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Fleas and Ticks in Carnivores From a Domestic–Wildlife Interface: Implications for Public Health and Wildlife

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Abstract

Fleas and ticks are parasites of wild and domestic mammals, and can be vectors of several pathogens. In rural areas, domestic carnivores such as the domestic dog (*Canis lupus familiaris* L.), may act as a "bridge" between natural areas and human settlements where ectoparasites can be used as a metric of such link. The aim of this study was to identify fleas, ticks, and *Rickettsia* spp., collected from domestic and wild carnivores in a natural reserve and surrounding human settlements in Central Chile, using morphological keys and molecular analysis. We surveyed 170 households from which 107 dogs and eight cats were sampled. From the natural reserve, we sampled two chilla foxes (*Pseudalopex griseus* Gray), two lesser grison (*Galictis cuja* Molina), three kodkods (*Leopardus guigna* Molina), and four dogs. From dogs, we collected *Ctenocephalides felis* Bouché, *Ctenocephalides canis* Curtis, *Pulex irritans* L., and *Rhipicephalus sanguineus* s.l. Latreille; *C. felis* was the most frequent ectoparasite. Cats were infested only by *C. felis* and *Rh. sanguineus* s.l. From wild carnivores, we obtained *C. canis* and *P. irritans*, the latter being most frequent. Molecular analysis of *P. irritans* detected 10 haplotypes and two main clades, which tended to separate fleas from wild and domestic hosts. Molecular analysis of *ompA* and *ompB* genes confirmed the presence of *Rickettsia felis* in fleas collected from owned dogs and cats, which could represent a potential risk factor of *R. felis* transmission in the area.

Key words: *Ctenocephalides, Pulex irritans, Rhipicephalus sanguineus, Rickettsia*, wild–domestic interface

In pristine landscapes, relationships between hosts and parasites are maintained in ecological balance. This balance can be broken by anthropogenic disturbance, mostly based on activities such as agriculture, cattle herding, forestry, urbanization, among others (Daszak et al. 2000). As host–parasite interactions are disturbed, this may alter transmission dynamics, driving infection events into novel hosts (Gortazar et al. 2014). This exchange can be observed in ectoparasites; for example, fleas from domestic animals can be found in wild carnivores, particularly in rural areas where this interface facilitates encounters between wild and domestic species (Araújo et al. 1998, Cerqueira and Silva 2000, Szabó et al. 2003, Dobler and Pfeffer 2011).

In this domestic–wild interface, free-ranging domestic dogs (*Canis lupus familiaris*) can act as a bridge, translocating ectoparasites (and their pathogens) between wildlife, domestic animals, and humans (Cerqueira and Silva 2000, Dobler and Pfeffer 2011). Hard ticks (Ixodidae) and fleas are ectoparasites that can be translocated by dogs, and they have a polyxenous natural behavior (i.e., can infest a broad number of species; Krasnov 2008), and are the most common ectoparasites of domestic carnivores (Curtis 2000). Furthermore, after mosquitoes, ticks are the most important arthropod related to vector-borne diseases derived from viruses, bacteria, protozoa, and helminthes (Parola and Raoult 2001). Additionally, ticks can inoculate toxins in the host that may cause paralysis (Parola and Raoult 2001). Together with ticks, fleas are ectoparasites of public health concern, related to the transmission of zoonotic pathogens such as *Bartonella henselae*, *Rickettsia typhi*, and *Rickettsia felis* (Curtis 2000).

Moreover, fleas are intermediate hosts in the cycle of worms, including the intestinal tapeworm *Dipylidium caninum*, which occasionally causes dipylidiasis in humans (Curtis 2000).

Different species of *Rickettsia* are zoonotic pathogens that can be transmitted by ticks and fleas (Angelakis and Raoult 2011a, 2011b). These microorganisms are intracellular gram-negative bacteria, usually related to specific vectors. Nevertheless, the emerging zoonotic pathogen *R. felis* has been reported worldwide in the cat flea *Ctenocephalides felis* Bouché, and has also been identified in other arthropods, including the fleas *Ctenocephalides canis* Curtis and *Xenopsylla cheopis* Rothschild, the tick *Rhipicephalus sanguineus* s.l. Latreille, and some mites (Oliveira et al. 2008, Reif and Macaluso 2009, Abarca et al. 2013a). In humans, *R. felis* infection is easily confused with other rickettsial infections due to its unspecific symptoms, usually represented by maculopapular rash, fever, headache, and myalgia; thus, it may be frequently misdiagnosed (Hun and Troyo 2012).

In Chile, available information concerning fleas and ticks in both domestic and wild carnivores is scarce (González-Acuña and Guglielmone 2005, Beaucournu et al. 2014). Until now, four tick and five flea species have been reported in dogs in Chile, including the ticks *Rh. sanguineus* s.l., *Amblyomma tigrinum* Koch, *A. triste* Koch, and *Ornithodoros megnini* Dugés; and the fleas *C. felis*, *C. canis*, *Pulex irritans* L., *Tunga penetrans* L., and *Echidnophaga gallinacea* Westwood (Alcaíno et al. 2002, González-Acuña et al. 2008, Beaucournu et al. 2014). For wild carnivores, ectoparasite reports only include one tick (*A. tigrinum*) and five flea species: *C. felis*, *C. canis*, *P. irritans*, *P. simulans* Baker, and *Nosopsyllus fasciatus* Bosc d'Antic (González-Acuña and Guglielmone 2005, Beaucournu et al. 2014).

Reports in Chile concerning *Rickettsia* spp. are mostly recent; the first records are represented by the description of *R. conorii* antibodies in dogs (López et al. 2007), and the molecular detection of *R. felis* in *C. felis* from cats (Labruna et al. 2007). In 2013, Abarca et al. (2013a) reported *R. felis* in *Rh. sanguineus* s.l. collected from dogs in central and northern Chile. Finally, *Candidatus* 'Rickettsia andeanae' was detected in the tick *A. tigrinum*, collected from rural dogs in Arica (northern Chile) and Angol (southern Chile; Abarca et al. 2013b).

Considering the lack of available information, the potential risk for human and animal health, and the increasing contact between domestic and wild carnivores, this study attempts to explore fleas, ticks, and their pathogens, collected from domestic and wild carnivores present in a natural–rural interface in central Chile. Our objectives were to: 1) identify flea and tick species present in domestic carnivores from human settlements located near a natural reserve, 2) identify flea and tick species present in wild carnivores from a natural reserve, 3) explore the presence of *Rickettsia* spp. in fleas and ticks from wild and domestic carnivores, and 4) evaluate dog features potentially associated with the presence of fleas.

Materials and Methods

Study Area

The study was conducted in Paine municipality, Metropolitan Region, central Chile. The study area was composed of 13 human settlements located in a rural landscape and the Altos de Cantillana natural reserve (NR; Fig. 1), which has one of the highest peaks of the coastal mountain range (2,300 m.a.s.l). According to a recent human census, the total number of households in this area is 2,624, with 10,005 inhabitants (INE 2005). Based on this, the estimated number of owned dogs would be 2,084 individuals (human:dog ratio of 4.8; Astorga et al. 2015). Three of these human settlements

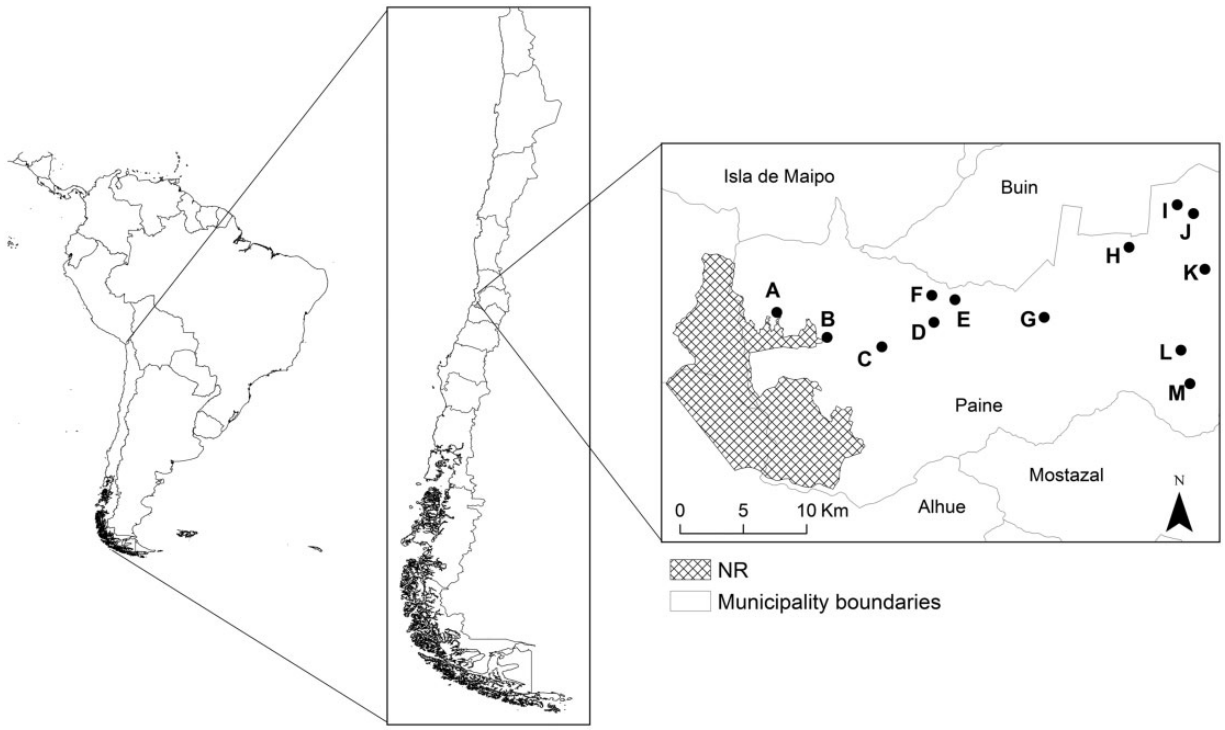

Fig. 1. Study area. Surveyed area included localities located <4 km from the Altos de Cantillana Natural Reserve (NR, gridded polygon): A—Rangue, B—Los Hornos/Aculeo, C—Pintúe; and localities >4 km from the NR: D—Abrantes, E—Vínculo, F—Peralillo, G—Champa, H—Rural Paine, I—Tránsito, J—Aparición, K—Rural Huelquén, L—Culitrín, and M—Chada.

are located less than 4 km from the NR (−33.903° S, −70.989° W; Fig. 1); nevertheless, all sampled localities had similar rural characteristics (i.e., human density, basic services availability). The NR is located 70 km southwest of Santiago, Chile's capital and the most populated city of the country. Principal activities in the study area include agriculture, agro-industry, mining, livestock production, tourism, and firewood and compost extraction (EULA-Chile 2004). Most of the natural areas are composed by mediterranean, sclerophyllous scrubland, and deciduous forest (EULA-Chile 2004). The coldest month is July (mean temperature 7.6 °C), and the warmest is January (mean temperature 20.3 °C; EULA-Chile 2004). Mean annual precipitation is ∼400 mm, but higher values can be detected at higher altitudes (data obtained from the General Direction of Civil Aeronautics and Meteorology of Chile, Rangue Meteorological Station). Hunting within the reserve and surrounding areas is prohibited (Fig. 1; localities A-G), except for invasive species considered harmful to the ecosystem by the national hunting law, such as rabbits (*Oryctolagus cuniculus* L.) and hares (*Lepus europaeus* Pallas; MINAGRI 1996).

Fleas and Ticks Sampling in Households

Fleas and ticks were collected from March to November, 2012, and from January to April, 2013, totaling 170 households (representing 6.5% of the total households). Houses were selected based on a random sampling, discarding households were no inhabitants were present at the survey time. In each household, we conducted a face-to-face questionnaire to one adult (>18 years old), to which we asked for an oral consent for the questionnaire and for pet sampling (dogs and cats). To maintain the respondents' privacy, all data was managed anonymously. The questionnaire was approved by the Facultad de Ecología y Recursos Naturales, Universidad Andrés Bello. For each sampled household dog, we recorded five dog variables: gender (male, female), function (pet and guarding, hunting and herding), external antiparasitic treatment in the past six months (yes, no), confinement management (permanent: the dog never goes out of the property without direct supervision, partial: the dog is allowed to go out of the property at any periodicity), and breed (mixed-breed, hound-type, other breeds). The variables function and confinement were included in order to explore the potential access of dogs to public and natural areas, because herding and hunting dogs, and dogs with partial confinement would have higher access to these areas. To classify different breed-types, we grouped under the category "hound-type" those breeds commonly used for hunting proposes (e.g., Beagle, English Pointer, Foxhound, and Greyhound), including a local mixed-breed called *zorrero*, used for rabbit and fox hunting, which is a cross-breed of hound-type breeds. Breeds were assigned using as reference the Kennel Club Breed List (www.thekennelclub.org.uk). Fleas and ticks extraction in all hosts was performed following a standardized protocol: one person conducted the animal physical immobilization, and another one collected fleas and ticks for ∼10 min, searching in the abdomen, tail base, neck, and ears. Parasites were removed with fine forceps and preserved with 70% ethanol in 2-ml vials.

Fleas and Ticks Sampling from the Natural Reserve

There are no houses within the NR; therefore, the collection of ticks and fleas from carnivores inside the NR was developed based on in situ live trapping (permissions are detailed in the acknowledgments section). Captures were performed using Tomahawk traps and rabbit carcasses as bait. Once captured, animals were sedated using a combination of ketamine and xylazine, following previous protocols (Arnemo and Søli 1992, Acosta et al. 2007, Acosta-Jamett et al. 2010). Parasite collection followed the same methodology used for household pets. If domestic carnivores were incidentally trapped, they were sampled with physical immobilization.

Morphological Identification

Parasite identification was performed at the Ecosystem Health Laboratory, Universidad Andrés Bello, Santiago, Chile. Using a magnifying glass and microscope, ectoparasites were identified and classified by gender, genus, and species according to taxonomic keys developed for fleas (Smit 1958, Acosta and Morrone 2003, Linardi and Costa Santos 2012), and for ticks (Barros-Battesti et al. 2006). The identification of *Ctenocephalides* spp. was done based on the shape of the head, length of the first spine of the genal comb, number of bristles on the lateral metanotal area, and the number of short stout bristles of the dorsal margin of the hind tibia. For *Pulex*, based on morphological features we could only identify the gender, using as principal diagnostic characters the rounded shape of the head, and the absence of forehead tubercle and pronotal comb (Smit 1958). Concerning ticks, morphological identification was developed using diagnostic characters such as the shape of basis capituli and the presence of festoons. We randomly selected flea specimens to support the morphological identification (at least one specimen of each species host), which were clarified using 10% KOH, dehydrated in a series of ethanol washes (70%, 80%, 96%, and 100%), and finally mounted using the mounting medium Euparal (Bioquip products, Rancho Dominguez, CA).

Molecular Analysis of *Pulex* spp

In order to confirm *Pulex* spp. identification, at least one flea specimen morphologically identified as *Pulex* spp. from each host was included in the molecular analysis. We assumed that all *Pulex* spp. from the same host belonged to the same species. Genomic DNA was extracted from the macerated flea's body using Qiagen DNAeasy Tissue Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Concentration and quality (A_{260}/A_{280} ratio) of eluted DNA were determined by absorption spectroscopy using a Synger Mx Microplate Reader (Bio-Tek Instruments, Winooski, VT). The COII region of mitochondrial DNA was amplified using polymerase chain reaction (PCR) for a 780 bp DNA fragment with A-tLEU and B-tLYS primers (Liu and Beckenbach 1992). Polymerase chain reactions were carried out using GoTaq Green Master Mix of Promega, in a final volume of 25 µl of the following solution: 9.5 µl of ultra-pure water (DNase and RNase free), 1 µl of each primer (10 µM), and 1 µl of template DNA (≈ 50 ng/µl). Amplification was conducted following the protocol described by Liu and Beckenbach (1992), with some modification (33 cycles with an annealing temperature of 47 °C for 1 min). Polymerase chain reaction products were visualized on a 1% agarose electrophoresis gel, and later purified and sequenced at Macrogen (Seoul, South Korea). Sequences were preliminary classified by BLAST analysis (GenBank http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with *P. simulans* and *P. irritans* (GenBank AF332503 and AF424041, respectively). For the molecular identification of *Pulex* spp., we developed a phylogenetic analysis with 25 flea species obtained from GenBank, with the aim of observing the phylogenetic association between our samples with previously described *Pulex* sequences, and with its closest species. *Pulex irritans* sequence (GenBank AF424041) formed a clade with 100% bootstrap with all the haplotypes obtained in the present study. We used the three closest species to this clade for the analysis (*Cediopsylla inaequalis inaequalis*

Baker, *Synosternus pallidus* Taschenberg, and *Xenopsylla cheopis*; GenBank EU335977, JF966769, and HQ881589, respectively). In addition, we included in the analysis *P. simulans* (GenBank AF332503) and *C. felis* (GenBank HQ696929) sequences. The latter was included because it is described as the closest group to *P. simulans* (Dittmar de la Cruz and Whiting 2003). Sequences polymorphism and similarity were obtained using DnaSP 5.1 (Librado and Rozas 2009). Maximum likelihood phylogenetic analyzes were carried out in MEGA 6, with the Tamura 3-parameter model, with 1,000 bootstrap replicates, using *P. simulans* as outgroup (Tamura et al. 2013).

Quantitative Analysis

For household dogs, we determined the species of ectoparasites collected, their number, sex ratio, mean intensity, and prevalence. For prevalence (Prev, %: proportion of infested hosts in the sample) and mean intensity (MI: arithmetic mean of the number of individuals of a particular parasite species per infected host in a sample), confidence intervals (95% CI) were calculated, using Sterne method and bootstrap (BCa, of 2,000 bootstrap replicates), respectively (Rózsa et al. 2000). We explored the potential association of prevalence and mean intensity between detected flea species. Only for flea species, we developed association analyses between prevalence and mean intensity with the previously described five dog variables (Table 1). To explore if this potential associations were associated with the distance to the NR, we performed the same analyses within two dog-groups; dogs from household located >4 km from the NR, and from households located <4 km from the NR. Bootstrap *t*-test (based on 1,000 bootstrap replications) and Fisher exact test (two-sided) were used for the analysis of mean intensity and prevalence, respectively. Differences were considered significant at $P < 0.05$. Statistical analyzes were performed using Quantitative Parasitology 3.0 software, implemented for the web (Rózsa et al. 2000).

Molecular Detection of *Rickettsia* spp

We created pools with the same ectoparasite species, separated by household and by host (dog, cat, and wild carnivores), using 1–10 specimens by pool. Engorged ticks were considered as one pool. DNA of each macerated pool was extracted as described above. Pools were then processed by PCR, targeting the conserved gene encoding rickettsial citrate synthase (*gltA*) using CS-78 and CS-323 primers, amplifying a 267-pb fragment (Labruna et al. 2004). Polymerase chain reactions were carried out using GoTaq Green Master Mix of Promega in a final volume of 25 µl of the following solution: 11.5 µl of ultra-pure water (DNase and RNase free), 0.5 µl of each primer (10 µM), and 5 µl of template DNA (≈ 50 ng/µl). Amplification was conducted following the protocol described by Labruna et al. (2004). Products were visualized in a 1% agarose electrophoresis gel. Positive samples were amplified in a second PCR, targeting the outer membrane protein *omp*A gene using Rr-190.70p and Rr-190.701 primers, amplifying a 631-pb fragment (Roux et al. 1996). Polymerase chain reactions were carried out using Taq Platinum, in a final volume of 25 µl of the following solution: 17.9 µl of ultra-pure water (DNase and RNase free), 0.5 µl of each primer (10 µM), and 2 µl of template DNA (≈ 50 ng/µl). Amplification was conducted following the protocol described by Roux et al. (1996), with a modification (35 cycles of extension for 1 min at 72 °C). In each PCR, a negative control (purified water) and a positive control (*R. felis* DNA) were included, and the PCR products were finally visualized in a 1% agarose electrophoresis gel. *Omp*A positive samples were purified and sequenced (Macrogen, Seoul, South Korea). Sequences were manually edited by Bioedit

Table 1. Characteristics of sampled household dogs

Dog characteristics		Location				Total (107)	
		<4 km NR (71)		>4 km NR (36)			
		No.	%	No.	%	No.	%
Gender	Male	39	36.4	24	22.4	63	58.9
	Female	26	24.3	10	9.3	36	33.6
	N/A	6	5.6	2	1.9	8	7.5
Antiparasitic treatment (past 6 months)	Yes	36	33.6	17	15.9	53	49.5
	No	20	18.7	13	12.1	33	30.8
	N/A	15	14.0	6	5.6	21	19.6
Dog breed	Mixed-breed	44	41.1	25	23.4	69	64.5
	Hound-type	9	8.4	1	0.9	10	9.3
	Other breed	16	15.0	7	6.5	23	21.5
	N/A	2	1.9	3	2.8	5	4.7
Dog function	Pet	47	43.9	18	16.8	65	60.7
	Guarding	13	12.1	13	12.1	26	24.3
	Hunting	7	6.5	4	3.7	11	10.3
	Herding	4	3.7	0	0.0	4	3.7
	N/A	0	0.0	1	0.9	1	0.9
Type of confinement	Permanent	42	39.3	22	20.6	64	59.8
	Partial	20	18.7	10	9.3	30	28.0
	No confinement	9	8.4	2	1.9	11	10.3
	N/A	0	0.0	2	1.9	2	1.9

Number of sampled household dogs, frequency and percentage of variables, and surveyed localities, grouped according to location: <4 km from the NR (localities: Rangue, Los Hornos/Aculeo, and Pintué), and >4 km from the NR (localities: Abrantes, Vínculo, Peralillo, Champa, Rural Paine, Tránsito, Aparición, rural Huelquén, Culitrín, and Chada). Number of sampled dogs is showed in parentheses.

No., number of sampled hosts; N/A, no data available for the corresponding variable.

v7.2.5 software, and aligned by ClustalW tool (Thompson et al. 1994, Hall 1998). Sequences were preliminary classified by BLAST, and aligned with *R. felis* (GenBank EU012496, HM636635, DQ408668, AY727036), *R. rickettsii* (GenBank AY319290), *R. massiliae* (GenBank HM014444), *Candidatus* 'R. amblyommii' (GenBank KM262194), and with *Candidatus* 'R. asemboensis' (GenBank JN315977 and JN315971) sequences using MEGA 6 (Tamura et al. 2013). Nucleotide polymorphism and sequences similarity were obtained using DnaSP 5.1 (Librado and Rozas 2009). The best model for maximum likelihood phylogenetic analyzes, considering the data set, was selected using MEGA 6. Tamura 3-parameter model with uniform distribution was selected, with 1,000 bootstrap replicates using *R. rickettsii* (GenBank AY319290), *Candidatus* 'R. amblyommii' (GenBank KM262194), *Candidatus* 'R. asemboensis' (GenBank JN315971 and JN315977) and *R. massiliae* (GenBank HM014444) as outgroups (Tamura et al. 2013).

Selected *omp*A-positive samples were used for *omp*B amplification. Primers were designed by Gustavo Valbuena's laboratory (University of Texas Medical Branch, TX) to target different *Rickettsia* species (unpublished data). Forward primer *omp*B 3392 (GGTACTGTAACTTATTTAGGTAATGCA) and reverse primer *omp*B 3749 (CATTATCTTGTACTTTAATGGTTGTAGT) were used to amplify a 357 bp fragment of the *omp*B sequence. Briefly, 1 µM of each primer was added to a 20 µl final volume of PCR Supermix High Fidelity (Invitrogen). Amplification conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 2 min, 50 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplification products were separated by 1% agarose gel stained with SYBR safe and visualized with a UV-Transilluminator. Amplified fragments were sequenced by Sanger method at Macrogen USA. Sequences were manually edited by Bioedit v7.2.5 software, and aligned by ClustalW tool (Thompson et al. 1994, Hall 1998). For *omp*B phylogenetic reconstruction, we used maximum likelihood methods based on Tamura 3-parameter model, with 1,000 bootstrap replicates. We incorporated sequences of *R. felis* (GenBank CQ329879 and CQ385243), *Candidatus* 'R. asemboensis' (GenBank JN315972), *R. massiliae* (GenBank KT588062), *Candidatus* 'R. senegalensis' (GenBank KF666470), and *Candidatus* 'R. amblyommii' (GenBank KX198773) as outgroups (Tamura et al. 2013).

Results

Fleas and Ticks Collected from Household Pets

From all surveyed houses, 85% had at least one dog (144 of 170) from which 60.4% (87 of 144) were sampled. In 22 houses we sampled more than one dog (from two to four dogs), totaling 107 sampled household dogs, representing 5.1% of the estimated owned dog population. Most of the dogs (66.3%) were located in the NR surroundings (Fig. 1; Table 1). Fleas were found in 94.3% of the surveyed household dogs (101 of 107), totalizing 555 fleas (Table 2), corresponding to three species: *C. felis* 74.3% (75 of 101), *C. canis* 58.4% (59 of 101), and *Pulex* sp. 11.8% (12 of 101). Most of the *Pulex* sp. fleas (75%; 9 of 12) were located near the NR (Table 2; Fig. 2), whilst 50% of household dogs infested by *Pulex* sp. were used for hunting or herding (6 of 12), and 75% (9 of 12) had partial confinement. In household dogs, single-flea species infestation were common (58.4%; 59 of 101), mainly associated with *C. felis* infestations (58%, 35 of 59), followed by *C. canis* single infestations (35.6%, 21 of 59), and *Pulex* sp. single infestations (5.1%, 3 of 59). Double-flea species infestations (38.6%; 39 of 101) and triple infestations (2.9%; 3 of 101) were less frequent.

Ticks were collected from 21.5% of the household dogs (23 of 107), totalizing 139 specimens, all morphologically identified as *Rh. sanguineus* s.l., and included 24 females, 21 males, 85 nymphs, and 9 larvae. Ticks were collected from 4 female dogs (3 mixed-breed, 1 Poodle), 18 males (16 mixed-breed, 2 with no breed data), and 1 household dog with no gender data recorded. Six household dogs, four males and two females, were infested only by *Rh. sanguineus* s.l. (i.e., with no fleas; Table 2).

Additionally, fleas and ticks from eight household cats were collected. All eight cats sampled were parasitized by *C. felis*, obtaining 19 flea specimens (15 females, 4 males). One cat was parasitized by one adult female *Rh. sanguineus* s.l.

Ectoparasites Collected in the NR

Two adult male chilla foxes (*Pseudalopex griseus* Gray, Pg1 and Pg2), two adult lesser grisons (*Galictis cuja* Molina; male Gc1, female Gc2), and three adult kodkods (*Leopardus guigna* Molina; male Lg1, female Lg2, female Lg3) were captured and sampled in the NR. Additionally, four free-ranging dogs were captured (not a target capture) inside the NR; all were hound-type, adult males, and not neutered. All captured carnivores were infested by fleas and ticks, except the kodkods. Chilla fox Pg1 was infested only by *Pulex* sp. (six females, one male), and chilla fox Pg2 was infested by *C. canis* (one female, two males) and by *Pulex* sp. (one female). Both lesser grisons were infested only by *Pulex* sp. (Gc1: one female and one male, Gc2: one female). From captured dogs, we collected *C. felis* (four females), *C. canis* (seven females, three males), and *Pulex* sp. (three females), the latter collected only in one dog. *Rhipicephalus sanguineus* s.l. ticks (one female and two males) were collected from one dog (Fig. 2).

Table 2. Fleas and ticks collected from household dogs

Location	Household dogs																	
	C. felis					*C. canis*				*P. irritans*				*Rh. sanguineus* s.l.				
	No.	No. Infes	Total	F	M	No. Infes	Total	F	M	No. Infes	Total	F	M	No. Infes	Total	F	M	L/N
<4 km from NR	71	49	195	145	50	43	190	112	78	9	19	15	4	7	51	9	4	38
>4 km from NR	36	26	93	76	17	16	55	38	17	3	3	1	2	16	88	15	17	56
Total	107	75	288	221	67	59	245	150	95	12	22	16	6	23	139	24	21	94

Number of sampled household dogs according to infection, by species, ectoparasite gender, and location: <4 km from the NR (localities: Rangue, Los Hornos/Aculeo, and Pintué), and >4 km from the NR (localities: Abrantes, Vínculo, Peralillo, Champa, Rural Paine, Tránsito, Aparición, rural Huelquén, Culitrín, and Chada).

No., number of sampled hosts; No. Infes, number of infested hosts; Total, total number of collected ectoparasites; F, female ectoparasites; M, male ectoparasites; L/N, *Rhipicephalus sanguineus* s.l. larva and nymphs.

Molecular Analysis of *Pulex* spp

Twenty-three *Pulex* spp. fleas (15 females, 8 males) were included in the molecular analysis, with at least one specimen from each infested host (3 wild hosts and 12 domestic hosts; Table 3). We excluded only the specimen collected from the lesser grison Gc2, and one household dog, because both were reserved for morphological identification purposes (not included in this study). Analyzed specimens were identified as *P. irritans* (99–100% query coverage, 99–100% of identity; Table 3). Nevertheless, nine specimens (two from household dogs and all fleas from wild species) had an identity of 93–96%. The analysis detected 100 polymorphic sites, with 39 parsimony informative sites, with a nucleotide diversity of Pi: 0.034 ± 0.006, and with a divergence between domestic and wild hosts clades (Fig. 3). We detected 10 haplotypes with a diversity of Hd: 0.775, where 40% of them were shared: two among domestic dogs (haplotypes 2 and 3, Table 3) and two among wild carnivores (haplotypes 8 and 9, Table 3). Haplotype 2 was the most abundant in *P. irritans* from dogs (Table 3), haplotypes 8 and 9 were the most common in wild carnivore fleas. Haplotype 8 was shared between lesser grison and chilla foxes fleas (Fig. 3; Table 3). Haplotypes 4 and 7, corresponding to a household dog (host 51c) and a dog captured inside the NR (host 2, Table 3), respectively, did not match with either of the two *P. irritans* clades (Fig. 3). Nucleotide sequence data from this study are available in GenBank under the accession numbers KJ815061 to KJ815086.

Quantitative Analysis

Ctenocephalides felis was the most prevalent species in the surveyed household dogs (Prev = 70.1%; CI = 60.8–78.1; 75 of 107 dogs), with 288 specimens (221 females, 67 males), followed by *C. canis* (Prev = 55.1%; CI = 45.0–64.0; 59 of 107 dogs), with 245 specimens (150 females, 95 males), and *P. irritans* (Prev = 11.2%; CI = 6.4–18.0; 12 of 107 dogs), with 22 specimens (16 females, 6 males). The flea sex ratio (female: male) was 3.3: 1 for *C. felis*, 2.7: 1 for *P. irritans*, and 1.6: 1 for *C. canis*. Flea mean intensity was 3.8 for *C. felis* (CI = 3.1–4.9), 4.1 for *C. canis* (CI = 3.2–5.5), and 1.8 for *P. irritans* (CI = 1.2–2.7). In household dogs, significant differences were found between the prevalence of *C. felis* and *C. canis* ($P = 0.033$), between *C. felis* and *P. irritans* ($P < 0.0001$), and between *C. canis* and *P. irritans* ($P < 0.0001$). Significant differences were also detected in mean intensity of fleas, between *C. felis* and *P. irritans* ($P = 0.003$), and between *C. canis* and *P. irritans* ($P = 0.004$).

No significant differences were detected between total fleas and dog variables (i.e., gender, function, confinement, external antiparasitic treatment, breed-type; Table 1). Nevertheless, significant differences were detected between individual flea species and three dog variables: breed, function, and confinement. *Pulex irritans* was significantly more prevalent in hound-type dogs (Prev = 40.0%; CI = 15.0–70.9) compared with mixed-breed dogs (Prev = 10.1%; CI = 4.9–19.4; $P = 0.028$) or with other breeds (Prev = 0; $P = 0.0051$). In relation to dog function, *P. irritans* was more prevalent in dogs used for hunting or herding (Prev = 40%; CI = 19.1–66.8), compared with pet/guard dogs (Prev = 6.6%; CI = 2.9–13.6; $p = 0.0043$). In addition, *P. irritans* was more prevalent in dogs with partial confinement (Prev = 22%; CI = 11.7–37.7), than in dogs with permanent confinement (Prev = 3.1%; CI = 0.6–10.7; $P = 0.0017$). Significant differences were detected in mean

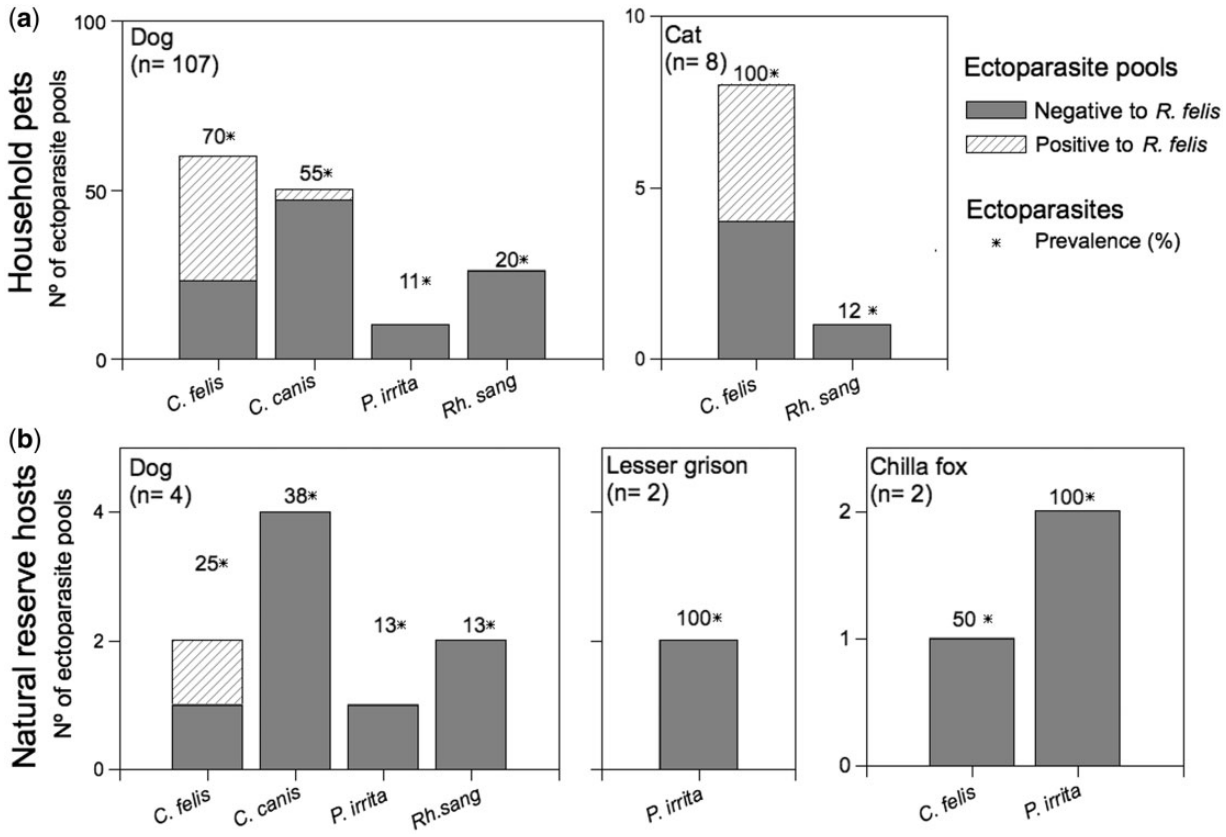

Fig. 2. Analyzed pools for molecular identification of *Rickettsia* spp. Number of analyzed ectoparasite pools by each host type (y axis) and ectoparasite species (x axis) are shown. Number of sampled hosts are shown in parentheses. Above each bar, prevalence (%) of each ectoparasite species is shown. (**a**) Household pets, i.e., household dogs and cats. (**b**) Hosts captured inside the natural reserve.

intensity of *C. canis* between mixed-breed dogs (MI = 4.1; CI = 3.1–6.3) and hound-type dogs (MI = 1.7; CI = 1.0–2.3; $P = 0.029$).

Analyses developed within dog-groups (>4 km from the NR and <4 km from the NR) for each flea species, detected significant differences in all the five dog variables. Specifically, in dogs located >4 km from the NR, significant differences were detected associated with dog breed type, where *C. felis* mean intensity was higher in mixed-breed dogs (MI = 3.15; CI= 2.3–4.1) than in other breeds (MI = 1; CI = 0; $P = 0.003$). Within the group located <4 km from the NR, *C. felis* presented a higher mean intensity in dogs with antiparasitic treatment (MI = 5.07; CI = 3.64–7.14) than in dogs without this treatment (MI = 2; CI = 1.3–2.7; $P = 0.012$). In *C. canis*, mean intensity was higher in mixed-breed dogs (MI = 4.83; CI = 3.4–7.4), than in hound-type dogs (MI = 1.67; CI = 1.0–2.3; $P = 0.029$). *Pulex irritans* was more prevalent in females (Prev = 26.9%; CI = 12.9–46.5) than in males (Prev = 2.6%; CI = 0.1–13.6; $P = 0.0054$). Similar to the global analyses, significant differences in *P. irritans* prevalence was detected in dogs associated with their confinement and their function. Prevalence of *P. irritans* was higher in dogs with partial confinement (Prev = 24.1%; CI = 11.5–4.3), than in dogs with permanent confinement (Prev = 4.8%; CI = 0.9–16.3; $P = 0.026$). *Pulex irritans* was more prevalent in dogs used for hunting or herding (Prev = 36.4%; CI = 13.5–66.7) compared with pet/guard dogs (Prev = 8.3%; CI = 3.3–18.1; $P = 0.027$).

In sampled household dogs, we detected a *Rh. sanguineus* s. l. prevalence of 21.5% (CI = 14.3–30.3; 23 of 107 dogs), with a mean intensity of six ticks per dog (CI = 4.1–9.5).

Table 3. Details of *P. irritans* haplotypes

Host type	Host Id.	Locality	Haplotype	Frequency (%)	Identity (%)
Household dog	31	A	1	4.3	95
Household dog	37	A	2	39.1	100
Household dog	41	A	2	39.1	100
Household dog	42	A	2	39.1	100
Household dog	51a	A	2	39.1	100
Household dog	7	C	2	39.1	100
Household dog	124a	H	2	39.1	100
Household dog	124b	H	2	39.1	100
Household dog	32	A	2	39.1	100
NR dog	2	NR	2	39.1	100
Household dog	37	A	3	8.7	99
Household dog	32	A	3	8.7	99
Household dog	51c	A	4	4.3	96
Household dog	52	A	5	4.3	99
Household dog	103	F	6	4.3	99
NR dog	2	NR	7	4.3	99
Lesser grison	Gc1	NR	8	13	94
Lesser grison	Gc1	NR	8	13	94
Chilla fox	Pg2	NR	8	13	94
Chilla fox	Pg1	NR	9	13	94
Chilla fox	Pg1	NR	9	13	94
Chilla fox	Pg1	NR	9	13	94
Chilla fox	Pg1	NR	10	4.3	93

Frequency of *P. irritans* haplotypes for COII, including host type (11 household dogs, one NR dog, and three wild carnivores), site of collection (locality), and frequency of each haplotype. Percentage of identity with *P. irritans* (GenBank AF424041) obtained in BLAST analysis is shown.

Host Id., host identification number; Locality, origin of samples, see Fig. 1.; Identity (%) is based on BLAST analysis.

Molecular Detection of *Rickettsia* spp

A total of 168 pools were created and analyzed (Fig. 2), including 155 pools from household domestic hosts (household dogs and cats), nine pools from dogs captured inside the NR, and four pools from wild carnivores. From all the pools, 72% (121 of 168) amplified for the *glt*A gene, and a fragment of 590 bp of the *omp*A gene was obtained in 57.1% (96 of 168) of the pools. As for some samples we obtained a shorter fragment (230 bp), sequences were cut to this length for *omp*A analyses. From these sequences, BLAST analysis showed a 46.8% match with *R. felis* from GenBank (45 of 96), with 100% of similarity with *R. felis*, an e-value of 0.00, and a 100% of query coverage (haplotypes 1, 2, 3, and 5). Haplotype 4 had 96% identity, an e-value of $2e^{-100}$, and 100% query coverage (Fig. 4a). Positive samples corresponded to *C. felis* and *C. canis* pools. None of the *Rh. sanguineus* s.l. pools were positive to the

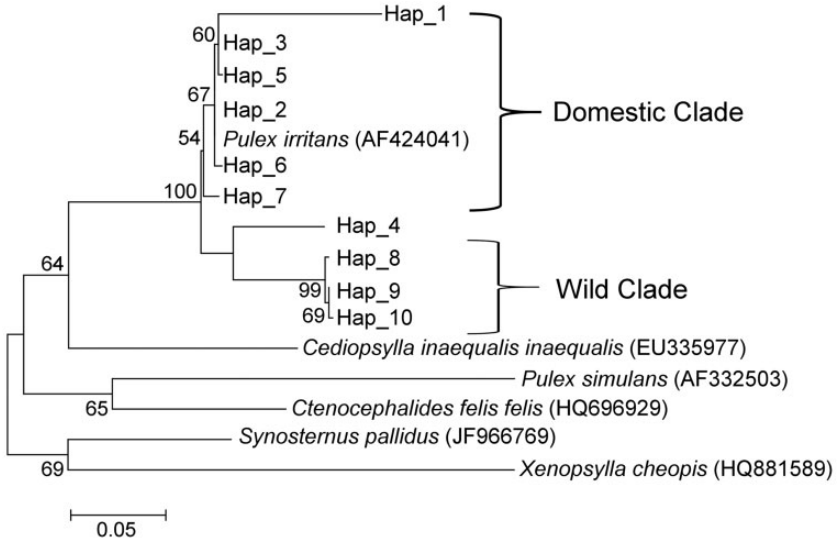

Fig. 3. *Pulex irritans* phylogenetic tree inferred by using the maximum likelihood method based on the Tamura-Nei model with gamma distribution conducted in MEGA6. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Hap—haplotypes from this study. For more details, see Table 3.

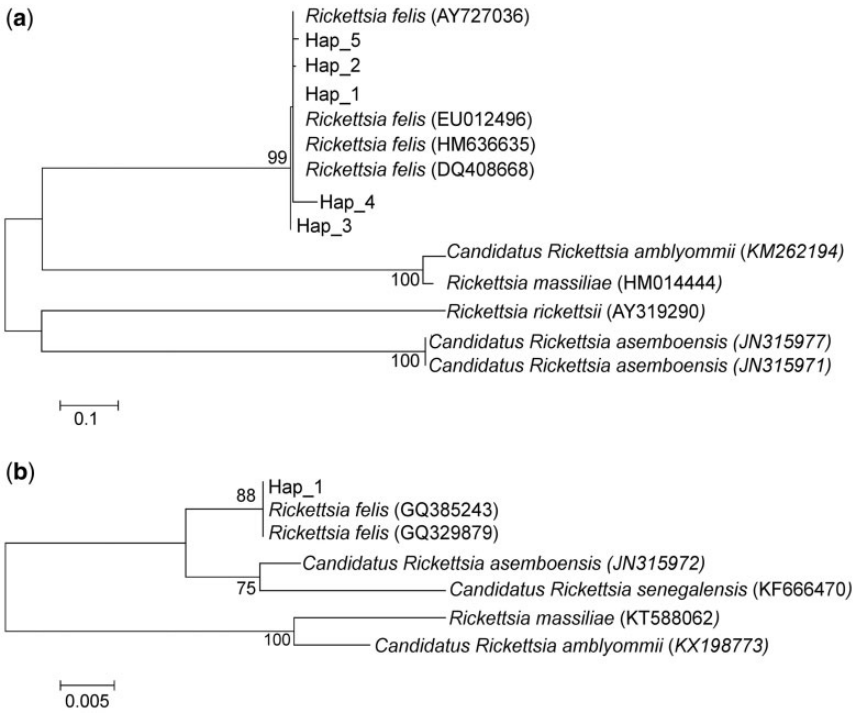

Fig. 4. *Rickettsia felis* phylogenetic tree, by maximum-likelihood, based on Tamura 3-parameter model conducted in MEGA6. (**a**) Molecular phylogenetic analysis for *omp*A gene. For tree construction, *R. rickettsii*, *Candidatus* 'R. amblyommi', *Candidatus* 'R. asemboensis', and *R. massiliae* were used as outgroups. (**b**) Molecular phylogenetic analysis for *omp*B gene. For tree construction, *Candidatus* 'R. senegalensis', *Candidatus* 'R. amblyommii', *Candidatus* 'R. asemboensis', and *R. massiliae* were used as outgroups. The percentage of trees in which the associated taxa clustered together is shown next to the branches. GenBank accession numbers are shown in parentheses. Hap—haplotypes from this study.

Rickettsia spp. detection. Most *Rickettsia* pools were grouped in the haplotype 1 (88.8%; 40 of 45). The sequences of the haplotypes reported here are available in GenBank, under accession numbers KU727817 to KU727821.

In order to confirm *R. felis* identity, from the 45 *omp*A-positive samples, we selected 32 for *omp*B amplification. From these, 28 amplified, and seven samples were sequenced for a 290 pb fragment. Sequences BLAST showed a 100% of similarity with a fragment coverage of 100% with *R. felis*, with an e-value of $2e^{-151}$ (Fig. 4b). These seven sequences were obtained from *C. felis* pools collected from household dogs. Sequence data from this haplotype is available in GenBank under the accession number KX532186.

Discussion

This study contributes to improve the scarce data available concerning ectoparasites from wild and domestic carnivores in Chile; in addition, it explores the potential dynamics involved in a domestic–wildlife interface, with further potential impacts for wildlife conservation and animal and human health. From all the four ectoparasite species collected, *C. canis* and *P. irritans* were present in both wild and domestic hosts, depicting the polyxenous natural behavior of fleas (Krasnov 2008). All ectoparasite species described here have been already reported in Chile (Muñoz and Casanueva 2001, Alcaíno et al. 2002, González-Acuña and Guglielmone 2005, González-Acuña et al. 2008, Beaucournu et al. 2014). Nevertheless, this is the first report of *P. irritans* in the lesser grison.

Pulex irritans was the less frequent flea species detected in dogs (11.8% in household dogs; 25% in dogs from the NR). In Chile, *P. irritans* has been previously reported with higher frequencies in dogs from Concepción city, in southern Chile (32.2%; Alcaíno et al. 2002), and in central insular territories (34.5%; González-Acuña et al. 2008). Also, it has been recorded in Santiago City (the capital of Chile), but with lower prevalence, suggesting an association between its abundance and rurality (Alcaíno et al. 2002). Most dogs infested with *P. irritans* in our study were managed unconfined, and were used for hunting or herding activities, thus, have access to broader wild areas. This is similar to previous reports of Gracia et al. (2008), who found that *P. irritans* infestation tended to increase in unconfined dogs. Unconfinement could favor dog encounters with wildlife, allowing the interspecies translocation of fleas at longer distances. In addition, the presence of ectoparasites, such as *P. irritans*, *C. felis*, and *C. canis* in wild carnivores, can be referred to anthropogenic influence, i.e., humans (and their dogs) intrusion to natural areas. For example, *C. canis* have been reported in the Iberian lynx (*Lynx pardinus* Temminck) and other sympatric carnivores associated with human settlements in Spain (Millán et al. 2007); the same pattern was reported for *C. felis* in opossums (*Didelphis marsupialis* L.) from Panama (Bermúdez et al. 2012). Moreover, given that *Ctenocephalides* is an Old World native genus, its presence in wild areas in the Americas can be attributed entirely to human disturbance (Lewis 1972, Dobler and Pfeffer 2011). In addition, many studies including ours, describe *C. felis* as the most common flea in dogs (Table 2; Alcaíno et al. 2002, González-Acuña et al. 2008, Escobar et al. 2011, Troyo et al. 2012). On the other hand, *P. irritans* originated in the Americas, and the whole *Pulex* genus have been suggested to originate in Central and South America (Hopla 1980). *Pulex irritans* is commonly associated to larger carnivores and humans (Smit 1958); due to this association with humans, this species is ubiquitous, present in all continents except the Antarctic (Lewis 1972). In addition, *P. irritans* adaptability may

have facilitate its actual globalized distribution, while its capable of infecting and prosper in different host (Smit 1958) with reported presence in wild and domestic hosts, as detected in the present study. Nevertheless, lower identity values (93–96%) of haplotypes from wild species (haplotypes 8, 9 and 10), and two haplotypes from dogs (haplotypes 1 and 4, the last one collected from a dog inside the NR), could be interpreted as the presence of a different species of *Pulex*, but this fact deserve further research. Nevertheless, the absence of sequences of other *Pulex* species in GenBank, and the strong bootstrap of the clade obtained through the phylogenetic analysis, allow us to assume that these specimens corresponded to *P. irritans*.

We suggest that wild carnivores would be more exposed to *Ctenocephalides* species in areas associated with humans and their domestic animals, and that dogs, particularly those free-ranging, hunting-type breed, and herding and hunting dogs, may act as parasite-transmission "bridges" (Salb et al. 2008). These dogs, while ranging unsupervised, may transport ectoparasites between human settlements and wild-natural areas, thus, between domestic and wild hosts (Dobler and Pfeffer 2011, Dantas-Torres et al. 2012). In our study site, ~40% of the owners managed their dogs with some level of unconfinement (Table 1). Interestingly, partial confinement was associated with higher *P. irritans* prevalence within the group located <4 km of the NR, and in the overall analysis. Herding and hunting dogs (function) and hound-type dogs (breed) were also associated to greater *P. irritans* prevalence. Nevertheless, these variables could be correlated creating a confounding effect, because dogs used for herding or hunting activities may tend to be hound-type breeds, and they all may be more susceptible to be managed unconfined.

The eco-epidemiological role of dogs is strongly influenced by local fine-scale and human features, such as individual dog exploratory behavior, health and care managements given by owners, landscape characteristics, among others (Sepúlveda et al. 2015). However, the influence of confinement in dogs' infestation, and the extended nonconfinement management, represents a context for plausible interspecific transmission of parasites. Indeed, molecular analysis of *P. irritans* COII gene contributed with interesting patterns to understand ectoparasites' dynamics in this interface. Here, *P. irritans* defined two main clades of fleas: the "wild" and the "domestic" clade (Fig. 3; Table 3); however, in the phylogenetic analysis, two haplotypes (7 and 4) did not match with neither of these clades. In particular, they corresponded to fleas collected from a dog inside the NR (Fig. 3; Table 3), and from a hound-type household dog (Table 3), both with greater access to the NR. These outcomes suggest interactions between domestic and wild animals, either by direct (e.g., predation or attacks) or indirect contact (e.g., use of the same resting areas). However, fleas can release offspring into the environment, which can interact, develop mixed progenies, and then infect diverse hosts; thus, we would need complex additional analyses to determine the time or type of interactions between hosts (Krasnov 2008). Even considering this limitation, the results of our exploratory study suggest a pattern, which could be explored in further studies, for example, through the amplification of hypervariable regions (Dittmar de la Cruz and Whiting 2003, Whiteman and Parker 2005).

The presence and abundance of ectoparasites in dogs and surrounding wildlife is associated not only with host diversity in the community; in fact, there are multiple factors influencing ectoparasite infection dynamics at local scale including host features, climate and landscape conditions, ownership management, among others (Szabó et al. 2003, Beck et al. 2006, Dantas-Torres and Otranto 2014). The great variability of these elements may explain in part the differences of our study with previous reports in Chile. Here, we only found *Rh. sanguineus* s.l; however, other tick species such as *A. tigrinum* have been described previously in dogs and in chilla foxes in central Chile (González-Acuña and Guglielmone 2005). The prevalence of *Rh. sanguineus* s.l. detected in our study was lower (21.5%) compared with other studies in Chile, reaching up to 75% in similar latitude (Abarca et al. 2013a). This situation could be mainly because *Rh. sanguineus* s.l. tends to be more abundant in urban areas (Costa et al. 2013). Half of our respondents reported ectoparasite treatment to their dogs in the past six months; however, according to the statistical analysis, this management did not have any major influence in ectoparasites prevalence. Moreover, mean intensity of *C. felis* in dogs located <4 km from the NR was higher in dogs that had been under antiparasitic treatment, indicating inaccuracy in owner responses or inadequate treatments. Another relevant element associated with parasite detection is sample size. Here, we did not find *C. felis* in lesser grison, although it has been reported before in this host (Beaucournu et al. 2014). This absence may be because we only captured and sampled two lesser grison.

Our study, through the amplification and analyses of *omp*A and *omp*B genes, confirmed the presence of *R. felis* in fleas from dogs and cats living in rural localities of central Chile, contributing with the first national report of this pathogen in the dog flea *C. canis*. It must be stated that *omp*B amplification was conducted in a random subsample, thus assuming that all other *omp*A-positive samples corresponded to *R. felis*. This information complements previous reports of *R. felis* in *C. felis* and in *Rh. sanguineus* s. l. in central Chile (Labruna et al. 2007, Abarca et al. 2013a). The percentage of positive pools (26.7%) was consistent, although slightly lower compared with previous reports from Latin America ranging between 35 to 40% (Bermúdez et al. 2011, Ramírez-Hernández et al. 2013, Horta et al. 2014). Labruna et al. (2007), based on molecular detection, reported 70% of *C. felis* positive to *R. felis* in cats of Santiago, Chile; however, all fleas collected in their study were from 22 cats living in a single household (Labruna et al. 2007).

Despite spatial closeness between domestic and wild carnivores in the study area, no rickettsial DNA was found in fleas from wild species. Nevertheless, this result could be related to our small sample size of wild animals. Previous studies described the presence of *R. felis* in *C. felis* from a bobcat (*Lynx rufus* Schreber; Azad et al. 1997), in rodents, and in opossums (*Didelphis* spp.; Panti-May et al. 2015). Therefore, considering that both rickettsial-positive flea species (*C. canis* and *C. felis*) are shared between wild and domestic carnivores, transmission between dogs and wild carnivores may be expected. A definitive vertebrate host or vector of *R. felis* has not been determined; however, *C. felis* have been suggested as the most competent vector for the pathogen (Hii et al. 2011). Until now, clinical signs had been confirmed only in humans (Brown and Macaluso 2016). Nevertheless, evidence of clinical disease in dogs was detected in Spain, where a dog and its owners developed clinical manifestation consistent with *R. felis* infection, corroborated through PCR analysis (Oteo et al. 2006). More recently, Hii et al. (2011) reported the presence of *R. felis* through PCR in a group of apparent healthy domestic dogs from a pound; based on these findings, the authors highlighted the possible role of dogs as reservoirs and sentinels for human infection. Previously, Richter et al. (2002) also detected *R. felis* infection in an asymptomatic dog owned by a person diagnosed with rickettsial infection. Thus, *R. felis* pathogenicity in dogs needs to be explored, as previous reports have presented contrasting outcomes: from a dog with clinical signs (Oteo et al. 2006), to apparently healthy individuals (Richter et al. 2002, Hii et al. 2011). Moreover, further studies should explore potential impacts

of rickettsial infections in animals, including wild and domestic species, and their potential role as reservoirs in an urban–wild landscape. It is possible that further sampling in domestic and wild fauna increase the number of species of ticks and fleas in this area, in addition to provide more data about pathogens or other microorganisms that infect these ectoparasites.

Our study contributes with novel data concerning ectoparasites in wild and domestic animals, and concerning *Rickettsia* species presence in this region, confirming that dogs and wild carnivores could share ectoparasites, and may potentially share pathogens, in a possible spillover–spillback dynamic. We suggest that the presence of dogs inside the NR and their frequent displacement between the anthropogenic matrix and natural areas, may facilitate the translocation of micro- and macroparasites. *Rickettsia felis* detection in fleas of domestic pets (dogs and cats) requires special attention from a public health approach. *Rickettsia felis* is a zoonotic agent, and represent a potential risk to human health, particularly considering that *C. felis* is widely distributed in domestic pets, and that can parasitize humans and wild species. Moreover, vertical transmission capacity of *R. felis* allows it to be maintained in several generations of *C. felis*, which would lead to a high incidence of fleas infected with *R. felis* close to human dwellings. Given the public health implications of the surveyed ectoparasites and their considerable prevalence, we consider dogs as a key eco-epidemiological link in the interface between humans, domestic animals, and wildlife.

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