Phlebotomine sand fly (Diptera, Psychodidae) survey in a dog shelter of Toledo, Castilla-La Mancha

Estudio de los flebotomos (Diptera, Psychodidae) en un refugio canino de Toledo, Castilla-La Mancha

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ABSTRACT

Leishmaniosis is considered the most important neglected tropical disease caused by protozoan parasites in Southern European countries such as Spain. Its main etiological agent is *Leishmania infantum* while *Phlebotomus perniciosus* and *Phlebotomus ariasi* are the main vectors in the country. During the first half of July 2021, an entomological survey was conducted in a dog shelter and its surroundings in the municipality of Toledo (Castilla-La Mancha). Sand fly sticky traps, made of A5-size white paper coated with castor oil, were placed in six different environments inside a 200 m (the mean flight distance of *Phlebotomus* sp.) radius from the animal shelter. Sticky traps were set for eleven consecutive trapping nights in each site. A total of 356 phlebotomine sand flies belonging to four species in two genera were identified. These species and their abundance in the traps were: *Sergentomyia minuta* (70.2%), *Phlebotomus perniciosus* (25.9%), *Phlebotomus sergenti* (2.8%), and *Phlebotomus papatasi* (1.1%). The following list gives a breakdown of the abundance of phlebotomine sand flies found in various locations: a burrow (45.1%), a maintenance warehouse (21.3%), a holm oak´s tree hole (15.6%), an abandoned cattle barnyard (10.3%), a dog kennels area (4.3%), and surroundings of a feral feline colony (3.3%). *Phlebotomus papatasi*, an anthropophagic species, was only found in crevices of the maintenance warehouse. Although dogs are considered the main *Leishmania* reservoir, this preliminary research shows the effect of different micro-environments in the surroundings of animal shelters on sand fly abundance and diversity, and the importance that other reservoirs could play as most documented captures were associated with glires (orders Rodentia and Lagomorpha) rather than dogs and cats.

Key words: *Phlebotomus*, *Sergentomyia*, microhabitat, leishmaniosis, Spain.

RESUMEN

La leishmaniosis es considerada como la enfermedad tropical protozoaria desatendida más importante en países del sur de Europa, como España. Su principal agente etiológico es *Leishmania infantum*, mientras que *Phlebotomus perniciosus* y *Phlebotomus ariasi* son los principales vectores en el país. Durante la primera quincena de julio de 2021 se realizó un estudio entomológico en un refugio canino y sus alrededores en el municipio de Toledo (Castilla-La Mancha). Se colocaron trampas adhesivas para flebotomos, hechas de láminas de papel blanco tamaño

A5 recubiertas de aceite de ricino, en seis entornos diferentes dentro de un radio de 200 m (la distancia media de vuelo de *Phlebotomus* sp.) desde el refugio de animales. Se colocaron trampas pegajosas durante 11 noches consecutivas de trampeo en cada punto de muestreo. Se identificaron un total de 356 flebotomos pertenecientes a cuatro especies y dos géneros. Estas especies y su abundancia en las trampas fueron: *Sergentomyia minuta* (70.2%), *Phlebotomus perniciosus* (25.9%), *Phlebotomus sergenti* (2.8%) y *Phlebotomus papatasi* (1.1%). La siguiente lista desglosa la abundancia de flebotomos encontrados en los diferentes lugares: una madriguera (45.1%), un almacén de mantenimiento (21.3%), una dendrotelma de encina (15.6%), una cabaña de ganado abandonada (10.3%), en una zona de perreras (4.3%) y en las inmediaciones de una colonia felina salvaje (3.3%). *Phlebotomus papatasi*, una especie antropófaga, sólo se encontró en las grietas del almacén de mantenimiento. Aunque se considera que los perros son el principal reservorio de *Leishmania*, esta investigación preliminar muestra el efecto de diferentes microambientes en el entorno de un refugio de animales en la abundancia y diversidad de los flebotomos y la importancia que podrían tener otros reservorios, ya que la mayoría de las capturas documentadas estuvieron asociadas a glires (órdenes Rodentia y Lagomorpha), y no a perros o gatos.

Palabras clave: *Phlebotomus*, *Sergentomyia*, microhábitat, leishmaniosis, España.

INTRODUCTION

Leishmaniosis are a group of tropical and subtropical vector-borne zoonotic diseases caused by intracellular parasites of the genus *Leishmania* Ross, 1903 (Kinetoplastida, Trypanosomatidae) that are transmitted to humans and other vertebrates by the bite of infected female sand flies (Diptera, Psychodidae). Leishmaniosis, caused by *Leishmania infantum* Nicolle, 1908, is an endemic disease in the Iberian Peninsula and Balearic Islands. Although many infected individuals do not have clinical signs, dogs, children, and immunosuppressed people are most vulnerable to developing this zoonosis (AMELA *et al*., 2012).

This vector-borne disease is considered an emerging and re-emerging situation of global public health concern (PAL *et al*., 2022). In fact, there has been an unusual rise of human cases in an urban area of the South-Western Madrid region since 2009, affecting hundreds of immunocompetent adults (FERNÁNDEZ-MARTÍNEZ *et al*., 2019) and causing the largest human outbreak of leishmaniosis in recent times on this continent (MOLINA *et al*., 2012). This outbreak was associated with the disturbance of the natural habitat of endemic sand fly species that also led to an overpopulation of lagomorphs, which acted as a sylvatic reservoir for the parasite (JIMÉNEZ *et al*., 2013). The dog (*Canis lupus familiaris* Linnaeus, 1758) is considered the main domestic reservoir of this parasite (GÁLVEZ *et al*., 2010), although other confirmed or potential urban, peri-urban and sylvatic reservoirs are considered, such as wild Leporidae (*Oryctolagus cuniculus* (Linnaeus, 1758), *Lepus granatensis* Rosenhauer, 1856) (MOLINA, 2012; GARCÍA *et al*., 2014; ORTEGA-GARCÍA *et al*., 2019), urban rodents (*Rattus norvegicus* Berkenhout, 1769*, Rattus rattus* Linnaeus, 1758) (GALÁN-PURCHADES *et al*., 2019, ZANET *et al*., 2014), cats (*Felis silvestris catus* Schreber, 1775) (AHUIR-BARAJA *et al*., 2021), as well as other reservoirs (MILLÁN *et al*., 2014).

Regarding the vector, 13 phlebotomine sand fly species (two of them with controversial status) have been described in Spain (BRAVO-BARRIAGA *et al*., 2022), but only *Phlebotomus perniciosus* Newstead, 1911 and *Phlebotomus ariasi* Tonnoir, 1921 are proven vectors of leishmaniosis in the territory (RIOUX *et al*., 1986). However, other *Phlebotomus* species are suspected to play an important role in the transmission of *L. infantum*, such as *Phlebotomus papatasi* Scopoli, 1786 or *Phlebotomus mascittii* Grassi, 1908, and even the originally considered herpetophilic species *Sergentomyia minuta* Rondani, 1843 (YAVAR *et al*., 2013; OBWALLER *et al*., 2016; MAIA & DEPAQUIT, 2016; GONZÁLEZ *et al*., 2020). Also, some emerging *Phlebovirus*, especially the Toscana virus (TOSV), are present in Spain and can be transmitted to humans by several autochthonous phlebotomine species, such as *Ph. perniciosus* or *Ph. ariasi* (GARCÍA-SAN MIGUEL *et al*., 2019). Sand flies are known to breed in a wide variety of urban, peri-urban, rural, and sylvatic terrestrial habitats; nevertheless, the environment where each sand fly species develops has not yet been accurately characterized (NÁJERA, 1946; FELICIANGELI, 2004; GUERNAOUI & BOUMEZZOUGH, 2009).

The design and further implementation of leishmaniosis control programs need to be supported, not only by an excellent knowledge of the epidemiology of the disease, but also by an increase in the accuracy of species identification (ALARCÓN-ELBAL *et al*., 2021) and in-depth knowledge of the biology of the vectors and reservoirs involved (WILSON *et al*., 2020). In relation to the latter, entomological surveys are essential to obtain information about species diversity, distribution, and density of sand flies, among others. Considering that animal shelters are characteristic sand fly habitats (TARALLO *et al*., 2010; PRIETO *et al*., 2021), the present study aimed to characterize the sand fly fauna in relation to different micro-environmental characteristics in and around a dog shelter in Toledo.

MATERIAL AND METHODS

Study area

Phlebotomine sand flies were captured between 1st to 12th of July 2021, in and around a dog shelter in a rural area of the municipality of Toledo, located on a terrace of the Tajo River, at around 100 meters to the main riverbank. Toledo is also the capital city of its homonymous province and of the Autonomous Community of Castilla-La Mancha (Central Spain). The study sight was characterized by a profound orography, with the presence of the "Arroyo de las Cañas", a dry riverbed occupied mainly by dried ground covering grasses. The area presents an arid meso-Mediterranean climate, with hot and dry summers that can be even higher than 44 °C, and cold and dry winters with temperatures below –13 °C. In 2021, a total of 113 mm of rainwater was recorded. During the twelve days trapping session, a temperature variation between 12.2 °C and 42.3 °C was recorded, with a mean temperature of 32.1 °C. No precipitation was documented, and the mean wind speed was 42.6 km/h (minimum of 33 and maximum of 62 km/h) (AEMET, 2022).

Sand fly collection and identification

Considering that adult sand flies are generally weak fliers, and they usually disperse no more than a few hundred meters from their breeding site (SERVICE, 2012; GÁLVEZ *et al*., 2021), a 200-meter radius buffer was set from the dog shelter. Thirteen sticky traps (A5 size pieces of paper coated on both sides with castor oil) were set in six different trapping sites inside this buffer: four in a maintenance warehouse inside the shelter, three inside dog's kennels, two in an abandoned barnyard outside the shelter, two inside a holm oaks' (*Quercus ilex* Linnaeus, 1753) tree hole, one in an abandoned shut where a feral feline colony was established, and one in a glire's burrow. This clade comprises both orders Rodentia and Lagomorpha. Due to the ambiguous state of the burrow's entrance, it was not possible to clearly state if it was a rodent or leporid den. As such, we prefer to consider this hole as "glire's burrow". Traps were set covering the entrance of the different resting sites, setting them inside or by placing them in a wooden stick in an upright position, completely blocking the opening, as for capturing every sand fly trying to get in or out of the resting site. To ensure the integrity and efficiency of the sticky traps, they were replaced the 6th of July, in mid-sampling.

The traps were retrieved and captured sand flies were sexed under a stereomicroscope and preserved separately in 70% ethanol until identification. Just before processing, individuals were placed in a 99% ethanol bath during 1 to 2 minutes and lightly moved, as to eliminate castor oil residues. Afterwards, males were identified by studying the external genitalia, whilst females were submerged in Marc-André solution for 24 hours for bleaching, and then mounted on Hoyer´s medium for identification under a microscope. Taxonomical identification was carried out according to TELLO FIERRO *et al*. (2014).

Data analysis

Data were analyzed employing the statics software RStudio (RSTUDIO, 2020). Total captures of sand flies were grouped by trapping site, species, and sex, and then were divided by the total amount of traps set in the field for each trapping site type inside the study area. A Kruskal-Wallis statistical test was carried out, employing as a descriptive variable the location of the different trapping sites and the described variable the relative abundance of captured phlebotomine sand flies.

RESULTS AND DISCUSSION

A total of 356 phlebotomine sand flies, belonging to four species in two genera, were identified: *Se. minuta* (252/356; 70.2%), *Ph. perniciosus* (93/356, 25.9%), *Ph. sergenti* (10/356, 2.8%) and *Ph. papatasi* (4/356, 1.1%) (Fig. 1A). These species were the same as previously found by GONZÁLEZ *et al*. (2017) in the leishmaniosis outbreak area in Southern Madrid. *Sergentomyia minuta* was the predominant species whilst *Ph. perniciosus* was the second in abundance, in accordance with other studies conducted in Central Spain with sticky traps (CONESA *et al*., 1999; GÁLVEZ *et al*., 2010; GÁLVEZ *et al*., 2011; PRIETO *et al*., 2021). However, *Ph. perniciosus* was the predominant species found in surveys of the Madrid area employing CDC light traps (GONZÁLEZ *et al*., 2017). This fact could be due to a more positive phototropic response of *Ph. perniciosus* than *Se. minuta* (ALEXANDER, 2000). Even though *Ph. ariasi* has been recorded in some areas across the Meseta Central (CONESA, 1999; GÁLVEZ *et al*., 2011), this species was not found in our study, nor in GONZÁLEZ *et al*. (2017). This could be due to a less resistance to arid climate, having been mostly associated with mountainous regions, although this species has also been reported in association with *Q. ilex* trees (GUERNAOUI & BOUMEZZOUGH, 2009), same as those present in the study area.

Based on a statistical Kruskal-Wallis test, we observed that there were significant differences in the total amount of captured phlebotomine sand flies among the different trapping sites (Chi-square = 13.709, df = 5, p-value = 0.018). The total relative abundance of sand flies per environmental site was distributed as follows: 45.1% in a glire's burrow, 21.3% in a maintenance warehouse, 15.6% in a holm oak´s tree hole, 10.3% in an abandoned cattle barnyard, 4.27% in dog kennels and 3.3% in a feral cat colony. Focusing only on the vector species *Ph. perniciosus*, its relative abundance was 58.7% in the glires' burrow, 20.6% in the maintenance warehouse, 13.0% in the dogs' kennel, 4.3% in the abandoned barnyard, and 3.3% in the feline colony (Fig. 1A). Specific recorded abundance, classified by species and sex per trap (mean number of captured sand flies per trapping type) are shown below (Fig. 1; Supplementary Material I).

Female sand flies generally lay their eggs in cracks and holes in the ground or in buildings, animal burrows and among tree roots (SERVICE, 2008). As seen in this preliminary study, a glire's burrow had the highest capture rate, specially of *Ph. perniciosus*, main vector of leishmaniosis in the Iberian Peninsula; it should be noted that it has been the sole trapping site where females of *Ph. perniciosus* were captured. Additionally, even though dogs are considered the main host of *L. infantum* (GÁLVEZ *et al*., 2010), and that animal shelters have been considered to be a key factor in the distribution of phlebotomine sand flies in peri-urban areas due to the presence of animals (PRIETO *et al*., 2021), it should be noted that the least amount of captures in this preliminary research was found associated to dog kennels and the feral feline colony. However, when focussing on the vector species, it is remarkable that *Ph. perniciosus* is the most abundant species in dogs' kennels (52.2%), tightly followed by *Se. minuta* (47.8%) (Fig. 1B). Nevertheless, dogs' kennels as a trapping site only represented 13.0% of the total amount of captured *Ph. perniciosus* (Fig. 1A).

Although dog shelters have been sometimes considered as focuses of phlebotomine sand flies (PRIETO *et al*., 2021), based on this study we suggest that dog presence is not the sole reason for this observation. Based on the exposed preliminary data, the greatest amount of captured sand fly densities, especially that of the vector species, *Ph. perniciosus* (58.7%), was associated with the presence of burrows, followed by a maintenance warehouse and a holm oak's tree hole, being the lowest reported captures associated within both dogs` kennels and feral cats' colonies. Phlebotomine sand flies' habitat preference is associated to multiple variables such as climate, presence of human dwellings, proximity to domestic animals or presence to organic matter in the soil, among other factors (GUERNAOUI & BOUMEZZOUGH, 2009). As such, we consider that the association between sand flies and animal shelters could not be mainly due to the presence of domestic animals but rather to several variables associated to those habitats. In this case, we considered that animal shelters in the Central Area of Spain are associated with several key factors that could increase sand fly population and that could be independent to

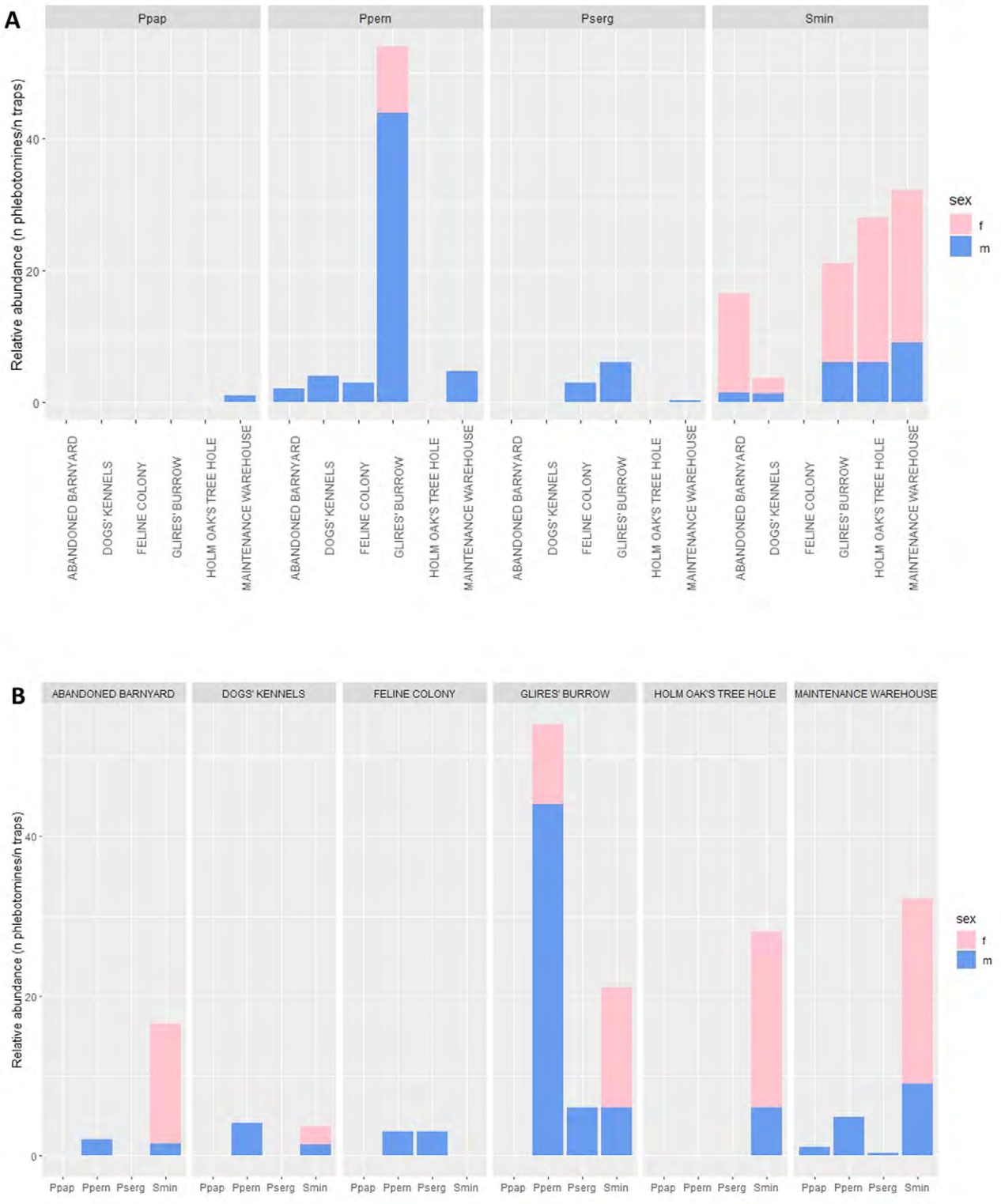

Fig. 1. Micro-environmental features associated with sand fly fauna relative abundance (captured individuals/number of established traps) present in Toledo's Dog Shelter and its surroundings (Castilla-La Mancha, Spain). *Ppap, Phlebotomus papatasi; Ppern, P. perniciosus; Pserg, P. sergenti; Smin, Sergentomyia minuta;* f, females; m, males. A. Relative abundance of captured phlebotomine sand flies classified by species and subclassified by micro-environment. B. Relative abundance of captured phlebotomine sand flies classified by micro-enviroments and subclassified by species.
Fig. 1. Microhábitats asociados a la abundancia relativa de flebotomos (individuos capturados/número de trampas instaladas) en el Centro de Acogida Animal de Toledo y sus alrededores (Castilla-La Mancha, España). *Ppap, Phlebotomus papatasi; Ppern, P. perniciosus; Pserg, P. sergenti; Smin, Sergentomyia minuta;* f, hembras; m, machos. A. Abundancia relativa de flebotomos clasificada por especies y subclasificada por microhábitat. B. Abundancia relativa de flebotomos clasificada por microhábitat y subclasificada por especies.

the presence of dogs or cats in the enclosure of an Animal Shelter. Firstly, animal shelters are often located in peri-urban areas, which have several biotic consequences such as the nearby presence of different sylvatic hosts (wild leporids, cervids, etc.) and the availability of soil (i.e. sand fly potential breeding sites), in contrast with pavemented urban environments. Secondly, animal shelters do not usually get many economical resources and they mostly get taken care of by volunteers or social services personnel. Consequently, due to lack of proper maintenance, animal shelters usually have structural deficiencies such as cracks and crevices that could be employed by phlebotomine sand flies as breeding and/or resting sites. In addition, the presence of other pests such as rodents, mainly due to the presence of animal food and water sources in the enclosure as well as a potential absence of proper pest control programs, may increase the availability of different blood sources for these hematophagous insects and as breeding or resting sites inside glires' burrows too. As such, animal shelters are conditioned by several variables that favour the presence of different micro-environments suitable for phlebotomine sand fly development, apart from the presence of dogs or cats. Based on these preliminary results, we consider that phlebotomine sand fly spatial modelling and control programs need to focus on addressing specific breeding and resting sites in a micro-scale level instead of just addressing meso- and macro-environmental variables.

Even though the captured individuals were identified to species level, we cannot differentiate the role of those micro-environments between adult resting sites or as breeding sites of preimaginal stages. For such a task, it should have been necessary to determine the presence of rotated genitalia in the case of male phlebotomine adults, as this characteristic is considered a key aspect for locating nearby phlebotomine sand fly breeding sites. This aspect was not considered during the development of the study, although it should have been. Male phlebotomine sand flies, as other Nematocera, enclose in the pupa with an unrotated genitalia. After a variable time among species after adult emergence, a 180° permanent rotation of the genitalia takes place (VOTYPKA *et al*., 2015). Identification of males with unrotated genitalia could then point the potential role of the sampling sites as also breeding sites for phlebotomine sand flies.

We would also like to point out the recent report of *Ph. papatasi* in the area, as its presence was previously unknown in the province of Toledo (BRAVO-BARRIGA *et al*., 2022). In this same article, these authors state the first detection of *Se. minuta* in this province, although it was previously reported in the literature by GILL COLLADO *et al.* (1989). Finally, we would just like to point out the capture of sand flies inside a holm oak tree, which are common breeding sites for other tree hole blood feeding Diptera such as *Aedes equinus* (Edwards, 1920) and *Aedes berlandi* (Séguy, 1921) (Diptera, Culicidae) (SÁNCHEZ COVISA *et al*., 1985), or *Culicoides haranti* Rioux, Descous & Pech, 1959 (Diptera, Ceratopogonidae) (AGUILAR YUSTE *et al*., 2021). Those same habitats in the case of phlebotomine sand flies could also act as breeding sites of immature stages as seen in studies conducted in tropical forests in Brazil (BAIATONE *et al*., 2011). However, to date the association between this natural micro-environment and sand flies has not been studied in depth in the Iberian Peninsula.

In conclusion, abundance of captured sand flies may be more dependent of micro-environmental variables than macro-environmental conditions. As such, the general association of phlebotomine sand flies' abundance and animal shelters could be biased by the presence of several factors such as the location of those enclosures in peri-urban areas and low budgets for maintenance and pest control programs. Further research needs to be conducted over the topic of sand flies micro-environmental breeding and resting sites preferences. A clear differentiation of the effect of environmental variables associated with animal shelters and the presence of dogs should be addressed. Further research is needed to evaluate this subject.

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Supplementary material I: Identified sand flies in the different micro-environments, view as the total amount of captured individuals (n) and its relative percentage (%). Data is classified as males, females and total amount of the different species and the total amount of phlebotomines captured.
Material Suplementario I: Flebotomos identificados en cada uno de los microhábitats estudiados, visualizado como el total relativo de individuos capturados (n flebotomos/n trampas) y su porcentaje relativo (%). Los datos están clasificados como machos, hembras y totales de las diversas especies y total de flebotomos capturados.

MICRO-ENVIRONMENTS															
Species	*Sex*	*ABANDONED BARNYARD*		*DOGS' KENNELS*		*FELINE COLONY*		*GLIRES' BURROW*		*HOLM OAK'S TREE HOLE*		*MAINTENANCE WAREHOUSE*		*TOTAL*	
		n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Ph. papatasi	Female	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	Male	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	4	100.00%	4	100.00%
	Total	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	4	100.00%	4	100.00%
Ph. perniciosus	Female	0	0.00%	0	0.00%	0	0.00%	10	100.00%	0	0.00%	0	0.00%	10	10.87%
	Male	4	4.88%	12	14.63%	3	3.66%	44	53.66%	0	0.00%	19	23.17%	82	89.13%
	Total	4	4.35%	12	13.04%	3	3.26%	54	58.70%	0	0.00%	19	20.65%	92	100.00%
Ph. sergenti	Female	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
	Male	0	0.00%	0	0.00%	3	30.00%	6	60.00%	0	0.00%	1	10.00%	10	100.00%
	Total	0	0.00%	0	0.00%	3	30.00%	6	60.00%	0	0.00%	1	10.00%	10	100.00%
Se. minuta	Female	30	15.87%	7	3.70%	0	0.00%	15	7.94%	44	23.28%	93	49.21%	189	75.60%
	Male	3	4.92%	4	6.56%	0	0.00%	6	9.84%	12	19.67%	36	59.02%	61	24.40%
	Total	33	13.20%	11	4.40%	0	0.00%	21	8.40%	56	22.40%	129	51.60%	250	100.00%
TOTAL	Female	30	15.08%	7	3.52%	0	0.00%	25	12.56%	44	22.11%	93	46.73%	199	55.90%
	Male	7	4.46%	16	10.19%	6	3.82%	56	35.67%	12	7.64%	60	38.22%	157	44.10%
	Total	37	10.39%	23	6.46%	6	1.69%	81	22.75%	56	15.73%	153	42.98%	356	100.00%