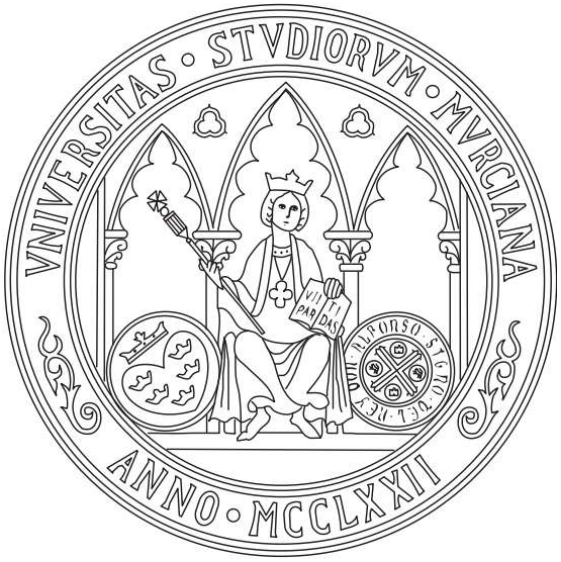

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Distribution, dispersion and blood-feeding preferences of *Phlebotomus* spp. (Diptera, Psychodidae) in microenvironments in southeast Spain: implications for transmission of *Leishmania infantum*

Distribución, dispersión y preferencias alimentarias de *Phlebotomus* spp. (Diptera, Psychodidae) en microambientes naturales en el sureste de España: implicaciones en la transmisión de *Leishmania infantum*

Dña. Clara Muñoz Hernández

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Memoria presentada por la Licenciada en Veterinaria

Clara Muñoz Hernández

Para optar al grado de Doctor en Ciencias Veterinarias con Mención Internacional

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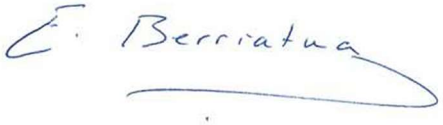
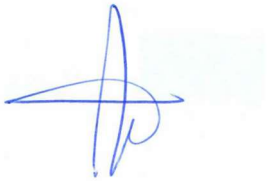
AUTORIZAN:

La presentación de la Tesis Doctoral titulada "Distribution, dispersion and blood-feeding preferences of *Phlebotomus* spp. (Diptera, Psychodidae) in microenvironments in southeast Spain: implications for transmission of *Leishmania infantum*" (Distribución, dispersión y preferencias alimentarias de *Phlebotomus* spp. (Diptera, Psychodidae) en microambientes naturales en el sureste de España: implicaciones en la transmisión de *Leishmania infantum*), realizada por Clara Muñoz Hernández, bajo nuestra supervisión y dirección, para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a veintiséis de julio de dos mil veinte.





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A mi familia

“Nada en la vida debe ser temido, solamente comprendido. Ahora es el momento de comprender más, para temer menos”

Marie Curie

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TABLE OF CONTENTS

SUMMARY **1**

RESUMEN **9**

GENERAL INTRODUCTION **17**

Introduction 19

Bibliographic review 22

Leishmaniasis 22

General context 22

Epidemiological situation of leishmaniasis in Spain 24

Human leishmaniasis 24

Canine leishmaniasis 25

Reservoirs of *Leishmania infantum* 25

Leishmania infantum in Murcia Region 27

Phlebotomine sand flies, vectors of *Leishmania* spp. 28

Sand fly taxonomy 28

Sand fly morphology 29

Sand fly biology and ecology, and vectorial competence of *Leishmania* spp. 30

Blood-feeding preferences of female sand flies 33

Sand fly species in Spain 36

Vector distribution and implications on leishmaniasis epidemiology 38

Entomological surveillance and quantitative studies of sand fly populations 39

References 40

OBJECTIVES **53**

CHAPTER 1: On how trap positioning affects phlebotomine sand fly density estimations **57**

Abstract 59

Introduction 59

Materials and methods 61

Results and discussion 63

References 70

CHAPTER 2: Investigations of *Phlebotomus perniciosus* sand flies in rural Spain reveal strongly aggregated and gender-specific spatial distributions and advocate use of light-attraction traps 73

Abstract 75

Introduction 75

Materials and methods 77

Results 81

Discussion 94

References 98

Supporting information 101

CHAPTER 3: Molecular xenomonitoring and host identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife park 105

Abstract 107

Introduction 107

Materials and methods 110

Results 116

Discussion 128

Conclusions 132

References 132

Supporting information 138

CHAPTER 4: A spatial ecology study in a high-diversity host community to understand blood-feeding behavior in *Phlebotomus* sand fly vectors of *Leishmania* 141

Abstract 143

Introduction 143

Materials and methods 146

Results 151

Discussion 155

Conclusions 158

References 159

GENERAL DISCUSSION 165

References 172

CONCLUSIONS 175

CONCLUSIONES 179

APPENDICES 183

Appendix 1. Sampling methodology and sand fly collection 185

Appendix 2. Phlebotomine sand fly species morphologically identified 186

Appendix 3. Scientific production from this doctoral thesis 188

INDEX OF FIGURES

GENERAL INTRODUCTION

Figure 1. Diagram of leishmaniasis epidemiology showing pathogen-host-vector-environment interactions involved in parasite transmission. Source: based on Oliveira *et al.* (2018) 20

Figure 2. Life cycle of *Leishmania* spp. that involves a biological vector (promastigote stage) and a vertebrate host (amastigote stage). Source: based on Esch and Petersen (2013) 22

Figure 3. Main morphological characters useful for sand fly species identification: head (A), external genitalia in males (B), spermatheca (C) and distal segments of abdomen (D) in females. Source: based on Depaquit and Léger (2017) 30

Figure 4. Sand fly life cycle: Egg - Larva (four instars) - Pupa - Adult. Source: based on Lawyer *et al.* (2017) 31

Figure 5. Percentage of identified bloodmeals in *P. perniciosus*, *P. ariasi* and *S. minuta*, from studies carried out in Spain (ESP), France (FRA), Italy (ITA) and Portugal (PRT), using molecular or serological techniques. Every bar corresponds to data of one study (first author, publication year, country and number of sand flies tested) 35

Figure 6. Distribution map of sand fly vectors of *L. infantum* in Spain: *P. ariasi*, *P. langeroni* and *P. perniciosus*. Source: based on Gil Collado *et al.* (1989), Gállego-Berenguer *et al.* (1992), González Peña (1994), Léger *et al.* (1995), Conesa Gallego *et al.* (1999), Lucientes *et al.* (2002), Aransay *et al.* (2004), Franco *et al.* (2010), Alcover *et al.* (2012), Durán-Martínez *et al.* (2013), Bravo-Barriga *et al.* (2016), Díaz Sáez *et al.* (2018), González *et al.* (2019), Gálvez *et al.* (2020) 37

CHAPTER 1: On how trap positioning affects phlebotomine sand fly density estimations

Figure 1. Depiction of trap location in the open-air dog kennel in southeast Spain using sticky (A-C) and CO2L traps (D-F). Sticky traps were set in parallel rows at the wire fence in 2015 (A) and in a stepwise rectangle arrangement at the wire fence (B), wooden smooth plank (B) and hung from a rope (C) in 2016. CO2L traps were placed in a stepwise arrangement at the wire fence in the two study years (D) and at the wooden smooth plank (E) and hung from a rope (F) in 2016 62

Figure 2. Sand fly species density in CO2L (sand flies/trap/night) and sticky traps (sand flies/m^2/night) according to trap location (rope, wire fence and wooden plank) and ground distance (15, 75 and 150 cm) 66

CHAPTER 2: Investigations of *Phlebotomus perniciosus* sand flies in rural Spain reveal strongly aggregated and gender-specific spatial distributions and advocate use of light-attraction traps

Figure 1. Locations of sand fly sampling premises and sites (A, B, C and D) in Murcia Region, southeastern Spain, used in the study of *P. perniciosus* distribution in 2016 79

Figure 2. Seasonal *P. perniciosus* sand fly distribution in 2016 in CDC light traps and mean indoor temperature and humidity at the times at which traps were put in place 89

CHAPTER 3: Molecular xenomonitoring and host identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife park

Figure 1. Geographical situation of Terra Natura Wildlife Park in Murcia city area in southeast Spain 110

Figure 2. Spatial distribution of sand fly sampling sites (A-H) (red points) and potential feeding host's location (yellow points). The latter represent areas within host's enclosures where they gathered at night (night grounds), when adult sand flies are most active..... 112

Figure 3. Degree of blood digestion in the sand fly gut. Engorged females were classified into five categories. Category 1 was considered as those bloodmeals with least digested, fresh, bright red blood occupying the whole abdomen and category 5 as those with the most digested blood 113

Figure 4. Spatial sand fly distribution according to the species frequency. Other species includes *P. papatasi*, *P. ariasi*, *P. sergenti* and *S. minuta*. Circle dimensions are proportional to sand fly abundance 117

Figure 5. Spatial sand fly distribution according to the gender. Circle dimensions are proportional to sand fly abundance..... 118

Figure 6. Spatial distribution of unfed, blood-fed and gravid female sand flies. Circle dimensions are proportional to sand fly abundance 119

CHAPTER 4: A spatial ecology study in a high-diversity host community to understand blood-feeding behavior in *Phlebotomus* sand fly vectors of *Leishmania*

Figure 1. Trap and host locations and sand fly feeding trajectories (represented as straight red lines at widths proportional to the number of specimens following the trajectory) in Terra Natura Wildlife Park, Murcia, southeast Spain..... 147

Figure 2. Flow diagram showing steps for calculating sand fly optimal feeding routes. Blue spheres represent spatial variables and yellow rectangles represent the applied GIS calculations ... 149

Figure 3. Optimal cumulative 'minimum-cost' routes for sand flies moving from trap F to potential feeding hosts..... 150

Figure 4. Distribution of minimum-cost sand fly trajectories to feeding hosts (solid red lines) and to other accessible hosts (dashed blue lines). Each blood-fed specimen was considered to have the choice of 30-33 trajectories to different hosts (solid red lines represent those eventually followed and dashed blue lines those that were not taken)..... 153

INDEX OF TABLES

GENERAL INTRODUCTION

Table 1. Wild, captive and domestic animals in Spain other than dogs and humans in which molecular and/or serological diagnosis has detected infection by *L. infantum* 27

Table 2. Taxonomic classification of sand fly species described in Spain 36

CHAPTER 1: On how trap positioning affects phlebotomine sand fly density estimations

Table 1. Percentage of sand fly positive traps and sand fly density according to trap type, location and distance to the floor, and number of sand fly specimens according to species and gender. A study in an open-air kennel in Murcia, southeast Spain 64

Table 2. Estimates from mixed negative binomial regression models investigating the relationship between sand fly density and trap location and distance to the floor, adjusted for climatic variables 68

CHAPTER 2: Investigations of *Phlebotomus perniciosus* sand flies in rural Spain reveal strongly aggregated and gender-specific spatial distributions and advocate use of light-attraction traps

Table 1. Absolute (relative to trap type) frequency of sand fly species in CDC light and sticky traps according to sand fly gender, month and trap environment 83

Table 2. Percentage of male and female *P. perniciosus*-positive CDC traps and abundance in positive traps in study sites 86

Table 3. Time-specific *P. perniciosus* abundance, percentage of positive CDC light traps and abundance in positive traps 88

Table 4. Relationship between on-site recorded environmental variables and the percentage of CDC light traps with *P. perniciosus* and the median and maximum number of male and female specimens in positive traps. 91

Table 5. Sex-specific incidence rate ratios (RR) from a negative binomial model of the relationship between CDC light trap *P. perniciosus* counts and distance to animals in flocks and kennel, adjusted for relative humidity, temperature, week, moon illumination, CO_2 concentration, rain and premise 93

Table S1. Premise and site-specific percentage of *P. perniciosus*-positive sticky traps, number of *P. perniciosus* collected, and median and maximum sand flies per m^2 and day in *P. perniciosus*-positive sticky traps 101

Table S2. Time-specific percentage of *P. perniciosus*-positive sticky traps, number of *P. perniciosus* collected, and median and maximum sand flies per m^2 and day in *P. perniciosus*-positive sticky traps 102

Table S3. Relationship between environmental variables recorded on site and the percentage of sticky traps with *P. perniciosus* and the median and maximum number of male and female specimens per m^2 and day in positive traps 103

CHAPTER 3: Molecular xenomonitoring and host identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife park

Table 1. Percentage of traps with at least one sand fly (% positive traps) and sand fly distribution according to species and sampling time, location and trap position and environmental features 120

Table 2. Percentage of traps with at least one sand fly (% positive traps) and sand fly distribution according to species, altitude and climatic variables 122

Table 3. Estimates of three negative binomial regression models examining the relationship between sand fly abundance and explanatory variables. Models differed in the outcome variable, which were abundance of: all sand flies (1), unfed *Phlebotomus* spp. females (2) and engorged and gravid *Phlebotomus* spp. females (3) 123

Table 4. Number (%) of bloodmeals and ectoparasiticide treatment according to the vertebrate host, sand fly species and sampling site 126

Table 5. Number of bloodmeals, census and biting rates according to the mammal and bird host families 128

Table S1. List of animal species and surface of their open-air enclosures in "Terra Natura" zoological park 138

Table S2. Environmental characteristics of the sampling sites and CDC light trap positioning .. 140

CHAPTER 4: A spatial ecology study in a high-diversity host community to understand blood-feeding behavior in *Phlebotomus* sand fly vectors of *Leishmania*

Table 1. Frequency of sand fly bloodmeals according to host location, median Euclidean distance (m) between hosts and light traps, and estimated median minimum sand fly feeding movement cost expressed as cost units (cu), in a study conducted in Terra Natura Wildlife Park, Murcia, Spain 152

Table 2. Estimates from a logistic regression model investigating the probability of *P. perniciosus*, *P. papatasi* and *P. ariasi* female sand flies taking a bloodmeal from different host species, adjusted for census and sand fly movement cost (cost unit) to reach the host 154

Table 3. Estimates from a logistic regression model investigating the probability of *P. perniciosus*, *P. papatasi* and *P. ariasi* female sand flies taking a bloodmeal from different host families, adjusted for census and sand fly movement cost (cost unit) to reach the host 155

SUMMARY

In this PhD dissertation we have studied the distribution, density and diversity of sand fly populations at a small geographical scale in animal groups from rural and periurban areas in Murcia Region. The importance of these hematophagous insects lies in their role as biological vectors of the protozoan *Leishmania* spp., the causative agent of leishmaniasis affecting humans and animals worldwide. This disease is mainly endemic in tropical and temperate zones, such as southern Europe. In this region, the most important species is *Leishmania infantum*, transmitted by sand flies of the genus *Phlebotomus*, including *Phlebotomus perniciosus* and *Phlebotomus ariasi* in Spain, and the main domestic reservoir is the dog (*Canis lupus familiaris*), which is particularly susceptible to infection. Canine leishmaniasis is one of the major parasitic diseases and a large percentage of dogs in endemic areas are infected. Human leishmaniasis has a lower impact in Europe, with a few thousand cases reported annually. In any case, disease control, which is limited by the absence of effective vaccines, focuses on measures against the vector and mainly on preventing dogs from becoming infected, using insecticides with repellent activity. Currently, it is not possible to act in sand fly breeding sites, since these are terrestrial and not well-characterized. Another aspect that hampers leishmaniasis control is the existence of domestic and wild animals other than dogs, acting as reservoirs in the transmission cycle of *L. infantum*.

Leishmaniasis is an emerging disease with a dynamic and changing epidemiology as a result of global warming, globalization, and anthropic changes in the environment. This has led to an increase in sand fly populations and colonization of new geographical areas, resulting in leishmaniasis emergence. Given the key role of sand flies in the transmission of *L. infantum*, it is paramount to improve our understanding of their spatial and temporal distribution and the environmental factors that govern sand fly populations. Biological and ecological traits of these insects contribute to their typically overdispersed distribution in the environment. They thrive in places where resources are available for sand fly development, such as hosts on which females can take bloodmeals and suitable breeding and resting sites. In Murcia Region, sand flies are widely distributed throughout the territory, with variable density and diversity depending on environmental conditions. Notwithstanding, it is necessary to further study the spatial distribution on a smaller

geographical scale, since no data are available, and it could help improve our understanding of the transmission risk and sand fly and leishmaniasis control.

For all the above-mentioned reasons, the main objective of this doctoral thesis is the study of the small-scale spatial distribution of phlebotomine sand flies in places with high vector densities that represent an infection risk for dogs and humans, such as rural and periurban areas in Murcia Region. Moreover, we analyze the transmission risk of *L. infantum* in these environments. These studies were based on intensive sand fly monitoring and sand fly density estimation, using both interception and attraction traps. In parallel to this work we carried out another study to assess how the sampling method may affect sand fly density estimations.

In **Chapter 1** we address precisely the study of how adult sampling methods, in particular the trap position with respect to distance to the floor and to a vertical surface, may affect sand fly density estimations. It was carried out in an outdoor dog kennel located in the periurban area of Murcia city during the summers of 2015-2016, using sticky and CO_2-light traps. They were placed at three distances from the ground (15, 75 and 150 cm) in the absence or presence of a vertical surface: wire fence and smooth wooden plank stood against the fence. The sampling lasted 48 nights and a total of 692 sand flies of the following species were captured: *Phlebotomus papatasi* (52%), *P. perniciosus* (32%), *Sergentomyia minuta* (15%) and *P. ariasi* (<1%). Regarding sticky traps, 54% of them were positive and density in this trap type was 3.0 sand flies/m^2/night. Instead, 78% of CO_2-light attraction traps collected at least one sand fly and density was 6.7 sand flies/trap/night. Multivariable statistical models were used to estimate the potential effect on sand fly density of trap placement (height and surface type), time of year and climatic factors during sampling (temperature, humidity and wind speed). Trap placement had a greater impact using sticky traps, as the negative association between sand fly density and trap distances to the ground and to the continuous vertical surface was confirmed. With attraction traps, the inverse relation between sand fly catches and trap height above the ground was also detected, but the effect was smaller, as the number of sand flies significantly decreased only in traps placed at 150 cm in open spaces far from a vertical surface, and at 75 cm and 150 cm in traps in the wire fence. The results of this study have important practical implications, since it emphasizes the need to consider trap distance

to the floor and existence of a solid vertical surface, especially when interception traps are used. There is a greater margin for positioning attraction traps in terms of distance to the ground and this may be advantageous to avoid animal interference. The analysis also showed the importance of considering climatic factors, especially wind speed, to accurately estimate sand fly density.

Chapter 2 describes the phenology and the spatial distribution of *P. perniciosus* populations at a small geographical scale in a rural area in northwest of Murcia Region. In particular, we analyze the relationship between sand fly abundance and distance to groups of animals in three sheep farms and one dog kennel, using an intensive sampling protocol with sticky and light traps. Traps were placed 5-740 m away from the animals for alternate weeks between May and October 2016, and a total of 576 light traps and 1,760 sticky traps were used. We collected 8,506 phlebotomine sand flies and identified five species: *P. perniciosus* (62%), *Phlebotomus sergenti* (23%), *P. papatasi* (8%), *S. minuta* (6%) and *P. ariasi* (1%). The vast majority of specimens (87%) were caught with light traps, and the relative abundance of each species varied depending on the trap type. The number of sand flies was higher in traps placed within the premises and next to the animals, except for *S. minuta*, which was more abundant in the field further away from the farms and the kennel. Differences were observed between males and females regarding the negative association between the abundance of *P. perniciosus* and distance to the animals. Females were more abundant in the traps placed where the animals were housed, while the number of males was greater in adjacent storage rooms. In addition, a positive relationship was detected between CO_2 concentration and female abundance, but not for males. These results demonstrate that *P. perniciosus* populations congregate inside the premises, allowing female sand flies to feed on sheep and dogs. Animal shelters can also provide ideal conditions for sand fly breeding, including high humidity and organic matter as larvae food. The spatial distributions of males and females suggest biological and behavioral differences, which highlight the need to analyze them separately. Finally, this study provides evidence that light traps are more suitable for assessing the spatial and temporal distribution of *P. perniciosus*, which is important for estimating the transmission risk of *L. infantum*.

In **Chapter 3** we describe the spatial distribution of sand flies collected in a periurban zoological park in Murcia city, discussing the epidemiological role of this place in the local transmission of *L. infantum*. For that purpose, we combined entomological surveillance, *L. infantum* infection rate in vectors and bloodmeal identification in females sand flies. The study was carried out in summers of 2016-2018 in eight georeferenced sampling sites adjacent to animal outdoor enclosures. We used a total of 111 light traps and 97% were found to be positive. Overall, 7,309 sand flies were captured, and five species were morphologically identified: *P. perniciosus* (67%), *P. papatasi* (16%), *S. minuta* (14%), *P. ariasi* (2%) and *P. sergenti* (<1%). Most sand flies were collected in five out of the eight selected sites. The sand fly spatial distribution was therefore variable, and depended on the species, sex and female physiological stage (unfed, blood-fed or gravid). Thus, we detected a significant positive association between the abundance of unfed females and the number of animals present in the vicinity of the traps, which can be possibly linked to the host-seeking behavior. In contrast, the number of blood-fed and gravid females was positively associated with traps placed in more sheltered locations and not to the animal census in the proximity, which could indicate a preference for finding undisturbed places to complete the blood digestion and egg laying. On the other hand, *P. perniciosus*, *P. papatasi* and *P. ariasi* females were found to feed on 15, 11 and 7 different animal species, respectively, corroborating the opportunistic feeding behavior of these insects. The most common hosts were the fallow deer (*Dama dama*) and the red deer (*Cervus elaphus*). Most of the analyzed bloodmeals belonged to mammals (98%), while the remaining 2% was blood from ostriches (*Struthio camelus*). Only 5% of females fed on canids. In addition, none of the 602 female sand flies analyzed for *L. infantum* infection by real-time PCR were positive. This study shows that anthropic changes in the environment, such as the presence of this zoological garden in a periurban area, can create the ideal conditions for development of sand fly populations. Notwithstanding, transmission cycle of *L. infantum* depends on interactions between infected vectors, reservoirs and susceptible hosts. Hence, these animal facilities do not imply a greater infection risk for neighboring residential areas, as most sand flies in the zoological park feed on hosts that are not *L. infantum* reservoirs, thus hindering the natural circulation of the parasite.

Chapter 4 aims to analyze the blood-feeding preferences of *P. perniciosus*, *P. papatasi* and *P. ariasi* in a high host richness environment, such as the zoological park where the previous work was carried out. Under these circumstances, the information provided by analyzing the percentage of bloodmeals of a given host alone, is not sufficient to demonstrate the preference for such animal species. It is necessary to take into account other variables, such as the host census of each available species and the movement cost for the sand fly to reach the different animals. Unlike other studies, we have used both factors in this study employing multivariable statistical modelling, and estimated the movement cost as a function of the travelling distance and altitude gradient saved by the insect to reach the host. It was assumed that sand flies have a small range of dispersal and display site fidelity, i.e., the place where sand flies were captured was also their breeding or resting site, which allowed the calculation of the distance travelled between the host and the sampling site in both directions. Most blood-fed females (87%) were captured in traps at distances under 100 m from the host from which they took bloodmeals, corroborating the small dispersal range of these insects. Although the most common species feeding on by sand flies were the red deer and the fallow deer, multivariable models including movement costs and host census, estimated that the feeding probability was maximum for red deer and common eland (*Taurotragus oryx*). These results suggest that, under similar circumstances and with a wide range of available animal hosts, *P. perniciosus*, *P. papatasi* and *P. ariasi* females prefer to feed on some species rather than others.

The findings obtained in the four studies comprising this doctoral thesis provide valuable information about the sand fly spatial distribution at a small geographical scale in certain rural and periurban environments in southeast Spain, and how this may influence the local epidemiology of *L. infantum*. We highlight the need for a holistic approach in the study of parasite transmission and control, involving factors linked to the vector (spatial distribution, density, feeding habits and *L. infantum* infection rate), and the host (species, census, accessibility and susceptibility to infection). Another remarkable aspect is the importance of the sampling method for estimating sand fly density. It is imperative when performing entomological monitoring studies to consider the trap type, the sampling intensity and the biological and ecological traits of phlebotomine sand flies.

There is an urgent need for further collaboration between entomologists and finding a universal method for sampling and reporting sand fly density to allow efficient and objective comparisons between studies. This will facilitate predictive modelling of sand fly distribution and *L. infantum* infection risk, as well as the development of dynamic and updated maps to help vector disease control.

RESUMEN

En esta tesis doctoral se ha estudiado la distribución, densidad y diversidad de las poblaciones de flebotomos a una escala geográfica pequeña en colectivos animales de zonas rurales y periurbanas de la Región de Murcia. La importancia de estos insectos hematófagos radica en el papel que desempeñan como vectores biológicos en el ciclo de transmisión del protozoo *Leishmania* spp., agente etiológico de la leishmaniosis que afecta a personas y animales. Esta enfermedad está distribuida a nivel mundial y es endémica principalmente en las zonas tropicales y zonas templadas, como es el caso del sur de Europa. En esta región, la especie endémica más importante es *Leishmania infantum*, transmitida por especies del género *Phlebotomus* (*P. perniciosus* y *P. ariasi* en España), y cuyo principal reservorio doméstico es el perro (*Canis lupus familiaris*), que es especialmente susceptible a la infección. La leishmaniosis canina es una de las enfermedades parasitarias más importantes, con un gran porcentaje de perros infectados. La leishmaniosis humana, en cambio, tiene un impacto menor en Europa, notificándose unos pocos miles de casos anualmente. En cualquier caso, el control de esta enfermedad se ve limitado por la ausencia de vacunas eficaces, centrándose en actuaciones frente al vector y, fundamentalmente, en evitar que los perros se infecten, empleando productos insecticidas con actividad repelente. Actualmente, no es posible actuar en los lugares de cría del insecto, ya que éstos son terrestres y no están bien caracterizados. Otro aspecto que dificulta el control de esta enfermedad es la presencia de otros reservorios domésticos y silvestres en el ciclo de transmisión de *L. infantum*, además del perro.

La leishmaniosis es una enfermedad emergente y su epidemiología se ve sometida a cambios, como consecuencia del calentamiento global, de la globalización y del impacto antrópico en el ambiente. Esto ha conllevado al aumento de tamaño de las poblaciones de flebotomos y a su expansión a nuevas áreas geográficas, lo que favorece asimismo el contacto entre vectores, reservorios y hospedadores susceptibles. Dado el papel clave de los flebotomos en la transmisión de *L. infantum*, es de vital importancia comprender mejor su distribución espacial y temporal, y los factores medioambientales que afectan a las poblaciones de estos dípteros. Las particularidades de la biología de estos insectos hacen que en el medio ambiente se distribuyan de forma heterogénea, abundando sobre todo en los lugares donde disponen de los recursos necesarios para su desarrollo, como

la presencia de hospedadores de los que las hembras hematófagas pueden alimentarse, y de adecuados lugares terrestres de cría y de reposo. En la Región de Murcia, estos insectos están distribuidos por todo el territorio, con una densidad y diversidad variable en función de las condiciones medioambientales. Sin embargo, es necesario profundizar en el estudio de la distribución espacial a una escala geográfica más pequeña, ya que no existen datos al respecto y podría facilitar un mejor conocimiento del riesgo de transmisión y del control de la leishmaniosis.

Por todo lo anteriormente mencionado, el objetivo principal de esta tesis doctoral es estudiar la distribución espacial a pequeña escala geográfica de flebotomos en lugares donde son abundantes y representan un riesgo de contagio para perros y personas, como son el ámbito rural y periurbano, y analizar el riesgo de transmisión de *L. infantum* en estos ambientes. Para estos trabajos se han realizado diversos muestreos entomológicos para la captura de flebotomos adultos, utilizando trampas adhesivas de intercepción y trampas luminosas de atracción, cebadas o no con dióxido de carbono (CO_2). Otro objetivo de esta tesis es valorar la importancia del método de captura empleado de cara a obtener una estimación precisa de la abundancia de flebotomos y de especies vectores de *Leishmania* spp. en particular.

El **Capítulo 1** aborda el estudio de la metodología de muestreo de flebotomos adultos. Se realizó en el recinto exterior de una perrera localizada en la zona periurbana de la ciudad de Murcia, con el objeto de estudiar cómo varía la densidad de flebotomos en relación con la colocación de las trampas de luz-CO_2 y adhesivas. Para tal fin, se evaluaron tres alturas (15, 75 y 150 cm) y la ausencia o presencia de una superficie vertical: valla metálica de alambre trenzado o tabla vertical de madera. El muestreo tuvo una duración de 48 días repartidos entre los meses de verano de 2015 y 2016, permitiendo capturar un total de 692 flebotomos de las siguientes especies: *Phlebotomus papatasi* (52%), *P. perniciosus* (32%), *Sergentomyia minuta* (15%) y *P. ariasi* (<1%). El 54% de las trampas adhesivas fueron positivas y registraron una densidad global de 3,0 flebotomos/m^2/noche. En cambio, el porcentaje de positividad de las trampas de atracción (luz-CO_2) fue mayor, al detectar flebotomos en el 78% de dichas trampas, y la densidad fue de 6,7 flebotomos/trampa/noche. Se emplearon modelos estadísticos multivariables para tener en cuenta el posible efecto de la altura y superficie de colocación

de las trampas en la estimación de la densidad de flebotomos, además de la época del año y de las condiciones climáticas durante el muestreo (temperatura, humedad y velocidad del viento). El lugar de colocación de la trampa tuvo un mayor impacto en las trampas adhesivas, pues se confirmó la asociación negativa entre la densidad y la distancia de la trampa al suelo y a una superficie vertical continua. En las trampas de atracción también se observó una relación inversa entre la densidad de las capturas y la distancia al suelo, pero el efecto fue menor, ya que el número de flebotomos disminuyó significativamente solo en las trampas colocadas a 150 cm del suelo en espacios abiertos, y a 75 cm y 150 cm en la valla metálica. Los resultados de este estudio tienen implicaciones prácticas importantes, pues destacan la necesidad de considerar la distancia al suelo y la presencia de una superficie vertical sólida, sobre todo cuando se emplean trampas adhesivas en los muestreos. Las trampas de atracción permiten un mayor margen de elección, y la posibilidad de colocarlas a cierta distancia del suelo evitaría la interferencia de animales. El análisis realizado pone de manifiesto la importancia de considerar los factores climáticos, en especial la velocidad del viento, para estimar de forma precisa la densidad de flebotomos.

En el **Capítulo 2** abordamos el estudio de la fenología y distribución espacial a escala geográfica pequeña de *P. perniciosus* en una zona rural del noroeste de la Región de Murcia. Específicamente, se analizó la relación entre la densidad de flebotomos y la distancia a núcleos de animales, concretamente a tres rebaños ovinos y una perrera. Se empleó un muestreo intensivo con trampas adhesivas y de luz. Ambos tipos de dispositivos se colocaron a 5-740 m de distancia de los animales durante semanas alternas entre los meses de mayo y octubre de 2016, empleando un total de 576 trampas de luz y 1.760 trampas adhesivas. Se capturaron 8.506 flebotomos pertenecientes a cinco especies: *P. perniciosus* (62%), *Phlebotomus sergenti* (23%), *P. papatasi* (8%), *S. minuta* (6%) y *P. ariasi* (1%). La gran mayoría de los especímenes (87%) se capturaron con las trampas de luz, y la abundancia relativa de cada especie varió dependiendo del tipo de trampa. El número de flebotomos capturados fue mayor en las trampas colocadas en el interior de las instalaciones y próximas a los animales, a excepción de *S. minuta*, cuya abundancia fue superior en zonas de campo más alejadas de las granjas y la perrera. Respecto a esta asociación negativa entre la abundancia de *P. perniciosus* y la distancia a

los animales, se observaron diferencias entre machos y hembras. Las hembras fueron más abundantes en las trampas colocadas en la inmediatez de los animales, mientras que los machos se capturaron en mayor número en los espacios adyacentes habilitados para almacenar material. Además, se observó una relación positiva entre la concentración de CO_2 y la densidad de las hembras, pero no así con la de los machos. Estos resultados demuestran que las poblaciones de *P. perniciosus* en el ámbito rural se concentran en el interior de las granjas y perrera, en proximidad a los colectivos de animales que sirven como fuente de alimento para las hembras, las cuales necesitan ingerir sangre para completar la maduración de los huevos. También en estos lugares pueden crearse las condiciones ideales de cría, con alta humedad y abundante materia orgánica para que las larvas pueden obtener alimento. Los patrones de distribución espacial de ambos sexos evidencian sus diferencias biológicas y de comportamiento, lo cual pone de manifiesto la necesidad de analizar sus distribuciones de forma separada. Por último, el estudio aporta evidencia de que las trampas de luz son más adecuadas a la hora de estudiar la distribución espacial y temporal de *P. perniciosus* que las trampas adhesivas, lo cual es importante para estimar el riesgo de transmisión de *L. infantum*.

En el **Capítulo 3** se describe la distribución espacial de los flebotomos en un parque zoológico periurbano de la ciudad de Murcia, analizando el papel epidemiológico que puede desempeñar dicho lugar en el ciclo de transmisión local de *L. infantum*. Para ello, se combinó la vigilancia entomológica junto con la determinación de la tasa de infección por *L. infantum* en el vector y de las fuentes de alimentación de las hembras de flebotomos. El estudio se realizó colocando trampas de luz en ocho puntos adyacentes a los recintos exteriores de los animales durante los meses de verano de 2016-2018. En total, se emplearon 111 trampas, de las que en el 97% se encontraron flebotomos. Se capturaron 7.309 especímenes de las siguientes especies: *P. perniciosus* (67%), *P. papatasi* (16%), *S. minuta* (14%), *P. ariasi* (2%) y *P. sergenti* (<1%). La mayor parte de las capturas se consiguió en cinco de los ochos lugares seleccionados. La distribución espacial de los flebotomos fue variable en función de la especie, del sexo y del estado reproductivo de las hembras (grávidas, no alimentadas o con sangre en el abdomen). De este modo, se observó una relación positiva entre la abundancia de las hembras no alimentadas (sin sangre) y el número de animales presentes en las inmediaciones de la trampa,

probablemente ligado a la búsqueda de hospedadores de los que alimentarse. En cambio, la abundancia de las hembras grávidas y recién alimentadas se asoció positivamente con las trampas colocadas en sitios más protegidos y no con el censo de animales en la proximidad de la trampa, lo que podría indicar prioridad por lugares tranquilos para la digestión de la sangre ingerida y para la puesta de huevos. Por otro lado, se observó que las hembras de *P. perniciosus*, *P. papatasi* y *P. ariasi* tomaron sangre de 15, 11 y 7 hospedadores distintos, respectivamente, lo que apoya el comportamiento oportunista de estos insectos a la hora de alimentarse. Las especies más comunes fueron el gamo (*Dama dama*) y el ciervo (*Cervus elaphus*). El 98% de las hembras analizadas se alimentaron de mamíferos, mientras que el 2% restante tomaron sangre de avestruces (*Struthio camelus*). Los cánidos representaron solamente el 5% de las alimentaciones. Además, no se detectó ADN de *L. infantum* en ninguno de los 602 flebotomos analizados. Los resultados de este estudio ponen de manifiesto que las modificaciones antrópicas en el ambiente, como la presencia de este zoológico en una zona periurbana, pueden generar las condiciones propicias para el desarrollo de las poblaciones de flebotomos. No obstante, dado que el ciclo de transmisión de *L. infantum* depende de la interacción entre vectores infectados, reservorios y hospedadores susceptibles, estas instalaciones no suponen un mayor riesgo para las zonas residenciales adyacentes, ya que la mayoría de flebotomos del zoológico se alimentan de hospedadores que no son reservorios de *L. infantum*, impidiendo, por tanto, la circulación del parásito.

Finalmente, el **Capítulo 4** tiene como objetivo analizar las posibles preferencias de *P. perniciosus*, *P. papatasi* y *P. ariasi* por alimentarse de especies concretas de hospedadores, en un ambiente con una elevada diversidad de mamíferos y aves próximas entre sí, como es el parque zoológico en el que se realizó el estudio anterior. En estas circunstancias, la información que proporciona el porcentaje de flebotomos que se alimentan de un hospedador determinado no es suficiente para confirmar la preferencia por dicha especie, sino que es necesario tener en cuenta otras variables, como el censo de cada especie hospedadora y el coste que supone para el flebotomo el desplazamiento hasta las distintas especies. A diferencia de otros estudios similares, en esta ocasión se tuvieron en cuenta ambos factores, calculándose el coste de desplazamiento en función de la distancia recorrida y del desnivel acumulado hasta el hospedador. Para tal fin, se

asumió que los flebotomos tienen un escaso radio de dispersión y muestran un "comportamiento de referencia", es decir, que el lugar donde se capturaron los flebotomos fue también su lugar de cría o de reposo, lo que permitió calcular la distancia recorrida desde el hospedador hasta el punto de captura y viceversa. El 87% de las hembras se capturaron en trampas situadas a menos de 100 m del hospedador del que habían ingerido sangre, lo que corroboraría su escaso radio de acción. Si bien las especies de las que más se alimentaron los flebotomos fueron el ciervo y el gamo, la probabilidad de alimentación, según los modelos de regresión logística que incluyeron el coste de desplazamiento y el censo, fue máxima en el ciervo y en el eland común (*Taurotragus oryx*). Estos resultados sugieren que, en circunstancias similares y teniendo acceso a una amplia variedad de hospedadores potenciales, los flebotomos de las especies *P. perniciosus*, *P. papatasi* y *P. ariasi* prefieren alimentarse de unas especies más que de otras.

Los hallazgos obtenidos a lo largo de los cuatro estudios que conforman esta tesis doctoral aportan una valiosa información sobre la distribución espacial a pequeña escala en determinados ambientes rurales y periurbanos del sureste de España, y cómo esto puede influir en la epidemiología local de *L. infantum*. Se pone de manifiesto la necesidad de adoptar un enfoque holístico en el estudio de la transmisión y control del parásito, teniendo en cuenta una amplia variedad de factores ligados al vector (distribución espacial, densidad, patrón de alimentación y tasa de infección) y al hospedador (especie, censo, accesibilidad y susceptibilidad a la infección). El otro aspecto destacable de la información derivada del trabajo realizado es la importancia del método de muestreo para obtener una medida realista de la densidad de flebotomos. En el diseño de los muestreos entomológicos, es necesario tener en cuenta el tipo de trampa, la intensidad de muestreo y las características biológicas y ecológicas de los flebotomos. Es urgente que la comunidad de entomólogos colabore para encontrar un método unificado de muestreo y publicación de los resultados, que permita comparar eficaz y objetivamente los distintos estudios. Todo ello facilitará la realización de modelos predictivos de la distribución de los flebotomos y del riesgo de infección, y la elaboración de mapas dinámicos que faciliten el control de las enfermedades vectoriales.

GENERAL INTRODUCTION

INTRODUCTION

Phlebotomine sand flies are hematophagous arthropods of great medical and veterinary importance, as vectors of several pathogens including protozoa (*Leishmania* spp.), bacteria (*Bartonella bacilliformis*) and viruses (*Phlebovirus*, *Vesiculovirus* and *Orbivirus*) (Depaquit *et al.*, 2010; Maroli *et al.*, 2013; Ready, 2013). In Spain, human and canine leishmaniasis is caused by *Leishmania infantum*, the main reservoir is the domestic dog and the proven vectors are *Phlebotomus perniciosus* and *Phlebotomus ariasi* (Amela *et al.*, 2012). Other domestic animals and wildlife may also be infected, although the reservoir role of most of them has not been demonstrated (Millán *et al.*, 2014; Risueño *et al.*, 2018; Alcover *et al.*, 2020). It is an endemic disease in the Iberian Peninsula and Balearic Islands, and whilst many infected individuals do not have clinical signs, dogs, children and immunosuppressed people are most vulnerable to developing disease (Amela *et al.*, 2012). However, leishmaniasis is an emergent disease and in the past decade, Spain experienced the highest disease incidence ever recorded in Europe affecting hundreds of immunocompetent adults in southwest Madrid. This outbreak was associated with urbanization of sand fly endemic areas that led to an overpopulation of lagomorphs, which acted as an unusual reservoir of the parasite (Molina *et al.*, 2012; Arce *et al.*, 2013; Jiménez *et al.*, 2014). This illustrates the great complexity of the *L. infantum* epidemiological cycle, which is regulated by interactions between the parasite, the host, the vector and the environment (Gradoni, 2018) (Figure 1). As a multifactorial vector-borne disease that affects humans and animals and it is strongly linked to the environment, the fight against leishmaniasis should be approached from a “One Health” perspective (Miró and López-Vélez, 2018; Martín-Sánchez *et al.*, 2020).

Given the decisive role of sand flies in the *L. infantum* transmission cycle, it is imperative to have a precise understanding of factors affecting its population dynamics, for a better insight of leishmaniasis epidemiology and control (Gálvez *et al.*, 2018). Sand flies breed in terrestrial microhabitats rich in organic matter and protected from desiccation, and plenty of places meet such conditions (Killick-Kendrick, 1999). Notwithstanding this, finding sand fly breeding places has always been challenging and unproductive, and this constitutes a major control limitation because it precludes environmental interventions to eliminate breeding sites. Hence, sand fly surveillance and

control strategies focus primarily on the adult stage (Feliciangeli, 2004). Adult sand flies are crepuscular and nocturnal, and are active from late spring to early autumn, being variable depending on latitude. Females are hematophagous and therefore responsible for parasite transmission, and they can feed from a great diversity of animal hosts (Maia *et al.*, 2015; Bravo-Barriga *et al.*, 2016; Cotteaux-Lautard *et al.*, 2016). Sand flies are poor fliers and concentrate close to breeding sites (Maroli *et al.*, 2013). They are well-adapted to different habitats and can thrive in sylvatic, rural, periurban and urban environments (Dvorak *et al.*, 2018). All these biological and ecological characteristics have a decisive impact on the epidemiology of leishmaniasis.

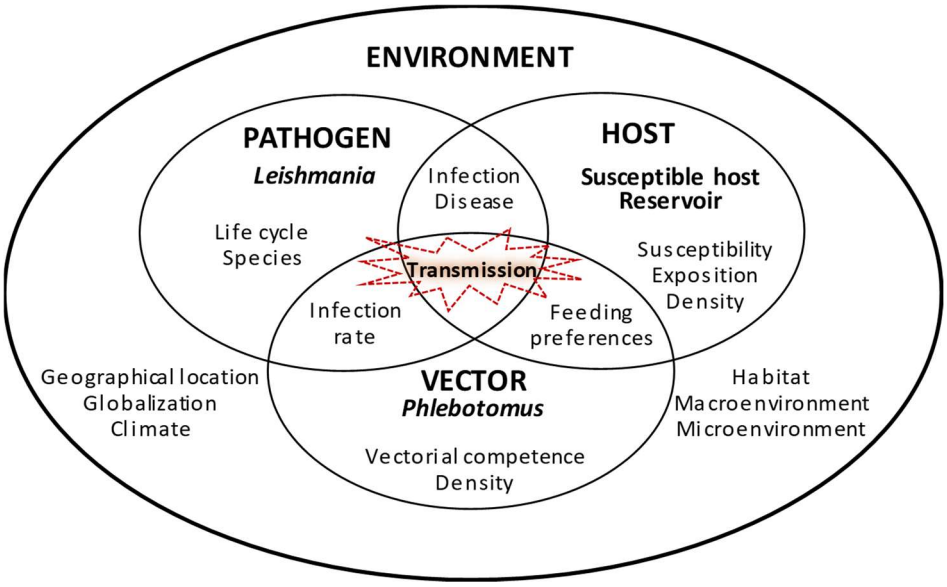

Figure 1. Diagram of leishmaniasis epidemiology showing pathogen-host-vector-environment interactions involved in parasite transmission. Source: based on Oliveira *et al.* (2018).

Murcia Region is endemic for human and canine leishmaniasis. Previous studies to this doctoral thesis have shown that sand flies are abundant throughout the province (Martínez-Ortega, 1985; Martínez Ortega and Conesa Gallego, 1987a) and the large-scale distribution of these insects is variable depending on macroclimate conditions (Risueño *et al.*, 2017). Moreover, microenvironmental factors were suggested to be responsible for differences observed in the small-scale distribution of sand flies (Risueño *et al.*, 2017). Our understanding of such factors is, however, not precise enough to allow predictions of sand fly distribution to be made and use this knowledge to enhance our ability to control sand fly populations and sand fly-borne infections. The aim of the work developed for this doctoral thesis is to improve our understanding of some of the aspects that govern sand

fly distributions on a small geographical scale in rural and periurban areas of Murcia Region; how this may vary according to the sand fly features such as gender and physiological status of females (unfed, blood-fed and gravid), and to the close presence of potential feeding hosts. Given that susceptibility to *L. infantum* infections is widely variable between host species, a further objective of this thesis was to investigate how sand fly feeding preferences may affect the epidemiology of infection. As a preliminary step to these investigations an experiment was performed to gain some understanding on adult sand fly sampling methods. Specifically, on how sand fly density estimations in a particular site are dependent on the way most commonly used interception and attraction traps are positioned.

BIBLIOGRAPHIC REVIEW

Leishmaniasis

General context

The term leishmaniasis encompasses a vector-borne disease complex caused by protozoan parasites of genus *Leishmania* spp. (Trypanosomatida: Trypanosomatidae), which infect cells of the reticuloendothelial system, mainly macrophages, of humans and other mammals (Ready, 2013). The transmission cycle involves a vertebrate host and a sand fly vector (Figure 2), where obligate intracellular amastigotes and motile extracellular promastigotes develop, respectively (Bates, 2007). There are 53 *Leishmania* species worldwide, of which 31 are mammalian parasites and, more specifically, 20 are pathogenic to humans (Akhoundi *et al.*, 2016).

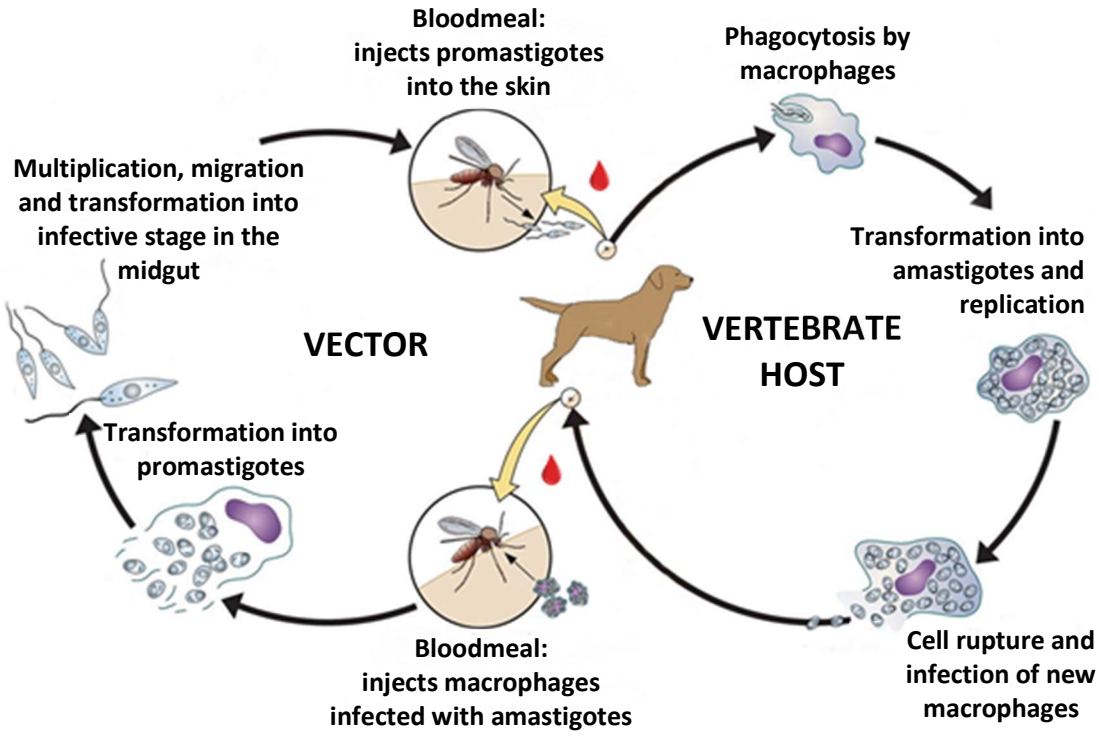

Figure 2. Life cycle of *Leishmania* spp. that involves a biological vector (promastigote stage) and a vertebrate host (amastigote stage). Source: based on Esch and Petersen (2013).

According to World Health Organization (WHO), leishmaniasis is endemic in 98 countries and remains one of the most neglected diseases worldwide, predominantly affecting the poorest countries (Alvar *et al.*, 2012). A total of 350 million people is estimated to be at risk of being infected, with an incidence of two million cases every year (OMS, 2010). Europe has the lowest proportion of clinical cases globally, with approximately 2% of the total (WHO, 2018). In that region, leishmaniasis is mainly caused by *Leishmania infantum*, with the domestic dog (*Canis lupus familiaris*) as the main reservoir; 2.5 million dogs may be infected by this species (Moreno and Alvar, 2002). However, transmission to humans of other *Leishmania* species has also been reported, such as *L. tropica* in Greece and *L. donovani* in Cyprus (Mazeris *et al.*, 2010; Christodoulou *et al.*, 2012; Antoniou *et al.*, 2013).

Leishmania spp. infection in humans has a wide clinical spectrum ranging from no symptoms at all to a life-threatening condition, depending to a great extent on the species involved and the host immune response. Localized cutaneous leishmaniasis is the mildest form, characterized by nodular and ulcerative lesions at the point of the vector's bite. But occasionally, infection also affects the mucous membranes and spreads causing diffuse cutaneous leishmaniasis, mucocutaneous leishmaniasis and visceral leishmaniasis. The latter is the most serious and fatal disease, responsible for more than 50,000 deaths annually (OMS, 2010). Leishmaniasis epidemiology is also strongly dependent on the parasite species and the presence of specific vectors and animal reservoirs (Ready, 2013). *Leishmania infantum* is the only endemic species in mainland Europe, but incidence is on the rise associated to an expansion of vector distribution and an increased exposure of susceptible hosts to infection (Maroli *et al.*, 2008; Miró *et al.*, 2012), as a result of environmental and anthropic changes, such as climate change, urbanization, deforestation, agriculture expansion or human and animal travelling (Aspöck *et al.*, 2008; Maia and Cardoso, 2015; Gradoni, 2018). Fortunately, our understanding of these distributional changes has been enhanced in recent times with modern Geographic Information Systems (GIS), that allow advanced analysis of the relationship between infection and ground and remotely sensed environmental information (Barón *et al.*, 2011; Gálvez *et al.*, 2011).

Epidemiological situation of leishmaniasis in Spain

Leishmaniasis is an endemic zoonotic disease that has now been reported in most regions of the Iberian Peninsula and Spanish islands. The epidemiological triad of leishmaniasis in Spain involves *L. infantum* as the etiological agent, sand fly species of genus *Phlebotomus* as vectors, namely *P. perniciosus*, *P. ariasi* and probably *P. langeroni*, and a wide variety of mammals that act as hosts for the parasite. Dogs are the main domestic reservoirs and therefore indirect sources of infection for humans (Amela *et al.*, 2012; Díaz Sáez *et al.*, 2018). Wildlife may also play an important epidemiological role as demonstrated in the previously mentioned recent human leishmaniasis outbreak in Madrid (Molina *et al.*, 2012; Jiménez *et al.*, 2014).

Human leishmaniasis

Human leishmaniasis was first detected in Spain in 1912, in a patient showing a visceral clinical manifestation. Disease incidence was high until the 1960s, mainly affecting children (Botet Fregola and Portús Vinyeta, 1993). With the improvement of sanitary and nutritional conditions leishmaniasis became an almost forgotten disease in subsequent decades. The situation changed in the 1980s and 1990s as a result of the human immunodeficiency virus (HIV) epidemic, when incidence rose dramatically among patients developing acquired immunodeficiency syndrome (AIDS) (Alvar, 1994). This crisis led to an activation of leishmaniasis surveillance and the disease was included in the list of notifiable diseases in 1982. Subsequently, after the "Red Nacional de Vigilancia Epidemiológica" (RENAVE) was created in 1995, notification of clinical cases became mandatory only in regions considered endemic. But from 2015, human leishmaniasis was again notifiable status at national level. Cases are currently recorded in the RENAVE and in the "Conjunto Mínimo Básico de Datos" (CMBD), which includes the national hospital admissions registry (Amela *et al.*, 2012). Comparison of both information systems highlights the significant low reporting of the disease to RENAVE (Amela *et al.*, 2012). Besides, a large number of the cutaneous cases are not diagnosed as they heal spontaneously or respond to treatments on an outpatient basis at local health centers, and therefore do not enter the CMBD casuistry (Herrador *et al.*, 2015). Regarding population susceptibility, children remain an important risk group, although a growing

number of adults are suffering from it, particularly immunosuppressed patients affected by concomitant diseases such as AIDS and cancer, or those receiving immunosuppressive treatment such as organ transplant patients. However, the human leishmaniasis outbreak in Madrid mainly affected immunocompetent adults (Arce *et al.*, 2013).

Canine leishmaniasis

Canine leishmaniasis is a multisystemic disease that can be fatal if untreated. Sick dogs can show a wide range of lesions and clinical signs, including skin lesions, cachexia, lymphadenomegaly, appetite loss, muscle atrophy, ocular signs, epistaxis and onychogryphosis, among others (Solano-Gallego *et al.*, 2009). On the other hand, molecular tools have evinced the high percentage of infected asymptomatic animals able to develop an effective cellular immune response against the parasite (Baneth *et al.*, 2008; Chitimia *et al.*, 2011). Prior to the advent of molecular tests, many of these animals were not considered infected because they do not develop a strong humoral response and detectable antibody tests by the most commonly used diagnostic tests.

There are no official data on the distribution and prevalence of canine leishmaniasis in the country. Epidemiological investigations carried out in several regions in Spain reported variable seroprevalences from 0% in Biscay to 57.1% in the Balearic Islands, depending on the geographical area, study population and methodology employed (Seguí, 1991; Miró *et al.*, 2012; Gálvez *et al.*, 2020). Recently, a systematic review along with a serological study in different regions in Spain have allowed us to get a general picture of canine seroprevalence in the country (Gálvez *et al.*, 2020). The provinces with the highest seroprevalence rates are mainly those in the Mediterranean area, in addition to Cádiz, Seville, Huelva, Cáceres and Ourense (Gálvez *et al.*, 2020).

Reservoirs of Leishmania infantum

A reservoir can be defined as one or more epidemiologically interconnected populations or environments, where the pathogen persists over time and is transmitted to other target populations (Haydon *et al.*, 2002). When many species can be infected, as occurs with *L. infantum*, vertebrate hosts can be classified as primary and secondary reservoirs and accidental hosts. Primary reservoirs can transmit the infection effectively

so that the parasite can persist indefinitely in the absence of other hosts (Quinnell and Courtenay, 2009). Secondary reservoirs may transmit the parasite to the vector but not efficiently enough to maintain *L. infantum* transmission in the population in the absence of the main reservoir. Instead, accidental hosts can also become infected but do not transmit the parasite to phlebotomine sand flies (Quinnell and Courtenay, 2009). The dog alone can maintain the parasite transmission, thus is the main domestic reservoir (Dantas-Torres, 2007). Dogs live in close relationship with humans and are one of the blood sources for sand flies (De Colmenares *et al.*, 1995). As mentioned above, they suffer from a chronic infection and a high percentage of infected dogs do not develop symptoms. Besides, asymptomatic dogs are also infectious to sand flies feeding on them (Molina *et al.*, 1994; Baneth *et al.*, 2008). However, some authors have indicated that transmission risk is greater in symptomatic animals, coinciding with a high parasitic load in blood (Manna *et al.*, 2004; Michalsky *et al.*, 2007).

Apart from dogs and humans, molecular and serological diagnoses in Spain have demonstrated *L. infantum* infection in numerous wild and domestic animals, including canids, felids, mustelids, lagomorphs, rodents and other mammals (Table 1) (Fernández-Bellon *et al.*, 2006; Martín-Sánchez *et al.*, 2007; Sastre *et al.*, 2008; Sobrino *et al.*, 2008; Ruiz-Fons *et al.*, 2013; Del Río *et al.*, 2014; Navea-Pérez *et al.*, 2015; Montoya *et al.*, 2016; Gonzálvez *et al.*, 2017; Millán, 2018; Miró *et al.*, 2018; Oleaga *et al.*, 2018; Risueño *et al.*, 2018; Galán-Puchades *et al.*, 2019; Ortuño *et al.*, 2019; Alcover *et al.*, 2020; Azami-Conesa *et al.*, 2020; Giner *et al.*, 2020). Wildlife usually develop a subclinical infection with a low parasitic burden, which has triggered a discussion about their role in the epidemiology of infection and the existence of an independent sylvatic transmission cycle that may or may not interact with a domestic one (Quinnell and Courtenay, 2009; Millán *et al.*, 2014; Tomassone *et al.*, 2018). In this sense, a recent study by Ortuño *et al.* (2019) reported identical *L. infantum* strains in dogs and wildlife, which suggested a common transmission cycle. However, the mere detection in tissues of *L. infantum* DNA does not necessarily imply that infected animals are parasite reservoirs. The ability to infect sand flies is a prerequisite to consider an animal species as parasite reservoir (OMS, 2010). Of the aforementioned species, such condition has only been demonstrated by xenodiagnosis in black rats (*Rattus rattus*), domestic cats (*Felis catus*), European rabbits (*Oryctolagus*

cuniculus) and Iberian hares (*Lepus granatensis*) (Quinnell and Courtenay, 2009; Molina *et al.*, 2012; Jiménez *et al.*, 2014). Regarding the two latter species, lagomorphs have been recognized to play a main role as active reservoirs of *L. infantum* in the outbreak of human leishmaniasis in Madrid, which was associated with the urban development of a traditionally agricultural area (Arce *et al.*, 2013).

Table 1. Wild, captive and domestic animals in Spain other than dogs and humans in which molecular and/or serological diagnosis has detected infection by *L. infantum*.

	Wildlife	**Captive animals**	**Domestic animals**
Canids	Red fox (*Vulpes vulpes*) Iberian wolf *(Canis lupus*)	Iberian wolf *(Canis lupus*) Swift fox (*Vulpes velox*)	-
Felids	Iberian lynx (*Lynx pardinus*) Wild cat (*Felis silvestris*)	Tiger (*Panthera tigris*)	Domestic cat (*Felis catus*)
Mustelids	Eurasian badger (*Meles meles*) Beech marten (*Martes foina*) Pine marten (*Martes martes*) Western polecat (*Mustela putorius*) European mink (*Mustela lutreola*) American mink (*Neovison vison*) Eurasian otter (*Lutra lutra*)	-	Domestic ferret (*Mustela putorius furo*)
Lagomorphs	European rabbit (*Oryctolagus cuniculus*) Iberian hare (*Lepus granatensis*) European hare (*Lepus europaeus*)	-	-
Rodents	Red squirrel (*Sciurus vulgaris*) Wood mouse (*Apodemus sylvaticus*) Algerian mouse (*Mus spretus*) House mouse (*Mus musculus*) Black rat (*Rattus rattus*) Brown rat (*Rattus norvegicus*)	-	-
Other mammals	Common pipistrelle (*Pipistrellus pipistrellus*) Common gennet (*Genneta genneta*) Egyptian mongoose (*Herpestes ichneumon*) Western European hedgehog (*Erinaceus europaeus*) White-toothed shrew (*Crocidura russula*)	Brown bear (*Ursus arctos*) Orangutan (*Pongo pygmaeus pygmaeus*) Bennett's wallaby (*Macropus rufogriseus rufogriseus*)	Horse (*Equus caballus*)

Leishmania infantum in Murcia Region

According to the human notifiable disease reporting data system ("Sistema de Información Sanitaria de Enfermedades de Declaración Obligatoria") of Murcia Region (http://www.murciasalud.es/), annual notifications of clinical leishmaniasis have not exceeded 9 cases since 1982, representing incidences between 0 and 0.9 cases/100,000 inhabitants. However, as occurs at national level, these data represent an

underestimation of the real proportion of the disease (Hernández-Torres *et al.*, 2015; Ortuño *et al.*, 2019). Regarding infection data, PCR prevalence in healthy blood donors was estimated at 8% (49/618). Most likely only a proportion of infected people have parasitemia, so this result is probably an underestimation of the real prevalence (Pérez-Cutillas *et al.*, 2015). Before the widespread use of molecular tools in epidemiological studies, asymptomatic infection in humans, which is associated with and effective cellular immunity, was studied by the Montenegro test, which measures the delayed hypersensitivity reaction to an intradermal injection of *Leishmania* spp. antigens (Marín Iniesta and Martín Luengo, 1982). In this sense, 44.2% people were positive to this technique in La Alpujarra, a rural area near Murcia Region (Acedo Sánchez *et al.*, 1996).

Concerning *L. infantum* infection in dogs, prevalence results were variable depending on the sample analyzed, the diagnostic technique used and the study population. It ranged from 3.7% by parasitological diagnosis in feral dogs (Marín Iniesta *et al.*, 1982) to 67% by PCR diagnosis in abandoned dogs tested in tissue samples (Chitimia *et al.*, 2011). *Leishmania infantum* infection in wildlife was first detected in Spain in 1982, particularly in three red foxes from Murcia Region (Marín Iniesta *et al.*, 1982). Since then, no further investigations in wildlife had been conducted in the province until the study of Risueño *et al.* (2018), in which subclinical infection was shown to be widespread in wildlife, including red fox, European rabbit, pine marten, wood mouse, black rat, wild cat and common genet, as well as an Iberian wolf from a zoological park. This study also suggested for the first time the existence in Spain of a sylvatic transmission cycle that interacts with the domestic one maintained by the dog, which may be a limiting factor for disease control.

Phlebotomine sand flies, vectors of *Leishmania* spp.

Sand fly taxonomy

Sand flies are hematophagous arthropods belonging to the order Diptera, suborder Nematocera, family Psychodidae and subfamily Phlebotominae (Maroli *et al.*, 2013; Akhoundi *et al.*, 2016). Among more than 900 sand fly species described in the Old World and New World, approximately 10% are vectors of pathogens (Ready, 2013). There is currently much controversy about the taxonomic classification of sand flies, which is still

far from being definitive (Akhoundi *et al.*, 2016). Several classifications have been proposed since the first systematic study of the genus *Phlebotomus* in 1911 by Newstead, differing in terms of classifying them into subfamily Phlebotominae or family Phlebotomidae, and in number of genera and subgenera (Maroli *et al.*, 2013; Akhoundi *et al.*, 2016). To date, the most commonly accepted classification is based on a conservative approach and includes the genera *Phlebotomus* spp. (13 subgenera), *Sergentomyia* spp. (10 subgenera), and *Chinius* spp. (4 species) in the Old World, and *Lutzomyia* spp. (26 subgenera and groups), *Warileya* spp. (6 species) and *Brumptomyia* spp. (24 species) in the New World (Lewis *et al.*, 1977; Young and Duncan, 1994; Akhoundi *et al.*, 2016). Sand flies belonging to *Phlebotomus* and *Lutzomyia* genera are the most important vectors of pathogens in the Old World and New World, respectively.

Sand fly morphology

Adult sand flies are small, hairy, and golden, brownish or grey colored insects measuring 2.5 to 3.5 mm, with long legs and piercing mouthparts. When at rest, sand flies hold their wings at 45° angle (V-shaped) above the abdomen (Gil Collado *et al.*, 1989; Killick-Kendrick, 1999; Lucientes *et al.*, 2005a). Like other insects, the sand fly body can be divided into head, thorax and abdomen. They are sexually dimorphic, since the distal abdominal segments of the males are modified and constitute the external genitalia (El-Hossary, 2006). Among the morphological structures with the greatest taxonomic value, the most commonly used are those found in the head and abdomen (Figure 3). Female sand flies can be identified according to the cibarium (number and shape of vertical and horizontal teeth, and presence or absence of a pigmented area), the pharynx (shape and size of the pharynx armature), antennae (relative size of segments and position and shape of ascoids), and the spermatheca (presence and number of rings, and shape and length of ducts). Morphological identification of males is done based on differences in the appendages of the external genitalia, including the shape of aedeagus, the length and shape of the coxite and style, the number and position of spines, the number of setae and the presence of a basal process in the coxite, among others (Lewis, 1982; El-Hossary, 2006).

However, morphological identification has some limitations, especially for identifying damaged specimens or cryptic species that are morphologically indistinguishable. Molecular tools, such as DNA sequencing, can complement traditional techniques (Depaquit, 2014). In addition, assessing protein profiles using MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) also allows precise identification of sand fly species (Dvorak *et al.*, 2014).

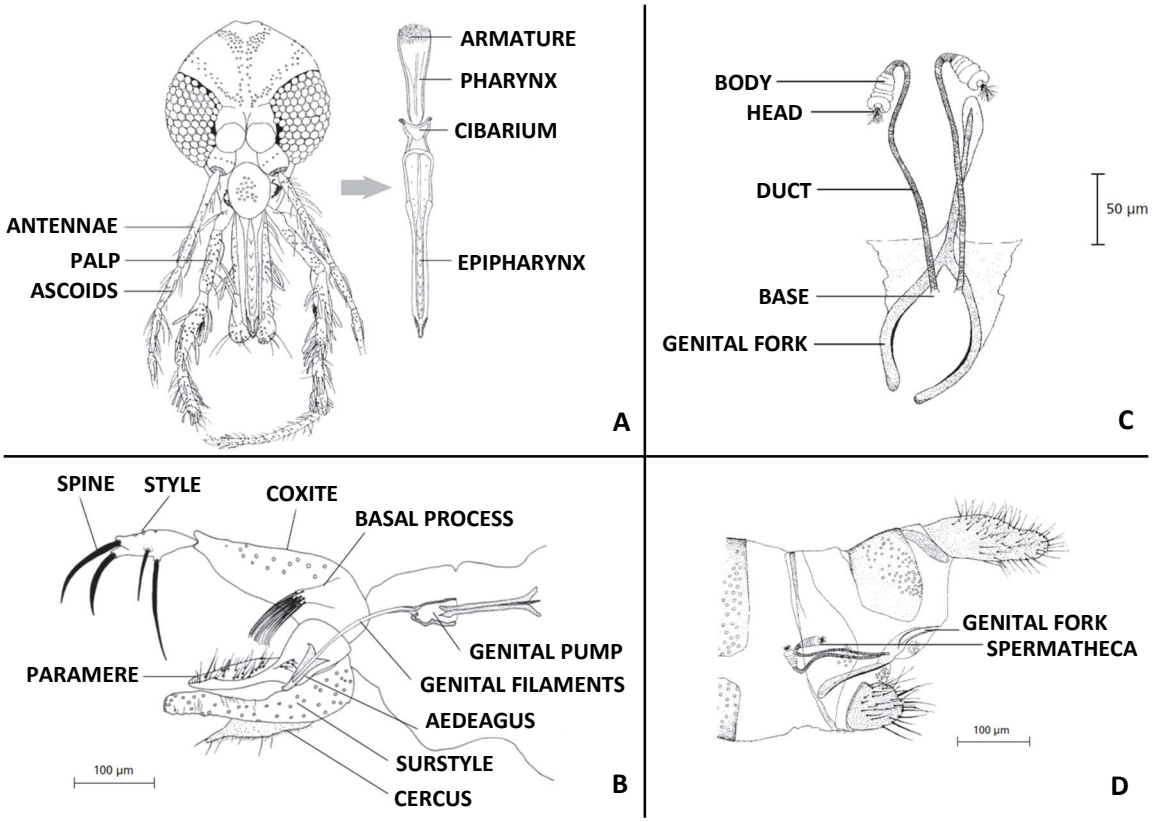

Figure 3. Main morphological characters useful for sand fly species identification: head (A), external genitalia in males (B), spermatheca (C) and distal segments of abdomen (D) in females. Source: based on Depaquit and Léger (2017).

Sand fly biology and ecology, and vectorial competence of *Leishmania* spp.

Phlebotomine sand flies undergo complete metamorphosis to reach the adult stage. Immature stages include egg, larvae (four instars) and pupa (Figure 4) (Killick-Kendrick, 1999). They are fully terrestrial insects and the time to complete a life cycle varies according to the species, geographical area and temperature (Volf and Volfova, 2011). Under laboratory conditions, *P. perniciosus* colonies, originating from Spain, take 41-47 days at 25-26°C from blood-feeding of females to the emergence of new adults (Volf and Volfova, 2011). Instead, development time from egg to adult stage for *Phlebotomus*

papatasi colonies can range between 28 days at 32°C and 246 days at 18°C (Kasap and Alten, 2005). In temperate zones, fourth-instar larvae enter diapause (dormancy period) in response to unfavorable environmental conditions (Killick-Kendrick, 1999).

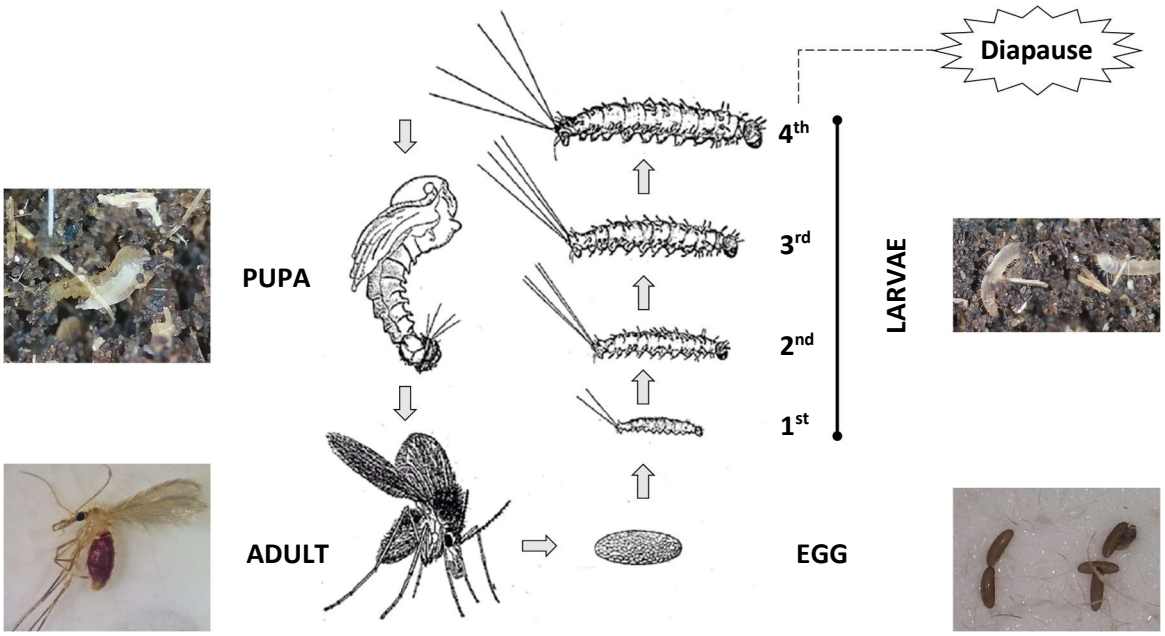

Figure 4. Sand fly life cycle: Egg - Larva (four instars) - Pupa - Adult. Source: based on Lawyer *et al.* (2017).

The precise environment where sand fly immature stages develop are not well characterized (Feliciangeli, 2004). Notwithstanding this, sand flies are known to breed in a wide variety of urban, periurban, rural and sylvatic habitats, including abandoned buildings, dwellings, burrows, farms, caves, rocks, walls, cracks, holes, leaf litter or hollow trees (Feliciangeli, 2004). Adequate sand fly breeding sites must provide moisture and organic matter for larvae to feed. Gravid females lay eggs in specific oviposition sites in response to pheromones of conspecific eggs previously laid in the same place and to other physical and chemical constituents of the site´s substrate or microenvironmental characteristics (Elnaiem and Ward, 1991; Killick-Kendrick, 1999).

Adults feed on sugar from plants or small insects, and females must also feed on blood that is necessary for the development of the eggs. Most sand fly species take only one bloodmeal or more if they cannot complete it, and some are able to produce viable eggs by autogeny without taking a bloodmeal (Killick-Kendrick, 1999; Shymanovich *et al.*, 2020). Sand fly activity is not uniform (Killick-Kendrick, 1999), and the occurrence of high

activity peaks depends on the study area (habitat, microenvironmental conditions) and the sand fly species, sex and reproductive stage in the case of the females (i.e., gravid, blood-fed or unfed). In eastern Spain, 84.7% of the total catches were achieved between 20:00 h and 23:00 h, coinciding with temperature and relative humidity between 18.7-21.6°C and 77.4-86.3%, respectively (Lucientes *et al.*, 2005b). In Murcia, *Phlebotomus sergenti* and *P. perniciosus* were active from 20:00 h to 06:00 h, showing two high activity peaks (Romera Lozano and Martínez Ortega, 1998). In Europe, the overall maximum activity peak for all species was between 23:00 h and 02:00 h, coinciding with the period of highest risk for *Leishmania* transmission (Alten *et al.*, 2016). During daytime, these insects remain sheltered in resting sites, which are usually fresh and humid microenvironments such as animal stables, houses, farms, caves, tree holes, leaf litter, rocks, burrows, cracks in walls or among vegetation (Killick-Kendrick, 1999). A deeper knowledge on the behavior of hematophagous females, in terms of where they prefer to feed and rest while blood is digested, may be very valuable for designing vector control strategies (Gálvez *et al.*, 2018). For instance, *P. ariasi* is predominantly exophagic and exophilic, i.e., feeding and resting occurs outdoors, respectively (Alvar Ezquerra, 2001).

Phlebotomine sand flies are poor flyers, tend to move in short hopping flights and are more active close to the ground and vertical surfaces, such as walls where they can easily go upwards (Faiman *et al.*, 2011; Maroli *et al.*, 2013). Flight speed does not usually exceed 1 m/s (Killick-Kendrick *et al.*, 1986), and wind is the main limiting factor for sand fly dispersal. Movement range is usually short, only a few hundred meters, so they are assumed not to disperse far from their breeding sites (Killick-Kendrick, 1999). Some authors have demonstrated that their dispersal distance range varies according to habitat, sand fly species and sex, although the maximum rarely exceeds one kilometer (OMS, 2010). However, using mark-release-recapture techniques, a *P. ariasi* female in France was shown to travel 2,200 m, possibly in search of food (Killick-Kendrick *et al.*, 1984). Other species, such as *P. papatasi* and *P. langeroni*, were captured 1,500 m from the release point in Egypt (Doha *et al.*, 1991).

Most phlebotomine species are not involved in *Leishmania* transmission, as vectors have to meet some requirements (Killick-Kendrick, 1999; OMS, 2010). They have to 1) feed on humans or susceptible hosts, 2) bite and be in contact with reservoirs of infection,

3) be infected with the same *Leishmania* that affects susceptible hosts, 4) transmit the parasite while feeding and 5) allow multiplication and development of the parasite. Parasite development in the vector takes place in the digestive tract of the insect and this can be investigated using molecular detection of parasite DNA and dissection and microscopy to confirm the location and development stage of *Leishmania* spp. (Seblova *et al.*, 2012). Fixation of promastigotes to the intestinal epithelium of the vector is one of the critical steps for *Leishmania* development, and mechanisms are different depending on the parasite and vector species (Volf and Myskova, 2007). For example, the main *L. infantum* vectors in Spain, *P. perniciosus* and *P. ariasi*, are permissive to other *Leishmania* species. Instead, *P. papatasi* and *P. sergenti* are considered specific vectors of *Leishmania major* and *L. tropica*, respectively, and do not transmit *L. infantum* (Dvorak *et al.*, 2018). Vector competence of permissive species has important epidemiological consequences, as it enables *Leishmania* spp. to spread into new areas where these vectors are present (Volf and Myskova, 2007).

The estimated infection rate in vectors (i.e., the percentage of sand flies infected with *Leishmania* spp.) is a good indicator of transmission intensity and is epidemiologically important. It depends on several factors, such as the epidemiological situation in the study area, the time of year, the vector´s blood-feeding patterns, female physiological status, and methodology employed to assess infection in the vector, and is highly variable in Spain (Martín-Sánchez *et al.*, 2006; Torina *et al.*, 2008; Maia *et al.*, 2009). In a leishmaniasis endemic foci in Catalonia, 38.7% of females found to be infected (Alcover *et al.*, 2012). Entomological surveillance of human leishmaniasis outbreak in southwest Madrid detected an overall infection rate in *P. perniciosus* of 58.5% (Jiménez *et al.*, 2013). In contrast, 0.33% of the same species presented *Leishmania* spp. DNA in Extremadura and 0.45% in Granada and Catalonia (Martín-Sánchez *et al.*, 2006; Bravo-Barriga *et al.*, 2016). In Málaga province, Morillas *et al.* (1996) found that 3.8% of dissected *P. perniciosus* and *P. ariasi* were infected with *L. infantum*.

Blood-feeding preferences of female sand flies

As stated above, adult females are responsible for pathogen transmission, as they need to take blood from vertebrate hosts. The small amount of blood ingested (0.5-0.9

µl) is rapidly surrounded in the midgut by the peritrophic matrix, where blood is digested by proteolytic enzymes (Pruzinova *et al.*, 2015; Dvorak *et al.*, 2018). Host-feeding patterns can be ascertained by analyzing these bloodmeals present in the sand fly abdomen, and is a very useful tool for understanding the interactions between pathogens, hosts and vectors (Kent, 2009). These investigations are of utmost ecological and epidemiological importance, since they can provide insight into diversity of blood sources as well as the vectorial competence in leishmaniasis outbreaks (Haouas *et al.*, 2007). In Algeria, high percentages of *Larroussius* females were shown to feed on small ruminants and equids, which are non-reservoirs of *L. infantum* and, therefore, could explain the low infection prevalence in sand flies (Bennai *et al.*, 2018). In addition, the level of exposure of hosts to sand fly bites can be estimated, and thus their potential implication in parasite transmission cycles as reservoirs (Pareyn *et al.*, 2020). In this sense, information about what host species are acting as reservoirs is required to establish efficient control measures in leishmaniasis foci (González *et al.*, 2017).

Phlebotomus perniciosus and *P. ariasi* have been found to exhibit an opportunistic behavior and feed on a wide range of hosts (Figure 5), depending on the habitat and host availability (Guy *et al.*, 1984; Branco *et al.*, 2013; Maia *et al.*, 2013, 2015; Bravo-Barriga *et al.*, 2016; Cotteaux-Lautard *et al.*, 2016; González *et al.*, 2017; Latrofa *et al.*, 2018; Abbate *et al.*, 2020; Iatta *et al.*, 2020). In Italy, the vast majority of *P. perniciosus* females took blood from existing animals in the shelters where the insects were captured (Bongiorno *et al.*, 2003), and particularly from lagomorphs (33%) (Abbate *et al.*, 2020). In contrast, 87% of the same species in Portugal fed mainly on rodents (Maia *et al.*, 2013). *Sergentomyia* species are vectors of *Leishmania* spp. infecting reptiles and have been traditionally considered as herpetophilic species, i.e., preference to feed on cold-blooded vertebrates. But some authors have noted that they sporadically feed on humans and other mammals (Figure 5). Besides, *Leishmania* DNA has been detected in several *Sergentomyia* species, suggesting the possible role of this genus in the transmission of *Leishmania* species pathogenic to humans and other mammals (Maia and Depaquit, 2016; González *et al.*, 2020). However, the mere detection of parasite DNA in sand flies is not enough to incriminate a species as a biological vector, as this fact does not demonstrate

that such species is able to transmit *Leishmania* to vertebrate hosts (Killick-Kendrick, 1999).

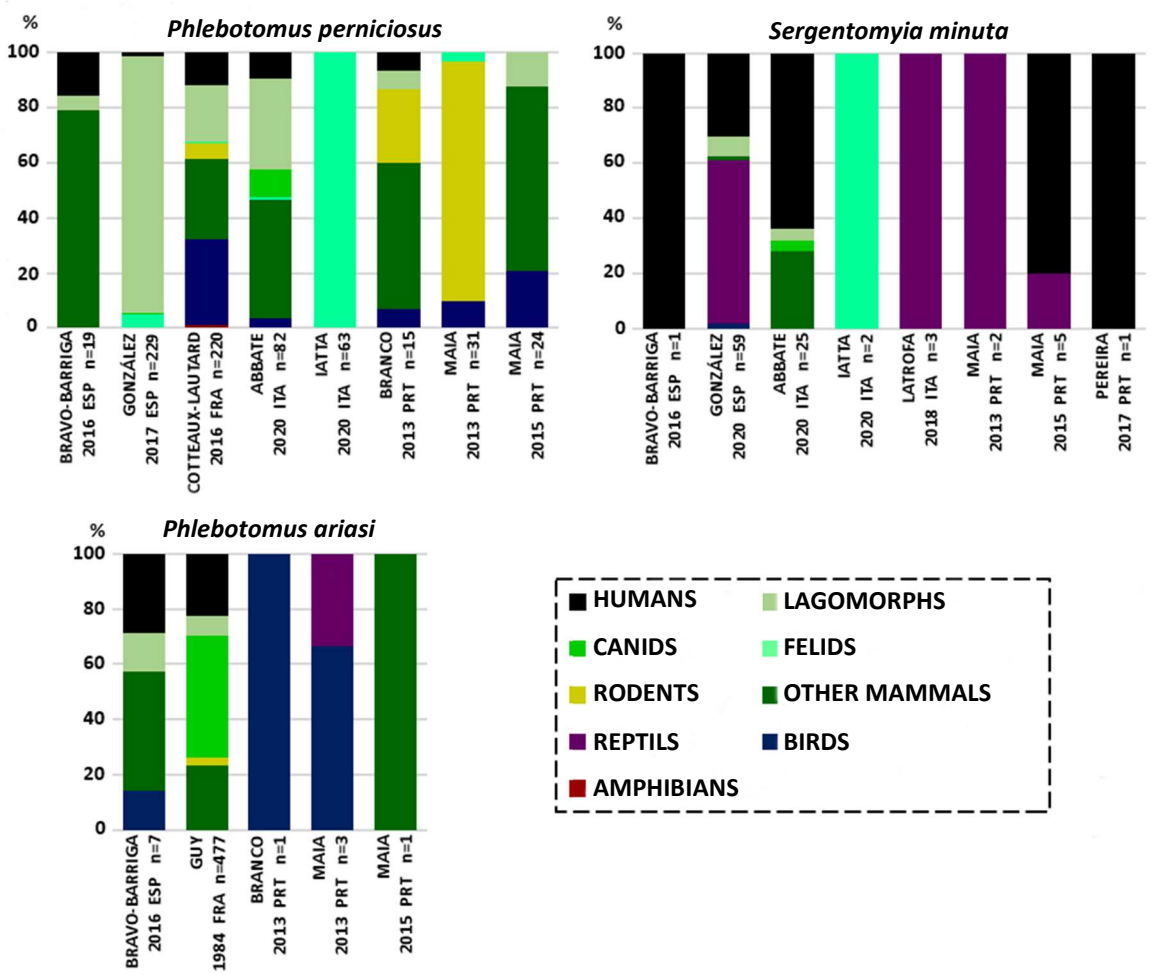

Figure 5. Percentage of identified bloodmeals in *P. perniciosus*, *P. ariasi* and *S. minuta*, from studies carried out in Spain (ESP), France (FRA), Italy (ITA) and Portugal (PRT), using molecular or serological techniques. Every bar corresponds to data of one study (first author, publication year, country and number of sand flies tested).

Identification of host-feeding preferences may be a challenging task, as relative frequencies alone do not provide enough information on preferences for a particular host (Svobodová *et al.*, 2003), and other factors that may influence host choice need to be considered. For this purpose, several authors have used the forage ratio, which takes into account the number of available hosts for each species (Bongiorno *et al.*, 2003; Rossi *et al.*, 2008; Maroli *et al.*, 2009), and can be defined as the proportion of bloodmeals of a given host divided by the relative abundance of such host at the sampling site. Values greater than one indicate preference for the analyzed host (Hess *et al.*, 1968). The host selectivity index (HSIx) takes into account the animal body mass and is calculated by

dividing the number of females fed on a given host by the available biomass (Agrela *et al.*, 2002; Rossi *et al.*, 2008; Maroli *et al.*, 2009). In addition to census and body mass, other factors that should be considered for understanding host-feeding preferences are distance to animals, size and defensive behavior of hosts, availability, carbon dioxide (CO_2) emissions, accessibility to skin, individual odors and use of insecticides or repellents, among others (Kent, 2009; Gebresilassie *et al.*, 2015; Cotteaux-Lautard *et al.*, 2016; Pareyn *et al.*, 2020).

Sand fly species in Spain

Since phlebotomine sand flies were first reported in El Escorial (Madrid) in 1909, numerous studies on sand fly distribution and morphology have been conducted in Spain (Gil Collado *et al.*, 1989). To date, 13 species have been described (Table 2), belonging to genera *Phlebotomus* (11 species grouped into 5 subgenera) and *Sergentomyia* (2 species) (Gil Collado *et al.*, 1989; Gállego-Berenguer *et al.*, 1992; Martínez Ortega *et al.*, 1992; Depaquit *et al.*, 1998).

Table 2. Taxonomic classification of sand fly species described in Spain.

GENUS ***Phlebotomus***		GENUS ***Sergentomyia***
Subgenus ***Paraphlebotomus***	Subgenus ***Larroussius***	Subgenus ***Sergentomyia***
P. alexandri	*P. ariasi*	*S. fallax*
P. chabaudi	*P. langeroni*	*S. minuta*
P. riouxi	*P. longicuspis*	
P. sergenti	*P. perniciosus*	
Subgenus ***Transphlebotomus***	Subgenus ***Phlebotomus***	
P. mascittii	*P. papatasi*	
Subgenus ***Anaphlebotomus***		
P. fortunatarum		

Nevertheless, the actual presence of *P. longicuspis* and *P. riouxi* in the country has been questioned, given the morphological similarity with *P. perniciosus* and *P. chabaudi*, respectively (Collantes and Martínez Ortega, 1997; Martín-Sánchez *et al.*, 2000; Lehrter *et al.*, 2017). Of the remaining 11 species, only *P. ariasi* and *P. perniciosus* are proven vectors of *L. infantum* (Lucientes-Curdi *et al.*, 1988; Guilvard *et al.*, 1996), although a recent study has shown the possible role of *P. langeroni* in the transmission of this parasite (Díaz Sáez *et al.*, 2018). Figure 6 shows the spatial distribution of *L. infantum* vectors in Spain. *Phlebotomus perniciosus* is the most abundant species of such genus in the Iberian

Peninsula and Balearic Islands, as is well-adapted to all types of environments, while *P. ariasi* prefers cold and humid habitats (Martínez Ortega and Conesa Gallego, 1987a; Gil Collado *et al.*, 1989). On the other hand, *P. langeroni* populations are preferentially found in arid environments, mainly associated with rabbit burrows (Lucientes *et al.*, 1995; Díaz Sáez *et al.*, 2018).

In Murcia Region, first investigations on distribution and phenology of sand fly populations were carried out in the 1980s, describing a total of 8 species: *P. alexandri*, *P. ariasi*, *P. chabaudi*, *P. longicuspis*, *P. papatasi*, *P. perniciosus*, *P. sergenti* and *S. minuta* (Martínez Ortega, 1984; Martínez-Ortega, 1985; Martínez Ortega and Conesa Gallego, 1987a, 1987b, 1987c). More recently, Risueño *et al.* (2017) performed an entomological survey in the whole region to analyze how environmental conditions affect vector abundance. A large number of these insects thrived in sheep farms and kennels, although the distribution on a large geographical scale was highly variable depending on climatic conditions (Risueño *et al.*, 2017).

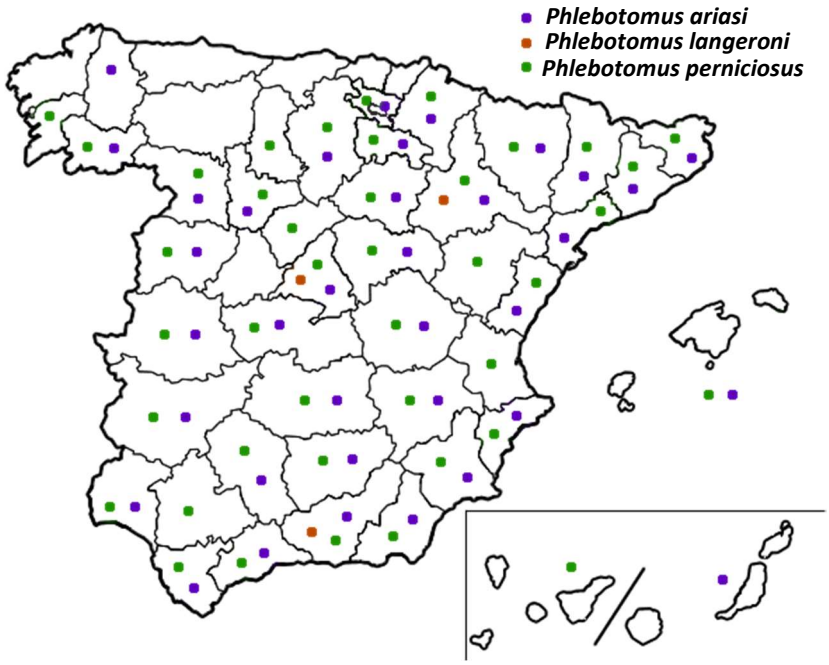

Figure 6. Distribution map of sand fly vectors of *L. infantum* in Spain: *P. ariasi*, *P. langeroni* and *P. perniciosus*. Source: based on Gil Collado *et al.* (1989), Gállego-Berenguer *et al.* (1992), González Peña (1994), Léger *et al.* (1995), Conesa Gallego *et al.* (1999), Lucientes *et al.* (2002), Aransay *et al.* (2004), Franco *et al.* (2010), Alcover *et al.* (2012), Durán-Martínez *et al.* (2013), Bravo-Barriga *et al.* (2016), Díaz Sáez *et al.* (2018), González *et al.* (2019), Gálvez *et al.* (2020).

Vector distribution and implications on leishmaniasis epidemiology

Phlebotomine sand flies are distributed in tropical and subtropical regions, as well as in temperate zones, occupying latitudes between 50°N and 40°S (Killick-Kendrick, 1999). As ectothermic arthropods, sand flies are governed by climatic conditions, being temperature the most determining factor in the seasonal activity of *L. infantum* vectors in Europe (Alten *et al.*, 2016). The minimum temperature at which sand flies are active is 17°C (Gálvez *et al.*, 2018). In temperate zones, they complete the life cycle during the most favorable time of the year, between May and November, and phenology depends on latitude and sand fly species. In southeast Spain, *P. ariasi* shows a monophasic pattern with an annual maximum in the warmer months in humid and high altitude areas, but also a biphasic pattern in more arid environments, with two maximum activity peaks in the temperate months and separated by the warm months (July and August) when abundance decreases dramatically (Martínez Ortega and Conesa Gallego, 1987a).

Mathematical models enable accurate predictions of sand fly distributions considering different climate change scenarios. In this sense, vectors are predicted to spread northwards in Europe, which may imply a higher risk for *Leishmania* transmission and establishment in those areas (Chalghaf *et al.*, 2018). Apart from spreading into new areas, changing climate conditions can also increase sand fly density, as annual activity period would be longer and the completion of the life cycle faster. In addition to factors affecting host and pathogen, vector distribution is one of the most important risk factors related to emerging leishmaniasis outbreaks in Europe (Medlock *et al.*, 2014).

However, despite evidence that infection risk is related to the sand fly abundance (Risueño *et al.*, 2018), other overlapping conditions are required for a successful parasite transmission, such as infection in vectors and availability of susceptible hosts and reservoirs as blood sources (ECDC and EFSA, 2018a). The absence of *L. major* in Spain illustrates this , because *Psammomys obesus* and *Meriones* spp. rodents are absent in Spain and are indeed the natural reservoir of this parasite in other regions, such as North Africa and the Middle East, where they are widely spread (Samy *et al.*, 2016).

Entomological surveillance and quantitative studies of sand fly populations

Vector control programs aim to disrupt parasite transmission to susceptible hosts. To this effect, different control strategies to reduce vector-host contact are available, such as the use of insecticides and repellents or environmental management (Gálvez *et al.*, 2018). The success of these control measures depends on a detailed understanding of vector population dynamics (OMS, 2010). As said before, sand fly distribution is spatially overdispersed, as they concentrate in places with suitable environmental conditions, depending on microclimate, vegetation, land use and presence of hosts, among others (Branco *et al.*, 2013; Rioux *et al.*, 2013; Ballart *et al.*, 2014). However, because of the difficulty in finding immature sand fly stages in the natural environment, most sand fly surveillance and monitoring studies focus on the adult stage (Alten *et al.*, 2015). These studies are useful for a number of reasons, including gathering information about pathogen circulation, host-feeding patterns, and assessment of control strategies (ECDC and EFSA, 2018b). Sampling design therefore depends on the study goal and entomologists can use a wide range of sand fly trapping methods (Alexander, 2000; Alten *et al.*, 2015).

The vast majority of investigations estimating sand fly distribution and density use interception and attraction traps (Alten *et al.*, 2015). The former captures a random selection of insects flying in the vicinity of the traps. The most commonly used are sticky traps, which consist of paper sheets impregnated with viscous substances such as castor oil. These are ideal for ecological studies and sand fly catches can be standardized as the number of sand flies/m^2, which enables comparison between studies. However, sticky traps are not effective in high-humidity environments and only dead specimens can be recovered, which hampers isolation of RNA viruses (Alexander, 2000; Alten *et al.*, 2015). On the other hand, attraction traps are based on lures, such as light, CO_2, odors or live baits that attract insects. Among them, the most commonly used trap is the miniature light trap developed by the Center for Disease Control and Prevention (CDC). Attraction range is limited to 2-6 m depending on the species (Killick-Kendrick *et al.*, 1985; Valenta *et al.*, 1995; Campbell-Lendrum *et al.*, 1999), though adding CO_2 to the light trap allow to attract insects at higher distances (Alexander, 2000; Alten *et al.*, 2015). Besides, more sand flies are collected compared to sticky traps and many of them remain alive.

Alternative configurations to the traditional light trap have been examined, including inverted orientation of the trap opening (Kline *et al.*, 2011) and LED (light-emitting diodes) technology with variations in color, wavelength and light intensity (Gaglio *et al.*, 2018; Lima-Neto *et al.*, 2018). For more information, Alexander (2000) and Alten *et al.* (2015) reviewed all entomological methodologies for adult and immature sand flies, including the aforementioned.

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OBJECTIVES

The **overall goal** of this doctoral thesis is to improve our understanding of the small-scale distribution and dispersal of *Leishmania* vectors, their host-feeding preferences and the consequences of the above aspects on the epidemiology of leishmaniasis in rural and periurban microenvironments of Murcia Region. The specific objectives are:

1. To investigate how the density of sand flies collected in a particular site is affected by the way traps are positioned, in terms of distance to the ground and to continuous or discontinuous vertical surfaces. This objective will be examined in **Chapter 1**.

2. To investigate the spatial and temporal distribution of *Phlebotomus perniciosus* on a small geographical scale in relation to distance to animal groups, in a high sand fly density rural environment. This objective will be examined in **Chapter 2**.

3. To investigate the spatial distribution of sand flies in a periurban zoological park and its potential involvement in the epidemiology of leishmaniasis in neighboring human residential areas. This objective will be examined in **Chapter 3**.

4. To investigate host-feeding preferences of female sand flies in a host species rich environment, taking into account the insect´s movement cost to reach potential hosts. This objective will be examined in **Chapter 4**.

CHAPTER 1

On how trap positioning affects phlebotomine sand fly density estimations

Abstract

There is a need for standardizing sand fly sampling methodology and guidance on trap positioning for quantitative sand fly studies. We investigated differences in sand fly density with sticky interception and CO_2-light attraction traps, in relation to trap distance to the ground and to the presence or absence of a continuous (wooden plank) or discontinuous (wire mesh) vertical surface adjacent to the trap. The study, conducted in a dog kennel in southeast Spain, lasted 48 days and collected 692 *Phlebotomus papatasi*, *Phlebotomus perniciosus*, *Phlebotomus ariasi* and *Sergentomyia minuta* specimens. There were no significant differences between species with respect to trap position. Overall, density in sticky traps was highest closest to the ground and next to the continuous vertical surface, followed sequentially by traps similarly placed adjacent to the wire mesh and those hanging from a rope across the kennel yard. In contrast, density in CO_2-light traps was highest in traps hanging from the rope near the ground, followed by those next to the continuous vertical surface. The overall negative relationship between sand fly density and ground distance was not significant for CO_2-light traps next to the continuous vertical surface. Modelling also suggested that sand flies do not use the wire mesh to move vertically.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are hematophagous vectors of pathogens of great medical and veterinary concern, including the protozoan *Leishmania* spp. (Trypanosomatida: Trypanosomatidae) and *Phlebovirus* (Bunyavirales: Phenuiviridae) (Dujardin *et al.*, 2008; Alkan *et al.*, 2013). Control of sand fly-borne infections relies on preventing transmission, and success is limited by a scarce understanding of sand fly and parasite dynamics in natural environments. More surveys are needed to assess environmental conditions influencing vector demographics and the risk of vector-borne infections (Muñoz *et al.*, 2019). Sand fly density surveys focus on trapping adult sand flies, either by interception, using castor oil-impregnated paper or polypropylene sheets (sticky traps), or by attraction with battery-operated light-suction traps, baited or not with CO_2 (Alexander, 2000; Alten *et al.*, 2015). Sticky traps collect a random sample of sand flies flying in the proximity of the trap and are ideal for ecological

studies (Alexander, 2000). Light traps bias species with high positive phototropism including most *Leishmania* spp. vectors, and their radius of action is less than 10 m (Killick-Kendrick *et al.*, 1985; Valenta *et al.*, 1995). CO_2 is a strong attractant particularly for host-seeking females (Muñoz *et al.*, 2018).

Sand fly density estimation depends on where and how traps are positioned. These are frail insects that concentrate in sheltered environments, and when foraging, they typically move forward along horizontal and vertical surfaces in short hopping flights (Faiman *et al.*, 2011a; Kirstein *et al.*, 2018). Faiman *et al.* (2011b) found that the number of *Phlebotomus sergenti* in residential neighborhoods in the Jordan Valley in Israel, was greater when sticky and light traps were placed against a wall or net barrier compared to those on a diamond wire fence. Moreover, density was inversely proportional to the height at which traps were placed, which ranged between 50 and 290 cm for sticky traps and ground level and 2 m for light traps. In Sicily, Gaglio *et al.* (2014) recorded a similar negative association between trap height and *Phlebotomus perniciosus* captures but not for *Sergentomyia minuta*. In Greece, Chaskopoulou *et al.* (2018) found no difference in *Phlebotomus perfiliewi* abundance in sticky traps placed between 50 and 150 cm from the ground but the number was lower in those situated at a height of 2 m. Other studies have shown that the spatial distribution of sand flies may vary significantly depending on the species, gender and adult female life stages, due to intrinsic ecological, biological and behavioral traits (Muñoz *et al.*, 2018, 2019). Understanding how sand flies behave in open spaces and how they deal with vertical surfaces is essential to study the insect's host approach behavior and to optimize and design effective vector control measures (Faiman *et al.*, 2011b; Chaskopoulou *et al.*, 2018; Kirstein *et al.*, 2018). This study was designed to further investigate the relationship between sand fly density and trap positioning in a *Leishmania infantum* endemic area in southeast Spain, where *Phlebotomus papatasi*, *P. perniciosus* and *S. minuta* are the predominant species (Risueño *et al.*, 2017). The study was performed over a relatively long time and used statistical modelling techniques to consider the potential confounding effect of climate and seasonality on sand fly density estimations.

Materials and methods

Study area and sand fly trapping design and identification

The study was carried out in a 415 m^2 open-air dog kennel, hosting 30-40 Beagle dogs at Murcia Veterinary School (38°00.501'N, 1°10.656'W). Sampling was performed during 48 nights (08:00 pm to 08:00 am) in September and October 2015, and in June, July and September 2016, using sticky interception traps and CO_2 and light attraction (CO2L) traps on alternate weeks to avoid interferences. The former consisted of A4-paper sheets impregnated in castor oil and the latter were miniature Centers for Disease Control (CDC) battery-operated light-suction traps (J.W. Hock Company, Gainesville, FL, USA) connected by a rubber tube to a polystyrene box containing 400 g of CO_2 dry ice.

Traps were placed at increasing distance to the ground in three different ways. The mean distances from the ground to the base of the sticky traps and to the light of CO2L traps were 15 cm, 75 cm and 150 cm, respectively. The way traps were placed were: (i) hanging from a rope across the kennel yard, (ii) directly on the perimeter woven wire fence and (iii) leaning on a vertical, smooth surface wooden plank that stood against the perimeter wire fence (Figure 1). Trap number, dimensions, disposition and location, and sampling time varied between years. In 2015, three CO2L traps and three rows of sticky traps (1.25 m^2/height) were hung on the wire fence at the three selected heights for 5 and 15 nights, respectively (Figure 1). In 2016, sticky traps were placed in the three places (rope, wire mesh and wooden plank) and heights for 7-8 nights, but they were set in a stepwise rectangle arrangement (0.75 m^2/height in the rope, 1.12 m^2/height in the wire mesh and 0.56 m^2/height in the wooden plank) (Figure 1). This was done to avoid trap rows below being in the way of sand flies possibly climbing vertically. In addition, CO2L traps were also placed in 2016 for 4-5 nights in the three different places and heights (Figure 1). The sampling effort was calculated as the sticky trap area (m^2) recovered or the number of CO2L traps, multiplied by the number of nights. The total study sampling effort was 110 m^2*nights for sticky traps and 54 traps*nights for CO2L traps.

Sand flies collected from traps were processed and identified morphologically as previously described (Muñoz *et al.*, 2018, 2019).

Figure 1. Depiction of trap location in the open-air dog kennel in southeast Spain using sticky (A-C) and CO2L traps (D-F). Sticky traps were set in parallel rows at the wire fence in 2015 (A) and in a stepwise rectangle arrangement at the wire fence (B), wooden smooth plank (B) and hung from a rope (C) in 2016. CO2L traps were placed in a stepwise arrangement at the wire fence in the two study years (D) and at the wooden smooth plank (E) and hung from a rope (F) in 2016.

Environmental data collection and statistical analysis

Temperature and relative humidity (RH) at the dog kennel were recorded at three-hour intervals using a thermohygrometer (Digital Logtag Haxo-8T, Templyzer) and used to calculate the nightly (8 pm to 8 am) mean temperature (°C) and RH (%). The mean and maximum wind speed (m/s) were measured using three anemometers (AZ 77535; Herter Instruments), which were placed on the wire fence adjacent to the traps at the three selected distances to the ground (Figure 1).

The sticky trap unit of analysis included all the traps set at a particular height and location, and positive traps were those with at least one sand fly. Sand fly density was the

number of sand flies collected divided by the sampling effort. Mixed effects negative binomial regression models were developed to investigate the relationship between sand fly density and trap position (the combination of trap distance to the floor and leaning surface), adjusted for climatic variables, month and sampling day (Kleinbaum *et al.*, 1998). Explanatory variables were included in the model as fixed effects except for day which was fitted as a random effect (Snijders and Bosker, 1999). Akaike´s information criteria (AIC) was used to compare the goodness of fit of models with the mean or the maximum wind speed and those with the lowest value were selected. Models were estimated using the maximum likelihood method using the glmer.nb function in in the lme4 package in R (https://cran.r-project.org/web/packages/lme4/lme4.pdf) (Bates *et al.*, 2015). For all analysis significance was considered when α=5% ($p<0.05$) for a two-tailed test.

Results and discussion

A total of 692 sand flies were collected and sand fly density was 3.0 (331/110) specimens/m^2/night for sticky traps and 6.7 (361/54) specimens/trap/night for CO2L traps (Table 1). Among the 682 successfully speciated sand flies, females and males represented 51% and 49%, respectively. *Phlebotomus papatasi* was the most numerous species (52%), followed by *P. perniciosus* (32%), *S. minuta* (15%) and *P. ariasi* (<1%). However, as shown in other studies (Kasap *et al.*, 2009; Signorini *et al.*, 2013; Risueño *et al.*, 2017), species frequencies varied according to trap type used, and *P. perniciosus* was the most abundant species in CO2L traps. This suggests that this species displays a higher positive phototropism than *P. papatasi* (Table 1). Notwithstanding this, CO_2 and light have been shown to enhance the trapping success of the latter species (Müller *et al.*, 2015).

Table 1. Percentage of sand fly positive traps and sand fly density according to trap type, location and distance to the floor, and number of sand fly specimens according to species and gender. A study in an open-air kennel in Murcia, southeast Spain.

TRAP						SAND FLIES									
Type	Location	Mean ground distance (cm)	No.	% positive	Area (m^2)	No.	Density	No. by species and gender							
								P. ariasi		*S. minuta*		*P. papatasi*		*P. perniciosus*	
								♂	♀	♂	♀	♂	♀	♂	♀
Sticky	Rope	15	7	71	5.0	15	3.0	0	0	2	2	4	5	2	0
		75	7	14	5.2	1	0.2	0	0	1	0	0	0	0	0
		150	7	0	5.2	0	0.0	0	0	0	0	0	0	0	0
	Wire fence[a]	15	23	91	27.4	218	7.9	0	0	12	12	92	81	7	12
		75a	15	73	18.7	20	1.1	0	0	5	0	4	8	2	1
		75b	8	12	8.5	1	0.1	0	0	0	0	0	0	1	0
		150a	15	20	18.7	5	0.3	0	0	1	0	0	1	1	2
		150b	8	12	8.1	1	0.1	0	0	0	0	0	0	0	0
	Wooden plank	15	8	88	4.5	39	8.7	0	0	11	1	15	6	5	1
		75	8	75	4.5	14	3.1	0	0	6	1	3	2	2	0
		150	8	62	4.4	17	3.8	0	0	7	3	4	1	0	2
	All		**114**	**54**	**110.2**	**331**	**3.0**	**0**	**0**	**45**	**19**	**122**	**104**	**20**	**18**
CO2L	Rope	15	4	100	-	70	17.5	0	1	1	5	10	6	24	20
		75	4	100	-	24	6.0	0	0	0	2	1	3	9	9
		150	4	50	-	3	0.8	0	0	0	0	1	2	0	0
	Wire fence	15	10	100	-	94	9.4	1	0	8	7	8	31	16	20
		75	10	80	-	39	3.9	0	0	4	2	7	3	6	17
		150	10	20	-	9	0.9	0	0	0	1	1	1	3	3
	Wooden plank	15	4	100	-	40	10.0	0	1	2	1	7	10	8	10
		75	4	100	-	56	14.0	0	0	1	2	12	16	7	18
		150	4	100	-	26	6.5	0	0	3	2	3	9	4	5
	All		**54**	**78**	**-**	**361**	**6.7**	**1**	**2**	**19**	**22**	**50**	**81**	**77**	**102**

[a] 75a and 150a: 2015 sticky trap disposition, placed in rows above other rows of sticky trap.
75b and 150b: 2016 sticky trap disposition, stepwise rectangle arrangement with no sticky traps below.

Sand fly density varied significantly according to trap position and depending on the trap type (Table 1). These differences, however, were similar across species (Figure 2). Overall sand fly density in sticky traps was much lower in traps hanging from the rope (1.0 sand flies/m^2/night) compared to those in the perimeter fence (3.3 sand flies/m^2/night) (Table 1). These results suggest that sand flies concentrated along the perimeter fence rather than in open kennel yard space. The irregular fence support structure at the base combined with an accumulation of grass and organic material around it, probably offered sand flies a suitable microenvironment. Moreover, the presence of the vertical, solid wood surface, would allow the insects to naturally wander further away from the ground explaining why comparatively more flies were found in sticky traps placed at higher altitudes in the plank than in those similarly placed on the mesh (Table 1). In contrast, sand fly density in CO2L traps was highest in traps hanging from the rope and close to the ground (17.5 sand flies/trap/night) followed by those on the wooden plank at middle and low distance from ground (14 and 10 sand flies/trap/night, respectively) (Table 1). Light traps hanging from the rope had a 360° attraction range compared to 180° for those on the wooden plank. The marked difference between sticky and CO2L traps would relate to the very essence of interception and attraction traps described before. Light and CO_2 are strong attractants stimulating sand flies to move in all directions from their natural resting places close to the ground. Possibly, in this study CO_2 was a more powerful attractant than light, since the distance between the traps hanging from the rope across the yard and the perimeter fence where sand flies concentrated, was 4-6 m, in the limit of light trap attraction range which was estimated at 2-6 m depending on the experimental design and sand fly species (Killick-Kendrick *et al.*, 1985; Valenta *et al.*, 1995; Campbell-Lendrum *et al.*, 1999).

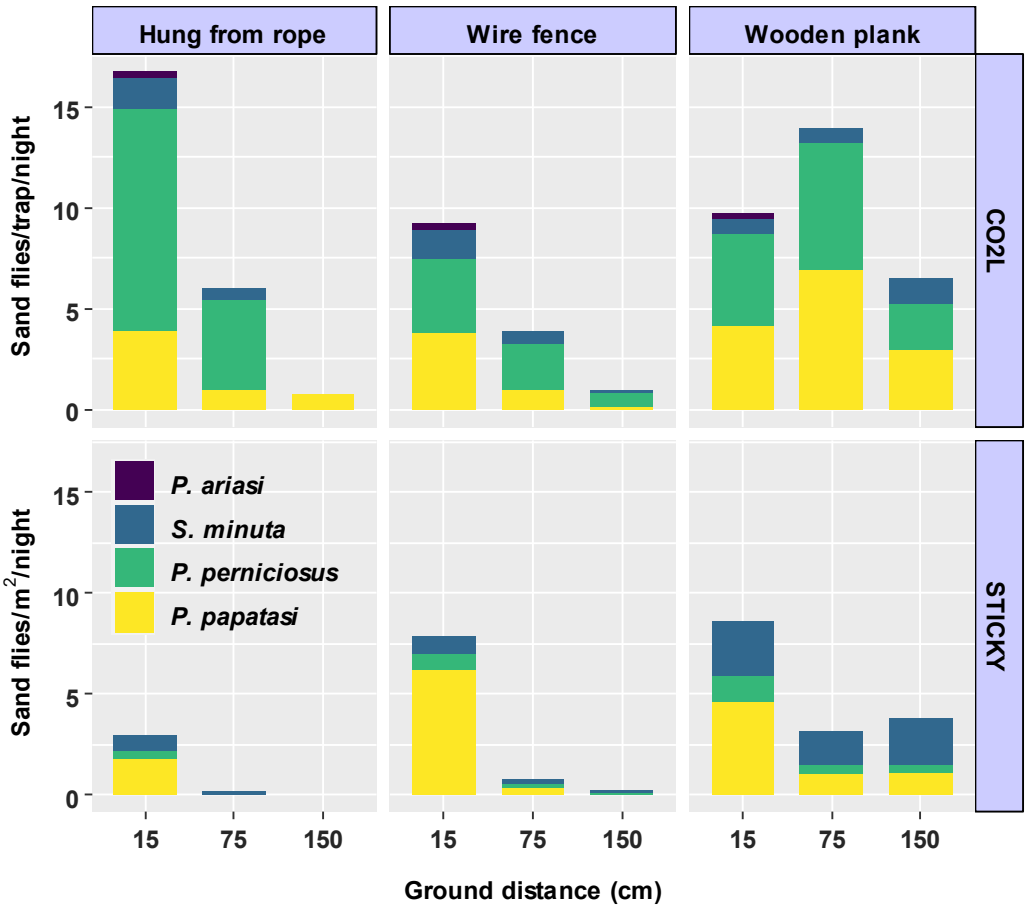

Figure 2. Sand fly species density in CO2L (sand flies/trap/night) and sticky traps (sand flies/m^2/night) according to trap location (rope, wire fence and wooden plank) and ground distance (15, 75 and 150 cm).

The multivariable model confirmed the negative association between sand fly density and distance to floor, particularly in sticky traps hanging from the rope or placed on the wire mesh. Interestingly, this relationship was similar for sticky traps in 2015 and 2016 when they were placed differently (in rows one above each other in 2015 and in a stepwise rectangle arrangement in 2016) (Figure 1; Table 2). This suggests that sand flies do not hop on the wire mesh to move vertically but fly instead. The effect of CO2L trap distance to the ground on sand fly density was smaller, as the number of specimens significantly decreased only in traps placed at 150 cm in open spaces far from a vertical surface, and at 75 and 150 cm in those in the wire fence. The model also reflected the importance of taking into account environmental factors to estimate density accurately. The relationship between climatic variables and sand fly density varied between traps and this is likely related to traps being used on different days with different climatic conditions. As reported in other studies (Branco *et al.*, 2013; Risueño *et al.*, 2017; Muñoz *et al.*, 2018)

density was negatively associated with wind speed and RH in sticky traps (Table 2). Increasing temperature was associated with higher density in CO2L traps. Environmental temperature regulates the sand fly cycle and seasonality, however, sand fly abundance at a particular time depends more on cumulative temperature over a period of time than on temperature on a particular day (Alten *et al.*, 2016).

In contrast to our findings, other authors have highlighted differences between species with respect to trap position. As previously mentioned, Gaglio *et al.* (2014) found differences between *P. perniciosus* and *S. minuta* abundance in relation to trap distance to the ground. In tropical forests, *Lutzomyia infraspinosa* sand flies were found at ground level while *Lutzomyia umbratilis* was also collected at the canopy level between 10 and 20 m (Rotureau *et al.*, 2006). Further studies with a larger sample size are required to investigate potential differences between sand fly species with respect to trap position.

The results of this study have important practical implications. They indicate that it is critical to consider distance to the ground and having an adjacent vertical surface when sampling sand flies with sticky interception traps. In contrast, it is less important to consider these two variables when sampling with CO2L attraction traps. Placing CO2L traps at some distance from the ground is advantageous to reduce animal interference, particularly small mammals, and ants that readily predate on these insects (Alexander, 2000).

Table 2. Estimates from mixed negative binomial regression models investigating the relationship between sand fly density and trap location and distance to the floor, adjusted for climatic variables.

Variable	Level	Sticky interception traps			CO2L attraction traps		
		Estimate	*SE*[a]	P value	Estimate	*SE*	P value
Intercept	(Intercept)	3.42	0.61	0.0000	0.87	0.53	0.1041
Location; mean ground distance (cm)[b]	Solid surface; 15	0.00			0.00		
	Solid surface; 75	-1.13	0.29	0.0001	0.21	0.23	0.3709
	Solid surface; 150	-0.84	0.26	0.0016	-0.57	0.29	0.0529
	Wire fence; 15	-1.00	0.26	0.0001	-0.30	0.30	0.3164
	Wire fence; 75	-	-	-	-1.21	0.34	0.0003
	Wire fence; 150	-	-	-	-2.59	0.43	0.0000
	Wire fence; 75a	-3.88	0.89	0.0000	-	-	-
	Wire fence; 75b	-3.28	0.39	0.0000	-	-	-
	Wire fence; 150a	-4.36	1.08	0.0001	-	-	-
	Wire fence; 150b	-4.58	0.58	0.0000	-	-	-
	Hung from rope; 15	-0.91	0.32	0.0049	0.61	0.32	0.0542
	Hung from rope; 75	-3.65	0.90	0.0000	-0.44	0.36	0.2263
	Hung from rope; 150	-	-	-	-2.74	0.62	0.0000
Maximum wind speed (m/s)	1.4 - 3.3	0.00			0.00		
	3.4 - 5.3	0.06	0.21	0.7888	-0.10	0.17	0.5629
	5.4 - 11.6	-0.65	0.31	0.0346	-0.03	0.28	0.9086
Mean relative humidity (%)	29.7 - 48.2	0.00			0.00		
	51.2 - 69.7	-0.72	0.32	0.0262	0.37	0.37	0.3244
	70.1 - 86.5	-0.70	0.38	0.0637	0.12	0.34	0.7171
Mean temperature (°C)	14.2 - 19.8	0.00			0.00		
	20.0 - 23.7	0.72	0.46	0.1197	0.89	0.30	0.0030
	24.3 - 30.1	0.34	0.55	0.5407	1.61	0.46	0.0004

Table 2 (continued). Estimates from mixed negative binomial regression models investigating the relationship between sand fly density and trap location and distance to the floor, adjusted for climatic variables.

Variable	Level	Sticky interception traps			CO2L attraction traps		
		Estimate	***SE***	**P value**	**Estimate**	***SE***	**P value**
Month-Year	September 2015	0.00			0.00		
	October 2015	-0.84	0.36	0.0202	1.01	0.55	0.0641
	June 2016	-2.00	0.40	0.0000	0.40	0.57	0.4839
	July 2017	-0.76	0.36	0.0370	0.20	0.50	0.6853
	September 2016	-1.10	0.42	0.0084	0.16	0.78	0.8387
Random error		**Variance**			**Variance**		
		0.08			0.04		

[a] SE: standard error.

[b] 75a and 150a: 2015 sticky trap disposition, placed in rows above other rows of sticky trap.
75b and 150b: 2016 sticky trap disposition, stepwise rectangle arrangement with no sticky traps below.

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CHAPTER 2

Investigations of *Phlebotomus perniciosus* sand flies in rural Spain reveal strongly aggregated and gender-specific spatial distributions and advocate use of light-attraction traps

Abstract

The spatial and temporal distribution of *Phlebotomus perniciosus* (Diptera: Psychodidae), the sand fly vector of pathogens of public and animal health importance, was investigated in a high sand fly density rural area in Spain using light-attraction and sticky-interception traps. Traps were placed inside animal buildings and outside at increasing distance from animals. A total of 8,506 sand flies were collected, 87% with light traps. Species frequency differed between trap types. The abundance of *P. perniciosus* decreased exponentially with increasing distance to animals and, while females were most common in the animal enclosure, males predominated in adjoining storage places. Increasing CO_2 concentration had an additional positive effect on female abundance only. Both male and female density increased with rising temperature, and there was some indication that females were more active than males at higher relative humidity. The study confirms that *P. perniciosus* aggregates around animal premises, although male and female distributions differ and should be analyzed separately to account for biological and behavioral differences. This provides further evidence that light traps offer an accurate estimation of the relative spatial and temporal abundance of *P. perniciosus*, conferring an added value for the study of this species and the risk of pathogen transmission.

Introduction

Phlebotomine sand fly species are hematophagous vectors of *Leishmania* spp. protozoa, phleboviruses and *Bartonella* spp. affecting humans and animals in the warm latitudes of the planet. In southeastern Spain, leishmaniasis caused by *Leishmania infantum* (Trypanosomatida: Trypanosomatidae) transmitted by *Phlebotomus perniciosus* is a major disease of dogs and immunosuppressed people (Pasquau *et al.*, 2005; de Ybáñez *et al.*, 2009). Toscana *Phlebovirus* (Bunyavirales: Phenuiviridae) infection associated with meningitis in people has also been reported (Martínez-García *et al.*, 2007) and quite possibly this infection remains underdiagnosed and its impact is unknown. The control of canine leishmaniasis in Europe is mainly based on the application of preventive insecticidal treatments to the dog. In spite of being highly efficacious under experimental conditions, the impact of these treatments in reducing the prevalence of *L. infantum* infection is limited because many dogs remain untreated or are not treated properly

(Goyena *et al.*, 2016). Infection risk is greatest for those living in rural and periurban areas where sand flies thrive, but it can vary significantly between zones depending on environmental conditions (Alonso *et al.*, 2010; Goyena *et al.*, 2016). A recent study indicates that vector abundance in Murcia Region in southeastern Spain is also spatially heterogeneous and correlates on a large geographical scale with *L. infantum* infection prevalence in people and dogs (Risueño *et al.*, 2017). These studies reinforce the need for detailed mapping of sand fly vectors as a means to improve the diagnosis and prevention of sand fly-borne infections.

In a study by Risueño *et al.* (2017), sheep farms and dog kennels were used as sentinels of sand fly abundance. Animal shelters are favorite places for sand flies (Dantas-Torres *et al.*, 2014), as they provide a plentiful source of blood for egg-producing adult females and suitable terrestrial breeding places. Other domestic and peridomestic environments where sand flies are commonly found include cellars, abandoned buildings, rubbish heaps, stone walls, caves and animal burrows, among others (Feliciangeli, 2004). For maximum benefit, sand fly abundance studies need common standard sampling approaches and statistical methods that take into account environmental factors affecting sand fly abundance. Investigating sand fly distributions relies on trapping the adult stages by interception or attraction (Alexander, 2000), because their terrestrial breeding sites are not well characterized (Killick-Kendrick, 1999). Sticky traps made of paper sheets impregnated in castor oil are the most popular interception trap. They capture a random selection of the sand flies flying in the immediate surroundings, which does not necessarily represent the resting sand fly population, and they tend to produce low sand fly yields. In contrast, light traps attract phototropic species including the *L. infantum* vectors *P. perniciosus* and *Phlebotomus ariasi* present within a few meters of the trap (Killick-Kendrick *et al.*, 1985; Valenta *et al.*, 1995; Alexander, 2000). In this sense, light traps may not necessarily provide a representative sample of the relative abundance of all species present in the environment, limiting their value in ecological studies, but they can be useful to measure relative changes in abundance over time and space (Alexander, 2000; Alten *et al.*, 2015). Trapping success can also be negatively influenced by rain, strong wind and moon illumination when using light traps (Alexander, 2000; Gebresilassie *et al.*, 2015). This study was designed to investigate the spatial and temporal distribution of *P.*

perniciosus on a small geographical scale, in and around animal premises in a high sand fly density area in southeastern Spain. An additional objective was to provide further evidence of the performance of light-attraction and sticky-interception traps in achieving this objective and the possible existence of differences between females and males in their spatial and temporal distributions. To this effect, we used a standardized sampling method, alternating the use of the two trap types in the same environments, and statistical methods that take into account the aggregated multifactorial nature of sand fly distributions. This study represents a continuation of the work commenced by Risueño *et al*. (2017) on a larger geographical scale, with the ultimate aim of contributing to elaborating regional, time-specific vector density maps that may be useful for vector and pathogen control.

Materials and methods

Study area, premises and design

The study lasted 23 weeks, from 16 May until 22 October 2016, and was carried out in four premises, three sheep farms and one hunting dog kennel located within 1.7-4 km of each other in the district of Archivel (municipality of Caravaca de la Cruz) in the west of Murcia Region (Figure 1). The municipality is situated at 38°1' N latitude and 2°0' E, has an average altitude of 900 meters above sea level (m.a.s.l.) and covers an area of 859 km^2, and the average annual precipitation is 325 mm. It has a permanent human population of approximately 1200 people with an economy based mainly on cereal crops and sheep and goat farming. In a recent study of sand fly abundance in Murcia Region, Archivel was the area with the highest sand fly abundance (Risueño *et al.*, 2017). In the present study, we used four of the premises investigated by these authors, who named them sheep farm 1 (SF1), SF2, SF3 and dog kennel 2 (DK2) (Risueño *et al.*, 2017) (Figure 1). Flock and kennel sizes were 80 sheep in SF1, 350 sheep in SF2, 410 sheep in SF3 and 20 dogs in DK2. Animals in SF1, SF2 and DK2 were kept in old (>100 years), poorly ventilated stone buildings and those in SF3 in a 32-year-old building with plastered brick walls that was better ventilated than SF1 and SF2. No insecticides were used in SF1 and SF3. Instead, to control fleas, in SF2 a 10% cypermethrin solution was used on the sheep bedding on 18 July and in DK2

dogs were administered a 2% imidacloprid spot-on pipette and kennel floors were fumigated with a deltamethrin solution in June.

In each of the premises, sand flies were monitored in four sites (A, B, C and D) (Figure 1) using one miniature Centers for Disease Control (CDC) battery-operated light trap (J. W. Hock Company, Gainesville, FL, U.S.A.) and five home-made sticky traps per site. Sticky traps were half A4 sheets of tracing paper measuring 210 mm × 148.5 mm, impregnated in castor oil, and were mounted on sticks or hung from pegs. Traps were placed close to walls, windows, fences, trees, bushes and wood piles in an attempt to cover the site's most representative microenvironments. The distance of traps to walls, floors, ceilings and the main group of animals in the premises (sheep or dogs) was measured.

In sheep farms, sites A and B were indoors and shared the airspace, site A was in the animal area and site B was where food and farming implements were stored. In contrast, sites C and D were outdoors, next to uninhabited or ruined stone buildings at a distance of 5-40 m and 400-740 m, respectively (Figure 1), from the premises or other animal holdings. It was not possible to follow the same protocol for sites A and D in the dog kennel. Site A in DK2 was the courtyard of the premise where half of the kennel dogs were kept, and site D was only 30 m from the premises and separated by 20 m from site C (Figure 1).

Light and sticky traps were put in place on alternate weeks to avoid interference between trap types, starting on weeks 1 and 2, respectively. Hence, the total number of weeks sampled with light and sticky traps was 12 and 11 weeks, respectively. Light traps were placed in collection sites on Monday, Wednesday and Friday mornings (and kept operational) for 24 h/day. Sticky traps were similarly placed on Mondays and Wednesdays and kept for 48 h each time. The aim was to collect sand flies from 576 light traps (1 trap × 3 days × 12 weeks × 4 premises × 4 sites) and 1,760 sticky traps (5 traps × 2 days × 11 weeks × 4 premises × 4 sites).

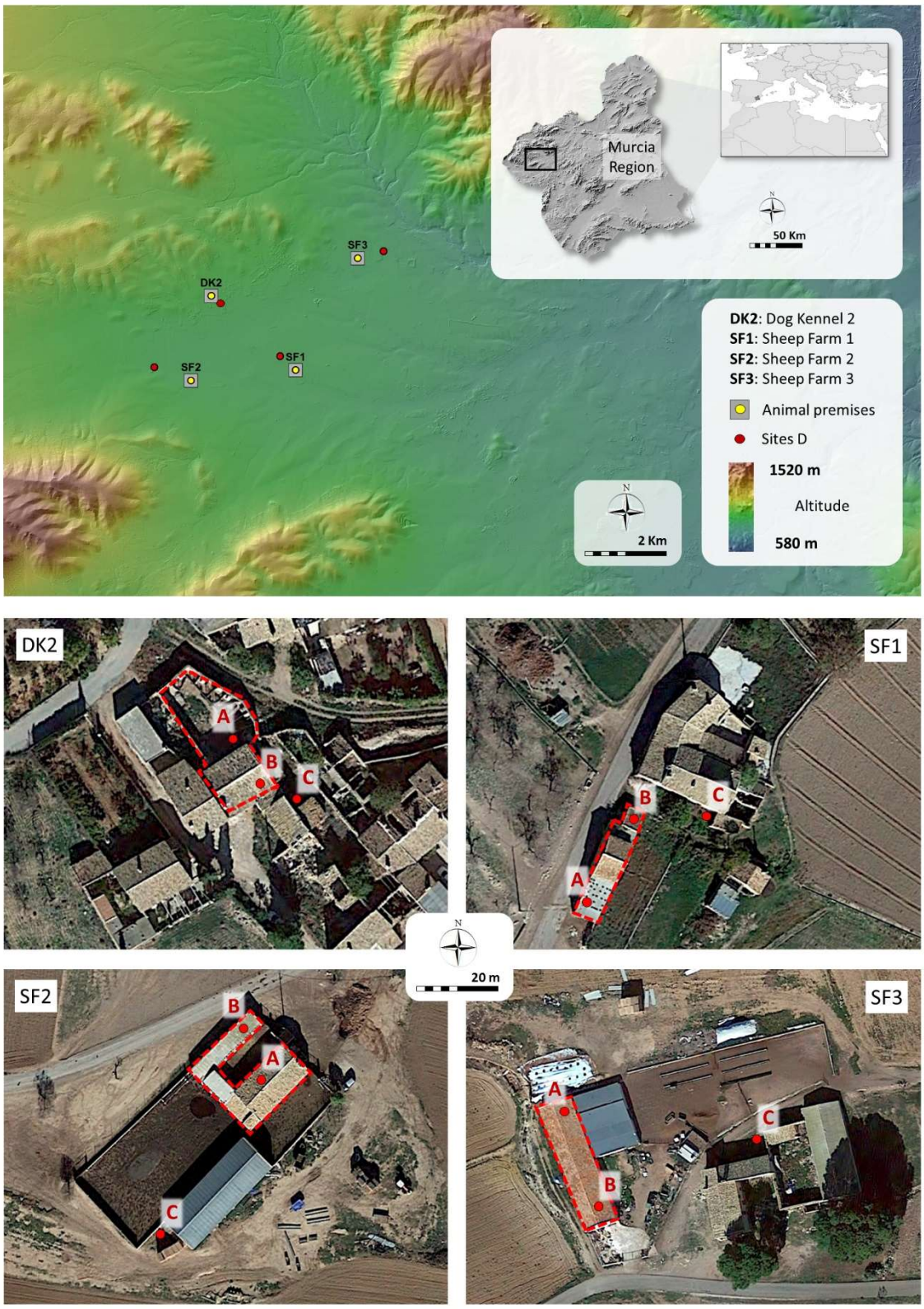

Figure 1. Locations of sand fly sampling premises and sites (A, B, C and D) in Murcia Region, southeastern Spain, used in the study of *P. perniciosus* distribution in 2016.

Sand fly counting, sexing and morphological speciation

Following sampling, light trap collection cups were kept at −20°C for at least 2 h to kill sand flies, which were then counted, sexed and stored in 96% ethanol until identification to species level. Sticky traps were stored at 4°C and within 3 days sand flies were collected and similarly stored in 96% ethanol until identified to species level.

Entomological keys were used to identify sand flies morphologically based on features of the aedeagus, stylopodite and coxopodite in males and the pharynx, cibarium and spermatheca in females (Lewis, 1982; Gállego-Berenguer *et al.*, 1992; Lawyer *et al.*, 2011).

Environmental data collection

Temperature (T) and relative humidity (RH) were recorded at sites A, B, C and D in each premises using a thermohygrometer (Digital Logtag Haxo-8T; Templyzer, INFOEXPO ACP S.L., Alicante, Spain) placed 2-3 m from the light trap, and measurements were taken every 3 h throughout the experiment. CO_2 concentration was measured with a portable CO_2 detector (AZ 77535; Herter Instruments, Barcelona, Spain) every time traps were placed and collected, and a sampling period average was calculated using both readings. Daily mean and maximum wind speeds at sites C and D were measured using an anemometer (Skywatch Eole; JDC Electronic). The daily percentage of moon illumination was obtained from a meteorological station situated in Benablón, 6 km from the village of Archivel (http://www.tutiempo.net/Calendario-Benablon-ES03123.html#Calendario).

Statistical and ecological data analysis

Sand fly and environmental data distributions were examined and compared using a Yates-corrected chi-square test or when required Fisher exact tests for proportions and the non-parametric Kruskal−Wallis test for medians.

Negative binomial regression for overdispersed count data was employed to investigate the relationship between male and female *P. perniciosus* abundances in light traps (outcome variable) and trap distance to the main group of animals in the premises (explanatory variable of interest), adjusted for other explanatory variables including trap distance to a surface (wall, ceiling or floor), T, RH, CO_2 concentration, moon illumination

percentage, wind speed, presence of rain, week and premises. The last two variables were used as proxies for unaccounted temporal and spatial factors potentially affecting vector abundance. Explanatory variables were fitted as categorical variables except for week, which was included as a polynomial with a quadratic term to allow for a nonlinear relationship with sand fly abundance.

A backward model-building strategy was used starting with a saturated model that incorporated all the explanatory variables. The potential confounding effect of explanatory variables on the relationship between sand fly count and distance to the main group of animals was then assessed by examining the degree of change in the regression coefficient of this variable before and after removing each of the other explanatory variables as recommended by Kleinbaum *et al.* (1998). Variables that changed the regression coefficient of any of the levels of the variable of interest by 10% or more were retained in the model. Parameters were estimated using the maximum likelihood method and were exponentiated to calculate incidence rate ratios. Differences were considered significant at alpha=5% ($p<0.05$) for a two-tailed test. All statistical analyses were performed using the r program (http://cran.r-project.org/).

Results

Sand fly species abundance in light and sticky traps according to sex, month and trap environment

The numbers of light and sticky trap collections recovered were 521 (90%) and 1,704 (97%), respectively, and most missing light trap catches were from site D in sheep flock 2, where the trap was stolen at the end of July and could not be replaced. The percentage of light and sticky traps with at least one sand fly were 64% and 12%, respectively. The total number of sand flies collected was 8,506 specimens, of which 59% were male and 41% female, and 87% of the specimens were caught with light traps. The relative (absolute) frequency of species was: 62% (5,240) *P. perniciosus*, 23% (1,963) *Phlebotomus sergenti*, 8% (654) *Phlebotomus papatasi*, 6% (514) *Sergentomyia minuta* and 1% (67) *P. ariasi* (Table 1). A further 1% (68) could not be identified. *Phlebotomus perniciosus* was more abundant in light traps, while *S. minuta*, *P. sergenti* and *P. papatasi* were relatively

more abundant in sticky traps, as, in general, were males (Table 1). The sand fly monthly distribution also differed according to trap type, and the relative abundance of sand flies in sticky traps was greater in August than that in light traps, as proportionally more *P. papatasi* and *P. sergenti* were captured in this month (Table 1). Moreover, differences in sand fly abundance between premises and sites were much greater in light traps compared with sticky traps (Table 1). Abundance in light traps was highest in SF1 followed by DK2, SF3 and SF2, and in site B, followed by A, C and D. Over 90% of sand flies, particularly *P. perniciosus*, came from sites A, B and C, and mostly from sites A and B. Abundance was greater for traps placed indoors, except for *S. minuta*. Species distribution also varied according to the premises. Most *P. ariasi* and more than half of *P. sergenti* individuals were captured in DK2. In contrast, the relative abundance of *P. papatasi* was greater in SF3 compared with DK2 and SF1, and similarly, the relative frequency of *P. perniciosus* was highest in SF1 and SF2 (Table 1).

Sand fly abundance varied depending on the distance of traps to animals and surfaces. Overall, it decreased with greater distance to animals, walls and ceilings (except in sticky traps) and increased with greater distance to the floor (Table 1), although there were some differences between species; for example, *S. minuta* was most abundant in traps further away from animals and closest to the floor.

Table 1. Absolute (relative to trap type) frequency of sand fly species in CDC light and sticky traps according to sand fly gender, month and trap environment.

Variable	Level	*P. ariasi*		*S. minuta*		*P. papatasi*		*P. perniciosus*		*P. sergenti*		All	
		Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky
Sex	Female	30 (1)	0 (0)	183 (6)	66 (29)	239 (7)	34 (15)	2187 (69)	75 (33)	548 (17)	52 (23)	**3187**	**227**
	Male	35 (1)	2 (<1)	203 (5)	62 (7)	235 (6)	146 (17)	2717 (65)	261 (30)	973 (23)	390 (45)	**4163**	**861**
Month	May	0 (0)	0 (0)	2 (7)	8 (57)	1 (3)	1 (7)	24 (83)	2 (14)	2 (7)	3 (21)	**29**	**14**
	June	9 (1)	0 (0)	74 (6)	48 (30)	64 (5)	16 (10)	1034 (78)	71 (44)	150 (11)	26 (16)	**1331**	**161**
	July	37 (1)	1 (<1)	150 (5)	16 (4)	204 (7)	66 (15)	1591 (54)	94 (22)	977 (33)	250 (59)	**2959**	**427**
	August	7 (<1)	1 (<1)	69 (4)	33 (10)	103 (6)	71 (21)	1073 (67)	79 (23)	357 (22)	156 (46)	**1609**	**340**
	September	11 (1)	0 (0)	87 (7)	23 (18)	92 (8)	23 (18)	974 (81)	75 (59)	35 (3)	7 (5)	**1199**	**128**
	October	1 (<1)	0 (0)	4 (2)	0 (0)	10 (4)	3 (17)	208 (93)	15 (83)	0 (0)	0 (0)	**223**	**18**
Premise	DK2	50 (3)	2 (1)	80 (5)	36 (9)	63 (4)	12 (3)	673 (40)	57 (15)	836 (49)	279 (72)	**1702**	**386**
	SF1	4 (<1)	0 (0)	146 (4)	14 (4)	151 (4)	61 (17)	2951 (81)	165 (47)	391 (11)	109 (31)	**3643**	**349**
	SF2	2 (1)	0 (0)	13 (3)	13 (16)	41 (10)	18 (23)	321 (78)	46 (58)	33 (8)	3 (4)	**410**	**80**
	SF3	9 (1)	0 (0)	147 (9)	65 (24)	219 (14)	89 (33)	959 (60)	68 (25)	261 (16)	51 (19)	**1595**	**273**
Site	A	5 (<1)	0 (0)	28 (1)	13 (4)	192 (8)	80 (24)	1819 (80)	122 (37)	219 (10)	112 (34)	**2263**	**327**
	B	40 (1)	1 (<1)	35 (1)	3 (1)	220 (7)	58 (17)	2059 (63)	62 (18)	940 (29)	218 (64)	**3294**	**342**
	C	10 (1)	1 (<1)	94 (9)	29 (13)	50 (5)	38 (16)	699 (63)	83 (36)	252 (23)	80 (35)	**1105**	**231**
	D	10 (2)	0 (0)	229 (33)	83 (44)	12 (2)	4 (2)	327 (48)	69 (37)	110 (16)	32 (17)	**688**	**188**
Location	Indoors	43 (1)	1 (<1)	51 (1)	5 (1)	400 (8)	132 (24)	3755 (71)	152 (28)	1073 (20)	255 (47)	**5322**	**545**
	Outdoors	22 (1)	1 (<1)	335 (17)	123 (23)	74 (4)	48 (9)	1149 (57)	184 (34)	448 (22)	187 (34)	**2028**	**543**
Distance to animals (m)	1 - 2	6 (<1)	1 (<1)	36 (1)	16 (2)	261 (7)	148 (22)	2945 (82)	185 (27)	333 (9)	331 (49)	**3581**	**681**
	4 - 5	33 (3)	-	18 (2)	-	47 (4)	-	435 (36)	-	665 (56)	-	**1198**	**-**
	10 - 30	26 (1)	1 (<1)	127 (6)	47 (19)	161 (8)	29 (12)	1322 (63)	86 (34)	461 (22)	89 (35)	**2097**	**252**
	400 - 740	0 (0)	0 (0)	205 (43)	65 (42)	5 (1)	3 (2)	202 (43)	65 (42)	62 (13)	22 (14)	**474**	**155**
Distance to wall (cm)	1	-	2 (1)	-	100 (13)	-	129 (16)	-	246 (31)	-	317 (40)	**-**	**794**
	5 - 15	45 (1)	0 (0)	176 (3)	20 (16)	418 (7)	30 (25)	3884 (67)	41 (34)	1235 (21)	31 (25)	**5758**	**122**
	20 - 40	10 (1)	0 (0)	186 (13)	3 (3)	49 (4)	19 (16)	895 (65)	34 (29)	238 (17)	63 (53)	**1378**	**119**
	50 - 70	10 (5)	0 (0)	24 (11)	5 (9)	7 (3)	2 (4)	125 (58)	15 (28)	48 (22)	31 (58)	**214**	**53**

Table 1 (continued). Absolute (relative to trap type) frequency of sand fly species in CDC light and sticky traps according to sand fly gender, month and trap environment.

Variable	Level	*P. ariasi*		*S. minuta*		*P. papatasi*		*P. perniciosus*		*P. sergenti*		All	
		Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky	Light	Sticky
Distance to ceiling (cm)	1 - 30	-	1 (1)	-	3 (2)	-	10 (7)	-	15 (11)	-	110 (79)	**-**	**139**
	40 - 90	35 (1)	0 (0)	30 (1)	2 (1)	137 (5)	25 (14)	1767 (65)	75 (43)	740 (27)	74 (42)	**2709**	**176**
	100 - 190	10 (<1)	0 (0)	58 (2)	2 (1)	183 (6)	87 (36)	2196 (76)	76 (32)	438 (15)	76 (32)	**2885**	**241**
	230 - 260	1 (<1)	0 (0)	4 (1)	0 (0)	90 (17)	10 (45)	364 (70)	3 (14)	58 (11)	9 (41)	**517**	**22**
Distance to floor (cm)	15 - 70	0 (0)	0 (0)	199 (45)	30 (39)	4 (1)	4 (5)	174 (40)	36 (47)	61 (14)	7 (9)	**438**	**77**
	75 - 140	24 (1)	1 (<1)	100 (4)	74 (16)	221 (8)	80 (18)	1904 (72)	133 (29)	413 (16)	167 (37)	**2662**	**455**
	150 - 205	41 (1)	1 (<1)	87 (2)	24 (4)	249 (6)	96 (17)	2826 (66)	167 (30)	1047 (25)	268 (48)	**4250**	**556**
All		**65 (1)**	**2 (<1)**	**386 (5)**	**128 (12)**	**474 (6)**	**180 (17)**	**4904 (67)**	**336 (31)**	**1521 (21)**	**442 (41)**	**7350**	**1088**

Spatial distribution of *P. perniciosus* males and females in light and sticky traps

Tables 2 and S1 (presented as supplementary material) show male and female *P. perniciosus* abundance in study sites in light and sticky traps, percentage of *P. perniciosus*-positive traps and median and maximum numbers of specimens in positive traps (1 m^2 in 1 day in sticky traps).

Light traps in site A captured more females and fewer males than those in site B except in DK2, where female abundance was greater in site B than site A, and in SF2, where male abundance was greater in site A compared with site B (Table 2). Differences in abundance between sites were attributable to the number of specimens in positive traps rather than the proportions of positive traps, which were very similar across sites (Table 2).

The proportion of male and female positive traps in SF1 and SF3 was greater in site C than in site D, and the opposite was the case in DK2 and SF2 (Table 2). Moreover, median male and female abundance in positive traps in SF1 was greater in site C compared with site D and did not differ significantly in other premises (Table 2). Overall, the proportion of positive traps and median abundance in positive traps in sites C and D were similar for males and females ($p>0.05$).

The spatial distribution of males and females in sticky traps across sites differed from that in light traps and male and female abundances were greatest in site A (Table S1). The proportion of male positive traps varied greatly between sites within and between premises, but the overall proportion and median male density in positive traps were similar across sites ($p>0.05$) (Table S1). In contrast, the estimated proportion of female positive traps was greatest in site A in all premises except in SF3, but the median density in positive traps was similar across sites.

Table 2. Percentage of male and female *P. perniciosus*-positive CDC traps and abundance in positive traps in study sites.

Premise	Site	No. traps	Males				Females				All sand flies			
			% P[a]	Total	Median	Max.	% P	Total	Median	Max.	% P	Total	Median	Max.
DK2	A	33	61	84	3	25	48	39	2	5	67	123	4	28
	B	34	82	258	7	40	76	118	4	24	85	376	9	47
	C	34	35	31	2	8	32	18	1	3	41	49	3	10
	D	34	59	65	3	9	56	60	2	17	65	125	5	19
	All	**135**	**59**	**438**	**3**	**40**	**53**	**235**	**2**	**24**	**64**	**673**	**5**	**47**
SF1	A	35	86	450	13	43	83	669	20	58	89	1119	34	90
	B	35	83	738	26	83	80	388	14	39	86	1126	37	100
	C	34	76	399	10	53	68	173	6	24	76	572	16	68
	D	32	56	113	4	32	34	21	1	6	59	134	4	34
	All	**136**	**76**	**1700**	**10**	**83**	**67**	**1251**	**8**	**58**	**78**	**2951**	**18**	**100**
SF2	A	33	64	103	2	28	64	110	3	15	73	213	5	36
	B	35	31	35	2	9	40	24	1	5	51	59	2	13
	C	33	27	16	2	4	12	5	1	2	33	21	2	4
	D	14	43	17	2	8	36	11	2	5	57	28	2	8
	All	**115**	**41**	**171**	**2**	**28**	**38**	**150**	**2**	**15**	**53**	**321**	**2**	**36**
SF3	A	34	56	91	3	24	71	273	7	55	71	364	10	79
	B	35	80	248	8	36	80	250	7	42	83	498	14	78
	C	33	42	43	3	8	36	14	1	2	52	57	2	8
	D	33	30	26	2	9	18	14	2	5	33	40	2	14
	All	**135**	**53**	**408**	**3**	**36**	**52**	**551**	**5**	**55**	**60**	**959**	**6**	**79**
All	A	135	67	728	4	43	67	1091	7	58	75	1819	9	90
	B	139	69	1279	8	83	69	780	4	42	76	2059	11	100
	C	134	46	489	3	53	37	210	2	24	51	699	4	68
	D	113	48	221	2	32	36	106	2	17	53	327	4	34
	All	**521**	**58**	**2717**	**4**	**83**	**53**	**2187**	**3**	**58**	**64**	**4904**	**6**	**100**

[a] % P: Percentage of positive traps (%).

Temporal distribution of *P. perniciosus* males and females in light and sticky traps and its association with indoor recorded T and RH

Phlebotomus perniciosus abundance was similarly seasonal in males and females in both light and sticky traps, although temporal trends differed between trap types (Tables 3 and S2). Relatively few specimens were collected in May and a sharp increase in abundance was observed in the second and third weeks of June in sticky and light traps, respectively. The number of specimens in light traps rose thereafter, reaching a maximum (784 specimens) in mid-July, decreased slowly thereafter until the end of August when it rose slightly (597 specimens), and remained above 450 specimens in September, dropping sharply to 190 specimens at the beginning of October (Table 3). The temporal trends in the proportion of positive light traps and the median abundance in these traps were very similar to that of the total abundance, except that the proportion of positive traps did not rise after peaking in July (Table 3).

In contrast to light traps, *P. perniciosus* abundance in sticky traps peaked in the last week of June (55 specimens), and this was followed by a progressive decline until the third week of August (22 specimens) and a significant rise at the end of August (37 specimens) and September (44 specimens). This rise was associated with a similar increase in the proportion of positive traps rather than in the mean number of specimens in these traps (Table S2).

Figure 2 depicts the temporal relationship between sand fly abundance in light traps and the mean T and RH on the day traps were put in place. The highest *P. perniciosus* median (11 specimens) corresponded to week 11, when the highest mean T (28°C) and lowest mean RH (34%) in the study were recorded (Figure 2). However, the *P. perniciosus* median abundance was much lower (2 specimens) in week 5, when T and RH were also high and low, respectively, and similar (10 specimens) in week 9, when both T and RH were high (Figure 2).

Table 3. Time-specific *P. perniciosus* abundance, percentage of positive CDC light traps and abundance in positive traps.

Month	Day[a]	Week[b]	No. traps	Males				Females				All sand flies			
				% P[c]	Total	Median	Max.	% P	Total	Median	Max.	% P	Total	Median	Max.
May	16	1	48	4	3	2	2	6	4	1	2	8	7	1	4
	30	3	48	42	56	2	9	23	20	2	3	44	76	3	12
June	13	5	48	55	341	4	83	55	193	3	58	64	534	5	100
	27	7	48	82	332	6	43	74	324	7	46	87	656	10	82
July	11	9	48	83	454	8	44	74	330	5	39	87	784	13	73
	25	11	48	88	324	7	42	85	268	5	52	94	592	11	78
August	8	13	45	72	218	6	45	74	258	5	51	85	476	9	62
	22	15	45	73	319	5	43	69	278	4	50	78	597	7	90
September	5	17	45	71	296	5	31	67	223	4	36	78	519	8	52
	19	19	45	60	253	5	53	53	202	3	55	65	455	6	79
October	3	21	45	56	109	3	15	58	81	2	14	69	190	4	24
	17	23	45	20	12	1	5	12	6	1	2	27	18	1	5

[a] First sampling day of the week.

[b] Study week.

[c] % P: Percentage of positive traps (%).

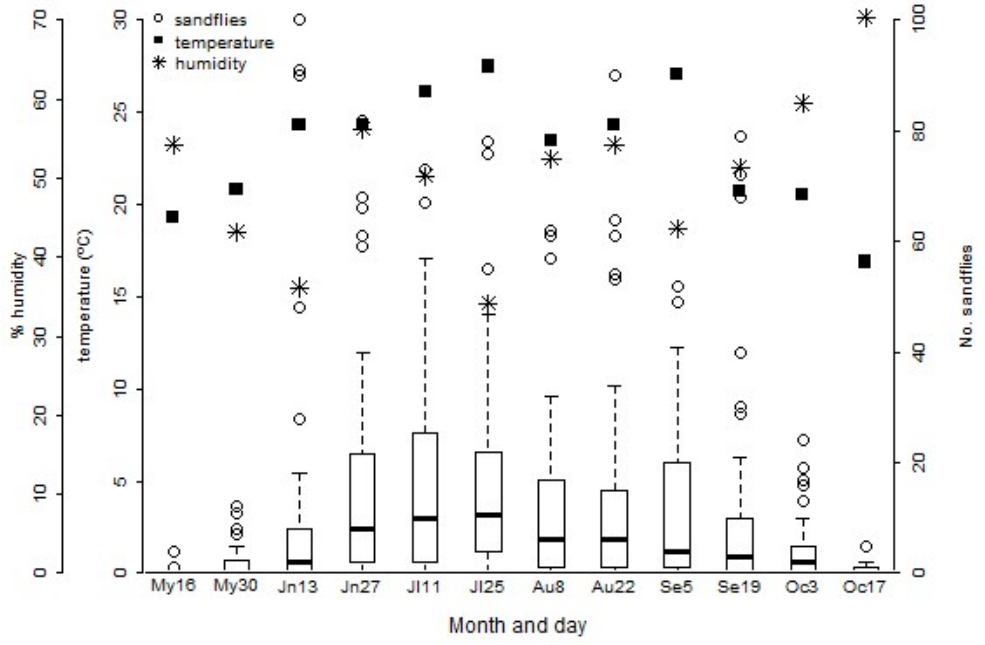

Figure 2. Seasonal *P. perniciosus* sand fly distribution in 2016 in CDC light traps and mean indoor temperature and humidity at the times at which traps were put in place.

Relationship between *P. perniciosus* abundance and temperature, relative humidity, CO_2 concentration, wind speed, and moon illumination

Further analysis of the relationship between *P. perniciosus* abundance and density in light and sticky traps, respectively, and on-site recorded T, RH, CO_2 concentration and wind speed are reported in Tables 4 and S3. The percentage of positive light traps was strongly associated with increasing T and decreasing RH, and median abundance in positive traps was similarly associated with T but only marginally so with RH for males (Table 4). Associations between T and RH and sticky trap catches were less clear; the percentage of positive sticky traps was associated with increasing T when considering males and females together only and with decreasing RH for both, although the relationship was linear only for females (Table S3). Mean wind speed was positively associated with median *P. perniciosus* abundance in light traps ($p<0.05$). Maximum wind speed was also associated with the percentage of *P. perniciosus*-positive light traps, but the relationship was not linear (Table 4). There was no relationship between maximum wind speed and median *P. perniciosus* density in sticky traps (Table S3).

The percentage of *P. perniciosus*-positive light traps in indoor sites A and B increased with CO_2 concentration up to CO_2 concentrations of 583-695 ppm (Table 4). In contrast, opposite relationships between median *P. perniciosus* abundance in positive traps and CO_2 concentration were found depending on sand fly gender; CO_2 level was negatively and positively associated with male and female abundances, respectively (Table 4). A similar positive relationship between CO_2 level and the percentage of female-positive sticky traps was observed, but it was not statistically significant (Table S3).

Overall, the percentage of moon illumination was negatively associated with the percentage of *P. perniciosus*-positive light traps and median abundance in these traps. However, when sites A and B (indoors, except in DK2) and C and D (outdoors) were analyzed separately, only the proportion of positive traps in sites A and B was related to moon illumination (Table 4). Associations between median *P. perniciosus* density in sticky traps and moonlight illumination were not significant (Table S3).

Table 4. Relationship between on-site recorded environmental variables and the percentage of CDC light traps with *P. perniciosus* and the median and maximum number of male and female specimens in positive traps.

Variable	Level	No. traps	Males				Females				All sand flies			
			% P[a]	Total	Median	Max.	% P	Total	Median	Max.	% P	Total	Median	Max.
Relative humidity (%)	20 - 30	15	87**	192	10*	42	87**	105	4	34	93**	297	14	76
	31 - 40	82	63	603	5	83	56	305	3	58	71	908	8	100
	41 - 50	154	60	728	4	45	52	606	3	46	64	1334	6	78
	51 - 60	194	58	900	4	53	56	907	4	55	65	1807	8	79
	61 - 73	76	41	294	4	43	39	264	2	50	49	558	6	90
Temperature (°C)	15 - 17	28	29	12	1	5	18	6	1	2	39	18	1	5
	18 - 19	29	7	3	2	2	3	2	2	2	7	5	3	4
	20 - 21	137	37	346	3	53	33	240	2	55	42	586	4	79
	22 - 23	48	67	145	3	16	56	98	2	20	77	243	4	30
	24 - 25	138	73	977	6	69	72	949	5	51	81	1926	8	91
	26 - 27	61	77**	484	5	44	72	498	5**	50	82	982	8	90
	28 - 30	80	75	750	8**	83	70**	394	4	58	83**	1144	10**	100
Mean wind speed (m/s)	0.0	73	49	152	3	30	48	103	1	24	59	255	4	54
	0.1 - 1.0	72	51	164	2	45	35	79	2	12	56	243	3	57
	1.1 - 2.0	22	64	152	7	43	45	47	4	13	64	199	8	53
	2.1 - 6.7	18	67	139	8**	41	67*	60	4*	14	78	199	9**	55
Maximum wind speed (m/s)	0.0 - 7.0	46	54	87	3	9	50	57	1	17	63	144	4	19
	7.1 - 13.0	53	40	86	2	30	28	54	2	24	45	140	3	54
	13.1 - 19.0	46	65*	278	4	45	61**	118	2	14	72*	396	4	57
	19.1 - 48.1	40	58	156	4	29	40	60	3	12	63	216	5	41
CO_2 concentration in sites A and B (ppm)	332 - 513	39	56	451	11**	83	62	220	3.5	58	67	671	9.5	100
	514 - 582	47	81	321	5.5	40	72	211	4	29	87	532	6	64
	583 - 695	54	83**	477	6	43	87**	528	6	42	91**	1005	13	78
	696 - 1952	74	77	670	8	44	82	866	9**	55	85	1536	19**	90

Table 4 (continued). Relationship between on-site recorded environmental variables and the percentage of CDC light traps with *P. perniciosus* and the median and maximum number of male and female specimens in positive traps.

Variable	Level	No. traps	Males				Females				All sand flies			
			% P[a]	Total	Median	Max.	% P	Total	Median	Max.	% P	Total	Median	Max.
Moon illumination (%)														
Sites A and B	0 - 25	63	76	510	7	43	75	374	4	39	87**	884	8	82
	26 - 50	65	80**	464	6	43	80**	534	5	51	86	998	11	73
	51 - 75	77	65	635	6	83	62	527	7	58	73	1162	10	100
	76 - 100	69	52	398	7	44	57	436	4	55	58	834	10	90
Sites C and D	0 - 25	51	43	158	3	45	37	48	2	12	49	206	4	57
	26 - 50	55	62	164	3	29	42	66	2	12	65	230	4	41
	51 - 75	75	36	172	4	43	35	91	2	13	44	263	4	53
	76 - 100	66	48	216	2	53	35	111	2	24	52	327	4	68

** p<0.05 and * p<0.10. Symbols placed in level with highest value.

[a] % P: Percentage of positive traps (%).

Multivariable modelling of *P. perniciosus* abundance in light traps

Models confirmed that both male and female *P. perniciosus* counts were negatively associated with trap distance to the main animal group for distances above 10 m (corresponding to sites C and D), and that male counts at 4-5 m were greater than at 1-2 m, although the difference was only marginally significant (Table 5). Sand fly abundance was also negatively associated with rain (not significantly in the case of males) and positively associated with temperature and with CO_2 concentration in females only. Moreover, there was remaining significant variation associated with week and premises. *Phlebotomus perniciosus* counts were also marginally associated with RH, but the relationship was positive for females and negative for males. Moon illumination was also related to abundance, but the trend was not conclusively positive or negative (Table 5). The model did not incorporate distances to wall, ceiling and floor, as these variables were strongly correlated to premises and site, or wind speed, as inclusion of this variable led to model convergence failure.

Table 5. Sex-specific incidence rate ratios (RR) from a negative binomial model of the relationship between CDC light trap *P. perniciosus* counts and distance to animals in flocks and kennel, adjusted for relative humidity, temperature, week, moon illumination, CO_2 concentration, rain and premise.

Variable	Level	Females				Males			
		RR	95% CI[a]		P value	RR	95% CI		P value
Humidity (%)	22 - 40	1.00	-	-	-	1.00	-	-	-
	41 - 60	1.74	1.21	2.50	0.0026	0.74	0.52	1.06	0.1006
	61 - 73	1.49	0.86	2.57	0.1571	0.58	0.33	1.01	0.0553
Temperature (°C)	15 - 21	1.00	-	-	-	1.00	-	-	-
	22 - 26	4.55	1.47	14.13	0.0087	4.51	1.61	12.59	0.0041
	27 - 30	7.68	2.21	26.70	0.0013	9.07	2.84	28.91	0.0002
Week		1.14	0.93	1.39	0.1249	1.14	0.93	1.39	0.2146
Week square		0.99	0.98	1.00	0.0159	0.99	0.99	1.00	0.0844
Moon illumination (%)	0 - 25	1.00	-	-	-	1.00	-	-	-
	26 - 50	0.81	0.54	1.19	0.2790	0.81	0.54	1.20	0.2918
	51 - 75	0.67	0.45	0.99	0.0449	0.66	0.44	0.98	0.0412
	76 - 100	1.37	0.90	2.11	0.1448	1.61	1.04	2.50	0.0310
CO_2 concentration (ppm)	333 - 450	1.00	-	-	-	1.00	-	-	-
	462 - 582	1.54	0.92	2.58	0.1012	1.21	0.73	2.02	0.4598
	583 - 696	2.25	1.32	3.85	0.0029	1.39	0.82	2.37	0.2227
	700 - 894	2.72	1.53	4.84	0.0007	1.82	1.02	3.25	0.0433
	903 - 1952	3.13	1.52	6.48	0.0021	1.35	0.63	2.88	0.4416
Presence of rain	No	1.00	-	-	-	1.00	-	-	-
	Yes	0.63	0.40	0.98	0.0406	0.90	0.57	1.42	0.6642

Table 5 (continued). Sex-specific incidence rate ratios (RR) from a negative binomial model of the relationship between CDC light trap *P. perniciosus* counts and distance to animals in flocks and kennel, adjusted for relative humidity, temperature, week, moon illumination, CO_2 concentration, rain and premise.

Variable	Level	Females				Males			
		RR	95% CI[a]		P value	RR	95% CI		P value
Premises	DK2	1.00	-	-	-	1.00	-	-	-
	SF3	2.39	1.59	3.58	0.0000	1.49	0.99	2.24	0.0541
	SF2	0.57	0.39	0.84	0.0050	0.47	0.32	0.69	0.0001
	SF1	4.41	3.06	6.35	0.0000	5.67	3.93	8.17	0.0000
Trap distance to animals (m)	1 - 2	1.00	-	-	-	1.00	-	-	-
	4 - 5	0.79	0.51	1.23	0.2955	1.53	0.98	2.38	0.0596
	10 - 30	0.43	0.31	0.58	0.0000	0.71	0.52	0.99	0.0409
	400 - 740	0.09	0.05	0.14	0.0000	0.26	0.16	0.41	0.0000

[a] 95% CI: 95% confidence interval.

Discussion

The low and variable percentage of sand fly-positive sticky traps reflects the large spatial overdispersion of sand fly populations and indicates that many more traps would be needed for a more precise estimation of their distribution in the microenvironments investigated in this study. This is in agreement with the study by Rioux *et al.* (2013) investigating the distribution and ecology of the natural sand fly population in the eastern French Pyrenees region. Moreover, a recent study of the seasonal patterns of *L. infantum* vectors in Mediterranean countries in Europe also reported significant variability in sand fly yields in sticky traps between countries and research groups (Alten *et al.*, 2016). With the exception of *L. infantum* vectors, *P. perniciosus* and *P. ariasi*, all other species were relatively more abundant in sticky traps, suggesting inherent differences in phototropism among species.

The intensive light trap sampling protocol used and the high abundance of *P. perniciosus* allowed an accurate estimation of its spatial distribution in relation to distance to farms and kennels, which was the main objective of this study. Abundance followed a negative gradient, decreasing the further away traps were placed from animal groups, with important differences between males and females. The latter were concentrated closer to animals, from which they can take bloodmeals for oogenesis. It is not clear why males were more abundant in adjoining storage places. They may be more stable environments for sand flies to rest and breed compared with animal enclosures which

have changing stocking rates, and in which the bedding is removed and floors disinfected periodically. In any case, it seems that both animal and contiguous storage spaces can maintain large vector populations, and this is important from a control perspective. Dog kennels can be hotspots of *L. infantum* infection (Foglia Manzillo *et al.*, 2013), but there is no similar evidence for sheep or other farm animal premises. Sheep may become infected with *L. infantum* (Gao *et al.*, 2015), but their ability to transmit infection to sand flies has not been investigated and they are not considered an important reservoir of the parasite (Quinnell and Courtenay, 2009). The low *P. perniciosus* counts in traps placed far away from the farms and kennel, close to abandoned and ruined buildings, suggests that these are not necessarily vector hotspots as often considered. They may offer adequate protected resting and breeding sites, but sand flies are poor fliers and females benefit from being close to a blood source. They can feed on birds and mammals and sand fly abundance in open farm land areas probably strongly depends on wildlife density. Animal dens and caves are considered good microenvironments for *P. perniciosus* and adult counts can be very high in these habitats, but quantitative data are missing (Killick-Kendrick, 1999) and this was not investigated in this study.

Phlebotomus perniciosus seasonality also differed between trap types and was similar for males and females in light traps. The temporal trend in light traps was unimodal with a single high-density peak in mid-July. In contrast, two similar peaks were observed in sticky traps at the end of June and end of September, particularly in males. The reasons for the differences in the seasonality according to trap type are unclear. Two almost identical *P. perniciosus* peaks in July and September were also described in Murcia Region in the 1980s using sticky traps (Martínez-Ortega, 1986), and in other low-latitude Mediterranean countries with light traps (Alten *et al.*, 2016). The latter authors considered multimodal distributions to be typical of longer sand fly seasons allowing several vector generations (Alten *et al.*, 2016). In laboratory conditions at 25-26°C, the duration of the life cycle of *P. perniciosus* from Murcia was 43 (41-47) days (Volf and Volfova, 2011). The number of sand fly generations in Archivel and elsewhere in Murcia Region has not been investigated. Using a degree-day model, Oshaghi *et al.* (2009) predicted one complete *P. papatasi* life cycle and development of the next generation up to late larval instar in the Germi district in Iran (39°1′ N latitude and 48°6′ E longitude;

altitude and rainfall 1,050 m.a.s.l. and 335 mm, respectively). Given the geographical and climatic similarities between these two distant regions, and allowing for differences between *P. perniciosus* and *P. papatasi* (Kasap and Alten, 2005; Volf and Volfova, 2011), it is possible that, in Archivel, many *P. perniciosus* populations are only able to complete one single generation and larval development of a second generation. Notwithstanding this, *P. perniciosus* abundance in light traps remained fairly high until mid-September, suggesting that some *P. perniciosus* populations were able to complete two generations. This is supported by the two peaks observed in sticky trap captures. However, the number of sand flies captured in sticky traps was comparatively small and consequently the precision of the estimated abundance was low.

Adult sand fly abundance peaks are probably the result of a combination of recent near-synchronous emergence of adults, which depends on climatic conditions several weeks before and on the trapping day. This would explain the less than perfect relationship between vector yields and T and RH in this study. Greatest *P. perniciosus* abundance in light traps was associated with T>24°C and RH of 35-60%. These conditions were met on several occasions during the study when vector abundance differed substantially. In the previous 2015 Murcia regional study, vector abundance correlated better with T and RH (Risueño *et al.*, 2017). Such differences are to be expected when comparing abundances in areas that are far apart and have substantially different climatic conditions.

Multivariable models indicated a strong relationship between female abundance and CO_2 concentration that was independent of trap distance to the main animal group. This relationship was less clear for males, which do not need to blood-feed. Sand fly catches are generally much greater when light traps are baited with CO_2. Stimuli that induce host-seeking responses of mosquitoes and probably also sand flies include CO_2, host odor, visual stimuli and body heat and vapor (Alexander, 2000). Sand flies approach hosts in a series of short flight hops, and walls or other surfaces facilitate this behavior. Light traps were placed close to vertical surfaces and abundance was negatively related to wall distance in the bivariate analysis, as reported before (Risueño *et al.*, 2017). Moonlight reduces the distance from which light traps are visible (Alexander, 2000). A previous study reported a strong negative association between moon illumination and *Phlebotomus*

orientalis yields in light traps in Ethiopia (Gebresilassie *et al.*, 2015), but this was not the case in the present study. The majority of sand flies were caught in traps placed indoors, where the effect of moonlight is likely to be smaller. This may also explain the absence of a negative association between wind speed and sand fly abundance. In contrast, strong prevailing winds were considered an important factor limiting sand fly abundance on a large geographical scale in Murcia Region (Risueño *et al.*, 2017).

Light and sticky traps also differed in the relative abundance of sand fly species. *Phlebotomus perniciosus* and *P. ariasi* were more common in light traps; in contrast, *S. minuta*, *P. sergenti* and *P. papatasi*, and males overall, were more frequent in sticky traps. Phototropism varies between species and is greater for *P. perniciosus* than for *S. minuta* (Alten *et al.*, 2016). Moreover, *S. minuta* was most abundant in sites furthest away from animal premises. This species is the vector of *Leishmania* spp. infecting lizards and may preferentially feed on them (Maia *et al.*, 2013). *Phlebotomus sergenti* and *P. papatasi* are vectors of *Leishmania tropica* and *Leishmania major*, respectively, in Northern Africa and the Middle East, where they are commonly found inside animal premises and cellars (Svobodová *et al.*, 2003). *Phlebotomus perniciosus*, *S. minuta*, *P. sergenti* and *P. papatasi* were also the most common species in the 2015 sand fly distribution study in Murcia Region, although their relative frequencies were not identical to those in the present study (Risueño *et al.*, 2017). Differences between studies could also be related to the sampling strategy, which was more intensive in the present study and covered a wider geographical area in Risueño *et al*. (2017).

In summary, the spatial and temporal distribution of sand flies in rural environments varies according to sand fly species, gender and trapping method. Sticky traps may provide low sand fly yields, limiting the statistical power of the study, and light traps are the preferred method for studying *P. perniciosus* distributions. Notwithstanding this, sticky traps are cheap and easy to prepare and, if sufficient are used, they provide an unbiased estimate of the active sand fly population in a particular place and time (Alexander, 2000; Alten *et al.*, 2015). *Phlebotomus perniciosus* were concentrated in animal premises, where male and female spatial distributions differed to some extent, and females but not males were increasingly attracted to rising CO_2 levels and were more abundant at higher RH. In

contrast, percentage moon illumination and wind speed did not significantly affect *P. perniciosus* density, probably because most were captured indoors.

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Supporting information

Table S1. Premise and site-specific percentage of *P. perniciosus*-positive sticky traps, number of *P. perniciosus* collected, and median and maximum sand flies per m^2 and day in *P. perniciosus*-positive sticky traps.

Premise	Site	No. traps	Males				Females				All sand flies			
			% P[a]	No.	Density		% P	No.	Density		% P	No.	Density	
					Median	Max.			Median	Max.			Median	Max.
DK2	A	109	14	25	8	48	5	7	16	32	17	32	16	48
	B	107	7	9	16	16	1	1	16	16	8	10	16	16
	C	109	8	9	16	16	2	2	16	16	8	11	16	32
	D	108	4	4	12	16	0	0	0	0	4	4	12	16
	All	**433**	**8**	**47**	**16**	**48**	**2**	**10**	**16**	**32**	**9**	**57**	**16**	**48**
SF1	A	110	21	51	16	56	10	16	8	32	25	67	12	80
	B	110	21	32	16	64	7	9	16	16	25	41	16	80
	C	106	9	19	16	96	3	3	16	16	12	22	16	96
	D	105	16	30	16	96	4	5	16	32	18	35	16	96
	All	**431**	**17**	**132**	**16**	**96**	**6**	**33**	**16**	**32**	**20**	**165**	**16**	**96**
SF2	A	107	7	7	8	16	6	6	8	16	10	13	16	16
	B	109	3	3	8	8	1	1	16	16	4	4	8	16
	C	100	6	6	16	16	4	4	16	16	8	10	16	32
	D	104	12	13	16	16	4	6	12	24	14	19	16	40
	All	**420**	**7**	**29**	**16**	**16**	**4**	**17**	**16**	**24**	**9**	**46**	**16**	**40**
SF3	A	102	4	5	16	16	4	5	8	16	8	10	12	16
	B	110	5	7	16	32	0	0	0	0	5	7	16	32
	C	100	17	32	16	144	6	8	8	24	20	40	16	144
	D	108	6	9	8	16	2	2	8	8	7	11	8	16
	All	**420**	**8**	**53**	**16**	**144**	**3**	**15**	**8**	**24**	**10**	**68**	**16**	**144**

Table S1 (continued). Premise and site-specific percentage of *P. perniciosus*-positive sticky traps, number of *P. perniciosus* collected, and median and maximum sand flies per m^2 and day in *P. perniciosus*-positive sticky traps.

Premise	Site	No. traps	Male				Females				All sand flies			
			% P[a]	No.	Density		% P	No.	Density		% P	No.	Density	
					Median	Max.			Median	Max.			Median	Max.
All	A	428	11	88	16	56	6	34	8	32	15	122	16	80
	B	436	9	51	16	64	2	11	16	16	11	62	16	80
	C	415	10	66	16	144	4	17	16	24	12	83	16	144
	D	425	9	56	16	96	2	13	12	32	11	69	16	96
	All	**1704**	**10**	**261**	**16**	**144**	**4**	**75**	**16**	**32**	**12**	**336**	**16**	**144**

[a] % P: Percentage of positive traps (%).

Table S2. Time-specific percentage of *P. perniciosus*-positive sticky traps, number of *P. perniciosus* collected, and median and maximum sand flies per m^2 and day in *P. perniciosus*-positive sticky traps.

Month	Day[a]	Week[b]	No. traps	Males				Females				All sand flies			
				% P[c]	No.	Density		% P	No.	Density		% P	No.	Density	
						Median	Max.			Median	Max.			Median	Max.
May	23	2	157	1	2	12	16	0	0	0	0	1	2	12	16
June	6	4	155	5	12	16	64	3	4	8	16	7	16	16	64
	20	6	156	15	38	16	80	8	17	16	32	22	55	16	80
July	4	8	157	13	37	16	48	6	16	16	32	17	53	16	48
	18	10	156	13	31	16	56	6	10	8	16	17	41	16	56
August	1	12	151	14	25	8	24	5	8	16	16	16	33	16	40
	15	14	153	10	20	16	32	1	2	8	8	10	22	16	32
	29	16	160	11	34	16	96	2	3	8	8	11	37	16	96
September	12	18	152	7	13	16	64	3	5	16	16	8	18	16	80
	26	20	151	16	38	16	144	3	6	16	32	17	44	16	144
October	10	22	156	5	11	16	32	3	4	16	16	8	15	16	32

[a] First sampling day of the week; [b] Study week; [c] % P: Percentage of positive traps (%).

Table S3. Relationship between environmental variables recorded on site and the percentage of sticky traps with *P. perniciosus* and the median and maximum number of male and female specimens per m^2 and day in positive traps.

Variable	Level	No. traps	Males				Females				All sand flies			
			% P[a]	No.	Density		% P	No.	Density		% P	No.	Density	
					Median	Max.			Median	Max.			Median	Max.
Relative humidity (%)	31 - 40	232	14**	50	16	80	6**	19	16	32	18**	69	16	80
	41 - 50	691	8	75	16	64	4	29	8	16	11	104	16	80
	51 - 60	394	11	75	16	96	3	16	12	32	12	91	16	96
	61 - 73	387	10	61	16	144	2	11	16	32	12	72	16	144
Temperature (°C)	15 - 17	77	1	1	16	16	0	0	0	0	1	1	16	16
	18 - 19	154	7	17	16	64	5	8	16	16	10	25	16	80
	20 - 21	228	11	39	16	144	2	6	16	32	12	45	16	144
	22 - 23	313	8	40	16	80	5	19	16	32	12	59	16	80
	24 - 25	387	12	77	16	64	4	22	12	32	14	99	16	64
	26 - 27	394	10	62	16	96	3	12	8	16	12	74	16	96
	28 - 30	151	14	25	8	24	5	8	16	16	16**	33	16	40
Mean wind speed (m/s)	0.0	168	15	46	16	144	4	8	8	24	15	54	16	144
	0.1 - 1.0	322	10	40	16	80	3	13	16	24	12	53	16	80
	1.1 - 2.0	86	9	13	16	96	5	4	16	16	12	17	16	96
	2.1 - 7.2	68	10	12	16	64	1	1	16	16	12	13	16	64
Maximum wind speed (m/s)	0.0 - 7.0	211	11	42	16	144	2	7	8	24	11	49	16	144
	7.1 - 13.0	191	10	26	16	64	3	6	16	16	12	32	16	64
	13.1 - 19.0	110	11	21	16	96	5	5	16	16	15	26	16	96
	19.1 - 48.1	132	13	22	16	48	5	8	16	24	15	30	16	48
CO_2 concentration in sites A and B (ppm)	332 - 513	138	11	20	16	48	2	4	16	16	12	24	16	48
	514 - 582	167	8	19	16	24	4	7	12	16	11	26	16	40
	583 - 695	223	14	51	16	64	5	13	16	32	16	64	16	80
	696 - 1952	237	11	45	16	56	6	20	8	32	15	65	16	80

Table S3 (continued). Relationship between environmental variables recorded on site and the percentage of sticky traps with *P. perniciosus* and the median and maximum number of male and female specimens per m^2 and day in positive traps.

Variable	Level	No. traps	Males				Females				All sand flies			
			% P[a]	No.	Density		% P	No.	Density		% P	No.	Density	
					Median	Max.			Median	Max.			Median	Max.
Moon illumination (%)														
Sites A and B	0 - 25	389	12	68	16	48	4	20	8	32	14	88	16	48
	51 - 75	79	9	8	8	32	4	3	8	16	13	11	8	32
	76 - 100	396	9	63	16	64	4	22	16	32	12	85	16	80
Sites C and D	0 - 25	385	12	78	16	144	3	17	16	32	13	95	16	144
	51 - 75	77	8	8	16	32	3	2	16	16	10	10	16	32
	76 - 100	378	8	36	16	80	3	11	8	16	10	47	16	80

** p<0.05. Symbols placed in level with highest value.

[a] % P: Percentage of positive traps (%).

CHAPTER 3

Molecular xenomonitoring and host identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife park

Abstract

The epidemiological cycle of zoonotic phlebotomine-borne *Leishmania infantum* is a complex system in which domestic animals and wildlife interact and participate in its maintenance and transmission. In this study, we combined entomological surveillance, xenomonitoring of *L. infantum* and identification of host feeding sources of engorged females to investigate the potential contribution of a periurban wildlife park to leishmaniasis in neighboring residential areas. Overall, 7,309 sand flies were collected in 111 trap-days during the summers of 2016-2018 in an endemic area in southeast Spain. Five different sand fly species were captured, with *Phlebotomus perniciosus*, the main *L. infantum* vector in this region, representing the most common species. Sand fly distribution was spatially heterogeneous in terms of species, sexes and female physiological stage (unfed, gravid and engorged females) and related to host distribution and management, and environmental features. None of the 602 sand flies analyzed for *L. infantum* infection by kinetoplast real-time PCR were positive. We used molecular tools to identify the vertebrate hosts of sand flies and identified 17 host species, mainly mammals. Human DNA was not identified in engorged sand flies. This study provides evidence that wildlife parks in southeast Spain are ideal grounds for sand fly vectors but do not necessarily increase *L. infantum* infection risk to humans and dogs living in surrounding residential areas. This is probably because vectors feed mostly on non-*L. infantum* competent hosts and this should be investigated for a better understanding of the contribution of wildlife parks to the local epidemiology of *L. infantum*.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are hematophagous insect vectors of *Leishmania infantum* in the Mediterranean basin, responsible for canine and human leishmaniasis (CanL and HumL, respectively) (Ready, 2013). The parasite targets macrophages causing a wide spectrum of clinical conditions ranging from localized cutaneous infections to a life-threatening multisystem disease (Alvar *et al.*, 2004, 2012). In Spain, CanL is one of the most important diseases of dogs in many parts of the country (Solano-Gallego *et al.*, 2001; Chitimia *et al.*, 2011). Moreover, HumL, typically observed in immunocompromised people, has recently taken epidemic proportions among

immunocompetent residents (Arce *et al.*, 2013; Roth-Damas *et al.*, 2017) with an increasing incidence of leishmaniasis records as first diagnosis in non-HIV-infected individuals (Herrador *et al.*, 2015). In central Spain, an important outbreak is associated with a high prevalence of asymptomatic infection in hares and wild rabbits (Molina *et al.*, 2012; Arce *et al.*, 2013; Jiménez *et al.*, 2014). This episode exemplifies the potential role of wildlife as a reservoir for this parasite and the risk of leishmaniasis emergence associated with environmental human interventions (Tomassone *et al.*, 2018).

Anthropization affects the distribution of insect vectors (Ferraguti *et al.*, 2016) and, consequently, the dynamics of transmission of vector-borne pathogens (Bradley and Altizer, 2007), including leishmaniasis (Desjeux, 2001). Periurban wildlife parks are man-made environments that simulate the natural habitat of wild animals kept in captivity. The variety of potential *L. infantum* reservoir hosts and park's architectural layouts may provide ideal conditions for sand flies to breed, and could have a strong influence in the epidemiology of leishmaniasis in endemic areas. Indeed, several studies have described leishmaniasis cases in wildlife kept in captivity (Sastre *et al.*, 2008; Libert *et al.*, 2012). In Spain, *Leishmania* infections have been reported in species maintained in captivity, including Bennett's wallabies (Ramírez *et al.*, 2013; Montoya *et al.*, 2016) and orangutans (Miró *et al.*, 2018). However, with few exceptions (Montoya *et al.*, 2016; Miró *et al.*, 2018), no attention has been paid to the sand fly abundance and *Leishmania* infection rates in wildlife parks.

Thirteen sand fly species have been described in Spain of which *Phlebotomus perniciosus*, *Phlebotomus ariasi* and probably *Phlebotomus langeroni* are vectors of *L. infantum* (Alcover *et al.*, 2014; Díaz Sáez *et al.*, 2018). Adult females are responsible for parasite transmission as they require a bloodmeal to produce eggs (Killick-Kendrick, 1999). Warm- and cold-blooded vertebrates have been recorded as potential blood sources of these vectors (Maia *et al.*, 2013, 2015; Cotteaux-Lautard *et al.*, 2016; Latrofa *et al.*, 2018). A number of approaches have been developed for the identification of the host origin of sand fly bloodmeals, including competitive enzyme-linked immunosorbent assays (De Colmenares *et al.*, 1995; Bongiorno *et al.*, 2003) and, more recently, molecular tools. Selecting appropriate methods for accurate diagnosis has been the focus of much attention. Several genes have been successfully used including mitochondrial cytochrome

b and cytochrome c oxidase subunit I (COI), 12S and 16S rRNA genes (Alcaide *et al.*, 2009; Branco *et al.*, 2013; Jiménez *et al.*, 2013; Maia *et al.*, 2013; Valinsky *et al.*, 2014; González *et al.*, 2015, 2017; Bravo-Barriga *et al.*, 2016; Cotteaux-Lautard *et al.*, 2016; Bennai *et al.*, 2018) and the nuclear prepronociceptin (PNOC) gene (Haouas *et al.*, 2007; Jaouadi *et al.*, 2018). Moreover, molecular diagnosis of *L. infantum* infection indicates that many of vertebrate species may become infected with the parasite, but their ability to transmit infection back to the vector and therefore their potential contribution to the epidemiology of infection has not been proven in most cases (Quinnell and Courtenay, 2009; Tomassone *et al.*, 2018). *Leishmania infantum* infection rates in the vector provide an indication of parasite transmission intensity. They are highly variable ranging from <1% to 59% in the Iberian Peninsula, depending on the area investigated, time of the year, vector's physiological stage, diagnostic procedure used, the epidemic situation and, probably, the predominant blood source in the area (Martín-Sánchez *et al.*, 2006; Maia *et al.*, 2009; Branco *et al.*, 2013; Jiménez *et al.*, 2013; Alcover *et al.*, 2014; Bravo-Barriga *et al.*, 2016; González *et al.*, 2017).

Recently, *L. infantum* DNA was diagnosed by PCR in skin samples from three wolves (*Canis lupus*) and one brown bear (*Ursus arctos*) from the Murcia city wildlife park, in southeast Spain (Ortuño *et al.*, 2018; Risueño *et al.*, 2018). The area is *L. infantum* endemic, and the estimated PCR prevalence was 67% in dogs (Chitimia *et al.*, 2011), 32% in free wildlife (mostly foxes, rabbits and rodents) (Risueño *et al.*, 2018) and 8% in human blood donors (Pérez-Cutillas *et al.*, 2015). Since it was not possible to collect samples for *L. infantum* diagnosis from a representative number of the animals in the park, we investigated the abundance, *L. infantum* infection prevalence and bloodmeal sources of sand flies in the park. This method allows us to understand its potential contribution to the epidemiology of leishmaniasis in neighboring residential areas.

Materials and methods

Study area and animals

The study was conducted in "Terra Natura" wildlife park in the periurban surroundings of Murcia city (Figure 1). The park occupied 16 hectares and accommodated approximately 200 wild mammals from 30 species, 15 reptiles from three species and 300 birds from 26 species (described as Table S1), kept in open-air enclosures (Figure 2). Most enclosures held individuals from one or two species only except enclosures 9 and 22, holding birds, and enclosure 11 with several African savannah mammals and ostriches (Table S1). The park also had two small, under cover infirmaries where animals needing veterinary care were kept during treatment periods.

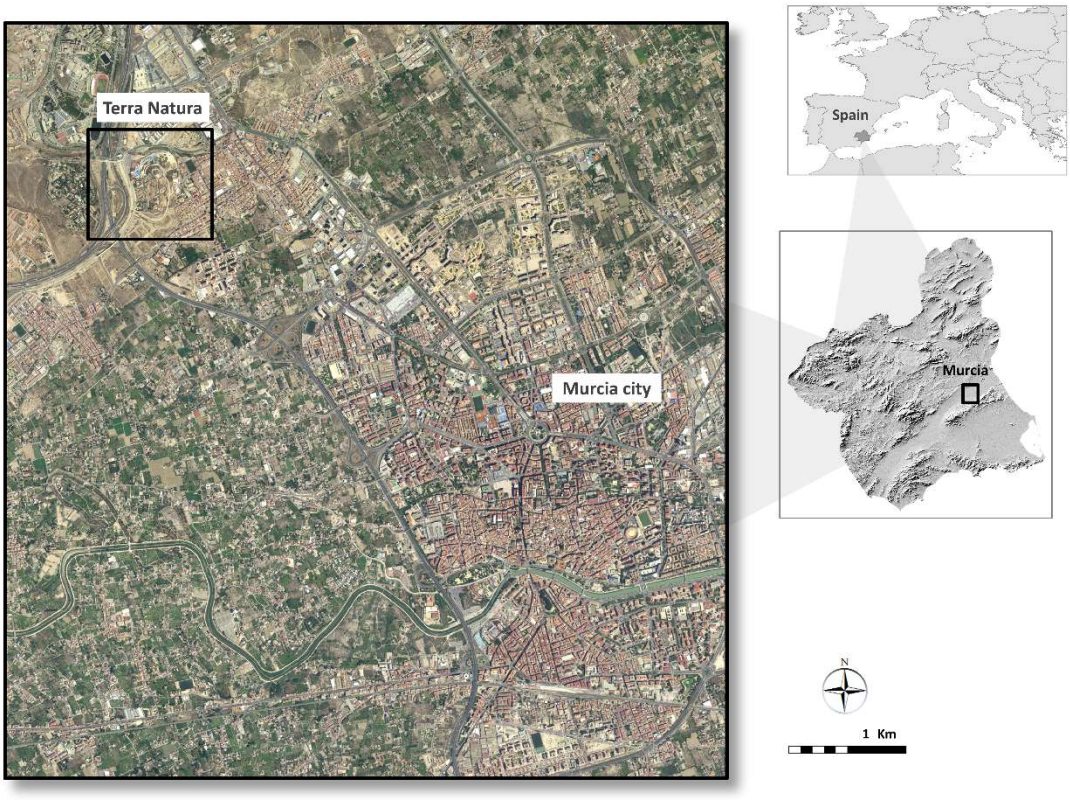

Figure 1. Geographical situation of Terra Natura Wildlife Park in Murcia city area in southeast Spain.

Vaccines against viral and bacterial infections and preventive anthelmintic and ectoparasitic treatments were selectively administered depending on the host species. Spot-on topical pipettes with the insecticide fipronil were applied to wolves and hyaenas once a month in the summer, and white rhinoceros were similarly treated with the pour-on insecticide cypermethrin. The injectable endectocide ivermectin was administered to brown bears and lions every 3 months and was alternated with oral anthelmintic fenbendazole on a six-monthly basis to treat white rhinoceros, primates, roe deer, sea lions, sitatunga, wildebeests and zebras. Other herbivore species were given fenbendazole every 6 months and carnivores received the cestocide praziquantel every 3 months.

Sand fly trapping

Sand flies were monitored in eight georeferenced sampling sites around the park (Figure 2). We used eight miniature Centers for Disease Control (CDC) battery-operated light-aspiration traps (J.W. Hock Company). Sand flies were trapped during 14 days (24 hr/day) in June and September 2016 (5 days), July 2017 (4 days), and June and September 2018 (5 days), totalling 112 trap-days. Altitude between sampling sites ranged from 80 to 98 m.a.s.l. (Table S2). Traps were placed adjacent to animal enclosures, and traps D and F were close to the infirmaries. They were hung at ground level against solid surfaces, under cover or in the open air. Vegetation cover within 5 m of the trap was visually assessed as being low, moderate or high (Table S2).

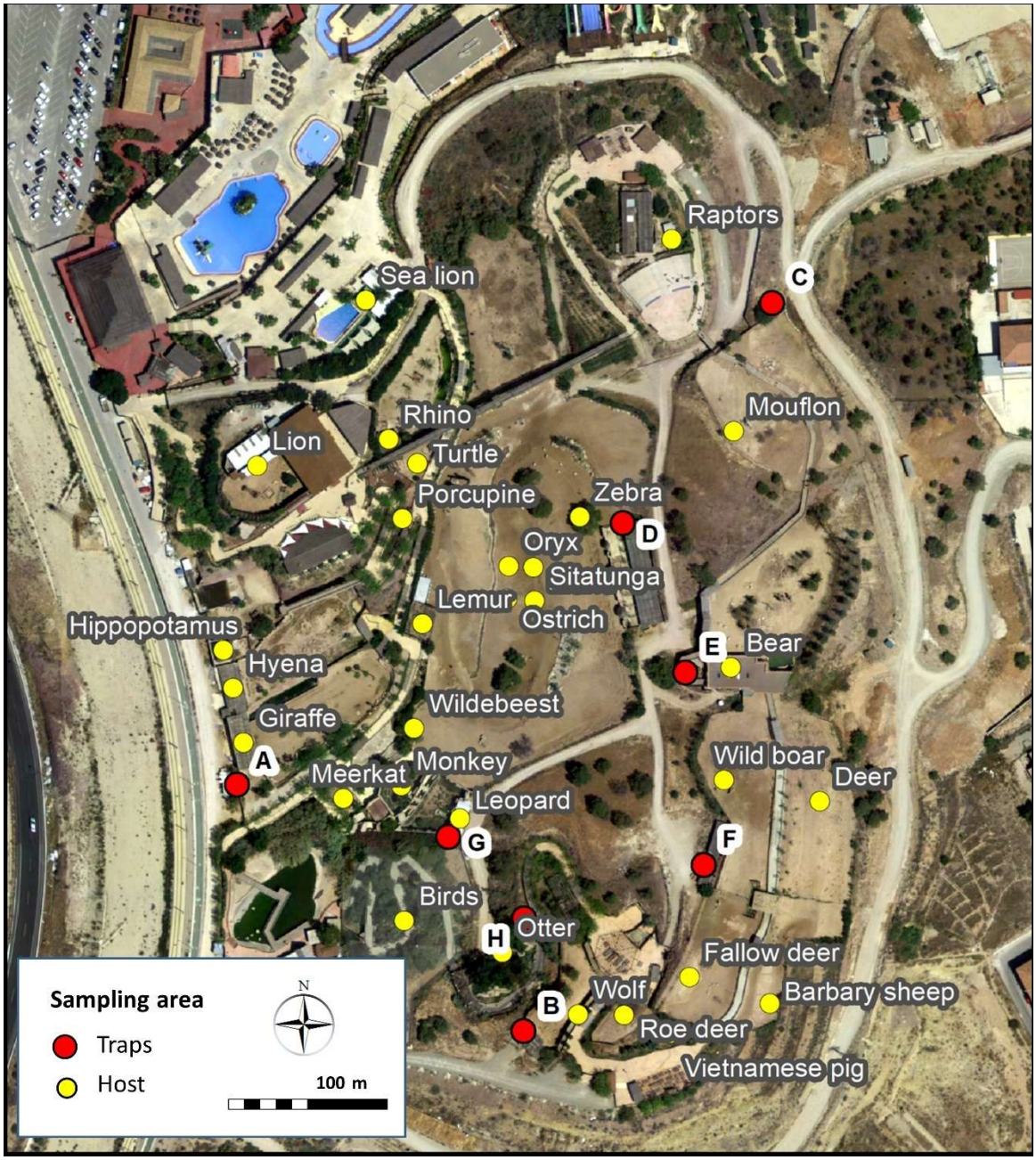

Figure 2. Spatial distribution of sand fly sampling sites (A-H) (red points) and potential feeding host's location (yellow points). The latter represent areas within host's enclosures where they gathered at night (night grounds), when adult sand flies are most active.

Sand fly identification

After collecting the traps, the net cages containing the arthropods were kept at −20°C for at least 2 h to kill the captures. Subsequently, sand flies were separated, counted and stored at −20°C in absolute ethanol until identified. Females were classified as gravid (with eggs in the abdomen), engorged (with a bloodmeal in the abdomen) and unfed (with neither blood nor eggs in the abdomen). Engorged sand flies were further classified into

five groups of increasing degree of blood digestion (Dolmatova and Demina, 1971), with sand flies in category 1 having the most abundant and brightest blood, and category 5 including those with the most digested, darkest and less abundant blood (Figure 3).

Figure 3. Degree of blood digestion in the sand fly gut. Engorged females were classified into five categories. Category 1 was considered as those bloodmeals with least digested, fresh, bright red blood occupying the whole abdomen and category 5 as those with the most digested blood.

All sand flies were morphologically identified using head (pharynx and cibarium) and genital structures (spermathecae in females and the external genitalia in males), using entomological keys (Martínez-Ortega and Conesa-Gallego, 1987; Gállego-Berenguer *et al.*, 1992). The sand fly head and terminal segments of the abdomen were dissected and then cleared and mounted using Marc-André solution and Hoyer's medium, respectively. The remaining sand fly body (thorax and abdomen) was stored at −20°C in absolute ethanol for genomic DNA extraction and DNA analysis to detect *L. infantum* infection and, for the case of engorged females, to identify the blood-feeding hosts.

DNA extraction

DNA was extracted using the 16 LEV Blood DNA Kit (Promega® ref: AS1290) in the Maxwell® semi-automated nucleic acid purification robot (Promega®). Samples included the thorax and abdomen of 602 field sand flies and 20 laboratory *L. infantum* infected and non-infected *P. perniciosus* used as DNA extraction and real-time PCR (rtPCR) positive and negative controls, respectively. Vials containing the sand flies were taken out of the freezer, and specimens were transferred to an empty tube. Remaining ethanol was allowed to evaporate before incubating them overnight at 56°C in the lysis buffer containing proteinase K. Sample preparation and manipulation was carried out under sterile conditions using a laminar flow cabin to avoid human DNA contamination.

Detection of *Leishmania infantum* DNA

The presence of *L. infantum* DNA was investigated in all 602 field specimens and in the 20 laboratory *L. infantum* infected and uninfected *P. perniciosus*, employing a rtPCR that amplified an approximately 120 base pair (bp) fragment of the kinetoplast DNA (kDNA) minicircle (Francino *et al.*, 2006), following Dantas-Torres *et al.* (2017). The rtPCR threshold cycle (Ct) was considered as a semi-quantitative measure of parasite DNA amplified. Samples were considered positive for Ct up to 38 since target sequences at this cycle approach a single copy (Mackay, 2007).

Host bloodmeal identification

PCR amplification and sequencing of a 648-bp fragment of the COI gene were used to identify the vertebrate hosts of 255 engorged females (Alcaide *et al.*, 2009). Briefly, a nested PCR with M13BCVFW and BCV-RV1 primers in the first reaction and M13BCV-FW and BCV-RV2 primers in the second reaction was used. Final amplicons were submitted to electrophoresis and visualized under UV light on a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen). Only sand flies collected before September 2018 were analyzed and included engorged specimens in bloodmeal digestion categories 1, 2 and 3, as using specimens with fresh blood maximizes host identification success (Haouas *et al.*, 2007; Martínez-de la Puente *et al.*, 2013; Valinsky *et al.*, 2014).

PCR products were sequenced in one or two directions using the M13BCV-FW and the M13BCV-FW and BCV-RV2 primers, respectively, by a commercial company (EZ-Seq, Macrogen Spain). Sequences were edited using the Sequencher v4.9 software (Gene Codes Corp., © 1991-2009), and electropherograms were visually analyzed to detect double peaks suggesting overlapping sequences corresponding to mixed species bloodmeals. Sequences were compared to those deposited in GenBank (National Center for Biotechnology Information Blast) and Barcode of Life Data Systems (BOLD). We assigned amplified vertebrate sequences to particular vertebrate species when agreement was ≥98% to sequences of known species.

Environmental data collection

Climatic information was obtained from La Alberca meteorological station (http://siam.imida.es/), distant 8 km from the wildlife park. The information included the hourly mean, maximum and minimum temperature (°C) and relative humidity (% RH), mean and maximum wind speed (m/s), wind direction (°) and rainfall (mm) from 22:00 to 03:00 h, coinciding with maximum sand fly activity (Romera Lozano and Martínez Ortega, 1998; Alten *et al.*, 2016). ArcGIS® (ESRI) was used to map the wildlife park and sampling sites.

Statistical analysis

The proportion of positive traps (with at least one specimen) and median female sand fly abundance in positive traps were compared across levels of explanatory variables, using Yates-corrected chi-squared test or Fisher's exact test when required, and the non-parametric Kruskal-Wallis test, respectively. The explanatory variables considered were trapping sites and date, mean temperature, % RH and wind speed, maximum wind speed, and trap-specific features including altitude, the number of animals within 10 m, proximity to the infirmary, roof cover, leaning surface, presence of vegetation and ground type (Table S2).

Three mixed negative binomial regression models were fitted to test the independent contribution of explanatory variables to three sand fly trap-day abundance variables: all sand flies and unfed or engorged and gravid *Phlebotomus* spp. females. Variables associated with the outcome in the bivariate analysis were considered and fitted as fixed effects, and trap was included as a random effect to assess remaining, unexplained trap variability (Snijders and Bosker, 1999). Because some trap variables were highly correlated (e.g. all traps under cover were in the high-altitude range, and those near the infirmary were the closest to the animals), to avoid collinearity these variables were not included in the same model. Instead, several models were developed and those with the lowest Akaike's information criteria (AIC) were considered the most parsimonious (Kleinbaum *et al.*, 1998). Significant differences were considered with $p<0.05$ for a double-sided test. Analyses were performed using R-project software (R Core Team, 2018).

Results

Frequency of sand flies: species and gender distribution

A total of 7,309 sand flies were collected. Overall, 97% (108/111, power in trap G failed one day) of the traps were positive for sand flies with a median (range) of 53 (4-313) specimens captured in these traps. Morphological identification was possible in 7,239 specimens (99%), and species frequency was 67% *P. perniciosus* (n=4,861), 16% *Phlebotomus papatasi* (n=1,204), 14% *Sergentomyia minuta* (n=1,045), 2% *P. ariasi* (n=114) and <1% *Phlebotomus sergenti* (n=15). The number of sand flies captured differed between traps, reaching the highest values in trap F (n=2,045) followed by traps B-E (range: 903-1,062) and traps A, G and H (range: 348-610). Females represented 40% of the specimens sexed, of which 23% contained visible blood in their abdomen, 13% were gravid, and 64% were unfed.

Species and gender frequency varied between traps. *Phlebotomus perniciosus* represented more than 75% of sand flies in traps C and D, but less than 56% of those collected in traps B and H (Figure 4). Likewise, the number of males and females collected was similar in traps B, C and H; instead, males predominated in other traps where the sex ratio (male/female) ranged from 1.44 to 2.33 (Figure 5). The distribution of females according to their abdominal content also varied according to the trap, and for example, the majority (>70%) of those in traps C and D were unfed females; instead, a large proportion of those in traps E and F were blood-fed females (32%). Similarly, the relative abundance of gravid females was generally low (4-18%) except in traps E and H that represented 21% and 22% of all females, respectively (Figure 6).

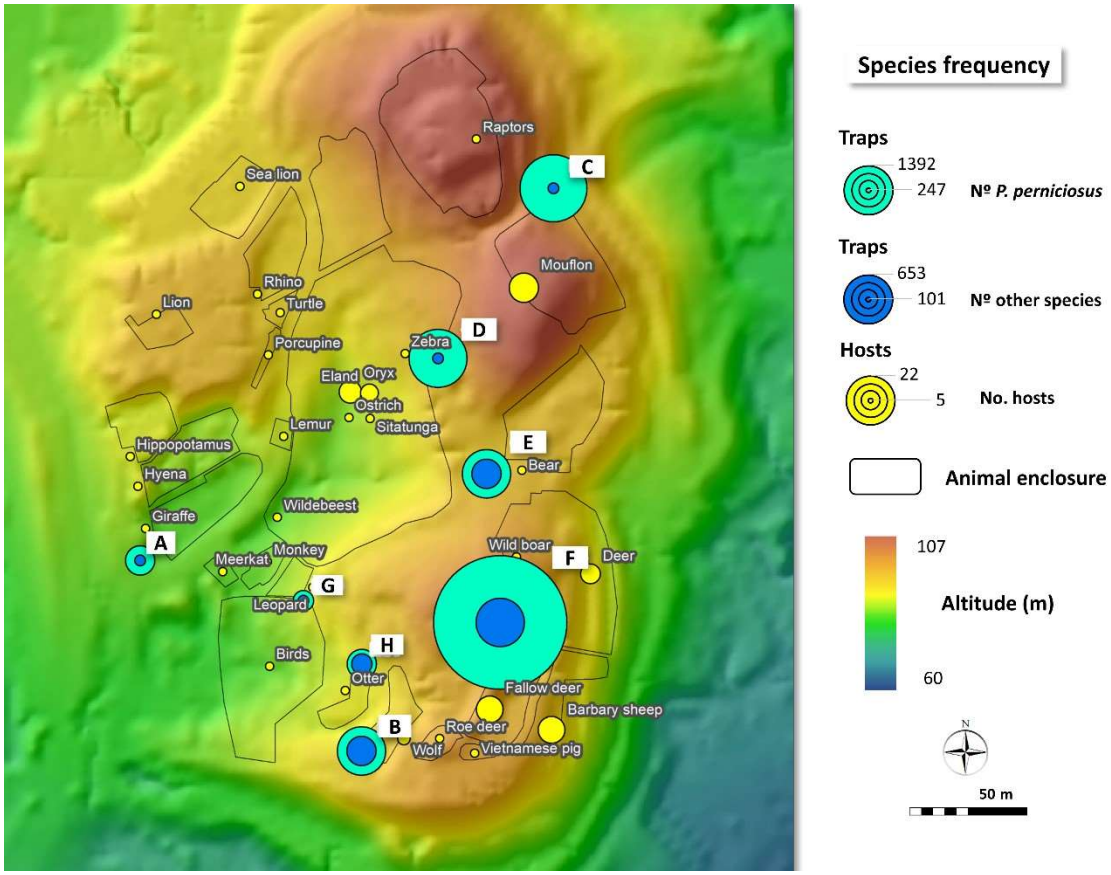

Figure 4. Spatial sand fly distribution according to the species frequency. Other species includes *P. papatasi*, *P. ariasi*, *P. sergenti* and *S. minuta*. Circle dimensions are proportional to sand fly abundance.

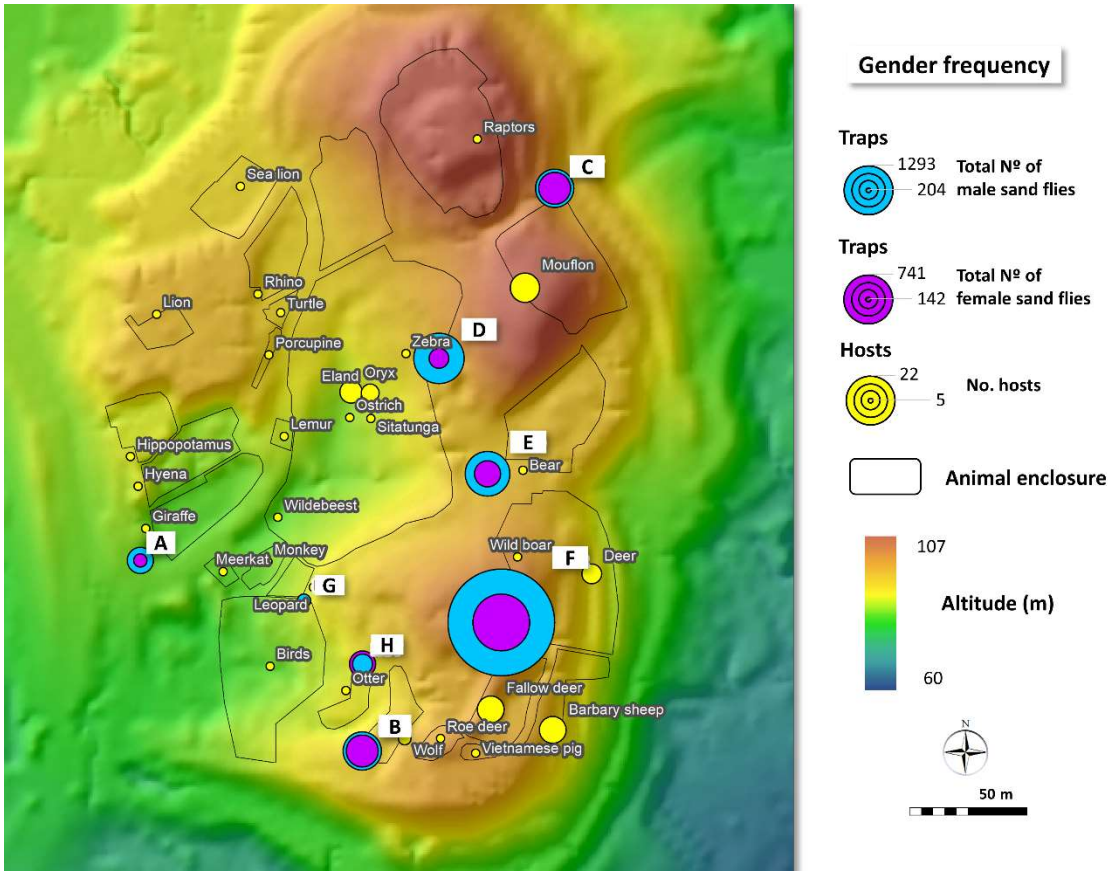

Figure 5. Spatial sand fly distribution according to the gender. Circle dimensions are proportional to sand fly abundance.

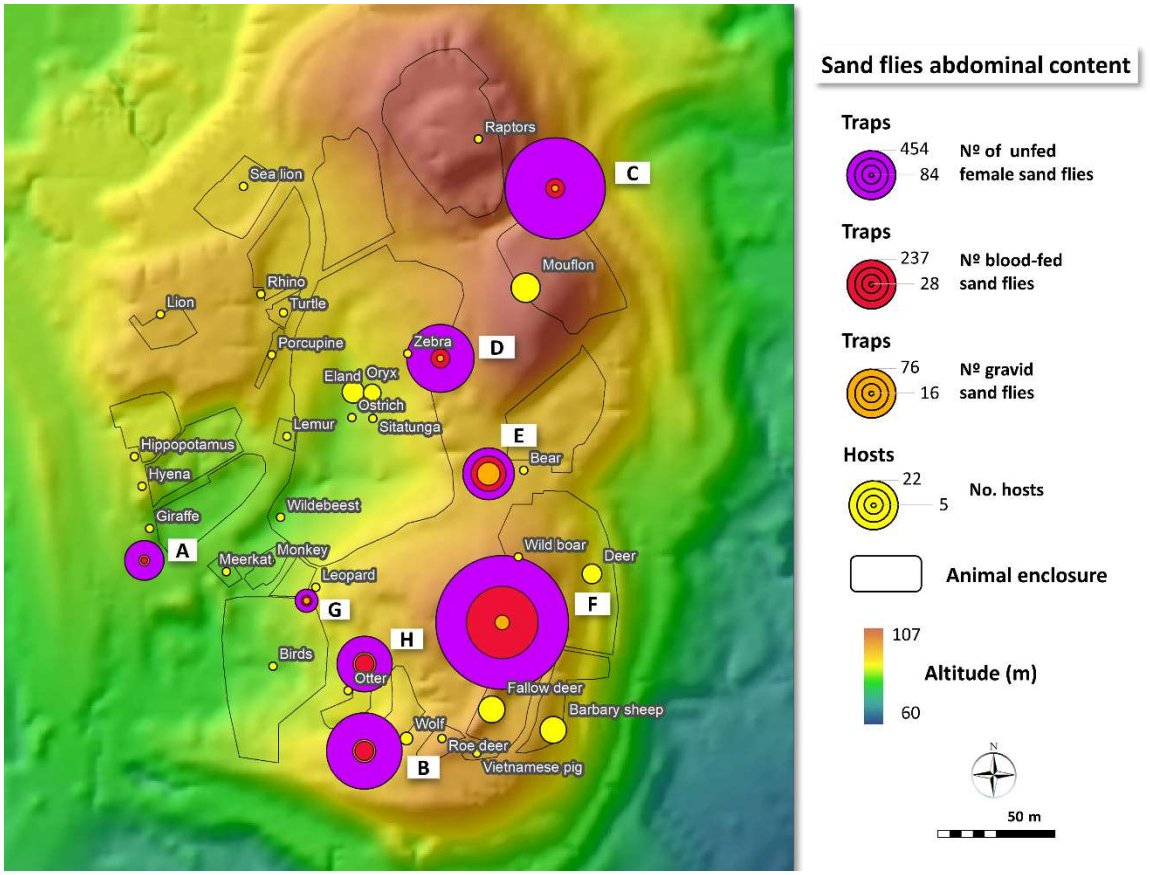

Figure 6. Spatial distribution of unfed, blood-fed and gravid female sand flies. Circle dimensions are proportional to sand fly abundance.

Relationship between female sand fly abundance, climate and environmental features

Bivariate analysis of median female sand fly abundance according to time and climatic and environmental factors of the trapping sites is presented in Tables 1 and 2. Median female abundance was highest in July 2017 and September 2018, and in trap F compared to other traps although there were differences depending on the species. The numerically highest median for *P. perniciosus* was in trap C, for *P. papatasi* in trap F and for *S. minuta* in trap B (Table 1). Moreover, median female abundance was positively associated with traps close to the infirmary, being under cover, animal census and % RH (Tables 1 and 2).

Table 1. Percentage of traps with at least one sand fly (% positive traps) and sand fly distribution according to species and sampling time, location and trap position and environmental features.

Variable	Level	No. traps	All females			*P. perniciosus* females			*P. papatasi* females			*S. minuta* females		
			% P	No. sand flies in positive traps		% P	No. sand flies in positive traps		% P[c]	No. sand flies in positive traps		% P	No. sand flies in positive traps	
				Total	Median (range)		Total	Median (range)		Total	Median (range)		Total	Median (range)
Sampling month and year	July 2016	23	96	344	13 (2-43)	96	172	6 (1-21)	83	91	3 (1-17)	74	69	3 (1-23)
	Sept. 2016	16	94	196	8 (2-32)	88	118	5 (1-26)	69	46	3 (1-9)	56	22	1 (1-10)
	July 2017	32	100	1006	30 (7-59)	100	665	17 (6-46)	91	137	4 (1-16)	94**	163	3 (1-20)
	June 2018	24	96	684	19 (3-126)	96	409	7 (2-90)	75	87	3 (1-20)	92	175	6 (2-37)**
	Sept. 2018	16	100	653	36 (10-92)**	88	343	24 (4-34)**	100**	211	11 (3-38)**	94	87	2 (1-24)
Sampling site	A	14	100	201	16 (2-28)	100	136	10 (1-23)	79	21	1 (1-5)	93	41	2 (1-12)
	B	14	100	385	32 (7-43)	100	131	10 (2-17)	64	51	6 (1-12)	100*	193	12 (3-37)**
	C	14	100	414	29 (6-59)	100	315	22 (5-46)**	93	43	3 (1-7)	64	21	2 (1-3)
	D	14	93	317	21 (2-56)	93	210	16 (2-36)	71	71	4 (1-22)	64	31	2 (1-9)
	E	14	100	361	17.5 (8-56)	100	225	11 (4-37)	100	81	5 (1-18)	93	45	3 (1-9)
	F	14	100	741	41 (12-126)**	100	437	19 (4-90)	100**	190	14 (4-38)**	93	99	3 (1-24)
	G	13	92	142	11 (2-23)	85	89	7 (2-16)	69	17	2 (1-4)	85	31	3 (1-6)
	H	14	93	322	29 (6-46)	93	164	11 (4-26)	93	98	7 (1-23)	79	55	3 (1-14)
Next to infirmary[a]	No	83	98	1825	20 (2-59)	96	1060	12 (1-46)	83	311	3 (1-23)	86	386	3 (1-37)
	Yes	28	96	1058	30 (2-126)**	96	647	16 (2-90)**	86	261	8 (1-38)**	79	130	3 (1-24)
Roofing	Absence	55	96	1050	18 (2-46)	95	520	10 (1-26)	76	187	3 (1-23)	89	320	3 (1-37)
	Presence	56	98	1833	29 (2-126)**	98	1187	16 (2-90)**	91**	385	5 (1-38)**	79	196	3 (1-24)
Leaning surface type	Heather fence	55	98	1498	21 (2-126)	96	977	13 (1-90)	85	271	3 (1-38)	84	192	3 (1-24)
	Concrete wall	14	93	317	21 (2-56)	93	210	16 (2-36)	71	71	4 (1-22)	64	31	2 (1-9)
	Stone wall	14	100	385	32 (7-43)	100	131	10 (2-17)	64	51	6 (1-12)	100*	193	12 (3-39)**
	Wooden post	14	100	361	18 (8-56)	100	225	11 (4-37)	100*	81	5 (1-18)	93	45	3 (1-9)
	Wire fence	14	93	322	29 (6-46)	93	164	11 (4-26)	93	98	7 (1-23)	79	55	3 (1-14)

Table 1 (continued). Percentage of traps with at least one sand fly (% positive traps) and sand fly distribution according to species and sampling time, location and trap position and environmental features.

Variable	Level	No. traps	All females			*P. perniciosus* females			*P. papatasi* females			*S. minuta* females		
			% P	No. sand flies in positive traps		% P	No. sand flies in positive traps		% P[c]	No. sand flies in positive traps		% P	No. sand flies in positive traps	
				Total	Median (range)		Total	Median (range)		Total	Median (range)		Total	Median (range)
No. animals[b]	2 - 4	41	95	665	17 (2-46)	93	389	9 (1-26)	80	136	2 (1-23)	86	127	3 (1-14)
	5 - 8	28	100	746	26 (7-56)	100	356	10 (2-37)	82	132	6 (1-18) **	96**	238	6 (1-37) **
	19 - 42	42	98	1472	30 (2-126)**	98	962	17 (2-90)**	88	304	5 (1-38)	74	151	3 (1-24)

** p-value <0.05, and * p-value <0.10. Symbols are placed in the level with highest value.

[a] Light trap placed next to open-air animal cages.

[b] Considering animal census from enclosures within 10 meters of the light trap.

[c] % P: Percentage of positive traps (%).

Table 2. Percentage of traps with at least one sand fly (% positive traps) and sand fly distribution according to species, altitude and climatic variables.

Variable	Level	No. traps	All females			*P. perniciosus* females			*P. papatasi* females			*S. minuta* females		
			% P[b]	No. sand flies in positive traps		% P	No. sand flies in positive traps		% p	No. sand flies in positive traps		% P	No. sand flies in positive traps	
				Total	Median (Range)		Total	Median (Range)		Total	Median (Range)		Total	Median (Range)
Altitude (m.a.s.l.)	≤ 90	55	96	1050	18 (2-46)	95	520	9.5 (1-26)	76	187	3 (1-23)	89	320	3 (1-37)
	> 90	56	98	1833	29 (2-126)**	98	1187	16 (2-90)**	91	385	5 (1-38)**	79	196	3 (1-24)
Mean temperature[a] (°C)	20.6 - 23	64	97	1699	20 (2-126)	95	947	11 (1-90)	83	390	4 (1-38)	84	323	3 (1-37)
	24 - 25.4	47	98	1184	26 (2-59)	98	760	14 (1-46)	85	192	4 (1-16)	83	193	3 (1-20)
Mean relative humidity[a] (%)	45 - 56.5	31	97	436	10 (2-74)	94	247	5 (1-62)	71	84	2 (1-15)	71	87	3 (1-13)
	57 - 66	48	98	1380	23 (3-126)	98	855	14 (2-90)	83	205	4 (1-20)	92*	279	3 (1-37)
	69 - 80	32	97	1067	33 (4-92)**	97	605	17 (1-46)**	94	283	6 (1-38)**	84	150	2 (1-24)
Mean windspeed[a] (m/s)	0.4 - 0.5	63	95	1579	21 (2-126)	95	852	10.5 (1-90)	86	387	4 (1-38)	81	302	3 (1-37)
	0.6 - 0.9	48	100	1304	26 (2-108)	98	855	15 (1-88)*	81	185	4 (1-16)	88	214	3 (1-20)
Maximum windspeed[a] (m/s)	1.2 - 1.5	63	95	1635	20 (2-126)	95	920	10 (1-90)	81	371	4 (1-38)	79	306	3 (1-37)
	1.7 - 3.5	48	100	1248	28 (2-59)	98	787	14 (1-46)*	88	201	4 (1-17)	90	210	3 (1-23)

** p-value <0.05 and * p-value <0.10. Symbols are placed in the level with highest value.

[a] Calculated as the average of hourly data from 22.00 h to 03.00 h of the following day.

[b] Percentage of positive traps (%).

The most parsimonious regression models included the number of hosts within a 10 m radius of the trap, traps being placed under a roof or otherwise and % RH (Table 3). Abundance of all sand flies or *Phlebotomus* females (unfed or engorged and gravid) was positively associated with % RH (Table 3). Total sand flies were also positively associated with host density within 10 m and with traps situated under a roof. In contrast, abundance of unfed sand flies was similarly associated with host density but not with traps being under a roof, and the opposite was the case of engorged and gravid female abundances, which were associated with traps placed under a roof and not with host density in the proximity of the trap (Table 3). In addition, models indicate substantial, remaining unexplained variation between traps; the standard deviation of the random effect ranged from 0.09 for the model for all flies to 0.23 in the model for unfed *Phlebotomus* females.

Table 3. Estimates of three negative binomial regression models examining the relationship between sand fly abundance and explanatory variables. Models differed in the outcome variable, which were abundance of: all sand flies (1), unfed *Phlebotomus* spp. females (2) and engorged and gravid *Phlebotomus* spp. females (3).

1) MODEL: All sand flies		**Estimate**	***SE*[a]**	**95% CI[b]**		**P value**
Fixed effects						
Intercept		2.29	0.38	1.55	3.03	<0.001
Roof cover	No	0.00				
	Yes	0.48	0.18	0.14	0.83	0.007
Animal census within 10 m.	2 - 4	0.00				
	5 - 8	0.37	0.20	0.02	0.75	0.061
	19 - 42	0.76	0.17	0.41	1.09	<0.001
Relative humidity (%)		0.02	0.01	0.01	0.031	<0.001
Random effect		SD[c]				
Trap		0.09				

2) MODEL: Unfed females[d]		**Estimate**	***SE*[a]**	**95% CI[b]**		**P value**
Fixed effects						
Intercept		0.57	0.437	0.28	1.41	0.189
Roof cover	No	0.00				
	Yes	0.13	0.28	0.41	0.66	0.650
Animal census within 10 m.	2 - 4	0.00				
	5 - 8	0.32	0.31	0.28	0.92	0.295
	19 - 42	0.84	0.27	0.32	1.360	0.002
Relative humidity (%)		0.03	0.01	0.02	0.04	<0.001
Random effect		SD[c]				
Trap		0.23				

Table 3 (continued). Estimates of three negative binomial regression models examining the relationship between sand fly abundance and explanatory variables. Models differed in the outcome variable, which were abundance of: all sand flies (1), unfed *Phlebotomus* spp. females (2) and engorged and gravid *Phlebotomus* spp. females (3).

3) MODEL: Blood-fed and gravid females[d]		Estimate	*SE*[a]	95% CI[b]		P value
Fixed effects						
Intercept		0.51	0.48	0.42	1.44	0.282
Roof cover	No	0.00				
	Yes	0.98	0.26	0.46	1.50	0.000
Animal census within 10 m.	2 - 4	0.00				
	5 - 8	0.13	0.30	0.45	0.72	0.659
	19 - 42	0.13	0.26	0.38	0.64	0.625
Relative humidity (%)		0.02	0.01	0.01	0.03	0.009
Random effect		*SD*[c]				
Trap		0.21				

[a] SE: Standard error.
[b] 95% CI: 95% confidence interval.
[c] SD: Standard deviation.
[d] It includes *P. perniciosus*, *P. ariasi*, *P. papatasi* and *P. sergenti*.

Sand fly *Leishmania infantum* infection

The number of field sand flies tested were 602 specimens including *P. perniciosus* (n=519), *P. ariasi* (n=32), *P. papatasi* (n=49) and *S. minuta* (n=2), comprising 435 blood-fed, 71 gravid and 96 unfed females. These sand flies were collected during the three study years in June (n=54), July (n=390) and September (n=158). *Leishmania infantum* DNA was not detected in any of the sand flies analyzed. In contrast, all 10 laboratory infected sand flies used as positive controls were rtPCR-positive. No evidence of contamination was found in the 10 infection-free laboratory *P. perniciosus* used as negative controls.

Vertebrate host identification

Vertebrate hosts of sand flies were successfully identified for 205 out of the 255 engorged females tested (80%). They included 160/194 *P. perniciosus*, 37/49 *P. papatasi*, and 8/10 *P. ariasi*. Blood belonged to 17 different host species (Table 4). Among the amplified sequences, 11 corresponded to *Canis lupus* belonging to either Iberian wolves (*C. l. signatus*) or dogs (*C. l. familiaris*) and two corresponded to *Sus scrofa*, either wild boar (*S. s. scrofa*) or Vietnamese pig (*S. s. domesticus*). Also, two sequences corresponding to *Panthera* spp. could correspond to either African lion or leopard. As expected, the

identification success decreased with increasing blood digestion and was 95%, 85% and 67% in sand flies in digestion categories 1, 2 and 3, respectively ($p<0.05$).

Phlebotomus perniciosus was found to feed on 15 different host species, and *P. papatasi* and *P. ariasi* fed on 11 and 7 species, respectively (Table 4). Fallow deer (n=57) and red deer (n=49) were the most common hosts of sand flies (Table 4). Sand flies feeding on cats were collected in all traps except trap A; instead, most of the sand flies feeding on other hosts were collected in one or two traps and the maximum was five traps (Table 4). We did not find evidence of mixed bloodmeals.

Table 4. Number (%) of bloodmeals and ectoparasiticide treatment according to the vertebrate host, sand fly species and sampling site.

Vertebrate hosts	Bloodmeal No. (%)	Insecticide treatment	Sand fly species			Trap							
			P. perniciosus	*P. papatasi*	*P. ariasi*	A	B	C	D	E	F	G	H
Scimitar-horned oryx (*Oryx dammah*)	20 (10)	No	17 (10.5)	3 (8)	-	2 (33)	-	-	6 (43)	7 (27)	-	2 (13)	3 (20)
Common eland (*Taurotragus oryx*)	16 (8)	No	12 (7)	3 (8)	1 (12.5)	-	-	-	2 (14)	11 (42)	-	2 (13)	1 (7)
Barbary sheep (*Ammotragus lervia*)	14 (7)	No	11 (7)	3 (8)	-	-	1 (7)	-	-	-	13 (12)	-	-
Mouflon (*Ovis aries*)	6 (3)	No	4 (2.5)	1 (3)	1 (12.5)	-	-	4 (67)	1 (7)	-	1 (1)	-	-
Blue wildebeest (*Connochaetes taurinus*)	1 (<1)	Ivermectin	-	1 (3)	-	-	-	-	-	-	-	-	1 (7)
Sitatunga (*Tragelaphus spekii*)	1 (<1)	Ivermectin	1 (1)	-	-	-	-	-	1 (7)	-	-	-	-
Fallow deer (*Dama dama*)	57 (28)	No	47 (29)	9 (24)	1 (12.5)	-	1 (7)	-	-	-	55 (50)	-	1 (7)
Red deer (*Cervus elaphus*)	49 (24)	No	36 (22)	12 (32)	1 (12.5)	-	1 (7)	-	-	5 (19)	34 (31)	5 (31)	4 (27)
Roe deer (*Capreolus capreolus*)	5 (2)	Ivermectin	2 (1)	1 (3)	2 (25)	-	2 (13)	-	-	-	2 (2)	1 (6)	-
Domestic cat (*Felis catus*)	11 (5)	No	8 (5)	2 (5)	1 (12.5)	-	1 (7)	1 (17)	1 (7)	1 (4)	1 (1)	4 (25)	2 (13)
African lion (*Panthera leo*)	1 (<1)	Ivermectin	1 (1)	-	-	1 (17)	-	-	-	-	-	-	-
Lion/Leopard (*Panthera* spp.)	2 (1)	No	2 (1)	-	-	-	-	-	-	-	-	1 (6)	1 (7)
Wild boar /Vietnamese pig (*Sus scrofa*)	2 (1)	No	2 (1)	-	-	-	-	-	-	-	2 (2)	-	-
Ostrich (*Struthio camelus*)	4 (2)	No	3 (2)	1 (3)	-	-	-	1 (17)	1 (7)	1 (4)	-	1 (6)	-

Table 4 (continued). Number (%) of bloodmeals and ectoparasiticide treatment according to the vertebrate host, sand fly species and sampling site.

Vertebrate hosts	Bloodmeal No. (%)	Insecticide treatment	Sand fly species			Trap							
			P. perniciosus	*P. papatasi*	*P. ariasi*	A	B	C	D	E	F	G	H
Iberian wolf/domestic dog[a] (*Canis lupus*)	11 (5)	Fipronil/ Unknown[a]	10 (6)	-	1 (12.5)	-	8 (53)	-	-	-	1 (1)	-	2 (13)
Giraffe (*Giraffa camelopardalis*)	3 (1)	No	3 (2)	-	-	3 (50)	-	-	-	-	-	-	-
Zebra (*Equus burchellii*)	1 (<1)	Ivermectin	-	1 (3)	-	-	-	-	1 (7)	-	-	-	-
White rhinoceros (*Ceratotherium simum*)	3 (1)	Cypermethrin Ivermectin	3 (2)	-	-	-	1 (7)	-	1 (7)	1 (4)	-	-	-
Total	**207 (100)**		**162 (100)**	**37 (100)**	**8 (100)**	**6 (100)**	**15 (100)**	**6 (100)**	**14 (100)**	**26 (100)**	**109 (100)**	**16 (100)**	**15 (100)**

[a] Dogs from residential areas adjacent to the park

Table 5 shows the number of bloodmeals, host family census and biting rates (No. of bloodmeals per host family). Census was not strongly associated with the number of bloodmeals, and biting rates (95% CI) were highest for the 31 members of the Cervidae at 3.6 (2.9-4.3) bloodmeals/animal, followed by the Rhinocerotidae (1 animal) at 3.0 (0-6.4) bloodmeals/animal and Canidae (5 animals) at 2.2 (0.9-3.5) bloodmeals/animal, and was only 0.8 (0.6-1.0) bloodmeals/animal for the Bovidae (70 animals) (Table 5).

Table 5. Number of bloodmeals, census and biting rates according to the mammal and bird host families.

Host family	No. bloodmeals	Census	Biting rate[a] (95% CI)
Bovidae	58	70	0.83 (0.62 - 1.04)
Cervidae	111	31	3.6 (2.9 - 4.3)
Felidae	14	29	0.48 (0.23 - 0.74)
Suidae	2	13	0.15 (0 - 0.37)
Struthionidae	4	6	0.67 (0.01 - 1.32)
Canidae	11	5	2.2 (0.9 - 3.5)
Giraffidae	3	3	1 (0 - 2.13)
Equidae	1	3	0.33 (0 - 0.99)
Rhinocerotidae	3	1	3 (0 - 6.4)

[a]Biting rate = No. of bloodmeals / family census.

Frequency of sand flies feeding on animals treated with insecticides

The percentage of sand fly feeds taken from animals receiving some kind of insecticide treatment was 13% (27/207). They included 12 feeds on animals receiving ivermectin, 11 feeds on wolves treated with fipronil or possibly dogs from outside the park with unknown insecticide treatment status, and four from white rhinoceros treated with cypermethrin (Table 4).

Discussion

Molecular xenomonitoring, defined as the identification of pathogens in the insect vectors rather than in hosts, represents a promising tool for parasite surveillance (Pilotte *et al.*, 2017). Here, we combined molecular xenomonitoring of *L. infantum* with entomological surveillance and identifications of vector bloodmeals to understand the epidemiology of leishmaniasis in a periurban wildlife park and its potential implication to neighboring human and dog populations in residential areas.

Human alteration of the landscape represents a key factor affecting the distribution and abundance of insect vectors (Ferraguti *et al.*, 2016), finally affecting the epidemiology of vector-borne pathogens. Here, we report a high vector density in a wildlife park, supporting the role of these modified environments providing ideal habitats for sand flies. There was predominance of *P. perniciosus* over other species, which is in line with the findings from surveys of sand fly species in Murcia Region (Martínez Ortega and Conesa Gallego, 1987; Risueño *et al.*, 2017). The spatial distribution of this and other species was strongly aggregated with marked differences between sites, sexes and female physiological stage-specific abundance. It is well established that adult sand flies congregate close to animals they feed on (Alexander, 2000; Munstermann, 2004). In farms and kennels in rural Murcia, *P. perniciosus* abundance was inversely proportional to the distance to the main animal group, with some distributional differences between males and females (Muñoz *et al.*, 2018). Here, the traps with largest captures were those next to the infirmary cages and a large proportion of bloodmeals corresponded to host species kept in these places. The abundance of vertebrates within 10 m of the trap was positively associated with the overall sand fly abundance and the abundance of unfed *Phlebotomus* spp. females, but not to the abundance of engorged and gravid *Phlebotomus* spp. females. Instead, engorged and gravid *Phlebotomus* spp. female abundance was larger in traps placed under roof cover. This suggests differential behavioral patterns of females depending on their physiological stage, with engorged and gravid females likely looking for sheltered areas to digest the bloodmeal and produce eggs, and unfed females preferring sites close to potential feeding hosts. Abundance of all sand fly stages was also positively associated with higher % RH, which may be favorable for sand fly survival, although previous studies of the relationship between % RH and *P. perniciosus* abundance in Murcia Region showed contrasting results (Risueño *et al.*, 2017; Muñoz *et al.*, 2018). The impact on vector distribution of selective administration of insecticides is difficult to assess because treated and untreated animals were scattered around the park and insecticide persistence declines with the time. Moreover, none of the treatment protocols used have a proven repellent efficacy against sand flies (Gálvez *et al.*, 2018). Finally, it is likely that some of the observed variability in sand fly abundance and particularly in the

frequency of engorged and gravid females was related to differences in site suitability as breeding and resting grounds for the insects.

The identification of the bloodmeal sources of arthropods is important for a better understanding of vector, host and pathogen interactions (Kent, 2009). Like in the present study, some authors highlight the inverse relationship between successful identification of vertebrate hosts and the extent of blood digestion in the insect's abdomen (Haouas *et al.*, 2007; Martínez-de la Puente *et al.*, 2013; Valinsky *et al.*, 2014), and this should be considered when performing feeding host identification studies. Our results support the role of mammals as the main bloodmeal sources of the sand fly species studied here, with ostrich representing the only bird species identified. Previous bloodmeal analysis has identified a variety of hosts including artiodactyls, rodents, lagomorphs, canids, felids, bats, humans, birds, reptiles and amphibians (Jiménez *et al.*, 2013; Maia *et al.*, 2013; Valinsky *et al.*, 2014; Bravo-Barriga *et al.*, 2016; Cotteaux-Lautard *et al.*, 2016; González *et al.*, 2017). These results support the opportunistic feeding behavior of sand flies. Interestingly, contrary to other studies conducted in Spain (Jiménez *et al.*, 2013; Bravo-Barriga *et al.*, 2016; González *et al.*, 2017), we did not detect the presence of human DNA in engorged sand flies, which may be due to the scarcity of humans in the park at night, when sand flies are most active. In this respect, we included information on host census, which allowed us to estimate relative biting rates in host species. This information is lacking in most studies conducted under natural conditions. Notwithstanding this, reporting the proportion of bloodmeals from different host species is not sufficient to assess blood-feeding preferences (Kent, 2009; Takken and Verhulst, 2013). Feeding behavior is conditioned by numerous factors that need to be considered before robust conclusions can be reached, including host availability and accessibility and selective use of insecticides. For this reason, in this study we reported the relative biting rate for each vertebrate host species, taking into account the abundance of each vertebrate in the park. From this information, it becomes apparent that cervids, rhinoceros and canids had the highest biting rates.

None of the specimens analyzed were infected with *L. infantum*, strongly suggesting that transmission in the park is very low. The absence of *L. infantum* infection in the vector is further evidence that vector abundance is not necessarily associated with high infection

risk. Vectors, susceptible hosts and *L. infantum* (pathogen) need to converge in time and space for efficient pathogen transmission (Kent, 2009). PCR detection of *L. infantum* in three wolves and one brown bear indicates that infection is present in the park, and canids are competent *L. infantum* reservoirs (Ortuño *et al.*, 2018; Risueño *et al.*, 2018). In addition, our results support the role of canines as common hosts of sand flies in the area. Due to the high genetic relatedness, it was not possible to discriminate between dog and wolf blood, but all the sand flies with canid blood were captured in the traps closest to the wolves' enclosure. Still, these species represented a small percentage of the animals in the park, and the majority of host species were presumably non-competent reservoirs of *L. infantum*, probably reducing the overall risk of infection. Further studies are needed to evaluate the reservoir role of most park host species. The phenomenon by which increased diversity of non-competent hosts results in reduced infection risk is known as infection “dilution effect”, and it has been widely studied in other arthropod-borne diseases (Keesing *et al.*, 2006). However, species diversity can be associated with both infection reduction and augmentation mechanisms with an often unpredictable net effect (Keesing *et al.*, 2006; Randolph and Dobson, 2012; Wood and Lafferty, 2013). Consequently, additional studies are necessary to understand how vertebrate and phlebotomine biodiversity affects the transmission dynamics of *L. infantum* and in particular its transmission to humans and domestic dogs.

In summary, the present study indicates that the wildlife park provides suitable conditions for sand flies but not necessarily for efficient *L. infantum* transmission. Factors driving this state of affairs are not well understood and probably include host´s susceptibility to infection and park layout features and management practices including animal concentration in infirmaries. The study also provided some evidence of differential spatial distribution of *Phlebotomus* spp. females depending on their physiological stage which may be useful to consider when implementing sand fly control measures in the park.

Conclusions

Periurban wildlife parks can provide ideal conditions for maintaining a large population of phlebotomine sand fly vectors. Although high vector abundance is not necessarily associated with increased *L. infantum* infection risk, given the circulation of this parasite in the area, it is still recommendable to apply a phlebotomine control program to reduce the risk of *Leishmania* spillover from domestic animals into park wildlife. The present situation in the park may be holding on a fine balance, and *Leishmania* surveillance should be part of its sanitary program.

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Supporting information

Table S1. List of animal species and surface of their open-air enclosures in "Terra Natura" zoological park.

Enclosure		Animal species (census[a])		Density (m^2/animal)
Number	**Surface (m^2)**			
1	376	African lion -*Panthera leo*- (6)		63
2	728	Hippopotamus -*Hippopotamus amphibius*- (3)		243
3	1203	Giraffe -*Giraffa camelopardalis*- (3)		401
4	1059	Spotted hiena -*Crocuta crocuta*- (2)	Striped hiena -*Hyaena hyaena*- (2)	265
5	569	Iberian wolf -*Canis lupus*- (5)		114
6	222	Roe deer -*Capreolus capreolus*- (1)		222
7	103	Vietnamese pig -*Sus scrofa*- (7)		15
8	2285	Mouflon -*Ovis aries*- (22)		104
9	3268	Griffon vulture -*Gyps fulvus*- (1) Black vulture -*Coragyps atratus*- (1) Turkey vulture -*Cathartes aura*- (1) Black kite -*Milvus migrans*- (5) Harris's hawk -*Parabuteo unicinctus*- (5) Steppe eagle -*Aquila nipalensis*- (1)	Saker falcon -*Falco cherrug*- (2) Peregrine falcon -*Falco peregrinus*- (1) Tawny owl -*Strix aluco*-(1) Indian eagle owl -*Bubo bengalensis*- (2) Eurasian eagle owl -*Bubo bubo*- (1) Barn owl -*Tyto alba*- (1)	149
10	131	African spurred tortoise -*Geochelone sulcata*- (6)	Leopard tortoise -*Stigmochelys pardalis*- (6)	11
11	9708	Bontebok -*Damaliscus pygargus*- (1) Common eland -*Taurotragus oryx*- (9) Lechwee -*Kobus leche*- (3) Ostrich -*Struthio camelus*- (6)	Scimitar-horned oryx -*Oryx dammah*- (15) Sitatunga -*Tragelaphus spekii*- (1) Wildebeest -*Connochaetes taurinus*- (1) Zebra -*Equus burchellii*- (3)	249
12	109	Porcupine -*Hystrix africaeaustralis*- (2)		55
13	882	Sea lion -*Zalophus californianus*- (2)		441
14	948	White rhinoceros -*Ceratotherium simum*- (1)		948
15	1716	Brown bear -*Ursus arctos*- (8)		215
16	738	Barbary sheep -*Ammotragus lervia*- (18)		41

Table S1 (continued). List of animal species and surface of their open-air enclosures in "Terra Natura" zoological park.

Enclosure		Animal species (census[a])		Density (m^2/animal)
Number	**Surface (m^2)**			
17	1068	Fallow deer -*Dama dama*- (18)		59
18	3349	Red deer -*Cervus elaphus*- (12)	Wild boar -*Sus scrofa*- (6)	186
19	242	De Brazzas monkey -*Cercopithecus neglectus*- (4)	Mantled guereza -*Colobus guereza*- (2)	40
20	232	Leopard -*Panthera pardus*- (1)		232
21	131	Meerkat -*Suricata suricatta*- (9)		15
22	2996	Black-crowned night heron -*Nycticorax nycticorax*- (125) Common crane -*Grus grus*- (2) Demoiselle crane -*Anthropoides virgo*- (2) Eurasian oystercatcher -*Haematopus ostralegus*- (3) Grey crowned crane -*Balearica regulorum*- (3) Greylag goose -*Anser anser*- (3) Griffon vulture -*Gyps fulvus*- (5)	Helmeted guineafowl -*Numida meleagris*- (6) Marabou stork -*Leptoptilos crumeniferus*- (6) Southern ground-hornbill -*Bucorvus leadbeateri*- (2) Turtledove -*Streptopelia* spp.- (>100) White stork -*Ciconia ciconia*- (8) Yellow-billed stork -*Mycteria ibis*- (4)	11
23	119	Ring tailed lemur -*Lemur catta*- (7)		17
24	340	Eurasian otter -*Lutra lutra*- (4)		85

[a] Calculated as the average of no. animals in 2016, 2017 and 2018.

Table S2. Environmental characteristics of the sampling sites and CDC light trap positioning.

Variable	Trap (No. sand flies collected)							
	A (515)	**B (912)**	**C (903)**	**D (1062)**	**E (914)**	**F (2045)**	**G (348)**	**H (610)**
Site altitude[a]	79.7	89.3	94.9	91.2	90.4	97.9	83.8	85.9
Vegetation	High	Low	Moderate	Low	Moderate	Low	Low	Moderate
Next to infirmary	No	No	No	Yes	No	Yes	No	No
Under cover	No	No	Corrugated iron	Corrugated iron	Wooden walkway	Corrugated iron	No	No
Trap leaning surface	Heather fence	Stone wall	Heather fence	Concrete wall	Wooden post	Heather fence	Heather fence	Wire fence
Ground type	Soil	Soil	Soil	Concrete	Soil	Concrete	Soil	Soil

[a] Meters above sea level (m.a.s.l.).

CHAPTER 4

A spatial ecology study in a high-diversity host community to understand blood-feeding behavior in *Phlebotomus* sand fly vectors of *Leishmania*

Abstract

Molecular studies indicate that phlebotomine sand flies (Diptera: Psychodidae) blood-feed on many vertebrate species, of which only a few are proven parasite reservoirs. Investigating sand fly vector feeding preferences is therefore important and requires taking into account the availability and accessibility of host species. In terms of the latter, it is necessary to consider the metabolic cost to the insect of reaching the host and moving on to a suitable breeding site. The present study used statistical modelling to compare the feeding patterns of *Phlebotomus perniciosus* (n=150), *Phlebotomus papatasi* (n=35) and *Phlebotomus ariasi* (n=7) on each of an average of 30 host species in a wildlife park in Murcia, Spain. Sand fly feeding movement costs were estimated as a function of the distance and altitude gradients saved by the insect, assuming that they displayed 'site fidelity'. Most (87%) engorged females were caught <100 m from the host on which they had fed. Although the percentage of bloodmeals was highest on fallow deer (*Dama dama*) (30%) and red deer (*Cervus elaphus*) (26%), the predicted feeding probability after considering movement cost was highest for red deer and common eland (*Taurotragus oryx*), and positively associated with host census. These results suggest that, under similar circumstances, sand flies prefer to feed on some host species more than on others.

Introduction

As vectors of the parasitic protozoan *Leishmania* spp. (Trypanosomatida: Trypanosomatidae) and other pathogens in tropical and subtropical latitudes, phlebotomine sand flies are a major veterinary and public health concern (World Health Organization, 2019). They breed in ubiquitous terrestrial habitats; adults feed on plant sugars and females require an additional bloodmeal for oogenesis, which they can take from a wide variety of mammal, bird and reptile species (Munstermann, 2004). Elucidating potential host feeding preferences is necessary for a better understanding of complex vector-pathogen-host relationships and the epidemiology of vector-borne infections (Keesing *et al.*, 2006; Kent, 2009). This is important in the context of sand flies and leishmaniasis because parasite susceptibility and transmissibility differ significantly among host species. Dogs are highly susceptible and represent the primary domestic reservoir of *Leishmania infantum*, the agent of zoonotic visceral leishmaniasis in the

Mediterranean sub-region, for which wild canids, lagomorphs and some rodents are also competent hosts (Tomassone *et al.*, 2018). The epidemiological roles of other vertebrates in which the parasite or its DNA have been analyzed by molecular methods remain uncertain and many are not considered competent reservoirs of *L. infantum* (Tomassone *et al.*, 2018).

Sand flies feed on many vertebrate species and are considered opportunistic blood feeders (Bongiorno *et al.*, 2003; Maia *et al.*, 2013; Cotteaux-Lautard *et al.*, 2016). However, not all the vertebrates are bitten with the same frequency and studies in mosquitoes have shown that preference for a particular host species can be variable between and within populations of the same insect species (Kent, 2009; Takken and Verhulst, 2013). Vector feeding choice is commonly related to host density; proxy measures that take into account the proportion of bloodmeals taken from a particular host species relative to its abundance include the 'forage ratio' (Hess *et al.*, 1968) and the 'feeding index' (Kay *et al*., 1979; Richards *et al.*, 2006). However, host and vector abundances are not necessarily linearly associated, and a small number of animals may be enough to sustain a comparatively large sand fly population (Risueño *et al.*, 2017).

An aspect traditionally neglected in investigations of feeding preferences in a multi-host scenario is the movement cost experienced by the insect in reaching an accessible host and subsequently finding a suitable breeding site. Movement cost may be seen as a function of the distance and altitude gradient saved by the insect along the trajectory it follows to reach the host and progress to a breeding site. Sand flies are not strong flyers, and wind speed and direction may also need to be considered in this calculation (Doha *et al.*, 1991). Methods to quantify spatial movement costs are available in computer-based geographical information systems (GIS) as an integral part of the econometrics of dynamic processes (Murekatete and Shirabe, 2018). They are commonly applied in wildlife studies (Desrochers *et al.*, 2011; Zeller *et al.*, 2012) and have been used to model the predictive dispersal of sand fly vectors in central Europe in the face of climate change (Fischer *et al.*, 2011a, 2011b).

The obvious limitation of insect movement cost calculations concerns ascertaining the feeding trajectory. Mark and recapture studies indicate that average dispersal areas for sand flies are small and breeding sites for most unfed females are close to the

collection point (Munstermann, 2004). The size of a dispersal area clearly depends on the environmental setting and the proximity of a blood source and may be different before and after the sand fly feeds. In the Cévennes mountains in France, engorged *Phlebotomus ariasi* stayed within 250 m of the release point, whereas unfed females travelled for 1000 m or more (Killick-Kendrick *et al.*, 1984). Similar studies showed mean flying distances for unfed *Lutzomyia* spp. (Diptera: Psychodidae) sand flies in a peridomestic area in South America of less than 100 m (Alexander, 1987; Morrison *et al.*, 1993; Casanova, Costa and Natal, 2005; Galvis-Ovallos *et al.*, 2018) and those for *Phlebotomus papatasi* in Egypt (Doha *et al.*, 1991) and a rural kibbutz community in Israel (Orshan *et al.*, 2016) ranged from 250 m to 950 m. Another factor that can be taken into account to estimate movement feeding costs is that, under natural circumstances, data suggest that sand flies and other insect vectors recognize a 'familiar area map', defined as an area in which they concentrate their activity and which they are able to memorize, and display breeding site fidelity by returning after feeding to lay eggs in the same area in which they developed as immature stages (Charlwood *et al.*, 1988; Renshaw *et al.*, 1994; Kelly and Dye, 1997; Campbell-Lendrum *et al.*, 1999; McCall *et al.*, 2001).

Periurban wildlife parks can be considered as experimental environments that provide adequate breeding sites and a wide diversity of potential blood sources for sand fly vectors in comparatively small spaces (Muñoz *et al.*, 2019). In these areas, it is possible to quantify host-feeding preferences in sand flies and to study the potential effects on pathogen transmission rates. Different authors have used zoological gardens to track post-bloodmeal flight distances of different insect vectors because these environments allow the determination of the distances between trap sites and host sources (Greenberg *et al.*, 2012; Tuten *et al.*, 2012). However, the costs borne by the insect in reaching an accessible host and subsequently finding a suitable breeding site were not considered. The present study used data on *Phlebotomus* vector abundances and feeding host diversity from a previous study (Muñoz *et al.*, 2019) to investigate sand fly vector feeding preferences, assuming that the majority of sand flies displayed site fidelity. The study estimated the probability that an engorged female collected in a light trap would feed from a particular host species when other species were available nearby, taking into account travelling costs, host census and environmental trapping site differences. This

study was conducted in three sand fly species, including *Phlebotomus perniciosus* and *P. ariasi*, which are the main vectors of *L. infantum* in Western Europe and North Africa, and *P. papatasi*, which plays a central role in the transmission of *Leishmania major* in North Africa and the Middle East (Ashford and Bettini, 1987).

Materials and methods

The wildlife park and its management

This study was conducted in a 16-ha wildlife park situated on the outskirts of the city of Murcia in southeast Spain (Figure 1) (Muñoz *et al.*, 2019), where *L. infantum* is endemic (Pérez-Cutillas *et al.*, 2015; Goyena *et al.*, 2016; Risueño *et al.*, 2018). The park was home to 200 wild mammals of 30 species, 15 reptiles of three species and 300 birds of 26 species, kept in large, earth-floored, open enclosures (Figure 1). To accommodate species-specific environmental and behavioral needs, most enclosures kept one or two host species only, except that compatible African savannah herbivores and ostriches, raptors and other birds were kept in three communal enclosures, respectively. The park also included two small infirmaries in which animals requiring veterinary attention stayed during treatment periods. None of the animals received sand fly-specific preventive treatments, although the insecticides fipronil and cypermethrin, used as topical pipettes and pour-on formulations, respectively, were applied to wolves and hyenas once per month from May to September, and cypermethrin was similarly administered to white rhinoceros. Moreover, the injectable endectocide ivermectin was administered at 3-6-month intervals to primates, white rhinoceros, roe deer, sea lions, sitatunga, wildebeests, zebras, brown bears and lions. Further details of the study area and animal management are available in Muñoz *et al.* (2019).

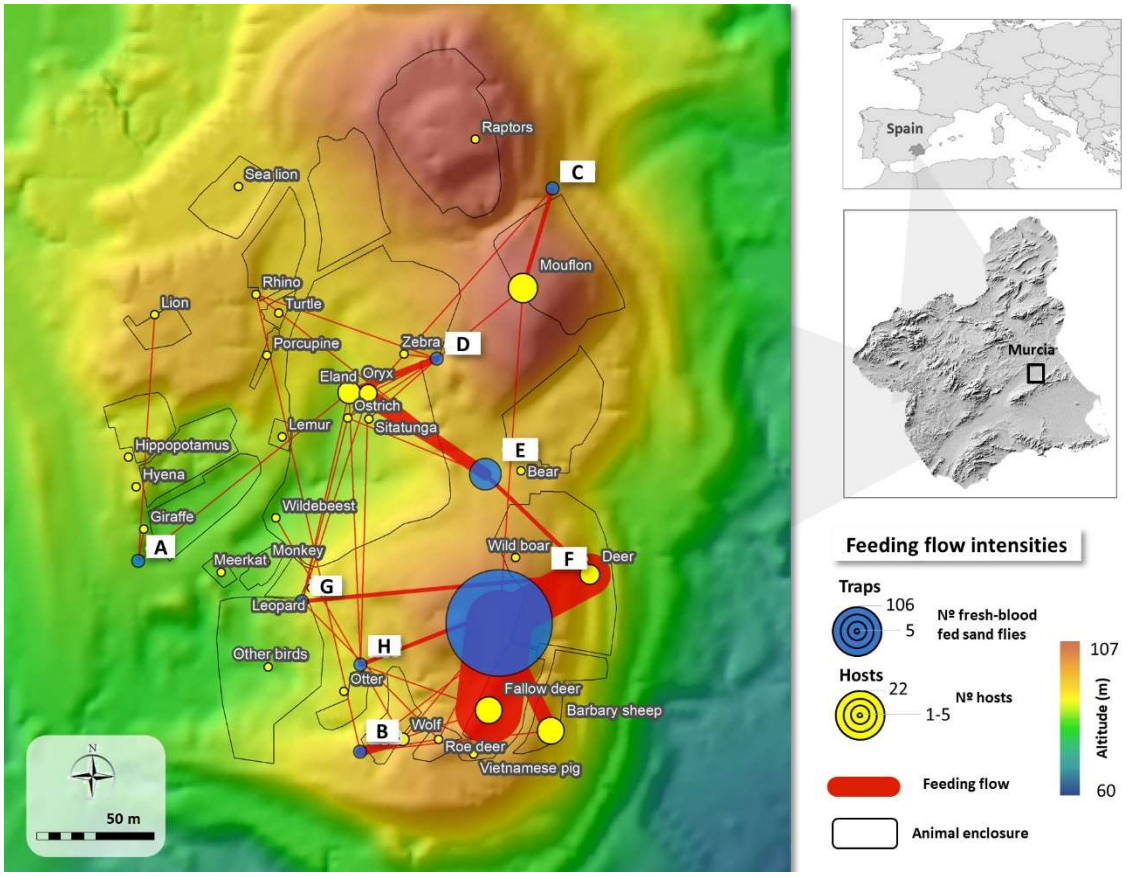

Figure 1. Trap and host locations and sand fly feeding trajectories (represented as straight red lines at widths proportional to the number of specimens following the trajectory) in Terra Natura Wildlife Park, Murcia, southeast Spain.

Entomological and bloodmeal studies

As reported by Muñoz *et al*. (2019), eight georeferenced sampling points scattered around the park were sampled for sand flies over 14 days (24 h/day) in the summers of 2016-2018, using miniature Centers for Disease Control (CDC) battery-operated light-aspiration attraction traps (J.W. Hock Co., Gainesville, FL, U.S.A.). Light traps have a small area of influence for sand flies (<2 m for *P. ariasi*) and are not considered to affect dispersal studies (Killick-Kendrick *et al.*, 1985). The median distance between study traps was 131 m (range: 41-283 m). Six of these traps (A, B, C, E, G and H) were placed on the perimeters of enclosures in which animals were kept, and the other two traps (D and F) were situated next to the infirmaries (Figure 1). Overall, 7,309 specimens were collected, of which 192 showed evidence of a recent bloodmeal in the abdomen [categories 1, 2 and 3 out of 5 in Muñoz *et al*. (2019)], including 150 *P. perniciosus*, 35 *P. papatasi* and seven *P. ariasi*. These engorged females were analyzed to identify the sources of their

bloodmeals by polymerase chain reaction amplification and sequencing of a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene using the method described by Alcaide *et al.* (2009). Vectors had fed on at least 15 different vertebrate host species, of which fallow deer (n=57), red deer (n=49), scimitar-horned oryx (n=20), common eland (n=16), Barbary sheep (n=14) and wolf (n=11) were the most common (Muñoz *et al.*, 2019).

Spatial analysis of trap and host locations and sand fly trajectories

A digital terrain model of the park with 1-m pixel resolution was obtained from a LIDAR cloud point available from the "Plan Nacional de Ortofotografía Aérea" (PNOA) (http://centrodedescargas.cnig.es/), by spatial interpolation in ArcGIS (ESRI, 2019). The spatial geoprocessing tool Model Builder in this GIS was then used to calculate distances and the cumulative movement costs of potential sand fly feeding trajectories for 28 mammal and reptile species and the two bird enclosures, assuming the insects recognized a 'familiar area map' and exhibited 'site fidelity'. Sand flies were considered to follow 'least-cost' trajectories [i.e. those that cost the least to complete (Adriaensen *et al.*, 2003)] from the trap to the host and back to the trap, which was not necessarily the straight line 'Euclidean' distance either way. Host location was considered as the site within the enclosure at which the host most frequently spent the night (night grounds) because this is the time during which adult sand flies are most active (Alten *et al.*, 2016).

The steps used to calculate optimal least-cost routes are presented in Figure 2. Movement cost was calculated for each 1-m^2 pixel as the weighted sum of distance and topographic costs, assigning 60% and 40% weightings to each, respectively, following an analytical hierarchy process approach (Sarı and Sen, 2017). This weighting combination proved to be a balanced option because for smaller topographic weights, low and medium slopes had little effect on movement costs, and greater topographic weights resulted in excessively long trajectories to save high slopes. Wind effect was excluded from this calculation after confirming that the average recorded wind speed during sampling nights was below 1 m/s, which does not interfere with sand fly flying (Doha *et al.*, 1991). Moreover, park surface features were not considered in cost calculations because the terrain was homogeneous, was earth-based with only small amounts of low vegetation

and included no concrete except in small areas such as the infirmary and indoor facilities available for some animals. The presence of vegetation around the trap was not associated with sand fly abundance (Muñoz *et al.*, 2018, 2019). Distance costs were arbitrarily set at 1 cost unit (cu) per pixel and similar topographic costs were considered proportional to the slope, using terrain orientation to determine the slope's sign (positive or negative for uphill and downhill trajectories, respectively). Subsequently, cumulative movement costs were estimated for all possible trajectories between traps and hosts and back to the trap, and those with the lowest cost were selected as the optimal. The number of potential trajectories per sand fly ranged between 30 and 33 depending on the presence or not of animals in the infirmary. When an individual from a certain host species was in the infirmary, sand flies with blood from that host were considered to have fed on the individual that was closer (in the infirmary or the host's enclosure). As an example, Figure 3 depicts minimum cost trajectories for sand flies moving from trap F to host enclosures.

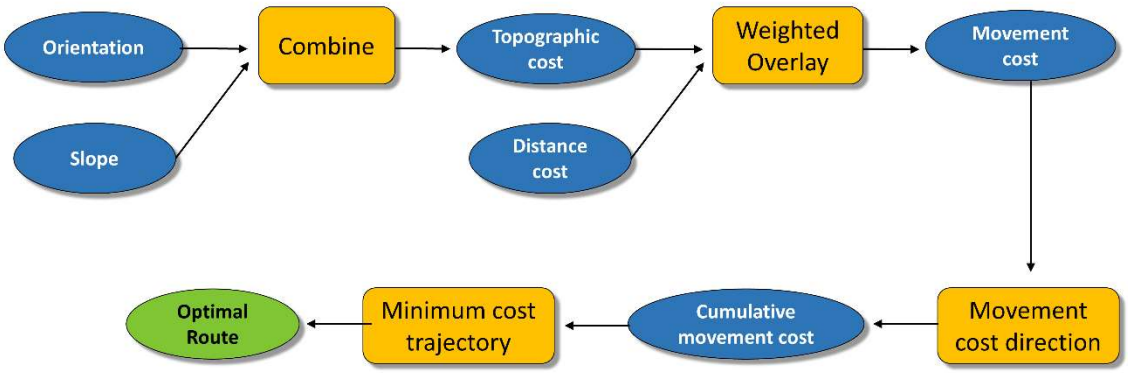

Figure 2. Flow diagram showing steps for calculating sand fly optimal feeding routes. Blue spheres represent spatial variables and yellow rectangles represent the applied GIS calculations.

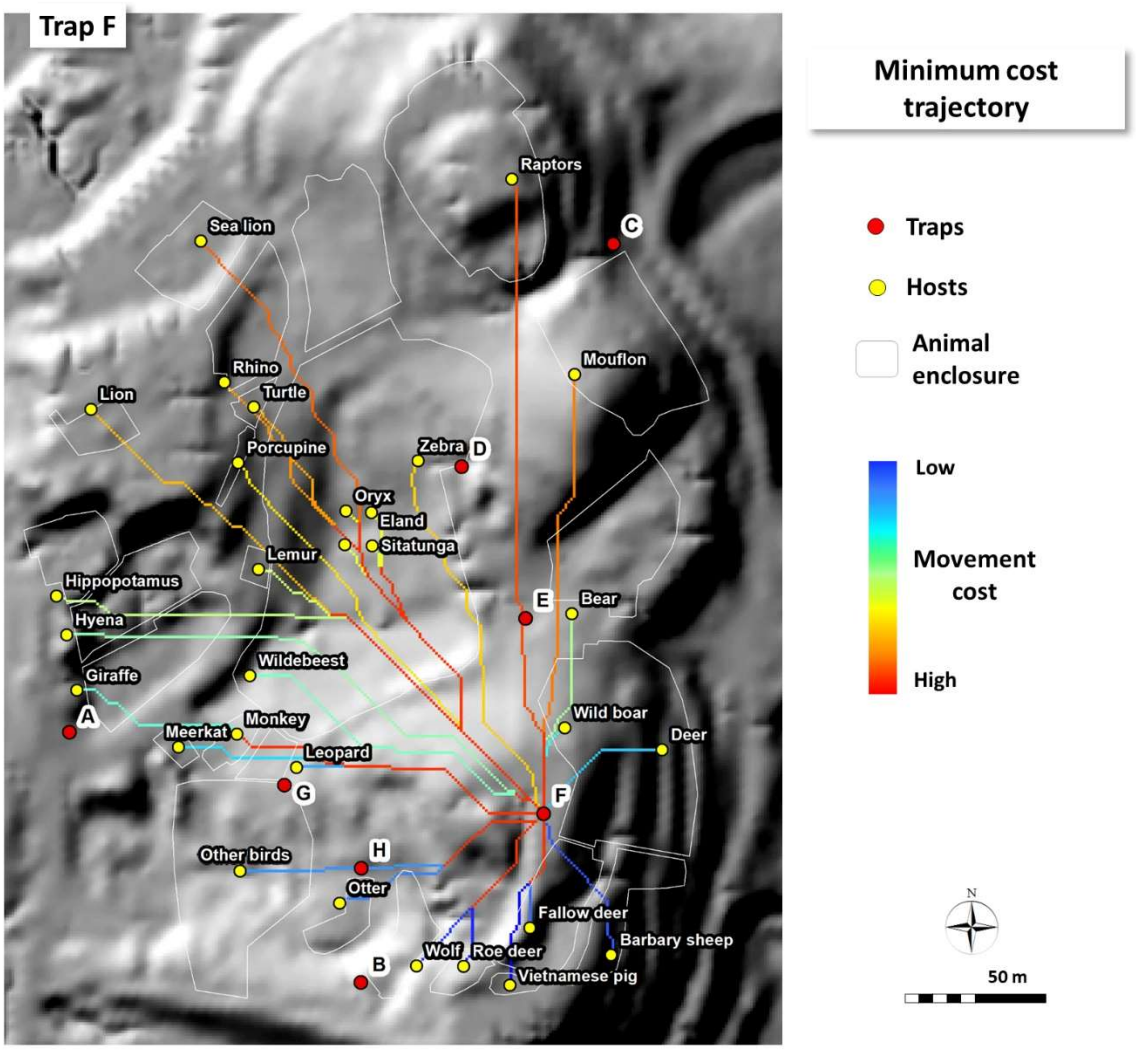

Figure 3. Optimal cumulative 'minimum-cost' routes for sand flies moving from trap F to potential feeding hosts.

Statistical analysis and modelling

Median distance and movement cost distributions were presented in histograms and differences between hosts with respect to these statistics were compared using the non-parametric Kruskal-Wallis test. Chi-squared analysis was used to compare the proportions of female sand flies feeding on insecticide-treated and untreated hosts. Collinearity between categorized distances and costs, host species and use of insecticides was assessed using contingency tables.

Random effects logistic regression models were developed to investigate the host-feeding probability of *P. perniciosus*, *P. papatasi* and *P. ariasi* females considered together, or of *P. perniciosus* or *P. papatasi* alone, using one of 30-33 available minimum

cost trajectories (dependent variable with two possible values: yes and no). Explanatory categorical variables included in the model were host species (seven levels: red deer, common eland, fallow deer, wolf, scimitar-horned oryx and other species) (model 1) or membership of a taxonomic family or birds (seven levels: Cervidae, Bovidae, Canidae, Felidae, Equidae, other mammals and birds) (model 2), movement cost (seven levels), host species census (three levels), year (three levels) and time of year (two levels: June-July and September). Sampling site (eight levels) was included in the model as a random variable to account for environmental differences between sites. Insecticide usage was not included in the model as a result of strong collinearity with host species because all species treated with ivermectin (the only treatment associated with reduced feeding probability) were in the 'other species' category of the host species variable. A forward model building strategy was used, fitting feeding host species first, followed by host census, year, time of year and finally movement cost. Models were estimated using the maximum likelihood and the likelihood ratio chi-squared test was used to estimate p-values; significance was assumed at $p<0.05$ in a two-tailed test. Only variables significantly associated with the outcome were retained in the model. Odds ratios were calculated by exponentiation of regression coefficients. These analyses were performed using lme4 library in R-Project Version 3.5.0. (https://www.rproject.org/) software.

Results

Distance and movement cost distributions for engorged females

The frequencies of engorged females in sampling sites (traps A to H) and feeding directions are shown in Figure 1. Table 1 provides the frequencies of host bloodmeals and median distances and feeding movement costs (cu) according to host location. The greatest number of engorged females was collected in trap F, in which most specimens had fed on fallow deer and red deer, and fewer or none fed on other hosts in the proximity, such as wild boars, Barbary sheep, wolves, otters, mouflons, Vietnamese pigs, bears and roe deer (Figure 1). Generally, blood from the host species closest to the trap was present in 25% (47/192) of engorged females; this percentage rose to 67% (128/192) for the four closest host species. The median distance between feeding hosts and light traps was 49 m and ranged from 6 m for fallow deer in the infirmary to 139 m for white

rhinoceros (Table 1); 87% and 99% of engorged females were caught within 100 m and 150 m, respectively, of the host on which they had fed.

Table 1. Frequency of sand fly bloodmeals according to host location, median Euclidean distance (m) between hosts and light traps, and estimated median minimum sand fly feeding movement cost expressed as cost units (cu), in a study conducted in Terra Natura Wildlife Park, Murcia, Spain.

Host species (census)	Animal location	Bloodmeals No. (%)	Euclidean distance (m)	Movement cost (cu)
Fallow deer, *Dama dama* (18)	INF	30 (16)	6	51
	NG	27 (14)	42	248
Red deer, *Cervus elaphus* (12)	NG	49 (26)	49	328
Scimitar-horned oryx, *Oryx dammah* (15)	INF	13 (7)	49	251
	NG	7 (4)	129	666
Common eland, *Taurotragus oryx* (9)	NG	13 (7)	68	395
	INF	3 (2)	40	251
Barbary sheep, *Ammotragus lervia* (18)	NG	14 (7)	57	363
Iberian wolf, *Canis lupus* (5)	NG	11 (6)	21	79
Mouflon, *Ovis aries* (22)	NG	5 (3)	49	300
	INF	1 (1)	15	18
Roe deer, *Capreolus capreolus* (1)	NG	5 (3)	62	376
Ostrich, *Struthio camelus* (6)	NG	4 (2)	81	457
Giraffe, *Giraffa camelopardalis* (3)	NG	3 (2)	15	80
White rhinoceros, *Ceratotherium simum* (1)	NG	3 (2)	139	834
Zebra, *Equus burchellii* (3)	NG	1 (1)	16	91
African lion, *Panthera leo* (6)	NG	1 (1)	118	654
Wildebeest, *Connochaetes taurinus* (1)	NG	1 (1)	81	471
Sitatunga, *Tragelaphus spekii* (1)	NG	1 (1)	43	234
All		**192 (100)**	**49**	**328**

Figure 4 presents two overlaid histograms depicting the observed feeding movement cost (solid red line) and similar average costs that would have been incurred if vectors had fed on other host species in the park (dashed blue line). The observed median cost (328 cu; range: 51-1408 cu) was significantly lower than the average median cost (834 cu; 45-2316 cu) associated with feeding on other host species ($p<0.05$). The low cost bars in Figure 4 correspond to sand flies feeding on animals in the infirmary, which accounted for 47 of a total of 192 (25%) bloodmeals, and included 30 of 57 from fallow deer, 13 of 20 from scimitar-horned oryx, three of 16 from common eland and one of six from mouflon (Table 1).

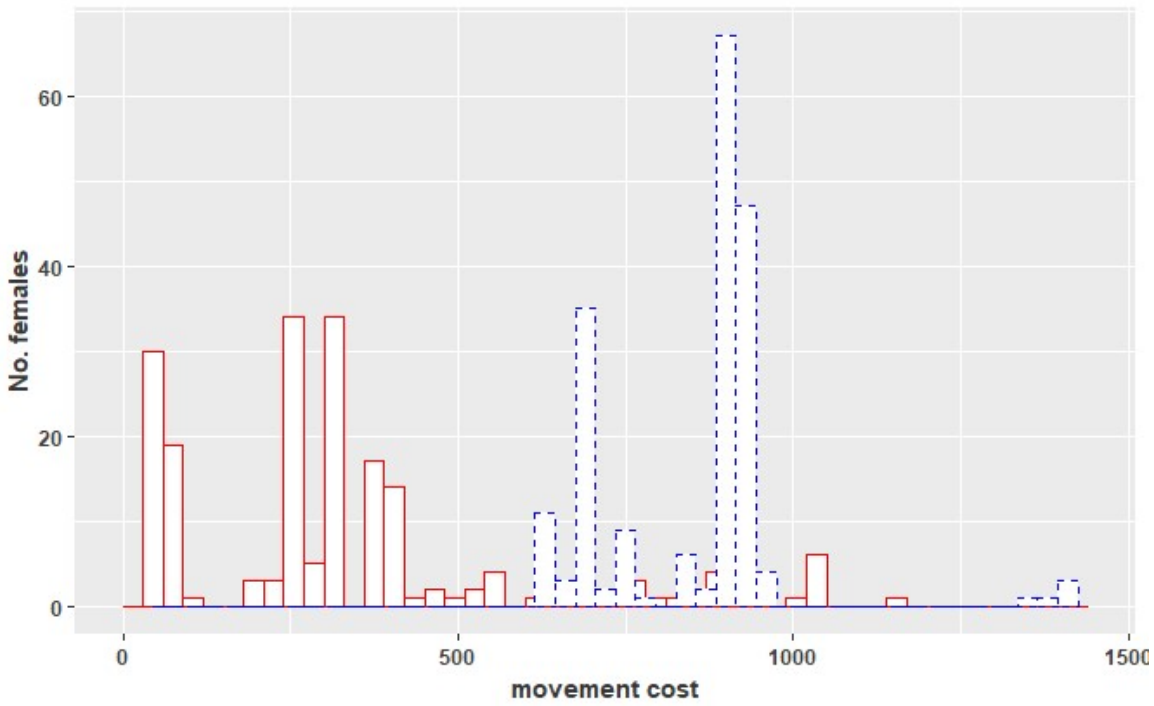

Figure 4. Distribution of minimum-cost sand fly trajectories to feeding hosts (solid red lines) and to other accessible hosts (dashed blue lines). Each blood-fed specimen was considered to have the choice of 30-33 trajectories to different hosts (solid red lines represent those eventually followed and dashed blue lines those that were not taken).

Relationship between feeding probability and insecticide use

The frequency of the receipt of insecticides in host species was 40% (12/30), represented by nine, two and one hosts treated with injectable ivermectin, topical fipronil and both topical cypermethrin and injectable ivermectin, respectively. Animals receiving ivermectin were significantly less likely to represent a blood source for sand flies compared with untreated animals ($p<0.05$). Thus, feeding probability was not associated with fipronil or cypermethrin treatments.

Multivariable analysis of *Phlebotomus* spp. host blood-feeding preferences

The logistic regression models indicated that the feeding probability of *P. perniciosus*, *P. papatasi* and *P. ariasi*, or of the first two species alone, differed between host species and was greatest for red deer and common eland (Table 2) and Cervidae (Table 3). It was also independently associated with decreasing movement costs and increasing host census (marginally in the host species model), varied between sampling sites and was not associated with year or time of year. The preference for red deer and common eland

rather than fallow deer after adjusting for feeding movement cost suggests that the high proportion of fallow deer feeds (Table 1) was to a great extent attributable to easy access to individuals in the infirmary. Incidentally, the paths extending from traps to fallow deer in the infirmary cannot be appreciated in Figure 1 because they are overlaid by the longer paths from traps to fallow deer night grounds.

Table 2. Estimates from a logistic regression model investigating the probability of *P. perniciosus*, *P. papatasi* and *P. ariasi* female sand flies taking a bloodmeal from different host species, adjusted for census and sand fly movement cost (cost unit) to reach the host.

Variable	Level	Estimate	*SE*[a]	*OR*[b]	95% CI[c]	P value
(Intercept)		2.59	0.65			<0.0001
Fixed variables						
Host species	Red deer	0.00		1.00		
	Common eland	-0.16	0.51	0.85	0.31, 2.33	0.7563
	Fallow deer	-1.05	0.34	0.35	0.18, 0.68	0.0020
	Scimitar-horned oryx	-1.06	0.45	0.35	0.14, 0.84	0.0196
	Barbary sheep	-1.56	0.34	0.21	0.11, 0.41	<0.0001
	Iberian wolf	-1.87	0.57	0.15	0.05, 0.47	0.0010
	Other animals	-3.94	0.44	0.02	0.01, 0.05	<0.0001
Host species census	1 - 3	0.00		1.00		
	4 - 10	-0.28	0.344	0.75	0.38, 1.48	0.4106
	11 - 23	0.76	0.433	2.15	0.92, 5.02	0.0776
Movement cost	45 - 51	0.00		1.00		
	79 - 111	-0.68	0.48	0.51	0.20, 1.30	0.1561
	166 - 285	-2.50	0.38	0.08	0.04, 0.17	<0.0001
	300 - 495	-3.41	0.43	0.03	0.01, 0.08	<0.0001
	524 - 691	-4.03	0.56	0.02	0.01, 0.05	<0.0001
	707 - 897	-5.62	0.55	0.00	0.00, 0.01	<0.0001
	904 - 1197	-5.39	0.58	0.00	0.00, 0.01	<0.0001
	1213 - 2316	-7.36	1.24	0.00	0.00, 0.01	<0.0001
Random variable	*SD*[d]					
Sampling site	0.7522					

[a] SE: standard error.
[b] OR: odds ratio.
[c] 95% CI: 95% confidence interval.
[d] SD: standard deviation.

Table 3. Estimates from a logistic regression model investigating the probability of *P. perniciosus*, *P. papatasi* and *P. ariasi* female sand flies taking a bloodmeal from different host families, adjusted for census and sand fly movement cost (cost unit) to reach the host.

Variable	Level	Estimate	*SE*[a]	*OR*[b]	95% CI[c]	P value
(Intercept)		0.92	0.57			0.1345
Fixed variables						
Host family	Cervidae	0.00		1.00		
	Bovidae	-0.67	0.22	0.51	0.33, 0.79	0.0026
	Canidae	-1.79	0.50	0.17	0.06, 0.44	0.0003
	Felidae	-3.27	1.11	0.04	0.00, 0.34	0.0033
	Equidae	-3.43	1.10	0.03	0.00, 0.28	0.0018
	Other mammals	-4.96	0.52	0.01	0.00, 0.02	<0.0001
	Birds	-1.27	0.58	0.28	0.09, 0.88	0.0287
Movement cost	45 - 51	0.00		1.00		
	79 - 111	0.16	0.58	1.18	0.38, 3.65	0.7752
	166 - 285	-2.88	0.44	0.06	0.02, 0.13	<0.0001
	300 - 495	-3.41	0.46	0.03	0.01, 0.08	<0.0001
	524 - 691	-4.44	0.62	0.01	0.00, 0.04	<0.0001
	707 - 897	-5.41	0.56	0.00	0.00, 0.01	<0.0001
	904 - 1197	-5.43	0.60	0.00	0.00, 0.01	<0.0001
	1213 - 2316	-7.27	1.18	0.00	0.00, 0.01	<0.0001
Host species census	1 - 3	0.00		1.00		
	4 - 10	1.04	0.34	2.84	1.46, 5.53	0.0021
	11 - 23	1.83	0.32	6.25	3.32, 11.8	<0.0001
Random variable	*SD*[d]					
Sampling site	0.6822					

[a] SE: standard error.
[b] OR: odds ratio.
[c] 95% CI: 95% confidence interval.
[d] SD: standard deviation.

Discussion

This study demonstrates that, given the choice, *P. perniciosus*, *P. papatasi* and *P. ariasi* prefer to bite some host species more than others. In particular, red deer and common eland were more likely targets than other herbivores, carnivores, reptiles and bird species. In addition, selected feeding trajectories were significantly shorter and required lower movement costs than might be expected by chance and sand flies tended to feed on higher-census host species. The analysis also revealed additional variation in abundances of engorged sand flies between traps, probably reflecting ecological differences in the sites' suitability for sand fly breeding and host access (Muñoz *et al*., 2019). These findings rely on the assumption that the insects bred in the area in which

they had developed as immature stages, as shown in other sand fly and mosquito species (Charlwood *et al*., 1988; Renshaw *et al*., 1994; Kelly and Dye, 1997; Campbell-Lendrum *et al*., 1999; McCall *et al*., 2001). These seem to be reasonable assumptions and are compatible with the observed heterogeneity in the spatial distribution of sand flies, suggesting that the park's population was made up of groups of individuals or colonies with relatively small dispersal areas. The present results are not necessarily extendable to other insect vectors. For example, in a study of the foraging range of *Culicoides* biting midges (Diptera: Ceratopogonidae), *Culicoides oxystoma* and *Culicoides imicola* females primarily fed on nearby horse, whereas *Culicoides kingi* travelled longer distances to feed on cattle (Bakhoum *et al.*, 2016).

Sand fly species are traditionally classified as preferentially anthropophilic or zoophilic (Lewis, 1971), and although *P. perniciosus*, *P. ariasi* and *P. papatasi* are attracted to humans and other animals, no studies have compared their preferences for large herbivores, including red deer and common eland. As for other insects, host finding and selection by sand flies rely on suitable semiochemical communication between the host and the insect, and among insects themselves (Dye *et al.*, 1991; Ward *et al.*, 1993; Quinnell and Dye, 1994; Kelly and Dye, 1997). Laboratory experiments showed that *Lutzomyia longipalpis* is attracted to volatile chemicals or kairomones released by hamsters (Oshaghi *et al.*, 1994). There is also evidence that *Lu. longipalpis* males arrive first on the host and release pheromones that attract females, a strategy that may maximize both feeding and mating success (Kelly and Dye, 1997). The reasons why sand flies preferentially target red deer and common eland are not clear. These belong to separate taxonomic families, the Cervidae and Bovidae, respectively, which suggests that sand flies do not have a strong preference for either family, although modelling results indicated that, overall, sand flies were more likely to feed on cervids than on bovines. As well as possible adaptive advantages, preferential feeding on certain host species may be influenced by numerous factors such as the host's behavior in response to sand fly approximation and bites, and anatomical differences, such as in size, skin thickness and presence of long hair. These features may be particularly important in the park, where host diversity and feeding choice were exceptionally large. Further, a large proportion of the blood-fed flies were collected in traps close to the infirmary, where the mobility of animals was reduced, and

it is possible that being sick made them easier targets for sand flies. This result highlights the need to administer preventive insect repellents to animals in such circumstances. Finally, feeding preference studies can be affected by area and time of year as a result of variations in the availability of the preferred host between places and seasons (Muñoz *et al.*, 2012). At this stage it is important to perform artificial blood-feeding experiments and to compare the nutritional value of the host's blood with associated sand fly life trait variables in order to improve current understanding of sand fly feeding preferences.

Differential host utilization by vectors has important implications in the transmission and control of *Leishmania* spp. There are no reports of *Leishmania* infecting cervids; however, by contrast, the parasite or its DNA have been recently detected in cattle, sheep and goats (Alam *et al.*, 2018; Han *et al.*, 2018; Paixão-Marques *et al.*, 2019), although their ability to infect vectors has not been investigated. Moreover, sand flies may readily feed on cattle (Svobodová *et al.*, 2009). In any case, as blood sources alone, ruminants would contribute to the maintenance of the large vector population in the wildlife park. In this respect, the selective use of ivermectin in some animals, including wildebeest, sitatunga, roe deer, lion, zebra and white rhinoceros (Muñoz *et al*., 2019), was associated with a lower feeding probability in the bivariate analysis. When modelling, ivermectin-treated host species were part of the 'other species category' in the host species variable and hence it was not possible to include in the model both this and a variable considering ivermectin treatment. Consequently, it was not possible to ascertain the independent contribution of ivermectin treatment to feeding probability, although this was not the objective of the study. Injectable ivermectin is an effective ectoparasiticidal with no repellent effect and it was shown to produce significant mortality of *P. papatasi* sand flies feeding on treated hamsters up to 10 days post treatment (Hanafi *et al.*, 2011). Also, it reduced survival of *Anopheles* mosquitoes (Diptera: Culicidae) feeding on treated cattle for up to 3 weeks (Lyimo *et al.*, 2017). Time lapses between ivermectin treatments and trapping dates in the present study were greater than 21 days in 88% of cases. Preventive treatments with other insecticides including fipronil and cypermethrin were not associated with reduced feeding, and neither product used alone is indicated against sand flies and leishmaniasis control in dogs. However, in a recent field study, fipronil baits given to *Rhombomys opimus* rodents, reservoirs of *L. major* in central Asia, significantly reduced

gravid female abundance (Poché *et al.*, 2018), and cypermethrin was found to be effective in controlling *Lu. longipalpis* sand flies when sprayed on the walls of human residences in Brazil (Barata *et al.*, 2011). It should be stressed, however, that none of the commercially available insecticide treatments against sand flies have repellent activity that is 100% effective, and studies in dogs suggest that sand fly biting frequencies and feeding preferences may vary among individuals of the same species (Risueño *et al.*, 2018, 2019). To account for the wide range of potential factors conditioning insect feeding choice, some experts advocate the use of the term 'host utilization' rather than 'feeding preference' (Kent, 2009).

In summary, this study provides statistical evidence of differential host utilization by sand fly vectors of *Leishmania* spp. Multivariable analysis allowed for controlling for important confounding factors, particularly the insect's feeding movement cost, revealing that red deer and common eland had a greater probability of being used as blood sources compared with other species. These species may represent useful models for the further investigation of host-vector interactions, and their susceptibility to *L. infantum* infection should be evaluated in order to gain better understanding of their potential contributions to its transmission cycle in the area. From a vector control perspective, widespread systematic treatment of hosts with insecticides would probably reduce sand fly feeding activity in the park; however, it could drive sand fly populations to nearby human and dog residential areas. Adequate sand fly control would also require the targeting of vector breeding sites, but these are widespread and not well characterized (Alexander, 2000).

Conclusions

Phlebotomus perniciosus, *P. ariasi* and *P. papatasi* vector sand flies in a multi-host wildlife park have feeding preferences based on host species, host census and proximity, and as yet uncharacterized intrinsic factors.

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GENERAL DISCUSSION

Leishmaniasis caused by *Leishmania infantum* is an emerging zoonotic disease affecting millions of people and animals worldwide (Dujardin *et al.*, 2008; Alvar *et al.*, 2012). The complex interactions between parasites, hosts and vectors, along with the environment, make the infection risk difficult to predict accurately. It requires a holistic approach including the study of sand fly vector populations. In this doctoral thesis we investigated key aspects of the small-scale geographical distribution, host-feeding preferences and *L. infantum* infection rates in sand fly vectors in rural and periurban environments in southeast Spain.

The work confirms that sand fly vectors thrive in these environments, particularly the main *L. infantum* vector *Phlebotomus perniciosus*. However, their distribution is highly heterogeneous and closely related to the proximity of animals from which female sand flies take bloodmeals. Moreover, sand fly distribution was also variable depending on the species, the gender and the physiological status of the female (unfed, blood-fed and gravid). Females may feed on a wide range of host species (Chapter 3) but given the choice they display preferences for certain species (Chapter 4). This has important epidemiological implications because not all host species are equally susceptible to *L. infantum* infection (Quinnell and Courtenay, 2009). Indeed, in spite of a high density of sand flies in the periurban zoological park surveyed, none were infected with the parasite and the majority had fed on large herbivores including the fallow deer and red deer, which are not considered a reservoir of infection (Chapter 3). It was concluded that in this scenario the periurban zoological park did not pose a significant risk of *L. infantum* infection for humans and dogs living in the neighboring residential areas. A further critical aspect for entomological surveillance investigated in these studies was the variability in sand fly relative abundance, sex ratio and diversity depending on the sampling site and the position and type of trap used (Chapter 2). Whilst sticky traps provide an unbiased estimate of sand fly species density in a particular spot, a big sampling effort is required in order to account for the large spatial overdispersion. Moreover, it is important to place these traps close to the ground and in the proximity of a solid vertical surface for best estimations of maximum sand fly density (Chapter 1). In contrast, light and CO_2 attraction traps are ideal to estimate mostly phototropic sand flies (Alexander, 2000; Alten *et al.*,

2015), including *P. perniciosus*, and are less affected by trap distance to the floor or presence of a solid vertical surface (Chapter 1).

The results from the work developed for this doctoral thesis highlight the need for: (i) a universal methodological framework for estimating and reporting sand fly density, (ii) a wider scale surveillance program to improve our understanding of sand fly distribution in areas with a high risk of sand fly-borne infections, (iii) the study of environmental factors that govern sand fly density, (iv) the development of dynamic maps of sand flies that aid evaluating the risk of sand fly-borne infections and (v) the design and application of intervention strategies to reduce sand fly density in risk areas and evaluate its effect on the risk of sand fly-borne infections.

(i) A universal methodological framework for estimating and reporting sand fly density

A wide range of trapping methods are available for entomological studies and its selection depends on the objectives of the study (Alexander, 2000; Alten *et al.*, 2015). Sticky and light traps are the most commonly used devices for adult density estimations (ECDC and EFSA, 2018a). Chapter 1 of the doctoral thesis provides evidence of the need to design standardized and uniform sampling protocols for sand fly quantitative studies, as density was highly variable at the same site depending on trap type and placement. In this sense, investigations should be based on well-designed sampling strategies, and should incorporate the collection of key parameters affecting sand fly density and employ advanced multivariable statistical methods to establish causal relationships. Data reporting needs to be standardized and should include a detailed description of the type and number/area of traps, the time in operation and the geolocation of the study. However, comparisons between studies are actually challenging to perform due to the lack of standard sampling programs and reporting, and therefore, more efforts need to be done to get a better standardization (ECDC and EFSA, 2018a).

(ii) Ongoing surveillance to improve our understanding of sand fly distribution

Entomological surveillance, aimed to provide information about presence, density and behavior of medically important arthropods, is an essential tool for a better understanding of *Leishmania* transmission risk and for controlling the vector (González *et al.*, 2017; Vaselek *et al.*, 2017). In this context, we detected the most ubiquitous sand fly

species described in the Iberian Peninsula, namely *Sergentomyia minuta*, *P. perniciosus*, *Phlebotomus sergenti*, *Phlebotomus papatasi* and *Phlebotomus ariasi* (Gil Collado *et al.*, 1989), with variable density depending on the area. As shown in other studies conducted in Spain, there was predominance of *P. perniciosus* over *P. ariasi*, which corroborates the important epidemiological role of the former species as the main vector of *L. infantum* in Murcia Region (Durán-Martínez *et al.*, 2013; Alcover *et al.*, 2014; Ballart *et al.*, 2014; Bravo-Barriga *et al.*, 2016; González *et al.*, 2017; Risueño *et al.*, 2017). Further sand fly monitoring is needed in areas where no data are available, and their highly scattered distribution should always be considered when sampling for these insects. Chapter 2 shows the high effectiveness of applying intensive protocols using light traps to accurately estimate the spatial and temporal distribution of *P. perniciosus* at a small geographical scale. Instead, for sticky traps, large amounts would be required to cover as many potential microenvironments occupied by sand flies as possible (Rioux *et al.*, 2013). However, design of sampling campaigns, in terms of trapping methods used, trapping frequency and area to be sampled, can be expensive and monitoring activities largely depends on available resources (Alten *et al.*, 2015).

(iii) The study of environmental factors that govern sand fly density

Chapters 2 and 3 of this doctoral thesis, which sample animal premises in rural and periurban environments, respectively, highlight the importance of environmental factors on sand fly density, being responsible for the scattered small-scale distribution of these insects in the natural environment, as sand flies can be more likely to thrive in some specific microhabitats compared to other nearby areas. Among other factors, the presence of warm-blooded animals on which female *Phlebotomus* sand flies can feed on seems to be determinant for sand fly distribution. However, these hematophagous insects are also able to settle in areas where the presence of fauna is unknown, such as caves or burrows (Killick-Kendrick, 1999; Díaz Sáez et al., 2018; Pareyn et al., 2019). Other studies conducted in Spain have also delved into the influence of climatic and environmental characteristics on the small-scale distribution of sand flies (Gálvez *et al.*, 2010; Barón *et al.*, 2011; Durán-Martínez *et al.*, 2013; Alcover *et al.*, 2014; Ballart *et al.*, 2014; Bravo-Barriga *et al.*, 2016; Risueño *et al.*, 2017), stressing the need to analyze the sand fly species separately to account for biological and behavioral differences between them (Ballart *et*

al., 2014; Bravo-Barriga *et al.*, 2016). Another study in Murcia Region remarked that macroclimate was determinant on sand fly distribution at large geographical scale, and anthropic environmental factors, such as animal building characteristics and husbandry practices, may affect the local sand fly density (Risueño *et al.*, 2017), though this entomological survey mainly focused on the large-scale distribution of sand flies. Based on our results, this type of studies should go a step further and consider differences between males and females (Chapter 2), as well as female physiological status (Chapter 3). In the sampled rural environment, only *S. minuta* was more abundant when the distance to the animal groups increased (Chapter 2). Despite sporadically feeding on mammals (Maia *et al.*, 2013, 2015; González *et al.*, 2020), *Sergentomyia* species are preferentially herpetophilic and its spatial distribution may be linked to the presence of wild reptiles living in rocky habitats or abandoned buildings (Prudhomme *et al.*, 2015).

(iv) The development of dynamic maps of sand flies that aid evaluating the risk of sand fly-borne infections

Results from entomological surveys of this doctoral thesis and other investigations can provide valuable information to elaborate distribution vector maps, which can help predict uncharted areas with a high parasite transmission risk. Maps must incorporate the analysis of a wide range of environmental features affecting sand fly density. This has already been performed for *P. perniciosus* and *P. ariasi* vector populations in different Mediterranean regions, including southern Italy (Rossi *et al.*, 2007), Granada (Barón *et al.*, 2011) and Madrid (Gálvez *et al.*, 2011) in southern and central Spain, respectively, and France (Chamaillé *et al.*, 2010; Hartemink *et al.*, 2011). The importance of geospatial analysis in understanding the distribution of *L. infantum* vectors is at the core of the VectorNet initiative (https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/vector-net), which is a European network focused of gathering data on medically important vectors to generate updated maps and investigate environmental determinants of vector distributions (ECDC and EFSA, 2018a, 2018b).

(v) The design and application of intervention strategies to reduce sand fly density in risk areas and evaluate its effect on the risk of sand fly-borne infections

Chapters 3 and 4 illustrate an integrated approach to the study of the epidemiological cycle of *L. infantum*, in which various factors must come together for effective parasite transmission. This approach can provide a valuable information to decide control strategies (González *et al.*, 2017). In this sense, the study carried out in the periurban zoological park combined entomological surveillance, *L. infantum* infection rates and host-feeding preferences in female sand flies collected, and the results suggested that this park may not act as a hotspot for *L. infantum* infection to neighboring residential areas, in spite of having a large population of vectors. It is now important to monitor sand fly density in these neighboring areas, characterize high vector density spots and then, evaluate the possibility of targeted application of insecticides. The same approach should be used in animal shelters and other potential hotspots for sand fly maintenance and amplification (Otranto and Dantas-Torres, 2013; Gálvez *et al.*, 2018). Other non-insecticide-based interventions should be considered, including habitat changes aimed at removing suitable resting and breeding conditions. This strategy was effectively used to control *Leishmania major* infections in the Middle East and North Africa and consisted in the destruction of the burrows of rodents that are reservoirs of the parasite *L. major* (Gradoni, 2018).

In conclusion, the results reported in this doctoral thesis represent a significant advancement in our understanding of sand fly distribution at a small geographical scale and sand fly feeding behavior, and how this can condition the risk of *L. infantum* infection. Besides, it highlights the need to consider vector biological and ecological traits for designing adequate entomological surveys and sand fly control strategies.

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CONCLUSIONS

FIRST: The sand fly species *Phlebotomus perniciosus*, *Phlebotomus ariasi*, *Phlebotomus papatasi*, *Phlebotomus sergenti* and *Sergentomyia minuta* are found in rural and periurban areas of Murcia Region, and the former species is the most abundant. This supports the epidemiological role of *P. perniciosus* as the main *Leishmania infantum* vector in southeast Spain.

SECOND: The estimation of adult sand fly density is affected by factors related to sampling, such as the trap type and placement. The highest densities are achieved in traps closest to the ground and to continuous vertical surfaces, especially when using sticky interception traps, and to a lesser extent with light and CO_2 attraction traps.

THIRD: In addition to the sampling method, adult sand fly density is affected by other factors extrinsic to the insect, such as climate, particularly wind speed, and proximity to animal groups, although the latter varies according to the sand fly species and gender.

FOURTH: Given the wide range of factors affecting sand fly catches, it is essential to standardize the sampling procedure so that the results are representative and comparable across studies. Likewise, estimations of sand fly density should be supported by multivariable statistical models including all these parameters.

FIFTH: The existence of a high number of phlebotomine sand flies in habitats with a high diversity of host species, such as periurban zoological parks, does not necessarily imply efficient transmission of *Leishmania infantum*, if sand fly vectors feed mainly on animals that are not reservoirs of the parasite, such as red deer (*Cervus elaphus*) and fallow deer (*Dama dama*).

SIXTH: The spatial distribution of adult female sand flies in a high host species richness-environment is not uniform, depending on the particular requirements of each female physiological status, such as seeking for a blood source or searching for adequate breeding sites. This may be useful to improve the design of environmental vector control strategies.

SEVENTH: The assessment of host-feeding preferences of sand flies needs to consider the movement cost of the insect, as well as the number of accessible hosts to feed from. This approach demonstrated that globally considered, *Phlebotomus perniciosus*, *Phlebotomus ariasi* and *Phlebotomus papatasi* prefer to feed on some host species rather than others.

CONCLUSIONES

PRIMERA: Las especies *Phlebotomus perniciosus*, *Phlebotomus ariasi*, *Phlebotomus papatasi*, *Phlebotomus sergenti* y *Sergentomyia minuta* están presentes en áreas rurales y periurbanas de la Región de Murcia, siendo la primera especie la más abundante. Este hecho apoya el papel epidemiológico de *P. perniciosus* como principal vector de *Leishmania infantum* en esta zona.

SEGUNDA: La estimación de la densidad de las poblaciones adultas de flebotomos se ve afectada por factores relacionados con el muestreo, tales como el tipo y lugar de colocación de las trampas. Las densidades más altas se obtienen en trampas próximas al suelo y a superficies verticales continuas, especialmente empleando trampas adhesivas de intercepción, y en menor medida con las de atracción por luz y CO_2.

TERCERA: Además del método de muestreo, la densidad de flebotomos adultos se ve afectada por otros factores extrínsecos al díptero, como el clima, particularmente el viento, y por la proximidad a colectivos de animales, aunque la influencia de este último factor varía en función de la especie e incluso del sexo de los flebotomos.

CUARTA: Dada la gran cantidad de factores que influyen en las capturas, es fundamental estandarizar el procedimiento de muestreo para que los resultados sean representativos y comparables entre estudios. Asimismo, las estimaciones de la densidad de flebotomos deben apoyarse en el empleo de modelos estadísticos multivariables que incluyan todos estos parámetros.

QUINTA: La presencia de un elevado número de flebotomos en hábitats con alta diversidad de especies animales, como los parques zoológicos periurbanos, no implica necesariamente una transmisión eficiente de *Leishmania infantum*, debido a que los vectores se alimentan principalmente de animales que no son reservorios del parásito, tales como ciervos (*Cervus elaphus*) y gamos (*Dama dama*).

SEXTA: La distribución espacial de las hembras adultas de flebotomos en un núcleo de alta diversidad biológica es heterogénea, dependiendo de las necesidades particulares de cada estado reproductivo, como es la búsqueda de hospedador o de lugares de cría. Esto puede ser de utilidad a la hora de diseñar el control medioambiental de vectores.

SÉPTIMA: La estimación de las preferencias alimentarias de los flebotomos ha de considerar el coste de desplazamiento del insecto, además del número de hospedadores accesibles de los que alimentarse, habiéndose demostrado que *Phlebotomus perniciosus*, *Phlebotomus ariasi* y *Phlebotomus papatasi*, considerados globalmente, muestran predilección por alimentarse más de unas especies que de otras.

APPENDICES

Appendix 1. Sampling methodology and sand fly collection

Figure A1. CDC light trap (rural area).

Figure A2. Sticky traps placed in indoor (left) and outdoor (right) locations (rural area).

Figure A3. Insect collection from a light trap.

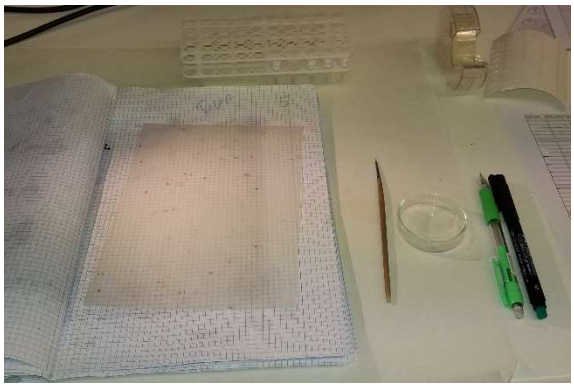

Figure A4. Sticky trap individually stored before sand fly collection in the laboratory.

Figure A5. Male sand fly.

Figure A6. Unfed female sand fly.

Figure A7. Blood-fed female sand fly.

Figure A8. Gravid female sand fly.

Appendix 2. Phlebotomine sand fly species morphologically identified

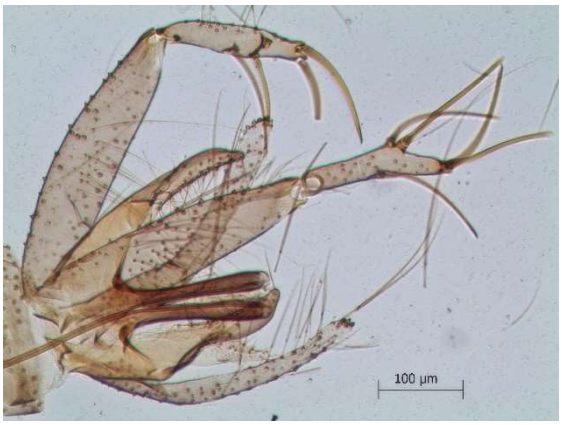

Figure A9. External genitalia of *Phlebotomus ariasi* male.

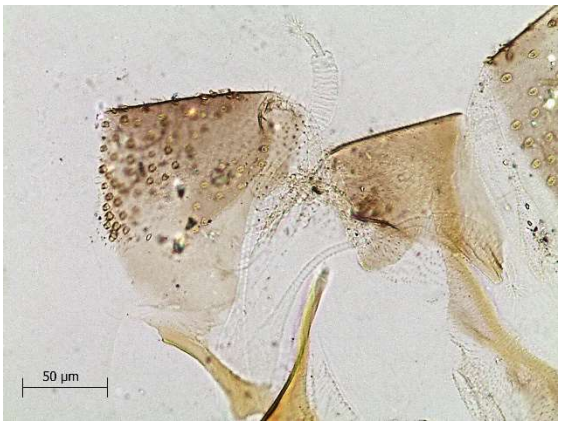

Figure A10. Spermatheca of *Phlebotomus ariasi* female.

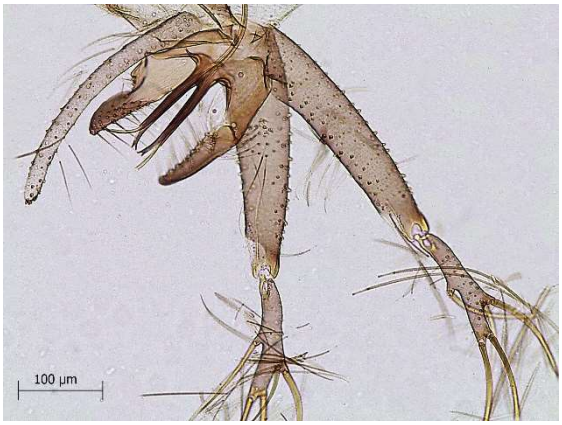

Figure A11. External genitalia of *Phlebotomus perniciosus* male.

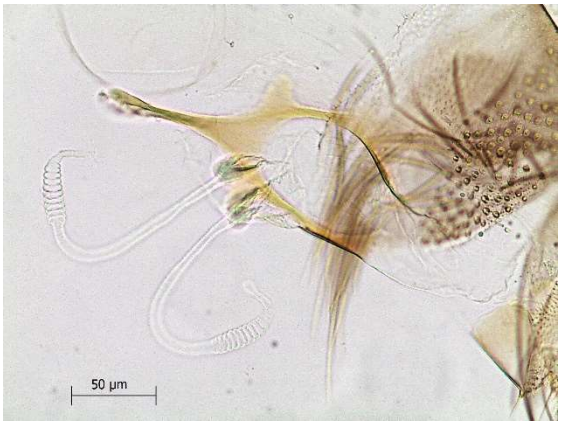

Figure A12. Spermatheca of *Phlebotomus perniciosus* female.

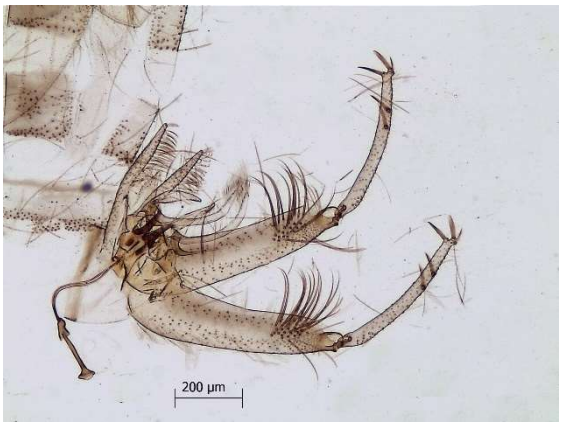

Figure A13. External genitalia of *Phlebotomus papatasi* male.

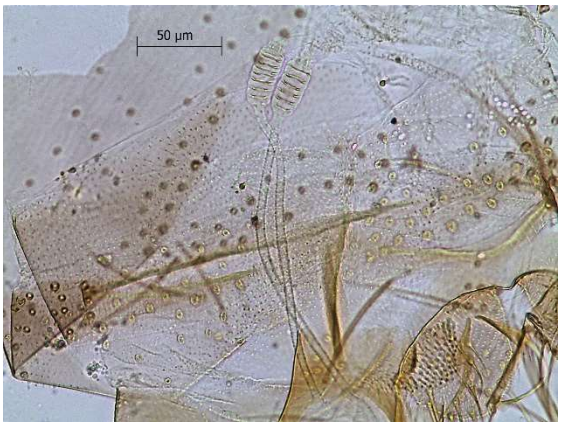

Figure A14. Spermatheca of *Phlebotomus papatasi* female.

Appendix 2 (continued). Phlebotomine sand fly species morphologically identified

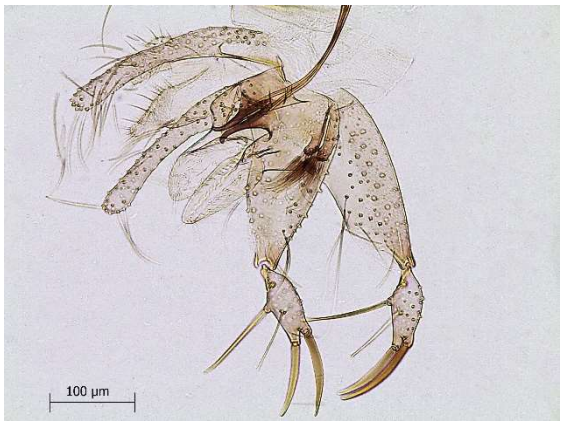

Figure A15. External genitalia of *Phlebotomus sergenti* male.

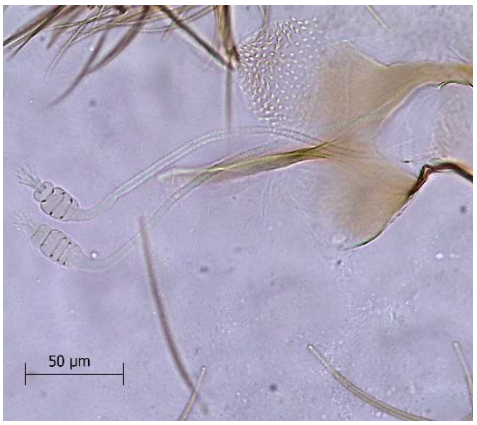

Figure A16. Spermatheca of *Phlebotomus sergenti* female.

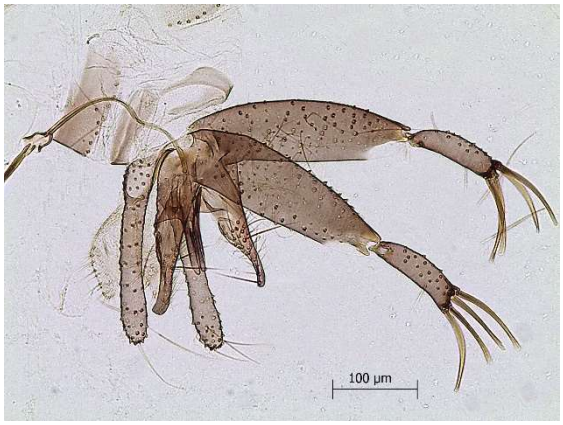

Figure A17. External genitalia of *Sergentomyia minuta* male.

Figure A18. Spermatheca of *Sergentomyia minuta* female.

Appendix 3. Scientific production from this doctoral thesis

❖ Articles:

Pérez-Cutillas, P., **Muñoz, C.**, Martínez-De La Puente, J., Figuerola, J., Navarro, R., Ortuño, M., Bernal, L. J., Ortiz, J., Soriguer, R. C. & Berriatua, E. (2020). A spatial ecology study in a high-diversity host community to understand blood-feeding behaviour in *Phlebotomus* sandfly vectors of *Leishmania*. *Medical and Veterinary Entomology*, *34*(2), 164-174.

Muñoz, C., Martínez-de la Puente, J., Figuerola, J., Pérez-Cutillas, P., Navarro, R., Ortuño, M., Bernal, L. J., Ortiz, J., Soriguer, R. & Berriatua, E. (2019). Molecular xenomonitoring and host identification of *Leishmania* sand fly vectors in a Mediterranean periurban wildlife park. *Transboundary and Emerging Diseases*, *66*(6), 2546-2561.

Muñoz, C., Risueño, J., Yilmaz, A., Pérez-Cutillas, P., Goyena, E., Ortuño, M., Bernal, L. J., Ortiz, J., Alten, B. & Berriatua, E. (2018). Investigations of *Phlebotomus perniciosus* sand flies in rural Spain reveal strongly aggregated and gender-specific spatial distributions and advocate use of light-attraction traps. *Medical and Veterinary Entomology*, *32*(2), 186-196.

❖ Communications to Conferences:

Muñoz, C., Martínez de la Puente, J., Figuerola, J., Navarro, R., Ortuño, M., Soriguer, R., Ortiz J. & Berriatua, E. (2018). City zoos in Mediterranean countries: a safe haven for *Leishmania infantum*? Oral communication. The 15th International Symposium of Veterinary Epidemiology and Economics. Chiang Mai (Thailand).

Muñoz, C., Navarro, R., Ortuño, M., Ortiz, J. & Berriatua, E. (2018). *Phlebotomus* sand fly distribution and preliminary results of *Leishmania infantum* infection rate in a zoological park. Oral communication. 1st International Caparica Congress on Leishmaniasis. Caparica (Portugal).

Risueño, J., **Muñoz, C.**, Yilmaz, A., Pérez-Cutillas, P., Goyena, E., Ortuño, M., Bernal, L.J., Ortiz, J., Alten, B. & Berriatua E. (2017). Small scale distribution of male and female *Phlebotomus perniciosus* in rural areas in southeast Spain assesed using sticky and light traps. Poster. 7th International Congress of the Society for Vector Ecology. Palma de Mallorca (Spain).

Muñoz, C., Risueño, J., Berriatua, E. & Ortiz, J. (2017). Sand fly counts in sticky and light traps: the influence of trap height and proximity to a flat surface. Poster. 7th International Congress of the Society for Vector Ecology. Palma de Mallorca (Spain).

Muñoz, C., Berriatua, E. & Ortiz, J. (2016). Estudio preliminar de los factores medioambientales que influyen en la abundancia de flebotomos (Diptera: Psychodidae) en el sureste español. Oral communication. II Jornadas Doctorales de la Universidad de Murcia. Murcia (Spain).

Muñoz-Hernández, C., Risueño, J, Berriatua, E. & Ortiz, J. (2016). Phlebotomine sandfly abundance is strongly affected by trap positioning height. Poster. The 3rd Conference on Neglected Vectors and Vector-Borne Diseases (EurNegVec) with Management Committee and Working Group Meetings of the COST Action TD1303. Zaragoza (Spain).