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Role of wild rabbits as reservoirs of leishmaniasis in a non-epidemic Mediterranean hot spot in Spain

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ABSTRACT

There is limited information regarding the role of wild mammals in the transmission dynamics of *Leishmania infantum*. A potential human leishmaniasis hot spot was detected in southern Spain that could not be explained solely by canine leishmaniasis prevalence. The aim of this work was to analyse the involvement of wild rabbits as the main factor affecting this Mediterranean hot spot. A survey of wild rabbits, dogs and sand flies was conducted in the human cases environment. A nearby region without clinical leishmaniasis cases was used as reference control. 51 wild rabbits shot by hunters were analysed by molecular techniques. 1100 sand flies were captured and morphologically identified. Blood collected from patients' relatives/ neighbours ($n = 9$) and dogs ($n = 66$) was used for molecular analysis and serology. In Mediterranean leishmaniasis hot spots such as Montefrío municipality (average incidence of 16.8 human cases per 100,000 inhabitants/year), wild rabbits ($n = 40$) support high *L. infantum* infection rates (100%) and heavy parasite burdens (average value: 503 parasites/mg) in apparently normal ear skin directly accessible to sand flies, enabling the existence of heavily parasitized *Phlebotomus perniciosus* females (12.5% prevalence). The prevalence of infection and median parasite load were very low among rabbits captured in Huéscar ($n = 11$), a human clinical leishmaniasis-free area for the last 18 years. *P. perniciosus* was the most abundant *Phlebotomus* species in all the domestic/peridomestic microhabitats sampled, both indoors and outdoors. Accordingly, leishmaniasis is clustering in space and time at this local scale represented by Montefrío due to the proximity of two competent host reservoirs (dogs and heavily parasitized wild rabbits) associated with overlapping sylvatic and domestic transmission cycles through the main vector, *P. perniciosus*. We highlight the usefulness of determining the prevalence of infection and parasite burden in wild rabbits as a control leishmaniasis measure with the advantage that the use of the ear offers.

1. Introduction

Leishmaniasis remains one of the world's most devastating neglected tropical diseases. According to the World Health Organization, up to 350 million people are at risk in 97 countries around the world. It is considered that approximately 12 million people are currently infected, and between 0.7 and 1 million new infections occur every year, of which an estimated 50,000–90,000 cases are visceral and 20,000–30,000 deaths occur annually (WHO, 2017).

The domestic dog is the main reservoir of *Leishmania infantum* Nicolle, 1908, the causative agent of canine leishmaniasis (CanL) and a variety of human leishmaniasis (HumL) clinical manifestations in the Mediterranean basin. In southwestern Europe, human incidence is low despite the high prevalence of CanL characterized by strong variation among micro-foci (Morales-Yuste et al., 2012; Vélez et al., 2019). Available data report HumL incidence in France was 0.22 cases per 100.000 inhabitants between 1999 and 2012 (Lachaud et al., 2013), whereas the burden of visceral leishmaniasis in Italy between 1982 and 2012 accounted for 3,122 cases (Gramiccia et al., 2013). The average annual hospitalization rate in Spain for leishmaniasis ranged from 0.4 between 1999 and 2003 (Valcárcel et al., 2008), to 0.56 between 1997 and 2011 (Herrador et al., 2015). This increasing trend was also reflected in a study over a 14-year period in southern Spain, where the annual incidence of autochthonous leishmaniasis increased from 0.12 per 100.000 inhabitants in 2003 to 3.93 in 2016 following a linear model (Martín-Sánchez et al., 2020). Underreporting and underdiagnosis have been found, particularly in cutaneous leishmaniasis (Merino-Espinosa et al., 2018).

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Although HumL and CanL forms are associated, pervasive infection of dogs with *L. infantum* does not necessarily imply a higher incidence of the disease in humans (Antoniou et al., 2013; Vélez et al., 2019; Otranto and Dantas-Torres, 2013). As a consequence, CanL prevalence alone could not explain the heterogeneous HumL incidence in southern Spain (Martín-Sánchez et al., 2020) and the Madrid epidemic outbreak (Molina et al., 2012; Aguado et al., 2013). The difficulty of finding an area where there are no dogs hinders the process of evaluating the potential contribution of other hosts to the epidemiology of leishmaniasis. Natural *Leishmania* infections have been found in healthy wild mammals (Díaz-Sáez et al., 2014; Millán et al., 2014; Navea-Pérez et al., 2015; Oleaga et al., 2018) suggesting a role in the transmission cycle. This involvement was actually confirmed for hares in epidemic outbreaks (Molina et al., 2012). However, there is limited information regarding the role of wild mammals in the natural transmission cycle of *L. infantum* in non-epidemic pictures; as a consequence, we hypothesize whether they are reservoirs or just incidental hosts. Knowledge of the role of reservoir hosts other than the dog contributes significantly to the establishment of rational control measures of leishmaniasis.

In Spain, 11 different species of sandflies have been identified and 3 proven *Leishmania infantum* vectors, *Phlebotomus perniciosus* Newstead, 1911, *Phlebotomus ariasi* Tonnoir, 1921 and *Phlebotomus langeroni* Nitzulescu, 1930 which can act in one same focus in sympatric conditions (Gil Collado et al., 1989; Sáez et al., 2018). They are found, to a greater or lesser extent, almost throughout the country, and their relative significance as vectors depends on their abundance and density, although we can certainly confirm that *P. perniciosus* is the main vector (Barón et al., 2011; Jiménez et al., 2013; Alcover et al., 2014; Sáez et al., 2018). *Phlebotomus langeroni* is associated with the existence of rabbit burrows and has been overlooked in most phlebotomine studies in Europe. Six sandfly species were present in this biotope, the five species most commonly found in Spain (*P. perniciosus, Phlebotomus papatasi* Scopoli, 1786, *P. ariasi, Phlebotomus sergenti* Parrot, 1917 and *Sergentomyia minuta* Rondani, 1943) and *P. langeroni* (Sáez et al., 2018). Transmission of the disease is linked to the presence of the vector, whose distribution is in turn closely related to ecological and bioclimatic factors, thus being sensitive to environmental changes related to human behaviour including urbanization, changes in land use or climate change (Barón et al., 2011; Molina et al., 2012; Alcover et al., 2014). The involvement of wild mammals in the transmission of *L. infantum* would imply the disappearance of borders between forest/agricultural lands/urbanized areas in the presence and spread of leishmaniasis.

We have recently explored *L. infantum* epidemiology in southern Spain through a One Health approach focused on estimating the HumL incidence in a spatiotemporal context and its association with the key actors in the transmission of the parasite. During a 14 years period, a linear increase in HumL cases was detected and no association was found with CanL, an endemic disease in the study area (Martín-Sánchez et al., 2020). No human clinical cases were found in 3 regions where CanL seroprevalence was high. A potential hot spot was detected and its high leishmaniasis incidence, which may be associated to the involvement of host species other than dogs, was the trigger for the present epidemiological survey.

This study was performed to investigate how the *Leishmania*-infected wild reservoirs affect the general and regional incidence patterns of leishmaniasis in order to deal with an important problem related to the relevance of domestic and sylvatic transmission cycles. The main objective is whether the wild animals influence the occurrence of human leishmaniasis, for which we selected two localities with the highest and lowest HumL incidence in our study area. Other components of the problem are the presence/absence of the vectors and the CanL prevalence. To answer this complex question, a survey of wild rabbits supplemented with others in dogs, humans and sandflies were conducted in a selected study area. Three recent human patients were used to establish the connection between all elements of the transmission chain.

2. Materials and methods

2.1. Study area

Montefrío (37°19′16″N and 4°00′40″W, altitude: 834 m a.s.l.) is one of the 174 municipalities of the province of Granada, in the southeast of Spain. The overall province has a population density of 72.4 inhabitants/km^2 and comprises 11 natural geographic regions. The municipality of Montefrío has a population of 5433 (2019) and a density of 21.6 inhabitants/km^2; it is located 60 km northwest of the city of Granada within the region of Loja (Fig. 1).

In the region of Loja, the annual incidence of autochthonous leishmaniasis was 0 until 2008 and rise from 1.5 in 2009 to 11.3 in 2016; Montefrío was the most affected municipality with an average incidence of 16.8 during that period (Martín-Sánchez et al., 2020).

In contrast, no cases were reported in the Huéscar region, the most septentrional region of the province. Huéscar municipality (37°48'34''N and 2°32'22''W, altitude: 953 m a.s.l.) has a population of 7253 (2019) and a density of 15.8 inhabitants/km^2 (Fig. 1); it has been used as reference control.

2.2. Description of the HumL cases from Montefrío

Three recent HumL patients from Montefrío were used to establish the connection between all elements of the transmission chain:

Case n° 1: In November 2015, a 6-months-old male who presented with fever, splenomegaly and hepatomegaly was diagnosed with visceral leishmaniasis (VL) via serological immunofluorescence antibody test (IFAT) (titre = 1/160).
Case n° 2: In March 2016, a 1-year-old male who presented with fever, pancytopenia, splenomegaly and hepatomegaly was diagnosed with VL via IFAT (titre = 1/640).
Case n° 3: In July 2016, a 52-year-old male who presented with fever, progressive weight loss and cervical lymphadenopathy was diagnosed with VL via histological sections and PCR.

These patients were hospitalized and successfully treated with liposomal amphotericin B (Ambisome®).

2.3. Wild rabbit survey

Fifty-one wild rabbits (*Oryctolagus cuniculus*) were shot by hunters in October-November 2018. Blood (taken by cardiac puncture), liver, spleen, one humerus and one ear lobe were collected from recently shot rabbits while of the non-recent ones, only one ear lobe was collected. The ear lobes measured on average 48 (95%CI 46–50) mm × 81 (95%CI 78–84) mm. Bone marrow was extracted by passing saline solution 0.9% through each clean humerus placed on a Petri dish. All ear lobes were examined for skin lesions and three approximately 5 × 5-mm^2 distant tissue sections were taken and weighed from each.

2.4. Sand fly collection, species identification and Leishmania infection rate in the vector

Sand flies were captured in July and September 2016 using CDC light traps and sticky traps. CDC traps were placed inside and outside dwellings, hanging one meter above the ground and left overnight. Sticky traps were placed in natural and artificial holes, outside dwellings and kept for a single day. The captured sand flies were then stored in 70% ethanol. Male and female specimens were morphologically identified and separated. The genitalia of female specimens was individually removed for morphological identification whereas the rest of the body was stored at −20 °C for DNA extraction and molecular analysis of *Leishmania* DNA. Morphological identification was carried out in accordance to Gil Collado et al. (1989), Martín-Sánchez et al. (2000) and

Fig. 1. Geographical location of the study area within the province of Granada in southern Spain.

Sáez et al. (2018) via examination under optical microscope, paying particular attention to the spermatheca in females and the external genitalia, mainly to the shape of the copulatory valves, in males. The gonotrophic cycle of the female sand flies was categorised as blood-fed, non-fed or gravid. Sand fly density (sand flies/CDCtrap/night or sand flies/m^2/night) and relative abundance (% specimens of a given species/total sand flies) data were estimated.

2.5. Survey of patients' relatives, neighbours and dogs

A survey was conducted in the home of each patient in which relatives, neighbours and their dogs (all from the same district within the town), which were called by the patient or his legal guardian, were attended. The selection criterion was sharing the patient's environment.

Blood collection from 9 patients' relatives/neighbours and 17 dogs was carried out in July and September 2016. Blood samples were split into two alliquots (whole blood with anticoagulant and serum) for PCR and serology (IFAT), respectively. Blood collection was also carried out on 13 dogs of Montefrío hunters hunters. In the town of Huéscar, blood was taken from 36 dogs from a local shelter that collects stray dogs.

2.6. Laboratory techniques

2.6.1. DNA extraction

Samples were processed in a room exclusively destined to DNA extraction. Each sand fly or tissue sample was placed in a sterile 1.5 mL Eppendorf tube, kept in liquid nitrogen for a few seconds and disrupted using a pestle. For blood samples, 200 μl of whole blood were used. DNA was extracted using the MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. Extraction controls (one for each ear lobe, 5 female sand flies or 5 blood samples) were used to monitor cross contamination: The extraction process was simultaneously applied to test tubes containing sterile water. After drying, DNA was resuspended in water and its quality and quantity were determined spectrophotometrically. For ear DNA, concentration was adjusted to 700 ng/ μL to use 5 μL in the PCR. The extracted DNA was kept at −20 °C.

2.6.2. Molecular analysis

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). PCR-ELISA detects *L. infantum* by amplifying a 75 bp fragment from the variable region of the *L. infantum* kDNA minicircle (Martín-Sánchez et al., 2002). It was performed using kits PCR-ELISA DIG Labeling and PCR-ELISA DIG Detection (Roche Diagnostics GmbH. Mannheim, Germany). In the PCR, primers 9 (forward): 5′-CAAAAGTCCCCACCAATCCC-3′ and 83 (reverse): 5′-AAACCCTGGTCTGGAGGCTTAG-3′ amplify a fragment labelled with digoxigenin at the following conditions: 3 min at 94 °C, then 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a final elongation of 3 min at 72 °C. The amplified fragment was detected on a streptavidin-coated microtitre plate, through hybridization for 1 h at 50 °C with the oligonucleotide probe specific to *L. infantum* 5′-CCA AAC AGG GCA AAA ACC-3′, labelled at the 5′-end with biotin, followed by ELISA using a peroxidase-labelled anti-digoxigenin antibody and ABTS as substrate. The results were read in a spectrophotometer at 405 nm. Samples returning absorbance values of ≥ 1 were considered positive. When optical density was ≥ 0.5 and <1, amplification was repeated using double amount of DNA.

GRANALEISH Multiplex qPCR (University of Granada, Spain) can differentiate between *L. infantum, L. tropica* and *L. major* and allows quantification of the parasite load (Merino-Espinosa et al., 2018). Primers *F, R* and the 3 Taqman probes were provided by the manufacturer. The following thermal profile has been used: 10 min at 95 °C, then 36 cycles of 30 s at 95 °C and 60 s at 60 °C. The number of parasites in every qPCR reaction was calculated through the interpolation of the cycle threshold (Ct) value in a standard curve. Every PCR run included the following negative controls: (i) PCR control test-tube; (ii) test-tube with *Leishmania* free *O. cuniculus* DNA, or DNA from a male sand fly; (iii) test-tubes with DNA of the extraction controls. DNA obtained from 1000 promastigotes of *L. infantum* (MHOM/ES/08/DP532) was used as positive control.

2.6.3. Serological analysis by indirect fluorescence antibody test

IFAT was carried out as previously described (Morales-Yuste et al., 2012). Briefly, a suspension of *L. infantum* promastigotes (2×10^6 cells/mL) strain MCAN/ES/91/DP204 (zymodeme MON-1 = GR-1) was

used as antigen. The antibody titre against *Leishmania* was determined in geometric dilutions from serum samples; a starting dilution of 1/10 was used. Rabbit anti-gamma globulin (ICN Biomedicals) was used as a conjugate at a concentration of 1/100 in Evans blue previously diluted at 1/104 with phosphate-buffered saline. A threshold titre of 1/160 or 1/80 was applied to confirm CanL or HumL, respectively.

2.7. Ethical statement

Ethical approval for the Project PI14-01024 was granted by the Research Ethics Committee of the University of Granada. Participants provided informed consent. Experiments were performed in accordance with regional guidelines and Directive 2010/63/EU.

3. Results

3.1. Survey of wild rabbits (Oryctolagus cuniculus)

40 wild rabbits were shot by hunters in Montefrío. All rabbits (100%) captured tested positive for *L. infantum* (40/40 ear lobe, 3/6 bone marrow and 4/6 spleen samples). The estimated average number of parasites reached to 9,692 parasites per mg of ear lobe or 780,058 parasites per 5 × 5 mm^2 tissue of ear lobe (wild rabbit n° 6, Table 1, Fig. 2). Only 12 of the 120 5 × 5-mm skin sections had parasite loads lower than 0.001 parasites (detectable with PCR-ELISA but not with qPCR). Seventeen animals (42.5%) had at least one lesion compatible with leishmaniasis in their ear lobe. The median parasite load was 503 parasites/mg in ear skin (29,023 parasites per 5 × 5 mm^2 tissue). No differences in parasite loads between lesions (17 lesions, average: 513 parasites per mg, IC95% 99–927) and healthy skin (103 skin sections without lesions, average: 439 parasites per mg, IC95% 41–838) were detected ($p = 0.887$).

None of the 11 wild rabbits (33 lobe ear samples) from Huéscar tested positive with qPCR whereas only 2/11 (18.2%) wild rabbits (2/33 skin samples) were positive with PCR-ELISA, showing residual parasites. All five ears with a lesion compatible with leishmaniasis were PCR negative.

Comparison of the parasites/ mg means in both localities by the *T* test for independent samples yielded statistically significant differences ($p = 0.006$).

3.2. Sand fly collection, species identification and Leishmania infantum infection rate in the vector

1100 sand flies belonging to four species of Phlebotomus genus (744 *Phlebotomus perniciosus*, 4 *Phlebotomus ariasi*, 21 *Phlebotomus sergenti* and 53 *Phlebotomus papatasi*) and 278 *Sergentomyia minuta*) were captured in the study.

In Montefrio, *P. perniciosus* density ranged from 0.8 to 94 Pp/CDC/night and 2.0 to 17.8 Pp/m^2/night using sticky traps, depending on the trap location. *Phlebotomus ariasi* was captured in low numbers while *P. papatasi* was absent (Table 2). *Leishmania infantum* DNA was found in 12.5% (8/64) *P. perniciosus* females (from 1 parasite/sand fly to 18,667 parasites/sand fly).

P. perniciosus density in Huéscar ranged from 2 to 156 Pp/CDC/night and 35.5 to 126.7 Pp/m^2/night using sticky traps depending on the trap location (Table 2). 4% (1/25) *P. perniciosus* females were infected by *L. infantum* with a low parasite load (1parasite/sand fly).

3.3. Survey of patients' relatives, neighbours and dogs

None of the 9 VL patients' relatives / neighbours investigated tested positive with IFAT or PCR. Seventeen dogs owned by VL patients' relatives and neighbours were investigated, four of them were positive for leishmaniasis. Eleven of the 13 dogs of the Montefrío hunters were positive for leishmaniasis (Table 3).

Of the 36 dogs in the Huéscar kennel, 5 had antibody titers ≥1/160. All dogs had a negative result in the PCR (Table 3).

4. Discussion

Leishmania infantum parasites are transmitted between hosts during blood-feeding by infected females of *Larroussius* subgenus characterised by their great opportunism (Rossi et al., 2008; Jiménez et al., 2013; Bravo-Barriga et al., 2016). Limiting prevalence in its principal reservoir host, the domestic dog, may result in a reduced risk of infection for the human population, provided no other reservoirs are involved. In recent years there has been an increasing interest in better understanding the role that wildlife can play in the transmission of leishmaniasis due to its particular relevance in the epidemiology and control programs of *L. infantum*.

Iberian hares (*Lepus granatensis*) and to a lesser extent wild rabbits (29 and 9% *L. infantum* infection prevalence, respectively), were recently deemed responsible for the outbreak of HumL occurred in Madrid, Spain (Molina et al., 2012; Moreno et al., 2014) but little is known about their involvement in non-epidemic areas. Wild rabbits are widely distributed across Europe and can be considered a pest sometimes whereas hare populations are usually small in the Iberian Peninsula (Millán et al., 2014).

A potential hot spot with a high HumL incidence was found in southern Spain (Martín-Sánchez et al., 2020) and its eventual association to the epidemiological involvement of wild rabbits is now investigated; the hare population in this locality is usually very small due to its orography. All the wild rabbits captured in Montefrío, a municipality with an average incidence of 16.8 human cases per 100,000 inhabitants/year (Martín-Sánchez et al., 2020), were infected with *L. infantum*, while the prevalence of infection was very low in the Huéscar rabbits, a human clinical leishmaniasis-free area for the last 18 years. In Huéscar, the hare population is higher and 16 animals were investigated, accounting for a prevalence of 37.5% (6 +/16) with residual parasite loads, except for 1 that showed a high parasite load (data not shown). Hares usually stay further away than rabbits from the limits between agricultural lands and urbanized areas, so these prevalence values may have less influence on the incidence of human leishmaniasis.

Other studies conducted suggest that *L. infantum* is widely spread in Spanish wild rabbits populations that showed heterogeneous prevalence values: overall *L. infantum* DNA prevalence has been estimated between 0.6 and 79.8% (Chitimia et al., 2011; Díaz-Sáez et al.,2014; García et al., 2014; de la Cruz et al.,2016; Ortega et al., 2017). A similar picture has been described in Italy and Greece (Abbate et al., 2019; Tsakmakidis et al., 2019). A variety of samples from the same animal can be used for molecular detection with different sensitivities due to differences in parasite burdens (Hernández et al., 2015; Corpas-López et al., 2016). In addition, the detection of the parasite does not imply that the host is involved in *Leishmania* epidemiology, hence the importance of quantifying parasite load. The highest proportion of positive rabbits observed using IFAT in some of these studies might occur due to the high prevalence figures of *Trypanosoma nabiasi* Railliet, 1895 whose antigens cross-react and consequently, the results of serological techniques must be taken with great caution (Díaz-Sáez et al., 2014, Merino-Espinosa et al., 2016). *Trypanosoma nabiasi* is a widespread parasite that has been found in Spanish wild rabbit populations, and the sympatric and syntrophic presence of both parasites must be taken into account in order to avoid any confusion (Merino-Espinosa et al., 2016; González et al., 2018).

Rabbit infectiousness to sand flies has been demonstrated by xenodiagnoses showing that *Leishmania* infected rabbits were able to transmit the parasite to *P. perniciosus* (Jimenez et al., 2014). Infectiousness to the sand fly vector has been associated with high parasite numbers in reservoir host (Courtenary et al., 2014; Pereira-Fonseca et al., 2017).

Leismania infantum was detected in all 120 5 × 5 mm ear skin sections of the rabbits from Montefrío hot spot. The median parasite load was

Table 1
Ear skin parasite burdens measured by qPCR in the 40 rabbits captured in Montefrío. Three approximately 5 × 5 mm^2 tissue sections were analyzed from each wild rabbit ear making a total of 120 analyzed sections. When there was a lesion, a sample of it was taken. For each rabbit, the average value, standard deviation of the average value (StDev), and minimum and maximum values of the parasitic loads detected in the 3 sections of the ear analyzed are shown.

Wild rabbit	Parasites/mg tissue		Parasites/μg DNA		Parasites/5 × 5 mm^2 tissue	
	Average (St Dv)	Minimum Maximum	Average (St Dv)	Minimum Maximum	Average (St Dv)	Minimum Maximum
1	12.6 (15.7)	3.2 30.8	7.5 (8.7)	1.3 17.4	1024.8 (1348.4)	186.6 2580.3
2	19.9 (30.8)	<0.001 55.4	17.8 (29.6)	<0.001 52	1927.5 (3013.8)	<0.001 5400.5
3	33.5 (7.6)	24.8 38.3	51.7 (19.6)	29.3 65.7	3334.6 (1296)	1875.3 4351.1
4	35.1 (47.1)	5.3 89.4	35.6 (43.6)	4.2 85.4	3797 (5003.2)	368.7 9538.1
5	129.1 (98.9)	28.6 226.4	155.5 (101)	49.2 250.1	11041.6 (9489.5)	3149.9 21570.8
6	9692.2 (8358.3)	41.2 14593.8	4188.4 (3676.5)	19.2 6965.8	780057.5 (679422.1)	7013 1.3E+006
7	29 (50)	<0.001 86.7	13.8 (23.6)	0.01 41	597 (1014.8)	0.2 1768.7
8	182.6 (72.1)	106.2 249.3	192.1 (114.1)	74.1 301.8	13415.6 (2200.5)	10944.8 15164.4
9	172.6 (71)	96.1 236.4	168.3 (57.9)	101.6 204.4	25294.6 (10400.3)	14085.6 34632
10	1043.5 (1453.3)	44.6 2710.7	225.9 (253.3)	14 506.5	79727.9 (117741.8)	2370.6 215231.2
11	639.7 (684.1)	200.5 1427.9	408.1 (488.5)	123.5 972.2	13765.9 (15802.3)	3780.2 31984.8
12	185.9 (302.7)	<0.001 535.2	10 (8.7)	<0.001 15.8	323.9 (301.7)	<0.001 597
13	0.01 (0.02)	<0.001 0.03	0.01 (0.01)	<0.001 0.02	0.2 (0.4)	<0.001 0.7
14	10.5 (18.1)	<0.001 31.4	6.5 (11.2)	<0.001 19.5	232 (400.5)	<0.001 694.5
15	1.6 (1.5)	<0.001 3	0.7 (0.8)	<0.001 1.6	33.8 (33.2)	<0.001 66.3
16	416.6 (650.1)	21.6 1167	107.3 (142.1)	17.7 271.1	5155.7 (7080.9)	495.8 13304
17	10.1 (12.7)	<0.001 24.4	10.3 (14.4)	<0.001 26.7	312.1 (430.3)	<0.001 803
18	92.3 (73.1)	33.2 174.1	24.2 (15.5)	10.1 40.7	5916.6 (6261.6)	704.7 12862.4
19	301.8 (335.5)	13.1 669.9	130.2 (142.1)	12.5 288	5316.4 (7995.2)	399.3 14541.8
20	68.8 (113.7)	0.8 199.9	19.5 (31.2)	0.4 55.5	975.1 (1546.1)	11.7 2758.5
21	173.1 (192.8)	<0.001 380.9	88.4 (78.8)	<0.001 155.2	3051 (3209.8)	<0.001 6399
22	34.7 (31.7)	5.3 68.3	14.4 (10.4)	4.1 24.9	872.1 (719.4)	183.2 1618.6
23	219.9 (342.8)	7.7 615.3	69.6 (104.3)	5.9 189.9	4107 (5547.9)	218.2 10460.2
24	16.8 (17)	0.3 34.3	8.3 (7.3)	0.2 14.4	304.9 (282.2)	7.9 569.6
25	1257.3 (1132.2)	22.1 2245.9	823.3 (716.3)	35.5 1435.6	32585.7 (31167.8)	812 63110.1
26	90.8 (42.1)	48.3 132.5	41.8 (27.1)	19.9 72.1	3046.7 (1112.6)	2201.4 4307.2
27	61.7 (106.9)	<0.001 185.2	22.9 (39.7)	<0.001 68.8	1191.5 (2063.7)	<0.001 3574.5
28	406.5 (621)	0.6 1121.3	101.3 (151)	0.3 274.8	9379.3 (12565.5)	14.5 23659.5
29	60 (75)	<0.001 144.1	27.9 (38.8)	<0.001 72.1	1343.8 (1753.7)	<0.001 3327.6
30	1.8 (1.8)	0.7 3.8	0.7 (0.6)	0.3 1.4	40.8 (36.4)	15.1 82.4
31	19.8 (16.6)	8.2 38.8	9.6 (9.5)	4 20.5	547.9 (603.3)	182.1 1244.3
32	8.8 (15)	<0.001 26.1	2.1 (3.6)	<0.001 6.3	187.6 (322.8)	<0.001 560.4
33	31.4 (38.9)	7 76.3	19.3 (24.8)	3.4 47.9	714.4 (914.4)	148.7 1769.4
34	125.4 (178.3)	0.6 329.6	37.4 (54.2)	0.3 99.6	2014.7 (2692.3)	13.7 5075.6
35	365.2 (441.6)	26.9 864.7	84.7 (82.8)	6.82 171.7	12158.3 (12423.8)	1088.1 25595.3
36	293.4 (393.7)		160.6 (198.4)		5210.6 (6499.6)	

(*continued on next page*)

Table 1 (*continued*)

Wild rabbit	Parasites/mg tissue Average (St Dv)	Minimum Maximum	Parasites/μg DNA Average (St Dv)	Minimum Maximum	Parasites/5 × 5 mm^2 tissue Average (St Dv)	Minimum Maximum
		57.4		45.4		1304.9
		747.9		389.7		12713.6
37	96.2 (91.9)	10.8	31.3 (18.4)	10.1	2748 (2993.8)	271.1
		193.5		43.3		6074.8
38	152.1 (188.4)	5.3	34.7 (37.3)	3.9	10063 (16076.7)	182.5
		364.5		76.1		28613.7
39	1064.6 (679.6)	433.5	401.9 (310.3)	65.2	69092.9 (77480.4)	11445.1
		1784		676.3		157168.5
40	2547.3 (3826.3)	3.5	1061.3 (1321.1)	2.7	49989.9 (75506.4)	186.6
		6947.6		2541.7		136866.9
Median parasite load	502.6	<0.001	220.4	<0.001	29022.5	<0.001
		14593.8		6965.8		1282407.4

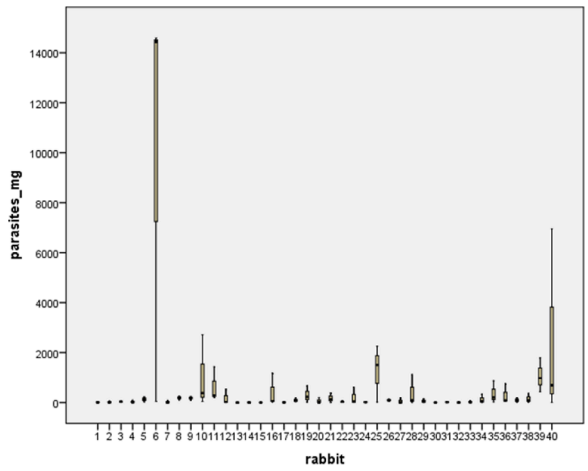

Fig. 2. Parasite loads found in rabbits from Montefrío expressed as parasites per milligram of ear tissue. Three approximately 5 × 5 mm^2 tissue sections were analyzed from each wild rabbit ear. Raw data are shown in Table 1.

502,600 parasites/g (29,023 parasites per 5 × 5 mm^2 tissue), a value much higher than that detected in skin of infected dogs by Courtenay et al. (2014) (119 parasites/g). According to these authors, the majority of natural infected dogs have loads $<10^6$ parasites per skin gram and are very rarely infectious to sand flies while others are super-spreaders (Courtenay et al., 2017). We detected a similar picture in these wild rabbits from which 20% (8/40) had parasite loads $>10^6$ parasites per skin gram at least in one of the 3 sections analysed (Fig. 2) and could act as super-spreaders.

There is usually a good correlation among dog parasite loads in blood, bone marrow, lymph node or hair, making parasitaemia and hair parasite load good biomarkers to monitor treatment efficacy in CanL (Manna et al., 2004; Belinchón-Lorenzo et al., 2013; Corpas-López et al., 2016). However, dog parasite loads in ear skin and bone marrow show different dynamics so that the first continued to increase over the time resulting in increased skin/bone marrow parasite load ratios in late infection (Courtenay et al., 2014). Tissue parasite burden heterogeneity could be behind different conventional PCR outcomes in rabbits: Ortega et al. (2017) found 79.8% skin, 66.7% hair and 26.2% spleen sample positivity. Although we could analyse multiple tissues in only 6 rabbits in the present study, the highest parasite loads were found in the skin of ears, followed by spleen and bone marrow, what would be in agreement with previous dog findings by Courtenay et al. (2014).

A poor correlation between the parasite load in the blood and the sand fly infection rates obtained by indirect xenodiagnoses has been observed in visceral leishmaniasis immunosuppressed human cases and the existence of additional sources of parasites, such as skin have been suggested (Molina et al., 2020). The skin is considered a major source of amastigotes for the sand flies in post-kala-azar dermal leishmaniasis [PKDL, caused by *Leishmania donovani* (Laveran et Mesnil, 1903) Ross, 1903] patients in Asia or Africa and CanL (Travi et al., 2001; Pereira-Fonseca et al., 2017; Mondal et al., 2019; Molina et al., 2020). Using serial xenodiagnoses to assess the infectivity of dogs naturally infected with *L. infantum,* Travi et al. (2001) showed that the ear skin was more heavily parasitized that that of the abdomen. Doehl et al. (2017) demonstrated that *L. donovani*-infected mice form heterogeneous skin parasite patches that govern infectiousness to sand flies with the involvement of intact-looking skin.

High parasite loads in ear skin, rather than the simple presence of parasites, has been suggested as the best marker to identify likely infectious individuals and potential reservoir populations (Svobodova et al., 2003; Courtenay et al., 2014; Pereira-Fonseca et al., 2017). Hosts with higher skin parasite load such as rabbits observed 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. Amastigotes in skin are directly accessible to sand flies, which are known to prefer safe, hairless parts of hosts such as the ear pinnae, and feed abundantly on them (Svobodova et al., 2003; Ready, 2013). In the present study, rabbit ears measured 48 × 81(95% CI: 46–50 × 78–84) mm on average and represents an extensive hairless exposed area of heavily parasitized skin to the bites of sand flies.

Phlebotomus perniciosus was the most abundant *Phlebotomus* species in all the domestic/peridomestic microhabitats sampled both indoors and outdoors (Table 2). Unfed, blood-fed and gravid females were found inside and outside dwellings. *Phlebotomus perniciosus* demonstrates an opportunistic feeding behaviour that allows its relationship with the multi-host species of *L. infantum*. This sand fly species breeds in wild sites, including wild rabbits burrows, and domestic environments allowing the overlapping of wild and domestic cycles (Barón et al., 2011; Alcover et al., 2014; Sáez et al., 2018). *Phlebotomus perniciosus* was the most abundant (61.0%) and densest species (8.4 individuals/trap/night) in wild rabbits burrows where *P. langeroni* seems to play a role in the parasite cycle (Saez et al., 2018). *Phlebotomus langeroni* was not captured inside dwellings or in their surroundings in the present study; therefore their role may not be directly relevant in the transmission to humans. *Phlebotomus perniciosus* abundance in rabbit burrows may well be benefited by increased oviposition of females that feed on rabbits. Furthermore, rabbits create an ideal environment for sand fly to thrive through the accumulations of organic matter where larvae feed (de Benito Martín et al., 1994).

According to Courtenay et al. (2017), the existence of subpopulations of mammalian (in the present study, wild rabbits) and sand fly "super-spreaders" provides the biological basis for the spatial and temporal clustering of clinical leishmaniasis cases, such as the hot spot identified in Montefrío.

5. Conclusions

In Mediterranean leishmaniasis hot spots, wild rabbits support high *L. infantum* infection rates and heavy parasite burdens in apparently

Table 2
Sand fly species collected in the Mediterranean human leishmaniasis hot spot represented by Montefrío and the human clinical leishmaniasis free area of Huéscar. N: Total number of specimens females and males; D: density of sand flies by site and species; A: Abundance of sand flies by site and species. Density with CDC traps expressed as "Sand flies/trap/night"; Density with sticky traps (ST) expressed as "Sand flies/m^2/night". in: CDC traps placed inside; out: CDC traps placed outdoor. All sticky traps were placed outdoor.

Collection site		Trap type, number and location		Phlebotomus perniciosus	Phlebotomus ariasi	Phlebotomus papatasi	Phlebotomus sergenti	Sergentomyia minuta	**Total**
Human leishmaniasis hot spot (Montefrío)	Case 1 (dwelling)	1 CDC in	N	28(10♀)	0	0	0	59(24♀)	87
			D	28				59	(34♀)
			A	32.2				67.8	87
									22.7
		1 CDC out	N	16(9♀)	1(1♀)	0	0	8(4♀)	25
			D	16	1			8	(14♀)
			A	64	4			32	25
									6.5
		12 ST	N	11(3♀)	0	0	0	68(19♀)	79
			D	7.4				45.7	(22♀)
			A	13.9				86.1	53.1
									20.6
	Case 1 (neighbouring house)	1 CDC out	N	6(6♀)	0	0	0	1(1♀)	7(7♀)
			D	6				1	7
			A	85.7				14.3	1.8
		8 ST	N	8(5♀)	0	0	0	5(5♀)	13
			D	8.1				5	(10♀)
			A	61.5				38.5	13.1
									3.4
	Town center	10 ST	N	22(11♀)	1(0♀)	0	0	6(3♀)	29
			D	17.7	0.8			4.8	(14♀)
			A	75.9	3.5			20.7	23.4
									7.6
	Case 2 (dwelling)	1 CDC out	N	11(10♀)	0	0	0	0	11
			D	11					(10♀)
			A	100					11
									2.9
		1 CDC in	N	94(18)	0	0	0	5(1♀)	99
			D	94				5	(19♀)
			A	95				5.1	99
									25.9
		19 ST	N	26(5♀)	0	0	4(0♀)	3(1♀)	33(6♀)
			D	11.0			1.7	1.3	14
			A	78.8			12.1	9.1	8.6
	Case 3 (dwelling)	4 CDC out	N	3(1♀)	1(1♀)	0	0	1(0♀)	5(2♀)
			D	0.7	0.3			0.3	1.3
			A	60	20			20	1.3
		16.5 ST	N	4(4♀)	0	0	0	2(2♀)	6(6♀)
			D	2				1	2.9
			A	66.7				33.3	1.5
	Total	9 CDC (2 in, 7 out)	N	158(54♀)	2(2♀)	0	0	74(30♀)	234
			D	17.6	0.2			8.2	(86♀)
			A	67.5	0.9			31.6	26
									59.4
		65.5 ST 8.1 m^2	N	71(28♀)	1(0♀)	0	4(0♀)	84(30♀)	160
			D	8.7	0.1		0.5	10.3	(58♀)
			A	44.4	0.6		2.5	52.5	19.7
									40.6
Human clinical leishmaniasis free area (Huescar)	kennel on the outskirts of town	2 CDC in	N	312(126♀)	1(0♀)	46(41♀)	14(13♀)	31(12♀)	404
			D	156	0.5	23	7	15.5	(192♀)
			A	77.2	0.2	11.4	3.5	7.7	202
									57.2
		10 ST	N	157(11♀)	0	6(1♀)	2(0♀)	20(15♀)	185
			D	126.7		4.8	1.6	16.1	(27♀)
			A	84.9		3.2	1.1	10.8	149.3
									26.2
	Outskirts of town	10 ST	N	44(4♀)	0	1(1♀)	1(1♀)	69(27♀)	115
			D	35.5		0,8	0,8	55.7	(33♀)
			A	38.3		0.9	0.9	60	92.8
									16.3
	Town center dwelling	1 CDC in	N	2(1♀)	0	0	0	0	2(1♀)
			D	2					2
			A	100					0.3
	Total	3 CDC in	N	314(127♀)	1(0♀)	46(41♀)	14(13♀)	31(12♀)	406
			D	104.7	0.3	15.3	4.7	10.3	(193♀)
			A	77.3	0.3	11.3	3.5	7.6	135.33
									57.51
		20 ST 2.5m^2			0				300
									(60♀)

(*continued on next page*)

Table 2 (*continued*)

Collection site	Trap type, number and location	Phlebotomus perniciosus	Phlebotomus ariasi	Phlebotomus papatasi	Phlebotomus sergenti	Sergentomyia minuta	**Total**
	N	201(15♀)		7(2♀)	3(1♀)	89(42♀)	121
	D	81.5		2.8	1.2	35.9	42.5
	A	67		2.3	1	29.7	

Table 3
Diagnosis of canine Leishmaniasis in dogs from Montefrío and Huéscar localities. [1]A dog with IFAT titre ≥1/2560. [2]The dog died a few days after the blood was drawn.

Site and number of dogs (N)		PCR result	IFAT titre ≥1/160	1/80	1/40	<1/20
Montefrío	Dogs owned by VL patients'relatives and neighbours *N* = 17	positive	1[1,2]	0	2	0
		negative	1	1	1	11
	Dogs owned by hunters *N* = 13	positive	2[1]	2	0	0
		negative	7	0	2	0
Huéscar	Kennel dogs *N* = 36	positive	0	0	0	0
		negative	5[1]	4	2	25

normal ear skin directly accessible to sand flies enabling the existence of heavily parasitized *P. perniciosus* females. The abundance of *P. perniciosus* in domestic, peridomestic and sylvatic microhabitats, and 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. Accordingly, leishmaniasis is clustering in space and time at this local scale represented by the municipality of Montefrío due to the proximity of two competent host reservoirs (dogs and heavily parasitized wild rabbits) associated with overlapping sylvatic and domestic transmission cycles through the main vector, *P. perniciosus*. We highlight the usefulness of determining the prevalence of infection and parasite burden in wild rabbits as a control leishmaniasis measure with the advantage that the use of the ear offers.

CRediT authorship contribution statement

Joaquina Martín-Sánchez: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Nieves Torres-Medina:** Resources, Investigation. **Francisco Morillas-Márquez:** Writing – review & editing. **Victoriano Corpas-López:** Formal analysis, Writing – original draft, Writing – review & editing. **Victoriano Díaz-Sáez:** Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

We declare no competing interests.

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