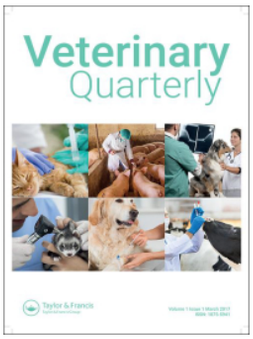

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Assessment of the exposure to *Phlebotomus perniciosus* and the presence of anti-*Leishmania infantum* antibodies in stray cats in an endemic region of Spain, and their potential correlation with environmental factors

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ABSTRACT

Phlebotomus perniciosus is a major vector of *Leishmania infantum* in the Mediterranean. While the seroprevalence of leishmaniosis in Spanish dogs and cats has been studied, data on the exposure of cats to *P. perniciosus* bites under natural conditions without repellents is limited. Stray cats could serve as sentinels for *L. infantum* and *P. perniciosus* exposure. This study analyzed sera from 204 apparently healthy stray cats, collected from January 2021 to January 2022, for antibodies against *P. perniciosus* saliva and *L. infantum* parasites. Anti-sand fly antibodies were detected in 40.69% of cats using an ELISA with the recombinant salivary protein SP03B of *P. perniciosus*. Seroprevalence of *L. infantum* infection was 23.52% by Western blot and 27.41% by ELISA, with an overall seroprevalence of 40.69% (95% CI 34.18–47.54%). This is the first assessment of antibody response to *P. perniciosus* saliva and *L. infantum* in naturally exposed stray cats in Spain. Further research is needed to examine the salivary antigens recognized by cats and to explore the relationship between *P. perniciosus* exposure and *L. infantum* infection severity in cats.

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1. Introduction

Leishmaniosis caused by *Leishmania infantum* is a zoonotic vector-borne disease endemic in southern Europe, Africa, Latin Central America, and Asia (Alvar et al. 2006). Domestic dogs serve as the primary reservoirs, exhibiting a wide spectrum of clinical signs, ranging from asymptomatic to mild, severe, or even fatal disease. Cats are recognized as potential contributors to the disease's epidemiology and are currently considered the most probable additional domestic reservoir in Europe (Maia and Campino 2011). They are frequently infected with this protozoan without displaying clinical manifestations (Spada et al. 2016).

A variety of diagnostic techniques are commonly employed to detect feline leishmaniosis, including molecular tests and serological techniques such as the immunofluorescent antibody test (IFAT), direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), and Western Blotting (WB) (Pennisi et al. 2015). However, an epidemiological study conducted in the Barcelona region revealed that subclinical infections of *L. infantum* in feline populations outnumber clinical infections in cat populations in endemic regions (Tabar et al. 2008). For a more accurate diagnosis, it is recommended to employ a combination of different diagnostic techniques (Alcover et al. 2021).

L. infantum is transmitted by several species of phlebotomine sand flies, with *Phlebotomus perniciosus* being the principal vector in the western part of the Mediterranean (Alten et al. 2016). In endemic regions, this species is generally considered the most abundant and dominant vector, although this may not hold true in cooler and more humid areas. It is active from May to November, with population density varying

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according to local endemic conditions, leading to temporal fluctuations in its presence (de Freitas Milagres and Maia 2024). In eastern Spain, the activity period of this species is highly variable, ranging from late March to mid-December. In the Mediterranean region, *P. perniciosus* exhibits a bimodal seasonal pattern, with peak activity in June and July and again from September to October (Lucientes-Curdi et al. 1991). Its activity period varies with environmental conditions, with the highest levels of activity occurring during the early part of the night, from sunset to midnight (Lucientes et al. 2005).

During a blood feeding, females of *P. perniciosus* inject immunogenic components of saliva into the host, leading to the development of anti-saliva antibodies (Lestinova et al. 2017). The antibody response to *P. perniciosus* salivary antigens in dogs from endemic areas has already been suggested as an epidemiological biomarker (Drahota et al. 2014; Kostalova et al. 2015, 2017; Quinnell et al. 2018; Velez et al. 2018). Several studies have revealed that levels of specific IgG antibodies against *Phlebotomus* saliva positively correlate with the number of blood-fed sand flies, decreasing at the end of the seasonal activity of these vectors (Kostalova et al. 2015; Quinnell et al. 2018). In contrast, a single study described the feline antibody response against *P. perniciosus* saliva (Pereira et al. 2019). Authors analyzed the cats' exposure to phlebotomine sand flies by detecting antibodies to *P. perniciosus* saliva and investigated the association between phlebotomine sand fly exposure and the presence of *Leishmania* infection in Portugal (Pereira et al. 2019).

Detecting anti-sandfly antibodies in a feline population is crucial for identifying exposure to sandfly-borne diseases like leishmaniasis, which can impact both animals and humans. By assessing the presence of these antibodies, researchers can evaluate the risk of disease transmission within the population, implement targeted preventive measures, and guide health management strategies. This information also supports epidemiological studies by providing insights into the prevalence of sandfly exposure and its potential impact on feline health.

Given the lack of studies on *L. infantum* infection and vector exposure in cats in endemic areas of *L. infantum* in Spain, the present study aimed to: (1) provide the first epidemiological data on the detection of anti- *P. perniciosus* antibodies in stray cats naturally exposed to sand flies in *L. infantum* endemic region; (2) investigate the seroprevalence of *L. infantum* infection in stray cats using two different serological techniques to evaluate the degree of exposure; and (3) evaluate the results of serological screenings by analyzing the presence of anti-*Leishmania* antibodies and the antibody response to *P. perniciosus* saliva with their possible relation to climatological parameters.

2. Material and methods

2.1. Animals

The study was carried out in the city of Zaragoza (41°38′58.8948″N, 0°53′15.7632″W, in the Aragon region of Spain). The study population included stray European shorthair cats captured in different areas of Zaragoza from January 2021 to January 2022 as part of a trap, neuter and release sterilization program that ran locally to control the stray colony cats. These cats did not receive any preventative measures such as drug administration. A complete physical examination was carried out before sampling. Only cats older than 1 year and classified as apparently healthy based on the general examination were included. This survey was encompassed under the Project License PI75/20 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals was undertaken according to the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.2. Sampling

Captured stray cats were anesthetized with a combination of dexmedetomidine (Dexdomitor®; 15 µg/kg, subcutaneous injection), ketamine (Anaestamine®; 5 mg/kg, subcutaneous injection) and methadone (Semfortan®; 0.3 mg/kg, subcutaneous injection). Data on the breed, age, gender and colony of origin of each cat were recorded. A complete physical examination was carried out before sampling. Additionally, the amounts of rainfall and average temperatures in the individual months of the study were included based on State Meteorological Spanish Agency (AEMET) (https://www.aemet.es/es/serviciosclimaticos/datosclimatologicos). Prior to collecting blood, the fur of the cats was trimmed around the jugular region. Sampling consisted of collecting 1 ml of blood aseptically by jugular venipuncture. The serum was separated and stored at − 20 °C until processing. Routine laboratory tests, such as a complete blood count and biochemistry profile, were not performed.

2.3. Diagnostic serological tests

Detection of specific anti-*Leishmania* antibodies (IgG) was performed using two in-house serological techniques: the ELISA and Western blotting (WB).

2.3.1. Detection of L. infantum antibodies by ELISA (ELISA-Leish)

An ELISA was performed on all sera as described previously by Riera et al. (1999), with some modifications. Briefly, each plate was coated with 20 µg/ml of crude antigen obtained from *L. infantum* promastigote forms (MHOM/MON-1/LEM 75) in 0.1 M carbonate/bicarbonate buffer (pH 9.6), and incubated overnight at 4 °C. A 100 µl aliquot of cat sera, diluted 1:200 in PBS containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M), was added to each well. The plates were then incubated for 1 h at 37 °C in a moist chamber, following which they were washed and 100 µl of

Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:20,000 in PBST-M was added. The plates were incubated for 1 h at 37 °C in the moist chamber and were washed again with PBST and PBS as described above. The substrate solution composed by ortho-phenylene-diamine and stable peroxide substrate buffer (Thermo Fisher Scientific) was added to 100 µl per well and developed for 20 ± 5 min at room temperature in the dark. The reaction was stopped by adding 100 µl of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (ELISA Reader Labsystems Multiskan, Midland, ON, Canada).

As a positive control (calibrator), each plate included serum from a cat from Spain diagnosed with feline leishmaniosis, confirmed by a positive *L. infantum* isolation using an NNN medium, and as a negative control, serum from a healthy, non-infected cat. The same calibrator sera (positive and negative controls) were used for all assays and plates, with a constant inter-assay variation of < 10%. Plates with an inter-assay variation of > 10% were discarded. All samples and controls were run in duplicate. The results were quantified as ELISA units (EU) compared to the positive control serum used as a calibrator and arbitrarily set at 100 EU. The cut-off was established at 13 EU (mean + 4 standard deviations [SD]) of values from 50 indoor cats from northern Spain (cats without contact with *P. perniciosus*) and the results above this value were considered to be positive (Alcover et al. 2021). Sera with an EU ≥ 100 were classified as clearly positive, with an EU ≥ 40 and < 100 as low positive, and with an EU > 13 and < 40 as very low positive.

2.3.2. Detection of L. infantum antibodies by western blotting (WB)

Anti-*Leishmania* antibodies were detected by WB using a whole antigen of *L. infantum* promastigotes (MHOM/FR/78/LEM75 zymodeme MON-1), as described by Riera et al. (1999) with some modifications. Antigen electrophoresis in 1% sodium dodecyl sulfate/15% polyacrylamide gels, together with molecular mass protein standards (Standard Low Range; Bio-Rad, Hercules, CA, USA), was performed on a Mini-Gel AE 6400 Dual Mini Slab Kit (ATTO Corp., Tokyo, Japan). The gels were run at 100 V for 1 h at room temperature.

Polypeptides were transblotted onto nitrocellulose sheets (0.45-mm pore size, HAWP 304 FO; Millipore Corp., Bedford, MA, USA), which were blocked with 20 mM Tris, 0.13 mM NaCl, pH 7.6 (TS) and 5% skimmed milk, overnight at 4 °C. The sheets were washed in TS and introduced into a multiscreen apparatus (Mini Protean II, Multiscreen Apparatus; Bio-Rad). Sera were diluted 1:200 in TS/1% skimmed milk and 0.2% Tween 20. Then 500 µl of each sample was introduced into each channel of the multiscreen apparatus and incubated for 2 h at 37 °C. Bound immunoglobulins were developed by incubation with a 1:1000 dilution of Protein A peroxidase conjugate (Thermo Fisher Scientific) for 1 h. After the sheets were washed three times with TST and a final time with TS, color reaction was developed with 4-chloro-1-naphthol (Thermo Fisher Scientific) and H_2O_2. The reaction was stopped with tap water after 30 min. The sera were considered to be positive when immunoreactivity from low-molecular-weight polypeptide fractions of 14 and/or 16 kDa from the *Leishmania* antigen was observed, as previously reported (Alcover et al. 2021).

2.3.3. Detection of anti-P. perniciosus saliva antibodies by ELISA (ELISA-Phlebo)

Anti-*P. perniciosus* IgG was measured in all sera samples by ELISA as previously described (Pereira et al. 2019) with some modifications. Briefly, each plate was coated with 1 µg/ml of recombinant protein rSP03B (Willen et al. 2019) in 0.1 M carbonate/bicarbonate buffer pH 9.6, (100 µl/well), and incubated overnight at 4 °C. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 6% dry skimmed milk for 1 h at 37 °C. Cat sera were diluted 1:100 in PBST with 2% dry PBST-M and 100 µl were added to each well. The plates were then incubated for 1 h at 37 °C in a moist chamber, then washed 3 times with PBST and 100 µl of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:5,000 in PBST-M was added. The plates were incubated for 45 min at 37 °C in the moist chamber and washed again with PBST. The substrate solution composed by ortho-phenylene-diamine and stable peroxide substrate buffer (Thermo Fisher Scientific) were added to each well and the reaction was developed for 5 min at room temperature in the dark. The reaction was stopped by adding 100 µl of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (ELISA Reader Labsystems Multiskan, Midland, ON, Canada). Each serum was tested in duplicate. Wells without serum (but coated with rSP03B) were used as blanks while serum samples from indoor cats living in non-endemic countries (Northern Switzerland), served as negative controls. The cut-off was established at 0.28 (mean + three SD of values from 50 indoor cats from Switzerland) and the results above this value were considered highly exposed to sand flies.

2.4. Statistical analysis

Statistical analysis was performed using IBM Corp., Armonk, USA, SPSS 26 software. For the numerical variables, a Kolmogorov-Smirnov normality test was carried out in order to determine their distribution. For all cases, it was found that these do not have a normal distribution. Therefore, non-parametric tests were used for comparisons and the results were recorded as medians and the IQR. A Spearman

Rank correlation test was performed to determine the correlation between the quantitative variables. Contingency tables were made to assess the potential association between the results of the ELISA tests (positive or negative), sex (male or female), Western blotting (negative, positive), and the four seasons of the year using Pearson's Chi-squared test. An unpaired Mann-Whitney test was performed to find differences between the results (positive, negative) of the ELISA analyses and environmental variables. All significance tests conducted were two-tailed, and p values less than 0.05 were deemed statistically significant.

3. Results

A total of 204 stray cats (114 males and 90 females) were included in the present study. No evident systemic (lymphadenomegaly, weight loss, pale mucous membranes, stomatitis, ocular lesions) or dermatological (bad appearance of the coat, exfoliative dermatitis, nodular dermatitis) signs compatible with *L. infantum* infection were detected during the examination.

Among the 204 cats, 26 (12.75%, 95% confidence interval [CI] 0.89 − 18.02%) were seropositive for *L. infantum* by WB only, 35 (17.20%, [CI] 12.60-22.93%) were seropositive for *L. infantum* by ELISA only, and 22 (10.78%, [CI] 7.23-15.79%) animals were positive for both techniques. When separated by gender, 33.3% of males and 23.8% of females were positive to the presence of anti-*Leishmania* antibodies detected by serological techniques. The overall seroprevalence of *L. infantum* was 40.69% (95% confidence interval [CI] 34.18 − 47.54%), considering a cat to be infected if it tested positive by at least one of the *L. infantum* diagnostic techniques. In the case of anti-*Leishmania* antibodies detected by ELISA, 57 stray cats were classified as positive (mean ± SD: 29 ± 34 EU), whilst the remaining cats were classified as seronegative (mean ± SD: 10 ± 2 EU). No doubtful results were obtained with this test for any cat. In the WB analysis, 19 cats showed only one 14 kDa mass band, 11 cats showed only one 16 kDa mass band and, finally 18 cats showed the two bands 14 kDa and 16 kDa (Table 1). Additionally, other non-diagnostic polypeptide fractions were also recognized, including peptide bands with mass 18, 24 and 36 kDa.

Out of 57 cats positive by ELISA to *L. infantum*, 52 animals were classified as very low positive, 2 as low positive and 3 animals as high positive. Among these 5 animals classified as low and high positive, 3 were also positive by WB (1 cat classified as low positive and 2 cats classified as clearly positive). Moreover, only a cat classified as low positive was also positive to the presence of anti-*P. perniciosus* antibodies, but negative to WB analysis. In the case of anti-*P. perniciosus* antibodies detected by the in-house ELISA, 83 animals were classified as seropositive (mean ± SD: 0.29 ± 0.08 OD), while 121 cats were classified as seronegative (mean ± SD: 0.25 ± 0.05 OD). The highest OD value among the seropositive cats was 0.68, observed in a female cat in July, while the lowest value was 0.29, recorded in a male cat in January. No inconclusive results were obtained from this test for any cat. To find out if there was a correlation between the optical densities (OD) of the two ELISA tests, a Spearman analysis was performed. No correlation was found (Figure 1). However, serological study shows that there was an association between results obtained by WB technique and ELISA *Leishmania* ($p = 0.003$).

Table 2 shows the correlation between ELISA results of anti-*Leishmania* IgG, anti-*Phlebotomus* IgG and atmospheric variables. Values OD for ELISA-Phlebo was positively correlated to average temperature ($p < 0.001$, $r = 0.276$). In Table 3, the association between qualitative variables are shown when the result of the serum IgG to *L. infantum* was analyzed. No differences between the sex, results of ELISA-Phlebo and season were found ($p > 0.05$).

Table 1. Summary of seropositivity based on different diagnostic tests from all cat colonies.

Period	Number of samples	Number of negative samples	Positive result by ELISA-Leish	Positive result by WB	Positive result by ELISA-Leish and WB	Positive result by ELISA-Phlebo	Positive result by ELISA-Leish and ELISA-Phlebo	Positive result by WB and ELISA-Phlebo	Positive result by WB, ELISA-Leish and ELISA-Phlebo
January 2021	18	6	2	2	1	4	1	1	1
February 2021	16	10	2	0	1	1	1	0	1
March 2021	21	15	2	0	0	0	2	1	1
April 2021	15	5	2	2	3	1	1	0	1
May 2021	18	7	3	0	1	1	4	2	0
June 2021	20	5	1	1	0	7	2	4	0
July 2021	15	5	2	0	0	4	2	0	2
August 2021	12	4	0	0	0	6	1	1	0
September 2021	9	3	0	0	0	5	1	0	0
October 2021	18	9	0	0	2	4	1	1	1
November 2021	21	6	1	4	1	5	1	1	2
December 2021	9	3	0	0	0	4	2	0	0
January 2022	12	1	1	4	4	0	0	2	0
Total	204	79	16	13	13	42	19	13	9

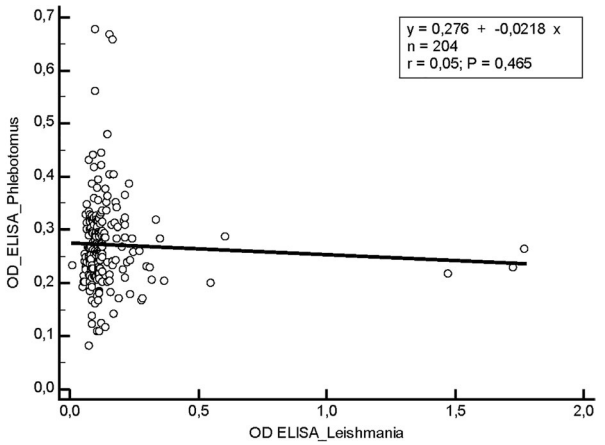

Figure 1. Correlation between the optical density obtained by the ELISA-Leish and the optical density obtained by the ELISA-Phlebo.

Table 2. Sperman rank correlation between ELISA results and atmospheric variables.

	ELISA-Leish	ELISA-Phlebo	Temperature	Rainfall
ELISA-Leish	1			
ELISA-Phlebo	0.575	1		
	0.039			
Temperature	0.313	0.000	1	
	-0.076	0.276		
Rainfall	0.775	0.722	0.072	1
	-0.026	0.020	-0.137	

Pearson's correlation coefficient (r) and statistical significance P value are shown.

Table 3. Chi square test between the results of the ELISA *Leishmania* IgG test and the study variables.

		Negative (%)	Positive (%)	P-value
Sex	Female	87 (73.6)	27 (23.8)	
	Male	60 (66.6)	30 (33.3)	0.157
Western blotting	Negative	121 (82.3)	26 (17.6)	
	Positive	35 (61.4)	22 (38.6)	0.003
ELISA-phlebotomus	Negative	92 (76)	29 (23.9)	
	Positive	55 (66.2)	28 (33.7)	0.153
Season	Winter	37 (68.5)	17 (31.4)	
	Spring	35 (63.6)	20 (63.3)	
	Summer	37 (78.7)	10 (21.1)	
	Autumn	38 (79.1)	10 (20.8)	0.21
Total		147 (72.1)	57 (27.9)	

Table 4. Chi square test between the results of the *Phlebotomus* IgG test and the study variables.

		Negative (%)	Positive (%)	P-value
Sex	Female	63 (55.2)	51 (44.7)	
	Male	58 (64.4)	32 (35.5)	0.199
Western Blotting	Negative	95 (60.9)	61 (39.1)	
	Positive	26 (45.8)	22 (45.8)	0.407
Season	Winter	36 (66.6)	18 (33.3)	
	Spring	41 (74.5)	14 (25.5)	
	Summer	18 (38.3)	29 (61.7)	
	Autumn	26 (54.1)	22 (45.8)	0.001
Total		121 (59.3)	83 (40.7)	

The association found between the results from anti-*Phlebotomus* IgG and sex, and the results from WB and the season are shown on Table 4. An influence of the season of the year on the levels of anti-*Phlebotomus* IgG was observed. The highest incidence of positive cats by ELISA was found in summer, followed by autumn ($p < 0.001$). As the presence of *P. perniciosus* in the environment and *Leishmania* infection in cats depends on atmospheric variables, an unpaired Mann-Whitney test was performed (Table 5). No association was found to the sex of cats or to results of WB method ($p > 0.05$). Cats that were seropositive for antibodies against *Phlebotomus* were identified in the months with the highest average temperature (median 18.6, IQR 13.1, $p = 0.005$). For the other study variables, no statistically significant differences were found ($p > 0.05$).

4. Discussion

To our knowledge, this study represents the first assessment of feline antibody response to *P. perniciosus* saliva in stray cats naturally exposed to the vector in Spain. Despite the increase of number of clinical cases reported in the literature, the epidemiological significance of cats in endemic areas remains incompletely understood.

Epidemiological studies have estimated different seropositivities in dogs infected with *L. infantum* in European mediterranean countries (Vilas-Boas et al. 2024). In this sense, serological studies conducted on cats in various regions of Spain report seroprevalences of *L. infantum* ranging from 1.29% to 60% (Martín-Sánchez et al. 2007; Ayllon et al. 2008) Serological tests and qPCR indicate the existence of *L. infantum* asymptomatic infection in apparently healthy stray cats in the city of Zaragoza, an endemic area in Spain (Alcover et al. 2021).

Understanding the distribution and dissemination of *L. infantum* requires detecting the presence of phlebotomine sand flies, especially *P. perniciosus*, which serves as the principal vector of *L. infantum* in western part of the Mediterranean area. The relationship between the detection of antibodies to *P. perniciosus* saliva and the presence of *Leishmania* infection in cats has been documented in Portugal (Pereira et al. 2019); this study reported 47.7% seropositivity to *P. perniciosus*, indicating that cats are frequently bitten by this sand flies. In our study, out of 204 cats, 83 were positive for anti-*P. perniciosus* IgG, representing more than 40% of our study population. These data confirm that high percentage of stray cats in endemic areas that may come into contact with the *Leishmania* vector.

Interestingly, only 33.7% of cats that tested positive by ELISA for anti-*P. perniciosus* IgG were also positive by ELISA for anti-*Leishmania* IgG and no statistically significant relationship was observed in correlations between results of these two ELISA tests. The recombinant salivary antigen SP03B was developed for screening of canine sera by ELISA and WB (Kostalova et al. 2015; Willen et al. 2019). In ELISA with feline antibodies, its positive correlation with whole salivary gland lysate of *P. perniciosus* was demonstrated (Pereira et al. 2019) but we cannot exclude a possibility that

Table 5. Unpaired Mann-Whitney test between the results of the *Phlebotomus* IgG, *Leishmania* IgG and environmental variables.

		Temperature			Rainfall		
		Median	IQR	P value	Median	IQR	P value
Phlebotomus	Positive	18.6	13.1	0.005	30.8	20.2	0.315
	Negative	12	8.4		20.8	30.2	
Leishmania	Positive	13.7	8.4	0.466	30.8	30.2	0.816
	Negative	13.7	12.8		20.8	27.5	

IQR: interquartile range.

the reaction SP03B with feline sera is suboptimal. High percentage of stray cats exposed to *P. perniciosus* saliva in endemic areas, such as the one in our study, may offer new perspectives on development of salivary antigens as epidemiological biomarkers for the distribution of *Leishmania*, rather than as biomarkers for active disease.

A previous epidemiological study on cats detected a higher presence of IgG during the period of sand fly activity (Pereira et al. 2019). Similar finding was described in dogs, particularly in longitudinal study by Kostalova et al. (2015) who demonstrated increase of specific IgG during summer season and decline during winter. In our study, there were significantly more individuals testing positive for IgG saliva compared to those testing negative, particularly during periods of higher average temperatures. This can be explained by the fact that warmer months in Zaragoza, from May to October, coincide with the activity season of *P. perniciosus* (Alten et al. 2016; Velez et al. 2018). This is further corroborated by the higher positive IgG saliva results detected in autumn and summer, which clearly correspond to the seasonal activity of *P. perniciosus*. In autumn, 45.8% of cats (out of 48) tested positive for *P. perniciosus* saliva, while in summer, the percentage rose to 61.7% (out of 47 cats).

In dogs with leishmaniosis, clinical manifestations of infection may range from mild or absent to severe and even fatal disease. This variability is thought to result from the host's cell-mediated immune response, which may be influenced by the dog's genetic background (Maia and Campino 2018). A similar pattern of humoral and cell-mediated adaptive immune response is observed in cats from endemic areas of *L. infantum* (Priolo et al. 2019). However, cats that develop leishmaniosis are often suspected of having impaired immunity due to comorbidities, which may be linked to immune suppression from drug therapy or the presence of concomitant diseases and/or co-infections (Garcia-Torres et al. 2022). It has recently been reported that both infective sand fly bites, whereby infectious mature metacyclic promastigotes are regurgitated and inoculated into the host and non-infective sand fly bites, where immature promastigotes are inoculated, can elicit Leishmania specific antibodies in animals (Gradoni et al. 2019; Cavalera et al. 2021).

Identifying and controlling reservoirs of *Leishmania* infection is crucial for managing leishmaniosis infection in both dogs and humans. Stray cats, being outdoor animals exposed to vector activity without any parasitic prevention, are of particular concern. Studies have shown that the density of *P. perniciosus* was positively correlated with the presence of cats (Alcover et al. 2013), highlighting the epidemiological significance of cats as potential reservoirs. Given this potential role of stray cats in sustaining and spreading *L. infantum* infection, monitoring their contact with the vector is essential, and detecting anti-*P. perniciosus* saliva antibodies proves to be a viable technique (Pereira et al. 2019). This information is crucial for the development of prophylactic measures aimed at reducing the prevalence of *Leishmania* infection and opens up the possibility of using anti-*P. perniciosus* saliva antibodies as an epidemiological biomarker.

The main limitations of this study include the reliance solely on serological tests to detect anti-*Leishmania* antibodies and the absence of molecular tests, which could identify infected but seronegative cats. Additionally, the study's retrospective nature limited the availability of EDTA blood samples for molecular testing. Moreover, stray cats were classified as apparently healthy based on physical examination alone, without supporting laboratory data from haematology and serum biochemistry. In contrast, the large number of samples obtained and the extended study period represent the key strengths of this research.

This study represents the initial assessment of feline antibody response to *P. perniciosus* saliva and their correlation with the presence of anti-*Leishmania* antibodies in stray cats naturally exposed to phlebotomine sand flies in Spain. While data suggest the potential utilization of stray cat populations as sentinel animals for the prevalence of *L. infantum* in endemic areas, further investigations are needed to characterize other salivary antigens of *P. perniciosus* recognized by cats bitten by this sand fly species and ascertain the utility of detecting anti-*P. perniciosus* saliva antibodies as an epidemiological biomarker.

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Data is available on request from the authors.

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