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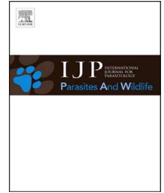

High *Trypanosoma cruzi* infection prevalence associated with minimal cardiac pathology among wild carnivores in central Texas

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ABSTRACT

Infection with the zoonotic vector-borne protozoal parasite *Trypanosoma cruzi* causes Chagas disease in humans and dogs throughout the Americas. Despite the recognized importance of various wildlife species for perpetuating *Trypanosoma cruzi* in nature, relatively little is known about the development of cardiac disease in infected wildlife. Using a cross-sectional study design, we collected cardiac tissue and blood from hunter-donated wildlife carcasses- including raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*) – from central Texas, a region with established populations of infected triatomine vectors and increasing diagnoses of Chagas disease in domestic dogs. Based on PCR analysis, we found that 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot). Although a histologic survey of right ventricles showed that 21.1% of 19 PCR-positive hearts were characterized by mild lymphoplasmocytic infiltration, no other lesions and no amastigotes were observed in any histologic section. DNA sequencing of the TcSC5D gene revealed that raccoons were infected with *T. cruzi* strain TcIV, and a single racoon harbored a TcI/TcIV mixed infection. Relative to other wildlife species tested here, our data suggest that raccoons may be important reservoirs of TcIV in Texas and a source of infection for indigenous triatomine bugs. The overall high level of infection in this wildlife community likely reflects high levels of vector contact, including ingestion of bugs. Although the relationship between the sylvatic cycle of *T. cruzi* transmission and human disease risk in the United States has yet to be defined, our data suggest that hunters and wildlife professionals should take precautions to avoid direct contact with potentially infected wildlife tissues.
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1. Introduction

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

Diverse wildlife species serve as reservoirs of *T. cruzi* across the parasite's range. Our understanding of the relative importance of wildlife reservoirs in the ecology and epidemiology of Chagas disease reflects vertebrate life history, especially as it relates to vector contact, and has been limited by the difficulty to collect large sample sizes of diverse wildlife taxa from across broad geographic areas. Although any mammalian species can potentially become infected with *T. cruzi* (Bern et al., 2011), the species that interact most frequently with kissing bugs have the opportunity to become infected or serve as the source of an infection. For example, in the southern United States, *Neotoma* spp. woodrats are well-recognized wild *T. cruzi* reservoirs, reflecting their association with nests commonly infested by triatomine nymphs and adults (Eads et al., 1963; Kjos et al., 2009). In South America, palm trees are an important ecological niche for contact between the *Rhodnius* genus of triatomines and opossums, which are a

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recognized reservoir of *T. cruzi* (Gaunt and Miles, 2000). With respect to the search effort, the ideal assessment of wildlife species' contributions to the enzootic transmission cycle would be to first have an unbiased assessment of triatomine feeding patterns, and then study those vertebrate species known to provide blood meals to the vector. A recent metaanalysis of triatomine feeding patterns suggests, however, that rather than innate preference for host species, host utilization by kissing bugs is dictated by the habitat they colonize (Rabinovich et al., 2011). Finally, with few exceptions (Barr et al., 1991a; Pietrzak and Pung, 1998; Roellig et al., 2009b), wildlife studies do not typically address how infection may relate to disease within infected individuals, likely because of sampling limitations.

The purpose of this study was to survey populations of raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*) in central Texas to determine infection prevalence and circulating *T. cruzi* strain types. Further, we aimed to study the relationships among parasite infection in blood, infection in different areas of the heart, and the manifestation of cardiac pathology. Each of these species has previously been shown to be exposed to or infected with *T. cruzi* (Burkholder et al., 1980; Brown et al., 2010; Kribs-Zaleta, 2010; Rosypal et al., 2010; Charles et al., 2013). Central Texas is a region where at least four species of infected triatomine species occur (Kjos et al., 2009; Curtis-Robles et al., 2015) and where human and canine Chagas disease have been diagnosed (Texas Department of State Health Services, 2015a, 2015b; Tenney et al., 2014). These particular wildlife species were selected for study due to their high population densities across Texas and 'varmint' status among ranch owners, which results in efforts to reduce population size through recreational hunting that may pose a human health risk from hunter contact with infected wildlife tissues (Yeager, 1961; Pung et al., 1995).

2. Materials and methods

2.1. Sampling

We conducted a cross-sectional wildlife study in January 2014 at a hunting check station for a recreational nuisance predator hunt in central Texas. This event was organized by a group of private landowners as a predator calling competition of animals considered pests to Texas ranches. Animals legally harvested by teams of hunters over a 24-h period were brought to a central check station where teams were awarded for their harvest. Our team collected samples from animals for which harvest location information was available. Animals in our study were harvested from 25 counties in central Texas and included raccoons, coyotes, foxes, and bobcats. We performed a field necropsy, at which time we removed the heart from each animal within 24 h of death and stored at −20°C until further processing.

In the laboratory, hearts were examined grossly and dissected in order to obtain an approximate 1 cm^3 section of each of the apex and right ventricular free wall for molecular testing. In some cases, portions of the cardiac tissue had been destroyed during harvest, and so paired samples of right ventricle and apex were not always possible. When present, blood clots were collected from within the chambers of the heart during the dissection and frozen until further processing occurred. A section of right ventricular free wall was prepared in 10% formalin for histological examination. Additionally, any gross lesions were described and preserved as above for histological examination.

2.2. Molecular work

2.2.1. DNA extraction and T. cruzi detection

DNA was extracted from heart apex, right ventricle, and blood clot samples using the Omega E. Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA). No-template controls were included in each set of DNA extractions, and molecular grade water was included as negative controls in PCR reactions. In order to detect presence of *T. cruzi* DNA, a 166-bp segment of the *T. cruzi* 195-bp repetitive satellite DNA was amplified using a Taqman qPCR reaction with Cruzi 1, 2, and 3 primers and probe (Piron et al., 2007; Duffy et al., 2013). This approach has previously been shown as both sensitive and specific for *T. cruzi* (Schijman et al., 2011). Reactions consisted of 5 μL of template DNA, primers at a final concentration of 0.75 μM each, 0.25 μM of probe, and iTaq Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA), in a total volume of 20 μL in a Stratagene MxPro3000 instrument (Agilent Technologies, Santa Clara, CA), following previously described thermocycling parameters (Duffy et al., 2013), except with a reduced, 3-min, initial denaturation. The DNA extracted from *T. cruzi* strain Sylvio X10 (American Type Culture Collection, Manassas, VA), as well as DNA extracted from *T. cruzi*-positive kissing bugs collected in Texas (*Triatoma gerstaeckeri*, *Triatoma lecticularia*, and *Triatoma sanguisuga*), served as positive controls. After each reaction, the machine-calculated threshold was visually confirmed as reliable, and all reaction curves were visually checked for appropriate shape indicating successful amplification. Internal laboratory validation tests have defined cycle threshold (Ct) values indicative of positive (<31), negative (>33), and equivocal (between 31 and 33) status.

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

2.2.2. Strain-typing using TcSC5D PCR

All positive samples that yielded Ct values lower than approximately 25 on the qPCR were subjected to an additional PCR and subsequent DNA sequencing in order to determine the *T. cruzi* discrete typing unit (DTU). We performed a PCR to amplify the TcSC5D putative sterol oxidase gene (Cosentino and Agüero, 2012). Reactions consisted of 1 μL extracted DNA, 0.75 μM of each primer, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a total volume of 15 μL. PCR products were visualized with gel electrophoresis as described above. Target amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and bi-directionally sequenced on either an ABI Prism® 3130 Genetic Analyzer or ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Florida DNA Sequencing Core Laboratory (Gainesville, FL). Resulting sequences and chromatographs were reviewed visually using Geneious version R7 (Kearse et al., 2012) and MEGA version 6 (Tamura et al., 2013) to confirm quality, align forward and reverse sequences, and examine the locations of key SNPs used to designate DTU (Cosentino and Agüero, 2012). Representative *T. cruzi* sequences were deposited to Genbank (Accession nos. KU705713-KU705715).

2.3. Histology

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

3. Results

3.1. T. cruzi detection

A total of 226 wild animals were included in our study, including bobcats (n = 14), coyotes (n = 84), foxes (n = 58), and raccoons (n = 70) from 25 counties in central and south Texas (Fig. 1). Paired right ventricle and apex samples were available from 219 animals, right ventricle only from 3 animals, and apex only from 4 samples. Blood clots from within heart chambers were recovered from 56 individuals. After the initial screening with qPCR, 45 samples were assigned an equivocal status and therefore subjected to additional, independent PCR, in which 41 (91.1%) were determined to be positive. Based on the results of our diagnostic algorithm, we determined that 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot; Table 1). Fifteen of the twenty-five sampled counties had at least one animal test positive for *T. cruzi* (Fig. 1).

3.2. T. cruzi tropism in hearts and blood

In comparisons of parasite molecular detection among multiple sample types from the same individuals (Fig. 2), across 219 individuals in which both right ventricle and apex were tested, we found that analysis of the two different tissues yielded the same result for 194 animals (88.6%), whereas the right ventricle tested positive and apex tested negative for 9 animals (4.1%) and the right ventricle tested negative and apex tested positive for 16 animals (7.3%). In 54 individuals, blood clot was also tested in addition to right ventricle and apex. We found that the blood clot tested positive when both right ventricle and apex tested positive in 7 of 8 animals (87.5%), whereas the blood clot tested positive when both right ventricle and apex tested negative in 7 of 38 animals (18.4%).

3.3. T. cruzi strain determination

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

3.4. Gross and histopathology

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

4. Discussion

We found widespread *T. cruzi* infection across a central Texas wildlife community (n = 226 individuals of 4 species), in which bobcat, coyote, and fox shared a similar prevalence of infection between 13 and 14%, whereas 70% of sympatric raccoons were infected. Infected animals occurred in 60% of the sampled counties within the 25-county central Texas study region. Previous raccoon studies in Texas have ranged from 24% hemoculture-positive of 25 raccoons sampled in west-central Texas (Schaffer et al., 1978) to 0% seroprevalence in nine raccoons sampled in southern Texas (Burkholder et al., 1980). However, in other southern states, studies based on hemoculture, analysis of blood smears, or serology have revealed infection rates in raccoons as high as 68% (Brown et al., 2010; Kribs-Zaleta, 2010). Similar to our estimate of infection prevalence based on molecular detection of the parasite, previous coyote studies in Texas have found 12.8%–14% seroprevalence (Burkholder et al., 1980; Grögl et al., 1984). Previous bobcat testing in Texas included 2 bobcats, one of which was seropositive (Burkholder et al., 1980). Although *T. cruzi* antibodies have been documented in foxes from other states (Rosypal et al., 2007; 2010), we are not aware of previous testing in foxes in Texas.

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

As shown previously in raccoons, parasite distribution may not be uniform throughout the heart. In a comparison of five separate biopsy sites within raccoon hearts, *T. cruzi* DNA was amplified more frequently from the endocardium of the interventricular septum, right ventricular wall, and the left atrium relative to the left ventricular wall and right atrium, but many infected individuals were positive at all tested sites (James et al., 2002). With over 88% congruence in infection status across two sample types, we found that both right ventricle and apex were nearly equally suitable samples for PCR-based detection of *T. cruzi* in naturally-infected wildlife. Further, we found that testing of blood revealed infection

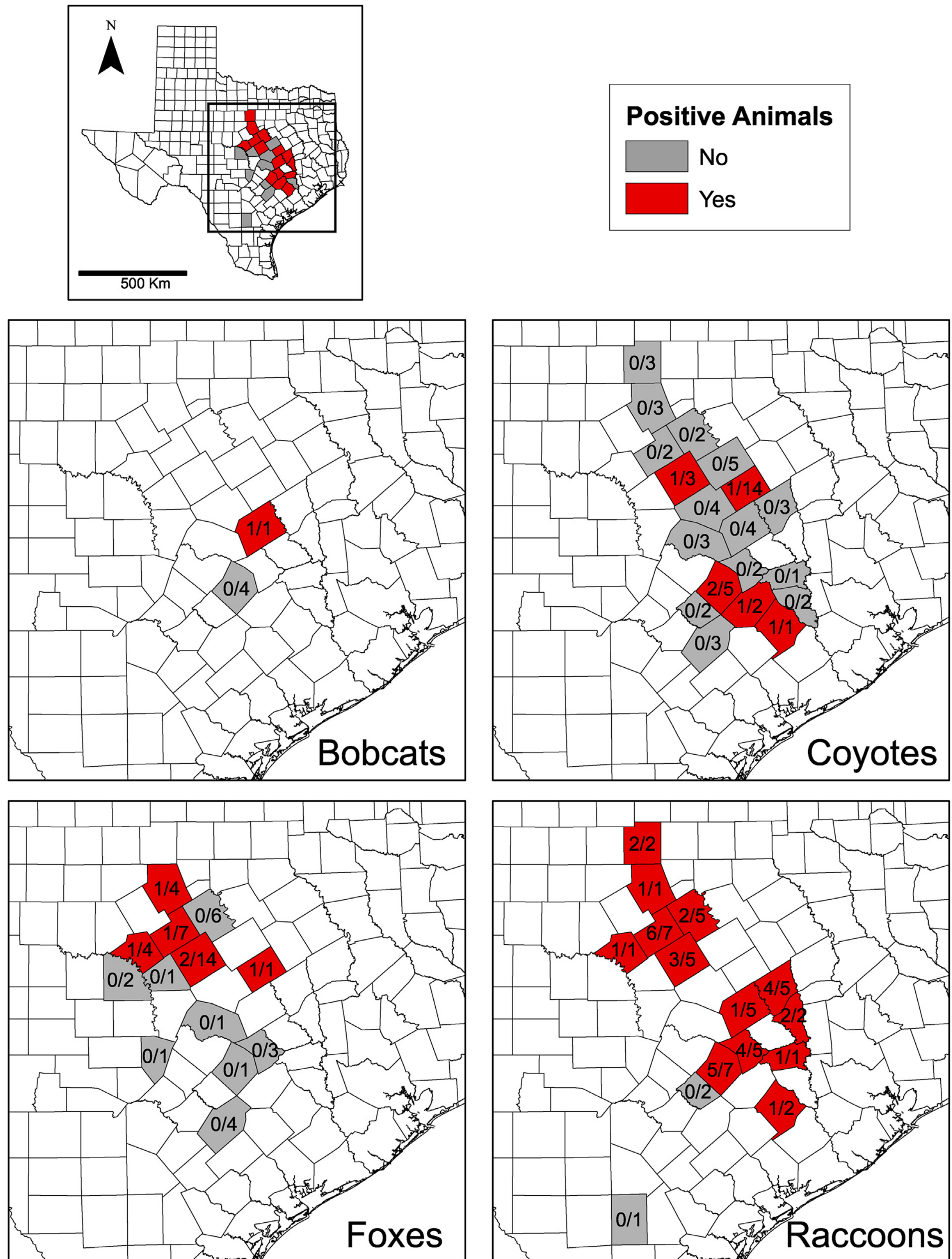

Fig. 1. Spatial occurrence and distribution of *T. cruzi* infected, hunter-harvested wildlife, 2014. Number of infected over total number of that species tested are shown by county.

in over 18% of animals with negative heart tissues, suggesting that these animals were acutely-infected or parasitemic.

Although *T. cruzi* DNA was readily detected in heart tissue of the wildlife community, no amastigotes were seen in the survey of right ventricle sections, and only 21.1% of PCR-positive animals that were histologically examined were characterized by minimal to

Table 1
T. cruzi infection prevalence as determined by PCR in hunter-harvested wildlife of central Texas, 2014. Number of positive samples over total number of that type of sample is shown with infection prevalence in parenthesis.

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

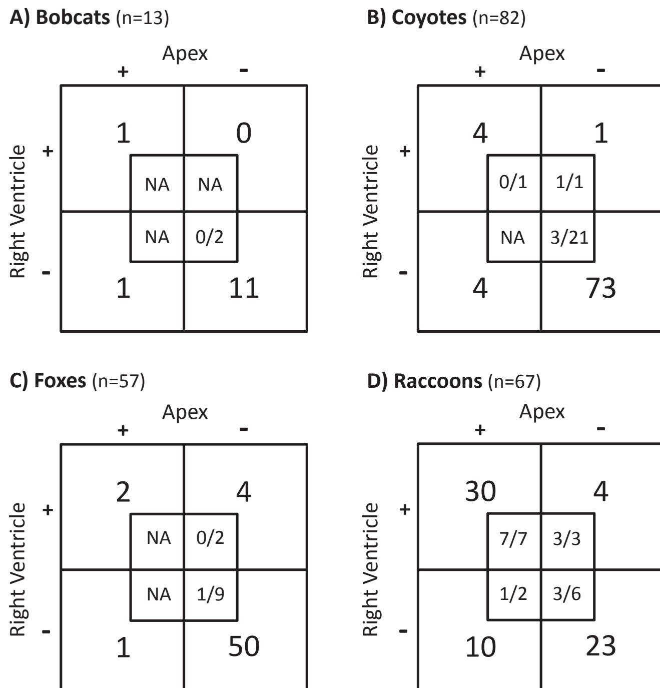

Fig. 2. Tissue-specific PCR results of *T. cruzi* testing. For each species, the outer 2 × 2 table cell values represent the number of animals with all possibilities of results based on analysis of right ventricle and apex. The nested cell data include the *T. cruzi* infection status of blood clots (number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

mild lymphoplasmacytic myocarditis. The minimal cardiac pathology may reflect that only a limited section of the heart was histologically evaluated, with only single section of right ventricle trimmed in. Additionally, a limited number of wildlife individuals were histologically examined. One previous study of infected raccoons also failed to detect more than “mild, multifocal, and interstitial inflammation”, possibly suggesting that the *T. cruzi* strain may not cause severe lesions in raccoons (Pietrzak and Pung, 1998). In contrast, a study of experimentally infected raccoons showed that a small sample of acutely infected raccoons displayed more severe lesions than chronically infected raccoons, and the authors suggested that wild-trapped raccoons displaying mild lesions may therefore be in the chronic stage of infection (Roellig et al., 2009b). Previous experimental infection of mice with a *T. cruzi* isolate from a raccoon from South Carolina resulted in PCR-detectable DNA in some tissue samples, but no parasites were observed in histological sections (Yabsley and Noblet, 2002). Alternately, our molecular results may reflect the presence of acutely infected, parasitemic animals with positive blood within vessels in the heart and absence of infection of the cardiac myocytes. However, at least four animals in our study had negative blood clot samples and positive heart tissue, suggesting that the cardiac muscle itself was infected. The relatively high infection prevalence in raccoons could be explained if they are able to sustain infections without severe chronic pathological implications, which is supported by the lack of cardiac lesions in our histological investigation. This has been shown for dogs (Barr et al., 1991b) and may therefore extend to other Canidae members, such as coyotes and foxes, which similarly failed to show cardiac lesions in our study. In contrast, the animals with severe myocarditis and those that died as a result of infection would be unlikely to be harvested by recreational hunters and therefore are not represented in our sample. However, it could also be possible that infected animals become ill and more susceptible to hunting; in which case, they would be overrepresented in this sampling effort. The clinical effects of *T. cruzi* infection in wildlife species require further study.

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

Current classification of the within-species genetic diversity of *T. cruzi* distinguishes six main discrete typing units (DTUs), TcI through TcVI (Zingales et al., 2009), which appear to have different ecological and epidemiological associations, as well as different geographical distributions (Zingales et al., 2012). Until recently, previous work in the southern United States has revealed exclusively TcI and TcIV in vector and wildlife samples (Roellig et al., 2008, 2013), and the small number of infected humans that have been typed were all TcI (Roellig et al., 2008), although a recent study of small rodents in Louisiana has detected a low prevalence of TcII (Herrera et al., 2015). In our study the vast majority of *T. cruzi* that infected the raccoons was TcIV (90.1% of animals), and one individual harbored a mixed TcI/TcIV infection. Similarly, a previous study has shown TcIV circulating in raccoons ($n = 5$) in the western part of Texas (Charles et al., 2013), and TcIV was the predominant strain found in raccoons in Georgia, Florida, and other eastern states (Roellig et al., 2013). The strain-typing assay we employed was based upon the amplification and sequencing of a single gene target (the TcSd5d gene (Cosentino and Agüero, 2012)) and therefore is an economical and efficient method relative to strain-typing approaches based upon multilocus sequence typing. However, this method was developed for typing of pure *T. cruzi* cultures, and does

not appear to be very sensitive for field-collected samples that contain mixed populations of DNA (i.e., a majority host DNA and minority parasite DNA). Among our samples, some were determined to be positive for *T. cruzi* on the basis of qPCR or conventional PCR to target a small gene fragment, yet negative on the strain-typing assay. The degree to which the proportion of TcI, TcIV, and mixed infections that we detected in raccoons reflects any preferential amplification in this assay is unknown. It has previously been shown that laboratory mice and rats infected with what is now known as TcIV from wildlife the US did not experience severe morbidity or mortality (Roellig and Yabsley, 2010), which may indicate a less virulent strain that could more easily be sustained in natural cycles. Nonetheless, while the significance of TcIV from a human and canine health perspective in the southern states is yet to be established, our identification of the raccoon as a reservoir of TcIV provides increased understanding of the natural history of this parasite strain.

Although the exact risk of *T. cruzi* transmission from infected wildlife to hunters is unknown, it has already been suggested by others that infected wildlife do pose a risk to hunters during field-dressing and skinning due to potential direct contact with infected materials (Yeager, 1961; Bern et al., 2011; Garcia et al., 2015). This study documents infection in a variety of wildlife species that are commonly hunted in Texas and other areas. Public health outreach efforts—including education about Chagas disease, triatomine bugs, and use of personal protective equipment when handing animals—directed to hunters should be undertaken in order to reduce risk to human public health.

Conflicts of interest

None.

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