International Journal for Parasitology: Parasites and Wildlife 17 (2022) 20–25

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Surveillance and genotype characterization of zoonotic trypanosomatidae in *Didelphis marsupialis* in two endemic sites of rural Panama

Vanessa J. Pineda [a,1], Kadir A. González [a,1], Milixa Perea [a], Chystrie Rigg [a], José E. Calzada [a,b], Luis F. Chaves [a], Vanessa Vásquez [a], Franklyn Samudio [a], Nicole Gottdenker [c,d,*], Azael Saldaña [a,e,**]

[a] Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES), Avenida Justo Arosemena, Panama, Panama
[b] Facultad de Medicina Veterinaria, Universidad de Panamá, Panama
[c] Center for the Ecology of Infectious Diseases, The University of Georgia, Athens, GA, USA
[d] Department of Veterinary Pathology, College of Veterinary Medicine, The University of Georgia, Athens, GA, USA
[e] Centro de Investigación y Diagnóstico de Enfermedades Parasitarias (CIDEP), Facultad de Medicina, Universidad de Panamá, Panama

ARTICLE INFO

Keywords:
Didelphis marsupialis
Trypanosoma cruzi
Trypanosoma rangeli
Leishmania sp. genetic characterization
Panama

ABSTRACT

Didelphis marsupialis has been reported as a competent reservoir for trypanosomatid parasites infections. The aim of this study was to measure *Trypanosoma cruzi, T. rangeli,* and *Leishmania* spp. infection rates and to characterize discrete typing units (DTUs) of *T. cruzi* in *D. marsupialis* from two Chagas disease endemic sites in Panama. Blood from 57 wild-caught *D. marsupialis* were examined from two rural communities, Las Pavas (N = 18) and Trinidad de las Minas (N = 39). Twenty-two (38.60%) opossums were positive for flagellates by general hemoculture. *T. cruzi* infection was confirmed by positive hemoculture and/or kDNA based PCR performed in 31/57 (54.39%) blood samples from opossums. *T. rangeli* infection was confirmed by hemoculture and/or TrF/R2-Primer PCR assay applied on 12/57 (21.05%) blood samples. Nine (15.79%) *D. marsupialis* harbored *T. cruzi/T. rangeli* coinfections. All opossums tested negative for *Leishmania* spp. by PCR assays based on kDNA and HSP70 gene amplification. There was a significant association between *T. cruzi* infection and site (Fisher exact test, p = 0.02), with a higher proportion of *T. cruzi* infected opossums in Las Pavas (77.78%, n = 14/18) compared to Trinidad de las Minas (43.59%, n = 17/39). A significant association was found between habitat type and *T. cruzi* infection in opossums across both communities, ($X^2 = 6.91$, p = 0.01, df = 1), with a higher proportion of *T. cruzi* infection in opossums captured in forest remnants (76%, 19/25) compared to peridomestic areas (37.5%, 12/32). *T. rangeli* detection, but not *T. cruzi* detection, may be improved by culture followed by PCR. TcI was the only DTU detected in 22 *T. cruzi* samples using conventional and real-time PCR. Eight *T. rangeli* positive samples were characterized as KP1(-)/lineage C. Trypanosome infection data from this common synanthropic mammal provides important information for improved surveillance and management of Chagas disease in endemic regions of Panama.

1. Introduction

Chagas disease and American cutaneous leishmaniasis, caused by vector-borne parasites in the family Trypanosomatidae, pose a significant health burden to many people throughout the Americas (de Lima et al., 2006). The protozoan parasite *Trypanosoma cruzi*, cause of Chagas disease, infects approximately 7 million people worldwide and cycles between many wild and domestic mammalian reservoir host species and triatomine vectors (Saldaña et al., 2005; WHO, 2021a). Based on genetic and biological characteristics, *T. cruzi* isolates have been divided into six discrete typing units (DTUs): TcI, TcII, TcIII, TcIV, TcV and TcVI (Zingales et al., 2012) and an additional bat-associated genotype TcBat (Marcili et al., 2009). All DTUs are infectious to humans with mounting evidence that different DTUs have distinct clinical presentations

* Corresponding author. Department of Veterinary Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA, USA.
** Corresponding author. Instituto Conmemorativo Gorgas de Estudios de la Salud, Avenida Justo Arosemena, Calle 35, Calidonia, 0816-02593, Panama.
E-mail addresses: gottdenk@uga.edu (N. Gottdenker), asaldana@gorgas.gob.pa (A. Saldaña).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijppaw.2021.12.002
Received 18 October 2021; Received in revised form 1 December 2021; Accepted 4 December 2021
Available online 6 December 2021

(Zingales, 2018). DTUs display genetic and biogeographic diversity, nevertheless, the association of these subpopulations with biological and epidemiological characteristics is not clearly defined (Izeta-Alberdi et al., 2016; Jansen et al., 2020). Identifying *T. cruzi* in wild reservoirs and their DTU/reservoir host relationships contribute to a better understanding of *T. cruzi* ecology and transmission patterns within a given geographic area (Jansen et al., 2017).

Originally, *T. cruzi* was limited to wild mammal reservoirs, but human settlements led to establishments and overlap of sylvatic and domestic mammal transmission cycle (Herrera and Urdaneta-Morales, 1992; Jansen et al., 2017). Throughout the Americas, natural *T. cruzi* infection has been identified in more than 180 species of mammals (Sousa, 1972; Noireau et al., 2009; Rodriguez and Loaiza, 2017). Across its range, the common opossum *Didelphis marsupialis,* an opportunistic mammal who adapts well to anthropogenic landscapes, is a reservoir host in the maintenance of *T. cruzi* circulation, serving as a link between wild and domestic cycle (Jansen et al., 2017; Pinho et al., 2000). Opossums acquire *T. cruzi* infection by feeding on triatomines or infected small mammals, mucous membrane triatomine bite wound contamination with infected triatomine feces, or contact with secretions of the anal glands of other infected opossums (Jansen et al., 2018). The main vector of Chagas disease in Panama, *Rhodnius pallescens*, frequently feeds from opossum blood (Pineda et al., 2008; Saldaña et al., 2012). *Trypanosoma rangeli,* which is also transmitted by *R. pallescens,* is not pathogenic in mammals, including humans, commonly co-circulates with *T. cruzi* in rural Panama, and may cause cross-reactivity with *T. cruzi* in some immunodiagnostic tests (Saldaña et al., 2005). In Panama, human infection with *T. rangeli* is up to 10 times more frequent than with *T.* cruzi (Sousa, 1972), thus justifying the search and characterization of this generalist hemoflagellate. In addition to its role as a *T. cruzi* reservoir, *D. marsupialis* can also play an important role in *Leishmania* spp. transmission (Travi et al., 1994, 1998b). *Leishmania* spp. are transmitted from wild mammal reservoirs to humans by phlebotomine sandfly vector bites (WHO, 2021b). *Leishmania* infections have been detected in *D. marsupialis* in Brazil (Cabrera et al., 2003; Schallig et al., 2007), and Colombia (Corredor et al., 1989; Travi et al., 1994, 1998a). *L. mexicana, L. infantum,* and *L. chagasi* infections, including co-infections with *T. cruzi*, have been detected in *D. marsupialis* in Venezuela (Viettri et al., 2018, 2019). Arguably, the role of *D. marsupialis* in *Leishmania* transmission is less well understood compared to its role in *T. cruzi* transmission throughout its range.

Although observations suggest *D. marsupialis* plays an important role in Chagas disease epizootiology in Panama, the last report being from the 1970s (Sousa, 1972), contemporary information regarding zoonotic trypanosomatidae infection prevalence and genotype in *D. marsupialis* across endemic rural landscapes in Panama is lacking. Accordingly, this study aims to evaluate the frequency of Trypanosomatid infection and the detection of *T. cruzi* DTUs in common opossums from sites near two rural communities in central Panama.

2. Materials and methods

2.1. Study area

We carried out a descriptive, cross-sectional study in peridomestic areas and forest remnants near communities of Las Pavas (LP), District of La Chorrera (9°6′15″N, 79°53′9″W, 50–156 m above sea level, near the west bank of the Panama Canal), and further west in Trinidad de Las Minas (TM), District of Capira (8° 46′32″N, 79° 59′45″W), 230 m above sea level. The potential vegetation at both sites is tropical rainforest (Holdridge, 1967), characterized by a mosaic of forest patches, riparian forest remnants, cattle pasture, regenerating forest, and human settlements. There is a marked dry season mid-December to March and a rainy season throughout the rest of the year.

2.2. Capture and sampling

For 5 consecutive nights (640 trap nights) during wet (November) and dry seasons (March) 2013-2015, *D. marsupialis* (N = 57) were captured using 32 Tomahawk traps distributed in four 150 m long transects separated by 50 m within each site (2 forest remnants located 1 and 3 km from each community, and a peridomestic site 300 m around houses), Fig. 1. Captured animals were anesthetized with Ketamine (5–7.5 mg/kg) and 500 µl-3 ml blood was drawn from the caudal vein of each opossum and placed in microtubes with EDTA.

2.3. Trypanosomatid diagnostics

Blood was cultured for trypanosomatids (Vásquez et al., 1997). DNA of blood samples collected at time of capture and blood samples cultured for six weeks after collection were extracted using a commercial kit (QIAamp® DNA Blood Mini Kit (Quiagen). PCR were performed using S35/S36 primers that amplify a 330 bp segment of the *T. cruzi* variable region minicircle and the primer pairs TrF/R2 that amplify a 620 bp fragment of *T. rangeli* snoRNA-c11 gene (Vallejo et al., 1999; Pavia et al., 2007). *T. cruzi* and *T. rangeli* positive samples were typed by a miniexon gene-based approach (Fernandes et al., 2001) and a PCR-RFLP approach targeting the COII gene (de Sá et al., 2013), respectively. *T. cruzi* DTUs were detected by a real time PCR strategy based on the amplification of a set of the specific markers SL-IR (TcI-TcIII), COII (TcII-TcIV), ND1 (TcV) and 18S rDNA (TcVI) that in combination discriminate all *T. cruzi* DTUs (Muñoz-San Martín et al., 2017). DNA samples from opossum blood were also tested for *Leishmania* spp. using a B1/B2 primers-PCR based assay that amplify a 750 bp kinetoplastic segment specific for *Leishmania Viannia* (Vergel et al., 2005) along with a PCR approach based on the amplification of a 1230 bp segment of the *Leishmania* sp. HSP70 gene (Montalvo et al., 2012).

2.4. Statistical analysis

RStudio (RStudio Team version 1.31, 2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/) was used for statistical analyses. Chi square or Fisher's exact test were used to evaluate any associations between *T. cruzi* infection, *T. rangeli* infection, *T. cruzi/T. rangeli* coinfection and site (TM vs LP), habitat type, season, year-season, and opossum sex. Basic descriptive and bivariate analyses were performed in the MASS package in R (and tables calculating the proportion of opossums infected with 95% confidence intervals using *confint* function (Venables and Ripley, 2004). We used the package 'fsmb' version 0.7.1 (Minakawa, 2007) for diagnostic agreement analysis (Cohen's kappa test).

2.5. Ethics

The Institutional Animal Care and Use Committee (014/CIUCAL-ICGES/13) of the Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES) approved this study, which was done in accordance with Law No. 23 of 15 January 1997 (Animal Welfare Assurance) of the Republic of Panama.

3. Results

The presence of *T. cruzi* and *T. rangeli* were evaluated in blood samples from 57 captured *D. marsupialis* (31 females, 25 males, and one unidentified sex) by PCR of DNA directly isolated from blood and by PCR performed on DNA samples obtained after 6 week of blood culture. Trypanosomes were detected in 35.09% (20/57, 95% CI; 22.91%, 48.87%) opossums by hemoculture.

Overall *T. cruzi* positivity in opossums (including individuals as positive who tested positive from non-cultured and cultured blood) was 54.39% (31/57, 95% CI; 41.59%,66.63%). Overall *T. rangeli* positivity in

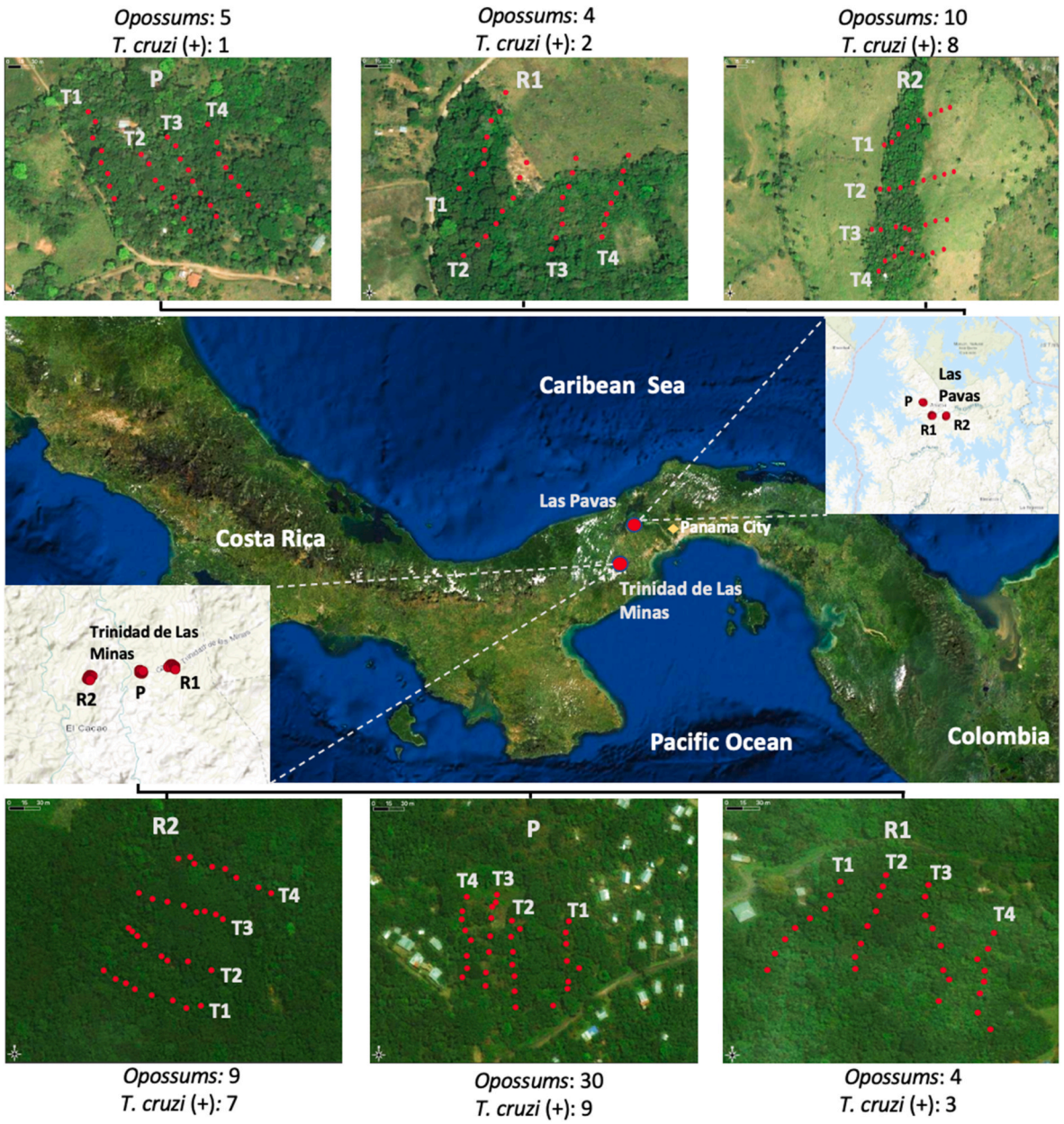

Fig. 1. Map showing the communities of Las Pavas (LP) (top set of images) and Trinidad de Las Minas (TM) (bottom set of images) with the number of opossums captured and infected with *T. cruzi* in the 3 collection sites in each community. A. Map with the geographic location of the LP and TM communities in the country of Panama. Satellite view of the P: Peridomicile (B), R1: remnant 1 (C) and R2: remnant 2 (D) collection site each with its 4 transects in the LP community. Satellite view of the P: Peridomicile (E), R1: remnant 1 (F) and R2: remnant 2 (G) collection site each with its 4 transects in the TM community.

opossums was 21.05% (12/57, 95% CI; 12.47%,33.29%). *T. cruzi/T. rangeli* coinfections were detected in 15.79% (9/57, 95% CI; 15.79%,27.36%) of opossums. Table 1 shows PCR results for *T. cruzi* and *T. rangeli* infection in opossums from each community.

There was a significant association between *T. cruzi* infection and community (X-squared = 4.51, df = 1, p-value = 0.03), with a higher proportion of *T. cruzi* infected opossums in LP (77.78%) than TM (43.59%). Across both communities, there was a significant association between habitat type and *T. cruzi* infection (Chi squared = 6.91, df = 1, p = 0.009) with a higher proportion of *T. cruzi*-infected opossums in forest remnants (19/25, 76%) compared to peridomiciliary sites (12/32, 37.50%). There was no significant association between community and *T. rangeli* infection (Fisher's exact test, p = 0.49) or *T. cruzi-T. rangeli* coinfection (Fisher's exact test, p = 0.44). Although there was a greater number of opossums captured at both sites in the wet season (N = 46) compared to the dry season (N = 11), there was no significant association between season of capture (wet vs dry) and *T. cruzi* infection (Fisher's exact test p-value = 0.52) or *T. rangeli* infection (Fisher's exact test p-value = 0.52), nor *T. cruzi-T. rangeli* coinfection (Fisher's exact test, p = 0.67). There was no significant association between opossum sex and *T. cruzi* infection (X-squared = 1.6, df = 1, p-value = 0.2059), *T. rangeli* infection (X-squared = 7.2547e-32, df = 1, p-value = 1), and *T. cruzi/T. rangeli* coinfection (Fisher's exact test, p = 1).

For *T. cruzi*, there was 70.9% crude agreement between PCR of cultured and non-cultured blood and a Cohen's Kappa of 0.415 (95% CI; 0.173, 0.656, Z = 3.06, p = 0.001), indicating moderate agreement. For *T. rangeli* detection, there was 81.18% crude agreement between cultured and non-cultured PCR diagnostic tests, and a Cohen's kappa of 0.068, 95% CI; −0.455, 0.590, Z = 0.247, p = 0.402, indicating slight agreement between the two methods. For coinfection, although there

Table 1
Trypanosoma cruzi and *T. rangeli* infections in *Didelphis marsupialis* from two Chagas disease endemic sites in Panama. Data show the following: infected/total number sampled, percent infected (%), and upper and lower bounds of the 95% confidence interval for infection.

Site	*T. cruzi*			*T. rangeli*			Coinfection		
	PD	Forest Remnant	Total	PD	Forest Remnant	Total	PD	Forest Remnant	Total
Las Pavas (LP)	3/5	11/13	14/18	3/5	2/13	5/18	2/5	2/13	4/18
	60.00	84.61	77.78	60.00	15.38	27.78	40.00	15.38	22.22
	23.07-	57.76-	54.78-	23.07-	4.33-	12.50-	11.76-	4.33-	9.00-
	88.20	95.67	91.0	88.24	42.23	50.87	76.93	42.23	45.21
Trinidad de las Minas (TM)	9/27	8/12	17/39	4/27	3/12	7/39	2/27	3/12	5/39
	33.33	66.67	43.59	14.81	25.00	0.1795	74.07	25.00	12.82
	18.64-	39.06-	29.30-	5.92-	8.89-	8.98-	2.05-	8.89-	5.60-
	52.17	86.19	59.02	32.48	53.23	32.67	23.37	53.23	26.71
Total	12/32	19/25	31/57	7/32	5/25	12/57	4/32	5/25	9/57
	37.50	76.00	54.39	21.87	20.00	21.05	12.50	20.00	15.79
	22.93-	56.57-	41.59-	11.02-	8.86-	12.47-	4.97-	8.86-	8.54-
	54.75	88.50	66.63	38.75	39.13	33.29	28.07	39.13	27.36

was 87% crude agreement between non cultured- and cultured blood PCR tests, there was slight agreement between the two methods (Cohen's kappa = 0.112, 95% CI; −0.421,0.689, Z = 0.446, p = 0.33).

Fifteen out of 31 *T. cruzi* positive samples by PCR were characterized as DTU 1 (8 from LP and 7 from TM). Nineteen out of 31 *T. cruzi* positive samples were successfully characterized as DTU 1 by real time PCR (8 from LP and 11 from TM). All *T. rangeli* positive samples (N = 8) were successfully characterized as a lineage corresponded to and KP1 (-)/lineage C. All 57 samples tested negative for *Leishmania* spp. by both the PCR targeting kinetoplastic DNA and the PCR assay based on the amplification of HSP70 gene.

4. Discussion

Didelphis opossums in our study are frequently infected with *T. cruzi* and *T. rangeli* in rural habitats in central Panama, with a higher percentage of *T. cruzi*-infected opossums (54.38%) than *T. rangeli*-infected opossums (21.05%). In studies from central Panama in 1932, *T. cruzi* was detected by parasitological methods in 24.6% of *D. marsupialis* (N = 81) (Clark and Dunn, 1932). In later studies in central Panama (early 1970's), trypanosome infection was detected by hemoculture in over 50% of *D. marsupialis,* with *T. cruzi* identified in 20% and *T. rangeli* in 28% of animals (Sousa, 1972). In our study, the higher *T. cruzi* infection rate in opossums compared to previous reports may be a true increase in prevalence, or due to improved molecular detection methods. Molecular assays are highly effective and sensitive for the detection of *T. rangeli* infection (Vallejo et al., 1999; Pavia et al., 2007; de Sá et al., 2013). In addition, these methods allow the evaluation of the genetic diversity of parasite populations, thus enabling a better understanding of the infection behavior among a given reservoir population.

Diagnostic agreement results between different methods (PCR direct from DNA extracted from blood and PCR from DNA extracted after hemoculture) suggest that *T. rangeli* detection, but not *T. cruzi* detection, may be improved by culture followed by PCR.

Throughout Latin America, *T. cruzi* infection rates in *D. marsupialis* range between 3% to over 80% (Herrera and Urdaneta-Morales, 1992; Grisard et al., 2000; Pinto et al., 2006; Rodriguez-Mongui et al., 2019; Magalhães et al., 2021). *T. cruzi* infection rates in our study are similar to opossums from rural landscapes of Costa Rica (Zeledón et al., 1970), the Brazilian Amazon (Roque et al., 2013; Magalhães et al., 2021), Colombia (Cantillo-Barraza et al., 2015, 2020; Rodriguez-Mongui et al., 2019) and Venezuela (Herrera and Urdaneta-Morales, 1992; de Lima et al., 2006).

Although no opossums were positive for *Leishmania* spp. infection by PCR, this does not mean that they do not play a role in *Leishmania* spp. transmission in the study area. Ardila et al. (2019) sampled 65 tissues (ear and tail biopsies) and blood from 19 *D. marsupialis* in a *Leishmania* endemic area of Colombia and did not detect *Leishmania* spp. (Ardila et al., 2019). *Leishmania* may not have been detected due to chance, and/or because low prevalence in opossums may require higher sample size/sample effort. Sample type may also play a role, as *Leishmania* DNA may be present in other tissues, but not in leukocytes in the peripheral blood sample. Also, *Leishmania* spp. infection may be rare or absent due to sandfly feeding preferences or low opossum-sandfly contact in the study area. Improved detection in future studies could include xenodiagnostics, serotesting for *Leishmania* exposure, multiannual longitudinal surveys, or more invasive sampling of additional tissues such as skin biopsies. There were significant differences in *T. cruzi* infection across community and habitat type. *D. marsupialis* abundance is favored by a greater number of forested patches, conserved areas, agricultural crops, which could explain the higher opossum trap success in the more forested TM compared to LP, where cattle pastures are extensive and forest remnants are smaller and irregularly distributed. Although it is more deforested, LP has a higher relative abundance of *Attalea* palms where *R. pallescens* vectors live. Opossums may occupy *Attalea* palms at a higher frequency in LP compared to TM, resulting in higher opossum-vector contact in LP, because in this pasture-dominated landscape, there are less alternative nesting and hiding places for opossums. The higher *T. cruzi* infection rates in opossums in forest remnants (Table 1) correlate with studies that found higher infection rates of *T. cruzi* in *R. pallescens* vectors in forest remnants compared to peridomiciliary sites (Gottdenker et al., 2012), suggesting more *T. cruzi*-triatomine contact occurs with opossums and possibly other competent reservoirs in these remnants (Travi et al., 1994; Ruiz-Pina and Cruz-Reyes, 2002; Roque et al., 2013; Orozco et al., 2013; Jansen et al., 2017; Rodriguez-Mongui et al., 2019).

Lower *T. rangeli* compared to *T. cruzi* infection in opossums correlates with previous studies in the vector *R. pallescens* describing a higher proportion of *T. cruzi*-infected vectors compared to *T. rangeli,* and similar *T. cruzi-T. rangeli* co-infection rates across habitat types (Gottdenker et al., 2016). Interestingly, studies from the same geographic region of LP show *T. rangeli* infection is relatively common in children (Saldaña et al., 2005). In this regard, little is known about the immune response induced by a *T. rangeli* infection, however it has been reported that immunization of dogs with *T. rangeli* antigens induces protection against Chagas infection (Basso et al., 2016). The search and genetic characterization of *T. rangeli* in other wild reservoirs is a requirement for understanding the eco-biology of this parasite.

Only TcI was detected in *D. marsupialis* in central Panama, corresponding to studies in other countries where TcI predominates in *D. marsupialis* (Legey et al., 2003; Jansen et al., 2017). Although *T. cruzi* circulates widely in Chagas disease endemic areas in Panama (Sousa et al., 2006; Samudio et al., 2007; Brandao et al., 2008; Saldaña et al., 2012), TcI is the predominant DTU reported to date in acute chagasic patients, and in triatomine species *R. pallescens* and *Triatoma dimidiata*. These results, the previous demonstration of TcI in both patients and triatomines (Sousa et al., 2006; Samudio et al., 2007; Prescilla-Ledezma

et al., 2021), and the fact that principal vectors of *T. cruzi* in Panama frequently feed on opossum blood (Pineda et al., 2008), show that *D. marsupialis* is an important reservoir in local *T. cruzi* transmission cycles. However, in Panama as in other regions, genetic heterogeneity present in TcI parasites of opossums as well as the presence of mixed trypanosome infections and DTU characterization in other wild mammalian reservoirs are critical to our understanding of *T. cruzi* infection ecology (Cura et al., 2010; Jansen et al., 2018; Prescilla-Ledezma et al., 2021). In our study, the KP1(-)/lineage C was the only genetic group found in eight *T. rangeli* positive samples, agreeing with the genotype reported so far in Panama (Salazar-Antón et al., 2009; Saldaña et al., 2018).

To summarize, this study contributes to understanding of parasite, host, and environmental relationships of Chagas and *Leishmania* infection across central Panama, allowing for comparison of wildlife infection rates across wider geographic areas and landscapes through the Americas.

Ethical statement

This study was approved by the institutional animal care and use committee: 014/CIUCAL-ICGES/13.

Funding

This research was funded by SENACYT, grant number Project COL11-043.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank José Montenegro, Roberto Rojas and Enrique Martínez for his support during fieldwork. This study was also supported by the Sistema Nacional de Investigación (SNI), Panama, awarded to Azael Saldaña and José E. Calzada as members of the SNI-SENACYT.

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