First finding of *Trypanosoma cruzi* II in vampire bats from a district free of domestic vector-borne transmission in Northeastern Argentina

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SUMMARY

Establishing the putative links between sylvatic and domestic transmission cycles of *Trypanosoma cruzi*, the etiological agent of Chagas disease, is of public health relevance. We conducted three surveys to assess *T. cruzi* infection in wild mammals from a rural and a preserved area in Misiones Province, Northeastern Argentina, which had recently been declared free of vector- and blood-borne transmission of human *T. cruzi* infection. A total of 200 wild mammals were examined by xenodiagnosis (XD) and/or polymerase chain reaction (PCR) amplification of the hyper-variable region of kinetoplast DNA minicircles of *T. cruzi* (kDNA-PCR). The overall prevalence of *T. cruzi* infection was 8%. Nine (16%) of 57 *Didelphis albiventris* opossums and two (7%) of 29 *Desmodus rotundus* vampire bats were positive by both XD and kDNA-PCR. Additionally, one *D. rotundus* positive for *T. cruzi* by kDNA-PCR tested positive by satellite-DNA-PCR (SAT-DNA-PCR). The *T. cruzi*-infected bats were captured indoors and in the yard of a vacant dwelling. All *D. albiventris* were infected with TcI and both XD-positive *D. rotundus* by TcII. Fifty-five opossum cubs within the marsupium were negative by XD. The mean infectiousness to the vector was 62% in *D. albiventris* and 50% in *D. rotundus*. Mice experimentally infected with a parasite isolate from a vampire bat displayed lesions typically caused by *T. cruzi*. Our study documents the presence of the genotype TcII in a sylvatic host for the first time in Argentina, and the occurrence of two transmission cycles of *T. cruzi* in a district free of domestic vector-borne transmission.

Key words: *Trypanosoma cruzi*, discrete typing unit, *Desmodus rotundus*, *Didelphis albiventris*.

INTRODUCTION

Trypanosoma cruzi, a flagellated protozoan that may infect any mammalian species and cause Chagas disease, is a major public health problem in Latin America with rising importance in developed countries due to international migration (Tibayrenc, 2009; Tarleton *et al*. 2014). *Trypanosoma cruzi* presents a complex population genetic structure classified into six discrete typing units (DTUs) ranging from TcI to TcVI (Miles *et al*. 2009; Tibayrenc, 2009; Zingales *et al*. 2009), and the recently described DTU denominated Tcbat (Marcili *et al*. 2009*a*). All DTUs may produce human Chagas disease, and show a heterogeneous distribution depending on geographic area, biome, mammalian host species and triatomine species involved (Zingales *et al*. 2012). Thus TcII, TcV and TcVI are closely associated with anthropogenic environments, although TcII had also been identified in wild mammals from several biomes (Lima *et al*. 2014); TcIII and TcIV with sylvatic transmission cycles, and TcI circulates in sylvatic and domestic cycles depending on geographic area (Yeo *et al*. 2005; Miles *et al*. 2009; Zingales *et al*. 2012). A major question of epidemiological relevance is whether both types of transmission cycles of *T. cruzi* are connected or independent. Identifying the circulating DTUs and the structure of sylvatic transmission cycles is also important to trace the origins of (re)emerging cases in areas under vector or disease surveillance (Miles *et al*. 2009).

The epidemiological role of many host species of *T. cruzi* is still unresolved and may vary with landscape features (Noireau *et al*. 2009; Jansen *et al*. 2015). Some mammalian host species are more exposed to *T. cruzi*, especially those that are nest-dwelling or use hollow trees, burrows and caves such as opossums, armadillos, rodents and bats (Noireau *et al*. 2009). Opossums and armadillos are considered the most ancient hosts of *T. cruzi*

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(Noireau *et al*. 2009). Rodents are frequently involved in sylvatic and (peri)domestic transmission cycles (Noireau *et al*. 2009; Orozco *et al*. 2014; Jansen *et al*. 2015). Synanthropic rats and mice may act as a link between sylvatic and domestic cycles (Gürtler and Cardinal, 2015).

Bats (Chiroptera) are reservoir hosts of numerous infectious agents, many of which are zoonotic (Calisher *et al*. 2006). Some of their ecological characteristics make them especially suitable for pathogen emergence or re-emergence: high densities; gregarious behaviour; use of multi-species refuges and spaces close to human dwellings, and high dispersal capacity (Calisher *et al*. 2006; Luis *et al*. 2013). These distinctive attributes enhance the transmission of pathogens between wild and domestic animals or humans in a context of increasing habitat fragmentation and loss (Molyneux *et al*. 2008). *Trypanosoma cruzi* frequently infects bats in the wild and may have derived from bat trypanosomes (Hamilton *et al*. 2012). As part of the Southern Cone Initiative for the interruption of vector- and blood-borne transmission of human *T. cruzi* infection, Misiones Province (Northeastern Argentina) was declared free of vector-borne transmission through *T. infestans* in 2011 (PAHO, 2012). The local host distribution of *T. cruzi* in sylvatic transmission cycles, including reservoir host species, vectors and DTUs implicated, remain unknown. To address this gap in knowledge, we assessed the occurrence of *T. cruzi* infection in sylvatic mammals from a well-defined area in Misiones; identified the circulating DTUs; assessed the hosts' infectiousness to the vector, and searched for triatomines in sites where the infected mammals had been caught.

MATERIALS AND METHODS

Study area

Field work was conducted in Capital (27°24′S 55°55′W) and Candelaria (27°22′S 55°34′W) Departments in Misiones Province, Argentina (Fig. 1). The study area belongs to the Southern Cone Mesopotamian savannah ecoregion. Originally composed of grasslands, marshes, woodlands and gallery forests (Viglizzo *et al*. 2006), the region has undergone major environmental changes and currently is characterized by cattle ranches, tree and "yerba mate" (*Ilex paraguariensis*) plantations. The climate is humid subtropical, with an average annual rainfall of 1500 mm uniformly distributed across the year. Annual temperatures averaged 22 °C. The study sites suffered an outbreak of sylvatic yellow fever affecting *Alouatta caraya* monkeys in 2009.

Field work in Capital Department was conducted at two sites: Santa Inés Ranch (SIR), a farm and cattle ranch including pine tree and "yerba mate"
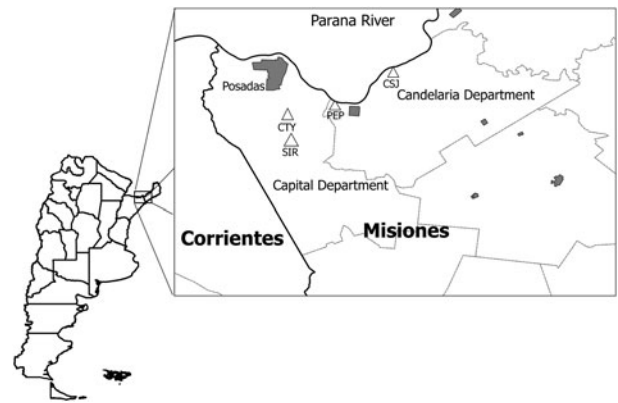

Fig. 1. Location of study sites in Misiones, Northeastern Argentina. CTY, Chacra Tuyuyu; PEP, Parque El Puma; SIR, Santa Inés Ranch; CSJ, Campo San Juan.

plantations, and small natural forest patches; and Chacra Tuyuyu (CTY), a small farm with citric plantations and a small natural forest patch surrounded by a gated community and grasslands. Field work in Candelaria Department was conducted in Parque El Puma (PEP), a 25 ha. multiple-use reserve situated in a rural area, and in Campo San Juan Reserve (CSJ), a well-preserved area of 5800 ha, including gallery forests, grasslands and fragments of "Urunday" (*Astronium balansae*) native forests. CSJ was transformed into a protected nature preserve in 2007, and had a few abandoned buildings with no permanent residents, few cattle and remnants of old plantations (Fig. 1).

Capture and handling of wild mammals

Captures were carried out by means of georeferenced traps and manual catches (Garmin Legend C; Garmin, Olathe, KS) in October 2012, June 2013 and April 2014. Tomahawk traps were deployed every 25 m along transect lines to capture medium-sized mammals (e.g. white-eared opossums). Traps were baited with beef or chicken scraps; checked every morning, and re-baited when appropriate. Small rodents and Woolly Mouse Opossums were caught using Sherman traps (H. B. Sherman Traps, Tallahassee, FL) deployed at ground level and on tree branches. Sherman traps were baited with seeds (oats, maize, seeds and local fruits), fresh (apple and banana) and dry fruits (walnuts, grape raisins) and pellets of a mixture of peanut butter, oatmeal and vanilla essence.

Bats were captured using mist nets (6·0 m wide, 2·6 m high, 38·0 mm black mesh; AFO Banding Supplies, Manomet, MA) opened at dusk and monitored every 30–40 min for 4 h. Four groups of two or three mist nets were placed in a zigzag formation at each capture site. The nets were set up in a cleared area free of vegetation where bat activity had previously been observed in CSJ and in PEP, and close

to a *D. rotundus* refuge in SIR. In addition, *D. rotundus* bats from a colony found in a vacant house at CSJ (close to the site where the nets were deployed) were captured using a hand capture net. Black and Golden Howler Monkeys were caught with tranquilizing darts (Pneudart Type P 1 mL) shot with an air rifle (Pneudart Model 178B). Capture and transit permits were obtained from the government of Misiones Province.

The captured animals were transported to the field laboratory in PEP where each individual was subjected to a pre-anaesthetic examination and then given parenteral anaesthesia for induction and inhalatory anaesthesia for maintenance. The former was performed with tiletamine clorhydrate and zolazepan clorhydrate (Zelazol®; Fort Dodge, Buenos Aires, Argentina) or ketamine clorhydrate (Vetaset; Fort Dodge) combined with dexmedetomidine (Pfizer) at the minimum dose appropriate to species and weight (West *et al.* 2007). Inhalatory anaesthesia with isoflurane was delivered with a vaporizer (IsoTec; Datex-Ohmeda GE Healthcare, Little Chalfont, UK) and medicinal O_2 ($0\cdot25$–3 L min^{-1}). The anaesthetized animals were maintained on thermic cushioned surfaces in a quiet and comfortable environment, with their eyes protected with ophthalmic lubricant solutions and covered with home-made eyecups. The vital signs during the anaesthetic procedure were monitored with a multiparametric patient monitor (Contec CMS 6000).

Each individual was sexed, weighed (Pesola®), marked with a numeric metal tag (National Band and Tag Company, Newport, KY), its general condition determined, and then released at the capture site after full recovery from anaesthesia. *Didelphis* opossums were assigned to stage class based on tooth eruption (Schweigmann *et al.* 1999).

Animals were bled by venepuncture and each blood sample was diluted 1:1 in 6 M guanidine hydrochloride, $0\cdot2$ M EDTA pH $8\cdot0$ buffer (GEB) for polymerase chain reaction (PCR) amplification. All specimens were tested by xenodiagnosis (XD) using 5–20 uninfected fourth-instar nymphs of *T. infestans* (depending on body size) contained in wooden boxes applied on the belly of the host for 25 min and checked for its degree of blood engorgement.

Road-killed mammals found in Candelaria routes during fieldwork; an adult male of *A. caraya* captured in a neighbouring locality, and mammals rescued at PEP were also examined for *T. cruzi* infection.

XD, parasite isolation and culture

The rectal contents from five XD bugs exposed to an identified host were pooled, diluted with physiological saline solution and examined microscopically (Zeiss, Oberkochen, Germany) at 400×

magnification 30 and 60 days post-exposure (Gürtler *et al.* 2007). The numbers of exuviae and of dead bugs in each box were recorded as indices of XD quality. When a pool was positive for trypanosomes, all bugs were re-examined individually to assess the individual host's infectiousness to the vector (i.e. the proportion of infected bugs fed on a given individual relative to the total number of insects examined for infection at least once, excluding the insects that died prior to the first examination). Estimates of infectiousness included specimens positive by XD or by kDNA-PCR from GEB samples.

For parasite culture, the rectal contents from two microscope-positive bugs from each XD-positive animal were inoculated separately into culture tubes that contained a biphasic medium, and then incubated at 28 °C and a relative humidity of 50% (Cardinal *et al.* 2008). Parasite growth was monitored weekly during 4 months until reaching 3×10^5 parasites mL^{-1}, and the isolates were cryopreserved in liquid nitrogen.

Molecular diagnosis and genotyping

Total DNA was extracted from GEB blood samples boiled for 15 min using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Infections with *T. cruzi* were identified by PCR amplification of the 330-basepair (bp) fragment from the kDNA minicircles of *T. cruzi* (kDNA-PCR) using primers and cycling conditions described (Burgos *et al.* 2005) with the incorporation of Taq Platinum® (Invitrogen, Carlsbad, CA, USA). Samples of 12 μL of PCR products were visualized under ultraviolet light after electrophoresis in 3% agarose gels containing GelRed® (Biotium, Inc., Hayward, CA). kDNA-PCR-positive hosts displayed the 330 bp band indicative of *T. cruzi* infection.

Samples from individual hosts that were XD-negative and kDNA-PCR-positive (in GEB samples) were subsequently tested by SAT-DNA-PCR, by sequence analyses of the 24S alpha ribosomal DNA (24Sα rDNA-PCR) or by kDNA-PCR of the rectal contents of XD bugs for confirmation of kDNA-PCR results. The rectal contents were diluted in 50 μL of deionized sterile water, boiled for 15 min., and DNA was extracted with DNAzol (Invitrogen) as described elsewhere (Cardinal *et al.* 2008).

Trypanosoma cruzi DTUs were identified in culture samples of each infected host by PCR-based strategies targeting the intergenic region of spliced-leader genes (SL-IRac, SLIRII, SL-IRI) and 24S alpha ribosomal DNA, and A-10 genomic markers as described by Burgos *et al.* (2007). For confirmation of DTU and phylogenetic analysis, PCR amplification and sequencing of the partial

sequences of CytB and H2B genes from two isolates obtained from two XD-positive *D. rotundus* was performed as described (Marcili *et al*. 2009*a*). The obtained amplifications were sequenced in an external service (Macrogen Inc, Korea) and compared with available sequences stored at GenBank using BLAST (Basic Local Alignment Search Tool). For phylogenetic analyses, the sequences obtained in the present study were aligned with available sequences of *T. cruzi* reference strains of all DTUs, from Colombian bats (Ramírez *et al*. 2014) and Brazilian samples (Marcili *et al*. 2009*a*; Cavazzana *et al*. 2010; Lima *et al*. 2015) (Table S1 – Supplementary Material). Alignments were performed using Clustal W and then were manually refined. The phylogenies were inferred using the Kimura two-parameter model and maximum-likelihood methods using Mega 6·06. The bootstrap was acquired from 1000 replicate trees.

Experimental infection and histopathology

To assess the pathogenicity and parasite amplification capability of bat trypanosomes, four Balb C strain mice were inoculated intraperitoneally with 10^5 parasites from the XD-positive *D. rotundus* bat (Murci 159). Each mouse was subsequently tested by XD as described above. Mice were anaesthetized with Zelazol® (20 mg kg^{-1}) 30 days post-inoculation and killed by cervical dislocation. Complete necropsies and histopathological analyses were performed. Blood samples were obtained by intracardiac puncture for haemoculture, and heart, spleen, liver and intestine were removed for histopathology. Tissues were fixed in 10% buffered formalin, dehydrated in absolute ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin and examined by light microscopy. Tissue parasite burden was classified in four levels: with no parasite nests (−); one to three nests (+); 3–6 (++), and more than six nests (+++). Inflammation of the myocardium (myocarditis) was scored according to Sun and Tarleton (1993), and skeletal muscle inflammation was based on a modification of a previously described method (Ben Younés-Chennoufi *et al*. 1988). Liver damage was evaluated by the presence of hepatocellular degeneration, necrosis, inflammation and hyperplasia of Kupffer cells. Spleen abnormalities included reactive changes and/or follicular hyperplasia.

Search of sylvatic triatomines

Searches for sylvatic foci of triatomine bugs were conducted by using mouse-baited sticky traps (Noireau traps), dissection of potential bug ecotopes, and tracking of opossums during 2 weeks in April 2014 at the sites where *T. cruzi*-infected opossums had previously been collected in 2012 and 2013. Baited sticky traps were deployed in hollow tree trunks (live or dead), fallen logs, and in the entrance of burrows on late evenings. All sites were georeferenced and identified with a numbered piece of cloth as described elsewhere (Ceballos *et al*. 2009). High tree holes were accessed by means of a telescopic ladder. Traps were left overnight and inspected early on the following morning.

Triatomine bug searches performed in association with opossum burrows and tree holes, seven *Didelphis* opossums were fitted with a spool-and-line tracking device at the site of capture (Miles *et al*. 1981), released in the evening, and tracked during the next day. Animals could not be tracked more than one day because the spool-and-line device was frequently dropped off when the animals entered some refuges or the line was used up. The burrows or tree holes in which the tracked animals sought shelter were marked with a coloured tape and georeferenced for subsequent searches of triatomine bugs by direct inspection or by deploying Noireau traps inside them.

Data analysis

Estimates of prevalence of infection excluded the 55 opossum cubs because they were not yet exposed to vector-borne infection. Proportions and 95% confidence intervals (95% CI) were estimated using Wilson's formula implemented in Epitools (Sergeant, 2015).

RESULTS

A total of 200 mammals from at least 18 species were captured, including 55 opossum cubs within the marsupial bag and 17 unidentified cricetid rodents (Table 1). The total catch effort was 1182 trap-nights with Tomahawk traps; 1479 trap-nights with Sherman traps and 92 mist net-hours. A total of 56 medium-sized mammals (all were *D. albiventris*) were captured with Tomahawk traps (4·7 per 100 trap-nights) (Table 2), eight of which were females carrying the 55 cubs. Additional catches included 19 micro-mammals (1·3 per 100 Sherman trap-nights), 39 bats (42 per 100 mist net-hours), 11 bats caught manually, ten mammals rescued at PEP, six road kills and four howler monkeys.

The composite prevalence of *T. cruzi* infection (as determined by XD or kDNA-PCR) over the 145 mammals examined was 8% (95% CI, 5–14%) (Table 1). The infected animals included nine (16%) *D. albiventris* opossums both kDNA-PCR- and XD-positive, and three (10%) *D. rotundus*, two of which were both kDNA-PCR and XD-positive and one was both kDNA-PCR- and SAT-DNA-PCR-positive. One (6%) *Sturnira lilium* bat (collected in the same site as the positive *D. rotundus*)

Table 1. Number of *T. cruzi*-infected wild mammals from Capital and Candelaria Departments, Misiones, Argentina, as determined by xenodiagnosis (XD) and kDNA-PCR

Scientific name	No. captured	XD		kDNA-PCR[a]		No. examined by kDNA-PCR or XD	No. positive by kDNA-PCR or XD
		No. examined	No. positive	No. examined	No. positive		
Alouatta caraya	4	4	0	4	0	4	0
Nasua nasua	5	0	0	5	0	5	0
Sphiggurus spinosus	2	1	0	2	0	2	0
Didelphis albiventris	57	56	9	57	9	57	9
Cavia aperea	1	0	0	1	0	1	0
Euphractus sexcinctus	2	1	0	2	0	2	0
Eira. barbara	1	1	0	1	0	1	0
Galictus cuja	1	0	0	1	0	1	0
Micoureus. cinereus	2	2	0	1	0	2	0
Artibeus lituratus	4	4	0	4	0	4	0
Sturnira lilium	16	16	0	16	0	16	0
Desmodus rotundus	29	29	2	29	3	29	3
Phyllostomus bilabiatum	1	1	0	1	0	1	0
Rodents	17	14	0	14	0	17	0
Dasypus novemcinctus	1	1	0	1	0	1	0
Lycalopex gymnocercus	1	0	0	1	0	1	0
Cerdocyon thous	1	0	0	1	0	1	0
Total	145[b]	130	11	141	12	145	12

[a] Only includes kDNA-PCR-positive samples confirmed by other more specific methods.
[b] Total does not include 55 opossum cubs within the mother's marsupium.

Table 2. Catch per unit effort (CPUE) and prevalence of *T. cruzi* infection in *D. albiventris* over time in the rural and preserved areas

Date of survey	Study area	No. trap-nights	No. captured	CPUE per 100 trap-nights (95% CI)	Prevalence of infection (95% CI)
October 2012	Rural	615	16	2·6 (1·6–4·2)	12 (3–36)
June 2013	Preserved	387	28	7·2 (5·1–10·3)	25 (13–43)
April 2014	Preserved	180	12	6·7 (3·9–11·3)	0
Total		1182	56	4·9 (3·7–6·1)	16 (9–28)

that was positive by kDNA-PCR of blood sample and of the rectal contents of XD bugs, failed to be confirmed by SAT-DNA-PCR or by sequence analyses of 24Sα-rDNA-PCR from GEB and XD bug rectal contents; thus this specimen was considered doubtful (i.e. negative for *T. cruzi*). All 55 opossum cubs and their mothers tested XD negative.

Parasite isolation through culture was successful in all XD-positive individuals. Among those positive by XD or kDNA-PCR, on average opossums infected 62% (95% CI, 54–69%) of nymphs whereas *D. rotundus* infected 50% (95% CI, 25–75%). Of 1605 bugs used in XD, 90% were alive on first examination and 10% of them moulted within the 60-day observation period. The *S. lilium* bat that was negative both by XD and SAT-DNA-PCR had two of five xenodiagnostic bugs positive by kDNA-PCR of rectal contents.

Didelphis opossums were found infected in CSJ (7), SIR (1) and CTY (1). The observed prevalence rates of infection in *Didelphis* from the rural area (12%; 95% CI, 3–35%) and the preserved area (18%; 95% CI, 9–32%) were not significantly different (χ^2 test, D.F. 1, $P = 0.58$) (Table 2). The

catch per unit effort (CPUE, per 100 trap-nights) of opossums was significantly greater in the preserved area (7·1; 95% CI, 5·2–9·5) than in the rural area (2·6; 95% CI, 1·6–4·2) (χ^2 test, D.F. 1, $P = 0·0003$) (Table 2). Similarly, rodents were slightly more abundant in the preserved area (1·6; 95% CI, 0·08–3·1) than in the rural area (1·1; 95% CI, 0·06–2·0).

All of the infected opossums were captured in 2012 and 2013 in the rural and in the preserved area, respectively, whereas the 12 opossums examined in 2014 (all from the preserved area, CSJ) were negative by XD and kDNA-PCR (Table 2). The infection prevalence in CSJ dropped from 25% (95% CI, 13–43%) in 2013 to 0% in 2014 despite catches were performed in same season and CPUE remained at similar levels (7·2 *vs* 6·6 per 100 trap-nights, respectively) (Table 2). *Trypanosoma cruzi* infection in *D. albiventris* steadily increased with developmental stage (Fig. 2).

A total of 50 bats from four species were captured (Table 1), 21 in the rural area and 29 in the preserved area. Vampire bats (*D. rotundus*) were most frequent (58%), followed by the frugivorous little yellow-shouldered bats (*S. lilium*) (32%), great fruit-eating bats (*Artibeus lituratus*) (8%), and Ipanema bats (*Pygoderma bilabiatum*) (2%). Four bats were kDNA-PCR-positive for *T. cruzi*, only three confirmed by SAT-DNA-PCR (Table 1). The three confirmed *T. cruzi*-infected vampire bats and the doubtful *S. lilium* were from the preserved area: three were captured by mist netting and one from the colony in the vacant dwelling. The prevalence of infection of vampire bats in CSJ was 21% (3/14; 95% CI, 8–48%) by kDNA-PCR and 14% (2/14; 95% CI, 4–40%) by XD.

TcI was identified in all infected *D. albiventris*, and TcII in both XD-positive specimens of *D. rotundus* (Table 2). For both CytB and H2B genes, the identity with TcII reference strains Esmeraldo and CBB was >98% (GenBank accession numbers are Murci159 CytB KU726546 and H2B KU726548, Murci204 CytB KU726547and H2B KU726549). We were not able to distinguish between TcII, TcV or TcVI in the XD-negative *D. rotundus* bat that was positive both by kDNA- and SAT-DNA-PCR. The phylogenetic analysis of CytB performed confirmed the two isolates were TCII and clustered together with Brazilian and Colombian bat strains (Fig. 3).

Heart and skeletal muscle of mice inoculated with the vampire parasite strain displayed mild to intense myocarditis and/or myositis with scattered amastigote nests (Fig. 4C and D). Calcification foci were observed in skeletal muscle. Liver and spleen exhibited non-specific reactive changes (Fig. 4A and B). Spleen microarchitecture showed reactive changes with enlargement of the lymphoid follicles and the liver suffered mild hepatitis with hyperplasia of

Kupffer cells. Intestine did not present relevant findings.

Searches for triatomines in SIR and CSJ included a total of 270 Noireau trap-nights, 45 ecotopes dissected, and seven opossums tracked. No triatomine was captured during these surveys.

DISCUSSION

Our results document the first finding of TcII in two *D. rotundus* vampire bats from Northeastern Argentina. In addition, we initially recorded a possible *T. cruzi* infection in one *S. lilium* bat captured in (peri)domestic habitats of the preserved area (as determined by kDNA-PCR of blood samples and from XD bug feces), which failed to be confirmed via the less sensitive SAT-DNA-PCR or 24Sα-rDNA-PCR.

Bats have been found infected with all *T. cruzi* DTUs (Marcili *et al*. 2009*a*; Cavazzana *et al*. 2010; García *et al*. 2012; Ramírez *et al*. 2014; Lima *et al*. 2015). TcII has been detected in various bat species from Brazil including the Atlantic Forest and Cerrado biomes (Lisboa *et al*. 2008; Lima *et al*. 2015), remnants of which reach our study area. In addition, TcII was identified in bats from Colombia, but not in *D. rotundus* vampire bats which were infected with TcI and TcIV (Ramírez *et al*. 2014).

In South America TcII occurs both in sylvatic habitats (Jansen *et al*. 2015) and in domestic transmission cycles from central and Southeastern Brazil and Colombia, and in the Gran Chaco region (Brisse *et al*. 2000; Cura *et al*. 2012; Zingales *et al*. 2012). In Brazil, besides the bats mentioned above, TcII was isolated in all biomes, including dogs residing in an area with residual peridomestic infestations adjacent to a conservation unit where the local wild mammals were infected with TcI (Rocha *et al*. 2012); coatis (*Nasua nasua*) from the Brazilian "cerrado" (Rocha *et al*. 2013), *Leontopithecus rosalia* and *Leontopithecus chrysomelas* monkeys and opossums from the Atlantic forest (Jansen *et al*. 2015; Lisboa *et al*. 2015), in six-banded armadillos (*Euphractus sexcinctus*) (Yeo *et al*. 2005), and in dogs and bats from the Amazon basin where no domestic transmission cycle occurs (Jansen *et al*. 2015; Lima *et al*. 2014, 2015). Our phylogenetic analyses of CytB sequences showed that the isolates clearly fitted into the TcII group. Further studies are required to elucidate whether the weakly supported phylogenetic relationships with Colombian bats strains inferred by this method, are congruent or not. The TcII-infected *D. rotundus* bats (including one with an undetermined DTU) and the dubious *S. lilium* were captured indoors and in the yard of a vacant dwelling from an old ranch which had been transformed into a protected area 5 years prior to our survey.

Fig. 2. Phylogenetic tree based on partial CytB gene sequences isolated from bats in this study and those obtained from Ramírez *et al*. (2014) and Marcili *et al*. (2009*a*, *b*) with their corresponding accession numbers at GenBank (in parenthesis). The phylogenies were inferred using the Kimura two-parameter model and maximum-likelihood methods. Numbers at nodes indicates bootstrap values derived from 1000 replicates. ■ Sequences obtained from this study.

The closest inhabited human dwelling at the time of our study was 5 km away. Given the observed association of TcII with domestic and sylvatic transmission cycles in other regions, combined with the extended lifespan (>10 years) and broad flight range (up to 10 km) of *D. rotundus* (Greenhall *et al*.

1983), the putative origins of TcII infections and transmission pathways in the study bats are several: the infected specimens could be part of a residual domestic transmission cycle, a domestic spill-over in the past, or part of an undetected local sylvatic cycle of TcII involving bats only or other
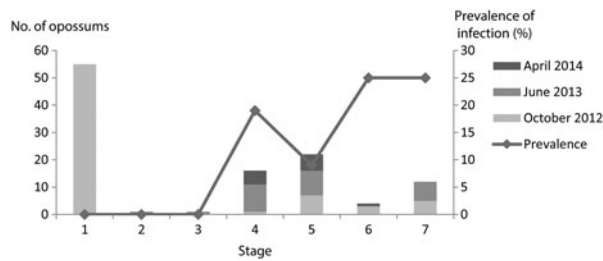
Fig. 3. Frequency of *D. albiventris* opossums captured and prevalence of *T. cruzi* infection according to stage of opossums and date of survey.

mammal species, as suggested by the increasing association of TcII with sylvatic transmission cycles in Amazonia. Bats may become infected by vertical transmission as in *Molossus molossus* (Añez *et al.* 2009), or by horizontal transmission when grooming (Thomas *et al.* 2007). The original source of parasites is also uncertain and may range from a domestic to a wild host.

Failure to confirm the dubious *T. cruzi* infection in *S. lilium* could be attributed to a lack of sensitivity (scarce *T. cruzi* DNA), or that the kDNA-PCR-positive outcome is detecting other bat trypanosomes (e.g. *Trypanosoma vespertilionis* and *Trypanosoma dionisi*). Our attempts to amplify the 24Sα rDNA fragment for further confirmation (Schijman *et al.* 2006) were unsuccessful. Given that both markers differ in the number of copies per genome, 24Sα rDNA-PCR is less sensitive than the kDNA-PCR, thus no conclusive identification of the trypanosome species involved could be achieved.

Although we did not detect triatomines or their signs in or around the vacant dwelling with the infected bats and in wild habitats, several species of Triatominae occur in Southern Misiones, including *Triatoma sordida*, *Triatoma melanosoma* (*syn T. infestans*), *Triatoma rubrovaria* and *Panstrongylus megistus* (Abalos *et al.* 1980; Carcavallo *et al.* 1999). Of special interest is *P. megistus*, which usually has wild colonies and frequently invades domestic premises in Southern Brazil (Litvoc *et al.* 1990). Wild colonies of *P. megistus* were found in refuges of opossums, monkeys, rodents, birds and bats (Lisboa *et al.* 2004), and in bromeliads, palm trees and hollow trees in the neighbouring Rio Grande do Sul State (Santos *et al.* 2013). *Panstrongylus megistus* has been occasionally found in domestic premises in Northeastern Argentina (Damborsky *et al.* 2001).

The haematophagous habits of *D. rotundus* suggest the possibility of an oral route of infection when the bat blood-feeds on a *T. cruzi*-infected mammal. Potential domestic mammalian hosts within the bats' broad home range within the study area included dogs, cats, cattle, equines and

sheep. Among these, dogs and cats are well-known reservoir hosts of *T. cruzi* throughout the Americas and are more infectious than domestic ungulates (Gürtler and Cardinal, 2015). In addition to the wild mammals we captured, the diverse suite of species inhabiting the gallery forest and grasslands include cebus monkeys (*Sapajus caî*), crab-eating raccoons (*Procyon cancrivorus*), lesser grisons (*Galictis cuja*), tayra (*Eira barbara*), margay cats (*Leopardus wiedii*), maned wolves (*Chrysocyon brachyurus*), and southern tamandua (*Tamandua tetradactyla*) (H. Argibay, unpublished observations). Some of these species are competent reservoir hosts of *T. cruzi* (Noireau *et al.* 2009) and a potential source of infection for vampire bats.

The infected *D. rotundus* bats were highly infectious to the vector (50%), and the *T. cruzi* isolate was pathogenic to mice and was further passed on to the vector. The histopathological findings (miocarditis and myositis with scattered amastigote nests) are consistent with an acute infection by *T. cruzi* (Sun and Tarleton, 1993). These results are relevant because the behaviour of *T. cruzi* strains in bats has rarely been investigated, and because some strains are known to be highly virulent, whereas others are weakly or non-pathogenic in experimentally inoculated mice (Martínez-Díaz *et al.* 2001; Roellig and Yabsley, 2010). Whether vampire bats are able to transmit *T. cruzi* when they blood-feed on susceptible mammals is unknown. Successful transmission occurred in *Trypanosoma evansi*, in which *D. rotundus* acted both as a reservoir host and a vector (Desquesnes *et al.* 2013). Because *T. cruzi* has been detected in the saliva and salivary glands of experimentally infected dogs and mice during the acute phase (Marsden and Hagstrom, 1966; Lopes *et al.* 1991), the probability of transmission from a *T. cruzi*-infected vampire bat to a susceptible mammal may not be excluded.

The finding of TcI-infected white-eared opossums was expected given previous findings within the same region (Bar *et al.* 1999; Vaz *et al.* 2007), and because *Didelphis* opossums are the most widely distributed sylvatic reservoir host of TcI throughout the Americas (Noireau *et al.* 2009; Marcili *et al.* 2009b; Zingales *et al.* 2012; Orozco *et al.* 2013). The prevalence of *T. cruzi* infection in the opossums (16%) was lower than elsewhere in the dry Chaco and surrounding areas (Bar *et al.* 1999; Yeo *et al.* 2005; Vaz *et al.* 2007; Alvarado-Otegui *et al.* 2012; Orozco *et al.* 2013), whereas their mean infectiousness to the vector (62%) was similarly high (Orozco *et al.* 2013). The infected opossums occurred both in the rural and preserved areas, and eventually may act as a bridge between (peri)domestic and sylvatic cycles. The drastic drop of opossum infection prevalence in 2014 reveals the large fluctuations in the sylvatic
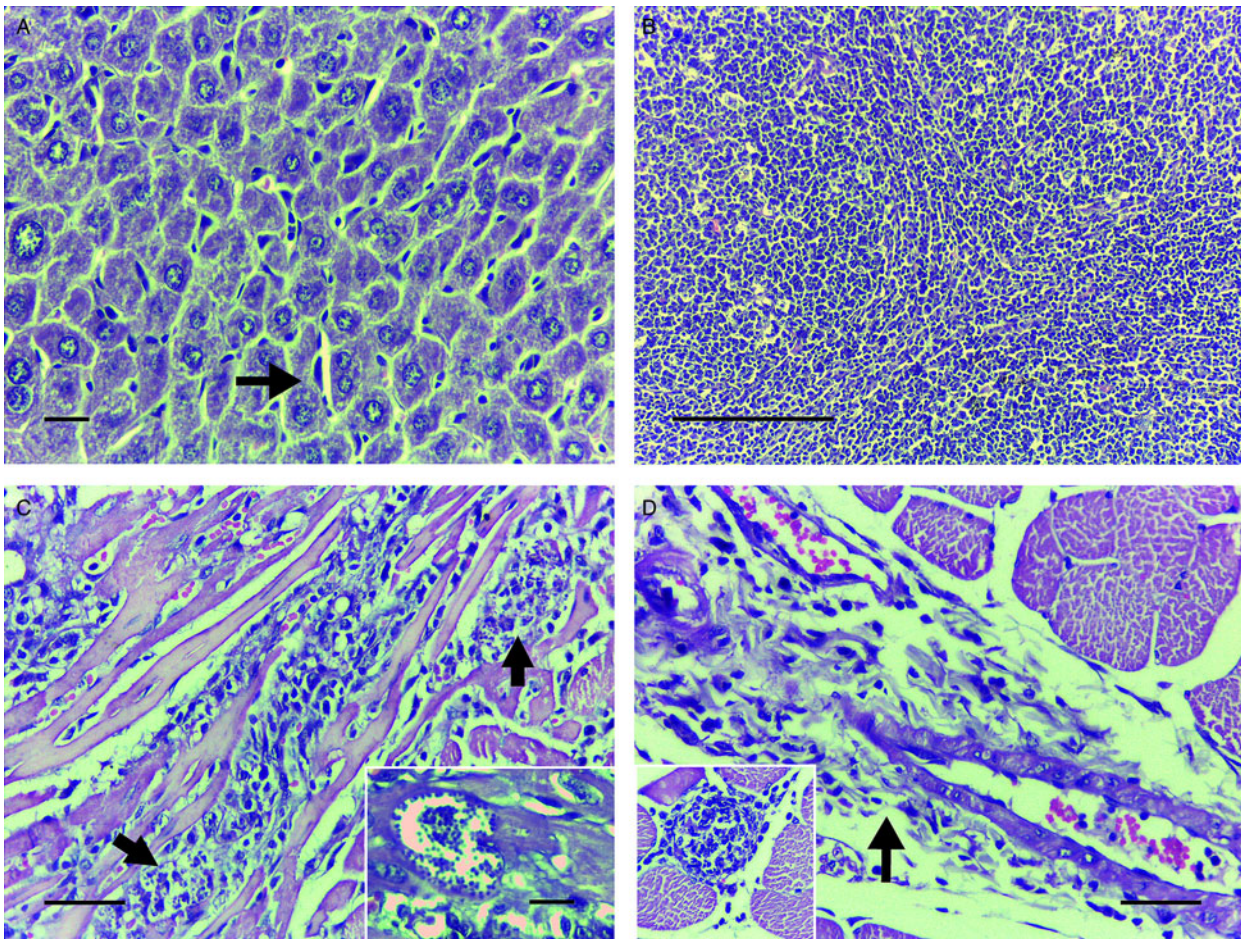
Fig. 4. Histopathology of the liver (A), spleen (B), heart (C) and skeletal muscle (D) of balb-C mice inoculated with *T. cruzi* parasites isolated from a XD-positive *D. rotundus*; (A) Liver showing hyperplasia of Kupffer cell lining sinusoids discreetly dilated (black arrow). A binucleated liver cell can be observed in the bottom left corner (scale bar 400 μm); (B) Spleen with folicular hyperplasia. Microarchitecture of the spleen with lymphoid follicles separated by rows of mature lymphocytes. Note on the left middle and at the top right side of the picture, in the centrofolicular area, numerous dendritic cells giving a starry sky image (scale bar 100 μm); (C) Heart section displaying an intense mononuclear inflammation. Black arrows point foci of inflammation with nuclear debris and amastigotes, oedema and rupture of cardiac muscle cells (scale bar 200 μm). Insert shows parasite nest scale bar 400 μm); (D) Neurovascular bundle of skeletal muscle with development of young collagen (arrow) around a vascular structure with isolated mononuclear cells and oedema (scale bar 200 μm). Insert shows foci of mononuclear inflammation (scale bar 200 μm).

transmission of *T. cruzi* even in a protected area not subject to anthropic disturbance. Such large variations may be overlooked by cross-sectional surveys with limited sample sizes.

Our results may have public health relevance. We found two different transmission cycles of *T. cruzi* involving mammalian host species that occupy preserved areas and (peri)domestic habitats. *Didelphis* opossums were infected in both areas, whereas vampire bats captured indoors or in the vicinities of a vacant house harboured a *T. cruzi* DTU found both in domestic and sylvatic habitats. The finding of vampire bats infected with a genotype of *T. cruzi* identified in other domestic and sylvatic reservoir hosts elsewhere, in a site with no permanent human residents considered to be free of domestic vector-borne transmission, deserves further research efforts.

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