



# Assessing the natural circulation of canine vector-borne pathogens in foxes, ticks and fleas in protected areas of Argentine Patagonia with negligible dog participation

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## ABSTRACT

We collected blood and/or ectoparasites from 49 South American grey foxes (*Lycalopex griseus*) and two Andean foxes (*L. culpaeus*) caught in two National Parks of southern Argentine Patagonia (Bosques Petrificados, BPNP; and Monte León, MLNP) where dogs are nearly absent (density < 0.01 dog/km<sup>2</sup>). Common ectoparasites were the flea *Pulex irritans* (88% prevalence) and the tick *Amblyomma tigrinum* (29%). Conventional PCR and sequencing of 49 blood samples, 299 fleas analysed in 78 pools, and 21 ticks revealed the presence of DNA of the following canine vector-borne pathogens: in grey foxes, *Rickettsia* sp. (3%), hemoplasmas (8%), including *Mycoplasma haemocanis*, and *Hepatozoon* sp. (50%); in *P. irritans*, *Bartonella* spp. (72% of flea pools from 76% of foxes), mostly *B. vinsonii* subsp. *berkhoffii* but also *B. rochalimae*, Anaplasmataceae (*Wolbachia* sp.; 60% and 54%), and *M. haemocanis/haemofelis* (29% and 18%); and in *A. tigrinum*, *Hepatozoon* sp. (33% of ticks in 4 of 7 foxes). No piroplasmid DNA was detected in any sample. Andean foxes were negative for all tested pathogens. Two different *Hepatozoon* haplotypes were detected: the most prevalent was phylogenetically associated with *H. felis*, and the other with *H. americanum* and related sequences. *Amblyomma tigrinum* and *Hepatozoon* sp. were more abundant and/or prevalent in BPNP than in colder MLNP, 300 km southwards, perhaps located close to the limit for tick suitability. *Bartonella v. berkhoffii* was also significantly more prevalent in fleas of foxes in BPNP than in MLNP. This study provides novel information about natural host-pathogen associations in wildlife, markedly extends the distribution area in South America of arthropods and vector-borne pathogens of veterinary and public health interest, and contributes preliminary evidence about the potential role of *A. tigrinum* and *P. irritans* as vectors, respectively, for potentially new species of *Hepatozoon* from *Lycalopex* spp. and for *M. haemocanis* that should be further investigated.

## 1. Introduction

Determining the impact of infectious diseases is a key issue in the conservation of wildlife (Scott, 1988). As most pathogens can infect more than one species and many wild carnivore species show solitary habits and a low frequency of intraspecific contacts, hindering pathogen transmission, it is often assumed that the epidemiology of disease agents of these species is driven by the existence of reservoirs (Woodroffe, 1999; Haydon et al., 2002; Power and Mitchell, 2004; Millán et al., 2009). In this sense, domestic dogs have been blamed as the origin of diverse epidemics affecting wild carnivores, such as the

paradigmatic canine distemper epidemic in Serengeti wildlife (Cleaveland et al., 2000), and the rabies outbreak in Ethiopian wolves (Randall et al., 2004). Leaving apart some studies where substantial epidemiological or molecular evidence strongly suggests a spill-over from dogs to wild counterparts (e.g. Gordon et al., 2015), the role of dogs as a source of pathogens infecting wildlife is often assumed without a solid scientific basis. Wild carnivores can also act as a source of pathogens, causing overt disease in domestic dogs. For example, the piroplasmids *Babesia microti*-like, *B. rossi* and *Rangelia vitalii*, that have restricted distribution areas and often cause severe disease in dogs but not in wild canids, may have their natural reservoir in the latter

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(Penzhorn, 2011; França et al., 2014; Checa et al., 2018).

The growing spread of humans and associated species into wild habitats is one of the main causes of interspecific pathogen transmission (Jones et al., 2013). As humans and their domestic animals now occur in almost every place on the planet, determining the pathogens that circulate naturally in wild carnivore populations without the participation of domestic reservoirs is challenging. Identifying these pathogens is valuable to determine whether they should be considered as potential disease threats for animal conservation. Pathogens circulating in wild populations without causing overt pathology could be considered part of a balanced host-parasite relationship from an evolutionary perspective (Mitchell, 1991; Hudson et al., 2006). Establishing the role of wild species as natural hosts for a given pathogen would also help to determine risk factors (e.g. outdoor access or use for hunting) and hence prevent transmission to dogs.

Searching for natural host-pathogen interactions should ideally be done in remote, scarcely populated areas without dogs. In these conditions, parasite transmission is more likely driven by intraspecific than by dog-wild canid contacts. To gain insight into natural carnivore-pathogen associations, the aim of the present study is to describe the presence of canine vector-borne pathogens (CVBP) in wild canids and associated fleas and ticks in remote areas with negligible interference from humans and dogs.

## 2. Material and methods

### 2.1. Study areas

The cold deserts of Santa Cruz province, in southern Argentine Patagonia, present several attributes suitable for our study. With agriculture nearly absent, the prevalent activity in the countryside is extensive sheep husbandry, where stocks graze free within large enclosures with little contact with shepherds during most of the year, and people concentrate in small towns or cities quite distant from each other (González and Rial, 2004). As a result, overall human density in Santa Cruz is 1.1 inhabitants/km<sup>2</sup>. Densities are much lower in the countryside where isolated, permanently inhabited houses are typically tens of kilometres apart.

The study was conducted in two protected areas of Santa Cruz (Fig. 1), featuring persistent westerly winds and an arid climate with annual rainfall not exceeding 300 mm. The Monumento Natural Bosques Petrificados National Park (BPNP; 78,000 ha; 47° 58' S, 67° 97' W) is located in the north of the province, 170 km west of the Atlantic coast. Winters are relatively mild (< 15 freezing days per year) and the mean annual temperature is 10 °C. Steppe of tussock grasses and shrubs covers most of the park, which also has large areas with very scarce vegetation or bare ground (Soriano, 1983). Monte León National Park (MLNP; 62,000 ha; 50° 12' S, 68° 56' W) is a coastal reserve 300 km south of BPNP. In MLNP the influence of Antarctic weather fronts is greater than in BPNP. The climate is colder, slightly wetter, and despite oceanic moderation, the mean annual temperature is 8.6 °C. As large open bare plains and extensive basaltic plateaus do not occur in MLNP, average shrub cover tends to be higher and average grass cover lower than in BPNP.

### 2.2. Model species

We studied CVBP in two sympatric species of neotropical foxes, the South American grey fox (*Lycalopex griseus*) and the Andean fox (aka culpeo) (*L. culpaeus*), and their ectoparasites. The grey fox is widespread in plains and mountains on both sides of the Andes in Chile and Argentina (Lucherini, 2016a), whereas the Andean fox is distributed throughout the Andes and hilly regions of South America from Colombia to Tierra del Fuego (Lucherini, 2016b). Andean foxes are scarce in both protected areas, with an estimated density < 0.1 ind/km<sup>2</sup>, whereas grey foxes are common and can occur at local densities of up to

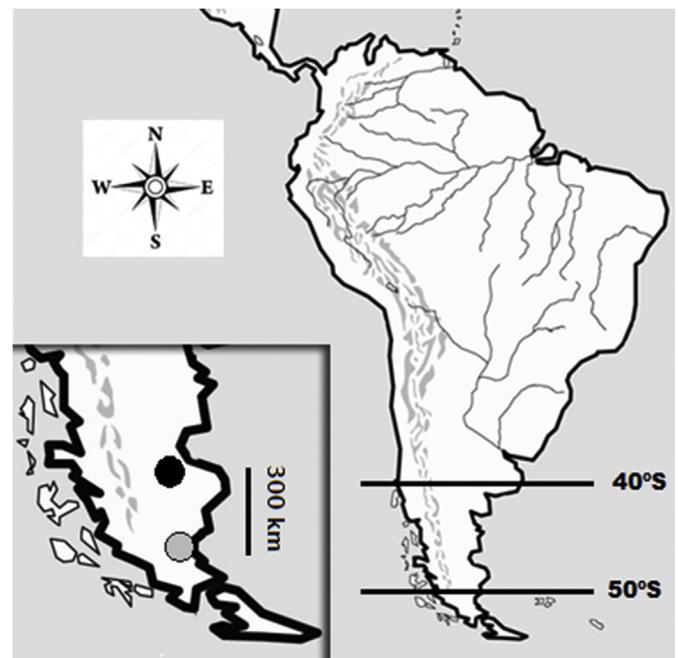


Fig. 1. Map of Latin America, showing the study areas in the insert. Black circle: Bosques Petrificados National Park; grey circle: Monte León National Park.

1 ind/km<sup>2</sup> (authors' unpublished data).

### 2.3. Domestic carnivores

We performed long-term monitoring and intensive field surveys of canid distribution and abundance during a 15-yr period (1998–2012) in BPNP and an 8-yr period in MLNP (2009–2016). Surveys included bait stations (Travaini et al., 2010), active searches for canid signs (footprints or faeces) and trapping campaigns. Although these surveys were primarily targeted at wild canids, we also recorded dog occurrence. The overall effort for surveying wild and domestic carnivores amounted to more than 5000 station or trap-nights and over 1000 km of cross-country surveys on foot. No sighting or sign was recorded for dogs or other domestic carnivores in the field in either area. Park visitors are not allowed to bring pets, although a few dogs and cats were owned by staff living in the parks. The mean ( $\pm$  SD) annual number of dogs in the few inhabited buildings of the parks was 2.5 ( $\pm$  1.5) dogs in BPNP ( $n = 17$ ), and 3.9 ( $\pm$  2.4) in MLNP ( $n = 12$ ), which yields a mean annual density of < 0.01 dog/km<sup>2</sup> in both areas. We recorded the number and habits of these domestic carnivores during the study period. According to the information provided by the owners, the mean annual percentage of dogs making occasional roams, and potentially making direct contact with wildlife, was 29% in BPNP and 3% in MLNP. The mean ( $\pm$  SD) annual number of cats in houses was 0.8 ( $\pm$  1.6) in BPNP, and 1.8 ( $\pm$  0.6) in MLNP. Therefore, mean annual densities of cats were also < 0.01 cat/km<sup>2</sup>. All cats were confined.

### 2.4. Field methods

Foxes were captured between 2010 and 2015 with Oneida Victor #1.5 soft-catch coil spring traps (Cleveland, OH, USA), and anaesthetized with a combination of tiletamine and zolazepam (Zoletil, Virbac, Spain). Foxes were sexed by external morphological characters, while their age was inferred from tooth wear. We caught 49 grey foxes (24 in BPNP and 25 in MLNP) and two Andean foxes (in BPNP) during several spring and fall trapping sessions (Table 1). All animals, either caught for the first time or recaptured were inspected for ectoparasites,

**Table 1**

Sex, age, and season of capture for 52 grey fox (*Lycalopex griseus*) capture events (49 individuals) in each study area. BPNP: Bosques Petrificados National Park; MLNP: Monte León National Park.

Area	BPNP	MLNP
Sex		
Male	18	13
Female	8	13
Age		
Juvenile	7	0
Adult	19	26
Season		
Spring	5	24
Autumn	21	0
Total	26	26

which were stored in 96% ethanol. Blood samples obtained from the femoral vein were obtained for 48 grey foxes and one Andean fox. Blood was applied (100 µl) to FTA™ Nucleic Acid Collection Cards (Whatman, Maidstone, Kent, UK), air dried and stored in sealed plastic bags. Three grey foxes were captured twice. Ectoparasites were collected during the second capture, and blood was also obtained from one individual that was recaptured in MLNP with a one-year interval; the events were considered independent (Table 1).

2.5. Laboratory methods

Ectoparasite identification was carried out using standard morphological criteria (Hopkins and Rothschild, 1953; Nava et al., 2017). A subset of fleas was preliminarily identified and reported by Sánchez et al. (2018), who found the species *Pulex irritans* and *Polygenis platensis* in a 20:1 ratio. Only *P. irritans* was included in the present study because it was the core species and its well-known vector capacity for some CVBD.

Total genomic DNA was extracted from a single 2 mm punch of the FTA™ Cards containing fox blood. DNA was also extracted from 299 fleas collected in the 46 infested foxes and analysed in 78 pools containing between one and five specimens each. DNA was also extracted from 21 individual half-ticks collected in seven grey foxes (five from BPNP, two from MLNP).

DNA extraction was performed using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions for tissue samples. FTA card punches and arthropods were mechanically crushed with a sterile micropestle or cut into smaller pieces with sterile scalpels, suspended in tissue lysis buffer and proteinase K and incubated for 1h at 56 °C. DNA was eluted in 200 µl of elution buffer.

We used molecular methods to confirm tick and flea species identification, as well as to compare DNA sequences with published data. Fragments of mitochondrial DNA of the 16S rRNA and cytochrome c oxidase II (*cox2*) regions were amplified for ticks and fleas, respectively (Supplementary File 1). Internal control PCR for canine genomic DNA was carried out for FTA Card samples targeting the *RPS19* gene. The

**Table 2**

Prevalence and abundance of ticks and fleas retrieved from grey foxes (*Lycalopex griseus*). n: number of captures. BPNP: Bosques Petrificados National Park; MLNP: Monte León National Park.

	n	<i>Pulex irritans</i>				<i>Amblyomma tigrinum</i>			
		Prevalence %	95% C.I.	Abundance	s.d.	Prevalence %	95% C.I.	Abundance	s.d.
BPNP	26	84.6	65.1–95.6	4.9	5.4	50.0	29.9–70.1	3.5	6.1
MLNP	26	92.3	74.9–99.1	6.6	6.9	7.7	0.9–25.1	0.1	0.3
Overall	52	88.5	76.6–95.6	5.7	6.3	28.8	17.1–43.1	1.7	4.5

presence of *Mycoplasma* sp., *Bartonella* sp., *Rickettsia* sp., Anaplasmataceae, Piroplasmida, and *Hepatozoon* sp. DNA was screened in fox blood and in fleas and ticks by conventional PCR targeting regions, using primers and with the conditions described in Supplementary File 1. Each reaction was carried out in a final volume of 25 µl using GoTaq® DNA Polymerase and 2 µl of template DNA. A positive control (*Mycoplasma haemocanis* from a dog, *Hepatozoon* sp. from a snake, *B. henselae* from a cat, *Anaplasma platys* from a dog, *Babesia ovis* from a sheep and *Rickettsia felis* from a flea) and two no template controls (without DNA) were used in each reaction. To avoid cross-contamination, the DNA extraction, mixing of DNA-free PCR reagents and the addition of the template DNA were carried out in separated areas with separate equipment and solutions. All PCR products were visualized on a 2% agarose electrophoresis gel, and later purified and sequenced at Macrogen (Seoul, South Korea). Sequences were compared with those available in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

New sequences reported here were uploaded to GenBank under accession numbers MK049948-MK049951, MK050553- MK050555, MK050558, MK060170-MK060178, MK063763-MK063782, MK064160-MK064164, and MK097143-MK097147.

2.6. Statistical and phylogenetic analyses

As climate and other environmental factors associated with latitude may affect the abundance of ectoparasite vectors (Linardi and Krasnov 2013), we examined the effects of study area and season on pathogen prevalence. We also explored the effect of host-related factors such as sex and age. We analysed differences in prevalence with generalized linear models (GLM), specifying binomial errors and logit link. When model fit was not possible due to very little variability in the response or redundancy/missing levels in predictors, we used standard contingency table analysis for binomial data. We also analysed differences in ectoparasite intensity using Poisson errors and log link. If needed, models were adjusted for overdispersion by setting the scale parameter as the deviance divided by its degrees of freedom. We assessed the percentage of deviance explained by the models. We compared alternative models containing different combinations of predictors (Burnham and Anderson, 2002) using the Akaike Information Criterion (AIC). We considered competing models those with ΔAIC ≤ 2.0.

PCR fragments were sequenced and manually assembled. All sequences obtained were compared to those of the GenBank database and aligned using the CLUSTALW algorithm (Geneious®). Phylogenetic trees were constructed based on maximum-likelihood analysis and the Tamura-Nei model with RAxML software version 1.5 (Stamatakis et al., 2008). The data set was resampled 1000 times to generate bootstrap values. The best model of evolution was selected by the program jModelTest2 (version 2.1.6) (Darriba et al., 2012), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). Genetic p-distances among the hemoplasmas were calculated with MEGA 7.0 (Tamura et al., 2007) using 616bp of the 18S ribosomal RNA gene.

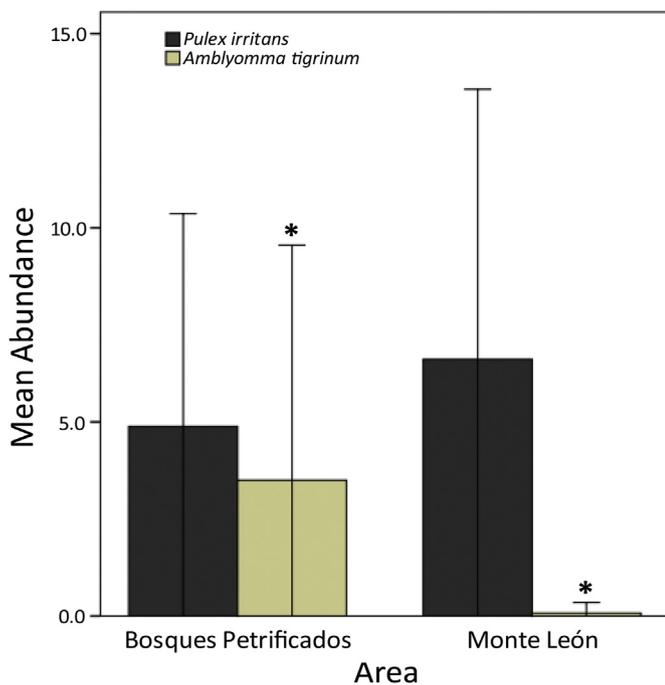


Fig. 2. Abundance of *Pulex irritans* and *Amblyomma tigrinum* in grey foxes depending on the study area. (\*) indicates significant differences.

### 3. Results

#### 3.1. Ectoparasites

Twenty-nine percent of grey foxes hosted ticks (Table 2). All ticks were identified morphologically as *Amblyomma tigrinum*. The sequencing of seven ticks resulted in amplicons of about 405 bp that had 99–100% identity with different sequences of that species, confirming the morphological identification. The prevalence of *A. tigrinum* was significantly higher in BPNP, where half of the foxes harboured ticks, compared to only about 8% of the individuals captured in MLNP (Supplementary File 2). Tick abundance in BPNP was also significantly higher than in MLNP (Fig. 2, Supplementary Files 3 and 4). As all foxes captured in MLNP were adult and were sampled in spring, the effects of age and season on prevalence and abundance of ectoparasites and pathogens were only examined in BPNP. No significant differences were found in tick prevalence depending on fox sex or season in that area (Supplementary File 2). Once the effect of area was controlled for, we found further significant effects of age (tick abundance in adults was higher than in juveniles) and season (tick abundance in spring was higher than in fall; Supplementary Files 3 and 4). The most parsimonious model kept all three effects, and a model containing study area and season was also competitive (Supplementary File 4). This model explained 20% of the variance in tick abundance.

Table 3

Prevalence of canine vector-borne pathogens in grey foxes (*Lycalopex griseus*). CI: confidence interval. BPNP: Bosques Petrificados National Park; MLNP: Monte León National Park.

	BPNP (n = 24)		MLNP (n = 24)		Total (n = 48)	
	Prevalence %	95% CI	Prevalence %	95% CI	Prevalence %	95% CI
<i>Bartonella</i> sp.	0	0–14.2	0	0–14.2	0	0–14.2
<i>Rickettsia</i> sp.	0	0–14.2	4.2	0–14.2	2.1	0–14.2
Anaplasmataceae	0	0–14.2	0	0–14.2	0	0–14.2
Haemotropic <i>Mycoplasma</i>	8.3	0.1–27.0	8.3	0.1–27.0	8.3	0.2–20.0
<i>Hepatozoon</i> sp.	95.8	78.9–99.9	4.2	0.1–20.2	50.0	35.2–64.8
<i>Babesia</i> sp.	0	0–14.2	0	0–14.2	0	0–14.2

Fleas were found on 89% of grey foxes (Table 2). Sequencing of five fleas identified morphologically as *P. irritans* yielded amplicons of between 530 and 630 bp, which showed 98% identity with different sequences of *P. irritans*. No statistical differences in flea prevalence were found across levels of environmental and host-related factors (Supplementary File 5). Mean flea abundance was greater in adults and females than in juveniles and males, respectively (Supplementary File 6). For grey foxes from the BPNP, mean flea abundance in fall was higher than in spring (Supplementary File 6). However, none of these differences were significant (Supplementary File 7).

Five females showed signs of reproductive activity at the time of capture. All lactating females were caught in MLNP. Two of them carried ticks, while we found none on non-breeding females from the same study area and the same season (Fisher exact test,  $p = 0.128$ ). All females from MLNP were infested with *P. irritans*. The mean ( $\pm$  SE) abundance of fleas on breeding females ( $2.8 \pm 0.8$ ) was significantly lower ( $t = 2.931$ ,  $df = 11$ ,  $p = 0.014$ ) than non-breeding females ( $13.8 \pm 2.9$ ). The two Andean foxes were infested with *A. tigrinum*, and one of them also hosted *P. irritans*.

#### 3.2. CVBP in foxes

DNA of three of the studied CVBP groups (*Rickettsia*, hemoplasmas and *Hepatozoon*) was found in grey foxes (Table 3). Hemoplasma DNA was found in two grey foxes from each area (Table 3). Three amplicons showed an identity of 99–100% with *M. haemocanis/haemofelis* (Mhc/Mhf) (a.n. KU645929), while the fourth was 99% identical to a hemoplasma detected in a wild rodent in Brazil (a.n. KT215636). Only one readable sequence of the *RNase-p* gene was obtained for the foxes positive to Mhc/Mhf, which was 98% similar to *M. haemocanis* (a.n. KU743386). All the hemoplasma-infected foxes were adult males. One of the positive foxes from MLNP was recaptured one year later and was PCR-negative in that event. The relationships between fox infection and hemoplasma and tick or flea prevalence and abundance were not significant (Supplementary Table 8).

Half of the studied grey foxes harboured *Hepatozoon* DNA. Of the 20 amplicons obtained, 19 (18 from BPNP and one from MLNP) were identical (Haplotype 1) and had 99% similarity with *H. felis* from an Indian cat (a.n. JN584475) but also with many other *H. felis* sequences such as the so-called “isolate Spain 2” (a.n. AY628681). A further amplicon from two foxes of BPNP (Haplotype 2) showed 98% identity with different *H. felis* sequences (e.g., a.n. AB983436), and 97% with *H. americanum*. Phylogenetic analysis, however, clustered this Haplotype 2 in the *H. canis* clade, in a branch with *Hepatozoon* sp. from Brazilian crab-eating foxes (*Cerdocyon thous*) and *H. americanum*, with 98% bootstrap value (Fig. 4). Haplotype 1 was grouped with *H. felis* sequences.

*Hepatozoon* prevalence was significantly and markedly higher in BPNP (96%) than in MLNP (4%; Supplementary Table 9). The effect of other predictors on *Hepatozoon* prevalence was not significant. The probability of a fox harbouring DNA of *Hepatozoon* and hosting *A. tigrinum* (0.85) was more than twice the prevalence in foxes not carrying

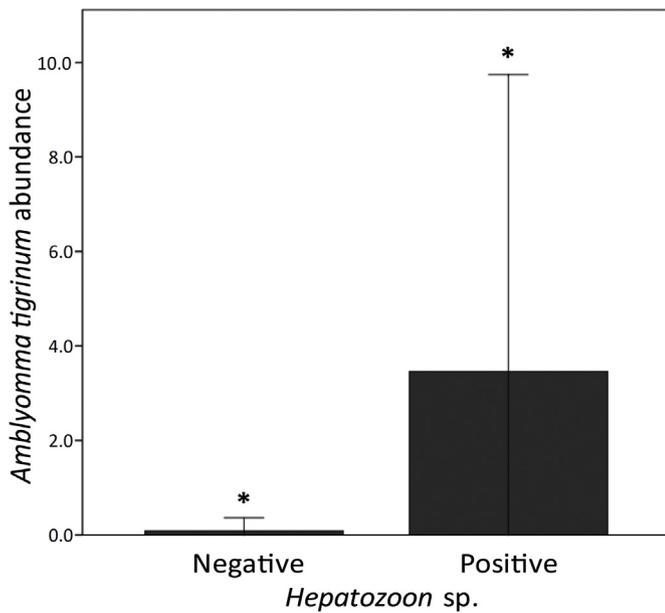


Fig. 3. Abundance of *Amblyomma tigrinum* in grey foxes depending on the *Hepatozoon* infection status of the fox. (\*) indicates significant differences.

ticks (0.37), and this effect was significant in the models (Supplementary Table 10). In foxes infected with *Hepatozoon* spp., the abundance of ticks was also significantly higher than in non-infected foxes (Fig. 3, Supplementary Table 8). There was no association between grey fox infection with *Hepatozoon* spp. and flea prevalence (Supplementary Table 8).

DNA from *Rickettsia* was found in one grey fox from MLNP. The 306

bp sequenced amplicon of the *gltA* gene was 99% similar to uncultured *Rickettsia* sp. clone 42-2 from a *Ctenocephalides felis* flea in Uganda (MF459640) and other *R. felis* isolates from around the world (e.g. MG893575). Unfortunately, our attempts to further characterize other gene fragments (*OmpA* and *OmpB*) were unsuccessful. DNA from *Babesia* or Anaplasmataceae was not amplified in any fox sample. The Andean fox for which blood was available was negative for all the pathogens tested.

### 3.3. CVBP in ectoparasites

DNA of *Bartonella* was found in 72% of flea pools from 76% of foxes hosting fleas (Table 4). Readable sequences revealed that the most prevalent species was *B. vinsonii* subsp. *berkhoffii* genotype III. Three different sequence types were identified, that differed in 1-2bp and that were detected in fleas from seven, 19 and one foxes, respectively (two foxes hosted fleas infected with two different sequence types). Nine pools showed a sequence that was 99% similar to a *B. rochalimae* sequence obtained from a dog in Palestine (a.n. KX420620). The prevalence of *B. v. berkhoffii* in fleas from foxes of BPNP (0.72) was significantly higher ( $\chi^2 = 4.54$ ,  $df = 1$ ,  $p = 0.033$ ) than in fleas from foxes of MLNP (0.38). In contrast, prevalence of *B. rochalimae* in fleas from foxes of MLNP (0.25) was higher than in those from BPNP (0.05), although the difference was not significant (Fisher's exact test,  $p = 0.226$ ). Up to 60% of the flea pools yielded positive reactions with the PCR for Anaplasmataceae. Sequencing revealed that the amplicons had 98–100% identity with DNA of diverse *Wolbachia* species, which are endosymbionts of different insects, including *P. irritans*. Hemoplasma DNA was detected in 37% of flea pools from 39% of grey foxes. All these pools hosted a sequence that was 100% identical to Mhc/Mhf. We found no strong association between the occurrence of hemoplasma in grey foxes and the fleas collected on them. The fleas from all infected

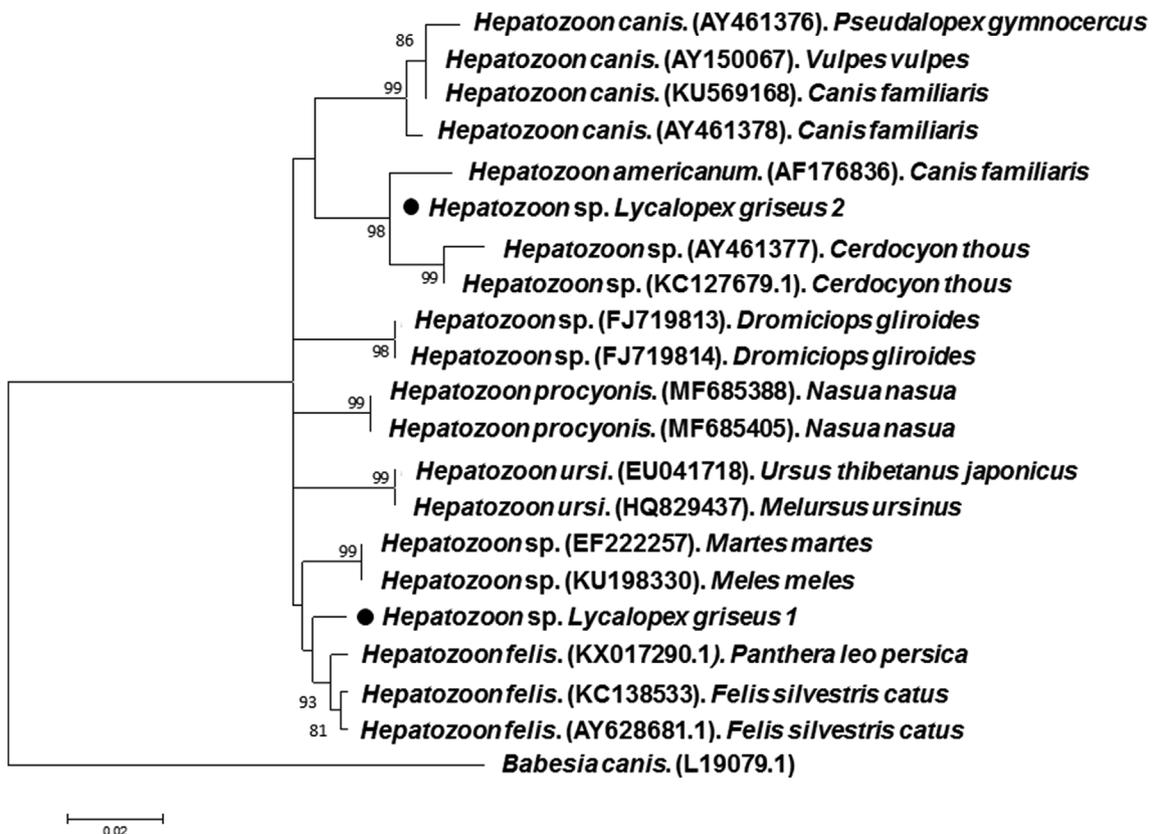


Fig. 4. Maximum-likelihood tree based on the Tamura-Nei model of selected sequences from *Hepatozoon* sp. The name of the sequence indicates the GenBank accession number and host species. The percentage of trees in which the associated taxa clustered together (bootstrap values) is shown next to the branches.

**Table 4**

Prevalence of canine vector-borne pathogens in *Pulex irritans* fleas collected from grey foxes (*Lycalopex griseus*). BPNP: Bosques Petrificados National Park; MLNP: Monte León National Park.

	BPNP		MLNP		Total	
	n = 22 foxes	n = 34 pools	n = 24 foxes	n = 44 pools	n = 46 foxes	n = 78 pools
	Positives (Prevalence %)					
<i>Bartonella</i> spp.	19 (86.3)	29 (85.2)	16 (66.6)	27 (61.3)	35 (76.1)	56 (71.7)
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	16 (72.7)	24 (70.5)	9 (37.5)	20 (45.4)	25 (54.3)	44 (56.4)
<i>B. rochalimae</i>	1 (4.5)	3 (8.8)	6 (25.0)	6 (13.6)	7 (15.2)	9 (11.5)
<i>Bartonella</i> sp.	2 (9.1)	2 (5.9)	1 (4.1)	1 (2.2)	3 (6.5)	3 (3.8)
<i>Rickettsia</i> sp.	0	0	0	0	0	0
Anaplasmataceae ( <i>Wolbachia</i> sp.)	12 (54.5)	18 (52.9)	13 (54.1)	29 (65.9)	25 (54.3)	47 (60.2)
<i>Mycoplasma</i> sp.	9 (40.9)	12 (35.2)	9 (37.5)	17 (38.6)	18 (39.1)	29 (37.2)

foxes were positive for hemoplasma, but fleas were also positive in one third of non-infected foxes. The co-occurrence hypothesis was therefore rejected (goodness of fit chi-square = 5.158, df = 1, p = 0.023). The three foxes with Mhc/Mhf DNA hosted fleas also with Mhc/Mhf DNA. In one case the amplicons obtained in the flea pools were identical to the amplicon from the host. In two other cases these were 99% similar. Unfortunately, the sequence obtained from the flea pool hosted by the fox positive for the hemoplasma related to a rodent *Mycoplasma* was unreadable. The flea pool of the single Andean fox infested with fleas was positive for hemoplasma and *Wolbachia*.

Seven ticks, belonging to four foxes (all from BPNP), hosted *Hepatozoon* DNA. All the positive ticks were engorged females, resulting in a significant difference in the prevalence between tick sexes (Fisher's exact test, p = 0.001). All ticks were negative for hemoplasma, piraplasms and *Rickettsia*.

#### 4. Discussion

Despite the relative small sample size, the present study revealed evidence of the presence of DNA of a range of vector-borne pathogens in foxes and associated ticks and fleas from a remote region in Patagonia, Argentina. The abundance of dogs in our study areas was up to four orders of magnitude lower than densities reported in rural areas of Africa (6–21 dogs/km<sup>2</sup>; Kitala et al., 2001), Asia (14–28 dogs/km<sup>2</sup>; Hossain et al., 2013; Belsare and Gommer, 2015) or Europe (1–37 dogs/km<sup>2</sup>; Schares et al., 2003; Krauze-Gryz and Gryz, 2014), where dogs are sometimes involved in pathogen transmission. Dog density tends to be lower in the less populated rural areas of temperate South America (Acosta-Jamett et al., 2010; Astorga et al., 2015). Using these regional values as a reference, dog density in BPNP and MLNP was 20 times lower than the minimum reported for 122 municipalities across Chile (0.2 dogs/km<sup>2</sup>; Astorga et al., 2015). Moreover, in our study areas dog trips away from houses were occasional and limited to a few individuals, compared, for example, to the estimated 67% of dogs roaming freely in rural areas of Chile (Acosta-Jamett et al., 2010). Sheep raising declined drastically during the last four decades, and many farms became inactive across large areas of Santa Cruz (Andrade, 2012), probably followed by a sudden drop in the number of dogs. Therefore, dog density is extremely low in the study areas and may also be quite low in the surrounding farms, especially around the BPNP. Few studies to date have been able to explore the presence of canine pathogens in carnivores inhabiting remote areas with such low densities of dogs.

Evidences of infection with some of the pathogens studied here were found in other little populated areas, like in a range of jackal and fox species from northern Africa (Maia et al., 2014) and in arctic foxes (Mascarelli et al., 2016). In consequence, we cannot discard that the pathogens studied here were brought to Patagonia by dogs during the

last two centuries. Currently, however, these pathogens might circulate by means of fox intra-specific transmission rather than by dog-to-fox transmission, indicating that grey foxes are competent hosts for the detected pathogens. The hypothesis that dogs are not (or not importantly) involved in the transmission of grey fox pathogens is further supported by the fact that dog and cat fleas (*C. canis* and *C. felis*) and ticks (*Rhipicephalus sanguineus* sensu lato) were not observed during our study. These species frequently infest wild carnivores in human-dominated landscapes (Millán et al., 2016; Clark et al., 2018) but also in natural areas subject to intense human pressure (Millán et al., 2007). Adults of the tick *A. tigrinum* are typically and only found in South American canids, while small rodents and birds are the principal hosts for immature stages (Nava et al., 2017). They have a wide distribution due to their ecological plasticity. To the best of our knowledge, the finding of specimens in MLNP expands their distribution area 600 km southwards (Gugliemone et al., 2000). Interestingly, the marked differences in abundance and prevalence between the areas studied here might indicate that the climate conditions of MLNP could be close to the boundaries of the ecological niche of this tick species. We observed ticks in seasons other than summer, which is consistent with previous observations (Gugliemone et al., 2000). Only the fleas *P. irritans* and *P. platensis* (a rodent flea) were retrieved in our sample of foxes (Sánchez et al., 2018). *Pulex irritans* was abundant through all seasons, indicating a strong association with foxes. This flea species is believed to have originated in South America (Buckland and Sadler, 1989) and can be very prevalent in populations of wild canids in isolated areas worldwide (e.g. Munkhzul et al., 2018).

Infection with an *H. felis*-like parasite appears to be a common feature in the studied grey fox populations, especially in the northern one (BPNP), where almost all foxes appeared to be infected. High rates of infection with *Hepatozoon* spp. have been observed in populations of wild canids worldwide. For example, prevalence ranging from 43% to 91% has been reported for crab-eating foxes in Brazil (de Sousa et al., 2017 and references therein). Other studies reported rates of infection of about 45% in red foxes (*Vulpes vulpes*) and golden jackals (*Canis aureus*) in Israel (Margalit Levi et al., 2018); 42% in diverse species of North Africa (Maia et al., 2014); and 70% in golden jackals from central Europe (Mitková et al., 2017). In the southern cone of South America, the only evidence of infection with *Hepatozoon* sp. was reported for a Pampas grey fox (*L. gymnocercus*) found ill in central Argentina (Giannitti et al., 2012). The two genotypes detected in the present study showed 99% (the most prevalent) and 97% identity with the sequence reported by Giannitti et al. (2012), although query cover was low (about 55%) due to the use of different sets of primers. Both our Haplotype 1 and the sequence reported by Giannitti et al. (2012) showed highest identity with *H. felis* isolate Spain 2, suggesting that there is an *H. felis*-like strain naturally circulating among *Lycalopex* spp. foxes in southern South America. The Haplotype 2 described here was, however,

included in a branch with sequences retrieved from the crab-eating foxes studied by different authors in Brazil that were most closely related to *Hepatozoon* sp. Curupira 2 and *H. americanum* (Criado-Fornelio et al., 2006; Almeida et al., 2013, de Sousa et al., 2017). *Hepatozoon canis* was also identified in Pampas grey foxes in Brazil (Criado-Fornelio et al., 2006). Therefore, it seems that up to three *Hepatozoon* species may be circulating among South-American canids. Further molecular and morphological characterization of the *H. felis*-like from *Lycalopex* spp. is pending.

We found a strong association between the probability of infection in foxes and the prevalence and abundance of *A. tigrinum*. In addition, we found *Hepatozoon* DNA in the analyzed *A. tigrinum*, although this does not prove vector competence. Other *Amblyomma*-*Hepatozoon* associations have been described before; for example, *A. ovale* is a confirmed vector for *H. canis* (Rubini et al., 2009). Together, our results suggest that *A. tigrinum* might be the vector of this *H. felis*-like strain, but further evidence is needed to confirm this.

Two species of *Bartonella* were identified in a sizeable proportion of flea pools. Both *B. rochalimae* and *B. vinsoni berkhoffii* are distributed worldwide and appear to have their natural reservoir in wild carnivores, especially canids, with fleas acting as vectors (Henn et al., 2009; Gabriel et al., 2009; Bai et al., 2016; Millán et al., 2016). To the best of our knowledge, none of these species have been found before in Argentina, not even in dogs. This probably reflects a scarcity of studies rather than true absence or low prevalence, since both species were detected in fleas and/or dogs in adjacent Chile (Pérez-Martínez et al., 2009; Müller et al., 2018). Our survey supports the role of fleas as vectors of these two pathogens. It is difficult to explain why we did not detect these species in mammal hosts, taking into account the high prevalence of infection in fleas. This may be caused by the way the blood samples were preserved, that prevented us from culturing the bacteria before applying PCR, which is strongly recommended to detect *Bartonella* (Gutiérrez et al., 2017). It is worth mentioning that *B. v. berkhoffii* genotype III can be highly pathogenic for dogs (Shelnutt et al., 2017), whereas *B. rochalimae* has recognized zoonotic capacity (Eremeeva et al., 2007).

DNA of a *Rickettsia* closely related to *R. felis* was found in a single fox. Unfortunately, our attempts to characterize the bacterium were unsuccessful, probably due to the use of FTA cards to preserve the samples. DNA of the zoonotic *R. felis* has been detected in many flea species but the European cat flea *C. f. felis* is the only known biological vector, although mosquitoes have been recently suspected to be able to transmit this bacterium (Angelakis et al., 2016).

Hemoplasma DNA was detected in some foxes and in a remarkable proportion of the flea pools analysed. *Mycoplasma haemocanis* infects dogs worldwide (Shapiro et al., 2017; Soto et al., 2017; Aktas and Ozubek, 2018), including Argentina (Mascarelli et al., 2016). As many as 77% of dogs from northern Argentina were infected with hemoplasmas; *M. haemocanis* was the most prevalent species (Mascarelli et al., 2016). *Mycoplasma haemocanis* can also be found in wild canids. For example, Cabello et al. (2013) found it to be prevalent in Darwin's fox (*L. fulvipes*) in southern Chile. The fact that *M. haemocanis* was found in up to 40% of the flea pools, including many foxes that were negative, and the observed association between the presence of DNA in hosts and their fleas, suggest potential transmission of this bacterium by fleas. The manner of transmission of *M. haemocanis* is unclear to date. It is generally believed that it is transmitted by the brown dog tick (*R. sanguineus* s.l., Novacco et al., 2010). The experimental transmission carried out by Seneviratna et al. (1973) was performed in pre-molecular times and was never replicated. Other findings indicate that an alternative way of transmission should exist. Aktas and Ozubek (2018) failed to detect *M. haemocanis* DNA in unfed *R. sanguineus* s.l. ticks in a shelter with 25% prevalence in dogs. The study of Cabello et al. (2013) was carried out in an island outside the distribution range of *R. sanguineus* s.l. and *A. tigrinum* in Chile. The present study also demonstrates that *M. haemocanis* circulates among wild foxes in the absence of *R.*

*sanguineus* s.l., and we did not find evidence of hemoplasma DNA in the *A. tigrinum* ticks analysed. In consequence, potential role of *P. irritans* as a vector for *M. haemocanis* deserves further exploration.

In summary, we have demonstrated that some CVBP naturally circulate in wild foxes without (or with negligible) participation of dogs. We provide novel information about host-pathogen associations in wildlife that points towards the existence of one or more *Hepatozoon* species typical of South American grey foxes. We also markedly extend the distribution area in South America of arthropods and vector-borne pathogens of veterinary and public health interest. We also contribute with preliminary evidence about the potential role of *A. tigrinum* and *P. irritans* as vectors for *H. felis*-like from *Lycalopex* spp. and *M. haemocanis*, respectively, that should be further investigated. Dog owners and veterinary practitioners of southern South America should be aware of the presence of these pathogens in the area and the risk of transmission to dogs, especially to those with outdoor access.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2018.11.007>.

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