HORIZONTAL AND VERTICAL TRANSMISSION EXCLUDED AS PRIMARY CAUSES OF *TRYPANOSOMA CRUZI* INFECTIONS IN RHESUS MACAQUES (*MACACA MULATTA*) HOUSED WITHIN A TEXAS BREEDING COLONY

A Thesis

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*) is a kinetoplasid protozoan parasite that infects both invertebrates and vertebrate hosts during its life cycle and is the causative agent for Chagas disease. Triatomine insects (kissing bugs) serve as vectors of *T. cruzi* and are distributed across the Americas including the southern United States. Kissing bugs feed on diverse wild animals, domestic animals, and humans, and hundreds of wild mammals serve as reservoirs of *T. cruzi* in nature. Across the southern US in regions where kissing bugs occur, biomedical research facilities housing non-human primates (NHPs) have increasingly been faced with *T. cruzi* infections among the NHPs, presenting major concerns for both the integrity of the animal models and for animal welfare. For this project, we tracked a population of *T. cruzi* seropositive rhesus macaques (*Macaca mulatta*) housed in an outdoor breeding colony in Texas to determine the potential for horizontal and vertical transmission. We determined the physical location of each seropositive animal within the colony at the time of their infection and also identified all animals housed with seropositive animals, including any offspring of seropositive dams. The animals housed with seropositive animals were deemed to be *T. cruzi*-associated monkeys (TAMs). The serostatus of all TAMs was determined through medical records or from the testing of archived banked serum samples. These serological data, along with spatial analyses of the seropositive and seronegative animals over time, were used to look for associations between animals that could support horizontal transfer, vertical transfer, or implicate environmental factors in the transmission of *T. cruzi* with the colony.

A total of 80 breeding colony animals housed between 1999 and 2018 were identified to be seropositive for *T. cruzi* antibodies. From these 80 animals, 688 individual TAMs were identified to have interacted in a total of 925 animal-to-animal association events during the

period covered in this study. These events were explored through the use of husbandry records, PCR, and identification of different discrete typing units (DTUs) to look for the potential of vertical and horizontal transmission within the colony. In review of the data related to the possibility of vertical transmission, there were 54 breeding-age, seropositive females identified in the colony that collectively produced 196 offspring. None of the offspring from these dams were found to be seropositive suggesting that vertical transmission either does not occur or is a rare event within this colony. In review of the data related to the possibility of horizontal transmission, it was found that horizontal transmission of *T. cruzi* may have occurred in as many as 25 of the 925 (2.70%) associations between naïve and seropositive animals, and that horizontal transmission may be the route of infection for as many as 25 of the 80 (31.25%) seropositive animals within the colony. These last two values are interpreted to represent the maximum number of horizontal transmission events that could have possibly occurred within the colony between 1999 and 2018, as these data are the result of a study-design that favored sensitivity over specificity, with regard to its inclusion of animals that were suspected to have become infected through horizontal transmission. Despite the fact that these values are likely overestimates of horizontal transmission within the colony, it is notable that even these data suggest horizontal transmission accounts for less than one-third of all *T. cruzi* infections and that horizontal transmission appears to be a very inefficient means of *T. cruzi* infection.

Spatial analysis using the location of the T. *cruzi* seropositive animals at the time of their seroconversion was undertaken to identify potential spatial clusters (hot spots) of infections within the colony and to determine if there were aspects of the environment that influenced the incidence of infection or if new infections occurred at equal rates throughout the colony as a whole. Data from these analyses identified a few specific animal rooms within the colony that

had increased incidences of infection. Notably, these hot spot rooms were adjacent to a wooded area and elevated storage sheds, both of which contained abundant leaf-litter and low growing vegetation which is presumed to have served as habitats for kissing bug vectors of the parasite. This presumption, that the colony infection rates are affected by increased exposure of animals to kissing bugs, was supported by the finding that there has been a marked decrease in the incidence of *T. cruzi* infections in the hot spot rooms adjacent to the wooded area since 2012 when the leaf-litter and low growing vegetation was cleared from this area as part of fire-mitigation efforts by the Center.

In conclusion, this study found that hot spots of *T. cruzi* infection within the breeding colony from 1999-2018 correlate with areas of the local environment that are appreciated to be prime habitats for the insect vectors that transmit the parasite. The potential contribution of the horizontal route of transmission to the overall number of *T. cruzi* infections within the colony was determined to be 31.25% of infections, at most, and there was no evidence for the vertical route of *T. cruzi* transmission identified within the colony. Collectively, these data suggest that exposure to the triatomine insect vectors is the primary route of *T. cruzi* infection in rhesus macaques and that direct animal-to-animal transmission of *T. cruzi* is likely to be minimal concern for captive colonies in south Texas. The findings from this study are instrumental towards advancing the management practices of NHP biomedical research facilities and breeding programs in the southern United States by providing novel data as to the risks associated with maintaining seropositive animals in a research setting and by contributing to the literature in support of environmental modifications to limit the exposure of NHPs to infected triatomines.

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NOMENCLATURE

KCCMR	Keeling Center for Comparative Medicine and Research
DTU	Discrete typing unit
TAM	*T. cruzi*-associated monkey
CBC	Complete blood count
NHP	Non-human primate
MFIA	Multiplexed Fluorometric ImmunoAssay®
ELISA	Enzyme-linked Immunosorbent Assay
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
ECG	Electrocardiogram
SIV	Simian Immunodeficiency Virus
CRL	Charles River Laboratories

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Trypanosoma cruzi*

Trypanosoma cruzi (*T. cruzi*) is a kinetoplasid protozoan parasite that infects both invertebrate and vertebrate hosts during its life cycle and is the causative agent for Chagas disease. It is considered a genetically heterogeneous species that has a wide variability in biological and biochemical characteristics [1]. *T. cruzi* has undergone evolutionary changes leading to discrete and stable subdivisions, which is, in part, responsible for the geographical and epidemiological differences noted. There is compelling evidence that natural recombination in *T. cruzi* is quite frequent [2]. Two models have been developed as possible explanations of the evolution of *T. cruzi* – the two hybridization and the three ancestor models. Both models incorporate two hybridization events that have led to the modern classification scheme of discrete typing units (DTUs) TcI through TcVI. Potentially a seventh genotype, TcBat, has been found only in bats until recently. A single contemporary human case was discovered in a child living in Columbia in 2012, and was identified in the heart tissues of mummies from Chile [2-6]. Although neither hybridization model incorporates TcBat, phylogenic analyses point to the placement of TcBat in a distinct cluster, closer to TcI [3]. The two hybridization model begins with two independent clades (TcI and TcII) originating from a common *T. cruzi* ancestor. This theory suggests that an ancient genetic exchange event between TcI and TcII produced TcIII and TcIV, followed by a second more recent hybridization event between TcII and TcIII to yield both TcV and TcVI. In the three ancestor model, three independent clades (TcI, TcII, and TcIII) are thought to originate from a common *T. cruzi* ancestor followed by a second more recent hybridization event between TcII and TcIII to

yield TcV and TcVI [3]. While TcIV is not specifically depicted in the three ancestor model to have undergone hybridization, it is in the same mitochondrial clade as TcIII, TcV, and TcVI; however, further research is required to understand the history of TcIV [3].

Each DTU has distinct characteristics in its ecological niche, including different vectors, distribution, host species, and clinical forms of disease [7]. While all DTUs are found in Latin America, both TcI and TcIV have the largest geographical distribution extending into North America. TcI is most frequently sampled in both the domestic and sylvatic cycle and is the dominant DTU responsible for the transmission of Chagas disease in endemic countries located north of the Amazon basin [4]. Studies of the remaining DTUs have implicated involvement in either the domestic or sylvatic cycle. DTUs TcII, V, and VI are more likely to be associated with the domestic cycle found in Bolivia, Argentina, Chile, and Uruguay. Finally, DTUs III and IV are mainly acquired from samples in rainforest sylvatic cycles, and it has been noted that clinical forms of disease from these DTUs are rare in humans [4, 7].

The lifecycle of *T. cruzi* involves an arthropod vector – a triatomine insect – and mammalian host (Figure 1). In the stercorarian or 'vector-fecal' route of transmission, an infected triatomine insect vector takes a blood meal from a host and releases metacyclic trypomastigotes in its feces near or around the site of the bite. Metacyclic trypomastigotes then enter the host through the site of the bite wound or intact mucous membranes. Once through the skin barrier, the trypomastigote invades various nucleated cell types adjacent to the site of entry. Once in the cytoplasm, metacyclic trypomastigotes undergo differentiation into the intracellular amastigote form that can multiply by binary fission within the cells of infected tissues. Eventually, the amastigotes differentiate back into trypomastigotes that can then be released into the circulation as bloodstream trypomastigotes. The circulating trypomastigotes can either invade other tissues or

be ingested by a triatomine bug during a blood meal. In the midgut of the triatomine vector, trypomastigotes transform into epimastigotes, which is the main replicating stage in the invertebrate host. These epimastigotes multiply and differentiate in the midgut, then migrate to the hindgut where they will further differentiate into metacyclic trypomastigotes, which are eventually excreted in the feces [1, 8].

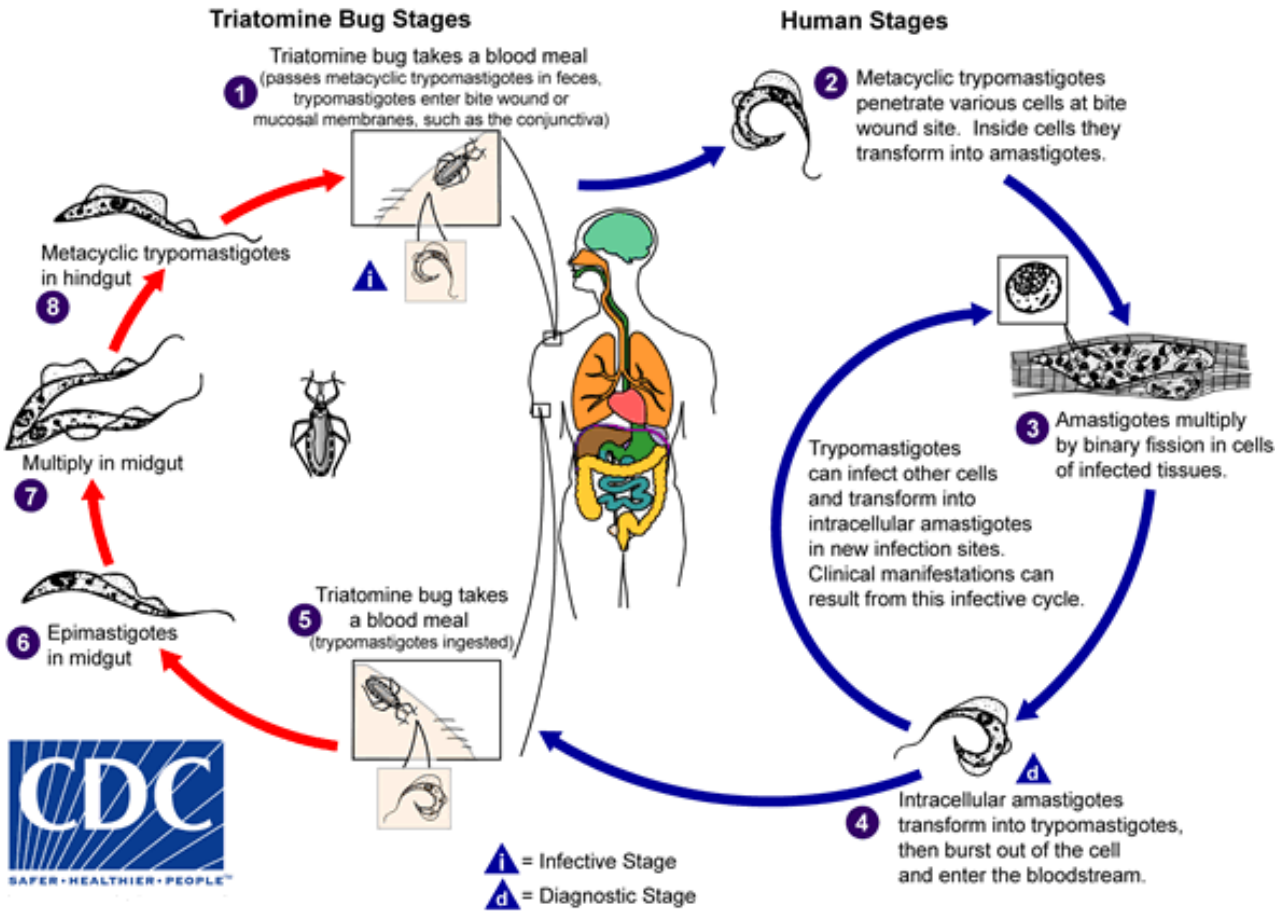

Figure 1. Life cycle of *Trypanosoma cruzi* with human host [8]. Figure courtesy of the Centers for Disease Control and Prevention's Division of Parasitic Diseases and Malaria (DPDM), Atlanta, Georgia.

1.2 Triatomines

Triatomine insects, commonly referred to as conenose, vampire, or kissing bug, are bloodsucking vectors found within the subfamily Reduviidae. There are more than 130 triatomine species in the Americas, many of which can be infected with and transmit *T. cruzi*. The two genera found in the United States are *Triatoma* and *Paratriatoma* [1, 9]. Eleven species of triatomine bugs, including *Triatoma gerstaeckeri*, *T. incrassata*, *T. recurve*, *T. rubida*, *T. rubrofasciata*, *T. sanguisuga*, and *Paratriatoma hirsute*, have been reported in the United States, inhabiting the entire southern half of the US, distributed from coast to coast. Eight of the eleven species of triatomine bugs have been known to harbor *T. cruzi* [1, 9]. In Texas, the two most abundant species of kissing bugs are *Triatoma gerstaeckeri* and *T. sanguisuga* [10].

All kissing bugs have complex life cycles that start from hatching into a wingless first stage, nymphs, which undergo molting through a total of five nymphal stages; each stage requires at least one blood meal in order to molt [9]. At the end of the fifth instar, the nymph will molt into a winged adult male or female that will continue to take blood meals. Triatomines often colonize the nests of rodents or small mammals, allowing for easy access to blood meals during the nymph stages. Triatomines can also colonize domestic environments. Accordingly, the high burden of Chagas disease in impoverished regions of Latin America may be attributed to poor quality housing, typically built with adobe, (i.e., unfinished brick) walls, and thatched roofs. These structures provide ample hiding places for the bugs during the day, and provide abundant meal sources for these nocturnal bugs [1].

In general, triatomines typically survive for a year, depending on feeding opportunities and climate [9]. Kissing bugs are primarily encountered within the United States from April to October, with their peak activity occurring in June and July [10]. During the summer, triatomines are most active and undergo dispersal flights at dusk in search of food or mates; a recent pilot field study

using radio telemetry found that a triatomine traveled as far as 20 meters, although triatomine dispersal is still poorly understood [9, 11]. Triatomines use multiple sensory systems to locate hosts and have been found to be attracted to both UV light and carbon dioxide exhaled by mammals. They are also able to detect infrared radiation, which allows them to orient towards hosts [9, 12]. Once a suitable host is located, kissing bugs will take a blood meal which, on average, lasts between 11 to 28 minutes depending on the insect species [9].

Differences have been noted between species of triatomines with regards to feeding and potential transmission of *T. cruzi*. Most human cases in Latin America involve kissing bugs taking a blood meal from the host and defecating, either in the bite wound or near mucous membranes, allowing for the spread of the parasite from their feces. However, a few studies of *Triatoma protracta* and *T. rubida* kissing bug species in the United States have shown that these species generally delay their defecation after feeding. This, in turn, makes them less efficient at transmitting the parasite as compared to their Latin American counterparts and thus breaking the transmission cycle [13]. Another interesting finding is that *T. cruzi* can induce behavioral changes within the triatomine. A study in Chile found that infected kissing bugs, *Mepraia spinolai*, were able to detect potential hosts faster, take faster blood meals, and defecate sooner after eating when compared to non-infected kissing bugs [14]. Although this modification in feeding behavior lends to the continued transmission of the parasite, it is important to note that this stercorarian transmission method has been shown in mathematical modeling studies to be very inefficient, with an estimated probability of transmission per contact with an infected triatomine to be 5.8×10^{-4} (95% CI: $[2.6, 11.0] \times 10^{-4}$) [15].

1.3 Chagas Disease Epidemiology in Humans

Although Chagas disease, commonly referred to as American trypanosomiasis, was not first characterized and studied until the turn of the century, parasite DNA has been found in mummified remains dating back 3-9,000 years ago in Peru [16]. Early evidence of the disease has also been discovered within the Rio Grande region of Texas in 2003. The mummified body of an adult male dating back to 1,150 BP (before present) via radiocarbon dating was found to have had an enlarged large intestine. Researchers were able to determine that the individual likely had Chagas megacolon as evidenced through the demonstration of *T. cruzi* DNA within the intestinal tissue [17]. This is the oldest known case of Chagas disease within the United States, demonstrating that *T. cruzi* has an extensive history of causing disease even in North America.

The Brazilian physician Dr. Carlos Chagas is credited with discovering the trypanosome in the hindgut of a blood-sucking insect while working in Brazil in 1909. Further investigation by Dr. Chagas led to the discovery of trypanosomes in the blood of a cat and that of a sick child that lived in the same dwelling [18]. Over the next few years, he was able to describe the pathophysiology of the disease in both acute and chronic infections in small mammals and humans. This early research and investigation by Dr. Chagas, has paved the way for further understanding of what could be considered one of the most devastating and often neglected tropical diseases of the Americas.

This parasite is endemic to the Americas with the ability to infect humans and non-human animals alike, creating a zoonotic disease potential. According to the Centers for Disease Control and Prevention (CDC), an estimated 8 million people are currently infected with *T. cruzi*, where the majority of cases are localized to Latin America [8]. The CDC estimates that there are currently 300,000 people infected with the chronic form of the disease living in the US [8]. While the vast majority of these cases likely became infected while traveling through parts of Latin America,

there have been a small number of case reports of autochthonous, or indigenous, infections occurring. A recent report detailed 28 autochthonous infections that have been documented within the United States from 1955 to 2015 [19].

1.4 Transmission Routes

Two transmission cycles have been described – the domestic and the sylvatic cycles. The domestic cycle results from direct human-vector contact, while the sylvatic cycle involves the interaction between the vector and wildlife. Although the domestic cycle is common in Latin America only, the sylvatic cycle plays a role in both Latin America and the United States. The most common route of infection in humans in Latin America is vector-borne transmission through fecal contact of an infected triatomine bug – stercorarian transmission. Infection occurs after the bug takes a blood meal and defecates in the bite wound or through fecal contamination of intact conjunctiva, or other mucous membranes [1]. As previously mentioned, the increased incidence in Latin America is thought to be linked to substandard living conditions, including the use of construction materials for housing such as thatch roof substrate, which provides excellent habitat conditions for triatomines. This route of transmission is not recognized to be common in North America, in large part due to differences in standards of living, but also because of species differences in both feeding and defecating practices that were previously described.

T. cruzi infections have been documented to occur through both blood and organ donation, congenital transmission, and on rare occasions from contaminated foods. The first documented case of transmission via blood transfusion was in 1952 [1]. In recent years, serologic screening for *T. cruzi* in blood donations has become standard practice. The American Association of Blood Banks reported in 2007-2018 that more than 2,400 people were confirmed positive through blood

donation screening [20]. Since 2000, case reports surfaced of transmission through organ donation in the United States. While instances of transmission have been associated with various organ types, it is thought that the risk from heart transplantations is higher than that of either the kidney or liver [21]. Any underlying cause of immunosuppression, as is medically induced for patients undergoing some types of organ transplantation, can exacerbate underlying or newly-acquired *T. cruzi* infections and result in severe manifestations of the disease [22].

Congenital transmission, or transmission passed from mother to fetus during pregnancy, is also of concern, especially in endemic areas where transmission occurs in about 5% of infants born to infected mothers [23]. Each DTU has distinct characteristics including clinical forms of disease. While all DTUs, except for TcIV, have been associated with congenital transmission, this type of transmission is more common when mothers are infected with TcII, TcV, or TcVI [7, 24]. While a woman may be chronically infected with *T. cruzi*, it does not necessarily mean that her baby will be born with a congenital infection. However, known factors that may enhance transmission during pregnancy include maternal parasitemia load, young maternal age, sustained vector exposure during pregnancy, coinfection with another immunosuppressive disease like HIV, maternal immune response, twin pregnancy, and the influence of genetic diversity of *T. cruzi* [20]. When congenital transmission does occur, the data suggests this occurs during the second or third trimester of pregnancy in either an acute infected or chronically infected mother. Approximately 10 to 40% of congenitally infected infants have clinical findings of Chagas disease at the time of birth or within the first few weeks of life. Of those, 5% of infants can have severe forms of the disease, including meningoencephalitis and myocarditis, ultimately leading to death [20]. Interestingly, congenital transmission has also been discovered to occur in infants whose mothers were congenitally infected themselves, in the absence of a vector [1, 20]. While the majority of

congenitally infected individuals remain clinically healthy throughout infancy, it is important to note that 20 to 40% of those individuals who are clinically normal at birth can develop cardiomyopathy at a later stage in life, in most cases decades later, leading to irreversible heart damage and disease [20].

Previous and early work with *T. cruzi* in animal models have demonstrated the presence of trypomastigotes in the milk of acutely experimentally infected mice and rats [25-27]. However, no animal studies have been able to demonstrate transmission from infected animals to their offspring via the ingestion of milk [27]. In humans, there have been two documented cases of possible breast milk transmission [27, 28]. In one of the cases, the transmission was thought to have occurred through the ingestion of blood through cracked skin surrounding the nipple, as no parasites were found in the milk. In the other case, transmission via breast milk ingestion was determined by the process of elimination of all other routes of transmission [27, 28].

Sporadic outbreaks of Chagas disease have been reported throughout Latin America in association with contaminated food and drink. Most of the reported outbreaks are small; however, at least one large outbreak occurred at a school in Venezuela that was linked to the ingestion of contaminated guava juice [29]. Just over 100 people, including both students and teachers, were confirmed to have antibodies to *T. cruzi*. Oral transmission of *T. cruzi* can occur circumstantially by ingestion of foods and/or beverages contaminated with feces, crushed bugs, and the secretions from anal glands of infected opossums (*Didelphis marsupialis*) [30, 31]. Experiments demonstrated that mice can become infected with *T. cruzi* when they are fed food contaminated with excrement from opossums [32]. Another hypothesized source of transmission is via undercooked animal meat or blood from contaminated animals [30, 32]. It has been proposed that oral transmission may be significant for infecting dogs in North America [33].

1.5 Clinical Disease in Humans

Regardless of the route of transmission, there are two phases of Chagas disease – an acute phase and a chronic phase. Some researchers point to a "third phase" referred to as the "indeterminate" phase. This phase occurs between the acute and chronic phases, in which the affected individuals are asymptomatic, but show parasitologic and/or serologic evidence of persistent infection [34]. The CDC describes the phases of the disease simply as acute and chronic indeterminate. The acute phase occurs after infection and is characterized by the presence of circulating trypomastigotes that can be visualized in peripheral blood or buffy coat smears. While this acute phase can last up to 3 months, most individuals remain either asymptomatic or present with mild, non-specific, self-limiting febrile illness. In some cases, individuals may present with a focal skin lesion called a chagoma or may exhibit prolonged eyelid edema called Romana's sign. The potential for each of these two early disease manifestations is determined by the site of inoculation from an infected triatomine [7, 35]. Generally speaking, the acute phase is not fatal; however, in rare circumstances, the acute phase may result in severe myocarditis or meningoencephalitis, which is more commonly seen in immunosuppressed patients or infants. Clinical signs in the majority of acute-phase cases will spontaneously resolve, even in the absence of treatment [7].

Following the acute phase, patients enter a chronic phase in which there is no detectable parasite in the peripheral blood. This is due to the fact that the parasite enters into a dormant stage within the tissues, predominantly in cardiac and other muscle tissue. During the chronic phase, most infected individuals remain asymptomatic, making them unaware of the infection, and remain clinically unrecognizable for life [7]. However, approximately 20-30% of chronically infected individuals will develop severe, life-threatening diseases involving the cardiac or gastrointestinal

systems over a period of 10 to 30 years [7, 8]. Evidence suggests that disease progression is likely a result of the inflammatory immune response of the host as well as *T. cruzi* strain virulence and/or genotype, as some discrete typing units of *T. cruzi* have tropisms for specific tissues [7, 35].

The most commonly identified clinical sequela, seen in 20-30% of chronically infected patients, is chronic chagasic cardiomyopathy. Early stages of chronic chagasic cardiomyopathy (CCM) are characterized by conduction abnormalities that eventually progress into ventricular tachycardia, heart blocks, and progressive dilated cardiomyopathy with congestive heart failure [7]. It is not uncommon for patients with CCM to experience sudden death.

Other less common sequelae of the chronic phase of Chagas disease involves the gastrointestinal tract. While gastrointestinal megasyndromes like megaesophagus and megacolon are uncommon, this manifestation of the disease can result in denervation, decreased motility, sphincter dysfunction, and eventual dilation of the gastrointestinal tract [7]. Involvement of the gastrointestinal tract is more commonly seen in Argentina, Bolivia, Chile, Paraguay, Uruguay, and parts of Brazil; again, this geographic distribution is thought to be due to differences in genotypes or strains of *T. cruzi* [35].

Individuals in the chronic phase of the disease are at risk for reactivation. Reactivation of disease is the reoccurrence of acute symptoms that occurs when the immune system of a chronically infected host is interrupted or suppressed, resulting in the reduced ability of the host to control the *T. cruzi* infection [36]. This has been reported to occur in infected individuals undergoing immune suppression for organ transplantation or who are immunocompromised due to an underlying disease like HIV/AIDS. A characteristic of reactivation is increased parasite multiplication and a return to detectable parasitemia at a level often higher than that of immunocompetent individuals [7]. In addition to acute Chagas myocarditis, other manifestations

of reactivation in organ transplant patients include inflammatory panniculitis and skin nodules. In HIV/AIDS patients, the most common organ system affected is the central nervous system (CNS). Pathologic findings include meningoencephalitis as well as *T. cruzi* brain abscesses that can be confused with CNS toxoplasmosis [1]. Other less common manifestations in HIV/AIDS patients include skin lesions and parasitic invasion of the peritoneum, stomach, or intestine [35].

People living in endemic areas are at an increased risk for reinfection. Researchers have shown that reinfection during the chronic phase in mice can cause more severe myocardial lesions; however, these cardiac changes were not seen in experiments of reinfection in the dog [37, 38]. In humans, reinfections are thought to have important effects on cardiomyopathy and the probability of congenital transmission, but little is known about the frequency of reinfection occurrence [39]. It has been proposed that reinfections during the chronic phase of the disease are due to a lack of adaptive immunity from the primary infection. Epidemiological modeling has been used to determine an estimate of the frequency of reinfection in endemic areas, specifically the Chaco province of rural Argentina. In this study investigators found that, on average, 3% (0.8%-5%), 14% (4%-18%), and 60% (21%-70%) of individuals were reinfected after 1, 5, and 30 years of the chronic disease phase, respectively [39]. Reinfection modeling has predicted that 20-30% of chronically infected individuals who develop cardiac disease are likely suffering from the highest reinfection rates. This subset of chronically infected individuals suffering from cardiac disease undergoes 1 to 2 reinfections every ten years [39].

1.6 Current Diagnostics

There are a number of different types of diagnostic assays utilized to detect exposure to, or infection with, *T. cruzi*. The optimal assay for diagnosis of the condition varies depending on the

phase of the disease; however it is widely accepted that individuals with antibodies are interpreted as currently being infected as self-cure is thought to be rare. The CDC provides guidance for health care providers concerning Chagas' disease diagnostic testing [1]. In the acute phase, the presence of circulating trypomastigotes within a fresh preparation of blood smear is diagnostic. Trypomastigotes may also be seen in the buffy coat or (in an acute phase infection with CNS involvement) in the cerebrospinal fluid. Another diagnostic assay used for *T. cruzi* diagnosis in the acute phase is polymerase chain reaction (PCR). This highly sensitive amplification technique allows for the detection of a low quantity of target *T. cruzi* DNA in the blood. PCR is also used to test for early infection following organ transplantation or after an accidental exposure [35, 40]. Hemoculture provides an additional testing option for acute phase diagnosis. The hemoculture process involves centrifugation of the blood sample to remove leukocytes that can inhibit the parasite, and enriched media to support the development and multiplication of existing parasites within the blood sample [41, 42]. This diagnostic test utilizes one of several types of standard parasitic media and is relatively sensitive during the acute phase; however, this testing requires 2 to 4 weeks to show replication [1].

Typically chronic phase infections rely on anti-*T. cruzi* antibody (IgG) serologic testing for diagnosis. PCR is not generally a useful test during the chronic phase as once the parasite enters into the host tissues as amastigotes, there is little to no circulating parasitic DNA for the PCR assay to detect. For this reason, negative PCR results do not constitute evidence of a lack of infection [1, 35]. Currently, there is no single gold-standard diagnostic test. The general consensus in the literature and current CDC recommendations state a positive result of two or more serological tests in parallel, preferably based on different antigens (whole parasite lysate and recombinant antigens, for example) are required for confirmation [1, 7, 35]. Diagnostic tests are generally run in parallel

or sequentially, starting with a screening test. Screening tests have high sensitivity and are done first to try and identify as many potential seropositive individuals as possible. Many rapid screening tests yield a qualitative result of either a suspect positive or negative. Screening tests are often followed up with confirmatory tests that have a higher specificity. The use of a two-step testing method is thereby much less likely to produce false-negative and false-positive results. The most common diagnostic tests used for humans are enzyme-linked immunosorbent based (ELISA) and immunofluorescent antibody (IFA) assays. By contrast, the CDC utilizes an FDA-approved enzyme immunoassay (EIA) that is based on recombinant *T. cruzi* antigens alongside an immunoblot, trypomastigote excreted secretion antigen (TESA), as their first-line tests for human disease [8].

Regardless of the serological assay used for testing, discordant results can occur in individuals. False-negative results can occur in infected individuals who have low antibody titers which are undetectable by less-sensitive tests. Additionally, false-negative results can occur if a patient has abundant antibody production, but the antibody being produced is specific to a strain/genotype of *T. cruzi* for which the assay has not been validated. This can occur due to the fact that sensitivity and specificity of serological assays can vary by geographic location, as a result of differences the immunological response to the various genotypes of *T. cruzi* [1, 7]. False-positive test results can occur when there is a cross-reaction with antigens from other parasites, in particular those of the trypanosomatid genus such as *Leishmania* that has many genetic similarities with *T. cruzi* antigens [1, 43]. Because there is no gold-standard assay for *T. cruzi*, it can be extremely difficult to diagnose in some individuals in the chronic phase of the disease. In some cases in both humans and animals, confirmation is based on tissues obtained during necropsy [1, 44].

In cases of disease reactivation to the acute phase, quantitative PCR assays like real-time PCR (RT-PCR) are useful to monitor patients. Quantitative RT-PCR testing can not only indicate a rising parasite number over time but is also more sensitive than conventional PCR [1]. According to CDC recommendations, molecular testing can also be used for suspected acute infections in the cases of transfusion and transplantation transmission, as well as for congenital Chagas disease diagnosis [8].

In 2013, computer-simulated models estimated that the total annual cost and burden of Chagas disease globally is approximately $627 million [45]. Newer models are able to estimate the costs associated with identification, diagnosis, treatment, and chronic disease costs and measured health effects [46]. Being able to identify and treat Chagas disease in earlier stages can potentially result in reduced transmission, better health outcomes, and cost savings of nearly $2.6 million in a single 2,000-person village in Latin America [46].

1.7 Treatments

Current treatment recommendations set forth by the CDC take into account the age of the patient and the phase of the disease. Treatment is advised for all of the following: individuals in the acute phase, those experiencing reactivation of the disease, congenitally infected babies, immunocompromised individuals, and chronically infected individuals that have not had any advanced heart disease. In Latin America, cure rates as high as 76% of acute-phase infections, 96% of congenitally infected infants, 62% of chronically infected children, and 37% chronically infected adults have been reported [47]. The use of the term “cure rate” in these studies may be misleading because there is not currently a gold standard testing method to determine infection status. As can be seen above, treatment has been shown to have a lower effectivity in chronically infected adult individuals compared to chronically infected children [47, 48].

Prior to 2017, there was no FDA approved treatment in the United States. However, the FDA has since approved the use of benznidazole, a nitroimidazole antimicrobial drug, labeled for use in pediatric patients 2 to 12 years of age [8]. This drug is often considered the first line of defense and works by inhibiting the parasite's ability to replicate DNA and is shown to be effective against both the trypomastigote and amastigote forms [47]. One other drug that is used outside of the United States is nifurtimox, a nitrofuran compound; this drug is currently under investigational use in the United States. Adverse effects of treatment from both drugs are common, although children seem to be more tolerant of higher doses and exhibit fewer side effects. Common side effects include nausea, vomiting, anorexia and weight loss, insomnia, and dose-dependent neuropathies. Hypersensitive skin reactions are one of the most commonly reported side effects, occurring in approximately 30% of patients treated with benznidazole [49]. There are some contraindications for the use of both these drugs in pregnant women and individuals with underlying liver or kidney disease, as these drugs are known to cause congenital abnormalities and the worsening of underlying disease, respectively. The use of both of these drugs in experimental animal studies have shown an increased prevalence of lymphoma. However, this increased risk has only been seen in a small subset of heart transplant recipients in humans [50].

The recommended clinical monitoring for patients with chronic phase *T. cruzi* infection includes an electrocardiogram, echocardiogram, 24-hour Holter monitoring, and thoracic radiographs (both at the time of diagnosis and annually thereafter) [47]. Chronically infected individuals that have started to display cardiac disease should not be treated with either benznidazole or nifurtimox due to an increased risk of side effects. Instead, these individuals are offered supportive care in the form of cardiac medications, pacemaker implants, and transplantation. In certain regions of Latin America, up to 30% of patients with *T. cruzi* infections

can exhibit both cardiac and digestive complications. In these cases, megaesophagus and/or megacolon may require surgical correction [47].

1.8 Disease in Other Animals

In addition to humans, numerous animal species are susceptible to T. *cruzi* infections. Since Carlos Chagas' discovery of *T. cruzi* in the domestic cat, there have been over 100 mammalian species reported to be natural hosts for *T. cruzi*, including at least 26 species of wildlife hosts in the United States [1, 51]. The broad host range of *T. cruzi* can serve as a reservoir for the parasite. This reservoir can potentially spillover, contributing to infections in other species like captive nonhuman primates (NHP), dogs, and humans here in the United States [51]. There are noted differences in primary reservoirs and transmission dynamics of *T. cruzi* by geographic location, even within the United States [1].

Naturally-infected animals within the United States range from the common house mouse (*Mus musculus*) to larger mammals like foxes (*Urocyon cinereoargenteus*) and coyotes (*Canis rufus*). While essentially all mammals are considered susceptible to infection, birds, reptiles, and amphibians are refractory due to their complement-mediated lysis and macrophage-induced killing of the parasite [1]. In the western United States, woodrats of the genus *Neotoma* serve as the most common reservoir; however, in the eastern United States, the prevalence of *T. cruzi* is highest in raccoons (*Procyon lotor*), opossums (*Didelphis virginiana*), armadillos (*Dasypus novemcinctus*), and skunks (*Mephitis mephitis*) [1]. In contrast to larger carnivorous mammals, smaller mammals such as woodrats are thought to become primarily infected through stercorarian transmission, as many triatomine species can be found living in the nests of woodrats. In addition, the insectivorous behavior of many animals like the woodrat and raccoon further increases the likelihood of infection

via the ingestion of infected bugs [1, 52]. While opportunistic carnivores feed on small mammals, some also feed on insects, and it is therefore probable that these animals are primarily infected via the oral route [53].

As seen with humans, not all infected wildlife show clinical manifestations of the disease. A recent study surveyed cardiac tissue and blood from hunter-harvested wildlife carcasses in central Texas in order to determine infection prevalence and to investigate the manifestation of cardiac pathology; results showed that while 21% of the PCR positive hearts were characterized by mild lymphoplasmacytic infiltration, no other lesions or amastigotes were observed in histologic sections [52]. It could be argued that the most severely-affected wildlife would not have been represented in a study that relied on hunter-harvests, given the need for animals to be active in order to be represented in the sample set. Regardless, it is known that different genetic strains of *T. cruzi* infect different species of wildlife, which may, in fact, lend to the differences in clinical manifestations of the disease. The two predominant strains of *T. cruzi* found within the United States are TcI and TcIV. In the United States, opossums and coyotes are predominately infected with TcI, raccoons infected with TcIV, and skunks are routinely identified to be infected with both DTUs [51, 52].

Domestic animals living within the same environment as these natural wildlife reservoirs are also at risk for the disease. Notably, the domestic dog can be asymptomatic or develop both an acute phase and a chronic phase of disease similar to what is seen in humans. Acute-phase mortalities have been seen in younger dogs and usually involve myocarditis and cardiac arrhythmias. Young dogs that survive the acute phase, along with adults that acquire infection with *T. cruzi*, may end up progressing to significant cardiac disease, typically involving dilation, electrocardiogram abnormalities and eventually, cardiac failure [1]. Dogs are more likely to

become infected with *T. cruzi* compared to humans throughout the Americas. Dogs can become infected through consuming the bug or being bitten while in their kennels, as kissing bugs are known to inhabit dog kennels [51, 54]. Supportive of this proposition is the finding that government working dogs that are housed in outside kennels are found to have a higher seroprevalence of *T. cruzi* compared to those dogs that are housed indoors [54].

1.9 Non-human Primates (NHP) and Chagas Disease

Non-human primates (NHP) play an important role in the sylvatic cycle of *T. cruzi* transmission in Latin America. It has been documented that some free-ranging NHP species play an important role as sylvatic hosts for *T. cruzi* [53, 55]. The natural habitation of these arboreal New World primates lends to their involvement as a reservoir as triatomines can be found within the trees and leaves. Some reports of naturally infected free-ranging lemurs (*Lemur catta*) and lion-tailed macaques (*Macaca silenus*) on St. Catherine's Island, Georgia, indicate that NHP can be asymptomatic like humans; although it is unknown if these animals will eventually develop cardiac disease [56, 57]. While there are no indigenous species of NHP found in the United States, there are a large number of primates housed in the United States for research purposes. Although little is known about the interface between wildlife and NHP within the United States, a recent surveillance study in Texas found that 80% of mesomammals (opossum, raccoon, and skunk) caught around an outdoor NHP breeding facility had *T. cruzi* DNA circulation in their blood [55]. The study also found high concentrations of *T. cruzi* DNA in the blood of these mesomammals, which suggests these species could serve as a source of infection to kissing bug vectors [55].

As a result of the warm weather and mild winters present in the southern United States, most of the NHP facilities in these locations utilize open-air housing systems for their animals

such as corncrib cages, corrals, and indoor-outdoor cages. While this form of housing is behaviorally beneficial to the animals, this type of housing also exposes the NHP to the same environment as the naturally infected wildlife reservoirs. In the United States, natural *T. cruzi* infection of outdoor housed NHPs have been reported since the 1970s [58]. Previously published surveys cite infection prevalence ranging from 2 to 10% in NHP facilities in the southern United States [55, 58, 59]. It has also been documented that NHP can be naturally infected with TcI and TcIV, the two most common *T. cruzi* strains found within wildlife reservoirs within the United States [55, 58, 59].

Reports have demonstrated a variety of disease outcomes in NHP, similar to those seen in humans, and the disease outcomes have been characterized as occurring in the acute, indeterminate, and chronic phases. Notably, chronic disease outcomes have been described as consisting of cardiac disease and, to a lesser extent, gastrointestinal disease [55, 59-68]. A subset of naturally infected baboons at the Southwest National Primate Center developed echocardiographic evidence of disease. Consistent with findings in humans, the primary finding in these animals was a nonspecific, lymphocytic myocarditis and tissue amastigotes were rarely seen [60, 64]. ECG and cardiac abnormalities have likewise been noted in a variety of both naturally and experimentally infected primates, including macaques, and chimpanzees [63]. Other case reports have demonstrated reactivation of Chagas disease and circulating parasitemia in NHP after immune compromise and suppression, either through experimental SIV infection, the use of corticosteroids, or tissue transplantation [59, 65-67]. There have been other documented cases of coinfection or immunosuppressant therapies that brought about acute and unusual clinical manifestations of Chagas disease such as cutaneous lesions, involvement with the central nervous system, or severe cardiac lesions similar to what has been reported in humans as well [3].

It is speculated that the most common route of transmission in NHP within the United States is through consuming infected triatomines. Macaques are known to actively forage for insects and have been observed not only handling, but also consuming triatomine bugs [56, 57] (unpublished data Hamer/Wilkerson).

While congenital transmission is well known in humans, there has been limited supporting evidence of it occurring in NHP. A single case report of congenital transmission in a squirrel monkey has been documented, which resulted in a live birth in 1975 [69]. In the case report of the squirrel monkey, the presence of trypanosomes was seen in a lymphocyte culture preparation from the offspring, warranting further investigation. *T. cruzi* like organisms that were identified on blood smear and by xenodiagnoses were used to infect both mice and VERO tissue culture cells. Notably, the dam and offspring in this case remained clinically normal and serological testing carried out by the CDC was inconclusive [69]. In addition to the lack of literature documenting congenital transmission in captive US populations, further support for the rarity of congenital *T. cruzi* infections in NHPs is the observation from wildlife researchers in the Atlantic coastal rainforest of Brazil that *T. cruzi* infection has not been identified in juvenile tamarins despite its prevalence in the adult animals from this area [53, 70]. A single study has suggested the possibility of congenital transmission after finding a mother-daughter pair of baboons was infected with *T. cruzi*, yet, it is unclear if this was due to maternal antibody interference since there was a lack of follow up testing for these animals [68].

It is also known that *T. cruzi* transmission can occur in humans through blood transfusions and organ transplantation procedures. While primates occasionally receive blood transfusions as part of clinical treatments and are routinely utilized for xenotransplantation studies, the potential for horizontal transfer of *T. cruzi* in research animals through these methods is likely minimal in

the United States. This is due to the fact that as awareness of the prevalence of *T. cruzi* infections in NHPs has become more recognized, it is becoming standard practice that blood and tissue donors get screened for *T. cruzi* prior to being confirmed as donor animals.

The fact that NHP can be infected naturally with *T. cruzi* carries severe implications for the NHP used in biomedical research, especially those being housed in outdoor facilities in the endemic southern United States. As previously mentioned, NHP are utilized for transplantation studies and HIV research in which they undergo immunosuppression. If a *T. cruzi* infected animal was used in these types of studies, reactivation of the parasite could easily confound the data from this research [59, 67]. Even in studies where immune suppression is not expected, such as most drug-safety trials, mild lesions associated with either the acute or chronic forms of Chagas disease have the potential to imply side-effects to the drugs being studied where none actually exist. Although disease implications are extremely important for breeding colonies in the southern United States where *T. cruzi* is endemic, the disease can potentially impact animals being used in research throughout the country. Animals in research are frequently sold and transported from other facilities, many of which are breeding colonies located in the South. There are case reports in the literature demonstrating this, one of which describes two geriatric NHP that originated from Texas displaying clinical signs of disease of *T. cruzi* years after being transported to a facility in the northwest United States [65].

The potential complications that Chagas disease can create for biomedical research emphasizes the importance of identifying and characterizing *T. cruzi* infected NHPs. While there may be a niche for seropositive NHP in biomedical research for further understanding Chagas disease and potential therapies, there is anecdotal information to suggest that most seropositive animals at facilities are being removed from colonies and breeding programs due to the potential

of horizontal transfer to other NHP. However, the actual risk posed by seropositive animals has not yet been characterized [55].

Further investigation into *T. cruzi* transmission within NHP is warranted and can potentially impact the future of NHP colony management. Specifically, if neither vertical nor horizontal transmission of the disease is identified to occur commonly amongst NHP then, the number of seropositive animals being culled from breeding colonies can likely be drastically decreased as these animals may continue to be valuable breeders.

For the project described within this thesis, we identified a subpopulation of *T. cruzi* seropositive rhesus macaques (*Macaca mulatta*) housed in an outdoor breeding colony in Texas. We determined the location of each seropositive animal within the colony at the time of their infection and also identified all animals that had ever been housed with seropositive animals, including any offspring of seropositive dams. These animals housed with seropositive animals were deemed to be *T. cruzi*-associated monkeys (TAMs). The serostatus of all TAMs was determined through medical records or from the retrospective testing of archived serum samples. We used this data to look for associations within and between sexes that could support horizontal transfer, vertical transfer, or implicate environmental factors. Trees and elevated storage sheds within the facility were both evaluated as potential environmental factors of influence on the risk of seroconversion though the use of spatial analysis.

Based on anecdotal observation, our three hypotheses for this study are as follows: (1) horizontal transfer is not a significant contributing risk factor for the spread of *T. cruzi* within outdoor-housed NHP in the southern United States, (2) vertical transfer is not a significant contributing risk factor for the spread of *T. cruzi* within naturally-infected NHP in the southern

United States, and (3) environmental factors do play a role in incidence *T. cruzi* infection within NHP breeding colonies of the southern United States.

CHAPTER 2
MATERIALS AND METHODS

2.1 Breeding Colony Animals

This study utilized data collected over 20 calendar years (1999 to 2018) from an Indian-origin, rhesus macaque (*Macaca mulatta*) breeding colony. The breeding colony was housed at the AAALAC accredited NHP facilities at The University of Texas, MD Anderson Cancer Center, Keeling Center for Comparative Medicine and Research (KCCMR) in Bastrop, TX. The annual census of the KCCMR rhesus colony over this time period ranged between approximately 750-1100 animals. The average female-to-male ratio of the entire colony through this same time was approximately 1.9:1, with an adult animal sex ratio of roughly 4.2:1 and an immature animal (animals less than 3 years of age) sex ratio around 1.1:1. The breeding colony has been considered a closed colony since 1983 in that 1982 was the last year animals born outside the colony were acquired for use in the breeding colony. Since 1991, the colony has been documented through serological means to be Specific Pathogen Free (SPF) for Macacine Alphaherpesvirus 1, Simian Immunodeficiency Virus, Systemic T-lymphotrophic Virus, and Simian Retroviruses 1, 2, and 5.

The cleaning, sanitation, transport, and feeding protocols of the Center have remained fundamentally consistent throughout the entire 20-year period of the study. Animals were fed a commercial monkey chow and provided daily enrichment in the form of fresh fruits, vegetables, and a variety of foraging materials (cereals, seeds, etc.). Water was provided ad libitum via automatic watering systems.

Throughout this 20-year period, the breeding groups typically consisted of between 5 to 10 adult females and one adult male. These groups were routinely reorganized every 5-6 years in efforts to promote the genetic diversity of the breeding colony as a whole. As per the standard

husbandry protocols of the Center, infants remained within their breeding groups until weaning age at approximately 7 months of age, at which time they were placed within a larger group of other weanling animals. These weanling animals remained housed together throughout their entire juvenile-stage of development or until they were sold. In general, the female juvenile animals that remained in the colony were placed into their first breeding groups at 3-4 years of age, while male juvenile animals that remained in the colony were placed into their first breeding groups at 5-7 years of age.

Starting at weaning age, each animal was given an annual physical examination to include routine blood work and serological screening. As rhesus macaques are seasonal breeders, the annual health examinations were consistently undertaken in late winter through spring each year in order to coincide with the weaning of infants and also to allow for these examinations to be performed during the early pregnancies of most adult females. Any serum obtained from these annual physical examinations that had not been utilized for diagnostic purposes was frozen at -80°C and archived. All routine husbandry, health monitoring, and serum archiving protocols were approved by the University of Texas, MD Anderson Cancer Center's IACUC, and followed the NIH standards established by the Guide for the Care and Use of Laboratory Animals [71].

2.2 Breeding Colony Facilities

The breeding colony was housed within a 381 acre, partially wooded, campus in Bastrop County in central Texas. The majority of the animals included in this study resided within a chain-linked fenced, 3.6-acre area identified as the 'main rhesus compound' (Figure 2). Housing within the main rhesus compound included 8 corncrib caging structures and 8 animal buildings with each of these buildings partitioned into 10 to 12 individual rooms around a central corridor.

Each corncrib structure housed a single breeding group and provided 360^{o} open-air access to the environment from late spring through early fall each year. Each of the rooms within the animal buildings housed either a juvenile group of animals, a single breeding group, or a group of single-sexed adult animals awaiting placement into breeding groups or sales. Between late spring and early fall of each year, the outside-facing barred walls of these rooms provided open-air access to the environment. Protective retractable panels were placed over the barred walls of these rooms between late fall through early spring each year. These panels were kept fully closed at night and on colder days but were partially retracted during the daytime hours as weather permitted. A second fenced area approximately 0.33 km north of the main rhesus compound was also used to house rhesus macaques prior to early 2018. This 0.56 acre 'north rhesus compound' contained two additional 12-room animal buildings identical to those of the main rhesus compound. These two buildings housed groups of animals similar to those described for the main rhesus compound buildings and likewise had retractable panels applied to their outside walls during the colder months of the year.

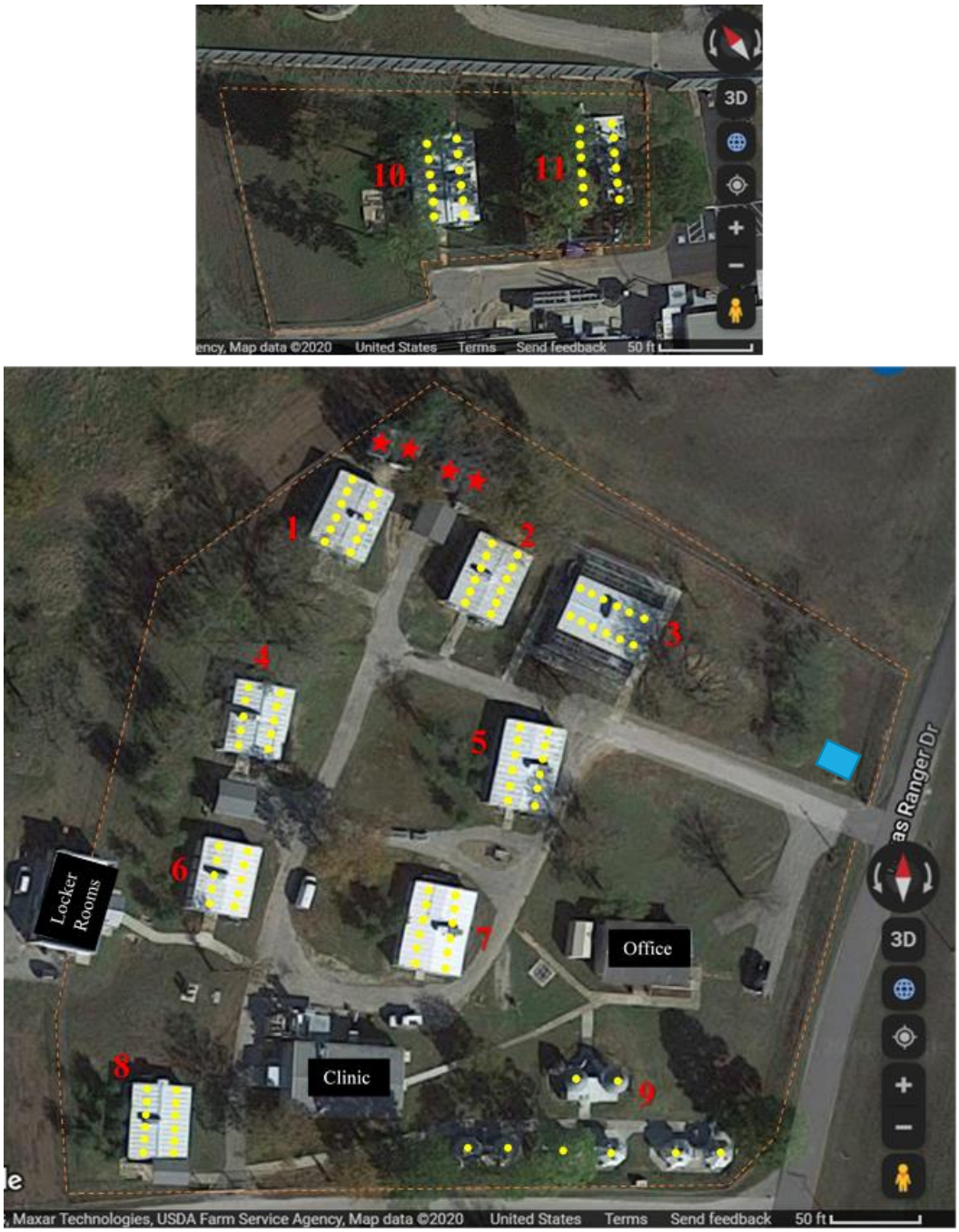

Figure 2. Satellite image of a portion of the KCCMR illustrating the two rhesus macaque colony compounds with animal building numbers labeled in red. The main rhesus compound (lower image) contains Buildings 1 through 8, the 8 corncrib structures (collectively referred to as 'Building 9'), a veterinary clinic, an administrative office, and a laundry/locker facility. Four elevated storage sheds are also located within the main rhesus compound (denoted with red stars). The previous location of a plant box of interest is denoted by the blue box in the northeast corner of the main rhesus compound. The north rhesus compound (upper image) contains Buildings 10 and 11. Yellow circles denote the center of each individual corncrib structure and also denote the individual rooms contained within each animal building. The fenced boundary of each compound is denoted with dashed orange lines. Photo credit: Map data © 2020 Google, Maxar Technologies.

Over the 20 years encompassing this study, the environmental features of the north rhesus compound remained essentially unchanged other than the continued growth of mature trees. In contrast, the main rhesus compound underwent several significant alterations to its environment over this same time period. The earliest of these environmental changes occurred between late 2001 and the middle of 2002 with the removal of approximately 6-10 mature trees and the construction of Building 3 within the main rhesus compound (Figure 2). Between late summer 2011 and spring 2012, a second alteration to the compound occurred as the KCCMR undertook intensive fire mitigation activities in response to wildfires that threatened the campus during the summer of 2011. Of possible significance here is that these activities included the cutting back of mature trees and the removal of the vast majority of ladder fuel (young trees, shrubs, and leaf litter) from the wooded area directly south of the main rhesus compound. Two more environmental changes began in late 2011 and continued through 2012. These included the construction of the laundry/locker room building on the west side of the main rhesus compound and the addition of concrete sidewalks around the corncrib housing structures. In the early fall of 2016, another environmental change occurred with the removal of several ornamental plant beds from the main rhesus compound. This activity was undertaken due to the fact that some of these structures had been identified as potential habitat/nesting sites for triatomine bugs. While most of these plant beds were small and contained little vegetation, there was one larger plant bed (approximately $15m^2$) of potential relevance to this study. This planter bed, located in the northeast corner of the main rhesus compound, had dense ground cover consisting of a cactus, various perineal plants, and a thick layer of leaf litter.

2.3 Serodiagnostic Assays

Two distinct commercially-available serodiagnostic methods were utilized to identify each animal as seropositive for *T. cruzi* antibodies for the purposes of this study. The first of the commercially-available testing methods involved a two-step process utilizing a suspension microarray (Macaque Chagas-Multiplexed Fluorometric ImmunoAssay® [MFIA], Charles River Laboratories, Wilmington, MA) followed by ELISA testing, as previously reported [55, 72]. For this testing method, frozen serum samples were thawed and homogenized at the KCCMR prior to being processed according to the manufacturer's instructions. In short, the beads, test sera, and assay reagents were added to a 96-well plate, incubated, and filter washed prior to being processed in a plate reader (Bio-Plex 200, Bio-Rad, Hercules, CA). Samples that were identified to be positive at the KCCMR were then shipped refrigerated overnight to the Charles River Laboratories, where a second MFIA test, identical to the first, was performed by the commercial laboratory. Samples confirmed to be positive by this second MFIA assay were subsequently tested at Charles River Laboratories using a proprietary ELISA assay. Samples were only identified to be seropositive by this method if all three tests confirmed seropositivity to *T. cruzi*.

The second of the commercially-available testing methods utilized a rapid, bench-top screening assay (Chagas Stat-Pak Assay®, Chembio Diagnostic Systems INC, Medford, NY) as previously reported [72]. This assay is a single-use immunochromatographic screening test for the detection of *T. cruzi* antibodies in human blood, serum, or plasma. For this testing method, frozen serum samples were thawed and homogenized at either the KCCMR or at Baylor College of Medicine prior to being processed according to the manufacturer's instructions. In short, 5 μL of serum followed by 6 drops of the diluent were placed into the sample well of the test device, and the results were read after 15 minutes of incubation. The presence of any line (regardless of

the strength of the line) in the prescribed testing area of the device was identified as a positive result.

2.4 Identification of Seropositive Animals and Year-of-Infection

As previously reported, mass serosurveillance of the KCCMR breeding colony animals for *T. cruzi* antibodies was first undertaken in 2013 and was performed a second time in 2015 with approximately 66% and 85% of the resident colony animals included in these two surveys, respectively [72]. Retrospective serological testing of archived serum samples was then undertaken in efforts to determine the *T. cruzi* serostatus of the remaining breeding colony animals present at the Center between the years 2012 through 2015. Between the years 2016 and 2018, *T. cruzi* serosurveillance was performed on all KCCMR breeding colony animals annually as part of their standard health examinations. Collectively these efforts resulted in the serological screening of 1899 of the 2166 animals that were housed at the Center between 2012 and 2018. No archived serum samples had been obtained from the remaining 267 animals prior to their departure from the colony.

In addition to the *T. cruzi* seropositive animals identified through the 2012-2018 colony surveys, a number of other animals that died or were sold from the breeding colony prior to 2012 had also been identified to be seropositive for *T. cruzi*. Specifically, archived serum samples had been screened for *T. cruzi* for the following purposes: to identify the seroprevalence of *T. cruzi* within the 2003 rhesus macaque breeding colony; to investigate the seroprevalence of *T. cruzi* in association with other disease states in the KCCMR rhesus macaques; and to support the diagnosis of Chagas disease in necropsied animals found to have histological lesions suggestive of chronic *T. cruzi* infection.

Once animals were identified to be *T. cruzi*-seropositive, the earliest year of *T. cruzi*-seropositivity was also determined for each animal, where possible. The earliest year of seropositivity was already established for the animals that seroconverted between 2013 and 2018 as a result of the data collected through the colony serosurveillance efforts. For animals that seroconverted prior to 2013, the earliest year of seropositivity was established through sequential serological screening of the archived serum samples in reverse chronological order. To do this, serological testing was performed on progressively older samples for each seropositive animal until the first seronegative result was identified. Once the earliest year of seropositivity was identified for each animal, the year of *T. cruzi* infection (year-of-infection) of each animal was then designated to be one year prior to its earliest seropositive serum result. As an example, an animal identified to be seropositive in 2013, 2012, and 2011 (i.e., with its first seronegative result in 2010) had 2010 designated as its year-of-infection. The rationale for this method of designating year-of-infection was based on three factors. First, it is our hypothesis that the primary cause of new *T. cruzi* infections to the colony animals each year is through the exposure of monkeys to the *T. cruzi*-infected triatomine bugs native to the area [55]. Second, is that the literature, along with our own historical experience at finding triatomine bugs, collectively suggest that the triatomine species common to the KCCMR area are almost exclusively active from late April to early October, with the greatest number of bugs present between June to August [10, 73, 74]. Third, is that the serum samples utilized in this study were collected as part of the annual health exams that are, for the vast majority of animals, completed between January and April each year. In summary, we propose that most KCCMR animals initially become *T. cruzi* infected between the late spring and summer but that these infections are not normally detected until the winter or early spring of the following year.

2.5 PCR Assessment of Seropositive Breeding Colony Animals

Between 2016 and early 2017, PCR analysis was performed using whole blood samples collected from all *T. cruzi*-seropositive breeding colony animals that were housed at the KCCMR during that time period [55]. The goal of these efforts was to identify the presence *T. cruzi* DNA in blood and to determine the DTU of these infections where possible. Since early 2017, additional PCR testing of select seropositive animals within the breeding colony has also been undertaken in support of other ongoing studies. These PCR assays were performed utilizing the protocols, primers, probes, and controls, as previously described [55]. In brief, DNA was extracted from EDTA-treated whole blood samples using a commercial extraction kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA). The DNA was subjected to a screening real-time quantitative PCR (qPCR) with primers and probes known to be sensitive and specific for all DTUs of *T. cruzi* [75-77]. All samples that were determined to be positive in the screening assay were then subjected to a multiplex probe-based qPCR for determination of DTU [78].

2.6 Identification of the Housing and Cage Mates for Seropositive Animals

Animal husbandry records documenting the month-to-month location of all animals within the KCCMR breeding colony were available for the time period October 1998 through January 2011 and also for the time period January 2012 to December 2018. These husbandry records, in conjunction with the medical records of the seropositive animals, were utilized to identify the housing location of each seropositive animal during the late spring and summer on their year-of-infection. The animal husbandry records were also used to identify all animals that were cohoused with any of the seropositive animals on or after the first day of April on the year each seropositive animal was determined to have become *T. cruzi* infected. Collectively the cage

mates to these *T. cruzi* seropositive animals, which included breeding partners, same-sexed social partners, and infants/offspring, have been termed *T. cruzi*-associated monkeys (TAMs).

2.7 Evaluation of *T. cruzi*-associated monkeys (TAMs)

After the TAMs for all seropositive animals were identified, efforts were made to document the serostatus of each TAM. The serostatus of the TAMs that had remained part of the KCCMR breeding colony until 2012, or later, was already known as a result of the data collected through colony serosurveillance efforts. The serostatus of the TAMs that had died or been sold from the colony prior to 2012 was established through serological testing of the last archived serum sample collected from each animal just prior to their departure from the colony. Any TAM that was identified to be seropositive underwent additional serological testing, as described above, in order to establish the earliest year of seropositivity for each animal. The housing location of each TAM on their year-of-infection and all the cage mates (secondary-TAMs) to these initial TAMS were also identified using animal records. The serostatus of these secondary-TAMs was then established through colony records or additional testing as needed.

Once the serostatus of the TAMs was established, the animal-to-animal associations between all seropositive animals and their TAMs were analyzed to look at four factors that were believed to possibly correlate with a higher risk of TAM seroconversion. These factors were as follows: 1) the total number of seropositive animals associated with each TAM; 2) the number of cumulative years each TAM was associated with one or more seropositive animals; 3) the total number of seropositive breeding partners associated with each TAM; and 4) the number of cumulative years each TAM was associated with one or more seropositive breeding partners.

In addition to these analyses, more detailed investigations of the associations between the seropositive animals and their TAMs were also undertaken to look for evidence of possible horizontal or vertical transmission occurring between the colony animals. These associations-of-interest included: 1) seropositive males and their male TAMs; 2) seropositive males and their female TAMs; 3) seropositive females and their male TAMs; 4) seropositive females and their female TAMs; and 5) seropositive dams and their offspring TAMs. These initial investigations looked for time and space links between the animals that could be suggestive of vertical or horizontal transmission of *T. cruzi*. For the purposes of this study, any TAM that was identified to have had its earliest year of seropositivity occur while housed with seropositive animals was considered 'linked' to that seropositive animal. Furthermore, any TAM that was identified to have had its earliest year of seropositivity occur within two years after having left shared housing with a seropositive animal was likewise considered linked to that seropositive animal. The 2-year time period was utilized here in order to account for any acute infections that may have occurred between a TAM and a seropositive animal just prior to their separation. More specifically, as the annual health exams and serum collections occurred just once a year, we considered the possibility that, with regard to horizontal transmission between animals, a TAM could become acutely infected with *T. cruzi* but may not have yet developed *T. cruzi* antibodies at the time of its first annual health exam following the separation of the TAM from the seropositive animal. For this same reason, we considered horizontal or vertical transmission to be possible causes of a *T. cruzi* infection in any infant that was born to a seropositive female and subsequently became seropositive within its first two years of life.

2.8 Evaluation of Environment

A study detailing the geographical location of newly-infected breeding colony animals was undertaken using the data from the 74 seropositive animals for whom the year-of-infection was known. The first goal of this investigation was to determine if there were specific areas within either animal compound that had higher ('hot spots') or lower ('cold spots') incidences of new *T. cruzi* infections than the colony in general. If areas of difference were identified within the compounds, a second goal of the study was to investigate the local environments for factors that could account for these differences.

As previously stated, it was our hypothesis that most new *T. cruzi* infections of the breeding colony animals occur during the late spring and summer months through the exposure of the monkeys to *T. cruzi*-infected triatomine bugs. With respect to this information, we documented the exact location or locations (corncrib structures or animal building rooms) of each of the 74 seropositive animals over a 5 month period (April to August) on the year each animal was identified to have become infected. In addition to this information, the corncrib structure or building room that housed the newly-infected animal was also given a numeric 'room-score' between 1 and 5, with the room-score representing the number of months the newly-infected animal spent in that location between April and August. Some newly-infected animals were housed in a single location throughout this entire 5-month period (room-score of 5) while other newly-infected animals moved one or more times between April and August, and the 5 points were divided across multiple room-scores correlating with the months spent in each location.

Two master maps of the colony were created for the tabulation of the room-scores. The first map combined the room-scores for animals identified to have become infected between the

years 1999-2010, and the second map combined the room-scores for animals identified to have become infected between the years 2012-2017. The division of the room-scores into two separate master maps was done for three reasons. The first was that no KCCMR husbandry data records existed for the time period between February and December 2011, as these records had previously been lost during a data transfer process. The lack of these records meant that none of the animals determined to have a year-of-infection of 2011 could be utilized for this exercise as their location(s) between April-August of 2011 were unknown. This fact resulted in a break in the continuity of the data set to pre and post-2011. The second reason was that the serological status for the colony as a whole was well-established for the period between 2012-2018, while the only serological information available for the colony prior to 2011 was data that was collected as part of a few small studies and through the retrospective testing of the known seropositive animals and their TAMs. The last, and potentially most significant, reason for the separation of data into two maps was due to the large number of environmental alterations that occurred within and around the main rhesus compound in 2011 through 2012. Specifically, we were interested to see if any changes occurred to the *T. cruzi* incidence rates within the compound in association with the timing of the fire-mitigation activities, the building of sidewalks around the corncrib structures, or the construction of the locker room/laundry facilities. After the cumulative room-scores were plotted on the two master maps, Google Maps© was used to determine the X and Y global-positioning-coordinates (GPS) of each individual building room or corncrib structure, and these data underwent spatial ‘hot-spot’ data analysis through the geographic information systems (GIS) using ESRI ArcGIS 10.3.1 Spatial Statistics toolbox (ESRI, Redlands, CA).

Triatomine insect species common to the KCCMR area often inhabit rodent and mesomammal nests, woody/leafy ground cover, and other dark confined spaces, and a study performed at the KCCMR had previously confirmed *T. cruzi*-infected triatomine bugs to be present at the Center [9, 55]. With this knowledge, we sought to identify environmental factors within the rhesus compounds that might be of potential significance to promoting the presence of triatomines and thereby the incidence of *T. cruzi* infections within the breeding colony. The first environmental factor we explored was a group of four elevated storage sheds located just north of Buildings 1 and 2 in the main rhesus compound (Figure 2). These structures were included as part of this environmental evaluation as triatomine bugs had previously been identified within and around the sheds and also due to the fact that rodents and mesomammals were known to routinely inhabit the leaf litter under these sheds. The second environmental factor we explored was trees within the rhesus compounds. While live trees are not generally considered a normal habitat of triatomine insects, it was hypothesized that the trees or their leaf litter might harbor rodent nests and, by extension, *T. cruzi*-infected triatomine populations, which could potentially affect the animals of the colony.

To evaluate the environmental influence of the storage sheds on the incidence of *T. cruzi* infections within the colony, a weighted metric termed ‘cumulative shed power’ was assigned to all animal rooms within 20 meters of a shed. The decision to use a distance of 20 meters as the outer limit of influence for these sheds was based on a study that explored the daily movement events of two triatomine species common to the KCCMR. In short, the results of that study identified these insects to have daily movement events ranging from 1 to 20 meters with an average of only 3.8 meters [11]. To calculate the shed power for a given animal room, the area of each shed (meters^2) was used as a proxy for its potential to harbor triatomine bugs while the closest measured

distance between the shed and animal room (meters) was used as a means to account for the likelihood of an interaction event between the insects and the monkeys. Division of the area of the shed by the distance between the shed and animal room resulted in a shed power for that one room from that one shed. For animal rooms that were within 20 meters of multiple sheds, each of the shed powers was calculated and then added together to find the cumulative shed power associated with each room as represented by the equation in Figure 3.

Shed Power = $\sum$ $([area(m)]/[distance\ (m)])^{\text{shed1-n}}$, where *n* is the farthest shed found within 20 meters

Tree Power = $\sum$ $([circumference\ (cm)]/[distance\ (m)])^{\text{tree1-n}}$, where *n* is the farthest tree found within 20 meters

Figure 3. Cumulative Shed Power and Cumulative Tree Power Equations

A similar approach was utilized to evaluate the environmental influence of trees on the incidence of *T. cruzi* infections within the colony. Here a weighted metric termed 'cumulative tree power' was assigned to all animal rooms within 20 meters of a tree. To calculate the tree power for a given animal room, the circumference of each tree (centimeters) measured at 1 meter off the ground was used as a proxy for its potential to provide habitat to wildlife hosts of triatomine bugs while the closest measured distance between the tree and animal room (meters) was used as a means to account for the likelihood of an interaction event between the insects and the monkeys. Division of the circumference of the tree by the distance between the shed and animal room resulted in a tree power for that one room from that one tree. For animal rooms that were within

20 meters of multiple trees, each of the tree powers was calculated and then added together to find the cumulative tree power associated with each room as represented by the equation in Figure 3.

2.9 Statistical Methods

VassarStats (VassarStats: Website for Statistical Computation, Richard Lowry, 2001-2020) was used to perform a z-ratio for the significance of the difference between two independent proportions, the number of seropositive animals that seroconverted between 2012 and 2018 after having been exposed to other seropositive animals, and the number of seropositive animals that seroconverted between 2012 and 2018 that did not have previous exposure.

The data was imported into JMP® (Pro Software, Version 15.0.0 SAS Institute Inc., Cary, NC, 1989-2019), and the relationship between potential risk factors and the serology status of TAMs was evaluated. The potential risk factors that were assessed included the number of seropositive animals a TAM was exposed to (1, 2, or ≥3), the time associated with seropositive animals in years (1, 2, ≥3), the number of seropositive breeding partners (0, 1, ≥2), and the time associated with seropositive breeding partners in years (1, 2, ≥3). Bivariable analysis using the chi-squared or Fisher's exact was used to identify putative risk factors. Factors with a $p < 0.05$ from the initial screening were used in a logistic regression model. Generalized linear mixed models were calculated, and factors with values of $p < 0.05$ were considered significant.

2.10 Spatial Statistics

Google Maps© was used to determine the X and Y coordinates of each individual room in the buildings housing NHP for spatial autocorrelation analysis. These coordinates, the number of seropositive/seronegative NHP, and the total number of cumulative months that seropositive

animals occupied individual rooms, were used for spatial data analysis to identify statistically significant hot spots and cold spots.

A local Moran's I measure of spatial autocorrelation, known as Local Indicator of Spatial Association (LISA), was used to distinguish clusters of cases, or "hot spots" [79]. With the use of a Moran scatterplot, interpretation of the significance of spatial clusters becomes possible; these are designated as "high-high" and "low-low" clusters. Spatial outliers are designated as "high-low" and "low-high."

Another class of local spatial autocorrelation was performed using Getis-Ord statistics in which spatial outliers are not considered. When using Getis-Ord statistics, a value larger than the mean suggests a high-high cluster or a hot spot, while a value smaller than the mean indicated a low-low cluster or cold spot [80].

CHAPTER 3

RESULTS

3.1 Seropositive Breeding Colony Animals

A total of 80 breeding colony animals housed at the KCCMR between 1999 and 2018 were identified to be seropositive for *T. cruzi* antibodies through both commercially-available testing methods. Fifty-nine of the 80 animals were female, and 21 were male (2.8:1 female-to-male ratio). Seventy-five of these 80 animals had already been identified to be seropositive prior to the start of the current study through the KCCMR colony serosurveillance efforts or through earlier retrospective investigations that utilized archived serum samples. The five remaining seropositive animals were identified from the serological testing of the TAMs that occurred as part of the current study.

Of the 80 seropositive animals that were included in this study, the year-of-infection was determined for 74 animals. The mean age of these 74 animals on their year-of-infection was 11.66 years (median 12 years, range 3-24 years, and S.E.M. 0.59). The year-of-infection could not be accurately determined for the remaining six animals due to the lack of consistently-archived serum samples prior to 2000, but four of the six animals were identified to be seropositive as early as 2000, and other the two animals were identified to be seropositive as early as 1989. Although the data from these six animals could not be utilized for some aspects of the study, the TAMs identified from these six animals, from 1999 onward, were included.

In that serostatus for the breeding colony as a whole was well characterized between 2012 and 2018, a number of additional analyses were also undertaken utilizing just this subset of animals. Of the 80 seropositive animals included in this study, 56 were determined to have

become infected between the years 2011 and 2017. Thirty-eight of these 56 animals were female, and 18 were male (2.1:1 female to male ratio). The mean age of these 56 animals on their year-of-infection was 11.30 years (median 10.5 years, range 3-24 years, and S.E.M. 0.73).

3.2 PCR-positive Breeding Colony Animals

Fifty of the 80 seropositive animals included in this study had also previously been identified as positive for *T. cruzi* infection through PCR analysis. For the 30 animals of unknown PCR status: 20 were animals had never been tested as they had departed from the colony prior to the start of PCR testing at the KCCMR in 2016; eight were animals that seroconverted in 2017 or 2018 that had not yet undergone PCR testing at the time of this report; and two were animals that had been previously tested but had not been identified to be PCR-positive. The DTU strain-typing of these infections was achieved for 44 of the 50 PCR-positive animals, with 24 animals identified as DTU I, 17 animals identified as DTU IV, and three animals identified to have a mixed (DTU I and DTU IV) infection.

3.3 *T. cruzi*-Associated Monkeys (TAMs)

The total number of unique TAMs identified from the 75 original seropositive animals was 656. There were 592 of the 656 TAMs that had archived serum samples available for testing. Out of the 64 TAMs for which serum was not available for testing, it is notable that 37 animals were less than one year of age, 22 animals were between one and three years of age, and five animals were three years of age or older at the time of their departure from the Center. Only five of the 592 TAMs that underwent serological testing were identified to be seropositive. These five seropositive TAMs were subsequently found to be associated with 54 TAMs (secondary-TAMs)

themselves. All 54 of these secondary-TAMs had previously been identified as TAMS to other seropositive animals, and all had already been determined to be seronegative for *T. cruzi* antibodies. Adding to the final tally of TAMs in this study was the finding that 33 of the original 75 seropositive animals had also been housed with another seropositive animal prior to seroconverting themselves. As such, these 33 original seropositive animals were also classified TAMs. In total, 624 of the 688 TAMS that were identified to have been present in the KCCMR colony between 1999 and 2018 had their serostatus established.

Of the 688 total TAMs identified for this study, a subset of 497 TAMs were present at the Center between the years 2012 and 2018. There were 449 of the 497 TAMs that had archived serum samples available for testing. Out of the 48 TAMs for which serum was not available for testing, 29 animals were less than one year of age, 17 animals were between one and three years of age, and two animals were three years of age or older at the time of their departure from the colony.

From the 624 TAMs identified from between 1999 and 2018 that underwent serological screening there were 38 animals identified to be seropositive (6.1% seroprevalence). For comparative purposes, of the 449 TAMs identified from between 2012 and 2018 that underwent serological screening there were 30 TAMs identified to be seropositive (6.7% seroprevalence). As the serostatus of the breeding colony as a whole was well established between 2012 and 2018, it was also possible to determine the incidence of seroconversion for animals that had never been previously exposed to a seropositive animal (non-TAMs). Serologic screening of 1450 non-TAMs housed at the Center between 2012 and 2018 identified 23 animals to be *T. cruzi*-seropositive (1.6%). The occurrence of seroconversion in TAMs was identified to be

significantly greater than the occurrence of seroconversion in non-TAMs for the time period between 2012 and 2018. (z-ratio 5.73; one-tailed probability < 0.001).

In efforts to try and illuminate what factors might account for the increased occurrence of seroconversion in TAMs as compared to non-TAMs, studies of the 624 TAMs were undertaken. The initial study was performed without consideration of the serostatus of the TAMs. This analysis identified the average number of seropositive animal associations per TAM to be 1.48 (range 1 to 9 animals) and the average cumulative time of association between seropositive animals and TAMs to be 2.9 years (range 2 months to 18 years).

Additional investigations were then performed in which the serostatus of the TAMs was taken into account. The first study here assessed four putative risk factors for TAM seroconversion by comparing the level of seropositive animal exposure and the incidence of seroconversion for all 624 TAMs (Table 1). Three of the four putative risk factors investigated showed patterns of data distribution which suggested that the more exposure a TAM has to seropositive animals, the greater the likelihood the TAM will become seropositive itself. Specifically, there were positive correlations between the incidence of seropositive TAMs and: 1) the cumulative amount of time a TAM spent with any seropositive animals (≤1 year: 1.30%; >1 to < 3 years: 3.10%; and ≥3 years: 11.70%); 2) the number of seropositive breeding partners of each TAM (0 animals: 5.04%; 1 animal: 6.12%; and ≥2 animals: 13.73%); and 3) the cumulative amount of time a TAM spent with seropositive breeding partners (≤1 year: 3.13%; >1 to < 3 years: 3.95%; ≥3 years: 13.08%). There was no direct positive correlation between the incidence of seropositive TAMs and the total number of seropositive animals to which each TAM was exposed (1 animal: 3.47%; 2 animals: 11.82%; and ≥3 animals: 9.91%). However, TAMs exposed to more than one seropositive animals had considerably higher incidence rates of

seroconversion than did the TAMs exposed to just a single seropositive animal. All were identified to be significantly different from one another, as detailed in Table 1.

Table 1. Potential risk factors for seroconversion of TAMs.

Putative Risk Factor		**Total TAMs** TAM numbers (% total TAMS)	**Seronegative TAMs** Numbers	**Seropositive TAMs** TAM Numbers (% TAMS in group)	**Mean (Median) Age TAM Seroconversion** Years	**P-value**
Total number of seropositive animals to which each TAM was exposed[1]	1 animal 2 animals ≥3 animals	403 (64.6%) 110 (17.6%) 111 (17.8%)	389 97 100	14 (3.47%) 13 (11.82%) 11 (9.91%)	11.25 (11.0)[3] 14.31 (13.0) 15.36 (15.0)	0.0005
Cumulative time (in years) each TAM was associated with seropositive animals[2]	≤ 1 >1 to <3 ≥3	230 (36.8%) 129 (20.7%) 265 (42.5%)	227 125 234	3 (1.30%) 4 (3.10%) 31 (11.70%)	10.50 (10.5) 12.75 (13.0) 13.61 (14.0)[3]	$1.16e^{-8}$
Number of seropositive breeding partners associated with each TAM[1]	0 animals 1 animal ≥2 animals	377 (60.4%) 196 (31.4%) 51 (8.2%)	358 184 44	19 (5.04%) 12 (6.12%) 7 (13.73%)	12.71 (13.0)[3] 13.08 (13.0) 16.71 (18.0)	0.0393
Cumulative time (in years) of exposure for each TAM with seropositive breeding partners[2]	≤ 1 >1 to <3 ≥3	64 (25.9%) 76 (30.8%) 107 (43.3%)	62 73 93	2 (3.13%) 3 (3.95%) 14 (13.08%)	11.50 (11.5) 18.67 (18.0) 13.93 (13.50)	$1.48e^{-3}$

[1]A Pearson's chi-square test was used for the statistical comparisons between TAMs exposed to different numbers of seropositive animals. [2]A Fisher's Exact one-tailed test was used for the statistical comparisons between TAMs with different time periods of exposure to seropositive animals. [3]One animal was excluded from the mean and median calculations as its age at seroconversion was undetermined.

After previously identifying ‘increased age’ to be a risk factor for *T. cruzi* seroconversion in the breeding colony animals, the mean and median age at seroconversion for the seropositive TAMs in each group was also calculated (Table 1) [72]. Positive correlations between the mean age of seroconversion and the incidence of seropositive TAMs were identified for: 1) the cumulative amount of time a TAM spent with any seropositive animals (10.50 years: 1.30%; 12.75 years: 3.10%; and 13.61 years: 11.70%); and 2) for the number of seropositive breeding partners of each TAM (12.71 years: 5.04%; 13.08 years: 6.12%; and 16.71 years: 13.73%). There was no direct positive correlation between the mean age of seroconversion and the incidence of seropositive TAMs with respect to the total number of seropositive animals to which each TAM was exposed (11.25 years: 3.47%; 14.31 years: 11.82%; and 15.36 years: 9.91%). However, TAMs exposed to more than one seropositive animals had significantly higher mean ages of seroconversion than did the TAMs exposed to just a single seropositive animal.

3.4 Investigations into the Possibility of Horizontal Transmission

In addition to investigating at the colony as a whole, there were four different animal-to-animal associations explored to look for data suggestive of horizontal *T. cruzi* transmission within the breeding colony (Table 2). First, the 80 seropositive animals and the 624 TAMs were arranged into their known associations (Table 2; ‘Seropositives’ and ‘TAMs’ columns). Equally important toward the process of determining the likelihood of horizontal transmission in the breeding colony, however, was the information that many of the TAMs had been exposed to more than one seropositive animal within each of these association groups. In that each exposure event between a seropositive animal and a TAM represented a potential opportunity for horizontal transmission to occur, the total number of exposure events for each association group

was also identified, and the average number of seropositive exposure events of each TAM in each association group was calculated (Table 2; 'TAM Events').

The next step in the evaluation of the colony for potential horizontal transmission involved identifying the number of linked-TAMs per association group. Initially, 38 TAMs were identified to be linked to a seropositive animal through time-and-space associations (Table 1) using the parameters as previously described. Ultimately though, there were only 29 of these animals included as linked-TAMs for the horizontal transmission evaluation. The paring down of these TAM numbers occurred as a direct result of DTU comparisons made between the TAMs and their respective seropositive animals. Specifically, pairs of animals that had previously been linked through time and space data but had *T. cruzi* infections of different DTUs were no longer considered linked. The logic behind this approach was that it should be expected that if horizontal transmission was responsible for a TAM seroconversion, then the TAM and its associated seropositive animal would both have *T. cruzi* infections comprised of the same DTU. In total, 17 of the linked pairs of animals had PCR data available for DTU comparisons. Eight comparisons identified matching DTUs between the two animals comprising each linked pair (Table 2; 'Matched DTU'). These data were interpreted to be supportive of possible horizontal transmission, and therefore these eight TAMs were included as part of 29 linked-TAMs along with the other 21 animals for which DTU comparisons were not possible (Table 2; 'Linked-TAMs'). For the other nine DTU comparisons, the DTUs of the TAMs did not match the DTUs of the seropositive animals to which they had been previously linked. Based on this information, these nine TAMs were not considered to have likely been infected through horizontal transmission, and these animals were excluded from the linked-TAMs counts (Table 2; 'Unlinked TAMs').

Table 2: Identification of links between TAMs and their associated seropositive animals.

Associations Explored	**Seropositives** # Animals Sex	**TAMs** # Animals Sex	**TAM Events[1]** # Events (Av. exposure)	**Linked-TAMs[2]** # TAMs	**Same Year Converted[3]** # TAMs	**Final-TAMs[4]** # TAMs (% TAM Events)	**Matched DTU[5]** # TAMs (% Final-TAMs)	**Unlinked TAMS[6]** # TAMs
Male-to-Male	5 Males	23 Males	28 (1.21)	2	2	1 (3.57%)	0 (0.0%)	0
Male-to-Female	21 Males	208 Females	232 (1.12)	4	2	3 (1.29%)	1 (33.3%)	2
Female-to-Male	59 Females	66 Males	135 (2.05)	5	0	5 (3.70%)	2 (40.0%)	2
Female-to-Female	59 Females	327 Females	530 (1.62)	18	4	16 (3.02%)	5 (31.3%)	5

[1]TAM Events are the total number of seropositive animal exposures the TAMs in each association group experienced with 'Av. Exposure' representing the average number of seropositive exposure events for each TAM in that association group. [2]TAMs were considered 'linked' to a seropositive animal when a TAM seroconverted while living with a seropositive animal or when a TAM seroconverted within 2 years of leaving shared housing with a seropositive animal. [3]Number of cohoused Linked TAMs from the previous column that were identified to have seroconverted on the same year. [4]Final-TAMs is the number of TAMs that potentially became infected through a horizontal transmission event and was calculated using the formula [Linked TAM number – (x/2)] where x was the TAM numbers from 'Same Year Converted' in the previous column. [5]DTUs of the TAM and its linked seropositive animal were confirmed to match between the pair. [6]Unlinked TAMs were associated with a seropositive animals through a time and space pattern suggestive of horizontal however the DTUs of the TAM and its associated seropositive animal did not match between the pair. Unlinked TAMs were not included in any of the calculations for Linked TAM animals or events.

The animal-to-animal association investigations also identified one other finding of potential importance in determining the likelihood of horizontal transmission occurring between animals. This was the finding that both animals in some of the linked pairs seroconverted on the same year (Table 2; 'Same Year Converted'). In the seropositive male-to-male TAM and seropositive male-to-female TAM association groups this occurred for 1 pair (2 animals) per group. In the seropositive female-to-female TAM association group, this occurred for 2 pairs (4 animals). This finding was problematic to the horizontal transmission calculations because the identity of the TAM and the seropositive animal in these pairs could not be established, and therefore both animals within each pair were initially included as part of the linked-TAM counts. With regard to horizontal transmission, however, only one of the animals from each pair should have been considered a TAM, with the other animal from each pair serving as the seropositive animal. To account for this overestimation of the TAMs potentially involved in horizontal transmission events, an additional number (Table 2; 'Final-TAMs') was calculated for each association group using the formula [Linked TAMs number – (x/2)] where x was the number of TAMs identified in each association group to have seroconverted in the same year. This Final-TAMs number was then used to calculate the maximum number of TAMs to have potentially been infected through horizontal transmission for the colony as a whole, and also for each association group. The maximum horizontal transmission occurrence rate for the colony as a whole, as well as for each association group, was then determined by dividing the Final-TAM numbers by the number of TAM events.

With regard to the colony as a whole, there were two findings related to the possibility of horizontal transmission. First, of the 80 seropositive TAMs in this study, 25 were identified to be Final-TAMs (31.25%) with time-and-space relationships to other seropositive animals that could

be consistent with horizontal *T. cruzi* transmission. However, it is notable that only eight Final-TAMs (10.00% of the 80 total TAMs) were confirmed to have *T. cruzi* infections comprised of the same DTU-strain as their associated TAM. The second finding here was that of the 925 total TAM events documented between seropositive animals and their TAMs, 25 Final-TAMs were identified to have potentially been infected through the horizontal *T. cruzi* transmission route. These data suggest that the horizontal *T. cruzi* transmission may occur in as many as 2.70% of all exposures between naïve and infected animals, but again, only eight Final-TAMs (0.86% of the 925 TAM events) were confirmed to have *T. cruzi* infections comprised of the same DTU-strain as their associated TAM.

With regard to comparisons for association groups, the final-TAMs numbers, the number of final-TAMs supported through DTU comparisons, and maximum seroconversion rates for each animal-to-animal association are detailed below. The remaining data obtained from these associations are provided in Table 2. Five seropositive male animals were associated with 23 male TAMS resulting in 28 TAM events (average seropositive exposures of 1.21 per TAM). From these 28 events, only one seropositive final-TAM was identified (no DTU data available) for a seroconversion rate of 3.57%. Sixteen seropositive male animals were associated with 208 female TAMS resulting in 232 TAM events (average seropositive exposures of 1.12 per TAM). From these 232 events, three seropositive final-TAMs were identified (one supported through DTU comparisons) for a seroconversion rate of 1.29%. Fifty-nine seropositive females were associated with 66 male TAMS resulting in 135 TAM events (average seropositive exposures of 2.05 per TAM). From these 135 events, five seropositive final-TAMs were identified (two supported through DTU comparisons) for a seroconversion rate of 3.70%. Fifty-nine seropositive female animals were associated with 327 female TAMs, resulting in 530 TAM events (average

seropositive exposures of 1.62 per TAM). Collectively of these 530 TAM events, 16 seropositive final-TAMs were identified (five supported through DTU comparisons), resulting in a maximum seroconversion rate of 3.02% for all possible horizontal transmission events.

3.5 Investigations into the Possibility of Vertical Transmission

Fifty-four of the 59 seropositive females identified in this study were of breeding age. After seroconverting, these 54 dams collectively produced a total of 196 live infants between 1999 and 2018. Of these 196 animals, 137 had archived serum samples available for testing, and all 137 samples were determined to be seronegative through the results of the serologic screening process.

3.6 Investigations into the Influence of the Environment on *T. cruzi* infection

Seventy-one of the 74 animals for which a year-of-infection had previously been determined were able to be utilized for the spatial hot-spot analysis of the animal compounds. In that no husbandry data records were available for the time period between February and December 2011, the three animals that were identified to have become infected in 2011 were excluded from this work. There were 18 of the 71 animals identified to have become infected between 1999 and 2010 that were utilized in one hot-spot analysis. The remaining 53 animals were identified to have become infected between 2012 and 2017 and were utilized for a second hot-spot analysis. Results from the 1999-2010 animal data identified several building-rooms and corncrib structures to be hot-spots within the main rhesus compound (Figure 4-A). Results from the 2012-2017 animal data identified several building-rooms to be hot-spots and other building-rooms to be cold-spots within the main rhesus compound (Figure 4-B). For the north rhesus

compound, all building-rooms were determined to be “Not Significant” for both time periods examined (data not shown).

Figure 4. Spatial hot-spot analysis for *T. cruzi* seroconversion in the main rhesus compound. Image A: Data obtained from analysis of animals that became infected 1999-2010. Image B: Data obtained from analysis of animals that became infected 2012-2017. Each colored dot represents an individual building room or individual corncrib structure. Legend: Red and orange dots represent animal housing with a significantly higher incidence of *T. cruzi* seroconversion than the colony in general. Blue and gray dots represent animal housing with a significantly lower incidence of *T. cruzi* seroconversion than the colony in general. Yellow dots represent animal housing with an incidence of *T. cruzi* considered average for the colony in general. Photo credit: Map data © 2014 Google. Image edited to remove red cars in parking lots.

Data obtained from the cumulative shed power calculations are presented in Figure 5. Building 1 and Building 2 were the only buildings in either animal compound to have cumulative shed power-values assigned to their rooms. This was due to the proximity of the four sheds to one another and the fact that the outer-limit-of-influence for the sheds had been previously set at 20 meters. The primary finding-of-interest from these analyses was that the five rooms with the highest cumulative shed powers for Buildings 1 and 2 were also the same five rooms identified as hot-spots on spatial analysis of the 1999-2010 animal data (Figure 4A).

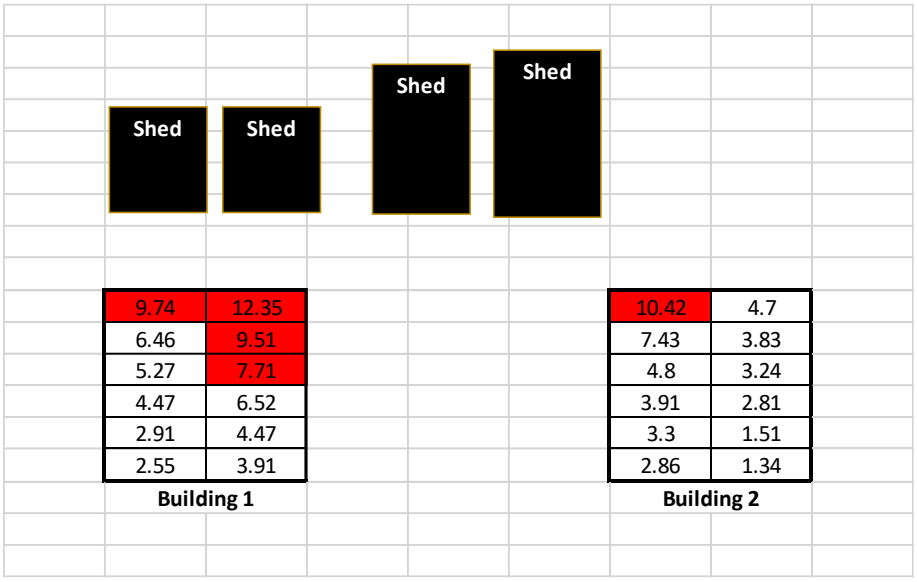

Figure 5. Cumulative shed power-values assigned to Building 1 and Building 2 rooms in the main rhesus compound. The four sheds are represented by the four black boxes at the top of the image. The two 12-room buildings are represented in the bottom half of the illustration. The illustration provides the approximate size and location of each shed relative to the two animal buildings. The number located within each of the building-rooms is the cumulative shed power-value assigned to each room. The five rooms with the largest cumulative shed power-values are highlighted in red.

There were 106 trees measured in the two compounds that were utilized in the cumulative tree power calculations (Figure 6). Due to the prevalence of trees within these compounds, every building-room and corncrib structure was assigned a cumulative tree power-value. There was minimal correlation identified between the cumulative tree power-values and the incidence of seroconversion as illustrated by the fact that 13 of the 15 rooms with the highest cumulative tree power-values, and 11 of the 15 rooms with the lowest cumulative tree power-values, were ultimately identified to be “Not Significant” on both spatial analyses.

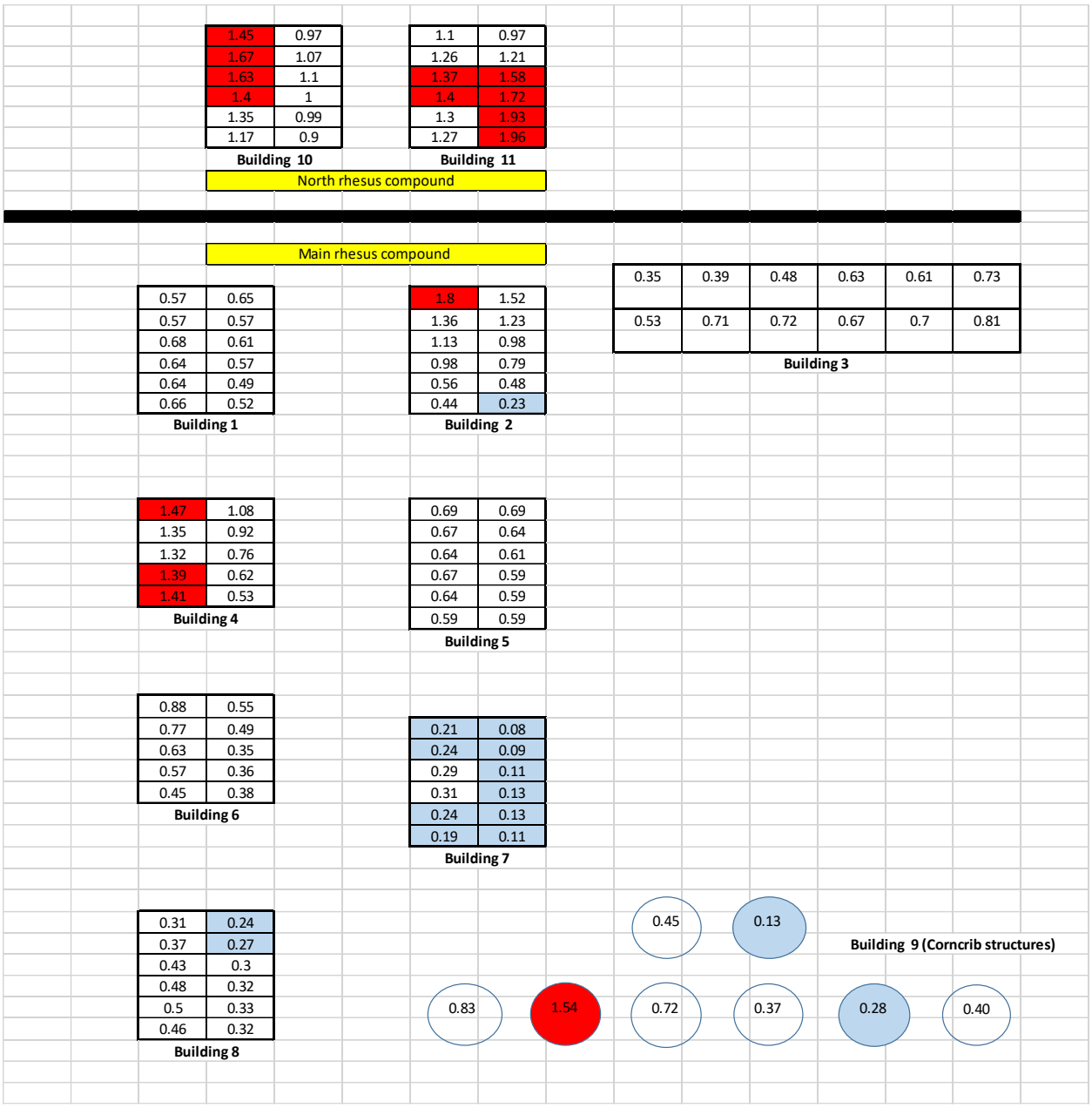

Figure 6. Cumulative tree power-values assigned to building-rooms and corncrib structures in the two animal compounds. The two buildings (Building 10 and 11) within the north rhesus compound are located at the top of the image. Buildings 1-8 and the 8 corncrib structure (collectively known as Building 9) are located at the bottom of the image and are laid out in a pattern to approximate their relative position within the main rhesus compound. Buildings are divided into 10 or 12 boxes and represent the rooms within each building. The number located within each of the building-rooms and corncrib structures is the cumulative tree power-value assigned to each room. The fifteen rooms with the highest cumulative tree power-values are highlighted in red. The fifteen rooms with the lowest cumulative tree power-values are highlighted in blue.

CHAPTER 4
DISCUSSION

The natural infections of caged NHPs in biomedical facilities in the southern US – where triatomine vectors occur locally – presents major challenges for animal welfare and biomedical research. Because there is uncertainty as to the risk that housing seropositive monkeys poses to others in the colony, we used retrospective data and samples obtained from a rhesus macaque breeding colony in south Texas to investigate the potential for horizontal and vertical transmission of *T. cruzi*. The results of these investigations suggest that the majority of *T. cruzi* infections for this colony appear to occur by mechanisms other than horizontal or vertical transmission. Specifically, we found significant spatial clusters of infection within key buildings at the facility that support environmental patterns of infection and may be related to triatomine infestation from the local environment.

Initial findings from the 2012-2018 data identified 22.9% (497/2166) of animals at KCCMR to be TAMs. Further comparisons of TAMs and non-TAMs from this time period found that TAMs have a significantly higher incidence of seroconversion and also found that the longer a TAM lived with a seropositive animal, or the more seropositive breeding partners a TAM had, the more likely a TAM was to have seroconverted.

While these findings could be interpreted to suggest animal-to-animal transmission is a significant contributor to the epidemiology of the pathogen, what also must be realized is that at least some of these data are confounded by the age of the animals. As demonstrated in Table 1, the percentage of TAMs that are seropositive increases with the increased mean age of the TAM with regards to the cumulative time in years that each TAM was associated with a seropositive animal, and the number of seropositive breeding partners associated with each TAM. This

finding is consistent with previous data collected from the KCCMR colony that suggested increased age is associated with an increased likelihood of seroconversion [72]. Given that rearrangement of breeding groups is utilized as part of the standard husbandry practices at the KCCMR and also the finding from 2012-2018 data that 22.9% of the animals within the colony are TAMs, it is logical that the older a KCCMR animal is, the greater its probability of having increased contact (through numbers or time) with a seropositive animal. In that these initial data could not be used to differentiate animal-to-animal transmission from environmental influences on transmission, further studies were conducted to try and identify the primary route of new *T. cruzi* infections occurring at the KCCMR.

Additional investigations into the possibility of horizontal transmission compared time-and-space interactions between naïve and seropositive animals within the KMMCR colony. Results from these analyses found that while as many as 31.25% of the total seropositive colony animals could have possibly become *T. cruzi*-infected through horizontal transmission, this route of transmission appears to be a very inefficient in that, at most, only 2.70% of the exposures between naïve and seropositive animals (TAM events) resulted in a novel infection. What needs to be noted here, however, is that both of the numbers above should be considered as the maximum possible upper limits for horizontal transmission in the colony due to the fact that the study was very generous in its definition of 'linked' animals that were included in the horizontal transmission calculations. The first aspect in which the study was perhaps overly inclusive in its definition of 'linked' is that a TAM was considered linked to a seropositive animal if a TAM seroconverted while living with a seropositive animal or if a TAM seroconverted for up to 2 years after leaving housing with a seropositive animal. The reasoning behind the 2-year, post-exposure timeframe for linked animals was to ensure all possible horizontal transmission events

from the colony were included for consideration. However, it is also acknowledged that by allowing the definition of linked animals to include any TAM that seroconverted for up to 2 years after leaving housing with a seropositive animal, it is likely that there may have been an overestimation of the actual total number of horizontal transmission events in the colony. The second aspect in which the study was perhaps overly inclusive was that animals were included as linked animals even when the DTU comparisons between the TAMs and their associated seropositive animal were not available. Specifically, of the 38 pairs of linked animals, DTU-comparisons were available for only 17 pairs. Of these 17 pairs, 8 pairs had matching DTUs while 9 pairs (>50%) did not. The 8 matched pair animals were included as linked animals along with the 21 paired TAMs for whom DTU identification was not able to be performed. If our initial testing is any indication, we might expect that at least half of the remaining 21 TAMs would also not match their seropositive animal either. Similar to what was mentioned above, while the inclusion of the animals of unknown DTU status as linked animals has ensured all possible horizontal transmission events within the colony were accounted for, it is likely that inclusion of these animals has led to an overestimation of the actual total number of horizontal transmission events in the colony.

We further investigated various types of animal-to-animal associations, beyond the scope of the average colony numbers mentioned above, for evidence of horizontal transmission specifically between certain populations of animals within the colony. These animal-to-animal associations, and the maximum possibility of horizontal transmission within each association, include male-to-male (3.57%), male-to-female (1.29%), female-to-male (3.70%), and female-to-female (3.02%) as summarized in Table 2. While three of these associations are largely comparable (around 3%), male-to-female is only 1.29%. While we do not have an explanation

for this finding, it is interesting to note that although not perfectly correlated: 1) the male-to-female association has the least number of average exposures (1.12 seropositive animals per TAM) and the lowest seroconversion rate at 1.29%; and 2) the female-to-male association has the highest number of average exposures (2.05) and the highest rate of seroconversion at 3.70%. Although these last two results could be interpreted to support the proposition that horizontal transmission of *T. cruzi* can occur between captive NHPs, the data collected from the study as a whole suggest that, if horizontal transmission does occur, it is not the primary route of novel infections for animals within this colony.

With regards to the possibility of horizontal transmission within the colony, the life cycle of *T. cruzi* needs to be taken into consideration. During the acute infection or reactivation in an individual, trypomastigotes circulate within the bloodstream before transforming to the amastigote tissue stage. Theoretically, an animal in the acute phase of infection may be able to transmit the parasite directly (without a vector) via blood contact. While this hypothetical scenario is possible, horizontal transmission within the KCCMR colony is not a foregone conclusion as exemplified by the following examples. The longest known association between a seronegative TAM and a seropositive animal was 18 years. This seronegative female TAM never seroconverted, despite having been associated with six seropositive animals, including seropositive breeding partners, over the course of 18 consecutive years. Likewise, the highest number of seropositive breeding partners one seronegative male TAM has was 9 over the course of 12 years. Additionally, one seronegative female though transcriptional error was inadvertently housed over a year with 8 other seropositive animals as part of a study using *T. cruzi* animals (7 other females and 1 breeding male). During this time period, the seronegative female TAM bred with the seropositive male, resulting in a live birth. This transcriptional error was due to

misreading a single digit in the animal identification number between a seronegative female and a seropositive female. Once the transcription error was discovered, the seronegative female was removed from the group and has remained seronegative for more than 5 years at the time of this report. Even though the potential for horizontal transmission within the colony is present, the transmission of *T. cruzi*, as demonstrated by these examples, remains unapparent.

No seropositive dam was identified to have transmitted *T. cruzi* infection to her offspring in this study based on antibody testing of 156 infants born to seropositive mothers. The timing of their samples was at approximately 7 to 8 months, post-weaning, a time when maternal antibodies would be expected to have waned. As in humans, maternal antibodies in infant macaques progressively decline until 6 to 12 months of age, with recent evidence that antibody levels are undetectable by 2 to 3 months of age [81-83]. As previously mentioned, each DTU has distinct characteristics including clinical forms of disease. In humans, case reports have identified all DTUs, except for TcIV, in association with congenital transmission [24]. However, congenital transmission is documented to commonly occur when the mother is infected with TcII, TcV, and TcVI [7]. Although TcI has been implicated in human congenital transmission cases, recent data suggests that protein kinase cascades, which are important to cell invasion of *T. cruzi*, are down-regulated in mammalian cells during TcI infection. This finding, coupled with known tissue tropisms, could explain lower levels of placental infection in TcI *T. cruzi* infections compared to other DTUs like TcVI [84]. We, therefore, propose that, with regards to the *T. cruzi* strains/DTUs common to the KCCMR (TcI and TcIV), vertical transmission either does not occur or occurs only as a very rare event in rhesus macaques. One limitation of our study was that 59 of the 196 infants (30.1%) derived from seropositive females did not have serum available for testing. However, we believe that, in addition to the findings mentioned above, the

validity of this proposal is also supported by the fact that of the 80 seropositive animals identified and characterized for this study, the youngest documented age of infection in the KCCMR colony is 3 years old.

There was strong environmental clustering of infections on campus as illustrated in the spatial analysis (Figures 4A and 4B). These clusters of infections varied to some extent within the main rhesus compound between the two time periods (1999-2010 and 2012-2018) examined for this study. Further investigation into the immediate surrounding areas of these hot and cold spots reveal some environmental factors that are potentially contributing to infections within the colony. Figures 4A and 5 both illustrate the impact of the elevated sheds. Hot spot analysis of 1999-2010 correlates almost perfectly for Buildings 1 and 2 with the cumulative shed power analysis. Based on these findings, all leaf litter underneath the elevated sheds have been removed and is currently being maintained at KCCMR.

We also investigated trees within the compound as potential environmental factors contributing to infections. No correlation between buildings and trees are found; however, this is not surprising as triatomine are not tree dwelling, rather they inhabit nests of small mesomammals. While small mammals may utilize any fallen leaf litter for nest building, the landscape within the compounds has always been well maintained and there is very little leaf litter associated with the trees at the time of peak bug movement in the spring/summer. Likewise, rodent control has been present and consistent throughout the 20 year time period of the study.

One of the most apparent changes in hot zones between the two spatial analyses is the corncribs. In the 1999-2010 spatial analyses, six of the eight corncrib structures were considered hot spots, but in the 2012-2018 spatial analyses, none of these structures were identified to be hot spots. Notably, the shift in clusters of infections occurred after the fire mitigation efforts of the

Center had been undertaken. In September 2011, Bastrop County suffered the most destructive wildfire in state history. As a preventative measure to help mitigate future fire-related damage, the small trees and bushes in the woods adjacent to the south side of the rhesus colony were removed. Perhaps even more importantly, nearly all the ladder fuel (dead vegetation, leaves, and debris) was also removed from the same area. Prior to these efforts, forestation with thick woody undergrowth extended right to the road opposite of the corncribs (Figure 7), which was approximately 11.67 meters from the nearest corncrib structure. After fire mitigation efforts, the wooded area has been cut back, and there is no undergrowth for about 35 meters (Figures 8A and B). This suggestion is supported by the finding that the hot spots with the greatest statistical certainty in Figure 4A are the 4 corncribs directly opposite of the old woods, while the hot spots with the least statistical certainty are the 2 corncribs directly north of the 4 corncribs with the greatest statistical certainty. Most convincingly, the two east most corncribs were not hot zones, as that corner across from these corncribs has always been tree and undergrowth free. It is also of note that the sidewalks around the corncribs do not appear to have any impact. These sidewalks were not present in the earlier time point and the two east-most corncribs were never documented to be a hot zone for the colony.

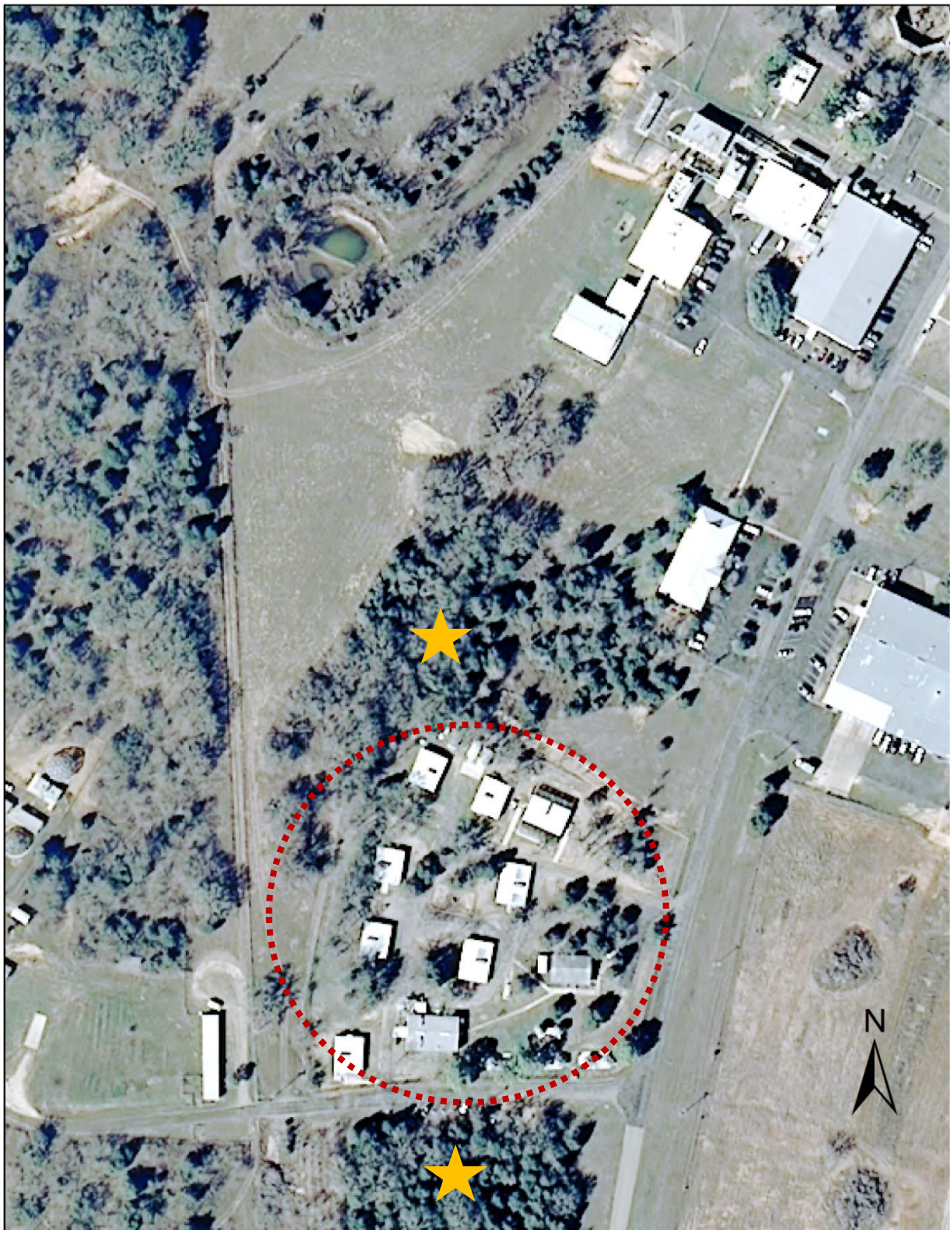

Figure 7. Satellite image from 2006 of the main rhesus compound indicated by the red dashed circles at the KCCMR. Notice the dense vegetation surrounding the colony as indicated by the stars. Photo credit: Map data © 2006 Google.

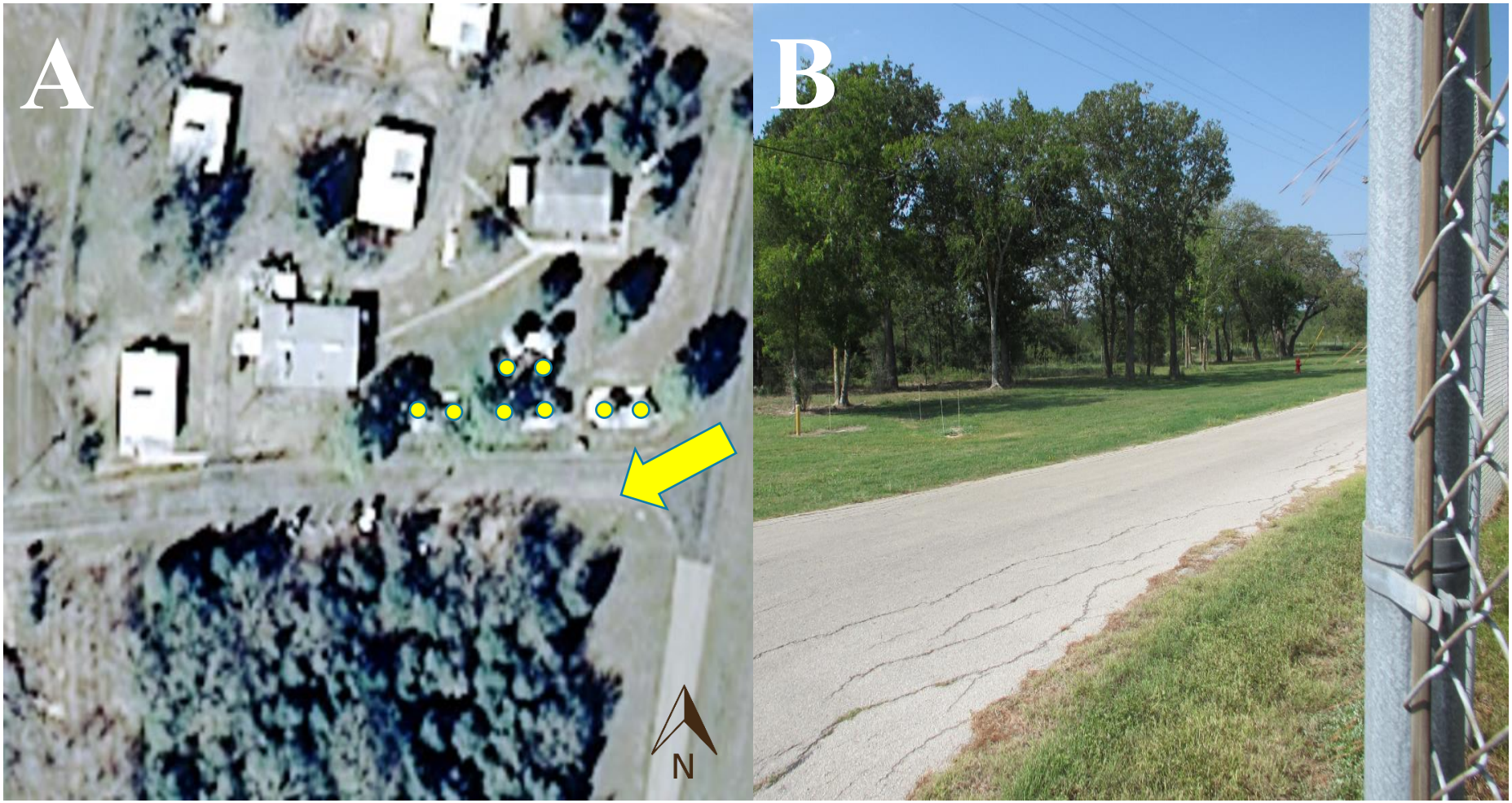

Figure 8. Images of main rhesus compound at KCCMR. Image A: Enlarged satellite image from 2006 of the main rhesus compound. Corncrib structures are denoted by the yellow dots. Notice the dense vegetation directly adjacent to the corncrib structures. The corner directly adjacent to the two eastern corncribs is bare. The yellow arrow indicated the direction Image B was captured. Image B: Current day (2020) image obtained of the once densely wooded area adjacent to the corn crib structures. Notice the sparse trees and well-maintained grass, free of underbrush and groundcover. Photo credit for left image: Map data © 2020 Google, Maxar Technologies.

The locker facility, which was also built between 2011 and 2012, also does not appear to affect the environment. Buildings 4, 6, and 8 are closest to the locker room facility. These buildings have never been a hot zone prior to construction nor after.

The hot spots associated with Building 1 in the 2012-2018 spatial analysis may be due to in part to the elevated sheds; however, it could also be due to the fact that the panels used for winterizing the buildings and corncrib structures are stored directly west of this building in the summer. These panels collect weeds and allow leaf accumulation under and around these structures. While these panel have been stored at the same location during the summer throughout the 20 years, the difference between the two spatial analyses may be due to the fact a more complete and refined data set of the colony was available for the 2012-2018 period as compared to the 1999-2010 period.

Finally, the southeast corner room of Building 3 has been identified as a hot spot for the 2012-2018 period. This could either be an anomaly of the data set or could be related to a large plant box that was present directly east of the building prior to 2016. This corner room hot spot was the closest animal room in Building 3 to the planter box. Since the removal of the planter box in 2016, no other animals have seroconverted while living in this corner room.

There were limitations encountered with a project of this scale. The first being that data collected from 1999 to 2011 was based on animals that had previously been identified as seropositive through other earlier studies or through TAM tracking. It is almost certain that not all seropositive animals that lived at KCCMR during this earlier period were identified. Subsequently, there may also be additional TAMs that were not accounted for. Despite limitation to the 1999-2010 data, it is considered likely that our lack of data for this time period is not biased to one specific area of the compounds and these data have provided novel and useful

information with regards to the spatial hot-spot statistics and environmental influences. In contrast, our serostatus data from 2012 to 2018 is largely complete giving us high confidence of the findings from these data. The data from 2012-2018 have also been supportive of the data acquired from the 1999-2010 time period, given similarities in some of the basic parameters between the two data sets.

While there was a small percentage of animals that were not able to be included in the study due to lack of serum, most of these animals were less than 3 years of age. This is significant in that the youngest age of infection for any KCCMR animal to date is 3 years old. Specifically, for the years 2012-2018, the serostatus of 87.7% of colony animals (1899/2166) was established. Of the 267 colony animals that were not able to be tested, only 3 animals were older than 3 years of age, accounting for only 0.13% of the entire colony present at the center during the time period. During this same time frame from 2012-2018, the serostatus of 90.3% of TAMs (449/497) was established. Out of the 48 TAMs that were not tested, only 2 were older than 3 years of age, accounting for 0.40% of TAMs present at the center during this time period. Further supporting the validity of the results from this study was the finding that the mean age of infection of the animals from 1999-2018 is similar to what we had identified in previous studies on other subsets of animals at KCCMR [72].

Although all samples and animals were confirmed seropositive through two testing methods using 3 different assays, initial serological testing of samples was performed using just one of the two assays. Potentially this can be a limitation if one assay is more sensitive, the other assay may have missed some seropositive animals. Chagas Stat-Pak Assay® sensitivity is reported as 98.5% and specificity is 96.0% in humans; however, the sensitivity and specificity of MFIA is not reported at this time [85]. The MFIA assay could be more sensitive than the Chagas

Stat-Pak Assay®, leading to more potentially missed seropositive animals when using the Chagas Stat-Pak Assay®. However, based on our numerous cross testing of our own samples, they appear very comparable. Both tests utilize proprietary antigens, thus direct comparisons of antigenic specificity between tests cannot be determined. It is also important to note, that while the Chagas Stat-Pak Assay® has been used for testing in NHP, it has not yet been validated in NHP, although the assay has also been widely used in wildlife and dogs [55, 72, 86-89].

Another limitation is that TAMs may have been missed due to incomplete husbandry records for 2011. However, the potential number of missed TAMs would be minimal as we did have the monthly husbandry records up to and including January 2011 and from January 2012 onward. If the animals moved housing locations more than once during this time period, they were not likely to have changed housing partners more than once as per the husbandry practices utilized by the KCCMR during this time period. By referring to both the January 2011 and January 2012 records, it is likely that essentially all the TAMs from this time period were able to be identified.

Another possible limitation occurred when determining the first year of seroconversion, in which back testing was performed on archived serum samples starting with the most recent year available, and tracing backwards until a negative result was obtained. It is possible that a negative result was due to the degradation of the target antigens within the archived serum instead of being truly seronegative. While this may be possible, degradation and diminished specimen quality over time is unlikely to have any statistical significance on detection of *T. cruzi* as has been recently demonstrated using plasma components in archived human samples stored up to 12 years [90]. This recent study utilized human samples dating back to 2006, however we have identified seropositive animals as old as 1989 through back testing. We acknowledge

however, if antibodies were low initially within the serum sample, they may not be detectable over time. For most animals, testing was concluded when a negative test result was reached. However, we may have prematurely stopped testing on some animals. While we have negative results preceeding to positive results prior to 2012, this was not consistent due to the lack of archived serum samples for some animals.

CHAPTER 5

SUMMARY AND CONCLUSION

In conclusion, our findings suggest environmental factors such as surrounding vegetation and raised foundation structures need to be taken into consideration for facilities housing outdoor NHPs in biomedical research facilities across the southern United States. While we cannot definitively say that Chagas disease is never horizontally transmitted between rhesus macaques in breeding groups, there is clear evidence that supports environmental factors are a leading contributor to the incidence of disease. Additional research focused on interrupting spillover from the sylvatic cycle and controlling wild mesomammals serving as potential reservoirs for *T. cruzi* warrants further investigation [55]. In the meantime, management efforts to reduce problem areas like small mammal burrows and reducing dense vegetation or other debris may help reduce the prevalence of triatomine bugs and thereby decrease infection rates in NHP with *T. cruzi*. Furthermore, with no evidence of congenital transmission of the parasite, infected monkeys may continue to be useful as breeding animals in the colony, as recent evidence has found a lack of significant adverse reproductive outcomes in seropositive dams and those offspring born to seropositive dams can be confidently used as *T. cruzi*-free research subjects [72].

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