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Research paper

Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain

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ABSTRACT

Leishmania infantum infection was investigated in 202 wild carnivores, rodents and lagomorphs in Southeast Spain using a real-time PCR (rtPCR) in skin and organ samples, mostly spleen. Lesions compatible with leishmaniosis were not observed in any of the animals. Prevalence defined as the percentage of rtPCR-positive animals was 32% overall, and 45% in foxes (n = 69), 30% in rabbits (n = 80) and stone martens (n = 10), 19% in wood mice (n = 16), 0% in black rats (n = 10) and ranged between 0% and 100% in other minoritarian species including badgers, wild cats, wolves, raccoons, genets and hares. Most infected rabbits were PCR-positive in skin and not in spleen samples and the opposite was the case for foxes ($p < 0.05$). *L. infantum* prevalence was lowest in spring following months of non-exposure to phlebotomine sand fly vectors, and spatially matched recently estimated *Phlebotomus perniciosus* vector abundance and the prevalence of subclinical infection in dogs and humans. Prevalence increased with altitude and was greater in drier and less windy South and West compared to the coastal Southeast of the study area ($p < 0.05$). Genetic diversity of *L. infantum* from foxes, investigated by PCR-restriction fragment length polymorphisms of kinetoplast DNA, revealed B genotype in all animals, which is frequent in people and dogs in the Iberian Peninsula and Morocco. The study provides further evidence that subclinical *L. infantum* infection is widespread in wildlife with prevalence depending on environmental factors and that parasite tissue tropism may vary according to host species. Moreover, it suggests that sylvatic and domestic transmission cycles are closely interconnected.

1. Introduction

Leishmaniosis caused by the protozoan *Leishmania infantum* is a severe vector-borne and emerging zoonotic disease that is endemic in the Mediterranean basin (Solano-Gallego et al., 2009, Michel et al., 2011). The domestic dog has historically been considered the main reservoir host of the parasite and canine leishmaniosis (CanL) has a high lethality rate in untreated dog. Humans are less susceptible to developing disease, although hundreds of human leishmaniosis (HumL) cases are reported in Europe every year (Dujardin et al., 2008; Antoniou et al., 2013). Control is mainly focused on the dog by the simultaneous use of several measures to prevent phlebotomine sand fly vector bites and enhance the host´s resistance to infection (Miró et al., 2017). However, the efficacy of these measures is only partial and leishmaniosis control remains a challenge. One of the main factors limiting CanL and HumL control is the existence of a sylvatic *L. infantum* transmission cycle in wildlife that interacts with the domestic cycle maintained by dogs (Quinnell and Courtenay, 2009; Molina et al.,

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2012). This possibility was recognized many decades ago when infection was occasionally diagnosed in foxes and rodents (Ashford and Bettini, 1987), using traditional methods lacking the sensitivity of molecular techniques, such as the polymerase chain reaction (PCR) tests. Numerous wildlife species have since been shown to be infected by PCR (Millán et al., 2014), but their importance as primary *L. infantum* reservoir hosts remains to be determined for most species (Quinnell and Courtenay, 2009).

Discussion concerning the participation of wild species in the transmission of *L. infantum* has renewed interest since the outbreak of HumL in southern Madrid associated to Iberian hares (*Lepus granatensis*) and wild rabbits (*Oryctolagus cuniculus*) (Molina et al., 2012; Jiménez et al., 2013). It is an example of how anthropogenic changes of ecosystems resulting in increased density of some wild species and, thus, the possibility of more contact with domestic animals and humans, can dramatically affect disease behavior and risk (Kurek et al., 2014; Hoverman and Searle, 2016; Tomassone et al., 2018). In fact, xenodiagnosis experiments incriminated lagomorphs as the source of infection to phlebotomine sand flies infecting humans in the Madrid outbreak. Unfortunately, such studies are rare in wild species because they require laboratories with active vector colonies and wildlife handling facilities.

Molecular epidemiological studies offer the opportunity for characterizing *Leishmania* spp. and strains and compare their distribution in hosts to improve the understanding of transmission cycles. There is a wide choice of techniques and molecular markers to quantify genetic variation at species and subspecies level (Akhoundi et al., 2016), but few such studies have been performed on *L. infantum* strains from wildlife. Del Río et al. (2014) compared the ribosomal Internal Transcribed Spacer 2 (ITS2) region of wildlife from Northern Spain and, also humans and dogs from Southeast Spain, finding a close similarity. Furthermore, the *L. infantum* strain from hares, rabbits, humans and phlebotomine sand flies in the Madrid focus shared the same ITS genotype (Chicharro et al., 2013). In contrast to ITS sequences, those in the variable region of kinetoplast (mitochondrial) DNA (kDNA) minicircles enable discriminating between strains, and since they are highly repetitive, PCR tests targeting these sequences are highly sensitive (Akhoundi et al., 2016). Cortes et al. (2006) used restriction fragment length polymorphism (RFLP) analysis on a PCR product of kDNA from dogs, humans and phlebotomine sand flies from Portugal, Brazil, East Africa and the Mediterranean Basin, and determined 16 different genotypes, named in alphabetical order from A to O.

The Región de Murcia (RM), where most of the wildlife in this study came from, is a *L. infantum* endemic area. Because of its comparatively large size (11,300 km^2) and geographical and climatic diversity, the prevalence of infection in dogs and humans and, also, the distribution of the main vector *Phlebotomus perniciosus*, vary significantly across the region (Pérez-Cutillas et al., 2015; Goyena et al., 2016; Risueño et al., 2017). No studies of *L. infantum* infection in wildlife in RM have been performed since it was first described in three foxes in the early 1980s (Marín Iniesta et al., 1982). A wider understanding of the epidemiology of infection must include studies in wildlife in their natural environment. Here we investigated *L. infantum* DNA prevalence in wild carnivores, rodents and lagomorphs from RM and neighboring zones, and performed kDNA-PCR-RFLP analysis on infected animals in order to compare with those from humans and provide evidence of a possible common sylvatic and domestic transmission cycle in the region.

2. Materials and methods

2.1. Study area and population

The study was part of a larger investigation into parasitic diseases in 202 dead wild carnivores, lagomorphs and rodents from Southeast Spain, including 188 animals from RM, 12 animals from Comunidad Autónoma de Valencia (CV), a region North of RM and 2 animals from Comunidad de Andalucía (CA) in the south of RM, collected between 2013 and 2015. Animals were 80 wild rabbits (*Oryctolagus cuniculus*), 69 red foxes (*Vulpes vulpes*), 16 wood mice (*Apodemus sylvaticus*), 10 stone martens (*Martes foina*), 10 black rats (*Rattus rattus*), 6 badgers (*Meles meles*), 4 wild cats (*Felis silvestris*), 3 Iberian wolves (*Canis lupus*), 2 racoons (*Procyon lotor*), 1 genet (*Genetta genetta*) and 1 Iberian hare (*Lepus granatensis*) (Table 1). With the exception of the wolves which belonged to a zoological park, animals had been killed by authorized hunters or found dead by local authorities following road traffic ac-

Table 1
Percentage of *L. infantum* rtPCR positives and distribution of Ct median (range) among wild carnivores, lagomorphs and rodents in Southeast Spain in.2013–2015.

Specie	All tissues				Skin				Organs			
	N	% +ves	95% CI	Ct values	N	% +ves	95% CI	Ct values	N	% +ves	95% CI	Ct values
Rabbit[a]	80	30	20–40	32 (26–37)	80	26	17-36	32 (26–37)	80	5	0–10	32 (26–37)
Fox[b]	69	45	33–57	33 (10–38)	45	9	1-17	30 (20–36)	69	39	28–51	33 (10–38)
Wood mice[c]	16	19	0–38	22 (22–23)	15	20	0-40	28 (25–29)	15	20	0–40	22 (21–23)
Stone marten[d]	10	30	2–58	34 (34–38)	7	14	0-40	34 (34–34)	10	20	0–45	34 (34–38)
Black rat[e]	10	0	0	–	8	0	0	–	10	0	0	–
Badger[f]	6	0	0	–	6	0	0	–	4	0	0	–
Wild cat[g]	4	25	0-67	36 (36–36)	2	50	0–100	36 (36–36)	4	0	0	36 (36–36)
Wolf[h]	3	33	0-87	35 (35–35)	3	33	0–87	35 (35–35)	0	–	–	–
Racoon[i]	2	0	0	–	0	0	0	–	2	0	–	–
Genet[j]	1	100	100	32 (32–32)	1	100	100	32 (32–32)	0	–	–	–
Hare[k]	1	0	0	–	1	0	0	–	1	0	–	–
All	202	32	25–38	33 (29–38)	168	19	13–25	32 (20–37)	195	18	13–24	33 (10–38)

[a]Oryctolagus cuniculus, [b]Vulpes vulpes, [c]Apodemus sylvaticus, [d]Martes foina, [e]Rattus rattus, [f]Meles meles, [g]Felis silvestris, [h]Canis lupus, [i]Procyon lotor, jGenetta genetta, [k]Lepus granatensis.

cidents, and they were necropsied at the University of Murcia. Tissue samples including skin, spleen, liver and lymph node were collected from 168, 190, 69 and 4 animals, respectively and stored at −20 °C until analyzed for *L. infantum* PCR diagnosis, as described below.

2.2. Environmental characterization of animal´s activity territories

The geographical coordinates of the place where animals from RM were collected were recorded and ArcGIS v.10 (ESRI, Redlands, USA) geographical information system (GIS) was employed to map their location, define the presumed activity territory of foxes and rabbits and extract environmental data to investigate the relationship with the animal´s PCR status. Activity areas considered were circular around the point location where animals were found, with an extension of 7 km^2 for foxes and 0.8 km^2 for rabbits, based on the average home range size for each species (Sanz et al., 2017). Environmental data included altitude acquired from the TERRA mission digital elevation model (DEM) (https://asterweb.jpl.nasa.gov/gdem.asp) and the mean monthly temperature, precipitation, percentage relative humidity (RH%) and maximum wind speed obtained from interpolated two-dimensional data layers (Ustrnul and Czekierda, 2005) created with point data from 45 weather stations in RM for the 2006–2015 period.

2.3. DNA purification from tissue samples and PCR diagnosis

Skin samples (50 mg) were incubated overnight at 56 °C in 350 µl buffered lysis solution (Tris 10 mM + EDTA 1 mM, pH = 8.0) containing proteinase K (10 µg/ml) and 10% sodium dodecilsulfate. DNA from the skin lysate and other tissues (50 mg) was extracted using an automated nucleic acid purification robot (Maxwell® 16, Promega), and a NanoDrop spectrophotometer (Thermo Scientific) was employed to determine DNA concentration and quality (ratio of absorbance at 260 nm and 280 nm). Good quality DNA samples (A260/A280 > 1.70) were analysed for highly repetitive *L. infantum* kDNA using a TaqMan probe real-time PCR (rtPCR) targeting a 140 bp DNA sequence as described by Mary et al. (2004). PCR amplification threshold cycles (Ct), at which near logarithmic product generation occurs, were used as a semi-quantitative measure of parasite DNA load (Gomes et al., 2008). Samples with Ct≤38 were considered positive as target quantities approach a single copy for this value (Mackay, 2007).

A selection of DNA from 15 rtPCR-positive samples with the lowest Ct including 5 rabbits, 8 foxes, 1 stone marten and 1 mouse were subsequently analysed with a conventional end-point PCR (PCR) amplifying a 447 bp kDNA minicircle sequence of *L. infantum* for RFLP analysis, according to Cortes et al. (2004). Electrophoresis of PCR-products was carried out in 1.5% agarose gel with 1X Tris-acetate-EDTA buffer and 3 µl of GreenSafe® during 1 h at 120 V and products were visualised under Ultraviolet (UV) light.

2.4. Restriction fragment length polymorphism analysis

Positive PCR-products were purified from the agarose gel with the NZY Gelpure kit (Nzytech). RFLP was performed with nine FAST-version endonucleases (*Rsa*I, *Vsp*I, *Hpa*II, *Bgl*II, *Bme1390*I, *Dde*I, *Pst*I, *Sfc*I, *Xap*I) as previously described (Cortes et al., 2006). Enzymatic digestions were carried out individually for each enzyme, and Portuguese *L. infantum* IMT310 strain belonging to genotype A was used as control. All reactions were carried out at 37 °C for 15 min, and the digested product was submitted to electrophoresis in 3% agarose gel in 1X TAE Buffer for 2 h and visualized under UV light. The combination of the digestion patterns from each restriction enzyme was then used to assign samples to a specific genotype of those previously reported by Cortes et al. (2006). To confirm findings, samples with good sequencing data were further analysed by *in silico* RFLP using the same panel of enzymes with the online program Restriction Mapper (http://www.restrictionmapper.org/).

2.5. Sequencing and sequence analysis

Purified PCR products were sequenced using the Sanger method by a commercial company (LIGHTrun™ Sequencing Service, GATC-biotech, Germany). Sequence identity was compared with those in the GenBank database. BioEdit Alignment Editor (version 7.2.5) and MAFFT multiple alignments with the alignment option G-INS-i (Katoh and Toh, 2008) were used to analyse the sequences.

2.6. Analysis of the relationship between PCR results and environmental and animal explanatory variables

The percentage of *L. infantum* rtPCR-positive samples and animals (defined as prevalence) and the Ct distribution in positive samples were calculated for all animals (Table 1), according to species, sex, age, body condition, year and origin, for foxes, rabbits, stone martens and wood mice (Table 2), and also in relation to environmental variables for foxes and rabbits (Table 3). For RM, origin comprised the main five geographical zones including the North (N), south (S), central (C), West (W) and Southeast (SE) zones (Fig. 1), as considered in previous CanL and HumL *L. infantum* and of *P. perniciosus* activity studies (Pérez-Cutillas et al., 2015; Goyena et al., 2016; Risueño et al., 2017). Climatic variables included May to October monthly averages, as this is the period of highest *P. perniciosus* abundance in RM (Risueño et al., 2017), and were categorized according to their distribution. Yates-corrected chi-squared test or when appropriate Fishers exact test, were used to compare the proportion of PCR-positives across levels of explanatory variables, the non-parametric Kruskal-Wallis test was employed to similarly compare median Cts in PCR positive animals and Spearman rank-coefficient test was used to study the correlation between environmental variables (Kirkwood and Sterne, 2003).

Logistic regression models were developed to investigate the multivariable relationship between rtPCR-positivity (binary outcome variable) in all animals and explanatory variables (Kleinbaum and Klein, 2010). A step-wise modeling approach was used and only variables significantly associated with the outcome were maintained in the final model. The maximum likely-hood model estimation methods were used and significance was taken for $p < 0.05$ for a double test. All analysis were performed using R program (https://www.r-project.org/).

3. Results

3.1. Prevalence of rtPCR positives and distribution of Ct values

Overall and skin and organ-specific PCR prevalence is presented in Table 1. From the 202 analysed animals, 64 (32%) were rtPCR positive including 45% of foxes, 30% of rabbits and stone martens, 0% of black rats and between 0% and 100% of minoritarian species (badgers, wild cats, wolves, raccoons, genets and hares) (Table 1). A total of 431 samples from the 202 animals were analysed and the percentage of rtPCR-positive samples was 16% (68/431), including 19% (32/168), 17% (33/190), 4% (3/69) and 0% (0/4) of skin, spleen, liver and lymph node samples, respectively. However, agreement between rtPCR results in different tissues was low and only 8% of animals tested positive in both skin and organ (spleen or liver) samples. Among rabbits and foxes positive to either skin or organs, the ratio of skin/organ positives were 20/3 for rabbits and 4/16 in foxes; so rabbits were much more likely to be positive in skin, whereas foxes were more likely to be positive in organs ($p < 0.05$). rtPCR prevalence in animals tested in

Table 2
L. infantum rtPCR prevalence (95% CI) in wildlife in southeast Spain in 2013-15, according to independent variables.

Variable	level	All animals		Foxes		Rabbits		Stone marten		Wood mice	
		No.	% + ves	No.	% + ves	No.	% + ves	No.	% + ves	No.	% + ves
Region[1]	RM	188	32 (25–39)	62	47 (34–59)	80	30 (20-40)	5	20 (0–55)	16	19 (0-38)
	CV	12	33 (7–60)	7	29 (0–62)		–	3	67 (13–100)	0	–
	CA	2	0	0	–	0	–	2	0	0	–
RM zones	Central	71	31 (20–42)	15	47 (21–72)	15	53 (28–79)	4	25 (0-67)	14	21 (0–43)
	Southeast	60	10 (2–18)	13	15 (0–35)	47	9 (1–16)	0	–	0	–
	South	37	65 (49–80)**	19	63 (41–85)	18	67 (45–88)**	0	–	0	–
	North	10	40 (10–70)	9	44 (12–77)	0		1	0	0	–
	West	10	40 (10–70)	6	67 (29–100)*	0		0	–	2	0
Season	Winter	48	60 (47–74)**	33	58 (41–74)	9	78 (51–100)	0	–	5	60 (17–100)*
	Spring	30	17 (3–30)	8	25 (0–55)	0		1	100	8	0
	Summer	10	60 (30–90)	6	83 (54–100)	0		0	–	1	0
	Autumn	17	29 (8–51)	9	56 (23–88)	0		1	0	2	0
Gender	Males	106	29 (21–38)	43	44 (29–59)	31	19 (5–33)	8	25 (0–55)	10	20 (0–45)
	Females	85	35 (25–45)	21	43 (22–64)	43	42 (27–57)*	2	50 (0–100)	6	17 (0–46)
Age	Juvenile	22	50 (29–71)	19	58 (36–80)	0	–	2	0	0	–
	Adult	69	33 (22–44)	47	36 (22–50)	0	–	8	38 (4–71)	0	–
Body	low	48	44 (30–58)	37	49 (33–65)	0	–	4	50 (1–99)	7	14 (0–40)
weight	optimum	40	33 (18–47)	27	37 (19–55)	0	–	4	25 (0–67)	9	22 (0–49)
Body	low	26	31 (13–49)	20	35 (14–56)	0	–	3	33 (0–87)	0	–
condition	optimum	25	44 (25–63)	19	47 (25–70)	0	–	2	50 (0–100)	0	–

[1]Región de Murcia (RM), Comunidad autónoma de Valencia (CV) and Comunidad autónoma de Andalucía (CA).
* $p < 0.10$, ** $p < 0.05$. Asterisk placed in the category with the largest percentage.

Table 3
Leishmania infantum rtPCR prevalence in foxes and rabbits fromm Region de Murcia (RM) in 2013-15 according to altitude and average rainfall, relative humidity, temperature and maximum wind speed in May to October for the period 2006-15.

Variable	level	No. of animals	% + ves.	95% CI	p-value
Altitude (masl[a])	5–54	49	10	2–19	< 0.0001
	95–294	34	38	22–55	
	343–736	36	56	39–72	
	802–1263	25	64	45–83	
Rain fall (mm[b])	22.1–24.0	58	60	48–73	< 0.0001
	24.1–25.9	85	21	12–30	
% relative humidity (RH)	58.3–61.5 (RH1)	78	58	47–69	< 0.0001
	61.7–67.5 (RH2)	65	12	4–20	
Temperature (°C)	18.9–22.3 (T1)	55	58	45–71	< 0.0001
	22.5–23.18 (T2)	88	24	15–33	
% RH; T (°C)[c]	RH1-T1	57	60	47–72	< 0.0001
	RH1-T2	22	55	34–75	
	RH2-T2	64	11	3–19	
Maximum wind speed (m/s)	7.3–8.4	36	53	36–69	< 0.0001
	8.5–9.4	45	62	48–76	
	9.5–10.6	62	10	2–17	

[a]meters above sea level, [b]millimeters, [c]combined relative humidity (RH%) and temperature (T).

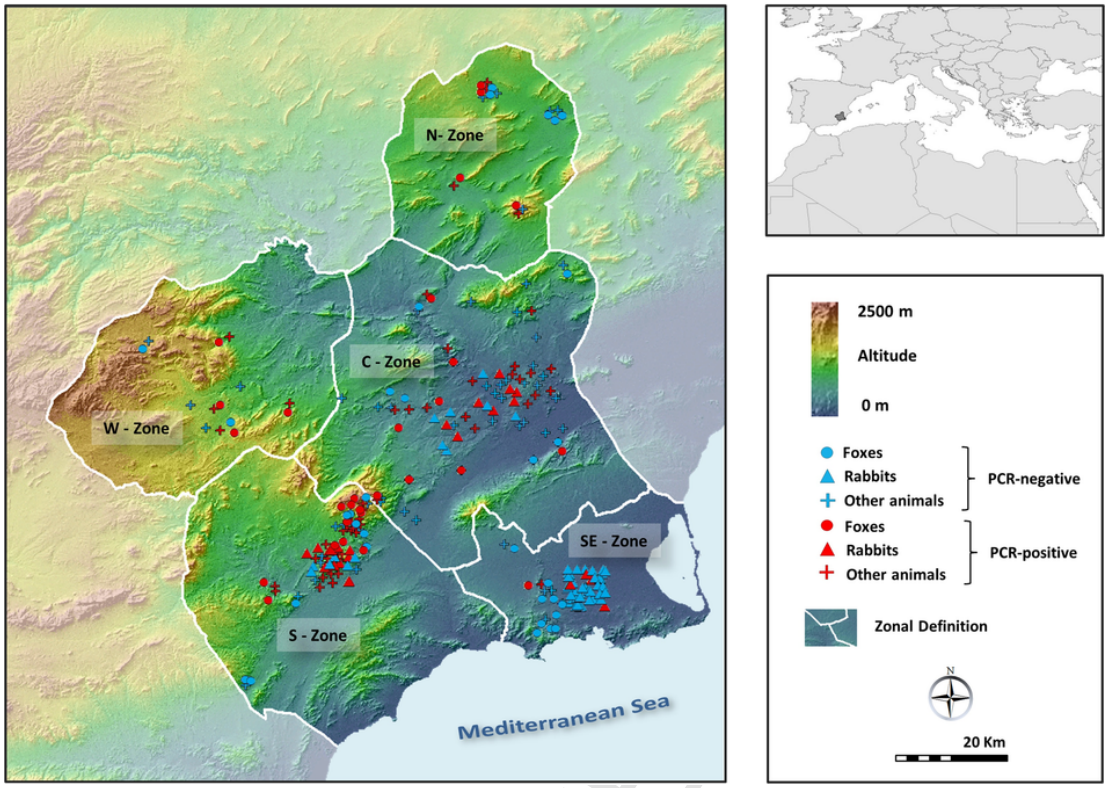

Fig. 1. Distribution of *Leishmania infantum* PCR positive and negative wildlife species in Región de Murcia (RM) in Southeast Spain in 2013-15.

one, two or three different tissues (skin or organs) were 31%, 35% and 27%, respectively ($p > 0.05$).

PCR threshold cycles in positive samples ranged from 10 to 38, and 21% of samples had Ct values greater than 35 (Table 1). There were no significant differences between animals or tissues in median Ct values although Ct values below 30 were only observed in foxes, rabbits and wood mice (Table 1).

3.2. Relationship between rtPCR-positivity and explanatory variables

Overall PCR prevalence was 32% in RM, 33% in CV and 0% in CA ($p > 0.05$) and differed significantly between RM zones ranging from 64% in the South to 10% in the South East of the region ($p < 0.05$) (Table 2; Fig. 1). Disparity in rtPCR prevalence between RM zones obeyed to environmental differences; specifically, rtPCR prevalence in foxes and rabbits, the most widely distributed species, was positively associated to altitude and negatively associated to rain fall, RH%, temperature and maximum wind speed (Table 3).

Moreover, there was evidence of seasonality and gender-specific differences in PCR prevalence and it was greatest in winter and lowest in spring ($p < 0.05$) and marginally greater in female compared to male rabbits ($p < 0.10$). rtPCR prevalence was independent of the animal's age, weight and body condition ($p > 0.05$) (Table 2).

The logistic regression model confirmed the independent association of rtPCR-positivity in RM and the geographical zone, season and host species. Infection risk was significantly greater in animals from the South than in those from the Southeast, in those collected in winter compared to the spring and in foxes compared to rodents (these results are not presented in tabular form).

3.3. Genotype analysis

Of the 15 rtPCR positive DNA samples analysed, 12 were positive to conventional PCR. Moreover, the intensity of the electrophoretic band was only strong enough for RFLP analysis in samples from 7 foxes. Combining the information from the RFLP digestions and the *in-silico* analysis allowed the assignation of the seven foxes to genotype B which corresponded to pattern I for all 9 enzymes tested (Cortes et al., 2006).

4. Discussion

Leishmania infantum infection was detected in foxes, rabbits, wood mice, stone martens, wild cats, wolves and genets, providing evidence that the parasite is present in a considerable proportion of native wildlife in Southeast Spain. There were significant differences in the prevalence of infection between host species, geographical areas and the season when animals were sampled. Species also differed with respect to the localization of infection in skin and organs.

All the species that tested *L. infantum* rtPCR-positive had been found infected or were PCR-positive in previous studies (Quinnell and Courtenay, 2009; Millán et al., 2014; Navea-Pérez et al., 2015). Except for a few foxes, rabbits and wood mice Ct values in other PCR-positive animals were high, indicating a low parasite load. This is typical of wildlife (Tomassone et al., 2018) as it is arguably, the result of evolutionary pressure selecting animals for an immunological response able to control and maintain low parasite levels. Moreover, infection in most rabbits was limited to the skin as shown in previous studies (García et al., 2014; Ortega et al., 2017), whilst in foxes it was mainly found in organs and not in the skin. In CanL subclinically infected dogs from RM *L. infantum* DNA is commonly found in both skin and organs (Chitimia et al., 2011a,b, c). Parasite visceralisation and subsequent disease development in dogs and humans is regulated by the host's im-

mune response (McCall et al., 2013, Baneth et al., 2008). Neither the rabbits nor the foxes in this study had clinical leishmaniosis and differences between them in the parasite's predominant location could be immunologically controlled or possibly, associated to different *L. infantum* strains and this should be further investigated. In any case, preferential tissue tropism of the parasite has important diagnostic implications in epidemiological studies of leishmaniosis in foxes and rabbits.

L. infantum rtPCR prevalence in RM, particularly in foxes, was greatest in the South and West, intermediate in the North and Central and lowest in the Southeast zones, exactly coinciding with the abundance of the vector *P. perniciosus* found in farms and dog kennels in rural areas in this region (Risueño et al., 2017). It suggests that vector density and *L. infantum* prevalence are positively correlated and that phlebotomine sand fly surveillance may be a useful indicator of the risk of *L. infantum* infection. However, phlebotomine sand flies can feed from a variety of hosts with different susceptibility to *L. infantum* infection; so, vector infection rates, host density and other factors are also required for assessing the risk of infection. CanL seroprevalence and HumL PCR prevalence in asymptomatic individuals in RM was also spatially heterogeneous and greatest in the South zone, strongly suggesting that this is a high *L. infantum* risk zone (Goyena et al., 2016; Pérez-Cutillas et al., 2015). Correlation between wildlife, dog and human prevalence in other zones was lower, probably because of small-scale geographical variation in vector and infection density. On a larger geographical scale however, it can be concluded that *L. infantum* prevalence and vector abundance in RM is highest in areas located 200 m or more above sea level, with comparatively low HR% and precipitation, moderately high spring-summer temperature and lower maximum wind speed (Goyena et al., 2016; Pérez-Cutillas et al., 2015; Risueño et al., 2017).

There was some evidence of a seasonal pattern in *L. infantum* prevalence in foxes and it was lower in spring compared to other seasons. Similarly, *L. infantum* PCR prevalence in apparently healthy dogs in Murcia was lower in spring compared to autumn (Chitimia et al., 2011a,b, c). Local adult *P. perniciosus* populations in RM are active from March to November (Martínez Ortega and Conesa Gallego, 1987; Risueño et al., 2017), and *L. infantum* infection rates in the vector in other parts of Spain are greatest at the end of the phlebotomine sand fly season (González et al., 2017). It would seem that some hosts infected in the summer and autumn are able to eliminate infection beyond PCR detection by spring, following several months of no exposure to infected sand flies.

The spatial and temporal correlation between *L. infantum* prevalence in wildlife, dogs and humans and, also, vector abundance does not necessarily imply that domestic and sylvatic *L. infantum* transmission cycles interact with each other. Comparative genetic analysis of *L. infantum* strains across wild and domestic hosts is useful in this respect. Foxes in the present study were classified as profile B in the kDNA RFLP analysis and this was the second most common in dogs and humans in Portugal after profile A (Cortes et al., 2006), and was the predominant in humans and dogs from Morocco (El Hamouchi et al., 2017). Ongoing studies in Murcia indicate that profile B is also the most common in dogs and people in this region (Ortuño et al., 2017). These findings suggest a link between both transmission cycles.

The potential for wildlife to maintain *L. infantum* infection endemicity in the absence of dogs is a matter of discussion (Tomassone et al., 2018). *L. infantum* was found in rodents from the island of Montecristo in Italy, where no dogs are present. Xenodiagnostic experiments have shown that several species including rodents, foxes and lagomorphs can readily transmit the parasite to the vector, indicating their potential to be primary reservoir hosts of infection (Quinnell and Courtenay, 2009; Molina et al., 2012; Jiménez et al., 2014). From an ecological point of view, several factors render them suitable reservoirs of *L. infantum*. Foxes, rodents and lagomorphs live in dens and burrows that are good sites for phlebotomine sand flies to breed and rest. Rabbits are a key prey species for most Mediterranean predators in the Iberian Peninsula, including the red fox (Cavallini and Volpi, 1996; Ferreras et al., 2011). In Southeast Spain Martínez-Carrasco et al. (2007) showed that foxes live in close contact with rabbits, sharing the same habitat. Moreover, foxes have adapted well to anthropic environments and have a diurnal-nocturnal daily cycle that coincides with vector activity (López-Martín, 2010; Cancio et al., 2017). Their opportunism and ability to track human and domestic animal discarded or unattended food are a characteristic trait of this species. Foxes and other wildlife species contact with phlebotomine sand flies in the domestic environment is likely to affect *L. infantum* transmission dynamics, and constitutes a further challenge for effective leishmaniosis control in Mediterranean countries.

5. Conclusions

Overlaping *L. infantum* infection distributions in wildlife, dogs and humans and vectors in Southeast Spain, reinforces the idea that the of infection risk is spatially heterogenous associated to specific environmental factors. The existance in wildlife of a parasite genotype typical of dogs and humans suggests that domestic and sylvatic cycles are interconected. This evidence should be taken into account for improving *L. infantum* control in Mediterranean countries.

Conflict of interest

The authors declare that there is no conflict of interest.

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