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Cytauxzoon europaeus, *Babesia vulpes,* and *Hepatozoon felis* circulating simultaneously in a European wildcat (*Felis silvestris*) population in northern Spain

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ABSTRACT

The Iberian population of the European wildcat (*Felis silvestris*) is undergoing a decline and is highly fragmented. This study aimed to determine the occurrence and identity of tick-borne parasites (piroplasms and *Hepatozoon* spp.) in Navarre, one of the strongholds for wildcats in Spain. DNA was extracted from spleen samples of 63 road-killed wildcats and analyzed using a suite of molecular protocols targeting the 18S rRNA and CytB gene fragments. Eighty percent of the wildcats tested positive for at least one parasite. *Cytauxzoon* spp. was detected in 65 % of the individuals. Molecular characterization and phylogenetic analysis confirmed the presence of *Cytauxzoon europaeus* in nine samples, representing the first report of this species in the Iberian Peninsula. *Hepatozoon* spp. was found in 46 % of the wildcats, and molecular characterization of four cases identified *Hepatozoon felis*. *Babesia vulpes* was detected in a single individual. Coinfection with *Cytauxzoon* sp. and *Hepatozoon* sp. was observed in 32 % of the wildcats, while one individual was coinfected with *C. europaeus* and *B. vulpes*. Occurrence of *H. felis* was higher in adult wildcats. No spatial structure or correlation with body condition was observed for any of the parasites. Given the widespread presence of *Cytauxzoon* and other parasites in this and other wildcat populations, their impact on wildcat health, if any, is likely to be minimal. However, wildcats appear to serve as important maintenance hosts for these parasites.

1. Introduction

The most important tick-borne parasites from the veterinary point of view are two groups of Apicomplexans: Piroplasmida (mainly *Babesia* spp., *Theileria* spp. and *Cytauxzoon* spp.) and Eucoccidiorida (*Hepatozoon* spp.). Piroplasmids are among the most prevalent vector-borne parasites of the phylum Apicomplexa (Schnittger et al., 2012; Yabsley and Shock, 2013) and infect a wide range of wild carnivores worldwide, including felines (Alvarado-Rybak et al., 2016). In Europe, three species of *Cytauxzoon* (i.e. *C. banethi*, *C. europaeus,* and *C. otrantorum*) have been described in wildcats (Panait et al., 2021). Likewise, more than 50 species of *Hepatozoon* have been described in mammals, including wildlife (Baneth et al., 2013; Hodžić and Alić, 2023). In Europe, *H. felis* and *H. silvestris* have been described in domestic and wild felids (Hodžić and Alić, 2023).

The European wildcat (*Felis silvestris* Schreber, 1775) is the most abundant native felid in Europe. Although it is classified as Least Concern by the IUCN, the Iberian population is declining and highly fragmented (García-Perea, 2002; Gerngross et al., 2023). In Spain, the species is considered Near Threatened at the national level (López-

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Table 1
Sample size, occurrence (%), and 95 % Confidence Intervals for *Cytauxzoon* spp. and *Hepatozoon* spp. in European wildcats, Navarre (northern Spain). *It indicates significant differences between groups ($P < 0.05$).

	n	*Cytauxzoon* spp.	*Hepatozoon* spp.	Coinfection
Adult males	28	71.4 (51.3–86.8)	57.1 (37.2–75.5)	42.8 (24.4–62.3)
Adult females	13	53.8 (25.1–80.1)	53.8 (25.1–80.1)	30.7 (9.1–61.4)
Overall adults	41	65.8 (49.4–79.9)	56.1 (39.7–71.5) *	39.0 (24.2–55.5)
Juvenile males	9	55.5 (21.2–86.3)	22.2 (2.8–60.0)	11.1 (0.3–48.2)
Juvenile females	13	69.2 (38.6–90.1)	30.1 (9.1–61.4)	23.3 (0.5–53.8)
Overall juveniles	22	63.6 (40.1–82.8)	27.7 (10.7–50.2) *	18.2 (0.5–40.2)
Total	63	65 (52.0–76.7)	46.0 (33.3–59.0)	31.7 (20.5–44.7)

Martín et al., 2007). However, the wildcat population in northern Spain appears to be stable (F. Urra, pers. obs.). The main threats to the Iberian wildcat population include hybridization with domestic cats, road mortality, and infectious diseases. Due to its close evolutionary relationship with domestic cats, both species share most, if not all, potential pathogens. Despite this, little is known about the pathogens infecting Iberian wildcats. Serological evidence of exposure or infection with various feline pathogens have been reported in wildcats from central Spain (Millán and Rodríguez, 2009; Candela et al., 2019; Nájera et al., 2021a; Calatayud et al., 2020) and Portugal (Duarte et al., 2012). Regarding vector-borne pathogens, nearly 80 % of 14 wildcats sampled in central Spain tested positive for *Cytauxzoon* DNA (León et al., 2017). *Cytauxzoon* spp. also circulates within some Iberian lynx (*Lynx pardinus*) metapopulations in southern Spain (Luaces et al., 2005; Millán et al., 2007; Nájera et al., 2021b) and in domestic cats (Díaz-Regañón et al., 2017). However, the precise identity of the *Cytauxzoon* species (one or more) infecting Iberian felines remains unresolved (Millán and Becker, 2021). With respect to other piroplasms, the only previous evidence of *Babesia* infection in Iberian wildcats comes from a single individual in southeastern Spain, in which a sequence related to an undescribed *Babesia* species found in foxes and jackals was identified (Ortuño et al., 2022). Infection with *Hepatozoon* spp., on the other hand, has been documented in domestic cats in Spain (Ortuño et al., 2008; Díaz-Regañón et al., 2017), and *Hepatozoon felis* was recently detected in three out of five wildcats in southeastern Spain (Ortuño et al., 2022).

Multihost pathogens such as piroplasmids can persist in wildlife reservoirs with occasional transmission to domestic animals. In particular, those transmitted by vectors do not require direct contact between individuals of different species. Therefore, continuous surveillance and molecular diagnostics are crucial for assessing potential spillover risks. In this study, we aimed to characterize the tick-borne apicomplexan parasites infecting a wildcat population in northern Spain, a region where parasitic infections in this species had never been studied. Specifically, we sought to identify, for the first time, the *Cytauxzoon* species circulating in Iberian felines and analyze potential risk factors for infection.

2. Material and methods

2.1. Field methods

The sample consisted of 63 road-killed wildcats (Table 1) collected in the frame of a monitoring program coordinated by the Autonomous Region of Navarre (Northern Spain; Fig. 1). Animals were collected by rangers and sent to the wildlife recovery center of Ilundain, where carcasses were frozen until necropsy. At necropsy, animals were sexed, aged according to the size of the animal and the teeth eruption pattern (Condé and Schauenberg, 1978), measured, and weighed, and a piece of spleen was collected and frozen until analysis. Ticks were also collected, preserved in 90 % ethanol, and morphologically identified (Estrada-

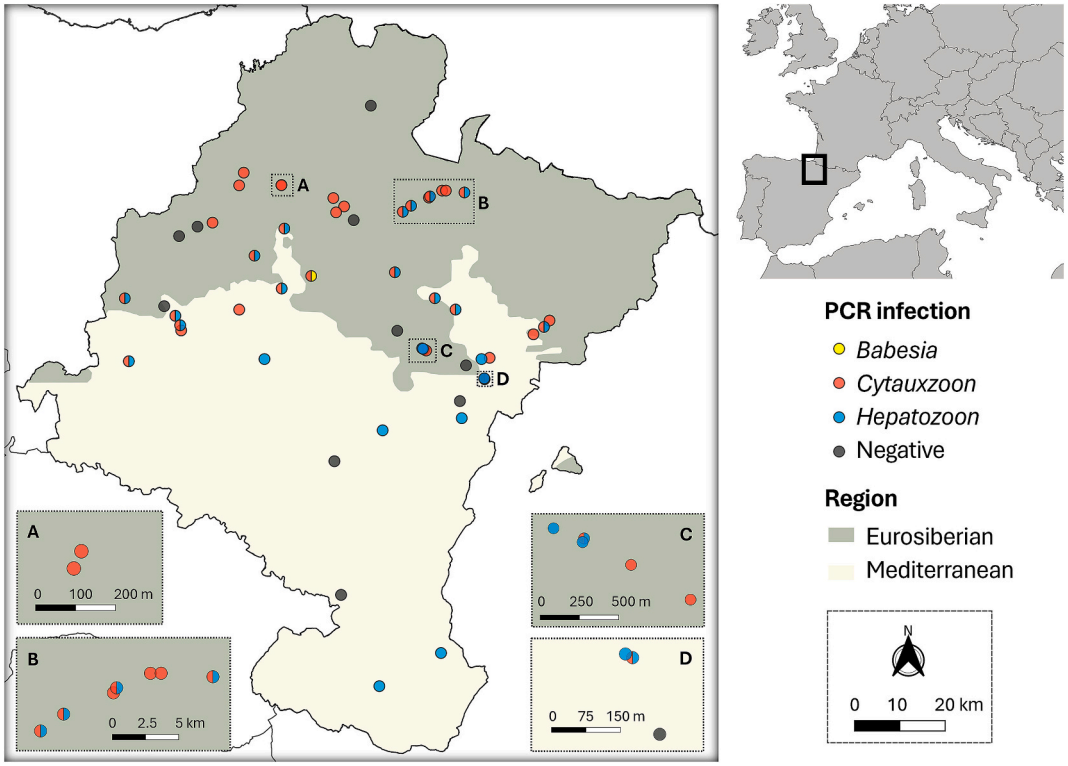

Fig. 1. Spatial distribution of the sampled wildcats in Navarre, north Spain. Colour dots represent PCR infection status regarding *Hepatozoon* spp. and piroplasmids (*Babesia* spp. and *Cytauxzoon* spp.).

Table 2
PCR conditions for the detection and characterization of piroplasmids and *Hepatozoon* spp. in European wildcats, Navarre (north Spain).

Target gene (protocol)	Primers (nucleotide sequence 5′-3′)	Method	Amplicon size	Annealing conditions	Reference
Piroplasmids / *Hepatozoon* spp.					
18S rRNA (#1)	PIRO F: CCAGCAGCCGCGGTAATTC PIRO R: CTTTCGCAGTAGTTYGTCTTTAACAAATCT	cPCR	360 bp	45 s at 64 °C	Tabar et al. (2008)
Hepatozoon spp.					
18S rRNA (#1)	HEPA F: GGTAATTCTAGAGCTAATACATGAGC HEPA R: ACAATAAAGTAAAAAACAYTTCAAAG	cPCR	574 bp	30 s at 50 °C	Almeida et al. (2012)
Piroplasmids					
18S rRNA (#1)	PIRO-A: AATACCCAATCCTGACACAGGG PIRO-B: TTAAATACGAATGCCCCCAAC	cPCR	408 bp	30 s at 58 °C	Olmeda et al. (1997)
18S rRNA (#2)	BTF1 (external): GGCTCATTACAACAGTTATAG BTR1 external): CCCAAAGACTTTGATTTCTCTC	nPCR	930 bp	20 s at 58 °C	Jefferies et al., (2007)
	BTF2 (internal): CCGTGCTAATTGTAGGGCTAATAC BTR2 (internal): GGACTACGACGGTATCTGATCG		800–850 bp	20 s at 62 °C	
Cytauxzoon spp.					
CytB (#1)	Cytaux_cytb_F1: CTTAACCCAACTCACGTACC Cytaux_cytb_R3: GGTTAATCTTTCCTATTCCTTACG	nPCR	1434 bp	1 min at 53 °C	Panait et al. (2021)
	Cytaux_cytb_Finn: ACCTACTAAACCTTATTCAAGCRTT Cytaux_cytb_Rinn: AGACTCTTAGATGYAAACTTCCC		1333 bp	1 min at 55 °C	

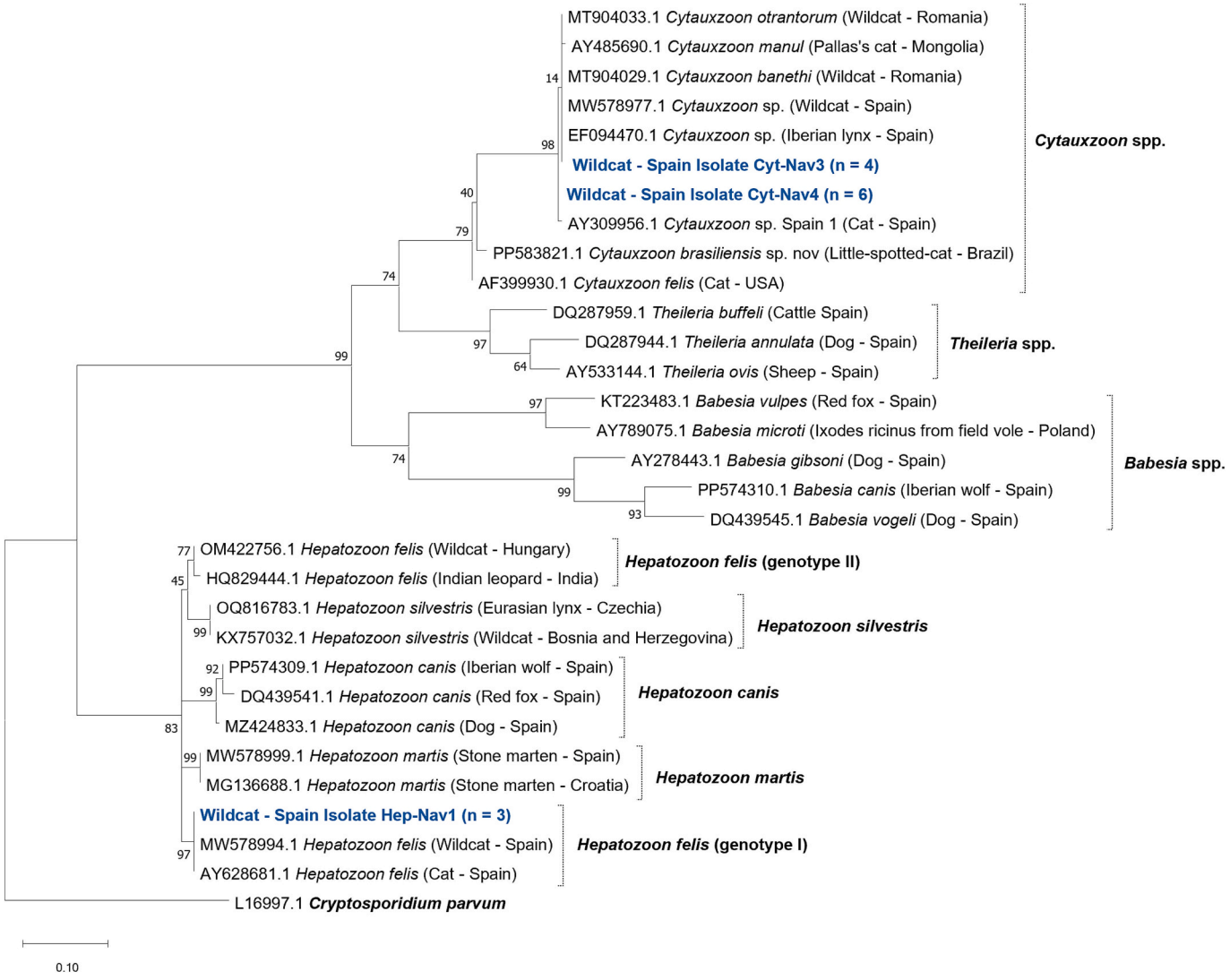

Fig. 2. Phylogenetic analysis of *Cytauxzoon* and *Hepatozoon* sequences obtained with primers PIRO-F / PIRO-R (*18S rRNA*, 313 bp) from wildcats and other relevant reference sequences deposited in GenBank. The evolutionary model used was the Hasegawa-Kishino-Yano model (HKY + G). *Cryptosporidium parvum* was included as an external outgroup.

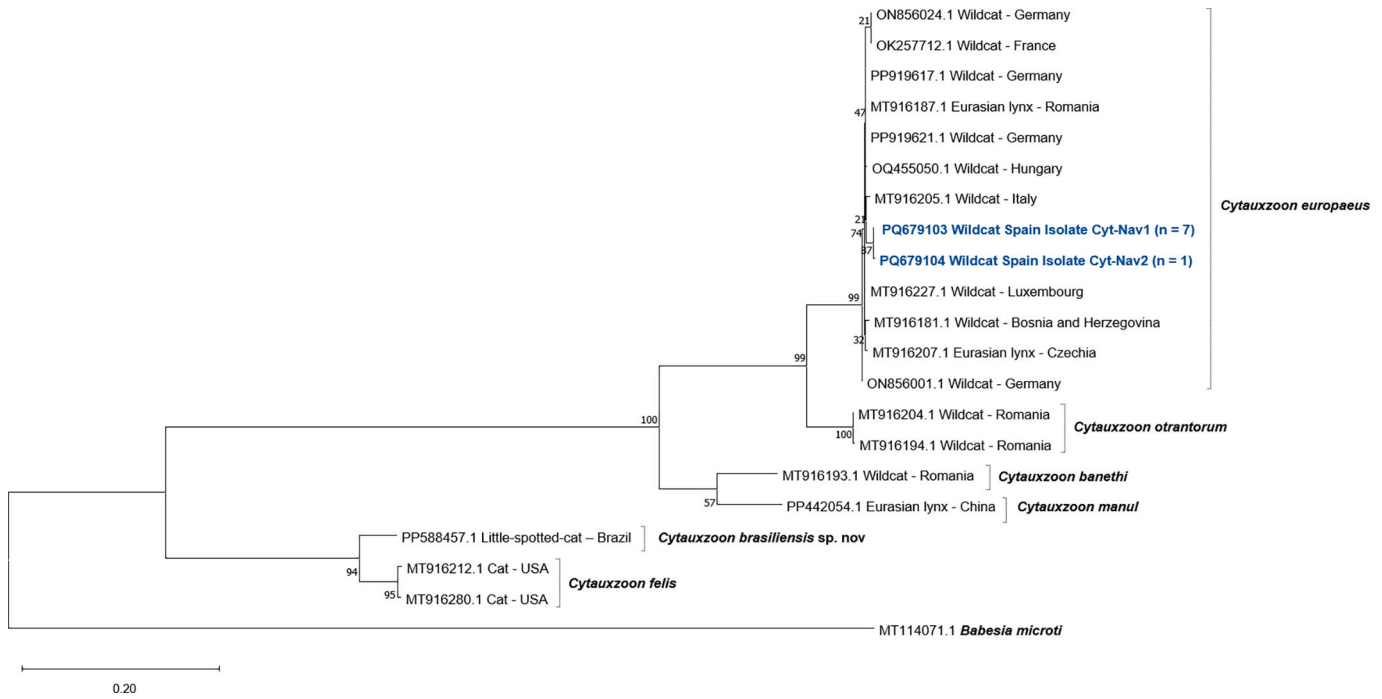

Fig. 3. Phylogenetic analysis of the different *Cytauxzoon* sequences (*CytB*, 913 bp) from wildcats and other relevant reference sequences deposited in GenBank. The evolutionary model used was the Hasegawa-Kishino-Yano model (HKY + G). *Babesia microti* was included as an external outgroup.

Peña et al., 2017; Millán et al., 2024). Additionally, body condition was estimated by calculating the body mass-to-tarsus length ratio. Animals were assigned to a bioclimatic region (Eurosiberian or Mediterranean). The Mediterranean region is characterized by a Mediterranean climate, with a summer drought period. Typically Mediterranean species include the holm oak (*Quercus rotundifolia*) and the kermes oak (*Q. coccifera*), which form holm oak forests and kermes oak scrublands, respectively. In the Eurosiberian region, there is no summer drought or it is attenuated. Eurosiberian species include the beech (*Fagus sylvatica*) and the downy oak (*Quercus pubescens*), which form beech forests and downy oak woodlands (Mapa de Vegetación Potencial de Navarra, Gobierno de Navarra: https://www.navarra.es/mapacultivos/htm/index.htm).

2.2. Laboratory methods

DNA was extracted from spleen samples using TriReagent® solution (Sigma-Aldrich, Burlington, MA, USA), according to the manufacturer's instructions. Briefly, 25 mg of spleen was cut into small pieces and homogenized in the TriReagent® solution with a 1 ml syringe and a 25G needle. The DNA was eluted in 30 µl of nuclease-free water and the Nanodrop One® spectrophotometer (ThermoScientific, Waltham, MA, USA) was used to measure the DNA concentration and purity ratios. DNA samples were then stored at −80 °C until further analysis.

DNA samples were tested using two protocols. Protocol #1 was carried out at the Instituto de Investigación en Recursos Cinegéticos (Ciudad Real, Spain) and consisted of an initial screening of the 63 wildcats with a conventional PCR (cPCR) to amplify a 360-bp common fragment of the piroplasmid and *Hepatozoon* spp. *18S rRNA* gene (primers PIRO-F / PIRO-R). To confirm the infection by one or more tick-borne parasites, additional cPCR assays using specific primers to amplify partial fragments of the *18S rRNA* gene for piroplasmids (PIRO-A / PIRO-B) and *Hepatozoon* spp. (HEPA-F / HEPA-R) were carried out in samples that tested positive for the first screening cPCR. Besides, the characterization of *Cytauxzoon* species in a selection of piroplasmid-positive samples was carried out with a nested PCR (nPCR) that amplifies 1333 bp of the cytochrome *b* (*CytB*) gene (Table 2). All PCR amplifications were carried out in a reaction volume of 25 µl, using 2× PCR Master Mix (Promega Corporation, Madison, WI, USA), 1 µl of each primer at 10 µM, 1 µl of DNA, and nuclease-free water. PCR amplicons were visualized by electrophoresis in 1.5 % agarose gels using GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Based on the bands visualized, a selection of the amplified PCR products of piroplasmids (*18S rRNA* and *CytB*) and *Hepatozoon* spp. (*18S rRNA*) were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Secugen S.L. (Madrid, Spain). All sequences were edited by visualizing the chromatogram using Chromas software version 2.6.6 (http://www.technelysium.com.au/chromas.html). The BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was then used to compare our nucleotide sequences with those previously deposited in GenBank. Phylogenetic analyses were carried out with the sequences amplified from each PCR assay (*Cytauxzoon*-specific *CytB* and several fragments of the *18S rRNA* for piroplasmids and/or *Hepatozoon* spp.). Alignments were performed using the ClustalW tool implemented in UGENE software (http://ugene.net) and Maximum Likelihood (ML) phylogenetic trees were generated using MEGA X software (Kumar et al., 2018). The selection of the ML trees was performed based on the Bayesian information criterion (BIC), and the evolutionary models with the lowest BIC were used. ML trees were constructed for each gene fragment by bootstrap analysis with 1000 replicates.

On the other hand, protocol #2 was carried out at the Veterinary Faculty, Universidad Complutense de Madrid, Spain, in 35 wildcats. Primer sequences, annealing conditions, and the expected size of amplicons are shown in Table 2. The reaction mixture was prepared as described elsewhere (Checa et al., 2019). PCR amplicons of the expected size (800–850 bp) were sent to the Genomics Unit (Universidad Complutense de Madrid) for purification and sequencing. The obtained sequences were analyzed using MEGA X program and compared with sequences available in GenBank© using the BLAST software.

The newly obtained sequences of tick-borne parasites from wildcats were submitted to the GenBank database under the accession numbers PQ679103- PQ679104 and PQ685062 (*Cytauxzoon europaeus*), and PQ685063 (*Babesia vulpes*).

Table 3
Summary of GLM best model results for prevalence for each host-parasite association. BC: Body condition.

	Hepatozoon sp.					Piroplasmida					Coinfection				
MODEL	BC	Age	Sex	Region	AIC	BC	Age	Sex	Region	AIC	BC	Age	Sex	Region	AIC
I	0.481	1.564	−0.229	−0.944	85.94	−0.039	0.024	0.844	1.120	84.15	−0.261	1.075	0.573	−0.001	78.63
	(0.714)	(0.785)	(0.612)	(0.612)		(0.733)	(0.768)	(0.644)	(0.643)		(0.771)	(0.865)	(0.673)	(0.638)	
	(0.501)	(0.047)**	(0.708)	(0.123)		(0.958)	(0.975)	(0.191)	(0.062)*		(0.734)	(0.214)	(0.394)	(0.998)	
II	0.469	1.499	–	−0.875	84.07	−0.053	–	0.848	1.197	82.16	−0.262	1.075	0.574	–	76.63
	(0.714)	(0.767)		(0.581)		(0.574)		(0.629)	(0.638)		(0.771)	(0.864)	(0.643)		
	(0.511)	(0.051)*		(0.132)		(0.926)		(0.178)	(0.060)*		(0.734)	(0.214)	(0.372)		
III	–	1.137	–	−0.985	83.79	–	0.676	–	1.029	83.33	–	1.204	0.500	–	75.82
		(0.588)		(0.573)			(0.598)		(0.610)			(0.720)	(0.637)		
		(0.053)*		(0.085)*			(0.259)		(0.091)*			(0.094)*	(0.432)		
IV	–	1.181	–	–	84.83	–	–	–	0.787	82.64	–	1.335	–	–	74.45
		(0.575)							(0.555)			(0.702)			
		(0.040)**							(0.157)			(0.057)*			

2.3. Statistical analysis

First, the data were analyzed using a generalized linear model with a binomial link function. The full model was:

$$\text{logit}\left(y_{ijkl}\right) = \mu + b_{ijkl} + a_i + s_j + r_k$$

where y_{ijkl} is the categorical observation (0 no presence, 1 presence) of the lth individual at the *ith* age, the *jth* sex and the *kth* region, μ is the general mean, b_{ijkl} is a covariate with physical condition, a_i is the ith age effect (2 levels), s_k is the jth sex effect (2 levels) and r_l is the kth region effect (2 levels). The generalized linear model was executed with the glm () function of the R environment (R Core Team, 2021). Once analyzed with the full model, a stepwise regression was performed to select the best model by using the Akaike Information Criterion (AIC) (Akaike, 1973) as the criterion for the elimination of variables. The analysis was implemented with the stepAIC() function of the MASS package (Venables and Ripley, 2002). The Cohen's kappa coefficient was calculated to determine the level of agreement between PCR protocols.

3. Results

Overall, 50 out of 63 wildcats (79.4 %) tested positive for at least one tick-borne parasite. Using protocol #1, 41 of 63 wildcats (65.1 %) were positive for piroplasmid DNA, while protocol #2 detected positivity in 18 of 35 individuals (51 %) (Table 1). When comparing the results from the 35 individuals analyzed by both protocols, all samples positive by protocol #2 were also positive by protocol #1 and, out of the 28 samples that tested positive with protocol #1, 18 were positive by protocol #2. The agreement between both techniques was moderate (Kappa = 0.42).

From protocol #1, sequencing of the *18S rRNA* gene fragments showed 24 sequences that were 100 % identical to those of *Cytauxzoon* sp. from domestic and wild European felids, but with no discrimination between the *Cytauxzoon* species described in European countries or elsewhere (Figs. 2 and S1). Instead, the molecular characterization of the *CytB* gene yielded nine sequences (99.9–100 % of identity among them) that showed the highest homology (identity: 99.1–99.3 %; query cover: 100 %) and clustered together with those of *C. europaeus* (Fig. 3).

Using protocol #2, sequencing identified *Cytauxzoon* sp. in 16 positive samples. The consensus sequence was submitted to the Genbank database under the accession number PQ685062 and subsequently compared with sequences available in the database. The obtained *Cytauzxoon* sp. *18S rRNA* gene sequences showed high sequence identity with *C. europeaus* isolated from wildcats in France (GenBank: MW727406.1, 99.8 % sequence identity, query cover 100 %) and Germany (GenBank: PP882683.1 and ON380460.1; 100 % sequence identity; query cover: 94–97 %). However, sequence comparison also revealed high identity with *Cytauxzoon felis* isolated form domestic cats in Switzerland (GenBank: KU306943.1 and KU306945.1, 99.8 % sequence identity, query cover: 100 %), and *Cytauxzoon* sp. from Italy (GenBank: OM004053.1, 99.8 % sequence identity, query cover: 100 %). In addition, protocol #2 identified one positive sample corresponding to *B. vulpes*. The sequence obtained in this study (GenBank accession number PQ685063) showed 100 % identity with *B. vulpes* isolates obtained from dogs (*Canis lupus familiaris*), red foxes (*Vulpes vulpes*) and a badger (*Meles meles*) in northern Spain (Genbank accession numbers: AF188001.1, MK585200.1, KT223483.1, P979536.1).

Hepatozoon spp. infection was confirmed in 29 wildcats (46.0 %; Table 1). The sequencing of partial fragments of the *18S rRNA* gene from four samples revealed the presence of *H. felis* in all cases. Specifically, the three sequences amplified with primers PIRO-F / PIRO-R were 100 % identical to *H. felis* isolates obtained from domestic cats (e.g., Spain -AY628681, Italy -KY649442-, Angola -MG386483, Uruguay -MT210598), wildcats (Spain -MW578994) and other wild felids from different countries (ON075470, PP528683). The remaining *H. felis* sequence (amplified with primers HEPA-F / HEPA-R) had a similarity of

Table 4
Observed occurrences and identified species of vector-borne Apicomplexan in European wildcats.

Reference	Country	n	*Cytauxzoon*		*Hepatozoon*		*Babesia*	
			+	Species	+	Species	+	Species
Willi et al., 2022	France	34	10	10 Ce	nt		nt	
Gallusova et al., 2016	Romania	12	6	n.c.	nt		nt	
León et al., 2017	Spain	14	11	n.c.	nt		nt	
Hodzic et al., 2018	Bosnia and Herzegovina	18	10	4 Ce	7 ($n = 9$)	3 Hf 2 Hs	1	n.c.
Hornok et al., 2022	Hungary	3	1	1 Ce	2	2 Hf		
Veronesi et al., 2016	Italy	21	4	4 Ce*	nt		0	
Panait et al., 2021	Germany	46	30	30 Ce	nt		nt	
	Romania	31	18	9 Ce, 7 Co, 2 Cb	nt		nt	
	Luxembourg	13	9		nt		nt	
	Czech Rep.	2	0		nt		nt	
Ortuño et al., 2022	Spain	5	1	n.c.	4	3 Hf, 1 Hm	1	
Grillini et al., 2023	Italy	19	4	4 Ce	8	6 Hf, 2 Hs	nt	
Obiegala et al., 2024	Germany	117	84	23 Ce	nt		nt	
This study	Spain	63	41	9 Ce	29	4 Hf	1 ($n = 25$)	1 Bv

nc: not characterized; nt: not tested for; Ce: *C. europaeus*; Co: *C. otrantorum*; Cb: *C. banethi*; Hf: *H. felis*; Hm: *H. martis*; Hs: *H. silvestris*; Bv: *Babesia vulpes*.

100 % with those obtained from domestic and wild felids of several continents (e.g., OL604173.1, OM256568.1, GQ377216.1 or OQ076291.1) and 99.04 % with the *H. felis* sequence retrieved from a cat in Spain (AY628681.1). As shown in the phylogenetic analyses based on the *18S rRNA*, our four sequences clustered with *H. felis* genotype I isolates from several countries and hosts, with a clear distinction with *H. silvestris* isolates detected in wildcats from easternmost European countries (Figs. 2 and S2).

Coinfection between *Cytauxzoon* sp. and *Hepatozoon* sp. was found in 20 (31.7 %) of the sampled wildcats. One further individual was coinfected with *C. europaeus* and *B. vulpes*.

The only significant variable influencing the occurrence of the studied parasites was age for *Hepatozoon* spp., with a higher prevalence in adults compared to juvenile wildcats (Table 3).

Ticks were collected from 15 wildcats. Of these, four had *Rhipicephalus sanguineus* sensu stricto, four *R. sanguineus* sensu lato, two *Rhipicephalus* sp., two *Ixodes ricinus*, three *Ixodes hexagonus*, two *I. ventalloi*, and one *Ixodes* sp.

4. Discussion

This study represents one of the largest investigations of tick-borne parasites in European wildcats conducted in Spain and across Europe (Veronesi et al., 2016; León et al., 2017; Ortuño et al., 2022; Hodzic et al. 2018; Gallusova et al., 2016; Hornok et al., 2022; Willi et al., 2022; Grillini et al., 2023; Obiegala et al., 2024; Table 4). Our findings align with previous studies, confirming that infection with *Cytauxzoon* spp. and/or *Hepatozoon* spp. is a common feature in European wildcats.

We detected a relatively high prevalence of *Cytauxzoon* sp. DNA. Most prior studies in Europe have been limited by small sample sizes (typically fewer than 20 individuals), making direct comparisons challenging. However, when compared to studies with larger sample sizes, our prevalence was similar to that observed in wildcats from Germany and Romania (Panait et al., 2021; Obiegala et al., 2024). In the Navarre population, *C. europaeus* was the only species identified. This species was recently described in wildcats from multiple European countries (Panait et al., 2021; Grillini et al., 2023). Our findings further support that *C. europaeus* is the only *Cytauxzoon* species reported in wildcats west of Romania.

Hepatozoon spp. infection was also frequent in the studied population, as previously reported (Hodzic et al., 2018; Ortuño et al., 2022; Hornok et al., 2022). Sequencing confirmed the presence of *H. felis* as the only identified species. However, this does not exclude the possibility of infection with other, less common *Hepatozoon* species, such as *H. silvestris* and *H. martis*, which have been documented in wildcats (Hodzic et al., 2018; Ortuño et al., 2022; Hornok et al., 2022). Infection with *H. felis* was more frequent in adult wildcats than in juveniles, likely due to prolonged exposure over time. However, in domestic cats, no age-related differences have been observed (Baneth et al., 2013), leading to the hypothesis of intrauterine transmission. In contrast, our findings suggest that alternative transmission routes, such as ingestion of paratenic hosts might play a role in wildcat infection with *H. felis*. However, this existence of this route has not been proven for *H. felis*.

Coinfection with *Cytauxzoon* and *Hepatozoon* species has been previously documented in wildcats (Hodzic et al., 2018; Ortuño et al., 2022; Hornok et al., 2022), as has coinfection with *Babesia* sp. and *Cytauxzoon* sp. (Hodzic et al., 2018). These frequent co-occurrences may result from shared tick vectors among these apicomplexan parasites. The vectors of *Cytauxzoon* spp. in Europe remain unidentified. In Eastern Europe, *Ixodes ricinus* has been proposed as the primary candidate (Gallusova et al., 2016; Hornok et al., 2022), which could also be the case in Navarre, particularly in the northern areas where suitable conditions exist for this tick species. However, other tick species may be involved, since *Cytauxzoon* spp. has also been detected in wildcats from Mediterranean Spain (León et al., 2017; Ortuño et al., 2022), where *I. ricinus* is absent. Similarly, the vector of *H. felis* has yet to be identified, though *Rhipicephalus sanguineus* s.l. has been proposed as a potential vector (Maia et al., 2014; Hornok et al., 2022). As mentioned, alternative transmission routes, such as transplacental transmission or ingestion of paratenic hosts, may play an important role for *H. felis*, but have not been reported for *Cytauxzoon* species. This could explain why apparent spatial clustering of *Cytauxzoon* cases in colder regions of Navarre was not observed for *Hepatozoon* spp.

In our study, a variety of tick species were recovered from both infected and non-infected wildcats. Since the samples were collected from road-killed individuals, and ectoparasites often leave the carcass once the animal has died, we could not establish tick-parasite associations. Notably, *I. ricinus* was not detected in any wildcat, while *I. hexagonus* was identified in three individuals. This tick species is considered the primary candidate for the transmission of *B. vulpes* to domestic and wild canids in the Iberian Peninsula, although its vector competence has not been confirmed (Camacho et al., 2001; Checa et al., 2019). Interestingly, we detected *B. vulpes* infection in one wildcat for the first time. *Babesia vulpes* (previously known as *Theileria annae* and *Babesia microti*-like piroplasm) was reported in two domestic cats from Portugal co-infected with FeLV (Criado-Fornelio et al., 2003). This hemoprotozoan parasite is pathogenic to domestic dogs (Miró et al., 2015) and has been reported in red foxes (*Vulpes vulpes*) across multiple European countries, as well as in the United States and Canada (Baneth et al., 2013). However, little is known about its occurrence and clinical relevance in felines. We suggest that future studies on piroplasms in wild felines use specific tests for different genera and species to detect rare

agents that would otherwise go undetected.

5. Conclusions

Our study confirms that *C. europaeus* circulates in Iberian wildcats and that coinfection with other vector-borne parasites, specially with *H. felis*, is common. Given the widespread presence of *Cytauxzoon* and other parasites in wildcat populations, we hypothesize that their impact on wildcat health is likely minimal. However, *Cytauxzoon* spp. is highly pathogenic to domestic cats, and wildcats may play an important role as reservoir hosts for this parasite, serving as sentinels for its presence in the ecosystem. Moreover, the wildcat is identified as a new host for *B. vulpes*, a parasite highly pathogenic for dogs.

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CRediT authorship contribution statement

Clara Muñoz-Hernández: Writing – review & editing, Visualization, Methodology. **Diego Villanúa:** Writing – review & editing, Resources, Investigation. **Rocío Checa:** Writing – review & editing, Investigation. **Marta Sánchez-Sánchez:** Writing – review & editing, Investigation. **Efrén Estévez-Sánchez:** Writing – review & editing, Investigation. **Alberto Moraga-Fernández:** Writing – review & editing, Investigation. **Fermín Urra:** Writing – review & editing, Investigation. **Guadalupe Miró:** Writing – review & editing, Resources, Investigation. **Isabel G. Fernández de Mera:** Writing – review & editing, Resources, Investigation. **Javier Millán:** Writing – original draft, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have nothing to declare.

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