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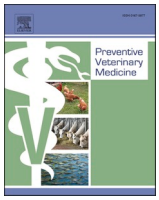

Epidemiological surveillance of *Leishmania infantum* in wild lagomorphs in Spanish Mediterranean ecosystems

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ABSTRACT

Wild lagomorphs play a key epidemiological role as reservoirs of *Leishmania infantum*, causative agent of the largest outbreak of human leishmaniosis in Europe to date. A large-scale survey study was conducted on wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations in Spanish Mediterranean ecosystems to evaluate the exposure of *L. infantum* and investigate potential risk factors associated with exposure to this zoonotic parasite. Between 2018 and 2021, a total of 631 wild lagomorphs (471 wild rabbits and 160 Iberian hares) were collected in Andalusia (southern Spain) and tested for antibodies against *L. infantum* using the indirect fluorescent antibody test (IFAT). Spleen samples from 563 of the wild lagomorphs sampled (441 wild rabbits and 122 Iberian hares) were also evaluated by real-time quantitative PCR (qPCR) for detection of *Leishmania* kDNA. Exposure to *L. infantum* (positive by IFAT and/or qPCR) was detected in 56.4 % (356/631; 95 %CI: 52.3–60.3) of the lagomorphs analyzed. Anti-*Leishmania* antibodies were found in 12.8 % (81/631; 95 % CI: 10.2–15.5) of the animals, and *L. infantum* kDNA was detected in 59.0 % (332/563; 95 %CI: 54.9–63.0) of the spleen samples tested. Phylogenetic analysis revealed high homology (99.9–100 %) between *L. infantum* sequences obtained and strains previously isolated from humans in Spain. While apparent seroprevalence was significantly higher in Iberian hares (19.4 %; 95 %CI: 13.3–25.5) compared to wild rabbits (10.6 %; 95 %CI: 7.9–13.4), no significant differences in prevalence were found between wild rabbits (61.0 %; 95 %CI: 56.5–65.6) and Iberian hares (51.6 %; 95 %CI: 42.8–60.5). At least one positive animal was found on 64.8 % (70/108) of the hunting grounds sampled, and a high-risk spatial cluster ($P < 0.001$) was also identified in central Andalusia. The multivariable analysis identified bioclimatic level (meso-Mediterranean climate) and the presence of goats on hunting grounds as risk factors potentially associated with *L. infantum* exposure in wild lagomorphs. This study

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shows high, widespread exposure, but heterogeneous distribution of *L. infantum* in wild lagomorph populations in Mediterranean ecosystems in southern Spain. The results point to the need to promote integrated surveillance programs for the detection of *Leishmania* spp. in wild lagomorphs in order to establish effective control measures against human leishmaniosis under a One Health approach.

1. Introduction

Leishmaniosis is a vector-borne disease caused by intracellular protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) transmitted by infected sand flies (WHO, 2023). Globally, this major zoonosis causes about one million new human cases and 30,000 deaths per year (WHO, 2023). Some European regions, where there has been a high reported incidence in humans and animals for decades, are considered hotspots for leishmaniosis (Maia et al., 2023). More specifically, the Mediterranean basin is an endemic area for *Leishmania* spp. circulation, with *Leishmania infantum* being the main species described in this region, and the dog (*Canis familiaris*) the most important domestic reservoir (Berriatua et al., 2021). Nevertheless, other domestic and wild mammals belonging to different families (e.g., Canidae, Felidae, Leporidae, Mustelidae, Muridae) can also act as potential reservoirs of *L. infantum* in the Mediterranean area (Millán et al., 2014; Cardoso et al., 2021).

The largest outbreak of human leishmaniosis ever reported in Europe occurred in Madrid (central Spain) in 2009, with more than 800 cases diagnosed (Arce et al., 2013; Mas et al., 2021). Epidemiological studies conducted during this epidemic outbreak confirmed a high circulation of *L. infantum* among wild lagomorph species, including Iberian hares (*Lepus granatensis*) and wild rabbits (*Oryctolagus cuniculus*), inhabiting areas surrounding municipalities affected by the leishmaniosis outbreak (Arce et al., 2013; García et al., 2014; Moreno et al., 2014). Xenodiagnostic studies on Iberian hares and wild rabbits captured in the area of the human outbreak also confirmed that these species are effective transmitters of *L. infantum* to *Phlebotomus perniciosus*, the main competent vector of this protozoan in Spain (Molina et al., 2012; Jiménez et al., 2014). Following that outbreak, a limited number of studies reported that *L. infantum* was circulating among wild lagomorphs in Spain and noted the complex network of competent wild hosts involved in the epidemiology of human leishmaniosis in anthropized areas (Chitimia et al., 2011; Ruiz-Fons et al., 2013; Díaz-Sáez et al., 2014). In the present study, a large-scale survey of wild lagomorph populations (Iberian hares and wild rabbits) in Iberian Mediterranean ecosystems was conducted to evaluate the exposure of *L. infantum* and to investigate potential risk factors associated with exposure to this zoonotic parasite.

2. Materials and methods

2.1. Study design and sample collection

A cross-sectional study was carried out to evaluate *L. infantum* exposure in wild lagomorphs from Andalusia (southern Spain; 36°N–38°60′N, 1°75′W–7°25′W). The study area (surface: 87,592 km^2) has a Mediterranean climate based on hot and dry summers and mild-to-cool and wet winters, with annual mean temperature and rainfall of 16 °C and 590 mm, respectively (CMAOT, 2009).

The sample size for wild rabbits was calculated with an estimated prevalence of 50 % (Ruiz-Fons et al., 2013), an accepted error of 5 % and a 95 % confidence interval (95 % CI), resulting in 385 animals to be sampled. Sampling sites were randomly selected from the sampling frame of the Andalusian Wildlife Disease Surveillance program (CAPMA, 2013), which divides this region into 23 hunting distribution areas (HDA) based on biological, physical and environmental habitat features and epidemiological criteria on presence and abundance of the large and small game species communities. In the present study, the selected sampling sites cover 11 HDA based on distribution and relative abundance of wild lagomorphs. Thus, between 2018 and 2021, a total of 471 wild rabbits from 38 hunting grounds (size range: 280–17497 ha) distributed across Andalusia were ultimately sampled (Fig. 1). In each hunting ground, between 5 and 25 (median: 12) wild rabbits were randomly selected. Convenience sampling was used for the Iberian hare, as its geographic distribution is limited to certain areas due to the recent decline in populations, primarily caused by the impact of myxomatosis on this species (García-Bocanegra et al., 2019, 2021). Samples were collected from all hunted Iberian hares, in all hunting grounds whenever possible. A total of 160 Iberian hares were collected (range: 1–11; median: 1) from 79 hunting grounds (size range: 309–15797 ha) during the same period.

The sampled animals were provided by legally authorized hunters. All individuals were inspected by members of our research team for macroscopic lesions compatible with transmissible diseases, including leishmaniosis. Then, blood samples were taken by intracardiac puncture or from the thoracic cavity. Subsequently, samples were centrifuged at 400 *g* for 15 min for serum collection. Additionally, spleens from 563 of the 631 sampled lagomorphs, including 441 wild rabbits and 122 Iberian hares, were collected. Both serum and spleen samples were stored at − 20 °C until serological and molecular analyses were performed.

An epidemiological questionnaire was performed by a direct interview with gamekeepers of each hunting ground. A total of 40 explanatory variables were selected to obtain information about potential risk factors related to *L. infantum* exposure in wild lagomorphs (Table 1). Additional data about hunting bags (number of individuals hunted per km^2) of the sampled hunting grounds were provided by the Regional Government of Andalusia. Also, meteorological information for each hunting ground [mean and maximum annual temperatures (°C), humidity (%), bioclimatic level (meso- or thermo-Mediterranean) mean annual rainfall (mm)] was collected from the closest official meteorological station (Spanish State Meteorological Agency, 2023).

2.2. Serological analysis

Sera from wild rabbits and Iberian hares were tested for anti-*Leishmania* antibodies using the indirect fluorescent antibody test (IFAT), as previously described (Moreno et al., 2014). Slides coated with *L. infantum* promastigotes (M/CAN/ES/97/10,445) and Fluorescein (FITC) AffiniPure Goat Anti-Rabbit IgG (Jackson Immunoresearch®, Cambridgeshire, UK) at 1:50 dilution were used. The criterion for a positive sample was based on the detection of a clear cytoplasmic and membrane fluorescence in promastigotes at a cut-off dilution of 1:50 (Moreno et al., 2014; Ortega et al., 2017). Positive sera were titrated by two-fold serial dilutions until negative results were obtained. Sera from an Iberian hare with confirmed *L. infantum* infection and from naïve rabbits were included on each slide as positive and negative controls, respectively (Moreno et al., 2014).

2.3. Molecular analysis

Total genomic DNA was extracted from 25 mg of spleen using the NucleoSpin® Tissue kit (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions. A 120-base-pair fragment from highly conserved regions of the kinetoplast DNA minicircle (kDNA) of *L. infantum* was amplified by real-time PCR (qPCR), using the primers LEISH-1 (5′-AACTTTTCTGGTCCTCCGGGTAG-3′) and LEISH-2 (5′ACCCCCAGTTTCCCGCC-3′), and the TaqMan-MGB probe (FAM-5′-AAAAATGGGTGCAGAAAT-3′-non-fluorescent quencher-MGB), as

previously described (Francino et al., 2006; Dantas-Torres et al., 2017). To determine the parasite load of each sample, a standard curve was constructed in duplicate with nine 10-fold serial dilutions ranging from 1000 ng to 10 fg of genomic DNA from *L. infantum* (reference strain: M/CAN/ES/97/10,445). The cut-off was defined as the threshold cycle (Ct) value at which the lowest amount of DNA was detected (Caraguel et al., 2011). Ct values lower than 37.6 were considered positive, and the number of parasites was calculated by interpolating the Ct values of the samples in the standard curve, on the basis that the genome of *L. infantum* has ~65 fg of DNA (Peacock et al., 2007; Dantas-Torres et al., 2017). This assay was developed for the detection of *L. infantum* kDNA (Francino et al., 2006) and has used a wide range of animal species (Ortega et al., 2017; Ortuño et al., 2022).

A selection of DNA from 20 positive qPCR samples (10 wild rabbits and 10 Iberian hares) with the lowest Ct values (≤28) were subsequently tested with conventional PCR (cPCR), using primers MC1 (5′-GTTAGCCGATGGTGGTCTTG-3′) and MC2 (5′-CACCCATTTTTCC-GATTTTG-3′) to amplify a 447-bp fragment of the *L. infantum* kDNA minicircle, following Cortes et al. (2004). The amplified PCR products of kDNA were purified using the QIAquick® PCR & Gel Cleanup Kit (Qiagen, Hilden, Germany) and sequenced by Stab Vida (Caparica, Portugal). Using the BLASTm tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the nucleotide sequences obtained were compared to evaluate similarity with sequences available in GenBank; multiple sequence alignments (using the MUSCLE tool) based on maximum likelihood (ML) estimation were also performed, using MEGA 11 software (Tamura et al., 2021). According to the lowest Bayesian Information Criterion (BIC), an ML tree (5000 replicates) was constructed following the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985), for which, a total of 26 nucleotide sequences were used, including those obtained in this study (n = 9) and other relevant *Leishmania* species available in GenBank (n = 17). In addition, a sequence of *Trypanosoma cruzi* (Accession number: M15512) was included as an outgroup.

2.4. Statistical analysis

Individual seroprevalence and prevalence of *L. infantum* were estimated by dividing the number of seropositive or positive animals by the total number of animals examined, with a 95 %CI (Thrusfield and Christley, 2018). Cut-off points for continuous variables were determined at the 33rd and 66th percentiles. Differences in parasite load between the two species were investigated using the Mann-Whitney U test. The level of agreement between diagnostic techniques was evaluated using Cohen's kappa statistic (κ) (McHugh, 2012), while the correlation between antibody titer and estimated parasite load was analyzed using the Kruskal-Wallis test. Univariable associations between the exposure to *L. infantum* (positivity to anti-*Leishmania* antibodies and/or *L. infantum* kDNA) and explanatory variables were analyzed using the Pearson's chi-squared test or Fisher's exact test, as appropriate (Table 1). Explanatory variables with $P \leq 0.10$ in bivariate analysis were selected as possible risk factors. Variables with a low response rate (<5 %) in at least one of the categories were excluded from the multivariable analysis. Collinearity between pairs of variables was also estimated using Cramer's V. When collinearity between variables was detected (correlation coefficient between variables > 0.6 and a $P \leq 0.05$), only the variable most clearly related to the epidemiology of *L. infantum* was included. Finally, a Generalized Linear Mixed Model (GLMM) was run using a binomial error distribution and a logit link function was performed with the lme4 R-package (Bates et al., 2015). To assess spatial differences, the "hunting ground" was added in the GLMM as a random effect. A backward stepwise strategy was performed, being the selection of the model made according to the Akaike's Information Criterion (AIC) in GLMM that retained variables with $P \leq 0.05$. Significance of the fixed effect variables was determined using car R-package (Fox and Weisberg, 2018). All statistical analyses were performed using R software version 4.1.3 (R Core Team, 2023) and significant differences were considered with $P \leq 0.05$ for a two-tailed test. The violin plot was

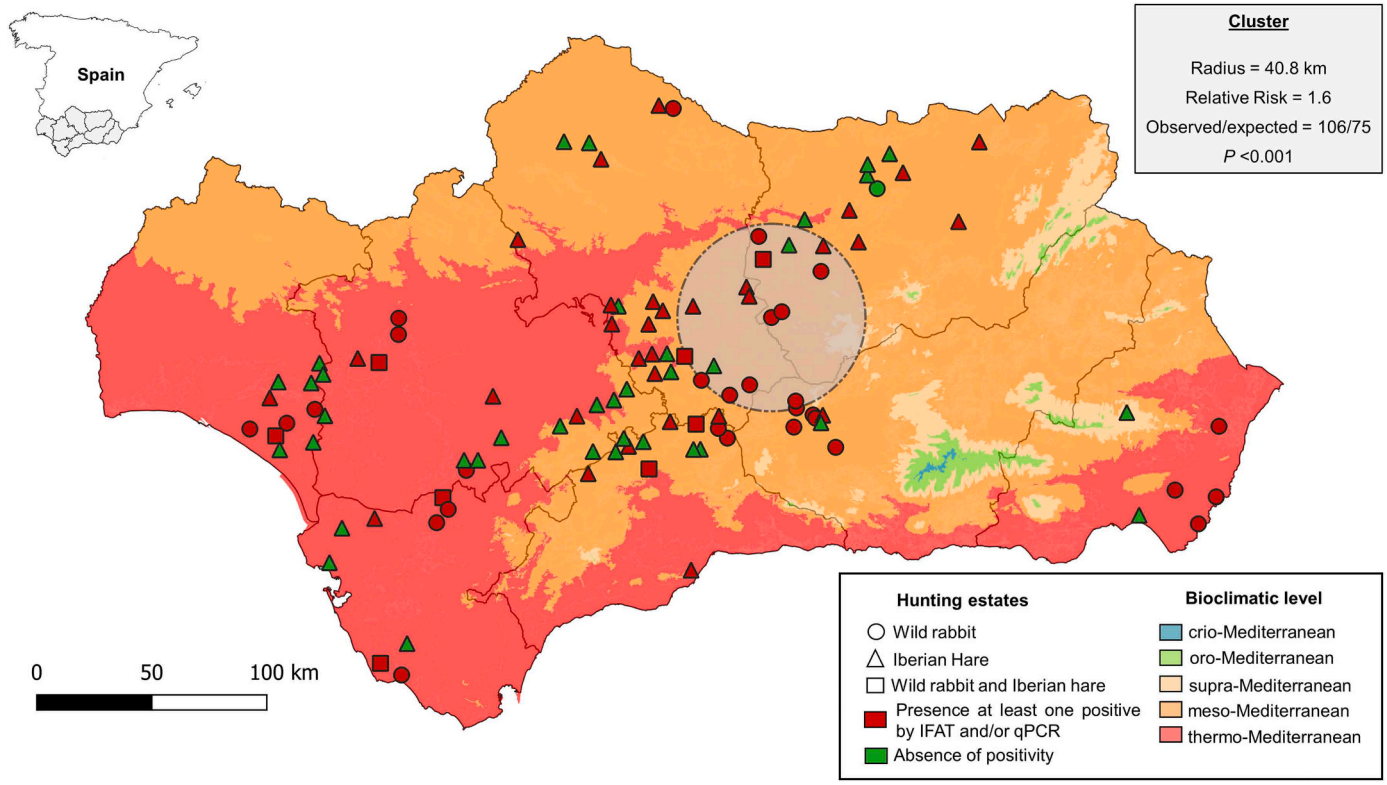

Fig. 1. Map of the geographical distribution of hunting grounds where wild rabbits (circles), Iberian hares (triangles) and both species (squares) were exposed to *L. infantum* (positivity to anti-*Leishmania* antibodies and/or *L. infantum* kDNA) in Andalusia (Southern Spain), including bioclimatic level. Gray dots represent the significant spatial cluster observed in the study area ($P < 0.001$).

Table 1
Exposure of *L. infantum* in wild lagomorphs from southern Spain based on serological and molecular analyses.

Variable	Category	Seroprevalence (IFAT)		Prevalence (qPCR)		Total exposure (IFAT and/or qPCR)		*P*-value[b]
		Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	
Species	Wild rabbit	50/471	10.6 (7.8–13.4)	269/441	61.0 (56.5–65.6)	278/471	59.0 (54.6–63.5)	0.030*
	Iberian hare	31/160	19.4 (13.3–25.5)	63/122	51.6 (42.8–60.5)	78/160	48.8 (41.0–56.5)	
Sex	Male	36/309	11.7 (8.1–15.2)	161/279	57.7 (51.9–63.5)	173/309	56.0 (50.5–61.5)	0.868
	Female	43/316	13.6 (9.8–17.4)	167/279	57.7 (54.1–65.6)	179/316	56.6 (51.2–62.1)	
Age	Adult	73/528	13.8 (10.9–16.8)	269/464	58.0 (53.5–62.5)	293/528	55.5 (51.3–59.7)	0.384
	Subadult	7/83	8.4 (2.5–14.4)	49/80	61.3 (50.6–71.9)	49/83	59.9 (48.5–69.6)	
	Young	1/20	5.0 (0.0–14.6)	14/19	73.7 (53.9–93.5)	14/20	70.0 (49.9–90.1)	
Kidney fat index	0	12/129	9.3 (4.3–14.3)	63/110	57.3 (48.0–66.5)	67/129	51.9 (43.3–60.6)	0.384
	1	28/171	16.4 (10.8–21.9)	90/162	55.6 (47.9–63.2)	98/171	57.3 (49.9–64.7)	
	2	14/118	11.9 (6.0–17.7)	68/109	62.4 (53.3–71.5)	72/118	61.0 (52.2–69.8)	
	3	12/80	15.0 (7.2–22.8)	47/71	66.2 (55.2–77.2)	50/80	62.5 (51.9–73.1)	
Bodyweight (kg)	≤ 0.9 kg	12/161	7.5 (3.4–11.5)	96/157	61.1 (53.5–68.8)	96/161	59.6 (52.1–67.2)	0.663
	> 0.9 kg	52/318	16.4 (12.3–20.4)	165/288	57.3 (51.6–63.0)	183/318	57.5 (52.1–63.0)	
Body length (cm)	19–37	9/133	6.8 (2.5–11.0)	80/128	62.5 (54.1–70.9)	82/133	61.7 (53.4–69.9)	0.442
	38–40	13/134	9.7 (4.7–14.7)	78/133	58.6 (50.3–67.0)	81/134	60.4 (52.2–68.7)	
	41–59	20/99	20.2 (12.3–28.1)	43/82	52.4 (41.6–63.3)	53/99	53.5 (43.7–63.4)	
Geographical area	Western	18/213	8.5 (4.7–12.2)	100/177	56.5 (49.2–63.8)	103/195	52.8 (45.8–59.8)	<0.001*
	Central	23/195	11.8 (7.3–16.3)	93/177	52.5 (45.2–59.9)	104/213	48.8 (42.1–55.5)	
	Eastern	40/223	17.9 (12.9–22.9)	139/209	66.5 (60.1–72.9)	149/223	66.8 (60.6–73.0)	
Mean temperature (°C)	12.3–16.7	40/241	16.6 (11.9–21.3)	134/204	65.7 (59.2–72.2)	149/241	61.8 (55.7–68.0)	0.024*
	16.8–17.5	29/184	15.8 (10.5–21.0)	98/167	58.7 (51.2–66.2)	105/184	57.1 (49.9–64.2)	
	17.6–18.5	9/189	4.8 (1.7–7.8)	91/177	51.4 (44.1–58.8)	92/189	48.7 (41.6–55.8)	
Mean annual rainfall (mm)	122–564	17/215	7.9 (4.3–11.5)	116/200	58.0 (51.2–64.8)	122/215	56.7 (50.1–63.4)	0.945
	565–600	48/220	21.8 (16.4–27.3)	110/180	61.1 (54.0–68.2)	125/220	56.8 (50.3–63.4)	
	601–1135	13/179	7.3 (3.5–11.1)	97/168	57.7 (50.3–65.2)	99/179	55.3 (48.0–62.6)	
Mean annual humidity (%)	≤65	30/201	14.9 (10.0–19.9)	103/179	57.5 (50.3–64.8)	110/201	54.7 (47.8–61.6)	0.483
	>65	7/87	8.0 (2.3–13.8)	41/79	51.9 (40.9–62.9)	43/87	49.4 (38.9–59.9)	
Bioclimatic level	Meso-Mediterranean	53/251	21.1 (16.1–26.2)	143/230	62.2 (55.9–68.4)	160/251	63.7 (57.8–69.7)	0.003*
	Thermo-Mediterranean	28/380	7.4 (4.8–10.0)	189/333	56.8 (51.4–62.1)	196/380	51.6 (46.6–56.6)	
Hunting season	2018–2019	3/61	4.9 (0.0–10.3)	24/46	52.2 (37.7–66.6)	25/61	41.0 (28.6–53.3)	0.007
	2019–2020	9/53	17.0 (6.9–27.1)	20/42	47.6 (32.5–62.7)	24/53	45.3 (41.9–58.7)	
	2020–2021	60/399	15.0 (11.5–18.5)	228/385	59.2 (54.3–63.1)	243/399	60.9 (56.1–65.7)	
	2021–2022	9/118	7.6 (2.8–12.4)	60/90	66.7 (56.9–76.4)	64/118	54.2 (45.3–63.2)	
Burrow density	High	55/421	13.1 (9.8–16.3)	233/395	59.0 (54.1–63.8)	248/421	58.9 (54.2–63.6)	0.887
	Medium	2/25	8.0 (0.0–18.6)	14/23	60.9 (40.9–80.8)	15/25	60.0 (40.8–79.2)	

(*continued on next page*)

Table 1 (*continued*)

Variable	Category	Seroprevalence (IFAT)		Prevalence (qPCR)		Total exposure (IFAT and/or qPCR)		*P*-value[b]
		Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	
	Low	14/71	19.7 (10.5–29.0)	40/60	66.7 (54.7–78.6)	44/71	61.9 (50.7–73.3)	
Distance to urban areas (Km)	<10	69/508	13.6 (10.6–16.6)	285/469	60.8 (56.4–65.2)	304/508	59.8 (55.6–64.1)	0.188
	10–20	1/6	16.7 (0.0–46.5)	2/6	33.3 (0.0–71.1)	2/6	33.3 (0.0–71.1)	
Presence of fleas	No	27/218	12.4 (8.0–16.8)	120/209	57.4 (50.7–64.1)	129/218	59.2 (52.7–65.7)	0.676
	Yes	38/239	15.9 (11.3–20.5)	137/231	59.3 (53.0–65.6)	146/239	61.1 (54.9–67.3)	
Presence of ticks	No	20/139	14.4 (8.6–20.2)	85/133	63.9 (55.8–72.1)	92/139	66.2 (58.3–74.1)	0.083
	Yes	45/318	14.2 (7.6–14.5)	172/307	56.0 (50.5–61.6)	183/318	57.5 (52.1–63.0)	
Presence of wildcat (*Felis silvestris silvestris*)	No	53/346	15.3 (11.5–19.1)	203/319	63.6 (58.4–68.9)	218/346	63.0 (57.9–68.1)	0.017
	Yes	18/171	10.5 (5.9–15.1)	84/159	52.8 (45.1–60.6)	89/171	52.0 (44.6–59.5)	
Presence of badger (*Meles meles*)	No	7/51	13.7 (4.3–23.2)	27/47	57.4 (43.3–71.6)	28/51	54.9 (41.3–68.6)	0.493
	Yes	64/466	13.7 (10.6–16.9)	260/431	60.3 (55.7–64.9)	279/466	59.9 (55.4–64.3)	
Presence of mongooses (*Herpestes ichneumon*)	No	18/118	15.3 (8.8–21.7)	77/117	65.8 (57.2–74.4)	82/118	69.5 (61.2–77.8)	0.006
	Yes	56/390	11.8 (10.9–17.8)	205/352	58.2 (53.1–63.4)	216/390	55.4 (50.5–60.3)	
Presence of genets (*Genetta genetta*)	No	18/98	18.4 (10.7–26.0)	52/93	55.9 (45.8–66.0)	58/98	59.2 (49.5–68.9)	0.965
	Yes	53/490	12.6 (8.1–13.6)	235/385	61.0 (56.2–65.9)	249/418	59.6 (54.9–64.3)	
Presence of marten (*Martes foina*)	No	24/175	13.7 (8.6–18.8)	90/157	57.3 (49.6–65.1)	97/175	55.4 (48.1–62.8)	0.191
	Yes	47/342	13.7 (10.1–17.4)	197/321	61.4 (56.0–66.7)	210/342	61.4 (56.2–66.6)	
Presence of weasel (*Mustela nivalis*)	No	15/117	12.8 (6.8–18.9)	54/101	53.5 (43.7–63.2)	60/117	51.3 (42.2–60.3)	0.043*
	Yes	56/400	14.0 (10.6–17.4)	233/377	61.8 (56.9–66.7)	247/400	61.8 (57.0–66.5)	
Presence of polecat (*Mustela putorius*)	No	30/262	11.5 (7.6–16.3)	151/237	63.7 (57.6–69.8)	160/262	61.1 (55.2–67.0)	0.426
	Yes	41/255	16.1 (11.6–20.6)	136/241	56.4 (50.2–62.7)	147/255	57.6 (51.6–63.7)	
Presence of Iberian lynx (*Lynx pardinus*)	No	68/440	15.5 (12.1–18.8)	259/421	61.5 (56.9–66.2)	278/440	63.2 (58.7–67.7)	<0.001
	Yes	3/77	3.9 (0.0–8.2)	28/57	49.1 (36.1–62.1)	29/77	37.7 (26.8–48.5)	
Presence of domestic cat (*Felis silvestris catus*)	No	7/80	8.8 (2.6–14.9)	48/75	64.0 (53.1–74.9)	49/80	61.3 (50.6–71.9)	0.711
	Yes	64/437	14.6 (11.3–18.0)	239/403	59.3 (54.5–64.1)	258/437	59.0 (54.4–63.7)	
Presence of domestic dog (*Canis familiaris*)	No	15/128	11.7 (6.2–17.3)	61/114	53.5 (44.4–62.7)	65/128	50.8 (42.1–59.4)	0.029*
	Yes	56/389	14.4 (10.9–17.9)	226/364	62.1 (57.1–67.1)	242/389	62.2 (57.4–67.0)	
Presence of cattle (*Bos taurus*)	No	63/375	16.8 (13.0–20.6)	221/360	61.4 (56.4–66.4)	238/375	63.5 (58.6–68.3)	0.001
	Yes	0/37	0.0	13/37	35.1 (19.8–50.5)	13/37	35.1 (19.8–50.5)	
Presence of domestic goat (*Capra aegagrus hircus*)	No	22/247	8.9 (5.4–12.5)	128/240	53.3 (47.0–59.6)	133/247	53.8 (47.6–60.1)	<0.001 *
	Yes	41/165	24.8 (18.3–31.4)	106/157	67.5 (60.2–74.8)	118/165	71.5 (64.6–78.4)	
Presence of sheep (*Ovies aries*)	No	36/188	19.1 (13.5–24.8)	117/179	65.4 (58.4–72.3)	124/188	66.0 (59.2–72.7)	0.055
	Yes	27/224	12.1 (7.8–16.3)	117/218	53.7 (47.1–60.3)	127/224	56.7 (50.2–63.2)	
Presence of farmed rabbit (*Oryctolagus cuniculus*)	No	59/464	12.7 (9.7–15.8)	255/436	58.5 (53.9–63.1)	273/464	58.8 (54.4–63.3)	0.455
	Yes	12/53	22.6 (11.4–33.9)	32/42	76.2 (63.3–89.1)	34/53	64.2 (51.2–77.1)	
Presence of domestic pig (*Sus scrofa domesticus*)	No	62/387	16.0 (12.4–19.7)	228/372	61.3 (56.3–66.2)	244/387	63.0 (58.2–67.9)	<0.001

(*continued on next page*)

Table 1 (*continued*)

Variable	Category	Seroprevalence (IFAT)		Prevalence (qPCR)		Total exposure (IFAT and/or qPCR)		*P*-value[b]
		Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	Number/ Overall[a]	% (IC95%)	
	Yes	1/25	4.0 (0.0–11.7)	6/25	24.0 (7.3–40.7)	7/25	28.0 (10.4–45.69	
Burrow deworming	No	38/250	15.2 (10.8–19.7)	151/229	65.9 (59.8–72.1)	159/250	63.6 (57.6–69.6)	0.071
	Yes	33/267	12.4 (8.4–16.3)	136/249	54.6 (48.4–60.8)	148/267	55.4 (49.5–61.4)	
Presence of swamps	No	63/476	13.2 (10.2–16.3)	265/440	60.2 (55.7–64.8)	283/476	59.5 (55.0–63.9)	0.909
	Yes	8/41	19.5 (7.4–31.6)	22/38	57.9 (42.2–73.6)	24/41	58.5 (43.5–73.6)	
Presence of waterholes	No	33/319	10.3 (7.0–13.7)	167/290	57.6 (51.9–63.3)	180/319	56.4 (51.0–61.9)	0.083
	Yes	38/198	19.2 (13.7–24.7)	120/188	63.8 (57.0–70.7)	127/198	64.1 (57.5–70.8)	
Presence of streams	No	25/239	10.5 (6.6–14.3)	133/213	62.4 (55.9–68.9)	142/239	59.4 (53.2–65.6)	0.989
	Yes	46/278	16.5 (12.2–20.9)	154/265	58.1 (52.2–64.1)	165/278	59.4 (53.6–65.1)	
The hunting estate is weeded	No	62/416	14.9 (11.5–18.3)	224/387	57.9 (53.0–62.8)	242/416	58.2 (53.4–62.1)	0.256
	Yes	9/101	8.9 (3.4–14.5)	63/91	69.2 (59.8–78.1)	65/101	64.4 (55.0–73.7)	
Cleaning watering places	No	38/306	12.4 (8.7–16.1)	160/287	55.7 (50.0–61.5)	170/306	55.6 (50.0–61.1)	0.033
	Yes	33/211	15.6 (10.7–20.5)	127/191	66.5 (59.8–73.2)	137/211	64.9 (58.5–714)	
Presence of artificial burrows	No	68/469	14.5 (11.5–17.7)	255/432	59.0 (54.4–63.7)	274/469	58.4 (54.0–62.9)	0.165
	Yes	3/48	6.3 (0.0–13.1)	32/46	69.6 (56.3–82.9)	33/48	68.8 (55.6–81.9)	
Crops intended for hunting	No	56/411	13.6 (10.3–16.9)	163/266	61.3 (55.4–67.1)	179/282	63.5 (57.9–69.1)	0.038
	Yes	15/106	14.2 (7.5–20.8)	124/212	58.5 (51.9–65.1)	128/235	54.5 (48.1–60.8)	
Lagomorph density (number of animal hunted/km^2)	High (51−100)	7/56	12.5 (3.8–21.2)	39/54	72.2 (60.3–84.2)	41/56	73.2 (61.6–84.8)	0.005
	Medium (26−50)	5/56	8.9 (1.5–16.4)	25/29	86.2 (73.7–98.8)	26/30	86.7 (74.5–98.8)	
	Low (11−25)	5/30	16.7 (3.3–30.0)	33/47	70.2 (57.1–83.39	34/56	60.7 (47.9–73.5)	
	Very low (0−10)	32/156	20.5 (14.2–26.9)	72/131	55.0 (46.4–63.5)	88/156	56.4 (48.6–64.2)	

Note: variables with a response rate < 5 % were not considered for the multivariable analysis.
[a] Missing values were omitted.
[b] *P*-value was obtained comparing "variables" with "total exposure".
[*] Variables included in the GLMM model.

constructed using the function ggbetweenstats from ggstatsplot R-package.

A spatial scan statistical analysis was applied using a Bernoulli model to detect significant clusters of high *L. infantum* exposure at hunting ground level, using SaTScan v.10.1.2 software. The number of Monte Carlo simulations was set to 1000 for the cluster scan statistic. SaTScan was used to estimate relative risk (RR), representing the relative frequency of cases (positive individuals to IFAT and/or qPCR) compared to baseline, for each cluster. Clusters were considered significant at $P \leq 0.05$.

3. Results

Overall, 356 out of 631 wild lagomorphs (56.4 %; 95 %CI: 52.3–60.3) tested positive for *L. infantum* exposure. A higher level of exposure was detected in wild rabbits (59.0 %; 278/471; 95 %CI: 54.6–63.5) compared to Iberian hares (48.8; 78/160; 95%CI: 41.0–56.5) (Table 1). None of the sampled individuals showed macroscopic lesions compatible with leishmaniosis. At least one positive animal was found on 64.8 % (70/108; 95 %CI: 55.8–73.8) of the hunting grounds sampled. Spatial analysis identified a high-risk spatial cluster (radius: 40.8 km; $P < 0.001$; RR = 1.6) that included 15 hunting grounds located in central Andalusia (Fig. 1).

The overall apparent seroprevalence of *L. infantum* was 12.8 % (81/631; 95 %CI: 10.2–15.5). Seropositivity in Iberian hares (19.4 %; 31/160; 95 %CI: 13.3–25.5) was significantly higher compared to wild rabbits (10.6 %, 50/471; 95 %CI: 7.9–13.4) ($P = 0.004$) (Table 1). In seropositive lagomorphs, a wide range of antibody titers was obtained, distributed as follows: 1:50 (44.4 %; 36/81), 1:100 (24.7 %; 20/81), 1:200 (9.9 %; 8/81), 1:400 (13.6 %; 11/81), 1:800 (4.9 %; 4/81), 1:1600 (1.2 %; 1/81) and 1:3200 (1.2 %; 1/81).

Molecular analysis showed an overall prevalence of 59.0 % (332/563; 95 %CI: 54.9–63.0). No statistically significant differences were found between wild rabbits (61.0 %; 269/441, 95 %CI: 56.5–65.6) and Iberian hares (51.6 %; 63/122, 95 %CI: 42.8–60.5) (Table 1). Ct values of qPCR in positive samples (≤37.6) ranged from 22.4 to 37.6 (median: 34.1). The estimated relative number of parasites per sample was correspondingly variable, ranging from 3.6 to 178,106.0 parasites/g

(median: 38.8). The parasite load was also significantly higher ($P < 0.001$) in Iberian hares (median: 154.1 parasites/g) compared to wild rabbits (median: 33.9 parasites/g) (Fig. 2). A total of 563 animals were analyzed by both IFAT and qPCR. However, only a slight concordance between the two techniques was found ($\kappa = 0.09$). Furthermore, no correlation was detected between antibody titers and estimated parasite load.

Fifteen of the 20 selected samples detected as positive by qPCR were also positive by conventional PCR. Of these, six wild rabbits and three Iberian hares showed a strong band in electrophoresis suitable for sequencing. After purification, these samples were successfully sequenced and registered in GenBank under accession numbers OQ969202-OQ969210. Sequencing analysis revealed high homology (99.9–100 %) between all isolates in the present study and two sequences of *L. infantum* previously detected in humans in a region bordering the study area (Fig. 3).

After applying the exclusion criteria, a total of seven of the 40 explanatory variables were selected for inclusion in the multivariable analysis (Table 1). The GLMM (AIC: 513.6; full model: 506.4) identified two risk factors significantly associated with exposure to *L. infantum*: bioclimatic level (meso-Mediterranean climate) and the presence of goats on the hunting ground (Table 2).

4. Discussion

To our knowledge, this is the largest epidemiological study to date to address the exposure of wild lagomorphs to *L. infantum*. The level of exposure to *L. infantum* detected in the present study (56.4 %) indicates high exposure of this protozoan in wild lagomorph populations in the Mediterranean ecosystem of southern Spain, and is higher than levels detected in previous surveys on wild lagomorphs in other Mediterranean countries, such as Greece (7.5 %; 36/483) (Tsakmakidis et al., 2019) or Italy (6.5 %; 6/92) (Abbate et al., 2019). These results in Mediterranean ecosystems in southern Spain are also higher than those reported in wild rabbits in eastern Spain during 2009–2010 (0.6 %; 1/162) (Chitimia et al., 2011). However, the level of exposure found in our study falls within the range (23.7–78.9 %) detected in the same lagomorph species during the human leishmaniosis outbreak in Madrid in 2009 (García et al., 2014; Moreno et al., 2014; Ortega et al., 2017; Ortega-García et al., 2019). Previous small-scale surveys conducted in our study area have also detected highly variable prevalence (20.7–100 %) in the two lagomorph species (Ruiz-Fons et al., 2013; Díaz-Sáez et al., 2014; Martín-Sánchez et al., 2021).

The large difference between the serological (12.8 %) and molecular (59.0 %) results obtained in the present study is noteworthy. Although IFAT has been shown to be highly sensitive and specific for monitoring the circulation of *Leishmania* spp. in wild lagomorphs (Moreno et al., 2014; WOAH, 2021), this serological technique may underestimate exposure to *Leishmania* spp. in wild asymptomatic reservoirs, which usually develop effective cell-mediated immunity after *Leishmania* spp. infection, so that the presence of antibodies may be low or undetectable (Solano-Gallego et al., 2001; Lachaud et al., 2002; Millán et al., 2014; Ortega-García et al., 2019). For this reason, it is recommended to use both molecular and serological diagnostic techniques in order to properly assess the exposure of *L. infantum* in wild asymptomatic lagomorphs.

A high rate of *L. infantum* positivity was detected on the hunting grounds sampled (64.8 %) indicating wide exposure to this protozoan among wild lagomorph populations in the study area. However, the parasite's geographical distribution was heterogeneous, as the spatial analysis identified a significant spatial cluster located in central Andalusia. Previous studies in this region have suggested that local climate conditions may favor an abundance of competent vectors of *L. infantum* in this area, increasing the exposure of hosts susceptible to this parasite (Barón et al., 2011; Martín-Sánchez et al., 2009, 2020). Indeed, this area has been identified as a hotspot for *L. infantum* circulation, with reports of high seroprevalence in dogs (33.3–47.3 %) and humans (12.9 %) (Aliaga et al., 2019; Martín-Sánchez et al., 2020). Recent studies have also highlighted the association between the prevalence of *L. infantum* in wild rabbits and clinical cases of human leishmaniosis within this cluster (Martín-Sánchez et al., 2021). In a similar vein, our results showed strong homology (99.9–100 %) between the *L. infantum* sequences detected in the present study and human sequences previously isolated in southern Spain (Fig. 2) (Ortuño et al., 2019). This suggests a common epidemiological transmission cycle of *L. infantum* involving humans and wild lagomorphs in Iberian Mediterranean ecosystems.

Interestingly, although the prevalence in rabbits (61.0 %) and hares (51.6 %) was similar, there were significant differences in seropositivity (19.4 % in hares *vs* 10.6 % in rabbits) and parasite load (median: 154.1 parasites/g in hares and 33.9 parasites/g in rabbits) between the two lagomorph species tested. These results are in agreement with previous studies suggesting that the higher seropositivity and parasite burden of *L. infantum* in Iberian hares compared to wild rabbits is mainly due to the development of humoral immunity in hares, which prevents effective control of *Leishmania* spp. infection (Moreno et al., 2014; Ortega-García et al., 2019). This finding indicates the important epidemiological role

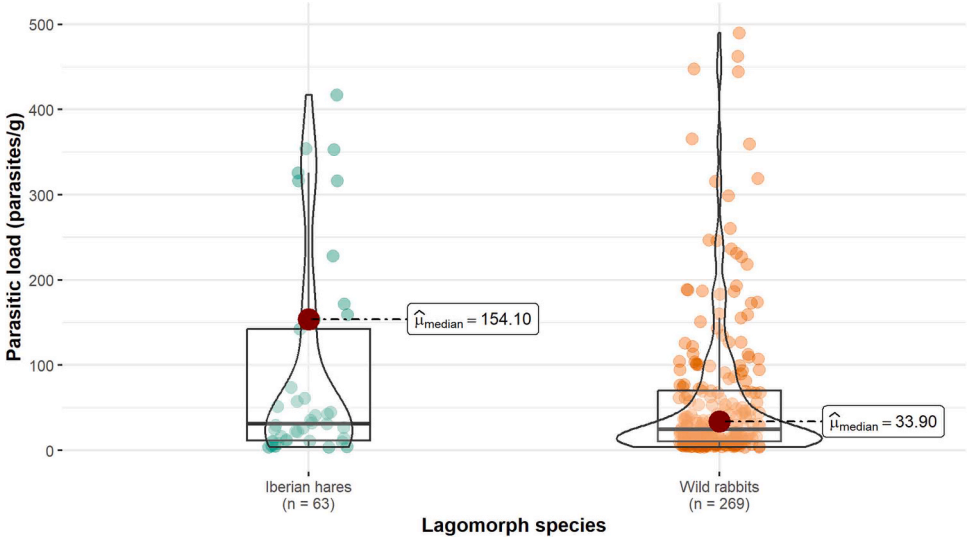

Fig. 2. Violin plots representing *L. infantum* parasite loads in Iberian hares and wild rabbits in the southern Iberian Peninsula.

Fig. 3. Phylogenetic relationship of *L. infantum* from wild rabbits and Iberian hares detected in this study and other kDNA reference sequences of *L. infantum* available in the GenBank database. The sequences obtained in this study are indicated by black circles for wild rabbits and black triangles for Iberian hares. Sequences are represented with their GenBank accession number, species of infected host (when known) and country of origin. Branches corresponding to partitions reproduced in less than 70 % of bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown above the branches (Felsenstein, 1985). (a) Brown bear, beech marten, rabbit, fox, genet, wolf, rat; (b) rabbit.

Table 2
Results of the generalized linear mixed model of potential risk factors associated with *L. infantum* exposure in wild lagomorphs in southern Spain including "hunting ground" variable as a random effect.

Variable	Category	OR (95 % CI)	*P*-value
Presence of domestic goat (*Capra aegagrus hircus*)	Yes	1.5 (1.2–6.2)	0.011
	No	*	*
Bioclimatic level	Meso-Mediterranean	2.9 (1.2–6.9)	0.022
	Thermo-Mediterranean	*	*

* Reference category.

of hares as natural reservoirs of *L. infantum* compared to wild rabbits in Iberian ecosystems (Jiménez et al., 2014; Molina et al., 2012).

Risk factor analysis revealed that *L. infantum* exposure was significantly higher in wild lagomorphs from areas with a meso-Mediterranean climate compared to those inhabiting thermo-Mediterranean areas. In regard to this, significant high exposure to *L. infantum* in domestic dogs in the study area has already been associated with a meso-Mediterranean climate as compared to other bioclimatic regions (Martín-Sánchez et al., 2009). The abiotic characteristics of this climate present the most favorable conditions (i.e., temperate temperatures, moderate humidity) for the breeding and development of sand flies (Martín-Sánchez et al., 2009; Barón et al., 2011). The GLMM model also showed that wild lagomorphs from hunting grounds where domestic goats were present were significantly more exposed to *L. infantum* (71.5 %) than those from grounds where this species was absent (53.8 %). Previous studies have suggested the likely role of domestic goats as competent reservoirs of *Leishmania* spp. (Bhattarai et al., 2010; Rezaei et al., 2022; Singh et al., 2013). The presence of livestock, including goats, has also been associated with a high abundance of competent sand fly populations, particularly females, which could increase exposure to *L. infantum* in other sympatric species, such as wild lagomorphs (Gálvez et al., 2010; Cortes et al., 2012; Dantas-Torres et al., 2014; Risueño et al., 2017; Villanueva-Saz et al., 2023). However, further studies are needed to assess the role of goats in the epidemiology of *L. infantum* in the Iberian Peninsula.

Some limitations of our study should be noted. First, given the low density of Iberian hares in the study area, convenience sampling had to be used, which may have led to selection bias. Second, there is no information available on the sensitivity and specificity of IFAT in the tested species, so the true seroprevalence could not be established. In this regard, further studies are warranted to evaluate the accuracy of IFAT for the diagnosis of *Leishmania* spp. in wild lagomorphs.

In conclusion, the results obtained in the present study indicate a high, widespread but heterogeneous exposure of *L. infantum* in wild rabbits and Iberian hares in Spanish Mediterranean ecosystems. We also provide information on the potential animal and public health

implications of these lagomorph species acting as natural reservoirs of *L. infantum* in the study area. Our findings point to the need to promote integrated surveillance programs for leishmaniosis under a One Health approach, making the monitoring of wild lagomorphs a priority, particularly in high-risk areas where *L. infantum* is actively circulating.

Ethics statement

No ethical approval was required since no animals were killed specifically for this study. All animals were legally hunted following the Spanish and European Union legislation No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

CRediT authorship contribution statement

Débora Jiménez-Martín: Writing – review & editing, Investigation, Conceptualization. **David Cano-Terriza:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Moisés Gonzálvez:** Writing – review & editing, Investigation, Conceptualization. **Sabrina Castro-Scholten:** Writing – review & editing, Investigation, Conceptualization. **Mercedes Domínguez:** Writing – review & editing, Investigation, Conceptualization. **Inmaculada Moreno:** Writing – review & editing, Investigation, Conceptualization. **Ignacio García-Bocanegra:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Remigio Martínez Pérez:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Jesús Barbero-Moyano:** Writing – original draft, Investigation, Conceptualization. **Leonor Camacho-Sillero:** Writing – review & editing, Investigation, Conceptualization.

Declaration of Competing Interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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