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Survey of infectious agents in the endangered Darwin's fox (*Lycalopex fulvipes*): high prevalence and diversity of hemotrophic mycoplasmas

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Abstract

Very little is known about the diseases affecting the Darwin's fox *(Lycalopex fulvipes)*, which is considered to be one of the most endangered carnivores worldwide. Blood samples of 30 foxes captured on Chiloé Island (Chile) were tested with a battery of PCR assays targeting the following pathogens: *Ehrlichia*/*Anaplasma* sp., *Rickettsia* sp., *Bartonella* sp., *Coxiella burnetti*, *Borrelia* sp., *Mycoplasma* sp., *Babesia* sp., *Hepatozoon canis*, *Hepatozoon felis*, *Leishmania donovani* complex, and Filariae. Analysis of the 16S rRNA gene revealed the presence of *Mycoplasma* spp. in 17 samples (56.7%, 95% Confidence Intervals= 38.2-73.7). Of these, 15 infections were caused by a *Mycoplasma* belonging to the *M. haemofelis*/*haemocanis* (Mhf/Mhc) group, whereas two were caused by a Mycoplasma showing between 89% and 94% identity with different *Candidatus* Mycoplasma turicensis (CMt) from felids and rodents hemoplasmas. Phylogenetic analysis grouped this sequence into the same clade as CMt and rodent hemoplasmas but without assigment to any subcluster, indicating that this may represent a new species. The analysis of the sequence of the RNA subunit of the RNase P gene of 10 of the foxes positive for Mhf/Mhc showed that eight were infected with *M. haemocanis* (Mhc), one with a *Mycoplasma* showing 94% identity with Mhc, and one by *M. haemofelis* (Mhf). One of the foxes positive for Mhc was infected with a *Ricketssia* closely related to *R. felis*. All foxes were negative for the other studied pathogens. Our results are of interest because of the unexpectedly high prevalence of *Mycoplasma* spp. detected, the variability of species identified, the presence of a potentially new species of hemoplasma, and the first time a hemoplasma considered to be a feline pathogen (Mhf) has been identified in a canid. Though external symptoms were not observed in any of the infected foxes, further

clinical and epidemiological studies are necessary to determine the importance of hemoplasma infection in this unique species.

Key words: Canidae, hemoplasma, *Hemobartonella*, *Pseudalopex fulvipes*, South America

1. Introduction

The Darwin's fox (*Lycalopex fulvipes*; syn. *Pseudalopex fulvipes*) is a rare member of the family Canidae endemic to Chile. It is classified as Critically Endangered by the IUCN, with a total population size of less than 250 adults (Jiménez et al., 2008). It has a distinct distribution with two subpopulations: at least 90% of the population occurs on Chiloé Island; on mainland Chile, a small subpopulation has been observed since 1975 in Nahuelbuta National Park, located about 600 km north of the island population. To date, no other subpopulations have been found in the remaining forest between the two locations.

Epidemics represent serious conservation threats for populations of endangered species because they can cause mortality, reduce host fitness and/or alter dispersal and movement patterns of infected animals (Scott, 1988). Since most individuals in an endangered population are seldom exposed to a pathogen because of the low rate of intra-specific interactions, there is little acquired immunity, such that when an epidemic occurs it tends to infect a large proportion of the population and mortality levels may be high (McCallum and Dobson, 1995). Therefore, monitoring the prevalence of disease should be a priority in conservation (Scott, 1988).

In spite of its critical conservation status, very little is known about the diseases affecting Darwin's fox. According to the IUCN, the greatest conservation threat to the Darwin's fox may be the presence of dogs in fox-inhabited areas, serving as potential vectors of disease (Jiménez et al., 2008). However, there is a profound lack of knowledge not only about the importance of diseases in fox mortality, but also about the pathogens infecting this species. To date, only three parasitological studies are available: one reporting the presence of antibodies against *Neospora caninum* in two

captive foxes (Patitucci et al., 2001), a report about the presence of dog lice in a fox (González-Acuña et al. 2007), and more recently, a copro-parasitological study of feces collected from the environment (Jiménez et al., 2012). The aim of the present study was to carry out a pilot survey of important canine pathogens infecting Darwin's fox in Chiloé Island, using stored blood samples.

2. Material and Methods

2.1. Sampling methods

Thirty stored blood samples collected between 2009 and 2012 for fox population genetic analyses in Chiloé Island (41°46'S 74°00'W) were included in this study. The sample consisted of 24 foxes older than one year (10 males and 14 females) and five foxes younger than one year (2 males, 3 females; three of these young foxes belonged to the same litter). Age and sex information was not available for one fox. Foxes were captured at ten different sites throughout Chiloé (Table 1, Figure 1) with box-traps. Traps were activated in the evening and checked the next morning at dawn. Individuals were anaesthetized with a combination of 10 mg/kg of ketamine (Imalgene, Merial, France) and 1 mg/kg of xylazine (Xilacina, Centrovet Ltda., Chile). Foxes were subjected to a basic external clinical evaluation by a veterinarian. About 200 µl of blood collected from the cephalic vein was added to 1000 µl of absolute ethanol. Ethanol preserves tissue and DNA by extracting water from the tissue. Once dried, DNA is quite stable even at room temperature. When DNA is dry, it forms an alpha structure, which is much more stable than the beta structure it forms when dissolved in a water-based solution (Wong et al. 2012). Foxes were released after full recovery at the capture site.

2.2. DNA isolation, qPCR amplification and sequencing

Twenty-five mg of peripheral whole blood was washed with 1ml of PBS to eliminate ethanol. DNA was isolated using a DNeasy® Blood & Tissue Kit (Qiagen) in a QIAcube according to manufacturer's instructions. Lysis was performed out of a QIAcube with 180ul of buffer ATL and 20ul of proteinase K (20mg/ml) at 56ºC for 2 hours. DNA was eluted in 200ul of AE buffer.

Real-time amplification of *Ehrlichia/Anaplasma* sp., *Rickettsia* sp., *Bartonella* sp., *Coxiella burnetii*, *Borrelia* sp., *Mycoplasma* sp., *Hepatozoon felis*, *H. canis*, *Babesia* sp., and Filariae were carried out in a final volume of 20µl using SYBR SELECT Master Mix (AB, LifeTechnologies), 4µl of DNA and a final primer concentration depending on the pathogen amplified (Table 2). The thermal cycling profile was 50 ºC 2 min and 95 ºC 10 min followed by 40 cycles at 95 ºC 15 s and 60 ºC 1 min. Real-time PCR specificity assessment was performed by adding a dissociation curve analysis at the end of the run. The target amplified for each pathogen and the primers used are shown in Table 2. Water was used as a negative PCR control and positive controls were obtained: (i) from commercial slides coated with cells infected with the pathogens (MegaScreen® FLUOEHRLICHIA c., MegaScreen® FLUOBABESIA canis, MegaScreen® FLUORICKETTSIA ri., MegaScreen® BARTONELLA h. (Megacor); (ii) from commercial DNA for *Borrelia burgdorferi* and *C. burnetii* (Genekam Biotechnology AG) and (iii) from clinical samples previously amplified and sequenced for *Mycoplasma* spp., *Hepatozoon felis*, *Hepatozoon canis* and Filariae (a cat infected with *Mycoplasma hemocanis*/*felis*; a cat infected with *H. felis,* a dog infected with *H. canis* and a dog infected with *D. immitis*). The eukaryotic 18S RNA Pre-Developed TaqMan Assay Reagents (AB, Live technologies) were used as an internal reference for genomic DNA amplification to

ensure (i) the proper PCR amplification of each sample and (ii) that negative results corresponded to true negative samples rather than to a problem with DNA loading, sample degradation or PCR inhibition. The real-time PCR products of *Mycoplasma* sp. and *Rickettsia* sp. positive samples were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (AB, Life Technologies) using the same primers. Quantitative *Leishmania* PCR was carried out according to the method described by Francino et al. (2006).

Sequences obtained were compared with the GenBank database (www.ncbi.nlm.nih.gov/BLAST) and the RDP database for 16S rRNA (http://rdp.cme.msu.edu/). The new *Mycoplasma* 16S rRNA and the RNase P gene nucleotide sequences were submitted to the EMBL database under accession numbers HF678195 and HF679526, respectively. The new *Rickettsia* ITS2 sequence was submitted to the EMBL database under accession number HG328363.

2.3. Genetic analysis of Mycoplasma *spp.*

Genetic distance P among the 15 *Mycoplasma* spp. was calculated with MEGA 4.0 (Tamura et al., 2007) using 334 bp of the 16S ribosomal RNA gene in 15 *Mycoplasma* spp.

2.4. Statistical analyses

Differences in prevalence depending on the fox sex and the capture area were compared using Fisher's exact test.

3. Results

All blood samples were negative by PCR targeting DNA of *Ehrlichia*/*Anaplasma* sp., *Bartonella* sp., *C. burnetti*, *Borrelia* sp., *Babesia* sp., *Hepatozoon* sp., *Leishmania* sp., and Filariae. Sequencing of the 16S rRNA gene revealed DNA of *Mycoplasma* spp. in seventeen foxes (56.7%, 95% Confidence Intervals= 38.2-73.7). Of these, fifteen cases were identified as *M. haemofelis*/*haemocanis* (Mhf/Mhc). The other two foxes were infected with the same hemoplasma species that only showed between 92.1% and 93.9% identity with CMt isolates from cats and between 89.1% and 93% with hemoplasmas of rodents (Table 3).

In order to further characterize the Mhf/Mhc positive samples, 10 of these were analyzed for the sequence of the RNA subunit of the RNase P gene. Eight showed 100% identity with *M. haemocanis* (Mhc), one showed 100% identity with *M. haemofelis* (Mhf), and another one showed 93.9% identity to the Mhc str. Illinois from a dog in the USA (CP003199).

Hemoplasma-infected foxes were found in eight of the 10 surveyed areas in the northern, central and southern areas of Chiloé, without differences in prevalence (in all cases Fishers'p>0.05), and without a clear geographical clustering of *Mycoplasma* spp. (Table 1, Figure 1). All the infected foxes were older than one year (prevalence in the adult age-class: 66.6%). No statistically significant differences were observed in prevalence depending on fox sex (Fishers'p>0.05). All the foxes were apparently healthy except for an old female with signs of emaciation, but this vixen was negative for hemoplasma infection. No clinical signs related to hemoplasmosis were recorded in any fox.

One fox positive for Mhc was also infected with a *Rickettsia* that showed 97% identity with different published sequences of *R. felis*.

4. Discussion

Foxes in this study showed little contact with the investigated infectious agents. This is a common feature of endangered carnivores (e.g. Millán et al., 2009; Almeida et al., 2013) and is explained by their solitary social system (Jiménez et al., 2008) that decreases the rate of intraspecific encounters. This naturally low rate of contact is probably accentuated by the currently small population size of the Darwin's fox. The fact that Chiloé is an island might also impair the transmission of infectious diseases from the continent.

However, our study revealed that infection by hemotrophic mycoplasmas is apparently a common feature of the Darwin's fox. Hemoplasmas are cell wall-less epicellular parasites of erythrocytes. Thus far, five hemoplasma species have been described in carnivores: Mhc is considered to be the causative agent of hemotrophic mycoplasmosis in dogs; *Candidatus* Mycoplasma haematoparvum (CMhp) was first isolated from a splenectomized dog with haemic neoplasia; Mhf, previously referred to as *Haemobartonella felis* large variant, is the causative agent of feline infectious anemia; *Candidatus* Mycoplasma haemominutum (CMhm), previously considered the small variant of *H. felis*, usually causes subclinical infections in cats; and *Candidatus* Mycoplasma turicensis (CMt) was described in a cat with hemolytic anemia (Skyes et al., 2005; Willi et al., 2005; Harvey., 2006).

Hemoplasma infections have been detected in a range of free-living felids (Willi et al., 2007; Munson et al., 2008; Hirata et al., 2012; Krengel et al., 2013) and more recently in one ursid, the black bear (*Ursus thibetanus japonicus*; Iso et al., 2013). In contrast, hemoplasma infection in a free-living wild canid has only been described in a

198 red fox (*Vulpes vulpes*) in Japan, which was found to be positive for a *Mycoplasma* sp.
199 belonging to the Mhf/Mhc group through the analysis of the 16S rRNA gene (Sasaki et
200 al., 2008). Among captive animals, two bush dogs (*Speothos venaticus*) were positive
201 for a *Mycoplasma* sp. closely related to CMhp, and two wolves (*Canis lupus*) were
202 positive for a *Mycoplasma* sp. closely related to CMhm, all of them from Brazilan zoos
203 (André et al., 2011).

204 The majority of the infections detected in the present study were caused by Mhc.
205 This hemoplasma infects dogs worldwide (Kenny et al., 2004). Dogs are latently
206 infected by Mhc until other factors, such as splenectomy or immunosuppression,
207 trigger overt disease (Kenny et al., 2004). All infected foxes showed good external
208 condition without signs of disease. The absence of clinical signs is also a typical feature
209 of hemoplasmosis in wild felids, and according to Willi et al. (2007) can be explained by
210 the possibility that the animals were sampled during a chronic carrier status and not
211 during acute infection. However, immune suppression caused by stress factors
212 (translocation or captivity), concurrent diseases, or corticoid treatment may induce
213 hemoplasma parasitemia and anemia in these wild carnivores (Guimaraes et al. 2007).

214 One individual was confirmed to be infected by Mhf. Recently, the sequencing of
215 the whole genome of Mhc allowed do Nascimento et al. (2012) to confirm that Mhf
216 and Mhc are different species infecting cats and dogs, respectively. We are not aware
217 of any report of infection of dogs with Mhf after the introduction of the analyses of the
218 RNA subunit of the RNase P gene, which allows the differentiation of the two species
219 of *Mycoplasma* (Tasker et al., 2003). Here, we present the first evidence that a canid
220 can be infected by Mhf. The present case is most likely the result of a spill-over from
221 domestic cats or some sympatric wild feline such as the kodkod (*Leopardus guigna*).

Two individuals were positive for a *Mycoplasma* sp. showing between 92% and 93.9% identity with previously reported sequences of CMt (Table 2). Other previously reported sequences of CMt presented a higher identity among them: between 95.1% and 99.7% (Table 2). This, along with the phylogenetic analysis, which clustered our isolate into a subcluster including CMt and hemoplasmas from rodents but did not clearly cluster our isolate with other CMt isolates, supports the hypothesis that this sequence may correspond to a new species of hemoplasma, though this hypothesis must be further validated (Drancourt and Raoult, 2005). The source of infection for the Darwin's fox is unknown. This may represent a *Mycoplasma* typical of this species or of South American canids, acquired after repeated consumption of rodents as part of their natural diet, which may explain why it was found in foxes sampled in two distant locations. Alternatively, this may be the result of a spillover from a domestic carnivore that later independently evolved on Chiloé Island. The latter scenario could also explain the sequence similar to Mhc detected in another fox.

No information is available about infection by hemoplasmas in domestic dogs sympatric to the Darwin's fox or elsewhere in Chile. The analysis of samples from these dogs will help to elucidate whether dogs are the source of the infection in foxes or if the infection is enzootically maintained in the fox population. Similarly, the analysis of domestic cat samples would be necessary to confirm if the Mhf and the new isolate found in Darwin's foxes are the result of a spill-over from cats. Also, molecular analyses of arthropods infesting dogs, cats, foxes, and other wild carnivores in Chiloé are necessary to determine patterns of hemoplasma transmission among carnivores on the island.

A *Rickettsia* closely related to *R. felis* was detected in one fox. *Rickettsia felis* has a worldwide distribution and is transmitted by the cat flea *Ctenocephalides felis*, whereas cats and opossums (*Didephis virginianus*) have been implicated as reservoirs in North America (Greene and Breitschwerdt, 2006). Apart from the mentioned species, in addition to humans, dogs and rats, no other mammal has been found to be infected by this pathogen (Reif and Macaluso, 2009). The fact that the *Rickettisa* sp. detected in this fox is most closely related to *R. felis* does not rule out the possibility of this being a different and perhaps unknown *Rickettisa* species, as was hypothesized in the case of a Pampas gray fox (*P. gymnocercus*) infected by a *Hepatozoon* sp. closely related to *H. felis* (Giannitti et al., 2012). Experimental infections of cats with *R. felis* produced subclinical infection (Wedincamp and Foil, 2000), but nothing is known about the potential pathogenicity for the Darwin's fox.

Taking into account the threat that diseases can pose for rare and isolated populations such as those of Darwin's fox, we believe that further studies are necessary to determine the importance of hemoplasmas and other infectious agents, not included in this survey, in this extremely endangered species.

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381 **Table 1**. Number of positives and *Mycoplasma* spp. detected in Darwin's foxes in each area of Chiloé Island, Chile. See Figure 1 for exact

382 location of each site and area.

Area	Sites included	Positive foxes /surveyed	*Mycoplasma* spp.*
North	1 to 5	9/18	3 Mhc, 1≈Mhc, 1 Mhf, 3 Mhf/Mhc, 1≈CMt
Center	6 and 7	1/4	1 Mhf/Mhc
South	8 to 10	7/8	5 Mhc, 1 Mhf/Mhc, 1≈CMt

383 *Mhc: *Mycoplasma haemocanis*; Mhf: *M. haemofelis*; Mhc/Mhf: *M. haemofelis*/*haemocanis*; ≈Mhc: *Mycoplasma* sp. showing 94% identity

384 with *M. haemocanis*; ≈CMt: *Mycoplasma* sp. showing between 92% and 94% identity with *Candidatus* Mycoplasma turicensis.

385 **Table 2.** Primers used for the molecular detection of pathogens in Darwins' foxes.

Pathogen	Region Amplified	Primer Forward (5'-3')	Primer Reverse (5'-3')	Reference	Final [primer] (µM)
Ehrlichia/Anaplasma sp.	16S rRNA	GCAAGCYTAACACATGCAAGTCG	CTACTAGGTAGATTCCTAYGCATTACTCACC	In-house design	0.5
Rickettsia sp.	ITS2	GCTCGATTGRTTTACTTTGCTGTGAG	CATGCTATAACCACCAAGCTAGCAATAC	In-house design	0.5/0.3
Bartonella sp.	ITS1	AGATGATGATCCCAAGCCTTCTG	CCTCCGACCTCACGCTTATCA	Modified from Maggi et al. (2005) and Gil et al. (2010)	0.3
Coxiella burnetti	16S rRNA	AAACCTTACCTACCCTTGACATCCTC	TCCCGAAGGCACCAAATCA	In-house design	0.3
Borrelia sp.	ITS2	GCGAGTTCGCGGGAGAGTA	C CATTCACCATAGACTCTTATTACTTTGACCA	In-house design	0.3
Mycoplasma sp.	16S rRNA	ATGTTGCTTAATTCGATAATACACGAAA	ACRGGATTACTAGTGATTCCAACTTCAA	In-house design	0.3/0.5
M. haemocanis/ *haemofelis*	RNAseP	CCTGCGATGGTCGTAATGTTG	GAGGRGTTTACCGCGTTTCAC	Modified from Tasker et al. (2003)	0.3
Babesia sp.	18S rRNA	GTGGCTTTTCCGATTCGTCG	TTCCTTTAAGTGATAAGGTTCACAAAACTT	In-house design	0.3
Hepatozoon felis	18S rRNA	CTTACCGTGGCAGTGACGGT	TGTTATTTCTTGTCACTACCTCTCTTATGC	In-house design	0.3
Hepatozoon canis	18S rRNA	CTTACCGTGGCAGTGACGGT	ATTGTTATTTCTTGTTACTACCTCTCTCAAAC	In-house design	0.3
Filariae	12S rRNA	TGACTGACTTTAGATTTTTCTTTGGAATAT	ATAAATYYATAAGCCAAATATATATCTGTTTTAAA	In-house design	0.3/0.5

386 **Table 3.** Pairwise sequence identity (as percentages) among 16S sequences of Candidatus *Mycoplasma turicensis* and hemoplasmas of rodents.

387 See Figure 1 for sequence accession numbers.

	ZD19 Darwin's Fox	CMt Cat1	CMt Leopard	CMt Cat2	CMt Lion	CMt Iriomote cat	CMt Wildcat	Mcc Mouse	Mhm Wild mouse
CMt Cat1	**93.9**	-							
CMt Leopard	**92.1**	96.7	-						
CMt Cat2	**93.6**	99.7	96.4	-					
CMt Lion	**93.3**	96.4	95.1	96	-				
CMt Iriomote cat	**92.1**	96.4	97.9	96	95.1	-			
CMt Wildcat	**92.4**	97	99.7	96.7	95.4	98.2	-		
Mcc Mouse	**93**	92.4	92.7	92.1	91.8	93.6	93	-	
Mhm Wild mouse	**89.1**	92.4	91.5	92.1	90.9	92.1	91.8	89.7	-
Msp Rat	**90**	91.5	89.7	91.2	90.6	89.7	90	90	89.1

388

389 **Figure legends.**

390 Figure 1. A: Known distribution areas of the Darwin's fox in Chile (shadow). B: Sampling
391 sites in Chiloé. 1: Chepu; 2: Ahuenco; 3: Lar; 4: Mechaico-San Antonio; 5: Palomar;
392 6: Rancho Grande; 7: Rahue; 8: Yaldad; 9: Chaiguata-Chaiguaco; 10: Emerenciana.
393 Protected areas are shaded.