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Rickettsia parkeri in free-ranging wild canids from Brazilian Pampa

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Summary

Spotted fevers are tick-borne diseases associated with various *Rickettsia* species. *Rickettsia parkeri* sensu stricto (s.s.) is the agent of an emerging eschar-associated rickettsiosis in humans from the USA and South American Pampa. Considering that *R. parkeri* s.s. is restricted to Americas and the potential role of dogs in the epidemiology of the disease, it is thus reasonable to hypothesize that wild canids could be involved in the enzootic cycle of this rickettsiosis. The aim of this work was to investigate the potential role of the wild canids from Pampa, *Cerdocyon thous* (crab-eating fox) and *Lycalopex gymnocercus* (Pampas fox), in the ecology of *R. parkeri* s.s. For that, 32 live-trapped free-ranging wild canids were sampled. Ticks were observed in 30 of the 32 foxes. Of the 292 ticks collected, 22 (7.5%) were positive by PCR for the presence of *R. parkeri* s.s. DNA. Also, 20 (62%) wild canids showed antibodies against *R. parkeri*. The results suggest that wild canids are involved in the enzootic cycle of *R. parkeri* s.s. in the Pampa biome and could be responsible for pathogen (and its vectors) dispersal.

KEYWORDS
Amblyomma, *Cerdocyon thous*, *Lycalopex gymnocercus*, rickettsiosis, spotted fever, tick

1 | INTRODUCTION

Spotted fevers are tick-borne diseases associated with various *Rickettsia* species. In the Americas, *Rickettsia parkeri* is the agent of an emerging febrile eschar-associated rickettsiosis in humans (Paddock et al., 2004). In addition to *Rickettsia parkeri* sensu stricto (s.s.), in last years, *R. parkeri*-like agents, such as *Rickettsia* sp. strain Atlantic Rainforest, have also emerged as public health issues (Spolidorio et al., 2010). In the USA, where approximately 40 human cases have been reported in recent years, the main vector of *R. parkeri* s.s. is the Gulf Coast tick, *Amblyomma maculatum* (Herrick et al., 2016; Sumner et al., 2007). In South America, *R. parkeri* s.s. has been incriminated as the major agent of spotted fever in the Uruguayan, Argentinian and Brazilian Pampa (Nava et al., 2008; Venzal et al., 2004; Weck et al., 2016).

In Pampa biome, the main tick vectors of *R. parkeri* s.s. are *Amblyomma triste* and *Amblyomma tigrinum* (Nava et al., 2008; Venzal et al., 2004; Weck et al., 2016), species traditionally found in domestic and wild canids. Recently, in a spotted fever case reported in the Brazilian Pampa (Weck et al., 2016), we observed that the patient's domicile and her dogs were frequently infested by *R. parkeri*-infected *A. tigrinum* ticks. Also, data from the literature indicates there is a high seroprevalence of antibodies against *R. parkeri* in dogs from the Pampa (Lado, Costa, Verdes, Labruna, & Venzal, 2015). The

Pampa biome, also known as Southern America Grasslands or "Campos", lies within the South Temperate Zone and has both subtropical and temperate climates with four well-characterized seasons and with well-distributed rainfall throughout the year (Roesch et al., 2009). Although the Pampa biome is frequently referred as a steppe, the biological uniqueness of the area leads some authors to suggest that Pampa should not be classified under the common accepted international physiographic terms (Overbeck et al., 2007). The vegetation is mainly characterized by grasslands, with sparse shrub and tree formations. The most part of Pampa's soil shows a sandy texture due to its sedimentary rock origin (Roesch et al., 2009).

Considering that *R. parkeri* s.s. is restricted to Americas and the potential role of domestic dogs in the enzootic cycle of the disease, it is thus reasonable to hypothesize that *R. parkeri* s.s. should evolve in close association with South American native wild canids, which in turn, may serve as potential reservoirs for the pathogen and its vectors. Although it has been suggested that wild carnivores could be associated with *R. parkeri* in the USA (Starkey et al., 2013), there are no data about potential natural host(s)/reservoir(s) of *R. parkeri* in South America. In the Pampa biome, the most abundant wild canids are *Cerdocyon thous* (crab-eating fox) and *Lycalopex gymnocercus* (Pampas fox). These canids, locally known as "graxaim" or "sorro", are very tolerant to changes in the environment, and can be found in cultivated areas, and even in the vicinity of urbanized areas (Beisiegel, Lemos, Azevedo, Queirolo, & Jorge, 2013; Gonçalves, Quintela, & Freitas, 2014; Queirolo, Kasper, & Beisiegel, 2013). Thus, the aim of this work was to investigate the potential role of the wild canids found in the Pampa, *C. thous* and *L. gymnocercus*, in the ecology of *R. parkeri* s.s.

2 | MATERIALS AND METHODS

2.1 | Study site and capture events

From December 2014 to December 2016, wild canids were captured in four municipalities within the Pampa biome in the Rio Grande do Sul (RS) state, southern Brazil: Santana do Livramento (30°25′53.1″S 55°28′09.9″W), Triunfo (29°51′58.1″S 51°21′54.5″W), Viamão (30°05′32.0″S 50°51′00.0″W) and Candiota (31°28′59.8″S 53°48′44.1″W) (Figure 1). *C. thous* and *L. gymnocercus* (Figure 2, Panels a and b) were captured using tomahawk live-traps. Once trapped, animals were anesthetized using an association of ketamine (10 mg/kg) and xylazine (1 mg/kg). Then, animals underwent a physical examination and collection of blood samples and ticks. Blood samples were collected by a single jugular vein puncture, using vacuum blood collection system and plastic tubes containing EDTA (approximately 4 ml blood) and serology tubes without anticoagulant (approximately 2 ml blood). Total blood and serum samples were stored at −20°C for further analysis. Ticks were manually collected from animals and immediately placed in ethanol. After full recovery from anaesthesia, animals were released at the site of capture. The study protocol was approved by our Committee for Animal Care and Experimentation (CEUA/IPVDF 28/2014) and by Brazilian biodiversity authorities (SISBIO 47357-3).

2.2 | Blood tests and detection of antibodies against *Rickettsia* spp.

Blood samples were submitted to haematological analysis in an automated veterinary haematology analyzer (Bio-1800 Vet®, Bioeasy Diagnostica S/A, Belo Horizonte, Brazil). The following parameters were analysed: red blood cells (RBC) count, haemoglobin (Hb), packed cell volume (PCV) and white blood cells (WBC) count. The reference values were obtained from Mattoso, Catenacci, Beier, Lopes, and Takahira (2012). Serum samples of wild canids were tested individually for the presence of antibodies against *Rickettsia* spp. by indirect immunofluorescence assay (IFA), as previously described (Labruna et al., 2007). As the antigen, five *Rickettsia* isolates from Brazil were used: *Rickettsia parkeri*, *Rickettsia rickettsii*, *Rickettsia amblyommatis*, *Rickettsia rhipicephali* and *Rickettsia bellii*. Samples that reacted at the screening dilution (1:64) were then titrated using serial 2-fold dilutions to determine endpoint titres. Reactions were performed using fluorescein-conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, MO, USA). For all reactions, negative and positive controls were included on each slide. Positive control sera were obtained from experimentally *Rickettsia*-infected dogs (Piranda et al., 2008); and negative controls were obtained from specific pathogen-free Beagle dogs.

2.3 | Ticks and molecular detection of *Rickettsia* spp.

Ticks were identified by morphologic dichotomous keys (Barros-Battesti, Arzua, & Bechara, 2006; Martins, Onofrio, Barros-Battesti, & Labruna, 2010). Parasitism prevalence and mean parasite abundance, and their respective confidence intervals, were calculated using the online tool QPweb (the web version of Quantitative Parasitology 3.0 software, available at www2.univet.hu/qpweb/qp10/). Ticks and fox blood samples were individually subjected to DNA extraction using the PureLink Genomic DNA MiniKit (Invitrogen, Carlsbad, CA, USA). DNA from ticks or blood samples was screened for *Rickettsia* spp. using a PCR assay for a 401 bp fragment of the citrate synthase (*gltA*) gene (Labruna et al., 2004), and positive samples were further tested for a 617 bp fragment of *ompA* from the spotted fever group (SFG) *Rickettsia* spp. (Regnery, Spruill, & Plikaytis, 1991). Positive samples were further analysed by PCR amplification of *htrA* fragment (549 bp; Labruna et al., 2004). Sequencing of PCR products from *ompA* and *htrA* was performed, and sequence comparisons were carried out using the BLAST algorithm.

3 | RESULTS

Of the 32 wild canids sampled, 27 were *C. thous* and five were *L. gymnocercus*. Only *C. thous* individuals were captured in the Triunfo, Santana do Livramento and Viamão municipalities, two in the

FIGURE 1 Setting for investigation of *Rickettsia parkeri* sensu stricto in wild canids from Brazilian Pampa. Rio Grande do Sul state, Brazil, and neighbouring countries. Light grey shading indicates the Pampa biome; dark grey shading indicates bodies of water; foxes indicate the sites of capture, as follows: (1) Santana do Livramento, (2) Candiota, (3) Triunfo and (4) Viamão municipalities

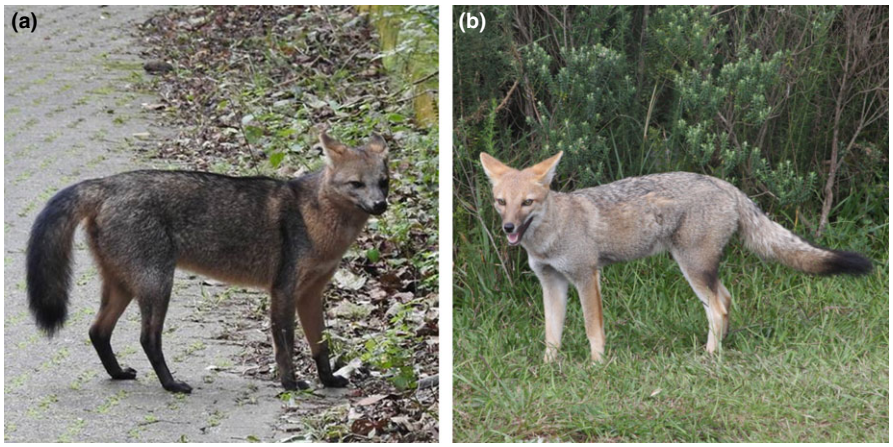

FIGURE 2 Wild canids sampled in this study. Panel a, *Cerdocyon thous* (crab-eating fox). Panel b, *Lycalopex gymnocercus* (Pampas fox)

first municipality and 11 in each of the latter two. Candiota was the only municipality in which *L. gymnocercus* was captured, resulting in five individuals and three additional *C. thous* specimens. All animals trapped were clinically healthy.

Ticks were observed attached (Figure 3, Panel a) in 30 of the 32 sampled wild canids (93.8% parasitism prevalence, CI 95% = 79.2–99.2). Only one *C. thous* and one *L. gymnocercus* from Candiota were not parasitized by ticks. Parasite infestation ranged from 0 to 29 ticks per fox (mean parasite abundance = 9.12; CI 95% = 6.62–12). The following ticks were recorded: 197 *Amblyomma aureolatum*, seven *A. tigrinum*, four *A. triste*, one *Amblyomma dubitatum*, 51 *Amblyomma* spp. larvae and 32 *Rhipicephalus microplus*. In one *C. thous*, we recorded an eschar at the tick (*A. aureolatum*) attachment site (Figure 3, Panel b).

Of the 292 ticks collected, 22 (7.5%) were positive in all PCR analyses for *Rickettsia* spp. Twenty *A. aureolatum* ticks from crab-eating foxes (15 from Santana do Livramento and five from Triunfo) and two *A. tigrinum* (one in a crab-eating fox and one in a Pampas fox) from Candiota were positive for *Rickettsia* spp. Blood samples of foxes were negative in all PCR analyses for *Rickettsia* spp. All sequences obtained from *ompA* and *htrA* rickettsial genes were identical and showed 100% identity to sequences from the *R. parkeri* s.s. clone RS (GenBank accession no. KX196265.1 and KX196266.1, respectively), which was previously detected in *A. tigrinum* ticks from dogs of an area endemic for spotted fever in the Brazilian Pampa (Weck et al., 2016). The infection rate of *R. parkeri* in ticks from wild canids was 10% for *A. aureolatum* and 28% for *A. tigrinum*.

Three of four regions investigated presented wild canids harbouring ticks positive for *R. parkeri*, and among the 32 wild canids sampled, 11 (34%) showed at least one tick positive for *R. parkeri*. Twenty-seven (84.3%) sera of wild canids tested by

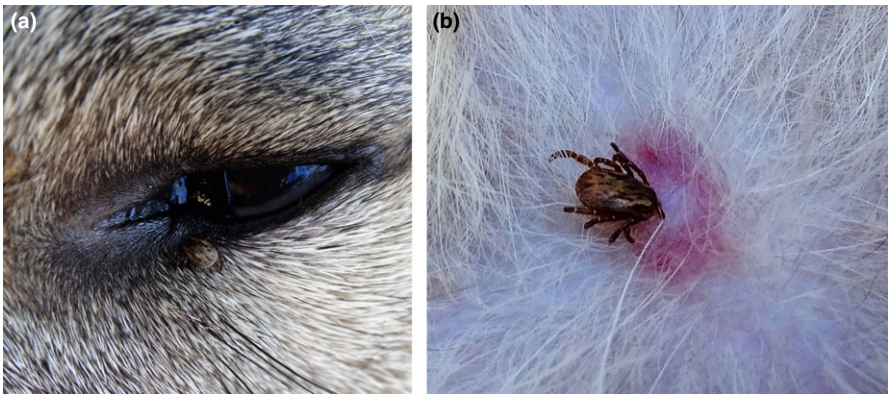

FIGURE 3 Ticks identified in wild canids from Brazilian Pampa. Panel a, *Amblyomma aureolatum* male tick attached near to the left eye of a crab-eating fox. Panel b, *A. aureolatum* male tick attached at the abdomen of a crab-eating fox. Note the eschar at the tick attachment site

IFA were positive for the presence of antibodies against *Rickettsia* spp. (titre > 1:64). Among these animals, 20 (74% of seropositive) showed a higher antibody titre against *R. parkeri* than against other rickettsial species. Despite the well-known cross-reactivity between *Rickettsia* species in serology tests, 11 canids showed antibody titres to *R. parkeri* that were more than 4-fold higher than endpoint titres to any other rickettsial antigen tested. Table 1 summarizes the information on the sampled wild canids, and the results of serology tests and of PCR analysis. Table S1 shows the results of antibody titration of each sampled animal against all antigens tested, as well as, the geometric mean titres (from each capture site) for each antigen tested. The average values of haematological parameters from sampled foxes were as follows: RBC 4.61 $\times$ 10^6/μl; Hb 12.92 g/dl; PCV 39.8%; WBC 15.11 $\times$ 10^3/μl. No animal showed abnormalities in haematological parameters in comparison with reference values. There was no difference between the average values of haematological parameters of seropositive and seronegative foxes ($p > .05$).

4 | DISCUSSION

Tick-borne diseases cannot be understood unless under the umbrella of one health approach (Oura, 2014), as it is directly associated with human, (wild and domestic) animals and the environment. Moreover, the risk of tick-borne diseases seems to be linked with some human activities, such as hunting and eco-tourism, as well as, the expansion of cites over wilderness areas (Bayles, Evans, & Allan, 2013; Bermúdez et al., 2016). These activities also increase the odds for contact between domestic and wild animals and create opportunities for parasites/pathogens exchange. In this sense, it is noteworthy that the majority of spotted fever cases in Rio Grande do Sul state, only part of Brazil located in the Pampa biome, occurred in people involved in illegal hunting using dogs (unpublished data kindly provided by Centro Estadual de Vigilância em Saúde—CEVS, State Health Department).

Despite the recent emergence of *R. parkeri* s.s. as the principal agent of spotted fever in the South American Pampa (Nava et al., 2008; Romer et al., 2011; Venzal et al., 2004; Weck et al., 2016, 2017), there is no epidemiological information concerning its potential natural host(s). Previous studies of *R. parkeri* rickettsiosis in the South American Pampa biome found that domestic dogs had a high frequency of *R. parkeri*-infected ticks and antibodies against *R. parkeri*. Moreover, in these areas, *R. parkeri*-infected ticks found in dogs belong to those tick species classically associated with wild carnivore hosts, particularly wild canids (Lado, Castro, Labruna, & Venzal, 2014; Weck et al., 2016).

Here, we report that the majority of wild canids from different sites of the Pampa biome showed high antibody titres against *R. parkeri* and that at least one-third of them were carrying *R. parkeri*-infected ticks. Concerning the potential role of wild canids as reservoirs of *R. parkeri* s.s. in the South American Pampa, we must take into account that the fox species investigated are as follows: (i) highly abundant in the Pampa (Beisiegel et al., 2013; Gonçalves et al., 2014; Queirolo et al., 2013); (ii) well adapted to live in habitats changed by humans and domestic animals (Beisiegel et al., 2013; Gonçalves et al., 2014; Queirolo et al., 2013); and (iii) are frequently parasitized by ticks already associated with spotted fever (Evans, Martins, & Guglielmone, 2000). Based on the above observations and the data reported here, it is plausible that wild canids are involved in the enzootic cycle of *R. parkeri* and have an important role in the dispersal of the ticks infected by *R. parkeri* in the Pampa biome.

We also report the detection of *R. parkeri* s.s. in *A. aureolatum* for the first time, a tick species widespread in Brazil, and previously associated with other spotted fever pathogens, such *Rickettsia rickettsii* and *Rickettsia parkeri*-like strain Atlantic Rainforest (Medeiros et al., 2011; Szabó, Pinter, & Labruna, 2013). So, these results place *A. aureolatum* tick as a vector species associated with the major distinct spotted fever pathogens involved in cases of spotted fever in Brazil.

Given that wild and domestic canids share habitats and ticks, we must also consider the potential role of domestic dogs in the epidemiology of *R. parkeri* in the Pampa. Indeed, as a part of another study using camera-traps in the same locality studied here (Santana do Livramento), we registered domestic and wild canids sharing the same habitat (Figure 4). Thus, domestic dogs can act as bridge-hosts (Sepúlveda et al., 2014), that is they carry the infected vector from the habitat of the natural host (foxes) to the habitat (houses) of the

TABLE 1 Wild canids sampled, serology tests and PCR analysis

Capture site	Wild canids species	Sample	Sex	Age	*Rickettsia* serology		*Rickettsia* PCR-positive ticks
					Species with the highest titre	Antibody titre	
Santana do Livramento	*C. thous*	C3	Male	Adult	*R. parkeri*	2,048	—
	C. thous	C5	Male	Juvenile	*R. parkeri*/*R. rickettsii*	64	1 *A. aureolatum* (*R. parkeri*)
	C. thous	C6	Male	Juvenile	*R. parkeri*	16,384	—
	C. thous	C7	Female	Juvenile	NR	—	2 *A. aureolatum* (*R. parkeri*)
	C. thous	C8	Female	Adult	*R. parkeri*	16,384	3 *A. aureolatum* (*R. parkeri*)
	C. thous	C9	Male	Adult	*R. parkeri*	512	2 *A. aureolatum* (*R. parkeri*)
	C. thous	C10	Female	Adult	*R. parkeri*	4,096	1 *A. aureolatum* (*R. parkeri*)
	C. thous	C11	Male	Adult	*R. parkeri*/*R. rickettsii*	256	—
	C. thous	C12	Male	Adult	NR	—	1 *A. aureolatum* (*R. parkeri*)
	C. thous	C13	Male	Adult	*R. parkeri*/*R. rickettsii*	256	—
	C. thous	C16	Male	Adult	*R. parkeri*	2,048	5 *A. aureolatum* (*R. parkeri*)
Triunfo	*C. thous*	C14	Male	Adult	*R. parkeri*/*R. rickettsii*	128	1 *A. aureolatum* (*R. parkeri*)
	C. thous	C15	Male	Adult	*R. rickettsii*	2,048	4 *A. aureolatum* (*R. parkeri*)
Viamão	*C. thous*	C17	Male	Adult	*R. parkeri*	256	—
	C. thous	C18	Male	Senile	*R. parkeri*	8,192	—
	C. thous	C19	Female	Senile	*R. parkeri*	4,096	—
	C. thous	C20	Male	Juvenile	*R. parkeri*	128	—
	C. thous	C21	Male	Adult	NR	—	—
	C. thous	C22	Male	Adult	*R. parkeri*	1,024	—
	C. thous	C23	Female	Adult	*R. parkeri*	16,384	—
	C. thous	C24	Male	Juvenile	*R. parkeri*	64	—
	C. thous	C26	Female	Juvenile	*R. parkeri*	512	—
	C. thous	C27	Male	Senile	*R. parkeri*	1,024	—
	C. thous	C28	Female	Juvenile	NR	—	—
Candiota	*C. thous*	C29	Female	Juvenile	*R. rhipicephali*	256	—
	C. thous	C31	Male	Adult	*R. parkeri*	256	—
	C. thous	C37	Male	Adult	*R. parkeri*/*R. rhipicephali*	8,192	1 *A. tigrinum* (*R. parkeri*)
	L. gymnocercus	C30	Female	Adult	*R. parkeri*	8,192	—
	L. gymnocercus	C32	Male	Adult	*R. parkeri*	512	—
	L. gymnocercus	C34	Female	Adult	*R. parkeri*	16,384	—
	L. gymnocercus	C38	Female	Adult	*R. parkeri*	2,048	1 *A. tigrinum* (*R. parkeri*)
	L. gymnocercus	C39	Male	Adult	NR	—	—

NR, Non reactive.

susceptible population (humans). In this context, both wild and domestic canids may serve as sentinels in future epidemiological studies on *R. parkeri* s.s. and spotted fever in the South American Pampa.

The results showed that *Rickettsia* spp. could infect and circulate among the great part of wild canids from Pampa. Also, antibody titres indicated that *R. parkeri* s.s. is the most prevalent *Rickettsia* species among sampled foxes. In addition, that both crab-eating fox and Pampas fox can harbour tick species associated with spotted fever, at least one-third of them carry *R. parkeri*-infected ticks. Thus, the results suggest that wild canids are involved in the enzootic cycle of *R. parkeri* s.s. in the Pampa biome and could be responsible for pathogen (and its vectors) dispersal.

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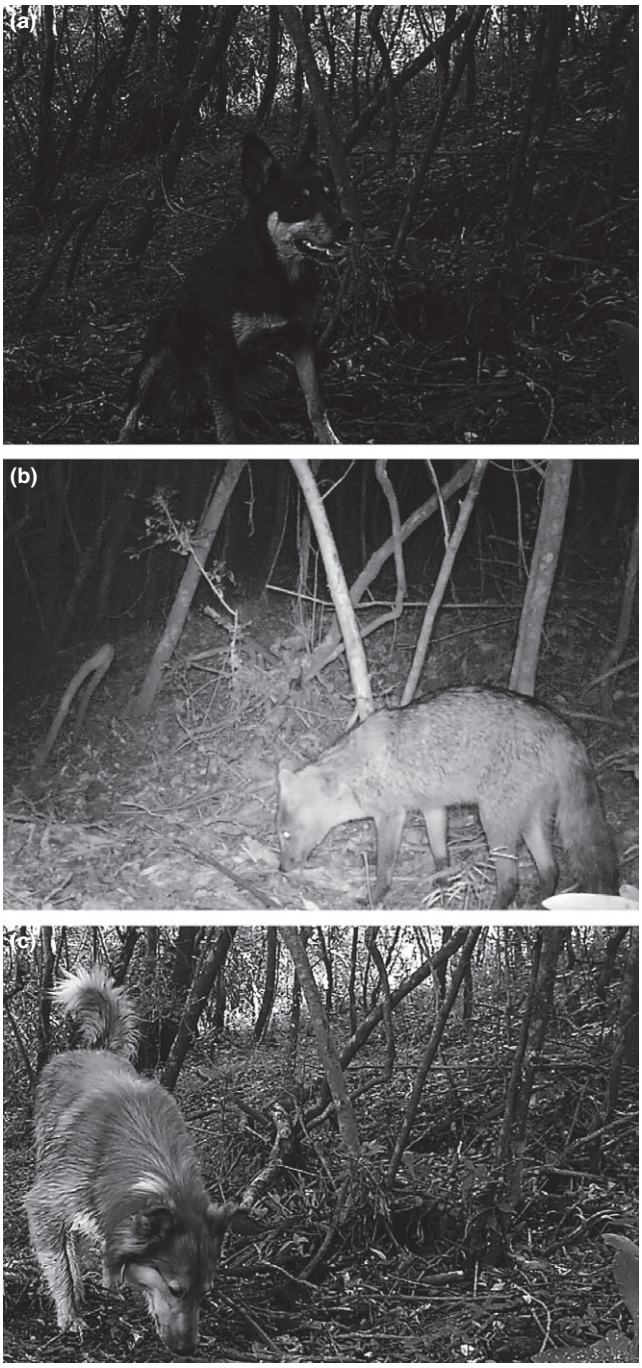

FIGURE 4 Camera trap records showing the habitat sharing between domestic dogs and wild canids in Santana do Livramento municipality. Panel a, domestic dog. Panel b, *Cerdocyon thous* (crab-eating fox), approximately 72 hr after the record of the image showed in Panel a. Panel c, domestic dog recorded approximately 5 days after the *C. thous* recorded in Panel b

Desenvolvimento Científico e Tecnológico (CNPq), Projeto RS Biodiversidade, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and INCT Entomologia Molecular.

CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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