Trypanosoma cruzi TcI and TcII transmission among wild carnivores, small mammals and dogs in a conservation unit and surrounding areas, Brazil

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SUMMARY

Aiming to better understand the ecological aspects of *Trypanosoma cruzi* transmission cycles, wild carnivores, small mammals and dogs were examined for *T. cruzi* infection in the Serra da Canastra National Park region, Brazil. Isolates were genotyped using mini-exon gene and PCR-RFLP (1f8 and H3) genomic targets. *Trypanosoma cruzi* transmission was well established in the area and occurred in both wild and peridomestic environments. Dog seroprevalence was 29·4% (63/214) and TcI and TcII genotypes, besides mixed infections were observed. Only TcI was detected in wild mammals. Marsupials displayed lower relative abundance, but a high prevalence of positive haemocultures (4/22), whereas rodents displayed positive haemocultures (9/113) mainly in the abundant *Akodon montensis* and *Cerradomys subflavus* species. The felid *Leopardus pardalis* was the only carnivore to display positive haemoculture and was captured in the same region where the small mammal prevalence of *T. cruzi* infection was high. Two canid species, *Chrysocyon brachyurus* and *Cerdocyon thous*, were serologically positive for *T. cruzi* infection (4/8 and 8/39, respectively), probably related to their capacity to exploit different ecological niches. Herein, dog infection not only signals *T. cruzi* transmission but also the genotypes present. Distinct transmission strategies of the *T. cruzi* genotypes are discussed.

Key words: transmission cycles, trophic network, reservoir, Discrete Typing Units, Chagas disease, Serra da Canastra National Park, Brazil.

INTRODUCTION

The aetiological agent of Chagas disease, *Trypanosoma cruzi*, is a multi-host parasite found in more than 100 mammalian species and capable of infecting almost all cell types (Noireau *et al*. 2009). Human infections have been generally associated with contact with the contaminated feces of infected triatomine bugs, besides blood transfusion, organ transplantations, congenital transmission and oral transmission. Indeed, this latter has been responsible for the most recent outbreaks in Brazil and is probably the most ancient route of infection among wild animals (Noireau *et al*. 2009, Shikanai-Yasuda and Carvalho, 2012). The continually new human cases demonstrate that numerous aspects of Chagas disease epidemiology still remain unclear, probably because the transmission cycles of the parasite are maintained in intricate transmission networks that embrace several mammalian and vector species, resulting in unique epidemiological scenarios.

Trypanosoma cruzi is a highly diverse complex of genetic lineages. The current nomenclatural consensus recognizes 6 major genotypes or 'Discrete Typing Units' (DTUs) within the taxon, *T. cruzi* I (TcI) to *T. cruzi* VI (TcVI) (Zingales *et al*. 2009). To date, all of them occur in Brazil, although with different geographical distribution patterns and ecological characteristics. TcI is described to be an ubiquitous lineage in view of the diversity of its hosts, vectors and habitats. The TcII lineage is reported to have a more restricted geographical distribution and to

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occur in focal transmission cycles. However, it has been described in several mammalian taxa and biomes (Lisboa *et al*. 2006, 2008; Herrera *et al*. 2008), suggesting that it may be much more widespread than is currently acknowledged. TcIII is found mainly in the Amazonia biome, although sparsely reported throughout the country. TcIV has been recorded in northern and northeastern Brazil, whereas TcVI has been found in the middlewestern and southern regions (Zingales *et al*. 2012). TcV is described to occur in Argentina, Bolivia, Paraguay and in northeastern Brazil (Araujo *et al*. 2011). This broad distribution in distinct mammalian host species suggests that we are far from understanding the dispersion strategies of each lineage and its consequence for the epidemiology of *T. cruzi* infection.

The importance of each mammalian species in the maintenance and dispersion of a multi-host parasite like *T. cruzi* will rely mainly on the ability of the parasite to persist in the mammalian host and be transmitted to the vector, besides the host's relative abundance. In this sense, we consider as reservoir a species or community responsible for the long term survival of a parasite in a given area (Ashford, 1996), in which the role of each host species should be interpreted at intervals of time and space as well as accounting for the community composition and environmental characteristics. Thus, the importance of domestic dogs as reservoirs of *T. cruzi* varies throughout Latin America. In northwestern Argentina, dogs displaying high parasitaemias and infectiousness to vectors for long periods have been recorded (Gurtler *et al*. 2007), whereas in most countries, including Brazil, dogs display high seroprevalence but rarely present high parasitaemia levels (Roque *et al*. 2008; Pineda *et al*. 2011; Xavier *et al*. 2012). With regard to small mammals, a considerable number of marsupial and rodent species have been found naturally infected (World Health Organization, 2002). From these, species of the opossum genus *Didelphis* have been generally pointed out as the main reservoir, mostly due to the fact that studies focused mainly on this synanthropic genus, thus discounting other mammal species that may compose the *T. cruzi* reservoir system within a given area.

Little is known about the role of carnivores in the *T. cruzi* transmission network, probably because the examination of free-ranging carnivores requires long-term and technically challenging studies. Data are available for 2 Procyonidae species, the raccoon (*Procyon lotor*) and the ring-tailed coati (*Nasua nasua*) (Herrera *et al*. 2008; Kribs-Zaleta, 2010), but for wild canid and felid species, the only available information is that they are exposed to the *T. cruzi* transmission cycles in different environments, as expressed by positive serological tests (Brown *et al*. 2010; Herrera *et al*. 2011). In essence, carnivores have great potential to be important reservoirs due to the high diversity in their ecological niches that might range from insectivorous to carnivorous diet in different forest strata and habitats (Nowak, 2005), favouring contact with different components of the *T. cruzi* transmission net. Besides, top predators can be bioaccumulators of parasites (Cleaveland *et al*. 2006), and this may be also the case for *T. cruzi*, since the oral transmission is a highly efficient route for this parasite. Along with their huge biomass and broad home range, these characteristics give them a great potential to amplify and spread the parasite populations.

The Serra da Canastra National Park (SCNP) is a natural landscape conservation unit in Minas Gerais state, one of the oldest known endemic areas for Chagas disease in Brazil. Herein, the aim of this study was to evaluate *T. cruzi* transmission in both peridomestic and sylvatic environments in the SCNP region. The role played by the different components of the *T. cruzi* reservoir net: wild carnivores, small mammals and sympatric domestic dogs and the maintenance of distinct *T. cruzi* lineages in the area are discussed.

MATERIALS AND METHODS

Study area

The study was conducted within the Serra da Canastra National Park – SCNP (UTM 23K 345499/7764402) and adjacent areas, in Minas Gerais state, southeastern Brazil (Fig. 1). It is an important remnant of the Cerrado biome and shelters huge populations of some vulnerable mammalian species, such as the maned wolf (*Chrysocyon brachyurus*) and the giant anteater (*Myrmecophaga tridactyla*). Many streams and rivers, including the São Francisco river, originate in the highlands of the SCNP. The vegetation is basically made up of highland grasslands, with some spots of stone fields, scrub savanna and riparian vegetation occurring sparsely alongside the river courses. The altitude varies from 700 to 800 meters above sea level in valleys and above 1000 meters on the plateau. The climate is tropical, the dry season occurs from March to October and the wet season from November to February. Annual rain precipitation ranges from 1200 to 1800 mm and average temperature is around 22–23 °C (IBAMA. Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis, 2005).

The park was created in 1972 with a total area of 2000 ha of which only 715 ha are managed by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), whereas the remaining areas are still privately owned. The park is surrounded by small rural properties (< 100 ha) whose economy is based on cattle ranching for artisanal cheese production and coffee plantations. Total rural population is 5500 inhabitants (Bizerril *et al*. 2011).

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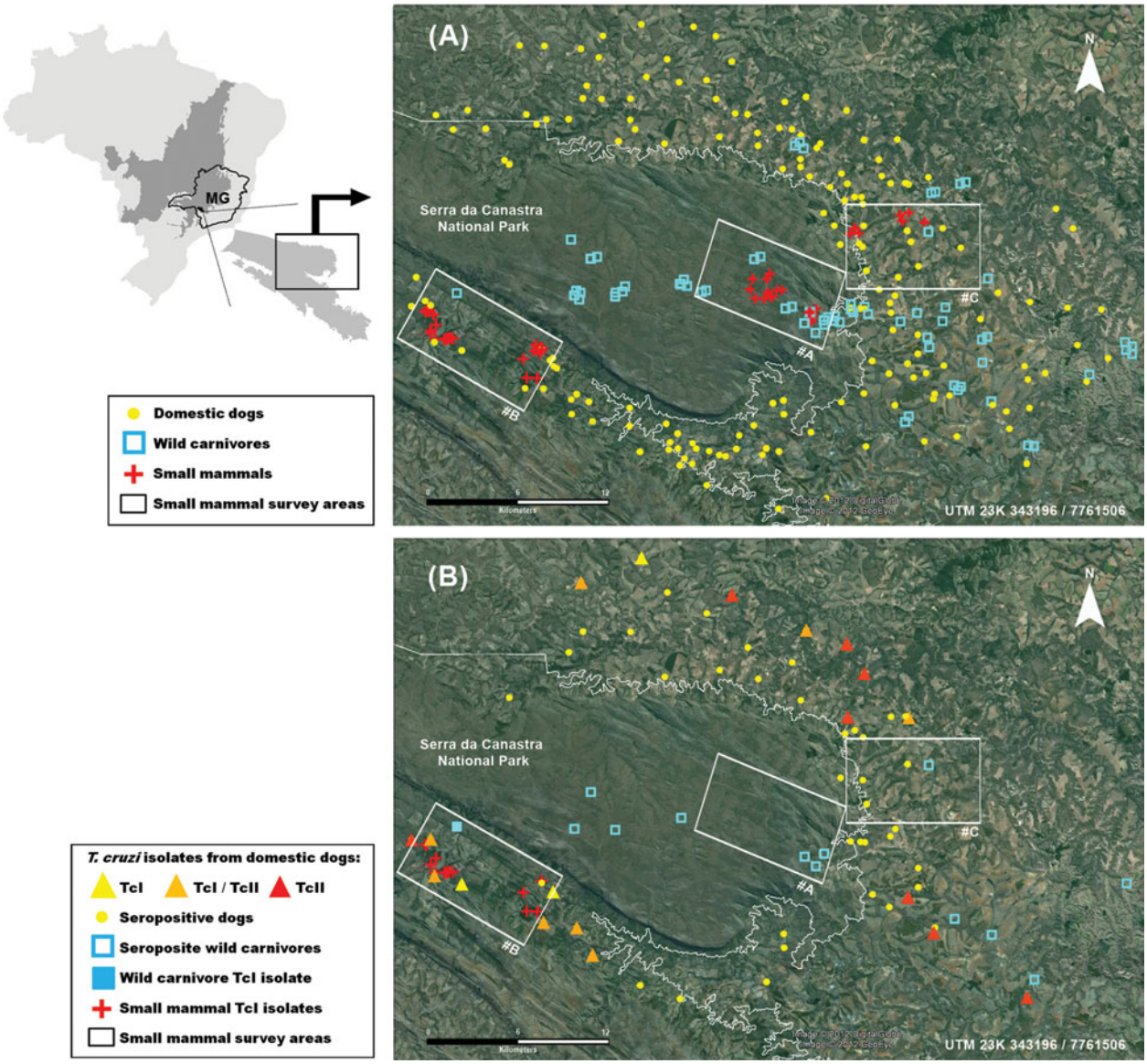

Fig. 1. Map of the spatial distribution of *Trypanosoma cruzi* infection in wild mammals from Serra da Canastra National Park (SCNP) and its surroundings. (A) All mammals sampled; (B) *T. cruzi* infected mammals. Triangles represent domestic dog isolates (genotypes according to the figure legend); blue squares represent infected wild carnivores and red crosses represent infected small mammals. The white squares indicate small mammal survey areas: #A – within SCNP, #B – Vão dos Cândidos region and #C – Cerradão/São Roque region. In the upper left figure the study site in Brazil, the grey shade corresponds to the limits of the Cerrado biome and the black contour shows Minas Gerais state (MG) limits.

Dog surveys

A house-to-house census of dogs was undertaken in farms located 5 to 20 km from the park border during annual rabies vaccination campaigns from 2007 to 2010. The annual average domestic dog population for the 4 years follow-up was 557 ± 59 individuals. After owner consent, blood samples were collected by puncture of the cephalic vein through a Vacutainer® system. Age class was based on the owner information and confirmed with dental condition status. We considered as juveniles dogs younger than 6 months and adults as the dogs older than that.

Our sample included 214 dogs, composed by 177 adults and 39 juveniles, ranging from 3 months to 14 years. The sex ratio was 3:1 (161 males/53 females). In the calculation of the prevalence of *T. cruzi* infection, resampled infected dogs were counted once.

The majority of adult dogs (71%) were used in cattle raising and slept outside the house. Owners also reported that their dogs hunt and that they go out by themselves for several consecutive days. The juvenile dogs were reported to be restricted only to peridomestic areas.

Capture of wild mammals

Wild carnivores. These were captured from March 2007 to August 2011 using box traps made with

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galvanized wire mesh baited with sardine and boiled chicken. Traps were disposed both inside the park and on adjacent farmlands. We immobilized the animals with an intramuscular injection of a combination of zolazepan and tiletamine (Zoletil®) at dosages of 3 mg/kg for maned wolves, 8·3 mg/kg for ocelots (*Leopardus pardalis*) and 10 mg/kg for hoary foxes (*Lycalopex vetulus*) and crab-eating foxes (*Cerdocyon thous*). We also used a subcutaneous injection of 0·04 mg/kg of atropine sulphate, whenever necessary. Anaesthetized animals were weighed, measured and had their teeth condition assessed in order to estimate age and were marked with ear-tags or radiocollars for individual identification. We took blood samples by puncture of the cephalic vein stored in Vacutainer® tubes for haemoculture and serological tests. Animals were released at the site of capture after recovery from anaesthesia. Total capture effort was 3819 traps/night.

Small wild mammals. These were captured using live traps (Sherman® – H. B. Sherman Traps, Tallahassee, FL, USA and Tomahawk® Tomahawk Live Traps, Tomahawk, WI, USA) baited with a mixture of banana, peanut butter, oat, bacon/sardines. Traps were set for 5 consecutive nights along linear transects, placed on the ground at 10 m intervals and alternating between trap type, in 3 field expeditions (May 2010, February 2011 and August 2011). Traps were placed into distinct habitat types inside SCNP (gallery forest, stone fields, savanna, and grasslands) as well as in 2 vicinal regions under anthropogenic influence, 'São Roque/Cerradão' and 'Vão dos Cândidos'. The last region is within the official limits of the SCNP, but not managed as a conservation unit, since there are still several privately owned farmlands. Total capture effort was 3126 traps-nights, equally distributed among the 3 expeditions. We calculated the relative abundance of small mammals as the number of individuals of each species divided by the total number of individuals multiplied by 100. Identification of specimens was based on external and cranial morphological characters and on karyological analyses as described by Bonvicino *et al*. (2005). Voucher specimens were deposited in the Mammal Collection of the National Museum – UFRJ (Rio de Janeiro, Brazil). Blood samples were collected by cardiac puncture after anaesthesia with an intramuscular injection of ketamine (10–30 mg/kg) associated with acepromazine (5–10 mg/kg) for rodents (proportion 9:1) or xylazine (2 mg/kg) for marsupials (1:1).

All animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian government environmental agency (Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO license number 18635–3) and was endorsed by the ethics committee of the Oswaldo Cruz Institute/FIOCRUZ (CEUA P-292–06), in accordance to Brazilian regulations. Appropriate biosecurity techniques and individual protection equipment were used during all procedures involving animals and biological sample collecting and handling.

Trypanosoma cruzi *infection*

The *T. cruzi* infection survey was performed by parasitological and/or serological methods. If insufficient blood was collected, priority was given to haemoculture. Parasitological tests were based on examination of fresh blood smears (microscopic analysis) and haemoculture (HC), the latter performed as follows: 0·3 ml of blood from each animal was cultured in 2 tubes containing Novy-McNeal-Nicole (NNN) medium with liver infusion tryptose (LIT) overlay. Tubes were examined every 15 days up to 5 months. When positive, parasites were amplified, cryopreserved and deposited in the Collection of Trypanosomatids from wild mammals, domestic animals and vectors – COLTRYP (Fundação Oswaldo Cruz, Rio de Janeiro-RJ, Brazil).

For the detection of anti-*T. cruzi* IgG antibodies in sera we used the Indirect Fluorescent Antibody Test (IFAT) as previously described by Camargo (1966) and the Enzyme-Linked Immunoabsorbent Assay (ELISA, Biomanguinhos, Rio de Janeiro-RJ, Brazil). We also searched for IgM antibodies through the IFAT to identify recent *T. cruzi* infection among HC-positive dogs and other dogs from the same farms. The antigen used in serological assays for all species was obtained from a *T. cruzi* isolate derived from dogs of this study area harvested from axenic culture. For small mammals, rodent sera were tested with a commercial anti-rat IgG conjugate (FITC, Sigma-Aldrich®, St Louis, MO, USA), whereas marsupial sera were tested with an intermediary anti-opossum serum raised in rabbits followed by a commercial anti-rabbit conjugate. Wild canids and felids were tested using domestic dog and cat conjugates, respectively. The cut-off value titre adopted for IFAT was 1:40 for dogs and marsupials and 1:10 for rodents (Herrera *et al*. 2005). The cut-off value for ELISA was optical absorbance $\geqslant$ 0·200 mean ± 3 S.D. For wild carnivores, the test was performed with a non-specific conjugate and since there are no available data on IFAT cut-off values for some carnivore species of this study, we performed a PCR in all ELISA-positive serum samples besides a subsample of ELISA negative samples ($n = 5$). Therefore, the cut-off value adopted was 1:20, as it was the lowest serum dilution in which parasites could be detected by PCR. Each reaction included 2 positive and 2 negative control sera.

In order to detect mixed-infection and/or cross-reaction with *Leishmania* spp. we performed an

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IFAT test using *L. infantum* and *L. braziliensis* parasites harvested from axenic culture as antigens (cut-off: 1:40). For wild and domestic canids, we also used the Rapid Test for Diagnosis of Canine Visceral Leishmaniasis (TR DPP®, BioManguinhos, Rio de Janeiro, Brazil).

Samples were considered positive when parasites were isolated by HC or when the sera showed reactivity in at least 2 of the serological tests. Samples that had IFAT-IgG concomitant positive values for *T. cruzi* and *Leishmania* sp. with non-corresponding ELISA, DPP or that displayed borderline results were attributed to cross-reaction and were considered indeterminate.

Trypanosoma cruzi *molecular characterization*

Genomic DNA was extracted from cultures and wild carnivore serum samples using standard phenol-chloroform protocols (Vallejo *et al*. 1999). Characterization was carried out in 3 steps: (1) multiplex PCR amplification of the mini-exon gene following conditions described by Fernandes *et al*. (2001) for the identification of 3 DTU *T. cruzi* groups: Tc1 (TcI – 200 basepairs), Tc2 (TcII/TcV/TcVI – 250 basepairs) and Zymodeme 3 (TcIII/TcIV- 150 basepairs), besides *T. rangeli* (100 basepairs) or mixed infections; (2) PCR amplification of nuclear 1f8 gene followed by restriction fragment length polymorphism (RFLP) analysis of fragments digested by Alw21I enzime (Rozas *et al*. 2007) to discriminate TcII from hybrids (TcV and TcVI) DTU's in isolates previously typed as Tc2 or mixed Tc1/Tc2 in mini-exon assays and (3) PCR-RFLP of histone H3/AluI (Westenberger *et al*. 2005) to rule out hybrids (TcV and TcVI) in mixed Tc1/Tc2 infections, as it could possibly overlap digested fragments in 1f8/Alw21I assay. Both PCR-RFLP 1f8/Alw21I and histone H3/AluI were performed with minor modifications in the conditions described by Rozas *et al*. (2007). Each reaction included a negative control and positive control samples from those *T. cruzi* strains representing the DTUs to be typed. PCR products were visualized in 2% agarose gel after ethidium bromide staining and visualized under ultraviolet light.

Spatial and statistical analyses

In order to verify the spatial distribution of trypanosomatid infection, locations of each individual captured were accessed through a hand-held GPS receiver using the WGS 84 Datum geodetic coordinate system. Locations were analysed in a Geographic Information System platform using GPS trackmakerPRO® software (Geostudio Tecnologia, Brazil) juxtaposed on a base map modified from Google earth® software (v. 6.2, Google Inc., USA)

To examine the distribution pattern of *T. cruzi*-infected dogs, the mean geographical distance was compared among infected dog locations ($n=76$) to the mean distance distribution across 10 000 randomly assigned samples of the same size using R 2.13 software. Spatial autocorrelation of seropositive dogs was tested with Moran's I. Maps with discriminated locality of infected hosts and parasite genotypes were also prepared. Statistical tests were conducted with $\alpha=0{\cdot}05$.

The degree of concordance between IFAT-IgG and ELISA assays was assessed by the kappa statistic using SYSTAT 11 for Windows. To test for the influence of sex and age class on *T. cruzi* infection rates in dogs the 3-dimensional Chi-square contingency table was used. We also compared the *T. cruzi* infection among domestic dogs, carnivores, rodents and marsupials and applied a Chi square test to verify whether infection rate is independent of taxonomic group.

RESULTS

Trypanosoma cruzi *infection in dogs*

Dogs are included in a well-established *T. cruzi* transmission cycle in all the geographical regions surrounding the Serra da Canastra National Park, as demonstrated by the high parasitological and serological *T. cruzi* infection prevalence (Table 1, Fig 1).

Trypanosoma cruzi was isolated from the blood of 19 dogs (7·9%, $n=214$), collected on the same expedition (September/2010). Genotyping revealed the presence only of 2 main *T. cruzi* lineages: TcI ($n=3$) and TcII ($n=8$), besides mixed TcI/TcII infections ($n=8$) (Figs 1 and 2). After 5 months, we re-examined 10 out of the 19 dogs that previously displayed positive HC and none of them tested positive. Among the remaining HC-positive dogs, 6 died and 3 disappeared.

Prevalence was calculated considering the total number of examined dogs during the study. A total of 63 dogs (29·4%) were seropositive for *T. cruzi* (Table 1), including 8 (12·7%) individuals that were co-infected with *Leishmania* sp. Once infected, *T. cruzi*-positive testing dogs maintained serological titres, as observed during re-sampling. Seventeen dog samples were considered indeterminate by the serological assays. We found no significant difference in the *T. cruzi* infection rate between the dogs' sex or age class (Chi square = 2·69; D.F. = 7; $P=0{\cdot}9$). The agreement between IFAT-IgG and ELISA was 78% with a kappa value of 0·522 (moderate agreement). ELISA presented higher sensitivity to detect the dog's acute phase since it was positive in 15 out of 19 HC-positive dogs, whereas IFAT detected infection in only 4 of them.

Six juvenile dogs (3–6 months old) displayed positive HC, demonstrating that *T. cruzi*

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Table 1. *Trypanosoma cruzi* infection assessment through serology (IgG – IFAT/ELISA) and haemoculture (HC) in mammals from the Serra da Canastra National Park and surrounding areas, Brazil

Species (common name)	*N*	Capture location[1]	Small mammals relative abundance (%)[2]	Serology (Positive/*N*)	HC (Positive/*N*)	Genotype
Akodon montensis	27	a,b	20·0	0/17	1/27	TcI
Akodon spp.[3]	13	a,b	9·6	0/5	1/13	TcI
Calomys spp.[4]	14	b	10·4	0/3	3/14	TcI
Cerradomys subflavus	15	a,b	11·1	0/13	4/15	TcI
Necromys lasiurus	28	a,b	20·7	0/21	0/28	–
Nectomys squamipes	3	a,b	2·2	0/3	0/3	–
Oligoryzomys spp.[5]	4	a,b	3·0	0/2	0/4	–
Oxymycterus delator	9	a,b	6·7	1/8	0/9	–
Total rodents	113		83·7	1/72 (1·4%)	9/113 (7·9%)	–
Caluromys philander	1	b	0·7	0/0	1/1	TcI
Didelphis albiventris	4	a,b	3·0	0/4	0/4	–
Gracilinanus agilis	4	a	3·0	0/2	0/4	–
Lutreolina crassicaudata	1	a	0·7	0/1	0/1	–
Marmosops incanus	5	b	3·7	4/4	3/5	TcI
Monodelphis spp.[6]	7	a,b	5·2	2/5	0/7	–
Total marsupials	22		16·3	6/16 (37·5%)	4/22 (18·1%)	–
Chrysocyon brachyurus (Maned wolf)	39	a,b	–	8/39	0/30	–
Cerdocyon thous (Crab-eating fox)	8	a,b	–	4/8	0/3	–
Lycalopex vetulus (Hoary fox)	10	a,b	–	0/10	0/6	–
Leopardus pardalis (Ocelot)	1	b	–	1/1	1/1	TcI
Conepatus semistriatus (Skunk)	2	a,b	–	0/0	0/2	–
Total wild carnivores	60		–	13/58 (22·4%)	1/42 (2·4%)	–
Canis lupus familiaris (Dog)	214		–	63/214 (29·4%)	19/214 (7·9%)	TcI (3) TcII (8) TcI-TcII (8)

[1] Capture location site: a, SCNP; b, Farmlands; (–), not applicable.
[2] Number of individuals of each species divided by the total number of individuals * 100.
[3] *Akodon* sp. (*n* = 6; a, b), *A. lindberghi* (*n* = 5; a), *A. cursor* (*n* = 1; a).
[4] *Calomys* sp. (*n* = 3; b) *C. tener* (*n* = 11; b).
[5] *Oligoryzomys* sp. (n = 2, b), *O. nigripes* (*n* = 1; a), *O. rupestris* (*n* = 1; a).
[6] *Monodelphis americana* (*n* = 3; b), *M. domestica* (*n* = 3; b) and *M. sorex* (*n* = 1; a).

transmission also occurs within the peridomestic environment. An active transmission in that region was confirmed by, among other factors, the serological conversion observed in 4 dogs 1 year after the first examination and the 4 HC-positive dogs that displayed concomitant positive IFAT-IgM, indicative of recent infection. Further, 28·3% (*n* = 46) of the dogs amid farms with HC-positive dogs had IFAT-IgM antibodies (corroborated by ELISA), suggestive of recent infection in this scenario. The *T. cruzi* infection in dogs was spatially autocorrelated (Moran's I: observed = 0·674; expected = – 0·004; S.D. = 0·076; *P*-value = 0) and not homogeneously distributed ($P < 0{\cdot}004$), suggesting that transmission occurred throughout the SCNP surrounding areas, though with hotspot transmission foci (Fig. 1, and see Supplementary Material, online version only).

Trypanosoma cruzi *infection in wild mammals*

A total of 60 wild carnivores belonging to 5 species were examined for *T. cruzi* infection. The only felid species examined, the ocelot, tested positive both in fresh blood preparations and at the first HC reading (7 days after blood culture) along with an elevated serology titre (1:160); altogether indicative of high parasitaemia levels. The ocelot's *T. cruzi* isolate was genotyped as TcI (Table 1).

The wild canids were exposed to infection as demonstrated by serology but none were parasitologically positive by HC suggesting they may not be infective to vectors. The crab-eating fox had the highest serum prevalence rate (50% – 4/8) followed by the maned wolf (20·5% – 8/39) (Table 1). Titres ranged from 1:20 to 1:80 (IFAT). All hoary fox samples were seronegative. Three (5·8%) maned wolf

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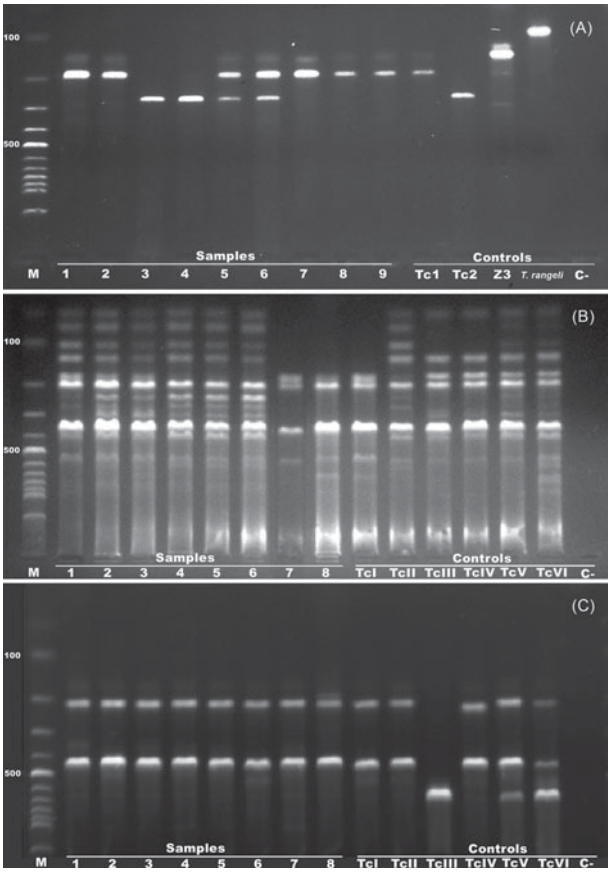

Fig. 2. *Trypanosoma cruzi* genotyping of domestic and wild mammal isolates from the Serra da Canastra National Park and surroundings, Brazil. (A) PCR products of the Mini-exon gene analysed by agarose electrophoresis gel stained with ethidium bromide. Lanes: M, Molecular weight markers (100 bp DNA ladder); 1–6, dog isolates; 7, wild carnivore isolate (*Leopardus pardalis*); 8, rodent isolate (*Akodon montensis*); 9, marsupial isolate (*Marmosops incanus*); Control samples: Tc1 (TcI – 200 bp), Tc2 (TcII/TcV/TcVI – 250 bp), Z3 (TcIII/TcIV – 150 bp), *T. rangeli* (100 bp). (B) PCR-RFLP products of 1f8 gene/Alw21I. Lanes: 1–3, dog isolates characterized as mixed Tc1/Tc2 in Mini-exon gene assay; 4–6, dog isolates characterized as Tc2; 7–8, dog isolates characterized as Tc1. Control samples: PCR-RFLP 1f8/Alw21I digestion patterns of TcI to TcVI. (C) PCR-RFLP products of histone H3/AluI. Lanes: 1–8, dog isolates characterized as mixed Tc1/Tc2 infection in Mini-exon gene assay. Control samples: PCR-RFLP H3/AluI digestion patterns of TcI to TcVI.

samples were considered indeterminate. The agreement between IFAT-IgG and ELISA was 82% with a kappa value of 0·628 (substantial agreement). The 2 specimens of the skunk (*Conepatus semistriatus*) examined only by HC tested negative.

Concerning the small mammals, relative abundances and fauna richness were comparable inside SCNP and farmlands (Table 1). The marsupials displayed high parasitaemia levels, mainly *Marmosops incanus* since 3 out of 4 seropositive individuals were positive on both fresh blood examination and HC. Positive HC was also achieved from the only captured *Caluromys philander*. Infection by IFAT was detected in the marsupial species *Marmosops incanus, Monodelphis americana* and *Monodelphis domestica* (prevalence of 37·5%), titres ranged from 1:40 to 1:320. Rodents from *Akodon, Calomys* and *Cerradomys* species also presented parasitaemias detected by HC in 9 individuals (Table 1). Of these positive rodents, we could collect sufficient blood to perform the IFAT assay from only 3 that tested negative. One *Oxymycterus delator* was the only seropositive sample (prevalence of 1·4%). The small mammal isolates were all characterized as TcI (Table 1, Fig. 1).

All *T. cruzi*-infected small mammals were collected in the 'Vão dos Cândidos' region as well as the infected ocelot (Fig. 1). The infected crab-eating foxes and 4 maned wolves were captured in farmland regions. Four serologically positive maned wolves displayed home range areas exclusively inside the SCNP, as confirmed by our parallel observations using radio telemetry techniques (May-Junior *et al*. 2009).

Comparing the 2 farmland areas, in the 'Vão dos Cândidos' region both small mammals and dogs presented high prevalence of positive HC (13/65–20% and 6/25–24%, respectively) whereas in the 'Cerradão/São Roque' region, neither small mammals ($n=7$) nor dogs ($n=21$) displayed positive HC. Overall, the proportion of *T. cruzi* infection varied significantly in different taxonomic groups. Domestic dogs tended to have more positive diagnoses than predicted by chance, whereas rodents had fewer positive diagnoses (Chi square = 21·56; D.F. = 3; $P<0·001$).

DISCUSSION

In the present study we describe a well-established and broadly distributed *T. cruzi* cycle in all geographical regions surrounding SCNP, which includes wild and domestic animals. The presence of dogs with high parasitaemia, as demonstrated by positive haemoculture (HC), contrasts with previous studies in Brazil, in which none or a minority of individuals displayed positive HC (Herrera *et al*. 2005; Roque *et al*. 2008; Xavier *et al*. 2012). The herein positive HC dogs reflect probably the initial phase of infection, typically characterized by a short period of detectable parasitaemia (Machado *et al*. 2001). A similar pattern in naturally infected dogs was observed in Monte Alegre, in the state of Pará, in Northern Brazil (Xavier *et al*. 2012). Besides, serological conversion observed during the follow up attested that they are continually being exposed to *T. cruzi* infections.

Dogs may become infected by the contaminative route (Gurtler *et al*. 2007) or by ingesting infected triatomines, a highly efficient transmission route

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(Pineda *et al*. 2011). Another possible infection route might be by hunting of infected small mammals (Herrera *et al*. 2011). Actually, with regards to the 2 areas where we simultaneously sampled for small mammals and dogs, dog infection was coincident with small mammal infection. In the SCNP, *T. cruzi* transmission might occur not only in the wild, but also in the peridomestic environment, as demonstrated by the infected juvenile dogs. Herein, besides acting as sentinels hosts, as already described in other Brazilian areas (Xavier *et al*. 2012), dogs were also able to signal the presence of the 2 main *T. cruzi* genotypes in the area, TcI and TcII.

In the SCNP, the huge distance among the infected dogs along with the fact that the foci of peridomiciliary vectors are residual and submitted to regular spraying rule out the existence of a *T. cruzi* transmission cycle supported solely by dogs. Thus, the finding of TcII genotype infecting only dogs, contrasting with TcI which was also found infecting wild mammals is an apparent paradox. At a first glance the explanation could rely on TcII circulation exclusively in mammalian groups not sampled in this study, such as armadillos and bats (Yeo *et al*. 2005; Lisboa *et al*. 2008). However, this seems an oversimplified explanation; a similar picture was observed in a Chagas Disease outbreak in Santa Catarina state, where TcII was found in humans and triatomines but not in the mammalian fauna (Roque *et al*. 2008; Steindel *et al*. 2008). Moreover, reports of TcII in different wild mammal species and biomes demonstrate that this genotype is also maintained in wild cycles (Lisboa *et al*. 2006; Herrera *et al*. 2008). This raises the question on where the TcII is hidden in nature. This could reflect the transmission strategy of this genotype. In analogy with the r-k ecological selection theory, the parasitaemia curve of TcII within its mammal hosts resemble an r strategist – one precocious and short period of high parasitaemia (Andrade and Magalhaes, 1996) and ultimately would impact on the dispersion strategy of this genotype in the wild. This kind of strategy does not impede TcII transmission in the wild, but hampers parasite detection, as a consequence, it may be underestimated in nature. Further, we cannot rule out the oddities in host-parasite interactions. For instance, the golden lion tamarin (*Leontopithecus rosalia*) maintains long-lasting TcII infection with high prevalence of positive HC throughout (Lisboa *et al*. 2006), whereas the opossum *Didelphis aurita* can control and even eliminate TcII in experimental conditions (Jansen *et al*. 1991). Here, we report for the first time TcII isolation and molecular characterization in dogs from Brazil, a well-studied *T. cruzi* host. This reinforces the view that the spectrum of mammal host infected by this genotype is currently underestimated.

Undoubtedly, from our results, none is more puzzling than the high number of dogs in early stages of infection in broadly distributed and unlinked areas simultaneously. This raises the idea that still unknown variables must be involved in the dispersion of *T. cruzi* among several host species and that these variables are included in a broader phenomenon. For instance, only recently El Niño Southern Oscillation (ENSO) and similar phenomena have been taken into account to influence living organisms (Hanf *et al*. 2011). Whatever the cause, the frequent occurrence of unsolved questions in biological systems may be related to our limitation in analysing them detached from a linear, Cartesian focus. Parasitic transmission nets are clearly complex systems since they are essentially dynamic, multivariate, non-linear and unpredictable, rendering a reductionist and deterministic focus interpretation of this phenomenon worthless. Indeed focusing these phenomena in the light of the chaos theory could perhaps fill the several gaps in the current knowledge of this issue (Mazzocchi, 2008). Taking into account the presence of several dogs geographically separated and recently infected by *T. cruzi* may be a stochastic phenomenon, or the signal that the maximum transmission fitness was achieved in that moment; indeed, a feature described to be the characteristic of the edge of the chaos.

The role of each wild mammal species in the *T. cruzi* transmission networks will depend on the ability of the parasite to be transmitted to its vector, as well as the abundance and distribution of its mammal host species. In SCNP, rodents and marsupials were shown to be equally important for the maintenance of *T. cruzi*. The marsupials, regardless of their low relative abundance, displayed high prevalence of positive HC, in particular the arboreal *Marmosops incanus* and *Caluromys philander*. Rodents also displayed positive HC, mainly in highly abundant species, i.e. the terrestrial *Akodon montensis* and *Cerradomys subflavus*, pointing to the epidemiological importance of these mammals in the local transmission net. Moreover, *T. cruzi* infection was spread among terrestrial and arboreal mammals, demonstrating that parasite transmission was occurring in both strata. We observed high *T. cruzi* infection rates in the area despite the low relative abundance of the common *Didelphis* genus, generally described as one of the most important and competent mammalian reservoir of the parasite (Yeo *et al*. 2005). This emphasizes the characteristic dynamics of *T. cruzi* transmission cycles that should be examined as an unique ecological system.

Herein, we observed that 3 wild carnivore species, the ocelot, the crab-eating fox and the maned wolf were infected by *T. cruzi* in the SCNP region, but they probably play distinct roles as a result of their pattern of *T. cruzi* infection and peculiar ecological characteristics. The ocelot was the only carnivore that displayed patent parasitaemia (positive fresh blood examination and HC). To the best of our knowledge this is the first report of an ocelot naturally infected

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with *T. cruzi*. In comparison to the other 2 carnivore species of this study, the ocelot is the one which better exemplifies the bioaccumulator role in a prey-predator chain since it has a more carnivorous diet, consuming mainly small mammalian prey, besides birds (Rocha-mendes *et al*. 2010). In fact, this infected ocelot was the only carnivore captured in the 'Vão dos Cândidos' region, where small mammals displayed high parasitaemia levels. Probably, *T. cruzi* infection in top predators with a more restricted carnivorous diet, such as the ocelot, is highly dependent on the prevalence of infection of the local mammal fauna that can be preyed, since infection by the contaminative route is less probable if we consider that this animal is nocturnal, very active and generally does not use dens (except during birthing). In the Pantanal, where small mammals had low infection rates, none of the 10 ocelots tested were positive in HC (Herrera *et al*. 2011).

The crab-eating fox seems to be highly exposed to *T. cruzi* infections, as demonstrated in this study and also in the Pantanal region of Brazil (Herrera *et al*. 2011). This might be related to its capacity to exploit different ecological niches. The crab-eating fox is known to be one of the most plastic carnivore species: it has an omnivorous diet – including insects and small mammals, opportunistic behaviour and is a habitat generalist (Juarez and Marinho, 2002). Also, it has great flexibility in the use of disturbed habitats (Michalski *et al*. 2006). These traits increase the probability of contact with a variety of components of the *T. cruzi* cycle pointing the crab-eating fox as a good sentinel for *T. cruzi* transmission areas.

The maned wolves were highly exposed and can also be considered a good sentinel for transmission. Accounting for its omnivorous diet and home range areas of 80 km^2 on average (Jacomo *et al*. 2009), this species can play a unique role that is to signal the transmission in large areas, in particular in wild environments which are generally difficult to access. This was the case of maned wolves from our study that signalled the *T. cruzi* transmission both inside SCNP and its surroundings. The distinct prevalence rates between them indicate that the transmission was occurring mostly outside of the conservation unit, given that only 16% (4/25) of the maned wolves that were captured and recaptured within the park area over the 5-year follow-up tested positive, whereas 28% (4/14) of the wolves from outside SCNP tested positive. Taken together with the finding that no other mammal captured inside the park was positive for *T. cruzi* infection, we can conclude that the *T. cruzi* cycle inside SCNP is less expressive than in its surroundings.

This study reports the current *T. cruzi* enzootic transmission in one of the oldest endemic areas for Chagas disease in Brazil. We surmise that the *T. cruzi* transmission is well established all around the SCNP region, and that this transmission includes 2 genotypes of the parasite: TcI and TcII. Therein, dogs, small mammals and carnivore species were shown to participate in the *T. cruzi* transmission net and parasite transmission was occurring in both arboreal and terrestrial strata, as well as in the peridomicile. An understanding of the peculiar characteristics of this net, as well as each host-parasite relationship, is the key to identify the risk of disease outbreaks. This is the first study to corroborate evidence that dogs can be used not only to report *T. cruzi* transmission areas but also the genotypes present in the area, which reinforces their role as sentinels for surveillance programmes. The observed *T. cruzi* eco-epidemiological profile should increase awareness of the necessity for continuous surveillance in order to prevent re-emergence of Chagas disease in this area.

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