Received: 15 January 2024 | Revised: 4 April 2024 | Accepted: 20 April 2024

DOI: 10.1111/zph.13139

SHORT COMMUNICATION

WILEY

Wild rabbits are *Leishmania infantum* reservoirs in southeastern Spain

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Funding information
Ministerio de Ciencia e Innovación, Spain (MICIU/AEI/10.1339/50110011033); FEDER, UE through the project, Grant/Award Number: PID2022-142230NB.100; Universidad de Granada/CBUA

Abstract

Objective: We contribute to the understanding of the transmission dynamics of *Leishmania infantum* suggesting the involvement of rabbits as wild reservoirs.

Results: The prevalence of infection was 86.0% (270/314 wild rabbits) ranging from 18.2% to 100% in natural geographical regions. The estimated average parasite load was 324.8 [CI 95% 95.3–554.3] parasites per mg of ear lobe ranging from 0 to 91,597 parasites/mg per tissue section.

Conclusions: A positive correlation was found between skin parasite load in wild rabbits and human incidence with evidence of the presence of the same *L. infantum* genotypes in rabbits and humans, providing new epidemiological and biological basis for the consideration of wild rabbits as a relevant *L. infantum* wild reservoir. Molecular parasite surveillance reflects the great genotypic variability of the parasite population in wild rabbits. Most of these genotypes have also been found to infect humans, dogs and sandflies in the region. Our findings also highlight that direct genotyping of the parasite in host tissues should be used for molecular surveillance of the parasite instead of cultured isolates.

KEYWORDS
Leishmania infantum, Mediterranean hotspots, parasite molecular surveillance, reservoir network, vector-borne diseases, wild rabbits

1 | INTRODUCTION

Leishmania infantum is a causative agent of visceral, cutaneous and mucosal human leishmaniasis as well as canine leishmaniasis in the Mediterranean basin (Martín-Sánchez et al., 2020). *Leishmania infantum* parasites are transmitted between hosts during blood-feeding by infected female sand flies (Diptera, Psychodidae), mainly of the *Larroussius* subgenus, characterized by their great opportunism. The dog is the main domestic reservoir of *L. infantum* (Morales-Yuste et al., 2022). Limiting the prevalence of *L. infantum* infection in the domestic dog may result in a reduced risk of infection for the human population, provided no other reservoirs are involved in the area. However, Mediterranean hotspots are associated with the presence of a network of wild and domestic reservoirs at the crossroads between sylvatic and urbanized areas for the spread of leishmaniasis (Martín-Sánchez et al., 2021;

Navea-Pérez et al., 2015). Wild rabbits (*Oryctolagus cuniculus*) have recently been associated with both epidemic outbreaks and non-epidemic hot spots (González et al., 2017; Martín-Sánchez et al., 2021) and are widely distributed across Europe (Millán et al., 2014).

Our objective was to investigate the prevalence of *L. infantum* infection in wild rabbits and its relationship with human incidence in an area were previous studies indicated no relationship between this and canine leishmaniasis prevalence (Martín-Sánchez et al., 2020). Parasite molecular surveillance by genotyping was carried out using markers that discriminate *L. infantum* strains such as the K26 gene (based on the amplicon size) and kDNA (PCR-sequencing) (Cortes et al., 2004; Haralambous et al., 2008).

Impacts

- *Leishmania infantum* prevalence and infection density in wild rabbits may be very high.
- A great parasite genotypic variability is detected in rabbits and these genotypes are also found infecting humans, dogs and sand flies.
- Wild rabbits may be an efficient reservoir of *L. infantum*.

2 | MATERIALS AND METHODS

Research on *O. cuniculus* was conducted in the area (Figure 1) in October–November 2018–2019. Abundance of wild rabbits ranged from very low to high across natural regions (Consejería de Medio Ambiente y Ordenación del Territorio, Andalusian government, 2019). Three hundred and fourteen wild rabbits were shot by hunters on hunting grounds during authorized rabbit hunting periods. Three 5×5-mm^2 tissue sections were collected from one ear of each animal (942 samples in total) and weighed.

DNA extracted from skin sections was qualitative and quantitative assessed the concentration adjusted to 700 ng/μL. Samples (5 μL DNA) were tested for *L. infantum* infection using a *L. infantum* PCR-ELISA and the GRANALEISH Multiplex qPCR (University of Granada, Spain, Trade Mark Number 3667362/5) according to Martín-Sánchez et al., 2020, 2021. Briefly, PCR-ELISA is a semi-quantitative technique that detects *L. infantum* by amplifying a 75 bp fragment from the variable region of the *L. infantum* kDNA minicircle. In the PCR, forward and reverse primers amplify a fragment labelled with digoxigenin. Detection is made on a streptavidin-coated microtitre plate, through hybridization with a specific *L. infantum* oligonucleotide probe labelled at the 5′-end with biotin, followed by ELISA using a peroxidase-labelled anti-digoxigenin antibody and ABTS as substrate; reading is made in a spectrophotometer at 405 nm. GRANALEISH Multiplex qPCR (University of Granada, Spain, Trade Mark Number 3667362/5) can differentiate between *L. infantum*, *L. tropica* and *L. major* and allows quantification of the parasite load. In the PCR, four primers (two forward and two reverse ones) and three TaqMan probes specific for each *Leishmania* species are provided by the manufacturer to be mixed with the others components of the PCR. The number of parasites in every qPCR reaction was calculated through the interpolation of the cycle threshold (Ct) value in a previously constructed *L. infantum* DNA standard curve (efficiency of 99.5%, $R^2 = 97.5\%$). A negative result was assigned when no amplification occurred or when Ct value was higher than 36.00; for the positive control with 1500 parasites the Ct was 17.00.

For parasite molecular surveillance, highly parasitized tissues (≥100 parasites/μg DNA) from wild rabbits, Iberian hares, dogs, sandflies (*Phlebotomus perniciosus*) and humans, and several cultivated isolates (Toledo et al., 2002) were amplified using primers targeting: (1) a 447 bp fragment of the minicircle kDNA used for sequencing as previously described by Cortes et al., 2004, and (2) a short nuclear repetitive region of the hydrophilic acylated surface protein B gene, known as k26 gene, used for the estimation of the product size by electrophoresis, as previously described by Haralambous et al., 2008. kDNA sequences were aligned, manually adjusted, and finally analysed using MEGA11 with three different methods of clustering (maximum likelihood -ML-, maximum parsimony -MP- and distance matrix analysis with both neighbour-joining -NJ- and unweighted pair group method using an arithmetic average -UPGMA-), and bootstrap analysis. Genetic distances among taxa were also measured using the Kimura 2-parameter model.

Statistical analyses were carried out with the IBM SPSS Statistics 20 software; for linear regression, the "Automated linear modelling" option was used.

Ethical approval is not required because we do not work with life animals. Rabbits are shot by hunters during authorized closed periods and it is the hunter himself who cuts off the rabbit's ear. Ethical approval for the One Health research was granted by the Research Ethics Committee of the University of Granada (Project PI14-01024).

3 | RESULTS AND DISCUSSION

The prevalence of *L. infantum* infection in the wild rabbit reservoir was 86.0% (270/314), both for the two PCR techniques and by PCR-ELISA, while it was 75.8% (238/314) for qPCR; this value ranged from 18.2% to 100% across natural geographical regions (Table 1). Only 14.0% of the 314 wild rabbits tested negative for *L. infantum* in all three tissue sections by both PCR techniques used. The percentage of rabbit ear sections tested positive was 72.2% (680/942). The estimated average parasite load was 324.8 [CI 95% 95.3–554.3] parasites per mg of ear lobe ranging from 0 to 91,597 parasites/mg per tissue section, a value much higher than that reported in the skin of infected dogs by Courtenay et al. (2014) (119 parasites/g) in Amazon Brazil. The average parasite load in rabbit ear skin varied between natural geographical regions, from 0.001 parasites/mg in the Huéscar region (where leishmaniasis cases have not been reported) to 1013 parasites/mg in the Loja region (Table 1),

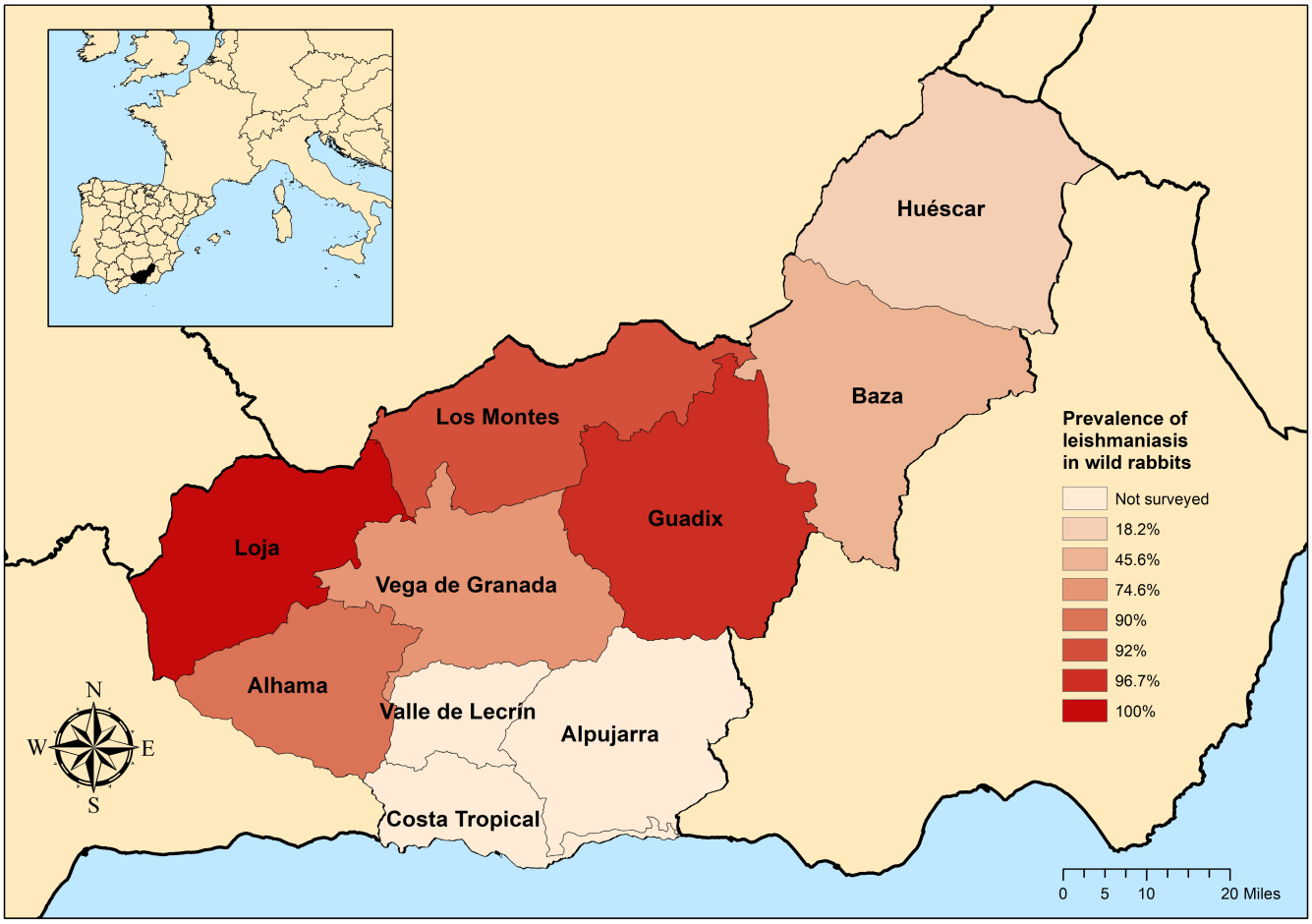

FIGURE 1 Geographical location of the study area showing its division into natural regions.

characterized by the highest human incidence of the study area (Martín-Sánchez et al., 2020). A positive linear association is observed between skin parasite load and human incidence providing a hypothetical epidemiological basis for the spatial clustering of the clinical disease (Pearson correlation: 0.791, $p = 0.034$). The following linear regression model could be fitted: Incidence = 0.701 + 0.02 × [Skin_Parasite_load], $p < 0.05$ (Supplementary file S1). Globally, in 9.2% wild rabbits, parasite loads $>10^6$ parasites/g skin were found. We hypothesize that these individuals are super-spreaders, making an analogy with the proposal of Courtenay et al., 2017. Differences between natural regions were detected (Table 1): in three regions, no super-spreaders were detected, while in the fourth region (Alhama), the probability of finding them was significantly lower than in the region of Loja ($p = 0.021$) characterized by the highest positivity rate of rabbits, with high parasite loads and by the highest incidence of human leishmaniasis in the study area. The majority of infected dogs have loads $<10^6$ parasites/g skin and are very rarely infectious to sand flies (Courtenay et al., 2014). These findings agree with the hypothesis that most parasites inhabit a few heavily infected hosts while the remaining hosts harbour few parasites. Sand flies become highly infected after feeding on hosts with high parasite loads and initiate more severe infections upon subsequent transmission (Courtenay et al., 2017).

Direct amplification of *L. infantum* kDNA was successful in 15 rabbits. Amplicons were subsequently sequenced and compared with other samples (two tissues from Iberian hares, one from dog, two from *P. perniciosus* and 13 from humans; four cultivated isolates from *P. perniciosus*, four from humans and one from dog) yielding a total of 42 kDNA sequences (421 bp), 17 haplotypes, four indels and 30 polymorphic sites (File S2). The comparison with previous published *L. infantum* sequences from southeastern Spain (Ortuño et al., 2019) provided 25 haplotypes for which phylogenetic relationships were analysed. The topologies of the ML, MP, NJ and UPGMA phylogenetic trees were highly congruent and highlighted two well bootstrap-supported clusters within *L. infantum* (Figure 2). Genetic distances between these clusters ranged from 0.011 to 0.042 with six fixed differences identified at nucleotide positions 66 (T/C), 182 (A/G), 303 (G/A), 343 (G/A), 352 (G/A) and 374 (G/A) whereas distances within clusters were 0.004 for cluster 2 and 0.004 to 0.030 for the most heterogeneous cluster 1. Genetic distances with *Labrus donovani* ranged from 0.147 to 0.166 and 44 synapomorphic characters were found (File S2). *Leishmania infantum* genotypes from wild rabbits showed widely dispersed and were also found in humans, dogs and sand flies.

Electrophoresis revealed 16 different k26 PCR product sizes (140 to 920 bp) for 90 samples. In the samples from rabbits, 12 k26

bands were identified that were also detected in the parasites from other vertebrate (humans, dogs and Iberian hares) and/ or sand fly hosts (*P. perniciosus*) in the area (Table 2) confirming results obtained with kDNA. Single band patterns were present in 72.5% of tissue parasites and 68.4% of cultured parasites. The largest bands (540–920 bp) were only present in parasite cultures. Other molecular typing studies targeting the k26 gene that included *L. infantum* isolates from Spain revealed PCR products sizes larger than 584 bp, in addition 626 and 920-bp fragments were the most frequent among in vitro parasites (Chicharro et al., 2013). This also highlights the importance of genotyping the parasite directly from the host tissues to perform molecular surveillance, thus avoiding the introduction of biases related to the selection of genotypes performed when culturing the parasite (Domagalska & Dujardin, 2020).

High parasite load in ear skin has been acknowledged as the best marker to identify infectious individuals and potential reservoir populations (Courtenay et al., 2014; Pereira-Fonseca et al., 2017). Hosts with higher skin parasite load such as the rabbits examined in this study have a greater potential to spread the disease, so that sand flies are at higher risk of becoming infected when exposed to them. The existence of subpopulations of super-spreader wild rabbits would support the spatial clustering of clinical leishmaniasis cases in southeastern Spain. Furthermore, these results contribute to a better knowledge of the *L. infantum* population circulating in the area, and reflect the great genotypic variability of the parasite population in wild rabbits. Most of these genotypes (6/7) have been found infecting humans (5), dogs (2), and sand flies (2) in the region (Figure 2). Our findings also highlight that direct genotyping of the parasite in host tissues should be used for parasite molecular surveillance instead of cultured isolates.

Iberian hares (*Lepus granatensis*) and to a lesser extent wild rabbits (29 and 9% *L. infantum* infection seroprevalence, respectively), were deemed responsible for the 2012–2018 outbreak of human leishmaniasis occurred in an urban area of southwestern Madrid, Spain (Molina et al., 2012; Moreno et al., 2014). Transmission of the parasite from these lagomorphs to sand flies was confirmed by xenodiagnoses (Jiménez et al., 2014; Molina et al., 2012) and high *L. infantum* loads were found in wild caught *P. perniciosus* females from the outbreak area (González et al., 2017). Other studies conducted in European endemic countries suggest that *L. infantum* is widely spread in wild rabbits populations that showed heterogeneous prevalence values (Abbate et al., 2019; Ortega et al., 2017; Tsakmakidis et al., 2019).

Wild rabbits are widely distributed across Europe and can be considered a pest sometimes (Millán et al., 2014). Díaz-Sáez et al., 2014 analysed the conditions that a reservoir host should meet and concluded that the rabbit appears to fulfil most of the parameters to be considered an effective reservoir host: it is an abundant, prolific, gregarious, territorial and nocturnal species with a long enough lifespan to ensure *L. infantum* transmission with seemingly limited or no clinical impact. The dispersion range of the male is larger than that of the female, reaching up to 7 ha, and its main biotope for the protection and reproduction is the warren

TABLE 1 Epidemiological characteristics of leishmaniasis by *Leishmania infantum* in southeastern Spain by natural regions: average incidence of human clinical cases in the 2003–2028 period expressed as cases per 100,000 inhabitants-year (Martín-Sánchez et al., 2020) and prevalence in wild rabbits showing parasitic loads in the ear skin. Wild rabbit densities (specimens/km^2) have been estimated in June 2018 by the Junta de Andalucía; June density is the highest throughout the year (Consejería de Medio Ambiente y Ordenación del Territorio, Andalusian government, 2019).

Natural regions	Abundance of wild rabbits June 2018 (specimens/km^2)	Incidence of human leishmaniasis (cases per 100,000 inhabitants-year) 2003–2018	Prevalence of infection in wild rabbits 2018–2019	Estimated average number of parasites per mg of ear rabbit	Prevalence of super-spreaders (parasite loads >10^6 parasites per skin gram)
Loja	53.7 (high)	2.72	100% (78/78)	1013.40	20.5% (16/78)
Los Montes	53.7 (high)	1.62	92.1% (58/63)	238.36	15.9% (10/63)
Guadix	6.0 (very low)	1.34	96.7% (29/30)	133.66	6.7% (2/30)
Alhama	53.7 (high)	0	90,0% (45/50)	53.29	2.0% (1/50)
La Vega	53.7 (high)	0.78	74.6% (53/71)	14.81	0% (0/71)
Baza	2.9 (very low)	1.52	45.5% (5/11)	8.95	0% (0/11)
Huescar	2.9 (very low)	0	18.2% (2/11)	0.001	0% (0/11)
All	-	1.24	86.0% (270/314)	324.8	9.2% (29/314)

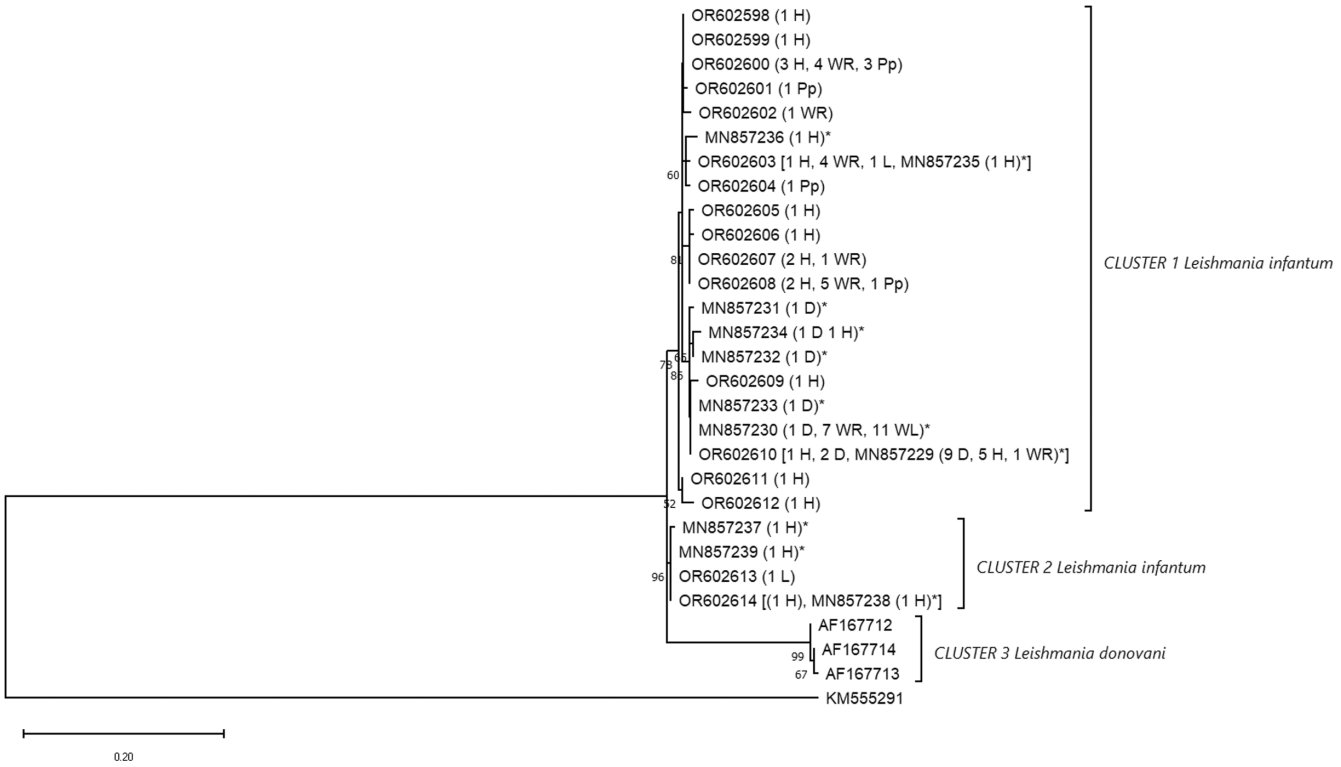

FIGURE 2 Maximum likelihood tree based on the kDNA sequences of 25 haplotypes of *Leishmania infantum* derived from this study and those published by Ortuño et al., 2019 (marked with *). Three haplotypes of *Leishmania donovani* published in GenBank are included. *Leishmania major* is used as the outgroup. H is human origin, Pp is *Phlebotomus perniciosus*, WR is wild rabbit, WL is other wild life, D is dog and L is *Lepus* sp.

TABLE 2 Molecular typing of parasites by electrophoresis of k26 gen: product size detected for 90 parasites (71 parasites from highly parasitized tissues from wild rabbits, hares, dogs, humans and female sand flies, and 19 cultivated isolates).

						Cultivated isolates		
K26 PCR products size in base pairs	**Wild rabbits**	**Iberian hares**	***Phlebotomus perniciosus***	**Dogs**	**Humans**	**Humans**	**Dogs**	***P. Perniciosus***
140	3	2	0	2	0	2	0	0
180	2	0	3	3	1	0	0	0
200	9	1	0	1	3	2	0	0
220	19	1	1	2	3	2	0	2
250	0	1	0	1	0	2	0	0
280	38	3	0	4	4	3	2	2
300	1	0	0	0	0	4	0	2
380	2	2	2	0	3	1	8	4
400	1	0	0	0	0	2	0	0
420	7	1	1	0	0	0	0	0
450	3	0	1	0	0	0	0	0
480	1	0	0	0	0	0	0	0
500	3	0	0	0	1	0	0	0
540	0	0	0	0	0	3	0	0
626	0	0	0	0	0	1	3	3
920	0	0	0	0	0	1	0	0
Parasite Number	51	3	4	5	8	6	8	5

that also constitute a suitable biotope for the vector. The presence of a high density of parasites on the rabbit's skin ensures parasite contact with the vector that uses telmophagy (pool feeding) as a feeding mechanism (Bouchet & Lavaud, 1999). The abundance of *P. perniciosus* in domestic, peridomestic and sylvatic microhabitats along with its opportunistic feeding behaviour allows its relationship with the multi-host species of *L. infantum* and the overlap of sylvatic and domestic habitats where dogs are the main host reservoir (Martín-Sánchez et al., 2021). Additionally, in this research, and in spite of sample size limitations, a positive correlation was found between skin parasite load in wild rabbits and human incidence with evidence of the presence of the same *L. infantum* genotypes in rabbits and humans, providing new epidemiological and biological basis for the consideration of wild rabbits as a relevant *L. infantum* wild reservoir.

ACKNOWLEDGEMENTS

We acknowledge support from MICIU/AEI/10.13039/501100011033 and FEDER, UE through the project PID2022-142230NB.100. Andrés Torres Llamas was supported by an FPI grant (PID202-2142230N-I00).

FUNDING INFORMATION

This study was funded by the Ministry of Science and Innovation, Spain, and FEDER, UE through the project PID2022-142230NB-I00. Funding for open access charge: Universidad de Granada/CBUA.

CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Martín-Sánchez, J., Díaz-Sáez, V., Morillas-Márquez, F., Corpas-López, V., Ibáñez-De Haro, P., Torres-Llamas, A., & Morales-Yuste, M. (2024). Wild rabbits are *Leishmania infantum* reservoirs in southeastern Spain. *Zoonoses and Public Health*, *00*, 1–7. https://doi.org/10.1111/zph.13139