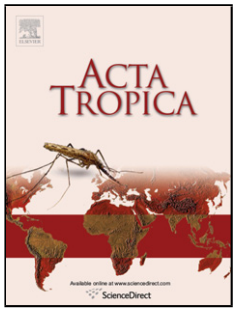

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PII: S0001-706X(15)30181-9
DOI: http://dx.doi.org/doi:10.1016/j.actatropica.2015.12.004
Reference: ACTROP 3792

To appear in: *Acta Tropica*

Received date: 22-9-2015
Revised date: 13-12-2015
Accepted date: 14-12-2015

Please cite this article as: Orozco, M.M, Enriquez, G.F, Cardinal, M.V., Piccinalli, R.V., Gürtler, R.E., A comparative study of Trypanosoma cruzi infection in sylvatic mammals from a protected and a disturbed area in the Argentine Chaco.Acta Tropica http://dx.doi.org/10.1016/j.actatropica.2015.12.004

A comparative study of *Trypanosoma cruzi* infection in sylvatic mammals from a protected and a disturbed area in the Argentine Chaco

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Running title: *Trypanosoma cruzi* in wild mammals from protected and disturbed areas

Highlights

- Sylvatic mammal infections were assessed in a protected and disturbed areas
- Infection was lower in the protected area and heterogeneous among species
- Infection among *Didelphis* opossums was nil in the protected area
- Fat-tailed opossums, bats and sigmodontine rodents had low infectiousness to the vector *Triatoma infestans*.
- Vampire and *Myotis* sp. bats were identified as hosts of *T. cruzi* in the Gran Chaco

Abstract

Understanding the complex epidemiology of *Trypanosoma cruzi* transmission cycles requires comparative studies in widely different environments. We assessed the occurrence of *T. cruzi* infection in sylvatic mammals, their infectiousness to the vector, and parasite genotypes in a protected area of the Argentine Chaco, and compared them with information obtained similarly in a nearby disturbed area. A total of 278 mammals from >23 species in the protected area were diagnosed for *T. cruzi* infection using xenodiagnosis, kDNA-PCR and nuclear satellite DNA-PCR (SAT) from blood samples. The relative abundance and species composition differed substantially between areas. *Didelphis albiventris* opossums were less abundant in the protected area; had a significantly lower body mass index, and a stage structure biased toward earlier stages. The capture of armadillos was lower in the protected area. The composite prevalence of *T. cruzi* infection across host species was significantly lower in the protected area (11.1%) than in the disturbed area (22.1%), and heterogeneous across species groups. The prevalence of infection in *Di. albiventris* and *Thylamys pusilla* opossums was significantly lower in the protected area (nil for *D. albiventris*), whereas infection in sigmodontine rodents was three times higher in the protected area (17.5 vs. 5.7%). Parasite isolates from the two xenodiagnosis-positive mammals (1 *Dasypus novemcinctus* and 1 *Conepatus chinga*) were typed as TcIII; both specimens were highly infectious to *Triatoma infestans*. Fat-tailed opossums, bats and rodents were kDNA-PCR-positive and xenodiagnosis-negative. *Desmodus rotundus* and *Myotis* bats were found infected with *T. cruzi* for the first time in the Gran Chaco.

Key words: *Trypanosoma cruzi*, *Desmodus rotundus*, *Myotis* sp., infectiousness, molecular diagnosis, protected areas

250 words

Introduction

Protected areas play a fundamental role in the conservation of natural ecosystems and can be among the most effective tools for protecting species from extinction and from the impact of human-induced threats (Naughton-Treves et al., 2005). These areas are expected to harbor a greater number of species than disturbed, exploited natural areas. In addition, protected areas can contribute to biodiversity conservation by maintaining ecological and evolutionary processes, diverse communities, viable populations, and natural areas large enough to be resilient to large-scale disturbances and long-term changes (Noss, 1992).

The richness of ecological communities can affect the occurrence and intensity of circulation of multiple pathogens by decreasing the risk of transmission with increasing diversity of host species, giving room to a "dilution effect" (Keesing et al., 2006; Schmidt and Ostfeld, 2001). Most insect vectors feed on host species that differ in reservoir host competence. Through the dilution effect, the presence of vertebrate hosts with a lower capacity to infect the vectors that feed on them would dilute the contribution of highly competent reservoirs (Schmidt and Ostfeld, 2001). Conversely, anthropogenic landscape disturbance could increase pathogen transmission (Daszak et al., 2001; Gottdenker et al., 2012). A recent meta-analysis showed that pathogen prevalence significantly decreased with increasing biodiversity (Civitello et al., 2015).

Trypanosoma cruzi, the etiologic agent of Chagas disease, infects more than 180 mammalian species from 7 orders and 25 families in the Americas (Jansen and Roque, 2010; Noireau et al., 2009), and has been classified into six main discrete typing units (DTUs) (Zingales et al., 2012). Marsupials (e.g., *Didelphis albiventris*), edentates (e.g. *Dasypus novemcinctus*), rodents and carnivores are the most common sylvatic hosts in the Gran Chaco region (Alvarado-Otegui et al., 2012; Ceballos et al., 2006; Orozco et al., 2013; Yeo et al., 2005) where Chagas disease and other neglected infectious diseases are hyperendemic (Gürtler et al., 2007b; Hotez et al., 2012). Ongoing large-scale changes in land use and habitat fragmentation throughout the Gran Chaco and other affected regions may have impacted heavily on the structure and functioning of the sylvatic transmission cycles of *T. cruzi.* Evidence on the presumable relevance of anthropic disturbance on sylvatic transmission cycles still is sparse (Ceballos et al., 2006; Vaz et al., 2007).

Dasypus novemcinctus armadillos and *Didelphis* opossums (including *Di. marsupialis*, *Di. aurita* and *Di. albiventris*) are the most widespread sylvatic hosts of *T. cruzi*; they are usually infected with *T. cruzi* III (TcIII) and *T. cruzi* I (TcI), respectively, and display large infectiousness to the vector *T. infestans* in the Gran Chaco and elsewhere (Alvarado-Otegui et

al., 2012; Ceballos et al., 2006; Diosque et al., 2004; Orozco et al., 2013; Yeo et al., 2005). Both TcV and TcVI are prevalent in domestic environments where *Triatoma infestans* is the primary vector of *T. cruzi* (Enriquez et al., 2012; Zingales et al., 2012). In the Argentine and Paraguayan Chaco, TcI was isolated from domestic dogs and cats, *Didelphis* opossums, and more rarely from *T. infestans* (Cardinal et al., 2008; Diosque et al., 2004; Enriquez et al., 2014; Orozco et al., 2013). TcIII was frequently found in *Da. novemcinctus* and *Chaetophractus* spp. armadillos, *Conepatus chinga* skunk and in the terrestrial marsupial *Monodelphis domestica* (Yeo et al., 2005; Ceballos et al., 2006; Alvarado-Otegui et al, 2012), and rarely in domestic dogs and *T. infestans* (Cardinal et al., 2008). This pattern agrees with the general notion that arboreal transmission cycles include opossums and TcI whereas terrestrial transmission include armadillos and TcIII (Gaunt and Miles, 2000; Yeo et al., 2005; Cardinal et al., 2008; Llewellyn et al., 2009). However, it has not yet been possible to demonstrate a strict association between DTUs and mammalian host species, and mixed infections of *T. cruzi* genotypes produced by differential histotropism may occur in mammals (Burgos et al., 2010). The complexity of host-vector interactions is compounded by the fact that several species of triatomine bugs may transmit different genotypes of *T. cruzi* in enzootic cycles potentially including a wide variety of mammalian hosts.

This study sought to assess the prevalence of *T. cruzi* infection and infectiousness to the vector *T. infestans* in a large sample of sylvatic mammals from a protected area, and compare these results with information obtained similarly in a nearby disturbed area within the same municipality (Orozco et al., 2013; Orozco et al., 2014). In the disturbed area, the composite prevalence of *T. cruzi* infection was high both in *Da. novemcinctus* (57.7%) and *Di. albiventris* opossums (38.1%) which were much more infectious to *T. infestans* than other host species. Among other 18 mammalian species examined, some had lower prevalence (the armadillos *Euphractus sexcinctus*, *Tolypeutes matacus* and *Chaetophractus vellerosus*, and the marsupial *Thylamys pusilla*) (Orozco et al., 2013). In both areas, 24 rodents from eight species were positive for *T. cruzi* infection by molecular methods, but the intensity of bug rectal infection was below the detection limit of xenodiagnosis (Orozco et al., 2014).

There is some evidence indicating that habitat fragmentation may increase the contact rates between sylvatic or domestic hosts of *T. cruzi* and triatomine bugs (Vaz et al., 2007; Xavier et al., 2012), and disturbed areas have more abundant populations of some highly competent reservoir hosts (e.g., opossums). Therefore, based on the putative influence of the "dilution effect", habitat fragmentation and disturbance, we expected that i) the prevalence of *T. cruzi* infection in the protected area would be lower than in the nearby disturbed area, and

ii) additional mammalian host species may participate in the sylvatic transmission of *T. cruzi* in the protected area.

Materials and methods

The field work was conducted in the protected area "Pampa del Indio Provincial Park" (26º 13' S; 60º 00' W), Pampa del Indio municipality (Chaco province, northeastern Argentina). The disturbed area (a rural section of 450 km^2) was described elsewhere (Orozco et al., 2013); small patches of native forest were close to the houses, usually subjected to intense human pressure. The prevalence of house infestation with *T. infestans* was 46% (Gurevitz et al., 2013) and the infection with *T. cruzi* was high (22-29%) in domestic bugs, dogs and cats (Cardinal et al., 2014) before a community-wide residual insecticide spraying conducted in November 2007.

Both areas are located in an ecotone between the wet and dry Chaco regions. The protected area (8,633 ha) is located about 30 km from the disturbed area; it had no human settlements within its limits, and was entirely surrounded by rural communities. The protected area included a primary forest of *Schinopsis balansae, Schinopsis lorentzii, Aspidosperma quebracho-blanco, Prosopis alba, Prosopis nigra* and *Tabebuia* spp.; gallery forests with bromeliads, savanna, marshes and small ponds, and is home to a diverse fauna (Bodrati et al., 2000).

In the protected area, wild mammals were caught during three-week surveys conducted in July 2009, November 2009 and July 2010. Two local collaborators assisted us in the captures. Medium-sized mammals were live-captured with Tomahawk traps set every 50 m along line transects. Armadillos also were live-captured with camouflaged home-made traps placed in the entrance of burrows. Local collaborators located burrows within the area specified by us, set up the traps, and inspected them several times at dusk and dawn. The home-made traps were built with iron mesh; are funnel-shaped and narrow (close to the average width of armadillo shell). The backdoor falls after when the armadillo enters, and it cannot turn or walk in reverse. Rodents and small marsupials were caught with Sherman traps arranged in pairs (one on the ground and the other at 1-2 m high on the trees) every 5 m along transect lines. All traps were checked and baited every morning using beef or chicken scraps for Tomahawk traps, and seeds, fruits and peanut butter pellets for Sherman traps. Bats were caught using mist nets (6.0 m wide, 2.6 m high, 38.0 mm black mesh, AFO Banding Supplies) placed in a zigzag pattern, opened at dusk and monitored every 30-40 min. for 6 h.

Capture sites were georeferenced (Garmin Legend C) and mammals were transported to the field laboratory. Capture and handling procedures were described in detail elsewhere (Orozco et al., 2013). Biosafety and animal processing procedures were performed according to protocols approved by the Dr. Carlos Barclay Ethical Committee. Wildlife permits (including transit permits for biological samples) were obtained from the provincial government through "Natural Resources Agency of Chaco".

Parenteral and/or inhalatory anesthetics were used for induction and maintenance of general anesthesia. For initial immobilization, parenteral anesthesia was performed with tiletamine clorhydrate and zolacepam clorhydrate (Zelazol; Fort Dodge, Buenos Aires, Argentina) at the minimum dose appropriate to species and weight (Kreeger and Arnemo, 2007). For maintenance, animals were given inhalatory anesthesia with Isoflurane delivered with a vaporizer (IsoTec; Datex-Ohmeda GE Healthcare, Little Chalfont, United Kingdom) and medicinal O_2 (0.25–3 L/min). Anesthetized animals were maintained on thermic cushioned surfaces in a quiet and comfortable environment, and their eyes were protected with ophthalmic lubricant solutions and covered with home-made eyecups.

Animals were sexed, measured from snout to base of tail, tail length, weighed with Pesola®, and marked with numeric metal tags (National Band & Tag co.). *Didelphis* opossums were assigned to stage class (I to VII) based on tooth eruption (Schweigmann *et al*., 1999). The body mass indices (BMI) of *Di. albiventris* and *Th. pusilla* was calculated as the ratio between the specimen's weight (in kg) and the square of body length (in m).

All mammals were bled by venipuncture and examined by xenodiagnosis using a variable number of uninfected *T. infestans* third- or fourth-instar nymphs; 3-5 bugs were applied to small specimens (< 250 g), and 20 to medium-sized specimens. The animals were released at the capture site once they fully recovered from anesthesia.

At the field laboratory, an aliquot of blood was diluted 1:1 in guanidine hydrochloride-EDTA buffer (GEB) and another one was centrifuged at 503 *g* for 15 min. for serum collection. Two heparinized microhematocrit tubes per animal were filled with blood and centrifuged at 10,000 rpm for 5 min.; the packed cell volume (PCV) value was measured by means of a graduated scale (i.e., microhematocrit reader, Rolco). The refractometric plasma total solids (TS) (g/dL) were determined in serum from each microhematocrit tube using a hand-held refractometer.

The rectal contents from two xenodiagnostic bugs fed on each animal were examined individually at 400× magnification (Zeiss) at 30 days post-exposure, and if negative, the remainder insects fed on the same mammal host were analyzed in pools of 4-5 insects each

(Gürtler et al., 2007a). When the first two bugs or any pool were positive, feces from of all insects in the positive pool were re-examined individually to assess the infectiousness of the individual host to the vector; the latter was estimated as the number of infected bugs fed on a given individual divided by the total number of insects examined for infection at least once, excluding bugs that died prior to the first examination. Bugs negative at 30 days post-exposure were re-examined individually at 60 days post-exposure. The numbers of exuviae and dead bugs in each box were recorded as a measure of xenodiagnosis quality and to indicate that blood-feeding had taken place. A total of 40 mammals were examined only by xenodiagnosis (3 *Th. pusilla*, 27 small rodents, and 10 bats) owing to insufficient blood samples.

For parasite culture, the rectal contents from xenodiagnosis-positive bugs were inoculated in biphasic medium (brain-heart-infusion and nutrient agar mixed with defibrinated rabbit blood) and incubated at 28°C and 50% relative humidity; parasite growth was monitored microscopically once a week for 4 months (Lauricella et al., 2005). Isolates were cryopreserved in liquid nitrogen.

DNA from GEB blood samples of the sylvatic mammals was tested by a polymerase chain reaction through amplification of the 330 base-pair fragment from the kinetoplast DNA minicircles of *T. cruzi* (kDNA-PCR) using primers and cycling conditions described elsewhere (Burgos et al., 2005). DNA was extracted using the DNeasy Blood & Tissue Kit following the manufacturer's instructions (QIAGEN Sciences, Maryland, USA). For further confirmation of *T. cruzi* infection, samples from all animals that were xenodiagnosis-negative and kDNA-PCR-positive (in GEB samples) were subsequently tested by nuclear satellite DNA-PCR (SAT-DNA-PCR) or by kDNA-PCR of the rectal contents of xenodiagnosis-negative triatomine bugs. DNA was extracted from the rectal contents of the xenodiagnostic bugs as described (Orozco et al., 2013). Parasite DTUs were identified in culture-derived DNA samples of each infected animal using a PCR-based strategy (Burgos et al., 2007) as described elsewhere (Orozco et al., 2013).

Data analysis

Chi-square tests were used to compare the frequency of capture of small fat-tailed and white-eared opossums, bats and rodents between study areas, and the stage structure of white-eared opossums between areas. White-eared opossums were grouped into juveniles (stages I-III), pre-adults (stage IV), and adult animals (stages V-VII). Kruskal-Wallis tests were used to compare the distribution of weight, BMI, tail length and body length in opossums from each

study area. Comparisons of TS and PCV in marsupials from both areas were performed with t tests. All tests were implemented in Stata 12 (StataCorp LP, College Station, TX).

Results

Capture of wild mammals

A total of 278 sylvatic mammals from at least 23 genera and/or species were captured in the protected area (Table 1), including 3 species of opossums, 3 species of armadillos, 2 species of carnivores, 5 genera and/or species of bats, and 10 genera and/or species of rodents. Species richness was slightly higher in the protected area (at least 23 species) than in the disturbed area (at least 20 species). The relative composition of the most frequent catches (rodents, bats, white-eared opossums and armadillos) differed in a highly significantly way between areas ($\chi^2 = 191.8$, df = 4, $p < 0.001$). White-eared opossums were more frequently caught in the disturbed area, whereas the opposite occurred in rodents and bats. Only three armadillos were caught in the protected area (1 *Da. novemcinctus,* 1 *To. matacus* and 1 *Eu. sexcinctus*) compared with 64 specimens in the disturbed area (26 *Da. novemcinctus,* 16 *To. matacus,* 16 *Chaetophractus vellerosus,* 5 *Eu. sexcinctus,* 1 *Ch. villosus*). The only skunk (*Co. chinga*) captured anywhere was an apparently old specimen trapped in the protected area.

The total capture effort was similar between areas: a total of 7,419 trap-nights in the protected area (Table 2) and 7,746 trap-nights in the disturbed area (Orozco et al., 2013). The total number of trap-nights using Tomahawk traps (4,385 versus 4,615, respectively) and Sherman traps (2,734 versus 3,131) were also similar. In the protected area, the mean catch per unit effort (CPUE) using Sherman traps (6.18 micro-mammals per 100 trap-nights) was 12 times greater than that of medium-sized mammals caught using Tomahawk or home-made traps (0.52 animals per 100 trap-nights). The latter varied very little across surveys, whereas the catch of rodents was remarkably higher during the 2010 winter. A total of 81 bats were captured in 216 mist net-hours (37 animals per 100 net-hours) (Table 2).

In *Didelphis* opossums, the stage distribution was not significantly different between areas ($\chi^2 = 2.75$, df = 2; $p = 0.25$) (Figure 1A) and sexes ($\chi^2 = 3.23$; df = 2; $p = 0.19$). Stage IV opossums (pre-adults) prevailed in both areas (46.7-47.6%). A large fraction of opossums (30.9%) from the disturbed area were stage V and VI, whereas most specimens (40%) from the protected area were stage II and III. The median BMI was significantly higher in the disturbed area relative to the protected area (Kruskal-Wallis, $\chi^2 = 8.4$, df = 1; $p = 0.004$) (Table 3, Figure 1B), showing clear differences among stages I-III and IV. The distribution of body weight, body length and tail length were significantly different between areas (Kruskal–

Wallis tests, $p < 0.05$). TS and PCV values were normal and similar between areas across all species examined (Table 3, Supplementary Table).

All fat-tailed opossums captured were assumed to be adults in both areas. The BMI ranged from 1.6 to 3.3 (n = 17) in the protected area, and from 1.7 to 3.6 (n = 20) in the disturbed area (Table 3). The distributions of BMI, weight, body length, tail length, PVC and TS in fat-tailed opossums were not significantly different between areas (Kruskal–Wallis test, $p > 0.05$).

Trypanosoma cruzi infection

Only 2 (0.7%) of 278 mammals from the protected area were xenodiagnosis-positive, including 1 *Da. novemcinctus* and 1 *Co. chinga.* However, a total of 29 xenodiagnosis-negative mammals were kDNA-PCR-positive, including 22 small rodents (2 *Ak. montensis*, 1 *Ak. toba*, 6 *Ne. lasiurus*, 5 *Ca. callosus*, 3 *Gr. chacoensis*, 2 *Oecomys* sp., 2 *Ol. chacoensis* and 1 *Ol. nigripes*) (Orozco et al., 2014), 1 *Euphractus sexcinctus*, 2 *Th. pusilla*, 1 *De. rotundus* and 3 *Myotis* sp. Confirmation of *T. cruzi* infection of kDNA-PCR-positive, xenodiagnosis-negative mammals was sought through two additional techniques (see footnotes of Table 4): using SAT-DNA-PCR we confirmed the infections of 1 *Eu. sexcinctus* armadillo, 1 *De. rotundus* bat, 1 *Oligoryzomys chacoensis* and 1 *Oecomys* sp. Using kDNA-PCR of the rectal contents of xenodiagnostic bugs we confirmed the infections of 8 rodents (1 *Ne. lasiurus*, 2 *Ca. callosus*, 2 *Gr. chacoensis*, 1 *Oecomys* sp., 1 *Ol. chacoensis* and 1 *Ol. nigripes*, as described in (Orozco et al., 2014), and 1 *Myotis* bat. The remainder kDNA-PCR-positive mammals were negative by the additional PCRs.

The overall prevalence of *T. cruzi* infection determined by xenodiagnosis (0.7%; 95% confidence interval, CI, 0.2-2.6%) in the protected area was 20 times lower than that detected by kDNA-PCR of blood samples (13.0%; 95% CI, 9.0-18.0%) (Table 4). The composite prevalence of *T. cruzi* infection (as determined by either method) in the protected area across all host species was 11.1% (95% CI, 7.7-15.5%). On average, of the 1,641 and 2,591 bugs used in xenodiagnosis in the protected and disturbed areas, 92% and 91% of the bugs survived to the first inspection, respectively, and 7.2% and 14.2% of the nymphs molted within the 60-day observation period, respectively.

These prevalence rates of infection differ from those obtained in the disturbed area: 14.6% (95% CI, 9.5-19.7%) by xenodiagnosis, and 20.3% (95% CI, 14.5-26.1%) by kDNA-PCR (Table 5). The composite prevalence of infection was significantly higher in the disturbed area (22.1%; 95% CI, 16.3-28.0%) than in the protected area (11.1%; 95% CI, 7.7-

15.5%) (χ^2= 9.34, df = 1, p < 0.05). The same trend was recorded in *Di. albiventris* opossums (38.1% versus 0.0%, respectively) but not in *Th. pusilla* (χ^2= 1.05; df = 1; p = 0.31). Rodent and bat infections were more frequent in the protected area, but the differences were marginally significant among rodents (χ^2 = 3.28; df = 1; p = 0.07) and non-significant among bats (χ^2 = 0.29; df = 1; p = 0.59). The prevalence of infection among rodents was 7.4% (n = 27) in July 2009, 11.8% (n = 17) in November 2009, and 17.6% (n = 108) in November 2010. Fat-tailed opossums *Th. pusilla*, bats and rodents were negative by xenodiagnosis in both areas.

Both xenodiagnosis-positive specimens of *Da. novemcinctus* and *Co. chinga* were highly infectious to *T. infestans* nymphs: 95% and 100%, respectively. *T. cruzi* parasites were successfully isolated by culture and identified as TcIII, both showing the 200-bp band for the SL-IRac leader sequence and the 125-bp band for the 24sα ribosomal DNA-HnPCR sequence.

In the disturbed area, all of the 12 xenodiagnosis-positive opossums were infected with TcI, and all parasite isolates from 12 *Da. novemcinctus*, 1 *Ch. vellerosus*, and 1 *To. matacus* armadillos were TcIII (Orozco et al. 2013). We were not able to identify the DTUs of kDNA-PCR-positive, xenodiagnosis-negative animals.

Discussion

Our study documents a significantly lower overall prevalence of *T. cruzi* infection in sylvatic hosts from the protected area in comparison with a nearby disturbed area, but the pattern was heterogeneous across host species. Despite *Didelphis* sp. is a well-known reservoir host throughout its distribution range, the prevalence of infection in the protected area was nil and nearly so was for *Th. pusilla* opossums. Conversely, overall infection in rodents was three times higher in the protected area (although not significantly so), and bats displayed minor differences between areas, with nil infectiousness to *T. infestans*. The only one *Da. novemcinctus* captured in the protected area was positive and showed very high infectiousness to *T. infestans* (95%). The patterns were therefore complex, varied with the host species considered, and did not completely conform to the expectations on the combined influence of the "dilution effect", habitat fragmentation and disturbance (see below). We also report the occurrence of *T. cruzi* infection in *De. rotundus* and *Myotis* bats for the first time in the Gran Chaco, and confirm that fat-tailed opossums, bats and several sigmodontine rodent species had low, subpatent infectiousness to *T. infestans* in both study areas.

One of the main limitations to draw definite conclusions on the disturbed/protected area comparison is that the relative abundance and composition of the target host species

examined (opossums, armadillos and rodents) differed substantially between areas, and therefore the number of specimens examined was rather limited and variable over time despite conducting three sizable trapping surveys in the protected area. A greater number of medium-sized mammals (including white-eared opossums) were caught in the disturbed area. This larger catch success may in part be related to the assistance of skilled local hunters with knowledge on wildlife trails and burrow locations. However, the higher abundance of white-eared opossums (and especially of armadillos) in the disturbed area is unlikely to represent an artefact, as opossums tend to fare better in disturbed areas and in fragmented landscapes such as those of the Atlantic Forest (Vaz et al., 2007). It is rather unclear whether the much lower relative abundance of armadillos in the protected area reflects their actual abundance (as the less frequent armadillo tracks recorded suggest) or is related to widely different catchability coefficients between disturbed and protected areas. This is a pervasive issue that affects between-habitat comparisons of wildlife abundance (Skalski et al., 2005). Similarly, based on local inventory lists and the well-preserved status of the forest, we assumed that the diversity of hosts in the protected area was larger. The total number of rodent species was likely underestimated because only *T. cruzi*-positive rodent specimens were taxonomically identified and this undermined the estimation of host diversity indices and infection prevalence by species. The interpretation of our results is also limited by the lack of information on the vector species involved in the transmission of TcI and TcIII, their population abundance and distribution, host associations, and infection with *T. cruzi*. Anthropogenic changes in land use may lead to increases or decreases in vector abundance (e.g., Sutherst, 2004). Further research on these issues is needed to understand how rodent infection rates with *T. cruzi* are sustained in the protected area.

Our results may be compared to other related findings. Gottdenker et al. (2012) investigated how anthropogenic land use change influenced the bloodmeal distribution of *Rhodnius pallescens* in Panama. Bloodmeal composition differed across habitat types, with a greater frequency of host blood sources recorded in early or mid-secondary fragments and peridomestic habitats. Marsupials (e.g., *Didelphis*) comprised the majority of blood meals in peridomestic habitats whereas blood meals on tamanduas and especially sloths predominated across all habitats, and armadillos were not studied (Gottdenker et al. 2012). Vaz et al. (2007) compared the patterns of *T. cruzi* infection among small wild mammals residing in continuous forest and in fragmented habitats of the Atlantic Rain Forest in Brazil. The fragmentation process apparently favored *T. cruzi* transmission, and increased the abundance of competent host species which displayed subpatent parasitemia. In contrast, *Didelphis* opossums from the

disturbed area in Chaco showed high levels of infection and infectiousness, whereas those from the protected area were not infected. *Di. albiventris* were abundant in peridomestic habitats and less abundant than rodents in the protected area, likewise the patterns recorded by Vaz et al. (2007). Unlike in our study, *Di. albiventris* was found infected with *T. cruzi* in a protected, well-preserved area in Brazil (Herrera et al., 2005).

Didelphis albiventris opossums were not infected with *T. cruzi* in the protected area despite the use of homogeneous diagnostic procedures across both areas and host species, including high-quality xenodiagnostic tests as shown by the levels of blood-feeding, survival and molting of the test bugs. Moreover, our current results of *T. cruzi* infection for white-eared opossums and other wild mammals agreed closely with those obtained by a sensitive assay based on trans-sialidase inhibition, showing high co-reactivity and co-negativity (Sartor et al., 2013). Another process that may contribute to explain the different patterns recorded is that *Didelphis* opossum populations display large fluctuations in abundance and stage structure over time (Telford and Tonn, 1982), both of which affect pathogen prevalence. In our study, white-eared opossums from the protected area showed signs of having a lower relative abundance and a younger stage distribution than in the disturbed area, which would decrease overall pathogen prevalence under equilibrium conditions. Moreover, in another rural area of the dry Chaco, the prevalence of *T. cruzi* infection in opossums increased with increasing stage and displayed a long-term declining trend over decades along with dramatic deforestation and land use change (Ceballos et al., 2006; Schweigmann et al., 1999). In our disturbed area, the small patches of forest and the diffuse ecotone with peridomestic areas may allow increased host-vector contact with a variety of triatomine species and other competent hosts.

TcI is a highly diverse DTU frequently found in opossums and rodents residing in sylvatic habitats (Cura et al., 2012; Guhl and Ramírez, 2011; Llewellyn et al., 2009). TcI infection occurred in approximately 6% of *Triatoma sordida* collected in (peri)domestic habitats, and bug infections were locally aggregated in partial coincidence with a hotspot of marsupials, armadillos and rodents infected with TcI or TcIII (Macchiaverna et al., 2015). TcI was isolated from *T. cruzi*-seropositive dogs, cats and *Didelphis* opossums from the disturbed area (Enriquez et al., 2014; Orozco et al., 2013).

Population vitality and health may influence pathogen prevalence, but these processes did not play any apparent role in our study. White-eared and small fat-tailed opossums showed no differences between areas in selected health indicators (i.e., TS and PVC), whereas the BMI and other body metrics of white-eared opossums were greater in the disturbed area,

especially among stages I-IV. This pattern may result from the greater availability and accessibility of food items in peridomestic habitats, and perhaps may affect reservoir host competence over time (Vaz et al., 2007). Whether white-eared opossums and other mammals residing in disturbed environments may be subject to higher stress levels that affect their immune response, infection risk and intensity of parasitemia is unknown.

Most of the sigmodontine rodent species with confirmed *T. cruzi* infections occurred in the protected area, including three specimens of *Gr. chacoensis*, which was the only infected rodent species found in the disturbed area (Orozco et al., 2014). *Gr. chacoensis* appears to have broad habitat selection patterns and is fairly frequent in the Chaco region, where it may eventually contribute to sylvatic and peridomestic transmission of *T. cruzi* via a vector or predator-prey link. Although rodents had a three times greater overall infection prevalence in the protected area, the number of rodents examined in the disturbed area was rather limited and statistically significant differences were not detected.

All positive rodents had subpatent infectiousness to *T. infestans* in both areas, which was remarkably lower than that of white-eared opossums and armadillos. While the susceptibility and parasitemia of experimentally-inoculated rats and mice depends on the strain of host and *T. cruzi* (Macedo and Pena, 1998; Camargos et al. 2000), synanthropic rodents (*Rattus rattus*, *R. norvegicus*, *Mus musculus*) usually are very important reservoir hosts of *T. cruzi* (Herrera and Urdaneta-Morales 1997; Gürtler and Cardinal, 2015). In contrast, wild rodents frequently showed low or nil parasitemia and subpatent infectiousness in South America (Herrera et al., 2005; Orozco et al., 2014). In our study, subpatent infectiousness may be related to the season when captures were performed (usually in winter); the fact that most of the specimens examined were adults, and the natural course of infection progressing to a chronic phase with undetectable infectiousness, as in some laboratory mouse strains and experimentally-infected *C. callosus* (Borges et al., 1992; Mello et al., 1979).

The single specimens of the nine-banded armadillo *Da. novemcinctus* and skunk *Co. chinga* caught in the protected area were infected with TcIII and were highly infectious to the vector, as recorded in the Paraguayan Chaco (Yeo et al., 2005). Specifically in the disturbed area, parasite isolates from 14 xenodiagnosis-positive armadillos were TcIII, and no skunk was captured there (Alvarado-Otegui et al., 2012; Orozco et al., 2013). The only armadillo caught in the protected area does not allow any conclusion regarding the between-area comparison. However, *Da. novemcinctus* are widespread natural reservoir hosts of TcIII with a high prevalence throughout the Americas (Yeo et al., 2005; Llewellyn et al., 2009), and therefore most likely were the main sylvatic reservoir hosts of TcIII in both study areas. In

contrast, skunks were extremely rare according to local hunters and our trapping surveys. Skunks usually seek shelter in underground burrows, and have been found using ground dens dug by other animals and sharing dens with other species. Some burrows were simultaneously occupied by opossums, skunks and armadillos, while mice, snakes, and other invertebrates cohabit parts of the same dens (Feldhamer et al., 2003). The candidate vector of TcIII most likely is *Panstrongylus geniculatus*, a triatomine species with a very broad distribution range across the Americas, which we caught in a local armadillo burrow (Alvarado-Otegui et al., 2012).

The occurrence of *T. cruzi* in bats was confirmed by molecular techniques. Of interest is the finding of a *T. cruzi*-infected vampire bat from a large colony situated in the well of a rural house located at 50 m from the protected area. This capture site was 200 m away from where the three *Myotis* bats positive by kDNA-PCR were caught, in the visitors' area of the park. *Desmodus rotundus* and *My. nigricans* were shown to cohabit in caves (Witt and Fabian, 2010), which suggests the possibility of overlapping habitat use and joint exposure to various pathogens, especially in edge areas subjected to anthropogenic disturbance.

The hematophagous habits of *De. rotundus* and the location of its colonies in peridomestic areas (in close contact with domestic dogs, other mammals and triatomines) suggest vampire bats might have become infected through feeding on *T. cruzi*-infected domestic mammals or by vector-borne transmission. The latter pathway appears to be less likely because the source house of the infected bat, other neighboring houses and the rural area adjacent to the park were all negative for *T. infestans* from September 2008 on (Yael Provecho et al., unpublished), but other sylvatic triatomine species may be infected in the surrounding area. Vampire bats could also become infected with *T. cruzi* by licking their skins contaminated with infected triatomine feces during grooming activities (Thomas et al., 2007); by vertical transmission; and by oral transmission since its oral mucosa would not represent a barrier to trypanosomes, likewise *T. evansi* (Hoare, 1965). Although domestic dogs from the same houses were seronegative for *T. cruzi* infection, *De. rotundus* bats have a large home range and were recently found infected with TcI and TcIV in Colombia (Ramirez et al., 2014). In *Myotis* bats, *T. cruzi* infections may have been acquired through the ingestion of infected triatomines as these bats are insectivorous and inhabit both natural and anthropogenic environments. Whether the infected *De. rotundus* and *Myotis* sp. also harbored Tcbat (a *T. cruzi* genotype found in *Myotis* and *Noctilio* bats; Marcili et al., 2009) and their role in the eco-epidemiology of *T. cruzi* needs to be further investigated.

Infectiousness to the vector in the study mammals could vary depending on the course of infection and disease, season, physiological state of the host, and other factors (Desquesnes and de Lana, 2010; Dowell, 2001). How variable is host infectiousness among different ecoregions mostly remains unknown. Since we have not been re-examined the study individuals, results for host infectiousness should be interpreted cautiously. Opossums and armadillos in the Chaco region showed high infectiousness regardless of stage and season, but there is a lack of detailed knowledge on the course *T. cruzi* infection in these and in other sylvatic hosts.

Our xenodiagnostic tests of wild mammals from Argentina have used *T. infestans* nymphs, a species of rare occurrence in sylvatic habitats and in association with the wild hosts here examined. The sensitivity of xenodiagnosis depends on the vector species used and other factors affecting vector competence including *T. cruzi* genotypes (Noireau et al., 2009). Sylvatic transmission cycles may include multiple triatomine bug species, and the exact vector species associated with each wild host species frequently remains unclear. Comparative studies of naturally-infected wild hosts xenodiagnosed with *T. infestans* and different species of triatomine bugs either revealed marginal differences in infectiousness between vector species (Minter et al., 1978; Moreira and Perlowagora-Szumlewicz, 1997), or the superiority of *T. infestans* third-instar nymphs relative to *T. sordida* and *Triatoma guasayana* fifth instars (the putative sylvatic vectors) fed on a *Di. albiventris* opossum naturally infected with TcI (Schweigmann et al., 1997). We conclude that xenodiagnostic tests using *T. infestans* are very effective at least for opossums and armadillos infected with TcI and TcIII.

Our study shows a complex relationship between the prevalence of *T. cruzi* infection in sylvatic mammals and the degree of habitat preservation depending on the host species considered. Protected areas with a large biodiversity may maintain both competent and non-competent reservoir host species some of which have a lower prevalence of *T. cruzi* infection than in disturbed areas. The conservation of natural landscapes and biodiversity are expected to reduce the contact rates among humans, vectors and highly competent reservoir hosts, and therefore contribute to decrease the risk of infection with *T. cruzi* (Gottdenker et al., 2012; Xavier et al., 2012). Translating our current results into human risk is beyond the scope of the study, and would require specific measures on the human-vector(s) contact rates. More evidence from integrative, comprehensive studies that include both vector and mammal infections with *T. cruzi* over time and space are needed to draw definite conclusions on

whether the combined effects of dilution, habitat fragmentation and disturbance are as pervasive as expected.

Acknowledgements

We are grateful to Flavia Netto, Marina Leporace, Yael Provecho, Julian Alvarado-Otegui, Lucía Maffey, Juan Pablo Arrabal, Jessie Pinchoff, Rebecca Levine, Ruben Bárquez, Margarita Bisio, Alejandro Schijman, Juan M. Burgos, Leonardo Lanati, Marta Lauricella and Raúl Stariolo for field or laboratory assistance. Special thanks to Catalino Alfonso, Jose Lescano and their families for their invaluable support during fieldwork. Daniel Portal and Susy Gutierrez kindly provided field accommodation in Pampa del Indio Provincial Park. This study was supported by awards from TDR (UNICEF/PNUD/WB/WHO), Agencia Nacional de Promoción Científica y Tecnológica (PICT and PICTO-Glaxo), PIP CONICET and University of Buenos Aires. MMO, RVP, MVC and REG are members of CONICET Researcher's Career. The age/size/body mass and infection prevalence per mammal/opossum is available on request.

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Figure 1. A. Stage distribution of white-eared opossums *Di. albiventris* in the protected and disturbed area. **B**. Distribution of Body Mass Index (BMI) of white-eared opossums in the protected and disturbed area, Pampa del Indio, Chaco, 2009-2010. Ref. DA: disturbed area, PA: protected area. Dots above DA plots are outliers beyond the the 90% confidence bounds.

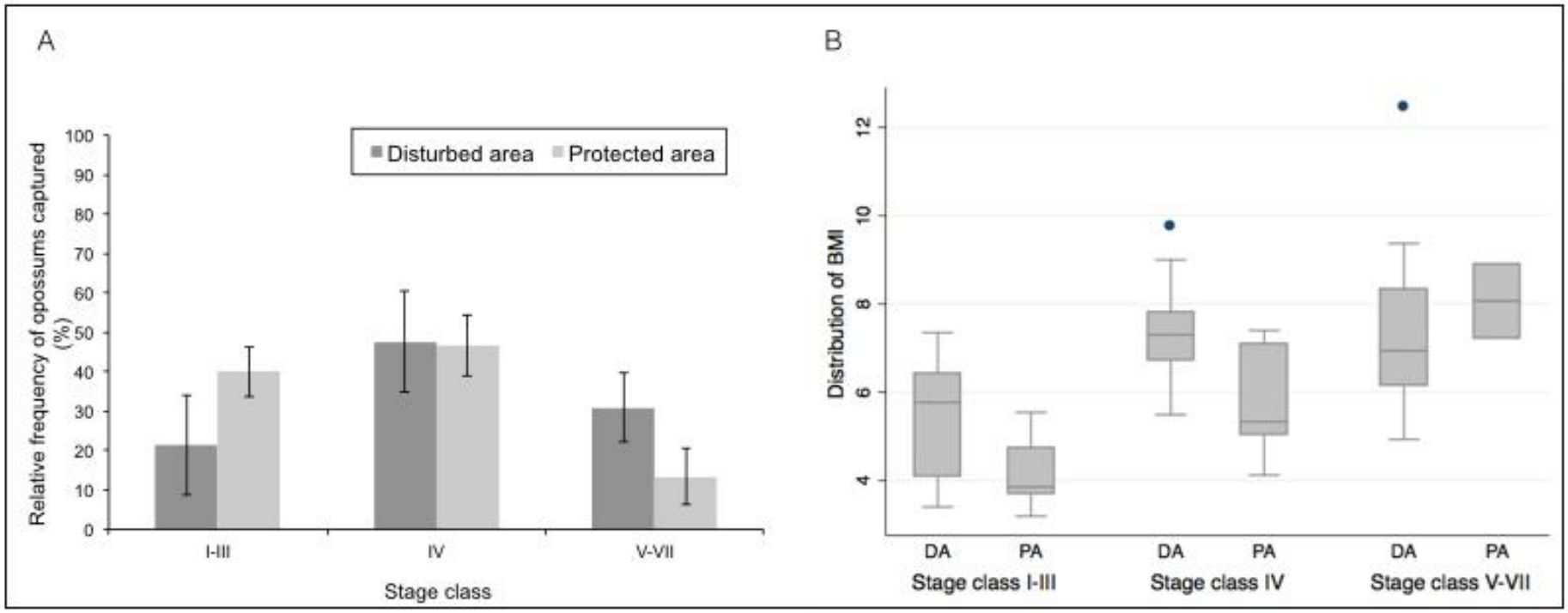

Table 1. Comparative catch of wild mammals in disturbed and protected areas of Pampa del Indio, Chaco, 2009-2010.

Host order	Scientific name	Common name	No. captured (%)	
			Disturbed area[a]	Protected area
Cingulata	*Dasypus novemcinctus*	Nine-banded armadillo	26 (13.3)	1 (0.4)
	Tolypeutes matacus	Three-banded armadillo	16 (8.2)	1 (0.4)
	Chaetophractus vellerosus	Screaming hairy armadillo	16 (8.2)	0
	Euphractus sexcinctus	Yellow armadillo	5 (2.6)	1 (0.4)
	Chaetophractus villosus	Big hairy armadillo	1 (0.5)	0
Didelphimorphia	*Didelphis albiventris*	White-eared opossum	43 (22.1)	15 (5.4)
	Thylamys pusilla	Small fat-tailed opossum	21 (10.8)	20 (7.2)
	Monodelphis sp.	Short-tailed opossum	1 (0.5)	0
	Philander opossum	Four-eyed opossum	0	3 (1.1)
Rodentia	Various species	Small rodents	38 (19.5)[1]	152 (54.7)[2]
	Galea musteloides	Common yellow-toothed cavy	1 (0.5)	0
	Ctenomys sp.	Tuco-tuco	1 (0.5)	0
Carnivora	*Nasua nasua*	Ring-tailed coati	5 (2.6)	0
	Procyon cancrivorus	Crab-eating raccoon	4 (2.1)	0
	Cerdocyon thous	Crab-eating fox	4 (2.1)	3 (1.1)
	Leopardus geoffroyi	Geoffroy's cat	4 (2.1)	0
	Conepatus chinga	Hog-nosed skunk	0	1 (0.4)
Chiroptera	Various species		5 (2.6)[3]	81 (29.1)[4]
Lagomorpha	*Sylvilagus brasiliensis*	Brazilian rabbit	3 (1.5)	0
Pilosa	*Myrmecophaga tridactyla*	Giant anteater	1 (0.5)	0
Total			195	278

[a] Data reported in Orozco et al., 2013.

[1]Including at least 6 species of rodents: *Akodon toba, Calomys callosus, Graomys chacoensis, Oligoryzomys nigripes, Oligoryzomys chacoensis, Oligoryzomys flavescens*. [2] Including at least 10 species of rodents: *Akodon montensis, Akodon toba, Necromys lasiurus, Calomys callosus, Calomys musculinus, Graomys chacoensis, Holochilus chacarius, Oecomys mamorae, Oligoryzomys nigripes, Oligoryzomys chacoensis*. [3] Including at least 4 genus or species of bats: *Myotis* sp., *Dasypterus ega, Eumops perotis, Molossops temminckii.* [4] Including at least 5 species of bats: *Myotis* sp., *Myotis nigricans, Myotis riparius, Eumops patagonicus, Desmodus rotundus*.

Table 2. Mean catch per unit effort (CPUE±SE) over time in the protected area of Pampa del Indio, Chaco, 2009-2010.

	Tomahawk traps			Sherman traps			Pitfall traps			Mist nets		
Date of fieldwork	No. of trap-nights	No. captured	CPUE*±SE	No. of trap-nights	No. captured	CPUE*±SE	No. of trap-nights	No. captured	CPUE*±SE	No. of net-hours	No. captured	Individuals/ 100 net-hours
July 2009	1,439	9	0.63±0.20	750	28	3.73±0.69	150	2	1,33±0.93	108	24	22
November 2009	1,379	6	0.44±0.18	1,132	17	1.50±0.36	150	0	0,00	108	57	53
July 2010	1,567	8	0.51±0.18	852	124	14.55±1.20	nd	nd	nd	nd	nd	nd
Total	4,385	23	0.52±0.10	2,734	169	6.18±0.46	300	2	0,67±0.47	216	81	37

*Animals per 100 trap-nights.

Ref: nd: not done.

Table 3. Comparative BMI, body weight, body length, tail length, PVC and TS in marsupials from the protected and disturbed areas, Chaco, 2009-2010.

Measure or index	*Didelphis albiventris*				*Thylamys pusilla*			
	Protected area		Disturbed area		Protected area		Disturbed area	
	No. examined	Mean±SD	No. examined	Mean±SD	No. examined	Mean±SD	No. examined	Mean±SD
BMI	15	5.4±1.6	41	6.9±1.7	17	2.5±0.4	20	2.3±0.5
Weight (g)	15	443.3±232.0	41	835.8±396.3	17	20.9±4.4	20	19.0±5.1
Body length (cm)	15	27.9±4.3	42	34.1±5.4	20	9.2±0.9	20	9.2±1.1
Tail length (cm)	15	25.2±3.7	42	29.8±6.3	20	10.7±0.8	20	10.2±0.9
PVC (%)	7	36.6±7.1	17	35.7±5.5	9	45.3±2.4	4	42.5±5.7
TS (g/dl)	5	6.8±0.7	15	7.0±0.9	9	6.9±0.6	3	6.2±0.7

Table 4. Prevalence of *Trypanosoma cruzi* infection determined by xenodiagnosis, kDNA-PCR and by either method in sylvatic mammals from Pampa del Indio protected area, Chaco, 2009-2010.

Order	Species	Infection prevalence by (No. examined)			Positive by both methods	Positive only by xenodiagnosis	Positive only by kDNA-PCR	Negative by both methods
		Xenodiagnosis	kDNA-PCR	Composite				
Cingulata	*Dasypus novemcinctus*	100.0 (1)	100.0 (1)	100.0 (1)	1	0	0	0
	Tolypeutes matacus	0.0 (1)	0.0 (1)	0.0 (1)	0	0	0	1
	Euphractus sexcinctus	0.0 (1)	100.0 (1)	100.0 (1)	0	0	1[a]	0
Didelphimorphia	*Didelphis albiventris*	0.0 (15)	0.0 (15)	0.0 (15)	0	0	0	15
	Thylamys pusilla	0.0 (20)	11.8 (17)	10.0 (20)	0	0	2[b]	15
	Philander sp.	0.0 (3)	0.0 (3)	0.0 (3)	0	0	0	3
Rodentia	Various species*[1]	0.0 (153)	17.5 (126)	14.4 (153)	0	0	22[c]	104
Carnivora	*Cerdocyon thous*	0.0 (3)	0.0 (3)	0.0 (3)	0	0	0	3
	Conepatus chinga	100.0 (1)	100.0 (1)	100.0 (1)	1	0	0	0
Chiroptera	Various species*[2]	0.0 (80)	5.7 (70)	5.0 (80)	0	0	4[d]	66
Total		0.7 (278)	13.0 (238)	11.1 (278)	2	0	29	207

[a] Positive by SAT-DNA-PCR.

[b] Negative by SAT-DNA-PCR and kDNA-PCR from xenodiagnostic bugs.

[c] Includes two positive by SAT-DNA-PCR, 8 positive by kDNA-PCR from xenodiagnosis bugs, and 13 negative by both techniques.

[d] One positive by SAT-DNA-PCR, 1 positive by kDNA-PCR from xenodiagnosis bugs, and 2 negative by both techniques.

*[1]Rodentia species: *Akodon montensis, Akodon toba, Necromys lasiurus, Calomys callosus, Calomys musculinus, Graomys chacoensis, Holochilus chacarius, Oecomys* sp., *Oligoryzomys nigripes* and *Oligoryzomys chacoensis*.

*[2] Chiroptera species: *Myotis nigricans, Myotis riparius, Myotis* sp., *Eumops patagonicus, Desmodus rotundus*.

Table 5. Comparative prevalence of *Trypanosoma cruzi* infection determined by xenodiagnosis and kDNA-PCR in the protected and disturbed area, Pampa del Indio, Chaco, 2009-2010.

		Protected area			Disturbed area		
		Infection prevalence by (No. examined)			Infection prevalence by (No. examined)		
Order	Species	Xenodiagnosis	kDNA-PCR	Composite	Xenodiagnosis	kDNA-PCR	Composite
Cingulata	*Dasypus novemcinctus*	100.0 (1)	100.0 (1)	100.0 (1)	48.0 (25)	56.0 (25)	57.7 (26)
	Tolypeutes matacus	0.0 (1)	0.0 (1)	0.0 (1)	12.5 (16)	0.0 (16)	12.5 (16)
	Chaetophractus vellerosus	nc	nc	nc	6.3 (16)	6.3 (16)	6.2 (16)
	Euphractus sexcinctus	0.0 (1)	100.0 (1)	100.0 (1)	0.0 (5)	20.0 (5)	20.0 (5)
Didelphimorphia	*Didelphis albiventris*	0.0 (15)	0.0 (15)	0.0 (15)	29.3 (41)	35.7 (42)	38.1(42)
	Thylamys pusilla	0.0 (20)	11.8 (17)	10.0 (20)	0.0 (20)	25.0 (20)	25.0 (20)
	Philander sp.	0.0 (3)	0.0 (3)	0.0 (3)	nc	nc	nc
Rodentia	Various species*[1]	0.0 (153)	17.5 (126)	14.4 (153)	0.0 (37)	5.7 (35)	5.4 (37)[b]
Carnivora	*Cerdocyon thous*	0.0 (3)	0.0 (3)	0.0 (3)	0.0 (4)	0.0 (4)	0.0 (4)
	Conepatus chinga	100.0 (1)	100.0 (1)	100.0 (1)	nc	nc	nc
Chiroptera	Various species*[2]	0.0 (80)	5.7 (70)	5.0 (80)	0.0 (8)	0.0 (8)	0.0 (8)
Total		0.7 (278)	13.0 (238)	11.1 (278)	14.6 (185)	20.3 (187)	22.1 (190)

Ref: nc: not captured.