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Importance of individual analysis of environmental and climatic factors affecting the density of *Leishmania* vectors living in the same geographical area: the example of *Phlebotomus ariasi* and *P. perniciosus* in northeast Spain

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Abstract. The aim of the present study was to determine the role of specific environmental and climatic factors affecting the distribution and density of *Phlebotomus ariasi* and *P. perniciosus*, the proven vectors for *Leishmania infantum* in Spain. An entomological study was carried out in July 2006 in the province of Lleida with sticky traps set in their diurnal resting places at altitudes ranging from 86 to 1,755 m above the mean sea level (339 sites were sampled). Bivariate analysis revealed that factors such as altitude, bioclimatic zone, temperature, precipitation, sampling site (site relative to settlement, site situation, site category), wall vegetation, particular environment (in this case a natural park), general environment, adjacent natural vegetation and land cover were significantly associated with sand fly densities. The multivariate model for *P. perniciosus* revealed that its density was affected by site and land cover. Specifically, paved driveways correlated negatively with vector density (Incidence Risk Ratio (IRR): 0.41) and arable land cover correlated positively (IRR: 4.59). In the case of *P. ariasi*, a significant correlation was observed with the altitude and bioclimatic zone, with density increasing at >800 m above the mean sea level (IRR: 3.40) and decreasing in the meso-Mediterranean bioclimatic zone (IRR: 0.08). Both species were mostly found in agricultural and forest areas far from domestic environments. However, the two species correlated differently with altitude, bio-climate, vegetation, temperature and precipitation, which emphasises the importance of their individual analysis in studies regarding risk of leishmaniasis transmission.

Keywords: *Phlebotomus ariasi*, *Phlebotomus perniciosus*, environmental factors, climatic factors, leishmaniasis, Spain.

Introduction

Leishmaniasis, one of the world's most neglected diseases (WHO, 2010), is a parasitic disease affecting man and other mammals. It is transmitted by the bite of female phlebotomine sand flies (Molina et al., 1994; Morillas et al., 1996; Killick-Kendrick, 1999; Ashford, 2000; Gállego, 2004). In Mediterranean European foci, leishmaniasis is caused by *Leishmania infantum*, and the domestic dog is the main reservoir host. Proven vectors are *Phlebotomus* sand flies belonging to the subgenus *Larroussius*: *P. ariasi*, *P. perniciosus*, *P. neglectus*, *P. perfiliewi* and *P. tobbi* (Killick-Kendrick, 1999; Ready, 2010; WHO, 2010; Maroli et al., 2013). In Spain the vectorial capacity has been demonstrated for two species, *P. ariasi* and *P. perniciosus* (Rioux et al., 1986; Morillas et al., 1996; Portús et al., 2007). Until the 1980s, it was suspected that only one species was responsible for *Leishmania* transmission in a specific focus, but in Spain and Portugal it has been demonstrated that *P. perniciosus* and *P. ariasi* share the vectorial role in the same geographical areas (Pires, 1984; Rioux et al., 1986).

Studies carried out on the richness of sand fly species in Spain have identified 10 species of the *Phlebotomus* genus and two of *Sergentomyia*. One species is endemic to the Canary Islands (*P. fortunatarum*), where *S. fallax* has also been found, three are present only in the southern parts of the Iberian Peninsula (*P. alexan-*

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dri, *P. chabaudi*, *P. riouxi*), one is found in the center and the north (*P. langeroni*), while one is exclusive to the northeast (*P. mascittii*) (Gil Collado et al., 1989; Gállego Berenguer et al., 1992; Depaquit et al., 1998; Pesson et al., 2004; Barón et al., 2008). The others (*P. papatasi*, *P. ariasi*, *P. perniciosus*, *P. sergenti*, *S. minuta*) have the widest distribution. This focalised geographical distribution has been related with climatic and environmental characteristics, but only a few studies have investigated in-depth the influence of such variables on the local distribution of sand flies (Gálvez et al., 2010a; Barón et al., 2011).

Anthropic factors, including demographic pressure, urbanisation and exploitation of land for agriculture, have been thought to affect leishmaniasis distribution (Desjeux, 2001; Gállego, 2004; Colwell et al., 2011). The opportunistic character of *L. infantum* and co-infections with the human immunodeficiency virus (HIV) have influenced the re-emergence of the disease (Ashford, 2000). Also, changes in precipitation and temperature regimes related to climate change are thought to have impacted its distribution (de La Rocque et al., 2008; Ready, 2010; WHO, 2010; Maroli et al., 2013). Long-term climate change could lead to suitable conditions for the occurrence of sand fly species in areas previously free of them (Kuhn, 1999; Aspöck et al., 2008; Ready, 2010) and for the expansion of leishmaniasis and its sand fly vectors (Rispail et al., 2002; Maroli et al., 2008; Ready, 2008; WHO, 2010).

The presence of vectors in areas currently free of leishmaniasis is considered to pose a significant risk for the emergence of the disease in temperate Europe (Ready, 2010). Nevertheless, few studies have been published regarding the factors influencing the development of the *L. infantum* life cycle in proven vectors such as *P. ariasi* (Rioux et al., 1985). However, environmental and climatic factors affecting the presence and density of vectors have been reported recently (Gálvez et al., 2010a; Barón et al., 2011; Ozbel et al., 2011).

In recent years, an increase in canine leishmaniasis has been observed in central and southern Spain (Martin-Sánchez et al., 2009; Gálvez et al., 2010b) as well as the presence of the disease also in the north (Amusategui et al., 2004; Miró et al., 2012), including the Pyrenean region of Lleida province (Ballart et al., 2013). However, only one partial entomological survey has been carried out in the latter area (Gállego et al., 1990) and no data on the factors influencing the distribution of leishmaniasis vectors in this region are available. The aim of the present study was to determine the roles of specific environmental and climatic factors in the density and distribution of sand fly species, particularly the leishmaniasis vectors and consequently the possible spread of the disease in Lleida province.

Materials and methods

Study area

The province of Lleida is situated in the northeast of Spain (Catalonia) bordering Andorra and France in the north. It covers 12,173 km^2 and had a population of 470,496 inhabitants in 2006 (IDESCAT Institut d'Estadística de Catalunya; http://www.idescat.cat). The area is is divided into 13 administrative divisions (counties) and delimited by four fixed corners situated at 42° 51.7' N, 0° 42.5' E; 41° 16.5' N, 0° 25.5' E; 42° 18.4' N, 1° 50.6' E; and 41° 40.3' N, 0° 19.3' E (Fig. 1). The altitude ranges widely from just over the mean sea level (MSL) to 3,143 m, which results in notable differences in variables like temperature, rainfall, vegetation, etc. and thus covers a great variety of potential sand fly habitats. As further seen in Fig. 1, the climate varies from Mediterranean, high-mountain weather in the north to continental in the central depression and is defined by six bioclimatic zones: meso-Mediterranean and supra-Mediterranean in the Ebro basin (Mediterranean region) and montane, subalpine, alpine and coline in the Pyrenees (Medioeuropean region) (Rivas-Martínez, 1983).

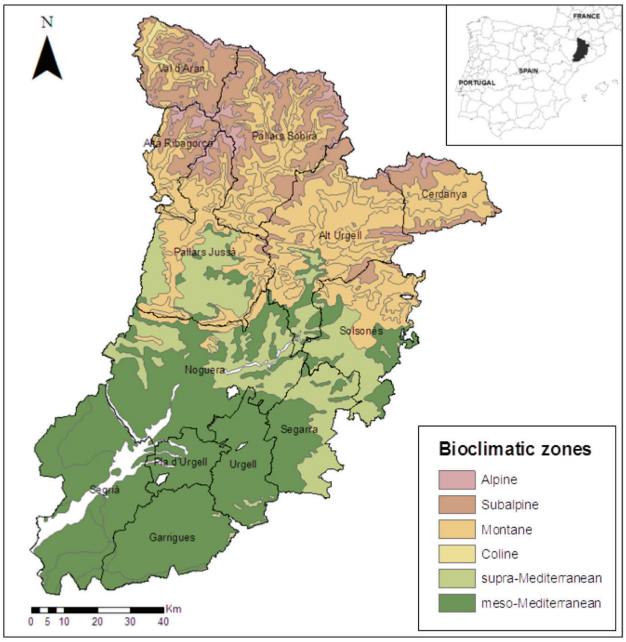

Fig. 1. The Lleida province: counties and bioclimatic zones.

Table 1. Baseline information for the different counties of Lleida province.

County	Points surveyed	Traps recovered	Adhesion surface (m^2)	Altitude range (m)	Geographical coordinates
Alt Urgell	42	535	42.8	400-1,700	42º 21.5’ N; 1º 27.8’ E
Alta Ribagorça	13	187	15.0	800-1,600	42º 24.6’ N; 0º 44.6’ E
Garrigues	35	442	35.4	200-800	41º 31.3’ N; 0º 52.2’ E
La Cerdanya	2	40	3.2	900-1,000	42º 26.0’ N; 1º 55.7’ E
Noguera	30	280	22.4	200-1,000	41º 47.5’ N; 0º 48.5’ E
Pallars Jussà	33	484	38.7	300-1,500	42º 10.1’ N; 0º 53.8’ E
Pallars Sobirà	43	529	42.3	500-1,400	42º 24.7’ N; 1º 07.9’ E
Pla d’Urgell	1	14	1.1	200-300	41º 37.9’ N; 0º 53.8’ E
Segarra	36	392	31.4	300-800	41º 40.2’ N; 1º 16.4’ E
Segrià	24	275	22.0	0-500	41º 37.2’ N; 0º 37.6’ E
Solsonès	31	287	23.0	600-1,400	41º 59.8’ N; 1º 31.3’ E
Urgell	28	379	30.3	200-700	41º 39.0’ N; 1º 08.5’ E
Vall d’Aran	21	256	20.5	600-1,800	42º 42.2’ N; 0º 47.8’ E
Total	339	4,100	328.0	0-1,800	-

Sand fly collection and identification

A cross-sectional study was carried out in July 2006. Sand flies were captured using sticky traps (20 x 20 cm sheets of paper covered with castor oil) set in their diurnal resting places (Rioux et al., 1969) and recovered after 4 days. Due to the extent and physical characteristics of the region, the traps were positioned along transects following the main roads in order to cover as much territory as possible (Rioux et al., 1969). A total of 4,100 sticky traps were recovered from 339 stations (Table 1). The specimens were removed with a brush and fixed in 95º ethanol for 2 days in order to dissolve the oil and were then definitively conserved in 70º ethanol until identification on the basis of morphological characters according to Gállego Berenguer et al. (1992). Males of all species and *Sergentomyia minuta* females were identified with a stereo microscope and *Phlebotomus* females were mounted in Hoyer’s medium and identified with an optical microscope.

Environmental and meteorological variables

The characteristics of the stations were recorded in a PDA (Palm Tungsten T5) using Pendragon Form version 5.0 software (PSC; Libertyville, USA) and a global positioning system (GPS) (Tom Tom Wireless GPS MK II) (Table 2). Maps were designed using ArcGIS version 9.2 software (ESRI; Redlands, USA). Temperature and rainfall data were provided by the *Servei Meteorològic de Catalunya* (MeteoCat) from the 40 meteorological stations existing in the area. Data from the closest meteorological station were assigned for each sampling site using the ArcGis spatial join-and-relate tool and included temperature and precipitation mean values for different periods (see the appendix). Precipitation for the sampling period (day-1 to day-4) was introduced as a dichotomous variable (presence/absence). Altitude data for each geocoded collection site were extracted from a 90 m resolution CGIAR Digital Elevation Model (Jarvis et al., 2008). In the same way, a 2.5 m resolution CORINE shape from the CNIG (*Centro Nacional de Información Geográfica*) was used to extract the land cover as well as the bioclimatic zone associated with each sampling site (Rivas Martínez, 1983).

The total set of variables considered in relation to location, collection method, habitat, environment and fauna are listed in Table 2.

Statistical analysis

The abundance of sand fly species was estimated as densities (number of sand flies per m^2 trap) (Gálvez et al., 2010a; Ozbel et al., 2011). The factors and categories considered are shown in the appendix. The effect of different variables on the density of *P. perniciosus* and *P. ariasi* was assessed by generalised linear models based on negative binomial distribution. All computations were performed using R free software (R Development Core Team, 2012) and the function *glm.nb*, which is available in the MASS package. The exponential transformation of estimates provided by these models can be understood as an incidence risk ratio (IRR) as was discussed and interpreted in a previous study (Gálvez et al., 2010a). Thus, the IRR approach derived from our models describes how like-

Table 2. Sampling site variables.

Location			
Site		Closest settlement	
Altitude		Road	
Altitude method		Distance to settlement	
Comments on location			
Collection			
Collection method		Traps recovered	
Traps set		Date/time recovered	
Date/time set		Traps on ground	
Comments on collection		Wetness of paper	
		Holes in paper	
		Snails	
		Still sticky	
Habitat			
Site relative to settlement		Wall construction	
Situation of site		Hole construction	
Site category		Hole interior	
Aspect		Wetness of hole	
Slope		Vegetation on wall	
Sheltered		Well present	
Water course		Refuse bin present	
Water flowing		Comments on habitat	
Environment			
Natural park		Adjacent land cover (predominant)	
General environment		Adjacent land cover (second predominant)	
Nearby natural vegetation (100 m-1 km)		Adjacent arable (predominant)	
Nearby natural vegetation (0-100 m)		Adjacent arable (second predominant)	
Adjacent natural vegetation (100 m-1 km)		Adjacent garden (predominant)	
Adjacent natural vegetation (0-100 m)		Adjacent garden (second predominant)	
Fauna			
Dogs	Cattle	Goats	Ducks
Cats	Sheep	Rabbits	Pigeons/Doves
Equines	Pigs	Chickens	Other large birds

ly it is to find the vector in a given factor level compared to the reference level. For continuous variables, the IRR was interpreted in the same way but here the risk changes per unit of that variable, e.g. if an IRR equal to 1.2 is found for altitude (in meters), it means that the chances of finding the vector increases by 20% per meter. In contrast, if a factor has two levels, an IRR equal to 1.2 means that the chances of finding the vector increases by 20% in level two compared to level one. Bivariate models were computed for a set of key variables, both numerical and categorical. The statistical significance of exponential coefficients was evaluated using two tests: the probability that each coefficient differs from zero was computed through a z-statistic (McCullagh and Nelder, 1989), while a likelihood-ratio test (through a χ^2 statistic) was performed to assess if the coefficients from a given model were different from those from the null model or, equivalently, to assess whether at least one of the estimated exponential coefficients differed from zero (McCullagh and Nelder, 1989).

Variables resulting in p-values of <0.2 in the bivariate analysis were selected to fit a multivariate model for each vector (Gálvez et al., 2010a; Ballart et al., 2013). In the case of closely correlated variables (temperature and precipitation) that could potentially create interaction or confusion in the multivariate analysis, we retained those from the sampling period (mean daily temperature and daily precipitation of Sampling Day 1 (traps set) to Day 4 (traps recovered)). The variables resulting in P-values of ≤0.05 were considered statistically significant.

Table 3. Baseline information for sand flies captured in Lleida province.

Species	Number of flies	Sex ratio[a]	Relative abundance[b]	Relative frequency[c]	Density[d]
Phlebotomus ariasi	330	1:3	2.6	22.7	1.0
Phlebotomus perniciosus	1,491	1:3	11.7	40.7	4.5
Phlebotomus sergenti	52	1:2	0.4	4.7	0.2
Phlebotomus papatasi	253	1:1	2.0	11.5	0.8
Sergentomyia minuta	10,651	1:1	83.4	47.2	32.5
Total	12,777	1:1	-	-	39.0

[a] Female to male; [b] relative number of species related to the total number of sand flies captured (%); [c] relative number of positive stations related to the total number of stations sampled (%); [d] number of specimens captured per m² sticky trap.

Results

Descriptive analysis

A total of 12,777 sand fly specimens were captured, out of which material five species were identified. Information on the number of specimens captured, sex ratio, relative abundance, frequency and density are provided in Table 3. In the present study, the leishmaniasis vectors *P. ariasi* and *P. perniciosus* were captured in 10 and 11, respectively, out of the 13 administrative divisions of the province. *P. ariasi* was present at altitudes from 273 to 1,620 m above the MSL, and *P. perniciosus* from 94 to 1,630 m above the MSL (Fig. 2). In the present study, *P. perniciosus* was detected in 138 sampling sites (41%) with a density of 4.5 specimens/m², while *P. ariasi* was present in 77 (23%) showing the low density of only 1 specimen/m².

Bivariate analysis

The results obtained in the bivariate analysis of factors associated with *P. perniciosus* and *P. ariasi* densities are shown in the appendix. Statistically significant results (P ≤0.05) were found for both species, which were affected differently by the factors. The density of *P. perniciosus* was positively correlated with the following variables: bioclimatic zones, temperature, site relative to settlement, situation of sites, site category and adjacent natural vegetation. A negative correlation was found for: altitude, precipitation and the fact of being a natural park environment. The density of *P. ariasi* was positively correlated with: altitude, precipitation, site relative to settlement, wall vegetation, the fact of being a natural park environment, general environment, adjacent natural vegetation and the corine land cover. The density was negatively correlated with bioclimatic zones and temperature.

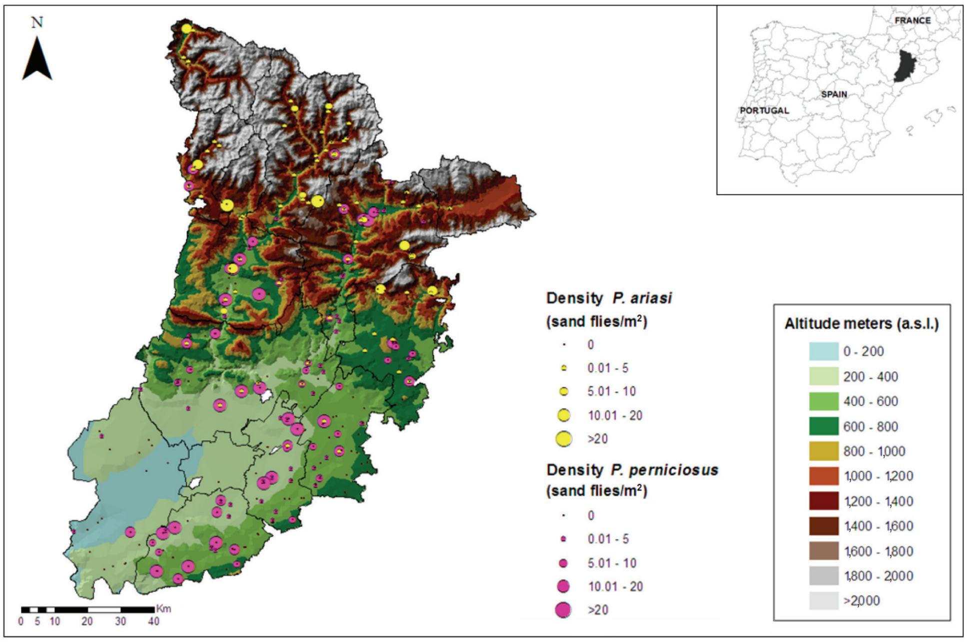

Fig. 2. Density of *P. ariasi* and *P. perniciosus* at different altitudinal ranges in the counties of Lleida province.

Multivariate analysis

The final model for *P. perniciosus* revealed that the site situation and corine land cover affect vector density, specifically paved driveway correlated negatively (IRR: 0.41) and arable land cover correlated positively (IRR: 4.59) (Table 4). In the case of *P. ariasi*, the altitude and bioclimatic zone displayed a significant correlation with vector density increasing over 800 m (IRR: 3.40) and decreasing in the meso-Mediterranean bioclimatic zone (IRR: 0.08) (Table 4).

Discussion

The motivation to extend the entomological survey in 1987 by Gállego et al. (1990) to cover the whole province is the lack of subsequent studies after 1987 despite human leishmaniasis cases appearing in the official epidemiological bulletins (http://gencat.cat/salut/depsalut; Ballart et al., 2012a). In the present study, despite the increased number of sampling sites, we did not come across any species not previously found there. As in other Spanish areas, the species captured in the greatest number was *S. minuta* followed by the vector *P. perniciosus* (Martínez-Ortega, 1985; Morillas et al., 1996; Gálvez et al., 2010a). These two ubiquitous species are found in a wide variety of zones ranging from semi-arid to sub-humid (Martínez Ortega, 1985; Gállego et al., 1990; Aransay et al., 2004; Rioux, 2006) and from up to 1,500 m above MSL (Ballart et al., 2012b), which would explain the high degree of detection in our study. Nevertheless, it should be taken into account that results can be influenced by the method of capture, especially in the case of *P. ariasi*, the other *L. infantum* vector in the Iberian Peninsula that, due to its phototropism, shows a higher abundance when light traps, as CDC traps from the Centers for Disease Control and Prevention (CDC) in the US, are used (Gállego Berenguer et al., 1992; Portús et al., 2007). Of the other two species, *P. papatasi* has an affinity for per-arid and arid climates, which are present throughout the mesogen area of the Palaearctic region, while *P. sergenti* prefers semi-arid and arid zones of the Mediterranean regions (Gállego et al., 1990; Aransay et al., 2004; Boussaa et al., 2010).

In general, the use of sticky traps influences not only the relative abundance of different species captured, but also the sex ratio providing more *Phlebotomus* males than females (Gállego Berenguer et al., 1992; Boussaa et al., 2010) or the opposite in the case of *S. minuta*, which is probably due to its feeding (her-

Table 4. Estimates of the multivariate linear model based on the negative binomial distribution.

Variable	Multivariate analysis		
	IRR[a]	(95% CI)[b]	P-value
Phlebotomus perniciosus			
Situation of site			
Paved road	Ref.[c]		
Paved drive	0.41	(0.19-0.88)	0.022
Unpaved track	1.32	(0.58-2.99)	0.492
Other	0.89	(0.42-1.92)	0.766
Corine land cover			
Rural, industrial or commercial area	Ref.[c]		
Arable	4.59	(1.79-11.76)	0.001
Pasture	3.58	(0.87-14.63)	0.075
Garrigue shrubs	3.78	(0.95-14.95)	0.057
Forestry	1.62	(0.39-6.69)	0.502
Phlebotomus ariasi			
Altitude			
<800m	Ref.[c]		
≥800m	3.40	(1.15-10.05)	0.026
Bioclimatic zones			
Other	Ref.[c]		
Supra-Mediterranean	1.12	(0.35-3.57)	0.844
Meso-Mediterranean	0.08	(0.02-0.39)	0.001

[a]Risk ratio; [b]95% confidence intervals; [c]reference category.
Variables with a P-value ≤0.05 were considered significant.

petophilic) and breeding habits (Martínez Ortega, 1985; Gil Collado et al., 1989; Gállego Berenguer et al. 1992; Boussaa et al., 2010; Gálvez et al., 2010a).

The results obtained show that the two vector species of *L. infantum* are present in Lleida province. Neither species was found in the Pla d'Urgell county, where it was only possible to sample one station. *P. ariasi* was not present in the southern counties (Garrigues and Segrià), probably because this species mainly inhabits humid or sub-humid zones with cold winters (Martínez Ortega, 1985; Gállego et al., 1990; Rispail et al., 2002; Aransay et al., 2004; Rioux, 2006), while *P. perniciosus* was not found in Vall d'Aran, which is the coldest and wettest county of Lleida province.

Several studies have recently reported the influence of environmental and climatic conditions on the distribution of leishmaniasis vectors (Rossi et al., 2007; Gálvez et al., 2010a; Ozbel et al., 2011; Queiroz et al., 2012; Branco et al., 2013). This study is the first to show the effect of these variables separately on *P. ariasi* and *P. perniciosus* living in sympatric conditions when captured with sticky traps in a given region showing a great variation of environmental conditions. As could be observed in the bivariate analysis, the only factors to influence the density and distribution of both vectors were site relative to settlement, general environment and corine land cover, while the other variables affected the two species differently.

P. perniciosus showed a wide altitudinal range and though present in all the bioclimatic zones was found mainly <800 m above MSL, showing a preference for meso-Mediterranean and supra-Mediterranean bioclimates (70% of the positive stations were in the Mediterranean region), as reflected in the bivariate analysis (see the appendix). In contrast, *P. ariasi*, which was present at altitudinal ranges similar to *P. perniciosus*, was found >800 m above MSL and in coline, subalpine and montane bioclimates (73% of the positive stations were in the Medioeuropean region). In the multivariate analysis (see Table 4), only altitude and bioclimatic zones were significantly correlated with *P. ariasi*, whose density increased 3.4-fold >800 m above MSL (and decreased 0.08-fold at the meso-Mediterranean level). Since other researchers have studied both species together and in bioclimatic zones that do not exactly match those of our study, no data are available for comparison (Gálvez et al., 2010a; Barón et al., 2011). It is generally accepted that altitude and bioclimate, two closely related variables, have an influence on sand fly distribution (Ferreira et al., 2001; Guernaoui et al., 2006; Gálvez et al., 2010a; Barón et al., 2011; Ballart et al., 2012b), and that the two Spanish leishmaniasis vectors are found along a climatic gradient ranging from humid to semi-arid (de la Rocque et al., 2008). Yet, we were able to demonstrate that the vector correlation with altitude and bioclimate differed according to whether species preference was for humid or semi-arid zones.

The variables of altitude and bioclimate are closely correlated with changes in vegetation and meteorological conditions (Elnaiem et al., 1998; Guernaoui et al., 2006; Ozbel et al., 2011). The influence of temperature has been considered not only on the biology and ecology of sand flies (rate of egg production, development of the juvenile stages, number of annual generations, feeding activity, period of activity and survival of adults), but also on the development of the parasite inside the vector (Rioux et al., 1969; Elnaiem et al., 1998; Ready, 2008; Martín Sánchez et al., 2009; Hartemink et al., 2011). Rainfall has been associated with the activity period of sand flies, particularly in some South American foci (Queiroz et al., 2012; Quintana et al., 2012). In the present study, both species were found in a similar range of climatic conditions. Thus, for example, *P. perniciosus* was captured at mean daily temperatures (sampling day-1 to sampling day-4) in a range of 12.5-28.3 °C and *P. ariasi* at 11.4-28.0 °C. In spite of this, the two vectors differed in their correlation with meteorological variables (temperature and rainfall). In the bivariate analysis, *P. perniciosus* increased significantly with the different temperature measures considered (see appendix) and correlated negatively with monthly and annual rainfall measures, while the opposite occurred with *P. ariasi*. The lowest temperature for *P. ariasi* activity should be about 15 °C (Ashford and Bettini, 1986) and its optimal nocturnal temperature 19-21 °C (Rioux et al., 1969), but no published data exist for *P. perniciosus*. In our study, we found both species at lower temperatures (mean daily temperatures of 12.5 °C and 11.4 °C, respectively). However, regardless of species, none of these variables were found to be statistically significant in the multivariate analysis.

It is assumed that in temperate regions, higher temperatures may shorten larval development and extend the breeding season of existing sand flies or allow new species to become established where low temperatures have hitherto prevented their over-wintering (Maroli et al., 2008), while declining autumn temperatures, together with the shortening day, induce diapauses of fourth instar larvae (Ready and Croset, 1980). Nevertheless, conflicting results have been obtained by different authors on the influence of temperature on the presence, abundance or density of sand flies, depending on the species analysed or the bioclimatic area considered (Martín Sánchez et al., 2009; Chamaillé et al., 2010; Gálvez et al., 2010a). These results indicate that temperature is not the only explanation for sand fly distribution and density (Gállego, 2004; Gage et al., 2008; Colwell et al., 2011).

In the bivariate analysis, significantly higher densities of both vector species were found in the areas between villages, and in the case of *P. perniciosus*, also at the village edge, which corresponds with other studies carried out in Spain (Gálvez et al., 2010a; Barón et al., 2011). The increased presence of vectors between villages could be favoured by the higher availability of oviposition and resting sites as well as the presence of a greater variety of animals constituting potential blood meals, considering the opportunistic feeding behaviour of both species (Guy et al., 1984; de Colmenares et al., 1995; Rossi et al., 2008). In fact, in the Lleida Pyrenees area, we have found a high percentage of *Leishmania* seropositive dogs among the canine population living in conditions favourable for transmission (rural dogs, living in kennels and sleeping outdoors) (Ballart et al., 2013).

As shown in the bivariate and multivariate analysis, *P. perniciosus* was found mainly on unpaved tracks and in places such as farm properties or gardens, rather than on paved roads and driveways, which implies a preference for quiet places with food facilities, as observed by Gálvez et al. (2010a). This result corresponds with the maximum detection of *P. perniciosus* in farm buildings in a rural environment far away from villages. No significant differences were found in the case of *P. ariasi*, even if this species was also found preferentially far away from villages. This result could be related to the positive correlation of this species with natural parks, unlike *P. perniciosus*, probably due to a preference for more natural environments and exophilic requirements. The two species also showed different positive correlation with flora: *P. ariasi* with mountain pine and oak, while *P. perniciosus* preferred Aleppo pine and garrigue shrubs. This is related to their altitudinal and bioclimatic distribution, which has been described for the South of France (Rioux et al., 1969; Hartemink et al., 2011). Sand fly abundance prediction maps showing a different predominance of both species according to altitude and vegetation have been obtained in southern France, *P. ariasi* being predominant in the forested foothills and *P. perniciosus* at lower altitudes (Hartemink et al., 2011).

Other variables such as wall and hole constructions and hole interior conditions did not seem to affect the density of these vector species, in agreement with other authors (Gálvez et al., 2010a). Nevertheless, Barón et al. (2011) proposed PVC piping holes as a feasible control measure against leishmaniasis due to the decreased finding of *P. perniciosus* in this kind of hole construction. Unlike other reports (Coleman et al., 2007; Gálvez et al., 2010a), we found the aspect and shelter conditions of the sampling site not to be correlated with vector density. In contrast to other studies in Spain or Morocco, which found the greatest densities of these sand flies in places where livestock or birds are present (Guernaoui and Boumezzough, 2009; Gálvez et al., 2010a; Barón et al., 2011), we did not find the presence of animals associated with vector density. The presence of dogs did not favour the presence of either vector species as observed by Gálvez et al. (2010a) and Barón et al. (2011), which is surprising considering the epidemiology of the disease caused by *L. infantum* with the dog acting as a reservoir in a domestic zoonotic cycle. However, the opportunistic trophic behaviour of the vectors may have influenced these results (Guy et al., 1984; de Colmenares et al., 1995; Rossi et al., 2008).

The incrimination of a potential leishmaniasis vector in a given area has usually involved the species present in the highest density (Martínez-Ortega, 1985). Studies in Spain have concluded that the potential vector of *L. infantum* in this country is *P. perniciosus*. Densities of 4-6 specimens/m^2 are considered to imply an increased risk of leishmaniasis emergence in France and Spain (Rioux et al. 1977; Martín-Sánchez et al., 2009). In the present study, *P. perniciosus* showed a higher density than *P. ariasi* and reached the minimal density considered a risk for transmission. Nevertheless, results regarding sand fly composition species may vary depending on the sampling techniques used (Gállego Berenguer et al., 1992) and also studies on the presence of kinetoplastida in sand flies have demonstrated the vectorial capacity of *P. ariasi* populations with low densities (Rioux et al., 1986; Lucientes et al., 1988; Guilvard et al., 1996; Morillas et al., 1996). We found the two vector species together in 46 sampling sites (14%) where they could be acting in the same geographical area in the transmission of the disease, as observed in other regions of Portugal and Spain (Pires, 1984; Rioux et al., 1986; Lucientes et al., 1988).

Leishmaniasis is a climate-sensitive disease affected by changes in rainfall, atmospheric temperature and humidity, which can strongly impact on the ecology of vectors and reservoir hosts by altering their distribution and influencing their activity, survival and population sizes (Elnaiem et al., 1998; Killick Kendrick, 1999; Aspöck et al., 2008; Gage et al., 2008; Ready, 2010). In the Mediterranean region, most leishmaniasis foci are between 5 °C and 10 °C in January isotherms and between 20 °C and 30 °C in July (Ashford and Bettini, 1986). Relatively small changes in temperature can have a considerable effect on vectorial capacity, due to alterations in the frequency of bloodmeals (Ready, 2010) and the development cycle of *Leishmania* promastigotes in sand flies (Rioux et al., 1985). Precipitation determines sand fly survival and promotes adult emergence and appropriate oviposition sites (Gage et al., 2008). In this study, *P. ariasi* and *P. perniciosus* differed in their correlation with temperature, precipitation, altitudinal and bioclimatic zones and vegetation. While *P. perniciosus* is considered the principal potential vector of leishmaniasis in the southern area of Lleida, *P. ariasi* could play this role in the northern, Pyrenean region. In fact, we have previously found *L. infantum* DNA in *P. ariasi* specimens (Alcover et al., 2012) and demonstrated the existence of an autochthonous focus of canine leishmaniasis in this area (Ballart et al., 2012a, 2013).

Global climate change could alter the dynamics of leishmaniasis transmission, leading to variation in distribution, emergence or re-emergence. It would thus be of interest to analyse how the present results on vector distribution and density could be affected by climate change, considering that Spain is undergoing trend of increasing temperatures (3.7 °C/100 years) based on the 1980-2006 period (Brunet et al., 2009). A related issue that needs to beaddressed is the hypothesis of an extension of *P. perniciosus* towards northern areas as this region aquires more tolerable summer temperatures, while it undergoes a decline in the South (Kuhn, 1999). There could also be an increase in the activity period and vectorial competence of *P. ariasi* in northern areas. Observations in that direction have been made in the centre and southern parts of Spain (Martín Sánchez et al., 2009; Gálvez et al., 2010a). This study could be the basis of future research on mapping the establishment risk for emerging leishmaniasis in the study area as has been done in Europe and South America (Hartemink et al., 2011; Foley et al., 2012).

Conclusions

The bivariate analysis provided information about the risk factors affecting the density of *P. ariasi* and *P. perniciosus*. Both species were found mainly in agricultural areas and forests far from the domestic environment. However, the two species correlated differently with altitude, bioclimate, vegetation, temperature and precipitation, which emphasises the importance of their individual analysis with regard to the risk of leishmaniasis transmission. A constant monitoring of sand fly vectors in new leishmaniasis foci is crucial for evaluating the geographical expansion of the disease. Studies on the parasitism of these species are required to gain more insight into their involvement in the transmission of *L. infantum* in Lleida province.

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Appendix

Factors studied in the cross-sectional study and results obtained with bivariate analysis.

Variable	Sampling site		*P. perniciosus*		*P. ariasi*	
	Number of sampling stations	Percent of stations	IRR[a] (95%CI)[b]	P-value	IRR[a] (95%CI)[b]	P-value
Altitude (m)[c]	700 (86-1,755)	438-949*	0.99 (0.99-0.99)	<0.001	1.00 (1.002-1.003)	<0.001
Altitude (m)						
<800	221	65%	Ref. category		Ref. category	
≥800	118	35%	0.21 (0.12-0.37)	<0.001	4.98 (2.67-9.28)	<0.001
Bioclimatic zone						
Coline, montane, subalpine	142	42%	Ref. category		Ref. category	
Supra-Mediterranean	76	22%	2.90 (1.44-5.85)	0.003	0.43 (0.22-0.87)	0.019
Meso-Mediterranean	121	36%	3.93 (2.13-7.25)	<0.001	0.02 (0.01-0.07)	<0.001
Mean daily temperature (ºC)[c]						
Sampling day-1 (traps set)	22.3 (11.4-28.3)	5.1**	1.18 (1.12-1.25)	<0.001	0.89 (0.84-0.94)	<0.001
to day-4 (traps recovered)	21.1 (11.1-27.7)	5.2**	1.18 (1.12-1.25)	<0.001	0.89 (0.84-0.94)	<0.001
Mean monthly temperature (ºC)[c]						
(The month before sampling day-1)	10.4 (2.0-15.0)	4.5**	1.24 (1.16-1.31)	<0.001	0.87 (0.81-0.93)	<0.001
Mean annual temperature (ºC)[c]						
(The year before sampling day-1)	-2.7 (-7.3-0.3)	-2.4**	1.33 (1.19-1.49)	<0.001	0.72 (0.64-0.81)	<0.001
Mean minimal winter temp. (ºC)[c]						
Daily precipitation						
Absent	136	40%	Ref. category		Ref. category	
Present	203	60%	1.11 (0.62-1.95)	0.730	1.46 (0.74-2.86)	0.265
Mean monthly precipitation (mm)[c]						
(The month before sampling day-1)	1.7 (0.03-5.8)	1.6**	0.69 (0.58-0.82)	<0.001	1.88 (1.56-2.27)	<0.001
Mean annual precipitation (mm)[c]						
(The year before sampling day-1)	15.2 (5.2-34.1)	6.7**	0.89 (0.85-0.92)	<0.001	1.16 (1.11-1.21)	<0.001
Site relative to settlement						
Within	124	37%	Ref. category		Ref. category	
Edge	112	33%	3.59 (1.88-6.84)	<0.001	1.13 (0.52-2.46)	0.751
Between	103	30%	4.69 (2.43-9.06)	<0.001	3.58 (1.67-7.68)	0.001
Situation of site						
Paved road	169	50%	Ref. category		Ref. category	
Paved drive	60	18%	1.16 (0.54-2.47)	0.701	0.60 (0.24-1.48)	0.268
Unpaved track	44	13%	2.48 (1.06-5.79)	0.034	0.36 (0.12-1.03)	0.057
Other (garden, farm property, other)	66	19%	2.36 (1.14-4.88)	0.020	0.58 (0.24-1.38)	0.220
Site category						
Embankment	164	48%	Ref. category		Ref. category	
Wall	82	24%	1.07 (0.54-2.11)	0.841	0.70 (0.31-1.58)	0.394
Farm building	10	3%	6.06 (1.20-30.44)	0.028	0.05 (0.00-1.28)	0.070
Other	83	25%	1.32 (0.67-2.61)	0.411	0.72 (0.32-1.61)	0.425
Aspect						
Other (not applicable)	24	8%	Ref. category		Ref. category	
North-facing	93	27%	0.37 (0.11-1.18)	0.092	0.76 (0.18-3.09)	0.708
East-facing	31	9%	0.45 (0.11-1.78)	0.255	1.07 (0.20-5.58)	0.927
South-facing	156	46%	0.37 (0.12-1.13)	0.082	2.62 (0.70-9.81)	0.150
West-facing	35	10%	0.60 (0.15-2.27)	0.451	1.69 (0.34-8.20)	0.513

continued

Variable	Sampling site		*P. perniciosus*		*P. ariasi*	
	Number of sampling stations	Percent of stations	IRR[a] (95%CI)[b]	P-value	IRR[a] (95%CI)[b]	P-value
Sheltered						
Not sheltered	267	79%	Ref. category		Ref. category	
Sheltered	42	12%	0.81 (0.34-1.87)	0.609	0.64 (0.23-1.79)	0.399
Unsure	30	9%	0.54 (0.20-1.48)	0.236	0.65 (0.20-2.15)	0.487
Drain hole wall construction						
Other	39	12%	Ref. category		Ref. category	
Stone	272	80%	1.09 (0.35-3.39)	0.877	2.94 (0.84-7.39)	0.098
Brick	28	8%	1.10 (0.31-3.92)	0.880	1.41 (0.29-6.68)	0.662
Drain hole construction						
Other	7	2%	Ref. category		Ref. category	
Unlined	179	53%	3.67 (0.47-28.68)	0.212	8.60 (0.46-160.52)	0.148
Brick lined	23	7%	2.08 (0.21-20.55)	0.527	4.88 (0.20-114.54)	0.322
Cement pipe	61	18%	5.85 (0.70-48.75)	0.101	4.31 (0.21-86.30)	0.338
Plastic pipe	69	20%	3.87 (0.47-31.93)	0.206	3.97 (0.20-78.81)	0.363
Hole interior						
Other (bare and other)	10	3%	Ref. category		Ref. category	
Dust	264	78%	1.33 (0.25-8.88)	0.730	0.51 (0.07-3.34)	0.482
Dust with vegetation	36	11%	0.90 (0.18-6.89)	0.904	0.48 (0.05-3.95)	0.497
Soil with vegetation	29	8%	0.37 (0.05-2.48)	0.308	0.30 (0.03-2.65)	0.277
Wall vegetation						
No	266	78%	Ref. category		Ref. category	
Yes	73	22%	0.86 (0.43-1.69)	0.665	2.90 (1.36-6.15)	0.005
Natural park						
No	269	79%	Ref. category		Ref. category	
Yes	70	21%	0.10 (0.05-0.22)	<0.001	3.24 (1.52-6.90)	0.002
General environment						
Other settlement	44	13%	Ref. category		Ref. category	
Rural agriculture and forestry	106	31%	4.37 (1.77-10.74)	0.001	5.44 (1.83-16.17)	0.002
Rural village	189	56%	1.61 (0.69-3.76)	0.263	1.74 (0.61-4.69)	0.292
Adjacent natural vegetation						
None	31	9%	Ref. category		Ref. category	
Mountain pine	17	5%	0.40 (0.08-1.95)	0.527	7.43 (1.43-38.39)	0.016
Oak	86	26%	2.12 (0.75-6.01)	0.154	3.42 (1.02-11.41)	0.045
Aleppo pine	31	9%	5.20 (1.50-18.02)	0.009	1.29 (0.29-5.68)	0.727
Garrigue shrubs	119	35%	4.57 (1.68-12.43)	0.003	1.18 (0.36-3.88)	0.775
Valley alder	31	9%	1.14 (0.32-4.05)	0.837	2.92 (0.69-12.22)	0.140
Other	24	7%	0.87 (0.22-3.43)	0.844	0.07 (0.00-1.13)	0.061
Corine land cover						
Rural, industrial/commercial area	42	12%	Ref. category		Ref. category	
Arable	189	56%	5.47 (2.30-12.98)	<0.001	3.57 (1.08-11.72)	0.035
Pasture	37	11%	1.16 (0.37-3.67)	0.792	22.06 (5.68-89.88)	<0.001
Garrigue shrubs	27	8%	2.18 (0.63-7.50)	0.212	5.52 (1.21-25.15)	0.027
Forestry	44	13%	1.59 (0.53-4.73)	0.402	13.10 (3.39-50.53)	<0.001

continued

Variable	Sampling site		*P. perniciosus*		*P. ariasi*	
	Number of sampling stations	Percent of stations	IRR[a] (95%CI)[b]	P-value	IRR[a] (95%CI)[b]	P-value
Dogs						
Not seen	230	68%	Ref. category		Ref. category	
Seen	109	32%	1.41 (0.78-2.55)	0.252	0.81 (0.40-1-65)	0.576
Cats						
Not seen	291	86%	Ref. category		Ref. category	
Seen	48	14%	1.31 (0.58-2.89)	0.510	0.68 (0.26-1.77)	0.428
Farm animals[d]						
Not seen	265	78%	Ref. category		Ref. category	
Seen	74	2%	1.91 (0.98-3.70)	0.056	2.04 (0.94-4.41)	0.068
Birds[e]						
Not seen	273	81%	Ref. category		Ref. category	
Seen	66	19%	1.63 (0.81-3.28)	0.166	0.55 (0.23-1.29)	0.170

*Interquartile range (IQR); **Standard deviation (SD); [a]Incidence Risk Ratio; [b]95% Confidence Interval; [c]Variable used as continuous in the model (for these variables, number is substituted by mean (with minimum and maximum values in brackets) and % by the IQR or, alternatively, the SD; [d]Horses, cattle, sheep, pigs, goats; [e]Chickens, ducks, pigeons and other large birds. Variables with a P-value ≤0.05 were considered significant.