Trypanosoma cruzi infection in mammals in Florida: New insight into the transmission of *T. cruzi* in the southeastern United States

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ABSTRACT

In Latin America, synanthropic mammalian reservoirs maintain *Trypanosoma cruzi*, a parasitic protozoan, where they facilitate the transmission of the parasite to humans and other reservoir hosts in peridomestic settings. In the United States, raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*) are known synanthropic *T. cruzi* reservoir hosts; however, the role these species have in the peridomestic transmission cycle in the US is not well understood. This study aimed to identify the suite of mammalian reservoirs of *T. cruzi* in Florida. We also compared infection prevalence in raccoon populations sampled from within and outside of the estimated distribution of the common *T. cruzi* vector in Florida to gain insight into how the arthropod vector distribution impacts the distribution of infected reservoirs in the state. Finally, to investigate the impact of peridomestic landscapes on parasite prevalence, we compared the prevalence of *T. cruzi*-infected raccoons and opossums across five paired peridomestic and sylvatic sites. We live-trapped and collected peripheral blood samples from 135 raccoons, 112 opossums, 18 nine-banded armadillos (*Dasypus novemcinctus*), and nine species of rodents in north central Florida. Using quantitative PCR methods, we found that raccoons (42.2%, 95% CI [34.2–50.7%]) and opossums (50.9%, 95% CI [41.8–60.0%]) were infected with *T. cruzi* and the prevalence across habitats was similar for both raccoons (peridomestic: n = 77, 44.2%, 95% CI [33.6–55.3%], sylvatic: n = 58, 39.7%, 95% CI [28.1–52.5%]) and opossums (peridomestic: n = 66, 48.5%, 95% CI [36.8–60.3%], sylvatic: n = 46, 54.3%, 95% CI [40.2–67.8%]). Raccoons sampled outside the estimated distribution of *Triatoma sanguisuga* were not infected with *T. cruzi* (n = 73, 0.0%, 95% CI [0.0–5.0%]). Our study did not indicate that peridomestic habitats in Florida maintained a higher infection prevalence than their sylvatic counterparts; however, we did find a difference in prevalence within vs. outside the estimated vector distribution in Florida.

1. Introduction

Vector-borne zoonotic pathogen transmission often depends on complex dynamics among vector, host, and ecosystem (Bradley and Altizer, 2007; Gürtler and Cardinal, 2015; Haydon et al., 2002; Hudson et al., 2002; Kilpatrick and Randolph, 2012; Woolhouse et al., 2005). *Trypanosoma cruzi*, a vector-borne protozoan zoonotic pathogen and the causative agent of Chagas disease in humans and canines across the Americas, is no exception (Bern et al., 2019; Rassi et al., 2012). Chagas disease in the United States (US) is an emerging infectious disease for humans and domestic canines; however, its transmission is complex and poorly understood (Beatty and Klotz, 2020; Bern et al., 2011, 2019; Rassi et al., 2012). The parasite, *Trypanosoma cruzi*, is a generalist, and in the US infects at least 27 mammalian species across a wide variety of habitats and landscapes (Bern et al., 2011, 2019; Brown et al., 2010; Busselman and Hamer, 2022; Hodo and Hamer, 2017; Jansen and Roque, 2010). In the US, *T. cruzi* is maintained in wildlife populations via consumption of the infected vector, cutaneous vector-borne transmission, and vertical transmission pathways (Bern et al., 2011, 2019; Brown et al., 2010; Hodo and Hamer, 2017; Torhorst et al., 2022).

Human modified landscapes are known to amplify the transmission of vector-borne zoonotic disease (Bradley and Altizer, 2007; Brearley et al., 2013; Cardozo et al., 2021). The aggregation of reservoir hosts at concentrated resources near humans can cause an increase in vector-borne disease transmission as higher reservoir densities can attract and maintain high vector densities and therefore maintain transmission and increase the risk of spill over to humans (Bradley and Altizer, 2007; Brearley et al., 2013; Cardozo et al., 2021; Civitello et al., 2018; Eisen et al., 2012; Ostfeld, 2011; Shaman, 2007). For *T. cruzi*, one mechanism of pathogen amplification could be the aggregation of human-adapted species like raccoon (*Procyon lotor*) and Virginia opossum (*Didelphis virginiana*), in habitats where the Triatomine kissing bugs occur (Bern et al., 2019; Bradley and Altizer, 2007; Fidino et al., 2016, 2021; Prange et al., 2004; Wright et al., 2012). In the US there are 11 described species of Triatomine, with 10 species from the genus *Triatoma* and one species from the genus *Paratriatoma* (Bern et al., 2011, 2019; Klotz et al., 2014). In Florida, *Triatoma sanguisuga* is the common *T. cruzi* vector (Klotz et al., 2014). This vector commonly feeds on human adapted species like raccoons and opossums, and is commonly found in human households including in the southeastern US (Dumonteil et al., 2020; Waleckx et al., 2014). The confluence of *T. sanguisuga* and human adapted wildlife near human modified landscapes could lead to the amplification of the parasite around human households.

The complexity of the *T. cruzi* disease system is evident throughout its endemic range in the Americas. In Latin America, anthropogenic landscape alterations are known to facilitate the transmission cycle of *T. cruzi* to humans, human-commensal wildlife, and companion animals, such as domestic dogs (*Canis familiaris*) via congregation of triatomine vectors at a central location (Alarcón de Noya et al., 2022; Cardozo et al., 2021; Castañera et al., 1998; Gürtler and Cardinal, 2015; Jansen et al., 2015; Jansen and Roque, 2010; Kjos et al., 2009; Noireau et al., 2009; Vaz et al., 2007). In the US far less is understood about how *T. cruzi* reservoirs impact the transmission of the parasite (Bern and Montgomery, 2009; Dorn et al., 2007; Gürtler and Cardinal, 2015; Hodo and Hamer, 2017; Majeau et al., 2020; Montgomery et al., 2016; Rodriguez et al., 2021). Recent surveillance efforts in the southwestern US aid our understanding of *T. cruzi* transmission in this ecologically unique region (Hodo and Hamer, 2017; Rodriguez et al., 2021); however, to better understand how anthropogenic changes to the landscape may impact the transmission of the parasite in other parts of the US, further reservoir and vector surveillance is needed, particularly in the southeastern US. In the southeastern US, human commensal mammals are infected with *T. cruzi* across a wide variety of landscapes including sylvatic, peridomestic, and urban settings (Bern et al., 2011, 2019; Hodo and Hamer, 2017; Majeau et al., 2020). Furthermore in the southeastern US, there are reports of *T. cruzi* infected raccoons in urban New Orleans, Louisiana, indicating a viable transmission cycle in locations of high human density which has important One Health implications (Majeau et al., 2020). Given the complexity of the disease system, we endeavored to understand the role of vector and host in the subtropical ecosystem of Florida.

In this study we sought to understand how human adapted mammalian reservoirs and vectors impact the distribution and spatial ecology of *T. cruzi*. We used a multiscale spatial approach to determine: (a) which mammalian species are infected with *T. cruzi* in Florida, (b) if the estimated regional distribution of the triatomine vector influences the distribution of infected mammalian reservoirs in Florida, and (c) whether infection among human commensal mammals is impacted by their association with human households as predicted by studies of other wildlife diseases. We hypothesized that infected raccoons would not be found outside of the known range of *T. sanguisuga* in Florida. We further hypothesized that peridomestic habitats close to human households would facilitate high infection prevalence and influence the infection status of both raccoons and opossums. This study sought to further the current understanding of the spatial disease ecology of *T. cruzi* among Florida's native reservoir hosts and the distribution of the parasite at the periphery of its southern distribution. As an emerging vector-borne zoonotic pathogen in the US, an understanding of the prevalence in reservoir hosts has critical human and veterinary health implications.

2. Methods

2.1. Study design

In this study, we sampled mesomammals and rodents between January 2021 and October 2021 in the Gulf Coast Forest ecosystem of north central Florida. To address the first objective in this study, raccoons were sampled outside of the estimated distribution of the common triatomine vector in Florida, *T. sanguisuga*, from six key or barrier islands in south Florida. Raccoons were sampled from these areas in order to understand if the regional distribution of the vector influenced the distribution of infected reservoirs (Fig. 1). The distribution of triatomines in Florida was adapted from multiple sources (Beatty, 2022; Beatty et al., 2022; Flores-Ferrer et al., 2018; Gourbière et al., 2012; Thurman et al., 1948) (Fig. 1); however, Pinellas, Sarasota, and Broward County, FL was excluded due to the ambiguity of the species identification of the collected specimen (Thurman et al., 1948). Raccoon collection sites from outside of the triatomine range were selected by USDA/APHIS/Wildlife Services based on their program needs.

To address the subsequent objectives in this study, mammals were sampled at 10 sites categorized as either peridomestic or sylvatic, making five pairs of peridomestic and sylvatic sites. A paired sites sampling scheme was used in order to understand if reservoir host infection prevalence was higher in human modified vs. sylvatic landscapes. Scientific collection permits were obtained from the Florida Fish and Wildlife Conservation Commission, and permission was obtained from the appropriate land managing agencies or private landowners at locations where sampling took place.

Peridomestic sites were located at households with known triatomine domiciliation, human-triatomine interaction within a household, or triatomines observed at close proximity (<50m) to the household. At two of the peridomestic sites, the inhabitants of the household were either bitten by a kissing bug and/or found kissing bugs close to their sleeping quarters. Mammals trapped at peridomestic sites had a range of capture distance between 87m and 232m from the household (Fig. 1). Location information of peridomestic households was defined at the county level to maintain privacy of the landowners.

Sylvatic sites were paired with peridomestic sites based on habitat similarity and distance from the peridomestic site. The centroid of each sylvatic site was at least 2000m from the paired peridomestic site to exclude capture of the same individuals. Raccoons and opossums in sylvatic sites had a range of distance of capture to the nearest inhabited household between 599m and 2,004m (Fig. 1). Habitat types across both peridomestic and sylvatic sites included mixed hardwood-pine forests, natural pine forests, hardwood hammock, hardwood swamp, and bottom land swamp. Raccoons and opossums were the only two species with sufficient sample sizes that allowed for comparison of prevalence between peridomestic and sylvatic habitats.

2.2. Animal handling and sample collection

The trapping, handling, and sample collection methods of the live sampled mammals were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC Study #202011195), the Florida Fish and Wildlife Conservation Commission (Permit #LSSC-21-00021 and LSSC-19-00008), and the governing biologist of each state managed property. Raccoon, Virginia opossum, coyote (*Canis latrans*), and nine-banded armadillo (*Dasypus novemcinctus*) from north central Florida were live trapped using 20 or 21 Tomahawk live traps (series 106 and 207) (Tomahawk Live Trap, Tomahawk, WI), baited with both wet and dry cat food, over an eight-to-twelve-night period. Ground dwelling rodents were live trapped using 100 Sherman live traps baited
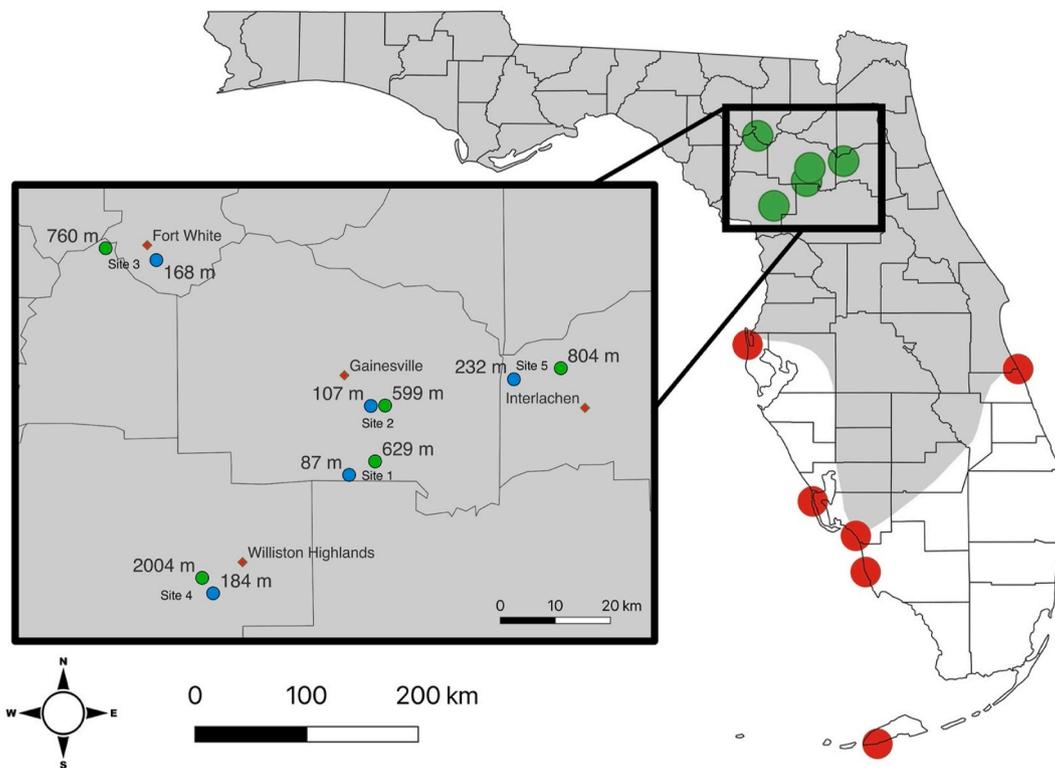
Fig. 1. Sampling scheme for the multiscale spatial analysis depicting the sampled sites in Florida. In the state-wide map, green circles indicate a sampled area within the estimated distribution of *Triatoma sanguisuga* in Florida (gray shaded region) and red circles indicate a sampled site outside of this distribution. The inset depicts the paired site design (peridomestic = blue, sylvatic = green, nearest township to the sampled area = red diamonds, and the average distance of capture from the nearest inhabited household = site associated meter distance). The map was created using QGIS Geographic Information System version 3.22.5-Białowieża, htt p://qgis.osgeo.org. County layers map (TIGER/Line Shapefile, 2016, state, Florida, Current County Subdivision State-based) and city locations (TIGER/Line Shapefile, Current, State, Florida, Places) was accessed from the United States Census Bureau, https://catalog.data.gov/dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with cotton balls and mixed bird seed (H.B. Sherman Traps, Tallahassee, FL, USA). Eastern gray squirrels (*Sciurus carolinensis*) were trapped using eight Tomahawk live traps (series 102) baited with corn, whole peanuts, and sunflower seeds. Gray squirrel traps were set each morning at sunrise then closed at sunset and were monitored for captures every 4 h. Samples were also collected from hunter harvested eastern gray squirrels from one sylvatic site and one peridomestic site. Traps for meso-mammals and ground dwelling rodents were opened each evening 1 h before sunset and closed prior to sunrise.

To facilitate handling of mesomammals, each sampled species was chemically immobilized following the methods found in Kreeger and Arnemo (2012). Virginia opossums were chemically immobilized using a combination of 10 mg/kg Ketamine and 2 mg/kg Xylazine, raccoons were immobilized using a combination of 3 mg/kg Telazol and 2 mg/kg Xylazine, and nine-banded armadillos were immobilized using a com-bination of 40 mg/kg Ketamine and 1 mg/kg Xylazine. Following immobilization, biological samples were collected from each species, and each was immediately released or allowed to fully recover prior to their release at the original location of capture. The single coyote captured was manually restrained, and its eyes were covered to reduce stress. Rodents were manually restrained using the scruffing method. Gray squirrels were manually restrained and handled following the methods of Koprowski (2002). Each sampled individual was semi-permanently marked using hair dye to identify previously trapped individuals and prevent rehandling animals. The marks remained throughout the trapping period, and any recaptured individuals were immediately released at the location of capture.

Peripheral blood was collected from mesomammals by puncturing the skin between the middle digits of the forepaw with a medical lancet. Peripheral blood was drawn from ground dwelling rodents by

puncturing the submandibular vein using a medical lancet and from squirrels by clipping the toenail from the hind paw of each squirrel. One to 3ul of peripheral blood was collected onto a Nobuto strip and stored at room temperature until extraction (Sterlitech Cooperation, Auburn, WA, USA).

Raccoons collected outside the estimated vector distribution were sampled by USDA/APHIS/Wildlife Services personnel during the regular course of their duties from six barrier and key islands of south Florida (Honeymoon Island State Park, Cayo Costa State Park, Lovers Key State Park, Sebastian Inlet State Park, Rookery Bay National Estuarine Research Reserve, Naval Air Station Key West). These raccoons were euthanized during control efforts on sea turtle and shorebird breeding habitat and beaches in accordance with the practices of USDA/APHIS/Wildlife Services. Armadillos were also sampled by USDA/APHIS/Wildlife Services as part of routine control efforts at MacDill Naval Air Station in Tampa, Florida and Naval Air Station Pensacola, Florida. Peripheral blood from raccoons and armadillos was collected by USDA/APHIS/Wildlife Services personnel via needle and syringe inserted into the heart cavity (USDA, 2017, 2013). Several drops of blood from each sampled raccoon and armadillo were placed on individual Nobuto strips, which were then stored at room temperature until total DNA extraction.

2.3. DNA extraction

Total DNA was extracted using a Gentra PureGene extraction kit following the manufacturer's protocol with the following minor modi-fications (Qiagen Puregene, Qiagen, Hilden, Germany). Peripheral blood on Nobuto strips was incubated for 30-min in 300uL of red blood cell lysis solution. The Nobuto strip was then incubated for 24 h in 600uL of PureGene cell lysis solution at 56 °C to lyse the white blood cells bound

to the Nobuto strip. Finally, 10uL of Proteinase K (ProtK) (20mg/uL) was added to each sample containing cell lysis solution and Nobuto strip and incubated at 56 °C for 24 h (Themo Fisher Scientific, Waltham, MA). Total DNA was washed and isolated following the manufacturer's recommended protocol (Qiagen Puregene, Qiagen, Hilden, Germany). Pelleted DNA was resuspended in 50uL of DNA hydration solution (TE buffer) and at 4 °C stored short term until amplification.

2.4. Trypanosoma cruzi detection and DTU determination

Trypanosoma cruzi infection was identified using a qPCR (quantitative polymerase chain reaction) assay which detected the nucleic acid of the parasite following previously described methods (Duffy et al., 2013; Ramírez et al., 2017). We inferred *T. cruzi* infection based on the identification of *T. cruzi* nucleic acid in peripheral blood (Curtis-Robles et al., 2018a; Torhorst et al., 2022; Zecca et al., 2020). Detection was based on a 166bp segment of *T. cruzi* satellite DNA (satDNA) amplified using a 2x Roche Fast Start universal master mix with the forward primer Cruzi1, reverse primer Cruzi2, and TaqMan probe Cruzi3 PrimeTime® 5′ 6-FAMTM/ZENTM/3′ IB®FQ (IDT, Coralville, Iowa). The 166bp satDNA segment was from the 195bp tandem repeating satDNA unit that has been used as a molecular diagnostic tool to identify *T. cruzi* in infected domestic dogs, wildlife, and triatomines (Curtis-Robles et al., 2016, 2017, 2018b; Torhorst et al., 2022; Zecca et al., 2020). Each reaction (20μL) contained master mix (18μL) and host template DNA (2uL) (20ng/uL). The prepared master mix also contained a separate exogenous amplification control (10x Exo IPC Mix, 50 IPC Exo) which contained primers and a probe, labeled with fluorophore VIC (5′ - VIC™/TAMRA™ Quencher - 3′) (Thermo Fisher Scientific, Waltham, MA). An exogenous amplification control was used in this reaction to identify host template DNA amplification inhibition, which could have led to false positives. A negative control (blanked with molecular grade water), an exogenous amplification positive control (containing molecular grade water and exogenous amplification control), and a positive control dilutions series from a cell culture isolate of TcI were used to verify each assay. To exclude contamination by the positive control, the positive controls and master mix reagents were stored separate from the tested gDNA samples. Each rtPCR *T. cruzi* detection assay was performed using an ABI 7500 FAST Real Time PCR machine (Thermo Fisher Scientific, Waltham, MA).

A sample was considered *T. cruzi* infected when the Ct value was ≤38 (Beatty et al., 2021; Torhorst et al., 2022). Any sample that amplified between the Ct values of 38–40 was labeled as suspect positive, and these samples were rescreened. If amplification occurred at the second attempt, these samples were then considered *T. cruzi* infected (Beatty et al., 2021; Torhorst et al., 2022). Samples that did not amplify the exogenous control were rescreened at a template DNA dilution series of 1:5 and 1:10 to eliminate amplification inhibition within the sample.

The samples which screened positive for *T. cruzi* were then subject to DTU determination via a multi-stepped, multiplexed qPCR (Beatty et al., 2021; Cura et al., 2015; Torhorst et al., 2022). Each DTU determination assay was performed using an ABI 7500 FAST Real Time PCR machine (Thermo Fisher Scientific, Waltham, MA). For each reaction, 2uL of template (18μL) (20ng/uL) was added to each well with the prepared master mix. The following reactions were performed to identify the infecting DTU: SL-MTq intended to identify TcI, the TcII/TcV/TcVI complex, and the TcIII/TcIV complex, while the 24Sα-III/IV MTq was used to differentiate TcIII from TcIV (Cura et al., 2015). The 18S-COII MTq assay was not used in this study, as there were no florescent indications of this DTU complex (Cura et al., 2015). DTU identification was discontinued after 3 unsuccessful amplification attempts. Each assay was verified with a water negative control and the positive controls of the DTU intended to be identified in the specified assays described in Cura et al. (2015).

2.5. Statistical analysis

2.5.1. Host prevalence and confidence interval calculations

Prevalence values in this study were calculated by dividing the number of infected individuals by the total number of individuals sampled within the estimated distribution of the common vector, *T. sanguisuga*, in Florida (Fig. 1). The sample size needed to report host prevalence values was calculated for each sampled species using EPI tools, and true prevalence values reported in Hodo and Hamer (2017), and associated citations (Humphry et al., 2004; Sergeant, 2018). Infection prevalence and Wilson 95% confidence intervals were calculated for those species that achieved true host prevalence detection levels or had at least one individual infected in the sampled population (Brown et al., 2001; Humphry et al., 2004; Sergeant 2018; "Epitools – Calculate confidence limits for a sample prop" n.d.). Wilson 95% Confidence Interval (CI) were calculated for each prevalence value using EPI tools (Brown et al., 2001; "Epitools - Calculate confidence limits for a sample prop," n.d.; Sergeant, 2018).

2.5.2. Kruskal-Wallis test for differences in infection prevalence

A nonparametric Kruskal-Wallis test was performed using EPI tools to identify if statistically significant differences in infection prevalence existed between peridomestic and sylvatic raccoons or opossums (Sergeant, 2018; "Statistical analysis of numeric data," 2022). The response variable in this analysis was infection prevalence per site and the explanatory variables were species (raccoon or opossum) and habitat (peridomestic or sylvatic). In this test a prevalence value was calculated for each species-habitat pair (sylvatic-raccoons, peridomestic-raccoons, sylvatic-opossums, and peridomestic-opossums) at each of the five paired sites, creating 20 individual prevalence values (5 at each paired site for the 2 species). The mean rank score was calculated for each species-habitat pair and analyzed as part of the Kruskal-Wallis test which generated p-values to identify statistical significance (p ≤ .05).

3. Results

3.1. Survey of mammalian reservoirs

In total, 13 species were tested for the presence of *T. cruzi* across the 10 sites in north central Florida. The 13 species sampled includes nine species from the order Rodentia, and four mesomammal species (Table 1). Of the rodents collected, two of the sampled species were infected with *T. cruzi* (Table 1). Cotton mice (*Peromyscus gossypinus*) were infected at a low prevalence (n = 1/170 (0.6%), 95% CI [0.1–3.3%]) but the DTU was not typeable in the one infected individual (Table 1). Eastern gray squirrels were also infected with *T. cruzi* (n = 2/38 (5.3%), 95% CI [1.5–17.3%]) and TcIV was the infecting DTU for both individuals (Table 1). No infected Florida mouse (*Podomys floridanus*) individuals were identified (Table 1). One nine-banded armadillo was infected with *T. cruzi* which was collected from Naval Air Station Pensacola, Pensacola, FL (n = 1/18 (5.6%), 95% CI [1.0–25.8%]), but the infecting DTU was not identified in this individual (Table 1). Virginia opossums (n = 57/112 (50.9%), 95% CI [41.8–60.0%]) and raccoons (n = 57/135 (42.2%), 95% CI [34.2–50.7%]) collected from north central Florida were infected with *T. cruzi* at the highest prevalence values across the species collected (Table 1). Of the infected opossums the only DTU identified in the infected population was TcI (Table 1). From infected raccoons, the infecting DTU type was identified as either TcI or TcIV and no mixed infections were identified (Table 1). For raccoons, TcI was only found in peridomestic habitats, and TcIV was found in both habitats. The raccoons sampled from the key or barrier islands were not included in this estimate, as they were outside of the currently understood distribution of the vector in Florida. Only four of 13 sampled species had sample sizes sufficient to estimate true host infection prevalence (Virginia opossum, raccoon, cotton mouse, and the Florida mouse) (Table 1). The other nine species did not have adequate sample

Table 1

Prevalence and DTU identification of *Trypanosoma cruzi* in reservoir hosts native to Florida. Species with an asterisk are known *T. cruzi* reservoirs; comprehensive reservoir lists have been provided by Bern et al. (2011, 2019) and Hodo and Hamer (2017). Citations provide the true prevalence value that was used to calculate the sample size needed to estimate true host infection prevalence in this study. Those species where no induvial were found to be infected and that did not reach the sample size needed to report true host prevalence are reported as N/A.

Species	Individuals Infected/ Captured	Infection prevalence [Wilson 95% CI]	DTU Identified, Prevalence, [Wilson 95% CI]	Sample size needed to estimate true host infection prevalence
Virginia opossum * (*Didelphis virginiana*)	57/112	50.9% [41.8%–60.0%]	TcI, 55/112, 49.1% [40.0–58.2%]	36 Parrish and Mead (2010)
Raccoon * (*Procyon lotor*)	57/135	42.2% [34.2–50.7%]	TcI, 3/135, 2.2%, [0.8–6.3%] TcIV, 8/135, 5.9%, [3.0–11.3%]	67 Curtis-Robles et al. (2016)
Nine-banded armadillo * (*Dasypus novemcinctus*)	1/18	5.6% [1.0–25.8%]	N/A	34 Hodo and Hamer (2017)
Coyote * (*Canis latrans*)	0/1	N/A	N/A	N/A
Eastern gray squirrel * (*Sciurus carolinensis*)	2/38	5.3% [1.5–17.3%]	TcIV, 2/38, 5.3%, [1.5–17.3%]	42 Hodo and Hamer (2017)
Cotton mouse * (*Peromyscus gossypinus*)	1/170	0.6% [0.1–3.3%]	N/A	66 Herrera et al. (2015)
Eastern woodrat * (*Neotoma floridana*)	0/9	N/A	N/A	N/A
Florida mouse (*Podomys floridanus*)	0/63	0% [0.0–5.7%]	N/A	42 Hodo and Hamer (2017)
Golden mouse (*Ochrotomys nuttalli*)	0/8	N/A	N/A	N/A
Eastern harvest mouse (*Reithrodontomys humulis*)	0/2	N/A	N/A	N/A
Hispid cotton rat * (*Sigmodon hispidis*)	0/10	N/A	N/A	N/A
House mouse * (*Mus musculus*)	0/6	N/A	N/A	N/A
Norway rat * (*Rattus norvegicus*)	0/1	N/A	N/A	N/A

sizes to estimate true host infection prevalence.

3.2. Trypanosoma cruzi infection prevalence inside and outside the vector distribution

We did not find evidence of *T. cruzi* infection in raccoons outside the estimated distribution of *T. sanguisuga* in Florida. An infection prevalence of 42.2% (n = 135, 95% CI [34.2–50.7%]) was identified from the raccoons collected within the distribution of the vector in Florida (Table 1) but no individuals (n = 73, 0.0%, 95% CI [0.0–5.0%]) were found to be infected from the six sampled sites on key or barrier islands of south Florida.

3.3. Influence of habitat on Trypanosoma cruzi in wildlife

3.3.1. Prevalence in peridomestic vs sylvatic Virginia opossums

In total, 112 adult and subadult Virginia opossums were sampled, and 57 individuals were infected with *T. cruzi* in north central Florida (50.9%, 95% CI [41.8–60.0%]). Infected opossums were found across the sampled time period and at each of the five paired sites (Table 2). The infection prevalences for peridomestic (n = 66, 48.5%, 95% CI [36.8–60.3%]) and sylvatic opossums (n = 46, 54.3%, 95% CI [40.2–67.8%]) were not statistically different (Table 2). In the peridomestic locations, the highest infection prevalence was identified at site 3 (84.6%, 95% CI [57.8–95.7%]), and the lowest prevalence was identified at site 5 (22.2%, 95% CI [9.0–45.2%]) (Table 2). Across the sylvatic sites, the highest infection prevalence was identified at site 5 (100.0%, 95% CI [56.6–100.0%]) and lowest at site 4 (25.0%, 95% CI [4.6–69.9%]) (Table 2). The largest difference in the infection prevalence between paired peridomestic and sylvatic sites was identified at site 5 (Table 2). A Kruskal-Wallis analysis indicated that there was no significant difference in infection prevalence between the species-habitat combinations ($\chi^2(3) = 2.33$, $p = .507$) (peridomestic-opossum: mean rank score = 11.3, sylvatic-opossum: mean rank score = 13.3).

Table 2

Infection prevalence for Virginia opossums across paired sites. Paired sites are numbered with their corresponding ID. The location of each paired site is referenced in Fig. 1.

Paired site ID:	Sampled individuals:	Peridomestic infection prevalence [Wilson 95% CI]	Sampled individuals:	Sylvatic infection prevalence [Wilson 95% CI]
1	4	50.0% [15.0–85.0%]	8	50.0% [21.5–78.5%]
2	17	47.1% [26.2–69.0%]	12	50.0% [25.4–74.6%]
3	13	84.6% [57.8–95.7%]	17	52.9% [31.0–73.8%]
4	14	50.0% [26.8–73.2%]	4	25.0% [4.6–69.9%]
5	18	22.2% [9.0–45.2%]	5	100.0% [56.6–100.0%]
Total:	66	48.5% [36.8–60.3%]	46	54.3% [40.2–67.8%]

3.3.2. Analysis of raccoons

In this study 135 adult and subadult raccoons were collected and 57 were infected with *T. cruzi* across the paired sites in north central Florida (42.2%, 95% CI [34.2–50.7%]). Infected individuals were identified across the sampled time and at each sampled site (Table 3). The infection prevalence for peridomestic raccoons (44.2%, 95% CI [33.6–55.3%]) and sylvatic raccoons (39.7%, 95% CI [28.1–52.5%]) was not found to be statistically different (Table 3). The highest infection prevalence at peridomestic sites was identified at site 1 (72.0%, 95% CI [52.4–85.7%]) and the lowest at site 5 (22.2%, 95% CI [6.3–54.7%]) (Table 3). Across the sylvatic sites the highest infection prevalence was identified at site 3 (66.7%, 95% CI [30.0–90.3%]) and there were two locations that had similarly low infection prevalence, site 1 (25.0%, 95% CI [8.9–53.2%]) and site 5 (25.0%, 95% CI [7.1–59.1%]) (Table 3). The largest difference between the infection prevalence between paired peridomestic and sylvatic site, was site 1 (Table 3). A Kruskal-Wallis

Table 3
Infection prevalence for raccoons across paired sites. Paired sites are numbered with their corresponding ID. The location of each paired site is further in Fig. 1.

Paired site ID:	Sampled individuals:	Peridomestic infection prevalence [Wilson 95% CI]	Sampled individuals:	Sylvatic infection prevalence [Wilson 95% CI]
1	25	72.0% [52.4–85.7%]	12	25.0% [8.9–53.2%]
2	21	28.6% [13.8–50.0%]	16	37.5% [18.5–61.4%]
3	18	38.9% [20.3–61.4%]	6	66.7% [30.0–90.3%]
4	4	25.0% [4.6–69.9%]	16	50.0% [28.0–72.0%]
5	9	22.2% [6.3–54.7%]	8	25.0% [7.1–59.1%]
Total:	77	44.2% [33.6–55.3%]	58	39.7% [28.1–52.5%]

analysis indicated that there was no significant difference in infection prevalence between the species-habitat combinations ($\chi^2(3) = 2.33$, $p = .507$) (peridomestic-raccoon: mean rank score = 8.0, sylvatic-raccoon: mean rank score = 9.4).

4. Discussion

In Florida, Virginia opossums (50.9%), raccoons (42.2%), nine-banded armadillos (5.6%), eastern gray squirrels (5.3%), and cotton mice (0.6%) were infected with *T. cruzi* (Table 1). This study reports true host infection prevalence for four of the 13 sampled mammal species from north central Florida (Table 1) and is the first report of armadillos, eastern gray squirrels, and cotton mice infected with *T. cruzi* in Florida (Bern et al., 2011; Hodo and Hamer, 2017) (Table 1). Raccoons and opossums are known *T. cruzi* reservoirs (Brown et al., 2010; Hodo and Hamer, 2017), and the reported infection prevalence for these two species appears to be among the highest reported for these species in the US (Brown et al., 2010; Hodo and Hamer, 2017; Majeau et al., 2020; Yabsley and Noblet, 2002). The identified infection prevalence for raccoon and opossums in this study is similar to the serological prevalence of exposure reported in other studies conducted in the southeastern US (Brown et al., 2010; Yabsley and Noblet, 2002). The high *T. cruzi* infection prevalence for raccoons and opossums in this study further emphasizes their importance as wildlife reservoirs in the transmission cycle of this parasite in the southeastern US.

The identification of *T. cruzi* infected armadillos is not surprising as they are a reservoir in the transmission cycle of *T. cruzi* across other endemic regions of North, Central, and South America (Bern et al., 2011; Yeo et al., 2005). However, in this study, armadillos were infected at a low prevalence (5.6%), which suggests they do not play the key role as a reservoir in Florida as they do in other locations across the Americas. In this study temporal variation in blood parasitemia due to the complexity of the *T. cruzi* infection cycle within in the host may have led to an underestimate in the reported prevalence. The infection prevalence identified in armadillos in this study does, however, further reinforce the importance of raccoons and opossums in the transmission of *T. cruzi* in Florida.

In total, nine rodent species were sampled in this study, two of which, eastern gray squirrels and cotton mice, were infected with *T. cruzi* in north central Florida (Table 1). Rodent reservoirs play a critical role in the maintenance and transmission of the *T. cruzi* in the southwestern US, however, we did not find evidence of this relationship in Florida (Bern et al., 2011; Hodo and Hamer, 2017). Eastern gray squirrels are a known *T. cruzi* reservoir in the southeastern US, and this study is the first report of *T. cruzi* infected gray squirrels in Florida (Olsen et al., 1964; Steele and Koprowski, 2001) (Table 1). As eastern gray squirrels are a known human commensal, this synanthropic

mammal species is likely playing a role in the maintenance and transmission of *T. cruzi* in human modified landscapes (Koprowski, 2005; Nixon and Hansen, 1987; Steele and Koprowski, 2001). Previous studies using blood meal analysis of *T. sanguisuga* have shown *T. cruzi* infected *T. sanguisuga* that have taken a blood meal from eastern gray squirrels, reinforcing their role in the transmission cycle of the parasite (Dumonteil et al., 2020; Waleckx et al., 2014).

Our results suggest that cotton mice and Florida mice in Florida do not play a key role in the maintenance and transmission of *T. cruzi* as they do in other southeastern states, particularly Louisiana (Bern et al., 2011; Ghersi et al., 2020; Herrera et al., 2015; Hodo and Hamer, 2017). Although cotton mice were infected with *T. cruzi* in this study it was at a much lower prevalence than in Louisiana (Ghersi et al., 2020; Herrera et al., 2015). These discrepancies could be explained by the differences in each study's sampled locations and by how the vector may be associated with the cotton mice of urban New Orleans, Louisiana versus more rural setting of north central Florida in this study (Ghersi et al., 2020; Herrera et al., 2015). Despite the large sample size, we did not find evidence of infection in Florida mice in this study (Table 1). This mouse species is almost exclusively associated with gopher tortoise burrows. These burrows are utilized by many other species which could provide consistent blood meals to *T. sanguisuga*, a generalist feeder (Klotz et al., 2014; Waleckx et al., 2014). However, the infection status of the Florida mouse would suggest that they do not contact the vector in these burrows, giving us further insight into the ecology of the vector.

This study suggests that the presence or absence of the vector has a direct impact on the distribution of infected reservoirs (Beatty, 2022; Beatty et al., 2022) which further reinforces the strong host-vector relationship between raccoons, opossums, and *T. sanguisuga* in Florida. These findings have human and veterinary health implications because it identifies areas with low transmission risk of *T. cruzi* in Florida. We identified an infection prevalence of 42.2% in raccoons sampled in north central Florida while no infected raccoons were identified from the key and barrier islands of south Florida. We collected an ample sample size of raccoons from both sampled areas to accurately estimate host prevalence values (Humphry et al., 2004; Sergeant, 2018). The sampled locations in the key and barrier islands of south Florida are outside the estimated distribution of *T. sanguisuga* in Florida (Flores-Ferrer et al., 2018; Gourbière et al., 2012; Thurman et al., 1948) (Fig. 1), and located in habitat not well suited for this species. With little known about the ecology of *T. sanguisuga* in Florida, these findings further support the estimated distribution of this triatomine vector in the state.

We do not exclude the possibility that the triatomine vector could be present but not infected with the parasite at these locations. However, investigations in the US and Florida have shown that triatomines are commonly infected with *T. cruzi* (Beatty, 2022; Beatty et al., 2022; Curtis-Robles et al., 2018b; Klotz et al., 2014) suggesting that *T. cruzi* negative raccoons sampled in the barriers islands are not exposed to the triatomine vector. Barrier islands in Florida are not prime habitat for triatomines and are typically small, with sand dunes and mangrove estuaries, tidal flats, and maritime forests with high salinity. Reports of *T. sanguisuga* from the barrier islands of Georgia have been described but these islands are much larger and have more diverse habitats that would allow the vector to establish populations (Roden et al., 2011). In Florida, much of the area surrounding each sampled island is highly urbanized, which could also exclude the vector and sylvatic infected hosts from establishing novel transmission cycles as they disperse on the landscape.

Contrary to our expectations, we did not find a higher infection prevalence in either raccoons or opossums surrounding peridomestic households from north central Florida. Our analysis suggests that human influenced peridomestic habitats do not drive *T. cruzi* prevalence in this region. Among our sampled cohort there was widespread and high infection prevalence among both raccoons and opossums regardless of habitat or proximity to human households in rural north central Florida. While the reason for widespread infection prevalence in north central Florida remains unknown, the Gulf Coast Forest ecosystem has abundant

resources available to species like raccoons and opossums, and therefore these mesomammals occur at high densities in both sylvatic and peri-domestic habitats. In addition, *T. sanguisuga* is also a habitat generalist and establishes itself across a wide variety of habitats and feeds on a wide variety of hosts (Curtis-Robles et al., 2018b; Dorn et al., 2007; Waleckx et al., 2014). The possibility of abundant resources and high densities of *T. sanguisuga* across the sampled areas, both sylvatic and peridomestic, likely amplify the pathogen prevalence in human adapted reservoirs, specifically raccoon and Virginia opossums.

The *T. cruzi* DTUs, TcI and TcIV, were identified in the sampled raccoon populations and TcI was the only DTU identified in opossums. These findings support the known DTU-host associations for both reservoirs and show that both DTUs are maintained across the landscape in both peridomestic and sylvatic transmission cycles (Bern et al., 2011; Roellig et al., 2009; Torhorst et al., 2022; Zecca et al., 2020). Both DTUs are known to infect domestic dogs and cause pathology in those infected (Curtis-Robles et al., 2017, 2018a). Identifying TcI in peridomestic raccoons and opossums is of public health concern as TcI is the only current DTU identified in autochthonous cases of human *T. cruzi* infection in the US (Garcia et al., 2017). Our inability to identify the infecting DTU in the majority of the infected proportion of raccoons, the infected armadillo and cotton mouse suggests the need to further understand the genetic variation found among DTUs of the southeastern US.

In conclusion, this study furthers the current understanding of the interaction between the protozoan parasite *T. cruzi,* and its native mammalian reservoir hosts in the southeastern US. This study reports host level infection prevalence values for raccoons and opossums in north central Florida. The infection prevalence reported for both species emphasizes their importance in the transmission and maintenance of *T. cruzi* in the studied area. Our results show that raccoons in Florida were not infected outside of the distribution of the vector adding to our current understanding and assumption of the parasite and vector's range in the state. The prevalence values identified in raccoons and opossums are consistent with previous exposure prevalence and the parasite appears to be ubiquitous across the peri-urban-sylvatic gradient. This result indicates a high hazard of transmission of the parasite to other reservoirs, humans, and domestic companion animals across multiple habitats in north central Florida.

Declaration of competing interest

The authors declare that they have not conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.06.009.

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