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Hemoplasmas Are Endemic and Cause Asymptomatic Infection in the Endangered Darwin's Fox (*Lycalopex fulvipes*)

Sophia Di Cataldo,[a] Ezequiel Hidalgo-Hermoso,[b] Irene Sacristán,[a] Aitor Cevidanes,[a] Constanza Napolitano,[c,d] Claudia V. Hernández,[e] Fernando Esperón,[f] Darío Moreira-Arce,[g] Javier Cabello,[h] Ananda Müller,[i,j] Javier Millán[k,l,m]

[a]Programa de Doctorado en Medicina de la Conservación, Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago, Chile
[b]Conservation and Research Department, Parque Zoológico Buin Zoo, Buin, Chile
[c]Departamento de Ciencias Biológicas y Biodiversidad, Universidad de Los Lagos, Osorno, Chile
[d]Instituto de Ecología y Biodiversidad, Santiago, Chile
[e]Laboratorio de Diagnóstico Veterinario, HCV, Escuela de Medicina Veterinaria, Universidad Andrés Bello, Lo Pinto, Chile
[f]Centro de Investigación en Sanidad Animal, Valdeolmos, Spain
[g]Laboratorio de Estudios del Antropoceno, Facultad de Ciencias Forestales, Universidad de Concepción, Concepción, Chile
[h]Facultad de Medicina Veterinaria, Universidad San Sebastián, Puerto Montt, Chile
[i]Department of Biomedical Sciences, Ross University School of Veterinary Medicine, Basseterre, Saint Kitts and Nevis
[j]Instituto de Ciencias Clínicas Veterinarias, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile
[k]Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago, Chile
[l]Instituto Agroalimentario de Aragón-IA2, Universidad de Zaragoza-CITA, Zaragoza, Spain
[m]Fundación Agencia Aragonesa para la Investigación y el Desarrollo, Zaragoza, Spain

ABSTRACT *Mycoplasma haemocanis* is prevalent in the endangered Darwin's fox (*Lycalopex fulvipes*) in its main stronghold, Chiloé Island (Chile). The origin of the infection, its dynamics, its presence in other fox populations and the potential consequences for fox health remain unexplored. For 8 years, hemoplasmal DNA was screened and characterized in blood from 82 foxes in Chiloé and two other fox populations and in 250 free-ranging dogs from Chiloé. The prevalence of *M. haemocanis* in foxes was constant during the study years, and coinfection with "*Candidatus* Mycoplasma haematoparvum" was confirmed in 30% of the foxes. Both hemoplasma species were detected in the two mainland fox populations and in Chiloé dogs. *M. haemocanis* was significantly more prevalent and more genetically diverse in foxes than in dogs. Two of the seven *M. haemocanis* haplotypes identified were shared between these species. Network analyses did not show genetic structure by species (foxes versus dogs), geographic (island versus mainland populations), or temporal (years of study) factors. The probability of infection with *M. haemocanis* increased with fox age but was not associated with sex, season, or degree of anthropization of individual fox habitats. Some foxes recaptured years apart were infected with the same haplotype in both events, and no hematological alterations were associated with hemoplasma infection, suggesting tolerance to the infection. Altogether, our results indicate that *M. haemocanis* is enzootic in the Darwin's fox and that intraspecific transmission is predominant. Nevertheless, such a prevalent pathogen in a threatened species represents a concern that must be considered in conservation actions.

IMPORTANCE *Mycoplasma haemocanis* is enzootic in Darwin's foxes. There is a higher *M. haemocanis* genetic diversity and prevalence in foxes than in sympatric dogs, although haplotypes are shared between the two carnivore species. There is an apparent tolerance of Darwin's foxes to *Mycoplasma haemocanis*.

KEYWORDS Canidae, *Lycalopex*, *Mollicutes*, risk factors, South America

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Address correspondence to Sophia Di Cataldo, sophidica@hotmail.com.

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The Darwin's fox (*Lycalopex fulvipes*) is an endangered carnivore native to Chile. Its distribution range includes three metapopulations: one on Chiloé Island, with an estimated population of 412 mature individuals, and two isolated mainland populations (Nahuelbuta Park and Valdivian Coastal Range) with about 227 mature individuals in total (1). The presence of dogs in and around parks comprises one of the major threats to the conservation of this carnivore (2). The presence of free-ranging dogs in the entire Darwin's fox distribution range has been documented (3), and the populations in the continent are completely surrounded by human-dominated lands. Rural dogs in Chile are usually allowed to range freely (4, 5) and often lack any kind of prophylactic treatment or veterinary care (6). The naive behavior of the Darwin's fox can predispose it to interactions with dogs, ending in physical attacks and potential pathogen transmission (7).

Hemotropic mycoplasmas (also called hemoplasmas) are small bacteria that attach to the surface of red blood cells of mammals (8). These species are widely distributed and can infect humans (9), domestic animals (including dogs and cats) (10), and wildlife (including wild carnivores) (11, 12). Three species of mycoplasmas have been described in canids: *Mycoplasma haemocanis*, "*Candidatus* Mycoplasma haematoparvum," and "*Candidatus* Mycoplasma turicensis" (10, 13–15). Hemoplasma infection in dogs can cause acute and chronic hemolytic anemia, with results ranging from asymptomatic infection or slight lethargy to death; *M. haemocanis* is the most pathogenic species (16). Thus far, no clinical signs have been reported in hemoplasma-infected wild canids, and the pathological and epidemiological relevance for wildlife remains unknown. The transmission route of hemoplasmas is still under debate. Some species infecting dogs and cats are believed to be vector borne, but direct and/or vertical transmission has been demonstrated for others (17, 18). *M. haemocanis* has recently been proved to be transmitted vertically (19) in a dog, although it was classically considered to be transmitted by the brown dog tick, *Rhipicephalus sanguineus* (20). Nevertheless, since there are no canine ticks on Chiloé Island, other ways of transmission (i.e., direct transmission) must be operating.

During a molecular disease survey, Cabello et al. (21) reported an unexpected high prevalence of hemoplasma DNA among 30 free-living Darwin's foxes captured in Chiloé in the period from 2009 to 2012. Sequencing showed that 80% of the sequences obtained corresponded to *M. haemocanis*, another to *M. haemofelis*, and one to an as-yet-uncharacterized *Mycoplasma* sp., which was later found to be shared with domestic cats and the wild cat guigna (*Leopardus guigna*) in Chile (22). All the foxes studied by Cabello et al. (21) were apparently healthy and were negative for almost every other of the nine vector-borne pathogen groups for which these animals were tested. Pathogen transmission is hindered in small populations of solitary species such as the Darwin's fox because of their low rate of interspecific contact (23). Therefore, the high prevalence of infection reported by Cabello et al. (21) would support the hypothesis that hemoplasmas more likely persist in foxes based on an interspecific (i.e., dog-to-fox) rather than an intraspecific (fox-to-fox) transmission pathway. Considering that poorly managed free-ranging dogs are abundant in rural parts of Chile (24), including those areas sustaining Darwin's fox populations, a role of the domestic dog as a reservoir and source of infection for the fox is a likely scenario.

Different epidemiological questions regarding hemoplasma infection in the Darwin's fox remain. Whether Darwin's foxes in Chiloé and mainland populations are reservoir or spillover hosts for *M. haemocanis* is still unknown. It has been observed in wild felids that the domestic cat would act as a source host of hemoplasma (25, 26). However, proving a role as reservoir for a pathogen is complex. In order to disentangle the origin of hemoplasma infection in the Darwin's fox, we took different approaches. If these bacteria are being transmitted from dogs to foxes, we predicted that shared sequences would be found in both hosts, with greater hemoplasma genetic diversity in dogs. We also expected a higher prevalence of infection in foxes living closer to human settlements. We also aimed to provide some insights into risk factors and effects of

TABLE 1 Nucleotide sequence types detected in hemoplasmas in blood samples from Darwin's foxes and rural sympatric dogs and their closest GenBank sequences

ntST	Animal(s) in which detected (*n*)	Closest sequence	Species	Identity (%)	Host	Country
1	Dog from Chiloé (10), fox from Chiloé (17), fox from Nahuelbuta (2)	EF416566	*Mycoplasma haemocanis*	100	*Canis lupus familiaris*	Switzerland
2	Fox from Chiloé (1)	AF197337	*Mycoplasma haemocanis*	99.92	*Canis lupus familiaris*	USA
3	Fox from Chiloé (1)	GQ129116	*Mycoplasma haemocanis*	99.92	*Canis lupus familiaris*	Italy
4	Fox from Chiloé (1), dog from Chiloé (1)	GQ129116	*Mycoplasma haemocanis*	99.84	*Canis lupus familiaris*	Italy
5	Fox from Chiloé (2)	DQ825458	*Mycoplasma haemofelis*	99.92	*Lynx lynx*	Switzerland
6	Fox from Chiloé (1)	MK064162	*Mycoplasma haemocanis*	99.77	*Pulex irritans* on *Lycalopex culpaeus*	Argentina
7	Dog from Chiloé (1)	KY117659	*Mycoplasma haemocanis*	99.73	*Canis lupus familiaris*	Chile

hemoplasma infection in the Darwin's fox and to determine whether the pathogen is present in other Darwin's fox populations. The ultimate goal of our study was to determine if hemoplasma infection should be considered a disease threat for the last Darwin's foxes.

RESULTS

Molecular detection and characterization. The overall observed prevalence of *Mycoplasma* DNA for the whole study period was 56.6% (95% confidence interval [CI] = 46.0% to 67.3%; 47 foxes positive). All the newly obtained 16S rRNA sequences showed between 99.7 and 100% identity with published sequences of *M. haemocanis*/*M. haemofelis* (Table 1). Sequencing of a portion of the RNase P gene (accession no. CP003199) indicated 100% identity with *M. haemocanis* sequences in all cases. Species-specific primers confirmed coinfection with "*Ca*. Mycoplasma haematoparvum" in nine of the 30 *M. haemocanis*-positive foxes for the period from 2013 to 2017 (30.0%, 95% CI = 13.6% to 46.4%). In the foxes from the mainland, *M. haemocanis* DNA was found in two individuals from Nahuelbuta, one of which was coinfected with "*Ca*. Mycoplasma haematoparvum," and in one from Valdivia (Fig. 1). Six of the recaptured foxes were positive for *M. haemocanis* (two of which were coinfected with "*Ca*. Mycoplasma haematoparvum"), and one was negative at both capture events. Another fox, which was positive for *M. haemocanis* at both capture events, did not present coinfection with "*Ca*. Mycoplasma haematoparvum" at the first sampling but 2 years later was coinfected with this species (Table 2).

Sixty dogs were positive for *Mycoplasma* sp. DNA (24.0%; 95% CI = 18.7% to 29.3%) across Chiloé Island (Fig. 1). Of the fifty-three readable sequences, 53.2% corresponded to *M. haemocanis*/*M. haemofelis* and 46.8% to "*Ca*. Mycoplasma haematoparvum." *M. haemocanis* prevalence in foxes was significantly higher than in dogs ($\chi^2 = 15.908$, $P < 0.001$), whereas "*Ca*. Mycoplasma haematoparvum" prevalence was not significantly different between species ($\chi^2 = 2.641$, $P > 0.1$). We did not analyze dogs for potential *M. haemocanis*/"*Ca*. Mycoplasma haematoparvum" coinfections, but even if all dogs positive for "*Ca*. Mycoplasma haematoparvum" were coinfected with *M. haemocanis*, prevalence of *M. haemocanis* in foxes would still be significantly higher than in dogs ($\chi^2 = 28.9$, $P < 0.001$).

Only one of the foxes surveyed in the period from 2009 to 2017 period was parasitized by a tick (a larvae of *Ixodes sigelos*), a typical species of pudu (*Pudu puda*), an endemic deer from Chile, and none hosted fleas. No ticks were recovered from dogs, and 57 individuals (25.2%; 95% CI = 19.6% to 30.8%) hosted fleas.

Genetic analysis. Sequencing of nearly 900 bp the 16S rRNA gene from 25 foxes and 12 dogs revealed the presence of seven different nucleotide sequence types (ntST), with 99.9% identity among them and between 99.7% and 99.9% identity with other *M. haemocanis* sequences (Fig. 2). Two of the seven ntST were shared between foxes and dogs (Table 1). ntST-1 was the most frequent; it was detected in 29 individuals (19 foxes and 10 dogs). The other ntST shared between species (ntST-4) was detected in one dog and one fox. ntST-5 was detected in two foxes, whereas the other four ntST were found in only a single individual, either a fox ($n = 3$) or a dog ($n = 1$). Five of the recaptured

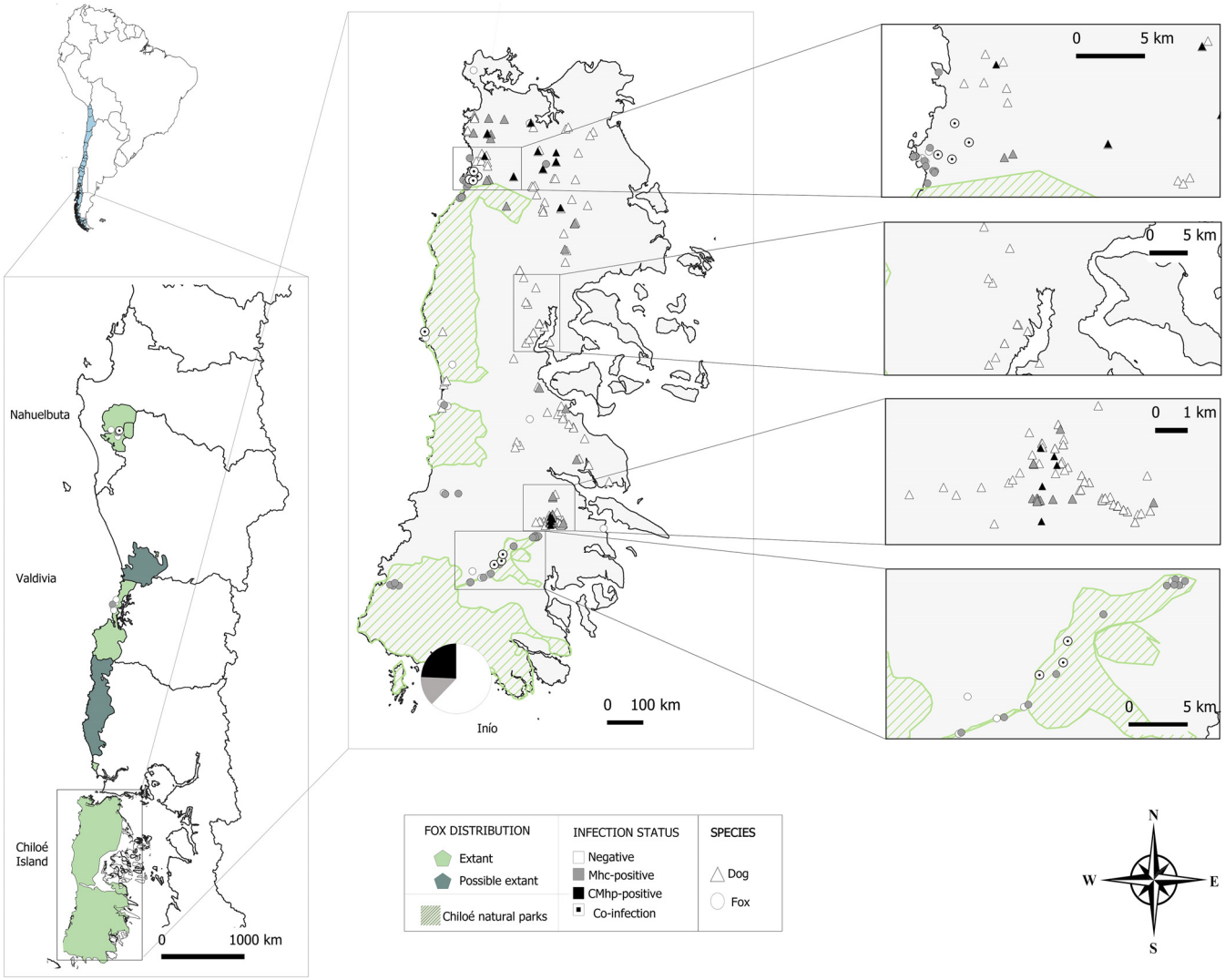

FIG 1 Map of the study areas, showing hemoplasma infection status in the surveyed Darwin's foxes and rural dogs. Dogs from Inio, a small fishing village in the south of Chiloé, are shown pooled in a pie chart.

foxes presented ntST-1 in both capture events, while the other two presented a different ntST (Table 2). All these sequences were placed in the *M. haemocanis*/*M. haemofelis* clade in the phylogenetic tree (Fig. 2).

The haplotype diversity (Hd) in foxes was 0.427 (standard deviation [SD] = 0.122), the nucleotide diversity (Pi) was 0.00116 (SD = 0.00040), and the average number of nucleotide differences (k) was 0.547. In dogs, Hd was 0.345 (SD = 0.172), Pi was 0.00108 (SD = 0.00057), and k was 0.727. The network analysis showed no genetic structure between dogs and foxes (F_{ST} = 0.02919, $P > 0.05$; nearest-neighbor statistic [Snn] = 0.51243, $P > 0.05$) (Fig. 3) and no geographic structure between the island and mainland populations (F_{ST} = 0.030079, $P > 0.5$; Snn = 0.83647, $P > 0.5$) (Fig. 3). The most prevalent ntST was present through the entire sampling period, and in concordance, no genetic structure was detected among years (F_{ST} = 0.03809, $P > 0.05$; Snn = 0.0790, $P > 0.05$) (Fig. 3).

Risk factor analysis. Models indicated that prevalence of infection of *M. haemocanis* was significantly higher in adult foxes than in juveniles (62.3% versus 20.0%; *Z* value = −2.247, $P < 0.05$) (Table 3). No other risk factor was related to the probability of *M. haemocanis* infection (Fig. 4). Prevalence of *M. haemocanis*-"*Ca*. Mycoplasma

TABLE 2 Hemotropic mycoplasma infection status in Darwin's foxes recaptured during the study period and *Mycoplasma haemocanis* nucleotide sequence type detected in each capture event

Fox	Yr	Site	Season	Age	*M. haemocanis* status	*M. haemocanis* ntST	"*Candidatus* M. haematoparvum" status
1	2014	Chiloé	Spring	Adult	+	4	−
	2016	Chiloé	Winter	Adult	+	1	+
2	2013	Nahuelbuta	Spring	Adult	−	−	−
	2016	Nahuelbuta	Summer	Adult	−	−	−
3	2014	Chiloé	Spring	Juvenile	+	1	−
	2016	Chiloé	Winter	Adult	+	1	−
4	2015	Chiloé	Fall	Adult	+	1	+
	2017	Chiloé	Fall	Adult	+	1	+
5	2016	Chiloé	Fall	Adult	+	3	−
	2017	Chiloé	Fall	Adult	+	1	−
6	2014	Chiloé	Winter	Adult	+	1	−
	2015	Chiloé	Fall	Adult	+	1	−
7	2013	Chiloé	Fall	Adult	+	1	+
	2014	Chiloé	Spring	Adult	+	1	+
8	2014	Chiloé	Winter	Adult	+	1	−
	2015	Chiloé	Fall	Adult	+	1	−

haematoparvum" coinfections did not differ depending on fox age ($\chi^2 = 0.099$, $P > 0.5$).

Prevalence was also higher in adult dogs than in juveniles ($\chi^2 = 63.2$, $P < 0.01$), and no sex-related differences were found ($\chi^2 = 1.7$, $P > 0.05$).

Hematological analysis. No significant differences were found in the hematological and biochemical variables evaluated depending on the sex or age of the animals, so the samples were pooled for further comparisons. None of the studied variables differed between the infection pattern groups that were compared.

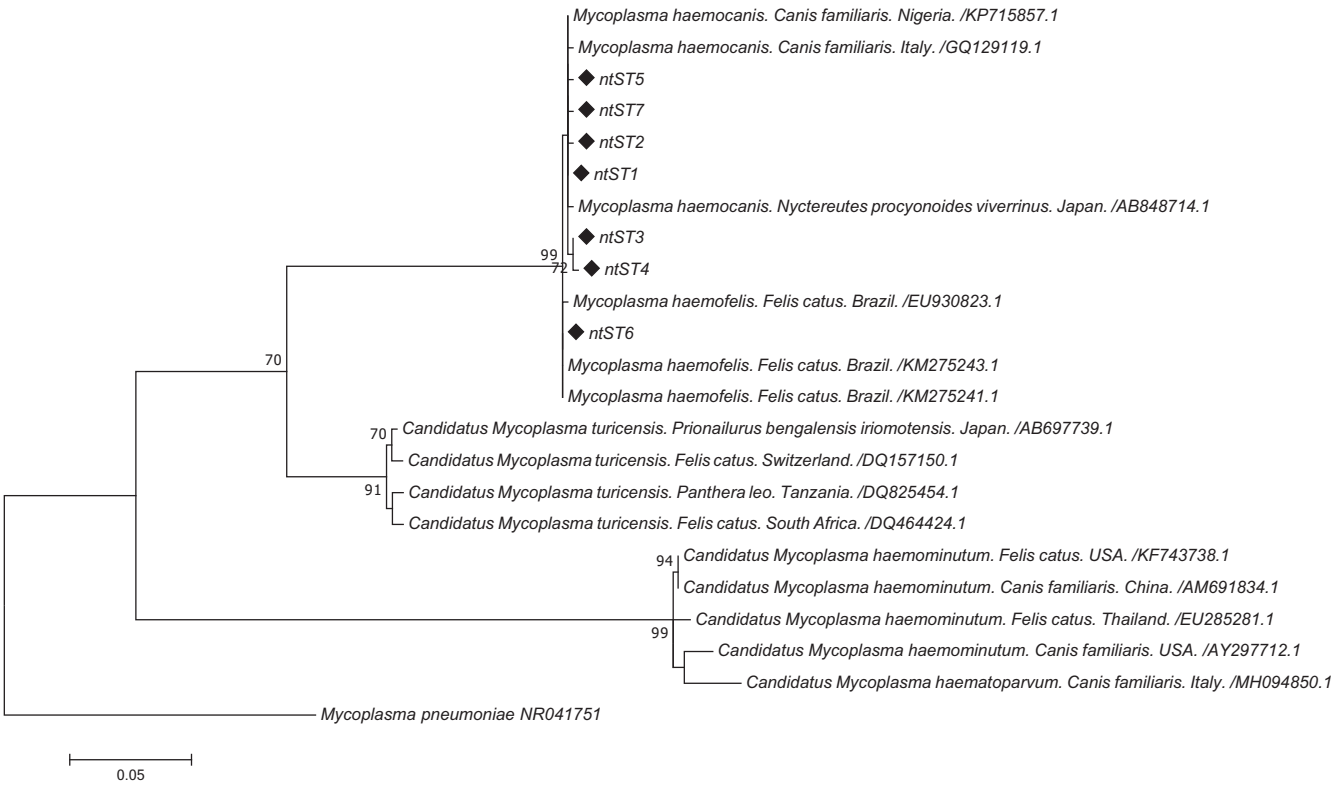

FIG 2 Maximum-likelihood tree of the 16S rRNA gene (893 bp) of *Mycoplasma haemocanis* for Darwin's foxes and domestic dogs. A *Mycoplasma pneumoniae* sequence was used as an outgroup. Bootstrap values of ≥70 are given at the nodes of the tree. Diamonds mark the nucleotide sequence types (ntST) from our study.

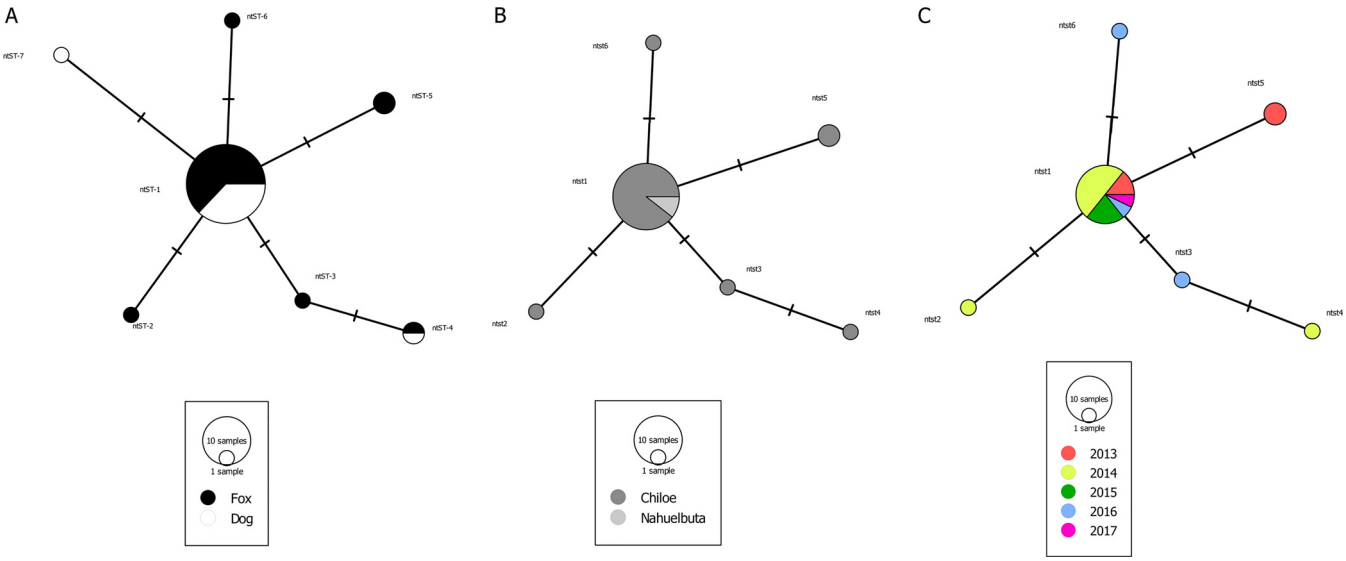

FIG 3 Median joining network of the 16S gene (893 bp) of *Mycoplasma haemocanis* in rural dogs and Darwin's foxes. Each circle in the network corresponds to a different nucleotide sequence type (ntST), the sizes of the circles correspond to ntST frequencies, the colors of the circles correspond to the two host species (Darwin's foxes and rural dogs) (A), two geographic sampling sites (Chiloé Island and Nahuelbuta) (B), and years of sampling (C). The networks in panels B and C were performed with fox samples only.

DISCUSSION

Our survey revealed that *M. haemocanis* was present in the Darwin's fox samples through the entire study period, indicating a constant exposure of the species to *M. haemocanis*. Moreover, the prevalence was higher in the endangered Darwin's fox than in dogs. Although the observed prevalence in dogs is in the range of that found in a previous study in Chile (15) and even higher than the rates of infection detected elsewhere (20, 27), the higher prevalence in foxes may be explained by a greater risk of exposure and/or different susceptibility to the infection.

The sharing of the most frequent ntST of the 16S rRNA gene of *M. haemocanis* in dogs and foxes suggests cross-infection between these species. Whether dogs are the main source of infection for Darwin's foxes cannot be proved with our data. However, considering that dogs markedly outnumber foxes and that dogs are moved large distances by their owners (24), it could be assumed that dogs were the origin of *M. haemocanis* infection for the fox. This is also supported by the fact that ntST-1 was found in foxes from both Chiloé Island and Nahuelbuta, which can be explained only by the movement of dogs between the continent and Chiloé. However, since this pathogen was introduced in the population, fox-to-fox transmission seems to be frequent now. This is supported by the higher prevalence in foxes than in dogs, the higher haplotype diversity detected in foxes, and the fact that the most prevalent ntST was found in foxes during all the studied years. This last observation would be better explained by intraspecific transmission than by periodic spillovers from dogs. Persistent intraspecific transmission of hemoplasmas following spillover from domestic animals has been also observed in felids (22, 26).

TABLE 3 Best model representing multivariate relationships between predictor variables and detection of *Mycoplasma haemocanis* in Darwin's foxes, using logistic regression analysis

Variable	Estimate ± SE	Z value[a]	AIC	Deviance	df	Hosmer-Lemeshow test *P* value
Intercept	0.32 ± 0.58	0.56	76.134	68.134	59	
Age juvenile	−2.07 ± 0.92	−2.24*				0.8
No. of houses	−0.17 ± 0.07	−2.30*				
Distance to nearest house	0.02 ± 0.02	1.41				

[a]*, $P = 0.01$.

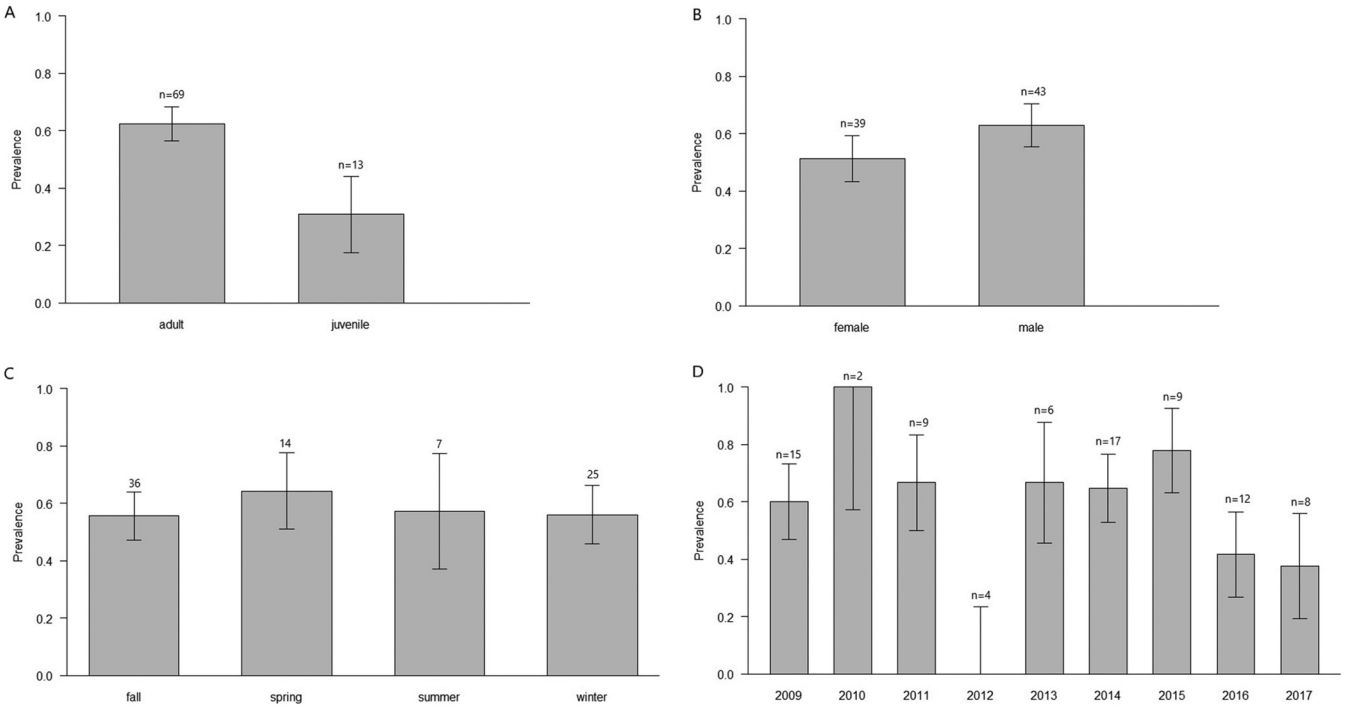

FIG 4 Prevalence of *Mycoplasma haemocanis* depending on different intrinsic and extrinsic variables in Darwin's foxes. (A) Age groups; (B) sex groups; (C) seasons; (D) study years.

The higher prevalence observed in adult foxes compared to juveniles coincides with studies of free-ranging dogs (9) and cats (28) and with the data of this study in rural dogs of Chiloé. This indicates that both foxes and dogs face an increasing possibility of exposure to the pathogen with age. This, together with the apparent lack of seasonality and interannual differences in the prevalence indicates that *M. haemocanis* infection behaves enzootically in the Darwin's fox. The data from the recaptured individuals further confirmed the widespread nature of the infection in foxes and that foxes can be infected more than once, as proven by the finding of foxes reinfected by a different haplotype. Host sex does not appear to be a risk factor for infection with *M. haemocanis* in foxes, which concurs with previous studies in domestic dogs (27, 29).

The observed prevalence of "*Ca*. Mycoplasma haematoparvum" in dogs in our study is in the range of that reported in Argentina (30) and higher than that reported in the above-metioned city dogs in Chile (15). Unfortunately, we were unable to characterize "*Ca*. Mycoplasma haematoparvum" further to confirm whether this species is also shared by both hosts.

The lack of effect of landscape features on hemoplasma prevalence in the Darwin's fox is in disagreement with a previous study on feline hemoplasmas in a threatened sympatric felid, the guigna (22). Our results likely reflect a heterogeneity of the risk of transmission of a multihost pathogen that may be using more than a single method of transmission (horizontal, vertical, and/or vector borne) and/or more than a single arthropod vector.

Infections with hemoplasmas appear to occur as chronic conditions in domestic species (27, 29). This seems to be the case in the Darwin's fox, as indicated by the high prevalence observed in the absence of clinical signs and the high proportion of foxes that were found to be infected by the same ntST in both capture events, which had up to 3 years of difference between them (although the possibility of periodic reinfections with the same ntST cannot be ruled out). The chronicity could explain the absence of hematological alterations associated with the presence of the pathogen. The possible tolerance of the Darwin's fox to *M. haemocanis* is

supported by the genetic variability of the pathogen in the fox population, with up to six different ntST detected in 25 individuals, which according to Kutzer and Armitage (31) may reflect some degree of tolerance to the infection. Kutzer and Armitage (31) also proposed that tolerance to a pathogen will confer a fitness advantage to the host, which could fluctuate by pathogen load and intrinsic factors of the host, reasons that may explain why we did not detect any risk factor associated with the infection other than age. Nevertheless, it is worth noting that when the apparent tolerance is lost, subclinical infections can lead to a reduction in host fitness, reproduction, survival, or dispersal, any of which can be detrimental for the population of an endangered species such as the Darwin's fox (32).

Previous studies identified coinfection with other agents as a risk factor for becoming infected with hemoplasmas (27, 33), and it is known that the presence of other agents can aggravate the pathogenicity of a primary agent. For example, distemper outbreaks in African lions were exacerbated by concomitant *Babesia* spp. (34). We did not investigate coinfections with other vector-borne pathogens because our preliminary survey indicated absence of all the other main canine vector-borne pathogens (21). Moreover, a recent study showed that the same foxes studied here were not exposed to canine distemper virus (CDV) (35). Therefore, it appears that coinfection is not an important driver of hemoplasma infection for this species. Rynkiewicz et al. (36) proposed that a host that has a tolerance response to a pathogen will have higher fitness due to the avoidance of the energetic cost of clearing an infection, which seems to be the case for the fox according to the hematological analyses. Nevertheless, asymptomatic infection could revert, triggered by other concomitant infectious or noninfectious causes.

Despite the small sample size, we confirmed the presence of both canine hemotropic mycoplasmas in foxes from both mainland metapopulations. This could be relevant during health assessment protocols before potential fox translocations (37). In these two mainland populations, two other sympatric anthropophilic and more-abundant fox species, the Andean fox (*Lycalopex culpaeus*) and the gray fox (*L. griseus*), could add a potential bridge between dogs and Darwin's foxes, increasing the complexity of interspecific transmission of this multihost pathogen.

Infectious diseases can pose a threat to the survival of endangered species, especially those such as the Darwin's fox, which are losing their natural habitat and encountering dogs more frequently, increasing the opportunities for pathogen transmission (23, 38). Therefore, information about the health of their populations and the evaluation of risk factors for infection are imperative.

Conclusion. We showed that the Darwin's fox can sustain a constant prevalence of infection with a relatively high genetic diversity of *M. haemocanis* and with apparent lack of associated pathology. Nevertheless, although hemotropic mycoplasmas may seem asymptomatic, the constant circulation of this disease agent in the reduced Darwin's fox population is a concern. The apparent tolerance of foxes to hemoplasma infection could be disrupted due to other factors that could be favored by poorly managed sympatric dogs. This disease risk is enhanced by the rapid habitat loss and degradation that the Darwin's fox is currently suffering (39).

MATERIALS AND METHODS

Study areas and field techniques. The samples included in this study were obtained between 2009 and 2017. The samples from 2009 to 2012 corresponded to the 30 fox blood samples from Chiloé Island included in the previous survey by Cabello et al. (21). From 2013 to 2017, we collected 52 additional fox samples in the three populations of Darwin's fox: the Nahuelbuta area ($n = 5$) (37°45′S, 73°00′W), comprising Nahuelbuta National Park and surrounding native forests; the Valdivian Coastal Range ($n = 5$) (40°07′S, 73°33′W); and Chiloé Island ($n = 42$) (42°S, 74°W) (Fig. 1). Throughout the years of our study, seven foxes in Chiloé and one in Nahuelbuta were recaptured once. One individual was juvenile at the initial sampling and adult when recaptured, while all the others were adults in both capture events.

Foxes were captured using Tomahawk traps baited with chicken or canned fish. Traps were activated at dusk and checked the next morning at dawn. Foxes were anesthetized with a combination of 1 mg/kg

xylazine (xylazine 2%; Centrovet, Chile) plus 10 mg/kg ketamine (Ketostop; DragPharma, Chile) or with 0.04 mg/kg dexmedetomidine (Dexdormitor; Zoetis, Chile) plus 5 mg/kg ketamine. The latter was reversed with 0.4 mg/kg atipamezole (Antisedan; Zoetis, Chile). A veterinarian performed an external clinical evaluation of the anesthetized animals. Blood was obtained from the cephalic, saphenous, or jugular vein. Foxes were classified as juveniles (less than 1 year) or adults (older than 1 year) based on tooth eruption.

Between the years 2015 and 2018, 250 rural dogs were sampled across Chiloé (Fig. 1). Blood was collected individually after the consent of the owner. Whole blood from dogs and foxes was placed in EDTA tubes. When possible, whole blood was sent by courier to our laboratory for hematological analyses. Otherwise, it was stored at –20°C. For serum biochemistry analysis, sera of foxes were extracted and stored at –20°C when possible. Dogs were classified as juveniles (less than 1 year) or adults (older than 1 year) based on tooth eruption.

All captures were made with the permission of the Servicio Agricola y Ganadero of the Chilean Government under permits 1262/2009, 2263/2010, 206/2012, 3155/2013, 1492/2014, 3363/2015, 3035/2016, 2288/2016, and 5029/2017. The study was approved by the authorities on bioethics of Universidad Andres Bello (permit no. 08/2016).

Molecular detection and characterization. DNA was isolated from the 302 fox and dog blood samples using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. All the newly obtained samples from foxes and dogs were initially screened for *Mycoplasma* spp. by a conventional PCR targeting a 391-bp fragment of the 16S rRNA gene (40). Amplicons were selected and assigned to a nucleotide sequence type (ntST). Since *M. haemocanis* and *M. haemofelis* are undistinguishable based on the characterization of the 16S rRNA gene alone, we confirmed that all positive samples corresponded to *M. haemocanis* by a conventional PCR targeting a 175-bp fragment of the RNase P gene. All these PCR protocols were as described by Millán et al. (40). The complete 16S rRNA gene (~1,400 bp) was then characterized in all the *M. haemocanis*-positive foxes and in two positive dogs per hemoplasma ntST (Table 4). In order to detect if foxes had so-far-undetected coinfections with "*Ca*. Mycoplasma haematoparvum," all the sequences corresponding to *M. haemocanis*/*M. haemofelis* from 2013 to 2017 were screened through a "*Ca*. Mycoplasma haematoparvum"-specific protocol targeting a 112-bp fragment of the 16S rRNA gene as described by Martínez-Díaz et al. (41). Positive controls were obtained from clinical samples of *M. haemocanis* and "*Ca*. Mycoplasma haematoparvum" from previously sequenced dog blood samples, and ultrapure water was used as a negative PCR control. Two percent agarose gel electrophoresis was performed, and PCR products were visualized under an UV transilluminator. All positive samples were sequenced by Macrogen, and the sequences obtained were compared with sequences deposited in the GenBank database (NCBI).

The sequences of the 16S rRNA genes obtained from foxes and dogs were aligned using ClustalW executed in Geneious Prime 2019.2.1 (Biomatters Ltd.). To determine genetic relationships between the sequences from wild and domestic canids, we constructed a maximum-likelihood phylogenetic tree using MEGA 7.0.26 (42) and median joining networks using PopART (43). A network containing the sequences from dogs and Darwin's foxes from this study was used to infer genetic relationships among hemoplasma species. A second network was developed to infer relationships according to the sampling site (Chiloé versus Nahuelbuta only, since the sequence from Valdivia was not readable) using only the fox sequences. Finally, a network considering the year of fox sampling was used to infer the dynamics of infection in the Darwin's fox. The genetic structure was estimated using the pairwise Phi_{ST} test implemented in Arlequin (44) (level of significance assessed with 1,000 permutations) and the nearest-neighbor statistic Snn (45) executed in DnaSP.5 (46). An analysis of the nucleotide polymorphisms of 16S rRNA sequences obtained was performed using the software DnaSP.5 (46) in order to determine the genetic differentiation of hemoplasmas among hosts.

Hematology and serum chemistry analyses. The harsh climatic conditions in southern Chile and distant locations of many study sites prevented the proper preservation of some of the blood and serum samples until laboratory analyses. Therefore, hematological and biochemical variables were obtained for 31 of the foxes during the study period. Seven hematological variables (hematocrit, red blood cell, platelet and total leukocyte counts, hemoglobin concentration, mean corpuscular volume, and mean corpuscular hemoglobin concentration) were calculated using a HumaCount 80 cell counter (Human GmbH, Germany). Relative leukocyte differentiation was estimated by microscopic observation after Diff-Quick staining. Fourteen biochemical variables were analyzed using a BA400 Analyzer (BioSystem SA, Barcelona, Spain). Measurement units and variables included are listed in Table 5.

Data analysis. *M. haemocanis* presence/absence was binary coded and examined with a set of models of possible intrinsic and extrinsic variables affecting animal exposure to hemoplasmas. We included generalized linear models considering age, sex, and their interaction in relation to *M. haemocanis* infection. A chi-square test was used to determine differences in infection depending on these factors in dogs and to determine differences in prevalence between foxes and dogs.

Spatial analyses were performed only in Chiloé to test the effect of extrinsic variables on the *M. haemocanis* presence/absence, due to insufficient sample sizes in the other study areas. In order to analyze if foxes' exposure to hemoplasmas depends on spillover events from dogs more than on intraspecific transmission (47), we used a model considering landscape anthropization in the fox home range area. We created a buffer zone around the fox capture site based on individual fox locations and the home range size described for the species (3.06 km^2 for males and 2.72 km^2 for females) (48). Independent variables were as follows: presence/absence of houses in the buffer area, total number of houses in the buffer area, distance of the capture site to the nearest house, land use, and proportion of vegetation cover in the buffer area (49). To determine if pathogen exposure showed temporal variation

TABLE 4 Genes targeted and primers used for PCR screening and characterization of hemotropic mycoplasmas in Darwin's foxes and rural dogs in Chile

Target	Primer sequences	Primer names	Fragment length (bp)	Purpose	Reference
Mycoplasma sp. 16S rRNA	5′-ATGTTGCTTAATTCGATAATACACGAAA-3′ (forward), 5′-ACRGGATTACTAGTGATTCCAACTTCAA-3′ (reverse)	Mycop16S rRNA-F, Mycop16S rRNA-R	384	Screening	40
Mycoplasma sp. 16S rRNA (seminested)				Characterization	This study
Template	5′-AGAGTTTGATCCTGGCTCAG-3′ (forward), 5′-TACCTTGTTACGACTTAACT-3′ (reverse)	HemoF1, HemoR2	1,428		
First	5′-ATATTCCTACGGGAAGCAGC-3′ (forward), 5′-TACCTTGTTACGACTTAACT-3′ (reverse)	HemoF2, HemoR2	1,107		
Second	5′-GCCCATATTCCTACGGGAAGCAGCAGT-3′ (forward), 5′-GTTTGACGGGCGGTGTGTACAAGACC-3′ (reverse)	HemMycop16S-322s, HemMycop16S-1420as	1,029		
Third	5′-GYATGCMTAAYACATGCAAGTCGARCG-3′ (forward), 5′-CTCCACCACTTGTTCAGGTCCCCGTC-3′ (reverse)	HemMycop16S-41s, HemMyco16S-938as	870		
M. haemocanis/*M. haemofelis* RNase P	5′-CCTGCGATGGTCGTAATGTTG-3′ (forward), 5′-GAGGRGTTTACCGCGTTTCAC-3′ (reverse)	RNAseP-F, RNAseP-R	175	Characterization	40
"*Ca*. Mycoplasma haematoparvum" 16S rRNA	5′-GGAATCACTAGTAATCCYGTGTCAGCTATAT-3′ (forward), 5′-AATTAAATACGGTTTCAACTAGTACGTTTCTTT-3′ (reverse)	Mycoplasma Species-F, *Candidatus* Mycoplasma haematoparvum-R	112	Characterization	41

TABLE 5 Hematological and serum chemistry values in Darwin's foxes from Chiloé Island

Variable (unit)[a]	Sample size	Median value	SD	Range
WBC (μl)	31	15,850	5,020	5,960–29,600
RBC (μl)	31	5,870,000	957,455	3,310,000–7,880,000
HB (g/liter)	31	13.9	2.14	7.3–18.7
HTO (%)	31	42.3	7	23.9–59.0
MCV (fl)	25	72	23.31	16.4–90.0
MCH (fl)	31	23.6	0.97	21.8–26.3
MCHC (g/liter)	31	32.3	1.7	27–35.5
PLT (μl)	31	304,000	109,250	141,000–580,000
N (μl)	25	12,648	3,845	4,410–18,480
L (μl)	30	1,738	1,717	752–9,400
M (μl)	30	510	1,167	138–6,150
E (μl)	26	150	681	0–3,071
Ca (mmol/liter)	26	2.46	0.39	1.35–2.94
P (mmol/liter)	28	1.95	3.50	0.1–11.3
BUN (mmol/liter)	28	9.11	5.02	4.82–27.4
Crea (mmol/liter)	28	0.07	0.3	0.04–0.18
Bil (μmol/liter)	21	3.93	2.57	1.71–13.68
Gluc (mmol/liter)	28	2.6	1.69	0.06–6.16
Chol (mmol/liter)	28	5	1.11	2.92–8.02
ALP (IU/liter)	22	40	76.59	3–227
ALT (IU/liter)	26	77	33.40	30–152
AST (IU/liter)	26	83	53.46	21–225
GGT (IU/liter)	24	2	4	1–21
Prot (g/liter)	25	76	11	45–96
Glob (g/liter)	23	40	73	32–58
Alb (g/liter)	28	30	66	17.2–47

[a]WBC, white blood cells, RBC, red blood cells; HB, hemoglobin; HTO, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; Ca, calcium; P, phosphorus; BUN, blood urea nitrogen; Crea, creatinine; Bil, bilirubin; Gluc, glucose; Chol, cholesterol; ALP, alkakine phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; Prot, total proteins; Glob, globulins; Alb, albumin.

(e.g., epizootic or seasonal), a third model, including the variables season and year, was developed (50). Finally, a full model considering all the mentioned variables and a null model were developed. All variables were compared with presence/absence of *M. haemocanis* using generalized linear models. Variables were initially analyzed with univariate models, and then the variables with significant *P* values were included in multivariate models. The Akaike information criterion (AIC) was used for model selection, and its fit was assessed using the Hosmer-Lemeshow goodness-of-fit test (51).

Hematological and biochemical variables were compared for each of the following fox infection statuses: hemoplasma-positive versus -negative foxes, *M. haemocanis*-infected foxes versus *M. haemocanis*- and "*Ca*. Mycoplasma haematoparvum"-coinfected foxes, and *M. haemocanis*- and "*Ca*. Mycoplasma haematoparvum"-coinfected foxes versus negative foxes. We used the Shapiro-Wilk normality test to determine if the data were distributed normally. The two-sample Mann-Whitney U test was performed to determine differences among the variables. Following the "Guidelines for the Determination of Reference Intervals in Veterinary Species" of the American Society for Veterinary Clinical Pathology (available at https://cdn.ymaws.com/www.asvcp.org/resource/resmgr/QALS/Other_Publications/RI_Guidelines_For_ASVCP_webs.pdf), the median, standard deviation, and range values were provided for each parameter. All data analyses were performed using R software version 3.4.1 (52).

Data availability. The new sequences obtained in the present study were submitted to GenBank with accession numbers MN164349 to MN164353.

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