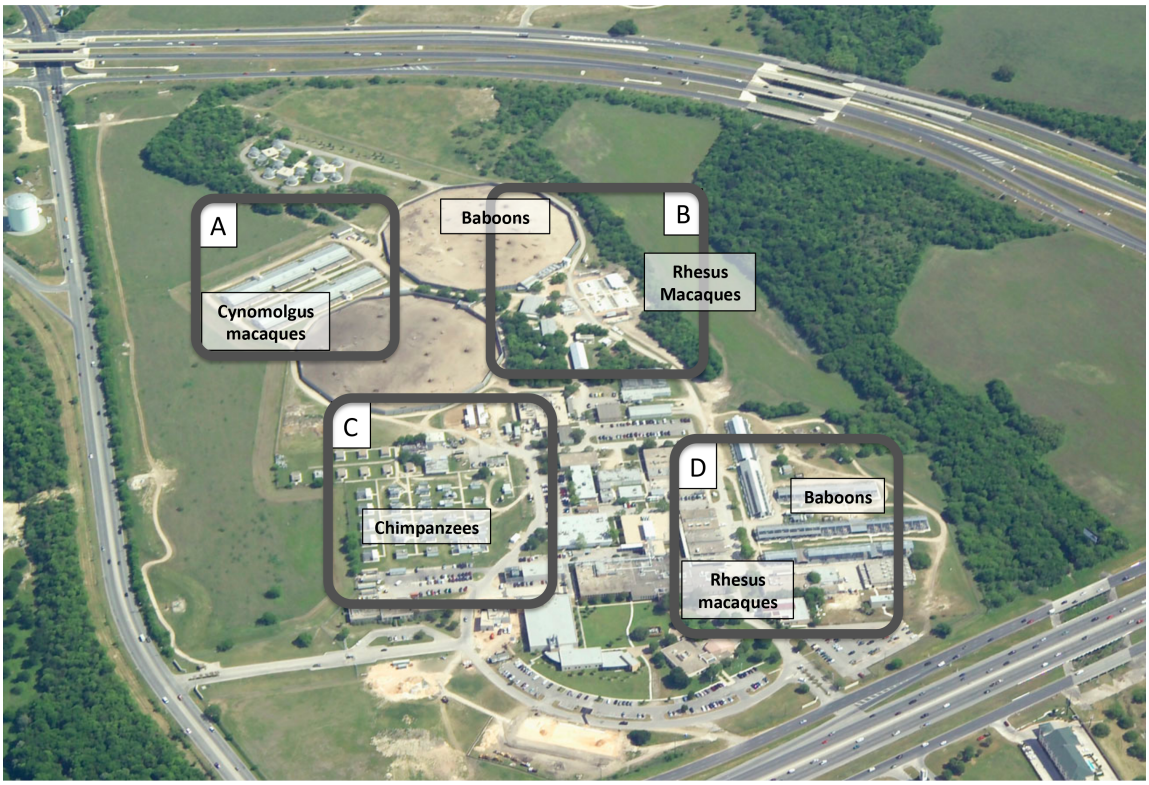


**Figure 5.1 Aerial photograph of primate facility with sampling areas.** Aerial photograph of the Southwest National Primate Research Center in San Antonio, TX showing sylvatic habitat and surrounding highway systems bordering urban areas. Boxes represent unique sampling areas and labels indicate species of primates housed in each area. Reprinted with permission from (Hodo et al., 2017).

### *5.2.2 Rat Collection*

Through collaboration with the SNPRC's pest control service, we salvaged roof rat carcasses that were collected as part of routine pest control activities from May-July and October-November, 2015. These rats were trapped in snap traps or found dead by pest control personnel, presumably following ingestion of poison baits from bait boxes within the facility. Rats were collected across the facility, which was divided into 4 general zones

(Figure 5.1), with the pest control service focusing their rat control efforts on areas with known high rat activity during the study period. Rats were stored at -20° C for up to 3 weeks before being transferred to Texas A&M University. We dissected the carcasses in Biosafety Level 2 laboratory conditions, recording species, sex, and post-mortem condition. Post-mortem condition was recorded on a 1-5 scale, with a score of 1 representing minimal autolysis, progressively increasing up to 5 for marked decomposition. Heart and clotted blood from within the ventricles were collected from animals in adequate post-mortem condition. Vertebrate use was secondary and therefore exempted from oversight by the Institutional Animal Use and Care Committees at Texas A&M University and the Texas Biomedical Research Institute.

*5.2.3 Trypanosoma cruzi Detection*

The DNA was extracted from heart and blood samples using a commercial kit according to the manufacturer's protocol (E.Z.N.A. Tissue DNA Kit; Omega Bio-Tek, Norcross, GA) but with an overnight lysis period. The extracted DNA was subjected to two independent PCR protocols. For the specific detection of *T. cruzi*, a 166-bp segment of the *T. cruzi* 195-bp repetitive satellite DNA was amplified using a probe-based real-time PCR with Cruzi 1/2 primers and 6-carboxyfluorescein (FAM)-labeled probe, Cruzi 3, as described (Piron et al., 2007), but with an initial denaturation time of 3 minutes. This assay has previously been shown to be a best performing method in an international PCR study (Schijman et al., 2011), being sensitive and specific for all strain types of *T. cruzi*, including TcI, TcIV (Schijman et al., 2011), and TcII (Moreira et al., 2013), the strain types found in the US. Based on internal laboratory validations, the cutoff for positive samples was

determined to be a quantification cycle value of 32 or less. Additionally, a nested traditional PCR using genus-level primers targeting a fragment of the 18S RNA-encoding gene of *Trypanosoma* species (Noyes et al., 1999) was performed on each sample to allow for sequencing of positive results and potential detection of other species of trypanosomes. This primer set has been used to detect and characterize novel trypanosomes in a variety of species while also detecting known trypanosomes such as *T. rangeli*, *T. dionisii,* and all strain types of *T. cruzi* (Cottontail et al., 2014; L. Lima et al., 2015b; Ocaña-Mayorga et al., 2015; Pinto et al., 2012). DNA extractions, primary and secondary amplifications, and product analyses were performed in separate dedicated laboratory areas. A negative control was included in each set of DNA extractions and one or more water negative controls were used in every PCR reaction as contamination controls. The DNA from *T.cruzi* Sylvio X10 clone4 (American Type Culture Collection, Manassas, VA), which is strain type TcI, served as a positive control. For the qPCR, positive and negative controls always gave expected results. For the nested PCR, if either the positive or negative control did not perform as expected, the entire plate was re-run, and expected results were always obtained on the second attempt.

Additionally, because of concerns about PCR inhibition, 10% of the negative rat samples (n=15), selected across a variety of autolysis scores and dates of extraction, were "spiked" with a low concentration (1:10^6 dilution) of *T. cruzi* positive control DNA and run on the qPCR with a negative water control and positive control of the same concentration as that in the spiked samples.

*5.2.4 Vector Surveillance*

Active nighttime kissing bug surveillance was performed during two different visitations to the facility in summer 2015 using active searches and stationary white cloth sheets with dry ice and UV lights, methods which have successfully been used by us to collect kissing bugs in other areas across Texas. Surveillance was conducted between 9pm and midnight for one night during each visit with a four-person team. Four stations with lights, sheets, and dry ice were set up in an area between sylvatic habitat and a building housing rhesus macaques where animals have seroconverted in years past (Area B, Figure 5.1) and the stations and immediate vicinity were actively checked for bugs 3-4 times per hour. In between checking the stations, team members patrolled the facility (Areas A, B, and D; Figure 5.1) with flashlights to actively search walls and sidewalks for bugs. For passive surveillance, after providing an informational lecture about Chagas disease and distributing outreach materials at the start of the study period, we enlisted the help of facility personnel. Additionally, during the month of October, the facility's pest control operators, acting on their own initiative, erected 4x4-foot white glue boards under fluorescent lights nightly along the perimeter fence facing the sylvatic habitat and checked for insects each morning; these were not actively monitored overnight.

*5.2.5 Sample Size Analysis*

We calculated the detectable level of parasite prevalence using the equation for sample size to detect disease in a large (infinite) population: $n = \ln(\alpha)/\ln(q)$ where $n$ = required sample size, $q$ = 1-minimum expected prevalence, and alpha = 0.05 (for 95% confidence level) (Dohoo et al., 2003).

## 5.3 Results

In total, 152 roof rats were collected over the 5-month study period (Table 5.1). Rats were collected from four main areas spread across the facility (A-D), within and around cages of all the species of primates and in food storage areas (Figure 5.1). Sixty-nine of the rats (45.4%) were male, 75 (49.3%) were female, and the sex of 6 rats (6.9%) could not be determined. Sixteen of the 87 rats (18.4%) were classified as juveniles based on immaturity of external genitalia, and the rest were adults. Distribution of the degree of autolysis (scored from 1-5) was as follows: 1 - 2.6%, 2 – 23%, 3 – 35.5%, 4 – 15.1%, 5 – 23.7%. A total of 145 of the 152 roof rats were tested; the remaining 7 were too advanced in autolysis to determine sex and identify organs. Heart tissues were collected from all 145 rats, and clotted blood was collected from 61.

None of the 145 rat hearts or 61 blood samples tested positive for *T. cruzi* with conventional or qPCR. We were able to detect *T. cruzi* in all 15 of the spiked samples and the Cq values were approximately equal to that of the positive control containing the same concentration of *T. cruzi* DNA, while the negative control was negative, demonstrating a lack of PCR inhibition. This sample size of 145 individuals affords the detection of a disease prevalence of 0.020 with a confidence level of 95%.

We did not collect any kissing bugs in a combined total of 7 hours of vector surveillance activities during the 2 nights we visited the facility in June and July, though a number of other species of bugs were observed. Facility personnel observed no kissing bugs on site during the duration of the study, though 3 bugs of other species suspected to be kissing bugs were collected.

**Table 5.1. Demographics of rats collected and *T. cruzi* PCR results.** Reprinted with permission from (Hodo et al., 2017).

| Collection Month | # Rats Collected | Sex | | | Collection Location | | | | | *T. cruzi* positives |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M | F | Unk | A | B | C | D | Unk | |
| May | 22 | 10 | 10 | 2 | 0 | 3 | 1 | 12 | 6 | 0 |
| June | 51 | 24 | 23 | 4 | 0 | 12 | 1 | 35 | 3 | 0 |
| July | 20 | 7 | 13 | 0 | 2 | 5 | 0 | 12 | 1 | 0 |
| Oct | 39 | 15 | 22 | 2 | 0 | 13 | 0 | 26 | 0 | 0 |
| Nov | 20 | 13 | 7 | 0 | 0 | 3 | 0 | 17 | 0 | 0 |
| Total | 152 | 69 | 75 | 8 | 2 | 36 | 2 | 102 | 10 | 0 |

## 5.4 Discussion

Our inability to detect *T. cruzi* DNA in a sample of 145 rats indicates that the prevalence of *T. cruzi* infection in roof rats at this facility is low (<2%) or zero, suggesting that this species may not serve as an important wildlife reservoir of *T. cruzi* at this time. Further, neither our active vector surveillance nor passive surveillance by facility personnel and pest managers yielded any kissing bugs from the site, but our active surveillance efforts were limited, primarily due to security constraints by the facility. In contrast, our statewide kissing bug citizen submission program received hundreds of kissing bugs from the greater San Antonio area during the same time period (Curtis-Robles et al., 2015; Texas AM UniversityTexas, 2015). Although it seems most likely that the infection of adult primates at this facility results from contact with kissing bug vectors that our sampling failed to detect, alternative modes of transmission have not been fully investigated. For example, *T. cruzi* has been identified by PCR in blood-sucking lice of the suborder Anoplura at the same primate facility, but transmission of the parasite by lice remains to be demonstrated (Argañaraz et al., 2001).

Previously published evaluations of *R. rattus* for *T. cruzi* infection in endemic areas of Latin America have found infection prevalence via PCR, microscopy, or culture ranging

from 5-57% (Edgcomb and Johnson, 1970; Galuppo et al., 2009; L. Herrera and Urdaneta-Morales, 1997; M. M. Lima et al., 2012; Pinto et al., 2006; Ramsey et al., 2012), and seropositive rates up to 73% (M. M. Lima et al., 2012), but there is little published on the infection prevalence of this species in the US. Charles et al. (2012) detected infection via PCR in a single *R. rattus* from Uvalde county, Texas, but this was the only member of this species tested in the study. Interestingly, old world rats such as *R. rattus* are the natural hosts of the non-pathogenic trypanosome *Trypanosoma conorhini* and are implicated in the spread of this parasite and its associated vector *Triatoma rubrofasciata* around the world, most likely via transport in shipping vessels (Dujardin et al., 2015; Hoare, 1972). It has been proposed that *T. cruzi* could be spread in a similar fashion (Dujardin et al., 2015), but thus far there have been no reports of infection of rats with *T. conorhini* in the continental US, and reports of *T. rubrofasciata* are limited to Florida and Hawai'i (Bern et al., 2011).

The lack of apparent infection in rats in our study may reflect that this species is not important in the local transmission ecology of *T. cruzi*, or, may result from limitations to our study design. First, we timed our study from May-July to coincide with the period of peak kissing bug activity as previously documented in our study region in Texas (Curtis-Robles et al., 2015; Pippin, 1970), and from October-November, when infection should be established in reservoirs. The timing of this survey, however, may have only provided the ability to detect infection in rats that resulted from active transmission during the current study year and not previous transmission seasons. Roof rats have an average life span in the wild of about a year, with an annual mortality rate of 91 to 97% (Nowak, 1991), so it is likely that very few of our sampled rats were alive during the previous year's period of kissing bug activity and parasite transmission. If there was reduced vector activity during the study year,

as suggested by the lack of vector detection in active or passive surveillance, then the lack of apparent infection in rats may reflect an overall low year for transmission due to unmeasured biotic or abiotic factors. Second, the interval between death and preservation of carcasses varied among specimens, but could have been up to 2 days in some cases. At the time of heart collection, 40% of rat carcasses were scored >4/5 on the autolysis scale (moderate-marked to marked autolysis). However, this is unlikely to have significantly affected the outcome of testing as the qPCR we used targets a very short fragment of DNA and thus is relatively resistant to effects of sample degradation. A final limitation of the study is that our active bug surveillance efforts were limited, and future efforts should include additional active trapping as well as excavation of woody debris and other potential kissing bug harborage sites along the perimeter of the facility across an extended time frame.

Kissing bugs have been collected from around the outdoor cages of this facility in years past (Gleiser et al., 1986), however, since 2011 pest control efforts have been intensified, including measures such as reducing debris and bug harborage sites and mowing a wide perimeter around outdoor enclosures, sealing of outside doors, and perimeter application of diatomaceous earth. Anecdotally, personnel have reported a reduction in bug activity overall and of incidence of new *T. cruzi* infections of primates as a result of these measures. However, animals are not routinely tested for *T. cruzi* at this facility until they are sold or enrolled in a study, so while we know there are seropositive animals present as a result of prior studies, we do not know if, in fact, bug activity and incidence of infections have been reduced. Thus, in the absence of systematic data on incidence of seroconversions, it is not possible to ascertain what impact these increased vector control measures have had on the incidence of *T. cruzi* at this facility.

A reservoir is defined as one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population (Haydon et al., 2002). Thus, based on our results, roof rats are unlikely to serve as important as local reservoirs of *T. cruzi* at this facility. Increased bug and rodent control efforts may have successfully reduced the frequency of transmission, but it is likely that other mammals are serving as wild reservoirs of *T. cruzi*, given the facility's location in San Antonio, a known hotspot for Chagas disease transmission with a high number of infected vectors and canine and human cases (Curtis-Robles et al., 2015; Kjos et al., 2008; Sarkar et al., 2010; Tenney et al., 2014; Texas Department of State Health Services, 2016b). The facility is partially surrounded by a brushy area with a dry creek on one side, and facility personnel have reported the presence of a number of mesomammals, such as skunks, raccoons, opossums, and armadillos, all of which have been implicated as important reservoirs of *T. cruzi* in the United States (Bern et al., 2011; Brown et al., 2010; Charles et al., 2012), in addition to rabbits and squirrels, the reservoir competency of which is largely unknown. A primate facility in North Carolina isolated *T. cruzi* from raccoons on its property and from an opossum in the surrounding area following detection of *T. cruzi* in a squirrel monkey (Karsten et al., 1992). It is likely that multiple wild species as well as some nonhuman primates serve as the local reservoirs of *T. cruzi.* Efforts to characterize the reservoir community should include a multidisciplinary approach to data collection and analysis, with interventions that can simultaneously answer questions about reservoir importance while providing direct benefits in control of the parasite (Viana et al., 2014). Future work should include expanded efforts to trap and test additional species of mammals, as well as increased efforts to confirm vector presence.

# 6. *TRYPANOSOMA CRUZI* INFECTION DYNAMICS IN PRIMATES, WILDLIFE, AND VECTORS AT A TEXAS NON-HUMAN PRIMATE FACILITY

## 6.1 Introduction

*Trypanosoma cruzi*, the causative agent of Chagas disease, is widespread throughout the Americas from the southern United States to northern Argentina, infecting over 200 species of mammals. This vector-borne protozoal parasite is maintained in domestic and sylvatic transmission cycles and is a major human and canine health problem. The complexity of the sylvatic transmission cycles, which involve multiple genetic strains of the parasite maintained by a diverse community of wildlife hosts, present one of the major challenges to Chagas disease control and prevention. *T. cruzi* is divided into 7 strain types or discrete typing units (DTUs): TcI-TcVI and TcBat, which are reportedly associated with differing clinical manifestations, reservoir host species, and geographical locations. (Barr et al., 1991c; 1991b; Duz et al., 2014; Jansen et al., 2015; J. D. Ramírez et al., 2010) In the US, TcI and TcIV are the most commonly reported strain types (Bern et al., 2011; Roellig et al., 2013). Diverse mammalian wildlife species serve as reservoirs for *T. cruzi* in the US (Bern et al., 2011; Hodo and S. A. Hamer, 2017). The importance of an animal species as a reservoir is related to its infectiousness to vectors as well as its local abundance and may vary across geographical locations (Gürtler and Cardinal, 2015; Haydon et al., 2002; Hodo and S. A. Hamer, 2017). Once an animal is infected, the parasite circulates in the blood during the parasitemic acute stage, after which it localizes in various organs. The specific dynamics of parasitemia frequency, degree, and duration are not well-described for most animal hosts.

*T. cruzi* is transmitted by triatomine bugs, which acquire infection by blood feeding on an infected mammal and pass the infective stage of the parasite in their feces which can contaminate the bite wound or nearby mucous membranes. Additionally, oral transmission through the ingestion of infected bugs is thought to be important in wildlife, dogs, and NHPs (Barr, 2009; Desquesnes, 2017; Dorn et al., 2012; Rocha et al., 2013). Transmission can also occur congenitally and through blood transfusion or organ transplant. There are 11 species of kissing bugs in the US, and the highest species diversity of triatomines is found in Texas (Bern et al., 2011). In Texas, adult triatomines are most active and most often encountered by humans during the warmest months, from May – September (Curtis-Robles et al., 2015; Pippin, 1970).

Some free-ranging neotropical non-human primate (NHP) species are important sylvatic hosts of the *T. cruzi* (Jansen et al., 2017; Lisboa et al., 2015), and natural infection has also been described in captive New and Old World NHPs in areas where the parasite and vectors are found (Bommineni et al., 2009; Dorn et al., 2012; Minuzzi-Souza et al., 2016; Williams et al., 2009). The southern US is home to a number of NHP research, breeding, and holding facilities and animals housed with outdoor access are at risk for exposure to infected triatomine vectors and thus infection with *T. cruzi*. As in humans and dogs, infection in NHPs is characterized by acute, indeterminate, and chronic stages with a subset of infected animals developing a lethal cardiomyopathy or, less commonly, severe gastrointestinal issues (Bonecini-Almeida et al., 1990; Carvalho et al., 2003).

Natural *T. cruzi* infection of NHPs in the US has been reported since the 1970s (Cicmanec et al., 1974; Kasa et al., 1977), but awareness and surveillance have increased in recent years. Published surveys report infection prevalence ranging from 2-10% in NHP

facilities in the southern US (Dorn et al., 2012; Kasa et al., 1977; Pisharath et al., 2013). Additionally, because animals are often transported across the country from breeding or holding facilities in the South, *T. cruzi* infection is a concern in NHPs housed in non-endemic areas as well (Dickerson et al., 2014). Both DTUs TcI and TcIV have been documented in NHPs in the US (Roellig et al., 2013), but sample sizes are low and there have been no published studies to correlate strain type with clinical outcome.

Incidental infection of captive NHPs presents problems for the biomedical industry. While infected animals may remain subclinical for life, seropositive NHPs are often removed from the pool of animals used in research and breeding due to concerns about the confounding effects of infection on biomedical research and concerns over potential risk to other NHPs. However, while concerns about confounding are valid, the risk posed by seropositive NHPs to others in the colony has yet to be definitively characterized through studies of infectiousness to vectors or documented instances of direct transmission. Additionally, little is known about the role of wildlife reservoirs and influence of the surrounding sylvatic environment on the transmission dynamics at NHP facilities. In the absence of effective vaccines and drugs against *T. cruzi*, interventions must be aimed at interrupting the transmission cycle from reservoir to vector and from vector to NHP. Additionally, in order to develop informed guidelines for the management of infected NHPs, it is necessary to quantify the infectious potential of seropositive animals to determine whether they may serve as reservoirs. Thus, the objectives of this study were to characterize the transmission cycles of *T. cruzi* at a NHP facility by investigating infection dynamics in NHPs and local wildlife, documenting vector activity, and determining host-DTU associations.

## 6.2 Materials and Methods

### *6.2.1 Facility*

The MD Anderson Michale E. Keeling Center for Comparative Medicine and Research (KCCMR) is located on 381 mostly wooded acres in Bastrop county in central Texas (Figure 6.1). The facility maintains an Indian-origin rhesus macaque (*Macaca mulatta*) breeding colony of approximately 980 animals, as well as housing several other species of primates. The animals comprising the rhesus macaque breeding colony are all housed in open-air enclosures. The KCCMR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AALACI) since 1979 and all animals are housed in full compliance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The KCCMR also maintains an approved PHS Assurance through the Office of Laboratory Animal Welfare. We visited the facility for mammal trapping and vector surveillance 8 times from July – September 2016 at 1-2 week intervals.

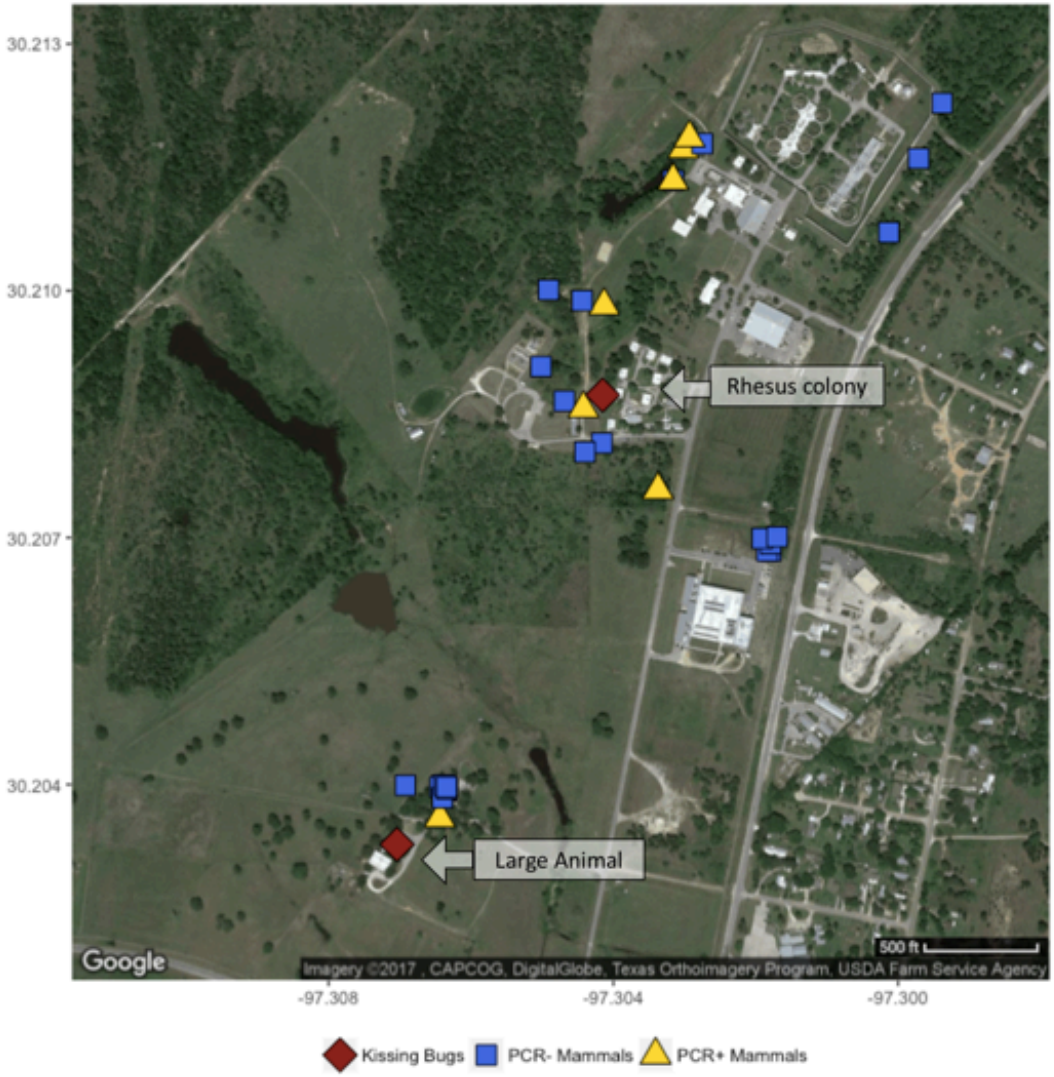


**Figure 6.1. Satellite image of KCCMR facility indicating the locations where *T. cruzi* PCR positive and negative mammals were trapped and where kissing bugs were found.** Created in R using ggplot2 and ggmap packages (Kahle and Wickham, 2013). Google map image from: http://maps.googleapis.com/maps/api/staticmap?center=30.20756,-97.30475&zoom=16&size=640x640&scale=2&maptype=satellite&language=en-EN&sensor=false

### *6.2.2 Primates*

The KCCMR rhesus macaque breeding colony has been a closed colony since 1983 and no outside animals have been added to this group since that time. The colony has been documented through serological means to be Specific Pathogen Free (SPF) for

Cercopithecine Herpesvirus 1, Simian Immunodeficiency Virus, Systemic T-lymphotrophic Virus and Simian Retroviruses 1, 2, and 5 since 1991. Surveillance for *T. cruzi* exposure in the macaque colony was first performed in 2013 and has been conducted yearly since 2015. All macaques used in this study are identified to be seropositive for *T. cruzi* for at least two consecutive years using a suspension microarray from a commercial laboratory, (Macaque Chagas-Multiplexed Fluorometric ImmunoAssay [MFIA], Charles River Laboratories, city, state), run in conjunction with an ELISA at the same commercial laboratory. From July 2016 – Jan 2017, we received up to 2 ml of whole blood from these seropositive animals and subjected it to a series of PCRs (see below) and used these samples to determine the presence and strain type of *T. cruzi* DNA circulating in the blood. After the first round of sampling in July 2016, each animal that was not positive on both the *T. cruzi* qPCR and strain typing qPCR was resampled at the next opportunity. This was repeated until Jan 2017, with each seropositive animal being sampled from 1 to 4 times. All blood collections made for this study were approved through The University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC; protocol #0806-RN01).

DNA was extracted from whole blood treated with the anticoagulant EDTA using a commercial spin-column extraction kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA) according to the manufacturer's tissue extraction protocol but with a final elution volume of 50 ul and following instructions for scaling up to larger volumes of starting material. The larger volume required passing the lysate through the filter in portions because it would not all fit in the column at the same time, then extraction proceeded according to instructions. For the first set of blood samples we initially extracted from 0.5 ml of blood,

then followed this with a second extraction from 1 ml of the original blood sample. For each subsequent set of samples, we extracted from 1 ml of blood.

Extracted DNA was first subjected to a sensitive real-time quantitative PCR (qPCR) for the specific detection of *T. cruzi* using the Cruzi 1/2 primers and a 6-carboxyfluorescein (FAM)-labeled probe, Cruzi 3, as previously described (Piron et al., 2007; J. C. Ramírez et al., 2015), but with an initial denaturation time of 3 min. Based on internal laboratory validations, the cutoff for suspect positive samples was determined to be a quantification cycle threshold (Ct) value of 36 or less. DNA-negative controls and a positive control of DNA extracted from pure culture of Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection, Manassas, VA; DTU TcI) were included in all reactions. This qPCR served as a screening test. Next, all suspect positive samples, as well as some that were negative, were subjected to a multiplex probe-based qPCR for determination of DTU (Cura et al., 2015). This PCR targets the spliced leader intergenic region (SL-IR) and reliably distinguishes between DTUs TcI, TcII/V/VI, and TcIII/IV. A follow-up qPCR can be used to confirm TcIII vs TcIV identity as some TcIII isolates can hybridize with the probes for both TcIII and TcIV (Cura et al., 2015), but because our samples hybridized only with the TcIV probe and TcIII has never been identified in the US, we did not run the follow-up PCR for these samples. Negative controls (water) and positive controls of DNA extracted from *T. cruzi* strain Sylvio X10 CL4 (DTU TcI, details above) and *T. cruzi*-infected *Triatoma sanguisuga* from Texas (DTU TcIV) were included in all reactions, and positive control of DNA extracted from *T. cruzi* Y-strain (ATCC 50832, American Type Culture Collection, Manassas, VA; DTU TcII) was added for reactions run later in the study. All positive controls performed as expected for each reaction. Samples that gave negative results on the

SL-IR qPCR under standard conditions were re-run on this qPCR using two additional treatments: (*i*) 1:10 dilution of the DNA template, and (*ii)* 2 times the volume of DNA template to afford additional opportunity to ascertain the strain type in samples that were suspect positive in the screening qPCR.

Statistical analyses were performed in R (R Core Team, 2014). For statistical analysis, NHPs were considered PCR-positive when they were positive on both qPCRs. Assessed variables were NHP sex and NHP age. NHPs were divided into two age groups, less than 16 years of age and 17 years and older, with 22 individuals in each group. Bivariable analysis using the chi-squared or Fisher's exact tests was performed to evaluate the relationship between these variables and PCR positivity. Bivariable analysis was also performed to evaluate the relationship between age and sex and infecting DTU of PCR-positive NHPs. All risk factors with $P$ value $\leq 0.25$ in bivariable analysis were further investigated with logistic regression using the GLM (generalized linear model) method in R. Values of $P \leq 0.05$ were considered significant. Odds ratios and their 95% confidence intervals were calculated for the risk factors included in the final models. An exact binomial test was used to compare the proportion of NHPs infected with DTU TcI with those infected with TcIV.

### *6.2.3 Wildlife*

Small mammals were trapped using Sherman live traps (H.B. Sherman Traps, Tallahassee, FL) spaced approximately 10 m apart and baited with sunflower seeds. During each of 8 visits we set from 60 to 160 traps in 3-5 different areas for one night. Medium mammals were trapped using Tomahawk live traps (Tomahawk Live Trap, Hazelhurst, WI)

baited with a combination of canned cat food, tuna, sardines, peanut butter, bacon grease, and marshmallows. Thirteen Tomahawk traps were set during the first two visits and 14 were set during the remaining visits. Trap locations (Figure 6.1) were chosen based on the appearance of suitable habitat and previous trapping success.

The location, species, sex, and weight of all captured mammals was recorded. Small mammals were transferred to a plastic container and euthanized via an inhaled overdose of isoflurane anesthetic agent (IsoFlo, Zoetis, Parsippany, NJ). Death was confirmed via absence of heart beat and respiration, and immediately after death, blood was collected via intracardiac puncture (exsanguination, secondary method of euthanasia) and stored in a microcentrifuge tube, then the thorax was opened and the heart was collected. In the case of pregnancy, fetuses were euthanized individually via intracoelomic injection of potassium chloride (KCl). Medium-sized mammals were weighed while in the trap and the known weight of the trap was subtracted for dose calculations. Medium-sized mammals were anesthetized via an intramuscular injection of tiletamine HCl and zolazepam HCl (Telazol, Zoetis, Parsippany, NJ) at 10 mg/kg for raccoons and skunks (Kreeger and Arnemo, 2012), and 30 mg/kg for opossums (Stoskopf et al., 1999). Once unconscious (as measured by lack of voluntary movement and palpebral response), animals were euthanized via an intracardiac injection of approximately 50 mg/kg potassium chloride (KCl). Blood (1-3 ml) was collected via intracardiac puncture and stored in a microcentrifuge or vacutainer tube with no additive, and the thorax was opened to collect the heart.

Wildlife trapping, handling, and euthanasia were conducted in compliance with Texas Parks and Wildlife Division scientific collections permit SPR-0512-917, Texas A&M University Institutional Animal Care and Use Committee AUP #2015-0088, and MD

Anderson IACUC 1581-RN00. Additionally, we received rodents found dead by the facility's pest control personnel, the use of which was deemed exempt from IACUC oversight.

Hearts and blood were transported from the field on ice and processed in the laboratory. The blood was centrifuged and serum was separated from the clot. Hearts were examined for gross abnormalities and dissected to allow visualization of all four chambers. Samples were collected for both histopathology and PCR from left and right ventricles and left and right atria. Samples for histopathology were fixed in 10% neutral buffered formalin, and samples for PCR were minced, pooled, and frozen until extraction.

We extracted DNA from 500 µl of clotted blood and approximately 0.5 $cm^3$ of heart using a commercial extraction kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA) following the manufacturer's protocol for tissue extraction but with an overnight lysis and final elution volume of 50 µl. A negative control was included with each batch of extractions. The *T. cruzi* screening qPCR was run on all samples as described above, and the strain type qPCR was run on all samples positive on the screening qPCR.

*6.2.4 Triatomine Vectors*

Active nighttime kissing-bug surveillance was performed during each of 8 visits to the facility by using active searches and stationary white cloth sheets with UV lights and occasionally dry ice; we have used all these methods previously to collect kissing bugs in other areas across Texas (Curtis-Robles, Hamer, unpublished data). Bug surveillance was conducted for 3-3.5 hours beginning around 9:00 pm for one night during each visit by a 3-person team. Three to four trapping stations with lights, sheets, +/- dry ice were set up in

areas between sylvatic habitat, buildings housing NHPs; these trapping stations and immediate vicinity were actively checked for bugs 3 to 4 times each hour. Between checks of the stations, team members patrolled the facility with flashlights to actively search walls and sidewalks for bugs. Additionally, passive vector surveillance was conducted by facility personnel, who submitted bugs as they encountered them during the course of normal duties over the summer months of 2015 and 2016.

Bugs were identified to species (Lent and Wygodzinsky, 1979), measured, sexed, externally decontaminated with bleach, and dissected to isolate the hindguts. The guts were subjected to DNA extraction using a commercial kit and tissue extraction protocol, and to screening and strain-typing qPCRs for *T. cruzi* detection and characterization as described above. Additionally, extracted DNA from the bugs was subjected to several PCRs targeting the vertebrate cytochrome b gene to determine blood meal source as previously described (Curtis-Robles et al., 2017b). We made several attempts with the same or multiple different PCR primer sets to increase the chances of assigning a blood meal host in the face of degraded DNA contained in aged blood meals. Additionally, although we decontaminated the external surface of insects prior to dissection, performing multiple PCRs to confirm results served to improve certainty that the amplified host species DNA was not the result of contamination. Separate PCRs using the "herp" primer set (Cupp et al., 2004; G. L. Hamer et al., 2009), "BM" primer set (Boakye et al., 1999; G. L. Hamer et al., 2009; Kjos et al., 2013), and "mammalian a" primer set (G. L. Hamer et al., 2009; Kjos et al., 2013; Molaei et al., 2006) were performed using 1.5 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 15 μL using the published cycling conditions. DNA-negative water

controls and positive controls of DNA extracted from tissue of white-tailed deer (*Odocoileus virginianus*), opossum (*Didelphis virginiana*), or cynomolgus macaque (*Macaca fascicularis*) were included in all PCR batches. Amplicons were visualized on 1.5% agarose gels with ethidium bromide, and amplicons of expected product sizes were sequenced using Sanger sequencing (Eton Bioscience Inc., San Diego, CA) in both directions. Forward and reverse strands were aligned in MEGA7 (Kumar et al., 2016) and the consensus region was compared with other published sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

## 6.3 Results

### *6.3.1 Primates*

We received blood samples from all known seropositive macaques housed at the facility that were alive throughout the duration of the study (n=41): 31 females (73%) and 10 males (27%), age range 4-23 years. Because our goal was to ascertain circulating parasite strain in seropositive individuals, which often required repeated blood draws to detect parasite DNA, we sampled 14 animals once, 10 animals twice, 8 animals three times, and 13 animals four times. For the most part, animals were only resampled if the previous samples were not PCR-positive on the second assay, but in a few cases animals were resampled before complete results were obtained, resulting in resampling after determination of PCR-positivity. At the end of the study, 33/41 (80%) macaques had at least one PCR-positive blood sample for which DTU was determined (Table 6.1). Overall, 13/44 were DTU-positive on the 1$^{st}$ attempt, 12/30 on the 2$^{nd}$ attempt, 5/18 on the 3$^{rd}$ attempt, and 4/13 on the 4$^{th}$ attempt (Figure 6.2). Two additional monkeys had a suspect positive result on the screening

qPCR, but did not generate a positive result on the SL-IR assay, despite multiple attempts. A total of 10 seropositive monkeys were PCR-negative on 4 different blood samples. Of the remaining PCR-negative monkeys, one died after the first round of blood sampling, and 3 were sampled only 3 times. The lowest Ct value on the screening qPCR for positive monkeys was 27, equivalent to approximately 150 parasite equivalents/ml of blood as estimated from a standard curve of quantified pure culture of *T. cruzi* epimastigotes, strain Sylvio X10 clone 4. The highest Ct value on the screening qPCR (in which the same sample was confirmed positive on the SL-IR assay) was 33.35, equivalent to less than 1.5 parasites equivalents/ml of blood (Figure 6.3).

In bivariable analysis to determine significant predictors of NHP PCR-positive status among seropositive individuals, age group and sex had *P* values of 0.053 and 0.08, respectively and were included in the logistic regression model. As estimated by logistic regression, neither age group (*P*-value = 0.13) nor sex (*P*-value = 0.057) were significantly associated with PCR-positive status.

While a slightly greater number of monkeys were infected with TcI (n=18) than TcIV (n=14), this difference was not statistically significant (p-value = 0.45). Two additional monkeys were co-infected with both TcI and TcIV. In bivariable analysis to determine associations between infecting DTU and other demographic factors, only sex was determined to be significant using a Fisher exact test (*P*-value 0.045). No male monkeys were infected with TcIV alone, though one male monkey was co-infected with both TcI and TcIV.

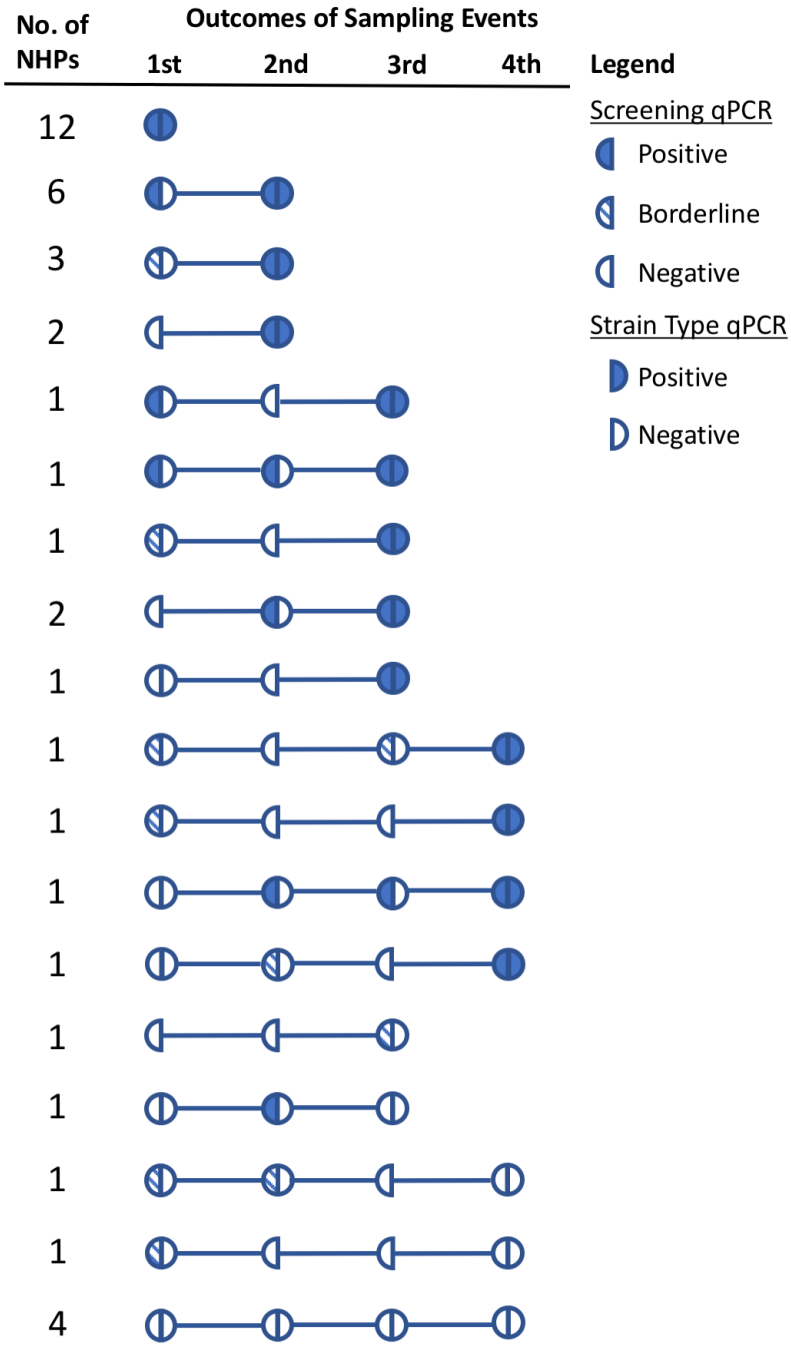


**Figure 6.2 Graphical demonstration of sampling efforts and qPCR results for NHP blood samples.** The number of NHPs with each specific sampling and results profile is in the lefthand column. Semicircles depict outcomes of qPCR assays as described in the legend.

**Table 6.1. Demographic data and PCR positivity of 41 seropositive rhesus macaques.**

| | | *N* | PCR-positive (%) |
|---|---|---|---|
| Overall | | 41 | 33 (80%) |
| Age (years) | | | |
| | 19-23 | 15 | 13 (87%) |
| | 15-18 | 10 | 10 (100%) |
| | 10-12 | 11 | 6 (55%) |
| | 4-8 | 5 | 4 (80%) |
| Sex | | | |
| | Female | 31 | 27 (87%) |
| | Male | 10 | 6 (60%) |
| Strain Type | | | |
| | TcI | 18 | |
| | TcIV | 13 | |
| | TcI + TcIV | 2 | |

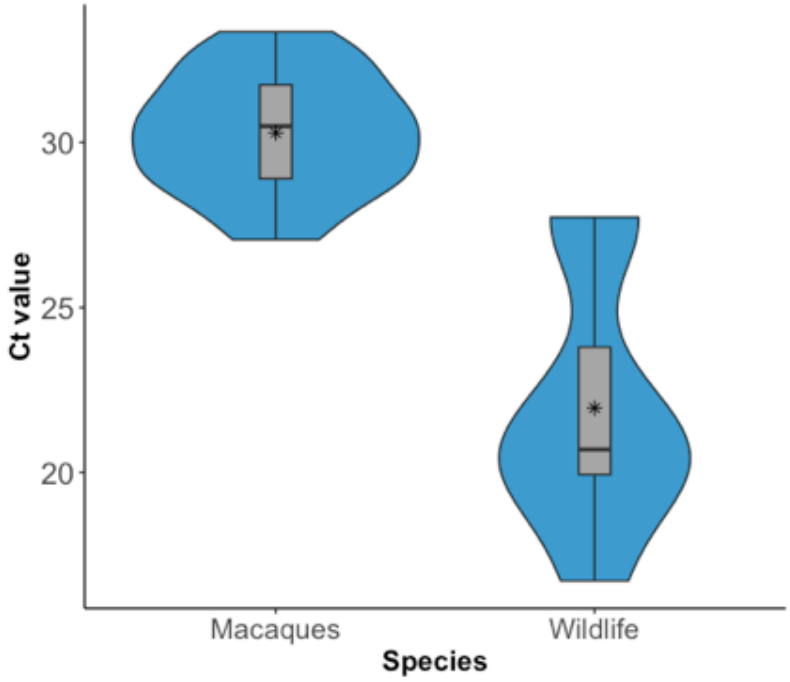


**Figure 6.3. Violin and box plots demonstrating higher Ct values in blood of macaques than in blood of wildlife, representing lower concentrations of circulating parasite DNA in macaques.**

*6.3.2 Mammals*

Over 8 visits to the facility (780 total trap nights), we captured 38 mammals of 5 species in multiple locations across the facility grounds, all less than 0.5 mile from the rhesus colony (Figure 6.1). We also received 8 roof rats (*Rattus rattus*) and 1 white-footed mouse (*Peromyscus leucopus*) from on-site pest control personnel (Table 6.2).

Overall, 8 of 10 mesomammals were PCR-positive for *T. cruzi* in blood and/or heart. *T. cruzi* DNA was not detected in hearts or blood of any of 4 rodent species of (Table 6.2). All 8 of the infected mesomammals had *T. cruzi* DNA circulating in the blood, with Ct values ranging from 16 to 27 (Figure 6.3), equal to approximately 300 to 300,000 parasite equivalents/ml. Of these, 6 also had infected heart tissue. Both raccoons were infected with DTU TcIV, and the 4 *T. cruzi*-positive opossums were infected with TcI (Table 6.2), based on both blood and heart for individuals in which both tissues were positive. Of the 2 positive skunks, 1 was infected with TcI and the other with TcIV (Table 6.2), based on both blood and heart.

**Table 6.2. Mammals collected from primate facility grounds summer 2016.**

| **Species** | **Common name** | **No. tested** | ***T. cruzi* + (%)** | **+ Heart** | **+ Blood** | **Strain type** |
|---|---|---|---|---|---|---|
| *Didelphis virginianus* | Virginia opossum | 5 | 4 (80%) | 3 | 4 | TcI |
| *Procyon lotor* | Raccoon | 2 | 2 (100%) | 1 | 2 | TcIV |
| *Mephitis mephitis* | Striped skunk | 3 | 2 (67%) | 2 | 2 | TcI,TcIV |
| *Sigmodon hispidus* | Cotton rat | 27 | 0 (0%) | | | |
| *Neotoma floridana* | Woodrat | 1 | 0 (0%) | | | |
| *Rattus rattus* | Roof rat | 8 | 0 (0%) | | | |
| *Peromyscus* sp. | Mouse | 1 | 0 (0%) | | | |

*6.3.3 Triatomine Vectors*

A total of 6 bugs were collected from the facility from Sept 2015 to Aug 2016. We conducted 25 total hours of vector surveillance over 8 nights from July-Sept 2016, averaging 3 hours per night. In September 2015, we received 3 kissing bugs that were collected by facility staff. During the summer of 2016, 1 bug was collected by facility staff, and we found 2 bugs during nighttime active search efforts (Table 6.3). Five of the 6 bugs were collected from inside or just outside of the same building (VLS1), a shower room located just on the edge of the rhesus colony (Figure 6.1). *T. sanguisuga* (n=4) was the species most often collected (Table 6.3) and we also collected 1 each of *Triatoma gerstaeckeri* and *Triatoma lecticularia*. Only the *T. sanguisuga* collected in July 2016 was PCR-positive for *T. cruzi*, and was infected with DTU TcI. Blood meal analysis of the extracted DNA from the hindguts of the bugs resulted in amplification of human DNA from one bug - a *T. sanguisuga* collected from a locker room in August 2016. No other bugs generated a sequenced amplicon on any of 3 CytB primer sets despite multiple attempts.

**Table 6.3. Kissing bugs collected from the primate facility grounds 2015-2016.**

| Species | Sex | Month | Location | *T. cruzi* status | Blood meal |
|---|---|---|---|---|---|
| *Triatoma sanguisuga* | M | Sept 2015 | VLS1 shower room | Neg | - |
| *Triatoma gerstaeckeri* | F | Sept 2015 | VLS1 shower room | Neg | - |
| *Triatoma sanguisuga* | F | Sept 2015 | Large animal | Neg | - |
| *Triatoma lecticularia* | M | June 2016 | VLS1 shower room | Neg | - |
| *Triatoma sanguisuga* | M | July 2016 | Outside VLS1 | Pos, TcI | - |
| *Triatoma sanguisuga* | F | Aug 2016 | VLS1 entry | Neg | Human |

## 6.4 Discussion

Our findings illustrate a robust transmission cycle of *T. cruzi* involving NHPs, local wildlife species, and triatomine vectors on the campus of a nonhuman primate facility in central Texas. Importantly, we document high concentrations of *T. cruzi* DNA in the blood of infected mesomammals in close proximity to NHP enclosures (Figure 6.1). While PCR does not demonstrate the presence of whole, viable parasites, PCR-positive blood samples suggest that the animal could be parasitemic and thus serve as a source of infection to blood-feeding kissing bug vectors. Real-time PCR can be used to compare relative concentrations of parasite DNA in samples and has been used to quantify parasitemia in previous studies (Caldas et al., 2012). We found that all of the wild medium-sized mammals in which *T. cruzi* was detected had relatively high quantities of *T. cruzi* DNA circulating in their blood (300 to 300,000 parasite equivalents/ml). This is in contrast to the NHPs, in which concentrations of parasite DNA, when present, were orders of magnitude lower (all were ≤ 150 parasite equivalents/ml), and were only detected intermittently in some individuals (Figures 6.2, 6.3). These findings suggest that mesomammals such as raccoons, opossums, and skunks in proximity to NHP facilities likely play important roles as local reservoirs of *T. cruzi*, while NHPs themselves may be less likely to be infectious to vectors.

The proportion of PCR positive females was higher than for males, though this was not a statistically significant finding. A larger sample of male monkeys would be useful to strengthen this observation. Differences related to sex may be related to hormonal effects (eg. pregnancy) or to other social factors resulting in stress to the immune system. In general, more research is needed to better characterize the parasitemia dynamics of chronically infected NHPs.

While parasite DNA concentration was low in the blood of the seropositive non-human primates, we were able to determine the infecting DTU for 77%. Previous reports of DTU in NHPs in the US are limited, with 2 rhesus macaques in Texas harboring TcI, and free-ranging lemurs in Georgia harboring TcIV (Roellig et al., 2013). TcI was detected in captive NHPs in a zoological park in Brazil (Minuzzi-Souza et al., 2016). We document both DTUs TcI and TcIV in this population of rhesus macaques, with two individuals being co-infected with both DTUs simultaneously. More work is needed to correlate DTU with clinical outcome in NHPs, which could have important implications for management of infected animals, as well as for their use as animal models of *T. cruzi* infection in humans. In the US, only TcI and isolates from the TcII/V/VI complex have been detected in autochthonous human cases; TcIV has not been documented in humans in the US.

Certain wildlife species are known to be associated with specific DTUs (Bern et al., 2011; Hodo and S. A. Hamer, 2017; Jansen et al., 2017). While our sample size was small, we documented TcIV infection in raccoons and TcI in opossums, as expected based on previous reports (Bern et al., 2011; Roellig et al., 2013). Both DTUs have been reported in skunks (Charles et al., 2012; Roellig et al., 2008), and this was consistent with our findings as well. These DTU-host associations may have important implications for public health and the role of certain species of reservoirs. However, because the NHPs were infected almost equally with both TcI and TcIV, we were not able to pinpoint any specific species of mesomammal as more important than the others in contributing to NHP infection.

Interestingly, while infection was very common in mesomammals, we did not detect *T. cruzi* in any of the rodent species we tested, even though these animals were captured in the same location and during the same time period as the mesomammals. This is consistent

with our findings at another NHP facility in Texas, in which *T. cruzi* was not detected in any of 156 *R. rattus* (Hodo et al., 2017). *T. cruzi* and triatomine bugs have long been associated with woodrats (*Neotoma* spp.) in the US (Charles et al., 2012; Packchanian, 1942), but we only captured and tested one woodrat, which was negative. Several studies have reported *T. cruzi* infection in other species of rodents, though most are represented by small sample sizes (Burkholder et al., 1980; Charles et al., 2012; C. P. Herrera et al., 2015; Navin et al., 1985). *T. cruzi* transmission cycles are characterized by regional heterogeneity, and the important reservoirs likely differ across geographical areas (Hodo and S. A. Hamer, 2017). The absence of *T. cruzi* infection in the rodent population in the face of active infection in other species suggests that rodents are not an important part of the reservoir population at this facility.

We were able to document the ongoing presence of triatomine vectors at this facility over two summers, though the number of triatomines we collected was low. The low capture success for bugs was not surprising, given that triatomines are nocturnal, elusive, and notoriously difficult to collect using standard entomological methods (Curtis-Robles et al., 2015; Kjos et al., 2013). Kissing bugs seek opportunistic harborage sites during periods of inactivity, and habitat features such as ornamental landscape plants, cracks in structures, woody debris, and undeveloped neighboring areas may provide harborage sites for bugs and increase the local risk of exposure. Interestingly, 5/6 of the bugs collected were all found in the same building, a shower room on the border between sylvatic habitat and the rhesus colony. There are severeal NHP enclosures in immediate proximity to this building, and while no triatomines have been recovered from the NHP enclosures directly, it's very likely that the animals would readily consume insects, leaving few in the environment to be discovered by the staff. The relatively low infection prevalence among the bugs we collected

(1/6, 17%) compared with other prevalences reported in Texas (50-63%) (Curtis-Robles et al., 2015; Kjos et al., 2009b) is likely an artifact the very small sample size. Of 44 bugs that were tested from elsewhere in the same county, 21 (47.7%) were *T. cruzi* positive (Curtis-Robles, unpublished data).

We detected human DNA in one of the bugs collected from the facility via one PCR, but were unable to confirm this with a second primer set. Human blood meals have been frequently documented in bugs collected in the US (Gorchakov et al., 2016; Klotz et al., 2014; Waleckx et al., 2014), though contamination is always a concern. None of the bugs we collected had a particularly fresh blood meal, and our limited success in blood meal analysis is likely related to degraded DNA. Future work involving testing of additional bugs and the use of advanced whole-genome sequencing methods is likely to uncover additional important information on vector feeding behavior.

In conclusion, we documented low and intermittent concentrations of circulating *T. cruzi* DNA among seropositive NHPs; high *T. cruzi* infection prevalence among mesomammals in contrast to absence of infection in rodents; and the ongoing presence of triatomine vectors at an NHP facility with active *T. cruzi* transmission. Primates and mesomammals were infected with both DTUs TcI and TcIV. Our findings add important components needed to understand the transmission cycles of *T. cruzi* in the southern US, and in particular at NHP facilities. Interventions to block transmission to NHPs should be aimed at interrupting the sylvatic cycles involving mesomammals and triatomine vectors, with rodents being less important. Future work should involve more thorough investigation of the infectiousness dynamics of infected NHPs and wildlife, and behavioral studies of local triatomine species to determine the best intervention strategies.

# 7. SUMMARY

*Trypanosoma cruzi* is characterized by a complex transmission cycle involving a great diversity of vertebrate hosts and vector species. Much of the ecology and epidemiology of the disease remains unknown, especially in the southern US. This dissertation provides information toward filling in some of these knowledge gaps, and highlights areas where further work is still needed. In this dissertation, I characterize the prevalence of *T. cruzi* in domestic dogs in animal shelters across the state, explore the presence of trypanosomes in Texas bats, describe infection and pathology in coyotes and raccoons, and investigate transmission cycles at non-human primate (NHP) facilities involving NHPs, wildlife, and vectors. For each of these projects, I determined the infecting discrete typing unit (DTUs) of *T. cruzi* in each of the mammalian hosts involved. This strain type data, together with overall prevalence estimates and quantification of circulating parasite DNA, is important in the characterization of particular species as reservoirs of *T. cruzi* in this region, and for characterization of potential pathologic outcomes of infection.

We found an overall *T. cruzi* seroprevalence of 18.2% in dogs in shelters across Texas, with prevalence estimates in individual shelters ranging from 5.4 – 29.5%. This study represents one of the most geographically extensive surveys of *T. cruzi* in dogs in the US and our results indicate that dogs across Texas are frequently exposed to *T. cruzi*. Additionally, the findings reinforce the need for better options for diagnosis and treatment of infected animals. Discordance between serologic tests is an issue that impedes accurate diagnosis of *T. cruzi* exposure.

A small percentage (1.1%) of dogs had evidence of parasite DNA in their blood confirmed by 2 qPCR assays. This low prevalence of circulating parasite DNA may have implications for the role of dogs as reservoirs. However, these results were based on extraction from only a small volume of blood, and additional work is needed to better characterize the infectious potential of dogs. Most of the PCR-positive dogs were infected with DTU TcI, with only 1 dog infected with TcIV. Further work is needed to determine whether there is an association between infecting DTU and clinical disease outcome.

Trypanosomes were detected in 15 of 593 (2.6%) bats, with a single bat positive for *T. cruzi*. This is lower than reports from Central and South America. Our findings likely represent a conservative estimate of the true prevalence, given the aged nature of some carcasses and testing of only cardiac tissue. Although we detected *T. cruzi* in only a single evening bat (*Nycticeius humeralis*), this must be considered in the context of the overall population size of these bats and how they move across the landscape. While *T. cruzi* infection in bats was rare, bats may nonetheless serve as reservoirs if they are part of a community in which the pathogen can be permanently maintained and transmitted (Haydon et al., 2002). Bats have the potential to play important roles in the spread of parasite across great distances during migration. The *T. cruzi*-infected bat in our study harbored TcI, the only strain type definitively associated with human infection in the US thus far (Roellig et al., 2008).

In addition to the single *T. cruzi* infection, we detected *T. dionisii* in 9/593 bats (1.5%) of 3 species (*T. brasiliensis*, *A. pallidus*, *P. hesperus*). *T. dionisii* is a well-known trypanosome of bats in South America and Europe (Molyneux, 1991), but has not before been detected in North America. Based on the 18S rRNA gene fragment we sequenced, the

Texas bat *T. dionisii* sequences all grouped with the New World isolates of *T. dionisii*. Further, we detected three unique variants that were uniform within each of the three infected bat species. Variants differed among species even within the same geographical area. Additional genetic analyses may further characterize the ecological importance of these host-parasite associations. As an unexpected finding, based on sequencing of two gene regions (18S rRNA and 24Sα rRNA), we detected bats infected with *Blastocrithidia* sp., a genus of trypanosome associated with the alimentary tract of insects of the order Heteroptera, which has not previously been isolated from mammals. *Blastocrithidia* spp. may be capable of travelling systemically within bats following the consumption of an infected insect, but the degree of transience and outcome of such an event is unknown.

Future work to explore the trypanosomes of bats of the US should focus on acquiring a larger sample size of diverse species of bats, especially from counties along the US-Mexico border, and include sequencing of additional gene segments for more detailed phylogenetic analysis, as well as attempts to culture isolates. Through these efforts, advances could be made to expand the knowledge base of host associations, genetic diversity, and geographical range of bat-associated trypanosomes.

In a cross-sectional study of hunter-harvested coyotes and raccoons of Central Texas and culled coyotes of South Texas, we found that coyotes, while less likely to be infected with *T. cruzi* than raccoons, were associated with more severe pathology and with DTU TcI. The findings in this study may have important implications for the association of *T. cruzi* DTU with resulting pathology, as well as for the reservoir potential of coyotes and raccoons. We also provide further support for the association of DTU TcIV with raccoons in the US. While raccoons are known to maintain high levels of parasitemia into the chronic stages of

infection, more research into parasitemia dynamics of coyotes is needed to determine their contribution to the reservoir system of *T. cruzi* in the US.

Studies designed to investigate *T. cruzi* transmission dynamics at NHP facilities were conducted at two separate locations. At the first location, we received 145 roof rats (*Rattus rattus*) from the facility's pest control personnel and tested hearts and blood via PCR for *T. cruzi*. None of the animals was positive, indicating that the prevalence of *T. cruzi* infection in roof rats at this facility was low (<2%) or zero, suggesting that this species was not serving as an important reservoir during the study period. Additionally, we performed limited vector surveillance for triatomines, but did not collect any throughout the summer. A limitation of this study was the lack of documented ongoing *T. cruzi* transmission during the study period, in the absence of systematic surveillance of the NHPs.

At the second NHP facility, we were able to conduct a more thorough investigation, involving the NHPs themselves, wildlife trapped on the facility grounds, and bugs collected by facility personnel or by us during nighttime vector surveillance. Serological surveillance by the facility documented ongoing transmission evidenced by seroconversion of primates over the study period. We documented low and intermittent concentrations of circulating *T. cruzi* DNA among seropositive NHPs; high *T. cruzi* infection prevalence among mesomammals in contrast to absence of infection in rodents; and the ongoing presence of triatomine vectors at an NHP facility with active *T. cruzi* transmission. Primates and mesomammals were infected with both DTUs TcI and TcIV. Our findings add important components needed to understand the transmission cycles of *T. cruzi* in the southern US, and in particular at NHP facilities. . Interventions to block transmission to NHPs should be aimed at interrupting the sylvatic cycles involving mesomammals and triatomine vectors, with

rodents being less important. Future work should involve more thorough investigation of the infectiousness dynamics of infected NHPs and wildlife, and behavioral studies of local triatomine species to determine the best intervention strategies.

The research presented here advances the knowledge of *T. cruzi* disease ecology and epidemiology in the southern US, particularly in Texas, especially in relation to the mammalian hosts of the parasite. Further research should continue to investigate associations between DTU and pathology. Additionally, it will be important to further quantify the infectiousness of candidate reservoir species and target other components of the reservoir competence equation.

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