UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

El papel de la fauna silvestre como especies centinela del género Leishmania

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Iris Azami Conesa

DIRIGIDA POR

María Teresa Gómez Muñoz

Madrid

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POR

Iris Azami Conesa

DIRECTORA

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A mis padres.

Por Yasmina y mis abuelas.

AGRADECIMIENTOS

A lo largo de la Tesis Doctoral te enfrentas a muchos momentos en los que tienes que parar un segundo, respirar y coger fuerzas delante de una hoja en blanco. Ahora es uno de ellos. Sois muchos los que me habéis acompañado y apoyado en estos años, y no puedo estar más agradecida de tener esta red de personas que me han comprendido, me han guiado y me han ayudado en cada pequeño paso que he dado hasta llegar hoy aquí.

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Todas estas personas han sido fundamentales en el desarrollo de esta Tesis Doctoral.

¡Muchísimas gracias!

"La evolución es un proceso caótico y creativo"

Lynn Margulis (1938- 2011)

ÍNDICE

LISTADO DE ABREVIATURAS

µl: microlitros, unidad de medida de volumen

µm: micrómetro o micra, unidad de medida de longitud

µM: micromolar, unidad de medida de concentración

”: segundos

˚**:** grados

º C: grados centígrados, unidad de medida de temperatura

A: adenina, base nitrogenada

a.C.: antes de Cristo

ADN: ácido desoxirribonucleico

C: citosina, base nitrogenada

CDC: *Centers for Disease Control and Prevention* o Centro para el Control y la Prevención de Enfermedades

CERI: Centro de Estudio de Rapaces Ibéricas

dNTP: desoxiribonucleótidos- difosfato

ECDC: *European Centre for Disease Prevention and Control* o Centro Europeo de Prevención y Control de Enfermedades

EDO: enfermedad de declaración obligatoria

EDTA: ácido etilendiaminotetraacético

ELISA: *enzyme-linked immunosorbent assay* o ensayo de inmunoadsorción ligado a enzima

EWDA: *European Wildlife Disease Association*

G: guanina, base nitrogenada

GREFA: Grupo para la Rehabilitación de la Fauna Autóctona y su Hábitat

hsp70: *heat shock protein 70* o proteína de choque térmico 70

ICT: inmunocromatogragía

IFAT: *immunofluorescence antibody test* o prueba de inmunofluorescencia indirecta

ITS1: *internal transcribed spacer 1* o espaciador transcrito interno 1

JCR: *Journal Citation Reports*

kDNA: *kinetoplast deoxyribonucleic acid* o ácido desoxirribonucleico del kinetoplasto

km: kilómetros, unidad de medida de longitud

LC: leishmaniosis cutánea

LMC: leishmaniosis mucocutánea

LV: leishmaniosis visceral

mg: miligramos, unidad de medida de peso

$MgCl_2$: cloruro de magnesio

msnm: metros sobre el nivel del mar

N: norte

n.a.: no analizado

nPCR: *nested polymerase chain reaction* o reacción en cadena de la polimerasa anidada

OMS: Organización Mundial de la Salud

OMSA: Organización Mundial de Sanidad Animal

pb: pares de bases nitrogenadas, medida de longitud de un fragmento genético

PCR: *polymerase chain reaction* o reacción en cadena de la polimerasa

pH: potencial de hidrógeno, medida de alcalinidad o acidez de una disolución

Q: *quartile* o cuartil

qPCR: *quantitative PCR* o PCR cuantitativa

rtPCR: *real time PCR* o PCR en tiempo real

S: sur

SARS-CoV-2: *Severe Acute Respiratory Syndrome Coronavirus 2* o Coronavirus de tipo 2 causante del síndrome respiratorio agudo severo

s.f.: sin fecha

Spp.: hace referencia a todas las especies dentro de un género.

SOCEPA: Sociedad Española de Parasitología

SSUrRNA: *Small subunit ribosomal ribonucleic acid* o subunidad pequeña ribosomal de ácido ribonucleico

Syn: sinónimo

T: timina, base nitrogenada

TAE: Tris, Acetato y EDTA

Taq: enzima polimerasa comercial procedente de la bacteria *Thermus aquaticus*

UCM: Universidad Complutense de Madrid

UV: ultravioleta

VIH: virus de la inmunodeficiencia humana

WDA: *Wildlife Disease Association*

WOAH*: World organization of animal health* u Organización mundial de sanidad animal

LISTADO DE FIGURAS

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LISTADO DE PUBLICACIONES

Esta Tesis Doctoral es un compendio de trabajos publicados en revistas científicas indexadas y con revisión por pares, todos ellos relacionados con la presencia de *Leishmania* spp. en fauna silvestre. A continuación, se muestra el listado de los trabajos incluidos:

- **Azami-Conesa, I.**, Martínez-Díaz, R. A., González, F., & Gómez-Muñoz, M. T. (2020). First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe. *Research in Veterinary Science*, *132*, 172–176. https://doi.org/10.1016/j.rvsc.2020.06.019

- **Azami-Conesa, I.**, Sansano-Maestre, J., Martínez-Díaz, R. A., & Gómez-Muñoz, M. T. (2021). Invasive species as hosts of zoonotic infections: The case of american mink (*Neovison vison*) and *Leishmania infantum*. *Microorganisms*, *9*(7), 1–10. https://doi.org/10.3390/microorganisms9071531

- **Azami-Conesa, I.**, Pérez-Moreno, P., Matas Méndez, P., Sansano-Maestre, J., González, F., Mateo Barrientos, M., & Gómez-Muñoz, M. T. (2023). Occurrence of *Leishmania infantum* in Wild Mammals Admitted to Recovery Centers in Spain. *Pathogens*, *12*(1048), 1–11. https://doi.org/doi.org/10.3390/pathogens12081048

- **Azami- Conesa, I.,** Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in non-endemic areas: Is *Leishmania tropica* circulating in wildlife in Spain?. bioRxiv 2024.03.16.585353. https://doi.org/10.1101/2024.03.16.585353. Prepint.

- **Azami- Conesa, I.,** Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in nonendemic areas: first detection of *Leishmania tropica* in wildlife in Spain. *Transboundary and Emerging Diseases,* 2024 (8259712), 1-10. https://doi.org/10.1155/2024/8259712

RESUMEN / SUMMARY

RESUMEN

Leishmania es un género de parásitos protozoarios capaces de afectar a humanos y animales domésticos y silvestres. Se transmite a través de la picadura de las hembras de flebotomos, un insecto díptero hematófago, provocando diferentes manifestaciones clínicas en el humano dependiendo de la especie de parásito y el estado inmunitario del hospedador.

En los últimos años se ha incrementado el interés por conocer cómo la fauna silvestre puede albergar a estos parásitos, pudiendo actuar como reservorios. En el presente trabajo, se han analizado diferentes especies de animales silvestres procedentes de distintas Comunidades Autónomas de España para determinar la presencia de *Leishmania* spp. Además, se ha determinado la sensibilidad y especificidad de distintas dianas del ADN utilizando PCR convencional.

Como parte de los resultados, se ha detectado por primera vez en Europa la presencia de *Leishmania infantum* en murciélago urbano común (*Pipistrellus pipistrellus*), siendo una especie ampliamente distribuida en Europa y muy relacionada con ambientes urbanos y peri-urbanos. Alrededor del 60% de los animales analizados fueron positivos al menos en una de las muestras estudiadas (bazo, pelo y coágulo de sangre de corazón). Además, el pelo aportó resultados esperanzadores, donde aproximadamente la mitad de los individuos positivos lo fueron a esta muestra no invasiva.

Asimismo, se ha detectado ADN de *L. infantum* en animales exóticos invasores, como el visón americano (*Neovison vison*), que supone una amenaza para los ecosistemas de ribera de la península ibérica, no solo por la competencia por los recursos naturales con otras especies autóctonas, sino también por la capacidad de albergar patógenos transmisibles a otros animales y a los humanos, como es el caso de *L. infantum*. El 90% de los animales analizados procedentes de la Comunidad Valenciana resultaron positivos a este parásito en muestras de bazo. Este órgano es uno de los más sensibles para detectar *L. infantum* debido a su localización visceral. Además, se analizaron las muestras con diferentes dianas del ADN para PCR, siendo la *SSUrRNA* y la *Repeat region* las más sensibles en comparación con la *ITS1* y el *kDNA*.

Otros animales silvestres como el tejón europeo (*Meles meles*), el erizo europeo (*Erinaceus europaeus*) y la ardilla roja (*Sciurus vulgaris*), entre otros, fueron analizados mediante PCR como posibles hospedadores de *L. infantum*. En este caso, todos los animales procedían de centros de recuperación de fauna silvestre de la Comunidad de Madrid, Castilla-La Mancha, Castilla y León y Comunidad Valenciana. Se detectó al parásito en tres de las nueve especies animales analizadas, siendo el tejón europeo en el que se observó una mayor presencia de *L. infantum* (35.7% de los individuos), seguido del erizo europeo (14.29%) y la ardilla roja (11,53%). Se analizaron muestras de bazo, piel, conjuntiva oral y conjuntiva ocular (estas dos últimas solo en erizos europeos)

resultando más sensible el bazo. También se estudiaron diferentes regiones del ADN, siendo la *SSUrRNA* y la *Repeat region*, una vez más, las más sensibles.

Por último, se ha detectado por primera vez en España la presencia de *Leishmania tropica* en animales silvestres autóctonos. Esta especie es propia de países vecinos como Marruecos o Túnez, pero nunca antes se había demostrado su presencia en nuestro país, más allá de algunos casos importados en personas. No debe resultar extraño este hallazgo, puesto que desde hace varias décadas el flebotomo responsable de la transmisión de *L. tropica* está establecido en la península ibérica, y solo era cuestión de tiempo que se detectaran los primeros casos autóctonos. El gato montés (*Felis silvestris*) ha sido el animal en el que se ha encontrado dicho parásito, siendo 18 los animales analizados en muestras de bazo y piel, y donde siete de los 18 resultaron positivos a *Leishmania* spp. De los siete, un animal fue positivo a *L. tropica* en muestras de piel mientras que otro individuo presentaba una secuencia mixta de *L. tropica* y *L. infantum*, pudiendo corresponder con una infección mixta o a un híbrido entre ambas especies. Este hallazgo abre la puerta al control y la prevención de esta especie de *Leishmania* foránea, que suele presentar una clínica cutánea, y donde los profesionales sanitarios, veterinarios y autoridades deben trabajar en conjunto para detectar, tratar y prevenir las infecciones con esta especie en la región.

La presente Tesis Doctoral ha puesto de manifiesto la presencia de *Leishmania* spp. en animales silvestres de la península ibérica, los cuales podrían investigarse como centinelas de la parasitosis, además de evaluar su posible papel como reservorio en futuros brotes epidemiológicos de leishmaniosis.

SUMMARY

Leishmania is a genus of protozoan parasites capable of affecting humans and domestic and wild animals. It is transmitted through the bite of female phlebotomine sandflies, a hematophagous dipteran insect, causing different clinical manifestations in humans depending on the parasite species and the immune status of the host.

In recent years, there has been increasing interest in how wildlife can harbour these parasites and act as reservoirs. In the present work, different species of wild animals from different Autonomous Communities of Spain have been analysed for the presence of *Leishmania* spp. In addition, the sensitivity and specificity of different DNA targets have been determined using conventional PCR.

As part of the results, the presence of *Leishmania infantum* has been detected for the first time in Europe in common urban bats (*Pipistrellus pipistrellus*), a species widely distributed in Europe and closely related to urban and peri-urban environments. About 60% of the animals tested were positive for at least one of the samples tested (spleen, hair and heart blood clot). In addition, hair provided encouraging results, where half of the positive individuals were positive to this non-invasive sample.

L. infantum DNA has also been detected in invasive exotic animals, such as the American mink (*Neovison vison*), which poses a threat to the river ecosystems of the Iberian Peninsula, not only because of competition for natural resources with other native species, but also because of its capacity to harbour pathogens transmissible to other animals and humans, as is the case of *L. infantum*. Ninety percent of animals tested from the Valencian Community were positive for this parasite in spleen samples. This organ is one of the most sensitive for detecting *L. infantum* due to its visceral location. In addition, samples were analysed by PCR with different DNA targets, being *SSUrRNA* and *Repeat region* the most sensitive compared to *ITS1* and *kDNA*.

Other wild animals such as the European badger (*Meles meles*), European hedgehog (*Erinaceus europaeus*) and red squirrel (*Sciurus vulgaris*), among others, were tested as potential hosts of *L. infantum*. In this case, all animals came from wildlife recovery centres in Madrid, Castilla-La Mancha, Castilla y León and Valencia. The parasite was detected in three of the nine animal species tested, with the European badger showing the highest presence of *L. infantum* (35.7% of individuals), followed by the European hedgehog (14.29%) and the red squirrel (11.53%). Samples of spleen, skin, oral conjunctiva and ocular conjunctiva (the latter two only in European hedgehogs) were analysed, with the spleen being more sensitive. Different DNA regions were also studied, with *SSUrRNA* and *Repeat region* being, once again, the most sensitive.

Finally, the presence of *Leishmania tropica* in native wild animals has been detected for the first time in Spain. This species is native to neighbouring countries such as Morocco and Tunisia, but its

presence in Spain had never been demonstrated before, with the exception of a few imported cases in humans. This finding should not be surprising, since the phlebotomine sandfly responsible for the transmission of *L. tropica* has been established in the Iberian Peninsula for several decades, and it was only a matter of time before the first autochthonous cases were detected. The wildcat (*Felis silvestris*) was the animal in which the parasite was found, with 18 animals tested using spleen and skin samples, and where seven of the 18 were positive for *Leishmania* spp. Of these seven, one animal was positive for *L. tropica* in skin samples while another individual had a mixed sequences of *L. tropica* and *L. infantum* and may correspond to a mixed infection or a hybrid between the two species. This finding opens the way to the control and prevention of this foreign *Leishmania* species, which usually presents with a cutaneous clinic, and where health professionals, veterinarians and authorities must work together to detect, treat and prevent infections with this species in the region. This PhD thesis has highlighted the presence of *Leishmania* in wild animals in the Iberian Peninsula, which could be investigated as sentinels of this parasitosis, as well as evaluating their potential role as a reservoir in future epidemiological outbreaks of leishmaniasis.

CAPÍTULO 1:
INTRODUCCIÓN

1.1. GÉNERO *LEISHMANIA*

1.1.1. CARACTERÍSTICAS GENERALES Y CICLO DE VIDA

El género *Leishmania* es un grupo de organismos protozoarios incluidos dentro del orden Kinetoplastida y la familia Trypanosomatidae que fue descrito por primera vez en 1903 por el patólogo escocés sir William Boog Leishman en la India (Steverding, 2017). Este género abarca más de 20 especies consideradas los agentes causales de la leishmaniosis humana, siendo un grupo heterogéneo en cuanto a distribución geográfica y animales a los que parasitan (Azami-Conesa et al., 2021a). Sin embargo, a pesar de las diferencias entre ellas, estas especies comparten características morfológicas y biológicas.

El parásito tiene al menos dos morfotipos que se encuentran en todas las especies del género, siendo esenciales para el correcto desarrollo del ciclo. Las diferencias entre ambos son diversas, de manera que los amastigotes son intracelulares obligados, esféricos u ovoides y miden entre 2 y 5 µm, presentan un núcleo redondeado y central, un kinetoplasto con forma alargada o de bastón, un cuerpo basal y axonema presentes, pero no tienen flagelo libre. Por el contrario, los promastigotes tienen un largo flagelo libre que les proporciona movimiento, siendo de forma alargada y fina, de entre 14 y 20 µm de largo y de 2 a 4 µm de ancho. En esta última forma se observa el núcleo en posición central y el kinetoplasto muy próximo, así como el axonema y el cuerpo basal (Figura 1) (Llanos-Cuenta, 2013).

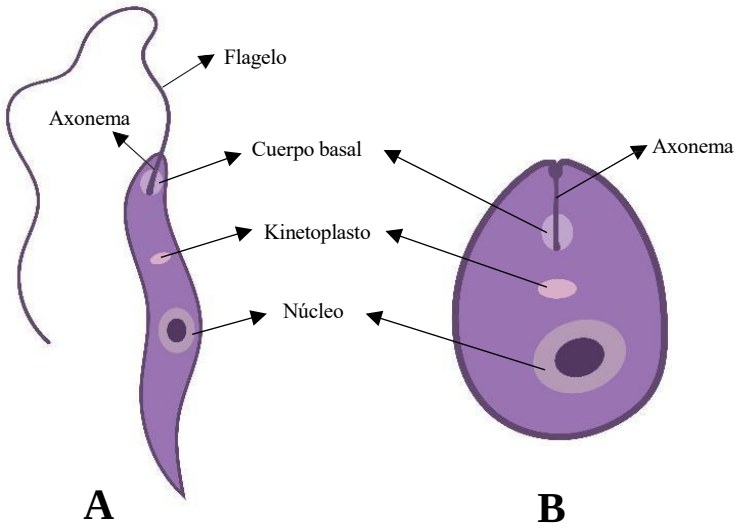

Figura 1. A: Promastigote de *Leishmania*. B: Amastigote de *Leishmania*. Diseño propio creado con Biorender.com.

El kinetoplasto es uno de los orgánulos más significativos de estos parásitos. Es común a todos aquellos organismos pertenecientes al orden Kinetoplastida, incluyendo a géneros como *Trypanosoma* o *Crithidia*, y se trata de una acumulación de ADN mitocondrial exclusiva de estos parásitos. La posición de este orgánulo, así como del flagelo y el núcleo, se han empleado para identificar las diversas fases de estos parásitos (Halliday et al., 2020). Además, el kinetoplasto es habitualmente utilizado como marcador o diana para la detección de estos organismos mediante la técnica de la reacción en cadena de la polimerasa (PCR por sus siglas en inglés) debido a su elevada especificidad y sensibilidad (Aransay et al., 2000).

El ciclo de vida de los parásitos del género *Leishmania* es complejo, ya que presenta fases de desarrollo en animales mamíferos y fases de desarrollo en insectos, principalmente flebotominos, más comúnmente denominados flebotomos, que actúan como vectores (Figura 2). Durante este ciclo, el parásito infecta al mamífero a través de la picadura de los flebotomos, insectos del orden Diptera y de la familia Psychodidae (Bates, 2007). La fase infectante para el mamífero es el promastigote metacíclico, que se inocula a través de la saliva del flebotomo, entrando, así, en contacto con los macrófagos y otras células, como las células dendríticas del mamífero, que iniciarán el proceso de fagocitosis para eliminar al patógeno. Los promastigotes se situarán en un fagosoma, que se fusiona con los endosomas y lisosomas, formando así un fagolisosoma, donde cambia el morfotipo de promastigote a amastigote. En el interior de este fagolisosoma se ataca al parásito mediante la acción de enzimas lisosomales y diversas toxinas, como los radicales libres de oxígeno o los intermediarios de nitrógeno (óxido nítrico, entre otros) (Matlashewski, 2001). Sin embargo, los amastigotes son capaces de evadir la acción de estas sustancias mediante diversos mecanismos, como por ejemplo la inhibición selectiva de la señal de transducción (Descoteaux et al., 1992) o la inhibición transitoria de la fusión de los fagosomas y los endosomas (Olivier et al., 1998), consiguiendo sobrevivir a las acciones ofensivas de la célula del mamífero, continuando con su ciclo vital. Superada la acción destructiva de la célula hospedadora, el amastigote tiene la capacidad de multiplicarse en el interior de los macrófagos mediante fisión binaria hasta llegar a provocar la lisis de la célula hospedadora, liberando así una elevada cantidad de amastigotes que infectarán nuevas células sanas (Matlashewski, 2001). El vector se infectará al ingerir sangre con macrófagos parasitados con los amastigotes, que llegarán al sistema digestivo del flebotomo, donde provocarán la lisis de la célula mamífera ingerida, liberándose en el estómago. En este momento del ciclo, el parásito se transformará a la forma promastigote, capaz de fijarse con su flagelo y multiplicarse en distintas zonas del intestino del insecto, según la especie de *Leishmania* y, tras su maduración (promastigote metacíclico), se trasladará hasta la probóscide, concretamente a las glándulas salivares, a la espera de ser liberados en un nuevo mamífero durante el momento de la picadura (U.S. Disease Control and Prevention. Laboratory Identification of Parasites of Public Health Concern., 2017).

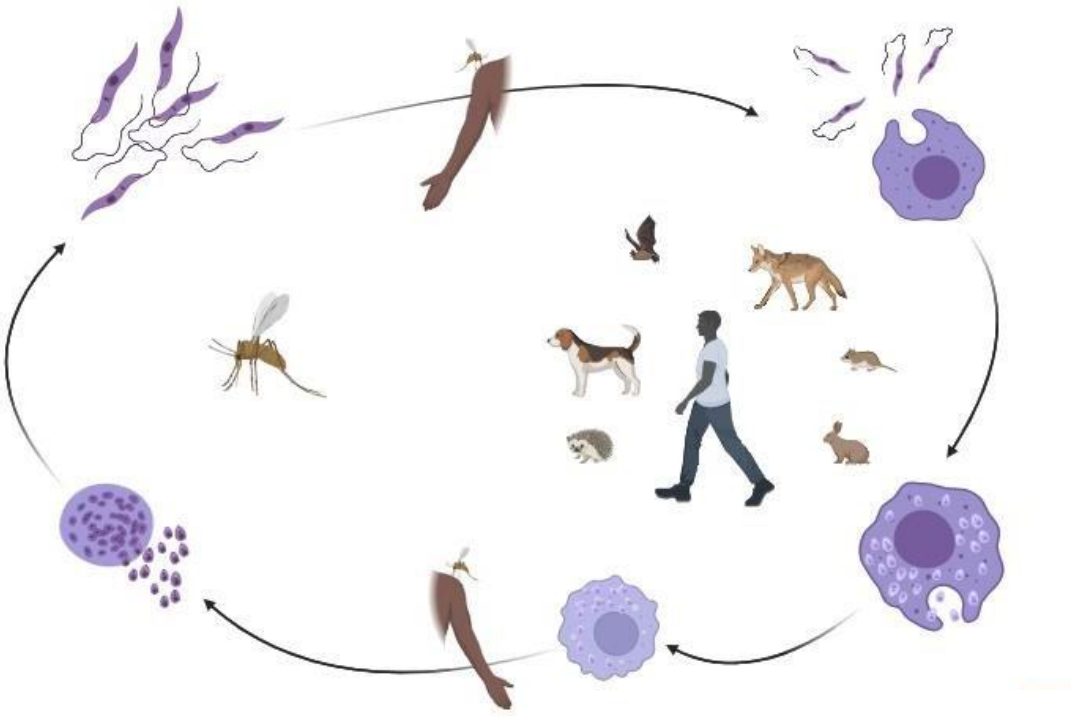

Figura 2. Ciclo biológico de *Leishmania*. Diseño propio creado con Biorender.com.

1.1.2. CLASIFICACIÓN DEL GÉNERO *LEISHMANIA*

El género *Leishmania* abarca un gran número de especies, muchas de ellas históricamente vinculadas al ser humano. Los primeros registros de este parásito en el hombre datan del 3.500 a.C., encontrándose ADN de *Leishmania* en momias del Antiguo Egipto. Asimismo, se han encontrado numerosos registros en antiguos escritos que describen patologías compatibles con la enfermedad que causa el parásito, especialmente de su manifestación cutánea (Akhoundi et al., 2016; Maxfield & Crane, 2019).

Establecer una taxonomía fiable y definitiva sobre el grupo no es una tarea fácil, debido a las variadas manifestaciones clínicas que se presentan y al elevado número de animales que infecta, pero actualmente existe un amplio consenso en clasificar a los parásitos del género *Leishmania* en el Reino Protista (Haeckel, 1866), phylum Euglenozoa (Cavalier-Smith, 1993), clase Kinetoplastea (Honigberg, 1963 emend. Vickerman, 1976), dentro del orden Trypanosomatida (Kent, 1880) y la familia Trypanosomatidae (Döflein, 1901) (Akhoundi et al., 2016; Armúa-Fernández & Venzal, 2019). No podemos olvidar que los protozoos y otros eucariotas son aún objeto de revisión

taxonómica (Adl et al., 2005) y algunos autores insisten en la dificultad de llegar a un consenso en el caso de los protozoos (Ruggiero et al., 2015). En una de las revisiones más recientes, se sugiere la presencia de dos dominios principales en los eukariotas: Diaphoretickes y Amorphea. El infrarreino Excavata, que engloba a tripanosomátidos y metamonádidos, se situaría dentro del dominio Diaphoretickes junto con las algas microscópicas y la mayor parte de los protozoos, tales como apicomplejos, ciliados y una minoría de amebas. Por otro lado, el dominio Amorphea englobaría la mayoría de las amebas, hongos y animales (Adl et al., 2019). También debemos tener en cuenta que las técnicas moleculares son de gran ayuda para la clasificación de organismos y, en general, más fiables y sensibles que las técnicas utilizadas en el momento de la descripción de nuevas especies, hace décadas en la mayoría de los casos (Schönian et al., 2010).

Dentro de la familia Trypanosomatidae podemos encontrar tanto parásitos monoxenos (aquellos con un solo hospedador, en su mayoría insectos) como parásitos heteroxenos (aquellos con la necesidad de pasar por dos hospedadores). Estos últimos son agentes causales de enfermedades tanto en mamíferos como en reptiles o plantas y, por tanto, son los más estudiados dentro del grupo. Todos ellos son organismos flagelados, entre los que encontramos 3 géneros: *Phytomonas, Trypanosoma* y *Leishmania* (Akhoundi et al., 2016; Votýpka et al., 2013).

El género *Phytomonas* abarca organismos parásitos de más de 100 especies de plantas, como el tomate, el café o la palma, siendo de gran relevancia debido a las pérdidas económicas que causa en la agricultura (Jaskowska et al., 2015; Kaufer et al., 2017). Dentro del género *Trypanosoma* encontramos parásitos de mamíferos que se transmiten mediante insectos hematófagos y que causan enfermedades en el ser humano de gran importancia, como la enfermedad de Chagas en América Central y del Sur (*Trypanosoma cruzi*) y la enfermedad del sueño en África (*Trypanosoma brucei gambiense* y *Trypanosoma brucei rhodesiense*) (Kaufer et al., 2017).

El género *Leishmania* es uno de los más ampliamente distribuidos y diversos dentro de los tripanosomátidos, abarcando más de 50 especies parásitas de mamíferos, entre ellos el ser humano, así como especies cuyos hospedadores son reptiles. Tradicionalmente, las especies de *Leishmania* se habían clasificado según tres criterios: las características morfológicas, los hospedadores que afectaban y los ciclos de vida que presentaban. Sin embargo, desde la aparición y mejora de los métodos moleculares, la clasificación y sistemática de este amplio grupo ha sufridos diversos cambios, siendo una herramienta de ayuda, combinada con los criterios biológicos anteriores (Klatt et al., 2019; Kostygov & Yurchenko, 2017). Actualmente se reconocen dos grandes clados dentro del género: *Euleishmania* y *Paraleishmania* y dentro de cada de uno se encuentran los diferentes subgéneros (Klatt et al., 2019).

En el caso de *Euleishmania*, está aceptado por la mayoría de los investigadores que incluye los subgéneros *L.* (*Leishmania*), *L.* (*Sauroleishmania*), *L.* (*Viannia*) y *L.* (*Mundinia*), siendo los tres primeros los que engloban a la mayor parte de las especies del género (Figura 3). Sin embargo, *Paraleishmania* levanta una mayor controversia debido a la heterogeneidad de sus integrantes. Muchos autores le consideran el "cajón de sastre" del género, ya que incluye algunas especies que tradicionalmente pertenecían al género *Endotrypanum*, que abarcaba inicialmente a tripanosomátidos endoeritrocíticos descritos en su mayoría en la Guayana Francesa (Espinosa et al., 2018).

Las especies de *Leishmania* que afectan a mamíferos se engloban, principalmente, dentro de los subgéneros *L.* (*Leishmania*) y *L.* (*Viannia*), con un total de 53 especies actualmente reconocidas dentro de estos grupos. Estas especies están ampliamente distribuidas, estando 29 de ellas presentes en el continente americano (clásicamente llamado Nuevo Mundo), 20 en África, Europa y Asia (Viejo Mundo), tres en las dos áreas de distribución anteriores, mientras que solo una especie del subgénero *L.* (*Mundinia*) ha sido descrita de forma exclusiva en Australia, (Klatt et al., 2019; Panahi et al., 2023). En torno a 20 de estas especies están descritas en el ser humano, causando distintas manifestaciones clínicas de la enfermedad conocida como leishmaniosis. Todas ellas son comunes a diversas especies de mamíferos, tratándose de organismos zoonóticos de elevada relevancia a nivel mundial.

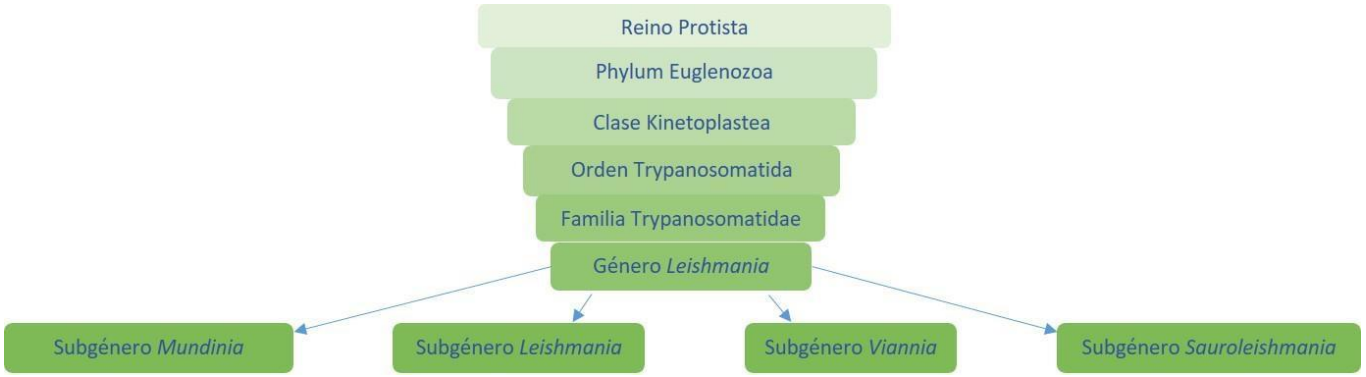

Figura 3. Clasificación del género *Leishmania*. Elaboración propia

1.1.3. ESPECIES ZOONÓTICAS RELEVANTES

Según el Centro para el Control y la Prevención de Enfermedades (CDC) se define a una enfermedad zoonótica como aquella capaz de transmitirse entre los animales y los seres humanos. Puede estar causada por diversos patógenos, entre los que se encuentran con gran frecuencia los parásitos, entre ellos el género *Leishmania* (Centers for Disease Control and Prevention One Health office, s.f.).

Uno de los continentes más afectados por la leishmaniosis es América, en especial América del Sur y Centroamérica (Tabla 1). En esta amplia región, una de las especies más estudiadas y más extensamente distribuida es *L.* (*Viannia*) *braziliensis*, abarcando desde México hasta Argentina, describiéndose la infección principalmente en humanos, perros, caballos, gatos, así como en numerosas especies de fauna silvestre, como murciélagos, armadillos y varias especies de roedores (Azami-Conesa et al., 2021a; Ratzlaff et al., 2023).

En Europa, África y Asia podemos encontrar especies tan relevantes como *L. tropica*, principalmente en el norte de África, Oriente Próximo y esporádicamente en Grecia, *L. donovani* en África central, Oriente Medio y determinadas regiones de Asia, o *L. major* en países como Kenia, Etiopía, Túnez o Israel (Tabla 1). Estas especies se han descrito en humanos, gatos, murciélagos, primates o roedores (Azami-Conesa et al., 2021a; Cardoso et al., 2021; El Idrissi Saik et al., 2022).

Sin embargo, la especie más ampliamente distribuida y con mayor número de grupos de animales afectados es *L. infantum*. Se ha descrito tanto en el continente americano como en Europa, Asia y África. Es un parásito claramente zoonótico teniendo en cuenta tanto la sintomatología que produce en el hombre y otras especies, como la elevada prevalencia descrita en humanos, perros, gatos, tejones, murciélagos, zorros o lobos, entre otros. *L. infantum* se puede encontrar principalmente en la cuenca mediterránea (tanto norte de África como sur de Europa), en América del Sur, Oriente Próximo, India, Sudeste Asiático o Asia central (Tabla 1) (Akhoundi et al., 2016; Azami-Conesa et al., 2021a; Cardoso et al., 2021).

Por último, cabe destacar a *L.* (*Sauroleishmania*) *tarentolae*, un parásito considerado, hasta el momento, propio de reptiles, pero que en los últimos años se ha detectado tanto en perros como en humanos en varias regiones de Italia (Iatta et al., 2021; Mendoza-Roldan et al., 2021; Pombi et al., 2020). Se considera no patógena en el hombre, pero podría tener numerosas implicaciones en los nuevos tratamientos contra la leishmaniosis humana (Taylor et al., 2010).

Tabla 1. Especies zoonóticas de *Leishmania* y su distribución. Adaptación propia de Akhoundi et al., 2016.

SECCIÓN	SUBGÉNERO	ESPECIE	ZONA GEOGRÁFICA
Euleishmania	*Leishmania*	*L. aethiopica*	Este de África
		L. amazonensis syn. *L. gamhami*	Centro de América del Sur
		L. donovani syn. *L. archibaldi*	África central, sur de Asia, Oriente Próximo, India y China
		L. infantum syn. *L. chagasi*	Cuenca mediterránea (sur de Europa y norte de África), Oriente Próximo, Asia central, centro y sur de América.
		L. major	Centro y sur de África, Oriente Próximo y Asia central.
		L. mexicana	Norte América y centro de América del Sur
		L. tropica syn. *L. killicki*	Centro y norte de África, Oriente Próximo, Asia central e India
		L. venezuelensis	Norte de América del Sur
	Viannia	*L. braziliensis*	América del Sur y Centroamérica
		L. guyanensis	Norte de América del Sur y Centroamérica
		L. lainsoni	Centro de América del Sur
		L. lindenbergi	Centro de América del Sur
		L. naiffi	Centro de América del Sur y Centroamérica
		L. panamensis	América del Sur y Centroamérica
		L. peruviana	Centro de América del Sur
		L. shawi	Centro de América del Sur
Paraleishmania	*L. enriettii complex*	*L. colombiensis*	Norte de América del Sur
		*L. martiniquensis**	Sudeste asiático
		*L. siamensis**	Europa, Sudeste asiático y América del Norte

Syn: sinónimo; * clasificación taxonómica sin consenso.

1.1.4. LEISHMANIOSIS HUMANA

La leishmaniosis humana afecta actualmente a más de un millón de personas al año en todo el mundo, especialmente en zonas tropicales, subtropicales y la cuenca mediterránea, encontrándose en más de 90 países. Más de un billón de personas viven en zonas endémicas y, por tanto, están expuestas a la infección. La Organización Mundial de la Salud (OMS) considera a la leishmaniosis como una de las denominadas "Enfermedades Olvidadas" o "Neglected Diseases" debido a su distribución en países en vías de desarrollo, lo que dificulta el acceso a un diagnóstico precoz y tratamiento adecuado, además de una falta importante de recursos económicos destinados a la investigación de la enfermedad y desarrollo de nuevos fármacos. Anualmente, la OMS destina grandes esfuerzos humanos y económicos para frenar la transmisión y mejorar el acceso a los tratamientos en zonas endémicas, con numerosas campañas informativas de detección y tratamiento de la infección en zonas comprometidas (Akhoundi et al., 2016; World Health Organization, 2010).

Existen tres formas clínicas principales de la infección en el ser humano: leishmaniosis cutánea (LC), leishmaniosis mucocutánea (LMC) y leishmoniasis visceral (LV). Cada una de ellas presenta un cuadro clínico propio y es provocada por distintas especies del parásito, aunque algunas especies pueden producir varias formas clínicas.

La LC es la forma clínica más habitual, con una estimación de entre 600 000 y un millón de casos nuevos al año a nivel mundial, provocando daños cutáneos visibles, lo que provoca la estigmatización y aislamiento social de las personas que lo padecen. Son numerosas las especies de *Leishmania* capaces de provocar esta forma clínica, siendo *L. tropica*, *L. major* y *L. infantum* las habituales en el norte de África, Asia central y Oriente Próximo y *L. amazonensis, L. guyanensis, L. panamensis, L. peruviana, L. mexicana* y *L. braziliensis* las propias del continente americano (Desjeux, 2004; Saidi et al., 2023), como se observa en la Figura 4. Esta forma clínica se caracteriza por la aparición de lesiones cutáneas en la zona de la picadura del vector, donde se produce la multiplicación de los amastigotes. Estas lesiones pueden ser difusas en aquellos individuos donde se produce una inhibición de la respuesta inmune mediada por receptor, provocando lesiones de mayor tamaño y con aspecto lepromatoso y afección de los nervios periféricos de la zona. A este tipo de lesiones se les denomina leishmaniosis cutánea difusa (Desjeux, 2004).

En el caso de la afección mucocutánea (LMC), se caracteriza por la destrucción parcial o total de tejido mucoso, especialmente de nariz, paladar y faringe, provocando daños irreversibles y con un elevado coste psico-social para el afectado. Esto se produce por la dispersión de los amastigotes desde la zona cutánea hasta las capas mucosas de la región nasofaríngea (Yadav et al., 2023). Las especies de *Leishmania* que habitualmente provocan este tipo de sintomatología son *L. braziliensis,*

L. amazonensis, L. guyanensis y *L. peruviana* en América del Sur y Central, donde es más habitual, pero esporádicamente lo encontramos en países asiáticos, africanos o europeos causada por *L. donovani, L. infantum, L. major, L. tropica* y *L. aethiopica*, aunque con menor frecuencia, siendo más habitual en pacientes inmunodeprimidos (Figura 4) (Desjeux, 2004; World Health Organization & Control of neglected tropical diseases, 2023a; Yadav et al., 2023).

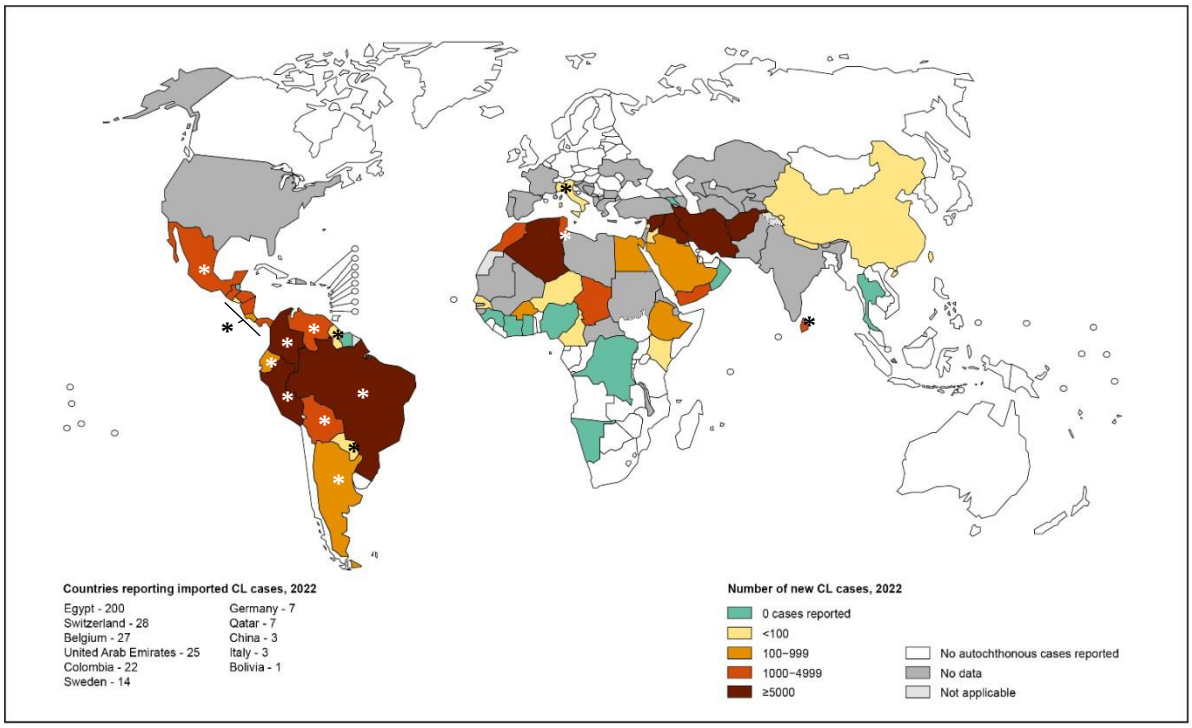

Figura 4. Mapa de distribución de leishmaniosis cutánea y mucocutánea. *: solapamiento de LC y LMC. Adaptación del mapa de la OMS 2023.

La clínica de la LV difiere en gran medida de las anteriores debido a los órganos donde habitualmente se acantona el parásito y a la sintomatología que presenta. En este caso, se observan alteraciones clínicas sistémicas, como fiebre, pérdida de peso, esplenomegalia, hepatomegalia, anemia severa o linfoadenopatías, debido a la presencia de los amastigotes en ganglios linfáticos, médula espinal, hígado y bazo, principalmente (Desjeux, 2004). En este caso se produce la translocación de los amastigotes desde la zona cutánea de picadura hasta estos órganos, donde provoca un granuloma debido a la parasitación de las células del órgano afectado y la respuesta inflamatoria que lo acompaña (Costa et al., 2023). Se trata de la forma clínica de mayor gravedad, ya que presenta tasas de mortalidad muy elevadas en personas y animales sin tratamiento adecuado. Esta enfermedad está provocada principalmente por *L. infantum* y *L. donovani*, tanto en Asia como en Europa, África y América (Figura 5) (World Health Organization & Control of neglected tropical diseases, 2023b). Además, en ciertas regiones de Asia, especialmente en India y sudeste asiático, los pacientes pueden

desarrollar recidivas con una afectación cutánea, denominada leishmaniosis post Kala-azar, que se caracteriza por la aparición de máculas y pápulas eritematosas en cara, extremidades superiores y tronco. Esto se produce generalmente en pacientes inmunocompetentes curados de LV provocada por *L. donovani* y en pacientes inmunodeprimidos con LV por *L. infantum*, unos 2 o 3 años después de recibir tratamiento frente a la enfermedad (Costa et al., 2023; Desjeux, 2004).

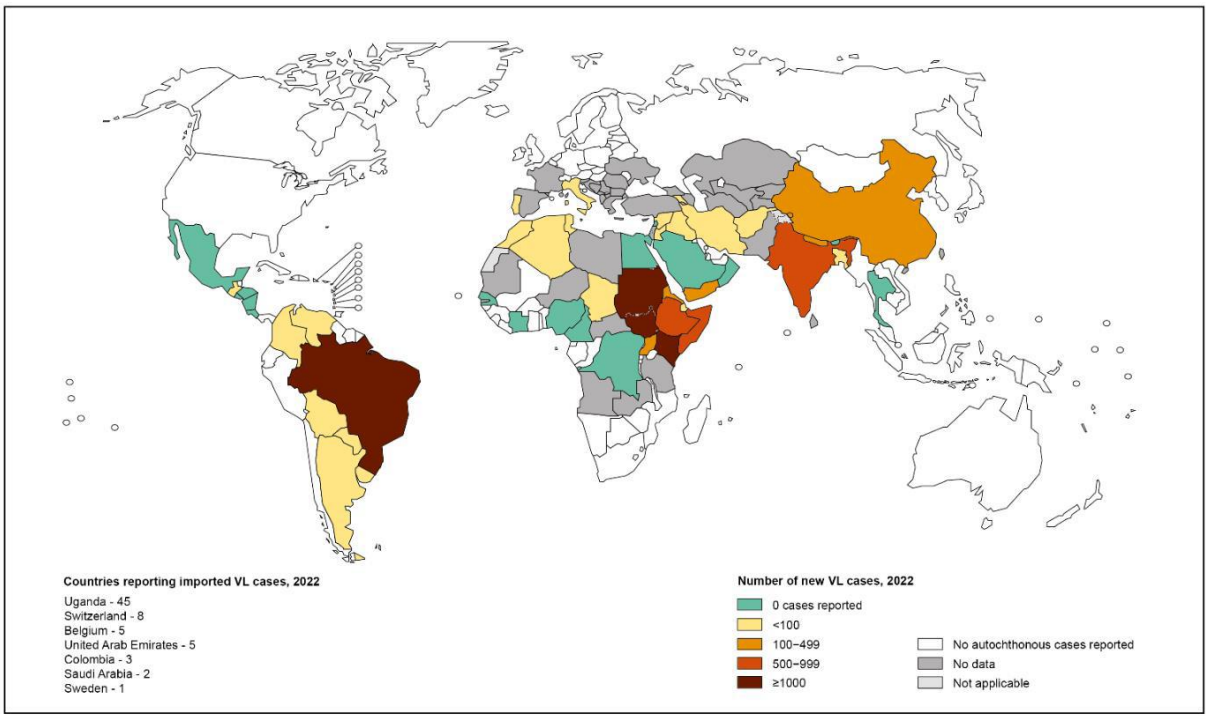

Figura 5. Mapa de distribución de leishmaniosis visceral. Fuente: OMS 2023

Estas manifestaciones clínicas tienen una especial relevancia en personas inmunocomprometidas, como los pacientes VIH-positivo o personas con tratamientos inmunosupresores, que además suelen presentar una sintomatología agravada por diversas co-infecciones (Costa et al., 2023).

Sin embargo, en numerosas ocasiones, las personas parasitadas no presentan sintomatología, y esto dependerá principalmente del estado inmunológico del paciente, (especialmente en zonas endémicas), de la carga parasitaria y de la especie de *Leishmania*. Además, algunas personas son más propensas a desarrollar síntomas que otras (Saidi et al., 2023).

En relación con el diagnóstico, siempre se va a realizar mediante técnicas de laboratorio que pongan de manifiesto la presencia del parásito en el individuo, pero la clínica y los datos epidemiológicos facilitan la sospecha (de Paiva-Cavalcanti et al., 2015; Morales-Yuste et al., 2022). Las técnicas inmunológicas como la inmunocromatografía (ICT), la inmunofluorescencia indirecta (IFAT por sus siglas en inglés) o el enzimoinmunoanálisis de adsorción (ELISA por sus siglas en inglés) son muy

utilizadas para detectar los anticuerpos específicos contra *Leishmania*, utilizando suero como muestra principal y obteniendo los resultados con relativa rapidez. Sin embargo, presentan algunas desventajas como un elevado porcentaje de falsos positivos por la reacción cruzada de otros patógenos o su limitada sensibilidad cuando el grado de parasitación es bajo (de Paiva-Cavalcanti et al., 2015; Morales-Yuste et al., 2022; Solano-Gallego et al., 2009). En las últimas décadas, la detección mediante PCR es una de las técnicas diagnósticas por excelencia, en especial la PCR en tiempo real (rtPCR), debido a su elevada sensibilidad y especificidad, la rápida obtención de resultados y que permite realizar un seguimiento del paciente y de la eficacia del tratamiento, cuantificando la carga parasitaria mediante la PCR cuantitativa (qPCR) (Solano-Gallego et al., 2009). Para estas técnicas se pueden usar numerosas muestras como sangre, tejido esplénico o hepático, tejido cutáneo, aspirado linfático o muestra de médula ósea, entre otros (Azami-Conesa et al., 2021a; Morales-Yuste et al., 2022). Además de estas técnicas, otras como la microscopía, la histopatología o el cultivo de promastigotes también son metodologías eficaces, aunque actualmente menos frecuentes para diagnóstico (Mathison & Bradley, 2023; Morales-Yuste et al., 2022).

Por último, actualmente el tratamiento contra la leishmaniosis es uno de los mayores retos a los que se enfrentan los investigadores del área, ya que no existe un tratamiento universal, los efectos citotóxicos de los fármacos actuales en los pacientes son graves y la respuesta al tratamiento depende de numerosas variables como la especie de *Leishmania*, el estado inmunológico del paciente o la carga parasitaria (Pradhan et al., 2022). En humanos, el tratamiento de elección para LC y LV son los antimoniales pentavalentes (antimoniato de meglumina o estibogluconato de sodio) por vía intravenosa o intramuscular o la anfotericina B liposomal en el caso de LC y LMC (Pradhan et al., 2022; Wu et al., 2022). Sin embargo, en los últimos años son numerosos los grupos de investigación que trabajan sobre alternativas efectivas y menos tóxicas para estos fármacos, obteniendo resultados esperanzadores (Bouabdallah et al., 2022).

1.1.5. RESERVORIOS ANIMALES DEL GÉNERO *LEISHMANIA*

Las especies zoonóticas de *Leishmania* se han descrito en numerosos animales que pueden actuar como reservorios o actores clave en el mantenimiento del parásito en determinadas áreas, facilitando así su presencia en zonas urbanas, peri-urbanas o rurales. El animal más estudiado y hospedador habitual de 13 especies de *Leishmania* es el perro. La leishmaniosis canina es endémica en todas las zonas anteriormente citadas, tratándose de una de las enfermedades parasitarias más importantes de este animal. Se trata de una enfermedad crónica y grave, que cuando presenta síntomas se caracterizan por una afectación cutánea y/o visceral severa, donde se desarrollan alteraciones como úlceras y dermatitis, inflamación de ganglios linfáticos, afectación ocular y diseminación a bazo,

hígado y médula espinal, junto con pérdida de peso, anemia severa y enfermedad renal crónica en las fases más avanzadas de la parasitosis. En la península ibérica, zona endémica de leishmaniosis, se han declarado más de 1100 casos nuevos en perros entre 2005 y 2020 según datos de la Organización Mundial de Sanidad Animal (WOAH por sus siglas en inglés) (Baneth & Solano-Gallego, 2022; Maia et al., 2023; Müller et al., 2022).

Sin embargo, no todos los animales donde se ha detectado este parásito pueden ser considerados reservorios, ya que el término reservorio solo se puede aplicar, según la OMS, a aquellos que son lo suficientemente abundantes y viven el suficiente tiempo como para que interactúen con el vector y sean capaces de mantener y desarrollar al parásito en su interior (World Health Organization, 2010). Además, existen otras consideraciones, como que al menos el 20% de los individuos deben estar parasitados, el parásito se tiene que mantener el tiempo necesario en el organismo y estar disponible en la piel o sangre para el vector y, por último, que el parásito presente en el reservorio y en el humano sean de la misma especie. Por tanto, en muchas ocasiones se habla de "potenciales reservorios" más que de reservorios propiamente dichos, debido a la complejidad de estudiar y demostrar todas estas características en un animal (Ashford, 1996; Roque & Jansen, 2014). Sin embargo, en los últimos años cada vez es más frecuente el uso de diversos términos para hacer referencia a los distintos tipos de reservorios u hospedadores de *Leishmania*, como pueden ser "hospedadores de mantenimiento" que son aquellos mamíferos sin síntomas ni signos pero capaces de mantener el parásito en su interior; "hospedadores de ampliación" o "amplifier hosts" en inglés, que son aquellos animales que favorecen la transmisión al vector porque tienen carga parasitaria suficiente como para que los parásitos estén disponibles en sangre y/ o piel; u "hospedadores de punto final", donde los parásitos no se pueden transmitir al vector por diversos motivos (Ashford, 1996).

Aparte de todos estos términos, los animales que actualmente se consideran reservorios principales de *Leishmania* son los siguientes:

1. Perro: se trata del principal reservorio de *L. infantum*, y se han descrito otras 12 especies de *Leishmania* en ellos. No solo se encuentran perros con *Leishmania* en países endémicos, sino también en otras regiones como Estados Unidos o Centroeuropa debido al movimiento de estos animales por diversos países. Se estima que en torno al 50% de los perros infectados son asintomáticos, lo que aumenta la dificultad de control de esta parasitosis (Baneth & Solano-Gallego, 2022; Solano-Gallego et al., 2009; World Health Organization, 2010).
2. Liebre (*Lepus granatensis*) y conejo silvestre (*Oryctolagus cuniculus*): reservorios de *L. infantum* en Europa, en concreto en la península ibérica y Grecia (Jiménez et al., 2014; Ortuño et al., 2019; Tsokana et al., 2016).

3. Gran Jerbo (*Rhombomys opimus*): principal reservorio de *L. major* en Asia central. No solo alberga esta especie, sino también otras no zoonóticas como *L. gerbilli* (Bakhshi et al., 2013; World Health Organization, 2010).
4. Jird Gordo (*Psammomys obesus*): es un roedor propio del este de Asia y el norte de África que se alimenta principalmente de cultivos de cereal, estando presente en zonas peri-urbanas y rurales, donde participa como reservorio principal de *L. major* (Ghawar et al., 2011; World Health Organization, 2010).
5. Hiracoideos: mamíferos placentarios involucrados en la transmisión de *L. tropica* en África y Oriente Próximo (Lemma et al., 2009; Pareyn et al., 2019; World Health Organization, 2010).
6. Perezoso de dos dedos (*Choloepus didactylus*): principal reservorio de *L. guyanensis* en Brasil y Guayana Francesa, así como de *L. shawi* en la región amazónica. Estrechamente relacionado con comunidades rurales de esos países (Dedet et al., 1989; Lainson et al., 1989; World Health Organization, 2010).
7. Osos hormiguero amazónico (*Tamandua tetradactyla*): en Brasil es el reservorio más importante de *L. guyanensis* (Caldart et al., 2021; Calvopina et al., 2004; World Health Organization, 2010).

Además de éstas, numerosas especies animales, tanto domésticas como silvestres, han sido descritas como hospedadores del género *Leishmania*, pudiendo actuar como especies centinela, que se definen como aquellos organismos capaces de poner de manifiesto el riesgo existente para la salud humana, animal o ambiental de un determinado patógeno u otro efecto perjudicial. La información obtenida a partir de estas especies centinela resulta de gran utilidad para llevar a cabo medidas preventivas o detectar de manera precoz los posibles brotes de leishmaniosis (Aguirre, 2009). Cabe destacar que muchas de ellas son habituales en zonas urbanas, peri-urbanas o rurales, y por tanto pueden estar en contacto estrecho y cercano con el ser humano, lo que incrementa en gran medida la posibilidad de que presenten papeles relevantes en el mantenimiento del ciclo de vida de este parásito y faciliten su transmisión al ser humano (Alcover et al., 2020, 2021; Ashford, 1996; Azami-Conesa et al., 2021a; Cardoso et al., 2021; Galán-Puchades et al., 2019; Kushwaha et al., 2022; Montaner-Angoiti & Llobat, 2023; Roque & Jansen, 2014; Tsokana et al., 2020; World Health Organization, 2010).

1.2. TRANSMISIÓN Y DISTRIBUCIÓN DE *LEISHMANIA* SPP.

La leishmaniosis es una enfermedad vectorial, cuya transmisión está determinada por la presencia y capacidad de los vectores para mantener el ciclo en los diferentes escenarios donde se desarrolla (Bates, 2007). Además de éstos, existen numerosos factores que favorecen la presencia de la enfermedad.

1.2.1. FLEBOTOMOS: CARACTERÍSTICAS GENERALES Y DISTRIBUCIÓN GEOGRÁFICA

Los organismos responsables de la mayor parte de las transmisiones de *Leishmania* son los flebotomos, insectos del orden Diptera, suborden Nematocera, familia Psychodidae y subfamilia Phlebotominae (Akhoundi et al., 2016). Estos insectos hematófagos están involucrados en la transmisión, no solo de *Leishmania*, sino también de otros patógenos como virus, bacterias y otros protozoos. Los estudios taxonómicos más recientes describen más de 1000 especies distintas de flebotomos, tanto en el Viejo Mundo, con 538 especies incluidas en 9 géneros, como en el Nuevo Mundo con 488 especies de 23 géneros. De todos ellos, los géneros más habituales implicados en el ciclo de vida de este parásito son *Phlebotomus* y *Sergentomyia* en Europa, África y Asia, y *Lutzomyia* en América (Rodrigues & Galati, 2023).

La importancia del vector radica en la necesidad del parásito en desarrollar ciertas fases de su ciclo en el interior de este animal. Cuando el flebotomo ingiere sangre de un mamífero parasitado, los amastigotes de *Leishmania* alcanzan el sistema digestivo, donde las diferencias en temperatura y pH con el mamífero provocan el cambio morfológico a promastigotes procíclicos, capaces de replicarse. Pasados unos días, la digestión de la sangre del mamífero provoca un nuevo cambio en el parásito, pasando a la forma de promastigote nectomonado, con capacidad de migración, dirigiéndose a la zona anterior del sistema digestivo. Con este movimiento, el parásito evita la salida del vector por medio de la defecación. Una vez en el nuevo destino, los promastigotes sufren un nuevo cambio de forma a promastigotes leptomonados, forma replicativa, que, tras varios ciclos de multiplicación, pasarán a la forma de promastigote metacíclico, que será la fase infectante para el vertebrado (Bates, 2007).

Los flebotomos, por tanto, son parte esencial del ciclo de *Leishmania* y por ello se habla de un proceso de "coevolución" entre ambos organismos. Existen registros fósiles tanto de flebotomos como de los parásitos en las mismas zonas geográficas, evidenciando la relación y la

similar distribución que han presentado a lo largo de millones de años, aunque existen ciertas dudas sobre el término correcto a usar para definir esta relación, ya que distintos autores hablan de co-asociación, interacción y co-especiación (Akhoundi et al., 2016; Esseghir et al., 2000).

Sin embargo, la sola presencia de estos parásitos en el interior de un flebotomo no puede considerarse rasgo característico de su papel como vector de una especie en concreto. Es necesario que se observen una serie de condiciones, como los comportamientos alimenticios del insecto, la capacidad para desarrollar todas las fases del ciclo del parásito en su interior, la habilidad para transmitir a un nuevo vertebrado dicho protozoo y la concurrencia en espacio y tiempo con el hospedador definitivo, entre otras (Akhoundi et al., 2016). Actualmente existen alrededor de 35 especies de flebotomos consideradas vectores probados de *Leishmania*, mientras que hay otras 63 especies de las que se sospecha un papel crucial en la transmisión de más de 40 especies de este parásito. En la zona geográfica denominada "Viejo Mundo" el género *Sergentomyia* se relaciona con la transmisión de especies de *Sauroleishmania*, mientras que el vector más habitual de las especies que afectan a mamíferos sería *Phlebotomus*. Sin embargo, en América este último papel lo realizan las especies de género *Lutzomyia*. Algunas especies de flebotomos están ligadas a una sola especie de *Leishmania*, denominándose vectores restrictivos, como es el caso de *Phlebotomus papatasi* que solo es capaz de transmitir *L. major*. Esto se debe a la presencia de receptores específicos en el sistema digestivo de estos insectos (Akhoundi et al., 2016; Labbé et al., 2023). Por otro lado, se han estudiado algunas especies de flebotomos, que en condiciones controladas de laboratorio son capaces de albergar distintas especies del parásito (vectores permisivos), aunque solo se ha demostrado la infección natural con *L. infantum* a *Luztomyia longipalpis* y *L. tropica* a *P. perniciosus* (Bongiorno et al., 2019; Labbé et al., 2023; Vaselek & Volf, 2019).

Las hembras de estos dípteros son hematófagas, al igual que pasa en otras muchas especies de dípteros, y por tanto van a ser las implicadas en la transmisión, mientras que los machos son de hábitos fitófagos. Las hembras necesitan el aporte nutritivo de la sangre para el correcto desarrollo y maduración de los huevos. La periodicidad diaria con la que las hembras se alimentan depende de la especie y del animal del que se alimenten con mayor frecuencia. En la mayor parte de las especies, los picos de movimiento se producen durante el amanecer y el atardecer, pero aquellos individuos que se encuentren en madrigueras, cuevas o selvas frondosas, pueden alimentarse durante las horas centrales del día. Los lugares de reposo y cría de los flebotomos son variados, siendo los más habituales las madrigueras, huecos de los troncos de los árboles, en el suelo bajo la hojarasca, en termiteros o incluso bajo las piedras en el caso de especies que se encuentren en zonas selváticas o rurales, mientras que en zonas urbanas y periurbanas se pueden encontrar en drenajes de carretera, basuras, agujeros en el suelo o huecos de edificaciones. La selección de los vertebrados dependerá de los niveles de dióxido de carbono, el olor y los hábitos y

tamaño del hospedador. Los flebotomos son capaces de detectar todos estos factores a la hora de seleccionar al animal (Alten et al., 2015; Bray & Hamilton, 2007; Ready, 2013). Las distintas especies de flebotomos, por tanto, se pueden encontrar en diversos nichos ecológicos, teniendo en cuenta su limitado rango de vuelo, de aproximadamente 1,5 km por día (Akhoundi et al., 2016).

Abarcan ecosistemas tan variados como las selvas tropicales en América o las zonas desérticas y semidesérticas en el Viejo Mundo. Además, su amplia distribución mundial abarca desde los 50º de latitud N (sur de Canadá, norte de Francia y Mongolia) hasta los 40º de latitud S, excluyendo Nueva Zelanda y las islas del Pacífico. En cuanto a la altitud, se pueden encontrar desde zonas más bajas que el nivel del mar, como en el área del Mar Muerto, hasta los 3500 msnm en Afganistán (Alten et al., 2015).

En Europa se estima la presencia de alrededor de 10 especies de flebotomos relevantes desde el punto de vista sanitario, siendo la mayoría vectores demostrados de *Leishmania*. En el caso concreto de España, los primeros flebotomos descritos eran de la especie *Phlebotomus ariasi* y datan de 1909, pero el mayor número de estudios sobre estos insectos se realizaron entre los años 1970 y 1990, con un nuevo repunte a partir del brote de leishmaniosis humana en la Comunidad de Madrid en 2009. A lo largo de todos estos años, se han descrito un total de 14 especies diferentes de flebotomos en España, incluidas en los dos géneros habituales en Europa, *Phlebotomus* y *Sergentomyia* (Bravo-Barriga et al., 2022; González et al., 2023).

Las especies más ampliamente distribuidas en España son *P. perniciosus* (en todas las provincias excepto Vizcaya y Gran Canaria) (Figura 6), *P. ariasi* (en todas las provincias excepto A Coruña, Pontevedra, Palencia y Tenerife) (Figura 7) y *S. minuta* (en todas las provincias excepto A Coruña, Palencia y País Vasco) (Bravo-Barriga et al., 2022), siendo las dos primera las consideradas de mayor relevancia en la transmisión de *L. infantum* en el país (Alonso Capitán & Vázquez Torres, 2013; Amela et al., 2012; European Centre for Disease Prevention and Control, 2023).

Además, en los últimos años, otras especies de flebotomos han sufrido una notable expansión en la Península Ibérica, como *P. sergenti,* vector de *L. tropica,* y en menor medida *P. perfiliewi*, recientemente descrito en Mallorca (González et al., 2023), y *P. papatasi,* vectores de *L. tropica, L. infantum* y *L. major* en países cercanos como Marruecos, Argelia, Túnez e Italia, entre otros (Calzolari et al., 2022; ECDC, 2023; Karmaoui, 2020), como se comentará posteriormente con mayor profundidad.

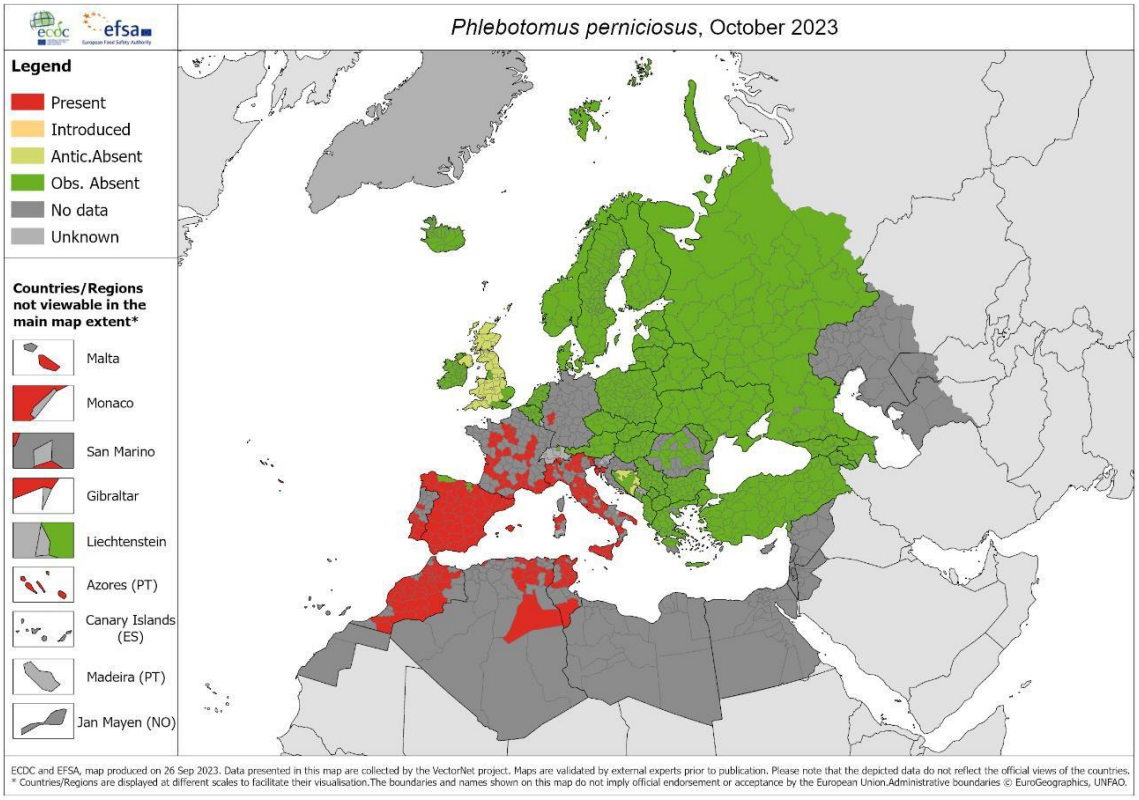

Figura 6. Mapa de distribución de *Phlebotomus perniciosus*. Fuente: ECDC, 2023

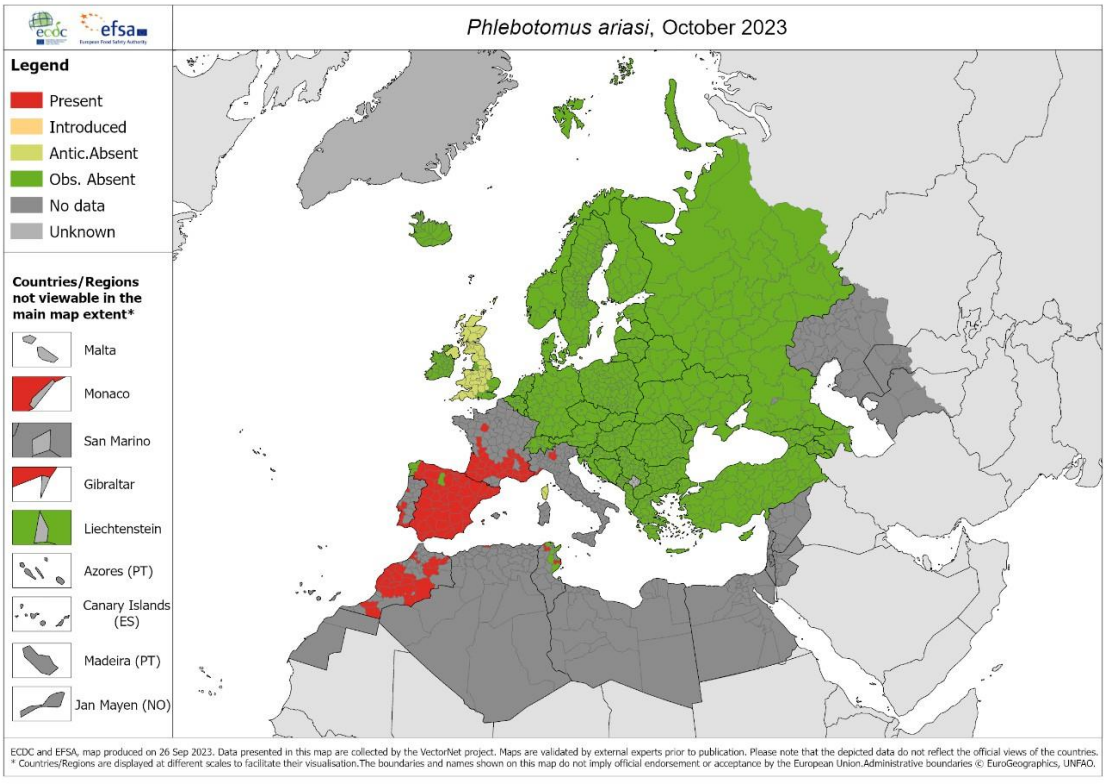

Figura 7. Mapa de distribución de *Phlebotomus ariasi*. Fuente: ECDC, 2023

1.2.2. VÍAS DE TRANSMISIÓN ALTERNATIVAS

A pesar de que la picadura de flebotomo es la vía más habitual de transmisión de *Leishmania*, en los últimos años se han descrito numerosas formas alternativas en las que el parásito puede pasar a un nuevo hospedador vertebrado, incluso sin la necesidad de que intervenga el vector.

Sin embargo, la mayoría de estas vías de transmisión son solo sugeridas por los autores, con la excepción de la vía vertical transplacentaria y la vía sexual, ambas probadas en perros (Boggiatto et al., 2011; Franssen et al., 2022; Naucke & Lorentz, 2012; Silva et al., 2009), en roedores (Martín-Sánchez, Torres-Medina, et al., 2020) y en humanos (Boehme et al., 2006; Mescouto-Borges et al., 2013; Toepp et al., 2019). En todos estos casos, se informa de resultados positivos a PCR, ELISA, IFAT y/ o examinación microscópica en individuos recién nacidos, siendo las madres positivas también al parásito. Estos hallazgos implican un mayor esfuerzo en el control y prevención de la enfermedad, en especial en zonas no endémicas del parásito, donde es posible su llegada por el movimiento global de personas y animales domésticos, ya que muchos de estos informes se dan en países como Alemania o Estados Unidos, que no son considerados endémicos de la parasitosis (Boehme et al., 2006; Boggiatto et al., 2011; Franssen et al., 2022; Naucke & Lorentz, 2012; Toepp et al., 2019).

Otras vías de transmisión sugeridas son:

- Pulgas: Coutinho y Linardi (2007) estudian el papel de estos insectos en la transmisión de *Leishmania* procedente de perros positivos. Observaron promastigotes por microscopía, obtuvieron resultados positivos a PCR e IFAT e inocularon un macerado de estas pulgas a roedores, obteniendo resultados positivos en dichos animales. Sin embargo, remarcan la necesidad de nuevos estudios para demostrar esta nueva vía de transmisión.
- Jejenes: varios autores han reportado la presencia de distintas especies de *Leishmania* en individuos del orden Ceratopogonidae, tanto en Asia como América, sugiriendo un posible papel relevante en la transmisión (Rebêlo et al., 2016; Sunantaraporn et al., 2021).
- Mordedura de perro: esta posible vía de transmisión se ha estudiado en perros, donde la mordedura de otro individuo pudo haber sido la fuente de parasitación en un país no endémico (Naucke et al., 2016).
- Ingesta de flebotomos: sugerida por De Oliveira et al. (2015), que la estudian en murciélagos insectívoros positivos a *Leishmania* en Brasil, los cuales se alimentan habitualmente de estos insectos. Esta vía de transmisión ha sido demostrada en otros tripanosomátidos, como *T. cruzi*, tanto en humanos como en otros animales, aunque es necesario tener en cuenta las numerosas diferencias entre los ciclos de vida de ambos parásitos (Barreto-de-Albuquerque et al., 2015; de Noya & González, 2015).

1.2.3. RELEVANCIA DE *LEISHMANIA* SPP. EN ANIMALES SILVESTRES

Los perros y otros animales domésticos han sido estudiados durante décadas en relación con su posible papel como reservorios y actores importantes en la transmisión y mantenimiento del ciclo de *Leishmania*. Esto se debe a su cercanía al ser humano, y a la posible implicación que pueden tener en brotes de leishmaniosis humana (Alcover et al., 2020; Amela et al., 2012; Cardoso et al., 2021). Sin embargo, en los últimos años se ha incrementado considerablemente el número de estudios sobre el posible papel de otros animales, más allá de los domésticos, que puedan estar implicados en el ciclo de vida de especies zoonóticas de *Leishmania*. Esto supone modificar numerosas estrategias de control y vigilancia de esta parasitosis, donde la perspectiva "One Health" ("Una Salud" en español) resulta de gran importancia debido a la propia naturaleza de la enfermedad, que implica al ser humano, a diversos vectores y a numerosas especies animales y, donde los cambios ambientales y socioeconómicos suponen factores de riesgo para el desarrollo de brotes epidémicos. Una de las características del género *Leishmania* es su presencia en tres tipos diferentes de ciclos: selvático, doméstico y urbano. En todos ellos se encuentran involucradas especies zoonóticas del parásito, permitiendo así la interacción entre los distintos ciclos (Hong et al., 2020; Quaresma et al., 2011). Estos continuos contactos entre los participantes de los diferentes ciclos de la parasitosis se deben, en parte, al aumento de la exposición de los humanos a zonas con mayor abundancia de animales silvestres, fruto de la fragmentación de los hábitats, el incremento de la urbanización hacia zonas rurales, el aumento de las actividades relacionadas con la agricultura y el ecoturismo, así como al movimiento de los animales silvestres a zonas urbanas y peri-urbanas en busca de alimento. Todos estos escenarios han provocado un aumento de la presencia de patógenos zoonóticos en humanos, entre ellos *Leishmania*, en especial en las zonas endémicas (Aguirre & Tabor, 2008; Roque & Jansen, 2014).

Además, el cambio climático y el movimiento global de personas y animales incrementa el riesgo de expansión de esta parasitosis a nuevas zonas, tradicionalmente consideradas como no endémicas, donde ya se realizan programas de vigilancia de flebotomos, como es el caso de Alemania u otros países del centro de Europa (Oerther et al., 2020). El establecimiento de los vectores, así como la presencia del parásito y de animales (tanto domésticos como silvestres) que puedan mantener el ciclo biológico, son cada vez más estudiados, habiéndose descrito ya varios casos de transmisión autóctona en estos países (Maia et al., 2023).

1.2.4. DISTRIBUCIÓN GEOGRÁFICA DE *LEISHMANIA* SPP. EN ANIMALES SILVESTRES

La presencia de *Leishmania* en animales silvestres es conocida desde hace décadas, siendo larga la lista de especies capaces de albergar al parásito. Además, en los últimos años, ha crecido notablemente el interés de los investigadores por revelar el posible papel de estos animales como potenciales reservorios y como parte importante en brotes humanos registrados. Esto implica un mayor esfuerzo en el control y vigilancia de la parasitosis, ya que muchas de las medidas tomadas en los últimos años pueden verse alteradas por el papel de los animales silvestres en brotes humanos. Todas las especies zoonóticas de *Leishmania* han sido descritas en animales silvestres, en todas las regiones endémicas del parásito (Azami-Conesa et al., 2021a; Cardoso et al., 2021; Millán et al., 2014a).

El orden Carnivora es uno de los más estudiados, probablemente por su relación con los perros, uno de los reservorios más importantes de *Leishmania* spp. Especies como el zorro cangrejero (*Cerdocyon thous*), el mapache (*Procyon cancrivorous*), el coatí (*Nasua nasua*), el lobo de crin (*Chrysocyon brachyurus*), el ocelote (*Leopardus pardalis*), el jaguar (*Panthera onca*) y el puma (*Puma concolor*), entre otros, han sido descritos como hospedadores de *L. infantum, L. mexicana, L. braziliensis* y *L. amazonensis* en América (Azami-Conesa et al., 2021a; Dahroug et al., 2010; De Almeida Curi et al., 2006; Luppi et al., 2008; Telleria et al., 1999; Voltarelli et al., 2009).

En Europa, Asia y África los carnívoros también han sido ampliamente estudiados, detectando *L. infantum* principalmente en animales como el lobo gris (*Canis lupus*), el gato montés (*Felis silvestris*), la gineta común (*Genetta genetta*), el meloncillo (*Herpestes ichneumon*), la nutria europea (*Lutra lutra*), el lince ibérico (*Lynx pardinus*), la garduña (*Martes foina*), la marta (*Martes martes*), el tejón europeo (*Meles meles*), el visón europeo (*Mustela lutreola*), el turón europeo (*Mustela putorius*), el oso pardo (*Ursus arctos*) o el zorro rojo (*Vulpes vulpes*), todos ellos estudiados en España y otros países mediterráneos, como Italia o Grecia. Además, se ha descrito *L. infantum* en el chacal común (*Canis aureus*) en países como Georgia, Rumanía, Irán e Israel (Alcover et al., 2020; Babuadze et al., 2014; Battisti et al., 2020; Mitková et al., 2017; Mohebali et al., 2016; Oleaga et al., 2018; Sastre et al., 2008; Sobrino et al., 2008; Talmi-frank et al., 2010).

Otro de los grupos más estudiados es el de los roedores, en parte debido a su próxima relación con entornos urbanos y peri-urbanos pero, además, por el papel como reservorios de alguno de ellos conocido desde hace tiempo. En este grupo encontramos varias especies de jerbo (*Rhombomys opimus, Gerbillus andersoni, G. nanus*), así como el ratón común o doméstico (*Mus musculus*), entre otros, capaces de albergar *L. tropica, L. donovani* y *L. major*, mientras que *L. infantum* ha sido descrito en roedores más ligados al hombre, como la ardilla roja (*Sciurus vulgaris*), la rata gris (*Rattus novergicus*), la rata negra (*R. rattus*), el ratón de campo (*Apodemus sylvaticus*), el ratón

argelino (*M. spretus*) o el ratón común. Todos ellos se han estudiado en Europa principalmente, pero también en África (tanto en países del norte del continente como en Kenia y Etiopía) y Asia, en especial en Oriente Próximo (Alcover et al., 2020; Echchakery et al., 2017; Galán-Puchades et al., 2019; Helhazar et al., 2013; Martín-Sánchez et al., 2020b; Navea- Pérez et al., 2015). En cuanto a América, las especies de *Leishmania* endémicas se han encontrado en roedores como el puercoespín paraguayo enano (*Coendou spinosus*), el capibara (*Hydrochoerus hydrochaeris*) o las ratas gris y negra, entre otras muchas especies, en el caso de *L. infantum*, y en más de 45 especies diferentes de roedores en el caso de *L. mexicana*, *L. amazonensis* y las especies del grupo *Leishmania* (*Viannia*) (Azami-Conesa et al., 2021a; De Castro Ferreira et al., 2015; Richini-Pereira et al., 2014).

Más allá de los humanos, otros primates han sido investigados como potenciales reservorios, tanto por encontrarse cerca de poblaciones humanas rurales en América como por su similitud al ser humano. Estos estudios revelan la presencia de *L. braziliensis, L. amazonensis* y *L. mexicana* en primates, como el mono araña (*Ateles paniscus*), el mono aullador negro (*Allouatta caraya*) o el mono capuchino (*Cebus apella*), mientras que *L. infantum* se ha detectado en un mayor número de especies de primates, más de 10 en total (Azami-Conesa et al., 2021a; Malta et al., 2010; Martínez et al., 2020; Voltarelli et al., 2009). En África también se han descrito primates infectados con *L. major*, como gorilas (*Gorilla gorilla*) y babuinos (*Papio cynocephalus anubis*), entre otros, en países como Kenia o Camerún (Gicheru et al., 2009). En el caso de Europa, los animales parasitados se encontraban en zoológicos, donde se observaron signos clínicos compatibles con la parasitosis por *Leishmania* y donde cabe destacar al orangután de Borneo (*Pongo pygmaeus*) infectado con *L. infantum* en España (Miró et al., 2018).

Otro de los órdenes a destacar es el de los quirópteros, especialmente en América, donde la variedad, abundancia y hábitos alimenticios de estos animales, ha levantado mucho interés en cuanto al papel que pueden jugar en el ciclo de *Leishmania*. Más de 12 especies de estos animales han sido descritas como posibles hospedadores de *L. braziliensis* y otras especies de *Leishmania* (*Viannia*). Además, se han descrito 14 especies infectadas con *L. amazonensis*, 13 especies con *L. mexicana* y alrededor de 18 especies diferentes con *L. infantum*. En todos los casos, se analizaron tanto animales de hábitos frugívoros como insectívoros, siendo los menos habituales los hematófagos (Azami-Conesa et al., 2021a; Castro et al., 2020; De Oliveira et al., 2015; Gómez-Hernández et al., 2017; Savani et al., 2010). En el caso del Viejo Mundo, se han descrito algunas especies de murciélagos positivos a *L. tropica* y *L. major* en Etiopía, Kenia y Egipto (Azami-Conesa et al., 2021a; Kassahun et al., 2015), mientras que en Europa solo se ha detectado en España, siendo esta primera descripción parte de la presente Tesis Doctoral (Azami-Conesa et al., 2020).

Tanto en Europa como en el norte de África se han estudiado varias especies de erizo como hospedadores de *L. infantum* y *L. major,* ya que sus hábitos son compatibles con las horas de mayor actividad de los vectores y cada vez es más habitual encontrarlos cerca de comunidades humanas.

Concretamente en España, se han analizado muestras de erizo europeo (*Erinaceus europaeus*) que resultaron positivas a *L. infantum* en diversos tejidos (Alcover et al., 2021; Azami-Conesa et al., 2023; Muñoz-Madrid et al., 2013). En el caso del erizo moruno (*Atelerix algirus*), ha sido estudiado en zonas endémicas de leishmaniosis de Túnez y Argelia, donde se han encontrado evidencias de la presencia de *L. infantum* y *L. major* (Chemkhi et al., 2015; Souguir-Omrani et al., 2018). Esta última especie ha sido descrita también en el erizo del desierto (*Paraechinus aethiopicus*) y en el erizo orejudo (*Hemiechinus auritus*) (Chemkhi et al., 2015; Pourmohammadi & Mohammadi-Azni, 2019; Rouhani et al., 2014; Souguir-Omrani et al., 2018; Tomás-Pérez et al., 2014).

En los últimos años, los lagomorfos han sido uno de los órdenes que más se ha estudiado como potencial reservorio de *Leishmania*, especialmente en Europa, y en concreto en España. Esto se debe al papel fundamental que presentaron estos animales en uno de los últimos brotes relevantes de leishmaniosis humana en Europa, que se inició en julio de 2009 en la Comunidad de Madrid. El incremento significativo de casos en humanos hizo saltar todas las alarmas y, tras el estudio de los datos epidemiológicos de la zona, no se observó un aumento en la prevalencia en perros u otros reservorios conocidos. Tras numerosos estudios, se determinó que los animales que podrían haber participado en la dispersión de estos parásitos por zonas urbanas eran la liebre y el conejo europeo, cuyas poblaciones habían sufrido un incremento muy significativo, debido a la presencia de nuevas zonas de refugio y alimentación por la creación de un parque urbano de grandes dimensiones, así como por la ausencia de depredadores. Este parque, situado entre los municipios de Fuenlabrada, Leganés, Getafe y Humanes, fue clave para la transmisión del parásito, ya que era una zona donde convivían en espacio y tiempo ser humano, reservorios y vectores (Arce et al., 2017). Tras este brote, numerosos investigadores pusieron el foco en estos animales, realizando varios estudios, incluyendo estudios serológicos, moleculares y de xenodiagnóstico, para finalmente sugerir el papel relevante de los lagomorfos como reservorios de *L. infantum* en España (Jiménez et al., 2014; Molina et al., 2012; Ortega-García et al., 2019).

Sin embargo, no solo en Madrid se ha descrito la presencia de este parásito en liebres. Es el caso de Grecia, donde se ha detectado *L. donovani* en liebres europeas en el 23,5% de los animales estudiados (Tsokana et al., 2016), o el de Colombia, donde *L. braziliensis* ha sido descrito en un ejemplar de conejo brasileño (*Sylvilagus brasiliensis*) (Alexander et al., 1998).

Además, cabe destacar la presencia de *Leishmania* en otros animales en América, como son: didelfimorfos, como las zarigüeyas (*Didelphis* spp.) (*L. baziliensis, L. amazonensis* y *L. infantum*) (Santiago et al., 2007); los osos hormigueros (*Myrmecophaga tridactyla*) y osos meleros (*Tamandua tetradactyla*) del orden Pilosa (*L. mexicana, L. amazonensis* y *L infantum*) (De Araújo et al., 2013; Richini-Pereira et al., 2014); y los armadillos (*Dasypus septemcinctus*) con *L. infantum* (Richini-Pereira et al., 2014). Por último, en Europa también se ha descrito *L. infantum* en una especie de marsupial, el walabí (*Macropus rufogriseus*) (Montoya et al., 2016).

1.2.5. EL PAPEL DE LAS ESPECIES EXÓTICAS INVASORAS EN ESPAÑA

Uno de los mayores riesgos a los que se enfrentan los ecosistemas en la actualidad es la introducción de especies exóticas invasoras. Estas especies afectan directa o indirectamente a las comunidades en las que se establecen de manera permanente. Esta problemática se produce por la competencia por los recursos alimentarios, el refugio y, especialmente, por la dispersión de patógenos, en la que estos animales exóticos pueden tener una gran implicación (Cassan et al., 2018; MAGRAMA, 2013). Sin embargo, a pesar de su transcendencia, son escasos los estudios que tengan como objetivo analizar el posible papel que juegan las especies exóticas invasoras en la epidemiología de las enfermedades zoonóticas. En el caso de España, el Ministerio para la Transición Ecológica y el Reto Demográfico tiene registradas 13 especies de mamíferos consideradas invasoras en la península ibérica (más 3 especies en las Islas Canarias), de las cuales, únicamente el visón americano (*Neovison vison*) ha sido seleccionado como posible implicado en la transmisión y dispersión de ciertos patógenos (MITECO, 2024). En concreto, esta especie ha sido descrita como hospedador de algunos virus, como Influenza A y SARS-CoV-2 (Gholipour et al., 2017; Mahdy et al., 2020), así como de algunos parásitos, como *Toxoplasma gondii* o varias especies de helmintos no zoonóticos (Martínez-Rondán et al., 2017; Ribas et al., 2018). Además, como parte de la presente Tesis Doctoral, se ha estudiado la presencia de *L. infantum* en esta especie exótica invasora, hallándose prevalencias relativamente elevadas en el este de España (Azami-Conesa et al., 2021b). Fuera de nuestras fronteras, se ha descrito la presencia de *L. infantum* en visones americanos en granjas, en concreto en Grecia, donde, al igual que en España y otros muchos países, se introdujo esta especie para el negocio de la peletería (Filioussis et al., 2018; Tsakmakidis et al., 2019). El problema radica principalmente en que numerosos ejemplares consiguen escapar de estar granjas o son liberados intencionadamente en los ecosistemas, provocando la dispersión de poblaciones, así como el incremento de contacto con animales silvestres, domésticos y humanos, pudiendo jugar papeles relevantes en el mantenimiento y diseminación de parasitosis como leishmaniosis y otras enfermedades zoonóticas (Cassan et al., 2018).

Sin embargo, estos mustélidos no son los únicos estudiados dentro del amplio grupo de los invasores. Los roedores también han sido objeto de análisis, como es el caso del ratón común o doméstico y la rata negra en Senegal, donde se consideran especies foráneas e invasoras. El estudio de Cassan et al. (2018) revela la presencia de *L. major* y *Trypanosoma lewisi* en estos dos roedores, pudiendo ser responsables de la dispersión y transmisión de estos parásitos, el primero claramente zoonótico, y el segundo más ligado a las poblaciones animales, pero del que ya se han reportado algunos casos en humanos.

A pesar de estos estudios, resulta insuficiente la información que se tiene acerca de la implicación de estas especies en sus nuevos hábitats donde, generalmente, llegan de la mano del ser humano, y donde resultan un problema cada vez mayor, tanto ecológica como sanitariamente.

1.2.6. INTRODUCCIÓN DE ESPECIES FORÁNEAS DE *LEISHMANIA* EN ESPAÑA

España es uno de los países considerados endémicos en cuanto a la transmisión y presencia de *L. infantum*. Hasta el año 2024 la única especie de este parásito considerada autóctona es *L. infantum*, cuyos vectores habituales son *P. perniciosus* y *P. ariasi*. La distribución geográfica tanto de vectores como del parásito cada vez es mayor, estando más presente en la región central y mediterránea (especialmente Comunidad Valenciana, Islas Baleares, Murcia y Comunidad de Madrid), pero cuya presencia ya se ha detectado en toda la zona norte del país, que clásicamente se había considerado libre de leishmaniosis (Amela et al., 2012; Centro Nacional de Epidemiología, 2022).

Sin embargo, en los últimos años cada vez es más habitual la detección de especies no autóctonas en personas con leishmaniosis, principalmente debido al movimiento global de personas, que facilita la presencia de estas especies en lugares diferentes a los habituales. En el caso concreto de España, la tasa de incidencia de casos de leishmaniosis humana (tanto autóctona como importada) en 2022 fue de 0.61 por cada 100 000 habitantes, siendo 291 casos autóctonos y 13 importados (Centro Nacional de Epidemiología, 2022). En muchos casos no se llega a determinar la especie concreta de *Leishmania* que está causando la infección, pero cuando se ha realizado dicho diagnóstico, se han detectado especies como *L. braziliensis, L. guyanensis, L. major, L. donovani* y *L. tropica* (Fernández-Arévalo et al., 2022; Pérez-Ayala et al., 2009).

Debido a la presencia de especies distintas a *L. infantum* en los casos importados, cada vez son más los investigadores que plantean la posibilidad de que se produzca una transmisión local de especies no presentes aún en España, en concreto de aquellas que proceden de países cercanos como Marruecos, Argelia o Túnez, donde *L. tropica* y *L. major* son endémicas, y cuyas condiciones climáticas cada vez son más similares a las de España. Estas condiciones facilitan la presencia y dispersión de los vectores principales de dichas especies foráneas en la península ibérica, como es el caso de *P. sergenti* (Figura 8), vector principal de *L. tropica*. *P. sergenti* se ha descrito desde hace varios años en prácticamente todas las provincias del país y su establecimiento y expansión es hoy en día una realidad (Baghad et al., 2020; Barón et al., 2013; Echchakery et al., 2020; European Centre for Disease Prevention and Control and European Food Safety Authority., 2019; Fernández-Arévalo et al., 2022; Gijón-Robles et al., 2018; Tomás-Pérez et al., 2014).

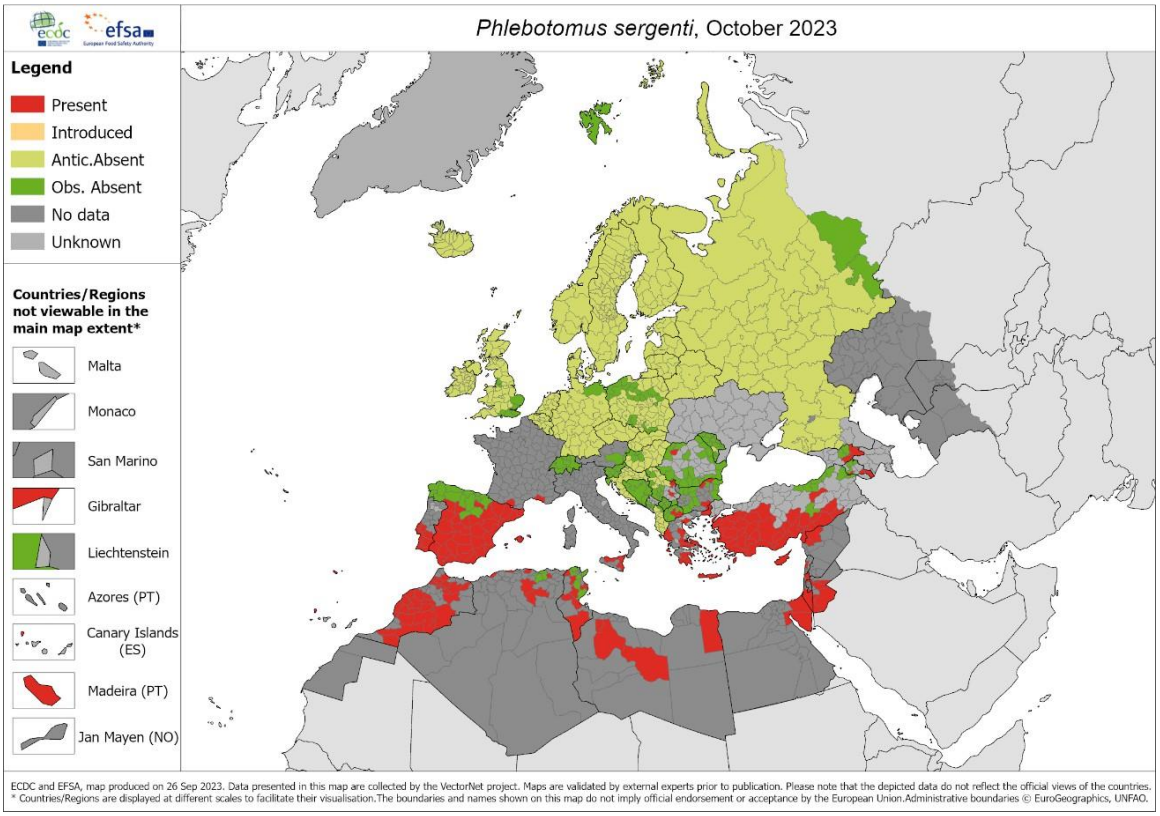

Figura 8. Mapa de distribución de *Phlebotomus sergenti*. Fuente: ECDC, 2023

En cuanto a los motivos de dicha expansión, la mayoría de los autores hablan de los movimientos humanos, de animales y de enseres como uno de los principales motivos de la ampliación de las zonas de distribución, tanto de vectores como de patógenos. Estos movimientos entre países, junto con la modificación de las condiciones climáticas en prácticamente todas las regiones del mundo, hacen más probable la presencia de nuevos patógenos en zonas no endémicas (Dujardin et al., 2008; Maroli et al., 2013; Shaw, 2007).

En el caso concreto de la leishmaniosis, en los últimos 20 años se ha observado un incremento relativamente elevado de los casos a nivel mundial. Esto se debe a numerosos motivos, donde cabe destacar el movimiento de personas debido a los conflictos bélicos, las condiciones socioeconómicas o el turismo masivo. Otros factores son el cambio climático, que provoca un aumento de las zonas de estabilización de los vectores, la falta de recursos económicos disponibles para el desarrollo de nuevos tratamientos, lo que obliga a utilizar fármacos no del todo efectivos y contra los que hay cada vez más resistencias, y, por último, la falta de recursos y medios para frenar de manera efectiva la transmisión mediante una detección precoz de las personas infectadas (Dujardin et al., 2008; Shaw, 2007).

Por todos estos motivos, cada vez se hace más urgente la detección temprana de introducción de especies foráneas de *Leishmania*, así como de sus vectores, en nuevas zonas de expansión, como es

el caso de España, donde la amplia expansión de *P. sergenti* se ha señalado como el principal factor de riesgo para la transmisión de *L. tropica*. Además, se ha identificado en España una línea genética del flebotomo muy común en zonas endémicas de leishmaniosis de Marruecos (Barón et al., 2013; Merino-Espinosa et al., 2016). Esta situación, probablemente, ha facilitado la detección en fauna silvestre de *L. tropica* por primera vez en la península ibérica, y cuya publicación es parte de la presente Tesis Doctoral (Azami-Conesa et al., 2024).

CAPÍTULO 2:
JUSTIFICACIÓN, HIPÓTESIS Y OBJETIVOS

2.1. JUSTIFICACIÓN

Las enfermedades zoonóticas han cobrado gran importancia en los últimos años y, en especial, la leishmaniosis ha sido objeto de numerosos estudios desde diversos enfoques. En el caso de esta Tesis Doctoral, se pretende analizar la presencia de este parásito en distintos animales silvestres, que se encuentran en zonas urbanas y/o peri-urbanas, lo que puede aportar información sobre la situación epidemiológica de *Leishmania* más allá de los casos notificados en perros y humanos. En estos últimos, se trata de una enfermedad de declaración obligatoria (EDO), por lo que se realiza un diagnóstico y tratamiento relativamente temprano en España (Centro Nacional de Epidemiología, 2022). En el caso de los perros, el uso de medidas preventivas, como collares antiparasitarios y otros productos, hace que el desarrollo de la leishmaniosis esté cada vez más controlado (Baxarias et al., 2022). Todo ello hace que los brotes de esta enfermedad puedan estar relacionados con otros factores, como la presencia de fauna silvestre en áreas circundantes y/o la dispersión de nuevas especies del parásito en nuestro país. Por tanto, el estudio y análisis de diferentes animales silvestres puede aportar información relevante para el control y detección de futuros brotes de leishmaniosis, así como la detección de la introducción de especies foráneas del parásito.

2.2. HIPÓTESIS

Teniendo en cuenta la justificación anterior, se plantea la siguiente hipótesis:

Los animales silvestres urbanos, peri-urbanos y que habitan cerca de las zonas naturales de recreo endémicas de leishmaniosis podrían estar parasitados con el protozoo zoonótico *L. infantum.* Estos animales podrían actuar como potenciales reservorios de la enfermedad, facilitando la transmisión al hombre y favoreciendo el mantenimiento del parásito en determinadas zonas. Además, estos animales silvestres podrían emplearse como especies centinela de la leishmaniosis y de la situación epidemiológica, teniendo en cuenta su mayor contacto con el vector, principal factor de riesgo para la aparición y persistencia de la enfermedad. Considerando la posible presencia del parásito en los animales silvestres a estudiar, no se ha determinado el órgano en el que éste se encuentra en mayor cantidad para ser detectado, por lo que la hipótesis de partida es que son más fáciles de encontrar en órganos donde teóricamente se acantonan, como la piel o el bazo. Dentro de este planteamiento debemos tener en cuenta que existen técnicas que serán más sensibles, y por tanto capaces de detectar mejor la presencia de *Leishmania*, y técnicas de mayor especificidad que serán de elección a la hora de determinar la especie de *Leishmania*. Ambas son de utilidad en el contexto de la vigilancia epidemiológica.

2.3. OBJETIVOS

El objetivo principal de esta Tesis es analizar la presencia de *Leishmania* en distintas especies de fauna silvestre poco estudiadas en relación con esta parasitosis. En concreto, especies presentes en ambientes urbanos y/o peri-urbanos, donde se evalúa su relevancia como potenciales reservorios y/ o especies centinela para la detección de *Leishmania* en España. Para ello se han especificado los siguientes objetivos concretos:

- **Objetivo 1.** Determinar el papel de mamíferos silvestres en los que aún no se ha estudiado la presencia de *Leishmania*, pero que cumplen las condiciones de longevidad y contacto frecuente con el vector y con el ser humano para poder considerarse potenciales reservorios. Análisis de la utilidad de diferentes órganos diana para la detección del parásito.

- **Objetivo 2.** Estudiar el papel de las especies exóticas invasoras que se encuentran en zonas urbanas, peri-urbanas o de recreo en relación con el posible papel como reservorios o especies centinela de la parasitosis. Análisis de las dianas de PCR más adecuadas para los objetivos planteados.

- **Objetivo 3**. Análisis del posible papel como reservorios o especies centinela de varias especies de mamíferos en los que se ha detectado previamente la presencia de *Leishmania*. Estimación de la prevalencia empleando un mayor número de ejemplares de cada especie.

- **Objetivo 4.** Estudio sobre la utilidad de diversas dianas de PCR en relación con la sensibilidad y la especificad con el fin de confirmar la presencia de especies foráneas de *Leishmania* en la península ibérica.

CAPÍTULO 3:
METODOLOGÍA

3.1. ANIMALES Y DISTRIBUCIÓN

Como se ha mencionado en el apartado de objetivos, en esta Tesis Doctoral se pretende analizar la presencia de *Leishmania* spp. en diferentes especies de animales silvestres, en concreto de mamíferos procedentes de áreas rurales y periurbanas de España, con el fin de aportar luz a su posible papel como reservorios o especies centinela de la enfermedad.

Según datos recientes, España cuenta con 107 especies de mamíferos terrestres, 94 de ellas presentes en la parte continental europea. En estas especies se incluyen ejemplares introducidos, endémicos y autóctonos, siendo estos últimos los más numerosos (Delibes & Palomo, 2007). Por tanto, resulta importante seleccionar de manera adecuada a aquellas especies que cumplan una serie de requisitos para analizar su posible papel en el mantenimiento del ciclo biológico de *Leishmania* es el país. Los criterios de inclusión que se han seguido son los siguientes:

1. Mamíferos silvestres de vida libre que ingresen en centros de recuperación de fauna silvestre o recogidos por las autoridades competentes en caso de accidente o colisión con vehículos.
2. Animales que fallezcan por causas naturales o en centros de fauna por causas ajenas a la investigación.
3. Mamíferos cuyas áreas de distribución se solapen con las zonas de presencia de los flebotomos.
4. Especies que se encuentren cerca de poblaciones urbanas o de áreas recreativas, con posible contacto con humanos y/o animales domésticos.

La selección de los animales viene dada inicialmente por los centros de fauna silvestre con los que se colabora, presentes en distintas Comunidades Autónomas, permitiendo así el acceso a animales con diferentes patrones de distribución, todos ellos de la península ibérica. Los centros de fauna silvestre que han participado en los distintos estudios llevados a cabo dentro de la presente Tesis doctoral se encuentran en la Comunidad de Madrid, la Comunidad Valenciana, Castilla y León y Castilla-La Mancha.

Gran parte de las muestras proceden de GREFA (Grupo para la Rehabilitación de la Fauna Autóctona y su Hábitat) ubicado en Majadahonda (Madrid), así como del Centro de Recuperación de Fauna Silvestre “La Granja de El Saler” en Valencia. Además, se ha contado con la colaboración de Mini-zoo, Guadalajara; “El Chaparrillo” en Ciudad Real y el CERI (Centro de Estudio de Rapaces Ibéricas) en Toledo.

Los animales proceden de tres líneas diferentes de trabajo, dentro de estos centros de fauna. Por un lado, animales que ingresan por trauma o enfermedad en centros de recuperación de fauna y, que por

causas naturales o bajo el control veterinario, fallecen en las instalaciones. Por otro lado, animales que fallecen por accidente con vehículos, y que son trasladados a los centros de fauna correspondientes en cada provincia. Por último, animales que pertenecen al grupo de Especies Exóticas Invasoras, y que según la normativa europea (European Parliament, 2014), nacional (Ministerio de Agricultura Alimentación y Medio Ambiente, 2013) y provincial (Conselleria Medio Ambiente, Agua, 2009) son objeto de control y erradicación, y que, por tanto, son eutanasiados bajo supervisión veterinaria estricta.

Cabe destacar la limitación que supone el hecho de trabajar con fauna silvestre, en cuanto al número de animales de los que se dispone, debido a que esto dependerá exclusivamente de los que ingresen en dichos centros. No debemos olvidar que en estos estudios siempre va a haber un sesgo de selección difícil de evitar, puesto que no se analizan animales silvestres sanos en libertad, aunque este grupo se encuentra parcialmente representado por los animales atropellados y exóticos invasores.

3.2. RECOGIDA Y PROCESADO DE LAS MUESTRAS BIOLÓGICAS

La recogida de muestras de los animales se realizó en instalaciones apropiadas, como salas de necropsia o lugares similares, donde primero se observaban los animales en busca de posibles lesiones compatibles con leishmaniosis, y después se determina el peso, sexo y estado de desarrollo del animal, en aquellos casos en los que fuera posible. Una vez obtenidos estos datos, se recogieron muestras de diferentes órganos, entre los que se incluye bazo, piel de oreja, pelo, hígado y coágulo de sangre del corazón, para su posterior análisis. Estas muestras eran identificadas y recogidas en tubos Eppendorf individuales mediante el empleo de cuchillas estériles desechables. En aquellos animales (erizos europeos, principalmente) donde se pretendía obtener información sobre la utilidad de muestras no invasivas, como pelo, hisopo ocular e hisopo oral, se tomaron las muestras bajo anestesia de los animales vivos, procedimiento que fue llevado a cabo por parte de los veterinarios de los centros de recuperación, dentro de las actuaciones de rutina de control y revisión de los animales seleccionados y sin perjudicar la salud del animal. Todas estas muestras fueron conservadas a -20 ºC hasta el momento de su procesado en el laboratorio.

En cuanto a la temporalidad, la recogida de muestras se dio principalmente entre los meses de febrero a octubre, coincidiendo con la época de mayor actividad de los animales y, por tanto, con

un mayor número de ingresos en los centros de fauna. Esta época coincide con los meses en los que los flebotomos son más activos.

3.3. DETECCIÓN DE *LEISHMANIA* spp. MEDIANTE PCR Y SECUENCIACIÓN

Para detección de *Leishmania* e identificación de la especie del parásito en las muestras procedentes de los animales se utilizó como principal técnica la PCR convencional, seguida de secuenciación, empleando diversas dianas de amplificación en cada caso.

Para ello, primero se seleccionaban las muestras a analizar, especialmente los animales objeto de estudio, así como los órganos y tejidos de interés. A continuación, se procedía a la extracción de ADN usando kits comerciales de extracción por columnas (NZY Tissue gDNA Isolation kit, NZYTech, Lisboa, Portugal). Siguiendo las instrucciones del fabricante, se utilizaban entre 10 y 15 mg de bazo o 20 mg de otros tejidos, con una elución final de ADN de entre 50 µl en buffer de elución para incrementar la concentración de ADN. Las diferentes cantidades de tejido utilizadas vienen dadas por la necesidad de evitar inhibidores enzimáticos presentes en el bazo, que puedan alterar los resultados de la detección de ADN. En todos los casos se utilizaron controles de extracción, siendo cultivo de promastigotes de *L. infantum*, *L. braziliensis* o *L. major* los controles positivos y agua autoclavada el control negativo. El ADN obtenido se conservó a -20 ºC hasta su análisis.

Para el análisis de las muestras por PCR, se utilizaron distintas dianas del ADN, tanto mitocondriales como nucleares, así como PCR anidadas (nPCR) en algunos casos, con el objetivo de aumentar la sensibilidad de la técnica, y obtener mejores resultados. Para poder realizar la PCR es necesario incluir una pareja de cebadores o primers para cada diana del ADN objeto de estudio, los cuales son pequeños fragmentos de ADN de alrededor de 20 oligonucleótidos o pares de bases (pb), complementarios del extremo de cada hebra del ADN que se pretende detectar, determinando así el tamaño del amplicón obtenido. Para el desarrollo de la PCR se utilizó una enzima ADN polimerasa comercial, procedente de la bacteria *Thermus aquaticus* (Taq), que incluye el tampón necesario para el correcto pH al que trabaja la enzima, los iones divalentes $MgCl_2$, así como los cuatro desoxiribonucleótidos- difosfato (dNTP) necesarios para la formación de las nuevas hebras de ADN, que son adenina (A), timina (T), citosina (C) y guanina (G). El uso de estos complejos comerciales reduce de manera notable la posibilidad de contaminación de la PCR, así como el margen de error que pueda deberse a la manipulación humana de los compuestos a la hora de realizar la técnica. La enzima utilizada en este caso fue HotStart Supreme NZY Taq II 2x (NZYTech, Lisboa, Portugal),

empleando 12,5 µl por cada muestra. A esto se añadían entre 0.4 µl y 1 µl de cada cebador a 10 µM, y entre 2,5 µl y 5 µl de ADN, dependiendo de la diana y el protocolo a seguir, y agua libre de DNAsas hasta un volumen final de 25 µl. Todo el proceso realizado, así como los cebadores y dianas utilizadas, se pueden ver en la Figura 9 y la Tabla 2.

Para poder iniciar la PCR, es necesario un primer paso de activación de la enzima ADN polimerasa, que se consigue a 95 ºC durante 5 minutos ("hot start"), seguido de tres fases repetidas de forma cíclica hasta 40 veces, que incluyen la desnaturalización, anillamiento y elongación de las nuevas moléculas de ADN, y que varían según los cebadores utilizados. Por último, se incluye una etapa de extensión, a 72 ºC durante 10 minutos, antes de finalizar la PCR. En todas la PCRs realizadas se añadieron controles positivos y negativos, siendo, una vez más, ADN procedente de cultivo de promastigotes de *L. infantum*, *L. major* o *L. braziliensis* y agua autoclavada, respectivamente.

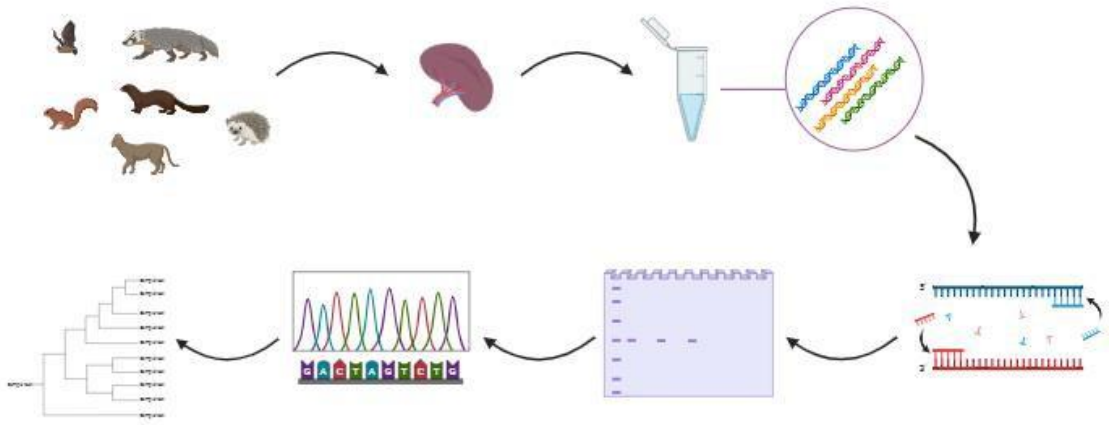

Figura 9. Proceso de recogida, procesado y análisis de las muestras mediante PCR convencional y secuenciación. Diseño propio creado con Biorender.com.

Una vez obtenidos los amplicones, es necesario ponerlos de manifiesto mediante una electroforesis en gel de agarosa para poder ver los resultados obtenidos y, así, seleccionar las muestras que requieren secuenciación. Para ello, se realiza un gel de agarosa, que contiene tampón TAE (Tris, acetato y EDTA) y agarosa del 1%. A este gel de agarosa se le añadió SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA, EE. UU.) para observar los resultados bajo luz ultravioleta (UV), tras la realización de la electroforesis.

Aquellas muestras que presentaron banda compatible con el número de pb esperado, se consideraron positivas y se seleccionaron para la secuenciación.

Tabla 2. Dianas del ADN, oligonucleótidos y condiciones de las PCR.

DIANA ADN	OLIGONUCLEÓTIDOS	TAMAÑO AMPLICÓN	CONDICIONES PCR	REFERENCIA
*SSUrRNA**	Ext R221 5′-GGTTCCTTTCCTGATTTACG-3′	603pb	94 ºC — 30” 60 ºC — 30” 72 ºC — 30”	(Cruz et al., 2002)
	Ext R332 5′-GGCCGGTAAAGGCCGAATAG-3′			
	Int R223 5′- TCCATCGCAACCTCGGTT-3′	358pb	94 ºC — 30” 65 ºC — 30” 72 ºC — 30”	
	Int R333 5′- AAAGCGGGCGCGGTGCTG-3′			
*ITS1**	Ext LITSR 5′-CTGGATCATTTTCCGATG-3′	320 pb	94 ºC — 30” 53 ºC — 30” 72 ºC — 15”	(Echchakery et al., 2020)
	Ext L5.8S 5′-ACACTCAGGTCTGTAAAC-3′			
	Int SAC 5′-CATTTTCCGATGATTACACC-3′	280 pb	94 ºC — 30” 57 ºC — 30” 72 ºC — 15”	
	Int VAN2 5′-CGTTCTTCAACGAAATAGG -3′			
*hsp70**	Ext HSP70-F25 5′-GGACGCCGGCACGATTKCT-3′	1286 pb	94 ºC — 40” 61 ºC — 60” 72 ºC — 120”	(Pereira et al., 2020; Van der Auwera et al., 2013)
	Ext HSP70-R1310 5′-CCTGGTTGTTGTTCAGCCACTC-3′			
	Int HSPF 5′-GACAACCGCCTCGTCACGTTC-3′	741 pb	94 ºC — 40” 61 ºC — 60” 72 ºC — 60”	
	Int HSPR 5′-GTCGAACGTCACCTCGATCTGC-3′			
Repeat region	T2 5`-CGGCTTCGCACCATGCGGTG-3`	250 pb	94 ºC — 30” 61 ºC — 30” 72 ºC — 15”	(Piarroux et al., 1995)
	B4 5`-ACATCCCTGCCCACATACGC-3`			

*: nPCR. Ext: cebadores externos. Int: cebadores internos.

En la etapa de secuenciación, se contó con el servicio de la empresa Macrogen (Madrid), así como con el Servicio de Secuenciación de la Unidad de Genómica de la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid (Madrid). En Macrogen se utilizó un termociclador “DNA

Engine Tetrad 2 Peltier" (BIO-RAD) y el kit comercial ABI BigDYE® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, MA, USA), siguiendo el protocolo del fabricante. En el caso del Servicio de Secuenciación de la UCM, se utilizó el termociclador 3730xl DNA Analyzer (Applied Biosystems) y el mismo kit comercial que en Macrogen.

Una vez obtenidas las secuencias, se analizaron manualmente para su alineamiento y comparación con las depositadas en la base de datos GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/), mediante la herramienta BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), gracias al programa MEGA: Molecular Evolutionary Genetics Analysis versión X (MEGA X) (Kumar et al., 2018).

Una vez obtenidas las secuencias de aquellas muestras positivas, se depositaron en GenBank para hacerlas accesibles, siempre y cuando tuvieran más de 200 pb. Todas las secuencias están identificadas con un número de acceso, en su correspondiente publicación científica.

En algunos casos, se han utilizado árboles filogenéticos para una mejor comprensión y exposición de los resultados. Para la realización de estas figuras se utilizó el programa MEGA X, donde se incluyeron las secuencias obtenidas en el estudio, así como otras secuencias obtenidas de GenBank de *Leishmania* spp. próximas filogenéticamente y secuencias de *T. cruzi* como referencia de grupo externo. Este análisis filogenético se realizó siguiendo la Estimación de Máxima Verosimilitud (Maximum Likelihood Method) basado en el modelo de Tamura- Nei (Tamura & Nei, 1993) y con 2000 repeticiones para que el análisis sea más robusto (Bootstrap).

3.4. MAPAS

En aquellas publicaciones donde se han realizado mapas para indicar la procedencia y/o resultados de los animales analizados, se ha utilizado el programa AutoCad (Autodesk® Inc., San Francisco, CA, USA).

3.5. ANÁLISIS ESTADÍSTICO

Los resultados obtenidos en los diferentes estudios realizados fueron analizados estadísticamente mediante análisis de factores de riesgo, utilizando la herramienta digital gratuita WinEpi (Work in Epidemiology) (De Blas, 2023). En todos los análisis de estadística descriptiva se asumió un factor de confianza del 95% y se obtuvieron los límites de confianza para el nivel mencionado.

CAPÍTULO 4:
RESULTADOS

4.1. OBJETIVO 1

Determinar el papel de mamíferos silvestres en los que aún no se ha estudiado la presencia de *Leishmania*, pero que cumplen las condiciones de longevidad y contacto frecuente con el vector y con el ser humano para poder considerarse potenciales reservorios. Análisis de la utilidad de diferentes órganos diana para la detección del parásito.

4.1.1. RESUMEN

En los últimos años se ha incrementado de manera notable el número de estudios en los que se analizan distintas especies de animales silvestres como posibles reservorios de *Leishmania* spp. En el caso concreto de España, este incremento está relacionado, entre otros motivos, con el brote de leishmaniosis humana de la Comunidad de Madrid, donde se planteó que los conejos y las liebres pudieron tener un papel fundamental en el aumento de los casos en las personas (Jiménez et al., 2014; Molina et al., 2012). Los principales grupos analizados en España han sido carnívoros y roedores, quizás por su similitud con el perro, en el primer caso, y con su amplia distribución, en el segundo, además de ser dos grupos que incluyen reservorios importantes de leishmaniosis (Alcover et al., 2020; Ortega et al., 2017; Ortuño et al., 2019; Risueño et al., 2018).

Sin embargo, existen numerosas especies de animales silvestres que se encuentran cerca de ambientes urbanos habitualmente, y que podrían albergar al parásito zoonótico. Uno de estos grupos de animales muy abundantes en ambientes urbanos y peri-urbanos son los quirópteros (orden Chiroptera). De hecho, en América del Sur y África se ha estudiado desde hace algunos años a estos mamíferos como actores importantes en el mantenimiento del ciclo silvestre de varias especies de *Leishmania*, entre ellas *L. braziliensis, L. major, L. mexicana* y *L. tropica* (Berzunza-Cruz et al., 2015; Castro et al., 2020; De Castro Ferreira et al., 2017; De Oliveira et al., 2015; Kassahun et al., 2015). A pesar de esto, en Europa no han sido objeto de análisis, con la excepción de la publicación de Millán et al. del año 2014, donde no se encontraron murciélagos positivos en el área de estudio (Millán et al., 2014b).

Teniendo en cuenta que España, al igual que otros países de la cuenca mediterránea, presenta prevalencias relativamente elevadas de animales silvestres infectados por *L. infantum*, resulta interesante analizar a los murciélagos urbanos comunes (*Pipistrellus pipistrellus*) como posibles hospedadores del parásito, ya que el estudio anterior realizado en el país se centró en murciélagos de Schreibers (*Miniopterus schreibersii*), que habitan cuevas alejadas de los núcleos urbanos. Para ello, se analizaron un total de 27 murciélagos procedentes de distintas localidades de la Comunidad de

Madrid, de los cuales se recogieron muestras de bazo, pelo del abdomen y coágulo de sangre de corazón. Estas muestras se analizaron mediante PCR y secuenciación, utilizando la diana *Repeat region* del ADN, que presenta elevada sensibilidad y especificidad para la detección de *L. infantum*. El 59,2% de los animales estudiados (16 de 27) resultaron positivos al menos a una muestra, siendo el bazo el que arrojó un mayor número de positivos (14 de los 16) seguido del pelo (7 de los 16) y del coágulo de sangre (6 de los 16). Estos resultados suponen la primera descripción de *L. infantum* en el murciélago común y la primera descripción en Europa.

4.1.2. ARTÍCULO

Este artículo científico ha sido publicado en una revista con revisión por pares y se encuentra incluido en la presente Tesis Doctoral.

- **Azami-Conesa, I.,** Martínez-Díaz, R.A., González, F., Gómez-Muñoz, M.T. (2020). First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe. Res. Vet. Sci. 132: 172-176. DOI: 10.1016/j.rvsc.2020.06.019.
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First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe

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ABSTRACT

Leishmania infantum is a protozoan causing leishmaniasis in humans and in dogs, among other animals, which is an endemic disease in the Mediterranean basin. In recent years, the role of wildlife as a possible reservoir of the disease was analyzed and several species of carnivores were reported to have the highest infection rates, with foxes and wolves being the more widely studied species; the role of rabbits and hares as reservoirs of leishmaniasis has also been described. In addition, several studies highlighted the role of bats as suitable hosts for *Leishmania* species (*L. braziliensis*, *L. major*, *L. mexicana*, and *L. tropica*) in South and Central America and Africa, but no *Leishmania* spp. infection in Chiroptera has been reported in Europe. In this study, samples from spleen, hair, and blood were analyzed to detect *L. infantum* DNA in bats from the Community of Madrid (Spain). Infection by *L. infantum* was detected in 59.2% of the bats studied ($n = 16/27$), with the spleen being selected as the site for detection, yielding 14/16 positive results (87.5% sensitivity), followed by hair ($n = 7/16$) and blood ($n = 6/16$). In two animals, samples from all three anatomical sites tested positive (7.4% of the total animals), while in four animals the spleen and hair samples tested positive (14.8%), in one animal the blood and hair samples tested positive (3.7%), and in another animal the blood sample only tested positive (3.7%). This is the first report of *L. infantum* detection in the common urban bat (*Pipistrellus pipistrellus*) in Europe.

1. Introduction

Leishmania spp. is a parasitic protozoan belonging to the order Kinetoplastida, which includes over 20 species that cause a vector-borne disease transmitted by phlebotomine sand flies (order Diptera, family Psychodidae) with a worldwide distribution (Europe, Africa, the Americas and Asia) and an endemic presence in more than 90 countries (Maxfield and Crane, 2019). Three different clinical forms of leismaniasis have been described, namely, cutaneous, mucocutaneous and visceral diseases, with the last one being endemic in the Mediterranean basin. The life cycle includes the amastigote stage, which multiplies in phagocytic mononuclear cells from a wide range of vertebrate animals, including humans, and the promastigote form, which multiplies in the digestive tract of phlebotomines, inoculating them to different vertebrate hosts during female blood meals. The World Health Organization (WHO) estimates one million new cases per year in people around the world, making leishmaniasis one of the most relevant yet neglected parasitic diseases (https://www.who.int/leishmaniasis/en/).

Leishmania infantum is present throughout the entire territory of Spain, including the Balearic and Canary Islands, due to the distribution of vectors and hosts and the suitable climatic conditions. The main vectors are *Phlebotomus perniciosus* and *Phlebotomus ariasi*, which have a wide distribution in the country and therefore any area is susceptible to the infection. Nevertheless, some regions of Spain have a greater prevalence, such as the Valencian Community, the Balearic Islands, Catalonia, Andalusia, and the Community of Madrid. In fact, in the Community of Madrid, there has been an active human leishmaniasis outbreak since 2009, although with a regressive tendency at present (Arce et al., 2017; Suárez Rodríguez et al., 2012).

Dogs are the species most widely studied with regard to leishmaniasis because they can be severely affected by the disease and are considered the main reservoir of *L. infantum*. However, the relevance of other animal species as reservoirs, such as wildlife, has been investigated and the presence of *L. infantum* has been reported in many species, including foxes, wolves, wild cats, bears, rats, hedgehogs, squirrels, jackals, genets, mice, martens, minks, rabbits, and hares (Alcover et al., 2020; Ortega et al., 2017; Ortuño et al., 2019; Risueño et al., 2018) (Table 1). During the aforementioned outbreak in Madrid,

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Table 1
Wildlife species in which L. *infantum* has been detected in Europe using PCR techniques in different tissues. Prevalence rates depend on the target tissue.

Species	Samples	Prevalence (%)	Reference
Western Mediterranean mouse (*Mus spretus*)	Liver, spleen, skin, heart blood	42.9	Alcover et al., 2020
American mink (*Mustela vison*)	Liver, spleen, skin, heart blood	100	Alcover et al., 2020
Beech marten (*Martes foina*)	Spleen, liver, skin	100	Ortuño et al., 2019
	Liver, spleen, skin, heart blood	50	Alcover et al., 2020
	Skin, organs	14–20	Risueño et al., 2018
Brown bear (*Ursus arctos*)	Spleen, liver, skin	100	Ortuño et al., 2019
European rabbit (*Oryctolagus cuniculus*)	Spleen, liver, skin	47–59	Ortuño et al., 2019
	Spleen, skin, hair	26.2–79.8	Ortega et al., 2017
	Skin, organs	5–26	Risueño et al., 2018
Long-tailed field mouse (*Apodemus sylvaticus*)	Spleen, liver, skin	0–50	Ortuño et al., 2019
	Skin, organs	20	Risueño et al., 2018
Red fox (*Vulpes vulpes*)	Spleen, liver, skin	19–73	Ortuño et al., 2019
	Skin, organs	39–45	Risueño et al., 2018
Common genet (*Genetta genetta*)	Spleen, liver, skin	100	Ortuño et al., 2019
	Skin	100	Risueño et al., 2018
Western European hedgehog (*Erinaceus europaeus*)	Liver, spleen, skin, heart blood	34.4	Alcover et al., 2020
Iberian hare (*Lepus granatensis*)	Spleen, skin, hair	10.1–55.7	Ortega et al., 2017
Brown rat (*Rattus norvegicus*)	Spleen, liver, skin	40–100	Ortuño et al., 2019
Red squirrel (*Sciurus vulgaris*)	Liver, spleen, skin, heart blood	20	Alcover et al., 2020
Wild cat (*Felis silvestris*)	Skin, organs	0–50	Risueño et al., 2018
Grey wolf (*Canis lupus*)	Spleen, liver, skin	50	Ortuño et al., 2019
	Skin	33	Risueño et al., 2018

new reservoirs were described in the area, e.g., hares and rabbits, who were studied after verifying that cases in dogs had not increased (Jiménez et al., 2014; Molina et al., 2012). Thus, a relationship between human cases and wildlife infection was suspected. Today studies on wildlife and its role as a possible reservoir of leishmaniasis have become increasingly important, especially studies that focus on wildlife living close to urban areas and sharing ecological niches with vectors and other hosts.

A few studies from South and Central America and Africa have analyzed the role of bats (order Chiroptera) as potential reservoirs, since they coexist with phlebotomines in the same caves. In Mexico, Berzunza-Cruz et al. (2015) detected infection with *L. mexicana* in approximately 10% of bats out of a total of 420 analyzed, using samples from the spleen, liver, skin, and heart. De Castro Ferreira et al. (2017) investigated bats from Brazil and found the presence of DNA from *L. braziliensis* in 6.4% of the skin samples examined. De Oliveira et al. (2015) analyzed bats from strictly urban areas of Brazil and found a high percentage of samples positive for *Leishmania* spp. from skin and spleen (47.9% and 37.6%, respectively), while Castro et al. (2020) found 4.8% of the skin samples and 54.7% of the liver samples positive for *Leishmania* spp. In Ethiopia, Kassahun et al. (2015) described the presence of *L. major* and *L. tropica* in approximately 5% of spleen samples from captured bats. These data highlight the relevance of these mammals as hosts of *Leishmania* spp. in different parts of the world.

In contrast to the number of studies from South and Central America, in Europe, infection by *Leishmania* spp. in bats has been poorly studied, despite the high number of bat species throughout the continent. The presence of the parasite was investigated in Spain by Millán et al. (2014), who studied 35 Schreiber's bats (*Miniopterus schreibersii*) from caves in Catalonia, but they did not find *L. infantum* DNA in blood samples using polymerase chain reaction (PCR). In the present study, we analyzed the presence of *L. infantum* in common pipistrelle bats (*Pipistrellus pipistrellus*) from Spain.

2. Material and methods

2.1. Animals and origin of the samples

A total of 27 common pipistrelle bats (*Pipistrellus pipistrellus*) from the Community of Madrid and adjoining provinces were analyzed in the study (Fig. 1). All of them were admitted to the wildlife recovery center of GREFA (Group for the Rehabilitation of the Autochthonous Fauna and their Habitat; Madrid, Spain) because of illness or trauma, from September 2018 to December 2019. Samples were obtained only from those animals that died of natural causes or that were humanely euthanized, minimizing pain and distress, according to veterinary criteria, following sickness or injury in cases where recovery or reintroduction into the wild was impossible. Death animals were kept frozen until samples were obtained. Protocols and procedures were supervised by the veterinary staff of GREFA, following the AVMA guidelines for the euthanasia of animals (Leary et al., 2020). The maps in Fig. 1 were elaborated using the free software gvSIG (Version: 2.3.1–2501, gvSIG Association).

Bat species were identified on the basis of morphological measurements: body length, body and tail length, wingspan, ear length, and arm wing length (Schmieder et al., 2015). All the samples belonged to the same species, the common pipistrelle bat.

The distribution of *P. pipistrellus* in Europe is shown in Fig. 1. It is widely distributed across the Mediterranean basin, where *L. infantum* is also present in a large part of the territory. In the Community of Madrid two species of phlebotomines that can act as vectors of the disease are present: *P. perniciosus* and *P. ariasi* (European Centre for Disease Prevention and Control and European Food Safety Authority, 2019).

2.2. DNA isolation

Samples from spleen, abdominal hair, and blood clots from the heart were aseptically collected from each animal and frozen at −20 °C until DNA isolation. DNA was extracted using the commercial kit DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol, and including positive and negative controls in each batch. The DNA obtained was stored at −20 °C until use.

2.3. PCR of L. infantum

DNA of *L. infantum* was detected by amplification of a DNA repeat region with conventional PCR using the primers T2 and B4 (Piarroux et al., 1995) and HotStart Taq© Master Mix Kit (QIAGEN, Hilden, Germany). DNA from the bone marrow of a dog infected with *L. infantum*, kindly supplied by Dr. Luis Cardoso, was used as positive control for the PCR and autoclaved water was used as negative control. The temperature profile was: 15 min at 95 °C for activation of the enzyme, followed by 30 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, and 1 min at 72 °C for extension, with a final

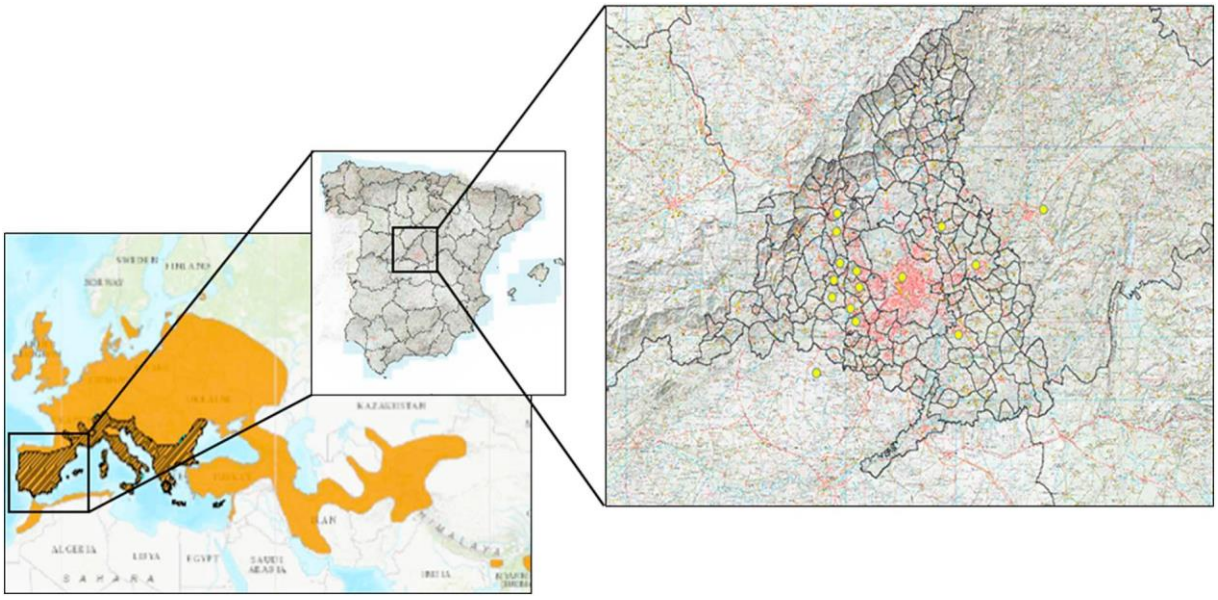

Fig. 1. Distribution of *P. pipistrellus* and *L. infantum* in Europe (modified map from Hutson et al., 2008) and amplified maps of Spain and the Community of Madrid. The black striped area indicates the overlapping of *L. infantum* and *P. pipistrellus*. The origin of the animals is highlighted with yellow dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Detection of *L. infantum* in samples from spleen, hair, and blood of bats analyzed in this study. GenBank accession numbers of positive samples are included. Sample 19/0698 displayed a sequence length of less than 200 bp. Sequence ID refers to the sequence with the highest homology to the sequences obtain in the present study.

Sample ID	Spleen	Blood clot	Hair	GenBank acc. no.	% Homology (% query cover)	Sequence ID
18/6515	+	–	–	MN994838	100 (100)	FR796463.1
19/0698	+	–	–	Short sequence	–	–
18/6640	+	–	+	MN994839	98.4 (92)	KU680922.1
18/6843	–	–	–	–	–	–
19/0873	–	–	–	–	–	–
19/3434	–	–	–	–	–	–
19/4545	–	–	–	–	–	–
19/3236	+	–	+	MN994840	100 (100)	FR796463.1
19/3302	+	–	–	MN994846	99.1 (100)	FR796463.1
19/2784	+	+	+	MN994841	100 (100)	KU680931.1
19/2949	–	–	–	–	–	–
19/2386	–	–	–	–	–	–
19/1553	–	–	–	–	–	–
18/6649	+	–	+	MN994842	99.1 (100)	FR796463.1
18/6490	+	+	+	MN994843	99.6 (100)	FR796463.1
18/6311	+	+	–	MN994844	99.6 (100)	FR796463.1
19/5525	–	–	–	–	–	–
19/5839	–	+	+	MN994849	99.6 (100)	FR796463.1
19/5561	–	–	–	–	–	–
19/5760	+	–	+	MN994848	100 (100)	KU680931.1
19/5612	–	–	–	–	–	–
19/5540	+	+	–	MN994847	99.6 (100)	FR796463.1
19/6520	–	+	–	MN994851	99.1 (99)	FR796463.1
19/6370	+	–	–	MN994850	99.6 (100)	FR796463.1
19/6585	+	–	–	MN994852	99.5 (100)	KU680931.1
19/6556	+	–	–	MN994845	98.2 (100)	FR796463.1
19/6445	–	–	–	–	–	–
Total	14/27	6/27	7/27			

extension step at 72 °C for 10 min. The PCR products were visualized under UV light after electrophoresis in 1.5% agarose stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc., California, USA).

2.4. Sequencing and alignment

After PCR amplification, positive samples were cleaned using the ExoSAP-IT commercial kit (Exonuclease I/Shrimp Alkaline Phosphatase, Applied Biosystems). Amplicons were sequenced in both directions at the Genomic Unit of the University Complutense of Madrid (Genomic Unit, Faculty of Biological Sciences, UCM, Madrid, Spain) with a 3730xl DNA Analyzer (Applied Biosystems) and employing the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

The sequences obtained were aligned using Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, Wisconsin, USA) and the chromatograms were manually checked. The consensus sequences were compared with previously published sequences using the BLAST algorithm of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Consensus sequences were submitted to GenBank for accession number identification.

2.5. Statistical analysis

The sensitivity of the PCR was evaluated according to the Youden approach with a confidence level of 95% and using the online software WinEpi (Working in Epidemiology) (http://www.winepi.net/). Animals with one or more sites testing positive in the PCR were considered as true positives, while animals with no site testing positive in the PCR were considered as true negatives. When more than one site was used to calculate sensitivity, the results were interpreted in parallel.

3. Results

3.1. Origin of the animals

The animals in this study had been sent to the GREFA Centre from different towns in the Community of Madrid, with the exception of two animals that came from Toledo and Guadalajara, two provinces close to Madrid (Fig. 1).

3.2. Detection of L. infantum

In total, 27 bats were analyzed in this study. Samples from spleen, hair, and blood clots were assessed separately (Table 2). Overall, 16 of

the animals tested positive in at least one of the anatomical sites analyzed, yielding a prevalence of *L. infantum* infection of 59.2%. Regarding the animals that tested positive in more than one sample, it is noteworthy that only two of them tested positive in all three anatomical sites: spleen, hair, and blood, i.e., 12.5% of the infected animals (7.4% of the total) (Table 2). The most common anatomical sites in animals with more than one PCR-positive sample were the spleen and hair (n = 4, 25% of infected animals, 14.8% of the total).

3.3. Evaluation of anatomical sites for PCR of L. infantum

The spleen was the site yielding the highest number of positive results (n = 14); however, in two animals that tested positive, the infection was not detected using samples from this anatomical location. These preliminary results indicate a sensitivity of 87.5% (71.3–103.7%, 95% CI) when the spleen is selected as the target site for detection, considering the total number of infected animals.

Samples from hair and blood clots were also useful for the detection of *L. infantum,* since seven samples from hair and six from blood tested positive, indicating a sensitivity of 43.8% (19.4–68.1%, 95% CI) and 37.5% (13.8–61.2%, 95% CI), respectively (i.e., 25.9% and 22.2% of the total, respectively). The highest sensitivity level was obtained when combining two sites in parallel for detection: spleen and blood clot (100%, 95% CI = 100–100%) (Table 3).

The specificity of the PCR was 100% when taking into account the results and the homology of the obtained sequences with other *L. infantum* sequences available at GenBank (vide infra).

3.3.1. Sequences and GenBank submission

The consensus sequences were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession number MN994838–MN994852 (Table 2). The obtained sequences showed 98–100% homology with *L. infantum*.

4. Discussion

In Europe, there are 45 species of Chiroptera living in urban, peri-urban, and rural environments. Among them, *P. pipistrellus* is one of the most widely distributed throughout the continent, maintaining its population at a stable level. The IUCN Red List category of this species is “Least Concern”. It is also the most common species of Chiroptera usually admitted to animal recovery centers across Europe, where more than 2500 bats are admitted each year and finally rescued and rehabilitated in 25 European countries. Their diet is based on insects and they live close to human and domestic animals, occupying cracks, hollows and trees, and breeding in buildings and other human infrastructures (Guardiola and Fernández, 2007; Barova and Streit, 2018). For these reasons, *P. pipistrellus* has a higher risk of infection with *L. infantum* in endemic areas, especially when taking into account its wide distribution, which overlaps with the main vector.

Several species of bats have been analyzed for the presence of *Leishmania* spp. in Central and South America and Africa (Berzunza-Cruz et al., 2015; De Castro Ferreira et al., 2017; De Lima et al., 2008; Kassahun et al., 2015) but the presence of bats infected with *Leishmania* spp. has not been reported in Europe. There is one study on the potential role of bats as hosts of *L. infantum* in Europe (Millán et al., 2014), but the authors analyzed one species only, *Miniopterus schreibersii*, and did not find *Leishmania* DNA. The data obtained in the present study reveal the presence of *L. infantum* in bats originating from the center of Spain, representing the first report in Europe. The fact that Millán et al. (2014) analyzed only peripheral blood could explain the differences found between the two studies, since peripheral blood seems to be less sensitive for detection compared with other anatomical sites, according to our results. Furthermore, the location in the two studies is different: the study by Millán et al., (2014) was carried out in sylvatic areas while our study comprises bats from urban and peri-urban locations, where the infection is common.

The data from the present study showed a higher number of positive results obtained from spleen than from hair and blood samples. The fact that spleen samples are positive in infected animals is not surprising in view of the biological cycle of *L. infantum*, whose amastigote forms remain in the spleen of the host in the chronic phase. By contrast, in recently infected animals, the incidence of parasites in skin or blood is frequent, especially when skin lesions are present. In other studies comparing sites for detection of *L. infantum* infection, the distribution of the parasite varies with the host, with the spleen being the preferred site in dogs and several mammals (Reis et al., 2013; Alcover et al., 2020), while for Leporidae it was hair and skin (Ortega et al., 2017). Some authors explain the difference between positive samples from different anatomical sites as a variation in the immune response of each individual, depending on the progression and distribution of the parasite as well as on the general condition of each animal (Maia and Campino, 2008; Reis et al., 2013). In the present study, the time of infection of the analyzed bats was unknown, but no skin lesions were found on them and, therefore, hair samples were randomly taken from the abdominal area. The absence of macroscopic lesions in bats infected with different species of *Leishmania* is a common finding (Berzunza-Cruz et al., 2015; Kassahun et al., 2015; De Oliveira et al., 2015; De Castro Ferreira et al., 2017). Studies comparing the presence of *Leishmania* spp. in several tissues from bats (Berzunza-Cruz et al., 2015; De Oliveira et al., 2015) reported the presence of DNA also in spleen, even when the species detected were *L. mexicana* and *L. amazonensis*, respectively, which usually cause cutaneous leishmaniosis. Moreover, the mode of *Leishmania* spp. infection in bats is unknown, although the feeding of some *L. infantum* vectors (*P. perniciosus*) on these animals has been demonstrated (Cotteaux-Lautard et al., 2016). However, it has been suggested that insectivorous bats could be infected via the digestive tract, as can occur occasionally in *Trypanosoma cruzi* (De Oliveira et al., 2015; De Rezende et al., 2017), and this might influence the main location of the parasite in the host. More studies are necessary to provide in-depth knowledge of this aspect.

The use of hair samples is a new and promising approach for the diagnosis of *L. infantum,* since Muñoz-Madrid et al. (2013) described it as a noninvasive and effective approach for the detection of the parasite. We also found a high number of positive samples from bat hair (25.9%), and confirm this site as a potential source of samples for diagnosis. Hair samples offer multiple advantages for the detection of *L. infantum* in live animals, although with a lower sensitivity.

The results obtained suggest that European bats are a suitable host of *L. infantum* in Spain and probably other European endemic areas. In recent years, several authors have selected the Community of Madrid as a study area due to the recent outbreak of human leishmaniasis, where rabbits and hares were described as new hosts of *L. infantum* (González et al., 2018; Jiménez et al., 2014; Molina et al., 2012; Ruiz- Fons et al., 2013). According to our findings, bats may also play a role in the life cycle of this parasite in peri-urban environments, and more studies of this mammal are necessary. Another point to be taken into consideration is the influence of *L. infantum* on the health of the infected animals.

Table 3
Evaluation of the anatomical sites for detection of *L. infantum* by PCR. The combinations of results from two anatomical sites interpreted in parallel are also shown. The Youden approach was used to establish the sensitivity at 95% confidence intervals.

Sites for PCR of *L. infantum* samples	Sensitivity	(95% CI)
Spleen	87.5%	71.3%–103.7%
Hair	43.8%	19.4%–68.1%
Blood clot	37.5%	13.8%–61.2%
Spleen + Hair	93.8%	91.9%–105.6%
Spleen + Blood clot	100%	100%–100%
Hair + Blood clot	62.5%	38.8%–86.2%

Being a recently described infection, it should be explored as a possible cause of sickness in endangered species of bats.

5. Conclusion

This is the first report in Europe of *L. infantum* in bats. It is an important finding as this is the most common bat in urban and peri-urban areas of Spain and other European countries. To detect the presence of the parasite in endemic areas, the spleen should be the target site for analysis in dead animals, while hair can be used in live animals. These results will help to establish control measures if more outbreaks of leishmaniasis occur, which can be expected in view of global warming and the potential increase of vector-borne diseases.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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References

Alcover, M.M., Ribas, A., Guillén, M.C., Berenguer, D., Tomás-Pérez, M., Riera, C., Fisa, R., 2020. Wild mammals as potential silent reservoirs of *Leishmania infantum* in a Mediterranean area. Prev. Vet. Med. 175. https://doi.org/10.1016/j.prevetmed.2019.104874.

Arce, A., Estirado, A., Ordobás, M., Moratilla, L., García, N., Pérez, A.M., Gil, E., Aranguez, E., García Puente, E., Martínez, A.M., 2017. Brote de leishmaniasis en la zona suroeste de la Comunidad de Madrid: detección e investigación epidemiológica. In: Dirección General de Salud Pública, de S, C. (Eds.), Brote de Leishmaniasis En Fuenlabrada y Otros Municipios de La Comunidad de Madrid. El Papel de Las Liebres y Los Conejos Como Reservorios, Madrid, pp. 105–112.

Barova, S., Streit, A., 2018. Action Plan for the Conservation of all Bat Species in the European Union 2018–2024. Brussels, Belgium.

Berzunza-Cruz, M., Rodríguez-Moreno, Á., Gutiérrez-Granados, G., González-Salazar, C., Stephens, C.R., Hidalgo-Mihart, M., Marina, C.F., Rebollar-Téllez, E.A., Bailón-Martínez, D., Balcells, C.D., Ibarra-Cerdeña, C.N., Sánchez-Cordero, V., Becker, I., 2015. *Leishmania* (*L.*) *mexicana* infected bats in Mexico: novel potential reservoirs. PLoS Negl. Trop. Dis. 9. https://doi.org/10.1371/journal.pntd.0003438.

Castro, L.S., Dorval, M.E.C., Matheus, L.M.D., Bednaski, A.V., Facco, G.G., Silveira, M., Santos, C.F., Gontijo, C.M.F., Oliveira, A.P.G., Ferreira, E.C., 2020. *Leishmania* presence in bats in areas endemic for leishmaniasis in central-West Brazil. Int. J. Parasitol. Parasite. Wildl. 11, 261–267. https://doi.org/10.1016/j.ijppaw.2020.02.008.

Cotteaux-Lautard, C., Leparc-goffart, I., Berenger, J.M., Plumet, S., Pages, F., 2016. Phenology and host preferences *Phlebotomus perniciosus* (Diptera: Phlebotominae) in a focus of Toscana virus (TOSV) in south of France. Acta Trop. 153, 64–69. https://doi.org/10.1016/j.actatropica.2015.09.020.

De Castro Ferreira, E., Pereira, A.A.S., Silveira, M., Margonari, C., Marcon, G.E.B., de Oliveira França, A., Castro, L.S., Bordignon, M.O., Fischer, E., Tomas, W.M., Dorval, M.E.C., Gontijo, C.M.F., 2017. *Leishmania* (*V.*) *braziliensis* infecting bats from Pantanal wetland, Brazil: first records for *Platyrrhinus lineatus* and *Artibeus planirostris*. Acta Trop. 172, 217–222. https://doi.org/10.1016/J.ACTATROPICA.2017.05.012.

De Lima, H., Rodríguez, N., Barrios, M.A., Ávila, Á., Cañizales, I., Gutiérrez, S., 2008. Isolation and molecular identification of *Leishmania chagasi* from a bat (*Carollia perspicillata*) in northeastern Venezuela. Mem. Inst. Oswaldo Cruz 103, 412–414. https://doi.org/10.1590/s0074-02762008000400018.

De Oliveira, F.M., Costa, L.H.C., de Barros, T.L., Rauschkolb Katsuda Ito, P.K., Colombo, F.A., de Carvalho, C., Pedro, W.A., Queiroz, L.H., Nunes, C.M., 2015. First detection of *Leishmania* spp. DNA in Brazilian bats captured strictly in urban areas. Acta Trop. 150, 176–181. https://doi.org/10.1016/j.actatropica.2015.07.010.

De Rezende, M.B., Herrera, H.M., Carvalho, C.M.E., Carvalho Anjos, E.A., Ramos, C.A.N., De Araújo, F.R., Torres, J.M., De Oliveira, C.E., 2017. Detection of *Leishmania* spp. in bats from an area of Brazil endemic for visceral Leishmaniasis. Transbound. Emerg. Dis. 64, 36–42. https://doi.org/10.1111/tbed.12597.

European Centre for Disease Prevention and Control and European Food Safety Authority, 2019. Phlebotomine sandflies maps [WWW Document]. In: Stock. ECDC, URL. https://www.ecdc.europa.eu/sites/default/files/images/map-Phlebotomus_perniciosus distribution July 2019.png (accessed 3.9.20).

González, E., Molina, R., Jiménez, M., 2018. Rabbit trypanosome detection in *Phlebotomus perniciosus* sand flies from the leishmaniasis outbreak in Madrid, Spain. Acta Trop. 187, 201–206. https://doi.org/10.1016/j.actatropica.2018.08.011.

Guardiola, Á., Fernández, M.P., 2007. *Pipistrellus pipistrellus* (Schreber, 1774). In: Palomo, L.J., Gisbert, J., Blanco, J.C. (Eds.), Atlas y Libro Rojo de Los Mamíferos Terrestres de España. Madrid, pp. 199–202.

Hutson, A., Spitzenberger, F., Aulagnier, S., Coroiu, I., Karataş, A., Juste, J., Paunovic, M., Palmeirim, J., Benda, P., 2008. *Pipistrellus pipistrellus*. IUCN Red List Threat. Species 2008, 8235. https://doi.org/10.2305/IUCN.UK.2008.RLTS.T17317A6968203.en.

Jiménez, M., González, E., Martín-Martín, I., Hernández, S., Molina, R., 2014. Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? Vet. Parasitol. 202, 296–300. https://doi.org/10.1016/j.vetpar.2014.03.027.

Kassahun, A., Sadlova, J., Benda, P., Kostalova, T., Warburg, A., Hailu, A., Baneth, G., Volf, P., Votypka, J., 2015. Natural infection of bats with *Leishmania* in Ethiopia. Acta Trop. 150, 166–170. https://doi.org/10.1016/j.actatropica.2015.07.024.

Leary, S., Underwood, W., Anthony, R., Cartner, S., Grandin, T., Greenacre, C., Gwaltney-Brant, S., McCrackin, M.A., Meyer, R., Miller, D., Shearer, J., Turner, T., Yanong, R., 2020. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition. Schaumburg, Illinois.

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. https://doi.org/10.1016/j.vetpar.2008.07.028.

Maxfield, L., Crane, J.S., 2019. Leishmaniasis, StatPearls. StatPearls Publishing [WWW Document]. URL. https://www.ncbi.nlm.nih.gov/books/NBK531456/ (accessed 6.9.20).

Millán, J., López-Roig, M., Cabezón, O., Serra-Cobo, J., 2014. Absence of *Leishmania infantum* in cave bats in an endemic area in Spain. Parasitol. Res. 113, 1993–1995. https://doi.org/10.1007/s00436-014-3855-3.

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet. Parasitol. 190, 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006.

Muñoz-Madrid, R., Belinchón-Lorenzo, S., Iniesta, V., Fernández-Cotrina, J., Parejo, J.C., Serrano, F.J., Monroy, I., Baz, V., Gómez-Luque, A., Gómez-Nieto, L.C., 2013. First detection of *Leishmania infantum* kinetoplast DNA in hair of wild mammals: application of qPCR method to determine potential parasite reservoirs. Acta Trop. 128, 706–709. https://doi.org/10.1016/j.actatropica.2013.08.009.

Ortega, M.V., Moreno, I., Domínguez, M., de la Cruz, M.L., Martín, A.B., Rodríguez-Bertos, A., López, R., Navarro, A., González, S., Mazariegos, M., Goyache, J., Domínguez, L., García, N., 2017. Application of a specific quantitative real-time PCR (qPCR) to identify *Leishmania infantum* DNA in spleen, skin and hair samples of wild Leporidae. Vet. Parasitol. 243, 92–99. https://doi.org/10.1016/j.vetpar.2017.05.015.

Ortuño, M., Latrofa, M.S., Iborra, M.A., Pérez, P., Bernal, L.J., Risueño, J., Muñoz, C., Bernal, A., Sánchez, P.F., Manuel, L., Giada, S., Carla, A., Cortes, S., Campino, L., Otranto, D., Berriatua, E., 2019. Genetic diversity and phylogenetic relationships between *Leishmania infantum* from dogs, humans and wildlife in south - East Spain. Zoonoses Public Health 0, 1–13. https://doi.org/10.1111/zph.12646.

Piarroux, R., Fontes, M., Perasso, R., Gambarelli, F., Joblet, C., Dumon, H., Quilici, M., 1995. Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. Mol. Biochem. Parasitol. 73, 249–252. https://doi.org/10.1016/0166-6851(95)00097-k.

Reis, L.E.S., Coura-Vital, W., Roatt, B.M., Bouillet, L.É.M., Ker, H.G., Fortes de Brito, R.C., Resende, D. de M., Carneiro, M., Giunchetti, R.C., Marques, M.J., Carneiro, C.M., Reis, A.B., 2013. Molecular diagnosis of canine visceral leishmaniasis: a comparative study of three methods using skin and spleen from dogs with natural *Leishmania infantum* infection. Vet. Parasitol. 197, 498–503. https://doi.org/10.1016/j.vetpar.2013.07.006.

Risueño, J., Ortuño, M., Pérez-cutillas, P., Goyena, E., Maia, C., Cortes, S., Campino, L., Bernal, L.J., Muñoz, C., Arcenillas, I., Martínez-rondán, F.J., Gonzálvez, M., Collantes, F., Ortiz, J., Martínez- Carrasco, C., Berriatua, E., 2018. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet. Parasitol. 259, 61–67. https://doi.org/10.1016/j.vetpar.2018.05.012.

Ruiz- Fons, F., Ferroglio, E., Gortázar, C., 2013. *Leishmania infantum* in free-ranging hares, Spain, 2004-2010. Euro Surveill 30, 80–81. https://doi.org/10.1016/j.pt.2019.10.006.

Schmieder, D.A., Benítez, H.A., Borissov, I.M., Fruciano, C., 2015. Bat species comparisons based on external morphology : a test of traditional versus geometric morphometric approaches. PLoS One 10, 8–13. https://doi.org/10.5061/dryad.705q4.

Suárez Rodríguez, B., Isidoro Fernández, B., Santos Sanz, S., Sierra Moros, M.J., Molina Moreno, R., Astray Mochales, J., Amela Heras, C., 2012. Situación epidemiológica y de los factores de riesgo de transmisión de *Leishmania infantum* en España. Rev. Esp. Salud Publica 86, 555–564. https://doi.org/10.4321/S1135-57272012000600002.

CAPÍTULO 4: RESULTADOS

4.2. OBJETIVOS 2

Estudiar el papel de las especies exóticas invasoras que se encuentran en zonas urbanas, peri-urbanas o de recreo en relación con el posible papel como reservorios o especies centinela de la parasitosis. Análisis de las dianas de PCR más adecuadas para los objetivos planteados.

4.2.1. RESUMEN

En Europa existen numerosas especies de organismos considerados exóticos invasores, los cuales son originarios de otras zonas del planeta y en el nuevo hábitat donde se encuentran tienen un efecto negativo sobre el ecosistema. Estos daños dependen del tipo de organismo, así como de la interacción que presenten con el entorno donde se sitúen. Dentro de este grupo de organismos invasores, encontramos numerosas especies de mamíferos que, como en el caso de visón americano (*Neovison vison*), alteran de manera muy significativa las poblaciones de otros animales autóctonos debido a la competencia por los recursos alimenticios y de refugio. Concretamente, los animales que se ven afectados principalmente por la presencia del visón americano son la nutria europea (*Lutra lutra*) y el amenazado visón europeo (*Mustela lutreola*), todos ellos carnívoros acuáticos, que residen en ecosistemas de ribera, provocando la inclusión del visón americano en programas de control y erradicación, según la normativa europea (Bonesi & Palazon, 2007; Sidorovich et al., 1999).

En España la presencia de estos mamíferos en libertad se detectó por primera vez en la década de 1970, siendo hoy en día numerosas las poblaciones de visón americano que se han asentado tanto en el norte como en el este del país (Ruiz-Olmo et al., 1997).

La presencia de estos animales en zonas de recreo naturales, donde las personas y animales domésticos acuden con relativa frecuencia, hace que hayan sido objeto de estudio en cuanto a su relación con patógenos zoonóticos. Por tanto, el objetivo de este estudio fue determinar la presencia de *L. infantum* en estos animales silvestres, como potenciales hospedadores del parásito y actores importantes en el mantenimiento del ciclo silvestre.

Se analizaron muestras de bazo de 22 visones americanos de los cuales 19 resultaron positivos a *L. infantum*. Para este estudio se utilizaron 4 dianas del ADN (*SSUrRNA, ITS1, kDNA* y *Repeat region*), que a su vez fueron comparadas para determinar su sensibilidad. Para ello, se utilizaron dos tipos de muestra: por un lado, un cultivo de promastigotes de *L. infantum* y, por otro, muestra de médula ósea de un perro parasitado. Bajo las condiciones y protocolos seleccionados, las dianas más sensibles resultaron ser *Repeat region* y *kDNA* (con la pareja de cebadores RV1/RV2), seguidos de la PCR anidada de *SSUrRNA*. En cuanto a las muestras de bazo de los visones americanos, la *Repeat region*

resultó la más idónea para detectar la presencia de *L. infantum* (19 positivos), quedando en segunda posición la diana *SSUrRNA* (2 positivos). Esto resultados obtenidos en visones americanos concuerdan con otros estudios realizados con estos animales en Grecia y España (Alcover et al., 2020; Filioussis et al., 2018; Tsakmakidis et al., 2019). La diferencia de resultados obtenidos con respecto al análisis del cultivo y la muestra de perro, en especial con respecto al *kDNA*, podría deberse a la variabilidad que presenta por su naturaleza multicopia, lo que hace que pequeños cambios en la secuencia se traduzcan en una imposibilidad para amplificar el ADN, otorgando una elevada diversidad de resultados en la bibliografía. También podría influir la carga parasitaria de las muestras analizadas, ya que el cultivo, así como la médula ósea de perro, presentaban elevadas cantidades de parásitos, lo cual no es habitual en un animal silvestre.

4.2.2. PONENCIAS

- **Azami- Conesa, I**., Sansano- Maestre, J., Martínez- Díaz, R.A., Gómez- Muñoz, M.T. "American mink (*Neovison vison*) as potential reservoir of *Leishmania infantum*. 69th International Wildlife Disease Association (WDA)/ 14th European Wildlife Disease Association (EWDA) Virtual Conference. Cuenca (España), del 31 de agosto al 2 de septiembre de 2021. Comunicación oral.

4.2.3. ARTÍCULO

- **Azami- Conesa, I**., Sansano- Maestre, J., Martínez- Díaz, R.A., Gómez- Muñoz, M.T. (2021). Invasive Species as Hosts of Zoonotic Infections: The Case of American Mink (*Neovison vison*) and *Leishmania infantum*. *Microorganisms*, 9 (7), 1513. DOI: 10.3390/microorganisms9071531.

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Article

Invasive Species as Hosts of Zoonotic Infections: The Case of American Mink (*Neovison vison*) and *Leishmania infantum*

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Abstract: *Leishmania infantum* produces an endemic disease in the Mediterranean Basin that affects humans and domestic and wild mammals, which can act as reservoir or minor host. In this study, we analyzed the presence of the parasite in wild American minks, an invasive species in Spain. We screened for *L. infantum* DNA by PCR using five primer pairs: Two targeting kinetoplast DNA (kDNA), and the rest targeting the ITS1 region, the small subunit of ribosomal RNA (SSU) and a repetitive sequence (Repeat region). The detection limit was determined for each method using a strain of *L. infantum* and a bone marrow sample from an infected dog. PCR approaches employing the Repeat region and kDNA (RV1/RV2 primers) showed higher sensitivity than the other PCR methods when control samples were employed. However, only PCR of the Repeat region and nested PCR of SSU (LnSSU) detected the parasite in the samples, while the other three were unable to do so. The majority of the analyzed animals (90.1%) tested positive. American mink may act as an incidental host of the disease for other mammals and should be further investigated, not only for their negative impact on the local fauna, but also as carriers of zoonotic diseases.

Keywords: American mink; ITS; kDNA; leishmaniasis; one health; hosts; Repeat region; SSUrRNA; wild carnivore; zoonosis

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1. Introduction

The American mink (*Neovison vison*) is a mustelid native of North America that was introduced in Europe for fur farming. Throughout recent decades, accidental or deliberate escape of mink from farms caused the establishment of stable feral populations. In Spain, the first observation of feral animals was detected at the end of the 1970s, and at present, there are stable populations distributed throughout the northwest and east of the Iberian Peninsula (North of Galicia, Cantabria, and Teruel-Castellón) [1]. The presence of this mammal on the banks of rivers supposes a strong competition for resources with other aquatic carnivores such as the European otter (*Lutra lutra*) or the endangered European mink (*Mustela lutreola*) [2]. For this reason, American mink is included in the European list of invasive species and is subjected to control and eradication programs [3]. Moreover, American mink can act as a carrier of multiple pathogens, including several nematode species [4], protozoans such as *Toxoplasma gondii* [5], and viruses such as influenza A [6] and SARS-CoV-2 [7]. These pose potential health risks for man and also for autochthonous freshwater carnivores in areas where there is an overlap in the distribution with this alien species.

Leishmania infantum is one of the etiological agents of zoonotic leishmaniasis, which is an endemic parasite of the Mediterranean Basin. It is mainly transmitted by phlebotomine

sandfly bites [8], although other ways of transmission, such as sexually or transplacentally, have been proven in dogs, which are considered the main reservoir for human *Leishmania* parasites [9]. The disease has several forms, with the cutaneous (CL) and visceral (VL) forms being the most common. More than one billion people are at risk of leishmaniasis in endemic areas around the world, and 30,000 new cases of VL are estimated every year. In addition to the high number of infected humans and animals that develop the disease, there are also a significant amount of people, dogs, and other animals carrying asymptomatic infections [10].

Occasionally, conditions that favor the expansion of the disease, including spatial and temporal coincidence of humans, vectors, and potential reservoirs appear, lead to human outbreaks. The ideal habitats for vector expansions are warm areas rich in organic material, such as caves or burrows where animals breed, including wild mammals [11].

When leishmaniasis outbreaks appear, an exhaustive search of potential reservoirs and vectors begins, and monitoring activities can reveal new hosts for the parasite. In the case of Spain, an outbreak starting in 2009 in Madrid highlighted the importance of wildlife as potential reservoirs of the disease, specifically lagomorphs [12]. Thenceforward, several studies reported the presence of *Leishmania* in different species of wildlife, including wolfs, foxes, mustelids, bats, hedgehogs, and rodents [13–18]. Despite the number of articles studying the role of wildlife as reservoirs of leishmaniasis, invasive species are not widely studied thus far. Invasive species are relevant not only for other wild species, but also for humans, considering the growing approach to urban places, and the potential role as reservoirs of vector-borne pathogens [19]. The American mink is in the order Carnivora, which includes many species considered as reservoirs and/or hosts suffering the disease. The presence of *L. infantum* has been reported in mink farms in Greece [20,21] and in one wild American mink killed by a car in Spain [17], which suggest its role as a host of *L. infantum*.

In this article, we explored the role of wild communities of American mink as a host of *L. infantum* by PCR using five primer pairs to detect the DNA of the parasite.

2. Materials and Methods

2.1. Samples and Area of Study

Spleen samples from American minks of three different river basins (Mijares, Palancia, and Turia) located in the Valencian Community (Spain) were analyzed (Figure 1). The animals were hunted in the context of a European LIFE project for the conservation of the European mink (LIFE Lutreola Spain, LIFE13 NAT/ES/001171), which include the eradication of the American mink among its objectives, due to the high menace they impose on the autochthonous fauna. The American mink is categorized as an alien invasive species in Europe [22], and European [23], National [24], and regional legislation [25] regulate the control and/or eradication programs of invasive species, such as *Neovison vison*, according to the status in each country. One of the objectives of the LIFE13 NAT/ES/001171 and the Spanish strategy for control and eradication of the American mink is to capture and euthanize as many animals as possible. The total number of animals captured in the Valencian community in previous control programs was 220 in 8–10 years, which means that the present sample approaches the highest number of animals possibly subject to eradication per year, although a precise number of animals in the area is not available. More information on the distribution of stable populations of feral American minks in Spain can be found elsewhere [26]. The animals were captured from February to October 2016 and were humanely euthanized, minimizing pain and distress, according to veterinary or control program criteria (animals which could not be reintroduced into the wild) and following the recommendations of the Spanish strategy for the control and eradication of the American mink. Euthanasia was carried out by the veterinary staff of the wildlife recovery center "La Granja de El Saler" (Valencia, Spain), following the AVMA guidelines for euthanasia of animals [27]. Biometric measurements were taken from each animal and

a necropsy was performed. A sample from the spleen of each animal was kept frozen (−80 °C) until further analysis.

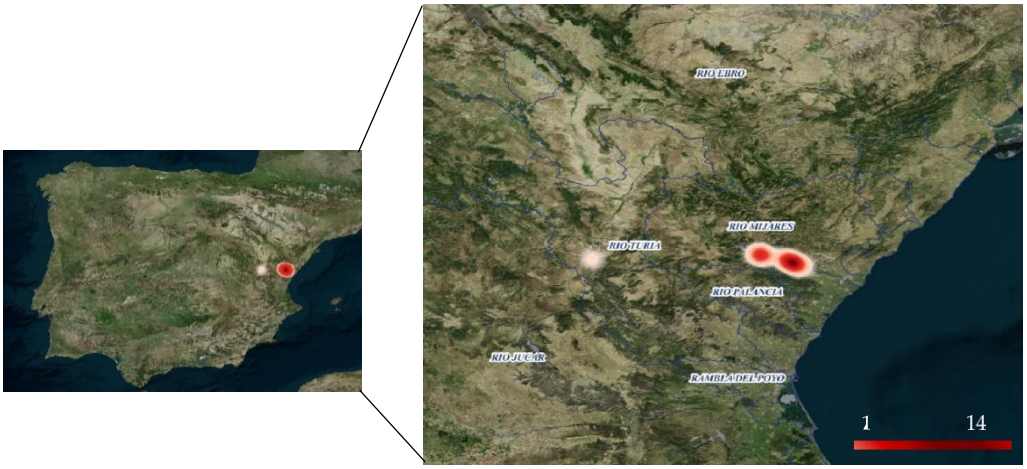

Figure 1. Geographic area where the animals were captured. The color intensity indicates the number of animals captured in each area (see bar on bottom).

2.2. Culture of Leishmania and Determination of the Detection Limit

A culture of the strain "JPC" (MCAN/ES/98/LLM-722) of *L. infantum* generously supplied by Dr. J.M. Requena was used to calculate the detection limit of the PCR protocols employed. The isolate was grown in RPMI containing 15% fetal bovine serum (Sigma, St. Louis, MO, USA) and 10 µg/mL of hemin (Acros Organics, Geel, Belgium). In the exponential phase of the culture (day 3 post-inoculation), promastigotes were counted in a Neubauer chamber using 1:1 Trypan blue (4%) in 10% formaldehyde. One million promastigotes were employed for DNA isolation. The parasites were recovered by centrifugation at 800× *g* for 5 min. The sediment was washed three times with phosphate-buffered saline (PBS) solution, followed by centrifugation in the same manner. We isolated DNA from the pellets obtained using a commercial DNA extraction kit (NZY Tissue gDNA Isolation kit; NZY Tech, Lisbon, Portugal), following the manufacturer's protocol. Bound DNA was eluted in 50 µL of elution buffer and stored at −20 °C until use.

The limit of detection was evaluated by conventional PCR, using four different targets (Repeat region, kDNA, ITS1, and SSUrRNA) by 1/10 serial dilutions, up to 10^{-7}-fold dilutions, using DNA from the *L. infantum* culture. The initial DNA concentration was quantified by spectrophotometry (260/280 nm ratio) using a basic Eppendorf BioSpectrometer® (Eppendorf AG, Hamburg, Germany), and 20 µg/mL was obtained.

To evaluate the detection limit of each PCR protocol, and also as a positive control of each PCR reaction, DNA from the bone marrow of a dog infected with *L. infantum,* kindly supplied by Prof. Luis Cardoso (UTAD, Vila Real, Portugal), was used. Again, 1/10 serial dilutions, up to 10^{-7}-fold dilutions, were employed to compared the five PCR protocols.

2.3. DNA Isolation of Spleen Samples and PCR

DNA was isolated from 15 mg of spleen tissue, employing the NZY Tissue gDNA Isolation kit, according to the tissue protocol instructions, and later eluted in 50 µL of elution buffer. Positive and negative controls (*L. infantum* culture and water, respectively) were included in each batch.

Each DNA sample was subjected to five protocols of PCR to detect the parasite: Two protocols targeting kinetoplast DNA (kDNA) [28,29], one protocol targeting the ITS region [30], a nested PCR targeting the small subunit of the ribosomal RNA (LnPCR SSU) [31,32], and a protocol amplifying a Repeat region of the parasite [33]. In all cases, the reaction was carried out in 25 µL, employing 12.5 µL of the HotStart Supreme NZY Taq II (NZY Tech, Lisbon, Portugal) and 1 µL of each primer at 10 µM. For all of the protocols,

2.5 µL of DNA was employed, except for the SSU, which used 5 µL of DNA in the first PCR and 5 µL of 1/40 dilution of PCR product in the second PCR. The specific information of each target, including the temperature profiles following adaptation suggested by manufacturer, and primers sequences is available in Table 1. An initial step of 95 °C for 5 min was used to activate the enzyme and a final step of 72 °C for 10 min for elongation was used in all of the protocols. As a positive control for the amplifications, DNA from the abovementioned bone marrow was employed, whilst water was used as a negative control. Electrophoresis was carried out in 1.5% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc., San Francisco, CA, USA) and later visualized under UV light. After each PCR protocol, the samples were considered positive if a band of the expected size was observed in the gel.

Table 1. PCR targets and primer pairs and conditions employed in each PCR protocol.

Target	Oligonucleotides (5^t-3^t)	Expected Size of the Amplicon	PCR Conditions	N° Cycles	Reference
Repeat region	T2 (CGGCTTCGCACCATGCGGTG) B4 (ACATCCCTGCCCACATACGC)	250 bp	94 °C—30″ 61 °C—30″ 72 °C—15″	35	[33]
kDNA	RV1 (CTTTTCTGGTCCCGCGGGTAGG) RV2 (CCACCTGGCCTATTTTACACCA)	140 bp	94 °C—30″ 62 °C—30″ 72 °C—20″	35	[28]
kDNA	13A (GTGGGGGAGGGGCGTTCT) 13B (ATTTTACACCAACCCCCAGTT)	120 bp	94 °C—30″ 60 °C—30″ 72 °C—20″	30	[29]
ITS1	LITSR (CTGGATCATTTTCCGATG) L5.8S (TGATACCACTTATCGCACTT)	320 bp	94 °C—30″ 53 °C—30″ 72 °C—15″	35	[30]
SSU	Ext R221 (GGTTCCTTTCCTGATTTACG) Ext R332 (GGCCGGTAAAGGCCGAATAG)	603 bp	94 °C—30″ 60 °C—30″ 72 °C—30″	35	[31]
	Int R223 (TCCATCGCAACCTCGGTT) Int R333 (AAAGCGGGCGCGGTGCTG)	358 bp	94 °C—30″ 65 °C—30″ 72 °C—30″	32	[32]

Ext, external primers; Int, internal primers.

2.4. Sequencing

Positive samples were cleaned using an ExoSAP-IT commercial kit (Exonuclease I/Shrimp Alkaline Phosphatase, Applied Biosystems, Foster City, CA, USA). Amplicons were sequenced in both directions at the Genomic Unit of Complutense University of Madrid with a 3730 × L DNA analyzer (Applied Biosystems), using a BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems). The obtained sequences were aligned employing Lasergen SeqMan software version 7.0.0 (DNASTAR, Madison, WI, USA) and manually checked. The consensus sequences were compared with available sequences using the BLAST algorithm of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 16 April 2021). Sequences of more than 200 bp were submitted to GenBank for accession number identification.

3. Results

3.1. Samples

In total, samples from 22 captured animals were obtained during the eradication program of invasive species in 2016, in the context of a LIFE project to protect the European mink. Data were recorded from each animal, including date of capture, age, sex, weight, length, and pregnancy in the case of females (Table 2). With the exception of one juvenile male, the rest of the individuals were adults—nine males and 10 females, two of them pregnant. The animals did not show any clinical signs of disease.

Table 2. Data from the animals captured.

Animal ID	Date	Sex	Age	Weight (g)	Length (cm)	River
68/16	8 March 2016	F	Ad	680	57	Mijares
26/16	10 February 2016	F	Ad	689	58	Mijares
192/16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
56/16	1 March 2016	M	Ad	1180	62	Mijares
23/16	10 February 2016	F	Ad	793	58.9	Mijares
197/16	20 October 2016	M	Ad	950	63	Palancia
66/16	6 March 2016	F	Ad	600	51	Mijares
201/16	22 October 2016	F	Ad	660	55.5	Palancia
55/16	1 March 2016	M	Ad	980	56	Mijares
41/16	18 February 2016	M	Ad	901	59	Mijares
62/16	4 March 2016	F	Ad	640	48	Mijares
1023/16	22 May 2016	M	Juv	878	62	Turia
77/16	14 March 2016	M	Ad	1180	64	Mijares
212/16	26 October 2016	F	Ad	650	58	Palancia
213/16	27 October 2016	M	Ad	810	58	Palancia
35/16	16 February 2016	M	Ad	1109	63.5	Mijares
29/16	14 February 2016	F *	Ad	721	57	Mijares
37/16	17 February 2016	M	Ad	1604	69	Mijares
30/16	14 February 2016	F *	Ad	583	52	Mijares
28/16	14 February 2016	F	Ad	637	56	Mijares
331/16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1001/16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., no data recorded; F, female; Juv, juvenile; M, male; * pregnant.

3.2. PCR Detection Limit

The amount of DNA amplified with each protocol varied among the tested procedures. The most sensitive protocols were PCR of the Repeat region and PCR of kDNA following the protocol of Lachaud et al. [28], while the other three protocols varied between the culture and the bone marrow samples from a positive dog.

3.2.1. PCR Detection Limit Using a Reference Strain

PCRs of the Repeat region and kDNA (RV1/RV2 primers) were able to detect 0.5 promastigotes of *L. infantum* (equivalent to 0.05 pg DNA) (Table 3), while LnPCR of the SSU amplified a minimum of one promastigotes of the parasite (0.1 pg DNA), since a doubled amount of DNA was employed in LnPCR SSU. PCRs of the ITS region and kDNA (13A/13B primers) were able to detect a minimum of five promastigotes (0.5 pg DNA).

Table 3. PCR amplification from the DNA obtained from the *L. infantum* strain using the protocols employed for the detection and identification of *L. infantum*.

		Ten-Fold Dilutions from the Original DNA Sample						
PCR Protocol	**DNA from Culture (20 µg/mL)**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
PCR Repeat region	+	+	+	+	+	+	−	−
PCR kDNA(RV1/RV2)	+	+	+	+	+	+	−	−
LnPCR SSU	+	+	+	+	+	+	−	−
PCR kDNA(13A/13B)	+	+	+	+	+	−	−	−
PCR ITS1	+	+	+	+	+	−	−	−

PCR of the kDNA region includes primers in parentheses to differentiate the two protocols applied. The first column includes the results with DNA without dilution, and each column adds 1/10 dilution from the original DNA. +, positive; −, negative.

3.2.2. PCR Detection Limit Using DNA from the Bone Marrow of an Infected Dog

When DNA from the bone marrow of a positive dog was employed, PCR of the Repeat region and of kDNA (RV1/RV2 primers) were again the most sensitive protocols, displaying positive results even at a 10^{-4}-fold dilution from the original DNA (Table 4). PCR of the ITS region showed positive results up to 10^{-3} dilution from the original DNA, while PCR of kDNA (13A/13B primers) and LnPCR SSU appeared less sensitive, amplifying only at a maximum of a 10^{-2}-fold dilution from the original DNA sample.

Table 4. PCR amplification from bone marrow DNA of a positive dog using the five PCR protocols employed for detection and identification of *L. infantum*.

		Ten-Fold Dilutions from the Original DNA Sample						
PCR Protocol	**Bone Marrow DNA**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
PCR Repeat region	+	+	+	+	+	−	−	−
PCR kDNA(RV1/RV2)	+	+	+	+	+	−	−	−
LnPCR SSU	+	+	+	−	−	−	−	−
PCR kDNA(13A/13B)	+	+	+	−	−	−	−	−
PCR ITS1	+	+	+	+	−	−	−	−

PCR of kDNA region includes primers in parentheses to differentiate the two protocols applied. The first column includes results with DNA without dilution, and each column adds 1/10 dilution from the original DNA. +, positive; −, negative.

3.3. PCR Results from Mink Spleen Samples

In total, 22 samples were analyzed by the five PCR protocols. Only PCR of the Repeat region and LnPCR of SSU rendered positive results, while the other three techniques amplified only the positive controls included in each reaction set. Nineteen samples were positive to PCR of the Repeat region, while only two samples were positive to LnPCR SSU (Table 5).

Table 5. PCR amplification from spleen samples using the five PCR protocols used for the detection and identification of *L. infantum*. PCR of the kDNA region includes primers in parentheses to differentiate the two protocols employed.

Animal ID	**PCR Repeat Region**	**LnPCR SSU**	**PCR kDNA (RV1/RV2)**	**PCR kDNA (13A/13B)**	**PCR ITS**
68/16	+	−	−	−	−
26/16	+	−	−	−	−
192/16	+	−	−	−	−
56/16	+ *	−	−	−	−
23/16	+	−	−	−	−
197/16	+	−	−	−	−
66/16	+	−	−	−	−
201/16	+	−	−	−	−
55/16	+	−	−	−	−
41/16	+	−	−	−	−
62/16	+	−	−	−	−
1023/16	+	−	−	−	−
77/16	+ *	−	−	−	−
212/16	−	−	−	−	−
213/16	+ *	−	−	−	−
35/16	−	−	−	−	−
29/16	+	+	−	−	−
37/16	+	−	−	−	−
30/16	+ *	+	−	−	−
28/16	+	−	−	−	−
331/16	+	−	−	−	−
1001/16	+ *	−	−	−	−

* Weak PCR band and short sequence; +, positive; −, negative.

3.4. Sequences Obtained

The sequences obtained in this study were deposited to GenBank under accession numbers MW972061–MW972074, MW945401, and MW945402. Fourteen 217 bp sequences from the Repeat region were obtained, while four samples displayed shorter sequences and could not be sent to GenBank. The obtained sequences from the Repeat region shared 100% identity with *L. infantum* sequence L42479. The two 322 bp sequences from the SSU region shared 100% identity with *L. infantum* sequence MN757921 from an isolate obtained from a dog.

4. Discussion

Herein, we reported a high percentage of animals infected with the *L. infantum*, but according to the results obtained employing the different PCR targets, and considering the detection limit obtained, it is probable that the animals carried a low amount of parasites in their bodies. All positive animals, except one, were adults, and they had exposure to at least one full season of sandfly activity. An explanation for the low parasite load could be that the context in which minks interact with sandfly populations in the wild limits the amount of bites they are exposed to and, consequently, the dose of promastigotes that each animal received. Alternatively, a transient infection with the parasite could be the cause of the low parasitic load. American minks have a lifespan of 10–12 years in the wild and they live in colonies, which facilitates host–sandfly contact. These characteristics, together with the high percentage of infection, the presence of *Phlebotomus perniciosus* in the area [34], and the identity of *L. infantum* DNA sequence from a dog, support their role as a host of the parasite and they should be considered for future studies.

There are a large number of domestic and wild animals in Europe, besides dogs, in which *L. infantum* has been described [35]. Among them, wild carnivores are the most studied and are considered suitable reservoirs for the parasite, since specific DNA has been detected in many of them, including canids and felids [35]. In addition, several species of mustelids have been reported with *L. infantum* DNA: European mink (*Mustela lutreola*), Eurasian otter (*Lutra lutra*), Beech marten (*Martes foina*), European pine marten (*Martes martes*), domesticated ferrets (*Mustela putorius furo*), European badger (*Meles meles*), and European polecat (*Mustela putorius*) [13,15,21,36–38].

A high rate of infection has been reported in farmed American minks (21.4% of analyzed animals), even at a young age [20]. In another study, 20% of older farmed minks were positive by serology [21], although lower values were found employing PCR of the ITS region (2.1%). Stress and conditions of the farms (temperature and humidity) may favor the development and expansion of sandflies. Whereas the presence of *L. infantum* seems to be frequent in farmed animals, only one study detected a wild American mink infected with the parasite by qPCR in Spain, but the authors only tested one animal [17]. In all cases, parasites were detected in samples from the spleen, and from the liver of one of them. Apart from this single report of DNA in one exemplar, the role of American minks as incidental host of the parasite in wild communities has not been investigated until the present article.

In this study, we employed different targets to detect the parasite as a multi-locus approach to assess the identity of the obtained sequences in comparison to other *L. infantum* sequences from humans and animals. Unfortunately, only two of them displayed positive results. Under our conditions, PCR using the Repeat region and kDNA (RV1 and RV2 primers) were the most sensitive techniques for detecting DNA from parasite cultures and from the bone marrow of a positive dog. Using DNA samples from cultures, in which the amount of parasites is easier to quantify, LnPCR of SSU showed the same limit of sensitivity. When employing PCR from the ITS region, a lower percentage of positivity has also been reported by other authors, including lower values of PCR detection in farmed American minks compared to serology [21]. Lower sensitivity employing the ITS target was also found in samples from hedgehogs, while PCR using kDNA and the Repeat region as the target displayed better results, with 6/24 samples positives by PCR kDNA and 13/26

positives by nested PCR of the Repeat region [39]. In agreement with the results obtained by Chemkhi et al. [39], PCR amplifying the Repeat region was the best approach to detect positivity in the samples employed in the present study.

Other authors have also compared different protocols of amplification, with the results varying between studies, although most of them agreed in pointing out that kDNA is a high-sensitivity target due to its multicopy nature, with thousands of maxicircles and several dozen of minicircles [40]. Lachaud et al. [28] compared six different primer pairs to detect the parasite in the blood of infected animals, three of them targeting nuclear DNA, and three more targeting mitochondrial DNA (kinetoplast minicircles). The authors found a limit of detection of 0.2 parasites per reaction tube employing the primers targeting the Repeat region, the same value reported in the present study using the same primer pairs. However, primers 13A and 13B were more sensitive, according to the authors, while in the present study, they were the less sensitive. A strong inhibition was reported by Lachaud et al. [28] when employing PCR of the Repeat region, but in the present study, no signs of inhibition were found, a fact that could be due to differences between the polymerases or DNA extraction protocols applied in each study. When tissues are employed to isolate DNA, results may vary from one individual to another, or even between animal species [28]. Indeed, spleen samples are full of inhibitors or competitors, and manufacturers recommend in many protocols to reduce the amount of tissue processed.

Albuquerque et al. [41] compared four PCR methods, two targeting kinetoplast DNA and two targeting nuclear DNA. According to the authors, LnPCR SSU was able to detect a higher number of samples from the bone marrow of clinically and sub-clinically infected animals, while ITS1 showed the lowest level of detection. The two mentioned PCR protocols were also employed in the present study, and the obtained results agree with those reported by the authors. None of the primer pairs targeting kDNA were employed in the present study and no comparison between results could be done.

Concerning the negative results obtained with both kDNA PCR protocols, they might be due to negligible changes in DNA sequences being enough to avoid amplification from the samples, since they represent a more variable target than the Repeat region or the SSU region [40]. Indeed, several approaches using kDNA as targets can be found in the literature and the results varied with the primers employed. De Oliveira et al. [42] reported that primers 13A and 13B had the worst performance among four primers derived from kDNA sequences, and employing DNA from parasite strains, a fact that is in agreement with the results obtained in the present study.

Taking into account our results, we believe that the employment of more than one target to detect *L. infantum* is necessary. Different sensitivities of the protocols, or minimal differences in the DNA sequences of more variable targets, could interfere with the results.

This is the first report of the detection of *L. infantum* in communities of wild American minks. The American mink is an invasive species that menaces the European mink, but it also harbors several zoonotic infections [4–7,17,20,21]. For these reasons, they could be used as sentinel species to monitor the epidemiological status of zoonotic pathogens in the regions where they inhabit, and this approach could be included in European mink conservation programs.

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References

1. Ruíz-Olmo, J.; Palazón, S.; Bueno, F.; Bravo, C.; Munilla, I.; Romero, R. Distribution, status and colonization of the American mink (*Mustela vison*) in Spain. *J. Wildl. Res.* **1997**, *2*, 30–36.
2. Sidorovich, V.; Kruuk, H.; Macdonald, D.W. Body size, and interactions between European and American mink (*Mustela lutreola* and *M. vison*) in Eastern Europe. *J. Zool.* **1999**, *248*, 521–527. [CrossRef]
3. Bonesi, L.; Palazón, S. The American mink in Europe: Status, impacts, and control. *Biol. Conserv.* **2007**, *134*, 470–483. [CrossRef]
4. Martínez-Rondán, F.J.; Ruiz de Ybáñez, M.R.; Tizzani, P.; López-Beceiro, A.M.; Fidalgo, L.E.; Martínez-Carrasco, C. The American mink (*Neovison vison*) is a competent host for native European parasites. *Vet. Parasitol.* **2017**, *247*, 93–99. [CrossRef] [PubMed]
5. Ribas, M.P.; Almería, S.; Fernández-Aguilar, X.; De Pedro, G.; Lizarraga, P.; Alarcia-Alejos, O.; Molina-López, R.; Obón, E.; Gholipour, H.; Temiño, C.; et al. Tracking *Toxoplasma gondii* in freshwater ecosystems: Interaction with the invasive American mink (*Neovison vison*) in Spain. *Parasitol. Res.* **2018**, *117*, 2275–2281. [CrossRef]
6. Gholipour, H.; Busquets, N.; Fernández-Aguilar, X.; Sánchez, A.; Ribas, M.P.; De Pedro, G.; Lizarraga, P.; Alarcia-Alejos, O.; Temiño, C.; Cabezón, O. Influenza A Virus surveillance in the invasive American Mink (*Neovison vison*) from freshwater ecosystems, Northern Spain. *Zoonoses Public Health* **2017**, *64*, 363–369. [CrossRef]
7. Mahdy, M.A.A.; Younis, W.; Ewaida, Z. An overview of SARS-CoV-2 and animal infection. *Front. Vet. Sci.* **2020**, *7*, 596391. [CrossRef]
8. Molina, R.; Jiménez, M.; García-Martínez, J.; San Martín, J.V.; Carrillo, E.; Sánchez, C.; Moreno, J.; Alves, F.; Alvar, J. Role of asymptomatic and symptomatic humans as reservoirs of visceral leishmaniasis in a Mediterranean context. *PLoS. Negl. Trop. Dis.* **2020**, *14*, e0008253. [CrossRef] [PubMed]
9. Naucke, T.J.; Lorentz, S. First report of venereal and vertical transmission of canine leishmaniosis from naturally infected dogs in Germany. *Parasit. Vectors* **2012**, *5*, 1–5. [CrossRef]
10. WHO. Health Topics: Leishmaniasis. Available online: https://www.who.int/health-topics/leishmaniasis (accessed on 4 April 2021).
11. Millán, J.; Ferroglio, E.; Solano-Gallego, L. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. *Parasitol. Res.* **2014**, *113*, 2005–2014. [CrossRef]
12. Molina, R.; Jiménez, M.I.; Cruz, I.; Iriso, A.; Martín- Martín, I.; Sevillano, O.; Melero, S.; Bernal, J. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. *Vet. Parasitol.* **2012**, *190*, 268–271. [CrossRef] [PubMed]
13. Muñoz-Madrid, R.; Belinchón-Lorenzo, S.; Iniesta, V.; Fernández-Cotrina, J.; Parejo, J.C.; Serrano, F.J.; Monroy, I.; Baz, V.; Gómez-Luque, A.; Gómez-Nieto, L.C. First detection of *Leishmania infantum* kinetoplast DNA in hair of wild mammals: Application of qPCR method to determine potential parasite reservoirs. *Acta Trop.* **2013**, *128*, 706–709. [CrossRef] [PubMed]
14. Risueño, J.; Ortuño, M.; Pérez-cutillas, P.; Goyena, E.; Maia, C.; Cortes, S.; Campino, L.; Bernal, L.J.; Muñoz, C.; Arcenillas, I.; et al. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. *Vet. Parasitol.* **2018**, *259*, 61–67. [CrossRef] [PubMed]
15. Oleaga, A.; Zanet, S.; Espí, A.; Pegoraro de Macedo, M.R.; Gortázar, C.; Ferroglio, E. *Leishmania* in wolves in northern Spain: A spreading zoonosis evidenced by wildlife sanitary surveillance. *Vet. Parasitol.* **2018**, *255*, 26–31. [CrossRef]
16. Azami-Conesa, I.; Martínez-Díaz, R.A.; González, F.; Gómez-Muñoz, M.T. First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe. *Res. Vet. Sci.* **2020**, *132*, 172–176. [CrossRef]
17. Alcover, M.M.; Ribas, A.; Guillén, M.C.; Berenguer, D.; Tomás-Pérez, M.; Riera, C.; Fisa, R. Wild mammals as potential silent reservoirs of *Leishmania infantum* in a Mediterranean area. *Prev. Vet. Med.* **2020**, *175*, 104874. [CrossRef]
18. Navea-Pérez, H.M.; Díaz- Sáez, V.; Corpas-López, V.; Merino-Espinosa, G.; Morillas-Márquez, F.; Martín- Sánchez, J. *Leishmania infantum* in wild rodents: Reservoirs or just irrelevant incidental hosts? *Parasitol. Res.* **2015**, *114*, 2363–2370. [CrossRef]
19. Otranto, D.; Iatta, R.; Baneth, G.; Cavalera, M.A.; Bianco, A.; Parisi, A.; Dantas-Torres, F.; Colella, V.; McMillan-Cole, A.C.; Chomel, B. High prevalence of vector-borne pathogens in domestic and wild carnivores in Iraq. *Acta Trop.* **2019**, *197*, 105058. [CrossRef] [PubMed]
20. Filioussis, G.; Petridou, E.; Papadopoulos, D.; Karavanis, E.; Morgan, E.; Billinis, C.; Papadopoulos, E. Hemorrhagic pneumonia in neonatal minks in Greece concomitant with *Leismania infantum* detection. *Pol. J. Vet. Sci.* **2018**, *21*, 405–408. [CrossRef] [PubMed]
21. Tsakmakidis, I.; Pavlou, C.; Tamvakis, A.; Papadopoulos, T.; Christodoulou, V.; Angelopoulou, K.; Dovas, C.I.; Antoniou, M.; Anastasakis, C.; Diakou, A. *Leishmania* infection in lagomorphs and minks in Greece. *Vet. Parasitol. Reg. Stud. Reports.* **2019**, *16*, 100279. [CrossRef]
22. Regulation (EU) 2016/1141 of 13 July 2016 Adopting a List of Invasive Alien Species of Union Concern Pursuant to Regulation (EU) No 1143/2014 of the European Parliament and of the Council. Available online: http://data.europa.eu/eli/reg/2014/1143/oj (accessed on 25 June 2021).

23. Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the Prevention and Management of the Introduction and Spread of Invasive Alien Species. Available online: http://data.europa.eu/eli/reg_impl/2016/1141/oj (accessed on 25 June 2021).
24. Real Decreto 630/2013, de 2 de Agosto, por el que se Regula el Catálogo Español de Especies Exóticas Invasoras. Available online: https://www.boe.es/eli/es/rd/2013/08/02/630 (accessed on 25 June 2021).
25. Decreto 213/2009, de 20 de Noviembre, del Consell, por el que se Aprueban Medidas Para el Control de Especies Exóticas Invasoras en la Comunitat Valenciana. [2009/13396]. Available online: https://dogv.gva.es/es/eli/es-vc/d/2009/11/20/213/ (accessed on 25 June 2021).
26. Melero, Y.; Santulli, G.; Gómez, A.; Gosàlbez, J.; Rodríguez-Refojos, C.; Palazón, S. Morphological variation of introduced species: The case of American mink (*Neovison vison*) in Spain. *Mamm. Biol.* **2012**, *77*, 345–350. [CrossRef]
27. Leary, S.; Underwood, W.; Anthony, R.; Cartner, S.; Grandin, T.; Greenacre, C.; Gwaltney-Brant, S.; McCrackin, M.A.; Meyer, R.; Miller, D.; et al. *AVMA Guidelines for the Euthanasia of Animals: 2020 Edition*; AVMA: Schaumburg, IL, USA, 2020; pp. 97–99.
28. Lachaud, L.; Marchergui-Hammami, S.; Chabbert, E.; Dereure, J.; Dedet, J.P.; Bastien, P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J. Clin. Microbiol.* **2002**, *40*, 210–215. [CrossRef]
29. Souguir-Omrani, H.; Chemkhi, J.; Fathallah-Mili, A.; Saadi-BenAoun, Y.; BelHadjAli, I.; Guizani, I.; Guerbouj, S. *Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) hedgehogs: Possible reservoirs of endemic leishmaniases in Tunisia. *Infect. Genet. Evol.* **2018**, *63*, 219–230. [CrossRef] [PubMed]
30. El Tai, N.O.; Osman, O.F.; El Fari, M.; Presber, W.; Schönian, G. Genetic heterogeneity of ribosomal internal transcribed spacer (ITS) in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms (sscp) and sequencing. *Trans. R. Soc. Trop. Med. Hyg.* **2000**, *94*, 1–5. [CrossRef]
31. Van Eys, G.J.; Schoone, G.J.; Kroon, N.C.; Ebeling, S.B. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. *Mol. Biochem. Parasitol.* **1992**, *51*, 133–142. [CrossRef] [PubMed]
32. Cruz, I.; Cañavate, C.; Rubio, J.M.; Morales, M.A.; Chicharro, C.; Laguna, F.; Jiménez- Mejías, M.; Sirera, G.; Videla, S.; Alvar, J. A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in coinfected patients with human immunodeficiency virus. *Trans. R. Soc. Trop. Med. Hyg.* **2002**, *96*, 185–189. [CrossRef]
33. Piarroux, R.; Fontes, M.; Perasso, R.; Gambarelli, F.; Joblet, C.; Dumon, H.; Quilici, M. Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. *Mol. Biochem. Parasitol.* **1995**, *73*, 249–252. [CrossRef]
34. European Centre for Disease Prevention and Control and European Food Safety Authority. Mosquito Maps. Stockholm: ECDC. 2018. Available online: https://www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps (accessed on 5 July 2021).
35. Cardoso, L.; Schallig, H.; Persichetti, M.F.; Pennisi, M.G. New Epidemiological Aspects of Animal Leishmaniosis in Europe: The Role of Vertebrate Hosts Other than Dogs. *Pathogens* **2021**, *10*, 307. [CrossRef] [PubMed]
36. Battisti, E.; Zanet, S.; Khalili, S.; Trisciuoglio, A.; Hertel, B.; Ferroglio, E. Molecular Survey on Vector-Borne Pathogens in Alpine Wild Carnivorans. *Front. Vet. Sci.* **2020**, *7*, 1–9. [CrossRef]
37. Del Río, L.; Chitimia, L.; Cubas, A.; Victoriano, I.; De la Rúa, P.; Gerrikagoitia, X.; Barral, M.; Muñoz-García, C.I.; Goyena, E.; García- Martínez, D.; et al. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. *Prev. Vet. Med.* **2014**, *113*, 430–435. [CrossRef]
38. Giner, J.; Villanueva-Saz, S.; Alcover, M.M.; Riera, C.; Fisa, R.; Basurco, A.; Yzuel, A.; Trotta, M.; Fani, C.; Verde, M.T.; et al. Treatment and follow-up of a domestic ferret (*Mustela putorius furo*) with clinical leishmaniosis caused by *Leishmania infantum*. *Vet. Parasitol. Reg. Stud. Rep.* **2020**, *21*, 100423. [CrossRef]
39. Chemkhi, J.; Souguir, H.; BelHadjAli, I.; Driss, M.; Guizani, I.; Guerbouj, S. Natural infection of Algerian hedgehog, *Atelerix algirus* (Lereboullet 1842) with *Leishmania* parasites in Tunisia. *Acta Trop.* **2015**, *150*, 42–51. [CrossRef] [PubMed]
40. Akhoundi, M.; Downing, T.; Votýpka, J.; Kuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; et al. *Leishmania* infections: Molecular targets and diagnosis. *Mol. Aspects Med.* **2017**, *57*, 1–29. [CrossRef] [PubMed]
41. Albuquerque, A.; Campino, L.; Cardoso, L.; Cortes, S. Evaluation of four molecular methods to detect *Leishmania* infection in dogs. *Parasit. Vectors* **2017**, *10*, 57. [CrossRef] [PubMed]
42. De Oliveira, D.M.; Lonardoni, M.V.C.; Theodoro, U.; Silveira, T.G.V. Comparison of different primes for PCR-based diagnosis of cutaneous leishmaniasis. *Braz. J. Infect. Dis.* **2011**, *15*, 204–210. [CrossRef]

4.3. OBJETIVOS 3

Análisis del posible papel como reservorios o especies centinela de varias especies de mamíferos en los que se ha detectado previamente la presencia de *Leishmania*. Estimación de la prevalencia empleando un mayor número de ejemplares de cada especie.

4.3.1. RESUMEN

El carácter zoonótico y la forma de transmisión a través de los flebotomos de *L. infantum* hace que podamos encontrar al parásito en el organismo de un gran número de especies. Los animales más estudiados tradicionalmente han sido los domésticos, en especial el perro, en el que son ampliamente conocidos los efectos que tiene esta parasitosis sobre su organismo. Sin embargo, el papel que pueden desarrollar los animales silvestres como centinelas respecto a la presencia de *Leishmania* spp. en diferentes ambientes no es tan claro, aunque a día de hoy, son cada vez más numerosos los estudios que centran sus esfuerzos en ampliar ese conocimiento.

En Europa, varias especies de carnívoros, como los zorros (*Vulpes vulpes*), los chacales (*Canis aureus*) o los lobos (*Canis lupus*), han sido ampliamente estudiados debido a su similitud con los perros o a que han sido señalados como potenciales reservorios, pero existen numerosas especies de las que aún se desconoce el papel que puedan jugar en el mantenimiento y dispersión de este parásito (Cardoso et al., 2021; Roque & Jansen, 2014).

Además, la elección de las muestras a analizar, así como la metodología a usar, son de extrema importancia, ya que se detecta en la bibliografía una amplia variación de resultados debido, probablemente, a diferencias en la metodología de análisis y toma de muestras. Esto se suma a la baja parasitemia que podrían presentar los animales silvestres, lo que dificulta más todavía la detección del parásito. Por tanto, en este estudio, se pretende hacer un análisis amplio, donde se estudian un total de 123 animales mamíferos, de 9 especies diferentes muy comunes en la península ibérica, como son el erizo europeo (*Erinaceus europaeus*), el erizo moruno (*Atelerix algirus*), la ardilla roja (*Sciurus vulgaris*), el tejón europeo (*Meles meles*), el meloncillo (*Herpestes ichneumon*), la garduña (*Martes foina*), la comadreja común (*Mustela nivalis*), el lirón careto (*Elyomis quercinus*) y el turón europeo (*Mustela putorius*) de donde se obtuvieron muestras de bazo y piel de oreja, y se analizaron tres dianas diferentes de ADN: *Repeat region, SSUrRNA* e *ITS1*, siguiendo protocolos de PCR anidada en los dos últimos casos, con el fin de incrementar la sensibilidad. Las muestras se seleccionaron debido a la capacidad que tiene *L. infantum* de acantonarse en el bazo y a que la oreja es una zona muy accesible para el flebotomo. Asimismo, las dianas elegidas, presentaron elevada sensibilidad a la hora de detectar el parásito en fauna silvestre, tanto en la bibliografía consultada

(Azami-Conesa et al., 2021a; Cardoso et al., 2021) como en los trabajos realizados anteriormente, y que se incluyen en esta Tesis Doctoral. Se excluyó la diana *kDNA* por dos razones, en primer lugar, por amplificar un fragmento muy pequeño que resulta difícil de secuenciar y no puede enviarse para su publicación en GenBank, y en segundo lugar porque en ensayos previos, a pesar de mostrar elevada sensibilidad en muestras de perro y cultivos de promastigotes, no resultó igual de sensible en tejidos de animales silvestres (bazo).

Además, se ha analizado la utilidad de determinadas muestras que no requieran la muerte del individuo, y que sean poco invasivas, como son la conjuntiva ocular (tomando la muestra con un hisopo) y la mucosa oral (hisopo), ya que uno de los principales problemas a la hora de analizar a la fauna silvestre es que la mayoría de los estudios toman las muestras *post morten*, pero pocos emplean muestras *ante mortem*. Estas técnicas fueron usadas con anterioridad por otros autores, donde se obtuvieron resultados esperanzadores (Aschar et al., 2016; Souguir-Omrani et al., 2018). Sin embargo, en el presente estudio no se obtuvieron resultados positivos en ninguno de los ocho erizos europeos analizados. Esto podría deberse al manejo a la hora de tomar las muestras, a las diferencias propias de las especies de animales o a la baja parasitemia que pudieran presentar los animales analizados.

En conjunto, el 14,84% de los animales analizados resultaron positivos a *L. infantum* en al menos una muestra (bazo o piel) y en una o más dianas del ADN. Los animales positivos pertenecían a las especies de erizo europeo, ardilla roja y tejón europeo exclusivamente. En cuanto al tipo de muestra, en esta ocasión el bazo presentó un mayor número de positivos (12/124) que la piel (9/113), lo cual concuerda con resultados obtenidos en otros estudios previos (Azami-Conesa et al., 2020; Gomes et al., 2020; Ramos et al., 2013; Sobrino et al., 2008). En relación a las dianas del ADN analizadas, la *Repeat Region* y la *SSUrRNA* resultaron más sensibles que la *ITS1*, acorde con la bibliografía y estudios previos de nuestro grupo incluidos en la presente Tesis Doctoral (Azami-Conesa et al., 2020, 2021b; Chemkhi et al., 2015; Lachaud et al., 2002a).

Este estudio pone el foco en los animales silvestres, una vez más, como posibles centinelas de la situación epidemiológica de la leishmaniosis, así como en la importancia de hacer estudios amplios con respecto a las muestras y dianas a utilizar.

4.3.2. PONENCIAS

- **Azami- Conesa, I.,** Sansano- Maestre, J., González, F., Gómez- Muñoz, M.T. "Occurrence of *Leishmania infantum* in unusual wild mammals from Spain". 15th International Congress of Parasitology. Copenhague (Dinamarca), del 21 al 26 de agosto de 2022. Comunicación en formato póster.

4.3.3. ARTÍCULO

- **Azami- Conesa, I.,** Pérez-Moreno, P., Matas- Méndez, P., Sansano- Maestre, J., González, F., Mateo- Barrientos, M., Gómez- Muñoz, M. T. (2023). Occurrence of *Leishmania infantum* in Wild Mammals Admitted to Recovery Centers in Spain. *Pathogens*, 22 (1048). DOI: doi.org/10.3390/pathogens12081048.
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CAPÍTULO 4: RESULTADOS

pathogens

Article

Occurrence of *Leishmania infantum* in Wild Mammals Admitted to Recovery Centers in Spain

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Abstract: Zoonotic leishmaniasis caused by *Leishmania infantum* is distributed worldwide and affects humans and domestic and wild mammals. In Europe, specifically in the Mediterranean basin, leishmaniasis is endemic due to the concurrence of the phlebotomine vectors and reservoir mammals, including carnivorous wildlife species and other less studied wild species. In this article, spleen, skin, and eye or oral swabs taken from 134 wild mammals admitted to five wildlife recovery centers in Spain were used. PCR employing fragments of the Repeat region, ITS1, and SSUrRNA were used for detection, and positive samples were processed for sequencing. *L. infantum* was detected in three out of the nine species analyzed, including European hedgehog, European badger, and red squirrel, with percentages ranging from 11.53 to 35.71%, depending on the species. Most of the species showed higher percentages of positivity in spleen samples than in skin samples. A small number of animals from the remaining six species tested negative, including Algerian hedgehog, stone marten, least weasel, garden dormouse, western polecat, and Egyptian mongoose. Hedgehogs and badgers are good candidates for consideration as epidemiological sentinels and pose a higher risk as potential reservoirs of leishmaniasis based on their percentage of infection and wide distribution.

Keywords: *L. infantum*; wild mammals; PCR; European hedgehog; red squirrel; European badger

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1. Introduction

Leishmaniasis is a zoonotic disease caused by a protozoan parasite of the genus *Leishmania*. The Genus includes more than 20 zoonotic species with a worldwide distribution. *Leishmania infantum* is the most frequently reported species in the Mediterranean basin, causing a life threatening disease in humans and animals [1–3]. Cutaneous and visceral leishmaniosis (CL and VL, respectively) can occur [4]. In the first case, the promastigote stage of the parasite infects phagocytic cells from the skin and, eventually, the infected cell bursts, liberating amastigote forms to infect surrounding cells, while systemic disease (VL) occurs when infected cells and amastigotes spread via the blood to infect phagocytic cells in the bone marrow, liver, spleen, and lymph nodes [1]. The main route of transmission is the bite of a phlebotomine sandfly (order Diptera, family Psychodidae), though they are minor

routes of infection, such as venereal or vertical transmission, which is proven in canids, or the oral route, which is described in hamsters and suggested in insectivorous bats [2,5,6]. About ten species of the genus *Phlebotomus* have been described as capable of transmitting *L. infantum* in southern Europe, with the most common being *P. perniciosus* and *P. ariasi*, which have a wide distribution in countries such as Italy, Portugal, France, and Spain [7,8]. Humans and domestic animals, such as dogs, have been the most widely studied reservoirs due to their high prevalence and relevance, but in the last few decades, several domestic and wildlife species have been analyzed to investigate their ability to maintain the biological cycle of the parasite [2,3,9]. The role of each host species in the spread of leishmaniosis in urban, peri-urban, and rural environments needs to be clarified. The first step should be the identification of potential hosts and, eventually, their ability to play a relevant role in outbreaks of this parasitosis, which can be proven through xenodiagnoses experiments. This approach was employed during the large outbreak of leishmaniosis in Madrid, when hares were proven to play a role in the maintenance of the cycle [10].

In Europe, wild carnivores, like foxes (*Vulpes vulpes*), jackals (*Canis aureus*), and wolves (*Canis lupus*), have been extensively studied due to their similarity to dogs, but other species, such as European hedgehog (*Erinaceus europaeus*), European badger (*Meles meles*), common bat (*Pipistrellus pipistrellus*), rodents (*Mus musculus*, *Rattus rattus*, etc.), red squirrel (*Sciurus vulgaris*), European and American minks [11,12] and martens (*Martes martes*), among others, have tested positive for *L. infantum* via both molecular and/or serological methods [2,3], although most of these studies employed few animals of each species. The growing interest in these species' roles as potential pathogen reservoirs is caused by the increasing interaction between wildlife and urban and peri-urban environments due to the fragmentation of their habitats, the extension of peri-urban areas into typically wild territories, and the increase in eco-tourism activities. These situations led to increased interactions between wildlife, humans, domestic hosts, and vectors [9].

Regarding the diagnostic techniques employed for the detection of the parasite, serology is mainly used in humans and dogs, as it requires blood and is a minimally invasive procedure. However, in some of the wildlife species studied, serology was complemented with molecular techniques, with the most employed methods being PCR and qPCR [2]. Numerous targets of DNA have been described for use in the detection of *L. infantum*, with kDNA and SSUrRNA being the most commonly used due to their high sensitivity, but other targets, such as the Repeat region, also displayed high sensitivity, and the ITS1 target is preferred due to its higher specificity [10,11,13]. Blood, liver, and spleen are common samples used in DNA extraction and PCR, while in recent years, samples obtained via less invasive methods, such as hair and oral or ocular swabs, have been used [2].

Considering that Spain is a country with high levels of prevalence of *L. infantum* in Europe and the Mediterranean area [14], the aim of this work was to analyze the occurrence of the parasite in wild mammals that are potential reservoirs of leishmaniosis in wild, urban, and peri-urban areas. For this purpose, different samples from the animals and several DNA targets were employed. The usefulness of less invasive techniques in testing wild species is evaluated in this work.

2. Materials and Methods

2.1. Origin of Animals and Samples

Samples were obtained, at convenience, from wildlife recovery centers in Spain. In total, 123 animals from 3 different mammal species were studied, including 83 European hedgehogs (*Erinaceus europaeus*), 26 red squirrels (*Sciurus vulgaris*), and 14 European badgers (*Meles meles*). The minimum number of animals included in each sample to detect the disease in these three species was calculated by employing previously described percentages of infection for each species: $n = 11$ for European badger (26% of infections) [15], $n = 11$ for red squirrel (25% of infections) [13], and $n = 8$ for European hedgehog (34.4% of infections) [13]. The online resource Working in Epidemiology (WinEpi) was employed to complete the calculation [16].

In addition, a small number (n = 11) of other six species were included in the study: Egyptian mongoose (*Herpestes ichneumon*), Algerian hedgehog (*Atelerix algirus),* stone marten (*Martes foina*), least weasel (*Mustela nivalis*), garden dormouse (*Elyomis quercinus*), and western polecat (*Mustela putorius*).

The animals were admitted between November 2015 and February 2023 to five wildlife recovery centers: GREFA (Group of Rehabilitation of the Autochthonous Fauna and their Habitat; Madrid, Spain), "La Granja de El Saler" (Valencia, Spain), Mini-zoo (Guadalajara, Spain), "El Chaparrillo" (Ciudad Real, Spain), and CERI "Iberian birds of prey Studies Center" (Toledo, Spain). The areas of origin of the animals were the regions of Madrid, Castilla–La Mancha, Castilla y León, and the Valencian Community, Spain (Table S1). All animals were admitted to the recovery centers due to illness or trauma, and most of them (128/134) were roadkill or humanely euthanized following veterinary criteria and the AVMA guidelines for the euthanasia of animals [17]. Samples from skin, spleen, and/or liver were obtained from the dead animals and kept frozen until analysis. Six of the European hedgehogs recovered from their injuries and were reintroduced into the wild, but before that stage and during veterinary examination, oral and ocular swab samples were taken and kept frozen. In addition, oral and ocular swab samples were taken at the time of admission from two of the dead hedgehogs (Table 1).

Table 1. Wild animal species analyzed for the presence of *L. infantum*. Numbers of animals and types of samples are included.

Scientific Name	Number of Animals (n)	Type and Number of Samples			
		Ear Skin	Spleen	Ocular Swab	Oral Swab
Erinaceus europaeus	83	64	75	8	8
Sciurus vulgaris	26	25	24 *	-	-
Meles meles	14	14	14 *	-	-
Atelerix algirus	3	3	3	-	-
Martes foina	2	2	2	-	-
Mustela nivalis	2	2	2	-	-
Eliomys quercinus	1	1	1	-	-
Mustela putorius	1	-	1	-	-
Herpestes ichneumon	2	2	2	-	-
TOTAL	134	113	124	8	8

* One animal analyzed using sample of liver tissue, instead of spleen, due to extensive damage caused to the spleen.

2.2. DNA Extraction

DNA was isolated from the samples using 10 (spleen) or 20 mg (ear skin or liver) of tissue sample based on the kit manufacturer's recommendations. The Thermo Scientific GeneJet Genomic DNA Purification kit (Thermo Fisher Scientific, MA, USA) was employed, and the manufacturer's protocol was followed, except for DNA elution, which was carried out in 60 µL of elution buffer, instead of 100 µL. Sample derived from *L. infantum* promastigotes in culture and DNase free water were employed as positive and negative controls, respectively, in each batch.

2.3. PCR of L. infantum and Sequencing

DNA isolated from each sample was used in the detection of *L. infantum* using three different targets of DNA: Repeat region, SSUrRNA, and ITS1 [18–20]. The latter was only performed in samples positive at the other two targets, as its sensitivity was lower [11]. In

the case of SSUrRNA and ITS1, a nested PCR (nPCR) was used. In all instances, the reaction was carried out in 25 µL, using 12.5 µL of the Supreme NZYTaq II (NZY Tech, Lisbon, Portugal) and 1 µL of each primer at 10 µM. For the Repeat Region and ITS1, 2.5 µL of DNA was used, and for the SSUrRNA, 5 µL of DNA was employed. The second reactions of both nPCRs were carried out using 5 µL of 1/40 dilution of the first PCR product or 1/20 dilution in case of faint positive reactions. For the Repeat region, primers T2 and B4 [20] were used; for SSUrRNA, primers R221 and R332 were employed for the outer section, and primers R223 and R333 were employed for the inner section [18]. Finally, for ITS1, outer primers LITSR and L5.8S and inner primers SAC and VAN2 were selected [19]. Moreover, positive (*L. infantum* promastigotes DNA) and negative (DNase free water) controls were employed in each reaction. For all PCR reactions, an initial step at 95 °C for 5 min was used to activate the enzyme, and a last step at 72 °C for 10 min was added for the elongation step. The intermediate steps, as well as other features, are described elsewhere [11]. Electrophoresis was carried out on 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and visualized under UV light. Positive samples displayed a band on the gel related to the expected size of each target, including positive controls.

2.4. DNA Sequencing and Alignments

All positive samples were cleaned and sequenced by a commercial company (Macrogen Spain, Madrid) using DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) and ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), following the manufacturer's protocol. The obtained sequences were manually checked and aligned with Molecular Evolutionary Genetics Analysis (MEGA X) software and FinchTV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA). The consensus sequences of each positive sample were then compared with other available sequences using the BLAST tool (National Library of Medicine, Rockville, MD, USA) [21]. All obtained sequences of more than 200 base pairs were submitted to GenBank for accession number identification.

2.5. Phylogenetic Trees

Phylogenetic trees were constructed using sequences available from GenBank, together with sequences obtained in this study that were of sufficient quality and length. Only trees for SSUrRNA and ITS1 regions are shown, since there were enough sequences from other *Leishmania* species obtained from different authors available at GenBank. The Repeat region was only employed for detection and estimation of the percentage of homology.

Sequences of the ITS1 region had a minimum length of 290 nucleotides, and a sequence of *Trypanosoma cruzi* was included as an out-group reference. Sequences of the SSUrRNA region with a minimum length of 320 nucleotides were included, and a sequence of *Trypanosoma cruzi* was included as an out-group reference. In addition, sequences of *L. infantum* and other species of *Leishmania* derived from different sources were included to verify similarity. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura–Nei model [22]. The tree with the highest log likelihood was shown. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a pairwise distance matrix estimated using the Maximum Composite Likelihood (MCL) approach and selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured based on the number of substitutions per site. The analysis involved 26 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted via MEGAX [23]. Bootstrap of 2000 replicates was employed to estimate the reliability of the trees.

2.6. Maps

The maps were created by taking as reference the map of prevalence of *Leishmania* spp. reported in wild animals [14] and the distribution of each animal species in Spain based on the atlas and red book of terrestrial mammals of Spain [24]. The AutoCAD program (Autodesk® Inc., the Landmark, San Francisco, CA, USA) was employed to observe the overlaps between the two maps.

2.7. Statistical Analysis

A descriptive statistical analysis with absolute (n) and relative frequency of infection (%) was carried out, and a confidence level of 95% was estimated by employing the free online tool WinEpi (Working in Epidemiology) [16].

3. Results

3.1. Ocurrence of Leishmania spp. in Different Animal Species

In total, 19 of the 128 necropsied animals (14.84%, CI 8.68–21%) tested positive for *L. infantum* in at least one sample (spleen or skin) and one PCR target (Table 2 and Table S2). Positives were found in spleen or ear skin, while oral and ocular swabs (n = 8) were all negative. Only two animals tested positive in both spleen and skin: a breeding European hedgehog and an adult European badger (Table S2). The highest percentage of infection was found in European badgers (35.71%, CI 10.63–60.8%), followed by European hedgehogs (14.29%, CI 6.5–22.07%) and red squirrels (11.53%, CI 0.0–23.8%).

Table 2. Animal species and samples evaluated for *L. infantum* by employing different PCR targets. Number of positive samples and percentage of positivity is shown.

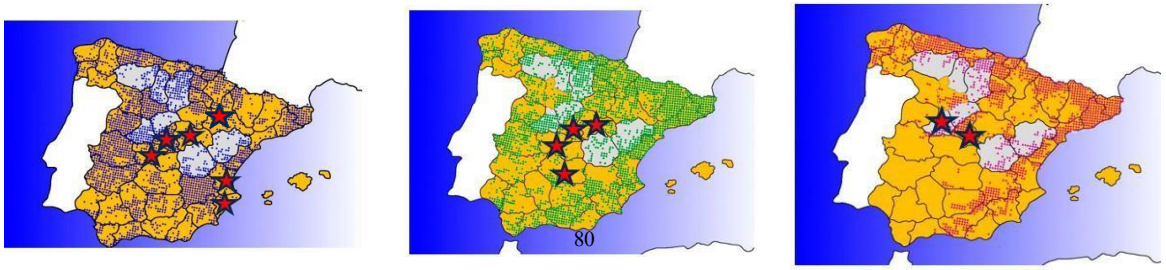
Species	Spleen (+/*n*)			Ear Skin (+/n)			TOTAL (% Value, 95% CI)
	Repeat Region	SSUrRNA	ITS1	Repeat Region	SSUrRNA	ITS1	
Erinaceus europaeus	2/75	3/75	1/5	6/64	1/64	1/7	11/77 (14.29%, CI 6.5–22.07%)
Sciurus vulgaris	3/24	1/24	0/3	0/25	0/25	N.A.	3/26 (11.53%, CI 0–23.8%)
Meles meles	0/14	4/14	3/4	2/14	0/14	0/2	5/14 (35.71%, CI 10.63–60.8%)
Atelerix algirus	0/3	0/3	N.A.	0/3	0/3	N.A.	0/3 (0%, CI 0–63.16%)
Mustela nivalis	0/2	0/2	N.A.	0/2	0/2	N.A.	0/2 (0%, CI 0–77.64%)
Martes foina	0/2	0/2	N.A.	0/2	0/2	N.A.	0/2 (0%, CI 0–77.64%)
Eliomys quercinus	0/1	0/1	N.A.	0/1	0/1	N.A.	0/1 (0%, CI 0.0–95%)
Mustela putorius	0/1	0/1	N.A.	N.A.	N.A.	N.A.	0/1 (0%, CI 0.0–95%)
Herpestes ichneumon	0/2	0/2	N.A.	0/2	0/2	N.A.	0/2 (0%, CI 0.0–77.64%)
TOTAL	5/124	8/124	4/12	8/113	1/113	1/9	19/128 (14.84%, CI 8.68–21%)

n: number of animals. N.A.: not analyzed. Eight oral and ocular swabs from European hedgehog were negative for *L. infantum* PCRs and are not included in the table.

3.2. Geographic Origin of the Samples and Risk of Infection

The area of leishmaniosis reported in humans and animals includes most of the Iberian Peninsula, which is in coincidence with the distribution of some of the species that tested positive in this study, namely European badger and European hedgehog, while red squirrel displayed a smaller area of distribution (Figure 1). The provinces in which animals were

found belong to Madrid, Comunidad Valenciana (Valencia and Alicante), Castilla–La Mancha (Guadalajara, Toledo, Ciudad Real), and Castilla y León (Ávila), and their locations are marked with a star in the figure. Most of the animals were found in peri-urban areas, since many of them were roadkill, although some red squirrels and hedgehogs were also recovered from streets and urban gardens (Table S1).



(**a**) (**b**) (**c**)

Figure 1. Map showing the distribution of species that tested positive for *L. infantum* in Spain (dotted areas). Areas colored in orange are recognized as having endemic leishmaniasis, according to ECDC, 2022. (**a**) Distribution areas of European hedgehog; (**b**) distribution areas of European badger; (**c**) distribution areas of red squirrel. Red stars indicate the origin of the samples used in the present study.

3.3. Sensitivity of PCRs

A total of 253 samples were first analyzed at the Repeat region and the SSUrRNA fragment, and only positive samples obtained via these previous PCR protocols were tested at the ITS1 region (n = 20) (Table S2).

Considering all samples, the Repeat Region and the SSUrRNA PCRs displayed similar sensitivity, since 13 and 9 samples were positive, respectively. In total, 4.03% (5/124) and 7.08% (8/113) of positive samples were obtained in spleen and skin, respectively, when employing the Repeat region compared to 6.45% (8/124) and 0.89% (1/113) of positive samples rendered via the SSUrRNA PCR. The European badger displayed a higher number of positive samples when employing the SSUrRNA PCR of the spleen, while higher number of positives were obtained via the Repeat region PCR using the skin in European hedgehogs. The ITS1 tested positive in 4/12 and 1/9 samples in spleen and skin, respectively.

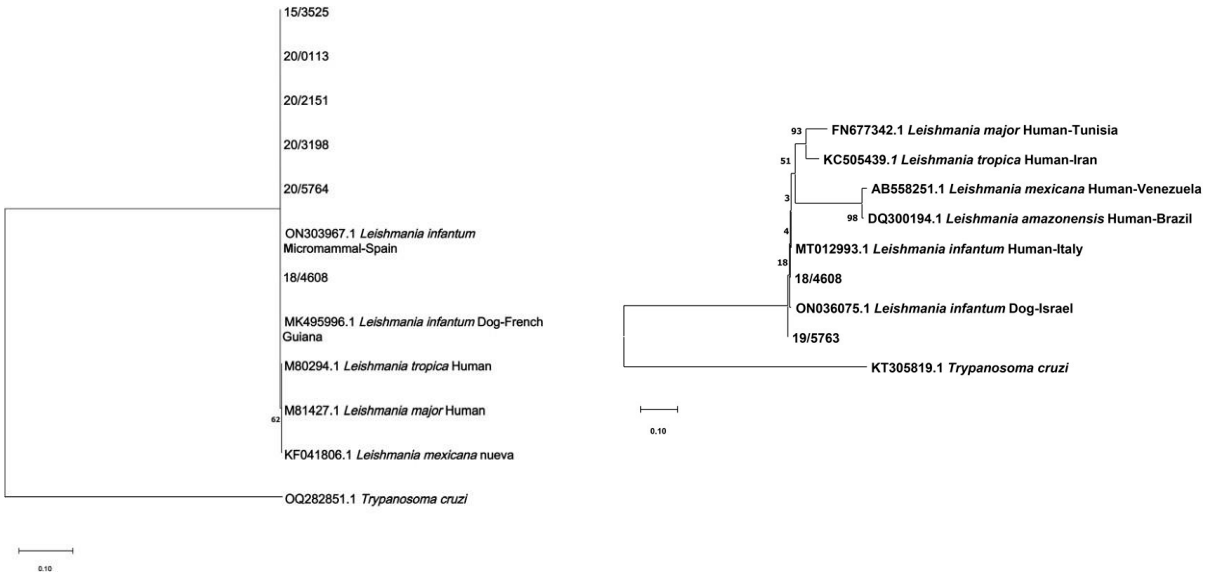
3.4. Sequences

All samples positive to PCRs were sequenced and submitted to GenBank, although faint reactions did not render a legible sequence. The accession numbers are as follows: OP594494–OP594504 for sequences of the Repeat region; OP588913–OP588918 for sequences of the SSUrRNA; OP588937 and OP588938 for samples positive at ITS1 PCR.

A total of 6 sequences of the SSU target, 11 of the Repeat region, and 2 of the ITS1 target were successfully aligned in both directions and subjected to BLAST analysis. The rest of PCR positive samples rendered short sequences, since the signal was low. All of the samples corresponded to *L. infantum*, with percentages of homology being 99–100%.

3.5. Phylogenetic Trees

Phylogenetic trees were constructed by employing sequences from the two selected targets after cutting the sequences of the primers employed via the PCRs (Figure 2). The Repeat region was excluded since it had very short sequences. All sequences obtained for SSUrRNA had 320 bp and were identical, since it is a conserved region. These sequences were also identical to reference sequences from GenBank, a sequence from a dog derived in French Guiana (acc. number MK495996), and a sequence from a micromammal derived in Spain (acc. number ON303967).



(**a**) (**b**)

Figure 2. Phylogenetic trees of SSUrRNA (**a**) and ITS1 (**b**) fragments. The trees were constructed by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura–Nei model and selecting the superior log likelihood value. The trees are drawn to scale, with branch lengths measured based on the number of substitutions per site. A bootstrap of 2000 replicates was employed.

The two sequences of the ITS region were employed to construct the phylogenetic tree with 290 bp. Although ITS1 sequences were grouped very close together in the phylogenetic tree, a slight difference was found between both sequences in this study. One sequence obtained from the spleen of a European hedgehog (18/4608) was closer to a *L. infantum* sequence derived from a human in Italy, while the other sequence obtained from the skin of a European hedgehog (19/5763) was aligned in a slightly more distant position.

4. Discussion

In this study, a high number of samples derived from wild animals were analyzed to determine the presence of *L. infantum*. Most of them had already been studied previously for the presence of *L. infantum*, but either a different target (kDNA) was employed in some of the studies [25] or a small number of samples or animals were included. In the present study, the most relevant studied species are the red squirrel, the European badger, and the European hedgehog, given the number of animals from each species that tested positive and their wide distribution in Spain and other Mediterranean countries.

The results obtained for the European badger, with 35.71% of animals testing positive, are in line with other studies conducted in endemic or peri-endemic areas of Spain and Italy, where results ranged from 26 to 53.3%. In both cases, spleen samples were analyzed (and, in some cases, liver), with different DNA targets, ITS2, and kDNA [15,25]. This diversity of targets may influence the results, as not all targets are equally sensitive. In our case, positive results were obtained via SSUrRNA nPCR, as a target had high sensitivity when a nested PCR was employed, as was demonstrated in other articles with other wildlife species [11,18,19,26]. In the present study, a substantial percentage of the animals were found to be infected, all of which were roadkill animals from peri-urban environments. Badgers are long-living animals, and for that reason, their role as potential reservoirs of the disease is relevant [24].

In the case of hedgehogs, most studies focused on species of the genera *Atelerix* and *Paraechinus* [27–29], with only two studies working with samples from *Erinaceus europaeus* [13,30]. In our case, the latter species is the one that tested positive for *L. infantum*,

with 14.29% of samples being positive at the spleen or ear skin. These results are slightly lower than those obtained in the studies carried out by Muñoz-Madrid et al. [30] (100% positive results in hedgehog hair, albeit using only one specimen) or Alcover et al. [13] (34.4% positive spleen and skin samples). In both cases, the target used for *L. infantum* detection was kDNA, which could have influenced this difference in results. kDNA is one of the most widely used targets in the detection of *L. infantum*, albeit with highly variable results, ranging from studies with no positive samples to studies with remarkably high percentages of positivity [11,13,31]. The animals analyzed in the present study came from peri-urban areas, and it is common to see them near human settlements. They inhabit nests, and these habitats could be protected places in which phlebotomine sandflies live, as is often the case in other small mammals' nests [24].

Red squirrels have scarcely been investigated for the presence of *L. infantum*, except for one study conducted in Catalonia, in which the authors found 25% of the animals to be infected [13]. In our study, 11.53% of red squirrels were positive, i.e., a slightly lower result, but this result could also be attributed to the origin of the animals. The analyzed specimens in this study were from urban or peri-urban areas, and they inhabited gardens and parks with trees, which can be considered as potential risk areas in case leishmaniosis outbreaks occur, as they are suitable habitats for the reproduction and maintenance of sandflies [24].

In the present study, several targets were used for the detection of *L. infantum* infection: the Repeat region and a fragment of the SSUrRNA. SSUrRNA displayed high sensitivity, according to previous studies, but better results were observed in hedgehogs and red squirrels employing the Repeat region, and this latter finding agrees with previous studies that employed this last target and obtained high percentages of positivity in common urban bats and American minks [11,32]. Other authors who employed the Repeat region found higher numbers of positive results compared to other DNA targets, such as kDNA, Mini-exon or SSUrRNA [29,33]. Authors such as Lauchaud et al. [33] have described a high degree of inhibition when using the Repeat region as a target for the detection of *L. infantum* in humans, albeit with variable results in other animal species. It appears that the Repeat region works differently for distinct animal species, a fact also observed in the present study.

Regarding the results obtained via ITS1, we found a small number of positive results, given that we only used this target in those samples that were positive for other DNA sites. There are numerous scientific articles that discuss the detection of *L. infantum* with ITS1, and the authors always highlight the high specificity of this region, but it has a lower level of sensitivity [19,34,35]. Indeed, this was the case for a previous report of *Leishmania* in hedgehogs [29]. Therefore, it is particularly useful when sequencing and determining the species or genetic variants of *Leishmania* present in the infected animals, but it has the disadvantage of lower sensitivity. In our study, we only obtained five positive samples for this target, despite performing a nested PCR to increase the sensitivity.

The results obtained in this study showed a higher percentage of positives using spleen samples (n = 12/124) compared to other tissue samples, such as skin (n = 9/113) (9.52% vs. 7.96%) (Table S2). This result agrees with other studies, in which this organ was the preferred site (the most sensitive target) for the detection of *Leishmania* [32,34,36,37]. However, it is necessary to handle this type of sample with care, as it can have a high number of inhibitory enzymes and proteins, according to DNA extraction protocols, and this situation can lead to false negative results. In this study, DNA extractions of these samples were performed using a lower amount of tissue (10 mg) than other samples, as indicated in the manufacturer's instructions. In any case, the spleen, as well as other animal tissues, such as the lymph nodes or bone marrow, accumulate high numbers of parasites, which have a high tropism toward these areas, where they easily multiply [38].

Another tissue sample frequently used for the detection of *L. infantum* in this study and other studies is the skin, but the results are highly variable. This issue could be due to the difficulty of detecting these parasites in the absence of lesions. In the case of the animals studied here, none presented lesions compatible with *L. infantum*, which might be

one reason that *L. infantum* is detected in skin samples less frequently than in the spleen. However, this observation was not true in hedgehogs, which displayed a higher percentage of positivity in skin (10.93% vs. 6.67%). Another explanation is that the distribution of the parasite depends on the analyzed species. Our findings agree with other studies, in which hedgehogs show similar or even higher percentages of positivity in skin than in spleen samples [28,39].

It would be interesting to analyze a larger number of less invasive samples, such as eye and oral swabs, which, in this study, have not yielded positive results, but encouraging results can be found in the literature, mainly in dogs and hedgehogs [27,38]. The drawback would be that samples are difficult to obtain, as experienced handling is needed to obtain a good sample capable of yielding conclusive results. Another reason that explained the negative results of these samples is that the animals may not have a large number of parasites, and only one of the two animals analyzed using skin and spleen tested positive in spleen. Again, no lesions were observed in the oral or ocular mucosae, and many of the animals (6/8) were released in the wild.

Considering the overlapping distribution of wild species positive for *L. infantum* infection with the distribution of the parasite in the Iberian Peninsula, we considered that the species with the highest risk of being potential reservoirs are badgers and hedgehogs. Also, we would like to highlight that the information provided in this article is relevant to developing conservation strategies and management of wildlife populations. Finally, the research of zoonotic diseases in wildlife species make them sentinels that can be used to explore the epidemiological situation of endemic or emerging diseases.

5. Conclusions

The present study analyzed the occurrence of *L. infantum* in nine wild species of mammals, three of which tested positive for the parasite. The European badger was the species with the highest percentage of infection (35.71%). Lower percentages were found in red squirrel (11.53%) and European hedgehog (14.29%). Considering the distribution of the wild species analyzed and the parasite in the Iberian Peninsula, European hedgehogs and European badger could pose a higher risk of being potential reservoirs of leishmaniasis. Wild animals could act as sentinels to evaluate the epidemic status and predict emerging situations.

Supplementary Materials: The following supporting information can be downloaded via the following link: https://www.mdpi.com/article/10.3390/pathogens12081048/s1, Table S1: Data of the animals employed in this study; Table S2: PCR results by animal sample and target.

Author Contributions: Conceptualization, M.T.G.-M. and M.M.B.; methodology, I.A.-C., P.P.-M., J.S.-M., F.G. and P.M.M.; software, I.A.-C.; validation, I.A.-C., P.P.-M., J.S.-M. and F.G.; formal analysis, I.A.-C. and M.T.G.-M.; investigation, I.A.-C., P.P.-M., J.S.-M., F.G., M.T.G.-M., M.M.B. and P.M.M.; resources, M.T.G.-M.; data curation, M.T.G.-M. and I.A.-C.; writing—original draft preparation, I.A.-C.; writing—review and editing, M.T.G.-M.; visualization, M.T.G.-M.; supervision, M.T.G.-M.; project administration, M.T.G.-M.; funding acquisition, M.T.G.-M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study because the actions carried out were not experimental procedures; instead, they were part of the daily activities of a wildlife recovery center, such as performing necropsies on dead animals and taking samples for diagnosis, which occurred in accordance with the following local legislation and annual permits: DGPFEN/SEN/avp_21_103_bis, DECRETO 259/2004, and Ley 2/1991 Comunidad Autónoma de Madrid (annual permits).

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequences obtained in this study were deposited at GenBank with the following accession numbers: OP594494–OP594504 for sequences of the Repeat region; OP588913–OP588918 for sequences of the SSUrRNA; OP588937 and OP588938 for samples positive at ITS1 PCR.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Maxfield, L.; Corley, J.E.; Crane, J.S. *Leishmaniasis*; StatPearls Pulblishing: Treasure Island, FL, USA, 2022.
2. Azami-Conesa, I.; Gómez-Muñoz, M.T.; Martínez-Díaz, R.A. A Systematic Review (1990–2021) of Wild Animals Infected with Zoonotic *Leishmania*. *Microorganisms* **2021**, *9*, 1101. [CrossRef]
3. Cardoso, L.; Schallig, H.; Persichetti, M.F.; Pennisi, M.G. New Epidemiological Aspects of Animal Leishmaniosis in Europe: The Role of Vertebrate Hosts Other than Dogs. *Pathogens* **2021**, *10*, 307. [CrossRef] [PubMed]
4. Steverding, D. The History of Leishmaniasis. *Parasites Vectors* **2017**, *10*, 82. [CrossRef] [PubMed]
5. Coutinho, M.T.Z.; Linardi, P.M. Can Fleas from Dogs Infected with Canine Visceral Leishmaniasis Transfer the Infection to Other Mammals? *Vet. Parasitol.* **2007**, *147*, 320–325. [CrossRef] [PubMed]
6. de Oliveira, V.V.G.; Alves, L.C.; da Silva, V.A. Vias de Transmissão Da Leishmaniose Visceral Em Mamíferos. *Cienc. Rural* **2015**, *45*, 1622–1628. [CrossRef]
7. Alten, B.; Maia, C.; Afonso, M.O.; Campino, L.; Jiménez, M.; González, E.; Molina, R.; Bañuls, A.L.; Prudhomme, J.; Vergnes, B.; et al. Seasonal Dynamics of Phlebotomine Sand Fly Species Proven Vectors of Mediterranean Leishmaniasis Caused by *Leishmania infantum*. *PLoS Neglected Trop. Dis.* **2016**, *10*, e0004458. [CrossRef]
8. Sáez, V.D.; Morillas-Márquez, F.; Merino-Espinosa, G.; Corpas-López, V.; Morales-Yuste, M.; Pesson, B.; Barón-López, S.; Lucientes-Curdi, J.; Martín-Sánchez, J. *Phlebotomus langeroni* Nitzulescu (Diptera, Psychodidae) a New Vector for *Leishmania infantum* in Europe. *Parasitol. Res.* **2018**, *117*, 1105–1113. [CrossRef]
9. Roque, A.L.R.; Jansen, A.M. Wild and Synanthropic Reservoirs of *Leishmania* Species in the Americas. *Int. J. Parasitol. Parasites Wildl.* **2014**, *3*, 251–262. [CrossRef]
10. Molina, R.; Jiménez, M.I.; Cruz, I.; Iriso, A.; Martín-Martín, I.; Sevillano, O.; Melero, S.; Bernal, J. The Hare (*Lepus granatensis*) as Potential Sylvatic Reservoir of *Leishmania infantum* in Spain. *Vet. Parasitol.* **2012**, *190*, 268–271. [CrossRef]
11. Azami-Conesa, I.; Sansano-Maestre, J.; Martínez-Díaz, R.A.; Gómez-Muñoz, M.T. Invasive Species as Hosts of Zoonotic Infections: The Case of American Mink (*Neovison vison*) and *Leishmania infantum*. *Microorganisms* **2021**, *9*, 1531. [CrossRef]
12. Giner, J.; Villanueva-Saz, S.; Fernández, A.; Gómez, M.A.; Podra, M.; Lizarraga, P.; Lacasta, D.; Ruiz, H.; Aranda, M.D.C.; Jimenez, M.; et al. Detection of Anti–*Leishmania infantum* Antibodies in Wild European and American Mink (*Mustela lutreola* and *Neovison vison*) from Northern Spain, 2014–2020. *J. Wildl. Dis.* **2022**, *58*, 198–204. [CrossRef] [PubMed]
13. Alcover, M.M.; Ribas, A.; Guillén, M.C.; Berenguer, D.; Tomás-Pérez, M.; Riera, C.; Fisa, R. Wild Mammals as Potential Silent Reservoirs of *Leishmania infantum* in a Mediterranean Area. *Prev. Vet. Med.* **2020**, *175*, 104874. [CrossRef]
14. Berriatua, E.; Jumakanova, Z.; Muñoz, C.; Ortuño, M.; Pérez-Cutillas, P. *Surveillance, Prevention and Control of Leishmaniases in the European Union and Its Neighbouring Countries*; ECDC: Stockholm, Sweden, 2022. [CrossRef]
15. Del Río, L.; Chitimia, L.; Cubas, A.; Victoriano, I.; De la Rúa, P.; Gerrikagoitia, X.; Barral, M.; Muñoz-García, C.I.; Goyena, E.; García-Martínez, D.; et al. Evidence for Widespread *Leishmania infantum* Infection among Wild Carnivores in Periendemic Northern Spain. *Prev. Vet. Med.* **2014**, *113*, 430–435. [CrossRef] [PubMed]
16. de Blas, I. *Win Epi: Working in Epidemiology*; Facultad de Veterinaria, Universidad de Zaragoza: Zaragoza, Spain, 2006; Available online: http://www.winepi.net/ (accessed on 30 July 2023).
17. Leary, S.; Underwood, W.; Anthony, R.; Cartner, S.; Grandin, T.; Greenacre, C.; Gwaltney-Brant, S.; McCrackin, M.A.; Meyer, R.; Miller, D.; et al. *AVMA Guidelines for the Euthanasia of Animals: 2020 Edition*; AVMA: Schaumburg, IL, USA, 2020; pp. 97–99.
18. Cruz, I.; Cañavate, C.; Rubio, J.M.; Morales, M.A.; Chicharro, C.; Laguna, F.; Jiménez-Mejías, M.; Sierra, G.; Videla, S.; Alvar, J. A Nested Polymerase Chain Reaction (Ln-PCR) for Diagnosing and Monitoring *Leishmania infantum* Infection in Patients Co-Infected with Human Immuno-Deficiency Virus. *Trans. R. Soc. Trop. Med. Hyg.* **2002**, *96*, S185–S189. [CrossRef] [PubMed]
19. Echchakery, M.; Chicharro, C.; Boussaa, S.; Nieto, J.; Carrillo, E.; Sheila, O.; Moreno, J.; Boumezzough, A. Molecular Detection of *Leishmania infantum* and *Leishmania tropica* in Rodent Species from Endemic Cutaneous Leishmaniasis Areas in Morocco. *Parasites Vectors* **2017**, *10*, 454. [CrossRef] [PubMed]
20. Piarroux, R.; Fontes, M.; Perasso', R.; Gambarelli, F.; Joblet, C.; Dumon, H.; Quilici, M. Phylogenetic Relationships between Old World *Leishmania* Strains Revealed by Analysis of a Repetitive DNA Sequence. *Mol. Biochem. Parasitol.* **1995**, *73*, 249–252. [CrossRef] [PubMed]
21. Basic Local Alignment Search Tool. Available online: https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 20 May 2022).
22. Tamura, K.; Nei, M. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526.

23. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
24. Palomo, L.J. The IUCN Red List of Threatened Species. 2022. Available online: https://www.iucnredlist.org/ (accessed on 16 July 2023).
25. Battisti, E.; Zanet, S.; Khalili, S.; Trisciuoglio, A.; Hertel, B.; Ferroglio, E. Molecular Survey on Vector-Borne Pathogens in Alpine Wild Carnivorans. *Front. Vet. Sci.* **2020**, *7*, 1. [CrossRef]
26. Ortega, M.V.; Moreno, I.; Domínguez, M.; de la Cruz, M.L.; Martín, A.B.; Rodríguez-Bertos, A.; López, R.; Navarro, A.; González, S.; Mazariegos, M.; et al. Application of a Specific Quantitative Real-Time PCR (qPCR) to Identify *Leishmania infantum* DNA in Spleen, Skin and Hair Samples of Wild Leporidae. *Vet. Parasitol.* **2017**, *243*, 92–99. [CrossRef]
27. Souguir-Omrani, H.; Chemkhi, J.; Fathallah-Mili, A.; Saadi-BenAoun, Y.; BelHadjAli, I.; Guizani, I.; Guerbouj, S. *Paraechinus Aethiopicus* (Ehrenberg 1832) and *Atelerix Algirus* (Lereboullet 1842) Hedgehogs: Possible Reservoirs of Endemic Leishmaniases in Tunisia. *Infect. Genet. Evol.* **2018**, *63*, 219–230. [CrossRef] [PubMed]
28. Tomás-Pérez, M.; Khaldi, M.; Riera, C.; Mozo-León, D.; Ribas, A.; Hide, M.; Barech, G.; Benyettou, M.; Seghiri, K.; Doudou, S.; et al. First Report of Natural Infection in Hedgehogs with *Leishmania major*, a Possible Reservoir of Zoonotic Cutaneous Leishmaniasis in Algeria. *Acta Trop.* **2014**, *135*, 44–49. [CrossRef] [PubMed]
29. Chemkhi, J.; Souguir, H.; Bel, I.; Ali, H.; Driss, M.; Guizani, I.; Guerbouj, S. Natural Infection of Algerian Hedgehog, *Atelerix algirus* (Lereboullet 1842) with *Leishmania* Parasites in Tunisia. *Acta Trop.* **2015**, *150*, 42–51. [CrossRef] [PubMed]
30. Muñoz-Madrid, R.; Belinchón-Lorenzo, S.; Iniesta, V.; Fernández-Cotrina, J.; Parejo, J.C.; Serrano, F.J.; Monroy, I.; Baz, V.; Gómez-Luque, A.; Gómez-Nieto, L.C. First Detection of *Leishmania infantum* Kinetoplast DNA in Hair of Wild Mammals: Application of qPCR Method to Determine Potential Parasite Reservoirs. *Acta Trop.* **2013**, *128*, 706–709. [CrossRef]
31. Ruiz-Fons, F.; Ferroglio, E.; Gortázar, C. *Leishmania infantum* in free-ranging hares, Spain, 2004–2010. *Eurosurveillance* **2013**, *18*, 20541. [CrossRef] [PubMed]
32. Azami-Conesa, I.; Martínez-Díaz, R.A.; González, F.; Gómez-Muñoz, M.T. First Detection of *Leishmania infantum* in Common Urban Bats *Pipistrellus pipistrellus* in Europe. *Res. Vet. Sci.* **2020**, *132*, 172–176. [CrossRef] [PubMed]
33. Lachaud, L.; Marchergui-Hammami, S.; Chabbert, E.; Dereure, J.; Dedet, J.P.; Bastien, P. Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis. *J. Clin. Microbiol.* **2002**, *40*, 210–215. [CrossRef]
34. Gomes, J.; Rocha, H.; Carvalho, C.; Bandeira, V.; Fonseca, C.; Rosalino, L.M.; Cunha, M.V. Molecular Detection and Characterization of *Leishmania infantum* in Free-Ranging Egyptian Mongoose (*Herpestes ichneumon*). *Int. J. Parasitol. Parasites Wildl.* **2020**, *11*, 158–162. [CrossRef]
35. Kassahun, A.; Sadlova, J.; Benda, P.; Kostalova, T.; Warburg, A.; Hailu, A.; Baneth, G.; Volf, P.; Votypka, J. Natural Infection of Bats with *Leishmania* in Ethiopia. *Acta Trop.* **2015**, *150*, 166–170. [CrossRef]
36. Sobrino, R.; Ferroglio, E.; Oleaga, A.; Romano, A.; Millan, J.; Revilla, M.; Arnal, M.C.; Trisciuoglio, A.; Gortázar, C. Characterization of Widespread Canine Leishmaniasis among Wild Carnivores from Spain. *Vet. Parasitol.* **2008**, *155*, 198–203. [CrossRef]
37. Ramos, R.A.; Ramos, C.A.; Santos, E.M.; Araújo, F.R.; Carvalho, G.A.; Faustino, M.A.; Alves, L.C. Quantification of *Leishmania infantum* DNA in the Bone Marrow, Lymph Node and Spleen of Dogs. *Rev. Bras. Parasitol. Vet.* **2013**, *22*, 346–350. [CrossRef]
38. Aschar, M.; de Oliveira, E.T.B.; Laurenti, M.D.; Marcondes, M.; Tolezano, J.E.; Hiramoto, R.M.; Corbett, C.E.P.; Da Matta, V.L.R. Value of the Oral Swab for the Molecular Diagnosis of Dogs in Different Stages of Infection with *Leishmania infantum*. *Vet. Parasitol.* **2016**, *225*, 108–113. [CrossRef]
39. Pourmohammadi, B.; Mohammadi-Azni, S. Short Communication Molecular Detection of *Leishmania major* in *Hemiechinus auritus*: A Potential Reservoir of Zoonotic Cutaneous Leishmaniasis in Damghan, Iran. *J. Arthropod-Borne Dis.* **2019**, *13*, 334–343.

CAPÍTULO 4: RESULTADOS

4.4. OBJETIVO 4

Estudio sobre la utilidad de diversas dianas de PCR en relación con la sensibilidad y la especificad con el fin de confirmar la presencia de especies foráneas de *Leishmania* en la península ibérica.

4.4.1. RESUMEN

En las parasitosis transmitidas por artrópodos, la presencia de los vectores es el factor determinante para que el parásito esté presente y se transmita en una región geográfica determinada y, esto, en las últimas décadas, ha tomado una importancia mayor debido al cambio climático, que tiene como consecuencia el aumento del número de vectores, y a la globalización, que generan una dispersión de personas, animales, objetos y, por ende, de vectores. Esta situación, ha sido puesta de manifiesto por numerosos autores en el caso de los vectores de *Leishmania* spp. en la península ibérica, donde es ya habitual encontrar especies de flebotomos capaces de transmitir diferentes especies del parásito, como son *Phlebotomus sergenti* y *P. papatasi*, vectores de *L. tropica* y *L. major* respectivamente (Berriatua et al., 2023; Cunze et al., 2019). Sin embargo, hasta el momento solo se habían descrito unos pocos casos de leishmaniosis cutánea importada, causada por *L. tropica*, en personas que habían viajado a Marruecos (Knöpfel et al., 2018), pero en ningún caso existe evidencia de transmisión autóctona de una especie diferente a la endémica *L. infantum*. Cabe destacar que son varios los países vecinos donde otras especies de *Leishmania* son habituales y causan numerosas infecciones tanto en humanos como en otros animales. Así sucede en Marruecos, Turquía y, de forma eventual, Grecia (Ait Kbaich et al., 2017; Frank et al., 1993; Tunalı & Özbilgin, 2023), por lo que no sería extraño hallar su presencia en España. En el último estudio realizado en la presente Tesis doctoral se detectó *L. tropica* en fauna silvestre autóctona en España. Se ha descrito que animales silvestres próximos genéticamente a los animales domésticos son capaces de albergar los mismos patógenos que estos últimos, como es el caso del gato montés (*Felis silvestris*), analizado en este estudio. Asimismo, esta especie ha sido descrita como hospedadora de *L. infantum* en la península ibérica con anterioridad, aunque en todos los estudios los animales analizados eras escasos (3 o 4 individuos) (Del Río et al., 2014; Oleaga et al., 2018; Risueño et al., 2018). Con relación a las dianas utilizadas en este estudio, se seleccionaron la *SSUrRNA* por su elevada sensibilidad, como ya se ha mencionado, además de la *ITS1* y la *hsp70* (heat shock protein 70). Estas dos últimas son altamente específicas, a la hora de diferenciar entre especies del parásito. En todos los casos, las muestras analizadas fueron piel de oreja y bazo (con la excepción de dos animales, donde esta última muestra no se encontraba en condiciones adecuadas). En esta ocasión se analizaron un total de 18 gatos monteses, procedentes de diversas provincias de Castilla y León y Castilla-La Mancha, de los cuales siete

fueron positivos a *Leishmania* spp. en muestras de oreja, empleando la diana *SSUrDNA* y donde dos fueron positivos también en bazo. De estos siete, 2 individuos fueron positivos a *L. infantum* tanto en oreja como en bazo¸ un animal fue positivo a *L. tropica* empleando las dianas *ITS1* y *hsp70* y otro individuo presentaba secuencias compatibles con *L. tropica* y *L. infantum*. De los tres animales restantes solo se obtuvo resultado positivo a la diana *SSUrRNA*, la cual tiene alta sensibilidad, pero no es capaz de diferenciar a nivel de especie.

Al igual que ocurre en los gatos domésticos analizados por otros autores, la piel de oreja resultó más sensible que el bazo, especialmente en los individuos con *L. tropica*, debido probablemente al tipo de leishmaniosis cutánea, propia de *L. tropica* (Baneth et al., 2022; Can et al., 2016). Este hallazgo supone la primera descripción de *L. tropica* en animales silvestres en la península ibérica, donde la transmisión autóctona de esta especie del parásito podría ser hoy en día una realidad.

4.4.2. PONENCIAS

- **Azami- Conesa, I**., Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. Primera descripción de *Leishmania tropica* en gato montés en España. SOCEPA 2024 - XXIII Congreso de la Sociedad Española de Parasitología. Sevilla (España), del 2 al 5 de septiembre de 2024. Comunicación oral.

4.4.3. ARTÍCULO

- **Azami- Conesa, I.,** Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in non-endemic areas: Is *Leishmania tropica* circulating in wildlife in Spain? bioRxiv 2024.03.16.585353; DOI: https://doi.org/10.1101/2024.03.16.585353. Prepint. (Disponible en Anexos)
- **Azami- Conesa, I.,** Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in non-endemic areas: first detection of *Leishmania tropica* in wildlife in Spain. *Transboundary and Emerging Diseases*, 2024 (8259712), 1-10; DOI: https://doi.org/10.1155/2024/8259712.
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Research Article

Wildlife as a Sentinel for Pathogen Introduction in Nonendemic Areas: First Detection of *Leishmania tropica* in Wildlife in Spain

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Leishmaniasis is a chronic global arthropod-borne zoonotic disease produced by several species of *Leishmania* with cutaneous, mucocutaneous, and visceral clinical manifestations. In Spain, only *Leishmania infantum* has been reported so far, although other species of *Leishmania*, such as *L. tropica* and *L. major*, are present in surrounding countries. The aim of this work is to analyze the occurrence of *Leishmania* spp. infection in European wildcats (*Felis silvestris*) as sentinels, including their genotypic characterization. Necropsies of 18 road-killed wildcats were conducted. Samples of ear skin and spleen were taken for DNA isolation and PCR of the highly sensitive *SSU-rDNA* target. Subsequent PCR tests were performed using more specific targets for the determination of *Leishmania* species: *hsp70* and *ITS1*. Positive samples were sequenced, and phylogenetic trees were constructed. Seven wildcats were found positive for *Leishmania* spp. Based on the *hsp70* and *ITS1* sequences, an animal was found to be infected only with *L. tropica* in ear skin samples, while two cats were found to be infected with *L. infantum* in both the ear skin and the spleen. In one animal, a clear sequence of *L. infantum* ITS1 and a sequence of *L. tropica hsp70* were obtained from the ear skin. Since *hsp70* and *ITS1* sequencing was not possible in three cats, the species of *Leishmania* infecting them was not determined. This is the first report of autochthonous infection with *L. tropica* in the Iberian Peninsula. Health care professionals, including physicians, dermatologists, and veterinarians, must be aware of this for a correct diagnosis, treatment, and management of possible coinfections.

1. Introduction

Leishmaniasis is a global zoonotic disease transmitted by the bite of phlebotomine insects, with more than 1,000,000 new cases of the cutaneous form and 30,000 new cases of the visceral form estimated every year, in 92 endemic countries [1]. The clinical presentation in humans is variable and includes cutaneous (CL), mucocutaneous (MCL), visceral (VL), and post kala-azar dermal leishmaniasis (PKDL). The disease is caused by several species of *Leishmania*, with the *L. donovani* complex, the *L. major* complex, and the *L. tropica* complex being the most frequent in Africa, Asia, southern Europe, South America, and the Middle East, while the *L. mexicana* complex, the *L. guyanensis* complex, and the *L. braziliensis* complex are the most diagnosed in the America. The CL form of the disease is the most common and is responsible for the stigmatization and social isolation of thousands of people, particularly women, while VL is usually lethal without treatment and is caused by *L. donovani* in Africa and Europe, and *L. infantum* in southern Europe, west and central Asia, and the Americas. MCL is characterized by the destruction of mucous tissue of the nose,

palate, and pharynx and can severely affect the daily life of thousands of people [2, 3]. Human leishmaniasis is more prevalent in low-income countries with displaced populations due to war, social, or economic conflicts and is classified by the WHO as a neglected tropical disease (NTD). This scenario makes early diagnosis and treatment of the disease, as well as the introduction of preventive measures, difficult [3].

In Europe, VL is caused by *L. infantum*, with humans and dogs as the target species. The infection is more prevalent in southern countries such as Portugal, Spain, Italy, and Greece. Dogs are heavily affected by this zoonotic species [4] and are considered the main reservoir. Human infections mainly affect immunocompromised patients (such as HIV+ or recipients of solid organ transplants) as well as occasional reports in elderly and children [5]. Several studies have stressed the paramount importance of wildlife in maintaining *Leishmania* infection [6, 7]. Wildcats (*Felis silvestris*) are susceptible to the same pathogens as domestic cats and therefore could be potential reservoirs for the infection. However, the studies conducted have been scarce [8, 9, 10] with a limited number of animals analyzed in each study ($n = 4$, $n = 3$, $n = 4$, respectively). The three studies identified *L. infantum* using a small fragment of kDNA or qPCR from the internal transcribed spacers-5.8 ribosomal DNA 2 (ITS2) regions.

Despite suitable bioclimatic conditions and vectors available for anthroponotic *L. tropica* (*Phlebotomus sergenti*) and *L. major* (*P. papatasi*) [11, 12], only *L. infantum* has been reported in the Iberian Peninsula [6, 12, 13]. However, *L. major* has recently been reported in a cat from Lisbon, Portugal [14]. Moreover, it is worth noting that *L. tropica* has been previously reported in Greece [15] and the three species (*L. infantum*, *L. tropica*, and *L. major*) are present in Morocco [16] as well as in other countries bordering Europe [17]. The availability of a larger number of wildcats allowed us to identify the presence of *Leishmania* in various tissues and genetically characterize the samples. The aim of this work is to analyze the occurrence of *Leishmania* spp. infection in wildcats as sentinels, including their genotypic characterization.

2. Materials and Methods

2.1. Animals, Samples, and Geographic Area. In this study, samples of 18 European wildcats (*F. silvestris*) that were killed on the road during 2022 and 2023 in the Spanish provinces of Ávila, Burgos, Ciudad Real, Guadalajara, Soria, and Valladolid, were analyzed. Samples from the skin and spleen were selected, since they are the preferred sites for CL and/or VL *Leishmania* amastigotes. All road-killed wildcats were included in the study period. Animals were transported to the wildlife recovery centers of Castilla y León (Burgos, Valladolid) and Castilla-La Mancha (Ciudad Real, Guadalajara), systematic necropsies were performed, and the presence/absence of lesions annotated. A portion of the ear skin and spleen was taken and frozen at −20°C for further processing. The dates, age, sex, and location (geographic coordinates where the dead animals were found) were included in the database.

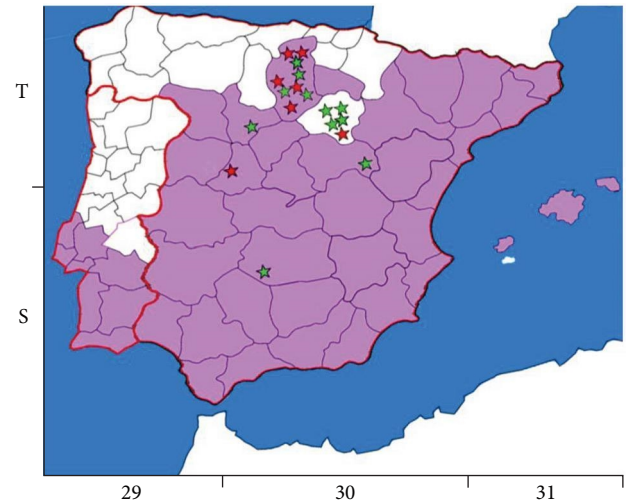

FIGURE 1: Map of the Iberian Peninsula with U.T.M. geographic coordinates. The purple area corresponds to the distribution of *P. sergenti* in 2022 and 2023 available at ECDC [18]. Red stars indicate the detection and identification of *Leishmania* spp. by *SSU-rDNA* sequences. Green stars indicate the absence of *Leishmania* spp. in the sampled cats.

2.2. Maps. The map shown in Figure 1 was created taking as reference the European Centre for Disease Prevention and Control (ECDC) maps of distribution in Europe of *P. sergenti*, the main vector of *L. tropica*, in 2022 and 2023 [18] and the Universal Transverse Mercato (U.T.M.) geographic coordinates of the closest villages to where the wildcats analyzed in this study were found. The AutoCAD program (Autodesk® Inc., Landmark, San Francisco, CA, USA) was used to observe the overlaps between the two maps.

2.3. DNA Isolation. DNA was isolated from the spleen (10 mg) and the skin of the ears (20 mg) using the NZYTech Tissue gDNA isolation kit (NZYTech, Portugal) and following the manufacturer's instructions. DNA elution was performed in 60 μL of elution buffer instead of 100 μL to increase DNA concentration. The positive and negative controls were used in each batch of the experiment. The positive control consisted of samples derived from *L. major* (clone V1: MHOM/IL/80/Friedlin) or *L. braziliensis* (MHOM/BR/75/M2904) promastigotes in culture (1×10^6 promastigotes), while the negative control consisted of water free of DNase. The DNA content was measured on a Multiskan GO microplate spectrophotometer with μDrop™ plates (Thermo Fisher Scientific, MA, USA). DNA from cultures of *L. major* and *L. braziliensis* was used as internal controls for sequencing also. These two isolates were chosen because they were absent from Spain.

2.4. PCR of Small Subunit Ribosomal RNA (SSU-rDNA), Internal Transcribed Spacers-5.8 Ribosomal DNA 1 (ITS1), and Heat Shock Protein 70 (hsp70) Targets. DNA samples from the skin of the ear and spleen of the 18 cats were used for *Leishmania* DNA detection. DNA from different species of *Leishmania* was also used as an internal control for PCR and sequencing: *L. major* (clone V1: MHOM/IL/80/Friedlin)

and *L. braziliensis* (MHOM/BR/75/M2904). Only samples with good DNA quality (absorbance ratio at 260/280 nm > 1.7) were used for all PCRs. All PCR conditions have been previously described and adapted to the working conditions recommended by the enzyme manufacturer (Supreme NZYTaq II 2x Master Mix, NZYTech, Lisbon, Portugal).

The SSU-rDNA target was chosen as the first PCR because of its higher sensitivity compared to other PCR targets. Nested PCRs (nPCR) to amplify a 358 bp fragment of the *SSU-rDNA* target were performed using primers R221 (5′-GGTTCCTTTCCTGATTTACG-3′) and R332 (5′-GGC CGGTAAAGGCCGAATAG-3′) for the outer section and primers R223 (5′-TCCATCGCAACCTCGGTT-3′) and R333 (5′-AAGCGGGCGCGGTGCTG-3′) for the inner section [19], following previously described conditions [20].

Primers L5.8S (5′-ACACTCAGGTCTGTAAAC-3′) and LITSR (5′-CTGGATCATTTTCCGATG-3′) were used for the outer section of the nPCR for *ITS1* region and primers SAC (5′-CATTTTCCGATGATTACACC-3′) and VAN2 (5′-CGTTCTTCAACGAAATAGG-3′) were used for the inner section to amplify a fragment of 280–330 bp, which allows species determination, as previously described [20, 21].

The nPCR used for *hsp70* amplified a fragment of 741 bp and was based on the primers and methods previously described for phylogenetic purposes [14, 22]. Briefly, the external primers HSP70-F25 (5′-GGACGCCGGCACGAT TKCT-3′) and HSP70-R1310 (5′-CCTGGTTGTTGTTCAG CCACTC-3′) were used at 0.8 μM each, and the internal primers HSPF (5′-GACAACCGCCTCGTCACGTTC-3′) and HSPR (5′-GTCGAACGTCACCTCGATCTGC-3′) were used at 0.4 μM each. In the first PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s, and 72°C for 120 s were used, while in the second PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s, and 72°C for 60 s were used.

All PCR reactions were performed in 25 μL using 12.5 μL of Supreme NZYTaq II 2x Master Mix. An initial step of 95°C for 5 min to activate the enzyme and a final elongation step of 72°C for 10 min to allow the elongation of the PCR products were used in all PCR reactions. In the first PCR, 5 μL of DNA was used, while in the second PCR reactions, 5 μL of a 1 : 50 dilution of each first PCR product were used for *hsp70*, while a 1 : 40 dilution of the first PCR product was used for *SSU-rDNA* and *ITS1*. The results of nPCR were visualized on 1% agarose gels stained with SYBR™ safe DNA gel staining (Invitrogen, Thermo Fisher Scientific, MA, USA) under UV light.

2.5. Sequencing and Alignment. Samples containing the amplicons of the expected size were sent to the facilities of Macrogen Spain (Madrid, Spain) for Sanger sequencing in forward and reverse directions. The resulting sequences were aligned using Molecular Evolutionary Genetics Analysis v.11 software (MEGA XI) [23] and FinchTV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA), manually checked, and subjected to BLAST analysis using the nucleotide Basic Local Alignment Search Tool (BLAST; National Library of Medicine, Rockville, MD, USA). Only sequences with more than 150 bp that were clear in both directions were sent to GenBank for accession numbers. PCR and sequencing were repeated at least twice for each sample to ensure results.

2.6. Phylogenetic Trees. Phylogenetic trees were constructed using the sequences obtained in the present study and other sequences available from GenBank. Only the trees for *hsp70* and *ITS1* are shown because the *SSU-rDNA* target is a conserved region and only a difference of 1–2 bp was observed between the sequences obtained. In all trees, a sequence from *Trypanosoma cruzi* was included as an outgroup reference. Additionally, other sequences of *Leishmania* spp. from GenBank and sequences from *L. infantum* and the species of *Leishmania* mentioned above were included from cultures to verify similarity (*L. major* and *L. braziliensis*). The sequences had a minimum length of 292 nucleotides for the *ITS1* region and 635 nucleotides for *hsp70*. Evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model [24]. The tree with the highest logarithmic likelihood was displayed. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a pairwise distance matrix estimated using the maximum composite likelihood (MCL) approach and selecting the topology with the superior log-likelihood value. The trees were drawn to scale, and the lengths of the branches were measured on the basis of the number of substitutions per site. The analysis included 13–18 nucleotide sequences depending on the tree. The included codon positions were 1st + 2nd + 3rd + noncoding. All positions that contained gaps and missing data were eliminated. Evolutionary analyzes were conducted via MEGA XI [23]. A bootstrap of 2,000 replicates was used to assess the reliability of the trees.

2.7. Statistical Analysis. A descriptive statistical analysis with absolute (n) and relative frequency of infection (%) was carried out, with a confidence level of 95%, using the free online tool WinEpi (Working in Epidemiology) and considering that the diagnostic techniques used were perfect (nPCR of the *SSUrDNA*). Odds ratio analysis was used to analyze the risk factors "sex" and "*Leishmania* infection" [25].

3. Results

3.1. Detection and Identification of Leishmania spp. in Wildcats. In this study, samples of the ear skin and spleen of 18 wildcats were analyzed, except for the spleen of two animals that were in poor condition when the cats were found (Table 1). None of the animals showed lesions comparable to those produced by *Leishmania* infections. Taking into account all animals positive for *Leishmania* PCR, 38.89% (16.37%–61.41% and 95% confidence interval) were found to be infected. A nonsignificant higher proportion of infection was found in males (OR = 27.17, 95% CI: 0.33, 2213.63), since only males were found infected, but we must consider that we obtained a smaller number of samples from females ($n = 3/18$).

Table 1: Age, sex, U.T.M. geographic coordinates, date of collection, and *Leishmania* infection of the studied wildcats.

Sample	Age	Sex	Date	U.T.M. geographic coordinates	*Leishmania* infection
262	Adult	M	20-04-2022	30T 439302, 4683502	+
263	Adult	M	21-04-2022	30T 308223, 4488924	+
268	Adult	M	22-06-2022	30T 438633, 4729501	+
272	Adult	M	13-07-2022	30T 370885, 4584961	−
275	Adult	M	15-07-2022	30T 427252, 4615635	+
276	Adult	M	15-07-2022	30T 510221, 4613053	−
310	Adult	M	21-12-2022	30T 538981, 4592797	−
317	Adult	M	24-02-2023	30T 455150, 4702613	−
318*	Adult	M	13-03-2023	30T 397133, 4707688	+
319	Adult	F	13-03-2023	30T 464371, 4722997	−
320	Adult	M	23-03-2023	30T 548263, 4605806	−
323	Adult	M	28-04-2023	30T 544058, 4577807	+
365	Adult	M	08-09-2023	30T 480564, 4725184	+
366	Adult	M	08-09-2023	30T 553424, 4621448	−
367	Adult	F	08-09-2023	30T 457990, 4653201	−
375	Adult	M	05-10-2023	30S 386031, 4251590	−
376	Adult	F	05-10-2023	30T 401098, 4678137	−
378*	Adult	nd	05-10-2023	30T 592304, 4494125	−

*Spleen not analyzed. M, male; F, female; nd, not determined.

Table 2: Results of endpoint PCR using samples from the ear skin and spleen and three targets for the amplification of *Leishmania* DNA.

Sample	Ear skin			Spleen		
	SSU-rDNA	*ITS1*	*hsp70*	*SSU-rDNA*	*ITS1*	*hsp70*
262	+	−	−	−	−	−
263	+	$+^{2}$	$+^{4}$	+	$+^{2}$	$+^{4}$
268	+	$+^{2}$	$+^{3}$	−	−	−
275	+	$+^{1}$	$+^{3}$	−	−	−
318	+	−	−	nd	nd	nd
323	+	$+^{2}$	$+^{4}$	+	$+^{2}$	$+^{4}$
365	+	−	−	−	−	−

Sequence identities are identified with super index numbers (1–4). nd, not determined; 1, *ITS1* sequence 100% identity with *L. tropica*; 2, *ITS1* sequence 100% identity with *L. infantum*; 3, *hsp70* sequence 99.86%–100% identity with *L. tropica*; and 4, *hsp70* sequence 99.24%–100% identity with *L. infantum*.

All *Leishmania* spp. infected animals were found in areas where *P. sergenti*, the main vector of *L. tropica* was identified, except for one animal in which *L. infantum* was determined in both ear skin and spleen (Figure 1).

Seven of the 18 animals were found to be positive by *Leishmania* PCR for *SSU-rDNA*. All were positive on the skin of the ears, and only two were also positive in the spleen samples (Table 2).

3.2. Sequences and GenBank Accession Numbers. The nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. Sequences from *L. major* and *L. braziliensis* isolates were deposited in the GenBank database with accession numbers PP389522, PP389523, and PP389533.

The sequences obtained from *SSU-rDNA* had 295–327 bp and differ by only one or two nucleotide positions. Nine sequences were obtained from the animals analyzed, seven from the skin of the ears, and two from the spleen. All of them displayed 100% query coverage and 99.69%–100% identity with other *Leishmania* spp. sequences available at GenBank, including *L. infantum* and *L. tropica*, among other species (accession numbers MN757921and KF302745).

ITS1 sequences of 236–247 bp were obtained from the ear skin of four animals (263, 268, 275, and 323), two of which (263 and 323) also gave sequences when DNA from the spleen was used (Table 2). Three sequences displayed 100% query coverage and 100% identity with sequences from *L. infantum* (accession number MT416168, samples from ear skin of animals 263, 268, and 323, and samples from spleen of animals 263 and 323), while one sequence (from wildcat 275 ear skin) displayed 100% query coverage and 100% identity with sequences from *L. tropica* (accession number MH595857, isolate R24, Iran).

Sequences obtained from *hsp70* amplification yielded four sequences from the ear skin and two sequences from

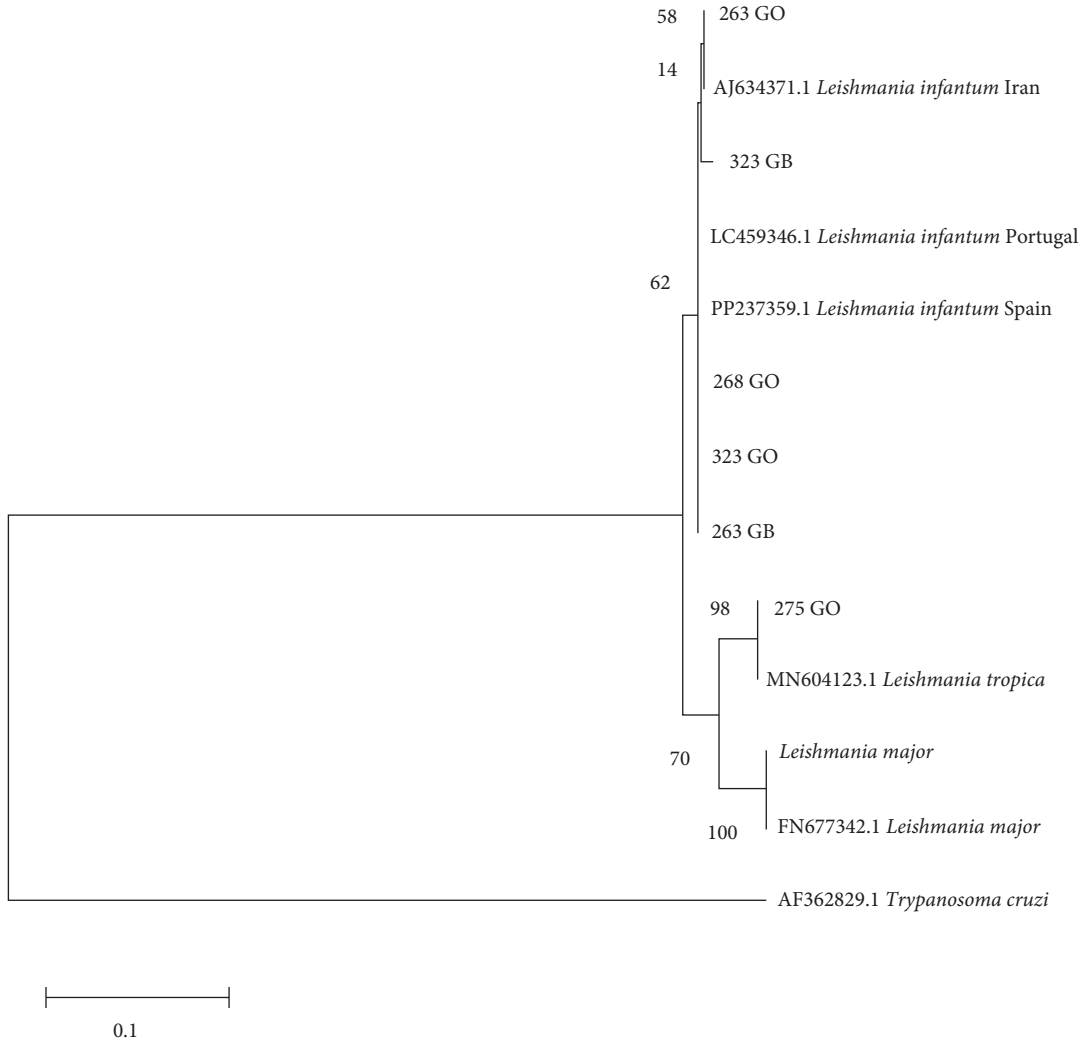

FIGURE 2: Phylogenetic tree of the *ITS1* region. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model. The tree with the highest logarithmic likelihood (−828.31) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 13 nucleotide sequences. There were a total of 293 positions in the final data set. GB, sample of the spleen; GO, sample from the skin of the ear.

the spleen with 635–711 bp. Of these, four sequences showed 100% coverage and 99.24%–100% identity with *L. infantum* (accession number OR136937, strain MHOM/IT/99/ISS1898). Sequences 275 from the ear skin showed 100% coverage and 100% identity with *L. tropica* (accession number MK335938, voucher ISS3183), while sequence obtained from the ear skin of 268 showed 100% coverage and 99.86 identity with *L. tropica* (acc. n LN907846, strain MHOM/IL/80/SINGER and others) and 99.72% identity with *L. donovani* (accession numbers MH202961 and JX021427; Table 2). Double peaks were observed at three and five positions of the sequences of the spleen of animal 323 and the skin of the ears of animal 263, respectively, indicating mixed sequences.

It is noteworthy to point out that *Leishmania* DNA was not detected by none of the three PCR methods in the spleens of cats with cutaneous infection with *L. tropica*. In one animal (275), the sequences from the *ITS1* and *hsp70* targets showed 100% identity with *L. tropica*. Two animals displayed sequences compatible with *L. infantum* on the skin of the ears and the spleen (263 and 323), and another wildcat was positive only on the skin of the ears with sequences compatible with *L. infantum* and *L. tropica* (268). Finally, in three animals (262, 318, and 365), the sequences could only be assigned to the *Leishmania* genus because they were only positive for *SSU-rDNA* (Table 2).

3.3. Phylogenetic Trees. Phylogenetic analysis of the sequences shows strong support for the clusters found with the two targets, in agreement with the percentage of identity of the sequences obtained. *ITS1* sequences from this study were grouped with *L. infantum* (samples 263, 268, and 323 from the skin of the ears and samples 263 and 323 from the spleen) or *L. tropica* (sample 275; Figure 2).

In the phylogenetic tree generated with the *hsp70* sequences obtained in the present study, two of them (samples 268 and 275 from the ear skin) clustered with *L. tropica*

and three of them (samples 263 and 323 from the ear skin and 263 spleen) clustered with *L. infantum* sequences recovered from GenBank (Figure 3). One of the *L. tropica* sequences was obtained from the same animal (275) that rendered the *L. tropica ITS1* sequence, while the other *L. tropica hsp70* sequence was obtained from an animal (268) that rendered the *L. infantum ITS1* sequence.

4. Discussion

In the present study, seven out of 18 wildcats (38.89%) were found to be positive for *Leishmania* infection, a value consistent with those found in previous studies [8, 10] although lower than the infection prevalence reported by Oleaga et al. [9] with all three wildcats tested being positive for *Leishmania*. *L. infantum* is endemic in the Iberian Peninsula [12, 13], but no infections of *L. tropica* in autochthonous species have been reported. Even considering the limitations of the study, including the low parasitic loads commonly found in wildlife and the number of animals, the information is of value, taking into account that the population of wildcats in Spain is considered relict and they are animals difficult to see unless they are sick or death, which is the case [26].

The risk of introduction of *L. tropica* in the Iberian Peninsula has been highlighted several times, considering the presence and distribution of the main vector, *P. sergenti*, and the identification in Spain of one genetic line of *P. sergenti* commonly found in Morocco [27, 28] where CL by *L. tropica* is endemic [21, 29, 30]. Large areas in Europe are considered suitable for *L. tropica* [11], and hot spots for foci of *L. tropica* have been suggested [27]. In addition, the possibility of *L. tropica* transmission by *P. perniciosus* has been pointed out [31, 32].

The most common targets used for the detection of *Leishmania* spp. infection are *SSU-rDNA* and kDNA with similar values of sensitivity [6]. However, other targets are useful for phylogenetic studies and determination of *Leishmania* spp., such as *ITS1*, *cytB*, *g6pdh*, and *hsp70* [14, 20, 21, 22, 29, 33, 34]. In the present study, *ITS1* and *hsp70* have been used to confirm the finding of *L. tropica* in autochthonous fauna, and sequences of *L. tropica* with *ITS1* and *hsp70* targets are reported. *L. tropica sequences* were detected in at least two wildcats positive for PCR with targets *hsp70* and/or ITS1, widely used for phylogenetic studies. Our finding extends the reported presence of the species in Greece [15] and extends its distribution to the Iberian Peninsula for the first time.

The isolation of alive *Leishmania* was not feasible considering the source of material, but it is also noteworthy that in our study the skin samples of the ears showed more positive results than the spleen samples ($n = 7/18$ vs. $n = 2/16$). This finding is consistent with the fact that *L. tropica* infections are primarily associated with clinical forms of skin in humans, where the persistence of the parasite is close to the site of natural infections transmitted by the sandfly, although the parasite can also be found less frequently associated with VL, mainly in immune suppressed people [35]. Imported CL cases from *L. tropica* have been sporadically reported in Spain after travelling to Morocco [36], but no previous autochthonous cases have been reported in Spain or Portugal. Apparently, *L. tropica* is expanding its distribution, and the main risk factor for the appearance of the disease is the presence of the vector [37]. It should be noted that some of the wildcats came from latitudes over 42° N (e.g., animal 365, geographic coordinates U.T.M. 30T 480564, 4725184) and during the last decade, *P. sergenti* has expanded to the northern areas of the Iberian Peninsula, according to ECDC maps of distribution [18].

In endemic areas, such as Israel, domestic cats appear to be more susceptible to infection with *L. tropica* than dogs [38]. A study of stray cats in Turkey showed that all samples with *ITS1* amplification were infected with *L. tropica*, while only one was coinfected with *L. infantum* [39]. Similarly, in the present study, while one animal showed infection by *L. tropica* only, another wildcat showed *hsp70* and *ITS1* sequences consistent with coinfection by *L. infantum* and *L. tropica* or by a hybrid of *L. infantum* and *L. tropica*. Mixed infections with more than one *Leishmania* species have also been reported in other endemic areas, hosts, and *Leishmania* species, such as in Brazil (*L. infantum* and *L. braziliensis*) in one dog [40] and 8/30 dogs [41], rodents (10% of *L. infantum* and *L. braziliensis*) [42], hedgehogs (*L. major* and *L. infantum*) in 8/12 animals and another coinfected with *L. tropica* [43], and human clinical cases [44, 45] of the new world. The possibility of hybridization between *Leishmania* species cannot be ruled out, as *L. tropica* shows a higher rate of natural hybrid formation than other species [46]. Indeed, at least one natural hybrid of *L. donovani* and *L. aethiopica* has been described in Ethiopia employing *ITS1*, *hsp70*, and cysteine proteinase B (*cpb*) targets [47]. The authors suggested that hybridization could take place in a vector permissive to both species, as it has been described in *P. pernicious* for *L. infantum* and *L. tropica* [31, 32]. One hybrid of *L. infantum* and *L. major* was also described in a natural infection in Portugal [48]. However, to further investigate the existence of hybrids, isolates from infected animals are necessary [49, 50], and in our study, they could not be obtained [51].

5. Conclusions

This is the first report of *L. tropica* infection in autochthonous wildlife in the Iberian Peninsula, extending the presence of the species in continental Europe. The main vector of *L. tropica* has been identified in Spain during the last decade, and its distribution has increased northward each year. There are no conclusive data on the zoonotic transmission of *L. tropica*, and the actual spread of the infection to other species is not yet known and should be investigated. Our results support the sentinel value of wild species in the detection of previously unreported infections. In addition, the presence of *L. tropica* in Spain should be communicated to health professionals, including physicians, dermatologists, and veterinarians, considering that *L. tropica* can produce both CL and VL in humans and pets. Awareness of the

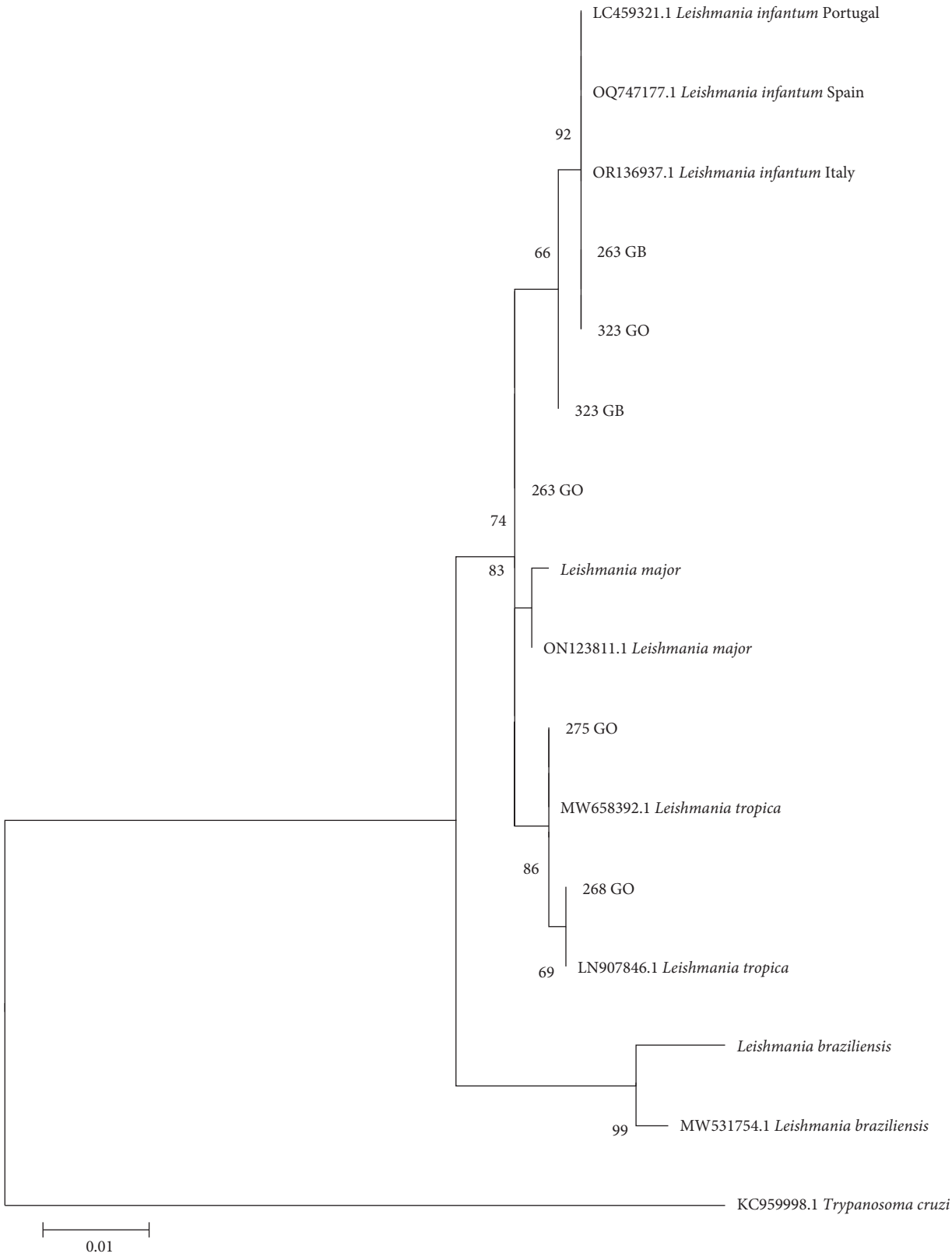

FIGURE 3: Phylogenetic tree of the *hsp70* region. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model. The tree with the highest log-likelihood (−1,328.38) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences. There were a total of 635 positions in the final dataset. Evolutionary analyzes were carried out in MEGA11. GB, sample of the spleen. GO, sample from the skin of the ear.

possibility of *L. tropica* infections and *L. infantum*/*L. tropica* coinfections is necessary to ensure the appropriate diagnosis and management of clinical cases.

Data Availability

Sequences obtained in the present study have been deposited in the GenBank database under accession numbers: PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. The sequences of the isolates of *L. major* and *L. braziliensis* are PP389522, PP389523, and PP389533.

Ethical Approval

The use of samples for parasitological investigations was approved by regional authorisation of the Junta de Castilla y León, reference: "AB/is", File "AUES/CYL/001/2021," and of Castilla-La Mancha, reference: DGPFEN/SEN/avp_21_103_bis.

Disclosure

A preprint version of the manuscript with doi https://www.biorxiv.org/content/10.1101/2024.03.16.585353v2 has been published on bioRxiv [51].

Conflicts of Interest

The authors declare that there is no conflicts of interest with respect to the publication of this article.

Authors' Contributions

Marta Mateo Mateo Barrientos, Iris Azami-Conesa, and María Teresa Gómez-Muñoz contributed to conceptualization. Iris Azami-Conesa, Paula Pérez-Moreno, and María Teresa Gómez-Muñoz contributed to methodology and formal analysis. Marta Mateo Barrientos, Pablo Matas Méndez, and Javier Carrión contributed to collection of data and biological samples. Iris Azami-Conesa and María Teresa Gómez-Muñoz contributed to writing the original draft. Pablo Matas Méndez, Javier Carrión, Iris Azami-Conesa, Marta Mateo Barrientos, María Teresa Gómez-Muñoz, and José María Alunda contributed to interpretation of data. Marta Mateo Barrientos and María Teresa Gómez-Muñoz contributed to supervision. José María Alunda, Marta Mateo Barrientos, and María Teresa Gómez-Muñoz contributed to resources. All authors critically reviewed the manuscript, intellectually contributed to the content, and approved the final version. Marta Mateo Barrientos and María Teresa Gómez-Muñoz contributed equally to this work and share last authorship.

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References

[1] WHO: health-topics/leishmaniasis, https://www.who.int/health-topics/leishmaniasis#tab=tab_1[Internet].WHO:health-topics/leishmaniasis, 2024.

[2] M. Akhoundi, K. Kuhls, A. Cannet et al., "A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies," *PLOS Neglected Tropical Diseases*, vol. 10, no. 3, Article ID e0004349, 2016.

[3] "WHO: neglected diseases, leishmaniasis," 2023, https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html.

[4] A. Montoya, R. Gálvez, R. Checa et al., "Latest trends in *L. infantum* infection in dogs in Spain, Part II: current clinical management and control according to a national survey of veterinary practitioners," *Parasites & Vectors*, vol. 13, Article ID 205, 2020.

[5] D. A. Zacarias, N. Rolão, F. A. de Pinho et al., "Causes and consequences of higher *Leishmania infantum* burden in patients with kala-azar: a study of 625 patients," *Tropical Medicine and International Health*, vol. 22, no. 6, pp. 679–687, 2017.

[6] I. Azami-Conesa, M. T. Gómez-Muñoz, and R. A. Martínez-Díaz, "A systematic review (1990-2021) of wild animals infected with zoonotic *Leishmania*," *Microorganisms*, vol. 9, no. 5, Article ID 1101, 2021.

[7] L. Cardoso, H. Schallig, M. F. Persichetti, and M. G. Pennisi, "New epidemiological aspects of animal leishmaniosis in europe: the role of vertebrate hosts other than dogs," *Pathogens*, vol. 10, no. 3, Article ID 307, 2021.

[8] L. Del Río, L. Chitimia, A. Cubas et al., "Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain," *Preventive Veterinary Medicine*, vol. 113, no. 4, pp. 430–435, 2014.

[9] A. Oleaga, S. Zanet, A. Espí, M. R. Pegoraro de Macedo, C. Gortázar, and E. Ferroglio, "*Leishmania* in wolves in northern Spain: a spreading zoonosis evidenced by wildlife sanitary surveillance," *Veterinary Parasitology*, vol. 255, pp. 26–31, 2018.

[10] J. Risueño, M. Ortuño, P. Pérez-Cutillas et al., "Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain," *Veterinary Parasitology*, vol. 259, pp. 61–67, 2018.

[11] S. Cunze, J. Kochmann, L. K. Koch, K. J. Q. Hasselmann, and S. Klimpel, "Leishmaniasis in Eurasia and Africa: geographical distribution of vector species and pathogens," *Royal Society Open Science*, vol. 6, no. 5, Article ID 190334, 2019.

[12] E. Berriatua, P. Pérez-Cutillas, A. González Vidal, and O. Briët, "The spatial relationship between the presence and absence of *Leishmania* spp. and leishmaniasis, and phlebotomine sand fly vectors in Europe and neighbouring countries," 2023, European Centre for Disease Prevention and Control, https://www.ecdc.europa.eu.

[13] R. Gálvez, A. Montoya, I. Cruz et al., "Latest trends in *Leishmania infantum* infection in dogs in Spain, Part I: mapped seroprevalence and sand fly distributions," *Parasites & Vectors*, vol. 13, Article ID 204, 2020.

[14] A. Pereira, R. Parreira, J. M. Cristóvão et al., "Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers,"

Infection, Genetics and Evolution, vol. 77, Article ID 104069, 2020.

[15] C. Frank, M. Hadziandoniou, F. Pratlong, A. Garifallou, and J. A. Rioux, "*Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 87, no. 2, pp. 184-185, 1993.

[16] A. M. Kbaich, I. Mhaidi, A. Ezzahidi et al., "New epidemiological pattern of cutaneous leishmaniasis in two pre-Saharan arid provinces, southern Morocco," *Acta Tropica*, vol. 173, pp. 11–16, 2017.

[17] V. Tunalı and A. Özbilgin, "Knock, knock, knocking on Europe's door: threat of leishmaniasis in Europe with a focus on Turkey," *Current Research in Parasitology & Vector-Borne Diseases*, vol. 4, no. 4, Article ID 100150, 2023.

[18] "European centre for disease prevention and control and European food safety authority," 2023, Phlebotomine sandflies maps [internet]. Stockholm: ECDC; 2022 https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps [Internet].

[19] I. Cruz, C. Cañavate, J. M. Rubio et al., "A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immuno-deficiency virus," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 96, no. Supplement 1, pp. S185–S189, 2002.

[20] I. Azami-Conesa, J. Sansano-Maestre, R. A. Martínez-Díaz, and M. T. Gómez-Muñoz, "Invasive species as hosts of zoonotic infections: the case of American mink (*Neovison vison*) and *Leishmania infantum*," *Microorganisms*, vol. 9, no. 7, Article ID 1531, 2021.

[21] M. Echchakery, C. Chicharro, S. Boussaa et al., "Molecular detection of *Leishmania infantum* and *Leishmania tropica* in rodent species from endemic cutaneous leishmaniasis areas in Morocco," *Parasites & Vectors*, vol. 10, no. 1, Article ID 454, 2017.

[22] G. Van der Auwera, I. Maes, S. De Doncker et al., "Heat-shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics," *Eurosurveillance*, vol. 18, no. 30, Article ID 20543, 2013.

[23] K. Tamura, G. Stecher, and S. Kumar, "MEGA11: molecular evolutionary genetics analysis Version 11," *Molecular Biology and Evolution*, vol. 38, no. 7, pp. 3022–3027, 2021.

[24] K. Tamura and M. Nei, "Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees," *Molecular Biology and Evolution*, vol. 10, no. 3, pp. 512–526, 1993.

[25] Win Epi, "Working in epidemiology," 2024, Ignacio de Blas. [Internet] Accesed on January 9th http://www.winepi.net/.

[26] J. M. López-Martín, F. J. García, A. Such et al., "Atlas y Libro Rojo de los Mamíferos Terrestres de España," 2007, Dirección General para la Biodiversidad -SECEM-SECEMU, Madrid. [Internet]. [cited 2023 Dec 6]. Available from: https://www.miteco.gob.es/es/biodiversidad/temas/inventarios-nacionales/inventario-especies-terrestres/inventario-nacional-de-biodiversidad/ieet_mamif_atlas.html.

[27] S. D. Barón, F. Morillas-Márquez, M. Morales-Yuste et al., "Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in south-western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917," *Parasitology*, vol. 140, no. 11, pp. 1413–1421, 2013.

[28] G. Merino-Espinosa, V. Corpas-López, R. Callejón-Fernández et al., "Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and their epidemiological implications," *Tropical Medicine & International Health*, vol. 21, no. 5, pp. 630–641, 2016.

[29] S. El Kacem, M. Ait Kbaich, O. Daoui et al., "Multilocus sequence analysis provides new insight into population structure and genetic diversity of *Leishmania tropica* in Morocco," *Infection Genetics and Evolution*, vol. 93, Article ID 104932, 2021.

[30] I. El Idrissi Saik, C. Benlabsir, H. Fellah, M. Lemrani, and M. Riyad, "Transmission patterns of *Leishmania tropica* around the Mediterranean basin: could Morocco be impacted by a zoonotic spillover?" *PLOS Neglected Tropical Diseases*, vol. 16, no. 1, Article ID e0010009, 2022.

[31] G. Bongiorno, T. Di Muccio, R. Bianchi, M. Gramiccia, and L. Gradoni, "Laboratory transmission of an Asian strain of *Leishmania tropica* by the bite of the southern European sand fly *Phlebotomus perniciosus*," *International Journal for Parasitology*, vol. 49, no. 6, pp. 417–421, 2019.

[32] S. Vaselek and P. Volf, "Experimental infection of *Phlebotomus perniciosus* and *Phlebotomus tobbi* with different *Leishmania tropica* strains," *International Journal for Parasitology*, vol. 49, no. 11, pp. 831–835, 2019.

[33] I. Mohammadpour, G. R. Hatam, F. Handjani, F. Bozorg-Ghalati, D. PourKamal, and M. H. Motazedian, "*Leishmania* cytochrome b gene sequence polymorphisms in southern Iran: Relationships with different cutaneous clinical manifestations," *BMC infectious diseases*, vol. 19, Article ID 98, 2019.

[34] A. Fernández-Arévalo, C. Ballart, J. Muñoz-Basagoiti et al., "Autochthonous and imported tegumentary leishmaniasis in Catalonia (Spain): Aetiological evolution in the last four decades and usefulness of different typing approaches based on biochemical, molecular and proteomic markers," *Transboundary and Emerging Diseases*, vol. 69, no. 3, pp. 1404–1418, 2022.

[35] M. Maroli, M. D. Feliciangeli, L. Bichaud, R. N. Charrel, and L. Gradoni, "Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern," *Medical and Veterinary Entomology*, vol. 27, no. 2, pp. 123–147, 2013.

[36] N. Knöpfel, L. Noguera-Morel, D. Azorin, F. Sanz, A. Torrelo, and A. Hernández-Martín, "Cutaneous *Leishmania tropica* in children: report of three imported cases successfully treated with liposomal amphotericin B," *Journal of the European Academy of Dermatology and Venereology*, vol. 32, no. 1, pp. e8–10, 2018.

[37] P. Gijón-Robles, N. Abattouy, G. Merino-Espinosa et al., "Risk factors for the expansion of cutaneous leishmaniasis by *Leishmania tropica*: Possible implications for control programmes," *Transboundary and Emerging Diseases*, vol. 65, no. 6, pp. 1615–1626, 2018.

[38] G. Baneth, Y. Nachum-Biala, O. Adamsky, and I. Gunther, "*Leishmania tropica* and *Leishmania infantum* infection in dogs and cats in central Israel," *Parasites & Vectors*, vol. 15, Article ID 147, 2022.

[39] H. Can, M. Döşkaya, H. G. Özdemir et al., "Seroprevalence of *Leishmania* infection and molecular detection of *Leishmania tropica* and *Leishmania infantum* in stray cats of İzmir, Turkey," *Experimental Parasitology*, vol. 167, pp. 109–114, 2016.

[40] M. F. Madeira, A. Schubach, T. M. P. Schubach et al., "Mixed infection with *Leishmania* (*Viannia*) *braziliensis* and *Leishmania*

(*Leishmania*) *chagasi* in a naturally infected dog from Rio de Janeiro, Brazil," *Transactions of The Royal Society of Tropical Medicine and Hygiene*, vol. 100, no. 5, pp. 442–445, 2006.

[41] N. Alves Souza, R. Souza Leite, S. de Oliveira Silva et al., "Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil," *Acta Tropica*, vol. 193, pp. 12–17, 2019.

[42] E. de Castro Ferreira, I. Cruz, C. Cañavate et al., "Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World," *BMC Veterinary Research*, vol. 11, Article ID 71, 2015.

[43] H. Souguir-Omrani, J. Chemkhi, A. Fathallah-Mili et al., "*Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) hedgehogs: possible reservoirs of endemic leishmaniases in Tunisia," *Infection, Genetics and Evolution*, vol. 63, pp. 219–230, 2018.

[44] S. Shirian, A. Oryan, G. R. Hatam, and Y. Daneshbod, "Mixed mucosal leishmaniasis infection caused by *Leishmania tropica* and *Leishmania major*," *Journal of Clinical Microbiology*, vol. 50, no. 11, pp. 3805–3808, 2012.

[45] A. Badirzadeh, M. Mohebali, S. Sabzevari et al., "Case report: first coinfection report of mixed *Leishmania infantum*/*Leishmania major* and human immunodeficiency virus-acquired immune deficiency syndrome: report of a case of disseminated cutaneous leishmaniasis in Iran," *The American Journal of Tropical Medicine and Hygiene*, vol. 98, no. 1, pp. 122–125, 2018.

[46] I. Louradour, T. R. Ferreira, K. Ghosh, J. Shaik, and D. Sacks, "In vitro generation of *Leishmania* Hybrids," *Cell Report*, vol. 31, no. 2, Article ID 107507, 2020.

[47] S. Odiwuor, S. De Doncker, I. Maes, J. C. Dujardin, and G. Van der Auwera, "Natural *Leishmania donovani*/*Leishmania aethiopica* hybrids identified from Ethiopia," *Infection Genetics and Evolution*, vol. 11, no. 8, pp. 2113–2118, 2011.

[48] C. Ravel, S. Cortes, F. Pratlong, F. Morio, J.-P. Dedet, and L. Campino, "First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major*," *International Journal for Parasitology*, vol. 36, no. 13, pp. 1383–1388, 2006.

[49] A. Ozbilgin, G. Culha, F. Y. Zeyrek et al., "Cutaneous and visceral tropisms of *Leishmania tropica*/*Leishmania infantum* hybrids in a murine model: First report of hybrid *Leishmania* strains isolated in Turkey," *International Journal of Infectious Diseases*, vol. 16, Article ID e165, 2012.

[50] F. Bruno, G. Castelli, B. Li et al., "Genomic and epidemiological evidence for the emergence of a putative *L. infantum* hybrid with unusual epidemiology in Northern Italy," *mBio*, vol. 15, no. 7, Article ID e0099524, 2024.

[51] I. Azami-Conesa, P. Matas Méndez, P. Pérez-Moreno et al., "Wildlife as a sentinel for pathogen introduction in nonendemic areas: first detection of *Leishmania tropic*a in wildlife in Spain," BiorXiv, 2024.

CAPÍTULO 5:
DISCUSIÓN

5.1. DISCUSIÓN INTEGRADORA

El carácter zoonótico de la leishmaniosis, entre otros motivos, hace que sean muy numerosas las investigaciones llevadas a cabo sobre esta parasitosis, incluyendo estudios sobre el organismo, el ciclo biológico o los vectores. Sin embargo, es menos conocido el papel que la fauna silvestre, lo que genera un amplio campo de estudio poco explorado aún. Por ello, en esta Tesis Doctoral se han seleccionado animales silvestres residentes en España, cuyas áreas de distribución permiten la interacción con los humanos, los animales domésticos y los flebotomos. Además, se ha pretendido estudiar diferentes dianas de ADN para contribuir al análisis de las muestras de una manera más eficaz, seleccionando a su vez tejidos donde se cree más probable la detección del parásito. Asimismo, el análisis de varias regiones de ADN permite también detectar especies de *Leishmania* que no se han descrito anteriormente circulando en fauna autóctona en la península ibérica, más allá de los casos importados en humanos.

5.1.1. ANIMALES SILVESTRES COMO POTENCIALES RESERVORIOS DE *LEISHMANIA* SPP.

El enfoque One Health ha ganado un gran peso en los últimos años en la investigación sanitaria, poniendo de manifiesto la importancia de las interrelaciones existentes entre el humano, los animales y el ambiente, así como la necesidad de realizar estudios multidisciplinares para obtener resultados que abarquen todos estos campos. En el caso concreto de la leishmaniosis, esta aproximación incluye el análisis de los distintos factores que pueden influir en la epidemiología y dispersión de estos organismos, como los distintos vectores, el efecto del cambio climático y el papel que juega la fauna silvestre (Blake & Betson, 2016; Hong et al., 2020; Martín-Sánchez et al., 2020a).

Algunos estudios analizan el potencial papel de los animales silvestres en el ciclo de *Leishmania*, tanto en Europa como en otros continentes. Sin embargo, son muchos los aspectos que se desconocen y, sobre todo, muchas las técnicas y tipo de muestras analizadas, lo que dificulta aún más el trabajo con estos individuos (Roque & Jansen, 2014). A esto debemos sumar el escaso acceso a muestras en buenas condiciones de los animales silvestres, ya que solo una mínima parte ingresan en centros de recuperación de fauna o son recogidos por las autoridades competentes tras su muerte, por lo que, en general, estos estudios suelen contar con un número de muestras limitado o se deben alargar durante varios años para poder obtener un número significativo de animales. En el caso de la presente Tesis, se ha contado con la afortunada colaboración de varios centros de recuperación de fauna, lo que ha permitido tener acceso a diversas especies de animales, entre ellos el murciélago común urbano (*P. pipistrellus*). Otras especies de murciélago han sido analizadas en América del Sur y África

(Berzunza-Cruz et al., 2015; De Castro Ferreira et al., 2017; De Lima et al., 2008; Kassahun et al., 2015) pero en Europa no se había demostrado la presencia de *Leishmania* en estos animales, a pesar de la existencia de un estudio anterior, donde Millán et al. (2014b) analizaron varios ejemplares de *Miniopterus schreibersii* sin obtener resultados positivos a *L. infantum*. En nuestro caso, un 59,2% de los individuos analizados presentaron ADN de *L. infantum* al menos en una de las muestras analizadas (bazo, pelo abdominal y coágulo de sangre de corazón) (Azami- Conesa et al., 2020), siendo superior a lo obtenido en otros estudios sobre este parásito en murciélagos, donde se encontraron evidencias de la presencia de *L. braziliensis, L. major* y *L. mexicana* (Berzunza-Cruz et al., 2015; De Castro Ferreira et al., 2017; Kassahun et al., 2015). Parte de las diferencias halladas entre los resultados de los distintos estudios podría explicarse por las diferentes dianas de ADN utilizadas y las variadas especies de murciélago analizadas, así como la procedencia de éstos, ya que en nuestro caso se trata de animales procedentes de zonas urbanas y peri-urbanas, mientras que en otros casos se trata de animales selváticos. Sin embargo, nuestros resultados se asemejan a los obtenidos en otros de nuestros estudios, con animales de la misma zona (Comunidad de Madrid, Castilla y León y Castilla-La Mancha), como los tejones europeos (*M. meles*) con un 33% de animales positivos (Azami-Conesa et al., 2023). En este mismo artículo, obtuvimos un 12,76% de los erizos europeos (*E. europaeus*) y el 11,56% de las ardillas rojas (*S. vulgaris*) también positivos a *L. infantum*, donde incluimos individuos de las zonas anteriores, así como de la Comunidad Valenciana. Además, en la detección de *Leishmania* en gato montés (*F. silvestris*) obtuvimos un 38,89% de los animales positivos (Azami-Conesa et al., 2024) siguiendo los mismos protocolos, también con animales procedentes de Castilla y León y Castilla-La Macha. Previamente otros estudios obtuvieron resultados similares en los gatos monteses, del 25% en el caso de Del Río et al. (2014) y de Risueño et al. (2018), con 4 ejemplares analizados en cada estudio, o del 100% en el estudio de Oleaga et al. (2018) donde tan solo se analizaron 3 ejemplares procedentes de Asturias.

En el caso de los tejones, los resultados concuerdan con los obtenidos en otras publicaciones españolas e italianas, donde se encuentra entre el 26 y 53,3% de los animales positivos a *L. infantum* (Battisti et al., 2020; Del Río et al., 2014). Por su parte, los erizos europeos no han sido excesivamente estudiados, y solo encontramos dos artículos donde los analizan obteniendo entre el 34,4 y el 100% de los animales positivos a *L. infantum*, siendo superiores estos porcentajes a los nuestros (Alcover et al., 2020; Muñoz-Madrid et al., 2013). Esto mismo ocurre en el caso de la ardilla roja que, a pesar de ser un animal ampliamente distribuido en España (Purroy, 2007), solo encontramos una publicación en la que se analice la presencia del parásito, donde se obtuvo un resultado del 25% de los animales analizados positivos a *L. infantum* (Alcover et al., 2020). Esto es superior a lo obtenido en nuestra investigación, aunque podría deberse a la procedencia de los propios animales, ya que se lleva a cabo en Cataluña y con

ejemplares urbanos, mientras que en nuestro caso son animales procedentes de zonas rurales en su mayoría.

Uno de los grupos de silvestres menos estudiados es el de las especies consideradas como exóticas invasoras. Dentro de esta calificación encontramos al visón americano (*N. vison*), muy común en las riberas de ríos españoles, en especial en la Comunidad Valenciana, Galicia y Cantabria, y que suponen un problema relevante en los ecosistemas donde habitan por la competencia directa con otros animales autóctonos, como la nutria (*L. lutra*) o el visón europeo (*M. lutreola*) (Ruiz-olmo et al., 1997; Sidorovich et al., 1999). En el estudio incluido en esta Tesis se analizaron un total de 22 individuos, de los cuales 19 fueron positivos a *L. infantum* (90,1%). Alcover et al. (2020) obtuvieron también un resultado positivo en el único ejemplar analizado de esta especie en España, mientras que otros estudios realizados en granjas de cría de visón americano en Grecia se presentaron resultados algo menores, en torno al 20% (Filioussis et al., 2018; Tsakmakidis et al., 2019). Sin embargo, en estos últimos se utilizó como diana la *ITS*, de menor sensibilidad y con la que en nuestro caso obtuvimos un menor porcentaje de positivos, posiblemente porque no fue capaz de detectar las bajas concentraciones de ADN que suelen tener las muestras de fauna silvestre. Otros animales han sido estudiados en esta investigación, como el erizo moruno (*A. algirus*), la comadreja (*M. nivalis*), la garduña (*M. foina*), el lirón careto común (*E. quercinus*), el turón (*M. putorius*) y el meloncillo (*H. ichneumon*), con un número de individuos escaso, entre uno y tres de cada especie, y cuyos resultados fueron negativos en todos los individuos a la infección por *L. infantum* (Azami-Conesa et al., 2023), a pesar de que en la bibliografía existen evidencias de la presencia de este parásito en todos ellos (Chemkhi et al., 2015; Del Río et al., 2014; Oleaga et al., 2018; Ortuño et al., 2019; Risueño et al., 2018; Sobrino et al., 2008; Souguir-Omrani et al., 2018), excepto en la comadreja y el lirón careto. Cabe destacar que todas las muestras de los diversos animales analizados y que resultaron positivas, fueron secuenciadas y comparadas con secuencias depositadas en GenBank, obteniendo homologías del 100% con *L. infantum* y, en el caso del gato montés, con *L. tropica*. Los diversos estudios existentes acerca del posible papel como reservorios o, al menos, como especies centinela de la fauna silvestre, entre los que se incluyen los de esta Tesis Doctoral, no hacen más que evidenciar la necesidad de conocer el estado de la parasitosis en estos animales para poder manejar una información amplia y veraz, que nos permita dar una respuesta rápida y eficaz a posibles brotes de leishmaniosis humana, así como para conocer la distribución que este parásito podría tener en el medio natural.

5.1.2. UTILIDAD DE LA DETECCIÓN DE *LEISHMANIA* SPP. MEDIANTE PCR

Para la detección de la infección por *Leishmania* existen numerosas técnicas, como la serología, el xenodiagnóstico, la microscopía o el cultivo, pero, sin duda, la metodología más utilizada en los últimos años es la PCR debido a su elevada sensibilidad, su rapidez en la obtención de resultados y la variada información que se puede obtener a partir de esta técnica. Además, actualmente existen variantes de la PCR convencional que incrementan los beneficios, como la sensibilidad o la especificidad. Una de estas variantes es la PCR anidada, que permite una mayor sensibilidad de los resultados, aunque el riesgo de contaminación es mayor (Hong et al., 2020).

En el desarrollo de la PCR, sea cual sea la variante, es importante la selección de la diana a utilizar, es decir, el fragmento de ADN que se quiere amplificar, ya que esto determinará la sensibilidad y especificidad, la fiabilidad y el éxito de los resultados (Lachaud et al., 2002b). Es por esto que uno de los objetivos de esta Tesis Doctoral es el análisis de diferentes dianas del ADN utilizadas frecuentemente en la detección en fauna silvestre, para conocer la sensibilidad y especificidad de cada una, bajo las condiciones y protocolos usados en las muestras que manejamos. Algunas de las más utilizadas (Akhoundi et al., 2017; Azami-Conesa et al., 2021a; Lachaud et al., 2002b; Pereira et al., 2020) y seleccionadas para nuestros estudios fueron:

- *Repeat region*: codifica para la región repetitiva del ADN nuclear con elevada sensibilidad y con alta especificidad para *L. infantum*.
- *kDNA*: codifica para el ADN mitocondrial del kinetoplasto, exclusivo del orden Kinetoplastida, que otorga gran sensibilidad.
- *SSUrRNA*: codifica para la subunidad pequeña del ribosoma (SSU o 18S). Habitualmente utilizada en PCR anidada. De gran sensibilidad, pero con menor capacidad de diferenciar especies dentro del género.
- *ITS1*: codifica para los espaciadores internos de transcripción. Situado entre SSU y LSU (subunidad ribosomal grande, 28S). Empleada para genotipado por su especificidad.
- *Hsp70*: Heat- Shock Protein 70. Codifica para la proteína de choque térmico 70. Con alta especificidad.

Los resultados obtenidos varían en función de la muestra utilizada, seleccionando en nuestro caso cultivo de promastigotes de *L. infantum* o médula ósea de perro infectado con *L. infantum*, para analizar la capacidad de detección de las dianas (con la excepción de la *hsp70*), siendo en ambos casos mayor la sensibilidad de la *Repeat region* y el *kDNA*, seguido de cerca por la *SSUrRNA* y en último lugar la *ITS1* (Tablas 3 y 4 en Azami-Conesa et al., 2021b). En el caso de la *Repeat*

region, esta elevada sensibilidad la observamos también en otros artículos de esta investigación al utilizar muestras de murciélago y de visón americano (Azami-Conesa et al., 2020, 2021b), así como en otros animales como tejones, erizos y ardillas, donde esta diana resultó, en general, más sensible que la *SSUrRNA* (Azami-Conesa et al., 2023). La alta sensibilidad de la *Repeat region* concuerda con resultados obtenidos por otros autores al compararlo con diversas dianas (Chemkhi et al., 2015; Lachaud et al., 2002b) (Tabla 3). Sin embargo, la *Repeat region* tiene el inconveniente de que solo amplifica la especie *L. infantum*. El *kDNA* es una de las regiones más utilizadas en la detección de *Leishmania* y los resultados obtenidos en cultivo y médula ósea confirman una elevada sensibilidad. No obstante, al utilizar muestras de visón americano, no obtuvimos ningún resultado positivo, a pesar de la elevada prevalencia que nos indican otras dianas en los mismos individuos (Tabla 3). Algunos autores indican que pequeños cambios en la secuencia del parásito podrían modificar notablemente la sensibilidad con esta diana, lo cual se ve reforzado por la elevada variedad de resultados en los trabajos que utilizan estos protocolos (Azami-Conesa et al., 2021a; Cardoso et al., 2021). Cabe destacar otro aspecto relevante a la hora de trabajar con el *kDNA* y se trata de la elección de los primers. Existe una amplia selección de cebadores para amplificar esta región, por lo que la elección resulta a veces compleja y puede ser determinante a la hora de obtener resultados satisfactorios (Albuquerque et al., 2017; De Oliveira et al., 2011; Lachaud et al., 2002b). En el caso de la *SSUrRNA*, la mayor parte de los autores coinciden en que se trata de una diana altamente sensible, lo que encaja con los resultados obtenidos en nuestros estudios, como es el caso del estudio en gato montés, donde obtuvimos un 38,9% de los resultados positivos (Azami-Conesa et al., 2024), aunque con la excepción de los visones americanos, donde fueron escasos los positivos obtenidos (2 de 22) en comparación con los de la *Repeat región* (20 de 22) (Azami-Conesa et al., 2021b). En cuanto a la *ITS1*, los límites de detección fueron menores si los comparamos con la *Repeat region* y el *kDNA* al usar cultivo de promastigotes y médula ósea de animal infectado. Esto ocurre también al usar las muestras de visones americanos, donde no se obtuvieron resultados positivos. No obstante, en el caso de erizos, tejones, ardillas y gato montés, se obtuvieron resultados satisfactorios, teniendo en cuenta que se analizaron solo los ejemplares que resultaron positivos a otras dianas como la *Repeat Region* y la *SSUrRNA* (Tabla 3). Si lo comparamos con estudios previos, observamos que son numerosos los que indican bajas sensibilidades de la *ITS1*, incluido en visones americanos (Tsakmakidis et al., 2019) y en erizos (Chemkhi et al., 2015). Sin embargo, esta diana presenta una elevada especificidad por lo que resulta de gran utilidad en estudios filogenéticos y para identificar hasta el nivel de especie (Echchakery et al., 2017; Pereira et al., 2020).

Otra de las dianas ampliamente utilizadas a la hora de diferenciar entre especies de *Leishmania* es la *hsp70*, utilizada en nuestro caso, en aquellos gatos monteses positivos a *SSUrRNA* (Azami-Conesa et al., 2024), presentando una alta especificidad, aunque una menor sensibilidad según nuestros datos

(Tabla 3), lo que nos permite detectar especies foráneas del parásito y que ha sido muy utilizada en estudios filogenéticos por diversos autores (Fernández-Arévalo et al., 2022; Pereira et al., 2020; Van der Auwera et al., 2013).

Tabla 3. Porcentajes de animales positivos a *L. infantum* y/o *L. tropica* en relación con la diana de ADN utilizada en los diferentes estudios que se incluyen

	Murciélago urbano común	**Visón Americano**	**Tejón europeo**	**Erizo europeo**	**Ardilla roja**	**Gato montés**
Repeat region	59.2%	90.1%	14,29%	8,43%	11,54%	n.a.
kDNA	n.a.	0%	n.a.	n.a.	n.a.	n.a.
SSUrRNA	n.a.	9,09%	28,57%	4,81%	3,85%	38,9%
ITS1*	n.a.	0%	21,43%	2,04%	0%	22,2%
hsp70*	n.a.	n.a.	n.a.	n.a.	n.a.	22,2%

n.a.: no analizado. *: PCR realizada solo en animales positivos a otras dianas.

La elección del tipo de muestra a analizar resulta igual de importante que lo mencionado anteriormente respecto a los fragmentos de ADN a amplificar. En la bibliografía encontramos una elevada variedad de muestras capaces de poner de manifiesto la presencia de este parásito en los hospedadores mediante PCR, pero, dependiendo de la especie de *Leishmania* y del tipo de animal, algunas resultan de mayor utilidad.

Para la detección de *L. infantum* es habitual utilizar muestras de bazo, hígado, médula ósea, aspirado de ganglio linfático y sangre periférica, debido al desarrollo visceral que presenta esta parasitosis y, en el caso de la sangre, a la necesidad de estar disponible para el vector (Azami-Conesa et al., 2021a). En animales silvestres, es habitual que estas muestras procedan de individuos fallecidos por diversas causas, como atropellos, traumatismos severos o enfermedad, por lo que resulta sencillo obtener este tipo de muestras, la mayoría de ellas altamente invasivas. Si nos centramos en la sensibilidad que presentan las distintas muestras, se piensa que varía notablemente dependiendo de la especie animal, la procedencia del individuo, la fase de la infección, así como la respuesta inmunitaria del propio hospedador (Millán et al., 2014a; Risueño et al., 2018), lo cual hemos podido comprobar con nuestros propios resultados, donde los resultados positivos varían notablemente según el animal, desde el 90% de positivos en bazo en visón americano al 6,02% en bazo de los erizo europeos. Es escasa la información que encontramos sobre el desarrollo de la parasitosis en animales silvestres, pero si nos basamos en cómo y dónde se localizan los amastigotes en los perros (principal reservorio), podríamos comprender las altas sensibilidades en muestras como el bazo, donde *L. infantum* se acantona, provocando cambios en la microestructura del órgano

en sus fases más avanzadas. También es habitual emplear como órgano diana la piel, tejido involucrado en la transmisión al flebotomo, siendo el primero parasitado en las etapas iniciales de la infección. Y, por último, la sangre, donde se presupone que se encuentran los macrófagos parasitados a disposición del vector (Morales-Yuste et al., 2022). La elevada sensibilidad de las muestras de bazo la podemos observar también en los animales estudiados en los artículos que se presentan en este trabajo, ya que supone uno de los tejidos con mayores porcentajes de resultados positivos en murciélagos, visón americano, tejón europeo, erizo europeo y ardilla roja, como se puede observar en la tabla 4, siendo los porcentajes de parasitación en bazo bastante menores en el gato montés, aunque en este último caso quizás sea debido a la presencia de *L. tropica*, de mayor tropismo cutáneo (Azami-Conesa et al., 2020, 2021b, 2023, 2024). Estos resultados siguen la línea de otros estudios, donde también se ha detectado una elevada sensibilidad en bazo de murciélagos (a pesar de que las especies detectadas eran diferentes a *L. infantum* y con alto carácter cutáneo en humanos) (Berzunza- Cruz et al., 2015; De Oliveira et al., 2015), en visón americano (Alcover et al., 2020; Filioussis et al., 2018; Tsakmakidis et al., 2019), en el único estudio que analiza bazo de erizo europeo y ardilla roja (Alcover et al., 2020) y en tejón europeo (Battisti et al., 2020; Del Río et al., 2014). En el caso del gato montés, también es posible que la mayor sensibilidad de las muestras de piel de oreja sea debida a la propia respuesta inmunitaria del animal o por la similitud al gato doméstico, donde las lesiones cutáneas por *L. infantum* son habituales (Batista et al., 2023; Cardoso et al., 2021; Pennisi et al., 2013; Pennisi & Persichetti, 2018). De hecho, la piel de oreja ha resultado ser una muestra interesante en el caso del gato montés, con una mayor sensibilidad frente al bazo (7 animales positivos frente a 2, respectivamente), lo que podría deberse a la presencia de *L. tropica*, cuyo desarrollo cutáneo es más frecuente, así como en el caso de erizos y tejones, donde 9 de los 113 animales tuvieron resultados positivos en piel a *L. infantum*. Sin embargo, en las ardillas, este tejido no arrojó resultados positivos en nuestro estudio, a pesar de haber 3 individuos positivos a bazo, y de lo obtenido por Alcover et al. (2020), donde 2 de los 12 animales analizados fueron positivos en piel. En el caso de los murciélagos, se estudió la sensibilidad de la sangre procedente de coágulo de corazón, seleccionada por las publicaciones científicas donde se analizaba la sangre periférica de diferentes especies de estos animales (De Lima et al., 2008; De Rezende et al., 2017; Gómez-Hernández et al., 2017). Seis de los 27 animales fueron positivos (22.22%), lo que implica una menor sensibilidad que en el caso del bazo, pero similar a la del pelo (Azami-Conesa et al., 2020), y similar a lo obtenido en otros artículos llevados a cabo en América del Sur y Centroamérica, como en el caso de Gómez- Hernández et al. (2017) o del estudio de De Rezende et al. (2017) donde el 8% y el 30% de las muestras de sangre analizadas, respectivamente, fueron positivas a distintas especies de *Leishmania*, entre ellas *L. infantum,* a pesar de haberse realizado en otras especies de quirópteros y con dianas diferentes a las utilizadas en nuestro estudio.

No debemos olvidar que para la recolección de las muestras anteriores se usan técnicas altamente invasivas, requiriendo en la mayoría de las ocasiones la muerte del animal para poder obtenerlas, con la excepción de la sangre, la punción esplénica ecoguiada y el aspirado de ganglio linfático. Por ello, en los últimos años, encontramos autores como Muñoz-Madrid et al. (2013), Ortega et al. (2017) y una de las publicaciones de esta Tesis Doctoral (Azami-Conesa et al., 2020), donde se analiza la utilidad del pelo para la detección de *L. infantum*, obteniendo en todos los casos resultados remarcables como son el 20,6%, alrededor del 80% y del 44% de resultados positivos (Tabla 4), respectivamente. Estos datos son cuanto menos esperanzadores, ya que la obtención de muestras no invasivas puede resultar de gran utilidad a la hora de realizar estudios epidemiológicos relacionados con esta parasitosis y su situación en la fauna silvestre. No obstante, otras muestras no invasivas, como la conjuntiva oral y ocular en erizos, han sido analizados en nuestros estudios sin encontrar resultados positivos, a pesar de que uno de los individuos analizados fue positivo a *L. infantum* en bazo. Este tipo de muestras podría resultar interesante, pero acarrea algunos inconvenientes como la toma de la propia muestra que requiere experiencia previa o que la parasitación del individuo sea baja y esta muestra no resulte útil por tener menor sensibilidad, y quizás sea de más utilidad en el caso de animales con signos clínicos evidentes. En la literatura encontramos dos artículos que obtuvieron resultados positivos con estas muestras, en perro y erizo moruno (Aschar et al., 2016; Souguir-Omrani et al., 2018).

Tabla 4. Porcentaje de animales positivos según la muestra a analizar mediante PCR.

	Murciélago urbano común	**Visón americano**	**Tejón europeo**	**Erizo europeo**	**Ardilla roja**	**Gato montés**
Bazo	51,85%	90,1%	28,57%	6,02%	11,54%	11,11%
Piel de oreja	n.a.	n.a.	14,29%	6,02%	0%	38,9%
Pelo	25,93%	n.a.	n.a.	n.a.	n.a.	n.a.
Coágulo de sangre	22,22%	n.a.	n.a.	n.a.	n.a.	n.a.

n.a.: no analizado

Cabe destacar la ausencia de lesiones características en los animales analizados, a pesar de la evidencia que podemos encontrar en la bibliografía de lesiones cutáneas, especialmente en animales como chacales, lobos, zorros y roedores, alguno de ellos considerados reservorios (Echchakery et al., 2017; Helhazar et al., 2013; Karayiannis et al., 2015; Moemenbellah-Fard et al., 2003; Mohebali et al., 2016; Oleaga et al., 2015). Esto nos hace pensar que el nivel de parasitación en las especies estudiadas sea bajo. Aun así, las técnicas empleadas han resultado

de utilidad en esta Tesis, tanto para detectar animales positivos (*Repeat region*, *SSUrDNA*), como para determinar la especie de *Leishmania* (*ITS1, hsp70*).

5.1.3. EXPANSIÓN DE LAS ESPECIES DE *LEISHMANIA*

L. infantum ha sido, hasta el momento, la única especie de este género con transmisión autóctona en España. A pesar de ello, desde hace varios años se detectan habitualmente poblaciones de flebotomos que son vectores principales de otras especies de *Leishmania*, como *P. sergenti*, principal vector de *L. tropica*, una especie foránea (Barón et al., 2013; Berriatua et al., 2023; Gálvez et al., 2020; Merino-Espinosa et al., 2016). Además, en países cercanos, como Marruecos, Túnez o Turquía, esta especie del parásito está asentada y ampliamente distribuida (Echchakery et al., 2020; El Idrissi Saik et al., 2022; El Kacem et al., 2021). Por ello, no es de extrañar que, desde hace un tiempo, varios investigadores hayan puesto el foco en la posibilidad de que *L. tropica* y otras especies de *Leishmania* sean capaces de establecerse en la península ibérica, teniendo en cuenta, además, que la climatología cada vez es más similar a la que encontramos en nuestros vecinos del norte de África (Barón et al., 2013; Cunze et al., 2019; Gálvez et al., 2020; Merino-Espinosa et al., 2016).

Tanto la presencia del vector como la del parásito, son factores críticos para que ocurra una transmisión local de una especie foránea y esto mismo se establece en España (Barón et al., 2013). Por un lado, *P. sergenti* lleva varios años presente en el país, extendiéndose progresivamente, y se han reportado casos importados de *L. tropica* en personas que habían viajado a zonas endémicas, como los casos clínicos descritos por Knöpfel et al. (2018) en Madrid, con claros signos clínicos de leishmaniosis cutánea y diagnóstico molecular de *L. tropica*. Asimismo, es altamente probable que los movimientos globales de personas y animales entre los países vecinos faciliten la llegada de esta especie del parásito a la península ibérica y otros países del sur de Europa, como Italia, donde se sugiere el traslado de perros del norte de África como posible vía de entrada (Gramiccia & Gradoni, 2005).

La fauna silvestre puede jugar un papel crucial en la detección de la llegada de especies foráneas de *Leishmania*, ya que arrojan información sobre la situación epidemiológica en una región determinada, en especial en aquellas regiones poco pobladas, como es el caso de Castilla y León, donde la detección de *L. tropica* en nuestro estudio abre las puertas a la posibilidad de que este parásito se encuentre circulando más habitualmente de lo que se pensaba hasta el momento. De los 18 gatos monteses analizados, 7 fueron positivos a *Leishmania* spp. De ellos, un individuo presentaba infección por *L. tropica*, según los datos obtenidos con la *ITS1* y la *hsp70*, siendo ambas dianas muy específicas y permitiendo la diferenciación a nivel de especie. Otros dos ejemplares fueron claramente positivos a *L. infantum*, tanto en piel como en bazo. Y un tercer gato montés presentó resultados mixtos de *L. infantum* y *L. tropica*, lo que podría indicar una infección mixta o una

hibridación de ambas especies. Los tres animales restantes fueron positivos a *SSUrRNA* exclusivamente, diana que no es capaz de diferenciar correctamente entre especies de *Leishmania*. Además, cabe destacar que los individuos que presentaban *L. tropica* y la infección mixta, solo fueron positivos a las muestras de piel, lo que podría reforzar los resultados, teniendo en cuenta el carácter preferentemente cutáneo de la especie no autóctona, al menos en humanos (Maroli et al., 2013).
Para comprender estos resultados, podemos fijarnos en estudios anteriores donde se reportan casos de infecciones mixtas entre diversas especies, como es el caso de *L. infantum* y *L. braziliensis* en roedores y perros (Alves Souza et al., 2019; De Castro Ferreira et al., 2015), en erizos con *L. major* y *L. infantum* (Souguir-Omrani et al., 2018) o de *L. tropica* con *L. infantum* en gatos domésticos (Can et al., 2016), por lo que no resulta descabellado pensar que si las dos especies comparten espacio y tiempo, podrían provocar este tipo de infección en los gatos monteses.

Otra posibilidad es la hibridación, demostrada *in vitro* en *L. tropica*, y cuya tasa de hibridación natural es ampliamente superior a la de otras especies de este género (Louradour et al., 2020). Además, se ha demostrado que los flebotomos capaces de transmitir *L. infantum* son susceptibles de albergar y actuar como vectores de *L. tropica*, como es el caso de *P. perniciosus*, vector habitual de *L. infantum*, y que ha demostrado capacidad *in vitro* de albergar *L. tropica* (Bongiorno et al., 2019; Vaselek & Volf, 2019). Sin embargo, *P. sergenti* presenta una alta exclusividad con *L. tropica* y, hasta el momento, no se ha detectado otra especie del parásito en su interior (Barón et al., 2013; Berriatua et al., 2023; Gramiccia & Gradoni, 2005; Merino-Espinosa et al., 2016).

CAPÍTULO 6:

CONCLUSIONES

Como resultado de los estudios realizados en la presente Tesis Doctoral se han obtenido las siguientes conclusiones:

1. Se ha descrito por primera vez en Europa la presencia de *Leishmania infantum* en murciélago urbano común en áreas urbanas y periurbanas con una alta prevalencia. Se trata de la especie de quiróptero más abundante de la península ibérica, cuyos hábitos de vida permiten que cohabiten en el mismo nicho ecológico el vector, el parásito, el hombre y otros reservorios, como el perro.

2. El tejón europeo, el erizo europeo, la ardilla roja, el murciélago urbano común y el gato montés son capaces de albergar *Leishmania* spp. en diferentes órganos del cuerpo y con porcentajes de parasitación notables. Estos animales están ampliamente distribuidos por la península ibérica, pudiendo tener un papel relevante en el mantenimiento del ciclo rural del parásito.

3. El visón americano procedente de la Comunidad Valenciana ha presentado una elevada prevalencia de *Leishmania infantum*. Esto supone que las especies exóticas invasoras podrían jugar un papel importante en la permanencia y transmisión de este parásito en zonas de ribera, donde el vector se encuentra de manera habitual.

4. El bazo ha resultado ser el órgano más sensible para la detección de *Leishmania* spp. mediante PCR convencional, en relación a otras muestras invasivas frecuentemente utilizadas, con la excepción del gato montés, donde la piel resultó más sensible. La predisposición cutánea o visceral de cada especie de *Leishmania* debe tenerse en cuenta a la hora de analizar los resultados.

5. Las muestras no invasivas como el pelo resultaron positivas en los murciélagos urbanos comunes, reforzando la posibilidad de usar este tipo de muestras para la detección de *Leishmania infantum*.

6. A la vista de los resultados obtenidos, existen diferencias significativas a la hora de elegir la diana de ADN a utilizar. La *SSUrRNA* y la *Repeat region* resultan adecuadas para la detección de *Leishmania* spp. debido a su elevada sensibilidad. Sin embargo, si el objetivo es diferenciar especies del género, sería más efectivo utilizar la *hsp70* o la *ITS1*, ambas con gran especificidad.

7. Se ha detectado por primera vez en España la presencia de *Leishmania tropica* en fauna silvestre. Hasta la fecha, *Leishmania infantum* era la única especie del género con presencia en fauna autóctona en la península ibérica. El establecimiento de una especie foránea en nuestro territorio supone un nuevo reto epidemiológico, donde profesionales de la salud humana, animal y ambiental deben trabajar conjuntamente para la detección, prevención y control de esta nueva especie.

BIBLIOGRAFÍA

Adl, S. M., Bass, D., Lane, C. E., Lukeš, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., del Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., … Zhang, Q. (2019). Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. *Journal of Eukaryotic Microbiology*, *66*(1), 4–119. https://doi.org/10.1111/jeu.12691

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., … Taylor, M. F. J. R. (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology*, *52*(5), 399–451. https://doi.org/10.1111/j.1550-7408.2005.00053.x

Aguirre, A. A. (2009). Wild canids as sentinels of ecological health: A conservation medicine perspective. *Parasites and Vectors*, *2*(SUPPL.1), 1–8. https://doi.org/10.1186/1756-3305-2-S1-S7

Aguirre, A. A., & Tabor, G. M. (2008). Global factors driving emerging infectious diseases: Impact on wildlife populations. *Annals of the New York Academy of Sciences*, *1149*, 1–3. https://doi.org/10.1196/annals.1428.052

Ait Kbaich, M., Mhaidi, I., Ezzahidi, A., Dersi, N., El Hamouchi, A., Riyad, M., Akarid, K., & Lemrani, M. (2017). New epidemiological pattern of cutaneous leishmaniasis in two pre-Saharan arid provinces, southern Morocco. *Acta Tropica*, *173*(March), 11–16. https://doi.org/10.1016/j.actatropica.2017.05.016

Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M., Granouillac, B., Gradoni, L., & Sereno, D. (2017). *Leishmania* infections: Molecular targets and diagnosis. *Molecular Aspects of Medicine*, *57*, 1–29. https://doi.org/10.1016/j.mam.2016.11.012

Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., & Sereno, D. (2016). A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. *PLoS Neglected Tropical Diseases*, *10*(3), 1–40. https://doi.org/10.1371/journal.pntd.0004349

Albuquerque, A., Campino, L., Cardoso, L., & Cortes, S. (2017). Evaluation of four molecular methods to detect *Leishmania* infection in dogs. *Parasites & Vectors*, *10:57*. https://doi.org/10.1186/s13071-017-2002-2

Alcover, M. M., Ribas, A., Guillén, M. C., Berenguer, D., Tomás-Pérez, M., Riera, C., & Fisa, R. (2020). Wild mammals as potential silent reservoirs of *Leishmania infantum* in a Mediterranean area. *Preventive Veterinary Medicine*, *175*. https://doi.org/10.1016/j.prevetmed.2019.104874

Alcover, M. M., Riera, M. C., & Fisa, R. (2021). Leishmaniosis in Rodents Caused by *Leishmania infantum*: A Review of Studies in the Mediterranean Area. *Frontiers in Veterinary Science*, *8*(August). https://doi.org/10.3389/fvets.2021.702687

Alexander, B., Lozano, C., Barker, D. C., McCann, S. H. E., & Adler, G. H. (1998). Detection of *Leishmania* (*Viannia*) *braziliensis* complex in wild mammals from Colombian coffee plantations by PCR and DNA hybridization. *Acta Tropica*, *69*(1), 41–50. https://doi.org/10.1016/S0001-706X(97)00114-9

Alonso Capitán, M., & Vázquez Torres, M. C. (2013). Impactos del Cambio Climático en la Salud. M. Alonso Capitán & M. C. Vázquez Torres (eds.). *Ministerio de Sanidad, Asuntos Sociales e Igualdad.*

Alten, B., Ozbel, Y., Ergunay, K., Kasap, O. E., Cull, B., Antoniou, M., Velo, E., Prudhomme, J., Molina, R., Banuls, A. L., Schaffner, F., Hendrickx, G., Van Bortel, W., & Medlock, J. M. (2015). Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe. *Bulletin of Entomological Research*, *105*(6), 664–678. https://doi.org/10.1017/S0007485315000127

Alves Souza, N., Souza Leite, R., de Oliveira Silva, S., Groenner Penna, M., Figueiredo Felicori Vilela, L., Melo, M. N., & de Andrade, A. S. R. (2019). Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil. *Acta Tropica*, *193*(February), 12–17. https://doi.org/10.1016/j.actatropica.2019.02.016

Amela, C., Suarez, B., Isidoro, B., Sierra, M. J., Santos, S., & Simón, F. (2012). Evaluación del Riesgo de Transmisión de Leishmania infantum en España (2012th ed.). *Centro de Coordinación de Alertas y Emergencias Sanitarias (CCAES). Dirección General de Salud Pública, Calidad e Innovación. Ministerio de Sanidad, Servicios Sociales e Igualdad*.

Aransay, A. M., Scoulica, E., & Tselentis, Y. (2000). Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. *Applied and Environmental Microbiology*, *66*(5), 1933–1938. https://doi.org/10.1128/AEM.66.5.1933-1938.2000

Arce, A., Estirado, A., Ordobás, M., Moratilla, L., García, N., Pérez, A. M., Gil, E., Aranguez, E., García Puente, E., & Martínez, A. M. (2017). Brote de leishmaniasis en la zona suroeste de la Comunidad de Madrid: detección e investigación epidemiológica. In C. de S. Dirección General

de Salud Pública (Ed.), *Brote de Leishmaniasis en Fuenlabrada y otros municipios de la Comunidad de Madrid. El papel de las liebres y los conejos como reservorios* (2017th ed., pp. 105–112). http://www.madrid.org/bvirtual/BVCM017962.pdf

Armúa-Fernández, M. T., & Venzal, J. M. (2019). Leishmaniosis: breve puesta al día. *Veterinaria (Montevideo)*, *55*(211), 29–36. https://doi.org/10.29155/vet.55.211.5

Aschar, M., de Oliveira, E. T. B., Laurenti, M. D., Marcondes, M., Tolezano, J. E., Hiramoto, R. M., Corbett, C. E. P., & Da Matta, V. L. R. (2016). Value of the oral swab for the molecular diagnosis of dogs in different stages of infection with *Leishmania infantum*. *Veterinary Parasitology*, *225*, 108–113. https://doi.org/10.1016/j.vetpar.2016.06.005

Ashford, R. W. (1996). Leishmaniasis reservoirs and their significance in control. *Clinics in Dermatology*, *14*(5), 523–532. https://doi.org/10.1016/0738-081X(96)00041-7

Azami-Conesa, I., Gómez-Muñoz, M. T., & Martínez-Díaz, R. A. (2021a). A systematic review (1990–2021) of wild animals infected with zoonotic *Leishmania*. *Microorganisms*, *9*(5). https://doi.org/10.3390/microorganisms9051101

Azami-Conesa, I., Martínez-Díaz, R. A., González, F., & Gómez-Muñoz, M. T. (2020). First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe. *Research in Veterinary Science*, *132*, 172–176. https://doi.org/10.1016/j.rvsc.2020.06.019

Azami-Conesa, I., Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in non-endemic areas: first detection of *Leishmania tropica* in wildlife in Spain. *Transboundary and Emerging Diseases,* 2024 (8259712), 1-10; https://doi.org/10.1155/2024/8259712.

Azami-Conesa, I., Pérez-Moreno, P., Matas Méndez, P., Sansano-Maestre, J., González, F., Mateo Barrientos, M., & Gómez-Muñoz, M. T. (2023). Occurrence of *Leishmania infantum* in Wild Mammals Admitted to Recovery Centers in Spain. *Pathogens*, *12*(1048), 1–11. https://doi.org/doi.org/10.3390/pathogens12081048

Azami-Conesa, I., Sansano-Maestre, J., Martínez-Díaz, R. A., & Gómez-Muñoz, M. T. (2021b). Invasive species as hosts of zoonotic infections: The case of american mink (*Neovison vison*) and *Leishmania infantum*. *Microorganisms*, *9*(7), 1–10. https://doi.org/10.3390/microorganisms9071531

Babuadze, G., Alvar, J., Argaw, D., de Koning, H. P., Iosava, M., Kekelidze, M., Tsertsvadze, N., Tsereteli, D., Chakhunashvili, G., Mamatsashvili, T., Beria, N., Kalandadze, I., Ejov, M., &

Imnadze, P. (2014). Epidemiology of Visceral Leishmaniasis in Georgia. *PLoS Neglected Tropical Diseases*, *8*(3), 1–8. https://doi.org/10.1371/journal.pntd.0002725

Baghad, B., Razanapinaritra, R., Maksouri, H., El Bouri, H., Outlioua, A., Fellah, H., Lemrani, M., Akarid, K., Martin-Sanchez, J., Chiheb, S., & Riyad, M. (2020). Possible introduction of *Leishmania tropica* to urban areas determined by epidemiological and clinical profiles of patients with cutaneous leishmaniasis in Casablanca (Morocco). *Parasite Epidemiology and Control*, *9*, e00129. https://doi.org/10.1016/j.parepi.2019.e00129

Bakhshi, H., Oshaghi, M. A., Abai, M. R., Rassi, Y., Akhavan, A. A., Mohebali, M., Hajaran, H., Mohtarami, F., Mirzajani, H., & Maleki-Ravasan, N. (2013). MtDNA cytb structure of *Rhombomys opimus* (Rodentia: Gerbellidae), the main reservoir of cutaneous leishmaniasis in the borderline of Iran-Turkmenistan. *Journal of Arthropod-Borne Diseases*, *7*(2), 173–184. PMID: 24409443; PMCID: PMC3875884.

Baneth, G., Nachum-Biala, Y., Adamsky, O., & Gunther, I. (2022). *Leishmania tropica* and *Leishmania infantum* infection in dogs and cats in central Israel. *Parasites and Vectors*, *15*(1), 1–9. https://doi.org/10.1186/s13071-022-05272-0

Baneth, G., & Solano-Gallego, L. (2022). Leishmaniasis. *Veterinary Clinics of North America - Small Animal Practice*, *52*(6), 1359–1375. https://doi.org/10.1016/j.cvsm.2022.06.012

Barón, S. D., Morillas-Márquez, F., Morales-Yuste, M., Díaz-Sáez, V., Gállego, M., Molina, R., & Martín-Sánchez, J. (2013). Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in south-western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917. *Parasitology*, *140*(11), 1413–1421. https://doi.org/10.1017/S0031182013000942

Barreto-de-Albuquerque, J., Silva-dos-Santos, D., Pérez, A. R., Berbert, L. R., de Santana-van-Vliet, E., Farias-de-Oliveira, D. A., Moreira, O. C., Roggero, E., de Carvalho-Pinto, C. E., Jurberg, J., Cotta-de-Almeida, V., Bottasso, O., Savino, W., & de Meis, J. (2015). *Trypanosoma cruzi* infection through the oral route promotes a severe infection in mice: New disease form from an old infection? *PLoS Neglected Tropical Diseases*, *9*(6), 1–21. https://doi.org/10.1371/journal.pntd.0003849

Bates, P. A. (2007). Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. *International Journal for Parasitology*, *37*(10), 1097–1106. https://doi.org/10.1016/j.ijpara.2007.04.003

Batista, J. F., Neto, F. das C. R. M., Lopes, K. S. P. do P., Sousa, C. M. G., Alcântara, D. S., Baêta, S. de A. F., Alves, M. M. de M., & de Mendonça, I. L. (2023). Clinical, hematological, biochemical, and histopathological evaluations in domestic cats (*Felis catus*) infected by

Leishmania infantum. *Revista Brasileira de Parasitologia Veterinaria*, *32*(3), 1–10. https://doi.org/10.1590/S1984-29612023037

Battisti, E., Zanet, S., Khalili, S., Trisciuoglio, A., Hertel, B., & Ferroglio, E. (2020). Molecular Survey on Vector-Borne Pathogens in Alpine Wild Carnivorans. *Frontiers in Veterinary Science*, *7*(January), 1–9. https://doi.org/10.3389/fvets.2020.00001

Baxarias, M., Homedes, J., Mateu, C., Attipa, C., & Solano-Gallego, L. (2022). Use of preventive measures and serological screening tools for *Leishmania infantum* infection in dogs from Europe. *Parasites and Vectors*, *15*(1), 1–11. https://doi.org/10.1186/s13071-022-05251-5

Berriatua, E., Pérez-Cutillas, P., González Vidal, A., & Briët, O. (2023). *European Centre for Disease Prevention and Control. The spatial relationship between the presence and absence of Leishmania spp. and leishmaniasis, and phlebotomine sand fly vectors in Europe and neighbouring countries.* www.ecdc.europa.eu

Berzunza-Cruz, M., Rodríguez-Moreno, Á., Gutiérrez-Granados, G., González-Salazar, C., Stephens, C. R., Hidalgo-Mihart, M., Marina, C. F., Rebollar-Téllez, E. A., Bailón-Martínez, D., Balcells, C. D., Ibarra-Cerdeña, C. N., Sánchez-Cordero, V., & Becker, I. (2015). *Leishmania* (L.) *mexicana* Infected Bats in Mexico: Novel Potential Reservoirs. *PLoS Neglected Tropical Diseases*, *9*(1). https://doi.org/10.1371/journal.pntd.0003438

Blake, D. P., & Betson, M. (2016). One Health: parasites and beyond. *Parasitology*, *144*, 1–6. https://doi.org/10.1017/S0031182016001402

Boehme, C. C., Hain, U., Novosel, A., Eichenlaub, S., Fleischmann, E., & Löscher, T. (2006). Congenital visceral leishmaniasis. *Emerging Infectious Diseases*, *12*(2), 359–360. https://doi.org/10.3201/eid1202.050449d

Boggiatto, P. M., Gibson-Corley, K. N., Metz, K., Gallup, J. M., Hostetter, J. M., Mullin, K., & Petersen, C. A. (2011). Transplacental transmission of *Leishmania infantum* as a means for continued disease incidence in North America. *PLoS Neglected Tropical Diseases*, *5*(4). https://doi.org/10.1371/journal.pntd.0001019

Bonesi, L., & Palazon, S. (2007). The American mink in Europe: Status, impacts, and control. *Biological Conservation*, *134*(4), 470–483. https://doi.org/10.1016/j.biocon.2006.09.006

Bongiorno, G., Di Muccio, T., Bianchi, R., Gramiccia, M., & Gradoni, L. (2019). Laboratory transmission of an Asian strain of *Leishmania tropica* by the bite of the southern European sand fly *Phlebotomus perniciosus*. *International Journal for Parasitology*, *49*(6), 417–421. https://doi.org/10.1016/j.ijpara.2018.12.009

Bouabdallah, S., Cianfaglione, K., Azzouz, M., Batiha, G. E. S., Alkhuriji, A. F., Al-Megrin, W. A. I., Ben-Attia, M., & Eldahshan, O. A. (2022). Sustainable Extraction, Chemical Profile, Cytotoxic and Antileishmanial Activities In-Vitro of Some Citrus Species Leaves Essential Oils. *Pharmaceuticals*, *15*(9). https://doi.org/10.3390/ph15091163

Bravo-Barriga, D., Ruiz-Arrondo, I., Peña, R. E., Lucientes, J., & Delacour-Estrella, S. (2022). Phlebotomine sand flies (Diptera, Psychodidae) from Spain: an updated checklist and extended distributions. *ZooKeys*, *1106*, 81–99. https://doi.org/10.3897/zookeys.1106.81432

Bray, D. P., & Hamilton, J. G. C. (2007). Host odor synergizes attraction of virgin female *Lutzomyia longipalpis* (Diptera: Psychodidae). *Journal of Medical Entomology*, *44*(5), 779–787. https://doi.org/10.1603/0022-2585(2007)44[779:HOSAOV]2.0.CO;2

Caldart, E. T., Pinto-Ferreira, F., de Matos, A. M. R. N., Pascoal, A. T. P., Bertão-Santos, A., Mitsuka-Breganó, R., & Navarro, I. T. (2021). Evaluation of an active and early surveillance methodology for visceral leishmaniasis by molecular detection in road-killed wild fauna. *Revista Brasileira de Parasitologia Veterinaria*, *30*(2), 1–11. https://doi.org/10.1590/s1984-29612021026

Calvopina, M., Armijos, R. X., & Hashiguchi, Y. (2004). Epidemiology of leishmaniasis in Ecuador: Current status of knowledge - A review. *Memorias Do Instituto Oswaldo Cruz*, *99*(7), 663–672. https://doi.org/10.1590/S0074-02762004000700001

Calzolari, M., Romeo, G., Bergamini, F., Dottori, M., Rugna, G., & Carra, E. (2022). Host preference and *Leishmania infantum* natural infection of the sand fly *Phlebotomus perfiliewi* in northern Italy. *Acta Tropica*, *226*(July 2021), 106246. https://doi.org/10.1016/j.actatropica.2021.106246

Can, H., Döşkaya, M., Özdemir, H. G., Şahar, E. A., Karakavuk, M., Pektaş, B., Karakuş, M., Töz, S., Caner, A., Döşkaya, A. D., Iz, S. G., Özbel, Y., & Gürüz, Y. (2016). Seroprevalence of *Leishmania* infection and molecular detection of *Leishmania tropica* and *Leishmania infantum* in stray cats of I˙zmir, Turkey. *Experimental Parasitology*, *167*, 109–114. https://doi.org/10.1016/j.exppara.2016.05.011

Cardoso, L., Schallig, H., Persichetti, M. F., & Pennisi, M. G. (2021). New epidemiological aspects of animal leishmaniosis in Europe: The role of vertebrate hosts other than dogs. *Pathogens*, *10*(3), 1–46. https://doi.org/10.3390/pathogens10030307

Cassan, C., Diagne, C. A., Tatard, C., Gauthier, P., Dalecky, A., Bâ, K., Kane, M., Niang, Y., Diallo, M., Sow, A., Brouat, C., & Bañuls, A. L. (2018). *Leishmania major* and *Trypanosoma lewisi* infection in invasive and native rodents in Senegal. *PLoS Neglected Tropical Diseases*, *12*(6), 1–21. https://doi.org/10.1371/journal.pntd.0006615

Castro, L. S., Dorval, M. E. C., Matheus, L. M. D., Bednaski, A. V., Facco, G. G., Silveira, M., Santos, C. F., Gontijo, C. M. F., Oliveira, A. P. G., & Ferreira, E. C. (2020). *Leishmania* presence in bats in areas endemic for leishmaniasis in central-west Brazil. *International Journal for Parasitology: Parasites and Wildlife*, *11*(February), 261–267. https://doi.org/10.1016/j.ijppaw.2020.02.008

Centers for Disease Control and Prevention One Health office. (n.d.). *Zoonotic Diseases*. CDC. Disponible en: www.cdc.gov/one-health/about/about-zoonotic-diseases.html

Centro Nacional de Epidemiología. (2022). *Informe epidemiológico sobre la situación de la leishmaniasis en España. Año 2022*.

Chemkhi, J., Souguir, H., Ali, I. B. H., Driss, M., Guizani, I., & Guerbouj, S. (2015). Natural infection of Algerian hedgehog, *Atelerix algirus* (Lereboullet 1842) with *Leishmania* parasites in Tunisia. *Acta Tropica*, *150*, 42–51. https://doi.org/10.1016/j.actatropica.2015.06.009

Conselleria Medio Ambiente, Agua, U. y V. (2009). *Decreto 213/2009, de 20 de Noviembre, del Consell, por el que se Aprueban Medidas Para el Control de Especies Exóticas Invasoras en la Comunitat Valenciana*. DOGV Núm. 6151 de 24.11.2009. Disponible en: https:// dogv.gva.es/es/disposicio-consolidada?signatura=013210/2009&idioma=es&L=1&url_lista=

Costa, C. H. N., Chang, K. P., Costa, D. L., & Cunha, F. V. M. (2023). From Infection to Death: An Overview of the Pathogenesis of Visceral Leishmaniasis. *Pathogens*, *12*(7). https://doi.org/10.3390/pathogens12070969

Coutinho, M. T. Z., & Linardi, P. M. (2007). Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? *Veterinary Parasitology*, *147*(3–4), 320–325. https://doi.org/10.1016/j.vetpar.2007.04.008

Cruz, I., Cañavate, C., Rubio, J. M., Morales, M. A., Chicharro, C., Laguna, F., Jiménez-Mejías, M., Sirera, G., Videla, S., & Alvar, J. (2002). A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immunodeficiency virus. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *96*, 185–189. https://doi.org/10.1016/s0035-9203(02)90074-x

Cunze, S., Kochmann, J., Koch, L. K., Hasselmann, K. J. Q., & Klimpel, S. (2019). Leishmaniasis in Eurasia and Africa: Geographical distribution of vector species and pathogens. *Royal Society Open Science*, *6*(5). https://doi.org/10.1098/rsos.190334

Dahroug, M. A. A., Almeida, A. B. P. F., Sousa, V. R. F., Dutra, V., Turbino, N. C. M. R., Nakazato, L., & de Souza, R. L. (2010). *Leishmania* (*Leishmania*) *chagasi* in captive wild felids in Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *104*(1), 73–74. https://doi.org/10.1016/j.trstmh.2009.08.003

De Almeida Curi, N. H., Miranda, I., & Talamoni, S. A. (2006). Serologic evidence of *Leishmania* infection in free-ranging wild and domestic canids around a Brazilian National Park. *Memorias Do Instituto Oswaldo Cruz*, *101*(1), 99–101. https://doi.org/10.1590/s0074-02762006000100019

De Araújo, V. A. L., Boité, M. C., Cupolillo, E., Jansen, A. M., & Roque, A. L. R. (2013). Mixed infection in the anteater *Tamandua tetradactyla* (Mammalia: Pilosa) from Pará State, Brazil: *Trypanosoma cruzi*, *T. rangeli* and *Leishmania infantum*. *Parasitology*, *140*(4), 455–460. https://doi.org/10.1017/S0031182012001886

De Blas, I. (2023). *Win Epi: Work in Epidemiology*. WinEpi. 10.1093/oxfordjournals.molbev.a040023

De Castro Ferreira, E., Cruz, I., Cañavate, C., de Melo, L. A., Pereira, A. A. S., Madeira, F. A. M., Nogueira Valério, S. A., Cunha, H. M., Paglia, A. P., & Ferreira Gontijo, C. M. (2015). Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World. *BMC Veterinary Research*, *11*(1), 1–7. https://doi.org/10.1186/s12917-015-0392-y

De Castro Ferreira, E., Pereira, A. A. S., Silveira, M., Margonari, C., Marcon, G. E. B., de Oliveira França, A., Castro, L. S., Bordignon, M. O., Fischer, E., Tomas, W. M., Dorval, M. E. C., & Gontijo, C. M. F. (2017). *Leishmania* (*V.*) *braziliensis* infecting bats from Pantanal wetland, Brazil: First records for *Platyrrhinus lineatus* and *Artibeus planirostris*. *Acta Tropica*, *172*, 217–222. https://doi.org/10.1016/J.ACTATROPICA.2017.05.012

De Lima, H., Rodríguez, N., Barrios, M. A., Ávila, Á., Cañizales, I., & Gutiérrez, S. (2008). Isolation and molecular identification of *Leishmania chagasi* from a bat (*Carollia perspicillata*) in northeastern Venezuela. *Memorias Do Instituto Oswaldo Cruz*, *103*(4), 412–414. https://doi.org/10.1590/s0074-02762008000400018

De Noya, B. A., & González, O. N. (2015). An ecological overview on the factors that drives to *Trypanosoma cruzi* oral transmission. *Acta Tropica*, *151*(1), 94–102. https://doi.org/10.1016/j.actatropica.2015.06.004

De Oliveira, D. M., Lonardoni, M. V. C., Theodoro, U., & Silveira, T. G. V. (2011). Comparison of different primes for PCR-based diagnosis of cutaneous leishmaniasis. *Brazilian Journal of Infectious Diseases*, *15*(3), 204–210. https://doi.org/10.1016/S1413-8670(11)70176-3

De Oliveira, F. M., Costa, L. H. C., de Barros, T. L., Rauschkolb Katsuda Ito, P. K., Colombo, F. A., de Carvalho, C., Pedro, W. A., Queiroz, L. H., & Nunes, C. M. (2015). First detection of *Leishmania* spp. DNA in Brazilian bats captured strictly in urban areas. *Acta Tropica*, *150*, 176–181. https://doi.org/10.1016/j.actatropica.2015.07.010

De Paiva-Cavalcanti, M., de Morais, R. C. S., Pessoa-e-Silva, R., Trajano-Silva, L. A. M., Gonçalves-de-Albuquerque, S. da C., Tavares, D. de H. C., Brelaz-de-Castro, M. C. A., Silva, R. de F. e., & Pereira, V. R. A. (2015). Leishmaniases diagnosis: An update on the use of immunological and molecular tools. *Cell and Bioscience*, *5*(1), 1–10. https://doi.org/10.1186/s13578-015-0021-2

De Rezende, M. B., Herrera, H. M., Carvalho, C. M. E., Carvalho Anjos, E. A., Ramos, C. A. N., De Araújo, F. R., Torres, J. M., & De Oliveira, C. E. (2017). Detection of *Leishmania* spp. in Bats from an Area of Brazil Endemic for Visceral Leishmaniasis. *Transboundary and Emerging Diseases*, *64*, 36–42. https://doi.org/10.1111/tbed.12597

Dedet, J. P., Gay, F., & Chatenay, G. (1989). Isolation of *Leishmania* species from wild mammals in French Guiana. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *83*(5), 613–615. https://doi.org/10.1016/0035-9203(89)90374-X

Del Río, L., Chitimia, L., Cubas, A., Victoriano, I., De la Rúa, P., Gerrikagoitia, X., Barral, M., Muñoz- García, C. I., Goyena, E., García- Martínez, D., Fisa, R., Riera, C., Murcia, L., Segovia, M., & Berriatua, E. (2014). Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. *Preventive Veterinary Medicine*, *113*, 430–435. https://doi.org/10.1016/j.prevetmed.2013.12.001

Delibes, M., & Palomo, L. J. (2007). *Atlas y Libro Rojo de los mamíferos Terrestres de España*. J. Palomo, J. Gisbert, & J. M. Blanco (eds.). Dirección General para la Biodiversidad-SECEMU

Descoteaux, A., Matlashewski, G., & Turco, S. J. (1992). Inhibition of macrophage protein kinase C-mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. *The Journal of Immunology*, *149*(9), 3008–3015. https://doi.org/10.4049/jimmunol.149.9.3008

Desjeux, P. (2004). Leishmaniasis: Current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases*, *27*(5), 305–318. https://doi.org/10.1016/j.cimid.2004.03.004

Dujardin, J. C., Campino, L., Cañavate, C., Dedet, J. P., Gradoni, L., Soteriadou, K., Mazeris, A., Ozbel, Y., & Boelaert, M. (2008). Spread of vector-borne diseases and neglect of leishmaniasis, Europe. *Emerging Infectious Diseases*, *14*(7), 1013–1018. https://doi.org/10.3201/eid1407.071589

Echchakery, M., Chicharro, C., Boussaa, S., Nieto, J., Carrillo, E., Sheila, O., Moreno, J., & Boumezzough, A. (2017). Molecular detection of *Leishmania infantum* and *Leishmania tropica* in rodent species from endemic cutaneous leishmaniasis areas in Morocco. *Parasites & Vectors*, *10:454*. https://doi.org/10.1186/s13071-017-2398-8

Echchakery, M., Chicharro, C., Boussaa, S., Nieto, J., Ortega, S., Carrillo, E., Moreno, J., & Boumezzough, A. (2020). Molecular identification of *Leishmania tropica* and *L. infantum* isolated from cutaneous human leishmaniasis samples in central Morocco. *Journal of Vector Borne Diseases*, *57*(1), 71–77. https://doi.org/10.4103/0972-9062.308804

El Idrissi Saik, I., Benlabsir, C., Fellah, H., Lemrani, M., & Riyad, M. (2022). Transmission patterns of *Leishmania tropica* around the Mediterranean basin: Could Morocco be impacted by a zoonotic spillover? *PLoS Neglected Tropical Diseases*, *16*(1), 1–14. https://doi.org/10.1371/journal.pntd.0010009

El Kacem, S., Kbaich, M. A., Daoui, O., Charoute, H., Mhaidi, I., Ejghal, R., Barhoumi, M., Guizani, I., Bennani, H., & Lemrani, M. (2021). Multilocus sequence analysis provides new insight into population structure and genetic diversity of *Leishmania tropica* in Morocco. *Infection, Genetics and Evolution*, *93*(February), 104932. https://doi.org/10.1016/j.meegid.2021.104932

Espinosa, O. A., Serrano, M. G., Camargo, E. P., Teixeira, M. M. G., & Shaw, J. J. (2018). An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. *Parasitology*, *145*(4), 430–442. https://doi.org/10.1017/S0031182016002092

Esseghir, S., Ready, P. D., & Ben-Ismail, R. (2000). Speciation of *Phlebotomus* sandflies of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the mediterranean subregion. *Biological Journal of the Linnean Society*, *70*(2), 189–219. https://doi.org/10.1006/bijl.1999.0393

European Centre for Disease Prevention and Control. (2023). *Vector distribution status changes in terms of spatial units since the previous update*. ECDC. Disponible en: www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps

European Centre for Disease Prevention and Control and European Food Safety Authority. (2019). *Phlebotomine sandflies maps*. Stockholm: ECDC. Disponible en: www.ecdc.europa.eu/sites/default/files/images/map-Phlebotomus_perniciosus distribution July 2019.png

European Parliament. (2014). *Regulation (EU) No 1143/2014*. Council of 22 October 2014 on the Prevention and Management of the Introduction and Spread of Invasive Alien Species.

Fernández-Arévalo, A., Ballart, C., Muñoz-Basagoiti, J., Basarte, L., Lobato, G., Arnau, A., Abras, A., Tebar, S., Llovet, T., Lami, P., Pratlong, F., Alsina, M., Roe, E., Puig, L., Muñoz, C., & Gállego, M. (2022). Autochthonous and imported tegumentary leishmaniasis in Catalonia (Spain): Aetiological evolution in the last four decades and usefulness of different typing approaches based on biochemical, molecular and proteomic markers. *Transboundary and Emerging Diseases*, *69*(3), 1404–1418. https://doi.org/10.1111/tbed.14107

Filioussis, G., Petridou, E., Papadopoulos, D., Karavanis, E., Morgan, E., Billinis, C., & Papadopoulos, E. (2018). Hemorrhagic pneumonia in neonatal minks in Greece concomitant with *Leismania infantum* detection. *Polish Journal of Veterinary Sciences*, *21*(2), 405–408. https://doi.org/10.24425/122608

Frank, C., Hadziandoniou, M., Pratlong, F., Garifallou, A., & Rioux, J. A. (1993). *Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *87*(2), 184–185. https://doi.org/https://doi.org/10.1016/0035-9203(93)90482-6

Franssen, S. U., Sanders, M. J., Berriman, M., Petersen, C. A., & Cotton, J. A. (2022). Geographic Origin and Vertical Transmission of *Leishmania infantum* Parasites in Hunting Hounds, United States. *Emerging Infectious Diseases*, *28*(6), 1211–1223. https://doi.org/10.3201/eid2806.211746

Galán-Puchades M.T., Gómez-Samblás M., Suárez-Morán J.M., Osuna A., Sanxis-Furió J., Pascual J., Bueno-Marí R., Franco S., Peracho V., Montalvo T., Fuentes M.V. (2019). Leishmaniasis in Norway Rats in Sewers, Barcelona, Spain. *Emerg Infect Dis.,* 25(6), 1222-1224. https://doi.org/10.3201/eid2506.181027

Gálvez, R., Montoya, A., Cruz, I., Fernández, C., Martín, O., Checa, R., Chicharro, C., Migueláñez, S., Marino, V., & Miró, G. (2020). Latest trends in *Leishmania infantum* infection in dogs in Spain, Part I: Mapped seroprevalence and sand fly distributions. *Parasites and Vectors*, *13*(1), 1–12. https://doi.org/10.1186/s13071-020-04081-7

Ghawar, W., Toumi, A., Snoussi, M.-A., Chlif, S., Zâatour, A., Boukthir, A., Bel Haj Hamida, N., Chemkhi, J., Diouani, M. F., & Ben-Salah, A. (2011). *Leishmania major* Infection Among *Psammomys Obesus* and *Meriones Shawi*: Reservoirs of Zoonotic Cutaneous Leishmaniasis in Sidi Bouzid (Central Tunisia). *Vector-Borne and Zoonotic Diseases*, *11*(12), 1561–1568. https://doi.org/10.1089/vbz.2011.0712

Gholipour, H., Busquets, N., Fernández-Aguilar, X., Sánchez, A., Ribas, M. P., De Pedro, G., Lizarraga, P., Alarcia-Alejos, O., Temiño, C., & Cabezón, O. (2017). Influenza A Virus Surveillance in the Invasive American Mink (*Neovison vison*) from Freshwater Ecosystems, Northern Spain. *Zoonoses and Public Health*, *64*(5), 363–369. https://doi.org/10.1111/zph.12316

Gicheru, M. M., Jeneby, M. M., Macharia, J. C., Carlsson, H. E., & Suleman, M. A. (2009). Prevalence of antibodies and cell mediated immune response against *Leishmania major* in feral nonhuman primates from Kenya. *Acta Tropica*, *109*(2), 136–140. https://doi.org/10.1016/j.actatropica.2008.09.020

Gijón-Robles, P., Abattouy, N., Merino-Espinosa, G., El Khalfaoui, N., Morillas-Márquez, F., Corpas-López, V., Porcel-Rodríguez, L., Jaaouani, N., Díaz-Sáez, V., Riyad, M., & Martín-Sánchez, J. (2018). Risk factors for the expansion of cutaneous leishmaniasis by *Leishmania tropica*: Possible implications for control programmes. *Transboundary and Emerging Diseases*, *65*(6), 1615–1626. https://doi.org/10.1111/tbed.12914

Gomes, J., Rocha, H., Carvalho, C., Bandeira, V., Fonseca, C., Rosalino, L. M., & Cunha, M. V. (2020). Molecular detection and characterization of *Leishmania infantum* in free-ranging Egyptian mongoose (*Herpestes ichneumon*). *International Journal for Parasitology: Parasites and Wildlife*, *11*(February), 158–162. https://doi.org/10.1016/j.ijppaw.2020.02.001

Gómez-Hernández, C., Bento, E. C., Rezende-Oliveira, K., Nascentes, G. A. N., Barbosa, C. G., Batista, L. R., Tiburcio, M. G. S., Pedrosa, A. L., Lages-Silva, E., Ramírez, J. D., & Ramirez, L. E. (2017). *Leishmania* infection in bats from a non-endemic region of leishmaniasis in Brazil. *Parasitology*, *144*(14), 1980–1985. https://doi.org/10.1017/S0031182017001500

González M. A., Ruiz-Arrondo I., Gutiérrez-López R., Barceló C., Miranda M. Á. (2023) First Record of *Phlebotomus* (*Larroussius*) *perfiliewi* (Diptera: Psychodidae), Vector of *Leishmania infantum* and Phleboviruses, in Spain. *Diversity*, 15(3), 400. https://doi.org/10.3390/d15030400

Gramiccia, M., & Gradoni, L. (2005). The current status of zoonotic leishmaniases and approaches to disease control. *International Journal for Parasitology*, *35*(11–12), 1169–1180. https://doi.org/10.1016/j.ijpara.2005.07.001

Halliday, C., Yanase, R., Catta-Preta, C. M. C., Moreira-Leite, F., Myskova, J., Pruzinova, K., Volf, P., Mottram, J. C., & Sunter, J. D. (2020). Role for the flagellum attachment zone in *Leishmania* anterior cell tip morphogenesis. *PLoS Pathogens*, *16*(10), 1–26. https://doi.org/10.1371/journal.ppat.1008494

Helhazar, M., Leitão, J., Duarte, A., Tavares, L., & Pereira da Fonseca, I. (2013). Natural infection of synathropic rodent species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra-Portugal. *Parasites & Vectors*, *8*(88). https://doi.org/10.1186/1756-3305-6-88

Hong, A., Zampieri, R. A., Shaw, J. J., Floeter-Winter, L. M., & Laranjeira-Silva, M. F. (2020). One health approach to leishmaniases: Understanding the disease dynamics through diagnostic tools. *Pathogens*, *9*(10), 1–24. https://doi.org/10.3390/pathogens9100809

Iatta, R., Mendoza-Roldan, J. A., Latrofa, M. S., Cascio, A., Brianti, E., Pombi, M., Gabrielli, S., & Otranto, D. (2021). *Leishmania tarentolae* and *Leishmania infantum* in humans, dogs and cats in the Pelagie archipelago, southern Italy. *PLoS Neglected Tropical Diseases*, *15*(9). https://doi.org/10.1371/journal.pntd.0009817

Jaskowska, E., Butler, C., Preston, G., & Kelly, S. (2015). *Phytomonas*: Trypanosomatids Adapted to Plant Environments. *PLoS Pathogens*, *11*(1), 1–17. https://doi.org/10.1371/journal.ppat.1004484

Jiménez, M., González, E., Martín-Martín, I., Hernández, S., & Molina, R. (2014). Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? *Veterinary Parasitology*, *202*, 296–300. https://doi.org/10.1016/j.vetpar.2014.03.027

Karayiannis, S., Ntais, P., Messaritakis, I., Tsirigotakis, N., Dokianakis, E., & Antoniou, M. (2015). Detection of *Leishmania Infantum* in red foxes (*Vulpes vulpes)* in Central Greece. *Parasitology*, *142*, 1574–1578. https://doi.org/10.1017/S0031182015001158

Karmaoui, A. (2020). Seasonal Distribution of *Phlebotomus papatasi*, Vector of Zoonotic Cutaneous Leishmaniasis. *Acta Parasitologica*, *65*(3), 585–598. https://doi.org/10.2478/s11686-020-00201-6

Kassahun, A., Sadlova, J., Benda, P., Kostalova, T., Warburg, A., Hailu, A., Baneth, G., Volf, P., & Votypka, J. (2015). Natural infection of bats with *Leishmania* in Ethiopia. *Acta Tropica*, *150*, 166–170. https://doi.org/10.1016/j.actatropica.2015.07.024

Kaufer, A., Ellis, J., Stark, D., & Barratt, J. (2017). The evolution of trypanosomatid taxonomy. *Parasites and Vectors*, *10*(1), 1–17. https://doi.org/10.1186/s13071-017-2204-7

Klatt, S., Simpson, L., Maslov, D. A., & Konthur, Z. (2019). *Leishmania tarentolae*: Taxonomic classification and its application as a promising biotechnological expression host. *PLoS Neglected Tropical Diseases*, *13*(7), 1–29. https://doi.org/10.1371/journal.pntd.0007424

Knöpfel, N., Noguera-Morel, L., Azorin, D., Sanz, F., Torrelo, A., & Hernández-Martín, A. (2018). Cutaneous *Leishmania tropica* in children: report of three imported cases successfully treated with liposomal amphotericin B. *Journal of the European Academy of Dermatology and Venereology*, *32*(1), e8–e10. https://doi.org/10.1111/jdv.14434

Kostygov, A. Y., & Yurchenko, V. (2017). Revised classification of the subfamily Leishmaniinae (Trypanosomatidae). *Folia Parasitologica*, *64*, 1–5. https://doi.org/10.14411/fp.2017.020

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Kushwaha, A. K., Scorza, B. M., Singh, O. P., Rowton, E., Lawyer, P., Sundar, S., & Petersen, C. A. (2022). Domestic mammals as reservoirs for *Leishmania donovani* on the Indian subcontinent: Possibility and consequences on elimination. *Transboundary and Emerging Diseases*, *69*(2), 268–277. https://doi.org/10.1111/tbed.14061

Labbé, F., Abdeladhim, M., Abrudan, J., Araki, A. S., Araujo, R. N., Arensburger, P., Benoit, J. B., Brazil, R. P., Bruno, R. V., Rivas, G. B. da S., de Abreu, V. C., Charamis, J., Coutinho-Abreu, I. V., da Costa-Latgé, S. G., Darby, A., Dillon, V. M., Emrich, S. J., Fernandez-Medina, D., Gontijo, N. F., … McDowell, M. A. (2023). Genomic analysis of two phlebotomine sand fly vectors of *Leishmania* from the New and Old World. In *PLoS Neglected Tropical Diseases,* 17 (4). https://doi.org/10.1371/journal.pntd.0010862

Lachaud, L., Chabbert, E., Dubessay, P., Dereure, J., Lamothe, J., Dedet, J. P., & Bastien, P. (2002a). Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. *Parasitology*, *125*(3), 197–207. doi: 10.1017/s0031182002002081.

Lachaud, L., Marchergui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J. P., & Bastien, P. (2002b). Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *Journal of Clinical Microbiology*, *40*(1), 210–215. https://doi.org/10.1128/jcm.40.1.210-215.2002

Lainson, R., Braga, R. R., De Souza, A. A. A., Pôvoa, M. M., Ishikawa, E. A. Y., & Silveira, F. T. (1989). *Leishmania* (*Viannia*) *shawi* sp. N., a parasite of monkeys, sloths and procyonids in Amazonian Brazil. *Annales de Parasitologie Humaine et Comparee*, *3*, 200–207.

Le Rutte, E. A., van Straten, R., & Overgaauw, P. A. M. (2018). Awareness and control of canine leishmaniosis: A survey among Spanish and French veterinarians. *Veterinary Parasitology*, *253*(January), 87–93. https://doi.org/10.1016/j.vetpar.2018.01.013

Lemma, W., Erenso, G., Gadisa, E., Balkew, M., Gebre-michael, T., & Hailu, A. (2009). A zoonotic focus of cutaneous leishmaniasis in Addis Ababa, Ethiopia. *Parasites & Vectors*, *2*(60), 1–8. https://doi.org/10.1186/1756-3305-2-60

Llanos-Cuenta, A. (2013). Leishmaniasis. In A. Werner (Ed.), *Parasitología Humana* (1st ed.). McGraw-Hill. ISBN: 978-607-15-0876-8

Louradour, I., Ferreira, T. R., Ghosh, K., Shaik, J., & Sacks, D. (2020). In Vitro Generation of *Leishmania* Hybrids. *Cell Reports*, *31*(2). https://doi.org/10.1016/j.celrep.2020.03.071

Luppi, M. M., Malta, M. C. C., Silva, T. M. A., Silva, F. L., Motta, R. O. C., Miranda, I., Ecco, R., & Santos, R. L. (2008). Visceral leishmaniasis in captive wild canids in Brazil. *Veterinary Parasitology*, *155*(1–2), 146–151. https://doi.org/10.1016/j.vetpar.2008.04.024

MAGRAMA. (2013). Ministerio de Agricultura, Alimentación Y Medio Ambiente. Real Decreto 630/2013, de 2 de agosto, por el que se regula el Catálogo español de especies exóticas invasoras. *Boletín Oficial Del Estado*, *185*, 56764–56786.

Mahdy, M. A. A., Younis, W., & Ewaida, Z. (2020). An Overview of SARS-CoV-2 and Animal Infection. *Frontiers in Veterinary Science*, *7*(December). https://doi.org/10.3389/fvets.2020.596391

Maia, C., Conceição, C., Pereira, A., Rocha, R., Ortuño, M., Muñozid, C., Jumakanova, Z., Pérez-Cutillas, P., Özbel, Y., Töz, S., Baneth, G., Monge-Maillo, B., Gasimov, E., Van der Stede, Y., Torres, G., Gossner, C. M., & Berriatua, E. (2023). The estimated distribution of autochthonous leishmaniasis by *Leishmania infantum* in Europe in 2005–2020. *PLoS Neglected Tropical Diseases*, *17*(7), 1–25. https://doi.org/10.1371/journal.pntd.0011497

Malta, M. C. C., Tinoco, H. P., Xavier, M. N., Vieira, A. L. S., Costa, É. A., & Santos, R. L. (2010). Naturally acquired visceral leishmaniasis in non-human primates in Brazil. *Veterinary Parasitology*, *169*(1–2), 193–197. https://doi.org/10.1016/j.vetpar.2009.12.016

Maroli, M., Feliciangeli, M. D., Bichaud, L., Charrel, R. N., & Gradoni, L. (2013). Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health. *Medical and Veterinary Entomology*, *27*, 123–147. https://doi.org/10.1111/j.1365-2915.2012.01034.x

Martín-Sánchez, J., Rodríguez-Granger, J., Morillas-Márquez, F., Merino-Espinosa, G., Sampedro, A., Aliaga, L., Corpas-López, V., Tercedor-Sánchez, J., Aneiros-Fernández, J., Acedo-Sánchez, C., Porcel-Rodríguez, L., & Díaz-Sáez, V. (2020a). Leishmaniasis due to *Leishmania infantum*: Integration of human, animal and environmental data through a One Health approach. *Transboundary and Emerging Diseases*, *67*(6), 2423–2434. https://doi.org/10.1111/tbed.13580

Martín-Sánchez, J., Torres-Medina, N., Corpas-López, V., Morillas-Márquez, F., & Díaz-Sáez, V. (2020b). Vertical transmission may play a greater role in the spread of *Leishmania infantum* in synanthropic *Mus musculus* rodents than previously believed. *Transboundary and Emerging Diseases*, *67*(3), 1113–1118. https://doi.org/10.1111/tbed.13436

Martínez-Rondán, F. J., Ruiz de Ybáñez, M. R., Tizzani, P., López-Beceiro, A. M., Fidalgo, L. E., & Martínez-Carrasco, C. (2017). The American mink (*Neovison vison*) is a competent host for native European parasites. *Veterinary Parasitology*, *247*(October), 93–99. https://doi.org/10.1016/j.vetpar.2017.10.004

Martínez, M. F., Kowalewski, M. M., Giuliani, M. G., Acardi, S. A., & Salomón, O. D. (2020). Molecular identification of *Leishmania* in free-ranging black and gold howler monkeys (*Alouatta caraya*) in northeastern Argentina. *Acta Tropica*, *210*(January), 105534. https://doi.org/10.1016/j.actatropica.2020.105534

Mathison, B. A., & Bradley, B. T. (2023). Review of the Clinical Presentation, Pathology, Diagnosis, and Treatment of Leishmaniasis. *Lab Medicine*, *54*(4), 363–371. https://doi.org/10.1093/labmed/lmac134

Matlashewski, G. (2001). *Leishmania* infection and virulence. *Medical Microbiology and Immunology*, *190*(1–2), 37–42. https://doi.org/10.1007/s004300100076

Maxfield, L., & Crane, J. S. (2019). Leishmaniasis. In *StatPearls*. StatPearls Publishing. Disponible en: www.ncbi.nlm.nih.gov/pubmed/30285351

Mendoza-Roldan, J. A., Latrofa, M. S., Iatta, R., R. S. Manoj, R., Panarese, R., Annoscia, G., Pombi, M., Zatelli, A., Beugnet, F., & Otranto, D. (2021). Detection of *Leishmania tarentolae* in lizards, sand flies and dogs in southern Italy, where *Leishmania infantum* is endemic: hindrances and opportunities. *Parasites and Vectors*, *14*(1), 1–12. https://doi.org/10.1186/s13071-021-04973-2

Merino-Espinosa, G., Corpas-López, V., Callejón-Fernández, R., Porcel-Rodríguez, L., Díaz-Sáez, V., Gállego, M., Ballart, C., Molina, R., Jiménez, M., Morillas-Márquez, F., & Martín-Sánchez, J. (2016). Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and their epidemiological implications. *Tropical Medicine and International Health*, *21*(5), 630–641. https://doi.org/10.1111/tmi.12686

Mescouto-Borges, M. R. M., Maués, É., Costa, D. L., da Silva Pranchevicius, M. C., & Romero, G. A. S. (2013). Congenitally transmitted visceral leishmaniasis: Report of two Brazilian human cases. *Brazilian Journal of Infectious Diseases*, *17*(2), 263–266. https://doi.org/10.1016/j.bjid.2012.10.017

Millán, J., Ferroglio, E., & Solano-Gallego, L. (2014a). Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. *Parasitology Research*, *113*, 2005–2014. https://doi.org/10.1007/s00436-014-3929-2

Millán, J., López-Roig, M., Cabezón, O., & Serra-Cobo, J. (2014b). Absence of *Leishmania infantum* in cave bats in an endemic area in Spain. *Parasitology Research*, *113*(5), 1993–1995. https://doi.org/10.1007/s00436-014-3855-3

Ministerio de Agricultura Alimentación y Medio Ambiente. (2013). *Real Decreto 630/2013, de 2 de Agosto, por el que se Regula el Catálogo Español de Especies Exóticas*. «BOE» Núm. 185, de 3 de Agosto de 2013. Disponible en: www.boe.es/eli/es/rd/2013/08/02/630

Miró, G., Troyano, A., Montoya, A., Fariñas, F., Fermín, M. L., Flores, L., Rojo, C., Checa, R., Gálvez, R., Marino, V., Fragío, C., & Martínez-Nevado, E. (2018). First report of *Leishmania infantum* infection in the endangered orangutan (*Pongo pygmaeus pygmaeus*) in MadridSpain. *Parasites & Vectors*, *11*(185). https://doi.org/10.1186/s13071-018-2772-1

MITECO. (2024). *Catálogo Español de Especies Exóticas Invasoras- Mamíferos*. Ministerio Para La Transición Ecológica y El Reto Demográfico. Disponible en: www.miteco.gob.es/es/biodiversidad/temas/conservacion-de-especies/especies-exoticas-invasoras/ce_eei_mamiferos.html

Mitková, B., Hrazdilová, K., Amico, G. D., Duscher, G. G., Suchentrunk, F., Forejtek, P., Gherman, M., Matei, I. A., Monica, A., Daskalaki, A. A., Mihalca, A. D., Votýpka, J., Hulva, P., & Modrý, D. (2017). Eurasian golden jackal as host of canine vector-borne protists. *Parasites & Vectors*, *10*(183), 1–11. https://doi.org/10.1186/s13071-017-2110-z

Moemenbellah-Fard, M. ., Kalantari, M., Rassi, Y., & Javadian, E. (2003). The PCR- based detection of *Leishmania major* infections in *Meriones libycus* (Rodentia: Muridae) from southern Iran. *Annals of Tropical Medicine and Parasitology*, *97*(8), 865–873. https://doi.org/10.1179/000349803225002651

Mohebali, M., Arzamani, K., Zarei, Z., Akhoundi, B., Hajjaran, H., Raeghi, S., Heidari, Z., Motavalli-Haghi, S. M., Elikaee, S., Mousazadeh-Mojarrad, A., & Kakoei, Z. (2016). Canine Visceral Leishmaniasis in Wild Canines (Fox , Jackal , and Wolf) in Northeastern Iran Using Parasitological , Serological , and Molecular Methods. *Journal Arthropod-Borne Dis*, *10*(December), 538–545. PMID: 28032106; PMCID: PMC5186744.

Molina, R., Jiménez, M. I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., & Bernal, J. (2012). The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. *Veterinary Parasitology*, *190*, 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006

Montaner-Angoiti, E., & Llobat, L. (2023). Is leishmaniasis the new emerging zoonosis in the world? *Veterinary Research Communications*, *0123456789*. https://doi.org/10.1007/s11259-023-10171-5

Montoya, A., De Quadros, L. P., Mateo, M., Hernández, L., Gálvez, R., Alcántara, G., Checa, R., Jiménez, M. Á., Chicharro, C., Cruz, I., & Miró, G. (2016). *Leishmania infantum* infection in Bennett's wallabies (*Macropus rufogriseus rufogriseus*) in a spanish wildlife park. *Journal of Zoo and Wildlife Medicine*, *47*(2), 586–593. https://doi.org/10.1638/2014-0216.1

Morales-Yuste, M., Martín-Sánchez, J., & Corpas-López, V. (2022). Canine Leishmaniasis: Update on Epidemiology, Diagnosis, Treatment, and Prevention. *Veterinary Sciences*, *9*(387). https://doi.org/10.3390/vetsci9080387

Müller, A., Montoya, A., Escacena, C., de la Cruz, M., Junco, A., Iriso, A., Marino, E., Fúster, F., & Miró, G. (2022). *Leishmania infantum* infection serosurveillance in stray dogs inhabiting the

Madrid community: 2007–2018. *Parasites and Vectors*, *15*(1), 1–8. https://doi.org/10.1186/s13071-022-05226-6

Muñoz-Madrid, R., Belinchón-Lorenzo, S., Iniesta, V., Fernández-Cotrina, J., Parejo, J. C., Serrano, F. J., Monroy, I., Baz, V., Gómez-Luque, A., & Gómez-Nieto, L. C. (2013). First detection of *Leishmania infantum* kinetoplast DNA in hair of wild mammals: Application of qPCR method to determine potential parasite reservoirs. *Acta Tropica*, *128*(3), 706–709. https://doi.org/10.1016/j.actatropica.2013.08.009

Naucke, T. J., Amelung, S., & Lorentz, S. (2016). First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. *Parasites & Vectors*, *9*(256), 1–4. https://doi.org/10.1186/s13071-016-1551-0

Naucke, T. J., & Lorentz, S. (2012). First report of venereal and vertical transmission of canine leishmaniosis from naturally infected dogs in Germany. *Parasites and Vectors*, *5*(1), 67. https://doi.org/10.1186/1756-3305-5-67

Navea- Pérez, H. M., Díaz- Sáez, V., Corpas- López, V., Merino- Espinosa, G., Morillas- Márquez, F., & Martín- Sánchez, J. (2015). *Leishmania infantum* in wild rodents : reservoirs or just irrelevant incidental hosts ? *Parasitology Research*, *114*, 2363–2370. https://doi.org/10.1007/s00436-015-4434-y

Oerther, S., Jöst, H., Heitmann, A., Lühken, R., Krüger, A., Steinhausen, I., Brinker, C., Lorentz, S., Marx, M., Schmidt-Chanasit, J., Naucke, T., & Becker, N. (2020). Phlebotomine sand flies in Southwest Germany: An update with records in new locations. *Parasites and Vectors*, *13*(173). https://doi.org/10.1186/s13071-020-04058-6

Oleaga, A., Vicente, J., Ferroglio, E., Pegoraro de Macedo, M. R., Casais, R., Del Cerro, A., Espí, A., García, E. J., & Gortázar, C. (2015). Concomitance and interactions of pathogens in the Iberian wolf (*Canis lupus*). *Research in Veterinary Science*, *101*, 22–27. https://doi.org/10.1016/j.rvsc.2015.05.010

Oleaga, A., Zanet, S., Espí, A., Pegoraro de Macedo, M. R., Gortázar, C., & Ferroglio, E. (2018). *Leishmania* in wolves in northern Spain: A spreading zoonosis evidenced by wildlife sanitary surveillance. *Veterinary Parasitology*, *255*(January), 26–31. https://doi.org/10.1016/j.vetpar.2018.03.015

Ortega-García, M. V., Salguero, F. J., Rodríguez-Bertos, A., Moreno, I., García, N., García-Seco, T., Luz Torre, G., Domínguez, L., & Domínguez, M. (2019). A pathological study of *Leishmania infantum* natural infection in European rabbits (*Oryctolagus cuniculus*) and Iberian

hares (*Lepus granatensis*). *Transboundary and Emerging Diseases*, *66*(6), 2474–2481. https://doi.org/10.1111/tbed.13305

Ortega, M. V., Moreno, I., Domínguez, M., de la Cruz, M. L., Martín, A. B., Rodríguez-Bertos, A., López, R., Navarro, A., González, S., Mazariegos, M., Goyache, J., Domínguez, L., & García, N. (2017). Application of a specific quantitative real-time PCR (qPCR) to identify *Leishmania infantum* DNA in spleen, skin and hair samples of wild Leporidae. *Veterinary Parasitology*, *243*(May), 92–99. https://doi.org/10.1016/j.vetpar.2017.05.015

Ortuño, M., Latrofa, M. S., Iborra, M. A., Pérez, P., Bernal, L. J., Risueño, J., Muñoz, C., Bernal, A., Sánchez, P. F., Manuel, L., Giada, S., Carla, A., Cortes, S., Campino, L., Otranto, D., & Berriatua, E. (2019). Genetic diversity and phylogenetic relationships between *Leishmania infantum* from dogs , humans and wildlife in south - east Spain. *Zoonoses Public Health*, *00*, 1–13. https://doi.org/10.1111/zph.12646

Panahi, E., Stanisic, D. I., Skinner, E. B., Faddy, H. M., Young, M. K., & Herrero, L. J. (2023). Detection of *Leishmania* (*Mundinia*) *macropodum* (Kinetoplastida: Trypanosomatidae) and heterologous *Leishmania* species antibodies among blood donors in a region of Australia with marsupial *Leishmania* endemicity. *International Journal of Infectious Diseases*, *130*, 42–47. https://doi.org/10.1016/j.ijid.2022.10.006

Pareyn, M., Van Den Bosch, E., Girma, N., Van Houtte, N., Van Dongen, S., Van Der Auwera, G., Massebo, F., Shibru, S., & Leirs, H. (2019). Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochollo , a hotspot in southern Ethiopia. *PLoS Neglected Tropical Diseases*, *13*(8), 1–19. https://doi.org/10.1371/ journal.pntd.0007667

Pennisi, M. G., Hartmann, K., Lloret, A., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hosie, M. J., Lutz, H., Marsilio, F., Möstl, K., Radford, A. D., Thiry, E., Truyen, U., & Horzinek, M. C. (2013). Leishmaniosis in cats: ABCD guidelines on prevention and management. *Journal of Feline Medicine and Surgery*, *15*(7), 638–642. https://doi.org/10.1177/1098612X13489229

Pennisi, M. G., & Persichetti, M. F. (2018). Feline leishmaniosis: Is the cat a small dog? *Veterinary Parasitology*, *251*(January), 131–137. https://doi.org/10.1016/j.vetpar.2018.01.012

Pereira, A., Parreira, R., Cristóvão, J. M., Castelli, G., Bruno, F., Vitale, F., Campino, L., & Maia, C. (2020). Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers. *Infection, Genetics and Evolution*, *77*. https://doi.org/10.1016/j.meegid.2019.104069

Pérez-Ayala, A., Norman, F., Pérez-Molina, J. A., Herrero, J. M., Monge, B., & López-Vélez, R. (2009). Imported leishmaniasis: A heterogeneous group of diseases. *Journal of Travel Medicine*, *16*(6), 395–401. https://doi.org/10.1111/j.1708-8305.2009.00341.x

Piarroux, R., Fontes, M., Perasso, R., Gambarelli, F., Joblet, C., Dumon, H., & Quilici, M. (1995). Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. *Molecular and Biochemical Parasitology*, *73*, 249–252. https://doi.org/10.1016/0166-6851(95)00097-k.

Pombi, M., Giacomi, A., Barlozzari, G., Mendoza-Roldan, J., Macrì, G., Otranto, D., & Gabrielli, S. (2020). Molecular detection of *Leishmania* (*Sauroleishmania*) *tarentolae* in human blood and *Leishmania* (*Leishmania*) *infantum* in *Sergentomyia minuta*: unexpected host-parasite contacts. *Medical and Veterinary Entomology*, *34*(4), 470–475. https://doi.org/10.1111/mve.12464

Pourmohammadi, B., & Mohammadi-Azni, S. (2019). Molecular Detection of *Leishmania major* in *Hemiechinus auritus*, A Potential Reservoir of Zoonotic Cutaneous Leishmaniasis in Damghan, Iran. *Journal of Arthropod-Borne Diseases*, *13*(3), 334–343. https://doi.org/10.18502/jad.v13i3.1542

Pradhan, S., Schwartz, R. A., Patil, A., Grabbe, S., & Goldust, M. (2022). Treatment options for leishmaniasis. *Clinical and Experimental Dermatology*, *47*(3), 516–521. https://doi.org/10.1111/ced.14919

Purroy, F. J. (2007). Sciurus vulgaris (Linnaeus, 1758). In L. J. Palomo, J. Gisbert, & J. C. Blanco (Eds.), *Atlas y Libro Rojo de los Mamíferos Terrestres de España* (pp. 378–380). Dirección General para la Biodiversidad-SECEM-SECEMU.

Quaresma, P. F., Rêgo, F. D., Botelho, H. A., da Silva, S. R., Moura, A. J., Neto, R. G. T., Madeira, F. M., Carvalho, M. B., Paglia, A. P., Melo, M. N., & Gontijo, C. M. F. F. (2011). Wild, synanthropic and domestic hosts of *Leishmania* in an endemic area of cutaneous leishmaniasis in Minas Gerais State, Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *105*(10), 579–585. https://doi.org/10.1016/j.trstmh.2011.07.005

Ramos, R. A. N., Ramos, C. A. do N., Santos, E. M. de S., Araújo, F. R. de, Carvalho, G. A. de, Faustino, M. A. da G., & Alves, L. C. (2013). Quantification of *Leishmania infantum* DNA in the bone marrow, lymph node and spleen of dogs. *Revista Brasileira de Parasitologia Veterinaria, 22(3), 346–350.https://doi.org/10.1590/s1984-29612013000300005*

Ratzlaff, F. R., Osmari, V., da Silva, D., de Paula Vasconcellos, J. S., Pötter, L., Fernandes, F. D., de Mello Filho, J. A., de Avila Botton, S., Vogel, F. S. F., & Sangioni, L. A. (2023).

Identification of infection by *Leishmania* spp. in wild and domestic animals in Brazil: a systematic review with meta-analysis (2001–2021). *Parasitology Research*, *122*(7), 1605–1619. https://doi.org/10.1007/s00436-023-07862-y

Ready, P. D. (2013). Biology of phlebotomine sand flies as vectors of disease agents. *Annual Review of Entomology*, *58*, 227–250. https://doi.org/10.1146/annurev-ento-120811-153557

Rebêlo, J. M., Rodrigues, B. L., Bandeira, M. D., Moraes, J. L., Fonteles, R. S., & Pereira, S. R. (2016). Detection of *Leishmania amazonensis* and *Leishmania braziliensis* in *Culicoides* (Diptera, Ceratopogonidae) in an endemic area of cutaneous leishmaniasis in the Brazilian Amazonia. *Journal of vector ecology : journal of the Society for Vector Ecology*, *41*(2), 303–308. https://doi.org/10.1111/jvec.12227

Ribas, M. P., Almería, S., Fernández-Aguilar, X., De Pedro, G., Lizarraga, P., Alarcia-Alejos, O., Molina-López, R., Obón, E., Gholipour, H., Temiño, C., Dubey, J. P., & Cabezón, O. (2018). Tracking T*oxoplasma gondii* in freshwater ecosystems: interaction with the invasive American mink (*Neovison vison*) in Spain. *Parasitology Research*, *117*(7), 2275–2281. https://doi.org/10.1007/s00436-018-5916-5

Richini-Pereira, V. B., Marson, P. M., Hayasaka, E. Y., Victoria, C., da Silva, R. C., & Langoni, H. (2014). Molecular detection of *Leishmania* spp. in road-killed wild mammals in the Central Western area of the State of São Paulo, Brazil. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, *20*(1), 1–7. https://doi.org/10.1186/1678-9199-20-27

Risueño, J., Ortuño, M., Pérez-cutillas, P., Goyena, E., Maia, C., Cortes, S., Campino, L., Bernal, L. J., Muñoz, C., Arcenillas, I., Martínez-rondán, F. J., Gonzálvez, M., Collantes, F., Ortiz, J., Martínez- Carrasco, C., & Berriatua, E. (2018). Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife , dogs and humans associated to vector abundance in Southeast Spain. *Veterinary Parasitology*, *259*(April), 61–67. https://doi.org/10.1016/j.vetpar.2018.05.012

Rodrigues, B. L., & Galati, E. A. B. (2023). Molecular taxonomy of phlebotomine sand flies (Diptera, Psychodidae) with emphasis on DNA barcoding: A review. *Acta Tropica*, *238*. https://doi.org/10.1016/j.actatropica.2022.106778

Roque, A. L. R., & Jansen, A. M. (2014). Wild and synanthropic reservoirs of *Leishmania* species in the Americas. *International Journal for Parasitology: Parasites and Wildlife*, *3*(3), 251–262. https://doi.org/10.1016/j.ijppaw.2014.08.004

Rouhani, S., Mirzaei, A., Spotin, A., & Parvizi, P. (2014). Novel identification of *Leishmania*

major in *Hemiechinus auritus* and molecular detection of this parasite in *Meriones libycus* from an important foci of zoonotic cutaneous leishmaniasis in Iran. *Journal of Infection and Public Health, 7(3), 210–217. https://doi.org/10.1016/j.jiph.2013.12.002*

Ruggiero, M. A., Gordon, D. P., Orrell, T. M., Bailly, N., Bourgoin, T., Brusca, R. C., Cavalier-Smith, T., Guiry, M. D., & Kirk, P. M. (2015). A higher level classification of all living organisms. *PLoS ONE*, *10*(4), 1–60. https://doi.org/10.1371/journal.pone.0119248

Ruiz-Olmo, J., Palazon, S., Bueno, F., Bravo, C., Munilla, I., & Romero, R. (1997). Distribution , Status and Colonization of the American Mink Mustela in Spain. *J. Wildl. Res.*, *2*(1), 30–36.

Saidi, N., Blaizot, R., Prévot, G., Aoun, K., Demar, M., Cazenave, P. A., Bouratbine, A., & Pied, S. (2023). Clinical and immunological spectra of human cutaneous leishmaniasis in North Africa and French Guiana. *Frontiers in Immunology*, *14*(July), 1–13. https://doi.org/10.3389/fimmu.2023.1134020

Santiago, M. E. B., Vasconcelos, R. O., Fattori, K. R., Munari, D. P., Michelin, A. de F., & Lima, V. M. F. (2007). An investigation of *Leishmania* spp. in *Didelphis* spp. from urban and peri-urban areas in Bauru (São Paulo, Brazil). *Veterinary Parasitology*, *150*(4), 283–290. https://doi.org/10.1016/j.vetpar.2007.09.026

Sastre, N., Francino, O., Ramírez, O., Enseñat, C., Sánchez, A., & Altet, L. (2008). Detection of *Leishmania infantum* in captive wolves from Southwestern Europe. *Veterinary Parasitology*, *158*(1–2), 117–120. https://doi.org/10.1016/j.vetpar.2008.08.008

Savani, E. S. M. M., de Almeida, M. F., de Oliveira Camargo, M. C. G., D'Auria, S. R. N., Silva, M. M. S., de Oliveira, M. L., & Sacramento, D. (2010). Detection of *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Leishmania*) *infantum chagasi* in Brazilian bats. *Veterinary Parasitology*, *168*(1–2), 5–10. https://doi.org/10.1016/j.vetpar.2009.10.019

Schönian, G., Mauricio, I., & Cupolillo, E. (2010). Is it time to revise the nomenclature of *Leishmania*? *Trends in Parasitology*, *26*(10), 466- 469469.https://doi.org/10.1016/j.pt.2010.06.013

Shaw, J. (2007). The leishmaniases - survival and expansion in a changing world . A mini-review. *Mem Inst Oswaldo Cruz*, *102*(August), 541–547. https://doi.org/10.1590/s0074-02762007000500001

Sidorovich, V., Kruuk, H., & Macdonald, D. W. (1999). Body size, and interactions between European and American mink (*Mustela lutreola* and *M. vison*) in Eastern Europe . *Journal of Zoology*, *248*(4), 521–527. https://doi.org/10.1111/j.1469-7998.1999.tb01051.x

Silva, F. L., Oliveira, R. G., Silva, T. M. A., Xavier, M. N., Nascimento, E. F., & Santos, R. L. (2009). Venereal transmission of canine visceral leishmaniasis. *Veterinary Parasitology*, *160*(1–2), 55–59. https://doi.org/10.1016/j.vetpar.2008.10.079

Sobrino, R., Ferroglio, E., Oleaga, A., Romano, A., Millan, J., Revilla, M., Arnal, M. C., Trisciuoglio, A., & Gortázar, C. (2008). Characterization of widespread canine leishmaniasis among wild carnivores from Spain. *Veterinary Parasitology*, *155*, 198–203. https://doi.org/10.1016/j.vetpar.2008.05.003

Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M. G., Ferrer, L., Bourdeau, P., Oliva, G., & Baneth, G. (2009). Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Veterinary Parasitology*, *165*(1–2), 1–18. https://doi.org/10.1016/j.vetpar.2009.05.022

Souguir-Omrani, H., Chemkhi, J., Fathallah-Mili, A., Saadi-BenAoun, Y., BelHadjAli, I., Guizani, I., & Guerbouj, S. (2018). *Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) hedgehogs: Possible reservoirs of endemic leishmaniases in Tunisia. *Infection, Genetics and Evolution*, *63*(December 2017), 219–230. https://doi.org/10.1016/j.meegid.2018.05.029

Steverding, D. (2017). The history of leishmaniasis. *Parasites and Vectors*, *10*(1), 1–10. https://doi.org/10.1186/s13071-017-2028-5

Sunantaraporn, S., Thepparat, A., Phumee, A., Sor-Suwan, S., Boonserm, R., Bellis, G. & Siriyasatien, P. (2021) *Culicoides* Latreille (Diptera: Ceratopogonidae) as potential vectors for *Leishmania martiniquensis* and *Trypanosoma* sp. in northern Thailand. *PLOS Neglected Tropical Diseases* 15(12): e0010014. https://doi.org/10.1371/journal.pntd.0010014

Talmi-frank, D., Kedem-Vaanunu, N., King, R., Bar-Gal, G. K., Edery, N., Jaffe, C. L., & Baneth, G. (2010). *Leishmania tropica* infection in Golden Jackals and Red Foxes, Israel. *Emerging Infectious Diseases*, *16*(12), 1973–1975. https://doi.org/10.3201/eid1612.100953

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpazees. *Molecular Biology and Evolution*, *10*(3),512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Taylor, V. M., Muñoz, D. L., Cedeño, D. L., Vélez, I. D., Jones, M. A., & Robledo, S. M. (2010). *Leishmania tarentolae:* Utility as an in vitro model for screening of antileishmanial agents. *Experimental Parasitology*, *126*(4), 471–475. https://doi.org/10.1016/j.exppara.2010.05.016

Telleria, J., Bosseno, M. F., Tarifa, T., Buitrago, R., Martinez, E., Torrez, M., Le Pont, F., & Brenière, S. F. (1999). Putative reservoirs of *Leishmania amazonensis* in a sub-andean focus of Bolivia identified by kDNA-polymerase chain reaction. *Memórias Do Instituto Oswaldo Cruz*, *94*(1), 5–6. https://doi.org/10.1590/S0074-02761999000100002

Toepp, A. J., Bennett, C., Scott, B., Senesac, R., Oleson, J. J., & Petersen, C. A. (2019). Maternal *Leishmania infantum* infection status has significant impact on leishmaniasis in offspring. *PLoS Neglected Tropical Diseases*, *13*(2), 1–16. https://doi.org/10.1371/journal.pntd.0007058

Tomás-Pérez, M., Khaldi, M., Riera, C., Mozo-León, D., Ribas, A., Hide, M., Barech, G., Benyettou, M., Seghiri, K., Doudou, S., & Fisa, R. (2014). First report of natural infection in hedgehogs with *Leishmania major*, a possible reservoir of zoonotic cutaneous leishmaniasis in Algeria. *Acta Tropica*, *135*(1), 44–49. https://doi.org/10.1016/j.actatropica.2014.03.018

Tsakmakidis, I., Pavlou, C., Tamvakis, A., Papadopoulos, T., Christodoulou, V., Angelopoulou, K., Dovas, C. I., Antoniou, M., Anastasakis, C., & Diakou, A. (2019). *Leishmania* infection in lagomorphs and minks in Greece. *Veterinary Parasitology: Regional Studies and Reports*, *16*(100279). https://doi.org/10.1016/j.vprsr.2019.100279

Tsokana, C. N., Sokos, C., Giannakopoulos, A., Birtsas, P., Valiakos, G., Spyrou, V., Athanasiou, L. V., Rodi Burriel, A., & Billinis, C. (2020). European Brown hare (*Lepus europaeus*) as a source of emerging and re-emerging pathogens of Public Health importance: A review. *Veterinary Medicine and Science*, *6*(3), 550–564. https://doi.org/10.1002/vms3.248

Tsokana, C. N., Sokos, C., Giannakopoulos, A., Mamuris, Z., Birtsas, P., Papaspyropoulos, K., Valiakos, G., Spyrou, V., Lefkaditis, M., Chatzopoulos, D. C., Kantere, M., Manolakou, K., Touloudi, A., Rodi Burriel, A., Ferroglio, E., Hadjichristodoulou, C., & Billinis, C. (2016). First evidence of *Leishmania* infection in European brown hare (*Lepus europaeus*) in Greece : GIS analysis and phylogenetic position within the *Leishmania* spp. *Parasitology Research*, *115*, 313–321. https://doi.org/10.1007/s00436-015-4749-8

Tunalı, V., & Özbilgin, A. (2023). Knock, knock, knocking on Europe's door: Threat of leishmaniasis in Europe with a focus on Turkey. *Current Research in Parasitology and Vector-Borne Diseases*, *4*(100150). https://doi.org/10.1016/j.crpvbd.2023.100150

U.S. Disease Control and Prevention. Laboratory Identification of Parasites of Public Health Concern. (2017). *Leishmaniasis*. Disponible en: www.cdc.gov/dpdx/leishmaniasis/index.html

Van der Auwera, G., Maes, I., De Doncker, S., Ravel, C., Cnops, L., Van Esbroeck, M., Van Gompel,

A., Clerinx, J., & Dujardin, J. C. (2013). Heat-shock protein 70 gene sequencing for *Leishmania* species typing in european tropical infectious disease clinics. *Eurosurveillance*, *18*(30), 20543. https://doi.org/10.2807/1560-7917.ES2013.18.30.20543

Vaselek, S., & Volf, P. (2019). Experimental infection of *Phlebotomus perniciosus* and *Phlebotomus tobbi* with different *Leishmania tropica* strains. *International Journal for Parasitology*, *49*(11), 831–835. https://doi.org/10.1016/j.ijpara.2019.05.009

Voltarelli, E., Arraes, S., Perles, T., Lonardoni, M., Teodoro, U., & Silveira, T. (2009). Serological survey for *Leishmania* sp. infection in wild animals from the municipality of Maringá, Paraná State, Brazil. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, *15*(4). https://doi.org/ISSN 1678-9199

Votýpka, J., Suková, E., Kraeva, N., Ishemgulova, A., Duží, I., Lukeš, J., & Yurchenko, V. (2013). Diversity of Trypanosomatids (Kinetoplastea: Trypanosomatidae) Parasitizing Fleas (Insecta: Siphonaptera) and Description of a New Genus *Blechomonas* gen. n. *Protist*, *164*(6), 763–781. https://doi.org/10.1016/j.protis.2013.08.002

World Health Organization. (2010). Control of the leishmaniases. *World Health Organization Technical Report Series*, *949*, 22–26. WHO (eds.). ISBN: 978 92 4 120949 6

World Health Organization, & Control of neglected tropical diseases. (2023a). *World: Endemicity of cutaneous leishmaniasis (CL), 2022*. World Health Organization. Disponible en: www.who.int/data/gho/map-gallery-search-results?&maptopics=910b5dfc-ce2e-4440-8b43-8d83f4a85485&term=leishmaniasis

World Health Organization, & Control of neglected tropical diseases. (2023b). *World: Endemicity of visceral leishmaniasis (VL), 2022*. World Health Organization. Disponible en: www.who.int/data/gho/map-gallery-search-results?&maptopics=910b5dfc-ce2e-4440-8b43-8d83f4a85485&term=leishmaniasis

Wu, Y., Jiang, M., Li, S., Waterfield, N. R., & Yang, G. (2022). Establish an allele-specific real-time PCR for *Leishmania* species identification. *Infectious Diseases of Poverty*, *11*(1). https://doi.org/10.1186/s40249-022-00992-y

Yadav, P., Azam, M., Ramesh, V., & Singh, R. (2023). Unusual Observations in Leishmaniasis—An Overview. *Pathogens*, *12*(2). https://doi.org/10.3390/pathogens12020297

ANEXOS

OTRAS PUBLICACIONES CIENTÍFICAS

- **Azami-Conesa, I.**, Gómez-Muñoz, M. T., & Martínez-Díaz, R. A. (2021). A systematic review (1990–2021) of wild animals infected with zoonotic *Leishmania*. *Microorganisms*, *9*(5). DOI: https://doi.org/10.3390/microorganisms9051101

- **Azami- Conesa, I.,** Matas- Méndez, P., Pérez- Moreno, P., Carrión, J., Alunda, J.M., Mateo-Barrientos, M., Gómez- Muñoz, M.T. (2024). Wildlife as a sentinel for pathogen introduction in non-endemic areas: Is *Leishmania tropica* circulating in wildlife in Spain? bioRxiv 2024.03.16.585353; DOI: https://doi.org/10.1101/2024.03.16.585353. Prepint.

microorganisms

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Review

A Systematic Review (1990–2021) of Wild Animals Infected with Zoonotic *Leishmania*

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Abstract: Leishmaniasis are neglected diseases caused by several species of *Leishmania* that affect humans and many domestic and wild animals with a worldwide distribution. The objectives of this review are to identify wild animals naturally infected with zoonotic *Leishmania* species as well as the organs infected, methods employed for detection and percentage of infection. A literature search starting from 1990 was performed following the PRISMA methodology and 161 reports were included. One hundred and eighty-nine species from ten orders (i.e., Carnivora, Chiroptera, Cingulata, Didelphimorphia, Diprotodontia, Lagomorpha, Eulipotyphla, Pilosa, Primates and Rodentia) were reported to be infected, and a few animals were classified only at the genus level. An exhaustive list of species; diagnostic techniques, including PCR targets; infected organs; number of animals explored and percentage of positives are presented. *L. infantum* infection was described in 98 wild species and *L.* (*Viania*) spp. in 52 wild animals, while *L. mexicana*, *L. amazonensis*, *L. major* and *L. tropica* were described in fewer than 32 animals each. During the last decade, intense research revealed new hosts within Chiroptera and Lagomorpha. Carnivores and rodents were the most relevant hosts for *L. infantum* and *L.* (*Viannia*) spp., with some species showing lesions, although in most of the studies clinical signs were not reported.

Keywords: *Leishmania*; host; reservoir; wildlife; wild mammal; zoonoses; one health

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1. Introduction

Included in the group known as neglected tropical diseases, the leishmaniases are a group of diseases caused by flagellated protozoan parasites from more than 20 species belonging to the genus *Leishmania*. The disease can appear with a variety of clinical pictures, depending on the species involved, the geographic region and the response of the host. Most people and animals infected by the parasite do not develop symptoms but, if present, the disease can follow three basic clinical forms in humans: cutaneous, mucocutaneous and visceral, while in animals, only cutaneous and/or visceral forms are observed. Leishmaniasis is a vector-borne disease transmitted by phlebotomine sand flies (order Diptera, family Psychodidae) with a worldwide distribution (Europe, Africa, the Americas, Asia, and one species recently described in Australia) and an endemic presence in more than 90 countries [1]. There are an estimated 350 million people at risk of *Leishmania* infection. The World Health Organization (WHO) estimates more than one million new cases per year in people around the world, making it one of the most relevant yet neglected parasitic diseases (https://www.who.int/leishmaniasis/en/, accesed on 8 January 2021). Leishmaniasis is one of the leading causes of morbidity in the world among infectious diseases and one of the leading causes of death among tropical diseases [2]. The disease is present in 98 of the 200 countries that collaborate with the WHO, and information is regularly updated by the organisation [3]. At least 39 described species of

Leishmania can be found in the literature, in addition to a significant number of informal or incomplete citations [4,5]. Many authors consider that some of these species should be synonymous and that the *Leishmania* taxonomy should be revised and simplified [6]. A list with the zoonotic species of the genus *Leishmania* along with their main characteristics is shown in Table 1.

Table 1. Zoonotic species of the genus *Leishmania* and their main characteristics (adapted from Ahoundi et al., 2016 and 2017 [4,5]).

Section	Subgenus	Species	Clinical Disease in humans	Geographic Area
Euleishmania	*Leishmania*	***L. aethiopica***	CL, DCL	Ethiopia, Kenya
		L. amazonensis	CL, DCL, MCL	Bolivia, Brazil and Venezuela
		L. donovani	VL, PKDL	Central Africa, South Asia, Middle East, India and China
		L. infantum	VL, CL	North Africa, South Europe, Middle East, Central Asia and North, Central and South America
		L. major	CL	Central and North Africa, Middle East and Central Asia
		L. mexicana	CL, DCL	USA, Mexico, Ecuador, Peru and Venezuela
		L. tropica	CL, VL	Central and North Africa, Middle East, Central Asia and India
		L. venezuelensis	CL	Northern South America
	Viannia	***L. braziliensis***	CL, MCL	Brazil, Bolivia, Peru, Guatemala and Venezuela
		L. guyanensis	CL, MCL	Bolivia, Brazil, French Guyana and Suriname
		L. lainsoni	CL	Brazil, Bolivia and Peru
		L. lindenbergi	CL	Brazil
		L. naiffi	CL	Brazil, French Guyana
		L. panamensis	CL, MCL	Brazil, Panama, Venezuela and Colombia
		L. peruviana	CL, MCL	Peru, Bolivia
		L. shawi	CL	Brazil
Paraleishmania		***L. colombiensis***	CL, VL	Colombia

Zoonotic species included in the systematic review are in bold. Clinical forms in humans: CL: cutaneous leishmaniasis; DCL: Diffuse cutaneous leishmaniasis; VL: visceral leishmaniasis; MCL: muco-cutaneous leishmaniasis; PKDL: Post-kala-azar dermal leishmaniasis.

All species of the genus follow a biological cycle with the same pattern, alternating amastigote forms that replicate intracellularly in the vertebrate host, and promastigote forms that reproduce in the digestive system of the insect vector (Figure 1). Sand flies (mainly *Phlebotomus* and *Lutzomya* genera) become infected while feeding on a parasitised reservoir. Through the bite, they ingest macrophage-bearing blood and tissue with amastigotes. Natural vectors have been experimentally proven to be highly susceptible, and one or two parasites are sufficient to initiate an infection [7]. For a species of sand fly to be a vector of zoonotic *Leishmania,* it must meet five conditions: (1) be anthropophilic; (2) feed from reservoir hosts in cycles of zoonotic transmission; (3) be infected in nature with the same *Leishmania* species that infects humans; (4) harbour the complete development of the parasite until it becomes infective; (5) be capable of transmitting the parasite through the bite [8].

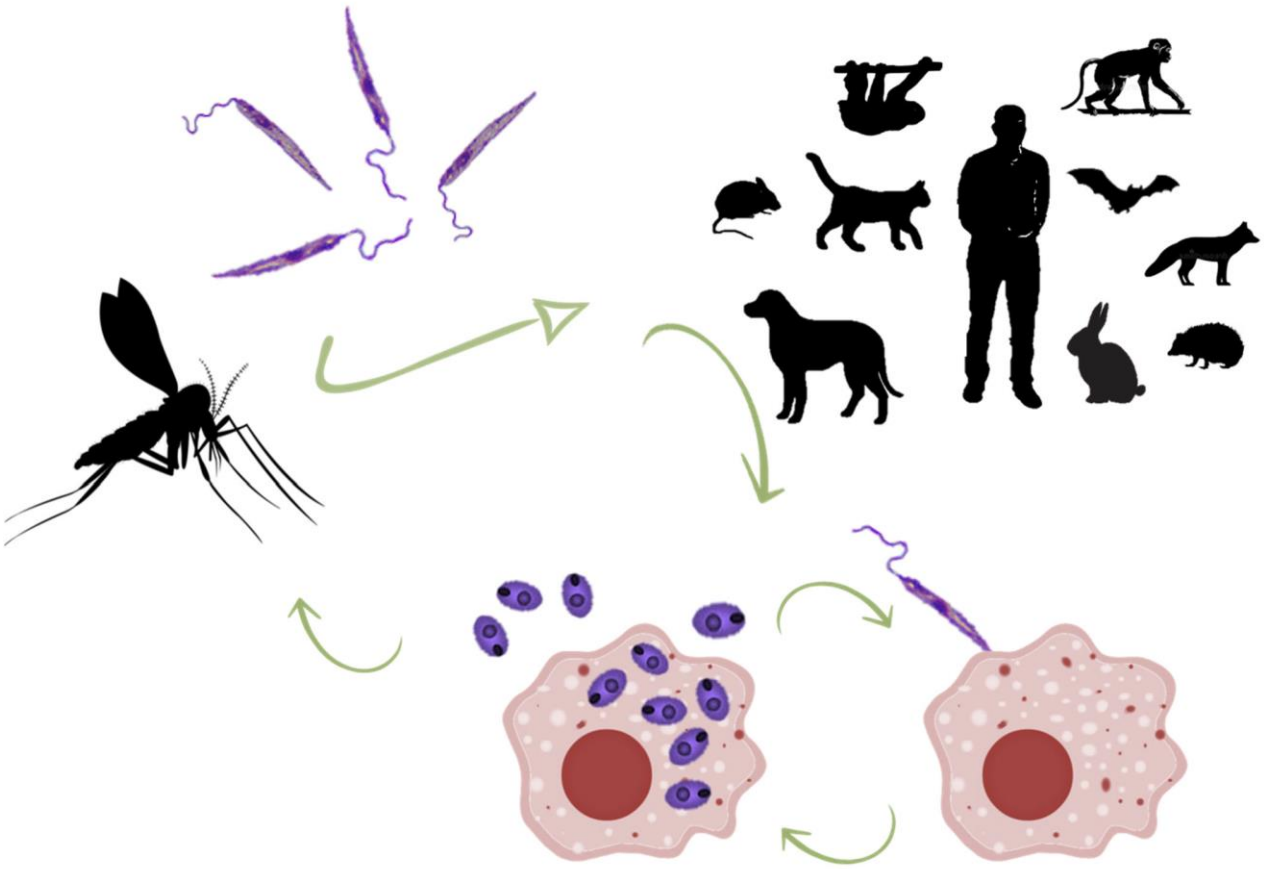

Figure 1. Life cycle of *Leishmania*: some of the wild animals found infected with the parasite are included.

Other infectious routes, such as venereal and vertical transmission, have been proved for *L. infantum* in a canine host [9], and it is seriously considered in humans [10]. Biting is a route suspected for canids [11], and the oral route has been confirmed in hamsters [12], which has been suggested to be associated with the ingestion of micromammals by common genets and servals [13], or the ingestion of phlebotomines by insectivorous bats [14]. In another study, the presence of *Leishmania* amastigotes and promastigotes in the faeces of gorillas have been reported [15]. Indeed, translocation of bacteria from the gut to distant locations helped by dendritic cells is a phenomenon widely studied in the human microbiome nowadays [16,17].

A combination of strategies is required for the prevention and control of the disease, including early diagnosis and prompt and effective treatment, vector control, effective disease surveillance, control of animal reservoir hosts and social mobilisation and strengthening partnerships [18]. Domestic animals have been widely studied and, traditionally, dogs are considered the main animal reservoir, and cats and equines have been found in several studies infected with the parasite [19]. However, dogs were found with similar or even lower prevalence than wildlife during some human outbreaks, probably due to preventive measures applied [20,21]. For these reasons, the investigation of the role of wildlife in the infectivity and potential transmission of the parasite is an important step in order to control future outbreaks, and to monitor the endemicity of certain areas. A change in the factors influencing the abundance of vectors (i.e., deforestation, climatic change, and new urbanised areas), or the presence of potential animal reservoirs in a spatial and temporal coincidence with humans, are essential factors in the appearance of outbreaks.

According to the WHO [22], the term "reservoir" should be used only for animals that are sufficiently abundant and long-lived to be a food source for sand flies and that maintain intense contact with the sand fly vector in its environment. Several characteristics are necessary: (1) more than 20% of the specimens should be infected; (2) the course of infection should be long; (3) parasites should be available in the skin or blood in sufficient numbers to be taken up by the sand flies; (4) the parasites in the reservoirs and humans should be the same [23]. Reservoir hosts usually represent a large proportion of the mammalian biomass [23]. Roque and Jansen [24] applied the terms "maintenance hosts" for mammals than can be infected and maintain the infection and "amplifier hosts" for mammals that, besides maintaining the infection, may favour the transmission (for example with more parasites in the blood and skin for longer periods). In this context, many of the wild animals mentioned in this review could be considered as maintenance hosts that may serve as secondary reservoirs, if adequate conditions for disease dissemination are present, while only a few could be considered as amplifier hosts (i.e., animals proved to infect the vector or with high prevalence values and close proximity with humans).

This paper presents updated information on wildlife as potential reservoir hosts for all zoonotic *Leishmania* species, following a systematic review from 1990 to nowadays. Previous reviews should be examined for partial and prior information [19,23–27].

2. Methods

This systematic review was carried out following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [28]. The main objective of this review was the identification of potential reservoirs of zoonotic species of *Leishmania*. Specific objectives were to (1) identify wild animal species naturally infected with zoonotic species of *Leishmania*; (2) recover information on the organs infected; (3) recover information on the techniques employed for detection and identification; (4) report data on the prevalence obtained in each study. These wild species should be investigated when an outbreak of leishmaniasis is present, or to monitor the endemicity of the disease in certain areas.

2.1. Search Strategy and Databases

Three databases were employed: PubMed (Medline), Scopus and Web of Science (WoS). The terms of search were "*Leishmania*" AND "wild" AND "host" OR "mammal". The information was retrieved from 1990 to 27 February 2021 and included only articles in English and zoonotic species of *Leishmania* (Table 1). Each author revised one database and eliminated reports according to the exclusion criteria. Duplicates were removed from the list before the employment of Mendeley to upload the selected articles. After the screening, the articles selected for inclusion were deeply analysed according to the species of *Leishmania*: *Leishmania* (*Viannia*) spp. were analysed by MTGM, *Leishmania infantum* was analysed by IAC and the rest of the species were analysed by RAMD. When doubts arose, the three authors discussed them and reached a consensus.

2.2. Exclusion Criteria

Automatic tools were employed to exclude some of the articles, while others were screened by the authors. Keywords for exclusion in Scopus were: animal experiment, animal model, mice, inbred C57BL, protozoan proteins, Bagg albino mouse, insect vectors, signal transduction, gene expression regulation, drug effect, inducible nitric oxide synthase, protein function, upregulation, wild type, macrophages, enzyme activity, mice knockout, cytokine production, Interleukin 10, Interleukin 4, mutant, mice inbred BALB C, unclassified drug, C57BL mouse, gene deletion, mutation, Th1 cell, cytokine, chemistry, Interleukin 12, protein expression, gamma interferon, arginase and CD4+T lymphocyte. Areas excluded in WoS were: research experimental medicine, virology, genetics heredity,

biophysics, mycology, endocrinology metabolism, forestry, haematology, plant sciences, evolutionary biology, fisheries, oncology, physiology, polymer science and respiratory system.

Articles in Spanish, Portuguese, Turkish or French were excluded. Reviews, books and chapters of books, opinion articles, conference papers and letters were also excluded from the systematic review. Other criteria for exclusion were the following: experimental infections; clinical cases (except first citations); articles dealing with wild-type and genetically modified parasites; articles dealing only with human and/or domestic animals samples; articles dealing only with vectors or xenodiagnosis; articles dealing only with isolates obtained in previous studies; articles dealing only with phylogeny; studies with negative results to *Leishmania* infections; articles without enough information on the identification of *Leishmania* species; non-zoonotic species of *Leishmania*.

In total, 151 references were retrieved from the search of the databases, and ten more articles were found from other sources, and included (references from previous research articles and reviews) (Figure 2). All information contained in the tables was obtained from these articles, but additional references are included for the background. Recorded variables are included in the Supplementary Materials: host, number of animals sampled, organs analysed, method of detection, prevalence and geographic area.

Due to the variability found among the studies and taking into account that wild animals are not easy to sample, a meta-analysis was not conducted. The main objective of this review was to update the list of potential reservoirs of the parasite and, for that reason, even studies with only one animal of a certain species were included.

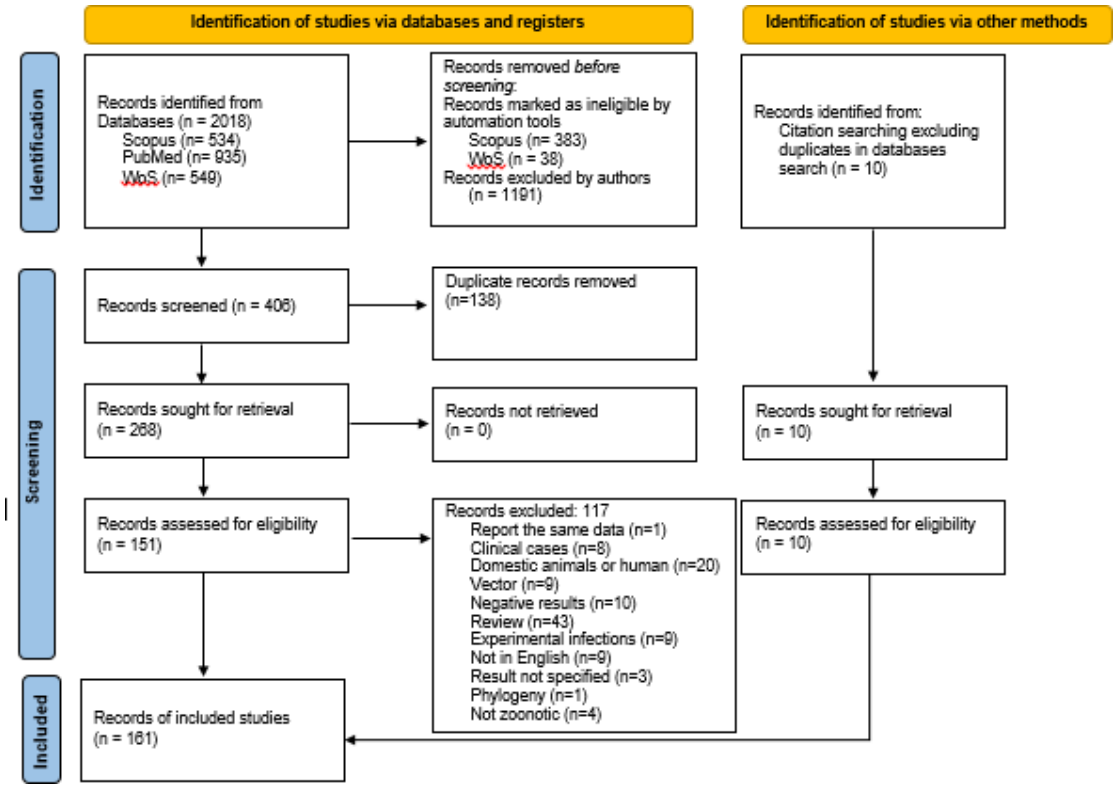

Figure 2. PRISMA 2020 flow diagram for the present systematic review.

3. Results

3.1. Result of the Search

The database search identified 2018 records, 534 from Scopus, 935 from PubMed and 549 from WoS. After removal of articles by automation tools and by authors using title and abstract, duplicates were removed. Exclusion criteria were further applied and an outcome of 161 articles was reached: 151 articles retrieved from databases and ten from other sources (Figure 2).

3.2. Wild Animals Infected with Zoonotic Leishmania (Viannia) spp.

Among the species of *Leishmania* described in the Americas, *L. braziliensis* is one of the most widely investigated. *L. (Viannia) braziliensis* is the species more extensively distributed, and it has been described in Latin American countries, from Mexico to more southern countries. Data from wildlife include not only Brazil but also Venezuela, Colombia, Honduras, Belize, Peru, Panama and Argentina (Supplementary Material File S1) [3,5]. Endemic leishmaniasis were present in several locations, such as the states of Sao Paulo and Minas Gerais in Brazil, especially where primary forest was substituted for human settlements [13]. Places such as coffee, banana or sugar cane plantations, ecotourism areas, or even chicken ruins and stables were known as breeding sites for the vector, and wild animals also became infected there [29–31]. Most of the studies were carried out in Brazil, especially in these endemic areas, whereas for other countries, such as Colombia, Peru, Bolivia, Venezuela or Argentina, infections were reported occasionally (Supplementary Material File S1).

Blood and skin were the sites of detection in many of the published epidemiological works, since they are the most accessible for the hematophagous vector (Supplementary Material File S1). However, when spleen, liver or bone marrow (BM) were included among the tissues analysed, prevalence rose, as the parasite tends to remain in these locations, even for species of *Leishmania* causing preferred cutaneous or mucocutaneous manifestations such as *L. (Viannia) braziliensis* or *L. mexicana*. Only one study employed oral swabs to successfully detect the DNA of the parasite [32].

Before the wide introduction of DNA amplification by PCR, fewer sensitive techniques were employed for the detection of *Leishmania*. Direct diagnosis of the parasite, such as examination of biopsies or imprints from skin or other organs, and culture in specific media were tested in rodents, marsupials and sloths in the past [24]. In fact, they are still being used in some studies today, although less frequently [30,31,33,34]. Serology has been extensively employed in domestic animals, but also in wildlife, as a sensitive and indirect evidence of *Leishmania* infection [30,35,36], and is the preferred method when studying the presence of *Leishmania* in wild carnivores [37] (Supplementary Material File S1). Xenodiagnosis by exploring transmission of the vector was rarely employed [29], although this approach could prove the reservoir character of the hosts. Finally, experimental infections of wild and synanthropic rodents and monkeys was an approach not frequently used and will not be treated here, because it is beyond the scope of this review [24].

Since PCR became a routine technique to detect *Leishmania* in tissues, the list of infected hosts has lengthened. Brandao-Filho et al. [30] compared three diagnostic tests with spleen samples from 203 animals (rodents and marsupials) and found kDNA PCR over three times more sensitive (17.6%) than traditional methods such as microscopy of imprints (5.7%) or culture (1.3%). Serology seems to be less sensitive than molecular techniques, as it was shown for the prevalence of *Leishmania* in *Didelphis marsupialis* (8.1% by serology vs. 20% by PCR) [38] and when small mammals were analysed (5% serology vs. 8.8–23.2% by PCR) [39].

A vast number of primers, methods and targets for PCR detection can be found in the literature [5], but a few of them are widely employed in epidemiological studies in wildlife by several authors. Some targets are recognised as highly sensitive; this is the case

for kinetoplast DNA (kDNA) which has more than 10,000 copies per cell [40]. PCR of kDNA has been employed for the detection of the parasite, followed by other approaches to determine the species of *Leishmania* present such as sequencing, RFLP or hybridisation (Supplementary Material File S1). In some papers, the employment of PCR that amplify ITS1 or SSU allows also for the classification at the species level [5,14].

Animals infected with zoonotic species of *L. (Viannia)* belong to the orders Carnivora, Cingulata, Chiroptera, Didelphimorphia, Lagomorpha, Pilosa, Primata and Rodentia, this last order being the most extensively studied.

Carnivores are usually blamed to be reservoirs of different species of *Leishmania*, and the scarce number of studies carried out revealed the presence of *Leishmania* (*Viannia*) spp. *L. braziliensis* DNAwas amplified by PCR in crab-eating dogs [34] and in Molina's hog-nose skunks, this last species being pointed out as a reservoir by the authors, since a strain was isolated from one animal [41]. Specific antibodies employing serological techniques, such as direct agglutination test (DAT), were found in high percentages in hoary foxes, ring-tailed coatis and crab-eating racoons (50–100%), but the authors tested a small number of the animals for each species [37] (for details, see Supplementary Material File S1).

The number of studies exploring the presence of *Leishmania* in bats has increased since 2013, when Shapiro et al. found DNA of the parasite in blood, liver and skin by PCR [42]. Since then, four more studies found *L.* (*Viannia*) spp. DNA in more than eight species of bats including hematophagous, insectivorous and frugivorous individuals [32,43–45].

Armadillos (order Cingulata) have been examined for *Leishmania* infection and found positive by PCR and culture plus zymodeme analysis in blood, spleen and liver [46] and in another study by PCR of the kDNA region, but the species of *Leishmania* present were not further investigated [34]. Regarding Lagomorpha, only one study demonstrated the presence of *L. braziliensis* in tapetis (*Sylvvilagus braziliensis*) in Colombia, employing xenodiagnosis and PCR followed by hybridisation [29] (Supplementary Material File S1).

The presence of *L. braziliensis* has previously been reported in sloths, rodents and marsupials, and the reservoir character of these groups has been shown by several authors in the past [13,23]. The order Didelphimorphia, especially the white-eared opossum (*Didelphis albiventris*), was the focus of at least sixteen studies employing diverse PCRs, culture and serology, with highly variables percentages of infection [21,29,30,34,37–39,47–55] (Supplementary Material File S1). In addition, xenodiagnosis was successful in this species as well as in the woolly-mouse opossum (*Micoureus demerarae*), which reinforces their role as main reservoirs of leishmaniasis [29,48]. One study found three two-toed-sloths (*Choloepus hoffmani*) infected with *L. panamensis* in Panama [56].

Rodents are the group most widely explored regarding *Leishmania* infections, both in natural and experimental conditions. The presence of *L. braziliensis* and other zoonotic species of the subgenus *Viannia* has been reported in 27 species including *Rattus rattus*, *Cerradomys subflavus, Necromys lasiurus*, *Nectomys squamipes* and *Mus musculus,* the latter being the species more often investigated [21,29–31,33–37,39,47,49,50,53,55,57–63] (Supplementary Material File S1). This may be due to the fact of several reasons: their probable role as relevant reservoirs of leishmaniasis for humans, their proximity and high prevalence values (rats and domestic mouse), their abundance in ecological niches where phlebotomines reproduce, or to the successful attempts when xenodiagnosis or strain isolation were employed. Prevalence values varied from 1.2% to 100% depending on the sampling area, the sample size, the organs analysed, the diagnostic procedures employed for detection and characterisation and, probably, also on the age and lifespan of the sampled animals. Xenodiagnosis was successful in synanthropic species, such as black rats, and wild species, such as *Melanomys caliginosus* and *Mycroryzomys minutus.* The parasite was isolated by culture from *Akodon* spp., *Agouti paca, C. subflavus, N. lasiurus, R. rattus* and *Sigmodon hispidus*, but the PCR of kDNA was the preferred method for detection. When RFLP or sequencing was applied after PCR, the species could be determined, being *L. braziliensis* and *L. guyanensi* the most frequent. *L. naiffi*, *L. shawi* and *L. lainsoni* were obtained from rodents. Minor zoonotic species, such *L. shawi* and *L. naiffi,*

were detected in species of the genus *Trichomys* [36], and *L. lainsoni* was mainly found in the big rodent paca (*Agouti paca*) [57] (Supplementary Material File S1). Less frequent species of *Leishmania*, such as *L. peruviana,* were obtained from rodents and Didelphimorphia in Peru [47].

Only four studies were performed on primates employing PCR of KDNA, miniexon or ITS regions, some followed by sequencing or RFLP [34,37,64,65], while DAT was only employed in one study. *Leishmania* (*Viannia*) spp. was found between 8.6–100% of the animals analysed, with *L. braziliensis* being present, when identified to the species level [37].

All these investigations are summarized in Table 2.

Table 2. Wild animals reported infected with zoonotic *Leishmania* (*Viannia*) species. Organs or tissues where the parasite was detected are indicated, as well as the techniques employed for detection. *L* (*Viannia*) species are as follows: Lb: *L. braziliensis*, Lg: *L. guyanensis*, LVsp: *Leishmania* (*Viannia*) spp., Lsp: *Leishmania* sp., Lpa: *L. panamensis*, Lpe: *L. peruviana*, Ln: *L. naiffi*, Ls: *L. shawi* and Ll: *L. lainsoni*.

Host	Prevalence	Organs/Tissue Analysed	Methods for Detection	*Leishmania* (*Viannia*) species	Country	References
Order Carnivora						
Cerdocyon thous (crab-eating fox)	20–100%	blood, serum	PCR (kDNA), DAT	LVsp	Brazil	[34] [37]
Conepatus chinga rex (Molina's hog-nose skunk)	50%	Skin + liver + spleen	Inoculation to hamster, isoenzyme analysis, hybridisation, PCR (kDNA)	Lb	Bolivia	[41]
Lycalopex (Pseudalopex) vetulus (hoary fox)	100%	serum	DAT	LVsp	Brazil	[37]
Nasua nasua (ring-tailed coati)	50%	serum	DAT	LVsp	Brazil	[37]
Procyon cancrivorous (crab-eating raccoon)	50%	serum	DAT	LVsp	Brazil	[37]
Order Cingulata						
Dasypus novemcinctus (armadillo)	15.6%	blood, LN, liver, skin, spleen	Culture, zymodeme analysis	Lb	Brazil	[46]
Dasypus sp.	100%	blood	PCR (kDNA)	LVsp	Brazil	[34]
Order Chiroptera						
Artibeus planirostris (frugivorous)	4.3%	skin	PCR (kDNA), PCR (HSP70) + RFLP, PCR (G6DP) + sequencing	Lb	Brazil	[43]
Cynomops planirostris (insectivorous)	11.1%	liver, skin	PCR (kDNA), nPCR (SSU) + sequencing	Lb	Brazil	[44]
Desmodus rotundus (hematophagous)	3.2%	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Lb	Brazil	[43]
Eumops perotis (insectivorous)	5.6%	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Lb	Brazil	[45]
Glossophaga soricina (insectivorous)	0.9–40%	blood, liver, spleen	PCR (ITS1) + RFLP, PCR (kDNA, PCR (Cyt B) + sequencing	Lb	Brazil	[42,44,45]
Lasiurus cinereus (insectivorous)	20%	liver, skin	PCR (kDNA), nPCR (SSU) + sequencing	Lb, LVsp	Brazil	[44]
Molossus molossus (insectivorous)	44–25%	blood	PCR (ITS1) + RFLP, PCR (kDNA), PCR (Cyt b) + sequencing	Lb	Brazil	[42,45]
Platyrrhinus lineatus (frugivorous)	13.3%	skin	PCR (kDNA), PCR (HSP70) + RFLP, PCR (G6DP) + sequencing	Lb	Brazil	[43]
Several species: *Artibeus lituratus, Carollia perspicillata, Diphylla ecaudata and Glossophaga soricina*)	19.8%	oral swab	PCR (SSU) + sequencing	LVsp	Brazil	[32]
Order Didelphimorphia						
Didelphis albiventris (white-eared opossum)	1.6–50%	blood, BM, liver, serum, skin (tail/ear), spleen	culture, imprints, isoenzymes, PCR (kDNA), qPCR (kDNA) PCR (ITS1), PCR (HSP70), PCR (HSP70) + RFLP, PCR (ITS) + RFLP, nPCR (SSU) + sequencing, IFAT, DAT	Lb, LVsp, Lg, Lpe	Peru, Brazil	[21,30,37–39,47,50–54]

Didelphis marsupialis (common opossum)	20–33.3%	ear	PCR (kDNA), hybridisation, xenodiagnoses vector	Lb, LVsp	Colombia	[29,49]
Didelphis sp.	90%	blood	PCR (kDNA), culture	LVsp	Brazil	[34]
Marmosa sp.	16.7–25%	skin, spleen	PCR (kDNA), smears, culture	LVsp	Brazil	[30,39]
Gracilinanus agilis (agile gracile opossum)	1.4–75%	blood, BM, liver, skin (tail/ear), spleen	PCR (kDNA), PCR (HSP70), PCR (HSP70) + RFLP, PCR (ITS) + RFLP	Lb, LVsp, Lg	Brazil	[21,55]
Marmosops incanus (grey slender opossum)	50%	ear skin	PCR (HSP70) + RFLP		Brazil	[21]
Temerarae (woolly mouse opossum)	66.7%	ear	PCR (kDNA), hybridisation, xenodiagnoses vector	Lb	Colombia	[29]
Monodelphis domestica	25%	skin, spleen	PCR (kDNA)	LVsp	Brazil	[39]
Micoureus pagaruayanus (woolly-mouse opossum)	4.2–11.6%	skin	PCR (kDNA), qPCR (kDNA), nPCR (SSU), nPCR (G6DP)	Lb, LVsp	Brazil	[48]
Micoureus sp.	100%	blood	PCR (kDNA)	LVsp or Lsp.	Brazil	[34]
Order Lagomorpha						
Sylvilagus brasiliensis (tapeti)	100% (*n* = 1)	ear	PCR (kDNA), hybridisation, xenodiagnoses vector	Lb	Colombia	[29]
Order Pilosa						
Choloepus hoffmani (two-toed sloth)	75%	blood	Culture, PCR (kDNA), PCR (HSP70)	Lpa	Panama	[56]
Order Primates						
Alouatta caraya (black howler)	8.3%	ear tissue	PCR (ITS) + RFLP + sequencing	Lb, LVsp	Argentina	[64]
Aotus azarai (Azara's night monkey)	44.4%	blood, spleen	PCR (miniexon) + RFLP + sequencing	Lb	Argentina	[65]
Callithrix sp.	100%	blood	PCR (kDNA)	LVsp	Brazil	[34]
Cebus apella (tufted capuchin)	100%	serum	DAT	LVsp	Brazil	[37]
Order Rodentia						
Agouti paca (paca)	100%	skin	culture, isoenzymes, inoculation hamster	Ll	Brazil	[57]
Akodon arviculoides	4%	spleen	smears, PCR (kDNA)	LVsp	Brazil	[30]
Akodon cursor	9.7%	liver, skin (tail), spleen	culture (liver and skin), PCR (kDNA)	Lb	Brazil	[31]
Akodon sp.	2.6%	blood, skin	culture + isoenzymes, PCR	LVsp	Peru	[47]
Cerradomys (sin. *Oryzomys) subflavus*	7.8–50%	BM, liver, skin (tail/ear), spleen	culture (skin), PCR (kDNA), PCR (kDNA) + hybridisation, nPCR (SSU) + sequencing, PCR (HSP70) + RFLP	Lb, LVsp	Brazil	[31,39,50,53,57]
Calomys expulsus	3.3%	liver	PCR (kDNA)	LVsp	Brazil	[55]
Dasyprocta azarae (Agouti)	75%	serum	DAT		Brazil	[37]
Holochilus scieurus	7.1–15%	skin, spleen	imprints, PCR (kDNA)	LVsp	Brazil	[30,39]
Melanomys caliginosus	21.4%	ear	PCR (kDNA) + hybridisation	Lb	Colombia	[29]
Microryzomys minutus	50%	ear	PCR (kDNA) + hybridisation	Lb	Colombia	[29]
Mus musculus	55–100%	blood, BM, liver, skin (tail/ear), spleen	PCR (kDNA) + RFLP, nPCR (SSU) + sequencing	Lb, LVsp	Brazil	[34,50,59]
Necromys (sin. Bolomys) lasiurus	4.9–100%	BM, liver, skin (tail/ear), spleen	culture, imprints, PCR (kDNA), PCR (kDNA) + RFLP, serodeme, isoenzyme, PCR (ITS) + RFLP, PCR (D7 24Sα rRNA =	Lb	Brazil	[30,35,50,59,60]

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			trypanosomatids) & PCR (ITS) + sequencing, nPCR (SSU) + sequencing			
Nectomys squamipes	7.2–28.1%	skin, spleen	culture, smears, serodeme, isoenzyme, PCR (ITS) + RFLP, PCR (kDNA), serology rK39 Ag, inoculation to hamster, zymodeme	Lb, LVsp	Brazil	[30,39,60]
Oecomys trinitatus	100% (*n* = 1)	ear skin	PCR (kDNA)	LV sp	Colombia	[49]
Oligoryzomis nigripes	26.8%	liver	PCR (kDNA)	LV sp	Brazil	[55]
Oxymyicterus dasytrichus	33.3%	liver	culture, PCR (kDNA), PCR (HSP70) + RFLP	Lb	Brazil	[31]
Phyllotis andinum	1.2%	blood, skin	culture + isoenzymes, PCR	LVsp, Lpe	Peru	[47]
Proechymis sp.	100% (*n* = 1)	liver, skin	PCR (kDNA)	LVsp	Brazil	[61]
Rhipidomys macrurus	29.6%	ear skin	PCR (D7 24Sα rRNA trypanosomatids) and PCR ITS + sequencing	Lb	Brazil	[35]
Rattus norvegicus (brown rat)	26.9–66.6%	blood, BM, liver, skin (tail/ear), spleen,	nPCR (SSU) + sequencing	Lb	Brazil	[50,62]
Rattus rattus (black rat)	2.5–50%	blood, BM, liver, skin (tail/ear), spleen	culture, hybridisation, smears, serodeme, isoenzyme, PCR (kDNA), PCR (kDNA) + RFLP, PCR (ITS) + RFLP, nPCR (SSU)+ sequencing, PCR (HSP70) + RFLP, serology rK39 Ag	Lb, LVsp	Brazil, Colombia, Venezuela	[29,30,33,39,50,53,57,60,63]
Sigmodon hispidus (hispid cotton rat)	0.3%-100% (*n* = 1)	blood, ear skin	culture, PCR (kDNA), PCR (kDNA) + RFLP or hybridisation	Lb, LVsp	Venezuela Colombia	[33] [49]
Trichomys apereoides	6.3–15.6%	liver, skin (tail/ear)	PCR (HSP70) + RFLP	Lb, Lg	Brazil	[21]
Trichomys fosteri	2.5%	spleen	PCR (kDNA), PCR (HSP70)	Ln	Brazil	[36]
Trichomys inermis	3%	spleen	PCR (kDNA), PCR HSP70	Ls	Brazil	[36]
Trichomys laurentis	2–3.9%	spleen	PCR (kDNA), PCR (HSP70)	Lb, Ls, Ln, Lg	Brazil	[36]
Trichomys sp.	100% (*n* = 1)	blood	PCR (kDNA)	LVsp	Brazil	[34]
Zygodontomys bruneus	100% (*n* = 1)	ear skin	PCR (kDNA)	LVsp	Colombia	[49]

BM: bone marrow; Cytb: cytochrome B; DAT: direct agglutination test; FML: fucose-mannose ligand; G6DP: glucose e phosphate dehydrogenase; HSP70: heat shock protein 70 kDa; IHC: immunohistochemistry; IC: immunochromatography; ELISA: enzyme immune assay; IFAT: immunofluorescence assay; ITS: internal transcriber spacer; kDNA: kinetoplast DNA; LN: lymph node; nPCR: nested PCR; qPCR: quantitative PCR; RFLP: restriction fragment length polymorphism; SSU: small subunit of ribosomal RNA.

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In previous reviews [23,24], other species of wild animals were found infected with *Leishmania* (*Viannia*) spp., such as rodents (*Coendu* sp., *Rhipidomys leucodactylus*, *Heteromys dermarestianus*, *Proechymis semispinosus*, *Trichomys pachyurus*), sloths (*Choleopus didactylus*, *Bradypus infuscatus*, *Bradypus tridactylus*), anteanters (*Tamandua tetradactyla*), primates (*Aotus trivirgatus*, *Cebus apella*, *Chiropotes satanas*, *Sanguinus geoffroyi*) and carnivores (*Nasua nasua*, *Potos flavus*). For more details, see previous articles dealing with leishmaniasis in the Americas [23,24].

3.3. Wild Animals Infected with Leishmania amazonensis.

L. amazonensis was described in countries from Central and South America, where data were available, including Costa Rica, Panama, Venezuela, Colombia, Ecuador, Peru, Argentina, Uruguay, French Guiana, Surinam, Brazil and Bolivia, the last two countries being the most widely studied [3,5]. This review includes updated information of wild animals infected with the parasite from surveys carried out in Brazil, Bolivia and Argentina.

Among carnivores, *L. amazonensis* was detected only in one of two skunks analysed from a focus of leishmaniasis in Bolivia [41]. More information was retrieved from three studies including several species of bats [14,45,66]. Most of the species analysed were insectivorous bats, but the parasite was also detected in hematophagous (*Desmodus rotundus*), nectarivorous and omnivorous species. Prevalence values varied from 1% to 25%, probably depending on the geographic area, the species and the organs or techniques employed for detection. Higher values were observed in *Sturnira lilium* and *Eumops auripendulus* from urban areas and remnants of primitive forest of Sao Paulo (Brazil), employing nested PCR from liver and spleen (Supplementary Material File S2).

Primates and opossum have been scarcely reported with *L. amazonensis* in the last 30 years, but the species was recently detected by PCR of the ITS region in the ear tissue of 2.8% of 209 black howler monkeys (*Alouatta caraya*) from Argentina [64]. In addition, a clinical case of a spider monkey (*Ateles paniscus*) from a zoo in Brazil, which showed weight loss and pale mucous membranes, was further confirmed by PCR and RFLP from blood [67]. *L amazonensis* DNA was also detected in 1.1% of the analysed woolly-mouse opossum (*Marmosa paraguayanus*) from Brazil [48] (Supplementary Material File S2).

Three studies from Bolivia and Brazil reported the presence of *L. amazonensis* in blood or skin (tail or ear) by PCR followed by sequencing, in 7.1–33.3% of the analysed rodents (*Hylaeamys, Oryzomys, Akodon, Necromys* and *Olygoryzomis* genera) [35,41,68]. Some of these rodents displayed old lesions including scars on the tail or ear [68].

Animals found infected with *L. amazonensis*, as weel as the techniues employed, are summarized in Table 3.

Table 3. Wildlife that reported positive for *Leishmania amazonensis*. Organs or tissues where the parasite was detected are indicated, as well as the techniques employed for detection.

Host	Prevalence	Organs/Tissue Analysed	Methods for Detection	Country	Reference
Order Carnivora					
Conepatus chinga rex (Molina's hog-nose skunk)	50%	liver, skin and spleen	Inoculation to hamster, Isoenzyme typing, PCR (kDNA) PCR (trypanosomatids) + hybridisation	Bolivia	[41]
Order Chiroptera					
Artibeus lituratus (nectarivorous)	1.6%	liver, skin and spleen	nPCR (SSU), qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14,66]
Artibeus planirostris (nectarivorous)	n.s.	skin	qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14]
Desmodus rotundus (haematofagous)	n.s.	liver, spleen	qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14]
Eumops glaucinus (insectivorous)	8.3%	liver, skin and spleen	nPCR (SSU), qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14,66]
Eumops auripendulus (insectivorous)	25%	liver, spleen	nPCR (SSU)	Brazil	[66]
Eumops perotis (insectivorous)	5.6%	blood	PCR (kDNA), PCR (Cyt b) + sequencing	Brazil	[45]
Glossophaga soricina (insectivorous)	2.8–4.2%	blood, liver and spleen	nPCR (SSU), PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45,66]
Molossus molossus (insectivorous)	1–1.6%	blood, liver and spleen	nPCR (SSU), PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45,66]
Molossus rufus (insectivorous)	1%	liver, skin, spleen	nPCR (SSU), qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14,66]
Myotis nigricans (insectivorous)	2.9%	liver, spleen	nPCR (SSU), qPCR (kDNA), PCR (ITS1) + RFLP	Brazil	[14,66]
Nyctinomops laticaudatus (insectivorous)	10%	liver, spleen	nPCR (SSU)	Brazil	[66]
Phyllostomus hastatus (omnivorous)	2.9%	blood	PCR (kDNA), PCR (Cyt b) + sequencing	Brazil	[45]
Platyrrhinus lineatus (omnivorous)	18.2%	blood, spleen	qPCR (kDNA), PCR (ITS1) + RFLP, PCR (kDNA), PCR (Cyt b) + sequencing	Brazil	[14,45]
Sturnira lilium (nectarivorous)	25%	liver, spleen	nPCR (SSU)	Brazil	[66]
Order Didelphimorphia					
Marmosa (Micoureus) paraguayanus (woolly-mouse opossum)	1.1%	skin	PCR (kDNA), qPCR (kDNA), nPCR (SSU), PCR (G6PD), sequencing	Brazil	[48]
Order Primata					
Alouatta caraya (black howler monkey)	2.8%	ear tissue	PCR (ITS) + RFLP + sequencing	Argentina	[64]
Ateles paniscus (spider monkey)	100%	blood	PCR (kDNA), PCR (ITS) + RFLP	Brazil	[67]
Order Rodentia					
Akodon spp.	7.1%	blood	PCR (kDNA) + hybridisation	Bolivia	[41]
Necromys (sin. *Bolomys) lasiurus*	20%	ear skin	PCR-D7 24Sα rRNA (trypanosomatids) and PCR (ITS) + sequencing	Brazil	[35]
Oligoryzomys spp. (rice rat)	25%	blood	PCR (kDNA) + hybridisation	Bolivia	[41]
Hylaeamys (Oryzomys) acritus	33.3%	tail skin	PCR (kDNA) + sequencing	Bolivia	[68]
Oryzomys nitidus	13.3%	tail skin	PCR (kDNA) + sequencing	Bolivia	[68]

BM: bone marrow; Cyt B: cytochrome B; G6DP: glucose 6 phosphate dehydrogenase; ITS: Internal transcriber spacer; kDNA: kinetoplast DNA; nPCR: nested PCR; n.s.: not specified; qPCR: quantitative PCR; RFLP: restriction fragment length polymorphism; SSU: small subunit of ribosomal RNA.

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Further information on other species infected with *L. amazonensis* can be found in previous reviews [23,24], and include rodents (*Dasyprocta* spp., *Oligoryzomis* spp., *Orzyomis melanotis*, *Proechymis* spp., *Trichomys apereoides*, *Sciurus vulgaris*), carnivores (*Cerdocyon thous*, *Potos flavus*), anteaters (*Tamandua tetradactyla*), marsupials (*Didelphis marsupialis*, *Metachirus nudicaudatus*, *Philander opossum* and *Marmosa cinerea*) and primates (*Saguinus geoffroyi*).

3.4. Wild Animals Infected with Leishmania mexicana

In this review, data regarding infection with *L. mexicana* in wild animals were mainly from the USA and Mexico, two countries where the parasite is frequently reported, but data from rodents and carnivores from Brazil and Bolivia were also included (Supplementary Material File S3). The species was present in other American territories such as Venezuela, Colombia, Ecuador and all countries in Central America [3,5].

According to the data from the systematic review, thirty-one species of wild animals from six orders were found to be infected with *L. mexicana*. In carnivores, only one out of two Molina's hog-nosed skunks (*Conepatus chinga rex*) were infected with *L. mexicana* in Bolivia, and the parasite was isolated by inoculation in hamster and subsequently analysed by isoenzyme analysis and hybridisation [41]. Samples from seven grey foxes (*Urocyon cinereoargenteus)* were analysed by ELISA, showing 100% prevalence in Mexico [69] (Supplementary Materials File S3). Both species should be considered as sentinel or even reservoirs, due to the parasite's isolation and high values of positivity.

Thirteen species of bats (order Chiroptera) were also found infected with the parasite in Mexico, employing skin, heart, liver and spleen in a PCR of kDNA and SSU [5]. The authors found infection rates ranging from 4–100% of the animals [70].

In rodents, eleven species were infected in ten different surveys, with *Handleyomis* (sin. *Oryzomis*) *melanotis*, *Ototylomis phyllotis*, *Peromyscus yucatanicus* and *Sigmodon hispidus* being the species with the higher levels of infection (100% in at least one study), which may indicate their role as reservoirs of the disease [33,58,71–78]. Within the order Didelphimorphia, the Mexican mouse opossum (*Marmosa mexicana*) [71] and the northern anteater [79] were found to be infected in Mexico employing PCR.

The order Primates was less explored, and only indirect evidence of the infection was reported by serology (ELISA, IFAT and Western blot). A prevalence of 5–37.5% was found in two species of howler monkeys (*Alouatta palliate* and *A. pigra*) in Mexico [80].

The base of the tail was the election site for detection or isolation of *L. mexicana* in rodents and marsupials, with 100% of infection in many studies in which animals with lesions were sampled [71–73,77], but the ear and foot were also included [76] (Supplementary Materials File S3). When other organs were investigated, such as liver, spleen, kidney or heart, they were also infected, but at lower percentages (11–66.7%) [72]. Heart, liver, spleen and skin were also employed to find infections in Chiroptera [70], while lymph nodes, lung, spleen, liver and kidney tissues were used in the northern anteater, with DNA detection by PCR only in spleen [79].

In general, wild animals showed mild clinical signs of leishmaniasis and no external signs were reported in the orders Carnivora, Chiroptera, Pilosa and Primates. On the contrary, rodents and marsupials were reported with cutaneous clinical signs in most of the surveys, including swollen skin, depigmentation, ulcers, alopecia and erythema, mainly at the base of the tail. This fact can be explained because the authors were searching for lesions to find reservoirs of the disease [71,72,77].

Wild animals infected with *L. mexicana*, along with techniques and organs or tissues positive to the parasite are shown in Table 4.

Table 4. Wild animals infected with *L. mexicana*. Organs or tissues where the parasite was detected, as well as the techniques employed, are indicated.

Host	Prevalence	Organs/Tissue Analysed	Methods for Detection	Country	References
Order Carnivora					
Conepatus chinga rex (Molina's hog-nosed skunk)	50%	Liver + skin + spleen (macerate)	Inoculation to hamster, isoenzyme analysis, hybridisation	Bolivia	[41]
Urocyon cinereoargenteus (fox)	100%	serum	ELISA	Mexico	[69]
Order Chiroptera					
Pteronotus personatus	25%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Artibeus jamaicensis	5.8%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Artibeus lituratus	7.3%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Carollia sowelli	4.4%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Choeroniscus godmani	23.1%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Desmodus rotundus	7.1%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Dermanura phaeotis	8.1%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Glossophaga commissarissi	75%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Glossophaga soricina	26.9%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Leptonycteris curasoae	50%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Phyllostomus discolor	100% (n = 1)	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Stumira lilium	11.1%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Stumira ludovici	4%	heart, liver, skin and spleen	PCR (kDNA), PCR (SSU)	Mexico	[70]
Order Didelphimorphia					
Marmosa mexicana (Mexican mouse opossum)	66.7%	base of the tail	PCR (kDNA)	Mexico	[71]
Order Pilosa					
Tamandua mexicana (northern tamandua)	6.3%	spleen	PCR (ALAT), PCR (ITS1) + sequencing	Mexico	[79]
Order Primates					
Alouatta palliate (mantled howler monkey)	5%	serum	ELISA, IFAT and WB	Mexico	[80]
Alouatta pigra (Guatemalan black howler)	37.5%	serum	ELISA, IFAT and WB	Mexico	[80]
Order Rodentia					
Heteromys gaumeri	46.3%	base of the tail	PCR (kDNA)	Mexico	[71]
Heteromys desmarestianus	100%	base of the tail	PCR (kDNA)	Mexico	[71]

Neotoma micropus (woodrats)	7.3–50%	skin, ear tissue	Culture, PCR (kDNA), Culture of lesions + PCR + isoenzyme analysis of cultures	USA	[73] [74]
Neotoma floridana (eastern woodrat)	100%	ear, foot	Smears, PCR	USA	[76]
Handleyomys (*Oryzomys) melanotis*	65–100%	skin (base-tail, lesions), liver and spleen	Culture, Mab, imprints and PCR (kDNA)	Mexico	[71,72,75,77]
Ototylomis phyllotis	75.5–100%	skin (base-tail), liver	Culture, Mab, imprints and PCR (kDNA)	Mexico	[71,72,75]
Peromyscus attwateri	100% (n = 1)	skin (neck)	PCR (ITS1) + sequencing	USA	[78]
Peromyscus yucatanicus	28.6–100%	skin (base-tail), heart and kidney, liver, spleen	Culture, PCR (kDNA) and imprints	Mexico	[71,72]
Rattus rattus (black rat)	2.9–19%	blood	Culture, PCR (kDNA) + RFLP/hybridisation	Venezuela, Brazil	[33] [58]
Reithrodontomys gracilis	66.6%	skin (base-tail)	Culture, PCR (kDNA)	Mexico	[71]
Sigmodon hispidus (cotton rat)	58.8–100%	liver, skin (base-tail, lesion) and spleen	Imprints, culture, Mab and PCR (kDNA)	Mexico	[71,72,75,77]
Trichomys apereoides	27.8%	blood	PCR (kDNA) + hybridisation	Brazil	[58]

ALAT: alanine transaminase; ELISA: enzyme immune assay; IFAT: Immunofluorescence assay; ITS: internal transcriber spacer; kDNA: kinetoplast DNA; Mab: monoclonal antibodies; SSU: small subunit of ribosomal RNA.

In previous reviews, several species from publications prior to 1990 were reported to be infected with *L. mexicana* (*Agouti paca*, *Marmosa robinson*, *Nyctomys sumichrasti*, *Oryzomis capito*, *Proechymis* spp., *Reithrodontomys gracilis*) [23,24].

3.5. Wild Animals Infected with Leishmania infantum (L. chagasi)

L. infantum is the most globally distributed of all species of zoonotic *Leishmania*. Australia is considered free of *L. infantum*, but the protozoan is present on almost all continents with available data, including Southern Europe, Africa, Asia and the Americas from north (excluding Alaska and Canada) to south. African countries and Brazil report more than 90% of the human VL cases around the world, but detailed characterised focusses are more frequently reported in Brazil and Mediterranean countries (North Africa and South Europe) [3,5].

Techniques employed to detect infection with *L. infantum* in different parts of the world are similar to those previously described for *L. braziliensis*. Serology was mainly employed in carnivores, primates and occasionally in marsupials or other species, such as rodents or Lagomorpha (Supplementary Materials Files S4 and S5), while the rest of the species were examined preferentially by PCR. Among the serological techniques, ELISA, IFAT, DAT or rapid test (rK39) were extensively employed. The most frequent target, again, was kDNA, but other targets, such as SSU and ITS1 and the repeat region, were also used in several studies and animal species. Less frequently used targets include cytochrome B (Cyt B), HSP70, ITS2, glyceraldehyde phosphate hydrogenase (GAPDH) and α-tubulin (for details, see Supplementary Materials File S4). Xenodiagnosis or culture were employed only in a few occasions.

Blood, skin, liver and spleen were the most employed tissues for PCR detection, but heart, lungs, lymph nodes, intestines, kidney and bone marrow were also used in several studies. Blood was more frequently employed in carnivores, marsupials and primates, because it is easier to obtain, while other tissues were accessible only during post-mortem examinations or after fatal clinical cases or euthanasia of the animals. This was the case for rodents, some bats, several clinical cases of carnivores, and road-killed mammals. Hair and eye swabs were also successfully tested in some studies (Supplementary Materials Files S4 and S5).

3.5.1. *L. infantum* in the Americas.

According to the literature, eight orders of wild animals are infected with *L. infantum* in the Americas: Carnivores, Chiroptera, Cingulata, Didelphimorphia, Lagomorpha, Pilosa, Primates and Rodentia (Supplementary Materials File S4). Carnivores were the most widely studied, mainly because domestic and wild carnivores are considered reservoirs of the disease, but also because clinical cases are more frequently reported in them, both in nature and in zoological parks [24,25]. The crab-eating fox (*Cerdocyon thous*) is a widespread carnivore in South America that can act as a reservoir of leishmaniasis for humans, since it can be found in forest locations as well as in residential areas. It was found to be infected with *L. infantum* or exposed to the parasite (positive serology) in several publications, some of which were clinical cases, and thus were not considered in this study. The percentage of infection varied widely among the studies when including more than one animal (4–75%), and exposure to the parasite was demonstrated by serology (i.e., ELISA, IFAT), while culture, microscopy of smears, PCR followed by sequencing and inoculation of hamsters were employed to detect the parasite [81–89]. Several organs and tissues tested positive via PCR: bone marrow, heart, lymph nodes, liver, lungs, skin and spleen. Mainly serological test were employed in the maned wolf (*Chrysocyon brachyurus*) with prevalence values from 10% to 75% depending on the study [82,84,85,88–90], while the parasite was found only in bone marrow and skin by PCR [84,85]. In the bush dog (*Speothos venaticus*), several techniques were employed including serology, culture of isolates, PCR, smears and histopathology. While most studies reported results from only one or two animals, only three studies analysed a higher number (4–6) and found 33.3% positives using PCR (blood) and 60–100% of the animals positive by serology

[84,85,89–91] (Supplementary Materials File S4). The potential transmission to the vector was demonstrated in manned wolves and bush dogs [89], which reinforces their role as reservoirs.

Several studies investigated free-ranging carnivores by serology using a direct agglutination test (DAT), and positive values were found in tayras (*Eira barbara*), lesser grison (*Galictis cuja*) and coatis (*Nasua nasua*) in Brazil at high serum dilutions (≥1:1280) [92]. In carnivores kept in captivity, serology was also employed to demonstrate the presence of antibodies against the parasites in ocelots (*Leopardus pardalis*), hoary foxes (*Lycalopex–Pseudalopex vetulus*), jaguars (*Panthera onca*), Siberian tigers (*Panthera tigris altaica*), African lions (*Panthera leo*) and cougars (*Puma concolor*) (Supplementary Material File S4) [84,90,93,94]. Clinical signs of VL were more frequent in wild canids compared to wild felines and included weight loss, anaemia, lymph node enlargement, vomiting, diarrhoea and polydipsia/polyuria, which were described in some of the animals from the previously mentioned species, such crab-eating foxes [85], bush dogs [84,85], hoary foxes [84], Siberian tigers and maned wolves [84,90]. African lions were reported to test positive for the first time by PCR (kDNA) followed by RFLP, but the animal did not show clinical signs [94]. Finally, *Leishmania* infection (probably *L. infantum*) was found in the kidney of one road-killed crab-eating raccoon (*Procyon cancrivorus*) in Brazil by PCR followed by sequencing [86].

The DNA of *L. infantum* was found in at least 17 species of bats in nine studies, including one hematophagous species and several frugivorous, omnivorous or insectivorous ones [14,32,44,45,66,95–98]. The feeding habits of the animals were relevant, since the oral route was suggested for transmission in animals feeding on insects, including the vector of leishmaniasis [14]. They also shared the same ecological niche in bat caves and probably other locations. Values of infection varied widely, from less than 1% to 100% of the analysed bats, being infected mainly in the blood but also in the liver, skin, spleen and even in oral swab samples [32]. PCR followed by RFLP or sequencing was employed in the studies and, when sensitive primers were employed, a prevalence higher than 30% was usually obtained [95].

A small number of species of the orders Cingulata and Pilosa were found to be infected with *L. infantum* in Brazil. The lesser anteater (*Tamandua tetradactyla*) was reported to test positive by PCR (kDNA) in blood and bone marrow in 2013 [99] and again in 2014, together with giant anteaters (*Myrmecophaga tridactyla*) and one seven-banded armadillo (*Dasypus septemcinctus*) found dead on the roads in Brazil, employing PCR from several tissues [86].

Marsupials were studied in several surveys by PCR or serology (Supplementary Material File S4). The white-eared opossum (*Didelphis albiventris*) was analysed by six groups in Brazil, who found the parasite in blood, bone marrow, lungs, kidney, skin and spleen by culture or PCR (kDNA, ITS1 or SSU) and sequencing or RFLP [38,39,50,54,86,100,101], with percentages of infection between 6.3% and 22.2%. The big-eared opossum (*Didelphis aurita*) was positive at a low percentage in Brazil by PCR, spleen imprints and serology (rK39), and one of the animals displayed spleen enlargement, but no other clinical signs were recorded from the rest [102]. In Brazil as well as in Colombia and Venezuela, the common opossum (*Didelphis marsupialis*) was widely analysed [102–107]. Two studies in Colombia demonstrated the transmission of isolates from common opossums to hamster, highlighting their role as reservoirs of *L. infantum* [105,106]. The parasite was found in several tissues employing PCR (kDNA, SSU and ITS1) followed by hybridisation or RFLP [38,103,107]. Two studies in Brazil employed serology and PCR simultaneously. In the first one, the authors found 9–21.6% of the animals positive using serology, and only 5% positive by PCR–RFLP [38], while the other study analysed 112 individuals of two species (i.e., white-eared and big-eared opossums), and found high percentages of positivity (71–91.6%) with both techniques (see Supplementary Materials File S4 for details).

Lagomorphs were scarcely reported as exposed to *L. infantum* in the Americas, with one European hare (*Lepus europaeus*) found positive in Brazil by DAT and with a low antibody titre (1:320) [92].

Infection with *L. infantum* in primates were studied in five surveys, and eleven species were reported with DNA of the parasite. Several species of captive primates

showed high prevalence values when employing PCR (kDNA) in an endemic area of Brazil including brown howler monkeys (*Alouatta guariba*), black-headed night monkeys (*Aotus nigriceps*), black-fronted titi (*Callicebus nigrifons*), golden-bellied capuchin (*Cebus xanthosternos*), golden-headed lion tamarin (*Leontopithecus chrysomelas*), bald-faced saki (*Pithecia irrorata*) and emperor tamarin (*Saguinus imperator*). Among them, one black-fronted titi was found dead with clinical signs compatible with leishmaniasis, but the rest did not show clinical signs [108]. On the other hand, free-ranging howler monkeys (*Alouatta caraya*) sampled at the marginal area of an endemic region from Argentina displayed low values of prevalence (6.3%) [64]. Two other studies found indirect evidence of infection with the parasite using DAT in one white-tufted-ear marmoset (*Callithrix jacchus*) [109] and 26.9% of the black-tufted marmosets (*Callithrix penicillata*) [109]; the last study also employed PCR of the skin. Positive serology was detected in twenty-two percent of red howler monkeys (*Alouatta seniculus*) in French Guiana, and data were further confirmed by PCR (110).

Rodents occupied most of the attention of researchers investigating *L. infantum* in the Americas, and twelve studies fulfilled the inclusion criteria of this systematic review [21,36,39,50,53,58,86,92,101,106,107,110]. Most of the studies employed different PCR approaches in several tissues, although in one study the authors detected antibodies. Brazilian guinea pigs were reported to be infected in Brazil by PCR in heart tissue [86]. The infection was also found in two species of porcupines from Brazil: the prehensile tailed porcupine (*Coendu–Sphiggurus villosus*) by serology (DAT) [92], which is indirect evidence of the parasitism, and the Paraguayan hairy dwarf porcupine (*Coendou–Sphiggurus spinosus*) by PCR and sequencing from several tissues Supplementary Materila File S4) [86]. Agouties were also reported to test positive for *L. infantum* in the spleen (16.7%), skin and blood by PCR [36,110]. The giant rodent capybara (*Hydrochoerus hydrochaeris*) was positive in the lungs by PCR and sequencing [86]. Several species from wild mice, rats and cricetidae of the genera *Cerradomys*, *Clyomis*, *Holochilus*, *Hylaeamys*, *Nectomys*, *Oryzomys*, *Proechymis*, *Rhipidomys* and *Trichomys* were reported to be infected with *L. infantum* in several surveys, and in previous studies the potential role as reservoir of some of them was indicated [24]. The authors employed distinct approaches of PCR followed by RFLP, hybridisation or sequencing [21,36,39,50,58,106,110].

Synanthropic rodents, such as the house mouse (*Mus musculus*), the black rat (*Rattus rattus*) and the brown rat (*Rattus norvegiccus*) were investigated in Brazil and Venezuela. Researchers found 20% of house mice to be infected [50], while the prevalence in black rats varied widely, with values from 0.1% to 100% using several approaches of PCR followed by hybridisation, RFLP or sequencing [21,39,50,53,58,107]. Almost 17% of brown rats were positive by nPCR and sequencing [50]. These synanthropic species of rodents could act as relevant reservoirs of leishmaniasis, since they were infected at high percentages and share habitats with humans.

Additional species were analysed in other surveys or in some of the previous studies in which the authors could not characterise the parasite at the species level (Supplementary Material File S4) [44,91,111–118]. Indirect evidence of *Leishmania* spp. was found in the USA using rapid tests (rk30 antigen) in several wild carnivores such as coyotes (*Canis latrans*, 1.6%), American red foxes (*Vulpes fulvus*, 9.1%) and grey foxes (*Urocyon cinereoargenteus*, 2%) [111,112]. On the other hand, DNA of *Leishmania* spp. was detected in several species, including carnivores, such as South American grey foxes (*Lycalopex–Pseudalopex griseus*) [113] and Neotropical otters (*Lontra longicaudis*), and some species of primates, such as black-headed night monkeys (*Aotus nigriceps*), black-bearded sakis (*Chiropotes satanas*) and grey-woolly monkeys (*Lagothrix cana*) in Brazil [114]. Among rodents, *Leishmania* spp. DNA was reported in the blood of a red-tailed squirrel (*Sciurus granatensis*) in Venezuela [103]. All this information is summarized in Table 5.

Table 5. Wild animals infected with *L. infantum* (sin. *L. chagasi*) and *Leishmania* spp. in the Americas. Organs or tissues positive to the parasite, as well as techniques employed, are indicated.

Host	Prevalence	Organs/Tissue Analysed	Method of Detection	Country	Reference
Order Carnivora					
Cerdocyon thous (crab-eating fox)	4–100%	BM, heart, liver, lung, mesenteric LN, serum skin and spleen	Smears, ELISA, culture, PCR, inoculation to hamster, IFAT, xenodiagnosis vector, PCR (kDNA) + sequencing, PCR (kDNA) + sequencing *	Brazil	[81–87,89]
Chrysocyon brachyurus (maned wolf)	10–75%	BM, serum and skin	ELISA, IFAT, PCR (kDNA), PCR (kDNA) + sequencing, IC rk39, xenodiagnoses in vector	Brazil	[82,84,85,88–90]
Eira barbara (tayra)	n.s.	serum	DAT (n = 3)	Brazil	[92]
Galictis cuja (lesser grison)	n.s.	serum	DAT (n = 3)	Brazil	[92]
Leopardus pardalis (ocelot)	75%	serum	ELISA,	Brazil	[90]
Lycalopex (*Pseudalopex*) *vetulus* (hoary fox)	33.3%	BM, serum	IFAT, ELISA, PCR (kDNA)	Brazil	[84]
Nasua nasua (coati)	n.s.	serum	DAT (n = 2)	Brazil	[92]
Speothos venaticus (bush dogs)	33.3–100%	blood, LN, serum, skin, spleen and other tissues (liver, kidney, lung and large intestine)	PCR (kDNA), PCR (kDNA) + sequencing, histopathology, IHC, ELISA, IFAT, IC rk39, xenodiagnoses in vector	Brazil	[84,85,89–91]
Panthera onca (jaguar)	20–50%	blood, LN and serum	PCR (kDNA) + RFLP, ELISA, IC	Brazil	[90,93]
Panthera tigris altaica (Siberian tiger)	50%	serum	ELISA, IC	Brazil	[90]
Panthera leo (lion)	50–100%	blood, serum	PCR (kDNA) + RFLP, ELISA	Brazil	[90,94]
Puma concolor (cougar)	71.4%	blood, LN	PCR (kDNA) + RFLP	Brazil	[93]
Procyon cancrivorus (crab-eating racoon)	33.3%	kidney	PCR (kDNA) + sequencing *	Brazil	[86]
Order Chiroptera					
Artibeus planirostris (frugivorous)	7.4–16.7%	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45,95]
Artibeus lituratus (frugivorous)	40.9%	blood	qPCR (kDNA)	Brazil	[95]
Desmodus rotundus (hematophagous)	50%	liver, skin	qPCR (kDNA), PCR (ITS1) + RFLP, PCR (kDNA), nPCR (SSU) + sequencing	Brazil	[14,44]
Carollia perspicillata (frugivorous)	3–27.3%	blood, spleen	Culture, qPCR (kDNA), qPCR (SSU), PCR (kDNA), PCR (ITS2) + sequencing	Venezuela, Brazil and French Guiana	[95–97]
Eumops perotis (insectivorous)	11.1%	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45]
Eptesicus furinalis (frugivorous)	100% (n = 1)	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45]
Glossophaga soricina (nectarivorous)	0.7–100%	blood, liver and spleen	nPCR (SSU), PCR (kDNA), PCR (Cyt B) + sequencing, qPCR (kDNA), PCR (kDNA) and nPCR (SSU) + sequencing	Brazil	[44,45,66,95]
Myotis nigricans (insectivorous)	33.3%	liver	PCR (kDNA) + nPCR (SSU) + sequencing	Brazil	[44]
Molossus molossus (insectivorous)	0.5%–100%	blood, liver and spleen	nPCR (SSU), qPCR (kDNA), PCR (ITS1) + RFLP, PCR (kDNA), PCR (Cyt B) + sequencing, PCR (kDNA) + nPCR (SSU) + sequencing	Brazil	[14,44,45,66]
Molossus pretiosus (insectivorous)	21.1%	liver, skin	PCR (kDNA) + nPCR (SSU) + sequencing	Brazil	[44]
Molossus rufus (insectivorous)	20–100%	liver, spleen	qPCR (kDNA), PCR (ITS1) + RFLP, nPCR (SSU) + sequencing	Brazil	[14,44]

Molossidae spp. (insectivorous)	40%	liver, skin	PCR (kDNA)+ nPCR (SSU) + sequencing	Brazil	[44]
Nyctinomops laticaudatus (insectivorous)	40%	liver, skin	PCR (kDNA) and nPCR (SSU) + sequencing	Brazil	[44]
Nyctinomops macrotis (insectivorous)	60%	liver, skin	PCR (kDNA) + nPCR (SSU) + sequencing	Brazil	[44]
Platyrrhynus lineatus (frugivorous)	15.4%	blood	qPCR (kDNA)	Brazil	[95]
Phyllostomus hastatus (omnivorous)	5.9%	blood	PCR (kDNA), PCR (Cyt B) + sequencing	Brazil	[45]
Phyllostomus discolor (omnivorous)	100% (*n* = 1)	blood	qPCR (kDNA)	Brazil	[95]
Pteronotus parnellii (insectivorous)	100% (*n* = 1)	blood	PCR (SSU), PCR (GAPDP)	Brazil	[98]
Bats (n.s.)	0.1%	oral swab	PCR (SSU) + sequencing *	Brazil	[32]
Order Cingulata					
Dasypus septemcinctus (seven-banded armadillo)	100% (*n* = 1)	liver	PCR (kDNA) + sequencing *	Brazil	[86]
Order Didelphimorphia					
Didelphis albiventris (white-eared opossum)	6.3–22.2%	blood, BM, liver, lung, kidney, skin and spleen	Culture, PCR (ITS1) + RFLP, PCR (kDNA), PCR (kDNA) + sequencing, nPCR (SSU) + sequencing, PCR (kDNA), PCR (ITS1)	Brazil	[100] [39,50,54,86]
Didelphis aurita (big-eared opossum)	6.3%	LN, serum and spleen	Spleen smears, PCR (kDNA) + hybridisation IC rk39	Brazil	[102]
Didelphis marsupialis (common opossum)	7.1–40.5%	blood, BM, liver, serum, skin and spleen	smears, Culture, inoculation to hamster + isoenzyme, Mab, PCR (kDNA) + hybridisation, IFAT, DAT, PCR+RFLP, nPCR (SSU), PCR (ITS1)	Brazil, Colombia and Venezuela	[38,103–107]
Didelphis sp. *D. albiventris* *D. aurita*	91.6%	blood, BM	PCR (kDNA) ELISA, FML-ELISA, smears, culture	Brazil	[101]
Order Lagomorpha					
Lepus europaeus (European hare)	n.s.	serum	DAT (n = 1)	Brazil	[92]
Order Pilosa					
Myrmecophaga tridactyla (giant anteater)	33.3%	heart, kidney, lung and mesenteric LN	PCR (kDNA) + sequencing *	Brazil	[86]
Tamandua tetradactyla (lesser anteater)	50–100%	BM, liver, lung and mesenteric LN	PCR (kDNA), PCR (ITS1) + sequencing	Brazil	[99] [86]
Order Primates					
Alouatta caraya (black howler)	3.7%	ear tissue	PCR (ITS) + RFLP + sequencing	Brazil, Argentina	[64]
Alouatta guariba (brown howler monkey)	12.5	blood	PCR (kDNA)	Brazil	[108]
Alouatta seniculus (red howler monkey)	22.2%	blood	PCR (kDNA), PCR (ITS2), PCR (SSU), IC	French Guiana	[119]
Aotus nigriceps (black-headed night monkey)	100% (*n* = 1)	blood	qPCR (kDNA)	Brazil	[108]
Callicebus nigrifons (black-fronted titi)	33.3%	blood, liver, lung, intestine and spleen	qPCR (kDNA), IHC,	Brazil	[108]
Callithrix jacchus (white-tufted-ear marmoset)	100% (*n* = 1)	serum	DAT	Brazil	[92]
Callithrix penicillata, C. jacchus	26.9%	blood, skin	DAT, PCR + sequencing	Brazil	[109]
Cebus xanthosternos (golden-bellied capuchin)	60%	blood	qPCR (kDNA)	Brazil	[108]

Leontopithecus chrysomelas (golden-headed lion tamarin)	20%	blood	qPCR (kDNA)	Brazil	[108]
Pithecia irrorata (bald-faced saki)	50%	blood	qPCR (kDNA)	Brazil	[108]
Saguinus imperator (emperor tamarin)	100%	blood	qPCR (kDNA)	Brazil	[108]
Order Rodentia					
Cavia aperea (Brazilian guinea pig)	25%	heart	PCR (kDNA) + sequencing	Brazil	[86]
Cerradomys (Oryzomys) subflavus	25%	BM, liver and spleen	nPCR (SSU) + sequencing	Brazil	[50]
Coendu (Sphiggurus) villosus (prehensile tailed porcupine)	n.s.	serum	DAT (n = 2)	Brazil	[92]
Coendou (Sphiggurus) spinosus (Paraguayan hairy dwarf porcupine)	20%	heart, kidney, liver and spleen	PCR (kDNA) + sequencing	Brazil	[86]
Clyomis laticeps	5.2%	spleen	PCR (kDNA) + PCR (HSP70)	Brazil	[36]
Dasyprocta azarae	16.7%	spleen	PCR (kDNA) + PCR (HSP70)	Brazil	[36]
Dasyprocta sp.	n.s.	blood, skin	PCR (kDNA) + PCR (ITS), PCR (HSP70) + sequencing	Brazil	[110]
Holochilus scieurus	10%	skin, spleen	PCR (kDNA)	Brazil	[39]
Hydrochoerus hydrochaeris (capybara)	50%	lung	PCR (kDNA) + sequencing	Brazil	[86]
Mus musculus (house mice)	20%	BM, liver, tail–ear skin and spleen	nPCR (SSU) + sequencing	Brazil	[50]
Nectomys squamipes	7%	skin, spleen	PCR (kDNA)	Brazil	[39]
Proechymis canicollis	8.8%	skin, spleen	PCR + hybridisation	Colombia	[106]
Proechymis cuvieri	n.s.	blood, skin	PCR (kDNA) + PCR (ITS), PCR (HSP70) + sequencing	Brazil	[110]
Rhipidomys mastacalis	28.5%	liver	PCR (HSP70) + RFLP	Brazil	[21]
Rattus norvegicus (brown rat)	16.7%	liver, tail–ear skin,	nPCR (SSU) + sequencing	Brazil	[50]
Rattus rattus (black rat)	0.1–100%	blood, BM, liver, skin and spleen	PCR (kDNA), PCR (kDNA) + hybridisation, PCR (HSP70) + RFLP, PCR (kDNA), nPCR (SSU) + sequencing, PCR (HSP70) + RFLP	Venezuela Brazil	[21,39,50,53,58,107]
Trichomys apereoides	6.3–11.1%	skin, ear skin	PCR (kDNA) + hybridisation PCR (HSP70) + RFLP	Brazil	[21,58]
Trichomys laurentis	1%	spleen	PCR (kDNA)	Brazil	[36]

Wild animals infected with ***Leishmania* spp.** in the Americas

Host	Prevalence	Organs/tissue analysed	Method of detection	Country	Reference
Order Carnivora					
Canis latrans (coyote)	1.6%	serum	IC rAgK39	USA	[112]
Cerdocyon thous (crab-eating fox)	15.3–100%	blood, serum and skin	qPCR (kDNA), IFAT, IC	Brazil	[114,115]
Chrysocyon brachyurus (maned wolf)	42.9%	blood	qPCR (kDNA)	Brazil	[114]
Lontra longicaudis (neotropical otter)	50%	blood	qPCR (kDNA)	Brazil	[114]
Lycalopex (Pseudalopex) griseus (South American grey fox)	37.5%	blood	PCR (kDNA) + sequencing	Argentina	[113]
Lycalopex (Pseudalopex) vetulus (hoary fox)	7.1–50%	blood, serum	qPCR (kDNA), IFAT	Brazil	[114] [115]
Nasua nasua (coati)	50%	blood	qPCR (kDNA), IFAT	Brazil	[114,116]
Puma concolor (cougar)	100% (n = 1)	blood	qPCR (kDNA)	Brazil	[114]
Spheotos venaticus (bush dog)	33.3–100%	Blood, serum, liver and LN	ELISA, PCR (kDNA)	Brazil	[91,117]

Vulpes fulvus (American red fox)	9.1%	serum	IC rK39	USA	[112]
Urocyon cinereoargenteus (gray fox)	2%	serum	IC rK39	USA	[111]
Order Chiroptera					
Molossus molossus (insectivorous)	7.4%	liver	PCR (kDNA)+ nPCR (SSU) + sequencing	Brazil	[44]
Molossus pretiosus (insectivorous)	5.2%	liver	PCR (kDNA)+ nPCR (SSU) + sequencing	Brazil	[44]
Nyctinomops macrotis (insectivorous)	6.7%	liver	PCR (kDNA)+ nPCR (SSU) + sequencing	Brazil	[44]
Order Pilosa					
Myrmecophaga tridactyla (giant anteater)	36.4%	blood	qPCR (kDNA)	Brazil	[114]
Tamandua tetradactyla (lesser anteater)	33.3%	blood	qPCR (kDNA)	Brazil	[114]
Order Primates					
Alouatta guariba (brown howler monkey)	37.5%	blood	PCR (kDNA)	Brazil	[91]
Aotus nigriceps (black-headed night monkey)	20%	blood	qPCR (kDNA)	Brazil	[114]
Chiropotes satanas (black-bearded saki)	50%	blood	qPCR (kDNA)	Brazil	[114]
Lagothrix cana (gray-woolly monkey)	33.3%	blood	qPCR (kDNA)	Brazil	[114]
Leontopithecus chrysomelas (golden-headed lion tamarin)	16.7%	blood	qPCR (kDNA)	Brazil	[114]
Order Rodentia					
Rattus rattus (black rat)	9.1%	serum	IFAT	Dominican Republic	[118]
Sciurus granatensis (red-tailed squirrel)	100% (*n* = 1)	blood	nPCR (SSU)	Venezuela	[103]

BM: bone marrow; Cyt B: cytochrome B; DAT: direct agglutination test; FML: fucose-mannose ligand; GADPH: glyceraldehyde phosphate dehydrogenase; HSP70: heat shock protein 70kDa; IHC: immunohistochemistry; IC: immunochromatography; ELISA: enzyme immune assay; IFAT: immunofluorescence assay; ITS: internal transcriber spacer; kDNA: kinetoplast DNA; LN: lymph node; nPCR: nested PCR; n.s.: not specified; qPCR: quantitative PCR; RFLP: restriction fragment length polymorphism; SSU: small subunit of ribosomal RNA. * Probably *L. infantum*, according to the sequence.

The following species have been reported to be infected with *L. infantum* in the Americas in previous reviews: the rodent *Proechymis spinosus*, the Brazilian porcupine (*Coendu prehensilis*) and the fennec fox (*Vulpes zerda*). More information can be retrieved from the abovementioned reviews [23–25,27].

3.5.2. *L. infantum* in Wild Animals from Europe, Asia and Africa

Carnivores, bats, wallabies, hedgehogs, lagomorphs and rodents tested positive for *L. infantum* via antibody detection or PCR in several countries in South Europe (Croatia, France, Greece, Italy, Portugal, Spain, Romania), North Africa (Morocco, Tunisia) and Asia (Georgia, Iran, Israel, Saudi Arabia), Spain being the country with the highest number of studies of wild animals (Supplementary Material File S5).

L. infantum infection was reported in a large list of wild carnivores including 18 species. The golden jackal (*Canis aureus*) was positive in four studies at low percentages. The lowest values were found in Georgia using a rapid test (2.6%) [120] and in Romania employing PCR and sequencing of the ITS1 region from bone marrow samples (3%) [121]. In Iran, 11.6% of the jackals were found positive by serological test (DAT), and subsequent smears and culture from skin lesions, lymph nodes, spleen and liver were furthered characterised by PCR and sequencing [122]. Only 7.8% of the animals were found positive by PCR using blood samples [123]. The target or the technique used in each study could influence the results obtained by the different authors.

The wolf was the focus of research of many zoonoses including *L. infantum* infections. In this review, nine studies reported the animal to be positive for the parasite, although three of them included a low number of animals (three or less). In Croatia, only one wolf was reported positive by PCR and sequencing of the cysteine protease B in lymph nodes [124]. The remaining authors employed PCR of the kDNA region to detect the infection and, in some cases, RFLP and sequencing were later applied. Prevalence values from 33% to 50% were found in Spain when using spleen, skin or lymph nodes as samples [125–129] including one study conducted in a non-endemic region [127]. The percentage of infected animals was lower when blood (9%) [130] or hair (4.1%) [131] were used to detect the parasite. In Italy, 25% of the animals were infected when samples from spleen were analysed [132]. Skin lesions were reported only in one study [127].

A smaller number of individuals from wild cats (*Felis silvestris*) [127,128,133] and genets (*Genetta genetta*) [125,127–129,133,134] tested positive to *L. infantum* in samples from skin, liver or spleen employing PCR of the kDNA. The percentage of infection in both species reached 100% of the sampled animals, although wild cats showed 25% as the minimum value [128,133], while genets displayed a 10% prevalence in blood or spleen samples [134]. In two studies, additional PCR and sequencing of the ITS2 region were carried out [129,133].

Several studies were conducted employing similar approaches to detect *L. infantum* in various species of carnivores. The parasite was found in the spleen or blood of 28.6% of Egyptian mongoose (*Herpestes ichneumon*) from Spain [125] and 4.7% (only spleen) from Portugal [135]. Seventy percent of otters' (*Lutra lutra*) spleens [136] and 25% of Iberian lynxes' (*Lynx pardinus*) samples (spleen and blood) [135] were found to be infected in two studies. Six surveys reported the presence of *L. infantum* in hair, liver, spleen or lymph nodes of 29–100% of sampled beech martens (*Martes foina*) in Spain [127–129,131,133,137]. Values between 30% and 62% were found in pine martens (*Martes martes*) using the same techniques [127,133,134]. Badgers (*Meles meles*) were found infected in the liver or spleen in Italy (53%) [132] and Spain (26%) [133]. European minks (*Mustela lutreola*) were found to be infected with values of 50% in Spain using a similar methodology [133] but at lower percentages (e.g., 2.1%) when ELISA or PCR of the ITS1 were employed in Greece [136]. Moreover, 20% of pole cats (*Mustela putorius*), 45% of tigers (*Panthera tigris*) in a zoo, 20% of red squirrels (*Sciurus vulgaris*) and one individual of each species of American mink (*Mustela vison*) and brown bear (*Ursus arctos*) were reported to be infected with the protozoa [129,133,137,138].

The DNA of *L. infantum* was found in at least 17 species of bats in nine studies, including one hematophagous species and several frugivorous, omnivorous or insectivorous ones [14,32,44,45,66,95–98]. The feeding habits of the animals were relevant, since the oral route was suggested for transmission in animals feeding on insects, including the vector of leishmaniasis [14]. They also shared the same ecological niche in bat caves and probably other locations. Values of infection varied widely, from less than 1% to 100% of the analysed bats, being infected mainly in the blood but also in the liver, skin, spleen and even in oral swab samples [32]. PCR followed by RFLP or sequencing was employed in the studies and, when sensitive primers were employed, a prevalence higher than 30% was usually obtained [95].

A small number of species of the orders Cingulata and Pilosa were found to be infected with *L. infantum* in Brazil (Supplementary Material File S4). The lesser anteater (*Tamandua tetradactyla*) was reported to test positive by PCR (kDNA) in blood and bone marrow in 2013 [99] and again in 2014, together with giant anteaters (*Myrmecophaga tridactyla*) and one seven-banded armadillo (*Dasypus septemcinctus*) found dead on the roads in Brazil, employing PCR from several tissues [86].

Marsupials were studied in several surveys by PCR or serology (Supplementary Material File S4). The white-eared opossum (*Didelphis albiventris*) was analysed by six groups in Brazil, who found the parasite in blood, bone marrow, lungs, kidney, skin and spleen by culture or PCR (kDNA, ITS1 or SSU) and sequencing or RFLP [38,39,50,54,86,100,101], with percentages of infection between 6.3% and 22.2%. The big-eared opossum (*Didelphis aurita*) was positive at a low percentage in Brazil by PCR, spleen imprints and serology (rK39), and one of the animals displayed spleen enlargement, but no other clinical signs were recorded from the rest [102]. In Brazil as well as in Colombia and Venezuela, the common opossum (*Didelphis marsupialis*) was widely analysed [102–107]. Two studies in Colombia demonstrated the transmission of isolates from common opossums to hamster, highlighting their role as reservoirs of *L. infantum* [105,106]. The parasite was found in several tissues employing PCR (kDNA, SSU and ITS1) followed by hybridisation or RFLP [38,103,107]. Two studies in Brazil employed serology and PCR simultaneously. In the first one, the authors found 9–21.6% of the animals positive using serology, and only 5% positive by PCR–RFLP [38], while the other study analysed 112 individuals of two species (i.e., white-eared and big-eared opossums), and found high percentages of positivity (71–91.6%) with both techniques (see Supplementary Materials File S4 for details).

Lagomorphs were scarcely reported as exposed to *L. infantum* in the Americas, with one European hare (*Lepus europaeus*) found positive in Brazil by DAT and with a low antibody titre (1:320) [92].

Infection with *L. infantum* in primates were studied in five surveys, and eleven species were reported with DNA of the parasite. Several species of captive primates showed high prevalence values when employing PCR (kDNA) in an endemic area of Brazil including brown howler monkeys (*Alouatta guariba*), black-headed night monkeys (*Aotus nigriceps*), black-fronted titi (*Callicebus nigrifons*), golden-bellied capuchin (*Cebus xanthosternos*), golden-headed lion tamarin (*Leontopithecus chrysomelas*), bald-faced saki (*Pithecia irrorata*) and emperor tamarin (*Saguinus imperator*). Among them, one black-fronted titi was found dead with clinical signs compatible with leishmaniasis, but the rest did not show clinical signs [108]. On the other hand, free-ranging howler monkeys (*Alouatta caraya*) sampled at the marginal area of an endemic region from Argentina displayed low values of prevalence (6.3%) [64]. Two other studies found indirect evidence of infection with the parasite using DAT in one white-tufted-ear marmoset (*Callithrix jacchus*) [109] and 26.9% of the black-tufted marmosets (*Callithrix penicillata*) [109]; the last study also employed PCR of the skin. Positive serology was detected in twenty-two percent of red howler monkeys (*Alouatta seniculus*) in French Guiana, and data were further confirmed by PCR (110).

Rodents occupied most of the attention of researchers investigating *L. infantum* in the Americas, and twelve studies fulfilled the inclusion criteria of this systematic review [21,36,39,50,53,58,86,92,101,106,107,110]. Most of the studies employed different PCR approaches in several tissues, although in one study the authors detected antibodies. Brazilian guinea pigs were reported to be infected in Brazil by PCR in heart tissue [86]. The infection was also found in two species of porcupines from Brazil: the prehensile tailed porcupine (*Coendu–Sphiggurus villosus*) by serology (DAT) [92], which is indirect evidence of the parasitism, and the Paraguayan hairy dwarf porcupine (*Coendou–Sphiggurus spinosus*) by PCR and sequencing from several tissues (Supplementary Material File S4) [86]. Agouties were also reported to test positive for *L. infantum* in the spleen (16.7%), skin and blood by PCR [36,110]. The giant rodent capybara (*Hydrochoerus hydrochaeris*) was positive in the lungs by PCR and sequencing [86]. Several species from wild mice, rats and cricetidae of the genera *Cerradomys*, *Clyomis*, *Holochilus*, *Hylaeamys*, *Nectomys*, *Oryzomys*, *Proechymis*, *Rhipidomys* and *Trichomys* were reported to be infected with *L. infantum* in several surveys, and in previous studies the potential role as reservoir of some of them was indicated [24]. The authors employed distinct approaches of PCR followed by RFLP, hybridisation or sequencing [21,36,39,50,58,106,110].

Synanthropic rodents, such as the house mouse (*Mus musculus*), the black rat (*Rattus rattus*) and the brown rat (*Rattus norvegiccus*) were investigated in Brazil and Venezuela. Researchers found 20% of house mice to be infected [50], while the prevalence in black rats varied widely, with values from 0.1% to 100% using several approaches of PCR followed by hybridisation, RFLP or sequencing [21,39,50,53,58,107]. Almost 17% of brown rats were positive by nPCR and sequencing [50]. These synanthropic species of rodents could act as relevant reservoirs of leishmaniasis, since they were infected at high percentages and share habitats with humans.

Additional species were analysed in other surveys or in some of the previous studies in which the authors could not characterise the parasite at the species level (Supplementary Material File S4) [44,91,111–118]. Indirect evidence of *Leishmania* spp. was found in the USA using rapid tests (rk30 antigen) in several wild carnivores such as coyotes (*Canis latrans*, 1.6%), American red foxes (*Vulpes fulvus*, 9.1%) and grey foxes (*Urocyon cinereoargenteus*, 2%) [111,112]. On the other hand, DNA of *Leishmania* spp. was detected in several species, including carnivores, such as South American grey foxes (*Lycalopex–Pseudalopex griseus*) [113] and Neotropical otters (*Lontra longicaudis*), and some species of primates, such as black-headed night monkeys (*Aotus nigriceps*), black-bearded sakis (*Chiropotes satanas*) and grey-woolly monkeys (*Lagothrix cana*) in Brazil [114]. Among rodents, *Leishmania* spp. DNA was reported in the blood of a red-tailed squirrel (*Sciurus granatensis*) in Venezuela [103].

Seventeen studies reported the infection in the red fox (*Vulpes vulpes*), with lower prevalence values found in France (9–15%) and Georgia (2.6%) and higher in southern European Mediterranean countries, such as Italy (12.3–40%), Greece (59.5%) or Spain (12–74%) (Supplementary Materals File S5) [122,125,127–129,131,132,139–146]. The high numbers of publications might be due to the epidemiological relevance of this animal because it is a widespread species, which inhabit different ecosystems where the parasite life cycle can be completed, from forest to areas close to human settlements. One study carried out in Iran reported intermediate values (28.6%) using serology, cultures, smears and PCR-sequencing from lymph nodes, skin and spleen [122]. Serology (ELISA, IFAT) was first employed in Italy [139] and then in Georgia (recombinant antigen rK39 rapid test) [120], but since 2000, PCR was the most widely used technique to detect the infection [122,125,127–129,131,132,140–143,145,146]. Although no clinical signs were reported in most of the studies, the majority of the animals from the study carried out in Greece (63.8%) showed at least 2–3 clinical signs compatible with canine VL including low weight, dermatitis, skin lesions, alopecia, esplenomegaly, enlargement of lymph nodes and onychogryphosis [143].

The infection was demonstrated also in Bennett's wallabies in a zoo in Madrid (Spain) using serology (rk39) and PCR followed by sequencing of the ITS1 and ITS2 regions.

Thirty- three percent of the animals were infected in several tissues including blood, kidneys, lymph nodes, liver, lungs, skin and spleen [147]. In addition, two North West Bornean orangutan (*Pongo pygmaeus*) with clinical signs of visceral leishmaniasis were found to be infected using PCR (ITS1) of the bone marrow and serology [148].

Over the last decade, new reservoirs of leishmaniasis in Europe have been reinforced through investigation with bats, lagomorpha and hedgehog species, enlarging the list of wild animals infected with the parasite. Only one study in Europe demonstrated the presence of *L. infantum* in spleen, hair and blood of 51.9% common urban bats (*Pipistrellus pipistrellus*) in Spain, using PCR and sequencing of the repeat region [149]. One hundred percent of Algerian hedgehogs were found infected in two studies in Tunisia [150,151]. The authors employed smears, PCR and RFLP and sequencing of several targets. Spleen, liver, kidney, heart, lymph nodes, blood and eye swabs were positive to all of the techniques employed. In Spain, the European hedgehog was found to be infected using ELISA and qPCR of the kDNA region, finding higher values of infection in the spleen than in skin samples [137]. The parasite was also detected in a hair sample from one animal [131].

The DNA of *L. infantum* was first detected in 2013 in the spleen of European hares (*Lepus europaeus*) and Iberian hares (*Lepus granatensis*) from Spain, with 43.6% of the animals infected [152], since the outbreak of human leishmaniasis in Madrid motivated research on wild reservoirs. Since then, other studies were conducted finding the parasite in spleen, hair and skin with molecular (PCR of several targets), histological (direct antibody fluorescence assay) and serological analyses (IFAT) in Spain [153,154], Italy [155,156] and Greece [136]. Similar approaches were used for the European rabbit (*Oryctolagus cuniculus*), which was investigated in six studies from Spain [128,129,153,154,157,158]. The authors found positive values ranging from 0.6% to 59%, depending on the time of the year, the sampling area or the techniques employed (i.e., smears, ELISA, IFAT, PCR). In general, serology displayed lower percentages of infection than PCR. In Italy and Greece, lower values of infected animals were obtained, but as previously pointed out, this might be due to the techniques employed (serology or PCR of ITS) [136,145].

Wild and synanthropic rodents were always the focus of researchers interested in *L. infantum* epidemiology, similar to what happened with other species of *Leishmania*. In Spain, the wood mouse (*Apodemus sylvaticus)* was positive with values of 20–50%, depending on the study, in several tissues [128,129,159]. The authors employed culture and smears [159], but also PCR followed by RFLP or sequencing of ITS1, ITS2, kDNA and SSU regions. Blood and spleen from shrews (*Crocidura russula*) were found to be infected at a low prevalence [160] as well as Algerian mice (*Mus spretus*), in percetanges from 4.3% to 42.9% using PCR in several tissues as well as serology [137,160]. In Iran, 39% of the sampled shor-tailed bandicoot rat (*Nesokia indica*) were positive in smears of skin and spleen, and later characterised as *L. infantum* by nested PCR of the kDNA when compared with reference isolates [161].

Synanthropic rodents were studied in eight surveys displaying high prevalence values for *Leishmania* infection. In most of them, *L. infantum* was found in the skin, liver and spleen of house mice (*Mus musculus*) and brown rats (*Rattus norvegicus*) in Portugal and Morocco [162,163], with animals displaying skin lesions in both studies. Smears were employed in Portugal while PCR and sequencing were employed in both studies, although different targets were selected (kDNA, ITS1, SSU). Prevalence values ranged from 22% to 33.3% in mice and 33% in rats. In Spain, 50% of house mice were found to test positive using PCR and sequencing of blood, bone-marrow and skin samples [159]. Brown rats were found infected using similar methologies, with 33–100% of animals positive in Spain [129,131] and 5.9% in Greece [164]. Black rats (*Rattus rattus*) were found infected in 18.2% of sampled animals in Saudi Arabia [163], 15.5% in Italy [165], 33.3% in Spain [157] and 7.5% in Morocco [161].

A summary of the techniques and organs found infected wit *L. infantum* in wild animals from Europe, Asia and Africa is summarised in Table 6.

Table 6. Wild animals reported to be positive for *L. infantum* from Europe, Asia and Africa. Organs or tissues where the parasite was detected are indicated, as well as the techniques employed for detection.

Host	Prevalence	Organs/Tissue Analysed	Methods for Detection	Country	References
Order Carnivora					
Canis aureus (golden jackal)	3–11.6%	blood, BM, liver, LN, serum, spleen	qPCR (ITS1), PCR (kDNA), IC rk39, smear, culture, PCR (α-tubulin and GAPDH)	Georgia, Israel, Iran and Romania	[120–123]
Canis lupus (grey wolf)	6–100%	blood, hair, liver, LN, skin, serum, spleen	PCR (cysteine protease B), qPCR (kDNA), PCR (kDNA) + RFLP, PCR (ITS2) + RFLP, PCR (kDNA) + sequencing, ELISA	Croatia, Italy, Spain	[124–132]
Felis silvestris (wildcat)	25–100%	liver, LN, skin, spleen	qPCR (kDNA), PCR (ITS2) + sequencing, PCR (kDNA) + sequencing, qPCR (kDNA) + RFLP + sequencing	Spain	[127,128,133]
Genetta genetta (common genet)	10–100%	blood, liver, skin and spleen	PCR (kDNA) + RFLP, qPCR (kDNA), PCR (ITS2) + sequencing, PCR (kDNA) + sequencing, qPCR (kDNA) + RFLP + sequencing, PCR (kDNA & ITS2) + RFLP	Spain	[125,127–129,133,134]
Herpestes ichneumon (Egyptian mongoose)	4.7–28.6%	blood, spleen,	PCR (kDNA) + RFLP, PCR (kDNA) + sequencing, PCR (ITS1)	Spain, Portugal	[125] [135]
Lutra lutra (Eurasian otter)	70%	spleen	PCR (kDNA) + sequencing	Spain	[127]
Lynx pardinus (Iberian lynx)	25%	blood, spleen,	PCR (kDNA) + RFLP	Spain	[125]
Martes foina (beech marten)	29–100%	liver, LN, hair, skin and spleen	qPCR (kDNA), qPCR (ITS2) + sequencing, PCR (kDNA) + sequencing, qPCR (kDNA) + sequencing, PCR (kDNA & ITS2) + RFLP	Spain	[127–129,131,133,137]
Martes martes (European pine marten)	30–62%	blood, liver, spleen	PCR (kDNA) + RFLP, qPCR (kDNA), qPCR (ITS2) + sequencing, PCR (kDNA) + sequencing	Spain	[127,133,134]
Meles meles (European badger)	26–53%	liver, spleen	qPCR (kDNA), PCR (ITS2) + sequencing, PCR (kDNA) + sequencing	Spain, Italy	[132,133]
Mustela lutreola (European Mink)	2.1–50%	liver, spleen, serum	qPCR (kDNA), PCR (ITS2) + sequencing, PCR (ITS1), ELISA	Greece, Spain	[133,136]
Mustela putorius (European polecat)	25%	liver, spleen	qPCR (kDNA), PCR (ITS2) + sequencing	Spain	[133]
Mustela vison (American mink)	100% (n = 1)	liver, spleen	qPCR (kDNA)	Spain	[137]

Panthera tigris (Tiger)	25%	serum, LN and swab (oral, conjunctival and nasal)	IFAT, qPCR	Italy	[138]
Sciurus vulgaris (red squirrel)	20%	liver, skin, pleen	qPCR (kDNA)	Spain	[137]
Ursus arctos (brown bear)	100% (*n* = 1)	liver, skin, spleen	PCR (kDNA), PCR (ITS2) + RFLP	Spain	[129]
Vulpes vulpes (red fox)	2.6–74.6%	blood, BM, hair, liver, LN, skin, spleen, serum	PCR (Repeat Region), PCR (kDNA), PCR (kDNA) + RFLP, qPCR (kDNA), qPCR (ITS2) + sequencing, qPCR (ITS1) + RFLP, PCR (α-tubulin and GAPDH) + sequencing, PCR (kDNA) + RFLP, PCR (kDNA) + sequencing, PCR (ITS2) + RFLP, PCR (ITS1) + sequencing, ELISA, IFAT, WB, IC rk39, smear, culture	France, Georgia and Greece, Iran, Italy and Spain	[120,122,125,127–129,131,132,139–146]
Order Chiroptera					
Pipistrellus pipistrellus (common urban bat)	59.2%	blood clot, hair, spleen	PCR (Repeat region) + sequencing	Spain	[149]
Order Diprotodontia					
Macropus rufogriseus (Bennett's wallaby)	33.3%	blood, BM, liver, lung, LN, kidney, skin, spleen	PCR (ITS1 and ITS2) + sequencing, IC rk39	Spain	[147]
Order Eulipotyphla					
Atelerix algirus (Algerian hedgehog)	100%	blood, eye swab, heart, kidney, liver, LN, skin, spleen	PCR (kDNA), PCR (ITS1), PCR (mini-exon), PCR (Repeat region), PCR (SSU), smear	Tunisia	[150,151]
Erinaceus europaeus (European hedgehog)	34.4–100%	hair, serum, skin, spleen	qPCR (kDNA), ELISA	Spain	[131] [137]
Order Lagomorpha					
Lepus europaeus (European hare)	0.9–43.6%	blood, spleen, serum	PCR (kDNA) + RFLP, PCR (ITS1), PCR (ITS1) + sequencing, ELISA, IFAT	Greece, Italy and Spain	[136,152,155,156]
Lepus granatensis (Iberian hare)	10.1–100%	hair, skin, spleen, serum	PCR (kDNA) + RFLP, qPCR (kDNA), nPCR (SSU), IFAT, DFA	Spain	[152–154]
Oryctolagus cuniculus (European rabbit)	0.6–59%	blood, BM, hair, heart, liver, LN, skin, spleen, serum	qPCR (kDNA), PCR (ITS1) + RFLP, ELISA, nPCR (SSU), qPCR (kDNA) + RFLP + sequencing, PCR (kDNA) + RFLP, PCR (ITS2) + RFLP, PCR (ITS1), smears, culture, IFAT, DFA, ELISA, IC rk39	Greece, Italy and Spain	[128,129,136,145,153,154,157,158]
Order Primates					

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Pongo pygmaeus (north west Bornean orangutan)	100%	BM, serum	Microscopy, IFAT, nPCR (ITS1)	Spain	[148]
Order Rodentia					
Apodemus sylvaticus (wood mouse)	20–50%	blood, BM, liver, skin, spleen	[illegible] (kDNA) + RFLP + sequencing, PCR (ITS2) + RFLP, smear, culture	Spain	[128,129,15[illegible]
Crocidura russula (white-toothed shrew)	13.3%	blood and/or spleen	qPCR (kDNA)	Spain	[160]
Mus musculus (house mouse)	22–50%	blood, BM, liver, skin, spleen	qPCR (kDNA) + sequencing, PCR (ITS1) + sequencing, PCR-ELISA (kDNA), nPCR (SSU and ITS1) + sequencing, smear	Morocco, Portugal and Spain	[162,163]
Mus spretus (Algerian mouse)	4.3–42.9%	blood, liver, skin, spleen and serum	qPCR (kDNA), ELISA	Spain	[137,160]
Nesokia indica (short-tailed bandicoot rat)	39%	liver, skin, spleen,	nPCR (kDNA), smear	Iran	[162]
Rattus norvegicus (brown rat)	5.9–100%	hair, liver, skin, spleen	nPCR (SSU), nPCR (ITS1) + sequencing, qPCR (kDNA), PCR (kDNA), PCR (kDNA) + RFLP, PCR (ITS2) + RFLP, smear	Greece, Morocco, Portugal and Spain	[129,131,162,163,166]
Rattus rattus (black rat)	7.5–33.3%	blood, BM, liver, skin, spleen	PCR (kDNA) + sequencing, PCR (ITS1) + sequencing, PCR-ELISA (kDNA), nPCR (SSU), nPCR (ITS1) + sequencing, smear, culture, inoculation to hamster, isoenzymes	Italy, Morocco, Saudi Arabia and Spain	[159,163–165]

BM: bone marrow; Cyt b: cytochrome B; DFA: direct fluorescence antibody assay; GADPH: glyceraldehyde phosphate dehydrogenase; IC: immunochromatography; ELISA: enzyme immune assay; IFAT: immunofluorescence assay; ITS: internal transcriber spacer; kDNA: kinetoplast DNA; LN: lymph node; nPCR: nested PCR; qPCR: quantitative PCR; RFLP: restriction fragment length polymorphism; SSU: small subunit of ribosomal RNA; WB: Western blot.

In previous reviews, other species were reported infected with *L. infantum* or were described as clinical cases: domestic ferrets (*Mustela putorius furo*), corsac foxes (*Vulpes corsak*), raccoon dogs (*Nyctereutes procyonoides*), Mediterranean monk seals (*Monachus monachus*), Persian jirds (*Meriones persicus*), Syrian hamsters (*Mesocricetus auratus*), grey hamsters (*Cricetulus migratorius*) and porcupines (*Hystrix* sp.). Detailed information can be found in specific reviews [19,26,27].

3.6. Wild Animals Infected with L. major

L. major infections extended through Asian and African countries [3,5], and nineteen studies were carried out from 1990 in wild animals in Algeria, Tunisia, Iran, Israel, Ethiopia, Kenya, Cameroon and Morocco (Supplementary Material File S5).

In the included studies, the most employed techniques to detect *L. major* were PCR of the kDNA (nine studies) and ITS (nine studies) regions, although SSU and the repeat regions were also employed, mainly followed by RFLP and/or sequencing (four and two studies, respectively). Only seven studies exclusively employed the skin to search for the parasite, but the rest of the studies employed also other anatomical sites such as liver, spleen, heart, blood, kidney, lymph nodes, eye swabs or even feces. Noteworthy were the higher prevalence values observed in smears compared with the PCR of the kDNA in some studies, probably due to the methodology employed, since the DNA was extracted from fixed smears [161,167–171] (for details, see Supplementary Materials File S5).

The infection was demonstrated in the orders Chiroptera, Eulipotyphla, Primates, and Rodentia, the last group, again, being the most widely studied. Only one species of bat (*Nycteris hispida*) was reported in Ethiopia to be infected with *L. major* in the spleen by qPCR and sequencing of the kDNA and ITS regions [172]. The DNA of the parasite was found in several organs and tissues in three species of hedgehogs in Algeria, Iran and Tunisia including spleen, skin, heart, kidney, liver, blood and eye swab. A hundred percent prevalence was reported in two studies carried out on the Algerian hedgehog (*Atelerix algirus*) in Tunisia [150,151], while 36.8% was reported in Algeria by serology and PCR–RFLP of the kDNA region employing the spleen and skin [173] (Supplementary Materials File S5). Two studies, including the long-eared hedgehog (*Hemiechinus auritus*), reported prevalence rates ranging from 33%, using nPCR of the ITS and smears of the skin [173], to 53.3%, employing nPCR of the kDNA from the skin and smears from skin, liver and spleen [174]. The desert hedgehog (*Paraechinus aethiopicus*) was found to be infected with *L. major* in two studies. The first study employed qPCR of the kDNA region from spleen and skin as well as serology [175], and the other reported the infection in the kidney, blood, liver, eye swab and lymph node of one animal by qPCR of the kDNA, SSU and repeat regions [151]. The high values of infection found in these animals suggest their role as reservoirs, and these animals should be monitored in endemic areas.

One study investigated the immune response to *L. major* of three species of primates in Kenya (*Cercopithecus mitis*, *Chlorocebus aethiops* and *Papio cynocephalus anubis*) including humoral (ELISA and Western blot) and cellular responses (lymphoproliferative assay) [176]. The authors include 57–213 individuals per technique and found that 60–77% of the animals were previously exposed to the parasite. Surprinsingly, one study found parasites (amastigote and promastigotes forms) and DNA of *L. major* in the feces of gorillas (*Gorilla gorilla*) in Cameroon, and the authors pointed to the ingestion of phlebotomines by the animals [15]. However, no other method to measure exposure (serology) or presence of the parasite in organs was employed.

Eleven species of rodents were reported as infected with the parasite in nine studies from Iran, and another one from Israel (Supplementary Material File S5), following the same tendency of other zoonotic species of *Leishmania* included in the systematic review. The higher prevalence was found in *Meriones libycus* by PCR of kDNA in the skin [177], which is also the species most studied regarding *Leishmania* infections in Iran [167–171,173,177,178]. Other species of *Meriones* (*M. hurrianae*, *M. persicus* and *M tristrami*) and *Microtus* (*M. guentheri* and *M. socialis*) were reported to be infected with parasites with

values of 5.7–58.3% [161,168,171,179]. *Mus musculus* was found to be infected with low percentages of infection (2.3–33%) in three studies carried out in Iran and Israel. However, PCR of the ITS region from skin samples, and smears from skin, liver and spleen, were used instead of PCR of kDNA, [161,179]. *Nesokia indica* was found to be infected in three studies from Iran, ranging from 8% in skin by smears and PCR of the ITS region [168] to values higher than 61% employing smears and PCR of skin, liver and spleen tissues [161,167]. The same techniques (PCR of the ITS and kDNA regions) were employed in three studies in Iran to detect *L. major* infections in *Tatera indica* from skin, liver or spleen, with values of prevalence ranging from 3,7 to 50% [168,171,178]. The great gerbil (*Rhombomys opimus*) was reported infected with the parasite in Iran [168,178] using smears, PCRs and inoculation of hamsters (see Supplementary Materials File S5 for details). The high prevalence found in many of these rodent species points to their reservoir role.

Most of the studies did not find clinical signs in infected animals, or the authors did not look for them; however, skin lesions were recorded in *Meriones libycus* from Iran [177].

All the information concerning *L. major* infection in wild animals is summarised in Table 7.

In previous reviews, other species were reported infected with *L. major* in the past including primates (*Cercopithecus aethiops*) and rodents (*Xerus rutilus*, *Gerbillus pyramidum*, *Tatera gambiana*, *Tatera robusta*, *Taterillus emini*, *Meriones crassus*, *Meryones meridianus*, *Meryones shawi*, *Psammomis obesus*, *Praomys erythroleucus* and *Mastomys natalensis*) [23].

Table 7. Wild animals reported positive for *L. major*. Organs or tissues where the parasite was detected, and the techniques employed are indicated.

Host	Prevalence	Organs/Tissue Analysed	Methods for Detection	Country	References
Order Chiroptera					
Nycteris hispida	100% (*n* = 1)	spleen	qPCR (kDNA and ITS1) + sequencing	Ethiopia	[172]
Order Eulipotyphla					
Atelerix algirus (Algerian hedgehog)	36.8–100%	blood, eye swab, heart, kidney, liver, LN, skin, spleen	qPCR (kDNA), PCR (kDNA), PCR (ITS1) + RFLP, nPCR (kDNA), PCR (ITS1) + sequencing + RFLP, PCR (mini-exon) + sequencing + RFLP, nPCR (Repeat region) + sequencing + RFLP, PCR (SSU) + sequencing, smear, ELISA, WB	Algeria, Tunisia	[150,151,175]
Hemiechinus auritus (long-eared hedgehogs)	33.3–53.3%	liver, skin, spleen	nPCR (ITS1) + sequencing, nPCR (kDNA), semi-nPCR (kDNA), smear	Iran	[173,174]
Paraechinus aethiopicus (desert hedgehog)	40–100%	blood, eye swab, kidney, liver, LN, skin, spleen, serum	qPCR (kDNA), PCR (ITS1) + RFLP, PCR (kDNA and SSU) + sequencing, nPCR (Repeat region) + RFLP + sequencing, ELISA, WB	Algeria, Tunisia	[151,175]
Order Primates					
Cercopithecus mitis (syke's monkeys)	67.2%	serum	ELISA, WB	Kenya	[176]
Chlorocebus aethiops (vervet monkeys)	60.6%	serum	ELISA, WB, lymphocyte proliferation assay	Kenya	[176]
Gorilla gorilla (gorilla)	13.2%	faeces	qPCR (SSU), qPCR (SSU) + sequencing, PCR (ITS) + sequencing, PCR (Cytb) + sequencing	Cameroon	[15]
Papio cynocephalus anubis (olive baboons)	77.2%	serum	ELISA, WB	Kenya	[176]
Order Rodentia					
Gerbillus nanus	11.8%	liver, skin, spleen	PCR (kDNA), smear	Iran	[171]
Meriones hurrianae	7.7%	liver, skin, spleen	PCR (kDNA), smear	Iran	[171]
Meriones libycus	5.7–100%	liver, skin, spleen	PCR (kDNA), nPCR (ITS1), PCR (ITS1) + RFLP + sequencing, semi-nPCR (kDNA), PCR (Cytb) + sequencing, nPCR (ITS1) + sequencing, nPCR (ITS2) + RFLP, smear, inoculation to hamster, inoculation to BALB/c mice	Iran	[167–170,173,177,178]
Meriones persicus	33%	skin	PCR (ITS1) + RFLP + sequencing, smear	Iran	[168]
Meriones tristrami	58.3%	skin	PCR (ITS1) + RFLP	Israel	[179]
Microtus guentheri	16.5%	skin	PCR (ITS1) + RFLP	Israel	[179]
Microtus socialis	50%	liver, skin, spleen	smear	Iran	[161]
Mus musculus (house mouse)	2.3–33%	liver, skin, spleen	PCR (ITS1) + RFLP, smear	Israel, Morocco and Iran	[161,179]
Nesokia indica	8–63.4%	liver, skin, spleen	PCR (ITS1) + RFLP + sequencing, PCR (kDNA), nPCR (kDNA), smear	Iran	[161,167,168]
Rhombomys opimus (great gerbil)	13.4–33%	skin	PCR (ITS1) + RFLP + sequencing, semi-nPCR (kDNA), PCR (Cytb) + sequencing, smear, IHC, inoculation to hamster, inoculation to BALB/c mice	Iran	[168,169]
Tatera indica	3.7–50%	liver, skin, spleen	PCR (kDNA), PCR (ITS1) + RFLP + sequencing, semi-nPCR (kDNA) + sequencing, PCR (Cytb), smear	Iran	[168,169,171]

Cytb: cytochrome B; IHC: immunohistochemistry; ELISA: enzyme immune assay; ITS: internal transcriber spacer; kDNA: kinetoplast DNA; LN: lymph node; nPCR: nested PCR; qPCR: quantitative PCR; RFLP: restriction fragment length polymorphism; SSU: small subunit of ribosomal RNA; WB: Western blot.

3.7. Wild Animals Infected with Leishmania tropica

Four studies conducted in Ethiopia [175,180], Kenya [181] and Egypt [182] demonstrated the presence of *L. tropica* DNA in the spleen of rodents and bats. One hundred and sixty-three bats (*Cardioderma cor*) were analyzed in Ethiopia by qPCR of the kDNA and ITS1 regions and 4.9% were found to be infected [175]. Using the same techniques, the authors found prevalences from 9.9% to 20% in the rodents *Acomys* sp. *Arvicanthis niloticus* and *Gerbillus nanus* [180] (Supplementary Materials File S5). In Egypt, *L. tropica* was found in 14.3% of the analyzed Anderson's gerbils (*Gerbillus andersoni*) with clinical signs by smears and PCR of the ITS1 region [182]. Finally, employing nested PCR and sequencing of the SSU and the ITS1 regions, 22% of the sampled house mice (skin) were found infected with *L. tropica* in Morocco [163].

The species *Procavia capensis* and *Arvicanthis niloticus* were reported in previous reviews infected with *L. tropica* [23].

3.8. Wild Animals Infected with Leishmania donovani

In Africa, rodents were infected with *L. donovani* in three studies. One employed serology and found 5.5% of African grass rats (*Arvicanthis niloticus*) to be positive by ELISA in Sudan [183], while another study found 18.2% of black rats (*Rattus rattus*) with clinical signs carrying the parasite in Saudi Arabia [164], employing culture, smear and inoculation of hamster. The other study found DNA of the *L. donovani* complex in the spleen in 15.3% of *Mastomys erythroleucus*, 7.7% of *Gerbilliscus nigricaudus* and 17.4% of *Arvicanthis niloticus* from Ethiopia using PCR of the kDNA and ITS regions [180].

Finally, one study obtained a prevalence of 23.5% of the *L. donovani* complex in European hares from Greece by nested PCR of the ITS region, employing the spleen of 166 animals [184] (Supplementary Materials File S5).

Details of infection with *L. donovani* of *Felis serval* can be found in a previous review [23].

Some of the previously mentioned studies obtained positive results for *Leishmania* spp., but they could not further determine the species (see Supplementary Materials File S5). The spleen of other four species of bats (*Glauconycteris variegate*, *Miniopterus arenarius*, *Neoromicia somalica* and *Scotophilus colias*) were found to be infected with *Leishmania* spp. in Ethiopia by PCR [175]. In a similar approach, 40% of the analyzed rodents of *Aethomys* spp. were also positive [180]. Smears of liver and spleen and indirect haemagglutination test were employed to detect the parasite in 40% of the sand cat (*Felis margarita*) in Saudi Arabia [185]. The same techniques were employed to detect *Leishmania* spp. infections in *Gerbillus pyramidum* and *Rattus norvegicus* in Egypt [186].

In previous reviews, other species of mammals, such as *Crycetomys gambianus*, *Heterohyrax brucei* and *Dendrohyrax arboreus*, were reported to be infected with *L. aethiopica* [23].

4. Conclusions

Knowledge of the role of wild animals as suitable hosts or reservoirs of *Leishmania* zoonotic species is essential in order to apply control measues or monitoring programmes. In this review, a systematic search of wild animals infected with zoonotic species of *Leishmania* was conducted, starting from 1990 and following PRISMA methodology. One hundred and eighty-nine species of wild animlas from ten orders (i.e., Carnivora, Chiroptera, Cingulata, Didelphimorphia, Diprotodontia, Lagomorpha, Eulipotyphla, Pilosa, Primates and Rodentia) were included in the review. Rodents and carnivores were the orders more widely explored, being the most probable main reservoirs, and also the ones presenting more clinical signs. *L. infantum* was the most widely distributed species, both geographically and in the range of species, followed by *L.* (*Viannia*) *braziliensis*, but this fact could be due to the more exhaustive investigation on these species.

More studies on the role of infected wild animals are necessary in order to implement specific measures when an outbreak of the disease appears.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/9/5/1101/s1, Supplementary File S1: Detailed information on wild animals infected with *Leishmania* (*Viania*) spp.; Supplementary File S2: Detailed information on wild animals infected with *Leishmania amazonensis;* Supplementary File S3: Detailed information on wild animals infected with *Leishmania mexicana;* Supplementary File S4: Detailed information on wild animals infected with *Leishmania infantum* in the Americas; Supplementary File S5: Detailed information on wild animals infected with *Leishmania* spp. from Europe, Africa and Asia.

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References

1. Maxfield: L.; Crane, J.S. *Leishmaniasis*; StatPearls Pulblishing: 2019. Available online: https://www.ncbi.nlm.nih.gov/books/NBK531456/ (accessed on 6 September 2020).
2. Bern, C.; Maguire, J.H.; Alvar, J. Complexities of assessing the disease burden attributable to leishmaniasis. *PLoS Negl. Trop. Dis.* **2008**, *2*, e313.
3. World Health Organization. *Leishmaniasis Situation and Trends*; Global Health Observatory data; World Health Organization: Geneva, Switzerland, 2018.
4. Akhoundi, M.; Kuhls, K.; Canne, A.; Votýpka. J.; Marty, P.; Delaunay, P.; Sereno, D. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. *PLoS Negl. Trop. Dis*. **2016**, *10*, e0004349.
5. Akhoundi, M.; Downing, T.; Votýpka, J.; KKuhls, K.; Lukeš, J.; Cannet, A.; Ravel, C.; Marty, P.; Delaunay, P.; Kasbari, M.; et al. *Leishmania* Infections: Molecular Targets and Diagnosis. *Mol. Asp. Med*. **2017**, *57*, 1–29, doi:10.1016/j.mam.2016.11.012.
6. Schönian, G.; Mauricio, I.; Cupolillo, E. Is it time to revise the nomenclature of *Leishmania*? *Trends Parasitol*. **2010**, *26*, 466–469.
7. Pruzinova, K.; Sadlova, J.; Seblova, V.; Homola, M.; Votypka, J.; Volf, P. Comparison of bloodmeal digestion and the peritrophic matrix in four sand fly species differing in susceptibility to *Leishmania donovani*. *PLoS ONE* **2015**, *10*, e0128203.
8. World Health Organization. *Control of the Leishmaniases*; WHO Technical Report Series; World Health Organization: Geneva, Switzerland, 2010; pp. 1–186.
9. Naucke, T.J.; Lorentz, S. First Report of Venereal and Vertical Transmission of Canine Leishmaniosis from Naturally Infected Dogs in Germany. *Parasit. Vectors* **2012**, *5*, 1–5, doi:10.1186/1756-3305-5-67.
10. Guedes, D.L.; van Henten, S.; Cnops, L.; Adriaensen, W.; Johan Griensven, J. Sexual Transmission of Visceral Leishmaniasis: A Neglected Story. *Trends Parasitol.* **2020**, *36*, 950–952, doi:10.1016/j.pt.2020.08.002.
11. Naucke, T.J.; Amelung, S.; Lorentz, S. First Report of Transmission of Canine Leishmaniosis through Bite Wounds from a Naturally Infected Dog in Germany. *Parasit. Vectors* **2016**, *9*, 1–4, doi:10.1186/s13071-016-1551-0.
12. Coutinho, M.T.Z.; Linardi, P.M. Can Fleas from Dogs Infected with Canine Visceral Leishmaniasis Transfer the Infection to Other Mammals? *Vet. Parasitol*. **2007**, *147*, 320–325, doi:10.1016/j.vetpar.2007.04.008.
13. Lainson, R. Ecological Interactions in the Transmission of the Leishmaniases. *Phil. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **1988**, *321*, 389–404, doi:10.1098/rstb.1988.0099.
14. De Oliveira, F.M.; Costa, L.H.C.; de Barros, T.L.; Ito, P.K.R.K.; Colombo, F.A.; Carvalho, C.; Pedro, W.A.; Queiroz, L.H.; Nunes, C.M. First Detection of *Leishmania* Spp. DNA in Brazilian Bats Captured Strictly in Urban Areas. *Acta Trop.* **2015**, *150*, 176–181, doi:10.1016/j.actatropica.2015.07.010.
15. Hamad, I.; Forestier, C.L.; Peeters, M.; Delaporte, E.; Raoult, D.; Bittar, F. Wild Gorillas as a Potential Reservoir of *Leishmania major*. *J. Infect. Dis.* **2015**, *211*, 267–273, doi:10.1093/infdis/jiu380.
16. Rescigno, M.; Urbano, M.; Valzasina, B.; Francolín, M.; Rotta, G.; Bonasio, R.; Granucci, F.; Kraehenbuhl, J.-P.; Ricciardi-Castagnoli, P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2001**, *2*, 361–367.
17. Rodríguez, J.M. The origin of human milk bacteria: Is there a bacterial entero-mammary pathway during late pregnancy and lactation? *Adv. Nutr.* **2014**, *5*, 779–784.
18. World Health Organization. Leishmaniasis Facksheet. Available online: https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis (accessed on 8 January 2021).
19. Cardoso, L.; Schallig, H.; Persichetti, M.F.; Pennisi, M.G. New Epidemiological Aspects of Animal Leishmaniosis in Europe: The Role of Vertebrate Hosts Other than Dogs. *Pathogens* **2021**, *10*, 1–46.

20. Miró, G.; Müller, A.; Montoya, A.; Checa, R.; Marino, V.; Marino, E.; Fuster, F.; Escacena, C.; Descalzo, M.A.; Gálvez, R. Epidemiological Role of Dogs since the Human Leishmaniosis Outbreak in Madrid. *Parasit. Vectors* **2017**, *10*, 209, doi:0.1186/s13071-017-2147-z.
21. Quaresma, P.F.; Rego, F.D.; Botelho, H.A.; da Silva, S.R.; Moura, A.J., Jr.; Teixeira Neto, R.G.; Madeira, F.M.; Carvalho, M.B.; Paglia, A.P.; Melo, M.N.; et al. Wild, synanthropic and domestic hosts of *Leishmania* in an endemic area of cutaneous leishmaniasis in Minas Gerais State, Brazil. *Trans. R. Soc. Trop. Med. Hyg.* **2011**, *105*, 579–585.
22. World Health Organization. *Control of the Leishmaniases*: *Report of a WHO Expert Committee*; WHO Report Series; World Health Organization: Geneva, Switzerland, 1990; Volume 793.
23. Ashford, R.W. Leishmaniasis Reservoirs and Their Significance in Control. *Clin. Dermatol.* **1996**, *14*, 523–532.
24. Roque, A.L. R.; Jansen, A.M. Wild and Synanthropic Reservoirs of *Leishmania* Species in the Americas. *Int. J. Parasitol. Parasit. Wildl.* **2014**, *3*, 251–262, doi:10.1016/j.ijppaw.2014.08.004.
25. Souza, T.D.; Turchetti, A.P.; Fujiwara, R.T.; Paixão, T.A.; Santos; R.L. Visceral Leishmaniasis in Zoo and Wildlife. *Vet. Parasitol.* **2014**, *200*, 233–241, doi:10.1016/j.vetpar.2013.12.025.
26. Millán, J.; Ferroglio, E.; Solano-Gallego, L. Role of Wildlife in the Epidemiology of *Leishmania infantum* infection in Europe. *Parasitol. Res.* **2014**, 2005–2014, doi:10.1007/s00436-014-3929-2.
27. Quinnell, R.J.; Courtenay, O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. *Parasitology* **2009**, *136*, 1915–1934, doi:10.1017/s0031182009991156.
28. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *PLoS Med*. **2021**, *18*, e1003583, doi:10.1371/journal.pmed.1003583, PMID: 33780438.
29. Alexander, B.; Lozano, C.; Barker, D.C.; McCann, S.H.E.; Adler, G.H. Detection of *Leishmania* (*Viannia*) *braziliensis* Complex in Wild Mammals from Colombian Coffee Plantations by PCR and DNA Hybridization. *Acta Trop.* **1998**, *69*, 41–50, doi:10.1016/S0001-706X(97)00114-9.
30. Brandão-Filho, S.P.; Brito, M.E.; Carvalho, F.G.; Ishikaw, E.A.; Cupolillo, E.; Floeter-Winter, L.; Shaw, J.J. Wild and Synanthropic Hosts of *Leishmania* (*Viannia*) *braziliensis* in the Endemic Cutaneous Leishmaniasis Locality of Amaraji, Pernambuco State, Brazil. *Trans. R. Soc. Trop. Med. Hyg.* **2003**, *97*, 291–296, doi:10.1016/S0035-9203(03)90146-5.
31. Tonelli, G.B.; Tanure, A.; Rego, F.D.; Carvalho, G.M.L.; Stumpp, R.; Ássimos, G.R.; Campos, A.M.; Lima, A.C.V.M.; Gontijo, C.M.F.; Paz, G.F.; et al. *Leishmania* (*Viannia*) *braziliensis* Infection in Wild Small Mammals in Ecotourism Area of Brazil. *PLoS ONE* **2017**, *12*, 1–10, doi:10.1371/journal.pone.0190315.
32. Lucas, J.; Lourenço, M.; Minuzzi-souza, T.T.C.; Silva, L.R.; Oliveira, A.M. High Frequency of Trypanosomatids in Gallery Forest Bats of a Neotropical Savanna. *Acta Trop.* **2018**, *177*, 200–206.
33. De Lima, H.; De Guglielmo, Z.; Rodriguez, A.; Convit, J.; Rodriguez, N. Cotton rats (*Sigmodon hispidus*) and black rats (*Rattus rattus*) as possible reservoirs of *Leishmania* spp. in Lara State, Venezuela. *Mem. Inst. Oswaldo Cruz* **2002**, *97*, 169–174.
34. Trüeb, I.; Portela, R.D.; Franke, C.R.; Carneiro, I.O.; Ribeiro, G.J.; Soares, R.P.; Barrouin-Melo, S.M. *Trypanosoma cruzi* and *Leishmania* sp. Infection in Wildlife from Urban Rainforest Fragments in Northeast Brazil. *J. Wildl. Dis.* **2018**, *54*, 76–84, doi:10.7589/2017-01-017.
35. Cardoso, R.M.; De Araújo, N.S.L.; Romero, G.A.S.; Souza, T.T.C.M.; Dietrich, A.G.; Mendes, J.D.; Reis, M.L.; Ferreira, J.B.C.; Hecht, M.M.; Gurgel-Gonçalves, R. Expanding the Knowledge about *Leishmania* Species in Wild Mammals and Dogs in the Brazilian Savannah. *Parasit. Vectors* **2015**, *8*, 1–8, doi:10.1186/s13071-015-0780-y.
36. Cássia-Pires, R.; Boité, M.C.; D'Andrea, P.S.; Herrera, H.M.; Cupolillo, E.; Jansen, A.M.; Roque, A.L.R. Distinct *Leishmania* Species Infecting Wild Caviomorph Rodents (Rodentia: Hystricognathi) from Brazil. *PLoS Negl. Trop. Dis.* **2014**, 8, doi:10.1371/journal.pntd.0003389.
37. Voltarelli, E.M.; Arraes, S.M.A.A.; Perles, T.F.; Lonardoni, M.V.C.; Teodoro, U.; Silveira, T.G.V. Serological survey for *Leishmania* sp. Infection in wild animals from the municipality of Maringá, Paraná state, Brazil. *J. Venom. Anim. Toxins Incl. Trop. Dis.* **2009**, 15, 732–744.
38. Schallig, H.D.F.H.; da Silva; E.S.; van der Meide, W.F.; Schoone, G.J.; Contifjo, C.M.F. *Didelphis marsupialis* (Common Opossum): A Potential Reservoir Host for Zoonotic Leishmaniasis in the Metropolitan Region of Belo Horizonte (Minas Gerais, Brazil). *Vector Borne Zoonotic Dis.* **2007**, *7*, 387–393, doi:10.1089/vbz.2006.0651.
39. Lima, B.S.; Dantas-Torres, F.; de Carvalho, M.R.; Marinho-Junior, J.F.; de Almeida, E.L.; Brito, M.E.; Gomes, F.; Brandão-Filho, S.P. Small mammals as hosts of *Leishmania* spp. in a highly endemic area for zoonotic leishmaniasis in North-Eastern Brazil. *Trans. R. Soc. Trop. Med. Hyg.* **2013**, *107*, 592–597.
40. Aransay, A.M.; Scoulica, E.; Tselentis, Y: Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. *Appl. Environ. Microbiol.* **2000**, *66*, 1933–1938.
41. Telleria, J.; Bosseno, M.F.; Tarifa, T.; Buitrago, R.; Martinez, E.; Torrez, M.; Le Pont, F.; Brenière, S.F. Putative reservoirs of *Leishmania amazonensis* in a Sub-Andean focus of Bolivia identified by kDNA-polymerase chain reaction. *Mem. Inst. Oswaldo Cruz* **1999**, *94*, 5–6.
42. Shapiro, J.T.; da Costa Lima Junior, M.S.; Dorval, M.E.; de Oliveira, F.A.; Cepa Matos, M.F.; Bordignon, M.O. First record of *Leishmania braziliensis* presence detected in bats, Mato Grosso do Sul, southwest Brazil. *Acta Trop*. **2013**, *128*, 171–174.

43. Castro Ferreira, E.; Pereira, A.A.S.; Silveira, M.; Margonari, C.; Marcon, G.E.B.; França, A.O.; Souza, L.; Castro, L.S.; OscarBordignon, M.; Fischer, E.; et al. *Leishmania* (*V.*) *braziliensis* Infecting Bats from Pantanal Wetland, Brazil: First Records for *Platyrrhinus lineatus* and *Artibeus planirostris*. *Acta Trop.* **2017**, *172*, 217–222, doi:10.1016/j.actatropica.2017.05.012.
44. Castro, L.S., Dorval, M.E.C.; Matheus, L.: M.D.; Bednaski, A.V.; Facco, G.G.; Silveira, M.; Santos, C.F.; Gontijo, C.M.F.; Oliveira, A.P.G.; Ferreira, E.C. *Leishmania* Presence in Bats in Areas Endemic for Leishmaniasis in Central-West Brazil. *Int. J. Parasitol. Parasit. Wildl.* **2020**, *11*, 261–267, doi:10.1016/j.ijppaw.2020.02.008.
45. Gómez-Hernández, C.; Bento, E.C.; Rezende-Oliveira, K.; Nascentes, G.A.N.; Barbosa, C.G.; Batista, L.R.; Tiburcio, M.G.S.; Pedrosa, A.L.; Lages-Silva, E.; Ramírez, J.D; et al. *Leishmania* Infection in Bats from a Non-Endemic Region of Leishmaniasis in Brazil. *Parasitology* **2017**, *144*, 1980–1985, doi:10.1017/S0031182017001500.
46. Naiff, R.D.; Freitas, R.A.; Naiff, M.F.; Arias, J.R.; Barrett, T.V.; Momen, H.; Grimaldi Júnior, G. Epidemiological and Nosological aspects of *Leishmania naiffi* Lainson & Shaw, 1989. *Mem Inst. Oswaldo Cruz* **1991**, *86*, 317–321.
47. Llanos-Cuentas, E.A.; Roncal, N., Villaseca, P.; Paz, L.; Ogusuku, E.; Perez, J.E.; Cáceres, A.; Davies, C.R. Natural infections of *Leishmania peruviana* in animals in the Peruvian Andes. *Trans. R. Soc. Trop. Med. Hyg.* **1999**, *93*, 15–20.
48. Quintal, A.P.; Ribeiro, E.S.; Rodrigues, F.P.; Rocha, F.S.; Floeter-Winter, L.M.; Nunes, C.M. *Leishmania* spp. in *Didelphis albiventris* and *Micoureus paraguayanus* (Didelphimorphia: Didelphidae) of Brazil. *Vet. Parasitol.* **2011**, *176*, 112–119.
49. Ocampo, C.B.; Ferro, M.C.; Cadena, H.; Gongora, R.; Pérez, M.; Valderrama-Ardila, C.H.; Quinnell, R.J.; Alexander, N. Environmental Factors Associated with American Cutaneous Leishmaniasis in a New Andean Focus in Colombia. *Trop. Med. Int. Health* **2012**, *17*, 1309–1317, doi:10.1111/j.1365-3156.2012.03065.x.
50. de Castro Ferreira, E.; Cruz, I.; Cañavate, C.; de Melo, L.A.; Pereira, A.A.S.; Madeira, F.A.M.; Nogueira Valério, S.A.; Cunha, H.M.; Paglia, A.P.; Ferreira Gontijo, C.M. Mixed Infection of *Leishmania infantum* and *Leishmania braziliensis* in Rodents from Endemic Urban Area of the New World. *BMC Vet. Res.* **2015**, *11*, 1–7, doi:10.1186/s12917-015-0392-y.
51. Silva, E.M.; Alves, L.C.; Guerra, N.R.; Farias, M.P.; Oliveira, E.L.; de Souza, R.C.; da Cunha, C.; Ramos, R.A.; Porto, W.J. *Leishmania* spp. in *Didelphis* spp. from Northeastern Brazil. *J. Zoo Wildl. Med.* **2016**, *47*, 942–944, doi:10.1638/2015-0148.1.
52. Paiz, L.; Donalisio, M.R.; Richini-Pereira, V.B.; Motoie, G.; Castagna, C.L.; Tolezano, J.E. Infection by *Leishmania* spp. in Free-Ranging Opossums (*Didelphis albiventris*) in an Environmentally Protected Area Inhabited by Humans in Southeastern Brazil. *Vector Borne Zoonotic Dis.* **2016**, *16*, 728–730, doi:10.1089/vbz.2016.2001.
53. Pereira, A.A.S.; De Castro Ferreira, E.; Da Rocha Lima, A.C.V.M.; Tonelli, G.B.; Rêgo, F.D.; Paglia, A.D.; Andrade-Filho, J.D.; Paz, G.F.; Gontijo, C.M.F. Detection of *Leishmania* spp in Silvatic Mammals and Isolation of *Leishmania* (*Viannia*) *braziliensis* from *Rattus rattus* in an Endemic Area for Leishmaniasis in Minas Gerais State, Brazil. *PLoS ONE* **2017**, *12*, 1–9, doi:10.1371/journal.pone.0187704.
54. Donalisio, M.R.; Paiz, L.M.; da Silva, V.G.; Richini-Pereira, V.B.; von Zuben, A.P.B.; Castagna, C.L.; Motoie, G.; Hiramoto, R.M.; Tolezano, J.E. Visceral leishmaniasis in an environmentally protected area in southeastern Brazil: Epidemiological and laboratory cross- sectional investigation of phlebotomine fauna, wild hosts and canine cases. *PLoS Negl. Trop. Dis.* **2017**, 11, e0005666, doi:10.1371/journal. pntd.0005666.
55. Brandão, E.M.V.; Samanta, C.C.; Xavier, J.G.; Carvalhaes, P.S.; D'andrea, Lemos, F.G.; Azevedo, F.C.; Cássia-Pires, R.; Jansen, A.M.; Roque, A.L.R. Trypanosomatids in Small Mammals of an Agroecosystem in Central Brazil: Another Piece in the Puzzle of Parasite Transmission in an Anthropogenic Landscape. *Pathogens* **2019**, *8*, 1–15, doi:10.3390/pathogens8040190.
56. González, K., Calzada, J.E.; Saldaña, A.; Rigg, C.A.; Alvarado, G.; Rodríguez-Herrera, B.; Kitron, U.D.; Adler, G.H.; Gottdenker, N.L.; Chaves, L.F.; et al. Survey of Wild Mammal Hosts of Cutaneous Leishmaniasis Parasites in Panamá and Costa Rica. *Trop. Med. Health* **2015**, *43*, 75–78, doi:10.2149/tmh.2014.
57. Silveira, F.T.; Lainson, R., Shaw; J.J., Braga, R.R.; Ishikawa, E.E.; Souza, A.A. Cutaneous leishmaniasis in Amazonia: Isolation of *Leishmania* (*Viannia*) *lainsoni* from the rodent *Agouti paca* (Rodentia: Dasyproctidae), in the state of Para, Brazil. *Rev. Inst. Med. Trop. Sao Paulo* **1991**, *33*, 18–22.
58. Oliveira, F.S., Pirmez, C.; Pires, M.Q.; Brazil, R.P.; Raquel, S. Pacheco. PCR-Based Diagnosis for Detection of *Leishmania* in Skin and Blood of Rodents from an Endemic Area of Cutaneous and Visceral Leishmaniasis in Brazil. *Vet. Parasitol.* **2005**, *129*, 219–227, doi:10.1016/j.vetpar.2005.01.005.
59. de Freitas, T.P.; D'Andrea, P.S.; de Paula, D.A.; Nakazato, L.; Dutra, V.; Bonvicino, C.R.; de Almeida, A.d.B.P.F.; Boa-Sorte, E.d.; Sousa, V.R.F. Natural infection of *Leishmania* (*Viannia*) *braziliensis* in *Mus musculus* captured in Mato Grosso, Brazil. *Vector Borne Zoonotic Dis*. **2012**, *12*, 81–83.
60. Brito, M.E.F.; Andrade, M.S.; Mendonça, M.G.; Silva, C.J.; Almeida, E.L.; Lima, B.S.; Félix, S.M.; Abath, F.G.C.; da Graça, G.C.; Porrozzi, R.; et al. Species Diversity of *Leishmania* (*Viannia*) Parasites Circulating in an Endemic Area for Cutaneous Leishmaniasis Located in the Atlantic Rainforest Region of Northeastern Brazil. *Trop. Med. Int. Health* **2009**, *14*, 1278–1786, doi:10.1111/j.1365-3156.2009.02361.x.
61. De Bruijn, M.H.L.; Barker, D.C. Diagnosis of New World leishmaniasis: Specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. *Acta Trop*. **1992**, *52*, 45–58.
62. Marcelino, A.P.; Ferreira, E.C.; Avendanha, J.S.; Costa, C.F.; Chiarelli, D.; Almeida, G.; Moreira, E.C.; Leite, R.C.; dos Reis, J.K.P.; Gontijo, C.M.F. Molecular Detection of *Leishmania braziliensis* in *Rattus norvegicus* in an Area Endemic for Cutaneous Leishmaniasis in Brazil. *Vet. Parasitol.* **2011**, *183*, 54–58, doi:10.1016/j.vetpar.2011.06.019.

63. Vasconcelos, I.A.; Vasconcelos, A.W.; Fe Filho, N.M.; Queiroz, R.G.; Santana, E.W.; Bozza, M.; Sallenave, S.M.; Valim, C.; David, J.R.; Lopes, U.G. The identity of *Leishmania* isolated from sand flies and vertebrate hosts in a major focus of cutaneous leishmaniasis in Baturité, northeastern Brazil. *Am. J. Trop Med. Hyg.* **1994**, *50*, 158–164.
64. Martínez, M.F.; Kowalewski, M.M.; Giuliani, M.G.; Acardi, S.A.; Salomón, O.D. Molecular Identification of *Leishmania* in Free-Ranging Black and Gold Howler Monkeys (*Alouatta caraya*) in Northeastern Argentina. *Acta Trop.* **2020**, *210*, 105534, doi:10.1016/j.actatropica.2020.105534.
65. Acardi, S.A.; Rago, M.V.; Liotta, D.J.; Fernandez-Duque, E.; Salomón, O.D. *Leishmania* (*Viannia*) DNA Detection by PCR-RFLP and Sequencing in Free-Ranging Owl Monkeys (*Aotus azarai azarai*) from Formosa, Argentina. *Vet. Parasitol.* **2013**, *193*, 256–259, doi:10.1016/j.vetpar.2012.12.012.
66. Savani, E.S., de Almeida, M.F., de Oliveira Camargo, M.C., D'Auria, S.R., Silva, M.M., de Oliveira, M.L.; Sacramento, D. Detection of *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Leishmania*) *infantum chagasi* in Brazilian bats. *Vet. Parasitol*. **2010**, *168*, 5–10.
67. De Lima, V.M.F.; Santiago, M.E.B.; Sanches, L.dC.; de Lima, B.D. Molecular diagnosis of *Leishmania amazonensis* ina captive spider monkey in Bauru, Sao Paulo, Brazil. *J. Zoo Wildl. Med*. **2012**, *43*, 943–945, doi:10.1638/2012-0059R1.1.
68. Kerr, S.F.; Emmons, L.H.; Melby, P.C.; Liu, C.; Perez, L.E.; Villegas, M.; Miranda, R. *Leishmania amazonensis* infections in Oryzomys acritus and Oryzomys nitidus from Bolivia. *Am. J. Trop. Med. Hyg*. **2006**, *75*, 1069–1073.
69. Zamora-Ledesma, S.; Hernández-Camacho, N.; Villagrán-Herrera, M.E.; Sánchez-Moreno, M.; Concha-Valdez, F.G.; Jones, R.W.; Moreno-Pérez, M.A.; Camacho-Macías, B. Presence of Trypanosomatid Antibodies in Gray Foxes (*Urocyon cinereoargenteus*) and Domestic and Feral Dogs (*Canis lupus familiaris*). *Vet. Parasitol. Reg. Stud. Rep.* **2016**, *5*, 25–30.
70. Berzunza-Cruz, M.; Rodríguez-Moreno, A.; Gutiérrez-Granados, G.; González-Salazar, C.; Stephens, C.R.; Hidalgo-Mihart, M.; Marina, C.F.; Rebollar-Téllez, E.A.; Bailón-Martínez, D.; Balcells, C.D.; et al. *Leishmania* (*L.*) *mexicana* Infected Bats in Mexico: Novel Potential Reservoirs. *PLoS Negl. Trop. Dis.* **2015**, *9*, 1–15, doi:10.1371/journal.pntd.0003438.
71. Van Wynsberghe, N.R.; Canto-Lara, S.B.; Sosa-Bibiano, E.I.; Rivero-Cardenas, N.A.; Andrade-Narvaez, F.J. Comparison of small mammal prevalence of *Leishmania* (*Leishmania*) *mexicana* in five foci of cutaneous leishmaniasis in the State State of Campeche, Mexico. *Rev. Inst. Med. Trop. Sao Paulo* **2009**, *51*, 87–94.
72. Van Wynsberghe, N.R.; Canto-Lara, S.B.; Mian-Centeno, A.G.; Itza-Ortiz, M.F.; Andrade-Narvaez, F.J. Retention of *Leishmania* (*Leishmania*) *mexicana* in naturally infected rodents from the State of Campeche, Mexico. *Mem. Inst. Oswaldo Cruz* **2000**, *95*, 595–600.
73. Kerr, S.F.; McHugh, C.P.; Dronen, N.O., Jr. Leishmaniasis in Texas: Prevalence and seasonal transmission of *Leishmania mexicana* in *Neotoma micropus*. *Am. J. Trop. Med. Hyg*. **1995**, *53*, 73–77.
74. Raymond, R.W.; McHugh, C.P.; Witt, L.R.; Kerr, S.F. Temporal and Spatial Distribution of *Leishmania mexicana* Infections in a Population of *Neotoma micropus*. *Mem. Inst. Oswaldo Cruz* **2003**, *98*, 171–180, doi:10.1590/S0074-02762003000200002.
75. Canto-Lara, S.B.; Van Wynsberghe, N.R.; Vargas-González, A.; Ojeda-Farfán, F.F.; Andrade-Narváez, F.J. Use of Monoclonal Antibodies for the Identification of *Leishmania* Spp. Isolated from Humans and Wild Rodents in the State of Campeche, Mexico. *Mem. Inst. Oswaldo Cruz* **1999**, *94*, 305–309, doi:10.1590/S0074-02761999000300005.
76. McHug, C.P.; Thies, M.L.; Melby, P.C.; Yantis, L.D.; Raymond, R.W.; Villegas, M.D.; Kerr, S.F. Short report: A disseminated infection of *Leishmania mexicana* in an eastern woodrat, *Neotoma floridana*, collected in Texas. *Am. J. Trop. Med. Hyg*. **2003**, *69*, 470–472.
77. Chable-Santos, J.B.; Van Wynsberghe, N.R.; Canto-Lara, S.B.; Andrade-Narvaez, F.J. Isolation of *Leishmania* (*L.*) *mexicana* from Wild Rodents and Their Possible Role in the Transmission of Localized Cutaneous Leishmaniasis in the State of Campeche, Mexico. *Am. J. Trop. Med. Hyg.* **1995**, *53*, 141–145, doi:10.4269/ajtmh.1995.53.141.
78. Kipp, E.J.; Mariscal, J.; Armijos, R.X.; Weigel, M.; Waldrup, K. Genetic Evidence of Enzootic Leishmaniasis in a Stray Canine and Texas Mouse from Sites in West and Central Texas. *Mem. Inst. Oswaldo Cruz* **2016**, *111*, 652–654, doi:10.1590/0074-02760160225.
79. Muñoz-García, C.I.; Sánchez-Montes, S.; Villanueva-García, C.; Romero-Callejas, E.; Díaz-López, H.M.; Gordillo-Chávez, E.J.; Martínez-Carrasco, C.; Berriatua, E.; Rendón-Franco, E. The Role of Sloths and Anteaters as *Leishmania* Spp. Reservoirs: A Review and a Newly Described Natural Infection of *Leishmania mexicana* in the Northern Anteater. *Parasitol. Res.* **2019**, *118*, 1095–1101, doi:10.1007/s00436-019-06253-6.
80. Rovirosa-Hernández, M.J.; Cortes-Ortíz, L.; García-Orduña, F.; Guzmán-Gómez, D.; López-Monteon, A.; Caba, M.; Ramos-Ligonio, A. Seroprevalence of *Trypanosoma cruzi* and *Leishmania mexicana* in free-ranging howler monkeys in southeastern Mexico. *Am. J. Primatol*. **2013**, *75*, 161–169, doi:10.1002/ajp.22094.
81. Courtenay, O.; Quinnell, R.J.; Garcez, L.M.; Dye, C. Low Infectiousness of a Wildlife Host of *Leishmania Infantum*: The Crab-Eating Fox Is Not Important for Transmission. *Parasitology* **2002**, *125*, 407–414, doi:10.1017/S0031182002002238.
82. Almeida Curi, N.H. de; Miranda, I.; Talamoni, S.A. Serologic Evidence of *Leishmania* Infection in Free-Ranging Wild and Domestic Canids around a Brazilian National Park. *Mem. Inst. Oswaldo Cruz* **2006**, *101*, 99–101, doi:10.1590/s0074-02762006000100019.
83. Gomes, R.B.; Mendonca, I.L.; Silva, V.C.; Ruas, J.; Silva, M.B.; Cruz, M.S.P.; Barral, A.; Costa, C.H.N. Antibodies against *Lutzomya longipalpis* saliva in the fox *Cerdocyon thous* and the sylvatic cycle of *Leishmania chagasi*. *Trans. R. Soc. Trop. Med. Hyg.* **2007**, *101*, 127–133.

84. Luppi, M.M.; Malta, M.C.C.; Silva, T.M.A.; Silva, F.L.; Motta, R.O.C.; Miranda, I.; Ecco, R.; Santos, R.L. Visceral Leishmaniasis in Captive Wild Canids in Brazil. *Vet. Parasitol.* **2008**, *155*, 146–151, doi:10.1016/j.vetpar.2008.04.024.
85. Jusi, M.M.G.; Starke-Buzetti, W.A.; de Sousa Oliveira, T.M.F.; da Silva Tenório, M.; de Oliveira de Sousa, L.; Zacarias Machad, R. Molecular and Serological Detection of *Leishmania* spp. in Captive Wild Animals from Ilha Solteira, SP, Brazil. *Rev. Bras. de Parasitol. Veterinária* **2011**, *20*, 219–222.
86. Richini-Pereira, V.B.; Marson, P.M.; Hayasaka, E.Y.; Victoria, C.; da Silva, R.C.; Langoni, H. Molecular Detection of *Leishmania* Spp. in Road-Killed Wild Mammals in the Central Western Area of the State of São Paulo, Brazil. *J. Ven. Anim. Tox. Trop. Dis.* **2014**, *20*, 1–7, doi:10.1186/1678-9199-20-27.
87. Almeida, J.C.; Melo, R.P.B.; Kim, P.C.P.; Guerra, N.R.; Alves, L.C.; Costa, D.F.; Alves, C.J.; Porto, W.J.N.; Mota, R.A. Molecular and Serological Investigation of Infectious Diseases in Captive and Free-Range Crab-Eating Fox (*Cerdocyon thous*-Linnaeus, 1776) from Northeastern Brazil. *Acta Parasitol.* **2018**, *63*, 184–189, doi:10.1515/ap-2018-0021.
88. de Almeida Curi, N.H.; Coelho, C.M.; Malta, M.C.C.; Magni, L.M.V.; Sábato, M.A.L.; Araújo, A.S.; Lobato, Z.I.P.; Santos, J.L.C.; Santos, H.A.; Ragozo, A.A.M.; et al. Pathogens of Wild Maned Wolves (*Chrysocyon brachyurus*) in Brazil. *J. Wildl. Dis.* **2012**, *48*, 1052–1056, doi:10.7589/2011-10-304.
89. Mol, J.P.S.; Soave, S.A.; Turchetti, A.P.; Pinheiro, G.R.G.; Pessanha, A.T.; Malta, M.C.C.; Tinoco, H.P.; Figueiredo, L.A.; Gontijo, N.F.; Paixão, T.A.; et al. Transmissibility of *Leishmania infantum* from Maned Wolves (*Chrysocyon brachyurus*) and Bush Dogs (*Speothos venaticus*) to *Lutzomyia longipalpis*. *Vet. Parasitol.* **2015**, *212*, 86–91, doi.org/10.1016/j.vetpar.2015.08.024.
90. Tolentino, N.; Pinheiro, G.R.G.; Ottino, J.; Rodrigues de Oliveira, A.; Coelho, C.M.; Tinoco, H.P.; Fujiwara, R.T.; Santos, R.L.; Ribeiro, V.M. Serological Evidence of *Leishmania* Infection by Employing ELISA and Rapid Tests in Captive Felids and Canids in Brazil. *Vet. Parasitol. Reg. Stud. Rep.* **2019**, *17*, 100308, doi:10.1016/j.vprsr.2019.100308.
91. Lombardi, M.C.; Turchetti, A.P.; Tinoco, H.P.; Pessanha, A.T.; Soave, S.A.; Malta, M.C.C.; Paixão, T.A.; Santos, R.L. Diagnosis of *Leishmania Infantum* Infection by Polymerase Chain Reaction in Wild Mammals. *Pesquisa Vet. Brasil.* **2014**, *34*, 1243–1246, doi:10.1590/S0100-736X2014001200017.
92. Paiz, L.M., Fornazari, F.; Menozzi, B. Serological Evidence of Infection by *Leishmania* (*Leishmania*) *infantum* (Synonym: *Leishmania* (*Leishmania*) *chagasi*) in Free-Ranging Wild Mammals in a Nonendemic Region of the State of Sa˜o Paulo, Brazil. *Vector Borne Zoonotic Dis.* **2015**, *11*, 667–673, doi:10.1089/vbz.2015.1806.
93. Dahroug, M.A.; Almeida, A.B.; Sousa, V.R.; Dutra, V.; Turbino; N.C., Nakazato, L.; de Souza, R.L. *Leishmania* (*Leishmania*) *chagasi* in captive wild felids in Brazil. *Trans. R. Soc. Trop. Med. Hyg*. **2010**, *104*, 73–74.
94. Dahroug, M.A.; Almeida, A.B.; Sousa, V.R.; Dutra, V.; Guimarães, L.D.; Soares, C.E.; Nakazato, L.; de Souza, R.L. The first case report of *Leishmania* (*leishmania*) *chagasi* in *Panthera leo* in Brazil. *Asian Pac. J. Trop. Biomed*. **2011**, *3*, 249–250, doi:10.1016/S2221-1691(11)60036.
95. Rezende, M.B. de; Herrera, H.M.; Carvalho, C.M.E.; Carvalho Anjos, E.A.; Ramos, C.A.N.; de Araújo, F.R.; Torres, J.M.; de Oliveira, C.E. Detection of *Leishmania* spp. in Bats from an Area of Brazil Endemic for Visceral Leishmaniasis. *Trans. Emerg. Dis.* **2017**, 64, e36–e42, doi:10.1111/tbed.12597.
96. Medkour, H.; Davoust, B.; Dulieu, F.; Maurizi, L.; Lamour, T.; Marié, J.L.; Mediannikov, O. Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to *Leishmania infantum* in French Guiana. *PLoS Negl. Trop. Dis.* **2019**, 13, e0007456, doi:10.1371/journal.pntd.0007456.
97. De Lima, H.; Rodriguez, N.; Barrios, M.A.; Avila, A.; Canizales, I.; Gutierrez, S. Isolation and molecular identification of *Leishmania chagasi* from a bat (*Carollia perspicillata*) in northeastern Venezuela. *Mem. Inst. Oswaldo Cruz* **2008**, *103*, 412–414.
98. Pereira da Costa, A.; Costa, F.B.; Soares, H.S.; Ramirez, D.G.; de Carvalho, E.T.K.; Gennari, S.M.; Marcili, A. *Trypanosoma cruzi* and *Leishmania infantum chagasi* Infection in Wild Mammals from Maranhão State, Brazil. *Vector Borne Zoonotic Dis*. **2015**, *15*, 656–666, doi:10.1089/vbz.2015.1771.
99. de Araujo, V.A.; Boite, M.C.; Cupolillo, E.; Jansen, A.M.; Roque, A.L. Mixed infection in the anteater *Tamandua tetradactyla* (Mammalia: Pilosa) from Para State, Brazil: *Trypanosoma cruzi*, *T. rangeli* and *Leishmania infantum*. *Parasitology* **2013**, *140*, 455–460.
100. Humberg, R.M.; Oshiro, E.T.; Cruz, M.S.; Ribolla, P.E.; Alonso, D.P.; Ferreira, A.M.; Bonamigo, R.A.; Tasso, N., Jr.; de Oliveira, A.G. *Leishmania chagasi* in opossums (*Didelphis albiventris*) in an urban area endemic for visceral leishmaniasis, Campo Grande, Mato Grosso do Sul, Brazil. *Am. J. Trop. Med. Hyg.* **2012**, *87*, 470–472.
101. Santiago, M.E.; Vasconcelos, R.O.; Fattori, K.R.; Munari, D.P.; Michelin, A.F.; Lima, V.M. An investigation of *Leishmania* spp. in *Didelphis* spp. from urban and peri-urban areas in Bauru (Sao Paulo, Brazil). *Vet. Parasitol*. **2007**, *150*, 283–290.
102. Carreira, J.C.; da Silva, A.V.; de Pita, P.D.; Brazil, R.P. Natural infection of *Didelphis aurita* (Mammalia: Marsupialia) with *Leishmania infantum* in Brazil. *Parasit. Vectors* **2012**, *5*, 111.
103. Viettri, M.; Herrera, L.; Aguilar, C.M.; Morocoima, S.; Reyes, J.; Lares, M.; Lozano-Arias, D.; García-Alzate, R.; Chacón, T.; Feliciangeli, M.D.; et al. Molecular Diagnosis of *Trypanosoma cruzi*/*Leishmania* Spp. Coinfection in Domestic, Peridomestic and Wild Mammals of Venezuelan Co-Endemic Areas. *Vet. Parasitol. Reg. Stud. Rep.* **2018**, *14*, 123–130, doi:10.1016/j.vprsr.2018.10.002.
104. Corredor, A.; Gallego, J.F.; Tesh, R.B.; Pelaez, D.; Diaz, A., Montilla; M.; Palau, M.T. *Didelphis marsupialis*, an apparent wild reservoir of *Leishmania donovani chagasi* in Colombia, South America. *Trans. R. Soc. Trop. Med. Hyg*. **1989**, *83*, 195–195.
105. Travi, B.L.; Jaramillo, C.; Montoya, J.; Segura, I.; Zea, A.; Goncalves, A.; Velez, I.D. *Didelphis marsupialis*, an important reservoir of *Trypanosoma* (*Schizotrypanum*) *cruzi* and *Leishmania* (*Leishmania*) *chagasi* in Colombia. *Am. J. Trop. Med. Hyg*. **1994**, *50*, 557–565.

106. Travi, B.L.; Osorio, Y.; Becerra, M.T.; Adler, G.H. Dynamics of *Leishmania chagasi* infection in small mammals of the undisturbed and degraded tropical dry forests of northern Colombia. *Trans. R. Soc. Trop. Med. Hyg*. **1998**, *92*, 275–278.
107. Zulueta, A.M.; Villarroel, E.; Rodriguez, N.; Feliciangeli, M.D.; Mazzarri, M.; Reyes, O.; Rodriguez, V.; Centeno, M.; Barrios, R.M.; Ulrich, M. Epidemiologic Aspects of American Visceral Leishmaniasis in an Endemic Focus in Eastern Venezuela. *Am. J. Trop. Med. Hyg.* **1999**, *61*, 945–950, doi:10.4269/ajtmh.1999.61.945.
108. Malta, M.C.; Tinoco, H.P.; Xavier, M.N.; Vieira, A.L.; Costa, E.A.; Santos, R.L. Naturally acquired visceral leishmaniasis in non-human primates in Brazil. *Vet. Parasitol*. **2010**, *169*, 193–197.
109. Paiz, L.M.; Motoie, G.; Richini-Pereira, V.B.; Langoni, H.; Menozzi, B.D.; Tolezano, J.E.; Donalisio, M.R. Antibodies and molecular detection of *Leishmania* (*Leishmania*) *infantum* in samples of free-ranging marmosets (Primates: Callicitrichidae: *Callithrix* spp.) in an area of canine visceral leishmaniasis in southeastern Brazil. *Vector Borne Zoonotic Dis.* **2019**, *19*, 249–254.
110. Moreno, E.S.; Sabioni, L.A.; Moraes de Seixas, M.M.; de Souza Filho, J.A.; Marcelino, A.P. Paloma Helena Fernandes Shimabukuro. Evidence of a Sylvatic Enzootic Cycle of *Leishmania infantum* in the State of Amapá, Brazil. *Rev. Da Soc. Brasil. Med. Trop.* **2020**, *53*, 13–15, doi:10.1590/0037-8682-0169-2019.
111. Rosypal, A.C.; Tripp, S.; Lewis, S.; Francis, J.; Stoskopf, M.K.; Larsen, R.S.; Lindsay, D. Survey of antibodies to *Trypanosoma cruzi* and *Leishmania* spp. in gray and red fox populations from North Carolina and Virginia. *J. Parasitol.* **2010**, *96*, 1230–1231.
112. Rosypal, A.C.; Alexander, A., Byrd, D.; Weaver, M.; Stewart, R.; Gerhold, R.; Houston, A.; vanWhy, K.; Dubey, J.P. Survey of antibodies to *Leishmania* spp. in wild canids from Pennsylvania and Tennesee. *J. Zoo Wildl. Med*. **2013**, *44*, 1131–1133.
113. Millán, J.; Travaini, A.; Zanet, S.; López-Bao, J.V.; Trisciuoglio, A.; Ferroglio, E.; Rodríguez, A. Detection of *Leishmania* DNA in Wild Foxes and Associated Ticks in Patagonia, Argentina, 2000 Km South of Its Known Distribution Area. *Parasit. Vectors* **2016**, *9*, 1–7, doi:10.1186/s13071-016-1515-4.
114. Reis, F.; Minuzzi-Souza Mariana, T.T.C.; Renata, N.; De Morais, I.O.B.; De Lima, T.M.; Hecht, M.; Nitz, N.; Gurgel-Gonçalves, R. Trypanosomatid Infections in Captive Wild Mammals and Potential Vectors at the Brasilia Zoo, Federal District, Brazil. *Vet. Med. Sci.* **2020**, *6*, 248–256, doi:10.1002/vms3.216.
115. Brandao, E.M.V.; Xavier, S.C.C.; Rocha, F.L.; Lima, C.F.M.; Roque, A.L.R. Wild and Domestic Canids and Their Interactions in the Transmission Cycles of *Trypanosoma cruzi* and *Leishmania* spp. in an Area of the Brazilian Cerrado. *Pathogens* **2020**, *9*, 1–19, doi:10.3390/pathogens9100818.
116. Porfirio, F.E.O.; Santos, F.M.; Carvalho de Macedo, G.; Barreto, W.T.G.; Campos, J.B.V.; Meyers, A.C.; André, M.R.; Perles, L.; Oliveira, C.E.; ChagasXavier, S.C.D.; et al. Maintenance of *Trypanosoma cruzi*, *T. evansi* and *Leishmania* spp. by Domestic Dogs and Wild Mammals in a Rural Settlement in Brazil-Bolivian Border. *Int. J. Parasitol. Parasit. Wildl.* **2018**, *7*, 398–404, doi:10.1016/j.ijppaw.2018.10.004.
117. Lima, V.M.F.; Fattori, F.R.; Apargecida de Fátima Michelin, A.F.; Nogueira, F.S.; de Oliveira e Souza, L. Evidence of *Leishmania* spp. Antibodies and DNA in Bush Dogs (*Speothos venaticus*) in Brazil. *J. Zoo Wildl. Med.* **2009**, *40*, 91–94.
118. Johnson, R.N.; Young, D.G.; Butler, J.F.; Bogaert-Diaz, H. Possible Determination of the Vector and Reservoir of Leishmaniasis in the Dominican Republic. *Am. J. Trop. Med. Hyg.* **1992**, *46*, 282–287, doi:10.4269/ajtmh.1992.46.282.
119. Medkour, H.; Davoust, B.; Levasseur, A.; Mediannikov, O. Molecular Evidence of *Leishmania infantum* and *Leishmania guyanensis* in Red Howler Monkey (*Alouatta seniculus*) from French Guiana. *Vector Borne Zoonotic Dis*. **2019**, *12*, 896–900.
120. Babuadze, G.; Alvar, J.; Argaw, D.; de Koning, H.P.; Iosava, M.; Kekelidze, M.; Tsertsvadze, N.; Tsereteli, D.; Chakhunashvili, G.; Mamatsashvili, T.; et al. Epidemiology of Visceral Leishmaniasis in Georgia. *PLoS Negl. Trop. Dis.* **2014**, *8*, 1–8, doi:10.1371/journal.pntd.0002725.
121. Mitková, B.; Hrazdilová, K.; Amico, G.D.; Duscher, G.G.; Suchentrunk, F.; Forejtek, P.; Gherman, M.; Matei, I.A., Ionică, A.M.; Daskalaki, A.A.; et al. Eurasian Golden Jackal as Host of Canine Vector-Borne Protists. *Parasit. Vectors* **2017**, 10, 1–11, doi:10.1186/s13071-017-2110-z.
122. Mohebali, M.; Arzamani, K.; Zarei, Z.; Akhoundi, B.; Hajjaran, H.; Raeghi, S.; Heidari, Z.; Motavalli-Haghi, S.M.; Elikaee, S.; Mousazadeh-Mojarrad, A.; et al. Canine Visceral Leishmaniasis in Wild Canines (Fox, Jackal and Wolf) in Northeastern Iran Using Parasitological, Serological, and Molecular Methods. *J. Arthopod Borne Dis.* **2016**, *10*, 538–545.
123. Talmi-Frank, D.; Kedem-Vaanunu, N.; King, R.; Bar-Gal, G.K.; Edery, N.; Jaffe, C.L.; Baneth, G. *Leishmania tropica* Infection in Golden Jackals and Red Foxes, Israel. *Emerg. Infect. Dis.* **2010**, *16*, 1973–1975, doi:10.3201/eid1612.100953.
124. Beck, A.; Beck, R.; Kusak, J.; Gudan, A.; Martinkovic, F.; Artukovic, B.; Hohšteter, M.; Huber, D.; Marinculic, A.; Grabarevic, Z. A Case of Visceral Leishmaniosis in a Gray Wolf (*Canis lupus*) from Croatia. *J. Wildl. Dis.* **2008**, *44*, 451–456, doi:10.7589/0090-3558-44.2.451.
125. Sobrino, R.; Ferroglio, E.; Oleaga, A.; Romano, A.; Millan, J.; Revilla, M.; Arnal, M.C.; Trisciuoglio, A.; Gortázar, C. Characterization of Widespread Canine Leishmaniasis among Wild Carnivores from Spain. *Vet. Parasitol.* **2008**, *155*, 198–203, doi:10.1016/j.vetpar.2008.05.003.
126. Oleaga, A.; Vicente, J.; Ferroglio, E.; Pegoraro de Macedo, M.R.; Casais, R.; Del Cerro, A.; Espí, A.; García, E.J.; Gortázar, C. Concomitance and Interactions of Pathogens in the Iberian Wolf (*Canis lupus*). *Res. Vet. Sci.* **2015**, *101*, 22–27, doi:10.1016/j.rvsc.2015.05.010.
127. Oleaga, A.; Zanet, S.; Espí, A.; Raquel, M.; De Macedo, P.; Gortázar, C.; Ferroglio, E. *Leishmania* in Wolves in Northern Spain: A Spreading Zoonosis Evidenced by Wildlife Sanitary Surveillance. *Vet. Parasitol.* **2018**, *255*, 26–31, doi:10.1016/j.vetpar.2018.03.015.

128. Risueño, J.; Ortuño, M.; Pérez-Cutillas, P.; Goyena, E.; Maia, C.; Cortes, S.; Campino, L.; BernalL, J.; Muñoz, C.; ArcenillasI, L.; et al. Epidemiological and Genetic Studies Suggest a Common *Leishmania infantum* Transmission Cycle in Wildlife, Dogs and Humans Associated to Vector Abundance in Southeast Spain. *Vet. Parasitol.* **2018**, *259*, 61–67, doi:10.1016/j.vetpar.2018.05.012
129. Ortuño, M.; Latrofa, M.S.; Iborra, M.A.; Pérez, P.; Bernal, L.J.; Risueño, J.; Muñoz, C.; Bernal, A.; Sánchez-Lopez, P.F.; Segovia, M.; et al. Genetic Diversity and Phylogenetic Relationships between *Leishmania infantum* from Dogs, Humans and Wildlife in South - East Spain. *Zoonoses Public Health* **2019**, *66*, 961–973, doi:10.1111/zph.12646.
130. Sastre, N.; Francino, O.; Ramírez, O.; Enseñat, C.; Sánchez, A.; Altet, L. Detection of *Leishmania infantum* in Captive Wolves from Southwestern Europe. *Vet. Parasitol.* **2008**, *158*, 117–120, doi:10.1016/j.vetpar.2008.08.008.
131. Muñoz-Madrid, R.; Belinchón-Lorenzo, S.; Iniesta, V.; Fernández-Cotrina, J.; Parejo, J.C.; Serrano, F.J.; Monroy, I.; Baz, V.; Gómez-Luque, A.; Gómez-Nieto, L.C. First Detection of *Leishmania infantum* Kinetoplast DNA in Hair of Wild Mammals: Application of QPCR Method to Determine Potential Parasite Reservoirs. *Acta Trop.* **2013**, *128*, 706–709, doi:10.1016/j.actatropica.2013.08.009.
132. Battisti, E.; Zanet, S.; Khalili, S.; Trisciuoglio, A.; Hertel, B.; Ferroglio, E. Molecular Survey on Vector-Borne Pathogens in Alpine Wild Carnivorans. *Front. Vet. Sci.* **2020**, *7*, 1–9, doi:10.3389/fvets.2020.00001.
133. Del Río, L.; Chitimia, L.; Cubas, A.; Victoriano, I.; De la Rúa, P.; Gerrikagoitia, X.; Barral, M.; Muñoz-García, C.I.; Goyena, E.; García-Martínez, D.; et al. Evidence for Widespread *Leishmania infantum* Infection among Wild Carnivores in *L. infantum* Periendemic Northern Spain. *Prev. Vet. Med.* **2014**, *113*, 430–435, doi:10.1016/j.prevetmed.2013.12.001.
134. Millán, J.; Zanet, S.; Gomis, M.; Trisciuoglio, A.; Negre, N.; Ferroglio, E. An Investigation into Alternative Reservoirs of Canine Leishmaniasis on the Endemic Island of Mallorca (Spain). *Trans. Emerg. Dis.* **2011**, *58*, 352–357, doi:10.1111/j.1865-1682.2011.01212.x.
135. Gomes, J.; Rocha, H.; Carvalho, C.; Bandeira, V.; Fonseca, C.; Rosalino, L.M.; Cunha, M.V. Molecular Detection and Characterization of *Leishmania infantum* in Free-Ranging Egyptian Mongoose (*Herpestes ichneumon*). *Int. J. Parasitol. Parasit. Wildl.* **2020**, *11*, 158–162, doi:10.1016/j.ijppaw.2020.02.001.
136. Tsakmakidis, G.; Pavlou, C.; Tamvakis, A.; Papadopoulos, T.; Christodoulou, V.; Angelopoulou, K.; Dovas, C.; Antoniou, M.; Anastasakis, C.; Diakou, A. *Leishmania* infection in lagomorphs and minks in Greece. *Vet. Parasitol. Reg. Stud. Rep*. **2019**, 16. 100279, doi:10.1016/j.vprsr.2019.100279.
137. Alcover, M.M.; Ribas, A.; Guillén, M.C.; Berenguer, D.; Tomás-Pérez, M.; Riera, C.; Fisa, R. Wild Mammals as Potential Silent Reservoirs of *Leishmania infantum* in a Mediterranean Area. *Prev. Vet. Med.* **2020**, 175, doi:10.1016/j.prevetmed.2019.104874.
138. Calavera, M.A.; Latta, R.; Laricchiuta, P.; Passantino, G.; Abramo, F.; Mendoza-Roldan, J.A.; Otranto, D.; Zatelli, A. Clinical, haematological and biochemical findings in tigers infected by *Leishmania infantum*. *BMC Vet. Res*. **2020**, 214, doi:10.1186/s12917-020-02419-y.
139. Mancianti, F.; Mignone, W.; Galastri, F. Serologic Survey for Leishmaniasis in Free-Living Red Foxes (*Vulpes vulpes*) in Italy. *J. Wildl. Dis.* **1994**, *30*, 454–456, doi:10.7589/0090-3558-30.3.454.
140. Criado-Fornelio, A.; Gutierrez-Garcia, L.; Rodriguez-Caabeiro, F.; Reus-Garcia, E.; Roldan-Soriano, M.A.; Diaz-Sanchez, M.A. A Parasitological Survey of Wild Red Foxes (*Vulpes vulpes*) from the Province of Guadalajara, Spain. *Vet. Parasitol.* **2000**, *92*, 245–251, doi:10.1016/S0304-4017(00)00329-0.
141. Dipineto, L.; Manna, L.; Baiano, A.; Gala, M.; Fioretti, A.; Gravino, A.E.; Menna, L.F. Presence of *Leishmania infantum* in Red Foxes (*Vulpes vulpes*) in Southern Italy. *J. Wildl. Dis.* **2007**, *43*, 518–520, doi:10.7589/0090-3558-43.3.518.
142. Davoust, B.; Mary, C.; Marié, J.L. Detection of *Leishmania* in Red Foxes (*Vulpes vulpes*) from Southeastern France Using Real-Time Quantitative PCR. *J. Wildl. Dis.* **2014**, *50*, 130–132.
143. Karayiannis, S.; Ntais, P.; Messaritakis, I.; Tsirigotakis, N.; Dokianakis, E.; Antoniou, M. Detection of *Leishmania infantum* in Red Foxes (*Vulpes vulpes*) in Central Greece. *Parasitology* **2015**, *142*, 1574–1578, doi:10.1017/S0031182015001158.
144. Lledó, L.; Giménez-Pardo, C.; Saz, J.V.; Serrano, J.L. Wild Red Foxes (*Vulpes vulpes*) as Sentinels of Parasitic Diseases in the Province of Soria, Northern Spain. *Vector Borne Zoonotic Dis*. **2015**, *15*, 743–749, doi:10.1089/vbz.2014.1766.
145. Abbate, L.M.; Arfuso, F.; Napoli, E.; Gaglio, G.; Giannetto, S.; Latrofa, M.S.; Otranto, D.; Brianti, E. *Leishmania infantum* in Wild Animals in Endemic Areas of Southern Italy. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *67*, 101374, doi:10.1016/j.cimid.2019.101374.
146. Medkour, H.; Laidoudi, Y.; Marié, J.L.; Fenollar, F.; Davoust, B.; Mediannikov, O. Molecular Investigation of Vector-Borne Pathogens in Red Foxes (*Vulpes vulpes*) from Southern France. *J. Wildl. Dis.* **2020**, *56*, 837–850, doi:10.7589/2019-09-234.
147. Montoya, A.; Pérez De Quadros, L.; Mateo, M.; Hernández, L.; Gálvez, R.; Alcántara, G.; Checa, R.; Jiménez, M.Á.; Chicharro, C.; Israel Cruz, G.M. *Leishmania infantum* infection in Bennett's wallabies (*Macropus rufogriseus rufogriseus*) in a spanish wildlife park. *J. Zoo Wildl. Med.* **2016**, *47*, 586–593, doi:10.1638/2014-0216.1.
148. Miró, G.; Troyano, A.; Montoya, A.; Fariñas, F.; Fermín, M.L.; Flores, L.; Rojo, C.; Checa, R.; Gálvez, R.; Marino, V.; et al. First report of *L. infantum* infection in the endangered orangutan (*Pongo pygmaeus pygmaeus*) in Madrid, Spain. *Parasit. Vectors* **2018**, *11*, 1–7, doi: 10.1186/s13071-018-2772-1.
149. Azami-Conesa, I.; Martínez-Díaz, R.A.; González, F.; Gómez-Muñoz, M.T. First Detection of *Leishmania infantum* in Common Urban Bats *Pipistrellus pipistrellus* in Europe. *Res. Vet. Sci.* **2020**, *132*, 172–176, doi:10.1016/j.rvsc.2020.06.019.
150. Chemkhi, J.; Souguir, H.; BelHadjAli, I.; Driss, M.; Guizani, I.; Guerbouj, S. Natural Infection of Algerian Hedgehog, *Atelerix algirus* (Lereboullet 1842) with *Leishmania* Parasites in Tunisia. *Acta Trop.* **2015**, *150*, 42–51, doi:10.1016/j.actatropica.2015.06.009.

151. Souguir-Omrani, H.; Chemkhi, J.; Fathallah-Mili, A.; Saadi-BenAoun, Y.; BelHadjAli, I.; Guizani, I.; Guerbouj, S. *Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) Hedgehogs: Possible Reservoirs of Endemic Leishmaniases in Tunisia. *Infect. Genet. Evol.* **2018**, *63*, 219–230, doi:10.1016/j.meegid.2018.05.029.
152. Ruiz- Fons, F.; Ferroglio, E.; Gortázar, C. *Leishmania infantum* in Free-Ranging Hares, Spain, 2004–2010. *Eurosurveillance* **2013**, *30*, 80–81, doi:10.1016/j.pt.2019.10.006.
153. Ortega, M.V.; Moreno, I.; Domínguez, M.; de la Cruz, M.L.; Martín, A.B.; Rodríguez-Bertos, A.; López, R.; Navarro, A.; González, S.; Mazariegos, M.; et al. Application of a Specific Quantitative Real-Time PCR (QPCR) to Identify *Leishmania infantum* DNA in Spleen, Skin and Hair Samples of Wild Leporidae. *Vet. Parasitol.* **2017**, *243*, 92–99, doi:10.1016/j.vetpar.2017.05.015.
154. Ortega-García, M.V.; Salguero, F.J.; Rodríguez-Bertos, A.; Moreno, I.; García, N.; García-Seco, T.; Torre, G.L.; Domínguez, L.; Domínguez, M.A Pathological Study of *Leishmania infantum* Natural Infection in European Rabbits (*Oryctolagus cuniculus*) and Iberian Hares (*Lepus granatensis*). *Trans. Emerg. Dis.* **2019**, *66*, 2474–2781, doi:10.1111/tbed.13305.
155. Ebani, V.V.; Poli, A.; Rocchigiani, G.; Bertelloni, F.; Nardoni, S.; Papini, R.A.; Mancianti, F. Serological Survey on Some Pathogens in Wild Brown Hares (*Lepus europaeus*) in Central Italy. *Asian Pac. J. Trop. Med.* **2016**, *9*, 465–469, doi:10.1016/j.apjtm.2016.03.032.
156. Rocchigiani, G.; Ebani, V.V.; Nardoni, S.; Bertelloni, F.; Bascherini, A.; Leoni, A.; Mancianti, F.; Poli, A. Molecular Survey on the Occurrence of Arthropod-Borne Pathogens in Wild Brown Hares (*Lepus europaeus*) from Central Italy. *Infect. Genet. Evol.* **2018**, *59*, 142–147, doi:10.1016/j.meegid.2018.02.005.
157. Chitimia, L.; Muñoz- García, C.I.; Sánchez-Velasco, D.; Lizana, V.; Del Río, L.; Murcia, L.; Fisa, R.; Rierad, C.; Giménez-Fonte, P.; Jiménez-Montalbán, P.; et al. Cryptic Leishmaniosis by *Leishmania infantum*, a Feature of Canines Only? A Study of Natural Infection in Wild Rabbits, Humans and Dogs in Southeastern Spain. *Vet. Parasitol.* **2011**, *181*, 12–16, doi:10.1016/j.vetpar.2011.04.016.
158. Díaz-Sáez, V., Merino-Espinosa, G.; Morales-Yuste, M.; Corpas-López, V.; Pratlong, F.; Morillas-Márquez, F.; Martín-Sánchez, J. High Rates of *Leishmania infantum* and *Trypanosoma nabiasi* Infection in Wild Rabbits (*Oryctolagus cuniculus*) in Sympatric and Syntrophic Conditions in an Endemic Canine Leishmaniasis Area: Epidemiological Consequences. *Vet. Parasitol.* **2014**, *202*, 119–127, doi:10.1016/j.vetpar.2014.03.029.
159. Navea-Pérez, H.M.; Díaz- Sáez, V.; Corpas- López, V.; Merino- Espinosa, G.; Morillas- Márquez, F.; Martín- Sánchez, J. *Leishmania infantum* in Wild Rodents: Reservoirs or Just Irrelevant Incidental Hosts? *Parasitol. Res.* **2015**, *114*, 2363–2370, doi:10.1007/s00436-015-4434-y.
160. Millán, J. Molecular Investigation of Vector-Borne Parasites in Wild Micromammals, Barcelona (Spain). *Parasitol. Res.* **2018**, *117*, 3015–3018.
161. Pourmohammadi, B.; Mohammadi-Azni, S.; Kalantari, M. Natural Infection of *Nesokia indica* with *Leishmania major* and *Leishmania infantum* Parasites in Damghan City, Northern Iran. *Acta Trop.* **2017**, *170*, 134–139, doi:10.1016/j.actatropica.2017.03.001.
162. Helhazar, M.; Leitão, J.; Duarte, A.; Tavares, L.; da Fonseca, I.P. Natural Infection of Synathropic Rodent Species *Mus musculus* and *Rattus norvegicus* by *Leishmania infantum* in Sesimbra and Sintra-Portugal. *Parasit. Vectors* **2013**, *6*, 1–6.
163. Echchakery, M.; Chicharro, C.; Boussaa, S.; Nieto, J.; Carrillo, E.; Sheila, O.; Moreno, J.; Boumezzough, A. Molecular Detection of *Leishmania infantum* and *Leishmania tropica* in Rodent Species from Endemic Cutaneous Leishmaniasis Areas in Morocco. *Parasit. Vectors* **2017**, *10*, 1–8, doi:10.1186/s13071-017-2398-8.
164. Ibrahim, E.A.; Al-Zahrani, M.A.; Al-Tuwaigri, A.S.; Al-Shammary, F.J.; Evans, D.A. *Leishmania* Infecting Man and Wild Animals in Saudi Arabia. 9. The Black Rat (*Rattus rattus*) a Probable Reservoir of Visceral Leishmaniasis in Gizan Province, South-West Saudi Arabia. *Trans. R. Soc. Trop. Med. Hyg.* **1992**, *86*, 513–514, Available online: https://academic.oup.com/trstmh/article/86/5/513/1922532 (accessed on 25 February, 2021).
165. Zanet, S.; Sposimo, P.; Trisciuoglio, A.; Giannini, G.; Strumia, F.; Ferroglio, E. Epidemiology of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in Absence of Domestic Reservoir and Definitive Hosts. *Vet. Parasitol.* **2014**, *199*, 247–249, doi:10.1016/j.vetpar.2013.10.023.
166. Papadogiannakis, E.; Spanakos, G.; Kontos, V.; Menounos, P.G.; Tegos, N.; Vakalis, N. Molecular Detection of *Leishmania infantum* in Wild Rodents (*Rattus norvegicus*) in Greece. *Zoonoses Public Health* **2010**, *57*, 2009–2011, doi:10.1111/j.1863-2378.2009.01264.x.
167. Pourmohammadi, B.; Motazedian, M.H.; Kalantari, M. Rodent Infection with *Leishmania* in a New Focus of Human Cutaneous Leishmaniasis, in Northern Iran. *Ann. Trop. Med. Parasitol.* **2008**, *102*, 127–133, doi:10.1179/136485908X252223.
168. Akhoundi, M.; Mohebali, M.; Asadi, M.; Mahmodi, M.R.; Amraei, K.; Mirzaei, A. Molecular Characterization of *Leishmania* spp. in Reservoir Hosts in Endemic Foci of Zoonotic Cutaneous Leishmaniasis in Iran. *Folia Parasitol.* **2013**, *60*, 218–224, doi:10.14411/fp.2013.024.
169. Masoumeh, A.; Kourosh, A.; Mohsen, K.; Hossein, M.M.; Qasem, A.; Djaefar, M.F.M.; Esmaeil, N.M.; Tahereh, D. Laboratory Based Diagnosis of Leishmaniasis in Rodents as the Reservoir Hosts in Southern Iran, 2012. *Asian Pac. J. Trop. Biomed.* **2014**, 4, S575–S580, doi:10.12980/APJTB.4.2014APJTB-2014-0199.
170. Nezamzadeh-Ezhiyeh, H.; Mirhendi, H.; Jafari, R.; Veysi, A.; Rassi, Y.; Oshaghi, M.A.; Arandian, M.H.; Abdoli, H.; Bahrami, S.; Ramazani, A.R.Z.; et al. An Eco-Epidemiological Study on Zoonotic Cutaneous Leishmaniasis in Central Iran. *Iran. J. Public Health* **2021**, *50*, 350–359, doi:10.18502/ijph.v50i2.5350.

171. Azizi, K.; Moemenbellah-Fard, M.D.; Fakoorziba, M.R.; Fekri, S. *Gerbillus nanus* (Rodentia: Muridae): A New Reservoir Host of *Leishmania major*. *Ann. Trop. Med. Parasitol.* **2011**, *105*, 431–437, doi:10.1179/1364859411Y.0000000036.
172. Kassahun, A.; Sadlova, J.; Benda, P.; Kostalova, T.; Warburg, A.; Hailu, A.; Baneth, G.; Volf, P.; Votypka, J. Natural Infection of Bats with *Leishmania* in Ethiopia. *Acta Trop.* **2015**, *150*, 166–170, doi:10.1016/j.actatropica.2015.07.024.
173. Rouhani, S.; Mirzaei, A.; Spotin, A.; Parvizi, P. Novel Identification of *Leishmania major* in *Hemiechinus auritus* and Molecular Detection of This Parasite in *Meriones libycus* from an Important Foci of Zoonotic Cutaneous Leishmaniasis in Iran. *J. Infect. Public Health* **2014**, *7*, 210–212]17, doi:10.1016/j.jiph.2013.12.002.
174. Pourmohammadi, B.; Mohammadi-Azni, S. Molecular Detection of *Leishmania major* in *Hemiechinus auritus*, A Potential Reservoir of Zoonotic Cutaneous Leishmaniasis in Damghan, Iran. *J. Arthropod Borne Dis.* **2019**, *13*, 334–343, doi:10.18502/jad.v13i3.1542.
175. Tomás-Pérez, M.; Khaldi, M.; Riera, C.; Mozo-León, D.; Ribas, A.; Hide, M.; Barech, G.; Benyettoub, M.; Seghirib, K.; Doudou, S.; et al. First Report of Natural Infection in Hedgehogs with *Leishmania major*, a Possible Reservoir of Zoonotic Cutaneous Leishmaniasis in Algeria. *Acta Trop.* **2014**, *135*, 44–49, doi:10.1016/j.actatropica.2014.03.018.
176. Gicheru, M.M.; Jeneby, M.M.; Macharia, J.C.; Carlsson, H.E.; Suleman, M.A. Prevalence of Antibodies and Cell Mediated Immune Response against *Leishmania major* in Feral Nonhuman Primates from Kenya. *Acta Trop.* **2009**, *109*, 136–140, doi:10.1016/j.actatropica.2008.09.020.
177. Moemenbellah-Fard, M.D.; Kalantari, M.; Rassi, Y.; Javadian, E. The PCR- Based Detection of *Leishmania major* Infections in *Meriones libycus* (Rodentia: Muridae) from Southern Iran. *Ann. Trop. Med. Parasitol.* **2003**, *97*, 865–873, doi:10.1179/000349803225002651.
178. Najafzadeh, N.; Sedaghat, M.M., Sultan, S.S.; Spotin, A.; Zamani, A.; Taslimian, R.; Yaghoubinezhad, A.; Parvizi, P. The Existence of Only One Haplotype of *Leishmania major* in the Main and Potential Reservoir Hosts of Zoonotic Cutaneous Leishmaniasis Using Different Molecular Markers in a Focal Area in Iran. *Revista Soc. Brasil. Med. Trop.* **2014**, *47*, 599–606, doi:10.1590/0037-8682-0164-2014.
179. Faiman, R.; Abbasi, I.; Jaffe, C.; Motro, Y.; Nasereddin, A.; Schnur, L.F.; Torem, M.; Pratlong, F.; Dedet, J.P.; Warburg, A. A Newly Emerged Cutaneous Leishmaniasis Focus in Northern Israel and Two New Reservoir Hosts of *Leishmania major*. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2058, doi:10.1371/journal.pntd.0002058.
180. Kassahun, A.; Sadlova, J.; Dvorak, V.; Kostalova, T.; Rohousova, I.; Frynta, D.; Aghova, T.; Yasur-Landaud, D.; Lemma, W.; Hailu, A.; et al. Detection of *Leishmania donovani* and *L. tropica* in Ethiopian Wild Rodents. *Acta Trop.* **2015**, *145*, 39–44, doi:10.1016/j.actatropica.2015.02.006.
181. Massamba, N.N.; Mutinga, M.J.; Kamau, C.C. Characterisation of *Leishmania* Isolates from Laikipia District, Kenya. *Acta Trop.* **1998**, *71*, 293–303, doi:10.1016/S0001-706X(98)00071-0.
182. Doha, S.A.; Shehata, M.G.; Fahmy, A.R.; Samy, A.M. Natural and Experimental Evidence of Viscerotropic Infection Caused by *Leishmania tropica* from North Sinai, Egypt. *J. Egypt. Soc. Parasitol.* **2014**, *44*, 425–434, doi:10.12816/0006481.
183. Mukhtar, M.M.; Sharief, A.H.; El Saffi, S.H.; Harith, A.E.; Higazzi, T.B.; Adam, A.M.; Abdalla, H.S. Detection of Antibodies to *Leishmania donovani* in Animals in a Kala-Azar Endemic Region in Eastern Sudan: A Preliminary Report. *Trans. R. Soc. Trop. Med. Hyg.* **2000**, *94*, 33–36, doi:10.1016/S0035-9203(00)90429-2.
184. Tsokana, C.N.; Sokos, C.; Giannakopoulos, A.; Mamuris, Z.; Birtsas, P.; Papaspyropoulos, K.; Valiakos, G.; Spyrou, V.; Lefkaditis, M.; Chatzopoulos, D.C. et al. First Evidence of *Leishmania* Infection in European Brown Hare (*Lepus europaeus*) in Greece: GIS Analysis and Phylogenetic Position within the *Leishmania* spp. *Parasitol. Res.* **2016**, *115*, 313–321, doi:10.1007/s00436-015-4749-8.
185. Morsy, T.A.; Al-Dakhil, M.A.; El-Bahrawy, A.F. Natural *Leishmania* infection in sand cats captured in Riyadh district, Saudi Arabia. J. *Egypt. Soc. Parasitol*. **1999**, *29*, 69–74, PMID: 12561884.
186. Morsy, T.A.; el Shazly, A.M.; el Kady, G.A.; Sabry, A.H.; Handousa, A.A.; Ragheb, D.A.; Kotb, M.M. Natural *Leishmania* infections in two stray dogs and two *Gerbillus pyramidum* in Dakahlia Governorate, Egypt. *J. Egypt Soc. Parasitol*. **1994**, *24*, 383–394.

Wildlife as a sentinel for pathogen introduction in non-endemic areas: Is *Leishmania tropica* circulating in wildlife in Spain?

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Abstract

Background

Leishmaniasis is a global arthropod-borne zoonotic disease of chronic course and cutaneous, mucocutaneous, and visceral clinical manifestations. In the Iberian Peninsula, only *Leishmania infantum* has been reported, although other species of *Leishmania*, such as *L. tropica* and *L. major*, are present in surrounding countries.

Aim

The aim of this work is to analyse the occurrence of *Leishmania* spp. infection in wildcats as sentinels, including their genotypic characterization.

Methods

Necropsies of 18 roadkill wildcats were carried out. Samples from ear skin and spleen were taken, and DNA isolation and PCR analysis of the highly sensitive *SSUrDNA* target were conducted. Subsequent PCR tests were conducted using more specific targets for *Leishmania* species determination: *hsp70* and *ITS1*Positive samples were sequenced, and phylogenetic trees were constructed.

Results

Seven out of 18 wildcats were found positive to *Leishmania* spp. in ear skin; of them, only two were positive in spleen samples. . Based on the *hsp70* and *ITS1* sequences, one animal was found to be infected solely with *L. tropica* in ear skin samples, while two cats were found to be infected with *L. infantum* in both ear and spleen. Another animal was found to be co-infected with *L.*

infantum and *L. tropica* in ear skin. The *Leishmania* species could not be determined in three cats as the targeted *SSUrDNA* is only specific to the genus.

Conclusions

This is the first report of autochthonous *L. tropica* infection in the Iberian Peninsula. Health care professionals, including physicians, dermatologist and veterinarians must be aware of this for a correct diagnosis, treatment, and management of possible co-infections.

Introduction

Leishmaniasis is a global zoonotic disease transmitted by the bite of phlebotomine insects, with more than 1,000,000 new cases diagnosed annually in 92 endemic countries [(1)]. Clinical presentation in humans is variable and includes cutaneous (CL), mucocutaneous (MCL), visceral (VL), and Post Kala-azar dermal leishmaniasis (PKDL). The disease is caused by several species of *Leishmania*, with *L. donovani* complex, *L. major* and *L. tropica* complex being the most frequent in Africa, Asia, southern Europe, South America, and the Middle East, while *L. mexicana* complex, *L. guyanensis* complex and *L. braziliensis* complex are the most diagnosed in the Americas. The cutaneous form of the disease is the most common and is responsible of the stigmatization and social isolation of thousands of people, particularly women, while VL is usually lethal without treatment and is caused by *L. donovani* in Africa and Europe, and *L. infantum* in southern Europe and the Americas. MCL is characterized by the destruction of mucous tissue of the nose, palate, and pharynx, and can severely affect the daily life of thousands of people [(2,3)]. Human leishmaniasis is more prevalent in low-income countries with displaced populations due to war, social or economic conflicts and is classified by WHO as a neglected tropical disease (NTD). This scenario makes early diagnosis and treatment of the disease, as well as the introduction of preventive measures, difficult (3)].

In Europe, VL is caused by *L. infantum* with both humans and dogs as target species. The infection is more prevalent in southern countries, such as Portugal, Spain, Italy, and Greece. Dogs are heavily affected by this zoonotic species [(4)] and are considered the main reservoir. Human infections mainly affect immunocompromised patients (such as those HIV+ or recipients of solid organs transplants) as well as occasional reports in elderly and children [(5)]. Several studies have stressed the paramount importance of wildlife in maintaining *Leishmania* infection [6,7]. Wildcats (*Felis silvestris*) are susceptible to the same pathogens as domestic cats and therefore could be potential reservoirs for the infection. However, studies conducted have been scarce [8–10], with a limited number of animals analysed in each study (n=4, n=3, n=4, respectively). The three studies identified *L. infantum* using a small fragment of kDNA or qPCR of the ITS2 regions.

Despite suitable bioclimatic conditions and available vectors for the anthroponotic *L. tropica* (*Phlebotomus sergenti*) and *L. major* (*P. papatasi*) [11,12], only *L. infantum* has been reported in the Iberian Peninsula [6,12,13]. However, *L. major* has been recently reported in a cat from Lisbon, Portugal [14]. Moreover, it is worth noting that *L. tropica* has been previously reported in Greece [15] and the three species (*L. infantum, L. tropica* and *L. major*) are present in Morocco [16], as well as in other countries bordering Europe [17]. Availability of a larger number of wildcats allowed us to identify the presence of *Leishmania* in various tissues and genetically characterize the samples.

Material and methods

Animals, samples, and geographic area

Samples from 18 wild cats that were road killed during 2022 and 2023 in the Spanish provinces of Burgos, Valladolid, Soria, Ávila, Guadalajara, and Ciudad Real were analysed in this study.

The animals were transported to the wildlife recovery centres of Castilla y León and Castilla-La Mancha, systematic necropsies were performed, and the presence/absence of lesions annotated. A portion of the ear skin and the spleen were taken and frozen at -20°C for further processing. Date, age, sex, and locality (geographic coordinates where the dead animals were found) were included in the data base.

Maps

The map was created by taking as reference the maps of distribution in Europe of *Phlebotomus sergenti*, the main vector of *L. tropica*, in 2022 and 2023 [18], and the geographic locations of the wildcats analysed in this study. The AutoCAD program (Autodesk® Inc., the Landmark, San Francisco, CA, USA) was employed to observe the overlaps between the two maps.

DNA isolation

DNA was isolated from spleen (10 mg) and ear skin (20 mg) using the NZYTech Tissue gDNA isolation kit (NZYTech, Portugal) and following the manufacturer's instructions . DNA elution was conducted in 60 µL of elution buffer instead of 100 µL to increase DNA concentration. Positive and negative controls were used in each batch of the experiment. The positive control consisted of samples derived from *L. major* (clone V1: MHOM/IL/80/Friedlin) or *L. braziliensis* (MHOM/BR/75/M2904) promastigotes in culture ($1x10^6$ promastigotes), while the negative control consisted of DNase-free water. DNA content was measured in a Multiskan™ GO microplate spectrophotometer with µDrop™ plates (Thermo Fisher Scientific, MA, USA). DNA from cultures of *L. major* and *L. braziliensis* were obtained and used as internal controls for PCRs and sequencing.

PCR of Small subunit ribosomal RNA (SSU-rDNA), Internal Transcribed spacers-5.8 ribosomal DNA 1 (ITS1), and heat shock protein 70 (hsp70) targets

DNA samples from ear skin and spleen of the 18 cats were used for *Leishmania* DNA detection. DNA from different *Leishmania* species was also used as an internal control for PCR and sequencing: *L. major* (clone V1: MHOM/IL/80/Friedlin) and *L. braziliensis* (MHOM/BR/75/M2904). Only samples with good DNA quality (absorbance ratio at 260/280 nm > 1.7) were used for all PCRs. All methods have been previously described and adapted to the working conditions recommended by the enzyme manufacturer (Supreme NZYTaq II 2x Master Mix, NZYTech, Lisbon, Portugal).

Nested PCRs (nPCR) to amplify a 358 bp fragment of the *SSU-rDNA* target were performed using primers R221 (5´- GGTTCCTTTCCTGATTTACG-3´) and R332 (5´- GGCCGGTAAAGGCCGAATAG-3´) for the outer section, and primers R223 (5´- TCCATCGCAACCTCGGTT-3´) and R333 (5´- AAAGCGGGCGCGGTGCTG-3´) for the inner section [19], following previously described conditions [20].

Primers L5.8S (5´- ACACTCAGGTCTGTAAAC-3´) and LITSR (5´- CTGGATCATTTTCCGATG-3´) were used for the outer section of the *ITS1* region and primers SAC (5´- CATTTTCCGATGATTACACC-3)´and VAN2 (5´- CGTTCTTCAACGAAATAGG -3´) were used for the inner section to amplify a fragment of 280-330 bp, as previously described [20,21].

The nPCR used for *hsp70* amplified a fragment of 741 bp and was based on the primers and methods previously described for phylogenetic purposes [14,22]. Briefly, external primers HSP70-F25 (5´-GGACGCCGGCACGATTKCT-3´) and HSP70-R1310 (5´-CCTGGTTGTTGTTCAGCCACTC-3´) were used at 0.8 µM each , and internal primers HSPF (5´-GACAACCGCCTCGTCACGTTC-3´) and HSPR (5´-GTCGAACGTCACCTCGATCTGC-3´) were used at 0.4 µM each. In the first PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s and 72°C for 120 s were used, while in the second PCR, 40 cycles of 94°C for 40 s, 61°C for 60 s and 72°C for 60 s were used.

All PCR reactions were performed in 25 µL using 12.5 µL of Supreme NZYTaq II 2x Master Mix. An initial step of 95°C for 5 min to activate the enzyme, and a final elongation step of 72°C for 10 min to allow the elongation of PCR products were employed in all PCR reactions. In the first PCR, 5 µL of DNA was used, while in the second PCR reactions 5 µL of a 1:50 dilution from each first PCR product were employed for *hsp70*, while a 1:40 dilution of the first PCR product was used for SSU-rRNA and ITS1.

The results of the nPCR were visualized in 1% agarose gels stained with SYBR™ safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, MA, USA) under UV light.

Sequencing and alignment

Samples containing the amplicons of the expected size were sent to the facilities of Macrogen Spain (Madrid, Spain)for Sanger sequencing. Samples were purified and sequenced using a DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD) and the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), following the manufacturer's instructions. The resulting sequences were aligned using Molecular Evolutionary Genetics Analysis v.11 software (MEGA XI) [(23)] and FinchTV 1.4.0 software (Geospiza, Inc.; Seattle, WA, USA), manually checked, and subjected to BLAST analysis using the nucleotide Basic Local Alignment Search Tool (BLAST) (National Library of Medicine, Rockville, MD, USA).

Only sequences with more than 150 bp that were clear in both directions were sent to GenBank for accession numbers.

Phylogenetic trees

Phylogenetic trees were constructed using the sequences obtained in the present study and other sequences available at GenBank. Trees for *hsp70* and *ITS1* are shown because *SSU-rDNA* target is a conserved region and only a difference of 1-2 bp was observed between the sequences obtained.

In all trees, a sequence from *Trypanosoma cruzi* was included as an out-group reference. In addition, other sequences of *Leishmania* spp. from GenBank and sequences from *L. infantum* and the above-mentioned species of *Leishmania* from cultures were included to verify similarity (*L. major,* and *L. braziliensis*). Sequences had a minimum length of 236 nucleotides for *ITS1* region and 635 nucleotides for *hsp70*. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura–Nei model [24]. The tree with the highest log likelihood was displayed. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Neighbour-Joining and BioNJ algorithms to a pairwise distance matrix estimated using the Maximum Composite Likelihood (MCL) approach and selecting the topology with the superior log likelihood value. Trees were drawn to scale, with branch lengths measured based on the number of substitutions per site. The analysis included 13-18 nucleotide sequences,

depending on the tree. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted via MEGA XI [23]. A bootstrap of 2000 replicates was used to assess the reliability of the trees.

Statistical analysis

A descriptive statistical analysis with absolute (n) and relative frequency of infection (%) was carried out, with a confidence level of 95%, using the free online tool WinEpi (Working in Epidemiology) and considering that the diagnostic techniques employed were perfect (nPCR of the *SSUrDNA*). Odds ratio analysis was used to analyse the risk factors "sex" and "*Leishmania* infection" [25].

Results

Detection and identification of Leishmania *spp. in wild cat*

Samples from ear skin and spleen of eighteen wildcats have been analysed in this study, except for the spleen of two animals that were in bad condition when the cats were found (Table 1). None of the animals displayed lesions comparable to those produced by *Leishmania* infections. Considering all the animals positive for *Leishmania* PCR, 38.89% (16.37-61.41%, 95% Confidence interval) were found to be infected. A non-significant higher proportion of infection was found in males (OR= 27.17, 95% CI 0.33, 2213.63), since only males were found infected, but we must consider that we obtained a smaller number of samples from females (n=3/18).

Table 1. Age, sex, geographical location, date of collection and *Leishmania* infection of the studied wildcats.

	Age	Sex	Date	Location	*Leishmania* infection
262	Adult	M	20-04-2022	Villagonzalo Pedernales (Burgos)	+
263	Adult	M	21-04-2022	Bonilla de la Sierra (Ávila)	+
268	Adult	M	22-06-2022	Sedano (Burgos)	+
272	Adult	M	13-07-2022	Cogeces de Íscar (Valladolid)	-
275	Adult	M	15-07-2022	Berlangas de Roa (Burgos)	+
276	Adult	M	15-07-2022	Torreblancos (Soria)	-
310	Adult	M	21-12-2022	Almazán (Soria)	-
317	Adult	M	24-02-2023	Temiño (Burgos)	-
318 *	Adult	M	13-03-2023	Castrillo de Riopisuerga (Burgos)	+
319	Adult	F	13-03-2023	Hermosilla (Burgos)	-
320	Adult	M	23-03-2023	Cubo de la Solana (Soria)	-
323	Adult	M	28-04-2023	Adradas (Soria)	+
365	Adult	M	08-09-2023	Cascajares de Bureba (Burgos)	+
366	Adult	M	08-09-2023	Martialay (Soria)	-
367	Adult	F	08-09-2023	Retuerta (Burgos)	-
375	Adult	M	05-10-2023	Fuencaliente (Ciudad Real)	-
376	Adult	F	05-10-2023	Pedrosa del Príncipe (Burgos)	-
378 *	Adult	nd	05-10-2023	Peralejo (Guadalajara)	-

*Spleen not analyzed. M: male; F: female. nd: not determined.

Seven of the eighteen animals were found positive by *Leishmania* PCR for SSU-rDNA. All were positive at the ear skin, and only two were also positive in spleen samples (Table 2).

Table 2. Results of end-point PCR using samples from ear skin and spleen and three targets for *Leishmania* DNA amplification. Sequences identities are identified with super index numbers (1-4).

Sample	Ear skin			Spleen		
	SSU-rDNA	*ITS1*	*hsp70*	*SSU-rDNA*	*ITS1*	*hsp70*
262	+	-	-	-	-	-
263	+	+[2]	+[4]	+	+[2]	+[4]
268	+	+[2]	+[3]	-	-	-
275	+	+[1]	+[3]	-	-	-
318	+	-	-	nd	nd	nd
323	+	+[2]	+[4]	+	+[2]	+[4]
365	+	-	-	-	-	-

nd: not determined. 1: *ITS1* sequence 100% identity with *L. tropica*; 2: *ITS1* sequence 100% identity with *L. infantum*; 3: *hsp70* sequence 99.86-100% identity with *L. tropica*; 4: *hsp70* sequence 99.24-100% identity with *L. infantum.*

Sequences and GenBank accession numbers

Nucleotide sequences from wildcats obtained in this study were deposited in the GenBank database under accession numbers PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. Sequences from *L. major* and *L. braziliensis* isolates were deposited in the GenBank database with accession numbers PP389522, PP389523, and PP389533.

Sequences obtained from *SSU-rDNA* had 295-327 bp and differ by only 1 or 2 nucleotide positions. Nine sequences were obtained from the analysed animals, seven from the ear skin and two from the spleen. Seven of the obtained sequences displayed 100% query coverage and 99.69-100% identity with other *L. infantum* sequences available at GenBank (acc. n MN757921, obtained from *Canis lupus*, Brazil). Sequences obtained from the ear skin of animals 365 and 323 showed 100% query coverage and 99.69-100% identity, respectively, with sequences from *L. major*, *L. enrietti, L. infantum, L. mexicana, L. guyanensis* or *L. tropica* (acc. n MT560279, MK737948, MK495994, KM061042, X53913, KF302745), being identical except one nucleotide.

ITS1 sequences of 236-247 bp were obtained from the ear skin of four animals (263, 268, 275, 323), two of which (263 and 323) also gave sequences when DNA from the spleen was used (Table 2). Three sequences displayed 100% query coverage and 100% identity with sequences from *L. infantum* (acc. n MT416168, samples from ear skin of animals 263, 268, and 323, and samples from spleen of animals 263 and 323), while one sequence (from ear skin of wildcat 275) displayed 100% query coverage and 100% identity with sequences from *L. tropica* (acc. n MH595857, isolate R24, Iran).

Sequences obtained from *hsp70* amplification yielded four sequences from the ear skin and two sequences from the spleen with 635-711 bp. Of these, four sequences showed 100% coverage and 99.24-100% identity with *L. infantum* (acc. n OR136937, strain MHOM/IT/99/ISS1898). The other two sequences (268 and 275 from ear skin) displayed 100% coverage and 99.86 and 100% identity, respectively, with *L. tropica* (acc. n LN907846, strain MHOM/IL/80/SINGER, and MK335938, voucher ISS3183) (Table 2). Double peaks were observed in three and five positions of sequences from the spleen of animal 323 and ear skin of animal 263, respectively, indicating mixed sequences.

Considering all sequences obtained with *SSUrDNA*, *ITS1* and *hsp70* targets, *L. tropica* tends to be better amplified in samples from ear skin than in samples from spleen. In one animal (275) sequences from the *ITS1* and *hsp70* targets showed 100% identity with *L. tropica*. Two animals displayed sequences compatible with *L. infantum* at ear skin and spleen (263 and 323), and another wildcat was positive only at ear skin with sequences compatible with *L. infantum* and *L. tropica* (268). Finally, in three animals (262, 318 and 365) the sequences could only by assigned to *Leishmania* genus because they were only positive for *SSUrDNA* (Table 2).

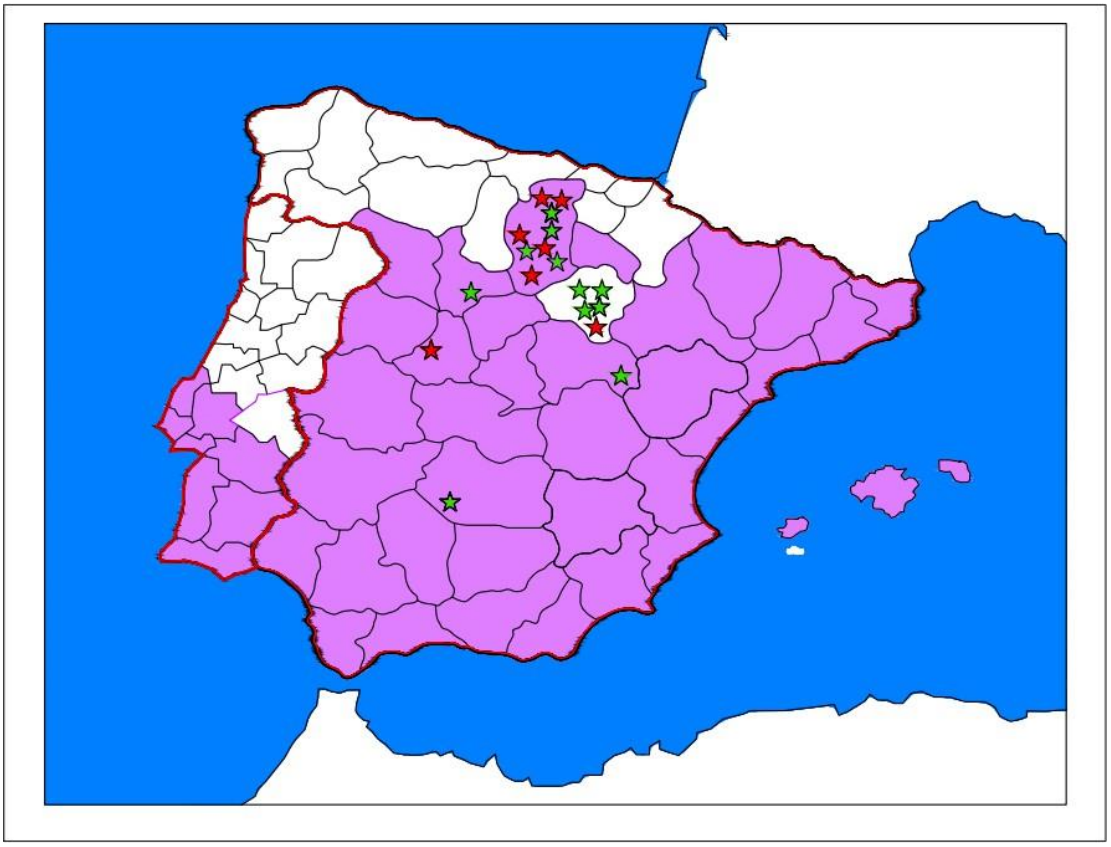

Figure 1. Map of the Iberian Peninsula. Fuchsia coloured area corresponds to the distribution of *Phlebotomus sergenti* in 2022 and 2023. Red stars indicate the detection and identification of *Leishmania* spp. by *hsp70* and *ITS1*sequences. Green stars indicate the absence of *Leishmania* spp. in the sampled cats.

Phylogenetic trees

Phylogenetic analysis of the sequences shows strong support for the clusters found with the two targets, in agreement with the percentage of identity of the obtained sequences. *ITS1* sequences from this study were grouped with *L. infantum* (samples 263, 268 and 323 from ear skin and samples 263 and 323 from spleen) or *L. tropica* (sample 275) (Figure 2).

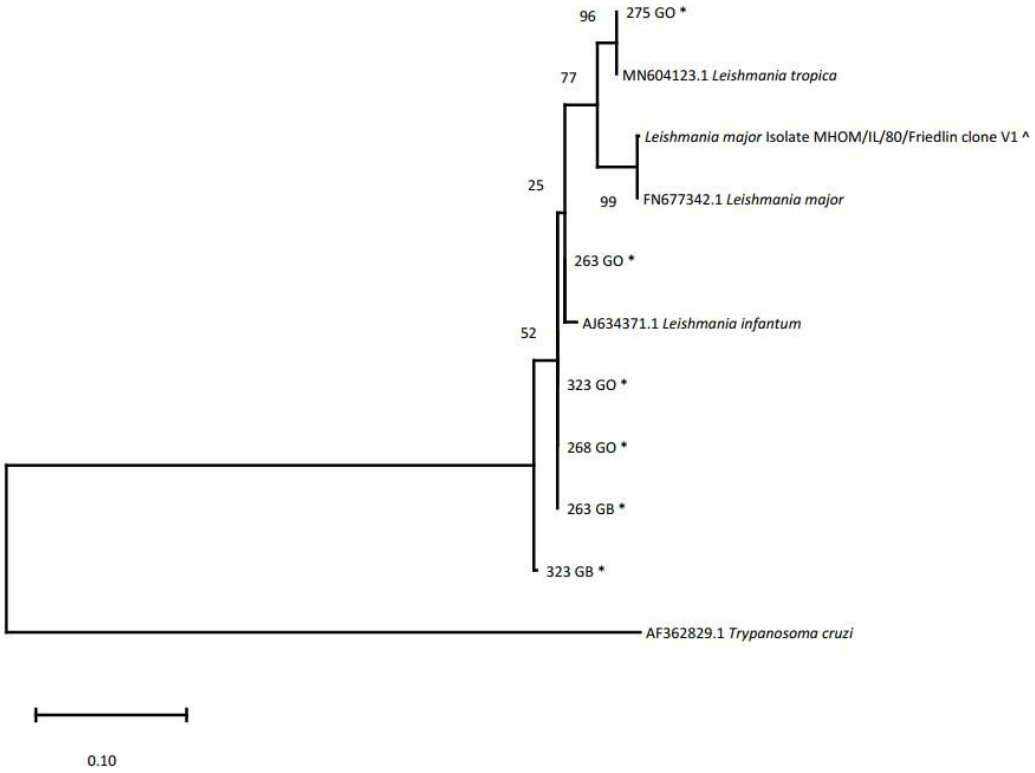

Figure 2. Phylogenetic tree of the *ITS1* region. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-841.79) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. There were a total of 292 positions in the final dataset. *****: Sequences from positive samples of this study. **^**: Sequences from isolates sequenced in the present study. GB: sample from spleen. GO: sample from ear skin.

In the phylogenetic tree generated with *hsp70* sequences obtained in the present study, two of them (samples 268 and 275 from ear skin) clustered with *L. tropica* and three of them (samples 263 and 323 from ear skin and 263 spleen) clustered with *L. infantum* sequences retrieved from GenBank (Figure 3). One of the *L. tropica* sequences was obtained from the same animal (275) that rendered *L. tropica ITS1* sequence, while the other *L. tropica hsp70* sequence was obtained from an animal (268) that rendered *L. infantum ITS1* sequence.

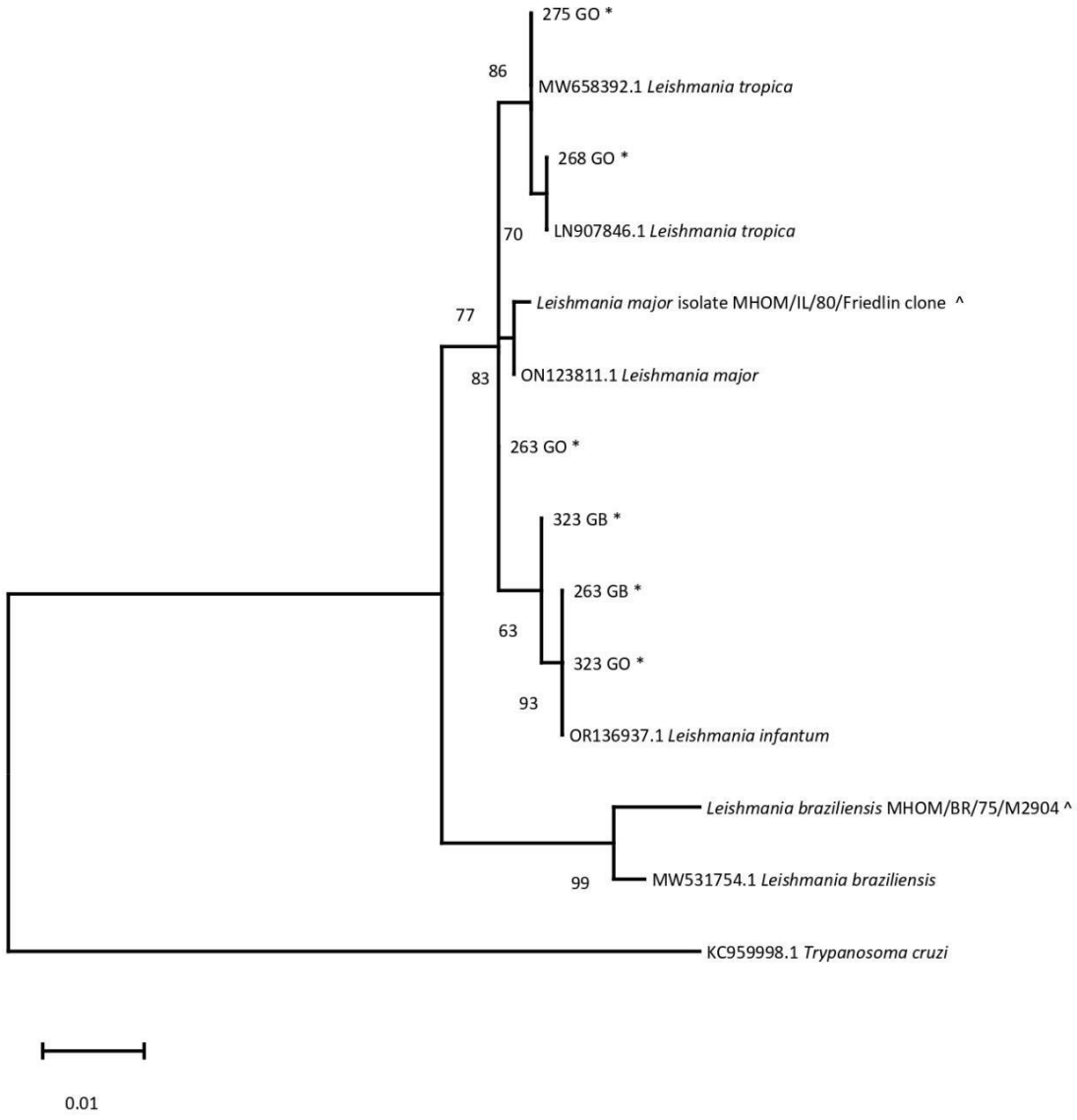

Figure 3. Phylogenetic tree of the *hsp70* region. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log-likelihood (-1328.37) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 nucleotide sequences. There were a total of 635 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. *****: Sequences obtained from positive samples of this study. **^**: Sequences from isolates sequenced in the present study. GB: sample from spleen. GO: sample from ear skin.

Discussion

In the present study, seven out of 18 wildcats (38.89%) were found positive to *Leishmania* infection, a value consistent with those found in previous studies [8,10] although lower than the infection prevalence reported by Oleaga et al. [9] with all three wildcats tested being positive for *Leishmania*. *L. infantum* is endemic in the Iberian Peninsula [12,13], but no infections of *L. tropica*

in autochthonous species have been reported. The risk of introduction of *L. tropica* in the Iberian Peninsula has been highlighted several times, considering the presence and distribution of the main vector, *P. sergenti*, and the identification in Spain of one genetic line of *P. sergenti* commonly found in Morocco [26,27] where CL by *L. tropica* is endemic [21,28,29]. Large areas in Europe are considered suitable for *L. tropica* [11] and hot spots for foci of *L. tropica* have been suggested [26].

The most common targets employed for detection of *Leishmania* spp. infection are *SSU-rDNA* and kDNA, with similar values of sensitivity [6]. However other targets are useful for phylogenetic studies and determination of *Leishmania* spp., such as *ITS1*, *cytB*, *g6pdh*, and *hsp70* [14,20–22,28,30,31]. In the present study, *ITS1* and *hsp70* have been employed to confirm the finding of *L. tropica* in autochthonous fauna, and sequences of *L. tropica* with *ITS1* and *hsp70* targets are reported. Sequences of *L. tropica* were detected in at least two wildcats positive to PCR with *hsp70* and/or *ITS1* targets, widely employed for phylogenetic studies. Our finding extends the reported presence of the species in Greece [32] and extends its distribution to the Iberian Peninsula for the first time.

Isolation of alive *Leishmania* was not feasible considering the source of material but it is also noteworthy than in our study ear skin samples displayed more positive results than spleen samples (n=7/18 *vs*. n=2/16). This finding is consistent with the fact that *L. tropica* infections are associated mainly with cutaneous clinical forms in humans, where parasite persistence is close to the site of natural infections transmitted by the sandfly, although the parasite can be also found less frequently associated with VL, mainly in immune suppressed people [33]. Imported cases of CL by *L. tropica* have been sporadically reported in Spain after travelling to Morocco [34] but no previous autochthonous cases have been reported in Spain or Portugal. Apparently, *L. tropica* is expanding its distribution and the main risk factor for the appearance of the disease is the presence of the vector [35]. It should be pointed out that some of the wildcats came from latitudes over 42º N (*e.g.* Pedrosa del Príncipe, N42°14'56.8") and during the last decade, *P. sergenti* has expanded to northern areas of the Iberian Peninsula, according to ECDC maps of distribution [18].

In endemic areas, such as Israel, domestic cats appear to be more susceptible to infection with *L. tropica* than dogs [36]. A study of stray cats in Turkey showed that all samples with *ITS1* amplification were infected with *L. tropica*, while only one was co-infected with *L. infantum* [37].Similarly, in the present study, while one animal showed infection by *L. tropica* only, another wildcat showed *hsp70* and *ITS1* sequences consistent with coinfection by *L. infantum* and *L. tropica*. Mixed infection with more than one *Leishmania* species have also been reported in other endemic areas, hosts and *Leishmania* species, such as in Brazil (*L. infantum* and *L. braziliensis*) in one dog [38] and 8/30 dogs [39], rodents (10% of *L. infantum* and *L. braziliensis*) [40], hedgehogs (*L. major* and *L. infantum*) in 8/12 animals and another co-infected with *L. tropica* [41], and human clinical cases [[42,43] of the New World. The possibility of hybridization between *Leishmania* species cannot be ruled out as *L. tropica* shows a higher rate of natural hybrid formation than other species [44]. However, to further investigate the existence of hybrids, isolates from infected animals are necessary [45,46], and in our study, isolates from these animals could not be obtained.

Conclusions

This is the first report of *L. tropica* infection in autochthonous wildlife in the Iberian Peninsula thus extending the presence of the species in continental Europe. The main vector of *L. tropica*

has been identified in Spain during the last decade and its distribution has increased northwards each year. There are no conclusive data on the zoonotic transmission of *L. tropica* and the actual spread of the infection to other species is not yet known and should be investigated. Our results support the sentinel value of wild species in the detection of not-previously reported infections. Moreover, the presence of *L. tropica* in Spain should be communicated to health professionals, including physicians, dermatologists, and veterinarians, considering that *L tropica* can produce both CL and VL in humans and pets. Awareness on the possibility of *L. tropica* infections and *L. infantum*/*L. tropica* coinfections is necessary to ensure appropriate diagnosis and management of clinical cases.

Ethical statement: The use of the samples for parasitological investigations was approved by regional authorisation of the Junta de Castilla y León, reference: "AB/is", File "AUES/CYL/001/2021" and of Castilla-La Mancha, reference: DGPFEN/SEN/avp_21_103_bis.

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Data availability: Sequences obtained in the present study have been deposited at GenBank Database under accession numbers: PP389513-PP389521 for *SSU-rDNA*, PP177368-PP177373 for *ITS1*, and PP397157-PP397162 for *hsp70*. Sequences of the *L. major* and *L. braziliensis* isolates are PP389522, PP389523, PP389533.

Ethical Committee

The employment of the samples for parasites investigation has been approved by permits of Junta de Castilla y León, reference: "AB/is", File "AUES/CYL/001/2021" and of Castilla-La Mancha, reference: DGPFEN/SEN/avp_21_103_bis.

Conflict of interest: None.

Authors' contributions:
MMB, IAC & MTG-M: conceptualization; IAC, PPM & MTG: methodology and formal analysis; MMB, PM & JC: collection of data and biological samples; IAC, MTG-M: writing the original draft; PMM, JC, IAC, MMB, MTG-M & JMA: interpretation of data; MMB & MTG-M: supervision; JMA, MMB & MTG-M: resources. All authors critically reviewed the manuscript, intellectually contribute to the content, and approved the final version.

References

1. https://www.who.int/health-topics/leishmaniasis#tab=tab_1 [Internet]. WHO: health-topics/leishmaniasis.

2. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis. 2016;10(3):1–40.

3. https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html [Internet]. 2023. WHO: neglected diseases, leishmaniasis.

4. Montoya A, Gálvez R, Checa R, Sarquis J, Plaza A, Barrera JP, et al. Latest trends in *L. infantum* infection in dogs in Spain, Part II: Current clinical management and control according to a national survey of veterinary practitioners. Parasit Vectors. 2020 Apr 21;13(1).

5. Zacarias DA, Rolão N, de Pinho FA, Sene I, Silva JC, Pereira TC, et al. Causes and consequences of higher *Leishmania infantum* burden in patients with kala-azar: a study of 625 patients. Tropical Medicine and International Health. 2017 Jun 1;22(6):679–87.

6. Azami-conesa I, Gómez-Muñoz MT, Martínez-Díaz RA. A systematic review (1990–2021) of wild animals infected with zoonotic *Leishmania*. Vol. 9, Microorganisms. MDPI AG; 2021.

7. Cardoso L, Schallig H, Persichetti MF, Pennisi MG. New Epidemiological Aspects of Animal Leishmaniosis in Europe : The Role of Vertebrate Hosts Other than Dogs. 2021;1–46.

8. Del Río L, Chitimia L, Cubas A, Victoriano I, De la Rúa P, Gerrikagoitia X, et al. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. Prev Vet Med. 2014;113(4):430–5.

9. Oleaga A, Zanet S, Espí A, Raquel M, Macedo P De, Gortázar C, et al. *Leishmania* in wolves in northern Spain : A spreading zoonosis evidenced by wildlife sanitary surveillance. Vet Parasitol [Internet]. 2018;255(January):26–31. Available from: https://doi.org/10.1016/j.vetpar.2018.03.015

10. Risueño J, Ortuño M, Pérez-Cutillas P, Goyena E, Maia C, Cortes S, et al. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet Parasitol. 2018 Aug 15;259:61–7.

11. Cunze S, Kochmann J, Koch LK, Hasselmann KJQ, Klimpel S. Leishmaniasis in Eurasia and Africa: Geographical distribution of vector species and pathogens. R Soc Open Sci. 2019 May 1;6(5).

12. Berriatua E, Pérez-Cutillas P, González Vidal A, Briët O. European Centre for Disease Prevention and Control. The spatial relationship between the presence and absence of *Leishmania* spp. and leishmaniasis, and phlebotomine sand fly vectors in Europe and neighbouring countries. 2023; Available from: www.ecdc.europa.eu

13. Gálvez R, Montoya A, Cruz I, Fernández C, Martín O, Checa R, et al. Latest trends in *Leishmania infantum* infection in dogs in Spain, Part I: Mapped seroprevalence and sand fly distributions. Parasit Vectors. 2020 Apr 21;13(1).

14. Pereira A, Parreira R, Cristóvão JM, Castelli G, Bruno F, Vitale F, et al. Phylogenetic insights on *Leishmania* detected in cats as revealed by nucleotide sequence analysis of multiple genetic markers. Infection, Genetics and Evolution. 2020 Jan 1;77.

15. Frank K HMPFGARJ. *Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. Trans R Soc Trop Med Hyg. 1993;87(2):184–5.

16. Ait Kbaich M, Mhaidi I, Ezzahidi A, Dersi N, El Hamouchi A, Riyad M, et al. New epidemiological pattern of cutaneous leishmaniasis in two pre-Saharan arid provinces, southern Morocco. Acta Trop. 2017 Sep 1;173:11–6.

17. Tunalı V, Özbilgin A. Knock, knock, knocking on Europe's door: Threat of leishmaniasis in Europe with a focus on Turkey. Vol. 4, Current Research in Parasitology and Vector-Borne Diseases. Elsevier B.V.; 2023.

18. https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/phlebotomine-maps [Internet]. 2023. European Centre for Disease Prevention and Control and European Food Safety Authority. Phlebotomine sandflies maps [internet]. Stockholm: ECDC; 2022, 2023. Available from:

19. Cruz I, Cañavate C, Rubio JM, Morales MA, Chicharro C, Laguna F, et al. Diagnosis A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring *Leishmania infantum* infection in patients co-infected with human immuno-deficiency virus. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2002;S185–9.

20. Azami-Conesa I, Sansano-Maestre J, Martínez-Díaz RA, Gómez-Muñoz MT. Invasive species as hosts of zoonotic infections: The case of American mink (*Neovison vison*) and *Leishmania infantum*. Microorganisms. 2021 Jul 1;9(7).

21. Echchakery M, Chicharro C, Boussaa S, Nieto J, Carrillo E, Sheila O, et al. Molecular detection of *Leishmania infantum* and *Leishmania tropica* in rodent species from endemic cutaneous leishmaniasis areas in Morocco. Parasit Vectors. 2017;10(1):1–8.

22. Van Der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Esbroeck M Van, et al. Heat-shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics [Internet]. Available from: www.eurosurveillance.orghttp://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20543

23. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021 Jul 1;38(7):3022–7.

24. Tamura K& NM (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512–526. Mol Biol Evol. 1993;10:512–26.

25. Ignacio de Blas. http://www.winepi.net/. Win Epi: Working in Epidemiology.

26. Barón SD, Morillas-Márquez F, Morales-Yuste M, Díaz-Sáez V, Gállego M, Molina R, et al. Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in south-western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917. Parasitology. 2013;140(11):1413–21.

27. Merino-Espinosa G, Corpas-López V, Callejón-Fernández R, Porcel-Rodríguez L, Díaz-Sáez V, Gállego M, et al. Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and their epidemiological implications. Tropical Medicine and International Health. 2016 May 1;21(5):630–41.

28. El Kacem S, Ait Kbaich M, Daoui O, Charoute H, Mhaidi I, Ejgha R, et al. Multilocus sequence analysis provides new insight into population structure and genetic diversity of *Leishmania tropica* in Morocco. Infection, Genetics and Evolution. 2021;september:104932.

29. Saik IEI, Benlabsir C, Fellah H, Lemrani M, Riyad M. Transmission patterns of *Leishmania tropica* around the Mediterranean basin: Could Morocco be impacted by a zoonotic spillover? Vol. 16, PLoS Neglected Tropical Diseases. Public Library of Science; 2022.

30. Mohammadpour I, Hatam GR, Handjani F, Bozorg-Ghalati F, Pourkamal D, Motazedian MH. *Leishmania* cytochrome b gene sequence polymorphisms in southern Iran: Relationships with different cutaneous clinical manifestations. BMC Infect Dis. 2019 Jan 29;19(1).

31. Fernández-Arévalo A, Ballart C, Muñoz-Basagoiti J, Basarte L, Lobato G, Arnau A, et al. Autochthonous and imported tegumentary leishmaniasis in Catalonia (Spain): Aetiological evolution in the last four decades and usefulness of different typing approaches based on biochemical, molecular and proteomic markers. Transbound Emerg Dis. 2022 May 1;69(3):1404–18.

32. Frank C HMPFGARJ. *Leishmania tropica* and *Leishmania infantum* responsible for cutaneous leishmaniasis in Greece: sixteen autochthonous cases. Trans R Soc Trop Med Hyg. 1993;87(2).

33. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Vol. 27, Medical and Veterinary Entomology. 2013. p. 123–47.

34. Knöpfel N, Noguera-Morel L, Azorin D, Sanz F, Torrelo A, Hernández-Martín A. Cutaneous *Leishmania tropica* in children: report of three imported cases successfully treated with liposomal amphotericin B. Vol. 32, Journal of the European Academy of Dermatology and Venereology. Blackwell Publishing Ltd; 2018. p. e8–10.

35. Gijón-Robles P, Abattouy N, Merino-Espinosa G, El Khalfaoui N, Morillas-Márquez F, Corpas-López V, et al. Risk factors for the expansion of cutaneous leishmaniasis by *Leishmania tropica*: Possible implications for control programmes. Transbound Emerg Dis. 2018 Dec 1;65(6):1615–26.

36. Baneth G, Nachum-Biala Y, Adamsky O, Gunther I. *Leishmania tropica* and *Leishmania infantum* infection in dogs and cats in central Israel. Parasit Vectors. 2022 Dec 1;15(1).

37. Can H, Döşkaya M, Özdemir HG, Şahar EA, Karakavuk M, Pektaş B, et al. Seroprevalence of Leishmania infection and molecular detection of *Leishmania tropica* and *Leishmania infantum* in stray cats of I˙zmir, Turkey. Exp Parasitol. 2016 Aug 1;167:109–14.

38. Madeira MF, Schubach A, Schubach TMP, Pacheco RS, Oliveira FS, Pereira SA, et al. Mixed infection with *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *chagasi* in a naturally infected dog from Rio de Janeiro, Brazil. Trans R Soc Trop Med Hyg. 2006 May;100(5):442–5.

39. Alves Souza N, Souza Leite R, de Oliveira Silva S, Groenner Penna M, Figueiredo Felicori Vilela L, Melo MN, et al. Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil. Acta Trop. 2019 May 1;193:12–7.

40. Ferreira E de C, Cruz I, Cañavate C, de Melo LA, Pereira AAS, Madeira FAM, et al. Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World. BMC Vet Res. 2015 Mar 20;11(1).

41. Souguir-Omrani H, Chemkhi J, Fathallah-Mili A, Saadi-BenAoun Y, BelHadjAli I, Guizani I, et al. *Paraechinus aethiopicus* (Ehrenberg 1832) and *Atelerix algirus* (Lereboullet 1842) hedgehogs: Possible reservoirs of endemic leishmaniases in Tunisia. Infection, Genetics and Evolution. 2018 Sep 1;63:219–30.

42. Shirian S, Oryan A, Hatam GR, Daneshbod Y. Mixed mucosal leishmaniasis infection caused by *Leishmania tropica* and *Leishmania major*. J Clin Microbiol. 2012 Nov;50(11):3805–8.

43. Badirzadeh A, Mohebali M, Sabzevari S, Ghafoori M, Arzamani K, Seyyedin M, et al. Case Report: First coinfection report of mixed *Leishmania infantum*/*Leishmania major* and human immunodeficiency virus-acquired immune deficiency syndrome: Report of a case of disseminated cutaneous Leishmaniasis in Iran. American Journal of Tropical Medicine and Hygiene. 2018;98(1):122–5.

44. Louradour I, Ferreira TR, Ghosh K, Shaik J, Sacks D. In Vitro Generation of *Leishmania* Hybrids. Cell Rep. 2020 Apr 14;31(2).

45. Ozbilgin A, Culha G, Zeyrek FY, Töz S, Gündüz C, Kurt Ö, et al. Cutaneous and visceral tropisms of *Leishmania tropica*/*Leishmania infantum* hybrids in a murine model: First report of hybrid *Leishmania* strains isolated in Turkey. International Journal of Infectious Diseases. 2012 Jun;16:e165.

46. Bruno F, Castelli G. Genomic and epidemiological evidence for the emergence of a putative *L. donovani*/*L. infantum* hybrid with unusual epidemiology in Northern Italy. Available from: https://doi.org/10.1101/2023.08.09.552585