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Epidemiology and control of canine leishmaniosis: characterization of a previously undescribed endemic area in Catalonia and CaniLeish® vaccine field trial

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EPIDEMIOLOGY AND CONTROL OF CANINE LEISHMANIOSIS: CHARACTERIZATION OF A PREVIOUSLY UNDESCRIBED ENDEMIC AREA IN CATALONIA AND CANILEISH® VACCINE FIELD TRIAL

Memòria presentada per Maria Rita Perdigão Velez per optar al títol de doctor per la Universitat de Barcelona

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CERTIFICA:

Que el presente trabajo de investigación titulado: "Epidemiology and control of canine leishmaniosis: characterization of a previously undescribed endemic area in Catalonia and CaniLeish® vaccine field trial", presentado por la licenciada en Medicina Veterinária Maria Rita Perdigão Velez, ha sido realizado en ISGlobal, en la Sección de Parasitología del Departamento de Biología, Sanidad y Medio Ambiente de la Universitat de Barcelona y en el Hospital Veterinari Canis bajo su dirección y cumple las condiciones exigidas para ser presentado y defendido como Tesis Doctoral.

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ABSTRACT

Leishmaniosis is an important vector-borne zoonosis caused by *Leishmania infantum*. The disease is widespread across several continents and endemic in the Mediterranean region. The domestic dog is the main vertebrate reservoir for the parasite and control of canine leishmaniosis (CanL) is deemed to be essential for the control of human cases of the disease. Due to the heterogeneous distribution of infection in endemic areas, epidemiological surveillance should be carried out focally, including both screening of canine populations and vector detection, the two determinant factors for parasite survival and expansion.

CanL control measures are usually directed at the canine reservoir through the detection and treatment of infected individuals, as well as disease prevention through insecticide treatments and/or canine immunoprophylaxis. Vaccination against CanL is relatively recent and evidence of its impact in infection control at the community level is still insufficient. This is also the case for CaniLeish® vaccine, the first CanL vaccine to be licensed in Europe, in 2011. Pre-licensing studies were performed exclusively in homogeneous populations of beagle dogs, experimentally infected or introduced in endemic areas, and very little is known regarding this vaccine's performance in native and heterogeneous dog populations from *L. infantum* endemic areas.

The study presented in this thesis is divided into two parts. The first consists of a CanL epidemiological study in Girona province, a previously uncharacterized region of north-eastern Spain. The results obtained confirmed the endemicity of CanL in Girona province, characterized by a high prevalence of *L. infantum* infection in dogs (19.5%), together with the detection of a significant proportion of asymptomatic infected individuals (93.2%). The increase of dogs' age and lower altitude of the kennel location were identified as risk factors. The two antigens tested to assess dog exposure to *Phlebotomus perniciosus* (SGH and rSP03B salivary antigens) proved to be suitable, with specific antibodies showing a marked decrease during the non-transmission season, which allowed detection of recent host exposure to vectors. In addition, detected levels of antibodies against both SGH and rSP03B were associated with seropositivity to *L. infantum*.

The second part of this thesis describes a one year field trial of CaniLeish® vaccine, performed in a native heterogeneous canine population from Girona province. These dogs were kept in their natural housing conditions throughout the study and were naturally exposed to an *L.*

infantum transmission season. Results showed that CaniLeish® vaccine induces the production of non-specific antibodies interfering with the serological diagnosis of *L. infantum* infection in dogs and that this interference could have a greater impact between one and four months post-vaccination. Vaccine trial results did not confirm CaniLeish® reported efficacy in preventing active *L. infantum* infection or clinical disease in dogs during the first year post-vaccination. These results were supported by an apparently short-lived vaccine-induced cellular mediated immunity, assessed in this study through the quantification of gamma-interferon (IFN-γ) produced by trial dogs at one and nine months post-vaccination.

The results presented in this thesis support the need for maintaining and extending epidemiological surveillance in CanL endemic areas, in order to better characterize current CanL distribution and to anticipate possible *L. infantum* expansion trends. Additionally, further CaniLeish® evaluation studies are needed, together with active vaccine surveillance, to definitely assess the utility of this vaccine in CanL control at the community level in *L. infantum* endemic areas.

LIST OF ACRONYMS

ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
BCG	Bacillus Calmette-Guérin
BUN	Blood urea nitrogen
CanL	CanL – Canine leishmaniosis
CBC	Complete blood count
CLWG	Canine Leishmaniasis Working Group
CMI	Cellular mediated immunity
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FML	Fucose-mannose ligand
GGT	Gamma-glutamyl transferase
HL	Human leishmaniosis
ICT	Immunochromatographic test
IFAT	Immunofluorescence antibody test
Ig	Immunoglobulin(s)
IFN-γ	Gamma interferon
IL-2	Interleukin-2
iNOS	Inducible nitric oxide synthase
kDNA	Kinetoplast DNA
LiESP	Purified excreted-secreted proteins of *L. infantum*
LST	Leishmanin skin test
MDP	Muramyl-dipeptide
MHC	Major histocompatibility complex
MHC II	Major histocompatibility complex class II
NO	Nitric oxide
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PCR	PCR – Polymerase chain reaction
PSA	Parasite surface antigen
qPCR	Quantitative real time polymerase chain reaction
QuilA	Saponin adjuvant isolated from *Quillaja saponaria*

rSP03B	43-kDa yellow-related recombinant protein
SGH	Salivary gland homogenate
SLA	Soluble *Leishmania* antigens
SNOAPAD	Standardized Nomenclature of Animal Parasitic Diseases
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-α
UPC	Urinary protein-to-creatinine (ratio)
VE	Vaccine efficacy

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1. INTRODUCTION

1. INTRODUCTION

1.1. THE LEISHMANIASES

The term “leishmaniases” is used to describe a wide spectrum of clinical presentations caused by vector-transmitted protozoan parasites of the genus *Leishmania* Ross, 1903 (Kinetoplastida, Trypanosomatidae) (Gradoni, 2018).

In humans, *Leishmania* infections have diverse clinical presentations: visceral leishmaniosis (VL), post-kala-azar dermal leishmaniosis (PKDL), cutaneous leishmaniosis (CL), diffuse cutaneous leishmaniosis (DCL), mucocutaneous leishmaniosis (MCL) and mucosal leishmaniosis (ML), CL being the most common (Akhoundi *et al*., 2017).

Leishmania parasites have a worldwide distribution and leishmaniases are present on all continents except Antarctica (Bañuls *et al*., 2007) (Figure 1). According to the WHO Global Health Observatory, 94 countries and territories were considered to be endemic for leishmaniases in 2016 (WHO, 2018a). An estimated 700.000 to 1 million new human cases and 20.000 to 30.000 deaths occur annually due to infections by *Leishmania* (WHO, 2018b).

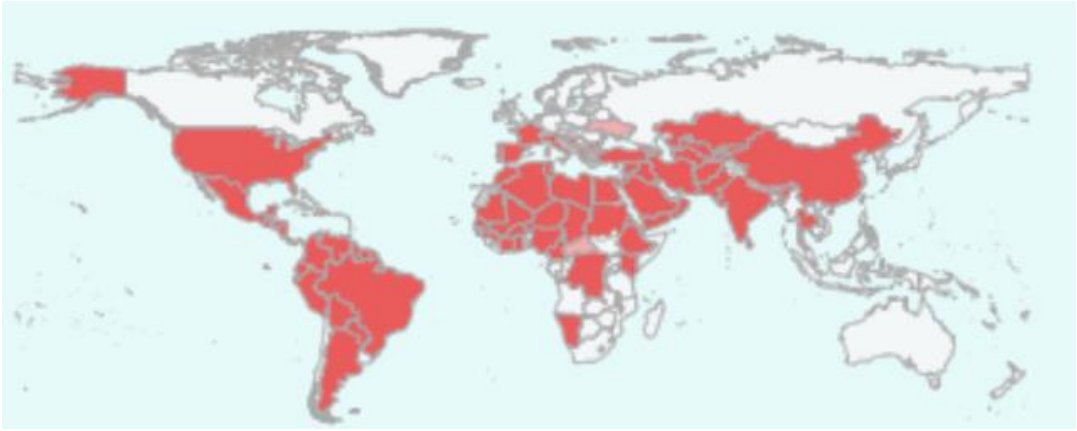

Figure 1. Geographical distribution of the human leishmaniases (available at: http://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html).

In Spain, the notification of human leishmaniosis (HL) was mandatory at national level from 1992 to 1995. Following this, a new decentralized surveillance system based on the political structure of the autonomous regions was implemented. HL was classified as a regional endemic disease and no longer of mandatory notification in non-endemic Autonomous Communities (Real Decreto 2210/1995). Since 2015, it is again of mandatory notification at national level (Orden SSI/445/2015).

HL is hypoendemic in the country, with 0.41 cases registered per 100,000 inhabitants (Alvar *et al*., 2012) (Figure 2). The disease is considered to be under-declared and the sub notification of cases to the National Surveillance System is estimated to be approximately 50% (Suarez Rodríguez *et al*., 2012). The Spanish Centralized Hospital Discharge Database recorded 3442 new cases of leishmaniosis amongst the 8010 HL hospitalization records between 1997 and 2011 (Herrador *et al*., 2015). In addition, there has been an epidemic outbreak in the Community of Madrid that began in July 2009 (Boletín Epidemiológico Comunidad Madrid, 2011; CCAES, 2012; Molina *et al*., 2012b).

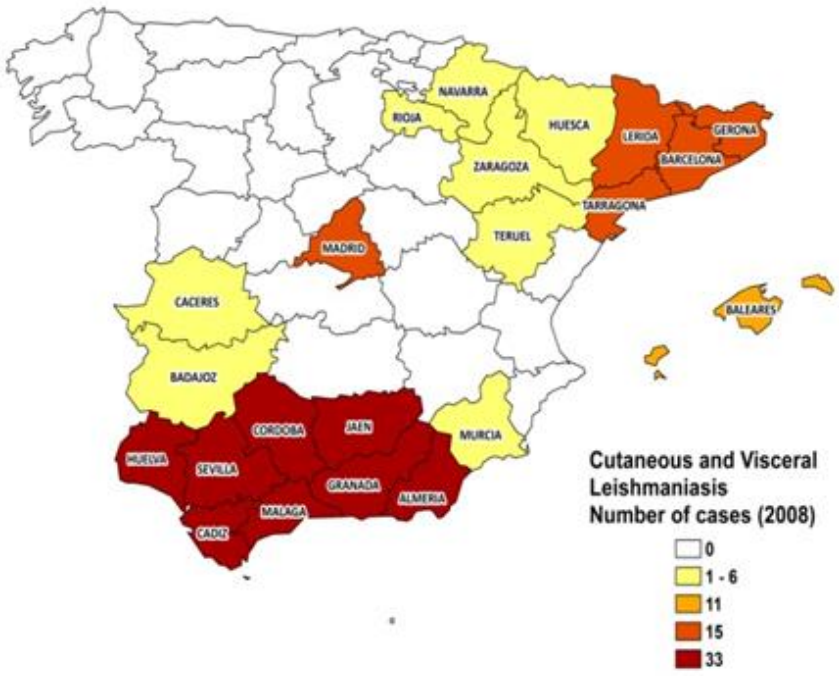

Figure 2. Distribution of HL in Spain (in Alvar *et al.*, 2012)

1.2. THE PARASITE

From the 54 *Leishmania* species described so far, 31 are known to be parasites of mammals (Akhoundi *et al*., 2017, 2016) and at least 21 species are pathogenic for humans (Akhoundi *et al*., 2016; WHO, 2010).

Isoenzyme analysis is considered the gold standard method for typing *Leishmania* species (Rioux *et al.*, 1990). More recently, DNA and protein based methods have been developed for the identification of these parasites with multilocus microsatellite typing and sequencing most commonly used (Akhoundi *et al*., 2017; Lachaud *et al*., 2017; Maurício, 2018; Talmi-Frank *et al*., 2010; van der Auwera and Dujardin, 2015).

An updated taxonomic classification of the genus *Leishmania* is presented in Figure 3.

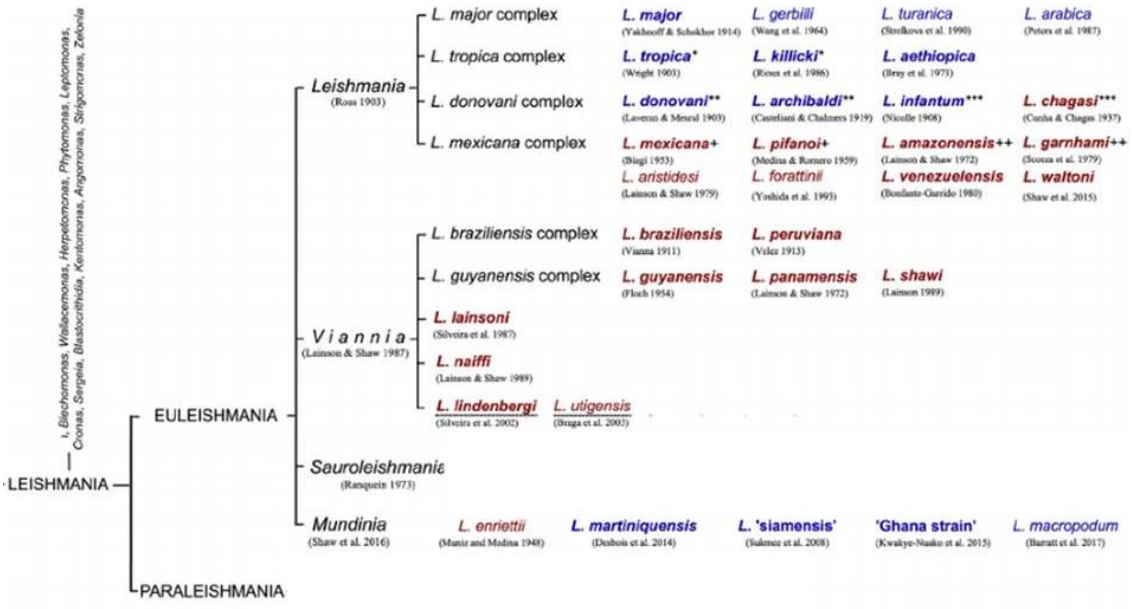

Figure 3. Taxonomic classification of the genus *Leishmania* (adapted from Akhoundi *et al.*, 2017). Information is missing for the *Paraleishmania* section and *Sauroleishmania* subgenus. Asterisk (*) and plus (+) signs denote synonymous species. Underlined: No final classification. **Bold**: Human pathogenic species. *Leishmania* names in quotation marks are unofficial names without formal descriptions. Old and New world species are highlighted in blue and red respectively. *L. siamensis* and *L. martiniquensis* have been found also in the New World.

The species included in the subgenus *Leishmania* multiply in the vectors' midgut and foregut and are present in Eurasia, Africa and the Americas whilst members of the *Viannia* subgenus multiply in the vector's hindgut and are restricted to the American continent (Lainson and Shaw, 1987). The parasites most commonly responsible for VL belong to the *Leishmania* subgenus, while both groups contain parasites causing CL. In recent years, new species have been described in unexpected hosts and locations, including some being isolated from humans (Cotton, 2017).

Leishmania infantum is the species with the widest geographical distribution. It is present in 47 countries throughout Europe, Africa, Asia and Central and South America (WHO, 2010), and is the only autochthonous species in Spain (Alvar *et al*., 2012). Isoenzyme analysis shows that there is a high diversity within *L. infantum* species, with 39 identified zymodemes (Pratlong *et al*., 2013; Pratlong, pers.com.), although the most prevalent is MON-1 (Gallego *et al*., 2001). In Spain, 32 zymodemes have been isolated from patients with leishmaniases, most of them HIV-positive patients (Chicharro *et al*., 2003; Gallego *et al*., 2001; Martín-Sánchez *et al*., 2004; Pratlong *et al*., 2013).

1.2.1. Transmission

Transmission of *Leishmania* parasites is mainly vectorial, through the bite of Phlebotomine sand flies (Diptera, Psychodidae) (Gállego, 2004; Killick-Kendrick, 1999; Maroli *et al.*, 2013). Sand fly vectors in Eurasia and Africa belong mainly to the *Phlebotomus* genus, while *Lutzomyia* spp. are responsible for transmission in the American continent (Akhoundi *et al*., 2016; Maroli *et al*., 2013). In the New World, the vectorial role of *Psychodopygus* and *Nyssomyia* genus is also considered (Maia and Depaquit, 2016). From the 800 known species, 98 are suspected or proven vectors of *Leishmania* parasites (Maroli *et al*., 2013).

Other phlebotominae (*Sergentomyia* genus) and arthropoda (ticks, biting midges and others) have also been taken into account as possible vectors of *Leishmania* (Dantas-Torres, 2011; Maia and Depaquit, 2016; Slama *et al*., 2014; Solano-Gallego *et al*., 2012). Nevertheless, due to the sole molecular detection of the parasites in the majority of cases, the confirmation of these species fulfilling all the criteria to be considered vectors of *Leishmania* parasites pathogenic to mammals is yet to be proven (Maia and Depaquit, 2016).

In the Mediterranean region, eight *Phlebotomus* species have been incriminated as vectors of *L. infantum* according to conventional criteria (Killick-Kendrick, 1999; WHO, 2010): *P. ariasi*, *P. balcanicus*, *P. kandelakii*, *P. langeroni*, *P. neglectus*, *P. perfiliewi*, *P. perniciosus* and *P. tobbi* (reviewed in Alten *et al*., 2016). All belong to the subgenus *Larroussius*, except *P. balcanicus*, which is a member of the *Adlerius* subgenus.

The vector species historically present in Spain are *P. ariasi* and *P. perniciosus,* from which the parasite has been isolated in Andalusia, Aragon, Catalonia and Madrid regions (Alcover *et al*., 2012; González *et al*., 2017; E. Guilvard *et al*., 1996; Lucientes-Curdi *et al*., 1988; Martín-Sánchez *et al*., 2006, 1994; Morillas-Márquez *et al*., 1991; Rioux *et al*., 1986; Sanchez *et al*., 1995). In addition, *L. infantum* DNA has been detected in *P. perniciosus* in Extremadura (Bravo-Barriga *et al*., 2016). Furthermore, a stable population of *P. langeroni* infected with *L. infantum* was recently detected by PCR in the south of the country (Sáez *et al*., 2018), showing that additional overlooked vector species may exist.

Vectorial transmission is influenced by the presence and density of sand flies which, in turn, is highly influenced by abiotic factors, showing a positive correlation with environmental temperature and a negative association with relative humidity (Dantas-Torres *et al*., 2014; Gálvez *et al*., 2010a; Tarallo *et al*., 2010). However, in a study carried out in Spain separately analysing two vector species, *P. ariasi* and *P. perniciosus*, opposite correlations were observed for each of the species (Ballart *et al*., 2014), highlighting the importance of performing individual species analyses. *P. ariasi* favoured humid or sub-humid areas, whilst *P. perniciosus* was more abundant in semi-arid zones (Aransay *et al*., 2004; Ballart *et al*., 2014; Gállego *et al*., 1990).

The sand fly activity period in Spain is variable and can extend from the end of March to the middle of December in the south of the country (Morillas-Márquez *et al*., 1983), although in central and northern regions the sand fly activity season is considered to be from June to October (González *et al*., 2017). This also corresponds to the period of higher potential risk for *L. infantum* transmission in the Mediterranean region (Alten *et al*., 2016). *P. perniciosus* shows a diphasic seasonal trend with two recognized abundance peaks in July and September, while *P. ariasi* presents a monophasic abundance cycle, peaking in August (Alten *et al*., 2016; Gálvez *et al*., 2010a).

1.2.2. Epidemiological cycles

Depending on the species, *Leishmania* parasites can present an anthroponotic or a zoonotic life cycle (Ashford, 2000; Bañuls *et al*., 2007; Gállego, 2004). Most *Leishmania* species known to cause disease in humans are considered to present a zoonotic epidemiological cycle (Gramiccia and Gradoni, 2005) or to have recent zoonotic origins (Ashford, 2000). In fact, the possibility or demonstration of animal reservoirs for anthroponotic species has been reported (Dereure *et al*., 1991; Kassahun *et al*., 2015; Singh *et al*., 2013).

A great number of animals have been found infected with *Leishmania* species and are considered parasite hosts, but only a minority is suspected of having a possible role in parasite maintenance and transmission in a particular scenario (i.e. parasite reservoirs). *Leishmania* reservoirs show regional and temporal variation, and only a local study including ecological and parasitological analysis could determine whether a species may serve as a reservoir in a given environment (Roque and Jansen, 2014). In the Americas, *Leishmania* hosts belong to the orders Didelphimorphia, Cingulata, Pilosa, Rodentia, Primata, Carnivora, and Chiroptera, whilst in the Old World implicated animal orders are Carnivora, Hyracoidea, Rodentia and Lagomorpha (Gramiccia and Gradoni, 2005; Roque and Jansen, 2014).

In the Mediterranean region, the cycle is zoonotic and domestic, with the dog acting as the principal reservoir of *L. infantum*. Other animal species, proven or suspected reservoirs of *L. infantum* in Europe have been revised by Millán *et al*. (2014) and Pennisi (2015) and are listed on Table 1. For the large majority, there is no evidence that these can act as reservoirs (Portús *et al*., 2002; Quinnell and Courtenay, 2009), but cats, black rats, foxes, hares and rabbits are considered to be able to maintain a wild or domestic epidemiological cycle (Jiménez *et al*., 2014; Maroli *et al*., 2007; Marín-Iniesta *et al*., 1982; Martín-Sánchez *et al*., 2007; Molina *et al*., 2012b; Pozio *et al*., 1985; Zanet *et al*., 2014).

Table 1. Alternative host species and possible reservoirs of *L. infantum* in Europe.

Species	Proven infectiousness to sand flies*/Isolation of parasites**	References
Badger (*Meles meles*)		del Río *et al.*, 2014
Beech marten (*Martes foina*)		Muñoz-Madrid *et al*., 2013
Black rat (*Rattus rattus*)	*/**	Helhazar *et al*., 2013; Morillas-Márquez *et al*., 1985; Muñoz-Madrid *et al*., 2013; Navea-Pérez *et al*., 2015; Pozio *et al*., 1985; Zanet *et al*., 2014
Brown rat (*Rattus norvegicus*)		Helhazar *et al*., 2013
Domestic cat (*Felis catus domesticus*)	*/**	Ayllon *et al*., 2008; Hervás *et al*., 1999; Maia *et al*., 2010; Maroli *et al*., 2007; Martín-Sánchez *et al*., 2007; Millán *et al*., 2011; I. Moreno *et al*., 2014; Solano-Gallego *et al*., 2007
Domestic goat (*Capra hircus*)		Fisa *et al*., 1999; Portús *et al*., 2002
Domestic ferret (*Mustela putorius furo*)		Brianti *et al*., 2005
Domestic horse (*Equus caballus*)	**	Fernández-Bellon *et al*., 2006; Gama *et al*., 2014; Portús *et al*., 2002; Rolão *et al*., 2005; Solano-Gallego *et al*., 2003
Domestic sheep (*Ovis aries*)		Fisa *et al*., 1999; Portús *et al*., 2002
Egyptian mongoose (*Herpestes ichneumon*)		Sobrino *et al.*, 2008
European brown hare (*Lepus europaeus*)		Rocchigiani *et al*., 2018; Ruiz-Fons *et al*., 2013; Tsokana *et al*., 2016
European mink (*Mustela lutreola*)		del Río *et al*., 2014
European rabbits (*Oryctolagus cuniculus*)	*/**	Díaz-Sáez *et al*., 2014; Jiménez *et al*., 2014; I. Moreno *et al*., 2014; Ortega *et al*., 2017; Risueño *et al*., 2018
Genet (*Genetta genetta*)		del Río *et al*., 2014; Millán *et al*., 2011; Oleaga *et al*., 2018;

		Risueño *et al*., 2018; Sobrino *et al*., 2008
Golden jackal (Canis aureus)		Hervás *et al*., 1996
Grey wolf (*Canis lupus*)		Muñoz-Madrid *et al*., 2013 Oleaga *et al*., 2015; Risueño *et al*., 2018; Sobrino *et al*., 2008
Hedgehog (*Erinaceous europaeus*)		Muñoz-Madrid *et al*., 2013
House mouse (*Mus musculus*)		Helhazar *et al*., 2013; Navea-Pérez *et al*., 2015
Iberian hare (*Lepus granatensis*)	*/**	Molina *et al*., 2012b; I. Moreno *et al*., 2014; Ortega *et al*., 2017; Ruiz-Fons *et al*., 2013
Iberian lynx (*Lynx pardinus*)		Sobrino *et al.*, 2008
Algerian mouse (*Mus spretus*)		Millán, 2018
Otter (*Lutra lutra*)		Oleaga *et al*., 2018
Pine marten (*Martes martes*)		del Río *et al*., 2014; Millán *et al*., 2011; Oleaga *et al*., 2018
Pole cat (*Mustela putorius*)		del Río *et al.*, 2014
Red fox (*Vulpes vulpes*)	**	Abranches *et al*., 1984; Criado-Fornelio *et al*., 2000; del Río *et al*., 2014; Fisa *et al*., 1999; Marín-Iniesta *et al*., 1982; Muñoz-Madrid *et al*., 2013; Oleaga *et al*., 2018; Piantedosi *et al*., 2016; Portús *et al*., 2002; Risueño *et al*., 2018; Sobrino *et al*., 2008; Verin *et al*., 2010
Stone marten (*Martes foina*)		del Río *et al*., 2014; Oleaga *et al*., 2018; Risueño *et al*., 2018
White-toothed shrew (*Crocidura russula*)		Millán, 2018
Wild cat (*Felis silvestris*)		del Río *et al*., 2014; Oleaga *et al*., 2018; Risueño *et al*., 2018
Wood mouse (*Apodemus sylvaticus*)		Navea-Pérez *et al*., 2015; Risueño *et al*., 2018

Detection of *L. infantum* by serological and/or molecular diagnostic techniques.
*Infectiousness to sand flies has been proven by xenodiagnoses
**Isolation of the parasite by culture

1.3. CANINE LEISHMANIOSIS

Canine leishmaniosis (CanL) is an important veterinary disease, present in at least 50 countries (Alvar *et al*., 2004). Kaszak *et al*. (2015) have estimated that CanL affects more than 2.5 million dogs in more than 70 countries, although other authors assert that these figures occur in the Mediterranean basin alone (Moreno and Alvar, 2002). In this region, it has been estimated that 50 to 80% of the canine population is infected and that the prevalence of the disease ranges between 2% and 5% (Saridomichelakis, 2009).

The term CanL is usually used to refer to the infection by *L. infantum,* although dogs can also be infected by other *Leishmania* species, which are listed in Table 2. Being the main known reservoir of *L. infantum* (Dantas-Torres, 2007; Gállego, 2004; Otranto *et al*., 2017; Ready, 2010), dogs assume a crucial role in the maintenance of the parasite's life cycle. Likewise, surveillance and control of infected canids in endemic regions is essential for the management of CanL, as well as for the reduction of HL (Gavgani *et al.*, 2002).

In dogs, the zymodeme MON-1 of *L. infantum* is the most prevalent, although 17 out of 39 zymodemes have been characterized in dogs' strains (Aït-oudhia *et al*., 2011; Gallego *et al*., 2001; Pratlong *et al*., 2013).

Table 2. *Leishmania* species reported in the domestic dog (*Canis familiaris*)

Leishmania spp.	Geographical distribution	References
L. aethiopica	Sudan	Dereure *et al.*, 2003
L. amazonensis	Brazil	Tolezano *et al.*, 2007
L. arabica	Saudi Arabia	Peters *et al.*, 1986
L. braziliensis	South America	Aguilar *et al.*, 1987; Marquez *et al.*, 2017; Mayrink *et al.*, 1979; Pirmez *et al.*, 1988; Reithinger and Davies, 1999; Vélez *et al.*, 2012
L. colombiensis	Venezuela	Delgado *et al.*, 1993; Reithinger and Davies, 1999
L. donovani	East Africa; Sudan	Dereure *et al.*, 2003; Mutinga *et al.*, 1980; Sukkar *et al.*, 1981
L. guyanensis	Colombia, Ecuador	Reithinger and Davies, 1999; Santaella *et al.*, 2011
L. infantum	Africa, America, Asia, Europe	Gállego, 2004[1]
L. major	Egypt, Saudi Arabia	Elbihari *et al.*, 1987; Pratlong *et al.*, 2009
L. mexicana	Ecuador, USA	Hashiguchi *et al.*, 1991; Mayrink *et al.*, 1979; Reithinger and Davies, 1999
L. panamensis	Colombia, Costa Rica, Ecuador, Panama	Dereure *et al.*, 1994; Vélez *et al.*, 2012; Reithinger and Davies, 1999
L. peruviana	Peru	Llanos-Cuentas *et al.*, 1999; Reithinger and Davies, 1999
L. pifanoi	Ecuador	Dantas-Torres, 2009; Reithinger and Davies, 1999
L. tropica	India, Iran, Morocco, Syria	Dereure *et al.*, 1991; Lemrani *et al.*, 2002; Pratlong *et al.*, 2009

Adapted from Alvar *et al.*, 2004; Dantas-Torres, 2009; Dantas-Torres *et al,.* 2012; Gállego, 2004; Reithinger and Davies, 1999; Solano-Gallego *et al.*, 2009

[1]other references are included along the manuscript

The northward expansion of CanL in Europe, along with the possible spread of human cases, is well documented (Ballart *et al.*, 2013b; Gramiccia and Gradoni, 2005; Maroli *et al.*, 2013; Medlock *et al.*, 2014). Surveys performed at border areas between CanL endemic and non-endemic regions prove that the infection is present in locations where it had not been documented before (Ballart *et al.*, 2013a; Capelli *et al.*, 2004; Cassini *et al.*, 2013; Dumitrache *et al.*, 2016; Vaselek *et al.*, 2017). Also, mathematical models and predictive risk maps forecast an expansion of *Leishmania* vectors due to climate change and anthropogenic impact on the landscape (Espejo *et al.*, 2015; Fischer *et al.*, 2010; Koch *et al.*, 2017).

New cases of CanL have been registered in non-endemic areas where no vectorial transmission exists, mainly as a result of dog movement to endemic countries (Dandrieux *et al.*, 2018; Maia and Cardoso, 2015). In some cases, however, autochthonous canine infection has been proven, most certainly through direct contact (Boggiatto *et al.*, 2011; Gibson-Corley *et al.*, 2008; Karkamo *et al.*, 2014; Naucke and Lorentz, 2012; Svobodova *et al.*, 2017; Tánczos *et al.*, 2012). Therefore, CanL in Europe is no longer only a problem of Mediterranean countries (Dujardin *et al.*, 2008; Pennisi, 2015; Ready, 2010). Although leishmaniosis is included in the OIE list of notifiable diseases (OIE, 2018), this is not clearly reflected in the European or country-level legislation (BOE, 2014; Official Journal of the European Union, 2012), meaning that a common European strategy for leishmaniosis surveillance and control does not exist.

The work described in this thesis is set in a European Mediterranean country and refers exclusively to canine infection by *L. infantum*; therefore, the term CanL will, for the reminder of this dissertation, used to refer strictly to infection of dogs by this *Leishmania* species.

The terminology used to describe the disease caused by *Leishmania* parasites changes according to authors' personal preference. The term "leishmaniasis" is frequently used to refer to the human disease, while "leishmaniosis" is more commonly applied to the veterinary condition (Miró and López-Vélez, 2018). In this dissertation, the guidelines of the Standardized Nomenclature of Animal Parasitic Diseases (SNOAPAD) will be followed, and the term "leishmaniosis" will be applied both for the veterinary and the human diseases (Kassai, 2006; Kassai *et al.*, 1988). "Leishmaniosis" is also the term used by the World Organization for Animal Health to refer to the canine infection by *Leishmania* species (OIE, 2014).

1.3.1. Clinical presentation

The course of *L. infantum* infection in the canine host is highly influenced by the host's immune response (more details in section 1.3.5), which introduces a high individual variability into the clinical outcome (Hosein *et al*., 2017). Likewise, factors such as parasitic burden, virulence of *Leishmania* strain, previous infections or coinfections can also affect the polarity of clinical manifestations (Saridomichelakis, 2009; Solano-Gallego *et al.*, 2009). Consequently, dogs with CanL can present a broad range of clinical signs and clinicopathological abnormalities, which are usually nonspecific (Paltrinieri *et al.*, 2016; Solano-Gallego *et al.*, 2011). The incubation period since infection and until the appearance of clinical disease in dogs can last from three months to seven years (Alvar *et al*., 2004; Miró *et al*., 2008; Oliva *et al*., 2006; Solano-Gallego *et al*., 2001a). CanL is a multisystemic disease that can potentially affect any organ or tissue, as well as present a diffuse or localized progression. The most common clinical signs observed in "classical" CanL are progressive weight loss, generalized lymphadenomegaly, onycogryphosis and non-pruritic exfoliative dermatitis (Solano-Gallego *et al.*, 2011, 2009). Non regenerative anaemia, mild thrombocytopenia, leukogram changes and indicators of renal dysfunction and/or inflammatory immune response are the most frequently detected clinicopathological alterations (Foglia Manzillo *et al.*, 2013; Paltrinieri *et al.*, 2016). Clinical signs and laboratory abnormalities observed in CanL are presented in Tables 3 and 4.

Table 3. Clinical signs and lesions reported in CanL distributed by organic system

Clinical signs and lesions	References*
<u>General</u> ○ **Poor body condition** ○ **Exercise intolerance** ○ Lethargy ○ Loss of appetite ○ Pale mucous membranes ○ Fever	
<u>Reticuloendothelial</u> ○ **Generalized lymphadenopathy** ○ **Splenomegaly**	Santana *et al.*, 2008
<u>Cutaneous</u> ○ **Non-pruritic exfoliative dermatitis with or without alopecia** ○ **Onychogryphosis**	Ferrer *et al.*, 1988; Lombardo *et al.*, 2014

○ Erosive-ulcerative dermatitis ○ Nodular dermatitis ○ Papular dermatitis ○ Pustular dermatitis ○ Mucosal ulceration ○ Nasal hyperkeratosis ○ Footpad hyperkeratosis	
<u>Ophthalmic</u> ○ Blepharitis ○ Conjunctivitis ○ Keratoconjunctivitis ○ Anterior uveitis	Naranjo *et al.*, 2005; Peña *et al.*, 2008
<u>Musculoskeletal</u> ○ **Muscular atrophy** ○ **Atrophic masticatory myositis** ○ Skeletal myositis	
<u>Renal</u> ○ Polyuria and polydipsia	
<u>Cardiac</u> ○ Myocardial lesions	Martínez-Hernández *et al*., 2017
<u>Coagulation/vascular</u> ○ **Epistaxis**	
<u>Articular</u> ○ Arthritis ○ Lameness	Agut *et al.*, 2003; Santos *et al.*, 2006
<u>Digestive</u> ○ Vomiting ○ Diarrhoea	Adamama-Moraitou *et al.*, 2007; Pinto *et al.*, 2011
<u>Neurological</u>	Giannuzzi *et al.*, 2017; Márquez *et al.*, 2013; Zobba *et al.*, 2017

The most common clinical signs are marked in **bold**.

*References presented in the table correspond to specific studies; general references for clinical signs and lesions were: Ciaramella *et al.*, 1997; Foglia Manzillo *et al.*, 2013; Koutinas and Koutinas, 2014; Noli and Saridomichelakis, 2014; Paltrinieri *et al.*, 2016, 2010; Saridomichelakis, 2009; Solano-Gallego *et al.*, 2011, 2009.

Table 4. Main laboratory findings associated with CanL

Laboratory findings	References
Hemogram ○ **Mild to moderate non-regenerative anaemia** ○ **Thrombocytopenia** ○ Leukocytosis or leukopenia Biochemical parameters ○ Renal azotaemia (↑BUN and creatinine) ○ Hepatic dysfunction (↑ALT, ALP and GGT) ○ **Hyperglobulinemia** ○ **Hypoalbuminemia** ○ Decreased albumin/globulin ratio Serum protein electrophoresis ○ **Polyclonal gammopathy** (less frequently, oligoclonal or monoclonal) Immunological parameters ○ Positive antinuclear antibody titres Urinalysis ○ Proteinuria ○ Increased UPC ratio ○ Decreased urine specific gravity	Koutinas and Koutinas, 2014; Maia and Campino, 2018; Meléndez-Lazo *et al*., 2018; Noli and Saridomichelakis, 2014; Paltrinieri *et al*., 2016, 2010; Saridomichelakis, 2009; Solano-Gallego *et al*., 2011, 2009

The most common laboratory findings are marked in **bold**.

Clinical staging systems for CanL have been proposed by two working groups, the Canine Leishmaniasis Working Group (CLWG) and LeishVet (Roura *et al.*, 2013; Solano-Gallego *et al.*, 2009). These have the purpose of providing clinically useful information for therapeutic decisions and for prognostic purposes. However, independent peer validation of these suggested systems is still lacking (Noli and Saridomichelakis, 2014).

An important feature of canine *L. infantum* infection is the high prevalence of asymptomatic dogs, particularly in CanL endemic areas (Alvar *et al*., 2004; Ballart *et al*., 2013a; Baneth *et al*., 2008; Fisa *et al*., 1999; Solano-Gallego *et al*., 2001a). Again, this is a result of the individual's immune response, which in some cases is capable of controlling the parasite (Solano-Gallego *et al.*, 2000). Asymptomatic infected dogs are characterized by parasite detection (either by

direct or indirect techniques) in the absence of symptoms or clinicopathological abnormalities (Molina *et al.*, 1994; Paltrinieri *et al.*, 2016). Apart from immunological resistance, the asymptomatic state can also denote an early stage of infection, in which case a later development of clinical signs is expected (Fisa *et al.*, 2001; Miró *et al.*, 2012). Although parasite clearance has been mentioned in dogs (Fisa *et al.*, 1999), there is no reliable evidence of it (Cavaliero *et al.*, 1999; Manna *et al.*, 2008b; Solano-Gallego *et al.*, 2016a), meaning that asymptomatic infected individuals are at risk of developing clinical disease throughout their lives (Alvar *et al.*, 2004; Baneth *et al.*, 2008). Furthermore, asymptomatic dogs assume a particularly important role from an epidemiological perspective, as they represent overlooked reservoirs of *L. infantum* in endemic areas. Previous studies have already demonstrated that these dogs are infectious to sand flies (Borja *et al.*, 2016; de Mendonça *et al.*, 2017b; Laurenti *et al.*, 2013; Molina *et al.*, 1994; Quinnell and Courtenay, 2009) and capable of maintaining the infection in the canine population (eventually also being the source for human transmission).

1.3.2. Diagnosis

As mentioned, an important concept in *L. infantum* infection, especially in endemic areas, is that parasite detection on the vertebrate host does not equate to active disease (Moreno and Alvar, 2002). A vast number of dogs are capable of controlling the infection either in a latent form or a transitory state, leading to eventual parasite clearance (Fisa *et al.*, 1999; Miró *et al.*, 2012). This adds complexity to CanL diagnosis which, in some cases, will require the use of multiple diagnostic tests (Morales-Yuste *et al.*, 2012; Otranto *et al.*, 2009).

Furthermore, the techniques used for CanL diagnosis must be adapted to each situation. Dogs presented to veterinary practitioners are commonly symptomatic individuals, and the challenge here may be to exclude differential diagnosis and to confirm that the clinical signs observed are produced by *L. infantum* infection, regardless of parasite detection (Paltrinieri *et al.*, 2016; Solano-Gallego *et al.*, 2011). Specific laboratory tests aimed at etiologic diagnosis include direct methods (cytology, histopathology, parasite culture, molecular tests, and, less frequently, xenodiagnosis) and indirect techniques (serology and cellular immune response evaluation) (Gomes *et al.*, 2008; Maia and Campino, 2008; Maroli *et al.*, 2010; Noli and Saridomichelakis, 2014; Rodríguez-Cortés *et al.*, 2010; Solano-Gallego *et al.*, 2009; Travi *et al.*, 2018). Description of these techniques, with a special emphasis on the ones used for

prevalence surveys, will be addressed later, under the "Epidemiologic studies" chapter (section 1.3.4).

Due to the variety of possible outcomes after *L. infantum* infection (Noli and Saridomichelakis, 2014), the differentiation between exposure and disease through the available diagnostic methods can be particularly difficult. In any case, clinical examination should be the first step of CanL diagnosis (Solano-Gallego *et al.*, 2009). In endemic areas, and due to the serious consequences of a delayed diagnosis, it is recommended that the disease be investigated in any dog presenting with even a single CanL-associated clinical sign (Noli and Saridomichelakis, 2014).

1.3.3. Control

Several methods have been proposed for CanL prevention and control, both at the individual and at the population levels. These strategies are mainly focused at: 1) reducing the number of infected and infectious animals by early detection and treatment of infected dogs, and 2) avoiding new CanL infections by applying insecticides to both infectious and naïve dogs and/or through immunomodulation and vaccination (Alvar *et al*., 2004; Maroli *et al*., 2010; Miró *et al*., 2017b; Otranto and Dantas-Torres, 2013; Quinnell and Courtenay, 2009; Reguera *et al*., 2016; Ribeiro *et al*., 2018; Travi *et al*., 2018).

First line treatment regimens are based on the association of a leishmanicidal drug (pentavalent antimony meglumine antimoniate or miltefosine) and a leishmaniostatic (allopurinol) (Solano-Gallego *et al.*, 2009). More recently, immunomodulators aimed at reducing parasite burden and boosting the host's immune response [domperidone (Gómez-Ochoa *et al.*, 2009) or P-MAPA, a protein aggregate of magnesium-ammonium phospholinoleate-palmitoleate anhydride (Melo *et al.*, 2014; Santiago *et al.*, 2013)] have also been added to the classical therapeutic protocols.

Treatment of dogs with CanL has the aim of improving diseased dogs' quality of life and extending their life expectancy, while it also has an impact on the parasite load, thus reducing dogs' infectiousness to sand flies (Otranto and Dantas-Torres, 2013). Post-treatment decrease in infectiousness has been shown by xenodiagnosis studies for a few chemotherapeutic protocols (reviewed in Travi *et al.*, 2018), proving that this is an effective method for reducing

infection risk at the population level. However, no CanL treatment has proved to achieve consistent parasite clearance and clinical improvement is only transitory (Manna *et al*., 2008b; Torres *et al*., 2011), with affected dogs usually needing recurring treatment cycles (Solano-Gallego *et al.*, 2009). For this reason, and because the assessment of an individual's infectiousness for diagnostic purposes is not feasible, permanent use of topical insecticides is recommended in animals known to be infected (Noli and Saridomichelakis, 2014).

Culling of infected dogs has been the approach used in some countries as a method for leishmaniosis control. In Brazil, where cases of HL have been recently expanding to urban areas, treatment of affected dogs is not allowed and CanL detection campaigns, followed by culling of seropositive dogs, is the control method recommended by the Ministry of Health (Ministério da Saúde Brasileiro, 2014). However, besides being an unethical procedure, there is no real evidence supporting its efficacy in reducing the number of CanL or HL cases (Costa, 2011; Costa *et al*., 2013; Courtenay *et al*., 2002; Dye, 1996; Reithinger *et al*., 2004). Furthermore, indiscriminate dog culling may have a detrimental effect if, by removing seropositive resistant animals responsible for increasing herd immunity, it induces an increase in disease transmission (Fox *et al.*, 1971). It is currently known that only a small proportion of dogs are responsible for most transmission (Courtenay *et al.*, 2002) and that infectiousness correlates with parasite load, especially on ear skin biopsies (Courtenay *et al.*, 2014), while serological tests seem to be unable to discriminate the most infectious dogs (de Mendonça *et al*., 2017b). Therefore, the development of a diagnostic test able to differentiate between infected (which may not be responsible for transmission) and infectious dogs is essential to efficiently direct control efforts in areas of high transmission (Courtenay *et al*., 2014; de Mendonça *et al*., 2017b; Duthie *et al*., 2018).

The application of topical insecticides with proven efficacy against sand flies is still considered the most effective method for preventing *L. infantum* infection in dogs (Brianti *et al.*, 2014; Courtenay *et al.*, 2009; Foglia Manzillo *et al.*, 2006a; Goyena *et al.*, 2016; Killick-Kendrick *et al.*, 1997; Maroli *et al.*, 2001; Guadalupe Miró *et al.*, 2007; Molina *et al.*, 2012a; Otranto *et al.*, 2013). However, due to the need of repeated applications (spot-on preparations and sprays) or product replacement (dog collars), owner compliance is critical to achieve a satisfactory coverage and efficacy (Maia *et al*., 2018; Reithinger *et al.*, 2004). The effect of systemic insecticides administered to dogs in the reduction of CanL and HL is under research (Gomez *et al.*, 2018; Gomez and Picado, 2017) and it can prove to be an effective method to aid infection control in endemic countries. Insecticides can also be used in the environment, through indoor residual spraying (IRS) of houses and animal shelters (Alexander and Maroli, 2003; Maroli *et*

al., 2010), and novel vector control methods, such as attractive toxic sugar baits, are under development (Qualls *et al.*, 2015).

Preventive treatments aimed at stimulating the dog's immune system can be unspecific (immunomodulators) or specific (vaccines). Domperidona, described before as a complementary therapy for CanL, is also used as a preventive treatment in *L. infantum* endemic regions (Sabaté *et al.*, 2014). There are currently three licensed vaccines for CanL: Leish-Tec® (Hertape) in Brazil and CaniLeish® (Virbac) and LetiFend® (LETI) in Europe (Reguera *et al.*, 2016). A fourth vaccine was commercialized in Brazil for 11 years, having been withdrawn in 2014 by the Brazilian Ministry of Agriculture due to lack of evidence for its effectiveness (MAPA, 2014). The vaccines available for CanL will be assessed later, under the section "Commercially approved vaccines against CanL" (section 1.3.6).

Control of *L. infantum* infection directed at the domestic dog can prove insufficient in some settings, where other animal species could be implicated in the maintenance and transmission of zoonotic leishmaniosis (Antoniou *et al*., 2013; del Río *et al*., 2014; Millán *et al*., 2014; Navea-Pérez *et al*., 2015; Zanet *et al*., 2014) (see Table 1). However, in the majority of species other than dogs, a reservoir status for the parasite has not been proven (Courtenay *et al*., 2009; Portús *et al*., 2002). Particularly noteworthy is the recent case of Fuenlabrada (Madrid region, Spain), where Iberian hares (*Lepus granatensis*) and European rabbits (*Oryctolagus cuniculus*) were identified as the animal reservoirs for *L. infantum* parasites (Jiménez *et al.*, 2014; Molina *et al.*, 2012b) in the largest HL community outbreak in Europe (Arce *et al.*, 2013).

Alternative routes have been confirmed for *L. infantum* transmission between dogs: transplacental (Boggiatto *et al.*, 2011; da Silva *et al.*, 2009; Vida *et al.*, 2016), venereal (Silva *et al.*, 2009) and blood transfusion (Owens *et al.*, 2001). These routes assume importance mostly in non-endemic countries (Karkamo *et al.*, 2014; Naucke and Lorentz, 2012), since transmission by sand fly bites in endemic areas is much more effective than any other route (Saridomichelakis, 2009). Based on these findings, breeding dogs from endemic areas should be tested and any infected animal should be excluded from reproduction. Likewise, any blood donors should be regularly tested for *L. infantum* infection (Miró *et al.*, 2017b).

1.3.4. Epidemiological studies

According to the CLWG, an "exposed" dog is clinically healthy and presents low-titer anti-*Leishmania* antibodies in the absence of parasite isolation (either by cytological, histological, parasitological or molecular methods). The presence of specific anti-*Leishmania* antibody titers together with parasite detection characterizes an "infected" dog (Paltrinieri *et al.*, 2010). However, in CanL endemic areas, molecular parasite detection in peripheral blood or skin during the infection transmission season should be carefully interpreted and may not be sufficient to classify a dog as infected (Solano-Gallego *et al.*, 2011). Infected dogs can be asymptomatic, which can be indicative of a "resistant" state or early infection, as mentioned previously. In these cases, resistance can be more accurately ascertained through the detection of an effective anti-*Leishmania* cellular-mediated immune response (Solano-Gallego *et al.*, 2000). Infected symptomatic dogs are considered "diseased", which means they present CanL (Paltrinieri *et al.*, 2010). These definitions are essential to better understand the information yielded by different epidemiological studies.

The distribution of *L. infantum* in canine populations from endemic regions is highly heterogeneous (Maia et al., 2018; Pennisi, 2015). Reported infection prevalence can vary substantially within a country, as described in a study which compiled published data from France, Italy, Portugal and Spain (Franco *et al.*, 2011). In this study, observed point CanL prevalences within these endemic countries varied from 0 to more than 80%. In Spain, reported canine *L. infantum* infection seroprevalence varies from 1.6% in the northwest (Miró and Molina, 2006) to 34.6% in the south (Morillas-Márquez *et al.*, 1996), with a range of intermediate values reported across the territory (Acedo Sánchez *et al*., 1996; Alcover *et al*., 2013; Amela *et al*., 1995; Amusategui *et al*., 2004; Arnedo Pena et al., 1994; Ballart *et al*., 2013a; Botet and Portús, 1993; Cabezón *et al*., 2010; Couto *et al*., 2010; Encinas Grandes *et al*., 1988; Fisa *et al*., 1999; Gálvez *et al*., 2010b; Goyena *et al*., 2016; Lepe *et al*., 2000; Martín-Sánchez *et al*., 2009; G. Miró *et al*., 2007; Miró *et al*., 2017a, 2012; Morales-Yuste *et al*., 2011; Morillas-Márquez *et al*., 1996; Nieto *et al*., 1992; Segovia and Martin-Luengo, 1985; Solano-Gallego *et al*., 2001a).

Reasons for this heterogeneous distribution can be related to factors affecting parasite or vector prevalence, as well as host-related factors. Additionally, study design and the diagnostic techniques used can also influence the results of epidemiological surveys (Franco *et al.*, 2011).

Diagnostic methods most frequently used in epidemiological studies are serological and molecular (Ballart *et al*., 2013a; Cabezón *et al*., 2010; Fisa *et al*., 1999; Maia *et al*., 2016; Miró *et al*., 2012; Solano-Gallego *et al*., 2001a), but parasite detection by direct exam or culture, as well as tests for specific cellular immunity assessment are also used (Fernández-Bellon *et al*., 2008; Iniesta *et al*., 2002; Solano-Gallego *et al*., 2000). Epidemiological surveys usually include large numbers of animals to be sampled under field conditions, whereby diagnostic tests used for this purpose should be easy to perform and interpret, low-cost, and either applicable in field settings or by making use of samples that can be easily transported.

Serological methods are one of the most commonly employed approaches for detection of *L. infantum* infection (Solano-Gallego *et al.*, 2014). Serology detects canine humoral response to *L. infantum*, which can occur as early as one month after an infective phlebotomine bite (Moreno and Alvar, 2002), although it has been reported to go up to 22 months in some cases (Oliva *et al.*, 2006). Therefore, serological tests are indicators of host-parasite contact, but not of parasite presence (Solano-Gallego *et al.*, 2009). These methods are considered to be good predictors of the onset of clinical signs, and good indicators of active infection, as diseased dogs tend to present significantly higher levels of anti-*Leishmania* antibodies (Oliva *et al.*, 2006). Enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFAT), two of the most frequently used serological quantitative methods, are the screening tests recommended by the World Organization for Animal Health for prevalence and surveillance studies (OIE, 2014). Research on anti-*L. infantum* immunoglobulin (Ig) isotypes and IgG subclasses has attempted to correlate serological profiles with protective cellular mediated immunity (CMI) and infection outcome (reviewed in Maia and Campino, 2018). Results across studies tend to provide evidence of an increase in all *Leishmania*-specific Ig subtypes in symptomatic dogs, when compared to asymptomatic individuals (Iniesta *et al*., 2005; Reis *et al*., 2006; Rodríguez-Cortés *et al*., 2007a). Similar trends are reported for IgG subclasses (Iniesta *et al*., 2005; Laia Solano-Gallego *et al*., 2001b), although some authors have found a clear correlation between IgG2 levels and disease progression (Cardoso *et al*., 2007; Iniesta *et al*., 2007). Lack of consistency across IgG subclasses studies could be a result of the low specificity of the polyclonal antisera commercially available and used in most laboratories (Day, 2007). When monoclonal antibodies to IgG were used, no substantial difference between subclasses was detected (Quinnell *et al*., 2003a; Strauss-Ayali *et al*., 2007). Serological qualitative methods or rapid immunochromatographic tests (ICT), which are easy to perform and provide immediate results, are also used in veterinary daily practice and in epidemiological studies. However, positive qualitative results should be followed by a quantitative test to

accurately assess the level of infection and for follow-up purposes (Noli and Saridomichelakis, 2014).

Molecular diagnostic techniques are used to detect parasite DNA in different organs or tissues. Likewise, a positive result in a conventional polymerase chain reaction (PCR) test confirms the presence of *Leishmania*, but has no predictive value on the infection outcome (Otranto *et al.*, 2009). Alternatively, quantitative PCR (qPCR) methods can be used to assess parasite loads in different organs and to provide useful information for follow-up on positive animals (Francino *et al.*, 2006). Molecular techniques are highly specific, but their sensitivity depends on the sample used (Solano-Gallego *et al*., 2016a) and on the target DNA sequence (Akhoundi *et al*., 2017; Paltrinieri *et al*., 2016). Bone marrow, lymph node and skin usually harbour high parasite loads and provide higher sensitivity for PCR techniques (Noli and Saridomichelakis, 2014; Solano-Gallego *et al.*, 2011). The use of non-invasive samples, such as conjunctival swabs (Di Muccio *et al.*, 2012; Strauss-Ayali *et al.*, 2004), cerumen (Belinchón-Lorenzo *et al*., 2016) and hair (Belinchón-Lorenzo *et al*., 2013; Corpas-López *et al*., 2016), has proved to yield good results and could be considered as a useful diagnostic alternative for large-scale field studies. Peripheral blood is usually regarded as a less sensitive sample (Solano-Gallego *et al.*, 2011), but studies show that the detection of *Leishmania* DNA in blood is far more common than has been previously recognized (Francino *et al*., 2006; Rodríguez-Cortés *et al*., 2007b). The application of qPCR to blood specimens significantly increases the detection sensitivity (Francino *et al.*, 2006) and this method is considered adequate to complement serological results in large-scale epidemiological field studies (Maia *et al*., 2009; Solano-Gallego *et al*., 2016a).

Parasite detection by direct microscopic examination or parasite culture is highly specific, but these techniques present low sensitivity. Although still frequently used in veterinary practice (Bourdeau *et al.*, 2014) and recommended by several authors as the first line diagnostic test (Gharbi *et al.*, 2015; Paltrinieri *et al.*, 2010; Saridomichelakis, 2009), direct visualization of amastigotes on lymph node smears or skin lesions imprints depends greatly on parasite load, on the examiner's experience and may be unfeasible in field settings. Parasite culture is laborious, time-consuming and can take several weeks to provide definitive results (Maia and Campino, 2008); for these reasons, it is generally used for research purposes only (Miró *et al.*, 2008).

The detection of *Leishmania*-specific CMI has also been used in epidemiological studies. In field settings, it can be assessed by the leishmanin skin test (LST) (Cardoso *et al.*, 1998). This

technique identifies exposed and usually resistant animals (Solano-Gallego *et al.*, 2000), as dogs with active CanL (and the expected immunosuppression) frequently test negative to LST (Solano-Gallego *et al.*, 2005). Likewise, LST can be used to assess exposure and resistance to the parasite at the community level, but cannot be used to identify infected or diseased individuals (Iniesta et al., 2002). LST is easy to perform and inexpensive, which makes it adequate for testing a large number of animals in field conditions. However, a follow-up is needed after 72h and a possible iatrogenic induction of false positives can occur after repeated inoculations (Fernández-Bellon *et al.*, 2005). Additionally, leishmanin antigen for veterinary use has not been internationally standardised and is not available commercially worldwide (OIE, 2014), which may hamper its application. Some of the most common laboratory-based CMI techniques include the lymphoproliferation assay (LPA) and the quantification of specific *Leishmania*-induced cytokines produced by canine lymphocytes. Although these techniques also evaluate CMI, they seem to measure distinct parameters of the cellular-mediated response, as their results only partially overlap with each other and when compared to LST (Fernández-Bellon *et al*., 2005; Iniesta *et al*., 2002). Unlike LST, and being *in vitro* assays, there is no risk of iatrogenic-induced false-positive results with repeated testing. However, these are complex and time-consuming techniques that require access to laboratory facilities and several days of sample processing. The recent development of easier-to-perform techniques that make use of whole blood instead of isolated peripheral blood mononuclear cells (PBMC) may allow its use in field settings (Zribi *et al.*, 2017).

Detection of host-vector contact can provide useful information to complement studies of vector population dynamics and host-vector interactions, to assess the risk of *Leishmania* infection (Carvalho *et al.*, 2015; Rohoušová *et al.*, 2005; Vlkova *et al.*, 2011) and to measure the effectiveness of vector-control programmes (Clements *et al.*, 2010; Gidwani *et al.*, 2011). Exposure of vertebrate hosts to sand flies can be assessed by the detection of antibodies against sand fly saliva in the hosts' blood, a method that has proven to be highly specific (Vlkova *et al.*, 2011) and was successfully used as a marker of exposure to *L. infantum* vectors (Kostalova *et al.*, 2015; Martín-Martín *et al.*, 2014). The recent development of a rapid ICT which detects host contact with *P. perniciosus* can provide a valuable tool for testing canine populations in field settings (Willen *et al*., 2018).

1.3.5. Immune response to *Leishmania infantum* in dogs

After the bite of an infected sand fly, *L. infantum* metacyclic promastigotes are inoculated under the host's skin. After first contact with the skin immune system, parasites are phagocytized by macrophages, neutrophils and dendritic cells and transported to regional lymph nodes (Hosein *et al*., 2017). From this point, the infection outcome will vary depending on vector, parasite and host factors (Saridomichelakis, 2009).

Parasite virulence is determined by its species and strain, as well as the number of promastigote forms inoculated (Saridomichelakis, 2009). Sand fly saliva injected during the bite also stimulates the host's immune system (Abdeladhim *et al.*, 2014), although its role in the establishment of infection or protection is still under debate (Collin *et al.*, 2009; Rohoušová *et al.*, 2011). The host's immune response is crucial to infection progression, although the precise immune mechanisms responsible for resistance or susceptibility are still unknown (Hosein et al., 2017). The wide spectrum of possible outcomes varies from an immediate local elimination of the parasite by the host's macrophages to a multisystemic overt disease (Solano-Gallego *et al.*, 2009).

1.3.5.1. Host genetics

It is currently accepted that a host's genetics play an important role in susceptibility or resistance to CanL (Baneth *et al.*, 2008). Epidemiological studies in canine populations from CanL endemic areas have shown that mongrels and autochthonous breeds tend to show a higher resistance to infection than exotic breeds (Cortes *et al*., 2012; Fisa *et al*., 1999; Sanchez-Robert *et al*., 2005; Solano-Gallego *et al*., 2000).

Genetic studies have identified genotypes that may be involved in susceptibility to *L. infantum* infection, for example the DLA-DRB1*01502 allele of the major histocompatibility complex class II (MHC II) (Quinnell *et al*., 2003b), the *Slc11α1* gene (Altet *et al*., 2002), or the canine β-defensin-1 (CBD1) gene (da Silva *et al*., 2017). TAG-8-141 haplotype of *Slc11α1* gene was found to be significantly associated with susceptibility to the disease in boxers (Sanchez-Robert *et al*., 2005), as well as in other breeds (Sanchez-Robert *et al*., 2008), while other authors found no difference in gene expression between phenotypically resistant and susceptible dogs (Bueno *et*

al., 2009; Turchetti *et al*., 2015). Genome-wide studies have also found evidence of high heritability and of genetic determinants for disease progression (Quilez *et al.*, 2012; Utsunomiya *et al.*, 2015).

Other individual non-genetic factors, such as nutritional status, physiologic state (e.g. pregnancy) and concomitant infections and parasitism, have a recognized role in the susceptibility to CanL (Baneth *et al.*, 2008). A recent study found a significant association between low levels of serum vitamin D and symptomatic *L. infantum* infection in dogs, although it has not yet been determined whether the avitaminosis is a cause or a consequence of CanL (Rodríguez-Cortés *et al*., 2017).

1.3.5.2. Innate immune response

The immediate inflammatory reaction produced at the skin level after parasite inoculation by sand flies is a non-specific immune response mediated by macrophages, dendritic cells and neutrophils (Hosein *et al*., 2017). These cells are able to phagocytise and destroy *Leishmania* parasites at the site of inoculation and, together with other molecular components of the innate immune system, are capable of innitiating the development of a long lasting specific adaptive immune response (Hosein *et al*., 2017; Kumar *et al*., 2009; Reis *et al*., 2010).

The role of neutrophils or polymorphonuclear cells (PMN) is still under debate; while it is known that these cells are able to phagocytise and kill *Leishmania* promastigotes, the inability of PMN to completely clear the parasite may favour the infection (Pereira *et al.*, 2017).

Macrophages are the parasite's main host cell and transporter, through which the parasite can multiply and invade other organic systems. Simultaneously, macrophages are also the most effective cells in parasite killing, whenever their effector mechanisms are activated (Saridomichelakis, 2009).

Toll-like receptors (TLRs) are membrane receptors usually expressed on sentinel cells such as macrophages and dendritic cells that recognize pathogen-associated molecular patterns (PAMPs) from infectious agents, including parasites like *Leishmania* (Hosein *et al.*, 2015). The role of TLRs is not completely understood, but they seem to up-regulate pro-inflammatory responses in infected macrophages resulting in parasite killing, while shaping adaptive immunity (Kumar *et al.*, 2009).

1.3.5.3. Acquired immune response

a) Cellular immune response

L. infantum amastigotes are phagocytized and transported inside macrophages from the skin inoculation point to regional lymph nodes. There, macrophages present parasite antigens to T lymphocytes, via their MHC molecules (Saridomichelakis, 2009). An effective immune response after *L. infantum* infection is assumed to be mediated by cytokines released by activated T lymphocytes, namely gamma-interferon (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) (Carrillo and Moreno, 2009; Chamizo *et al*., 2005; Pinelli *et al*., 1995, 1994). These cytokines will then upregulate macrophage ability to kill intracellular *Leishmania* parasites through the activation of inducible nitric oxide synthase (iNOS) and consequent production of nitric oxide (NO), responsible for amastigote apoptosis (Panaro *et al.*, 2008). In susceptible dogs, this mechanism is not elicited and parasite multiplication inside macrophages goes unrestricted, resulting in the lysis of infected cells and the release of amastigotes, which are then able to infect other macrophages (Saridomichelakis, 2009).

L. infantum-specific CMI is characterized by a mixed lymphocyte T helper type 1 (Th1) and type 2 (Th2) responses, in which a predominance of one type or the other will define host susceptibility or resistance to infection (Hosein *et al*., 2017; Koutinas and Koutinas, 2014; Panaro *et al*., 2009; Saridomichelakis, 2009). Resistant animals usually show a predominant Th1 profile, with the corresponding production of protective cytokines (IFN-γ, TNF-α and IL-2), while susceptible dogs seem to display a mixed Th1-Th2 response, together with a decrease of $CD4^+$ T-cells and reduced cytokine release (Saridomichelakis, 2009). $CD8^+$ cytotoxic T-cells are also considered important for host immunity, as they are able to lyse infected macrophages in resistant dogs (Pinelli *et al.*, 1995). However, higher levels of $CD8^+$ T cells have also been found in lymph nodes and peripheral blood of symptomatic dogs (Giunchetti *et al.*, 2008; Moreno *et al.*, 1999). The $CD4^+/CD8^+$ ratio, which reflects the relative changes in both T-cell subset population numbers, is usually diminished in diseased animals and is considered by some authors as a good indicator of susceptibility (Papadogiannakis *et al.*, 2010); nonetheless, other studies have found no relationship between this ratio and disease progression (Miranda *et al.*, 2007; Rosypal *et al.*, 2005).

While the majority of studies tried to characterize *Leishmania*-specific CMI in peripheral blood, some authors demonstrated that immune response to the parasite is organ-specific (Alexandre-Pires *et al.*, 2010; Barbosa *et al.*, 2011; Foglia Manzillo *et al.*, 2006b; Lage *et al.*, 2007; Quinnell *et al.*, 2001; Reis *et al.*, 2009; Rodríguez-Cortés *et al.*, 2016; Sanchez *et al.*, 2004; Strauss-Ayali *et al.*, 2007). Results revealed that different organs may simultaneously present Th1, Th2 or mixed Th1-Th2 profiles, and that these may change throughout the course of the infection (reviewed in Maia and Campino, 2012). These studies highlight the complexity of the CMI developed by the canine host to *L. infantum* infection and provide a possible explanation to the discrepancies observed across different studies which only focused on circulating cytokines.

b) Humoral immune response

Production of specific antibodies against *L. infantum* is not protective and is frequently marked in susceptible dogs, as opposed to resistant infected dogs, which usually present normal or only a moderate increase in antibody levels (Paltrinieri *et al.*, 2010). Amongst Ig isotypes, IgG predominate, whereas IgM, IgE and IgA usually show lower concentrations (Saridomichelakis, 2009) or present contradictory results across studies (Reis *et al*., 2006; Rodríguez-Cortés *et al*., 2007b). In addition to the direct correlation between humoral immune response and the host clinical status, specific anti-*Leishmania* Ig levels are also directly related with tissue parasite density (Reis *et al.*, 2006). In addition to the absence of protective effect, a strong humoral response is one of the main causes of CanL pathogenesis, due to type II (antibody-mediated) and III (immune complex-mediated) hypersensitivity reactions (Brandonisio *et al.*, 1990; Lopez *et al.*, 1996). Immune complex deposition frequently induces renal, muscular, articular, ocular, dermal and vascular damage, while the production of autoantibodies can cause anaemia, thrombocytopenia and myositis (Hosein et al., 2017; Saridomichelakis, 2009).

1.3.6. Commercially approved vaccines against canine leishmaniosis

Given the limited efficacy of chemotherapy in CanL treatment, there is a pressing need for the development of effective prophylactic measures. Mathematical models have identified canine vaccination as the most effective method for reducing both CanL and HL in *L. infantum* endemic areas (Dye, 1996).

The "ideal" CanL vaccine should induce a strong and long-lasting Th1-dominated immunity to control infection progression, as well as a significant reduction in dogs' infectiousness to sand flies (Gradoni, 2015). Additionally, it should be able to protect equally against infection and disease (Alvar *et al*., 2013). Although there are currently three licensed vaccines for CanL (Leish-Tec® in Brazil and CaniLeish® and LetiFend® in Europe), these criteria are not fully met or, in some cases, additional studies are needed to confirm them. Furthermore, the diversity of study designs used to evaluate these vaccines, for which no standardization exists concerning study population characteristics, intervention methods or techniques used to assess infection, restrict any comparison of vaccine performance (Wylie *et al.*, 2014). Assuming a high enough efficacy of current vaccines, an important challenge to *Leishmania* control through vaccination is uptake. To be most effective, vaccination coverage would need to be sufficient to induce herd immunity, whereby a significant reduction in infection at the reservoir and vector levels would induce protection of non-vaccinated individuals and humans (Fox *et al.*, 1971). Finally, CanL vaccination introduces a potential added challenge to the screening and diagnosis of a complex disease (Solano-Gallego *et al.*, 2017a). It is known that the strong seroconversion induced by some CanL vaccines can be detected by current serological diagnostic tests (Marcondes *et al.*, 2013; Martin *et al.*, 2014). The implications of this are the incorrect classification of false-positive individuals (vaccinated but not infected) or, as no vaccine is 100% effective, the difficulty in correctly identifying vaccinated and infected dogs (Solano-Gallego *et al.*, 2017a). In this section, currently available information on each of the marketed vaccines against CanL is outlined.

1.3.6.1. Leishmune®

Leishmune® (Fort Dodge Wyeth, later Zoetis, Brazil) was the first licensed vaccine for CanL, having been registered in Brazil in 2004. It is a second-generation vaccine, composed of the GP36 glycoprotein fraction of *L. donovani*, which bears a fucose-mannose ligand (FML), and the QuilA adjuvant (a plant-derived saponin isolated from the bark of *Quillaja saponaria*) (Borja-Cabrera *et al.*, 2002). Vaccination protocol consists of 3 vaccine doses administered subcutaneously every 21 days to dogs 4 months old or older, followed by annual boosters (Zoetis, n.d.).

Leishmune® vaccine proved to be well-tolerated in a large safety trial which included 600 dogs and where no severe adverse reactions were observed; likewise, mild adverse reactions registered after the first vaccine dose were transient and dissipated before the following vaccine administration (Parra *et al.*, 2007).

Several clinical trials have been conducted, showing vaccine-specific seroconversion and an effective cellular response, either evaluated through LST (Borja-Cabrera *et al.*, 2008, 2002; da Silva *et al.*, 2000) or by *in vitro* characterization of lymphocyte populations and cytokine production in vaccinated dogs (Araújo *et al.*, 2011; Costa-Pereira *et al.*, 2015; de Lima *et al.*, 2010; Moreira *et al.*, 2016). Phase III field studies were performed in CanL endemic areas and included 117 (da Silva *et al.*, 2000) and 85 dogs (Borja-Cabrera *et al.*, 2002), although FML antigen alone and not the complete Leishmune® formulation with the QuilA adjuvant was used in the first study. Vaccine performance was evaluated through an FML-ELISA assay, LST and CanL-induced morbidity and mortality rates. Vaccine efficacy (VE) and protection against infection, severe disease and death due to CanL (Borja-Cabrera *et al.*, 2008) were 76% and 92%, respectively, for the first study (da Silva *et al.*, 2000) while 80% VE and 95% vaccine protection were achieved in the second study (Borja-Cabrera *et al.*, 2002).

When formulated with a higher adjuvant concentration, Leishmune® also proved to be effective on the immunotherapy of experimental CanL (Borja-Cabrera *et al.*, 2010, 2004; Santos *et al.*, 2007). Leishmune® was considered a transmission-blocking vaccine based on the assumption that, as no CanL clinical signs or *Leishmania* DNA could be detected in vaccinated dogs, these could not be infectious to sand flies (Nogueira *et al.*, 2005). For the same purpose, Saraiva *et al.* (2006) demonstrated that FML-induced antibodies present in dog sera were capable of inhibiting *L. donovani* and *L. chagasi* procyclic promastigote-binding to dissected *L.*

longipalpis midguts. In a comparative study of Leishmune® and Leish-Tec® vaccines, 5.1% (2/39) of Leishmune®-vaccinated dogs were infectious to sand flies by xenodiagnosis, against a 36.6% (11/30) infectiousness in the control group (Fernandes *et al.*, 2014).

Vaccination of dogs with Leishmune® was claimed to reduce CanL and HL incidence in Brazilian endemic areas (Palatnik-de-Sousa *et al.*, 2009), which in the case of an increased vaccine coverage, could make it more effective in controlling *Leishmania* infection than dog culling, the method currently adopted by the Brazilian Ministry of Health (Ministério da Saúde Brasileiro, 2014). Conversely, a possible cross-reaction of Leishmune®-vaccinated dogs with the official Brazilian serological tests for CanL screening (Marcondes *et al*., 2013, 2011) would impede the use of this vaccine in areas where seropositive dog culling is regularly performed; however, a sero-cross-reactivity was not confirmed by all studies (Ribeiro *et al.*, 2015).

In 2014, the Brazilian Ministry of Agriculture decided to withdraw Leishmune® vaccine's production and marketing license due to lack of effectiveness evidence in phase III trials (MAPA, 2014).

1.3.6.2. Leish-Tec®

Leish-Tec® (Hertape Calier Saúde Animal, Brazil) is formulated with a recombinant protein A2 from *L. donovani* amastigotes and saponin as vaccine adjuvant. It was licensed in Brazil in 2007 and is currently the only authorized CanL vaccine in that country. It should be administered to dogs of 4 months or older and the primary vaccination course consists of 3 doses, administered subcutaneously at 21-day intervals, followed by annual boosters (Hertape, 2015).

Although Leish-Tec® was shown to induce protective immunity in beagle dogs against a high dose intravenous infection with *L. chagasi*, the parasite was isolated in culture from bone marrow samples of 4 out of 7 vaccinated dogs. The appearance of clinical signs was delayed in the vaccine group (1 year post-infection) when compared with the control group (3-6 months) (Fernandes *et al.*, 2008). A comparative study between Leishmune® and Leish-Tec® found no significant differences between vaccines in elicited humoral response or infection and transmission rates; the only difference detected was a higher rate of adverse reactions in the Leish-Tec® group (Fernandes *et al.*, 2014).

Side effects after Leish-Tec® administration were not found to be severe in a safety analysis, which registered a 3.09% rate of mild, site-specific, adverse reactions in vaccinated dogs, against a 0.68% rate in placebo animals (Toepp *et al.*, 2017). Leish-Tec® did not induce unspecific seroconversion in the large majority of vaccinated animals (69/70), showing no cross-reactions with anti-*Leishmania* promastigote antigen (LPA) or the Dual Path Platform test (DPP), based on the recombinant antigens K39 and K26 (de Souza Testasicca *et al.*, 2014). Vaccination also significantly reduced the infectiousness of dogs to sand flies, as demonstrated by xenodiagnosis (Fernandes *et al.*, 2014; Regina-Silva *et al.*, 2016).

The first Leish-Tec® field trial included more than 500 dogs, equally allocated to vaccine and control groups. Animals were followed up by clinical, serological and parasitological exams over 18 months and a significant reduction in the number of CanL cases was observed in the vaccine group. Different VE were estimated according to results in different diagnostic tests and varied from 58.1% to 80.8% (Regina-Silva *et al.*, 2016). A more recent efficacy trial, apart from presenting several methodological inaccuracies, showed very few advantages in the use of Leish-Tec® vaccine. Although reporting a significant difference in incidence of infection between vaccine (27%; 40/151) and control (42%; 33/78) groups, described a two-fold higher proportion of diseased dogs amongst the immunized animals (44%; 18/40) when compared to the placebo group (21.2%; 7/33) (Grimaldi *et al.*, 2017).

1.3.6.3. CaniLeish®

CaniLeish® vaccine (Virbac, France) was released in Europe in 2011 (EMA, 2011). It is a second generation vaccine and is composed of purified excreted-secreted proteins of *L. infantum* (LiESP) and a saponin adjuvant (QA-21) from a purified fraction of *Quillaja saponaria* (Moreno *et al.*, 2012). Vaccination protocol consists of one vaccine dose administered subcutaneously to animals older than 6 months every 21 days for a total of 3 doses, followed by single dose annual boosters (EMA, 2011). According to the pharmacovigilance data reported by Virbac in October 2015, more than 1.8 million doses of CaniLeish® have been sold during the first 3.5 years of marketing in the European Economic Area, Switzerland and Tunisia (Breton *et al.*, 2015).

Several studies focusing on the LiESP antigen associated with another adjuvant (muramyl-dipeptide, MDP) have been published prior to CaniLeish® release (Bourdoiseau *et al.*, 2009;

Holzmuller *et al.*, 2005; Lemesre *et al.*, 2007, 2005), demonstrating a good protective effect in vaccinated dogs. The first study performed on CaniLeish® measured the impact of a primary vaccination course in beagle dogs on selected humoral and cellular markers of immunity (Moreno *et al.*, 2012). Results showed that only vaccinated dogs produced antibodies to both LiESP and parasite surface antigen (PSA), with a bias towards an IgG2 profile in the presence of PSA. Vaccination also induced a proper CMI, with *in vitro* isolated PBMC from vaccinated animals showing a specific T cell response and IFN-γ production when exposed to soluble *Leishmania* antigens (SLA). Monocyte-derived macrophages from the vaccinated group presented, when infected with *L. infantum* promastigotes and exposed to autologous lymphocytes, an increased parasite killing capacity, inducible nitric oxide synthase (iNOS) expression and NO_2 production. The same immunity markers were evaluated at different time points during the first year after vaccination (Moreno *et al.*, 2014), showing that a similar immune profile persisted during this period of time.

One year after completing the vaccine primary course and before the annual booster, an infectious intravenous challenge was performed and animals were monitored for a year (Martin *et al.*, 2014). As in the previous studies, the same humoral and cellular patterns were observed, showing that vaccinated dogs maintained a protective CMI throughout the study and consistently presented seroconversion after exposure to total *L. infantum* antigens, while in the control group this humoral recognition was only observed in infected animals.

The pre-licensing randomised efficacy field trial of CaniLeish® included 90 beagle dogs introduced in two CanL endemic areas in Italy and Spain (Oliva *et al.*, 2014). From these, 46 animals were randomly assigned to the vaccine group and 44 were kept as controls. The vaccination phase was held in controlled conditions, during which vaccine safety was assessed by regular clinical examinations and serological responses to vaccination were quantified. Observed vaccine side effects were local oedema and crusting followed by local alopecia, all resolving spontaneously. Animals were then transferred to the study sites and naturally exposed to *L. infantum* vectors for 2 years. The results of this study demonstrated significant differences between groups in the number of dogs showing active infection (33.3% in the control group vs. 12.2% in the vaccine group) and the number of symptomatic cases (23.1% in the control group vs. 7.3% in the vaccine group). However, no significant difference was observed in the proportion of dogs presenting a positive PCR result on at least one occasion throughout the trial, confirming that the vaccine does not prevent parasite invasion of “deep” tissues (Martin *et al.*, 2014). From the positive PCR dogs, some reverted to a *Leishmania*-free status during the observation period, which was considered more frequent in the vaccine

group than in placebo dogs. Based on these results, CaniLeish® VE in preventing clinical signs was 68.4% and the vaccine protection level, defined as the percentage of non-symptomatic vaccinated animals, was 92.7%. An odds ratio of 3.8 expressed the difference in the prevention of clinical disease between the groups. An important additional conclusion of this study is that IFAT alone cannot be used to test vaccinated dogs for *Leishmania* infection, as animals from this group consistently presented positive titres due to vaccine-induced antibodies.

A more recent study, which evaluated the efficacy of two insecticide dog collars and CaniLeish® vaccine in the prevention of CanL in highly endemic areas, found no statistically significant differences in the frequency of active *L. infantum* infections between vaccinated (n=54) and control (n=55) groups (Brianti *et al*., 2016). This trial enrolled mixed breed dogs that were kept in 4 dog kennels in CanL endemic regions of Italy for one year. *L. infantum* infection was assessed through PCR in bone marrow and skin samples. Positive detections were followed by positive IFAT titres and asymptomatic cases were also registered. Similarly, no differences were observed in the frequency of symptomatic infection between vaccine and control individuals.

The infectiousness potential of infected CaniLeish®-vaccinated dogs was assessed through xenodiagnosis (Bongiorno *et al.*, 2013). Ten beagle dogs at different stages of *L. infantum* infection were enrolled in the study (6 vaccinated animals and 4 controls). The results showed no difference in the rate of sand fly infection in symptomatic dogs between groups, but infectiousness levels were considered lower in the vaccinated cohort.

Starita *et al.* (2016) evaluated the impact of CaniLeish® vaccination in several haematological, biochemical and serological parameters of healthy canine blood donors. Slight hyperproteinaemia and a rise in some globulin fractions were the only observed changes detected in vaccinated dogs. Serologic diagnosis of *L. infantum* infection with IFAT proved unreliable, as it was not able to differentiate between vaccine and infection-induced antibodies, confirming the results presented in previous studies (Martin *et al.*, 2014; Oliva *et al.*, 2014).

1.3.6.4. LetiFend®

LetiFend® (Laboratorios LETI, Spain) was licensed in Europe in February 2016 (EMA, 2016). It is a recombinant vaccine containing a chimerical protein (protein Q) formed by five antigenic fragments from four different *L. infantum* proteins (ribosomal proteins LiP2a, LiP2b and LiP0 and the histone H2A), to which no adjuvant has been added (Carcelén *et al*., 2009). Vaccination protocol consists of one vaccine dose, followed by annual boosters, and should only be used in dogs aged 6 months or older (EMA, 2016).

During phase II clinical trials, immunization results with protein Q were not consistent. Its use with live bacillus Calmette-Guérin (BCG) adjuvant provided an estimated 90% protection against infection and CanL-induced morbidity (Molano *et al.*, 2003). However, BCG is not considered a safe adjuvant in dogs, as it frequently induces local pain, skin irritation, abscesses, ulcers and, occasionally, hypersensitivity reactions (Poot *et al.*, 2009; Reguera *et al.*, 2016). Protein Q was then combined with 6 different adjuvants, and the results showed that protection was not induced by any of the candidate vaccines, suggesting that live BCG could be responsible for the protective effect against *L. infantum* infection in dogs (Poot *et al.*, 2009). Finally, the use of protein Q with no adjuvants (which corresponds to LetiFend® formulation) was able to demonstrate a protective effect in vaccinated dogs in a third clinical trial (Carcelén *et al.*, 2009).

The LetiFend® phase III trial included 549 dogs (275 vaccinated and 274 controls) exposed to natural infection in two CanL endemic areas in France and Spain during a two-year period. Humoral response to protein Q antigen and SLA, prevalence of infection defined by presence of the parasite in lymphoid organs and clinical assessment of all animals were performed at pre-determined time-points. According to the results of this field study, LetiFend® showed a 72% VE in the prevention of CanL clinical signs and reduced the likelihood of confirmed CanL cases or development of clinical signs in vaccinated dogs vs. placebo dogs in 5 and 9.8 times, respectively (Fernández Cotrina *et al.*, 2018). Additionally, no general or local adverse effects were observed after LetiFend® administration during laboratory or field studies (Carcelén *et al.*, 2009; Fernández Cotrina *et al.*, 2018) and vaccination does not seem to elicit false-positive results in *L. infantum* serological diagnostic tests (Iniesta *et al*., 2016).

2. OBJECTIVES

2. OBJECTIVES/OBJECTIVOS

The main objective of this thesis is to obtain independent results on the efficacy of canine leishmaniosis vaccine CaniLeish® under field conditions in a population of native dogs from an endemic region.

El objetivo general del presente trabajo es obtener datos independientes acerca de la eficacia de la vacuna CaniLeish® frente a la leishmaniosis canina, dirigidos a supervisar el control de la enfermedad, a través de un estudio independiente y en condiciones de campo en una población de perros natural de zona endémica.

The specific objectives are:

Los objetivos específicos son:

1. To characterize the distribution of *Leishmania infantum* infection in the canine population of Girona province (Catalonia, north-eastern Spain).

   Estudiar la distribución de la leishmaniosis canina por *Leishmania infantum* en la provincia de Girona (Cataluña, noreste de España).

2. To determine possible risk factors associated with canine leishmaniosis infection in dogs in the study area

   Determinar los posibles factores de riesgo asociados a la distribución de la leishmaniosis canina en el área de estudio.

3. To investigate the exposure of dogs to *Leishmania infantum* vectors through the detection of anti-sand fly saliva antibodies in the canine host.

   Analizar la exposición de los perros a los flebotomos vectores de *Leishmania infantum* a través de la detección de anticuerpos frente a su saliva.

4. To assess the possible impact of vaccination with CaniLeish® in *Leishmania infantum* serological surveillance of canine populations from endemic areas.

   Determinar el posible impacto de la vacunación con CaniLeish® en estudios serológicos y en la vigilancia de la leishmaniosis canina, causada por *Leishmania infantum*, en áreas endémicas.

5. To evaluate the performance of a vaccine against CanL (CaniLeish®) in preventing active *Leishmania infantum* infection in dogs from endemic areas under field conditions.

   Evaluar la eficacia de la vacuna CaniLeish® en la prevención de la leishmaniosis canina de una población natural de perros de zona endémica.

3. MATERIALS AND METHODS

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3.1. STUDY REGION

The present study took place in Girona province (42°10'0"N, 2°40'0"E; area of 5,910 km^2), the most north-eastern province of Catalonia region (Spain). It is delimited by the Mediterranean Sea (to the east), France (to the north), and by Barcelona and Lleida Catalan provinces (to the south and west, respectively) (Figure 4). The province occupies a surface of 5,910 km² and it is divided into nine counties with altitudes ranging from zero meters above sea level (m a.s.l.) to 2,910 m a.s.l. (Table 5).

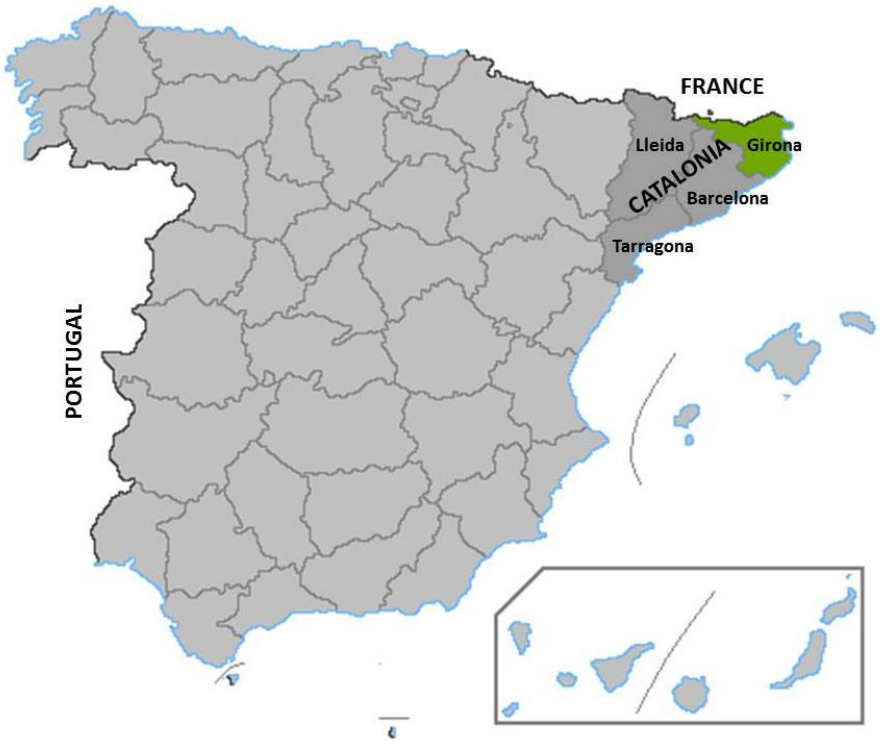


Figure 4. Location of the province of Girona (available at: https://nn.wikipedia.org/wiki/Provinsen_Girona#/media/File:Location_Girona_province.png)

Habitats and climates vary from Mediterranean on the coast to Alpine in the Pyrenees. Annual mean temperatures range from 16°C in the southern counties to 5°C in the north of the province, though maximum and minimum temperatures can reach 39°C and -16°C, respectively. Mean relative humidity varies from 61% to 81% and average annual rainfall ranges from 550 mm to 1350 mm (Servei Meteorològic de Catalunya, 2016) (Table 6).

Table 5. Girona counties. Geographical position (2015) and demography (2017).

Counties	Altitude (m a.s.l.)	Longitude (E)		Latitude (N)		Surface (km^2)	Population (inhabitants)	Population density (inhab/km^2)
		Oriental extrem	Occidental extrem	Septentrional extrem	Meridional extrem			
Girona counties	0-2,909	3°19'59,94"	1°57'27,27"	42°29'0,09"	41°39'4,42"	5,910.00	755,716	127.87
Alt Empordà	0-1,451	3°19'59,94"	2°32'37,13"	42°29'0,09"	42°5'57,10"	1,357.54	139,705	102.91
Baix Empordà	0-533	3°14'7,08"	2°53'13,71"	42°8'24,18"	41°45'14,81"	701.69	132,906	189.41
Cerdanya*	1,088-2,914	2°0'59,08"	1°34'57,72"	42°30'20,01"	42°16'51,03"	546.69	17,623	32.24
Garrotxa	146-1,557	2°46'53,44"	2°20'1,90"	42°20'7,35"	42°0'50,86"	734,62	56,184	76.48
Gironès	34-988	2°59'18,89"	2°37'55,45"	42°7'6,23"	41°45'32,00"	575.6	188,083	326.76
Pla de l'Estany	96-880	2°54'19,72"	2°38'17,22"	42°12'48,52"	42°1'59,10"	262.78	31,738	120.78
Ripollès	690-2,909	2°33'35,26"	1°57'27,27"	42°26'24,29"	42°6'30,27"	956.62	24,999	26.13
Selva	0-1,705	2°58'18,95"	2°24'28,49"	42°3'36,44"	41°39'4,42"	995.04	167,837	168.67
Osona**	400-1,697	2°30'29,01"	1°59'12,40”	42°10'38,55"	41°43'39,39”	1,245.20	156,572	125.7

Source: Institut d'Estadística de Catalunya (http://www.idescat.cat/pub/aec/200; https://www.idescat.cat/pub/?id=aec&n=208) and Diputació de Girona (https://www.enciclopedia.cat)

*Cerdanya county is part of Girona (eleven municipalities in the oriental region) and Lleida (six municipalities) provinces.
**Osona county belongs principally to Barcelona province, but there are three municipalities in the oriental region that are within the boundary of Girona province.

Table 6. Metereological data from Girona counties (2016).

Counties	Stations	Altitude (m)	Temperature (°C)					Precipitation (mm)	Humidity (%)	Wind speed (m/seg)	
			Annual average	Average maximum	Average minimum	Absolute maximum	Absolute minimum	Annual	Relative	Average speed	Dominant direction
Alt Empordà	Roses	24	16.6	21.6	11.8	35.5	-0.9	545.9	65	3	N
Baix Empordà	la Bisbal d'Empordà	29	15.4	22.1	9.1	36.6	-3.4	603.8	73	1.9	S
Cerdanya	Das	1.097	8.7	17.8	0.8	34.3	-12.7	553.5	69	2.5	E
Garrotxa	Olot*	433	:	:	:	38.2	:	:	:	:	:
Gironès	Girona	72	14.8	22.6	7.8	36.3	-7	701.2	77	1.3	S
Pla de l'Estany	Banyoles	176	15.4	21.6	10.1	37.6	-2.6	658.3	71	2	NW
Ripollès	Sant Pau de Segúries	852	10.4	17.6	4.4	33.8	-7.5	936.2	80	1.2	SW
Selva	Anglès	150	14.8	22.3	8.1	39.3	-5.8	653.1	74	1.3	W

Source: Departament de Territori i Sostenibilitat, Servei Meteorològic de Catalunya, Institut d'Estadística de Catalunya (http://www.idescat.cat/pub/?id=aec&n=214&t=2016)

*Olot - Pla de Baix (DC), inactive since the 21st of July 2016

3.2. STUDY LOCATIONS AND STUDY POPULATION

For this work, a total of 593 dogs living in 36 kennels and 26 localities in the province of Girona were sampled from 2012 to 2016. These dogs were all included in the *L. infantum* seroprevalence study, described in section 3.3. The study of humoral immune response to vector saliva and CaniLeish® vaccine trials only included animals sampled between 2015 and 2016, totalling 406 dogs distributed across 21 kennels and 16 localities. Sampling and recruitment of dogs for these studies is described in sections 3.2.1 and 3.2.2. The work reported in these sections does not include field work performed before 2015.

3.2.1. Preliminary assessment of *Leishmania infantum* infection in the study locations

Field work for the project started in July 2015 by a serological survey in 16 dog kennels distributed over 11 localities.

A total of 300 dogs were sampled and tested for the detection of anti-*L. infantum* antibodies. This cross-sectional study was intended to evaluate the level of infection at the different dog kennels and to estimate point *L. infantum* infection prevalence (Annex 1).

During September 2015, sand fly surveys were performed in order to confirm the presence of *L. infantum* vectors in the study locations. One to two CDC light traps were placed for one night in each dog kennel. Collected sand flies were identified and the different phlebotomine species abundance and frequency were registered. Sand fly species captured at each dog kennel are described in Annex 2.

The following serological survey took place in February 2016 and included 293 dogs. From these, 106 were dogs sampled for the first time, the majority of them belonging to five additional dog kennels in four different localities. The remaining 187 corresponded to dogs sampled for the second time. By comparison with the first survey, annual point *L. infantum* infection incidences were estimated (Annex 1). Apart from the locations selection, the 2016 survey also allowed for a pre-selection of individuals to be enrolled in the vaccine field trial.

3.2.2. Location and individual selection for the study of humoral immune response to vector saliva and CaniLeish® vaccine trial

In February 2016, dogs from 18 dog kennels and 12 localities were enrolled in the vaccine field trial. The selection of dog kennels was based on the detection of canine *L. infantum* infection cases and the presence of phlebotomine vectors. Thus, one dog kennel sampled in July 2015 (Annex 1; kennel 15) and one dog kennel sampled in February 2016 (kennel 21) were not included in the study due to the absence of cases of *L. infantum* infection. A third kennel was also excluded when the dog owner refused to participate in the vaccine trial (kennel 17).

Individuals were selected according to their serological result to *L. infantum* (only seronegative dogs were enrolled in the vaccine study), followed by additional criteria, as described in Figure 5.

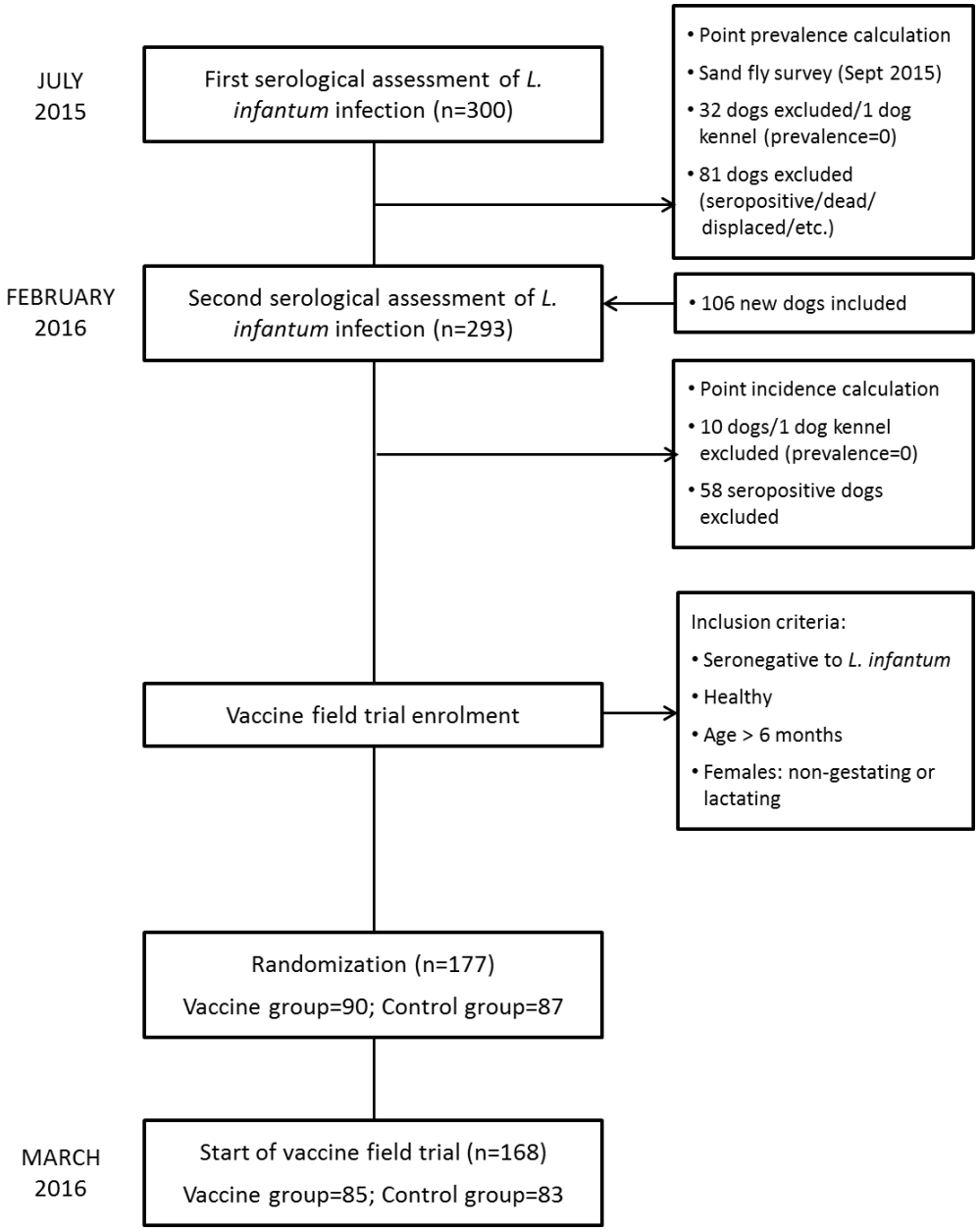


Figure 5. Flow chart of pre-vaccination screening and criteria followed for selection of individuals for the vaccine field trial.

Due to the reduced number of dogs in some dog kennels, their geographical proximity and the similar infection prevalence and/or incidence estimated for kennels in the same localities, a decision was made to group dogs by locality instead of dog kennels. This way, trial dogs were divided by “locations” in the following studies, as shown in Figure 6. Dog density per study location varied between 4 and 23.

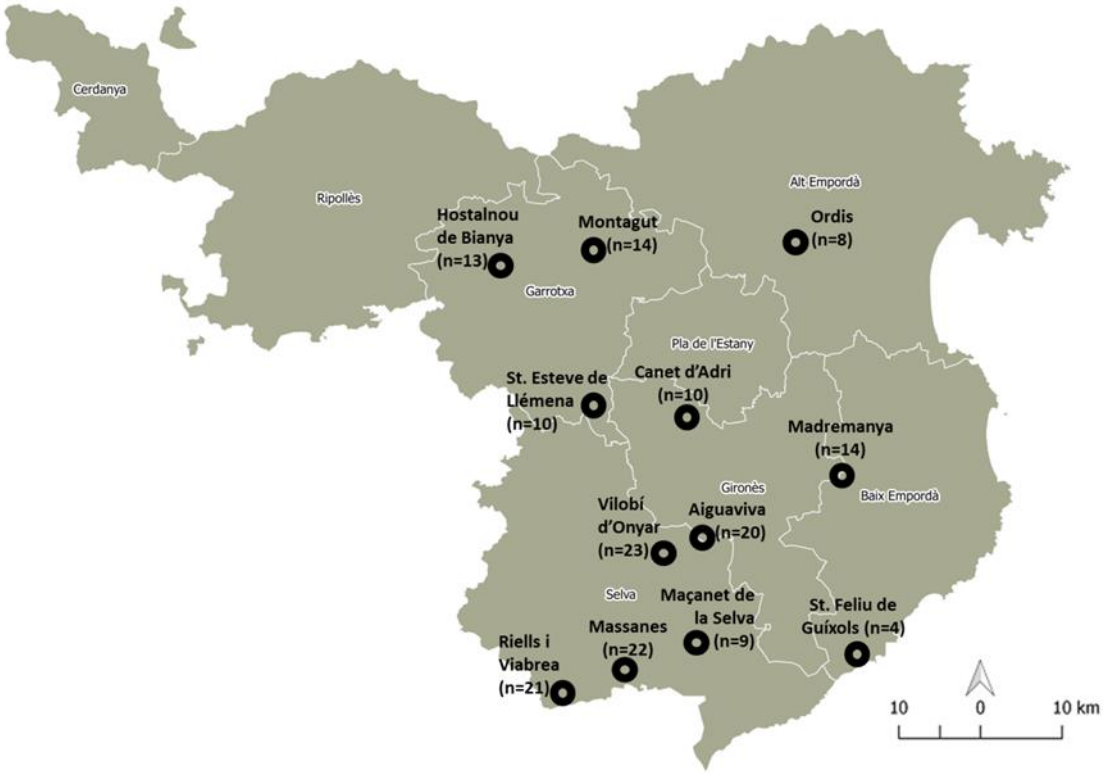


Figure 6. Map of Girona province. Field trial locations are marked in black circles; the number of study dogs per location (n) is presented.

The vaccine study population was initially composed of 177 dogs (90 in the vaccine group and 87 in the control group), from which 168 (85 vaccine and 83 control) completed the first vaccination phase and were included in the field trial. The majority of the dog population was composed of hunting dogs (87.1% of the vaccine group and 83.9% of the control group), but breeding (8.9% of the total dog population), racing (6.5%) or pet dogs (0.6%) were also represented; no statistically significant differences in dog purpose between groups were observed (χ^2=3.66, p=0.3). Crossbred dogs represented 55.3% of the vaccine group and 45.8% of the control group (χ^2=1.52, p=0.218), while 55.3% and 65.1% of the vaccine and control group, respectively, were males (χ^2=1.67, p=0.196). In total, 60.1% of the dog population were males and this high male/female ratio can be explained by the exclusion of gestating or lactating females, either at the beginning of the study or expected to be so during the duration of the trial. Mean dog age in the vaccine group was 3.3 years (SD=2.9) and in the control group was 3.4 years (SD=3.0), ranging from 6 months to 11 years (χ^2=6.58, p=0.832).

All animals were kept in open-air facilities, mostly in rural and periurban areas. Furthermore, no specific anti-sand fly insecticide treatments were applied in any location, providing the ideal conditions for dog exposure to *L. infantum* vectors.

3.3. SEROPREVALENCE OF CANINE *Leishmania infantum* INFECTION IN GIRONA PROVINCE AND IDENTIFICATION OF RISK FACTORS FOR THE INFECTION

The *L. infantum* seroprevalence study performed in Girona province included data from canine serological surveys performed throughout the province between 2012 and 2016. From these, 406 individuals were sampled in the context of this study, in July 2015 and February 2016, as described in section 3.2.1.

3.3.1. Study design

Study individuals were recruited through local veterinarians registered in the regional veterinary association (Col.legi Oficial de Veterinaris de Girona – COVGi). After an informative talk about CanL, a number of professionals were willing to participate by being the link between their clients and the project researchers. Dog owners consisted mostly of wild boar hunters, who usually keep large packs of dogs, allowing the sampling of several animals in the same location. Four cross-sectional surveys were conducted between April 2012 and March 2016 in different locations of north-eastern and Pyrenean areas of Spain, in Girona province, including 36 sampling stations in 26 localities (Figure 7). Blood samples were collected from all animals by cephalic or jugular venepuncture to 5 mL EDTA tubes and preserved at -40°C until further processing.

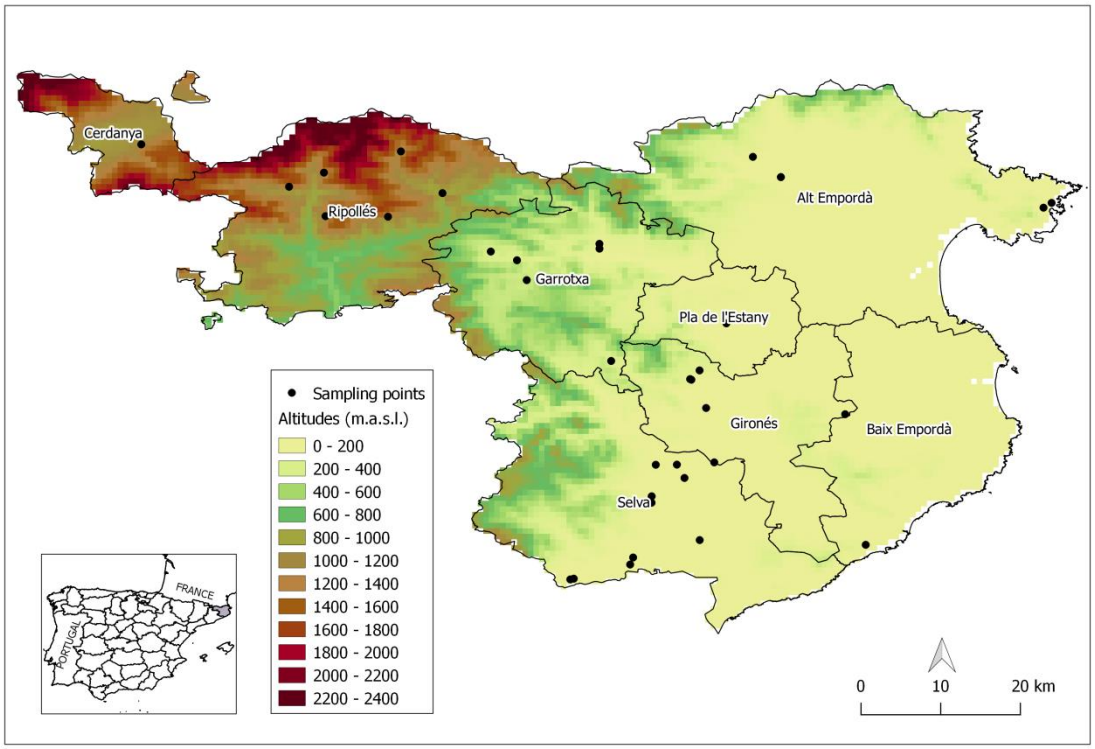


Figure 7. Map of altitudinal distribution in Girona province. Sampling locations are marked as black dots.

3.3.2. Serological detection of *Leishmania infantum* infection

Plasma was obtained and preserved at -40°C. Samples were analysed by an in-house enzyme-linked immunosorbent assay (ELISA) for the presence of anti-*L. infantum* antibodies, using a technique previously described (Ballart *et al*., 2013a; Fisa *et al*., 2001; Iniesta *et al*., 2002; Riera *et al*., 1999). Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar®) previously coated with sonicated whole promastigotes at a protein concentration of 20 µg/mL in 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase ((1:30,000, Sigma®) was used as conjugate and reactions were stopped with H_2SO_4 3M when a pre-determined positive control serum reached an optical density of 450 read at 450 nm. Sample optical densities were then read at 492 nm. All samples were run in duplicate and calibrator, positive and negative serums were included in all plates. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The cut-off was established at 24U.

3.3.3. Data collection

In addition to sample collection, information was gathered from each sampling location (geographical coordinates, altitude, county, nearest locality, type of habitat, and presence of other domestic and farm animals) and each animal's individual characteristics (sex, age, breed, given use, type of night shelter, and presence of visible CanL clinical signs). Clinical exams were performed by veterinarians and the criteria for classifying dogs as "symptomatic" were the detection of the following clinical signs: weight loss, lymphadenomegaly, periocular or diffuse alopecia, onychogryphosis, ocular lesions, and/or pale mucous membranes. Dog owners were asked about their previous knowledge of CanL, as well as control measures regularly taken to prevent the disease. This data was then used to determine possible risk factors associated with CanL seroprevalence in the population studied. Data collected from each sampled dog and the questionnaire completed by the dog owners is presented in Annex 3.

3.3.4. Statistical analysis

True seroprevalence was calculated following the method described in Cortes *et al*. (2012). The formula used was: true prevalence (TP) = [apparent prevalence (AP) – 1 + test specificity (Sp)] / [test sensitivity (Se) – 1 + Sp]. Confidence intervals for true prevalence were also calculated with the following formula: TP 95%CI = 1.96 x √ [AP x (1-AP) / sample size (n) x (Se+Sp-1)]. The *L. infantum* in-house ELISA has a specificity of 90% and a sensitivity of 85%, when the chosen cut-off is used. These values were calculated based on a population of 77 dogs (Fisa *et al*., 2001; Iniesta *et al*., 2002). Reference positivity status for *L. infantum* infection was determined by parasite detection (culture and/or direct exam and/or PCR). ELISA sensitivity and specificity analysis is provided in Annexes 4 to 6.

The relationship between CanL seropositivity and a series of individual and location variables was assessed through a mixed logistic regression model. The choice of variables to analyse, as well as the categories defined, were based on those used in previous publications (Ballart *et al*., 2013a; Gálvez *et al*., 2010b; Martín-Sánchez *et al*., 2009) and adapted to the characteristics of the present study. In summary, the covariates considered in the analysis were: altitude (<800/>800 m.a.s.l.), type of habitat (rural or between villages/periurban or at the edge of villages/urban or inside villages), presence of other animal species (yes/no), sex (male/female),

age (<1 to 13 years, introduced as a continuous variable), breed (purebred/crossbred), use given (hunting/breeding/others, with "others" including racing and pet dogs), night shelter (indoors/outdoors), dog owner knowledge of preventive methods against CanL (yes/no), dog owner use of preventive methods against CanL (yes/no) and dog owner use of prophylactic methods against other arthropods (yes/no).

A bivariate logistic regression analysis was performed, in which the relationship between the outcome variable ("dog seropositivity") and each explanatory variable listed above was assessed individually. Statistical significance was set at $p<0.05$. This was followed by a multivariable mixed logistic regression analysis, in which non-significant covariates ($p>0.05$) were sequentially deleted through a backward stepwise selection method until a final model was obtained. In this model, "Locality" was introduced as a random-effects variable to account for geographic clustering of the data (Alonso *et al*., 2010; Ballart *et al*., 2013a) and the year of the survey was included as a fixed-effects variable.

The association between CanL seroprevalence calculated per dog shelter and owner's perception of risk of infection (graded in percentage categories from 0 to 90-100%) was assessed through a Spearman's coefficient correlation.

All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX, USA).

3.4. EVALUATION OF DOG EXPOSURE TO *Phlebotomus perniciosus* THROUGH THE DETECTION OF ANTI-SAND FLY SALIVA ANTIBODIES IN THE CANINE HOST

3.4.1. Study design

The study included a population of 176 dogs distributed across 12 locations, as described in section 3.2.2. Individuals were sampled before, during and after the *L. infantum* transmission season, at 5 pre-determined time points: February, August and October 2016, and January and April 2017.

3.4.2. Sand flies and salivary proteins

A colony of *P. perniciosus* was reared under standard conditions as described previously (Volf and Volfova, 2011). Salivary glands were dissected from 4–6 day-old females, pooled at a concentration of 1 salivary gland per 1 µl of 20 mM Tris buffer with 150 mM NaCl and stored at -80 °C. The *P. perniciosus* 43 kDa yellow-related recombinant protein (rSP03B, Genbank accn. DQ150622) was obtained from Apronex s.r.o. (Prague, Czech Republic) and quantified by the Lowry method (Bio-Rad Laboratories, Inc.) following the manufacturer's protocol.

3.4.3. Serological detection of dog exposure to sand flies

Anti-*P. perniciosus* IgG was measured by an in-house enzyme-linked immunosorbent assay (ELISA) as described previously (Kostalova *et al*., 2015). All samples from a single dog were processed in the same plate. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (40 ng per well, equivalent to 0.2 salivary gland) or with rSP03B (5 µg/ml) in 20 mM carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4 °C. Plates were then blocked with 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine plasma were diluted 1:200 for SGH and 1:100 for rSP03B in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. The reaction was stopped with 10% H_2SO_4 and absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Tecan®). Each sample was tested in duplicate and positive and negative controls were included in each plate. To account for the variability between plates, sample OD readings were normalized by dividing them by the mean OD of positive controls run in the same plate (Sanchez *et al*., 2002). The normalized OD values were multiplied by 100. Positivity cut-offs were calculated as the mean plus 3 standard deviations from 14 dog samples from a non-endemic area.

3.4.4. Serological detection of *Leishmania infantum* infection

The serological technique used to assess *L. infantum* infection was an in-house ELISA described in section 3.3.2.

3.4.5. Statistical analysis

Statistical analyses were performed using R software (http://cran.r-project.org/) and Stata 15 software (StataCorp LP, College Station, TX, U.S.A.).

Correlations between IgG responses to *P. perniciosus* SGH and rSP03B and between each one of the salivary antigens and anti-*L. infantum* IgG levels were tested by the Spearman rank correlation test. Median OD values between time points were compared using the Wilcoxon signed rank sum test.

The relationship between anti-SGH and anti-rSP03B antibodies and sampling month, *L. infantum* infection status and location was tested by fitting multilevel linear regression models, taking into account the correlation between repeated measures of the same dogs over time. In the models, log-transformed anti-saliva or rSP03B normalized OD values were considered as continuous dependent variables and sampling month, *L. infantum* infection and location as categorical predictor variables. In order to assess variations in OD between the first sampling month and those following, “February 2016” was set as reference level for this variable. Likewise, the locality with the lowest median OD (“Aiguaviva”) was considered to be the reference for the variable location. Finally, “seronegative” was set as the reference level for the variable *L. infantum* infection. The random component included dog and time to allow for variation at the intercept (between dogs) and the slope (over time). The inclusion of “dog” as a random effects variable significantly improved both models, with a between dog variance of 48% for SGH and of 47% for the rSP03B model. A $p<0.05$ was considered to indicate statistical significance.

3.5. CANILEISH® VACCINE FIELD TRIAL: IMPACT OF VACCINATION IN SEROPREVALENCE STUDIES AND VACCINE EVALUATION IN NATIVE DOG POPULATIONS

3.5.1. Study design

At the beginning of the trial, in March 2016, 168 dogs were selected from a population of 406 dogs, as described in section 3.2. Dog owners were previously informed of all details of the study and signed an informed consent before the start of the trial.

Inclusion criteria for the vaccine study were those recommended by CaniLeish® manufacturer: seronegativity to *L. infantum*, normal clinical exam, minimum age of 6 months, and non-gestating or lactating females (either at the beginning of the study or expected to be so during the trial). Furthermore, any dogs that had ever received a vaccine for CanL were also excluded (Figure 5).

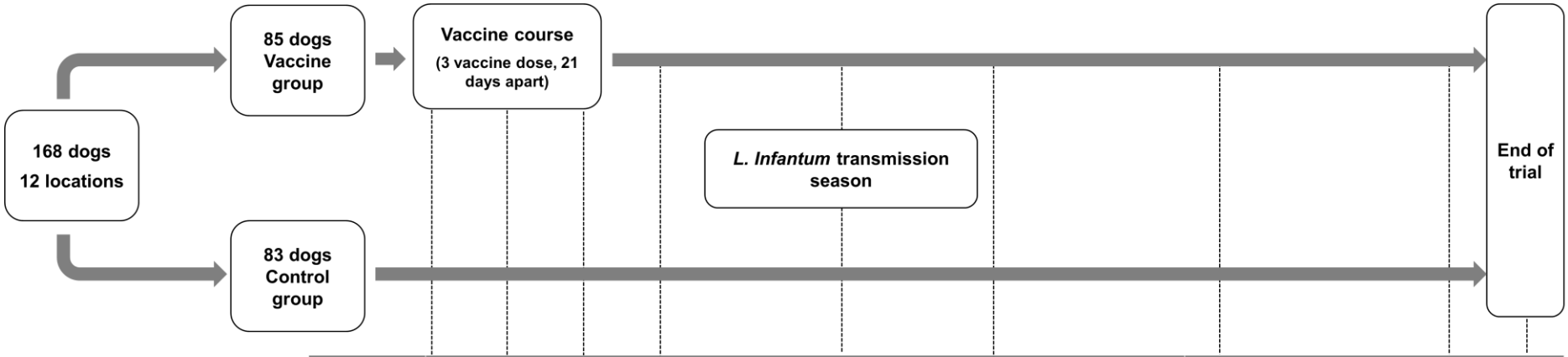
Sample size was calculated assuming a 1:1 ratio between the two experimental groups, an expected 17.6% incidence of *L. infantum* infection in the control group, 3.6 times fewer cases of active infection in the vaccine group, 10% estimated losses during 1 year trial, a power of 0.8 and a significance level of 0.05 in a two-sided test. Final sample size was constrained by the number of individuals available and the limitations of the research team to follow a larger group of dogs during the one year trial.

Selected animals were distributed over 12 locations, with the number of individuals per location ranging from 4 to 23 (Figure 6). Dogs were randomly assigned to either vaccine (n=85) or control (n=83) groups. As different locations had shown distinct infection levels, individual allocation to study groups was first stratified per dog kennel and then randomized. This way, an even proportion of dogs were assigned to each study group in each location, avoiding possible result bias introduced by distinct infection pressures. After vaccination, dogs were kept with their owners, under their usual housing and husbandry conditions, and naturally exposed to an *L. infantum* transmission season.

As recommended by CaniLeish® vaccine manufacturer, all dogs from both groups were dewormed with a mixture of febantel, pyrantel pamoate and praziquantel prior to vaccination.

Individuals from the vaccine group received a three dose vaccine course, 21 days apart. Dogs in the control group did not receive any vaccine dose.

The vaccine field study took place between March 2016 and May 2017. During this period, dogs were sampled at pre-determined time points, at which different samples were obtained. Blood was collected from the cephalic or jugular veins to EDTA tubes (for serology and clinical blood analysis) or heparin tubes (for peripheral blood mononuclear cells (PBMC) isolation and cellular-mediated immunity tests). Lymph node samples were collected by fine needle aspiration and placed in 100 µL of sterile 0.9% sodium chloride solution. Blood and lymph node samples were frozen at -40°C and PBMC were preserved in liquid nitrogen until processing. Follow-up samples from the same individual were analysed in parallel. The field study chronogram, which describes sampling months, number of dogs sampled at each time point, type of sample collected and laboratory tests performed, is presented in Figure 8



	2016										2017				
	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Timepoints	Vac1	Vac2	Vac3	1M		4M		6M			9M			12M	
Days	-42	-21	0	28		120		180			270			365	
No. dogs	168 (85V+83C)	165 (85V+80C)	168 (85V+83C)	167 (85V+82C)		33 (17V+16C)		161 (81V+80C)			152 (75V+77C)			145 (71V+74C)	33 (20V+13C)
Samples collected	WB	WB	WB	WB		WB		WB			WB			WB	WB LN aspirate
Tests performed	Serology PBMC CBC BQ PTG	Serology	Serology	Serology PBMC		Serology		Serology			Serology PBMC			Serology	LN qPCR CBC BQ PTG

Figure 8. Vaccine field trial chronogram. Sampling months, number of dogs sampled, samples collected and tests performed at each time point are presented.
Vac1: first vaccine dose (also T0); Vac2: second vaccine dose; Vac3: third vaccine dose; 1M: 1 month after the third vaccine dose; 4M, 6M, 9M and 12M: as in 1M; WB: whole blood; PBMC: peripheral blood mononuclear cells; CBC: complete blood count; BQ: biochemical plasma profile; PTG: protein electrophoretogram; LN aspirate: aspirate of popliteal lymph node; LN qPCR: real-time PCR on lymph node aspirate.

3.5.2. Vaccine safety assessment

After each vaccine dose, dog owners were asked to monitor their dogs and to report any adverse clinical signs observed to the researchers. Periodical revisions by the veterinarians of the team were also performed.

3.5.3. Serological follow-up

The serological technique used to assess *L. infantum* infection is described in section 3.3.2.

Serological assessments were performed at eight time points throughout the study (Figure 8). An increase of 4-fold ELISA units when compared with the same individual's basal values (ELISA units measured at T0) was considered evidence of seroconversion to *L. infantum* (Paltrinieri *et al*., 2016; Solano-Gallego *et al*., 2011, 2009).

3.5.4. Molecular assessment

L. infantum qPCR on lymph node samples was performed in suspected cases of CanL and in the last sampling time point for seropositive dogs. DNA was extracted from lymph node aspirates using the High Pure PCR Template Preparation Kit (Roche®), following the manufacturer's instructions. Quantitative PCR was performed in all samples as described elsewhere (Martín-Ezquerra *et al*., 2009; Mary *et al*., 2004) with minor modifications. Briefly, PCR mix reaction was prepared with 5 µL of DNA, 10 µL of master mix (FastStart Universal Probe Master (ROX), Roche®), 10 µM of *Leishmania* primers (direct 5'-CTTTTCTGGTCCTCCGGGTAGG-3' and reverse 5'-CCACCCGGCCCTATTTTACACCAA-3', supplied by StabVida®) and 5 µM TaqMan® probe (FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA), designed to target a kinetoplast DNA (kDNA) sequence, and 1 µL of H_2O. Eukariotic 18S rRNA was used as endogenous control (VIC™/MGB probe, primer limited, Thermo Fisher Scientific®). Amplifications and detection were performed in an ABI7900 device (Applied Biosystems) and the thermal cycling profile was 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 sec, and 60°C for 1 min. All samples were analysed in triplicate and positive (DNA from *L. infantum* MHOM/FR/95/LEM3141 strain) and

negative controls were included in all qPCR reactions. Parasite quantification was performed by comparison with a standard curve generated with *L. infantum* DNA extracted from 1 x 10^6 parasites/mL by using serial dilutions from 10^5 to 1 parasites/mL.

3.5.5. Clinical follow-up

All dogs were clinically assessed before the beginning of the vaccine trial. This included a clinical exam, complete blood count (CBC), renal and hepatic function assessment, and serum protein electrophoretogram. In addition to allowing an assessment of each dog's health status at T0, these results were kept as a baseline to compare with subsequent exams throughout the study.

The clinical exam included inspection of general body condition, hydration status, skin, hair and nail condition, mucosae, external lymph nodes and ocular lesions. Owners were asked about any recent disease, visible weight loss, anorexia, exercise intolerance, polyuria/polydipsia, vomit or diarrhoea. Clinical assessments were repeated throughout the field trial whenever there was a suspicion of CanL, either detected by the researchers during follow-up visits or by the dog owners. At the end of the trial, a thorough clinical exam was performed to all dogs. Likewise, blood analyses were repeated whenever needed to confirm a CanL case and at the end of the study for all seropositive dogs.

Due to the nonspecific clinical presentation of CanL, dogs were considered symptomatic only if two or more clinical signs compatible with the disease were observed (Fisa *et al*., 1999). The same criterion was followed for any detected laboratory changes.

3.5.6. Evaluation of vaccine-induced cellular mediated immunity (CMI)

PBMC were obtained from each individual at three time points: before the first vaccine dose (T0), 4 weeks after the 3rd vaccine dose (M1) and 9 months after vaccination completion (M9) (Figure 8). Only dogs with samples for the 3 time points studied were included in the CMI assessment, in a total of 152 individuals (75 in the vaccine group and 77 in the control group).

Samples of heparinized whole blood were processed within 4h of collection. PBMC were isolated by centrifugation with a density gradient medium (Lymphoprep™; Stemcell Technologies), frozen in foetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen until processing (Requena *et al*., 2015).

For assessment of antigen-specific cytokine responses, samples from the same dog were processed together. PBMC were slowly thawed, washed and left for an overnight rest at 37°C in 5% CO_2. On the following day, cells were counted on a TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc.) and incubated in 96-well culture plates at a density of 10^6 cells/mL as described elsewhere (Rodríguez-Cortés *et al*., 2017). Briefly, PBMC were incubated with 10 µg/mL soluble *L. infantum* antigen (LSA), or 2.5 µg/mL concanavalin A (ConA) (Sigma Aldrich®), or culture media (unstimulated, negative control) for a period of 5 days at 37°C in 5% CO_2. On the fifth day, plates were centrifuged and supernatants were collected and stored at -40°C.

Interferon-γ (IFN-γ) concentration was determined on PBMC supernatants by a sandwich-ELISA. The Canine IFN-γ DuoSet ELISA kit (R&D Systems) was used, following the manufacturer's instructions. All samples were processed in duplicate and a standard curve was included in all plates, with a range of IFN-γ concentrations from 0 to 2000 pg/mL. Optical densities were determined at 450 nm, with wavelength correction set to 570 nm. IFN-γ concentrations were calculated using a four parameter logistic curve produced in GraphPad Prism® version 5.3. To obtain the specific IFN-γ concentration for each sample, readings from the unstimulated cell supernatant were subtracted from the LSA-stimulated cell supernatant. All plates presented a coefficient of determination (R^2) above 0.99.

3.5.7. Definition of canine leishmaniosis case

Classification of trial dogs' infection status was determined based on the results of serological tests, presence of clinical signs, and detection of CBC or plasma biochemical abnormalities compatible with CanL. Any suspicion of *L. infantum* infection detected by the researchers during sample collection or the dog owners throughout the trial period would be further evaluated. Apart from these reported cases, and because all analyses were performed in parallel at the end of the field study, identification of CanL cases was mainly performed in April 2017.

A confirmed case of leishmaniosis was defined as:

- Seroconversion to *L. infantum*, defined as a 4-fold increase in ELISA units when compared with basal values (ELISA units measured at T0) for the same individual and,

- Detection of *L. infantum* DNA in lymph node samples and,

- Presence of clinical signs or laboratory abnormalities compatible with CanL.

In addition, animals without detectable clinical signs or laboratory findings, but showing evidence of seroconversion and presence of *Leishmania* DNA in lymph nodes at the last time point (12M) were also considered confirmed cases of active *L. infantum* infection.

3.5.8. Study endpoint

Cases of CanL confirmed during the field study were submitted to treatment and follow-up or euthanasia, according to the dog owner's decision.

3.5.9. Statistical analysis

All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX, USA). Continuous variables included in this study did not present a normal distribution and normality could not be achieved by data transformation. Therefore, non-parametric statistical tests were used to compare between and within group results for continuous variables. Comparisons between groups at each time point were tested by Mann-Whitney *U* test. Longitudinal comparisons within groups were tested by Wilcoxon signed-rank test. Statistical significance of difference in proportions between groups was tested by the Pearson Chi-square test.

4. RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. SEROPREVALENCE OF CANINE *Leishmania infantum* INFECTION IN GIRONA PROVINCE AND IDENTIFICATION OF RISK FACTORS FOR THE INFECTION

This study has been accepted for publication in the journal Preventive Veterinary Medicine and is available in article format in Annex 7.

4.1.1. Resumen

La Cuenca mediterránea es una región endémica de la leishmaniosis canina (LCan), donde representa un importante problema veterinario y plantea problemas de salud humana. Sin embargo, la distribución de la enfermedad es heterogénea y no todos los países y lugares han sido estudiados y caracterizados por igual. Este trabajo describe la situación de la LCan en la provincia de Girona (Cataluña, España), para la cual no existen datos reportados y publicados. Entre 2012 y 2016, se realizaron cuatro encuestas transversales de seroprevalencia en toda la provincia, incluyendo 36 estaciones de muestreo en 26 localidades y un total de 593 perros. Para cada animal también se recolectaron variables individuales y de ubicación. Además, cada propietario respondió a un cuestionario sobre sus conocimientos previos de la LCan y los métodos preventivos utilizados. Las muestras de sangre se analizaron mediante un ELISA *in house* y se utilizó un modelo de regresión logística mixta para evaluar la relación entre las variables predeterminadas y la seropositividad del perro. Se utilizó la correlación de Spearman para evaluar la asociación entre el riesgo de LCan percibido por los dueños y la seroprevalencia de *L. infantum* en un lugar determinado. La seroprevalencia global estimada para la provincia de Girona fue del 32,8% (IC 95%: 28,2-37,5), de los cuales solo el 6,8% de los perros (10/146) se consideraron sintomáticos. La edad del perro [OR = 1.21 (IC 95%: 1.11-1.31); p <0.001] y la altitud [OR = 0.02 (IC 95%: 0.001-0.19); p = 0.001] fueron identificados como factores de riesgo para la infección. Se espera que los resultados obtenidos en este estudio ayuden a la implementación de programas de control dirigidos en áreas endémicas de

LCan en toda Europa, así como proporcionen datos adecuados para el diseño de mapas de evaluación de riesgo de la enfermedad más idóneos.

4.1.2. Background

Spain is an endemic country for CanL and, as observed in other endemic areas, the distribution of the infection is highly heterogeneous throughout the territory (Miró and Molina, 2006). For this reason, Mediterranean endemic regions would benefit greatly from CanL directed control efforts, targeted at areas with higher levels of infection. CanL seroprevalence in owned dogs in Spain ranges from 1.6% in the northwest (Miró and Molina, 2006) to 34.6% in the south (Morillas-Márquez *et al*., 1996), with a range of intermediate values reported across the territory (Alcover *et al*., 2013; Ballart *et al*., 2013a; Martín-Sánchez *et al*., 2009; Miró *et al*., 2017a; Solano-Gallego *et al*., 2001a). Nevertheless, as in other Mediterranean countries, the map of CanL distribution in Spain is far from complete, with many regions still lacking documented information.

Catalonia, in the north-east of Spain, is considered one such endemic area for CanL. Here, like in other regions, identifying locations for the implementation of CanL directed control programmes is constrained by the heterogeneous distribution of the infection and the lack of published data on CanL prevalence. Historically, the south of Catalonia was known for the presence of well-established and important foci of CanL (Fisa *et al*., 1999; Portús, 2007) but recent studies in northern areas such as the Lleida region (Ballart *et al*., 2013a) showed that the infection is more widespread than previously thought. Furthermore, results of a recently published questionnaire-based study suggest that Girona province, in the north-east of Catalonia, may be an endemic area of CanL (Lladró *et al*., 2017). In a survey of local veterinarians, the general opinion was that CanL is well established throughout the province and the number of autochthonous cases has risen in the last years. Additionally, Girona province shares a range of physical and climatic characteristics with other Mediterranean locations and is therefore an appropriate example for CanL epidemiological studies.

The objectives of this study were to provide the first data on CanL seroprevalence for Girona province, which could also confirm the hypothesis of CanL endemicity in this region suggested by Lladró *et al*. (2017), and to identify possible individual and location

risk factors associated with the infection in the study area which can be applied in the control of CanL in other Mediterranean endemic regions.

4.1.3. Results

Descriptive analysis of the study population

A total of 593 blood samples were obtained from dogs distributed throughout the north-east and Pyrenean areas of Spain, in Girona province, with sampling points ranging from 1 to 10 per county (Table 7).

Sampling sites were mainly rural (corresponding to 50.1% of the dog sample) and periurban (41.8% of sampled dogs), with dog density per site ranging from 3 to 34. Altitudes ranged from 50 to 1300 m a.s.l., with the majority of dogs living below 800 m a.s.l. (83%). Most dogs were hunting animals (78.9%), but breeding (16%), shelter (2.5%), racing (2.2%) and pet dogs (0.3%) were also represented. A large number of sampled dogs were born in Girona province (60.4%) and were not reported to have left the region. All animals included in the study were kept with other dogs in open kennels during the day time, and the majority were also kept outdoors at night (87.9%). There were other animal species kept in close proximity to 49.6% of the sampled dogs. These included cats, horses, cows, goats and pigs. Observed age average was 3.6 years (SD=2.9), 58.9% of the dogs were males and 55.4% were crossbred.

Table 7. True seroprevalence for canine *Leishmania infantum* infection observed in each locality and overall true seroprelavence calculated per county and for Girona province.

County	Locality	No. sampling points	No. sampled dogs (No. positive dogs)	No. seropositive dogs (%)	True seroprevalence % (95% CI)
Alt Empordà	Cadaqués	2	29 (12)	41.4	41.8
	Darnius	2	18 (6)	33.3	31.1
	Ordis	1	31 (9)	29.0	25.4
	Total	**5**	**78 (27)**	**34.6**	**32.8 (20.6-45.0)**
Baix Cerdanya	Urtx	**1**	**30 (1)**	**3.3**	**0**
Baix Empordà	Sant Feliu de Guíxols	**1**	**19 (10)**	**52.6**	**56.8 (30.9-82.8)**
Garrotxa	Hostalnou de Bianya	2	21 (3)	14.3	5.7
	Montagut	2	55 (16)	29.1	25.5
	Olot	1	12 (5)	41.7	42.2
	Sant Esteve de Llémena	1	21 (2)	9.5	0
	Total	**6**	**109 (26)**	**23.9**	**18.5 (9.2-27.7)**
Gironès	Aiguaviva	1	30 (4)	13.3	4.4
	Canet d'Adri	3	44 (35)	79.6	92.7
	Madremanya	1	20 (3)	15.0	6.7
	Sant Gregori	1	24 (13)	54.2	58.9
	Total	**6**	**118 (55)**	**46.6**	**48.8 (38.4-59.2)**
Plà de l'Estany	Banyoles	**1**	**15 (7)**	**46.7**	**48.9 (19.7 -78.0)**
Ripollés	Bruguera	1	9 (0)	0	0
	Camprodon	1	8 (0)	0	0
	Ogassa	1	32 (0)	0	0
	Serrat	1	6 (0)	0	0
	Setcases	1	3 (0)	0	0
	Ventola	1	13 (0)	0	0
	Total	**6**	**71 (0)**	**0**	**0**
Selva	Brunyola	1	10 (0)	0	0
	Maçanet de la Selva	1	17 (3)	17.7	10.2
	Massanes	2	29 (3)	10.3	0.5
	Riells i Viabrea	2	31 (7)	22.6	16.8
	Sta Coloma de Farners	2	32 (0)	0	0
	Vilobí d'Onyar	2	34 (7)	20.6	14.1
	Total	**10**	**153 (20)**	**13.1**	**4.1 (0-10.3)**
Total for Girona province		**36**	**593 (146)**	**24.6**	**19.5 (15.5-23.5)**

Dog owners' perception on CanL and use of preventive measures

The majority of dog owners showed previous knowledge of CanL (93.9%) and approximately half of them knew preventive methods against CanL (57.6%), although only 27.3% had ever used them (Table 8).

Table 8. Results of the questionnaire asked to dog owners regarding their knowledge of canine leishmaniosis and the methods used to prevent the infection (n=33).

Question	No. replies (%)
Have you ever heard of CanL?	
Yes	31 (93.9)
No	2 (6.1)
In your opinion, how great is the risk of any of your dogs having CanL throughout their lives?	
0%	4 (12.1)
5%	9 (27.3)
10%	5 (15.2)
20%	7 (21.2)
50%	1 (3.0)
50-90%	4 (12.1)
90-100%	3 (9.1)
Do you know of any measures to protect your dogs against CanL?	
Yes	19 (57.6)
No	14 (42.4)
Do you use any measure to protect your dogs against CanL?	
Yes	9 (27.3)
No	24 (72.7)
If YES, which method do you use? (n=9)	
Collar	4 (44.5)[2]
Spot-on	3 (33.3)[2]
Others[1]	2 (22.2)[2]
If NO, why not? (n=24)	
Unawareness	14 (58.3)[2]
Do not believe it works	3 (12.5)[2]
Too expensive	2 (8.3)[2]
Do not believe there is CanL	1 (4.2)[2]
Others/no answer	4 (16.7)[2]
Do you use any measure to protect your dogs against other arthropods (e.g. ticks, fleas, etc.)	
Yes	25 (75.8)
No	8 (24.2)
If YES, which method do you use? (n=25)	
Pour-on	12 (48.0)[2]
Sprays	2 (8.0)[2]
Spot-on	1 (4.0)[2]
Others[1]	10 (40.0)[2]

[1] Includes the use of others or multiple preventive measures.

[2] Percentage based on the total for the subgroup YES or NO of the previous answer.

Only a small number of dog owners believed that their dogs were not at risk of contracting CanL during their lifetime (12.1 %), with the majority of them believing that the risk of CanL ranged from 5 to 20% (63.7%). The result of the Spearman's correlation showed a positive association between dog owners' perceived risk of CanL infection and CanL seroprevalence (r_s=0.5046; *p*=0.0027).

Prophylactic methods against CanL, when used, included dog collars (44.5%), spot-on (33.3%) and combined insecticide treatments (22.2%). Vaccination against CanL or immunomodulatory prophylactic treatments had not been used by any of the dog owners. The main reasons given for not using any preventive method against CanL were unawareness (58.3%) and not believing that prophylaxis worked (12.5%).

CanL study results

From the 593 dogs analysed, 146 were considered seropositive by ELISA. Apparent seroprevalence at the sampling point level ranged from 0% to 79.6%, with a total apparent seroprevalence calculated for Girona province of 24.6% (95% CI: 21.2-28.3). Considering these values, the estimated true CanL seroprevalence for Girona province was 19.5% (95%CI: 15.5-23.5). Estimated seroprevalence at the county level ranged from 0 to 56.8%. Results for all localities and counties are summarized in Table 7.

Only 10 out of 146 seropositive dogs were considered symptomatic (6.8%). Observed clinical signs included onychogryphosis (n=9), weight loss (3), skin wounds (3), diffuse alopecia (2), popliteal lymphadenomegaly (2), periocular alopecia (2), and ocular lesions (2).

Bivariate statistical analysis

One of the dog kennels included in the seroprevalence study (Banyoles, Plà de l'Estany) was excluded from the statistical analysis, following the criteria used in similar studies (Ballart *et al*., 2013a). This is a shelter kennel that collects stray dogs, which means that some of the individual data, as well as owners' perception of CanL, could not be collected. Therefore, the statistical analysis included 578 individuals and 25 localities. Results of the bivariate analysis are summarized in Table 9. Dogs' age and location altitude (p<0.001) showed a very strong relationship with dog seropositivity. In our population, a bimodal CanL seroprevalence distribution according to age was observed, with a first peak at 3-4 years and a second at 7-8

years old, with the risk of infection rising by each year of life [OR=1.18 (95%CI: 1.09-1.27)] and decreasing at altitudes above 800 m a.s.l. [OR=0.012 (95%CI: 0.002-0.07)]. Also, according to the results, being a crossbred dog raises the risk of infection [OR=2.19 (95%CI: 1.18-4.06); *p*=0.013] and the use of unspecific insecticides against arthropods has a protective effect [OR=2.94 (95%CI: 1.58-5.45); *p*=0.001]. All the other variables (sex of the dog, type of habitat, dog purpose, type of nocturnal refuge, presence of other animal species, owner's knowledge of prophylactic measures against CanL and the regular application of these methods) showed no statistically significant relationship with dog seropositivity.

Multivariable mixed model

The final multivariable mixed logistic regression model identified age of the dog and altitude of the location as the explanatory variables that affect dog seropositivity. According to this model, the odds of being infected rise in 1.21 per each year of life [(95%CI: 1.11-1.31); *p*<0.001] and decrease at locations above 800 m a.s.l. [OR=0.02 (95%CI: 0.001-0.19); *p*=0.001]. The final model explains 53.7% of the total variance of the outcome variable, of which 42% is explained by the fixed effects terms and 11.7% by the random effects variable.

Table 9. Number of dogs analysed and *Leishmania infantum* seropositivity observed for each category of the explanatory variables, followed by the results of the bivariate analysis expressed in odds ratios (OR). Statistically significant variables (p<0.05) are marked with (*).

Explanatory variables and categories	No. dogs analysed	No. seropositive dogs (% seropositive dogs)	Bivariate analysis OR (95% CI)	p-value
Altitude (m a.s.l.)				
< 800	492	144 (29)	Ref	
>800	109	6 (6)	0.012 (0.002-0.07)	<0.001*
Type of habitat				
Rural	297	70 (24)	Ref	
Periurban	228	51 (22)	1.64 (0.93-2.88)	0.082
Urban	53	19 (36)	0.97 (0.42-2.22)	0.934
Presence of other animals (other than dogs)				
Yes	302	73 (24)	Ref	
No	299	78 (26)	1.10 (0.58-2.08)	0.763
Sex				
Male	338	87 (26)	Ref	
Female	240	53 (22)	0.88 (0.56-1.38)	0.581
Age (years)			1.18 (1.09-1.27)	<0.001*
<1	53	6 (11)		
1	83	12 (14)		
2	91	13 (14)		
3	79	20 (25)		
4	51	16 (31)		
5	42	10 (24)		
6	42	12 (29)		
7	33	15 (45)		
8	33	14 (42)		
9	15	3 (20)		
10	18	5 (28)		
11	10	4 (40)		
12	3	0 (0)		
13	1	1 (100)		
Breed				
Purebred	258	51 (20)	Ref	
Crossbred	320	89 (28)	2.19 (1.18-4.06)	0.013*
Use given				
Hunting	468	118 (25)	Ref	
Breeding	95	21 (22)	2.28 (0.78-6.63)	0.130
Others[1]	15	1 (7)	0.20 (0.02-1.73)	0.145
Night shelter				
Outdoors	506	126(25)	Ref	
Indoors	72	14 (19)	0.50 (0.20-1.23)	0.131
Owner knows preventive measures against CanL				
Yes	362	103 (28)	Ref	

No	216	37 (17)	0.58 (0.29-1.19)	0.138
Owner has used preventive measures against CanL				
Yes	140	46 (33)	Ref	
No	438	112 (26)	1.24 (0.63-2.43)	0.539
Owner has used prevention methods against other arthropods				
Yes	472	112 (24)	Ref	
No	106	28 (26)	2.94 (1.58-5.45)	0.001*

[1] Includes racing (n=13) and pet dogs (n=2).

4.1.4. Discussion

Until now, data on CanL in north-eastern and Pyrenean areas of Spain is scarce and fragmented. The only published study regarding CanL in Girona province is a questionnaire-based survey of veterinary practitioners working in the region (Lladró *et al*., 2017). This work provided the first data from a previously recognized, but non-documented CanL endemic area in north-east Spain and highlights gaps in the epidemiological picture in Mediterranean regions considered to be endemic for CanL (Ready, 2017). The veterinary survey showed that new cases of CanL in autochthonous animals were diagnosed annually, including some asymptomatic cases detected by CanL pre-vaccination screening (Lladró *et al*., 2017). The present study confirms the suspected endemicity of CanL in the region, providing results for canine seroprevalence, as well as an overview of the infection distribution throughout Girona province. Preliminary exploratory surveys showed the presence of phlebotomine vectors in the surroundings of many of the sampling points (Annex 2), confirming that all conditions are present for a complete *L. infantum* biologic cycle to be maintained in this region. In addition, the characterisation of all individuals and locations included in the study allowed for the identification of risk factors associated with CanL distribution.

As previously mentioned, there was an active search for individuals to be enrolled in the study, assisted by local veterinarians. There was therefore a constraint in the distribution and type of animals recruited, depending on the availability of veterinary practitioners' clients willing to participate. As a result, the dog population was mainly composed of hunting dogs. These animals have inherent characteristics, such as the fact that they are usually kept with other dogs in open kennels, in rural or periurban settings, and generally do not have the same type of veterinarian monitoring as pet dogs. Therefore, this type of population is usually considered

a good sentinel for CanL (Ballart *et al*., 2013a; Cabezón *et al*., 2010). As similar hunting activities take place throughout Mediterranean areas in Europe, it can be expected that comparable dog populations are widespread. An overestimation of the overall infection prevalence can however occur due to an expected lower incidence in urban centres, mostly explained by a decreased probability of contact between dogs and sand fly vectors (Ballart *et al*., 2013a). Additionally, there was an increased difficulty in recruiting dogs from higher altitude regions, mainly because these areas are more inhospitable and less populated. Consequently, dogs living at locations above 800 m a.s.l. are less represented.

Some degree of spatial clustering may have been introduced by sampling several dogs in the same kennel or locality. This could also have had a clustering effect on the positive results, as higher dog densities tend to favour the transmission of the parasite, especially if some of the dogs are already infected (Alonso *et al*., 2010). Nevertheless, in the present study we have used similar dog populations in the different sampling points, allowing comparison between them. Additionally, this methodology has also been used in similar studies describing other regions of Spain (Alcover *et al*., 2013; Ballart *et al*., 2013a). In the statistical analysis, the potential clustering effect was dealt with by introducing "Locality" as a random-effects term in the final multivariable mixed logistic regression model.

The serological technique used to measure antibody levels to *L. infantum* was an in-house ELISA. ELISA is one of the methods recommended by the World Organization for Animal Health for performing CanL surveillance studies and to determine prevalence of infection (OIE, 2014), the other one being the indirect immunofluorescent antibody test (IFAT). Unlike IFAT, ELISA is easy to perform and interpret, being particularly useful in field study settings, where a large number of samples must be analysed (Maia and Campino, 2008). In addition, this ELISA has been widely used for CanL diagnosis, as well as in other CanL epidemiological studies (Alcover *et al*., 2013; Ballart *et al*., 2013a; Fernández-Bellon *et al*., 2008; Fisa *et al*., 2001; Iniesta *et al*., 2002; Riera *et al*., 1999; Rodríguez-Cortés *et al*., 2010; Solano-Gallego *et al*., 2005).

The overall estimated seroprevalence for Girona province was 19.5%, ranging from 0 to 56.8% across the different counties. These results are in accordance with previous reports for other regions of Spain, as well as the Mediterranean basin (Ballart *et al*., 2013a; Cortes *et al*., 2012; Maroli *et al*., 2008; Ntais *et al*., 2013). A series of CanL seroprevalence surveys undertaken in France, Italy, Spain and Portugal between 1971 and 2006 showed an overall seroprevalence of 23.2%, with point prevalences of 0% and higher than 80% in some locations (Franco *et al*., 2011). These values are comparable to the ones obtained in the present study and correspond

to the previous claims of the heterogeneous distribution of the disease. However, as pointed out by Franco *et al*. (2011), caution must be taken when comparing studies with different experimental designs and different criteria used in the selection of the target dog population, as this can introduce significant variations in seroprevalence results. A common European strategy for leishmaniosis surveillance and control would aid the implementation of standardized methodology. However, although leishmaniosis is currently listed as a notifiable disease by the World Organization for Animal Health (OIE, 2018), this is not clearly reflected in the European or Spanish legislation (BOE, 2014; Official Journal of the European Union, 2012).

From the 146 seropositive dogs, only 10 (6.8%) showed clinical signs compatible with CanL and more than 50% presented low standard ELISA units (inferior to 50U). This can be explained by the cryptic nature of the infection and the wide clinical spectrum it can present, ranging from asymptomatic or mild symptomatic cases to very severe clinical stages (Solano-Gallego *et al*., 2009). There is also the possibility that some of the dogs are in an early stage of infection (Fisa *et al*., 2001; Miró *et al*., 2012) or are immunologically resistant and only transiently seropositive, eventually showing spontaneous clearance of the parasite (Fisa *et al*., 1999). In such populations, serological techniques could have a lower sensitivity (Otranto *et al*., 2009). It is also known that, in endemic areas, only a small proportion of dogs display symptoms of CanL, while the majority of infected dogs do not show any clinical evidence of the disease (Baneth *et al*., 2008). It is believed that the high prevalence of asymptomatic infected dogs, comparable to that observed in Lleida province (other north-eastern and Pyrenean region studied in Spain), is strong evidence for a well-established CanL focus in Girona province (Ballart *et al*., 2013a). In the present study, clinical signs compatible with CanL were also identified in 10 out of 447 seronegative dogs (2%), illustrating the lack of specificity of the disease's clinical presentation and the added difficulty in detecting affected dogs. As mentioned before, the ability of serological tests to detect infected animals is limited, especially in endemic settings, and a small number of seronegative asymptomatic infected dogs should be expected, as previously reported in other studies (Iniesta *et al*., 2002; Otranto *et al*., 2009; Solano-Gallego *et al*., 2011). These animals can harbour parasites in the skin, detectable by PCR (Otranto *et al*., 2009), and could also be infectious to sand flies, as has been demonstrated for asymptomatic seropositive dogs (Molina *et al*., 1994; Quinnell and Courtenay, 2009). Considering this, any control programme for CanL should be based on multiple diagnostic methods, as serology alone can prove to be insufficient in detecting all infected and infectious dogs.

In the present study, the risk of infection increased with dogs' age. This is an individual factor commonly reported as being positively related with *L. infantum* infection (Alonso *et al*., 2010; Ballart *et al*., 2013a; Cortes *et al*., 2012; Gálvez *et al*., 2010b; Maresca *et al*., 2009; Martín-Sánchez *et al*., 2009; Miró *et al*., 2012), and which can be explained by an incremental risk of exposure to infected sand flies. The bimodal CanL seroprevalence distribution observed has been previously described by other authors (Gálvez *et al*., 2010b; Miró *et al*., 2012). This pattern suggests that *L. infantum* may be able to infect immunologically vulnerable animals at an earlier age, followed by a later infection of resistant animals either by cumulative exposure or due to concomitant diseases that weaken the dogs' immune system (Miranda *et al*., 2008).

According to the results, altitude shows a negative correlation with *L. infantum* infection. This is mainly related to the bioclimatic needs of the phlebotomine vector species present in Spain, *Phlebotomus perniciosus* and *P. ariasi* (Rioux *et al*., 1986). Altitude is known to be closely linked to temperature, precipitation and land cover (Barón *et al*., 2011; Rivas-Martínez, 1983). In temperate regions, as atmospheric temperature rises, a higher biting rate is expected (Hartemink *et al*., 2011), therefore increasing the risk of sand fly bites to vertebrate hosts. Simultaneously, a shorter extrinsic incubation period (Hartemink *et al*., 2011) and a more effective development of the parasite inside the vector (Rioux *et al*., 1985) are observed, raising the risk of *L. infantum* infection. Also, an increased altitude may provide a more hostile environment for sand fly survival (Gálvez *et al*., 2010a), not only because of the more extreme bioclimatic conditions, but also due to a possible scarcity of vertebrate hosts. However, a relationship between altitude and risk of CanL infection was not observed in the neighbouring province of Lleida (Ballart *et al*., 2014, 2013a) or in France (Chamaillé *et al*., 2010), where both vector species are present and show different altitudinal preferences. In these areas, *P. perniciosus* is known to occupy ecological niches commonly below 800 m a.s.l., while *P. ariasi* shows a higher abundance above this altitude. Therefore, it would be of particular interest to perform entomological studies and risk factor analysis associated with the vector populations present in the study area. This could also help to improve the ability of the present model to predict the outcome variable. One of the possible reasons for the moderate performance of the final statistical model presented (which explains only 53.7% of variance of the outcome variable) is the absence of data on the abiotic factors mentioned above, which are known to have an important impact on sand fly populations, and indirectly on *L. infantum* infections (Dantas-Torres *et al*., 2014; Gálvez *et al*., 2010a).

The present study failed to detect an effect of type of habitat (rural/urban) or access to night shelter, which several other authors identified as significantly related to *L. infantum* infection

(Ballart *et al*., 2013a; Cortes *et al*., 2012; de Almeida *et al*., 2012; Gálvez *et al*., 2010b; Martín-Sánchez *et al*., 2009; Oliveira *et al*., 2016). According to these studies, dogs that live in rural habitats and are left outdoors at night show an increased risk of infection. In this study, the high percentage of dogs living in rural/periurban areas and kept permanently outdoors may not have allowed detection of such an effect. Also, periurban areas are increasingly described as the most suitable ecosystems for sand flies, due to the ideal microclimate offered by house gardens associated with the abundance of vertebrate hosts (Alvar *et al*., 2004; Ballart *et al*., 2013a).

Results from the bivariate statistical analysis identified dog breed and the use of general insecticide treatment against arthropods as variables associated with dog seropositivity. In the first case, crossbred dogs would be at higher risk of infection [OR=2.19 (95%CI: 1.18-4.06); p=0.013]. However, previous studies have shown that this should not be the case, as crossbred, autochthonous dogs tend to be more resilient to *L. infantum* infection (Alvar *et al*., 2004; Solano-Gallego *et al*., 2000). This is even more noticeable when the purebred dogs belong to exotic breeds like boxers and beagles (both represented in this study), known for their higher sensitivity to CanL (Solano-Gallego *et al*., 2009). The effect of dog breed was absent in the mixed model, showing that the previous results were most probably induced by confounding factors related to the kennel locations (e.g. altitude) or dog owners' attitudes (e.g. use of prophylactic measures against CanL). The non-use of generalist insecticide preventive methods against arthropods was also identified as a risk factor for *L. infantum* infection [OR=2.94 (95%CI: 1.58-5.45); p=0.001], while the use of specific prophylaxis against CanL failed to show a protective effect (p=0.539). Again, this may be related to confounding factors, such as a possible partial effect of some insecticides against phlebotomine vectors, even though they may not be licensed for sand fly prevention. Additionally, the improper use of specific sand fly prevention treatment, such as failure to apply it to all dogs or to maintain it during the whole transmission season, may impair the protective effect of these products (Courtenay *et al*., 2009). Once again, the effect of this variable lost significance in the multivariable analysis and was not included in the final statistical model.

The majority of dog owners showed previous knowledge of CanL and to be aware of preventive methods for the infection. Although a positive correlation was observed between owners' perceived risk of infection and CanL seropositivity at the dog kennel level, only 27.3% of dog owners stated that they regularly used CanL prophylactic measures. This result is in accordance with those reported by Lladró *et al*. (2017), in which all veterinary practitioners working in Girona province recommended at least one preventive measure against CanL,

though the majority did not believe that dog owners protected their dogs properly. When used, the most frequent prophylactic methods applied against CanL were dog collars and spot-on insecticides, as recommended by veterinarians. However, most owners did not keep their dogs indoors at night and did not report the use of vaccination against CanL or immunomodulatory agents, as also suggested by veterinarians (Lladró *et al*., 2017). Our studyshows that the implementation of prophylactic measures by dog owners should be reinforced in order to reduce *L. infantum* transmission between dogs, as well as to reduce the public health risk (Miró and López-Vélez, 2018).

4.2. EVALUATION OF DOG EXPOSURE TO *Phlebotomus perniciosus* THROUGH THE DETECTION OF ANTI-SAND FLY SALIVA ANTIBODIES IN THE CANINE HOST

This study has been published in the journal Parasites & Vectors and is available in article format in Annex 8.

4.2.1. Resumen

La transmisión de *Leishmania infantum* se hace a través de la picadura de vectores flebotomos. Por lo tanto, el monitoreo del contacto entre el hospedador y el vector representa una herramienta epidemiológica importante que podría usarse para evaluar la efectividad de los programas de control de vectores en áreas endémicas. Estudios previos han demostrado que los anticuerpos caninos contra la saliva de los flebotomos son marcadores específicos de la exposición a los vectores de *Leishmania*. Sin embargo, la validación de este método debe ampliarse a poblaciones heterogéneas de perros naturales de áreas endémicas de LCan. En este estudio, se siguieron durante 14 meses 176 perros que viven en 12 lugares diferentes de un área endémica para *L. infantum* en el noroeste de España (provincia de Girona). Las muestras de sangre a utilizar para evaluar la respuesta inmune humoral canina al homogeneizado de glándulas salivales (SGH) y a la proteína recombinante "yellow-related" de 43 kDa (rSP03B) de *Phlebotomus perniciosus*, uno de los vectores confirmados de *L. infantum* presente de forma natural en esta región, fueron tomadas en 5 momentos de muestreo predeterminados (Febrero, Agosto y Octubre de 2016, Enero y Abril de 2017). Simultáneamente, se evaluó la infección por *L. infantum* en todos los perros por serología (ELISA *in house*). La relación entre los anticuerpos anti-SGH y anti-rSP03B con el mes de muestreo, la infección por *L. infantum* y la localidad se estudió mediante el ajuste de modelos de regresión lineal multinivel. La dinámica de las IgG caninas anti-saliva de SGH y rSP03B siguió las tendencias esperadas de actividad de *P. perniciosus* en la región. Se detectaron asociaciones estadísticamente significativas para ambos antígenos salivales entre la exposición al vector y el mes de muestreo o la seropositividad del perro a *L. infantum*. La correlación entre anticuerpos caninos contra SGH y rSP03B fue moderada. Los resultados confirman la presencia frecuente de vectores de LCan en el área de estudio así como la aplicabilidad de las

pruebas ELISA basadas en SGH y rSP03B para estudiar la exposición canina a *P. perniciosus* en áreas endémicas de *L. infantum*.

4.2.2. Background

The detection of anti-sand fly salivary antibodies in the blood of vertebrate hosts has proven to be highly specific (Rohoušová *et al*., 2005) and was successfully used as a marker of exposure to *L. infantum* vectors (Martín-Martín *et al*., 2014; Vlkova *et al*., 2011). In CanL endemic areas, monitoring the canine IgG response to sand fly saliva can be a useful epidemiological tool (Kostalova *et al*., 2015; Vlkova *et al*., 2011), complementing studies of vector population dynamics and host-vector interactions, as well as enabling the assessment of risk of *Leishmania* infection (Carvalho *et al*., 2015; Marzouki *et al*., 2011; Rohoušová *et al*., 2005). Furthermore, it can be used to measure the effectiveness of vector-control programmes and to assist in the design of better control strategies for the disease (Clements *et al*., 2010; Gidwani *et al*., 2011).

Originally, sand fly whole salivary gland homogenates (SGH) were used to investigate the presence of anti-sand fly saliva antibodies in vertebrate hosts (Gidwani *et al*., 2011; Volf and Rohoušova, 2001). However, its use in large-scale studies is impaired by technical limitations (Lestinova *et al*., 2017). Additionally, the use of SGH in vector exposure tests may reduce the specificity of detection due to a possible cross-reactivity with saliva of sympatric and closely related sand fly species (Lestinova *et al*., 2015).

An alternative to the use of SGH is the identification of species-specific salivary proteins that can be expressed in recombinant forms and produced in large quantities for use in large-scale epidemiological studies (Drahota *et al*., 2014; Kostalova *et al*., 2017). Recent studies identified *P. perniciosus* yellow-related protein rSP03B as the most promising candidate to replace SGH in the detection of host markers of exposure to this vector species (Kostalova *et al*., 2017, 2015; Martín-Martín *et al*., 2014). This recombinant protein has been tested and validated in dogs and other animals in cross-sectional studies (Kostalova *et al*., 2017; Martín-Martín *et al*., 2014), as well as in a canine longitudinal study (Kostalova *et al*., 2015), but no information exists on the seasonal dynamics of either SGH or rSP03B in natural heterogeneous dog populations from endemic areas.

Therefore, the objectives of this study were (i) to investigate the dynamics of *P. perniciosus* and their relative density in a previously uncharacterized CanL endemic area through the detection of anti-saliva IgG in dogs; and (ii) to evaluate the performance of both SGH and rSP03B antigens as markers of exposure to *P. perniciosus* in natural canine populations.

4.2.3. Results

Seasonal dynamics of IgG response against salivary proteins from Phlebotomus perniciosus

Median values of normalized ELISA OD values for SGH ranged from 9.04 (range: 3.94–66.23) in January 2017 to 18.51 (7.93–100.58) in August 2016 (Table 10). For rSP03B, median OD values varied between 12.21 (6.75–53.71) and 19.53 (10.64–124.01) in January 2017 and August 2016, respectively. With both antigens, median OD readings raised from basal values in February 2016 (10.11 and 14.67 for SGH and rSP03B, respectively) to peak in August (18.51 and 19.53 for SGH and rSP03B, respectively), sustained higher readings in October (11.15 and 15.31 for SGH and rSP03B, respectively), and descended again to basal levels in January (9.04 and 12.21 for SGH and rSP03B, respectively) and April 2017 (9.54 and 13.44 for SGH and rSP03B, respectively). Median normalized ELISA OD results obtained per month for both SGH and rSP03B are described in Table 10 and plotted in Figure 9.

Table 10. Median values of normalized OD readings for SGH and rSP03B obtained per sampling month in all locations

Variable	N	SGH	rSP03B
		Median (Range)	Median (Range)
February 2016	174	10.11 (5.49–49.62)	14.67 (7.36–41.24)
August 2016	33	18.51 (7.93–100.58)	19.53 (10.64–124.01)
October 2016	164	11.15 (5.56–86.44)	15.31 (6.15–112.54)
January 2017	154	9.04 (3.94–66.23)	12.21 (6.75–53.71)
April 2017	148	9.54 (5.25–62.59)	13.44 (6.27–36.22)

N: number of dogs sampled per sampling month

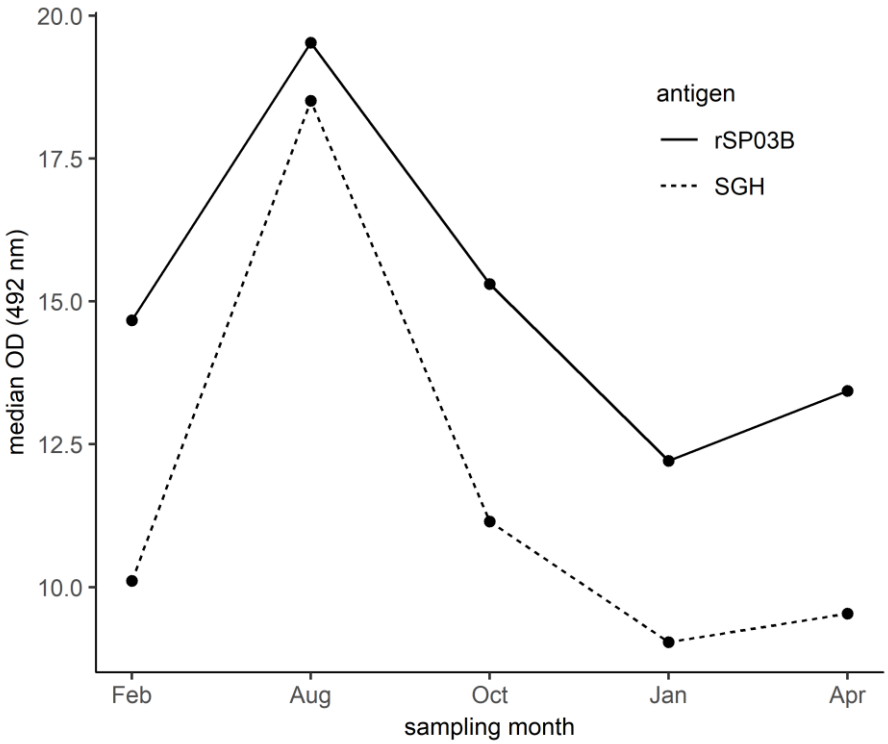


Figure 9. Dynamics of anti-*P. perniciosus* salivary proteins IgG response in dogs from an endemic area during a sand fly activity season. Values presented refer to the normalized OD medians obtained at each sampling month for all dogs and locations. Statistically significant differences in median OD between two consecutive months are marked with an asterisk (p<0.05).

Cut-off values were set at 13 for SGH and 22 for rSP03B. When these were applied to the OD readings obtained in August 2016, 75.76% (25/33) of the dogs were positive to anti-SGH IgG, and 36.36% (12/33) to anti-rSP03B antibodies. In October, these values dropped to 35.98% (59/164) for SGH and 18.9% (31/164) for rSP03B. During the non-transmission season (considered to extend from November to May), the percentage of seropositive dogs ranged from 14.29% (25/175) in February 2016 to 17.57% (26/148) in April 2017 for SGH and 8.44% (13/154) in January 2017 to 12.16% (18/148) in April 2017 for rSP03B.

Correlation results for IgG response between SGH and rSP03B were r_S = 0.54 (95% CI: 0.48–0.60, p<0.001) (Figure 10).

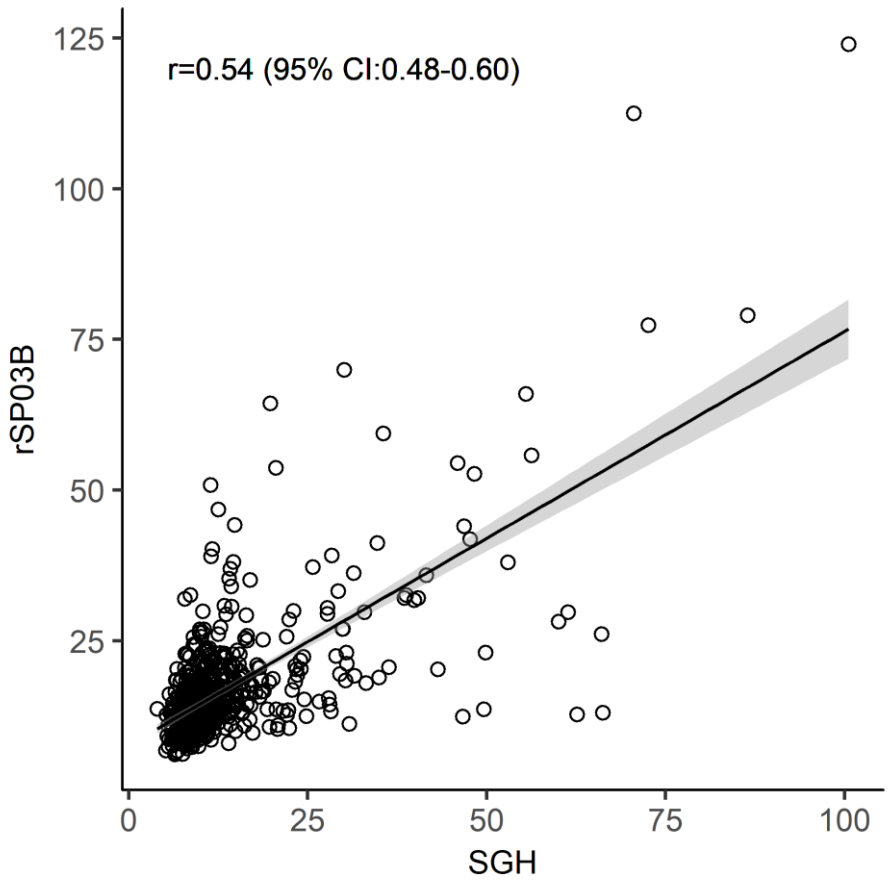


Figure 10. Correlation between IgG recognizing SGH and rSP03B protein in dogs naturally exposed to *P. perniciosus*. Results from both SGH and rSP03B are presented in normalized OD (r_S = 0.54; 95% CI: 0.48–0.60, p<0.001)

Dogs' exposure to Phlebotomus perniciosus in the study area

Exposure of dogs to phlebotomine vectors showed some variation according to the location. Median OD readings varied from 9.11 (range: 5.25–20.57) to 14.14 (7.44–55.45) for SGH ELISA and from 12.71 (7.53–64.44) to 17.87 (8.39–112.54) for rSP03B. Minimum median values of response to both SGH and rSP03B corresponded to the same location (Aiguaviva), but maximum median values were registered in different sites for each antigen (Sant Feliu de Guíxols for SGH and Montagut for rSP03B) (Table 11). Figure 11 presents the dynamics of dogs' IgG response to SGH (Figure 11a) and rSP03B (Figure 11b) in each locality.

The percentage of anti-sand fly saliva seropositive dogs per location, defined as the number of dogs that showed a positive IgG titre at least once during the study period, ranged from

13.33% (1/8) in Ordis to 100% in Canet d'Adri (8/8) and Sant Feliu de Guíxols (4/4) for SGH, and from 8.16% (1/12) in Hostalnou de Bianya to 100% (4/4) in Sant Feliu de Guíxols for rSP03B. Total anti-sand fly saliva seropositivity calculated for the study area was 49.43% (87/176) for anti-SGH IgG and 28.98% (51/176) for anti-rSP03B antibodies.

Dogs' exposure to Phlebotomus perniciosus and Leishmania infantum infection

Correlation results between antibody response to *P. perniciosus* saliva and *L. infantum* were low both for SGH (r_S = 0.27, 95% CI: 0.19–0.35, p<0.001) and rSP03B protein (r_S = 0.25, 95% CI: 0.18–0.32, p<0.001).

Table 11. Median values of normalized OD readings for SGH and rSP03B obtained per sampling location at all time points

Variable	*n* (Range)	Geographical coordinates	SGH	rSP03B
			Median (Range)	Median (Range)
Ordis	8 (7–9)	42°13'37.7''N, 2°54'24.1''E	9.14 (6.45–45.95)	15.16 (8.35–54.50)
Madremanya	14 (12–15)	41°58'47.0''N, 2°58'7.2''E	11.22 (6.79–49.84)	14.49 (8.95–43.99)
Vidreres	8 (7–9)	41°47'27.4''N, 2°45'0.4''E	10.59 (7.80–16.86)	13.46 (8.58–40.23)
Massanes	21 (20–23)	41°45'15.3''N, 2°38'44.0''E	9.31 (5.67–62.59)	16.35 (7.82–55.81)
Hostalnou de Bianya	12 (11–14)	42°13'26.0''N, 2°26'9.7''E	8.75 (5.35–33.16)	13.19 (6.27–46.82)
Montagut	13 (7–15)	42°14'7.7''N, 2°35'57.6''E	12.01 (3.94–72.61)	17.87 (8.39–112.54)
St. Esteve de Llémena	9 (9–10)	42°3'35.1''N, 2°37'1.4''E	9.49 (6.23–22.40)	14.18 (9.12–22.46)
Canet d'Adri	8 (4–10)	42°1'53.7''N, 2°44'15.3''E	10.61 (6.52–100.58)	14.03 (7.36–124.01)
Aiguaviva	19 (16–22)	41°54'27.2''N, 2°46'19.0''E	9.11 (5.25–20.57)	12.71 (7.53–64.44)
St. Feliu de Guíxols	4	41°47'2.3''N, 2°59'58.7''E	14.14 (7.44–55.45)	16.73 (8.57–65.97)
Riells i Viabrea	20 (18–21)	41°43'59''N, 2°33'39.3''E	10.02 (6.07–66.23)	13.43 (8.59–35.31)
Vilobí d'Onyar	23 (22–23)	41°53'3.2''N, 2°43'38.6''E	9.13 (5.17–16.49)	13.05 (6.15–38.07)

n: mean number of dogs sampled in each location

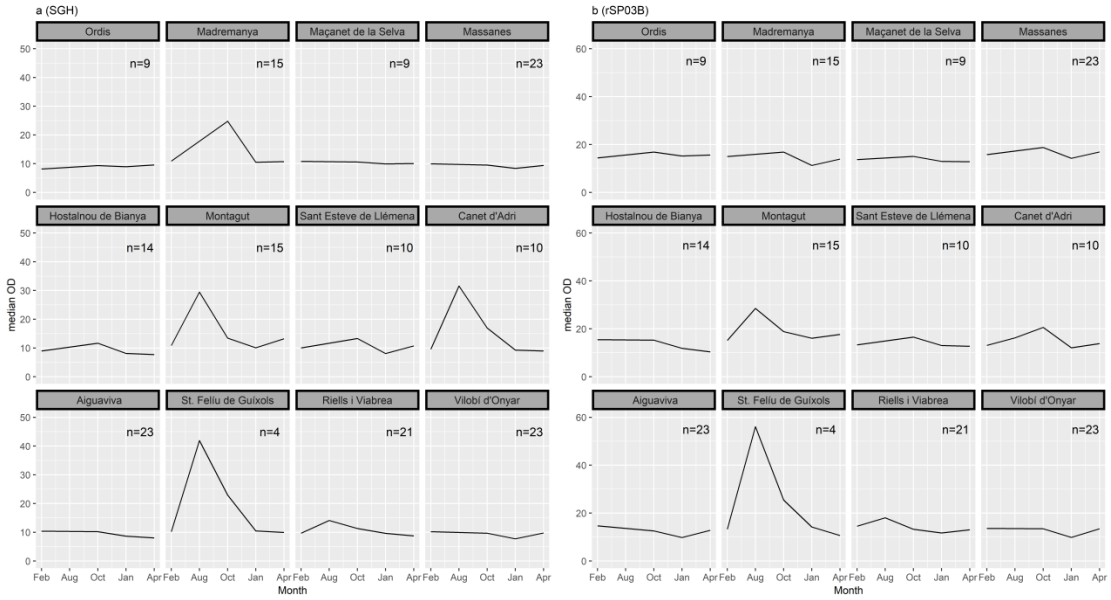


Figure 11. Dynamics of dogs' IgG recognizing SGH (a) and rSP03B protein (b) in the different sampling locations during a sand fly activity season. Values presented refer to the normalized OD medians obtained at each sampling month.

Multilevel analysis of the relationship between anti-Phlebotomus perniciosus salivary proteins, month and location and Leishmania infantum seropositivity

The multilevel model results confirmed the annual dynamics of anti-salivary proteins IgG responses. When compared to the first sampling month (February 2016), IgG responses to SGH significantly rose in August (t = 8.55, df = 491, p<0.001) and October (t = 6.49, df = 491, p<0.001) and dropped in January (t = -2.49, df = 491, p=0.013) and April 2017 (no significant difference when compared to February 2016). As expected, the highest log OD estimate was observed in August 2016 and the lowest in January 2017 (Table 12). The same trend was observed in the model run for the rSP03B protein, with comparable levels of significance (Table 13). There were no significant differences in IgG responses for both antigens between each sampling location and the one set as reference, except for Montagut, where significantly higher OD levels were observed for SGH (t = 2.28, df = 166, p=0.024) and rSP03B (t = 2.13, df = 164, p=0.035). According to the multilevel model, seropositivity to *L. infantum* proved to be associated with a rise in anti-salivary proteins OD values for both SGH (t = 2.5, df = 491, p=0.013) and rSP03B (t = 2.15, df = 493, p=0.032).

Table 12. Estimates of the multilevel linear regression model of the relationship between log transformed normalized SGH OD values and sampling time, location and dog seropositivity to *L. infantum*. "Dog" was included as a random effects variable

Variable	Levels	Estimate	SE	*p*-value[a]
Intercept		2.40	0.06	< 0.001
Sampling month	February 2016	Ref	–	–
	August 2016	0.54	0.06	< 0.001
	October 2016	0.20	0.03	< 0.001
	January 2017	-0.06	0.03	0.013
	April 2017	-0.01	0.03	0.666
Location	Aiguaviva	Ref	–	–
	Ordis	0.07	0.11	0.562
	Madremanya	0.08	0.10	0.427
	Vidreres	0.10	0.11	0.393
	Massanes	0.07	0.09	0.441
	Hostalnou de Bianya	-0.08	0.10	0.404
	Montagut	0.22	0.10	0.024
	St. Esteve de Llémena	-0.03	0.11	0.786
	Canet d'Adri	-0.02	0.11	0.891
	St. Feliu de Guíxols	0.16	0.15	0.308
	Riells i Viabrea	0.03	0.09	0.703
	Vilobí d'Onyar	-0.02	0.09	0.791
L. infantum seropositivity	Seronegative	Ref	–	–
	Seropositive	0.10	0.04	0.013

SE: standard error
[a]Level of significance of $p<0.05$ was used

Table 13. Estimates of the multilevel linear regression model of the relationship between log transformed normalized rSP03B OD values and sampling time, location and dog seropositivity to *L. infantum*. "Dog" was included as a random effects variable

Variable	Levels	Estimate	SE	*p*-value[a]
Intercept		2.79	0.06	< 0.001
Sampling month	February 2016	Ref	–	–
	August 2016	0.39	0.06	< 0.001
	October 2016	0.09	0.03	0.003
	January 2017	-0.13	0.03	< 0.001
	April 2017	-0.06	0.03	0.016
Location	Aiguaviva	Ref	–	–
	Ordis	0.06	0.10	0.563
	Madremanya	-0.04	0.09	0.652
	Vidreres	-0.03	0.10	0.783
	Massanes	0.05	0.08	0.533
	Hostalnou de Bianya	-0.16	0.09	0.074
	Montagut	0.18	0.09	0.035
	St. Esteve de Llémena	-0.10	0.10	0.287
	Canet d'Adri	-0.05	0.10	0.641
	St. Feliu de Guíxols	-0.19	0.14	0.173
	Riells i Viabrea	-0.08	0.08	0.302
	Vilobí d'Onyar	-0.06	0.08	0.399
L. infantum seropositivity	Seronegative	Ref	–	–
	Seropositive	0.07	0.03	0.032

SE: standard error
[a]Level of significance of $p<0.05$ was used

4.2.4. Discussion

The quantification of anti-sand fly saliva antibodies in vertebrate hosts of *L. infantum* has been previously shown to be an effective way of measuring exposure to the parasite vectors (Martín-Martín *et al*., 2014). In the case of dogs, the most frequent host and reservoir of *L. infantum*, this has been proven for *P. perniciosus* (Kostalova *et al*., 2017; Vlkova *et al*., 2011), as well as for other sand fly species (Hostomska *et al*., 2008; Rohoušová *et al*., 2015; Sima *et al*., 2016). These markers of exposure can then be applied in host-vector epidemiological studies, in *L. infantum* infection risk assessment, and to assist in the design of control strategies for the disease. Therefore, it is important to validate these techniques in natural, heterogeneous populations from endemic areas, in which a higher individual variability is expected.

Phlebotomus perniciosus activity period in Spain shows two main peaks, the first in June-July and the second in September-October. These peaks also correspond to the periods of highest *L. infantum* transmission (Gálvez *et al*., 2010a; González *et al*., 2017; Morillas-Márquez *et al*., 1983). This trend was identified in our study and corresponds to the rise in anti-saliva antibody levels observed between August and October. Humoral immune response to *P. perniciosus* saliva elicited in experimentally bitten dogs showed that antibody levels significantly rose after 2–4 weeks of continued exposure, peaking in week 5 (Vlkova *et al*., 2011). In our field study, the highest IgG levels were in August, which clearly corresponded to the June-July *P. perniciosus* expected activity peak. Similarly, the high IgG readings obtained in October are likely to correspond to *P. perniciosus* second peak of activity. The lower rise in antibody levels observed at this time point can be explained by an earlier sampling at the beginning of October, which may have hindered the display of a complete seroconversion. The high overall levels of seropositivity to anti-sand fly saliva antigens, especially for SGH (49.43%), strongly support the CanL endemicity status for the region (Lladró *et al*., 2017). These results also validate both SGH and rSP03B as suitable antigens to assess exposure to *P. perniciosus* in natural canine populations from endemic areas.

An important remark when analysing the longitudinal dynamics of anti-sand fly saliva IgG in the study dog population is that there was a clear basal antibody level before the transmission season. After the expected rise in humoral response during summer months, IgG levels returned again to basal levels. These results show that, though exposed to repetitive bites during several months, dogs from endemic areas do not sustain high anti-saliva IgG levels

throughout the year, allowing the detection of recent exposure to sand flies in natural populations. Similar results were recently reported in a longitudinal field study in Brazil, where canine IgG against *Lutzomyia longipalpis* saliva were evaluated (Quinnell *et al*., 2018). Our study identified the same trends for both SGH and rSP03B, which reinforces the suitability of recombinant antigens in detecting recent exposure to phlebotomine vectors in endemic settings, particularly when considering the use of these tests in large-scale studies for vector control interventions (Marzouki *et al*., 2015; Souza *et al*., 2010).

Antibodies recognizing both SGH and rSP03B followed similar dynamics throughout the field study. However, the correlation between the two antigens was only moderate (r_S = 0.54; 95% CI: 0.48–0.60, p<0.001). Even so, available studies show that rSP03B is the most promising surrogate for SGH as a marker of exposure to *P. perniciosus* in the canine host. It has presented high levels of correlation with SGH in both experimentally (Drahota *et al*., 2014) and naturally bitten dogs (Kostalova *et al*., 2017, 2015; Martín-Martín *et al*., 2014). Two apyrase proteins (rSP01B and rSP01) have also shown a good correlation with SGH (Drahota *et al*., 2014). However, in a study where these three recombinant proteins presented similarly high correlations with SGH, rSP03B presented the lowest data dispersion and was considered a better option (Martín-Martín *et al*., 2014). These results were confirmed in a field trial, where single rSP03B demonstrated a higher correlation coefficient with SGH than the combination of rSP03B with rSP01 (Kostalova *et al*., 2015).

A similar correlation between SGH and rSP03B to the one obtained in the present study has been observed before in Umbria region (central Italy) (r_S=0.56; 95% CI: 0.38–0.71, p<0.001; n=96), in a screening study of dog exposure to *P. perniciosus* across European CanL endemic foci (Kostalova *et al*., 2017). A possible reason for these discordant results may be the presence of other closely related phlebotomine species which could induce cross-reactivity with the SGH (Volf and Rohoušova, 2001). In some parts of Catalonia, *P. perniciosus* is sympatric with *P. ariasi*, also a proven vector of *L. infantum* (E Guilvard *et al*., 1996). Due to the close relationship between *P. perniciosus* and *P. ariasi*, both belonging to the subgenus *Larroussius*, it is expected that they share similar salivary antigens (Anderson *et al*., 2006). When comparing the percentage of seropositive dogs detected by both methods during the study, results for SGH are higher (49.43%) than for rSP03B (28.98%). Also, median results per sampling location show differences between SGH and rSP03B: in some cases, the trend between antigens is very similar (e.g. sera from Sant Feliu de Guíxols); in other cases, there is a recognizable peak in anti-SGH IgG, while anti-rSP03B IgG shows no change (e.g. sera from Madremanya). These differences can also be observed over time in the same location, with

humoral responses to SGH and rSP03B peaking in different months along the transmission season (e.g. Canet d'Adri). We may hypothesize that SGH, because it contains more proteins than the single-antigen rSP03B, will more likely cross-react with antibodies against *P. ariasi*, inducing a stronger unspecific reaction to this vector species. It would also mean that the prevalence of sand fly species responsible for *L. infantum* transmission in the province varies according to the location, and possibly in the same location throughout the transmission season, for which it would be interesting to perform further entomological studies in the region.

Correlation indexes between levels of antibodies against both salivary antigens and *L. infantum* infection were low [SGH: r_S=0.27 (95% CI: 0.19–0.35, p<0.001); rSP03B: r_S=0.25 (95% CI: 0.18–0.32, p<0.001)]. Similar low correlations have been described before between sand fly bites and human visceral leishmaniasis (VL), while stronger correlations are reported between human cutaneous leishmaniasis (CL) and recent vector exposure (reviewed in Lestinova *et al*., 2017). This can be explained by VL's longer incubation period and/or the differences in host immune responses to cutaneous and visceral infection (Kedzierski and Evans, 2014). Results from some studies in human populations also suggest that the repeated contact with non-infected sand flies could be correlated with markers of protection for VL (Andrade and Teixeira, 2012). Partial protection against *L. major*, an agent of CL, has also been achieved in immunized mice by the bites of uninfected sand flies (Kamhawi *et al.*, 2000). However, another study with BALB/c mice demonstrated that this type of immunity is limited to short-term exposure and questioned the efficacy of sand fly saliva-induced protection against *Leishmania* infection in CL endemic areas (Rohoušová *et al*., 2011). CanL follows a pattern which is more similar to VL than to CL, therefore a low correlation between humoral responses to sand fly saliva and *Leishmania* would be expected (Vlkova *et a*l., 2011). However, results of the multilevel linear regression model show a positive and statistically significant relationship between *P. perniciosus* bites and a seropositive status for *L. infantum*, both for SGH and rSP03B. Similar results have been described in other longitudinal field studies on both canine anti-*P. perniciosus* and anti-*L. longipalpis* IgG dynamics (Kostalova *et al*., 2015; Quinnell *et al*., 2018). Unlike cross-sectional surveys, longitudinal studies are able to detect the relationship between a higher number of sand fly bites at a given time point and a subsequent *L. infantum* infection. Therefore, this type of study is likely to better explain the relationship between these two events, which can take place several months apart.

4.3. IMPACT OF CANILEISH® VACCINATION IN *Leishmania infantum* INFECTION SEROPREVALENCE STUDIES

4.3.1. Resumen

El desarrollo de vacunas efectivas contra las leishmaniosis humana y canina es un objetivo para la comunidad científica. Sin embargo, deben considerarse y evaluarse los posibles efectos secundarios de la vacunación, preferiblemente antes de la autorización y comercialización de la vacuna. Uno de estos posibles efectos es la reacción cruzada de los anticuerpos inducidos por la vacuna con las pruebas serológicas comunes utilizadas para la detección de la infección por *Leishmania infantum*. Se han realizado estudios longitudinales sobre el tipo de perfil humoral inducido por las vacunas para la leishmaniosis canina comercializadas en Brasil, pero poco se sabe sobre la situación en Europa. En este estudio, se realizó un seguimiento anual de 85 perros vacunados con CaniLeish® y 83 perros control no vacunados. Se tomaron muestras de sangre de todos los animales en puntos de muestreo predeterminados: antes de iniciar la vacunación, inmediatamente antes de cada una de las dos dosis de vacuna siguientes (a intervalos de 21 días) y a los uno, cuatro, seis, nueve y 12 meses después de finalizar la primovacunación. Todas las muestras se analizaron mediante un ELISA *in house* para determinar la presencia de anticuerpos anti-*L. infantum*. La respuesta humoral detectable mediante el método de diagnóstico serológico utilizado fue significativamente mayor en el grupo de los perros vacunados en comparación con el grupo control ($p < 0,01$) hasta un mes después de la vacunación. Los resultados muestran que los anticuerpos inducidos por la vacuna CaniLeish® reaccionan de forma cruzada con una prueba serológica de uso común para el diagnóstico de la infección natural por *L. infantum*. Se discuten las implicaciones de esta interferencia, con especial énfasis en un posible impacto negativo en los estudios de vigilancia de la leishmaniosis canina.

4.3.2. Background

Vaccination is considered one of the most effective methods of controlling CanL and, indirectly, human leishmaniosis (HL) (Palatnik-de-Sousa, 2012; Palatnik-de-Sousa *et al*., 2009). Mathematical models have shown that this method is more effective than treatment or culling of infected dogs (Dye, 1996). A vaccine for CanL should induce a strong, parasite-specific and long-lasting cellular mediated immunity to control infection progression, as well as to block *Leishmania infantum* transmission to sand fly vectors by significantly reducing parasite burden at the vertebrate host level (Courtenay *et al*., 2014; Gradoni, 2015). A possible side effect of most vaccines is the stimulation of humoral immunity and the consequent induction of antibody production (Solano-Gallego *et al.*, 2017a). These can be vaccine-specific, which would not be detected by common serological tests for *L. infantum* infection diagnosis. However, vaccines can also elicit the production of non-specific antibodies that cross-react with standard diagnostic tests (Marcondes *et al*., 2013). In these cases, vaccinated individuals cannot be differentiated from naturally infected ones (Marcondes *et al*., 2011).

CaniLeish® vaccine (Virbac) was licensed in Europe in 2011 (EMA, 2011). It is a second generation vaccine, composed of purified excreted-secreted proteins of *L. infantum* (LiESP) and a saponin adjuvant (QA-21) from a purified fraction of *Quillaja saponaria* (Moreno *et al*., 2012). The pre-licensing CaniLeish® field trial (Oliva *et al*., 2014) showed that vaccine efficacy in the prevention of CanL clinical signs is 68.4% and the risk of developing active disease is reduced by 3.6 times in vaccinated dogs (EMA, 2011). This field trial also reported that vaccine-induced antibodies were detected by a diagnostic IFAT, which was confirmed by two later vaccine follow-up reports (Montoya *et al*., 2017; Oliva *et al*., 2014; Sagols *et al*., 2013).

Speed Leish K™, a qualitative immunocromatographic test (ICT), is the pre-vaccination screening method recommended by the CaniLeish® vaccine manufacturer (Virbac, n.d.). Because in vaccine follow-up studies it showed no (Sagols *et al*., 2013) or low (Montoya *et al*., 2017) cross-reactivity with vaccine-induced antibodies, its use as diagnostic tool able to discriminate between vaccinated and infected individuals has been proposed. However, reports of this ICT performance are not consistent (Ferroglio *et al*., 2013; Solano-Gallego *et al*., 2014) and its sensitivity in *L. infantum* detection has been questioned (Solano-Gallego *et al*., 2017a).

In the present study, a one year follow-up of CaniLeish® vaccinated dogs was performed and an in-house ELISA test was used to measure anti-*L. infantum* antibodies at pre-determined time points. The results reported are expected to provide information on the possible impact of CaniLeish® vaccination on *L. infantum* seroprevalence studies and to motivate a reflection on the need to rethink CanL research and control measures in endemic areas where vaccination has been implemented.

4.3.3. Results

Humoral response to whole *L. infantum* antigen in the trial groups is presented in Figure 12. During the immunization course, vaccinated dogs showed a progressive increase in anti-*L. infantum* antibody levels, which peaked at 1 month post-vaccination. Differences between groups at T2 [median (vaccine group) = 16.2; median (control group) = 14.0; z=-3.120; p=0.002], T3 [median (vaccine) = 24.1; median (control) = 14.1; z=-7.149; p<0.001] and T4 [median (vaccine) = 32.3; median (control) = 16.6; z=-7.052; p<0.001] were considered statistically significant. At T2, 27.1% (23/85) of vaccinated dogs were considered seropositive [in comparison with 8.8% (7/80) in the control group], at T3 seropositivity was 50.6% (43/85) in vaccinated dogs [against 10.8% (9/83) in controls], and at T4 the proportion of seropositive vaccinated dogs was 74.1% (63/85) [20.7% (17/82) in controls]. Differences in the proportions of seropositive individuals between vaccine and control groups at these sampling points (T2 to T4) were considered statistically significant (p<0.01).

After this, antibody levels between groups followed a similar trend, with no statistically significant differences detected between groups, except for T7 (in January 2017, 9 months post-vaccination) [median (vaccine) = 16.7; median (control) = 14.2; z=-2.010; p=0.044].

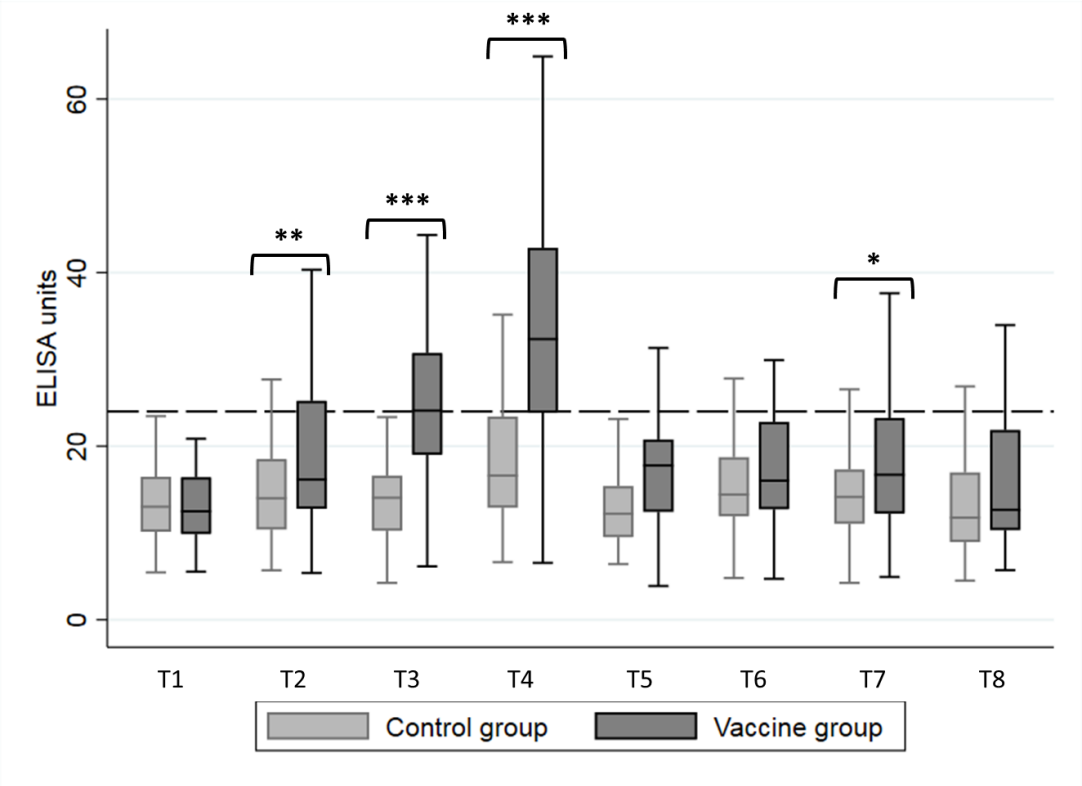


Figure 12. Median and interquartile range ELISA units observed in control and vaccine groups at each sampling point. Statistically significant differences between groups assessed by a Mann-Whitney *U* test are marked with asterisks: *p <0.05; **p<0.01; ***p<0.001. The horizontal dashed line marks the ELISA cut-off, set at 24U. Outlier results are not represented in the figure.

4.3.4. Discussion

The development of effective vaccines for CanL and HL should be the ultimate goal for disease control. (Dye, 1996; Palatnik-de-Sousa, 2012; Palatnik-de-Sousa *et al*., 2009). However, despite the obvious advantages of vaccination, possible drawbacks of its implementation should also be considered. Vaccines which are unable to block parasite transmission have the detrimental effect of "masking" vaccinated asymptomatic carriers (Miró *et al*., 2017b). These animals, although showing a lower susceptibility to developing active *L. infantum* infection or clinical disease, can still harbour the parasite and play a potential role in maintaining its life cycle in endemic areas (Solano-Gallego *et al*., 2001a). Furthermore, vaccines with low or only moderate efficacy do not prevent disease in all vaccinated dogs, and these are a potential diagnostic challenge (Solano-Gallego *et al*., 2017a).

"Differentiating between infected and vaccinated animals" (DIVA) is a well-known concept in veterinary vaccinology (Liu *et al*., 2013; Solano-Gallego *et al*., 2017a). According to the DIVA principle, veterinary vaccines should be produced in such a way that they allow serological differentiation between vaccinated and infected animals. This differentiation can be achieved by the non-interference with standard serological techniques or through the development of specific diagnostic tests, which should present high specificity and sensitivity (Schmitt, 2005). In any case, this aspect must be considered during the development of any new vaccine.

The impossibility of distinguishing between vaccinated and naturally infected dogs can introduce considerable problems to disease or infection diagnosis and surveillance, especially in endemic areas (Solano-Gallego *et al.*, 2017a). CanL serves as a good example of this situation. The diversity of possible infection outcomes and the high proportion of asymptomatic infected animals (Baneth *et al*., 2008), make CanL a diagnostic challenge that often requires the use of multiple diagnostic methods (Morales-Yuste *et al*., 2012; Otranto *et al*., 2009). After the introduction of Leishmune® in Brazil, the first licensed CanL vaccine, several studies pointed out the possibility of vaccine-induced antibodies cross-reacting with CanL official diagnostic tests (Marcondes *et al*., 2013, 2011). In a country where detection and culling of infected dogs is the control measure established by the Ministry of Health (Ministério da Saúde Brasileiro, 2014), this would pose a risk to healthy vaccinated dogs, which could be mistakenly identified as naturally infected individuals and removed (Marcondes *et al*., 2013). A more recent study demonstrated that Leishmune® vaccinated dogs did not test positive with the fast agglutination screening test (FAST) or the direct agglutination test (DAT) (Ribeiro *et al*., 2015), which could then be used as confirmatory diagnostic methods for seropositive vaccinated dogs. Meanwhile, Leishmune® vaccine was withdrawn from the market by the Brazilian Ministry of Agriculture due to lack of effectiveness evidence in phase III trials (MAPA, 2014) and no other cross-reactivity studies were performed.

CaniLeish® vaccine was licensed in Europe in 2011. Results of preliminary studies on the vaccine's safety and efficacy showed that vaccine-induced antibodies could cross-react with commonly used serological techniques like IFAT and ELISA (Martin *et al*., 2014; Oliva *et al*., 2014). The use of IFAT to test for *L. infantum* infection in CaniLeish® vaccinated dogs was not recommended, as these animals consistently presented positive titres due to vaccine-induced antibodies (Oliva *et al*., 2014). This has also been confirmed by a long-term follow-up of owned CaniLeish® vaccinated dogs, in which 31.9-40.3% and 3.2% of individuals tested positive on IFAT one month and one year after vaccination, respectively (Montoya *et al*., 2017), while

another study reported 80% seropositivity with IFAT one month after the first annual vaccine booster (Sagols *et al*., 2013).

Results from the longitudinal study presented here show that a similar situation occurs when ELISA is used. In this study, vaccine-induced antibodies were detected by a commonly used *L. infantum* diagnostic ELISA technique 3 weeks after the first vaccine dose and continued to rise until they peaked one month after vaccination completion. At this time point, 74.1% of vaccinated dogs would be classified as seropositive to *L. infantum*. Three months later (4 months after the third vaccine dose), antibody levels in the vaccine and control groups did not show a statistically significant difference and the same non-significant results were observed at 6 and 12 months post-vaccination. These results show that CaniLeish® vaccinated dogs have a high probability of testing positive by ELISA test until one month post-vaccination. Unfortunately, the absence of data between this time point and the following one (4 months post-vaccination), which showed a marked decrease in vaccine-induced humoral immunity (in comparison with the control group), did not allow detection of an antibody inflexion point. It should also be mentioned that the reduced number of animals tested 4 months after vaccination (n=33) could be a possible reason for the failure in detecting a significant difference between groups. Statistically significant differences in ELISA results between groups were again detected 9 months after vaccination. However, because this assessment was preceded by two sampling points where no significant differences between groups were observed and since it corresponds to a post-transmission season sampling, the observed difference cannot be clearly attributed to a vaccine effect.

As mentioned before, follow-up studies of CaniLeish® vaccinated dogs receiving their annual vaccine boosters (up to the 4th annual booster in one of the studies) revealed that these individuals also cross-reacted with IFAT testing (Montoya *et al*., 2017; Sagols *et al*., 2013). Although it is expected that the same occurs with ELISA, it would be interesting to confirm it and to evaluate the duration and magnitude of interference with this diagnostic test.

The impact of the interaction between vaccine and serological diagnostic tests at the individual dog level is well documented. Several reports describing CanL cases in CaniLeish® vaccinated dogs highlight the added complexity in the diagnosis of these animals (Ceccarelli *et al*., 2016; Gavazza *et al*., 2016; Solano-Gallego *et al*., 2017b). Unfortunately, no information exists on the impact of vaccination on *L. infantum* infection serological surveillance. Diagnostic techniques for CanL large-scale surveys should be simple to perform and interpret, low-cost and highly sensitive and specific. Due to the variable clinical presentation of *L. infantum* infection,

quantitative tests which can provide an assessment of infection stages across the community should be favoured over qualitative ones. Finally, survey techniques must be applicable to the whole studied population so that results can be compared and conclusions can be drawn. Considering these points, quantitative serological methods remain the best tools for *L. infantum* infection mass-screening surveys (OIE, 2014) and are commonly used in epidemiological studies on CanL in Spain (Alcover *et al*., 2013; Amela *et al*., 1995; Ballart *et al*., 2013a; Fisa *et al*., 1999; Gálvez *et al*., 2010b; Martín-Sánchez *et al*., 2009; Miró *et al*., 2012; Morales-Yuste *et al*., 2011; Morillas-Márquez *et al*., 1996). Rapid qualitative serological techniques, aside from only providing positive vs. negative results, can also show lower sensitivity in infection detection (Maia and Campino, 2018). In the case of Speed Leish K™, the CaniLeish® recommended pre-vaccination screening and post-vaccination diagnostic test, reported performance results are not consistent. Although a preliminary comparative study of this ICT with IFAT and Western blot (WB) showed very high test sensitivity and specificity (Ferroglio *et al*., 2013), a later study did not confirm these results, considering Speed Leish K™ inferior to all the quantitative serological tests evaluated (Solano-Gallego *et al*., 2014). The use of a less sensitive screening test in epidemiological studies, even when it holds the advantage of not cross-reacting with vaccine-induced antibodies, compromises infection detection and yields false lower prevalence and incidence rates. Likewise, the use of these tests in pre-vaccination screening produces apparent vaccine failure due to vaccination of previously infected dogs (Solano-Gallego *et al*., 2017a).

Taking into consideration the results presented here and others previously obtained, a critical appraisal of the methods currently used for CanL epidemiological surveillance must be performed. The need to either change or complement the currently used diagnostic techniques and/or to develop new, more efficient, diagnostic methods capable of differentiating between vaccinated and naturally infected individuals is urgently needed.

4.4. EVALUATION OF CANINE LEISHMANIOSIS VACCINE CANILEISH® UNDER FIELD CONDITIONS IN NATIVE DOG POPULATIONS FROM AN ENDEMIC AREA OF SPAIN

4.4.1. Resumen

La vacunación es considerada como el mejor método para controlar la leishmaniosis, una importante enfermedad parasitaria y zoonótica transmitida por vectores. Los perros domésticos son el principal reservorio de *Leishmania infantum*, el agente etiológico de la infección. Por lo tanto, la inmunización de perros contra el parásito podría ser una forma efectiva de detener la transmisión del parásito y de reducir la incidencia de infección en la población canina, así como la transmisión a humanos. Con este objetivo, en los últimos 14 años fueron autorizadas cuatro vacunas para la leishmaniosis canina, dos en Brasil y dos en Europa. Sin embargo, el uso de vacunas solo parcialmente efectivas puede tener un efecto perjudicial "enmascarando" a los animales portadores asintomáticos vacunados, que pueden albergar al parásito y ser responsables de la transmisión de *L. infantum* a individuos susceptibles. Después de siete años en el mercado europeo, se han publicado muy pocos estudios sobre la seguridad y eficacia de la vacuna CaniLeish®. En el presente estudio, se realizó un ensayo de campo aleatorio de dicha vacuna ® durante un año en un área endémica de leishmaniosis canina. El estudio incluyó 168 individuos seleccionados de una población de perros nativa y heterogénea. Los criterios de inclusión en el estudio fueron los recomendados por el fabricante de la vacuna. El seguimiento serológico, molecular y clínico de los individuos permitió la detección de casos de infección activa por *L. infantum* en ambos grupos. Simultáneamente, se realizó la cuantificación de interferón-γ en tres puntos de muestreo para evaluar la inmunidad celular inducida por la vacuna contra *L. infantum*. No se observaron reacciones adversas graves en perros vacunados. Los resultados no mostraron diferencias en el número o gravedad de los casos de infección activa por *L. infantum* entre el grupo de perros vacunado y el grupo control. Un mes después de la vacunación, los niveles de interferón-γ inducidos por el parásito presentaban valores significativamente más altos en el grupo vacunado, en comparación con los niveles pre-vacunación ($p < 0,001$) o con el grupo control en el mismo punto de muestreo ($p = 0,001$). Sin embargo, 9 meses después de la finalización de la vacunación, las diferencias en esta citoquina entre los dos grupos se consideraron estadísticamente no significativas ($p = 0,078$). Los resultados obtenidos sugieren el fallo de la

vacuna CaniLeish® en la prevención de la infección activa en perros de áreas endémicas y expuestos naturalmente al parásito. Esto podría explicarse por una reducción temprana en la inmunidad celular inducida por la vacuna, que no protegería a los perros vacunados durante toda la extensión de la presunta cobertura de la vacuna.

4.4.2. Background

Vaccination is seen as one of the optimal methods for controlling *L. infantum* infection (Dye, 1996; Palatnik-de-Sousa, 2012; Palatnik-de-Sousa *et al*., 2009) and the development of effective vaccines against both CanL and human leishmaniosis (HL) has been a goal for the scientific community. A vaccine for CanL should induce a strong and long-lasting Th1-dominated cellular immunity to control infection progression, while simultaneously reducing parasite burden in dogs in order to decrease their infectiousness to sand flies (Gradoni, 2015). Furthermore, it should be equally effective in protecting against infection or disease (Alvar *et al*., 2013).

The first vaccines for CanL were licensed in Brazil, where leishmaniosis has a significant impact on human health. Leishmune® (Zoetis, Brazil) a second-generation vaccine composed of the fucose-mannose ligand (FML) glycoprotein complex of *L. donovani* and a saponin adjuvant (Borja-Cabrera *et al*., 2002) was licensed in 2004. Leish-Tec® (Hertape Calier, Brazil) is formulated with a recombinant protein A2 from *L. donovani* amastigotes associated to saponin and was released in 2007 (Fernandes *et al.*, 2008). After 10 years of use, the Leishmune® production and marketing licence was withdrawn by the Brazilian Ministry of Agriculture due to lack of efficacy evidence in phase III trials (MAPA, 2014).

The European Medicines Agency (EMA) granted a marketing licence to the first CanL vaccine in 2011 (EMA, 2011). CaniLeish® (Virbac, France) is a second generation vaccine composed of purified excreted-secreted proteins (LiESP) of *L. infantum* and a saponin adjuvant (Moreno *et al*., 2012). In 2016, LetiFend® (Laboratorios LETI, Spain), a recombinant vaccine consisting of a chimerical protein (protein Q) with no added adjuvants (Carcelén *et al.*, 2009), also received marketing permission for the European region (EMA, 2016). In both cases, licensing was granted based on the safety and efficacy results of a single field study (Fernández Cotrina *et al*., 2018; Oliva *et al*., 2014).

According to pharmacovigilance data reported by Virbac in October 2015, more than 1.8 million doses of CaniLeish® had been sold during the first 3.5 years of marketing in the European Economic Area, Switzerland and Tunisia (Breton *et al*., 2015). However, few studies have been published since the preliminary phase II research (Martin *et al*., 2014; Moreno *et al*., 2014, 2012) and the only phase III trial performed before a licence was granted (Oliva *et al*., 2014). Very little is known about the vaccine's effect in heterogeneous dog populations from endemic areas. In addition, after 7 years on the European market, CaniLeish® safety and efficacy have been questioned by veterinarians and the general public. Cases of CanL in vaccinated dogs have been reported (Ceccarelli *et al*., 2016; Gavazza *et al*., 2016), and the performance of the recommended pre-vaccination screening method has presented inconsistent results (Solano-Gallego *et al*., 2017b).

The present study consists of a one-year randomized CaniLeish® vaccine field trial performed in a CanL endemic area with a heterogeneous and autochthonous canine population. Dogs of both sexes, different ages and various breeds have been included. Inclusion criteria were the same as recommended by the vaccine's manufacturer for dog vaccination and were followed for both experimental groups. The objective of this study was to evaluate CaniLeish® vaccine under real field conditions in a representative population of dogs bred in a CanL endemic area.

4.4.3. Results

From the 177 dogs initially enrolled in the vaccine study, 168 completed the vaccination phase (95%) [85 dogs in the vaccine group (94.4%) and 83 in the control group (95.4%)]. No statistically significant differences in dogs' characteristics were detected between groups, as described in section 3.2.2 (pages 48-49).

Vaccine safety

No severe adverse reactions were observed in vaccinated dogs. One case of transient anorexia and apathy following first vaccine dose administration was reported, which was not observed again in the same animal following the second or third vaccination dose. No other adverse reactions were reported.

Humoral and molecular detection of L. infantum

The dynamics in humoral response to whole *L. infantum* antigen during the one year vaccine trial in both groups was previously described in section 4.3.3.

In April 2017 (one year post-vaccination), 35 individuals were seropositive for *L. infantum* and were further tested by qPCR on lymph node samples (21 in the vaccine group and 14 in the control group). From these, 19 (54.3%) presented a positive qPCR result [9 vaccine (42.9%) and 10 control (71.4%)], with parasite loads ranging from less than one parasite/mL to $1.24x10^7$ parasites/mL. No statistically significant differences were detected in lymph node parasite load ($z=1.31$, $p=0.1903$) or in the incidence of positive results ($\chi2=2.76$, $p=0.096$) between groups.

Vaccine-induced CMI

At the pre-vaccination sampling point, 28.1% of the trial dogs (43/153) presented *L. infantum*-specific IFN-γ production (20 dogs in the vaccine group and 23 in the control group). Measurable IFN-γ concentrations at this time point ranged from 2.50 to 7317.25 pg/mL.

Levels of IFN-γ in vaccine and control groups throughout the study are presented in Figure 13. Median IFN-γ levels for the control group were equal to zero (range: 0 to 7317.25 pg/mL) in the 3 sampling points tested and no differences were detected in this group between time points ($p>0.05$). Dogs in the vaccine group showed a marked increase in IFN-γ levels one month after vaccination completion (median=38.95 pg/mL; range: 0 to 5136.58 pg/mL), considered to be significantly higher when compared to pre-vaccination results ($z=-6.624$, $p<0.001$). At 9 months after vaccination, IFN-γ levels in the vaccine group had dropped considerably (median=12.74 pg/mL; range: 0 to 6235.92 pg/mL), but maintained significantly higher results than pre-vaccination levels ($z=-2.931$, $p=0.003$). Differences between vaccine and control groups were only considered significant at the 1M time point ($z=-3.297$, $p=0.001$). No statistically significant differences in IFN-γ levels were detected between groups at the pre-vaccination ($p=0.730$) or 9M time points ($p=0.078$).

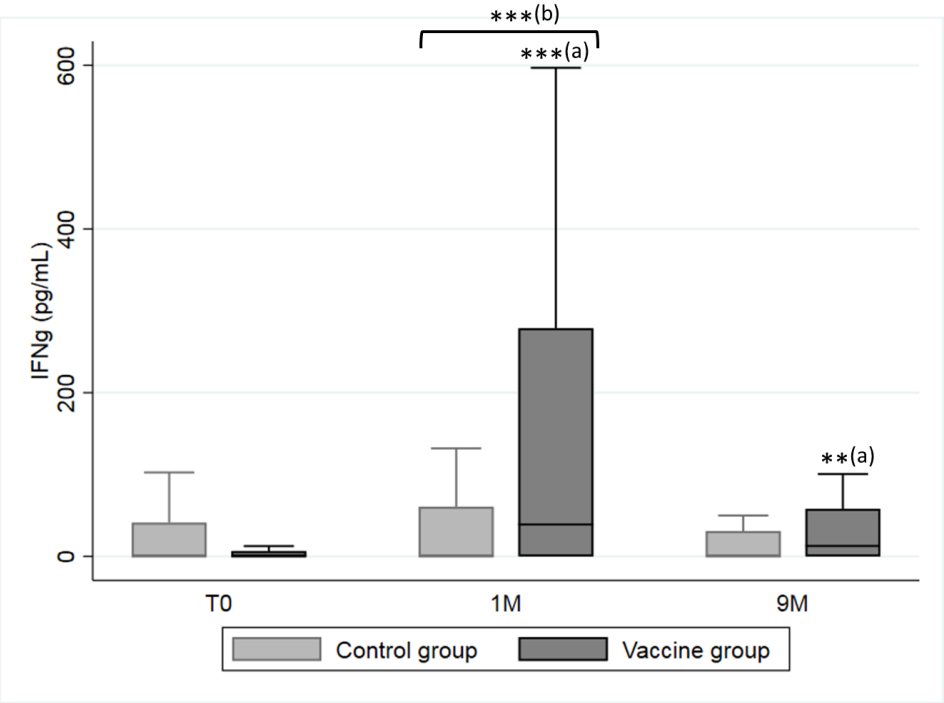


Figure 13. Median and interquartile ranges of IFN-γ levels observed in the vaccine and control groups at 3 time points: before vaccination (T0), one month after vaccination completion (1M) and 9 months after vaccination completion (9M). (a) indicates a statistically significant difference in the vaccine group between T0 and each time point; (b) indicates a statistically significant difference between trial groups at the same time point. ** p<0.01; *** p<0.001. Outside results (above the 3rd quartile) are not presented in the figure.

Clinical assessment of trial dogs

At the end of the vaccine trial, 87.6% of dogs (127/145) were considered asymptomatic for CanL [62 dogs in the vaccine group (87.3%) and 65 in the control group (87.8%)]. The remaining 18 individuals (12.4%) showed two or more clinical signs compatible with CanL [9 in the vaccine group (12.7%) and 9 in the control group (12.2%)]. These were mainly characterized by localized or multifocal lymphadenomegaly (detected in 100% of symptomatic dogs) and pale mucous membranes (50% of symptomatic dogs). Other observed clinical signs were dermatological lesions (38.9%), poor body condition (27.8%) and ocular alterations (22.2%).

Laboratory exams after T0 were only performed in dogs suspected of CanL and in seropositive dogs at the end of the trial. At 12M, 37.1% of the analysed dogs (13/35) were considered healthy (zero or one laboratory changes compatible with CanL) (11 in the vaccine group and 2 in the control group), while 42.9% (7 dogs in the vaccine group and 8 dogs in the control

group) presented 2 or 3 analytical alterations, and 20.0% (3 vaccine and 4 control) showed 4 to 6 laboratory abnormalities.

Results of clinical exams and laboratory analysis were used as additional information for defining *L. infantum* active infection in trial dogs. Table 14 describes clinical and laboratory alterations found in confirmed cases of CanL.

Confirmed cases of CanL in the vaccine and control groups

During the vaccine field trial, two dogs were diagnosed with CanL (one in the vaccine group and one in the control group). The remaining individuals were evaluated one year after vaccination completion (April 2017) for seropositivity against *L. infantum*. From these, 33 dogs showed positive anti-*L. infantum* antibody levels (20 in the vaccine (28.2%) and 13 in the control (17.6%) groups) in one of the two post-transmission season serological assessments (January and April 2017). These 35 dogs were further assessed by *L. infantum* DNA detection in lymph node samples by qPCR and clinico-laboratory evaluation. Only dogs that met the parameters previously defined for *L. infantum* active infection were considered to be confirmed CanL cases. From these, 4 CanL cases were observed in vaccinated dogs (5.6%; 4/71) and 4 in control individuals (5.4%; 4/74). The results showed no difference in the development of active *L. infantum* infection between the two study groups (Table 14).

Table 14. Profile of dogs diagnosed as confirmed cases of canine leishmaniosis.

Treatment group	Dog ID	*L. infantum* ELISA units[a]	Parasite detection in LN[b] (parasites/mL)	Clinical findings	Laboratory findings
Control	5-009	109.81	Positive ($3.05x10^3$)	No clinical signs.	- Anemia - Hypoalbuminemia
	8-004	227.61	Positive ($1.24x10^7$)	- Poor body condition - Exfoliative dermatitis - Pale mucous membranes - Lymphadenomegaly	- Anemia - Increased renal parameters (BUN and CREA) - Hypoalbuminemia - Decreased A/G ratio
	12-003[c]	108.20	Positive ($1.43x10^4$)	- Poor body condition - Diffuse alopecia - Lymphadenomegaly	- Anemia - Leukocytosis - Hypoalbuminemia
	23-022	151.13	Positive (2.11x10)	- Ocular purulent discharge - Lymphadenomegaly	- Hyperproteinemia - Hypoalbuminemia - Decreased A/G ratio
Vaccine	2-013	159.67	Positive ($4.76x10^2$)	- Poor body condition	- Hypoalbuminemia
	8-025	158.94	Positive ($2.00x10^4$)	- Lymphadenomegaly	- Hypoalbuminemia
	18-003[d]	173.62	Positive ($1.35x10^4$)	- Lymphadenomegaly - Skin lesions	No laboratory findings.
	22-003	204.06	Positive ($1.39x10^6$)	- Pale mucous membranes	- Anemia - Hyperproteinemia - Mild hypoalbuminemia

[a]ELISA units measured at 12M, except when specified otherwise; the ELISA cut-off considered was 24U.

[b]qPCR performed in lymph node samples at 12M, except when specified otherwise.

[c]CanL diagnosis in May 2017

[d]CanL diagnosis in October 2016

BUN: blood urea nitrogen; CREA: creatinine; A/G ratio: albumin/globulin ratio

4.4.4. Discussion

The present study, a multi-site randomized vaccine trial, had the objective of evaluating CaniLeish® vaccine in field conditions in a native heterogeneous population of dogs living in an *L. infantum* endemic region.

Canine seropositivity to *L. infantum* at the end of the trial was detected in 75% (9/12) of the trial locations, demonstrating the presence of infection in most dog kennels. The study of dog exposure to sand fly saliva in the area showed a high incidence of reactivity to sand fly saliva antigens and a homogeneous vector presence in the trial locations (Velez *et al*., 2018a).

Due to the duration of the present field trial, which included only one *L. infantum* transmission season, detection of CanL clinical cases was not expected. The mean period between infection and development of clinical disease was reported to be 7 months, ranging from 3 to 14 months (Oliva *et al*., 2006), but it can extend to years in resistant individuals (Baneth *et al*., 2008). For this reason, the studied outcome was active *L. infantum* infection and not clinical CanL. Additionally, previous longitudinal studies of natural *L. infantum* infection have shown that once an individual reaches a high specific antibody level, the evolution will be inevitably towards the development of clinical disease (Oliva *et al*., 2014, 2006). The expected effect of vaccination on the development of active infection is reported by the CaniLeish® vaccine manufacturer, which states that the risk of developing active disease is reduced by 3.6 times in vaccinated dogs (EMA, 2011), and this was the parameter used to compare vaccine and control groups.

CaniLeish® vaccine proved to be safe in the dog population studied. Apart from one single case of transient apathy and anorexia, no other adverse effects were reported by dog owners or observed by the researchers. It should be noticed, however, that the study population was mainly composed of robust crossbred or purebred hunting dogs weighing between 15 and 25 kg, which may be less likely to show discomfort. In a questionnaire-based survey of veterinary practitioners working in the Girona region, 82% of vaccine appliers reported adverse reactions, ranging from the most commonly observed local swelling and pain, to cases of anaphylactic shock. However, as also pointed out by the study's authors, the attribution of these adverse effects to vaccine administration was based on veterinarians' criteria and confirmation of the cause of clinical signs may not have been pursued on all occasions (Lladró *et al*., 2017). In the present study, severe adverse effects were not observed, which is in accordance with previous vaccine safety reports (Breton *et al*., 2015; Marino *et al*., 2017).

In the present study, a whole promastigote in-house ELISA was used as a diagnostic test for *L. infantum* infection (Ballart *et al*., 2013a; Riera *et al*., 1999; Velez *et al*., 2018b). By measuring the humoral immune response to *L. infantum*, quantitative serological tests are considered reliable indicators of active infection and good predictors of the onset of clinical signs (Oliva *et al*., 2006). In dogs, high levels of specific IgG antibodies against *L. infantum* have been related to the active phase of the disease and to the onset of pathophysiological disorders (Oliva *et al*., 2006; Pinelli *et al*., 1994). In a CanL longitudinal field study, it was observed that serology and parasite culture were the best predictors of progression to active *L. infantum* infection, with highly seropositive dogs showing no return to a parasite-free status (Oliva *et al*., 2006). Seroconversion is defined as a 4-fold increase in sequential samples from the same dog (Paltrinieri *et al*., 2010) or a 3-fold increase in the cut-off value of a well-standardized diagnostic test (Solano-Gallego *et al*., 2011, 2009). In endemic areas, the median time between the establishment of progressive infection and seroconversion was estimated to be 10.5 months (ranging from 4 to 22 months) (Oliva *et al*., 2006).

Specific humoral response to vaccine antigens has not been evaluated in this trial for two reasons. First, because it has been characterized in all previous CaniLeish® studies (Martin *et al*., 2014; Moreno *et al*., 2014; Moreno *et al*., 2012; Oliva *et al*., 2014), showing consistent results. Second, because no correlation was observed between CaniLeish®-induced IgG profile and protection in vaccinated dogs (Oliva *et al*., 2014). Considering these points, and because the main aim of this study was the characterization of *L. infantum* infection in the study population, a complete description of the humoral immune profile induced by the vaccine was not performed.

Molecular detection of the parasite was performed in lymph node samples to confirm the diagnosis of active *L. infantum* infection in seropositive dogs at the end of the trial. Although the levels of seropositivity considered for infection diagnosis in the study were very conservative and clear indicators of progressive infection, the detection of the parasite in a target organ validated the serological results. On the other hand, the detection of parasite DNA in lymph nodes in the absence of seroconversion would not have been considered as a definitive confirmation of infection. Reversion from a PCR positive result in “deep” tissues to a *Leishmania*-free status has been documented before (Oliva *et al*., 2014, 2006; Paltrinieri *et al*., 2010), showing that molecular detection alone, especially in the presence of low parasite loads, cannot be used to perform a definitive CanL diagnosis.

IFN-γ is considered to be a high-quality biomarker of immunogenicity and protection against *Leishmania* infection (Carrillo and Moreno, 2009; Reis *et al*., 2010). It is considered the key cytokine involved in the activation of macrophages and the killing of intracellular *L. infantum* amastigotes, in collaboration with other immune mechanisms (Carrillo and Moreno, 2009). High levels of IFN-γ are associated with host resistance to *L. infantum* infection (Chamizo *et al*., 2005; Reis *et al*., 2010; Solano-Gallego *et al*., 2016b) and it is used as a marker of response to CanL therapy (Manna *et al*., 2008a; Martínez-Orellana *et al*., 2017). It has also been quantified as a marker of protection in previous vaccine studies, both for CaniLeish® (Martin *et al*., 2014; Moreno *et al*., 2014; Moreno *et al*., 2012), and for other vaccines (Costa-Pereira *et al*., 2015; de Lima *et al*., 2010; Fernandes *et al*., 2008; Resende *et al*., 2013). Apart from providing an indication of vaccine-induced CMI, the quantification of IFN-γ in this study also allowed assessment of previous exposure to *L. infantum* in the trial population. According to the results obtained in the pre-vaccination evaluation, almost 30% of dogs demonstrated a positive IFN-γ response when exposed to SLA, which indicates recognition of *L. infantum*. It is also an indicator of resistance in the trial dogs, showing that some of the individuals enrolled in the vaccine trial were probably naturally immune to the parasite. This degree of resistance is expected in canine populations from endemic areas (Baneth *et al*., 2008), although its effect may be difficult to quantify and account for when setting a field trial.

The diversity of methods described for IFN-γ quantification may induce variability in reported results (Cortese *et al*., 2013; Holzmuller *et al*., 2005; Moreno *et al*., 2012; Rodríguez-Cortés *et al*., 2016, 2007a). Previous CaniLeish® studies have measured this cytokine production through the ELISpot assay (R&D Systems), which quantifies the proportion of IFN-γ producing T cells after stimulation with SLA. The commercial ELISA kit used in this study provides a direct quantification of IFN-γ concentration in the supernatant of lymphocytes cultured with SLA and has been used in previous CanL studies (Martínez-Orellana *et al*., 2017; Solano-Gallego *et al*., 2016b). Although a direct comparison of results obtained with different methods is not possible, the detection of significant changes at different time points or between trial groups can still be performed. Levels of IFN-γ measured in the vaccine group one month after vaccination completion showed a marked increase when compared to the pre-vaccination time point or to parallel results in the control group. As mentioned before, this corresponds to the moment when vaccine-induced immunity should be established (EMA, 2011), and illustrates the stimulation of cellular immune response in vaccinated individuals. A similar significant difference was observed between vaccine and control groups 3 weeks after vaccination completion in a previous CaniLeish® study (Moreno *et al*., 2012). IFN-γ concentrations were measured again 9 months after vaccination,

showing that the cytokine levels in the vaccine group were still significantly higher than in the pre-vaccination phase, but did not show a statistically significant difference when compared to the control group at the same time point. Results from previous CaniLeish® studies have shown a statistically significant difference in the proportion of IFN-γ producing cells between vaccine and control dogs at 6 months post-vaccination (Moreno *et al*., 2014) and no detectable difference one year post-vaccination (Martin *et al*., 2014; Moreno *et al*., 2014). In these studies, the 9 month post-vaccination time point was not assessed. If the results of this and the two CaniLeish® previous studies are gathered, it can be inferred that the levels of IFN-γ may drop to non-significant levels between 6 and 9 months post-vaccination. However, unlike the two studies mentioned, the present study was performed in field conditions and animals were naturally exposed to one *L. infantum* transmission season, which may also have interfered in IFN-γ levels. Nevertheless, 3 months after the end of the transmission season and of possible interactions with *L. infantum* parasites, vaccinated dogs did not show evidence of significant differences in IFN-γ production when compared to the control group. A short-lived vaccine-induced CMI which fails to be protective during the whole period of expected vaccine coverage could explain the lack of difference in detected CanL cases between vaccine and control groups observed at the end of this study. However, care should be taken in the over-interpretation of a single parameter as it is known that IFN-γ is only part of a complex network of regulatory and counter-regulatory interactions involving multiple cells and cytokines (Hosein *et al*., 2017; Reis *et al*., 2010). Further studies on the immune response developed by trial individuals would be needed to fully characterize vaccine-induced CMI.

Cases of *L. infantum* active infection were defined by the assessment of multiple parameters. Serological screening at the end of the vaccine trial allowed for the detection of potential cases. These were further analysed by molecular detection of *L. infantum* in lymph nodes and by characterization of general laboratory parameters (CBC, renal and hepatic biochemical profiles and serum protein electrophoretogram). The combined information provided by all these assays allowed for the identification of active *L. infantum* infection cases. Two individuals, one in each trial group, were identified as diseased during the study. The remaining six (3 in each group) were detected at the end of the trial. According to previous vaccine studies in natural conditions, where a continued parasite challenge is present, it is unlikely that these individuals reverted to a negative state (Oliva *et al*., 2014). The CaniLeish® vaccine manufacturer reports an efficacy of 68.4% in the prevention of clinical signs of CanL and a protection level, defined as the percentage of vaccinated animals which do not develop clinical signs, of 92.7%. These results were obtained during the vaccine pre-licensing field study in a homogeneous population of

naïve beagle dogs, 5 to 7.5 months old (Oliva *et al*., 2014). In the Oliva *et al*. study, four cases of active *Leishmania* infection were recorded at 12 months post-vaccination; one in the vaccine group (2.4%) and three in the control group (7.7%). All these dogs progressed to symptomatic active infection in the following months. In the present trial, no differences in the number or severity of active infection cases were observed between vaccine and control groups one year post-vaccination. Although the reduced number of CanL cases observed demands caution in the interpretation of the results of this trial, they are supported by the results of a recent field study, which compared the efficacy of the CaniLeish® vaccine and two insecticide dog collars in the prevention of CanL. After one year of study, although different protection efficacies could be determined for each insecticide collar, no difference was detected in the number of CanL cases between CaniLeish® vaccinated dogs and the control group (Brianti *et al*., 2016). Again, the total number of CanL cases detected in the trial presented by Brianti *et al*. was low, which may have impaired the detection of a difference between groups. However, considering the results obtained in both studies, if such a difference exists it is unlikely to be the one claimed by the vaccine's manufacturer.

The ultimate step to assess the efficacy of a vaccine against CanL is the phase III field trial, with native canine populations from endemic areas, where vaccinated and control dogs are exposed to natural infection by sand fly bites (Reis *et al*., 2010). However, in contrast to laboratory experimental challenge, natural infection depends on many variable factors related to the canine host, the vector and the parasite. According to Solano-Gallego *et al*. (2009), only an estimated 1/3 of dogs living in CanL endemic areas will be susceptible to infection during the course of their lives. This implies that, at the time of enrolment for a vaccine field trial, a high proportion of individuals testing negative for *L. infantum* are already resistant to the parasite and will be worthless in terms of vaccine effect assessment. Likewise, the proportion of individuals lost during a field trial cannot be accurately ascertained beforehand. In the present study, the expected loss to follow-up was 10%, based on preliminary assessments performed on the same dog population. However, at the end of the study, 18% of the initial dog sample had been lost. This was mainly due to deaths related to hunting activities and animal movement to other dog kennels. Another important factor of variability in field trials is vector-related. Sand fly populations are highly influenced by biotic and abiotic factors (Ballart *et al*., 2014; Gálvez *et al*., 2010a; Hartemink *et al*., 2011), which change annually. Some of these factors, such as temperature, are also known to influence *L. infantum* development inside the vector (Rioux *et al*., 1985). Likewise, it is impossible to predict the success of natural parasite transmission in a given area and year. For these reasons, field trials with privately owned dogs are challenging and

their success difficult to predict. Nevertheless, they represent the closest situation to a “real life” scenario, allowing for a more realistic assessment of vaccine performance.

5. GENERAL DISCUSSION

5. GENERAL DISCUSSION

The work presented in this thesis aimed to extend the current knowledge of canine *L. infantum* infection epidemiology and control. In a first phase, a survey of the dog population of Girona province allowed the first description of *L. infantum* infection in a previously uncharacterized region in the north-east of Spain, a CanL endemic country. An assessment of individual and environmental risk factors associated with canine *L. infantum* infection in the studied population provided additional information which can be applied to the design of directed control measures. The indirect detection of host-vector contact through markers of exposure to sand fly saliva completed the characterization of *L. infantum* infection in the province.

The epidemiological study was followed by a field trial of CaniLeish®, the first CanL vaccine licensed in Europe in 2011. In addition to evaluating the possible role of CaniLeish® in CanL control, this work also looked at the potential impact of vaccination on the seroepidemiological vigilance of *L. infantum* infection.

5.1. EPIDEMIOLOGICAL STUDIES OF CANINE *Leishmania infantum* INFECTION

Epidemiological vigilance is essential for the study and monitoring of infectious diseases, as well as for the planning and implementation of directed control measures (WHO, 2014). This is certainly the case for *L. infantum* infection, the distribution of which in canine populations from endemic regions is highly heterogeneous (Gradoni, 2018; Pennisi, 2015). The expected existence of hyperendemic foci within an endemic region or country justifies the implementation of epidemiological surveys at a finer scale, in order to identify these "hotspots" of canine *L. infantum* infection (Ballart *et al*., 2013b; Capelli *et al*., 2004). This would also be fundamental for controlling HL, as these CanL hyperendemic foci correspond to higher reservoir concentration areas, thus increasing the risk of human infection (Sevá *et al*., 2016). Surveys of *L. infantum* infection and vector presence are needed to predict possible parasite geographic expansion (Ballart *et al*., 2013b; Maia and Cardoso, 2015; Maroli *et al*., 2008; Ntais *et al*., 2013) and to anticipate and minimize the impact of disease outbreaks (Arce *et al*., 2013). In addition to *L. infantum* infection incidence and prevalence, the characterization of associated risk factors is essential for the design of accurate risk assessment maps for the

disease (Franco *et al*., 2011; Hartemink *et al*., 2011; Pigott *et al*., 2014). The information gathered by these studies not only allows the selection of significant variables to introduce into the maps, but also provides suitable data for them.

The study presented in section 4.1 of this thesis, an *L. infantum* infection prevalence study in Girona province (Catalonia, north-eastern Spain), is an example of this type of epidemiological survey. Although surrounded by proven CanL endemic areas (Ballart *et al*., 2013a; Pomares *et al*., 2016; Portús *et al*., 2007), very little was known about the status of the disease in Girona province. The only previously published study, by Lladró *et al*. (2017), presented results from a questionnaire-based survey of veterinary practitioners working in the region and reported the diagnosis of autochthonous cases of CanL in the province. Amongst the respondents, 75% confirmed between one and 20 cases of CanL annually, most of which had not been diagnosed before. Also, almost 50% of the veterinarians believed that the disease had been presenting a rising trend in the previous 10 years. The study presented in this thesis confirmed the endemicity of *L. infantum* infection in Girona and once again demonstrated the heterogeneity in CanL distribution throughout an endemic region. Characteristics of a stable CanL focus were identified, such as a significant infection prevalence detected in the canine population, associated to a high number of asymptomatic individuals, which is indicative of the adaptation of local dogs to the parasite (Baneth *et al*., 2008). Furthermore, contact between the canine host with phlebotomine vectors was demonstrated (section 4.2), proving that all conditions for the local maintenance of a complete *L. infantum* life cycle are gathered (Maroli *et al*., 2008). Canine infection distribution in Girona province seems to be influenced by landscape and/or climatic factors, as indicated by the close relationship found between altitude and infection. Simultaneously, at the individual level, dog's age showed a positive association with *L. infantum* infection. The overall estimated seroprevalence for Girona province was 19.5%, which is in accordance to others reported recently in Spain (Alcover *et al*., 2013; Ballart *et al*., 2013a; Gálvez *et al*., 2010b; Morales-Yuste *et al*., 2011), as well as in other countries of the Mediterranean basin (Cortes *et al*., 2012; Maroli *et al*., 2008; Ntais *et al*., 2013). Observed point seroprevalences of 0 to 80% are comparable to those reported in a study which compiled results from CanL seroprevalence surveys undertaken in France, Italy, Spain and Portugal between 1971 and 2006 (Franco *et al*., 2011), confirming the previous claims of the heterogeneous distribution of the disease.

Individual and location factors partially explain the differences in reported CanL prevalence in endemic regions. Genetic factors can determine resistance or susceptibility to *L. infantum* infection, and this can be correlated with specific breeds (Quilez *et al*., 2012; Sanchez-Robert

et al., 2008, 2005; Solano-Gallego *et al*., 2000). Age of the host can also be considered a risk factor, as observed in Girona province and previously documented in several CanL studies (Alonso *et al*., 2010; Cortes *et al*., 2012; Fisa *et al*., 1999; Gálvez *et al.*, 2010b; Miranda *et al*., 2008; Miró *et al.*, 2012), as well as for HL, in which children under 5 years old are considered to be at higher risk of *L. infantum* infection (Gradoni, 2018; WHO, 2010). Location risk factors are usually determined by the edaphoclimatic conditions needed by sand flies (Barón *et al*., 2011; Dantas-Torres *et al*., 2014; Gálvez *et al*., 2010a; Tarallo *et al.*, 2010), as well as by vertebrate host densities (Sharma and Singh, 2008). The interaction between these variables will determine the presence and abundance of infected competent vectors, the main risk factor for *L. infantum* transmission (Maroli *et al*., 2013; Suarez Rodríguez *et al*., 2012).

The design of epidemiological surveys can also introduce significant variations in *L. infantum* infection seroprevalence results (Franco *et al.*, 2011). Different criteria in the selection of the target dog population or in its geographical distribution, as well as differences in the diagnostic techniques used, usually allow little or no comparison between studies in different areas or in the same region over a period of time (Morales-Yuste *et al*., 2012). Access to a representative sample of a region's canine population can prove to be difficult in countries where dog registration is not mandatory and no population census exists. Alternative sources of study individuals can be veterinary practices (Goyena *et al*., 2016; Maia *et al*., 2015; Morales-Yuste *et al*., 2011), dog owners associations (hunters, breeders, etc.) or dog shelters (Cabezón *et al*., 2010; Miró *et al*., 2012; Santi *et al*., 2014). Any of these options is not optimal and is expected to introduce bias in CanL prevalence results (Botet and Portús, 1993; Franco *et al*., 2011). Since the end of the compulsory anti-rabies vaccination in Catalonia (Escola d'Oficis Catalunya, 2017), for which campaigns were organized that gathered a significant proportion of the dog population, it has become more difficult to perform large-scale surveys. For the work presented in this thesis, and in order to obtain a representative sample of the province's canine population, dog recruitment was performed through local veterinarians. The target dog population were not dogs attending the veterinary practice, but mostly composed of hunting packs kept outdoors in rural or periurban settings, where CanL was expected to be present and prevalent as shown in other studies (Alcover *et al*., 2013; Ballart *et al*., 2013). Their inherent characteristics make them good sentinels for CanL (Ballart *et al*., 2013a; Cabezón *et al*., 2010) and the consistent use of the same type of canine population throughout this study, as well as in similar studies in Spain (Alcover *et al*., 2013; Ballart *et al*., 2013a), allowed for comparisons to be made. Nevertheless, the bias introduced by dog selection and geographic constraints is

assumed in this work (discussed in detail in pages 75-76 of section 4.1) and should be accounted for in future surveys.

As already mentioned, the choice of diagnostic methods also represents an important source of variability, which should be considered when comparing results obtained in different studies (Morales-Yuste *et al*., 2012). The most frequently used diagnostic methods in CanL epidemiological surveys are serological and molecular. While the former identify antibodies and are a reflection of the host's humoral immune response to *L. infantum*, the latter detect parasite DNA in a host's tissues, regardless of the level of host-parasite interaction (Solano-Gallego *et al.*, 2009). Both present advantages and drawbacks but, most importantly, the two methods provide different information that should be interpreted accordingly (Solano-Gallego *et al*., 2011).

Serological methods are widely used and represent the most frequently reported screening method in CanL epidemiological studies (Alcover *et al*., 2013; Ballart *et al*., 2013a; Cortes *et al*., 2012; Gálvez *et al*., 2010b; Miró *et al*., 2017a). However, their performance is greatly influenced by technical aspects and population-related parameters (de Mendonça *et al*., 2017a; Dye *et al*., 1993; Greiner and Gardner, 2000; Morales-Yuste *et al*., 2012). By detecting anti-*L. infantum* antibodies and not the parasite, serological tests are unable to differentiate between infected, diseased, resistant and/or vaccinated individuals (Greiner and Gardner, 2000). Thus, in endemic areas, where 50% to 80% of the canine population is expected to be infected or to have been exposed to the parasite (Noli and Saridomichelakis, 2014) and there is a likely predominance of asymptomatic dogs (Baneth *et al.*, 2008), a method capable of discerning between the different levels of parasite exposure would certainly provide more useful results. The higher prevalence of *L. infantum* infection observed in endemic areas, together with the complexity of infection staging, can impact the sensitivity and specificity of serological tests (de Mendonça *et al*., 2017a; Greiner and Gardner, 2000). A test's specificity will be reduced by the high number of false-positive results yielded by immune individuals (de Mendonça *et al*., 2017a), either naturally exposed or vaccinated (Marcondes *et al*., 2011), and possible cross-reactions with other common canine infectious agents, such as *Ehrlichia canis*, *Babesia canis* or *Hepatozoon canis* (Morales-Yuste *et al*., 2012). Likewise, infected dogs with prepatent infections, which have not yet seroconverted, will test negative (Dye *et al*., 1993; Oliva *et al*., 2006; Otranto *et al*., 2009). The duration of this seronegative prepatent period usually ranges between 1 and 22 months (Moreno and Alvar, 2002), but can be longer in dogs showing a subpatent condition, characterized by an apparent "parasite silencing" (Oliva *et al*., 2006). The impact of these factors in serological tests performance can vary across different

populations and subpopulations in an endemic area, as well as in the same population over time, according to changes in infection dynamics (Greiner and Gardner, 2000). In addition to these sources of variability, the accuracy of each serological method is distinct, and differences in detection are observed when using whole *Leishmania* antigen or recombinant antigens, either alone or combined (reviewed in Duthie *et al*., 2018). Finally, both quantitative and qualitative serological methods are available, and the choice between these should be based on the study purpose and resources. Quantitative methods provide a measurement of antibody levels, which can be correlated with infection severity and used to identify diseased individuals, as these tend to present significantly higher levels of anti-*Leishmania* antibodies (Oliva *et al.*, 2006). When only a dichotomous result is required, qualitative methods may prove to be a better option, with the additional advantages of not requiring trained personnel or laboratory facilities and providing almost immediate results (Ribeiro *et al*., 2018). Claims of a possible lower diagnostic sensitivity provided by qualitative methods (Morales-Yuste *et al*., 2012; Quinnell *et al*., 2013; Rodríguez-Cortés *et al*., 2013; Solano-Gallego *et al*., 2014) may not apply to more recently developed techniques (Duthie *et al*., 2018; Travi *et al*., 2018).

Molecular techniques show high specificity which, unlike serological methods, is not affected by the prevalence of infection in the study area (de Mendonça *et al*., 2017a). However, their diagnostic sensitivity depends on the method and sample used (Solano-Gallego *et al*., 2016a) and on the target DNA sequence (Lachaud *et al*., 2001, 2002a, 2002b; Paltrinieri *et al*., 2016). Also, the use of PCR alone is not recommended for screening clinically healthy dogs in CanL endemic areas, where it should be used and interpreted together with serological and clinical findings for a correct diagnosis (Solano-Gallego *et al*., 2011). Kinetoplast DNA (kDNA) is the most used target for detection of *Leishmania* because of its multicopy nature and high sensitivity, but ribosomal DNA (rDNA) and protein-coding genes can also be used for diagnosis (Akhoundi *et al*., 2017). The use of qPCR instead of conventional PCR methods allows the reliable measurement of DNA products and provides higher detection sensitivity (Francino *et al*., 2006). Finally, PCR performance is dependent on the sample used, as parasite burdens will differ according to the organic tissue. Peripheral blood does not seem to provide enough sensitivity to be used as a sole method (Maia *et al*., 2009; Solano-Gallego *et al*., 2011), while a higher incidence of false-positives may be expected during the *L. infantum* transmission season due to natural contamination or transient infection (Maia and Campino, 2008). Tissues harbouring larger parasite burdens, for instance, bone marrow, lymph nodes or skin (Courtenay *et al*., 2014; Noli and Saridomichelakis, 2014; Solano-Gallego *et al*., 2011), may be more difficult to collect in field conditions and in a large number of animals. The validation of

non-invasive samples, such as conjunctival swabs (Di Muccio *et al*., 2012; Strauss-Ayali *et al*., 2004), would prove highly beneficial in large-scale epidemiological studies. Another significant step towards the application of molecular techniques to field studies is the development of methods capable of amplifying DNA at constant temperatures, avoiding the use of sophisticated equipment or the need for specialized personnel (Travi *et al*., 2018). Techniques such as loop-mediated isothermal amplification (LAMP) (Chaouch *et al*., 2013; Gao *et al*., 2015) or recombinase polymerase amplification (RPA) (Castellanos-Gonzalez *et al*., 2015) may prove to be effective ways of allowing access to molecular methods in the field and point-of-care.

The choice of diagnostic methods for CanL epidemiological studies must be based on the proposed study objectives, cost, ease of sampling required and the characteristics of the target canine population (Duthie *et al*., 2018; Gomes *et al*., 2008; Greiner and Gardner, 2000). In general, the diagnostic tests needed for large-scale surveys of *L. infantum* infection in CanL endemic areas should be easy to use, low-cost, highly sensitive (Duthie *et al*., 2018), and showing high specificity in the differentiation between infected dogs (both symptomatic and asymptomatic) and healthy immune individuals (either naturally exposed to the parasite or vaccinated) (de Mendonça *et al*., 2017a). A "gold standard" diagnostic test for *L. infantum* surveillance in natural populations from endemic areas does not exist and, depending on the study goal, the possible use of more than one technique is advised (Maia and Campino, 2018, 2008; Morales-Yuste *et al*., 2012).

The *L. infantum* diagnostic method used in the prevalence study in Girona province (section 4.1) was a whole promastigote antigen in-house ELISA, a quantitative serological method. There were several reasons for this choice. First, this method was developed by members of this research group and was standardized and consistently used for CanL diagnosis and survey of autochthonous canine populations (Alcover *et al*., 2013; Ballart *et al*., 2013a, 2012; Fernández-Bellon *et al*., 2008; Fisa *et al*., 2001; Iniesta *et al*., 2002; Riera *et al*., 1999; Rodríguez-Cortés *et al*., 2017, 2010, Solano-Gallego *et al*., 2016b, 2005, 2016a). Together with IFAT, ELISA is one of the methods recommended by the World Organization for Animal Health for performing CanL surveillance studies and to determine the prevalence of infection (OIE, 2014). However, unlike IFAT, the ELISA is easy to perform and provides objective results, which are not conditioned by subjective operator interpretation (Solano-Gallego *et al*., 2014). Whole parasite antigen ELISA is considered suitable for the serological diagnosis of CanL in both symptomatic and asymptomatic dogs (Mettler *et al*., 2005); by contrast, IFAT shows very low diagnostic sensitivity in asymptomatic individuals (Adel *et al*., 2016; Mettler *et al*., 2005; Rodríguez-Cortés *et al*., 2013). These characteristics make ELISA particularly useful in endemic

field study settings and for processing large numbers of samples (Maia and Campino, 2008). Additionally, techniques based on whole crude *Leishmania* antigen are usually associated with a higher diagnostic sensitivity than monospecific tests, mainly due to a greater capacity in detecting the heterogeneous range of individual immune responses expected to be present in endemic settings (Morales-Yuste *et al*., 2012). However, this type of antigen is likely to exhibit less specificity due to cross-reactivity with other canine infectious agents, mainly tick-transmitted diseases (Solano-Gallego *et al*., 2014); nonetheless, the frequent cross-reactions with other *Leishmania* or Trypanosomatidae species observed in other endemic regions (Ferreira *et al*., 2007; Paz *et al*., 2018) are not as great a concern in Europe. Finally, an ELISA was used to allow comparison with other CanL prevalence studies in the nearby regions of Catalonia and Andorra and in the Balearic Islands (Alcover *et al*., 2013; Ballart *et al*., 2013a). The use of molecular techniques is not yet sufficiently established in large-scale epidemiological studies and few results have been published to allow extensive comparison. Furthermore, as mentioned before, samples which provide higher sensitivity for PCR parasite detection are either too invasive or complex to collect in field settings. Blood samples do not provide enough diagnostic sensitivity for cross-sectional surveys (Maia *et al*., 2009; Solano-Gallego *et al*., 2016a) and non-invasive samples, for example, hair or conjunctival swabs are still under validation (Corpas-López et al., 2016; Di Muccio *et al.*, 2012; Strauss-Ayali *et al*., 2004). Being an endemic region, the inherent characteristics of *L. infantum* infection in the canine population of Girona province were anticipated to have an impact on ELISA performance, as discussed above. This negative impact was expected to be minimized in an assay standardized with very similar dog populations; nevertheless, as population characteristics are not stable (Greiner and Gardner, 2000), there would always be potential associated error. Likewise, assay-related characteristics would also influence the results. This error source was accounted for by calculating and reporting true seroprevalence results, which took into consideration the diagnostic test's sensitivity and specificity (section 4.1).

The study presented in section 4.2 evaluated the contact of dog populations in Girona province with *P. perniciosus* through the quantification of antibodies produced against sand fly saliva in dogs. Besides confirming the presence of phlebotomine vectors in the surveyed locations, this is the first longitudinal study performed with *P. perniciosus* saliva antigens in natural canine populations from CanL endemic areas, with significant results contributing to the validation of these techniques in field conditions. These methods are useful epidemiological tools and can be applied in endemic areas to complement studies of vector population dynamics, as markers of host exposure, to assess the risk of *Leishmania* transmission, or to measure the

effectiveness of vector-control programmes (reviewed in Lestinova et al., 2017). Two salivary antigens were used in parallel: total salivary gland homogenate (SGH) and the recombinant protein rSP03B, a yellow-related protein present in *P. perniciosus* saliva. Although SGH is considered the "gold standard", its use in large-scale epidemiological surveys is impaired by technical limitations, such as the difficulty in maintaining a large enough sand fly colony and the laborious work of dissecting large numbers of salivary glands (Lestinova *et al*., 2017). Additionally, protein composition of SGH can vary depending on sand fly age and maintenance temperature (Volf and Tesar, 2000), as well as individually and between colonies from different geographic locations (Rohoušová *et al*., 2012). Finally, the use of SGH in diagnostic tests may reduce the specificity of detection due to a higher probability of cross-reactivity with saliva of sympatric sand fly species (Volf and Rohoušova, 2001). The recombinant rSP03B is considered to be the most promising candidate to replace SGH as a marker of exposure to *P. perniciosus* and studies have been conducted to validate this antigen in the field (Kostalova *et al*., 2017, 2015; Martín-Martín *et al*., 2014). Results obtained in the longitudinal study presented in this thesis (section 4.2) showed that canine humoral response (IgG) against SGH and rSP03B followed the expected trends for *P. perniciosus* activity in the region, confirming the applicability of both antigens as markers of exposure to *L. infantum* vectors in natural populations from endemic areas. Notably, it was observed that, despite the repeated exposure to sand flies during the vector activity season, dogs from endemic regions showed a marked decrease in IgG levels during the non-transmission season. This finding demonstrates the capability of salivary antigens in detecting recent host exposure, which can be particularly relevant to studies assessing the effectiveness of vector-control programmes (Clements et al., 2010).

The correlation observed between SGH and rSP03B was only moderate, which could be explained by the co-occurrence of *P. ariasi*, the other *L. infantum* vector species present in Spain. Due to its close relation to *P. perniciosus* (both belonging to the *Larroussius* subgenus), IgG produced against the bite of *P. ariasi* could potentially be detected by *P. perniciosus* SGH antigen. Unfortunately, due to the lack of colonies of sand fly species co-occurring with *P. perniciosus* (Lawyer *et al*., 2017), this possible cross-reaction has not yet been tested. Characterization of sand fly populations in the study area and comparison of SGH-rSP03B correlations between locations of single *P. perniciosus* occurrence and co-occurrence of both species could provide useful information to clarify this hypothesis. Nevertheless, because both species are proven vectors of *L. infantum*, possible cross-reactions between *P. perniciosus* and *P. ariasi* would not diminish the usefulness of both antigens in host-vector-parasite studies.

Finally, the results obtained in the study described in section 4.2 support the conclusions of section 4.1 regarding the endemicity status of Girona province for CanL. The detection of *L. infantum* vectors in an area where autochthonous CanL cases were confirmed and seropositive asymptomatic individuals were also found, fulfil the criteria for an indisputable leishmaniosis focus (Maroli *et al*., 2008). Despite some degree of variation observed across the different locations, the exposure of dogs to phlebotomine vectors was detected throughout the study area. Overall seropositivity to SGH and rSP03B during the trial was 49.4% and 29.0%, respectively. Differences between the two antigens could be explained by the previously suggested cross-reactivity with *P. ariasi*. Nevertheless, as also mentioned before, the observed seropositivity would still indicate contact with one of the two *L. infantum* vector species present in the region, which is equally significant for the proposed study objectives. The recent development of a rapid ICT containing rSP03B antigen, which demonstrated a good correlation with SGH ELISA, as well as a high diagnostic sensitivity and specificity in experimentally exposed dogs (Willen *et al*., 2018), shows that this is a promising line of research in the field of vector epidemiology.

5.2. DOG VACCINATION AND THE CONTROL OF CANINE LEISHMANIOSIS

Vaccination is considered to be one of the best methods for controlling leishmaniosis (Dye, 1996), either alone or in combination with insecticide treatments (Sevá *et al*., 2016). A vaccine for CanL should be able to induce a strong and long-lasting Th1-dominated cellular immunity to control infection progression, while simultaneously reducing parasite burden in dogs and their infectiousness to sand flies (Courtenay *et al*., 2014; Gradoni, 2015). Additionally, it should be equally effective in protecting against infection or disease (Alvar *et al*., 2013). The success of vaccination at the population level is closely linked with vaccine efficacy and coverage, which when high enough, would be able to induce herd immunity and indirect protection of non-vaccinated individuals and humans (Fox *et al.*, 1971). When effective, the advantages of vaccination are well recognized; however, failure to provide proper protection against infection can have the detrimental effect of “masking” vaccinated asymptomatic carriers, which can harbour the parasite and represent a source for transmission to naïve individuals; both other dogs and humans (Miró *et al*., 2017b). Another possible negative effect of vaccination is the cross-reaction of vaccine-induced antibodies with serological techniques commonly used to diagnose *L. infantum* infection (Marcondes *et al*., 2013). This interference

with standard tests hinders the diagnosis of vaccinated and infected individuals (which assumes a greater relevance if vaccine efficacy is only moderate or low) and can have an important impact on *L. infantum* seroprevalence surveys.

Unlike previous CaniLeish® vaccine studies, which were carried out under experimental laboratory conditions or with beagle dogs introduced into endemic areas, the studies described in sections 4.3 and 4.4 focused on an independent evaluation of CaniLeish® vaccine in field conditions and with native dogs in an endemic region. This vaccine has been licensed in Europe since 2011 (EMA, 2011) and very little has been published since the pre-licensing phase II and III trials. The studies presented in this thesis were performed with native canine populations from endemic areas, naturally exposed to phlebotomine vectors and to *L. infantum* infection. The recommended procedures for pre-vaccination screening and vaccination inclusion criteria provided by the vaccine's manufacturer were followed. The vaccine trial was designed to mimic the conditions of field vaccination as closely as possible.

The study of the possible impact of CaniLeish® vaccination in *L. infantum* infection seroprevalence studies is presented in section 4.3. Humoral immune response to the parasite was longitudinally assessed in the trial population over one year and antibody responses obtained in vaccinated and non-vaccinated dogs were compared. Results demonstrated a clear non-specific vaccine-induced seroconversion in the group treated with CaniLeish®, which cross-reacted with a standard diagnostic in-house ELISA. Vaccine-induced humoral immune response started increasing after the first vaccine dose, peaking one month after vaccination completion; it is possible that the duration of this false-seropositive state lasted until 4 months after vaccination. Similar results had been previously reported for IFAT and the use of this method in the diagnosis of *L. infantum* in CaniLeish® vaccinated dogs is not recommended (Martin *et al*., 2014; Montoya *et al*., 2017; Oliva *et al*., 2014 ; Sagols *et al*., 2013). A comparable situation was identified after the introduction of Leishmune® in Brazil, the first licensed CanL vaccine (Borja-Cabrera *et al*., 2002). Studies performed several years after its commercialization showed evidence of the cross-reaction of vaccine-induced antibodies with CanL official diagnostic tests (Marcondes *et al*., 2013, 2011). Detection and culling of infected dogs is the control measure adopted by the Ministry of Health in Brazil (Ministério da Saúde Brasileiro, 2014), meaning that, for a number of years, healthy Leishmune®-vaccinated dogs could have been mistakenly identified as naturally infected individuals and removed (Marcondes *et al*., 2013). This is not the case in Europe; however, there is now an added complexity in CanL diagnosis introduced by vaccination, which is extensive to CanL surveillance studies (Solano-Gallego *et al*., 2017a).

Vaccination with CaniLeish® is recommended for dogs living in CanL endemic areas. The fact that vaccination could be extensively implemented in areas where the most common diagnostic methods are unable to distinguish between vaccinated and infected individuals raises a number of pertinent questions. The use of traditional quantitative serological methods in epidemiological studies may need to be revised. Their use in association with other diagnostic methods (e.g. molecular techniques) has been previously recommended (Maia and Campino, 2018, 2008; Morales-Yuste *et al*., 2012), although this practice raises the cost and duration of surveillance programmes. Bourdoiseau *et al*. (2009) suggested the monitoring of LiESAP specific IgG2 to distinguish sera samples of LiESAP-MDP vaccinated dogs (a vaccine formulation with the same antigen as CaniLeish®, but with a different adjuvant) from infected individuals in large-scale studies in the field; however, the ability to diagnose vaccinated and infected dogs would not be improved by this approach. The replacement of quantitative serological methods by qualitative or molecular ones could also present some drawbacks, already discussed in this chapter. In the specific case of Speed Leish K™, the pre-vaccination screening and post-vaccination diagnostic method recommended by CaniLeish®, reported performance results are not consistent across studies (Ferroglio *et al*., 2013; Solano-Gallego e*t al*., 2014), raising doubts about its suitability for large-scale epidemiological surveys.

An additional aspect to consider when performing surveys in CanL endemic areas where vaccination is implemented would be the need to collect extra information from vaccinated individuals. For immunized dogs, it would be important to know which vaccine had been used, if the initial vaccination protocol had been followed and when had the last vaccine booster been administered. This information would then support the interpretation of diagnostic tests. Finally, the development of new, highly sensitive serological diagnostic tests (or the improvement of existing ones), capable of detecting infection but not vaccine-induced humoral response, should be a priority. In such cases, the same detection method could be applied to the whole canine population and used alone or in conjunction with other diagnostic methods. However, the continuous development and possible licensing of new CanL vaccines makes the development of a "universal" diagnostic test difficult to achieve. Since 2016, another CanL vaccine, LetiFend® (Laboratorios LETI, Spain), was licensed in Europe (EMA, 2016). According to results available until now, vaccination with LetiFend® does not seem to induce cross-reactions with the most common serological diagnostic tests (Iniesta *et al*., 2016).

The evaluation study of CaniLeish® vaccine in field conditions is provided in section 4.4. The vaccine proved to be safe in the study population, in accordance with previous vaccine reports (Breton *et al*, 2015; Marino *et al*., 2017). Nevertheless, it should be mentioned that the trial

canine population was mainly composed of 15 to 20 kg robust hunting dogs, which may not be representative of the wide range of dog sizes and breeds presented for CanL vaccination in veterinary practices. Study of vaccine-induced CMI was performed through the quantification of *L. infantum*-specific IFN-γ production by canine PBMC. This cytokine is considered to be a high-quality biomarker of a Th1-predominant cellular immune response, and an indicator of immunogenicity and protection against *Leishmania* infection in dogs (Carrillo and Moreno, 2009; Reis *et al*., 2010). Vaccination with CaniLeish® induced a marked production of specific IFN-γ, detectable one month after vaccination conclusion, with levels of this cytokine in the vaccine group being significantly higher when compared to control dogs or with both groups at T0. However, although higher levels of IFN-γ were still present in vaccinated dogs 9 months after immunization, these were not considered statistically different from the ones observed in the control group at the same time point. Similar results were reported in previous CaniLeish® studies, where differences in the number of IFN-γ producing cells between vaccine and control groups were detected 6 months post-vaccination, but not 12 months post-vaccination (Martin *et al*., 2014; Moreno *et al*., 2014). This could mean that CaniLeish® vaccine may not able to elicit a long enough protection against infection, but only a transiently effective CMI against *L. infantum*. A similar situation is described by de Luca and Macedo (2016) regarding some HL vaccine candidates, which although presenting satisfactory results in immunotherapeutic interventions, mainly characterized by Th1-type effector cells, could not generate memory T cells and an effective immunological memory. It could also be that IFN-γ quantification is insufficient for characterizing the immune response to *L. infantum*, known to be modulated by a complex network of regulatory and counter-regulatory interactions involving multiple cells and cytokines (Hosein *et al*., 2017; Reis *et al*., 2010). Further studies would be needed to fully assess the cellular immune response in vaccinated dogs and confirm its intensity and persistence.

In the CaniLeish® vaccine study presented in section 4.4, no differences were found in number or intensity of active *L. infantum* infections between vaccine and control groups one year post-vaccination. In the context of this study, “active infection” was defined as an established and progressive *L. infantum* infection, either symptomatic or asymptomatic, but that is expected to inevitably lead to clinical disease. This state was characterized by the concomitant evidence of specific-*L. infantum* seroconversion, defined as a 4-fold increase in ELISA units when compared with the same individual’s basal values (Paltrinieri *et al*., 2010), detection of parasite’s DNA in lymph node samples and, in the majority of cases, by the presence of clinical or laboratory changes compatible with CanL. Using these criteria, 5.6% of vaccinated dogs (4/71) and 5.4%

of control dogs (4/74) were diagnosed with active *L. infantum* infection during one year follow-up. These results confirm those presented in a previous field study, which compared the efficacy of CaniLeish® and two insecticide dog collars in the prevention of CanL. After one year, although different protection efficacies were determined for each insecticide collar, no difference was detected in the number of CanL cases between CaniLeish® and control groups (Brianti *et al*., 2016). As in the Brianti *et al*. (2016) study, the reduced sample size and trial duration of the present study may have impaired the detection of a vaccine effect.

Another aspect deemed essential for an effective CanL vaccine is its capacity to reduce infectiousness at the vertebrate host level, blocking *L. infantum* transmission to the sand fly vector. The work presented in this thesis did not focus on this particular aspect of CaniLeish® vaccination; however, results on the number and severity of active *L. infantum* infection cases observed in the treated group strongly suggest that the vaccine does not stop or reduce parasite transmission to the vector. Vaccinated dogs presented the same incidence of infection as the control group, with similar levels of specific humoral response, parasite load in lymph nodes and presence of clinical or laboratory abnormalities. Nevertheless, infectiousness was not assessed in any group either by quantification of skin parasite loads (Courtenay *et al*., 2014) or xenodiagnosis (Fernandes *et al*., 2014). The only previous xenodiagnosis study performed in CaniLeish® vaccinated dogs did not find any difference in the proportion of infectious vaccinated and non-vaccinated individuals progressing to active *L. infantum* infection, although a reduction in the infectiousness burden was observed in the vaccine group (Bongiorno *et al*., 2013). Nonetheless, the reduced number of dogs included in the mentioned study (6 vaccinated and 4 controls) did not allow for any conclusions to be drawn regarding a possible impact of vaccination on parasite transmission.

The results of CaniLeish® field study described in section 4.4 of this thesis do not corroborate a significant role of this vaccine in the control of *L. infantum* infection in endemic areas. According to these results, CaniLeish® vaccine does not seem to meet the criteria proposed for an effective immunization against *Leishmania* parasites (Alvar *et al*., 2013; Gradoni, 2015). At the individual level, there is little evidence that vaccination enhances a long enough Th1-dominated cellular immune response capable of protecting against infection progression during one year. This is suggested not only by the early decrease observed in IFN-γ levels in vaccinated dogs, but also by a failure in detecting difference in frequency or severity of active *L. infantum* infection cases between vaccinated and non-vaccinated animals. The apparent vaccine inefficacy in protecting against infection or disease (previously reported by Brianti *et al*., 2016), together with the proven infectiousness of infected vaccinated dogs to sand flies

(Bongiorno *et al*., 2013), is also suggestive of an apparently negligible impact of CaniLeish® in blocking *L. infantum* transmission in canine populations from endemic areas, as well as to humans. Further studies are needed to corroborate these findings and to unequivocally determine whether recommendations for CaniLeish® vaccine use in canine *L. infantum* infection prophylaxis in endemic areas should be maintained.

Field evidence of CanL vaccines effectiveness in reducing CanL and HL is essential to truly assess the usefulness of such control measures (Weinberg and Szilagyi, 2010). Studies of vaccine efficacy, designed to demonstrate the advantages of vaccination at the individual level, do not provide clear information on the impact of such interventions in *Leishmania* infection epidemiology. The only published example of population-level evaluation of vaccine impact was performed for Leishmune® in Brazil, showing that in areas where vaccination had been adopted, a decrease in the number of seropositive dogs and in the incidence of HL had been observed (Palatnik-de-Sousa *et al*., 2009). This study reported results on CanL and HL cases detected before and after vaccine introduction in regions subject to different vaccination coverage rates. Official reports from the Ministry of Health's Centre for Zoonosis Control and data from the vaccine's manufacturer and local veterinary practices were used. Surprisingly, ten years later, the production and marketing license for Leishmune® vaccine would be withdrawn by the Brazilian Ministry of Agriculture, under claims of lack of effectiveness evidence in phase III trials (MAPA, 2014). Epidemiological surveillance should be considered an essential procedure after any veterinary vaccine licensing to confirm safety and efficacy rates reported in phase II and III trials, thus avoiding long-term commercialization of suboptimal or ineffective medicines. Importantly, these results would also provide solid information to the general public, who could then make informed decisions on whether to use these products. So far, topical insecticides applied to the canine reservoir are the only prophylactic method showing field evidence in the reduction of both CanL and HL (Gavgani *et al*., 2002).

5.3. STUDY LIMITATIONS

As already mentioned, the main limitation of the canine *L. infantum* serological survey presented in section 4.1 was access to dogs. The selection of an unbiased and representative sample of dogs from a whole province can prove to be problematic, given the cessation of anti-rabies vaccination campaigns and the lack of systematic canine registration. As also discussed before, alternative methods for recruitment of individuals will inevitably incur in some type of selection bias. Furthermore, the analysis of individual and environmental risk factors associated with the infection imposed the need for working with compliant dog owners. The recruitment method chosen involved the local veterinarians, who were the link between the research team and their clients. However, instead of sampling dogs attending the veterinary practices, the study focused on dog populations living in rural and periurban areas, which were considered to be a better sentinel for *L. infantum* infection. Sample selection and geographic bias introduced by the study design were assumed by the research team and discussed in section 4.1 (pages 75-76).

The follow-up study of vaccinated dogs presented in section 4.3 was performed with individuals enrolled in the vaccine study presented in section 4.4. Likewise, the study was designed as a case-control trial, where both groups were exposed to natural *L. infantum* infection. Apart from a possible humoral response produced by vaccination, dogs could also have developed a humoral response to infection, which is not possible to differentiate from the vaccine-induced one. Interactions between vaccine-induced and parasite-induced humoral responses are not studied and represent an added hindrance when analysing the data. The study described in section 4.3 attempted to mimic possible cross-sectional seroprevalence studies, this way providing an insight into the impact of the presence of vaccinated dogs in any surveyed population. However, the only way of isolating a possible vaccine effect was by comparing vaccine and control groups, and this method is not able to provide conclusive results due to the confounding effect of natural infection. Furthermore, as observed in the study results, even humoral responses from the control group failed to show stability during the non-transmission season, when presence of the parasite is not expected, and may reflect previous host-parasite contacts. Alternative study designs are described to evaluate antibody levels in vaccinated dogs and their cross-reactivity with diagnostic methods (Marcondes *et al.*, 2013, 2011). In these studies, insecticide-impregnated collars were placed on trial dogs to avoid infection during the follow-up period, this way isolating a possible effect of vaccination

on antibody titres at any given time point. Another possible way of performing such studies would be by vaccinating dogs in non-endemic areas, this way reducing the chances of infection and controlling for this confounding effect.

The vaccine field study presented in section 4.4 also endured some limitations. Sample size was strongly conditioned by the number of available dogs, as well as by the research team's capacity to perform the follow-up on a large number of dogs dispersed over 12 different localities in Girona province. Additionally, the one-year duration of the vaccine trial also hampered the detection of CanL cases. The outcome of *L. infantum* infection in dogs is known to be variable and to present long incubation periods, reported to range from 3 months to 7 years (Miró *et al*., 2008; Oliva *et al*., 2006; Solano-Gallego *et al*., 2001a). In canine populations from endemic areas, which present some degree of immune adaptation to the parasite, infection progression can be even less predictable. These factors placed marked constraints on the study power to detect a possible vaccine effect.

Additional limitations were mainly related to those inherent in long-term field studies and with the heterogeneity observed in autochthonous canine populations. Study samples selected from natural communities are more representative of the general population and the results obtained are more applicable to "real-life" conditions. However, they also represent an added difficulty in controlling for external factors and confounders that may interfere in the study results and are not directly related with the effect to be measured. Some of these limitations were discussed in section 4.4 (pages 112-113). Solutions for overcoming the impact of these interferences could be a considerable increase in sample size or by an exclusive recruitment of young dogs, not exposed to previous *L. infantum* transmission seasons. However, while the first option would make the study unfeasible, the second would diminish population representativeness, because vaccination for CanL is recommended for dogs of all ages, and not exclusively for 6-month-old puppies.

5.4. FUTURE WORK

Epidemiological characterization of canine *L. infantum* infection in Girona province should be maintained and extended in the future. Sampling of urban dog populations and of dogs in areas less represented in this survey could complement and improve the accuracy of reported infection prevalence. Likewise, it would be important to maintain the surveillance in the

currently characterized locations, in order to identify future changes in vector or infection distribution trends. Maintaining the same diagnostic method would be important to allow comparisons to be made, even if additional tests were added to the surveys. Comparing information from HL and CanL geographic incidence could be effectual in recognizing the importance of implementing *L. infantum* control measures at the reservoir level.

The study of risk factors associated with *L. infantum* infection would also need further investigation. Although an altitudinal trend was identified, observations in the field strongly suggested that the infection is focally distributed, with the occurrence of clearly demarked CanL "hotspots". The high infection incidence observed in these locations, although certainly influenced by altitudinal-related factors such as temperature, humidity and land cover, cannot be explained solely by these conditions. Therefore, the inclusion of other environmental variables, as well as information from entomological surveys and investigation of alternative reservoir species should be added to the analysis of risk factors for *L. infantum* infection.

Extending the epidemiological surveillance to vector surveys would also assist in clarifying some questions raised during the study of dog exposure to sand fly vectors. The hypothesis of possible cross-reactions between *P. perniciosus* and *P. ariasi* in anti-vector saliva serological studies should be clarified. Due to the difficulties in maintaining *P. ariasi* colonies, a possible way of investigating this could be by the characterization of field sand fly populations in areas where these diagnostic tests have been previously used. This way, comparisons in serological results could be made between areas of single species occurrence and of co-occurrence of both *Phlebotomus* species. The estimation of *L. infantum* burdens in sand fly vectors would also provide meaningful information to assess the risk of dog and human infection and compare it with individual sand fly exposure. Finally, entomological studies would also provide data for building vector distribution and density maps in the region.

Concerning CaniLeish® vaccine studies, further research is needed to confirm the results reported in this thesis. Amplifying the dog sample size and extending the duration of the vaccine field trial would provide more robust conclusions. It would also be interesting to use the PBMC samples collected during this field trial to better characterize a vaccine-induced cellular immune response to *L. infantum* infection. Quantification of additional cytokines or characterization of predominant lymphocyte populations at one and 9 months post-vaccination would provide useful information and help to determine the duration of effective vaccine immunity. The extension of the vaccine field trial would also allow for a follow-up of non-specific vaccine-induced humoral immunity after annual vaccine boosters, and provide

information that is currently non-existent for ELISA and insufficient for IFAT. Nevertheless, as mentioned before, possible interactions with natural exposure to *L. infantum* could impede interpretation of results. An alternative study design could prove to be a better option for assessing this parameter in field dog populations.

6. CONCLUSIONS

6. CONCLUSIONS/CONCLUSIONES

A. EPIDEMIOLOGICAL STUDIES OF CANINE *Leishmania infantum* INFECTION IN THE PROVINCE OF GIRONA / ESTUDIOS EPIDEMIOLÓGICOS DE LA INFECCIÓN CANINA POR *Leishmania infantum* EN LA PROVINCIA DE GERONA

1. Girona province shows evidence of a stable and endemic focus of CanL, characterized by a high *Leishmania infantum* seroprevalence observed in dogs (19.5%), together with a large number of asymptomatic cases (93.2%).

   La provincia de Gerona muestra evidencias de ser un foco estable y endémico de leishmaniosis canina, caracterizado por una elevada seroprevalencia de *Leishmania infantum* observada en perros (19,5%) junto a una gran cantidad de casos asintomáticos (93,2%).

2. The increase of dogs' age and lower altitude of the kennel location were identified as risk factors for *Leishmania infantum* infection in the population studied.

   El incremento de la edad de los perros y la disminución de la altura en donde estaban ubicadas las perreras se han identificado como factores de riesgo para la infección por *Leishmania infantum* en la población estudiada.

3. The correlation found between SGH and rSP03B salivary antigens was moderate, but both antigens proved to be suitable to assess exposure to *Phlebotomus perniciosus* in native and heterogeneous canine populations from leishmaniosis endemic areas.

   La correlación entre los antígenos salivales SGH y rSP03B ha sido moderada, pero ambos antígenos han demostrado ser adecuados para evaluar la exposición a *Phlebotomus perniciosus* en poblaciones caninas naturales y heterogéneas de un área endémicas de leishmaniosis canina.

4. Despite exposure to repeated sand fly bites during their period of activity, anti-saliva immunoglobulin G levels in the study dogs show a marked decrease during the non-transmission season, which allows detection of recent host exposure to vectors and their implementation in studies assessing the effectiveness of vector control programs.

   A pesar de la exposición a repetidas picaduras de los flebotomos durante su período de actividad, los niveles de inmunoglobulina G anti-saliva de los perros analizados en el área endémica presentan una marcada disminución durante la temporada de no transmisión, lo que permite la detección de la exposición reciente del hospedador a los vectores y su implementación en estudios que evalúen la efectividad de los programas de control de vectores.

5. Levels of antibodies against both SGH and rSP03B salivary antigens detected in the dog population analyzed are associated with seropositivity to *Leishmania infantum.*

   Los niveles de anticuerpos frente a los antígenos salivares SGH y y rSP03B detectados en la población canina analizada están asociados a la seropositividad frente a *Leishmania infantum.*

B. DOG VACCINATION AND THE CONTROL OF CANINE LEISHMANIOSIS / LA VACUNACIÓN DE PERROS Y EL CONTROL DE LA LEISHMANIOSIS CANINA

1. Vaccination with CaniLeish® induces the production of non-specific antibodies after the first vaccine dose administration, peaking one month after vaccination completion.

   La vacunación con CaniLeish® induce la producción de anticuerpos inespecíficos después de la primera dosis de administración de la vacuna y alcanza un máximo un mes después de finalizada la vacunación.

2. The cross-reactivity shown with an in-house ELISA, a test commonly used for the detection of *Leishmania infantum* infection, interferes with infection and disease diagnosis, as well as with seroepidemiological surveys of canine leishmaniosis.

La reactividad cruzada mostrada al aplicar un ELISA convencional comúnmente utilizado para la detección de la infección por *Leishmania infantum* interfiere en el diagnóstico de la infección y enfermedad, así como en las encuestas seroepidemiológicas de la leishmaniosis canina.

3. The widespread use of canine leishmaniosis vaccines in endemic regions may impose the need to change current surveillance methodologies.

El uso generalizado de las vacunas frente a la leishmaniosis canina en regiones endémicas puede suponer la necesidad de cambiar las metodologías actuales de vigilancia.

4. CaniLeish® vaccine induces a marked and specific cellular mediated immunity in vaccinated dogs, measured by the production of interferon gamma against soluble *Leishmania* antigens, detectable one month after vaccination but not at nine month post-vaccination, when no statistically significant differences between vaccinated and control groups were observed; this suggests that the cellular immunity induced by the vaccine may not be effective throughout the year of alleged vaccine coverage.

La vacuna CaniLeish® produce una marcada y específica inmunidad mediada por células en los perros vacunados, medida por la producción de interferón gamma contra antígenos solubles de *Leishmania*, un mes después de la vacunación y sin mostrar diferencias estadísticamente significativas entre el grupo control y el vacunado a los nueves meses post-vacunación, lo que sugiere que la inmunidad celular inducida por la vacuna no sería efectiva durante todo el año de su supuesta cobertura.

5. Vaccination with CaniLeish® did not protect dogs against *Leishmania infantum* infection or clinical disease during the first year post-vaccination and does not seem to meet the criteria needed for an effective canine leishmaniosis vaccine.

La vacunación con CaniLeish® no protegió a los perros contra la infección por *Leishmania infantum* o la enfermedad clínica durante el primer año post-vacunación y no cumpliría con los criterios necesarios para ser considerada una vacuna eficaz contra la leishmaniosis canina.

7. REFERENCES

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8. ANNEXES

ANNEX 1. Results of serological *Leishmania infantum* infection surveys performed in the study population for the vaccination studies in July 2015 and February 2016. Estimated point seroprevalences and incidences obtained are reported.

			July 2015					February 2016			
Locality	Dog kennel	Geographical coordinates	No. of dogs sampled	No. of seropositive dogs	No. of seronegative dogs	No. of doubtful results[1]	Estimated seroprevalence (%) (95%CI)	No. of dogs sampled	No. of seropositive dogs	Estimated seroprevalence (%) (95%CI)[2]	Estimated annual incidence (%) (95%CI)[3]
Ordis	**1**	N 42° 13' 37.7'' E 2° 54' 24.1''	30	5	21	4	16.7 (5.6-34.7)	24	5		20.0 (6.8-40.7)
Madremanya	**2**	N 41° 58' 47.0'' E 2° 58' 7.2''	18	0	14	4	0	19	2		11.1 (1.4-34.7)
Maçanet de la Selva	**3**	N 41° 47' 27.4'' E 2° 45' 0.4''	17	1	14	2	5.9 (0.1-28.7)	13	3		18.8 (4.0-45.6)
Massanes	**4**	N 41° 45' 15.3'' E 2° 38' 44.0''	11	1	10	0	9.1 (0.2-41.3)	12	0		0
	5	N 41° 45' 53.8'' E 2° 39' 0.1''	14	1	12	1	7.1 (0.2-33.9)	13	1		7.7 (0.2-36.0)
Hostalnou de Bianya	**6**	N 42° 13' 26.0'' E 2° 26' 9.7''	5	0	4	1	0	7	2		40.0 (5.3-85.3)
	7	N 42° 12' 39.2'' E 2° 28'35.0''	13	1	11	1	7.7 (0.2-36.0)	10	0		0
Montagut	**8**	N 42° 14' 7.7'' E 2° 35' 57.6''	30	7	20	3	23.3 (9.9-42.3)	21	3		13.0 (2.8-33.6)
	9	N 42° 13' 41.8'' E 2° 35' 58.1''	21	4	16	1	19.0 (5.4-41.9)	15	6		35.3 (14.2-61.7)
Sant Esteve de Llémena	**10**	N 42° 3' 35.1'' E 2° 37' 1.4''	20	0	18	2	0	16	0		0
Canet d'Adri	**11**	N 42° 1' 53.7'' E 2° 44' 15.3''	16	8	4	4	50.0 (24.7-75.3)	8	4		50.0 (15.7-84.3)

	12	N 42° 2' 43.9'' E 2° 44' 59,2''	11	5	2	4	45.5 (16.7-76.6)	5	2		33.3 (4.3-77.7)
	13	N 42° 1' 57.0'' E 2° 44' 8.6''	16	6	2	8	37.5 (15.2-64.6)	9	5		50.0 (18.7-81.3)
Aiguaviva	**14**	N 41° 54' 27.2'' E 2° 46' 19.0''	27	1	23	3	3.7 (0.1-19.0)	25	1		3.8 (0.1-19.6)
Sta. Coloma de Farners	**15**	N 41° 50' 39.4'' E 2° 40' 48.4''	32	0	32	0	0	Excluded from the vaccine study			
St. Feliu de Guíxols	**16**	N 41° 47' 2.3'' E 2° 59' 58.7''	19	7	9	3	36.8 (16.3-61.6)	9	5		41.7 (15.2-72.3)
Olot	**17**	N 42° 10' 19'' E 2° 30' 29''	No information					12	5	41.7 (15.2-72.3)	
Riells I Viabrea	**18**	N 41° 43' 59'' E 2° 33' 39.3''	No information					31	7	22.6 (9.6-41.1)	
Vilobí d'Onyar	**19**	N 41° 53' 3.2'' E 2° 43' 38.6''	No information					23	5	21.7 (7.5-43.7)	
	20	N 41° 54' 15.4'' E 2° 42' 57.1''	No information					11	2	18.2 (2.3-51.8)	
Brunyola	**21**	N 41° 54' 25.9'' E 2° 42' 9.9''	No information					10	0	0	
TOTAL	**21**		**300**	**47**	**212**	**41**	**15.7 (11.7-20.3)**	**293**	**58**	**21.8 (13.7-32.0)**	**17.6 (12.9-23.3)**

[1]Doubtful results correspond to individuals who tested positive to an in-house ELISA and negative to a commercial ELISA assay (Leiscan®); these individuals were retested in February 2016.

[2]Point seroprevalences were not estimated for the second survey.

[3]Calculated as the no. of new cases (seropositive dogs in 2016) divided by the number of individuals at risk (seronegative and doubtful individuals in July 2015).

ANNEX 2. Sand fly species identified at each dog kennel after one night capture with CDC light traps.

Locality	Dog kennel	Geographical coordinates	Altitude (m a.s.l.)	No. of dogs	No. CDC light traps recovered/night	Sand fly species captured
Ordis	**1**	N 42° 13' 37.7'' E 2° 54' 24.1''	104	30	1	---
Madremanya	**2**	N 41° 58' 47.0'' E 2° 58' 7.2''	139	18	2	*P. perniciosus* *P. ariasi* *S. minuta*
Maçanet de la Selva	**3**	N 41° 47' 27.4'' E 2° 45' 0.4''	73	17	1	*S. minuta*
Massanes	**4**	N 41° 45' 15.3'' E 2° 38' 44.0''	88	11	2	*P. perniciosus* *S. minuta*
	5	N 41° 45' 53.8'' E 2° 39' 0.1''	117	14	2	---
Hostalnou de Bianya	**6**	N 42° 13' 26.0'' E 2° 26' 9.7''	396	5	2	---
	7	N 42° 12' 39.2'' E 2° 28'35.0''	355	13	2	*P. perniciosus*
Montagut	**8**	N 42° 14' 7.7'' E 2° 35' 57.6''	254	30	2	*P. ariasi*
	9	N 42° 13' 41.8'' E 2° 35' 58.1''	258	21	2	*P. perniciosus* *P. ariasi* *S. minuta*
Sant Esteve de Llémena	**10**	N 42° 3' 35.1'' E 2° 37' 1.4''	283	20	2	*S. minuta*
Canet d'Adri	**11**	N 42° 1' 53.7'' E 2° 44' 15.3''	231	10	2	*P. perniciosus* *P. ariasi* *S. minuta*
	12	N 42° 2' 43.9'' E 2° 44' 59,2''	248	16	1	*P. perniciosus* *S minuta*
	13	N 42° 1' 57.0'' E 2° 44' 8.6''	234	12	2	*P. perniciosus* *P. ariasi* *S. minuta*
Aiguaviva	**14**	N 41° 54' 27.2'' E 2° 46' 19.0''	148	27	1	---

Sta. Coloma de Farners	**15**	N 41° 50' 39.4'' E 2° 40' 48.4''	128	32	2	*P. perniciosus*
Sant Feliu de Guíxols	**16**	N 41° 47' 2.3'' E 2° 59' 58.7''	84	19	2	*P. perniciosus*

P. perniciosus: *Phlebotomus perniciosus*; *P. ariasi*: *Phlebotomus ariasi*; *S. minuta*: *Sergentomyia minuta*

ANNEX 3. Data collected from each sampled dog and questionnaire performed to the dog owner during the *Leishmania infantum* seroprevalence study in Girona province.

<table>
<tr><th>DATOS DEMOGRÁFICOS DEL PERRO</th><th colspan="2">DATOS DEL VETERINARIO</th><th colspan="2">CUESTIONARIO DEL PROPIETARIO DEL PERRO</th></tr>
<tr><td>Nombre</td><td colspan="2">Apellidos y Nombre</td><td colspan="2">Apellidos y Nombre</td></tr>
<tr><td>Longitud del pelo:
Corto ☐ Medio ☐ Largo ☐</td><td colspan="2">Dirección</td><td colspan="2">Dirección</td></tr>
<tr><td>Color del pelo</td><td colspan="2">Ciudad</td><td colspan="2">Longitud Latitud</td></tr>
<tr><td>Sexo: M ☐ H☐
Si hembra, ¿está preñada?</td><td colspan="2">Provincia y comarca</td><td colspan="2">Ciudad</td></tr>
<tr><td rowspan="2">Fecha de nacimiento / /</td><td colspan="2">Código postal</td><td colspan="2">Provincia y comarca</td></tr>
<tr><td colspan="2">Teléfono</td><td colspan="2">Código postal</td></tr>
<tr><td>Edad (años)</td><td colspan="2" rowspan="2">e-mail</td><td colspan="2">Teléfono</td></tr>
<tr><td>Peso (kg)</td><td colspan="2">e-mail</td></tr>
<tr><td>Raza</td><td rowspan="2">SIGNOS CLÍNICOS COMPATIBLES CON LA LEISHMANIOSIS CANINA</td><td>DATOS EXTRACCIÓN</td><td colspan="2">¿Ha oído hablar de la leishmaniosis visceral?: Sí ☐ No ☐</td></tr>
<tr><td>País de origen</td><td>Formulario No.</td><td colspan="2" rowspan="2">¿Qué posibilidad cree que tiene su perro de padecer leishmaniosis a lo largo de su vida?
0% ☐ 5% ☐ 10% ☐ 20% ☐ 50% ☐ 50-90% ☐ 90-100% ☐</td></tr>
<tr><td>Región de origen</td><td rowspan="2"></td></tr>
<tr><td>Provincia de origen</td><td rowspan="2">Ninguno ☐
Inflamación de ganglios linfáticos ☐
Dermatitis furfurácea ☐
Alopecia ☐
Úlceras ☐
Onicogriposis ☐
Epistaxis ☐
Palidez mucosas ☐
Lesiones oculararares ☐
Esplenomegalia ☐
Pérdida de peso ☐
Otro ☐ (especificar):</td><td colspan="2" rowspan="2">¿Conoce algún método para proteger a su perro de las picadas de los flebotomos? Sí ☐ No ☐</td></tr>
<tr><td>RESIDENCIA HABITUAL DEL PERRO</td><td rowspan="2">Tipo de muestra
Suero ☐
Muestra cutánea ☐
Sangre periférica ☐
Ganglio linfático ☐
Médula ósea ☐
Otra ☐
Si otra, especificar:</td></tr>
<tr><td>Dirección</td><td colspan="2" rowspan="2">Si SÍ, ¿ha oído hablar de alguna medida para proteger a su perro contra la LV?
Sí ☐ No ☐</td></tr>
<tr><td>Localidad</td></tr>
<tr><td>Comarca Provincia</td><td rowspan="2">TEST DIAGNÓSTICO</td><td colspan="2" rowspan="2">¿Ha utilizado alguna medida para proteger a su perro contra la LV?
Sí ☐ No ☐
Si SÍ, ¿cuáles?</td></tr>
<tr><td>Long Lat</td></tr>
<tr><td>Reside en:
En vivienda ☐ En exterior ☐ En perrera ☐</td><td rowspan="2">Microscopio ☐
ELISA ☐
IFAT ☐
WB ☐</td><td rowspan="2">Collar ☐
Scalibor ☐
Otro ☐ (especificar):

Spot-on ☐
Advantix ☐ Expot ☐
Otro ☐ (especificar):</td><td rowspan="2">Otros ☐ (especificar):

¿Aplica a su perro algún otro insecticida para protegerlo de la picada de otros insectos o de garrapatas?
Sí ☐ No ☐</td></tr>
<tr><td>Hábitat usual:
Urbano ☐ Periurbano ☐ Rural ☐
Plano ☐ Montaña ☐</td></tr>
<tr><td>Refugio nocturno/vivienda:
Exterior ☐ Interior ☐</td><td></td><td></td><td></td></tr>
</table>

<table>
<tr><td>Ocupación del perro:
Mascota ☐ Guardia ☐ Ovejero ☐ Cazador ☐
Perrera ☐ Vagabundo ☐ Otro ☐ (especificar):</td><td>Cultivo in vitro ☐
PCR ☐
Otro ☐ (especificar)</td><td>Spray ☐
Duowin ☐
Otro ☐ (especificar):</td><td>Si SÍ, proporcione detalles</td></tr>
<tr><td>Vive con otros perros: Sí ☐ No ☐
Si SÍ, ¿cuántos?</td><td rowspan="3">Resultado
Positivo ☐ Negativo ☐
Título
Observaciones</td><td>Shampoo ☐
(especificar):</td><td rowspan="3">Si NO aplica profilaxis, ¿por qué?
No lo puedo encontrar en la tienda ☐
Demasiado caro ☐
No creo que funcione ☐
La LV no es importante ☐
Otro (especificar) ☐</td></tr>
<tr><td>Vive con otros animales: Sí ☐ No ☐
Si SÍ, ¿cuáles?</td><td>Jarabe LeisGuard®☐</td></tr>
<tr><td>Desplazamientos/viajes: Sí ☐ No ☐
Si SÍ, detalle de los lugares
Si SÍ, periodo</td><td>Vacuna CaniLeish® ☐</td></tr>
</table>

ANNEX 4. Dataset used for the *Leishmania infantum* in-house ELISA sensitivity and specificity analysis.

Dog code	Parasite detection result*	ELISA result (U)	Reference
B1	Positive	337	Iniesta *et al*., 2002
B2	Positive	31	
B3	Negative	40	
B4	Positive	177	
B5	Positive	58	
B6	Positive	174	
B7	Positive	247	
B8	Positive	17	
B9	Positive	13	
B10	Positive	252	
B11	Positive	209	
B12	Negative	33	
B13	Negative	8	
B14	Negative	11	
B15	Positive	30	
B16	Negative	27	
B17	Negative	2	
B18	Negative	12	
B19	Positive	118	
B20	Positive	16	
B21	Positive	24	
B22	Positive	69	
B23	Positive	42	
B24	Positive	20	
B25	Positive	40	
B26	Positive	75	
B27	Positive	29	
B28	Positive	90	
B29	Positive	24	
B30	Positive	69	
B31	Positive	12	
B32	Negative	13	
B33	Positive	258	
B34	Positive	348	
B35	Positive	11	
B36	Positive	12	
B37	Negative	18	
B38	Positive	375	
1	Positive	53	Fisa *et al*., 2001
2	Positive	100	
3	Positive	38	
4	Positive	100	
5	Positive	107	
6	Positive	84	
7	Positive	147	
8	Positive	27	
9	Positive	86	

10	Positive	86
11	Positive	183
12	Positive	153
13	Positive	355
14	Positive	197
15	Positive	200
16	Positive	298
17	Positive	48
18	Positive	102
19	Positive	132
NC1	Negative	17
NC2	Negative	11
NC3	Negative	12
NC4	Negative	7
NC5	Negative	6
NC6	Negative	10
NC7	Negative	9
NC8	Negative	23
NC9	Negative	6
NC10	Negative	7
NC11	Negative	9
NC12	Negative	8.5
NC13	Negative	9
NC14	Negative	7
NC15	Negative	14
NC16	Negative	11
NC17	Negative	7
NC18	Negative	9
NC19	Negative	7
NC20	Negative	8.5

*Dogs were classified as positive if parasite culture, direct examination and/or PCR (on lymph node, bone marrow and/or PBMC) were positive.

ANNEX 5. *Leishmania infantum* in-house ELISA sensitivity and specificity analysis (Stata output)

Cutpoint	Sensitivity	Specificity	Correctly Classified	LR+	LR-
(>= 2)	100.00%	0.00%	62.34%	1.0000	
(>= 6)	100.00%	3.45%	63.64%	1.0357	0.0000
(>= 7)	100.00%	10.34%	66.23%	1.1154	0.0000
(>= 8)	100.00%	27.59%	72.73%	1.3810	0.0000
(>= 8.5)	100.00%	31.03%	74.03%	1.4500	0.0000
(>= 9)	100.00%	37.93%	76.62%	1.6111	0.0000
(>= 10)	100.00%	51.72%	81.82%	2.0714	0.0000
(>= 11)	100.00%	55.17%	83.12%	2.2308	0.0000
(>= 12)	97.92%	65.52%	85.71%	2.8396	0.0318
(>= 13)	93.75%	72.41%	85.71%	3.3984	0.0863
(>= 14)	91.67%	75.86%	85.71%	3.7976	0.1098
(>= 16)	91.67%	79.31%	87.01%	4.4306	0.1051
(>= 17)	89.58%	79.31%	85.71%	4.3299	0.1313
(>= 18)	87.50%	82.76%	85.71%	5.0750	0.1510
(>= 20)	87.50%	86.21%	87.01%	6.3437	0.1450
(>= 23)	85.42%	86.21%	85.71%	6.1927	0.1692
(>= 24)	85.42%	89.66%	87.01%	8.2569	0.1627
(>= 27)	81.25%	89.66%	84.42%	7.8542	0.2091
(>= 29)	79.17%	93.10%	84.42%	11.4792	0.2238
(>= 30)	77.08%	93.10%	83.12%	11.1771	0.2461
(>= 31)	75.00%	93.10%	81.82%	10.8750	0.2685
(>= 33)	72.92%	93.10%	80.52%	10.5729	0.2909
(>= 38)	72.92%	96.55%	81.82%	21.1458	0.2805
(>= 40)	70.83%	96.55%	80.52%	20.5416	0.3021
(>= 42)	68.75%	100.00%	80.52%		0.3125
(>= 48)	66.67%	100.00%	79.22%		0.3333
(>= 53)	64.58%	100.00%	77.92%		0.3542
(>= 58)	62.50%	100.00%	76.62%		0.3750
(>= 69)	60.42%	100.00%	75.32%		0.3958
(>= 75)	56.25%	100.00%	72.73%		0.4375
(>= 84)	54.17%	100.00%	71.43%		0.4583
(>= 86)	52.08%	100.00%	70.13%		0.4792
(>= 90)	47.92%	100.00%	67.53%		0.5208
(>= 100)	45.83%	100.00%	66.23%		0.5417
(>= 102)	41.67%	100.00%	63.64%		0.5833
(>= 107)	39.58%	100.00%	62.34%		0.6042
(>= 118)	37.50%	100.00%	61.04%		0.6250
(>= 132)	35.42%	100.00%	59.74%		0.6458
(>= 147)	33.33%	100.00%	58.44%		0.6667
(>= 153)	31.25%	100.00%	57.14%		0.6875
(>= 174)	29.17%	100.00%	55.84%		0.7083
(>= 177)	27.08%	100.00%	54.55%		0.7292
(>= 183)	25.00%	100.00%	53.25%		0.7500
(>= 197)	22.92%	100.00%	51.95%		0.7708
(>= 200)	20.83%	100.00%	50.65%		0.7917
(>= 209)	18.75%	100.00%	49.35%		0.8125
(>= 247)	16.67%	100.00%	48.05%		0.8333
(>= 252)	14.58%	100.00%	46.75%		0.8542
(>= 258)	12.50%	100.00%	45.45%		0.8750
(>= 298)	10.42%	100.00%	44.16%		0.8958
(>= 337)	8.33%	100.00%	42.86%		0.9167
(>= 348)	6.25%	100.00%	41.56%		0.9375
(>= 355)	4.17%	100.00%	40.26%		0.9583
(>= 375)	2.08%	100.00%	38.96%		0.9792
(> 375)	0.00%	100.00%	37.66%		1.0000

ANNEX 6. Area under the ROC curve (95%CI) for the *Leishmania infantum* in-house ELISA (Stata output)

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Obs	ROC Area	Std. Err.	—Asymptotic Normal— [95% Conf.	Interval]
77	0.9508	0.0210	0.90958	0.99200

ANNEX 7. Article accepted for publication in the journal Preventive Veterinary Medicine (doi: 10.1016/j.prevetmed.2018.10.015) (uncorrected proof format)

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Seroprevalence of canine *Leishmania infantum* infection in the Mediterranean region and identification of risk factors: The example of North-Eastern and Pyrenean areas of Spain

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ABSTRACT

The Mediterranean basin is an endemic region for canine leishmaniosis (CanL), where it represents a major veterinary problem and raises human health concerns. However, the distribution of the disease is heterogeneous and not all countries and locations have been equally studied and characterized. This work describes the situation of CanL in Girona province (Catalonia, Spain), for which no data has been previously reported, and presents a relevant study to exemplify other areas with similar characteristics across the region. Four cross-sectional seroprevalence surveys were performed from 2012 to 2016 throughout the province, including 36 sampling stations in 26 localities and a total of 593 dogs. For each animal, individual and location variables were also collected. Additionally, each dog owner answered a questionnaire about their knowledge of CanL and preventive methods used. Blood samples were analysed by an in-house ELISA and a mixed logistic regression model was used to assess the relationship between pre-determined variables and dog seropositivity. A Spearman's correlation was used to assess the association between dog owners' perceived risk of CanL and *Leishmania infantum* seropositivity in dogs at a given location. The overall true seroprevalence estimated for Girona province was 19.5% (95%CI: 15.5–23.5), of which only 6.8% (10/146) were considered symptomatic. Age of the dog [OR = 1.21 (95%CI: 1.11-1.31); p < 0.001] and altitude [OR = 0.02 (95%CI: 0.001-0.19); p = 0.001] were identified as risk factors for the infection. The results obtained in this study are expected to aid in the implementation of directed control programmes in CanL endemic areas throughout Europe, as well as to provide suitable data for the design of better risk assessment maps of the disease.

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic parasitic disease caused by *Leishmania infantum*, widely distributed in the Mediterranean area (Dujardin et al., 2008). In this region, *L. infantum* transmission is mainly vectorial through the bite of phlebotomine sand flies of the genus *Phlebotomus*, subgenus *Larroussius*. The domestic dog is the main vertebrate reservoir of the parasite (Alvar et al., 2004). CanL is a multisystemic disease that can present variable, usually unspecific, clinical signs. However, in endemic regions, the high proportion of asymptomatic dogs favours the unnoticed spread of *L. infantum* infection in the dog population (Baneth et al., 2008). Asymptomatic seropositive dogs are at risk of developing the clinical disease throughout their lives (Baneth et al., 2008) and are infectious for sand flies, which makes them permanent and unnoticed reservoirs of the parasite for other dogs and humans (Molina et al., 1994). Likewise, the early detection of these asymptomatic carriers is crucial for the control of the disease

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both in endemic and in non-endemic areas, as it is known that the infection is spreading to northern European regions through dog movement (Maia and Cardoso, 2015).

Spain is an endemic country for CanL and, as observed in other endemic areas, the distribution of the infection is highly heterogeneous throughout the territory (Miró and Molina, 2006). For this reason, Mediterranean endemic regions would benefit greatly from CanL directed control efforts, targeted at areas with higher levels of infection. CanL seroprevalence in owned dogs in Spain ranges from 1.6% in the northwest (Miró and Molina, 2006) to 34.6% in the south (Morillas-Márquez et al., 1996), with a range of intermediate values reported across the territory (Alcover et al., 2013; Ballart et al., 2013; Goyena et al., 2016; Martín-Sánchez et al., 2009; Miró et al., 2017; Solano-Gallego et al., 2001). Nevertheless, as in other Mediterranean countries, the map of CanL distribution in Spain is far from complete, with many regions still lacking documented information.

Catalonia, in the north-east of Spain, is considered one such endemic area for CanL. Here, like in other regions, identifying locations for the implementation of CanL directed control programmes is constrained by the heterogeneous distribution of the infection and the lack of published data on CanL prevalence. Historically, the south of Catalonia was known for the presence of well-established and important foci of CanL (Fisa et al., 1999; Portús et al., 2007) but recent studies in northern areas such as the Lleida region (Ballart et al., 2013) showed that the infection is more widespread than previously thought. Furthermore, results of a recently published questionnaire-based study suggest that Girona province, in the north-east of Catalonia, may be an endemic area of CanL (Lladró et al., 2017). In a survey of local veterinarians, the general opinion was that CanL is well established throughout the province and the number of autochthonous cases has risen in the last years. Additionally, Girona province shares a range of physical and climatic characteristics with other Mediterranean locations and is therefore an appropriate example for CanL epidemiological studies.

The objectives of this study were to provide the first data on CanL seroprevalence for Girona province (Catalonia, north-eastern Spain), which could also confirm the hypothesis of CanL endemicity in this region suggested by Lladró et al. (2017), and to identify possible individual and location risk factors associated with the infection in the study area which can be applied in the control of CanL in other Mediterranean endemic regions.

2. Materials and methods

2.1. Study area and population

Girona province (42°10′0″N, 2°40′0″E; area of 5,910 km^2) is located in the north-east of Catalonia (Spain). It is delimited by the Mediterranean Sea (to the east), France (to the north), and by Barcelona and Lleida Catalan provinces (to the south and west, respectively), all endemic for CanL. Girona is divided into nine counties with altitudes ranging from zero meters above sea level (m a.s.l.) to 2,910 m a.s.l. Habitats and climates vary from Mediterranean on the coast to alpine in the Pyrenees. Annual mean temperatures range from 16 °C in the southern counties to 5 °C in the north of the province, though maximum and minimum temperatures can reach 39 °C and -16 °C, respectively. Mean relative humidity varies from 61% to 81% and average annual rainfall ranges from 550 mm to 1350 mm (Servei Meteorològic de Catalunya, 2016).

Study individuals were recruited through local veterinarians registered in the regional veterinary association (Col.legi Oficial de Veterinaris de Girona – COVGi). After an informative talk about CanL, a number of professionals were willing to participate by being the link between their clients and the project researchers. Dog owners consisted mostly of wild boar hunters, who usually keep large packs of dogs, allowing the sampling of several animals in the same location. Four cross-sectional surveys were conducted between April 2012 and March 2016 in different locations of north-eastern and Pyrenean areas of Spain, in Girona province, including 36 sampling stations in 26 localities (Fig. 1).

2.2. Sample collection and serological techniques

Blood samples from all animals were collected by cephalic or jugular venepuncture to 5 mL EDTA tubes. Plasma was obtained and preserved at −40 °C. Samples were analysed by an in-house enzyme-linked immunosorbent assay (ELISA) for the presence of anti-*L. infantum* anti-

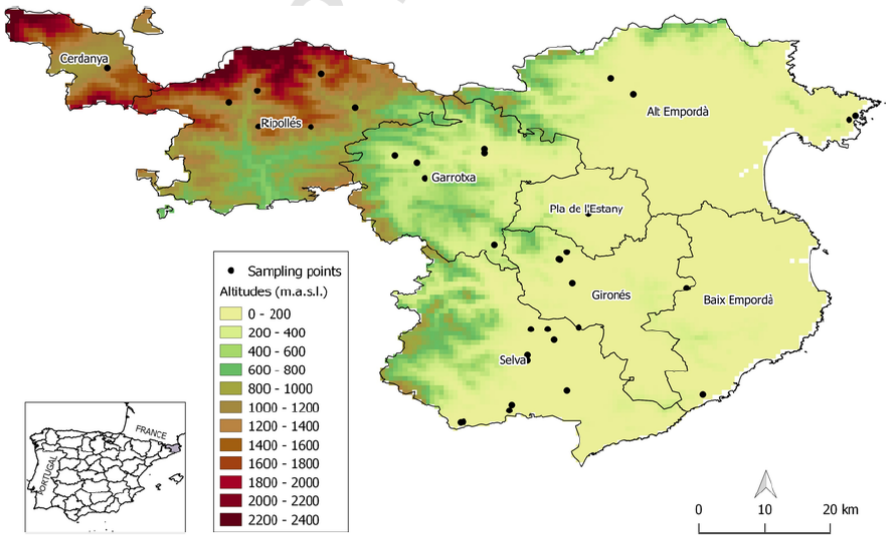

Fig. 1. Map of altitudinal distribution in Girona province. Study sampling locations are marked as black dots.

bodies, using a technique previously described (Ballart et al., 2013; Riera et al., 1999). Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar®) previously coated with sonicated whole promastigotes at a protein concentration of 20 μg/ml in 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase ((1:30,000, Sigma®) was used as conjugate and reactions were stopped with H_2SO_4 3 M when a pre-determined positive control serum reached an optical density of 450 read at 450 nm. Sample optical densities were then read at 492 nm. All samples were run in duplicate and calibrator, positive and negative serums were included in all plates. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The cut-off was established at 24U.

2.3. Data collection

In addition to sample collection, information was gathered from each sampling location (geographical coordinates, altitude, county, nearest locality, type of habitat, and presence of other domestic and farm animals) and each animal's individual characteristics (sex, age, breed, given use, type of night shelter, and presence of visible CanL clinical signs). Clinical exams were performed by veterinarians and the criteria for classifying dogs as "symptomatic" were the detection of the following clinical signs: weight loss, lymphadenomegaly, periocular or diffuse alopecia, onychogryphosis, ocular lesions, and/or pale mucous membranes. Dog owners were asked about their previous knowledge of CanL, as well as control measures regularly taken to prevent the disease. This data was then used to determine possible risk factors associated with CanL seroprevalence in the population studied.

2.4. Statistical analysis

True seroprevalence was calculated following the method described in Cortes et al. (2012). The formula used was: true prevalence (TP) = [apparent prevalence (AP) – 1 + test specificity (Sp)] / [test sensitivity (Se) – 1 + Sp]. Confidence intervals for true prevalence were also calculated with the following formula: TP 95%CI = 1.96 x $\sqrt{}$ [AP x (1-AP) / sample size (n) x (Se + Sp-1)].

The relationship between CanL seropositivity and a series of individual and location variables was assessed through a mixed logistic regression model. The choice of variables to analyse, as well as the categories defined, were based on those used in previous publications (Ballart et al., 2013; Gálvez et al., 2010a; Martín-Sánchez et al., 2009) and adapted to the characteristics of the present study. In summary, the covariates considered in the analysis were: altitude (<800/>800 m.a.s.l.), type of habitat (rural or between villages/periurban or at the edge of villages/urban or inside villages), presence of other animal species (yes/no), sex (male/female), age (<1 to 13 years, introduced as a continuous variable), breed (purebred/crossbred), use given (hunting/breeding/others, which includes racing and pet dogs), night shelter (indoors/outdoors), dog owner knowledge of preventive methods against CanL (yes/no), dog owner use of preventive methods against CanL (yes/no) and dog owner use of prophylactic methods against other arthropods (yes/no).

A bivariate logistic regression analysis was performed, in which the relationship between the outcome variable ("dog seropositivity") and each explanatory variable listed above was assessed individually. Statistical significance was set at $p < 0.05$. This was followed by a multivariable mixed logistic regression analysis, in which non-significant covariates ($p > 0.05$) were sequentially deleted through a backward stepwise selection method until a final model was obtained. In this model, "Locality" was introduced as a random-effects variable to account for geographic clustering of the data (Alonso et al., 2010; Ballart et al., 2013) and the year of the survey was included as a fixed-effects variable.

The association between CanL seroprevalence calculated per dog shelter and owner's perception of risk of infection (graded in percentage categories from 0 to 90–100%) was assessed through a Spearman's coefficient correlation.

All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX, USA). Maps were produced in QGIS Desktop version 2.18.11.

3. Results

3.1. Descriptive analysis of the study population

A total of 593 blood samples were obtained from dogs distributed throughout the north-east and Pyrenean areas of Spain, in Girona province, with sampling points ranging from 1 to 10 per county (Table 1).

Sampling sites were mainly rural (corresponding to 50.1% of the dog sample) and periurban (41.8% of sampled dogs), with dog density per site ranging from 3 to 34. Altitudes ranged from 50 to 1300 m a.s.l., with the majority of dogs living below 800 m a.s.l. (83%). Most dogs were hunting animals (78.9%), but breeding (16%), shelter (2.5%), racing (2.2%) and pet dogs (0.3%) were also represented. A large number of sampled dogs were born in Girona province (60.4%) and were not reported to have left the region. All animals included in the study were kept with other dogs in open kennels during the day time, and the majority were also kept outdoors at night (87.9%). There were other animal species kept in close proximity to 49.6% of the sampled dogs. These included cats, horses, cows, goats and pigs. Observed age average was 3.6 years (SD = 2.9), 58.9% of the dogs were males and 55.4% were crossbred.

3.2. Dog owners' perception on CanL and use of preventive measures

The majority of dog owners showed previous knowledge of CanL (93.9%) and approximately half of them knew preventive methods against CanL (57.6%), although only 27.3% had ever used them (Table 2).

Only a small number of dog owners believed that their dogs were not at risk of contracting CanL during their lifetime (12.1%), with the majority of them believing that the risk of CanL ranged from 5 to 20% (63.7%). The result of the Spearman's correlation showed a positive association between dog owners' perceived risk of CanL infection and CanL seroprevalence ($r_s = 0.5046$; $p = 0.0027$).

Prophylactic methods against CanL, when used, included dog collars (44.5%), spot-on (33.3%) and combined insecticide treatments (22.2%). Vaccination against CanL or immunomodulatory prophylactic treatments had not been used by any of the dog owners. The main reasons given for not using any preventive method against CanL were unawareness (58.3%) and not believing that prophylaxis worked (12.5%).

3.3. CanL study results

From the 593 dogs analysed, 146 were considered seropositive by ELISA. Apparent seroprevalence at the sampling point level ranged from 0% to 79.6%, with a total apparent seroprevalence calculated for Girona province of 24.6% (95% CI: 21.2–28.3). The L. *infantum* in-house ELISA has a specificity of 90% and a sensitivity of 85%, when the chosen cut-off is used. These values were calculated based on a population of 77 dogs (Fisa et al., 2001; Iniesta et al., 2002). Reference positivity status for L. *infantum* infection was determined by parasite detection (culture and/or direct exam and/or PCR) (provided as supplementary material). Considering these values, the estimated true

Table 1
True seroprevalence for canine *Leishmania infantum* infection observed in each locality and overall true seroprelavence calculated per county and for Girona province.

County	Locality	No. sampling points	No. sampled dogs (No. positive dogs)	No. seropositive dogs (%)	True seroprevalence % (95% CI)
Alt Empordà	Cadaqués	2	29 (12)	41.4	41.8
	Darnius	2	18 (6)	33.3	31.1
	Ordis	1	31 (9)	29.0	25.4
	Total	5	78 (27)	34.6	32.8 (20.6-45.0)
Baix Cerdanya	Urtx	**1**	**30 (1)**	3.3	**0**
Baix Empordà	Sant Feliu de Guíxols	**1**	**19 (10)**	52.6	56.8 (30.9-82.8)
Garrotxa	Hostalnou de Bianya	2	21 (3)	14.3	5.7
	Montagut	2	55 (16)	29.1	25.5
	Olot	1	12 (5)	41.7	42.2
	Sant Esteve de Llémena	1	21 (2)	9.5	0
	Total	6	109 (26)	23.9	18.5 (9.2-27.7)
Gironès	Aiguaviva	1	30 (4)	13.3	4.4
	Canet d'Adri	3	44 (35)	79.6	92.7
	Madremanya	1	20 (3)	15.0	6.7
	Sant Gregori	1	24 (13)	54.2	58.9
	Total	6	118 (55)	46.6	48.8 (38.4-59.2)
Plà de l'Estany	Banyoles	**1**	15 (7)	46.7	48.9 (19.7 -78.0)
Ripollés	Bruguera	1	9 (0)	0	0
	Camprodon	1	8 (0)	0	0
	Ogassa	1	32 (0)	0	0
	Serrat	1	6 (0)	0	0
	Setcases	1	3 (0)	0	0
	Ventola	1	13 (0)	0	0
	Total	6	71 (0)	0	0
Selva	Brunyola	1	10 (0)	0	0
	Maçanet de la Selva	1	17 (3)	17.7	10.2
	Massanes	2	29 (3)	10.3	0.5
	Riells i Viabrea	2	31 (7)	22.6	16.8
	Sta Coloma de Farners	2	32 (0)	0	0
	Vilobí d'Onyar	2	34 (7)	20.6	14.1
	Total	**10**	153 (20)	**13.1**	4.1 (0-10.3)
Total for Girona province		36	593 (146)	24.6	19.5 (15.5-23.5)

CanL seroprevalence for Girona province was 19.5% (95%CI: 15.5–23.5). Estimated seroprevalence at the county level ranged from 0 to 56.8%. Results for all localities and counties are summarized in Table 1.

Only 10 out of 146 seropositive dogs were considered symptomatic (6.8%). Observed clinical signs included onychogryphosis (n = 9), weight loss (3), skin wounds (3), diffuse alopecia (2), popliteal lymphadenomegaly (2), periocular alopecia (2), and ocular lesions (2).

3.4. Bivariate statistical analysis

One of the dog kennels included in the seroprevalence study (Banyoles, Plà de l'Estany) was excluded from the statistical analysis, following the criteria used in similar studies (Ballart et al., 2013). This is a shelter kennel that collects stray dogs, which means that some of the individual data, as well as owners' perception of CanL, could not be collected. Therefore, the statistical analysis included 578 individuals and 25 localities. Results of the bivariate analysis are summarized in Table 3. Dogs' age and location altitude ($p < 0.001$) showed a very strong relationship with dog seropositivity. In our population, a bimodal CanL seroprevalence distribution according to age was observed, with a first peak at 3–4 years and a second at 7–8 years old, with the risk of infection rising by each year of life [OR = 1.18 (95%CI: 1.09–1.27)] and decreasing at altitudes above 800 m a.s.l. [OR = 0.012 (95%CI: 0.002-0.07)]. Also, according to the results, being a crossbred dog raises the risk of infection [OR = 2.19 (95%CI: 1.18–4.06); $p = 0.013$] and the use of unspecific insecticides against arthropods has a protective effect [OR = 2.94 (95%CI: 1.58–5.45); $p = 0.001$]. All the other variables (sex of the dog, type of habitat, dog purpose, type of nocturnal refuge, presence of other animal species, owner's knowledge of prophylactic measures against CanL and the regular application of these methods) showed no statistically significant relationship with dog seropositivity.

3.5. Multivariable mixed model

The final multivariable mixed logistic regression model identified age of the dog and altitude of the location as the explanatory variables that affect dog seropositivity. According to this model, the odds of being infected rise in 1.21 per each year of life [(95%CI: 1.11–1.31); $p < 0.001$] and decrease at locations above 800 m a.s.l. [OR = 0.02 (95%CI: 0.001-0.19); $p = 0.001$]. The final model explains 53.7% of the total variance of the outcome variable, of which 42% is explained by the fixed effects terms and 11.7% by the random effects variable.

4. Discussion

Until now, data on CanL in north-eastern and Pyrenean areas of Spain is scarce and fragmented. The only published study regarding CanL in Girona province is a questionnaire-based survey of veterinary practitioners working in the region (Lladró et al., 2017). This work provided the first data from a previously recognized, but non-documented CanL endemic area in north-east Spain and highlights gaps in the epidemiological picture in Mediterranean regions considered to be endemic for CanL (Ready, 2017). The veterinary survey showed that new cases of CanL in autochthonous animals were diagnosed annually, including some asymptomatic cases detected by CanL pre-vaccination screening (Lladró et al., 2017). The present study confirms the suspected endemicity of CanL in the region, providing results for canine seroprevalence, as well as an overview of the infection distribution throughout Girona province. Preliminary exploratory surveys showed the presence of phlebotomine vectors in the surroundings of many of the sampling points (authors' unpublished data), confirming that all

Table 2
Results of the questionnaire asked to dog owners regarding their knowledge of canine leishmaniosis and the methods used to prevent the infection (n = 33).

Question	No. replies (%)
Have you ever heard of CanL?	
Yes	31 (93.9)
No	2 (6.1)
In your opinion, how great is the risk of any of your dogs having CanL throughout their lives?	
0%	4 (12.1)
5%	9 (27.3)
10%	5 (15.2)
20%	7 (21.2)
50%	1 (3.0)
50-90%	4 (12.1)
90-100%	3 (9.1)
Do you know of any measures to protect your dogs against CanL?	
Yes	19 (57.6)
No	14 (42.4)
Do you use any measure to protect your dogs against CanL?	
Yes	9 (27.3)
No	24 (72.7)
If YES, which method do you use? (n = 9)	
Collar	4 (44.5)[b]
Spot-on	3 (33.3)[b]
Others[a]	2 (22.2)[b]
If NO, why not? (n = 24)	
Unawareness	14 (58.3)[b]
Do not believe it works	3 (12.5)[b]
Too expensive	2 (8.3)[b]
Do not believe there is CanL	1 (4.2)[b]
Others/no answer	4 (16.7)[b]
Do you use any measure to protect your dogs against other arthropods (e.g. ticks, fleas, etc.)	
Yes	25 (75.8)
No	8 (24.2)
If YES, which method do you use? (n = 25)	
Pour-on	12 (48.0)[b]
Sprays	2 (8.0)[b]
Spot-on	1 (4.0)[b]
Others[a]	10 (40.0)[b]

[a] Includes the use of others or multiple preventive measures.
[b] Percentage based on the total for the subgroup YES or NO of the previous answer.

conditions are present for a complete *L. infantum* biologic cycle to be maintained in this region. In addition, the characterisation of all individuals and locations included in the study allowed for the identification of risk factors associated with CanL distribution.

As previously mentioned, there was an active search for individuals to be enrolled in the study, assisted by local veterinarians. There was therefore a constraint in the distribution and type of animals recruited, depending on the availability of veterinary practitioners' clients willing to participate. As a result, the dog population was mainly composed of hunting dogs. These animals have inherent characteristics, such as the fact that they are usually kept with other dogs in open kennels, in rural or periurban settings, and generally do not have the same type of veterinarian monitoring as pet dogs. Therefore, this type of population is usually considered a good sentinel for CanL (Ballart et al., 2013; Cabezón et al., 2010). As similar hunting activities take place throughout Mediterranean areas in Europe, it can be expected that comparable dog populations are widespread. An overestimation of the overall infection prevalence can however occur due to an expected lower incidence in urban centres, mostly explained by a decreased probability of contact between dogs and sand fly vectors (Ballart et al., 2013). Additionally, there was an increased difficulty in recruiting dogs from higher altitude regions, mainly because these areas are more inhospitable and less populated. Consequently, dogs living at locations above 800 m a.s.l. are less represented.

Table 3
Number of dogs analysed and *Leishmania infantum* seropositivity observed for each category of the explanatory variables, followed by the results of the bivariate analysis expressed in odds ratios (OR). Statistically significant variables ($p < 0.05$) are marked with (*).

Explanatory variables and categories	No. dogs analysed	No. seropositive dogs (% seropositive dogs)	Bivariate analysis	
			OR (95% CI)	*p*-value
Altitude (m a.s.l.)				
< 800	492	144 (29)	Ref	
>800	109	6 (6)	0.012 (0.002-0.07)	<0.001*
Type of habitat				
Rural	297	70 (24)	Ref	
Periurban	228	51 (22)	1.64 (0.93-2.88)	0.082
Urban	53	19 (36)	0.97 (0.42-2.22)	0.934
Presence of other animals (other than dogs)				
Yes	302	73 (24)	Ref	
No	299	78 (26)	1.10 (0.58-2.08)	0.763
Sex				
Male	338	87 (26)	Ref	
Female	240	53 (22)	0.88 (0.56-1.38)	0.581
Age (years)			1.18 (1.09-1.27)	<0.001*
<1	53	6 (11)		
1	83	12 (14)		
2	91	13 (14)		
3	79	20 (25)		
4	51	16 (31)		
5	42	10 (24)		
6	42	12 (29)		
7	33	15 (45)		
8	33	14 (42)		
9	15	3 (20)		
10	18	5 (28)		
11	10	4 (40)		
12	3	0 (0)		
13	1	1 (100)		
Breed				
Purebred	258	51 (20)	Ref	
Crossbred	320	89 (28)	2.19 (1.18-4.06)	0.013*
Use given				
Hunting	468	118 (25)	Ref	
Breeding	95	21 (22)	2.28 (0.78-6.63)	0.130
Others[a]	15	1 (7)	0.20 (0.02-1.73)	0.145
Night shelter				
Outdoors	506	126(25)	Ref	
Indoors	72	14 (19)	0.50 (0.20-1.23)	0.131
Owner knows preventive measures against CanL				
Yes	362	103 (28)	Ref	
No	216	37 (17)	0.58 (0.29-1.19)	0.138

Table 3 (*Continued*)

Explanatory variables and categories	No. dogs analysed	No. seropositive dogs (% seropositive dogs)	Bivariate analysis	
			OR (95% CI)	*p*-value
Owner has used preventive measures against CanL				
Yes	140	46 (33)	Ref	
No	438	112 (26)	1.24 (0.63-2.43)	0.539
Owner has used prevention methods against other arthropods				
Yes	472	112 (24)	Ref	
No	106	28 (26)	2.94 (1.58-5.45)	0.001*

[a] Includes racing (n = 13) and pet dogs (n = 2).

Some degree of spatial clustering may have been introduced by sampling several dogs in the same kennel or locality. This could also have had a clustering effect on the positive results, as higher dog densities tend to favour the transmission of the parasite, especially if some of the dogs are already infected (Alonso et al., 2010). Nevertheless, in the present study we have used similar dog populations in the different sampling points, allowing comparison between them. Additionally, this methodology has also been used in similar studies describing other regions of Spain (Alcover et al., 2013; Ballart et al., 2013). In the statistical analysis, the potential clustering effect was dealt with by introducing "Locality" as a random-effects term in the final multivariable mixed logistic regression model.

The serological technique used to measure antibody levels to *L. infantum* was an in-house ELISA. ELISA is one of the methods recommended by the World Organization for Animal Health for performing CanL surveillance studies and to determine prevalence of infection (OIE, 2014), the other one being the indirect immunofluorescent antibody test (IFAT). Unlike IFAT, ELISA is easy to perform and interpret, being particularly useful in field study settings, where a large number of samples must be analysed (Maia and Campino, 2008). In addition, this ELISA has been widely used for CanL diagnosis, as well as in other CanL epidemiological studies (Alcover et al., 2013; Ballart et al., 2013; Fernández-Bellon et al., 2008; Fisa et al., 2001; Iniesta et al., 2002; Riera et al., 1999; Rodríguez-Cortés et al., 2010; Solano-Gallego et al., 2005).

The overall estimated seroprevalence for Girona province was 19.5%, ranging from 0 to 56.8% across the different counties. These results are in accordance with previous reports for other regions of Spain, as well as the Mediterranean basin, (Ballart et al., 2013; Ntais et al., 2013; Cortes et al., 2012; Maroli et al., 2008). A series of CanL seroprevalence surveys undertaken in France, Italy, Spain and Portugal between 1971 and 2006 showed an overall seroprevalence of 23.2%, with point prevalences of 0% and higher than 80% in some locations (Franco et al., 2011). These values are comparable to the ones obtained in the present study and correspond to the previous claims of the heterogeneous distribution of the disease. However, as pointed out by Franco et al. (2011), caution must be taken when comparing studies with different experimental designs and different criteria used in the selection of the target dog population, as this can introduce significant variations in seroprevalence results. A common European strategy for leishmaniosis surveillance and control would aid the implementation of standardized methodology. However, although leishmaniosis is currently listed as a notifiable disease by the World Organization for Animal Health (OIE, 2018), this is not clearly reflected in the European or Spanish legislation (BOE, 2014; Official Journal of the European Union, 2012).

From the 146 seropositive dogs, only 10 (6.8%) showed clinical signs compatible with CanL and more than 50% presented low standard ELISA units (inferior to 50U). This can be explained by the cryptic nature of the infection and the wide clinical spectrum it can present, ranging from asymptomatic or mild symptomatic cases to very severe clinical stages (Solano-Gallego et al., 2009). There is also the possibility that some of the dogs are in an early stage of infection (Fisa et al., 2001; Miró et al., 2012) or are immunologically resistant and only transiently seropositive, eventually showing spontaneous clearance of the parasite (Fisa et al., 1999). In such populations, serological techniques could have a lower sensitivity (Otranto et al., 2009). It is also known that, in endemic areas, only a small proportion of dogs display symptoms of CanL, while the majority of infected dogs do not show any clinical evidence of the disease (Baneth et al., 2008). It is believed that the high prevalence of asymptomatic infected dogs, comparable to that observed in Lleida province (other north-eastern and Pyrenean region studied in Spain), is strong evidence for a well-established CanL focus in Girona province (Ballart et al., 2013). In the present study, clinical signs compatible with CanL were also identified in 10 out of 447 seronegative dogs (2%), illustrating the lack of specificity of the disease's clinical presentation and the added difficulty in detecting affected dogs. As mentioned before, the ability of serological tests to detect infected animals is limited, especially in endemic settings, and a small number of seronegative asymptomatic infected dogs should be expected, as previously reported in other studies (Iniesta et al., 2002;. Otranto et al., 2009; Solano-Gallego et al., 2001). These animals can harbour parasites in the skin, detectable by PCR (Otranto et al., 2009), and could also be infectious to sand flies, as has been demonstrated for asymptomatic seropositive dogs (Molina et al., 1994; Quinnell and Courtenay, 2009). Considering this, any control programme for CanL should be based on multiple diagnostic methods, as serology alone can prove to be insufficient in detecting all infected and infectious dogs.

In the present study, the risk of infection increased with dogs' age. This is an individual factor commonly reported as being positively related with *L. infantum* infection (Alonso et al., 2010; Ballart et al., 2013; Cortes et al., 2012; Gálvez et al., 2010a; Maresca et al., 2009; Martín-Sánchez et al., 2009; Miró et al., 2012), and which can be explained by an incremental risk of exposure to infected sand flies. The bimodal CanL seroprevalence distribution observed has been previously described by other authors (Gálvez et al., 2010a; Miró et al., 2012). This pattern suggests that *L. infantum* may be able to infect immunologically vulnerable animals at an earlier age, followed by a later infection of resistant animals either by cumulative exposure or due to concomitant diseases that weaken the dogs' immune system (Miranda et al., 2008).

According to the results, altitude shows a negative correlation with *L. infantum* infection. This is mainly related to the bioclimatic needs of the phlebotomine vector species present in Spain, *Phlebotomus perniciosus* and *P. ariasi* (Rioux et al., 1986). Altitude is known to be closely linked to temperature, precipitation and land cover (Baron et al., 2011; Rivas-Martínez, 1983). In temperate regions, as atmospheric temperature rises, a higher biting rate is expected (Hartemink et al., 2011), therefore increasing the risk of sand fly bites to vertebrate hosts. Simultaneously, a shorter extrinsic incubation period (Hartemink et al., 2011) and a more effective development of the parasite inside the vector (Rioux et al., 1985) are observed, raising the risk of *L. infantum* infection. Also, an increased altitude may provide a more hostile environment for sand fly survival (Gálvez et al., 2010b), not only because of the more extreme bioclimatic conditions, but also due to a possible scarcity of vertebrate hosts. However, a relationship between altitude and risk of CanL infection was not observed in the neighbouring province of Lleida (Ballart et al., 2014, 2013) or in France (Chamaillé

et al., 2010), where both vector species are present and show different altitudinal preferences. In these areas, *P. perniciosus* is known to occupy ecological niches commonly below 800 m a.s.l., while *P. ariasi* shows a higher abundance above this altitude. Therefore, it would be of particular interest to perform entomological studies and risk factor analysis associated with the vector populations present in the study area. This could also help to improve the ability of the present model to predict the outcome variable. One of the possible reasons for the moderate performance of the final statistical model presented (which explains 53.7% of variance of the outcome variable) is the absence of data on the abiotic factors mentioned above, which are known to have an important impact on sand fly populations, and indirectly on *L. infantum* infections (Dantas-Torres et al., 2014; Gálvez et al., 2010b).

The present study failed to detect an effect of type of habitat (rural/urban) or access to night shelter, which several other authors identified as significantly related to *L. infantum* infection (Ballart et al., 2013; Cortes et al., 2012; de Almeida et al., 2012d; Gálvez et al., 2010a; Martín-Sánchez et al., 2009; Oliveira et al., 2016). According to these studies, dogs that live in rural habitats and are left outdoors at night show an increased risk of infection. In this study, the high percentage of dogs living in rural/periurban areas and kept permanently outdoors may not have allowed detection of such an effect. Also, periurban areas are increasingly described as the most suitable ecosystems for sand flies, due to the ideal microclimate offered by house gardens associated with the abundance of vertebrate hosts (Alvar et al., 2004; Ballart et al., 2013).

Results from the bivariate statistical analysis identified dog breed and the use of general insecticide treatment against arthropods as variables associated with dog seropositivity. In the first case, crossbred dogs would be at higher risk of infection [OR = 2.19 (95%CI: 1.18–4.06); p = 0.013]. However, previous studies have shown that this should not be the case, as crossbred, autochthonous dogs tend to be more resilient to *L. infantum* infection (Alvar et al., 2004; Solano-Gallego et al., 2000). This is even more noticeable when the purebred dogs belong to exotic breeds like boxers and beagles (both represented in this study), known for their higher sensitivity to CanL (Solano-Gallego et al., 2009). The effect of dog breed was absent in the mixed model, showing that the previous results were most probably induced by confounding factors related to the kennel locations (e.g. altitude) or dog owners' attitudes (e.g. use of prophylactic measures against CanL). The non-use of generalist insecticide preventive methods against arthropods was also identified as a risk factor for *L. infantum* infection [OR = 2.94 (95%CI: 1.58–5.45); p = 0.001], while the use of specific prophylaxis against CanL failed to show a protective effect (p = 0.539). Again, this may be related to confounding factors, such as a possible partial effect of some insecticides against phlebotomine vectors, even though they may not be licensed for sand fly prevention. Additionally, the improper use of specific sand fly prevention treatment, such as failure to apply it to all dogs or to maintain it during the whole transmission season, may impair the protective effect of these products (Courtenay et al., 2009). Once again, the effect of this variable lost significance in the multivariable analysis and was not included in the final statistical model.

The majority of dog owners showed previous knowledge of CanL and to be aware of preventive methods for the infection. Although a positive correlation was observed between owners' perceived risk of infection and CanL seropositivity at the dog kennel level, only 27.3% of dog owners stated that they regularly used CanL prophylactic measures. This result is in accordance with those reported by Lladró et al. (2017), in which all veterinary practitioners working in Girona province recommended at least one preventive measure against CanL, though the majority did not believe that dog owners protected their dogs properly. When used, the most frequent prophylactic methods applied against CanL were dog collars and spot-on insecticides, as recommended by veterinarians. However, most owners did not keep their dogs indoors at night and did not report the use of vaccination against CanL or immunomodulatory agents, as also suggested by veterinarians (Lladró et al., 2017). Our study, being an example for other Mediterranean endemic areas, shows that the implementation of prophylactic measures by dog owners should be reinforced in order to reduce *L. infantum* transmission between dogs, as well as to reduce the public health risk (Miró and López-Vélez, 2018).

5. Conclusions

According to the results presented, Girona province shows characteristics of a stable, endemic focus of CanL: a high *L. infantum* seroprevalence observed in dogs, together with a large number of asymptomatic cases and the presence of the sand fly vector. The majority of these dogs are autochthonous and have never left the province. Dogs' age and altitude were identified as risk factors for the disease, providing additional information to complement the design of risk assessment maps for *L. infantum* infection as well as for the implementation of CanL control measures in endemic areas across the Mediterranean basin.

Ethics approval

The research protocol was submitted to the Ethics Committee on Animal Experimentation (CEEA) of University of Barcelona, which considered that an ethical approval was not required for this study. The project was also submitted to and approved by ISGlobal Internal Scientific Committee (ISC). All dog owners were informed about the research protocol and signed an informed consent allowing for sample and data collection.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2018.10.015.

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Parasites & Vectors

RESEARCH Open Access

Seasonal dynamics of canine antibody response to *Phlebotomus perniciosus* saliva in an endemic area of *Leishmania infantum*

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Abstract

Background: Canine leishmaniosis (CanL) is an important zoonotic parasitic disease, endemic in the Mediterranean basin. In this region, transmission of *Leishmania infantum*, the etiological agent of CanL, is through the bite of phlebotomine sand flies. Therefore, monitoring host-vector contact represents an important epidemiological tool, and could be used to assess the effectiveness of vector-control programmes in endemic areas. Previous studies have shown that canine antibodies against the saliva of phlebotomine sand flies are specific markers of exposure to *Leishmania* vectors. However, this method needs to be further validated in natural heterogeneous dog populations living in CanL endemic areas.

Methods: In this study, 176 dogs living in 12 different locations of an *L. infantum* endemic area in north-east Spain were followed for 14 months. Blood samples were taken at 5 pre-determined time points (February, August and October 2016; January and April 2017) to assess the canine humoral immune response to whole salivary gland homogenate (SGH) and to the single salivary 43 kDa yellow-related recombinant protein (rSP03B) of *Phlebotomus perniciosus*, a proven vector of *L. infantum* naturally present in this region. Simultaneously, in all dogs, *L. infantum* infection status was assessed by serology. The relationship between anti-SGH and anti-rSP03B antibodies with the sampling month, *L. infantum* infection and the location was tested by fitting multilevel linear regression models.

Results: The dynamics of canine anti-saliva IgG for both SGH and rSP03B followed the expected trends of *P. perniciosus* activity in the region. Statistically significant associations were detected for both salivary antigens between vector exposure and sampling month or dog seropositivity to *L. infantum*. The correlation between canine antibodies against SGH and rSP03B was moderate.

Conclusions: Our results confirm the frequent presence of CanL vectors in the study area in Spain and support the applicability of SGH- and rSP03B-based ELISA tests to study canine exposure to *P. perniciosus* in *L. infantum* endemic areas.

Keywords: Canine leishmaniosis, *Phlebotomus perniciosus*, Saliva proteins, Markers of exposure, Longitudinal study, North-east Spain

Background

Leishmania infantum (Kinetoplastida: Trypanosomatidae) is the causative agent of canine leishmaniosis (CanL), a zoonotic vector-transmitted disease widespread in the Mediterranean region, as well as in other parts of the world [1–3]. Prevalence of *L. infantum* infection in canine populations from endemic areas is highly heterogeneous [4], and not all infected dogs will ever develop clinical signs of the disease [5]. However, infected asymptomatic dogs could act as a reservoir of the parasite and are capable of transmitting *L. infantum* to other dogs, as well as to humans [6, 7].

The transmission of the parasite is mainly vectorial, through the bite of phlebotomine sand flies. In the Mediterranean basin, eight species of the genus *Phlebotomus* have been implicated as vectors of *L. infantum*, according to conventional criteria. From these, all except one belong to the subgenus *Larroussius* [8]. In Spain, CanL transmission is mainly shared by *P.* (*L.*) *perniciosus* and *P.* (*L.*)

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ariasi [9, 10], with the second species having a narrower distribution but being responsible for maintaining the infection at higher altitudes [11, 12]. Recently, *L. infantum* DNA was also found in another *Larroussius* species, *P. langeroni*, in the south of the country [13].

The detection of anti-sand fly salivary antibodies in the blood of vertebrate hosts has proven to be highly specific [14] and was successfully used as a marker of exposure to *L. infantum* vectors [15, 16]. In CanL endemic areas, monitoring the canine IgG response to sand fly saliva can be a useful epidemiological tool [15, 17], complementing studies of vector population dynamics and host-vector interactions, as well as enabling the assessment of risk of *Leishmania* infection [14, 18, 19]. Furthermore, it can be used to measure the effectiveness of vector-control programmes and to assist in the design of better control strategies for the disease [20, 21].

Originally, sand fly whole salivary gland homogenates (SGH) were used to investigate the presence of anti-sand fly saliva antibodies in vertebrate hosts [20–22]. However, its use in large-scale studies is impaired by technical limitations [23]. Additionally, the use of SGH in vector exposure tests may reduce the specificity of detection due to a possible cross-reactivity with saliva of sympatric and closely related sand fly species [24].

An alternative to the use of SGH is the identification of species-specific salivary proteins that can be expressed in recombinant forms and produced in large quantities for use in large-scale epidemiological studies [25, 26]. Recent studies identified *P. perniciosus* yellow-related protein rSP03B as the most promising candidate to replace SGH in the detection of host markers of exposure to this vector species [16, 17, 26]. This recombinant protein has been tested and validated in dogs and other animals in cross-sectional studies [16, 26], as well as in a canine longitudinal study [17], but no information exists on the seasonal dynamics of either SGH or rSP03B in natural heterogeneous dog populations from endemic areas.

Therefore, the objectives of this study were (i) to investigate the dynamics of *P. perniciosus* and their relative density in a previously uncharacterized CanL endemic area through the detection of anti-saliva IgG in dogs; and (ii) to evaluate the performance of both SGH and rSP03B antigens as markers of exposure to *P. perniciosus* in natural canine populations.

Results

Seasonal dynamics of IgG response against salivary proteins from *P. perniciosus*

Median values of normalized ELISA OD values for SGH ranged from 9.04 (range: 3.94–66.23) in January 2017 to 18.51 (7.93–100.58) in August 2016 (Table 1). For rSP03B, median OD values varied between 12.21 (6.75–53.71) and 19.53 (10.64–124.01) in January 2017 and August 2016, respectively. With both antigens, median OD readings raised from basal values in February 2016 (10.11 and 14.67 for SGH and rSP03B, respectively) to peak in August (18.51 and 19.53 for SGH and rSP03B, respectively), sustained higher readings in October (11.15 and 15.31 for SGH and rSP03B, respectively), and descended again to basal levels in January (9.04 and 12.21 for SGH and rSP03B, respectively) and April 2017 (9.54 and 13.44 for SGH and rSP03B, respectively). Median normalized ELISA OD results obtained per month for both SGH and rSP03B are described in Table 1 and plotted in Fig. 1.

Table 1 Median values of normalized OD readings for SGH and rSP03B obtained per sampling month in all locations

Variable	*N*	SGH	rSP03B
		Median (Range)	Median (Range)
February 2016	174	10.11 (5.49–49.62)	14.67 (7.36–41.24)
August 2016	33	18.51 (7.93–100.58)	19.53 (10.64–124.01)
October 2016	164	11.15 (5.56–86.44)	15.31 (6.15–112.54)
January 2017	154	9.04 (3.94–66.23)	12.21 (6.75–53.71)
April 2017	148	9.54 (5.25–62.59)	13.44 (6.27–36.22)

Abbreviation: *N* number of dogs sampled per sampling month

Cut-off values were set at 13 for SGH and 22 for rSP03B. When these were applied to the OD readings obtained in August 2016, 75.76% (25/33) of the dogs were positive to anti-SGH IgG, and 36.36% (12/33) to anti-rSP03B antibodies. In October, these values dropped to 35.98% (59/164) for SGH and 18.9% (31/164) for rSP03B. During the non-transmission season (considered to extend from

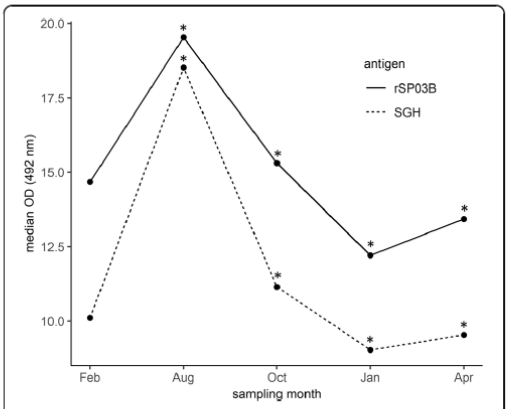

Fig. 1 Dynamics of anti-*P. perniciosus* salivary proteins IgG response in dogs from an endemic area during a sand fly activity season. Values presented refer to the normalized OD medians obtained at each sampling month for all dogs and locations. Statistically significant differences in median OD between two consecutive months are marked with an asterisk ($P < 0.05$)

November to May), the percentage of seropositive dogs ranged from 14.29% (25/175) in February 2016 to 17.57% (26/148) in April 2017 for SGH and 8.44% (13/154) in January 2017 to 12.16% (18/148) in April 2017 for rSP03B.

Correlation results for IgG response between SGH and rSP03B were r_S = 0.54 (95% CI: 0.48–0.60, P < 0.001) (Fig. 2).

Dogs' exposure to *P. perniciosus* in the study area

Exposure of dogs to phlebotomine vectors showed some variation according to the location. Median OD readings varied from 9.11 (range: 5.25–20.57) to 14.14 (7.44–55.45) for SGH ELISA and from 12.71 (7.53–64.44) to 17.87 (8.39–112.54) for rSP03B. Minimum median values of response to both SGH and rSP03B corresponded to the same location (Aiguaviva), but maximum median values were registered in different sites for each antigen (Sant Feliu de Guíxols for SGH and Montagut for rSP03B) (Table 2). Figure 3 presents the dynamics of dogs' IgG response to SGH (Fig. 3a) and rSP03B (Fig. 3b) in each locality.

The percentage of anti-sand fly saliva seropositive dogs per location, defined as the number of dogs that showed a positive IgG titre at least once during the study period, ranged from 13.33% (1/8) in Ordis to 100% in Canet d'Adri (8/8) and Sant Feliu de Guíxols (4/4) for SGH, and from 8.16% (1/12) in Hostalnou de Bianya to 100% (4/4) in Sant Feliu de Guíxols for rSP03B. Total anti-sand fly saliva seropositivity calculated for the study area was 49.43% (87/176) for anti-SGH IgG and 28.98% (51/176) for anti-rSP03B antibodies.

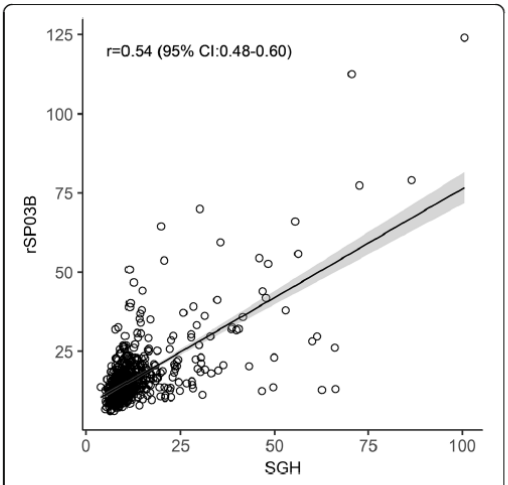

Fig. 2 Correlation between IgG recognizing SGH and rSP03B protein in dogs naturally exposed to *P. perniciosus*. Results from both SGH and rSP03B are presented in normalized OD (r_S = 0.54; 95% CI: 0.48–0.60, P < 0.001)

Dogs' exposure to *P. perniciosus* and *L. infantum* infection

Correlation results between antibody response to *P. perniciosus* saliva and *L. infantum* were low both for SGH (r_S = 0.27, 95% CI: 0.19–0.35, P < 0.001) and rSP03B protein (r_S = 0.25, 95% CI: 0.18–0.32, P < 0.001).

Multilevel analysis of the relationship between anti-*P. perniciosus* salivary proteins, month and location and *L. infantum* seropositivity

The multilevel model results confirmed the annual dynamics of anti-salivary proteins IgG responses. When compared to the first sampling month (February 2016), IgG responses to SGH significantly rose in August (t = 8.55, df = 491, P < 0.001) and October (t = 6.49, df = 491, P < 0.001) and dropped in January (t = -2.49, df = 491, P = 0.013) and April 2017 (no significant difference when compared to February 2016). As expected, the highest log OD estimate was observed in August 2016 and the lowest in January 2017 (Table 3). The same trend was observed in the model run for the rSP03B protein, with comparable levels of significance (Table 4). There were no significant differences in IgG responses for both antigens between each sampling location and the one set as reference, except for Montagut, where significantly higher OD levels were observed for SGH (t = 2.28, df = 166, P = 0.024) and rSP03B (t = 2.13, df = 164, P = 0.035). According to the multilevel model, seropositivity to *L. infantum* proved to be associated with a rise in anti-salivary proteins OD values for both SGH (t = 2.5, df = 491, P = 0.013) and rSP03B (t = 2.15, df = 493, P = 0.032).

Discussion

The quantification of anti-sand fly saliva antibodies in vertebrate hosts of *L. infantum* has been previously shown to be an effective way of measuring exposure to the parasite vectors [16]. In the case of dogs, the most frequent host and reservoir of *L. infantum*, this has been proven for *P. perniciosus* [15, 26], as well as for other sand fly species [27–29]. These markers of exposure can then be applied in host-vector epidemiological studies, in *L. infantum* infection risk assessment, and to assist in the design of control strategies for the disease. Therefore, it is important to validate these techniques in natural, heterogeneous populations from endemic areas, in which a higher individual variability is expected.

Phlebotomus perniciosus activity period in Spain shows two main peaks, the first in June-July and the second in September-October. These peaks also correspond to the periods of highest *L. infantum* transmission [30–32]. This trend was identified in our study and corresponds to the rise in anti-saliva antibody levels observed between

Table 2 Median values of normalized OD readings for SGH and rSP03B obtained per sampling location at all time points

Variable	*n* (Range)	Geographical coordinates	SGH	rSP03B
			Median (Range)	Median (Range)
Ordis	8 (7–9)	42°13'37.7"N, 2°54'24.1E	9.14 (6.45–45.95)	15.16 (8.35–54.50)
Madremanya	14 (12–15)	41°58'47.0"N, 2°58'7.2"E	11.22 (6.79–49.84)	14.49 (8.95–43.99)
Vidreres	8 (7–9)	41°47'27.4"N, 2°45'0.4"E	10.59 (7.80–16.86)	13.46 (8.58–40.23)
Massanes	21 (20–23)	41°45'15.3"N, 2°38'44.0"E	9.31 (5.67–62.59)	16.35 (7.82–55.81)
Hostalnou de Bianya	12 (11–14)	42°13'26.0"N, 2°26'9.7"E	8.75 (5.35–33.16)	13.19 (6.27–46.82)
Montagut	13 (7–15)	42°14'7.7"N, 2°35'57.6"E	12.01 (3.94–72.61)	17.87 (8.39–112.54)
St. Esteve de Llémena	9 (9–10)	42°3'35.1"N, 2°37'1.4"E	9.49 (6.23–22.40)	14.18 (9.12–22.46)
Canet d'Adri	8 (4–10)	42°1'53.7"N, 2°44'15.3"E	10.61 (6.52–100.58)	14.03 (7.36–124.01)
Aiguaviva	19 (16–22)	41°54'27.2"N, 2°46'19.0"E	9.11 (5.25–20.57)	12.71 (7.53–64.44)
St. Feliu de Guíxols	4	41°47'2.3"N, 2°59'58.7"E	14.14 (7.44–55.45)	16.73 (8.57–65.97)
Riells i Viabrea	20 (18–21)	41°43'59"N, 2°33'39.3"E	10.02 (6.07–66.23)	13.43 (8.59–35.31)
Vilobí d'Onyar	23 (22–23)	41°53'3.2"N, 2°43'38.6"E	9.13 (5.17–16.49)	13.05 (6.15–38.07)

Abbreviation: *n* mean number of dogs sampled in each location

August and October. Humoral immune response to *P. perniciosus* saliva elicited in experimentally bitten dogs showed that antibody levels significantly rose after 2–4 weeks of continued exposure, peaking in week 5 [15]. In our field study, the highest IgG levels were in August, which clearly corresponded to the June-July *P. perniciosus* expected activity peak. Similarly, the high IgG readings obtained in October are likely to correspond to *P. perniciosus* second peak of activity. The lower rise in antibody levels observed at this time point can be explained by an earlier sampling at the beginning of October, which may have hindered the display of a complete seroconversion. The high overall levels of seropositivity to anti-sand fly saliva antigens, especially for SGH (49.43%), strongly support the CanL endemicity status for the region [33]. These results also validate both SGH and rSP03B as suitable antigens to assess exposure to *P. perniciosus* in natural canine populations from endemic areas.

An important remark when analysing the longitudinal dynamics of anti-sand fly saliva IgG in the study dog

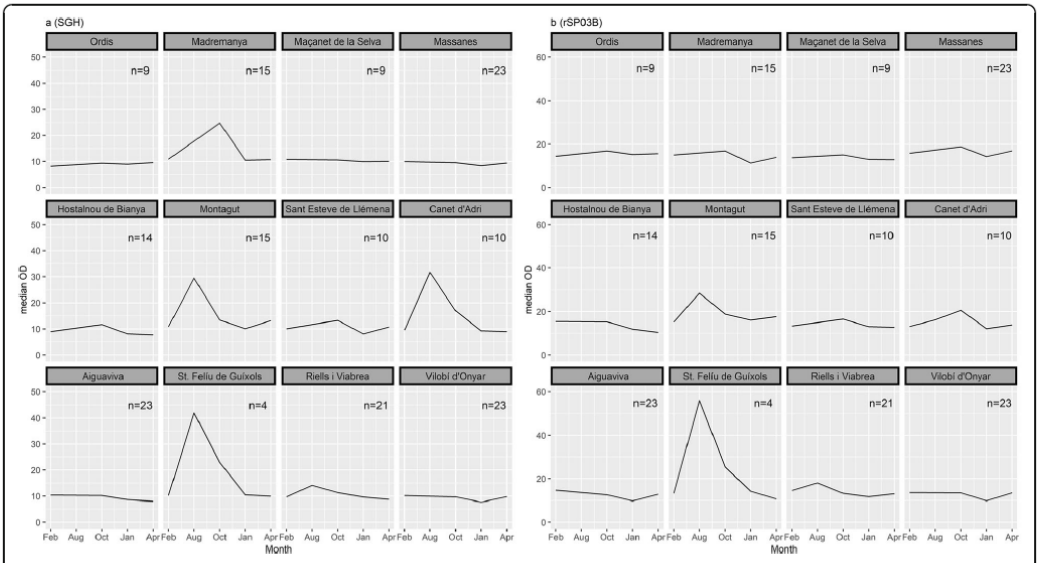

Fig. 3 Dynamics of dogs' IgG recognizing SGH (**a**) and rSP03B protein (**b**) in the different sampling locations during a sand fly activity season. Values presented refer to the normalized OD medians obtained at each sampling month

Table 3 Estimates of the multilevel linear regression model of the relationship between log transformed normalized SGH OD values and sampling time, location and dog seropositivity to *L. infantum*. "Dog" was included as a random effects variable

Variable	Levels	Estimate	SE	*P*-value[a]
Intercept		2.40	0.06	<0.001
Sampling month	February 2016	Ref	–	–
	August 2016	0.54	0.06	<0.001
	October 2016	0.20	0.03	<0.001
	January 2017	-0.06	0.03	0.013
	April 2017	-0.01	0.03	0.666
Location	Aiguaviva	Ref	–	–
	Ordis	0.07	0.11	0.562
	Madremanya	0.08	0.10	0.427
	Vidreres	0.10	0.11	0.393
	Massanes	0.07	0.09	0.441
	Hostalnou de Bianya	-0.08	0.10	0.404
	Montagut	0.22	0.10	0.024
	St. Esteve de Llémena	-0.03	0.11	0.786
	Canet d'Adri	-0.02	0.11	0.891
	St. Feliu de Guíxols	0.16	0.15	0.308
	Riells i Viabrea	0.03	0.09	0.703
	Vilobí d'Onyar	-0.02	0.09	0.791
L. infantum seropositivity	Seronegative	Ref	–	–
	Seropositive	0.10	0.04	0.013

Abbreviation: *SE* standard error
[a]Level of significance of *P*-value < 0.05 was used

Table 4 Estimates of the multilevel linear regression model of the relationship between log transformed normalized rSP03B OD values and sampling time, location and dog seropositivity to *L. infantum*. "Dog" was included as a random effects variable

Variable	Levels	Estimate	SE	*P*-value[a]
Intercept		2.79	0.06	<0.001
Sampling month	February 2016	Ref	–	–
	August 2016	0.39	0.06	<0.001
	October 2016	0.09	0.03	0.003
	January 2017	-0.13	0.03	<0.001
	April 2017	-0.06	0.03	0.016
Location	Aiguaviva	Ref	–	–
	Ordis	0.06	0.10	0.563
	Madremanya	-0.04	0.09	0.652
	Vidreres	-0.03	0.10	0.783
	Massanes	0.05	0.08	0.533
	Hostalnou de Bianya	-0.16	0.09	0.074
	Montagut	0.18	0.09	0.035
	St. Esteve de Llémena	-0.10	0.10	0.287
	Canet d'Adri	-0.05	0.10	0.641
	St. Feliu de Guíxols	-0.19	0.14	0.173
	Riells i Viabrea	-0.08	0.08	0.302
	Vilobí d'Onyar	-0.06	0.08	0.399
L. infantum seropositivity	Seronegative	Ref	–	–
	Seropositive	0.07	0.03	0.032

Abbreviation: *SE* standard error
[a]Level of significance of *P*-value < 0.05 was used

population is that there was a clear basal antibody level before the transmission season. After the expected rise in humoral response during summer months, IgG levels returned again to basal levels. These results show that, though exposed to repetitive bites during several months, dogs from endemic areas do not sustain high anti-saliva IgG levels throughout the year, allowing the detection of recent exposure to sand flies in natural populations. Similar results were recently reported in a longitudinal field study in Brazil, where canine IgG against *Lutzomyia longipalpis* saliva were evaluated [34]. Our study identified the same trends for both SGH and rSP03B, which reinforces the suitability of recombinant antigens in detecting recent exposure to phlebotomine vectors in endemic settings, particularly when considering the use of these tests in large-scale studies for vector control interventions [35, 36].

Antibodies recognizing both SGH and rSP03B followed similar dynamics throughout the field study. However, the correlation between the two antigens was only moderate (r_S = 0.54; 95% CI: 0.48–0.60, P < 0.001). Even so, available studies show that rSP03B is the most promising surrogate for SGH as a marker of exposure to *P. perniciosus* in the canine host. It has presented high levels of correlation with SGH in both experimentally [25] and naturally bitten dogs [16, 17, 26]. Two apyrase proteins (rSP01B and rSP01) have also shown a good correlation with SGH [25]. However, in a study where these three recombinant proteins presented similarly high correlations with SGH, rSP03B presented the lowest data dispersion and was considered a better option [16]. These results were confirmed in a field trial, where single rSP03B demonstrated a higher correlation coefficient with SGH than the combination of rSP03B with rSP01 [17].

A similar correlation between SGH and rSP03B to the one obtained in the present study has been observed before in Umbria region (central Italy) (r_S = 0.56; 95% CI: 0.38–0.71, P < 0.001; n = 96), in a screening study of dog exposure to *P. perniciosus* across European CanL endemic foci [26]. A possible reason for these discordant results may be the presence of other closely related phlebotomine species which could induce cross-reactivity with the SGH [22]. In some parts of Catalonia, *P. perniciosus* is sympatric with *P. ariasi*, also a proven vector of *L. infantum* [10]. Due to the close relationship between *P. perniciosus* and *P. ariasi*, both belonging to the subgenus *Larroussius*, it is

expected that they share similar salivary antigens [37]. When comparing the percentage of seropositive dogs detected by both methods during the study, results for SGH are higher (49.43%) than for rSP03B (28.98%). Also, median results per sampling location show differences between SGH and rSP03B: in some cases, the trend between antigens is very similar (e.g. sera from Sant Feliu de Guíxols); in other cases, there is a recognizable peak in anti-SGH IgG, while anti-rSP03B IgG shows no change (e.g. sera from Madremanya). These differences can also be observed over time in the same location, with humoral responses to SGH and rSP03B peaking in different months along the transmission season (e.g. Canet d'Adri). We may hypothesize that SGH, because it contains more proteins than the single-antigen rSP03B, will more likely cross-react with antibodies against *P. ariasi*, inducing a stronger unspecific reaction to this vector species. It would also mean that the prevalence of sand fly species responsible for *L. infantum* transmission in the province varies according to the location, and possibly in the same location throughout the transmission season, for which it would be interesting to perform further entomological studies in the region.

Correlation indexes between levels of antibodies against both salivary antigens and *L. infantum* infection were low [SGH: r_S = 0.27 (95% CI: 0.19–0.35, $P < 0.001$); rSP03B: r_S = 0.25 (95% CI: 0.18–0.32, $P < 0.001$)]. Similar low correlations have been described before between sand fly bites and human visceral leishmaniasis (VL), while stronger correlations are reported between human cutaneous leishmaniasis (CL) and recent vector exposure (reviewed in [23]). This can be explained by VL's longer incubation period and/or the differences in host immune responses to cutaneous and visceral infection [38]. Results from some studies in human populations also suggest that the repeated contact with non-infected sand flies could be correlated with markers of protection for VL [39]. Partial protection against *L. major*, an agent of CL, has also been achieved in immunized mice by the bites of uninfected sand flies [40]. However, another study with BALB/c mice demonstrated that this type of immunity is limited to short-term exposure and questioned the efficacy of sand fly saliva-induced protection against *Leishmania* infection in CL endemic areas [41]. CanL follows a pattern which is more similar to VL than to CL, therefore a low correlation between humoral responses to sand fly saliva and *Leishmania* would be expected [15]. However, results of the multilevel linear regression model show a positive and statistically significant relationship between *P. perniciosus* bites and a seropositive status for *L. infantum*, both for SGH and rSP03B. Similar results have been described in other longitudinal field studies on both canine anti-*P. perniciosus* and anti-*L. longipalpis* IgG dynamics [17, 34]. Unlike cross-sectional surveys, longitudinal studies are able to detect the relationship between a higher number of sand fly bites at a given time point and a subsequent *L. infantum* infection. Therefore, this type of study is likely to better explain the relationship between these two events, which can take place several months apart.

Conclusions

The results of this study confirmed the applicability of both anti-*P. perniciosus* SGH and rSP03B IgG as markers of exposure to *L. infantum* vectors in natural dog populations from an endemic area. Canine humoral response to both antigens is compatible with the annual sand fly activity dynamics expected for the region. Significantly lower IgG levels were observed during the non-transmission season; despite the repeated exposure to sand flies during the summer months, there is a return to basal IgG levels in these dog populations during the winter. The comparative performance of SGH and rSP03B showed a moderate correlation, which might be explained by the occurrence of cross-reactions of SGH with other closely related sympatric sand flies. Further longitudinal studies in natural canine populations from endemic areas, together with entomological studies, should be carried out in order to corroborate this hypothesis. Nevertheless, both antigens are expected to detect only vectors of *L. infantum*, confirming their suitability for host-vector-parasite studies. Finally, the overall results support the CanL endemicity status for the study region, which had already been suggested by previous studies [33].

Methods

Experimental design

The study included a heterogeneous population of 176 dogs distributed by 12 locations in Girona Province (Catalonia, northeast of Spain), an area endemic for CanL [33]. These dogs were enrolled in a canine leishmaniosis vaccine field trial, but no statistically significant differences in *L. infantum* infection between groups were observed either during or at the end of the trial. These were all owned dogs, used mainly for hunting, but some breeding and racing individuals were also included. All animals were kept in large packs in open-air facilities, mostly in rural and periurban areas. Furthermore, no specific anti-sand fly insecticide treatments were applied, providing conditions for dog exposure to the vector. Dog density per study location varied between 4–23. The dogs were followed from February 2016 to April 2017 and blood samples, obtained by venepuncture and placed in 5 ml EDTA tubes, were collected at 5 pre-determined time points (Table 1). Plasma was obtained and stored at -40 °C until processing.

Sand flies and salivary proteins

A colony of *P. perniciosus* was reared under standard conditions as described previously [42]. Salivary glands were

dissected from 4–6 day-old females, pooled at a concentration of 1 salivary gland per 1 μl of 20 mM Tris buffer with 150 mM NaCl and stored at -80 °C. The *P. perniciosus* 43 kDa yellow-related recombinant protein (rSP03B, Genbank accn. DQ150622) was obtained from Apronex s.r.o. (Prague, Czech Republic) and quantified by the Lowry method (Bio-Rad, Hercules, California, USA) following the manufacturer's protocol.

Serological detection of dog exposure to sand flies

Anti-*P. perniciosus* IgG was measured by an in-house enzyme-linked immunosorbent assay (ELISA) as described previously [17]. All samples from a single dog were processed in the same plate. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (40 ng per well, equivalent to 0.2 salivary gland) or with rSP03B (5 μg/ml) in 20 mM carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4 °C. Plates were then blocked with 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine plasma were diluted 1:200 for SGH and 1:100 for rSP03B in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. The reaction was stopped with 10% H_2SO_4 and absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Each sample was tested in duplicate and positive and negative controls were included in each plate. To account for the variability between plates, sample OD readings were normalized by dividing them by the mean OD of positive controls run in the same plate [43]. The normalized OD values were multiplied by 100. Positivity cut-offs were calculated as the mean plus 3 standard deviations from 14 dog samples from a non-endemic area.

Serological detection of *L. infantum* infection

All samples were tested for the presence of IgG against *L. infantum* through an in-house enzyme-linked immunosorbent assay (ELISA), using a technique described previously [44, 45]. Again, serial samples from a single dog were tested in parallel on the same plate. Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar® Corning®, New York, USA) previously coated with sonicated whole promastigotes at a protein concentration of 20 μg/ml in 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase (1:30,000, Sigma-Aldrich®, St. Louis, Missouri, USA) was used as conjugate and reactions were stopped with H_2SO_4 3M when a pre-determined calibrator control serum reached an optical density of 450 at 450 nm. Sample optical densities were read at 492 nm. All samples were run in duplicate and the calibrator, positive and negative sera were included in all plates. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The positivity cut-off was established at 24U.

Statistical analysis

Statistical analyses were performed using R software (http://cran.r-project.org/) and Stata 15 software (StataCorp LP, College Station, TX, USA).

Correlations between IgG responses to *P. perniciosus* SGH and rSP03B and between each one of the salivary antigens and anti-*L. infantum* IgG levels were tested by the Spearman rank correlation test. Median OD values between time points were compared using the Wilcoxon signed rank sum test.

The relationship between anti-SGH and anti-rSP03B antibodies and sampling month, *L. infantum* infection status and location was tested by fitting multilevel linear regression models, taking into account the correlation between repeated measures of the same dogs over time. In the models, log-transformed anti-saliva or rSP03B normalized OD values were considered as continuous dependent variables and sampling month, *L. infantum* infection and location as categorical predictor variables. In order to assess variations in OD between the first sampling month and those following, "February 2016" was set as reference level for this variable. Likewise, the locality with the lowest median OD ("Aiguaviva") was considered to be the reference for the variable location. Finally, "seronegative" was set as the reference level for the variable *L. infantum* infection. The random component included dog and time to allow for variation at the intercept (between dogs) and the slope (over time). The inclusion of "dog" as a random effects variable significantly improved both models, with a between dog variance of 48% for SGH and of 47% for the rSP03B model. A *P*-value of < 0.05 was considered to indicate statistical significance.

Abbreviations

CanL: Canine leishmaniosis; CL: Human cutaneous leishmaniasis; ELISA: Enzyme-linked immunosorbent assay; IgG: Immunoglobulin G; OD: Optical density; rSP03B: 43 kDa yellow-related recombinant protein; SGH: Salivary gland homogenate; VL: Human visceral leishmaniasis

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding authors upon reasonable request.

Authors' contributions
RV, LW, JC, PV and MG designed the study; RV, ED and MG performed the fieldwork; RV, TS and LW performed the lab work; TS and RV analysed and interpreted the data; RV and MG wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The research protocol was submitted to the Ethics Committee on Animal Experimentation (CEEA) of University of Barcelona, which considered that an ethical approval was not required for this study. The project was also submitted to and approved by ISGlobal Internal Scientific Committee (ISC). All dog owners were informed about the research protocol and signed an informed consent allowing for sample and data collection.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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