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ORIGINAL PAPER

Assessment of the role of small mammals in the transmission cycle of tegumentary leishmaniasis and first report of natural infection with *Leishmania braziliensis* in two sigmodontines in northeastern Argentina

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Abstract
To contribute to the knowledge of the role of small mammals in the transmission cycle of tegumentary leishmaniasis caused by *Leishmania braziliensis*, we studied the small mammal community and its temporal and spatial association with phlebotominae, as well as small mammal infection by *Leishmania* spp. by PCR-RFLP analyses in an endemic area of northeastern Argentina. Ten small mammal samplings were conducted (2007–2009, 7506 Sherman trap nights and 422 cage trap nights). In two of these samplings, 16 capture stations each one consisting of a CDC light trap to capture phlebotominae, two to four Sherman traps and two cage traps were placed. We found co-occurrence of phlebotominae and small mammal captures in four stations, which were all the stations with small mammal captures and yielded 97% (2295 specimens, including 21 gravid females) of the total phlebotominae captures, suggesting that small mammals may provide a potential source of blood for phlebotominae females. One *Didelphis albiventris* and two *Rattus rattus* were associated with high captures of *Nyssomyia whitmani*, vector of *L. braziliensis* in the study area. The PCR-RFLP analyses confirm the presence of *L. braziliensis* in two sigmodontine small mammals (*Akodon* sp. and *Euryoryzomys russatus*) for the first time in Argentina, to our knowledge.

Keywords Small mammals · *Nyssomyia whitmani* · Co-occurrence · *Leishmania braziliensis* infection

Introduction

Leishmaniases are vector-borne diseases. The etiologic agents involved are parasites of the genus *Leishmania*, and different species of phlebotominae were confirmed as vectors. Mammals, such as rodents, edentates, marsupials, canids, nonhuman primates, and also humans, have been incriminated as possible reservoirs of *Leishmania* spp. in different regions of the world, depending on the eco-epidemiological context (Grimaldi and Tesh 1993; Ready 2008; W.H.O. 2010).

In Argentina, leishmaniases are considered emerging zoonotic diseases. The two clinical manifestations are visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL), which includes cutaneous and mucosal leishmaniases. TL has endemic and epidemic patterns in Argentina; *Leishmania braziliensis* is the main causative agent and the

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phlebotominae *Nyssomyia neivai*, *Ny. whitmani*, *Evandromyia cortellezziii/Ev. sallesi*, and *Migonemyia migonei* are involved as vectors in different scenarios (Salomón et al. 2008, 2009), while the reservoir or reservoirs have not been identified yet (Salomón et al. 2008).

Roque and Jansen (2014) reviewed available data on *Leishmania* reservoirs among wild and synanthropic species in the Americas, considering a potential reservoir when the authors demonstrated the retention of infection or the potential to transmit the parasite to vectors, while a parasite host is a species which was found infected but its competence in transmission to vectors was not demonstrated. Among other groups, many species of didelphimorphs and rodents were found infected with different *Leishmania* species. Between the didelphimorphs, *Didlephis marsupialis* and *D. albiventris* were described as potential reservoirs of *L. infantum*, and were demonstrated to effectively transmit the infection to vectors. *D. marsupialis* was also described as potential reservoir of *L. guyanensis* and *D. albiventris* of *L. peruviana*. Many wild and synanthropic species of rodents were found infected with *Leishmania* and are considered the main reservoirs of the *L. mexicana* complex. Caviomorphs from the genus *Proechimys* and *Thrichomys* were found infected by various *Leishmania* species and *P. semispinosus* from Colombia experimentally infected with *L. panamensis* transmitted the infection to vectors. The synanthropic rodent *Rattus rattus* was involved as a potential reservoir of *L. braziliensis* in Brazil and Venezuela (Roque and Jansen 2014).

The determination of reservoirs of *Leishmania* spp. responsible for the cutaneous forms of human leishmaniasis is in general difficult, and conclusions about potential reservoirs are based on accumulation of evidence, because some species may be accidentally infected but do not play a significant role in the transmission cycle. According to the World Health Organization (2010), a vertebrate species must meet certain criteria to be considered a reservoir of *Leishmania* spp. Among these criteria is a significant source of blood to vectors (which depends on body size, abundance, and longevity) and to be in intimate contact with them (for example, in burrows or shelters). Transmission may also be affected by environmental conditions and behavioral or social characteristics of vectors and reservoirs (Haydon et al. 2002; Chaves et al. 2007). Another approach is to consider that a multispecies assemblage with distinct and transient degrees of transmissibility competence throughout infection may act as a reservoir system, instead of few "hot species" with high transmissibility competence (Haydon et al. 2002; Roque and Jansen 2014).

The aim of this work was to contribute to the knowledge of the role of small mammals in the transmission cycle of TL caused by *Leishmania braziliensis*. For this purpose, we studied the temporal and spatial association between small mammals and phlebotominae in an endemic area of TL transmission in northeastern Argentina. We also analyzed small mammal infection with *Leishmania* spp. by PCR-RFLP analysis of spleen samples and skin lesions if these were present.

Materials and methods

Study area

The region belongs to the Selva Paranaense ecoregion (Rodriguez and Silva 2012). The altitude varies from 140 to 240 m above sea level. The climate is subtropical without a dry season, with annual rainfall ranging between 1600 and 2000 mm and an average annual temperature of 20 °C (Martínez-Crovetto 1963).

The study was conducted in the south of the city of Puerto Iguazú, northeastern Argentina. Most of the study was conducted in farms of a rural area (2H), but we also sampled a forest military area (FMA), the Reserva Natural Militar Puerto Península in the south edge of 2H, and in the Parque Nacional Iguazú (PN) in the east–southeast edge of 2H (Fig. 1).

The rural area was a forest of primary and secondary vegetation, which suffered wood extraction since 1950. During 2004, there was intense deforestation, with the subsequent installation of farms and the rise of TL human infection (Salomón et al. 2009). The main productive activity was small-scale family subsistence agriculture and farm animal breeding, mainly pigs and chickens (Nuñez 2009). Most of TL cases reported in Puerto Iguazú during 2003 and 2004 (85%, 36 cases) were associated with this area (Salomón et al. 2009).

Small mammal community description

Ten small mammal samplings were conducted: one every 3 months from summer 2007 to spring 2008 (eight samplings) plus two additional trappings, in autumn and winter 2009. Three to five farms placed in the 2H area were included in each sampling. Areas with lower levels of human intervention were included in three samplings, in winter 2008 and autumn 2009 (FMA, three sites) and in spring 2008 (FMA and PN, five sites).

Six habitats were defined according to vegetation characteristics and land use: (1) "Buildings," corresponding to dwellings, henhouses, pig sheds, storage rooms, and other human constructions; (2) "Cleared areas," areas around buildings; (3) "Crops," culture land placed in the vicinity of buildings (usually manioc and maize crops); (4) "Herbaceous vegetation," characterized by high grass and shrub cover; (5) "Forest areas," referring both to rainforest vegetation patches placed in 2H, and FMA and PN rainforest areas; (6) "Border areas" defined as edges between Cleared areas or roads and Forest or Crop areas, characterized by high cover of herbaceous vegetation and

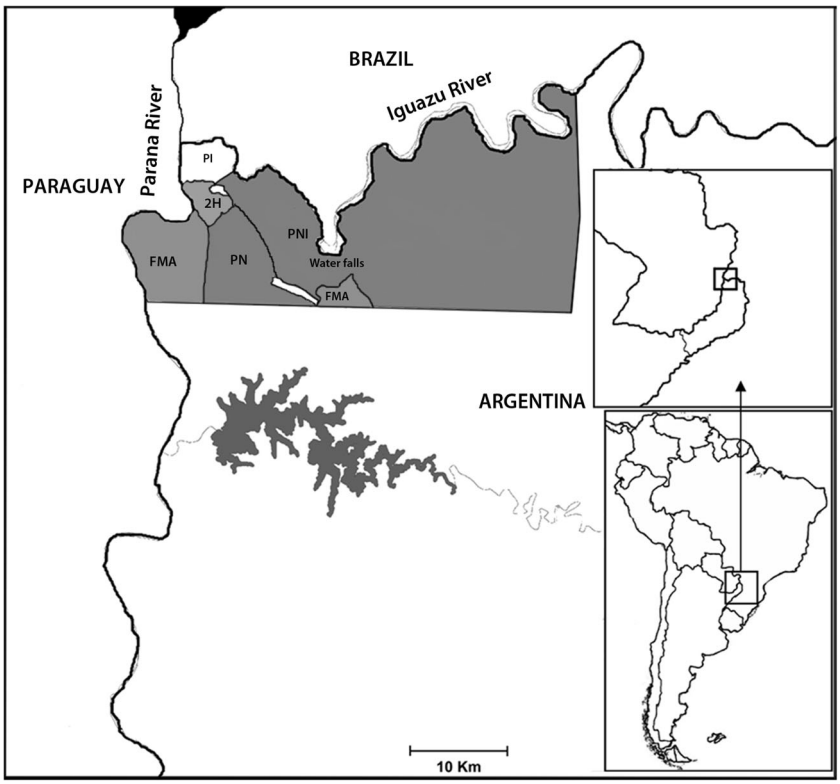

Fig. 1 Study area, in the northeastern border of Argentina (bottom right). 2H, "2000 ha" (rural area); FMA, forest military area; and PN, natural protected areas, including Parque Nacional Iguazú and other protected areas. PI, urban area of Puerto Iguazú

trees. In the 2H area, all types of habitats were represented and sampled, while only Forest and Border areas were sampled in FMA, and only Forest in PN.

At least three transects of 15 Sherman traps were placed in all habitats, and at least two cage traps in Buildings and Forest habitats. The total sampling effort was of 7506 Sherman trap nights and 422 cage trap nights. Traps were active for three consecutive nights and were inspected at morning. Sherman traps were baited with a mixture of peanut butter, oatmeal, and fat, and cage traps were baited with meat and carrot (due to differences in the target species between traps). Captured mammals were euthanized and samples from spleen were taken, in addition to visual inspection in search of skin lesions (wounds, scars, alopecia, and skin ulcer) that might be due to infection with *Leishmania* spp. (De Lima et al. 2002). They were preserved for subsequent taxonomic identification conducted at the National Museum of Natural Science "Bernardino Rivadavia". PCR-RFLP analyses were performed for detection and typification of *Leishmania* spp. from spleen tissue and skin lesion samples. Individuals were humanely sacrificed following the procedures and protocols approved by the Argentine Law for Animal Care 14346 and Ethics Committee for Research on Laboratory Animals, Farm and Obtained from Natures of National Council of Science and Technical Research (CONICET; resolution 1047, section 2, annex II).

For small mammal species, the relative density index (RDI) was estimated as the number of individuals captured divided by the number of trap nights and was calculated for each sampling discriminating by type of habitat.

Assessment of co-occurrence of small mammals and phlebotominae

In order to study whether small mammals and phlebotominae co-occur in space and time, simultaneous samplings for both groups were conducted in autumn and winter 2009. At each capture station in the Forest habitat, a CDC light trap to capture phlebotominae and a grid of 2×2 m of two to four Sherman traps and two cage traps around the light trap were placed (six stations in autumn 2009 and five stations in winter 2009). In the Building habitat, light traps were placed in henhouses or pig sheds and small mammal traps nearby (five stations in winter 2009). Phlebotominae and small mammal traps were active for three consecutive nights and were inspected for captures at morning.

Small mammals were collected and processed as described above (see community description). Collected insects were kept at −20 °C until processing. Phlebotominae were removed from the rest of the material; specimens were cleared with lacto-phenol and

identified according to the keys of Galati (2003), separated by sex and counted.

Detection of *Leishmania* infection by PCR-RFLP techniques

Two PCR-RFLP protocols were used; the first assay for *Leishmania* detection was conducted under conditions suggested by Marfurt et al. (2003) which has an expected 220–443 bp product (Fme 5′-TAT TGG TAT GCG AAA CTT CCG-3′ and Rme 5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′ primers), and targets the mini-exon gene present in tandem repeats in all species of the *Leishmania*. The reaction was carried out with 5 μL of extracted DNA in a final volume of 50 μL containing 1× PCR Buffer (200 mM Tris-HCl, pH 8), 0.1 mM EDTA, 1 mM DTT, 50% glycerol (*v*/*v*) (Invitrogen), 1 mM MgCl2 (Invitrogen), 10% DMSO (SIGMA), 0.2 mM dNTP Mix, 0.5 μM of each primer, and 1.4 U Taq polymerase (Invitrogen). Up to 10 μL of the amplified product was analyzed by 2% agarose horizontal gel electrophoresis at 5 V/cm, stained with SYBR® Safe (0.5 μg/mL) and visualized with a Safe Imager™ 2.0 Blue-Light Transilluminator (470 nm). *L.* (*V.*) *braziliensis* reference strain (MHOM/BR/1975/2903) was employed as positive control. The RFLP assay was carried out with Hae III restriction enzyme (5′-GGCC-3′ Promega) by digesting 10 μL of PCR reaction and visualized under the conditions mentioned above.

The second method used followed that of Montalvo et al. (2012), (F25 5′-GGA CGC CGG CAC GAT TKC T-3′ and R617 5′-CGA AGA AGT CCG ATA CGA GGG A-3′ primers), recognizes as target the gene encoding 70-kDa heat shock protein (Hsp70), with an expected product of 593 bp for all *Leishmania* spp. The reaction was carried out with 5 μL of extracted DNA in a final volume of 50 μL containing 1× PCR Buffer (200 mM Tris-HCl, pH 8), 0.1 mM EDTA, 1 mM DTT, 50% glycerol (*v*/*v*) (Invitrogen), 2 mM MgCl2 (Invitrogen), 2.5% DMSO (SIGMA), 0.2 mM dNTP Mix, 0.5 μM of each primer, and 1.4 U Taq polymerase (Invitrogen). Up to 10 μL of the amplified product was analyzed by 2% agarose gel electrophoresis; *L.* (*V.*) *braziliensis* reference strain (MHOM/BR/1975/2903) was employed as a positive control. For the RFLP assay, a new protocol (Rusman 2016) was developed employing the Sau3AI restriction enzyme (5′-GATC-3′ Promega) by digesting 5 μL of PCR reaction and visualized under the conditions mentioned above. The correspondence of the products obtained after Sau3AI enzyme digestion with those expected (210 + 160 bp) was performed by estimating the band size as compared to a CienMarker molecular weight marker (Biodynamics®) (Fig. 2).

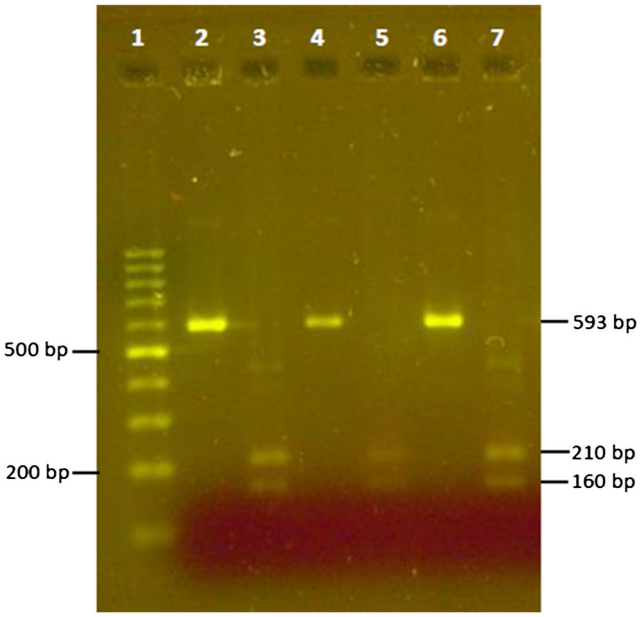

Fig. 2 Sau3AI RFLP analysis. Samples: *Akodon* spp. (lanes 2 and 3), *Euryoryzomys russatus* (lanes 4 and 5), *L.* (*V.*) *braziliensis* reference strain MHOM/BR/1975/2903 (lanes 6 and 7). Lane 1: MW (100 bp molecular weight ladder). Lanes 2, 4, and 6: PCR products. Lanes 3, 5, and 7: Sau3AI digested PCR products

Results

Small mammal samplings

A total of 123 individuals of eight species of small mammals belonging to two different mammal groups (Rodentia and Didelphimorphia) were captured. Among rodents, the introduced commensal rodents *Mus musculus* ($n = 29$) and *Rattus rattus* ($n = 14$) and four native sigmodontines, *Akodon spp.* ($n = 68$), *Oligoryzomys nigripes* ($n = 4$), *Euryoryzomys russatus* ($n = 1$), and *Thaptomys nigrita* ($n = 1$), were captured. *Akodon* individuals could not be identified at a specific level because the species present in the study area, *A. montensis* and *A. paranaensis*, are cryptic (Geise et al. 2005). Two didelphids were captured, *Didelphis albiventris* ($n = 4$) and *Didelphis aurita* ($n = 2$). A total of 2363 phlebotominae were captured, the most abundant species being *Nyssomyia whitmani* ($n = 2182$), followed by *Migonemyia migonei* ($n = 143$). Less represented species were *Psathyromyia bigeniculata* ($n = 15$), *Pintomyia monticola* ($n = 12$), *Pi. pessoai* ($n = 3$), *Sciopemyia sordelli* ($n = 2$), *Pa. Ianei* ($n = 2$), *Ny. neivai* ($n = 1$), and 3 individuals of the genus *Brumptomyia*.

The RDI of small mammal species varied from 0 to 8.3% and differed among seasons and habitats. The higher abundance and species richness were found in winter, and the peak of abundance was due to the increase in *Akodon* spp. (Fig. 3).

Regarding habitat distribution, *R. rattus* was mainly captured in Buildings, and only on two occasions in the Cleared

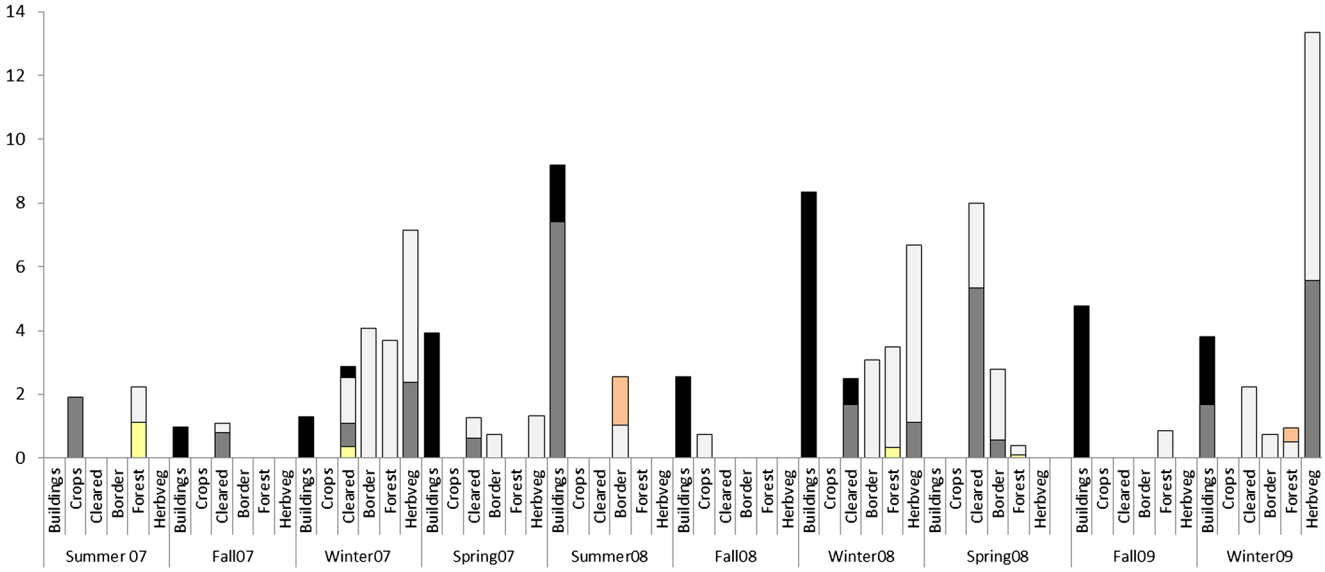

Fig. 3 Seasonal variation of the most captured small mammals from summer 2007 to winter 2009 in Puerto Iguazú, northeaster Argentina: *Akodon* spp. (light gray bars), *Mus musculus* (dark gray bars), *Rattus rattus* (black bars), *Oligoryzomys nigripes* (yellow bars), and *Didelphis albiventris* (orange bars). Y axis: RDI = (number of individuals/number of trap nights)×100. Less captured species, *Euryoryzomys russatus*, *Thaptomys nigrita*, and *D. aurita* are not shown. Habitats were described in "Materials and methods." Herbveg, Herbaceous vegetation

areas, near Buildings. This species was present in eight of ten samplings and in all seasons. *Mus musculus* was the most captured commensal species; it was present in all habitats sampled except in Forest and was frequently captured in Cleared and Herbaceous vegetation areas (Fig. 3). The most captured sigmodontine was *Akodon* spp., present in all habitats except in Buildings. It was found with the highest abundance in Forest, Border, and Herbaceous vegetation areas. The second sigmodontine in abundance was *O. nigripes*, although represented by few specimens, and was captured in Forest and Cleared areas (Fig. 3). The specimens of *E. russatus* and *T. nigrita* were found in Herbaceous vegetation (summer 2008) and in Forest (winter 2009), respectively. Regarding the didelphids, *D. albiventris* was captured in Border and Forest, and *D. aurita* in Forest.

A total of nine individuals with skin lesions were found, five *R. rattus* (captured in Buildings, three specimens in winter and two in autumn), three *Akodon* spp. (two captured in Herbaceous vegetation in winter and one in Border area in spring), and one *D. albiventris* (captured in a forest vegetation patch in winter). Skin lesions in *R. rattus* were observed with alopecia in the snout and below the eyes. In the case of *D. albiventris*, skin lesions were found in the snout area. The three specimens of *Akodon* spp. presented wounds in the tail.

Small mammals and phlebotominae co-occurrence

The stations positive for mammals and phlebotominae were 4 and 12, respectively. All positive small mammal stations reported phlebotominae captures, constituting 97% of the total phlebotominae captures. In the Forest habitat, *Akodon* spp. was captured simultaneously with the phlebotominae *Pa. bigeniculata* and *Pa. Ianei* (autumn 2009, Forest habitat placed in FMA) and the opossum *D. albiventris* was captured simultaneously with the phlebotominae *Ny. whitmani*, *Mg. migonei*, *Ny. neivai*, *Pa. bigeniculata*, *Pi. pessoai*, and *Pi. monticola* (winter 2009, Forest habitat placed in a rainforest vegetation patch from the rural area). In Buildings, two individuals of *R. rattus* were captured in two different stations, simultaneously with phlebotominae *Ny. whitmani*, *Mg. migonei*, *Pa. bigeniculata*, *Pi. pessoai*, and *Pi. monticola* (winter 2009) (Table 1).

Molecular analyses

Two spleen samples analyzed by the PCR-RFLP protocol, which targets the Hsp 70 protein, were positive for *Leishmania* (*Viannia*) *braziliensis*, one from an individual of *Akodon* spp. and one of *Euryoryzomys russatus*, both captured in Herbaceous vegetation, in spring and summer, respectively (n analyzed = 110, representing the 89.4% of the total small mammal captures). All the skin lesion samples analyzed were negative (n analyzed = 6) in this analysis. For the PCR-RFLP analysis following the protocol conducted under conditions suggested by Marfurt et al. (2003), all tissue samples were negative.

Discussion

This work describes a small mammal species present in an area with endemic TL transmission, their association with

Table 1 Results from each site where small mammals were captured along with phlebotominae. Between brackets is shown the number of gravid females. *Akodon* spp. was captured in a station placed in a rainforest vegetation area (FMA); *Didelphis albiventris* in a rainforest vegetation patch from the rural area and both *Rattus rattus* in Building habitat, placed in the rural area. The light traps in Building habitat were placed in henhouses or pig sheds

Small mammal	Phlebotominae species						
	Ny. whitmani	*Mg. migonei*	*Pa. bigeniculata*	*Pa. Ianei*	*Pi. pessoai*	*Pa. montícola*	*Ny. neivai*
Akodon spp.			2	2			
Didelphis albiventris	1144 (12)	74	2		2	5	1
Rattus rattus	837 (8)	55	6		1		
Rattus rattus	149 (1)	12	2			1	

the phlebotominae *Ny. whitmani*, the vector previously involved in the transmission of *L. braziliensis* in the 2H area (Salomón et al. 2009), and to our knowledge confirms for the first time in Argentina the presence of *Leishmania braziliensis* in two sigmodontine small mammals, one of the genus *Akodon* and *Euryoryzomys russatus*. *Leishmania braziliensis* is the same species found in TL human cases of Puerto Iguazú (unpublished data), so these rodents would be involved in the transmission chain, although it is not possible to specify if they are potential reservoirs or incidental hosts. The finding of two different rodent species infected favors the hypothesis of a multihost wildlife reservoir responsible for maintaining transmission of *Leishmania* (*Viannia*) *braziliensis* (Haydon et al. 2002; Roque and Jansen 2014; Andrade et al. 2015).

Some of the small mammals captured in this study have been previously found positive for *L. braziliensis* or *L. amazonensis* in the Americas: *D. albiventris*, *R. rattus*, and *M. musculus*. With respect to sigmodontines, infection has been previously reported in species of *Akodon* and *Oryzomys* (Roque and Jansen 2014). In this study, *R. rattus* and *D. albiventris* were found temporally and spatially associated with *Ny. whitmani*, one of the criteria to be considered as reservoirs of *Leishmania*. According to the captures of insect gravid females, these mammal species may provide a potential blood source for phlebotominae, as was suggested for rodent and opossum species by Fonteles et al. (2009) through the analysis of blood meals of females of *Ny. whitmani*.

Although phlebotomine sampling took place only in autumn and winter, according to previous studies, *Ny. whitmani* is present in the 2H area throughout the year (Fernández et al. 2012), enhancing the probability of interaction with small mammals. Our findings agree with other studies that reported joint captures of phlebotominae and these mammal species, such as Ferreira et al. (2015) who found infected small mammals (*R. rattus*, *M. musculus*, *D. albiventris*, and other species), and mentioned that in the same area and period, *Ny. whitmani* was the main phlebotominae species captured; and Cutolo et al. (2014), who reported captures of phlebotominae of the genus *Evandromyia* associated with *D. albiventris* nests in an urban area of Brazil.

We also registered the presence of *Mg. migonei* and *Ny. neivai* which are proposed as *L. braziliensis* vectors (Salomón et al. 2008). Further, *Ny. neivai* was the phlebotomine involved as the main vector in a TL focus in a rural neighborhood placed 50 km away from 2H (Salomón et al. 2001). Consistent with previous studies (Salomón et al. 2009; Fernández et al. 2012), the second species in order of abundance was *Mg. migonei*.

Mg. migonei is a permissive *L. infantum* vector (Guimarães et al. 2016). *Ny. whitmani* and *Mg. migonei* were also found infected with *L. infantum* in the study area, but further investigations will be necessary to define them as specific or permissive vectors of *L. infantum*, and their actual role in the transmission of both tegumentary and visceral leishmaniases in the study area (Moya et al. 2015).

A conclusion regarding the cause of the skin lesions found in this study in small mammals cannot be reached, but these findings suggest the importance of conducting extensive studies for detecting skin lesions along with the analysis of blood meals of female phlebotominae. When employing the Marfurt et al. (2003) protocol, all samples were negative in PCR analysis. Although this protocol has an acceptable analytical sensitivity (Acardi et al. 2010, 2013), differences observed in the present study with respect to the protocol of Montalvo et al. (2012) could probably be associated with intrinsic factors of each PCR (primers match/mismatch) related to local strains of *Leishmania braziliensis*.

The search of *Leishmania* infection would be best performed jointly with the study of other zoonoses because some of the captured species are described as primary transmitters, reservoirs, and mechanical vectors of zoonotic diseases as Hantavirus Pulmonary Syndrome (Padula et al. 2007) and leptospirosis (Vanasco et al. 2003), among others.

Further, the captures of small mammals reported in this article were performed during the years 2007–2009, a period of low incidence of human TL in the same region (Salomón et al. 2016). Therefore, future research on small mammal infections associated with human TL outbreaks would provide more information about their role in the transmission cycle of *Leishmania* spp.

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Compliance with ethical standards Individuals were humanely sacrificed following the procedures and protocols approved by the Argentine Law for Animal Care 14346 and Ethics Committee for Research on Laboratory Animals, Farm and Obtained from Natures of National Council of Science and Technical Research (CONICET; resolution 1047, section 2, annex II).

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