



Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain



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ABSTRACT

Leishmania spp. infection was investigated in tissue samples of wild carnivores from the Spanish Basque Country (BC), by PCR and DNA sequencing. The region is at the northern periphery of *Leishmania infantum* endemic Iberian Peninsula and infection in the dog (reservoir) or other species has not been previously reported. *Leishmania* kinetoplast DNA was detected by real-time PCR (rtPCR) in 28% (44/156) of animals. Specifically, in 26% of Eurasian badgers ($n = 53$), 29% of foxes ($n = 48$), 29% of stone martens ($n = 21$) and in 25–50% of less numerous species including genets, wild cats, pole cats, European mink and weasels. Infected animals particularly badgers, were most prevalent in the southernmost province of the BC (Araba) in areas dominated by arable land. Subsequent amplification and sequencing of a fragment of the rRNA internal transcribed spacer 2 (ITS2) from a subset of rtPCR positives samples confirmed the species as *L. infantum*, showing a high sequence homogeneity with ITS2 sequences of *L. infantum* from dogs and humans from southern Spain. In summary, this study reports for the first time *L. infantum* infection in wild carnivores from the BC including in stone martens, pole cats and minks in which infection has not been previously described. It supports the need to study infection in dogs and people in this region and is an example of the value of infection surveillance in wildlife to assess potential risks in the domestic environment and their role in spreading infections in non-endemic areas.

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1. Introduction

Leishmania infantum infection transmitted by phlebotomine sand flies is endemic in the Mediterranean basin and causes zoonotic canine visceral leishmaniasis (CanL),

which has a high fatality rate in untreated patients. Sub-clinical infection is however, very common and has been detected in numerous domestic and wild animal species, particularly since sensitive PCR diagnosis is available (Miró et al., 2008). The recognition of infection in wildlife has stimulated debate about a sylvatic *L. infantum* transmission cycle operating independently or interacting with a domestic cycle maintained by dogs (Quinnell and Courtenay, 2009). In Spain alone, *L. infantum* DNA have been detected

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in foxes, wolves, Egyptian mongooses, genets, Iberian lynx, pine martens, weasels, rabbits and hares (Criado-Fornelio et al., 2000; Sobrino et al., 2008; Sastre et al., 2008; Chitimia et al., 2011a; Millán et al., 2011; Molina et al., 2012). Hares were found to be infectious to sand flies suggesting they could be a potential reservoir for infection (Molina et al., 2012). Furthermore, the high prevalence of infected hares coincided with an outbreak of human leishmaniosis in the outskirts of Madrid (Aguado et al., 2013).

Of interest in *L. infantum* epidemiology in Europe is that infection may be spreading its natural range northwards, as there is evidence of clinical cases in dogs and presence of vector species in areas of northern Italy and Spain, previously considered non endemic (Morosetti et al., 2009; Ballart et al., 2012, 2013). The reasons for this have not been fully established and could be related to climatic, environmental and societal factors. Climate change over the last decades may be responsible for the emergence of vector borne diseases locally and its effect would be greatest along the cold latitudinal and altitudinal periphery of naturally endemic zones (Kilpatrick and Randolph, 2012). The Atlantic (Cantabrian) coastal region in Northern Spain including the Basque Country (BC), is at the northern periphery of the Iberian *L. infantum* endemicity zone. Autochthonous cases of visceral leishmaniosis have not been reported in this region and two recent surveys in northern Spain described 0–8% seroprevalence in dogs including, 0% in a dog shelter in the BC (Amusatogui et al., 2004; Miró et al., 2012). Vector sand fly species *Phlebotomous perniciosus* and *Phlebotomous ariasi* have been detected in northern Spain and the latter was identified in the BC southernmost region of Araba (Aransay et al., 2004).

The present study was part of a wider study of diseases in wildlife in the Spanish BC. The aim was to investigate *Leishmania* infection by PCR analysis of target tissues from wild carnivores including canines, mustelids and viverrid species, to compare DNA sequences of positive animals with those isolated from dogs and people of endemic areas in Spain, in order to gain evidence for the need of future studies in this area.

2. Materials and methods

2.1. Study population, samples and animal data

Leishmania infection was investigated by real time PCR (rtPCR) in a macerate of liver and spleen from 156 dead wild carnivores from the BC, including badgers, foxes, stone martens, genets, pine martens, wild cats, pole cats, minks, weasels and wolves (Table 1). The BC is 7234 km², includes the provinces of Bizkaia (also referred in the literature as Biscay), Gipuzkoa and Araba (Álava) and has a human population of 2.2 million people (Fig. 1.). Animals had been hunted or found dead between 2001 and 2006 and were submitted to a post-mortem examination prior to taking organ tissue samples for investigating several infections, including *Leishmania* spp. Tissue macerates were frozen at -20° until DNA was extracted for PCR analysis. Species, gender, age (adult or juvenile) and date, locality and geographical coordinates of animal locations were included in a data base for further analysis. Dog and human DNA

Table 1

L. infantum rtPCR prevalence among wild carnivores, in the Basque Country in Spain in 2001–2006.

| Variable | N | % PCR positive | 95% confidence interval |
|---------------------------|-----|----------------|-------------------------|
| Badger ^a | 53 | 26 | 15, 38 |
| Fox ^b | 48 | 29 | 18, 44 |
| Stone marten ^c | 21 | 29 | 9, 48 |
| Genet ^d | 10 | 40 | 10, 70 |
| Pine marten ^e | 10 | 30 | 2, 58 |
| Wild cat ^f | 4 | 25 | 0, 67 |
| Pole cat ^g | 4 | 25 | 0, 67 |
| Mink ^h | 2 | 50 | 50, 100 |
| Weasel ⁱ | 2 | 0 | 0, 100 |
| Wolf ^j | 2 | 0 | 0, 100 |
| All | 156 | 28 | 21, 35 |

^a *Meles meles*.

^b *Vulpes vulpes*.

^c *Martes foina*.

^d *Genetta genetta*.

^e *Martes martes*.

^f *Felis silvestris*.

^g *Mustela putorius*.

^h *Mustela lutreola*.

ⁱ *Mustela nivalis*.

^j *Canis lupus*.

samples used to compare *Leishmania* spp. sequences were from asymptotically infected individuals from south-east Spain (Chitimia et al., 2011a).

2.2. PCR diagnosis and sequencing of *Leishmania* spp.

DNA from tissue macerates was recovered using an automated nucleic acid purification robot (Maxwell[®] 16, Promega) and its concentration and quality were analysed with a spectrophotometer (Nanodrop). *Leishmania* spp. DNA was initially investigated with a TaqMan probe rtPCR targeting highly repeated kinetoplast sequences (Mary et al., 2004; Martín-Ezquerria et al., 2009). A subset of DNA from rtPCR-positive samples was further tested with a conventional PCR test amplifying the *Leishmania* spp. discriminative rRNA internal transcribed spacer 2 (ITS2) (de Almeida et al., 2011). PCR tests were done in duplicate using 300 ng of high quality template DNA (A260/A280 ≥ 1.7) per PCR reaction. Samples from *L. infantum* clinical cases and uninfected dogs were used as positive and negative amplification controls respectively. Rt-PCR amplification threshold cycles (C_T), defined as the cycle at which near logarithmic product generation occurs, were used as a semi-quantitative measure of parasite DNA load (Gomes et al., 2008). Samples with $C_T=1-34$ were considered clear positives and those with $C_T=35-38$ were deemed weakly positive as target quantities approach a single copy in this range (Mackay, 2007).

PCR amplified ITS2 fragments were purified and sequenced using BigDye, v.3.1 from Applied Biosystems. Sequences were assembled, edited and aligned using MEGA software (Tamura et al., 2007). ITS2 sequences amplified in infected dogs and humans, and *Leishmania* sequences downloaded from GenBank (GU045592, FJ753391 and FJ948447) were included in the alignment to verify the identity of the *Leishmania* species. Standard genetic indices such as haplotype (gene) diversity (h) and

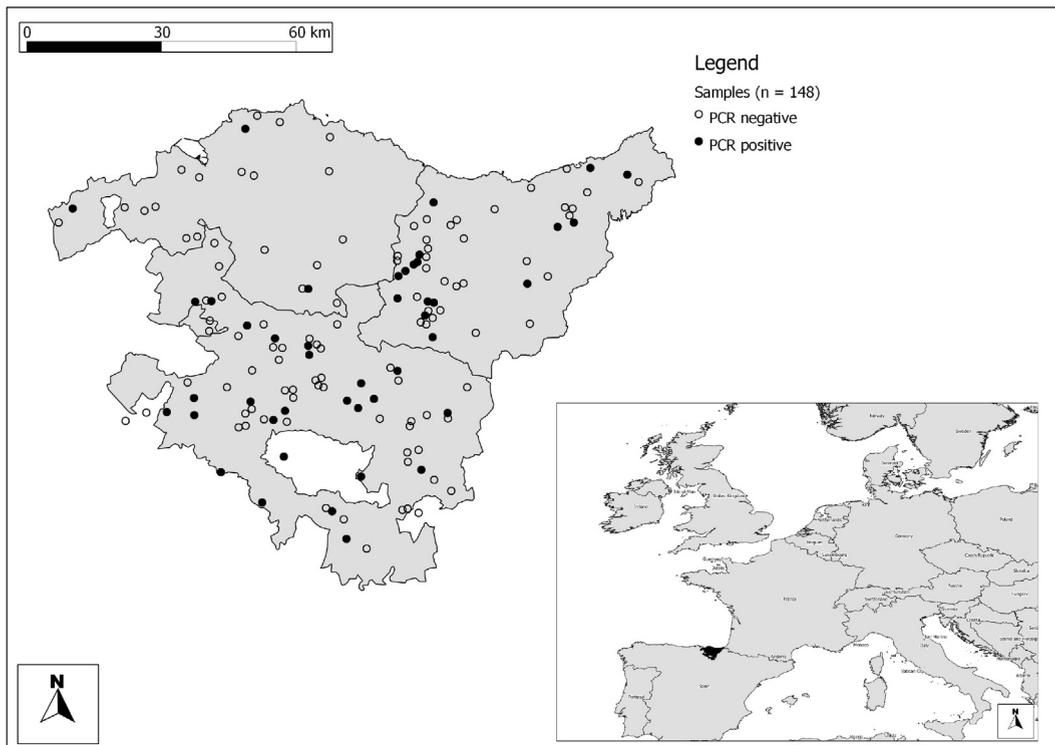


Fig. 1. Geographical distribution of *Leishmania infantum* positive and negative wild carnivores in the Basque Country in Spain in 2001–06.

nucleotide diversity (π) were calculated with DnaSP, v. 5.10.01 (Librado and Rozas, 2009)

2.3. Climatic and environmental data from wildlife territories

Open source geographical information system (GIS) (gvSIG, Generalitat Valenciana) was used to plot on a digital map of the BC the point location where animals were found and to obtain quantitative environmental data from the animal' presumptive territory of activity. Animal' activity territories were defined as circular areas around the point locations with a specific area (km^2) for each animal species equal to the arithmetic mean of maximum and minimum ranges obtained from 14 published ecological studies and the Encyclopaedia of Iberian Vertebrates (reviewed by Victoriano, 2011). These were 300 km^2 for wolves, 50 km^2 for wild cats, 10 km^2 for European mink and genets, 7 km^2 for foxes, 6 km^2 for badgers, 3.4 km^2 for pole cat, 3 km^2 for stone martens and 0.69 km^2 for pine martens.

Data from each territory included altitude, ambient temperature, rainfall, human population density, land cover and soil type (Table 2). Land cover and soil types data were obtained as georeferenced images from CORINE land cover data (at 250 m spatial resolution; <http://terrestrial.eionet.europa.eu/CLC2000>) and the Basque Government's Spatial Data (scale 1: 25,000 infrastructure; <http://www.geo.euskadi.net/>), respectively. Land use and soil types areas in each animal territory were extracted using the gvSIG "intersect" tool and used

to calculate territory land use and soil type percentages. Climatic and demographic data were available at the municipality level from the Spanish Agency of Meteorology (<http://www.aemet.es/>), and used to calculate municipality-specific mean annual temperatures and rainfall, and human population density. Animal territories were assigned climatic and demographic means corresponding to the municipality where animals were found.

2.4. Statistical analysis

EpiInfo 2000 (CDC, Atlanta) and R 2.15.0 (R-project open software) were used to study variable frequency distributions, correlations, compare proportions with the chi-squared test, medians using the non-parametric Kruskal–Wallis test and investigate the multivariable relationship between badger *L. infantum* PCR-results and animal and climatic and environmental variables using logistic regression analysis. In logistic models, the dichotomous dependent variable was the animal PCR result (positive or negative), whereas independent variables were other dog and environmental variables associated to the PCR results in the univariate analysis with a p -value < 0.15 (Table 2). These variables were modelled as categorical variables with 2 or 3 levels defined according to their frequency distribution. A step-wise model building approach was used and variables remaining in the model were those significantly associated with the outcome. Model estimation was based on the maximum likelihood, p values were

Table 2

Median (interquartile range) of environmental and climatic variables in *L. infantum* PCR negative and positive wild carnivore activity areas in the Basque Country in Spain in 2001–06.

| Variable | All species ^a (n = 147) | | | Badger ^b (n = 47) | | |
|------------------------------------|------------------------------------|------------------|---------|------------------------------|------------------|---------|
| | PCR status | | P value | PCR status | | P value |
| | Negative | Positive | | Negative | Positive | |
| Altitude (m) | 506 (254–626) | 488 (305–591) | 0.950 | 507 (292–610) | 542 (473–697) | 0.137 |
| Mean annual temperature (°C) | 11.9 (11–13.1) | 11.9 (11.1–12.5) | 0.417 | 11.5 (11.2–12.8) | 11.4 (10.8–12.3) | 0.339 |
| Annual rainfall (mm) | 1220 (914–1519) | 1050 (799–1490) | 0.171 | 1173 (798–1369) | 786 (717–1112) | 0.019 |
| Human density (p/km ²) | 77 (19–285) | 60 (13–162) | 0.252 | 39 (40–530) | 19 (11–162) | 0.270 |
| Land cover types (%) | | | | | | |
| Arable land ^c | 0 (0–43) | 0 (0–56) | 0.557 | 0 (0–53) | 45 (0–87) | 0.044 |
| Pastures ^d | 22 (0–43) | 7 (0–37) | 0.266 | 26 (0–54) | 0 (0–0) | 0.013 |
| Coniferous forest | 3 (0–26) | 0 (0–24) | 0.364 | 4 (0–31) | 0 (0–8) | 0.051 |
| Broad-leaved forest | 6 (0–19) | 2 (0–24) | 0.638 | 5 (0–21) | 7 (0–56) | 0.190 |
| Mixed forest | 0 (0–10) | 0 (0–3) | 0.179 | 0 (0–5) | 0 (0–0) | 0.110 |
| Any forest type | 35 (14–54) | 26 (5–56) | 0.448 | 35 (20–46) | 11 (4–66) | 0.650 |
| Scrub and bush ^e | 6 (0–17) | 7 (0–18) | 0.684 | 7 (0–16) | 6 (0–15) | 0.508 |
| Soil types (%) | | | | | | |
| Alluvial | 5 (0–18) | 4 (0–19) | 0.933 | 6 (1–18) | 4 (1–52) | 0.960 |
| Sandstone | 1 (0–12) | 2 (0–11) | 0.702 | 1 (0–11) | 2 (0–26) | 0.297 |
| Limestone, marl limestone | 45 (10–75) | 56 (22–86) | 0.169 | 42 (11–82) | 47 (26–75) | 0.401 |
| Slate, shale, gabbro | 9 (0–45) | 0 (0–29) | 0.122 | 22 (0–62) | 0 (0–11) | 0.083 |

^a *Meles meles*, *Vulpes vulpes*, *Martes foina*, *Genetta genetta*, *Martes martes*, *Felis silvestris*, *Mustela putorius*, *Mustela nutreola*, *Mustela nivalis*, *Canis lupus*.

^b *Meles meles*.

^c Corine Land Codes (CLC): 211 (non-irrigated arable land), 212 (irrigated arable land), 221 (vineyards), 242 (complex cultivation patterns) and 243 (Agricultural land with areas of natural vegetation).

^d CLC: 231 (pastures) and 321 (natural grasslands).

^e CLC: 322 (Moors and heathland), 323 (esclerophyllous vegetation), 324 (transitional woodland scrub).

calculated with the chi-squared test and significance was set at $p < 0.05$ for a double test.

3. Results

3.1. *L. infantum* rtPCR prevalence and relationship with animal and environmental variables

None of the animals showed typical CanL lesions at post-mortem examination. *L. infantum* rtPCR positive animals were detected among all species except weasels and wolves. The percentage of PCR-positives was 28% (44/156), ranging among positives from 25% to 50% according to species (Table 1). PCR threshold cycles among positive samples ranged from 28 to 38 and 55% of samples had $C_T \geq 35$. PCR positivity was independent of sex, age and year and season of capture ($p > 0.05$), and was marginally associated to the province; PCR prevalence was 33% (25/76) in Araba, 31% (15/49) in Gipuzkoa and 13% (4/31) in Bizkaia ($p = 0.10$). The greater prevalence in Araba was primarily due to the high proportion of PCR positive badgers in this province (12/28) compared to Gipuzkoa (1/5) and Bizkaia (1/20) ($p < 0.05$). In contrast, the proportion of PCR positive foxes was numerically greatest in Gipuzkoa (9/26), followed by Araba (4/17) and Bizkaia (1/5) ($p > 0.05$). Median C_T differed between species ($p < 0.05$) and was lower for foxes compared to badgers and stone martens ($p < 0.05$) and similar between the two latter species.

Activity zones of positive and negative PCR wild carnivores differed with respect to some environmental variables (Table 2). Globally, areas of PCR-positives were marginally drier and with lower human population density than those of PCR-negative animals. Moreover,

PCR-positive badger territories had significantly lower rainfall, more arable land, less grassland and marginally less coniferous forest and slate, shale, gabbro, compared to negative Badger territories (Table 2), and the latter soil type was also less abundant in areas occupied by PCR-positive stone martens ($p < 0.05$). There was a strong correlation between environmental variables; for example drier areas were coldest, those with a high proportion of arable land had a little pasture and a low percentage of slate, shale and gabbro. As a result, in the logistic regression analysis the animal's PCR-status was not significantly associated to any combination of two or more environmental variables (results not tabulated).

3.2. DNA sequence analysis of *L. infantum* from wild carnivores, dogs and humans

Table 3 presents the percentage of RT-PCR samples of wild carnivores that were subsequently submitted to ITS2 amplification and sequencing. ITS2 fragments of wild carnivores, dog and human fragments had 99–100% sequence homogeneity among each other and with the consensus *L. infantum* sequence.

Differences between *L. infantum* sequences were attributable to one or two single nucleotide polymorphism (SNP) (Fig. 2 in Appendix A): a thymine (T) transversion for adenosine (A) (referred to as T83A transversion) in a fox, a guanine (G) transversion for cytosine (C) in nucleotide position 181 (C181G transversion) in two foxes, a T286C transition in a badger and two transitions in positions 332 and 335 (G332A and G335A) observed in a human. Consequently, five additional ITS2 sequences (sequences 2–6) were defined (Table 3), three of which were present in two

Table 3

Percentage of ITS-2 PCR amplifications and sequencing among RT-PCR-positive wildlife, dogs and humans. The distinct ITS2 sequences found in each species are also indicated.

| | No. of samples | No. (%) amplification | No. (%) sequences | ITS2 sequence ^h |
|---------------------------|----------------|-----------------------|-------------------|----------------------------|
| Badger ^a | 15 | 11 (73) | 5 (45) | 1, 3 |
| Fox ^b | 11 | 9 (82) | 5 (56) | 1, 2, 5 |
| Stone marten ^c | 6 | 4 (67) | 2 (50) | 1 |
| Genet ^d | 2 | 2 (100) | 1 (50) | 1 |
| Pine marten ^e | 3 | 3 (100) | 2 (67) | 1 |
| Wild cat ^f | 1 | 1 (100) | 1 (100) | 4 |
| Pole cat ^g | 1 | 1 (100) | 1 (100) | 3 |
| Dog | 9 | 9 (100) | 6 (67) | 1 |
| Human | 12 | 12 (100) | 5 (42) | 1, 4, 6 |
| All | 60 | 52 (87) | 28 (54) | 1–6 |

^a *Meles meles*.

^b *Vulpes vulpes*.

^c *Martes foina*.

^d *Genetta genetta*.

^e *Martes martes*.

^f *Felis silvestris*.

^g *Mustela putorius*.

^h see Supplementary Material for ITS2 sequence characteristic.

or more individuals. The overall h value for all *L. infantum* sequences was 0.833 (SD = 0.127), while π was 0.0035 (SD = 0.0009).

4. Discussion

The present study provides evidence for *L. infantum* infection in wild carnivores including stone martens, pole cats and minks, in which infection have not been previously reported. There are no reports of autochthonous CanL cases in the BC, although no region-wide epidemiological studies have been carried out to confirm this. PCR prevalence was greatest in Araba which is geographically and climatically closest to CanL endemic areas in Central Spain, and entomological studies found the *L. infantum* vector species, *P. ariasi* in this province (Aransay et al., 2004). *L. infantum* PCR prevalence in tissue of wild carnivores from other parts of Spain ranged from 5% to 74% and was 20% in the Cantabric region, west of Bizkaia (Criado-Fornelio et al., 2000; Sobrino et al., 2008; Millán et al., 2011).

Detection of *L. infantum* DNA in the animal tissues does not necessarily imply active infection or host infectiousness to sandflies, thereafter xenodiagnosis is necessary to demonstrate the latter (Gradoni et al., 1983; Pozio et al., 1985; Maroli et al., 2007; Molina et al., 2012). It is likely that the success of host to sandfly transmission depends on blood and skin parasite load. Most positive samples in the present study had high Ct values suggesting low parasite load. This contrast to generally lower Ct values and presumably larger parasite burdens, found in many asymptomatic dogs in Murcia, where PCR prevalence was estimated at 67% with the same rtPCR protocol (Chitimia et al., 2011a). Moreover, Ct differences between wildlife and dogs could also be due to variation in the rtPCR's efficiency as a result for example, of nucleotide changes in primer and/or probe target kinetoplast sequences.

None of the epidemiological studies of *L. infantum* infection in wildlife rule out dog involvement as a source

of infection for wildlife (Quinell and Courtenay, 2009). Differences between *L. infantum* strains isolated from wildlife and dogs could suggest independent domestic and sylvatic life cycles. Two studies addressing this issue provided evidence of different PCR-restriction fragment length polymorphism (RFLP) patterns in dogs and several wild carnivore species (Sobrino et al., 2008; Millán et al., 2011). ITS-2 sequence comparison in the present revealed only small differences between some *L. infantum* samples due to isolated transitions and transversions. They could indicate the existence of multiple different copies of the ITS-2 sequence in the same isolate (de Almeida et al., 2011). These findings and those from other studies indicate the ITS2 region has a low discriminatory power within *L. infantum* isolates (de Almeida et al., 2011) therefore other markers and available methods with higher within-species discrimination power should be tested (Schönian et al., 2011).

The wide distribution of CanL in the Mediterranean basin is a clear indication of the vector's ability to occupy a variety of ecosystems in these latitudes; however, its distribution at a local level may be overdispersed, associated to environmental characteristics (Alonso et al., 2010). Sandfly breeding sites and particularly those of *P. ariasi* are poorly characterised (Killick-Kendrick, 1999). In this respect, the most remarkable finding in the present study was a positive association between arable land and infected badgers. Further studies including additional habitat variables would need to test this association. Arguably, agricultural land may be able to support a greater density of badgers and sandflies, and favour contact with a domestic CanL cycle. However, data on *L. infantum* prevalence in dogs in Araba is not available so this remains speculative.

CanL is commonly seasonal and age related (Miró et al., 2008) and PCR studies in subclinically infected dogs in southeast Spain indicated a greater percentage of positives in the autumn following the sandfly season, compared to spring (Chitimia et al., 2011b). The relatively small number of wildlife examined and low infection challenge in the BC could partly account for the lack of an association between PCR results and age and year and season of capture observed in the present study.

In summary, this study reports a considerable prevalence of *L. infantum* infected wild carnivores which showed approximately the same ITS2 sequence as in dogs and humans from southern Spain. Since there are no previous reports about the infection in the area, it is not possible to know whether infection is of recent introduction or not and whether its incidence is stable or changing.

5. Conclusions

L. infantum infection is most likely present in wildlife in the Basque Country in northern Spain, and further studies need to be carried out to evaluate the parasite prevalence in local dogs. This would be advantageous from a veterinary and public health perspective and may also improve our understanding of the parasite sylvatic cycle and its relationship to a domestic cycle. Further surveillance of infection in wild carnivores and sandflies would also be beneficial to estimate infection incidence and providing evidence of

a possible expansion of *L. infantum* natural distribution to cooler areas as a consequence of climatic changes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.prevetmed.2013.12.001>.

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